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Research Article

Improved Accumulation and Tolerant to Heavy Metals Triggered by Over-Expressing AtGCS from *Arabidopsis Thaliana* in *E. coli*

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Abstract: GSH is one of the most ubiquitous proteins known to provide protection against toxic heavy metals. A gene-AtGCS encoding Arabidopsis γ-glutamylcysteine synthetase was introduced into *Escherichia coli* (BL21) by over-expression of TrxA-AtGCS fusion protein and analyzed the tolerant capacity to heavy metal by transformed *E. coli*. As comparison, the strain over-expressing TrxA was selected as the control. The results showed that the growth of *E. coli* cells over-expressing TrxA-AtGCS was much better than the control cells expressing TrxA under 1 mM Cd^{2+}, Zn^{2+} and Cu^{2+} heavy metals respectively. Meanwhile, the higher bioaccumulation of Cd^{2+}, Zn^{2+}, Cu^{2+} and GSH content were observed in the strain over-expressing TrxA-AtGCS. It could be concluded that over-expression of AtGCS offered a promising heavy metals resistance of *E. coli* with superior heavy metals accumulation and GSH content.

Keywords: Accumulation, AtGCS, *E. coli*, heavy metals tolerance, GSH content

INTRODUCTION

Heavy metals pollution of soils and waters, mainly caused by mining and the burning of fossil fuels, is a major environmental problem, which impacted many organisms by stress-induced formation of Reactive Oxygen Species (ROS) (Nies, 1999; Nriagu and Pacyna, 1998). The best-known detoxification molecule, which serves as storage place as well as transportation molecules in cells, is glutathione (L-γ-glutamyl-L-cysteinylglycine, GSH) (Chan and Cherian, 1992; Foyer and Noctor, 2005; Meister, 1995). GSH seems to be the first defense against metal toxicity. GSH functions as an antioxidant itself and as a component of the detoxifying enzyme system containing oxidase and reductase. Glutathione is also required for synthesis of proteins and nucleic acids, maintenance of enzymes in their active forms and maintenance of cell membranes. Production of GSH has been suggested to play a protective role against metal toxicity (Szalai *et al.*, 2009). Thus, increase of glutathione content may increase the cells' tolerance to heavy metal stress. In eukaryotes, GSH is synthesized intracellular in two ATP-dependent steps catalyzed by γ-glutamylcysteine synthetase (GSH1, γ-GCS; EC 6.3.2.2) and glutathione synthetase (GSH2, GS; EC 6.3.2.3) and γ-ECS is feedback inhibited by GSH. The two enzymes have been isolated and characterized from several plants, yeast and etc. (Kim *et al*., 2003; Rawlins *et al.*, 1995).

In the past decades, in order to sequester, precipitate or alter such oxidation state of various heavy metals, the use of microorganisms has been extensively studied. Attempts to enhance the metal content of bacterial cells have been made by expression of Metallothioneins (MTs) to increase the affinity and biosorptive capabilities for heavy metals (Chaturvedi *et al.*, 2012; Sauge-Merle *et al.*, 2012), over-expressing metal-binding peptides or proteins such as poly-histidines (Sousa *et al*., 1996) and metal binding motif (Pazirandeh *et al*., 1998). Nevertheless, few researches focus on manipulating the expression of enzymes involved in glutathione, which maybe a good approach to enhance heavy-metal tolerance in *E. coli*.

To gain more insight into the protective role of AtGCS gene in heavy metal tolerance of *E. coli* and whether and how it could enhance the metal-retaining ability of bacterial cells more evidently, AtGCS was expressed as a fusion protein with TrxA to obtain a stable over-expressed protein. The experiment was carried out with transformed *E. coli* in 1 mM Cd^{2+}, Zn^{2+} and Cu^{2+} respectively. The heavy metal tolerance and

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accumulation were examined with the glutathione content assay comprehensively.

MATERIALS AND METHODS

Materials: *E. coli* TransDH5α and BL21, used as expression host strains, were cultured aerobically in Luria-Bertani medium (LB). The expression vector used in this study was pETM-20, which contains a lac promoter for isopropyl *β*-D-Thiogalactopyranoside (IPTG) inducible, high-level TrxA-fusion protein expression and a cleavage site for Tobacco Etch Virus (TEV) protease.

Cloning and generation of expression vectors: *Arabidopsis thaliana* AtGCS (γ-glutaylcysteine synthetase, Genbank accession no. AT4G23100) gene was amplified from *A. thaliana* total RNA using RT-PCR as the specific primers: sense 5´-AGCTATATATA*CCATGG*CGC-3´ (*NcoI*), antisense 5´-CCG*CTCGAG*TTAGTACAGCA-3´ (*XhoI*). The PCR product was digested with appropriate restriction enzymes as indicated in italics and ligated into pETM-20 to construct the plasmid of pETM-20-AtGCS. The resulting plasmid was transformed into *E. coli* cells BL21.

Expression and purification of recombinant fusion protein: *E. coli* BL21 harboring plasmid pETM-20-AtGCS was used for expression and purification of TrxA-AtGCS. Cells transfected with pETM-20 of deletion RAGE were used for expression of TrxA as a control. Cells were grown to OD_{600}~0.5 at 37°C and induced by adding IPTG to a final concentration of 1 mM overnight at 30°C with gentle shaking. Cell pellets were resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) supplemented with 2 mg/mL lysozyme. The translucent suspension was centrifuged at 15000 g for 20 min. Proteins were purified by Ni-NTA resin affinity column chromatography. AtGCS recombinant proteins were obtained from the TrxA-fusion proteins by cleavage with TEV protease.

Heavy metal tolerance analysis of transformed *E. coli*: For assessment of heavy metal tolerance on solidified medium, *E. coli* cultures were incubated at 37°C until cells reached the midlog phase (OD_{600} = 0.5). Each strain was separately inoculated into 100 ml of LB medium containing 1 mM $CdCl_2$, 1 mM $ZnCl_2$ or 1 mM $CuCl_2$ with or without 1 mM IPTG induction for the duration of the experiment. Growth rate was analyzed by monitoring the increase in absorbance at 600 nm at 4 h intervals for 24 h.

Heavy metal accumulation assay and intracellular glutathione determination: Overnight cultures of *E. coli* BL21 were diluted to OD_{600} = 0.5 and incubated at 37°C for 24 h containing 1 mM IPTG and metal ions at the desired concentration. The biomass was separated by centrifugation at 5 000g for 10 min and the supernatant were analyzed for residual metal concentration on an atomic absorption spectroscopy (Thermo Electron Corp. USA). Heavy metal content was expressed as the weight of metal per weight of the wet cells (milligram per milligram of wet weight).

For determination of intracellular glutathione, the pellets were resuspended and intracellular GSH measurements were made by using a GSH assay kit (Sigma, USA). The results are expressed as nmol of intracellular glutathione per mg of total protein.

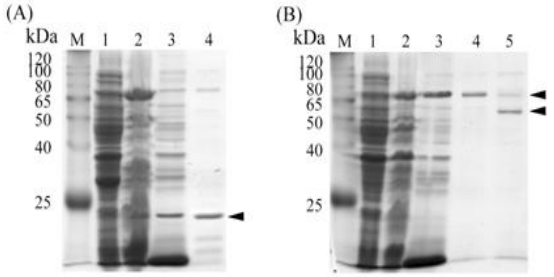

Fig. 1: Expression and purification of recombinant proteins as followed by SDS-PAGE. (A) TrxA, (B) TrxA-AtGCS. Lane 1 and 2, total crude extract of uninduced or induced cells; lane 3, cell lysate supernatant of induced cells; lane 4 and 5, purified TrxA-AtGCS and AtGCS cleavage by TEV protease indicated by arrows. M, molecular mass markers

Statistical analysis: All data were subjected to statistical analysis. All experiments were carried out in triplicate and three samples in each individual determination were examined each time. The values shown in the figures and table are mean values ±SD.

RESULTS AND DISCUSSION

Expression and purification of TrxA-AtGCS recombinant protein in *E. coli*: To determine whether the recombinant constructs were successfully overproduced of fusion protein, plasmid pETM-20 encoding AtGCS was constructed and introduced into *E. coli* BL21 strains. The fusion proteins were fused with thioredoxin (TrxA) and affinity purified on Ni-agarose matrix. The empty vector of pETM-20 with deletion of RAGE expressing TrxA was used as a control. As shown in Fig. 1, by induction with 1 mM IPTG, it was detected approximately 14 and 100 kDa bands which were the expected sizes of the translated product of TrxA and TrxA-AtGCS in transformed *E. coli* cells by SDS-PAGE determination,, which were absent in noninduced cells. By cleavage with TEV protease, the AtGCS protein was obtained with expected molecular mass of 58 kDa. These data suggest that the recombinant constructs were properly

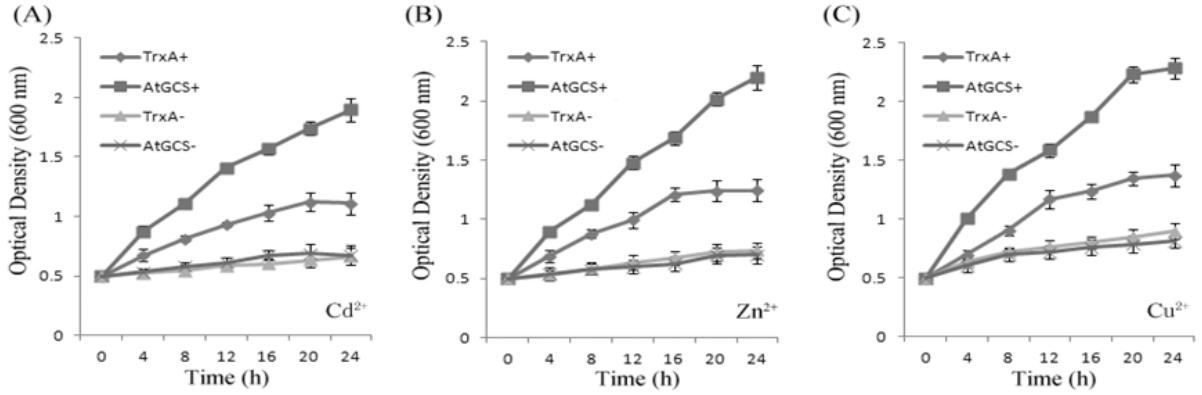

Fig. 2: Growth curves for *E. coli* strains over-expressing TrxA and TrxA-AtGCS with (+) or without (-) IPTG induction in the presence of 1 mM Cd^{2+} (A), 1 mM Zn^{2+} (B) and 1 mM Cu^{2+} (C) heavy metal stresses

Table 1: Accumulation of heavy metal ions and glutathione content by *E. coli* BL21 strains over-expressing TrxA-AtGCS

	Amount of heavy metal accumulated by bacteria [mg of metal/mg of cells (wet weight)][a]			Intracellular glutathione concentration [nmol of glutathione/mg of total cellular protein][a]		
Strains	Cd^{2+}	Zn^{2+}	Cu^{2+}	Cd^{2+}	Zn^{2+}	Cu^{2+}
TrxA	7.2±1.8	9.3±2.3	8.8±2.4	10.8±3.6	13.1±2.4	12.5±4.3
TrxA-AtGCS	68.7±6.6	49.5±5.1	53.7±4.9	54.6±6.8	49.6±5.5	55.2±7.2

[a]: Initial amount ions added were 1 mM; data were presented as means±standard deviations

transcribed the translated thus resulting in the overproduction of the recombinant proteins.

Heavy metal tolerance of *E. coli* cells over-expressing TrxA-StGCS-GS fusion protein: To determine whether AtGCS could enhance the heavy metal tolerance of *E. coli*, the effects of heavy metal stress on growth of *E. coli* cells transformed with TrxA-AtGCS were examined. Under normal conditions, there were no differences in growth rate among *E. coli* cells transformed with TrxA and TrxA-AtGCS, whether or not there was IPTG induction (data not shown). When there was a heavy metal stress, the growth rate of all four transformed strains was seriously damaged to almost the same low level without IPTG induction. On the contrary, although there was a little enhancement of cells expression of TrxA, expression of AtGCS reached a higher grown stage within 24 h (Fig. 2). The results suggested that the cells over-expressing AtGCS could overcome the deleterious effects of cadium, zinc and copper toxicity to some content. In *E. coli*, a similar function has also been shown for MTs, poly-histidines and metal binding motif proteins (Chaturvedi *et al.*, 2012; Sauge-Merle *et al.*, 2012; Sousa *et al.*, 1996; Pazirandeh *et al.*, 1998; An *et al.*, 2006).

Heavy metal bioaccumulation and cellular GSH level by transformed strains over-expressing TrxA-AtGCS: To determine why and how the expression of AtGCS recombinant protein enhanced the growth of *E. coli* under heavy metal stress, the ability of cells expressing TrxA-AtGCS fusion to remove heavy metals from solutions was tested and compared to that of the control bacteria. Induced *E. coli* cells were incubated for 24 h under 1 mM Cd^{2+}, Zn^{2+} and Cu^{2+} pressure. As shown in Table 1, higher levels of bioaccumulation were measured with the strains over-expressing TrxA-AtGCS under such three kinds of heavy metal ions. No obvious accumulation of heavy metal ions was detected in the control cells over-expressing TrxA. The differences were more distinctive when cells suffered from Cd^{2+} stress. It could be concluded that the over-expression of TrxA-AtGCS could more efficiently remove Cd^{2+}, Zn^{2+} and Cu^{2+}.

Furthermore, the effects of heavy metal on cellular glutathione levels of the two *E. coli* strains were evaluated (Table 1). The GSH concentrations of cells over-expressing TrxA-AtGCS were relatively stable and GSH was about five-fold that produced by the strains over-expressing TrxA under the same heavy metal stressful conditions in 24 h. The results indicated that the high content of cellular GSH could prevent *E. coli* cells from harmful heavy metal stress damage to some degree. It can be speculated that such high glutathione levels by over-expression of AtGCS overcoming the inhibition feedback by GSH, resulted in the higher accumulation of heavy metal ions in *E. coli* cells. These results coincided with the previous study that GSH was important for residual metal resistance in *E. coli* (Helbig *et al.*, 2008).

CONCLUSION

The applications of genetic engineering in the modification of the microorganisms have become a promising way for increasing the efficiency of the

bioremediation processes for heavy metals removal (Kim *et al.*, 2005). GSH, an important antioxidant involved in the heavy metal stress response, is synthesized by the consecutive actions of γ-glutamylcysteine synthetase and glutathione synthetase (Anderson, 1998). Because the activity of the former is feedback regulated by GSH, it partially restores impaired growth of *E. coli*. Nevertheless, by over-production of γ-GCS, there might be no feedback inhibition of GSH. Thus, we try to analyze the relative importance of over-expression of AtGCS in enhancement of *E. coli* under heavy metal stressful conditions. In TrxA-AtGCS-transformed cells, production of recombinant protein allowed *E. coli* to survive under Cd^{2+}, Zn^{2+} and Cu^{2+} more consistently, which was lethal to cells expressing TrxA. This improved resistance of *E. coli* cells to heavy metal stresses was relative to more efficient bioaccumulation of Cd^{2+}, Zn^{2+} and Cu^{2+} and higher levels of glutathione content. It could be concluded that AtGCS played an essential role in protecting *E. coli* cells against the toxic effects of Cd^{2+}, Zn^{2+} and Cu^{2+}.

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