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ORIGINAL PAPER

GintABC1 encodes a putative ABC transporter of the MRP subfamily induced by Cu, Cd, and oxidative stress in *Glomus intraradices*

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Abstract A full-length cDNA sequence putatively encoding an ATP-binding cassette (ABC) transporter (*GintABC1*) was isolated from the extraradical mycelia of the arbuscular mycorrhizal fungus *Glomus intraradices*. Bioinformatic analysis of the sequence indicated that *GintABC1* encodes a 1513 amino acid polypeptide, containing two six-transmembrane clusters (TMD) intercalated with sequences characteristics of the nucleotide binding domains (NBD) and an extra N-terminus extension (TMD0). GintABC1 presents a predicted TMD0-(TMD-NBD)$_2$ topology, typical of the multidrug resistance-associated protein subfamily of ABC transporters. Gene expression analyses revealed no difference in the expression levels of *GintABC1* in the extra- vs the intraradical mycelia. *GintABC1* was up-regulated by Cd and Cu, but not by Zn, suggesting that this transporter might be involved in Cu and Cd detoxification. Paraquat, an oxidative agent, also induced the transcription of *GintABC1*. These data suggest that redox changes may be involved in the transcriptional regulation of GintABC1 by Cd and Cu.

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Introduction

The ATP-binding cassette (ABC) family of transporters has a widespread distribution in nature (Higgins 1992), with multiple representatives in a given genome. For instance, 150 have been described in *Arabidopsis thaliana* and 30 in *Saccharomyces cerevisiae* (Martinoia et al. 2002; Jungwirth and Kuchler 2006; Verrier et al. 2008). These two facts highlight the importance these transporters have in biological systems and hint at their functional versatility. In fact, ABC transporters are involved in a stunning variety of cellular processes, such as maintenance of mitochondrial function, peroxisome biogenesis, export of Fe/S clusters, heavy metal detoxification, removal of toxic catabolic compounds, or pheromone transport, among others (Borst et al. 1999; Ketchum et al. 2001; Martinoia et al. 2002; Chen et al. 2007b; Footitt et al. 2007). This is possible due to the broad range of substrates of the ABC transporters: ions, nutrients, phospholipids, peptides, and even whole proteins, in a process that is coupled to the hydrolysis of ATP (Higgins 1992).

In spite of the broad range of substrates, ABC transporters share the same structural architecture, consisting of two transmembrane domains (TMDs), each of them consisting of six helices, and two nucleotide binding domains (NBDs), responsible for ATP binding and hydrolysis (Higgins 1992). The NBDs present two conserved sequences responsible for ATP binding, Walker A (GXXGXGK(S/T)) and Walker B ($RX_{6-8}hyd_4D$), which

are shared with other ATPases (Walker et al. 1982). Between these two motifs is the signature sequence or C-loop (LSXGX(K/R)), unique to ABC transporters. The different subfamilies of ABC transporters differ on how the NBDs and TMDs are organized. Their arrangement varies from the multidrug resistance-associated protein (MRP) subfamily, which presents a single coding frame in which the organization is $(TMD\text{-}NBD)_2$ (Borst et al. 1999), to transporters associated with antigen processing proteins in which two genes codify for a functional protein, each with a TMD-NBD arrangement (van Endert et al. 2002), to bacterial ABCs in which each of the four elements is codified as a separate coding frame (Davidson and Chen 2004).

In fungi, the most common ABC transporters are the so-called full-size ABC transporters, in which the required arrangement of domains is contained in one polypeptide chain (Del Sorbo et al. 2000). They are largely confined to the multidrug resistance (MDR), MRP, and pleiotropic drug resistance (PDR) protein subfamilies. At the structural level, members of the MDR and MRP present the characteristic $(TMD\text{-}NBD)_2$ topology, while members of the PDR subfamily present the reverse topology $(NBD\text{-}TMD)_2$. Moreover, some MRPs present an extra N-terminus extension (NTE), containing a TMD0 consisting of five helices and involved in protein sorting (Mason and Michaelis 2002). The diversity of function of these transporters is manifest not only at the subfamily level but also in individual members of the subfamily (for a review, see Jungwirth and Kuchler 2006).

Members of the MRP subfamily transport drug complexes with more soluble products such as glutathione, taurocholate, or glucosides (Li et al. 1996; Klein et al. 2000; Gerk et al. 2007), and in this form, the drugs are eliminated from the cytosol. This aspect hints at the physiological role of MRP transporters, since establishing complexes with soluble products is one of the main processes by which lipophilic compounds, such as some xenobiotics (herbicides, anti-cancer drugs, etc.) or products from the metabolism, are detoxified (Bock et al. 1987). In addition, MRP transporters are also involved in heavy metal tolerance, participating in the transport to the vacuole of Cu/Cd-glutathione or Cu/Cd-phytochelatin complexes (Ortiz et al. 1995; Li et al. 1996). Removal of this transport activity in yeasts results in hypersensitivity to Cd (Wemmie et al. 1994).

In spite of their importance and abundance, no ABC transporter has been identified in mycorrhizal fungi to date. Arbuscular mycorrhizal (AM) fungi are able to tolerate a wide range of metal concentrations in soils, thereby protecting plants from metal toxicity. Although a body of literature exists on the effects of heavy metals on arbuscular mycorrhiza (Leyval et al. 1997; Meharg 2003; Göhre and Paszkowski 2006), only a few studies focus on the fungal side of the symbiosis, and even fewer on the molecular basis of heavy metal homeostasis in AM fungi (González-Guerrero et al. 2009). In a recent study using a combination of transmission electron microscopy and energy-dispersive X-ray spectroscopy, we showed that upon exposure of the AM fungus *Glomus intraradices* to high concentrations of either Cu, Zn, or Cd, the cytoplasmic concentrations of heavy metals were kept low, whereas vacuoles had the highest intracellular concentrations of heavy metals (González-Guerrero et al. 2008). Accumulation of heavy metals in the AM fungal vacuoles implies the presence of a number of heavy metal transporters involved in loading these organelles. However, to date, only two heavy metal transporters have been described to some extent in AM fungi: the cation diffusion facilitator (CDF) Zn transporter GintZnT1 that might be involved in Zn detoxification in the fungal vacuole (González-Guerrero et al. 2005) and a Cu-regulated ZRT/IRT-like protein (ZIP) transporter whose function remains to be ascertained (Ouziad et al. 2005; Hildebrandt et al. 2007). In this work, we report the isolation and expression analysis of *GintABC1*, the first ABC transporter gene described to date in AM fungi. *GintABC1* presents an expression pattern consistent with a role in Cu, Cd, and oxidative stress protection.

Materials and methods

Biological material

G. intraradices 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 was grown together with the AM fungus *G. intraradices* Smith & Schenck (DAOM 197198, Biosystematic Research Center, Ottawa, Canada), in two-compartment Petri dishes. Cultures were initiated in the "root compartment", 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 extraradical hyphae growing in HCs started the transition from absorptive to sporulative phase (6–8 weeks).

G. intraradices treatments

Different experiments were set up to analyze the effect of the metals Cu, Cd, and Zn, and also of the oxidative agent paraquat, on *GintABC1* expression in the extraradical mycelia (ERM) of *G. intraradices*. Each metal or paraquat

was applied as a pulse to the M-C medium once the ERM were well established on it (transition from absorptive to sporulative phase). $CuSO_4$, $CdSO_4$, $ZnSO_4$, or paraquat was applied to their respective HCs to obtain final concentrations of 5 mM Cu, 0.45 mM Cd, 7.5 mM Zn, or 0.5 mM paraquat. This was done by distributing 500 µl of each filter-sterilized stock solution dropwise, so that evenly diffusion of the added solutions was ensured. The time point just before metal/paraquat addition is referred to "Time 0". After the pulse, ERM were harvested at time points 6 h, 12 h, 24 h, and 7 days.

Sample recovery and RNA extraction

ERM from the different HCs were recovered by blending (5 s 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). Mycorrhizal carrot roots were recovered from the RCs 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 binocular microscope. Mycorrhizal root colonization was confirmed by visual observation of the fungal structures under a stereomicroscope after trypan blue staining (Phillips and Hayman 1970). Samples of ERM or roots were immediately liquid nitrogen-frozen and stored at −80°C until used. RNAs were extracted using the RNeasy Plant Mini Kit (Qiagen, Germantown, MD, USA) following the manufacturer's instructions.

Cloning and sequence analysis of *GintABC1*

A genomic fragment of 917 bp with homology to MRP transporters was identified in a *G. intraradices* sequence database (http://darwin.nmsu.edu/~plammers/glomus; Bago et al. 2003). The 3′ and 5′ ends were obtained by rapid amplification of cDNA ends (RACE) using the SMART RACE cDNA Amplification kit (Clontech, PaloAlto, CA, USA), the *GintABC1* specific primers ABC1-A (for the 3′ end) 5′-AGAGATAGAGAAACAAGTCAACCTG-3′ or ABC1-B (for the 5′ end) 5′-TAATCATAATACCCACAC CAGCATA-3′, and 1 µg total RNA from ERM grown under standard conditions in compartmented plates. The amplified cDNAs were cloned in the pYES2.1 TOPO TA vector (Invitrogen, Carlsbad, CA, USA).

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). The Kimura two-parameter method was used to estimate distances, and the phylogenetic analysis was performed by the neighbor-joining method by using PHYLIP (Felstein 1993). The relative support of the different clades was determined based upon 100 bootstrap trees. The phylogenetic tree was displayed with the help of the TREEVIEW program (Page 1996). Predicted topology of the protein was obtained with the program TM-pred (Hofmann and Stoffel 1993).

Analysis of gene expression

Gene expression was studied by quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) using 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), according to the manufacturer's protocol. The primer set used to amplify *GintABC1* in the synthesized cDNAs were ABC1-A and ABC1-B whose specificity for amplification of *G. intraradices* nucleic acids was confirmed by performing conventional RT-PCR on RNA from *G. intraradices* ERM and from non-mycorrhizal carrot roots. 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 a 5-min incubation at 95°C to activate the hot-start recombinant Taq DNA polymerase, followed by 35 cycles of 30 s at 95°C, 45 s at 55°C, and 45 s 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°C to 100°C) after the final cycle of the PCR. The efficiency of the primer set was evaluated by performing real-time 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′-TGTTAATAAAAATCGGTGCGT TGC-3′ and RMR: 5′-AAAACGCAAATGATCAACC GGAC-3′. Real-time PCR determinations were carried out with RNA extracted from three independent biological samples, with the threshold cycle (C_t) determined in triplicate. The relative levels of transcription were calculated by using the $2^{-\Delta\Delta ct}$ method (Livak and

Schmittgen 2001). Data were subjected to ANOVA and, when appropriate, to the Tukey's honestly significant difference test ($P<0.05$). In all RT-PCR reactions, a non-RT control was used to detect any possible DNA contamination.

Results

A 917-bp DNA fragment with homology to MRP transporters was identified by exploring a *G. intraradices* sequence database (clone Bgl53-T7). This fragment comprises at least one intron and two exons. In order to obtain the complete coding sequence, 3′ and 5′ RACE were performed in total RNA using exon-specific primers. *In silico* translation of the full-length *GintABC1* cDNA revealed an open reading frame encoding a 1513 amino acid polypeptide with a predicted molecular weight of 172 kDa (Fig. 1a). Comparison with the amino acid sequence databases showed that it is related to fungal members of the MRP subfamily of ABC transporters, presenting the highest degree of homology to the yeast cadmium factor 1 protein (ycf1) from *S. cerevisiae* (Szczypka et al. 1994), with 44% identity and 61% similarity.

In silico analysis of the protein topology reveals 17 TMDs in the deduced amino acid sequence of *GintABC1*, with a predicted $(TMD\text{-}NBD)_2$ topology, each TMD consisting of six helices, and a TMD0 domain consisting of five helices (Fig. 1b). This domain architecture is typical of ABC transporters belonging to the MRP subfamily, such as the ycf1 from *S. cerevisiae*. The NBDs present the classical Walker A and B and the C-loop motives, at positions 646, 748, and 736, respectively, for NBD1 and at positions 1293, 1408, and 1396, respectively, for NBD2. The only difference with the common signature motives of MRP transporters refers to the C-loop motif of NBD2. Instead of the residue leucine of the common LSXGX(K/R) motif, there is a phenylalanine.

A phylogenetic analysis of GintABC1 with representative members of the MDR, MRP, and PDR subfamilies of fungal ABC transporters clearly demonstrates that GintABC1 is grouped into the MRP subfamily (Fig. 2). Thus, GintABC1 can be classified as a new member of this subfamily.

To get some insight into the putative roles of GintABC1 in *G. intraradices*, its expression profile was analyzed by real-time RT-PCR. Firstly, to determine whether *GintABC1*, as other members of the MRP subfamily, is regulated by heavy metals, its gene expression was assessed in ERM which had been exposed for different periods of time to Cu, Cd, or Zn. The regulation pattern varies among the three metals (Fig. 3). While the expression level of *GintABC1* steadily increases along the 7-day period after Cd addition, it reaches a nearly fourfold induction 12 h after Cu exposure, progressively decreasing thereafter. On the contrary, *GintABC1* expression was not affected by the supplementation with Zn.

Given that MRP transporters have also been reported to be regulated by oxidative stress (Maher et al. 2007), the effect of paraquat, an intracellular superoxide generator, on *GintABC1* expression was also analyzed. As it is shown in Fig. 4, the addition of 500 μM paraquat to *G. intraradices* ERM induces an increase in *GintABC1* transcription levels at all time points analyzed. A twofold induction was detected 12 and 24 h after the addition of paraquat, and a threefold up-regulation was observed after 7 days exposure.

Since some fungal ABC transporters, such as the vacuolar MRP-like transporter1 from *Candida albicans*, are involved in fungal virulence (Theiss et al. 2002), to learn more about the putative roles of GintABC1, we investigated whether it was differentially expressed in the ERM and the structures the fungus develops inside the roots. This was done by analyzing *GintABC1* expression on RNA from ERM developed in the HC of the split-Petri dishes and on RNA from the *G. intraradices*-colonized carrot roots developed in the root compartment, from which ERM had been removed under the dissection microscope. While no transcripts were detected in non-mycorrhizal carrot roots, *GintABC1* transcripts were detected in the ERM and in the carrot mycorrhizal roots lacking ERM, with no significant differences between the expression levels in the intra- and extraradical fungal structures (Fig. 5).

Discussion

At low levels, heavy metal cations are essential for the completion of multiple biological processes, such as oxidative respiration or free radical control (Fraústro da Silva and Williams 2001). However, at slightly higher levels they have deleterious effects, such as promoting oxidative damage to membranes or DNA. For this reason, living beings have developed a complex system to control and maintain heavy metal homeostasis, which involves metal transporters, metallochaperones, and metallothioneins (Cherian and Chan 1993; O'Halloran and Culotta 2000; Eide 2004; Argüello et al. 2007). Here, we describe *GintABC1*, a *G. intraradices* gene encoding an ABC transporter, whose gene product might be involved in Cd and Cu tolerance.

AM fungi are able to withstand high levels of Cu, Cd, and Zn, both when growing in high-metal soils in natural or polluted environments and also when developing in *in vitro* cultures (Pawlowska and Charvat 2004; Chen et al. 2007a).

a

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   1  MLCRDKDGWG PWRPDLPNQQ FPDFTDCFEE GVILTSVNLL LIVLGFYEIR
  51  RYSKKHAVLP LHALNNWHNI AITITLYLLI ALSIINFVST LWSDWQLLNI
 101  MVISSFINLF TMFVAYSVYK KSYTHSHVSS PVLLLYWLFY LIAHILKLRT
 151  LVLMGYASKP FYFFTTLFST ILVLVVFVLE LLPKPQSDYE LINGDDNLNC
 201  PEESANIFSR STFYWMTPLM KLGHQKFLTM DDLWNLDPQY RSKKISEDFD
 251  VAWNKELKKK NPSLLRAITL TFGGQFAFAA AFKAVQDILN FVQPQLLGEL
 301  MEFVNSQRDR ETSQPAYRGY CIAILMFVTA VIQTMFLHQY FQLCFISGMR
 351  VKAALVTAIY QKAFKLSNTS RQKSTVGEIV NHMSVDAQEL MDLFTYLHIA
 401  WSGPLQIILA LYFLHQTMGV STYAGVGIMI MMVPVNAYLA NKMKILQKKQ
 451  MKNKDERIKL MVSLYNEILN GIKVIKLYAW EQAFLKKVRN DLELKTLKRL
 501  GYLYAVQSFT WTSTVSHLFP IFTPFLVSFA TFAVYVLISN SPLTVQVVFV
 551  AIPLFNLLQF PLAVFPSVIT SIIEASVALR RVEEYLTSEE LDPKAVIRQG
 601  YYDTEDERSE LVPVKNGTFG WGNSGEAVLE DINLSVKKGE LVAIVGKVGA
 651  GKSSLLSSLL GEMEKIGGEV IVKGHVAYVH QTPWIMNATL RDNITFGYEY
 701  KPELYDEIIE ACALKPDIAI LPGGDLTEIG EKGINLSGGQ KARVALARAV
 751  YARADVYLFD DTLSAVDAHV GKHIFDKVVG SNGILRTKAR IFVTHGIHYL
 801  SKTDSVVMMR DGKIIEQGHF DSLMKLKSEL FNLIDEFGQQ EESNNLLDDE
 851  PPDDPEELMP LAYETDEVAT DQRSEETVSQ LRERRVSVPS IHRRASTATV
 901  KNESKREQQK NELITKEEMA KGSVSWQVYS SYLKSCGVVT ITFWIITLVI
 951  SQGIQVATNV FLKYWSSEES NERILLYFVI YGLLGLLFSL MVIFQTIVLW
1001  VFCFFRAARK LHHQMLDGVI RSPMSFFDTT PLGRILNRFS KDIYTIDELL
1051  PRIFAGYFRT FFVVLSTIFV ISFSTPLFII LIIPMTFMYI YIQTYYLSTS
1101  RELKRLDSVT RSPIYAHFQE TLGGLTTIRA FQQMNRFIRD NETKLDVNQK
1151  AYFPSFSSNR WLAVRLEFLG SIIIFGAAIF SVISVLTTGN IDAGLVGLSV
1201  SYALSVTQAL NWAVRQFCEI ETNIVSVERV KEYIDLPSEA PVVIQDNRPD
1251  PTWPQNGLIE YQNYSTRYRQ GLELVLKGVS FVINPREKVG IVGRTGAGKS
1301  SLTLSLFRLI EAVDGAILMD GVDISKIGLY DLRSRLTIIP QDPILFEGTV
1351  EFNLDPFETH DEVEIWQALQ SAHLKDYISK LEGKLHAKIL EGGDNFSQGQ
1401  RQLLCLARAL LRRSNIIVLD EATACVDVET DFQIQNTIRN EFNWATLLCI
1451  AHRLRTIIDY DRVLVLDEGN VVEFDTPYNL LQNPNSLFYK LCEQSNEFDY
1501  LKDLATKNHS PKR
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b

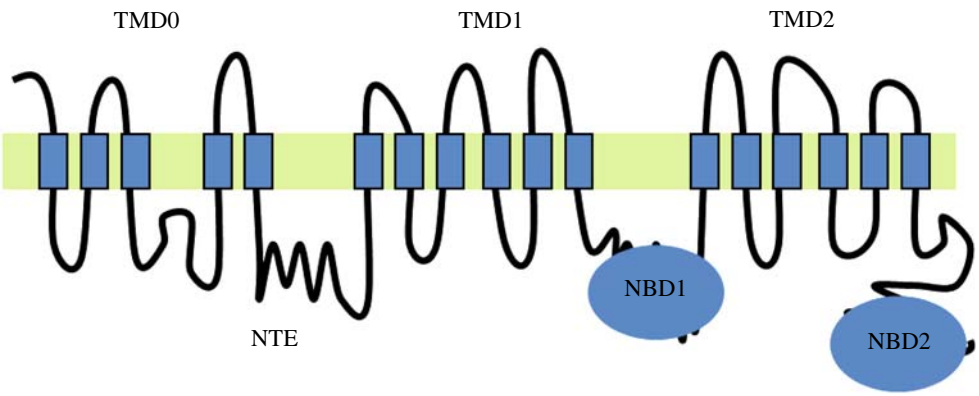

Fig. 1 **a** Amino acid sequence of GintABC1. *Underlined amino acids* indicate predicted transmembrane domains. *Continuous line boxes* indicate the position of the Walker A and B motives. *Discontinuous line boxes* indicate the position of the C-loops. **b** Topology model of GintABC1

Energy-dispersive X-ray spectroscopy of heavy metal-treated AM fungi indicates that once the metals enter the cytosol, they are preferentially accumulated in vacuoles, what would involve the action of specific transporters (González-Guerrero et al. 2008, 2009). To date, only two heavy metal transporters from AM fungi are known, the CDF transporter from *G. intraradices* GintZnT1 and a ZIP transporter from the same fungus (González-Guerrero et al. 2005; Ouziad et al. 2005). However, no member of these two families has been shown to be able to transport Cu, although an effect of Cu in gene expression of the ZIP transporter has been indicated (Hildebrandt et al. 2007).

GintABC1 represents the first ABC transporter described so far in an AM fungus. Bioinformatic analysis of the

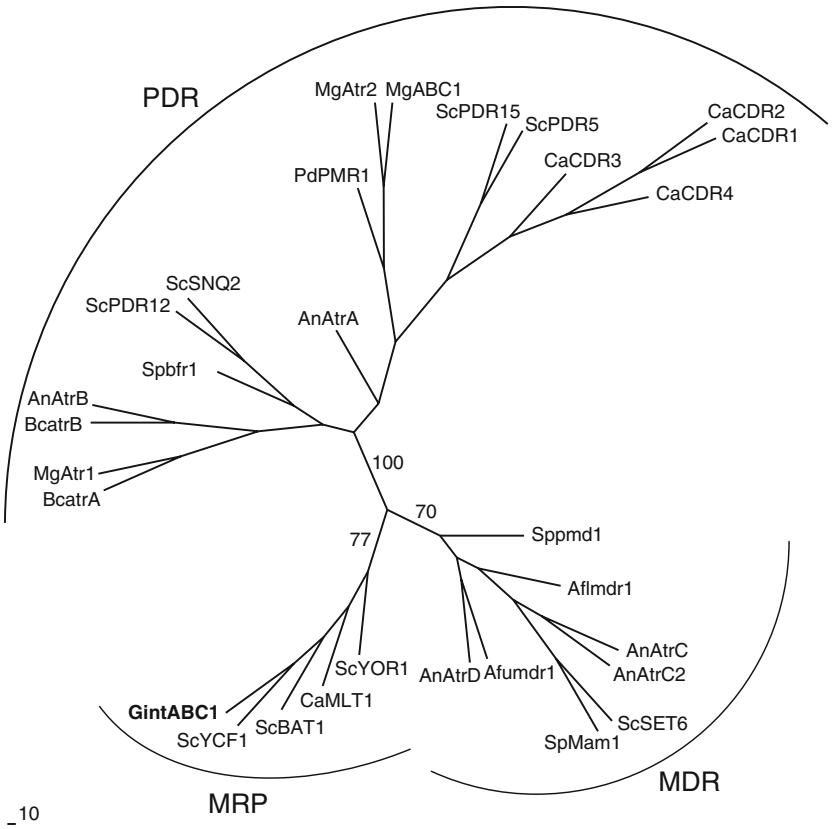

Fig. 2 Unrooted phylogenetic tree of fungal full-size ATP-binding cassette (ABC) transporters. Sequences were obtained from GenBank with the following accession numbers: *Aspergillus flavus* (Aflmdr1: U62931), *Aspergillus fumigatus* (Afumdr1: U62934), *Aspergillus nidulans* (AnAtrA: Z68904; AnAtrB: Z68905; AnAtrC: AF071410; AnAtrC2: AF082072; AnAtrD: AF071411), *Botryotina fuckeliana* (BcatrA: Z68906; BcatrB: AJ006217); *Candida albicans* (CaCDR1: X77589; CaCDR2: U63812; CaCDR3: U89714; CaCDR4: AF044921; CaMLT1: AF110027), *Glomus intraradices* (GintABC1: GQ249346), *Magnaporthe grisea* (MgABC1: AF032443), *Mycosphaerella graminicola* (Mgatr1: AJ243112; Mgatr2: AJ243113), *Penicillium digitatum* (PdPMR1: AB010442), *Saccharomyces cerevisiae* (ScYCF1: Z48179; ScBAT1: Z73153; ScYOR1: Z73066; ScSET6: Z28209; ScPDR5: L19922; ScPDR12: U39205; ScPDR15: U32274; ScSNQ2: X66732), and *Schizosaccharomyces pombe* (SpMam1: U66305; Spbfr1: S76267; Sppmd1: D10695)

GintABC1 amino acid sequence classifies this protein in the MRP subfamily of ABC transporters. The predicted secondary structure further supports GintABC1 as a member of the MRP subfamily consisting of two six-transmembrane clusters resembling the TMDs, intercalated with sequences characteristics of the NBDs containing the Walker A and B motives and a C-loop situated between the two Walker boxes. The Walker motifs in the two NBDs and the C-loop of NBD1 contain the canonical sequences of these motifs. However, the C-loop of NBD2 presents a phenylalanine instead of the critical leucine residue. This type of C-signature motif is usually called a "degenerated C-loop", and it has been previously described for other ABC transporters (Chen et al. 2004). Particularly, the C-loop of NBD2 in GintABC1 (FSQGQR) is identical to the one of another ABC transporter, the sulfonylurea receptor (SUR1). Based on this asymmetry, it has been proposed that one NBD in the complex hydrolyses ATP to provide the energy for translocation, while the other one only binds ATP as a regulatory domain (Yang et al. 2003). GintABC1 sequence also presents an NTE containing a transmembrane TMD0 domain consisting of five helices, as some MRPs, such as *S. cerevisiae* ycf1 do (Mason and Michaelis 2002).

The similarity between ycf1 and GintABC1 and the fact that ycf1 plays a key role in heavy metal detoxification in *S. cerevisiae* suggest a role for GintABC1 in heavy metal tolerance. Our gene expression studies hint at GintABC1 playing a role in Cu and Cd tolerance. After a short exposure to Cd or Cu, *GintABC1* is up-regulated, with higher expression levels reached in the Cd treatments. However, Zn did not induce its transcription. This expression pattern is consistent with that of the AtMRP3 transporter of *A. thaliana* implicated in Cd tolerance (Tommasini et al. 1998). *AtMRP3* transcription was shown to be strongly induced by Cu and Cd, but not by Zn (Bovet et al. 2003; Zientara et al. 2009). Up-regulation by Cd has been also reported for the *AtMRP6* of *A. thaliana* and for the *CrMRP2* transporter of *Chlamydomonas reinhardtii*,

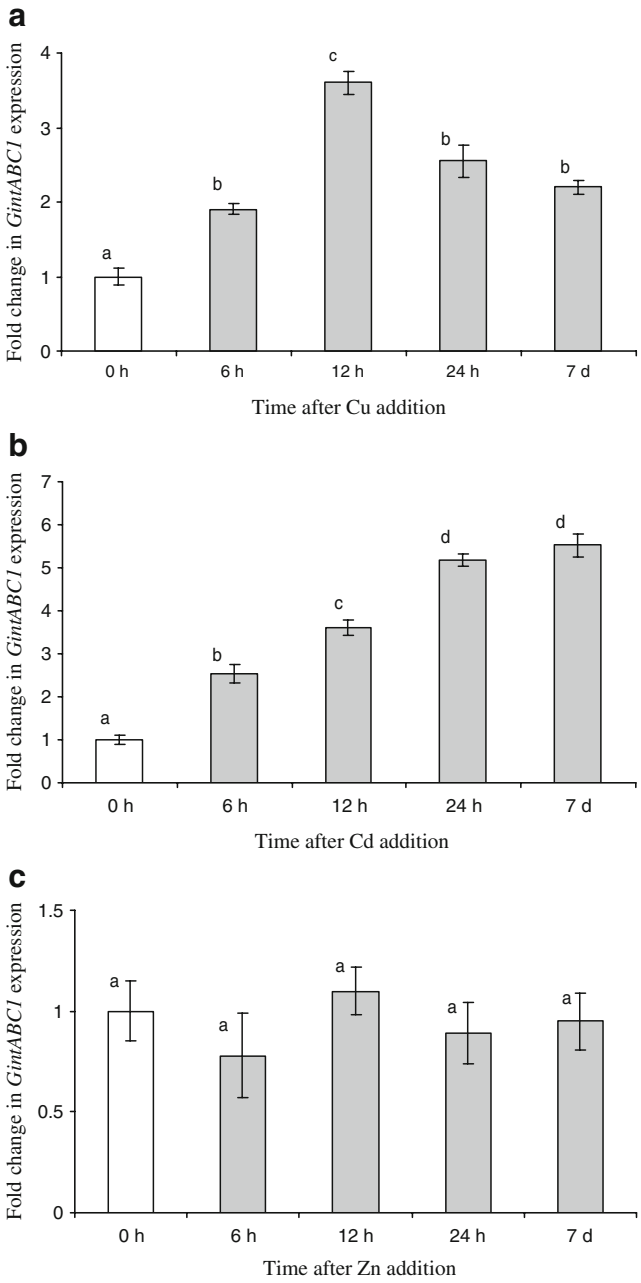

Fig. 3 Time course analysis of *GintABC1* expression in *Glomus intraradices* extraradical mycelia grown in M-C medium after the addition of 5 mM Cu (**a**), 0.45 mM Cd (**b**), or 7.5 mM Zn (**c**). *Bars* represent SD of the means of three independent biological replicates. Data not sharing a letter in common differ significantly ($P<0.05$) according to the Tukey's honestly significant difference test

both involved in Cd transport and detoxification (Wang and Wu 2006; Gaillard et al. 2008). Increased transcript levels of MRP transporters in the presence of high Cu concentrations suggest a role for these transporters in Cu detoxification; however, their Cu transporting activity has not been yet demonstrated. In *S. cerevisiae*, deletion of Ycf1 revealed that this protein was not required for Cu tolerance (Szczypka et al. 1994); however, growth of the

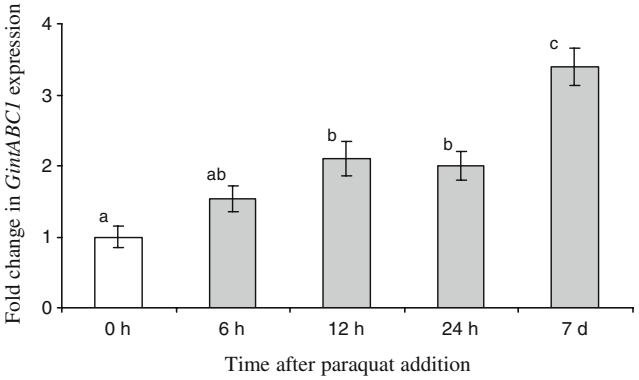

Fig. 4 Time course analysis of *GintABC1* expression in *Glomus intraradices* extraradical mycelia grown in M-C medium after the addition of 500 μM paraquat. *Bars* represent SD of the means of three independent biological replicates. Data not sharing a letter in common differ significantly ($P<0.05$) according to the Tukey's honestly significant difference test

Δycf1 mutant cells in the presence of high Cu concentrations might be due to the action of other transporters and cellular detoxification mechanisms.

GintABC1 induction by Cd and Cu follows a pattern similar to that of Cd and Cu accumulation in the vacuoles of *G. intraradices* when supplemented with these metals (González-Guerrero et al. 2008). These data suggest that in *G. intraradices*, like in other eukaryotes, the MRP transporter encoded by *GintABC1* might play a key role in Cd and Cu detoxification. Given that Cu is an active redox metal that induces oxidative stress in *G. intraradices* (González-Guerrero et al. 2007; Benabdellah et al. 2009) and that transcription of *GintABC1* is induced by oxidative stress, up-regulation of *GintABC1* expression in the presence of Cu might be also due to the oxidative response elicited by Cu.

Currently, substrate specificity for most of the MRPs is still missing. There is evidence that the *S. cerevisiae*

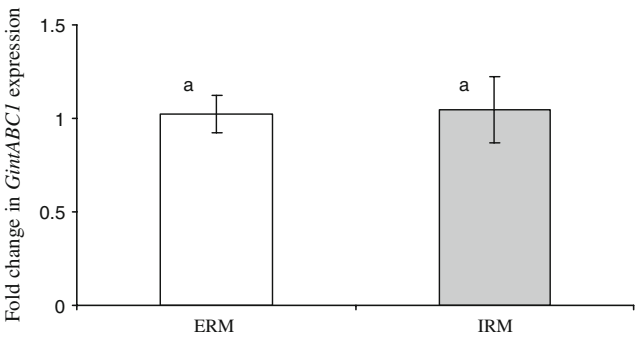

Fig. 5 *GintABC1* expression in the extraradical and intraradical mycelia of *Glomus intraradices*-colonized carrot roots in monoxenic culture. *Bars* represent SD of the means of three independent biological replicates. Data not sharing a letter in common differ significantly ($P<0.05$) according to the Tukey's honestly significant difference test

Ycf1 transports across the vacuolar membrane bis-glutathione-Cd complexes (Li et al. 1997), while *C. reinhardtii CrMRP2* transporter transports heavy metal-phytochelatin complexes (Wang and Wu 2006). Given the higher similarity of GintABC1 to Ycf1 than to CrMRP2 (61% vs 44% similarity), it is tempting to speculate that in *G. intraradices*, metals might be detoxified by complexation with glutathione and translocation of the metal-glutathione$_2$ complexes into vacuoles. However, further studies are needed to determine the substrates transported by GintABC1.

Transcriptional regulation by oxidative stress is a common feature of MRP transporters in mammals and yeast (Rebbeor et al. 2002; Maher et al. 2007). Given the wide range of processes involved in oxidative stress responses, we can only speculate about the physiological role of GintABC1 in free radical control. One possibility is that the depletion of reduced glutathione caused by free radicals might be sensed as an accumulation of glutathione complexes that need to be exported. Alternatively, given the broad range of substrates that MRP can transport oxidative stress might result in the accumulation of a damaging molecule which, after conjugation, is detoxified by GintABC1. Interestingly, it has been observed that oxidative stress and heavy metals are able to induce the expression of glutathione-S-transferases (Dixon et al. 2002; Waschke et al. 2006), enzymes which are responsible for glutathione conjugation. However, further experiments, such as determining substrate specificity and subcellular localization of GintABC1, are needed to define the specific role of this ABC transporter in oxidative stress protection and heavy metal homeostasis in *G. intraradices*.

In summary, in the present study, we report that GintABC1, the first MRP transporter identified in a mycorrhizal fungus, might play a key role in the protection against Cu and Cd toxicity. Our results also suggest that GintABC1 is involved in the control of the redox status of the ERM of *G. intraradices*. However, further studies are needed to fully understand the role of GintABC1 in AM fungi and to assess the role of this transporter in the adaptation to soils with high levels of Cu or Cd.

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