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Original Research

Physiological and Proteomic Analysis of Seed Germination under Salt Stress in Mulberry

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Abstract

Background: Salinity is the main abiotic stress that affects seed germination, plant growth and crop production. Plant growth begins with seed germination, which is closely linked to crop development and final yields. *Morus alba* L. is a well-known saline-alkaline tree with economic value in China, and the most prominent method of expanding mulberry tree populations is seed propagation. Understanding the molecular mechanism of *Morus alba* L. salt tolerance is crucial for identifying salt-tolerant proteins in seed germination. Here, we explored the response mechanism of mulberry seed germination to salt stress at physiological and protein omics levels. **Methods**: Tandem mass tag (TMT)-based proteomic profiling of *Morus alba* L. seeds germinated under 50 mM and 100 mM NaCl treatment for 14 days was performed, and the proteomic findings were validated through parallel reaction monitoring (PRM). **Results**: Physiological data showed that salt stress inhibited the germination rate and radicle length of mulberry seeds, decreased the malondialdehyde (MDA) content and significantly increased superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) activities. Then, a TMT marker technique was used to analyze the protein groups in mulberry seeds with two salt treatment stages, and 76,544 unique peptides were detected. After removing duplicate proteins, 7717 proteins were identified according to TMT data, and 143 (50 mM NaCl) and 540 (100 mM NaCl) differentially abundant proteins (DAPs) were screened out. Compared with the control, in the 50 mM NaCl solution, 61 and 82 DAPs were upregulated and downregulated, respectively, and in the 100 mM NaCl solution, 222 and 318 DAPs were upregulated and downregulated, respectively. Furthermore, 113 DAPs were copresent in the 50 mM and 100 mM NaCl treatments, of which 43 were upregulated and 70 were downregulated. Gene Ontology (GO) annotation and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis revealed that the DAPs induced by salt stress during mulberry seed germination were mainly involved in photosynthesis, carotenoid biosynthesis and phytohormone signaling. Finally, PRM verified five differentially expressed proteins, which demonstrated the reliability of TMT in analyzing protein groups. **Conclusions**: Our research provides valuable insights to further study the overall mechanism of salt stress responses and salt tolerance of mulberry and other plants.

Keywords: *Morus alba* L.; proteomic; seed germination; salt stress; tandem mass tag; parallel reaction monitoring

1. Introduction

Soil salinization is among the most serious abiotic factors responsible for decreasing crop yields worldwide; the damage is mainly due to high sodium salt concentrations (NaCl or Na_2SO_4), which damage plants through osmotic stress, oxidative stress, ionic stress, nutritional deficiency, and other adverse conditions [1]. According to statistics, more than 60 million hm^2 of arable land worldwide is threatened by varying degrees of salinization, accounting for approximately 20% of the world's total area of irrigated land [2]. Due to improper irrigation practices, poor fertilizer application and industrial pollution, the salinized land area will continue to increase, and it is predicted that more than half of the arable land will be salinized by 2050 [3]. Soil salt stress not only causes direct ionic toxicity to plants, but also indirectly induces osmotic stress and oxidative stress, inhibition of cell division and proliferation, and affects plants at the physiological, biochemical, and molecular levels [4,5]. These adverse physiobiochemical and molecular conditions simultaneously leads to inhibits plant growth, and its development. Many crops and vegetables, such as *Vigna aconitifolia* [6], *Beta vulgaris* [7] and *Avena sativa* [8], are very sensitive to salt stress. In addition, salt stress adversely affects the growth and development of many woody plants, such as *Populus* [9], *Eucalyptus grandis* [10] and *Camellia sinensis* [11], reducing the yield and quality of plants and causing very large economic losses. Previous studies have shown that plants are most sensitive to salinity during the seed germination and seedling stages; salt stress produces osmotic stress and excessive ion toxicity, causing retarded plant development and inhibiting tissue differentiation and growth [12]. The germination rate, shoot length and root length of seeds at the germination stage are extremely sensitive to salt stress in plants [13]. Salt stress leads to increased Na^+ and Cl^- accumulation in the plant seed hypocotyl and endosperm, which hinders seed germination, seedling growth, and DNA, RNA and protein synthesis [14,15]. It has been

reported that salt stress delays seed germination in tomato varieties, inhibits cottonseed germination and endogenous melatonin accumulation, reduces the content of α-amylase and β-galactosidase in cotton seeds and decreases the germination rate of *Suaeda salsa* seeds [16–18]. Over the years, researchers have explored various approaches, such as operating seed biopriming techniques [19] and nanoparticles [20], to identify salt-tolerant genes, cultivate salt-tolerant varieties and alleviate the impact of salt stress on plants. Currently, due to the high degree of salinization and poor agricultural production conditions in Songnen Plain of China, the popularization of mulberry is expected to transform saline-alkali land into fertile land [21]. Therefore, understanding the molecular and physiological mechanisms of mulberry salt tolerance is necessary for maintaining mulberry productivity.

Morus alba L. is an important perennial woody plant, and its leaves are the main food source for silkworms. Mulberry has high nutritional and economic value and has various uses in ecology, food and medicine [22]. Its high content of active ingredients and high resistance to adversity stresses, such as drought, salinity and flooding, make mulberry an excellent material for researching stress in woody plants [23] and one of the ecological tree species with great potential for afforestation, soil and water conservation, bioremediation of pollutants, land desertification and saline land management [24,25]. In depth research on genes related to mulberry stress signaling has been conducted. For example, under ultraviolet-B and dark stress, mulberry leaves activate the cell redox and ER quality control systems, enhancing protein synthesis and weakening N-glycan biosynthesis in the ER to resist damage [26]. The activities of superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) in mulberry can eliminate Cd stress-induced oxidative stress [27]. After abscisic acid (ABA) biosynthesis is activated in mulberry, some drought-responsive genes (such as *CBP6A*, *MGST3*, *ERF1B*, etc.) are also activated [28]. In recent years, mulberry planting has been performed for the ecological restoration of land, which is of great significance for restoring and reconstructing vegetation in saline-alkali land and to improve the ecological environment. Seed sowing propagation is an important agricultural production method for mulberry propagation. Mulberry seeds in the germination stage are extremely sensitive to salt stress, and China contains a large area of saline-alkali land, which urgently needs to be utilized. It is necessary to study the response mechanism of mulberry seeds to salt stress. Proteins are directly involved in the stress response of plants. Studying the response of mulberry to salt stress conditions at the protein level can provide a powerful tool for revealing the physiological mechanism of mulberry stress resistance.

Furthermore, physiological responses in cells are produced by proteins, and proteomics, a global analysis strategy, is the key to studying gene functions [29]. Proteomic analysis can provide information on multiple processes in complex events, such as seed germination and seed filling. Seed germination is the very beginning of plant life and is among the most critical and decisive stages in the plant growth cycle, which determines the formation of adult plants and the final crop yield [30,31]. Analyzing seed germination through proteomics is mainly carried out by sequencing plants, such as *Oryza sativa* [32], *Brassica napus* [33], *Populus* [34], *Jatropha curcas* [35], *Araucaria* [36] and *Platanus orientalis* [37]. Relatively few studies have focused on the germination of seeds from *Morus alba*. In recent years, the molecular basis of salt responses and tolerance during seed germination has been investigated using proteomic approaches in some crops and woody plant species, including *Triticum aestivum* [38], *Zea mays* [39], *Sorghum bicolor* [40] and *Ulmus pumila* [41]. The results showed that the abundance of H_2O_2 scavenging enzymes decreased under salt stress, and seed priming with ascorbic acid could improve the salt tolerance of durum wheat; MFT (MOTHER OF FT ANDTFL1) was upregulated by the repressor of the GA signaling pathway under salt treatment through inhibition of ABA-positive transcription factor ABI5, which promoted maize seed germination; under salt stress conditions, the germination of salt-sensitive sweet sorghum germplasm was significantly inhibited. With the publication of the mulberry genome sequence, genome-wide transcriptome and proteome analyses can be performed with mulberry [22,42]. However, previous studies on mulberry have mainly focused on the tolerance of transgenic mulberries to drought stress [28], transcriptomic analysis of mulberry roots under cadmium stress [43] and molecular mechanisms of copper stress in mulberry leaves and identification of quality resources in resistant species [44]. The molecular mechanism for the proteomic response of mulberry to salt stress during seed germination remains unclear. Therefore, this study aimed to identify salt stress-responsive proteins during mulberry seed germination at the physiological and proteomic levels and to provide some new insights to further understand the abiotic stress response of mulberry and mulberry breeding and cultivation.

2. Materials and Methods

2.1 Mulberry Seed Germination under Salt Stress Treatment

The experimental material of this study is “Yuesang No. 11” provided by Botanical Garden of Zhongkai University of Agriculture and Engineering. First, the mature mulberries were harvested; the seeds were washed; mature, full and uniform seeds were selected; and the seeds were stored at 4 °C for subsequent experiments. The seeds were soaked at room temperature for 24 h and the soaking solution was discarded. The seeds were then surface disinfected with 75% ethanol and sodium hypochlorite solution (1% effective chlorine content). 75% ethanol was soaked

for 5 min, rinsed with sterile water 3 times [17], sodium hypochlorite solution was soaked for 30 min, rinsed with sterile water 3 times, and the moisture on the surface of the seeds was absorbed with filter paper. The samples were placed in a sterile 90 mm diameter Petri dish covered with three layers of filter paper. The control group (CK) was treated with a proper amount of sterilized water (approximately 12 mL/dish) and 50 and 100 mM NaCl solutions. Each dish was placed with 100 seeds for germination, and biological reproduction was carried out three times. After 14 days, the seedlings were sampled for physiological analysis and protein extraction.

2.2 Physiological Analysis of Mulberry Seed Germination

Mulberry seeds germination began after 2 days of treatment (seeds were considered germinated when radicles appeared), and germinating seeds were observed and counted every 48 hours to calculate the germination percentage. After 14 days, mulberry seedlings from different treatments were collected and rinsed with deionized water. The germination rate and radicle length of mulberry seedlings were determined, and the final germination rate was the number of seeds germinated divided by the total number of seeds used for the test $\times$ 100.

In this experiment, SOD, POD, CAT activities as well as H_2O_2 content and MDA content were detected using biological assay kits A001-1-1, A084-3, A007-1, A064-1 and A003-1-1 respectively (Nanjing Jiancheng Bioengineering Institute, Nanjing,China) according to the manufacturer's instructions. SOD activity was determined by measuring the absorbance at 550 nm using the xanthine oxidase method. In addition, the POD activity was determined by gauging the absorbance at 420 nm using guaiacol and H_2O_2 as substrates, and CAT activity was determined by measuring the absorbance at 405 nm using H_2O_2 as a substrate. The malondialdehyde content was determined through a thiobarbituric acid reactive substance (TBARS) assay. Three biological replicates were detected.

2.3 Protein Extraction, Peptide Enzymatic Hydrolysis and Peptide Desalination

Mulberry seedling samples that were stored at –80 °C were ground into a fine powder, and four volumes of phenol extraction buffer (1% protease inhibitor and 2 mmol/L EDTA, 10 mmol/L dithiothreitol) were added for ultrasonic cracking. Add the same amount of Tris balanced phenol, centrifuge at 5500 $\times$ g at 4 ℃ for 10 min, add 5 times of 0.1 mol/L ammonium acetate/methanol for precipitation for 12 h, and wash with methanol and acetone respectively. The extracted protein was quantified by bicinchoninic acid disodium (BCA) method.

The extracted proteins were subjected to trypsin (317107 Promega) hydrolysis (protein/trypsin, 50/1) using the method of filter-aided proteome preparation (FASP) described by Wisniewski *et al.* [45]. Add the total protein solution into a protein concentrator (10K MWCO, 0.5 mL Pierce™ Protein Concentrator, Thermo Scientific, Waltham, MA, USA), dilute the sample with 8 mol/L urea, and after reduction and alkylation, digest it with trypsin at 37 °C overnight. After digestion, centrifuge at 10,000 $\times$ g for 15 min, collect the filtrate, and then filter and wash it twice with 50 μL NH_4HCO_3 solution. Then, 10% trifluoroacetic acid (TFA) was added, and when the final concentration was 0.4%, the reaction was terminated. The proteolytic product was steamed in a rotary evaporator until the volume was less than 50 μL, and then buffer A (64% acetonitrile, 0.2% TFA) was added to about 200 μL for later use. The peptide desalting was carried out by PierceTM C-18 desalting column (PierceTM C-18 Spin columns, Thermo Scientific, USA). After the desalting column was activated by methanol, the enzymolysis product was added to the desalting column, centrifuged at 1000 $\times$ g for 30 s, and the filtered sample was returned to the desalting column and centrifuged again. The filtrates were combined, concentrated and dried.

2.4 TMT Labeling and Peptide Fractionation

100 μg of peptide was extracted from each sample for labeling according to TMT labeling kit instructions (Thermo Fisher Scientific, USA). The mulberry seedlings from the three groups under different salt stresses are labeled. Each group included three biological replicates. The mulberry seedling peptides from the control treatment were labeled with mass 126, 127N and 127C isobaric TMT tags, while the seedling peptides from the 50 mmol salt treatment were labeled with mass 128N, 128C and 129N isobaric TMT tags, and the 100 mmol salt treatment was labeled with mass 129C, 130N and 130C isobaric TMT tags.

Each set of labeled peptide fragments was uniformly mixed and fractionated using the Reverse-Phase High pH Peptide Fractionation Kit (Thermo Scientific, USA). The TMT-labeled digested sample was fractionated into 10 fractions using a gradient of acetonitrile elution using the Pierce™ High pH Reverse-Phase Peptide Fractionation Kit (Thermo scientific, USA) according to the manufacturer's instructions.

2.5 LC-MS/MS Analysis

Each fraction was injected and analyzed by Nano LC-MS/MS. The peptide mixture was loaded onto a reversed-phase trap column (Thermo Scientific Acclaim PepMap100, 100 μm $\times$ 2 cm, nanoViper C18) that was connected to the C18-reversed-phase analytical column (Thermo Scientific Easy Column, 10 cm long, 75 μm inner diameter, 3 μm resin) in buffer A (0.1% Formic acid) and separated with a linear gradient of buffer B (84% acetonitrile and 0.1% Formic acid) at a flow rate of 300 nL/min. After column separation, all samples were analyzed by mass spectrometry using a Q-Exactive mass spectrometer (Proxeon Biosystems, Thermo Fisher Scientific, USA).

2.6 Protein Identification and Quantification

Raw data for the Q-Exactive spectrometry analysis were obtained as RAW files. Identification and quantitative analyses were performed by searching the database using Mascot version 2.2 and Proteome Discoverer version 1.4. The UniProt_Lycopersicon_esculentum Mulberry_35921_20211214.fasta (35921 sequences, downloaded on 14 December 2021) database was searched. Trypsin was selected as a random cleavage enzyme, and the parameter of max missed cleavages was set to 2. Carbamidomethyl (C), TMT 9 plex (N-term), and TMT 9 plex (K) was selected as fixed modifications. The variable modifications were set to oxidation (M), TMT 9 plex (Y). The peptide mass tolerance was set to $\pm$20 ppm and the fragment mass tolerance was 0.1 Da. The database schema was used as a decoy for calculating the false discovery rate (FDR). The screening criteria for plausible peptides were FDR $\leq$0.01. DAPs were screened using a power analysis Student's t test $p < 0.05$ (n = 3) and the criterion of a fold change greater than 1.2 (thresholds of fold changes $\geq$1.2 for up-regulation proteins and the thresholds of fold changes $\leq$0.83 for down-regulation proteins).

2.7 Bioinformatics Analysis

All annotation and DAP classification of Gene Ontology (GO) features were performed using the Blast2GO (https://www.blast2go.com/) program. The Automatic Annotation Server was used to perform Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotations [46]. The significance of protein enrichment of GO functional annotations and KEGG pathway annotations was evaluated by Fisher's exact test.

2.8 PRM Analysis

The peptide information suitable for PRM analysis was imported into Xcalibur software (Thermo scientific, USA) to establish the PRM method. An equivalent amount of digested peptide of approximately 1 μg from each sample was removed and mixed with an equivalent amount of standard peptide of 20 fmol (PRTC:ELGQSGVDTYLQTK) for detection. PRM analyses were performed on a Q-Exactive Plus mass spectrometer equipped with an Easy nLC-1200 system (Thermo Fisher Scientific, Bremen, Germany). The liquid chromatographic column was equilibrated with 95% solution A (0.1% formic acid). 2 μL of each sample was injected into the column at a flow rate of 300 nL/min for gradient separation. The gradient of the liquid phase separation was as follows: linear gradient of 5–10% solution B (acetonitrile solution of 0.1% formic acid) for 0–2 min, followed by 10–30% solution B for 2–45 min, followed by 30–100% solution B for 45–55 min; finally, solution B was kept at 100%.

PRM analysis of samples separated by HPLC was performed for 60 min using a Q-Exactive HF mass spectrometer (Thermo Scientific, USA). The analytical column was a homemade tip column (75 μm $\times$ 200 mm, 3 μm-C18). Finally, Skyline software 3.5.0 (Thermo Scientific, USA) was used to statistically analyze the PRM data.

2.9 Statistical Analysis

Analysis and examination of the obtained experimental data were performed using Excel (Microsoft office professional plus 2010; Microsoft corporation, Redmond, WA, USA) and SPSS software 17.0 (SPSS Inc., Chicago, IL, USA). All data are the mean $\pm$ standard deviation (SD) of three repetitions, and the differences among different treatments were compared by one-way ANOVA and LSD. The level of significance was set at $p < 0.05$, and a very significant difference was set at $p < 0.01$ for all tests.

3. Results

3.1 Physiological Responses of Mulberry Seeds to Salt Stress During Germination

The germination percentage reflects the resistance of the seed germination process to abiotic stress. As shown in Fig. 1A, the germination rate of mulberry seeds increased significantly with increasing incubation time. Different concentrations of the same treatment days had different effects on the germination of mulberry seeds. With 0 mmol/L salt concentration, the germination rate continued to increase and reached the highest rate of 94.33% at day 14. With the 50 mmol/L salt treatment, the germination rate of seeds increased slowly at 4 days, increased rapidly from 4 to 8 days, and then increased slowly again from 8 to 14 days, and the germination rate of seeds at day 14 was 50%, which decreased by 44.33% compared with that of the control. With the 100 mmol/L salt treatment, the seed germination rate increased slowly from 0 to 8 days, but both were slower than the control and 50 mM NaCl stress treatments, and the seed germination rate on day 14 was 36.67%, which was decreased by 57.66% compared to that of the control. The germination rate of mulberry seeds was significantly lower than the germination rate of the control on the same day when the salt concentration was 50 mmol/L and 100 mmol/L. The growth of embryonic roots is crucial for plants to properly develop into seedlings out of the soil. As shown in Fig. 1B, the embryo root length increased with increasing embryo root growth days at the same concentration. Under 0 mmol/L salt, the embryo root length of mulberry seeds increased significantly, and the longest radicle length was 32.20 mm on the 14th day. With the 50 mmol/L salt stress treatment, the radicle length increased slowly from 0 to 6 days and rapidly from 6 to 14 days and basically stopped increasing after day 14. After 14 days, the length basically stopped increasing. At a salt concentration of 100 mmol/L, mulberry embryo roots basically did not grow. The length of the mulberry radicles varied at the same time for different concentrations. Compared to the control, both the 50 mmol/L and 100 mmol/L salt treatments significantly reduced the radicle length on the same day.

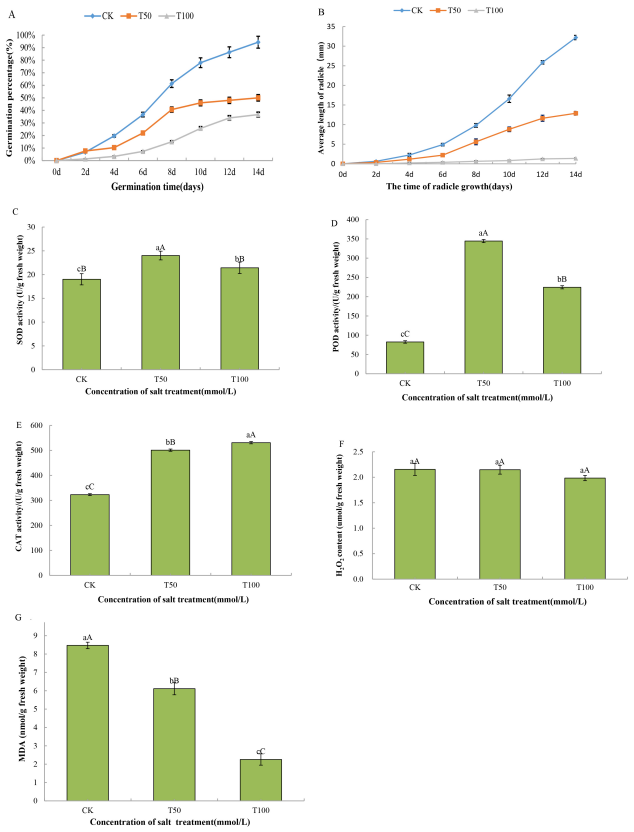

Fig. 1. Detection of the physiological parameter of mulberry seeds at different salt treatment stages. (A) 14-day germination rate of mulberry seeds. (B) Radicle length of mulberry seeds in 14 days. (C) Superoxide dismutase (SOD) activity. (D) Peroxidase (POD) activity. (E) Catalase (CAT) activity. (F) H_2O_2 content. (G) Malondialdehyde (MDA) content. Note: Three replicates were performed. The values are presented as the means ± SDs. Significant differences are expressed by different lowercase letters ($p < 0.05$), and very significant differences are expressed by different capital letters ($p < 0.01$).

In response to salt stress, antioxidant enzymes (GST, POD, SOD, DHAR, APX and CAT) were differentially accumulated during seed germination of mulberry. Therefore, we studied the physiological indicators of salt stress, including the determination of SOD, POD and CAT activities and the content of H_2O_2 and MDA. The results are shown in Fig. 1. Under different salt treatment conditions, compared with the control, SOD, POD and CAT in mulberry seeds under salt stress exhibited significantly increased activities (Fig. 1C–E), SOD activity increased by 1.26-fold and 1.13-fold, POD activity increased by 4.17-fold and 2.72-fold, and CAT activity increased by 1.55- and 1.64-fold, respectively. The effect of different levels of salt stress on H_2O_2 content was not significant and did not differ significantly among treatments (Fig. 1F). However, the MDA content in mulberry seeds decreased significantly with increasing salt concentration (Fig. 1G), thus alleviating the damage caused by salt to the cell membrane.

3.2 Identification of the Proteins in Mulberry Seeds under Salt Stress during Germination

TMT-based quantitative proteomics was used to analyze the dynamic changes in proteins in mulberry seeds. A total of 76,544 unique peptides were detected, and 7717 proteins were identified based on TMT data after removing duplicate proteins. We counted the molecular weights of the identified proteins (Fig. 2A), and the results showed that there were 811 (10.51%) of 10.0–20.0 kD, 1123 (14.55%) of 20.0–30.0 kD, 1193 (15.46%) of 30.0–40.0 kD, and 1067 (13.83%) of 40.0–50.0 kD, 959 (12.43%) were 50.0–60.0 kD, and 90 (1.17%) were larger than 200.0 kD. The numbers of proteins with 1 peptide, 2–5 peptides, 6–10 peptides and more than 11 unique peptides were 2224, 3207, 1351 and 935, respectively. In addition, the number of peptides corresponding to almost 70% of the identified proteins was ≥2, which indicated that the MS data could be further analyzed (Fig. 2B). Proteins with peptide sequence coverage changes of 40%–100%, 30%–40%, 20%–30%, 10%–20%, and 10% accounted for 9.33%, 7.62%, 11.77%, 22.68% and 48.61%, respectively (Fig. 2C). The peptide sequences of most proteins have good coverage, and the results show that the protein data are accurate and reliable.

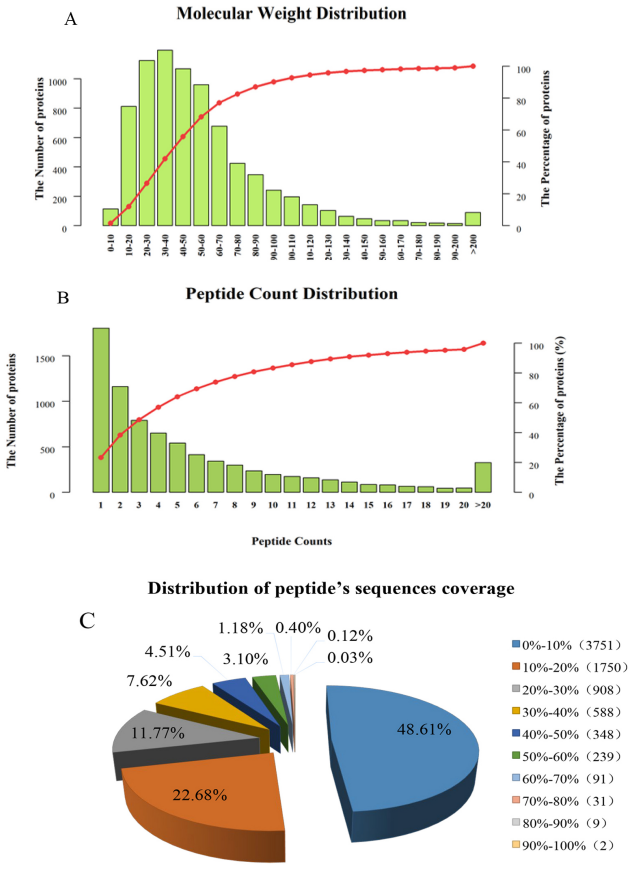

Fig. 2. The overall distribution of mulberry seed proteins identified by TMT. (A) Protein molecular weight distribution. (B) Peptide count distribution. (C) Distribution of peptide sequence coverage.

3.3 Identification and Analysis of DAPs

In this study, with the mulberry seedling protein sample (CK group) as the control, the quantitative intensity of mass spectrometry of the mulberry seedling protein sample in the 50 mmol/L NaCl and 100 mmol/L NaCl salt-treated groups was compared with the protein sample in the CK group. The mean value of three biological replicates was used as the fold difference of protein expression. A fold-change cut off of $\geq$1.2 and $p < 0.05$ for upregulated proteins and $\leq$0.83 and $p < 0.05$ for downregulated proteins was applied to identify the DAPs between salt-treated and control plants (Fig. 3). A total of 143 DAPs were identified in salt-treated (50 mmol/L NaCl) and control (CK) mulberry seeds, of which 61 DAPs were upregulated and 82 were downregulated (Fig. 3A). Then, 540 DAPs were identified in salt-treated (100 mmol/L NaCl) and control (CK) mulberry seeds, of which 222 DAPs were upregulated and 318 DAPs were downregulated (Fig. 3B). It is obvious that the DAP of the 100 mmol/L NaCl treatment is more than three times higher than that of the 50 mmol/L NaCl treatment, indicating that a high salt concentration has a greater impact on seed germination.

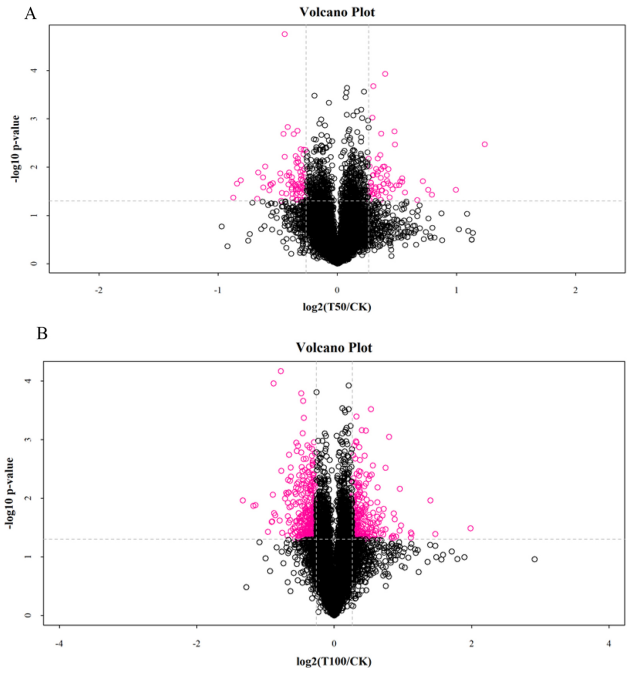

Fig. 3. Analysis of DAPs in mulberry seeds. (A) The volcano plots of DAPs in T50 vs. CK. (B) The volcano plots of DAPs in T100 vs. CK.

A total of 113 DAPs were screened in both salt treatments, of which 43 DAPs were upregulated and 70 DAPs were downregulated. By analyzing the hierarchical clustering analysis of DAP in different treatments, every three replicates of CK and salt-treated samples were clustered together separately with high reproducibility. Furthermore, the protein expression abundance and expression levels in mulberry seeds differed significantly between the CK and salt-treated samples (Fig. 4). At the same time, the functional analysis of the differentially abundant proteins screened from the two salt-stressed mulberry seeds showed that the differentially expressed proteins mainly belonged to reactive oxygen species (ROS) scavenging, stress defense, energy production, carbohydrate metabolism, transcription and translation, growth and development, signal transduction, substance transport, protein synthesis, folding and degradation categories (Table 1). Statistics of differential proteins include molecular weight, fold difference and *p* value.

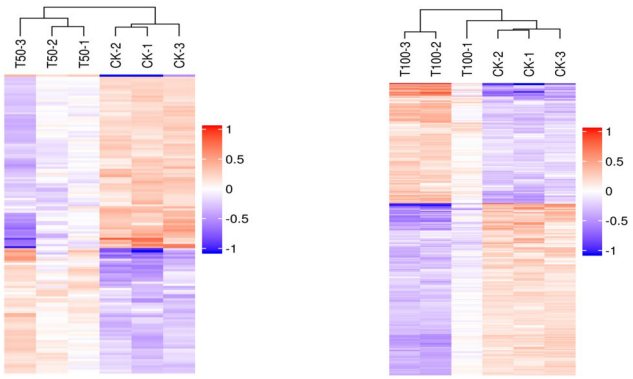

Fig. 4. Hierarchical clustering analysis of the DAPs in different treatments.

3.4 Functional Annotation of DAPs

In the GO enrichment analysis of DAP in T50 vs. CK, DAP was significantly enriched into 66 functional GO terms, of which 40 belonged to biological processes (BPs), 25 to molecular functions (MFs), and 1 to cellular components (CCs). Among the top 20 enriched GO terms (Fig. 5A), carotenoid biosynthesis, tetraterpenoid biosynthesis, tetraterpene metabolism, carotenoid metabolism, lipid metabolism, and transcription elongation from the RNA polymerase II promoter were the most significantly enriched GO terms in the BP category. In the MF category, hydrolase activity (acting on ester bonds), polygalacturonase activity, secondary activity transmembrane transporter activity, nuclease activity and hydrolase activity were the most significantly enriched GO terms. Moreover, the most important term for GOs in the CC category is the extracellular region. GO enrichment analysis of the DAPs identified in T100 vs. CK resulted in a significant enrichment of 122 functional GO terms, of which 50 belonged to BPs, 46 to MFs, and 26 to CCs. In the top 20 enriched GO terms (Fig. 5B), cellular glucan metabolism, glucan metabolism, cellular polysaccharide metabolism, carotenoid biosynthesis, and abscisic acid-activated signaling pathway were the most significantly enriched GO terms

Table 1. Partial DAPs of mulberry seeds induced by salt stress.

Function classification	Accession	Description	MW [kDa]	Fold Change		*p*-value
				T50/CK	T100/CK	
Reactive oxygen scavenging and stress defense	W9QU60	Peroxidase	34.19	1.379	1.678	0.024
	W9RZP5	Superoxide dismutase	26.41	1.276	1.405	0.036
	A0A1D8KVU3	Dihydroflavonol reductase 1	38.30	0.793	0.775	0.008
	W9QH65	Glutathione peroxidase	26.50	1.268	1.361	0.024
	W9T1J8	SHSP domain-containing protein	24.41	1.206	1.323	0.003
	W9RN30	Jacalin-type lectin domain-containing protein	15.71	1.321	1.740	0.017
	W9RJC9	Putative disease resistance protein RGA4	128.22	0.833	0.800	0.030
	W9SE40	Sulfiredoxin	13.76	0.827	0.773	0.002
	W9RK87	Spermine synthase	43.61	1.258	1.284	0.024
	W9SCX0	Lycopene beta cyclase	56.26	0.768	0.806	0.029
	W9QK12	Lycopene epsilon cyclase	59.98	0.827	0.725	0.026
	W9RBR2	Allene oxide synthase	57.48	0.649	0.538	0.042
	W9RAN9	Anthocyanin 5-aromatic acyltransferase	52.24	0.795	0.685	0.028
Energy production	W9S8G4	Lipase	45.45	1.458	1.866	0.030
	W9QX55	Lipoxygenase	86.97	0.821	0.758	0.009
	Q09X21	Photosystem II CP43 reaction center protein	51.79	0.827	0.783	0.048
	Q09X17	Photosystem I P700 chlorophyll a apoprotein A1	83.06	0.807	0.724	0.029
	W9QPP1	Oxygen-evolving enhancer protein 3-2	24.70	0.628	0.545	0.019
	W9R2T4	Glucose-1-phosphate adenylyltransferase	59.46	1.249	1.299	0.020
	W9RFS1	Short-chain type	64.75	1.397	1.413	0.011
	W9SKC6	Flavin-containing monooxygenase	47.43	0.736	0.543	0.034
	W9R035	Frataxin	22.18	1.292	1.279	0.035
	W9R2T4	Glucose-1-phosphate adenylyltransferase	59.46	1.249	1.299	0.020
	W9RCL2	Lipase_3 domain-containing protein	58.76	1.288	1.437	0.036
	W9QJ14	Sphingoid base hydroxylase 2	30.37	1.290	1.458	0.015
	W9RFT7	Alpha-carbonic anhydrase domain-containing protein	30.62	0.718	0.644	0.023
Carbohydrate metabolism	W9QF53	Cellulose synthase	120.43	0.829	0.830	0.024
	W9RH68	Endoglucanase	55.19	0.802	0.741	0.019
	W9SJJ0	Alpha-L-fucosidase 1	58.36	1.218	1.277	0.003
	W9S5L8	Fructose-1,6-bisphosphatase	44.87	0.810	0.744	0.029
	W9R8E3	Putative polygalacturonase	63.38	1.442	1.426	0.033
Transcription and translation	W9R0R0	Histone	53.57	0.721	0.748	0.013
	W9SN28	Histone H1	23.17	0.687	0.628	0.023
	W9RWW0	Transcriptional corepressor SEUSS	108.92	0.785	0.583	0.023
	W9SG17	BHLH domain-containing protein	39.45	0.719	0.658	0.021
	W9QUN0	Transcription initiation factor IIF subunit alpha	52.71	0.817	0.801	0.025
	W9RKQ5	HMG1/2-like protein	15.88	0.736	0.762	0.002
	W9RJ53	High mobility group B protein 2	16.04	0.755	0.735	0.001
	W9QWT1	Peptidylprolyl isomerase	24.58	0.794	0.731	0.025
	W9QWT8	Glyceraldehyde-3-phosphate dehydrogenase B	147.81	0.795	0.713	0.034
	W9STM4	Btz domain-containing protein	73.82	0.771	0.695	0.016
	W9SN10	AT-hook motif nuclear-localized protein	31.51	0.631	0.621	0.045
	W9QMK8	TCP domain-containing protein	38.21	0.752	0.679	0.015
	W9RVK1	SWI/SNF chromatin-remodeling complex subunit snf22	246.07	0.803	0.750	0.008
	W9SBM7	U3 small nucleolar RNA-associated protein 15-like protein	58.89	0.788	0.725	0.028

Table 1. Continued.

Function classification	Accession	Description	MW [kDa]	Fold Change		*p*-value
				T50/CK	T100/CK	
Growth and development	W9R8E1	GDSL esterase/lipase	38.53	1.695	1.822	0.010
	W9RZ71	Aspartic proteinase nepenthesin-2	63.51	1.457	1.571	0.030
	W9RMA6	Beta-galactosidase	88.18	1.644	1.785	0.022
	W9RY25	Subtilisin-like protease	87.92	1.310	1.359	0.011
	W9QYC8	GDSL esterase/lipase	41.61	1.216	1.203	0.023
	W9SHN8	GDSL esterase/lipase	40.75	1.409	1.446	0.011
	W9RD56	GDSL esterase/lipase 6	39.63	1.432	1.543	0.002
	W9RG64	Purple acid phosphatase	50.47	1.344	1.368	0.039
	W9QMY5	Purple acid phosphatase	50.11	1.345	1.382	0.025
	W9S1A8	Pectinesterase	62.59	0.813	0.784	0.013
	W9SKF4	Caffeic acid 3-O-methyltransferase	29.05	0.657	0.647	0.016
Signal transduction	W9RBX0	Putative receptor-like protein kinase	91.76	1.333	1.388	0.007
	W9RZ35	Putative L-type lectin-domain containing receptor kinase S.5	76.53	1.204	1.248	0.037
	W9QVJ6	Protein kinase domain-containing protein	31.02	0.796	0.702	0.002
	W9RA31	Systemin receptor	127.65	0.806	0.811	0.046
	W9R8I4	G-type lectin S-receptor-like serine/threonine-protein kinase	134.05	0.794	0.800	0.048
Transport proteins	W9R5Q9	Putative cyclic nucleotide-gated ion channel 8	85.60	0.790	0.783	0.037
	W9SAJ0	ABC transporter G family member 28	112.05	1.247	1.334	0.049
	W9RS36	ABC transporter G family member 8	66.42	0.790	0.746	0.048
	W9RGK8	Nitrate transporter 1.1	29.38	0.755	0.680	0.023
	W9SDQ8	Nitrate transporter 1.3	66.41	0.768	0.639	0.020
	W9QFD5	Protein DETOXIFICATIO	59.23	1.213	1.301	0.031
	W9RWS8	Transmembrane ascorbate ferrireductase 1	25.28	1.232	1.226	0.047
	W9QQQ4	Putative sulfate transporter 3.3	74.52	0.806	0.789	0.046
	W9QN95	Protein translocase subunit SecA	118.21	0.829	0.750	0.016
	W9S950	CMP-sialic acid transporter 2	44.79	1.355	1.335	0.006
	W9SK42	t-SNARE coiled-coil homology domain-containing protein	56.65	1.394	1.414	0.017
Protein synthesis, folding and degradation	W9SIU2	30S ribosomal protein S21	20.85	0.824	0.735	0.012
	W9QWL4	50S ribosomal protein L34	16.60	0.680	0.683	0.030
	W9SXH8	Carboxypeptidase	58.05	1.335	1.451	0.012
	W9SPH4	Ribonuclease 3	23.25	2.357	2.638	0.015

in the BP category. Protein dimerization activity, protein heterodimerization activity, DNA binding, isoprenoid binding, alcohol binding, and abscisic acid binding were the most significantly enriched GO terms in the MF category. The most significantly enriched GO terms in the CC category were protein–DNA DNA packaging complex, DNA packaging DNA packaging complex, nucleosome, chromatin and chloroplast part.

3.5 KEGG Pathway Enrichment Analysis of DAPs

Furthermore, DAPs were also annotated according to the KEGG pathway. KEGG enrichment analysis of T50 vs. CK DAPs showed that 55 pathways were enriched, including photosynthesis, chlorocyclohexane and chlorobenzene degradation, fluorobenzoate degradation, toluene degradation, and prodigiosin biosynthesis. Notably, only one pathway, carotenoid biosynthesis, was significantly enriched among these enriched pathways (Fig. 6A). In the KEGG pathway enrichment analysis of DAPs of T100 vs. CK, a total of 147 pathways were enriched, of which 7 were significantly enriched; these pathways mainly related to photosynthesis, systemic lupus erythematosus, carotenoid biosynthesis, alcoholism, mineral absorption, plant hormone signal transduction and necroptosis (Fig. 6B).

3.6 PRM-Based Validation of TMT Data

To validate the TMT proteomic data, we selected five differentially abundant proteins for PRM quantification (Table 2). PRM identified five candidate differentially abundant proteins, including cellulose synthase, glyceraldehyde-3-phosphate dehydrogenase B, superoxide dismutase, glutathione peroxidase, and photosystem II CP43 reaction center protein. The PRM results exhibited consistency in the differential expression patterns of the selected proteins with the TMT data. In 50 mmol/L NaCl solution, the expression levels of glutathione per-

Table 2. Comparison of the quantitative results between TMT and PRM.

Accession	Gene name	Description	TMT		PRM	
			T50/CK	T100/CK	T50/CK	T100/CK
W9QF53	L484_012515	Cellulose synthase	0.829	0.830	0.835	0.455
W9QWT8	L484_024833	Glyceraldehyde-3-phosphate dehydrogenase B	0.795	0.713	0.682	0.175
W9RZP5	L484_013984	Superoxide dismutase	1.276	1.405	1.363	1.868
W9QH65	L484_024293	Glutathione peroxidase	1.268	1.361	1.016	1.508
Q09X21	psbC	Photosystem II CP43 reaction center protein	0.827	0.783	0.648	0.196

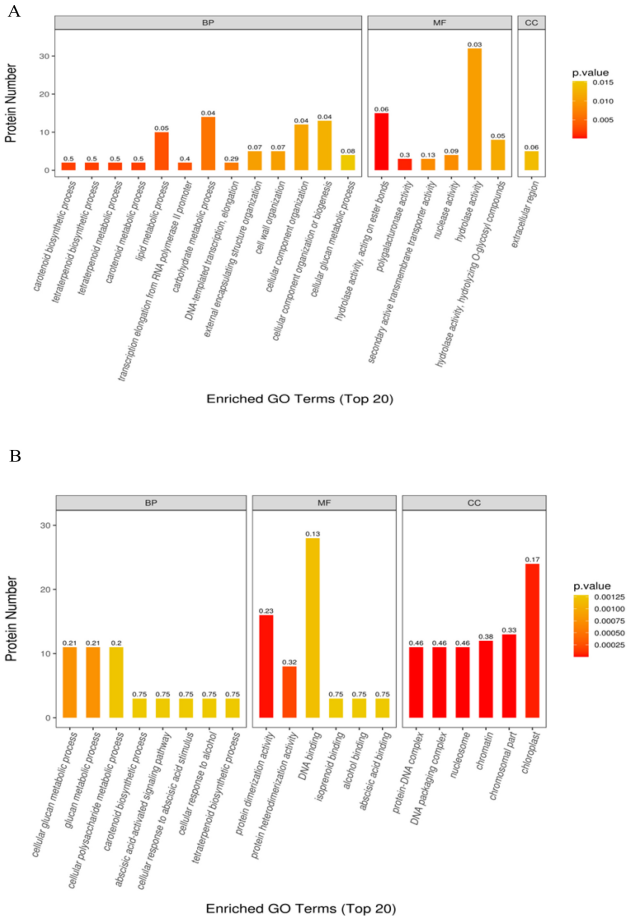

Fig. 5. Gene ontology annotation of all differentially abundant proteins identified in mulberry seeds. The top 20 significantly enriched GO terms in (A) T50 vs. CK and (B) T100 vs. CK.

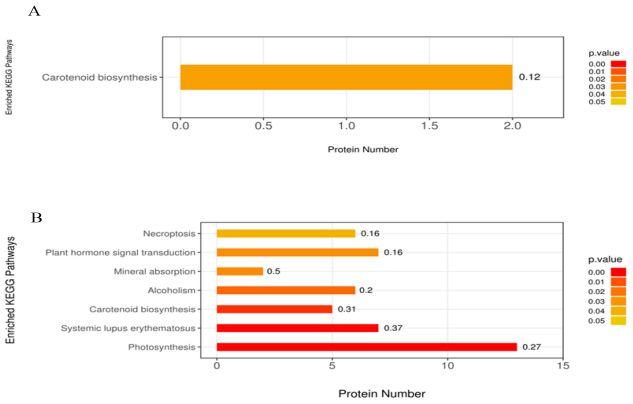

Fig. 6. KEGG pathway enrichment analysis of DAP in mulberry seeds. The significantly enriched KEGG pathways in DAPs from (A) T50 vs. CK, (B) T100 vs. CK. The abscissa is the number of proteins involved in metabolic pathway; the ordinate is metabolic pathway. The color gradient represents the *p*-value below 0.05. The darker the color (closer to red), the smaller the *p*-value, and the more significant is the enriched KEGG pathways. The number on the bar graph indicates the rich factor, referring to the percentage of differential proteins that are involved in this pathway.

oxidase and superoxide dismutase increased significantly in PRM and TMT analysis, while the expression levels of cellulose synthase, glyceraldehyde 3-phosphate dehydrogenase B and photosystem II CP43 reaction center decreased significantly in PRM and TMT analysis. In the 100 mmol/L NaCl solution, the expression of cellulose synthase, glyceraldehyde-3-phosphate dehydrogenase B and photosystem II CP43 reaction center protein was downregulated in the PRM data and did not change according to the TMT results. Overall, the consistency of PRM and TMT results validates the credibility of TMT in proteomic analysis.

4. Discussion

Seed germination and seedling growth are among the most sensitive stages in plant life, representing the first contact with water and soil, whereas salt stress affects seed germination and seedling growth [47]. In the current study, we identified salt stress-responsive proteins during mulberry seed germination at the proteomic level by the TMT method. A total of 143 DAPs were identified when treated with 50 mmol/L NaCl solution, of which 61 were upregulated and 82 were downregulated, and 540 DAPs were identified when treated with 100 mmol/L NaCl solution, of which 222 proteins were upregulated and 318 were downregulated. A total of 113 DAPs were screened in the two salt treatments, of which 43 proteins were upregulated and 70 proteins were downregulated. Functional analysis of DAPs screened from mulberry seeds treated with salt stress showed that the differentially expressed proteins mainly belonged to ROS scavenging, stress defense, energy production, carbohydrate metabolism, transcription and translation, growth and development, signal transduction, sub-

stance transport, protein synthesis, folding and degradation. In the following section, we will discuss the differential proteins that fall into these functional categories.

4.1 Salt Stress Induces Changes in ROS Scavenging and Stress Defense

During seed germination, respiration produces large amounts of ROS, and salt stress accelerates the accumulation of ROS, resulting in an imbalance between ROS production and detoxification. In response to this oxidative stress, an effective antioxidant system can scavenge excess ROS [48]. ROS can also contribute to the endosperm weakening during germination as a result of the cell wall loosening, but uncontrolled ROS production can lead to cell damage, resulting in seed deterioration, retarded germination and early seedling development, and the production of some antioxidants, such as SOD, POD, CAT, glutathione-related enzymes and dehydrogenases, to reduce ROS-induced cell damage in plants [49]. In this study, physiological data showed that the activities of SOD, POD, and CAT in mulberry seedling samples continued to increase under NaCl-induced salt stress. It has been reported that the activities of antioxidant enzymes, such as SOD, POD, CAT, and glutathione-related enzymes, generally increase in plants under oxidative stress conditions, resulting in enhanced tolerance to stress [50]. Proteomics research in this study showed that antioxidant-related proteins, such as peroxidase (W9QU60), superoxide dismutase (W9RZP5) and glutathione peroxidase (W9QH65), were significantly upregulated during seed germination under salt stress. Moreover, the changes in peroxidase, superoxide dismutase and glutathione peroxidase were higher under 100 mM NaCl treatment than under salt stress of 50 mM NaCl. These results may indicate that the plants suffer from relatively severe oxidative stress and need to initiate antioxidant enzyme systems to scavenge excessive ROS. Under the induction of 50 mM NaCl and 100 mM NaCl, the MDA content decreased from 1.39-fold to 3.81-fold compared with the control. MDA is mainly produced by polyunsaturated fatty acid peroxides and is an important indicator of cell membrane lipid peroxidation and plasma membrane damage caused by biotic and abiotic stresses [51]. In general, the lower the MDA content is, the less damage the plasma membrane incurs. The results of this study showed that the damage to the plasma membrane in mulberry seeds was relatively mild, indicating that mulberry seedlings exhibit significant salt tolerance under salt stress.

Salt stress can induce the production of ROS and damage plant cells, and to regulate ROS levels, many defense proteins are induced during plant responses to biotic or abiotic stresses [52]. In this study, in response to changes in ROS scavenging and stress defense induced by salt stress, 15 DAPs (7 upregulated and 8 downregulated) were screened under salt stress (50 mM NaCl), while under 100 mM NaCl salt stress, 40 DAPs were screened (24 upregulated and 16 downregulated); a total of 13 DAPs were screened in both salt treatments (5 upregulated and 8 downregulated). Heat shock proteins (HSPs), also known as heat stress proteins, play an important role in regulating the disease resistance and stress response of plants [53]. sHSP is the plant heat shock protein with the smallest molecular weight, and this protein mainly protects other proteins from various stresses in plants through molecular chaperones. A large number of studies have shown that sHSP members can respond to various external stresses [54]. Overexpression of the rice sHSP member Os HSP16.9 can improve the salt resistance and drought resistance of rice plants [55]. Overexpression of the sHSP member Ms HSP17.7 in alfalfa can improve the defense ability of alfalfa plants against heat, drought, and antioxidant stresses [56]. In this study, the expression of sHSP domain-containing protein (W9T1J8) was upregulated, and the fold change ratio increased with increasing salt concentration, which indicated that the upregulated expression of sHSP domain-containing protein was a defense mechanism of mulberry seed germination in response to salt stress. Furthermore, KEGG enrichment analysis of DAPs showed that seed germination under salt stress was mainly enriched in carotenoid biosynthesis. Carotenoids are natural pigments that exhibit antioxidant activity, play an important role in the production of plant hormones and can effectively protect plant cells [57]. Lycopene cyclization is an important part of the carotenoid biosynthetic pathway in plants and is catalyzed by both lycopene β-cyclase (LCY-β) and lycopene ε-cyclase (LCY-ε) [58]. In this study, proteins related to carotenoid biosynthesis, such as zeta-carotene desaturase (W9QKC9), lycopene ε cyclase (W9QK12) and lycopene β cyclase (W9SCX0), were significantly downregulated. Studies have shown that downregulated IbLCY-ε increases the synthesis of carotenoids in the nonembryonic callus of transgenic sweet potato and enhances the tolerance of sweet potato to environmental stress and the ability to grow on marginal land [59]. The above results further indicate that salt stress induces changes in DAP that were related to ROS scavenging and stress defense; thus, the tolerance and antioxidant capacity of mulberry seeds to salt stress were maintained during germination.

4.2 Salt Stress Induces Changes in Energy Production and Carbohydrate Metabolism

In plants, glycolysis and the tricarboxylic acid (TCA) cycle are the main features of carbohydrate and energy metabolism, which not only satisfy energy demands but also produce many essential cofactors and other substrates of metabolism [60]. In this study, to examine changes in energy production and carbohydrate metabolism induced by salt stress, 26 DAPs (9 upregulated and 17 downregulated) were screened under a salt stress of 50 mM NaCl, 77 DAPs (31 upregulated and 46 downregulated) were screened under a 100 mM NaCl salt stress, and a total

of 17 DAPs (8 upregulated and 9 downregulated) were screened under both salt treatments. Glyceraldehyde 3-phosphate dehydrogenase catalyzes the conversion of 3-phosphoglyceraldehyde to 1,3-diphosphoglyceric acid and plays an important role in sugar metabolic pathways in plants [61]. Phosphoglycerate translocase plays an important role in glycolysis by catalyzing the conversion of phosphate from 3-phosphoglycerate to a second carbon to form 2-phosphoglycerate. In this study, the proteomic analysis results showed that related proteins involved in glycolysis and the tricarboxylic acid cycle, including short-chain type (W9RFS1), 2,3-diphosphoglycerate nondependent phosphoglycerate translocase (W9SCE4), 3-phosphoglyceraldehyde dehydrogenase (W9SF99), and phosphoenolpyruvate carboxylase 2 (W9RNP2), were significantly upregulated, while fructose diphosphate aldolase (W9RMK1) was significantly downregulated, indicating that when most identified glycolytic enzymes exhibited increased expression, the production of more NADH was promoted during mulberry seed germination under salt stress; then, the oxidative phosphorylation process by mitochondria began for ATP synthesis, providing the necessary energy for normal cell metabolism under salt stress and playing an important role in the process of improving salt tolerance of mulberry. Moreover, seeds require energy for germination and growth, and lipids are the most efficient form of energy storage in plant seeds [62]. Plant lipases are responsible for the hydrolysis of acylglycerides during lipid processing and can hydrolyze the oil stored in the seeds to provide energy and protection [63]. The expression of lipase (W9S8G4) was significantly upregulated in this study, indicating that this lipase may have enhanced the salt tolerance of mulberry seeds by providing energy and thus promoting germination. It has been found that the overexpression of *Arabidopsis* lithium-tolerant lipase increased salt tolerance in transgenic *Arabidopsis* plants, allowed seed to germinate in the presence of NaCl, and stimulated nutritional growth, flowering, and fruit set in the presence of NaCl [64]. Photosynthesis is a fundamental process that promotes the nutritional growth and development of plants, providing the necessary energy for seed germination and early seedling establishment [65]. It has been shown that during seed development, photosynthesis plays an important role in seed maturation; furthermore, inhibition of photosynthesis in seeds results in delayed germination and reduced seed storability, as well as reduced seedling vigor [66]. In this study, proteins related to photosynthesis cytochrome f (Q09X04), chloroplast heme oxygenase (A0A076K090) and oxygen precipitation enhancing protein 3-2 (W9QPP1) were significantly downregulated, indicating that photosynthesis-related proteins may be inhibited by salt stress during seed germination.

In addition, carbohydrate metabolism regulates the synthesis and conversion of sugars, and environmental stresses can disrupt carbohydrate metabolism in plant species [67]. It has been reported that glycoside hydrolase is among the key enzymes in carbohydrate metabolism, which can affect cellulose biosynthesis, such as β-D-xylosidase and xyloglucan transglycosidase/hydrolase, which is involved in sugar biosynthesis and remodeling, and glycoside hydorlase has been identified in the seed development process of many species [68]. Altering the expression levels of glycoside hydrolase genes can regulate cellulose levels and thus biomass production [69]. Glycoside hydrolase genes are upregulated in rice in response to biotic and abiotic stress treatments, indicating their role in stress adaptation [70]. Various proteins related to carbohydrate metabolism in this study, including the putative xyloglucan endoglucosylase/hydrolase protein 32 (W9SJJ4) and β-D-xylosidase 1 (W9R6G8), were significantly upregulated, indicating that these proteins have an important role in adaptation to salt stress during mulberry seed germination.

4.3 Salt Stress Induces Changes in Transcription, Translation and Growth and Development

In this study, the changes in transcription, translation and growth and development caused by salt stress were examined, and 32 DAPs were screened under 50 mM NaCl stress treatment (11 upregulated and 21 downregulated), 116 DAPs (50 upregulated and 66 downregulated) were screened under 100 mM NaCl salt stress treatment, and a total of 22 DAPs were screened in both salt treatments (10 upregulated and 12 downregulated). Seed germination is a complex trait influenced by many genetic, endogenous and environmental factors and is driven by many cellular processes, including transcription and translation [71]. Proteins involved in the regulation of transcriptional and translational activities are important because oxidative stress induced by high NaCl concentrations may affect protein integrity, indicating that the production of new proteins is essential to maintain plant growth [72]. In this study, we found that various transcription and translation factor-related proteins were significantly upregulated, especially under 100 mM NaCl stress, including transcription factor VIP1 (W9RA57), nuclear transcription factor Y subunit C-9 (W9QV28), translation initiation factor IF-2 (W9RQB9), and eukaryotic translation initiation factor 5A (W9S2P4). This indicates that salt stress may affect the process of initiating protein translation during seed germination in *Morus alba*. It has been shown that under stress conditions, epigenetic changes (i.e., DNA methylation and histone modifications can regulate the expression of important genes associated with stress tolerance and can activate the transcription of genes that respond to abiotic stresses [73]. Histones can be involved in promoting the unwinding, replication and transcription of DNA as well as stabilizing chromatin structure under salinity [60]. It has been reported that SRT-like histone deacetylases in *Arabidopsis* inhibit transcription, thereby reducing excessive H_2O_2 accumulation and DNA damage under salt stress and enhancing salt tolerance

during seed germination in Arabidopsis [74]. High mobility group proteins (HMG) are widely involved in gene expression and regulation, including DNA replication, transcription, recombination, and DNA repair processes [75]. In the present study, histone (W9R0R0), histone H1 (W9SN28) and high mobility group B protein 2 (W9RJ53) were significantly downregulated, indicating that these proteins may inhibit transcription for germination of mulberry seeds, resulting in better adaptation to salt stress.

Studies have shown that GDSL esterase/lipase is widely distributed in plants, is mainly involved in the regulation of growth and development, and plays an important role in seed germination [76,77]. It has been reported that GDSL esterase/lipase is overexpressed in transgenic plants [78]. The overexpression of *Arabidopsis* LTL1, a salt-inducible gene encoding a GDSL motif lipase, also improved salt tolerance in yeast and transgenic plants [79]. The expression levels of GDSL esterase/lipase (W9R8E1) and GDSL esterase/lipase 6 (W9RD56) were significantly upregulated in this study, indicating that these proteins may promote the tolerance of mulberry seed germination to salt stress. *Aspartic proteinases* (APs) are involved in the degradation of storage proteins, hydrolyze seed storage proteins to provide amino acids for seed germination, and play an important regulatory role in plant growth and development, disease resistance, and adversity stress processes [80]. Studies have shown that the *Fagopyrum esculentummoench* AP member Fe AP9 is upregulated in response to darkness, drought and salicylic acid stress [81]. Overexpressing the grape AP gene *VlAP17* can improve salt tolerance and drought tolerance by protecting the integrity of the plasma membrane and increasing the root quality of seedlings [82]. In this study, the expression of aspartic proteinase nepenthesin-2 (W9RZ71) and bark storage protein A (W9RDI0) were significantly upregulated, which indicated that the salt tolerance of mulberry seeds might be enhanced by hydrolyzing and storing protein during germination.

4.4 Salt Stress Induces Changes in Signal Transduction and Substance Transport

In this study, we studied the changes in signal transduction and substance transport caused by salt stress. We screened 14 DAPs (7 upregulated and 7 downregulated) under salt stress (50 mM NaCl) and 51 DAPs (16 upregulated and 35 downregulated) under salt stress (100 mM NaCl), while 17 DAPs (7 upregulated and 10 downregulated) were screened in two salt treatments. Seed germination determines the beginning of the seed plant life cycle, which is a complex process that involves various signal transduction pathways [83]. Receptor-like kinase is a common key signaling molecule in many stress reactions that can mediate the response of plants to development and environmental stimuli [84]. Receptor-like protein kinase may play a role in transducing extracellular information into cells. The kinase consists of an extracellular domain, transmembrane domain and cytoplasmic-located serine/threonine protein kinase domain [85]. Studies have shown that the plant hormone abscisic acid (ABA) regulates seed germination, plant growth and development, while protein kinase may positively regulate ABA signal transduction in seed germination and seedling growth [86]. In this study, many protein kinases related to signal transduction were found to be significantly upregulated, including putative receptor-like protein kinase (W9RBX0), receptor-like protein kinase HERK 1 (W9RFT8), putative serine/threonine protein kinase (W9RNL9), serine/threonine protein kinase PRP4-like protein (W9RJN3) and putative LRR receptor-like serine/threonine protein kinase (W9SN33), suggesting that these proteins may play a critical role in the germination of mulberry seeds. Under stress, plants induce transporters to transport signal molecules to express some stress resistance genes and cope with adverse environments [87]. In wheat roots under NaCl stress, several K^+ transporters, such as voltage-gated potassium channels, cyclic nucleotide-gated potassium channels and ABC transporters, increased; thus, increasing potassium absorption is an important strategy to regulate root ion homeostasis to cope with salt and alkali stress [88,89]. As a potential nonselective cation transporter, cyclic nucleotide gated channels (CNGCs) can participate in the absorption and/or transport of monovalent and possible divalent cations and play a role in plant defense and seed germination [90]. It has been reported that CNGC10 negatively regulates the salt tolerance of *Arabidopsis thaliana*, indicating that it may inhibit the salt tolerance of *Arabidopsis thaliana* seed germination by participating in mediating Na^+ transport [91]. In this study, the estimated cyclic nucleotide gated ion channel 8 (W9R5Q9) was significantly downregulated, while the ABC transporter G family member 28 (W9SAJ0) was significantly upregulated. However, studies have shown that the ABC transporter is directly powered by ATP, which can transport complex organic substances against the concentration gradient. ATP is essential for plant growth and development and plays an important role in seed development, seed germination, abiotic stress response and the interaction between plants and the environment [92,93]. Overexpressing *Arabidopsis thaliana* BCG 36 reduces the sodium content in buds and roots, thus endowing the plant with salt tolerance [94]. Early studies have shown that salt stress mainly reduces NO_3^- absorption by regulating different nitrate transporters [95]. The expression of the nitrate transporter gene family (*OsNRT*) in rice is downregulated, which indicates that salt stress affects its expression level, which may inhibit the accumulation of NO_3^- during rice seed germination [96]. In this study, nitrate transporter 1.1 (W9RGK8) was significantly downregulated, which may indicate that the accumulation of NO_3^- under salt stress was also inhibited. These results indicate that these substance transport signaling molecules may respond to salt stress through dif-

ferent expression patterns.

4.5 Salt Stress Induces Changes in Protein Synthesis, Folding and Degradation

In this study, to examine the changes in protein synthesis, folding and degradation caused by salt stress, we screened 7 DAPs (2 upregulated and 5 downregulated) under salt stress (50 mM NaCl) and 39 DAPs (9 upregulated and 30 downregulated) under salt stress (100 mM NaCl), while 5 DAPs (3 upregulated and 2 downregulated) were screened under two salt treatments. Proteins enable life activities, and when seeds germinate, the change in soluble protein content can reflect various information on protein synthesis, denaturation and degradation in cells. In many plants, such as *Arabidopsis* [97], *Ricinus communis* [98] and *Phoenix dactylifera* [99], protein folding and stability involve protein synthesis, including ribosomal proteins. Ribosomes consist of two parts, namely, ribosomal protein (RP) and ribosomal RNA. RP not only maintains the configuration of RNA but also participates in the synthesis, transportation and localization of proteins [100]. Studies have shown that under Na_2CO_3 stress, almost all the genes involved in protein synthesis were downregulated in *Puccinellia tenuiflora* seedlings, which indicated that the protein synthesis mechanism of *Puccinellia tenuiflora* was inhibited by Na_2CO_3 stress [101]. Additionally, the abundance of $NaHCO_3$ and NaCl was reduced in ribosomal proteins in tomato [102] and Arabidopsis [103]. Moreover, 30S ribosomal protein S1 in annual ryegrass and 50S ribosomal protein in perennial ryegrass decreased under salt stress, which affected the folding and synthesis of protein in annual and perennial ryegrass [104]. In this study, many of the proteins involved in protein synthesis were significantly downregulated, including the ribosomal protein family 30S ribosomal protein S21 (W9SIU2), 50S ribosomal protein L28 (W9QPT4), 50S ribosomal protein L34 (W9QWL4) and 60S ribosomal protein L28-1 (W9QUV3), indicating that the protein synthesis mechanism of mulberry is inhibited by salt stress during seed germination, thus affecting protein folding and synthesis.

Studies have shown that ubiquitin-binding enzymes are highly involved in proteolysis, protein modification and protein degradation, including abiotic stress, and play an important role in all aspects of plant growth, development and cell processes [105]. It has been reported that overexpressing the soybean ubiquitin-binding enzyme gene *GmUBC2* can enhance salt tolerance by regulating the expression of abiotic stress-responsive genes in *Arabidopsis thaliana* [106]. DnaJ plays an important role in the NaCl tolerance of *Arabidopsis thaliana* and is a molecular chaperone involved in many cellular processes, including the regulation of protein folding, protein degradation, protein activity and protein aggregation prevention [107]. In this study, proteins related to protein folding and degradation were significantly upregulated, including ubiquitin-binding enzyme E2 27 (W9RHB5), DnaJ-like protein (W9QJ41) and peptidyl prolyl cis-trans isomerase (W9RAD0). This result indicated that the folding and stability of these proteins might be enhanced to cope with the salt tolerance of mulberry seed germination.

5. Conclusions

This study explored the response mechanism of mulberry seed germination to salt stress at the physiological and proteomic levels. Physiological data showed that salt stress inhibited the germination rate and radicle length of mulberry seeds, decreased the content of MDA, and significantly increased the activities of SOD, POD and CAT. Then, the TMT marker technique was used to analyze the protein group of mulberry seeds in two salt treatment stages, and 76,544 unique peptides were detected. After removing duplicate proteins, 7717 proteins were identified according to TMT data. Through proteomic analysis, we obtained 143 (50 mM NaCl) and 540 (100 mM NaCl) DAPs associated with mulberry seed germination. Furthermore, a total of 113 DAPs were treated with 50 mM NaCl and 100 mM NaCl solutions, of which 43 were upregulated and 70 were downregulated. KEGG analysis showed that the proteins involved in photosynthesis, carotenoid biosynthesis and plant hormone signal transduction were usually enriched in mulberry seeds. Finally, five differential proteins were verified by PRM, which validated the credibility of TMT in proteomic analyses. The data presented herein provide new insights into plant salt-responsive proteins, with potential implications for enhancing the salt tolerance of mulberry plants. Future research requires further analysis at the RNA and RT-PCR levels for deeper molecular level studies to provide a more in-depth functional study of the genes and proteins involved in salinity tolerance.

Availability of Data and Materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

YW conceived and designed this study; YW, WJ, JC, WG and YL performed the experiments, interpreted the results and wrote the article; YW, WJ, WG, YL and CL revised the article and analyzed the data. All the authors read and approved the final version.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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