

This is the post-peer reviewed version of the following article:

Li, L., Meng, Y., Cao, Q., Yang, Y., Wang, F., Jia, H., Wu, S. & Liu X. (2016). Type 1 metallothionein (ZjMT) is responsible for heavy metal tolerance in *Ziziphus jujuba*. *Biochemistry Moscow*, 81(6), 565–573.

<http://dx.doi.org/10.1134/s000629791606002x>

Downloaded from [e-publications@UNE](mailto:e-publications@UNE) the institutional research repository of the University of New England at Armidale, NSW Australia.

## **Type 1 Metallothionein (ZjMT) Is Responsible for Heavy Metal Tolerance in *Ziziphus jujuba***

TYPE 1 METALLOTHIONEIN IN *Ziziphus jujuba*

Lan-Song Li<sup>1,2</sup>, Yu-Ping Meng<sup>2</sup>, Qiu-Fen Cao<sup>2,3\*</sup>, Yong-Zhen Yang<sup>3</sup>, Fan Wang<sup>2</sup>,  
Hu-Sheng Jia<sup>3</sup>, Shu-Biao Wu<sup>2,4\*</sup>, and Xu-Guang Liu<sup>1,3\*</sup>

<sup>1</sup>College of Chemistry and Chemical Engineering, Taiyuan University of Technology, 030024 Taiyuan, China; fax: +86-351-6014138; E-mail: liuxuguang@tyut.edu.cn

<sup>2</sup>Biotechnology Research Center, Shanxi Academy of Agricultural Sciences, 030031 Taiyuan, China; fax: +86-354-6215341; E-mail: qiufengcao@163.com

<sup>3</sup>Taiyuan University of Technology, Key Laboratory of Interface Science and Engineering in Advanced Materials, Ministry of Education, 030024 Taiyuan, China

<sup>4</sup>University of New England, School of Environmental and Rural Science, NSW 2351 Armidale, Australia; fax: +61-2-6773-3922; E-mail: shubiao.wu@une.edu.au

Received November 16, 2015

Revision received February 3, 2016

\* To whom correspondence should be addressed.

**Abstract**—Metallothioneins (MTs) are a family of low molecular weight, cysteine-rich, metal-binding proteins that are able to make cells take up heavy metals from the environment. Molecular and functional characterization of this gene family improves understanding of the mechanisms underlying heavy metal tolerance in higher organisms. In this study, a cDNA clone, encoding 74-a.a. metallothionein type 1 protein (ZjMT), was isolated from the cDNA library of *Ziziphus jujuba*. At the N- and C-terminals of the deduced amino acid sequence of ZjMT, six cysteine residues were arranged in a CXCXXXCXCXXXCXC and CXCXXXCXCXXXCXC structure, respectively, indicating that ZjMT is a type 1 MT. Quantitative PCR analysis of plants subjected to cadmium stress showed enhanced expression of ZjMT in *Z. jujuba* within 24 h upon Cd exposure. *Escherichia coli* cells expressing ZjMT exhibited enhanced metal tolerance and higher accumulation of metal ions compared with control cells. The results indicate that ZjMT contributes to the detoxification of metal ions and provides marked tolerance against metal stresses. Therefore, ZjMT may be a potential candidate for tolerance enhancement in vulnerable plants to heavy metal stress and *E. coli* cells containing the *ZjMT* gene may be applied to adsorb heavy metals in polluted wastewater.

*Key words:* *Z. jujuba* Mill., metallothionein, heavy metal tolerance, heavy metal hyperaccumulation

Many higher plants have evolved the ability to tolerate trace elements including metals. These plants are often excluders, which exclude the metals by preventing them from entering the plants or minimizing root-to-shoot translocation of trace metals. On the other hand, a group of rare plants called hyperaccumulators have extremely high tolerance to trace elements, so that high concentration of these metals does not necessarily damage their cells [1]. The overall understanding of such tolerance at genomic level is still limited due to the great complexity of tolerance and limited availability of sequence information from hyperaccumulator species, albeit the physiological and genetic mechanisms underlying heavy metal tolerance have been explored during recent decades [2]. Nevertheless, genes involved in metal tolerance have been identified in the hyperaccumulators, and many of them are functionally associated with metal uptake (ZIP family), metal vacuolar sequestration (MTP family), metal remobilization (NRAMP family), xylem loading/unloading of metal (YSL family), and the synthesis of metal ligands (NAS family) [3-9].

Metallothioneins (MTs) are a family of low molecular weight, cysteine-rich, metal-binding proteins [10, 11]. They are involved in metalloregulatory processes that include cell growth and reproduction [12], and they are present in a vast range of taxonomic groups representing a high-heterogeneity sequence without general homology [13]. In angiosperms, MTs consist of four types, MT1, MT2, MT3, and MT4, with different levels of expressions in varying tissues during plant development and with different metabolic functions [14]. It has been reported that MTs showed high binding capability to heavy metals and are able to remove heavy metals even in low concentrations [15-17]. Although the role of MTs in Cd detoxification was considered to be secondary as compared with phytochelatins and stress proteins [18], the importance of this protein family in Cd detoxification has been demonstrated at least in some of plant species such as wheat and rice [14, 19].

Chinese jujube (*Ziziphus jujuba* Mill.) is a unique and economically important fruit tree. It is native to, and has a long history of cultivation, in China. The species is well known for its tolerance to drought, salinity, and different environmental conditions in the field. Although limited investigations have been conducted in the drought tolerance of *Ziziphus* species [20, 21], the molecular mechanisms underlying the

tolerance to abiotic stress is still largely unknown in the *Ziziphus* species such as Chinese jujube. The isolation and identification of potential genes responsible for the tolerance will provide essential insight into the understanding of the molecular mechanisms in these tolerant plants.

We hypothesize that the MT gene family present in *Z. jujuba* is responsible for its tolerance to abiotic stress, and bacteria such as *E. coli* expressing the gene will be a potential candidate for the remediation of heavy metal contaminated wastewater. In this study, a novel metallothionein type 1 gene (*ZjMT*) was isolated and cloned from the cDNA library of *Z. jujuba*, and the differential expression of *ZjMT* in cultured *Z. jujuba* seedlings stressed with different levels of cadmium was analyzed. We found that *ZjMT* expressed in heterologous *E. coli* also enhances tolerance to, and the accumulation of, heavy metals, showing potential application perspective in the treatment of heavy metal wastewater.

## MATERIALS AND METHODS

**Plant material and treatments.** The stress tolerant *Z. jujuba* Mill. “Hupingzao”, grown under field conditions at Pomology Institute of Shanxi Academy of Agricultural Sciences (Taiyuan, Shanxi, China), was used in the present study. The seeds were surface-sterilized using 0.1% (w/v) mercuric chloride for 10 min and washed thoroughly with sterile water. They were germinated in Petri dishes containing sterile wet blotting paper. Seedlings were transferred to pots 6 days after germination and grown in a greenhouse under controlled environmental conditions: temperature at  $25 \pm 1^\circ\text{C}$ , relative humidity of 65-70%, and light density of  $\sim 2500$  lx at 12 : 12 h dark/light cycle. Thirty-day-old seedlings of *Z. jujuba* Mill. were subjected to stress of 100 mM  $\text{CdCl}_2$  in 1/2 MS solution (pH 6.0) for 6, 24, and 48 h. Control seedlings were grown in 1/2 MS solution. Harvested leaves at appropriate time points were quick-frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  until required for RNA isolation.

**Construction of cDNA library from Chinese jujube.** Total RNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) method [22], and

the quality was assessed on a denaturing 1.5% agarose gel. mRNA was isolated with the PolyATtract mRNA Isolation System II kit (Promega, USA) following manufacturer's instruction. The purified mRNA was then reverse-transcribed, and double-stranded cDNA was synthesized using the cDNA Library Construction Kit (Invitrogen, USA). The cDNA was ligated to the SalI adapters, digested with NotI, and size-fractionated by a column, and then the resulting fragments of more than 500 bp were ligated into the NotI-SalI-cut pSPORT1 vector according to the manufacturer's instructions (Invitrogen). Ligated products were transformed into *E. coli* strain DH5 $\alpha$  (Invitrogen) to generate a cDNA library.

**cDNA library screening and isolation of ZjMT from *Z. jujuba*.** The cDNA clones were plated onto LB plates. Recombinant plasmids were isolated, NotI-SalI digested, and cDNA inserts sequenced. Protein prediction was performed by the alignment of the sequences obtained with homologous sequences in ExPASy (<http://expasy.org>) and GenBank by blastx (<http://www.ncbi.nlm.nih.gov>). The signal peptides were detected using the SignalP 3.0 software (<http://www.cbs.dtu.dk/services/SignalP/>). Sequence alignment of ZjMT proteins from different species was performed using the ClustalX-2.1 program with the default settings [23] and then processed with the online BOXSHADE 3.21 software ([http://www.ch.embnet.org/software/BOX\\_form.html](http://www.ch.embnet.org/software/BOX_form.html)).

**Quantitative PCR.** Approximately 1  $\mu$ g of RNA was reverse-transcribed with PrimeScript<sup>®</sup> RT Master Mix kit (TaKaRa, China), and SYBR<sup>®</sup> Premix Ex Taq<sup>TM</sup>II (TaKaRa) was used to perform quantitative PCR. Amplifications were carried out on an ABI 7300 Real Time PCR System (Applied Biosystems, USA) at a final volume of 20  $\mu$ l containing 1  $\mu$ l of diluted cDNA template (100 ng/ $\mu$ l), 8 pmol of each primer, 10  $\mu$ l SYBR<sup>®</sup> Premix Ex Taq<sup>TM</sup>II, 0.4  $\mu$ l of Rox, and 7.8  $\mu$ l of ddH<sub>2</sub>O. The *ZjH3* gene was used as the housekeeping gene [24]. The ZjMT primers were designed using the Primer Premier 5.0 software. Both the primers for *ZjH3* (forward primer 5'-GAGGAAGCAACTGGCAACTAAGG-3' and reverse primer 5'-ACCAGCCTCTGGAATGGAAGTTTG-3') and *ZjMT* (forward primer 5'-ACGAATTCTGATGTCTGGCTGTGG-3' and reverse primer 5'-TATCCCGGGATTTGCACGTGCATG-3') were synthesized by Shanghai Sangon,

Ltd. (China). PCR amplification was performed under the following cycle conditions: 30 s at 94°C, followed by 40 cycles of 5 s at 95°C, 31 s at 60°C. PCR amplification was performed with samples representing six seedlings, each in triplicates. The results are expressed as the relative expression of the *ZjH3* gene using the  $2^{-\Delta\Delta CT}$  method [25].

***ZjMT* cDNA cloning and transformation in *E. coli*.** Forward primer 5'-CTGGATCCATGTCTGGCTGTGGTTGT-3' and reverse primer 5'-AGAAGCTTTCATTTGCACGTGCATG-3' were designed and synthesized by Shanghai Sangon to amplify the full-length *ZjMT* cDNA coding sequence using recombinant plasmid from cDNA library as a template.

The PCR was performed in a 50- $\mu$ l reaction mixture containing 5  $\mu$ l of 10 $\times$  buffer, 1.5 U Taq polymerase (TaKaRa), 10 pmol of each primer, 50 ng of DNA template, and 50  $\mu$ mol of dNTPs. The PCR amplification was performed under the following conditions: 95°C for 5 min, 35 cycles at 94°C for 30 s, 52°C for 30 s, 72°C for 45 s, followed by 1 cycle at 72°C for 5 min. PCR products were purified using the Gel Extraction Kit (Qiagen, Germany), and cloned into pGEX-4T-2 vector containing the GST gene (GE Healthcare, USA). The pGEX-4T-2-*ZjMT* fusion plasmid was transformed into *E. coli* strain BL21(DE3), and pGEX-4T-2-plasmid containing transformant was used as a negative control. Putative clones containing the fusion product were screened by digestion with BamHI/HindIII and PCR amplification under the same conditions as described previously.

**Expression and analysis of *ZjMT* in *E. coli*.** *Escherichia coli* cells containing pGEX-4T-2/pGEX-4T-2-*ZjMT* plasmids or negative control were inoculated in LB medium containing 50 mg/ml of ampicillin and incubated at 37°C overnight with shaking. During the incubation, different concentrations of isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) were added to induce expression of the fusion protein when OD<sub>600</sub> of the bacterial culture reached 0.5. The *Escherichia coli* cells were then harvested by centrifugation at 9000g for 5 min at 4°C. The harvested cells in the loading buffer (20 mM Tris-HCl, pH 8.0, 4% (w/v) SDS, 10% (v/v)  $\beta$ -mercaptoethanol, 20% (v/v) glycerol, 0.04% (v/v) bromophenol blue) were heated in a boiling water bath for 5-10 min and subjected to electrophoresis on a 15% SDS-polyacrylamide gel to determine the expression of the fusion protein.

The harvested cells were also disrupted by sonication, and the supernatant was transferred onto a column of Glutathione Resins (TaKaRa) to purify the fusion protein. The purity and concentration of the protein were assessed by 15% SDS-PAGE.

**Metal tolerance and accumulation in the cells containing recombinant ZjMT.** Metal tolerance and bioaccumulation of heavy metals ( $\text{Cd}^{2+}$ ,  $\text{Cr}^{3+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Ni}^{2+}$ , and  $\text{Cu}^{2+}$ ) in *E. coli* containing pGEX-4T-2/pGEX-4T-2-ZjMT plasmids were assayed. Seed culture (500  $\mu\text{l}$ ) was added into 50 ml of MJS medium with 50  $\mu\text{g}/\text{ml}$  of ampicillin and incubated at 37°C. IPTG (0.1 mM) and different concentrations of heavy metals were added to the culture when its density ( $\text{OD}_{600}$ ) reached 0.5. The cells were further incubated, and  $\text{OD}_{600}$  values were measured to assess the growth of the *E. coli* cells under the heavy metal addition treatments. When induced for 4 h in the present of IPTG, the cells were harvested by centrifugation at 9000g for 10 min at 4°C, washed three times with 5 mM HEPES buffer, and vacuum freeze-dried. The cell pellets were digested overnight with 4 ml of 70%  $\text{HNO}_3$  at 42°C and diluted with 6 ml of deionized water. Heavy metal concentrations were then determined using an AA-6800 atomic absorption spectrophotometer (Shimadzu, Japan). Bioaccumulation capacity was presented as the amount of metals in mg accumulated per kilogram of dry cells. All the experiments were repeated three times under identical conditions.

**Statistical analysis.** The data were statistically analyzed using the SPSS software package (version 16.0; SPSS IBM, USA). Data are expressed by means and corresponding standard errors. One-way ANOVA was performed to compare expression of ZjMT in leaves of *Z. jujuba* under cadmium stress. Two-way ANOVA was performed to test the effect of the *ZjMT* gene and metal concentrations on ion accumulation in *E. coli* cells. Tukey's *post hoc* test was used to compare the means. The difference was considered significant when *P* value was <0.05.

## RESULTS

**cDNA and putative protein of *Z. jujuba* metallothionein 1 gene.** A cDNA clone with a sequence of 225 bp (GenBank Accession No. AB513130), coding for a

polypeptide of 74 a.a. was isolated from the cDNA library of *Z. jujuba*. Based on blast and alignment analysis, the deduced protein sequence was homologous to the metallothionein proteins of other plant species in the database. Therefore, it was designated as the *Z. jujuba* metallothionein 1 gene (*ZjMT*). The deduced amino acid sequence of *ZjMT* contained six Cys-Xaa-Cys motifs; three of each were identified in the N- (CXCXXXCXCXXXCXC) and C-terminal (CXCXXXCXCXXXCXC) domains respectively, which is the common feature of the type 1 MT proteins reported in plants. No cysteine was present in the regions between the cysteine-rich domains, which is also typical of MT proteins (Fig. 1). When the *ZjMT* protein was subjected to analysis by ExPASy software, it was found to be rich in neutral residues with 17.6% glycine, 16.2% cysteine, and 12.2% serine, and its molecular weight was 7.376 kDa with theoretical isoelectric point (pI) of 4.83 and mean hydrophobic value of -0.377. However, no signal peptide was detected by the Signal P 3.0 software.

High similarities were observed between the amino acid sequences of *ZjMT* and its homologs. Respectively, *ZjMT* showed 72.6% similarity to *Betula platyphylla* MT, 67.6% to *Fagus sylvatica* MTII, 66.7% to *Nelumbo nucifera* MT2b, 65.3% to *Sesbania drummondii* MT2, 64.4% to *Pyrus pyrifolia* MT, 61.6% to *Salix matsudana* MT1A, 60.8% to *Pisum sativum* MT1, and 60.6% to *Camellia sinensis* MT1 (Fig. 1).

**Expression of *ZjMT* gene in response to cadmium.** Quantitative PCR was performed to analyze the expression of *ZjMT* under cadmium stress, and the results are shown in Fig. 2. A basal level of MT synthesis was seen at the beginning of the experiment. In response to cadmium exposure, *ZjMT* exhibited higher expression levels in the leaves of *Z. jujuba* compared with that prior to the stress treatment. Among various exposure times, the strongest response appeared at 24 h with exposure to 100 mM Cd<sup>2+</sup>, then the expression levels decreased significantly at 48 h.

**Expression of *ZjMT* cDNA in *E. coli*.** A 35-kDa protein was expressed in the *E. coli* transformed by the recombinant plasmid pGEX-4T-2-*ZjMT*. This is consistent with the calculated molecular weight of the fusion protein, indicating the expression of *ZjMT*. An increased level of its expression was also seen by IPTG induction compared with that prior to IPTG addition into the culture (Fig. 3).

**Metal tolerance and ion accumulation in *E. coli*.** To assess the role of *ZjMT*

in metal tolerance, *E. coli* cells containing pGEX-4T-2-ZjMT or pGEX-4T-2 (as a control) were subjected to different levels of metal stress. Under normal conditions, the growth pattern of cells containing pGEX-4T-2-ZjMT was similar to that of control. However, under heavy metal stress, *E. coli* cells expressing ZjMT showed greater proliferation than the cells containing only pGEX-4T-2, indicating the ability of ZjMT to tolerate heavy metals, particularly copper and cadmium (Fig. 4).

The ability of *E. coli* cells expressing ZjMT (pGEX-4T-2-ZjMT) to accumulate heavy metals was also investigated using *E. coli* cell line pGEX-4T-2 as a control. The concentrations of the metal ions in the *E. coli* cultures were analyzed to assess such bioaccumulations. The overall results showed that recombinant *E. coli* cells had significantly higher metal bioaccumulation capacity than the control group for all the metals analyzed, as shown in Fig. 5. Meanwhile, the concentrations of the metals contained in the culture significantly contributed to the levels of metal accumulations in both recombinant and control cells. In all the groups except that treated with Mn, higher concentration of metals in the culture resulted in higher levels of metal accumulations in the cells. The increases in the accumulation ability of recombinant *E. coli* cells expressing ZjMT were different in the groups treated with different metals. The culture of *E. coli* expressing ZjMT had up to 7.99-fold of  $Zn^{2+}$  compared with that in the control, which was the highest relative accumulation among all the metals. In contrast, the culture of *E. coli* expressing ZjMT contained only 1.34-fold of  $Cu^{2+}$  compared with that in the control, which was the lowest accumulation among all the metals. Overall, the relative metal adsorption capacity of the *E. coli* expressing ZjMT was in the order  $Zn^{2+} > Mn^{2+} > Cr^{3+} > Ni^{2+} > Cd^{2+} > Cu^{2+}$ . When the absolute concentrations of metals in the culture were measured, *E. coli* cells expressing ZjMT showed the maximum bioaccumulation capacities: Ni at 323 mg/kg dry weight culture (DW), Cr at 1145 mg/kg DW, Mn at 734 mg/kg DW, Cd at 696 mg/kg DW, Cu at 3075 mg/kg of DW, and Zn at 354 mg/kg DW. Accordingly, the absolute metal adsorption capacity of the *E. coli* expressing ZjMT was in the order  $Cu^{2+} > Cr^{3+} > Mn^{2+} > Cd^{2+} > Zn^{2+} > Ni^{2+}$ .

## DISCUSSION

Metallothioneins (MTs) are Cys-rich proteins with low molecular weight that are involved in metal tolerance of diverse living organisms. In animals, MTs were found to play a key role in metal detoxification and homeostasis [17], the protection of cells against oxidative stress [26], and the scavenging of free radicals [27]. The affinity and bioaccumulation capacity of MT for heavy metals in aquatic species make these organisms possible candidates to serve as an indicator of environmental exposure to metals and also as a biomarker of metal pollution. It has been reported that the exposure of aquatic organisms to metals induced MT expression. Numerous studies have shown the bioaccumulation capacities of aquatic species such as rare minnow [28], mudskipper [29], Mediterranean clam [30], and blue crab [31]. MT genes have also been reported in various plant species, such as *Arabidopsis thaliana* [32], *Olea europaea* L. [33], and *Helianthus annuus* [34]. While mammalian MTs have highly conserved regions [35], plant MTs contain distinctive arrangement(s) of Cys residues although their sequences, and their functions may vary [16]. Despite the confirmation of the presence of MT genes in plants, conclusive demonstration of their precise cellular roles in the sequestration/homeostasis of heavy metals has been lacking.

Chinese jujube is highly tolerant to abiotic stresses such as cold, drought, salinity, and alkalinity. However, the mechanisms underlying such stresses have not been extensively investigated. In the present study, we isolated and characterized the ZjMT in the species for the first time. It is highly homologous to the MTs of *Betula platyphylla* and *Fagus sylvatica*. The alignment of the deduced amino acid sequence of ZjMT with those from other plants revealed that it belongs to the cysteine-rich protein family that is typical of MT-1 genes. The fact that ZjMT expressed in *E. coli* significantly enhanced the growth of the cells in comparison with the control under heavy metal stress indicates that the *ZjMT* gene promotes heavy metal tolerance. This was manifested by the higher heavy metal accumulation in ZjMT-expressing *E. coli* cells compared with the control. Furthermore, the seedlings of *Z. jujuba* exposed to Cd<sup>2+</sup> showed higher expression of the *ZjMT* gene. This also confirmed the heavy metal tolerance role of ZjMT *in vivo*. Similar tolerance nature of the plant MT genes has also

been reported elsewhere [32-34, 36]. Therefore, the ability of the *ZjMT* gene to tolerate heavy metals can at least partially explain the stress tolerance of *Z. jujuba* under different cultivation conditions.

Experimental results have shown that the expression of the reported *MT*-like genes from plants could be induced by various environmental factors other than metals, such as cold, drought, and salt stress [36-38]. The functions of plant *MT* genes thus appear to be diversified. Nevertheless, the molecular mechanisms underlying the diverse tolerance of *MTs* to environmental stresses remain largely unknown in plants [16]. It was demonstrated in the present study that IPTG-induced over-expression of the *ZjMT* gene in *ZjMT*-containing *E. coli* enhanced tolerance to not only essential metals such as Mn, Cu, Ni, and Zn, but also to non-essential metals such as Cd and Cr. As this tolerance of *MT* has also been shown in other species [33, 34, 39, 40], we suggest that the cysteine residue motifs in *ZjMT* may be responsible for the metal ion binding and sequestration ability [13, 15-17].

Heavy metal contamination is a worldwide environmental concern, and the risk posed to humans is increasing. Although some metals at low levels are essential for metabolisms in organisms, over-accretion of heavy metals in cells is toxic. Industries involving metal plating, mining, leather processing, and photography generate large amounts of heavy metal-containing wastes [41]. Much such waste is discharged into the environment, which inevitably poses threats to ecological systems, plant and animal production, and eventually to human health. Various measures have been applied to minimize such contamination, and removal of heavy metals from contaminated water is one of the most important measures [42, 43]. Biosorption of heavy metals using materials of microbial or plant origin has been considered as an innovative technology for removal of such pollutants [44]. For example, *E. coli* is biosorptive and has recently been used to adsorb heavy metals [45, 46]. Enhanced uptake and bioaccumulation of mercury by *E. coli* have been achieved through genetic engineering to produce a recombinant strain [47]. In our study, enhanced expression of the *ZjMT* gene in *E. coli* showed increased accumulations of essential and non-essential metal ions, confirming its important role in metal detoxification. The recombinant *E. coli* cells expressing *ZjMT* protein also showed high heavy metal tolerance and binding capacity. Therefore,

the ZjMT-expressing *E. coli* can be potentially used in heavy metal sequestration from the environment, especially from aqueous media such as wastewater systems.

In conclusion, a metallothionein gene (*ZjMT*) was isolated from the cDNA library of *Z. jujuba*, and its function was characterized using *in vitro* as well *in vivo* approaches in the present study. *ZjMT* is a type 1 metallothionein and is induced in *Z. jujuba* plants grown under cadmium-stress conditions. Expression of the *ZjMT* gene in *E. coli* showed evident heavy metal tolerance and increased accumulations of essential and non-essential metal ions. Therefore, the *ZjMT* gene can be used as a candidate for transformation to other vulnerable plants to enhance their tolerance to heavy metal stress, and the recombinant *E. coli* strain can be potentially used in heavy metal sequestration from the environment such as in wastewater systems. The results obtained in the present study confirmed our hypothesis that the *MT* gene in *Z. jujuba*, i.e. *ZjMT*, is responsible for its tolerance to abiotic stress, at least partially, and the recombinant *E. coli* expressing the gene will be a potential candidate for the remediation of heavy metal contaminated wastewater. Nevertheless, due to the complexity of the mechanisms underlying heavy metal tolerance and other abiotic stresses, thorough investigation of such mechanisms in plants is warranted in future studies.

This work was supported by Shanxi Provincial Key Innovative Research Team in Science and Technology (2015013002-10), Innovation Initiative Program in Science and Technology, Shanxi Academy of Agricultural Sciences (zzcx22) and Youth Foundation of Taiyuan University of Technology (No. 2012L019).

## REFERENCES

1. Verbruggen, N., Hermans, C., and Schat, H. (2009) Molecular mechanisms of metal hyperaccumulation in plants, *New Phytol.*, **181**, 759-776.
2. Chao, Y.-E., Zhang, M., Feng, Y., Yang, X.-E., and Islam, E. (2010) cDNA-AFLP analysis of inducible gene expression in zinc hyperaccumulator *Sedum alfredii* Hance under zinc induction, *Environ. Exp. Bot.*, **68**, 107-112.

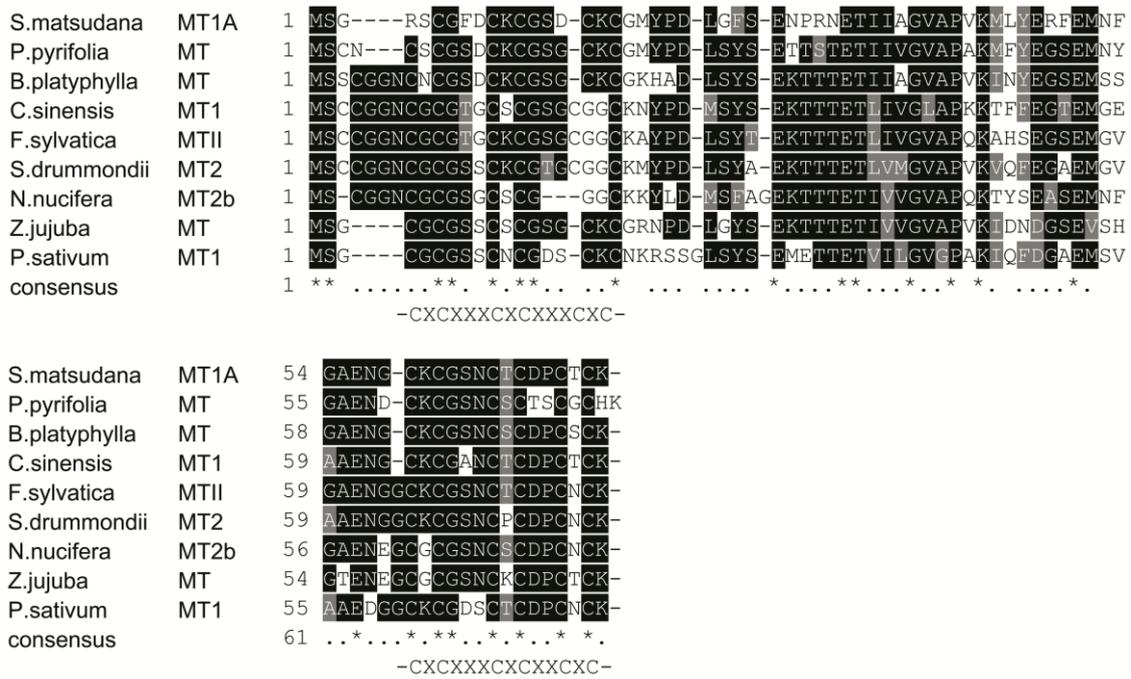
3. Becher, M., Talke, I. N., Krall, L., and Kramer, U. (2004) Cross-species microarray transcript profiling reveals high constitutive expression of metal homeostasis genes in shoots of the zinc hyperaccumulator *Arabidopsis halleri*, *Plant J.*, **37**, 251-268.
4. Kramer, U., Talke, I. N., and Hanikenne, M. (2007) Transition metal transport, *FEBS Lett.*, **581**, 2263-2272.
5. Talke, I. N., Hanikenne, M., and Kramer, U. (2006) Zinc-dependent global transcriptional control, transcriptional deregulation, and higher gene copy number for genes in metal homeostasis of the hyperaccumulator *Arabidopsis halleri*, *Plant Physiol.*, **142**, 148-167.
6. Weber, M., Trampczynska, A., and Clemens, S. (2006) Comparative transcriptome analysis of toxic metal responses in *Arabidopsis thaliana* and the Cd<sup>2+</sup>-hypertolerant facultative metallophyte *Arabidopsis halleri*, *Plant Cell Environ.*, **29**, 950-963.
7. Hammond, J. P., Bowen, H. C., White, P. J., Mills, V., Pyke, K. A., Baker, A. J. M., Whiting, S. N., May, S. T., and Broadley, M. R. (2006) A comparison of the *Thlaspi caerulescens* and *Thlaspi arvense* shoot transcriptomes, *New Phytol.*, **170**, 239-260.
8. Van De Mortel, J. E., Villanueva, L. A., Schat, H., Kwekkeboom, J., Coughlan, S., Moerland, P. D., Van Themaat, E. V. L., Koornneef, M., and Aarts, M. G. M. (2006) Large expression differences in genes for iron and zinc homeostasis, stress response, and lignin biosynthesis distinguish roots of *Arabidopsis thaliana* and the related metal hyperaccumulator *Thlaspi caerulescens*, *Plant Physiol.*, **142**, 1127-1147.
9. Gendre, D., Czernic, P., Conejero, G., Pianelli, K., Briat, J. F., Lebrun, M., and Mari, S. (2007) *TcYSL3*, a member of the *YSL* gene family from the hyper-accumulator *Thlaspi caerulescens*, encodes a nicotianamine-Ni/Fe transporter, *Plant J.*, **49**, 1-15.
10. Margoshes, M., and Vallee, B. L. (1957) A cadmium protein from equine kidney cortex, *J. Am. Chem. Soc.*, **79**, 4813-4814.

11. Bataineh, Z. M., Heidger, P. M., Jr., Thompson, S. A., and Timms, B. G. (1986) Immunocytochemical localization of metallothionein in the rat prostate gland, *Prostate*, **9**, 397-410.
12. Thirumoorthy, N., Kumar, K. M., Sundar, A. S., Panayappan, L., and Chatterjee, M. (2007) Metallothionein: an overview, *World J. Gastroenterol.*, **13**, 993-996.
13. Vallee, B. L. (1991) Introduction to metallothionein, *Methods Enzymol.*, **205**, 3-7.
14. Ernst, W. H. O., Krauss, G. J., Verkleij, J. A. C., and Wesenberg, D. (2008) Interaction of heavy metals with the sulphur metabolism in angiosperms from an ecological point of view, *Plant Cell Environ.*, **31**, 123-143.
15. Kagi, J. H. R., and Schaffer, A. (1988) Biochemistry of metallothionein, *Biochemistry*, **27**, 8509-8515.
16. Cobbett, C., and Goldsbrough, P. (2002) Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis, *Annu. Rev. Plant Biol.*, **53**, 159-182.
17. Coyle, P., Philcox, J. C., Carey, L. C., and Rofe, A. M. (2002) Metallothionein: the multipurpose protein, *Cell. Mol. Life Sci.*, **59**, 627-647.
18. Sanita di Toppi, L., and Gabbriellini, R. (1999) Response to cadmium in higher plants, *Environ. Exp. Bot.*, **41**, 105-130.
19. Shim, D., Hwang, J. U., Lee, J., Lee, S., Choi, Y., An, G., Martinoia, E., and Lee, Y. (2009) Orthologs of the class A4 heat shock transcription factor HsfA4a confer cadmium tolerance in wheat and rice, *Plant Cell*, **21**, 4031-4043.
20. Maraghni, M., Gorai, M., Neffati, M., and Labeke, M. C. V. (2014) Differential responses to drought stress in leaves and roots of wild jujube, *Ziziphus lotus*, *Acta Physiol. Plant.*, **36**, 945-953.
21. Kulkarni, M., Schneider, B., Raveh, E., and Tel-Zur, N. (2010) Leaf anatomical characteristics and physiological responses to short-term drought in *Ziziphus mauritiana* (Lamk.), *Sci. Hortic.*, **124**, 316-322.
22. Kotoda, N., Wada, M., Komori, S., Kidou, S., Abe, K., Masuda, T., and Soejima, J. (2000) Expression pattern of homologues of floral meristem identity genes

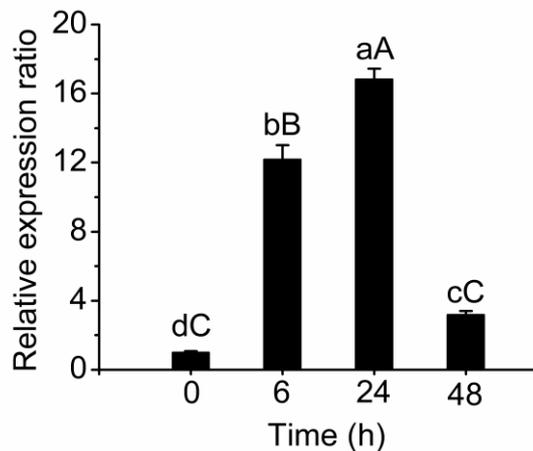
- LFY* and *API* during flower development in apple, *J. Am. Soc. Hortic. Sci.*, **125**, 398-403.
23. Larkin, M. P., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J., and Higgins, D. G. (2007) Clustal W and Clustal X version 2.0, *Bioinformatics*, **23**, 2947-2948.
  24. Sun, H. F., Meng, Y. P., Cui, G. M., Cao, Q. F., Li, J., and Liang, A. H. (2009) Selection of housekeeping genes for gene expression studies on the development of fruit bearing shoots in Chinese jujube (*Ziziphus jujuba* Mill.), *Mol. Biol. Rep.*, **36**, 2183-2190.
  25. Livak, K. J., and Schmittgen, T. D. (2001) Analysis of relative gene expression data using realtime quantitative PCR and the  $2(\Delta\Delta C(T))$  method, *Methods*, **25**, 402-408.
  26. Hansen, B. H., Romma, S., Garmo, O. A., Olsvik, P. A., and Andersen, R. A. (2006) Antioxidative stress proteins and their gene expression in brown trout (*Salmo trutta*) from three rivers with different heavy metal levels, *Comp. Biochem. Phys. C*, **143**, 263-274.
  27. Atif, F., Kaur, M., Yousuf, S., and Raisuddin, S. (2006) *In vitro* free radical scavenging activity of hepatic metallothionein induced in an Indian freshwater fish, *Channa punctata* Bloch, *Chem. Biol. Interact.*, **162**, 172-180.
  28. Wang, C., Zhang, F., Cao, W., and Wang, J. (2014) The identification of metallothionein in rare minnow (*Gobiocypris rarus*) and its expression following heavy metal exposure, *Environ. Toxicol. Pharmacol.*, **37**, 1283-1291.
  29. Han, Y. L., Zhang, S., Liu, G. D., Long, L. L., Wang, Y. F., Yang, W. X., and Zhu, J. Q. (2015) Cloning, characterization and cadmium inducibility of metallothionein in the testes of the mudskipper *Boleophthalmus pectinirostris*, *Ecotoxicol. Environ. Safety*, **119**, 1-8.
  30. Santovito, G., Boldrin, F., and Irato, P. (2015) Metal and metallothionein distribution in different tissues of the Mediterranean clam *Venerupis philippinarum* during copper treatment and detoxification, *Comp. Biochem. Phys. C*, **174-175**, 46-53.

31. Lavradas, R. T., Hauser-Davis, R. A., Lavandier, R. C., Rocha, R. C. C., Pierre, T. D. S., Seixas, T., Kehrig, H. A., and Moreira, I. (2014) Metal, metallothionein and glutathione levels in blue crab (*Callinectes* sp.) specimens from southeastern Brazil, *Ecotoxicol. Environ. Safety*, **107**, 55-60.
32. Murphy, A., and Taiz, L. (1995) Comparison of metallothionein gene expression and nonprotein thiols in ten *Arabidopsis* ecotypes, *Plant Physiol.*, **109**, 945-954.
33. Dundar, E., Sonmez, G. D., and Unver, T. (2015) Isolation, molecular characterization and functional analysis of OeMT2, an olive metallothionein with a bioremediation potential, *Mol. Genet. Genom.*, **290**, 187-199.
34. Tomas, M., Pagani, M. A., Andreo, C. S., Capdevila, M., Atrian, S., and Bofill, R. (2015) Sunflower metallothionein family characterization. Study of the Zn(II)- and Cd(II)-binding abilities of the HaMT1 and HaMT2 isoforms, *J. Inorg. Biochem.*, **148**, 35-48.
35. Klaassen, C. D., Liu, J., and Choudhuri, S. (1999) Metallothionein: an intracellular protein to protect against cadmium toxicity, *Annu. Rev. Pharmacol.*, **39**, 267-294.
36. Yan, L., Yue, Y. C., Shu, G. Y., and Wei, M. T. (2015) Cloning and characterization of *HbMT2a*, a metallothionein gene from *Hevea brasiliensis* Muell. Arg differently responds to abiotic stress and heavy metals, *Biochem. Biophys. Res. Commun.*, **461**, 95-101.
37. Sun, H. K., Jeong, J. C., Ahn, Y. O., Lee, H. S., and Kwak, S. S. (2014) Differential responses of three sweet potato metallothionein genes to abiotic stress and heavy metals, *Mol. Biol. Rep.*, **41**, 6957-6966.
38. Akashi, K., Nishimura, N., Ishida, Y., and Yokota, A. (2004) Potent hydroxyl radical scavenging activity of drought-induced type-2 metallothionein in wild watermelon, *Biochem. Biophys. Res. Commun.*, **323**, 72-78.
39. Liu, J., Shi, X., Qian, M., Zheng, L., Lian, C., Xia, Y., and Shen, Z. (2015) Copper-induced hydrogen peroxide upregulation of a metallothionein gene, *OsMT2c*, from *Oryza sativa* L. confers copper tolerance in *Arabidopsis thaliana*, *J. Hazard. Mater.*, **294**, 99-108.

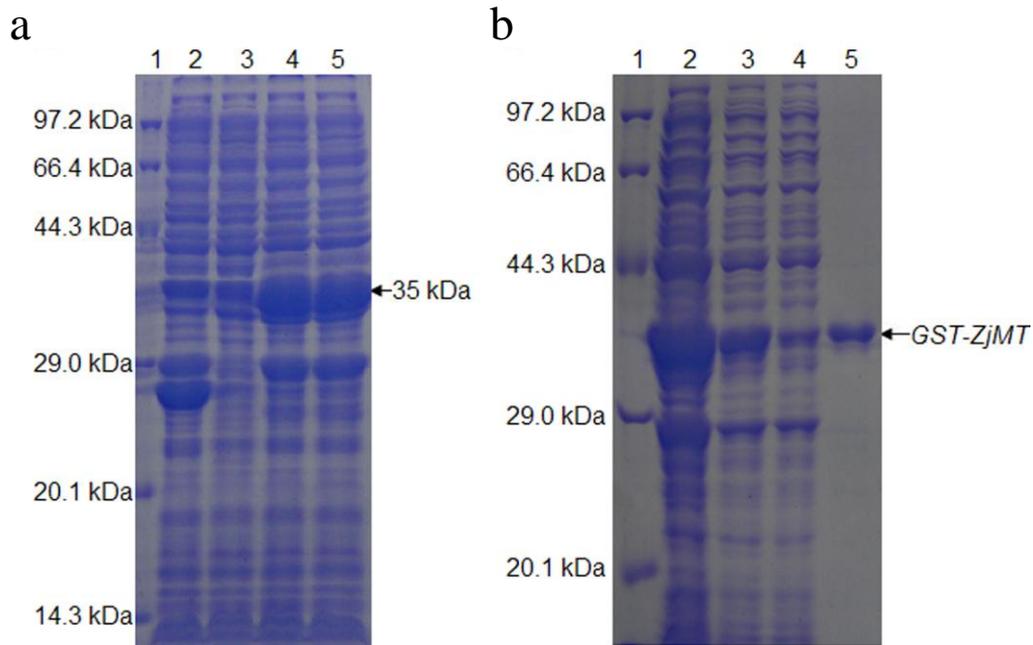
40. Gu, C. S., Liu, L. Q., Deng, Y. M., Zhu, X. D., Huang, S. Z., and Lu, X. Q. (2015) The heterologous expression of the *Iris lactea* var. *chinensis* type2 metallothionein *IlMT2b* gene enhances copper tolerance in *Arabidopsis thaliana*, *Bull. Environ. Contam. Toxicol.*, **94**, 247-253.
41. Wu, G., Kang, H., Zhang, X., Shao, H., Chu, L., and Ruan, C. (2010) A critical review on the bio-removal of hazardous heavy metals from contaminated soils: issues, progress, eco-environmental concerns and opportunities, *J. Hazard. Mater.*, **174**, 1-8.
42. Ai, P. L., and Aris, A. Z. (2014) A review on economically adsorbents on heavy metals removal in water and wastewater, *Rev. Environ. Sci. Biotechnol.*, **13**, 163-181.
43. Sen, A., Pereira, H., Olivella, M. A., and Villaescusa, I. (2015) Heavy metals removal in aqueous environments using bark as a biosorbent, *Int. J. Environ. Sci. Technol.*, **12**, 391-404.
44. Srivastava, S., Agrawal, S. B., and Mondal, M. K. (2015) A review on progress of heavy metal removal using adsorbents of microbial and plant origin, *Environ. Sci. Pollut. Res. Int.*, **22**, 15386-15415.
45. Kim, S., Song, M. H., Wei, W., and Yun, Y. S. (2015) Selective biosorption behavior of *Escherichia coli* biomass toward Pd(II) in Pt(IV)-Pd(II) binary solution, *J. Hazard. Mater.*, **283**, 657-662.
46. Khan, Z., Nisar, M. A., Hussain, S. Z., Arshad, M. N., and Rehman, A. (2015) Cadmium resistance mechanism in *Escherichia coli* P4 and its potential use to bioremediate environmental cadmium, *Appl. Microbiol. Biotechnol.*, **99**, 10745-10757.
47. Lin, K. H., Chien, M. F., Hsieh, J. L., and Huang, C. C. (2010) Mercury resistance and accumulation in *Escherichia coli* with cell surface expression of fish metallothionein, *Appl. Microbiol. Biotechnol.*, **87**, 561-569.



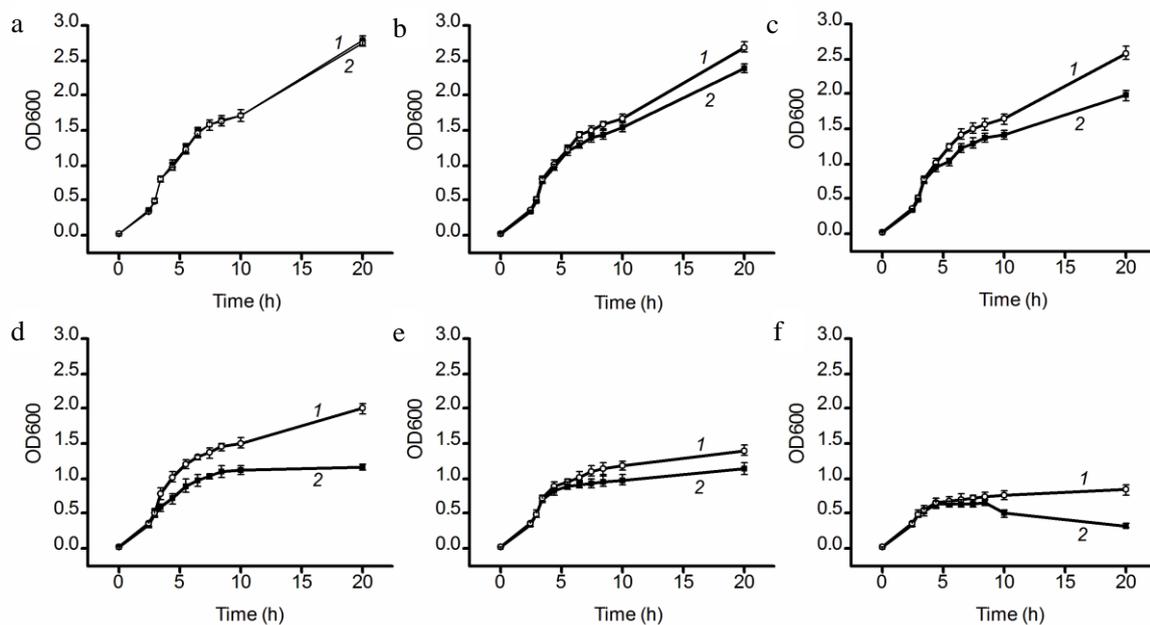
**Fig. 1.** Alignment of the deduced MT amino acid sequence of *Z. jujuba* using ClustalX software. MT sequences from eight other species (*Betula platyphylla*, *Fagus sylvatica*, *Nelumbo nucifera*, *Sesbania drummondii*, *Pyrus pyrifolia*, *Salix matsudana*, *Pisum sativum*, and *Camellia sinensis*) are available in GenBank. Identical amino acids are marked with asterisks (\*), and related amino acids with a single dot (·).



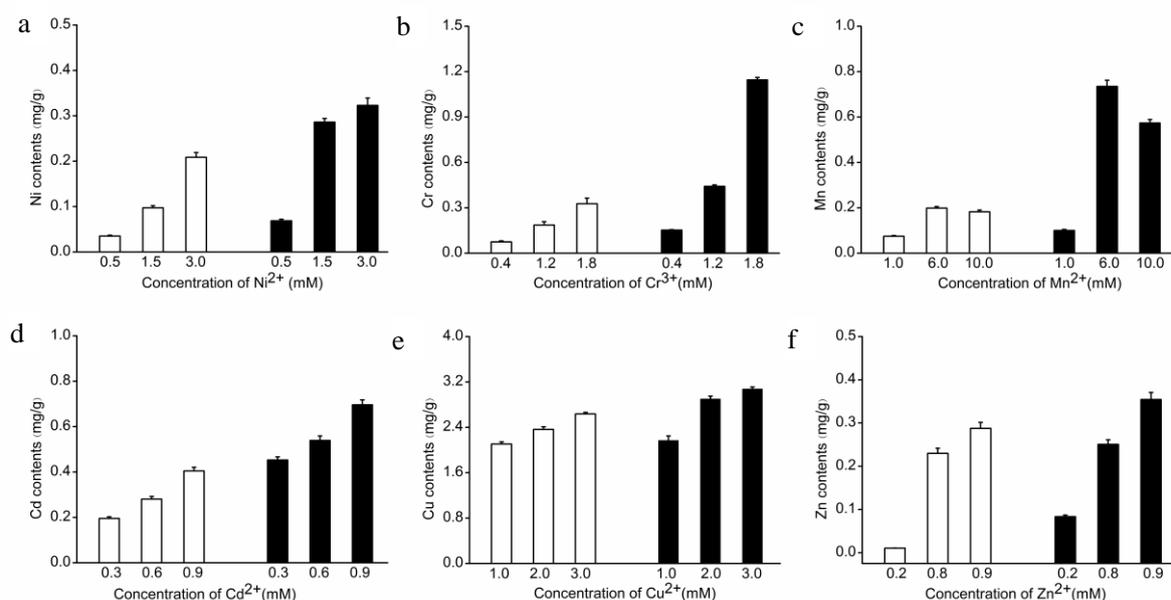
**Fig. 2.** mRNA expression level of ZjMT in *Z. jujuba* after exposure to 100 mM Cd<sup>2+</sup>. Expression was measured by SYBR Green I RT-PCR. The relative ZjMT expression level as expressed by 2<sup>-ΔΔCT</sup> was determined for each group, and vertical bars represented the mean ± S.E. (n = 6).



**Fig. 3.** SDS-PAGE analysis of GST-ZjMT fusion protein in *E. coli* cells. a) GST-ZjMT fusion protein in pGEX-4T-2-ZjMT/BL21 before and after IPTG induction. Lanes: 1) protein marker; 2) total protein of pGEX-4T-2/BL21 after IPTG induction; 3) total protein of pGEX-4T-2-ZjMT/BL21 without IPTG induction; 4, 5) total protein of pGEX-4T-2-ZjMT/BL21 after IPTG induction. b) Purification of GST-ZjMT fusion protein by Glutathione Resins. Lanes: 1) marker; 2-4) total protein of pGEX-4T-2-ZjMT/BL21; 5) GST-ZjMT protein purified by Glutathione Resins.



**Fig. 4.** Effects of heavy metal concentrations on growth of *E. coli* BL21 cells containing pGEX-4T-2-ZjMT (circles) or pGEX-4T-2 (squares). a) No metals; b) 400 μM Cu<sup>2+</sup>; c) 800 μM Cu<sup>2+</sup>; d) 1600 μM Cu<sup>2+</sup>; e) 400 μM Cd<sup>2+</sup>; f) 800 μM Cd<sup>2+</sup>. The OD<sub>600</sub> values with error bars represent the means and S.E. of three independent experiments.



**Fig. 5.** Bioaccumulation of heavy metals by *E. coli* containing pGEX-4T-2-ZjMT (solid columns) or pGEX-4T-2 (blank columns) in responses to respective metal concentrations in the culture and *ZjMT* expression in the *E. coli* cells: a) Ni(II); b) Cr(III); c) Mn(II); d) Cd(II); e) Cu(II); f) Zn(II). The columns represent means with error bars showing S.E. of three independent experiments. The main effects of *ZjMT* gene, concentration of heavy metals, and the interaction between the expression of the *ZjMT* gene and metal concentrations on ion accumulations in *E. coli* cells were analyzed using the GLM model in SPSS. The main effects were significant at the levels of  $P < 0.001$  on the accumulations of all ions with exception of  $\text{Cu}^{2+}$ , in which  $\text{Cu}^{2+}$  accumulation was significantly affected by  $\text{Cu}^{2+}$  concentration in the culture at the level of  $P < 0.01$ . Interactions between the expression of *ZjMT* gene and metal concentrations in the culture were significant at the level of  $P < 0.001$  for  $\text{Ni}^{2+}$ ,  $\text{Cr}^{3+}$ , and  $\text{Mn}^{2+}$  and  $P < 0.01$  for  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$ , while no such interaction was detected for  $\text{Cd}^{2+}$ . The metal contents in the cells are expressed on dry matter basis.