Mycorrhiza (2007) 17:327–335
DOI 10.1007/s00572-007-0108-7

ORIGINAL PAPER

GintMT1 encodes a functional metallothionein in *Glomus intraradices* that responds to oxidative stress

M. González-Guerrero · C. Cano · C. Azcón-Aguilar · N. Ferrol

Received: 4 September 2006 / Accepted: 20 December 2006 / Published online: 3 February 2007

Abstract A full-length metallothionein (MT) gene (*GintMT1*) was isolated from *Glomus intraradices* extraradical mycelium. This is the first MT gene reported in the genus *Glomus*, third in the Glomeromycota. Functional analysis of *GintMT1* in a MT-defective *Saccharomyces cerevisiae* strain indicates that it encodes a functional MT. Gene expression analyses revealed that the transcript levels of *GintMT1* were elevated in mycelia treated with 5 mM Cu or paraquat but inhibited in mycelia treated with 50 μM Cu or 450 μM Cd. The elevated expression of *GintMT1* in the 5 mM Cu-treated mycelia together with the ability of *GintMT1* to provide tolerance to a Cu-sensitive yeast suggests that *GintMT1* might afford protection against Cu. Induction of *GintMT1* expression by paraquat and 5 mM Cu, treatments that also produced an oxidative damage to the fungal membranes, suggests that *GintMT1* may play a role in the regulation of the redox status of the extraradical mycelium of *G. intraradices*.

Keywords Arbuscular mycorrhizal fungi · Heavy metals · Lipid peroxidation · Metallothionein · Oxidative stress

Introduction

Various heavy metals (HM), such as Zn, Cu, Mn, Ni, and Co, are essential micronutrients required for development of living organisms, whereas others such as Cd, Pb, and Hg, have no known biological function and are toxic even at very low concentrations. Accumulation of HM in the soil from either natural sources such as volcanoes and continental dusts, or human activities like mining, combustion of fossil fuels, metal-working industries, etc., have detrimental effects on soil organisms and therefore, on ecosystem functioning.

There is evidence that arbuscular mycorrhizal (AM) fungi are present in HM polluted sites (Weissenhorn et al. 1995; del Val et al. 1999a; Chen et al. 2003), and several studies have shown a positive impact of mycorrhizal establishment on plant resistance to HM, the resulting effect depending on plant growth conditions, AM fungal isolate involved, and HM concentration (del Val et al. 1999b; Hildebrandt et al. 1999; Rivera-Becerril et al. 2002). The occurrence of AM fungi in metal-contaminated soils provides evidence of adaptation and tolerance of these microorganisms to toxic metals, although it has been shown that this naturally occurring tolerance decreases after a few generations in the absence of HM (Malcová et al. 2003). The constitutive and adaptive mechanisms of mycorrhizas to contaminated soils and the contribution of AM symbiosis to soil remediation have been recently reviewed (Meharg 2003; Gohre and Paszkowski 2006). Nevertheless, current knowledge on the molecular basis of HM tolerance in AM fungi is still in its infancy.

AM fungi, like other organisms, must have evolved a range of mechanisms including binding to extracellular materials, regulation of transport processes, and/or intracellular sequestration to maintain the concentration of essential metals within a physiological range and control the toxicity of nonessential HM ions. The ability of the AM fungal hyphae to sequester HM not only in the mucilaginous zone of the hyphal wall and in the cell wall but also inside the

M. González-Guerrero · C. Cano · C. Azcón-Aguilar · N. Ferrol (✉)
Departamento de Microbiología del Suelo y Sistemas Simbióticos,
Estación Experimental del Zaidín,
Consejo Superior de Investigaciones Científicas,
C. Profesor Albareda 1,
18008 Granada, Spain
e-mail: nferrol@eez.csic.es

hyphal cytoplasm has been described (González-Chavez et al. 2002). The finding that glomalin, a glycoprotein copiously produced by AM fungi, efficiently sequesters both in vivo and in vitro different HM has led to hypothesize that glomalin may be one of the molecules involved in HM sequestration both at the hyphal cell wall and in the soil (González-Chavez et al. 2002). At the genetic level, just two genes involved in HM homeostasis have been analyzed in detail in AM fungi: the Zn transporter *GintZnT1* from *Glomus intraradices* involved in vacuolar Zn compartmentalization (González-Guerrero et al. 2005) and the metallothionein gene *GmarMT1* from *Gigaspora margarita* that may provide protection against Cu (Lanfranco et al. 2002). Recently, several as-yet-uncharacterized gene sequences coding for enzymes potentially catalyzing the detoxification of reactive oxygen species were found in a suppression subtractive hybridization library from hyphae of *G. intraradices* grown in high versus low Zn (Ouziad et al. 2005).

Metallothioneins (MTs) constitute an extensive and diverse family of small cysteine-rich proteins that bind metals via the thiol groups of their cysteine residues and may play a role in the intracellular sequestration of HM. MTs are distributed throughout all the kingdoms, and have been traditionally classified, based on the arrangement of their cysteine residues, into two classes (Fowler et al. 1987; Kojima 1991). Class I MTs are widespread in vertebrates, whereas class II MTs include all those from plants and fungi as well as from nonvertebrate animals. Because MTs efficiently bind metals and some MT genes are positively regulated by metals, they are thought to play a role in cellular HM detoxification and homeostasis (Cobbett and Goldsbrough 2002). Nonetheless, a growing amount of evidence suggests an alternative role for MTs as protectants from oxidative damage (Tamai et al. 1993), a function that results from the capability of the thiolate groups to be reversible oxidized (Maret 2003). With the aim of getting further insights into the putative roles of MTs in AM fungi, regulation of a new MT gene from *G. intraradices* by Cu and Cd was analyzed, and the possibility that its induction could be linked to oxidative stress caused by these metals was studied.

Materials and methods

AM monoxenic cultures

AM monoxenic cultures were established as described by St-Arnaud et al. (1996). Briefly, a carrot (*Daucus carota* L., clone DC2) Ri-T DNA transformed root organ culture was grown together with the AM fungus *G. intraradices* Smith & Schenck (DAOM 197198, Biosystematic Research Centre, Ottawa, Canada) in two-compartment Petri dishes. Cultures were initiated in the "root compartment", which contained a minimal culture medium ("M medium") gelled with 0.35% (w/v) phytagel (Sigma-Aldrich, St. Louis; Chabot et al. 1992). Fungal hyphae, but not roots, were allowed to grow over the plastic barrier to the second compartment (the "hyphal compartment"), which contained the gelled M medium described above but without sucrose ("M-C medium"; St-Arnaud et al. 1996). Plates were incubated in the dark at 24°C.

Treatments of *G. intraradices* extraradical mycelium in monoxenic cultures

Cu, Cd, or paraquat, an oxidative agent, were applied as a pulse to the M-C medium once the extraradical mycelium was well established on the hyphal compartment (transition from absorptive to sporulative phase, typically after 7–8 weeks after culture establishment). $CuSO_4$, $CdSO_4$, or paraquat were applied to their respective hyphal compartments to obtain final concentrations of 50 μM Cu, 5 mM Cu, 0.45 mM Cd, or 500 μM paraquat. This was done by distributing 500 μl of each filter-sterilized stock solution drop wise so that even diffusion of the added solutions was ensured. Time point just before HM/paraquat addition is referred to "Time 0." After the pulse, the extraradical mycelium was harvested at time points 12 h, 24 h, and 7 d. Control plates received 500 μl sterile H_2O.

Extraradical mycelia recovery and RNA extraction

Extraradical mycelium from the different hyphal compartments was recovered by blending (5 sec. high speed, 5 min. with occasional low speed pulses) the culture medium in 10 mM sodium citrate (pH 6) and collecting the mycelia with a 50 μm sieve under sterile conditions (Bago et al. 1999). The mycelium was immediately liquid nitrogen frozen and stored at −80°C until used. RNAs were extracted using the RNeasy Plant Mini Kit (QIAGEN, Maryland, USA) following manufacturer's instructions.

Gene isolation, cloning, and sequence analyses

The 3′ end of *GintMT1* was identified in the *G. intraradices* EST database (Accession number BI452270) and obtained from Dr. M. Harrison (Noble Foundation, Ardmore, OK, USA). The 5′ end was obtained by rapid amplification of cDNA ends (RACE) using the SMART RACE cDNA Amplification kit (Clontech, Palo Alto, CA, USA), the *GintMT1*-specific primer MT1 5′-AGTTACATGCTCCTG GAGAACACTT-3′ and 1 μg total RNA from extraradical mycelia grown in control plates. The amplified cDNA was cloned in the pGEM-T Easy vector (Promega, Madison,

USA). *GintMT1* was then excised from the pGEM-T Easy vector by NotI digestion and cloned into the NotI-linearized yeast expression vector pFL61 (Minet et al. 1992).

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 Basic Local Alignment Search Tool (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 ΔCUP1 strain of *Saccharomyces cerevisiae* DTY113 (α ura3-50 leu2-3,112 trp1-1 gal1 his Δcup61; Ecker et al. 1986) was transformed with pFL61 (negative control) or a properly oriented pFL61-*GintMT1* construct. Transformants were selected by auxotrophy to uracile in SD medium. For the metal tolerance tests on agar plates, a drop (10^5 cells) of a liquid culture of the transformants in selective media was placed on SD plates containing or not containing 50 μM $CdSO_4$ or 500 μM $CuSO_4$. Plates were grown aerobically for 3 d at 30°C.

Gene expression analyses

GintMT1 gene expression was studied by real-time polymerase chain reaction (RT-PCR; iCycler iQ, BioRad, Hercules, CA, USA). cDNAs were obtained from 1 μg of DNase-treated total RNA from the different treatments in a 20 μl reaction containing 200 units of SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and 100 ng of random hexamers, according to the manufacturer's protocol. The primer set used to amplify *GintMT1* in the synthesized cDNAs were MT1 and MT2 (5′-GGAGA TAGTTGTCAATGTACAGGTG-3′). Each 25-μl reaction contained 1 μl of a 1:10 dilution of the synthesized cDNA, 200 mM dNTPs, 200 nM each primer, 3 mM $MgCl_2$, 2.5 μl 1×SyBR Green (Molecular Probes, Eugene, OR, USA), and 0.5 U Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA) in 1×PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl).

The PCR program consisted of 5 min incubation at 95°C to activate the hot-start recombinant Taq DNA polymerase, followed by 35 cycles of 30 sec at 95°C, 45 sec at 55°C and 45 sec at 70°C, where the fluorescence signal was measured. The specificity of the PCR amplification procedure was checked with a heat dissociation protocol (from 70 to 100°C) after the final cycle of the PCR. The efficiency of the primer set was evaluated by performing RT-PCR on several dilutions of plasmid DNA. The results obtained for the different treatments were standardized to the 18S rRNA levels, which were amplified with the primers: RMF: 5′-TGTTAATAAAAATCGGTGCGTTGC-3′ and RMR: 5′-AAAACGCAAATGATCAACCGGAC-3′. RT-PCR determinations were performed on three independent biological samples from three replicate experiments. Three technical replicates for each biological sample were included in the iCycler. The relative levels of transcription were calculated by using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). In all RT-PCR reactions, a non-RT control was used to detect any possible DNA contamination.

Lipid peroxidation

Lipid peroxidation was measured by the thiobarbituric acid-reacting substances method (Beuge and Aust 1978). Briefly, 20 mg extraradical mycelium were homogenized in 60 μl of sample buffer containing 50 mM potassium phosphate pH 7.8, 10% glycerol, 0.1 mM ethylenediamine tetraacetic acid, 0.1% Tritón X-100, and 5 mM Dithiothreitol. The suspension was filtered through two layers of Miracloth and centrifuged for 20 min at 27,000 g at 4°C. The supernatant was collected, added to five volumes of a solution consisting of 15% trichloroacetic acid, 0.375% thiobarbituric acid, and 0.01% hydroxytoluene butilate in 0.25 N HCl, and incubated for 15 min in boiling water. Samples were then left to cool and centrifuged for 5 min at 2,500 g. The amount of malondialdehyde reacting with thiobarbituric acid in the supernatant was determined by measuring the OD 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 samples.

Results

Sequence analysis of *GintMT1*

A 335 bp cDNA fragment corresponding to the 3′ end of a putative MT gene was identified in a *G. intraradices* EST database (accession number BI452270) and obtained from Dr. M. Harrison (Noble Foundation, Ardmore, OK, USA). The 5′end of this gene was obtained by 5′-RACE using a gene-specific primer and cDNA from *G. intraradices* extraradical mycelium as template. The full-length gene, named *GintMT1*, contained a 213-nucleotide open reading frame, a 5′UTR of 73 bp, and a 3′UTR of 187 bp. Several

stop codons upstream the ATG initiation codon and the presence of a TGA stop codon well before the end of the cDNA indicates that the cDNA sequence is indeed the full length (Fig. 1a). The deduced amino acid sequence of *GintMT1* consists of 71 amino acids with a predicted molecular mass of 7.2 kDa, and containing 13 cysteine residues (Fig. 1a). BLAST searches in the protein sequence database indicated that *GintMT1* is related to gene members of the MT family, showing 55 and 48% similarity to the MT genes of the AM fungi *Gigaspora rosea* and *G. margarita* (Stommel et al. 2001; Lanfranco et al. 2002), respectively.

GintMT1 contains two cysteine-rich domains separated by a 24-amino acid region. The N-terminal domain contains six cysteine residues showing the following pattern: Cys-X-Cys-X_3-Cys-X-Cys-X_3-Cys-X-Cys, and the C-terminal domain presents seven cysteine residues arranged as follows: Cys-X_4-Cys-X-Cys-X_2-Cys-X-Cys-X_4-Cys-X-Cys (Fig. 1a). This sequence type fits with the pattern presented by class II MTs. The *GintMT1* cysteine arrangement is closely shared by the other two known mycorrhizal MTs and by a putative MT from *Coprinus cinereus* (Fig. 1b), resulting in the conserved arrangements Cys-X-Cys-X_3-Cys-X-Cys-$X_{2–3}$-Cys-X-Cys at the N terminus and Cys-X_4-Cys-X-Cys-$X_{2–3}$-Cys-X-Cys-X_4-Cys-X-Cys at the C terminus. Additionally, some ESTs from ectomycorrhizal fungi encoding putative MTs such as the EST clone AAS19463 from *Paxillus involutus* or the *Hebeloma cylindrosporum* sequence CK995302, have the same C-terminal domain (data not shown).

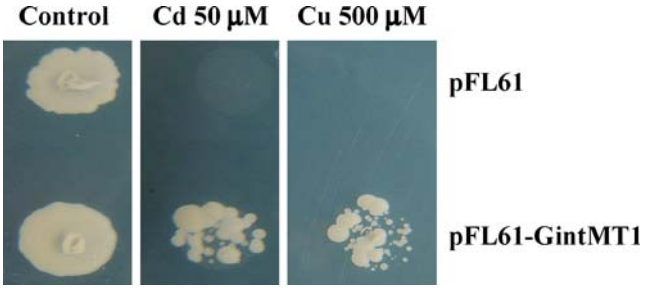

Fig. 2 Complementation of a yeast strain defective at the MT locus *CUP1* by *GintMT1*. DTY113 cells were transformed with pFL61-*GintMT1* or with the empty vector pFL61 and grown in SD medium supplemented with 50 µM Cd or 500 µM Cu

GintMT1 encodes a functional MT

The ability of *GintMT1* to function as a MT was assessed in a yeast strain carrying a deletion of the MT gene *CUP1*, mutation which renders the yeast strain sensitive to both Cu and Cd (Ecker et al. 1986). As shown in Fig. 2, transformation of this yeast strain with a plasmid carrying the *GintMT1* gene under the control of the constitutive yeast phosphoglycerate kinase promoter complemented the defect of the ΔCUP1 mutant to grow in media containing 500 µM Cu or 50 µM Cd. Restoration by *GintMT1* of the function

a

```
   1 taatacgactcactatagggcaagcagtggtaacaacgcagagtacgcggggaaaaataa

  61 aaaaaaggcataatgtctacaaaaggagatcaaattaactgtcaatgtggagatagttgt
   1              M  S  T  K  G  D  Q  I  N  C  Q  C  G  D  S  C
                                              DOMAIN I

 121 caatgtacaggtgtttgtacctgcggtaaatcttctactactaccactaaagctaccgaa
  17  Q  C  T  G  V  C  T  C  G  K  S  S  T  T  T  T  K  A  T  E

 181 acaaaagactccaagagtggcgaaactcataaagcatgtggaaataaatcttgtacttgc
  37  T  K  D  S  K  S  G  E  T  H  K  A  C  G  N  K  S  C  T  C

 241 agcgaatgcaagtgttctccaggagcatgtaactgcggttcgaaatgatttaaatgatt
  57  S  E  C  K  C  S  P  G  A  C  N  C  G  S  K  *
         DOMAIN II

 301 catcaaatatcctatgatgcatctgttatgacatctgttacgtcgcatgaaaatatcata
 361 cccttatttagtatattttagtattagtattatttatttttttttttcaattgatgtatt
 421 aactctatcatcgaattcttaacatttatttttcaataaaaccatttgacaagtg
```

b

```
GmarMT          ----MCQ-----NCKCGSACQCGTNCTCPKDYTTSSTTQQQQQSTDTQKKTASCGVSTCR 51
GiroMT          ----MCD-----DCKCGSSCQC-TNCACVK--TTKQNFVDKQIETDP-----SCGVSTCN 43
CcMT            MIFDFTTPVDLHECKCGTSCSCN-PCTCG--------------DAKPQSSTASCGSSSCT 45
GintMT1         ----MSTKGDQINCQCGDSCQCTGVCTCGKS-STTTTKATETKDSKSGETHKACGNKSCT 55
                    :       :*:** :*.*   *:*               .:..     :** .:*
GmarMT          CGEVCKCTKGNCKC--- 65
GiroMT          CGENCSCTQGACKC--- 57
CcMT            CGD-CKCKPGECKC--- 58
GintMT1         CSE-CKCSPGACNCGSK 71
                *.: *.*. * *:*
```

Fig. 1 **a** Nucleotide and deduced amino acid sequence of *GintMT1*. The two cysteine-rich domains appear *underlined*. **b** Multiple alignment of GintMT1 with other known fungal MT genes (*GintMT1* Acc. no. AM040753; *GmarMT1* Acc. no. AJ421527; *GrosMT1* Acc. no. BE057027, and *CcMT* Acc. no. DY852723)

lost by the *CUP1* deletion indicates that *GintMT1* encodes a functional MT that is able to sequester metal ions.

HM regulation of *GintMT1* expression

To investigate whether *GintMT1* could play a role in HM detoxification/homeostasis in *G. intraradices*, *GintMT1* expression was analyzed in extraradical mycelium that had been exposed to Cu (50 μM and 5 mM) or Cd (0.45 mM) for 12 h, 24 h, and 7 d. As *GintMT1* expression in mycelia from control plates was the same at all the time points analyzed, gene expression data are referred to the expression levels detected in mycelia from control plates at time 0. Exposure of extraradical mycelia to 50 μM Cu inhibited transiently *GintMT1* expression (Fig. 3a). Relative to the control mycelia, *GintMT1* transcript levels decreased 12 and 24 h after the addition of 50 μM Cu but returned to the levels detected in the untreated mycelia 7 d after metal addition. However, exposure to 5 mM Cu clearly induced an increase in *GintMT1* transcripts. The level of these transcripts was already slightly higher 24 h after the addition of 5 mM Cu (1.8-fold increase) and further increased (fivefold) 7 d later. By contrast, exposure of the extraradical mycelium of *G. intraradices* to Cd inhibited *GintMT1* transcription at all time points examined.

Regulation of *GintMT1* by oxidative stress

To gain more information about the putative roles of *GintMT1* and taking into consideration that MTs may also play a role in scavenging reactive oxygen species, the effect of paraquat, an intracellular superoxide generator, on *GintMT1* expression was analyzed. Addition of paraquat to the extraradical mycelium of *G. intraradices* clearly induced an increase of *GintMT1* transcript levels at all time points analyzed. A twofold induction was detected 12 and 24 h after the addition of paraquat, and a ninefold up-regulation was observed after 7 d exposure (Fig. 3b). These

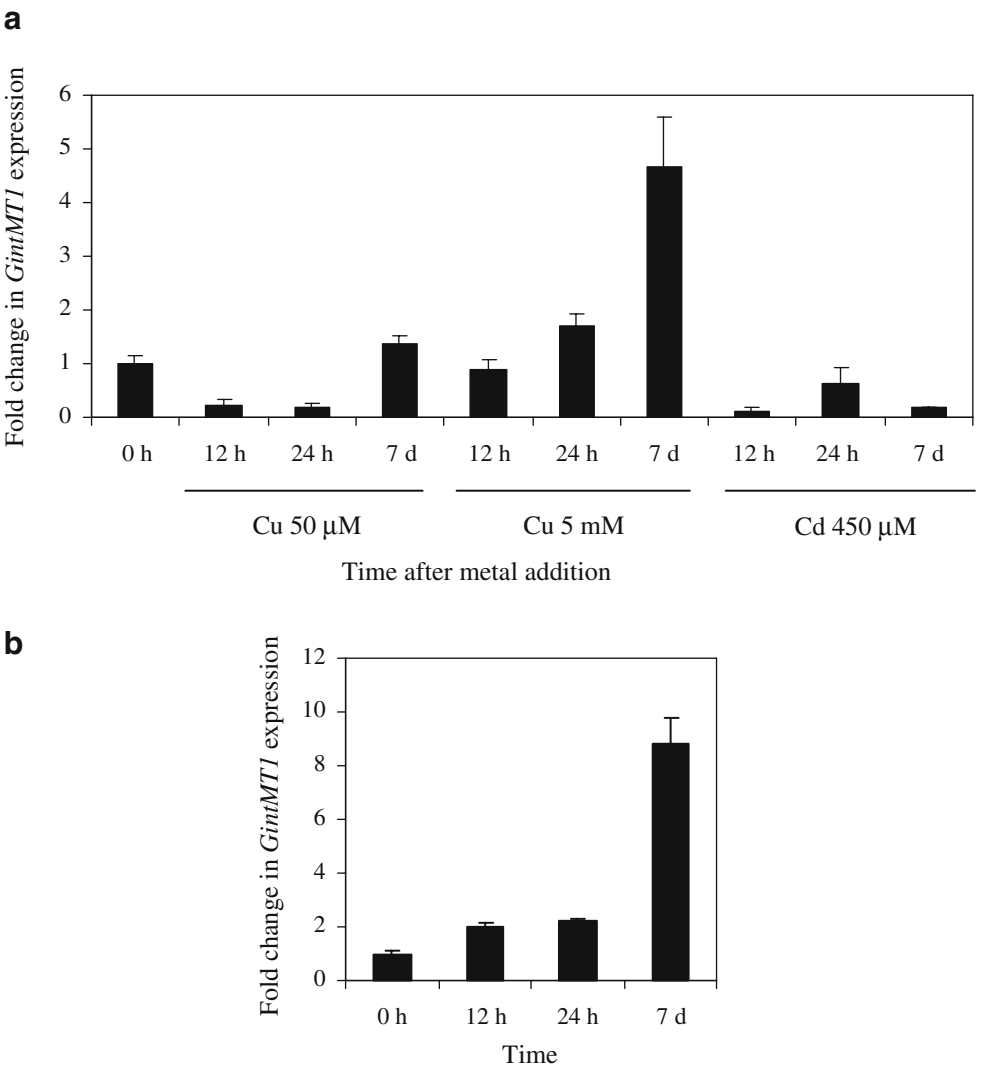

Fig. 3 **a** Time course analysis of *GintMT1* expression in *G. intraradices* extraradical mycelium grown in M-C medium after the addition of 50 μM Cu, 5 mM Cu or 0.45 μM Cd. **b** Time course analysis of *GintMT1* expression in *G. intraradices* extraradical mycelium grown in M-C medium after the addition of 500 μM paraquat. Gene expression was studied by RT-PCR using specific primers for 18S rRNA and *GintMT1*. The relative levels of transcription were calculated by using the $2^{-\Delta\Delta C_T}$ method. Bars represent SD of the means of three independent experiments

data suggest that *GintMT1* may act as an antioxidant in *G. intraradices*.

Analysis of the oxidative stress caused by HM exposure

The finding that *GintMT1* transcript levels increased in mycelia exposed to paraquat and the consideration that some HM have been reported to cause oxidative stress in different organisms (de Vos et al. 1992; Gallego et al. 1996; Yamamoto et al. 1997) prompted us to check whether regulation of *GintMT1* gene expression by Cu and Cd could be linked to the oxidative status of the fungus.

Oxidative stress was estimated by measuring the level of oxidative damage of membrane lipids in the Cu- and Cd-treated mycelia, more precisely by using the levels of malondialdehyde formed by the alteration of polyunsaturated acids due to peroxidation. In control mycelia, lipid peroxidation did not change during the time-course experiment (data not shown). Relative to the control mycelia, addition of 5 mM Cu caused a great increase in lipid peroxidation at all time points analyzed (Fig. 4a); however at 50 μM Cu, lipid peroxidation was only increased 1 week after the addition of the metal. Exposure of *G. intraradices* extraradical mycelia to Cd increased the level of lipid peroxidation at all time points analyzed, being this increase higher 24 h post-addition of the metal. As expected, exposure of the mycelia to paraquat clearly increased lipid peroxidation (Fig. 4b).

Discussion

The results presented in this paper describe the characterization of a new MT gene from *G. intraradices* that provides tolerance to a Cu-sensitive yeast strain and is transcriptionally regulated by Cu, Cd, and paraquat in the extraradical mycelia of *G. intraradices*.

Although the most widely accepted role for MTs is metal detoxification, several studies have indicated that MTs play a role in the protection against the effect of

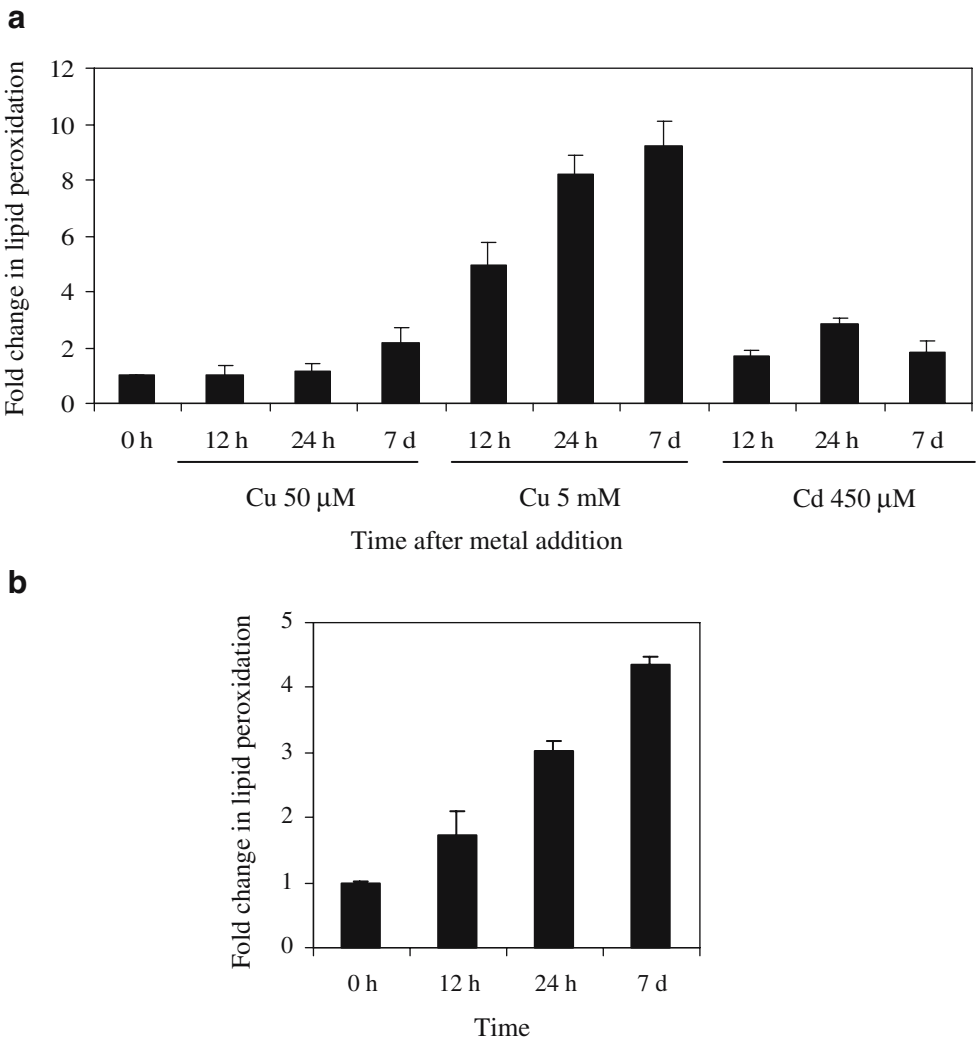

Fig. 4 **a** Lipid peroxidation levels in *G. intraradices* extraradical mycelium grown in M-C medium after the addition of 50 μM Cu, 5 mM Cu or 450 μM Cd. **b** Lipid peroxidation levels in *G. intraradices* extraradical mycelium grown in M-C medium after the addition of 0.5 mM paraquat. Data are expressed as the relative amount of malondialdehyde referred to the controls. Bars represent SD of the means of three independent experiments

reactive oxygen species. In fact, the MT protein itself acts as an antioxidant as it is a potent scavenger of hydroxyl radicals (Andrews and Geiser 1999). Our expression analyses revealed that the transcript levels of *GintMT1* were elevated in mycelia treated with 5 mM Cu or paraquat but inhibited in mycelia treated with 50 μM Cu or 450 μM Cd. The elevated expression of *GintMT1* in the 5 mM Cu-treated mycelia together with the ability of *GintMT1* to provide tolerance to a Cu-sensitive yeast suggests that *GintMT1* might afford protection against Cu, as it was hypothesized for the MT gene *GmarMT1* of *G. margarita* (Lanfranco et al. 2002). However, down-regulation by a lower Cu concentration as well as by Cd suggests that the main role of *GintMT1* in *G. intraradices* is not related to HM detoxification, as it could be concluded from the yeast complementation assays showing the ability of *GintMT1* to bind both Cu and Cd. Up-regulation of *GintMT1* expression by paraquat indicates that in *G. intraradices*, expression of this MT gene is related to the levels of reactive oxygen species, as it was confirmed by the oxidative damage induced by paraquat in the extraradical mycelia, and suggests a role for *GintMT1* in maintaining the fungal redox balance. Consistent with this protective function, MT synthesis is consistently induced by a variety of agents and conditions producing oxidative stress in different organisms (Bonneton et al. 1996; Murphy et al. 1999; Chen et al. 2004).

Because Cu is a redox-active metal that can be toxic through its participation in Fenton or Haber-Weiss reactions producing hydroxyl radicals (Halliwell and Gutteridge 1989), *GintMT1* might be involved in maintaining the redox balance either by sequestering Cu and preventing deleterious Fenton reactions or by directly scavenging deleterious oxygen radicals. The finding that treatment of the mycelia with 5 mM Cu increased the level of lipid peroxidation 12 h after Cu addition whereas the induction of *GintMT1* was not evidenced till 12 h later suggests a direct role for *GintMT1* in scavenging reactive oxygen species under these experimental conditions.

Cd, a metal without redox properties per se, causes an increase in the levels of lipid peroxidation. Consistent with this observation, it has been reported in various organisms that Cd indirectly induces oxidative stress by depleting free-radical scavengers such as glutathione and protein-bound sulphydryl groups (Schutzendubel and Polle 2002). However, down-regulation of *GintMT1* expression in both the Cd- and 50 μM Cu-treated mycelia suggests an alternative role for *GintMT1*, which does not necessarily involve HM sequestration or oxidative stress protection. Down-regulation of MT gene expression by various metals has been reported in different plant (Kawashima et al. 1991) and animal species (Costello et al. 2004) and has led to the hypothesis that MTs might be involved in the scavenging and transfer of HM to metalloproteins (Costello et al. 2004; Feng et al. 2005) or in stress signalling (Wong et al. 2004). Both of the above-described roles could be valid for *GintMT1*. In the case of acting as a metal scavenger, the inhibitions would be interpreted as the response to a situation in which the levels of cytosolic Cu rise and therefore, there is no need for a high affinity chelating system. Cd inhibition would be caused by its particular chemistry, which allows this element to mimic some bioelements such as Zn and Cu (Nath et al. 1984; Kaur et al. 2006). The other explanation suggests that *GintMT1* inhibition would contribute to potentiate the generation of a free-radical-mediated signal, in a similar way than the rice MT OsMTb2 (Wong et al. 2004). Transient down-regulation OsMTb2, a reactive oxygen scavenger, during the oxidative burst phase during stress signalling is explained as a way to potentiate the accumulation of reactive oxygen species, which function as signals that trigger plant response to stress. However, with the available data, it is not possible to attribute a biological role to *GintMT1* inhibition. Nevertheless and regardless of the explanation for the inhibition of *GintMT1* transcription, it is clear that once a certain level of oxidative damage is reached, as evidenced by the level of membrane lipid peroxidation, *GintMT1* transcription is enhanced.

In the plant pathogenic fungi *Magnaportha grisea* (Tucker et al. 2004) and *Uromyces fabae* (Jakupovic et al. 2006), MTs have been involved in the plant–fungus interaction. The MT-like protein NMT1 of *M. grisea*, which acts as a powerful antioxidant, is essential for appressorium-mediated penetration of intact leaf surfaces and plays a novel role in fungal cell-wall biochemistry that is required for fungal virulence (Tucker et al. 2004). Future studies on the regulation of *GintMT1* along the life cycle of *G. intraradices* might shed some light on its role in the interaction of the AM fungus with its host plant.

In conclusion, all these data together suggest that *GintMT1* might play a key role in the regulation of the redox status of the extraradical mycelia of *G. intraradices* through either its metal chelation activity or its thiol groups, which might contribute to the pool of cytosolic thiols that regulate fungal redox status.

Acknowledgements This work was funded by the EU project Genomyca (QLK5-CT-2000-01319). Manuel González-Guerrero was supported by a FPU fellowship from the Spanish Ministry of Education, Culture and Sports. We are grateful to Dr. Maria Harrison for providing the *GintMT1* partial clone and Dr. Simone Ottonello for the gift of the *S. cerevisiae* strain DTY113. We also want to thank Drs. José Miguel Barea and Alberto Bago for helpful discussions and Ascensión Valderas for excellent technical assistance.

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Andrews GK, Geiser J (1999) Expression of the mouse metal-lothionein-I and -II genes provides a reproductive advantage during maternal dietary zinc deficiency. J Nutr 129:1643–1648

Bago B, Pfeffer PE, Douds DD, Brouillette J, Bécard G, Shachar-Hill Y (1999) Carbon metabolism in spores of the arbuscular mycorrhizal fungus *Glomus intraradices* as revealed by nuclear magnetic resonance spectroscopy. Plant Physiol 121:263–270

Beuge JA, Aust SD (1978) Microsomal lipid peroxidation. Meth Enzymol 52:302–310

Bonneton F, Théodore L, Silar P, Maroni G, Wegnez M (1996) Response of *Drosophila* metallothionein promoters to metallic, heat shock and oxidative stress. FEBS Lett 380:33–38

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

Chabot S, Bécard G, Piché Y (1992) Life cycle of *Glomus intraradix* in root organ culture. Mycologia 84:315–321

Chen BD, Li XL, Tao HQ, Christie P, Wong MH (2003) The role of arbuscular mycorrhiza in zinc uptake by red clover growing in a calcareous soil spiked with various quantities of zinc. Chemosphere 50:839–846

Chen L, Wu W, Dentchev T, Zeng Y, Wang J, Tsui I, Tobias JW, Bennett J, Baldwin D, Dunaief JL (2004) Light damage induced changes in mouse retinal gene expression. Exp Eye Res 79:239–247

Cobbett C, Goldsbrough P (2002) Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. Annu Rev Plant Biol 53:159–182

Costello LC, Guan Z, Franklin RB, Feng P (2004) Metallothionein can function as a chaperone for zinc uptake transport into prostate and liver mitochondria. J Inorg Biochem 98:664–666

del Val C, Barea JM, Azcón-Aguilar C (1999a) Diversity of arbuscular mycorrhizal fungus populations in heavy-metal-contaminated soils. Appl Environ Microbiol 65:718–723

del Val C, Barea JM, Azcón-Aguilar C (1999b) Assessing the tolerance to heavy metals of arbuscular mycorrhizal fungi isolated from sewage sludge-contaminated soils. Appl Soil Ecol 11:261–269

de Vos RCH, Vonk MJ, Vooijs R, Schat H (1992) Glutathione depletion due to copper-induced phytochelatin synthesis due to oxidative stress in *Silene vulgaris*. Plant Physiol 98:853–858

Ecker DJ, Butt TR, Sternberg EJ, Neeper MP, Debouck C, Gorman JA, Crooke ST (1986) Yeast metallothionein function in metal ion detoxification. J Biol Chem 261:16895–16900

Feng W, Cai J, Pierce WM, Franklin RB, Maret W, Benz FW, Kang YJ (2005) Metallothionein transfers zinc to mitochondrial aconitase through a direct interaction in mouse hearts. Biochem Biophys Res Commun 332:853–858

Fowler BA, Hildebrand CE, Kojima Y, Webb M (1987) Nomenclature of metallothionein. Experientia Suppl 52:21

Gallego SM, Benavides MP, Tomaro ML (1996) Effect of heavy metal ion excess on sunflower leaves: evidence for involvement of oxidative stress. Plant Sci 121:151–159

Gohre V, Paszkowski U (2006) Contribution of the arbuscular mycorrhizal symbiosis to heavy metal phytoremediation. Planta 223:1115–1122

González-Chavez C, D'Haen J, Vangronsveld J, Dodd JC (2002) Copper sorption and accumulation by the extraradical mycelium of different *Glomus* spp. (arbuscular mycorrhizal fungi) isolated from the same polluted soil. Plant Soil 240:287–297

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 Genet Biol 42: 130–140

Halliwell B, Gutteridge JMC (1989) Free radicals in biology and medicine. Clanderon, Oxford, UK

Hildebrandt U, Kaldorf M, Bothe H (1999) The zinc violet and its colonization by arbuscular mycorrhizal fungi. J Plant Physiol 154:709–717

Jakupovic M, Heintz M, Reichmann P, Mendgen K, Hahn M (2006) Microarray analysis of expressed sequence tags from haustoria of the rust fungus *Uromyces fabae*. Fungal Genet Biol 43:8–19

Kaur J, Sharma N, Attri S, Gogia L, Prasad R (2006) Kinetic characterization of zinc transport process and its inhibition by cadmium in isolated rat renal basolateral membrane vesicles: in vitro and in vivo studies. Mol Cell Biochem 283:169–179

Kawashima I, Inokuchi Y, Chino M, Kimura M, Shimizu N (1991) Isolation of a gene for a metallothionein protein from soybean. Plant Cell Physiol 32:913–916

Kojima Y (1991) Definitions and nomenclature of metallothioneins. Meth Enzymol 205:8–10

Lanfranco L, Bolchi A, Ros E, Ottonello S, Bonfante P (2002) Differential expression of a metallothionein gene during the presymbiotic versus the symbiotic phase of an arbuscular mycorrhizal fungus. Plant Physiol 130:58–67

Livak K, Schmittgen T (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta}C_T$ method. Methods 25:402–408

Malcová R, Rydlová J, Vosátka M (2003) Metal-free cultivation of *Glomus* sp. BEG 140 isolated from Mn-contaminated soil reduces tolerance to Mn. Mycorrhiza 13:151–157

Maret W (2003) Cellular zinc and redox states converge in the metallothionein/thionein pair. J Nutr 133:1460S–1462S

Meharg AA (2003) The mechanistic basis of interaction between mycorrhizal associations and toxic metal cations. Mycol Res 107:1253–1265

Minet M, Dufour ME, Lacroue F (1992) Complementation of *Saccharomyces cerevisiae* auxotrophic mutants by *Arabidopsis thaliana* cDNAs. Plant J 2:417–422

Murphy BJ, Andrews GK, Bittel D, Discher DJ, McCue J, Green CJ, Yanovsky M, Giaccia A, Sutherland RM, Laderoute KR, Webster KA (1999) Activation of metallothionein gene expression by hypoxia involves metal response elements and metal transcription factor-1. Cancer Res 59:1315–1322

Nath R, Prasad R, Palinal VK, Chopra RK (1984) Molecular-basis of cadmium toxicity. Prog Food Nutr Sci 8:109–163

Ouziad F, Hildebrandt U, Schmelzer E, Bothe H (2005) Differential gene expression in arbuscular mycorrhizal-colonized tomato grown under heavy metal stress. J Plant Physiol 162:634–649

Rivera-Becerril F, Calantzis C, Turnau K, Caussanel JP, Belimov AA, Gianinazzi S, Strasser RJ, Gianinazzi-Pearson V (2002) Cadmium accumulation and buffering of cadmium-induced stress by arbuscular mycorrhiza in three *Pisum sativum* L. genotypes. J Exp Bot 53:1177–1185

Schutzendubel A, Polle A (2002) Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization. J Exp Bot 53:1351–1365

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

Stommel M, Mann P, Franken P (2001) EST-library construction using spore RNA of the arbuscular mycorrhizal fungus *Gigaspora rosea*. Mycorrhiza 10:281–285

Tamai KT, Gralla EB, Ellerby LM, Valentine JS, Thiele DJ (1993) Yeast and mammalian metallothioneins functionally substitute for yeast copper-zinc superoxide dismutase. Proc Natl Acad Sci USA 90:8013–8017

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

Tucker SL, Thornton CR, Tasker K, Jacob C, Giles G, Egan M, Talbot NJ (2004) A fungal metallothionein is required for pathogenicity of *Magnaporthe grisea*. Plant Cell 16:1575–1588

Weissenhorn I, Mench M, Leyval C (1995) Bioavailability of the heavy metals and arbuscular mycorrhiza in a sewage-sludge-amended sandy soil. Soil Biol Biochem 27:287–296

Wong HL, Sakamoto T, Kawasaki T, Umemura K, Shimamoto K (2004) Down-regulation of metallothionein, a reactive oxygen scavenger, by the small GTPase OsRac1 in rice. Plant Physiol 135:1447–1456

Yamamoto Y, Hachia A, Matsumoto H (1997) Oxidative damage to membranes by a combination of aluminum, and iron in suspension-cultured tobacco cells. Plant Cell Physiol 38:133–1339