Proteomic analysis of the meristematic root zone in contrasting genotypes reveals new insights in drought tolerance in rice

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Abstract
Drought is responsible for major losses in rice production. Root tips contain meristematic and elongation zones that play major roles in determination of root traits and adaptive strategies to drought. In this study we analysed two contrasting genotypes of rice: IR64, a lowland, drought-susceptible, and shallow-rooting genotype; and Azucena, an upland, drought-tolerant, and deep-rooting genotype. Samples were collected of root tips of plants grown under control and water deficit stress conditions. Quantitative proteomics analysis resulted in the identification of 7294 proteins from the root tips of IR64 and 6307 proteins from Azucena. Data are available via ProteomeXchange with identifier PXD033343. Using a Partial Least Square Discriminant Analysis on 4170 differentially abundant proteins, 1138 statistically significant proteins across genotypes and conditions were detected. Twenty enriched biological processes showing contrasting patterns between two genotypes in response to stress were detected through gene ontology enrichment analysis. This included identification of novel proteins involved in root elongation with specific expression patterns in Azucena, including four Expansins and seven Class III Peroxidases. We also detected an antioxidant network and a metallo-sulfur cluster assembly machinery in Azucena, with roles in reactive oxygen species and iron homeostasis, and positive effects on root cell cycle, growth and elongation.

KEYWORDS
contrasting genotypes, drought, Oryza sativa, plant proteomics, rice, root tip, water-deficit stress

Abbreviations: BP, biological process; DAP, differentially abundant protein; GO, gene ontology; PLS-DA, partial least squares-discriminant analysis; ROS, reactive oxygen species; RWC, relative water content; VIP, variable of importance in projection.

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INTRODUCTION

Rice (Oryza sativa L.) is a staple food for nearly half of the world population [1]. Drought is a multidimensional environmental constraint and is responsible for major losses in rice production throughout the world [2]. Drought affects plant growth, development, and performance, eventually leading to less productivity [3]. Therefore, development of plants capable of tolerating conditions of water deficit while maintaining a high yield is a topic of international interest among geneticists and breeders [4]. There is considerable demand for a deep understanding of plant strategies in response to drought, in order to develop strategies for the preservation of their growth and survival in the face of water deficit [5].

Plant roots are the primary plant organs which detect soil condition alterations, and they play major roles in plant adaptation and productivity in water-limited environments [6, 7]. Hence, understanding the morphological, physiological, and molecular characteristics of the adaptation of roots to water stress will help to find better solutions for improving breeding programs for drought tolerance and enhancing yields under stress. Moreover, a deep-rooting system may help plants to avoid water stress by enabling them to acquire more water and nutrient resources while sufficient moisture remains available in deeper soil layers [8]. Therefore, knowledge of mechanisms regulating root structure may be useful for improving water use efficiency in crops under dry conditions [9]. Root tips contain meristematic and elongation zones wherein cells divide, grow and elongate, and are the most important parts of the roots in the determination of root traits and adaptive strategies to water-deficit stress [10].

Quantitative proteomic studies of roots of contrasting genotypes subjected to water deficit provide valuable insights into plant responses, and provide information on the biological pathways that participate in adaptation processes, eventually leading to stress-responsive and functional protein biomarker discovery [11]. Several proteomic studies have been conducted on roots of contrasting genotypes under drought in wheat [12], tomato [13], rice [14], barley [15], soybean [16], pearl millet [17], and sunflower [18]. There are also numerous proteomics studies which have been performed on rice roots under drought [14, 19–24]. However, these studies were mainly focused on whole roots and hence may have missed important signaling events localized to the root tips, which are considered to be of great functional significance.

Lowland rice accounts for 80% of the total rice produced worldwide, is generally grown under flooded conditions, and is susceptible to drought stress due to its shallow root distribution. Upland rice, however, is mainly planted in unbounded fields without irrigation facilities. Hence, upland rice, having encountered a greater risk of drought, has accumulated relatively greater morphological and genetic variations contributing to its drought resistant traits, including the development of deeper roots for enhanced water uptake [25].

In the present study, we selected two well-known contrasting genotypes of rice to compare their root proteomic responses under water stress: IR64, which is a lowland, drought susceptible, and shallow-rooting genotype; and Azucena, which is a traditional, upland, drought tolerant and deep-rooting genotype. We investigated the proteomic response of distal tips of IR64 and Azucena root under water-deficit and well-watered conditions, using TMT-based quantitative proteomic analysis to reveal candidate proteins associated with drought-adaptation strategies in rice roots.

EXPERIMENTAL SECTION

2.1 Plant material, water deficit treatment, and root tip sampling

Seeds of two contrasting genotypes of rice (Oryza sativa L.), IR64 (an indica-type, lowland, cultivated, shallow-rooting, and drought-sensitive genotype) and Azucena (a japonica-type, upland, traditional, deep-rooting, and drought-tolerant genotype) [26] were provided by the IRRI International Rice Genebank Collection (IRGC) in the Philippines. After seed germination on water-soaked filter paper, the uniformly-sized 7-day-old seedlings were transferred to Yoshida hydroponic solution at 22 to 25°C, relative humidity of 85% and a photoperiod of 16 h for 2 weeks. Two 20-day-old seedlings were transferred to root boxes (dimensions: 25 cm x 3 cm x 40 cm L x W x H) filled with a mix ratio of 1:1:2 of clay, peat moss, and sand, respectively. In a randomized design, water deficit treatment was imposed on 35-day-old plants by withholding water for 14 days where the level of field capacity was reduced to 25% to 35%. The control plants were watered regularly, with 200 plants, contained in 100 root boxes, grown for each treatment, and field capacity measured twice a day during the treatment period for at least 40 root boxes randomly [27].

At the end of the water-stress treatment, seminal root tips (5 mm) were dissected and collected into 1.5 mL tubes, immediately snap-frozen in liquid nitrogen, and then stored at -80°C. Root tip sections of approximately 60 plants were pooled as a biological replicate. Three biological replicates were considered for each genotype/condition. Sampling was performed on different dates (all between 8 and 11 a.m.)
to account for day-to-day variation within each genotype/condition and other environmental variables.

2.2 Preparation of protein samples

Total protein was isolated from 100 mg root tip tissue using Invitrogen Trizol Reagent (Thermo Fisher Scientific) as previously described [28]. Briefly, the powdered tissues were homogenized by Trizol and insoluble materials were removed by centrifugation at 13,000 × g for 10 min at 4°C. The supernatant solution was incubated with chloroform and then centrifuged for 15 min at 13,000 × g at 4°C. After removing supernatant RNA and DNA phases, absolute ethanol was added to the remaining protein phase followed by inverting for 1 min, incubating for 5 min at room temperature, and centrifuging for 5 min at 2000 × g at 4°C. The extracted proteins were precipitated by adding cold absolute acetone and incubating for 10 min at room temperature. Using 13,000 × g centrifuging, the extracted proteins were pelleted. The resulting pellets were washed with 0.3 M guanidine hydrochloride in 95% ethanol solution three times, followed by washing in absolute ethanol and then re-air-drying.

2.3 TMT labelling

The 12 biological samples (three well-watered IR64, three water-stressed IR64, three well-watered Azucena, three water-stressed Azucena) were labelled across four separate 10plex TMT labelling kits, as part of a larger study. Labels were assigned to samples as follows: TMT1, 126 = IR64 control 1, 127N = IR64 control 2, 127C = IR64 drought 1; TMT2, 126 = IR64 drought 2, 127N = IR64 drought 3, 127C = IR64 control 3; TMT3, 126 = Azucena control 1, 127N = Azucena control 2, 127C = Azucena control 3; TMT4, 126 = Azucena drought 2, 127N = Azucena drought 3, 127C = Azucena control 3.

Dried peptides from each sample were re-suspended in 100 mM HEPES buffer (pH 8.8) and their concentrations were measured using the peptide assay kit (Thermo Scientific- Rockford, IL, USA). TMT labelling was performed on 50 μg of peptides from each sample with 0.8 mg of reagent per tube. Labelling was performed with continuous vortexing for 1 h at room temperature and remaining TMT reagent was quenched by addition of hydroxylamine. Before pooling the samples, and to ensure equal amounts of total peptides are pooled from all samples, a “label check” experiment was performed by mixing 1.5 μL of each individually labelled TMT sample. The label check sample was vacuum dried, then desalted using Stage tips (Empore SDB-RPS). Samples were reconstituted in 2% ACN, 0.1% formic acid in water after vacuum dried, and analysed by LC-coupled to a mass spectrometer (Q-Exactive, Thermo Fisher, USA). A normalization factor was obtained from the label check experiment and the TMT-labelled peptide samples were pooled at a 1:1 ratio across all samples and vacuum dried. The samples were cleaned by desalting with C18 solid-phase extraction (SPE, Sep-Pak, Waters) and vacuum centrifuged to complete dryness.

After resuspending the peptide mixture in loading buffer (5 mM ammonia solution (pH 10.5), peptides were separated by High pH Reversed Phase chromatography using a Zorbax 300 Extend C18 column (3.5 μm particles, 2.1 × 150 mm), an Agilent 1260 HPLC system equipped with quaternary pump, a degasser and a Multi-Wavelength Detector (MWD) (set at 210, 214, and 280-nm wavelength). The separations were carried out on a 55-min linear gradient from 3% to 30% acetonitrile in 5 mM ammonia solution pH 10.5 at a flow rate of 0.3 mL/min Fractions were consolidated and dried by vacuum centrifugation, re-suspended in 0.1% formic acid [29].

2.4 Nanoflow liquid chromatography - Tandem mass spectrometry (Nano LC- MS/MS)

NanoLC-MS/MS was performed on a Q Exactive Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA, USA) coupled to an EASY-nLC1000 nanoflow HPLC system (Thermo Scientific, San Jose, CA, USA). Reversed-phase chromatography of labelled peptides was performed on an in-house packed reverse-phase column (75 μm × 15 cm Halo 2.7 μm 160 Å, Halo C18, Advanced Materials Technology) with linear gradients of Solvent A (97.9% water/2% acetonitrile/0.1% formic acid) and solvent B (99.9% acetonitrile/0.1% formic acid); 0% to 30% B over 110 min with a flow rate of 300 nL/min across the gradient. The Q Exactive mass spectrometer was operated in data-dependent acquisition (DDA) mode. A 2.6 kV electrospray voltage was applied via a liquid junction upstream of the column. Peptide precursors from 350 to 1850 m/z were scanned at 70k resolution with an AGC target value of 1 × 106. The ten most intense ions from the preceding survey scan were fragmented by Higher-Energy Collisional Dissociation (HCD) using normalized collision energy of 35 with an isolation width of 0.7 m/z. Only precursors with charge state +2 to +4 to MS/MS analysis. The MS method had a minimum signal required value of 2.5 × 104 for MS2 triggering, an AGC target value of 2 × 105 for MS2 and a maximum injection time of 250 ms for MS2, MS/MS scan resolution was set at 70,000, and dynamic exclusion was set to 90 s [30].

The raw mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository [31] with the dataset identifier PXD033343.

2.5 Database searching, peptide quantification, and statistical analysis

Processing of MS/MS raw spectrum data files was performed in Proteome Discoverer V2.1 (Thermo Scientific, San Jose, CA) using Mascot (Matrix Science, UK) for peptide to spectrum matching against the most recent predicted protein database of Oryza sativa ssp. japonica cv. Nipponbare from RAP-DB (IRGSP-1.0-2019-08-29, 42, 260 predicted protein sequences) (https://rapdb.dna.affrc.go.jp/). The MS1 tolerance was set to ± 10 ppm and the MS/MS tolerance to 0.02 Da. Carbamidomethylation of cysteine was set as fixed and oxidation of methionine and carboxamidation of protein N-terminal as variable modifications. The percolator algorithm was used to calculate statistics including FDR and posterior error probabilities, and search results were filtered to a peptide FDR of < 1%. Relative
quantitation of proteins was obtained through pairwise comparison of TMT reporter ion signal to noise (S/N) ratios using TMTPrepPro [32]. Proteins which were identified in all six biological replicates of one variety were considered for further analysis. In pairwise comparison tests, relative quantitation of protein abundance was derived from the ratio of the TMT labels detected in each condition and genotype, and differentially expressed proteins were identified based on Student t-tests between well-watered and water-stressed ratios. Differential expression required a t-test p-value < 0.05 [33, 34].

2.6 | Bioinformatics analysis of proteomic data

Proteins with a p-value < 0.05 were considered to be differentially increased or decreased in abundance. Pearson correlation and partial least square discrimination analysis (PLS-DA) [35] was carried out using the Web-based MetaboAnalyst software. PLS-DA was performed to find the greatest contributions of variables to the differences between samples based on the variable of importance in projection (VIPs) score value. GO enrichment analysis was performed using Cytoscape software (version 3.8.2) with the ClueGO V2.5.7 plug-in [36]. The p-value was calculated by two-sided hyper-geometric tests, and Benjamini-Hochberg adjustment was used for multiple test correction. GO terms with a p-value < 0.05 were considered significant. Heatmaps to illustrate proteins with significant differences in abundance between genotypes and conditions were generated using heatmap and RColorBrewer packages in the R environment.

2.7 | Parallel reaction monitoring analysis

A total of twelve drought-responsive proteins obtained from TMT-based quantitative proteomics were selected for targeted Parallel Reaction Monitoring (PRM) quantification using the same sample materials described earlier. Skyline ver. 20.1.1.175 was used to create an inclusion list of unique peptides, for each protein, with their mass to charge ratio [37]. The same Q-Exactive was operated in PRM mode, following the acquisition method and the parameters described in [38]. The obtained PRM data were imported into Skyline and subject to quality control analysis. The sum of areas of all transition peaks for each peptide were collected, log2-transformed, and a Student t-test analysis was used to compare stress versus control conditions.

3 | RESULTS

3.1 | Proteomic profiling of root tip under control and water stress conditions

In this study, two contrasting upland and lowland genotypes of rice (Azucena and IR64, respectively) were subjected to a 14-day water stress treatment. TMT-based quantitative proteomic profiling was performed on the root tips (5 mm) under well-watered and water-deficit conditions.

We identified 6307 and 7294 proteins from root tips of Azucena and IR64, respectively. Details of all proteins identified in this study and their abundance are provided in Table S1 for each genotype separately. Pearson correlation coefficients of the non-redundant total of 7815 proteins (reproducibly identified in at least one genotype under both control and stress conditions) are shown as a heatmap, representing the consistency between biological replicates of each sample (Figure 1A). Using a pairwise comparison (p-value < 0.05), 1759 and 3610 differentially abundant proteins (DAPs) between control and stress conditions were identified in the Azucena and IR64 genotypes, respectively. Details of all identified DAPs are provided in Table S2 (each genotype separately). The DAPs were visualized in a Venn diagram to distinguish between common and unique proteins belonging to each genotype (Figure 1B). A total of 560 and 2411 DAPs were unique to the Azucena and IR64, respectively, while 1199 proteins overlapped between the two genotypes.

3.2 | Multivariate data analysis of DAPs

A total of 1759 and 3610 DAPs were identified in the Azucena and IR64 genotypes in response to water stress, respectively. To find discriminating proteins with stronger effects on the separation of the genotypes (Azucena and IR64) and conditions (well-watered and water stress), a Partial Least Square Discriminant Analysis (PLS-DA) was applied to the nonredundant total of 4170 proteins which had significantly responded to water stress treatment in at least one genotype. The PLS-DA is a supervised model, which uses multivariate tools via linear combinations of original variables, and can predict class membership based on the largest predicted indicator variable. The prediction accuracies of this model were also assessed by cross-validation with different numbers of components. In the present study, the two-component system was employed for further analysis based on the values of accuracy (> 0.95), multiple correlation coefficients (R² > 0.98) and the explained variance in prediction (Q²) (> 0.96), revealing good discrimination and predictive ability of the model. PLS-DA also indicated the contribution of variables for the differences observed between the genotypes and conditions. The obtained score plot illustrated clustering of samples by genotypes and conditions (Figure 1C). The first two components accounting for 85.4% of the variation were sufficient for clear separations of the genotypes along component 1 and conditions along component 2. The PLS-DA analysis clearly delineated the distinct expression patterns between stress and normal conditions.

Proteins further from the origin were significantly important to the differences observed between groups and have a greater VIP score. A total of 1138 statistically significant responsive proteins with VIP scores greater than 1.0 were detected according to component 2 (Table S2), which can be attributed to the observed variability between genotypes and conditions.
Figure 1  (A) Distance matrix heatmap between all samples. Pearson correlation coefficients based on protein abundance between each pair of samples were shown as a heatmap and represented the consistency between biology replicates of each sample. A total of 7815 proteins identified in all six replicates (both conditions) of at least one genotype were used in this analysis. (B) Venn diagram indicating the number of unique and common DAPs in the root tips of IR64 and Azucena genotypes. (C) Partial Least Square Discriminant Analysis (PLS-DA) score plot revealing biological replicates closely aligned, and that 85.4% of the total variation among differentially abundant proteins could be attributed to treatment type. A total of 4170 proteins which significantly responded to water stress treatment, in at least one genotype, were considered. IRn, IR64 genotype under normal condition; IRs, IR64 genotype under water-deficit stress condition; AZn, Azucena genotype under normal condition; AZs, Azucena genotype under water-deficit stress condition.

3.3 Gene ontology (GO) enrichment and biological process (BP) analysis of responsive proteins based on the VIP score value

GO enrichment analysis was performed on the 1138 statistically significant responsive proteins with VIP scores greater than 1.0. GO terms with a p-value < 0.05 were considered significant. The sum of VIP score values of protein members of each enriched BP were calculated for abundant proteins in water stress conditions (increased in abundance in response to stress) and well-watered conditions (decreased in abundance in response to stress) of Azucena and IR64 genotypes, separately. From these, 22 significantly enriched BPs were identified showing contrasting patterns between two genotypes in response to water stress (Figure 2). Nine enriched BPs were abundant in IR64 in water stress conditions, including energy derivation by oxidation of organic compounds, tricarboxylic acid cycle, ATP generation, pyruvate metabolic process, hexose metabolic process, glyceraldehyde-3-phosphate metabolic process, gluconeogenesis, glutamine family amino acid biosynthetic process, and arginine metabolic process.

In Azucena, seven different BPs were abundant in water stress conditions including RNA export from nucleus, multicellular organism process, proteasomal ubiquitin-independent protein catabolic process, cellular oxidant detoxification, hydrogen peroxide catabolic process, iron-sulfur cluster assembly, and metallo-sulfur cluster assembly. We
also found some significant BPs for which the protein abundance decreased in response to water stress, such as ribosome biogenesis and RNA export from nucleus in IR64, and Golgi vesicle transport, nucleotide-sugar metabolic process, cytoplasmic translational initiation, protein localization to endoplasmic reticulum and post-transcriptional regulation of gene expression in Azucena (Figure 2).

We surveyed expression patterns of the members of five important BPs that were specifically upregulated in Azucena under stress treatment compared to IR64, including multicellular organism process, cellular oxidant detoxification, hydrogen peroxide catabolism process, iron-sulfur cluster assembly, and metallo-sulfur cluster assembly (Figure 2C). As shown in Figure 2C, out of 18 protein members of cellular oxidant detoxification and hydrogen peroxide catabolism process BPs, 15 proteins were significantly increased in abundance in Azucena in response to water stress, while unchanged or decreased in abundance in IR64. These include seven Class III peroxidases (PRX12, PRX15, PRX16, PRX61, PRX89, PRX91, and PRX123), two ascorbate peroxidases (APX1 and APX5), two methionine sulfoxide reductases (MSRA4 and MSRB5), along with glutaredoxin-C6, Late embryogenesis abundant protein 17 (OsLEA17), and Metallothionein-like protein 4C, all of which are involved in minimizing the deleterious effects of excess ROS production. These proteins play vital roles in a wide range of plant antioxidant networks that work together to detoxify ROS and maintain ROS homeostasis in different subcellular compartments, such as the protein-repairing methionine sulfoxide reductase pathway, ascorbate-glutathione cycle, and peroxidase and glutaredoxin pathways (Figure 4). Taken together, all these data reinforced the ability of Azucena to withstand oxidative stress through a highly efficient antioxidant defense system, contributing to its tolerance to long-lasting water stress and capability of coping with increasing ROS levels.

Another BP specifically upregulated in Azucena root tips was multicellular organism process. Through surveying the members of this BP, we found four expansin family proteins (EXPB4, EXPB6, EXPB7, and EXPB17) which were significantly increased in abundance in Azucena root tips in response to water stress, while unchanged or decreased in abundance in IR64 (Figure 3B). Expansin family members are involved in cell wall loosening and cell expansion and elongation in extracellular regions (Figure 4), which reinforces the importance of these proteins as candidates potentially involved in root length and response to water stress conditions. Moreover, seven class III peroxidases (PRX12, PRX15, PRX16, PRX61, PRX89, PRX91, and PRX123) located in the extracellular region (Figure 4) were also increased in abundance in Azucena root tips (Figure 2C). Based on major functions of class III peroxidases in either cell elongation and cell wall stiffening, they are also potentially important candidates for enhancing rice root elongation under drought.

As shown in Figure 3C, the BPs iron-sulfur cluster assembly and metallo-sulfur cluster assembly, which play important roles in enhancing drought tolerance and root growth, revealed an increase in abundance in Azucena and a decrease in abundance in IR64. Of the 19 protein members of these clusters, six proteins were specifically upregulated in Azucena while unchanged in IR64, including OsCASPL1D1, SDH2-1, OsISC37, OsISC12, Os03g0775500, and OsISC5. Additionally, 10 proteins were found to be decreased in in IR64 while unchanged in Azucena, namely OsFd4, Os07g0406800, RPS14, Os04g0596400, OsISC44, OsGLT1, OsFd3, GRXS1, OSPOLA, and ELP3 (Figure 3C).

3.4 Parallel reaction monitoring validation of protein abundance changes of drought-responsive proteins

Twelve candidate proteins associated with drought stress were selected for PRM analysis to validate DAPs obtained from proteomics analysis (Table 1, Figure 5). The results indicated that the differential changes in abundance of catalase isozyme A (Q0E4K1), Os01g0916400 protein (Q8RZW7), and Os02g0595800 protein (Q6Z1S1) agree with the proteomics data both in IR64 and Azucena. PRM analysis showed that the abundance of Os05g0151200 protein (B7F8G3), Os09g0491772 protein (B9G4B3), heat shock cognate 70 kDa protein 2, putative, expressed (Q84TA1), glutathione reductase, cytosolic (P48642), and leucine aminopeptidase (Q84TA3) changed significantly in IR64, which agrees with the proteomics result. Similarly, Os04g059800 protein (Q7XL7Z) was upregulated in...
FIGURE 3  (A) Heatmap showing the change in abundance patterns of the corresponding proteins (with gene names) belonging to cellular oxidant detoxification and hydrogen peroxide catabolic process BPs. Grey colour depicts proteins with no significant change under stress compared to control condition. (B) Heatmap showing the change in abundance patterns of the corresponding proteins (with their gene names) belonging to multi-cellular organism process BP. Grey colour depicts proteins with no significant change under stress compared to control condition. (C) Heatmap showing the change in abundance patterns of the corresponding proteins (with their gene names) belonging to iron-sulfur cluster assembly and metal-co-sulfur cluster assembly BPs. Grey colour depicts proteins with no significant change under stress compared to control condition. IRn, IR64 genotype under normal condition; IRs, IR64 genotype under water-stress condition; Azn, Azucena genotype under normal condition; Azs, Azucena genotype under water-stress condition.

Azucena both in proteomics and PRM results. The remaining proteins followed the same trend in proteomics and PRM results (up or down), even though these proteins were not statistically significantly changed either in proteomics or PRM results. Overall, the PRM results supported the TMT-based quantitative proteomics, which confirms the reliability of the datasets.

4 | DISCUSSION

In the present study we investigated the proteomic response of rice root tips to water stress in two contrasting genotypes; Azucena, a drought-tolerant genotype with a deep-rooting system, and IR64, a drought-susceptible genotype with a shallow-rooting system. In a previous study [39], we reported a higher level of leaf RWC, reduction in root and shoot dry weight, and a higher growth and elongation rate of roots under the 14-day water stress condition in Azucena compared to IR64.

In a related previous study [40] we performed transcriptomic analysis of Azucena and IR64 root tips, and found that Azucena appears to enhance cell growth as a means of attempting to avoid water stress, while IR64 appears to rely on cell wall thickening to maintain root integrity under water stress. A total of 288 differentially expressed genes were found to be co-localised with QTLs related to root system architecture, which had been previously reported under both drought stress and normal conditions. From a developmental biology perspective, root tips contain meristematic and elongating cells where cell division, growth and expansion occur. Thus, compared to other root parts, root tips play vital roles in response and adaptation to water stress and are enriched in gene products involved in several root traits such as length, thickness and angle [41]. The aim of this study was to develop a better understanding of key proteins with vital roles in adaptation strategies by stress tolerant rice genotypes under water-limited environments.

In this report, the proteomic analysis of the root tip led to the identification of > 6000 proteins in both genotypes, with many showing
abundance significantly increased or decreased under water stress conditions. Using PLS-DA, a multivariate dimensionality-reduction tool which is a useful feature selector and classifier for omics data analyses [42], we focused on the discriminating DAPs with stronger effects on the separation of the genotypes and conditions. The DAPs with VIP scores > 1 were involved in important biological pathways, and showed specific patterns in each genotype. Upregulation of energy derivation, ATP generation, and hexose and amino acid metabolic pathways in IR64 was observed, which may be a response to the lost energy in response to water stress, while in Azucena, ROS homeostasis and growth maintenance are the main strategies in the face of stress. We performed a detailed investigation on those pathways that belonged to Azucena, the stress -tolerant genotype, and found important proteins with possible roles in drought adaptation and deep-rooting systems that were involved in cellular oxidant detoxification, hydrogen peroxide catabolic process, multicellular organism process and iron-sulfur cluster assembly.

4.1 Highly efficient detoxification of reactive oxygen species as a successful strategy to adapt to water stress in root tips of Azucena

Our results showed that 18 statistically significant proteins (VIP score > 1) were involved in cellular oxidant detoxification and hydrogen peroxide catabolic process, and most of them belonged to the
A total of twelve drought-responsive proteins were selected for PRM validation including Os05g01512000 protein (B7FG3), Os09g0491772 protein (B9G4B3), Os04g0589800 protein (Q7XL7), catalase isozyme A (Q0E4K1), L-ascorbate peroxidase 1, cytosolic (Q0N21), heat shock cognate 70 kDa protein 2, putative, expressed (Q84TA1), cysteine desulfurase (Q6ERL4), glutathione reductase, cytosolic (P48642), ARF GAP-like zinc finger-containing protein ZIGA2, putative, expressed (Q10N88), leucine aminopeptidase (Q84TA3), Os01g0916400 protein (Q8RZW7), and Os02g0595800 protein (Q6Z151). * and ** show significant and non-significant differences, respectively, between control and drought-stressed samples indicated by t-test analysis for p-value < 0.05.

### TABLE 1
Comparing the abundance of several DAPs subjected to TMT-based quantitative proteomics and PRM

| Uniprot ID | IRn versus IRs | PRM | A2n versus A2s |
|------------|---------------|-----|----------------|
| B7FG3      | UP*           | UP* | UP*            |
| B9G4B3     | DOWN*         | DOWN* | DOWN*         |
| Q7XL7      | UP*           | UP* | UP*            |
| Q0E4K1     | UP*           | UP* | UP*            |
| Q0N21      | UP*           | UP* | UP*            |
| Q84TA1     | UP*           | UP* | UP*            |
| Q6ERL4     | UP*           | UP* | UP*            |
| P48642     | UP*           | UP* | UP*            |
| Q10N88     | DOWN*         | DOWN* | DOWN*         |
| Q84TA3     | UP*           | UP* | UP*            |
| Q8RZW7     | UP*           | UP* | UP*            |
| Q6Z151     | DOWN*         | DOWN* | DOWN*         |

A total of twelve drought-responsive proteins were selected for PRM validation including Os05g01512000 protein (B7FG3), Os09g0491772 protein (B9G4B3), Os04g0589800 protein (Q7XL7), catalase isozyme A (Q0E4K1), L-ascorbate peroxidase 1, cytosolic (Q0N21), heat shock cognate 70 kDa protein 2, putative, expressed (Q84TA1), cysteine desulfurase (Q6ERL4), glutathione reductase, cytosolic (P48642), ARF GAP-like zinc finger-containing protein ZIGA2, putative, expressed (Q10N88), leucine aminopeptidase (Q84TA3), Os01g0916400 protein (Q8RZW7), and Os02g0595800 protein (Q6Z151). * and ** show significant and non-significant differences, respectively, between control and drought-stressed samples indicated by t-test analysis for p-value < 0.05.

Azucena increased in abundance category. Fifteen proteins showed increased abundance in Azucena, including two ascorbate peroxidases (APX1 and APX5), two methionine sulfoxide reductases (MSRA4 and MSRB5), seven class III peroxidases (PRX12, PRX15, PRX16, PRX61, PRX89, PRX91, and PRX123), along with glutaredoxin-C6. Late embryogenesis abundant protein 17/OsLEA17), and Metallothionein-like protein 4C. These proteins are known to play important roles in plant antioxidant networks and work together to minimize the deleterious effects of excess ROS production (Figure 4). In a previous study, enzyme assays also indicated a greater extent of antioxidant activity in Azucena in response to water stress compared to IR64. Moreover, significant lower levels of H2O2, electrolyte leakage and thiobarbituric acid reactive substance contents under 14-day water-stress conditions had been previously recorded in Azucena in comparison with IR64 [39].

An important consequence of water stress is the closure of stomata leading to a restriction in CO₂ fixation and the excessive production of reactive oxygen species (ROS). ROS can be accumulated in different plant organs such as leaves, stems and roots. In rice, roots and stems seem to be two main organs of ROS production, which might be related to their adaptation to the aquatic environment [43]. ROS are involved in maintaining normal plant growth, and improving their tolerance to stress [44]. At times when plants are incapable of performing ROS scavenging, ROS levels may reach a high concentration and cause oxidative damage to proteins, DNA and lipids, eventually leading to growth retardation and cell death [45]. Under steady-state physiological conditions, ROS are detoxified by various antioxidative defence mechanisms [46]. The balance between the ROS production and detoxification may be disturbed by various abiotic and biotic stresses [47].

Drought-tolerant rice genotypes demonstrate comparatively higher oxidant detoxification activities than susceptible genotypes [39, 48–53]. An interesting report showed that roots of IR64 presented a greater level of H2O2 and lipid peroxidation, and reduced activity of the antioxidative system, under treatment with aluminium when compared to Azucena [54]. Although several drought-induced proteins involving in oxidative stress detoxification have also been reported via proteomic analysis in various plants [55], we still need to identify more of these proteins to better understand this very important mechanism for manipulation of sensitive genotypes towards enhancing stress tolerance. Furthermore, antioxidative enzymes include many proteins whose function in responding to stress has not yet been confirmed.

Our results showed that the abundance of two members of the ascorbate peroxidase family (APX1 and APX5) specifically increased in the root tip of Azucena under stress while remaining unchanged in IR64. APXs (belonging to the family of heme peroxidases) play a key role in catalysing the conversion of H₂O₂ into H₂O using ascorbate as a specific electron donor. APXs are components of the Ascorbate-Glutathione (ASC-GSH) cycle, or Foyer-Halliwell-Asada pathway, which is the most well-characterized route for H₂O₂ metabolism in plants [56].

Two methionine sulfoxide reductases involved in reversing methionine oxidation (MSRA4 and MSRB5) were increased in abundance only in the root tip of Azucena in response to stress. ROS is capable of oxidizing the lateral chain of amino acids leading to altering the functions and conformation for many signalling proteins. Methionine, a sulfur-containing amino acid, is sensitive to oxidative damage due to its high reactivity with ROS. Methionine can be readily oxidized to methionine sulfoxide (MetSO) by hydrogen peroxide, while methionine sulfoxide is reduced back to methionine by two types of methionine sulfoxide reductases (MSRs), A and B, specific to the S- and R-diastereomers of MetSO, respectively, through reduct- active cysteines (Figure 4) [57].

We also found a glutaredoxin enzyme (GRXC6) in Azucena, which is a member of the oxidant detoxification pathway. Glutaredoxins, along with the thioredoxins and peroxiredoxins, employ a three-step catalytic cycle to maintain an appropriate level of H₂O₂, and are part of a protective mechanism to prevent over-oxidation of the amino acids (especially cysteine), thus minimizing permanent protein damage (Figure 4) [58].

Seven class III peroxidases (PRX12, PRX15, PRX16, PRX89, PRX91, and PRX123) were identified as being increased in abundance in Azucena root tip in response to stress. Class III peroxidases, which are efficient components of the antioxidative system induced in response to environmental stress, such as drought, catalyse the oxidation of a variety of substrates by H₂O₂. Peroxidases catalyse the reduction of H₂O₂ by transporting electrons to various donor molecules [59, 60]. All together, these results highlight the importance of the ROS detoxification enzymes in rice root growth and adaptation to water stress.
4.2 DAPs associated with enhanced root elongation in Azucena under water stress conditions

We found that four β-expansin family proteins (EXPB4, EXPB6, EXPB7, and EXPB17) were significantly increased in abundance in Azucena root tips. These proteins were decreased in abundance or showed no significant changes in IR64. Expansins are extracellular proteins and are grouped into two related clades, the α- and β-expansins. They are known to play a role in cell wall loosening, by disturbing hydrogen bonds between cellulose microfibrils and matrix polymer, and turgor-driven cell expansion and elongation in plants, especially in roots [61, 62]. The role of both α- and β-expansins in root growth and elongation has been studied many times under different conditions in different plants such as soybean [63], Arabidopsis [64–66], Maize [67, 68], and rice [69, 70]. However, the α-expansins have been investigated more than the β-expansins. Recently, it has been reported that the coordinated action of these two expansin clades (α- and β-expansins) may be necessary to induce plant root elongation [71]. In the present study, we identified four β-expansins as proteins involved in root elongation (EXPB4, EXPB6, EXPB7, and EXPB17), for consideration in further analysis. The necessity of EXPB4 and EXPB6 for rapid internodal elongation of deep water rice during submergence, and their roles in the elongation of rice mesocotyl and coleoptile, have been reported [72, 73], but there is as yet no information concerning the role of these proteins in root elongation induction.

As mentioned before, seven class III peroxidases (PRX12, PRX15, PRX16, PRX61, PRX89, PRX91, and PRX123), another extracellular protein family, with specific upregulation in Azucena root tips, were detected in response to water stress and thus might be involved in root elongation. Class III peroxidases, in addition to oxidant detoxification function and ROS homeostasis, are involved in a broad range of plant growth and development. Peroxidase isoenzymes (which are the only H₂O₂-scavenging enzymes in the extracellular space) are located in the cell wall and apoplast, play key roles in cell wall cross-linking and loosening, and are responsible either for cell elongation or cell wall stiffening [60, 74, 75]. Peroxidases are glycoproteins synthesized in the
endoplasmic reticulum and are transported via the Golgi apparatus to the extracellular space. They regulate plant growth and defense under stress conditions through redox homeostasis. Recent work on a set of PRXs in Arabidopsis thaliana roots suggested the important role of PRXs in cell differentiation during root development [76]. There is close cooperation between ROS homeostasis and the early stages of the cell cycle [77] and thus root growth and development [44]. Differences in superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) content in the root tip of Arabidopsis significantly affect primary root growth and differentiation [78]. The balance between H$_2$O$_2$ and O$_2^-$ is required for cell division and elongation in the root tips through modulating the balance between cell proliferation and differentiation [76]. Once the level of H$_2$O$_2$ reaches a certain level, cells stop dividing and begin to elongate [78], while the accumulation of high concentrations of H$_2$O$_2$ leads to a variety of perturbations in growth and development. It has been reported that high levels of H$_2$O$_2$ in root tips resulted in abnormal root growth [79]. It has also been reported that ROS homeostasis is directly related to root apical meristem size, growth and maintenance [80, 81], lateral root development and elongation [82], crown root development, emergence and elongation [83], root hair elongation and development [84], and controlling the root tip stem cell activity [85, 86]. Several studies have shown that class III peroxidases mainly expressed in the cell wall of roots promoted cell elongation and root length in Arabidopsis [74, 87, 88], and one report showed a relationship between decreased root elongation in IR64 with increasing H$_2$O$_2$ content and lipid peroxidation, and lower levels of antioxidant system activities compared to Azucena, under treatment with aluminium [54].

Taken together, by integrating morphological data and root tip protein profile, we suggest four β-expansins and seven class III peroxidases as candidate proteins that are potentially involved in rice root elongation under water stress conditions, warranting further exploration of their potential to enhance drought adaptation in rice.

4.3 | The proteins involved in iron-sulfur cluster assembly machineries may enhance root growth and drought tolerance in Azucena

In our results it was clear that the BPs iron-sulfur cluster assembly and metallo-sulfur cluster assembly were increased in abundance in Azucena and decreased in abundance in IR64. We identified six proteins specifically increased in abundance in Azucena while not changed in IR64 (including OsCASPL1D1, SDH2-1, OsISC37, OsISC12, Os03g0775500, and OsISC5), and 10 proteins which were decreased in abundance in IR64 while not changed in Azucena (OsFd4, Os07g0406800, RPS14, Os04g0596400, OsISC44, OsGLT1, OsFd3, GRXS1, OSPOLA, and ELP3).

Fe-S clusters are prosthetic groups, built of iron atoms and acid-labile inorganic sulfide. The iron atoms of Fe-S clusters usually interact with cysteine residues of polypeptide chains [89]. The Fe-S cluster enables the proteins to function in many essential biological processes in higher plants such as iron homeostasis, electron transfer, catalysis, protein stabilization respiration, photosynthesis, nitrogen fixation, DNA repair, and metabolic pathways [89–91].

The proteins involved in the Fe-S cluster biogenesis machineries may represent an efficient way for the regulation of cellular processes in response to either alterations in the intracellular conditions or to extracellular stimuli [92]. Genes involved in iron-sulfur cluster assembly have been reported as targets of abiotic stress [93]. Previous findings have shown that Fe–S cluster assembly and iron homeostasis are regulated by drought stress in plants [94, 95] and there is still much more investigative work required to understand their role in response to drought.

These clusters also have regulatory roles, modulating gene expression in response to oxidative stress as a consequence of salinity and drought. Oxidative stress increases expression of a subset of Fe–S cluster assembly genes in rice roots, thereby improving stress tolerance. Fe–S cluster assembly genes have thus been considered as potential candidate genes for molecular breeding of rice to enhance stress tolerance [93]. An iron-sulfur cluster scaffold protein gene, when introduced into a salt-sensitive sweet potato, greatly increased its salt tolerance by regulating osmotic balance, protecting membrane integrity and photosynthesis, and activating the ROS scavenging system [96].

Iron homeostasis as a result of iron-sulfur cluster assembly is essential for crop productivity under stress conditions. Iron acts as an important cofactor of different antioxidant enzymes like ascorbate peroxidase (APX), peroxidase (PRX), and catalase (CAT) [97, 98], which play unique roles in ROS scavenging, thereby enhancing cell defense mechanisms against oxidative stress [99]. Moreover, iron homeostasis is integrated with the production of ROS, and has emerged as a major parameter controlling ROS homeostasis and oxidative stress [100, 101] in Arabidopsis root tips, which eventually plays major roles in root growth and architecture [102]. The root tip zone is considered as the main action site for iron homeostasis and is more sensitive than other zones, but the mechanism underpinning this remains largely unknown.

Iron homeostasis affects root growth through NO-mediated alterations in K$^+$ homeostasis in Arabidopsis [103]. Data analyses based on previous findings showed that there is an interaction between iron homeostasis and root system architecture in Arabidopsis, which is in part mediated by the H$_2$O$_2$/O$_2$ balance between the proliferation and differentiation zones in root tips. The levels of superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) content in the root tip significantly affect primary root growth and root system architecture remodelling [78], while disturbing iron homeostasis decreases primary root length and root meristem size [104].

5 | CONCLUDING REMARKS

Detailed analyses of the proteome responses of root tip to water stress in two contrasting genotypes of rice, IR64 (a susceptible and shallow-rooting genotype) and Azucena (a tolerant and deep-rooting genotype), led to the identification of a number of DAPs and pathways which
FIGURE 6  Schematic representation of the differential adaptation strategies of Azucena and IR64 in response to water stress

may be involved in water stress adaptation and root elongation. Using multivariate data analysis of DAPs, 1138 statistically significant stress responsive proteins were detected. In addition, 22 BPs specifically enriched in each genotype in response to water stress were obtained by GO enrichment analysis. This analysis revealed that the proteins involved in cellular oxidant detoxification, root elongation and iron-sulfur cluster assembly were specifically increased in abundance in root tips of Azucena, the tolerant genotype, in response to water stress. Through surveying the members of these pathways, we identified fifteen proteins with increased abundance in Azucena, including two ascorbate peroxidases (APX1 and APX5), two methionine sulfoxide reductases (MSRA4 and MSRB5), seven class III peroxidases (PRX12, PRX15, PRX16, PRX61, PRX89, PRX91, and PRX123), along with glutaredoxin-C6, Late embryogenesis abundant protein 17 (OsLEA17), and Metallothionein-like protein 4C. These proteins play important roles in plant antioxidant networks that work together to minimize the deleterious effects of excess ROS production. In addition, we identified four β-expansin family proteins (EXPB4, EXPB6, EXPB7, and EXPB17), and seven class III peroxidases, with specific upregulation in Azucena root tips which may play important roles in root elongation. Six proteins of the iron-sulfur cluster assembly pathway (OsCASPL1D1, SDH2-1, OsISC37, OsISC12, Os03g0775500, and OsISC5), which revealed an upregulated pattern in Azucena and a downregulated pattern in IR64, may also play roles in enhancing drought tolerance and root growth. In conclusion, our analyses showed that the efficient oxidant detoxification and iron and sulfur cluster assembly systems in Azucena under water stress led to ROS homeostasis and cell growth and elongation in root tips, which caused root growth and elongation, aimed at capturing water from deeper soil layers. In IR64, the ROS accumulation due to loss of the oxidant detoxification system led to oxidative stress, cell damage and root growth inhibition, as summarised in Figure 6.

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The raw mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD033343.

CONFLICT OF INTEREST
The authors declare no conflict of interest.
Mass Spectrometric data are available via ProteomeXchange with identifier PXD033343.

REFERENCES

1. Gross, B. L., & Zhao, Z. J. (2014). Archaeological and genetic insights into the origins of domesticated rice. Proceedings of the National Academy of Sciences of the United States of America, 111(17), 6190–6197.
2. Lesk, C., Rowhani, P., & Ramankutty, N. (2016). Influence of extreme weather disasters on global crop production. Nature, 529(7584), 84–87.
3. Micaletti, A., Naghavi, M. R., Toorchi, M., Zolla, L., & Rinalducci, S. (2018). Metabolomics and proteomics reveal drought-stress responses of leaf tissues from spring-wheat. Scientific reports, 8(1), 5710.
4. Martignago, D., Rico-Medina, A., Blasco-Escámez, D., Fontanet-Manzaneque, J. B., & Caño-Delgado, A. I. (2020). Drought resistance by engineering plant tissue-specific responses. Frontiers in Plant Science, 10, 1676.
5. Hamanishi, E. T., & Campbell, M. M. (2011). Genome-wide responses of leaf tissues from spring-wheat. Forestry, 84(3), 273–283.
6. Manavalan, L. P., Guttikonda, S. K., Nguyen, V. T., Shannon, J. G., & Nguyen, H. T. (2010). Evaluation of diverse soybean germplasm for root growth and architecture. Plant and Soil, 330(1), 503–514.
7. Prince, S. J., Song, L., Qiu, D., Maldonado Dos Santos, J. V., Chai, C., Krajewski, P., Ogrodowicz, P., Krystkowiak, K., Surma, M., Adamski, T., Bednarek, K., Marczak, L., Ciesiolka, D., Kuczynska, A., Mikolajczak, P., & Stobiecki, M. (2016). Analysis of drought-induced proteomic and metabolomic changes in barley (Hordeum vulgare L.) leaves and roots unravels some aspects of biochemical mechanisms involved in drought tolerance. Frontiers in Plant Science, 7, 1108.
8. Yu, X., Yang, A., & James, A. T. (2017). Comparative proteomic analysis of drought response in roots of two soybean genotypes. Crop and Pasture Science, 68(7), 609–619.
9. Ghatak, A., Chaturvedi, P., Bachmann, G., Valledor, L., Rasmak, Z., Bazargani, M. M., Bajaj, P., Jegadeesan, S., Li, W., Sun, X., Gruden, K., Varshney, R. K., & Weckwerth, W. (2020). Physiological and proteomic signatures reveal mechanisms of superior drought resilience in pearl millet compared to wheat. Frontiers in Plant Science, 11, 600278.
10. Gaffari, M., Toorchi, M., Valizadeh, M., & Komatsu, S. (2013). Differential response of root proteome to drought stress in drought sensitive and tolerant sunflower inbred lines. Frontiers in Plant Biology, 4(6), 609–617.
11. Agrawal, L., Gupta, S., Mishra, S. K., Pandey, G., Kumar, S., Chauhan, P. S., Chakrabarty, D., & Nautiyal, C. S. (2016). Elucidation of complex nature of PEG induced drought-stress response in rice root using comparative proteomics approach. Frontiers in Plant Science, 7, 1446.
12. Dhakarey, R., Raorane, M. L., Treumann, A., Peethambaran, P. K., Schendel, R. R., Saih, V. P. H., Hause, B., Bunzel, M., Henry, A., Kohli, A., & Riemann, M. (2017). Physiological and proteomic analysis of the rice mutant cpm2 suggests a negative regulatory role of jasmonic acid in drought tolerance. Frontiers in Plant Science, 8, 1903.
13. Mirzaei, M., Soltani, N., Sarhari, E., Pascovici, D., Keighley, T., Salekdeh, G. H., Haynes, P. A., & Atwell, B. J. (2012). Shotgun proteomic analysis of long-distance drought signaling in rice roots. Journal of Proteome Research, 11(11), 348–358.
14. Paul, S., Gayen, D., Datta, S. K., & Datta, K. (2015). Dissecting root proteome of transgenic rice cultivars unravels metabolic alterations and accumulation of novel stress responsive proteins under drought stress. Plant Science, 234, 133–143.
15. Raorane, M. L., Pabuayon, I. M., Varadarajan, A. R., Mutte, S. K., Kumar, A., Treumann, A., & Kohli, A. (2015). Proteomic insights into the role of the large-effect QTL qDTY 12.1 for rice yield under drought. Molecular Breeding, 35(6), 1–14.
16. Singh, A., Shamim, D. M., & Singh, K. N. (2013). Genotypic variation in root anatomy, starch accumulation, and protein induction in upland rice (Oryza sativa) varieties under water stress. Agricultural Research, 1(1), 24–30.
17. Sandar, M. M., Ruangsmi, R., Chutteang, C., Arunyanark, A., Toojinda, T., & Siangliw, J. L. (2022). Root characterization of Myanmar upland and lowland rice in relation to agronomic and physiological traits under drought stress condition. Agronomy, 12(5), 1230.
18. Fonta, J. E., Giri, J., Vejchasarn, P., Lynch, J. P., & Brown, K. M. (2022). Spatiotemporal responses of rice root architecture and anatomy to drought. Plant and Soil, 1–22.
19. Puertas, J., Alarcón, E. K., Davies, W. J., & Dodd, I. C. (2017). Applying drought to potted plants by maintaining suboptimal soil moisture improves plant water relations. Journal of Experimental Botany, 68(9), 2413–2424.
20. Xiong, J., Yang, Q., Kang, J., Sun, Y., Zhang, T., Margaret, G., & Ding, W. (2011). Simultaneous isolation of DNA, RNA, and protein from Medicago truncatula L. Electrophoresis, 32(2), 321–330.
21. Mirzaei, M., Pushpitha, K., Deng, L., Chitranshi, N., Gupta, V., Rajput, R., Mangani, A. B., Dheer, Y., Godinez, A., McKay, M. J., Kamath, K., Pascovici, D., Wu, J. X., Salekdeh, G. H., Karl, T., Haynes, P. A., Graham, S. L., & Gupta, V. K. (2019). Upregulation of proteolytic pathways and altered protein biosynthesis underlie retinal pathology in a mouse model of Alzheimer’s disease. Molecular Neurobiology, 56(9), 6017–6034.
22. Muralidharan, S., Thompson, E., Raftos, D., Birch, G., & Haynes, P. A. (2012). Quantitative proteomics of heavy metal stress responses in Sydney rock oysters. Proteomics, 12(6), 906–921.
31. Perez-Riverol, Y., Bai, J., Bandla, C., Garcia-Seisdedos, D., Hewapathirana, S., Kamatchinathan, S., Kundu, D. J., Prakash, A., Freiricks-Zipper, A., Eisenher, M., Walzer, M., Wang, S., Brazma, A., & Vitkain, J. A. (2022). The PRIDE database resources in 2022: A hub for mass spectrometry-based proteomics evidences. Nucleic Acids Research, 50(D1), D543–D552.

32. Mirzaei, M., Pascovici, D., Wu, J. X., Chick, J., Wu, Y., Cooke, B., Haynes, P., & Molloy, M. P. (2017). TMT one-stop shop: From reliable sample preparation to computational analysis platform. Methods in Molecular Biology, 1549, 45–66.

33. Kammers, K., Cole, R. N., Tiengwe, C., & Ruczinski, I. (2015). Detecting significant changes in protein abundance. EuPA Open Proteomics, 7, 11–19.

34. Margolin, A. A., Ong, S. E., Schenone, M., Gould, R., Schreiber, S. L., Carr, S. A., & Golub, T. R. (2009). Empirical Bayes analysis of quantitative proteomics experiments. Plos One, 4(10), e7454.

35. Saccenti, E., & Timmerman, M. E. (2016). Approaches to sample size determination for multivariate data: Applications to PCA and PLS-DA of omics data. Journal of Proteome Research, 15(8), 2379–2393.

36. Bindea, G., Mlecnik, B., Hackl, H., Charoentong, P., Tosolini, M., Kirilovsky, A., Fridman, W. H., Pages, F., Trajanowski, Z., & Galon, J. (2009). ClueGO: A Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. Bioinformatics, 25(8), 1091–1093.

37. MacLean, B., Tomazela, D. M., Shulman, N., Chambers, M., Finney, G. L., Frewen, B., Kern, R., Tabb, D. L., Liebler, D. C., & MacCoss, M. J. (2010). Skyline: An open source document editor for creating and analyzing targeted proteomics experiments. Bioinformatics, 26(7), 966–968.

38. Masoomi-Aladizgeh, F., McKay, M. J., Asar, Y., Haynes, P. A., & Atwell, B. J. (2022). Patterns of gene expression in pollen of cotton (Gossypium hirsutum) indicate downregulation as a feature of thermotolerance. The Plant Journal, 109(4), 965–979.

39. Abdirad, S., Majd, A., Irian, S., Hadidi, N., & Salekdeh, G. H. (2020). Differential adaptation strategies to different levels of soil water deficit in two upland and lowland genotypes of rice: A physiological and metabolic approach. Journal of the Science of Food and Agriculture, 100(4), 1458–1469.

40. Abdirad, S., Ghaffari, M. R., Majd, A., Irian, S., SoleymaniNiya, A., Daryani, P., Koobaz, P., Shoobar, Z.-S., Farsad, L. K., & Yazdanapanah, P. (2022). Genome-wide expression analysis of root tips in contrasting rice genotypes revealed novel candidate genes for water stress adaptation. Frontiers in Plant Science, 49.

41. Takehisa, H., Sato, Y., Igashishi, M., Abiko, T., Antonio, B. A., Kamatsuki, K., Minami, H., Namiki, N., Inukai, Y., Nakazono, M., & Nagamura, Y. (2012). Genome-wide transcriptome dissection of the rice root system: Implications for developmental and physiological functions. Plant Journal, 69(1), 126–140.

42. Ruiz-Perez, D., Guan, H., Madhivanan, P., Mathee, K., & Narasimhan, G. (2020). So you think you can PLS-DA? Bmc Bioinformatics [Electronic Resource], 21(Suppl 1), 2.

43. Yamauchi, T., Yoshioika, M., Fukazawa, A., Mori, H., Nishizawa, N. K., Tsutsumi, N., Yoshioika, H., & Nakazono, M. (2017). An NADPH oxidase RBOH functions in rice roots during Lysigenous Aerenchyma Formation under oxygen-deficient conditions. Plant Cell, 29(4), 775–790.

44. Huang, H., Ullah, F., Zhou, D. X., Yi, M., & Zhao, Y. (2019). Mechanisms of ROS regulation of plant development and stress responses. Frontiers in Plant Science, 10, 800.

45. Foyer, C. H., & Noctor, G. (2005). Redox homeostasis and antioxidant signaling: A metabolic interface between stress perception and physiological responses. Plant Cell, 17(7), 1866–1875.

46. Halliwell, B. (2006). Reactive species and antioxidants. Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. Plant Physiology, 141(2), 312–322.

47. Sharma, P., Jha, A. B., Dubey, R. S., & Pessarakli, M. (2012). Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. Journal of Botany, 217037.

48. Kibria, M. G., Hossain, M., Murata, Y., & Hoque, M. A. (2017). Antioxidant defense mechanisms of salinity tolerance in rice genotypes. Rice Science, 24(3), 155–162.

49. Lum, M. S., Hanafi, M. M., Rafii, Y. M., & Akmar, A. S. N. (2014). Effect of drought stress on growth, proline and antioxidant enzyme activities of upland rice. The Journal of Animal and Plant Sciences, 24(5), 1487–1493.

50. Mishra, S. S., & Panda, D. (2017). Leaf traits and antioxidant defense for drought tolerance during early growth stage in some popular traditional rice landraces from Koraput, India. Rice Science, 24(4), 207–217.

51. Nahar, S., Vemireddy, L. R., Sahoo, L., & Tanti, B. (2018). Antioxidant protection mechanisms reveal significant response in drought-induced oxidative stress in some traditional rice of Assam, India, India. Rice Science, 25(4), 185–196.

52. Saddique, M. A. B., Ali, Z., Sher, M. A., Farid, B., Ikrar, R. M., & Ahmad, M. S. (2020). Proline, total antioxidant capacity, and OsPSCS gene activity in radical and plumule of rice are efficient drought tolerance indicator traits. International Journal of Agronomy, 8682792.

53. Wang, X., Liu, H., Yu, F., Hu, B., Jia, Y., Sha, H., & Zhao, H. (2019). Differential activity of the antioxidant defence system and alterations in the accumulation of osmolyte and reactive oxygen species under drought stress and recovery in rice (Oryza sativa L.) tillering. Scientific Reports, 9(1), 8543.

54. Ma, B., Gao, L., Zhang, H., Cui, J., & Shen, Z. (2012). Aluminum-induced oxidative stress and changes in antioxidant defenses in the roots of rice varieties differing in Al tolerance. Plant Cell Reports, 31(4), 687–696.

55. Laxa, M., Liebthal, M., Telman, W., Chibani, K., & Dietz, K. J. (2019). The role of the plant antioxidant system in drought tolerance. Antioxidants (Basel), 8(4), 94.

56. Foyer, C. H., & Noctor, G. (2011). Ascorbate and glutathione: The heart of the redox hub. Plant Physiology, 155(1), 2–18.

57. Tarrago, L., Laugier, E., & Rey, P. (2009). Protein-repairing methionine sulfoxide reductases in photosynthetic organisms: Gene organization, reduction mechanisms, and physiological roles. Molecular Plant, 2(2), 202–217.

58. Farooq, M. A., Niazi, A. K., Akhtar, J., Saifullah, Farooq, M., Souri, Z., Karimi, N., & Rengel, Z. (2019). Acquiring control: The evolution of ROS-induced oxidative stress and redox signaling pathways in plant stress responses. Plant Physiology and Biochemistry, 141, 353–369.

59. Passardi, F., Theiler, G., Zamocky, M., Cosio, C., Rouhier, N., Teixeira, F., Margis-Pinheiro, M., Ioannidis, V., Penel, C., Falquet, L., & Dunand, C. (2007). Peroxibase: The peroxibase database. Phytochemistry, 68(12), 1605–1611.

60. Veljovic-Jovanovic, S. V., Kukavcica, B., Vidovic, M., Morina, F., & Menchhoff, L. (2018). Class III peroxidases: Functions, localization and redox regulation of isoenzymes. In D. K. Gupta, J. M. Palma & F. J. Corpas (Eds.), Antioxidants and antioxidant enzymes in higher plants (pp. 269–300). Cham: Springer.

61. Cosgrove, D. J. (1996). Plant cell enlargement and the action of expansins. Bioessays, 18(7), 533–540.

62. McQueen-Mason, S., & Cosgrove, D. J. (1994). Disruption of hydrogen bonding between plant cell wall polymers by proteins that induce wall extension. Proceedings of the National Academy of Sciences of the United States of America, 91(14), 6574–6578.
63. Lee, D. K., Ahn, J. H., Song, S. K., Choi, Y. D., & Lee, J. S. (2003). Expression of an expansin gene is correlated with root elongation in soybean. Plant Physiology, 131(3), 985–997.

64. Lin, C., Choi, H. S., & Cho, H. T. (2011). Root hair-specific EXPANSIN A7 is required for root hair elongation in Arabidopsis. Molecules and Cells, 31(4), 393–397.

65. Liu, W., Xu, L., Lin, H., & Cao, J. (2021). Two expansin genes, AtEXPA4 and AtEXPB5, are redundantly required for pollen tube growth and AtEXPA4 is involved in primary root elongation in Arabidopsis thaliana. Genes (Basel), 12(2), 249.

66. Pacifici, E., Di Mambro, R., Dello Ioio, R., Costantino, P., & Sabatini, (2001). Modification of expansin transcript levels in the maize primary root at low water potentials. Plant Physiology, 126(4), 1471–1479.

67. Wang, Y., Ma, N., Qiu, S., Zou, H., Zhang, G., Kang, Z., Wang, G., & Huang, J. (2014). Regulation of the I2-expsin gene OeEXP8 expression affects root system architecture in transgenic rice plants. Molecular Breeding, 34(1), 47–57.

68. Wu, Y., Thorne, E. T., Sharp, R. E., & Cosgrove, D. J. (2001). Microtubule network organization of polysaccharides from type I and type II primary cell walls. Journal of Plant Biology, 48(3), 304–310.

69. Wu, Y., Thorne, E. T., Sharp, R. E., & Cosgrove, D. J. (2001). Modification of expansin transcript levels in the maize primary root at low water potentials. Plant Physiology, 126(4), 1471–1479.

70. Yang, W., Xu, L., Lin, H., & Cao, J. (2021). Two expansin genes, AtEXPA4 and AtEXPB5, are redundantly required for pollen tube growth and AtEXPA4 is involved in primary root elongation in Arabidopsis thaliana. Genes (Basel), 12(2), 249.

71. Kam, M. J., Yun, H. S., Kaufman, P. B., & Chang, S. C. (2005). Two expansins, EXP1 and EXP2, are correlated with the growth and development of maize roots. Journal of Plant Biology, 48(3), 304–310.

72. Wu, Y., Thorne, E. T., Sharp, R. E., & Cosgrove, D. J. (2001). Modification of expansin transcript levels in the maize primary root at low water potentials. Plant Physiology, 126(4), 1471–1479.

73. Xiong, Q., Ma, N., Qiu, S., Zou, H., Zhang, G., Kang, Z., Wang, G., & Huang, J. (2014). Regulation of the I2-expsin gene OeEXP8 expression affects root system architecture in transgenic rice plants. Molecular Breeding, 34(1), 47–57.

74. ZhiMing, Y., Bo, K., XiaoWei, H., ShaOLei, L., YouHuang, B., WoNa, D., Ming, C., HuangYang, C., & Ping, W. (2011). Root hair-specific expansins modulate root hair elongation in rice. Plant Journal, 66(5), 725–734.

75. Kozlova, L. V., Nazipova, A. R., Gorshkov, O. V., Petrova, A. A., & Gorshkova, T. A. (2020). Elongating maize root: Zone-specific combinations of polysaccharides from type I and type II primary cell walls. Scientific Reports, 10(1), 10956.

76. Lee, Y., & Kende, H. (2001). Expression of I2-expsins is correlated with internodal elongation in deepwater rice. Plant Physiology, 127(2), 645–654.

77. Xiong, Q., Ma, B., Lu, X., Huang, Y. H., He, S. J., Yang, C., Yin, C. C., Zhao, H., Zhou, Y., Zhang, W. K., Wang, W. S., Li, Z. K., Chen, S. Y., & Zhang, J. S. (2017). Ethylene-inhibited jasmonic acid biosynthesis promotes mesocotyl/coleoptile elongation of etiolated rice seedlings. Plant Cell, 29(5), 1053–1072.

78. Passardi, F., Tognolli, M., De Meyer, M., Penel, C., & Dunand, C. (2006). Two cell wall-associated peroxidases from Arabidopsis influence root elongation. Planta, 223(5), 965–974.

79. Welinder, K. G. (1992). Superfamily of plant, fungal and bacterial peroxidases. Current Opinion in Structural Biology, 2(3), 388–393.

80. Tsukagoshi, H., Busch, W., & Benfey, P. N. (2010). Transcriptional regulation of ROS controls transition from proliferation to differentiation in the root. Cell, 143(4), 606–616.

81. de Simone, A., Hubbard, R., de la Torre, N. V., Velapann, Y., Wilson, M., Considine, M. J., Sopp, W. J. J., & Foyer, C. H. (2017). Redox changes during the cell cycle in the embryonic root meristem of Arabidopsis thaliana. Antioxidants & Redox Signaling, 27(18), 1505–1519.

82. Dunand, C., Crevecoeur, M., & Penel, C. (2007). Distribution of superoxide and hydrogen peroxide in Arabidopsis root and their influence on root development: Possible interaction with peroxidases. New Phytologist, 174(2), 332–341.

83. Ahammad, G. J., He, B. B., Qian, X. J., Zhou, Y. H., Shi, K., Zhou, J., Yu, J. Q., & Xia, X. J. (2017). 24-Epibrassinolide alleviates organic pollutants-retarded root elongation by promoting redox homeostasis and secondary metabolism in Cucumis sativus L. Environmental Pollution, 229, 922–931.

84. Tognetti, V. B., Bielach, A., & Hrtyan, M. (2017). Redox regulation at the site of primary growth: Auxin, cytokinin and ROS crosstalk. Plant, Cell & Environment, 40(11), 2586–2605.

85. Yu, Q., Tian, H., Yue, K., Liu, J., Zhang, B., Li, X., & Ding, Z. (2016). A P-Loop NTPase regulates quiescent center cell division and distal stem cell identity through the regulation of ROS homeostasis in Arabidopsis root. Plos Genetics, 12(9), e1006175.

86. Manzano, C., Pallero-Baena, M., Casimiro, I., De Rybel, B., Orman-Ligeza, B., Van Isterdael, G., Beeckman, T., Draye, X., Casero, N., & Del Pozo, J. C. (2014). The emerging role of reactive oxygen species signaling during lateral root development. Plant Physiology, 165(3), 1105–1119.

87. Steffens, B., Kovalev, A., Gorb, S. N., & Sauter, M. (2012). Emerging roots alter epidermal cell fate through mechanical and reactive oxygen species signaling. Plant Cell, 24(8), 3296–3306.

88. Steffens, B., Kovalev, A., Gorb, S. N., & Sauter, M. (2012). Emerging roots alter epidermal cell fate through mechanical and reactive oxygen species signaling. Plant Cell, 24(8), 3296–3306.

89. Balk, J., & Lobreau, S. (2005). Biogenesis of iron-“sulfur proteins in plants. Trends in Plant Science, 10(7), 324–331.

90. Balasubramanian, R., Chen, G., Bryant, D. A., & Golbeck, J. H. (2006). Regulatory roles for IscA and SufA in iron homeostasis and redox stress responses in the cyanobacterium Synechococcus sp. strain PCC 7002. Journal of Bacteriology, 188(9), 3182–3191.

91. Beinert, H., & Kiley, P. J. (1999). Fe-S proteins in sensing and regulatory functions. Current Opinion in Chemical Biology, 3(2), 152–157.

92. Coudurier, J., Touraine, B., Briat, J. F., Gaymard, F., & Rouhier, N. (2013). The iron-sulfur cluster assembly machineries in plants: Current knowledge and open questions. Frontiers in Plant Science, 4, 259.

93. Liang, X., Qin, L., Liu, P., Wang, M., & Ye, H. (2014). Genes for iron-sulfur cluster assembly are targets of abiotic stress in rice, Oryza sativa. Plant, Cell & Environment, 37(3), 780–794.

94. Song, Z., Yang, Y., Xu, J., Ma, R., & Yu, M. (2014). Physiological and transcriptional responses in the iron-sulfur cluster assembly pathway under abiotic stress in peach (Prunus persica L.) seedlings. Plant Cell, Tissue and Organ Culture, 117(3), 419–430.

95. Xu, L., Dong, Z., Chiniquy, D., Pierroz, G., Deng, S., Gao, C., Diamond, S., Simmons, T., Wipf, H. M., Caddell, D., Varoquaux, N., Madera, M. A., Hutmacher, R., Deutschbauer, A., Dahlberg, J. A., Guerinot, M. L., Purdom, E., Banfield, J. F., Taylor, J. W., & Lemaux, P. G. (2021). Genome-resolution metagenomics reveals role of iron metabolism in drought-induced rhizosphere microbiome dynamics. Nature Communication, 12(1), 3209.

96. Liu, D., Wang, L., Liu, C., Song, X., He, S., Zhai, H., & Liu, Q. (2014). An Ipomoea batatas iron-sulfur cluster scaffold protein gene, IbNFU1, is involved in salt tolerance. Plos One, 9(4), e93935.
97. Kumar, P., Tewari, R. K., & Sharma, P. N. (2010). Sodium nitroprusside-mediated alleviation of iron deficiency and modulation of antioxidant responses in maize plants. (plq002) AoB Plants.

98. Sharma, S. S., Kaul, S., Metwally, A., Goyal, K. C., Finkemeier, I., & Dietz, K. J. (2004). Cadmium toxicity to barley (Hordeum vulgare) as affected by varying Fe nutritional status. Plant Science, 166(5), 1287–1295.

99. Scandalios, J. G. (1990). Response of plant antioxidant defense genes to environmental stress. Advanced Genetics, 28, 1–41.

100. Briat, J. F., Duc, C., Ravet, K., & Gaymard, F. (2010). Ferritins and iron storage in plants. Biocimica et Biophysica Acta, 1800(8), 806–814.

101. Ravet, K., Touraine, B., Boucherez, J., Briat, J. F., Gaymard, F., & Cellier, F. (2009). Ferritins control interaction between iron homeostasis and oxidative stress in Arabidopsis. Plant Journal, 57(3), 400–412.

102. Shen, N., Hou, S., Tu, G., Lan, W., & Jing, Y. (2021). Transcription factor WRKY33 mediates the phosphate deficiency-induced remodeling of root architecture by modulating iron homeostasis in arabidopsis roots. International Journal of Molecular Sciences, 22(17), 9275.

103. Zhang, L., Li, G., Wang, M., Di, D., Sun, L., Kronzucker, H. J., & Shi, W. (2018). Excess iron stress reduces root tip zone growth through nitric oxide-mediated repression of potassium homeostasis in Arabidopsis. New Phytologist, 219(1), 259–274.

104. Reyt, G., Boudouf, S., Boucherez, J., Gaymard, F., & Briat, J. F. (2015). Iron- and ferritin-dependent reactive oxygen species distribution: Impact on Arabidopsis root system architecture. Molecular Plant, 8(3), 439–453.

SUPPORTING INFORMATION
Additional supporting information may be found online https://doi.org/10.1002/pmic.202200100 in the Supporting Information section at the end of the article.