Divalent nutrient cations: friend and foe during zinc stress in rice

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Abstract

Zn deficiency is the most common micronutrient deficit in rice but also a widespread industrial pollutant. It is unclear how rice responds to Zn depletion or excess, and which signaling molecules link the affected physiological processes. We therefore compared the physiological, transcriptomic and biochemical properties of rice plants subjected to Zn starvation or excess at early and later treatment stages. Both forms of Zn stress inhibited root and shoot growth. Several divalent cations (Fe, Cu, Ca, Mn and Mg) accumulated in Zn-depleted shoots, possibly due to the increased synthesis and activity of promiscuous Zn transporters and chelators. Gene Ontology enrichment analysis of 970 differentially expressed genes revealed overrepresentation of ion and oligopeptide transport, antioxidative defense and secondary metabolism. The expression of genes encoding Fe/Ca-binding peroxidases was activated after 3 days of Zn starvation, boosting the activity of ascorbate peroxidase and thus scavenging H_2O_2 more effectively to prevent leaf chlorosis. Conversely, excess Zn triggered the expression of genes encoding Mg-binding proteins (*OsCPS2/4* and *OsKSL4/7*) required for antimicrobial diterpenoid biosynthesis. We demonstrated the key components of crosstalk between Zn and other divalent cations under Zn stress conditions, leading to the regulation of gene expression and corresponding biochemical and physiological processes.

Running head: Zn stress induced physiological responses

Divalent nutrient cations: friend and foe during zinc stress in rice

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Abstract

Zn deficiency is the most common micronutrient deficit in rice but also a widespread industrial pollutant. It is unclear how rice responds to Zn depletion or excess, and which signaling molecules link the affected

physiological processes. We therefore compared the physiological, transcriptomic and biochemical properties of rice plants subjected to Zn starvation or excess at early and later treatment stages. Both forms of Zn stress inhibited root and shoot growth. Several divalent cations (Fe, Cu, Ca, Mn and Mg) accumulated in Zn-depleted shoots, possibly due to the increased synthesis and activity of promiscuous Zn transporters and chelators. Gene Ontology enrichment analysis of 970 differentially expressed genes revealed overrepresentation of ion and oligopeptide transport, antioxidative defense and secondary metabolism. The expression of genes encoding Fe/Ca-binding peroxidases was activated after 3 days of Zn starvation, boosting the activity of ascorbate peroxidase and thus scavenging H_2O_2 more effectively to prevent leaf chlorosis. Conversely, excess Zn triggered the expression of genes encoding Mg-binding proteins (*OsCPS2/4* and *OsKSL4/7*) required for antimicrobial diterpenoid biosynthesis. We demonstrated the key components of crosstalk between Zn and other divalent cations under Zn stress conditions, leading to the regulation of gene expression and corresponding biochemical and physiological processes.

Keywords: zinc deficiency, excess zinc, *Oryza sativa* L., nutrient homeostasis, iron, calcium, magnesium, reactive oxygen species, diterpenoids

Introduction

Zinc (Zn) is one of the eight essential micronutrients in plants required for normal development and defense (Marschner, 2011; Cabot*et al* ., 2019). Zn-binding proteins account for more than 10% of the eukaryotic proteome (Andreini, Banci, Bertini & Rosato, 2006) and include ˜300 Zn-binding enzymes belonging to all six major enzyme classes (Sagardoy, Morales, López-Millán, Abadía & Abadía, 2009). Zn acts as a structural, regulatory or catalytic cofactor for these proteins (McCall, Huang & Fierke, 2000; Saravanan, Vasu, Ghosh & Nagaraja, 2007). Well-known Zn-binding proteins include Zn-finger domain transcription factors, superoxide dismutase (SOD), carbonic anhydrase and RNA polymerase (Eide, 2011). They regulate a wide range of physiological processes, including gene expression, photosynthesis, protein synthesis, hormone production, and stress responses, and they also play key roles in carbohydrate, lipid and nucleic acid metabolism (Stoyanova & Doncheva, 2002; López-Millán, Ellis & Grusak, 2005; Hafeez, Khanif & Saleem, 2013; Cabot *et al* ., 2019). Zn deficiency and excess both trigger adverse effects on plant physiology and metabolism, so Zn levels must be tightly regulated and maintained within an optimal range (Clemens, 2001; Palmgren *et al* ., 2008; Ishimaru, Bashir & Nishizawa, 2011; Lin & Aarts, 2012).

Zn homeostasis in plants is controlled in part by F-group bZIP (basic leucine zipper) transcription factors that regulate *ZIP*transporter genes (zinc-regulated transporters, iron-regulated transporter-like protein). In Arabidopsis (*Arabidopsis thaliana*), two F-group bZIP proteins (AtbZIP19 and AtbZIP23) bind the zinc deficiency response element (ZDRE) located in the promoter region of a small group of Zn homeostatic genes encoding ZIP transporters (*AtZIP1 /3 /4 /5/ 9 /10 /12* and*AtIRT3*) and nicotianamine synthases (*AtNAS2 /4*) (Assunção *et al* ., 2010; Assunção *et al* ., 2013). Overexpressing either *AtbZIP19* or *AtbZIP23* in the Arabidopsis *bzip19bzip23* double mutant rescues the mutant's hypersensitivity to Zn deficiency (Assunção *et al* ., 2010; Lilay, Castro, Campilho & Assunção, 2019). Recently, AtbZIP19 and AtbZIP23 have been shown to act as Zn sensors because they contain a Zn sensor motif that can bind to Zn^{2+} ions, and mutation of this motif affects Zn binding and eventually leads to a constitutive transcriptional Zn deficiency response (Lilay *et al* ., 2020b). The rice (*Oryza sativa*) genome contains 16 paralogous *OsZIP*genes (Tiong *et al* ., 2015). Complementation assays in yeast have shown that OsZIP1/3/4/5/7/8/9 are functional Zn transporters (Ramesh, Shin, Eide & Schachtman, 2003; Ishimaru *et al* ., 2005; Yang, Huang, Jiang & Zhang, 2009; Lee *et al* ., 2010a; Lee, Kim, Lee, Guerinot & An, 2010b; Tan *et al* ., 2019; Huang *et al* ., 2020). OsZIP1/9 facilitate Zn uptake in roots whereas OsZIP4/5/7/8 control Zn translocation or distribution in shoots (Huang *et al* ., 2020). The F-bZIP-mediated Zn deficiency response in Arabidopsis is conserved in barley (*Hordeum vulgare*), wheat (*Triticum aestivum*) and rice (Castro *et al* ., 2017; Evens, Buchner, Williams & Hawkesford, 2017; Nazri, Griffin, Peaston, Alexander-Webber & Williams, 2017; Lilay *et al* ., 2020a). OsbZIP48 and OsbZIP50 are the rice F-bZIP homologs that regulate the Zn deficiency response (Lilay *et al* ., 2020a).

If Zn homeostasis fails, many physiological and metabolic processes are affected. Rice plants starved of Zn therefore display symptoms such as bronzed leaves, short internodes, fewer tillers, delayed development, and low grain yields (Yoshida & Tanaka, 1969; Widodo *et al* ., 2010). On the other hand, Zn toxicity symptoms include leaf chlorosis and the inhibition of growth and flowering (Silva, Vitti & Trevizam, 2014). The production of gibberellin (GA) and indole-3-acetic acid (IAA) is repressed by Zn starvation in tomato (*Solanum lycopersicum*) and maize (*Zea mays*) plants (Takaki & Kushizaki, 1977; Sekimoto, Hoshi, Nomura & Yokota, 1997), whereas the application of exogenous GA alleviates growth attenuation in rice seedlings exposed to excess Zn (Nag, Nag, Paul & Mukherji, 1984). Zn starvation and excess both disrupt the balanced production and removal of reactive oxygen species (ROS), leading to the accumulation of ROS and oxidative damage to major cellular structures (Apel & Hirt, 2004; Holler, Hajirezaei, Von Wirén & Frei, 2014; Feigl *et al* ., 2015). For example, Zn deficiency increases membrane permeability mainly by the peroxidation of unsaturated fatty acids (Cakmak, 2000). Exposure to photo-oxidative stress (light-dependent generation of ROS) therefore disrupts chlorophyll synthesis, photosynthetic electron transport and carbohydrate metabolism in chloroplasts (Bae, Oh, Rhee & Yoo, 2011).

Transcriptomic studies have shown that Zn starvation modulates the expression of genes encoding transporters, enzymes involved in the synthesis of phytosiderophores, organic acids and phytohormones (Suzuki*et al* ., 2012; Bandyopadhyay, Mehra, Hairat & Giri, 2017; Zeng, Zhang, Ding, Zhang & Zhu, 2019a). Previous studies in rice have mainly compared responses to Zn deficiency in different cultivars and varieties. For example, the transcriptomic profiles of roots (particularly crown roots) and shoots were compared in lines RIL46, IR55179 and Nipponbare (which tolerate Zn deficiency) and lines IR26, IR74 and IR64 (which do not) under normal conditions and Zn deprivation (Widodo *et al* ., 2010; Nanda, Pujol & Wissuwa, 2017; Zeng, Zhang, Ding & Zhu, 2019b). The unique gene expression profile of line RIL46 explained its ability to outperform line RIL74 in terms of root growth, the efflux of phytosiderophores and organic acids, and the uptake of Zn-chelator complexes in response to Zn starvation (Widodo *et al* ., 2010). The more efficient relocation of Zn to roots allowing the better maintenance of crown root growth in Zn-efficient rice genotypes was explained by the unique expression of various Zn-finger, hormone-responsive and transporter genes (Nanda *et al* ., 2017). Conversely, transcriptomic studies of rice seedlings exposed to excess Zn are rare. A comprehensive comparison of the rice transcriptome under normal conditions, Zn starvation and excess Zn is therefore needed to better understand how rice plants address Zn stress.

The regulation of Zn deficiency and homeostasis in Arabidopsis is closely linked to the regulation of other micronutrients (Lilay *et al* ., 2019). To determine whether this is also true in rice, we compared the physiological, transcriptomic and biochemical responses of rice plants to Zn deprivation and excess 3, 14 and 21 days after the onset of treatment (DAT) to identify links with the regulation of Mn, Fe, Cu, Ca and Mg. We focused on transcriptional regulators, metal chelators and transporters, ROS-dependent transcriptional reprogramming and the effect of Zn on secondary metabolism and defense responses.

Materials and Methods

Plant materials and Zn treatments

The rice line selected for this study (*Oryza sativa* L. cv. Kitaake) was chosen because its short life cycle (9 weeks) is particularly suitable for functional genomics experiments (Jain *et al* ., 2019). Seeds were surface sterilized with 1.25% NaOCl for 60 min, washed three times with Millipore water, soaked in Millipore water at 37°C for 24 h and germinated on moistened paper towels in the dark at 37°C for 38 h. The germinated seeds were sown in full-strength 1× Kimura B nutrient solution and maintained thereafter in a growth chamber with a 12-h photoperiod (30/25°C day/night temperature, light intensity = 220 µmol $m^{-2}s^{-1}$). The 1× Kimura B nutrient solution with normal Zn comprised 0.36 mM $(NH_4)_2SO_4$, 0.18 mM KNO_3, 0.55 mM $MgSO_4$, 0.18 mM KH_2PO_4, 61.20 µM Fe citrate, 0.37 mM $Ca(NO_3)_2$, 2.51 µM H_3BO_3, 0.20 µM $MnSO_4$, 0.2 µM $ZnSO_4$, 0.05 µM $CuSO_4$, and 0.05 µM H_2MoO_4 (Yoshida, 1976). After 7 days, the seedlings were transferred to full-strength 1× Kimura B nutrient solution containing three different Zn concentrations: Zn

deficient ([Zn] = 0.002 μM), normal Zn control ([Zn] = 0.2 μM) and excess Zn ([Zn] = 300 μM). During the treatment phase, the nutrient solution was changed every other day. Root and shoot samples were harvested separately at 3, 14 and 21 DAT for physiological, gene expression and biochemical analysis.

Physiological measurements

We recorded shoot height, root length, fresh weight (FW), dry weight (DW) and (relative) chlorophyll content at 3, 14 and 21 DAT from four biological replicates of 4-10 plants. The relative chlorophyll content in the leaves was measured with a SPAD 502 Plus chlorophyll meter (Spectrum Technologies, USA). For each plant, SPAD measurements were taken from three different points on the fully extended youngest leaf and the mean value was calculated. The chlorophyll a /$b$content was also measured at 3, 14 and 21 DAT using a SpectraMax ABS Plus microplate reader (Molecular Devices, USA) (Wintermans & De Mots, 1965). We ground 45-50 mg of the fully extended youngest leaf of each plant to homogeneity in 2 mL 50 mM sodium phosphate buffer (pH 6.8) at 4°C. We mixed a 40 μL aliquot of the leaf extract with 960 μL 100% ethanol in a 1.5 mL tube before incubating the mixture at 4°C in the dark for 30 min. After centrifugation at 1000 g for 15 min at 4°C, 800 μL of the supernatant was transferred to a quartz cuvette for absorbance measurements at 665 and 649 nm. The mean value for each sample was derived from four biological replicates and three technical replicates, and the chlorophyll concentration/content was calculated as previously described (Wintermans & De Mots, 1965).

Library preparation for transcriptome sequencing

Total RNA was isolated from root and shoot tissues using the Direct-zol RNA MiniPrep Kit (Zymo Research, USA) with two plants pooled as one biological replicate and three biological replicates per treatment or tissue. RNA concentration and purity were checked on a SimpliNano spectrophotometer (Biochrom, USA) to confirm samples fell within the ranges OD_{260}/OD_{280} = 1.8-2.2 and OD_{260}/OD_{230} [?] 2.0. RNA integrity and purity were determined by agarose gel electrophoresis (to confirm the presence of the 4.5-kb 28S rRNA and 1.9-kb 18S rRNA bands). RNA integrity and concentration were also assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA) to confirm a RIN value [?] 8.2). We then used 1 μg of total RNA per sample for the preparation of each sequencing library using the KAPA mRNA HyperPrep Kit (Roche, Switzerland) following manufacturer's protocol. Briefly, mRNA was captured from total RNA using magnetic oligo-dT beads and was fragmented to a mean library insert size of 100-200 bp by incubation for 8 min at 94°C in the presence of Mg^{2+}. First-strand cDNA was synthesized with random hexamer primers followed by second-strand cDNA synthesis (including dUTP markers), 3' polyadenylation of both strands and adaptor ligation via complementary 3' dTMP overhangs. The library fragments were size selected (300-400 bp) and amplified by PCR with the dUTP-marked strands excluded, allowing strand-specific sequencing. The amplified library was purified and quality checked using the Qsep100 DNA/RNA Analyzer (BiOptic, Taiwan). We prepared 18 RNA sequencing (RNA-Seq) libraries (two tissues x three Zn concentrations x three biological replicates) for sequencing (Biotools, Taiwan) on the NovaSeq 6000 platform (Illumina, USA).

Bioinformatics analysis of transcriptomic data

Post-sequencing, paired-end raw reads were retrieved in FASTQ format. FastQC and MultiQC were used to check the quality of the raw reads (Ewels, Magnusson, Lundin & Kaller, 2016). Read trimming steps, including adapter clipping and the removal of low-quality reads and bases, were applied using Trimmomatic v0.38 to obtain high-quality reads (Bolger, Lohse & Usadel, 2014). High-quality read pairs from each sample were mapped to the *Oryza sativa* Nipponbare IRGSP-1.0 reference genome using HISAT2 v2.1.0 (Kawahara *et al* ., 2013; Kim, Langmead & Salzberg, 2015). FeatureCounts v1.6.0 was used to count reads mapped to annotated genes in the reference genome (Liao, Smyth & Shi, 2014). To assess variation between samples, principal component analysis (PCA) scatter plots were generated with log(FPKM+1) (fragments per kilobase million) normalized reads using the prcomp function and ggplot2 package in R (Reimers & Carey, 2006; Wickham, 2016). The DESeq2 package in R was used to normalize the read counts into "Relative Log Expression" before the identification of differentially expressed genes (DEGs) between two sample groups (Love, Huber & Anders, 2014). DEGs were defined based on two criteria: (i) the average normalized

expression of three biological replicates recorded a $|\log_2$ fold change| [?]1 between two sample groups, and (ii) the Benjamini-Hochberg adjusted p-value was < 0.05. Functional Gene Ontology (GO) enrichment and pathway mapping analysis was performed on the DEGs identified by comparing the Zn deficiency/excess samples using agriGO v2.0 (Tian *et al* ., 2017) and MapMan (Thimm *et al* ., 2004), respectively. Heat maps showing fold changes in expression were generated using the heatmap.2 function of the gplots package in R (Warnes *et al* ., 2005).

Validation of DEGs by quantitative reverse transcription PCR (RT-qPCR)

Total RNA was isolated and quality checked as described above. For each sample, 1 μg of total RNA was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad, USA) and the first-strand cDNA was diluted 5× with Millipore water. Quantitative PCR (qPCR) was carried out on a CFX Connect Real-Time PCR Detection System (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad). We mixed 3 μL PCR-grade water, 10 μL 2× iQ SYBR Green Supermix, 1 μL 10 μM forward primer, 1 μL 10 μM reverse primer and 5 μL cDNA. We used primers that passed the primer efficiency test as shown in Table S1. Each reaction comprised an initial denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. Melting curves were generated to confirm primer specificity. Transcript levels in each sample were measured in four biological replicates (four plants were pooled per replicate) and the mean value was calculated from three technical repeats. The fold difference in expression was calculated using the $2^{-\Delta\Delta^{\tau}}$ method (Livak & Schmittgen, 2001). Although four housekeeping genes (*OsUbi1* , *OsUBQ5* , *$\epsilon E\Phi 1a$* and*18SrRNA*) were tested in this study, we chose *OsUbi1* (rice polyubiquitin) as the reference gene because it achieved the highest stability, lowest coefficient of variance and lowest M value (Mathur, 2014).

Elemental analysis

Root and shoot tissues were harvested separately for elemental analysis as previously described (Lin *et al* ., 2009). Briefly, the harvested tissues were washed with ice-cold 10 mM $CaCl_2$and Millipore water before drying at 70°C for 3 days. The dried samples were cut into fine pieces and digested with 0.5 N HNO_3. Multi-element analysis was carried out by inductively coupled plasma-optical emission spectrometry (ICP-OES) using an OPTIMA 5300 device (Perkin-Elmer, USA). Concentrations of elements in rice tissues were measured in four biological replicates (four plants were pooled per biological replicate).

Histochemical detection of hydrogen peroxide and superoxide

Hydrogen peroxide (H_2O_2) and superoxide (O_2^-) in rice leaves were detected by 3,3-diaminobenzidine (DAB) and nitroblue tetrazolium (NBT) staining, respectively. The staining solutions were prepared immediately before the experiments as recommended (Bournonville & Diaz-Ricci, 2011; Daudi & O'Brien, 2012). The fully-extended youngest leaves of rice plants in different Zn treatment groups were cut into two fragments, 2 cm in length, from the tip of the leaf blade. The fragments were immersed in 3 mL of staining solution and then infiltrated under vacuum for 5 min. The plates containing tissue samples were placed under light for 12-16 h. For clearer visualization, the leaf fragments were immersed in boiling 95% ethanol for 10-20 min to remove chlorophyll. Images of four biological replicates were captured against a plain white background.

Measurement of antioxidant enzyme activity

The activities of ascorbate peroxidase (APX), catalase (CAT) and glutathione reductase (GR) were measured as previously described (Foster & Hess, 1980; Nakano & Asada, 1981; Kato & Shimizu, 1987). For protein extraction, 0.04 g of finely-ground leaf material in a 10 mL Falcon tube was mixed with 4 mL 50 mM sodium phosphate buffer (pH 6.8) and centrifuged at 12000 g for 20 min at 4°C. The supernatant containing the protein extract was transferred to a fresh 2 mL tube for protein quantification using the Bradford method (Bradford, 1976) and the three antioxidant enzyme assays described below.

APX activity was measured as described by Nakano and Asada (1981). The reagents were added to a disposable cuvette in the following sequence: 1 mL 150 mM potassium phosphate buffer (pH 7.0), 1 mL 1.5 mM ascorbate (prepared fresh), 0.4 mL 0.75 mM EDTA, 0.5 mL 6 mM H_2O_2 (prepared fresh) and 0.1 mL protein extract. The cuvette was inverted to mix before measuring the absorbance at 290 nm for 1 min, with

Millipore water used as the blank. APX activity was calculated using the following formula: APX activity (units/mg protein) = ΔA_{290} × 3 (total assay volume in mL) × 40 (dilution factor) ÷ 2.8 (absorbance coefficient, mM^{-1} cm^{-1}) ÷ 1 (min) ÷ protein content (mg), where 1 unit is defined as 1 µmol of ascorbate consumed per minute.

CAT activity was measured as described by Kato and Shimizu (1987). The reagents were added to a quartz cuvette in the following sequence: 2.7 mL 100 mM sodium phosphate buffer (pH 7.0), 0.1 mL 1 M H_2O_2 (prepared fresh) and 0.2 mL protein extract. The cuvette was inverted to mix before measuring the absorbance at 240 nm for 1 min, with Millipore water was used as the blank. CAT activity was calculated using the following formula: CAT activity (units/mg protein) = ΔA_{240} × 3 (total assay volume in mL) × 20 (dilution factor) ÷ 40 (extinction coefficient, mM^{-1} cm^{-1}) ÷ 1 (min) ÷ protein content (mg), where 1 unit is defined as 1 nmol H_2O_2 consumed per minute.

GR activity was measured as described by Foster and Hess (1980). The reagents were added to a disposable cuvette in the following sequence: 1 mL 150 mM Tris-HCl buffer (pH 7.5), 0.3 mL 30 mM $MgCl_2$, 0.5 mL 3 mM GSSG (prepared fresh), 1 mL 0.45 mM NADPH (prepared fresh) and 0.2 mL protein extract. The cuvette was inverted to mix before measuring the absorbance at 340 nm for 1 min, with Millipore water was used as the blank. GR activity was calculated using the following formula: GR activity (units/mg protein) = ΔA_{340} × 40 (dilution factor) ÷ 1 (min) ÷ protein content (mg), where 1 unit is defined as 1 nmol NADPH oxidized per minute.

The final value for each sample was the mean of four biological replicates each comprising three technical replicates.

Statistical analysis

Statistical analysis was carried out using the lsmeans, multcomp and ggplot2 packages in R (Hothorn, Bretz & Westfall, 2008; Lenth, 2016; Wickham, 2016). We carried out two-way or three-way analysis of variance (ANOVA) as appropriate, followed by Tukey's post hoc test with different letters denoting a significant difference at $p < 0.05$.

Results

Zn deficiency and excess delay the growth of rice seedlings

To determine the physiological changes in rice seedlings under Zn stress, we exposed seedlings to three different Zn treatments: Zn deficient ([Zn] = 0.002 µM), normal Zn ([Zn] = 0.2 µM) and excess Zn ([Zn] = 300 µM). We recorded the responses at 3, 14, and 21 DAT (Figure 1). We observed no morphological changes at 3 DAT but root and shoot growth was delayed under both Zn deprivation and excess, resulting in shorter plants at 14 and 21 DAT (Figure 1A; Figure S1A). The roots were first to sense the Zn status, with the length, FW and DW progressively affected by the duration of treatment (Figure 1B, C; Figure S1B). A similar trend was observed for shoot height, FW and DW (Figure 1D, E; Figure S1C).

The Zn-deficient plants were also characterized by shorter leaf blades than controls, whereas the plants exposed to excess Zn displayed interveinal chlorosis near the leaf tip. The treatments had opposing effects on total chlorophyll levels at 14 DAT, with the Zn-deficient plants accumulating chlorophyll but excess Zn causing the loss of chlorophyll compared to control plants (Figure 1F). However, continued Zn starvation also resulted in the depletion of chlorophyll so that by 21 DAT both the Zn-deprived plants and those exposed to excess Zn had lower total chlorophyll levels than the control plants. This result was corroborated by the SPAD analysis and individual levels of chlorophylls *a* and *b* (Figure S1D-F).

To determine whether the Zn treatments affected Zn uptake and accumulation, we also measured the concentration of Zn in the roots and shoots. In plants exposed to excess Zn, the metal began to accumulate in both the roots and shoots by 3 DAT, and increased further with prolonged treatment at 14 and 21 DAT (Figure

1G, H). Even at 3 DAT, the Zn concentration in the shoots was well above the 400 mg/kg DW previously reported as critical toxicity (Silva *et al* ., 2014). On the other hand, Zn deficiency did not significantly reduce Zn concentrations in the roots or shoots (Figure 1G, H) but there was a slight decline, enough for the concentration in the shoots of Zn-deprived plants to fall below the previously reported 15 mg/kg DW critical deficiency concentration (Shanmugam, Tsednee & Yeh, 2012) at 14 and 21 DAT (Figure 1G). The observed physiological changes are therefore likely to reflect the intracellular Zn concentrations caused by Zn deprivation and excess, respectively.

RNA-Seq analysis reveals DEGs involved in metal ion homeostasis as a response to Zn deficiency or Zn toxicity

The Zn levels in the shoots and roots suggested that regulatory mechanisms are activated in response to Zn stress at 3 DAT, in agreement with several transcriptome-level studies of nutritional deficiency (Yang, Lin & Schmidt, 2010; Rodríguez-Celma *et al* ., 2013; Zhang*et al* ., 2017). We therefore collected root and shoot tissues at 3 DAT from the three different Zn treatment groups for RNA-Seq analysis. The mapped reads data for all 18 samples are shown in Table S2. The total reads per sample ranged from 37 million to 58 million. We mapped an average of 40,333,876 reads per sample (93.7%) to the reference genome, 38,946,803 of which (90.5%) mapped to a single genomic locus and were defined as uniquely mapped reads. The high percentage of uniquely mapped reads confirmed the quality of the samples.

PCA was carried out to evaluate variation among the samples (Figure S2). All 18 samples passed this assessment because the three biological replicates from each treatment were clustered together due to their relatively small variation. The first principal component (PC1, explaining 22.2% of the variance) separated shoot from root samples, indicating that the two tissues were characterized by distinct transcriptomic profiles. The second principal component (PC2, explaining 7.8% of the variance) separated root samples from different Zn treatment groups, indicating that the root transcriptome is more strongly affected by Zn levels than the shoot.

DEGs were compared in six groups (Table 1). The first four groups compared Zn deficiency (-Zn) or excess Zn (+Zn) with normal Zn (NZn) in roots (R) or shoots (S): -Zn/NZn_R, -Zn/NZn_S, +Zn/NZn_R and +Zn/NZn_S. In agreement with PC2, roots from the +Zn/NZn comparison showed a higher number of DEGs (820) than the corresponding shoots (163 DEGs). However, the -Zn/NZn comparison yielded similar numbers of DEGs in each tissue: 188 in roots, 186 in shoots (Table 1). The remaining two groups compared -Zn and +Zn in roots and shoots: -Zn/+Zn_R and -Zn/+Zn_S. This comparison was required to identify Zn-responsive genes that might fail to meet the $|\log_2$ fold change$|$ [?] 1 threshold in comparison with normal Zn conditions. For comprehensive analysis, we investigated 723 DEGs from the -Zn/+Zn_R comparison and 287 from the -Zn/+Zn_S comparison in more detail (Table 1; Figure 2A).

GO enrichment analysis of the -Zn/+Zn DEGs (Figure 2A) revealed several important biological processes affected by Zn treatments: (i) ion and oligopeptide transport, (ii) protection against ROS, (iii) primary metabolism (fatty acids, polysaccharides and amines), and (iv) secondary metabolism linked to defense responses (Figure 2B). The cellular component annotation showed that 197 DEGs were associated with cytoplasmic membrane-bound vesicles (Figure 2B). Interestingly, the molecular function annotation revealed DEGs encoding proteins that bind Fe, Ca and Mg ions (Figure 2B; Table S3A, B, C). These proteins were notable because they were also overrepresented in the biological processes related to ROS, secondary metabolism and defense, which are pertinent to Zn deficiency and Zn toxicity responses (Table S3D, E, F).

The expression of *OsZIP* genes is induced by Zn deficiency but suppressed by excess Zn

F-bZIP transcription factors that mediate Zn-deficiency responses in Arabidopsis are conserved in rice (Lilay *et al* ., 2020a). To enhance Zn uptake and transport under Zn-deficiency conditions, the rice F-bZIP proteins may induce the expression of *OsZIP* transporter genes by binding to the ZDRE motifs (RTGTCGACAY) in the corresponding promoters (Lilay *et al* ., 2020a). We therefore analyzed the expression profiles of *OsZIP*

and F-*OsbZIP* genes by RT-qPCR to confirm the RNA-Seq data at 3, 14 and 21 DAT (Figure 3). Among the 16 *OsZIP* genes in the RNA-Seq dataset, five (*OsZIP4* ,*OsZIP5* , *OsZIP7* , *OsZIP8* and *OsZIP9*) were upregulated by Zn deprivation but downregulated by excess Zn at 3 DAT (Figure 3A). The expression of *OsZIP4* , *OsZIP5* ,*OsZIP7* and *OsZIP8* at 3 DAT was validated by RT-qRCR, and consistent patterns were observed at 14 and 21 DAT (Figure 3C). However, the expression of their upstream regulatory genes (*OsbZIP48* ,*OsbZIP49* and *OsbZIP50*) was not significantly altered by either Zn deprivation or excess at 3 DAT (Figure 3B). RT-qPCR confirmed the unchanged level of *OsbZIP48* and *OsbZIP50* expression at 3 DAT, whereas *OsbZIP49* expression in shoots was induced by Zn deprivation but suppressed by excess Zn (Figure 3D). At 14 and 21 DAT, the expression of these *OsbZIP* genes was generally repressed by both Zn treatments, although *OsbZIP49* was induced in the shoots of Zn-deficient plants at 21 DAT (Figure 3D).

Divalent nutrient cations in shoots gradually accumulate in response to Zn deficiency

Transcriptome profiling also revealed changes in the expression of genes involved in the homeostatic regulation of other nutrient cations. We identified 17 DEGs encoding ion transporters or homeostatic proteins at 3 DAT (Figure 2B, 4A; Table S3G). This was substantiated by the MapMan transport annotation of root DEGs (Figure 4B; Table S4). For example, the expression of Na^+ and K^+transporter genes (Os04g0607600 and Os06g0701700) was induced in the roots of Zn-deficient plants at 3 DAT (Figure 4A; Table S3G). Conversely, genes encoding Mn^{2+} (*OsNRAMP1* ; Os07g0258400) and phosphate (Os08g0564000) transporters, an iron-related bHLH transcription factor 2 (Os01g0952800), and a Ca^{2+}/calmodulin domain containing protein (Os07g0633400) were downregulated in the roots of Zn-deficient plants at 3 DAT (Figure 4A; Table S3G, S4). In shoots, Zn deprivation induced the expression of a Na^+/H^+ transporter gene (Os05g0382200) but suppressed a Cu^{2+} transporter gene (Os01g0770700) compared to plants exposed to excess Zn (Figure 4A; Table S3G). Overall, these data show that many genes involved in Ca, Na, K, Mn, Fe and P nutrient transport were modulated in response to Zn stress.

Metals ions in plant tissues form complexes with ligands such as amino acids, oligopeptides and organic acids, for internal transport and storage (Verbruggen, Hermans & Schat, 2009). In addition to metal transporters, MapMan analysis also identified several DEGs encoding oligopeptide and amino acid transporters (Figure 4B; Table S4). Most were upregulated by excess Zn, including genes encoding proton-dependent oligopeptide transport (POT) family proteins (Os01g0871600, Os10g0109900, Os10g0148400 and Os01g0871500), an amino acid transporter (Os04g0201800), and oligopeptide transporters of the YELLOW STRIPE LIKE (YSL) family (Os02g0650300 and Os02g0649900). Genes required for the biosynthesis of these ligands were also affected: for example, the *OsNAS1* gene encoding nicotianamine synthase 1 (Os03g0307300) and *OsNAS2* (Os03g0307200) were upregulated in roots by excess Zn at 3 DAT but *OsNAS3* (Os07g0689600) was downregulated (Figure 4A; Table S3G). *OsNAS3* is more likely to be induced by Zn deprivation so its expression was validated by RT-qPCR along with the *OsNAAT1* gene encoding nicotianamine aminotransferase because both are involved in the synthesis of mugineic acid phytosiderophores (Suzuki *et al* ., 2006). *OsNAS3* and *OsNAAT1* were generally induced by Zn deficiency but repressed by excess Zn at all three time points (Figure 4C). The differential expression of genes responsible for ligand biosynthesis and metal-ligand transport is therefore likely to affect the availability and accumulation of metal nutrients in rice plants.

To confirm that Zn treatments influence the homeostasis of other nutrient ions in rice, we determined the concentrations of relevant macronutrients (P, Na, Ca, K and Mg) and micronutrients (Mn, Fe and Cu) in the roots and shoots of rice plants under different Zn treatments at 3, 14 and 21 DAT (Figure 4D; Figure S3). In the roots, only Mn, P and Na were affected by Zn treatments (Figure S3A, D, E) but all the tested nutrients except Na were affected in the shoots (Figure 4D). The concentration changes in shoots allowed us to assign the nutrients to four groups: (1) Fe, Cu and Ca only accumulated in response to Zn deficiency at 21 DAT; (2) Mn and Mg accumulated in response to Zn deficiency but were depleted by excess Zn at 14 and 21 DAT; (3) K was depleted by Zn deficiency but increased in response to excess Zn at 14 DAT; and (4) P accumulated in response to both treatments at 21 DAT (Figure 4D). The translocation rate of these

nutrients supported the observed concentration changes in the shoots (Figure S4). For example, in the first group, the enhanced translocation of Fe and Ca due to Zn deficiency at 21 DAT contributed to their elevated shoot concentrations (Figure S4B, F). Similarly, for the second group, the translocation rates of Mn and Mg mirrored their shoot concentrations at 21 DAT, when both increased in response to Zn deficiency but were depleted in the presence of excess Zn (Figure S4A, H). We evaluated the crosstalk between Fe, Ca, Mg and Zn in more detail because GO enrichment identified DEGs encoding proteins that bind to these divalent cations (Figure 2B).

The transcription of genes encoding Fe/Ca-binding peroxidases and the activity of APX in rice leaves are induced by Zn deficiency

Based on GO molecular function enrichment analysis, 44 DEGs encoding Fe-binding proteins and 22 encoding Ca-binding proteins were identified in the -Zn/+Zn comparison at 3 DAT (Figure 2B). To visualize the response to Zn treatments, we generated heat maps showing the fold change in expression (Figure 5A; Figure S5). Interestingly, 14 of the 44 genes encoding Fe-binding proteins (Figure 5A) and 13 of the 22 genes encoding Ca-binding proteins (Figure S5) were peroxidase genes. The 13 Ca-binding peroxidases completely overlapped with the 14 Fe-binding peroxidases (Table S3A, B) and were coordinately regulated in response to Zn stress (Figure 5A; Figure S5). Given that Zn deficiency markedly increased the shoot concentrations of Fe and Ca at 21 DAT, we hypothesized that some of the peroxidase genes were induced in rice shoots that accumulate modest levels of Fe and Ca during the early stage of Zn deficiency (Figure 4D). In the shoots, Zn deficiency caused modest increases in Fe levels at 3 and 14 DAT, and in Ca levels at 14 DAT (Figure 4D).

The presence of peroxidases indicated the potential involvement of ROS detoxification during Zn deficiency, so we tested leaf tissues from the three treatment groups for the presence of ROS and antioxidant enzyme activity after 3, 14 and 21 days (Figure 5B, C, D; Figure S6). At 3 DAT, DAB staining showed that H_2O_2 had not accumulated in the leaves of Zn-deficient plants or those exposed to excess Zn (Figure S6A), but more $H_2O_2$was present in the leaves exposed to excess Zn at 14 and 21 DAT (Figure 5B; Figure S6C). NBT staining to detect O_2^- yielded comparable results (Figure 5C; Figure S6B, D). The elimination of ROS involves enzymes such as APX, CAT and GR, which reduce oxidative stress (Cakmak, 2000). CAT and GR activity in shoots was generally reduced by excess Zn at 14 and 21 DAT, but there was no effect on the activity of APX (Figure 5D). Interestingly, although CAT activity in the shoots was reduced in response to Zn deficiency at 14 and 21 DAT, the opposite profile was observed for APX, which showed higher activity at these time points (Figure 5D).

In summary, these data show that early Zn deficiency leads to the modest accumulation of Fe and Ca in rice shoots, enhancing the expression of genes encoding Fe/Ca-binding proteins such as peroxidases to improve oxidative stress tolerance via the removal of H_2O_2.

Excess Zn in rice shoots induces the expression of genes encoding Mg-binding enzymes required for diterpenoid biosynthesis

Finally, we investigated the importance of crosstalk between Mg and Zn in rice plants by comparing Mg levels and Mg-related DEGs. In the presence of excess Zn, the concentration of Mg in rice shoots decreased slightly at 3 DAT, but more severely at 14 and 21 DAT (Figure 4D). GO molecular function enrichment analysis of the -Zn/+Z comparison revealed 12 DEGs encoding Mg-binding proteins at 3 DAT (Figure 2B). To visualize the response to Zn treatments, we generated heat maps showing the fold change in expression. This revealed that most of the genes were suppressed in the roots but induced in the shoots in response to both forms of Zn stress (Figure 6A). Eleven of the genes were related to terpene biosynthesis, eight for the synthesis of diterpenes (Os04g0179700, Os11g0474800, Os04g0178300, Os02g0570400, Os02g0571100, Os04g0612000, Os12g0491800 and Os02g0571300), two for sesquiterpenes (Os03g0362500 and Os08g0167800) and one for monoterpenes (Os04g0340300) (Table S3C). This suggests that the Zn-mediated changes in endogenous Mg levels alter the expression of genes encoding Mg-binding proteins, especially those encoding enzymes involved in terpene biosynthesis.

As an illustration, four genes in the diterpenoid pathway were concomitantly upregulated in shoots exposed

to excess Zn (Figure 6B; Table S3C). Two genes encoding upstream copalyl diphosphate synthases (*OsCPS2* and *OsCPS4*) were induced in shoots by excess Zn (Figure 6B). At the branching point of this pathway, the former enzyme catalyzes the conversion of geranylgeranyl diphosphate (GGPP) to *ent* -copalyl diphosphate (*ent* -CPP), and the latter enzyme catalyzes the formation of *syn* -CPP from the same substrate (Figure 6B). In the *ent* -CPP branch, the expression of a gene encoding *ent* -kaurene synthase-like enzyme 7 (*OsKSL7*) was upregulated by excess Zn (Figure 6B). OsKSL7 catalyzes the synthesis of precursors for phytocassanes A-E (Umemura *et al* ., 2003). In the *syn* -CPP branch, the *OsKSL4* gene was also induced by excess Zn (Figure 6B). OsKSL4 catalyzes the formation of precursors for momilactones A and B (Umemura *et al* ., 2003). Furthermore, the*OsKSL8* gene (responsible for the synthesis of a precursor for oryzalexin S) was modulated by excess Zn with a 0.86 $\log_2$fold change in expression (Figure 6B).

Taken together, our results show that excess Zn causes an early, slight decline in Mg levels in rice shoots, enhancing the expression of genes encoding Mg-binding proteins. In the diterpenoid biosynthesis pathway, these proteins are key enzymes responsible for the biosynthesis of two subtypes of important defense-related diterpenoids: momilactones A and B, and phytocassanes A-E.

Discussion

In this study, we compared the physiological, transcriptomic and biochemical responses of rice to normal Zn levels and two forms of Zn stress (Zn deprivation and excess) at 3, 14 and 21 DAT. The genes encoding Zn transporters *OsZIP4/5/7/8/9/10* were differentially expressed in response to Zn stress. The levels of Mn and Mg in rice shoots mirrored the expression of the *OsZIP* genes. The accumulation of Fe and Ca in response to Zn deficiency probably increase the activity of Fe/Ca-binding proteins related to ROS-mediated stress responses. Interestingly, the enhanced expression of genes encoding Mg-binding proteins in response excess Zn is related to the production of antifungal and other antimicrobial secondary metabolites.

The roles of F-bZIP transcription factors, ZIP transporters, nicotianamine and deoxymugineic acid in the response to Zn deficiency

Two F-bZIP proteins (AtbZIP19 and AtbZIP23) regulate the response to Zn deficiency in Arabidopsis by binding to ZDRE motifs in the promoters of Zn homeostatic genes such as those encoding ZIP transporters and nicotianamine synthase (Assuncao *et al* ., 2010; Assuncao *et al* ., 2013). Similar genetic modules are conserved in the response to Zn deficiency in barley, wheat and rice (Castro *et al* ., 2017; Evens*et al* ., 2017; Nazri *et al* ., 2017; Lilay *et al* ., 2020a). Recently, a complementation experiment showed that the rice F-bZIP transcription factor genes *OsbZIP48* and *OsbZIP50*(but not *OsbZIP49*) rescued hypersensitivity to Zn deficiency in the Arabidopsis double mutant *bzip19bzip23* (Lilay *et al* ., 2020a). In our study, the transcript profiles of *OsbZIP48* ,*OsbZIP49* and *OsbZIP50* in the roots and shoots of rice plants from different Zn treatment groups at 3, 14 and 21 DAT revealed that *OsbZIP48* and *OsbZIP50* were not induced by Zn deficiency at the three time points (Figure 3B, D). RNA-Seq analysis suggested that *OsbZIP49* was not affected by Zn deficiency at 3 DAT but RT-qPCR verification experiments showed that the gene was induced by Zn deficiency in the shoots at 3 and 21 DAT (Figure 3B, D). With the exception of *OsbZIP49* , our transcriptional data for*F-OsbZIP* genes generally agreed with Lilay *et al* . (2020a), who reported that the transcript levels of these three genes did not increase in response to Zn deficiency in rice roots or shoots at 21 DAT. In Arabidopsis, the expression of *AtbZIP19* and *AtbZIP23*was also not significantly increased by Zn deficiency (Assuncao *et al* ., 2010; Lilay *et al* ., 2019). Therefore, the regulation of these transcription factors may occur at the post-translational level (Assuncao *et al* ., 2013; Lilay *et al* ., 2019). In contrast, genes encoding the wheat F-TabZIP transcription factors (*TabZIPF1* , *TabZIPF3a* , *TabZIPF3b* and *TabZIPF4*) were induced in the roots and shoots of 2-week-old seedlings throughout a Zn deficiency treatment lasting 7 days (Evens*et al* ., 2017). The contrast between Arabidopsis and wheat suggests that F-bZIP proteins are regulated by different species-dependent mechanisms acting at the transcriptional, post-transcriptional or post-translational levels.

F-OsbZIP transcription factors are thought to regulate *OsZIP*genes to mediate adaptation to Zn deficiency (Assuncao *et al* ., 2010; Lilay *et al* ., 2020a). The rice ZIP transporter family, represented by 16 genes, is involved in the transport of various divalent cations, including Zn^{2+} (Ishimaru *et al* ., 2005; Tiong *et al* ., 2015). In this study, the expression of*OsZIP4/5/7/8/9* in roots and shoots was modulated in opposite directions by Zn deficiency (induction) and excess Zn (suppression) at the three time points (Figure 3A, C). This agrees with a previous study showing that *OsZIP4/5/8* were induced in rice roots and shoots by Zn deprivation (Suzuki *et al* ., 2012). These three *OsZIP*genes, expressed individually in yeast, were also able to complement a Zn-dependent growth mutant, confirming they encode functional Zn transporters (Ishimaru *et al* ., 2005; Yang *et al* ., 2009; Lee *et al* ., 2010a; Lee *et al* ., 2010b). *OsZIP7* was also induced in rice roots by Fe starvation and complemented a yeast mutant deficient for Fe uptake, confirming it encodes a functional Fe transporter (Yang *et al* ., 2009). However, another yeast complementation assay indicated that OsZIP7 is an influx transporter for Zn and cadmium (Cd) but not Fe (Tan *et al* ., 2019). OsZIP7 transports Zn and Cd upward in rice via xylem loading in roots and intervascular transfer in nodes (Tan *et al* ., 2019). Expression data suggest that OsZIP9 is involved in the regulation of Zn homeostasis in shoots (Shao, Xia, Yamaji, Shen & Ma, 2018), but two more recent studies provide strong evidence that OsZIP9 facilitates Zn uptake in rice roots during Zn deprivation (Huang *et al* ., 2020; Tan*et al* ., 2020). Besides Zn uptake, the synergistic action of OsZIP5 and OsZIP9 (encoded by duplicate genes) also modulates Cd uptake, with OsZIP9 taking on the prominent role (Tan *et al* ., 2020).

Nicotianamine is required for the chelation and transport of divalent cations, including Zn^{2+} (Higuchi, Kanazawa, Nishizawa, Chino & Mori, 1994). The synthesis of nicotianamine from S-adenosylmethionine is catalyzed by nicotianamine synthase (Higuchi*et al* ., 1994) encoded in rice by three paralogous genes: *OsNAS1* , *OsNAS2* and *OsNAS3* (Higuchi *et al* ., 2001). The expression of *OsNAS1* and *OsNAS2* in roots was induced by excess Zn at 3 DAT, whereas *OsNAS3* expression in roots was reduced by the same treatment (Figure 4A). A previous study showed that *OsNAS1* and *OsNAS2* were induced in rice roots and leaves in response to Fe deficiency, whereas *OsNAS3* was induced in roots but suppressed in leaves (Inoue *et al* ., 2003). In maize,*ZmNAS1* and *ZmNAS2* were induced in Fe-deficient roots, whereas *ZmNAS3* was suppressed (Mizuno *et al* ., 2003). Considering all these results together in the context of the well-known negative crosstalk between Fe and Zn (Cakmak, 2000),*OsNAS3* appears likely to be involved in the regulation of Zn homeostasis. Indeed, *OsNAS3* and *OsNAAT1* both encode enzymes required for the synthesis of mugineic acid phytosiderophores, and were induced by Zn deprivation but repressed by excess Zn in our experiments (Figure 4C).

Promiscuous Zn transporters and chelators increase the accumulation of multiple divalent cations in rice shoots under Zn deficiency conditions

In this study, the concentrations of Mn and Mg in shoots increased in response to Zn deficiency at 14 and 21 DAT, and the concentrations of Fe, Cu and Ca increased at 21 DAT (Figure 4D). The accumulation of these five nutrients in shoots reflected their translocation from roots under Zn deficiency conditions (Figure S4). To determine the molecular mechanisms underlying this finding, we monitored the dynamic expression profiles of genes annotated by GO enrichment analysis as “ion transport” and by MapMan as “transport” (Figure 4A, B; Table S3G; Table S4).

Both RNA-Seq and RT-qPCR data confirmed the upregulation of*OsZIP4/5/7/8/9* , *OsNAS3* and *OsNAAT1* in response to Zn deficiency at 3, 14 and 21 DAT (Figure 3A, C; Figure 4A, C).We did not observe the induction of any genes encoding Mn, Fe, Cu, Ca or Mg transporters in response to Zn deficiency at 3 DAT, but these genes were downregulated in the –Zn/+Zn comparison at 3 DAT (Table S3G; Table S4). Therefore, the translocation of nutrients to shoots and their accumulation in response to Zn deficiency may reflect the activity of OsZIP transporters and Zn chelators, including nicotianamine and deoxymugineic acid. Functional complementation in yeast mutants deficient for metal uptake demonstrated that ZIP transporters bind various divalent cations, including Zn^{2+}, Fe^{2+}, Mn^{2+} and Cd^{2+} (Guerinot, 2000). Among the transporters identified by our transcriptomic analysis, OsbZIP7 is a functional Fe transporter in rice because it complemented a yeast mutant deficient for Fe uptake (Yang *et al* ., 2009), and the expression of*OsZIP4* was

influenced by the availability of Zn^{2+}, Cu^{2+} and Mn^{2+} (Ishimaru *et al* ., 2005).

OsNAS1 and *OsNAS2* are induced by Fe starvation but *OsNAS3* was recently shown to be induced in various rice tissues in response to excess Fe (Aung *et al* ., 2019). Similarly, we observed the upregulation of *OsNAS3* in rice roots and shoots in response to Zn deficiency (Figure 4C). The accumulation of nicotianamine would increase the chelation and transport of various divalent cations, including Cu^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+}, $Fe^{2+}$and Mn^{2+} (Higuchi *et al* ., 1994; Lin & Aarts, 2012). Furthermore, nicotianamine is also the precursor of deoxymugineic acid (DMA), a mugineic acid phytosiderophore secreted from the roots to chelate Fe^{3+} in the rhizosphere for subsequent uptake (Suzuki *et al* ., 2006). We observed the significant upregulation of *OsNAAT1* expression in rice shoots in response to Zn deprivation (Figure 4C). YSL transporters interact with metal-phytosiderophore complexes to translocate Fe^{3+}, Cu^{2+} and Mn^{2+}, and this could also explain the accumulation of these nutrients in rice shoots under Zn deficiency conditions (Dai *et al* ., 2018) (Figure 4D).

In summary, to maximize Zn availability in a Zn-deficient rhizosphere, rice plants increase the expression of *OsZIP* genes, *OsNAS3*and *OsNAAT1* . However, the low availability of Zn in the rhizosphere and the promiscuity of OsZIP transporters and Zn chelators cause the accumulation of Mn, Fe, Cu, Ca and Mg, which are translocated to the rice shoots. These five nutrients follow two distinct trends with potential biological significance: (i) the accumulation of Fe, Cu and Ca in shoots increased significantly in response to Zn deficiency at 21 DAT; and (ii) Mn and Mg accumulated in response to Zn deficiency but were also depleted in the presence of excess Zn at 14 and 21 DAT (Figure 4D).

Modest Fe and Ca accumulation in shoots induces gene expression and APX activity to prevent leaf chlorosis caused by Zn deficiency

Our results showed that the expression of genes encoding Fe/Ca-binding peroxidases was induced in rice shoots during the early phase of Zn deficiency (Figure 4D; Figure 5A; Figure S3B, F). APX activity in the shoots increased in response to Zn deficiency at 14 and 21 DAT, mirroring the accumulation of H_2O_2 in leaves (Figure 5B, D). Changes in intracellular Zn and Fe levels in plants disrupt the balance between ROS production and elimination (Wang & Jin, 2007; Tripathi *et al* ., 2018). Zn deficiency (below 15 mg/kg DW) and Fe toxicity (400-1000 mg/kg DW) can both increase ROS production in plants (Vose, 1982; Shanmugam *et al* ., 2012). In addition to their individual effects, the crosstalk between Zn and Fe also affects ROS levels and the antioxidant system (Shanmugam *et al* ., 2012). The increase in Fe levels we observed under Zn deficiency conditions (Figure 4D) suggests that the crosstalk triggers further ROS production. An optimal level of Zn is required for the activity of antioxidant enzymes with Zn cofactors, which prevent the accumulation of excess ROS (Westin & Schaffner, 1988; Miller, 2012). Zn deficiency also triggers the ROS accumulation via the membrane-bound O_2^--generating NADPH oxidase (Cakmak & Marschner, 1988). The accumulation of Fe in plants increases the generation of ROS through diverse mechanisms (Shahid *et al* ., 2014). Notably, in the Fenton reaction, Fe^{2+} reduces H_2O_2 to the highly reactive hydroxyl free radical OH^* (Mittler, 2017).

Excessive ROS production is responsible for the typical phenotypes of Zn-deficient plants, such as leaf chlorosis and delayed growth (Wang & Jin, 2007; Shinozaki *et al* ., 2020) and we observed the same phenotypes in our study (Figure 1A, Figure S1). A recent model suggests that OH^* produced via the Fe-dependent Fenton reaction is the cause of Zn-deficient leaf chlorosis (Shinozaki *et al* ., 2020). More importantly, the study showed the importance of autophagy, conferred by a small number of autophagy-related proteins, in salvaging limited Zn reserves by degrading particular target proteins and organelles. The salvaged Zn supply is then redistributed to important Zn-binding enzymes such as Cu/Zn SOD in the chloroplasts. Following the redistribution of autophagy-derived Zn, the enzymatic activity of Cu/Zn SOD is restored and the chloroplast is protected from damage (Shinozaki*et al* ., 2020). Our results fit this model because APX scavenges H_2O_2, which is produced when Cu/Zn SOD catalyzes the dismutation of O_2^-(Figure 5B, D). Otherwise, the accumulation of H_2O_2 causes the inhibition and degradation of Cu/Zn SOD (Casano, Gomez, Lascano, Gonzalez & Trippi, 1997) and leads to the accumulation of OH^* via the Fenton reaction (Mittler, 2017).

Plants express a number of antioxidant enzymes to remove ROS (SOD, APX, CAT and GR) as well as producing antioxidant metabolites such as ascorbic acid, glutathione and phenolic compounds (Kasote, Katyare, Hegde & Bae, 2015). Three families of SOD are recognized based on their metal cofactors: Cu/Zn, Fe and Mn (Miller, 2012). The Cu/Zn SOD family is predominant in higher plants and members are found in the chloroplast and cytosol (Pilon, Ravet & Tapken, 2011). As expected, given that Zn is one of the cofactors of Cu/Zn SOD, Zn deficiency reduces the activity of this enzyme (Cakmak & Marschner, 1993; Wang & Jin, 2007). The activities of enzymes that scavenge H_2O_2(APX, CAT and GR) are also inhibited by Zn deficiency (Cakmak, 2000). However, higher activities of these antioxidant enzymes have been reported in Zn-efficient wheat and rice plants under Zn deficiency conditions (Cakmak *et al* ., 1997; Begum *et al* ., 2016). In the current study, we found that APX activity in shoots increased in response to Zn deficiency at 14 and 21 DAT (Figure 5D), in agreement with the accumulation of H_2O_2 in the leaves of Zn-deficient plants at 14 and 21 DAT (Figure 5B; Figure S6C).

The induction of genes encoding Fe/Ca-binding peroxidases after 3 days of Zn deprivation suggests that the higher APX activity we observed may reflect the increased abundance of these enzymes (Figure 5A, D). APX uses Fe as a cofactor, so the accumulation of this enzyme would sequester free Fe ions as well as scavenging more H_2O_2 (Santos *et al* ., 2019). These dual functions may have led to the evolutionary selection of APX as a response to Zn deficiency. This might also explain the opposite effects of Zn deficiency and excess on the total chlorophyll content of rice leaves at 14 DAT, with the content increasing in response to Zn deficiency (Figure 1F). In summary, rice plants starved of Zn begin to accumulate Fe and Ca in the shoots at 3 DAT, leading to the induction of peroxidase genes. This increases the activity of APX in the shoots at 14 and 21 DAT, helping to prevent Zn-deficient leaf chlorosis. However, if the Fe concentration in the shoot increases above the 400-1000 mg/kg DW threshold of critical toxicity during prolonged (e.g., 21 DAT) or severe Zn deficiency stress (Figure 4D), then the response becomes insufficient and chlorosis ensues.

The modest depletion of Mg in shoots exposed to excess Zn induces defense-related genes associated with diterpenoid biosynthesis

The growth hormone GA and defense-related phytoalexins and phytoanticipins are derived from the diterpenoid pathway, so the coordinated control of this pathway is needed for the efficient allocation of metabolic resources to growth or defense (Lu *et al* ., 2018). The pathway branches when GGPP is converted into one of the two stereoisomers *ent* -CPP and *syn* -CPP, with GA derived from the former and each branch also giving rise to different phytoalexins/phytoanticipins (Figure 6B). Although GA and defense-related diterpenoids are produced via the *ent* -CPP route, the rice genome encodes two *ent* -CPP synthases: *OsCPS1* (for GA) and *OsCPS2* (for defense-related diterpenoids). These allow the coordinated modulation of growth and defense in rice plants (Hayashi*et al* ., 2008). Intriguingly, our results suggested that an early response to excess Zn is a slight depletion of intracellular Mg levels in shoots, which induces the expression of five genes encoding Mg-binding proteins (Figure 4D; Table S3C). Four of these genes (*OsCPS4* and *OsKSL4* from the *syn* -CPP branch;*OsCPS2* and *OsKSL7* from the *ent* -CPP branch) encode enzymes that synthesize important defense-related diterpenoids: momilactones A and B, and phytocassanes A-E (Figure 6B) (Umemura*et al* ., 2003). Given that genes required for GA synthesis (*OsCPS1* and *OsKS1*) were not differentially expressed, we deduce that defense is prioritized in the presence of excess Zn at 3 DAT (Figure 6B).

When exposed to microbial infection, rice plants allocate metabolic resources to the synthesis of diterpenoid phytoalexins, including momilactones A and B, oryzalexins A-F, oryzalexin S and phytocassanes A-E, by inducing the corresponding genes (Lu *et al* ., 2018). For example, Shimizu *et al* . (2008) reported that the *OsCPS4* ,*OsKSL4* , *OsCPS2* and *OsKSL7* were induced within 12 h following exposure to a fungal elicitor, leading to the accumulation of momilactones and phytocassanes at 12-48 h. Furthermore, these genes were induced more rapidly when a variety resistant to the rice blast fungus*Magnaporthe oryzae* was inoculated with the pathogen, resulting in the earlier and stronger accumulation of momilactones A and B, and phytocassanes A-E, in resistant versus susceptible rice leaves (Hasegawa*et al* ., 2010). Momilactones and phytocassanes also accumulated in rice leaves following infection with the bacterial leaf blight pathogen,*Xanthomonas oryzae* (Klein *et al* ., 2015). However, given that the knockdown of *OsCPS4* and the overexpression of*OsCPS2*

reduces susceptibility to *X. oryzae* infection, phytocassanes are likely to be the most effective phytoalexins against this pathogen (Lu *et al* ., 2018). *OsCPS4* (or the*syn* -CPP route) also confers resistance to fungal non-host disease in rice (Lu *et al* ., 2018). Therefore, our findings could facilitate research aiming to improve the resistance of rice against microbial infection by Zn fortification.

Conclusion

This study has demonstrated the important relationship between Zn and other divalent nutrient cations (Fe, Cu, Ca, Mn and Mg) during Zn stress in rice (Figure 7). Zn deficiency causes these divalent cations to be translocated to rice shoots due to the increased activity of promiscuous Zn transporters and chelators. The accumulation of these cations during prolonged Zn deficiency is harmful to rice plants, especially Fe. Therefore, over time, rice plants have evolved to interpret modest changes in cation levels as important signals of early Zn stress. Early detection allows rice plants to respond immediately by reprogramming their transcriptional and metabolic processes. In certain cases, such readjustments serve both to reduce the intracellular levels of cations and mediate important Zn stress responses. Rice plants have achieved this delicate modulation by naturally selecting for the functional clustering of enzymes with the same cofactor. For example, the early detection of increasing Fe and Ca levels in shoots activates the transcription of genes encoding Fe/Ca-binding peroxidases, ultimately promoting the synthesis and activity of APX in the shoots. APX provides a good example of the dual-function metabolic adjustment mentioned above, because the increased availability of this enzyme sequesters Fe to prevent Fe-induced toxicity in the leaves while scavenging H_2O_2 more efficiently during periods of Zn deprivation to prevent leaf chlorosis. Similarly, the modest depletion of Mg in the shoots in response to excess Zn may act as a signal to activate defense-related diterpenoid biosynthesis pathways.

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Author Contributions

YL conceived and designed the research project. YC, JL, IT conducted the experiments. BC and YL contributed to data interpretation. BC, YC, KY and YL contributed to manuscript preparation. All authors have read and approved the final version of the manuscript.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Table 1 The number of differentially expressed genes (DEGs) in each comparison group: Zn deficiency (-Zn), normal Zn (NZn) and excess Zn (+Zn). R = root and S = shoot.

Comparison	Total DEGs^{+}	Upregulation	Downregulation
-Zn/NZn_R	188	116	72
-Zn/NZn_S	186	106	80
+Zn/NZn_R	820	182	638
+Zn/NZn_S	163	74	89
-Zn/+Zn_R	723	541	182
-Zn/+Zn_S	287	170	117

$^{+}$ A gene was considered as a DEG if |$\log_2$ fold change| between two sample groups was [?] 1 (adjusted p-value < 0.05).

Figure Legends

Figure 1 Rice physiology in response to Zn deficiency and excess at 3, 14 and 21 DAT. (A) Morphological comparison of shoots and roots of rice plants under -Zn, NZn and +Zn treatments at 3, 14 and 21 DAT (scale bar = 10 cm). Bar charts show the quantitative measurements of root length **(B)** , root dry weight **(C)** , plant height **(D)** , shoot dry weight **(E)** , total chlorophyll content **(F)** , shoot Zn concentration **(G)**and root Zn concentration **(H)** . Zn deficiency (-Zn) is represented by white bars, normal Zn (NZn) by gray bars and excess Zn (+Zn) by black bars. Zn concentration is indicated as mg per kg plant dry weight (DW). Two-way ANOVA and Tukey's post hoc test were used for all measurements, with different letters denoting a significant difference ($p < 0.05$).

Figure 2 An overview of RNA-Seq data comparing gene expression in rice seedlings in response to Zn deficiency and excess at 3 DAT. (A) The number of DEGs between Zn treatments is shown for roots and shoots.**(B)** GO enrichment analysis of DEGs in three groups: biological process (BP), cellular component (CC) and molecular function (MF). Enrichment significance is shown by the length of each bar, and gene numbers for each enriched GO term are indicated by the color intensity of the bars. A gene was considered to be differentially expressed if |$\log_2$ fold change| between -Zn and +Zn samples was [?] 1 (adjusted p-value < 0.05). -Zn = Zn deficient treatment and +Zn = excess Zn treatment.

Figure 3 Effects of Zn deficiency and excess on the expression of *F-OsbZIP* transcription factor and *OsZIP*transporter genes. RNA-Seq data showing fold changes in the expression of **(A)** 16 *OsZIP* genes, and **(B)** *OsbZIP48* ,*OsbZIP49* and *OsbZIP50* in the -Zn/NZn and +Zn/NZn comparisons for roots and shoots at 3 DAT. RT-qPCR data showing fold changes in the expression of **(C)** *OsZIP4* , *OsZIP5* ,*OsZIP7* and *OsZIP8* , and **(D)** *OsbZIP48* ,*OsbZIP49* and *OsbZIP50* genes at 3, 14 and 21 DAT. Upregulated genes are shown in red and downregulated genes in blue. In the sample comparison, R = root and S = shoot. For the RT-qPCR results, values for Livak fold difference are the mean (error bars indicate SD) of four biological replicates with *OsUbi1* as the housekeeping reference gene. Zn deficiency (-Zn) is represented by white bars, normal Zn (NZn) by gray bars and excess Zn (+Zn) by black bars. *bZIP* = gene encoding basic leucine zipper transcription factor, *ZIP* = gene encoding zinc-regulated transporter, iron-regulated transporter-like protein. Three-way ANOVA and Tukey's post hoc test were used for all measurements, with different letters denoting a significant difference ($p < 0.05$).

Figure 4 Effects of Zn deficiency and excess on the expression of nutrient transport-related genes and shoot nutrient concentrations.(A) RNA-Seq data showing fold changes in the expression of 17 GO-enriched ion transport genes in the -Zn/NZn and +Zn/NZn comparisons for roots and shoots at 3 DAT. **(B)** MapMan transport annotation results showing the expression change patterns of nutrient transport-related DEGs (-Zn/+Zn comparison) in the roots at 3 DAT.**(C)** RT-qPCR data showing fold changes in the expression of the mugineic acid phytosiderophore biosynthesis-related genes *OsNAS3*and *OsNAAT1* in the roots and shoots of rice plants under -Zn, NZn and +Zn conditions at 3, 14 and 21

DAT. **(D)** Shoot nutrient concentrations of rice plants under -Zn, NZn and +Zn conditions at 3,14 and 21 DAT. Upregulated genes are shown in red and downregulated genes in blue. In the sample comparison, R = root and S = shoot. For RT-qPCR results, values for Livak fold difference are the mean (error bars indicate SD) of four biological replicates with *OsUbi1* as the housekeeping reference gene. Zn deficiency (-Zn) is represented by white bars, normal Zn (NZn) by gray bars and excess Zn (+Zn) by black bars.*NAS* = nicotianamine synthase gene and *NAAT* = nicotianamine aminotransferase gene. Two-way ANOVA (for elemental analysis)/Three-way ANOVA (for RT-qPCR) and Tukey's post hoc test were used for all measurements, with different letters denoting a significant difference ($p < 0.05$).

Figure 5 Effects of Zn deficiency and excess on the expression of peroxidase genes, ROS formation and activities of H_2O_2-scavenging enzymes. (A)RNA-Seq data showing fold changes in the expression of 44 genes encoding GO-enriched iron-binding proteins in the -Zn/NZn and +Zn/NZn comparisons for roots and shoots at 3 DAT. DAB **(B)** and NBT **(C)**staining show the accumulation of $H_2O_2$and O_2^- respectively in leaves of rice plants under -Zn, NZn and +Zn conditions at 14 DAT (scale bar = 5 mm).**(D)** Enzymatic activities (units per mg protein) of APX, CAT and GR in shoots of rice plants under -Zn, NZn and +Zn conditions at 3, 14 and 21 DAT. Upregulated genes are shown in red and downregulated genes in blue. In the sample comparison, R = root and S = shoot. For enzymatic activity, Zn deficiency (-Zn) is represented by white bars, normal Zn (NZn) by gray bars and excess Zn (+Zn) by black bars. APX = ascorbate peroxidase, CAT = catalase, GR = glutathione reductase. Two-way ANOVA and Tukey's post hoc test were used for all measurements, with different letters denoting a significant difference ($p < 0.05$).

Figure 6 Effects of Zn deficiency and excess on the expression of genes encoding enzymes that synthesize defense-related diterpenoid compounds. (A) RNA-Seq data showing fold changes in the expression of 12 genes encoding GO-enriched magnesium-binding proteins in the -Zn/NZn and +Zn/NZn comparisons for roots and shoots at 3 DAT. Upregulated genes are shown in red and downregulated genes in blue. In the sample comparison, R = root and S = shoot. **(B)** The expression of five genes encoding enzymes that synthesize defense-related diterpenoid compounds may be enhanced by excess Zn. DEGs enhanced (*OsCPS2/4* and *OsKSL4/7/8*) or repressed (*OsKSL5*) by excess Zn are shown as red/pink or blue boxes, respectively, whereas constitutive genes (*OsCPS1* and*OsKSL1/2/10*) are shown as gray boxes. Stronger expression is represented by deeper color. GGPP = geranylgeranyl diphosphate, CPP = copalyl diphosphate, GA = gibberellin, *CPS* = copalyl diphosphate synthase gene, *KS* = kaurene synthase gene, *KSL* = kaurene synthase-like gene.

Figure 7 Model of rice response to Zn stress. When rice is starved of Zn, a range of divalent cations are translocated to the shoots due to the increased expression of promiscuous Zn transporters and chelators. The accumulation of these cations during prolonged Zn deficiency is harmful to rice plants, which have therefore evolved mechanisms to prevent cation accumulation and mediate important Zn deficiency responses. This is achieved by the functional clustering of enzymes with the same metal cofactor. For example, rice plants detect a slight increase in shoot Fe and Ca levels at the onset of Zn deficiency and induce genes encoding Fe/Ca-binding peroxidases (3 DAT). This increases the abundance and activity of ascorbate peroxidase (APX) in leaves at 14 and 21 DAT. APX requires Fe as a cofactor, so the greater abundance of APX sequesters free Fe (thus helping to prevent chlorosis, which in Zn-deficient leaves is triggered by excess H_2O_2 being converted into OH^* via the Fe-dependent Fenton reaction). The increase in APX activity also scavenges more H_2O_2 thus reducing the likelihood of chlorosis via two mechanisms. In response to excess Zn, genes encoding Mg-binding proteins are induced in rice shoots at 3 DAT, presumably due to the detection of falling intracellular Mg levels. These Mg-binding proteins include enzymes in the diterpenoid biosynthesis pathway, potentially leading to the accumulation of diterpenoid secondary metabolites such as oryzalexin S, momilactones A and B, and phytocassanes A-E, which kill fungal and bacterial pathogens. Mehler reaction is depicted by purple arrows and Fenton reaction by light blue arrows. -Zn = Zn deficiency, NZN = normal Zn, +Zn = excess Zn, GGPP = geranylgeranyl diphosphate, CPP = copalyl diphosphate, *CPS* = copalyl diphosphate synthase gene, *KSL* = kaurene synthase-like gene.

Supplementary Figure Legends

Figure S1 Rice physiology in response to Zn deficiency and excess at 3, 14 and 21 DAT. (A) Morphological comparison,**(B)** root fresh weight, **(C)** shoot fresh weight, and**(D)** SPAD (relative chlorophyll content) of rice plants under -Zn, NZn and +Zn conditions at 3, 14 and 21 DAT (scale bar=10cm).**(E)** Chlorophyll *a* and **(F)** chlorophyll *b*content of rice plants at 14 and 21 DAT. Two-way ANOVA and Tukey's post hoc test were used for all measurements, with different letters denoting a significant difference ($P< 0.05$). Zn deficiency (-Zn) is represented by white bars, normal Zn (NZn) by gray bars and excess Zn (+Zn) by black bars.

Figure S2 Principal component analysis scatter plot showing the relatively small variation between three biological replicates and relatively large variation between samples of different tissues and Zn treatments. -Zn = Zn deficiency, NZn = normal Zn control, +Zn = excess Zn, R = root and S = shoot. The numbers represent different biological replicates.

Figure S3 Root nutrient concentrations in rice plants under -Zn, NZn and +Zn conditions at 3, 14 and 21 DAT. Bar charts show concentration of the micronutrients **(A)** Mn, **(B)** Fe, and **(C)** Cu, as well as the macronutrients **(D)** P,**(E)** Na, **(F)** Ca, **(G)** K and **(H)** Mg. Two-way ANOVA and Tukey's post hoc test were used for all measurements, with different letters denoting a significant difference ($P< 0.05$). Zn deficiency (-Zn) is represented by white bars, normal Zn (NZn) by gray bars and excess Zn (+Zn) by black bars.

Figure S4 Translocation rate of nutrients in rice plants under -Zn, NZn and +Zn conditions at 3, 14 and 21 DAT. Bar charts show translocation rates of the micronutrients **(A)** Mn,**(B)** Fe, **(C)** Cu, as well as the macronutrients**(D)** P, **(E)** Na, **(F)** Ca, **(G)** K and**(H)** Mg based on the shoot concentration divided by the root concentration. Two-way ANOVA and Tukey's post hoc test were used for all measurements, with different letters denoting a significant difference ($P< 0.05$). Zn deficiency (-Zn) is represented by white bars, normal Zn (NZn) by gray bars and excess Zn (+Zn) by black bars.

Figure S5 RNA-Seq data showing fold change in the expression of 22 genes encoding GO-enriched calcium-binding proteins in the -Zn/NZn and +Zn/NZn comparisons for roots and shoots at 3DAT. Upregulated genes are shown in red and downregulated genes in blue. -Zn = Zn deficiency, NZn = normal Zn, +Zn = excess Zn, R = root and S = shoot.

Figure S6 Effects of Zn deficiency and excess on leaf ROS formation at 3 and 21 DAT. DAB staining showing the effects of H_2O_2accumulation in the leaves of rice plants under -Zn, NZn and +Zn conditions **(A)** at 3 DAT and **(C)** 21 DAT. NBT staining showing the effects of O_2^-accumulation in the leaves of rice plants under with -Zn, NZn and +Zn conditions **(B)** at 3 DAT and **(D)** 21 DAT (scale bar = 5mm). -Zn = Zn deficiency, NZn = normal Zn, +Zn = excess Zn.

Supplementary Table Captions

Table S1 Primers used for RT-qPCR amplifications.

Table S2 Statistics of total, total mapped and uniquely mapped reads per RNA-Seq sample.

Table S3 Gene list and expression details of enriched GO terms.

Table S4 MapMan transport annotation results showing the expression change patterns of nutrient transport-related DEGs identified between -Zn- and +Zn-treated plants in roots at 3DAT.