RESEARCH ARTICLE

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Enhancement of catalase and superoxide dismutase activities in transgenic *Escherichia coli* expressing rice metallothionein isoforms

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ABSTRACT
Metallothioneins (MTs) are small, ubiquitous Cys-rich proteins known to be involved in the homeostasis of essential metals, detoxification of toxic metals and protection against oxidative stress. In the present work transgenic *E. coli* overexpressing different rice MT isoforms including OsMT1, OsMT2, OsMT3 and OsMT4 showed higher tolerance against hydrogen peroxide stress. Moreover, the enzymatic activity of catalase (CAT) and superoxide dismutase (SOD) in the transgenic strains were higher than that in control strain when they were grown in the medium containing hydrogen peroxide. To understand the mechanistic action of MT on the removal of reactive oxygen species, the activity of CAT and SOD in the *E. coli* protein extract were assayed after addition of pure Zn^{2+}/MTs, Fe^{2+}/MT and apo/MT, whereas the activity of CAT and SOD did not change by the addition of apo/MT, the activity of CAT and SOD were increased after addition of Fe^{2+}/MTs and Zn^{2+}/MTs, respectively. These results open new insights into the role of MT as an activator of CAT and SOD by supplying metals.

Key words: Catalase, Hydrogen peroxide, Metallothionein, Rice, Superoxide dismutase

Introduction

Generation of reactive oxygen species (ROS) is one of the earliest responses of the cells to various biotic and abiotic stresses (Jajic et a., 2015). The different ROS, including superoxide, hydrogen peroxide, singlet oxygen and hydroxyl radicals are capable of inducing cellular damage by oxidation of proteins, inactivation of enzymes, alterations in gene expression, DNA injury and decomposition of biomembranes (Jajic et al., 2015; Lobo et al., 2010). Scavenging or detoxification of excess ROS is achieved by an efficient antioxidative system comprising of the non-enzymatic as well as enzymatic antioxidants (Das et al., 2014). The enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), enzymes of ascorbate-glutahione (AsA-GSH) cycle such as ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) (Das et al., 2014; Sharma et al., 2012). Ascorbate (AsA), glutathione (GSH), carotenoids, tocopherols, phenolics and metallothioneins (MTs) serve as potent non-enzymatic antioxidants within the cells (Sharma et al., 2012).

MTs are Cys-rich, low molecular weight proteins that can bind metals via the thiol groups of their Cys residues (Cobbet et al., 2002). MTs play important roles in maintaining the homeostasis of essential metals and metal detoxification (Yuan et al., 2008). In addition, it is very well documented that MTs involve in cellular defence against oxidative stress by functioning as an antioxidant (Kang et al., 2007). It has been hypothesized that MTs scavenge the superoxide radicals either independent of SOD or act as an activator of SOD by supplying metals, such as Cu^{2+} or Zn^{2+} to apo SOD (Go et al., 2001).

Plants have several MT isoforms which are classified into four types based on the arrangement of Cys residues. With few exceptions, a typical type 1 MT contains six Cys residues in their N-terminal CXC motifs (where X denotes any amino acid other than Cys). Type 2 MTs contain eight Cys residues at their N-terminus, arranged in the form of CC, CXC, and CXXC motifs. Type 3 MTs are small proteins with four Cys residues arranged in the form of CXXC and CXC motifs in their N-terminal Cys-rich region (Zhou et al., 2006). In contrast, the distribution of Cys residues within the C-terminus follows the consensus sequence CXCXXXCXCXXCXC, which is highly conserved in all three types of MTs (Freisinger et al., 2011). Type 4 MTs contain three Cys-rich regions, each containing five or six conserved Cys residues. Each type of MT exhibits a distinct spatial and temporal expression pattern in plant tissues during

development and possibly has different functions (Yang et al., 2015). Type 1 MT genes are predominantly expressed in both leaves and roots, whereas type 2 MT genes are expressed primarily in leaves, stems, and developing seeds. Type 3 MT genes are expressed in the leaves or in the ripening fruits, and the expression of type 4 MT genes are reported not only in seeds but also detected in reproductive organs and vegetative tissues (Leszczyszyn et al., 2013; Chyan et al., 2005)

Previously, we heterologously expressed and characterized four rice (*Oryza sativa*) genes encoding OsMT1 (Mohammadi-Nezhad et al., 2013), OsMT2 (Pirzadeh et al., 2016), OsMT3 (Shahpiri et al., 2015) and OsMT4 (Mohammadi-Nezhad et al., 2013) which belong to the type 1, type 2, type 3 and type 4, respectively. The *E. coli* cells expressing OsMTI-1b, OsMTI-2b and OsMTI-3a showed increased tolerance to Cd^{2+}, Ni^{2+} and Zn^{2+} and accumulated more metals compared with control strains. However, heterologous expression of OsMT4 had no significant effects on metal tolerance or ion accumulation. Here we aim to study the scavenging effect of these four MT isoforms. To this end, the CAT and SOD activity were determined in the *E. coli* cells which heterologously expressed these four OsMT isoforms. In addition, the purification of recombinant forms of OsMTs and preparation of Zn^{2+}/ and Fe^{2+}/OsMTs enabled us to study the effect of Zn^{2+}/ and Fe^{2+}/OsMTs on SOD and CAT activity using *in vitro* assays.

Materials and Methods

Heterologous expression of GST-OsMTs in E.coli

The cells harboring plasmids pET41a, pET-OsMT1 (Mohammadi-Nezhad et al., 2013), pET-OsMT2 (Pirzadeh et al., 2016), pET-OsMT3 (Shahpiri et al., 2015), and pET-OsMT4 (Mohammadi-Nezhad et al., 2013) were grown at 37 °C in 80 mL Luria-Bertani (LB) medium supplemented with 50 µg/ml kanamycin and 5 µg/ml chloramphenicol to an OD600 of about 0.6. At this OD cultures were induced by 100 µM Isopropyl β-D-1-thiogalactopyranoside (IPTG). To confirm heterologous expression of the proteins, 1 ml samples of culture medium were harvested by centrifugation 4 hours after addition of IPTG and frozen at – 80 °C until use. The frozen pellets were resuspended in 250 µl pre-cold 10 mM Tris–HCl, pH 8.0, disrupted by mild sonication at 4 °C and centrifuged at 12,000 g, for 20 min. The soluble proteins recovered in the supernatant phase were analyzed by 12 % SDS-PAGE and stained by Coomassie Brilliant Blue R-250 (Mohammadi-Nezhad et al., 2013).

Tolerance of E. coli cells to H_2O_2 and ethanol

Overnight cultures of different strains were inoculated into 80 mL of LB medium with desired antibiotics and grown at 37◦C under agitation. Protein expression was induced at an A600 of 0.6 by addition of 0.1 mM IPTG. After 20 min, culture flasks were supplemented with either 2 mM H_2O_2 or 4 % ethanol and bacterial growth was monitored by A600 measurements at 1 h intervals up to 12 h.

Determination of CAT activity

Overnight cultures of different strains were inoculated into 50 mL of LB medium with desired antibiotics and grown at 37◦C under agitation. Protein expression was induced at an A600 of 0.6 by addition of 0.1 mM IPTG. After 20 min, culture flasks were supplemented with 2 mM H_2O_2. The cells from whole volume were harvested 4 h after addition of IPTG. For extraction of soluble proteins first the cells were resuspended in 40 ml buffer A (50 mM Tris-HCl, 50 mM Glucose, pH 7.8). Then, the cells were harvested by centrifugation at 4°C for 20 min. The harvested cells were again resuspended in 3 ml buffer A containing 1mg/ml lysozyme. The cell suspensions were kept at room temperature for 15 min. Then 3 ml of buffer B (50 mM Tris-HCl, 50 mM KCl,1 % Triton X-100) was added and the cell suspensions were shaken for 2 h at 37 °C. Then the cell suspension was centrifuged (25 min, 4°C, 10000g) to remove cell debris. The supernatant containing soluble protein was transferred to clean tubes and kept in – 20 °C until use. The concentration of proteins was determined by the Bradford assay with bovine serum albumin (BSA) as standard (Bradford et al., 1976). The CAT activity was determined spectrophotometrically following H_2O_2 consumption at 240 nM (Aebi et al., 1974). A 500 µl reaction mixture containing 100 µg/ml extracted protein, 10 mM Tris-HCl, pH 7.4 was started with the addition of 20 µM H_2O_2. The reaction was monitored at 240 nm for 1 min and the rate of H_2O_2 consumption was calculated (using the extinction coefficient of 36 M^{-1} Cm^{-1}). One unit of CAT activity is defined as the degradation of 1 µmol H_2O_2/min.

Determination of SOD activity

The growth of bacteria and the extraction of soluble proteins from different strains were performed as described above. SOD activity was determined based on the ability of this enzyme to inhibit the reduction of nitro-blue tetrazolium by superoxide (Winterbourn et al. 1975). 500 µl reactions containing 1 mM nitroblue tetrazolium chloride (NBT), 3.75 mM EDTA, 100 µg/ml extracted proteins, 10 mM Tris-HCl, pH 7.4 were started by addition of 100 µM riboflavin. The tubes were placed below 15 W fluorescent lamps for 10 min. The reactions were stopped by switching off the light and covering the tubes with black cloth. A non-irradiated complete reaction mixture was served as a blank. Absorbance was recorded at 560 nm. The reaction containing all components except cell extract was used as negative control. The rate of reactions (Δ A260/min) was determined for both samples and negative control. The SOD activity correlates

with the percent of inhibition of reduction of NBT. The inhibition percent was calculated according to the equation (1).

$$\%\ inhibition\ = \frac{\Delta 260nm/\min(Negative\ Control) - \Delta 260nm/\min(Sample)}{\Delta 260nm/\min(Negative\ Control)} \quad (1)$$

Preparation of Zn/GST-OsMTs and Fe/GST-OsMTs

The production of recombinant GST-OsMTs was performed as described above. The purification of GST-MTs was performed using affinity chromatography as described previously14. To this end the extracted proteins were applied on to His Trap HP column (GE, Healthcare) pre-equilibrated with loading buffer (10 mM imidazole, 500 mM NaCl, 30 mM Tris–HCl, pH 8.0) and the bound proteins were eluted by 68.5–283 mM imidazole gradient. The quality of purification was tested by analyzing on 12 % SDS-PAGE and stained by Coomassie Brilliant Blue R-250. The pure fractions were transferred into 12 kDa molecular weight cutoff cellulose tubes (12 kDa molecular weight cut off, Sigma) and dialyzed against 10 mM Tris–HCl, pH 8.0, at 4 ◦C overnight to remove imidazole. The concentration of proteins was determined by Beer-Lambert law with the molar extinction coefficient of 46340, 46465, 46277, 44975 M-1cm-1 for GST-OsMT1, GST-OsMT2, GST-OsMT3 and GST-OsMT4, respectively. Apo-proteins were prepared by the methods previously described (Toriumi et al., 2005). For the preparation of apo-proteins, aliquots of 25 µM purified GST-MTs were acidified with HCl to pH 2.0 and the samples were then dialyzed against 0.1 N HCl, pH 2.0, to remove bound metal ions. Reconstitution with Zn^{2+} and Fe^{2+} was achieved by the addition of 10-mole equivalents of Zn^{2+} and Fe^{2+} followed by neutralization of the samples to pH 8.0 with 200 mM Tris. The unbound metals were removed by dialyzing against 10 mM Tris–HCl, pH 8.0, at 4◦C overnight.

The effect of Pure Zn^{2+}/ and Fe^{2+}/GST-OsMTs on the activity of CAT and SOD

The soluble protein fraction was extracted from control strain that was grown in the presence of 2 mM H_2O_2. The activities of CAT and SOD in this extract were determined before and after addition of 2 µM Apo/GST-OsMTs, Zn/GST-OsMTs or Fe/GST-OsMTs by the methods described above.

Results

Confirmation of heterologous expression of OsMTs in E. coli

The isoforms OsMT1 with 6 Cys, OsMT2 with 8 Cys, OsMT3 with 4 Cys at N-terminus of their amino acid sequences are characterized as members of type 1, type 2 and type3 MT, respectively. The isoform OsMT4 with 3 Cys-rich regions is known as a type 4 MT (Fig. 1A). The coding sequence of OsMT1, OsMT2, OsMT3 and OsMT4 were previously cloned into the expression vector pET41a containing coding sequences for an N-terminal fusion partner of glutathione-S-transferase (GST-tag), a 6 His-tag, and an S-tag (the complete tag was named GST) (Fig. 1B). Following induction with IPTG, the recombinant proteins GST, GST-OsMT1, GST-OsMT2, GST-OsMT3 and GST-OsMT4 were expressed in the soluble fraction of the E. coli cells carrying the pET41a (Control strain), pET41a-OsMT1 (R-OsMT1), pET-OsMT2 (R-OsMT2), pET-OsMT3 (R-OsMT3) and pET-OsMT4 (R-OsMT4), respectively. The theoretical molecular weight of GST, GST-OsMT1, GST-OsMT2, GST-OsMT3 and GST-OsMT4 were 35.5, 39.94, 43.9, 38.98 and 41.2 kDa, respectively. SDS-PAGE analysis showed sharp protein bands of expected molecular masses for these proteins (Fig. 2).

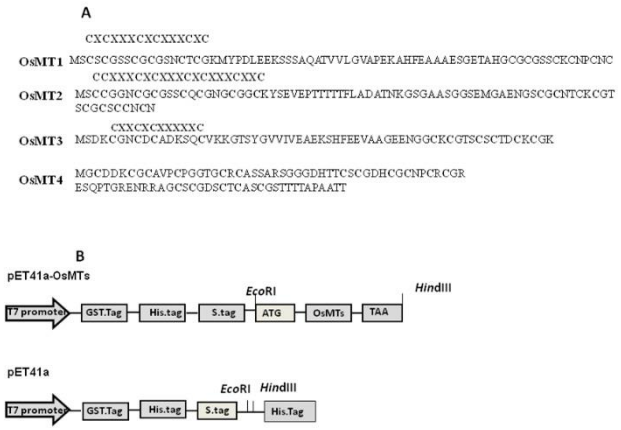

Figure 1. *Amino acid sequence and expression vector maps. (A) The amino acid sequence of OsMT1, OsMT2, OsMT3 and OsMT4. The Cys residues are shown as bold. (B) The map of pET41a-OsMTs and pET41a. The positions of His.tag, S.tag and GST.tag are shown in gray boxes.*

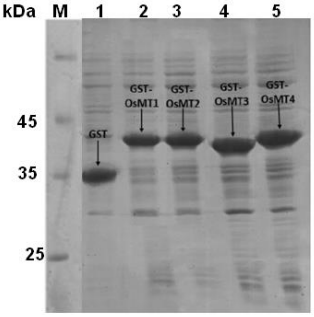

Figure 2. *SDS-PAGE analysis of GST and GST–OsMTs. Total proteins extracted from E. coli harboring pET41a (lane 1), pET41a-OsMT1 (lane 2), pET41a-OsMT2 (lane 3), pET41a-OsMT3 (lane 4) and pET41a-OsMT4 (lanes 5) 4 h after addition of IPTG.Tolerance of E. coli cells to H_2O_2 and ethanol stress.*

The final cell densities (cell density after 12 h) of strains R-OsMT1, R-OsMT2, R-OsMT3 and R-OsMT4 were similar

to that from control when the strains were grown in the medium with no stress (Fig. 3A). However, in the presence of 2 mM H_2O_2 whereas the density for control strain after 12 h reached 0.58, the densities for strains R-OsMT1, R-OsMT2, R-OsMT3 and R-OsMT4 were 1.07, 1.25, 0.76 and 1.25, respectively (Fig. 3B). These data show that the heterologous expression of GST-OsMT1, GST-OsMT2, GST-OsMT3 and GST-OsMT4 enhance the tolerance of E. coli cells to H_2O_2. In the presence of 4 % ethanol, the final cell densities for strains expressing OsMTs were almost similar to that from control strain indicating the expression of OsMTs does not affect the E. coli tolerance to ethanol (Fig. 3C).

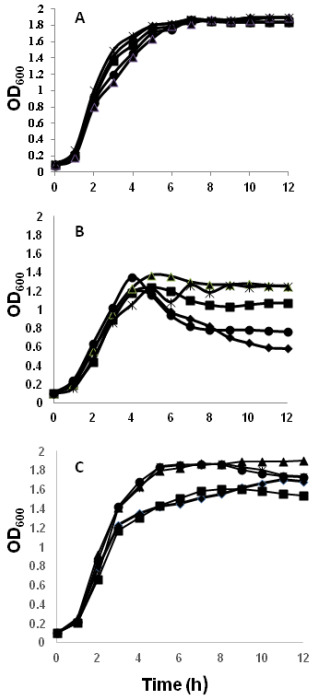

Figure 3. *Growth curves of control (♦), R-OsMT1 (■), R-OsMT2 (□), R-OsMT3 (●) and R-OsMT4 (▲) in the (A) control medium and in the medium containing (B) 2 mM H_2O_2 and (C) 4 % ethanol.*

The activities of CAT and SOD are enhanced in bacteria expressing GST-OsMTs

As shown in Fig. 4 A, the CAT activity in the control strain was 15.2±0.78 U mg-1 when it was grown in the medium containing 2 mM H_2O_2. This value significantly increased to 20.8±1, 23.6±0.8, 18±0.5, and 19.4±0.9 in the strains R-OsMT1, R-OsMT2, R-OsMT3 and R-OsMT4, respectively when they were grown in the same condition. The SOD activity was quantified for the extracts of control strain as well as strains expressing GST-OsMTs. The values are presented as percentage inhibition of formazan production. Higher inhibition of formazan production from NBT represents higher SOD activity. As shown in Fig. 4B, the SOD activity was 43±5 % (% of inhibition) for control strain which was grown in the medium containing 2 mM H_2O_2. This value significantly increased to 70±6, 67±3, 70±4 and 73±7 % in the strains R-OsMT1, R-OsMT2, R-OsMT3 and R-OsMT4, respectively when they were grown in the same condition. These results imply that the strains expressing GST-OsMTs might have obtained a more efficient antioxidant system with improved antioxidant enzyme activities.

The effect of Pure Zn^{2+}/ and Fe^{2+}/GST-OsMTs on the activity of CAT and SOD

The CAT activity was determined for the extract of control strain before and after addition of pure recombinant forms of either apo/GST-OsMTs as well as Fe^{2+}/GST-OsMTs. The CAT activity in the extract was 15 Umg-1 proteins. This value did not change by the addition of apo/GST-OsMTs (data not shown) (Fig. 5A). However, the CAT activity was increased to 19±1, 20±1, 20±1.5 and 20±1 after addition of Fe^{2+}/GST-OsMT1, Fe^{2+}/GST-OsMT2, Fe^{2+}/GST-OsMT3 and Fe^{2+}/GST-OsMT4, respectively. The SOD activity was 42±5 % (% of inhibition) in the extract of control strain (Fig. 5B). This value reached 55±5, 60±6.5, 57±4.5 and 68±8 % when Zn/GST-OsMT1, Zn^{2+}/GST-OsMT2, Zn^{2+}/GST-OsMT3 and Zn^{2+}/GST-OsMT4 were added to the extract, respectively. It should be noted that the addition of apo/GST-OsMTs did not affect the SOD activity. These data show that Fe^{2+}/GST-OsMTs and Zn^{2+}/GST-OsMTs behave as an activator for CAT and SOD by supplying Fe^{2+} and Zn^{2+}, respectively.

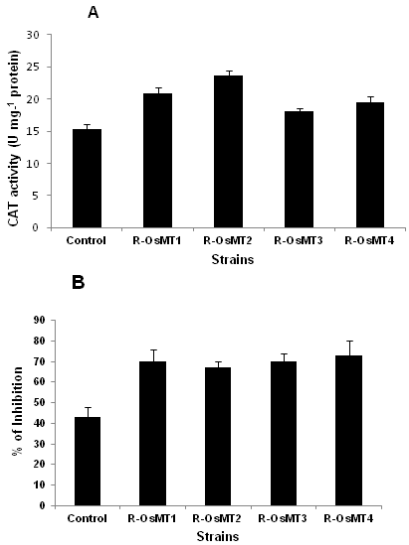

Figure 4. *The activity assays of antioxidant enzymes CAT (A) and SOD (B) in the control strain and the strains expressing OsMT; R-OsMT1, R-OsMT2, R-OsMT3 and R-OsMT4 when they were grown in the medium containing 2 mM H_2O_2. The data represent the average from three independent experiments with standard deviation.*

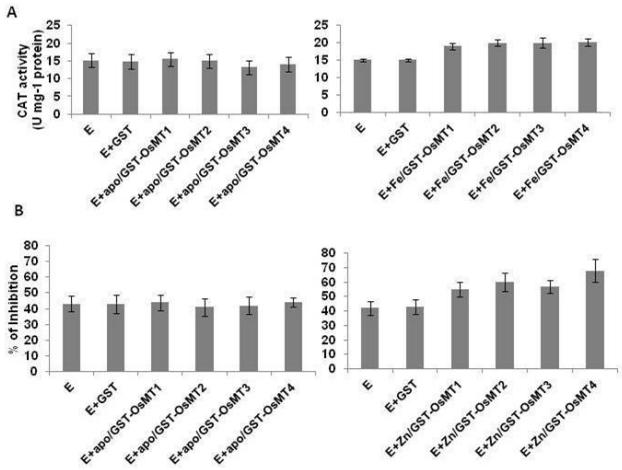

Figure 5. *The activity of (A) CAT and (B) SOD were assayed in E. coli protein extract after addition of pure recombinant apo/OsMTs, Fe/OsMTs or Zn/OsMTs. The data represent the average from three independent experiments with standard deviation.*

Discussion

During growth and development, plants have to cope with a range of different external stresses, such as low temperature, drought and high salinity. Most of these stresses cause increased ROS at a certain stage of stress (Zhu et al., 2009). The excessive accumulation of ROS can lead to the oxidative destruction of cells (Apel et al., 2004). Consequently, the cells have developed a variety of ROS-scavenging mechanisms including both non-enzymatic and enzymatic anti-oxidants (Birben et al. 2012). It has been reported that in the cells superoxide radicals (O_2^-) and hydrogen peroxide are scavenged through the catalytic activity of SOD and CAT, respectively (Chekulayeva et al., 2006)). Most of these enzymes play their function by binding metal ions as co-factors. CAT isoforms in prokaryotes and eukaryotes use Fe ion in their active site. However, SODs are found in three types (CuZn-SOD, Fe-SOD and Mn-SOD) based on the metal ions identified in their active sites (Tuteja et al., 2015). E. coli contains two cytoplasmic SOD isozymes, one each of the Mn- and Fe- types (MnSOD and FeSOD), and secretes a copper, zinc-type (CuZnSOD) to the periplasm (Imlay et al., 2008).

Recently, a number of studies have demonstrated MTs as being efficient scavengers of ROS. For instance, the expression of BrMT1 (Kim et al., 2007), CgMT1 (Obertello et al., 2007), or GhMT3a (Xue et al., 2009) decreased ROS production in transgenic plants. The Arabidopsis T-DNA insertion mutant, mt2a, had higher H_2O_2 levels than wild-type plants during cold stress (Zhu et al., 2009). The recombinant GhMT3a could scavenge ROS in an *in vitro* assay which suggests that the higher tolerance to abiotic stress in transgenic tobacco may be due to ROS scavenging by GhMT3a. However, in these studies, it is not clear whether MTs scavenge the ROS independent of ROS-scavenging enzymes or act as either an activator of ROS-scavenging enzymes by supplying metals or regulators of genes encoding ROS-scavenging enzymes.

In the present work, it was shown that the activities of SOD and CAT in the *E. coli* cells expressing different types of rice MT isoforms were higher than control in the medium containing H_2O_2. On the other hand, we revealed that the addition of pure Zn^{2+}/OsMTs and Fe^{2+}/OsMTs to the *E. coli* extract in an *in vitro* assay enhanced the activity of SOD and CAT, respectively. The activities of these enzymes were not affected by apo-MT. Therefore, it seems that OsMT isoforms can act as an activator for SOD and CAT isoforms by supplementation of Zn^{2+} and Fe^{2+}, respectively.

Conclusion

In this work, we showed that different rice MT isoforms despite their high metal binding constant can transfer metal ions to SOD and CAT. MTs may act as a temporary cellular reservoir, release metals in a process that are dynamically controlled by the cellular redox status. However, the analysis of the structural interaction of Zn/MTs with CuZnSOD or Fe/MTs with CAT is necessary to understand how Zn^{2+} and Fe^{2+} are transported by different MT isoforms to other proteins.

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