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Original research article

Expression analysis of new Metallothionein2-like protein under mercury stress in tomato seedling

Takeshi Nagata

Faculty of Science and Engineering, Setsunan University. 17-8 Ikedanakamachi, Neyagawa, Osaka 572-8508, Japan

Corresponding author: T. Nagata, E-mail: t-nagata@lif.setsunan.ac.jp, Phone: +81-72-839-8222, Fax: +81-72-839-8222

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Abstract: Plants including tomato produce several kinds of chelator proteins such as metallothioneins (MTs) for protection against Hg^{2+} toxicity. However, the mechanism of protection from Hg^{2+} is not perfectly clear. Hg^{2+} content subsequently was plateaued from days 1 to 7. Cell death and DNA digestion were not observed in the primary root in the presence of Hg^{2+} over the 7 days. The predicted protein sequences of 5 tomato type 2 MT-like (MT2-like) proteins were compared. The coding sequences of accession number Z68185 had no Cys-Cys motif in the N-terminal. However, the *Z68185* cDNA genetic recombinant showed high resistance to Hg^{2+} in bacteria. In tomato, the expression was observed in the roots, but not in the leaves or stems. mRNA of the MT2-like protein was measured in tomato seedlings exposed to 1 μM Hg^{2+}. The expression level did not increase until day 3, but increased expression was observed after day 5. These results suggest that new Metallothionein2-like protein express in root specific and it may trap mercury. Our results indicate that functional identification of an MT2-like protein will be useful for molecular breeding designed to improve plant tolerance to Hg^{2+}.

Keywords: Expression, Mercury, Metallothionein2-like protein, Tomato (*Solanum lycopersicum*)

Abbreviations: Hg^{2+}, mercury; MT, metallothionein; RT-PCR, reverse transcription - PCR

Introduction

Mercury (Hg^{2+}) is a widespread nonessential toxic metal, which is released into the environment by natural events and industrial incidents. In Japan, air-sea exchange of mercury is one of the problems (Narukawa et al. 2006). Their results indicate the concentration of total gaseous mercury over the sea was about 2.0 ng/ m^3. Hg^{2+} in atmosphere melts in rain and may fall in farm. Hg^{2+} is a heavy metal that is toxic to most plants (Patra and Sharma 2000). Trace elements act as important cofactors for many enzymes and function in cellular physical processes (Williams and Mills 2005). However, Hg^{2+} is a nonessential element in plants and generally considered toxic, because it displaces some essential elements and reacts with sulfur in amino acid side chains (Clemens 2001). We revealed the mercury effect to Solanum plants, *Nicotiana tabacum* (tobacco) (Nagata et al. 2006, 2009). The results indicated that Hg^{2+} concentrations of 1 to 10 μM caused the damage, such as inhibition of shoot growth and root elongation, and over 10 μM was lethal.

Nonessential heavy metals such as Hg^{2+} enter plants via passive diffusion and also through a low-affinity metal transporter with broad specificity (Cui et al. 2014). In addition, Hg^{2+} induces more severe oxidative stress by triggering production of reactive oxygen species (Clemens 2001) and damaging macromolecules (Heidenreich et al. 2001; Cargnelutti et al. 2006). Thus, Hg^{2+} directly damages proteins and DNA and ultimately causes cell death.

To avoid Hg^{2+}-induced damage, plants have evolved a heavy-metal resistant mechanism such as metallothioneins (MTs) to detoxify Hg^{2+}. Plants have developed a series of uptake, extrusion, chelation, and storage mechanisms to maintain essential trace element homeostasis and to alleviate heavy metal toxicity (Clemens et al. 2002). In particular, plants synthesize several kinds of chelator peptides, such as glutathione, phytochelatin, and MTs. Glutathione is a peptide that detoxifies toxic materials in plant cells (Sobrino-Plata et al. 2014). MTs are cysteine-rich,

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low-molecular weight proteins, which bind metal ions (Kagi and Schaffer 1988). Many plant MT-like genes have been described including those in maize (de Framond 1991), barley (Kille et al. 1991; Okumura et al. 1991), soybean (Kawashima et al. 1991), *Alabidopsis thaliana* (Takahashi 1991; Zhou and Goldbrough 1994), rice (Hsieh et al. 1995; Sasaki et al. 1994), wheat (Snowden and Gardner 1993), tobacco and alfalfa (Robinson et al. 1992).

These proteins have been subdivided into 3 categories based on the arrangement of cysteine residues within the N-terminal domain (Robinson et al. 1993). Class I MTs (MT1) exclusively have a Cys-x-Cys cluster (where x is an amino acid other than cysteine). Class II MTs (MT2) are very similar proteins but cannot be aligned with class I MTs (Kagi and Schaffer 1988) because they have a characteristic Cys-Cys cluster and appear to be ubiquitous. Most plant species tested synthesize (γ-EC)n G peptides, and these were called phytochelatins (PCs) and later termed class III MTs (Gekeler et al. 1989). At present, certain plants possess a complement of gene-encoded class I/class II MTs suggesting multiple roles in growth and development (Robinson et al. 1993; Schindler et al. 1992).

The MTs nucleotide sequences have been deposited in the EMBL Nucleotide Sequence Database under accession numbers Z68138, Z68185, Z68309, Z68310 (Giritch et al. 1998) and DQ996038 (Harvey et al. 2008). Whitelaw et al. (1997) showed that the tomato MT_A and MT_B genes were encoded DQ996038 and Z68138, which belong class II MTs. These mRNA were more abundant in leaves than in roots by northern blot analysis. The nucleotide sequences reported have been submitted to GenBank under accession numbers DQ996038 and Z68138, respectively. In addition, Giritch et al. (1998) showed Z68309 and Z68310 expression in tomato root; the expression levels were influenced by iron.

Solanum lycopersicum (tomato) is an important vegetable. Tomato is one of the sensitive vegetable plant to Hg^{2+} (Gauba et al. 2007). But, Cho and Park (2000) have reported that Hg^{2+} stress induced the activity of antioxidant enzyme, such as superoxide dismutase, catalase, and peroxidase. However, the protection mechanism of chelator peptides to Hg^{2+} toxicity is unclear. Hg^{2+} contamination is a serious environmental problem and greatly affects tomato growth and quality. The main objectives of this study were to achieve a better understanding of the Hg^{2+} stress to the tomato plantlets and the means by which MT2-like protein regulates tomato adaptation to Hg^{2+} stress.

Materials and Methods

Plants and growth conditions

The seeds of *S. lycopersicum* cv. Micro-tom (tomato) were obtained from Inplanta Innovations, Inc. (Tokyo, Japan) and surfaces sterilized in 70% ethanol for 2 min and in 10% commercial bleach with detergent (Kitchen Haiter, Kao, Tokyo, Japan) for 20 min. The seeds were then rinsed with sterilized water 3 times for 5 min each. The seeds were planted in Murashige and Skoog (MS) medium containing B5 vitamins with 0.8% agar and 3% sucrose (Murashige and Skoog 1962). All cultures were maintained at 25°C under a 16 h light / 8 h dark cycle. The 7-day-old seedlings were transferred to MS liquid medium without Hg^{2+} for 7 days. Plants were then transferred to new MS liquid medium supplemented with a final concentration of 1 μM Hg^{2+} and cultured in an MLR-350 growth chamber (Sanyo, Osaka, Japan). Leaves, stems, and roots were harvested, and their Fresh weights (FWs) were measured. Each organ was dried in an oven at 55°C for 17 h, and dry weights were measured. Moisture content was expressed as percentage of fresh mass.

Determination of Hg^{2+} content

After culturing at 25°C, the Hg^{2+} content in various plant tissues was determined by flameless cold-vapor atomic absorption spectrophotometry using an atomic mercury analyzer (RA-2A, Nippon Instruments, Tokyo, Japan) after the samples were digested with concentrated nitric acid (Pan-Hou et al. 2001).

DNA extraction and gel electrophoresis

Fresh root tissue was homogenized in liquid nitrogen using a motor and pestle. The samples were incubated at 65°C in an aliquot of cetyltrimethylammonium bromide (CTAB) buffer [2% (w/v) CTAB, 1.4 M NaCl, 100 mM EDTA, 100 mM Tris-HCl pH 8.0] for 30 min, and genomic DNA were isolated. The DNA samples (2 μg) were separated on a 2% agarose gel (Murray and Thompson 1980) containing GelRed (Wako Pure Chemical Ind., Osaka, Japan) and photographed under 500-nm light.

Microscopy

Cell viability assay was performed as described by Ma et al. (1997). Roots treated or untreated with Hg^{2+} for 7 days were placed in distilled water for 5 min, stained with 3 mg/ L propidium iodide (PI) solution for 15 min, and then rinsed with distilled water. Fluorescent images were captured with a LSM 710 laser-scanning microscope (Carl Zeiss, Tokyo, Japan). Samples were visualized under an epifluorescence illumination (excitation: 546 nm, emission: 615-619 nm) for the cell viability assay. All figures are representative of

staining detected in roots of three independent experiments, and the microscopic field over the specimen was randomly chosen.

Searching for the MT2-like protein coding gene in tomato and gene annotation

Published tomato MT2-like protein gene sequences (Accession nos. DQ996038, Z68138, Z68309, Z68310, and Z68185) in the NCBI database were used (http://www.ncbi.nlm.nih.gov/). The protein sequences were aligned by ClustalW (http://www.clustal.org/) and manually adjusted.

Hg^{2+} resistance of the MT2-like protein expressed in Escherichia coli (*E. coli*)

E. coli JM109, a protease-defective strain, was grown at 37°C in Luria-Bertani (LB) medium and used for routine plasmid propagation. When necessary, the medium was supplemented with 100 mg/ ml ampicillin. The recombinant plasmid pFCLIII-MT2, which carries the MT2-like protein coding tomato *Z68185* cDNA, was transferred into *E. coli* JM109. The plasmid pFCLIII-MT2 was kindly supplied by Dr. K. Aoki of Osaka Prefecture University.

E. coli transformed with the empty or constructed plasmid were precultured in LB liquid medium at 37°C, 100 rpm for 16 h. Resistance of bacteria to Hg^{2+} was determined on Petri dishes as described previously (Kiyono and Pan-Hou 1999). The zones of growth inhibition around the disks were measured after incubation at 37°C for 16 h.

RNA isolation and RT-PCR analysis

Seedlings of untreated and Hg^{2+}-treated plants were incubated for 1, 3, 5, and 7 days in MS liquid medium, and each organ was subjected to RT-PCR analysis. After the 7-day incubation under the different treatments, total RNA from leaves, stems, and roots was isolated using an RNeasy Plant Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. First-strand cDNA was synthesized from 1-μg total RNA using the oligo dT (18) primer and random primers (ReverTra Ace, TOYOBO, Osaka, Japan).

The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene (GenBank/DDBJ accession no. AAB51592.1) of tomato was used as the internal standard. Transcript accumulation was quantified as a value relative to accumulation of the *GAPDH* transcript. The *MT2-like protein* (Accession no. Z68185) sequence from tomato is published in the NCBI database (http://www.ncbi.nlm.nih.gov/page). Sequences to design the primers were chosen using Primer3 software (Rozen and Skaletsky 2000).

PCR was performed under the following conditions: predenaturation at 95°C for 1 min, followed by 25 cycles of 15 s at 95°C and 30 s at 60°C using the Thunderbird SYBR qPCR Mix (Toyobo, Osaka, Japan) and GeneQ (Bioer Technology, Tokyo, Japan). The PCR products were electrophoresed on a 0.8% agarose gel containing GelRed and photographed under a 500-nm light. The Thunderbird SYBR qPCR Mix and CFX96 (Bio-Rad, Tokyo, Japan) were used for the quantitative RT-PCR analysis. Fragment amplifications by PCR were performed under the following conditions: predenaturation at 95°C for 1 min, followed by 40 cycles of 15 s at 95°C and 30 s at 60°C in 20-μL reaction mixture. The primer sequences used for *MT2-like protein* gene were: 5′-ATGTCTG GCTGTGGAGGAAG-3′ (forward) and 5′-CTC CCTCGATGATGGTAAGG-3′ (reverse); *GAPDH* gene were: 5′-TTGAACTCGTCGCAGTGAAC-3′ (forward) and 5′-GGAAGTTTCCTCTGGGTTCC-3′ (reverse).

Statistical analysis

All experiments were performed at least three times. Data are presented as means of three replicates. Differences between each treatment were determined by Student's *t*-tests. A p-value<0.05 was considered significant.

Results

Hg^{2+} affects tomato growth, and moisture content

2-week-old tomato plants were transferred into MS liquid medium with or without Hg^{2+} to examine Hg^{2+} toxicity. The seedlings were harvested after 1, 3, 5, and 7 days of Hg^{2+} exposure (Fig. 1a, b). Hg^{2+}-treated plants were smaller than Un-treatment plants after 3 days of Hg^{2+} exposure.

The seedlings were separated, and their fresh weights were measured to organs (Fig. 2a). FWs of the leaves and stems of Hg^{2+}-treated plants were lower than those of untreated plants. FWs of the leaves and stems of Hg^{2+}-treated plants were approximately 62.2% and 77.8% compared with those of untreated plants, respectively. However, root FW was not significantly different between the Hg^{2+}-treated and untreated plants after 7 days of Hg^{2+} exposure, but root FW of Hg^{2+}-treated plants tended to be decreased than those of untreated plants during the early period. Hg^{2+} content in the leaves of Hg^{2+}-treated plants was greater until day 5 (Fig. 2b). Hg^{2+} was not detected in stems until day 7. Hg^{2+} content increased rapidly within 24 h in roots. However, content plateaued at FW of approximately 4 μg Hg^{2+} /g from days 1 to 7. As

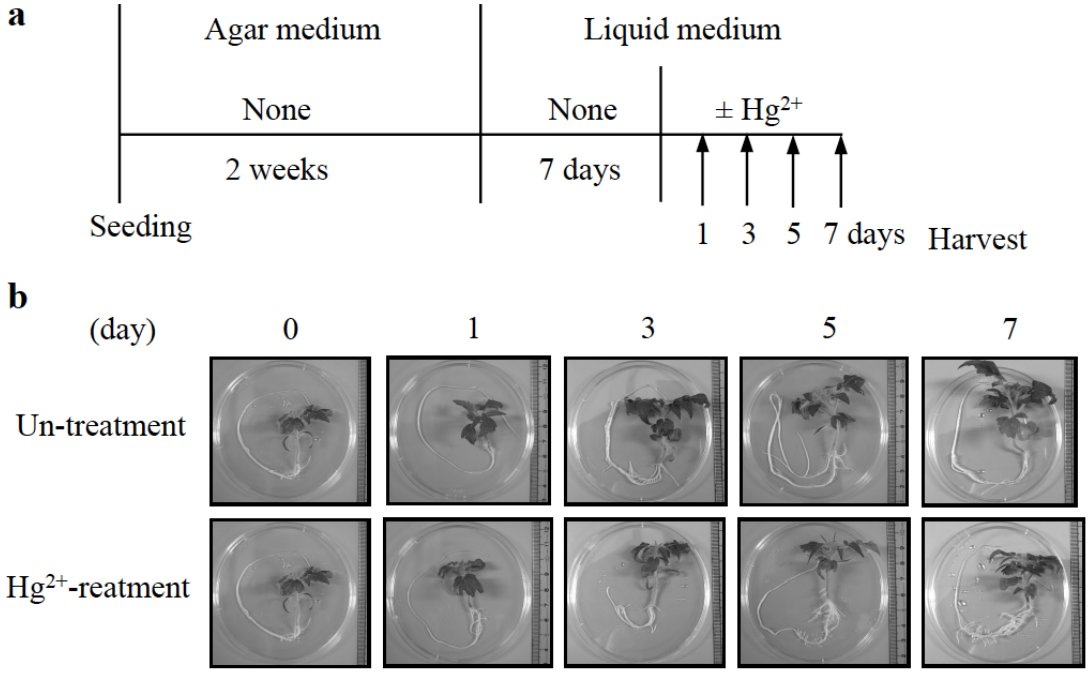

Fig. 1. Experimental schedules and effect of Hg^{2+} on phenotype of tomato seedling. The effect of Hg^{2+} on growth was evaluated by monitoring the fresh weight of plant tissues after incubation at 25°C. Three-week-old plantlets were exposed to MS liquid medium with or without 1 μM Hg^{2+}. (a) Schematic of the experimental timetable for Hg^{2+} treatment, (b) Phenotype of plants.

shown in Fig. 2c, the moisture content of Hg^{2+}-treated leaves and roots was decreased than that of untreated leaves and roots until day 3. In particular, moisture content was reduced in during the early period. The reduction in leaf and root moisture at the 24 h of exposure in the Hg^{2+}-treated plants was approximately 2% and 4% compared with those of untreated plants, respectively. The reduction of moisture content in stems of Hg^{2+}-treated plants was approximately 1.5% less than that of untreated plants until day 5 (Fig. 2c).

Hg^{2+} damages root cells but does not induce programmed cell death

Isolated genomic DNA was separated on a 2% agarose gel to investigate genomic DNA digestion, which is one of the symptoms of programmed cell death (Fig. 3a). Genomic DNA of the untreated and Hg^{2+}-treated plants was not digested at 7 days indicating that the toxicity of Hg^{2+} to root cell was not caused by programmed cell death. Cell viability was assessed in roots using propidium iodide (PI) staining. PI penetrates damaged cell membranes and intercalates with DNA and RNA to form a bright-red fluorescent complex seen in dead cells. The results showed dead cells in the root tips and hairy root of plants treated with 1 μM Hg^{2+} (Fig. 3b) for 7 days. Untreated root cells were almost not detected by fluorescence (Fig. 3c).

The MT2-like protein Z68185 has a unique N-terminal sequence

DQ996038, Z68138, Z68309, and Z68310 have predicted amino acid sequences with 14 cysteine residues, whereas the Z68185 coding protein has 13 cysteine residues resulting from a displaced N-terminal cysteine to glycine (Fig. 4a). Thus, the Z68185 coding protein has no Cys-Cys or Cys-x-Cys motif in the N-terminal. The amino acid sequence alignment and phylogenetic tree analyses of the Z68185 coding protein with other MT2-like proteins of tomato revealed high sequence identity with accession nos. Z68310 (78%), DQ996038 (73%), Z68309 (68%), and Z68138 (47%) (Table 1 and Fig. 4b). However, there are no reports on accession nos. Z68185 and its role is unclear.

The Z68185 coding protein confers tolerance to Hg^{2+} in E. coli

To investigate the involvement of *Z68185* coding protein in Hg^{2+} tolerance, we examined the viability of recombinant *E. coli* on plates with various concentra-

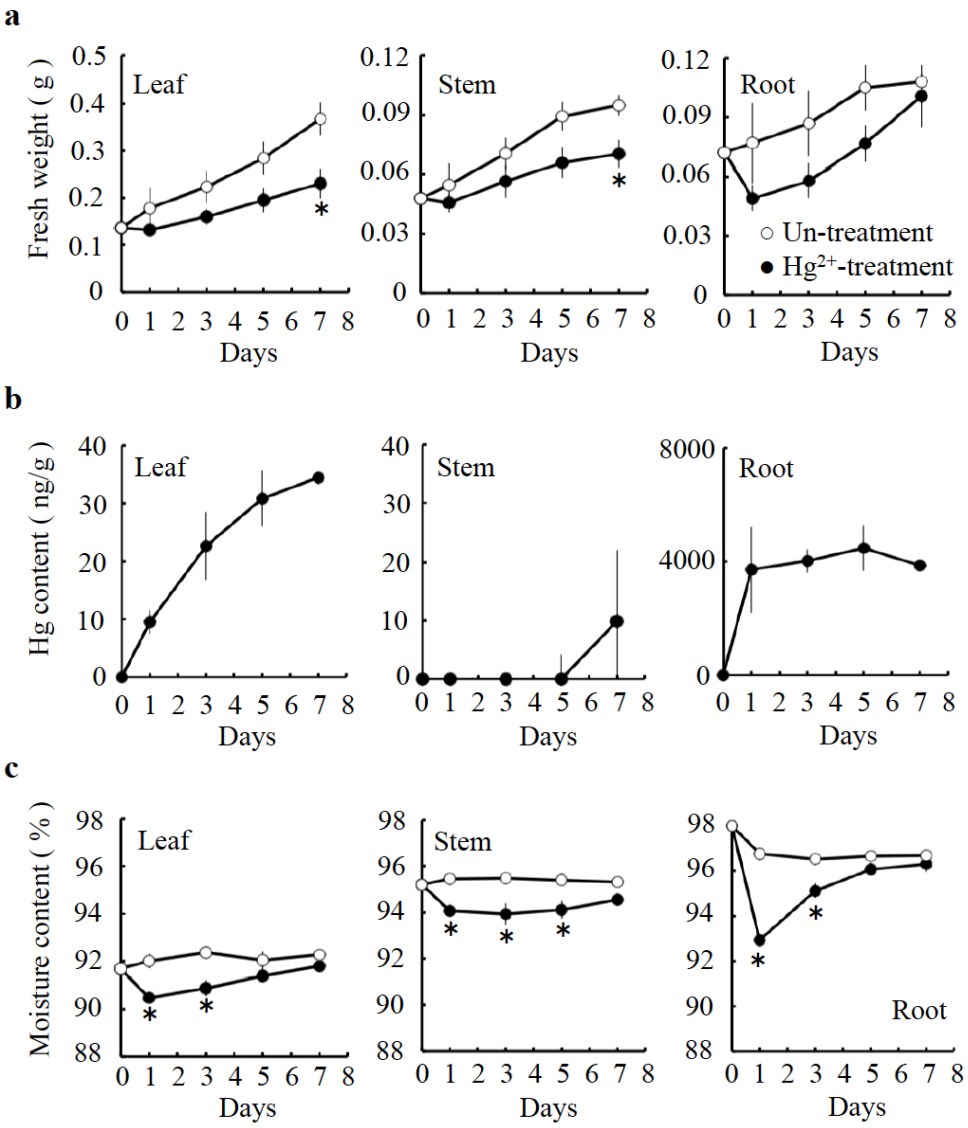

Fig. 2. Effect of Hg^{2+} on growth, accumulation of Hg^{2+} and moisture content. Two-week-old plantlets were exposed to MS liquid medium with or without 1 μM Hg^{2+}. (a) Fresh weight, (b) Hg^{2+} contents and (c) Moisture content. Data are expressed as means±standard deviations (SD; n= 4). Asterisks indicate a significant difference from untreated ($p < 0.05$).

tions of Hg^{2+}. Dose-dependent growth inhibition by Hg^{2+} was detected on the basis of the zone on LB agar medium. Growth of the vector control *E. coli* was strongly inhibited by 200 nmol Hg^{2+}, whereas *Z68185*-expressing *E. coli* maintained growth in up to 600 nmol Hg^{2+} (Fig. 4c). The *Z68185* cDNA genetic recombinant showed high Hg^{2+} resistance compared with the vector control, indicating that expression of the *Z68185* coding protein may detoxify Hg^{2+} in cells.

The Z68185 coding protein is expressed in tomato roots under an Hg^{2+}-free condition

Tomato seedlings were assayed by semi-quantitative and quantitative RT-PCR to identify the tissue expressing the mRNA of *Z68185* coding protein. A primer set that amplified a part of *Z68185* (bp positions 79–196 from the start codon) was constructed and tested by RT-PCR. The PCR product was detected as an apparent single band on agarose gel electrophoresis (data not shown).

The quantity of *Z68185* mRNA expressed by tomato plantlets not exposed to 1 μM Hg^{2+} was measured by semi-quantitative RT-PCR. Expression was observed in the roots, but not in the leaves or stems (Fig. 5a). Furthermore, quantitative RT-PCR showed that mRNA expression level of the *Z68185* coding protein in roots was approximately 10-fold

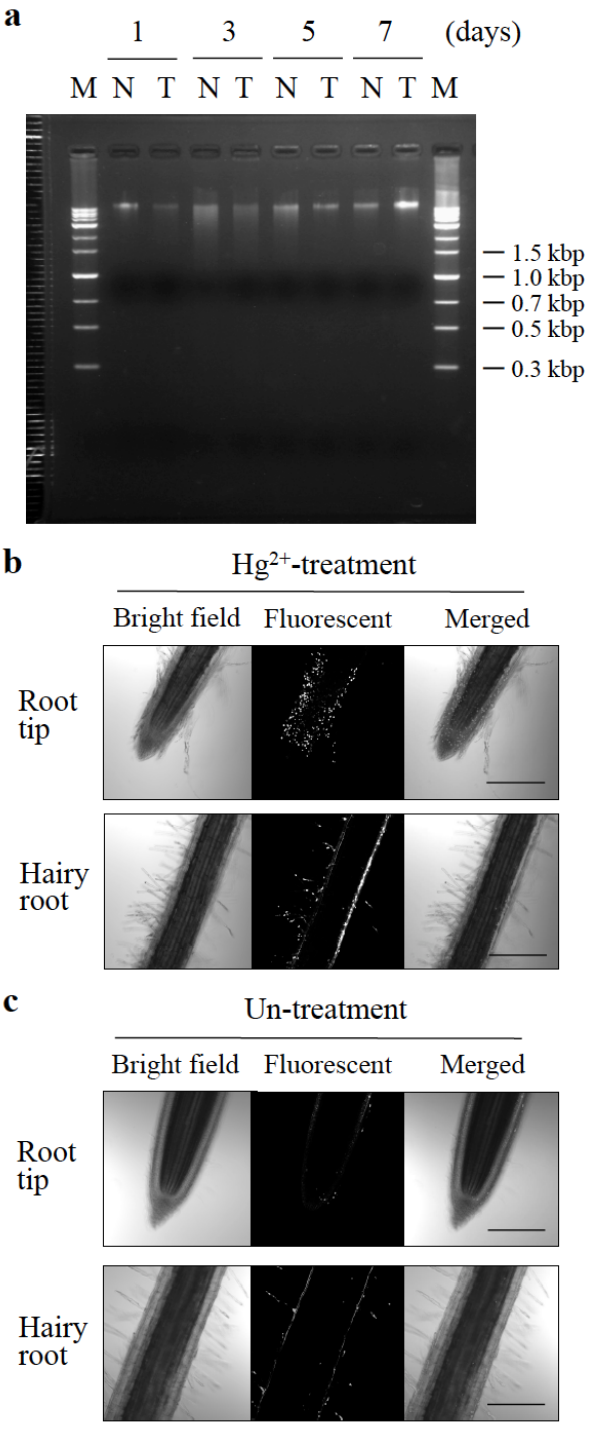

Fig. 3. Effect of Hg^{2+} on DNA degradation and cell viability in tomato root. (a) A 2-µg aliquot of total DNA was extracted from roots and electrophoresed on a 2% agarose gel. Three biological replications were performed for each. M: 100 bp DNA ladder marker. N: untreated, T: Hg^{2+}-treated. Propidium iodide (PI) staining of roots 7 days after transfer to liquid medium with (b) or without 1 µM Hg^{2+} (c). The root samples were washed with deionized water for 5 min and then incubated with 3 mg/L PI for 15 min. The stained roots were then rinsed in deionized water and observed under a laser-scanning microscope. Scale bar = 200 µm.

higher than that in leaves and stems (Fig. 5b), suggesting that *Z68185* mRNA expression is root-specific.

Hg^{2+} induces the Z68185 coding protein in tomato root

The *Z68185 mRNA* expression level was determined by semi-quantitative and quantitative RT-PCR to examine the response to Hg^{2+} toxicity. The primer set used to amplify a part of *Z68185* is described above.

The *Z68185* mRNA expression level in tomato seedlings exposed to 1µM Hg^{2+} was determined. Expression level did not change until day 3 (Fig. 6a, b). After 5 days of Hg^{2+} exposure, expression was approximately 4-fold higher than that of roots before Hg^{2+} exposure. However, mRNA in leaves and stems was not detected until day 7.

Discussion

Hg^{2+} is the most toxic nonessential element to plants (Patra and Sharma 2000), because it displaces some essential elements, such as zinc and manganese, and reacts with sulfur in amino acid side chains (Clemens 2001). As a result, Hg^{2+} reduces of crop yield. Tomato is an important vegetable. However, the mechanism that underlies Hg^{2+} toxicity is unclear. Ortega-Villasante et al. (2005) showed that Hg^{2+} causes physiological responses such as cell necrosis in alfalfa. In this study, we investigated Hg^{2+} phytotoxicity in tomato plantlets. We also investigated the effect of Hg^{2+} on physiological responses such as Hg^{2+} accumulation, root cell viability, and activity of the MT2-like protein against Hg^{2+} toxicity.

FW of leaves and stems exposed to Hg^{2+} for 7days was less than that of untreated leaves and stems (Fig. 2a). In contrast, FW of roots was not significantly different between the Hg^{2+}-treated and untreated plants after 7 days of Hg^{2+} exposure, but FW of Hg^{2+}-treated roots was less than that of untreated roots during the early period, suggesting that root weight was restored after 3 days of Hg^{2+} exposure. Hg^{2+} content rapidly increased in roots within 24 h and then stopped (Fig. 2b). It is suggesting that Hg^{2+} was trapped in roots was mainly bound to cell wall because Hg^{2+} ion was easy to interact with anionic compounds (Chen and Yang 2012) and some Hg^{2+} was translocated to the shoot via the xylem. It is known that Hg^{2+} enters plants via passive diffusion and through low-affinity metal transporter with broad specificity (Cui et al. 2014). Taken together, our results suggested that the Hg^{2+} phytotoxicity in tomato root increased in early period.

One of the most effective Hg^{2+} phytotoxic mechanisms in plants is water depletion. Because Hg^{2+}

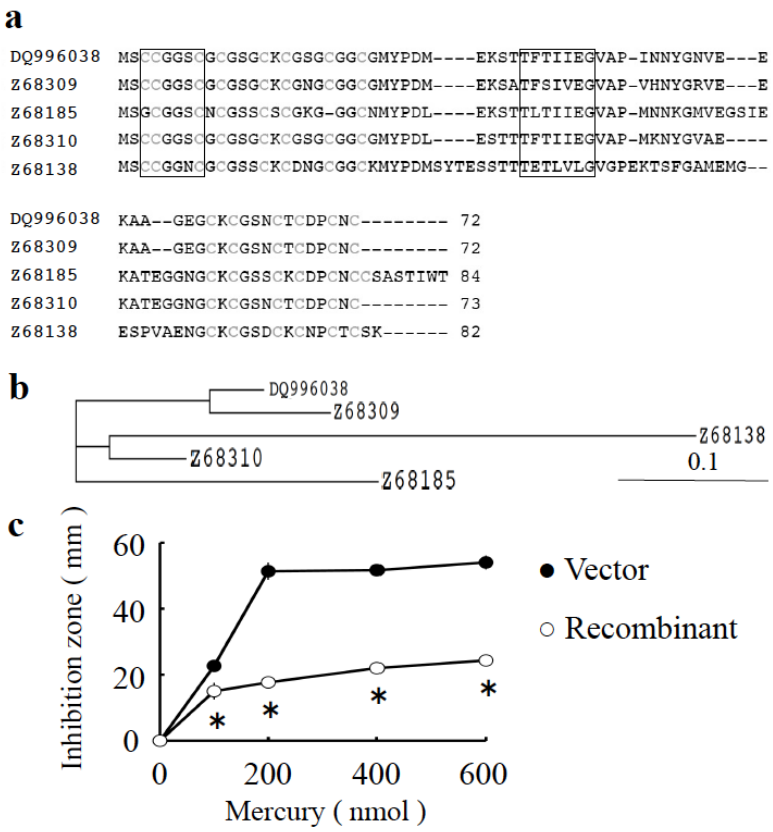

Fig. 4. Sequence analyses of tomato metallothionein2 (MT2)-like proteins and its contribution to Hg^{2+} resistance in *Escherichia coli*. (a) Alignment of the predicted protein sequences of the Z68185 tomato MT2-like protein with those of other tomato MT2-like proteins was performed using ClustalW (DDBJ). Orange characters indicate cysteine residues. (b) The unrooted phylogenetic tree was constructed on the basis of the deduced amino acid sequences of the 5 tomato MT2-like proteins. (c) The recombinant plasmid was constructed by inserting the *Z68185* gene into the vector plasmid pFLCIII. After a 16-h incubation at 37°C, the diameters of the inhibition zones (minus the 6 mm disk diameter) of the vector plasmid and the recombinant plasmid were measured on Petri dishes. All values are the means of triplicate determinations from 3 experiments. Data are means ± standard deviations (SD; n = 3). Asterisks indicate a significant difference from the vector control ($p < 0.05$).

Table 1. Comparison of amino acid sequence of Z68185 with other tomato MT2-like proteins

Accession nos.	Number of amino acids	% of amino acid sequence homology	Number of cysteine
Z68185	84	-	13
DQ996038	72	73.0	14
Z68138	82	47.0	14
Z68309	72	68.0	14
Z68310	73	78.0	14

disrupts the aquaporin water pore through local conformational changes (Hirano et al. 2010), the moisture content of Hg^{2+}-treated leaves and roots decreased compared with untreated leaves and roots until day 3 (Fig. 2c). The reductions in moisture content of leaves and roots after 24 h of Hg^{2+} exposure were approximately 2% and 4% compared with those of untreated plants, respectively. The reduction in root moisture content recovered after 5 days of Hg^{2+} exposure (Fig. 2c). This might due to express aquaporin.

Then, we isolated and separated fragmented

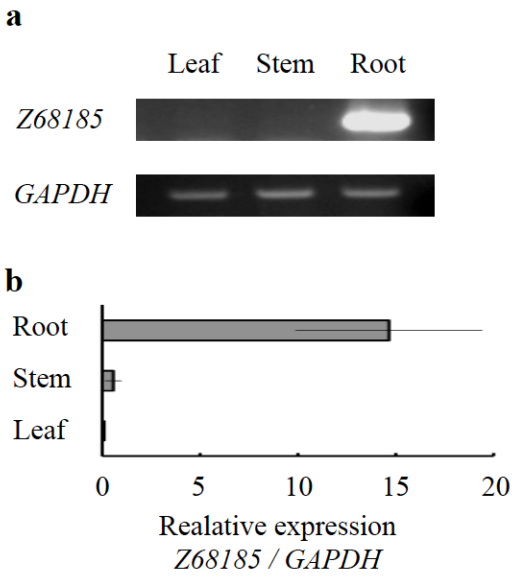

Fig. 5. Gene expression of tomato metallothionein2-like protein in various tissues. The expression levels of Z68185 transcripts were determined in 2-week-old plantlets. (a) Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis. (b) Quantitative RT-PCR analysis. Expression results are normalized against GAPDH expression. Total RNAs were independently prepared from 3 individual plants from each treatment. Data are means ± standard deviations (SD; n = 3).

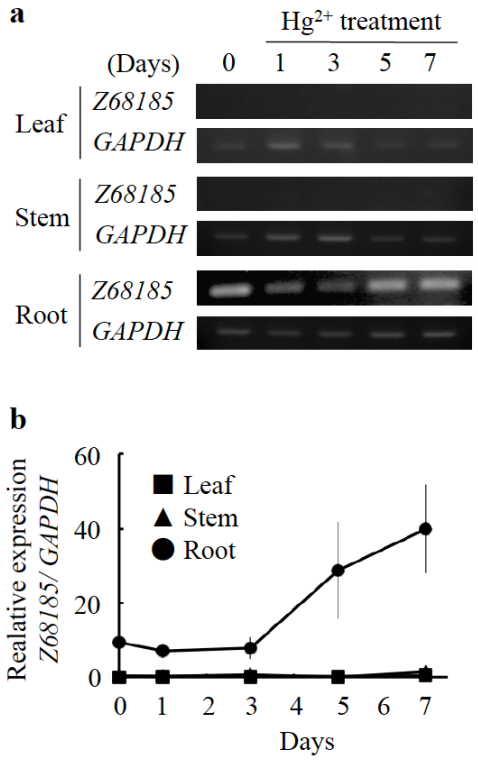

Fig. 6. Induction of tomato *metallothionein2-like protein* transcripts by Hg^{2+}. Two-week-old plantlets were exposed to Hg^{2+} for 7 days. (a) Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis. (b) Quantitative RT-PCR analysis. Expression results were normalized against *GAPDH* expression. Total RNAs were independently prepared from 3 individual plants from each treatment. Data are means ± standard deviations (SD; *n* = 3).

genomic DNA to investigate genomic DNA digestion, which is a marker of programmed cell death (Fig. 3a). The genomic DNA of Hg^{2+}-treated and untreated plants was not digested following 7 days of exposure. The dead cells in roots were assessed by PI staining. The results showed that dead cells in the roots treated with 1 µM Hg^{2+} for 7 days dramatically increased compared with that in untreated plants (Fig. 3b, c). Ortega-Villasante et al. (2005) grew alfalfa (*Medicago sativa*) in 30 µM Hg^{2+} for 7 days to trace early and small plant responses to Hg^{2+}. Their results revealed that cell necrosis increased after Hg^{2+} exposure for 6-24 h. However, we found no programmed cell death after exposure to Hg^{2+} for 7 days (Fig. 3a). Taken together, 7 days of Hg^{2+} exposure resulted in initiation of cell necrosis, but it did not lead to cell death in tomato roots. To our best knowledge, this is the first time that cell necrosis has been reported to be caused by 1 µM Hg^{2+}. We believe that a protective protein such as the MT2-like protein was synthesized for protection against Hg^{2+} phytotoxicity in root cells.

The predicted protein sequences of 5 tomato MT2-like proteins were compared (Fig. 4a). The coding sequences of accession nos. Z68185 and Z68138, which encode MT_B, showed 47.0% identity at the amino acid level (Fig. 4b and Table 1). Whitelaw et al. (1997) showed that MT_A and MT_B gene transcripts are more abundant in leaves than in roots of tomato plants grown without extra metal ions. All tomato MT2-like proteins except Z68185 have 14 cysteine residues, and these proteins have strong binding capacity for heavy metals (Giritch et al. 1998). Z68185 has not only Cys-Cys or Cys-x-Cys motif at the N-terminal, suggesting that the *Z68185* coding protein is a unique molecule compared with other MT2-like proteins. The $MT2_B$ protein of Cavendish banana (Musa acuminate) has also no Cys-Cys in the N-terminal (Liu et al., 2002). In future, these unique molecules may be found in other plants.

The *Z68185* coding protein, which has 13 cystein residues has not be cleared metal resistance ability, we examined in *E. coli* for tolerance against Hg^{2+} toxicity. Growth of the *Z68185* cDNA genetic recombinant was not inhibited by Hg^{2+} (Fig. 4c), suggesting that the MT2-like protein chelates Hg^{2+} in bacterial cells. The

expression of the MT2-like protein Z68185 in bacteria may prevent displacement of essential elements or the Hg^{2+} reaction with sulfur in amino acid side chains. And more, the result suggested that Z68185, which has an x-Cys motif in N terminal sequence, has binding ability for mercury. However, the expression level of the MT2-like protein in tomato remains unclear.

The MT2-like protein Z68185 expression was observed in the root but not in the leaves or stems (Fig. 5a, b). Interestingly, it is thought the protein was expressed constitutively. We next investigated induction of *Z68185* mRNA expression under Hg^{2+} exposure. Expression increased in roots after 3 days of Hg^{2+} exposure (Fig. 6a, b), which agreed with the growth restoration of roots after 3 days of Hg^{2+} exposure (Fig. 2a). These results suggest that Hg^{2+} damaged the root cells mainly within 3 days. This is the first time that the relationship between induction of an MT2-like protein and cell death in Hg^{2+}-exposed roots has been reported. And more, the 7 days expression was 4-fold higher than the first day. It is suggested that new MT2-like protein is included protection from mercury stress and is able to connect by mercury. More detailed studies will be required to identify the function and location of the MT2-like protein in plant. We have demonstrated one of the reasons that tomato is sensitive to Hg^{2+}. Thus far, no reports are available on strategies to breed Hg^{2+}-free tomatoes. To overcome Hg^{2+} sensitivity, Z68185, which is one of the MT2-like proteins, could be useful to trap Hg^{2+} in tomato roots.

In conclusion, new Metallothionein2-like protein express in root specific and it may trap mercury. These findings could help improve our understanding of mechanism of protection from Hg^{2+}. Generating Hg^{2+}-free tomato fruits is believed to be acceptable to the public. Thus, our results indicate that functional identification of new MT2-like protein will be useful for molecular breeding designed to improve plant tolerance to Hg^{2+} and reduce Hg^{2+} accumulation in tomato fruits in the future.

Acknowledgements

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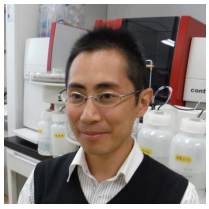

Dr. Takeshi Nagata is a plant physiologist and has studied bioaccumulation of heavy metals. He is interested in the metal homeostasis in vegetative plants.