Characterization of a CuZn superoxide dismutase gene in the arbuscular mycorrhizal fungus Glomus intraradices

M. González-Guerrero; E. Oger; K. Benabdellah; C. Azcón-Aguilar; L. Lanfranco; N. Ferrol. Characterization of a CuZn superoxide dismutase gene in the arbuscular mycorrhizal fungus Glomus intraradices. CURRENT GENETICS. 56(3) pp: 265-274.
DOI: 10.1007/s00294-010-0298-y

Characterization of a CuZn superoxide dismutase gene in the arbuscular mycorrhizal fungus *Glomus intraradices*

Manuel González-Guerrero, Elodie Oger, Karim Benabdellah, Concepción Azcón-Aguilar, Luisa Lanfranco, Nuria Ferrol

M. Gonza´lez-Guerrero _ K. Benabdellah _ C. Azco´n-Aguilar _N. Ferrol (&) Departamento de Microbiologı´a del Suelo y Sistemas Simbio´ticos, Estacio´n Experimental del Zaidı´n, CSIC, Profesor Albareda 1, 18008 Granada, Spain
e-mail: nuria.ferrol@eez.csic.es URL: http://www.eez.csic.es/

DOI: 10.1007/s00294-010-0298-y

Cite this article as:
González-Guerrero, M., Oger, E., Benabdellah, K. et al. Curr Genet (2010) 56: 265. doi:10.1007/s00294-010-0298-y

Abstract

To gain further insights into the mechanisms of redox homeostasis in arbuscular mycorrhizal fungi, we characterized a *Glomus intraradices* gene (*GintSOD1*) showing high similarity to previously described genes encoding CuZn superoxide dismutases (SODs). The *GintSOD1* gene consists of an open reading frame of 471 bp, predicted to encode a protein of 157 amino acids with an estimated molecular mass of 16.3 kDa. Functional complementation assays in a *CuZnSOD*-defective yeast mutant showed that GintSOD1 protects the yeast cells from oxygen toxicity and that it, therefore, encodes a protein that scavenges reactive oxygen species (ROS). *GintSOD1* transcripts differentially accumulate during the fungal life cycle, reaching the highest expression levels in the intraradical mycelium. *GintSOD1* expression is induced by the well known ROS-inducing agents paraquat and copper, and also by fenpropimorph, a sterol biosynthesis inhibitor (SBI) fungicide. These results suggest that GintSOD1 is involved in the detoxification of ROS generated from metabolic processes and by external agents. In particular, our data indicate that the antifungal effects of fenpropimorph might not be only due to the interference with sterol metabolism but also to the perturbation of other biological processes and that ROS production and scavenging systems are involved in the response to SBI fungicides.

Keywords

Arbuscular mycorrhizal fungi, Copper, Fenpropimorph, Oxidative stress, Paraquat, Superoxide dismutase

Introduction

Oxidative stress occurs when cellular defences are unable to cope with existing reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hydroxyl radical (OH·). ROS production is common to all aerobic organisms as an unavoidable consequence of respiration. Furthermore, ROS are also generated by exposure to exogenous factors, such as UV light and toxic compounds. An excess of ROS can cause damage to numerous cellular components, including nucleic acids and lipids. In recent years, it has become evident that ROS also play an important signaling role controlling and regulating some biological processes, such as growth, development, response to biotic and abiotic environmental stimuli and programmed cell death (Perrone et al. 2008; Scott and Eaton 2008). To control ROS toxicity and to enable ROS to act as signaling molecules, cells have developed a variety of antioxidant systems to efficiently scavenge ROS excess and to keep ROS production and scavenging systems in balance. The antioxidant defences include enzymes such as superoxide dismutases (SODs), catalases, peroxidases and low molecular weight compounds such as glutathione, ubiquinol and vitamins E, C and B6 (Moradas-Ferreira and Costa 2000; Herrero et al. 2008).

Superoxide dismutases are metalloenzymes that form a primary cellular antioxidant defence system by detoxifying oxygen radicals *via* conversion of superoxide radicals to oxygen and H_2O_2 (Fridovich 1995), which is then disproportionated to water by catalases or peroxidases (Ruis and Koller 1997). In eukaryotes, there are different forms of SODs, containing either Cu/Zn, Mn or Fe at their active sites and with different cellular localizations. CuZnSOD is predominantly located in the cytosol, while MnSOD is mainly present in the mitochondria and FeSOD is present in chloroplasts of some plant species (Fridovich 1997). CuZnSODs have been extensively studied in the budding yeast *Saccharomyces cerevisiae*, where it accounts for 90–95% of the total SOD activity of the cell (Gralla and Valentine 1991). This enzyme has been shown to play a key role in protection against metabolically and externally generated ROS (Zyracka et al. 2005).

There is compelling evidence that ROS and SODs play a central role in plant–pathogen interactions. In the plant, *CuZnSOD* expression is usually one of the earliest detectable plant responses to pathogen infection and a key element of the plant defence system (Apostol et al. 1989; Mandal et al. 2008). In phytopathogenic fungi, there are examples of SODs that contribute to virulence (Rolke et al. 2004), but there are also reports where SODs were shown to be dispensable for full virulence (Fang et al. 2002; Moore et al. 2002). There is also evidence that ROS production and antioxidant systems, including SODs, from the plant and/or the microorganism play a role in symbiotic associations (Pauly et al. 2006; Takemoto et al. 2007; Abbà et al. 2009).

Arbuscular mycorrhiza (AM), the most widespread terrestrial symbiosis, is a mutualistic association formed by roots of most land plants with fungi that belong to the Glomeromycota phylum (Smith and Read 2008). Symbiotic development results in the formation of tree-shaped fungal structures within plant cells. These structures, called arbuscules, are thought to be the main sites of nutrient and signal exchange between the two partners (Parniske 2008). An accumulation of H_2O_2 and catalase and peroxidase transcripts has been observed in arbuscule-containing root cells of different plant species (Blee and Anderson 2000; Lambais et al. 2003; Fester and Hause 2005). Far less is known about the antioxidant responses on the fungal partner. A gene encoding a CuZnSOD has been identified in the AM fungus *Gigaspora margarita* (Lanfranco et al. 2005). Since the highest transcription levels were detected in intraradical fungal structures, it was suggested that CuZnSOD might be an essential component in the plant/fungus dialogue necessary to reach structural and functional compatibility between the partners.

Concomitantly with the intraradical colonization, AM fungi develop an extraradical mycelium that overgrows the soil surrounding plant roots and exploits the soil for resources. The extraradical mycelium developing in the matrix soil is continuously exposed to variable environmental conditions and must quickly adapt to changing conditions. The development of AM in vitro cultures, that is the co-culture of transformed hairy roots and AM fungi, has allowed the investigation of soil-free extraradical hyphae and spores (Fortin et al. 2002). By using this experimental system, it has been shown that environmental stresses, such as exposure to Cu or mechanical damage, promote ROS formation in the extraradical mycelium of *Glomus intraradices* (Fester and Hause 2005; Benabdellah et al. 2009). To cope with this oxidative stress, AM fungi must activate their antioxidant defences. Among the few literature data on this topic, evidence has been provided for the participation of a metallothionein (González-Guerrero et al. 2007) and a glutaredoxin (Benabdellah et al. 2009) in the alleviation of oxidative damage in the AM fungus *G. intraradices*.

The aim of this work was to get further insights into the ROS scavenging systems in AM fungi and into their role in fungal morphogenesis and protection against exogenously induced ROS. For this purpose, we have cloned and characterized a *G. intraradices* gene encoding a CuZnSOD by analyzing its expression during the fungal life cycle and in the extraradical mycelia exposed to different ROS generating agents. Here, we present evidence that this gene is highly expressed in the intraradical phase and induced in response to Cu, the herbicide paraquat, and fenpropimorph, a sterol biosynthesis inhibitor (SBI) fungicide widely used in agriculture.

Materials and methods

Biological materials

Glomus intraradices monoxenic cultures were established as described by St-Arnaud et al. (1996). Briefly, a Ri-T DNA transformed carrot (*Daucus carota*) root was grown together with the AM fungus *G. intraradices* Smith & Schenck (DAOM 197198, Biosystematic Research Center, Ottawa, Canada), in bicompartmental Petri dishes. Cultures were initiated in the "root compartment" (RC), which contained the minimal medium (M medium) described by Chabot et al. (1992). Fungal hyphae, but not roots, were allowed to grow over the plastic barrier to the second compartment (the "hyphal compartment", HC), which contained M medium without sucrose (M–C medium) (St-Arnaud et al. 1996). Plates were incubated in the dark at 24°C until the fungus was well established in the HC. At this stage, the AM fungus was submitted to different types of stresses.

Stress treatments were performed by applying 500 µM paraquat, 50 µM copper or 10 mg/l fenpropimorph to the medium of the HC. For the paraquat and copper treatments, when the fungus was well established on the HC, 800 µl of filter-sterilized stock solutions were distributed drop wise on the agar M–C medium ensuring a homogenous distribution. The control plates received 800 µl of sterile ddH_2O. For the fungicide treatment, the fungus was grown on liquid medium. When the extraradical mycelium was well developed, the liquid medium was replaced with 12 ml of fresh liquid medium containing, or not, fenpropimorph dissolved in acetone (0.5 ml/l medium) to obtain a final concentration of 10 mg/l. The control plates only received the medium with acetone. The extraradical mycelium was harvested 6 h, 12 h, 24 h and 7 days after paraquat and Cu supplementation, and 2, 7 and 14 days after the fungicide supplementation. The extraradical mycelium from the different HCs was collected, immediately liquid nitrogen-frozen and stored at −80°C until used.

Mycorrhizal roots were recovered from the RC with forceps and gently washed under tap water to eliminate attached extraradical fungal hyphae and spores. The absence of extraradical fungal

mycelium was verified under a stereomicroscope. Mycorrhizal root colonization was confirmed by visual observation of the fungal structures under the microscope after trypan blue staining (Phillips and Hayman 1970).

Spores of the AM fungus *G. intraradices* were collected from pot cultures of mycorrhizal *Trifolium repens* by the wet sieving and decanting method, followed by sucrose centrifugation (Sieverding 1991).

The *S. cerevisiae* strain DTY116 (*Δsod1*) (α gal1 leu 2–3,112 his-cup1s trp1-1:*sod1* deletion:trp1ura3-50) was grown on either YPD (1% yeast extract, 2% peptone and 2% glucose) or minimal SD medium containing 2% glucose and supplemented with auxotrophic requirements.

Lipid peroxidation

Lipid peroxidation was measured by the thiobarbituric acid reacting substances method (Beuge and Aust 1978). About 15 mg of extraradical mycelium were homogenized in 200 µl of sample buffer containing 50 mM potassium phosphate pH 7.8, 10% glycerol, 0.1 mM ethylene diamine tetraacetic acid, 0.1% Triton X-100 and 5 mM dithiothreitol. After a centrifugation of 20 min at 4°C at 10,000*g*, the supernatant was collected and added of five volumes of a solution consisting of 15% trichloroacetic acid, 0.375% thiobarbituric acid, and 0.01% hydroxytoluene butylate in 0.25 N HCl. Samples were incubated for 15 min in boiling water and then cooled and centrifuged 5 min at 2,500 *g*. The amount of malondialdehyde (MDA) reacting with thiobarbituric acid in the supernatant was determined by measuring the optical density at 535 nm. Protein content was determined by the method of Bradford (1976) using BSA to standardize the assay. The amount of protein was used to normalize the peroxidation values. Data are means of three independent experiments carried out on three different biological replicates.

Gene isolation, cloning and sequence analyses

The full-length *GintSOD1* gene was identified in Genbank (Accession number BI452161), and PCR-amplified using the gene-specific primers SOD1 5′-CATAATGACTATTAAAGCTG-3′ and SOD2 5′-GCGGCCGC TTATTTGGTGATACCGATAA-3′, and cDNA obtained from RNA isolated from *G. intraradices* extraradical mycelium grown in control plates. PCR products were cloned in the pGEM-T Easy vector (Promega, Madison, USA). GintSOD1 was then excised from the pGEM-T Easy vector by *Not*I digestion and cloned into the *Not*I-digested shuttle yeast vector pYES2 to yield the p*GintSOD1* construct.

Nucleotide sequences were determined by *Taq* polymerase cycle sequencing by using an automated DNA sequencer (Perkin-Elmer ABI Prism 373). Computer database comparisons were performed using BLAST algorithm (Altschul et al. 1990) and computer translation by using the Translate tool from EXPASY Molecular Biology Server. Amino acid sequence comparisons were made with the BESTFIT program of the Genetics Computer Group (Madison, WI, USA). Multiple sequence alignments of translated gene sequences were carried out with the program CLUSTALW (version 1.5) (Thompson et al. 1994).

Functional complementation assays in yeast

The *S. cerevisiae SOD1Δ* strain DTY116 was transformed with either pYES2 (negative control) or the p*GintSOD1* construct, and transformants were selected by autotrophy to uracil in SD medium. In order to do the functional complementation assays, pYES2 and p*GintSOD1* containing strains were co-transformed with pGEV-trp (Gao and Pinkham 2000). This second plasmid allows

induction of the Gal promoter in the presence of 1 μM β-estradiol, thus avoiding the use of galactose as inducer in those strains that cannot use this sugar as carbon source. To test for reversal of lysine, methionine and cysteine auxotrophy of this *SOD1*-deficient yeast strain, transformants were plated on SD medium with and without these amino acids and 1 μM β-estradiol, and grown aerobically for 3 days at 30°C.

RNA extraction and real-time RT-PCR

RNA was extracted from extraradical structures and from mycorrhizal and non-mycorrhizal roots using the SV Total RNA Isolation System kit (Promega) or the RNeasy Plant Mini Kit (QIAGEN, MD, USA). RNA samples were routinely checked for DNA contamination by RT-PCR analyses using the RMF and RMR 18S rRNA primers (González-Guerrero et al. 2005).

First single-strand cDNA was primed by random hexamers using 100–1,000 ng of DNase-treated RNA. RNA samples were denatured at 65°C for 5 min and then reverse transcribed at 25°C for 10 min and 42°C for 50 min in a final volume of 20 μl containing 10 μl of total RNA, 10 μM random primers (Invitrogen, Carlsbad, CA, USA), 0.5 mM dNTPs, 10 U RNase inhibitor, 4 μl of 5× buffer, 2 μl 0.1 M DTT, and 1 μl of SuperscriptII (Invitrogen). The samples were precipitated with 1 (v/v) isopropanol and resuspended in 20 μl of water. The cDNA samples were standardized to the elongation factor 1-alpha gene levels (GenBank Accession No. DQ282611) using the primers GintEFfw (5′-GCTATTTTGATCATTGCCGCC-3′) and GintEFrev (5′-TCATTAAAACGTTCTTCCGACC-3′) with the following program: 95°C for 3 min (1 cycle), 92°C for 30 s, 63°C for 30 s, 72°C for 30 s (35 cycles).

The same reactions were performed with the gene-specific primers for *GintSOD1* (Fw: 5′-GTACTATTACTTTCATTCAGGA-3′ and Rev: 5′-AGTTCATGACCACCTTTACCAA-3′). Individual real-time RT-PCR reactions were assembled with oligonucleotide primers (0.15 μM each), 10 μl of 2× iQSYBR Green Supermix (Bio-Rad; containing 100 mM KCl, 40 mM Tris–HCl pH 8.4, 0.4 mM dNTPs, 50 U/μl i*Taq* DNA polymerase, 6 mM $MgCl_2$, 20 nM SYBR Green I, 20 nM fluorescein) plus 1 μl of the cDNA appropriate dilution in a final volume of 20 μl. The PCR cycling programme consisted of 5 min incubation at 95°C, followed by 35 cycles of 30 s at 95°C, 45 s at 55°C and 45 s at 70°C. Real-time RT-PCR was carried out with an iCycler iQ apparatus (Bio-Rad, Hercules, CA, USA). A melting curve (55–95°C) with a heating rate of 0.5°C per 10 s and a continuous fluorescence measurement was recorded at the end of every run to assess amplification product specificity (Ririe et al. 1997). All reactions were performed with three technical replicates. The comparative threshold cycle (C_t) method (Rasmussen 2001) was used to calculate the relative expression level of each gene. Standard curves were obtained using recombinant plasmids containing a fragment of the gene of interest.

Real-time RT-PCR assays were performed on three to five biological replicates. One replicate corresponded to a pool of three to five plates. Statistical analysis was performed using one-way ANOVA followed by the Tukey's HSD test with $P < 0.05$ as the significance cut-off.

Results

Sequence analysis of *GintSOD1*

A 650 bp *G. intraradices* EST, which displayed high similarity to *CuZnSOD*s of other organisms, was identified in Genbank (Accession number BI452161). This cDNA, named *GintSOD1*, contains a complete open reading frame of 471 nucleotides, a 5′ untranslated region of 39 bp and a 3′

untranslated region of 87 bp (data not shown). The predicted protein has 157 amino acid residues with a molecular mass of 16.3 kDa. The deduced amino acid sequence is closely related to fungal CuZnSOD homologs, showing the highest similarity to partial *CuZnSOD* gene sequences from different *Glomus* species (98–80% identity; Corradi et al. 2009), 73% similarity to the *G. margarita* homolog (Lanfranco et al. 2005) and approximately 70% similarity to sequences from ascomycotan CuZnSODs. An alignment of the GintSOD1 protein sequence with those from the AM fungus *G. margarita*, the ericoid mycorrhizal fungus *Oidiodendrom maius* and the ascomycetes *Paecilomyces tenuipes*, *Claviceps purpurea*, *S. cerevisiae* and *Aspergillus niger* revealed that it contains features characteristic of CuZnSOD proteins, such as the conserved histidine and aspartate residues essential for dismutation and metal binding (Fig. 1). GintSOD1 includes six histidine residues at positions 50, 52, 67, 75, 84 and 124, and an aspartate residue at position 87. GintSOD1 also has a conserved arginine residue located at position 147 which is believed to enhance the binding of the O_2^- to the copper atom. The two-cysteine residues in positions 61 and 150 are involved in disulfide bond formation. This is essential for maintaining the functional structure of the enzyme. Asparagine at position 90, believed to be involved in N glycosylation, is also conserved (Bordo et al. 1994).

```
G. intraradices      MT----IKAXAVFTPDKPSIKVGGTITFIQESENGPTEIDIKIEG-LAPGEHGFHIHEFG 55
G. margarita         MSQKSPTKAIAVLRPDKPDGTVDGTIVFTQEV--GKVTVDIDIKG-LTDGDHGFHIHEFG 57
O. maius             -----MVKAVAFIRGDS---KITGTVTFEQTDESAPTTITWNITGHDPNAKRGMHVHQFG 52
S. cerevisiae        -----MVQAVAVLKGDA---GVSGVVKFEQASESEPTTVSYEIAGNSPNAERGFHIHEFG 52
P. tenuipes          -----MVKAVCVLRGDA---KVGGTVTFEQESESAPTTITYDLTGNDPNAERGFHIHTFG 52
C. purpurea          -----MVKAVAVLRGDA---KVGGTVVFEQESESAPTTITWDITGNDANAKRGFHIHTFG 52
A. niger             -----MVKAVAVIRGDS---KVSGTVTFEQANENTPTTISWNITGHDANAERGFHVHQFG 52
                           :* ..:  *     : *.: * *      . :  .: *  . ..:*:*:* **

G. intraradices      DNTNGCTSAGPHFNPFGKTHGAPKDDDRHVGDLGNVTAGPDGKVATKITDDQIKLSGPNS 115
G. margarita         DNTNGCTSAGPHFNPHKKTHGGKDDENRHVGDLGNVKA-VNGVVKEQITDAIITLEGEYS 116
O. maius             DNTNGCTSAGPHFNPHGKTHGAPADENRHVGDLGNFETDAQGNGKGSVTDKLVKLIGPQS 112
S. cerevisiae        DATNGCVSAGPHFNPFKKTHGAPTDEVRHVGDMGNVKTDENGVAKGSFKDSLIKLIGPTS 112
P. tenuipes          DNTNGCTSAGPHFNPHGKTHGAPTDAARHVGDLGNVKTDAQGNAKGSIQDSQVKLIGPHS 112
C. purpurea          DNTNGCTSAGPHFNPHGKTHGAPTDEARHVGDLGNLETDGQGNAKGSVKDEHVKLIGPHS 112
A. niger             DNTNGCTSAGPHFNPFGKTHGAPEDDERHVGDLGNFKTDAEGNAVGSKQDKLVKLIGAES 112
                     * ****.********. ****.  *  *****:**. :  :*    .  *  :.* *  *

G. intraradices      VIGRTIVIHADVDDLGKGGHELSLKTGNAGDRLACGVIGITK 157
G. margarita         IIGRTVVVHEGIDDLGKGGHEFSLTTGNAGGRLACGVIGYLK 158
O. maius             VIGRTVVVHAGTDDLGKGGNDESLKTGNAGPRPACGVIGIAA 154
S. cerevisiae        VVGRSVVIHAGQDDLGKGDTEESLKTGNAGPRPACGVIGLTN 154
P. tenuipes          VIGRTVVVHAGTDDLGKGGNEESLKTGNAGPRPACGVIGVAN 154
C. purpurea          VIGRTVVIHAGTDDLGKGDNEESLKTGNAGPRPACGVIGISS 154
A. niger             VLGRTLVVHAGTDDLGRGGNEESKKTGNAGPRPACGVIGIAA 154
                     ::**::*:* . ****:*. : * .***** * ******
```

Fig. 1 Alignment of the amino acid sequence of the Cu,Zn SOD gene of *G. intaradices* with those of other fungal species. *G. margarita* (AJ640199); *O. maius* (CN200173); *S. cerevisiae* (NP012638); *P. tenuipes* (AAN75576); *C. purpurea* (CAC50073) and *A. niger* (A2QMY6). Identical amino acid residues are indicated by *asterisks* and similar amino acid residues by *colons*. Conserved amino acids important for enzyme structure and activity are *boxed* (Bordo et al. 1994)

Complementation of a *sod1* mutant yeast strain

To determine whether the isolated *G. intraradices* gene encodes a functional protein, the full-length cDNA was expressed in a yeast strain bearing a deletion of the *SOD1* gene (*sod1Δ*) encoding a cytosolic CuZnSOD. Phenotypes of the SOD1 deficiency in aerobically grown yeast include retarded growth rate and auxotrophy for cysteine, lysine and methionine (Liu et al. 1992), since the synthetic pathways for these amino acids contain steps that are hypersensitive to reactive oxygen (Liu et al. 1992; Slekar et al. 1996). To determine whether the *GintSOD1* gene product could restore cysteine, lysine and methionine synthesis to aerobically grown *SOD1*-deficient yeast, the full-length cDNA was cloned into the pYES2 vector and expressed in the mutant strain (Fig. 2). The

empty vector transformed *sod1Δ* cells could grow on complete medium but failed to grow in the absence of cysteine, lysine and methionine. The same strain expressing *GintSOD1* could grow with these amino acids absent from the medium. These results indicate that *GintSOD1* expression in aerobically grown *SOD1*-deficient yeast protects the cells from oxygen toxicity to the extent that cysteine, lysine and methionine synthesis is restored. Thus, *GintSOD1* is the functional homolog of the yeast CuZnSOD *sod1*.

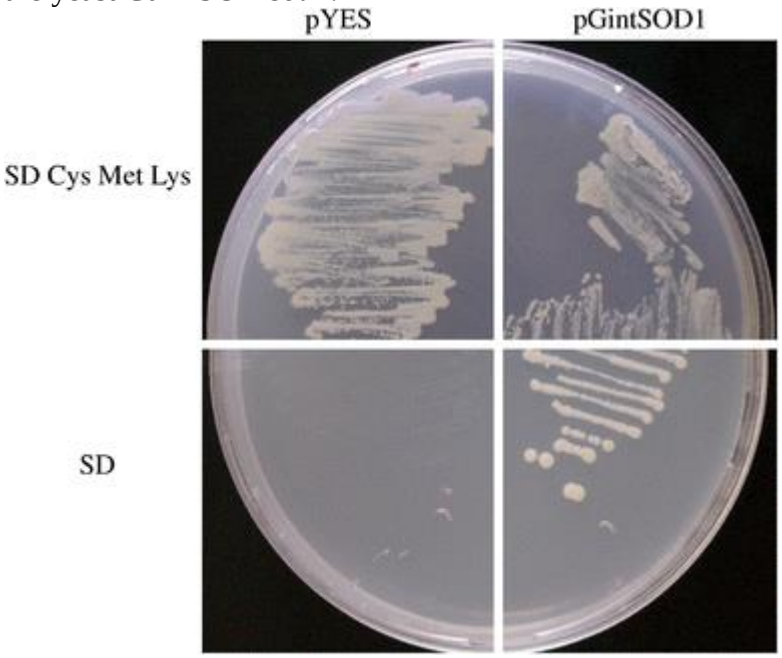

Fig. 2 Complementation of the yeast strain DTY116 defective at the Cu,Zn superoxide dismutase locus *SOD1* by *GintSOD1*. DTY116 cells were transformed with p*GintSOD1* or with the empty vector pYES2 and grown in SD medium supplemented or not with cysteine, lysine and methionine

GintSOD1 is strongly up-regulated during the symbiotic intraradical phase

To provide insights into the regulation of *GintSOD1*, its expression was assessed during the fungal life cycle. Quantitative expression analysis was performed by real-time RT-PCR on spores, extraradical mycelium and carrot mycorrhizal roots from which external hyphae were removed. Mycorrhizal colonization of the carrot roots was 30%. Specific amplification of the fungal material with the primers designed to amplify *GintSOD1* was confirmed by performing conventional PCR reactions on *G. intraradices* and carrot genomic DNAs and cDNAs (data not shown). Relative expression levels were calculated using spores as a reference sample (Fig. 3). A significant increase in *GintSOD1* expression levels was observed in the symbiotic stage of the fungal life cycle. In particular, the highest expression levels were observed in the intraradical fungal structures, that is, the sample of mycorrhizal roots from which external mycelia were eliminated.

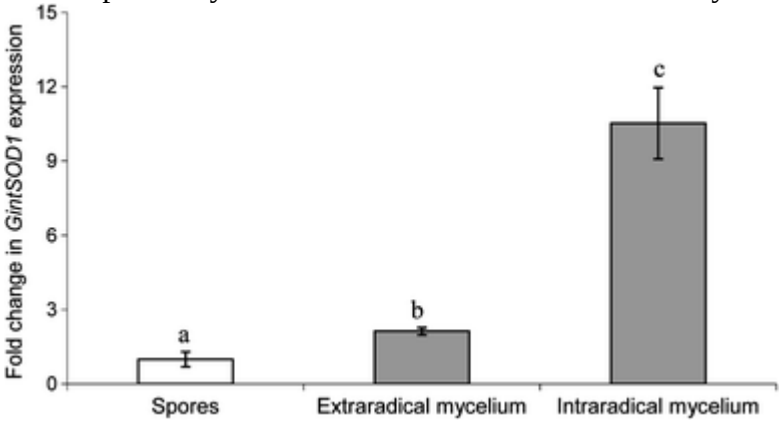

Fig. 3 *GintSOD1* expression in spores, extraradical and intraradical mycelium of *G. intraradices* developed in monoxenic cultures. Gene expression was studied by real-time RT-PCR. Data were calibrated by the expression values obtained for the gene encoding the elongation factor 1-α. Relative expression levels were calculated using spores as a reference sample. *Error bars* represent standard deviation of the mean of three independent experiments. Data not sharing a *letter* in common differ significantly ($P < 0.05$) according to the Tukey's HSD test

GintSOD1 mRNA is accumulated during oxidative stress

A time-course experiment of real-time PCR was set up to investigate the level of *GintSOD1* transcripts in response to paraquat. We used the same conditions in which paraquat was shown to induce an accumulation of ROS as well as an oxidative damage to the membrane lipids of *G. intraradices* extraradical mycelia (González-Guerrero et al. 2007; Benabdellah et al. 2009). Given that *GintSOD1* expression in mycelia from control plates did not change with time, gene expression data were referred to the expression levels detected in mycelia from control plates at time 0. Relative to the expression levels in the untreated mycelia, an increase in *GintSOD1* transcript levels was observed 12 h, 24 h and 7 days after exposure of the mycelia to paraquat, reaching a tenfold induction at the 7 days time-point (Fig. 4a).

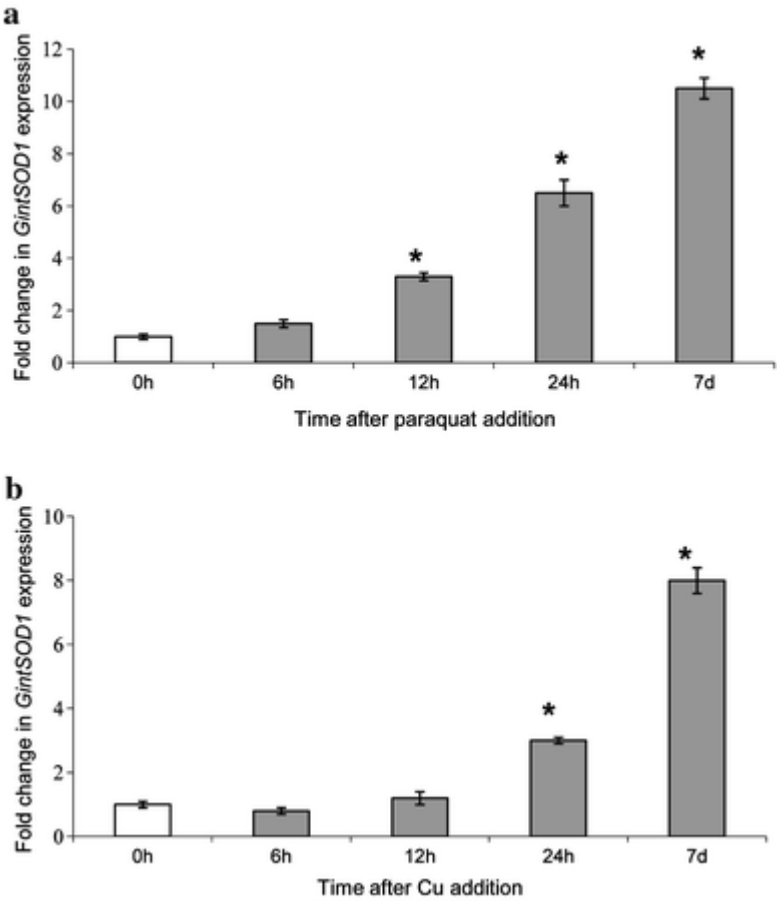

Fig. 4 Time-course analysis of *GintSOD1* expression in *G. intraradices* extraradical mycelium grown in M–C medium after the addition of 0.5 mM paraquat (**a**) or 5 mM Cu (**b**). Gene expression was studied by real-time RT-PCR. Data were calibrated by the expression values obtained for the gene encoding the elongation factor 1-α. Relative expression levels were calculated using as a reference sample mycelia from control plates at time 0. *Error bars* represent standard deviation of the mean of three independent experiments. *Asterisks* represent statistically significant ($P < 0.05$) in comparison to the control value at time 0

Since CuZnSODs have been involved in the cellular response to toxic levels of heavy metals (Adamis et al. 2004; Vallino et al. 2009) and Cu induces an oxidative stress to *G. intraradices* extraradical mycelia (González-Guerrero et al. 2007; Benabdellah et al. 2009), transcriptional regulation of *GintSOD1* by Cu was also investigated. Analysis of *GintSOD1* expression in mycelia exposed to 5 mM Cu revealed a significant increase in *GintSOD1* mRNA levels, in particular after 7 days of Cu application, when an eightfold increase was observed (Fig. 4b).

GintSOD1 response to fenpropimorph exposure

It has often been shown that fungicides treatment induces an oxidative stress (Bammert and Fostel 2000; Meyer et al. 2007). However, little information is available on the effects of fungicides on the biology of AM fungi as well as on the mechanisms of fungicide actions. We investigated therefore the impact of 10 mg/l fenpropimorph application on *G. intraradices* extraradical mycelium. Fenpropimorph, a SBI fungicide widely used in agriculture, was recently shown to have a strong impact on *G. intraradices* hyphal growth and spore production (Zocco et al. 2008; Campagnac et al. 2009) possibly through a perturbation of its sterol metabolism (Campagnac et al. 2009; Oger et al. 2009).

For this experiment longer exposure times (2, 7 and 14 days) were considered to let the fungicide exerts its effects. At first, the oxidative injury caused by fenpropimorph was determined by measuring the level of lipid peroxidation by using the MDA assay. The treatment of the extraradical mycelia with fenpropimorph induced a considerable increase in lipid peroxidation compared to the untreated sample (Fig. 5a). This increase was statistically significant after 7 and 14 days of fungicide application. Also in this case gene expression data were referred to the expression levels detected in mycelia from control plates at time 0 since *GintSOD1* expression in control plates did not change with time. An up-regulation of gene expression in the treated samples was observed at 7 and 14 days (Fig. 5b).

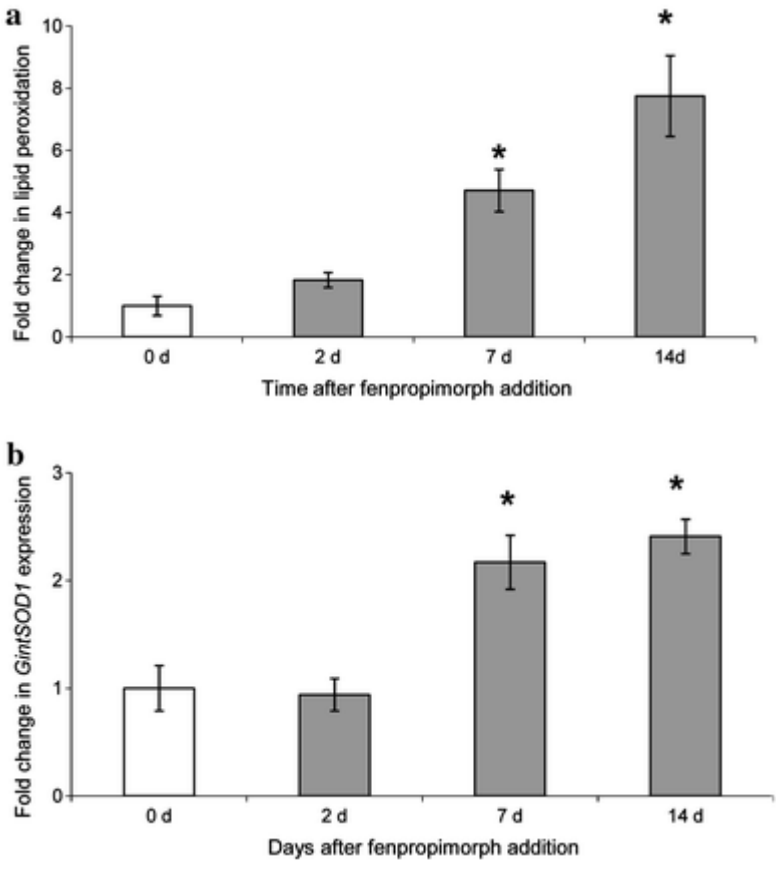

Fig. 5 **a** Lipid peroxidation levels in *G. intraradices* extraradical mycelium supplemented with 10 mg/l fenpropimorph. Data are expressed as the relative amount of malondialdehyde referred to the control at time 0. **b** Time-course analysis of *GintSOD1* expression levels in extraradical mycelium exposed to 10 mg/l fenpropimorph. Gene expression was studied by real-time RT-PCR. Data were calibrated by the expression values obtained for the gene encoding the elongation factor 1-α. Relative expression levels were calculated using as a reference sample mycelia from control plates at time 0. *Error bars* represent standard deviation of the mean of three independent experiments. *Asterisks* represent statistically significant ($P < 0.05$) in comparison to the control value at time 0

Discussion

Life under aerobic conditions is intimately linked with ROS production. ROS have been shown to be toxic, but also to function as signaling molecules. This biological paradox suggests the existence of finely tuned mechanisms that are important for the integrity and fitness of living organisms. There are few reports highlighting the importance of ROS homeostasis in AM fungi and in the establishment of the AM symbiosis. However, very little is known about the complex gene network involved in the regulation of ROS homeostasis in AM, that is, the mechanisms controlling the balance between the ROS-producing and ROS-scavenging enzymes. SOD activity was shown to increase in mycorrhizal roots (Palma et al. 1993; Arines et al. 1994; Ruiz-Lozano et al. 1996), however, the contribution of each partner is still unknown. In this work, we characterized a *G. intraradices* gene encoding a CuZnSOD to provide new data on the role of this enzyme in ROS homeostasis in AM.

Superoxide dismutase enzymes scavenge radicals by catalyzing the conversion of two of these radicals into H_2O_2 and molecular oxygen, thus being involved in the cellular defence strategies against oxidative damage by ROS (Natvig et al. 1996). Our yeast complementation assay reveals that *GintSOD1* indeed encodes a functional polypeptide that scavenges ROS. Differential accumulation of *GintSOD1* transcripts during the life cycle of the fungus and its induction in the extraradical mycelium by external ROS-inducer agents indicates a role for this enzyme in the detoxification of ROS generated both from metabolic processes and by external agents.

The expression pattern of *GintSOD1* during the fungal life cycle is consistent with earlier analyses of this gene sequence in the *Medicago truncatula–G. intraradices* association (Seddas et al. 2008, 2009), and resembles the expression profile of the orthologous gene of *G. margarita* in interactions with *M. truncatula* and *Lotus japonicus* (Lanfranco et al. 2005). Overall these data indicate that *SOD1* expression is strongly induced in the intraradical phase of the fungus, irrespective of the plant species and fungus involved in the association. Although the precise role of this enzyme in the symbiosis remains to be ascertained, it is likely that it plays a pivotal role in the relationship of the fungus with its host plant, as it has been recently described in the ericoid mycorrhizal fungus *O. maius* (Abbà et al. 2009). A significant decrease in the percentage of mycorrhization was in fact observed in roots colonized by the *O. maiusSOD1*-null mutant. These authors proposed that the lack of SOD1 causes an imbalance in the redox homeostasis during host colonization and an alteration in the delicate molecular dialogue between the two symbionts. Unfortunately, the lack of standardized protocols for the genetic transformation in AM fungi precludes us to follow a similar approach. It is worth to note that enhanced expression of *SOD1* was also observed in hyphae of different AM fungi upon exposure to root exudates (Lanfranco et al. 2005; Seddas et al. 2009) or analogs of strigolactones (Besserer et al. 2008). These data suggest that in AM fungi SOD1 might also be involved in the early stages of the interaction when a dialogue mediated by diffusible molecules between the plant and the fungus occurs.

Differential accumulation of *SOD1* transcripts at different stages of the fungal life cycle also suggests that this enzyme might play a role in fungal morphogenesis. Involvement of SOD1 in the control of morphogenetic processes has been demonstrated in other fungi, such as *Neurospora crassa* (Munkres 1992; Belozerskaya and Gessler 2006), *S. cerevisiae* (Liu et al. 1992) and, more recently, in the ericoid mycorrhizal fungus *O. maius* (Abbà et al. 2009). A significant decrease in conidiation was in fact observed in the *O. maiusSOD1*-null mutant.

Interestingly, some isolates of *G. intraradices* from a natural population were shown to harbor very divergent orthologous *SOD1* sequences (Corradi et al. 2009). It has been hypothesized that this high rates of protein evolution might be driven by evolutionary forces to facilitate interactions with the host plants at an organismic (colonization of root tissues) and/or ecosystem (widening the host range) level. All together these data point to a key role of SOD1 in the establishment of the symbiosis.

Data presented in this work also provide strong evidence for a role of GintSOD1 in protecting the fungus from the oxidative stress induced by environmental factors. Induction of SODs by the superoxide generators menadione and paraquat, as well as by heavy metals has been reported in other fungi (Culotta et al. 1995; Ott et al. 2002; Vallino et al. 2009). Accumulation of ROS and induction of an oxidative damage to the fungal membranes when the extraradical mycelia of *G. intraradices* were exposed to either paraquat or copper have been recently reported (González-Guerrero et al. 2007; Benabdellah et al. 2009). Therefore, up-regulation of *GintSOD1* transcripts in the paraquat- and Cu-treated mycelia indicates that its gene product might be involved in the detoxification of the ROS induced by these two external agents.

Evidence for a relationship between ROS and sterol metabolism has recently emerged. It has been demonstrated that the deletion of single genes in the ergosterol synthesis pathway rendered yeast cells more susceptible to oxidative stress (Branco et al. 2004; Thorpe et al. 2004). In addition, H_2O_2 was found to repress the ergosterol biosynthesis pathway through Pof14, a new member of the F-box protein family, which binds and decreases the activity of the squalene synthase ERG9, a key enzyme in ergosterol metabolism, leading to a decrease of the total cellular ergosterol levels in *Schizosaccharomyces pombe* (Tafforeau et al. 2006). Modulation of sterol level plays, therefore, a key role in adaptation to oxidative stress.

In this study, we also demonstrate that fenpropimorph exposure causes an oxidative stress in *G. intraradices* extraradical structures. An increase in the level of lipid peroxidation was observed 7 and 14 days after exposure. It is known that mitochondrial membranes contain a certain amount of sterols (Tuller and Daum 1995). A decrease in ergosterol levels in the inner mitochondrial membrane is likely to affect the mitochondrial electron transport leading to the formation of ROS. This physiological status is mirrored by an up-regulation of a number of genes related to oxidative stress in fungal cells exposed to SBI (Bammert and Fostel 2000; Meyer et al. 2007). Interestingly, the transcript abundance of *GintSOD1* correlates with the oxidative damage of the fungal membranes and H_2O_2 accumulation in the treated sample (E. Oger et al., unpublished data). These data suggest that antifungal effects of this SBI fungicide might not be only due to the interference with sterol metabolism, as recently described by Campagnac et al. (2009) and Oger et al. (2009), but also to the perturbation of other biological circuits within the fungal cells and suggest that ROS production and scavenging are involved in the response to SBI. Interestingly, *G. intraradices* colonization was shown to partially protect roots from oxidative stress induced by fenpropimorph (Campagnac et al. 2010). It is tempting to speculate that fungal antioxidant activities, including GintSOD1, may play a role in this protective effect.

In conclusion, data provided in this work indicate that in the AM fungus *G. intraradices* GintSOD1 is important not only as an antioxidant enzyme to overcome the oxidative stress induced by environmental factors but also for the establishment of the symbiotic association between the fungus and the plant.

Acknowledgments

This work was founded by the Consejería de Innovación, Ciencia y Empresa of the Junta de Andalucía, Spain (P06-CVI-02263), a Marie Curie Early stage Research Training Fellowship of the European Community's Sixth Framework Programme under contract number MEST-CT-2004-514213 and the bilateral project Italy–Spain HI2007-0229.

References

1. Abbà S, Khouja HR, Martino E, Archer DB, Perotto S (2009) SOD1-targeted gene disruption in the ericoid mycorrhizal fungus *Oidiodendron maius* reduces conidiation and the capacity for mycorrhization. Mol Plant Microbe Interact 22:1412–1421
2. Adamis PDB, Gomes DS, Pereira MD, Mesquita JFd, Pinto MLCC et al (2004) The effect of superoxide dismutase deficiency on cadmium stress. J Biochem Mol Toxicol 18:12–17
3. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410
4. Apostol I, Heinstein PF, Low PS (1989) Rapid stimulation of an oxidative burst during elicitation of cultured plant cells: role in defense and signal transduction. Plant Physiol 90:109–116
5. Arines J, Quintela M, Vilariño A, Palma JM (1994) Protein patterns and superoxide dismutase activity in non-mycorrhizal and arbuscular mycorrhizal *Pisum sativum* L. plants. Plant Soil 166:37–45
6. Bammert GF, Fostel JM (2000) Genome-wide expression patterns in *Saccharomyces cerevisiae*: comparison of drug treatments and genetic alterations affecting biosynthesis of ergosterol. Antimicrob Agents Chemother 44:1255–1265
7. Belozerskaya T, Gessler N (2006) Oxidative stress and differentiation in *Neurospora crassa*. Microbiology 75:427–431
8. Benabdellah K, Merlos MA, Azcón-Aguilar C, Ferrol N (2009) *GintGRX1*, the first characterized glomeromycotan glutaredoxin, is a multifunctional enzyme that responds to oxidative stress. Fungal Genet Biol 46:94–103
9. Besserer A, Becard G, Jauneau A, Roux C, Sejalon-Delmas N (2008) GR24, a synthetic analog of strigolactones, stimulates the mitosis and growth of the arbuscular mycorrhizal fungus *Gigaspora rosea* by boosting its energy metabolism. Plant Physiol 148:402–413
10. Beuge JA, Aust SD (1978) Microsomal lipid peroxidation. Methods Enzymol 52:302–310
11. Blee KA, Anderson AJ (2000) Defence responses in plants to arbuscular mycorrhizal fungi. In: Podila GK, Douds DD (eds) Current advances in mycorrhizae research. The American Phytopathological Society, St Paul, pp 27–44
12. Bordo D, Djinovic K, Bolognesi M (1994) Conserved patterns in the Cu,Zn superoxide dismutase family. J Mol Biol 238:366–386
13. Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
14. Branco MR, Marinho HS, Cyrne L, Antunes F (2004) Decrease of H_2O_2 plasma membrane permeability during adaptation to H_2O_2 in *Saccharomyces cerevisiae*. J Biol Chem 279:6501–6506

15. Campagnac E, Fontaine J, Lounès-Hadj Sahraoui A, Laruelle F, Durand R, Grandmougin-Ferjani A (2009) Fenpropimorph slows down the sterol pathway and the development of the arbuscular mycorrhizal fungus *Glomus intraradices*. Mycorrhiza 19:365–374
16. Campagnac E, Lounès-Hadj Sahraoui A, Debiane D, Fontaine J, Laruelle F, Garçon G, Verdin A, Durand R, Shirali P, Grandmougin-Ferjani A (2010) Arbuscular mycorrhiza partially protect chicory roots against oxidative stress induced by two fungicides, fenpropimorph and fenhexamid. Mycorrhiza 20:167–178
17. Chabot S, Bécard G, Piché Y (1992) Life cycle of *Glomus intraradix* in root organ culture. Mycologia 84:315–321
18. Corradi N, Ruffner B, Croll D, Colard A, Horák A, Sanders IR (2009) High-level molecular diversity of copper-zinc superoxide dismutase genes among and within species of arbuscular mycorrhizal fungi. Appl Environ Microbiol 75:1970–1978
19. Culotta VC, Joh HD, Lin SJ, Slekar KH, Strain J (1995) A physiological role for *Saccharomyces cerevisiae* copper/zinc superoxide dismutase in copper buffering. J Biol Chem 270:29991–29997
20. Fang GC, Hanau R, Vaillancourt L (2002) The *SOD2* gene, encoding a manganese-type superoxide dismutase, is up-regulated during conidiogenesis in the plant–pathogenic fungus *Colletotrichum graminicola*. Fungal Genet Biol 36:155–165
21. Fester T, Hause G (2005) Accumulation of reactive oxygen species in arbuscular mycorrhizal roots. Mycorrhiza 15:373–379
22. Fortin J, Bécard G, Declerck S, Dalpé Y, St-Arnaud M, Coughlan A, Piché Y (2002) Arbuscular mycorrhiza on root-organ cultures. Can J Bot 80:1–20
23. Fridovich I (1995) Superoxide radical and superoxide dismutases. Annu Rev Biochem 64:97–112
24. Fridovich I (1997) Superoxide anion radical (O2-.), superoxide dismutases, and related matters. J Biol Chem 272(30):18515–18517
25. Gao C, Pinkham J (2000) Tightly regulated β-estradiol dose-dependent expression system for yeast. Biotechniques 29:2–6
26. González-Guerrero M, Azcón-Aguilar C, Mooney M, Valderas A, MacDiarmid CW, Eide DJ, Ferrol N (2005) Characterization of a *Glomus intraradices* gene encoding a putative Zn transporter of the cation diffusion facilitator family. Fungal Gen Biol 42:130–140
27. González-Guerrero M, Cano C, Azcón-Aguilar C, Ferrol N (2007) *GintMT1* encodes a functional metallothionein in *Glomus intraradices* that responds to oxidative stress. Mycorrhiza 17:327–335
28. Gralla EB, Valentine JS (1991) Null mutants of *Saccharomyces cerevisiae* Cu,Zn superoxide dismutase: characterization and spontaneous mutation rates. J Bacteriol 173:5918–5920
29. Herrero E, Ros J, Belli G, Cabiscol E (2008) Redox control and oxidative stress in yeast cells. Biochim Biophys Acta 1780:1217–1235
30. Lambais MR, Rios-Ruiz WF, Andrade RM (2003) Antioxidant responses in bean (*Phaseolus vulgaris*) roots colonized by arbuscular mycorrhizal fungi. New Phytol 160:421–428
31. Lanfranco L, Novero M, Bonfante P (2005) The mycorrhizal fungus *Gigaspora margarita* possesses a CuZn superoxide dismutase that is up-regulated during symbiosis with legume hosts. Plant Physiol 137:1319–1330
32. Liu XF, Elashvili I, Gralla EB, Valentine JS, Lapinskas P, Culotta VC (1992) Yeast lacking superoxide dismutase. Isolation of genetic suppressors. J Biol Chem 267:18298–18302
33. Mandal S, Mitra A, Mallick N (2008) Biochemical characterization of oxidative burst during interaction between *Solanum lycopersicum* and *Fusarium oxysporum* f. sp. *lycopersici*. Physiol Mol Plant Pathol 72:56–61

34. Meyer V, Damveld RA, Arentshorst M, Stahl U, van den Hondel CA, Ram AF (2007) Survival in the presence of antifungals: genome-wide expression profiling of *Aspergillus niger* in response to sublethal concentrations of carpofungin and fenpropimorph. J Biol Chem 282:32935–32948
35. Moore S, De Vries O, Tudzynski P (2002) The major Cu, Zn SOD of the phytopathogen *Claviceps purpurea* is not essential for pathogenicity. Mol Plant Pathol 3:9–22
36. Moradas-Ferreira P, Costa V (2000) Adaptive response of the yeast *Saccharomyces cerevisiae* to reactive oxygen species: defences, damage and death. Redox Rep 5:277–285
37. Munkres KD (1992) Selection and analysis of superoxide dismutase mutants of *Neurospora*. Free Radic Biol Med 13:305–318
38. Natvig DO, Sylvester K, Dvorachek WH Jr, Baldwin JL (1996) Superoxide dismutases and catalases. In: Brambl R, Marzluf GA (eds) The Mycota III. Springer, Berlin, pp 191–209
39. Oger E, Ghignone S, Campagnac E, Fontaine J, Grandmougin-Ferjani A, Lanfranco L (2009) Functional characterization of a C-4 sterol methyl oxidase from the endomycorrhizal fungus *Glomus intraradices*. Fungal Genet Biol 46:486–495
40. Ott T, Fritz E, Polle A, Schützendübel A (2002) Characterization of antioxidative systems in the ectomycorrhiza-building basidiomycete *Paxillus involutus* (Bartsch) Fr. and its reaction to cadmium. FEMS Microbiol Ecol 42:359–366
41. Palma JM, Longa MA, del Rio LA, Arines J (1993) Superoxide dismutase in vesicular arbuscular mycorrhizal red clover plants. Physiol Plant 87:77–83
42. Parniske M (2008) Arbuscular mycorrhiza: the mother of plant root endosymbioses. Nat Rev Microbiol 6:763–775
43. Pauly N, Pucciariello C, Mandon K, Innocenti G, Jamet A, Baudouin E, Hérouart D, Frendo P, Puppo A (2006) Reactive oxygen and nitrogen species and glutathione: key players in the legume-*Rhizobium* symbiosis. J Exp Bot 57:1769–1776
44. Perrone GG, Tan S-X, Dawes IW (2008) Reactive oxygen species and yeast apoptosis. Biochim Biophys Acta 1783:1354–1368
45. Phillips JM, Hayman DS (1970) Improved procedures for cleaning roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Trans Br Mycol Soc 55:158–162
46. Rasmussen R (2001) Quantification on the LightCycler. In: Meuer S, Wittner C, Nakagawara K (eds) Rapid cycle real-time PCR, methods and applications. Springer, Heidelberg, pp 21–34
47. Ririe KM, Rasmussen RP, Wittwer CT (1997) Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. Anal Biochem 245:154–160
48. Rolke Y, Liu S, Quidde T, Williamson B, Schouten A, Weltring KM, Siewers V, Tenberge KB, Tudzynski B, Tudzynski P (2004) Functional analysis of H_2O_2-generating systems in *Botrytis cinerea*: the major Cu–Zn-superoxide dismutase (BCSOD1) contributes to virulence on French bean, whereas a glucose oxidase (BCGOD1) is dispensable. Mol Plant Pathol 5:17–27
49. Ruis H, Koller F (1997) Biochemistry, molecular biology, and cell biology of yeast and fungal catalases. In: Scandalios JG (ed) Oxidative stress and the molecular biology of antioxidant defenses. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp 309–342
50. Ruiz-Lozano JM, Azcón R, Palma JM (1996) Superoxide dismutase activity in non-mycorrhizal *Lactuca sativa* plants subjected to drought stress. New Phytol 134:327–333
51. Scott B, Eaton CJ (2008) Role of reactive oxygen species in fungal cellular differentiations. Curr Opin Microbiol 11:488–493
52. Seddas PM, Arnould C, Tollot M, Arias CM, Gianinazzi-Pearson V (2008) Spatial monitoring of gene activity in extraradical and intraradical developmental stages of

arbuscular mycorrhizal fungi by direct fluorescent in situ RT-PCR. Fungal Genet Biol 45:1155–1165
53. Seddas PMA, Arias CM, Arnould C, van Tuinen D, Godfroy O, Benhassou HA, Gouzy J, Morandi D, Dessaint F, Gianinazzi-Pearson V (2009) Symbiosis-related plant genes modulate molecular responses in an arbuscular mycorrhizal fungus during early root interactions. Mol Plant Microbe Interact 22:341–351
54. Sieverding E (1991) Vesicular-arbuscular mycorrhiza management in tropical agrosystems. Deutsche Gesellschaft für Technische Zusammenarbeit Nr. 224 Hartmut Bremer Verlag, Friedland
55. Slekar KH, Kosman DJ, Culotta VC (1996) The yeast copper/zinc superoxide dismutase and the pentose phosphate pathway play overlapping roles in oxidative stress protection. J Biol Chem 271:28831–28836
56. Smith S, Read DJ (2008) Mycorrhizal symbiosis. Academic Press, Amsterdam
57. St-Arnaud M, Hamel C, Vimard B, Caron M, Fortin JA (1996) Enhanced hyphal growth and spore production of the arbuscular mycorrhizal fungus *Glomus intraradices* in an in vitro system in the absence of host roots. Mycol Res 100:328–332
58. Tafforeau L, Le Blastier S, Bamps S, Dewez M, Vandenhaute J, Hermand D (2006) Repression of ergosterol level during oxidative stress by fission yeast F-box protein Pof14 independently of SCF. EMBO J 25:4547–4556
59. Takemoto D, Tanaka A, Scott B (2007) NADPH oxidases in fungi: diverse roles of reactive oxygen species in fungal cellular differentiation. Fungal Genet Biol 44:1065–1076
60. Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680
61. Thorpe GW, Fong CS, Alic N, Higgins VJ, Dawes IW (2004) Cells have distinct mechanisms to maintain protection against different reactive oxygen species: oxidative-stress-response genes. Proc Natl Acad Sci USA 101:6564–6569
62. Tuller G, Daum G (1995) Import of sterols into mitochondria of the yeast *Saccharomyces cerevisiae*. FEBS Lett 372:29–32
63. Vallino M, Martino E, Boella F, Murat C, Chiapello M, Perotto S (2009) Cu,Zn superoxide dismutase and zinc stress in the metal-tolerant ericoid mycorrhizal fungus *Oidiodendron maius* Zn. FEMS Microbiol Lett 293:48–57
64. Zocco D, Fontaine J, Lozanova E, Renard L, Bivort C, Durand R, Gransmougin-Ferjani A, Declerck S (2008) Effects of two sterol biosynthesis inhibitor fungicides (fenpropimorph and fenhexamid) on the development of an arbuscular mycorrhizal fungus. Mycol Res 112:592–601
65. Zyracka E, Zadrag R, Koziol S, Krzeplko A, Bartosz G, Bilinski T (2005) Yeast as a biosensor for antioxidants: simple growth tests employing a *Saccharomyces cerevisiae* mutant defective in superoxide dismutase. Acta Biochim Pol 52:679–684