Effects of Zn on plant tolerance and non-protein thiol accumulation in Zn hyperaccumulator *Arabis paniculata* Franch

Xiao-Wen Zeng, Lena Q. Ma, Rong-Liang Qiu, Ye-Tao Tang

1. Introduction

Although Zn is an essential element to plants, excessive Zn is toxic to plants, resulting in functional and structural disorders. Zinc concentrations in plant > 300 mg kg⁻¹ are considered toxic (Marschner, 1995). However, Zn hyperaccumulator plants can accumulate and tolerate much higher Zn in tissues (Baker and Brooks, 1989). Among these plants, *Thlaspi caerulescens* and *Arabidopsis halleri* are two examples, which hyperaccumulate both Zn and Cd (Lasat et al., 2000; Pence et al., 2000; Zhao et al., 2000).

Plants have developed defense strategies against heavy metals, such as avoidance, chelation and sequestration inside the cells, or efflux from the cytosol to the apoplast. In addition, chelation of heavy metal is achieved in plants by phytochelatins (PCs), which have a basic structure of \((\gamma\text{-Glu-Cys})^n\text{-Gly}\), where \(n = 2–11\) (Cobbett, 2000). While the potential role of non-protein thiols (NPTs) such as GSH and PCs in Cd uptake and complexation in Zn/Cd hyperaccumulator *Arabis paniculata* F. has been reported previously (Zeng et al., 2009), the role of NPTs in Zn defense system is poorly understood. Therefore, the objective of this study was to investigate the Zn accumulation and tolerance, and responses of non-protein thiol.
thiols in Zn hyperaccumulator A. paniculata under different Zn stress (0, 100, 250, 500, 1000 and 2000 μM Zn).

2. Materials and methods

2.1. Plant material and treatments

Seeds of A. paniculata were germinated in the mixture of sands and vermiculite with modified 20% Hoagland solution in a controlled room at 25°C and relative humidity of 60%. The modified 20% Hoagland solution contained (in μM): 796 Ca(NO3)2, 422 MgSO4·7H2O, 1300 KNO3, 100 NH4H2PO4, 9 H3BO3, 0.5 MnSO4·H2O, 0.08 CuSO4·5H2O, 0.15 ZnSO4·7H2O, 0.02 (NH4)2Mo7·4H2O, and 25 Fe-EDTA. A 10-h light period with average light intensity of 350 μmol m−2 s−1 was supplied by an assembly of fluorescent lamps. Six months after germination, seedlings were transferred to a modified 20% Hoagland solution for a month acclimation. The solution pH was maintained at 5.8 by adding 300 mM of 1 M methansulphonic acid. The nutrient solution was renewed every week and aerated continuously.

Before exposing to Zn treatments, the P concentration in nutrient solution was reduced from 100 to 50 μM to minimize precipitation of Zn–P minerals. The treatment consisted of six Zn concentrations (0, 100, 250, 500, 1000 and 2000 μM) for 3-w of exposure, and three replicates for each treatment.

2.2. Non-protein thiols analysis by HPLC

The extraction of NPTs in plant tissues was performed via the method of Sneller et al. (2000) and Sun et al. (2006). After harvest, the plant tissues were immediately frozen in liquid-N2 and stored in the dark at 4°C until analysis. Tissues were ground in liquid-N2 and then used for thiol extraction in 0.1% trifluoroacetic acid (2-morpholinoethanesulphonic acid). The homogenate was centrifuged at 25,000 × g for 15 min. To 1 ml of supernatant, equal volume of 10% (w/v) TCA containing 0.5% (w/v) TBA was added, mixed and heated at 95°C for 30 min. Samples were then cooled in ice and centrifuged at 10,000 × g for 10 min. The absorbance of the resulting supernatant was recorded at 532 nm and corrected for non-specific turbidity by subtracting the absorbance at 600 nm. The concentration of lipid peroxides, together with oxidatively modified plant proteins, were quantified and expressed as total thiobarbituric acid-reacting substances (TBARS) in terms of μmol g−1 fw using an extinction coefficient of 155 mM−1 cm−1.

2.4. Total and water-soluble Zn in plants

At harvest, plant roots were immersed into 20 mM Na2-EDTA for 15 min to remove the metals adhering to root surface, then rinsed with distilled water thoroughly (Tang et al., 2009). Plant samples for Zn determination were dried at 70°C for 3 d and digested with HNO3 and H2O2 (EPA Method 3050B). Total Zn concentration was determined by flame atomic absorption spectrophotometry (SpectraA 220FS, Varian Inc., USA). Water-soluble Zn was determined following Zhao et al. (1998). Ground dry plant tissues of 0.5 g were extracted with 20 ml of 1 mM MES (pH 6.0) by shaking for 2 h thoroughly. Extraction solution was filtered with 0.4 μM membrane filter prior to Zn analysis.

2.5. X-ray fraction (XRD) analysis

X-ray diffraction (XRD) using Nicolet computer-automated diffractometer. The X-ray source was a Cu anode operating at 35 kV and 20 mA using CuKα radiation with a diffracted beam graphitemonochromator. The powder of sample was mounted on a quartz holder. Scans were conducted from 2 to 60° at a rate of 1.8° 20 per min. Identification of the phase peaks was accomplished by comparing the observed XRD patterns to standards compiled by the Joint Committee on Powder Diffraction and Standards (JCPDS, 1980).

2.6. Statistical analyses

Statistical analyses were performed using the SPSS statistical package. Data were tested at significant level by one-way ANOVA. The subsequent multiple comparisons among means were examined based on the least significance difference (LSD) test at α = 0.05.

3. Results

3.1. Plant growth

To determine the effects of Zn on its Zn accumulation and tolerance, and responses of non-protein thiols, A. paniculata was grown in 20% Hoagland solution containing 0–2000 μM Zn for 3-w. After exposing to as high as 2000 μM Zn for 3-w, A. paniculata showed neither visible symptom of phytotoxicity nor reduction in the biomass (Table 1). In fact, Zn concentrations up to 500 μM increased its biomass. For example, the roots exposed to 100 and
Table 1
Fresh biomass of *A. paniculata* after 3-w of exposure to Zn (g pot$^{-1}$).

<table>
<thead>
<tr>
<th>Zn concentration (µM)</th>
<th>Shoot biomass</th>
<th>Root biomass</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>10.2 ± 1.58 AB</td>
<td>1.82 ± 0.27bc</td>
</tr>
<tr>
<td>100</td>
<td>12.1 ± 0.30 A</td>
<td>4.00 ± 0.28a</td>
</tr>
<tr>
<td>250</td>
<td>12.7 ± 0.62 A</td>
<td>3.36 ± 0.36a</td>
</tr>
<tr>
<td>500</td>
<td>11.2 ± 0.88 AB</td>
<td>2.94 ± 0.37ab</td>
</tr>
<tr>
<td>1000</td>
<td>9.62 ± 0.25 B</td>
<td>1.90 ± 0.27bc</td>
</tr>
<tr>
<td>2000</td>
<td>8.84 ± 0.60 B</td>
<td>1.79 ± 0.26c</td>
</tr>
</tbody>
</table>

Data were presented as mean ± S.E.; means followed by different letters in a given column indicate significant difference between treatments ($p<0.05$).

250 µM Zn accumulated 2.2- and 1.8-times more biomass than the control ($p<0.05$).

3.2. Zinc accumulation in plants

The total Zn concentrations in the plant shoots increased as Zn levels increased, which reached the maximum of 6030 mg kg$^{-1}$ (dw) at 2000 µM Zn (Fig. 1a). However, there was no significant difference in the root Zn concentrations in plants exposed to 250–2000 µM Zn, which ranged from 9025 to 12,892 mg kg$^{-1}$. To better understand the forms of Zn in *A. paniculata*, water-soluble Zn was determined in the plants (Fig. 1b). Similar to total Zn, water-soluble Zn in the shoots gradually increased as Zn increased from 100 to 2000 µM in the solution (Fig. 1b). Water-soluble Zn in the roots increased with increasing Zn concentrations from 100 to 1000 µM; however, it decreased as Zn concentrations increased from 1000 to 2000 µM (Fig. 1b). Though water-soluble Zn in the roots was greater than those in the shoots except for 2000 µM treatment (Fig. 1b), the proportion of water-soluble Zn in the shoots (58–73%) were greater than that in the roots (30–53%) (Fig. 1c). Among all treatments, the roots treated with 2000 µM Zn had the lowest soluble Zn at 30% (Fig. 1c). Given its highest Zn content (12,892 mg kg$^{-1}$), the dried-roots treated with 2000 µM Zn were analyzed using XRD. However, the analysis was unsuccessful due to the interference from high amounts of organic materials, so the ashed-roots were used in the XRD analysis. XRD pattern indicated that zinc sulphide (ZnS) and zinc oxide (ZnO) were present in the roots (Fig. 2).

3.3. Membrane stability index and lipid peroxidation in plant tissues

The extent of membrane damage was evaluated indirectly by analyzing solution conductivity, which measures electrolyte leak-
age from cells. Clearly Zn exposure had limited impact on the membrane stability index (MSI) in the shoots (Fig. 3a). It impacted the MSI in the roots only at the highest Zn treatment at 2000 \(\mu\text{M}\), which was 61% lower than that in the control (\(p < 0.05\)).

Formation of thiobarbituric acid-reactive substances (TBARs) is currently used as an index of lipid peroxidation in plants exposed to adverse environmental conditions. Among all treatments, Zn exposure did not enhance lipid peroxidation in plants tissues except that TBARs accumulation in the roots increased in 2000 \(\mu\text{M}\) Zn (Fig. 3b), which is consistent with the MSI data (Fig. 3a). For example, compared to the control, TBAR in the roots increased by 42% whereas the MSI in the roots reduced by 61% (\(p < 0.05\)).

### 3.4. Non-protein thiols in plant tissues

The NPTs analyzed in this experiment included GSH, cysteine, and PCs. Fig. 4 shows HPLC chromatograms for NPTs standard and the tissues of 0 and 1000 \(\mu\text{M}\) Zn treatment. Though no PC was detected in the shoots (Fig. 4b and c), GSH and cysteine were detected. Unlike the shoots, PCs and an unknown thiol eluted at 20.5 min (named X20.5) were detected in the roots (Fig. 4e). To better understand the changes in GSH and cysteine, their concentrations in the shoots and roots under different Zn stress were measured (Fig. 5). Increasing Zn concentrations in the plant did not induce higher GSH concentrations in the shoots or the roots (\(r < 0.09\)). The GSH concentration in the shoots was the lowest in the 1000 \(\mu\text{M}\) Zn treatment, which corresponded to the highest GSH concentration in the roots among all treatments (Fig. 5a). Though little change was observed in the cysteine concentrations in the shoots, increasing Zn levels induced cysteine synthesis in the roots with the highest levels observed in the 1000 and 2000 \(\mu\text{M}\) Zn treatments (\(p < 0.05\), Fig. 4b). Unlike GSH, Zn concentrations in the plant were correlated with cysteine concentrations in the shoots (\(r = 0.51\)) and the roots (\(r = 0.46\)).
4. Discussion

4.1. A. paniculata as a Zn hyperaccumulator

The highest Zn in the shoots of A. paniculata was 6030 mg kg$^{-1}$ Zn, which is lower than 10,000 mg kg$^{-1}$, the threshold for a Zn hyperaccumulator (Baker and Brooks, 1989). In a field investigation, A. paniculata accumulated 20,800 mg kg$^{-1}$ Zn, 434 mg kg$^{-1}$ Cd and 2300 mg kg$^{-1}$ Pb in the shoots growing in a mining soil containing 17.9% Zn, 0.42% Cd and 2.8% Pb (Tang et al., 2009). So the hyperaccumulation property of A. paniculata identified in the field was not replicated under our hydroponic system. Based on the frequency distribution of Zn values observed in the genus Thlaspi, which contains most Zn hyperaccumulators, 3000 mg Zn kg$^{-1}$ (dw) has been proposed to be a more suitable evolutionary definition than 10,000 mg kg$^{-1}$ (Broadley et al., 2007). Using the new definition, A. paniculata could be considered as a Zn hyperaccumulator.

In addition to low Zn accumulation, low Zn translocation from the roots to the shoots was also observed as indicated by translocation factor (TF; ratio of Zn concentrations in shoots to roots) of <1 in all treatments. It is possible that the Zn is complexed with organic acids or amino acids including cysteine, which is then sequestered in the root vacuoles (Jiang and Wang, 2008; Straczek et al., 2008), making it less available for xylem loading to the shoots. Similarly low TF was also reported in some populations of T. caerulescens (Zhao et al., 1998) and A. halleri (Sarret et al., 2002). The authors suggest that precipitation of Zn–P minerals on the root surface or in the apoplastic may be responsible. In our case, reduction of P concentration from 100 to 50 μM in the solution should help to minimize the precipitation of potential Zn–P minerals. However, complexation of Zn with S- and O-containing compounds was likely in our study. XRD may be derived from those S- and O-containing complexes, like Zn–thiols or Zn–organic acids under high temperature during ashing. Zinc complex sequestered in vacuoles may also contribute to the low Zn translocation observed in this study.

4.2. High Zn tolerance in A. paniculata

A. paniculata showed high tolerance to Zn stress. The fact that exposure to <500 μM Zn or 33 mg L$^{-1}$ Zn increased its plant biomass and exposure to 2000 μM Zn or 130 mg L$^{-1}$ Zn had no impact on its plant biomass was consistent with its high Zn tolerance. The Zn tolerance in A. paniculata is higher than T. caerulescens (Shen et al., 1997) and Sedum alfredii (Jin et al., 2008), both of which show phytotoxicity symptoms at 1000 μM Zn. The MSI and TBAR data also suggested A. paniculata possessed remarkable Zn tolerance. Impaired membrane stability (MSI) and high lipid peroxidation (TBAR) occurred only in the roots of A. paniculata exposed to the highest Zn level at 2000 μM (Fig. 3).

The Zn accumulated in A. paniculata was mostly present as water-soluble forms in the shoots (58–73%), which is similar to the values reported in A. halleri (Zhao et al., 2000). The high proportion of water-soluble Zn in the shoots of A. paniculata rules out apoplastic precipitation as an important detoxification mechanism. The data may suggest that the excess Zn may be complexed with soluble organic compounds and stored in the vacuoles (Sarret et al., 2002). The positive correlation between Zn concentrations and cysteine concentrations in the shoots of A. paniculata supports this hypothesis.

4.3. Cysteine instead of PCs was involved in Zn homeostasis in A. paniculata

Although PCs have a role in metal detoxification (Tennstedt et al., 2009), they are not involved in Cu, Zn and Ni hypertolerance (Verbruggen et al., 2009). In hyperaccumulators, PCs are mainly induced in the roots, in particular by Cd, but barely by Zn (Schat et al., 2002). Therefore, the responses of GSH and PCs to Zn exposure in A. paniculata were different from those of Cd exposure where both participated in Cd-sequestration in the roots (Zeng et al., 2009). It has been reported that the level of Arabidopsis PC synthase activation by a Zn–cysteine complex was 74-fold higher than a Cd–cysteine complex (Owen et al., 2002), which might be the reason why Zn failed to active PCs synthesis in A. paniculata.

Among the NPTs analyzed, cysteine was the only one, which may play a role in Zn tolerance in A. paniculata. Cysteine not only plays an important role in biological process as an extracellular reducing agent, but also in sequestration of several metals (Hernández-Allica et al., 2006). The present study showed both ZnS and ZnO were detected in the ashed-roots of A. paniculata in 2000 μM Zn treatment (Fig. 2). Similar result is also found in Zn hyperaccumulator Diploschistes muscorum, which shows the presence of zinc oxalate dihydrate and ZnS using the same technique (Sarret et al., 1998). The EXAFS spectroscopy analysis further suggests that the possible complexation of Zn to S-containing intracellular proteins is Zn–cysteine (Sarret et al., 1998). In addition, the significant positive correlation between Zn and cysteine observed in A. paniculata was consistent with this hypothesis. Therefore, the complexation of Zn with cysteine may contribute to the Zn-tolerance in A. paniculata.

Overall, the present study showed that A. paniculata exhibited high Zn tolerance where its biomass was not impacted by as high as 2000 μM Zn, and where it showed membrane stability and no lipid peroxidation except for the roots in 2000 μM Zn treatment during the 3–w growth. While GSH and PCs were not involved in Zn tolerance in A. paniculata, cysteine may play a role as evidenced by its positive correlation with Zn concentrations in the plants as well as the presence of ZnS in the ashed-roots.

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References
