Small Heat Shock Proteins Can Confer Tolerance to Nanomaterial-induced Toxicity

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Abstract. The expression of a small heat shock protein (sHSP) in plants and its possible function in conditions related to nanomaterial exposure were examined. Multiwalled carbon nanotubes (MWCNTs) and silver nanoparticles (AgNPs) induced toxicity that was indicated by the bending and curling of carrot leaf tissues. Both nanomaterials induced the expression of a small heat shock protein in carrot, DcHsp17.7, but reduced the level of a constitutive heat shock cognate 70. To examine the possible function of DcHsp17.7, the coding gene was heterologously expressed in *Escherichia coli*. Both nanomaterials reduced the viability of *E. coli* cell lines. However, the transgenic cell line heterologously expressing DcHsp17.7 showed higher levels of cell viability, compared with vector controls, when exposed to MWCNTs and, more notably, to AgNPs. To the best of our knowledge, this is the first study reporting the influence of nanomaterials on the expression of a plant sHSP and its possible function in conferring tolerance to nanomaterial stress.

Engineered nanomaterials with at least one dimension between 1 and 100 nm are classified into two categories: fullerenes that include single-walled and multiwalled carbon nanotubes and inorganic nanoparticles that have two dimensions between 1 and 100 nm (Rico et al., 2011). They are widely used in more than 500 consumer products in industries such as food, communication, medicine, cosmetics, and agriculture and are estimated in the world market to reach \$1 trillion in 2015 (U.S. Environmental Protection Agency, 2014). In agriculture, nanomaterials are used in the form of fertilizers, herbicides, and pesticides for efficient delivery to promote plant growth (Pérez-de-Luque and Rubiales, 2009). As a result of their small sizes, nanomaterials easily pass through cellular membrane, translocate to various intracellular compartments, and associate with various biomolecules and cellular structures (reviewed in Nair et al., 2010).

Despite some advantages of using nanomaterials, there is an increasing number of recent studies reporting the possible cytotoxic and genotoxicities of nanomaterials in

living organisms. In humans, nanomaterials can cause toxicity in various organs such as lung, dermis, and liver (reviewed in Donaldson and Poland, 2012). They trigger oxidative stress, proinflammatory responses (Prasad et al., 2013), endoplasmic stress response, and apoptosis (Bouwmeester et al., 2011). Recent studies have reported that the accumulation of certain nanomaterials could also cause toxic effects on plants. For example, single-walled carbon nanotubes induced the accumulation of H₂O₂ and the expression of genes related to oxidative stress such as ascorbate peroxidase 1 and mitochondrial superoxide dismutase 1 (Shen et al., 2010). At the gene level, CuO in the form of nanoparticles caused mutagenic DNA lesions in radish (*Raphanus sativus* L.) and ryegrass (*Lolium perenne* L. and *Lolium rigidum* L.; Atha et al., 2011). The conflicting results on the effects of nanomaterials appear to be because of the experimental conditions used in the studies such as the characteristics of the nanomaterials (type, size, and concentration) and the plants (species and growth stages).

Heat shock proteins (HSPs) are a group of proteins that accumulate under heat and other various abiotic stresses. Small HSPs (12 to 42 kDa in size) are unusually diverse in plants compared with other organisms. There are more than 30 sHSPs in plants (36 sHSPs in *Populus trichocarpa*; Waters et al., 2008), which is the largest number in living organisms, suggesting that sHSPs probably play important roles in sessile plants. The model protein DcHsp17.7, a sHSP in carrot (*Daucus carota* L.), accumulates under various stress conditions such as heat (Malik et al., 1999), cold (Song and Ahn, 2010), salinity (Song and Ahn, 2011), oxidation, water shortage (Ahn and Song, 2012), and heavy metal stresses (Lee

and Ahn, 2013). Furthermore, the transgenic *E. coli* heterologously expressing DcHsp17.7 showed a higher rate of survival compared with that of vector controls under these stresses, suggesting that DcHsp17.7 can confer tolerance to various abiotic stresses.

Carbon nanotubes are used to enhance germination of recalcitrant seeds. However, Tan and Fugetsu (2007) reported that MWCNTs caused aggregation of rice cell cultures. Furthermore, the nanomaterials also induced the expression of reactive oxygen species resulting in complete cell death in rice (Tan et al., 2009). Zucchini plants exposed to MWCNTs and AgNPs showed reduced biomass accumulation by 60% and 75%, respectively (Stampoulis et al., 2009). AgNPs possess broad-spectrum antibacterial and antifungal properties, thus protecting plants from various diseases (Panáček et al., 2009). However, Kumari et al. (2009) observed chromatin bridge, stickiness, and a disrupted metaphase resulting in abnormal cell division in *Allium cepa* L. treated with AgNPs. Panda et al. (2011) also reported that AgNPs induced cell death and DNA damage in *A. cepa* L. Despite the possible phytotoxicity caused by nanomaterials, the changes in gene expression induced by nanomaterials and the tolerance mechanism in plants against nanomaterials are not completely understood. In this study, we examined the expression of DcHsp17.7 induced by nanomaterials such as MWCNTs and AgNPs. Furthermore, the possible function of DcHsp17.7 in conferring tolerance to nanotoxicity was also examined using a transgenic *E. coli* heterologously expressing DcHsp17.7. To the best of our knowledge, this is the first study reporting nanomaterial-induced accumulation of plant sHSP and its possible function in conferring tolerance to nanotoxicity.

Materials and Methods

Dispersion of nanomaterials. MWCNTs (6 to 9 nm × 5 μm; diameter = 6.6 nm median) and AgNPs (diameter of less than 100 nm) from Sigma-aldrich (St. Louis, MO) were added to double-distilled water (200 mL for 0.2 g) containing Tween 20 (0.05%); subsequently, ultrasonication (420 W, 20 KHz, for a total of 30 min; repeats of 10 s ultrasonication and 30-s pause) was performed using a Sonomasher (S & T Science, Seoul, Korea). Stock solutions were diluted to the final concentration of 200 μg·mL⁻¹. After 5 h, the appearance of suspension was documented by photographs.

Acute toxicity of nanomaterials on carrot leaf tissue. Carrot plants (*Daucus carota* L. cv. Mussangochon) were grown in a controlled environmental chamber (19 to 21 °C, 8 to 16 h, night–day) with light from a fluorescent lamp at an intensity of 200 μE·m⁻²·s⁻¹ and a relative humidity of 60%. Leaf tissues from 3- to 2-month-old carrot plants were placed in MWCNT (up to 200 μg·mL⁻¹ with 0.01% Tween 20; Khodakovskaya et al., 2011) and AgNP solutions (up to 200 μg·mL⁻¹ with 0.01% Tween 20; Stampoulis

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Fig. 1. Dispersion of nanomaterials by Tween 20. (A) Multiwalled carbon nanotubes (MWCNTs) and (B) silver nanoparticles (AgNPs; 0.2 g each) were added to double-distilled water (200 mL) containing 0.05% Tween 20 and subjected to ultrasonication for 30 min. Resulting solutions were diluted with double-distilled water to specified concentrations in 200 mL. After 5 h, appearance of suspension was documented by photographs.

Fig. 2. Acute toxicity of nanomaterials on carrot leaf tissues. (A) Multiwalled carbon nanotubes (MWCNTs) and (B) silver nanoparticles (AgNPs) stock solutions (0.2 g in 200 mL double-distilled water containing 0.05% Tween 20) were prepared, as described previously. After dilution to specified concentrations with double-distilled water, leaf tissues from 2- to 3-month-old carrot plants were placed in the solution for 5 h, and photographs were taken from the side.

et al., 2009) for 5 h and photographs were taken from the side. In addition, carrot leaf tissues were also placed in activated carbon and Ag powder (up to 200 µg·mL⁻¹), the corresponding controls for MWCNTs and AgNPs, for 5 h to examine if these non-nanosized controls cause acute toxicity.

Immunodetection of DcHsp17.7 accumulation induced by nanomaterials. Carrot leaf

tissues treated with MWCNTs and AgNPs (up to 200 µg·mL⁻¹ with 0.01% Tween 20 for 5 h) as described previously were immediately frozen in liquid nitrogen and stored at -80 °C until analysis. To examine the accumulation of DcHsp17.7 induced by MWCNTs and AgNPs, proteins were extracted from carrot tissues, quantified by performing the Bradford assay (Bradford, 1976), and resolved

by performing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 17%) followed by immunoblot analysis using a polyclonal antibody raised against DcHsp17.7, as previously described (Ahn et al., 2004). Levels of a constitutive protein, which reacted to a polyclonal antibody raised against a recombinant heat shock cognate 70 in spinach (Enzo Life Sciences, Farmingdale, NY), were also examined under the same conditions. The sizes of the protein bands were ≈18 and 70 kDa for the two antibodies, respectively. Coomassie staining showed the entire protein profile in the presence of nanomaterials at equal protein loading. Experiments were performed three times with two to three leaves per condition and representative images are shown.

Generation of transgenic *E. coli* heterologously expressing DcHsp17.7. The DcHsp17.7 gene was inserted into pET11a vector using a previously described method (Kim and Ahn, 2009). Transformed *E. coli* BL21 (DE3) containing pET11a-DcHsp17.7 recombinant vector and vector control containing the unmodified pET11a expression vector were cultured overnight, diluted to 1:1000 with fresh Luria Broth (LB) medium containing ampicillin, and continuously incubated until the O.D. at 600 nm reached 0.6. Then, isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM to induce heterologous expression of DcHsp17.7. After 2 h of incubation, MWCNT and AgNP stock solutions (1 g·L⁻¹ with 0.05% Tween 20), prepared as described previously, were added to the final concentration of 200 µg·mL⁻¹. After incubation for 5 h, bacterial proteins were extracted using ultrasonication (420 W, 20 Khz for a total of 4 min and 40 s; repeats of 10 s ultrasonication and 30 s pause; Lee and Ahn, 2013) and quantified by performing the Bradford assay (Bradford, 1976). Bacterial proteins (30 µg per lane) were subjected to SDS-PAGE (17%) and immunoblot analysis using a polyclonal antibody raised against DcHsp17.7, as per a previously described method (Song and Ahn, 2011).

Bacterial cell viability in the presence of surfactants and nanomaterials. Overnight bacterial cell culture, dilution, and IPTG treatment (2 h) were performed for the transgenic cell line heterologously expressing DcHsp17.7 and vector control containing the unmodified pET11a expression vector, as described previously. To examine possible toxicity of surfactant, the two bacterial cell lines were cultured in 0.01% Tween 20 (El-Temsah and Joner, 2010) and SDS (Stampoulis et al., 2009). The stock solutions of activated carbon, Ag powder, MWCNTs, and AgNPs (0.1 g in 200 mL double-distilled water with 0.05% Tween 20) were prepared using ultrasonication and added to the two bacterial cell lines to the final concentration of 100 µg·mL⁻¹. Then, all the cell lines were cultured for an additional 5 h, diluted to 1:10⁻⁶, and plated on solid LB plates containing ampicillin. After an overnight incubation, the number of surviving colonies was counted and the rates of bacterial cell viability were

Fig. 3. Expression of DcHsp17.7 by nanomaterials. Carrot leaf tissues treated with (A) multiwalled carbon nanotubes (MWCNTs) and (B) silver nanoparticles (AgNPs; up to 200 µg/mL for 5 h) were prepared as described previously. Then, proteins were extracted, quantified, and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 17%) and immunoblot analysis using either a polyclonal antibody raised against DcHsp17.7 or a polyclonal antibody raised against spinach heat shock cognate (HSC) 70. The size of the bands was ≈18 and 70 kDa, respectively. Coomassie staining indicated equal loading of protein and overall protein profile in the presence of both nanomaterials.

Fig. 4. Heterologous expression of DcHsp17.7 in *Escherichia coli*. The DcHsp17.7 gene was inserted into pET11a expression vector and introduced into *E. coli* BL21 (DE3). Overnight cell culture was performed, as described in the “Materials and Methods” section. After 2 h of isopropyl β-D-1-thiogalactopyranoside (IPTG) treatment, multiwalled carbon nanotube (MWCNT) and silver nanoparticle (AgNP) stock solutions (0.2 g in 200 mL double-distilled water containing 0.05% Tween 20) were added to the final concentration of 200 µg·mL⁻¹, and bacterial cells were continuously cultured for 5 h. Protein was extracted by performing ultrasonication, and 30 µg of protein was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 17%) followed by immunoblot analysis using a polyclonal antibody raised against DcHsp17.7. The size of the band was ≈18 kDa. VC = vector control containing the unmodified pET11a expression vector.

calculated. A paired *t* test was performed to compare the differences in cell viability between the transgenic cell line heterologously expressing DcHsp17.7 and the vector control containing the unmodified pET11a expression vector.

Results and Discussion

Dispersion of nanomaterials. When preparing test solutions containing nanomaterials, it is important to maintain them well suspended throughout the time course. In this study, Tween 20 (El-Temsah and Joner, 2010) was added to the test solutions containing MWCNTs and AgNPs, and ultrasonication was performed. Our results showed that Tween 20 effectively prevented the aggregation of the two nanomaterials (Fig. 1). In the absence of Tween 20, both the nanomaterials floated up on the surface of the test solutions at all the tested concentrations. Based on this observation, Tween 20 was used to prepare the test solutions containing MWCNTs and AgNPs in this study.

Acute toxicity of nanomaterials on carrot leaf tissue. To examine if the expression of DcHsp17.7 is induced by nanomaterials, carrot leaf tissues were placed in MWCNT and AgNP solutions (up to 200 µg·mL⁻¹). After 5 h, all the tested leaves wilted and hung down, more severely in the presence of MWCNTs than that in the presence of AgNPs (Fig. 2), suggesting that nanomaterials can be toxic to plants. Adverse effects of nanomaterials on plants include reduction in germination, growth (seedlings, roots, and shoots), and biomass accumulation (reviewed in Rico et al., 2011). In addition, activated carbon and Ag powders, the corresponding controls for MWCNTs and AgNPs, did not cause any morphological changes at the same concentrations (data not shown).

Fig. 5. Effects of surfactant on bacterial cell viability. Overnight cell culture was performed as described in the “Materials and Methods” section. After 2 h of isopropyl β-D-1-thiogalactopyranoside (IPTG) treatment, (A) Tween 20 and (B) sodium dodecyl sulfate (SDS) were added up to the final concentration of 0.01% and cultured at 37 °C for 5 h with shaking. Then, the cells were diluted to 1:10⁶ and plated on solid Luria Broth (LB) plates containing ampicillin. After overnight incubation, the number of surviving colonies was counted, and the rates of cell viability were calculated. A paired *t* test was performed to compare the differences in cell viability between the transgenic *Escherichia coli* heterologously expressing DcHsp17.7 and the vector control containing the unmodified pET11a expression vector (**P* < 0.05; ***P* < 0.01). Black bars = vector control; gray bars = transgenic *E. coli* heterologously expressing DcHsp17.7.

A. Activated carbon

B. Ag powder

C. MWCNTs

D. AgNPs

Fig. 6. Enhanced tolerance of cells heterologously expressing DcHsp17.7 to nanomaterials. Overnight cell culture was performed as described in the “Materials and Methods” section. After 2 h of isopropyl β -D-1-thiogalactopyranoside (IPTG) treatment, (A) activated carbon, (B) silver (Ag) powder, (C) multiwalled carbon nanotube (MWCNT), and (D) silver nanoparticle (AgNP) stock solutions were added to the final concentration of $100 \mu\text{g}\cdot\text{mL}^{-1}$. After 5 h incubation with shaking, the cells were diluted to $1:10^{-6}$ and plated on a solid Luria Broth (LB) medium containing ampicillin. The number of surviving colonies was counted after overnight incubation at 37°C , and the rates of survival were calculated. A paired t test was performed to compare the differences in cell viability between the transgenic *Escherichia coli* heterologously expressing DcHsp17.7 and vector controls containing the unmodified pET11a expression vector (* $P < 0.05$; ** $P < 0.01$). Black bars = vector control; gray bars = transgenic *E. coli* expressing DcHsp17.7.

Expression of DcHsp17.7 by MWCNTs and AgNPs. HSPs accumulate under various abiotic stresses such as heat, cold, salinity, drought, oxidation, and heavy metals (reviewed in Wang et al., 2004). They primarily function as molecular chaperones that prevent cellular proteins from denaturing and/or promote refolding of partially denatured proteins, resulting in increased cell viability under stress conditions. In this study, we examined the expression of DcHsp17.7, a small HSP in carrot, after treatment with MWCNTs and AgNPs. In the absence of stress, the protein was not expressed in the leaf tissue (Fig. 3). However, the nanomaterials triggered the expression of DcHsp17.7. In the presence of MWCNTs, the level of DcHsp17.7 increased and then decreased as the concentration of

MWCNTs increased (Fig. 3A). The level of the protein continuously increased in the presence of AgNPs (Fig. 3B). On the other hand, the level of heat shock cognate (HSC) 70, a cytosolic protein that is constitutively and ubiquitously expressed in most cells, gradually decreased as the concentration of MWCNT increased. In the presence of AgNPs, HSC70 rapidly disappeared at $100 \mu\text{g}\cdot\text{mL}^{-1}$ and above, suggesting that nanomaterials such as MWCNTs and AgNPs can inhibit the expression of housekeeping genes. Coomassie staining indicated that MWCNTs and AgNPs did not significantly affect the overall protein profile in carrot leaf tissue.

There are only a couple of studies reporting nanomaterial-induced HSP gene expression in plants. Cerium oxide (CeO_2) nanoparticles

enhanced the accumulation of HSP70 in the roots of *Zea mays* L. (Zhao et al., 2012). At the gene level, Khodakovskaya et al. (2011) reported that MWCNTs induced the upregulation of stress-related genes in tomato (*Solanum lycopersicum* L.), including HSP90. These results indicate that some nanomaterials can trigger the expression of stress-related genes such as HSP genes. To the best of our knowledge, this is the first study reporting the nanomaterial-induced expression of a plant sHSP. Unlike large-molecular-weight HSPs, which require adenosine-5'-triphosphate (ATP) for their functions, the function of sHSPs is either unaffected or inhibited by ATP (Smykal et al., 2000). Considering the fact that the level of ATP decreases under stress conditions, leaving large molecular HSPs less active,

the function of sHSPs is likely to be very important, especially under prolonged and/or severe stress conditions.

Heterologous expression of DcHsp17.7. To examine the function of DcHsp17.7 under conditions related to nanomaterial exposure, the coding region of the *DcHsp17.7* gene was introduced into *E. coli* (BL21). Immunoblot analysis using a polyclonal antibody raised against DcHsp17.7 indicated that IPTG successfully induced the heterologous expression of DcHsp17.7 (Fig. 4). The level of the protein remained unchanged when exposed to MWCNTs and AgNPs. In our previous studies, the level of heterologously expressed DcHsp17.7 decreased under conditions of cold (Song and Ahn, 2010), salinity (Song and Ahn, 2011), oxidation (Ahn and Song, 2012), and in the presence of lead (Lee and Ahn, 2013). On the other hand, the level did not change during water shortage (Ahn and Song, 2012) and in the presence of arsenate (Lee and Ahn, 2013). Our results indicate that the stability of heterologously expressed DcHsp17.7 in transgenic *E. coli* depends on the type of abiotic stress applied to the bacterial cell.

Possible toxicity of surfactant on bacterial cell viability. The effect of surfactant on *E. coli* viability was examined. Tween 20 (up to 0.01%) slightly decreased the viability of both cell lines, vector controls, and transgenic cell lines heterologously expressing DcHsp17.7 to 87% and 89%, respectively (Fig. 5A). Another commonly used surfactant, SDS, dramatically decreased the bacterial cell viability of both the cell lines to lower than 20%, thus exhibiting cytotoxicity (Fig. 5B). However, the transgenic cell lines heterologously expressing DcHsp17.7 showed higher levels of survival, compared with the vector controls, in the presence of both surfactants, suggesting that DcHsp17.7 can confer tolerance to toxicity induced by surfactants.

Enhanced tolerance of transgenic cells heterologously expressing DcHsp17.7 to nanomaterials. The viability of the transgenic cell lines heterologously expressing DcHsp17.7 and vector controls was examined in the presence of MWCNTs and AgNPs. Corresponding non-nanopowdered controls, activated carbon and Ag powder, were first added to the cell lines. Activated carbon slightly lowered the cell viability to $\approx 90\%$ (Fig. 6A) with no significant difference in the cell viability between the transgenic and vector control cell lines. Ag powder reduced the cell viability to 76% and 88% in the vector controls and transgenic cell lines heterologously expressing DcHsp17.7, respectively (Fig. 6B), indicating that Ag is toxic to *E. coli*.

MWCNTs and AgNPs further reduced bacterial cell viability (Fig. 6C–D). MWCNTs lowered the survival rate to 29% and 35% in the vector controls and transgenic cell lines heterologously expressing DcHsp17.7, respectively. AgNPs lowered the survival rate to 52% and 82% for the two cell lines,

respectively. Our results indicate that MWCNTs are relatively more cytotoxic than AgNPs toward the bacterial cells. However, the heterologously expressed DcHsp17.7 can confer tolerance against both nanomaterials in transgenic *E. coli*. Considering the fact that the level of the heterologously expressed DcHsp17.7 did not change with MWCNTs (Fig. 4), the reduced viability of the transgenic cell lines expressing DcHsp17.7 with MWCNTs was probably not the result of the degradation of the protein. It is possible that the function of DcHsp17.7 is inhibited by MWCNTs. To the best of our knowledge, this is the first study reporting that a sHSP can provide protection against nanotoxicity.

Considering the increase in the accumulation of nanomaterials in our environments, understanding their actions and tolerance mechanisms in living organisms is very important to reduce possible toxicity of nanomaterials. The results of this study suggest that plant sHSPs can be expressed and that they can play a role in reducing the toxic effects of nanomaterials. Through genetic engineering, useful gene(s) can be introduced into plants and other organisms to confer tolerance against nanotoxicity. Further studies are required to examine the mechanism underlying the expression and function of HSPs under conditions related to nanomaterial exposures.

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