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

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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.

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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 (Albrecht 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, 1087-1845/S - see front matter © 2004 Elsevier Inc. All rights reserved. doi:10.1016/j.fgb.2004.10.007
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 Zn uptake in yeast, and expression of these transporters is highly induced under conditions of Zn 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 micro-nutrient, 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 phyla Glomeromycota (Schübler 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 112 plant species. AM fungi down-regulate a plasma membrane 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 ZnSO4 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 ZnSO4 (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 (Δznc1 Δcot1 Δhis3 Δleu2 trp1 ura3-52 zrc1::HIS3 cot1::Kanr'), DEY1551 (ade6 can1-100oc his3-11 leu2-3, 112, trp1-1 ura3-52 mscΔ), and the wild type DY1457 (zade6 can1-100oc his3 leu2 trp1 ura3) were used. The
**Schizosaccharomyces pombe** Δzfh::ura4 strain (h* ade6-M216 leu1-32 ura4-A18 can1-1), provided by Dr. S. Clemens (Leibniz Institut für 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 Δzfh::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 Edinburgh minimal medium (EMM) and HA2 (Table 1) with glucose as carbon source.

Cell number in liquid cultures was determined by measuring the optical density of cell suspensions at 600 nm (OD600) 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 BamHI/EcoRI-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,
200, 400, or 1000 µM ZnSO₄ 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 Ettinger et al. (2000). Complementation tests were performed in EMM media supplemented with 0, 50, 100, 200, 300, 400, or 500 µM ZnSO₄.

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 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: (OD₄₂₀ x 1000) / (min x ml of culture used x OD₆₀₀).

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 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 PCR 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 (Cₜ) 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⁻ΔΔCₜ 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. GinZnT1 belongs to the CDF family of heavy metal transporters

A 563 bp cDNA fragment corresponding to the 3’ end of GinZnT1 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 GinZnT1 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 GinZnT1. These domains are almost identical among GinZnT1 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) between helices IV and V and the C-terminal sequence, E-X-H-X-W-X-L-S-X8-H.

3.2. Ectopic expression of GinZnT1

To further characterize the GinZnT1 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 GinZnT1 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 (MacDarmid et al., 2003). However, GinZnT1 failed to complement the zrc1 cot1 phenotype.

The lack of complementation of the zrc1 cot1 mutant by GinZnT1 could be due to the high levels of transcription caused by a strong promoter, inadequate or defective transcription or translation of the GinZnT1 gene, or sorting of the protein to an inappropriate location in the cell. To address the issue of transcription levels, GinZnT1 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 GinZnT1 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 GinZnT1 gene in yeast.

To verify the translation and location of GinZnT1 protein in the zrc1 cot1 cell, a sequence encoding a triple

Fig. 1. Identification of a new CDF from G. intraradices. (A) Predicted topology of GinZnT1, as defined by SOSUI. (B) Multiple sequence alignment of GinZnT1 (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.
repeat of the hemagglutinin (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.
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 exposure on 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^(-ΔΔCt) method. Data represent the means of three replicates from a representative experiment. Error bars represent SD.

Fig. 4. 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^(-ΔΔCt) 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 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^(-ΔΔCt) 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 $\sim 60\%$ when the extraradical mycelium was grown in the presence of 75 $\mu$M Zn. However, a marked 5-fold inhibition was observed when the mycelia were cultured in the presence of 750 and 7500 $\mu$M 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 to transmembrane domains IV and V, and includes a histidine rich motif (HX$_4$ = 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$_2$-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 ($\sim 9 \mu$M), this medium is likely to be zinc replete. Hence, the induction of GintZnT1 upon addition of 75 $\mu$M 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.

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