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# Functional characterization of *tzn1* and *tzn2*-zinc transporter genes in *Neurospora crassa*

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**Abstract** Previous work from our laboratory involved the description of the *Neurospora* metal transportome, which included seven hypothetical zinc transporters belonging to the ZIP family. The aim of the present study was to make a comparative functional evaluation of two hypothetical zinc transporters named *tzn1* (NCU07621.3) and *tzn2* (NCU11414.3). Phenotypic analysis of Δ*tzn1* and Δ*tzn2* mutants and a double mutant (Δ*tzn1tzn2*) revealed that the deletion of *tzn1* causes aconidiation and a greater defect in growth than the single deletion of *tzn2*. Supplementation with zinc restores growth but not conidiation in Δ*tzn1* and Δ*tzn1tzn2*. TZN1 complemented a zinc-uptake-deficient *Saccharomyces cerevisiae* mutant (Δ*zrt1zrt2*) in zinc-deficient conditions, while *tzn2* restored growth upon supplementation with zinc (0.05 mM). Furthermore, the Δ*tzn1* mutant was found to have severely reduced zinc content indicating that *tzn1* functions as a key regulator of intracellular zinc levels in *Neurospora crassa*. Zinc uptake studies indicate *tzn1* is a specific transporter of zinc, while *tzn2* transports both zinc and cadmium. Quantitative RT-PCR showed up-regulation of *tzn1* (128-fold) under zinc-depleted conditions and down-regulation (>1,000-fold) in zinc-replete conditions. The present study indicates that the zinc transport proteins encoded by *tzn1* and *tzn2* are members of the zinc uptake system regulated by zinc status in *N. crassa*.

**Keywords** Zinc · Metal ion transporters · ZIP family · Aconidiation · *Neurospora crassa*

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## Introduction

*Neurospora crassa* is a multicellular filamentous fungus that has played a central role as a model organism in twentieth century genetics, biochemistry and molecular biology (Perkins 1992). Zinc constitutes 1/30,000 part of *N. crassa*'s mycelial dry weight, and zinc accumulation in *N. crassa* was first reported in 1949 by Anderson-Kotto and Hevesy (1949). Later studies were related to zinc accumulation at toxic concentrations (Sastry et al. 1962). A transport block in zinc uptake was reported in thee Zn-resistant mutants (Rama Rao and Maruthi Mohan 1997). Though zinc toxicity and resistance is well studied in *N. crassa*, zinc transport has not been investigated at the genetic level. The recently completed 39 MB genome sequence of *N. crassa* revealed over 9,826 genes without significant

matches for a large number of genes (41%) in the sequence databases (Galagan et al. 2003). Based on the in silico analysis of the *Neurospora* genome, a catalog of metal transporters referred to as the 'Metal Transportome' was organized from which seven putative zinc transporter genes belonging to the ZIP family were identified (Kiranmayi and Maruthi Mohan 2006).

Zinc transporters are classified into two metal transporter families the ZIP (ZRT/IRT-like protein) and the CDF (Cation Diffusion Facilitator) families. Several ZIP and CDF transporters have been characterized in mammals, yeast, nematode, fruit fly, zebra fish and many candidate genes have been identified by genome projects (Kambe et al. 2006; Eide 2006). The ZIP and CDF family members in mammalian system are referred by the solute carrier nomenclature system (SLC), as SLC39A and SLC30A respectively (Eide 2004; Palmiter and Huang 2004). ZIP family transporters increase intracellular zinc by promoting extracellular zinc uptake, and CDF family transporters reduce intracellular zinc by promoting zinc efflux from cells or into intracellular vesicles. Members of the ZIP family contain eight transmembrane domains (TMDs), with amino and carboxy terminal ends situated on the outer surface of the plasma membrane. The long loop region situated between TMD's III and IV contains conserved histidine residues characteristic to this family that are presumed to function as a zinc-binding site (Guerinot 2000). Also, the lysine residue (K), present in the loop region is responsible for zinc-induced ubiquitination of Zrt1 (Gitan and Eide 2000). Zinc transport has been most extensively studied in the yeast system, and this system served as the model for the identification of several zinc transporters in plants and animal systems. In *Saccharomyces cerevisiae*, there are four transporters that are involved in zinc uptake. Zrt1 is a high affinity transporter that works under low zinc conditions and Zrt2 is a low affinity transporter that plays a role in zinc acquisition under less severe zinc-limiting conditions (Zhao and Eide 1996a, b). The Fet4 transporter is involved in the low affinity uptake of iron and copper as well as zinc (Waters and Eide 2002). A fourth system of zinc uptake in yeast is likely to be the Pho84 high affinity phosphate transporter (Jensen et al. 2003). The human genome contains 14 members of ZIP family for maintaining zinc homeostasis. The ZIP1 transporter is the major zinc uptake system that is ubiquitously expressed in tissues of mice and humans (Eide 2004). TcZNT1, from the Zn/Cd-hyperaccumulating plant *Thlaspi caerulescens* was shown to mediate high-affinity zinc uptake and low-affinity cadmium uptake following expression in yeast (Pence et al. 2000). ZIP1, ZIP3 and ZIP4 from *Arabidopsis* restore zinc uptake function in the corresponding transporter mutant of *S. cerevisiae* (Δ*zrt1 zrt2*) and have been proposed to play a role in zinc transport (Grotz et al. 1998). In the present study the physiological role of two hypothetical zinc transporters in *N. crassa* (NCU07621.3 and NCU11414.3) was investigated.

## Materials and methods

### Strains, media and growth

*Neurospora crassa* and *S. cerevisiae* strains used in this study are listed in Table 1. The *N. crassa* cultures were grown in basal medium (BM) (Maruthi Mohan and Sastry 1983) for vegetative growth and synthetic crossing medium (SCM) was used for crossing (Westergaard and Mitchell 1947). Strains were grown in liquid or solid medium (supplemented with 1.5% agar). The concentration of zinc in BM was 3 μM unless otherwise mentioned. The zinc deficiency conditions were created by adding 1 μM TPEN [N,N,N′,N′-Tetrakis (2-pyridyl methyl) ethylene diamine] to the BM without zinc supplementation. Hyphal growth rates of *N. crassa* strains were measured in Ryan's race tubes at 28°C for 3d (Ryan et al. 1943). In liquid basal media growth was determined at end of experiments (3d) by removing excess moisture by pressing mycelial mats with filter papers and drying at 80°C for 3 h and recording weights. Metal content of mycelia was determined using Atomic Absorption Spectrophotometer (GBC–932 plus) after subjecting to wet acid digestion as described previously (Venkateswerlu and Sastry 1973).

The single knockouts (Δ*tzn1 and* Δ*tzn2*) were obtained from FGSC (Colot et al. 2006). For generation of double knockout mutant, Δ*tzn1* (mating type 'A') and Δ*tzn2* (mating type 'a') were crossed and ascospores were plated on BM-agar containing 1% Sorbose to obtain individual colonies. Single colony isolates with the aconidiation phenotype were

**Table 1** Primers, strains and plasmids used in the study

| Primers | Sequences | Restriction sites |
|---|---|---|
| CTzn1L | 5′ GCTAGCATGTCTTCTGCTGAGCCTTT 3′ | *NheI* |
| CTzn1R | 5′ GTTAACTCAAGCCCACTTGCCAAGCA 3′ | *HpaI* |
| CTzn2L | 5′ GCTAGCATGAACTGCCCTTCTCGCA 3′ | *NheI* |
| CTzn2R | 5′ GTTAACCTAAGCCCAAGCCCCCACC 3′ | *HpaI* |
| ActinL | 5′ ATGATCGGTATGGGCCAGAA 3′ | |
| ActinR | 5′ TGCCATAGAGATCCTTCCTGAC 3′ | |
| RtTzn1L | 5′ AGGGCTGGGACTCTTACTCC 3′ | |
| RtTzn1R | 5′ AGGTGGTGATGACCTTTTCG 3′ | |
| RtTzn2L | 5′ TTGATGCAGCTCTCCATGTC 3′ | |
| RtTzn2R | 5′ AGGAGACGCCGAATATGATG 3′ | |
| RtG3PDHL | 5′ TACATGCTCCGCTACGACAC 3′ | |
| RtG3PDHR | 5′ CACCAGTGGACTCGACAATG 3′ | |

| Strains | Genotype/Comment | Source |
|---|---|---|
| *Neurospora crassa* | | |
| Wild type | 74A-OR23-1A (#4200) *matA* | FGSC |
| #11301 | Δ*tzn1::hph+ matA* | FGSC |
| #11271 | Δ*tzn2::hph+ mata* | FGSC |
| #11272 | Δ*tzn2::hph+ matA* | FGSC |
| Δ*tzn1tzn2* | Δ*tzn1*::hph$^+$ Δ*tzn2*::hph$^+$ | This study |
| *Saccharomyces cerevisiae* | | |
| DY1457 [wild] | *MATα ade6 can1 his3 leu2 trp1 ura3* | Zhao and Eide (1996a) |
| ZHY3 [Δ*zrt1zrt2*] | *MATα ade6 can1 his3 leu2 trp1 ura3 zrt1::LEU2 zrt2::HIS3* | Zhao and Eide (1996b) |

| Plasmids | Comment | Source |
|---|---|---|
| pTA-*tzn1* | cDNA of *tzn1* in TA vector | This study |
| pTA-*tzn2* | cDNA of *tzn2* in TA vector | This study |
| YEp24 | Yeast expression vector | Zhao and Eide (1996a) |
| pMC5-HSET | Yeast expression vector with zrt1 | Zhao and Eide (1996a) |
| pMC5-*tzn1* | pMC5-HSET with cDNA of *tzn1* | This study |
| pMC5-*tzn2* | pMC5-HSET with cDNA of *tzn2* | This study |

selected, and genomic DNA was used to screen via PCR for *tzn1* and *tzn2* using gene-specific primers (Table 1). The actin gene (NCU04173.3) was used as a positive control.

*Saccharomyces cerevisiae* strains were routinely grown in YPD medium (1% Yeast extract, 2% Peptone, 2% Dextrose) at 28 ± 1°C for 2d. Low zinc medium (LZM-EDTA) was used to grow yeast strains under zinc-depleted conditions (Zhao and Eide 1996a). *Escherichia coli* DH5α cells were used in the routine propagation of plasmids. Standard molecular biology procedures for DNA isolation and manipulation were used as per the methods described previously (Sambrook et al. 1989) or as per the manufacturer's protocol. The oligonucleotides and plasmids used in this study are summarized in Table 1.

## Sample preparation for SEM analysis

*Neurospora crassa* mycelia from both the wild type and Δ*tzn1* strains were inoculated on carbon-deficient BM-agar plates and incubated for 12 and 24 h. Later, these samples were fixed with glutaraldehyde (4%) in

phosphate buffer (0.02 M, pH 6.9) for 1 h followed by thee washes with distilled water. The samples were then dehydrated by washing with an ethanol series (10, 20, 30, 50, 70, 90, and 100%) for 15 min each step, and the samples were then air-dried. The specimens were vapor coated with gold chloride and examined under a Scanning Electron Microscope (Hitachi S-806C).

### Construction of plasmids used for *S. cerevisiae* transformation

*Neurospora crassa* cDNA was prepared and used for the amplification of *tzn1* and *tzn2* genes using the primer pairs CTzn1L, CTzn1R and CTzn2L, CTzn2R (Table 1). The PCR products were stabilized in a T/A vector (Inst TA cloning kit, Fermentas) and their identity was confirmed by sequencing (MWG, Bangalore). The cDNA was then subcloned into the yeast expression vector pMC5-HSET containing the zrt1 promoter and the yeast-selectable marker *URA3*. The ZHY3 (Δ*zrt1zrt2*) yeast strain was transformed with the plasmids pMC5-*tzn1* and pMC5-*tzn2*. The ZHY3 strain was also transformed with the plasmids YEp24 and pMC5-*zrt1*, which were used as negative and positive controls respectively. Transformation was carried out according to the lithium acetate procedure (Gietz et al. 1995). All the transformants were selected on LZM-EDTA Leu-Ura dropout medium supplemented with 500 μM $ZnCl_2$.

### Zinc uptake

*Neurospora crassa* strains (wild type, Δ*tzn1,* Δ*tzn2,* Δ*tzn1tzn2*) were grown with increasing concentrations of $^{65}Zn$ (Specific activity: 2.05 mCi/gm). Mycelia were washed and pressed to rid them of excessive moisture, dried at 65°C for 2 h and sample weight was measured. Radioactive counts were measured using an integral $\gamma$-counter coupled to a $2'' \times 2''$ NaI (Tl) well type detector (GRS-201L, ECL). For short-term uptake, 3d mycelia were suspended in 10 ml BM containing 1 μM $^{65}Zn$ and incubated for different time intervals upto 60 min, and counts were then taken. For competition studies, mycelia were suspended in BM containing 1 μM $^{65}Zn$ and 10 μM other metal ions, incubated for 30 min and counts were taken.

### Quantitative real time-PCR of *tzn1* and *tzn2*

Total RNA was isolated using TRI reagent (Sigma) from 1d *N. crassa* grown in zinc-depleted and -replete conditions. The integrity of the RNA was checked on a 2% agarose gel, and quantification was performed at $A_{260nm}$ in a spectrophotometer (Biomate3, Merck). Total RNA was treated with RNase-free DNase I (Fermentas) to remove contaminating genomic DNA. Then, 2 μg of RNA was used to synthesize first-strand cDNA using H Minus M-MuLV reverse transcriptase (Revert Aid kit, Fermentas) by priming with the oligo-$d(T)_{18}$ primer. The fragments were amplified by PCR in a Smart cycler (Applied Biosystems) using Real Master Mix SYBR ROX (5 PRIME, USA). Primer design involved consideration of primer dimer formation, self-priming formation and primer melting temperature using the Primer 3.0 software (Rozen and Skaletsky 2000). The primers used are listed in Table 1. Cycling parameters consisted of 40 cycles of denaturation at 95°C for 20 s, annealing at 56°C for 30 s, and extension at 68°C for 30 s. Reaction specificity was determined for each reaction using the melt-curve analysis of the PCR product. This melt-curve was obtained by tracking the SYBR Green Fluorescence level during a gradual heating of the PCR products from 60°C to 95°C. Greater than 90% amplification efficiency was obtained for all the reactions. The amplified fragments were confirmed by gel electrophoresis and sequencing. Each reaction was carried out in triplicate. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as an internal control, and the level of expression of each transporter gene was normalized to that of G3PDH. There was no genomic contamination and no differences in the internal control for each sample. Analysis of relative gene expression data was performed using the $2^{(-\Delta\Delta Ct)}$ method (Livak and Schmittgen 2001).

### Statistical analysis

Data shown are mean values of triplicates from two separate experiments (±SD).

## Results

A comparative evaluation of NCU07621.3 (*tzn1*) and NCU11414.3 (*tzn2*) was performed. *N. crassa tzn1*

has an open reading frame (ORF) of 1,257 bp that encodes a 419-amino acid protein with eight transmembrane domains (TMDs). *Tzn1* shows 55% homology to the first characterized zinc transporter of *S. cerevisiae* (zrt1), while *tzn2* shows only 29% homology to zrt2. TMDs III and IV are separated by a region that is longer than any of the rest of the spacer regions between other domains. Interestingly, no introns were observed within the coding region of *tzn1*, in contrast to other metal transporter genes identified in *N. crassa.* The ORF of *tzn2* is 1,854 bp and encodes 597-amino acids, contains of nine TMDs and is interrupted by a 60 bp intron. Both genes (*tzn1* and *tzn2*) are located on linkage group IV and have a lysine (K) residue in the variable loop region predicted to be involved in post translational modification though ubiquitination (Gitan and Eide 2000).

## Phenotypic analysis and metal content of zinc transporter mutants

In the asexual life cycle of *N. crassa* chains of macroconidia are formed by budding and constriction of the aerial hyphae, several aconidiation mutants with defects in the above have been characterized (Davis 2000). Morphologically, Δ*tzn2* resembles the wild type but Δ*tzn1* and Δ*tzn1tzn2* show a distinct aconidiation phenotype and lacked aerial hyphae. In order to evaluate whether the aconidiation was due to zinc deficiency, Δ*tzn1* and Δ*tzn1tzn2* were grown in excess zinc. However, conidiospores were not observed, even upon extended incubation upto two weeks. Scanning electron microscopy (SEM) was performed to evaluate the morphological block in the Δ*tzn1* mutant. After 12 and 24 h growth in conidiation-inducing conditions, the wild type strain displayed abundant proconidial chains (Fig. 1a, b), while Δ*tzn1* did not produce conidia, this strain had hyphal morphology and aerial hyphae similar to the wild type (Fig. 1c, d).

The linear growth of wild type, **Δ***tzn1*, Δ*tzn2*, and Δ*tzn1tzn2* strains was compared on BM-agar medium in race tubes at 28°C for 3d. The growth of wild type and Δ*tzn2* strains was similar, whereas Δ*tzn1* and Δ*tzn1tzn2* showed very slow growth (Fig. 2a). When compared to the wild type 50% less growth was observed in these strains, even after supplementation with trace amounts of zinc (≤1.5 μM). Overall growth restoration in the knockout strains was observed with zinc supplementation (2–3 μM). Similar results were observed in liquid media (Fig. 2b). In order to determine whether this was a zinc specific effect, other metal ions were supplemented under zinc deficient conditions. None of the other metal

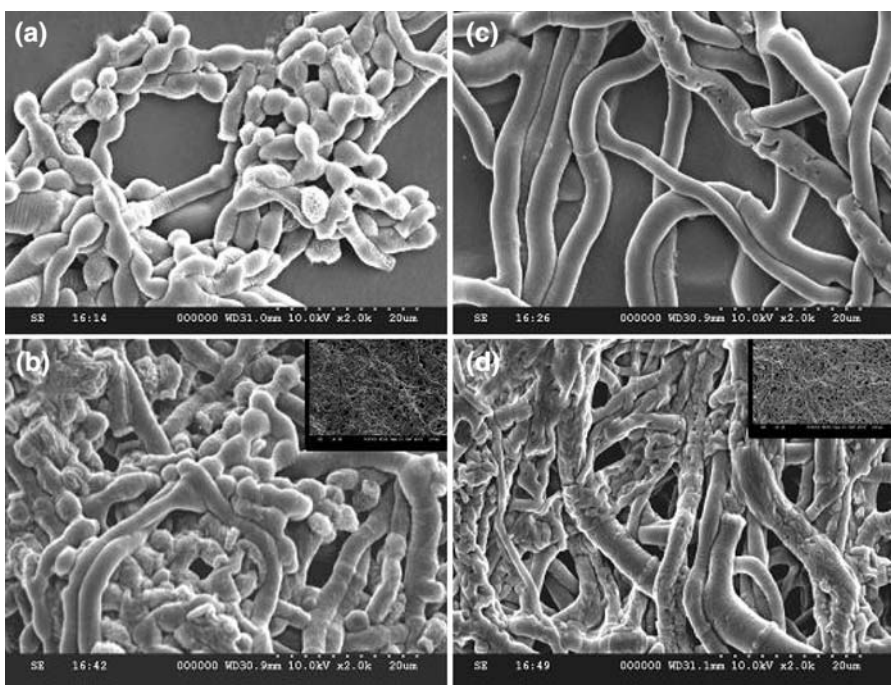



**Fig. 1** Scanning electron micrographs displaying wild type (**a**, **b**) and Δ*tzn1* (**c**, **d**) conidiation phenotypes. Mycelia were inoculated in carbon-deficient BM-agar plates, which is a conidiation inducing condition, and incubated for 12 h (**a**, **c**) and 24 h (**b**, **d**). Magnification: a, b, c, d—2000×; inset pictures—500×. Scale bars—20 μm. This phenotype was used to score progeny during double knockout generation

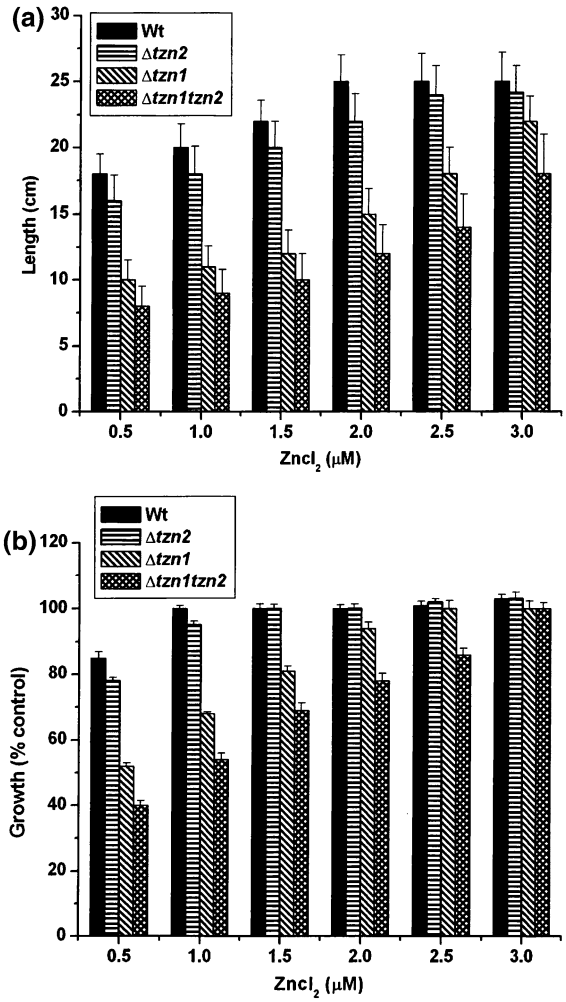



**Fig. 2** **a** Comparison of linear growth (in race tubes) of the wild type and the mutant strains (Δ*tzn1*, Δ*tzn2* and Δ*tzn1tzn2*) on BM agar with increasing concentrations of zinc at 28°C. **b** *N. crassa* strains were grown in 10 ml BM containing increasing concentrations of zinc at 28 ± 1°C for 3d. Growth in normal BM is used as a control

ions tested (Fe, Ni, Cd, Co, and Cu) restored growth (data not shown).

The total mycelial zinc content in wild type, Δ*tzn1*, Δ*tzn2*, and Δ*tzn1tzn2* strains was assayed after growth in BM with and without zinc supplementation. The data in Fig. 3 shows that in the presence of zinc (3 μM), there is a marginal decrease in zinc content in all the mutants. In zinc-deplete conditions, a 70% decrease in the zinc content of mycelia in Δ*tzn1* and Δ*tzn1tzn2* mutants was observed, whereas a ∼25% decrease was observed in Δ*tzn2* mutant.

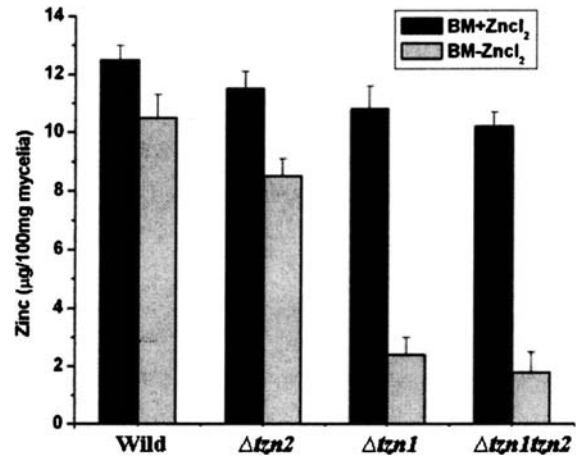



**Fig. 3** Zinc concentration in the mycelia of wild type and mutant strains (Δ*tzn1*, Δ*tzn2* and Δ*tzn1tzn2*) grown in BM with (3 μM $ZnCl_2$) and without zinc

## *Tzn1* restores the growth defect of *S. cerevisiae* (Δ*zrt1zrt2*)

To obtain further evidence for zinc transport function, *tzn1* and *tzn2* were expressed in a knockout model of *S. cerevisiae* (Δ*zrt1zrt2*). This mutant fails to grow under zinc deficient conditions and responds only to high amount of zinc (>1 mM). In a heterologous experiment using LZM-EDTA medium, *zrt1* (positive control) and *tzn1* restored the growth defect of the Δ*zrt1zrt2* mutant, while *tzn2* could restore growth only after supplementation with zinc (50 μM). YEp24 used as negative control showed growth at a high concentration from 1 mM zinc (Fig. 4).

## Zinc uptake by *tzn1* and *tzn2*

Zinc accumulation by *N. crassa* at end of 3d growth was studied using $^{65}Zn$ as a tracer. The data in Fig. 5a shows that zinc accumulation increases upto 20 μM in all the *N. crassa* strains. However, accumulation by Δ*tzn1* and Δ*tzn1tzn2* strains was markedly lower as compared to the wild type strain, which shows saturation at 20 μM zinc. *N. crassa* Δ*tzn2* accumulates comparatively more than other mutants (Fig. 5a).

In long-term zinc uptake during growth for 3d, zinc accumulation could also occur though other non-specific transport systems. Hence, the transport kinetics for short time intervals was studied using mycelial mats. Zinc uptake by wild type *N. crassa*

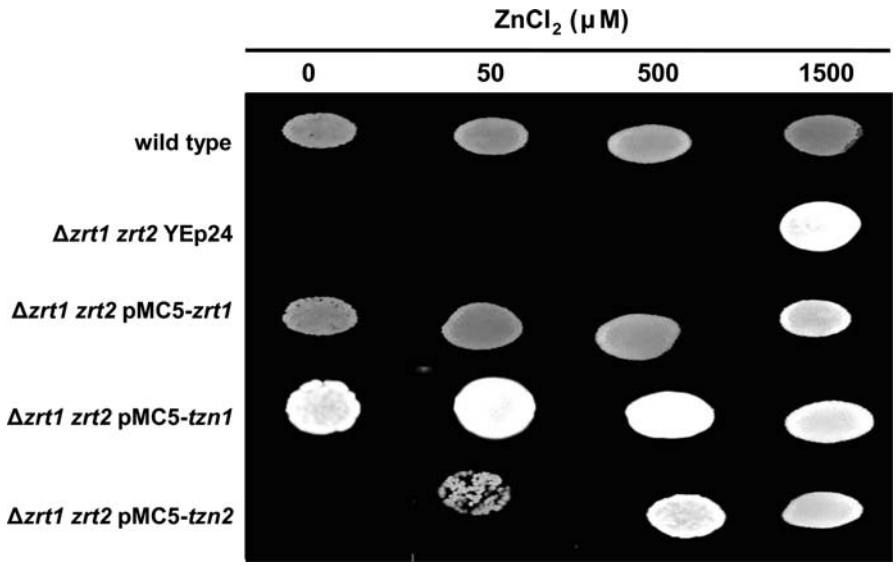



**Fig. 4** Complementation analysis of zinc uptake by the *N. crassa tzn1* and *tzn2* genes in *S. cerevisiae* ZHY3 (Δ*zrt1zrt2*), a double knockout of *zrt1* and *zrt2* zinc transporter genes. ZHY3 was transformed with the empty vector YEp24, pMC5-*zrt1*, pMC5-*tzn1* and pMC5-*tzn2*. *zrt1* was taken as positive control. A total of $10^4$ *S. cerevisiae* cells were spotted on LZM-EDTA medium with increasing concentrations of $ZnCl_2$. Plates were incubated for 2d at 30°C

was distinctly higher, showing near saturation around 50 mins at which a concentration of ~240 pmol/100 mg mycelia was observed. In sharp contrast, the Δ*tzn1* and Δ*tzn1tzn2* strains accumulated very low levels (30 pmol/100 mg) during the 60 min test period. During the same testing period, the Δ*tzn2* mutant was able to transport 90 pmol/100 mg (Fig. 5b).

To assess the substrate specificity of the uptake systems, other metal ions were included in excess (10-fold) and zinc uptake was investigated. Nickel, cobalt, copper and iron did not influence zinc accumulation in any of these strains. The addition of cold zinc alone inhibited uptake between 80 and 90% in wild type and mutant strains of *N. crassa*. However, cadmium showed 70–90% inhibition of zinc uptake in Δ*tzn1* and wild type strains, while only 20% inhibition was observed for Δ*tzn1tzn2* (Fig. 5c). In the case of Δ*tzn2*, there was no significant inhibition by cadmium.

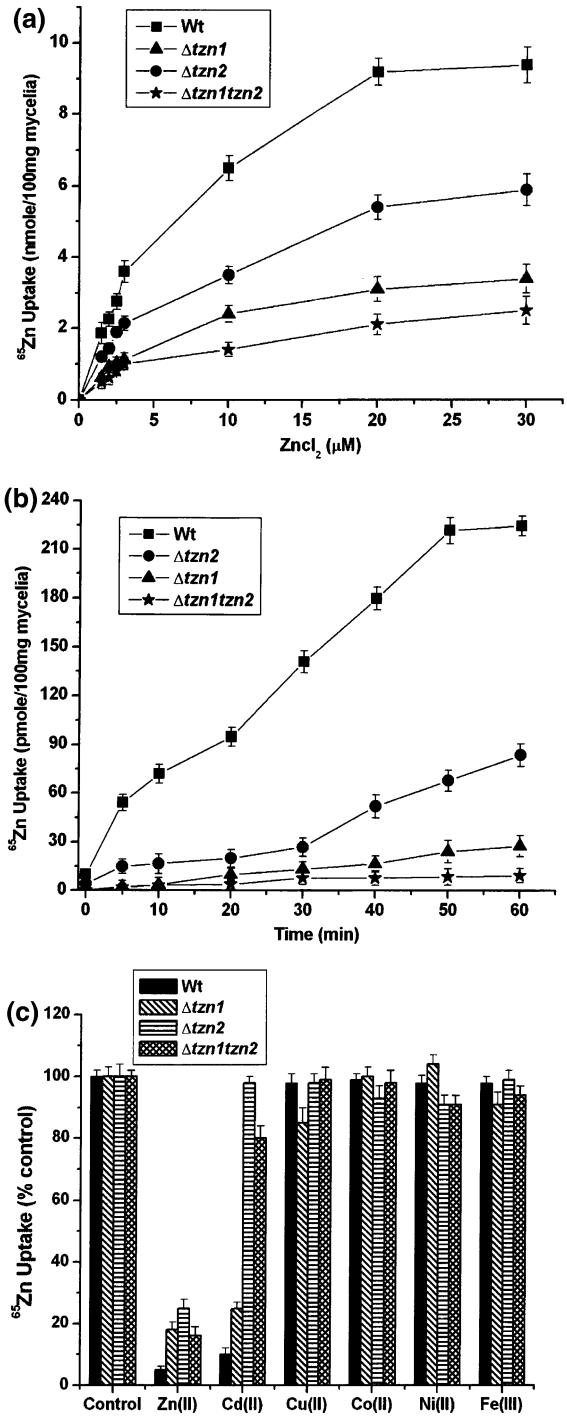
### Expression of tzn1 is regulated in a zinc-dependent manner

The expression of *tzn1* was studied under zinc-deplete and -replete conditions though quantitative Real Time PCR (qRT-PCR). The expression of *tzn1* was up-regulated 128-fold under zinc-depleted and down-regulated 1,024-fold under zinc-replete conditions (Table 2).

To investigate the stability of the *tzn1* transcript, *N. crassa* was grown under zinc deficient conditions (1d) and then supplemented with zinc (20 μM) and incubated upto 60 min. The results show a significant decrease 12-fold decrease in the *tzn1* transcript levels at 10 min, while at 60 min, a 798-fold decrease is observed (Table 2). Under similar conditions to those described above for *tzn1*, less expression of *tzn2* (4-fold during deplete and 8-fold during replete conditions) compared to *tzn1* was observed. With respect to the stability of the *tzn2* transcript, 15-fold less expression was observed at 60 min (Table 2).

## Discussion

The present study demonstrates that *tzn1* and *tzn2* function as zinc-regulated transporters responsible for the uptake of zinc in *N. crassa*. The Tzn1 and Tzn2 proteins of *N. crassa* seem to be orthologues of zrt1 and zrt2 of *S. cerevisiae* respectively. The growth of Δ*tzn2* was identical to the wild type strain, whereas the growth of the Δ*tzn1* mutant had a profound effect on growth that resembled the zrt1 mutation in *S. cerevisiae*. A series of zinc accumulation experiments performed at substrate concentrations between 0.5 and 30 μM confirmed the assumption that *tzn1* and *tzn2* are zinc transporters in *N. crassa*. A 60-min uptake assay indicated the importance of other transporters for zinc uptake. To test the specificity




◀ **Fig. 5** **a** *Neurospora crassa* strains were grown for 3d in BM containing increasing concentrations of $^{65}$Zn. **b** *N. crassa* strains were grown in zinc-depleted medium for 3d and mycelial mats were incubated with 1 μM 65Zn for the indicated time periods at 30°C. **c** *N. crassa* strains were grown for 3d under zinc-depleted conditions and later incubated in medium containing 65Zn (1 μM) and 10 μM of other metal ions for 30 min at 30°C. In all the experiments the mycelia were washed with deionized water and radioactivity was measured in a $\gamma$-counter

**Table 2** Quantitative real-time PCR analysis of *tzn1* and *tzn2*

| | *tzn1* | *tzn2* |
|---|---|---|
| Condition[a] | | |
| 1 μM TPEN | 128 ± 2↑ | 4 ± 1↑ |
| 250 nM Zinc | 2 ± 1↓ | 2 ± 1↓ |
| 1 μM Zinc | 8 ± 2↓ | 4 ± 1↓ |
| 5 μM Zinc | 1024 ± 4↓ | 8 ± 2↓ |
| Time[b] | | |
| 10 min | 12 ± 2↓ | 2 ± 1↓ |
| 30 min | 88 ± 3↓ | 8 ± 2↓ |
| 60 min | 798 ± 3↓ | 15 ± 3↓ |

[a] Wild type *N. crassa* grown under zinc-depleted and -replete conditions for 1d. Total RNA was isolated, from which cDNA was prepared, and the expression of *tzn1* and *tzn2* were quantified by qRT-PCR

[b] Wild type *N. crassa* was grown under zinc-deplete conditions for 1d, with zinc (20 μM) was added thereafter; mycelia were harvested at different time intervals. Total RNA was isolated, and the expression of *tzn1* and *tzn2* were quantified by qRT-PCR

of both these genes, the effect of other competing metal ions on zinc accumulation was studied. With the exception of cadmium, none of the other metal ions caused significant inhibition of zinc accumulation (Fig 5c) in Δ*tzn1*. This indicates that *tzn2* transports both zinc and cadmium, whereas *tzn1* is highly selective for zinc. Similar reports of zinc-specific transporters have been published for ZIP family members in rice (Ishimaru et al. 2005). Several reports indicated that cadmium mimics zinc in its ability to bind biological substrates, probably due to the similar electronic conformation that exists between the two elements (Palumaa et al. 2002). Regardless, cadmium is a toxic element; even though it can replace zinc structurally, it cannot replace it functionally (Hartwig 2001). Expression of *tzn1* and *tzn2* under zinc-depleted and zinc-replete conditions shows that *tzn1* is upregulated under depleted conditions and down-regulated under replete conditions. In comparison to the above, expression of *tzn2* was quantitatively much lower, but regulated by zinc levels under both of the above conditions. These results are similar to those obtained for zrt1 of

*S. cerevisiae* and *zrfB* of *A.fumigatus* (Zhao and Eide 1996b; Vicentefranqueira et al. 2005).

The aconidiation phenotype of Δ*tzn1* is similar to the fluffy (*fl*), fluffyoid (*fld*) and acon (acon-2, acon-3) mutants of *N. crassa*. In the fluffy mutant, the *fl* gene is responsible for white aerial growth without the production of conidia. This gene located on linkage group II encodes a protein that resembles C6 zinc cluster transcription factors of the Gal4p class (Bailey and Ebbole 1998). Fluffyoid (*fld*) and aconidiate (*acon-2*) do not produce minor constriction chains and are blocked early in development while acon-3 has defect in major constriction chain formation (Matsuyama et al. 1974; Springer and Yanofsky 1989). An interesting point to be noted herein is that in three of the mutants (*fld*, *acon-3*, Δ*tzn1*) have separate and non-identical loci on the same chromosome (Linkage group IV) of *N. crassa*, while acon-2 is located on linkage group III (Perkins et al. 1982). The above feature along with the SEM data suggests Δ*tzn1* to be a newer member of the aconidiation phenotypes. Excess zinc supplementation could not reverse the aconidiation phenotype of Δ*tzn1*, indicating that the defect is not due to zinc deficiency. This opens up newer questions regarding the role of *tzn1*, a membrane zinc transporter in conferring an aconidiation phenotype.

The presence of seven membrane transporters for zinc raises a fundamental question regarding their sub-cellular localization. In *S. cerevisiae*, the zinc transporters *ZRT1and ZRT2* are located on the plasma membrane, while *ZRT3*, *ZRC*, and *COT1* are localized to vacuolar membranes; *Msc-zrg17* is located in the endoplasmic reticulum (Eide 2006). As such we made an attempt to characterize *tzn1* using a GFP tagged expression system. However, the data obtained did not provide a decisive result, though broad membrane based expression was observed throughout. One noteworthy feature of overexpressing *tzn1* in the homologous system was that a 40% increase in zinc accumulation was observed as compared to the wild type strain of *N. crassa* (data not shown).

The present study in the model system of *N. crassa* was the first conducted with respect to the high affinity and low affinity zinc transporters in this organism. Furthermore, this is also the first study to investigate the functional assignment for any of the transition metal ion transporters in this model fungus. Although yeast model system is extensively used for the study of metal ion transporters, *Neurospora* offers several additional challenges due to its multicellularity and the fact that its differentiation is divided into asexual and sexual life cycles. The present study provides functional evidence for *tzn1* as a high affinity gene and *tzn2* as a low affinity gene involved in zinc uptake in *N. crassa*.

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