

ZAT11, a zinc finger transcription factor, is a negative regulator of nickel ion tolerance in *Arabidopsis*

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Abstract

Key message ZAT11, a Zinc Finger of *Arabidopsis Thaliana* 11, is a dual-function transcriptional regulator that positively regulates primary root growth but negatively regulates Ni²⁺ tolerance.

Abstract Zinc Finger of *Arabidopsis Thaliana* 11 (ZAT11) is a C₂H₂-type zinc finger protein that has been reported to function as an active transcriptional repressor. However, the biological function of ZAT11 remains unknown. Here we show that GFP-tagged ZAT11 is targeted to the nucleus. Analysis of plants expressing ZAT11 promoter-GUS showed that ZAT11 is highly expressed in roots and particularly in root tips. To identify the biological function of ZAT11, we constructed three independent lines of ZAT11 overexpressing transgenic plant (ZAT11 OE). ZAT11 OE enhanced the elongation of primary root but reduced the metal tolerance against nickel ion (Ni²⁺). The reduced Ni²⁺ tolerance of ZAT11 OE was correlated with

decreased accumulation of Ni²⁺ in plants. The decreased accumulation of Ni²⁺ in ZAT11 OE was caused by the reduced transcription of a vacuolar Ni²⁺ transporter gene. Taken together, our results suggest that ZAT11 is a dual function transcriptional regulator that positively regulates primary root growth but negatively regulates Ni²⁺ tolerance.

Keywords *Arabidopsis* · Nickel ion · Tolerance · ZAT11 · Zinc finger protein

Abbreviations

GFP Green fluorescent protein
GSH Glutathione
His Histidine
NA Nicotianamine
Ni²⁺ Nickel ion

X.-M. Liu and J. An contributed equally to this work.

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Introduction

Trace amounts of nickel ion (Ni²⁺) are essential for plant nutrition. However, excessive concentrations of nickel in the environment become phytotoxic through disruption of various enzyme activities, photosynthesis and mineral nutrition (Pandey and Sharma 2002; Samarakoon and Rausser 1979). Therefore, plants possess a range of cellular mechanisms for Ni²⁺ homeostasis and tolerance (Hall 2002). A general mechanism for Ni²⁺ detoxification in plants is the chelation of Ni²⁺ by ligands such as glutathione (GSH), nicotianamine (NA), histidine (His), or organic acids, and subsequent compartmentalization of the ligand–metal complexes (Cobbett 2000; Hall 2002). For

example, NA is synthesized from *S*-adenosylmethionine by the action of nicotianamine synthase (NAS) and heterologous expression of *TcNAS1* and *HvNAS1* in *Arabidopsis* resulted in increased NA production and improved tolerance to Ni^{2+} (Kim et al. 2005; Pianelli et al. 2005). Free His is another important Ni^{2+} chelator contributing to Ni^{2+} tolerance in plants. Some Ni^{2+} -hyperaccumulating plant species contain high levels of free His which exists in coordination with the Ni^{2+} ions. Exogenous Ni^{2+} elicits a dose-dependent increase of free His concentration in xylem of these hyperaccumulators (Kerkeb and Kramer 2003; Krämer et al. 1996). Ni^{2+} -nonaccumulating plant species were unable to uptake and accumulate exogenous Ni^{2+} . However, the plants acquired the ability to uptake and accumulate external Ni^{2+} by supplementation of His (Kerkeb and Kramer 2003; Krämer et al. 1996). Overexpression of an enzyme in His biosynthetic pathway is sufficient to enhance Ni^{2+} tolerance through the increase of endogenous free His in *Arabidopsis* (Ingle et al. 2005; Wycisk et al. 2004). Meanwhile, several transporters involved in Ni^{2+} transport including *Nicotiana* calmodulin-binding protein 4 (NtCBP4), *Arabidopsis* iron-regulated protein 2 (AtIREG2) and *Arabidopsis* iron-regulated transporter 1 (AtIRT1) have been identified (Arazi et al. 1999; Nishida et al. 2011; Schaaf et al. 2006). However, the transcriptional regulations of these Ni^{2+} transporter genes remain unclear.

Cys2/His2-type zinc finger proteins (C_2H_2 -ZFPs) represent one of the better-characterized classes of eukaryotic transcription factors (Ciftci-Yilmaz and Mittler 2008). Plant C_2H_2 -ZFPs comprise a large family and play critical roles in developmental processes and environmental stress tolerance through transcriptional regulation, posttranslational regulation and protein–protein interactions (Laity et al. 2001). The C1-2i subclass of the *Arabidopsis* C_2H_2 -ZFP family contains 20 members including ZAT6, ZAT7, ZAT10, ZAT11 and ZAT12. Members of this subclass are characterized by two zinc finger motifs, an invariant QA-LGGH motif in the zinc finger domain and an ERF-associated amphiphilic repression (EAR) motif in the C-terminal region. The EAR motif is believed to function as the transcriptional repression motif (Ciftci-Yilmaz and Mittler 2008; Takatsuji 1999). ZAT6 has been reported to be involved in root development and phosphate homeostasis. Overexpression of ZAT6 in *Arabidopsis* resulted in repression of primary root growth and a subsequent change in phosphate acquisition (Devaiah et al. 2007). Transgenic plants constitutively expressing ZAT7 showed enhanced tolerance to salt stress (Ciftci-Yilmaz et al. 2007). ZAT10 plays a dual role as a positive and negative regulator of numerous abiotic stresses (Mittler et al. 2006). ZAT12 plays a central role in reactive oxygen species (ROS) and abiotic stress signaling (Rizhsky et al. 2004). Recently, ZAT11 was

shown to be involved in oxidative stress-induced programmed cell death (Qureshi et al. 2013). However, the physiological function of ZAT11 in plant development and stress tolerance still remains unclear. Here, we report that ZAT11 is a positive regulator in primary root growth at normal condition but a negative regulator in Ni^{2+} tolerance at high Ni^{2+} condition. We suggest that ZAT11 represses Ni^{2+} tolerance by the transcriptional repression of a vacuolar Ni^{2+} transporter gene.

Materials and methods

Plant materials and growth conditions

All plants used in this study were in the Columbia-0 (Col-0) genetic background. T-DNA insertion mutants of ZAT11 (SALK_013996 and SALK_110012) were obtained from Arabidopsis Biological Resource Center. All seeds were surface sterilized, placed in the dark at 4 °C for 2 days, and then sown on 1/2 Murashige and Skoog (MS) agar plates containing 2 % sucrose. Seedlings were grown under a 16 h light/8 h dark cycle in a growth chamber at 22 °C, 75 % humidity and 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity.

Construction of transgenic plants

ZAT11 cDNA fused in-frame to 3X FLAG was subcloned into the plant binary vector pCAMBIA 1300, resulting in 35S::ZAT11-FLAG plasmid. The plasmid was introduced into *Agrobacterium tumefaciens* GV3101, and the transformed *Arabidopsis* plants were obtained by the *Agrobacterium*-mediated floral dip method (Clough and Bent 1998). Seeds were collected from infiltrated plants and transformants were selected by plating on MS medium containing 40 $\mu\text{g ml}^{-1}$ hygromycin. Homozygous lines of ZAT11-overexpressing transgenic plants were used for all experiments.

Histochemical GUS assay

The forward primer 5'-GAATTCGTAAAAA GAAAGCTTATG-3' and reverse primer 5'-GGATC CACTTCTTTGAGAATTAAGATC-3' were used to amplify ZAT11 promoter from genomic DNA (−1,285 to +3 bp from the translational start site). The amplified fragment was digested with *EcoRI*/*Bam*H1 and subcloned into pCAMBIA-1301 vector to yield a *PromZAT11::GUS* plasmid. The plasmid was transformed into *Arabidopsis* by the *Agrobacterium*-mediated floral dip method (Clough and Bent 1998). Histochemical localization of GUS activity was performed as previously described (Jefferson et al. 1987).

Subcellular localization of ZAT11

To ascertain the subcellular localization of ZAT11, *ZAT11* cDNA was subcloned into the p35S::smGFP expression vector to construct the plasmid, 35S::ZAT11-smGFP, for the expression of ZAT11 tagged with soluble modified GFP (smGFP) at its C-terminus under the control of *CaMV* 35S promoter. The plasmid was introduced into *Arabidopsis* protoplasts by polyethylene glycol (PEG)-mediated transformation or onion epidermal cells using the particle bombardment method as previously described (Lee et al. 2007; Varagona et al. 1992). Expression of the fusion constructs was monitored at 24 h after transformation by fluorescence microscopy using a fluorescence microscope (Carl Zeiss, Germany) as previously described (Lee et al. 2007).

Semi-quantitative and quantitative RT-PCR

Total RNA was extracted using a LiCl method (Liu et al. 2010) and rendered free of genomic DNA by digestion with RNase-Free DNase I (Promega, USA). First-strand cDNA was synthesized from 5 µg of total RNA in a 20 µl reaction mixture using cDNA synthesis kit (Fermentas, Canada). Semi-quantitative RT-PCR was carried out following cDNA synthesis using gene-specific primers as previously described (Liu et al. 2010). *Tubulin2* level was monitored as an internal control. Quantitative RT-PCR was performed as described (Han et al. 2012). Primer sequences are listed in Table S1.

Plant growth assays for stress tolerance

All *Arabidopsis* seeds used for experiments were grown and harvested at same condition. For stress tolerance analysis, seeds were grown on vertically placed 1/2 MS plates (with 2 % sucrose) for 4 days and then transferred to 1/2 MS plates containing H₂O₂, CdCl₂, Ni(NO₃)₂ or KNO₃ and grown for indicated time. The fresh weight and the root length of plants were measured.

The measurement of nickel ion content

Individual plants were harvested, rinsed in distilled water and dried at 80 °C for 48 h. The dried material was weighed and digested with concentrated HNO₃ for 90 min at 200 °C. Samples were then centrifuged at 2,000×g for 10 min and the clear supernatant was diluted tenfold with deionized water. Ni²⁺ content was measured by inductively coupled plasma-optical emission spectroscopy (Perkin Elmer, CT, USA).

Results and discussion

ZAT11 is a nuclear protein

ZAT11 has been expected to be localized in nucleus because ZAT11 was proposed to function as a transcriptional repressor (Ohta et al. 2001). However, the subcellular localization of ZAT11 has not yet been examined. To investigate the subcellular localization of ZAT11, we introduced 35S::ZAT11-smGFP plasmid into *Arabidopsis* protoplasts or onion epidermal cells for the expression of smGFP tagged ZAT11 fusion protein (Fig. 1). The fluorescence signal from ZAT11-GFP fusion protein was predominantly localized in the nucleus whereas the fluorescence signal of free GFP was detected throughout the whole cells in *Arabidopsis* protoplasts (Fig. 1b) as well as in onion epidermal cells (Fig. 1c). These results strongly indicate that ZAT11 is a nuclear localized transcriptional regulator. Most of C₂H₂-type ZFPs that contain two zinc finger motifs have been shown to bind directly to A(G/C)T

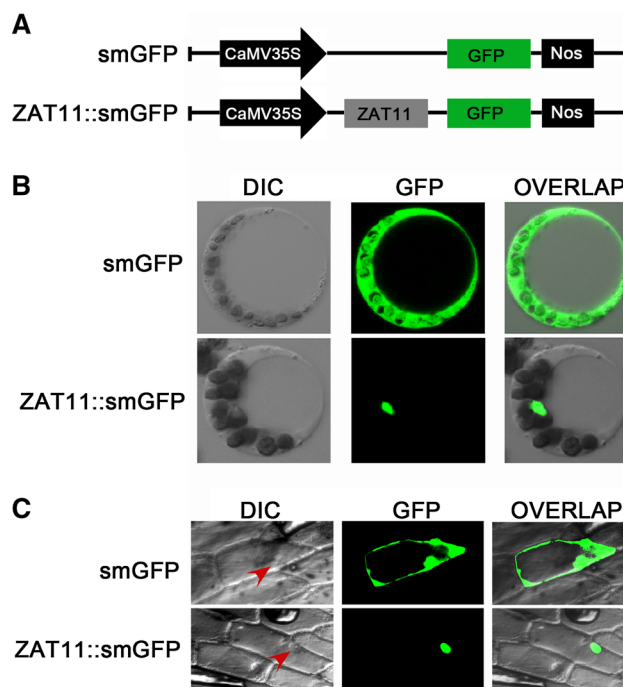


Fig. 1 The subcellular localization of green fluorescence protein (GFP) tagged ZAT11. **a** Schematic representation of the constructs used for subcellular localization. smGFP and ZAT11::smGFP represent the constructs for constitutive expression of soluble modified GFP and ZAT11-smGFP fusion protein, respectively. **b** Subcellular localization of the indicated proteins in *Arabidopsis* protoplasts. **c** Subcellular localization of the indicated proteins in onion epidermal cells. *DIC* differential interference contrast, *GFP* green fluorescent protein, *OVERLAP* overlap of DIC and GFP images. *Arrowheads* indicate the positions of nuclei

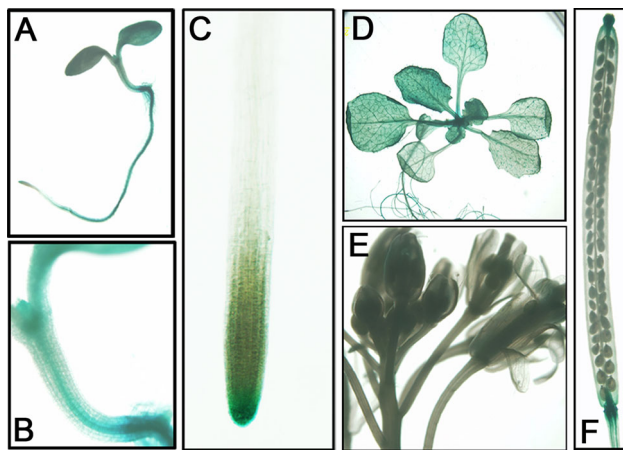


Fig. 2 The gene expression patterns of *ZAT11* in tissues. Histochemical analysis of GUS activity in *PromZAT11::GUS* transgenic *Arabidopsis* plants. **a** Five-day-old whole seedling; **b**, **c** close-up views of the hypocotyl (**b**) and primary root tip (**c**) of a 5-day-old seedling; **d** 2-week-old whole seedling; **e** flowers; **f** silique

repeats within an EP2 sequence (Sakamoto et al. 2004). However, we could not detect the binding of recombinant *ZAT11* protein to the EP2 sequence by electrophoretic mobility shift assay (data not shown). This result implies that *ZAT11* has different DNA binding property from other related C_2H_2 -type ZFPs.

Tissue-specific pattern of *ZAT11* gene expression

The tissue-specific expression pattern of *ZAT11* was determined by evaluating GUS reporter activity in tissues of transgenic plants expressing *PromZAT11::GUS*. Strong GUS activity was detected in cotyledons, primary roots and hypocotyls of seedlings (Fig. 2). Strong expression of *ZAT11* in root tip suggests that *ZAT11* may be involved in development or stress tolerance in root.

Primary root growth was increased in *ZAT11*-overexpressing transgenic plants

To identify the physiological function of *ZAT11*, we obtained two independent T-DNA insertion mutant lines of *ZAT11* (SALK_013996 and SALK_110012) from the Arabidopsis Biological Resource Center collection. However, these lines exhibited comparable transcript levels with wild type plant (Fig. S1) because of each T-DNA insertion in the 5'-untranslated region of *ZAT11*. Therefore, we performed a gain-of-function approach to investigate the physiological function of *ZAT11*. We constructed transgenic plants (*ZAT11* OE) constitutively overexpressing FLAG-tagged *ZAT11* under the control of *CaMV* 35S promoter (Fig. 3a). Three independent homozygous lines (OE #8, OE #11 and OE #20) showing high expression

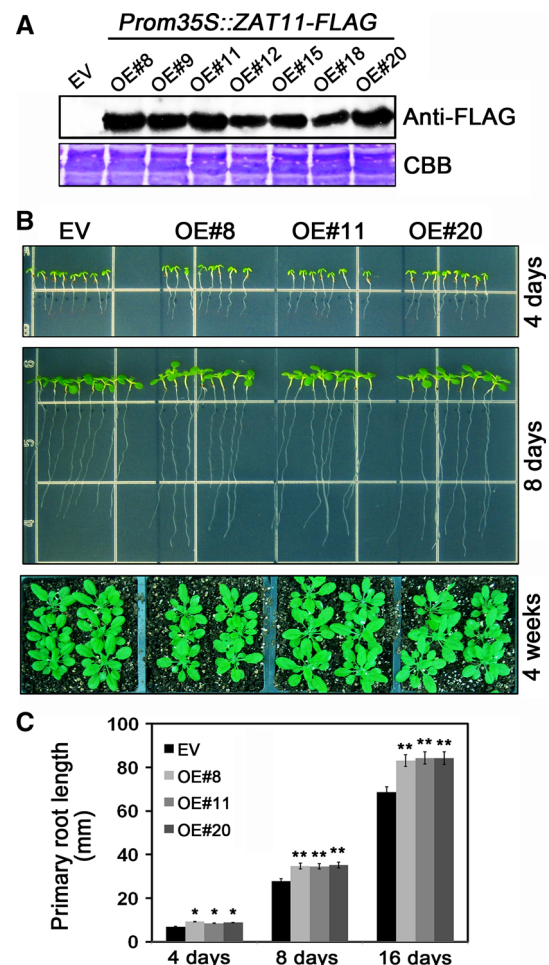


Fig. 3 Increased growth of primary root in *ZAT11* OE plants. **a** Western blot analysis of *ZAT11* protein levels in empty vector (EV) and several independent *Prom35S::ZAT11-FLAG* transgenic plant lines (OE). Total proteins (50 μ g) from 2-week-old seedlings were analyzed by immunoblot with an anti-FLAG antibody (Anti-FLAG). The Coomassie brilliant blue-stained membrane (CBB) is shown as a loading control. **b** The indicated lines were grown on 1/2 MS (*top* and *middle* rows) or in soil (*bottom* row). The photographs were taken at 4 days, 8 days and 4 weeks after germination as indicated. **c** Measurements of primary root length in plants grown on 1/2 MS-agar for 4, 8 and 16 days. Data are the mean \pm SE of three independent experiments. Asterisks indicate statistically significant differences compared with the corresponding EV as determined by Student's *t* test (* P < 0.05; ** P < 0.01)

levels of *ZAT11-FLAG* protein were chosen for further study. RT-PCR analysis showed that *ZAT11* transcripts were highly expressed in these transgenic lines (Fig. S2A). The primary roots were significantly longer in *ZAT11* OE plants than in empty vector plants (EV) (Fig. 2b, c). The growth difference at primary roots was observed at 4 days after germination (Fig. 3b), and much obvious at 16 days after germination (Fig. 3c). Microscopic analyses suggested that the elongated primary root of *ZAT11* OE was caused by the increased lengths of cells but not by the increased number of cells (Fig. S3). However, we could not

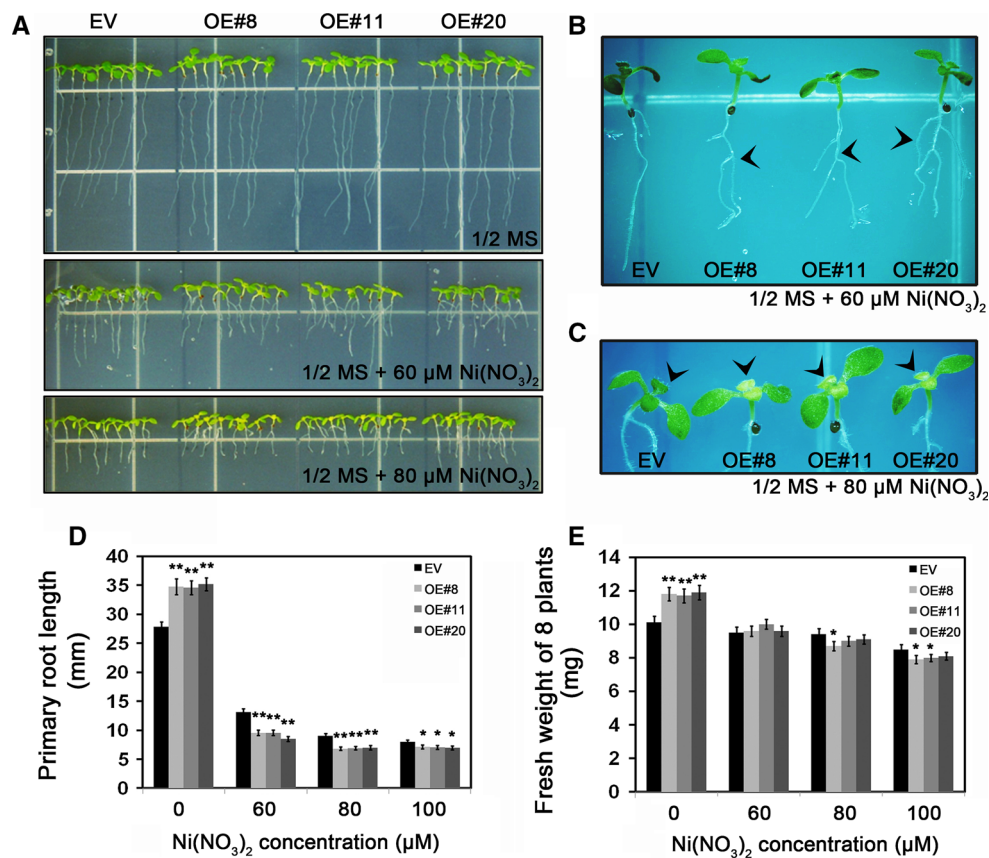


Fig. 4 Decreased Ni²⁺ tolerance in *ZAT11* OE plants. Four-day-old EV and *ZAT11* OE plants grown on 1/2 MS were transferred to plates containing the same medium supplemented with 0, 60, 80 or 100 μM Ni(NO₃)₂. **a** Photographs illustrating phenotypes of *ZAT11* OE plants at 4 days after transfer. **b** Increased lateral root numbers in *ZAT11* OE plants at 4 days after transfer to growth medium containing 60 μM Ni(NO₃)₂. Arrowheads indicate the position of lateral roots. **c** Primary leaves in *ZAT11* OE at 4 days after transfer to growth medium containing 80 μM Ni(NO₃)₂. Arrowheads indicate chlorosis in first leaves. **d** Measurements of primary root length of seedlings 4 days

after transfer to medium containing indicated concentrations of Ni(NO₃)₂. Data are the mean ± SE of three independent experiments. Asterisks indicate statistically significant differences compared with the corresponding EV as determined by Student's *t* test (**P* < 0.05; ***P* < 0.01). **e** Measurements of fresh weight of seedlings 4 days after transfer to medium containing indicated concentrations of Ni(NO₃)₂. Data are the mean ± SE of three independent experiments. Asterisks indicate statistically significant differences compared with the corresponding EV as determined by Student's *t* test (**P* < 0.05; ***P* < 0.01)

detect significant difference in leaves in soil-grown plants (Fig. 3b, bottom panel). These results imply that *ZAT11* may function as a positive transcriptional regulator for primary root growth at normal condition. The increased root growth phenotype of transgenic *ZAT11* OE plants is opposite to those of *ZAT6*, *ZAT7*, *ZAT10* and *ZAT12* overexpressing transgenic plants (Ciftci-Yilmaz et al. 2007; Devaiah et al. 2007; Mittler et al. 2006; Rizhsky et al. 2004). This difference strongly indicates that the physiological function of *ZAT11* could be different from those of other C₂H₂-type ZFPs in *Arabidopsis*.

ZAT11 overexpressing transgenic plants are sensitive to Ni²⁺

It has been reported that *ZAT11* transcript is highly induced by H₂O₂ (Gechev et al. 2005), and *ZAT11* may be involved

in oxidative stress-mediated programmed cell death (Qureshi et al. 2013). To determine whether *ZAT11* can confer the resistance against H₂O₂, we treated H₂O₂ to *ZAT11* OE. The elongated primary root phenotype of *ZAT11* OE was disappeared by the treatment of H₂O₂ (Fig. S2B), suggesting that *ZAT11* cannot protect the toxic effect of H₂O₂. Several metal ions are well known to induce free radicals including H₂O₂ and result in oxidative stress in plants (Maksymiec 2007). To determine whether *ZAT11* can confer the resistance against toxic metal ions, we examined the growth of *ZAT11* OE in the media containing different concentrations of CdCl₂ or Ni(NO₃)₂. The root growth of both plants was inhibited by the increase of CdCl₂ concentration. However, the primary roots of *ZAT11* OE were still longer than those of EV in media containing CdCl₂ (Fig. S4), indicating that *ZAT11* may not function in the resistance against CdCl₂ (Fig. S4). In contrast, three

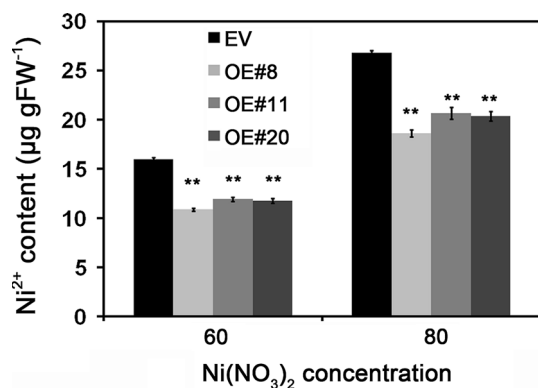


Fig. 5 Less accumulation of Ni²⁺ in *ZAT11* OE plants. Four-day-old EV and *ZAT11* OE plants grown on 1/2 MS were transferred to 1/2 MS containing 60 or 80 µM Ni(NO₃)₂. Ni²⁺ content was measured in roots at 2 weeks after transfer. Data are the mean ± SE of three independent experiments. Asterisks indicate statistically significant differences compared with the corresponding EV as determined by Student's *t* test (***P* < 0.01)

ZAT11 OE lines had shorter primary roots than EV plants in media containing 60, 80 or 100 µM Ni(NO₃)₂, whereas they had longer roots than EV plants in control media (Figs. 4a, d, S5). In addition, *ZAT11* OE plants showed increased numbers of lateral roots compared to EV plants in medium containing 60 µM Ni(NO₃)₂ (Fig. 4b). In addition, the first leaves of *ZAT11* OE plants showed chlorosis in media containing 80 µM Ni(NO₃)₂ whereas EV plants did not (Fig. 4c). Fresh weights of *ZAT11* OE plants were significantly reduced by the addition of Ni(NO₃)₂ to media (Figs. 4e, S5C). To rule out the possibility of toxicity due to high nitrate levels, we repeated the experiment under the same media containing twice the concentration of KNO₃ instead of Ni(NO₃)₂ (Fig. S6). The primary roots were longer in *ZAT11* OE than in EV plants regardless of the concentration of KNO₃. These data strongly suggest that *ZAT11* is a negative regulator of Ni²⁺ tolerance in *Arabidopsis*. Next, we compared Ni²⁺ content in roots of *ZAT11* OE and EV plants grown in media containing 60 or 80 µM Ni²⁺. The Ni²⁺ contents were significantly lower in *ZAT11* OE compared to EV (Fig. 5), suggesting *ZAT11* may be a negative regulator of Ni²⁺ accumulation in the internal compartments.

The transcript levels of a vacuolar Ni²⁺ transporter is decreased in *ZAT11* OE plants

An important mechanism contributing to Ni²⁺ resistance is the chelation of Ni²⁺ by GSH, NA or His. Genes encoding key enzymes involved in biosynthesis of GSH, NA or His molecules are *GSH1*, *NAS1* and *ATP-PRT1/2*, respectively. To test whether *ZAT11* is involved in the gene expression of these genes, we performed RT-PCR. However,

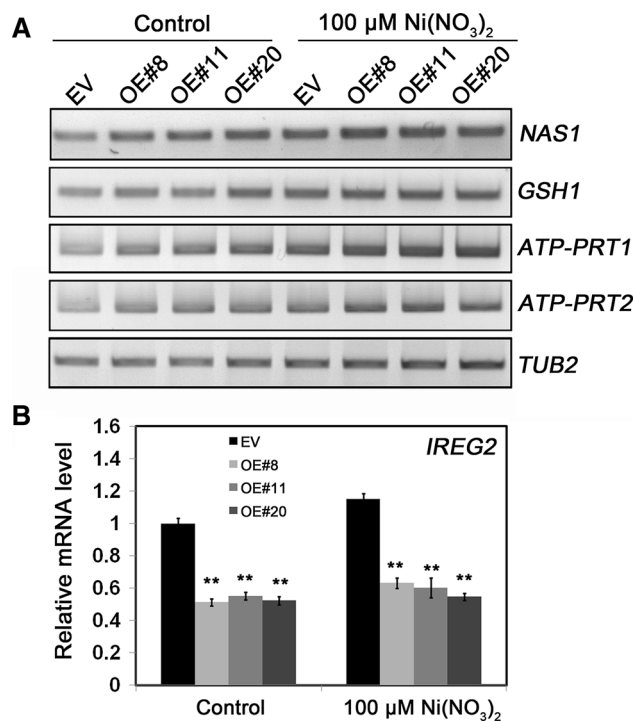


Fig. 6 Decreased expression of a vacuolar Ni²⁺ transporter in *ZAT11* OE plants. Four-day-old EV and *ZAT11* OE plants grown on 1/2 MS were transferred to fresh 1/2 MS without (Control) or with 100 µM Ni(NO₃)₂ supplement. Total RNA was extracted from the plants at 2 weeks after transfer. *Tubulin2* (*TUB2*) transcript level was measured as an internal standard for all PCR analyses. Shown in (a) are results of RT-PCR analysis of *NAS1*, *GSH1*, *ATP-PRT1* and *ATP-PRT2* levels. Shown in (b) is the quantitative PCR analysis of *IREG2* transcripts. Data are the mean ± SE of three independent experiments. Asterisks indicate statistically significant differences compared with the corresponding EV as determined by Student's *t* test (***P* < 0.01)

transcript levels of *GSH1*, *NAS1* and *ATP-PRT1/2* were not significantly different in *ZAT11* OE and EV plants and not changed by the supplement of Ni(NO₃)₂ (Fig. 6a).

Sequestration of Ni²⁺ in the vacuole is one of key mechanisms to confer Ni²⁺ resistance in plant (Schaaf et al. 2006). Vacuolar Ni²⁺ transporters include members of IREG family. Overexpression of an IREG family, *IREG2*, resulted in enhanced nickel tolerance and increased Ni²⁺ accumulation in roots (Schaaf et al. 2006). To test whether the reduced Ni²⁺ accumulation in *ZAT11* OE plants was correlated with the reduced gene expression of *IREG2*, we measured *IREG2* transcript level by quantitative PCR (Fig. 6). The level of *IREG2* transcript was significantly lower in *ZAT11* OE than in EV plant regardless of the presence of 100 µM Ni(NO₃)₂ in media (Fig. 6b). These results suggest that *ZAT11* is negatively involved in the transcription of *IREG2* gene. Further study would be required to examine whether *ZAT11* directly or indirectly regulates the transcription of *IREG2*.

In this study our results showed that ZAT11 positively regulates the elongation of primary root and negatively regulates the resistance against Ni^{2+} through the transcriptional control of a vacuolar Ni^{2+} transporter gene, *IREG2*. Our finding suggests the new biological function of a C_2H_2 -type zinc finger protein that is involved in the metal ion tolerance of plants.

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Conflict of interest The authors declare that they have no conflict of interest.

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