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Bioremediation of cadmium contaminated soil using symbiosis between leguminous plant and recombinant rhizobia with the *MTL4* and the *PCS* genes

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Abstract

Cadmium contamination in rice grains is one of the important issues in Asian countries. We have developed a novel bio-remediation system based on the symbiosis between leguminous plant and genetically engineered rhizobia. We designed two types of recombinant rhizobia, carrying two genes, synthetic tetrameric metallothionein (*MTL4*) and cDNA encoding phytochelatin synthase from *Arabidopsis thaliana* (*AtPCS*). The *MTL4* and *AtPCS* genes were transferred to *Mesorhizobium huakuii* subsp. *rengei* B3, which can infect and form nodules on Chinese milk vetch, *Astragalus sinicus*. The two genes were fused to the *nolB* or *nifH* promoter, which generated nodule specific expression of these genes in strain B3. The two recombinant strains, B3(pMPnolBMTL4nifHPCS) and B3::nifHMTL4(pMPnifHPCS), showed 25 and 12-fold increase in Cd concentration, in the free-living cells, respectively. When these recombinant strains established the symbiotic relationship with *A. sinicus*, the symbionts increased Cd accumulation in nodules by two-fold in hydroponic culture. The expression of the both *MTL4* and *AtPCS* genes showed additive effect on cadmium accumulation in nodules. We also applied these recombinant bacteria to rice paddy soil polluted with Cd (1 mg kg^{-1} dry weight soil). The accumulation of Cd increased not only in nodules but also in the roots of *A. sinicus* infected by the recombinant rhizobia. The accumulation of Cd in the plant roots infected by B3(pMPnolBMTL4nifHPCS) achieved three-fold than that by the wild-type B3. After two months of cultivation of the symbiont, a maximum of 9% of Cd in paddy soil was removed. Thus, the symbiosis will be useful in phytoremediation for heavy metals.

Keywords: Phytoremediation; Cadmium; Symbiosis; Leguminous plant; Rhizobia

1. Introduction

Cadmium, which arises mainly from mining electroplanting, smelting and other industrial activities, is one of the contaminating heavy metals in soil. Historically farmers in Asian countries, especially in Japan were confronted with a symptom, Itai-itai disease, for years. The Itai-itai disease resulted from rice grains containing large amounts of Cd supplied by river water from metalliferous mine. This disease has not yet been eradicated. Unlike many other pollutants, heavy metals are difficult to remove from environment. These metals cannot be chemically or biologically degraded, and are ultimately indestructible. The use of metal-accumulating plants to remove potential toxic metals, including Cd, from soil has been proposed. Although the process, "phytoremediation", may provide an economically viable solution for the remediation of metal-polluted sites, it has several problems that should be overcome. It requires a much longer time to remove pollutants than civil engineering works, such as soil exchange, or cannot be applied to high-level-metal-polluted sites, because of the sensitivity of plants to heavy metals. To shorten the treatment period and improve decontamination capacities of plants, the improvements of plant

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abilities by genetic engineering, that is, by modifying characteristics such as metal uptake, transport and accumulation of heavy metals as well as metal tolerance, opens up new possibilities for phytoremediation. To date, only a few cases have been reported in which one or more of these characteristics have been successfully altered. It would be of considerable interest if we could exploit the symbiotic relationship between leguminous plants and rhizobia for the improvement of plant abilities by introducing genetically engineered rhizobia to plant roots.

Rhizobia are Gram-negative bacteria that can establish a symbiotic relationship with leguminous plants. A successful infection by a single bacterium can lead to the formation, on a root of a legume, of a nitrogen-fixing nodule that contains more than 10^8 bacterial progenies (Downie, 1997). Recombinant rhizobia in each nodule on a root of a legume are advantageous for the expression of foreign genes that help to sequester heavy metals in contaminated soil. *Mesorhizobium huakuii* subsp. *rengei* strain B3 (Murooka et al., 1993; Nuswantara et al., 1999) is the bacterium that establishes a symbiotic relationship with *Astragalus sinicus*, the legume that has been used as green manure in rice fields in China and Japan, by eliciting formation of nitrogen-fixing root nodules (Chen et al., 1991). It would be advantageous if we could use this leguminous plant to increase fertilizer-nitrogen and, at the same time, to remove heavy metals from soil. In previous reports, we described the introduction of the gene encoding metal-binding protein, synthetic tetrameric metallothionein (*MTL4*) (Hong et al., 2000) or *Arabidopsis* phytochelatin synthase (*AtPCS*) (Rauser, 1995; Zenk, 1996; Cobbett, 2000), into *M. huakuii* subsp. *rengei* strain B3 (Sriprang et al., 2002, 2003). Each gene was expressed under the control of a bacteroid-specific promoter, *nifH* or *nolB* (Ruvkun et al., 1982; Perret et al., 1999). Resultant recombinant strains enhanced the accumulation of Cd in free-living cells. The MTL4 and PCS proteins were detected by immunostainings in bacteroids in mature nodules on *A. sinicus* roots (Sriprang et al., 2002, 2003).

In this study, we expressed both of *MTL4* and *AtPCS* in strain B3 in an effort to increase Cd content in bacteroid in nodules. We analyzed the Cd accumulation in free-living cells and nodules on roots carrying the two genes. We also applied this symbiosis to the decontamination of Cd in rice paddy soil polluted by Cd, and successfully demonstrated the applicability of the symbiosis for *in situ* phytoremediation.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The bacterial strains used were *Esherichia coli* DH5α (Sambrook and Russell, 2001) as the host for constructing various plasmid DNAs, and *M. huakuii* subsp. *rengei* B3 isolated from nodules of *A. sinicus* cv. Japan, which had been grown in a rice paddy in Hiroshima, Japan as the host through the experiments (Murooka et al., 1993; Nuswantara et al., 1999). The cloning vector pT7Blue T-vector was purchased from Novagen (San Diego, USA). The pMP220 plasmid DNA (Spaink et al., 1987) was used for the expression of the *MTL4* and *AtPCS* genes. *E. coli* cells were grown at 37 °C in LB broth (Sambrook and Russell, 2001) or on agar (1.5%; w/v) plates supplemented with appropriate antibiotics. Strain B3 was grown in TY medium (Beringer, 1974) and incubated at 30 °C.

2.2. Gene constructs

The 1.1-kb *nolB-MTL4* fusion gene from pT7nolBMTL4 (Sriprang et al., 2002) digested with *Eco*RI was ligated to pMPnifHPCS (Sriprang et al., 2003) digested with *Eco*RI followed by treatment with calf alkaline phosphatase. The resulting plasmid was designated as pMPnolBMTL4nifHPCS (Fig. 1). The restriction enzymes and molecular biological enzymes were purchased from TaKaRa Biotechnology Co., Ltd. (Otsu, Japan) and used according to the manufacturer's directions.

2.3. Electroporation of expression vectors into rhizobium strains

The expression vectors, pMPnolBMTL4nifHPCS and pMPnifHPCS, were transformed by electroporation (Hayashi et al., 2000) into strain B3 and mutant strain B3::nifHMTL4 (Sriprang et al., 2002), respectively. Transformants carrying pMPnolBMTL4nifHPCS or pMPnifHPCS were selected on TY agar medium supplemented with tetracycline (20 μg ml^{-1}). Several clones were isolated and the plasmid DNA in the clones was confirmed by restriction enzyme digestion.

2.4. Microaerobic culture of free-living rhizobia and quantitation of Cd

Cd accumulation in free-living rhizobia was analyzed under microaerobic conditions. A 10 ml cell culture (A_{600} 0.2–0.4) was transferred to a sealed 100 ml bottle. The sample was flushed with a mixture of 1% O_2 and 99% N_2 at 0.55 l min^{-1} for 5 min at room temperature. $CdCl_2$ was added to the bottle to obtain a final concentration of 30 μM and cells were grown at 28 °C with gentle shaking (100 rpm) for 40 h. For Cd extraction from bacterial cells, the cells were pelleted, washed twice in 5 mM HEPES (pH 7.1) containing 0.85% NaCl, dried at 65 °C for 24 h, and suspended overnight in 70% nitric acid (Romeyer et al., 1988). The concentration of Cd was measured directly in the soluble fraction using an atomic absorption spectrophotometer (AAS) (model AA-8500 MARK II; Jarrel Ash, Tokyo, Japan).

2.5. Nodule formation and Cd accumulation test in hydroponic culture

A. sinicus seeds were surface-sterilized, sown on 0.7% agar plate and incubated for three d in the dark at 25 °C.

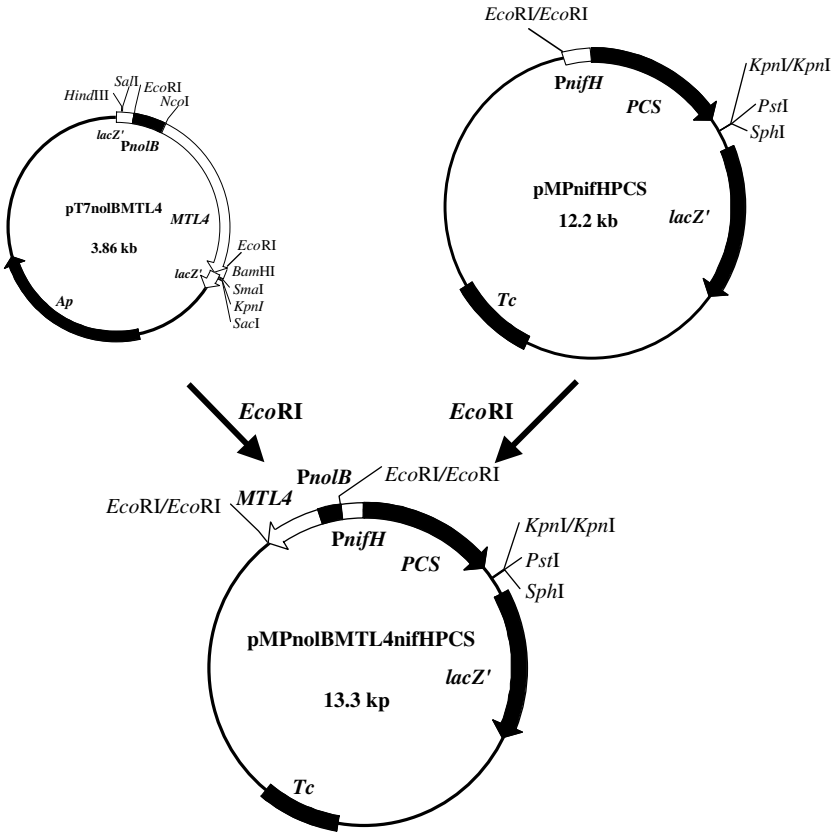

Fig. 1. Construction of *AtPCS* and *MTL4* expression vectors pMPnolBMTL4nifHPCS. Abbreviation: P*nifH*, the promoter of *nifH*; P*nolB*, the promoter of *nolB*; *Ap*, ampicillin resistant marker gene; *Tc*, tetracycline resistant marker gene; *lacZ*, β-galactosidase gene.

Seedlings were transferred to vermiculate soil in screw-capped glass bottles containing a nitrogen-free (NFR) medium (Murooka et al., 1993), and then inoculated with approximately 10^6 cells of wild-type B3 or recombinant B3. After two weeks of cultivation under light (16 h photoperiod per a day) at 25–28 °C, nodule formation observed. Plants with nodules were transferred to the NFR medium supplemented with 50 μM $CdCl_2$ for hydroponic culture. After two weeks of hydroponic culture, plants were harvested, washed in 10 mM EDTA and then in 0.1 N HCl, dried at 105 °C for 24 h, and suspended in 70% nitric acid. Cd concentration was measured directly from the soluble fraction using AAS.

2.6. Phytoremediation of Cd polluted soil

The soil remediation ability of the plant-bacteria symbiosis was tested using a rice paddy soil without Cd contamination, which was supplied with $CdCl_2$ solution to obtain a Cd concentration of 1 mg kg^{-1} dry weight soil. The pots (7.5 cm depth, 6 cm bottom diameter) contained a mixture of each 200 g of (wet weight) soil and approximately 10^6 cells of wild-type B3 or recombinant B3. Six seedlings of *A. sinicus*, which were three d old after germination, were transferred to one pot and grown under light (16 h photoperiod per a day) at 25–28 °C for two months. Shoots, roots and nodules were harvested from two month-old plants, washed in 10 mM EDTA (and further washed in 0.1 N HCl for roots and nodules), dried at 105 °C for 24 h and suspended overnight in 70% nitric acid. Cd concentration was measured directly from the soluble fraction using AAS.

3. Results

3.1. Accumulation of Cd in free-living cells

We designed two recombinant strains, of *M. huakuii* subsp. *rengei*, carrying two metal-binding protein genes, *MTL4* and *AtPCS*. Cultures of recombinant strains carrying each gene or both genes were supplemented with 30 μM $CdCl_2$ and incubated under microaerobic conditions. The amounts of Cd accumulated were 3.8 ± 1.5 μg mg^{-1} dry weight cells ($n = 3$) in the recombinant strain, namely B3(pMPnolBMTL4nifHPCS) cells, which is carrying both of *MTL4* and *AtPCS* genes, 2.6 ± 0.8 μg mg^{-1} dry weight cells ($n = 3$) in the single-recombinant strain, namely B3(pMPnifHPCS), and 0.15 ± 0.04 μg mg^{-1} dry weight cells ($n = 3$) in wild-type B3 (Fig. 2). The B3(pMPnolBMTL4nifHPCS) cells accumulated Cd 25- and 1.4-fold more than the wild-type B3 and B3(pMPnifHPCS), respectively. The amounts of

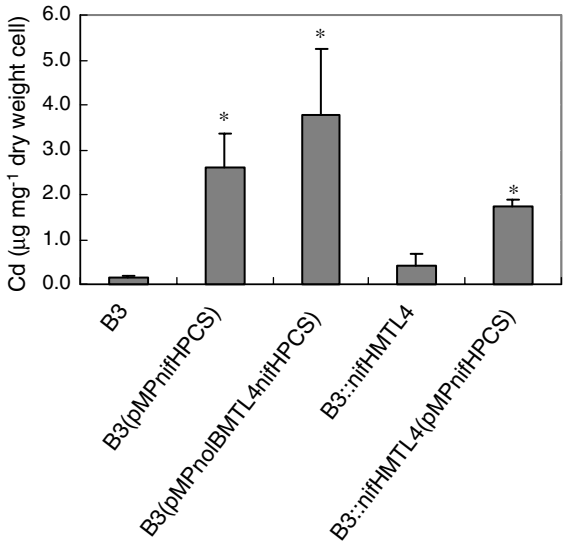

Fig. 2. Accumulation of Cd in the recombinant *M. huakuii* subsp. *rengei* B3 cells that expressed *AtPCS* and/or *MTL4* gene. Bacteria were grown under microaerobic conditions in TY medium plus 30 μM $CdCl_2$ for 40 h. The Cd concentrations shown are means (+ standard deviations) of results of three independent experiments in each case. The asterisks (*) indicate that the values are significantly ($P < 0.05$) higher than those for B3.

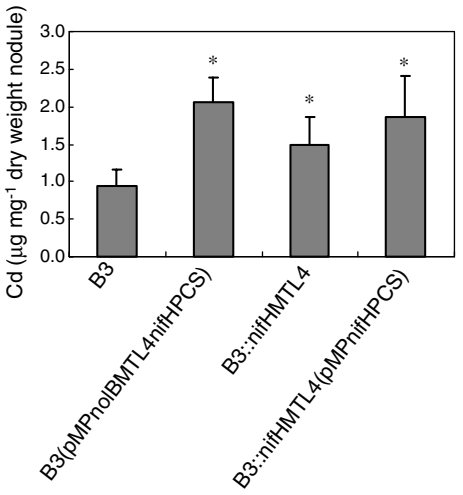

Fig. 3. Accumulation of Cd in nodules containing bacteroids from B3 and recombinant strain of B3 carrying *AtPCS* and/or *MTL4* gene. *A. sinicus* infected by B3 were cultured hydroponically in the NFR medium containing 50 μM $CdCl_2$ for two weeks. The asterisks (*) indicate that the values are significantly ($P < 0.05$) higher than those for B3.

Cd accumulated were 1.7 ± 0.2 μg mg^{-1} dry weight cells ($n = 3$) in another recombinant B3, carrying both of *MTL4* and *AtPCS*, namely the B3::nifHMTL4-(pMPnifHPCS), which was constructed by insertion of *nifHMTL4* in the chromosomal DNA and transferred a plasmid with the *AtPCS* gene under the *nifH* promoter, and 0.4 ± 0.3 μg mg^{-1} dry weight cells ($n = 3$) in the single-recombinant strain, namely B3::nifHMTL4 (Fig. 2). The B3::nifHMTL4(pMPnifHPCS) cells accumulated Cd 12- and 4.3-fold more than the wild-type B3 and B3::nifHMTL4, respectively. Thus, the expression of the two metal binding genes showed additive effect on accumulation of Cd in free-living bacterial cells.

3.2. Accumulation of Cd in nodules in hydroponic culture

The nodulation ability on *A. sinicus* root infected by constructed recombinant B3 strains, B3(pMPnolBMTL4-nifHPCS) and B3::nifHMTL4(pMPnifHPCS) was ascertained after two weeks of cultivation in sterilized vermiculite soil. The stability of transformed plasmids in nodule bacteroids was checked by PCR using nodules as template, after two months of cultivation (Sriprang et al., 2002). To assess whether the *MTL4* and *AtPCS* were expressed in bacteroids stably, we also performed immunostaining using MT antibody and anti-His antibody to detect the presence of MT and PCS-His-tag in bacteroids in nodules, respectively (Sriprang et al., 2002, 2003). Each gene was expressed and the produced proteins were detected in root nodules under the control of either *nifH* or *nolB* promoter. To examine whether the bacteroids in nodules show a high Cd accumulation ability, seedlings harboring several nodules were cultivated hydroponically for two weeks in the presence of 50 μM $CdCl_2$. The amounts of Cd accumulated in nodules containing bacteroids from B3(pMPnolBMTL4nifHPCS) and B3 were 2.1 ± 0.3 and 0.95 ± 0.2 μg mg^{-1} dry weight nodules, respectively (Fig. 3). Cd concentration in the nodules on root infected by B3(pMPnolBMTL4nifHPCS) increased by 2.2-fold compared with that from the wild-type B3. The recombinant strain conferred the two genes was more effective than a single recombinant strain, B3(pMPnifHPCS), which showed only 1.5-fold higher accumulation than that in the wild-type B3 (Sriprang et al., 2003). The amounts of Cd accumulated in nodules containing bacteroids from B3::nifHMTL4(pMPnifHPCS) and B3::nifHMTL4 were 1.9 ± 0.5 and 1.5 ± 0.4 μg mg^{-1} dry weight nodules, increased by 2.2 and 1.6-fold, respectively, compared with that from B3. Both of the two foreign genes contributed to the promotion of Cd uptake, resulting in the generation of effective Cd-accumulating plants.

3.3. Phytoremediation of Cd contaminated soil

We performed several tests in pots using rice paddy soil supplied Cd, to apply the symbiosis to phytoremediation. The Cd concentration in soils was adjusted to 1 mg kg^{-1} dry weight soil, which is lower than that in the free-living cell tests or hydroponic culture tests examined in this study. Even under the conditions of a rich nitrogen source and Cd contamination in soil, the *A. sinicus* seedlings formed nodules at 100–300 per pot. These numbers were similar to that in soil without Cd. The nodules might contain those derived from autochthonous rhizobia in rice paddy soil used in the study. The plants infected by recombinant B3 showed no severe growth inhibition (Fig. 4b and d). The shoots, roots and nodules were harvested and Cd concentration was measured (Fig. 5). Cd appears to be

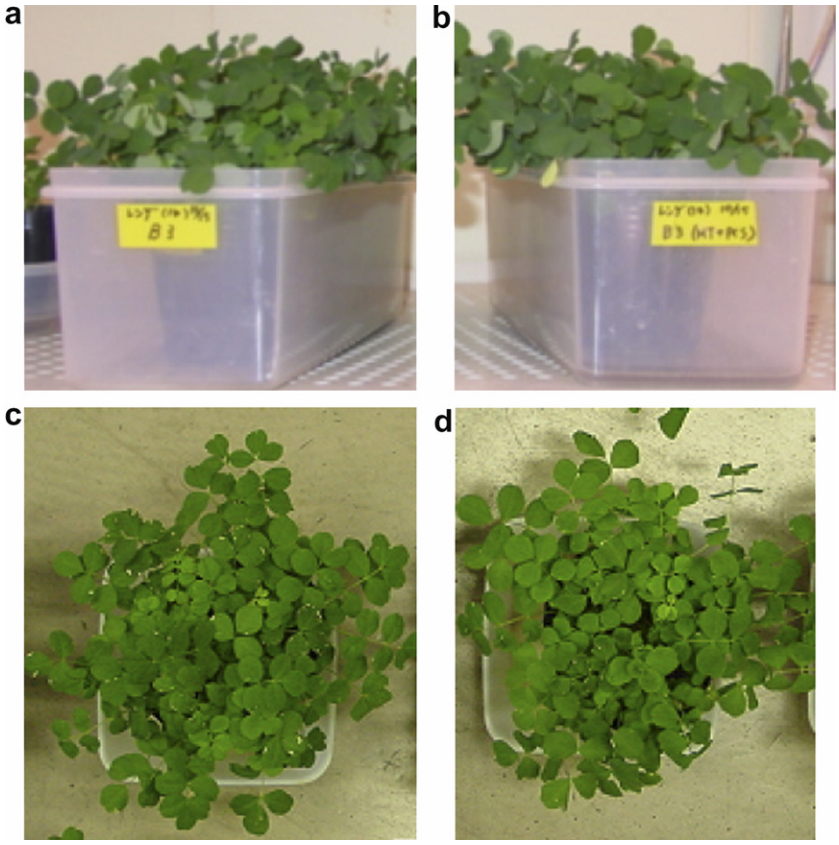

Fig. 4. Cultivation of the symbiosis in Cd-contaminated soil. Cd phytoremediation tests were performed using rice paddy soil. *A. sinicus* infected by *M. huakui* B3 (a, c), B3(pMPnolBMTL4nifHPCS) (b), or B3::nifHMTL4(pMPnifHPCS) (d) cells were cultivated in soil for two months.

accumulated in under ground tissues (nodules and root) rather than in the shoots of *A. sinicus*. No significant difference between the Cd concentrations in shoots of *A. sinicus* infected by wild-type B3 and those infected by recombinant strains were observed (Fig. 5a). The Cd concentrations in nodules and root of *A. sinicus* infected by B3(pMPnolBMTL4nifHPCS) and B3::nifHMTL4-(pMPnifHPCS) strains were higher than that infected by wild-type B3 (Fig. 5b and c). The Cd concentrations in nodules were 9.7 ± 2.4, 13 ± 5.1 and 13 ± 1.7 mg kg^{-1} dry weight, and those in root were 8.9 ± 3.2, 26 ± 6.0 and 22 ± 10 mg kg^{-1} dry weight, in *A. sinicus* in symbiosis with B3, B3(pMPnolBMTL4nifHPCS) and B3::nifHMTL4(pMPnifHPCS), respectively (Fig. 5b and c). Cd concentration was increased not only in nodules but also in root.

4. Discussion

We have developed a heavy-metal phytoremediation system based on the symbiosis between the genetically engineered rhizobia and its host plant. We have demonstrated the utilization of the symbiosis for the remediation of metal-polluted rice paddy soil. In our previous studies, we expressed the tetrameric metallothionein gene *MTL4*, or the phytochelatin synthase gene *AtPCS*, in *M. huakuii* subsp. *rengei* B3 to improve the metal-accumulating ability of the rhizobium (Sriprang et al., 2002, 2003). In this study, we generated recombinant B3 strains carrying both *MTL4* and *AtPCS* genes to obtain a much greater metal accumulation. We then estimated the possibility of *A. sinicus* infected by recombinant strains for phytoremediation of Cd-polluted rice paddy soil.

We found a significant enhancement of Cd accumulation in free-living rhizobium cells containing both of the *MTL4* and *AtPCS* genes compared with cells containing only *MTL4* or *AtPCS*. The stepwise improvement of Cd accumulation ability in single-recombinant strains with either one gene and recombinant strains with the two genes suggested that both metal-binding genes induced into B3(pMPnolBMTL4nifHPCS) and B3::nifHMTL4-(pMPnifHPCS) were successfully expressed in free-living cells. The single-recombinant B3 harboring pMPnifHPCS showed 17-fold more Cd accumulation than wild-type B3. Sriprang et al. (2002) reported that the single-recombinant B3 harboring pBBRnolBMTL4 showed 2.1-fold more Cd accumulated than wild-type B3. Taken together, the contribution of the *AtPCS* gene to Cd accumulation was much larger than that of *MTL4* gene on the pMPnolBMTL4nifHPCS.

Free-living B3(pMPnolBMTL4nifHPCS) and B3::nifHMTL4(pMPnifHPCS) cells accumulated 25- and 12-fold more Cd than wild-type B3, respectively. B3::nifHMTL4(pMPnifHPCS) cells showed a lower Cd

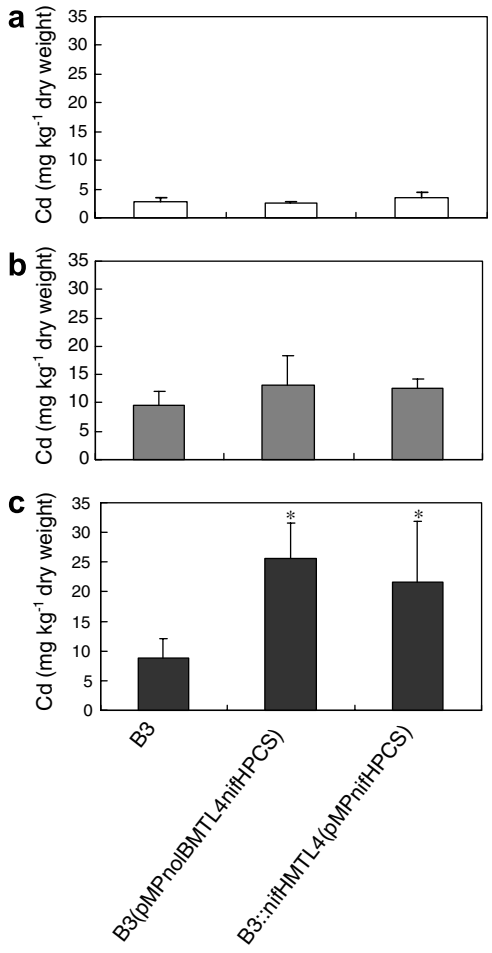

Fig. 5. Accumulation of Cd in *A. sinicus* infected by the recombinant *M. huakui* B3 cells in Cd contaminated rice paddy soil. Cd concentrations in shoot (a), nodules (b) and root (c) in *A. sinicus*. The asterisks (*) indicate that the values are significantly ($P < 0.05$) higher than those for B3.

accumulation capacity than B3(pMPnolBMTL4nifHPCS) cells, because a copy number of the *MTL4* gene in the chromosome of B3::nifHMTL4(pMPnifHPCS) is lower than that in the plasmid of B3(pMPnolBMTL4nifHPCS).

In a previous paper (Sriprang et al., 2002), we showed a slight enhancement of Cd accumulation in nodules containing recombinant B3 carrying the *MTL4* gene. In this study, the hydroponic Cd uptake test showed that Cd content in nodules carrying B3 (pMPnolBMTL4nifHPCS) and B3::nifHMTL4(pMPnifHPCS) increased significantly by approximately two-fold than that carrying wild-type B3. The genetic modifications in the rhizobium can be effective for the host plant. Cd^{2+} ions in liquid medium would pass through outer membrane of nodules and bacteroid membrane, and was taken up in bacteroids. The corporated Cd^{2+} ions were chelated by PCs and MTL4, consequently Cd accumulation in nodules was enhanced.

Although free-living B3(pMPnolBMTL4nifHPCS) cells accumulated much higher Cd than free-living B3::nifHMTL4(pMPnifHPCS) cells, Cd accumulation in the nodules derived from these two recombinant strains was almost similar level in the hydroponic test. It might be considered that the Cd amount provided to nodule was disproportionate to the high accumulation capacity in the bacteroid containing pMPnolBMTL4nifHPCS.

We applied *A. sinicus* symbiosis with recombinant rhizobia to the soil remediation and found a good growth of plants. The nodules containing B3(pMPnolBMTL4nifHPCS) and B3::nifHMTL4(pMPnifHPCS) showed a higher Cd concentration than those containing wild-type B3, indicating that the recombinant bacteroids contribute to an enhanced Cd accumulation not only in hydroponic culture but also in soil.

Beyond our expectation, Cd concentration increased not only in the nodules but also in the roots of *A. sinicus* infected by recombinant B3. The roots infected by B3(pMPnolBMTL4nifHPCS) achieved a 2.9-fold of Cd accumulation than those infected by wild-type B3. It is suggested that the foreign proteins, MTL4 and PCS, was produced in the nodules and also in rhizobia grown in soil around roots. The free-living recombinant B3 could contribute to the collection of Cd in soil and transport to legume roots. Generally, the rhizobium-legume symbiosis is initiated when flavonoids and related plant compounds induce the bacterium to produce molecular signals that stimulate nodule organogenesis (Fisher and Long, 1992). Free-living rhizobia in soil could move to the surface of leguminous roots by signal interaction. If recombinant B3 take up much more Cd from soil than wild-type B3, and transport them to the root surface, it might lead to a density gradient of the rhizobia in rhizosphere and the roots could take up a greater amount of Cd. In hydroponic culture, the Cd concentration in roots infected by B3(pMPnolBMTL4nifHPCS), 1.2 ± 0.2 μg mg^{-1} dry weight roots, was similar to that infected by wild-type B3, 1.1 ± 0.2 μg mg^{-1} dry weight roots. Since the plant roots in hydroponic culture were exposed by the liquid medium with high Cd concentration, recombinant rhizobia might not contribute to the collection of Cd in medium solution.

We achieved the maximum Cd removal from rice paddy soil containing Cd of 1 mg kg^{-1} dry weight soil, using the recombinant strain, B3(pMPnolBMTL4nifHPCS). The Cd concentrations in *A. sinicus* in symbiosis with B3(pMPnolBMTL4nifHPCS) were 2.6 ± 0.3 and 26 ± 6.0 mg kg^{-1} dry weight in shoots and root, respectively. The bioaccumulation factors (concentration in dry plant tissue/concentration in dry soil) of Cd were 2.6 for shoot and 26 for root, which were at least three or four times higher than that for shoot and root of general crop plant such as sorghum, cucumber, wheat and sweet corn cultivated in various Cd contaminated soil (0–60 Cd mg kg^{-1} dry weight soil) (An, 2004). At the maximum, we obtained biomasses of 1451 mg-dryweight pot^{-1} and Cd concentrations of 6.7 mg kg^{-1} dry weight (whole plant), i.e. the Cd content of 9.7 μg pot^{-1} in the entire plant. Accordingly, this result indicates that about 9% of Cd in the polluted soil is removed in 60 days, suggesting a great potential of the symbiosis for the phytoremediation of Cd-contaminated

soil. For improving Cd removal efficiency, the stimulation of nodulation and growth in root will be more effective and simple strategy. The overexpression of both *MTL4* and *AtPCS* in *M. huakuii* subsp. *rengei* B3 appears to be a promising method of improving the phytoremediation of soil contaminated by multiple heavy metals such as Cd, Cu, Ag, Hg and Zn, which form complexes with metallothionein and phytochelatin.

In this study, we developed a very useful symbiosis between the leguminous plant and the genetically modified rhizobium, in which multiple foreign genes could be coincidentally expressed. We also demonstrated the possibility to apply the genetically engineered plant for the remediation of metal-contaminated soil.

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