A Mutant Strain Arabidopsis thaliana that Lacks Vacuolar Membrane Zinc Transporter MTP1 Revealed the Latent Tolerance to Excessive Zinc

Miki Kawachi1, Yoshihiro Kobae1, Haruki Mori1, Rie Tomioka1, Youngsook Lee2, and Masayoshi Maeshima1,∗

1Laboratory of Cell Dynamics, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya, 464-8601 Japan
2Division of Molecular Life Sciences, Pohang University of Science and Technology, Pohang, 790-784 Korea

A mutant line of Arabidopsis thaliana that lacks a vacuolar membrane Zn²⁺/H⁺ antiporter MTP1 is sensitive to zinc. We examined the physiological changes in this loss-of-function mutant under high-Zn conditions to gain an understanding of the mechanism of adaptation to Zn stress. When grown in excessive Zn and observed using energy-dispersive X-ray analysis, wild-type roots were found to accumulate Zn in vacuolar-like organelles but mutant roots did not. The Zn content of mutant roots, determined by chemical analysis, was one-third that of wild-type roots grown in high-Zn medium. Severe inhibition of root growth was observed in mtp1-1 seedlings in 500 µM ZnSO₄. Suppression of cell division and elongation by excessive Zn was reversible and the cells resumed growth in normal medium. In mutant roots, a marked formation of reactive oxygen species (ROS) appeared in the meristematic zone, where the MTP1 gene was highly expressed. Zn treatment enhanced the expression of several genes involved in Zn tolerance: namely, the plasma membrane Zn²⁺-export ATPase, HMA4, and plasma and vacuolar membrane proton pumps. CuZn-superoxide dismutases, involved in the detoxification of ROS, were also induced. The expression of plasma membrane Zn-uptake transporter, ZIP1, was suppressed. The up- or down-regulation of these genes might confer the resistance to Zn toxicity. These results indicate an essential role of MTP1 in detoxification of excessive Zn and provide novel information on the latent adaptation mechanism to Zn stress, which is hidden by MTP1.

Keywords: Arabidopsis thaliana • Metal tolerance • MTP • Zinc transporter • Vacuole.

Abbreviations: AHA, Arabidopsis thaliana plasma membrane H⁺-ATPase; EDX, energy dispersive X-ray; HMA, heavy metal transporter; ICP-AES, inductively coupled plasma atomic-emission spectroscopy; GUS, β-glucuronidase; MTP, metal tolerant protein; ROS, reactive oxygen species; TEM, transmission electron microscopy; V-ATPase, vacuolar membrane H⁺-ATPase; VHA-a, subunit a of V-ATPase; V-PPase, vacuolar H⁺-pyrophosphatase; MES, 2-(N-morpholino)ethanesulfonate.

Introduction

Zinc is a micronutrient that is relatively abundant in plants and functions in a number of enzymes and proteins, including copper–zinc superoxide dismutase (CuZn-SOD), DNA polymerase, and zinc-finger transcription factors (Delhaize et al. 2003, Vallee and Auld 1990). In plants, Zn deficiency suppresses growth, stress tolerance and chlorophyll synthesis. Zn-deficient soil, which includes around 30% of the world’s cultivated soil, is a serious problem, as is excessive Zn in soil caused by soil pollution and fertilizers. Supraoptimal concentrations of Zn cause symptoms such as chlorosis in leaves (Krämer 2005, Broadley et al. 2007). In mammals, excessive Zn may be a cause of neuronal cell death and various diseases (Lee et al. 2002a). Therefore, the free cytoplasmic Zn concentration in living cells must be precisely controlled at the nanomolar level (Outten and O’Halloran 2001, Kambe et al. 2008).

Plants contain a multiplicity of Zn transporters and Zn-chelating proteins. Uptake of Zn from soil is mediated by ZIP (zinc–iron permease) on the plasma membrane of roots.

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A mutant strain of Arabidopsis thaliana that lacks ZIF1 (zinc-induced facilitator-1), a Zn-inducible protein in Arabidopsis halleri (Kim et al. 2009), is more sensitive to Zn than either of the single mutants. When grown on a high-Zn MS medium, root elongation was strongly suppressed to less than 50% of that of the wild type (Kobae et al. 2004). The same phenomenon was observed for the RNAi knockdown mutant of MTP1 in the Columbia strain of Arabidopsis thaliana (Desbrosses-Fonrouge et al. 2005). MTP1 has been demonstrated to play a crucial role in Zn tolerance in the hyperaccumulator Arabidopsis halleri (Drager et al. 2004) and Thlaspi goesingense (Gustin et al. 2009). These observations strongly suggest that MTP1 plays a key role in detoxification of excess Zn in both A. thaliana and A. halleri.

In previous studies, we examined the effect of Zn on the root growth of these plants. We grew the seedlings for 6 days on MS plates (30 µM ZnSO4) containing 500 µM Zn, but the growth of roots and shoots of the mtp1-1 knockout plants suffered Zn toxicity severely at, or above, 700 µM Zn++. The aim of the present study was to examine the physiological roles of MTP1 and the adaptation process of mtp1-1 plants under excessive Zn. Therefore, we used Zn at 500 µM as high-Zn hereafter, unless otherwise indicated.

### Results

#### Disruption of MTP1 affects tolerance to Zn

A T-DNA-insertion mutant of Arabidopsis thaliana (strain Wassilewskija), mtp1-1, was demonstrated to be sensitive to Zn and resistance was recovered when mtp1-1 was transformed with the MTP1 gene under the control of the 35S promoter (Kobae et al. 2004). Therefore, we examined the mutant and wild-type plants, and compared their responses to excessive Zn. The germination rate of wild-type and mutant seeds on MS plates was not affected by Zn, even at 1 mM. At 2 mM Zn++, the germination rate of mtp1-1 seeds was reduced to 55%, although all the wild-type seeds germinated (Fig. 1A). We grew the seedlings for 6 days on MS plates (30 µM ZnSO4) and then transferred them to MS plates containing ZnSO4 at the indicated concentrations. After 10 days, the growth of shoots and roots were measured as fresh weight (Fig. 1B). The wild-type plants grew normally in medium containing 500 µM Zn, but the growth of roots and shoots of mtp1-1 plants was suppressed to less than 50%.

Both the wild-type and the knockout plants suffered Zn toxicity severely at, or above, 700 µM Zn++. The aim of the present study was to examine the physiological roles of MTP1 and the adaptation process of mtp1-1 plants under excessive Zn. Therefore, we used Zn at 500 µM as high-Zn hereafter, unless otherwise indicated.

#### Suppressed root growth is restored by removal of excessive Zn

The initial and most evident symptom of Zn toxicity was the inhibition of root growth in mtp1-1 plants (Fig. 1B). Thus, we examined the effect of Zn on the root growth of these mutant plants (Fig. 2). When mtp1-1 seeds were germinated on the high-Zn MS medium, root elongation was strongly suppressed to less than 50% of that of the wild type (Kobae et al. 2004). The same phenomenon was observed for the RNAi knockdown mutant of MTP1 in the Columbia strain of Arabidopsis thaliana (Desbrosses-Fonrouge et al. 2005). MTP1 has been demonstrated to play a crucial role in Zn tolerance in the hyperaccumulator Arabidopsis halleri (Drager et al. 2004) and Thlaspi goesingense (Gustin et al. 2009). These observations strongly suggest that MTP1 plays a key role in detoxification of excess Zn in both A. thaliana and A. halleri.
suppressed (Fig. 2D, L). Furthermore, growth was suppressed in the mutant plants transferred to high-Zn plates after germinated on the MS plates (Fig. 2F). The seedlings were transferred to normal MS plates to examine the reversibility of growth inhibition. As shown in Fig. 2H, the roots and shoots resumed growth, indicating that the inhibition of root elongation by excessive Zn was not due to cell death in the meristem and elongation zone. In other words, neither apoptosis nor necrosis occurred in roots and shoots of mtp1-1 plants, even in the presence of 500 µM ZnSO₄.
Cell size in the root tip is reduced under Zn stress

We observed the morphology of the roots under Zn stress. The mtp1-1 seedlings were observed 6 d after sowing in high-Zn or normal medium. Roots grew well in the normal medium and the mean root length was 7.6±1.3 mm. However, the root length was only 1.2±0.4 mm in the high-Zn conditions. The average cell length in the root epidermis was 182 and 28 µm under normal and high-Zn conditions, respectively (Fig. 3A–C). When cell number was counted on photomicrographs of roots, the average cell number on a single vertical line in the root epidermis under high-Zn conditions was reduced to a quarter of that of the roots grown in normal medium. These results indicate that excessive Zn suppresses the cell proliferation and cell elongation in roots.

To observe the morphological changes of the meristematic zone, we stained the roots with propidium iodide, which visualizes the cell wall. There was no significant difference in the arrangement or shape of cells in the meristematic and elongation zones (Fig. 3E, F). These results indicate that the inhibition of root elongation is due to arrest of cell proliferation and cell elongation.

**Fig. 3** Reduction of cell size of mtp1-1 roots by Zn. Mutant seedlings were germinated in 0.5×MS liquid medium (A and B) or 0.5×MS liquid medium containing 500 µM ZnSO₄ (C and D) for 6 d and then photographed. Parts a, b, and c of panel A were enlarged (B, a–b). Panel D is an enlarged image of the root in panel C. Mutant seedlings were grown on MS plates for 6 d and then transplanted to MS liquid medium (E) or a high-Zn (500 µM ZnSO₄) medium (F) for 6 d. Roots were then stained with propidium iodide and the images were captured using a confocal laser microscope.
Zn stress induces formation of reactive oxygen species

Excess concentrations of heavy metals, such as cadmium and nickel, have been reported to induce formation of ROS, including superoxide, hydrogen peroxide and hydroxyl radicals, in plant tissues (Mittler 2002, Freeman et al. 2004). Oxidative stress results from the formation of ROS, which damages or kills cells. We further examined the damage caused by excessive Zn, especially the oxidative stress in the mtp1-1 seedlings. Twenty-day-old plants grown under the normal conditions were transferred to the high-Zn medium and grown for two more days. We examined the ROS formation in roots by staining with a ROS indicator, carboxy-H$_2$DCFDA, which is a membrane-permeable fluorogenic reagent. On oxidation, carboxy-H$_2$DCFDA becomes the highly green fluorescent DCF in the cells. No ROS were detected in the wild-type roots even in the high-Zn medium (Fig. 4). In mtp1-1 seedlings grown under high-Zn conditions, ROS were clearly detected in the meristematic and elongation zones, including the quiescent center, but not in the columella and the root cap cells (Fig. 4H, L). The results indicate that the Zn-sensitive mutant formed ROS in the root meristematic and elongation zones in response to excessive Zn. ROS were also detected in the root tissues grown in the high-Zn medium for 12 and 24 h, but the fluorescence strength was weak.

Expression of MTP1 in roots and leaves

To investigate which cells express MTP1 in wild-type A. thaliana, we cloned an upstream region that contained a 5’-non-coding region of 2,593 bp and a 63-bp section of the coding region of MTP1 gene. The promoter construct was fused to a β-glucuronidase (GUS) reporter enzyme gene and introduced into mtp1-1 A. thaliana. In the previous studies, the promoter–GUS constructs were introduced into the wild-type A. thaliana Columbia strain (Desbrosses-Fonrouge et al. 2005). T$_2$ transgenic lines were analyzed for patterns of GUS expression. No significant differences in expression were observed among four independent lines. The GUS signal was detected in whole tissues of 6-day-old seedlings (Fig. 5A). In 10- and 14-day-old plants, the staining was visible in roots but not in leaves (Fig. 5B, C). This was consistent with the results indicating that the Zn-sensitive mutant formed ROS in the root meristematic and elongation zones in response to excessive Zn.
with the results reported for the Columbia strain (Desbrosses-Fonrouge et al. 2005). Intense staining was detected in the apex of the primary (Fig. 5E) and lateral roots of 14-day-old plants (Fig. 5F). Thus, the site of high expression of MTP1 is consistent with the ROS formation in the high-Zn conditions, as shown in Figure 4. The root hairs (Fig. 5G), hydathode (Fig. 5H, I) and stomata cells also showed high GUS activity (Fig. 5I). When the reporter gene was introduced into the wild-type plants, expression of MTP1 was not detected in the stomata cells or root hairs (Desbrosses-Fonrouge et al. 2005).

Zn stress enhances expression of Zn transporters, proton pumps and superoxide dismutases

We determined the transcript levels of several genes, which are thought to be related to Zn homeostasis, in the wild-type and mtp1-1 plants under high-Zn conditions. Ten-day-old seedlings previously grown in the standard medium were transferred to the high-Zn medium and cultivated for a further 10 d.

In the wild-type plants, expression of MTP1 was higher in roots than in shoots and was not changed under Zn stress (Fig. 6A). As described above, ROS were generated in mutant roots in the high-Zn medium (Fig. 4). Among three isozymes of CuZn superoxide dismutase (CSD), CSD1 has been reported to be involved in detoxification of ROS in the cytosol and CSD2 in the chloroplasts (Kanematsu and Asada 1989, Bowler et al. 1992, Bueno et al. 1995). Therefore, the mRNA levels of CSD1 and CSD2 were also quantified. Zn stress enhanced the expression of both CSD1 and CSD2 in shoots and CSD1 in roots of the mtp1-1 mutant.

In roots of the mtp1-1 mutant, excessive Zn enhanced approximately twofold the expression of HMA4, which is a plasma membrane heavy metal ATPase (Axelsen and

Fig. 5 Expression patterns of MTP1 promoter–GUS transgenic plants. Seedlings were grown in normal medium for 6 d (A), 10 d (B) and 14 d (C), and stained for GUS activity at 37°C for 1 h. Scale bar = 2 mm (A–C). (D) Root–shoot junction region of 6-day-old seedling. (E) Tip of a main root. (F) Tip of a lateral root. Scale bar = 200 µm (D–F). (G) Root hair. Scale bar = 50 µm. (H) True leaf. Scale bar = 1 mm. (I) A portion of hydathode (white arrowhead) and stomata (black arrowheads) of a true leaf marked by a square in panel H. Scale bar = 50 µm.
**Fig. 6** Changes in mRNA levels of Zn transporters, proton pumps and superoxide dismutase under excess-Zn conditions. Wild-type (WT) and mtp1-1 plants grown on MS plates for 10 d were transplanted to MS plates (gray bars) or MS plates containing 500 µM ZnSO₄ (black bars). After 10 d, total RNA fractions were prepared from shoots and roots separately. The mRNA levels of genes for MTP1 (A), ZIP1 (B), HMA2 (C), HMA4 (D), HMA3 (E), cytosolic copper/zinc superoxide dismutase-1 (CSD1, F), chloroplast copper/zinc superoxide dismutase-2 (CSD2, G), V-ATPase subunit a (VHA-a, H), H⁺-PPase (VHP1, I), and plasma membrane H⁺-ATPase (AHA1, J) were determined by real-time PCR using specific primer pairs and normalized to that of actin 2. The primer pairs used in the present experiments are listed in Table SI. The obtained values are expressed relative to that in shoots of wild-type plants grown on MS plates. The data represent means ± SD for two experiments (n > 12). n.d., not determined. Asterisks indicate fold induction values that are significantly different (P < 0.001) in Zn-treated samples compared with control samples.
Palmgren 2001, Hussain et al. 2004). In mutant shoots exposed to excessive Zn, the mRNA levels of vacuolar membrane H^+ATPase (V-ATPase) subunit a (VHA-a), vacuolar membrane H^+-pyrophosphatase (VHPT), and plasma membrane H^+-ATPase (AHAA1) were significantly increased. These proton pumps energetically support the Zn export at the plasma membrane and the Zn uptake into vacuoles by Zn^{2+}/H^+ antiporters, respectively.

In contrast to these genes, ZIP1 transcript level was markedly decreased when grown on the high-Zn plates. ZIP1 is known to be a metal transporter involved in the uptake of metals into the cytosol at the plasma membrane (Grotz et al. 1998).

Arabidopsis thaliana HMA1, HMA2, HMA3 and HMA4 belong to the Zn/Co/Cd/Pb-ATPase group. HMA2 and HMA4 in the plasma membrane are involved in the long-distance translocation of Zn (Verret et al. 2004) and HMA3 in the vacuolar membrane is involved in Zn homeostasis (Becher et al. 2004, Gravot et al. 2004, Morel et al. 2009). In the mtp1-1 mutant, expression of HMA4 was enhanced twofold in roots and shoots and expression of HMA3 was decreased in roots by 50% on the high-Zn plates, although HMA2 showed no response to Zn stress (Fig. 6C, D, E), suggesting a difference in physiological roles between HMAs.

In the wild-type plants, expression of MTP1 was higher in roots than in shoots and was not changed under Zn stress (Fig. 6A). Zn stress did not affect the mRNA levels of most genes in the wild-type plants, except for ZIP1 and CSD2 (Fig. 6B, G). The expression level of ZIP1 was markedly reduced, to 30% of the control, under Zn stress conditions.

**Knockout of the MTP1 gene suppresses accumulation of Zn in plants**

To examine the role of the MTP1 transporter in the accumulation of Zn into vacuoles, we grew the mtp1-1 and wild-type seedlings in the normal or excess-Zn media. In the latter case, seedlings were grown in the MS medium supplemented with additional 80 µM ZnSO_4_ for 10 d. Contents of 13 metals were determined by inductively coupled plasma atomic-emission spectroscopy (ICP-AES). The contents of metals are shown in Table 1. In the normal MS plates, roots of the mtp1-1 plants accumulated Zn at 116 µg (g dry weight)^{-1}. The value was approximately half of that in the wild-type roots (207 µg g^{-1}). Under excess-Zn conditions, the Zn content of the mutant root was approximately one-third of that in the wild-type (387 µg g^{-1} in the knockout and 1060 µg g^{-1} in the wild-type). Reduced accumulation of Zn in the mtp1-1 roots indicates the actual contribution of MTP1 to Zn accumulation into vacuoles in planta.

In the wild-type plant, the Zn content of the shoots was one-third of that in the roots under either condition. In the mutant plants, the Zn content of the shoots under the normal and excess-Zn conditions were one-half and one, respectively. Zn content of the shoots did not differ between

**Table 1 Contents of metal elements in shoots and roots of wild-type and mtp1-1 mutant seedlings.**

<table>
<thead>
<tr>
<th>Additional 80 µM ZnSO_4</th>
<th>WT</th>
<th>mtp1-1</th>
<th>WT</th>
<th>mtp1-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn (µg/g)</td>
<td>207±32</td>
<td>1060±170</td>
<td>116±20</td>
<td>387±6</td>
</tr>
<tr>
<td>P (mg/g)</td>
<td>41.4±2.4</td>
<td>36.5±5.9</td>
<td>45.3±10.0</td>
<td>33.7±1.3</td>
</tr>
<tr>
<td>S (mg/g)</td>
<td>38.2±1.8</td>
<td>36.5±5.6</td>
<td>43.8±9.8</td>
<td>31.2±16.2</td>
</tr>
<tr>
<td>K (mg/g)</td>
<td>24.5±1.6</td>
<td>25.0±3.1</td>
<td>28.9±7.5</td>
<td>17.9±0.6</td>
</tr>
<tr>
<td>Fe (mg/g)</td>
<td>3.49±0.18</td>
<td>2.62±0.51</td>
<td>3.79±0.80</td>
<td>3.05±0.22</td>
</tr>
<tr>
<td>Ca (mg/g)</td>
<td>3.04±0.12</td>
<td>2.60±0.36</td>
<td>3.68±0.46</td>
<td>3.17±0.28</td>
</tr>
<tr>
<td>Na (mg/g)</td>
<td>2.62±0.17</td>
<td>2.69±0.41</td>
<td>2.88±0.75</td>
<td>1.70±0.04</td>
</tr>
<tr>
<td>Mg (mg/g)</td>
<td>1.80±0.17</td>
<td>1.56±0.21</td>
<td>1.85±0.35</td>
<td>1.30±0.05</td>
</tr>
<tr>
<td>Cu (µg/g)</td>
<td>10.3±0.34</td>
<td>7.94±1.08</td>
<td>13.6±4.0</td>
<td>10.9±0.7</td>
</tr>
<tr>
<td>Mn (µg/g)</td>
<td>94.7±4.4</td>
<td>99.7±16.5</td>
<td>86.6±14.1</td>
<td>97.1±4.1</td>
</tr>
<tr>
<td>B (µg/g)</td>
<td>64.5±19.0</td>
<td>9.00±2.10</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Cr (µg/g)</td>
<td>2.9±0.4</td>
<td>1.8±0.4</td>
<td>2.9±0.4</td>
<td>2.6±1.0</td>
</tr>
<tr>
<td>Al (µg/g)</td>
<td>555±230</td>
<td>336±189</td>
<td>494±227</td>
<td>379±209</td>
</tr>
</tbody>
</table>

Arabidopsis thaliana seedlings were grown in solid MS medium (30 µM ZnSO_4_) for 10 d and then transferred to the Gellan gum plates or the liquid medium containing MS salt supplemented with 80 µM ZnSO_4_ (final Zn^{2+} concentration, 110 µM). After further 10 d, shoots were collected from seedling in the plates and roots from seedlings in the liquid medium. Metals were extracted from dried tissues and determined by the ICP-AES method as described in Materials and Methods. The contents are expressed on the basis of dry weight of tissues. Values are mean of three independent experiments. n.d., not detectable (less than 1.2 ng g^{-1}).
the mutant and wild-type plants. The Zn contents under the normal and excess-Zn conditions were 66.4 and 402 µg g\(^{-1}\), in the mutant, and 72.2 and 362 µg g\(^{-1}\), in the wild type. This result suggests no effect of the lack of MTP1 on the shoot under these assay conditions for at least 10 d.

In the mutant roots under the excess-Zn conditions, contents of K and Na were decreased by approximately 40\% (Table 1). Treatment with Zn increased accumulation of Cu, Mg, P, S and Al more than twofold in the wild-type and mutant shoots. The contents of Fe, Cr and Ca in both shoots were also increased.

The B content in the mutant roots under either condition was decreased to less than the detectable amount (<1.2 ng g\(^{-1}\)), although the mutant shoots accumulated B at 33.2 and 11.2 µg g\(^{-1}\) under the normal and excess-Zn conditions, respectively. The MS medium contained 6.2 mg l\(^{-1}\) of B.

Morphological properties of root cells grown under high-Zn conditions

High accumulation of Zn in the wild-type plants grown in the high-Zn medium led us to examine the morphological changes in the intracellular and cell wall structure in plants after Zn treatment. Figure 7 shows the electron-micrographs of the epidermis regions of wild-type roots. These proliferating and growing cells in the root apex are young and only a few micrometers in diameter. The cells are rich in mitochondria and are not vacuolated. ER bodies are surrounded by a single ribosome-bearing membrane and exist in the epidermal cells of roots, hypocotyls and cotyledons of A. thaliana seedlings (Matsushima et al. 2003, Yamada et al. 2008). ER-body-like structures were observed in these cells (Fig. 7B, D). Zn treatment did not affect the morphology or number of the ER-body-like organelles.

Many small vesicles, less than 0.5 µm in diameter, were observed in the roots grown in the MS medium (Fig. 7A, B). These vesicles seem to be small vacuoles or pre-vacuolar compartments. These small vesicles were also observed in the roots grown under the high-Zn conditions. In the medium containing 500 µM ZnSO\(_4\), where the growth of the wild-type roots is not affected, the cells contained many electron-dense organelles of irregular shape (Fig. 7C, D). The rough ERs were well developed, especially in the roots grown in the high-Zn medium. These observations indicate that excessive Zn induces morphological changes in the ER, small vacuoles, prevacuolar compartments and electron-dense small vesicles of the root epidermal cells of wild-type plants. In contrast to the wild-type roots, the mtp1-1 roots showed no notable changes in cell structure (Fig. S1).

To determine the intracellular distribution of Zn taken into roots of wild-type plants, we analyzed the root tissues by a combination technique of transmission electron microscopy and energy-dispersive X-ray (TEM-EDX). For this technique, the tissue fixation is partly different from that for regular TEM. As a result, the structures of plasma and vacuolar membranes are not sharp. Fig. 8B shows a distribution map of Zn in the field of view shown in panel A. The EDX map of the wild-type root revealed that Zn was concentrated in specific vacuoles, in which several electron-dense particles of approximately 100 nm in diameter were observed. The EDX map of Zn was consistent with vacuoles or vacuolar-like organelles containing electron-dense particles, although the signal intensity of Zn was not high. Existence of Zn in the high-density part in the EDX map (Fig. 8B) was confirmed by the energy spectrum of EDX (Fig. 8C). Absorption peaks of the electron shells of K, L, and M of Zn could be detected. In addition to Zn, the spectrum shows the existence of Ca and P. Thus, it is estimated that the...
electron-dense particles are not crystals of Zn but aggregates of Zn and other components, such as organic acids and other metals. It should be noted that there are peaks of Pb and Cu, which were used for staining of thin sections and as a mesh for TEM. Peaks of Pb and Cu, but not of Zn, were detected in the EDX of the mtp1-1 roots (Fig. 8D). Thus, the Zn peaks are due to Zn accumulated in the wild-type roots grown in the high-Zn medium.

Discussion

Excessive Zn suppressed cell proliferation and elongation in mutant roots

The present study demonstrated that excessive Zn inhibited cell proliferation and subsequent cell elongation in mutant seedlings. The inhibition of cell proliferation was evidenced by a reduced number of cells in the roots. The average cell number on a single line of the root epidermis was reduced to one-quarter and the average cell length to one-sixth (Fig. 3). As a result, the length of mutant roots was suppressed to less than 16% by Zn stress. Growth of shoots and leaves was much less affected by excessive Zn (Fig. 2). The suppression of root elongation by Zn was removed when transferred to the normal medium. In the present study, we focused on the reversible inhibition of root elongation by excessive Zn, to understand the strategy of plants to survive excess-Zn conditions.

By using the mtp1-1 plants we can investigate physiological changes at relatively low Zn concentrations. Furthermore, it is clear that the dysfunction occurs in the vacuolar Zn uptake system. In addition, mtp1-1 plants provide novel information on the latent adaptation mechanism to Zn stress, which is hidden by MTP1.

Suppression of root elongation of the mutant seedlings by excessive Zn seems to be an adaptive response for tolerance to Zn stress since: (i) no severe damage or necrosis of the root tissues was observed; (ii) roots resumed growth after transfer to normal culture medium; and (iii) excessive Zn stimulated several genes (HMA2, HMA3, HMA4, CSD1, CSD2, VHA-a, VHP1 and AHA1), which might be involved in the avoidance of Zn stress. In addition to the stimulation of these genes, expression of ZIP1, a Zn uptake transporter (Grotz et al. 1998), was suppressed under the high-Zn conditions. ZIP1 is a plasma membrane Zn uptake transporter and expression of the gene is enhanced in roots under the Zn-deficient conditions (Grotz et al. 1998). Judging by the changes in expression of these genes, there might be a signaling pathway that is initiated by the hazard signal of excessive Zn to bring about protective responses.

Induction of ROS production by Zn

The present study demonstrated the involvement of ROS in the response of roots to Zn stress. ROS were produced in
the root meristematic zone but not in the other tissues (Fig. 4). The ROS level in this zone was detectable but may not be high enough to damage the tissues severely. ROS have a dual function in plant cells. As a toxic metabolite, ROS cause oxidative stress and serious damage to proteins, lipids and DNA. On the other hand, ROS act as signaling molecules that regulate cellular processes, such as apoptosis and defense to biotic stress (Mithöfer et al. 2004, Mittler 2002, Poschenrieder et al. 2006). In the latter case, ROS allow a plant to respond to stress, a prerequisite for adaptation to changes in physiological conditions. The concentration of ROS thus has to be balanced. The tissue specificity of ROS production is consistent with that of the expression of ROS thus has to be balanced. The tissue specificity of ROS changes in physiological conditions. The concentration of ROS has to be high enough to damage the tissues severely. ROS have a dual function in plant cells. As a toxic metabolite, ROS are known to be expressed in roots; namely, HMA2, HMA4 and MTP3. HMA2 and HMA4, heavy metal-transporting ATPases, participate in exclusion of metal ions through the plasma membrane (Eren and Argüello 2004, Hussain et al. 2004). MTP3, an orthologues of MTP1, is also involved in accumulation of cytosolic Zn into vacuoles (Arrivault et al. 2006). However, these three genes are not expressed in the meristematic zone of roots.

Phytochelatin is a well-known metal chelator which removes metal ions from sensitive sites of enzymes or proteins by sequestration. Accumulation of phytochelatins is triggered by exposure to various metal ions (Cobbet and Goldsbrough 2002, Roth et al. 2006). Phytochelatin synthase, AtPCS1, is expressed in roots but not in root tips (Lee et al. 2002b). Therefore, in the mature region of roots, HMA2, HMA4 and MTP3 might exclude excessive Zn from the cytosol to the extracellular space and vacuoles, and phytochelatin can also trap Zn ions. There is a possibility that a Zn ligand or a Zn–ligand complex is transported into vacuoles by ZIF1 (Hydon and Cobbett 2007). In contrast to these tissues, only MTP1 plays a role in exclusion of excessive Zn in the cytosol of the meristematic zone. Thus, Zn stress might increase the cytoplasmic Zn concentration in the root meristematic zone in the mtp1-1 mutant. This elevation of cytoplasmic Zn^{2+} might be a trigger of ROS production.

Adaptive responses of Zn- and stress-related genes to Zn stress

In addition to ROS production, expression of several genes was regulated in the wild-type and mtp1-1 plants under Zn stress. We examined isoforms of superoxide dismutases (SOD) that catalyze the dismutation of superoxide radicals to molecular oxygen and hydrogen peroxide. Plants have three types of SOD; namely, CuZn-SOD, Mn-SOD, and Fe-SOD. Mn-SOD and Fe-SOD are localized in mitochondria and chloroplasts, respectively (Jackson et al. 1978). There are three isozymes of CuZn-SOD (CSD); CSD1, CSD2 and CSD3, which are localized in the cytosol, chloroplast and peroxisome, respectively (Chu et al. 2005, Kliebenstein et al. 1998). Thus, CSD1 is the single candidate for elimination of ROS in the cytosol. Treatment with Zn doubled the mRNA level of CSD1 in the mutant plants, but not in the wild-type plants (Fig. 6). The increased CSD1 level may scavenge for ROS in the cytosol.

The reduction in the mRNA level of ZIP1 was significant both in the wild-type and mutant plants under the excess-Zn conditions. ZIP1 is a plasma membrane Zn uptake transporter of roots and its transcription is enhanced under Zn-deficient conditions (Grotz et al. 1998). Therefore, the suppression of ZIP1 expression is a logical response to excessive Zn. The mRNA level of HMA4, which is involved in long-distance Zn transport (Mills et al. 2005, Verret et al. 2004), was increased twofold in the mutant roots and shoots. This may be a reason for the altered shoot to root ratio of Zn content in the mutant (Table 1). Both HMA4 and HMA2 have been reported to be co-expressed in the vascular tissues (Hussain et al. 2004). Their double-knockout mutant plants showed symptoms of Zn deficiency under normal conditions. Thus these HMAS are thought to export Zn from the root symplasm into the apoplastic xylem. Recently, Hankene et al. (2008) reported that the cis-regulatory element of HMA4 and its triplication are essential for hyper-accumulation of metals. In contrast to HMA4, HMA2 showed no significant change under the high-Zn conditions (Fig. 6), suggesting a difference in their physiological roles. Expression of a gene for vacular membrane HMA3 was enhanced in the wild type by Zn (Fig. 6). This is consistent with a previous observation that HMA3 is involved in Zn homeostasis (Becher et al. 2004, Morel et al. 2009). However, HMA3 expression was notably suppressed under Zn stress in the mutant. The results suggest that HMA3 does not complement the physiological function of MTP1 in the mutant.

In the mutant shoots, the mRNA levels of three proton pumps were significantly increased (Fig. 6). Arabidopsis thaliana has 11 functional isoforms of the plasma membrane H^{+}-ATPase (Axelsen and Palmgren 2001, Harper et al. 1989, Palmgren 2001). AHA1 and AHA2 are expressed at high levels in virtually all tissues and organs, and these enzymes appear to function as housekeeping genes required for ion homeostasis (Gaxiola et al. 2007). AHA1 transcript is enriched in shoots compared to roots, whereas AHA2 is predominantly expressed in roots, especially in root hairs (http://www.geneinvestigator.com, Hruz et al. 2008). The transcription level of AHA1 was increased in shoots of the mtp1-1 mutant.
AHA1 may support an unknown \( \text{Zn}^{2+}/\text{H}^{+} \) antiporter system at the plasma membrane or maintain the cytosolic pH in the mutant shoots.

V-ATPase and V-PPase energetically support the secondary active transporters, including MTP1 and MTP3 (Sze et al. 2002, Gaxiola et al. 2007). Especially in the growing young tissues, V-PPase serves as a main proton pump in the vacuolar membrane (Maeshima and Yoshida 1989, Nakanishi and Maeshima 1998, Maeshima 2000). In mtp1-1 shoots, the mRNA levels of V-PPase and the subunit a of V-ATPase (VHA-a) were markedly increased in response to excessive Zn. VHA-a has multiple functions; namely, proton transport, membrane targeting, prevention of unproductive rotation of the head V, complex and interaction with aldolase (Forgac 2007). Therefore, increased levels of mRNAs of these proton pumps are reasonable to support the secondary active transporters of Zn and other metals accumulated in the mutant shoots. It might be concluded that these changes in gene expression are adaptive responses to the increased level of Zn in the wild-type and mutant plants.

**Alteration of contents of Zn and other metals in mutant seedlings**

Excessive Zn increased the Zn content in the wild-type plants approximately fivefold in both shoots and roots. Lack of MTP1 reduces the accumulation of Zn (Table 1). These mean, firstly, that A. thaliana roots accumulate Zn at a high level under an excess-Zn conditions, and, secondly, that the accumulation of Zn partly depends on MTP1 in the vacuolar membrane. Under excess-Zn conditions, the increased level of Zn in vacuoles was demonstrated by ICP-AES (Table 1). In the present experiment using TEM-DEX, Zn was shown to have accumulated in the vacuoles or vacuole-like organelles, which contained the electron-dense particles, in the wild-type roots (Fig. 8). Vesicular storage sites for Zn have been found in yeast and mammalian cells (Devirgiliis et al. 2004, Eide 2006, Haase and Beyersmann 2002). These membrane-bound vesicles have been designated as ‘zincosomes’. ‘Zincosomes’ are small vesicles and have been proposed to correspond to late endosomes. ‘Zincosomes’ have not been found in plant cells. In the present study, we detected Zn-accumulating vesicles, which were relatively large with a diameter of 1 μm. The existence of ‘zincosomes’ or Zn-specialized vacuoles in plant cells and the MTP1 proteins in its membrane remain to be examined in the future.

Analysis of metals revealed that the lack of MTP1 affected the contents of several metals in plants grown under excess-Zn conditions (Table 1). Contents of Cu, Cr and Ca in the mutant roots and of Cu, Ca, K, Mg, Na, P and S in the mutant shoots were increased by more than 20% when compared with the wild-type plants. Other metals, such as Al in the mutant shoots, and K and Na in the mutant roots, were decreased. Changes in metal contents may be related to a delay in growth of mutant plants by Zn stress. Furthermore, a high concentration of Zn may affect the transport activity of the other metals. Another possibility is that a change in Zn balance in the mutant plants affects the Zn signaling, which has been well studied in mammalian cells (Kambe et al. 2005), and alters the balance of metal ions through regulation of membrane transporters and/or metal-binding compounds. Biochemical mechanism and physiological implications of changes in the contents of metals, including a drastic decrease of B (Table 1), in the mtp1-1 plants remain to be examined.

In conclusion, the results of the present study revealed two points; namely, the physiological importance of MTP1 in A. thaliana and the molecular mechanism of response to Zn stress in the Zn-sensitive mutant. The present study, using a loss of function mutant of MTP1, discloses latent adaptive responses to Zn stress, which are hidden by MTP1. Excessive Zn inhibited the proliferation and elongation of the root meristematic cells but did not kill the cells. These protective and adaptive processes may be mediated by a signal substance(s) including ROS specifically formed in the root meristematic and elongation zones. It is concluded that MTP1 plays a crucial role in avoiding Zn stress in the root meristematic tissue before activation of Zn export systems and syntheses of phytochelatins.

**Materials and Methods**

**Plant materials and growth conditions**

Surface-sterilized seeds of A. thaliana (strain Wassilewskija) were germinated on sterile plates of Murashige-Skoog medium containing 2.5 mM MES-KOH, pH 5.7, 2% (w/v) sucrose, and 0.3% (w/v) Gelman gum (MS plate) or MS plates supplemented with different concentrations of ZnSO₄. Six- or 10-day-old seedlings were transferred to MS plates or MS media with different concentrations of ZnSO₄. The regular MS medium contained 30 μM ZnSO₄. For the germination test, seeds of the wild-type and mtp1-1 were sown on plates of 0.5×MS (half concentrations of MS salts) supplemented with ZnSO₄. After 8 d, the germinated seeds were counted. All plants were grown at 23°C under long-day conditions (light/dark regime of 16 h/8 h, cool-white lamp). A mutant line of mtp1-1 was obtained as described previously (Kobay et al. 2004). In some cases, the roots were photographed to count cell numbers in the epidermis.

**Histochemical assay of MTP1 promoter-MTP1::GUS gene expression**

A genomic sequence for the promoter region (~2593 bp from the first ATG) was isolated from genomic DNA by PCR using the primers 5′-CACCAGAGGGAACCTATTTATGGTGATGCTGTC-3′ (forward) and 5′-TTCATCAGATTTTCC AACATTAACCTCAACAT-3′ (reverse). The resulting
fragment was linked with cDNA for MTP1 and then ligated into a Gateway entry vector pENTER/D-TOPO with a TOPO cloning kit (Invitrogen, Carlsbad, CA, USA) and introduced into the binary vector pGWB203 (developed by Dr. Tsuyoshi Nakagawa, Shimane University, Japan), in order to produce a translational fusion product. The chimeric construct was introduced into Agrobacterium tumefaciens strain GV3101::PM90 by electroporation and used to transform mtp1-1 plants. Transformants were selected on plates containing 0.2 µg ml⁻¹ Cefotax (Chugai Pharmaceutical Co., Tokyo, Japan), 20 µg ml⁻¹ hygromycin, and 30 µg ml⁻¹ kanamycin. These MTP1 promoter-MTP1::GUS line seeds were selected and germinated on MS medium Gelan gum plates. Plants or plant tissues were incubated in 1.92 mM 5-bromo-4-chloro-3-indolyl-β-D-glucurinide, 0.5 mM K₃Fe(CN)₆, 0.5 mM K₄Fe(CN)₆, and 3% (w/v) Triton X-100 at 37°C for 1 h. The reaction was stopped by incubating in 70% ethanol. Samples were photographed using an Olympus SZ61 microscope (Tokyo, Japan).

**Propidium iodide staining**

Roots were incubated in 10 µg ml⁻¹ propidium iodide (Sigma Aldrich, Steinheim, Germany) for the staining of root cells walls. Propidium iodide red fluorescence was visualized with a Fluoview FV500 confocal laser-scanning microscope (Olympus) set at 561 nm for excitation and at 550 to 650 nm for emission.

**Imaging of reactive oxygen species**

*Arabidopsis thaliana* roots were stained with 10 µM carboxy-H₂DCFDA (C400; Molecular Probes, Eugene, OR, USA) for 15 min and then rinsed. A BX51 fluorescence microscope (Olympus) equipped with a green fluorescent filter (excitation 450–490 nm, emission 510–550 nm) was used for epifluorescence images. Images were captured with a DP70 CCD camera (Olympus).

**RNA preparation and mRNA quantification**

Shoots or roots were collected from *A. thaliana* seedlings and the root/shoot junction region was removed. The collected tissues were frozen in liquid nitrogen and homogenized with a mortar and pestle. RNA was extracted from the frozen tissue powder by the NucleoSpin™ RNA Plant Kit (Macherey-Nagel, Düren, Germany). First-strand cDNA was synthesized from 5 µg of total RNA in a 100-µl reaction medium by reverse transcription using an iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer’s protocol. Quantitative PCR analysis was conducted on a Thermal Cycler Dice Real-Time System (Takara Bio, Otsu, Japan) using a SYBR Premix Ex Taq kit (Takara Bio), according to the manufacturer’s instructions, for 40 cycles with denaturation at 95°C for 5 s and annealing at 60°C for 30 s. The amounts of mRNA species were calculated according to those of Actin2 as determined in parallel amplifications and shown as relative quantities in comparison to the standard mRNA preparation from shoots or roots of wild-type plants grown on the normal MS plates. Primer sets used for real-time PCR are listed in Table S1.

**Electron microscopy**

For transmission electron microscopy, 20-day-old roots were cut into 2-mm long pieces. The tissue was fixed overnight at 4°C in 2% paraformaldehyde of distilled EM grade (Merck, Germany) and 2% glutaraldehyde in 30 mM HEPES, pH 7.4, 100 mM NaCl, 2 mM CaCl₂, and then washed four times for 15 min each in 0.1 M cacodylic acid buffer, pH 7.4, and post-fixed in 1% OsO₄ for 4 h. After three 5-min washes in 0.1 M cacodylic acid buffer, pH 7.4, tissue was dehydrated through an ethanol series (30% to 100%) for 15 min each at 4°C up to 70% ethanol and then at room temperature. The tissue was infiltrated with an epoxy resin (Quetol-651; Nisshin EM Co., Tokyo, Japan) at 60°C for 2 d. Ultra thin sections (90 nm in thickness) were counter stained with uranyl acetate and lead citrate and viewed with a JEOL (Tokyo, Japan) JEM2010 transmission electron microscope.

**Determination of metal contents in plant tissues**

*Arabidopsis thaliana* seedlings were grown on the MS plates (30 µM ZnSO₄) for 10 d and then transferred to the Gellan gum plates or the liquid medium containing MS salt supplemented with an additional 80 µM ZnSO₄ (final Zn²⁺ concentration, 110 µM). After a further 10 d, shoots were collected from seedlings grown in the plates and roots from seedlings grown in the liquid medium. Shoots and roots (n ≥ 6 for shoots, n ≥ 15 for roots) were harvested and dried at 80°C for 48 h. Three sets of samples were prepared. Dried tissues (20 mg) were digested with ultra-pure concentrated HNO₃ for 22 min at 130°C using Teflon vessels (ETHOS-1600, Sorisole, Italy). Then the contents of metal elements were determined by an ICP-AES (inductively coupled plasma atomic-emission spectroscope) (type IRIS ICAP, Nippon Jarrell Ash, Tokyo, Japan). The analytical precision for element analysis was confirmed using the NIES standard (No. 1 and No. 9) to be better than 10% for all elements analyzed.

**Elemental composition analysis (TEM-EDX)**

For transmission electron microscopy (TEM), the root tips of 20-day-old seedlings were cut into 2-mm long pieces and fixed at 4°C in 2% glutaraldehyde and 2% paraformaldehyde in HEPES buffer (30 mM HEPES, 100 mM NaCl, 2 mM CaCl₂, pH 7.4) over night. After fixation, the tissues were dehydrated through an ethanol series (30% to 100%) for 15 min each and then infiltrated with an epoxy resin (Quetol-651; Nisshin EM Co.) at 60°C for 2 d. Tissues were sectioned on an ultramicrotome (2088 Ultrrotome V; LKB, Bromma, Sweden) equipped with a diamond knife,
mounted on formvar-coated copper grids and stained with Reynold’s lead citrate. TEM-EDX analysis was performed on a transmission electron microscope (JEM-2010; JEOL Co., Tokyo, Japan) equipped with an EDX analyzer (NORAN Voyager III; NORAN Instruments Inc., Middletown, WI, USA). The TEM-EDX analyses were carried out at an accelerating voltage of 200 kV in the scanning-TEM mode. X-ray spectra were taken for 100 s and elemental peak data were analyzed with an EDX analyzer.

**Supplementary data**

Supplementary data are available at PCP online. (This manuscript has supplementary data: Fig. S1 and Table S1.)

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**References**


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