Physiological response and transcriptome analysis of *Prunus mume* to early salt stress

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Abstract

Soil salinisation is an important factor limiting the cultivation and distribution of *Prunus mume*. Therefore, identifying the regulation mechanism of salt tolerance of *P. mume* will promote its cultivation and molecular breeding. In this study, the garden tree species *P. mume* ‘Meiren’ was investigated, with a soil NaCl content (w/w) of 0.3%. Photosynthetic gas exchange parameters, relative electrical conductivity, malondialdehyde, osmoregulation substances, and antioxidant enzyme activity were measured in the early (3d), middle (10d), and late (30d) stages of stress. Salt treatment was applied for 0 h, 6 h, 24 h, and 72 h; following this, the gene library was constructed from the leaves. The results showed that the CBL-CIPK, mitogen-activated protein kinase, calcium-dependent protein kinase, and abscisic acid signalling pathways were involved in salt stress signal transduction of *P. mume*. Furthermore, bHLH, WRPK, ERF, and MYB were identified as potential key transcription factor families of salt tolerance in *P. mume*. Combined with determined physiological responses, the results showed that early salt stress inhibited the activities of RuBisCO, chlorophyll synthase, glutamyl tRNA reductase, divinyl reductase, and magnesium chelatase; resultantly, this led to a reduction in the photosynthetic rate. Also, it was found that P5CS, SS, LEA, and dehydrin regulated the synthesis of proline, soluble sugar, and macromolecular protein to alleviate osmotic stress. POD gene plays an important role in scavenging reactive oxygen species due to its regulation of POD activity. The findings of this research reveal the internal mechanism of the physiological response and provide a foundation for the construction of a genetic regulatory network in response to salt stress in *P. mume*.

Keywords *Prunus mume* · Transcriptome · Signal transduction · Salt tolerance · QRT-PCR

Abbreviations

ABA	Absciscic acid
AKT	Potassium channel
ALDH	Aldehyde dehydrogenase
AQP	Aquaporin
bHLH	Basic Helix–Loop–Helix
bZIP	Basic leucine zipper
CAMTA	Calmodulin-binding transcription activator

CBL	Calcineurin B-like protein
CBF	Core binding factor
CDPK	Calcium-dependent protein kinase
CHX	Cation/H ⁺ exchangers
Ci	Intercellular CO ₂ concentration
CIPK	CBL-interacting protein kinase
CNGCs	Cyclic-nucleotide gated channels
DEGs	Differentially expressed genes
DHN	Dehydrin
ERF	ETS2 Repressor Factor
Gs	Stomatal conductivity
GTLs	GT element-binding-like proteins
HSP	Heat shock protein
LEA	Late embryogenesis abundant protein
MAPK	Mitogen-activated protein kinase
MDA	Malonic dialdehyde
MT	Metallothionein protein
MYB	Myeloblastosis
MYC	MYC proto-oncogene, bHLH transcription factor

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NHX	Sodium/hydrogen exchanger
NSCCs	Nonselective cation channels
P5CS	Pyrroline-5-carboxylate synthase
PLD	Phospholipase D
Pn	Net photosynthetic rate
POD	Peroxidase
PP2C	Protein phosphatase 2C
Pro	Free proline
PrxR	Peroxiredoxin
PS I	Photosystem I reaction centre
PYL	Pyrrolysine-tRNA
PYR	Pyramus
Rubisco	Ribulose-1,5-bisphosphate Carboxylase/oxygenase activase
SnRK	Serine/threonine-protein kinase
SOD	Superoxide dismutase
SS	Soluble sugar
SUSY	Sucrase synthase
TF	Transcription factor
Tr	Transpiration rate

Introduction

The salinisation of soil has become a major global resource concern and environmental issue. The salt content in soil affects the geographical distribution of plants in nature, whilst also limiting the productivity of plants (Zhu 2016; Agarwal et al. 2020). Increased concentrations of Na^+ and Cl^- affect plant metabolism and reduce the rate of photosynthesis, and consequently, slow down plant growth (Waqas et al. 2021; Zhao et al. 2020). In the process of long-term evolution and environmental adaptation, plants have formed a series of physiological and molecular mechanisms in response to salt stress, which can be divided into two categories: the first is salt tolerance, whereby effector molecules directly participate in changes to the physiological cellular metabolism, and therefore, contribute to salt tolerance, and the second is regulatory molecules, which are located upstream of effector molecules and are involved in signal transduction, primarily including various kinases and transcription factors comprising the signal transduction system (Chen et al. 2021).

Mei is a traditional, well-known flower in China, which has been cultivated for over 3000 years (Zhang et al. 2012). Soil salinity and alkali content are important factors that can limit the distribution of *P. mume* (Yang et al. 2018; Li 2009). The role of core binding factor (CBF) (NF-Y),

phospholipase D (PLD), late embryogenesis abundant protein (LEA), basic leucine zipper (bZIP), and small heat shock proteins (HSP) has been established in abiotic stress (Du et al. 2012; Yang et al. 2016; Peng et al. 2016; Wan et al. 2016). Overexpression of *PmLEA* can improve cold and drought resistance of tobacco (Bao et al. 2017). Moreover, CBF is also associated with cold resistance, salt tolerance, and antioxidant capacity of *P. mume* (Yang et al. 2016). However, the salt tolerance mechanism of *P. mume* has yet to be systematically studied, and the signal transduction pathway involved in salt tolerance remains unclear. To expose the mechanism of salt tolerance of *P. mume* and screen the key genes of salt tolerance, we aim to provide a theoretical basis for the breeding of new salt tolerant varieties and to promote the cultivation and distribution of *P. mume* in saline alkali soil.

Materials and methods

Plant material and salt treatment

The experimental aspect of this study was carried out in the nursery greenhouse of Beijing Forestry University (116.3°E, 40.0°N), between June–August. The annual branches of *P. mume* ‘Meiren’ were collected from one mother plant and grafted onto the same growing rootstock of *P. mume* ‘Meiren’. Seedlings with relatively uniform growth were planted in plastic flowerpots with a size of 35 cm × 30 cm × 35 cm in March, with trays arranged underneath the flowerpots; the planting density was 1 plant/pot. The culture medium was mixed by volume ratio of peat soil: perlite: river sand = 2:1:1, and the mass of each pot was 7.0 kg.

When each test seedling entered the vigorous growth period, the pot soil was controlled for 7 days to ensure the soil was dry and to facilitate the even distribution of salt during salt application. Once the pot soil was dry, 1500 ml of distilled water was poured into the control pots, whilst 1500 ml of salt solution was poured into the test pots, and the exuded water from the pots was poured into the pots again. The soil NaCl content (w / W) of control group and experimental group were 0.0% and 0.3% respectively, 3 replicates of each treatment were conducted. The soil moisture content was maintained at 60–80% by adding clean water every 5 days. During treatment 0d, 3d, 10d, and 30d, samples were taken in the morning for index determination, and 2 or 3 new middle and upper functional leaves were taken from different directions of each plant. At 0 h, 6 h, 24 h, and 72 h of salt treatment, new middle and upper functional leaves were taken for transcriptome sequencing and gene expression. The transcripts were marked as T0, T6, T24, and T72, and each treatment was

repeated 3 times, resulting in a total of 12 samples. The samples were immediately frozen in liquid nitrogen after collection and were stored at -80°C until use.

Physiological index determination

The direct leaching method was used to determine chlorophyll content, whilst the conductivity meter method was used to determine relative conductivity, and the determination of malondialdehyde (MDA) was achieved through the thiobarbituric acid method. The determination of soluble sugar was attained by the anthrone colorimetric method, acid ninhydrin colorimetry was used to determine free proline (Pro), and the Coomassie Brilliant Blue G250 staining method was used to determine soluble protein. In addition, the nitrogen blue tetrazolium (NBT) photoreduction method was implemented to determine superoxide dismutase (SOD) activity, and the guaiacol method was used to determine peroxidase (POD) activity (Li 2003).

The photosynthetic gas exchange parameters, such as net photosynthesis (Pn), transpiration (Tr), stomatal conductance (Gs), and intercellular CO_2 concentration (Ci), were measured by LI-6400 portable photosynthetic apparatus. The temperature was maintained at 30°C , the light intensity was $1,000\ \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, and the CO_2 concentration was $400\ \mu\text{mol}\cdot\text{mol}^{-1}$.

RNA extraction, library construction and data processing

RNA was extracted from the plant samples using RNAprep Pure Plant Kit (Polysaccharides and Polyphenolics-rich). Agarose gel electrophoresis was used to determine the extent of RNA degradation and contamination, whilst Nanodrop was used to detect the purity of the extracted RNA (OD260/280 ratio). In addition, a qubit accurately quantified the RNA concentration, and Agilent 2100 accurately detected RNA integrity. The cDNA library was sequenced using Illumina HiSeq 4000.

The raw image data files obtained by high-throughput sequencing were converted into original sequenced reads by CASAVA base calling analysis; the raw reads were then filtered to obtain clean reads to be used as the basis of subsequent analysis. The data processing steps were as follows: 1) reads with adapters were removed; 2) reads containing N (undetermined bases) greater than 10% were removed; and 3) low quality reads were removed ($\text{Qphred} \leq 20$ bases that accounted for more than 50% of the read length). HISAT software was selected to perform genomic localisation analysis on the filtered sequencing sequence. The raw reads were uploaded to NCBI, reference: PRJNA678761.

Screening and enrichment analysis of differentially expressed Unigene

RNA-seq expression quantification was based on the fragments per kilo base per million mapped reads (FPKM) values. Also, the negative binomial distribution model was used to calculate the hypothesis test probability (pvalue). The false discovery rate (FDR) was calculated to correct for multiple testing, as applicable for multi-hypothesis testing. In addition, the p value adjusted (padj) < 0.05 was used as a differential gene screening standard.

The Gene Ontology (<http://www.geneontology.org/>) was divided into three parts: molecular function, biological process, and cellular component (Young et al. 2010). Pathway enrichment analysis was based on the pathway in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Also, the hypergeometric test was used to identify the pathways that were significantly enriched in the differentially expressed genes compared with the entire genome background (Kanehisa 2008). Furthermore, KOBAS (2.0) was used for pathway enrichment analysis.

Transcript screening and statistics

Plant transcription factor prediction was carried out using iTAK software. The basic principle of this process relied on the use of the TF family and rules defined in the database to identify transcription factors through hmmscan in the HMMER suite. The identification and classification of transcription factors correspond to the method of Perez-Rodriguez et al. (2010).

qRT-PCR verification

cDNA was synthesised using Primer ScriptTM RT Master Mix. PCR was then performed based on the CFX Connect Real-time System (Bio-Rad, USA) using SYBR Primix Ex TaqTM II (Tli RNaseH Plus). The $25\ \mu\text{L}$ reaction system contained $2\ \mu\text{L}$ of template, $1\ \mu\text{L}$ of upstream and downstream primers ($10\ \mu\text{M}$) (Table 1), $8.5\ \mu\text{L}$ RNase Free dH_2O , and $12.5\ \mu\text{L}$ of SYBR Premix Ex TaqII (Takara). All experiments were performed as 3 biological replicates. The ubiquitin C (UBC) gene was used as the internal reference gene (Wang et al. 2014). The relative expression levels of the genes were calculated using the double standard curve method.

Table 1 Primer sequences for quantitative expression

Gene ID	Upstream primer sequence 5'-3'	Downstream primer sequence 5'-3'	Annealing temperature/ T _m	Amplification length/ bp
LOC103333990	TAAGCGTTAGGGAGCGAGTC	AGAAAGGTGGCGATTGAGG	55	473
LOC103333005	GTCCTAATGCCGTAAACCC	CTTCACTGCCAGCAAAATAA	55	326
LOC103344682	GTTTCACAACCTCTGACCCTT	GCATCATCTCACTACTCCCTC	55	148
LOC103323454	CTGCCATTTGATGACGCTA	CCAATCCTTGTTTTCTGCTT	55	395
LOC103323643	GGTCCCCAAAATAAGCAAG	TGTAACCCTAAGTTCAAGCA	55	262
LOC103323814	CGTTCCCAGATCCCTGTAT	GCACTTTGGTCCTTTCCAT	55	321
LOC103334364	AGCAGCAAGCAGCACATAA	TTCAATCTCGTCCAAAGCA	55	467
LOC103328315	ACTTCACTTTCTCCTTCGGTC	TGAGGTCCAGAGGGTCCAA	55	284
LOC103334065	ATCAAAGGGTTGTTCTGCC	TGGGTCAAGTTAGTGGTGG	55	126
LOC103327836	AGAGTAATGCTACCCGAAGA	CTGACAAGAAGGCAGAAAA	55	113
LOC103340164	TCCTTGACATTGGACCCTG	CTAATTTCTGCCCCACCCT	55	164
LOC103340279	CACGGATGACTCGTTGCTC	GTGCCTTGATGCCTTTGTA	55	116
AT5G53300	GCAGGTGGAGTGTTTCCTGTGA	GGCAGGGCTCCACTGTTCTTT	55	146

Results

Salt response: physiological characteristics

The photosynthetic gas exchange parameters, relative conductivity, MDA level, osmotic regulators, and antioxidant enzyme activities were measured at early-stage stress (3d), mid-stage stress (10d), and late-stage stress (30d) (Fig. 1a–d). The results of Pn, Gs, and SOD enzyme showed a downward trend over time; contrastingly, relative conductivity, free Pro, and POD enzyme activities showed an upward trend. MDA, soluble sugar, soluble protein, and Ci showed an initial increasing trend, followed by decreasing levels. On the other hand, Tr initially decreased, and then increased, but the value remained lower post-stress than pre-stress. Moreover, the physiological characteristics of *P. mume* in response to salt stress varied in different stress periods. In the early period of stress, the photosynthetic gas exchange parameters, relative conductivity, MDA, and soluble sugar content changed significantly ($P \leq 0.05$); in the mid-stage of stress, the indicators all changed with the exception of Ci, which returned to a normal value; in the late stage of stress, soluble sugar and Ci returned to normal levels, while Pn, Tr, and SOD showed values lower than those of control group, and relative conductivity, MDA, free proline, and soluble protein were higher than normal levels. Under salt stress, photosynthetic gas exchange, membrane system activity, osmotic adjustment substance, and antioxidant enzyme activity showed significant positive correlation (Fig. 1e).

The seedlings with relatively consistent growth were planted in individual flowerpots, and the NaCl content (w/w) of the soil for the control and test plants was 0.0% and

0.3%, respectively. The samples were collected at 0d, 3d, 10d, and 30d to determine each index. According to Duncan's test, different letters represent a different level of significance ($P \leq 0.05$). The correlation heat map was drawn using GraphPad Prism 8, whereby A represents photosynthetic gas exchange parameters, B represents membrane system related indexes, C represents osmotic adjustment substances, and D represents antioxidant enzyme activity.

Transcriptome sequencing analysis

To investigate the expression patterns of the *P. mume* leaves at 0 h, 6 h, 24 h, and 72 h of salt stress, four samples (T0, T6, T24, and T72) were transcribed using Illumina HiSeq 4,000. After removing the splice sequence, repetitive sequence, unknown reading segment, and low-quality reading segment, 66,150,124, 51,282,181, 54,613,020, and 52,907,542 clean reads were obtained, which covered 85.53%, 84.57%, 83.05%, and 83.59% of the genome sequence of *P. mume*, respectively (Table 2).

Gene expression analysis

A total of 64,916 transcripts were obtained from the 4 libraries constructed based on sequencing. The DEGs were filtered with expression levels $\log_2$ fold change > 1 or < -1 , q value < 0.005 in each pairwise comparison, and 5,161 genes were differentially expressed under salt stress (T6 vs. T0, T24 vs. T0, T72 vs. T0). Among these three groups, the greatest number of differentially expressed genes was 3140 at T72. In T6 vs. T0, the differentially expressed genes included more down-regulated genes than up-regulated

Fig.1 a Effects of salt stress on photosynthetic gas exchange parameters. b Effects of salt stress on malondialdehyde and relative conductivity. c Effects of salt stress on osmotic adjustment substances. d Effects of salt stress on antioxidant enzyme activity. e The

correlation heat map of four physiological indexes: photosynthetic gas exchange parameters, membrane system related indexes, osmotic adjustment substances and antioxidant enzyme activity

Table 2 Statistical results of Illumina HiSeq 4000 sequencing

Sample	Original reading	Filtered reads	Filter data/G	Error rate/%	Q30/%	GC/%	Coverage/%
T0	67,850,617	66,150,124	9.92	0.01	93.45	45.54	85.53
T6	52,550,703	51,282,181	7.69	0.01	93.44	45.41	84.57
T24	56,131,598	54,613,020	8.19	0.02	93.11	45.47	83.04
T72	54,398,926	52,907,542	7.94	0.02	93.08	45.74	83.59

genes, while at T24 vs. T0 and T72 vs. T0 the differentially expressed genes presented more up-regulation than down-regulation (Fig. 2a). The differential expression of genes varied at different stress time points. Compared with 0 h stress, a total of 527 genes were significantly differentially expressed at 6 h, 24 h, and 72 h salt stress. It is possible that these co-expressed genes are related to the salt response (Fig. 2b).

Hierarchical clustering based on gene expression patterns (Fig. 3a) identified T24 and T72 as being more likely to be clustered together. As the stress time increased, the gene expression patterns became closer. Hierarchical clustering was used to cluster the relative expression levels of differential genes log₂ (ratios) (Fig. 3b). The two most significant enrichment groups were Subcluster 61,371 genes and Subcluster 31,092 genes. At 6 h, a smaller

fluctuation was identified than at 0 h, which then increased or decreased significantly at 24 h. T72 followed essentially the same pattern as T24, as the expression of a considerable number of genes changed significantly at 24 h, and this trend of change was maintained at 72 h of stress.

Twelve genes were selected to verify the transcriptome sequencing results (Enclose Table 1), whereby strong positive correlation was determined between the results of RNA-seq and qRT-PCR (Fig. 4); the correlation coefficients R² in T6 vs. T0, T24 vs. T0, and T72 vs. T0 were 0.89, 0.90, and 0.88, respectively. This signified the reliability of the transcriptome sequencing data.

Fig. 2 **a** Histogram and **b** Venn diagram of differentially expressed genes. A total of 5161 genes were differentially expressed under salt stress; the histogram depicts the number of genes up-regulated or

down-regulated in T6 vs. T0, T24 vs. T0, and T72 vs. T0. The Venn diagram shows the number of differentially expressed genes overlapping from the three time points after salt stress

Fig. 3 **a** Hierarchical cluster analysis, using the FPKM value of the differential gene as the expression level; the areas with different colours represent different clustering grouping information. **b** Differential genetic clustering of the H-cluster method.

GO and KEGG analysis of differential genes

GO and KEGG pathway enrichment analyses were conducted for the differential genes. Significant enrichment was denoted when $FDR \leq 0.05$. The results found a lack of significant enrichment in T6 vs. T0. However, 17 functional groups were significantly enriched in T24 vs. T0, and the highest proportion of “organonitrogen compounds” in the biological processes class was 16.93%. In T72 vs. T0, a total of 62 functional groups were significantly enriched. The highest proportion of “cellular metabolic processes” in the biological processes class was 19.07%, the highest proportion of “thylakoids” in the cell composition class was 16.22%, and the highest proportion of “small molecule

binding” in the molecular function class was 6.10% (Fig. S1 and Fig. S2). The analysis showed that up-regulated transcripts in biological processes primarily derived from the pathways of “phosphate-containing compound metabolic process”, “protein metabolic process”, “protein modification process”, and “macromolecule modification”. In addition, up-regulated transcripts in cell composition were mostly related to photosynthesis, including “photosystem”, “photosynthetic membrane”, and “thylakoid”. In terms of molecular function, many up-regulated transcripts were related to enzyme activity, including “kinase activity”, “transferase activity”, “protein kinase activity”, and “phosphotransferase activity”, whilst others were related to substance synthesis, including “ATP

Fig. 4 Correlation between RNA-seq and qRT-PCR results. Twelve genes were randomly selected to verify the transcriptome sequencing results

binding”, “small molecule binding”, “nucleotide binding”, and “anion binding”.

The result of KEGG enrichment analysis showed 1150 differential genes belonging to 113 KEGG pathways in the T6 vs. T0 group, but a significant enrichment pathway was not identified. The T24 vs. T0 group had 1138 differential genes belonging to 106 KEGG pathways, of which the “photosynthesis-antenna protein” pathway was significantly enriched. Furthermore, the T72 vs. T0 group showed 1553 differential genes belonging to 116 KEGG pathways, and the “photosynthesis-antenna protein” pathway was also significantly enriched at 72 h (Fig. S3).

Salt response-related transcription factor

A total of 362 transcripts were identified from the 4 transcripts (T0, T6, T24, and T72), which belonged to 56 gene families. Of these families, 13 had more than 10 genes

(Fig. 5a). In the T6 vs. T0 group, differentially expressed transcription factors belonged to 29 families. The gene families with more than 5 genes included the basic helix–loop–helix (bHLH) gene family (11 entries), WRKY gene family (8 entries), MYB gene family (7 entries), and ethylene response factor (ERF) (7 entries). In the T24 vs. T0 group, differentially expressed transcripts belonged to 25 families; those that comprised more than 5 genes included the ERF gene family (17 entries), WRKY gene family (14 entries), bHLH gene family (8 entries), and the MYB gene family (7 entries). The T72 vs. T0 group had differentially expressed transcripts belonging to 31 gene families, and those that included more than 5 genes were the WRKY gene family (18 entries), bHLH gene family (11 entries), ERF (10 entries), DIVARICATA gene family (6 entries), and MYB gene family (5 entries). The T6 vs. T0, T24 vs. T0, and T72 vs. T0 ranges co-expressed transcripts belonging to 20 gene families, which are

Fig. 5 a 13 gene families with more than 10 transcription factors. b Differential expression of four transcription factor families under salt stress

possibly related to salt tolerance. Among these families, bHLH, WRPK, ERF, and MYB included the largest number of genes. Four of the gene families presented gene expression at 6 h, 24 h, and 72 h of stress (Fig. 5b). Moreover, the transcription factors up-regulated at 24 h and 72 h stress were more than that at 6 h stress, with the exception of the MYB gene family whereby the up-regulated genes at 72 h were lower than 6 h. However, the transcription factors down-regulated at 6 h of stress were more than that at 24 h and 72 h.

Identification of DEGs in different treatment periods

The genes that showed a statistically significant difference in expression levels at T6, T24, or T72, compared with those at T0, were enriched to reveal the specific genes and their functions that were significantly different in the early stage of salt tolerance in *P. mume*. The functional categories of the differentially expressed genes (DEGs) significantly up-regulated are shown in Fig. 6a. The results showed that a large number of transcription factors and hormone signal transduction genes were up-regulated at T6, T24, and T72. Sucrase synthase (SUSY) and photosystem I reaction centre (PS I) subunit genes of the metabolic signal transduction pathway were significantly up-regulated, indicating their considerable role in signal transduction in the early stage of salt tolerance. Several genes related to ion transport and reconstruction of ion balance, as well as genes related to the biosynthesis of

osmotic protective substances, reactive oxygen species scavenging, and stress resistance-related protein genes were also significantly up-regulated. The functional categories of DEGs with significantly down-regulated expression are shown in Fig. 6b. Numerous transcription factors and aquaporins (AQPs) related to dehydration protection and water transport were significantly down-regulated in T6, T24, and T72. These findings suggest that regulatory networks involving hormone signal transduction, ion transport and reconstruction of ion balance-related genes, biosynthesis of osmotic protective substances-, reactive oxygen species scavenging, and stress resistance-related genes regulated the early salt tolerance activities of *P. mume*.

Based on the enrichment of GO and KEGG, protein–protein interaction analysis of DEGs and TFs was undertaken to identify the key genes conveying salt tolerance in *P. mume*. It was possible that these structural genes were regulated by bZIP family transcription factors. In addition, cyclic-nucleotide gated channels (CNGCs), POD, and SUSY may contribute to salt tolerance of *P. mume* (Fig. 7).

Discussion

Genes related to ion transport and reconstruction of ion balance

Plants must undergo osmotic adjustments to rebuild the ion balance when under certain environmental adversity, like

Fig. 6 Heatmap comparison of **a** significantly up-regulated DEGs and **b** significantly down-regulated DEG, in three treatment periods. The functional categories of significantly over-represented DEGs were identified in T0 and T72

Fig. 7 Construction of protein–protein interaction network model of salt-tolerant candidate genes in *P. mume* using Cytoscape software

salt stress. Many genes were found to be involved in the reconstruction of ion balance in the gene expression profile of *P. mume*. The K^+ ion channel (AKT) was up-regulated at 24 h of salt stress, while the Mg^{2+} transporter was up-regulated at 6 h, 24 h, and 72 h of stress. In addition, the K^+ transporter was also up-regulated at 6 h, 24 h, and 72 h (Table 3a). Previous studies have shown that cation/H⁺ exchangers (CHX) may also participate in sodium efflux (Manohar et al. 2011). In the present study, a CHX18 gene was found to be up-regulated at 6 h and 72 h in the differential expression profile of *P. mume*, which may be involved in ion metabolism regulated by CHX. A comparison of the differential expression profiles of salt stress at 6 h, 24 h, and 72 h identified greater down-regulation of genes than up-regulation in the genes corresponding to ion

balance at 6 h stress, whilst greater up-regulation than down-regulation was established of the genes at 24 h and 72 h of stress. Ion metabolism and transport are significant areas in relation to the salt tolerance of *P. mume* because salt stress affects the ion balance, and the plant uses the process of ion transport to regain such balance.

Genes related to biosynthesis of osmotic protective substances

The differential expression profile of *P. mume* showed the up-regulation of SUSY genes after stress. Sugars, polyols, amino acids (such as Pro), and betaine are all non-toxic soluble substances, which accumulate in the cytoplasm in an external hypertonic environment and have no effect on cellular metabolism (Chen et al. 2002). Pyrroline-5-carboxylate synthase (P5CS) is a key enzyme for Pro synthesis and degradation; up-regulation of the P5CS gene was identified at 6 h of stress. The P5CS gene has been successfully overexpressed in many plants such as arabidopsis and tobacco, and can effectively improve the salt tolerance of transgenic plants (Cao et al. 2011; Huang et al. 2013). The differential expression profile showed only one mannitol dehydrogenase gene, which was down-regulated at 6 h and 24 h of stress (Table 3b). Subsequent data analysis found that most of the key genes encoding osmotic protective substances lacked significant expression changes. It is possible that these genes had not yet accumulated during the stress response process at the selected time ranges, and that they primarily assist the process of stress resistance.

Genes related to active reactive oxygen species scavenging and anti-stress proteins

Under salt stress, plant cells produce large amounts of reactive oxygen species due to hindered metabolism. These species include oxygen free radicals ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl free radicals ($\cdot OH$). Although a normal product of cellular reactions, an excessive amount of reactive oxygen species is harmful to cells as they can cause lipid peroxidation of cell membranes and DNA damage that leads to cell death (Luo et al. 2020). To overcome this potential issue, plants synthesise antioxidants, which are renowned for their free radical scavenging ability. Many antioxidant genes, such as POD, SOD, metallothionein protein (MT), glutathione sulfur transferase, aldehyde dehydrogenase (ALDH), and peroxiredoxin (PrxR), were detected in the differential expression profile of *P. mume* (Table 3c). In high-salt and arid environments, the ALDH gene family was up-regulated, indicating their correlation to the stress response in cotton (Guo et al. 2017). Data analysis of the antioxidant genes identified greater up-regulation of POD, MT, glutathione

Table 3 Key genes conveying salt tolerance in *P. mume*

Genes	T6VST0		T24VST0		T72VST0	
	Down -regulated	Up-regulated	Down-regulated	Up-regulated	Down -regulated	Up-regulated
a. Genes related to ion transport and reconstruction of ion balance						
V-ATPase	0	1	0	0	1	0
phospholipid-transporting ATPase	0	0	0	2	0	1
plasma membrane ATPase	1	0	0	0	0	1
NHX	3	0	0	1	0	1
AKT	1	0	0	1	1	0
potassium transporter	2	2	0	1	0	1
magnesium transporter	0	1	0	1	0	1
CHX	1	1	1	0	1	1
CNGCs	0	2	0	2	0	8
b. Genes related to biosynthesis of osmotic protective substances						
P5CS	0	1	0	0	0	0
sucrose synthase	0	1	0	1	0	1
mannitol dehydrogenase	1	0	0	0	1	0
c. Genes related to active oxygen scavenging and anti-stress proteins						
POD	5	4	0	3	1	4
SOD	0	0	3	0	3	0
MT	0	0	0	2	0	2
Glutathione S-transferase	3	1	4	2	2	9
ALDH	0	2	0	1	0	1
PrxR	0	0	3	0	2	0
HSP	18	1	18	1	16	1
disease resistance protein	6	7	0	13	1	14
d. Genes related to dehydration protection and water transport						
AQPs	5	1	1	0	5	2
LEA	0	0	0	2	1	4
DHN	0	0	0	1	0	1
e. Genes related to stress signal transduction						
MAPK	5	2	0	3	0	8
CDPK	0	3	0	3	0	5
phospholipase	2	3	1	2	3	2
CIPKs	7	1	0	2	1	2
CBLs	1	0	0	0	0	4
CAMTAs	0	0	0	1	0	1
PP2C	7	4	3	7	0	9
PYL	0	1	0	1	0	1
PYR	0	0	0	0	0	1
SnRKs	0	0	0	1	0	1
bZIP	1	1	1	1	1	1
MYB	2	5	1	6	2	3
MYC	1	0	0	1	1	0

sulfur transferase, and ALDH than down-regulation as the stress time increased;

In addition, regarding physiological response, SOD enzyme activity and expression of the SOD gene decreased after 24 h of stress, which implies that SOD was not the

key gene involved in salt tolerance of the *P. mume* in this study. This finding differs from previous results studying the salt stress tolerance of barley and arabidopsis, whereby the SOD gene was depicted as being highly involved in salt tolerance (Zhang et al. 2021; Zheleznova et al. 2020). This difference may be due to differences in salinity tolerance of species or treatment conditions. Therefore, the role of the SOD gene in the salt stress tolerance of *P. mume* needs further exploration and verification.

Genes related to dehydration protection and water transport

Two AQP genes were up-regulated after stress exposure, while 9 AQP genes were shown to be down-regulated after stress. The results of the study established an increase in the expression of LEA and DHN genes of *P. mume* at 24 h and 72 h of stress. LEA proteins possess high hydrophilicity and thermal stability, which enables stabilisation of the cell membrane structure, and can be used as molecular chaperones. Furthermore, LEA can be used to bind metal ions and prevent oxidation in cells, and induce expression under abiotic stresses including salt, drought, and cold temperatures (Cheng et al. 2021). DHN is a member of the LEA family; studies have found that over-expression of the DHN gene can enhance the response of Arabidopsis seeds to salt stress (Williams et al. 2015). In terms of *P. mume*, Du et al. (2012) identified 6 members of the DHN gene family in the *P. mume* genome. With the exception of PmLEA29, the expression levels of the remaining 5 DHN genes increased under salt stress (Table 3d). Therefore, dehydration protection is an important approach for salt tolerance of *P. mume*.

Genes related to stress signal transduction

In the calcineurin B-like protein-CBL-interacting protein kinase (CBL-CIPK) pathway, the CBL, CIPK, and calmodulin-binding transcription activator (CAMTA) gene families all showed changes in their expression levels. CBLs and CIPKs jointly participate in the process of ion stress signal transduction. Low K^+ content and high Mg^{2+} content generates an ion stress signal, which subsequently causes the interaction of transmembrane proteins with their respective target proteins through Ca^{2+} flow. This initiates cascade phosphorylation (CBLs and CIPKs) (Boudsocq et al. 2013), which results in corresponding changes to the transcription factors of downstream related genes. Alternatively, cascade phosphorylation results in direct interaction with Ca^{2+} transcription activators, such as CAMTAs and GT element-binding-like proteins (GTLs) (Pandey et al. 2013; Hua et al. 2012) to regulate the salt stress ionic

balance genes and the expression of transcription factors related to stress resistance.

In the signal transduction pathways of mitogen-activated protein kinase (MAPK) and calcium-dependent protein kinase (CDPK), these protein kinases were up-regulated at 6 h, 24 h, and 72 h of stress. It could be speculated that the MAPK cascade achieves osmotic balance by adjusting the osmotic solute, including the functionally interconnected protein kinases of MAPK, MAPK kinase (MAPKK), and MAPK kinase kinase (Table 3e). In the differential expression profile of *P. mume*, only changes in the expression of phospholipase were detected; yet the corresponding gene of its hydrolysate was not found. Overall, establishing whether salt stress initiates the phospholipase transduction pathway requires more in-depth research.

Analysis of the DEGs of *P. mume* showed more down-regulated genes of PP2C than up-regulated genes at 6 h of stress, whilst more up-regulated genes were detected at 24 h and 72 h than down-regulated genes. The gene expression of PYL, PYR, and SnRKs increased post-stress, which subsequently positively regulated the abscisic acid (ABA) signalling pathway (Soon et al. 2012). In pathways involving protein synthesis, salt stress signals were recognised by ABA and were then transmitted to downstream transcription factors, such as MYB and MYC. These intermediate proteins recognise each other due to cis-regulatory elements, like MYBRS and MYCRS which are drought responsive elements, on the promoters of osmotic stress-induced genes. This enables the regulation of gene expression in response to salt stress (Yong et al. 2019; Li et al. 2010). The expression of bZIP, MYB, and MYC transcription factors in *P. mume* varies significantly; however, these factors all participate in the ABA-dependent signal transduction pathway.

Analysis of the internal mechanism of physiological response

The internal mechanism of the physiological response of *P. mume* to salt stress was analysed according to the physiological response results alongside the expression of key genes (Table 3). In the early stage of stress, the photosynthetic gas exchange parameters of Pn, Gs, and Tr all decreased, while Ci increased; the initial decline of Pn to Ci reflects the efficiency of carbon carboxylation, and correlation with RuBisCO activity was identified to an extent (Nik et al. 2020). Two RuBisCO-related genes were identified in the differential gene spectrum of *P. mume*; these were down-regulated at 24 h and 72 h of salt stress, which was consistent with the physiological results, as salt stress inhibits the activity of Rubisco, which is the key photosynthetic enzyme of *P. mume*. Consequently, the photosynthetic rate was found to decrease. In addition,

P5CS and SUSY genes related to Pro synthesis were detected in the gene expression profile of *P. mume*; these genes regulate the synthesis of Pro and sucrose, promote the accumulation of Pro and soluble sugar under salt stress, and alleviate osmotic stress (Funck et al. 2020).

The results also portrayed the important role played by POD in the removal of reactive oxygen species from plant cells, and the regulation of POD enzyme synthesis by POD genes. POD enzyme activity increased after 10 days of stress. In terms of transcription level, POD genes were up-regulated at 6 h and 24 h of stress, and up-regulated genes were more than down-regulated genes. Therefore, gene expression can be viewed as related to protein synthesis, but not completely unified.

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Declarations

Conflict of interest All authors have read and approved this version of the article, and due care has been taken to ensure the integrity of the work. No conflict of interest exists in the submission of this manuscript.

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References

- Agarwal P, Khurana P (2020) TaZnF, a C3HC4 type RING zinc finger protein from *Triticum aestivum* is involved in dehydration and salinity stress. *J Plant Biochem Biot* 29:395–406
- Bao F, Du D, An Y, Yang W, Wang J, Cheng T, Zhang Q (2017) Overexpression of *Prunus mume* Dehydrin Genes in Tobacco Enhances Tolerance to Cold and Drought. *Front Plant Sci* 8.
- Boudsocq M, Sheen J (2013) CDPKs in immune and stress signaling. *Trends Plant Sci* 18:30–40
- Calestani C, Moses MS, Maestri E, Marmioli N, Bray EA (2015) Constitutive expression of the barley dehydrin gene *aba2* enhances *Arabidopsis* germination in response to salt stress. *Int J Plant Biol* 6(1):1039–1054
- Cao L, Yi MF, Sun ZY, Han L, Ju GS, Ma XR (2011) Site directed mutagenesis of the LpP5CS gene of *Lolium perenne* and its transformation in *Arabidopsis thaliana*. *Acta Pratacul Sin* 20(1):242–247
- Chen P, Zhao X, Tian GG, Yuan X, Li X, Li Z, Yu X, Hu R, Wang Y, Pei X et al (2021) C28 induced autophagy of female germline stem cells in vitro with changes of H3K27 acetylation and transcriptomics. *Gene* 766:145–150
- Chen THH, Murata N (2002) Enhancement of tolerance of abiotic stress by metabolic engineering of betaines and other compatible solutes. *Curr Opin Plant Biol* 5:250–257
- Cheng Z, Zhang X, Yao W, Zhao K, Liu L, Fan G, Zhou B, Jiang T (2021) Genome-wide search and structural and functional analyses for late embryogenesis-abundant (LEA) gene family in poplar. *Bmc Plant Biol* 21(1101).
- Du D, Zhang Q, Cheng T, Pan H, Yang W, Sun L (2012) Genome-wide identification and analysis of late embryogenesis abundant (LEA) genes in *Prunus mume*. *Mol Biol Rep* 40:1937–1946
- Funck D, Baumgarten L, Stift M, von Wiren N, Schoenemann L (2020) Differential Contribution of P5CS Isoforms to Stress Tolerance in *Arabidopsis*. *Front Plant Sci* 11(565134).
- Shi GY, Wang YM, Hua JP (2012a) Aquaporins and Salt Tolerance of Higher Plants. *J Agr Sci Tec* 14(4):31–38
- Goetz K, Naher J, Fettke J, Chmielewski F (2018) Changes of proteins during dormancy and bud development of sweet cherry (*Prunus avium* L.). *Sci Hortic-Amsterdam* 239:41–49
- Guo X, Wang Y, Lu H, Cai X, Wang X, Zhou Z, Wang C, Wang Y, Zhang Z, Wang K et al (2017) Genome-wide characterization and expression analysis of the aldehyde dehydrogenase (ALDH) gene superfamily under abiotic stresses in cotton. *Gene* 628:230–245
- Huang C, Jiang W, Yang L, Li Y, Yang C (2013) Response of antisense ScP5CS transgenic tobacco plants to PEG and NaCl treatments. *Crops* 3(2013):42–45
- Kanehisa M, Araki M, Goto S, Hattori M, Hirakawa M, Itoh M, Katayama T, Kawashima S, Okuda S, Tokimatsu T et al (2008) KEGG for linking genomes to life and the environment. *Nucleic Acids Res* 36:D480–D484
- Li C, Lv J, Zhao X, Ai X, Zhu X, Wang M, Zhao S, Xia G (2010) TaCHP: a wheat zinc finger protein gene down-regulated by abscisic acid and salinity stress plays a positive role in stress tolerance. *Plant Physiol* 154:211–221
- Li HS (2003) Principles and techniques of plant physiology and biochemistry experiment. Higher Education Press, Beijing, China
- Li QW (2009) Studies on Investigation of Wild Mei Germplasm Resources of Sichan Yunnan Tibet and Guizhou Province as well as Regional Tests of Hardy Mei Cultivars in North China. Beijing Forestry University.
- Luo Z, Szczepanek A, Abdel-Haleem H (2020) Genome-Wide Association Study (GWAS) Analysis of Camelina Seedling Germination under Salt Stress Condition. *Agronomy-Basel* 10(14449).
- Manohar M, Shigaki T, Hirschi KD (2011) Plant cation/H⁺ exchangers (CAXs): biological functions and genetic manipulations. *Plant Biol* 13:561–569
- Nik Muhammad Nasir NN, Khandaker MM, Mohd KS, Badaluddin NA, Osman N, Mat N (2020) Effect of Hydrogen Peroxide on Plant Growth, Photosynthesis, Leaf Histology and Rubisco Gene Expression of the *Ficus deltoidea* Jack Var. *deltoidea* Jack. *J Plant Growth Regul*.

- Pandey N, Ranjan A, Pant P, Tripathi RK, Ateek F, Pandey HP, Patre UV, Sawant SV (2013) CAMTA 1 regulates drought responses in *Arabidopsis thaliana*. *Bmc Genomics* 14.
- Peng T, Guo C, Yang J, Xu M, Zuo J, Bao M, Zhang J (2016) Overexpression of a Mei (*Prunus mume*) CBF gene confers tolerance to freezing and oxidative stress in *Arabidopsis*. *Plant Cell Tiss Org* 126:373–385
- Perez-Rodriguez P, Riano-Pachon DM, Correa LGG, Rensing SA, Kersten B, Mueller-Roeber B (2010) PInTFDB: updated content and new features of the plant transcription factor database. *Nucleic Acids Res* 38:1:D822–D827
- Przedpelska-Wasowicz EM, Wierzbicka M (2011) Gating of aquaporins by heavy metals in *Allium cepa* L. epidermal cells. *Protoplasma* 248:663–667
- Shi GY, Wang YM, Hua JP (2012b) Aquaporins and Salt Tolerance of Higher Plants. *J Agric Sci Technol* 14:31–38
- Soon F, Ng L, Zhou XE, West GM, Kovach A, Tan MHE, Suino-Powell KM, He Y, Xu Y, Chalmers MJ et al (2012) Molecular mimicry regulates ABA signaling by SnRK2 kinases and PP2C phosphatases. *Science* 335:85–88
- Sun X, Sun C, Li Z, Hu Q, Han L, Luo H (2016) AsHSP17, a creeping bentgrass small heat shock protein modulates plant photosynthesis and ABA-dependent and independent signalling to attenuate plant response to abiotic stress. *Plant Cell Environ* 39:1320–1337
- Wan X, Yang J, Li X, Zhou Q, Guo C, Bao M, Zhang J (2016) Overexpression of PmHSP17.9 in Transgenic *Arabidopsis thaliana* confers Thermotolerance. *Plant Mol Biol Rep* 34:899–908
- Wang L, Zhang C, Wang Y, Wang Y, Yang C, Lu M, Wang C (2018) *Tamarix hispida* aquaporin ThPIP2;5 confers salt and osmotic stress tolerance to transgenic *Tamarix* and *Arabidopsis*. *Environ Exp Bot* 152:158–166
- Wang XK, Huang JL (2015) Principles and techniques of plant physiology and biochemistry experiment. Higher Education Press, Beijing, China
- Waqas M, Chen Y, Iqbal H, Shareef M, Rehman HU, Bilal HM (2021) Synergistic consequences of salinity and potassium deficiency in quinoa: linking with stomatal patterning, ionic relations and oxidative metabolism. *Plant Physiol Bioch* 159:17–27
- Weng H, Yoo CY, Gosney MJ, Hasegawa PM, Mickelbart MV (2012) Poplar GTL1 Is a Ca²⁺/Calmodulin-Binding Transcription Factor that Functions in Plant Water Use Efficiency and Drought Tolerance. *Plos One* 7(3)
- Yang J, Wan XL, Guo C, Zhang JW, Bao MZ (2016) Identification and expression analysis of nuclear factor Y families in *Prunus mume* under different abiotic stresses. *Biol Plantarum* 60:419–426
- Yang JX, Li QW, Liu YX, Xu XL, Zhang QX, Li WG, Sun YF (2018) Effects of NaCl Stress on the Germination and Physiological Characteristics of *Prunus mume* and *Prunus davidiana* Seeds. *J Northwest Forestry Univ* 33(2):94–99
- Yong Y, Zhang Y, Lyu Y (2019) A MYB-Related Transcription Factor from *Lilium lancifolium* L. (LIMYB3) Is Involved in Anthocyanin Biosynthesis Pathway and Enhances Multiple Abiotic Stress Tolerance in *Arabidopsis thaliana*. *Int J Mol Sci* 20(319513).
- Young MD, Wakefield MJ, Smyth GK, Oshlack A (2010) Gene ontology analysis for RNA-seq: accounting for selection bias. *Genome Biol* 11(2).
- Zhai M, Sun Y, Jia C, Peng S, Liu Z, Yang G (2016) Over-expression of JrsHSP17.3 gene from *Juglans regia* confer the tolerance to abnormal temperature and NaCl stresses. *J Plant Biol* 59:549–558
- Zhang Q, Chen W, Sun L, Zhao F, Huang B, Yang W, Tao Y, Wang J, Yuan Z, Fan Get al (2012) The genome of *Prunus mume*. *Nat Commun* 3
- Zhang X, Zhang L, Chen Y, Wang S, Fang Y, Zhang X, Wu Y, Xue D (2021) Genome-wide identification of the SOD gene family and expression analysis under drought and salt stress in barley. *Plant Growth Regul*
- Zhao W, Faust F, Schubert S (2020) Potassium is a potential toxicant for *Arabidopsis thaliana* under saline conditions. *J Plant Nutr Soil Sc* 183:455–467
- Zheleznova N, Terashvili M, Bullis J, Stumpf M, Linh N, Geurts A, Lombard J (2020) Blood Pressure, Vascular Reactivity, and SOD Expression/Activity in Mas1 Receptor Knockout Rats in the Dahl Salt-Sensitive Genetic Background. *Faseb J* 341
- Zhu J (2016) Abiotic Stress Signaling and Responses in Plants. *Cell* 167:313–324

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