Plant Molecular Biology **54:** 805–815, 2004.

Arabidopsis metallothioneins 2a and 3 enhance resistance to cadmium when expressed in *Vicia faba* guard cells

Joohyun Lee[1,2,†], Donghwan Shim[1,†], Won-Yong Song[1,3], Inhwan Hwang[4] and Youngsook Lee[1,*]

[1]*National Research Laboratory of Phytoremediation, Division of Molecular Life Sciences, Pohang University of Science and Technology, Pohang 790-784, Korea (*author for correspondence; e-mail ylee@postech.ac.kr);* [2]*Current address: Biological Science Department, Dartmouth College, Hanover NH 03755-3576, USA;* [3]*Current address: Division of Applied Biology and Chemistry, College of Agriculture and Life Sciences, Chungnam National University, Daejeon 305-764, Korea;* [4]*Center for Plant Intracellular Trafficking, Pohang University of Science and Technology, Pohang 790-784, Korea;* [†]*These authors contributed equally to the work*

Received 18 December 2003; accepted in revised form 23 April 2004

Key words: Arabidopsis thaliana, cadmium resistance, localization, metallothionein, reactive oxygen species, *Vicia faba* guard cells

Abstract

The Arabidopsis metallothionein genes *AtMT1* and *AtMT2* confer Cd(II) resistance to Cd(II)-sensitive yeast, but it has not been directly shown whether they or other metallothioneins provide the same protection to plants. We tested whether *AtMT2a* and *AtMT3* can confer Cd(II) resistance to plant cells by introducing GFP- or RFP-fused forms into guard cells of *Vicia faba* by biolistic bombardment. AtMT2a and AtMT3 protected guard cell chloroplasts from degradation upon exposure to Cd(II), an effect that was confirmed using an FDA assay to test the viability of the exposed guard cells. AtMT2a- and AtMT3-GFP were localized in the cytoplasm both before and after treatment of *V. faba* guard cells or Arabidopsis protoplasts with Cd(II), and the levels of reactive oxygen species were lower in transformed guard cells than in non-transformed cells after Cd(II)-treatment. These results suggest that the Cd(II)-detoxification mechanism of AtMT2a and AtMT3 may not include sequestration into vacuoles or other organelles, but does involve reduction of the level of reactive oxygen species in Cd(II)-treated cells. Increased expression of AtMT2a and AtMT3 was observed in Arabidopsis seedlings exposed to Cd(II). Together, these data support a role for the metallothioneins AtMT2a and AtMT3 in Cd(II) resistance in intact plant cells.

Introduction

Metallothioneins (MTs) are proteins rich in cysteine residues that bind heavy metals and confer resistance to Cd(II) and Zn(II) in mammalian cells (Palmiter, 1998). Plants also have many *MT* genes and express some of these genes at high levels (Cobbett and Goldsbrough, 2002). The *MT1* and *MT2* genes from *Arabidopsis thaliana* (*AtMT1* and *AtMT2*) enhance resistance to Cu(II) and Cd(II) when expressed in yeast cells (Zhou and Goldsbrough, 1994). Exposure of Arabidopsis to various heavy metals, including Cu(II) and Cd(II), increases the expression of *AtMT1* and *AtMT2* messages (Murphy and Taiz, 1995). Although these data suggest that plant MT genes play an important role in resistance to Cu(II) and Cd(II), it has not been tested whether plant MTs confer resistance to heavy metals in intact plant cells. Several lines of indirect evidence suggest that MTs are mainly involved in Cu(II) resistance and in copper homeostasis: the increase in *AtMT2*

mRNA is higher in plants treated with Cu(II) than in those treated with any other heavy metal (Zhou and Goldsbrough, 1994; Murphy and Taiz, 1995), AtMT1 and AtMT2 protein levels increase upon exposure to Cu(II) in tissue-specific manners (Murphy *et al.*, 1997), and *MT* gene expression increases in senescing leaves together with a set of genes involved in copper homeostasis (Cobbett and Goldsbrough, 2002).

The availability of knockout plants has greatly facilitated the identification of the proteins involved in resistance to individual heavy metals. For example, phytochelatin synthase knockout plants are highly sensitive to Cd(II); based on this, Cd(II) resistance in plants is attributed mainly to phytochelatins (Howden *et al.*, 1995; Cobbett *et al.*, 1998). However, the many MT genes in plants most likely have overlapping functions (Cobbett and Goldsbrough, 2002), which precludes the use of available knockout plants for the identification of their functions. Therefore, the roles of plant MTs in heavy metal resistance remain less clear, and a method that could identify the contributions of individual MTs to the resistance to particular heavy metals would be highly valuable.

Heavy metals in the cytoplasm can be detoxified by several different pathways (Hall, 2002). They can be chelated by various ligands which often have many cysteine residues; they can be sequestered into the vacuole; or they can be transported out to the apoplast. Heavy metal resistance of plant cells may also include efficient repair of heavy metal-induced damage. Cd(II) has been reported to be sequestered into vacuoles as a step of detoxification (Wink, 1993). For example, Cd(II) bound to phytochelatin has been found in vacuoles of tobacco (Vogeli-Lange and Wagner, 1990), and Cd(II) is transported into vacuoles by a Cd^{2+}/H^{+} antiporter (Hirschi *et al.*, 2000). However, the sub-cellular localization of plant MTs has not been reported, partly due to their instability in the presence of oxygen, which limits our understanding of the MT mechanism of action.

Here we confirm that *AtMT2a* confers strong resistance to Cd(II) when expressed in yeast, and in addition, show that *AtMT2a* and *AtMT3* enhance Cd(II) resistance when overexpressed in guard cells of *Vicia faba*. Further, we show that these MTs protect plant cells from Cd(II)-toxicity by reducing the levels of reactive oxygen species (ROS) without apparent translocation from the cytoplasm.

Materials and methods

Screening of Cd(II)-resistance genes

A ycf1 mutant of *Saccharomyces cerevisiae*, DTY167 (*MATa ura3 leu2 his3 trp3 lys2 suc2 ycf::hisG*) was used to screen Cd(II)-resistance genes from an Arabidopsis leaf cDNA library cloned in pFL61 (Piao *et al.*, 1999). The library was introduced into DTY167 and transformants were selected on SG plates lacking uracil (SG-ura) containing 30–100 μM $CdCl_2$. The plasmids from the surviving yeasts were rescued in *E. coli*, extracted, and then sequenced. The sequences of *AtMT2a* and *AtMT3* genes were identical to those published (GenBank accession numbers S57861 and AF013959, respectively).

Heavy metal resistance tests of yeast strains

The pYES2/NT C vector with or without the *AtMT2a* or *AtMT3* genes was introduced into DTY167 and its isogenic wild type, DTY165. The transformants were spotted onto $CdCl_2$-containing half strength (1/2) SG-ura plates and grown for 3–5 days.

Plant material and growth conditions

Arabidopsis wild-type seeds (*Arabidopsis thaliana* ecotype Columbia 0) were surface sterilized, placed in the dark at 4 °C for 2 days, and then sown on plates of 1/2 Murashige and Skoog with 1.5% sucrose (Murashige and Skoog, 1962) at 18 °C. *Vicia faba* plants were grown in cycles of 16 h light and 8 h dark at 22 ± 2 °C in a greenhouse. Young mature leaves from 3- to 4-week-old plants were used in all experiments.

Northern analysis of Cd(II)-induced MT gene expression

Arabidopsis plants were grown vertically on 1/2 MS-agar plates supplemented with 1.5% sucrose for 2 weeks. The plates were then placed horizontally, and the roots were exposed to 10 ml of 10, 30, 50 μM $CdCl_2$ solution for 24 h. Subsequent

RNA preparation and Northern hybridization were carried out as described (Sambrook and Russell, 2001).

Fluorescent gene constructs of AtMTs

AtMT2a and AtMT3 were amplified by PCR and inserted into the BamHI single site located at the 5′ termini of GFP and RFP genes in the GFP and RFP vectors. The primers used were 5′GGATCCATGTCTTGCTGTGGAGGAAAC3′ and 5′GGATCCTCTTGCGGTGCAAGGATCAC3′ for the AtMT2a:GFP construct, and 5′GGATCCATGTCAAGCAACTGCGGAAG3′ and 5′GGATCCTGTTGGGGCAGCAAGTGCAG3′ for the AtMT3:GFP construct. For the AtMT2a:RFP construct, 5′GGATCCATGTCTTGCTGTGGAGGAAAC3′ and 5′GGATCCTCTTGCGGTGCAATGGATCAC3′ primers were used, and 5′GGATCCATGTCAAGCAACTGCGGAAG3′, and 5′GGATCCTGTTGGGGCAGCAATGTGCAG3′ were used for AtMT3:RFP. DNA manipulations were performed according to standard methods (Sambrook and Russell, 2001). The fidelity of all constructs was confirmed by sequencing.

Transient expression of genes in V. faba *and in Arabidopsis cells*

AtMTs:GFP and cytosolic RFP were co-expressed in Arabidopsis protoplasts and *V. faba* guard cells using PEG transformation (Jin *et al*., 2001) and bombardment (Jung *et al*., 2002), respectively. For PEG transformation, Arabidopsis protoplasts were isolated from seedlings grown in plates of 1/2 Murashige and Skoog with 1.5% sucrose. For biolistic bombardment, young mature leaves from 3- to 4-week-old *V. faba* plants were used.

Viability tests in V. faba *guard cells*

To test the viability of the transformed cells in the presence of Cd(II), *V. faba* guard cells were transformed with AtMTs:RFP by using the bombardment technique. The bombarded leaves were kept under darkness on wet filter paper for 2–3 days and then floated on 50 mM KCl, 10 mM Mes–KOH (pH 6.15) bathing medium supplemented with 10 μM $CdCl_2$ under darkness for 1 h. The abaxial epidermis was then peeled from the leaves and the number of chloroplasts in guard cells was counted using an Axioplan fluorescence microscope. In addition, fluorescein diacetate (FDA) was used as a viability probe at a final concentration of 50 μg/ml. We quantified the fluorescence level in guard cells using Adobe Photoshop 5.5 software as previously described (Park *et al*., 2003). In short, areas occupied by each transformed and non-transformed guard cell pair were delineated, the number of pixels and their green fluorescence intensity in the regions were measured, and then the mean value of green fluorescence of the region was obtained using the software.

Localization and translocation assay for AtMTs

To test localization, expression of GFP- or RFP-fused protein was monitored after 20 h using an Axioplan fluorescence microscope (Zeiss, Oberkochen, Germany). The filter sets used were Filter set 44 (excitation, BP 455–495; beamsplitter, FT500; emission, BP 505–555) for green fluorescent proteins and Filter set 20 (excitation, BP 540–552; beamsplitter, FT560; emission, BP 575–640) for red fluorescent proteins (Zeiss). To test whether AtMT proteins translocate in response to Cd(II) in plants, cells were observed at room temperature before and after a 1 h incubation in incubation solution (50 mM KCl and 10 mM MES, pH 6.2) containing 10 μM $CdCl_2$. Images were captured with a cooled charge-coupled device camera.

ROS assay

V. faba leaves bombarded with AtMTs:RFP were treated with 10 μM $CdCl_2$ for 1 h as described previously for the viability assay. Epidermal strips of *V. faba* were peeled and floated for 10 min on a 50 μM dichlorodihydrofluorescein diacetate (H_2DCF-DA) solution containing 1:100 diluted 10% (w/w) paraphenylene diamine (an anti-fading agent). Then, they were incubated for 30 min to remove ROS generated by the stripping stress. The green fluorescence was detected in transformed and non-transformed guard cells using a fluorescence microscope, and the mean intensity level was quantified using Adobe Photoshop 5.5 software as described above.

Results

Expression of AtMT2a and AtMT3 enhances Cd(II) resistance in Saccharomyces cerevisiae

To select genes that confer Cd(II) resistance, an Arabidopsis cDNA library was introduced into the DTY167 strain of *Saccharomyces cerevisiae* which is hypersensitive to Cd(II) due to a deletion of *YCF1*, a vacuolar sequester of Cd(II)-glutathione complexes. Transformed cells were grown on Cd(II)-containing 1/2 SG agar medium for 4 days. Surviving colonies were harvested, the plasmids were isolated from the colonies and amplified in *E. coli*. The plasmids were introduced again into the DTY 167 cells and resistance tests were repeated. The plasmids isolated from the surviving colonies contained cDNAs of *AtMT2a* and *AtMT3*, phytochelatin synthase, and some unknown genes.

To confirm that *AtMT2a* and *AtMT3* confer Cd(II)-resistance, we spotted four yeast strains on 1/2 SG medium containing $CdCl_2$: DTY167 transformed with *AtMT2a*, *AtMT3*, and the empty vector, and DTY 165, the isogenic wild type of DTY167, transformed with the empty vector. DTY167 yeast expressing *AtMT2a* and *AtMT3* grew better than either strain transformed with the empty vector (Figure 1, right).

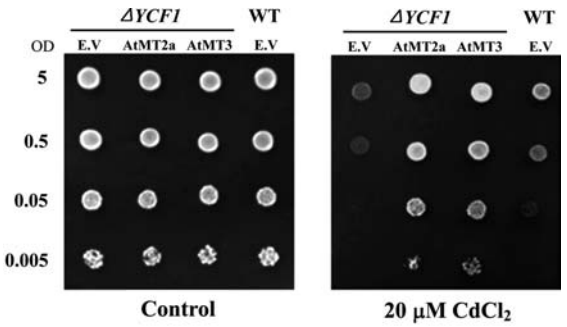

Figure 1. Expression of *AtMT2a* and *AtMT3* strongly enhances Cd(II)-resistance of YCF1-null mutant and wild-type yeast. *AtMT2a* or *AtMT3* was inserted into a pFL61 vector and transformed into DTY167 (YCF1-null) and DTY165 (wild type) yeast strains. Yeast cells were grown on 1/2 SG media lacking uracil with or without 20 μM $CdCl_2$ at 30 °C for 3 days. WT; wild type, Δycf1; YCF1-null mutant, EV; empty vector only.

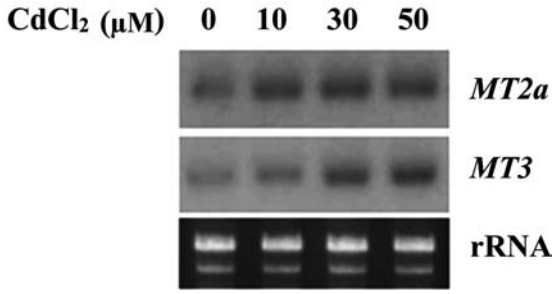

Figure 2. Northern blot analysis of *AtMT2a* and *AtMT3* expression in whole seedlings of Arabidopsis. Ethidium bromide staining of rRNA was used as a loading control.

AtMT2a and AtMT3 are induced by Cd(II) treatment in Arabidopsis

If AtMT2a and AtMT3 are involved in Cd resistance in Arabidopsis, their expression levels might increase upon Cd treatment. To test this possibility, we performed Northern blot analysis using RNA from 2-week-old whole seedlings. Treatments with 10, 30 and 50 μM $CdCl_2$ for 24 h caused upregulation of AtMT2a and ATMT3 expression (Figure 2). The level of expression of the AtMT2a gene seemed to reach saturation at 10 μM Cd(II) because it did not increase any further at higher concentrations of Cd(II), whereas the level of AtMT3 continued to increase with increasing Cd(II) concentration. In short, both MT genes were induced by Cd(II), but AtMT3 was induced more strongly than AtMT2a.

Expression of AtMT2a or AtMT3 enhances Cd(II)-resistance in Vicia faba *guard cells*

The resistance of transgenic plants to heavy metals is commonly tested by generating stable lines of transformants and growing them in medium containing the heavy metal. However, this procedure is time-consuming. For a fast and simple test for genes that confer Cd(II)-resistance to plant cells, we used *V. faba* guard cells transiently transformed by biolistic bombardment.

To test the role of *MT* genes in resistance to Cd(II), we bombarded *V. faba* guard cells with AtMT2a:GFP and AtMT3:GFP. The expression of the introduced genes was detected as green fluorescence in the cells at about 14 h after bombardment (Figure 3B and D), at which time the cells were treated with 10 μM $CdCl_2$ for 1 h. We

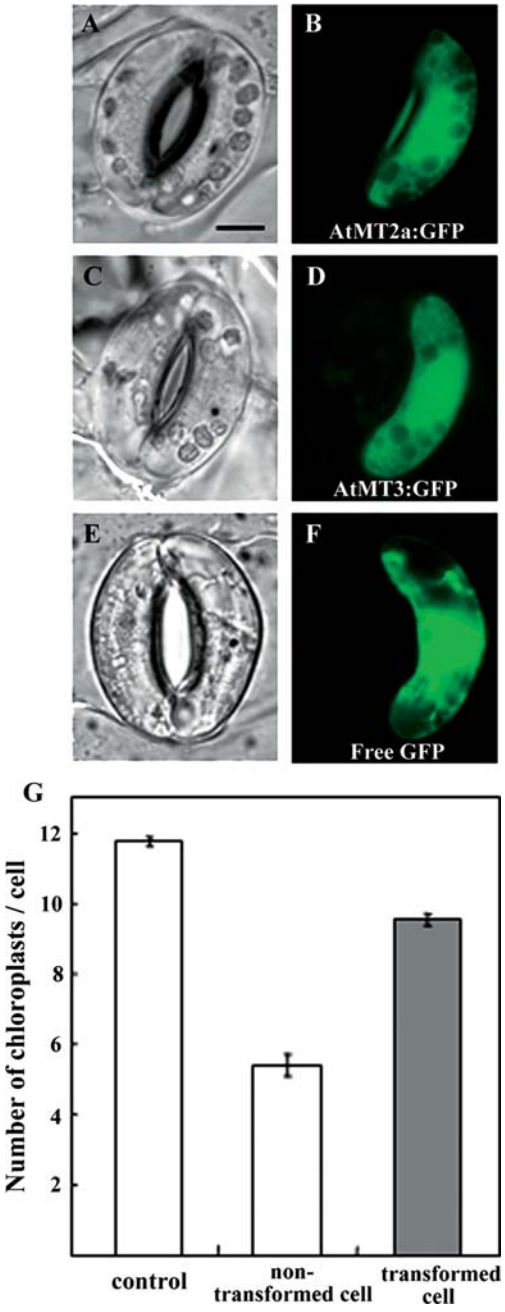

Figure 3. Expression of AtMT2a:GFP and AtMT3:GFP protects chloroplasts in *Vicia faba* guard cells treated with 10 μM $CdCl_2$ for 1 h. (A, C, E) Bright field images. (B, D, F) Fluorescent images of AtMT2a-GFP (B), AtMT3-GFP (D), and free GFP (F), from the cells shown in A, C, and E, respectively. The guard cells on the right express GFP-fused or free GFP proteins, as detectable by their green fluorescence. (G) Number of chloroplasts in the non-treated control, and Cd-treated AtMT3:GFP-transformed and their non-transformed neighbor guard cells ($n = 100$). Error bars represent SE. Scale bar = 10 μm.

selected guard cell pairs of which only one was transformed and the other was not, and compared the responses of the two cells of each stoma. Since a pair of guard cells is produced from division of a single guard mother cell, the neighboring cell provides a good control. While most of the cells expressing *AtMT2a* (Figure 3A and B, right) and *AtMT3* (Figure 2C and D, right) remained intact, many non-transformed neighboring cells lost their normal cellular organization (Figure 3A and C, left). Most strikingly, more chloroplasts remained intact in *AtMT2a*- or *AtMT3*-expressing cells than in non-transformed neighboring guard cells. Guard cells transformed with free *GFP* (Figure 3E, right) were not any more resistant to Cd(II) than their non-transformed neighbors (Figure 3E, left). Cells transformed with *AtMT3* had an average of 9.4 chloroplasts, whereas non-transformed neighboring cells had an average of 6.4 chloroplasts (Figure 3G). Thus, overexpressed *AtMT3* conferred Cd(II)-resistance to *V. faba* guard cells. Experiments with guard cells expressing *AtMT2a* showed a similar protective effect on chloroplasts against Cd(II)-toxicity (data not shown).

To confirm the AtMT3-mediated Cd(II)-resistance, fluorescein diacetate (FDA) was used as a viability probe. The intensity of the green fluorescence of FDA represents the viability of plant cells (Widholm, 1972); stronger intensity indicates a healthier cell. To detect cells expressing *AtMT* genes under a fluorescent microscope, *AtMT* genes were fused to the gene for red fluorescent protein (RFP) (Jach *et al*., 2001) and biolistically introduced into guard cells. Before Cd(II) treatment, normal guard cells showed strong green FDA fluorescence (Figure 4A and B). However, when Cd(II) was applied, non-transformed guard cells, detectable by the absence of red fluorescence, had weak (Figure 4D, left) or no (Figure 4G, left) green FDA fluorescence. In contrast, guard cells transformed with *AtMT3-RFP*, identified by the presence of red fluorescence (Figure 4E and H), showed strong green FDA fluorescence even after Cd(II)-treatment (Figure 4D and G, right). Guard cells transformed with free *RFP* had FDA fluorescence levels similar to those of their non-transformed neighbor cells in the presence of Cd(II) (Figure 4J and K).

We quantified the FDA fluorescence level in guard cells using Adobe Photoshop 5.5 software.

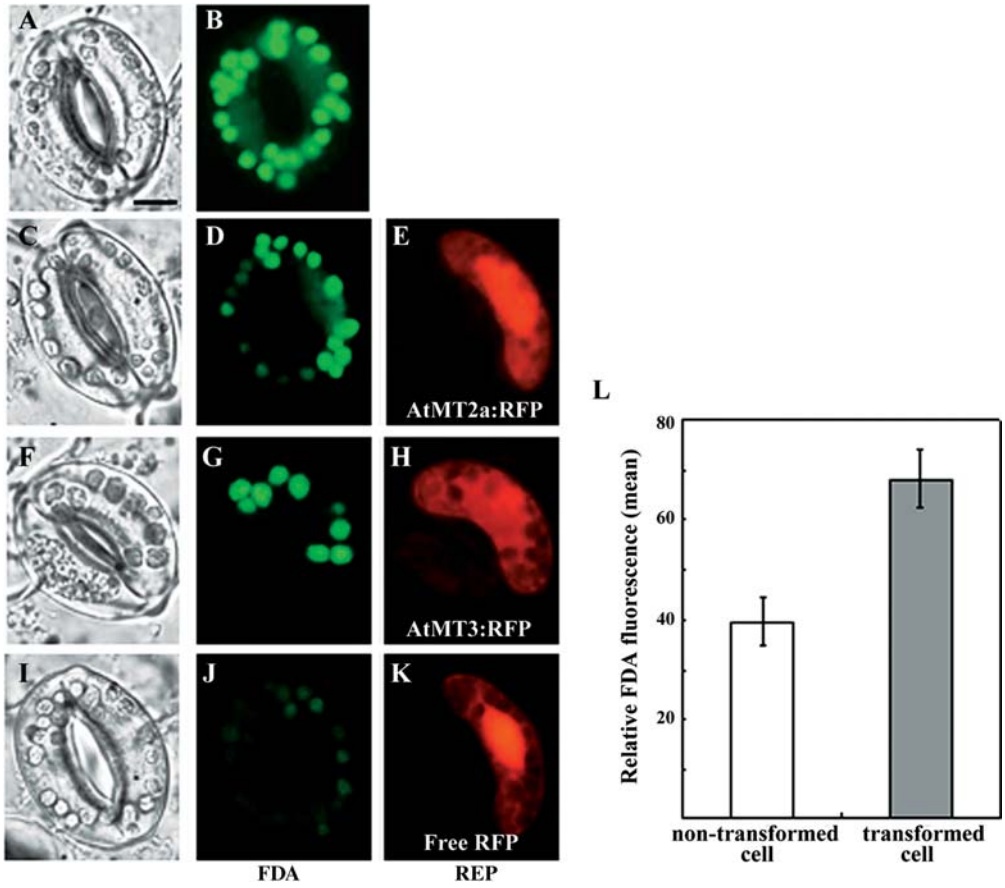

Figure 4. Viability tests with FDA of *Vicia faba* guard cells transformed with AtMT3-RFP. (A, B) Bright field (A) and fluorescent (B) images of a pair of non-transformed guard cells before Cd(II) treatment; (C–K) images of guard cells after 1 h of treatment with 10 μM $CdCl_2$; (C, F, I) bright field images; (D, G, J) FDA fluorescence images of cells shown in C, F, and I, respectively; (E, H, K) RFP fluorescence images of cells shown in C, F, and I, respectively. The guard cells on the right express AtMT3-RFP (E, H) or free RFP (K), as detectable by their red fluorescence. (L) The green fluorescence intensity of FDA from guard cells expressing AtMT3-RFP and their neighbor cells was quantified from microscopic images using Photoshop software ($n = 20$). Error bars represent SE. Scale bar = 10 μm.

Transformed cells had a mean fluorescence intensity of 68.2 (arbitrary unit) and the non-transformed guard cells had a mean fluorescence intensity of 39.5 (Figure 4L). This result confirmed that overexpressed *AtMT3* enhanced Cd(II)-resistance in *V. faba* guard cells. AtMT2a demonstrated a similar protective effect against Cd(II)-toxicity in guard cells (data not shown).

AtMT2a and AtMT3 are localized in the cytoplasm of transformed V. faba *guard cells*

To understand the mechanism by which AtMTs protect against Cd(II)-toxicity, we investigated the localization of AtMT2a and AtMT3 in *V. faba* guard cells. When *AtMT2a* and *AtMT3* fused with *GFP* under the 35S promoter were introduced into *V. faba* guard cells, the green fluorescence was co-localized with free RFP (Figure 5), which we have previously observed to localize in the cytoplasm of plant cells (data not shown). The same experiment with guard cells expressing *AtMT2a* showed similar cytoplasmic localization of AtMT2a (data not shown).

To test MT localization in other types of plant cells, Arabidopsis protoplasts were transformed with AtMT3:GFP. The green fluorescence was found to localize in the cytosol, but not in vacuoles or chloroplasts in mesophyll cells (Figure S1B) or in all other cell types observed (data not shown).

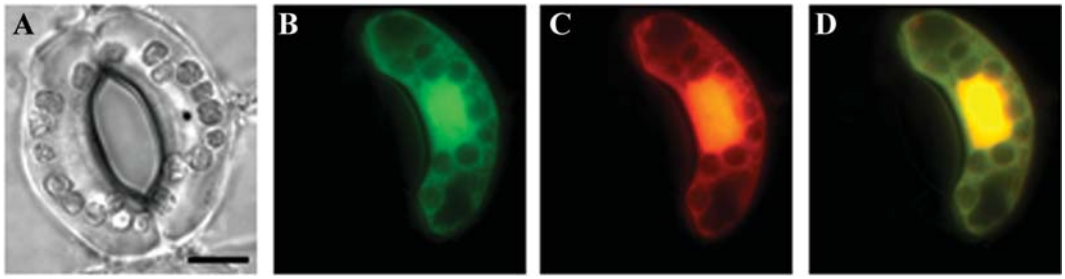

Figure 5. Localization of AtMT3 overexpressed in *Vicia faba* guard cells before Cd(II) treatment. (A) Bright field image; (B) green fluorescent image of AtMT3-GFP in the guard cells shown in A; (C) red fluorescent image of free RFP in the guard cells shown in A. AtMT3-GFP and free RFP are co-expressed in the guard cell on the right side; (D) overlap of B and C. Scale bar = 10 μm.

The same experiment with cells transformed with *AtMT2a* showed cytoplasmic localization of AtMT2a similar to that of AtMT3 (data not shown). Therefore we concluded that AtMT2a and AtMT3 are localized in the cytoplasm in transiently transformed *V. faba* guard cells and Arabidopsis cells in normal conditions before Cd(II) treatment.

AtMT2a and AtMT3 do not translocate from the cytoplasmic region in response to Cd(II) treatment

One previously reported mechanisms of Cd(II) detoxification in plant cells is vacuolar sequestration of Cd(II) bound to phytochelatin (Vogeli-Lange and Wagner, 1990). Since MTs, like phytochelatins, have many cysteine residues, we tested whether the MTs of Arabidopsis are also translocated to the vacuole after treatment of intact cells with $CdCl_2$. When *V. faba* guard cells transformed with AtMT2a:GFP and AtMT3:GFP were treated with 10 μM $CdCl_2$, the localization of AtMT2a-GFP and AtMT3-GFP did not change (Figure 6B and F); they continued to be colocalized with free RFP (Figure 6C and G), as shown by the overlap of the green and red fluorescence (Figure 6D and H). Even after 3 h of treatment with 1–100 μM $CdCl_2$, AtMT2a and AtMT3 did not change their localization (data not shown). Since their protective effect can be observed clearly after 1 h of

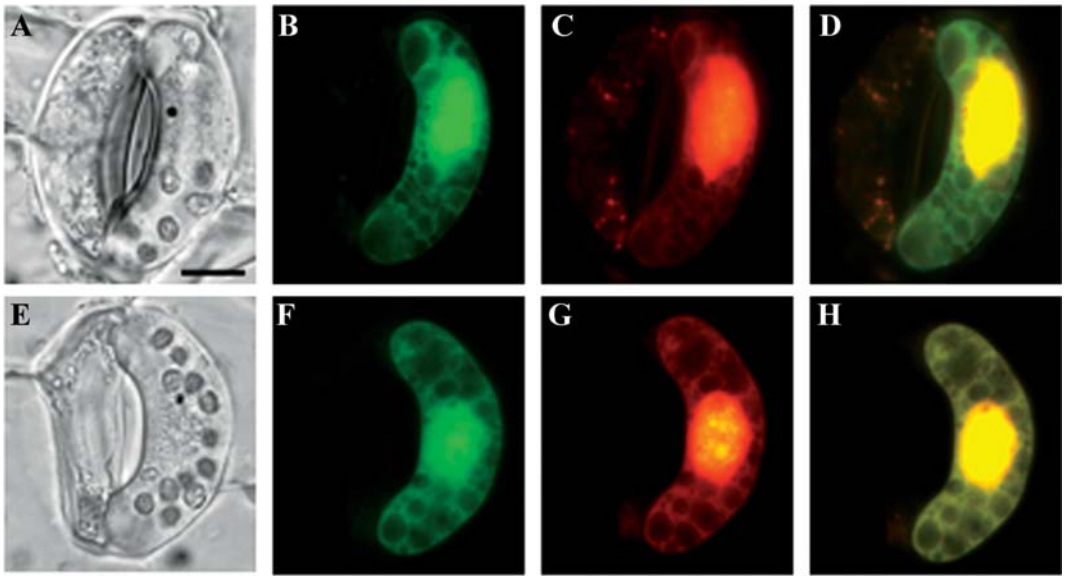

Figure 6. Localization of AtMT2a and AtMT3 overexpressed in *V. faba* guard cells after treatment with 10 μM $CdCl_2$(II) for 1 h. (A) Bright field image; (B) green fluorescent image of AtMT2a-GFP in the guard cells shown in A; (C) red fluorescent image of free RFP in the guard cells shown in A; (D) overlap of B and C; (E) bright field image; (F) green fluorescent image of AtMT3-GFP in the guard cells shown in E; (G) red fluorescent image of free RFP in the guard cells shown in E; (H) overlap of F and G. Scale bar = 10 μm.

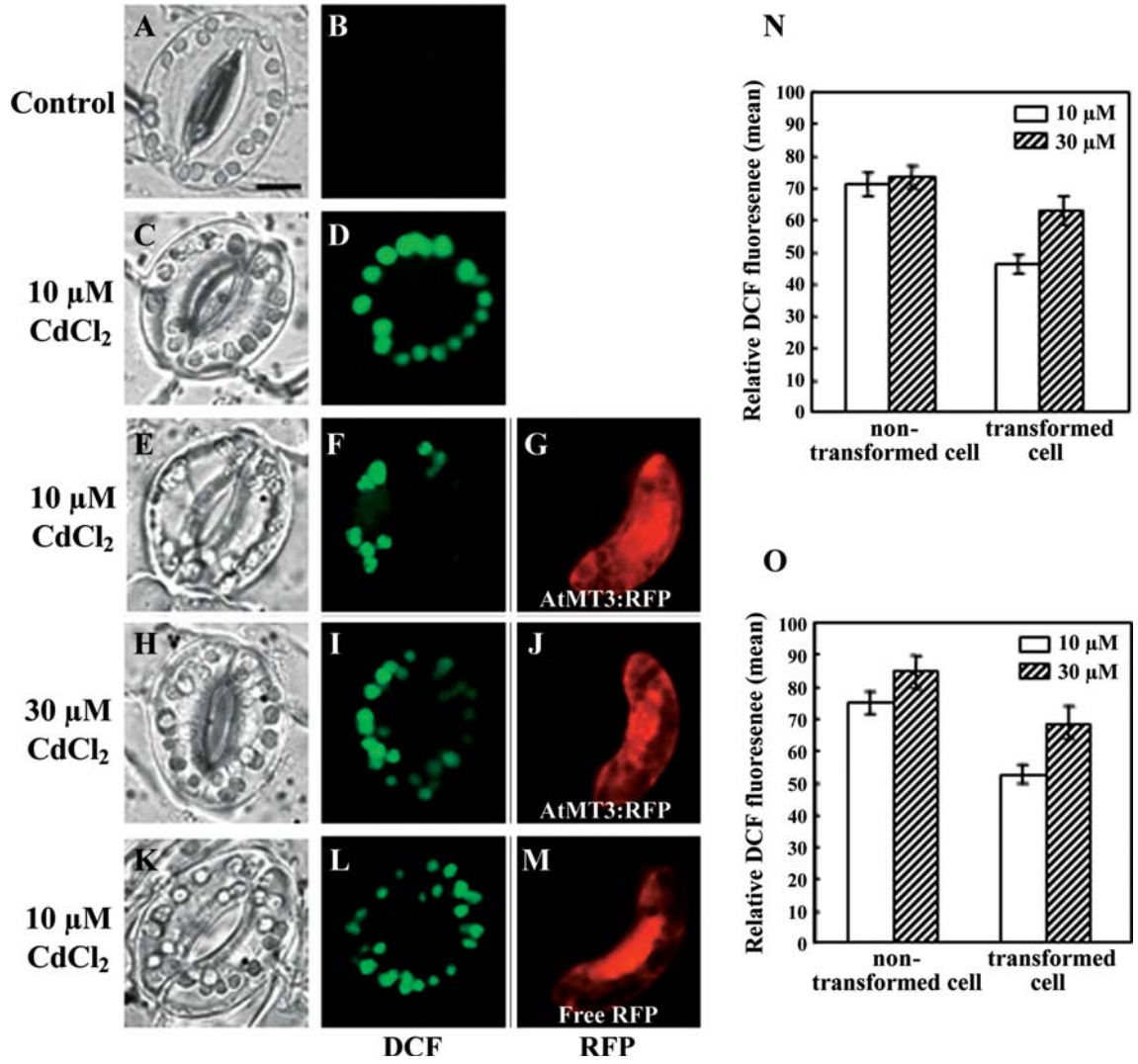

Figure 7. ROS contents of AtMT3-transformed and non-transformed guard cells before and after treatment with Cd(II). (A, B) Bright field and fluorescence images of a non-transformed pair of guard cells before heavy metal treatment after staining with H_2DCF. (C, D) Bright field and fluorescence images of a pair of non-transformed guard cells stained with DCF after treatment with 10 μM $CdCl_2$ for 1 h. (E) Bright field image of a guard cell transformed with AtMT3:RFP (right) and its neighbor (left) after treatment with 10 μM $CdCl_2$ for 1 h. (F) DCF fluorescence image of the same cells shown in E. (G) Red fluorescence image of AtMT3:RFP in the same cells shown in E. (H) Bright field image of a guard cell transformed with AtMT3:RFP (right) and its non-transformed neighbor (left) after treatment with 30 μM $CdCl_2$ for 1 h. (I) DCF fluorescence image of the same cells shown in H. (J) Red fluorescence image of AtMT3:RFP in the same cells shown in H. (K) Bright field image of a guard cell transformed with RFP (right) and its non-transformed neighbor (left) after treatment with 10 μM $CdCl_2$ for 1 h. (L) DCF fluorescence image of the same cells shown in K. (M) Red fluorescence image of RFP in the same cells shown in K. (N, O) DCF fluorescence in AtMT3:RFP(N)- and AtMT2a:RFP(O)-transformed and non-transformed guard cells after treatment with $CdCl_2$ for 1 h. The level of fluorescence was quantified by measuring the green fluorescence intensity of DCF from microscopic images using Photoshop software ($n = 20$). Error bars represent SE. Scale bar = 10 μm.

treatment with 10 μM $CdCl_2$ (Figures 3 and 4), we propose that AtMT2a and AtMT3 protect *V. faba* guard cells against Cd(II)-toxicity while localized in the cytosolic region.

To test the possible translocation of AtMT2a and AtMT3 in other cell types, Arabidopsis protoplasts from whole seedlings were transiently transformed with AtMT2a:GFP or AtMT3:GFP

simultaneously with free RFP (Figure S2). They were then treated with $CdCl_2$ for 1 h. The green fluorescence of both AtMT2a:GFP (Figure S2B) and AtMT3:GFP (Figure S2F) co-localized with the red fluorescence of free RFP (Figure S2C, D, G and H, respectively) in Arabidopsis protoplasts after $CdCl_2$ treatment. Even after 3 h of treatment with 1–100 μM $CdCl_2$, translocation of AtMT2a and AtMT3 was not detectable (data not shown). Therefore we propose that AtMT2a and AtMT3 localize in the cytoplasm in Arabidopsis protoplasts before and after Cd(II) treatment.

Transformed AtMTs prevent ROS generation in V. faba *guard cells*

Exposure of cells to Cd(II) induces generation of reactive oxygen species (Watanabe and Suzuki, 2002), and oxidative stress by ROS damages plant cells (Bethke and Jones, 2001). To test if overexpressed AtMT2a or AtMT3 decreased cell damage by Cd(II) in plant cells by reducing the level of ROS, we assayed ROS content in guard cells using dichlorodihydrofluorescein diacetate (H_2DCF-DA), which produces fluorescent DCF upon oxidation (Ohba *et al*., 1994). *V. faba* guard cells were bombarded with AtMT3:RFP and then incubated in a Cd(II)-containing bath solution for 1 h. Green DCF fluorescence was hardly detectable in intact guard cells without Cd(II) treatment (Figure 7B). However, the green fluorescence appeared in guard cells after $CdCl_2$ treatment (Figure 7D). When the guard cells in bombarded leaves were observed for DCF fluorescence after a 1 h treatment with 10 μM of Cd(II), the non-transformed cells showed strong green fluorescence (Figure 7F, left), whereas the neighboring cells transformed with AtMT3:RFP (Figure 7G) showed only weak fluorescence (Figure 7F, right). When Cd(II) concentration was increased to 30 μM. The cells transformed with AtMT3:RFP (Figure 7J) still had a lower fluorescence compared to neighboring non-transformed cells (Figure 7I, right). The mean values of DCF fluorescence observed at 10 and 30 μM Cd(II) were respectively 46 and 63 for transformed guard cells and 71 and 73 respectively for non-transformed guard cells (Figure 7N). The differences between transformed and non-transformed cells in the levels of fluorescence at 10 and 30 μM Cd(II) were statistically significant at $P < 0.01$. When Cd(II) concentration was increased to 50 μM, all guard cells showed high fluorescence and the difference between transformed and non-transformed cells was no longer significant (data not shown). Guard cells transformed with free RFP (Figure 7M) gave a level of DCF fluorescence that was similar to that of their non-transformed neighboring cells and unrelated to the concentration of Cd(II) employed (Figure 7L and data not shown). Similar experiments using AtMT2a:RFP resulted in similar results. The mean values of DCF fluorescence observed at 10 and 30 μM Cd(II) were respectively 53 and 69 for transformed guard cells and 75 and 85 respectively for non-transformed guard cells (Figure 7O). The differences between transformed and non-transformed cells in the level of fluorescence at 10 and 30 μM Cd(II) were statistically significant at $P < 0.01$. Thus, the results confirm that Cd(II) induces ROS generation and that overexpressed AtMT3 or AtMT2a decreases the level of ROS in guard cells treated with Cd(II).

Discussion

Cd(II) resistance in plant cells has been attributed mainly to phytochelatins since the discovery of phytochelatin synthase in plants (Clemens *et al*., 1999; Ha *et al*., 1999; Vatamaniuk *et al*., 1999). In this paper, we show that plant MTs can also contribute to Cd(II) resistance in plants as the expression of AtMT2a and AtMT3 was induced when Arabidopsis plants were exposed to Cd(II) (Figure 2) and *V. faba* guard cells overexpressing *AtMT2a* and *AtMT3* displayed enhanced Cd(II) resistance (Figures 3 and 4). We further showed that reduction of Cd(II)-induced ROS production is a mechanism by which MT protects the cells from Cd(II) toxicity (Figure 7). This effect of MTs does not seem to require translocation from the cytoplasm, since GFP-fused MTs that did not show apparent translocation after Cd(II) treatment (Figures 5 and 6) were able to confer improved Cd(II) resistance (Figures 3 and 4).

The resistance of transgenic plants to heavy metals is commonly tested by generating stable lines of transformants and growing them in a medium containing the heavy metal. However, this procedure is time-consuming. We therefore designed a new and faster method to test transformed cells for their resistance to abiotic stress

(Figure 3), which was to count the number of chloroplasts in guard cells transformed by biolistic bombardment. We found this method to be simple and easy, since *V. faba* guard cells are easily transformed by bombardment, express foreign genes at a high level, and have chloroplasts that are sensitive to stress. The validity of the method of counting chloroplasts for viability assay was confirmed with an FDA test (Figure 4).

Besides being a good model system, guard cells could also be a direct target for Cd toxicity, especially when Cd is dispersed in the atmosphere from industrial smoke or as a contaminant in rain. These cells have been shown to respond sensitively to Cd(II) treatment, leading to the hypothesis that plants attempt to reduce Cd-induced damage by closing their stomata, thereby reducing transpiration and, consequently, accumulation of the metal (Perfus-Barbeoch *et al.*, 2002).

In order to elucidate the function of a protein, it is important to know its location. This has been difficult for MT proteins, since cell fractionation destroys MT proteins that are unstable in the presence of oxygen. We showed that AtMT2a and AtMT3 fused to GFP or RFP are localized in the cytosol of *V. faba* and Arabidopsis cells (Figures 3–5 and S1), and that this localization did not change after Cd(II) treatment (Figures 6 and S2). While the protective effect of AtMT2a and AtMT3 was evident after treating the guard cells with 10 μM Cd(II) for 1 h, translocation was not detectable even after 3 h of treatment with higher concentrations of Cd(II), suggesting that these MTs are not sequestered into a compartment of low metabolic activity. Therefore, we suggest that Cd(II) bound to AtMT2a and AtMT3 most likely stays in the cytoplasm of the cell; this differs from Cd(II) bound to phytochelatin, which is sequestered into vacuoles. However, further studies using reliable techniques to detect MT proteins *in vitro* will be necessary to conclusively determine the localization of the MT proteins and their possible translocation out of the cytoplasm. It would be very interesting if indeed MTs and phytochelatins, which both contain many cysteine residues, are different in the final step of detoxification of Cd(II).

It has already been shown that Cd(II) induces the generation of ROS (Watanabe and Suzuki, 2002), but it had not previously been shown whether any Cd(II) detoxifier actually reduces the Cd-induced increase in ROS in plant cells. We clearly showed that AtMT3 reduced the level of ROS in a plant cell exposed to Cd(II) (Figure 7), and a similar effect was found for AtMT2a (Figure 7O). It is possible that AtMT2a and AtMT3 chelate Cd(II) in the cytoplasm, thereby blocking Cd(II) from freely interacting with cytoplasmic components or entering into organelles. This in turn would reduce the generation of ROS by the Cd(II)-induced disturbance of cellular processes, resulting in protection of the cell. Alternatively, MT protein itself might act as an antioxidant (Butt *et al.*, 1998). Consistent to this hypothesis, MT1 gene expression in Arabidopsis leaves is induced by H_2O_2 and intense light (Dunaeva and Adamska, 2001), which most likely elevates the ROS level in the leaf cells.

In summary, we have shown that AtMT2a and AtMT3 can contribute to Cd(II)-resistance when overexpressed in guard cells of *V. faba*, and its mechanism of detoxification apparently does not include sequestration of Cd(II) into organelles. In addition, our method of introducing the genes into guard cells and assaying the resistance of the transformed cells to abiotic stress provides a simple and fast way to evaluate candidate genes that may confer stress resistance.

Acknowledgements

We thank Dr. Dennis Thiele for the *YCF1*-null yeast line and its isogenic wild type, and Ms. Ki-Youb Park and Ms. Eunsook Jung for technical assistance. We also thank Ms. Jumok Park, Dr. Ildoo Hwang, and an anonymous reviewer for helpful suggestions. This work was supported by grants awarded to Y.L. from the National Research Laboratory program of the Ministry of Science and Technology of Korea and to I.H. from the Creative Research Initiative Program of the Ministry of Science and Technology of Korea (M10116000005-02F0000-00310).

References

Bethke, P.C. and Jones, R.L. 2001. Cell death of barley aleurone protoplasts is mediated by reactive oxygen species. Plant J. 25: 19–29.

Butt, A., Mousley, C., Morris, K., Beynon, J., Can, C., Holub, E., Greenberg, J.T. and Buchanan-Wollaston, V. 1998. Differential expression of a senescence-enhanced metallothio-

nein gene in *Arabidopsis* in response to isolates of *Peronospora parasitica* and *Pseudomonas syringae*. Plant J. 16: 209–221.

Clemens, S., Kim, E.J., Neumann, D. and Schroeder, J.I. 1999. Tolerance to toxic metals by a gene family of phytochelatin synthases from plants and yeast. EMBO J. 18: 3325–3333.

Cobbett, C. and Goldsbrough, P. 2002. Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. Annu. Rev. Plant Biol. 53: 159–182.

Cobbett, C.S., May, M.J., Howden, R. and Rolls, B. 1998. The glutathione-deficient, cadmium-sensitive mutants, cad2-1, of *Arabidopsis thaliana* is deficient in (gamma)- glutamylcysteine synthetase. Plant J. 16: 73–78.

Dunaeva, M. and Adamska, I. 2001. Identification of genes expressed in response to light stress in leaves of *Arabidopsis thaliana* using RNA differential display. Eur. J. Biochem. 268: 5521–5529.

Ha, S.-B., Smith, A.P., Howden, R., Dietrich, W.M., Bugg, S., O'Connell, M.J., Goldsbrough, P.B. and Cobbett, C.S. 1999. Phytochelatin synthase genes from Arabidopsis and the Yeast *Schizosaccharomyces pombe*. Plant Cell 11: 1153–116.

Hall, J.L. 2002. Cellular mechanism for heavy metal detoxification and tolerance. J. Exp. Bot. 53: 1–11.

Hirschi, K.D., Korenkov, V.D., Wilganowski, N.L. and Wagner, G.J. 2000. Expression of Arabidopsis CAX2 in tobacco. Altered metal accumulation and increased manganese tolerance. Plant Physiol. 124: 125–133.

Howden, R., Goldsbrough, P.B., Anderson, C.R. and Cobbett, C.S. 1995. Cadmium-sensitive, *cad1* mutants of *Arabidopsis thaliana* are phytochelatin deficient. Plant Physiol. 107: 1059–1066.

Jach, G., Binot, E., Frings, S., Luxa, K. and Schell, J. 2001. Use of red fluorescent protein from *Discosoma* sp. (dsRED) as a reporter for plant gene expression. Plant J. 28: 483–491.

Jin, J.B., Kim, Y.A., Kim, S.J., Lee, S.H., Kim, D.H., Cheong, G.W. and Hwang, I. 2001. A new dynamin-like protein, ADL6, is involved in trafficking from the trans-golgi network to the central vacuole in Arabidopsis. Plant Cell 13: 1511–1525.

Jung, J.Y., Kim, Y.W., Kwak, J.M., Hwang, J.U., Young, J., Schroeder, J.I., Hwang, I. and Lee, Y. 2002. Phosphatidylinositol 3- and 4-phosphate are required for normal stomatal movements. Plant Cell 14: 2399–2412.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant 15: 473–497.

Murphy, A. and Taiz, L. 1995. Comparison of metallothionein gene expression and nonprotein thiols in ten Arabidopsis ecotypes. Plant Physiol. 109: 945–954.

Murphy, A., Zhou, J., Goldsbrough, P.B. and Taiz, L. 1997. Purification and immunological identification of metallothioneins 1 and 2 from *Arabidopsis thaliana*. Plant Physiol. 113: 1293–1301.

Ohba, M., Shibanuma, M., Kuroki, T. and Noes, K. 1994. Production of hydrogen peroxide by transforming growth factor-beta 1 and its involvement in induction of egr-1 in mouse osteoblastic cells. J. Cell Biol. 126: 1079–1088.

Palmiter, R.D. 1998. The elusive function of metallothioneins. Proc. Natl. Acad. Sci. USA 95: 428–430.

Park, K. Y., Jung, J. Y., Park, J., Hwang, J. U., Kim, Y. W., Hwang, I., Lee, Y. 2003. A role for phosphatidylinositol 3-phosphate in abscisic acid-induced reactive oxygen species generation in guard cells. Plant Physiol. 132: 92–98.

Perfus-Barbeoch, L., Leonhardt, N., Vavasseur, A. and Forestier, C. 2002. Heavy metal toxicity: cadmium permeates through calcium channels and disturbs the plant water status. Plant J. 32(4): 539–548.

Piao, H.L., Pih, K.T., Lim, J.H., Kang, S.G., Jin, J.B., Kim, S.H. and Hwang, I. 1999. An Arabidopsis GSK3/shaggy-like gene that complements yeast salt stress-sensitive mutants is induced by NaCl and abscisic acid. Plant Physiol. 119(4): 1527–1534.

Sambrook, J. and Russell, D.W. 2001. Molecular Cloning: A Laboratory Manual, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Vatamaniuk, O.K., Mari, S., Lu, Y.P. and Rea, P.A. 1999. AtPCS1, a phytochelatin synthase from Arabidopsis: Isolation and in vitro reconstitution. Proc. Natl. Acad. Sci. USA 96: 7110–7115.

Vogeli-Langer, R. and Wagner, G.J. 1990. Sub-cellular localization of cadmium and cadmium-binding peptides in tobacco leaves. Implication of a transport function for cadmium binding peptides. Plant Physiol. 92: 1086–1093.

Watanabe, M. and Suzuki, T. 2002. Involvement of reactive oxygen stress in cadmium-induced cellular damage in *Euglena gracilis*. Comp. Biochem. Phys. C 131: 491–500.

Widholm, J.M. 1972. The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. Stain Technol. 47: 189–194.

Wink, M. 1993. The plant vacuole: a multifunctional compartment. J. Exp. Bot. 44: 231–246.

Zhou, J. and Goldsbrough, P.B. 1994. Functional homologs of fungal metallothionein genes from Arabidopsis. Plant Cell 6: 875–884.