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ORIGINAL ARTICLE

Mercury removal by engineered *Escherichia coli* cells expressing different rice metallothionein isoforms

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Abstract

Mercury is one of the more common and potentially most harmful toxic metals. Remediation using conventional physical and chemical methods is uneconomical and generates large volumes of chemical waste. Bioremediation of hazardous metals has received considerable and growing interest over the years. In the present work, genetically engineered *Escherichia coli* cells, which express four rice metallothionein (MT) isoforms as fusions with glutathione-S-transferase (GST), were tested for their ability to remove mercury. The results showed that the *E. coli* cells expressing OsMT1, OsMT2, OsMT3, and OsMT4 are able to remove 20, 13.7, 10, and 7 nmol Hg^{2+}/mg (dry weight) from the culture medium, respectively. The recombinant GST–OsMTs were purified using affinity chromatography. The UV absorption spectra and the results of 5,5-dithio-bis-(2-nitrobenzoic) acid (DTNB) assay recorded after the reconstitution of the apo-OsMTs with mercury confirmed that the different OsMT isoforms were able to form mercury complexes in vitro with different binding capacities and different binding strength.

Keywords *Escherichia coli* · Heterologous expression · Mercury · Metallothionein · Rice

Introduction

Mercury is one of the most toxic heavy metals in the environment and is released into environment due to both natural phenomena (volcanoes, degradation of minerals, or evaporation from soils) (Bhuiyan et al. 2011) and manmade processes (Driscoll et al. 2013).

At the cellular level, mercury exposure is associated with alterations in membrane permeability, changes in macromolecular structure, DNA damage, alterations in calcium homeostasis, and increased lipid peroxidation (Christie and Costa 1984; Bhargava et al. 2012). Exposure to mercury has harmful effects on human health and can result in death, mental retardation, dysarthria, blindness, neurological deficits, loss of hearing, developmental defects, and abnormal muscle tone (Karlen et al. 1997; Rossbach et al. 2000).

Mercury exists in different forms, but ionic mercury (Hg^{2+}) is the predominant form in soils and readily absorbed by plants (Stephan et al. 1996). Mercury is dominantly accumulated in roots, and only a small proportion of mercury is translocated to other parts of plants (Stephan et al. 1996; Cai and Ma 2003). The areal parts of plants are another important ways for absorption of mercury ions which is present in the air due to industrial mercury emission (Cai and Ma 2003). The treatment of plants with mercury inhibits the chlorophyll synthesis which could be due to the inhibition effect of enzyme protochlorophyllide oxidoreductase (Fosso-Kankeu and Mulaba-Bafubiandi 2014). Another mechanism by which mercury exerts toxic effects on the plants is the induction of generation of active oxygen species (ROS) and oxidative injury (Blencowe and Morby 2003; Davis et al. 2003; Fosso-Kankeu and Mulaba-Bafubiandi 2014). However, the mechanism of how mercury induces the oxidative stress is not completely understood (Cai and Ma 2003; Zhou et al. 2007).

Due to the harmful effects of mercury, the living organisms are equipped with different mechanisms to counteract mercury toxicity (Cai and Ma 2003). An extensively studied resistance system in a wide range of negative and positive bacteria is based on clustered genes in an operon (*Mer* operon) that allows bacteria to detoxify Hg^{2+} into volatile metallic mercury by enzymatic reduction (Mathema et al. 2011). Mercury

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methylation is known as a resistance/detoxification mechanism in certain bacteria (Trevors 1986). The production of extracellular compounds such as siderophores, metal-binding exopolysaccharides, and surfactants is another bacterial mechanism for heavy metal detoxification.

In plants, a group of signaling molecules such as nitric oxide, carbon monoxide, and salicylic acid has been identified to be involved in the regulation of mercury-induced oxidative stress and plant tolerance to mercury (Bilecen et al. 2005). Several genes regulated by mercury have been identified in plants (Heidenreich et al. 2001). For instance, the genes involved in the cell wall development as well as genes involved in response to oxidative stress are upregulated in response to mercury treatment (Cai and Ma 2003; Heidenreich et al. 2001; Hassinen et al. 2011). In addition, the genes encoding metallothioneins (MTs) and phytochelatin synthases (PCs) have been demonstrated to be upregulated in response to mercury (Hall 2002). MTs and PCs comprise two major classes of metal-binding peptides found in many eukaryotic organisms (Hall 2002; Cai and Ma 2003).

MTs are low molecular mass (4–14 kD) proteins with high Cys content. The Cys residues provide sulfhydryl ligands for coordination of bivalent metal ions (Janssens et al. 2009). Plants have multiple MT types that are generally divided into four distinct subgroups according to the arrangement of Cys residues (Morgan et al. 2007; Bhargava et al. 2012). Cys residues in the primary structures of types 1, 2, and 3 are organized into two Cys-rich domains and are separated by a Cys-devoid linker that varies in length depending on the type and source of MTs. The six Cys residues at the C-terminal in each type are arranged in a highly conserved pattern (CxCxxxCxCxxCxC). The variation in the Cys position is largely contained in the N-terminal Cys-rich region (Hassinen et al. 2011). Type 1 MTs generally contain six Cys residues in the N-terminal arranged in a CXC motif; type 2 MTs contain eight Cys residues arranged in CC, CXC, and CXXC motifs; and type 3 MTs contain four Cys residues with a CXXCXCXDXXC consensus sequence (Hassinen et al. 2011). The sequences of the type 4 MTs differ by having a three Cys domain, each containing five or six conserved Cys residues generally arranged as a CXC motif.

Multiple isoforms belonging to different MT types are present in rice (Cobbett and Goldsbrough 2002; Hassinen et al. 2011). Five isoforms are grouped as type 1 (OsMTI-1a, OsMTI-1b, OsMTI-4a, OsMTI-4b, and OsMTI-4c), three isoforms as type 2 (OsMTI-2a, OsMTI-2b, and OsMTI-2c), two isoforms as type 3 (OsMTII-3a and OsMTI-3b), and only one isoform is grouped as type 4 (Fang et al. 2010; Sabolić et al. 2010). Previously, we heterologously expressed and functional characterized four rice (*Oryza sativa*) genes encoding OsMT1 (Mohammadi Nezhad et al. 2013), OsMT2 (Pirzadeh and Shahpiri 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.

In the present work, we heterologously expressed OsMT1, OsMT2, OsMT3, and OsMT4 in *Escherichia coli* as a fusion protein with glutathione-S-transferase (GST) and examined their ability for protection of *E. coli* cells from mercury toxicity and their properties for mercury accumulation. In addition, the production and purification of considerable amounts of recombinant forms of these isoforms enabled us to study in vitro mercury-binding abilities of OsMT isoforms.

Materials and methods

Heterologous expression of GST–OsMTs in *E. coli*

The cells harboring expression construct pET41a-OsMT1 (Mohammadi Nezhad et al. 2013), pET41a-OsMT2 (Pirzadeh and Shahpiri 2016), pET41a-OsMT3 (Shahpiri et al. 2015), pET41a-OsMT4 (Mohammadi Nezhad et al. 2013), and empty pET41a (control) 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 OD_{600} of about 0.6. At this OD, cultures were induced by 100 μM Isopropyl β-D-1-thiogalactopyranoside (IPTG). To verify the heterologous expression of the proteins, 2 ml samples of culture medium were harvested by centrifugation 4 h after addition of IPTG and frozen at − 80 °C until use. The frozen pellets were resuspended in 250 μl precold 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.

Tolerance of transformed cells to Hg^{2+} and analysis of accumulated Hg^{2+}

Overnight cultures of different strains were inoculated into 80 ml of LB medium with the desired antibiotics and grown at 37 °C under agitation. Protein expression was induced at an A_{600} of 0.6 by addition of 0.1 mM IPTG. After 20 min, culture flasks were supplemented with 30 μM mercury nitrate (HgN_2O_6. H_2O_2) and bacterial growth was monitored by A_{600} measurements at 1-h intervals up to 12 h. For the analysis of the amount of accumulated Hg^{2+}, cells from 10 ml of culture at 0 and 6 h after metal addition were precipitated by centrifugation at 6000×*g* for 20 min. The supernatant was analyzed for Hg^{2+} using flame atomic absorption spectroscopy (FAAS).

Purification of GST–OsMTs

For large-scale production of proteins, the cells were grown in 500 ml of medium. Induction with IPTG and supplementation with 0.6 mM $ZnSO_4 \cdot 7H_2O$ were performed as explained previously. The cells from whole volume were harvested 4 h after addition of IPTG. The soluble proteins were extracted as explained above (part 2.1). The purification of extracted soluble proteins was performed using affinity chromatography. To this end, the extracted proteins were applied onto the His Trap HP column (GE Healthcare) preequilibrated with the 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. Aliquots of the protein fractions were analyzed by 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 cutoff, 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 46,340, 46,465, 46,277, and 44,975 M^{-1} cm^{-1} for GST–OsMT1, GST–OsMT2, GST–OsMT3, and GST–OsMT4, respectively.

Reconstitution of GST–OsMTs with Hg^{2+}

The preparation of apo-OsMTs was done using the methods previously described (Toriumi et al. 2005). Aliquots of 25 μM purified GST–OsMTs were acidified with HCl to pH 2.0, and the samples were then chromatographed on an HiTrap desalting column (GE Healthcare) equilibrated with 0.1 N HCl to remove the bound metal ions. Reconstitution with Hg^{2+} was achieved by the addition of 10 mol equivalents of Hg^{2+} followed by neutralization of the samples to pH 8.0 with 200 mM Tris. The unbound metals were removed by using HiTrap desalting columns equilibrated with 10 mM Tris–HCl pH 8.

Ultraviolet absorption spectroscopy

The ultraviolet absorption spectra of Hg^{2+}-incubated and apo forms of GST–OsMTs were scanned at room temperature in the range of 200–350 nm with 5-nm increments. All measurements were carried out using 500 μl of 10 mM Tris–HCl, pH 8.0, as blank.

Reaction with DTNB

The binding ability of GST–OsMTs toward Hg^{2+} were evaluated by the competitive reaction with 5,50-dithiobis(2nitrobenzoic) acid (DTNB) using Emoto method with slight modifications (Emoto et al. 1996). A 300-μl reaction mixture containing 10 mM Tris–HCl, pH 8.0, and 1.5 nmol of each protein (apo-MTs or Hg^{2+}/MTs) was placed in a quartz cuvette. The reaction was started by addition of 75 nmol of DTNB. The absorbance at 412 nm was recorded on a Beckman DU-530 spectrophotometer at 1-min intervals for 60 min at room temperature reflecting the formation of 5-thio-2-nitrobenzoate anion with a molar extinction coefficient of 14,140 M^{-1} cm^{-1}. As a blank, 300 μl of 10 mM Tris–HCl solution containing 75 nmol of DTNB were used. The pseudo initial velocity of each curve was calculated from absorbance versus the reaction time.

Results

Confirmation of the heterologous expression of GST–OsMTs in *E. coli*

The coding sequence of OsMT1, OsMT2, OsMT3, and OsMT4 (Fig. 1a) was 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 a 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-MT1), pET-OsMT2 (R-MT2), pET-OsMT3 (R-MT3), and pET-OsMT4 (R-MT4), respectively. The theoretical molecular weight of GST, GST–OsMT1, GST–OsMT2, GST–OsMT3, and GST–OsMT4 were 35.5, 39.9, 43.9, 38.98, and 41.2 kDa, respectively. The SDS-PAGE analysis showed sharp protein bands of expected molecular masses for these proteins (Fig. 2).

Tolerance of *E. coli* cells to mercury stress

The final cell densities (cell density after 12 h) of strains R-MT1, R-MT2, R-MT3, and R-MT4 were similar to that from control (A_{600} = 1.8) when the strains were grown in the medium with no stress (Fig. 3a). However, in the presence of mercury whereas the density for control strain after 12 h reached 0.95, the densities for strains R-MT1, R-MT2, R-MT3, and R-MT4 were 1.37, 1.74, 1.53, and 1.15, respectively (Fig. 3b). These data show that the heterologous expression of GST–OsMT1, GST–OsMT2, GST–OsMT3, and GST–OsMT4 enhances the tolerance of *E. coli* cells to mercury.

Accumulation of mercury by recombinant strains expressing OsMTs

To determine whether the enhanced of tolerance of R-MTs to mercury is accompanied by accumulation of this metal ion in these cells, the recombinant strains as well as control were grown in a liquid medium supplemented with mercury (Fig. 4). The concentrations of Hg^{2+} ions in the culture

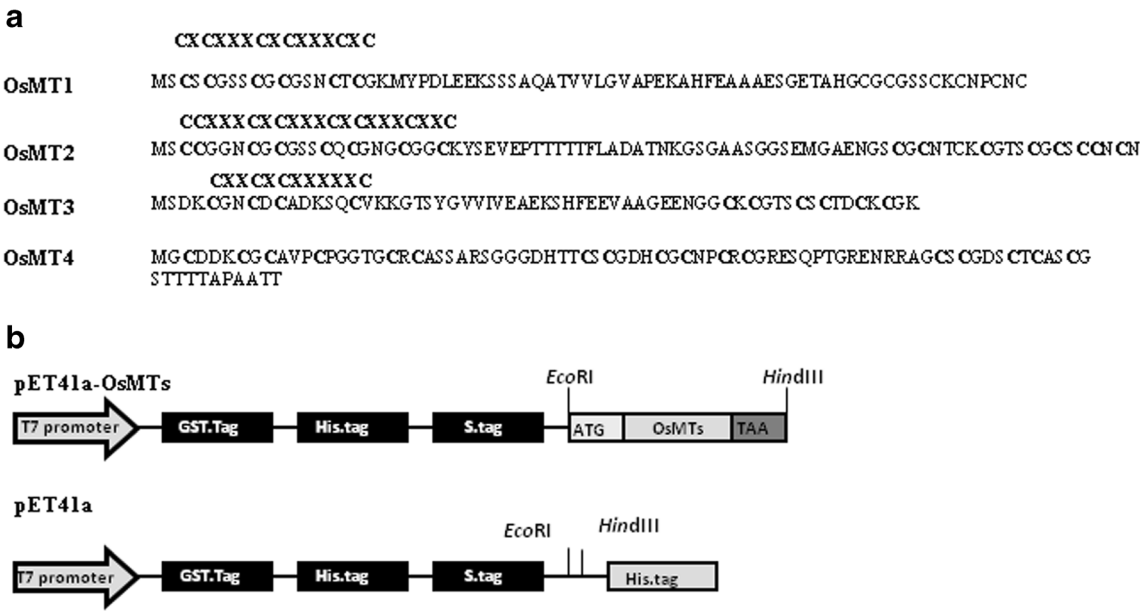

Fig. 1 Amino acid sequence and expression vector maps. **a** The amino acid sequence of OsMT1, OsMT2, OsMT3, and OsMT4. The Cys residues are shown as bold. **b** The map of pET41a-OsMTs and pET41a. The positions of His.tag, S.tag, and GST.tag are shown in gray boxes

medium were determined by atomic absorption 6 h after the addition of mercury to the cultures. Whereas in the culture medium of the control strain, the concentration of Hg^{2+} remained almost constant, the recombinant strains R-MT1, R-MT2, R-MT3, and R-MT4 were able to remove 20, 13.7, 10, and 7 nmol Hg^{2+}/mg (dry weight) from the culture medium, respectively. These results suggest that the heterologous expression of GST–OsMTIs enhances the capacities of *E. coli* cells for Hg^{2+} accumulation.

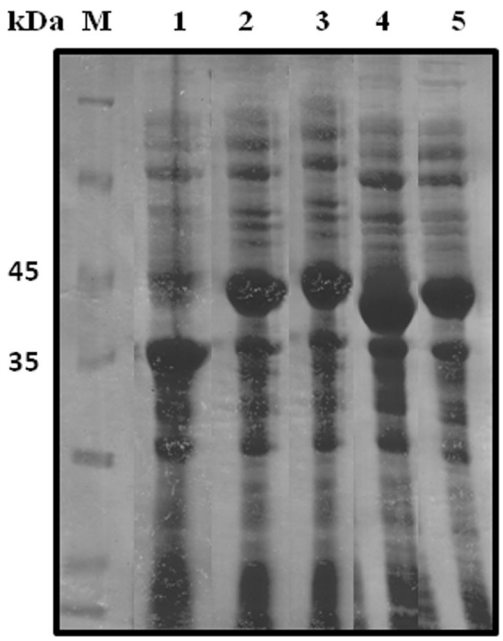

Fig. 2 SDS-PAGE analysis of GST and GST–OsMTs. Total proteins extracted from *E. coli* harboring pET41a (lane 1), pET41a-OsMT1 (lane 2), pET41a-OsMT2 (lane 3), pET41a-OsMT3 (lane 4), and pET41a-OsMT4 (lanes 5) 4 h after addition of IPTG

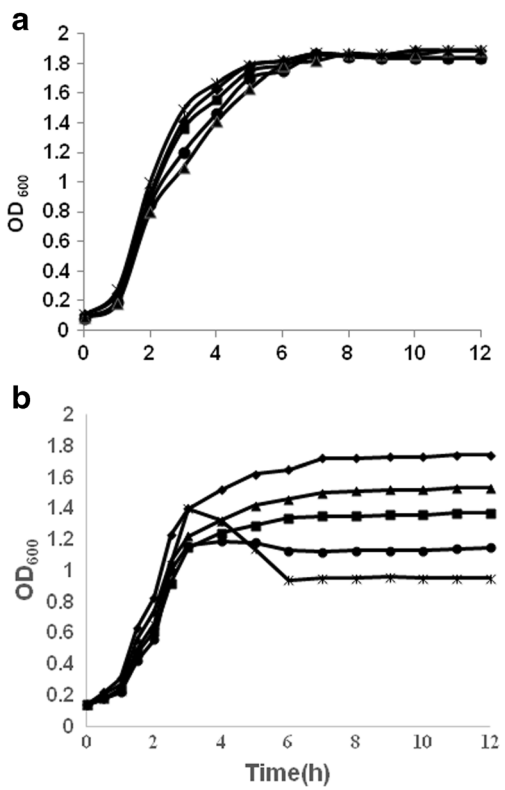

Fig. 3 Growth curves of control (asterisks), R-MT1 (filled squares), R-MT2 (filled diamonds), R-MT3 (filled triangles), and R-MT4 (filled circles) in the **a** medium with no mercury and **b** medium with 30 μM mercury nitrate

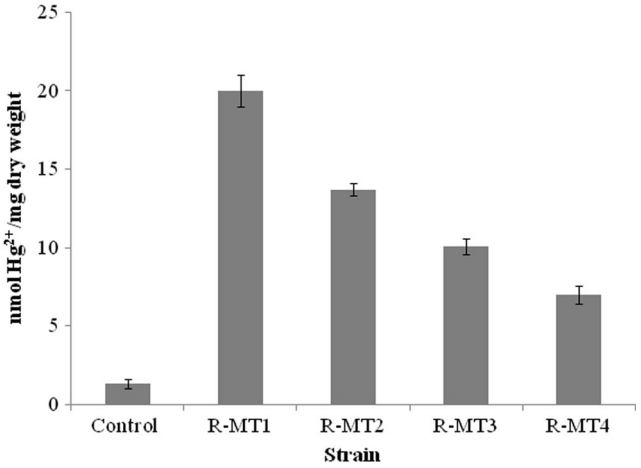

Fig. 4 Bioaccumulation of Hg^{2+} by strains R-MT1, R-MT2, R-MT3, and R-MT4 as well as control. Each data represents the mean ± SD obtained from two independent experiments with two replicates

Recombinant GST–OsMTs are able to bind mercury in vitro

The presence of Hg^{2+}-thiolate clusters was confirmed by recording the UV-spectra (200–350 nm). Figure 5 reveals the absorption spectra of fully protonated OsMTs (apo-OsMTs) and Hg^{2+}/OsMT complexes. In comparison to the apo forms, the complexes Hg^{2+}/OsMTs showed increased absorbance at 280 nm typical of Hg^{2+}-containing proteins (Faller et al. 2000). However, as shown in Fig. 5, the variation at 280 nm for Hg^{2+}/OsMT4 complex is very low. This variation remarkably increased for Hg^{2+}/OsMT1 and Hg^{2+}/OsMT2, indicating different OsMT isoforms have different capacities to bind Hg^{2+} ions.

The competitive reactions of Hg^{2+}/OsMTs with DTNB were used to investigate the accessibility of DTNB to protein sulfhydryls which relates to the structure and binding strength of metal to MTs (Ejnik et al. 2002; Wan and Freisinger 2009). The reaction was followed by determining the production rate of TNB at 412 nm. The initial velocities of Hg^{2+}/OsMTs were remarkably lower than that of their corresponding apo forms which verify that Hg^{2+} can bind to OsMT1, OsMT2, OsMT3, and OsMT4 (Fig. 6 and Table 1). Because of high binding strength of OsMT1, OsMT2, and OsMT3 toward Cd^{2+}, the Cd^{2+}/OsMT complexes were used as control in DTNB assays. As shown in Fig. 6a and Table 1, the initial velocities of reaction of Hg^{2+}/OsMT1 with DTNB was lower than that from Cd^{2+}/OsMT1, suggesting that the binding strength of OsMT1 toward Hg^{2+} is even higher than that toward

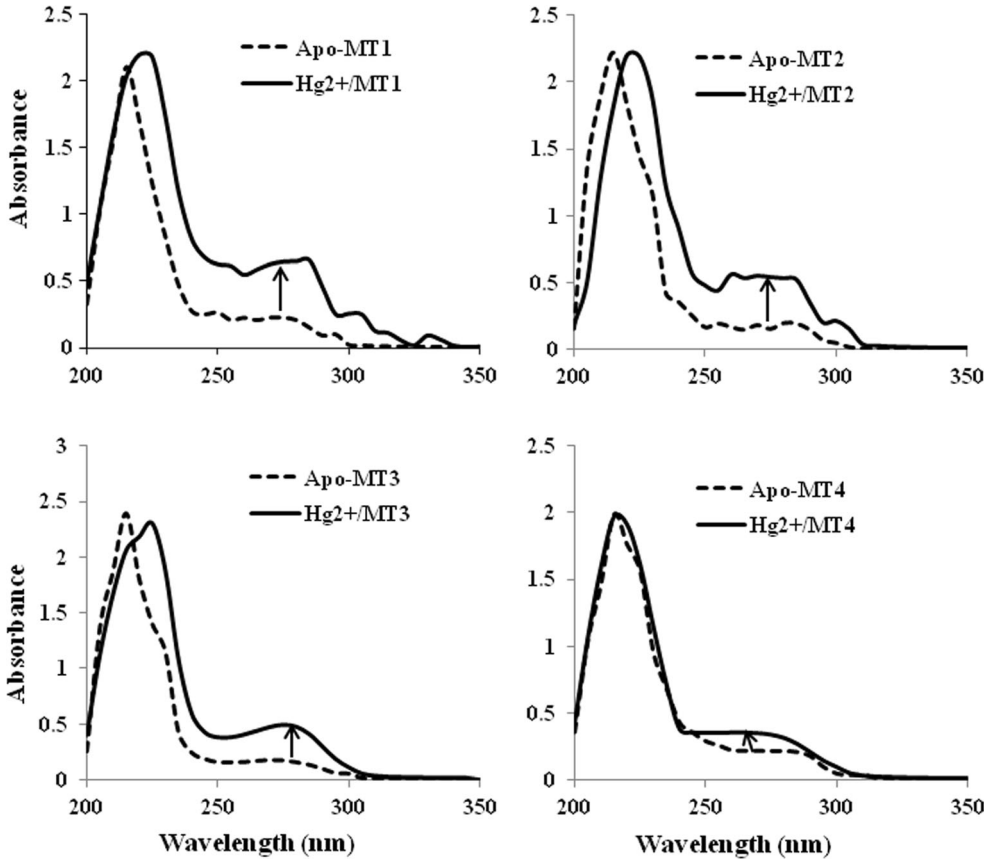

Fig. 5 The comparison between UV absorption spectra of $Hg2^{+}$/OsMT1, Hg^{2}/OsMT2, Hg^{2+}/OsMT3, and Hg^{2+}/OsMT4 (solid lines) with their corresponding apo forms (dashed lines). The arrows show increase in absorption

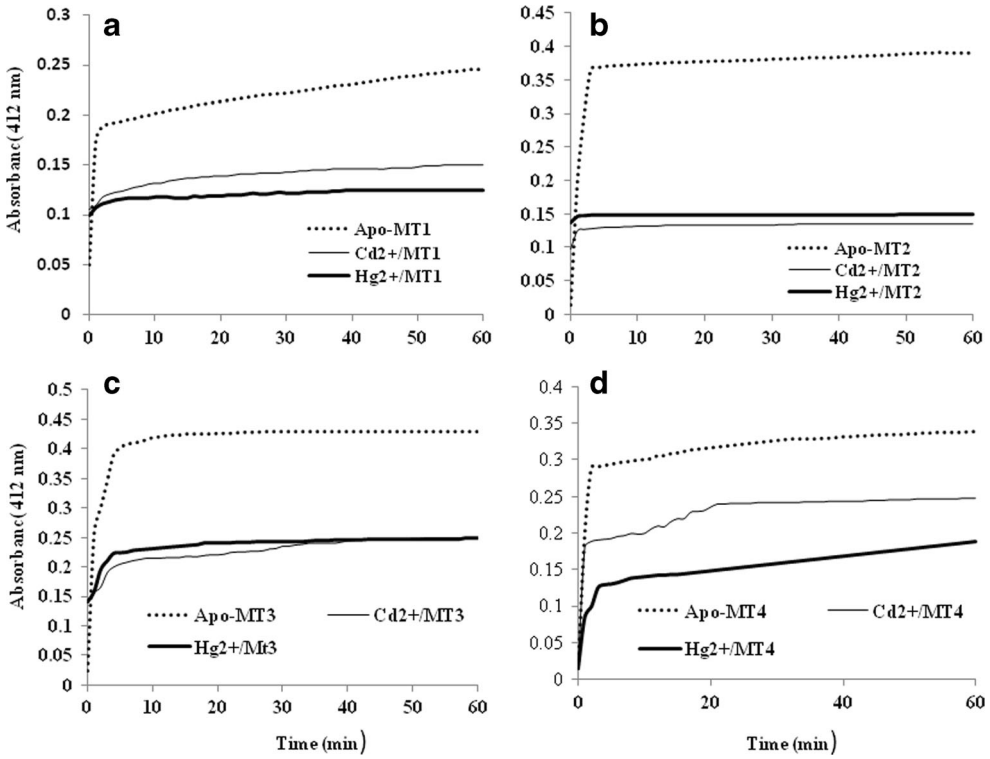

Fig. 6 The reactivity of **a** metal/OsMT1, **b** metal/OsMT2, **c** metal/OsMT3 and **d** metal/OsMT4 and their corresponding apo forms with DTNB

Cd^{2+}. The binding strength of OsMT2 and OsMT3 toward Hg^{2+} were almost similar to binding strength of these isoforms toward Cd^{2+} (Fig. 6b, c, Table 1). The initial velocity of reaction of Cd^{2+}/OsMT4 with DTNB is almost similar to that from apo-OsMT4, indicating that OsMT4 is not able to bind Cd^{2+}. Nevertheless, the reaction velocity of Hg^{2+}/MT4 was lower than its corresponding apo form, suggesting that OsMT4 can also bind Hg^{2+}. Based on the data presented in Table 1, different OsMT isoforms have different binding strength toward Hg^{2+} and was in the order of OsMT1/OsMT2 > OsMT3 > OsMT4.

Table 1 Initial velocities of reactions of apo/OsMTs and metal/OsMTs with DTNB

Metal/OsMTs	Initial velocity (nmol/min)
Apo/OsMT1	1780
Cd^{2+}/OsMT1	282
Hg^{2+}/OsMT1	233
Apo/OsMT2	1870
Cd^{2+}/OsMT2	233
Hg^{2+}/OsMT2	233
Apo/OsMT3	1760
Cd^{2+}/OsMT3	545
Hg^{2+}/OsMT3	545
Apo/OsMT4	1890
Cd^{2+}/OsMT4	1590
Hg^{2+}/OsMT4	776

Discussion

The presence of mercury in the environment has received a great deal of attention due to its highly toxic nature and translocation through the food chain (Driscoll et al. 2013; Rossbach et al. 2000). Since the first discovery of high level of methyl mercury in fish and shellfish in Minamata Bay (Japan) in 1956, the toxicity of mercury has been researched by scientists worldwide (Hachiya 2006). Bioremediation has been considered as a potentially low-cost, safe, and environmental friendly technology for remediation of contaminated sites with a wide range of pollutants (Pätsikkä et al. 2002; Mendoza-Cózatl et al. 2005). Biotransformation through bacterial reduction and volatization of mercurials mediated by the gene encoding mercury reductase, merA, has been the most actively studied processes for remediation of mercury from environment (Petrus et al. 2015). However, the use of this process is limited due the release of mercury vapor into the air which will be recycled to the ecosystem. Therefore, particular attention is given to the genetic engineering for expression new mechanism in bacteria cells for chelating and accumulation of mercury without releasing mercury vapor. Heterologous expression of proteins such as a de novo peptide sequence containing a metal-binding motif (Pazirandeh et al. 1998), synthetic phytochelatines with the repetitive metal-binding motif $(Glu\text{-}Cys)_nGly$ (Bae et al. 2001), and polyphosphate kinase and MTs (Ruiz et al. 2011) are examples of genetic engineering of *E. coli* for enhanced uptake and bioaccumulation of mercury.

MTs have been shown to bind mercury with a high affinity and form stable complexes with a variety of species (Wuhua et al. 1993). However, the expression of MTs in bacteria has encountered some difficulties due to their instability and degradation. Recently this problem has been minimized by expression of MTs as fusion proteins (Sauge-Merle et al. 2012; Tomas et al. 2016; Ma et al. 2011). Previously, we heterologously expressed four rice MT isoforms belonging to four different types MT in *E. coli* as a fusion protein with GST (Mohammadi Nezhad et al. 2013; Shahpiri et al. 2015; Pirzadeh and Shahpiri 2016). By this way, a considerable amount of proteins GST–OsMTs were found in the soluble fraction of *E. coli*. Here, we have shown that the expression of each of OsMT1, OsMT2, OsMT3, and OsMT4 confers enhanced tolerance to mercury in *E. coli* cells. However, the amount of tolerances of strains expressing different OsMT isoforms was different in response to Hg^{2+}. It seems that the *E. coli* cells expressing OsMT2 and OsMT4 showed the highest and lowest tolerance, respectively. The difference was also observed in the amount of mercury accumulated by different strains. In comparison to the control strain which removes 1.3 nmol/mg (dry weight) Hg^{2+} from the medium, the *E. coli* cells expressing OsMTs were able to remove 7–20 nmol/mg (dry weight) of mercury. These amounts were comparable with those that reported for *E. coli* cells expressing either MTs or mercury transporters (Lin et al. 2010; Pazirandeh et al. 1998). However, they are remarkably lower than the amounts accumulated by engineered *E. coli* cells which coexpress Hg^{2+} transporter together with either MT or phytochelatin synthase (Chen and Wilson 1997; Bae et al. 2001; Deng and Wilson 2001). Here, we also found that OsMT isoforms were able to bind Hg^{2+} in vitro. In agreement with our previous data, OsMT1, OsMT2,s and OsMT3 showed a high binding strength toward Cd^{2+}. The binding strength of these isoforms toward Hg^{2+} was almost similar to Cd^{2+} as shown with the DTNB assay. In addition, the data presented here demonstrate that the binding strength of different OsMT isoforms toward Hg^{2+} was different and follows: OsMT1/OsMT2 > OsMT3 > OsMT4. Based on spectrophotometer analysis, OsMT1 and OsMT2 seem to have more capacities to bind Hg^{2+} ions than OsMT3 and OsMT4 when their corresponding apo forms were exposed to saturated Hg^{2+} solution. The formation of Hg7-MT and Hg18-MT species have been previously illustrated for rabbit MTs, suggesting that mercury binds to different MT isoforms with different stoichiometry ratios (Wuhua et al. 1993). These differences between different OsMT isoforms could be due to differences in the Cys arrangement in their primary structure which has been proposed to confer differential metal-binding properties to different MTs (Vasak and Hasler 2000).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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