Spatial-temporal analysis of zinc homeostasis reveals the response mechanisms to acute zinc deficiency in *Sorghum bicolor*

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Summary

• Zinc (Zn) is an essential micronutrient in plants. The activity of copper/zinc superoxide dismutase (CSD) and carbonic anhydrase (CA) correlate with differences in Zn efficiency in plants; therefore, it is reasonable to hypothesize the existence of a Zn economy model that saves Zn for these essential Zn proteins during Zn deficiency. However, up to this point, direct evidence for the idea that CSD and/or CA might be priorities for Zn delivery has been lacking.

• Here, we investigated the spatial-temporal effects of acute Zn depletion and resupply by integrating physiological studies and molecular analyses using hydroponically grown *Sorghum*.

• The elevated expression of miR398 repressed CSD expression in roots, whereas the reduced expression of miR528 resulted in a relatively stable level of CSD expression in *Sorghum* leaves under Zn depletion.

• Spatial-temporal analysis after Zn resupply to previously depleted plants revealed that the expression and activity of CA were the first to recover after Zn addition, whereas the recovery of the activities of CSD and alcohol dehydrogenase (ADH) was delayed, suggesting that CA receives priority in Zn delivery over CSD and ADH. Our results also indicate that microRNAs (miRNAs) are important regulators of the response of Zn deficiency in plants.

Introduction

Zinc (Zn) homeostasis is a tightly regulated process because Zn is an essential micronutrient for all organisms and serves as a cofactor for >300 enzymes (Gonzalez-Guerrero *et al.*, 2005). Therefore, Zn is closely involved in a wide range of cellular processes, such as free radical defense, electron transport, protein and auxin biosynthesis, cell proliferation, and reproduction in plants. In plants, two major Zn proteins have been broadly studied: copper/zinc superoxide dismutase (Cu/Zn SOD, CSD) and carbonic anhydrase (CA). CSD plays an important role in protecting plants against oxidative damage. Zn deficiency reduces CSD activity and thereby results in reactive oxygen species (ROS) accumulation and oxidative damage (Sharma *et al.*, 2004). CA is directly involved in photosynthesis, facilitating the diffusion of CO₂ through the liquid phase of the cell to the chloroplast (Randall & Bouma, 1973). Zn deficiency drastically reduces CA concentrations and thereby inhibits photosynthesis and growth in spinach (Randall & Bouma, 1973). Another important Zn protein is alcohol dehydrogenase (ADH). ADH is the key enzyme in alcohol fermentation. Expression of ADH is known to increase under various abiotic stresses (Nanjo *et al.*, 2010). Cinnamyl alcohol dehydrogenase is also involved in lignin biosynthesis in plants. Zn deficiency reduces ADH activity, and thereby represses lignin biosynthesis and subsequently reduces plant growth (Li *et al.*, 2010).

It has been suggested that CA activity could be used as an indicator for diagnosing Zn deficiency in plants (Bar-Akiva & Lavon, 1969; Hacisalihoglu *et al.*, 2003). Hacisalihoglu *et al.* (2003) found that the expression and activity of CSD and CA correlate with differences in Zn efficiency in wheat. Therefore, it is reasonable to hypothesize a Zn economy model that saves Zn for these essential Zn proteins, such as CSD and CA, during Zn deficiency. However, up to this point, direct evidence for the idea that CSD and/or CA might be priorities for Zn delivery has been lacking.
Other Zn proteins include those with Zn finger domains such as transcriptional regulatory proteins (Ishimaru et al., 2005), which is the largest group of Zn-binding proteins in plants, and the Zn-binding catalytic activity proteins, such as P_{1B}-ATPase transporters (HMAs) and mitogen-activated protein kinases (MAPKs) (Broadley et al., 2007).

Two closely related members of the basic-region leucine-zipper (bZIP) transcription factor genes, bZIP19 and bZIP23, regulate Zn homeostasis in Arabidopsis by mediating the major response when Zn is limited (Assuncao et al., 2010). bZIP19 and bZIP23 are required for the up-regulation of cellular Zn uptake and assimilation mechanisms. Their requirement is highlighted by the up-regulated expression of ZIP transporters (including ZIP 1/3/5/9/10/12, IRT3) and the nicotianamine synthetases, NAS2 and NAS4, which contain one or more zinc deficiency response elements (ZDREs) in their promoters. These ZDREs allow the binding of bZIP19 and bZIP23 proteins. The metal-responsive element (MRE) represents another important metal-responsive motif in plants that is involved in modulating metal uptake and Zn deficiency (Qi et al., 2007). In vertebrates, metal-responsive element-binding transcription factor-1 (MTF-1) plays a central role in Zn homeostasis by binding to the MRE. However, MTF orthologs have not yet been found in plants.

MicroRNAs (miRNAs) are 19- to 23-nucleotide-long noncoding, endogenous RNAs that play a regulatory role in the cell by negatively affecting gene expression at the post-transcriptional level, either by endonucleolytic cleavage or by translational inhibition (Song et al., 2010). Numerous studies have indicated that miRNAs play important roles in various aspects of plant development and in plant responses to stresses, pathogen invasion, and mineral nutrient homeostasis (Jones-Rhoades et al., 2006; Abdel-Ghany & Pilon, 2008). Many miRNAs have been identified that are believed to be involved in modulating responses to heavy metal toxicity and micronutrient deficiency; however, miRNAs involved in the plant response to Zn deficiency are almost completely unknown (Jain & Brar, 2010).

Zinc is the most common crop micronutrient deficiency, particularly in high-pH soils with a low concentration of Zn in the bulk soil solution (Broadley et al., 2007). Classic symptoms of Zn deficiency include the presence of brown blotches and streaks, lack of photosynthetic activity, reduction in biomass, delayed maturity, and reduced yield (Wissuwa et al., 2006; Abdel-Ghany & Pilon, 2008). Many miRNAs have been identified that are believed to be involved in modulating responses to heavy metal toxicity and micronutrient deficiency; however, miRNAs involved in the plant response to Zn deficiency are almost completely unknown (Jain & Brar, 2010).

Materials and Methods

Plant materials and growth conditions

Seeds of Sorghum bicolor (L.) Moench cv BTx623 were kindly provided by Prof. Haichun Jing. To obtain seedlings, the seeds were sown under sterile conditions in Petri dishes with deionized water (dH₂O). Cultures were maintained at 22–25°C under a 16 h photoperiod. Five-day-old seedlings were transferred into Hoagland solution (pH 6.5 and Fe was supplied as Fe-EDTA) (Hoagland & Arnon, 1950) in a 51 plastic vessel (16 plantlets per vessel) for plant growth under the same culture conditions. For growth under Zn-depleted conditions, Zn was omitted from the medium, whereas 2 μM ZnSO₄ was added for the Zn-sufficient condition. The culture solution was replaced every 6 d. Each experiment was performed with at least three biological replicates. Sampling was partitioned into young leaves (latest three leaves), mature leaves, and roots. Material from three plants was pooled into a single sample, after which it was immediately frozen in liquid nitrogen and stored frozen until further analysis.

Inductively coupled plasma mass spectroscopy (ICP-MS) analysis

Sorghum bicolor seedlings were grown hydroponically in the presence of 2 μM ZnSO₄ or without added Zn. The treated roots were immersed in a solution containing 1 mM EDTA for 2 h and thoroughly rinsed with distilled water. The samples were oven-dried at 75°C for 48 h. The dried plant tissues were ground and digested in concentrated nitric acid for 2–3 d at room temperature. The samples were then boiled for 1–2 h until completely digested. After adding 4 ml of Millipore-filtered deionized water and a brief centrifugation, Zn content was determined using ICP-MS. Each experiment was repeated six times.

Antioxidant capacity

Five-day-old S. bicolor seedlings were grown in Hoagland solution without Zn for 45 d and subsequently transferred to normal Hoagland solution containing 2 μM ZnSO₄. After 1 and 5 d, the seedlings were harvested. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was used to estimate antioxidant capacity and was
measured using the method of Xie et al. (2008). Zn deficiency-induced oxidative damage (membrane liquid peroxidation) was estimated by measuring malondialdehyde (MDA) concentrations as described by Xu et al. (2010).

**Quantification of photosynthesis index**

The Chl content was measured after 45 d of growth using the method described by Arnon (1949). Light intensity-dependent electron transport rate (ETR) was measured after 45 d of growth using the method described by Freeman et al. (2010). The photosynthetic rate (Pn), intercellular CO2 concentration (Ci), and stomatal conductance (gs) were recorded using an intelligent portable photosynthesis system (LCpro+ ADC, Hoddesdon, UK). The stomatal limitation value (Ls) was calculated using the following formula: \[ Ls = \left(1 - \frac{C_i}{C_a}\right) \times 100\% \] according to Yin et al. (2006). These observations were recorded for eight plants per treatment.

**MiRNA microarray**

MicroRNA samples isolated from the young leaves and roots of control or stress-treated *S. bicolor* plants were used for miRNA microarray hybridization. Three replicates for each leaf and root, using a total of 12 arrays, were applied to compare the control and Zn-deficiency conditions. The detailed experimental procedure for microarray analysis is described in the Supporting Information Methods S1.

**MiRNA target prediction and cis-acting element analysis**

The rules used for target prediction were based on those suggested by Allen et al. (2005) and Schwab et al. (2005). The 2 kb region upstream of the stem-loop structure of these Zn deficiency-responsive miRNA genes was truncated to identify cis-element-responsive miRNA genes (Fig. S1). The detailed experimental procedure for miRNA target prediction and cis-acting element analysis is described in Methods S1.

**5′ RACE of miRNA cleavage**

For mapping the internal cleavage site in the predicted target genes of the miRNAs, RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) was performed using the GeneRacer Kit (Invitrogen). A modified procedure for RLM-RACE was performed following the GeneRacer Kit instructions as described previously (Song et al., 2009). The detailed experimental procedure for RLM-RACE is described in Methods S1.

**Reverse transcription polymerase chain reaction (RT-PCR) analysis of gene expression**

The RNA was extracted from the young leaves, mature leaves, and roots of control or stress-treated *S. bicolor* plants using TRizol (Gibco/BRL, Life Technologies, Grand Island, NY, USA). The RNA quality and integrity were checked before the cDNA was synthesized using the Bio-Rad Experion RNA StdSens analysis kit (Bio-Rad, Hercules, CA, USA). For the semi-quantitative RT-PCR, we performed control reactions using the 18S rRNA and ACT primers to ensure that an equal amount of RNA was used in each set of reactions. We optimized the cycle numbers to ensure that the amplification reaction was performed in the exponential phase. The quantitative RT-PCR (RT-qPCR) analysis was performed according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009). The detailed experimental procedure for the RT-qPCR analysis is described in Methods S1 and Fig. S1.

**Mature miRNA stem-loop RT-qPCR**

Total RNAs were extracted using TRizol reagent (Invitrogen) as described earlier. However, ethanol washes were avoided and nucleic acid precipitation steps were performed using 1 : 1 (v : v) isopropanol and 1 : 10 (v : v) 3 M sodium acetate (pH 5.2) to optimize small RNA molecule retrieval (Ravet et al., 2011). The stem-loop RT-qPCR was performed as previously described (Chen et al., 2005). Specific primers for each gene are listed in Table S1. All RT-qPCRs were replicated three times using templates prepared from three independent samples.

**Transient coexpression of miRNA and target gene in *Nicotiana benthamiana* leaves**

The precursor of *miR399c* was amplified from *Sorghum* genome DNA and cloned into a binary vector pTCK303. The corresponding target gene *UBC24* fragment was inserted into the same vector. The plasmids carrying precursors of *miR399c* and *UBC24* were infiltrated with separate or mixed cells as previously described (Lacombe et al., 2008). The total RNA of infiltrated leaves was isolated after 4 d of growth and used to synthesize the first cDNA as templates of RT-PCR, as mentioned earlier.

**Pi measurements**

Inorganic phosphate (Pi) concentrations were determined using the colorimetric method according to Itaya & Ui (1966).

**Quantification of hormones**

Indole-3-acetic acid (IAA) and jasmonic acid (JA) concentrations were determined using enzyme-linked immunosorbent assay (ELISA) methods according to Yang et al. (2001). The results are the means ± SE of six replicates.

**SOD activity**

Superoxide dismutase activity was determined by recording the inhibition of the formazan formation rate by the enzyme (Dhindsa et al., 1981). The reaction mixture consisted of 50 mM
phosphate buffer (pH 7.8), 10 mM methionine, 25 mM nitroblue tetrazolium (NBT; N6876, Sigma-Aldrich), 0.1 mM EDTA, 50 mM sodium carbonate, 2 μM riboflavin, and 100 μl enzyme in a volume of 3 ml. Reactions were conducted at 25°C under a light intensity of 120 μmol m⁻² s⁻¹ for 15 min. To distinguish the Cu/ZnSOD, FeSOD, and MnSOD isoforms, the sensitivity of Cu/ZnSOD to cyanide (3 mM) and the sensitivity of Cu/ZnSOD and FeSOD to H₂O₂ (5 mM) were measured. The absorbance was recorded at 560 nm. One unit of SOD activity was defined as the amount of enzyme required to inhibit the reduction rate of NBT by 50%. Protein concentrations were determined according to Bradford (1976) using BSA as a standard. For in-gel SOD activity analysis, 50 μg of native protein extract was fractionated on a 15% nondenaturing gel, which was then stained for activity as described by Beauchamp & Fridovich (1971). Each experiment was performed with at least three biological replicates, with identical results, and representative gels are shown.

CA activity

*Sorghum bicolor* seedlings were grown hydroponically in the presence of 2 μM ZnSO₄ or without added Zn. After 45 d, 2 μM ZnSO₄ was added to the medium of plants previously grown in the absence of Zn. CA activity was determined immediately after treatment by measuring the length of time required for the pH to change from 8.3 to 7.0 according to the method of Ignatova *et al.* (2011).

ADH activity

Suspensions extracted from the young and mature leaves were subjected to an assay of ADH activity according to the method described by Nanjo *et al.* (2010).

Statistical analysis

All experiments were repeated at least three times. The results are presented as the means ± SD. For statistical analysis, we used Student’s *t*-test (*P* < 0.05).

**Results**

Zn-deficiency tolerance assay

Zn-deficiency tolerance in *S. bicolor* was first tested in seedlings by measuring plant growth on Hoagland’s solution, which contains 2 μM ZnSO₄, a concentration shown to be sufficient for a range of plants. In the Zn-deficiency condition, we omitted Zn from the medium. The *Sorghum* plants grown in the Zn-sufficient condition (2 μM Zn) continued to grow vigorously over the duration of experiment. The seedlings grown without Zn showed no deficiency symptoms for the first 30 d. The first symptoms of deficiency became apparent after 30 d, when leaves started to exhibit brown blotches and streaks; growth gradually ceased after 45 d (Fig. 1a–c). The symptoms of Zn deficiency were largely reversible if Zn was added before 55 d. However, the deficient plants started to die after 70 d (Fig. S2a), at which time secondary effects started to dominate. Therefore, we selected the 45-d-old seedlings for analysis in this study.

After a 45-d exposure to Zn deficiency, the seedlings exhibited large reductions in plant height (Fig. 1b), leaf width (Fig. 1d), and biomass (Fig. 1e). Interestingly, although Zn deficiency reduced the root biomass by reducing the lateral root density, it increased the primary root length after 30 d of treatment, especially in 45 d (Figs 1c, S2b). After 45 d, the growth of the primary root in Zn-deficient seedlings gradually ceased, and necrosis even occurred at the root tip (not shown). As a result, the primary root length of the control plants was longer than that of the Zn-deficient seedlings (Fig. 1c). Zn content was drastically reduced in deficient plants and had dropped to below 15 μg g⁻¹ DW in leaves (Fig. 1f), a concentration that is considered below the typical critical deficiency threshold for most plants (Marschner, 1995).

Zinc deficiency has been previously shown to induce ROS accumulation and subsequent oxidative damage in plants (Yu & Rengel, 1999; Cakmak, 2000). The antioxidative capacity of *Sorghum* seedlings was evaluated by examining the DPPH radical-scavenging capacity and MDA concentration (Fig. 2). These tests showed similar results: Zn depletion depressed the radical-scavenging capacity and elevated the amount of oxidative damage as indicated by MDA content in mature leaves and roots, indicating that there is a correlation between Zn supply and antioxidative capacity in *Sorghum* plants. However, the antioxidative capacity was further reduced at 1 d after resupplying Zn to the Zn-deficient seedlings. This result is discussed in the following section.

**Zn deficiency reduces photosynthesis efficiency resulted from a nonstomatal limitation**

The leaves of Zn-deficient plants were slightly chlorotic and exhibited brown blotches and streaks in mature leaves. Chl content was reduced by 17.5 and 18.3% in young leaves and mature leaves, respectively, when compared with their Zn-sufficient counterparts (Fig. 3a). To evaluate the effects of Zn deficiency on the efficiency of photosynthesis, we analyzed the light intensity-dependent ETR and photosynthetic rate (*Pₙ*). Zn deficiency dramatically depressed the ETR in young leaves and especially mature leaves (Fig. 3b). Consistent with the reduced ETR, drastic reductions in *Pₙ* were also observed in *Sorghum* leaves (Fig. 3c). In summary, the photosynthesis efficiency was strongly affected by Zn depletion in *Sorghum*.

The stomata are an important limitation for photosynthesis efficiency. To investigate whether the reduction of photosynthesis efficiency in Zn-deficient *Sorghum* seedlings is the result of stomatal constraint or nonstomatal constraint, we measured intercellular CO₂ concentration (*Cᵢ*), stomatal conductance (*gₛ*), and stomatal limitation value (*Lₛ*). In contrast to *Pₙ*, Zn depletion markedly elevated *Cᵢ* in both young and mature leaves (Fig. 3d) and increased *gₛ* in young leaves (Fig. 3e). The *Lₛ*, which reflects the relationship between *gₛ* and the nonstomatal capacity for photosynthesis (Ball & Berry, 1982), was significantly lower in
Zn-deficient leaves (Fig. 3f). These results indicated that the decrease of $P_n$ in Zn-deficient *Sorghum* plants did not result from stomatal limitations.

Zn-responsive miRNAs in *Sorghum*

To investigate the responsive functions of miRNAs during Zn deficiency, miRNA expression in Zn-deficient *S. bicolor* was profiled using a microarray assay. In general, different plant species have similar mature miRNA sequences in the same miRNA families. They typically differ by only one to four nucleotides (Fig. S3). Thus, cross-hybridization among members from the same family and among orthologs across species could explain these microarray results (Zhang et al., 2008). In this study, a total of 19 Zn-responsive miRNAs belonging to eight families were identified to be responsive to Zn deficiency (Fig. 4a,b, Tables S2, S3, S4). It was found that miRNA members of the same family had similar expression profiles, most likely owing to their highly homologous sequences, which were difficult to distinguish even using hybridization-based methods (Zhang et al., 2008). Among the eight Zn-responsive miRNA families, only the *miR171* family (including *miR171a*, *miR171b*, *miR171d*, and *miR171b*) and *miR528* were significantly down-regulated in leaves. The other six miRNA families, including *miR408* in leaves, and five other miRNA families (*miR398*, *miR319*, *miR166*, *miR168*, and *miR399*) in roots were significantly up-regulated by Zn deficiency. These up-regulated miRNAs in roots include *miR398*, *miR319b*, *miR166a*, *miR166b*, *miR166c*, *miR166d*, *miR166f*, *miR166h*, *miR166i*, *miR166j*, *miR166k*, and *miR399c* (Fig. 4b). All of these miRNAs were experimentally confirmed using RT-qPCR, and the results were consistent with the microarray data (Fig. 4c), thereby indicating the reliability of our data.

Target genes were predicted for these Zn-responsive miRNAs, which encoded transcription factors and proteins associated with metabolic processes or stress responses (Fig. S4). We also investigated the potential metal stress-responsive cis-elements in the promoters of the Zn-miRNA genes. As shown in Fig. S5, MRE motifs occurred frequently in the promoter regions of Zn-responsive miRNAs. The ZDRE motifs are present in the *miR166a*, *miR166a*, *miR166b*, *miR166d*, *miR166f*, *miR166h*, *miR166i*, *miR166j*, *miR166k*, *miR168*, *miR398*, and *miR399c* promoters. These findings suggested that miRNAs played an important role in Zn-deficiency tolerance in *Sorghum* and highlighted a novel molecular mechanism of Zn-deficiency tolerance in plants.

miRNAs are involved in Zn-mediated development processes in *Sorghum*

Several Zn deficiency-induced miRNAs are involved in modulating growth and development in plants. *MiR408* targets *plantacyanin,*
which plays a role in ROS signaling, pathogen defense, and reproduction (Dong et al., 2005). MiR166 targets the Class III HD ZIP family proteins PHB, REV, and ATHB15, which affect root system development. MiR168 and miR171 target AGO1 and the GRAS family transcription factor, SCL6, respectively, and thereby modulate plant growth and development processes. MiR319b targets the basic helix–loop–helix (bHLH) transcription factor TCP2, which affects the biosynthesis of JA. To investigate whether the predicted target genes are actually regulated by Zn-miRNAs, the expression levels of the targets were measured in Zn-deficient Sorghum seedlings using RT-qPCR (Figs 4c, 5) and modified 5′-RNA ligase-mediated RACE (Fig. 6). As shown in Fig. 5, the profiles of the miRNAs and their target transcripts were complementary to one another, though not completely. Together, the negatively correlated expression patterns between miRNAs and their targets further validated the regulatory role of miRNAs on their targets and indicated the role of miRNAs in Zn-deficiency responses of Sorghum seedlings.

Because the miRNA expression analyses revealed differences in the expression levels of miRNA genes associated with the biosynthesis of and the response to phytohormones (e.g. miR319b affects the biosynthesis of JA by targeting TCP2, and miR166 regulated IAA responses by targeting Class III HD ZIP), we measured the plant concentrations of these hormones in Sorghum seedlings with or without Zn supplementation. As shown in Fig. 7, Zn deficiency markedly reduced the concentrations of auxin IAA and total JA (containing JA and methyl jasmonic acid (MeJA)) in both the roots and leaves. After 1 d of Zn re-supplementation, the concentrations of both JA and IAA recovered quickly in leaves, suggesting that these phytohormone concentrations were tightly regulated by Zn supply in plants. These results indicated that miRNAs play a part in regulating plant growth and development by affecting phytohormone biosynthesis and responses.

![Figure 2](image-url)  
**Fig. 2** Quantification of the free radical-scavenging capacities and oxidative damage in Sorghum bicolor seedlings grown hydroponically in the presence of 2 μM ZnSO4 (control) or without added Zn (Zn deficiency). (a) 2,2′-Diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging capacity. (b) Malondialdehyde (MDA) content. Values are given as means ± SD (n = 3). Control, 2 μM ZnSO4; Zn-, without Zn; 1 and 5 d after recovery denote the supplementation of 2 μM ZnSO4 to Zn-deficient seedlings (treated for 45 d) for 1 and 5 d before harvest. Columns labeled with different letters are significantly different at *P* < 0.05, Duncan’s multiple range test.

![Figure 3](image-url)  
**Fig. 3** Zinc (Zn) deficiency affects photosynthetic performance in Sorghum bicolor. (a) Leaf Chl content. (b) Light intensity-dependent electron transport rate (ETR). (c) Photosynthetic rate (*Pn*). (d) Intercellular CO2 concentration (*Ci*). (e) Stomatal conductance (*g*) and *Ls* (f) Stomatal limitation value (*Ls*). Values are given as the means ± SD (n = 8). Control, 2 μM ZnSO4; Zn-, without Zn. Asterisks indicate values significantly different from the Zn control. *Student’s t*-test with *P* < 0.05.
Zinc deficiency induced Pi accumulation by miRNA mediation pathway

Zinc deficiency-induced Pi accumulation in plants has been reported (Loneragan & Webb, 1993; Huang et al., 2000). However, the detailed molecular mechanism remains unclear. We measured Pi content in Zn-deficient Sorghum seedlings. As shown in Fig. 8(a), Zn depletion markedly improved Pi content, especially in mature leaves. The Pi concentrations gradually recovered after resupplying the Zn-deficient seedlings with Zn. In this study, we found that Zn deficiency up-regulated the expression of miR399c in Sorghum roots (Figs 4b,c, 8b). A bioinformatics analysis of the Sorghum genome, target validation by RACE (Fig. 8c) and coexpression analysis (Fig. 8d) suggested that miR399c targets the 5′-UTR of UBC24 (PHO2), a negative regulator of Pi transporters, including several Ph1 family Pi transporter genes (Aung et al., 2006). Therefore, the elevated expression of miR399c in Sorghum roots increases Pi accumulation by regulating the expression of PHO2. Three days after Zn resupplementation, the expression of miR399c was down-regulated, and the subsequent Pi accumulation gradually decreased in Sorghum leaves. A cluster of MREs (10 MRE/MRE-like) and one ZDRE occur in the region of 823–1668 bp of the miR399c promoter (Fig. S4). These results support the idea that miRNAs play a role in modulating Pi accumulation during Zn deficiency.

Zn depletion affects CSD function

In this study, we found that the expression levels of miR398 and miR528 affected CSD levels in Sorghum plants. As shown in Fig. 9, in Zn-deficient Sorghum, the down-regulated miR528 in young leaves increased the gene expression and enzyme activity of CSD (Figs 9a–c, 9j, S6a). However, the up-regulated miR398-mediated down-regulation of CSD and reduced CSD activity were observed in the roots of Sorghum exposed to Zn depletion (Fig. 9g–j). Interestingly, as a result of the sharp elevation of miR528, the expression and enzyme activity of CSD in both young and mature leaves further decreased at 1 d after Zn reintroduction.

Zn depletion depressed ADH activity

Alcohol dehydrogenase is an important Zn protein that is involved in alcohol fermentation and lignin biosynthesis in plants. We therefore analyzed the effect of Zn depletion on the gene expression and enzyme activity of ADH in Sorghum leaves. As shown in Fig. 10(a,b), Zn depletion down-regulated the expression of three ADH genes (Sb01g008730, Sb05g009350, and Sb04g037050) in both young and mature leaves. Enzyme activity assays indicated that ADH activity is strongly depressed under Zn deficiency (Figs 10c, S6b). After 5 d of resupplementation with Zn, neither the gene expression nor the enzyme activity of ADH recovered in the leaves (Fig. 10).

CA receives priority in Zn delivery after Zn reintroduction

Carbonic anhydrase is an important Zn protein in plants (Ignatova et al., 2011). We therefore analyzed the effects of Zn depletion on the transcript abundance and enzyme activity of CA in Sorghum plants. As shown in Fig. 11(a,b), Zn depletion
down-regulated the expression of three CA genes in both young and mature leaves. Similar to the transcript abundances, the CA activity was severely affected by Zn depletion (Figs 11c, S6c). In contrast to what was observed for CSD and ADH, after 1 d of resupplementation with Zn, both the gene expression and enzyme activity of CA recovered quickly in the leaves (Fig. 11).

These results indicate that Zn deficiency-induced photosynthesis efficiency inhibition resulted from a nonstomatal limitation.

Therefore, we speculated that the depressed CA activity might be responsible for photosynthesis efficiency inhibition in Zn-deficient Sorghum plants. As a result, we investigated the change in photosynthetic rate ($P_n$) with Zn resupply. As shown in Fig. 11(d), the fast rise in CA activity is also reflected in the very quick recovery of $P_n$ especially in young leaves. By contrast, the activities of CSD and ADH are delayed relative to $P_n$ (Figs 9–11). The recovery of CSD and ADH activity is even slower in the mature leaves. Together, these data indicate that
3' CUAUACCUGGCAGGUUAGU5' sbi-MiR171
5' GAUAUUGGGGCGGCUCUAAA3' s04g032570 (SCL6-IV)

3' CCCUUACUUCGGACCCGGC5' sbi-MiR166
5' CGGGAGAAGAUGUGGCCG3' sb03g02660 (ATHB15)

3' CCCUUACUUCGGACCCGGC5' sbi-MiR166
5' UGGGAUGAAGAUGUGCCGG3' sb08g02350 (PHB-2)

3' CCCUUACUUCGGACCCGGC5' sbi-MiR166
5' UGGGAUGAAGAUGUGCCGG3' sb01g050000 (REV-2)

Fig. 6 Experimental validation of the predicted mRNA targets for miR171 and miR166. The mRNA cleavage sites were determined by modified 5' RNA ligase-mediated rapid amplification of cDNA ends (RACE). The red boxes represent the exons, and the horizontal lines represent introns. Gray boxes represent 5'-UTRs, and gray boxes represent 3'-UTRs. The mRNA sequences and complementary microRNA (miRNA) sequences are shown. Vertical arrows indicate the 5' termini of miRNA-guided cleavage products, as identified by 5'-RACE, with the frequency of clones shown.

Watson–Crick pairing (vertical dashes) and G:U wobble pairing are indicated.

Earlier studies have found that Zn deficiency leads to Pi accumulation, which can reach toxic concentrations in plants. Huang et al. (2000) found that Zn deficiency induced the expression of genes encoding Pi transporters in plants grown in either P-sufficient or P-deficient conditions. In this study, we found that Zn deficiency induced the expression of miR399c in Sorghum roots. MiR399 can be regarded as Pi-starvation signals leading to URC24 (PHO2) suppression and thus derepressing the Pi-starvation response and Pi uptake capacity. MiR399 activity can be quenched by noncoding RNA IPS1 through a novel mechanism termed target mimicry (Franco-Zorrilla et al., 2007). MiR399 also serve as phloem-mobile long-distance signals communicating Pi status between shoots and roots (Lin et al., 2008; Pant et al., 2009). These results indicated that Zn deficiency induced Pi accumulation via an miRNA signaling pathway.

There are two types of factors that can inhibit photosynthesis efficiency: stomatal limitation and nonstomatal limitation (Farquhar & Sharkey, 1982). We found that Zn deficiency strongly inhibited photosynthesis efficiency in Sorghum seedlings, whereas it elevated Ci in both young and mature leaves and increased gs in young leaves. Our analysis of Ls indicated that it is significantly lower in Zn-deficient leaves, further confirming that the reduced photosynthesis efficiency in Zn-deficient Sorghum plants resulted from a nonstomatal limitation (Fig. 3). This result could be largely explained by the Zn-depleted decrease of CA activity. CA catalyzes reversible CO2 hydration and supplies Rubisco with CO2 (Ignatova et al., 2011). Depressed CA activity decreased the availability of CO2 for photosynthesis. The rapid recovery of CA activity after Zn resupplementation is
advantageous for the rapid recovery of photosynthesis and is an important adaptation mechanism of plant responses to Zn deficiency.

Two Zn-miRNAs, miR171 and miR166, target transcriptional factors and thereby regulate plant growth and development. MiR171 acts to negatively regulate shoot branching by targeting the GRAS gene family member, SCL6 (Ma et al., 2010; Hwang et al., 2011). MiR166 acts to negatively regulate auxin canalization by targeting the Class III HD ZIP family proteins PHB, REV, and ATHB15 (Zhong & Ye, 2001; Boualem et al., 2008; Ilegems et al., 2010). On the other hand, tryptophan biosynthesis is strictly dependent on Zn; as tryptophan is the amino acid precursor of auxin synthesis, impairment of auxin synthesis is expected to be a major reason for decreased auxin concentrations in plants. This finding could explain the phenotype of reduced lateral roots in Zn-depleted *Sorghum* roots. However, determining whether the increased length of the primary roots in the *Sorghum* roots grown in the Zn-deficient medium for 45 d is related to the expression of miR166 requires further investigation.

In this study, we also found that the expression levels of most miRNAs did not recover after Zn resupplementation, especially after 1 d of recovery (i.e. miR398, miR168, miR399c, miR319b, and miR171) (Figs 5, 8b, 9g). The differential expression patterns implied that miRNAs play a role in modulating Zn reallocation, a mechanism that requires further elucidation.

Phytohormone JA plays a role in pathogen and stress responses and in developmental processes. MiR319b targets the JA biosynthesis-related gene TCP2 and is up-regulated in Zn-depleted *Sorghum* roots. Accordingly, JA concentrations are markedly lower in Zn-depleted *Sorghum* seedlings. MiR408 targets plantacyanin, a copper blue protein whose function is not fully elucidated in plants (Dong et al., 2005). Nersissian et al. (1998) proposed that plantacyanin is involved in the oxidative burst that occurs during pathogen infection and in the cross-linking and insolubilization of cell wall materials, such as lignin and callose. The elevated expression of miR408 in *Sorghum* leaves was shown to inhibit plantacyanin and is therefore responsible for ROS signaling and pathogen defense in Zn-deficient plants (Broadley et al., 2007).

Zinc deficiency has been shown to reduce the expression and activity of CSD in wheat and maize (Hacisalihoglu et al., 2003; Sharma et al., 2004). In this study, we found that two miRNAs that target CSD showed opposite expression patterns: up-regulated miR398 reduced the expression and activity of CSD in roots, whereas down-regulated miR528 elevated the expression and activity of CSD in mature and especially young leaves. It is surprising that the expression of miR528 did not recover immediately after Zn resupplementation. Instead, the expression of miR528 peaked sharply at 1 d after Zn resupply, thereby resulting in a further decrease in CSD activity. In contrast to what was observed for CSD, CA recovered quickly after Zn resupplementation, even though Zn deficiency reduced the expression and activity of CA. The recovery of CA in leaves reflects the recovery of Zn concentrations in *Sorghum* plants (Figs 1f, 11). These results suggested that CA is the preferred target protein for Zn resupply, whereas CSD is not.

Our results support the viewpoint that plants regulate metal-binding proteins using miRNA mechanisms (Fig. S7). Higher
Zinc (Zn) depletion depresses copper/zinc superoxide dismutase (CSD) function by miR528 and miR398. *Sorghum bicolor* seedlings were grown hydroponically as described in Fig. 1. After 45 d, 2 μM ZnSO₄ was added to the medium of plants previously grown in the absence of Zn. Expression profile analysis of miR528 (a, d) and miR398 (g) and their corresponding target genes. Total soluble proteins (40 μg) were fractionated on a nondenaturing 15% acrylamide gel and stained for total superoxide dismutase (SOD) activity in young leaves (b), mature leaves (e), and roots (h), respectively. (c, f, i) SOD activity was measured immediately after treatment. Values are given as means ± SD (n = 3). (j) Experimental validation of the predicted mRNA targets for miR528 and miR398. The mRNA cleavage sites were determined by modified 5′ RNA ligase-mediated rapid amplification of cDNA ends (RACE). The red boxes represent the exons, and the horizontal lines represent introns. Gray boxes represent 5′-UTRs, and grey arrows represent 3′-UTRs. The mRNA sequences and complementary microRNA (miRNA) sequences are shown. Vertical arrows indicate the 5′ termini of miRNA-guided cleavage products, as identified by 5′-RACE, with the frequency of clones shown. Watson–Crick pairing (vertical dashes) and G:U wobble pairing are indicated. Control, 2 μM ZnSO₄; Zn–, without Zn; 1, 3, and 5 d after recovery denote the supplementation of 2 μM ZnSO₄ to Zn-deficient seedlings (treated for 45 d) for 1, 3, and 5 d before harvest; MSD, manganese superoxide dismutase; FSD, iron superoxide dismutase. Asterisks indicate values significantly different from the Zn control. *Student’s t-test with P < 0.05.

plants can prioritize the delivery of essential metal-binding proteins by down-regulating nonessential or replaceable metal-binding proteins (Abdel-Ghany & Pilon, 2008; Ravet et al., 2011). This process is an important adaptation mechanism in micronutrition homeostasis that allows plants to cope with varying micronutrition statuses and thereby broadens the range of conditions in which plants can thrive (Abdel-Ghany & Pilon, 2008). The identification and functional analysis of these key miRNAs would open up the possibility of exploring Zn-deficiency response mechanisms in plants and manipulating Zn use by regulating miRNA expression. Further research may also reveal miRNA-mediated signal transduction mechanisms for breeding Zn-efficient crops.

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Fig. 10 Zinc (Zn) depletion reduces alcohol dehydrogenase (ADH) activity. Expression profile analysis of three ADH genes (Sb01g008730, Sb05g009350, and Sb04g037050) in young leaves (a) and mature leaves (b). (c) ADH activity in Sorghum leaves. Sorghum bicolor seedlings were grown hydroponically in the presence of 2 μM ZnSO₄ (control) or without added Zn (Zn deficiency). Values are given as means ± SD (n = 3). Control, 2 μM ZnSO₄; Zn-, without Zn; 1, 3, and 5 d after recovery denote the supplementation of 2 μM ZnSO₄ to Zn-deficient seedlings (treated for 45 d) for 1, 3, and 5 d before harvest. Columns labeled with different letters are significantly different at P < 0.05, Duncan’s multiple range test.

Fig. 11 Zinc (Zn) deficiency reduces carbonic anhydrase (CA) activity in Sorghum bicolor. Expression profile analysis of three CA genes (Sb02g026930, Sb03g029180, Sb03g029170) in young leaves (a) and mature leaves (b). (c) CA activity was measured immediately after treatment. (d) Photosynthetic rate (Pn) in Sorghum leaves. Values are given as the means ± SD (n = 3). Control, 2 μM ZnSO₄; Zn-, without Zn; 1, 3, and 5 d after recovery denote the supplementation of 2 μM ZnSO₄ to Zn-deficient seedlings (treated for 45 d) for 1, 3, and 5 d before harvest. Columns labeled with different letters are significantly different at P < 0.05, Duncan’s multiple range test.
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**Fig. S1** Gene expression stability and pairwise variation of the candidate reference genes.

**Fig. S2** Zn deficiency depressed plant growth.

**Fig. S3** Similarity of sequences among miRNA members.

**Fig. S4** Sequences of predicted target genes.

**Fig. S5** Potential metal-responsive motifs in the promoters of Zn deficiency-responsive miRNAs.

**Fig. S6** Enzyme activities of SOD, ADH and CA in *Sorghum* leaves.

**Fig. S7** Potential regulatory network for Zn-responsive miRNAs.

**Methods S1** Supplemental materials and methods.

**Table S1** Primers used for RT-PCR analysis of the genes.

**Table S2** Zn deficiency-responsive miRNAs in the roots.

**Table S3** Zn deficiency-responsive miRNAs in the leaves.

**Table S4** Zn deficiency-responsive miRNA families and members.

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