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Proteomic investigation of Zn-challenged rice roots reveals adverse effects and root physiological adaptation

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Abstract

Aims Understanding the molecular responses of plant roots to the challenging environment contributes to engineering plants with improved stress tolerance. However, little has been done to understand rice (*Oryza sativa* L.) roots response to the toxic concentration of zinc (Zn) at the proteomic level. This study explored proteomic responses of young rice roots 5-6 days after sowing to 765 μ M of Zn in a hydroponic set-up after 4-day exposure.

Methods Dye staining method and spectrophotometry were chosen for physiological response investigations. ICP-MS was used to determine metal content in rice seedlings. Two-dimensional gel electrophoresis was used for the proteomic study of root tissue.

Results Elevated Zn reduced Mg translocation to the shoots and Mn uptake, decreased plant growth, and increased cell death of roots. Zn stimulated the biosynthesis of glutathione but decreased ROS and malondialdehyde levels. In total, 42 Zn-responsive proteins were identified. Proteins involved in redox regulation, defense response, sulfur metabolism, and proteolysis were induced, while those of energy production and cell wall biogenesis were decreased.

Conclusion Roots of *O. sativa* seedlings could tolerate certain Zn excess (765 μ M Zn for 4 days) by stimulating nutrient recycling and glycolysis, and production of defensive metabolites and proteins to maintain ion and redox homeostasis. Besides, adenosylhomocysteinase (AHCY) down-regulation may contribute to balanced transmethyations, and methionine salvage pathway appears important for the adaptation to Zn. Our results provide some new insight into the complex metabolic response of rice roots to Zn stress, which is related to both stress and defense mechanisms.

Keywords

Zn stress, *Oryza sativa* L., root growth inhibition, glutathione, cell death, comparative proteomics

Abbreviations

LC-MALDI-TOF/TOF MS	liquid chromatography tandem matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry
2-DE	two-dimensional gel electrophoresis
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
GSH	reduced glutathione

56	ROS	reactive oxygen species
57	GO	Gene Ontology
58	KEGG	Kyoto Encyclopedia of Genes and Genomes
59	PPI	protein-protein interaction
60	ICP-MS	inductively coupled plasma mass spectrometry
61	DAPs	differentially abundant proteins
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Introduction

Zinc (Zn) is one of the most abundant transition metal in the organisms, participating in various physiological processes in plants (Sadeghzadeh and Rengel 2011). Although Zn toxicity in crops is far less known than Zn deficiency, Zn concentration in agricultural soils (Simmons et al. 2005), and in the soil solution (Lock and Janssen 2003; Stephan et al. 2008) which determines Zn bioavailability and potential toxicity (Lock and Janssen, 2003), have accumulated in various regions due to different anthropogenic inputs to the concentrations exceeding that for optimal rice development ($5 - 20 \text{ mg Zn kg}^{-1}$ in soil (Fageria et al. 2011) and $2 - 7.5 \text{ mg Zn L}^{-1}$ in the soil solution (Wei et al. 2007)). At high environmental concentrations, Zn damage individual plants as well as their communities which result in a significant yield reduction (Wei et al. 2007).

Numerous studies have been conducted to understand the response of rice to elevated Zn. Plants grown under excess Zn have various growth defects (Song et al. 2011, 2014) which were proposed to be a secondary effect. Excess Zn leads to micro- and macronutrient deficiencies (De Souza Silva et al. 2014; Mehrabanjoubani et al. 2015).

A well-developed root system is a prerequisite for plant fitness and productivity. During environmental challenges, roots respond by adjusting their metabolism and architecture or by the toxic response like (senescence and cell death). Understanding these processes could contribute to improving plant stress tolerance avoiding yield losses (Seo et al., 2020). Root growth inhibition by excessive Zn concentrations in rice can be attributed to mitochondrial electron transport chain-induced ROS production (Chang et al. 2005; Song et al. 2011) and altered activities of antioxidative enzymes which lead to lipid peroxidation, decreased membrane integrity (Song et al. 2011), and ultimately apoptosis (Chang et al. 2005). Nag et al. (1984) also proposed that reduced growth of rice seedlings exposed to Zn was due to the decreased content of indole-3-acetic acid (IAA) and decreased activities of enzymes involved in food and energy utilization.

There is much less known about rice tolerance to elevated Zn exposure concentrations. For example, various plant metabolites, such as nicotianamine (Tsednee et al. 2014), metallothioneins (Liu et al. 2016b), and phytochelatins (Tennstedt et al. 2008), were suggested to be involved in Zn tolerance. Besides, cell wall Zn fixation could be vital (Van De Mortel et al. 2006; Muschitz et al. 2009).

In addition, quantitative trait loci (QTL) mapping revealed ten QTLs associated with Zn tolerance in rice (Liu et al. 2016a). Furthermore, several Zn-resistant genes were recognized by mutant and transgenic approaches. Among

them, Zn transporters (Lan et al. 2013; Menguer et al. 2013; Takahashi et al. 2012), genes involved in ROS detoxification (Kumar et al. 2012), and maintenance of plant hormone gibberellic acid, zeatin, and IAA levels (Sahoo et al. 2014; Tuteja et al. 2013) were shown to contribute to Zn tolerance in rice.

Although previous studies have given us valuable insights into rice responses to Zn stress, the molecular mechanisms of the Zn stress response in rice cannot be completely understood unless the Zn-induced proteomic changes are analyzed (Agrawal et al. 2009). To the best of our knowledge, the only study examining the proteomic response of rice to excessive Zn was conducted on leaves by Hajduch et al. (2001) who showed Zn damaged photosynthetic apparatus and caused oxidative stress. However, no proteomic work has been done to decipher the changes in protein profiling in rice roots when exposed to excessive Zn levels.

Therefore, the present work focuses on proteome responses of young rice roots following exposure to excessive Zn concentration (765 μ M Zn) in a growth medium by using two-dimensional SDS-PAGE analysis combined with protein identification by LC-MALDI-TOF/TOF MS. The results from the proteomic analysis were explained together with some whole organism endpoints and alterations at the biochemical level. The results provide additional information to existing knowledge on the molecular mechanism of Zn stress response in rice root which might be a basis for future studies aiming to improve the performance of rice under Zn stress.

Materials and methods

Plant material, growth conditions, and exposure to elevated Zn

The rice seeds (*Oryza sativa* L.) were surface sterilized in 10% of hydrogen peroxide for 20 min, followed by rinsing with distilled water thoroughly. The seeds were then soaked in distilled water for 48 h in the dark at the temperature of 30°C. The experiment was conducted in an environmentally controlled growth chamber with a temperature of 27°C/25°C (day/night) and photosynthetic photon flux density (PPFD) of 400 μ mol m⁻² s⁻¹ at a photoperiod of 16 h during the daytime, and relative humidity (RH) of about 70%. For the determination of growth parameters, germinated seeds were transferred onto nylon net fixed in plastic pots, touching distilled water, and left 3 to 4 days to reach approximately 4-5 cm and 1 cm in root and shoot length, respectively. Seedlings with the same vigor were selected, transferred onto the nylon net floating in the 2 L plastic pots (48 seedlings/pot, 1.5 cm between

seedlings), and pre-cultured for two days on full-strength Kimura B nutrient solution. To obtain materials for the proteomic and biochemical analysis, pre-soaked seeds were transferred onto the nylon net in the 6 L plastic pots (150 seeds/pot, 1.5 cm between seeds), left for 3-4 days, and pre-cultured for two days on the full-strength nutrients.

After two days (5-6 days after sowing (DAS)), the Zn treatment started by supplementing the nutrient solution with ZnCl_2 at the Zn concentrations of 0 (CK), 153 (Zn1), 765 (Zn2), or 1530 μM (Zn3). Nutrient solutions were additionally supplemented with 2 mM of 2-(N-morpholino)ethanesulfonic acid (MES, pH buffer) to prevent pH variation among treatments (Uraguchi et al. 2009), and the pH was adjusted to 5.5 by using KOH. Seedlings were grown for 4 days without aeration under the above-described conditions. All experiments were done in triplicates.

ICP-MS analysis of nutrient solutions and Zn speciation analysis

ICP-MS analysis of Zn availability in the nutrient solution was performed according to Famoso et al. (2010). The chemical speciation of Zn in the nutrient solutions was predicted by Visual MINTEQ (Version 3.1). The nominal exposure concentrations of Zn in the nutrient solution were close to those determined by ICP-MS (Supplementary Table S2). The centrifugation experiment and theoretical prediction by Visual MINTEQ showed Zn did not precipitate at any of the Zn treatments (Supplementary Table S2, S3). Visual MINTEQ further showed that the species of Zn in the culture media regardless of the treatment were mainly comprised of the following species: free Zn^{2+} , ZnSO_4 (aq), ZnEDTA^{-2} (Supplementary Table S3).

Determination of Zn, Cu, Fe, Mg, and Mn in rice seedlings

After harvest, the rice seedlings were washed with 10 mM ethylenediaminetetraacetic acid disodium salt (Na_2EDTA , pH = 5.5) for 10 min and then three times with deionized water (Gu et al. 2012). After washing, the seedlings were separated into roots and shoots and dried for 3 days at 70°C. The ion content in plant material was then analyzed according to Chen et al. (2010), without analyzing the washing solution. The results are shown in Supplementary Table S4.

Measurement of root and shoot biomass

After harvesting (9-10 DAS), 15 randomly selected seedlings (from 48 in the pot) from each treatment were selected, and the experiment was done in triplicates. The seedlings were photographed for root and shoot (stem and leaf blade) length measurement using the SmartRoot plugin (<http://www.uclouvain.be/en-smartroot>) for ImageJ (<http://rsbweb.nih.gov/ij/>) (Wayne Rasband National Institute of Health, Bethesda, MD, USA). Then, plants were divided into roots and shoots of which fresh weight (FW) was determined (roots or shoots from 15 seedlings were weighted together), and dry weight was measured after the plant material was oven-dried at 70°C.

Metabolites content measurements

For shoots, the photosynthetic pigment was extracted according to Chen et al. (2011), and the total chlorophyll content was calculated using formula proposed by Arnon (1949). For roots, the glutathione content and its reduced (GSH) or oxidized state (GSSG) were estimated using a total GSH/GSSG assay kit (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. The content of superoxide radical and hydrogen peroxide in roots was determined spectrophotometrically according to Chen et al. (2013). Flavonoid content was estimated by aluminum chloride colorimetric method according to Rahman et al. (2016), and the results were presented as absorbance at 510 nm relative to control treatment (Supplementary Fig. S3). The ethylene content in roots was measured by gas chromatography (GC). Frozen plant sample (0.1 g) was put into a 1 ml GS injection bottle where two drops of distilled water were added. The samples were left for 24 h at room temperature in the dark. Five µL of gas was withdrawn from the airspace and injected into a gas chromatograph equipped with a capillary column (HP-5, 30 m, 0.25 mm) and flame-ionization detector for ethylene determination with the temperature set to 230°C (Supplementary Fig. S1C).

Analysis of membrane damage and cell death in roots

Lipid peroxidation (malondialdehyde content) was determined according to the thiobarbituric acid (TBA) method described by Chen et al. (2013). The extent of Zn-induced cell death was estimated according to Denness et al.

(2011) with slight modifications. After harvesting, roots were washed with distilled water, and 200 mg of root material was soaked in Evans blue stain (0.25% w/v) for 10 min. Then, the roots were placed in Eppendorf tubes filled with distilled water and centrifuged at 15000 g to remove the excess stain on the roots. Washing was repeated until no presence of the stain was visible. The roots were then soaked in 1 mL of formamide for 24 h to release the stain from the roots. After 24 h, formamide was collected, and absorbance was measured at 600 nm.

Proteomics analysis in roots

Protein extraction, 2-DE electrophoresis, and gel imaging and analysis

Total proteins from rice roots were obtained by mixed TCA/acetone precipitation and phenol extraction method (Wu et al. 2014). Proteins were air-dried and dissolved in lysis buffer (8 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 1% IPG buffer pH 4–7) at room temperature, and protein content was determined according to Bradford (1976).

Two-dimensional SDS-PAGE was carried out according to Hu et al. (2014). The 500 µg of protein sample was firstly separated in the first dimension (isoelectric focusing (IEF)) using 18 cm long, pH 4-7 linear (GE Healthcare, Piscataway, NJ, USA) IPG strips, and the electrophoresis in the second dimension was performed on 12.5% SDS-polyacrylamide gels using a protean apparatus (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The gels were stained with Coomassie Brilliant Blue (CBB) R-250 in 10% acetic acid and finally destained with 10% acetic acid. Three independent biological replicates were performed for each treatment.

Gel images were acquired at a resolution of 600 dots per inch (dpi) by a scanner (Uniscan M3600, Beijing, China) and analyzed using PDQuest software (version 7.0, Bio-Rad, Hercules, CA, USA) according to Liu et al. (2011). After normalization and background subtraction, spots intensity from Zn-treated roots which changed by more than two-fold compared to the control, and passed Student's t-test ($p < 0.05$) were considered differentially abundant proteins (DAPs) and were further identified by LC-MALDI-TOF/TOF MS analysis.

Protein digestion and identification

Protein spots were manually excised from the stained gels and prepared for MALDI-TOF/TOF analysis according to Liu et al. (2011). MS analysis was conducted using a 4800 Plus MALDI-TOF/TOF Proteomics Analyzer (Applied Biosystems, USA). The spectra were calibrated with the flexAnalysis software (Version 3.2, Bruker-Daltonics). The measured tryptic peptide masses were searched against the National Center for Biotechnology Information non-redundant (NCBI nr, Bethesda, MD, USA) database, and selecting the taxonomy of green plants using MASCOT interface (Version 2.5; Matrix Science, London, UK). Search parameters for protein identification were as follows: no molecular weight restriction, one allowed miscleavage, fixed modifications of carbamidomethyl (C), variable modifications of oxidation (Met), peptide tolerance of 100 ppm, fragment mass tolerance of ± 0.4 Da, and peptide charge of 1+. MASCOT protein scores greater than 61 with the NCBI nr database were considered statistically significant ($p < 0.05$). The match was considered regarding a higher Mascot score, the putative functions, and differential abundance patterns on 2-DE gels. Keratin contamination was removed.

Protein classification

The functions of the identified proteins were searched against the UniProt database (<http://www.UniProt.org>), and/or NCBI protein database (<http://www.ncbi.nlm.nih.gov>). KEGG database (<http://www.genome.jp/kegg/pathway.html>) was used for placing the proteins in appropriate functional pathways, and CELLO2GO (<http://cello.life.nctu.edu.tw/cello2go/>) (Yu et al. 2014) was used for prediction of their subcellular localization. Web-based software agriGO was used for Gene Ontology (GO) enrichment analysis, (<http://bioinfo.cau.edu.cn/agriGO/>) (Du et al. 2010), and the generated GO terms were together with their corresponding p values exported to online tool REVIGO (<http://revigo.irb.hr/>) (Supek et al. 2011) which reduces redundant GO terms by grouping semantically similar GO terms into clusters and superclusters. The protein-protein interaction network and enriched KEGG pathways were analyzed by STRING (version 10.5, <https://string-db.org/>). Data were exported and visualized by Cytoscape (Version 3.5.1) (Shannon 2003), and the schematic model of differently abundant proteins was done in the open-source tool Inkscape (The Inkscape Team, 2019). Some figures were further modified in Photoshop (Version 13.0).

Statistical analysis

Results in figures and tables are expressed as the means $\pm$ standard error (SE) of three replicates. A one-way analysis of variance (ANOVA) followed by Fisher's Least Significant Difference (LSD) multiple comparisons using OriginPro 9.0.0 (Originlab Corp., Northampton, MA, USA) was used. The statistical significance level of the differences between the experimental data was set to $p < 0.05$.

Results

Effect of Zn on root and shoot growth, and total chlorophyll content

All the tested Zn concentrations (Zn1, Zn2, and Zn3) negatively affected seedling growth after 4-day exposure (from 5-6 DAS). While at Zn1 shoot growth and its chlorophyll content were primarily reduced, the higher two concentrations (Zn2 and Zn3) significantly suppressed both shoot, and even more root development (Fig. 1A, 1B). Shoot chlorosis was apparent at all Zn exposure concentrations which coincided with decreased total chlorophyll content (Fig. 1D). At the highest Zn concentration, leaf necrotic lesions were observed (Fig. 1C). Also, at Zn2 and Zn3, a reduced length of lateral roots was evident. Besides, roots were fragile at exposure to Zn3.

Effects of Zn on reactive oxygen species, lipid peroxidation, GSH content, and cell death in roots

In the roots of Zn-treated plants, neither $O_2^{\cdot-}$ nor H_2O_2 (except at Zn1) accumulated to the levels higher than control (Fig. 1E and 1F, respectively). At all Zn treatments, decreased MDA levels when compared to control indicate lower amount of peroxidised lipids (Fig. 1G). The opposite trend was observed for GSH content, where Zn caused an increase of reduced GSH (Fig. 1H) and its share in the total GSH (Fig. 1J). However, compared to control, cell death in roots slightly decreased at Zn1 but increased at the higher two Zn concentrations (Supplementary Fig. S1A).

Comparative proteomic analysis of Zn-exposed rice roots

Proteomic profile of rice roots in response to Zn

The 2-DE proteomic approach was used to identify metabolic pathways altered by Zn²⁺ (765 μ M) after four-day treatment (from 5-6 DAS). The concentration selected for the proteomic study was based on the first observed adverse effect concentration (OAEC) of Zn for root growth, that is reduced root growth and biomass (Fig. 1A, 1B). More than 400 reproducible protein spots were resolved over an isoelectric point (pI) ranging from 4 to 7 and molecular weight (MW) ranging from 9 to 90 kilo Daltons (kDa) (Fig. 2A). The information on peptide sequences for protein identification is summarized in Supplementary Table S5). Representative image of the 2-DE gel and close-up views of some randomly selected protein spots are shown in Fig. 2A and Fig. 2B, respectively.

Functional classification and subcellular localization of differentially abundant proteins in response to Zn stress in rice roots

The list of DAPs in rice roots exposed to Zn²⁺ is summarized in Table 1. The identified proteins were classified into six groups based on their biological functions. The largest group includes proteins involved in metabolism (amino acids, nucleic acids, carbohydrates secondary metabolites), followed by proteins involved in stress and defense (abiotic and biotic stress responses) and protein turnover (protein synthesis, degradation, and folding), and redox regulation and energy production. One protein is related to cell structure, while one protein has an unknown function (Fig. 3A, Table 1).

The majority of the DAPs are cytoplasmic, as predicted by the CELLO2GO predictor (<http://cello.life.nctu.edu.tw/cello2go/>). Other proteins localize in the nucleus, extracellular space, mitochondria, vacuole, and endoplasmic reticulum, while the location of some proteins is unknown (Fig. 3B).

Gene Ontology enrichment of down- or up-regulated proteins in response to Zn stress in rice roots

Gene ontology (GO) enrichment analysis for the biological process (BP) was done by web-based software AgriGO (Supplementary Fig. S2), and the results were further analyzed by exporting enriched GO terms together with their corresponding *p*-values to REVIGO and visualized as a circular treemap chart (data used for the generation of treemap chart are in Supplementary Table S6) (Fig. 4). Excess Zn mainly affected the abundance of stress-responsive proteins (abiotic and abiotic stimulus, and oxidative stress). Among other BPs, Zn also influenced the abundance of proteins involved in aminoglycan metabolism, cellular homeostasis, and iron ion transport.

Protein-protein interaction (PPI) analysis of down- or up-regulated proteins in response to Zn stress in rice roots

To explore a functional relationship between DAPs, a protein-protein interaction (PPI) network was generated (the detailed information on the interactions is available in Supplementary Table S6), and protein nodes were marked with colors that correspond to the selected enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (STRING database) (Fig. 5, Supplementary Table S8). Twenty-seven proteins constructed the PPI network, while 15 proteins were detached and, thus, excluded from the network (Fig. 5). The proteins were distributed into two sub-clusters, both interacting with triosephosphate isomerase (TPI).

The first protein sub-cluster (left) includes proteins involved in different metabolic pathways. While proteins participating in the metabolism of carbohydrates were mainly down-regulated by Zn, those proteins involved in sulfur, cysteine, and methionine metabolism were up-regulated. The majority of proteins directly or indirectly interacts with adenosylhomocysteinase (AHCY).

The second sub-cluster (right) includes proteins involved in protein folding and degradation, oxidative phosphorylation, and oxidative stress. Proteins located in the endoplasmic reticulum (ER) (protein disulfide isomerase-like 1-1 (PDI)) and mitochondria (NADH-ubiquinone oxidoreductase (NUO), ATP synthase subunit alpha (ATP1), mitochondrial F0-ATP synthase D chain (ATP0)) which are involved in protein synthesis and oxidative phosphorylation, respectively, were down-regulated. Meanwhile, ROS-scavenging enzymes and proteins involved in protein folding and proteolysis were mainly up-regulated. The two most interacting proteins were PDI and L-ascorbate peroxidase 2 (APX2).

Discussion

Growth, and metal content in rice seedlings exposed to elevated Zn

In the present work, we studied the effects of elevated Zn exposure (153 (Zn1), 765 (Zn2), or 1530 μ M (Zn3)) on rice after 4-day (from 5-6 DAS) hydroponic experiments. The concentrations were selected based on Wei et al. (2007), and are found in the soil solution of Zn-contaminated regions (Lock and Janssen 2003; Stephan et al. 2008).

Roots and shoots responded differently to elevated Zn in the nutrient media. While at low Zn excess (Zn1) shoots were more sensitive, the opposite was observed at the highest Zn concentration tested (Zn3). However, all the treatment negatively affected rice development as also indicated by the reduction in the total chlorophyll content which agreed well with the previous experiment on rice (Song et al. 2014; Wei et al. 2007).

Zn stress primarily results from Zn-induced deficiencies of other micro- and macronutrients (De Souza Silva et al. 2014; Ishimaru et al. 2008; Mehrabanjoubani et al. 2015). In this study, elevated Zn increased the content of Mg in the roots but reduced its content in the shoots which suggest that Zn affected its translocation (Supplementary Table S4). Mg is an essential co-factor of various enzymes, and is required for chlorophyll formation, and plays a key role in photosynthesis (Ding et al. 2006). An even more pronounced effect was observed for Mn that decreased in both roots and shoots which indicates that Zn interferes with Mn uptake (Supplementary Table S4). Mn might be part of the Fe plaque on the root surface which serves as a protective barrier against Zn uptake (Liu et al. 2010). Zn, however, increased in both parts of the seedling, but its translocation strongly decreased under Zn excess exposure (Supplementary Table S4). Based on the studies of Ishimaru et al. (2008, 2011), we speculate, in the present study, Zn altered the ratio between nicotianamine (NA) and deoxymugineic acid, favoring NA to reduce intra-cellular availability of Zn and to decrease its translocation to the shoots.

Proteomic and biochemical responses of rice roots under Zn exposure

To further understand the mechanism of Zn-induced growth reduction of rice roots as well as their adaptation strategies to excessive Zn, we performed proteomic analyses of roots. Based on the phenotype, notable growth inhibition of roots, we choose the treatment Zn2 (765 μ M). Analysis of 2-DE gels revealed that the abundance of 42

proteins changed more than 2-fold by Zn. It should be noted that only a certain portion of proteins was identified due to the methods applied, but likely more are changing in abundance. For example, Wang et al. (2020) examined the transcriptomic and proteomic response of rice to heat stress. After 24 h treatment, transcriptomic data revealed 3242 DEGs, and 58 DAPs were identified using the 2D-E approach. Nevertheless, based on GO enrichment analysis (Supplementary Fig. S6 and Fig. 4), we found rice roots responded to Zn stress by stimulating ROS-scavenging systems, metabolic adjustments, and ion and cellular homeostasis.

Zn stimulates glycolysis and metabolic pathways involved in sulfur metabolism in rice roots

Two isoforms of glycolytic enzyme triosephosphate isomerase (TPI) (spot 3101 and 3109) were up-regulated while two proteins which channel sucrose into the glycolytic pathway, that is sucrose synthase 1 (SUS) (spot 7813) and fructokinase-2 (FRK) (spot 2201), were down-regulated (Table 1) in Zn-challenged roots. It might be speculated that glucose derived from starch is preferred over sucrose and fructose as an initial substrate in the glycolytic pathway which might be due to the decreased synthesis and/or transport of sucrose to the roots (Herren and Feller 1997). These could cause energy and carbon limitations in roots, and activation of starch breakdown is needed to supply carbon for metabolic processes under Zn stress.

The simultaneous Zn-induced up-regulation of TPIs and up-regulation of two isoforms of cysteine synthase (spots 3302 and 3310) (Table 1), however, suggests TPI provided glyceraldehyde-3-phosphate (GAP) for cysteine synthesis via serine (Ros et al. 2014). The increased abundance of the two cysteine synthases explains increased GSH content measured biochemically in rice roots exposed to Zn (Fig. 1H) (Romero et al. 2014).

Cysteine is also a predecessor in the biosynthesis of methionine (Romero et al. 2014). In the present study, three proteins involved in methionine metabolism were up-regulated by excess Zn, S-adenosylmethionine synthase 2 (SAMS2, spot 5401), methylthioribose-1-phosphate isomerase (MRI1/IDI2) (spot 2405) and 1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase 2 (ARD2/IDI1) (spot 2004) (Table 1). While SAMS2 produces S-adenosylmethionine (SAM) from methionine and ATP, MRI1 and ARD2 participate in methionine biosynthesis via the salvage pathway (Yang cycle) that is responsible for the synthesis of several metabolites produced from SAM (Kosová et al. 2014). These results imply elevated Zn induced biosynthesis of phytosiderophores in rice roots which

is in agreement with Ishimaru et al. (2008). Besides, our biochemical data also showed that ethylene production increased in Zn-challenged roots (Supplementary Fig. S1C).

Zn causes cell wall modification in rice roots

SAM is also an essential co-factor of SAM-dependent methyltransferases that utilize methyl group, releasing S-adenosyl-L-homocysteine (SAH), a feedback inhibitor (Sauter et al. 2013). Excessive Zn was found to cause down-regulation of two major proteins involved in the removal of SAH, adenosylhomocysteinase (AHCY) (spot 5604) and putative adenosine kinase (ADK) (spot 1304) (Table 1). The former is a hub protein in the left cluster of the PPI network (Fig. 5), while the gene of the latter localizes on quantitative trait locus MT2.2 for Zn toxicity tolerance (Meng et al. 2017). We speculate that SAM was mainly consumed for ethylene and phytosiderophore production (discussed above) rather than transmethylation reactions, which results in a lower level of SAH. In agreement, a decreased abundance of SAM-dependent and the key enzyme involved in lignin biosynthesis tricin synthase 1 was detected (Table 1) (Li et al. 2013). In support, we detected a down-regulated protein abundance of chalcone-flavonone isomerase (CHI) (spot 2108) (Table 1) that synthesizes naringenin, a precursor metabolite for all other classes of flavonoids (Lam et al. 2017), and agreed well with a decreased relative flavonoid content under Zn measured in the present study (Supplementary Fig. S3).

Furthermore, a decreased abundance of sucrose synthase 1 (spot 7813) and fructokinase-2 (spot 2201) hints that reduced lignification was accompanied by a disturbed cell wall biogenesis and vascular tissue development (Hirose et al. 2008; Stein et al. 2018).

Taken together, the potential reductions in cell wall biosynthesis and lignification imply that Zn disturbed root cell generation. In addition, potentially poorly developed vascular tissue can partly explain the decreased root growth of rice seedling exposed to excessive Zn (Table 1).

Zn disturbs ATP production in rice roots

The proteomic analysis indicate that oxidative phosphorylation in rice roots was strongly affected by Zn, which is in agreement with Ahmed et al. (2017) and Glińska et al. (2016). Specifically, Zn decreased the abundance of subunits

building complex I (NADH-ubiquinone oxidoreductase (spot 9002)) and ATP synthase (ATP synthase subunit alpha (spot 5501) and mitochondrial F0-ATP synthase D chain (spot 1111)) of the mETC (Table 1).

It was demonstrated by Chang et al. (2005) that complex I participates in ROS-induced cell death in Zn-exposed rice roots. Also, decreased ATP production can result in a shortage of energy supply for the vegetative growth plant. Zn-perturbed energetic functions of mitochondria, and activation of the alternative respiratory pathway were in agreement with increased gene expressions of voltage-dependent anion channel (*OsVDAC1*) and alternative oxidase (*OsAOX1*), respectively (Supplementary S1. and Supplementary Fig. S4).

Mitochondrial dysfunction and potentially ROS-induced damage of mitochondrial proteins can be further speculated based on increased protein abundance of ubiquitin family protein (spot 704) (Table 1) which has been suggested to participate in ubiquitin-proteasome proteolysis of damaged mitochondrial proteins (Fujii et al. 2014). The most similar protein with known function in plants is *Arabidopsis thaliana* dominant suppressor of kar2 (DSK2), which has been recently shown to mediate selective autophagy, increasing the tolerance against carbon starvation and drought stress (Bassham and Clouse 2017).

Zn stimulates antioxidative defense in rice roots

In the present study, two superoxide dismutases (SOD) were induced by Zn treatment, i.e. superoxide dismutase [Cu-Zn] 1 (spot 7001) and chloroplastic superoxide dismutase [Cu-Zn] (spot 4013)) (Table 1). Interestingly, two isoforms of L-ascorbate peroxidase (APX1 and APX2 (spots 2009, 3110)) were differently regulated (Table 1). Based on the diverse effects of silencing different *APX* genes (Caverzan et al., 2012), APXs seem to modulate ROS (H_2O_2) signaling in Zn-challenged rice roots.

Moreover, two more proteins involved in redox regulation and detoxification of hydroperoxides were up-regulated by Zn, protein IN2-1 homolog B (spot 3312) and peroxiredoxin-2C (spot 5007) (Table 1). Transgenic *Arabidopsis* lines harboring rice IN2-1 homolog B (named OsGSTL2 by Hu et al. (2013)) were more resistant to different heavy metals and other abiotic stresses (Kumar et al. 2013), suggesting protein IN2-1 homolog B might be one of the key proteins contributing to abiotic stress tolerance in rice. Similarly, plants overexpressing peroxiredoxin-2C are more resistant to drought stress, with an alleviated degree of oxidative damage (Zhang et al.

2019). Additional membrane protection could be assumed based on the increased abundance of temperature stress-induced lipocalin 1 (spot 2111) (Table 1) (Wahyudi et al. 2018). Taken together, these results indicate Zn stimulated the antioxidative defense which explains higher reduced GSH content (Fig. 1H, 1J), and lower amount of ROS and peroxidised lipids (MDA content) (Fig. 1E, 1F, 1G) measured in the rice seedling roots when compared to the control. Besides, lower levels of these parameters might also result from increased cell death (Supplementary Fig. S1A) which most likely occurred at the tips of primary and lateral roots (Foreman et al. 2003), increasing the proportion of dead (PCD or necrosis) and, thus, metabolically inactive root cells (Miljuš-Djukić et al. 2013).

Zn causes hormone imbalance in rice roots

In addition to increased ethylene (ETH) synthesis (Supplementary Fig. S1C), two proteins involved in abscisic acid (ABA) and jasmonic acid (JA) signaling, respectively, were differentially regulated under excessive Zn in rice roots.

Zn excess seems to decrease ABA sensitivity and/or biosynthesis as indicated by down-regulated protein abundance of abscisic stress-ripening protein 5 (ASR5, spot 8004) (Table 1). Silencing ASR5 primarily resulted in the up-regulation of genes involved in PCD in Al-exposed rice roots (Arenhart et al. 2014).

In contrast, Zn up-regulated putative 12-oxophytodienoate reductase 5 (OPR) (spot 4509) (Table 1). OPR is one of the key enzymes in the JA biosynthesis pathway. JA may alleviate metal-induced oxidative stress by decreasing NADPH oxidase activity, stimulating accumulation of osmolytes, and activities and/or gene expressions of antioxidant enzymes (Sirhindi et al. 2016). Increased JA biosynthesis is in accordance with increased gene expression of JA-inducible transcription factor *OsJAmyb* (Supplementary S1. and Supplementary Fig. S1B).

The role of ETH under elevated Zn might be dual. On one hand, ETH stimulates the production of phytosiderophores to maintain ion homeostasis disturbed by high Zn (Wu et al. 2011). On the other hand, together with JA, ETH likely operates in Zn stress-induced senescence (Thao et al. 2015), which explains growth inhibition (Fig. 1A, 1B) and increased cell death (Supplementary Fig. S1A) in rice roots exposed to Zn.

Zn causes senescence and cell death in rice roots

Stress-induced senescence is considered a plant strategy to increase the probability of survival, during which plant recycles and mobilizes nutrients from degraded proteins, lipids, and nucleic acids to the still growing and/or storage sites (Sade et al. 2018).

In the present study, the abundance of copper transport protein ATX1 (spot 3012) was found up-regulated (Table 1) by high Zn. ATX1 is likely involved in Cu transport from the root parts with increased cell death to the still growing and developing parts of the rice seedling (Zhang et al. 2018).

Moreover, three proteolytic enzymes were up-regulated by Zn, oryzain gamma chain (spot 1201), aspartic proteinase oryzasin-1 (spot 1314), and putative subtilisin proteinase (spot 2813) (Table 1). Liu et al. (2008) reported that gene expressions of both cysteine (oryzain gamma chain) and aspartic proteases (aspartic proteinase oryzasin-1) were induced during senescence in rice flag leaves.

On the other hand, Zn decreased the abundance of protein disulfide isomerase-like 1-1 (PDIL1-1) (spot 1605) (Table 1) that absence in rice endosperm resulted in the endoplasmic reticulum (ER) stress and PCD (Han et al. 2012). PDIL1-1 might inhibit the stimulation of PCD by blocking vacuolar proteases (Kim et al. 2012; Li et al. 2006) as it was shown for Arabidopsis AtPDIL1;1 (Andème-Ondzighi et al. 2008). Recently, Xia et al. (2018) showed that OsPDIL1-1 is targeted by microRNA5144-3p, and suggested both as potential candidates for molecular breeding to improve abiotic stress tolerance of rice.

In accordance, decreased abundance of growth- and stress-related translationally-controlled tumor protein homolog (TCTP) (spot 102) (Table 1) was measured in rice roots challenged by Zn in the present study. TCTP has been suggested to prevent PCD by binding free cytosolic calcium in plants (Hoepflinger et al. 2013). Besides, decreased TCTP might promote ethylene-mediated growth inhibition (Tao et al. 2015).

Increased PCD by Zn was further explained by an up-regulated protein abundance of root-specific pathogenesis-related protein 10 (PR10) (spot 1024) and putative beta-alanine synthase (spot 7506) (Table 1). While rice PR10 plays a key role in PCD (Kim et al. 2011), beta-alanine synthase catalyzes the final step of pyrimidine degradation (Walsh et al. 2001). These results imply that increased RNA/DNA degradation (a hallmark of PCD) and nutrient recycling from nucleotides were taking place in rice roots exposed to excessive Zn.

Protein-protein interaction (PPI) network analysis

Analyzing protein-protein interacting networks has become increasingly important to unravel valuable new insights into biological processes under specific conditions. Among proteins in the interactome, so-called hub proteins (highly connected central proteins) are of special interest (Vandereyken et al. 2018).

In our study, 27 proteins (from total 42) were identified to build the PPI network (Fig. 5). Proteins were distributed into two main clusters, both interacting with triosephosphate isomerase (TPI).

In Cluster 1, the interaction between sucrose synthase 1 (SUS) and fructokinase-2 (FRK), and FRK and TPI is due to their functional link in the glycolysis. The most interacting protein in cluster 1 is adenosylhomocysteinase (AHCY) which catabolizes S-adenosyl-L-homocysteine (SAH) to homocysteine, and adenosine which is removed by adenosine kinase (ADK). Homocysteine is a precursor in cysteine and methionine synthesis which explains the interactions between AHCY and cysteine synthases (CS1 and CS2), and methylthioribose-1-phosphate isomerase (MRI1), respectively. The product of S-adenosylmethionine synthase 2 (SAMS), S-adenosylmethionine (SAM), is needed for SAM-dependent methyltransferases. Each SAM-dependent methylation will yield a feedback inhibitor SAH (Moffatt and Weretilnyk 2001). Tricin synthase 1 (TS) is a SAM-dependent enzyme involved in lignification. AHCY controls the level of SAH and, thus, the activity of TS. In short, the PPI analysis of cluster 1 suggested a complex regulation network between metabolisms of amino acids, carbohydrates, and secondary metabolites in roots of rice challenged by Zn, where AHCY seems to play the major role.

The TPI also interacts with protein disulfide isomerase-like 1-1 (PDI), located in cluster 2. Both proteins are potential targets of thioredoxine (TRX) (Marchand et al. 2010). TPI is sensitive to multiple redox modifications, and TRX may control PDI activity (Hameed and Sarma 2012). The interaction between TPI and PDI in the present study could be because PDI contains two TRX domains. Compared to cluster 1, proteins in cluster 2 are more connected (size of protein nodes). ATP synthase subunits (ATP0, ATP1), and putative NADH-ubiquinone oxidoreductase (NUO) are proteins involved in oxidative phosphorylation. Disturbances in mETC could result in excessive ROS production, which would explain mainly up-regulated oxidative stress-related proteins, such as cytosolic L-ascorbate peroxidase 2 (APX2), superoxide dismutase (SOD1, SODCP), and peroxiredoxin-2C (PRXC). SOD1 and SODCP interact with copper transport protein ATX1 (ATX1). These interactions can be due to ATX1 similarity with the copper chaperone for SOD (CCS) which delivers Cu to Cu/Zn-SODs (Chu et al. 2005). Both, ROS and decreased abundance of protein disulfide isomerase-like 1-1 (PDI) could give rise to unfolded and damaged proteins which explain interactions between ROS scavengers and PDI with proteins involved in the protein folding, putative heat

shock cognate 70 kDa protein (HSP) and putative chaperonin 21 (CPN). Interaction between PDI and PRXC, APX2, and APX1 could be due to their direct and indirect involvement in oxidation and reduction of protein disulfide bonds (Onda and Kobori 2014). Interaction of actin-7 (ACT7) with APX2, HSP, and aspartic proteinase oryzasin-1 (APO) may suggest cytoskeletal alterations mediated by pro- and/or anti-oxidants, molecular chaperones, and proteases in rice roots grown under to Zn excess. In short, PPI analysis of cluster 2 suggests a strong interplay between oxidative phosphorylation and ATP production, redox regulation, protein turnover, and cytoskeletal rearrangement in rice roots exposed to excess Zn.

Conclusion

Excess Zn treatments reduced seedling development and increased chlorosis. Zn stimulated the biosynthesis of GSH, leading to decreased content of ROS and lipid peroxides (MDA) in roots, the later likely resulting from the stimulation of the antioxidative defense and increased cell death. Zn disturbed metal homeostasis, affecting Mg translocation to the shoots and decreasing Mn uptake. The 2D-E analysis suggested that Zn caused carbon starvation, and induced mitochondrial and endoplasmic reticulum dysfunctions, resulting in elevated ROS production, decreased growth and increased cell death which were likely modulated by ethylene and translationally-controlled tumor protein homolog (TCTP) signaling pathways. The simplified schematic representation of DAPs in rice roots is shown in Fig. 6. To adapt to the Zn-imposed challenge, rice roots seemed to activate nutrient recycling and glycolysis to provide building blocks for the biosynthesis of metabolites important for ROS detoxification and ion homeostasis, and proteins needed for the maintenance of protein stability and redox homeostasis. Protein-protein interaction analysis hinted adenosylhomocysteinase (AHCY) is the major protein involved in metabolic changes in rice roots exposed to Zn, its down-regulation is potentially important to maintain balanced methylation potential. It is also worth emphasizing that some novel Zn-responsive proteins were identified, such as AHCY, tricin synthase 1, and TCTP. Besides, we discuss ubiquitin family protein (Os10g0542200) as a potential candidate protein in alleviating excess Zn and other abiotic stresses. The results provide additional information on the adverse effects and mode of interaction between excessive Zn and rice roots as well as their adaptation response which might be a basis for future studies.

CRedit authorship contribution statement

Martin Šimon: Conceptualization, Investigation, Formal analysis, Writing - Original Draft, Writing - Review & Editing. **Zhi-Jun Shen:** Methodology, Validation, Investigation. **Kabir Ghoto:** Investigation, Resources. **Juan Chen:** Conceptualization, Methodology, Validation, Investigation. **Xiang Liu:** Conceptualization, Methodology, Validation, Investigation. **Gui-Feng Gao:** Conceptualization. **Anita Jemec Kokalj:** Writing - Review & Editing. **Sara Novak:** Writing - Review & Editing. **Barbara Drašler:** Writing - Review & Editing. **Jing-Ya Zhang:** Resources. **Yan-Ping You:** Resources. **Damjana Drobne:** Supervision, Writing - Review & Editing. **Hai-Lei Zheng:** Conceptualization, Supervision, Writing - Review & Editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Spot ^a	ID (NCBI)	Protein name/Description	MW/pI		MP ^c	Score ^d	Fold ^e change	<i>p</i> - value ^g
			b theor.	b obser.				
Metabolism								
Amino acid and nucleic acid metabolism								
3310	108711069	Cysteine synthase, putative, expressed (CS)	34.4/5.3	31.3/5.5	13	401	↑2.8	0.001
3202	84028195	Cysteine synthase (CS)	33.9/5.4	30.2/5.6	15	1100	↑2.8	0.032
5401	3024122	S-adenosylmethionine synthase 2 (SAMS)	43.3/5.7	46.1/6.1	6	680	↑4.2	0.002
5604	77550703	Adenosylhomocysteinase (AHCY)	50.0/5.9	55.5/6.1	12	232	↓2.4	0.006
2405	313471327	Methylthioribose-1- phosphateisomerase (MRI1)	39.7/5.2	39.7/5.4	6	231	↑2.5	0.035
2004	122247504	1,2-dihydroxy-3-keto-5- methylthiopentene dioxygenase 2 (ARD2)	23.7/5.1	21.8/5.3	10	429	↑2.7	0.016
1304	125582940	Putative adenosine kinase (ADK)	37.4/5.1	40.5/5.2	11	811	↓2.1	0.006
7506	222637048	Putative beta-alanine synthases (BAS)	46.1/6.0	45.1/6.5	8	194	↑3.3	0.026
Carbohydrates and secondary metabolism								
7813	401140	Sucrose synthase 1 (SUS)	93.5/5.9	75.3/6.5	27	570	↓2.3	0.002
2201	158513662	Fructokinase-2 (FRK)	35.9/5.0	34.7/5.3	7	424	↓2.7	0.001
2207	113624111	Tricin synthase 1 (TS)	27.9/5.1	27.1/5.4	13	480	↓2.3	0.004

2108	75148163	Chalcone--flavonone isomerase (CFI)	24.0/5.2	24.6/5.3	5	266	↓3.8	0.029
<i>Energy metabolism</i>								
<i>Glycolysis and TCA cycle</i>								
3101	1351270	Triosephosphate isomerase, cytosolic (TPI)	27.3/5.4	23.2/5.5	10	534	↑2.7	0.005
3109	385717670	Triosephosphate isomerase (TPI)	27.3/5.4	23.1/5.7	12	753	↑2.9	0.018
6509	125590750	NAD-dependent isocitrate dehydrogenase c;1 (IDH)	40.5/7.6	46.7/6.	11	549	↑2.9	0.03
<i>Oxidative phosphorylation</i>								
9002	31433663	NADH-ubiquinone oxidoreductase, putative, expressed (NUO)	12.6/6.2	13.2/6.8	7	252	↓2.8	0.008
5501	353685290	ATP synthase subunit alpha (ATP1)	55.5/5.8	52.6/6.2	13	254	↓2.3	0.005
1111	113624019	Mitochondrial F0-ATP synthase D chain (ATP0)	19.7/5.2	18.1/5.2	5	91	↓2.9	0.017
<i>Protein turnover</i>								
704	31433330	Ubiquitin family protein, expressed (UB)	59.4/-	59.1/4.8	9	547	↑2.8	0.032
1201	115510968	Oryzain gamma chain (OGC)	39.6/6.7	26.8/4.9	4	199	↑4.6	0.003
1314	78099759	Aspartic proteinase oryzasin-1 (APO)	54.7/5.2	31.5/5.1	2	152	↑3.6	0.003
2813	113537832	Putative subtilisin-like proteinase (SUBT)	81.4/6.0	74.1/5.4	6	226	↑2.8	0.014

1605	77549143	Protein disulfide isomerase-like 1-1 (PDI)	57.0/5.0	56.1/5.1	11	201	↓2.5	0.003
		Heat shock cognate 70						
2702	108707463	kDa protein, putative, expressed (HSP)	71.5/5.1	66.6/5.3	14	515	↑4.7	0.012
1216	113631503	Putative chaperonin 21 (CPN)	25.5/6.0	25.2/5.1	4	208	↑2.5	0.01
		Bowman-Birk type						
1113	255672821	proteinase inhibitor A (BBI)	29.2/5.4	16.6/5.1	3	67	↓4.6	0.006
<hr/> Redox regulation <hr/>								
3312	75153256	Protein IN2-1 homolog B (IN2-1)	27.5/5.4	31.7/5.6	5	247	↑2.2	0.001
5007	75172153	Peroxiredoxin-2C (PRX)	17.3/5.6	14.5/6.1	4	131	↑2.2	0.011
7001	122170280	Superoxide dismutase [Cu-Zn] 1 (SOD1)	15.4/5.7	17.3/6.5	6	404	↑2.5	0.001
2209	75308965	L-ascorbate peroxidase 2, cytosolic (APX2)	27.2/5.2	26.3/5.3	10	878	↑3.7	0.022
4013	113624483	Superoxide dismutase [Cu-Zn], chloroplastic; Flags: Precursor (SODCP)	21.4/5.8	14.3/5.9	5	586	↑2.2	0.004
3110	108707558	L-ascorbate peroxidase 1, cytosolic, putative,expressed (APX1)	27.3/5.4	23.9/5.7	10	535	↓2.5	0.008
<hr/> Stress and defense <hr/>								
102	549063	Translationally-controlled tumor protein homolog	19.0/4.5	21.9/4.7	2	194	↓4.0	0.001

		(TCTP)						
		Os07g0609000 (stress responsive A/B Barrel domain containing protein) ^g						
6105	113611783		26.5/7.1	24.3/6.3	3	175	↑2.1	0.001
3012	113623203	Copper transport protein ATX1	8.6/-	9.7/5.5	3	178	↑2.3	0.028
8004	77548908	Absciscic stress-ripening protein 5 (ASR5)	15.5/6.2	20.1/6.7	4	630	↓2.1	0.01
2111	113536947	Temperature stress-induced lipocalin 1 (TIL)	22.3/5.2	20.2/5.3	6	273	↑2.3	0.001
1024	77556750	Root-specific pathogenesis-related protein 10 (PR10)	17.0/4.9	14.2/5.1	8	442	↑2.8	0.004
3208	31433336	Chitinase 8 (CHI)	27.9/6.1	24.2/5.5	6	195	↑2.9	0.002
4509	75119043	Putative 12-oxophytodienoatereductase 5 (OPR)	41.9/5.3	48.2/5.7	14	378	↑2.2	0.038
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<i>Cell structure</i>								
2401	148886771	Actin-7	41.9/5.2	44.5/5.4	3	555	↑2.2	0.013
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<i>Unknown function</i>								
1026	125543273	Hypothetical protein OsI_10917	11.3/5.1	12.9/5.0	4	111	↓3.9	0.012

The proteins that showed a significant change in abundance by a factor of > 2.0 ($p < 0.05$) are listed.

a Numbers correspond to the 2-DE gels shown in Figure 3A.

b Theoretical and observed MW (kDa) and pI values.

c The number of matched peptides (MP).

d The Mascot searched score against the database NCBIInr.

e Ratio between Zn and control. Up-regulated protein spots (↑ and red) and down-regulated protein spots (↓ and green) after Zn exposure.

f *p*-value (paired t test) based on 3 biological replicates.

g Description of the protein found on MSU Rice Genome Annotation Project (<http://rice.plantbiology.msu.edu/>) websites.

Table 1 Identification of differentially abundant proteins by at least 2-fold (*p* < 0.05) following excessive Zn exposure in rice seedling roots.

Fig. 1 Effects of various concentrations of Zn on rice growth and biochemical parameters. (A) The phenotype of rice seedlings exposed to different concentrations of Zn for 4 days (from 5-6 DAS). (B) Effect of elevated Zn on dry weight of roots and shoots. (C) Visible chlorosis and necrotic lesions on the leaves caused by Zn3 after 4 day-exposure. (D) Changes in total chlorophyll content. Effect of excess Zn on the contents of (E) superoxide (O₂⁻), (F) hydrogen peroxide (H₂O₂), (G) malondialdehyde (MDA) and (H) reduced glutathione (GSH), (I) oxidized

glutathione (GSSG), and (J) the ratio between GSH and GSH + GSSG. Different letters (a, b, c) above the bars indicate statistical differences ($p < 0.05$). (K) Abundance changes of proteins involved in antioxidative defense detected by 2D-E (Table 1). All results are expressed as the means $\pm$ standard error (SE) of three replicates.

Fig. 2 2-DE analysis of proteins extracted from the roots of excess Zn-treated rice seedlings. (A) Representative 2-DE gel image of rice roots treated with Zn₂ for 4 days (from 5-6 DAS). The molecular weight (MW) in kilo Daltons (kDa) and isoelectric point (pI) are indicated on the left and top of the representative gel, respectively. Protein spots with abundance changed by at least 2-fold ($p < 0.05$) were indicated by numbered red marks. (B) Close-up view of some differentially abundant protein spots. Those up-regulated marked with ‘U’ and those down-regulated marked with ‘D’.

Fig. 3 Biological function and subcellular localization distributions of the differentially abundant proteins in rice roots under Zn stress. Each identified protein listed in Table 1 was functionally classified based on their known or putative biological function (A) and subcellular localization (B).

Fig. 4 Treemap of enriched GO terms for the biological process. More significant GO-terms are represented by bigger pie chart slices. Excess Zn mainly affected the abundance of proteins which are stress-responsive (abiotic and abiotic stimulus, and oxidative stress), followed by “aminoglycan metabolism”, “cellular homeostasis” and “iron ion transport”.

Fig. 5 Protein-protein interaction network of differently abundant proteins induced by excess Zn in rice roots. The network was obtained by online-tool STRING using default settings. The disconnected proteins are not included in the figure. The red border of protein nodes represents proteins being up-regulated and green border those down-regulated. The size of protein nodes corresponds to the number of protein-protein interactions. The full names of the proteins can be found in Table 1.

Fig. 6 The simplified schematic representation of differentially abundant proteins in rice roots grown in basic Kimura nutrient solution supplemented with Zn²⁺ (not all the detected proteins are included). The proteins marked red or green were up-regulated or down-regulated, respectively. ADK: putative adenosine kinase; AHCY: adenosylhomocysteinase; APO: aspartic proteinase oryzasin-1; APX1: putative cytosolic L-ascorbate peroxidase 1; APX2: L-ascorbate peroxidase 2, cytosolic; ARD2: 1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase 2; ASR5: abscisic stress-ripening protein 5; ATP0: mitochondrial F0 ATP synthase D chain; ATP1: ATP synthase subunit alpha; ATX1: copper transport protein ATX1; BAS: putative beta-alanine synthases; CFI: chalcone--flavonone isomerase; CPN: putative chaperonin 21; CS: putative cysteine synthase and cysteine synthase; FRK: fructokinase-2 ; HSP: putative heat shock cognate 70 kDa protein; IN2-1: protein IN2-1 homolog B; MRI1: methylthioribose-1-phosphateisomerase; NUO: putative NADH-ubiquinone oxidoreductase; OGC: oryzain gamma chain; OPR: putative 12-oxophytodienoate reductase 5; PDI: protein disulfide isomerase-like 1-1; PR10: root-specific pathogenesis-related protein 10; PRXC: peroxiredoxin-2C; SAMS: S-adenosylmethionine synthase 2; SOD1: superoxide dismutase [Cu-Zn] 1; SODCP: chloroplastic superoxide dismutase [Cu-Zn]; SUS: sucrose synthase 1; TCTP: translationally-controlled tumor protein homolog; TIL: temperature stress-induced lipocalin 1;

TPI: triosephosphate isomerase, cytosolic and triosephosphate isomerase; TS: tricin synthase 1; UB: ubiquitin family protein. The detailed information on the proteins can be found in Table 1. DMA: deoxymugineic acid; GSH: reduced glutathione; JA: jasmonic acid; NA: nicotianamine. Broken black lines indicate some of the steps (proteins) in the pathway are not included, and broken blue lines designate the potential interplay between various components involved in signaling, stress and defense responses, and cell death. “→” indicates “positive regulation”, “-|” represents “inhibition”, red “↑” indicates increased and green “↓” decreased content of metabolites determined by biochemical assays. Purple “↑” indicates suggested by gene expression analysis.

Electronic Supplementary Material

S1. Materials and methods

Table S1 Forward and reverse primers used for gene expression analysis in the roots of rice seedlings.

Table S2 Comparison of nominal and actual Zn exposure concentrations in Kimura B solution.

Table S3 Calculated Zn species (%) in the Kimura B nutrient solution supplemented with Zn as predicted by Visual MINTEQ 3.1.

Table S4 Metal content in rice seedling roots and shoots exposed to elevated Zn in the nutrient solution. The values followed by the same letter in each row are not statistically different ($p < 0.05$).

Table S5 The detailed information of the peptide sequence, expected and observed m/z values, sequence coverage for the differentially abundant proteins identified by MALDI-TOF/TOF MS in the roots of rice seedlings exposed to Zn excess. Spot numbers correspond to those in Table 2.

Table S6 Enriched GO terms clustered into representative terms (based on semantic similarity) by REVIGO used for the generation of Figure 4.

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Table S7 The detailed information of interaction network among the differentially abundant proteins generated by String 10.5. used for the generation of Figure 5. The name of the nodes corresponds to that in Table 1 and Figure 5.

Table S8 KEGG pathway enrichment analysis for the 42 differentially abundant proteins obtained by STRING 10.5. Pathways in bold were used for marking the protein nodes in Figure 5. Protein abbreviations correspond to those in Table 1 and Figure 5.

Fig. S1 (A) Cell death, (B) transcript abundance of *OsJAmyb*, (C) ethylene content, and (4) protein abundance change of PR10 in rice seedling roots exposed to elevated Zn.

Fig. S2 Gene Ontology (GO) enrichment analysis on the differentially abundant proteins (DAPs) in rice seedling roots under elevated Zn for the biological process by AgriGO. Among them, most DAPs are involved in cellular and metabolic process, however, “response to stimulus” was highly overrepresented GO term.

Fig. S3 Relative flavonoid content (absorbance at 510 nm relative to the control) in rice seedling roots.

Fig. S4 (A) Transcript abundance changes of *OsVDAC1* and (B) *OsAOX1a* in rice seedling roots under elevated Zn.