Heterologous Expression of a Carrot Small Heat Shock Protein Increased *Escherichia coli* Viability under Lead and Arsenic Stresses

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Abstract. The expression and function of DcHsp17.7, a small heat shock protein (sHSP), in carrot (*Daucus carota* L.) was examined under lead [Pb(II)] and arsenic (arsenate) stresses. In a time course experiment, the level of DcHsp17.7 increased in carrot leaf tissue treated with lead ions or arsenate. To examine the function of DcHsp17.7, the DcHsp17.7 gene was cloned and introduced into *Escherichia coli*. Heterologous expression of DcHsp17.7 was confirmed by immunoblot analysis using a polyclonal antibody raised against DcHsp17.7. Lead ion and arsenate reduced bacterial cell viability. However, transgenic *E. coli* with accumulated DcHsp17.7 showed higher levels of survival under both lead ion and arsenate conditions compared with the vector control. Immunoblot analysis showed that the level of heterologously expressed DcHsp17.7 decreased under lead ion conditions, but remained the same under arsenate conditions. Our results suggest that DcHsp17.7 can confer tolerances to lead and arsenic stresses.

Heat shock proteins are a group of proteins expressed under heat and other abiotic stresses (reviewed in Lindquist and Craig, 1988). They are classified into five different families, HSP100, HSP90, HSP70, HSP60, and sHSP (12 to 42 kDa), based on their molecular masses. sHSPs are unusually abundant and diverse in plants (19 in *Arabidopsis thaliana*; Scharf et al., 2001; 23 in *Oryza sativa*; Waters et al., 2008; 36 in *Populus trichocarpa*; Waters et al., 2008), compared with other organisms (four in *Drosophila melanogaster*, 10 in *Homo sapiens*, 16 in *Caenorhabditis elegans*; reviewed in Haslbeck et al., 2005), suggesting that sHSPs may confer enhanced protection to abiotic stresses in sessile plants. sHSPs are divided into 11 subfamilies based on their cellular localization and sequence homology (Waters, 2013). There are six subfamilies in the cytoplasm/nucleus (CI to CVI), two in the mitochondria (MTI and MTII), and one each in the endoplasmic reticulum, peroxisome, and chloroplast. A number of studies have reported that sHSPs function as molecular chaperones by preventing protein denaturation and/or

restoring folding of partially denatured proteins under abiotic stresses (reviewed in Sun et al., 2002).

Our previous studies have reported that DcHsp17.7, a cytosolic (CI) sHSP in carrot (*Daucus carota* L.), is expressed under heat (Ahn et al., 2004), cold (Song and Ahn, 2010), salinity (Song and Ahn, 2011) as well as osmotic and oxidation (Ahn and Song, 2012) stresses. Transgenic carrot (Malik et al., 1999) and potato (*Solanum tuberosum* L.; Ahn and Zimmerman, 2006) plants overexpressing DcHsp17.7 showed increased thermotolerance. When heterologously expressed in *E. coli*, DcHsp17.7 increased survival rates in a transgenic cell line, compared with vector control, under various abiotic stresses (Ahn and Song, 2012; Kim and Ahn, 2009; Song and Ahn, 2010, 2011), functioning as a molecular chaperone. Our results suggested that DcHsp17.7 could confer tolerances to not only heat, but also multiple abiotic stresses.

In this study, we examined the expression and function of DcHsp17.7 under heavy metal (lead ion) and metalloid (arsenate) stresses. Heavy metal and metalloid contamination poses a serious health risk to all living organisms, including humans and plants. Lead ion enters the food chain through living organisms such as microorganisms, insects, and plants taking up lead from various sources including residues from mining of metalliferous ores, burning of leaded gasoline, and municipal sewage (Gisbert et al., 2003). Within the cell, lead ions can replace essential ions and thus disrupt redox balance (Wang et al., 2011). In plants, lead ion inhibits a number of metabolic processes, including mineral acquisition and photosynthesis (Brunet et al.,

2009). Arsenate compounds have been widely used in agriculture such as pesticides for insects, fungi, and rodents and wood preservatives (Murcott, 2012). The level of arsenic is rapidly increasing in groundwater and soils (Goswami et al., 2010). Inorganic forms of arsenic, i.e., arsenate and arsenite, are more toxic than the organic forms (Chakrabarty et al., 2009). However, the mechanism(s) of its uptake and translocation is largely unknown in plants.

The production of stress-tolerant proteins is one of the mechanisms that plants and other living organisms have developed to withstand heavy metal contamination. Phytochelatins and methallothioneins are the most well-known proteins that bind to and detoxify heavy metals in living organisms (Cobbett and Goldsbrough, 2002). HSPs have also been reported to accumulate under heavy metal conditions, such as lead (HSP70 and HSP60, *Solanum lycopersicum*, Wang et al., 2008; *Spinacia oleracea*, Wang et al., 2011) and arsenic (HSP70 in *Oryza sativa*, Goswami et al., 2010; HSP21 in *Crambe abyssinica*, Paulose et al., 2010; HSP23 in *Nicotiana tabacum*, Lee et al., 2012). These results suggest that HSPs may be involved in heavy metal stress tolerance in plants.

Materials and Methods

Plant materials and heavy metal treatment.

Carrot seeds (*Daucus carota* L. cv. Mussangochon) were sown in a commercial soil mix (Ssakanara; Minong, Seoul, Korea) and grown in a controlled environmental chamber (18 to 21 °C, 14-h photoperiod) with light supplied by a fluorescent lamp at an intensity of 200 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and a relative humidity of 60%. To examine the expression pattern of DcHsp17.7 under lead and arsenic conditions, leaf tissues detached from 2- to 3-month-old carrot plants were incubated in an 125-mL Erlenmeyer flask containing lead [lead(II) nitrate, $\text{Pb}(\text{NO}_3)_2$; Sigma-Aldrich, St. Louis, MO] or arsenic (sodium arsenate dibasic heptahydrate, $\text{Na}_2\text{HAsO}_4\cdot 7\text{H}_2\text{O}$; Sigma-Aldrich) solutions (at 1 mM up to 7 h; Lee et al., 2012; Yi et al., 2006). Samples were taken immediately after each time point, frozen in liquid nitrogen, and stored at $-80\text{ }^\circ\text{C}$ until analysis.

Protein extraction from carrot leaf tissue and immunoblot analysis. Protein extraction from carrot tissue, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblot analysis using a polyclonal antibody raised against DcHsp17.7 were performed as previously described (Ahn et al., 2004). Briefly, carrot leaf tissue (0.5 g) was homogenized in extraction buffer I (1 mL; 0.3% SDS, 200 mM dithiothreitol, 28 mM Tris-HCl, and 22 mM Tris-base). After incubation at 100 °C for 5 min, the lysate was mixed with extraction buffer II (100 μL ; 24 mM Tris-base, 476 mM Tris-HCl, 50 mM MgCl_2 , 1 $\text{mg}\cdot\text{mL}^{-1}$ DNase I, and 0.25 $\text{mg}\cdot\text{mL}^{-1}$ RNase A) followed by addition of trichloroacetic acid (50%) to precipitate the proteins. The protein pellet was washed with acetone (95%) three times and dissolved in extraction buffer I. The

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concentration of the protein sample was quantified using the Bradford assay (Bradford, 1976). Proteins (50 µg) from each sample were resolved on a SDS-PAGE gel (17%) and electroblotted to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA) followed by immunoblot analysis using a polyclonal antibody raised against DcHsp17.7, according to the instructions of the ECL Plus system (GE Healthcare Life Science, Buckinghamshire, U.K.). Chemiluminescent signals were detected on Hyperfilm (GE Healthcare Life Science) and quantified using a densitometer (IMAGER; Bioneer, Seoul, Korea). Experiments were performed three times with two to three leaves per condition.

DcHsp17.7 gene cloning in expression vector. The insert gene was generated by the polymerase chain reaction (one cycle at 94 °C for 5 min followed by 35 cycles of 94 °C for 40 s, 68 °C for 50 s, and 72 °C for 50 s) using a pair of DcHsp17.7 gene-specific (National Center for Biotechnology Information accession number X53851) primers (forward: 5'-GGGGGCATATGTCGATCATTCCAAGC-3', reverse: 5'-GGGGGGGCTAGCTTAACCAAGAAATATCAATGGC-3') and introduced into the Novagen pET11a expression vector (Merck KGaA, Darmstadt, Germany) at the *NdeI* and *NheI* restriction enzyme sites (underlined). The recombinant pET11a-DcHsp17.7 expression vector was introduced into *E. coli* (DH 5α; Enzymonics, Daejeon, Korea) through heat shock for 1 min at 42 °C (Kim and Ahn, 2009). After overnight incubation on the selection medium [solid Luria-Bertani medium (LB; BD Difco, Franklin Lakes, NJ) containing 100 µg·mL⁻¹ ampicillin], a positive colony was selected, and the sequence of the recombinant plasmid was confirmed. The recombinant pET11a-DcHsp17.7 plasmid was then transformed into the BL21 (DE3) cell line (Enzymonics) for gene expression.

Heterologous gene expression in *E. coli* and immunoblot analysis. Transgenic *E. coli* containing the recombinant pET11a-DcHsp17.7 plasmid and vector control containing the unmodified pET11a expression vector were cultured in LB (BD Difco) containing 100 µg·mL⁻¹ ampicillin overnight at 37 °C. Bacterial cell cultures were then diluted 1:1000 and continuously cultured. When the growth of bacterial cell cultures reached 0.6 at an OD₆₀₀, isopropylthio-β-galactoside (IPTG) was then added to a final concentration of 1 mM to express the heterologous DcHsp17.7 gene. After 2 h, bacterial cultures (50 mL) were centrifuged (2830 g at 4 °C for 20 min), and the resulting bacterial pellet was resuspended in extraction buffer (4 mL; 25 mM Tris-HCl pH 7.5, 300 mM NaCl, and 3 mM β-mercaptoethanol). Bacterial proteins were extracted by disrupting cells using ultrasonication (420 W, 20 KHz for a total of 4 min and 40 s; repeat of sonication for 10 s and pause for 30 s) using Sonomasher (S & T Science, Seoul, Korea). The lysate was centrifuged (20,900 g at 4 °C for 1 h), and the supernatant was removed. The bacterial protein concentration was measured using the Bradford assay (Bradford, 1976). To confirm heterologous accumulation of DcHsp17.7 in transgenic *E. coli*, bacterial proteins (30 µg per lane) were subjected to SDS-PAGE (17%) and immunoblot analysis as described previously.

Cell viability of *E. coli* under lead and arsenic stresses. Overnight *E. coli* cell culture, cell dilution, and additional cell culture were performed as described previously. After 2 h of IPTG treatment, transgenic cell lines accumulating DcHsp17.7 and vector control cell lines containing the unmodified pET11a vector were exposed to lead ion (up to 7 mM for 1 h) or arsenate (up to 50 mM for 2 h) followed by serial dilution of 1:10⁶.

Bacterial cells were then spread on solid LB plates containing 100 µg·mL⁻¹ ampicillin. After overnight incubation at 37 °C, the number of surviving colonies was counted, and the percentage of cell viability was calculated. The experiment was independently repeated at least three times (five plates per condition). A paired *t* test was performed to compare the difference in the level of cell viability between transgenic *E. coli* accumulating DcHsp17.7 and the vector control cell line containing the unmodified pET11a expression vector (***P* < 0.01).

Immunoblot analysis of heterologously expressed DcHsp17.7 under lead and arsenic stresses. Overnight cell culture, IPTG treatment, bacterial protein extraction, and protein quantification were performed as described previously. Heavy metals (lead ion up to 20 mM or arsenate up to 500 mM) were added to 2 mg bacterial proteins in 600 µL extraction buffer (25 mM Tris-HCl pH 7.5, 300 mM NaCl, and 3 mM β-mercaptoethanol) and incubated at 25 °C for 1 h or 2 h for lead ion and arsenate, respectively. Protein samples were then centrifuged (20,900 g at 4 °C for 1 h) to precipitate denatured proteins. The supernatant (22 µL per sample) was subjected to SDS-PAGE (17%) and immunoblot analysis using a polyclonal antibody raised against DcHsp17.7, as described previously. The relative levels of DcHsp17.7 signals were quantified using a densitometer (IMAGER). Experiments were performed three times with two samples per condition and the most representative images are shown.

Results and Discussion

DcHsp17.7 accumulated in carrot leaves under lead and arsenic conditions. A time-course immunoblot analysis was performed to examine the accumulation pattern of

Fig. 1. Accumulation pattern of DcHsp17.7 in carrot leaf tissues under lead and arsenic conditions. Carrot (*Daucus carota* L.) leaf tissues were exposed to lead ion or arsenate (1 mM in distilled water) for up to 7 h. Proteins were extracted under denaturing conditions, and an equal amount (50 µg protein per sample) was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis using a polyclonal antibody raised against DcHsp17.7. The size of the signal was ≈18 kDa.

DcHsp17.7 under lead and arsenic conditions. In non-stressed leaf tissue, DcHsp17.7 was not present or present at a low level (Fig. 1), as shown in our previous study (Ahn et al., 2004). On exposure to lead ion and arsenate, the level of DcHsp17.7 gradually increased (≈ 3 -fold). In arsenate-treated leaf tissue, the level of DcHsp17.7 decreased at a later time point (7 h). The expression of DcHsp17.7 suggests that the protein could be involved in lead and arsenic stress tolerance in plant cells.

Heterologously expressed DcHsp17.7 enhanced bacterial cell viability under lead and arsenic conditions. To examine if DcHsp17.7 can confer tolerance to lead and arsenic stresses, the *DcHsp17.7* gene was introduced into *E. coli*, which is

Fig. 2. Heterologous accumulation of DcHsp17.7 in transgenic *E. coli*. The coding sequence of the *DcHsp17.7* gene was inserted into the pET11a expression vector. The recombinant plasmid was then introduced into *E. coli* and heterologous expression of DcHsp17.7 was induced by isopropylthio- β -galactoside (IPTG) treatment. Bacterial proteins from the transgenic *E. coli* containing the recombinant pET11a-DcHsp17.7 expression vector and from the vector control cell line containing the unmodified pET11a vector were extracted using ultra-sonication. An equal amount (30 μ g per lane) of protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis using a polyclonal antibody raised against DcHsp17.7. The size of the signal was ≈ 18 kDa.

a convenient model to study protein functions. Studies have shown that some HSPs, whose heterologous expression increased cell viability in *E. coli* under abiotic stress conditions, also conferred tolerances to the stresses in overexpression transgenic plants. For example, Jiang et al. (2009) reported that a cytosolic CI sHSP, RcHsp17.8, in *Rosa chinensis* conferred tolerances to various abiotic stresses in *E. coli*, yeast, and *Arabidopsis*. More recently, Lee et al. (2012) reported that transgenic tobacco plants (*Nicotiana tabacum*) overexpressing alfalfa mitochondrial Hsp23 and transgenic *E. coli* heterologously expressing the protein showed increased tolerance to salinity and arsenate stresses. On IPTG treatment, DcHsp17.7 protein accumulated in the transgenic *E. coli* containing the pET11a-DcHsp17.7 recombinant vector (Fig. 2). Without IPTG treatment, the transgenic cell line did not produce the protein, suggesting that the heterologous expression of DcHsp17.7 was tightly controlled in *E. coli*. A control cell line containing the unmodified pET11a vector did not produce a signal regardless of IPTG treatment.

In our previous study, heterologously expressed DcHsp17.7 increased bacterial cell viability under a number of abiotic stresses (Ahn and Song, 2012; Kim and Ahn, 2009; Song and Ahn, 2010, 2011), suggesting that DcHsp17.7 may confer tolerance to multiple abiotic stresses. In this study, we examined the changes in the bacterial cell viability in the presence of heterologously expressed DcHsp17.7 under lead and arsenic stresses. Under the stresses, bacterial cell viability decreased in a concentration-dependent manner (Fig. 3). On a molar basis, lead ion reduced bacterial cell survival rates more than arsenate. However, the transgenic *E. coli*

expressing DcHsp17.7 showed a higher level of cell viability (by $\approx 10\%$ to 30%) under the both stress conditions compared with the vector control cells.

HSPs are molecular chaperones, which perform a number of activities such as protein folding/unfolding, assembly/disassembly, translocation, and degradation (Waters, 2013). Under stress conditions, HSPs detect unusual hydrophobic surfaces of denatured proteins and bind them either to correct the folding or to prevent from further degradation. Heavy metals reduce cellular proteins breaking disulfide bonds and some metals are reported to interact with hydroxyl and carboxyl groups (Sahr et al., 2005), destabilizing the normal structure and function of proteins. It is possible that DcHsp17.7 interacts with a broad range of bacterial proteins disrupted by lead ion or arsenate and performs molecular chaperone activity, resulting in increased cell viability.

Heterologously expressed DcHsp17.7 was more stable under arsenic than under lead stress. The level of heterologously expressed DcHsp17.7 in transgenic *E. coli* was examined under lead ion and arsenate stresses. Immunoblot analysis showed that the level of heterologously expressed DcHsp17.7 significantly decreased as the concentration of lead ion increased (Fig. 4A), although it remained unchanged under arsenate conditions (up to 500 μ M; Fig. 4B). Considering the fact that the bacterial cell viability decreased to less than 20% at 50 mM arsenate (Fig. 3B), it is noteworthy that the heterologously expressed DcHsp17.7 was very stable up to 500 μ M arsenate.

This study showed that heterologously expressed DcHsp17.7 can confer resistance to lead and arsenic stresses in transgenic *E. coli*. We have previously reported that

Fig. 3. Bacterial cell viability under lead and arsenic conditions. Bacterial cell culture was performed as described in “Materials and Methods.” After 2 h of isopropylthio- β -galactoside (IPTG) treatment, bacterial cell cultures were exposed to lead ion (up to 7 mM for 1 h) or arsenate (up to 50 mM for 2 h). After a serial dilution of $1:10^6$, bacterial cells were plated on solid LB medium containing ampicillin and incubated overnight. The number of surviving colonies was then counted, and the percentage of cell viability, compared with non-stressed control, was calculated. The error bars show the SEM. A paired *t* test was performed to compare the differences in cell viability between the transgenic *E. coli* accumulating DcHsp17.7 and the vector control cell line containing the unmodified pET11a expression vector (***P* < 0.01).

Fig. 4. Stability of heterologously accumulated DcHsp17.7 in *E. coli* under lead and arsenic conditions. Bacterial cell culture was performed as described in “Materials and Methods.” After 2 h of isopropylthio- β -galactoside (IPTG) treatment, bacterial proteins were extracted and exposed to lead ion (up to 20 mM for 1 h) or arsenate (up to 500 mM 2 h). Protein samples were then centrifuged and the supernatant containing soluble proteins was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblot analysis using a polyclonal antibody raised against DcHsp17.7. The molecular weight of the band was $\approx$ 18 kDa.

overexpression of DcHsp17.7 increased thermotolerance in transgenic carrot and potato plants (Ahn and Zimmerman, 2006; Malik et al., 1999). Heterologously expressed DcHsp17.7 also conferred tolerances to various abiotic stresses in transgenic *E. coli* as described previously. These results suggest that DcHsp17.7 may confer tolerances to multiple abiotic stresses, which are present in the actual field conditions. DcHsp17.7 could be useful for plant genetic engineering for multiple stress tolerance.

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