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(Article begins on next page)



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Cu,Zn superoxide dismutase and zinc stress in the metal-tolerant ericoid mycorrhizal fungus *Oidiodendron maius* Zn.

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Abstract

The sequence encoding a superoxide dismutase (SOD) was isolated from the cDNA library of a zinc-tolerant strain of the ericoid mycorrhizal fungus *Oidiodendron maius*, grown under zinc-stress conditions. Sequence homology to other SODs strongly suggests that it is a copper- and zinc-containing SOD. Functional complementation assays showed that the gene confers increased tolerance to zinc and copper stress to a Cu,ZnSOD-defective yeast mutant. Monitoring of transcript and protein levels following zinc stress suggests that OmSOD1 expression is controlled at the transcriptional level. The OmSod1 protein was found both in the cell extract and in the growth medium of viable fungal cultures. This is the first characterization of an extracellular Cu,ZnSOD in a mycorrhizal fungus. In nature, the presence of OmSod1 in the extracellular environment may also extend the protective role of this enzyme to the plant symbiont. This may be of particular interest from the perspective of using mycorrhizal fungi in bioremediation programmes.

Introduction

Superoxide dismutase (SOD) enzymes catalyze the breakdown of superoxide into hydrogen peroxide and water. They are central regulators of reactive oxygen species (ROS) levels (Fridovich, 1995). SODs have been categorized into different families on the basis of metal cofactors at the active site (Cu,ZnSODs, FeSODs, MnSODs, Fe,MnSODs, and NiSODs) and their evolutionary homology (Miller, 2004). Cu,ZnSOD is found almost exclusively in eukaryotic species and can be localized in the cytosol, in the outer mitochondrial space (Sturtz *et al.*, 2001) and, for plant cells, in the chloroplast. In addition, extracellular Cu,ZnSODs with distinctive features (EC-SOD) have been found in some mammalian species (Fattman *et al.*, 2003). SODs play a major role in the defense against toxic-reduced oxygen species, which are generated

as byproducts of many biological oxidations. The generation of ROS can be further increased due to environmental stress, including heavy metals such as zinc (Schützendübel & Polle, 2002).

Zinc is one of the most important trace elements in living organisms and is essential to the growth and development of microorganisms, plants, and animals. Nevertheless, zinc is potentially toxic at elevated levels, and most cells must maintain cellular zinc content within a very narrow window. Zinc-binding proteins are protective in situations of exposure to toxic metals. Cu,ZnSOD is one of the major proteins involved in the cellular response to elevated levels of zinc ions and its protective effects against zinc toxicity may involve both its metal-binding capacity and its enzymatic activity (Wei *et al.*, 2001).

If the involvement of SODs in heavy metals toxicity has been described by several authors in plants, animals and microorganisms (Chongpraditnun *et al.*, 1992; Yoo *et al.*, 1999; Vido *et al.*, 2001), information about filamentous fungi is scarce. However, SODs of filamentous fungi were reviewed by Natvig *et al.* (1996) and Frealle *et al.* (2005) and some research on their response to metal and oxidative stress has been carried out (Jacob *et al.*, 2001; Angelova *et al.*, 2005; Lanfranco *et al.*, 2005; Azevedo *et al.*, 2007).

Mycorrhizal fungi are ubiquitous soil microorganisms that associate with the roots of almost all land plants and play an important ecological role as plant health promoters (Smith & Read, 1997). Ericoid mycorrhizal (EM) fungi colonize the epidermal root cells of ericaceous plants to form a distinctive type of endomycorrhiza (Perotto *et al.*, 1995). EM plants colonize low mineral, acidic organic soils that can be high in toxic metals (Read, 1991) and a crucial role in plant survival has been ascribed to the presence of the mycorrhizal endophyte (Sharpley *et al.*, 2000). The mechanisms that allow EM fungi to tolerate toxic concentrations of heavy metals and to protect their host plants are poorly understood. To investigate these aspects, we have chosen as a model organism the metal-tolerant strain *Oidiodendron maius* Zn, an ascomycete that was originally isolated from soils heavily contaminated by industrial dusts (Martino *et al.*, 2000). Preliminary investigations showed that high concentrations of Zn in the growth medium could induce in *O. maius* Zn the production of extracellular SODs (Martino *et al.*, 2002). In the context of a symbiotic system, extracellular SOD

enzymes could play an important role in the defense of both partners against the oxidative stress generated by the presence of potentially toxic metal ions.

In this work, we have isolated the gene coding for a Cu,ZnSOD in *O. maius* Zn, investigated SOD responsiveness to Zn exposure at both gene and protein level, and carried out a functional analysis in a heterologous system.

Materials and methods

Fungal and yeast strains and growth media

Oidiodendron maius strain Zn was isolated in the Niepolomice Forest (Krakow, Poland) from the roots of *Vaccinium myrtillus* growing in experimental plots treated with metal-containing dusts (Turnau, 1988; Martino *et al.*, 2000). The fungus was grown in Czapek-pectin liquid medium as reported in Martino *et al.* (2002). Zinc (as ZnSO₄) was added at 5 or 10 mM concentration.

The Δ sod1 *Saccharomyces cerevisiae* mutant (yeast haploid deletion mutant collection – Open Biosystems, DTY116- Δ SOD1) has the following genotype: *MAT α , trp1-1::SOD1deletion::TRP1 leu2-3,-112 gal1 ura3-50 his-CUP1s*. The mutant was grown on rich YPD medium.

Evaluation of cell viability

The LIVE/DEAD BacLight Bacterial Viability Kit (L-7012, Molecular Probes) was used for the evaluation of mycelia viability in a time-course experiment. Clumps of fungal mycelium, grown in liquid medium in the absence or in the presence of 10 mM zinc ions, were carefully picked after 13, 19 and 25 days of growth, washed in phosphate-buffered saline (0.13 M NaCl in 10 mM Na₂HPO₄, pH 7.2) and stained, following the manufacturer's instructions. Samples were observed immediately under blue light using a Leica TCS SP2 confocal microscope. To assess autofluorescence, the mycelium was observed without previous staining. Heat-killed mycelium (boiled for 20 min) was also included as control.

RNA and DNA preparation

Mycelia RNA extraction was performed on two independent biological samples as described in Vallino *et al.* (2005). Before expression experiments, RNA was treated with RNase-free DNase (Invitrogen) and checked for DNA contamination using reverse transcriptase (RT)-PCR reactions as described in Lanfranco *et al.* (2002).

Genomic DNA was recovered from the supernatant obtained after LiCl precipitation in the RNA preparation. Four aliquots each of 5 µg of the genomic DNA were digested with *SacI*, *XhoI*, *HincII* and *ApoI*, size fractionated on a 1% agarose gel and blotted onto nylon membrane according to Sambrook *et al.* (1989). The Southern blot was hybridized using the same probe and the same detection system described for the DNA library screening.

Screening of the genomic library

In all, 40 000 plaques of a Lambda-DASH phage *O. maius* Zn genomic library (L. Bardi *et al.*, unpublished data) were blotted on a Hybond-N⁺ membrane (Amersham), according to Sambrook *et al.* (1989). The primary positive areas were subjected to a second and a third round of screening to isolate single positive plaques, used for phage DNA extraction with the Qiagen Lambda Mini Kit. Probe to detect *OmSOD1* gene was produced by PCR amplification on *O. maius* Zn genomic DNA using standard PCR conditions and the primers AH9F (5'-GTACCGTCACCTTCGAGCAG-3') and AH9R (5'-GGCAACGATGAGTCCCTAAAG-3'). The ECL Direct Nucleic Acid Labeling and Detection System (Amersham) was used for probe labelling and signal detection.

Phylogenetic analysis

The GenBank blastx search tool (Altschul *et al.*, 1997) was used to determine protein sequence similarities, and clustalx software was used for multiple sequence alignments (Thompson *et al.*, 1997). The mega 3.1 program (Kumar *et al.*, 2004) was used to exclude regions with ambiguous alignment and to perform the phylogenetic analysis using a distance method. The distance matrix and the neighbor-joining (NJ) tree were based on the Kimura's two-parameter model. Gaps were treated as missing data. Branch robustness was estimated by bootstrap (BP) analyses of 100.

Sequencing and sequence analysis

Phage DNA containing the *OmSOD1* gene was initially sequenced using primers AH9F and AH9R and then by primer-walking using new specific oligonucleotides. Sequences editing was performed using the program sequencher version 4.1.4 (Gene Codes Corporation).

The signalp 3.0 program (Bendtsen *et al.*, 2004) was used to search for signal peptides. Transcription factor binding sites were searched with the Transcription Element Search System (TESS) (Schug & Overton, 1998).

The genomic sequence has been deposited at the National Centre for Biotechnology Information (NCBI) GenBank (<http://www.ncbi.nlm.nih.gov>) under accession number EU386164.

Liquid isoelectric focusing, protein blotting and sequencing

Using a rotary evaporator system, culture filtrates from 25-day-old fungal cultures were concentrated fivefold and dialysed. A 50-mL aliquot was subjected to liquid-phase preparative IEF in a Bio-Rad Rotofor system (4 h, 12 W, 4% ampholines, pH 3–10). After measuring the pH, an aliquot of 200 µL for each rotofor fraction was used for protein precipitation, according to Perotto *et al.* (1998), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separation and blotting on a polyvinylidene difluoride (Bio-Rad) membrane as described in Martino *et al.* (2002). After staining with 0.1% Coomassie Blue, protein bands were cut from the membrane and analyzed using an automatic LF 3000 Protein Sequencer (Beckman). Protein sequences were analyzed through the blast (NCBI –Altschul *et al.*, 1997) and thefasta (Pearson & Lipman, 1988) searching program.

Protein extraction, immunoblotting and activity assay

Culture filtrates from the 25-day-old fungal cultures (40-mL aliquots) were also used for extracellular protein precipitation with 85% ammonium sulfate, and intracellular proteins were extracted from the corresponding mycelia using sodium phosphate 20 mM pH 7.5, polyvinylpyrrolidone 8%, phenyl methyl sulfonyl fluoride 2 mM, EDTA 1 mM.

Protein profiles obtained using SDS-PAGE (10% polyacrylamide, following Laemmli, 1970) were revealed by silver staining. Alternatively, proteins were blotted (45 min, 100 V) onto nitrocellulose paper (Amersham). An anti-Cu,ZnSOD rabbit antibody (EnVirtue Biotechnologies) was used. SOD activity was measured according to Krishnan *et al.* (2002). The program one-way anova (Tukey *post hoc* test) has been used for the statistical analysis.

Real-time RT-PCR analysis

cDNAs were obtained from 1.5 µg of total RNA using 200 U of SuperScript II RT (Invitrogen) and 250 ng random primers (Invitrogen). Real-time reactions were carried out in a final volume of 20 µL containing 1 × iQ SYBR Green Supermix (Bio-Rad), 0.3 µM of each oligonucleotide and 15 ng of cDNAs. The primers used were OmEff1 (5'-TGGGCGGTGTGGCAATCGAG-3') and OmEfr1 (5'-TCGTGGCAACGTCGCTGGTG-3') for *O. maius* Zn elongation factor (EF) gene (accession number CN200254) or AH9F and RealSOD (5'-GCCGTGTGGGTTGAAATGGG-3') for *OmSOD1*. The following program was run: 95 °C for 3 min (one cycle) and 95 °C for 15 s, 62 °C for 30 s (50 cycles) in an iCycler iQ™ real-time PCR detection system (Bio-Rad). Real-time PCR reactions were carried out in triplicate. The $\Delta\Delta C_t$ method was used to calculate relative *OmSOD1* expression level with the EF gene as a reference (Livak & Schmittgen, 2001).

Yeast complementation assays

The full-length *OmSOD1* cDNA sequence (accession number CN200173) was first amplified under standard PCR conditions using the NotI site-containing primers NOTSODF (5'-ATTGCGGCCGCAAGATGGTCAAGGCAGTTGC-3') and NOTSODR (5'-CGAGCGGCCGCCCATCTATGCAGCAATGCC-3'). The resulting product was digested with NotI and cloned into the dephosphorylated NotI site of the yeast expression vector pFL61 (Minet *et al.*, 1992). The pFL61-*OmSOD1* construct or the empty pFL61 vector were then transformed (Rose *et al.*, 1990) into a chemically competent $\Delta sod1$ *S. cerevisiae* mutant. Transformants were grown at 30 °C for 2–3 days on selective (minus uracil) synthetic dextrose (SD)-agar medium and then inoculated in liquid (minus uracil) SD medium. When the cells reached an OD_{600 nm} of 0.3 they were plated in triplicate on SD-agar plates containing or not ZnSO₄ (5 or 10 mM), CuSO₄ (25 or 100 µM), CdSO₄ (100 µM).

Results

Evaluation of fungal mycelium viability

Although *O. maius* Zn has been reported as a metal-tolerant fungal strain (Martino *et al.*, 2000), the viability of the mycelium exposed to metal ions has not been previously assessed. Figure 1 shows the fluorescent confocal images obtained in time course experiments, where mycelia grown in the presence or absence of 10 mM zinc ions were stained with the LIVE/DEAD BacLight Viability kit and

observed after 13, 19 and 25 days of growth. The proportion of green and red areas looks comparable in the control and in the treated samples, suggesting that mycelium viability is not affected by the presence of this high amount of zinc ions, at least in the conditions tested. Heat-killed mycelium uniformly fluoresced red (not shown) confirming that the red fluorescence is an indicator of cell death.

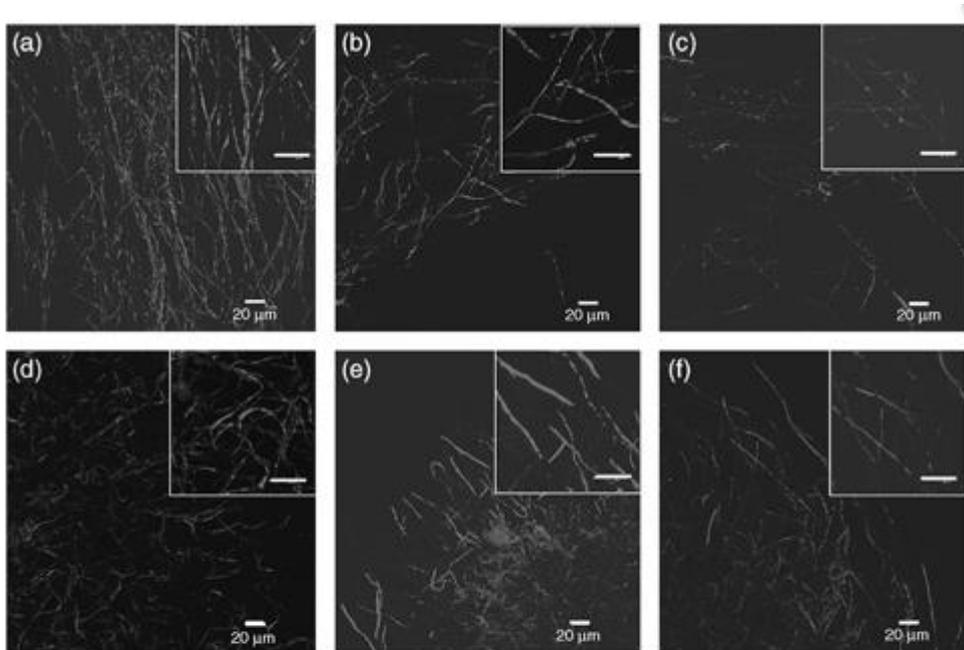


Figure 1. Confocal fluorescent images of *Oidiiodendron maius* Zn after staining with the LIVE/DEAD BacLight Bacterial Viability Kit. Mycelia were observed after 13 days (a, d), 19 days (b, e) and 25 days (c, f) days of growth in the absence (a, b, c) and in the presence (d–f) of 10 mM zinc ions. Green or red fluorescence in nuclei and cytoplasm indicates viability or cell death, respectively. Magnification bars are 20 μ m.

Identification and sequence analysis of *OmSOD1*

A previous study on *O. maius* Zn gene expression in the presence of 10 mM Zn^{2+} yielded an expressed-sequence tag clone matching Cu,ZnSOD sequences in gene databases (AH9 clone, accession number CN200173; Vallino *et al.*, 2005). The corresponding full-length cDNA, named *OmSOD1*, is 589 bp long and contains a complete ORF of 462 bp, with 5' and 3' nontranslated regions (not shown). The fungal origin of *OmSOD1* was confirmed by positive PCR amplifications of *O. maius* Zn genomic DNA with AH9F/AH9R primers. *OmSOD1*cDNA codes for a 154-amino acid peptide with a predicted molecular weight (MW) of 15.9 kDa and pI 6.42. The amino acid residues known to be responsible for binding to copper and zinc ions, the arginine residue which is believed to be necessary to guide the superoxide anion to the active site and the two cysteine residues involved in the formation of a

disulfide bond, were found in the *OmSOD1* sequence (Steinman, 1980; Chary *et al.*, 1990). No evidence for a signal peptide in the N-terminal sequence was obtained.

Querying the deduced amino acid sequence of *OmSOD1* against the NCBI protein database, the closest similarity was found with Cu,ZnSOD sequences from other filamentous fungi, with an identity up to 84% and a similarity up to 93% (such as *Chaetomium thermophilum*, *Chaetomium globosum*, *Aspergillus fumigatus*, *Aspergillus niger*, *Humicola lutea*).

The deduced amino acid *OmSOD1* sequence was aligned with 38 intracellular and extracellular Cu,ZnSOD protein sequences from several species ranging from fungi to humans and a NJ tree was constructed (Fig. 2). Six clades supported by high bootstrap values were identified: one clade grouped the extracellular SODs (EC-SOD) and the five other clades grouped Cu,ZnSODs from mammals, fishes, insects, plants and fungi, respectively. As expected, *OmSod1* clustered in the fungal clade.

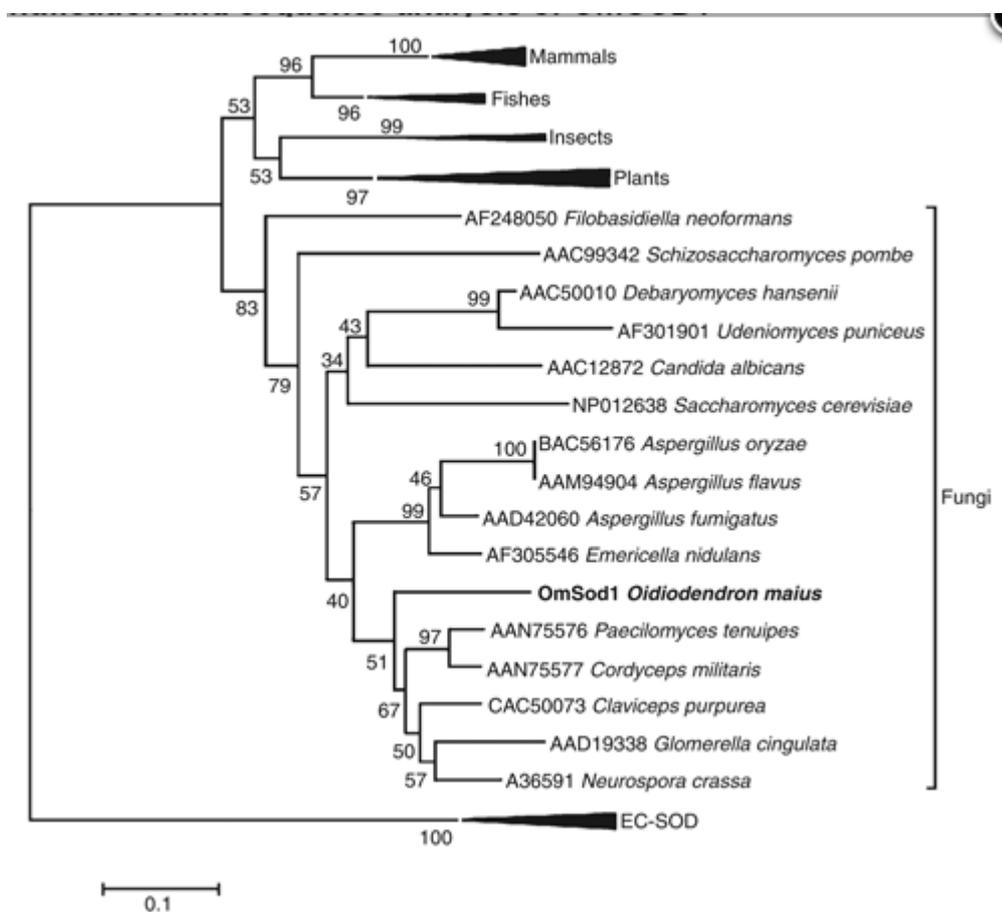


Figure 2. Phylogenetic analysis of OmSod1. NJ tree obtained from the alignment of *OmSOD1* gene product from *Oidiodendron maius* Zn (in bold) and Cu,ZnSODs and EC-SOD sequences of other organisms retrieved from database. (Fishes: AAO15363*Pagrus major*, O73872*Danio rerio*, AF469663*Oncorhynchus mykiss*. Mammals: BAC20347*Hylobates lar*, JQ0915*Mus musculus*, BAC20351*Cebus apella*, JC1192*Rattus norvegicus*, P00442*Bos taurus*, P00441*Homo sapiens*. Insects: P28755*Ceratitis capitata*, AF127156*Drosophila erecta*. Plants: JW0084*Glycine max*, AF061519*Arabidopsis thaliana*, CAC34448*Pinus sylvestris*, AAA33659*Pisum sativum*, CAA73929*Carica papaya*, BAA00799*Oryza sativa*. EC-SODs: CAA91785*Oryctolagus cuniculus*, AAB51106*Mus musculus*, NP_037012*Rattus norvegicus*, NP_003093*Homo sapiens*, NP_001076079*Bos taurus*). Bootstrap values are indicated (100 replicates). Branch lengths are proportional to genetic distance, which is indicated by a bar at the bottom left.

The sequence of the *OmSOD1* gene was obtained from an *O. maius* Zn genomic library. The comparison between genomic and cDNA sequences revealed that the *OmSOD1* gene contains four small introns. Southern blot analysis of genomic DNA indicated that a single gene encodes OmSod1 (not shown).

Biochemical characterization, sequencing, expression and activity of *O. maius* Zn Cu,ZnSOD

Residues 4–16 of the polypeptide encoded by *OmSOD1* matched exactly the N-terminus sequence of a Cu,ZnSOD protein, identified by microsequencing among other extracellular proteins specifically released by *O. maius* Zn in a zinc-containing medium (Martino *et al.*, 2002).

SDS-PAGE separation of rotofor fractions (pH 2–10) of *O. maius* Zn culture media revealed, for the zinc-treated samples, a general increase in the amount of secreted proteins, with a specific protein band of about 19 kDa in the basic fractions (pH 6–9.5) (Fig. 3).

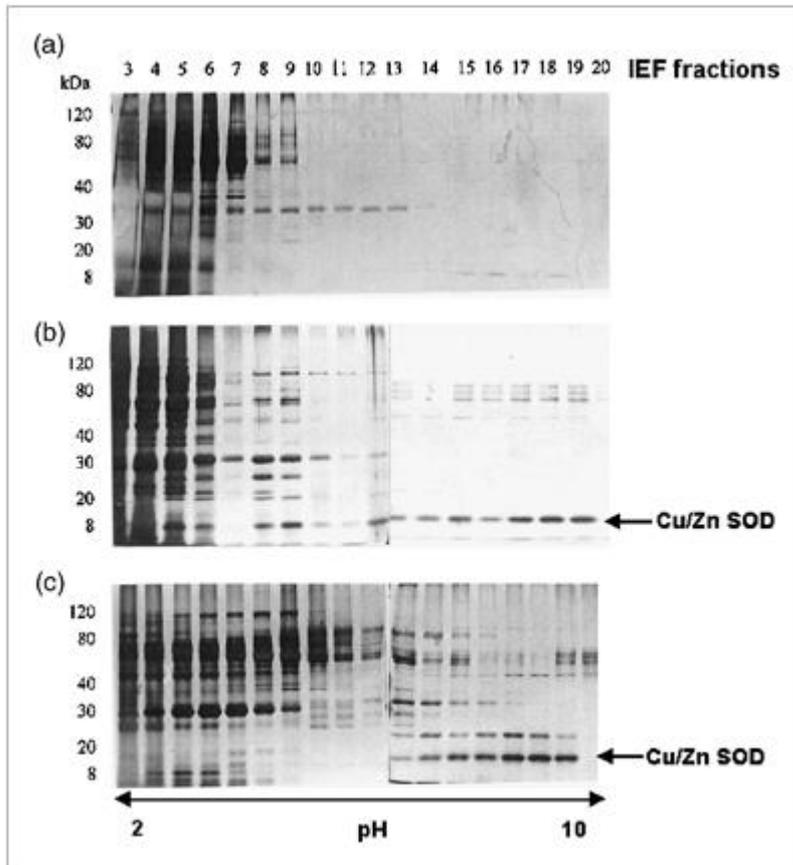


Figure 3.

SDS-PAGE separation of extracellular protein fractions obtained by liquid IEF (the pH of the IEF fractions ranged from 2 to 10): (a) control medium. (b) 5 mM zinc-added medium. (c) 10 mM zinc-added medium. New protein bands can be observed for the zinc-treated samples. Some prominent low-molecular-weight proteins fractionated in the basic pH range and among them a Cu,ZnSOD (arrow), identified through N-terminus sequence.

In immunoblot experiments, this extracellular protein band was positively labelled by an anti-Cu,ZnSOD antibody (Fig. 4a). Protein identity was confirmed through microsequencing, and the aminoacid sequence corresponded to that of the Cu,ZnSOD reported in Martino *et al.* (2002).

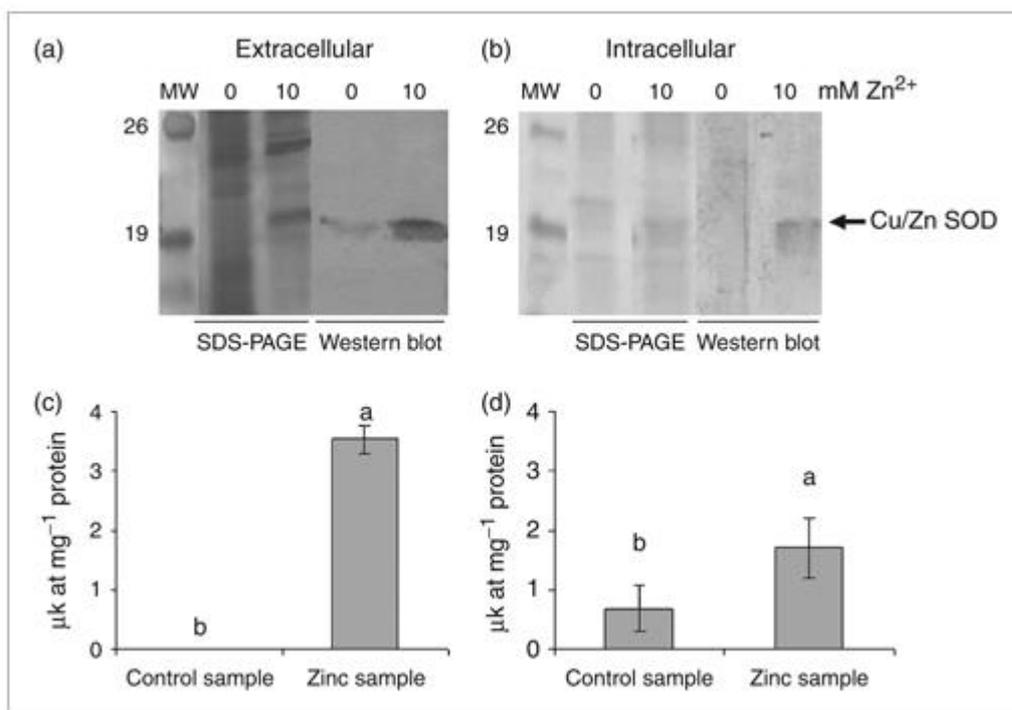


Figure 4.

SDS-PAGE and immunoblot analyses of (a) extracellular and (b) intracellular protein extracts after fungal growth in control and in 10 mM zinc-added medium. The protein band recognized by the Cu,ZnSOD antibody is indicated by an arrow. Ladders used are the broad molecular range, Bio-Rad, ranging from 203 to 7.4 kDa. (c) Extracellular and (d) intracellular SOD activity measured in the absence and in the presence of 10 mM zinc.

The same anti-Cu,ZnSOD antibody also revealed, in the samples exposed to zinc ions, the induction of a protein band of about 19 kDa in the intracellular protein extract (Fig. 4b). After 25 days of fungal culture, intracellular and extracellular total SOD activities were measured (Fig. 4c and d). When compared with the control samples, a significant ($P \leq 0.05$) 2.45-fold increase in the intracellular SOD activity was measured for the 10-mM zinc-treated samples. Similarly, extracellular SOD activity was very low and could not be detected in the control sample, whereas a strong enzyme activity was detected in the medium of the 10-mM zinc-treated sample.

***OmSOD1* gene expression**

A time course experiment of real-time RT-PCR was set up to investigate the level of *OmSOD1* transcripts in response to zinc. Specific primers were designed on *OmSOD1* and on *O. maius* EF gene, considered a housekeeping gene. cDNA

samples were prepared from fungal mycelia that were grown for the same number of days (25) but were treated with 10 mM Zn for 0, 4, 8 hours and 1, 2, 4, 7 and 10 days before harvesting. Samples were calibrated using the fungal EF transcript and the relative expression was calculated taking untreated mycelia as reference sample. As shown in Fig. 5, the expression of *OmSOD1* showed at first a rapid but moderate increase, followed by a more consistent rise.

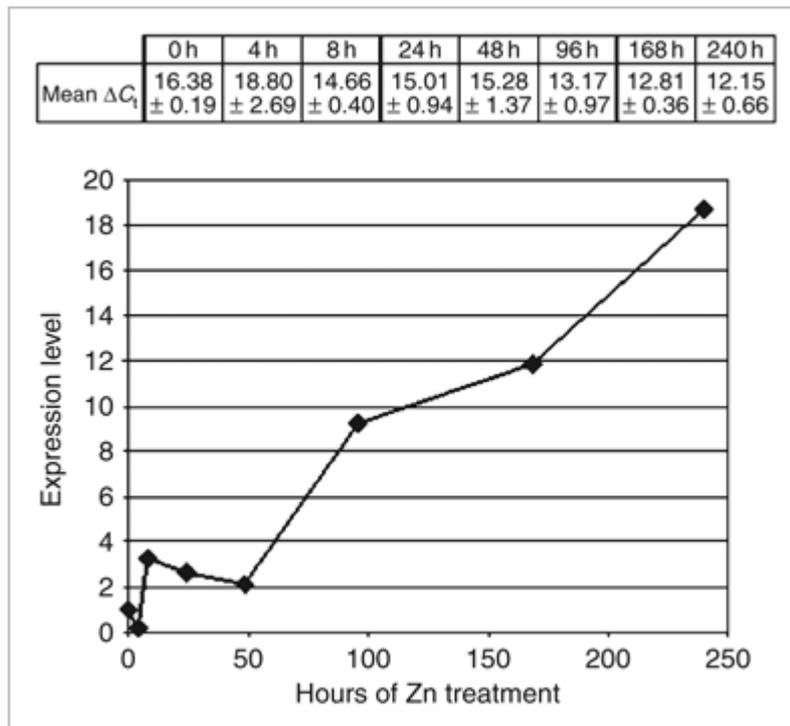


Figure 5.

Expression analysis of *OmSOD1*. Real-time RT-PCR analysis on *OmSOD1* mRNA from *Oidiodendron maius* Zn mycelia following zinc treatment. The fungus was grown for 25 days and treated with 10 mM zinc for 0–10 days. The ΔC_t mean values and the errors are indicated in the table (upper part) and the relative expression is shown in the graph (lower part). Relative expression levels were obtained with the $\Delta\Delta C_t$ method (see M Expression analysis of *OmSOD1*. Real-time RT-PCR analysis on *OmSOD1* mRNA from *Oidiodendron maius* Zn mycelia following zinc treatment. The fungus was grown for 25 days and treated with 10 mM zinc for 0–10 days. The ΔC_t mean values and the errors are indicated in the table (upper part) and the relative expression is shown in the graph (lower part). Relative expression levels were obtained with the $\Delta\Delta C_t$ method (see Materials and methods for details) and were normalized with respect to the untreated mycelium.aterials and methods for details) and were normalized with respect to the untreated mycelium.

Functional complementation assays

The yeast transformants, obtained as reported in Materials and methods, were plated onto SD-agar (-ura) plates containing or not Zn, Cu and Cd, and cell growth

was assayed (Fig. 6). Both types of transformants could grow on control and treated plates, but more colonies developed for *OmSOD1*-carrying cells than for cells carrying the vector alone in the presence of heavy metals. The difference in CFU numbers was quite high on the Zn- and Cu-containing medium (Fig. 6), whereas there was only a very slight increase on the Cd-containing medium (not shown).

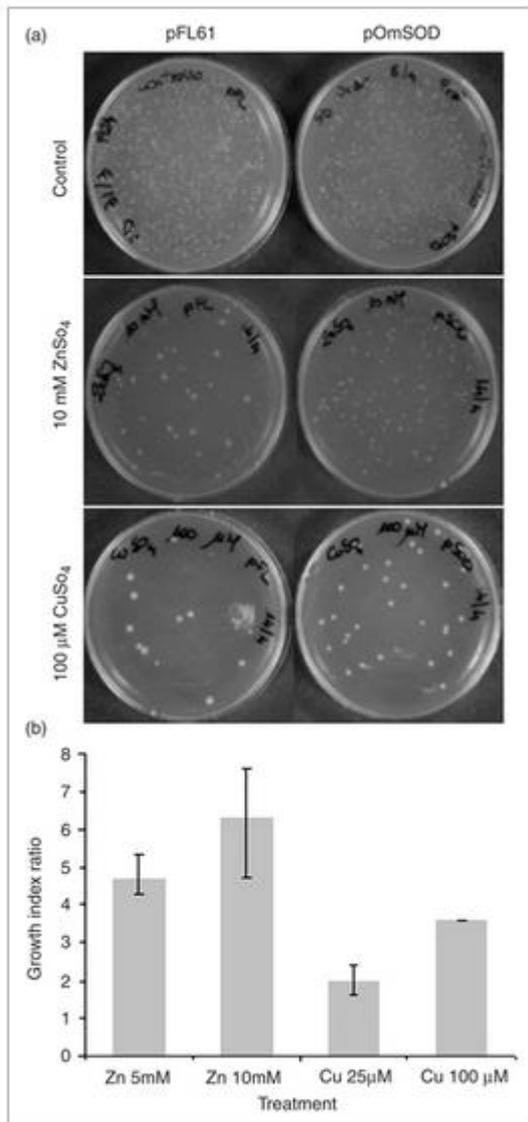


Figure 6.

Functional complementation assay of yeast Δ sod1 mutants with *OmSOD1*. (a) Yeast Δ sod1 mutants harboring either the empty vector (pFL61) or the pFL61-*OmSOD1* plasmid (pSOD) growing on SD (ura-) agar plates not amended (control) or amended with metal ions. (b) Growth performance of transformed *OmSOD1*-carrying yeasts plated on medium containing different heavy metals vs. vector-carrying yeasts. A growth index (no. of colonies on treated medium/no. of colonies on control medium) was calculated for *OmSOD1*-carrying yeast (GI_{SOD}) and for vector-carrying yeast (GI_{pFL61}). The growth index ratio was then obtained after normalization (GI_{SOD}/GI_{pFL61}).

Discussion

The EM species *O. maius* has been frequently found in symbiosis with host plants growing on soils naturally and industrially polluted by heavy metals (Lacourt *et al.*, 2000; Martino *et al.*, 2000). *Oidiodendron maius* strain Zn, in particular, exhibits a significant ability to grow at high Zn concentrations (Martino *et al.*, 2000). We found that treatments of *O. maius* Zn with high concentrations of zinc ions resulted in an increased amount and activity of both intracellular and extracellular SOD enzymes (Martino *et al.*, 2002; this study). *OmSOD1* was isolated from both cDNA (Vallino *et al.*, 2005) and genomic libraries of *O. maius* Zn. The deduced amino acid sequence revealed all the typical features of a Cu,ZnSOD protein, and the phylogenetic analysis showed that it clustered with other fungal Cu,ZnSOD proteins.

Cu,ZnSOD is known to occur as intracellular (cytosolic) and extracellular forms (EC-SOD). *OmSod1* shares typical features of cytosolic Cu,ZnSOD (e.g. the MW of 19 kDa, a value close to the predicted molecular mass of 15.9 kDa and conserved amino acids), and has no signal peptide. Nevertheless, protein expression and activity were consistently detected in the fungal culture filtrates. The deduced N-terminus sequence of *OmSOD1* matched exactly the protein sequences obtained for the extracellular enzyme, and the presence of a single-copy *OmSOD1* in the fungal genome supports the hypothesis that proteins induced by zinc and found both in the cell homogenate and in the culture medium are actually encoded by the same gene.

To test whether the presence of Cu,ZnSOD in the growth medium of zinc-treated samples resulted from cell lysis (caused by heavy metal stress) or from active release, we tested the viability of the fungal mycelium exposed to zinc ions using a fluorescent kit. The confocal images clearly showed that the extent of unviable fungal cells was low and comparable in control and treated samples. Thus, protein release in the growth medium as a result of cell lysis is unlikely.

The presence of noncanonical extracellular Cu,ZnSOD in culture filtrates and extracellular fluids has already been described in other organisms. *Aspergillus fumigatus* Cu,ZnSOD has no signal peptide, but it was also detected in culture filtrates (Holdom *et al.*, 1995, 2000). In extracellular washing fluid from Scots pine (*Pinus sylvestris* L.) needles, Steller & Wingsle (1994) found a Cu,ZnSOD, with features different from both the typical EC-SOD and the plant cytosolic and chloroplastic SOD (Karpinska *et al.*, 2001). There is evidence for mechanisms of protein export which do not involve the mediation of signal peptides (Binet *et al.*,

1997). We can thus speculate that OmSod1 may be one of these proteins, even though we have currently no clear clue about the export process.

Expression analyses by real-time RT-PCR showed that zinc regulates OmSod1 at the transcriptional level, and that transcripts accumulate over long-time exposure to the stress. A likely metal-responsive element (MRE) located at position -1788 (TGCGCTC) was found in the upstream sequence of *OmSOD1* gene. MRE sequences bind the transcriptional factor MTF-1, which is under the control of metals (such as Cu and Zn) and oxidative stress. MTF-1 also controls the expression of metallothioneins and a number of other genes directly involved in the intracellular sequestration and transport of zinc (Laity & Andrews, 2007). Although transcriptional induction of Cu,ZnSOD by metal ions has been described previously, zinc has been rarely investigated and, among fungi, exclusively in yeast (Lee *et al.*, 2002; Tarhan *et al.*, 2007). Similarly, an increase in SOD activity due to elevated zinc level was shown, among fungi, only in *Schizosaccharomyces pombe* (Tarhan *et al.*, 2007). Thus, we have shown for the first time the involvement of zinc in the induction of SOD transcription and activity in a filamentous fungus.

OmSod1 is directly involved in the cellular response to elevated levels of zinc and other metal ions. In fact, the introduction of *OmSOD1* gene in the yeast Δ sod1 mutant resulted in increased tolerance to zinc and copper stress. A heterologous system was used because, although transformation technology has recently been applied to *O. maius* (Martino *et al.*, 2007), knock-out mutants for specific genes are not yet available.

Cu,ZnSOD are usually found in the cytosol of eukaryotic cells, where they protect the cell against endogenous superoxide production. *Oidiodendron maius* Zn also releases OmSod1 in the extracellular environment, where it could deal with heavy metals and exogenous sources of superoxide. Upon exposure to different stress, ROS can form not only in the cell, from where they could diffuse through the plasma membrane, but also directly in the extracellular compartment. The presence of extracellular ROS has been demonstrated in green algae (Knauert & Knauer, 2008), plants (Langebartels *et al.*, 2002; Żróbek-Sokolnik *et al.*, 2009), fungi (Jarosz-Wilkolazka *et al.*, 1998) and lichens (Beckett *et al.*, 2003) exposed to heavy metals and other stress conditions. Thus, extracellular SODs may have a direct role in maintaining cell integrity against oxidative stress generated by environmental stress conditions as reported, concerning fungi, by Jaszek *et al.* (2006) and by Fink-Boots *et*

al. (1999). *Oidiodendron maius* Zn OmSod1 is the first extracellular Cu,ZnSOD described in a mycorrhizal association; it may have a special significance because it may extend its protective role to attenuate heavy metals damage to the host plant.

EM fungi play a pivotal role in protecting their host plant against heavy metal toxicity (Sharples *et al.*, 2000), but the mechanisms are poorly understood. We found that fungal strains isolated from polluted soils (including *O. maius* Zn) released fewer organic acids than fungi isolated from unpolluted soils, and were less effective in solubilizing metal ions from insoluble forms (Martino *et al.*, 2003). We speculated that a reduced solubilization of toxic metals by directly affecting the metal ion concentration in the soil environment, could represent a specific adaptation that allowed these fungi to protect, in polluted soils, not only their own cells, but also their host plants. The increased production of an extracellular Cu/Zn SOD may be an additional protection mechanism of these fungi because this protein, by binding metals and by performing its specific enzyme activity, could help both the fungus and the host plant to cope with the ROS formed in the extracellular medium, caused by metal pollution.

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