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Proteomic analysis of the regulatory network of salt stress in *Chrysanthemum*

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Abstract

Background Saline-alkali stress is one of the main abiotic stresses that constrains plant growth. Understanding the response mechanism of ornamental plants to saline-alkali stress is of great significance for improving saline-alkali landscape greening. *Chrysanthemum* is a good ornamental plant with strong resistance to stress, rich colors and easy management.

Results Using TMT quantitative proteomics technology, leave and root of *Chrysanthemum* that were either untreated or treated with 200 mM NaCl for 12 h, screened the differentially expressed proteins. The results showed that 66 and 452 differential proteins were present in leaves and roots after salt treatment, respectively. GO function is mainly related to carbohydrate and energy metabolism, hormone response, antioxidant response and membrane protein activity. The KEGG metabolic pathway is mainly concentrated in glycine metabolism, glutathione metabolic pathway, carbon fixation in prokaryotes, 2-oxy-carboxylic acid metabolism. Combining transcripto-proteomics, GO and KEGG analyses revealed significant enrichment in starch anabolic catabolism, redox processes, ion homeostatic transport, phenylpropane biosynthesis.

Conclusions Under salt stress, the active pathways of carbohydrate and energy metabolism and glutathione metabolism enable plants to accumulate more energy substances and improve antioxidant capacity, which may play a safeguarding role in maintaining growth and development and mitigating reactive oxygen species damage in *Chrysanthemum* under stress. The purpose of this study was to screen key proteins and regulatory networks through proteomic assay, and reveal the molecular mechanism of response to salt stress. The research not only provides resources for salt-tolerant breeding of *Chrysanthemum* but also offers theoretical support for agricultural production and ecological environmental protection.

Keywords Chrysanthemum, Salt stress, Proteomics, Transcriptome, Starch and energy metabolism, Glutathione metabolism

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Introduction

The growth and development of plants cannot be separated from the natural environment, and abiotic stresses such as drought and salinization have become important environmental factors restricting plant development and evolution [1]. At present, the global saline-alkali land is gradually expanding, and the area of saline-alkali land in China has reached 1×10^8 hm^2 [2]. The problem of soil salinization is increasingly severe, urgently necessitating in-depth research into the response mechanisms of plants to salt stress to improve their salt tolerance and adaptive capacity. Studies have shown that plant roots in salinized soil are difficult to absorb water from the soil, cellular metabolism is hindered and slowed down, and osmotic stress caused by the excessive accumulation of Na^+/K^+ leads to decreased water potential of plant roots and cell dehydration, which inhibits the cellular growth and amplification; The effect of ion toxicity on plant growth is slightly delayed. High concentration of Na^+ will inhibit the absorption of K^+, and K^+ can affect protein synthesis and enzyme activity in plants, resulting in disorder of physiological activities and growth obstruction of plants [3]; Due to the destruction of osmosis and ion balance in plants, the redox balance will be further broken, resulting in excessive accumulation of reactive oxygen species (ROS), resulting in oxidative damage, inhibiting normal plant growth, and eventually leading to plant death [4].

Plant salt tolerance is controlled by a variety of complex genetic factors, and generally responds to stress through morphological structure, physiological and biochemical reactions, and molecular metabolic pathways to ensure plant growth and development, which mainly include osmotic regulation, ion homeostasis regulation, active oxygen clearance system, and material metabolism. Starch is an important energy and nutrient reserve substance in plants, which is crucial for plant growth and development. The role of starch metabolism during plant salt stress is mainly reflected in energy supply, osmotic regulation and antioxidant defense [5, 6]. Studies have shown that multiple metabolic pathways are altered in plants under salt stress, especially carbohydrate metabolism such as tricarboxylic acid cycle, starch and sucrose metabolism, glycolysis [7, 8]; After metabolic decomposition, some sugars are converted into sucrose and glucose and transported to the cytoplasm to provide energy, and some are hydrolyzed into hexose or sugar-derived osmotic pressure regulating substances to help plants maintain osmotic balance and alleviate the damage caused by stress [9]. In the study of *Karelinia caspia*, it was found that plants under salt stress can enhance their salt resistance by accumulating secondary metabolic substances such as amino acids, sugars and organic acids, regulating osmotic balance and reactive oxygen homeostasis [10]. Starch metabolism during plant salt stress can also help plants adapt to the stress environment and maintain normal growth by regulating enzyme activity, gene expression, signal transduction and other pathways. At present, starch metabolism has been reported as an important factor in plant adaptation to salt stress, but the specific mechanism of starch synthesis and degradation under salt stress, the differences of starch metabolism patterns in different plant species, and the correlation between transcription and protein levels of starch metabolism related genes and proteins remain to be further studied.

Glutathione is a ubiquitous small molecule antioxidant in plants, which plays an important role in protein and nucleic acid biosynthesis, enzyme activity regulation, plant antioxidant and redox signal transduction [11]. The accumulation of reactive oxygen species (ROS) in plants under salt stress causes damage to plants. Glutathione reductase (GR) is a key enzyme in the ascorbate-glutathione cycle, which can reduce oxidized glutathione (GSSG) to reduced glutathione (GSH), thus providing reducing power for the removal of reactive oxygen species (ROS) and protecting plants from damage [12–14]. It was found that glutathione transferase (GSTs) can bind Na^+/K^+ ions to glutathione in plants under salt stress to reduce ion toxicity and enhance plant tolerance [15]; GSTs can also reduce the ROS concentration in plants by catalyzing the binding of ROS and glutathione, and reduce the damage caused by oxidative stress reaction under salt stress [16]. Exogenous application of β-glutathione can improve osmotic pressure tolerance and salt tolerance of plants under salt stress, and exogenous glutathione can also alleviate stress by regulating the activity of antioxidant enzymes to improve ROS clearance [16, 17]. These studies indicate that the glutathione metabolic pathway has an important role in the process of plant salt stress. However, since glutathione metabolism involves multiple enzymes and a complex regulatory network, a comprehensive discussion from multiple perspectives, such as molecular mechanisms, metabolic pathways, and signaling, is needed in order to comprehensively understand its regulatory mechanism in plant salt stress resistance.

The integrated analysis of transcriptomics, proteomics, metabolomics [18], and other omics, along with in-depth research on chloroplast genomics and pan-genomics, has led to significant breakthroughs in the deciphering of plant genomes, with omics technologies making remarkable progress [19–22]. At present, multi-omics analysis has been regarded as an effective means to analyze plant response to salt stress. Through multi-level data analysis, it is possible to gain a comprehensive understanding of the mechanisms by which plants resist salt stress, including gene expression regulation, changes in protein function, and alterations in metabolites. In maize, rice and

other food crops, great breakthroughs have been made in the study of the response mechanism to abiotic stress [23, 24]. The research on stress-resistant gene editing breeding in poplar has also made a breakthrough [25]. At present, the research on the salt stress condition of chrysanthemum mainly focuses on the physiological and genetic level, involving ion balance regulation, gene expression regulation, antioxidant enzyme activity, osmoregulatory substance change and so on [26–29]. According to transcriptome sequencing analysis, the response of chrysanthemum to salt stress was mainly through hormone metabolism, starch and energy metabolism, reactive oxygen species (ROS) clearance, phenylpropane biosynthesis and other processes [30–32]. The mechanism of starch metabolism and glutathione metabolism under salt stress of chrysanthemum is mainly focused on the transcriptional level. Few reports have revealed the effects of starch metabolism and glutathione metabolism on salt stress resistance of chrysanthemum from protein level. As the direct embodiment of plant physiological functions and life activities, proteins of different natures and types exhibit variations in their functions. Through proteomics technology, proteins associated with salt tolerance in chrysanthemum can be identified, which aids in understanding the metabolic and physiological changes in chrysanthemum under salt stress, and provides new strategies for enhancing the salt tolerance of chrysanthemum. In this study, *chrysanthemum* was used as the experimental material to screen differentially expressed proteins in plants and analyze the ways in which protein levels participate in starch and energy metabolism under salt treatment. Through differential protein analysis, it was verified that the glutathione metabolic pathway in the plant was mainly through regulating the activities of oxidation and reductase to improve the antioxidant ability, thereby reducing the damage of ROS to plants. The adaptive strategies of *chrysanthemum* salt stress were comprehensively analyzed by combining transcriptomic and proteomic data, which made the analysis results more specific and comprehensive. It is of great theoretical and practical significance to improve the salinity resistance of chrysanthemum, to provide technical support for the cultivation of new salt-tolerant varieties, and to provide scientific guidance for saline land restoration and agricultural production.

Materials and methods

Plant material and treatments

In this study, *Chrysanthemum*×*grandiflora* was used as plant material. The tissue culture seedlings with the root length of 2–3 cm were refined, and the seedlings were transplanted into nutrient soil after 2–3 days. The plants were treated with salt when they reached 9–10 leaves. The treatment group was irrigated with 60 mL 200 mM NaCl at 8 am, while the control group was not treated. 12 h later, leaves (CK-L) and roots (CK-R) of the control group were taken as samples (three biological replicates for each sample) along with leaves (S200-L) and roots (S200-L) of the treatment group, and a total of twelve samples were collected, frozen in liquid nitrogen, and stored at -80 until protein extraction. Materials used for transcriptome sequencing are treated in the same way.

Protein extraction and quantitative analysis

Tissue proteins were extracted by trichloroacetic acid (TCA)/acetone method [33]. Plant materials were ground into powder with liquid nitrogen, and mixed with TCA/acetone in a vortex. The powder was deposited at -20 ℃ for more than 4 h, centrifuged at 6000 g for 40 min, and the supernatant was discarded. Add proper amount of pre-cooled acetone to wash, repeat 3 times. After drying and precipitation, SDT lysate was added [34], precipitated by vortex suspension and bathed in boiling water for 5 min. After sonication, the sample was further incubated in a boiling water bath for 15 min, centrifuged at 14,000 g for 15 min, and the supernatant was filtered. Protein quantification was performed using the BCA method and stored at -20 °C. Twenty microliters of the extracted proteins were mixed with buffer and incubated in a water bath for 5 min before SDS-PAGE electrophoresis to assess protein quality. Proteolytic digestion was carried out according to the FASP method [35]. Protein labeling and mass spectrometry identification were performed following the instructions of the Thermo Scientific TMT labeling kit.

Protein data was analyzed using Proteome Discoverer 2.1 software (Thermo Fisher Scientific, 2014) for database searching, with a False Discovery Rate (FDR) of less than 0.01 as the criterion for filtering to obtain viable data. Proteins that meet the criteria of an expression fold change greater than 1.2 (up or down regulation) and a P-value (t-test) less than 0.05 are considered differentially expressed proteins. Bioinformatics analysis of the differentially expressed proteins was conducted using Gene Ontology (GO) functional annotation, KEGG pathway annotation, and protein clustering analysis.

Results and analysis

Protein data identification

Sequencing results generated a total of 750,302 spectra, with 87,349 matches to known spectra. A total of 24,899 peptides were identified in these known spectra, containing 21,825 specific peptides. Finally, a total of 6778 proteins were identified (Table 1). The results show that most peptides have a mass error range of -5ppm to 10ppm. This indicates the high quality accuracy of LC-MS data (Fig. 1A). Meanwhile, more than 70% of the identified proteins corresponded to a peptide number ≥ 2,

Table 1 Summary of identified proteins

Name	Information
Total Spectra	750,302
Matched Peptides Spetra	87,349
Peptides	24,899
Unique Peptides	21,825
Protein Group	6778

indicating that the results had high confidence (Fig. 1B), quality control assays indicate that the protein quality is good (Fig. S1-S13).

Table 2 Differentially expressed protein summary (filtered with threshold value of expression fold change and P value < 0.05)

Compare Group	Regulated Type	Fold Change > 1.2	*P* value < 0.05
S200_PL VS SCK_PL	Up-regulated	32	66
	Down-regulated	34	
S200_PR VS SCK_PR	Up-regulated	286	452
	Down-regulated	166	
SCK_PL VS SCK_PR	Up-regulated	967	2772
	Down-regulated	1805	
S200_PL VS S200_PR	Up-regulated	962	2774
	Down-regulated	1812	

Identification and analysis of differential proteins

Proteins with a fold change greater than 1.20 and a P-Value less than 0.05 were considered as significantly differentially abundant proteins (DAPs) for statistical analysis. The numbers of up-regulated and down-regulated proteins between the groups S200_PL VS SCK_PL、S200_PR VS SCK_PR、SCK_PL VS SCK_PR、S200_PL VS S200_PR were 32 and 34、286 and 166、967 and 1805、962 and 1812, respectively (Table 2). After salt treatment, the number of significantly differentially up and down-regulated proteins was basically the same in the same tissues, but the number of significantly differentially up and down-regulated proteins varied among tissues, with more significantly differentially differentiated protein DAPs in the roots, which were about six times as many as those in the leaves (Fig. 2A, B).

After salt treatment, there were only two DAPs with significant differences among the four comparison groups. S200_PL VS SCK_PL and S200_PR VS SCK_PR have 1 up-regulated DAP and 3 down-regulated DAPs (Fig. 3A-C), indicating that there may be differences in the proteins responding to salt stress in root and leaf proteins.

GO annotation analysis of daps

Enrichment analysis and screening were performed using P-Value < 0.05 as the standard. 512 functional items were identified by GO annotation in DAPs of S200_PL VS SCK_PL group, and the top 30 GO enrichment functions were selected for analysis, including 14 biological processes (BPs), 13 molecular functions (MFs), and 3 cellular components (CCs) (Fig. 4A). The BP class of GO annotations included the process of starch catabolism, response to salicylic acid and jasmonic acid, protein import into peroxisome membrane, and maintenance of protein location in cell; MF includes carbohydrate kinase activity, cadmium ion transmembrane transporter activity, glycosyltransferase activity, and strictosidine synthase activity; CC includes the chloroplast envelope, integral component of the membrane and the cytoplasmic side of the plasma membrane; The analysis results show that the starch catabolism process is significantly enriched, and the enrichment of proteins with carbohydrate kinase function is also notable. Additionally, the GO enrichment of salicylic acid and jasmonic acid biological processes is significant, with a relatively large number of related proteins, indicating that energy metabolism pathways and hormone metabolism pathways may play a major role in the salt stress resistance of *chrysanthemum* leaf tissues. In the S200_PR VS SCK_PR group DAPs, GO annotation identified 2364 functional items. The top 30 GO enrichment functions were selected for analysis, which included 16 biological processes (BPs), 8 molecular functions (MFs), and 6 cellular components (CCs) (Fig. 4B). The BP class of GO annotations primarily included

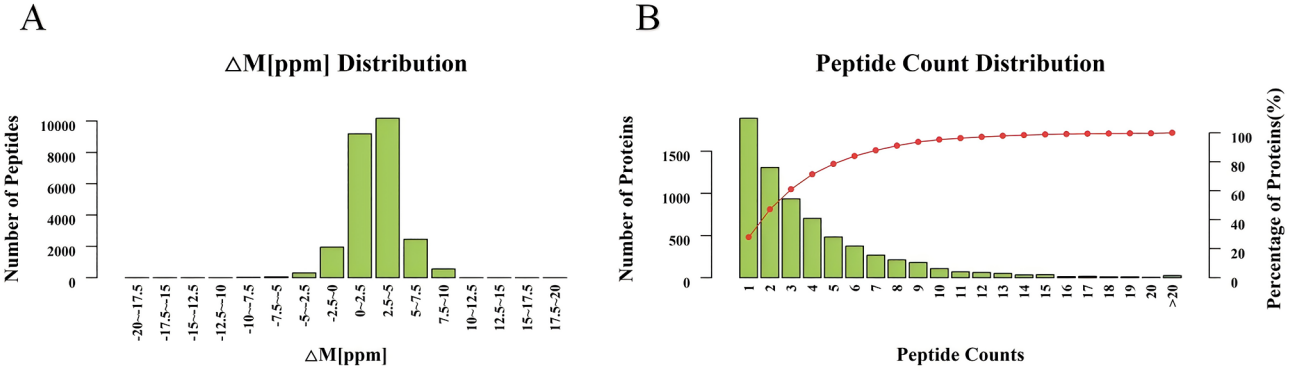

Fig. 1 The critical quality control of MS data. **A**. The mass error of distribution of IPs. **B**. The peptides count distribution of IPs

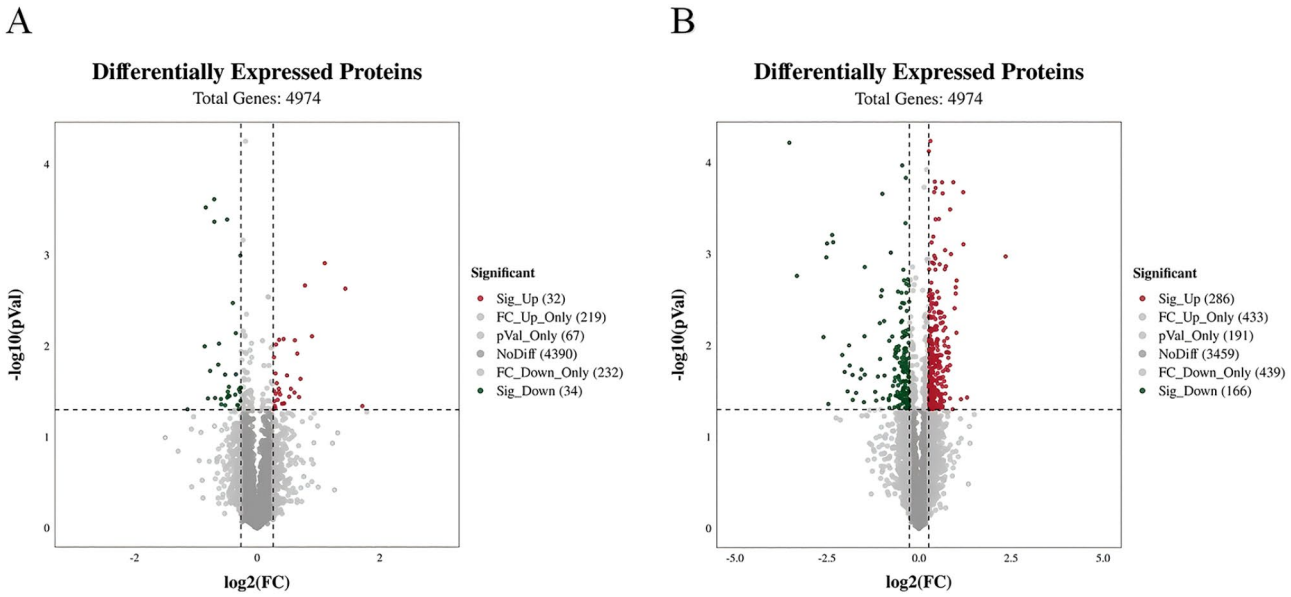

Fig. 2 Analysis of differentially abundant proteins in all compared groups. **A**. The volcano plots of DAPs in S200_PL VS SCK_PL. **B**. The volcano plots of DAPs in S200_PR VS SCK_PR

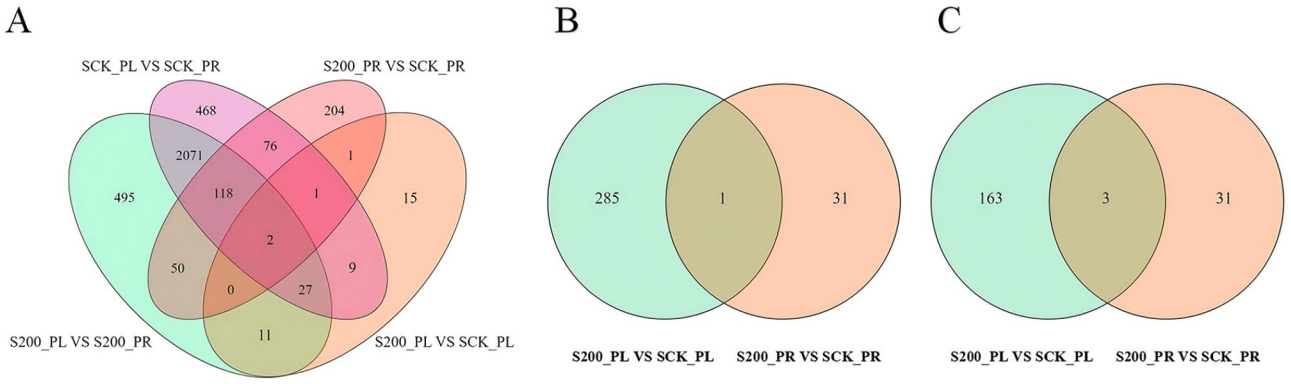

Fig. 3 Venn diagram of differentially abundant proteins under salt stress treatment between different control groups. **A**. Venn plot of DAPs of S200_PL VS SCK_PL、S200_PR VS SCK_PR、SCK_PL VS SCK_PR and S200_PL VS S200_PR. **B**. Number of up-regulated DAPs in Root VS Leaf in response to salt stres. **C**. Number of down-regulated Root VS Leaf in response to salt stress

cellular response to phosphate starvation, response to oxidative stress, isocitrate metabolic process, galactose metabolic process, citrate metabolic process, glutathione metabolic process, stomatal opening, and toxin catabolic process; MF includes aconitate hydratase activity, protein homodimerization activity; CC includes plant-type cell wall. Among them, there are more biological processes related to starch and energy metabolism, while antioxidant reaction is closely related to glutathione metabolism, which plays an important role in plant antioxidant defense.

KEGG annotation analysis of daps

To understand the major metabolic pathways involved in DAPs, the KEGG database was used to further analyze to understand the metabolic pathways enriched for these differential proteins. Using P-Value < 0.05 as the criterion for screening, S200_PL VS SCK_PL screened 10 significantly enriched KEGG pathways, among which the accumulation of Glycerolipid metabolism pathway (ko00561) was more obvious, and the metabolic pathway (ko01100) annotated the highest number of differentially expressed proteins, with a total of 12 proteins (Fig. 5A). S200_PR VS SCK_PR screened 14 significantly enriched KEGG pathways, among which the metabolic pathway (ko01100), glutathione metabolism (ko00480), carbon fixation pathway in prokaryotes (ko00720), 2-Oxocarboxylic acid metabolism (ko01210)and the citrate cycle (ko00020) (Fig. 5B) were involved in the response to salt stress in *chrysanthemum*.

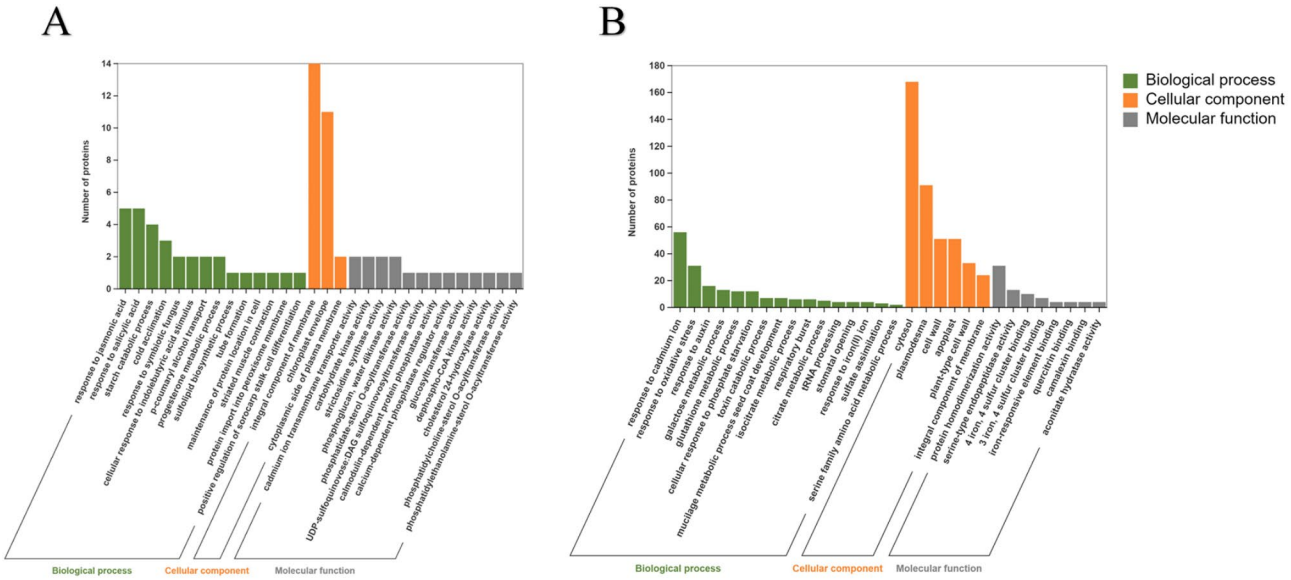

Fig. 4 Gene Ontology (GO) analysis of DAPs from different groups. The significantly enriched top 20 GO terms in **A**. S200_PL VS SCK_PL. **B**. S200_PR VS SCK_PR

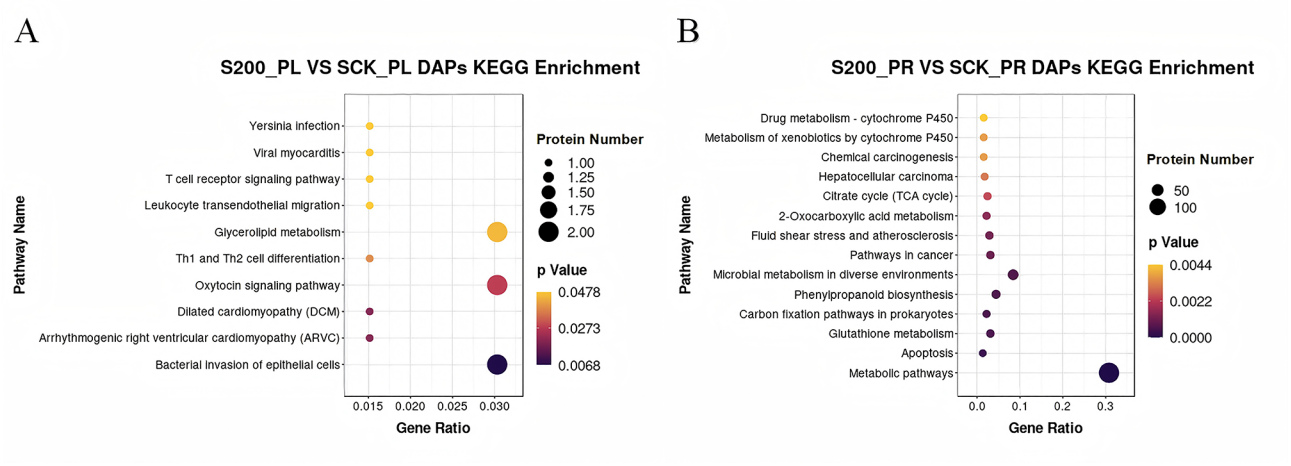

Fig. 5 Enriched KEGG pathways in DAPs from **A**. S200_PL VS SCK_PL; **B**. S200_PR VS SCK_PR

Pathways involved in the response of *Chrysanthemum* to salt stress

GO and KEGG analyses revealed significant changes related to carbohydrate metabolism and energy metabolism pathways in *chrysanthemum* leaf and root tissues following salt treatment. Three pathways, five DAPs, were enriched in leaf tissues for glycerolipid metabolism pathway (ko00561), amino sugar and nucleotide sugar metabolism (ko00520), and starch and sucrose metabolism (ko00500) (Fig. 6A); Gene.14,831 and Gene.12,722 have glucolipid transferase activity and phosphoacyltransferase activity, respectively, and participate in the process of glycerol ester metabolism; Gene.29,922 and Gene.12,160 exhibit L-arabinokinase (L-arabinose kinase) and chitinase activities, and their differential upregulated expression participates in the amino sugar and nucleotide sugar metabolism process; Gene.19,586 has starch synthase activity and is involved in the starch and sucrose metabolism process, promoting the synthesis of starch and sugars. The root tissues were enriched with 18 proteins from 3 pathways of Carbon fixation pathways in prokaryotes (ko00720), 2-Oxocarboxylic acid metabolism (ko01210) and Citrate cycle (ko00020) (Table S1), among which 6 DAPs were involved: IDHP, ILV5, CAPPB, ICDHP and PCKA were all up-regulated (Fig. 6B). Gene.17,273 and Gene.28,652 exhibit isocitrate dehydrogenase activity and are involved in all three pathways. Gene.23,155 and Gene.12,956 both have phosphoenolpyruvate carboxylase activity, Gene.14,577 has

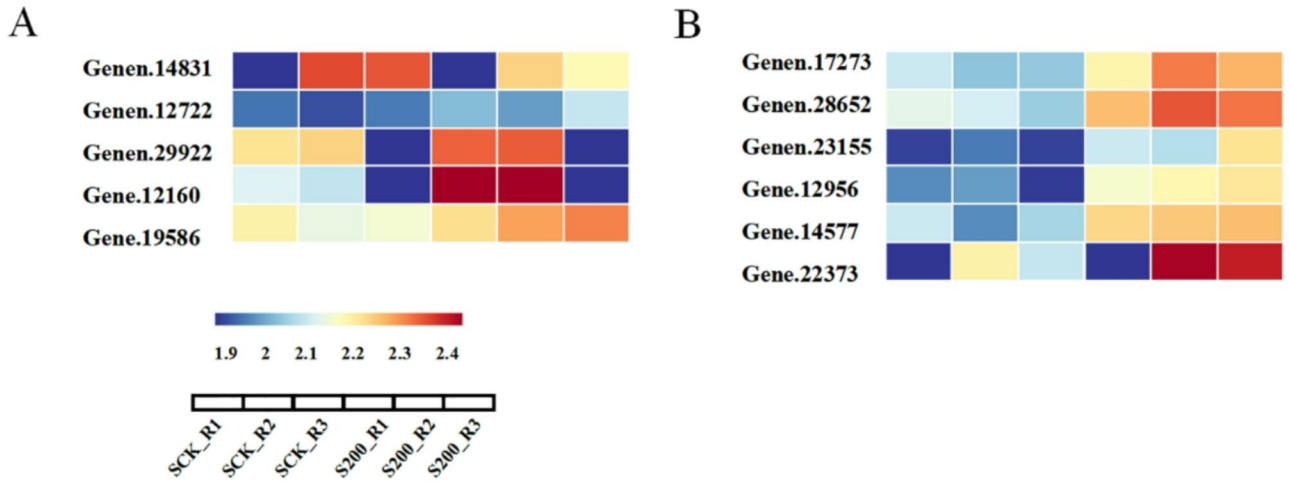

Fig. 6 Heatmap of clustering of DAPs associated with energy metabolism pathways in different tissues. **A**. DAPs heat map of leaf energy metabolism-related pathways. **B**. DAPs heat map of root energy metabolism-related pathways. The horizontal axis represents different sample names, with different colors indicating varying expression levels as detailed in the legend

ketol-acid reductoisomerase activity, and Gene.22,373 has phosphoenolpyruvate carboxylase activity.

The biological processes of salicylic acid and jasmonic acid related to plant hormone signal transduction pathway in leaf tissues under salt stress were different, which was consistent with the transcriptome data analysis. The analysis revealed a total of six DAPs involved in the salicylic acid and jasmonic acid biological processes: Gene.30,880 and Gene.26,095 are SSL6 proteins, Gene.17,073 is an Arath nrt1 PTR family protein with the function of transporting nitrate, amino acids, auxins, and abscisic acid, Gene.24,918 is an AB40G protein, Gene.11,037 is an FL3H2 protein, and Gene.22,316 is an RCA protein. Correlation analysis of these six DAPs with the remaining 60 DAPs in the leaf tissue (Fig. 7) showed that the three hormone pathway-related DAPs (Gene.30880, Gene.26095 and Gene.22316) in the core region all had interaction relationships with the other DAPs.

In proteomic analysis, two KEGG pathways glutathione metabolism pathway (ko00480) and biosynthesis of secondary metabolites pathway (ko01110) were found to be significantly enriched in root tissues during salt stress, as well as GO bioprocesses such as response to oxidative stress, glutathione metabolism process and other processes that are related to antioxidant processes. By analysis, it was found that glutathione metabolism was significantly enriched in both GO and KEGG, and the pathway showed up-regulated expression of several DAPs in the conversion of GSSG to GSH, including: isocitrate dehydrogenase (IDH), glutathione S-transferase (GST), and glucose-6-phosphate 1-dehydrogenase, whereas down-regulated expression of glutathione peroxidase (GPX), a DAP for the conversion from GSH to GSSG that resulted in enhanced GSH/GSSG signaling (Fig. 8). This increased the GSH level in the root tissue, effectively scavenging reactive oxygen species (ROS) and enhancing salt tolerance. In addition, 31 DAPs in the bioprocesses of the antioxidant response and 74 DAPs in the biosynthetic pathways of secondary metabolites existed in 19 identical proteins (Table S2), most of which were oxidoreductases (PER1, PER3, PER12, PER21, PER47, PER52, PER60 peroxidases, IDHP dehydrogenases, etc.) (Table 3). Plant oxidoreductases are an important component of the plant antioxidant system and can also scavenge plant reactive oxygen species (ROS) to reduce oxidative damage and enhance salt tolerance.

Correlation analysis of the proteome and transcriptome

To investigate whether there was a correlation between changes in *Chrysanthemum* proteins and mRNA after salt treatment, proteome DAPs were compared with published transcriptome DEGs data [32]. DAPs and DEGs with a fold change greater than 1.20 ($P<0.05$) were considered differentially up-regulated, while those with a fold change less than 0.83 ($P<0.05$) were considered differentially down-regulated. A total of 66 and 452 DAPs; 7880 and 3094 DEGs were screened in the S200_PL VS SCK_PL and S200_PR VS SCK_PR comparison groups. By joint analysis, the results showed that 21 and 43 DEGs/DAPs pairs in S200_PL VS SCK_PL and S200_PR VS SCK_PR, respectively (Fig. 9A, B) were differentially expressed in both proteomes and transcriptomes.

Based on the correlation analysis of trends in gene and protein expression changes in leaves and roots, the results can be categorized into four groups: (1) There were 12 and 14 pairs of DEGs/DAPs that were significantly different in both genes and proteins and had the same expression change trend in the S200_PL VS SCK_PL and S200_PR VS SCK_PR comparison groups;

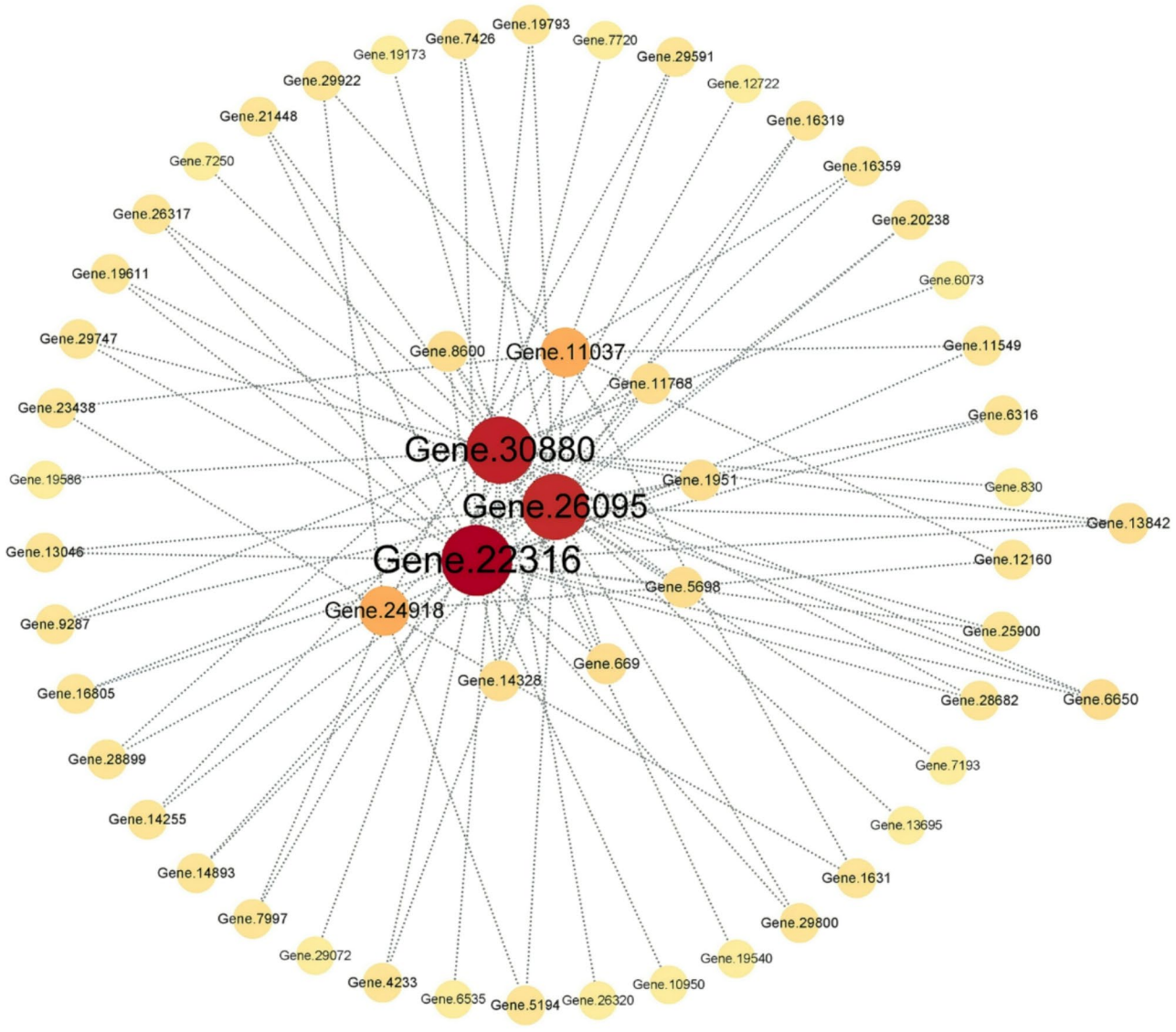

Fig. 7 Interaction network between leaf hormone-related proteins and DAPs. Each circle in the figure represents a protein, and the lines between the circles denote the interactions between different proteins

(2) There were 9 and 29 pairs of DEGs/DAPs that were significantly different in both genes and proteins with opposite trends of expression changes in the S200_PL VS SCK_PL and S200_PR VS SCK_PR comparison groups; (3) In the S200_PL VS SCK_PL and S200_PR VS SCK_PR comparison groups, there were 7859 and 3025 DEGs that showed significant differences in the transcriptomic data, respectively, while no significant differences were observed at the protein level; (4) In the S200_PL VS SCK_PL and S200_PR VS SCK_PR comparison groups, there were 45 and 409 DAPs that showed significant differences in the proteomic data, respectively, while no significant differences were observed at the transcriptomic level; The association of the two omics DEGs/DAPs is conducive to obtaining complete and complementary results. We focused on DEGs/DAPs with identical or opposite expression patterns at the protein and transcription levels and their biological processes involved in salt stress.

GO analysis of DEGs/DAPs for proteome-transcriptome association

By correlating the transcriptome and proteome, the results showed that 21 pairs of DEGs/DAPs in S200_PL VS SCK_PL were associated, with the same or opposite expression pattern and significantly expressed. GO analysis showed that the biological processes (BPs) of DEGs/DAPs in leaves mainly included phosphorylation, starch synthesis and catabolic processes, ion homeostatic transport, carbohydrate metabolism processes, lignin

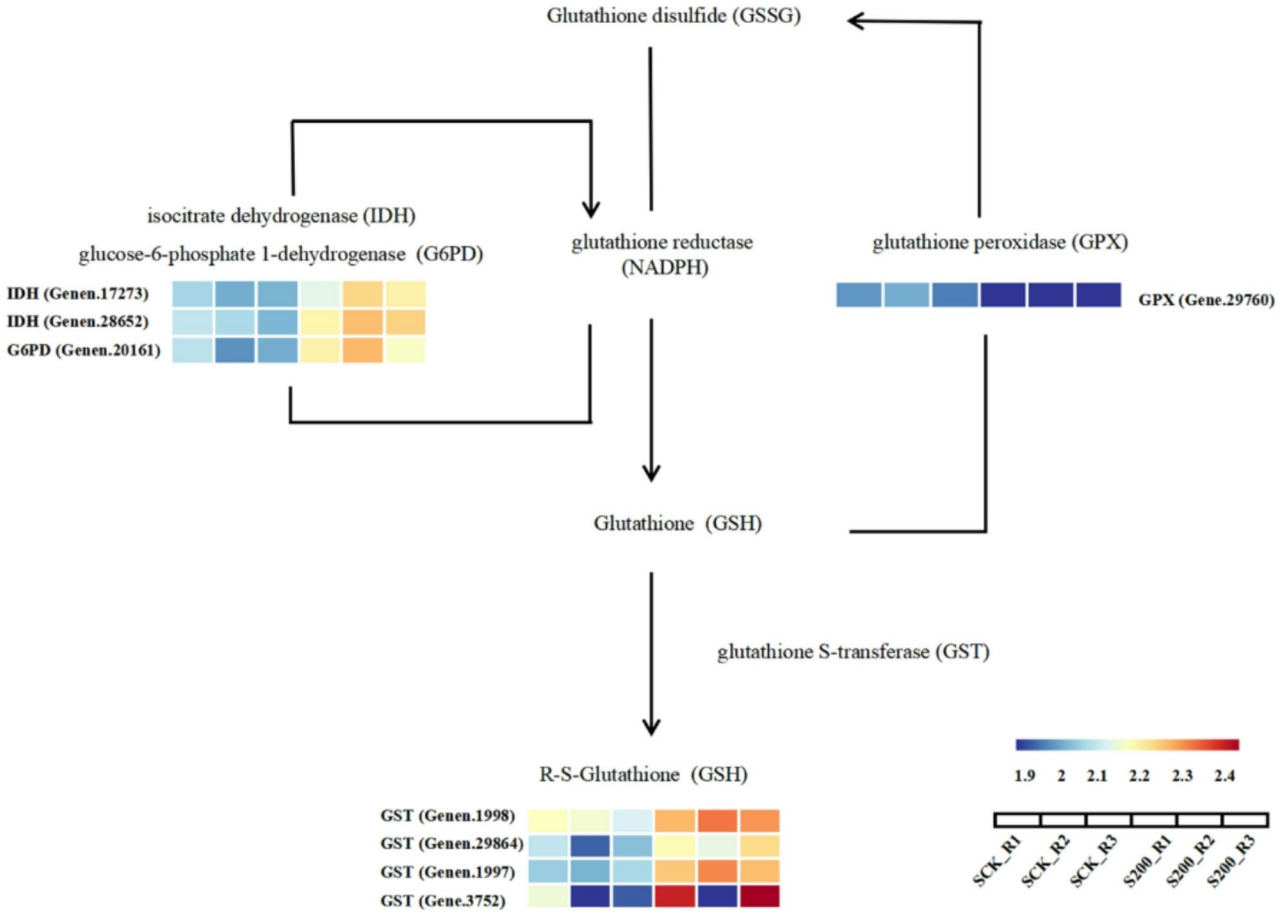

Fig. 8 Root DAPs participate in glutathione REDOX (GSH/GSSG) pathway heat map

Table 3 List of daps associated with antioxidant pathways found in roots related to salt stress response

Accession	Protein name	Protein abundance		Fold change	*P*-value	MW [kDa]	Calc. pI
		Control	Treat				
Gene.26,714	PER1_ARAHYCationic peroxidase	290.45	188.45	1.54	0.01	15.2	5.73
Gene.29,558	PER3_ARATHPeroxidase 3	163.43	110.03	1.49	0.0026	35.5	8.76
Gene.5383	PER47_ARATHPeroxidase 47	142.73	108.27	1.32	0.0078	33.2	6.54
Gene.25,541	PER52_ARATHPeroxidase 52	147.3	113.33	1.30	0.02	33.6	9.09
Gene.9331	PER21_ARATHPeroxidase 21	143.2	197.7	0.72	0.04	12.5	8.94
Gene.17,273	IDHP_MEDSAIsocitrate dehydrogenase	105	147.97	0.71	0.01	18.8	8.48
Gene.25,807	PER60_ARATHPeroxidase 60	173.85	246	0.71	0.01	36.4	9.39
Gene.10,741	PER12_ARATHPeroxidase 12	111.8	181.07	0.62	0.01	25.5	6.19

biosynthesis processes and oxidative stress responses; The cellular components (CCs) mainly consist of chloroplast, nucleus, cytoplasm, plastid and other components; The molecular functions (MFs) mainly includ ATP binding, kinase activity, catalytic activity, ion transport and binding and starch binding. In S200_PR VS SCK_PR, 43 pairs of DEGs/DAPs were associated. GO analysis showed that the biological processes (BPs) of DEGs/DAPs in roots mainly included redox process, cell wall organization, sulfate reduction, abscisic acid signaling pathway and glycolytic metabolism process. Cell components (CCs) mainly include cytoplasm, nucleus, cell wal, chloroplast, cell membrane. Molecular functions (MFs) mainly include ion transport and binding, ATP binding, kinase activity and catalytic activity, redox enzyme activity.

KEGG analysis of DEGs/DAPs for Proteome-Transcriptome association

By correlating the transcriptome and proteome, the results showed that 21 pairs of DEGs/DAPs in S200_PL VS SCK_PL were associated, with the same or opposite

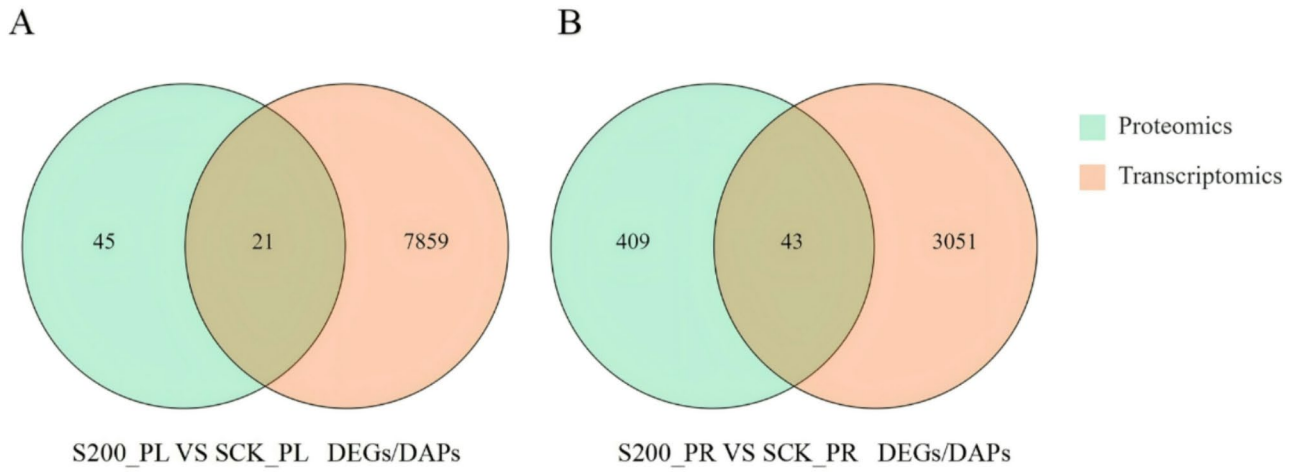

Fig. 9 Correlation venn diagram of DEGs and DAPs. **A**. DEGs/DAPs Venn diagram of proteome and transcriptome leaf. **B**. DEGs/DAPs Venn diagram of proteome and transcriptome root

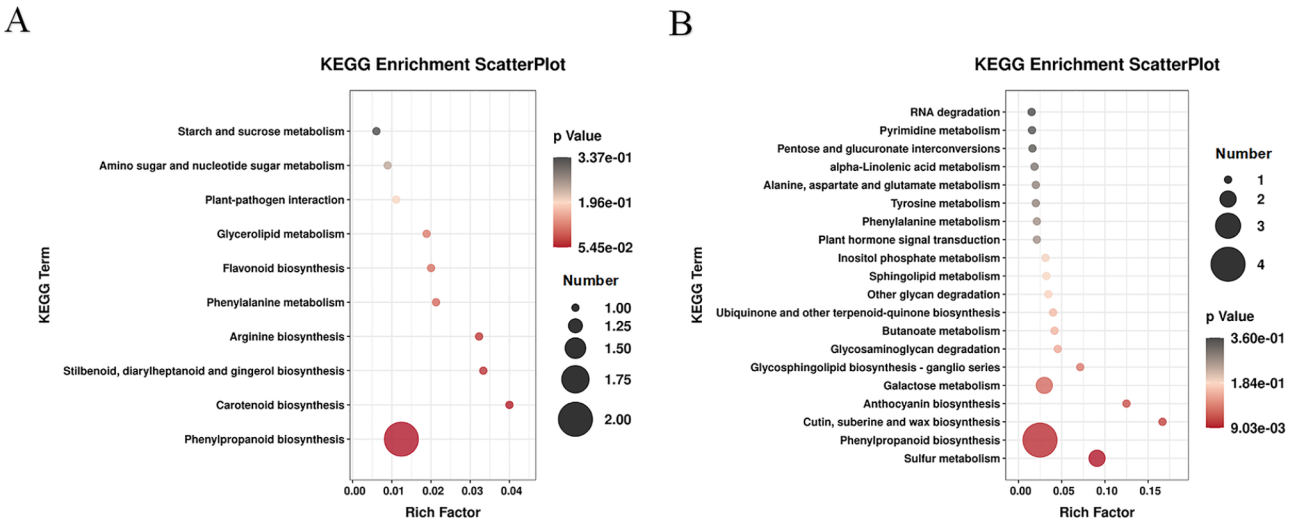

Fig. 10 KEGG enrichment analysis of DEGs/DAPs. **A**. DEGs/DAPs KEGG map of proteome and transcriptome leaves; **B**. DEGs/DAPs KEGG map of proteome and transcriptome roots

expression pattern and significantly expressed. KEGG analysis showed that 8 of the 21 associated DEGs/DAPs pairs in leaves were involved in the relevant KEGG signaling pathway. KEGG signaling pathway mainly includes phenylpropanoid biosynthesis pathway, carotenoid biosynthesis, flavonoid biosynthesis, starch and sucrose metabolism. In S200_PR VS SCK_PR, 43 pairs of DEGs/DAPs were associated. KEGG analysis showed that 39 of the 43 associated DEGs/DAPs pairs in the roots were involved in relevant KEGG signaling pathways, mainly includes sulfur metabolism pathway, phenylpropanoid biosynthesis pathway, galactose metabolism, ubiquinone and other terpenoid-quinone biosynthesis pathway, and tyrosine metabolism (Fig. 10A, B).

By comparing the results of transcriptomic and proteomic analyses, the combined omics analysis revealed that starch and energy metabolism are consistently involved in the plant's salt stress response process and are significantly enriched pathways. The phenylpropanoid biosynthesis pathway is a dominant metabolic pathway in the transcriptome, and it was also identified through correlation analysis. However, in proteomic analysis, the phenylpropanoid biosynthesis pathway is significantly enriched only in the roots, not in the leaf tissue. The plant hormone signaling pathways of salicylic acid and jasmonic acid showed significant changes in the transcriptome, but correlation analysis revealed that the plant hormone pathways were not significantly enriched. In proteomics, only a partial presence of differentially enriched proteins related to salicylic acid and jasmonic acid metabolism was observed in the leaf tissue. These changes may be related to the fact that the regulation

at the gene and protein levels in plants is not entirely consistent.

Discussion

Soil salinization is the main abiotic limiting factor for plant growth and development. It has been reported that most perennial flowers can adapt to salt stress environment through a variety of mechanisms, which include the synthesis of osmotic regulatory substances, the maintenance of ion balance, the regulation of antioxidant enzyme system activity, and the regulation of photosynthesis [28, 36, 37]. In this study, after applying a 200mM NaCl solution for 12 h, the Na^+ content in plant tissues began to rise, while the K^+ content showed a downward trend. After a period of time, the content of H_2O_2 in plant tissues significantly increased, indicating that osmotic and ionic stress gradually occurred, and corresponding oxidative defense measures were initiated [32].

Under salt stress, plants respond by altering the expression of proteins related to carbohydrate and energy metabolism, which is an important process for plants to resist stress [38, 39]. In this study, through GO/KEGG enrichment analysis, it was found that multiple pathways related to carbohydrate and energy metabolism were significantly enriched in *Chrysanthemum*, and the leaf tissues mainly consisted of carbohydrate metabolism and carbohydrate metabolism, involving multiple DAPs such as: SQD2, LPAT2, ARAK, CHIE, SSG1. The root tissues are mainly carbohydrate and energy metabolism, which involves multiple DAPs, such as: IDHP, ICDHP, PCKA are isocitrate dehydrogenase, pyruvate carboxylase and ketoacid reductoisomerase proteins in the tricarboxylic acid cycle metabolism pathway, respectively, among which isocitrate dehydrogenase (IDH) exists and is up-regulated in multiple metabolic pathways. It has been reported that isocitrate dehydrogenase, which is involved in energy metabolism, changes differently during plant stress resistance. Among them, IDH (isocitrate dehydrogenase) expression is up-regulated in *Arabidopsis* root systems under salt treatment to increase root salt resistance [40], Overexpression of IDH from maize in *Arabidopsis thaliana* also enhanced its salt resistance [41]. However, the differential changes of IDH under salt stress in different rice [42] and tomato [43] varieties indicate that while IDH can be involved in salt stress in different plant species, the regulatory patterns may vary. In this study, salt stress induced differential expression of multiple proteins related to carbohydrate and energy metabolism pathways, among them, isocitrate dehydrogenase (IDH) was up-regulated in the multiple metabolic pathways involved, promoting the accumulation of more carbohydrates, lipids, energy and other substances. The accumulation of these substances may play a crucial role in maintaining plant growth and development and osmotic regulation during the salt stress process in *Chrysanthemum*, thereby alleviating the damage caused by salt stress.

Glutathione (GSH) is a substance widely present in plant cells, which has antioxidant effects, maintains cellular redox balance, and regulates cellular osmotic pressure during plant salt stress. Glutathione (GSH), as an antioxidant, has been confirmed to participate in the regulation of plant growth under salt stress in various plant species, and GSH/GSSG, composed of reduced glutathione (GSH) and oxidized glutathione (GSSH), is one of the most abundant redox pairs in the plant cell, which can participate in intracellular redox balance and is essential for maintaining normal physiological functions under saline and alkaline stress, and can be participate in plant signaling and resistance to adversity stress as signals [44, 45]. In this study, it was found that glutathione metabolic pathway was significantly enriched in the root tissues after salt stress treatment, and the differentially expressed proteins were mainly enzymes in the mutual conversion process of oxidized glutathione (GSSG) and reduced glutathione (GSH), including: IDH dehydrogenase, GST glutathione-S transferase, glucose-6-phosphate, dehydrogenase promoted the production of reduced glutathione significantly up-regulated, whereas GPX glutathione peroxidase, which promotes oxidized glutathione production, was significantly down-regulated, thus promoting the GSH/GSSG ratio. Part of the antioxidant pathway, PER peroxidase, IDHP dehydrogenaseand ACO3M hydratase, were also up-regulated, and peroxidase was able to reduce oxidative damage by catabolizing reactive oxygen species during salt stress, as well as enhance plant salt tolerance through gene expression regulation, and changes in its activity were closely related to plant salt tolerance [46–48]. No similar phenomenon was found in leaf tissues, suggesting that the response of glutathione metabolic pathway and antioxidant pathway to salt stress only plays a leading role in the root. It has been reported that the increase of GSH level, GPX glutathione peroxidase, GST glutathione-S transferase and Gly I activity in tobacco is related to the salt stress tolerance of tobacco; Wild-type *Brassica napus L.* (canola) seedlings showed greater salt tolerance with double glutathione and cysteine synthesis under salt stress compared with mutants with GSH or Cys biosynthesis gene deficiency [49]; Similarly, the glutathione metabolism pathway has also been found to play a role in the salt stress response in plants such as rice, soybean and *Arabidopsis thaliana* [50–52]. Salt stress in rice significantly affects the expression of multiple genes related to glutathione metabolism, leading to a marked activation of the metabolic pathway; The expression level of *Le*GST14, a glutathione peroxidase in *Arabidopsis thaliana*, was increased under salt stress, and ROS clearance

was enhanced to improve plant salt tolerance; Exogenous application of GABA in soybean improved the metabolic pathway of glutathione, enhanced the antioxidant capacity and salt tolerance of soybean; These results suggest that glutathione metabolic pathways play an important role in the response of plants to salt stress. Some studies have reported that under salt stress, the glutathione metabolism pathway is significantly activated in the salt-tolerant rice variety HD961, whereas the glutathione content significantly decreases in the 9311 variety. Tomatoes show a weak response to exogenous GSH, whereas the GSH content in barley leaves is positively correlated with K+/Na+, and the application of exogenous GSH can mitigate the chlorophyll damage caused by salt stress and alleviate the stress-induced harm [53]; This demonstrates that there is variability in the response of different plant species to glutathione under salt stress. In the research progress of salt stress in *chrysanthemum*, the main metabolic pathways of resistance to salt and alkali stress in *chrysanthemum* leaves were found through transcriptomics sequencing analysis, including plant hormone metabolism, starch and energy metabolism, reactive oxygen species (ROS) clearance, phenylpropane biosynthesis and other processes [30–32]. Transcriptome sequencing in root showed that reactive oxygen species (ROS) and energy metabolic pathways were used to resist salt stress [32], while glutathione metabolic pathways were rarely reported. In this study, salt stress induced the differentially up-regulated expression of reductive glutathione (GSH) synthesis-related proteins and some peroxidase, while differentially down-regulated expression of oxidation-related glutathione (GSSG) synthesis-related proteins. The changes of GSH/GSSG and the enhancement of peroxidase activity could improve the antioxidant capacity of plants. During salt stress, *Chrysanthemum* may enhance its antioxidant capacity to scavenge excess reactive oxygen species within the body, thereby achieving resistance to the damage caused by stress.

Proteomics, as a technology to study the changes of proteins in the body, has an obvious advantage in obtaining quantitative information of temporal and spatial expression of plant proteins. In this study, DAPs after 12 h of salt treatment were analyzed, and it was found that all tissues produced DAPs after 12 h of salt stress, and the DAPs of root and leaf tissues were more different and differentiated, with more salt-responsive proteins in the root system of the plant as the first organ to receive salt signals, which suggests that there is a certain degree of spatiotemporal and temporal variability in the response to salt stress in the plant as a whole [54–56]. Correlation analysis of the transcriptome and proteomics showed that some genes were differentially altered at both the protein and transcript levels [32], but most of the differentially expressed genes were not significantly altered at the corresponding protein levels, indicating a weak correlation between the two histologies. Due to the fact that changes in gene transcription levels can rapidly respond to changes in the external environment, while the process of protein translation and modification may require more time. Additionally, the complexity of transcription, post-translational regulation, protein expression, and the intricate mechanisms of salt stress regulation are among the many factors that could lead to a result.

Conclusions

In this study, proteomic sequencing analysis of 200mM NaCl treated *Chrysanthemum* leaf and root tissues was performed, and the results of GO and KEGG analyses showed that starch and energy metabolism and glutathione metabolism pathway played an leading role in the salt response process. Starch, as an important energy storage substance in plants, its synthesis and decomposition are crucial for the energy supply and metabolic balance of plants. Under salt stress, the carbohydrate substances derived from starch decomposition provide energy for the cytoplasm and maintain cellular osmotic balance, thereby alleviating salt damage and enhancing the salt tolerance of *Chrysanthemums*. Glutathione and its metabolic pathway play a key role in plant antioxidant defense and abiotic stress. Under salt stress, glutathione metabolism pathway can reduce oxidative stress and ion toxicity by clearing reactive oxygen species, protecting cell structure and synergizing with other metabolic pathways. The starch and energy metabolism pathway and the glutathione metabolism pathway work together through multiple aspects such as antioxidant defense, energy supply, and material transport, collectively forming the salt resistance defense system of *Chrysanthemum*. Analysis revealed that the regulatory mechanisms in the leaves and roots of *Chrysanthemum* in response to stress differ, which may be due to the temporal differences in salt stress responses across different tissues leading to distinct response mechanisms. The changes at the transcriptional and protein levels also vary, confirming the complexity of plant regulation. This study, through the combined analysis of different tissues and multi-omics approaches, has conducted a multi-level analysis of the regulatory network underlying *Chrysanthemum* adaptation to salt stress. This provides a new perspective and research direction for the development of plant science, aiding in a deeper understanding of the adaptation mechanisms of *Chrysanthemums* to salt stress. It also contributes to the development of new molecular breeding strategies, promotes the cultivation of new *Chrysanthemum* varieties with stress resistance, and offers theoretical foundations and technical support for agricultural production and plant breeding.

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

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Author contributions
D.Z. designed the research and wrote the manuscript. Y.Z. and Y.B. provided guidance on the experiment. D.W., N. X, S.F., Y.Q. and S.W. reviewed and revised the manuscript. All authors read and approved the fnal manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
This article does not contain any studies with human participants or animals. The collection materials of the plants, complies the relevant institutional, national and international guidelines and legislation.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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