Available online at www.sciencedirect.com
SCIENCE DIRECT®
ELSEVIER
Fungal Genetics and Biology 42 (2005) 130–140
FUNGAL GENETICS AND BIOLOGY
www.elsevier.com/locate/yfgbi

Characterization of a *Glomus intraradices* gene encoding a putative Zn transporter of the cation diffusion facilitator family

Manuel González-Guerrero[a,*], Concepción Azcón-Aguilar[a], Michelle Mooney[b], Ascensión Valderas[a], Colin W. MacDiarmid[c], David J. Eide[b], Nuria Ferrol[a]

[a] *Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, CSIC, Profesor Albareda, 1, 18008 Granada, Spain*
[b] *Departments of Biochemistry and Nutritional Sciences, University of Missouri-Columbia, Columbia, MO 65211, USA*
[c] *Department of Biology, University of Missouri-St. Louis, 8001 Natural Bridge Road, St. Louis, MO 63121-4499, USA*

Received 7 May 2004; accepted 11 October 2004
Available online 10 December 2004

Abstract

A full-length cDNA (*GintZnT1*) encoding a putative Zn transporter was isolated from the extraradical mycelium of *Glomus intraradices*. Based on its sequence analysis, *GintZnT1* was classified as a member of the cation diffusion facilitator (CDF) family of heavy metal transporters. Functional analysis of *GintZnT1* was performed by heterologous expression in yeast mutants defective in different CDFs. Although Zn sensitivity of the mutants was not reverted, an effect of GintZnT1 on the labile regulatory Zn pool was detected by using a Zn-regulated β-galactosidase reporter gene. *GintZnT1* expression was studied in the extraradical mycelium obtained from a symbiotic root organ culture. *Gin ± ZnT1* was up-regulated in the extraradical mycelium of *G. intraradices* upon short-time exposure to Zn and when the mycelia were developed in 75 μM Zn supplemented plates. These data suggest a role of GintZnT1 in Zn compartmentalization and in the protection of *G. intraradices* against Zn stress.
© 2004 Elsevier Inc. All rights reserved.

Index Descriptors: Arbuscular mycorrhizal fungi; Zn homeostasis; Cation diffusion facilitator; Root organ culture

1. Introduction

Zinc plays a critical role in all the organisms, being an essential component of more than 300 enzymes, with representatives from the six major functional enzyme classes (Vallee and Auld, 1990). These include RNA polymerase, alkaline phosphatase, alcohol dehydrogenase, Cu/Zn superoxide dismutase, carbon anhydrase, and many proteases. In *Caenorhabditis elegans* and *Saccharomyces cerevisiae*, for example, 3 and 2%, respectively, of the protein sequences inferred from their genome, have Zn binding motifs (Albrecth et al., 1998; Bohm et al., 1997). Although an essential nutrient, Zn can be toxic if accumulated in excess. The mechanisms of Zn toxicity are unknown, but may involve competition for catalytic sites, for transporter proteins, etc. As a result, Zn homeostasis in living organisms is tightly controlled. In *Escherichia coli*, it has been shown that these cells strive to maintain essentially no free intracellular Zn (Outten and O'Halloran, 2001), indicating the exquisite balance between uptake and efflux systems despite changing extracellular conditions.

In eukaryotes, Zn homeostasis is largely due to the coordinated action of two families of transporters: Zrt-Irt-like proteins (ZIP) and cation diffusion facilitators (CDF). ZIPs derive their name from the first two members of the family to be described: the Irt1 iron transporter in *Arabidopsis thaliana* (Eide et al., 1996) and the Zrt1 zinc transporter in *S. cerevisiae* (Zhao and Eide,

* Corresponding author. Fax: +34 958 12 96 00.
E-mail address: manuelgg@eez.csic.es (M. González-Guerrero).

1087-1845/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.fgb.2004.10.007

1996a,b). To date, ZIP members have been found at all phylogenetic levels and are involved in the uptake of heavy metals from the extracellular media or the mobilization of intracellular stores (Gaither and Eide, 2001). CDFs are a family of heavy metal transporters, first described in the Gram-negative bacteria *Ralstonia metallidurans* strain CH4 (CzcD) (Nies, 1992), and in *S. cerevisiae* (Zrc1) (Kamizono et al., 1989). They have also been found in plants (Bloß et al., 2002) and animals (Huang et al., 2002). CDF family members mediate Zn efflux out of the cell (Palmiter and Findley, 1995) and may also facilitate Zn transport into intracellular compartments for detoxification, storage or to maintain essential functions (Anton et al., 1999; MacDiarmid et al., 2002).

Much of the current knowledge on Zn homeostasis comes from the study of the yeast *S. cerevisiae*. The ZIP transporters Zrt1 and Zrt2 are responsible for high affinity zinc uptake in yeast, and expression of these transporters is highly induced under conditions of zinc deficiency. A third plasma-membrane protein, Fet4, is thought to be responsible for some low-affinity uptake in zinc-replete conditions (Waters and Eide, 2002). Once inside the cell, CDF proteins (e.g., Msc2) transport Zn into different organelles to constitute metalloproteins (Li and Kaplan, 2001), or into the vacuole for future use in Zn deficiency conditions (Zrc1 and Cot1) (MacDiarmid et al., 2002). The vacuolar Zn reserve is mobilized by a third ZIP transporter, Zrt3, whose role is to transport Zn into the cytoplasm from the vacuole (MacDiarmid et al., 2000). Zn homeostasis is regulated at a transcriptional level by the transcription factor Zap1, which under low Zn concentration induces the expression of those promoters with zinc response elements (ZREs), such as *ZRT1*, *ZRT2*, *ZRT3*, and *ZRC1* (MacDiarmid et al., 2003).

Despite the importance of Zn as an essential micronutrient, information about the mechanisms of Zn homeostasis in fungi other than yeasts is still lacking. We are studying these mechanisms in arbuscular mycorrhizal (AM) fungi, ubiquitous soil-borne microorganisms that establish a mutualistic symbiosis with most plant species. AM fungi belong to the new fungal phylum Glomeromycota (Schüßler et al., 2001), and are obligate biotrophs whose life cycle completion depends on their ability to colonize the root of a host plant. AM symbiosis, a keystone to the productivity and diversity of terrestrial ecosystems, is present in more than 80% of land plants and represents the most widespread symbiosis on earth (Van der Heijden and Sanders, 2002). This symbiosis provides the plant with the possibility to explore a wider soil volume and enhance absorption of relatively immobile nutrients such as P, N, Zn, and Cu from deficient soils (Díaz et al., 1996; George et al., 1994; Johansen et al., 1992; Smith and Read, 1997). On the other hand, under conditions of high available Zn, the concentration of this element has been reported to be lower in mycorrhizal than in non-mycorrhizal plants (Bi et al., 2003; Joner and Leyval, 1997; Kothari et al., 1990; Kothari et al., 1991). Recent work on *Medicago truncatula* has indicated that AM fungi down-regulate a plasma membrane Zn transporter which was associated with a reduced level of Zn within the host plant tissues (Burleigh et al., 2003).

In spite of the importance of this dual role of AM fungi in Zn plant nutrition, nothing is known about the molecular mechanisms controlling Zn homeostasis in AM fungi. The aim of the present work was to clone and analyse the regulation of a *Glomus intraradices* Zn transporter, as a first step to gain insights into these mechanisms. In the present paper, we report the first Zn transporter described so far in arbuscular mycorrhizal fungi.

2. Materials and methods

2.1. Biological materials and culture conditions

AM monoxenic cultures were established as described by St-Arnaud et al. (1996). Briefly, clone DC2 of carrot (*Daucus carota* L.) Ri-T DNA transformed roots were cultured with the AM fungus *G. intraradices* Smith and Schenck (DAOM 197198, Biosystematic Research Center, Ottawa, Canada) in two-compartment petri dishes. Cultures were initiated in one-compartment ("root compartment") of each plate, which contained M medium (Chabot et al., 1992). Fungal hyphae, but not roots, were allowed to grow over to the second compartment ("hyphal compartment"), which contained M medium without sucrose (M-C medium). The plates were incubated in the dark at 24 °C until the transition from absorptive to sporulative phase was initiated (approximately 2–3 months).

At this stage, 75 and 7500 μM Zn pulses were applied to the extraradical mycelia by placing 500 μl $ZnSO_4$ stock solutions in droplets dispersed throughout the compartment, allowing diffusion to distribute the added metal evenly. The time of heavy metal addition was referred to time 0. Mycelia were harvested at time 0, 6, 12, and 24 h, and 7 day after the pulse.

For experiments involving growth in metal supplemented plates, M-C medium (which contained 9.3 μM zinc) was used unsupplemented (control treatment) or supplemented with $ZnSO_4$ (75, 750, or 7500 μM). The plates were incubated as described above until the transition from the absorptive to the sporulative phase.

For heterologous expression, the *S. cerevisiae* strains CM137 (αcan *1-100oc his3 leu2 trp1 ura3-52 zrc1::HIS3 cot1:Kan*^{r}), DEY1551 (*ade6 can1-100oc his3-11 leu2-3, 112, trp1-1 ura3-52* msc2Δ), and the wild type DY1457 (αade6 can1-100oc *his3 leu2 trp1 ura3*) were used. The

Schizosaccharomyces pombe Δ*zhf*::ura4 strain (*h^+ ade6-M216 leu1-32* ura4-Δ18 *can1-1*), provided by Dr. S. Clemens (Leibniz Institut fur Pflanzenbiochemie, Halle, Germany), was also used. CM137 is unable to grow in SD medium supplemented with more than 60 μM Zn (MacDiarmid et al., 2003), DEY1551 is unable to use glycerol–ethanol as carbon source when grown at 37 °C (Li and Kaplan, 2001), and Δzhf::ura4 is unable to grow in Edinburgh minimal medium (EMM) supplemented with more than 250 μM Zn (Clemens et al., 2002). *S. cerevisiae* strains were grown in SD, YPD (Sherman et al., 1986), YPGE (Li and Kaplan, 2001), or LZM (low zinc media) (Gitan et al., 1998) media supplemented with necessary auxotrophic requirements and either 2% glucose or galactose. *S. pombe* was grown in the standard culture media YES and EMM (Moreno et al., 1991) with glucose as carbon source.

Cell number in liquid cultures was determined by measuring the optical density of cell suspensions at 600 nm (OD_{600}) and converting to cell number with a standard curve.

2.2. Cloning and sequence analysis

The 3′ end of *GintZnT1* was identified in the gene database and obtained from Dr. Maria Harrison (Ardmore, OK, USA). The 5′ end of *GintZnT1* was obtained by rapid amplification of cDNA ends (RACE) by using the SMART RACE cDNA Amplification kit (Clontech, Palo Alto, CA, USA) and the *GintZnT1* specific primer ZnT1 (Table 1). 5′ RACE reactions were performed using 1 μg total RNA extracted from extraradical mycelium grown in M-C plates. The amplified cDNA was cloned in TOPO pYES2.1 His vector (Invitrogen, Carlsbad, California, USA). The full-length clone was obtained by ligating the 3′ and 5′ fragments in a *Bam*HI/*Eco*RI-digested pYES2 vector (Invitrogen, Carlsbad, California, USA) by homologous recombination in yeast to generate pGintZnT1. The primers used were ZnT2 and ZnT3 (Table 1) to generate the 5′ fragment; and ZnT4 and ZnT5 (Table 1) to generate the 3′ fragment.

An epitope-tagged version of GintZnT1 was obtained by inserting three copies of the *Haemophilus influenzae* hemagglutinin (HA) epitope at the C-terminus of the GintZnT1 in pYES2 by homologous recombination, to create pGintZnT1-HA. The primers used were HA1 and HA2 (Table 1).

The plasmid pZRC1-HA was used as a positive control in the yeast assays. This vector was obtained by cloning a C-HA-tagged *ZRC1* gene in the centromeric URA3 pFL38 vector, expressed under the promoter of *ZRC1* (Bonneaud et al., 1991).

The *S. pombe* vector pSGP72 (provided by Dr. S. Clemens, Leibniz-Institute für Pflanzenbiochemie, Halle, Germany) was used to generate pFyGintZnT1 by cloning *GintZnT1* into the *Xho*I and *Bam*HI sites of the vector.

High fidelity *Taq* polymerase (Roche Diagnostics, Mannheim, Germany) was used in the described PCR steps and all PCR products were sequenced to discard mutations.

Nucleotide sequences were determined by *Taq* polymerase cycle sequencing 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, Wis., USA), and multiple sequence alignments of translated gene sequences were carried out with the program CLUSTALW (version 1.5; Thompson et al., 1994). Transmembrane domains of GintZnT1 were predicted by using SOSUI (Hirokawa et al., 1998).

2.3. Heterologous expression

Saccharomyces cerevisiae transformations were performed using a lithium acetate-based method (Schiestl and Gietz, 1989). Cells transformed with pYES2, pGintZnT1 or pGintZnT1-HA were selected in SD medium by autotrophy to uracil and pDg2L (described below) transformants by leucine autotrophy. Those cotransformed with pGEV-trp, a plasmid which enables the transcription of gal promoters with the addition of β-estradiol and in absence of galactose (Gao and Pinkham, 2000), were selected by tryptophane autotrophy.

For phenotypic tests, CM137 transformants were plated on SD agar medium supplemented with 0, 50, 100,

Table 1
Primers used in this paper

Primer	Sequence
HA1	ATCACAACCACCAAAAGATATGACGACGACT CCCTCTAATTACCCATACGATGTTCCT
HA2	GCTCGAGCGGCCGCCAGTGTGATGGATATCT GCAGAATTGTTAAGCGTAATCTGGAA
RMF	TGTTAATAAAAATCGGTGCGTTGC
RMR	AAAACGCAAATGATCAACCGGAC
ZnT1	AGCAAGCAAGCAGTTTCGTGATTAT
ZnT2	CACTATAGGGAATATTAAGCTTGGTACCGAG CTCGGATCCCGCAGAGTTCGCGGGGGAAA
ZnT3	TTCATTTGCGACAAGTTCCA
ZnT4	GCAACTTTCTGATACGAAGCG
ZnT5	GCTCGAGCGGCCGCCAGTGTGATGGATATCT GCAGAATTCAATTTTTAATTAGAGGGAGTCG
ZnT6	ATGGCTTTATGTCTTAGTATTTTC
ZnT7	TCTCGTGACTCTTGTGCAGCC
ZnT8	GCACAACCAACTCCAAGGAT
ZnT9	AAAATCCGGTTGTCATC

The annealing temperature used for all primer sets was 55 °C.

200, 400, or 1000 μM $ZnSO_4$ and DEY1551 transformants in YP glycerol–ethanol media incubated at 37 °C. To regulate GintZnT1 transcription levels in the pGEV-trp transformants, the selective SD medium was supplemented with 0, 50, 100, 200, 400, 1000, 2000, 5000, and 10,000 μM β-estradiol. pSGP72 or pFy-GintZnT1 *S. pombe* transformants where obtained using the method described by Eitinger et al. (2000). Complementation tests were performed in EMM media supplemented with 0, 50, 100, 200, 300, 400, or 500 μM $ZnSO_4$.

2.4. β-Galactosidase assay

Wild type DY1457 cells were transformed with pDg2L, a plasmid carrying the ZRE sequence of Zrt1 fused upstream to the β-galactosidase gene of *E. coli* (Zhao et al., 1998), with either pYES2, pGintZnT1 or pZRC1-HA (positive control) and grown to exponential phase in SD medium. Cells were washed twice in LZM media and resuspended in LZM. Two clones of each type of transformant were assayed and the assay was repeated thrice.

β-Galactosidase activity was measured by the method of Guarente (1983) and expressed in Miller Units calculated as follows: $(OD_{420} \times 1000)/(\min \times \text{ml of culture used} \times OD_{600})$.

2.5. Immunoblot

Total protein extractions and immunoblots were performed as previously described (Gitan and Eide, 2000). Briefly, proteins isolated from yeast cells transformed with pYES2 or pGintZnT1-HA were run on a 10% acrylamide gel and transferred to nitrocellulose. The blot was probed with a 1:2000 dilution of a 12CA5 anti-HA antibody (Boehringer–Mannheim, Germany) followed by 1:5000 dilution of a horseradish peroxidase-conjugated goat anti-mouse antibody (Pierce, Illinois, USA). The blot/complexes were developed by enhanced chemiluminescence (ECL, Amersham, UK).

2.6. Indirect immunofluorescence microscopy

Indirect immunofluorescence microscopy was performed on pGintZnT1 or pYES2 transformed wild type DY1457 cells, grown overnight in selective SD medium with galactose as sole carbon source. The detection was done essentially as described by Pringle et al. (1991) with the following modifications. Cells were fixed in 10 volumes of cold methanol (−20 °C) for 30 min. Fixed cells were treated with glusulase to remove the cell wall and bound to polylysine-treated glass slides. The cells were blocked in PBS + 1 mg/ml BSA (PBS/BSA) by incubating at room temperature for 2 h and stained with 12CA5 antibody (1:200 dilution in PBS/BSA) at room temperature for 16 h. Following washing of the cells, goat anti-mouse IgG antibody coupled to ALEXA 488 (Molecular Probes, Eugene, Oregon, USA) was applied (1:200 dilution in PBS/BSA) and the cells were incubated at 37 °C for 1 h. The slides were then washed 10 times in PBS and viewed using epifluorescence.

2.7. RNA extractions and gene expression

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

GintZnT1 expression was studied by real-time RT-PCR by using iCycler (Bio-Rad, Hercules, California, USA). cDNAs were obtained from 1 μg of total DNase-treated RNA in a 10 μl reaction containing 4 U of Avian myeloblastosis virus reverse transcriptase (Finnzymes, Oy, Finland), 200 ng random hexamer primers, 1 mM each dNTP, 20 U of RNase inhibitor, and 1× reverse transcription buffer. The primer set used to amplify *GintZnT1* in the synthesized cDNAs were ZnT6 and ZnT7 (Table 1). Each 25 μl reaction contained 1 μl of a dilution 1:10 of the cDNA, 200 mM dNTPs, 200 nM each primer, 3 mM $MgCl_2$, 2.5 μl of 1× SyBR Green (Molecular Probes, Eugene, Oregon, USA), and 0.5 U Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, California, USA) in 1× PCR buffer (20 mM Tris–HCl, pH 8.4, 50 mM KCl).

The PCR program consisted in 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 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 on the different treatments were standardized to the 18S rRNA levels, which were amplified with the following primers: RMF and RMR (Table 1). Real-time PCR experiments were carried out three times, with the threshold cycle (C_T) determined in triplicate. The average for the triplicate of one representative experiment was used in all subsequent analysis. The relative levels of transcription were calculated by using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001).

Saccharomyces cerevisiae RNA was obtained by standard methods (Sherman et al., 1986). One step RT-PCR was performed using Access RT-PCR System from Pro-

mega (Promega, Madison, Wisconsin, USA) following manufacturer's instructions. The primer sets used to detect *GinZnT1* transcripts in yeast RNA were ZnT2–ZnT8, ZnT9-ZnT3, and ZnT4-ZnT5 (Table 1). In all RT-PCRs, a non-RT control was used to check DNA contaminations.

3. Results

3.1. *GintZnT1 belongs to the CDF family of heavy metal transporters*

A 563 bp cDNA fragment corresponding to the 3′ end of *GintZnT1* was identified by exploring a *G. intraradices* EST library (Accession No. BI452096) and the 5′ end was obtained by RACE using gene-specific primers. In silico translation of the full-length *GintZnT1* cDNA clone revealed an open reading frame encoding a 454 amino acid polypeptide with a predicted molecular weight of 54 kDa. Comparisons with the amino acid sequence databases showed that it is related to members of the CDF family, showing the highest homology to the vacuolar CDFs Zrc1 and Cot1 of *S. cerevisiae* (60 and 59% of similarity, respectively) and to the endoplasmic reticulum Zhf CDF of *S. pombe* (62% similarity).

The topology prediction programme SOSUI predicts six transmembrane domains in the deduced amino acid sequence of *GintZnT1*. These domains are almost identical among GintZnT1 and the three previously characterized fungal CDF proteins (Fig. 1). Moreover, there are two cytoplasmic motifs widely distributed in CDFs: the histidine-rich-motif $(HX)_8$ between helices IV and V and the C-terminal sequence, E-X-H-X-W-X-L-S-X_8-H.

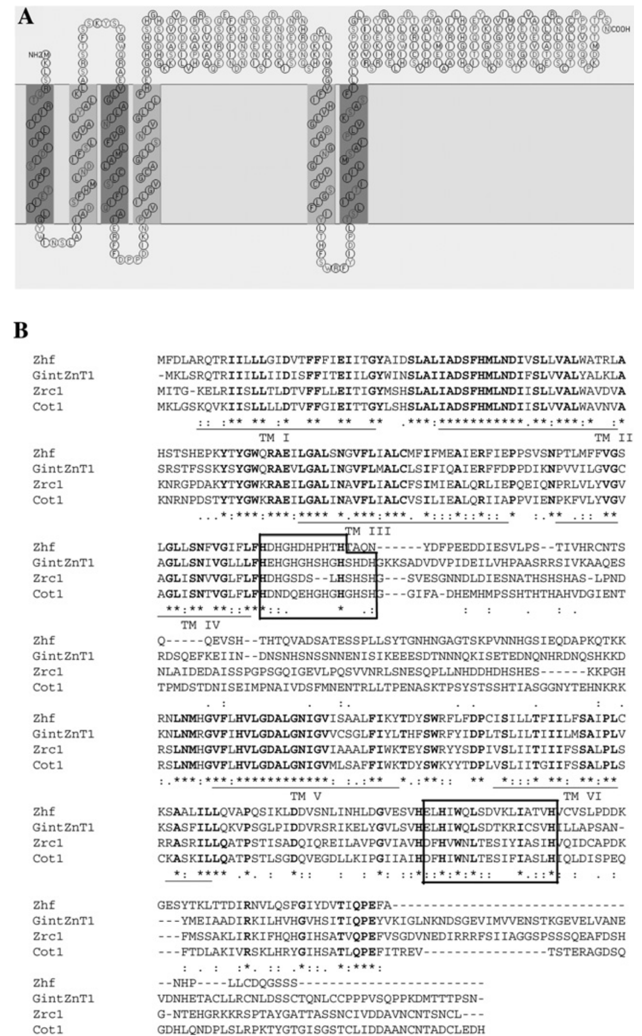

Fig. 1. Identification of a new CDF from *G. intraradices*. (A) Predicted topology of GintZnT1, as defined by SOSUI. (B) Multiple sequence alignment of GintZnT1 (Accession No. AJ574787), Zhf (Accession No. 1759680), Zrc1 (Accession No. 6323899), and Cot1 (Accession No. 6324892) by using the CLUSTALW method. The conserved CDF cytoplasmic motifs are boxed, the transmembrane domains are underlined and the conserved amino acids are shown in bold.

3.2. *Ectopic expression of GintZnT1*

To further characterize the *GintZnT1* gene product and to establish whether it has Zn transport activity, the cDNA was expressed in a series of yeast mutants defective in different CDFs. First, we tested whether *GintZnT1* can relieve the Zn sensitivity of the *zrc1 cot1* double mutant of *S. cerevisiae*, in which Zn accumulates in the cytoplasm due to a defect in the two vacuolar Zn transporters that prevents growth at concentrations higher than 60 μM Zn (MacDiarmid et al., 2003). However, *GintZnT1* failed to complement the *zrc1 cot1* phenotype.

The lack of complementation of the *zrc1 cot1* mutant by *GintZnT1* could be due to the high levels of transcription caused by a strong promoter, inadequate or defective transcription or translation of the *GintZnT1* gene, or sorting of the protein to an inappropriate location in the cell. To address the issue of transcription levels, GintZnT1 expression levels were regulated by cotransforming the mutant strain CM137 with pGintZnT1 and pGEV-trp, a plasmid that allows transcription of Gal promoters when exposed to β-estradiol. However, no complementation of the mutant phenotype was observed with any of the β-estradiol concentrations tested.

The issue of anomalous *GintZnT1* mRNA synthesis in the mutant yeast was analysed by RT-PCR. To verify the presence of the complete mRNA, an overlapping set of primers were used to amplify various regions of the cDNA. PCR products of the expected sizes were amplified in the pGintZnT1 transformants, but not in the strain transformed with the empty vector (Fig. 2A), verifying expression of the full-length *GintZnT1* gene in yeast.

To verify the translation and location of GintZnT1 protein in the *zrc1 cot1* cell, a sequence encoding a triple

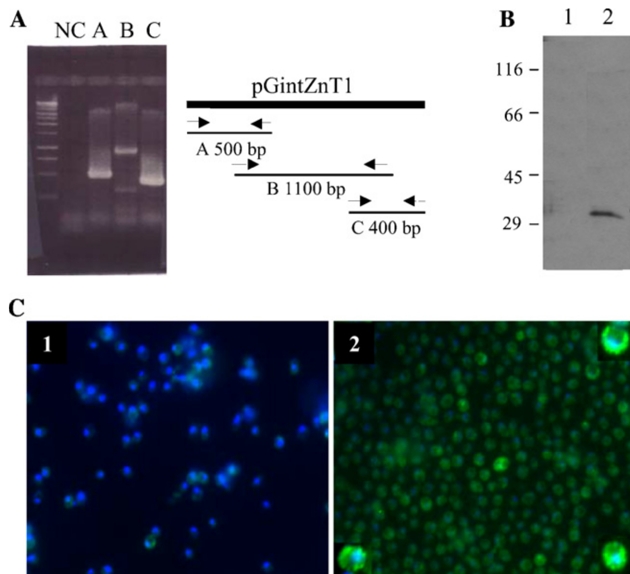

Fig. 2. Expression of *GintZnT1* in yeast. (A) Overlapping PCR of cDNA from a pGintZnT1-transformed *zrc1 cot1* mutant of *S. cerevisiae*. The primer set A corresponds to primers ZnT2-ZnT8, set B to ZnT9-ZnT3, and set C to ZnT4-ZnT5. Amplicon sizes are shown. NC, negative control (no reverse transcriptase added). (B) Western analysis of total protein extracts from a pYES2 (1) and pGintZnT1-HA (2) transformed *zrc1 cot1* mutant of *S. cerevisiae*. Total proteins were isolated from yeast transformants and separated with 12CA5 anti-HA antibody. Positions of molecular weight markers, in kiloDalton, are shown on the left. (C) Immunofluorescence localization of GintZnT1-HA in a *zrc1 cot1 S. cerevisiae* mutant strain. DAPI-stained nuclei are shown in blue and the anti-HA antibody in green. Panel 1 shows the pYES2 transformant (negative control) and panel 2 shows the pGintZnT1-HA transformant. Higher magnification of selected cells are shown in the corners of panel 2. Using differential contrast optics, the vacuole was apparent in these cells as a single large organelle (data not shown).

repeat of the hemaglutinin (HA) epitope was fused to the C-terminal end of the *GintZnT1* reading frame. Immunoblot analysis with an anti-HA antibody detected a band of approximately 30 kDa in protein extracts from pGintZnt1-HA transformants, whereas no protein was detected in the cells transformed with the vector alone (Fig. 2B). Although the size of the detected protein was smaller than expected, these data show that *GintZnT1* mRNA was translated in the heterologous system. Indirect immunofluorescence of strains expressing tagged protein detected signal distributed throughout the cell, but no apparent vacuolar membrane localization was observed (Fig. 2C). The lack of GintZnT1 accumulation in the vacuole membrane could explain why *GintZnT1* expression did not complement the zinc sensitivity of the *zrc1 cot1* mutant.

The GintZnT1 protein showed lower homology (47% similarity) to Msc2, a CDF Zn transporter which is believed to be localized in the endoplasmic reticulum/nuclear envelope. The non-vacuolar localization of GintZnT1-HA suggested that this protein may be accumulating in the early secretory pathway, and prompted us to test its ability to complement the *msc2* mutation. The *msc2* deletion strain shows a phenotype of decreased viability in YP glycerol–ethanol media at 37 °C, which is suppressed by the addition of extracellular Zn (Li and Kaplan, 2001). Transformation of DEY 1551 cells, which lack the Msc2 Zn transporter, with *GintZnT1* did not restore the growth defect of the mutant.

The ZAT1 Zn transporter from *A. thaliana* did not attenuate the increased Zn sensitivity of a *S. cerevisiae zrc1 cot1* strain, but complemented this phenotype in a *ΔSpZhf1* mutant strain of *S. pombe* (Bloß et al., 2002). The significant homology between *GintZnT1* and the *S. pombe* gene suggested that GintZnt1 might complement the Zhf1 mutant phenotype. To test this, we expressed *GintZnT1* in the *S. pombe ΔSpZhf1* strain and assayed zinc sensitivity. However, GintZnT1 did not affect the Zn sensitivity of the *Zhf1* strain.

As a more sensitive test for GintZnT1 function, we determined if *GintZnT1* expression could alter other measures of Zn homeostasis. While there are currently no direct assays of cytosolic labile zinc, measuring the activity of the zinc-responsive Zap1 transcriptional activator provides a convenient indirect assay of zinc homeostasis. Overexpression of the *ZRC1* gene increased the expression of a Zap1-responsive reporter gene (ZRE-LacZ) in yeast, consistent with the role of Zrc1 in sequestering zinc in the vacuole and reducing cytoplasmic zinc availability (Miyabe et al., 2001). To test if GintZnT1 might affect Zap1 activity and therefore yeast Zn homeostasis, the wild type strain DY1457 was cotransformed with the ZRE-LacZ gene and either pGintZnT1, pYES2 (negative control) or pZRC1-HA

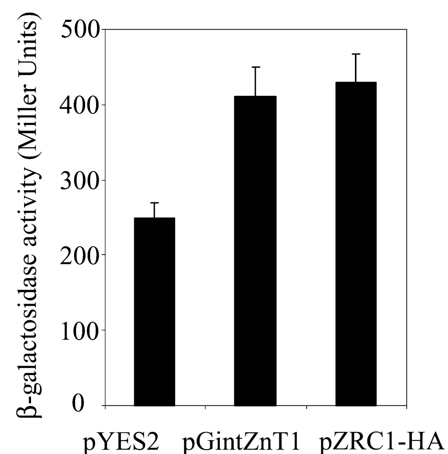

Fig. 3. Effects of *GintZnT1* expression on zinc-responsive reporter expression. The yeast strain DY1457 was transformed with either pYES2, pGintZnT1 or pZRC1-HA and cotransformed with the ZRE-lacZ reporter pDg2L. Cells of each strain were grown to log phase in zinc-replete medium with 2% galactose, then transferred to LZM with 2% glucose. β-Galactosidase activity was measured 2 h after inoculation into LZM. Data points represent the means of three replicates from two representative experiments. Error bars indicate SD.

(positive control). When cells of these strains were incubated in LZM for 2 h, *ZRC1* and GintZnT1 were observed to significantly increase β-galactosidase activity (Fig. 3). This observation suggests that expression of *GintZnT1* is able to reduce cytoplasmic Zn availability and activate Zap1.

3.3. Regulation of GintZnT1 expression in the extraradical mycelium of G. intraradices

Some CDF family members are known to be transcriptionally regulated in response to zinc availability, which is consistent with their roles in zinc homeostasis. If GintZnT1 is important for zinc homeostasis, the expression of this gene may respond to the concentration of zinc in the fungal environment. To determine the effect of zinc on *GintZnT1* expression, extraradical mycelia of *G. intraradices* grown in M-C media containing 9.3 μM Zn were exposed for different periods of time to 75 μM and 7.5 mM Zn and *GintZnT1* mRNA was quantified using real-time RT-PCR.

Upon exposure to a moderate Zn concentration (75 μM), an increase in *GintZnT1* mRNA was observed, reaching a 4-fold induction peak 12 h after the exposure. Recovery of *GintZnT1* expression at the 24 h time-point to almost the basal levels and the slight 1.5-fold induction detected one week after the addition of Zn, could be due to an adaptation of the mycelia to growing in a Zn-rich environment (Fig. 4A). Addition of a high Zn concentration (7.5 mM) to the mycelia caused a 4-fold induction of *GintZnT1* mRNA 6 h after the exposure, 6 h earlier than when the mycelia were exposed to 75 μM Zn. However, down-regulation of *GintZnT1* mRNA was observed at all the other time-points analysed after the addition of 7.5 mM Zn (Fig. 4B).

To study the regulation of *GintZnT1* gene expression when the mycelium is exposed for a long period of time to Zn, fungal mycelia were grown in M-C media supplemented with different zinc concentrations. The reason behind this change in the experimental setting (instead of using the system described above and letting the mycelia grow for a longer period of time after the addition of Zn) is that the transition from absorptive to sporulative phase takes place in about two weeks, which would not let study the effect of the long-term Zn exposure on *GintZnT1* gene expression at the same physiological level than in the previous experiment (when the fungus starts the transition from absorptive to sporulative phase). The results of the effect of the long-time

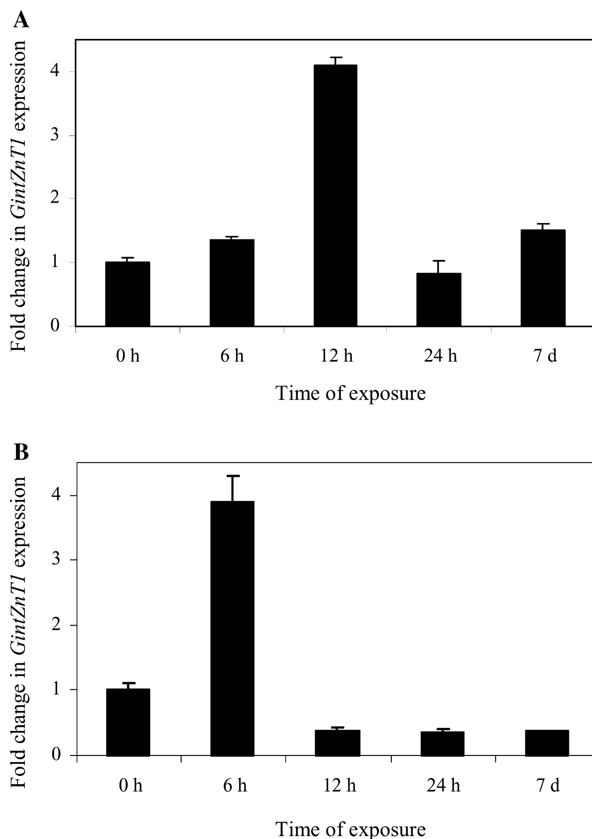

Fig. 4. *GintZnT1* gene expression in the extraradical mycelium of *G. intraradices* under a short term exposure to Zn. Total RNAs were extracted from extraradical mycelia of *G. intraradices* cultured in standard conditions and then exposed for 0, 6, 12, and 24 h or 7 day to 75 μM (A) or 7.5 mM Zn (B). RNAs were reverse transcribed and expression was assayed by quantitative real-time RT-PCR using gene-specific primers for *GintZnT1* and 18S rRNA. The fold change in *GintZnT1* gene expression induced by the metal treatments was calculated using the $2^{-\Delta\Delta C_T}$ method. Data represent the means of three replicates from a representative experiment. Error bars represent SD.

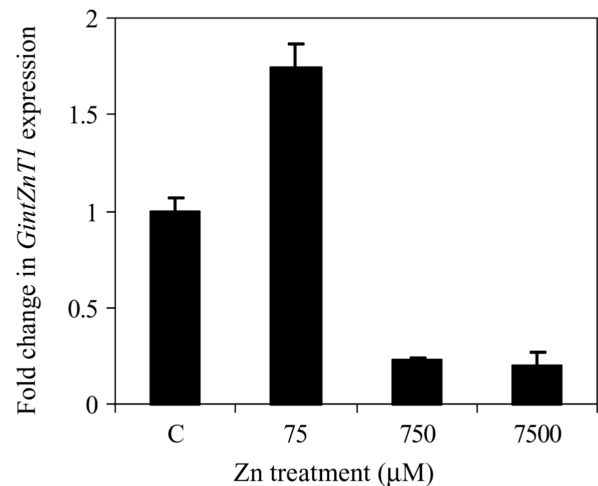

Fig. 5. *GintZnT1* gene expression in the extraradical mycelium of *G. intraradices* under a long term exposure to Zn. Total RNAs were extracted from extraradical mycelia of *G. intraradices* developed in M-C medium unsupplemented (C) or supplemented with either 75, 750, or 7500 μM Zn. RNAs were reverse transcribed and expression was assayed by quantitative real-time RT-PCR using gene-specific primers for *GintZnT1* and 18S rRNA. The fold change in *GintZnT1* gene expression induced by the metal treatments was calculated using the $2^{-\Delta\Delta C_T}$ method. Data represent the means of three replicates from a representative experiment. Error bars represent SD.

exposure to Zn are shown in Fig. 5. This analysis revealed that *GintZnT1* transcript levels consistently increased by ~60% when the extraradical mycelium was grown in the presence of 75 μM Zn. However, a marked 5-fold inhibition was observed when the mycelia were cultured in the presence of 750 and 7500 μM Zn. Overall, these data show the regulation of *GintZnT1* by the Zn levels surrounding the extraradical hyphae.

4. Discussion

Arbuscular mycorrhizal fungi play a key role in terrestrial ecosystems by forming a symbiotic association with plant roots. The success of this relationship is based on the improved nutrient supply and protection that both symbionts derive from their mutual association (Azcón-Aguilar and Barea, 1996; Smith and Read, 1997). Much research has been done at the physiological level in the last 30 years. Due to the obligate biotrophic character of these fungi and their coenocytic nature, progress on the molecular physiology of AM fungi has been much slower (Ferrol et al., 2004). Moreover, most research has been focussed on the transfer of phosphorus from the fungi to the plant, or how carbon is supplied to the fungi (Ferrol et al., 2002). Much less is known about micronutrients such as Zn, Cu, or Fe. In this paper we describe the cloning of *GintZnT1*, the first AM fungal gene to be implicated in zinc transport and homeostasis.

GintZnT1 encodes an apparent membrane protein, which we have assigned to the CDF family of zinc transporters based on examination of its sequence and general structural features. Most CDF family members are predicted to have six transmembrane domains and two cytoplasmic loop regions (Gaither and Eide, 2001). The first of these regions is localized between transmembrane domains IV and V, and includes a histidine rich motif ($HX_{n=3-6}$). Although the position and number of repeats is not strictly conserved within the CDF family of proteins, this feature is common to many of its members. The second cytoplasmic domain includes the more tightly conserved motif H-D/E-X-H-X-W-X-L-T-X_8-H. While the function of these two motifs is as yet unclear, they are present in the GintZnT1 protein. These structural similarities, together with the close sequence relationship between GintZnT1 and other characterized CDF family members, suggest a role for this protein in the homeostasis of Zn.

The characteristics of AM fungi prevent the use of most common strategies to assess the functionality of a cloned gene, such as generating strains lacking or overexpressing the gene of interest. To bypass this technical constrain, we assessed GintZnT1 function when expressed in two heterologous systems, *S. cerevisiae* and *S. pombe* although we have to keep in mind that this approach does not provide information about its physiological role . Despite the high similarity of the GintZnT1 protein to Zrc1, Cot1, and Zhf, *GintZnT1* did not complement the phenotypes of any of the yeast mutants used in the present study. The reason for the lack of effect of GintZnT1 in yeast is currently unknown. One possible explanation is the discrepancy between the predicted molecular mass of GintZnT1 and its apparent migration on SDS–PAGE; however, several other transporter proteins are known to migrate aberrantly by SDS–PAGE analysis. For example, the molecular mass of the glucose transporter GLUT1 based on its amino acid composition is predicted to be approximately 55 kDa, but the apparent migration, as measured by SDS–PAGE, in the absence of its N-linked oligosaccharide, is only 35 kDa (McMahon and Frost, 1995). The reason, however, for the discrepancy in the apparent migration of GintZnT1 is unclear at the present time. A more likely explanation for the lack of effect of GintZnT1 in the mutant yeasts is the lack of protein accumulation in the vacuolar membrane (Fig. 2). Our experiments with a zinc-responsive reporter construct indicated that GintZnT1 altered zinc homeostasis in a manner consistent with this protein acting to efflux or sequester cytoplasmic zinc. If the GintZnT1 protein does transport significant zinc out of the cytoplasm, the lack of effect on zinc tolerance of *S. cerevisiae* mutants would be due to sequestration of this zinc in a compartment which is unable to tolerate higher than normal zinc concentrations (such as the endoplasmic reticulum or Golgi).

Regulation of *GintZnT1* expression by Zn may also shed some light on its role in the extraradical mycelium of *G. intraradices*. In this paper, we have shown that this putative Zn transporter reaches higher expression levels 12 and 6 h after the addition of moderate and high Zn concentrations, respectively. Since zinc is abundant in basal fungal growth medium (~9 μM), this medium is likely to be zinc replete. Hence, the induction of *GintZnT1* upon addition of 75 μM zinc is most likely a response to zinc toxicity. These observations are consistent with a role for GintZnT1 as a zinc efflux system, expression of which is regulated according to zinc availability. Precedent for this view comes from studies of CDF family zinc transporters in mammalian systems. Expression of the major zinc efflux system, Znt1, is induced by excess zinc via the activity of the zinc-regulated transcription factor MTF1 (Langmade et al., 2000). The *GintZnT1* regulation pattern by Zn, resembling that of a MTF1 regulated response, leads us to hypothesize the existence of similar regulatory elements in *G. intraradices*. Future work on the isolation and characterization of the *GintZnT1* promoter will shed more light on this regulatory mechanism.

The role of GintZnT1 in Zn detoxification is also supported by the slight induction observed in the long term

assays when plates were supplemented with 75 μM Zn. Down-regulation of *GintZnT1* when *G. intraradices* was developed in the presence of higher Zn concentrations, as well as from the 12 h time-point in the 7.5 mM Zn pulse assay, may be caused by an impaired translation due to the excessive accumulation of Zn resulting from the exposure to these very high (and probably non-physiological) zinc concentrations.

The expression pattern of *GintZnT1* observed in the 75 μM time-course experiment, showing a maximum level of expression 12 h after Zn exposure, suggests that additional mechanisms must exist in *G. intraradices* to avoid Zn toxicity. As too much Zn is toxic, it is essential that the fungus responds rapidly when exposed to high Zn levels to prevent cytoplasmic Zn overaccumulation. In other organisms, this is achieved by the transcriptional and/or post-transcriptional regulation of a number of genes encoding Zn transporters (Eide, 2004). For instance, transcription of the Zn uptake systems Zrt1 and Zrt2 of *S. cerevisiae* is induced by Zn limitation thereby increasing the activity of these transporters (Zhao and Eide, 1996a,b). Moreover, when Zn-deficient cells, with high levels of Zrt1 are resupplied with Zn, this transporter is inactivated by endocytosis and vacuolar degradation (Gitan et al., 1998). These data indicate that one mechanism of protection against an excessive concentration of Zn in the medium would be a diminishment of the activity of Zn uptake systems, either by down-regulating the levels of transcription (Bird et al., 2004; Zhao and Eide, 1996a,b) or by post-translational modifications (Gitan et al., 1998; Wang et al., 2004). However, *GintZnT1* presents a slight induction 7 day after 75 μM Zn exposure and in the long term assay, what suggests that some Zn is still being incorporated in the cytoplasm, perhaps due to unspecific transporters, such as Fet4 in *S. cerevisiae*, altering Zn homeostasis. Therefore, the lower level of induction of *GintZnT1* mRNA observed after 12 h exposure to 75 μM Zn would be the result of the adaptation of *G. intraradices* to a situation with increased Zn levels in the medium, where multiple levels of regulation strive to keep cytoplasmatic Zn levels under control.

Further analysis of the role of GintZnT1 in protecting against zinc toxicity must await advancements in techniques to enable obtention of fungal mutant strains. However, based on its regulation by Zn and its effects on zinc homeostasis when expressed in *S. cerevisiae*, we propose that GintZnT1 transports zinc into an intracellular compartment or out of the fungal hypha. Thus, GintZnT1 could play a role in general zinc homeostasis (via zinc storage or efflux) or in supplying zinc to the plant cell (via efflux). The determination of the GintZnT1 protein location in the fungal hyphae and the characterization of the promoter will provide further information on the value of this protein to the fungal–plant symbiotic relationship.

Acknowledgments

We are grateful to Dr. Maria Harrison for providing the *GintZnT1* partial clone, Dr. Stephan Clemens for the gift of the *S. pombe* strains and plasmids and Dr. Bert Bago for providing the *G. intraradices* inoculum and excellent assistance on the establishment of the monoxenic cultures. We thank Custodia Cano and Olga Revelles for excellent technical assistance, and Dr. José Miguel Barea for helpful discussions. This work was funded by the EU project Genomyca (QLK5-CT-2000-01319) and the NIH Grant GM56285. Manuel González-Guerrero was supported by a FPU fellowship from the Spanish Ministry of Education, Culture, and Sports.

References

Albrecth, C., Geurts, R., Lapyrie, F., Bisseling, T., 1998. Endomycorrhizae and rhizobial Nod factors both require SYM8 to induce the expression of the early nodulin genes PsENOD5 and PsENOD12A. Plant J. 15, 605–614.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

Anton, A., Große, C., Reißmann, J., Pribyl, T., Nies, D.H., 1999. CzcD is a heavy metal ion transporter involved in regulation of heavy metal resistance in *Ralstonia* sp. strain CH34. J. Bacteriol. 181, 6876–6881.

Azcón-Aguilar, C., Barea, J.M., 1996. Arbuscular mycorrhizas and biological control of soil-borne plant pathogens—an overview of the mechanisms involved. Mycorrhiza 6, 457–464.

Bago, B., Pfeffer, P.E., Douds, D.D., 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, 263–271.

Bi, Y.L., Li, X.L., Christie, P., 2003. Influence of early stages of arbuscular mycorrhiza on uptake of zinc and phosphorus by red clover from a low-phosphorus soil amended with zinc and phosphorus. Chemosphere 50, 831–837.

Bird, A.J., Blankman, E., Stillman, D.J., Eide, D.J., Winge, D.R., 2004. The Zap1 transcriptional activator also acts as a repressor by binding downstream of the TATA box in ZRT2. EMBO J. 23, 1123–1132.

Bloß, T., Clemens, S., Nies, D.H., 2002. Characterization of the ZAT1p zinc transporter from *Arabidopsis thaliana* in microbial model organisms and reconstituted proteoliposomas. Planta 214, 783–791.

Bohm, S., Frishman, D., Mewes, H.W., 1997. Variations of the C2H2 zinc finger motif in the yeast genome and classification of yeast zinc finger proteins. Nucleic Acids Res. 25, 2464–2469.

Bonneaud, N., Ozier-Kalogeropoulus, O., Li, G.Y., Labouesse, M., Minvielle-Sebastia, L., Lacroute, F., 1991. A family of low and high copy replicative, integrative and single-stranded *S. cerevisiae E. coli* shuttle vectors. Yeast 7, 609–615.

Burleigh, S.H., Kristensen, B.K., Bechmann, I.E., 2003. A plasma membrane zinc transporter from *Medicago truncatula* is up-regulated in roots by Zn fertilization, yet down-regulated by arbuscular mycorrhizal colonization. Plant Mol. Biol. 52, 1077–1088.

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

Clemens, S., Bloss, T., Vess, C., Neumann, D., Nies, D.H., zur Nieden, U., 2002. A transporter in the endoplasmic reticulum of *Schizosaccharomyces pombe* cells mediates zinc storage and differentially affects transition metal tolerance. J. Biol. Chem. 277, 18215–18221.

Díaz, G., Azcón-Aguilar, C., Honrubia, M., 1996. Influence of arbuscular mycorrhizae on heavy metals (Zn and Pb) uptake and growth of *Lygeum spartum* and *Anthillis cytisoides*. Plant Soil 180, 241–249.

Eide, D., Broderius, M., Fett, J., Guerinot, M.L., 1996. A novel iron-regulated metal transporter from plants identified by functional expression in yeast. Proc. Natl. Acad. Sci. USA 93, 5624–5628.

Eide, D., 2004. The ABC of solute carriers. The SLC39 family of metal ion transporters. Eur. J. Physiol. 447, 796–800.

Eitinger, T., Degen, O., Böhnke, U., Müller, M., 2000. Nic1p., a relative of bacterial transition metal permeases in *Schizosaccharomyces pombe*, provides nickel ion for urease biosynthesis. J. Biol. Chem. 275, 18029–18033.

Ferrol, N., Azcón-Aguilar, C., Bago, B., Franken, P., Gollotte, A., González-Guerrero, M., Harrier, L.A., Lanfranco, L., van Tuinen, D., Gianinazzi-Pearson, V., 2004. Genomics of Arbuscular Mycorrhizal Fungi. Applied Mycology and Biotechnology an International Series. Elsevier Science B.V., Amsterdam.

Ferrol, N., Barea, J.M., Azcón-Aguilar, C., 2002. Mechanisms of nutrient transport across interfaces in arbuscular mycorrhizas. Plant Soil 244, 231–237.

Gaither, L.A., Eide, D., 2001. Eukaryotic zinc transporters and their regulation. BioMetals 14, 251–270.

Gao, C.Y., Pinkham, J.L., 2000. Tightly regulated β-estradiol dose-dependent expression system for yeast. BioTechniques 29, 2–6.

George, E., Römheld, V., Marschner, H., 1994. Contribution of Mycorrhizal Fungi to Micronutrient Uptake by Plants. Biochemistry of Metal Micronutrients in the Rhizosphere. Lewis Publishers, Boca Raton, Ann Arbor, London, Tokyo.

Gitan R.S, Eide, D., 2000. Zinc-regulated ubiquitin conjugation signals endocytsis of the yeast ZRT1 zinc transporter. Biochem. J. 346, 329–336.

Gitan, R.S., Luo, H., Rodgers, J., Broderius, M., Eide, D., 1998. Zinc-induced inactivation of the yeast ZRT1 zinc transporter occurs through endocytosis and vacuolar degradation. J. Biol. Chem. 273, 28617–28624.

Guarente, L., 1983. Yeast promoters and lacZ fusions designed to study the expression of cloned genes in yeast. Methods Enzymol. 101, 181–191.

Hirokawa, T., Boon-Chieng, S., Mitaku, S., 1998. SOSUI: classification a secondary structure prediction system for membrane proteins. Bioinformatics 14, 378–379.

Huang, L., Kirschke, C.P., Gitschier, J., 2002. Functional characterization of a novel mammalian zinc transporter, ZnT6. J. Biol. Chem. 277, 26389–26395.

Johansen, A., Jakobsen, I., Jensen, E.S., 1992. Hypal transport of ^{15}N-labelled nitrogen by a vesicula–arbuscular mycorrhizal fungus and its effect on depletion of inorganic soil N. New Phytol. 122, 281–288.

Joner, E.J., Leyval, C., 1997. Uptake of ^{109}Cd by roots and hyphae of a *Glomus mosseae Trifolium subterraneum* mycorrhiza from soil amended with high and low concentrations of cadmium. New Phytol. 135, 353–360.

Kamizono, A., Nishizawa, M., Teranishi, Y., Murata, K., Kimura, A., 1989. Identification of a gene conferring resistance to zinc and cadmium ions in the yeast *Saccharomyces cerevisiae*. Mol. Gen. Genet. 219, 161–167.

Kothari, S.K., Marschner, H., Römheld, V., 1990. Direct and indirect effects of VA mycorrhizal fungi and rhizosphere microorganisms on acquisition of mineral nutrients by maize (*Zea mays* L.) in a calcareous soil. New Phytol. 116, 637–645.

Kothari, S.K., Marschner, H., Römheld, V., 1991. Contribution of the VA mycorrhizal hyphae in acquisition of phosphorus and zinc by maize grown in a calcareous soil. Plant Soil 131, 177–185.

Langmade, S.J., Ravindra, R., Daniels, P.J., Andrews, G.K., 2000. The transcription factor MTF-1 mediates metal regulation of the mouse ZnT1 gene. J. Biol. Chem. 275, 34803–34809.

Li, L., Kaplan, J., 2001. The yeast gene *MSC2*, a member of the cation diffusion facilitator family, affects the cellular distribution of zinc. J. Biol. Chem. 276, 5036–5043.

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

MacDiarmid, C.W., Gaither, L.A., Eide, D., 2000. Zinc transporter that regulate vacuolar zinc storage in *Saccharomyces cerevisiae*. EMBO J. 19, 2845–2855.

MacDiarmid, C.W., Milanick, M.A., Eide, D., 2002. Biochemical properties of vacuolar zinc transport systems of *Saccharomyces cerevisiae*. J. Biol. Chem. 277, 39187–39194.

MacDiarmid, C.W., Milanick, M.A., Eide, D., 2003. Induction of the *ZRC1* metal tolerance gene in zinc-limited yeast confers resistance to zinc shock. J. Biol. Chem. 278, 15065–15072.

McMahon, R.J., Frost, S.C., 1995. Nutrient control of GLUT1 processing and turnover in 3T3-L1 adipocytes. J. Biol. Chem. 270, 12094–12099.

Miyabe, S., Izawa, S., Inoue, Y., 2001. The Zrc1 is involved in zinc transport system between vacuole and cytosol in *Saccharomyces cerevisiae*. Biochem. Biophys. Res. Commun. 282, 79–83.

Moreno, S., Klar, A., Nurse, P., 1991. Molecular genetic analysis of the fission yeast *Schizosaccharomyces pombe*. Methods Enzymol. 191, 795–823.

Nies, D.H., 1992. CzcR and CzcD, gene products affecting regulation of resistance to cobalt, zinc and cadmium (*czc* system) in *Alcaligenes eutrophus*. J. Bacteriol. 174, 8102–8110.

Outten, C.E., O'Halloran, T.V., 2001. Femtomolar sensitivity of metalloregulatory proteins controlling zinc homeostasis. Science 292, 2488–2492.

Palmiter, R.D., Findley, S.D., 1995. Cloning and functional characterization of a mammalian zinc transporter that confers resistance to zinc. EMBO J. 14, 639–649.

Pringle, J.R., Adams, A.E., Drubin, D.G., Haarer, B.K., 1991. Immunofluorescence methods for yeast. Methods Enzymol. 194, 565–602.

Schiestl, R.H., Gietz, R.D., 1989. High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. Curr. Genet. 16, 339–346.

Schüßler, A., Schwarzott, D., Walker, C., 2001. A new fungal phylum, the Glomeromycota: phylogeny and evolution. Mycol. Res. 105, 1413–1421.

Sherman, F., Fink, G.R., Hicks, J.B., 1986. Methods in Yeast Genetics. Cold Spring Harbor Lab. Press, Plainview, NY.

Smith, S.E., Read, D.J., 1997. Mycorrhizal Symbiosis. Academic Press, London.

St-Arnaud, M., Hamel, C., Vimard, B., Caron, M., Fortin, J.A., 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.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTALW: 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.

Vallee, B.L., Auld, D.S., 1990. Zinc coordination, function, and structure of zinc enzymes and other proteins. Biochemistry 29, 5467–5659.

Van der Heijden, M.G.A., Sanders, I.R., 2002. Mycorrhizal Ecology. Springer-Verlag, Berlin.

Wang, F., Dufner-Beattie, J., Kim, B., Petris, M.J., Andrews, G., Eide, D.J., 2004. Zinc-stimulated endocytosis controls activity of the mouse ZIP1 and ZIP3 zinc uptake transporters. J. Biol. Chem. 279, 24631–24639.

Waters, B.M., Eide, D.J., 2002. Combinatorial control of yeast FET4 gene expression by iron, zinc, and oxygen. J. Biol. Chem. 277, 33749–33757.

Zhao, H., Butler, E., Rodgers, J., Spizzo, T., Duesterhoeft, S., Eide, D., 1998. Regulation of zinc homeostasis in yeast by binding of the ZAP1 transcriptional activator to zinc-responsive promoter elements. J. Biol. Chem 273, 28713–28720.

Zhao, H., Eide, D., 1996a. The yeast *ZRT1* gene encodes the zinc transporter protein of a high-affinity uptake system induced by zinc limitation. Proc. Natl. Acad. Sci. USA 93, 2454–2458.

Zhao, H., Eide, D., 1996b. The *ZRT2* gene encodes the low affinity zinc transporter in *Saccharomyces cerevisiae*. J. Biol. Chem. 271, 23203–23210.