Journal of Plant Biology, February 2007, 50(1) : 1-7

Chloroplast-Targeted BrMT1 (*Brassica rapa* Type-1 Metallothionein) Enhances Resistance to Cadmium and ROS in Transgenic *Arabidopsis* Plants

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Metallothioneins (MTs) are low-molecular-weight, cysteine-rich proteins that bind to heavy metals. Type-1 MTs function under various abiotic stresses, including exposure to the cadmium ion. We have now isolated the *Brassica rapa* type-1 metallothionein gene (*BrMT1*) using yeast systems, and have found that it confers resistance to Cd in otherwise Cd-sensitive yeast. Using a constitutive CaMV35S promoter and an RbsS transit peptide, we successfully targeted BrMT1 to the chloroplasts of *Arabidopsis*. Overexpression in either the chloroplasts or the cytosol effectively detoxified cadmium and H_2O_2 stresses in transgenic *Arabidopsis*. In particular, the chloroplast-targeted BrMT1 was associated with a significant reduction in paraquat-induced chlorosis and the accumulation of H_2O_2. This is the first report regarding the effects of type-1 MT1 targeted to chloroplasts. Our results suggest that this may be applicable to the development of plants with enhanced tolerance against environmental stresses.

Keywords: BrMT1, cadmium, DAB staining, H_2O_2, metallothioneins, ROS

Metallothioneins (MTs) are low-molecular-weight, cysteine-rich metal-binding proteins (Hamer, 1986; Robinson et al., 1993). The proteins and genes of MT occur in plants, animals, and prokaryotes (Cobbett and Goldsbrough, 2002). Based on the arrangement of their Cys residues, these proteins are grouped into three classes: I, II, and III (Suzuki et al., 1993; Binz and Kägi, 1999; Cobbett and Goldsbrough, 2002). Class II includes the MTs from plants, fungi, and invertebrate animals. The plant Class II MT proteins can be further grouped into types 1 through 4, according to their amino acid sequences, particularly the positioning of the cysteine residues (Robinson et al., 1993; Cobbett and Goldsbrough, 2002). Type-1 MTs (MT1) have six Cys-Xaa-Cys motifs (where Xaa represents another amino acid), which are equally distributed in both the N- and C-termini. Approximately 40 amino acids are usually detected between the two domains, including the aromatic amino acids. However, some plant MT1s, from a variety of Brassicaceae members, lack this 40-amino acid spacer (Buchanan-Wollaston, 1994; Zhou and Goldsbrough, 1994). Instead, their cysteine-rich domains are separated by a spacer of less than 10 amino acids, which harbors no aromatic residues.

MTs play a role in the transport of metals, e.g., copper, zinc, and cadmium, in the human body, and also appear to function in the regulation of physiological processes. In animal systems, MTs confer resistance against Cd and H_2O_2 (Chubatsu et al., 1992), inhibit ultraviolet B-induced apoptosis (Baba et al., 1998), and modulate three fundamental processes: (1) the release of gaseous mediators, including the hydroxyl radical or nitric oxide; (2) apoptosis; and (3) the binding and exchange of heavy metals, including Zn, Cd, and Cu (Simpkins, 2000). In addition, they exert moderating effects on the immune system, thereby protecting against several human diseases, including cancer, circulatory and septic shock, coronary artery disease, and Alzheimer's disease (Simpkins, 2000).

In plants, many *MT* genes have been isolated and characterized, with the first report describing the purification of a wheat MT (Lang et al., 1987). Plant type-1 *MT* (*MT1*) genes are expressed abundantly in the roots and shoots (Foley et al., 1997; Cobbett and Goldsbrough, 2002). Transcript levels of *MT1s* increase dramatically in the senescing leaves of *Brassica napus* (Buchanan-Wollaston, 1994), *Arabidopsis* (Garcia-Hernandez et al., 1998), and rice (Hsieh et al., 1995). Expression of plant *MT1s* has also been induced by copper in *Arabidopsis* (Zhou and Goldsbrough, 1994), rice (Hsieh et al., 1995), wheat (Snowden et al., 1995), tobacco (Choi et al., 1996), *Fucus* (Morris et al., 1999), and *Posidonia oceanica* (Giordani et al., 2000). A variety of other stresses, including exposure to aluminum and cadmium, nutrient deprivation, and heat shock, can also induce *MT1* gene expression in both wheat and rice (Hsieth et al., 1995; Snowden et al., 1995). Finally, *Arabidopsis* MT1 confers cadmium resistance in yeast (Lee et al., 2004), as well as Cd tolerance and accumulation in *Arabidopsis* (Zimeri et al., 2005).

Cadmium, one of the most toxic non-essential elements with high mobility in plants, directly or indirectly inhibits primary physiological processes, such as photosynthesis, water relations, gas exchange, and respiration, and also disrupts plant mineral nutrition (van Assche and Clijsters, 1990; Sandalio et al., 2001; Seregin and Ivanov, 2001). The photosynthetic apparatus appears to be particularly sensitive to cadmium toxicity, even at very low concentrations (about 1% of the total leaf Cd) (Krupa, 1999; Seregin and Ivanov, 2001). Development of oxidative stress under toxic conditions has been fairly well documented, with reactive oxygen species (ROS) being indirectly generated via disruptions in the electron-transport chains, activation of lipoxygenase,

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and alterations in the structure or inhibition of antioxidative metalloenzymes (Sandalio et al., 2001).

Chinese cabbage (*Brassica rapa* ssp. *pekinensis*) is one of the most important vegetable crops in Korea. Its genome constitutes up to half that of *Brassica juncea*, a plant known to be most tolerant to heavy metals, including cadmium. However, little is known about the role of type-1 MT with regard to ROS in the chloroplasts. The objective of our study was to use a yeast system to identify Cd-resistance genes from *B. rapa*, and to introduce a *Brassica MT* gene into *Arabidopsis*, targeted to the chloroplasts. Cadmium tolerance and ROS generation also were assessed in transgenic *Arabidopsis* plants.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Plants of *B. rapa* L. ssp. *pekinensis*, inbred line 'Chiifu', were grown either in the greenhouse or in a growth chamber (22 ± 2°C, 16-h photoperiod from 150 μmol m^{-2} s^{-1} illumination). Seeds from *Arabidopsis thaliana* (L.) Heynh ecotype 'Columbia' and transformed *Arabidopsis* plants were sterilized twice with 70% EtOH, then sown in pots containing a mixture of peat moss, perlite, and cocopeat. The pots were incubated for 3 d at 4°C before being transferred for 3 to 4 weeks to either the greenhouse or a growth chamber (21±1°C under a continuous light intensity of 150 μmol m^{-2} s^{-1}). To evaluate resistance to cadmium, wild-type and transgenic *Arabidopsis* seeds were surface-sterilized, sown on plates of a 1/2 MS medium containing 1.5% sucrose and Cd (II), then maintained at 4°C for 3 d under darkness. After this chilling treatment, the plants were placed for 2 to 3 weeks in a growth chamber (21±1°C, continuous light).

Screening of Cadmium-Resistance Genes Using a Yeast Mutant

To clone cadmium-resistance genes from *B. rapa* using a functional cloning strategy, we generated a cDNA library from Cd-treated *B. rapa* shoots and inserted a root mRNA population into a yeast-expressing vector. The constructs were then transformed into the Cd-sensitive yeast mutant strain DTY167, which lacks the yeast cadmium factor (YCF1), and the resistant yeast clones were screened on Cd (40 μM)-containing media. The type-1 *Brassica rapa* metallothionein gene (*BrMT1*) was selected from the sequence analysis of 200 Cd-resistance genes in yeast. *BrMT1* was re-amplified via PCR using two primers: BrMT1-f: 5'-GGATCCATGGCAGGTTCTAACTG-3' and BrMT1-r: 5'-CTCTAGATTACTCGAGACAGCTGCAGCTGTCC-3'. The forward primer (BrMT1-f) harbors *Bam*HI, while the reverse primer (BrMT1-r) has two enzyme sites, *Xho*I and *Xba*I. The yeast expression vector, pYES2, was constructed using the *Bam*HI and *Xho*I sites.

BrMT1 mRNA Levels in Various Tissues and under Cd Stress

To examine the expression profile of *BrMT1* in Chinese cabbage, various tissues were collected from greenhouse-grown plants. For the stress treatment, three-week-old seedlings were exposed to 40 μM Cd for 24 h. Total RNAs were extracted and subjected to RT-PCR with the primer sets described above.

Arabidopsis Transformation

The RbcS transit peptide was inserted into pCAMBIA3301 at the *Eco*RI and *Bam*HI sites, and the *BrMT1* gene was then cloned into the *Bam*HI-*Xba*I site, yielding the *RbcS-BrMT1-GFP* construct. Two overexpressing vectors for *RbcS-BrMT1* and *BrMT1* were then generated from this construct. All constructs were transferred to *Agrobacterium tumefaciens* strain GV1301 via electroporation, then introduced into *A. thaliana* (Col-0) via floral-dipping (Cough and Bent, 1998; Yoon et al., 2004).

Cadmium-Resistance Test in Yeast

The recombinant plasmid of the pYES2 vector, with or without the *BrMT1* gene, was introduced into the DTY167 cells. Transformants were spotted onto $CdCl_2$-containing half-strength synthetic galactose (SG)-ura plates, and were grown for 3 to 5 d at 30°C.

Measurement of Chlorophyll Contents

Chlorophyll was extracted from fresh rosette leaves for 10 min, using 100% ethanol at 95°C. The extracts were then measured spectrophotometrically at 480, 648, and 664 nm. Chlorophyll contents were calculated via the Moran equation.

Paraquat Treatment

To determine whether the transgenic plants conferred tolerance against oxidative stress, we monitored visible leaf damage caused by various concentrations of paraquat. PQ was first dissolved in 1/10 MS containing 0.025% Tween 20 before the leaves were floated on this solution for 40 h under continuous light.

DAB Staining

To visualize H_2O_2 *in situ*, 3,3-diaminobenzidine (DAB) staining was conducted as described by Thordal-Christensen et al. (1997), with some modifications. Leaves of *A. thaliana* were excised, treated for 24 h with PQ and $CuSO_4$, then submerged through the abraded side in wells containing 5 mL of DAB solution (1 mg mL^{-1} DAB; pH 3.8) for 5 to 8 h at 25°C. The treatment was terminated by immersing the leaves for 10 min in boiling ethanol (95%). After cooling, the leaves were retained in ethanol and photographed.

RESULTS

Classification of BrMTs Based on Amino Acid Sequences and the Distribution of Conserved Cysteine Residues

In all, 200 Cd-resistance genes were identified from the surviving yeast clones and sequence analysis. Following their screening, four *B. rapa* metallothionein genes (*BrMTs*) were cloned (data not shown), and a type-1 gene, *BrMT1*, was

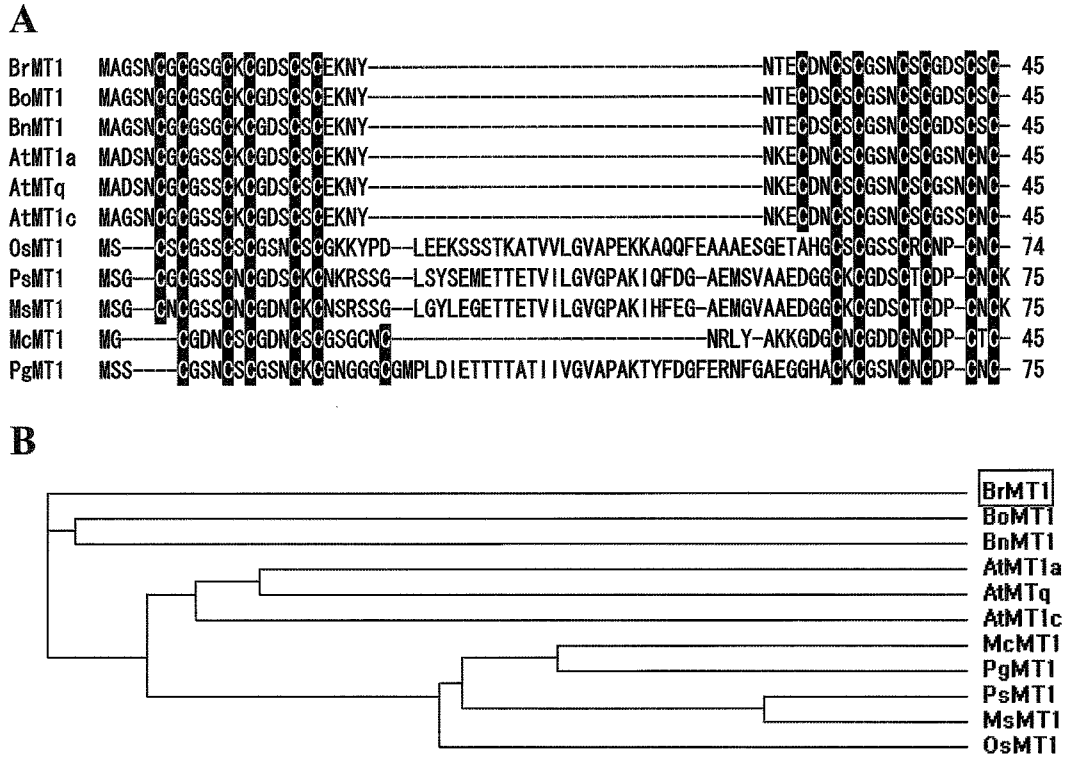

Figure 1. A. Alignment of BrMT1 (*B. rapa* ssp. *pekinensis*) with other plant type-1 metallothioneins. Dark-shadowed boxes indicate cysteine residues. Sequences were derived from BoMT1 (*Brassica oleracea*, AAL67438), BnMT1 (*B. napus*, AAA64489), AtMT1a (*A. thaliana MT1A*, NP_172239), AtMTq (*A. thaliana* metallothionein-like protein, AAA50251), AtMT1c (*A. thaliana MT1C*. NP_172240), PgMT1 (*Panax ginseng*, ABD73299), McMT1 (*Mesembryanthemum crystallinum*, AAC27529), PsMT1 (*Pisum sativum*, BAD18382), MsMT1 (*Medicago sativa*, AAF04584), and OsMT1 (*Oryza sativa*, AAC49626). Numbers in parentheses are GenBank identifications. Amino acid sequences are presented in single-letter code, and have been aligned by introducing gaps (---) to maximize homology. **B.** Neighbor-joining tree representing phylogenetic relationship among MT1s. Alignment of amino acid sequences was performed using CLUSTAL W (1.82) multiple sequence alignment program.

selected for further study. The deduced BrMT1 polypeptide comprised 45 amino acid residues and 2 six-Cys residues at both the N- and C-termini, which were separated by a central Cys-free spacer. All of the Cys residues were located in both terminal domains, in a Cys-X-Cys pattern, which is typical of plant type-1 MTs. A BLAST search of the predicted peptide in the NCBI database revealed a high degree of homology with other type-1 plant MTs (Fig. 1A). These results suggest that *BrMT1* encoded for a type-1 MT-like protein. BrMT1 evidenced the highest degree of identity (98%) with BoMT1 (*B. oleracea* MT1) and BnMT1 (*B. napus* MT1), while the lowest identity (28%) was found with MsMT1 (*M. sativa*, MT1) at the amino acid sequence levels. BrMT1 showed an overall identity of 87 to 91% with its counterparts in *Arabidopsis*, and could be grouped in the same clade with BoMT1 and BnMT1 (Fig. 1B). BrMT1 also harbored an extra Cys residue, as is also true for BoMT1, BnMT1, AtMT1a, AtMTq, and AtMT1c.

BrMT1 Expression Enhances Cd Resistance in *Saccharomyces cerevisiae*

Because *Arabidopsis* MT1 confers cadmium resistance in yeast (Lee et al., 2004), we expected BrMT1 to also induce Cd tolerance in yeast. Therefore, *BrMT1* genes were introduced into the *YCF1*-null (*ycf1*) yeast mutant strain DTY167, which is sensitive to $CdCl_2$, and a complementation test was conducted to confirm the $CdCl_2$ resistance of BrMT1. When

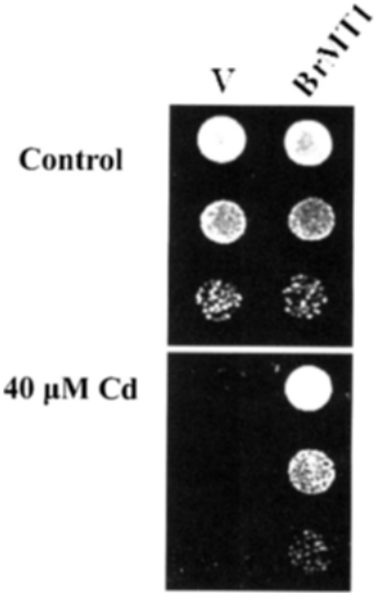

Figure 2. Cd resistance of *BrMT1*-overexpressing yeast. DTY167 yeast cells were transformed with empty vector (V) or *BrMT1*. Yeast strains were grown at 30°C for 3 d on plates without (Control) or with 40 μM Cd.

cultured on half-diluted (1/2) synthetic galactose (SG) plated with 40 μM $CdCl_2$, the *YCF1*-null yeast cells that contained an

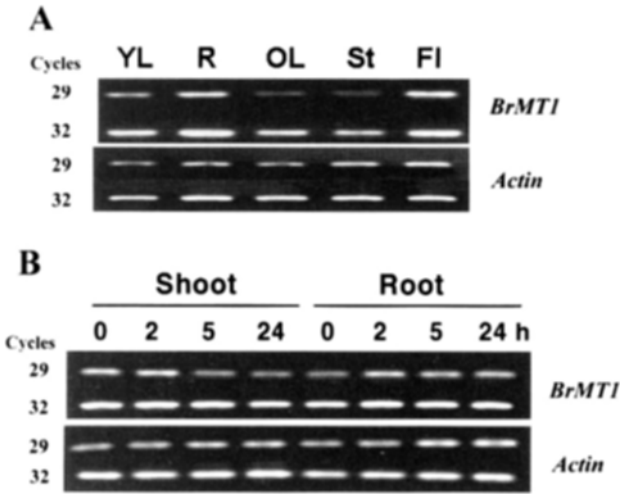

Figure 3. Expression of *BrMT1* in various tissues and under Cd stress. **A.** *BrMT1* mRNA levels in young leaf (YL), root (R), old leaf (OL), stem (St), and flower (Fl). **B.** *BrMT1* mRNA levels in shoot or roots exposed to 40 μM Cd for indicated times. RT-PCR was performed for either 29 or 32 cycles. *Actin* gene served as control for equal loading.

empty vector (DTY167-pYES2) did not grow, whereas the same cell strains transformed with *BrMT1* grew better than the wild-type yeast that harbored the empty pYES2 vector (Fig. 2).

BrMT1 mRNA Levels in Various Tissues and under Cd Stress

To examine the expression profile of *BrMT1* in Chinese cabbage, we performed RT-PCR with the same primer sets used for cloning. Transcript levels were high in the roots and flowers, but low in the old leaves and stems (Fig. 3A). Expression was moderate in the young leaves. Cadmium treatment increased the level of *BrMT1* in the roots, but not in the shoots (Fig. 3B), indicating that Chinese cabbage roots are sensitive to Cd.

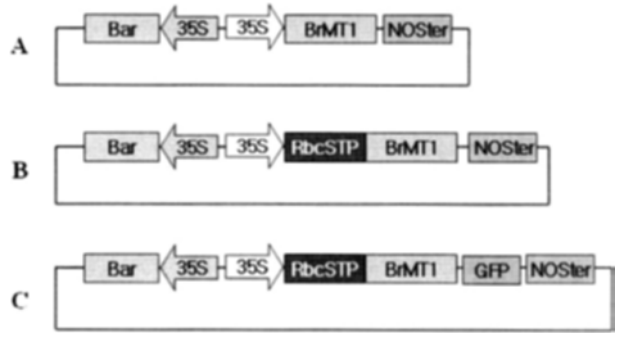

Figure 4. Vector constructs for *Arabidopsis* transformation. **A.** pCAMBIA-3301 vector containing *BrMT1*, *35S::BrMT1*. **B.** pCAMBIA-3301 vector containing RbcS transit peptide and *BrMT1* gene, *35S::RbcSTP-BrMT1*. **C.** pCAMBIA-3301 vector containing transit peptide, *BrMT1*, and *GFP* gene, *35S::RbcSTP-BrMT1-GFP*.

Production and Characterization of *Arabidopsis* Plants Expressing *BrMT1*

AtMT1 confers cadmium tolerance in *Arabidopsis* plants (Zimeri et al., 2005), and BrMT1 manifests a high degree of identity with *Arabidopsis* AtMT1s. To determine whether *BrMT1* expression would increase cadmium resistance, we introduced that gene into *A. thaliana*, which could be targeted either to the cytosol or to the chloroplasts (Fig. 4). Using genotype and phenotype analyses, we obtained eight independent homozygote T_3 lines for each construct. All subsequent experiments were conducted with these homozygote lines (at least 3 independent lines). Initially, we investigated whether the RbcS transit peptide (RbcSTP) could result in the targeting of BrMT1 to the chloroplasts, using *RbcSTP::GFP* plants as a control. Here, RbcSTP efficiently guided the GFP to the chloroplasts (Fig. 5A). This was confirmed via western blot analysis (Fig. 5B), although some of the proteins still remained in the cytosol.

Enhanced Cd Resistance in *BrMT1* and *RbcS-BrMT1* Plants

To ascertain whether *BrMT1* overexpression conferred Cd

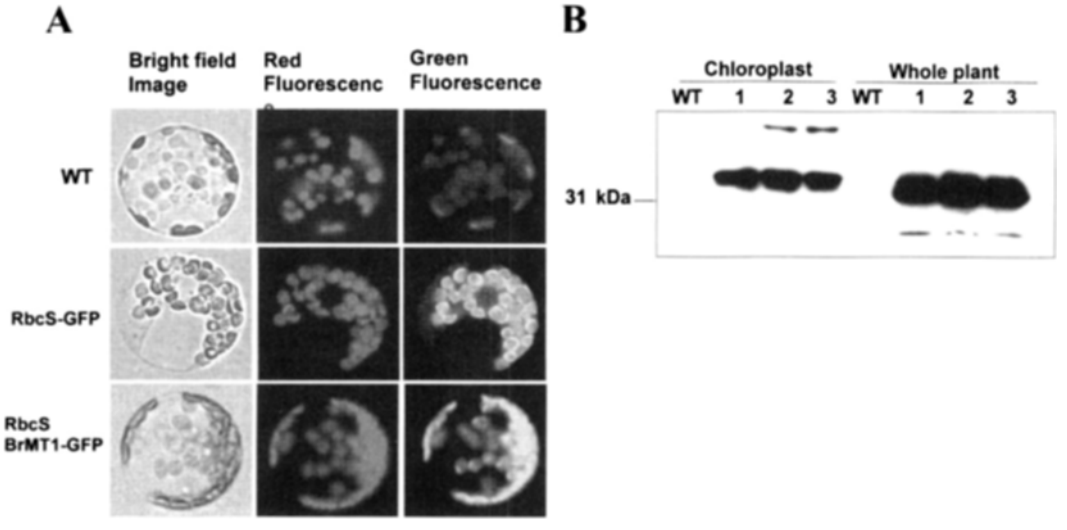

Figure 5. Fluorescence in chloroplasts and cytosol of protoplast isolated from transgenic *Arabidopsis* plants (**A**), and western blot analysis of GFP (**B**). Soluble proteins were extracted from transformed *Arabidopsis* chloroplast or from whole plant containing *35S::RbcSTP-BrMT1-GFP* construct. BrMT1-GFP was detected with GFP anti-body. WT, non-transformed *Arabidopsis*; Lanes 1 to 3, independent T_3 transgenic lines.

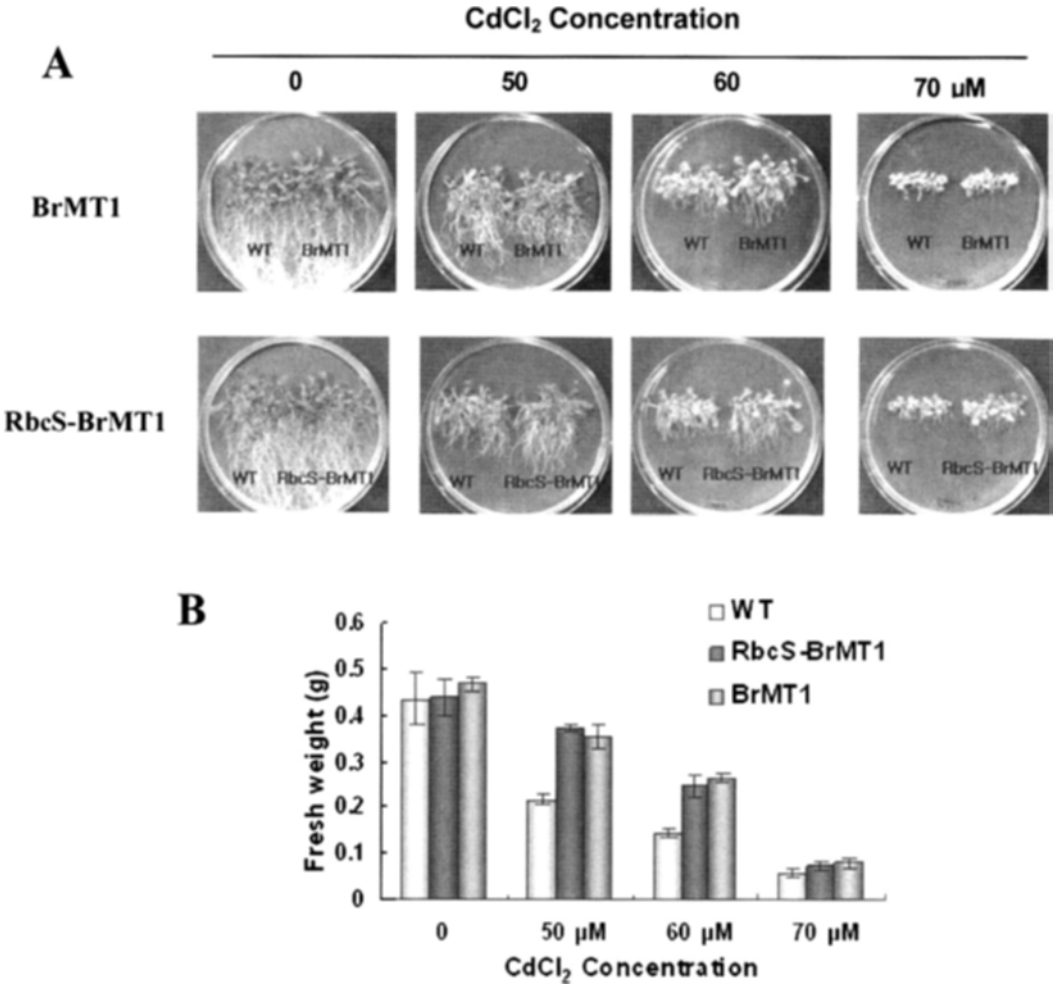

Figure 6. (**A**) Enhanced Cd resistance in T_3 generation of *BrMT1* and *RbcS-BrMT1* plants. Wild-type and transgenic plants were grown at 21°C for 2 weeks under continuous light on 1/2 MS agar medium containing various concentrations of $CdCl_2$. (**B**) Fresh weights of 3-week-old T_3 *BrMT1* and *RbcS-BrMT1* plants grown on ½ MS agar plates containing various concentrations of $CdCl_2$.

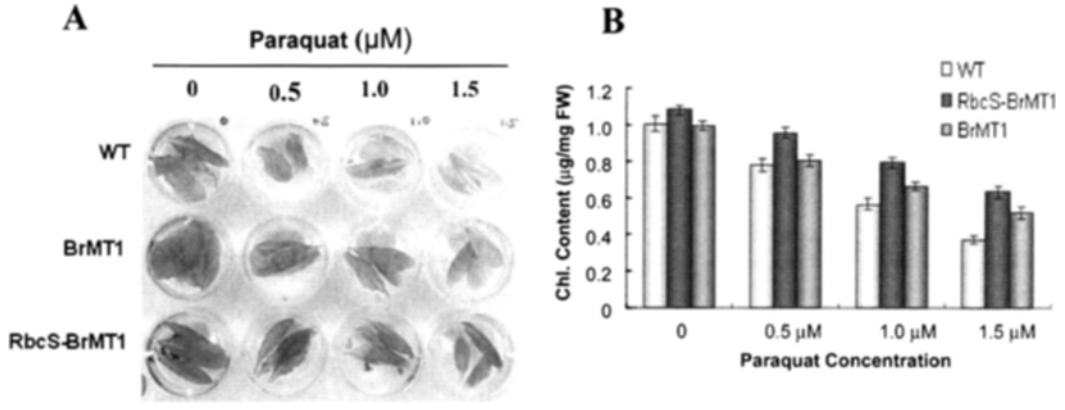

Figure 7. (**A**) Paraquat-bleaching experiment using leaves from wild-type, *BrMT1*, and *RbcS-BrMT1* plants. Leaves were placed for 40 h under continuous light on 1/10 MS medium containing 0.025% Tween 20 and 0.0, 0.5, 1.0, or 1.5 μM paraquat. (**B**) Chlorophyll contents illustrate paraquat tolerance based on resistance to PQ-mediated chlorophyll degradation, as examined from two independent lines for each construct.

resistance, *Arabidopsis* plants were grown in 1/2 MS agar plates containing 50 to 70 μM $CdCl_2$. Overexpressing plants proved resistant at 50 and 60 μl (Fig. 6A). Although the rosette leaves and roots from these transgenics grew better than those of the wild-type plants, no significant differences were found between the *BrMT1* and *RbcSTP-BrMT1* plants. This result indicates that Cd might exert similar effects in both the cytosol and the chloroplasts, when absorbed into the roots.

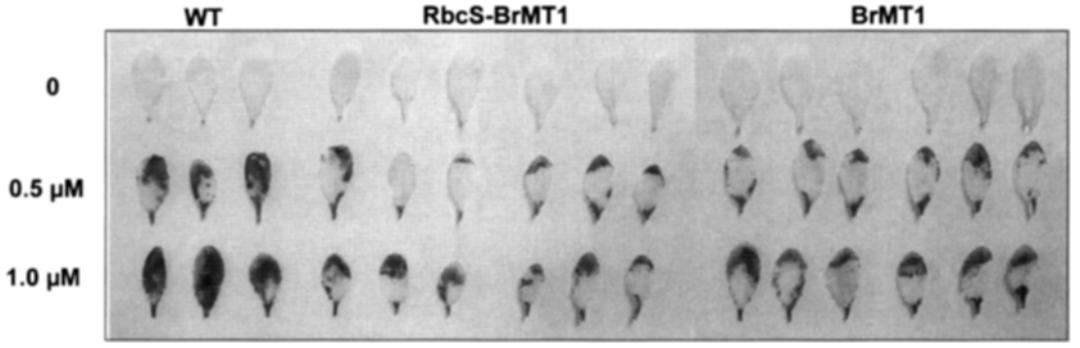

Figure 8. H_2O_2 accumulation in *Arabidopsis* leaves detected by 3,3-diaminobenzidine (DAB) staining. After 40 h of stabilization in water containing 0.5 or 1.0 μM paraquat, leaves were held for 5 to 8 h at 25°C under continuous light in solution of 1 mg mL^{-1} DAB-HCl.

Chloroplast-Targeted BrMT1 Are More Resistant to PQ-Induced Oxidative Stress than Cytosol-Targeted BrMT1

The paraquat (PQ) herbicide induces oxidative stress in plant leaves by generating reactive oxygen species (ROS), specifically in Photosystem I (PSI) of the chloroplasts. Here, the rosette leaves of three- to four-week-old *Arabidopsis* seedlings were floated for 40 h under continuous light on 1/10 MS plus 0.025% Tween 20, with or without PQ. Chlorosis became severe in the wild-type leaves, in proportion to the PQ concentration applied, but was less profound in leaves that expressed *BrMT1* (Fig. 7A). In particular, the chloroplast-targeted BrMT1 conferred a greater degree of chlorosis resistance than did the cytosol-localized BrMT1. Our measurements of chlorophyll contents confirmed the result of the bleaching experiment (Fig. 7B), suggesting that BrMT1 in the chloroplasts functions in PQ-mediated ROS tolerance.

H_2O_2 Accumulation Is Decreased in Chloroplast-Targeted BrMT1 Plants

To assess H_2O_2 accumulation *in vivo*, we stained leaves from wild-type and two transgenic *Arabidopsis* strains with 3,3-diaminobenzidine (DAB) after PQ treatment. Accumulations were remarkably lower in the chloroplast-targeted BrMT1 plants than in either the wild-type or cytosol-localized plants (Fig. 8). This demonstrates that the chloroplast-targeted BrMT1 may confer tolerance against PQ-mediated oxidative stress.

DISCUSSION

Metallothioneins bind to heavy metals, e.g., zinc, copper, and cadmium, thereby reducing their concentrations to physiological or nontoxic levels. The transcription of metallothionein genes is induced by both heavy metals and oxidative stress (Cobbett and Goldsbrough, 2002). *Arabidopsis* plants contain four types of MTs that are distinguished on the basis of amino acid sequence phylogeny analysis (Zimeri et al., 2005). The plant MTs vary in the cysteine spacing of their metal-ion binding domains. In addition, all four types harbor well-conserved, relatively long spacer sequences of 7 to 43 amino acids, which are significantly longer than the typical 3 amino-acid spacer sequences detected in mammalian MTs. These distinct spacer sequences affect cellular functions by interacting with other metal-ion processing proteins (Zimeri et al., 2005). Among these, the *MT1* genes assist plants in the processing of only particular heavy metals and metalloids (Guo et al., 2003; Zimeri et al., 2005). In this study, we cloned and characterized the Chinese cabbage type-1 metallothionein gene (*BrMT1*), and found that it has a high degree of similarity to its counterparts in *Arabidopsis* (Fig. 1). This suggests that BrMT1 may perform as many functions as AtMT1. However, it differs somewhat from the MT1 of rice, pea, *Medicago*, *Mesembryanthemum*, and ginseng in their spacings and positioning of Cys-residues. These variations may imply that MT1s from cruciferous crops function differently from those of other plants. As expected, *BrMT1* conferred Cd resistance in transformed yeast (Fig. 2), a result consistent with previous reports that the *Arabidopsis* metallothionein genes *AtMT1* and *AtMT2* confer Cd(II) resistance to Cd(II)-sensitive yeast (Lee et al., 2004).

Chloroplasts are considered sensitive to cadmium toxicity at very low Cd concentrations (i.e., about 1% of the total leaf Cd) (Krupa, 1999; Seregin and Ivanov, 2001). High cadmium contents in the leaves disrupt chlorophyll biosynthesis and the formation of the Photosystem, resulting in accelerated senescence, enhanced oxidative damage and chlorophyllase activity, and deficiencies in several essential ions, including Fe^{3+} and Mg^{2+} (Baryla et al., 2001). Under conditions of Cd toxicity, reactive oxygen species (ROS) are also indirectly generated via disturbances in the electron-transport chains, lipoxygenase activation, and alterations in the structure of or inhibition of the antioxidative metalloenzymes (Sandalio et al., 2001). To assess the effects of BrMT1 against Cd resistance as well as ROS reduction, we successfully introduced *BrMT1* to *Arabidopsis* chloroplasts using a constitutive CaMV35S promoter and an RbcS transit peptide (Fig. 4, 5). Overexpression in both the chloroplasts and cytosol resulted in similarly enhanced Cd resistance at 50 and at 60 mM $CdCl_2$ (Fig. 6). Although it is unknown why BrMT1 exerts the same influence in both chloroplasts and cytosol, this may be attributed to the incorporation of Cd in plant cells. Cadmium generally accumulates in the vacuoles and is transported as free ions or as a phytochelatin-Cd complex (Salt and Wagner, 1993; Salt and Rauser, 1995). However, *Euglena gracilis*, which lacks a plant-like vacuole, accumulates cadmium within the chloroplasts (Mendoza-Cózatal et al., 2002).

Although BrMT1s targeted into both chloroplasts and

cytosol manifested similar Cd-tolerance effects, its targeting to the former was more efficient with regard to the reduction in paraquat-mediated chlorosis, as well as the accumulation of -H_2O_2 (Fig. 7, 8). This suggests that BrMT1 can detoxify the H_2O_2 that results from PQ treatment. Therefore, chloroplast-targeting is effective in the detoxification of cadmium and ROS stress in chloroplasts. This method may also prove applicable toward developing plants with enhanced tolerance to environmental stresses. However, little is known about signal transduction for metallothionein gene expression in plants. In animals, induction of expression is mediated by the metal-responsive transcription factor 1 (MTF-1), an essential zinc finger protein that binds to specific DNA motifs, i.e., metal-response elements (Zhang et al., 2003). In future studies, plant homologues to animal MTF-1 will be identified and characterized.

ACKNOWLEDGMENT

This work was supported by a grant from the BioGreen 21 Program, Rural Development Administration, Korea.

Received September 26, 2006; accepted December 11, 2006.

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