RESEARCH ARTICLE

iTRAQ-based quantitative proteomic analysis of wheat roots in response to salt stress

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Salinity is a major abiotic stress that affects plant growth and development. Plant roots are the sites of salt uptake. Here, an isobaric tag for a relative and absolute quantitation based proteomic technique was employed to identify the differentially expressed proteins (DEPs) from seedling roots of the salt-tolerant genotype Han 12 and the salt-sensitive genotype Jimai 19 in response to salt treatment. A total of 121 NaCl-responsive DEPs were observed in Han 12 and Jimai 19. The main DEPs were ubiquitination-related proteins, transcription factors, pathogen-related proteins, membrane intrinsic protein transporters and antioxidant enzymes, which may work together to obtain cellular homeostasis in roots and to determine the overall salt tolerance of different wheat varieties in response to salt stress. Functional analysis of three salt-responsive proteins was performed in transgenic plants as a case study to confirm the salt-related functions of the detected proteins. Taken together, the results of this study may be helpful in further elucidating salt tolerance mechanisms in wheat.

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1 Introduction

Bread wheat (*Triticum aestivum* L.) is one of the world's most important cereal grain crops and is an important source of proteins, carbohydrates, and minerals, serving as the staple food source for 30% of the human population [1]. It is important to optimize wheat yield, but the achievement of this goal is under constant challenge because the crops are exposed to both biotic and abiotic stresses. Abiotic stresses, such as salinity, decrease wheat growth and productivity by reducing water uptake and causing nutrient disorders and ion toxicity in many regions. This finding has led to research on salt stress with the aim of increasing salt tolerance in wheat by genetic engineering.

The identification of novel salt-tolerant genes and gaining an understanding of their functions in salt stress adaptation will provide us with the basis for effective engineering strategies to improve crop stress tolerance [2]. The sequencing of the wheat genome offers a global view of wheat genes [3,5]. In addition, high-throughput transcriptomic studies have provided large amounts of data describing the mRNA expression levels of genes related to salt tolerance in many plants [6]. However, because of post-transcriptional events and post-translational modifications, the mRNA levels do not usually correlate with the protein expression levels, which are more directly related with the signaling and metabolic processes in response to salt stress conditions [6]. As a necessary and complementary approach in the postgenomic era, proteomics technologies have been utilized to study global protein expression levels in response to salt stress to obtain an understanding of the complex mechanisms of plant salt responses

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Significance of the study

In this study, the iTRAQ-based proteomic technique was employed to identify the DEPs from seedling roots of salt-tolerant and salt-sensitive genotypes in response to salt stress. One hundred and twenty-one NaCl-responsive DEPs were identified, and a synergetic mechanism explaining the response of roots to salt stress was proposed. A functional analysis of three salt-responsive proteins was performed in transgenic plants. More salt-responsive proteins were detected in this study using the highly sensitive iTRAQ-based proteomic technique, which is helpful for deciphering the molecular networks of plant salt tolerance and further elucidating the salt tolerance mechanisms in wheat. Moreover, proteomic studies could provide the foundation for detecting candidate salt-tolerance genes. *TaPPDK, TaLEA1* and *TaLEA2* may be useful for improving plant salt tolerance.

and tolerance [6]. In particular, the "isobaric tags for relative and absolute quantitation" (iTRAQ) technology coupled with liquid chromatography-quadrupole mass spectrometry (LC-MS/MS) enables the direct quantification and comparison of protein levels from samples with greater efficiency and accuracy [7, 8] compared with gel-based techniques. In 2011, the iTRAQ method was used only in one of 64 original research articles on plant salinity responsive proteomics [6]; however, from 2012–2016, 13 manuscripts analyzed ten plants, namely sugar beet, tomato, cucumber, maize, rice, cotton, *Arabidopsis*, *Brassica napus*, Halophyte *Halogeton glomeratus*, and halophyte *Tangut nitraria*, to determine the responses of different proteins to salt stress using the iTRAQ method (References in Additional file 1 in Supporting Information).

Plant roots in saline soil are the primary point of contact with ionic toxicity and osmotic stress, and they can rapidly respond to the stress to maintain functionality and transmit the stress signal to the shoot for appropriate changes in shoot function [9]. Using an integrated functional analysis of salt-responsive proteins identified in 34 plant species, researchers found that the highest percentage of stress- and defense-related proteins is expressed in the root compared with the leaf, shoots, unicells and seedlings [9]. A comprehensive survey of the root proteome in response to salinity stress will help improve our understanding of salt tolerance in wheat.

Using proteomic analyses, 905 salt-responsive proteins in the roots of 14 plant species were identified, which provided essential information at the protein level and a better understanding of the salt responses in roots, and highlighted the proteomic findings regarding the molecular mechanisms in the fine-tuned salt-responsive networks [9]. However, proteomic studies on wheat roots under salt stress are limited. Wang et al. [10] identified 49 differentially expressed salt-responsive proteins between seedling roots of wheat cultivars Shanrong No. 3 and Jinan 177 in response to treatment with 200 mM salt for 24 h. A comparative analysis of the proteomic dynamics between salt-tolerant and salt-sensitive wheat varieties with 2DE and MALDI-TOF-TOF MS showed that some proteins were salt responsive, with significant changes in expression in both varieties, as well as some variety-specific changes [11, 12]. The authors also found that some salt-responsive proteins were significantly up-regulated in salt-tolerant wheat in response to salt stress, whereas they were down-regulated in salt-sensitive wheat [11]. The root microsomal proteomes of wheat under salt stress were analyzed by 2DE and mass spectrometry. The results showed that the expression of a wheat V-H+-ATPase E subunit protein was increased in response to salt stress [13]. Further confirmation of its salt-tolerant function was provided, as overexpression of the wheat V-H+-ATPase E subunit by transgenic *Arabidopsis thaliana* was able to enhance seed germination, root growth and adult seedling growth in response to salt stress [13].

In this study, an iTRAQ-based proteomic technique was used to identify the salt stress responsive proteins in seedling roots of two wheat varieties. The results showed that the proteome of wheat roots under salt stress was complex and provided an improved understanding of the molecular mechanisms involved in the tolerance of the plant to salt stress. Meanwhile, the further functional elucidation of three DEPs, namely LEA1, LEA2 and PPDK, was performed by overexpressing the genes in *Arabidopsis*. The overexpression of *TaPPDK1*, *TaLEA1*, and *TaLEA2* could confer salt tolerance in transgenic plants, which validated the proteomic results and showed that the iTRAQ-based proteomic technique was reliable for identifying salt-tolerant genes in plants.

2 Materials and methods

2.1 Plant materials and NaCl treatment

The design of this study is shown in Additional file 2 (Supporting Information). Jimai 19, national approval wheat variety (2003014), is a major wheat cultivars grown in the Huanghai wheat production area and extends over 5 million hectare in China. Han 12 was approved by the Variety Approval Committee of Hebei Province of China in 2013, and is protected by national plant variety rights (announcement number CNA010879E). Wheat [*T. aestivum* L. cv. Jimai 19 (salt-sensitive) and Han 12 (salt-tolerant)] seeds were allowed to germinate in the dark in a thermostatically controlled

chamber at 25 ± 1°C for approximately 70 h. The germinated seeds were grown in plastic containers containing complete Kimura B nutrient solution [24] under white light (150 μmol photons m^{-2} s^{-1}; 14-h light/10-h dark photoperiod) at 25°C in a growth chamber. After the full development of the three leaf stage of each plant, the wheat seedlings were treated as follows: (i) control plants were grown in Hoagland's solution (Control), and (2ii) salt-treated plants were grown in Hoagland's solution plus 350 mM NaCl (Salt). NaCl was added directly to the nutrient solution at the beginning of the treatment. The solution was changed to a fresh solution each day. For the proteomic analysis, the roots from 60 control (untreated) and treated plants were harvested after 4 days of NaCl treatment and washed with distilled water three times before being immersed in liquid nitrogen; the samples were stored at –80°C for further use. Two independent biological replicates were performed for the 4-plex iTRAQ labeling experiment to validate the results.

2.2 Salt tolerance evaluation

The responses to salt stress were evaluated by measuring the chlorophyll content after 4 days of NaCl treatment. Leaf chlorophyll was extracted with 80% acetone, and the contents of the a and b types of chlorophyll were determined spectrophotometrically at 663 and 645 nm, respectively [12].

Jimai 19 and Han 12 were grown in saline-alkaline soil in natural fields and normal fields (ck) using a randomized complete block design with three replicates to evaluate the salt tolerance throughout the growing season. The area of each plot was 135 m^2. The average soil salt content was 0.4%. Wheat yields in saline-alkaline (Yield-salt) and normal fields (Yield-ck) were measured at harvest. The salt tolerance index (STI) was calculated as Yield-salt/Yield-ck.

2.3 iTRAQ and LC-MS/MS analysis

2.3.1 Protein preparation

Proteins were extracted using the protocol reported by Jiang et al. [14]. The fresh roots of each sample were ground into a fine power in liquid nitrogen. The resulting powder was suspended (100 mg in 1 mL) in chilled (−20°C) 10% TCA in acetone containing 0.07% dithiothreitol (DTT). The mixture was incubated at −20°C overnight. After centrifugation at 10 000 × *g* for 30 min at 4°C, the protein pellet was washed three times with chilled acetone. After centrifugation at 10 000 × *g* for 20 min between rinses, the supernatant was discarded and the pellet was vacuum-dried. Approximately, 2 mg of dried powder was solubilized (5:1) in SDT lysis buffer (4% SDS, 1 mM DTT, and 100 mM Tris-HCl, pH 7.6), and ultrasonicated on ice for 10 s and then incubated on ice for 15 s for a total of ten cycles. The solution was placed in a boiling-water bath for 5 min and centrifuged immediately at 14 000 × *g* for 30 min. The supernatant was used for protein qualification using the BAC-method.

2.3.2 Trypsin digestion and iTRAQ isobaric labeling

Proteins were digested according to the FASP method, as previously described [15]. Total protein samples (300 μg) diluted in 30 μL of SDT buffer (4% SDS, 1 mM DTT, and 100 mM Tris-HCl, pH 7.6) were incubated in boiling water for 5 min. After cooling to room temperature, 200 μL of UA buffer (8 M urea and 150 mM Tris-HCl, pH 8.0) was added, and the mixture was transferred onto a 30 kDa filter (Sartorius, Germany) for ultrafiltration. The samples were centrifuged at 14 000 × *g* for 15 min and washed again with UA buffer. Then, 100 μL of 50 mM iodoacetamide in UA buffer was added and vortexed for 1 min at 600 rpm. The samples were incubated for 30 min at room temperature in the dark and centrifuged at 14 000 × *g* for 20 min. Two wash steps with 100 μL UA buffer were performed with centrifugation at 14 000 × *g* for 20 min after each wash step. Then, 100 μL of DS buffer (50 mM triethylammonium bicarbonate, pH 8.5) was added to the filters and the samples were centrifuged at 14 000 × *g* for 10 min. This step was repeated twice. Finally, 2 μg of trypsin (Promega) in 40 μL of DS buffer was added to each filter. The samples were incubated at 37°C for 16–18 h. The filter unit was transferred to a new tube and centrifuged at 14 000 × *g* for 20 min. The resulting peptides were collected as a filtrate, and the peptide concentration was analyzed at OD280.

Subsequently, a 30-μg peptide mixture was labeled with iTRAQ reagents using the iTRAQ Reagent 8-plex mutiplex kit (Applied Biosystems, USA), according to the manufacturer's instructions. For each cultivar (i.e., wheat cv. Han 12 or Jimai 19), four samples were labeled with iTRAQ reagents: 113-, 114-, 115-, and 121-iTRAQ tags for the control and salt-treated replicate 1 and replicate 2 in cv. Han 12; and 116-, 117-, 118- and 119-iTRAQ tags for the control and salt-treated replicate 1 and replicate 2 in cv. Jimai 19. Two independent biological experiments were performed. The labeled samples were incubated at room temperature for 1 h. The peptide mixtures were then pooled and dried by vacuum centrifugation.

2.3.3 SCX chromatography separation of the iTRAQ-Labeled Peptides

The dried peptide mixture was reconstituted and acidified with 2 mL of buffer A (10 mM KH_2PO_4 in 25% of ACN, pH 3.0) and loaded onto a 4.6 × 100 mm Polysulfethyl column (5 μm, 200 Å, PolyLC Inc., Maryland, USA). The peptides were eluted at a flow rate of 1 mL/min with a gradient of 0−10% buffer B (500 mM KCl and 10 mM KH_2PO_4 in 25% of ACN, pH 2.7) for 2 min, 10−20% buffer B for 10 min, 20−45% buffer B for 5 min, and 45−100% buffer B for 5 min. The elution was monitored by determining the absorbance at 214 nm, and fractions were collected every 1 min.

The collected fractions (approximately 30 fractions) were finally combined into ten pools and desalted on C18 Cartridges (Empore SPE Cartridges C18 (standard density), 7-mm inner diameter, 3 mL volumes, Sigma). All of the fractions were dried and stored at –20°C until further analysis.

2.3.4 Nano-LC-MS/MS analysis

The samples were separated using a nanoscale HPLC system (EASY-nLC from Proxeon Biosystems) connected to an Orbitrap Q Exactive equipped with a nanoelectrospray source (Thermo Fisher Scientific). Each dried peptide sample was reconstituted in mobile phase A (0.1% formic acid) and then loaded onto an EASY C18 column (2 cm × 100 μm, 5 μm) (Thermo Scientific) with a flow rate of 15 μL/min. The peptides were then separated on an EASY C18 column (75 μm × 100 mm, 3 μm) (Thermo Scientific). Mobile phases A (0.1% formic acid) and B (84% ACN and 0.1% formic acid) were used to establish a 120 min gradient elution with a flow rate of 250 nL/min. The gradient began with 0 to 35% B for 100 min, increased to 100% B in the following 8 min, and hold at 100% B from 108 to 120 min. The HPLC effluent was directly electrosprayed into the mass spectrometer.

The Q Exactive MS instrument (Thermo Finnigan) was used in the data-dependent mode to automatically switch between one full-scan MS and 10 MS/MS acquisitions. After the accumulation of a 'target value' of 3×10^6, a survey of the full-scan MS spectra (m/z 300–1800) was obtained with a resolution R of 70 000 at m/z 200. In the octopole collision cell, the ten most intense peptide ions (charge states ≥ 2) were sequentially isolated to a maximum target value of 5×10^5 by pAGC (predictive Automatic Gain Control) and fragmented using higher-energy collisional dissociation (HCD) [16]. Dynamic exclusion was 40.0 s. The maximum allowed ion accumulation times were 100 ms for full scans and 60 ms for HCD. All HCD fragment ion spectra were recorded with a resolution of 17 500 at m/z 200. The normalized collision energy was 30 eV, and the under-fill ratio, which specifies the minimum percentage of the target value likely to be reached at the maximum fill time, was defined as 0.1%.

2.3.5 Sequence database search and data analysis

MS/MS spectra were searched against the Uniprot triticum database using Proteome Discoverer1.3 (Thermo) and the MASCOT engine (Matrix Science, London, U.K.; version 2.3.02). The following search parameters were used: peptide mass tolerance at ±20 ppm, MS/MS tolerance at 0.1 Da, trypsin enzyme with up to 2 missed cleavages, fixed modification of iTRAQ 8-plex (K), iTRAQ 8-plex (N-term), variable modification of oxidation (M), and a Reverse decoy database pattern. All reported data were based on 99% confidence levels for protein and peptide identification as determined by the false discovery rate (FDR) of no more than 1%; 2*N(decoy)/((N(decoy) + N(target)) was used as the formula for computing FDR protein identification and was supported by the identification of at least one unique peptide.

The protein ratios were normalized to each separate ratio in each experiment using the overall median ratio for all of the peptides in the sample. The ratio for a given protein was calculated by taking the average of all of the peptide ratios that were used to identify the protein. All the proteins identified in this study were provided in Additional file 3 (Supporting Information). The differentially expressed proteins were identified based on a *t*-test. Each protein was attributed a total of four expression ratios with corresponding p-values. A twofold cutoff and a *p*-value less than 0.05 were used to determine the significant changes in abundance for the regulated proteins.

2.4 Reverse transcription-PCR (RT-PCR)

DNaseI-treated RNA was used for first strand cDNA synthesis using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and oligo (dT)18 primers according to the manufacturer's protocol. Between 24 and 28 cycles of PCR amplification were performed using gene-specific primers. The *actin* gene was amplified as an internal control. The sequences of all primers used for the RT-PCR analysis can be found in Additional file 4 (Supporting Information).

2.5 Validation of the functions of three salt-tolerant candidate genes

2.5.1 Vector construction and transformation into Arabidopsis

The *TaLEA1*, *TaLEA2* and *TaPPDK1* genes were subcloned into the pCAMBIA3301 and pGFPGUS expression vectors. The gene-specific primers, restriction sites, expression vectors, and antibiotics that were used to construct the overexpression vectors are shown in Table 1.

The constructs were transformed into *Agrobacterium tumefaciens* GV3101 using the freeze–thaw method and then transformed into *Arabidopsis*. Transgenic *Arabidopsis* were selected using MS selective medium containing 25 mg^-/L phosphinothricin or 25 mg^-/L hygromycin and further confirmed by RT–PCR.

2.5.2 Evaluation of salt tolerance of transgenic Arabidopsis

The seeds of both the wild-type and the homozygous transgenic *Arabidopsis* were collected, sown on MS medium for the germination assay and root growth assay or planted in soil for the seedling salt-tolerance assay [17]. The experiments were performed in triplicate.

Table 1. Primers, restriction sites, expression vectors, and antibiotics used for constructs

Gene	Primer	Sequence (5′- 3′)	Restriction site	Expression vector	Antibiotic
TaLEA1	Forward	gaagatcttcATGGCCTCCAACCAGAACC	*Bgl II and PmlI*	pGFPGUS	Hygromycin
	Reverse	gtgCTAGTGATTCCTGGTGGTGGTG			
TaLEA2	Forward	catgccatggCCTCCAACCAGAACC	*NcoI and BglII*	pCAMBIA3301	Phosphinothricin
	Reverse	gaagatctTTACACCAAATGGGCGGAAA			
TaPPDK1	Forward	gaagatcttcATGCCGTCGGTTTCGAG	*Bgl II and PmlI*	pGFPGUS	Hygromycin
	Reverse	gtgTCAGACAAGGACCTGGGCT			

For the germination assay, seeds from the wild-type (WT) and transgenic plants were placed on MS agar plates saturated with distilled water or different concentrations of NaCl and incubated at 4°C for 72 h before being placed at room temperature (22°C) under cool-white light for germination. The seeds were considered germinated when the radicles had completely penetrated the seed coat. Germination was scored each day after the seeds were placed at room temperature.

For the root growth assay, transgenic and WT seeds were placed on MS agar plates for germination. Three days later, 30 germinated seedlings from each line were carefully transferred to new MS agar plates supplemented with 100 mM NaCl. Seedling root lengths were measured after 7 days of growth in the treatment medium.

For the seedling salt-tolerance assays, *Arabidopsis* seedlings were cultured in soil. Water was withheld for 4 weeks and 45-day-old plants were then well irrigated with a NaCl solution (350 mM) applied at the bottom of the pots. When the soil was completely saturated with the salt solution, the free NaCl solution was removed and the plants were cultured under normal conditions. After 8 days of salt treatment, the leaves from WT and transgenic plants were collected to determine the chlorophyll content according to the method described by Aono et al. [18]; the absorbances of chlorophyll extracted in acetone (80%) were measured at 663 and 645 nm using a Beckman DU800 spectrophotometer (Fullerton, CA, USA).

2.5.3 Gain-of-function test of TaPPDK1 in soybean hairy root system

The CDs sequence of *TaPPDK1* was cloned into the pGFPGUS vector between the SacI and XbaI sites downstream of the constitutive Cauliflower Mosaic Virus 35S promoter (replacing GFP). The pGFPGUS vector was used as a negative control. Both constructs were then transformed into Williams 82. The soybean hairy root transformation and salt treatments were performed as described [19]. Surface-sterilized soybean seeds were germinated on germination medium (3.16 g/L B5 medium, 2% sucrose, 0.6% agar, pH 5.8) for 4 days (16 h light/8 h dark). The Agrobacterium rhizogenes strain K599 containing the recombinant constructs was grown in yeast extract peptone medium containing 50 mg/L kanamycin and 200 μM acetosyringone at 28°C for 16 h. It was then used to infect the cotyledons through scalpel incisions. The cotyledons were co-cultivated with *A. rhizogenes* in the dark for 5 days on moist filter paper. After that, the infected cotyledons were transferred to root-inducing medium (4.3 g/L Murashige and Skoog (MS) medium, 1 × B5 vitamin, 3% sucrose, 250 mg/L cefotaxime and 50 mg/L kanamycin). After 2 weeks, cotyledons with roots emerging from the incision sites were transferred to new root-inducing medium with 150 and 200 mM NaCl or medium without NaCl as the untreated control. The root mass was weighed 2 weeks after treatment.

2.6 Statistical analysis

The data were subjected to Student's *t*-test analysis using SPSS statistical software 17.0 (SPSS Inc., USA).

3 Results

3.1 Plant responses to salinity stress

Salinity stresses reduced the chlorophyll content of Jimai 19 and Han 12 (Additional file 5A in Supporting Information). However, the adverse effect on Jimai 19 was greater than on Han 12. The leaves of the wild-type Jimai 19 turned yellow earlier than the leaves of Han 12 in response to salt stress (Additional file 5A in Supporting Information).Under salt stress, the chlorophyll content of Han 12 was 24% higher than the Jimai 19 seedlings (Additional file 5B in Supporting Information). When Jimai 19 and Han 12 were planted in saline-alkaline soil and normal soil in natural fields, the salt tolerance index (STI) of Han 12, as calculated by the yield in salt and salt-free conditions, was significantly higher than Jimai 19 in 2014 and 2015 (Additional file 6 in Supporting Information). Based on these results, it was suggested that Han 12 has a higher salt tolerance than Jimai 19.

3.2 Protein expression profiles in response to salt stress

One hundred and twenty-one differentially expressed proteins (DEPs) were identified from seedling roots of the salt-tolerant genotype Han 12 and the salt-sensitive genotype Jimai 19 in response to salt treatment (Additional file 7 in Supporting Information). The numbers of DEPs and their identified overlap in the sensitive and tolerant varieties grown under salt stress are illustrated by the Venn diagram shown

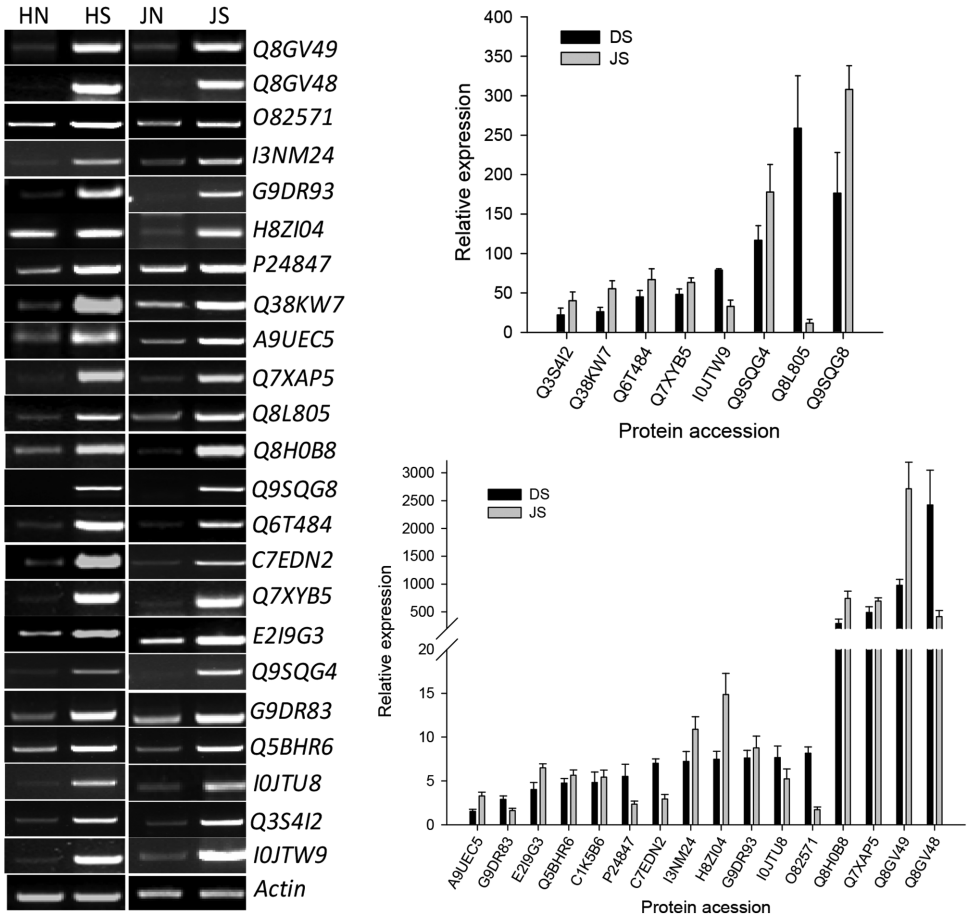

Figure 1. Gene expression analysis of a sample of DEPs by semi-quantitative RT-PCR (A) and qRT-PCR (B). The letters and numbers represent the root DEP accession numbers; actin was used as the internal standard. HN: Han 12 under normal condition; HS: Han 12 under salt stress; JN: Jimai 19 under normal condition; JS: Jimai 19 under salt stress.

in Additional file 8 (Supporting Information). Of the 50 proteins that were differentially expressed in both Jimai 19 and Han 12 in response to salt stress, 34 were up-regulated and 16 were down-regulated. Thirty-two proteins were differentially expressed (16 up-regulated and 16 down-regulated) in Jimai 19 in response to salt stress, and 39 (17 up-regulated and 22 down-regulated) were differentially expressed in Han 12.

The identified proteins were classified into several groups according to their function: stress and defense (39%), carbohydrate and energy metabolism (14%), transcription-related (16%), transport (7%), signaling (3%), protein synthesis (6%), protein degradation (4%), other functions (5%), and hypothetical or putative proteins with unknown functions (6%) (Additional file 9 in Supporting Information). More than one-third of the identified protein spots were stress and defense-related proteins, and most of them were up-regulated in response to salt stress (Additional file 10 in Supporting Information), including 22 DEPs in Han 12 and 25 in Jimai 19. The number of DEPs with different functions was similar in Han 12 and Jimai 19 grown under salt stress, particularly the up-regulated proteins (Additional file 10 in Supporting Information). These proteins may work together to obtain cellular homeostasis in roots and determine the overall salt tolerance of different wheat varieties grown under salt stress.

A sample of 33 up-regulated root proteins from both Jimai 19 and Han 12 grown under salt stress was collected for semi-RT-PCR and qRT-PCR to further characterize the gene expression patterns. There was a correlation between protein abundance and the results of the RT-PCR assay for 24 genes in salt-stressed Han 12 and Jimai 19 (Fig. 1).

3.3 Tests of salt-related gene functions

3.3.1 *TaPPDK1* overexpression confers salt tolerance in *Arabidopsis* and soybean root hair

Transgenic *Arabidopsis* plants overexpressing *TaPPDK1* (DEP accession number: Q7XYB5) were generated to elucidate the function of *TaPPDK1* in plants. The homozygous transgenic lines were selected for functional analysis. Transgenic *Arabidopsis* plants were detected by reverse transcription (RT)-PCR (Fig. 2C) and the GFP activity assay (Fig. 2B). *TaPPDK1* was overexpressed in the three transgenic *Arabidopsis* lines (Fig. 2C). When subjected to the NaCl treatments, the transgenic *Arabidopsis* plants showed a significant increase in relative root length and seed germination rate compared with the controls (Additional file 11 in Supporting Information).

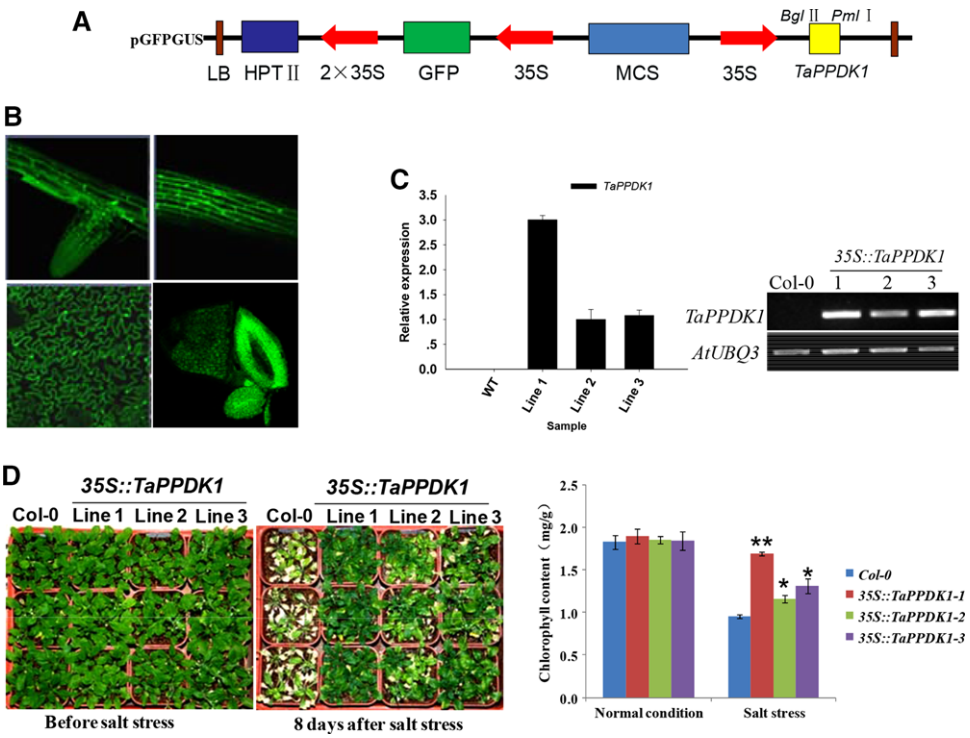

Figure 2. *TaPPDK1* overexpression confers salt tolerance in transgenic *Arabidopsis*. (A) Construction of the *TaPPDK1* vector. The *TaPPDK1* gene was subcloned into the pGFPGUS expression vector. (B) GFP protein expression in the root, stem, leaf and seed of *35S: TaPPDK1* transgenic *Arabidopsis*. (C) Detection of *TaPPDK1* mRNA in *35S: TaPPDK1* transgenic *Arabidopsis* using real-time PCR and semi-quantitative RT-PCR. The mRNA of the target gene accumulated in all three transgenic lines, but not in WT plants. (D) Salt tolerance of the *35S: TaPPDK1* plants. The phenotypes and chlorophyll contents of the wild-type and transgenic lines grown under normal and salt stress conditions are shown. Error bars represent the SD of three independent experiments, and the asterisks indicate a significant difference.

Consistent with these results, 45-day-old *Arabidopsis* plants were grown in soil irrigated with 350 mM NaCl. Approximately 8 days after NaCl treatment, both the wild-type and transgenic plants displayed chlorosis, but the chlorophyll content of the transgenic plants was higher than that of the WT plants (Fig. 2D).

We performed a gain-of-function test by expressing *TaPPDK1* in the hairy root culture of Williams 82 to further validate that *TaPPDK1* is a candidate salt tolerance gene. Expression of the transgene was confirmed by GUS staining and real-time PCR (Additional file 12c, e in Supporting Information). In the absence of NaCl treatment, there was no significant difference in the fresh root weights of the plants expressing *TaPPDK1* and green fluorescent protein (GFP; control) (Additional file 12b, d in Supporting Information). However, when subjected to 150 mM or 200 mM NaCl treatments, the roots transformed with *TaPPDK1* showed a significant increase in the fresh root weights compared with the control (Additional file 12b, d in Supporting Information), indicating that *TaPPDK1* overexpression can alleviate salt stress in soybean root hair.

Taken together, these results show that *TaPPDK1* overexpression confers salt tolerance in transgenic plants.

3.3.2 Overexpression of *TaLEA1* and *TaLEA2* confers salt tolerance in *Arabidopsis*

Transgenic *Arabidopsis* plants overexpressing the full-length *TaLEA1* (DEP accession number: Q8GV49) and *TaLEA2* (DEP accession number: Q8GV48) CDS were generated to validate the roles of *TaLEA1* and *TaLEA2* in mediating tolerance to salt stress (Fig. 3A and B). The homozygous transgenic lines overexpressing *TaLEA1* or *TaLEA2* and those overexpressing both genes were selected (Fig. 3C–E) for the salt tolerance analysis. The single-gene transgenic plants, that overexpressed *TaLEA1* or *TaLEA2*, showed a significant increase in the relative root length compared with the wild-type plants following the NaCl treatments; the transgenic plants also exhibited more tolerance to salt stress than the wild-type plants at the seedling stage (Additional file 13A, B in Supporting Information). The root length in the two-gene transgenic plants was increased compared with the wild-type plants (Additional file 13C in Supporting Information) and single-gene transformants (Fig. 3F). The two-gene transgenic plants also exhibited less chlorosis when grown in soil irrigated with 350 mM NaCl compared to the wild-type plants (Additional file 13C in Supporting Information) and single-gene transformants (Fig. 3G). These results suggested that the overexpression of *TaLEA1*, *TaLEA2* or both genes could confer salt tolerance in transgenic plants, and the plants overexpressing both genes showed more tolerance.

4 Discussion

Salinity can cause ion imbalance, hyperosmotic stress and oxidative damage in plants. Plants have evolved sophisticated mechanisms to cope with salinity stress, and a series of genes or proteins are involved in the plant response to salt stress. In a review of proteomic studies, the authors revealed the salt-responsive expression patterns of 905 proteins in the

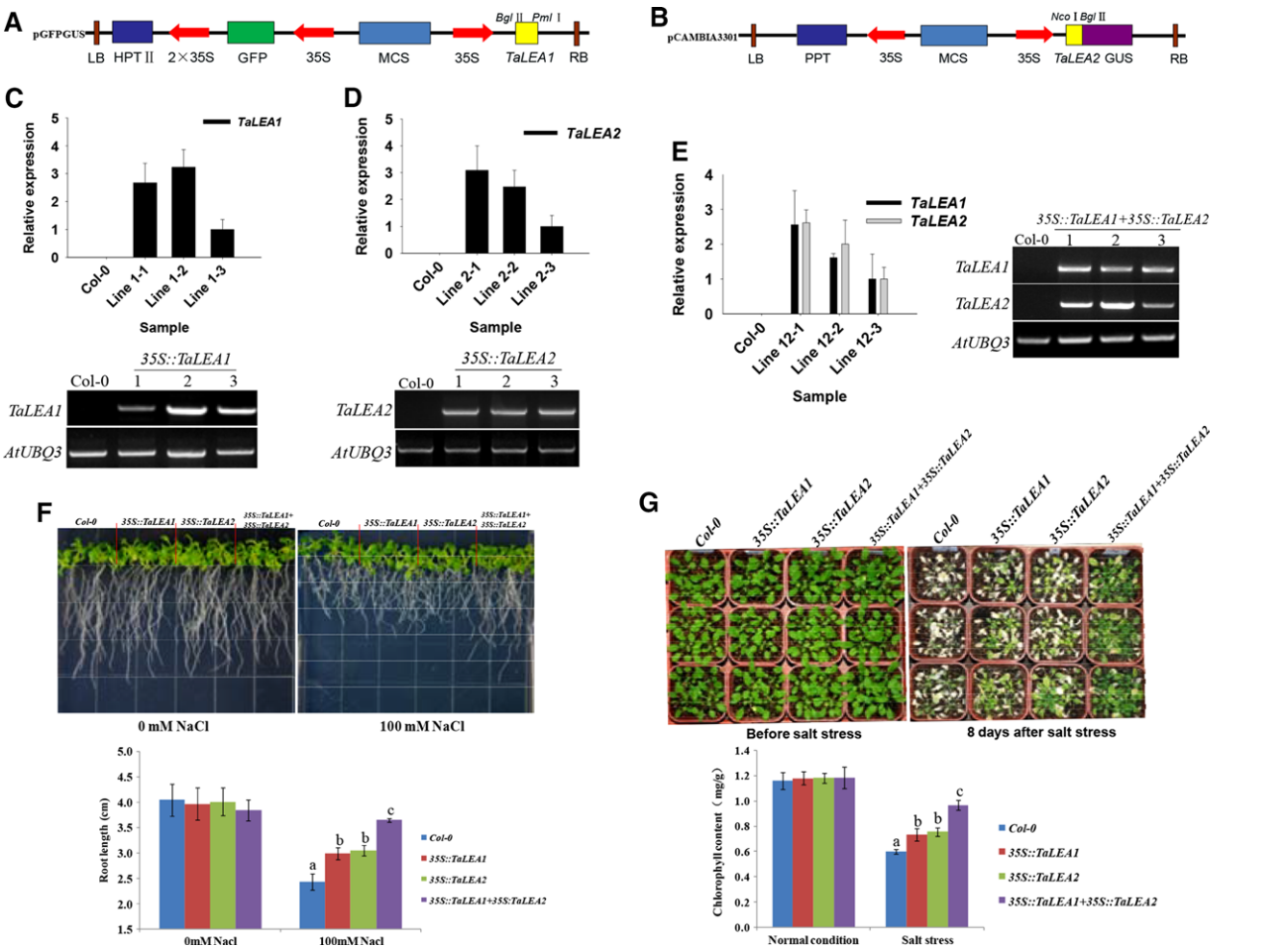

Figure 3. Overexpression of *TaLEA1*, *TaLEA2* or both genes confer salt tolerance in transgenic *Arabidopsis*, and the plants overexpressing both genes show more tolerance. (A and B) Construction of the *TaLEA1* and *TaLEA2* vectors. The *TaLEA1 and TaLEA2* genes were subcloned into the pGFPGUS and pCAMBIA3301 expression vectors. (C, D, E) Detection of *TaLEA1* and *TaLEA2* mRNAs in *35S: TaLEA1*, *35S: TaLEA2* and *35S: TaLEA1*+ *35S: TaLEA2* transgenic *Arabidopsis* using real-time PCR and semi-quantitative RT-PCR. The mRNAs of target gene accumulated in all three transgenic lines, but not in the WT plants. (F) The root length was increased in the transgenic plants compared with the wild-type plants. All values are the means (±SE) of 72 seedlings from three independent experiments (24 seedlings per experiment). (G) Salt tolerance of the *35S:TaLEA1*, *35S:TaLEA1* and *35S: TaLEA1*+ *35S: TaLEA2* plants. The phenotypes and chlorophyll contents of the wild-type and transgenic lines grown under normal and salt stress conditions are shown. Error bars represent the SD of three independent experiments, and the asterisks indicate a significant difference.

roots of 14 plant species, providing novel insight into the molecular regulatory pathways in the fine-tuned salt-responsive networks in roots [9]. Compared with these previously published proteomic data from plants under salt stress, 72 of the salt-responsive proteins identified in this study by iTRAQ (Additional file 14 in Supporting Information) were not previously detected in the roots of 14 plant species [9]. Of these 72 proteins, 18 were detected as salt stress-responsive proteins in the roots from three species and were reported in five proteomics studies from 2014 to 2016, and 17 were detected by iTRAQ (Additional file 14 in Supporting Information). With the development of highly sensitive technologies that cover the whole proteome, more and more salt-responsive proteins would be detected and allow us to decipher the molecular networks of plant salt tolerance.

4.1 Protein ubiquitination may occur in wheat roots under salt stress

Ubiquitin-like protein (C7AE90, down-regulated in Jimai 19) could covalently bind to target proteins by a cascade enzyme system consisting of Ub-activating (E1), -conjugating (E2), and -ligating (E3) enzymes [20]. SPOP (H8ZI04, up-regulated in Han 12 and Jimai 19) is a BTB (Bric-a-brac/Tramtrack/Broad complex) protein that is a member of the Cul3-based ubiquitin ligase family. F-box protein (I3NM24, up-regulated in Han 12 and Jimai 19) is one member of the well-defined RING E3 ligase Skp1/Cul1/F-box protein complex [21]. Coronatine insensitive 1 (E2I9G3, up-regulated in Han 12 and Jimai 19), an F-box protein that is essential for all of the jasmonate responses, interacts with

multiple proteins to form the SCFCOI1 E3 ubiquitin ligase complex and recruits jasmonate ZIM-domain (JAZ) proteins for degradation by the 26S proteasome [22]. The differential expression of these four proteins might indicate that protein ubiquitination occurred in wheat roots under salt stress. The consequences of protein ubiquitination include proteasomal degradation, protein-protein interactions and allosteric protein regulation, which play critical roles in regulating plant responses to abiotic stresses [23, 24]. The specificity of the ubiquitination pathway is mainly controlled by the substrate-recruiting E3 ubiquitin ligases, and consequently, E3 ligases facilitate responses to environmental stimuli by modulating the abundance of key downstream stress-responsive transcription factors [24].

4.2 Transcription factors may regulate stress-related genes to improve salt tolerance

BZIP-type transcription factor (C7EDN2) was up-regulated in both Han 12 and Jimai 19 in response to salt stress. BZIP (basic region/leucine zipper) type transcription factors interact with major cis-acting elements of Cor/Lea promoters [25, 26]; the expression of the Cor (cold-responsive or -regulated)/Lea (late embryogenesis-abundant) gene family is up-regulated under abiotic stress conditions, and the gene products function in stress tolerance [27, 28]. Consistent with this observation, Cold-regulated protein (Q8H0B8), LEA1 protein (Q8GV49), LEA2 protein (Q8GV48), and ABA-inducible protein (Q7XAP5) were all up-regulated in both Han 12 and Jimai 19 in response to salt stress. Moreover, Cold acclimation protein WCOR80 (P93609) and Group3 late embryogenesis abundant protein (A7VL27) were also up-regulated in the salt-tolerant wheat Han 12.

4.3 The response of PR proteins to salt stress

A total of 8 pathogen-related (PR) proteins were differentially expressed in response to salt stress in this study. PR proteins include many proteins that are categorized into 17 families. Chitinases, β-1,3-glucanases and thaumatin-like proteins are all PR proteins. In addition to their antipathogen function, some PR proteins respond to abiotic stress environments. In some cases, several PR proteins had a common response to one or more abiotic stresses [29, 31]. In this study, pathogenesis-related protein 5 (Q3S4I2), pathogenesis-related protein 4 (Q9SQG8), PR-4 (Q9SQG4), and class I chitinase (Q6T484) were all up-regulated in both Han 12 and Jimai 19 in response to salt stress. PR-4, PR-5 and class I chitinase also contributed to salt regulation in *Arabidopsis* [32] and winged Bean [33]. Endochitinase (Q41539), Thaumatin-like protein (Q8S4P7), Beta-1,3-glucanase (Q4JH28) and PR-4 (Q9SQG3) were up-regulated in Jimai 19. These PR proteins have different roles in plant salt tolerance, which may determine some aspects of the salt tolerance levels of the different varieties.

4.4 MIP transporters may improve the water uptake or conservation in response to salt stress

Membrane intrinsic proteins (MIP) transport water across cellular membranes and play vital roles in all organisms. Several membrane intrinsic proteins identified in this study displayed different responses to salt stress. Aquaporin (A9UEC5) was up-regulated, whereas delta tonoplast intrinsic protein TIP2;2 (Q6QU78) and plasma membrane intrinsic protein 3 (Q9M7C2) were down-regulated in both Han 12 and Jimai 19 in response to salt stress. Aquaporin (C9E2R2) was down-regulated in Han 12. These proteins may play different roles in wheat to enhance plant salt tolerance. In other plants, the different aquaporin isoforms had different responses to salt stress. Salt stress (200 mM NaCl) down-regulated HvPIP2;1 but had almost no effect on the expression levels of HvPIP1;3 or HvPIP1;5 [34]. Moreover, transgenic approaches established that over-expression of some MIP genes could change a plant's tolerance to salt stress. Over-expression of the wheat aquaporin genes TaAQP8 [35], TaNIP [36], TdPIP1;1 or TdPIP2;1 [37] enhanced salt stress tolerance in transgenic plants, and constitutive over-expression of soybean plasma membrane intrinsic protein GmPIP1;6 confered salt tolerance to soybeans [38]. MIPs mainly confer salt stress tolerance by regulating water uptake and distribution to plant tissues [39]. However, over-expression of a barley aquaporin, HvPIP2;1, increased salt sensitivity in transgenic rice plants [40]. The over-expression of aquaporins in this plant may increase membrane water permeability and thus decrease cellular water conservation during periods of salt stress. Consistent with this observation, the expression levels of *ZmPIP1* and *ZmPIP2* in maize [41] were also suppressed by salt.

4.5 Antioxidant enzymes protect against oxidative stress caused by high salinity

Salt tolerance in most crop plants is often highly correlated with a more efficient oxidative system [42, 43]. It was found that superoxide dismutase (O82571), ferritin (A4GSN5) and oxidoreductase NAD-binding domain containing protein (D8L9U1) were up-regulated in Han 12 and Jimai 19 in response to salt stress. Polyphenol oxidase (C0SPI2) was up-regulated in Han 12, and catalase (F1DKC1), peroxidase 7 (Q5I3F1), peroxidase 8 (Q5I3F0), and thioredoxin M-type (Q9ZP21) were up-regulated in Jimai 19. The enzymatic antioxidants include superoxide dismutase (SOD), which catalyzes the disproportionation of superoxide radicals and converts them to molecular oxygen and H_2O_2, and catalase (CAT), which subsequently detoxifies the H_2O_2 into H_2O and oxygen [44]. During salt stress, the reaction between ferrous iron and

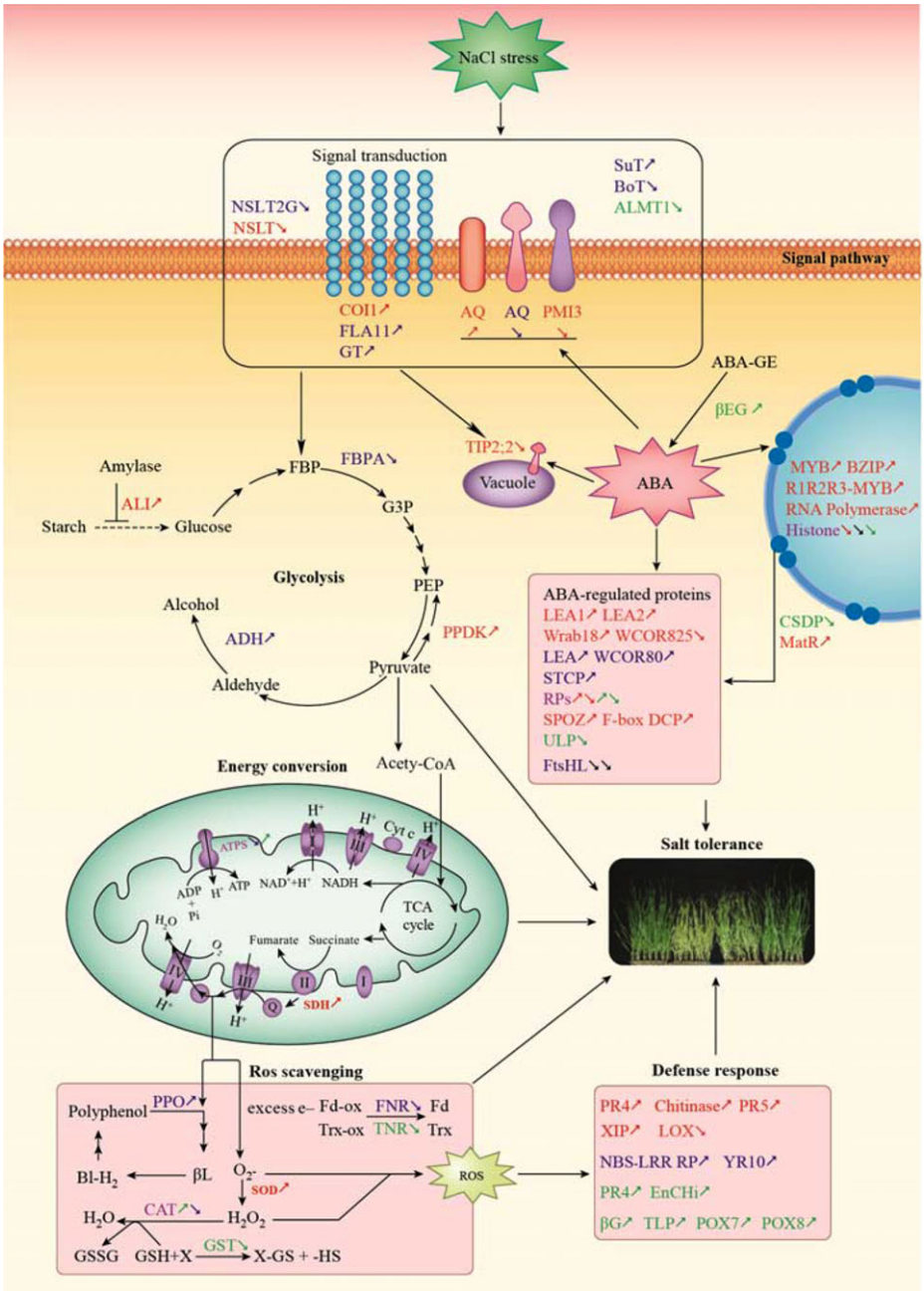

Figure 4. Model of the salt stress pathway in wheat root. Up or down-regulation in both Han12 and Jimai 19 is marked as red arrows; up- or down-regulation in Han12 is marked as blue arrows, and up or down-regulation both in Jimai 19 is marked as green arrows. SOD, superoxide dismutase; PR, pathogenesis –related protein; XIP, xylanase inhibitor; CAT, catalase; CSDP, cold shock domain protein 2; GST, glutathione S-transferase; LOX, lipoxygenase; LEA, late embryogenesis abundant protein; STCP, salt-tolerant correlative protein; NBS-LRR RP, NBS-LRR type resistance protein; PPO, polyphenol oxidase; FNR, ferredoxin-NADP(H) oxidoreductase; EnChi, endochitinase; TNR, Thioredoxin M-type; βG, beta-1,3-glucanase; TLP, thaumatin-like protein; POX, peroxidase; βEG, Glucan endo-1,3-beta-D-glucosidase; ALI, Dimeric alpha-amylase inhibitor; SDH, Succinate dehydrogenase subunit; PPDK, Pyruvate orthophosphate dikinase; ADH, Alcohol dehydrogenase; ATPS, ATP synthase; FBPA, Fructose-bisphosphate aldolase; BZIP, BZIP-type transcription factor; COI1, Coronatine insensitive 1-like protein; NSLT, Non-specific lipid-transfer protein; NSLT2G, Non-specific lipid-transfer protein; AQ, aquaporin; PMI, plasma membrane intrinsic protein; TIP, Delta tonoplast intrinsic protein; SuT, Sulphate transporter; GT, Glycosyltransferase; BOT, Boron transporter; AIMT, Aluminum-activated malate transporter; RP, ribosomal protein; F-boxDCP, F-box domain-containing protein; SPOZ, Speckle-type POZ; ULP, ubiquitin-like protein; FtsHL, FtsH-like protein.

H_2O_2 could result in the formation of hydroxyl radicals, the most dangerous type of ROS. Thus, the increased expression of ferritin could help to neutralize the ROS-induced damage [45]. Peroxidase (POX) and polyphenol oxidase (PPO) are the two major enzymes responsible for the oxidation of phenolic compounds [46]. PPO was up-regulated in Han 12 and POX was up-regulated in Jimai 19. Han 12 and Jimai 19 might use the different antioxidants to reduce the phenol accumulation in the roots under salt stress.

Our iTRAQ-based proteomic data showed that the ubiquitination-related proteins, transcription factors, pathogen-related proteins, membrane intrinsic protein transporters and antioxidant enzymes may work together to obtain cellular homeostasis in roots and determine the overall salt tolerance levels of different wheat varieties in response to salt stress (Fig. 4).

4.6 Validation of the function of genes encoding salt-responsive proteins

Each member of the molecular network involved in plant salt tolerance has its own special role. This high-throughput proteomic study could provide the foundation for detecting

candidate salt-tolerance genes. Our goal is to identify the new candidate genes with salt tolerance. PPDK was induced by salt stress in this study. There is little information available about the relation between plant PPDK and stress. Recently, it was found that microbes escape the effects of stress by re-engineering metabolic networks mediated by PPDK [47]. PPDK may be a new salt-tolerant gene with a special mechanism of stress tolerance. We also want to explore and determine the relationships of different members in the salt stress pathway summarized in this study. Single LEA protein is widely believed to protect cells from water stress. LEA1 and LEA2 were induced simultaneously by salt stress in this study. However, the additive effects of co-expression of two LEA genes have not been studied. Therefore, LEA1 protein (Q8GV49), LEA2 protein (Q8GV48) and pyruvate orthophosphate dikinase (PPDK) (Q7XYB5) were selected to validate their role in mediating tolerance against salt stress by overexpressing these genes in *Arabidopsis* and soybean hair root.

PPDK catalyzes a reversible reaction that converts ATP, Pi, and pyruvate to AMP, PPi, and PEP, respectively [48]. In C4 plants, this reaction regenerates the primary CO_2 acceptor PEP, but its role in C3 plants is not fully understood [49], particularly the role of PPDK in stress tolerance. C4 PPDK isoforms have been studied in cold conditions [50, 51] and following UV-B exposure [52]. There is little information available about the relation between C3 PPDK and stress. All types of water stress (drought, high salt, and mannitol treatment), as well as cold and low-oxygen stresses induce the expression of the PPDK protein in the roots of rice seedlings [53]. The levels of PPDK1 ubiquitination in rice root subjected to salt treatment were identified [54]. In this study, PPDK was upregulated in Han 12 and Jimai 19 in response to salt stress. The overexpression of *TaPPDK1* could confer salt tolerance in transgenic plants. For the first time, we validated the function of the C3 PPDK gene in salt tolerance through a wheat proteomic study.

LEA proteins are widely believed to protect cells from water stress. A number of putative mechanisms have been proposed, including ion sequestration, membrane binding and stabilization, redox balance as an antioxidant, buffering of hydrate water, and chaperone activity [55, 56]. Tunnacliffe and Wise [57] obtained evidence supporting the possibility that some LEA proteins act to prevent protein aggregation during water loss. When introduced into yeast, rice, and tomato, *Salvia miltiorrhiza* LEA proteins have been shown to confer increased resistance to salt stress [58–63]. In the wheat used in this study, the overexpression of *TaLEA1*, *TaLEA2* or both genes together could confer salt tolerance in transgenic plants, and the plants overexpressing both genes showed more tolerance, which validated the proteomic results and suggested that the cooperation of different members of the molecular network of plant salt tolerance enhanced wheat salt tolerance more than a single gene.

These results confirm that the proteomic approach used in this study was successful in predicting the functions of stress-related genes in wheat and uncovered their potential role in enhancing wheat growth under stress conditions. However, some genes may not be directly involved in plant salt tolerance, although they were induced or suppressed by salt stress. Therefore, the roles of these genes in salt tolerance need to be further validated in other experiments.

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