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SOD1-Targeted Gene Disruption in the Ericoid Mycorrhizal Fungus *Oidiodendron maius* Reduces Conidiation and the Capacity for Mycorrhization

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The genome sequences of mycorrhizal fungi will provide new opportunities for studying the biology and the evolution underlying this symbiotic lifestyle. The generation of null mutants at the wild-type loci is one of the best methods for gene-function assignment in the post-genomic era. To our knowledge, the generation of superoxide dismutase 1 (SOD1)-null mutants in the ericoid mycorrhizal fungus *Oidiodendron maius* is the first example of a gene-targeted disruption via homologous recombination in a mycorrhizal fungus. The disruption of *OmSOD1* by *Agrobacterium*-mediated transformation resulted in the presence of oxidative stress markers, even in the absence of external superimposed stresses, and an increased sensitivity to reactive oxygen species (ROS)-generating substances, especially to menadione. A reduction in conidiation and in the percentage of mycorrhization of *Vaccinium myrtillus* roots was also observed. The latter findings establish the pivotal role of SOD1 as an important factor in the relationship between *O. maius* and its symbiotic partner. The lack of this ROS-scavenger may cause an imbalance in the redox homeostasis during host colonization and an alteration in the delicate dialogue between the fungus and its host plant.

The complex communication that must occur during mycorrhizal symbiosis between the fungus and the plant is likely to be explained in the coming years through comparative genomics, because a large amount of information from the genome sequences of fungal saprotrophs, pathogens, and symbionts is now available (Martin et al. 2007). The release of the complete *Laccaria bicolor* genome (Martin et al. 2008) and the ongoing sequencing of the *Tuber melanosporum* and *Glomus intraradices* genomes are going to provide further opportunities for studying the biology and the evolution underlying this symbiotic lifestyle. Gene-function assignment then becomes the new challenge and depends on the development of high-throughput methodologies to test gene-function in vivo or in vitro. One such method is the generation of null mutants via homologous recombination at the wild-type loci by using inactivation cassettes.

The first evidence for genetic transformation of a mycorrhizal fungus (Barrett et al. 1990) was the successful transformation of the basidiomycete *Laccaria laccata* using protoplast polyethylene glycol-based transformation. Since then, other mycorrhizal species were stably transformed, i.e., several ectomycorrhizal fungi, all basidiomycetes, such as *Hebeloma cylindrosporum* (Combier et al. 2003; Pardo et al. 2002; Marmeisse et al. 1992), *Paxillus involutus* (Bills et al. 1995; Pardo et al. 2002), *Laccaria bicolor* (Bills et

al. 1999; Kempainen et al. 2005), *Suillus bovinus* (Hanif et al. 2002; Pardo et al. 2002), and *Pisolithus tinctorius* (Rodriguez-Tovar et al. 2005), and one ericoid mycorrhizal (ERM) fungus, the ascomycete strain *Oidiodendron maius* Zn (Martino et al. 2007). The random integration of a selectable marker into the host genome can lead to the creation of large collections of random mutants, which are then screened for a phenotype of interest. By contrast, homologous recombination has not yet been achieved for mycorrhizal fungi.

In this work, we have obtained the first targeted gene inactivation in a mycorrhizal fungus. *O. maius* is one of the most widely investigated ERM fungi. The symbiotic association between ericaceous plant roots and their endophytic fungi is known to facilitate the uptake by plants of organic nitrogen from recalcitrant organic compounds. Beside the nutritional benefits, ERM fungi also play a crucial role in the protection of their host plants against heavy metal toxicity through their ability of complexing and compartmentalizing excess toxic elements (Sharples et al. 2000; Turnau et al. 2007). In particular, *O. maius* Zn, which was isolated from plots contaminated with industrial dusts, has been demonstrated to tolerate concentrations of zinc (Zn) that cause a complete growth inhibition of isolates coming from unpolluted soils (Martino et al. 2000).

O. maius can be easily grown in vitro, reproducing asexually by forming conidia with just a single haploid nucleus. This characteristic makes this fungus a good candidate to study mutants, because uninucleated spores can germinate and produce a homokaryotic mycelium with all the nuclei carrying the mutation. Moreover, the haploid monokaryotic status implies that the modifications of the wild-type phenotype, if detectable, can be observed even when mutations are recessive.

Agrobacterium-mediated transformation (AMT) was recently used for random insertional mutagenesis to generate mutants of this fungus (Martino et al. 2007). In this work, we have used the same transformation method to specifically delete the single copy gene *OmSOD1*, the copper (Cu)- and zinc-containing superoxide dismutase (SOD) (EC 1.15.1.1.) of *O. maius* Zn (Vallino et al. 2009).

This gene has a twofold importance; it has been recently proposed to play a role in the differentiation processes associated with fungal symbiosis (Tanaka et al. 2006) and it is a key enzyme in the detoxification of the cell from reactive oxygen species (ROS), as described in many other fungi, such as *Saccharomyces cerevisiae* (Gralla and Valentine 1991), *Aspergillus nidulans* (Guelfi et al. 2003), *A. niger* (Todorova et al. 2007), *P. involutus* (Jacob et al. 2001), and *Schizosaccharomyces pombe* (Tarhan et al. 2007). ROS are by-products of normal metabolism, but their level dramatically increases in the presence of redox-active metals such as iron and Cu (Schutzendubel and Polle 2002) or superoxide ion generators such as the redox cycling agent menadione (Emri et al. 1999). Interestingly, the occurrence of activated oxygen and symptoms of oxidative injury have also been observed in organisms exposed to non-redox active metals like Zn or cadmium (Cd) (Schutzendubel and Polle 2002). In most cases, exposure to these metals initially resulted in a severe depletion of free-radical scavengers, such as thiol-containing compounds, and in increasing ROS levels (Dietz et al. 1999).

Given the functional dualism of the SOD1 protein, we took advantage of our experimental organism, which is both a mycorrhizal and a zinc-tolerant fungus to assess whether i) the lack of a ROS scavenger can alter its ability to establish a mycorrhizal symbiosis with the roots of its host plant and ii) the deletion of the *SOD1* gene can influence the fungal response to the oxidative stress caused directly by a ROS-generating agent such as menadione and indirectly by Zn and Cd.

RESULTS

Gene disruption of *OmSOD1*. To determine if *OmSOD1* is essential in *O. maius* for resistance to heavy metals and for mycorrhization, a deletion construct that replaces almost all the coding sequence of the *OmSOD1* gene with the hygromycin-resistance gene was designed and introduced into *O. maius* ungerminated conidia by AMT (Fig. 1A). Hygromycin-resistant colonies were screened by polymerase chain reaction (PCR) for homologous recombination. SOD1-null mutants were characterized by i) the absence of amplification with primer set 1 (described below), designed to amplify the DNA fragment replaced by the *OmSOD1* cassette and ii) a 5,376-bp-long amplicon obtained with primer set 3 (described below), designed on the two *OmSOD1* regions external to the targeting construct. Thirteen putative homologous recombinants out of the 857 screened transformants were hence identified. Genomic DNA of these homologous recombinants, the wild-type strain, and the ectopic transformant B3_123 were digested with *PstI* for Southern hybridization analysis. *PstI* has no recognition sites either in the *OmSOD1*

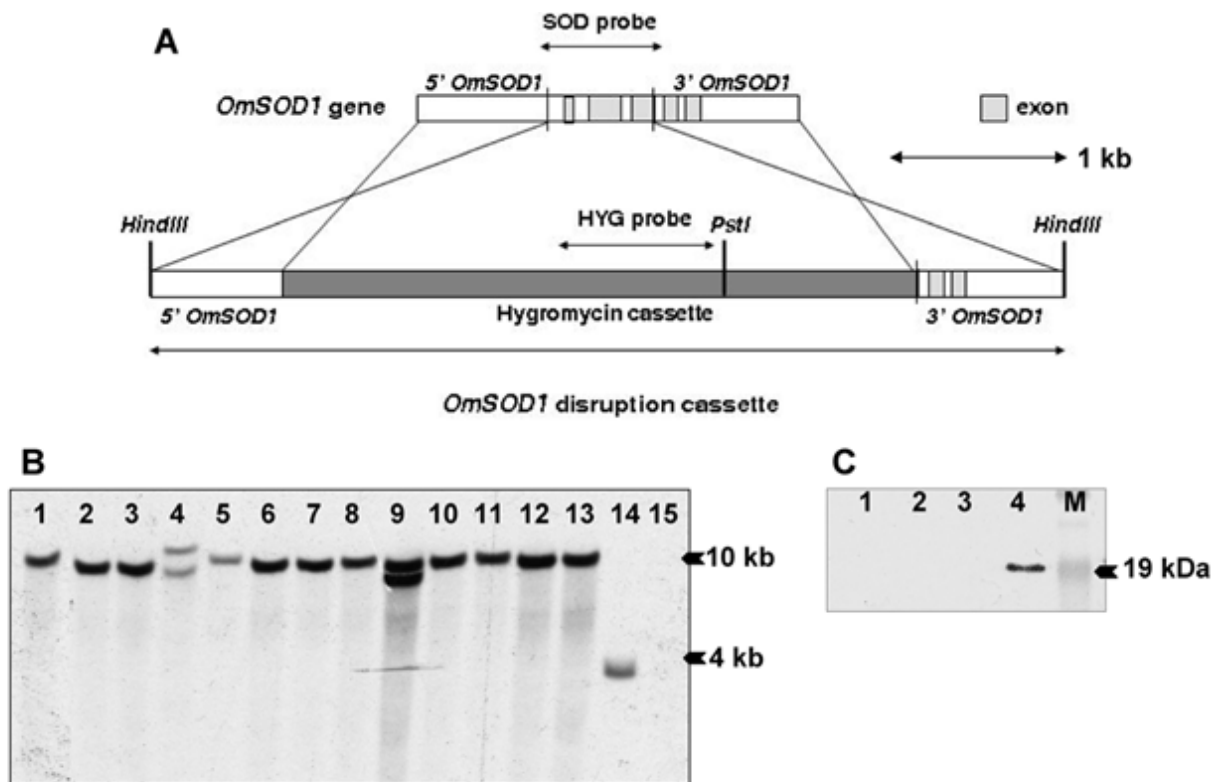


Fig. 1. Disruption cassette and Southern blot analysis. **A**, Upper part, the wild-type *OmSOD1* gene and the position of the five exons. Bottom part, the disruption cassette containing the 5' flanking sequence and the remaining nonfunctional 3' part of the *OmSOD1* gene. The hygromycin phospho-transferase (*hph*) gene and its promoter and terminator sequences are depicted as one unit within the disruption cassette to simplify the picture. The *SOD1* and *hph* gene fragments used as probes in Southern blotting are indicated by black arrowed bars. The position of the *PstI* recognition site is also shown. **B**, Southern blot analysis of the recombination event. Genomic DNA was restricted with *PstI* and was hybridized with *hph* probe. Lanes 1 through 13, polymerase chain reaction-selected SOD1-null mutants (C2_42, A4_57, D5_74, B5_75, C5_76, D5_83, D5_125, A4_95, D3_98, B4_97, C4_86, B1_84, C6_18); Lane 14, an ectopic transformant (B3_123); Lane 15, wild-type *Oidiodendron maius* Zn. The disruption cassette has integrated by homologous recombination in the genome of all the 13 transformants but, in B5_75 and D3_98, an ectopic integration also occurred. **C**, The Western blot analysis of 650 ng of total protein from the selected SOD1-null mutants and the wild type demonstrates the lack of SOD1 protein from SOD1-null mutants D5_83 (lane 1), D5_125 (lane 2), and A4_57 (lane 3), and the presence of a band at 19 kDa in the wild-type sample (lane 4). M = prestained protein molecular weight marker (Bio-Rad, Hercules, CA, U.S.A.).

gene or in the hygromycin phospho-transferase (*hph*) gene covered by the *hph* probe. The absence of signal on all the PCR-selected SOD1-null mutants after hybridization with the *OmSOD1*-specific probe confirmed the *OmSOD1* gene disruption (data not shown). The same blot was also probed with a fragment

of the hygromycin-resistance gene to verify the single integration of the construct at the *OmSOD1* locus (Fig. 1B).

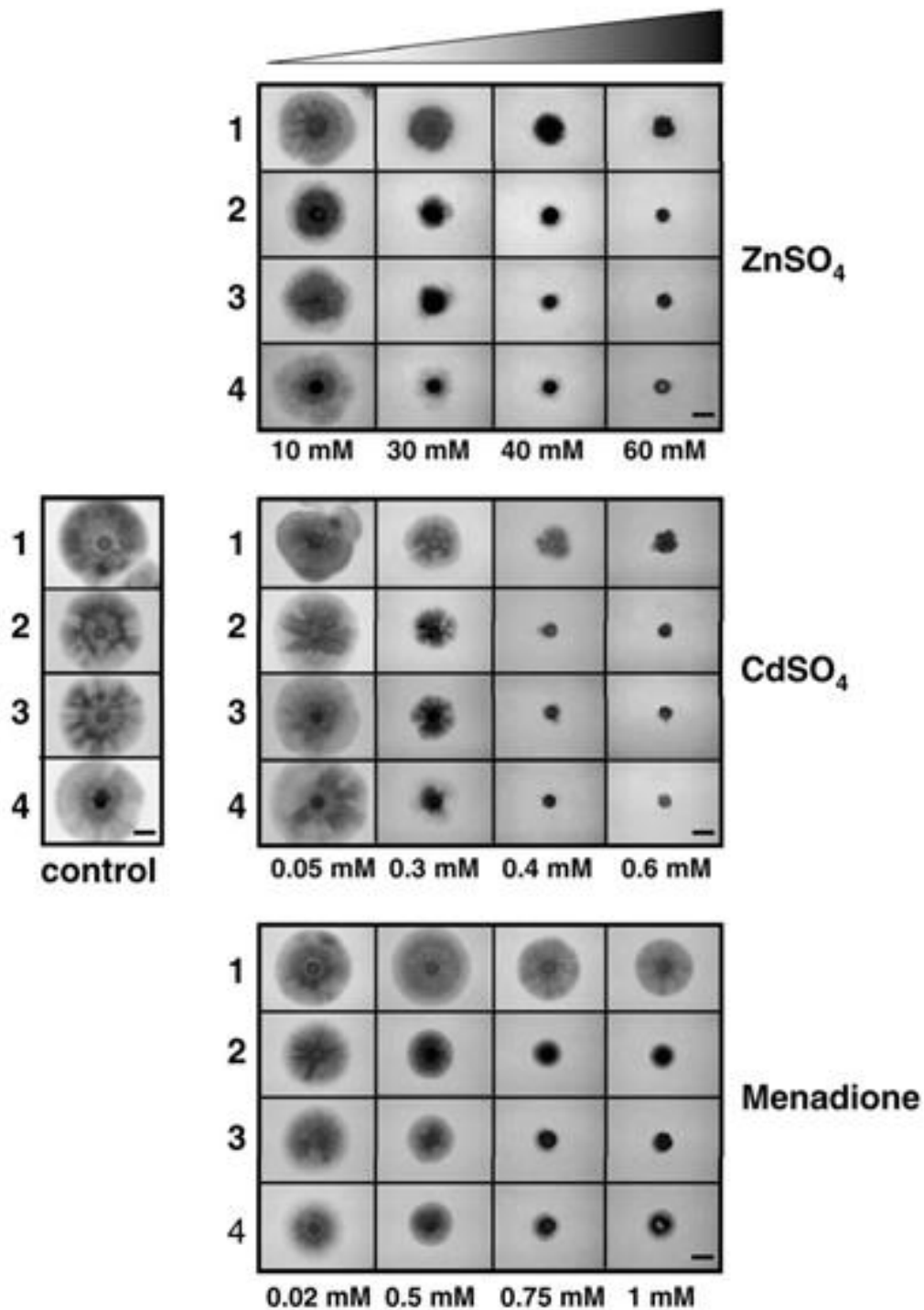


Fig. 2. Determination of oxidative stress tolerance of SOD1-null mutants. The wild-type strain and the mutants were grown on agar medium for 30 days in Czapek-glucose, amended or not with zinc (Zn), cadmium (Cd), and menadione. The concentration of heavy metals and menadione rises from left to right. Concentrations of 10, 30, 40, and 60 mM Zn, 0.05, 0.3, 0.4, and 0.6 mM Cd, 0.02, 0.5, 0.75, and 1 mM menadione were tested. All of the SOD1-null strains exhibited an increased sensitivity, especially to menadione, in respect to the wild-type. 1 = *Oidiodendron msius* Zn wild-type, 2 = D5_83, 3 = D5_125; A4_57. Bars = 1 cm.

Two copies of the expression assette were integrated in two of the SOD1-null mutants (B5_75 and D3_98), while the other 11 showed a single integration.

The gene replacement (GR) frequency was defined as the number of homologous recombinant colonies divided by the total number of the analyzed hygromycin-resistant colonies. Based on PCR and Southern analysis, the GR frequency obtained in this AMT is 1.52%, with *OmSOD1* homologous regions of 786 and 820 bp flanking the hygromycin-resistance cassette.

A4_57, D5_83, and D5_125 mutants were randomly chosen for further analyses among the 11 SOD1-null mutants with a single integration. After having confirmed the integration of the disruption cassette at the correct locus, we verified the loss of expression by Western blot analyses. The wild type and the three selected mutants were grown in the same culture conditions (in liquid 10 mM zinc-amended medium for 30 days) that had been shown by Vallino and colleagues (2009) to induce the expression of the SOD1 protein. Western blots showed a single band of the expected size in the wild type and no detectable SOD1 protein in the SOD1-null mutants (Fig.1C). Reverse transcription (RT)-PCR of the RNA isolated from the same samples used for Western blot analyses revealed that the *OmSOD1* gene was not transcribed in the mutants (data not shown).

SOD1-null mutants have a significant growth defect on stressing media.

The wild type and the three mutants were grown on heavy metal- and menadione-amended media to evaluate their ability to tolerate increasing concentrations of these stress-inducing substances (Fig. 2). All the fungi showed a similar radial growth on the control media, while the addition of Zn, Cd, and menadione adversely affected the SOD1-null mutants more than the wild type. Differences in the radial growth were clearly visible on Zn-, Cd-, and menadione-amended media starting from 30 mM, 0.3 mM, and 0.5 mM, respectively. Especially on menadione, the mutants showed almost no growth at the highest concentrations applied, whereas the wild type was still able to grow.

A reduced conidiation was observed in SOD1-null mutants.

Based on one-way analysis of variance (ANOVA), the number of conidia per surface unit produced by the SOD1-null mutants was significantly fewer in respect to the wild type. Conidia numbered 1.05 ± 0.11 (expressed in millions) per square centimeter for the wild type, while they numbered 0.61 ± 0.08 , 0.64 ± 0.06 , and 0.65 ± 0.03 for D5_125, D5_83, and A4_57, respectively. Thus, an average 1.5-fold reduction in conidiation capacity was recorded in the mutants.

ROS production.

Mycelial samples of the wild type and the mutants grown both in control and in stress-inducing media were examined for their ability to generate superoxide anions by reduction of nitroblue tetrazolium (NBT) to dark blue, water-insoluble formazan. Within 3 h after NBT treatment, hyphae of the mutants grown in control medium were clearly stained with dark-blue formazan spots, while no deposits were found in the wild type (Fig. 3). No significant differences were observed in the pattern and quantity of the deposits between the control and the stressed hyphae in either the mutants or the wild type (data not shown).

Plant growth parameters and degree of in vitro mycorrhization.

We investigated whether the SOD1-null mutants showed the same ability of the wild type in establishing in vitro mycorrhizal symbiosis with the hair roots of *Vaccinium myrtillus* and in influencing plant

development. Growth parameters (shoot length, shoot fresh weight, shoot dry weight, root length, root fresh weight, total leaf area, and internodal length) and mycorrhization percentages of *V. myrtillus* seedlings grown with the SOD1-null mutants were recorded after 3 months of cocultivation and were compared with the seedlings inoculated with the wild type (Table 1). Mycorrhization percentages were also recorded after 1.5 months of cocultivation (Table 1). The only statistically significant difference ($P < 0.05$) in the plant growth parameters was a slight reduction of the internodal length in the seedlings inoculated with the SOD1-null mutants in respect to those inoculated with the wild type, even if a high level of variation of this parameter (generally observed on cocultivation medium) was taken into account. On the other hand, the reduction in the degree of mycorrhization of SOD1-null mutants was always statistically significant ($P < 0.05$), both after 1.5 and after 3 months.

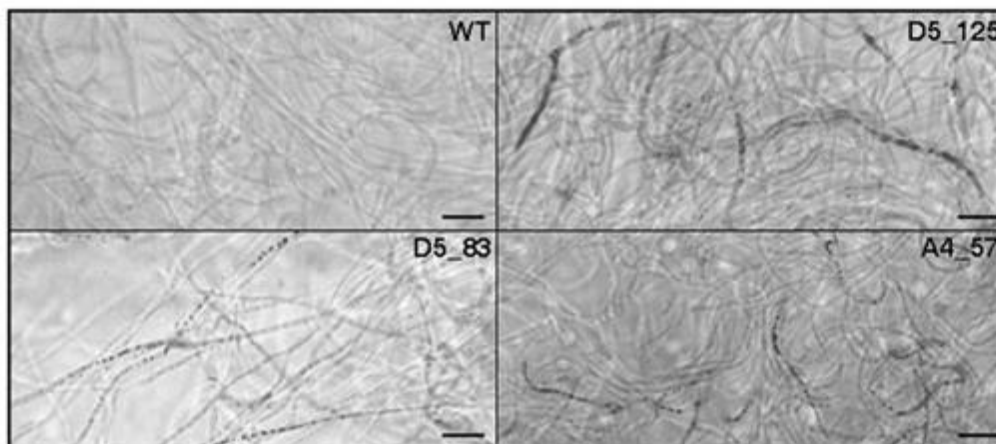


Fig. 3. Reactive oxygen species (ROS) production by *Oidioidendron maius* wild type and SOD1-null mutants in liquid control medium. Light microscope photographs of colony mycelia of *O. maius* Zn wildtype (WT) and SOD1 null mutants (D5_83, D5_125, and A4_57) grown on Czapek–glucose liquid medium and stained with 0.05% (wt/vol) nitroblue tetrazolium solution for 3 h. Formazan deposition was uniform in the cells of the disrupted mutants, even though the inability to merge pictures taken on different optical plans caused an apparently localized staining. On the contrary, no staining was observed in the wild-type strain. Bars = 10 μ m.

DISCUSSION

First targeted gene disruption in a mycorrhizal fungus.

Although mycorrhizae are critical elements of terrestrial ecosystems, we have just begun to understand the molecular interactions between mycorrhizal fungi and their host plants. Since the first global gene-expression data from ectomycorrhizas (Johansson et al. 2004; Voiblet et al. 2001), the genome sequencing program of *Laccaria bicolor* (Martin et al. 2008) combined with powerful bioinformatics tools has had a major impact on research on mycorrhizas. Comparison of the genomes of various pathogenic and saprobic fungi with the *Laccaria* genome will enable functional genomic studies with a focus on the complexity of plant-fungus associations (Martin et al. 2007). The complete inactivation of a gene is generally the first and most direct way to explore its function, but gene manipulation in mycorrhizal fungi is limited by the lack of effective methods for targeted gene replacement on a large scale and the high frequency of ectopic integrations of the transforming DNA molecule.

In this paper, we have described the first targeted gene replacement by AMT in a mycorrhizal fungus. The GR frequency of 1.52% obtained in *O. maius* with both flanking regions of about 800 bp is only slightly lower than the GR frequency observed in the nonmycorrhizal fungus *Aspergillus awamori* (1.75% with flanking regions of 500 bp and 4.04% with flanking regions of 1,000 bp) (Michielse et al. 2005), but it is very much lower than the GR frequencies observed in *A. niger* (7% with 500 bp and 19% with 1,000 bp)

(Meyer et al. 2007) or *Neurospora crassa* (9% with 500 bp and 21% with 1,000 bp) (Ninomiya et al. 2004) wild-type strains. Unfortunately, the genome of *O. maius* has not been sequenced, so the length of the flanks we used in the disruption cassette was the longest available based on the known sequence of the *OmSOD1* gene. A significant increase in the frequency may be achieved either by increasing the length of flanking regions or with the suppression of the nonhomologous end-joining (NHEJ) pathway, as observed in other filamentous fungi (da Silva Ferreira et al. 2006; Meyer et al. 2007; Ninomiya et al. 2004; Poggeler and Kuck 2006).

RNA silencing could be a reliable alternative to disruption experiments for the functional analysis of genes, especially in fungi having multinuclear hyphae or a low frequency of homologous recombination (Janus et al. 2007). This method has been recently used in the dikaryotic ectomycorrhizal fungus *Laccaria bicolor* to suppress the expression of the nitrate reductase (Kempainen et al. 2009). The RNA silencing of this gene resulted in fungal strains severely affected in their capacity to establish a symbiotic interaction with *Populus* spp. Investigations on specific genes mediating symbiotic events in mycorrhiza formation have hitherto been based exclusively on the study of nonmycorrhizal (Myc⁻) plant mutants interacting with arbuscular mycorrhizal (AM) fungi (Parniske 2004). On the fungal side, a collection of 10 *Hebeloma cylindrosporum* Myc⁻ mutants blocked at the early stages of ectomycorrhiza formation was obtained by Combier and colleagues (2004). Although the identification of which fungal genes were mutated to give this kind of phenotype was not undertaken, those results along with those reported by Keimann and associates (2009) confirmed the idea that genes essential for symbiosis establishment do exist in mycorrhizal fungi.

Martin and colleagues have recently used *Laccaria bicolor* whole-genome expression oligoarrays to demonstrate that numerous genes are differentially expressed during the symbiotic stage (Martin et al. 2008). The feasibility of targeted gene disruptions in a mycorrhizal fungus opens new possibilities to study the biological role of these symbiosis-regulated genes. These genes are, in fact, good candidates for being the primary targets for gene inactivation.

SOD1 and oxidative stress.

Superoxide dismutase enzymes scavenge radicals by catalyzing the conversion of two of these radicals into hydrogen peroxide and molecular oxygen, thus being involved in the cellular defense strategies against oxidative damage by ROS. Their induction by Zn, Cd, and menadione has been described in other fungi such as *A. niger* (Todorova et al. 2007), *Candida intermedia* (Fujs et al. 2005), *A. nidulans* (Guelfi et al. 2003), *Candida albicans* (Hwang et al. 2002), and *Cryptococcus neoformans* (Narasipura et al. 2002). The loss of SOD1 activity has various pleiotropic consequences on organisms, including slow growth, conditional auxotrophies, and DNA damage (Fridovich 1989, 1995). For example, a SOD1-null yeast strain was shown to be oxygen-sensitive, hypermutable, auxotrophic for lysine and methionine, and defective in sporulation (Liu et al. 1992). It is thus possible that each SOD increases fitness while not being essential for viability (Chary et al. 1994). As expected, *O. maius* Zn SOD1-null mutants showed an increased sensitivity to all the tested substances in respect to the wild type. Measurement of mycelium radial growth demonstrated that the disruption of *SOD1* caused the mutants to be more sensitive especially to menadione. This quinone, which undergoes intracellular redox cycling, generates a direct oxidative stress, and thus, it is thought to exert its toxicity in a different way from non-redox active substances, such as Zn and Cd, as demonstrated by a large-scale expression experiment on *Saccharomyces cerevisiae* (Thorsen et al. 2009).

Moreover, the mutants showed a significant reduction in the number of conidia per surface unit on control medium. A similar result was obtained in a study on the capacity for conidiation of *Neurospora crassa* SOD1-null mutants (Belozerskaya and Gessler 2006). In the same fungus, SOD were shown to be required for the germination of conidia and for a long lifespan (Munkres 1992).

Table 1. Plant-growth parameters and percentages of root colonization of *Laccinium mytilus* seedlings mycorrhized with the wild-type strain *Oidiodendron maius* Zn and three selected superoxide dismutase 1-null mutants^a

Parameters	Wild-type	A4 57	D5 125	D5 83
Shoot length (cm)	8.54 ± 1.44	6.34 ± 2.30	5.94 ± 1.61	6.44 ± 2.68
Shoot fresh weight (g)	0.058 ± 0.014	0.051 ± 0.021	0.043 ± 0.011	0.047 ± 0.032
Shoot dry weight (g)	0.016 ± 0.004	0.014 ± 0.007	0.012 ± 0.003	0.014 ± 0.009
Root length (cm)	8.64 ± 1.99	7.30 ± 1.96	6.76 ± 1.51	6.20 ± 1.66
Root fresh weight (g)	0.012 ± 0.004	0.011 ± 0.006	0.012 ± 0.003	0.012 ± 0.008
Total leaf area (cm ²)	4.99 ± 1.17	4.20 ± 1.66	3.33 ± 0.66	3.71 ± 2.29
Internodal length (cm)	0.34 ± 0.05	0.26 ± 0.04*	0.23 ± 0.04*	0.27 ± 0.06*
% mycorrhization after 1.5 months	4.67 ± 0.32	1.45 ± 0.45*	2.93 ± 0.17*	2.87 ± 0.69*
% mycorrhization after 3 months	6.71 ± 0.84	4.59 ± 1.06*	4.34 ± 1.41*	4.32 ± 1.13*

^a The results are expressed as the mean ± standard deviation. All the plant growth parameters were recorded after 3 months. Asterisks indicate statistically significant differences ($P < 0.05$) with the wild type.

Martino and colleagues (2002) have previously demonstrated that *O. maius*, as other eukaryotes, also features a manganese (Mn)-SOD (OmSOD2). Mn-SOD characterized in other fungi are generally located in mitochondria, while the Cu,Zn-SOD usually occur in the cytosol. Although both enzymes catalyze the disproportionation of superoxide to oxygen and hydrogen peroxide, their location in different cellular compartments has been shown to result in independent and unique roles in *Cryptococcus neoformans* var. *gattii* (Narasipura et al. 2005) and in *Saccharomyces cerevisiae* (Dziadkowiec et al. 2007). The increased sensitivity of the SOD1-null mutants to all the tested substances in respect to the wild type supports the hypothesis that multiple Mn-SOD and Cu,Zn-SOD have distinct roles in protecting cells from damaging reactions caused by ROS, which are presumably dismutated in the compartment in which they are generated. Therefore, although OmSOD2 is active in the mutants (data not shown), it cannot compensate for the lack of the OmSOD1. It is noticeable that strong staining by NBT was observed in the mutants with no appreciable differences between control and stressed hyphae. This result supports the idea that, although viable in atmospheric oxygen, SOD1-null mutants exhibit markers of oxidative stress even during normal cellular growth (Pereira et al. 2003; Reddi et al. 2009), and the NBT staining cannot distinguish those caused by external superimposed stress.

SOD1 and mycorrhization.

SOD are shown to be important not only as antioxidant enzymes, but they also play an additional role in fungal morphogenesis and in both the pathogenic and symbiotic interactions between fungi and other organisms (Scott and Eaton 2008). ROS are strongly implicated in oxidative damage but, paradoxically, small amounts of these reactive species have proven to be involved in important physiological functions, such as signal transduction associated with the control of gene expression and cell proliferation (Brar et al. 1999).

Once fungal pathogens have infected a host, they inevitably encounter ROS produced by host cells as well as ROS produced as a consequence of their own oxygen metabolism. During the oxidative burst, SOD play

a protective role by suppressing oxidative killing by the infected host. This up-regulation of fungal ROS-scavenger machinery during the early stages of the pathogenic interaction has been demonstrated in *Candida albicans* (Hwang et al. 2002), *Cryptococcus neoformans* (Cox et al. 2003) and *Colletotrichum acutatum* (Brown et al. 2008).

A host oxidative burst-like defense response, commonly observed when the plant is confronted with a pathogen, was also detected in compatible mycorrhizal associations (Garcia-Garrido and Ocampo 2002). Thus, even during symbiosis, a dialogue based on ROS modulation occurs between the fungus and its host plant. Tanaka and colleagues described a role for NADPH oxidase-generated ROS in regulating and maintaining the mutualistic interaction between a clavicipitaceous fungal endophyte, *Epichloe festucae*, and its grass host, *Lolium perenne* (Tanaka et al. 2006). This dialogue is essentially based on the superoxide anion that is rapidly converted to hydrogen peroxide either by spontaneous dismutation and/or by the catalytic activity of SOD.

Treatment of suspended cells of *Picea abies* (Norway spruce) with elicitors released from the ectomycorrhizal fungi *Amanita muscaria* and *Hebeloma crustuliniforme* suggested a rapid but transient production of ROS, mainly H₂O₂ (Salzer et al. 1996). Baptista and colleagues (2007) evaluated ROS production and the activities of plant SOD and catalase during the

early contact of the ectomycorrhizal fungus *Pisolithus tinctorius* with the roots of *Castanea sativa*. In AM associations, a stimulation of fungal respiratory activity and the concomitant induction of ROS-scavenging enzymes have also been reported (Fester and Hause 2005; Lanfranco et al. 2005; Tamasloukht et al. 2003; Salzer et al. 1999). In particular, an increased expression of *SOD1* was registered in *Glomus intraradices* during the root exudates perception by the fungus (Seddas et al. 2009).

The ability of both pathogenic and symbiotic fungi to maintain this redox dialogue with host plants is based on various ROS-scavenging systems, including SOD, catalases, peroxidases, glutathione peroxidases, and peroxiredoxins that, collectively, have an important role in ensuring a rapid turnover of the ROS homeostasis and fine-tuning the ROS-dependent communication between fungi and plants (Scott and Eaton 2008). How ROS signal within the cell and trigger differentiation processes still remains a key question.

The significant decrease in the percentage of mycorrhization in *SOD1*-null mutants observed in this work provides further evidence that *SOD1* is involved in the fungal morphogenetic responses to the symbiotic partner. In our case, the lack of the *SOD1* protein is proposed to cause an imbalance in the redox homeostasis during host colonization and an alteration in the delicate dialogue between *O. maius* and its host plant.

Summarizing, the most important technical advance in our study was the first successful disruption of a gene by homologous recombination in a mycorrhizal fungus. Besides assessing the percentage of homologous recombination in *O. maius* and characterizing the phenotype of *SOD1*-null mutants, we have also provided further insights into the role of *SOD1* as an important enzyme in the relationship between *O. maius* and its symbiotic partner.

MATERIALS AND METHODS

Fungal isolate and growth media.

The fungal isolate investigated in this study is *O. maius* Zn, deposited in the Mycotheca Universitatis Taurinensis, (Tourino, Italy) collection at the Department of Plant Biology, University of Turin (CLM1381.98). This strain was isolated from the Niepolomice Forest (25 km northeast of Krakow, Poland) from the roots of *V. myrtillus* growing in experimental plots treated with 5,000 tons km⁻² of dust containing high concentrations of Zn, Cd, and aluminium derived from industrial electro-filters. The characteristics of the site and the identification of this fungal isolate are described in more detail by Martino and associates (2000).

Fungi were grown in Czapek mineral medium supplemented with 2% (wt/vol) glucose (Sigma, St. Louis). The mineral medium contained NaNO₃ (3 g liter⁻¹), K₂HPO₄·3 H₂O (1.31 g liter⁻¹), MgSO₄·7 H₂O (0.5 g liter⁻¹), KCl (0.5 g liter⁻¹), FeSO₄·7 H₂O (0.01 g liter⁻¹). When required, the medium was supplemented with either Zn²⁺ (as ZnSO₄·7 H₂O, Fluka [St. Louis], 99% purity), Cd²⁺ (as CdSO₄·8 H₂O, Sigma, 98% purity) or menadione sodium bisulfite (95%, Sigma). The pH of solid media was adjusted to 4, using 1 M HCl, while liquid media were buffered at the same pH, using 20 mM MES (2-[N-morpholino] ethane sulphonic acid) before autoclaving.

Construction of the *OmSOD1* disruption cassette.

The *OmSOD1* disruption cassette was designed as a gene replacement vector for the removal of a 621-bp fragment of the *OmSOD1* gene (GenBank accession number EU386164; Vallino et al. 2009), from 136 bp upstream to 485 bp downstream of the start codon (Fig. 1A). The *OmSOD1* disruption cassette carried the 786-bp 5' *SOD1* flanking region (from 135 to 921 bp upstream of the start codon) and the 820-bp 3' *SOD1* flanking region (from 486 to 1,324 bp downstream of the start codon) amplified from genomic *O. maius* Zn DNA, separated by the hygromycin-resistance expression cassette containing the *A. nidulans* *gpdA* promoter, the *Escherichia coli* *hph* gene, and the *A. nidulans* *trpC* gene terminator region derived from the pAN7-1 plasmid (Punt et al. 1987). Four PCR reactions were set up—three primary reactions to generate the three parts of the disruption cassette and a secondary reaction intended to fuse the three fragments into a single 5,313-bp-long amplicon—according to the protocol described by Kuwayama and associates (2002). The three primary PCR reactions were carried out in a final volume of 50 µl containing 200 µM of each dNTP, 5 µM of each primer, 5 µl of 5× Phusion HF Buffer, and 0.5 units of Phusion high-fidelity DNA polymerase (Finnzymes, Espoo, Finland). The PCR program was as follows: 30 s at 98°C for 1 cycle, 10 s at 98°C, 45 s at 60°C, 45 s and 30 s (for each kbp) at 72°C for 35 cycles, 10 min at 72°C for 1 cycle. The 5' flanking region of *OmSOD1* was amplified with primers 1 and 2 and the 3' region with primers 3 and 4 (Table 2). The marker cassette DNA fragment was amplified with primer 5, complementary to primer 2, and primer 6, complementary to primer 3 (Table 2). During the fusion PCR, the 5' and 3' flanking regions were joined to the hygromycin cassette, and the final PCR product was amplified with the *Hind*III-tailed primers 1 and 4. The fusion PCR reaction was carried out using 30 ng of the purified 5' and 3' PCR products and 90 ng of the purified hygromycin cassette, following the same protocol previously described for the amplification of the three separate parts. Construction of the disruption cassette was confirmed by DNA sequencing.

AMT.

To create the pΔ*SOD1* gene disruption construct, the *Hind*III-tailed *OmSOD1* disruption cassette was cloned into the *Hind*III-digested pCAMBIA0380 (Cambia, Canberra, Australia) between the T-DNA borders. The vector was introduced into *Agrobacterium tumefaciens* LBA1100, which carries the virulence genes essential for T-DNA transfer. This *Agrobacterium tumefaciens* strain was used to transform the

ungerminated co- nidia of *O. maius* Zn according to the protocol described by Martino and associates (2007). The only modification to this protocol was that the pH of the induction medium was set at 5 instead of 6. A pilot transformation experiment demonstrated

Table 2. Primers for generating the *OmSOD1* disruption cassette by fusion polymerase chain reaction

Primer	Sequences
1 ^a	5'-GGACTTGGAGCAGACGGTGC-3'
2 ^b	5'-CTCCATGTTGGTAGTTGTGACAGGACAGGGATGTG <u>CGTGCCTGGAGTTTTGCC-3'</u>
3 ^b	5'-CAAGCCTACAGGACACACATTCATCGA <u>CCCCAGA</u> <u>GTGTTATTGGCGTAAGTCG-3'</u>
4 ^a	5'-CGAGCAOCTGGCTTAGAGGAGGC-3'
5	5'- <u>GGCAA</u> AACTCCAGGCACGCACATCCCTGTCCTGTC ACA <u>ACTACCA</u> ACATGGAG-3'
6	5'- <u>CGACTTACGCCAATAACACTCTGGGGG</u> TTCGATGAA TGTGTGTCCTGTAGGCTTG-3'

^a Primers 1 and 4 are complementary to the *OmSOD1* gene.

^b Primers 2 and 3 (and their reverse complementaries 5 and 6) are chimerical primers. The underlined part of each primer is complementary to the *OmSOD1* gene, while the other part is complementary to the region of the hygromycin phospho-transferase gene that is going to be fused with the *OmSOD1* gene.

that the number of transformants at pH 5 is almost 20-fold greater than at pH 6 (data not shown).

The transformants were isolated and transferred into 24-well plates with Czapek-Dox agar (1%, wt/vol) supplemented with hygromycin at 100 µg/ml.

PCR screening for SOD1-null mutants.

SOD1-null mutants were primarily identified by PCR. A small portion of each fungal colony was collected and boiled for 15 min in 20 µl of 10 mM Tris HCl, pH 8.2 and was vortexed for 1 min. Then, 2 µl were used directly for PCR amplification without any other purification. PCR amplification was carried out in a final volume of 20 µl, using REDTaq DNA polymerase (Sigma), following the manufacturer's protocol. We used two sets of primers: set 1, 5'-CGCCACCACAGCTC TAGAGT-3' and 5'-CACGGATAAGCTGGTCAAGC-3'; set 2,

5'-ATGCCTGAACTACCGCGAC-3' and 5'-GCAGTTCGG TTTCAGGCAGG-3'. The first set of primers was designed to amplify the DNA fragment to be replaced by the *hph* gene and removed from the disruptants.

The second was designed to amplify the *hph* gene. If the fungus was a nonhomologous transformant, an amplified product would be obtained with both sets of primers. If the transformant was a SOD1-null mutant, an amplified product would be obtained only with the second primer set.

Gene disruption by homologous recombination was further checked by PCR using specific primers (set 3) designed on two *OmSOD1* regions external to the targeting construct: forward, 5'-ACGTGGTAGGGGCTAGCAGA-3' and reverse, 5'-TGGTCTTGATATGGACAGTTC-3'. Amplification with these primers was diagnostic of a targeted gene replacement resulting from accurate homologous recombination at each end of the construct. In the wild-type strain and in the nonhomologous transformants, a 2,310-bp-long fragment was expected, while a 5,376-bp-long amplicon indicated the integration of the *OmSOD1* disruption cassette at the correct locus.

Southern blot.

The candidates for SOD1-null mutants were further confirmed by Southern hybridization analysis. Genomic DNA extracted from the PCR-selected SOD1-null mutants (5 µg), the wild type, and one ectopic transformant were digested with *Pst*I and were size-fractionated on a 1% (wt/vol) agarose gel. The separated restriction fragments were blotted onto nylon membrane following standard procedures (Sambrook and Russell 2001). Hybridization with a chemiluminescent detection system (ECL direct DNA labeling and detection system; GE Healthcare, Chalfont St. Giles, U.K.) was performed according to the manufacturer's recommendations. An *OmSOD1*-specific probe and a *hph*-specific probe were used to confirm the absence of a functional *OmSOD1* gene in the SOD1-null mutants and a single-copy integration of the disruption cassette in their genome.

Protein analysis.

Three randomly chosen SOD1-null mutants with a single-copy integration of the disruption cassette (D5_83, D5_125, and A4_57) and the wild type were also tested by Western blot, using a polyclonal rabbit anti-SOD1 primary antibody (Abcam plc, Cambridge). Proteins were extracted from 30-day-old mycelia grown in 10 mM Zn-amended liquid medium using 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 20 mM sodium phosphate buffer, pH 7.5, and 8% polyvinyl polypyrrolidone (wt/vol). Following protein quantification with a Bradford assay (Sigma), 650 ng were loaded on 12.5% acrylamide separating gels. Proteins were then electrotransferred for 50 min at 100 V onto Hybond-ECL nitrocellulose paper (GE Healthcare), and the membrane was hybridized overnight at 4°C with a 1:5,000 dilution of the primary antibody. A goat anti-rabbit alkaline phosphatase-conjugated antibody (Sigma) was then diluted 1:5,000 and was used as secondary antibody. Analytical gels were silver stained.

Determination of metal tolerance concentrations on agar medium.

To determine the concentrations of Zn, Cd, and menadione that cause a drastic decrease in radial growth as compared with the control, SOD1-null mutants and the wild type were grown in 9-cm (diameter) petri dishes containing the same mineral medium described above and different Zn (10 to 60 mM), Cd (0.05 to

0.6 mM), and menadione (0.02 to 1 mM) concentrations. Radial growth was recorded after 30 days of growth at 25°C in the dark. At least three replicates were used for each experimental condition.

Determination of the capacity of conidiation.

Conidia were harvested from 3-month agar cultures of both wild type and mutants by gently scraping cultures in 9-cm petri dishes flooded with 1 ml of sterilized distilled water. Conidia were counted in a Bürker counting chamber (Marienfeld, Germany). Harvest from each fungus was performed in triplicate, and the mean and standard error of the total number of conidia per surface unit (cm²) were reported.

NBT staining.

A total of 300 conidia of each fungus were inoculated in 12-well plates containing 2 ml of liquid medium in each well. After 20 days of growth at 25°C in the dark, 10 mM zinc, 0.05 mM cadmium, and 0.02 mM menadione were added into the wells. After 3 h of incubation, the medium was replaced by a 0.05% (wt/vol) NBT (Sigma) solution in 50 mM sodium phosphate buffer, pH 7.5 (Munkres 1990). After 3 h of staining in the dark, the small colonies were placed on microscope slides and were fixed with lactic acid for observation. The colonies were examined and photographed with a Primo Star (Zeiss, Oberkochen, Germany) compound microscope with a Moti-cam 2300 camera at ×1,000 magnification.

Mycorrhizal synthesis.

Endomycorrhizae were synthesized aseptically using a nutrient agar culture system (Villarreal-Ruiz et al. 2004). Pot experiments with sterile substrates were avoided due to technical problems encountered in previous experiments (e.g., high risk of contaminations, difficulty in determining fungal growth on the peat substrate). Plastic petri plates (15 cm in diameter, 2.5 cm in height) containing autoclaved modified Ingestad's solution (MISAG) (Ingestad 1971) were prepared as follows. Solution B contained NH₄NO₃ at 1.062 g liter⁻¹, KNO₃ at 0.372 g liter⁻¹, KH₂PO₄ at 0.286 g liter⁻¹, K₂SO₄ at 0.222 g liter⁻¹. Solution C (stock solution) contained HNO₃ at 1.6 g liter⁻¹, Ca(NO₃)₂ at 14.3 g liter⁻¹, Mg(NO₃)₂ at 26.0 g liter⁻¹, Fe₂(SO₄)₃ at 2.50 g liter⁻¹, MnSO₄ at 0.55 g liter⁻¹, H₃BO₃ at 0.57 g liter⁻¹, CuCl₂ at 0.032 g liter⁻¹, ZnSO₄ at 0.036 g liter⁻¹, Na₂MoO₄ at 0.007 g liter⁻¹. Solution B was mixed with solution C (diluted 1:100) in equal amounts. After the addition of 0.1% glucose, the pH was brought to 4.8 with KOH, and the medium was finally supplemented with 1% agar. Axenic *V. myrtillus* seedlings were obtained from surface-sterilized (70% ethanol, vol/vol, plus 0.2% Tween 20 for 3 min; rinsed twice with sterile water and 0.25% sodium hypochlorite for 15 min, with three additional sterile water rinses) bilberry seeds germinated on water and 1% agar petri plates in the dark for 2 weeks before transfer to the growth chamber for 1 month. Four mycelium plugs (5 mm in diameter) were removed from the leading edges of a 30-day-old fungal colony and were placed in the bottom half of a MISAG petri plate at a distance of 2 cm from one another. Three germinated *V. myrtillus* seed-

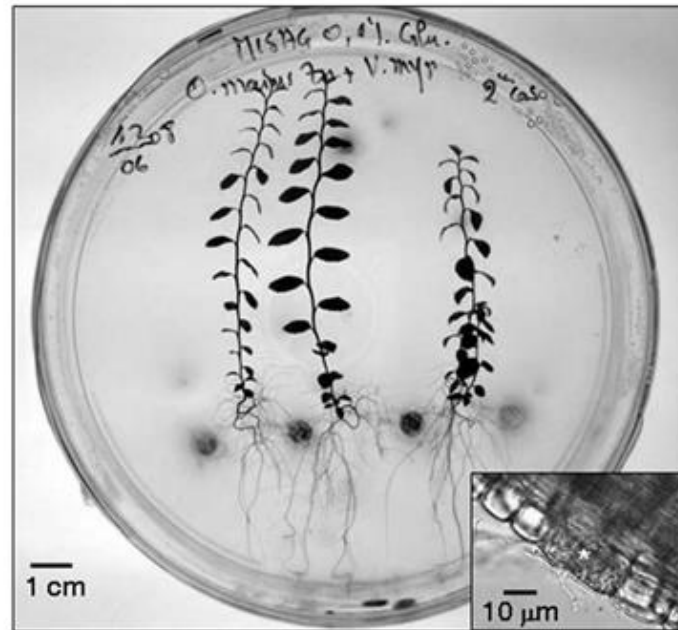


Fig. 4. In vitro mycorrhization system involving *Vaccinium myrtillus* seedlings and *Oidiodendron maius*. After a month of pregrowth in 9-cm petri dishes, three axenic germinated *V. myrtillus* seedlings were aseptically transferred in a 15-cm petri plate near four mycelium plugs. The root systems were observed after 1.5 and 3 months of incubation in a growth chamber (photoperiod 16 h, light at $170 \mu\text{mol m}^{-2} \text{s}^{-1}$, and 23°C day and 21°C night temperatures). *O. maius* has formed a cotton blue-stained coil (asterisk) in an epidermal cell (inset).

lings were then transferred aseptically in the MISAG plates near the mycelium plugs (1 cm distance between the plugs and the root system) (Fig. 4). Finally the plates were sealed and placed in a growth chamber (16-h photoperiod, light at $170 \mu\text{mol m}^{-2} \text{s}^{-1}$, temperatures at 23°C day and 21°C night).

Two independent experiments were set up. In the first, three *Vaccinium* seedlings were used for each fungus, and the root systems were observed after a 1.5-month incubation. In the second, three petri dishes, each containing three seedlings (for a total of nine replicates), were used for each fungus.

Plant growth parameters and degree of mycorrhization.

Plant growth parameters and the percentage of mycorrhization were recorded after 3 months to determine differences in root colonization between *O. maius* Zn wild-type and the SOD1-null mutants. Length (cm) and fresh weight (g) of each stem and root system were measured, and then, each stem was inserted between two glass plates and scanned. Lucia G imaging software (version 4.82, Nikon, Tokyo) was used to evaluate the total leaf surface (cm^2) and the internodal distance (cm). Finally, stems were dried at 70°C for 2 days and were weighed. Each *Vaccinium* hair-root system from the synthesis plates was cleared for 5 min with 10% (wt/vol) KOH in a water bath at 90°C , acidified with 0.1 M hydrochloric acid for 1 h, stained in 0.1 % (wt/vol) cotton blue for 15 min at 90°C , and de-stained overnight with 80% lactic acid (Fig. 4, inset). Whole roots were mounted in the destaining solution, observed using a Nikon Eclipse E400 optical microscope, and photographed. The magnified intersections method (Villarreal-Ruiz et al. 2004) was adapted to quantify the percentage of infection of *Vaccinium* hairy roots. For each fungus, the root system of *Vaccinium* seedlings was examined under the microscope using the rectangle around the cross-hair as intersection area at $200\times$ magnification. A total of 100 intersections per seedling root system were scored. Counts were recorded as percentage of root colonized (RC) by the fungus using the formula: $\text{RC}\% =$

$100 \times \Sigma$ of coils counted for all the intersections, where Σ is the number of epidermal cells for all the intersections.

Statistical analysis.

The angular transformation was applied to the mycorrhization percentages. Statistical tests were all carried out through one-way ANOVA and Tukey's post hoc test, using a probability level of $P < 0.05$.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

The *Tuber melanosporum* database:

mycor.nancy.inra.fr/IMGC/TuberGenome

Department of Energy Joint Genome Initiative *Glomus intraradices* page:

www.jgi.doe.gov/sequencing/statusreporter/psr.php?projectid=1606