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Expressing a bacterial mercuric ion binding protein in plant for phytoremediation of heavy metals

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ABSTRACT

A specific mercuric ion binding protein (MerP) originating from transposon Tn*MERI1* of *Bacillus megaterium* strain MB1 isolated from Minamata Bay displayed good adsorption capability for a variety of heavy metals. In this study, the Gram-positive MerP protein was expressed in transgenic Arabidopsis to create a model system for phytoremediation of heavy metals. Under control of an actin promoter, the transgenic Arabidpsis showed higher tolerance and accumulation capacity for mercury, cadium and lead when compared with the control plant. Results from confocal microscopy analysis also indicate that MerP was localized at the cell membrane and vesicles of plant cells. The developed transgenic plants possessing excellent metal-accumulative ability could have potential applications in decontamination of heavy metals.

1. Introduction

Toxic heavy metals have been released to biosphere through industrial activities and spread into a variety of environments, causing a severe threat to the human health. In particular, mercury is considered the most hazardous heavy metal on earth [1,2]. In response to toxic mercury compounds globally distributed on the ancient earth due to geological activities, microbes have developed resistance systems to overcome mercury poisoning. The *mer* operon is an intensively studied mercurial resistance system consisting of a cluster of linked genes involved in detection and regulation (*mer*R), recognition and mobilization (*mer*P, *mer*T, *mer*C), and enzymatic detoxification (*mer*A) [3,4]. Mercuric ion passes through the cell membrane via protein components such as MerP, MerT and MerC. The intracellular mercuric ion is then transformed into volatile metallic mercury Hg(0) by enzymatic reduction catalyzed by MerA, a NADPH-dependent reductase, and finally Hg(0) diffuses out of the cells via volatilization. This bacterial mercury resistance system represents a model for biological detoxification of heavy metals and offers valuable genetic elements for approaches in bioremediation. Previously, we identified the first mercury resistance transposon from Gram-positive bacteria [5,6]. The transposon, Tn*MERI1*, was harbored in the *Bacillus megaterium* strain MB1, which was isolated from mercury-polluted sediment sample. The structure of *mer* operon encoded in Tn*MERI1* is more complicated than those of Gram-negative bacteria and the genetic elements from Tn*MERI1* have been intensively studied [5–10].

Utilizing metal-binding proteins or peptides developed from metal-tolerant organisms to promote metal-accumulation ability of bacterial or plant hosts has recently been of great interest [11]. Since those peptides expressed outside the cytosol, allowing for the efficient metal-binding, the bacterial hosts exhibited better metal biosorption performance when the metal-binding proteins were expressed outside the cytosol [12]. The MerP of Gram-negative bacteria is a periplasmic protein that recognizes and binds to mercuric ion with a highly conserved domain possessing two cysteine residues for metal binding [13,14], thereby suggesting the feasibility of using MerP to improve the metal biosorption efficiency. The metal binding domain occurred in MerP was also found in other metalloproteins such as P-type ATPase [15,16]. In addition, the compact MerP protein is also an excellent model for metal-polypeptide recognition research [17]. Current

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evidence suggests that MerP protein is indispensable for the transport and may compartmentalize the toxic mercuric ion to form other components of periplasmic space [17]. MerP originating from Gram-positive bacterium possesses vicinal cysteines, while that of Gram-negative bacteria often contains two cysteines separated by two residues. This difference may affect the selectivity in heavy-metal binding [18]. Indeed, our previous study showed that MerP of Gram-positive bacterial system with adjoining cysteines indeed had better metal-binding ability than that with two separated cysteines [19].

While phytoremediation has recently been proposed as a low cost and environmentally friendly way to remove heavy metals from contaminated soils, genetic manipulation of plants would allow us to both optimize natural plant processes and further supplement them with novel traits from organisms outside the plant kingdom. Although phytovolatilization via *merA* reductase has been intensively studied for the control of mercury [21–25], the aforementioned Gram-positive MerP protein has not been expressed in plant to study the metal accumulation ability. In addition, a bacterial heavy-metal transporter, ZntA, has been successfully expressed in plasma membrane of the transgenic *Arabidopsis* [20]. The information encouraged us to further express the bacterial MerP protein in plant for heavy metal removal from soil environment. Since no natural plants have been identified reproducible to hyperaccumulate mercury, our transgenic plant may provide an optional strategy to tackle the mercury contaminated problem.

2. Materials and methods

2.1. Strains and plasmids

Bacillus megaterium MB1 encoded with mercury resistance transposon Tn*MERI1* was previously isolated from the sediment sample of Minamata Bay [5,6]. The total DNA of strain MB1 was used as the template for the amplification of *merP* gene. The pHm3 vector with a rice actin promoter [26] is a T-DNA based vector modified from pBI101 and was kindly provided by Prof. Lin, Jen-Hong (National Chung Hsing University).

2.2. Construction of Bacillus merP for plant expression

The designed primers for expressing bacterial *merP* gene in plant were modified from the study by Bizily et al. [1]. The sense primer, designated as AramerPF (5′-TCTAGAGCCACAATGATGTT GCTATCCCTTATGC-3′), contained a *Xba* I cloning site, an AGCCACA consensus sequence for plant translation, and an ATG start codon with the first 22 nucleotides of the *merP* gene coding sequence to prime the forward PCR reaction. The antisense primer, designated as AramerPR (5′-CCCGGGTTATTCGACGGGGAGACCCAAG T-3′), has a *Sma* I cloning site and anticodons to the last 23 nucleotides of *merP* coding sequence to prime the reverse PCR reaction. PCR was carried out for 35 cycles with denaturing at 95 °C for 1 min, annealing at 50 °C for 1 min, and extending at 72 °C for 1 min. The amplified fragment, *merP*, was cloned into pGEM-T Easy vector (Promega) to confirm the nucleotide sequence, and was then cleaved in the flanking *Xba* I and *Sma* I sites and was subsequently ligated to pHm3 to construct pHm3P (Fig. 1).

2.3. Agrobacterium thaliana transformation and cell growth

For plant transformation, pHm3P was introduced into the host strain *Agrobacterium tumefaciens* LBA4404 by electroporation procedures [27]. The wild-type *A. thaliana* (ecotype Columbia) was transformed with the pHm3P harboring *Agrobacterium* strain using the dipping method [28]. The transgenic plants were screened

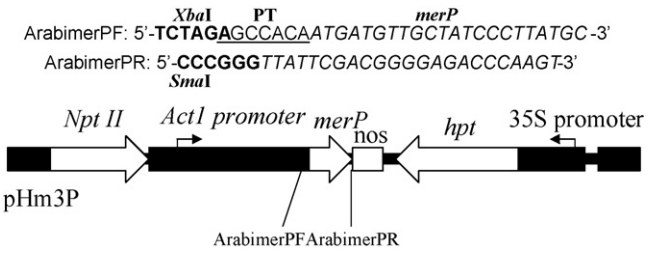

Fig. 1. PCR primers and strategy for modification of the *merP* gene to express in plant. Two PCR primers were used to add synthetic flanking sequences to the *merP* gene that have element necessary for plant expression. The sense primer, AramerPF, contained restriction endonuclease cloning sites, bacterial (BT) and plant translation signals (PT), an ATG start codon with the first 22 nt of the *merP* coding sequence to prime the forward PCR reaction. NptII; kanamycin resistance. Act1; rice actin promoter. hpt; hygromycin resistance.

in the Murashige and Skoog medium (4.3 g/l, Sigma, M5524, http://www.sigmaaldrich.com/sigma/formulation/m5524for.pdf) containing 50 mg/l of kanamycin. For the growth experiments of transgenic plants, seeds were sterilized and germinated on Murashige and Skoog medium containing mercury or other heavy metals. Seeds were grown at 22 °C with a 16 h light/8 h dark regime. The callus induction was carried out under 1× Gamborg's B5 medium (Caisson Laboratories), 2% (w/v) glucose, 1.7 mM Mes, pH 5.7, 0.8% (w/v) agar, 0.5 mg/L 2,4-D, and 0.05 mg/L of kinetin.

2.4. Test of gene expression

The total DNA extraction of transgenic plants was performed with Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, USA). The PCR reaction was performed with primer pair BmerPF (5′-CATATGATGTTGCTATCCCT TATGCTAG-3′) and BmerPR (5′-GGATCCTTATTCGACGGGGAGACCCAAG T-3′). To examine whether the bacterial *merP* was expressed in *A. thaliana* cells, total plant RNA was extracted and purified (SV Total RNA Isolation System, Promega). RT-PCR was performed with an RT-PCR kit (Access RT-PCR System, Promega) and the same primers pair for PCR reaction. As a positive control, total RNA was also purified from *B. megaterium* MB1 for comparison.

2.5. Preparation of MerP antibody

A N-terminal truncated MerP (B95P) with the deletion of putative signal peptides (the first 21 amino acids) was expressed in *E. coli* BL21 DE3 with pET-21b expression system. Protein purification was performed using DEAE column, phenyl sepharose column and Superdex 75 column. The antiserum against B95P was prepared from B95P immuned Rabbit. As the polyclonal antibody from the rabbit immunized by B95P was used, for reducing nonspecific interference, the anti-B95P serum was mixed with crude proteins of wild type *Arabidopsis* in advance. The mixture was used as specific antibody.

2.6. Fluorescence microscopy

To reveal the location of MerP protein in transgenic plant, observations were conducted on wild type and *merP* transgenic plant callus, 3 days after subculture. The processes of fixation and the immunollabeling of callus were modified from the study by Jauh et al. [29]. The fluorescence dye, Nile red was used as membrane marker [30]. Fluorescein (FITC)-conjugated AffiniPure Goat Anti-Rabbit IgG (H + L) was used as the second antibody. A laser scanning confocal microscope (Zeiss LSM 510) was used for scanning the fluorescence signals.

2.7. Assay of heavy metal accumulation

The Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) was employed to measure the heavy metal contents accumulated in wild and transgenic plant samples. For measuring mercury content, 0.5 g dry weight sample were digested with 10 ml 70% HNO_3 (Sigma) at 90 °C for 180 min. For measuring lead and cadmium contents, 0.5 g dry weight samples were digested with 10 ml 70% HNO_3 at 190 °C for 60 min and with 5 ml $HClO_4$ at 190 °C for 30 min.

3. Results and discussion

3.1. Expression of the Bacillus merP gene in transgenic A. thaliana

To examine whether the expression of *merP* gene in plants would increase heavy metal (e.g., Hg, Cd, Pb) resistance of the host cell, the *Bacillus merP* gene was introduced to *A. thaliana* by using T_i plasmid based vector pHm3-BmerP (Fig. 1). Transgenic plants were selected from kanamycin-amended Murashige Skoog (MS) medium plate. Among eight transgenic lines, no obvious differences were observed and all of the transgenic lines could survive in MS medium plates with 12.5 μM $HgCl_2$. One line of the transgenic plants, designated as BmerP-1, was selected for further study. To confirm the existence of *merP* gene in transgenic plant BmerP-1 on DNA level, total DNA from the third generation of BmerP-1 was extracted and PCR amplification was performed (Fig. 2a). An expected band of approximately 0.37 kbp DNA fragment was found from BmerP-1 while the band was not detected from wild type plant (Fig. 2a). Furthermore, dot hybridization of total DNA was also to re-confirm the existence of *merP* gene (data not shown). Expression of *merP*

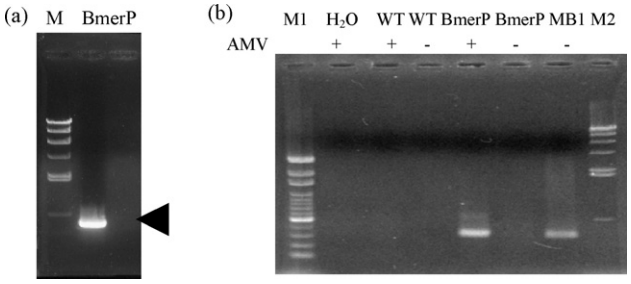

Fig. 2. (a) Confirmation of the existence of *merP* gene in transgenic plant by PCR. M, Lamada phage *Hind*III marker. BmerP, PCR product from the third generation of *merP* gene transgenic plant. The expected band of approximately 0.37 kbp DNA fragment was demonstrated by an arrow. WT, PCR product from wild-type plants. (b) Confirmation of the *merP* gene expression in plant. From left side: Lane 1, 100 bp ladder. Lane 2, ddH_2O as template. Lane 3, total RNA of wild-type plant with AMV reverse transcriptase addition as template. Lane 4, total RNA of wild-type plant without AMV reverse transcriptase addition as template. Lane 5, total RNA of BmerP-1 transgenic plant with AMV reverse transcriptase addition as template. Lane 6, total RNA of BmerP-1 transgenic plant without AMV reverse transcriptase addition as template. Lane 7, Total DNA of *Bacillus megaterium* MB1 as template. Lane 8, Ramada phage *Hind*III marker. The "+" and "−" marks indicate with and without the addition of AMV reverse transcriptase, respectively.

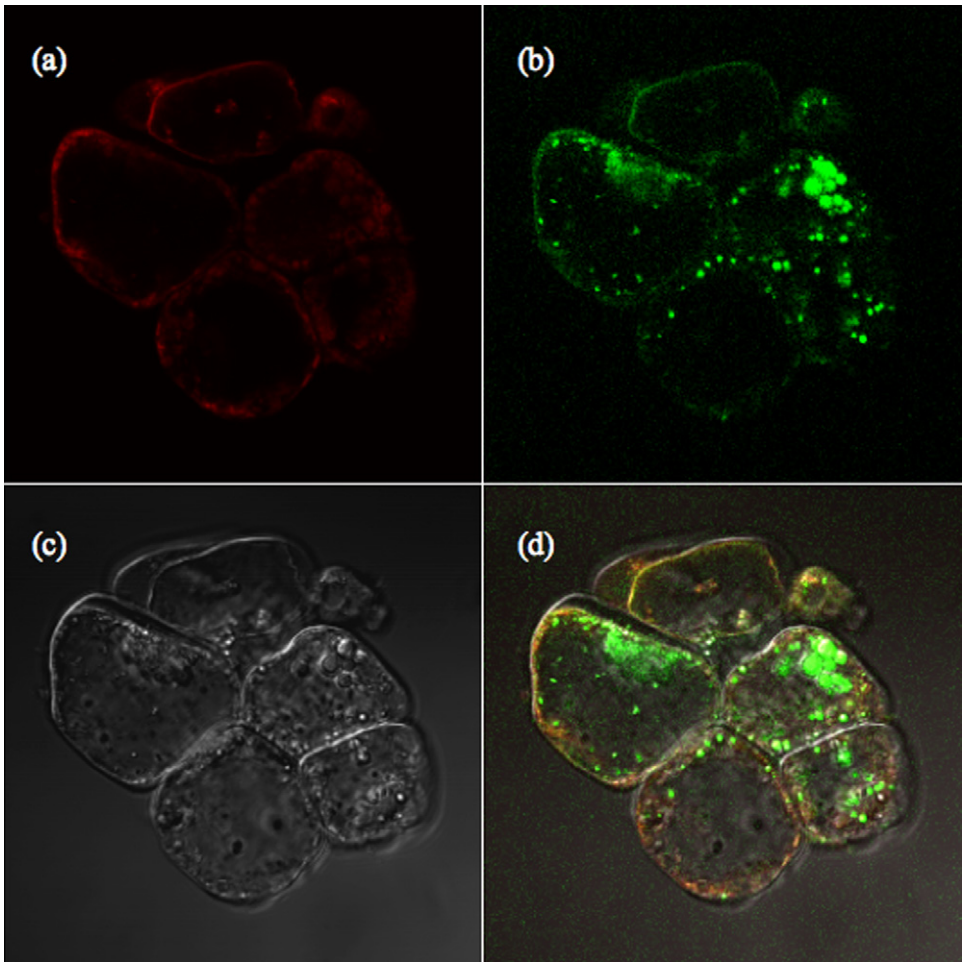

Fig. 3. Localization of MerP in transgenic plant. (a) Callus membrane was stained with Nile red (red). (b) MerP with antibody detected by Fluorescein (FITC)-conjugated AffiniPure Goat Anti-Rabbit IgG (H + L) (green). (c) White light. (d) Colocalization of fluorescent signals. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

gene on RNA level was examined by *merP* mRNA-targeted reverse transcription-PCR (Fig. 2b). Total RNA of both wild type and transgenic plants were extracted as the template. Primers used were the same as those for PCR made from sequences on both end of the gene to ensure the detection of fully transcribed messages. The total mRNA from mercury induced MB1 strain was used as a positive control. As demonstrated in Fig. 2b, only mRNA from transgenic plant showed the messages from the RT-PCR of expected size and no messages can be detected from wild type plant. These results confirm the stable harboring of the *Bacillus merP* gene in *A. thaliana* transgenic plant as well as its expressing under control of plant actin promoter.

3.2. Localization of MerP in transgenic plant

Plant calluses were analyzed by confocal microscopy to determine the cellular localization of the bacterial MerP. For simultaneous fluorescent detection of MerP and membrane, either the MerP specific antiserum (*green fluorescence*) or Nile red (*red fluorescence*) was used to stain the plant cells. Colocalization of red and green fluorescence signals indicates that MerP is localized at the cell membrane and vesicles of plant cells (Fig. 3). The localization of MerP on the original host strain, *Bacillus megaterium* MB1, was observed and the MerP signals also occurred on the bacterial surface (data not shown). Although MerP is known as a periplasmic protein in Gram-negative bacterial mercury resistance system, the location of Gram-positive bacterial MerP has not yet been precisely identified. It is interesting to observe the existence of MerP protein in Gram-positive bacteria because of the lack of periplasmic space [1]. It has been shown in the literature that, without any modification in signal peptide or coding region, a membrane protein of bacterial heavy metal transporter, ZntA, has been successfully expressed in plasma membrane of the transgenic *Arabidopsis* [20]. Similarly, observation by confocal microscopy suggests that MerP can also be localized at plasma membrane of transgenic *A. thaliana* without any modification in signal peptide or coding region. How and why bacterial membrane components function in plant cell are interesting topics that remain to be elucidated.

3.3. Heavy metal tolerance of the merP transgenic plant

Mercury tolerance of BmerP-1 was also examined. A control test was performed to show no differences between the transgenic lines and wild type plant on mercury-non-contained condition (Fig. 4). In the presence of 10 and 12.5 μM $HgCl_2$ on MS plate, the wild-type plants seeds did not germinate while the BmerP-1 seeds germinated and grew vigorously (Fig. 5a). When 15 μM $HgCl_2$ was added into the plate, both wild-type seeds and transgenic seeds could not germinate. To examine the feasibility of using the *merP* transgenic plant in phytoremediation of heavy metals other than mercury, the tolerance for lead and cadmium were also tested. In the presence of 15 μM $CdCl_2$, the growth of BmerP-1 was apparently better than that of wild-type plants (Fig. 5b). The same trend was observed for the case of 32.5 μM $PbCl_2$ (Fig. 5c). The results indicated that MerP may offer a protection effect in plants to avoid the toxicity of those heavy metals.

Fig. 4. A control test of tolerance assay showing the comparison of the growth of transgenic (merP-1 and merP-5) and wild type (WT) plants on the MS medium agar plate containing no mercury.

3.4. Heavy metals accumulation of the merP transgenic plant

Both transgenic and wild type plants were grown on the same medium containing 10 μM mercuric chlorides for 24 days to compare the mercury accumulation capability of those plants. The transgenic plants were able to grow with an average fresh weight of approximately 20 mg for each plant (Fig. 6a). In contrast, wild-type plant seeds did not germinate under the same culture conditions. The average mercury adsorption of the twenty-five BmerP-1 plants was 0.107 μg for each plant (Fig. 6b), giving an adsorption capacity of 5.35 μg/g fresh weight. The results confirm the accumulation of mercury in transgenic plants at a higher mercury concentration at which the wild-type plant was unable to survive. When the plants were grown on medium containing 15 μM cadmium chlorides for 12 days, the average fresh weight of BmerP-1 was 56% higher than that of wild type plants (Fig. 6a). Over the fifty plants examined, the BmerP-1 adsorbed 0.019 μg cadmium in average for each plant (Fig. 6b) with a capacity of 3.4 μg/g fresh weight, while the adsorption capacity of the wild-type plant was 5.0 μg/g fresh weight (0.018 μg total adsorption for each plant) (Fig. 6b). On the other hand, in the presence of 32.5 μM lead chloride, the fresh weight of BmerP-1 was 22% higher than that of wild type plants (Fig. 6a) and the average lead adsorption was 0.09 and 0.08 μg for each BmerP-1 and wild-type plants, respectively, with a capacity

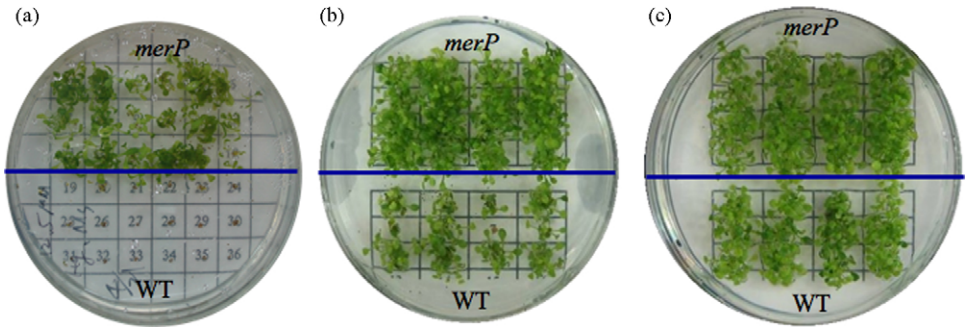

Fig. 5. Heavy metal tolerance assays. Growth comparison of transgenic (merP) and wild type (WT) plants grew in the MS medium agar plate containing (a) 10 μM mercuric chlorides, (b) 15 μM cadmium chlorides, and (c) 32.5 μM lead chlorides after 2 weeks.

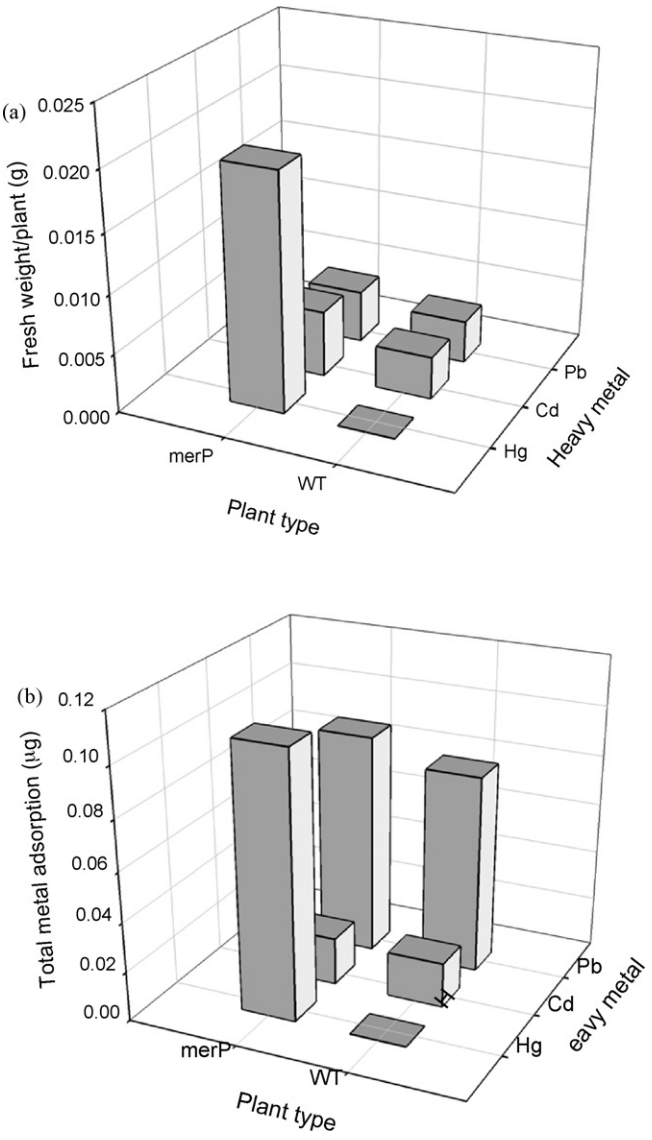

Fig. 6. Heavy metal biosorption in transgenic plant. For metal biosorption tests, seeds were germinated and grew on the MS medium containing 12.5 μM mercuric chlorides (for 24 days), 15 μM cadmium chloride (for 12 days), and 32.5 μM lead chloride (for 12 days). (a) Average fresh weight and (b) average metal adsorption obtained from 25 plants (in the presence of mercury) and 50 plants (in the presence of cadmium and lead). WT: wild-type plant (without merP expression); merP: transgenic plant with merP expression. The deviations of those duplicate or triplicate data were within the range pf 4–9%.

of 20.2 and 22.2 μg/g fresh weight (Fig. 6b). These results show that expressing MerP protein in *A. thaliana* could enhance the cell growth and metal tolerance of the host plant, resulting in a higher total metal accumulation. However, the metal adsorption capacity per fresh weight of the transgenic plant was similar or even slightly lower than the wild-type one. The total adsorption capacity of each transgenic plant was more than wild type, due to a larger amount of biomass for transgenic plant than that for wild type plant. However, the metal adsorption capacity per fresh weight in transgenic plants did not increase along with the increase in total adsorption capacity. This may be because the adsorption capacity and the amount of increased biomass were not linearly correlated. Nevertheless, the MerP protein expressed on the cell surface seemed to play a crucial role in protecting the cell from the toxicity of the three heavy metals examined. This result is consistent with the findings that MerP protein is dispensable for the transport and may function as a "mercury sponge" to compartmentalize the toxic mercuric ion [17].

By expressing the MerP protein in bacterial hosts [19] and plants, our studies showed that MerP of Gram-positive bacterial system with adjoining cysteines is functional in host cell for both metals binding and tolerance enhancement. This also demonstrates the potential of *merP* transgenic plant BmerP-1 in phytoremediation of a broad range of heavy metals in practice. Further analysis of tolerance for other kinds of heavy metals and their precise minimum inhibition concentrations still needs to be conducted.

4. Conclusions

The work demonstrated that expressing *Bacillus* MerP protein in plant is a feasible approach to develop more effective phyto-sorbent for metal removal from contaminated soil. This MerP-based genetically engineered plant for accumulation of mercury or other heavy metals may offer an alternative metal removal strategy in contrast to phytovolatilization via MerA reduction [21–25]. However, the follow-up treatment of metal-loaded biomass is also a critical problem and technology leading to cost-effective metal recovery from loaded biomass should be developed. Moreover, the protection effect provided by MerP also makes it a good candidate to combine with other mechanisms of engineered plants for detoxification of heavy metals contaminated environments.

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