Pol. J. Environ. Stud. Vol. 34, No. 3 (2025), 2263-2272
DOI: *10.15244/pjoes/186400* ONLINE PUBLICATION DATE: 2024-09-03

Original Research

Transcriptome Analysis of Maize under Salt Stress and Overexpression of ZmHSP20 Gene Confers Salt Tolerance in Tobacco

Chun-Lei Li[1], Ling-Cong Meng[2*], Yi Zhong[2], Ren-Hai Jiao[2], Chuan-Bo Sun[2], Gui-Hua Su[2], Zhi-Chao Sun[2], Yi-Chen Su[2*], Xiao-Ming Yu[1**]**

[1]College of Agronomy, Jilin Agricultural Science and Technology University, Jilin 132101, China
[2]Maize Research Institute, Jilin Academy of Agricultural Sciences/National Engineering Laboratory for Maize, Gongzhuling Jilin 136100, China

Received: 22 December 2023
Accepted: 22 March 2024

Abstract

Soil salinity and alkalinity present a serious threat to global agriculture. The objective of this study is to elucidate candidate genes associated with salt stresses in maize plant tissues through the utilization of transcriptome and transgene methods. Specifically, 8723 and P138 maize cultivars were employed as salt-tolerant and salt-sensitive materials, respectively. To verify the salt tolerance of maize materials and screen the best salt stress experimental concentration, different concentrations of salt stress treatment were carried out. Then, transcriptome analysis was performed based on the sequence data of seedlings and the salt tolerance related genes were identified. The findings revealed that high salt concentrations significantly inhibited the germination characteristics and seedling growth of maize. Notably, the gene expression profiles in response to salt stress differed between the two maize inbred lines during the seedling stage. After the functional enrichment and co-expression interaction network analysis, we found that *ZmHSP20* was a key gene that related to salt tolerance. In order to further verify the function of *ZmHSP20*, we constructed *ZmHSP20* overexpression lines. The results showed that *ZmHSP20* carrying tobaccos were more salt tolerant than the wild-type (WT). Additionally, the activity of antioxidant enzymes was significantly higher in the *ZmHSP20* tobacco seedlings than in the WT. This study lays the groundwork for further research on the functional aspects of *ZmHSP20* and presents an initial exploration of its role in conferring resistance to salt stress. Further investigations are required to reveal the mechanism by which *ZmHSP20* regulates resistance against salt stress in maize and tobacco.

Keywords: maize, salt stress, ZmHSP20, transcriptome, overexpression

*e-mail: suliu1111@163.com
**e-mail: yuxm446@nenu.edu.cn
***e-mail: boxuecao88@126.com

Introduction

Soil salinity is a major type of abiotic stress that limits crop productivity and affects plant growth and development. Salinity has degraded millions of hectares worldwide, seriously threatening food production in a third of the world's arable land [1]. The detrimental effects of water, salt, and drought stress on maize growth and yield have intensified in recent years, with salt stress, in particular, causing substantial damage and resulting in significant losses. Therefore, there is a need to explore strategies for improving maize salt tolerance.

Salinity induces three primary types of plant stresses - osmotic stress, ionic stress, and oxidative stress [2], primarily mediated by the accumulation of Na^+ in cells. The low K^+/Na^+ ratio ion imbalances inhibit plant growth. Plants employ various gene expression evolution mechanisms to adapt to salinity-induced stress [3], regulating critical biological processes, including signal transduction, energy metabolism, transcription, protein biosynthesis, membrane trafficking, and photosynthesis [4-7]. Plant responses to salt stress could be shaped by gene regulation. In addition to the known salinity tolerance genes (e.g., HKT1, SOS members, DREB2, APX, GR, GST), certain small proteins, such as the HSPs, enhance plant salt resistance [8, 9]. Notably, genes mediate salinity resistance in different ways. Physiological and transcriptomic analyses [10-13] have been utilized to analyze how plants cope with abiotic stresses. Although transcriptome sequencing is a powerful method for identifying novel transcripts and analyzing gene expression, this approach alone is not enough. Also, plant transgene is a good choice for varying candidate genes.

In this present study, we identified the distinct gene expression patterns in response to salt stress in the salt-tolerant and salt-sensitive maize using transcriptome data. The differentially expressed genes (DEGs) between the salt-tolerant and salt-sensitive varieties were identified and analyzed. The role of potentially salinity-related genes in maize was validated in tobacco. Our findings provide new knowledge on plant physiology under stress, especially the physiological mechanism of salt tolerance.

Materials and Methods

Plant Culture and Salt Stress Treatment

Salt-tolerant maize variety 8723 (designated as Tol) and salt-sensitive maize variety P138 (designated as Sens) were utilized in a study to investigate the mechanisms underlying salt tolerance in maize [14]. The seeds were sourced from the maize seed bank at Gansu Agricultural University. Seeds of the respective maize varieties were cultivated in solutions containing 0, 100, 200, and 300 mM NaCl to assess their salt resistance capabilities. The germination rate (GR), germination potential (GP), and germination index (GI) were evaluated after a five-day germination period following established protocols [15]. The 8723 and P138 maize inbred lines were then treated with 0, 100, 200, and 300 mM NaCl solutions. The experiment was performed in the greenhouse at 25±2ºC, 12/112 h light, and dark cycle under 60% relative humidity. NaCl solution was added every three days after the plants in control grew to the three-leaf stage (about seven days). The flag leaf of each plant was collected and preserved at −80ºC in liquid nitrogen till further analysis.

Determination of Antioxidant Enzymatic Activity

The level of antioxidant enzymes in flag leaf plant extracts was analyzed as described by Ahmad et al. [16]. Briefly, flag leaves were frozen in liquid nitrogen and then homogenized on ice using 10 mL of 50 mM phosphate buffer (pH 7.8). Following centrifugation at 4,000g for 15 minutes at 4ºC, the resulting supernatant was collected for the determination of enzyme activities. The activities of malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) were assessed using specific kits (kit Numbers: A003-1, A001-1, A007-1, A084-3, respectively) from Nanjing Jiancheng Bioengineering Institute, China, in accordance with the manufacturer's instructions [17].

Transcriptome Analysis

The optimal concentration of salt treatment for transcriptome analysis was selected based on the germination and antioxidant enzyme results. The total RNA of the flag leaves was extracted using a commercial kit (Takara, Dalian, China). Then, the purified total RNA was used to construct the sequence library. The mDNA sequence library was constructed using the NEBNext Ultra RNA Library Prep Kit for Illumina, following the manufacturer's instructions. A total of 12 samples (triplicates per group) were used for library construction (i.e., 8723-CK, 8723-T, P138-CK, and P138-T). Sequencing was then performed using an Illumina HiSeq X10 platform.

The raw sequence data were analyzed using the FastQC software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) under default parameters. The clean sequence data were mapped to the maize genome downloaded from the Maize Genome Database (MaizeGDB; https://www.maizegdb.org/). The reference genome was converted into an index using HISAT2 software and an improved Burrows–Wheeler transform (BWT) algorithm. Then, the gene expression level was determined based on fragments per kilobase per million bases (FPKM). Read counts data were normalized using TMM method, and p-value was calculated with the Poisson distribution model. The FDR (false discovery rate) is determined by p-value ranges in multiple tests. In this study, the threshold of $|\log2FC \text{ (fold change)}| > 2$ with q-value < 0.01 was selected as simulated biological

variation to determine whether a gene has significantly differential expression in the DEGseq2 analysis [18]. GO classification (GO database, http://www.geneontology.org.) and KEGG pathway (KEGG database, http://www.genome.jp/kegg/) methods were carried out for allocating genes to different functional categories and predicting their biological functions, respectively. The KEGG pathway enrichment and GO enrichment analysis were performed with the condition of corrected p value < 0.05 [19].

Subcellular Localization of Salt Stress-Related Protein

In order to ascertain the subcellular localization of a potential stress-related protein, constructs containing the 35S gene-GFP and 35S-GFP vectors were generated and introduced into tobacco epidermal cells through injection, following established protocols [20]. The fluorescence signals in the inner epidermal cells were observed using a confocal laser scanning microscope.

Construction of Tobacco Lines Overexpressing the Salt-Tolerant Gene

The full-length RNA of the potentially salt-related gene was reverse transcribed into the cDNA. Overexpression of salt-related genes in tobacco (*Nicotiana tabacum*) was induced through leaf disk transformation with *Agrobacterium*. The full-length cDNA of the salt-related gene was cloned into the modified binary vector pMV (derived from pBI121 vector) to form the pMV-EsFLS construct under the control of the CaMV 35S promoter. The plasmids were electroporated into Agrobacterium tumefaciens strain EHA105. Tobacco immature embryos (IEs) of 1.5 to 2.0 mm were isolated from ears harvested 10 d after pollination into 2.0 mL tubes with 1.8 mL inoculation medium and were infected with Agrobacterium suspension (inoculation medium with 200 µM of acetosyringone and Agrobacterium cells) for 5 min, then poured onto co-cultivation medium. Kanamycin (100 µg/mL) resistance was used as the marker to select the transformed plants, whereas the presence of the exogenous transgene was detected using PCR. Three transgenic tobacco plants overexpressing the salt-related gene were salt tolerant. Transgenic tobacco plants overexpressing empty pMV were used as the negative control.

Development of Transgenic Tobacco

For the germination assay, 25 seeds (per repeat) of transgenic salt-tolerant and WT tobaccos were surface sterilized using Xyz and placed on Petri plates containing 1/2 strength Murashige and Skoog (MS) basal medium supplemented with 0 and 50 mM NaCl, respectively. The Petri plates were kept in a growth chamber at 25ºC under a 16 h photoperiod for 7 days. The germination rates were recorded every day after sowing for 7 days. For root length measurements, germinated seeds were supplied with the same nutrients and grown at 0, 150, 175, and 200 mM NaCl stress for one additional week. The root length was measured after 14 days, separately from each experiment. The fresh weight of tobacco seedlings was also measured. In addition, the leaf MDA, SOD, POD, and CAT content were analyzed as described in the preceding sections.

qRT-PCR Analysis

For qPCR analysis, a 20 µl reaction volume was employed, consisting of 1 µl of cDNA template, 0.6 µl each of reverse and forward primers, 7.4 µl of ddH_2O, 0.4 µl of ROX, and 10 µl of the PCR master mix from Thermo Fisher Scientific (Waltham, MA, USA). Amplification was carried out in the ABI 7500 system, following the specified conditions: initial denaturation for 30 s at 95ºC, subsequent denaturation through 40 cycles for 5 s at 95ºC, and annealing at 60ºC for 30 s. All reactions, including control reactions, were performed in triplicate to ensure accuracy. The DNA quantity was determined using the $2^{-\Delta\Delta Ct}$ method.

Statistical Analyses

Data were analyzed using the SPSS software, version 15.0 (SPSS, Inc., Chicago, IL, United States). Differences between groups were analyzed using the Student's t-test. Statistical significance was set at $P<0.05$. Continuous normally distributed variables were expressed at mean± the Standard Deviation (SD).

Results and Discussion

Effects of Salt Stress on Germination

Salinity exerted a notable reduction in the germination rate of maize seeds, with the inhibitory impact of salt stress being more pronounced in the inbred maize line P138 compared to the inbred line 8723, as detailed in Table 1. No significant disparity in germination rates was observed between the inbred lines under non-stress conditions. Also, there was no significant difference in GR and GP of 8723 at 100 and 200 mM NaCl. However, the GR and GP of 8723 were significantly higher and lower at 100 and 200 mM NaCl than at 300 mM NaCl and under non-stress conditions, respectively. Conversely, for the P138 line, the GR, GP, and GI decreased significantly with an increase in salinity. As expected, the GR and GP of 8723 were significantly higher than P138 under the same salinity but not under non-saline conditions. The GI of both 8723 and P138 decreased significantly at high salinity.

Table 1. Effects of the salt stress on germination characteristics.

Type	Salt concentration (mM·L^{-1})	GR (%)	GP (%)	GI
8723	0	99.33±0.94 ^{a}	98.00±1.63 ^{a}	31.52±0.42 ^{a}
	100	96.67±0.94 $^{b\|*}$	95.33±0.94 $^{b\|*}$	30.58±0.56 $^{a\|*}$
	200	95.33±0.94 $^{b\|*}$	94.00±1.63 $^{b\|*}$	29.95±0.77 ^{ab}
	300	86.67±2.49 $^{c\|*}$	83.33±3.40 $^{c\|*}$	28.75±0.90 $^{b\|*}$
P138	0	100.00±0.00 ^{a}	96.67±0.94 ^{a}	31.86±0.53 ^{a}
	100	88.00±1.63 ^{b}	85.33±1.89 ^{b}	29.47±0.15 ^{b}
	200	82.67±1.89 ^{c}	82.00±1.63 ^{b}	28.18±0.81 ^{b}
	300	76.00±3.27 ^{d}	73.33±2.49 ^{d}	26.66±0.15 ^{c}

GR: germination rate; GP: germination potential; GI: Germination index.
Columns labeled with different letters are significantly different ($p<0.05$) in the same maize inbred line.
* $P<0.05$, compared with P138 at the same salt concentration.

Effects of Salt Stress on MDA, SOD, POD, and CAT Activities

MDA contents were higher in Sens than in Tol under all salinity levels. The MDA activity increased with salinity in both maize cultivars (Fig. 1 A). However, the activities of SOD, POD, and CAT reduced with salinity level (Fig. 1 B, C, and D).

Transcriptome Differences in Response to Salt Stress Between Two Maize Inbred Lines

RNA-seq generated between 47,213,066 and 77,151,924 clean reads for each library. The average percentage of Q20 and Q30 is 99.71% and 94.32, respectively (Table 2). Totally, 20,579 DEGs were identified in four comparisons: Tol-T vs. Tol-CK

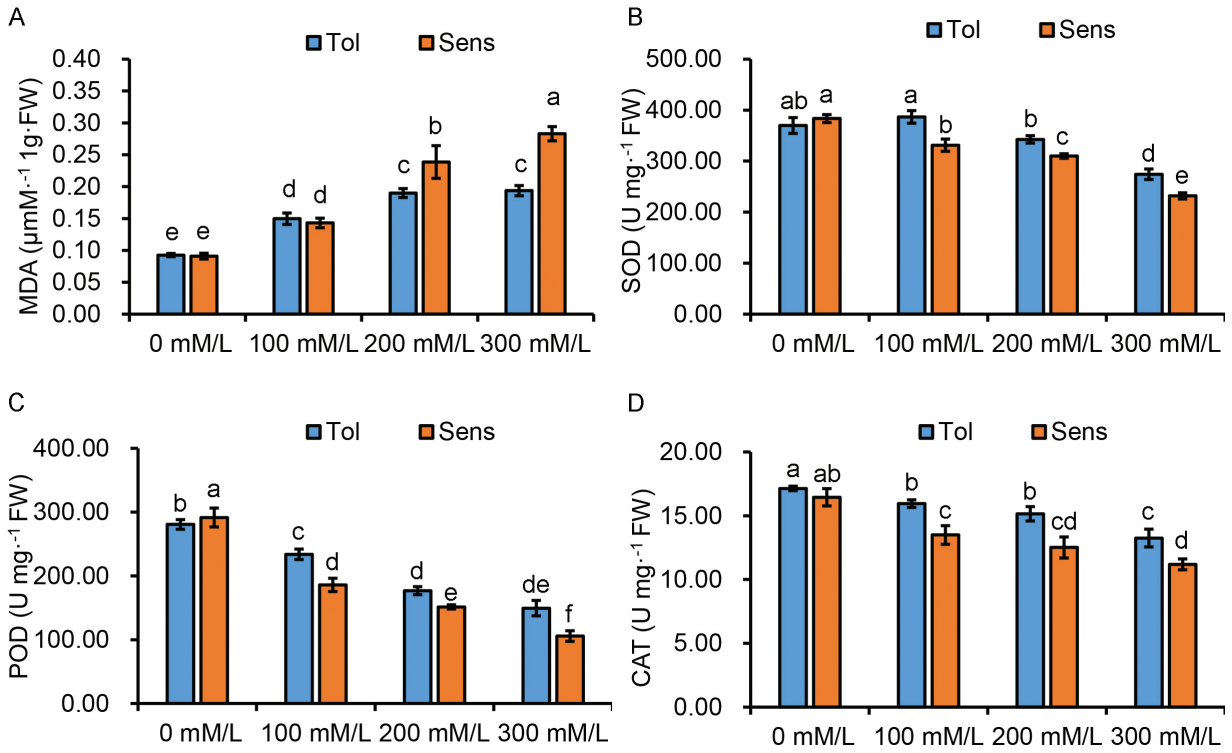

Fig. 1. Effects of salt stress on MDA, POD, SOD, and CAT activities in maize cultivars.
A, B, C, and D, the content of MDA, POD, SOD, and CAT in both maize cultivars. Tol, Salt-tolerant 8723; Sens, salt-sensitive P138. Histograms on top of which the same letter appears represent means that are not statistically different ($p>0.05$); different letters identify means that are significantly different ($p<0.05$).

Table 2. Sequence data quality control summary.

Sample	Raw Reads	Raw Bases	Clean Reads	Clean Bases	Error Rate	Q20	Q30	GC Content
8723_CK_1	62124854	8.68G	60888570	8.51G	0,02%	99,02%	94,49%	55,75%
8723_CK_2	78622158	10.98G	77151924	10.81G	0,02%	99,13%	94,75%	55,62%
8723_CK_3	61254488	8.56G	60072276	8.41G	0,02%	99,07%	94,33%	55,70%
P138_CK_1	52621482	7.35G	51516430	7.21G	0,03%	98,90%	94,06%	53,73%
P138_CK_2	48215962	6.73G	47213066	6.61G	0,03%	98,95%	94,49%	53,41%
P138_CK_3	54782136	7.65G	53664582	7.51G	0,02%	98,86%	94,37%	53,59%
8723_24_1	53621484	7.49G	52500798	7.31G	0,03%	97,89%	94,25%	54,00%
8723_48_1	54397826	7.60G	53369702	7.51G	0,02%	98,01%	94,75%	55,05%
8723_48_2	61024876	8.53G	59779968	8.41G	0,02%	98,16%	94,44%	54,33%
8723_48_3	60398520	8.44G	59184508	8.31G	0,02%	98,85%	94,49%	54,67%
P138_48_1	61782548	8.63G	60546896	8.51G	0,02%	98,83%	94,51%	55,17%
P138_48_2	66682482	9.32G	64862052	9.11G	0,03%	98,57%	92,73%	55,81%
P138_48_3	57689224	8.06G	56500824	7.91G	0,02%	98,96%	94,44%	55,41%

(2,239 up-regulated and 2,502 down-regulated), Sens-T vs. Sens-CK (5,921 up-regulated and 5,586 down-regulated), Tol-CK vs. Sens-CK (5,371 up-regulated and 4,676 down-regulated) and Tol-T vs. Sens-T (7,137 up-regulated and 6,920 down-regulated). GO and KEGG enrichment analyses were performed with all DEGs to further explore their functions. The GO enrichment results showed that, between T and CK, many up-regulated DEGs in Tol plants were enriched in "carbohydrate metabolic process" and "carbohydrate biosynthetic process"; lots of down-regulated DEGs (compared with CK) in Sens plants were enriched in "ribonucleoprotein complex biogenesis" and "photosynthesis" terms, when. Additionally, DEGs in Tol-T vs. Sens-T were significantly enriched in the "photosynthesis" term, and no significant GO terms found in the enrichment results of Tol-CK vs. Sens-CK (Fig. 2 A). The KEGG enrichment results showed that "Protein processing in endoplasmic reticulum" and "plant hormone signal transduction" were significantly enriched in Tol-T vs. Tol-CK; "Metabolic pathways", "Ribosome" and "Peroxisome" were significantly enriched in Tol-CK vs. Sens-CK; "Protein processing in endoplasmic reticulum", "Ribosome", "plant hormone signal transduction" etc. were significantly enriched in Tol-T vs. Sens-T; no pathways were significantly enriched in Sens-T vs. Sens-CK (Fig. 2 B). DEGs with high foldchange and at the core of the pathway were defined as candidate genes. Additionally, A PPI network of candidate DEGs was constructed (Fig. 2 C).

The top five genes with the highest degree in the PPI network were GRMZM2G037146_P01, GRMZM2G109814_P01, GRMZM2G110622_P01, gpm624 and bhlh103. Among the five genes, the annotation information of GRMZM2G037146_P01 and GRMZM2G109814_P01 is *ZmHSP20*. The expression of *ZmHSP20* in Tol was upregulated after salt stress treatment, but not in Sens plants. Therefore, we have reason to believe that ZmHSP20 played an important role in the salt stress tolerance of maize.

Salt Stress on ZmHSP20 Overexpressing Tobacco Seedlings

ZmHSP20 fusion protein was seen in the nucleus (Fig. 3 A), whereas the 35S-GFP (control) was distributed throughout the cells (Fig. 3 A). To test the function of *ZmHSP20*, we developed transgenic tobaccos overexpressing *ZmHSP20* (OE#1, OE#2, and OE#3). The overexpression of *ZmHSP20* in the OE#1, OE#2, and OE#3, but not in WT tobaccos, was confirmed using PCR (Fig. 3 B). To investigate the effect of *ZmHSP20* overexpression on germination, transgenic tobaccos were grown on 1/2 strength MS medium supplemented with 0 and 50 mM NaCl treatments. We found no significant GR difference between the WT and *ZmHSP20* tobacco seeds under non-saline condition (Fig. 3 C). However, the GR of tobacco seeds overexpressing *ZmHSP20* was significantly higher than that of the WT after 14 days in 100 mM NaCl stress conditions (Fig. 3 C). However, there was no significant reduction in the root length of *ZmHSP20* overexpressing seedlings and the WT under both saline and non-saline conditions (Fig. 3 D). There was also a significant difference in fresh plant weight between the WT and the *ZmHSP20* overexpression seedlings under 150, 175, as well as 200 mM NaCl stress and non-saline condition (Fig. 3 E).

The MDA content increased from 0 to 200 mM/L NaCl in WT, OE#1, OE#2, and OE#3. At the same salt stress concentration, the MDA level was higher in the WT group than in the *ZmHSP20* overexpression lines (Fig. 4 A). The SOD, POD, and CAT activity decreased

significantly in all the plant groups with an increase in salinity (Fig. 4 B, C, and D). Additionally, the activity of antioxidant enzymes was significantly higher in the *ZmHSP20* tobacco seedlings than in the WT seedlings.

Discussion

Salt stress induces the production and accumulation of reactive oxygen species (ROS), leading to irreversible oxidative damage in cells and impeding

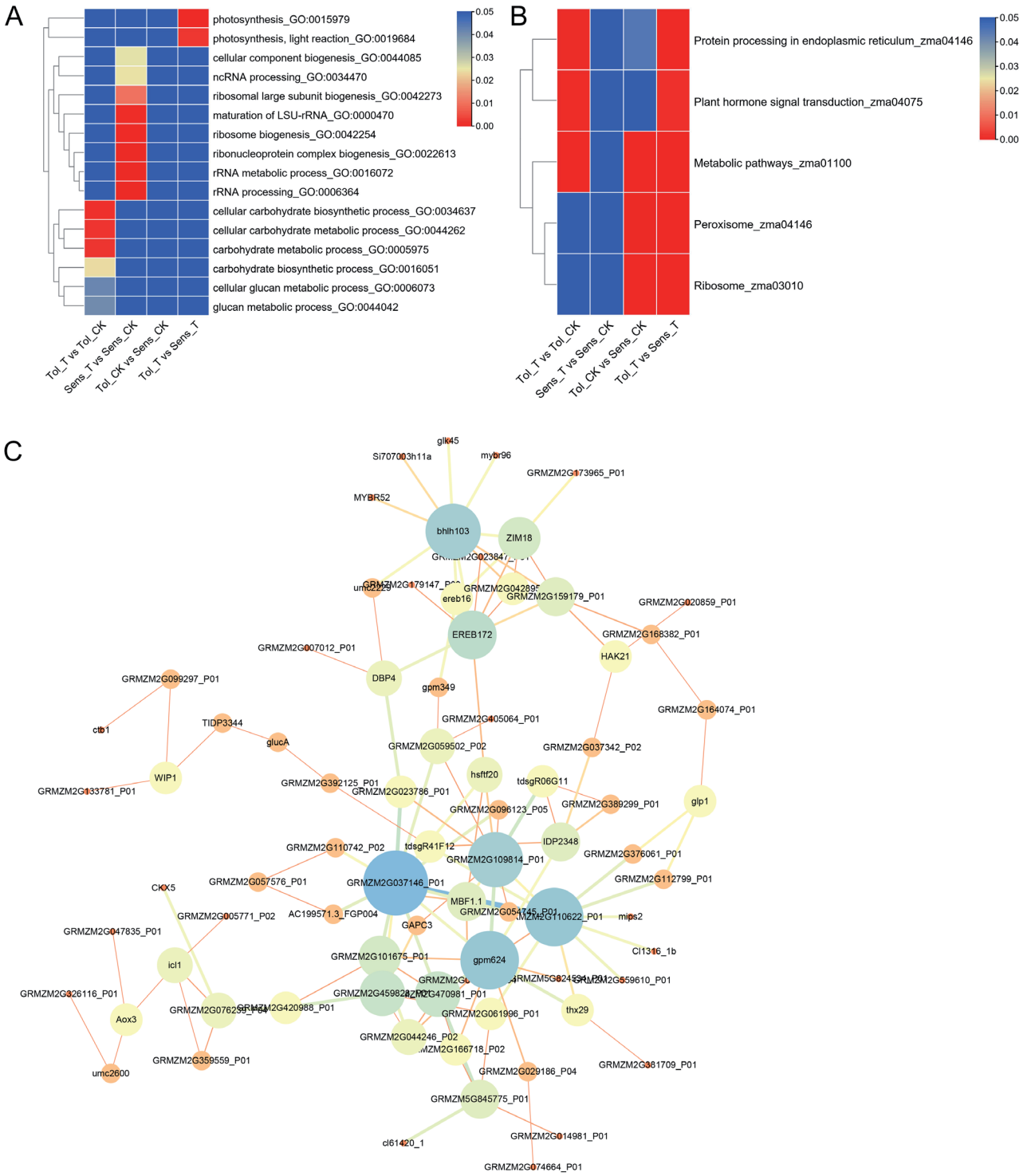

Fig. 2. Main results of transcriptome sequencing.
A and B, The GO and KEGG enrichment results. The redder the color, the smaller the significance value. Tol, Salt-tolerant 8723; Sens, salt-sensitive P138; T, salt treatment; CK, control. C, PPI network of candidate DEGs. The size of the nodes is relative to the number of connections, indicating that the more the number of connections is, the bigger is the size of the nodes.

plant growth [21]. Our findings revealed that under salinity conditions, GR, GP, GI, SOD, POD, and CAT activities were higher in Tol plants than in Sens under salinity, indicating that Tol plants respond to salt stress by increasing the activity of antioxidant enzymes. Additionally, heightened Malondialdehyde (MDA) activity suggested an increase in oxidative damage with rising salt concentration, particularly in Sens maize. Transcriptome analysis revealed that carbohydrate metabolism-related genes were differentially expressed between the Tol-T and Tol-CK groups but not in the Sens plants. Carbohydrate metabolism plays an important role in plant growth, development, and stress response [22]. Abiotic stress dysregulates the expression of genes involved in carbohydrate metabolism [23]. In our study, an upregulation of genes associated with carbohydrate metabolism was observed, likely as a response to the elevated energy demand induced by salt stress. Contrastingly, the dearth of gene abundance in the carbohydrate metabolism pathway in Sens maize suggested challenges in meeting the energy requirements to counteract salt stress.

Further interaction network analyses were performed to uncover the role of the *ZmHSP20* gene. Plants respond to biotic and abiotic stressors by regulating the expression of related genes. Many chaperones are considered HSP, one of the largest protein families in higher plants, and are essential in biotic and abiotic stress tolerance, particularly heat, drought, and salinity tolerance [24-28]. Research shows that *HSP17.0, HSP23.7*, and *HSP20* significantly enhance salt tolerance in rice [8, 25]. Overexpression of *Populus trichocarpa*

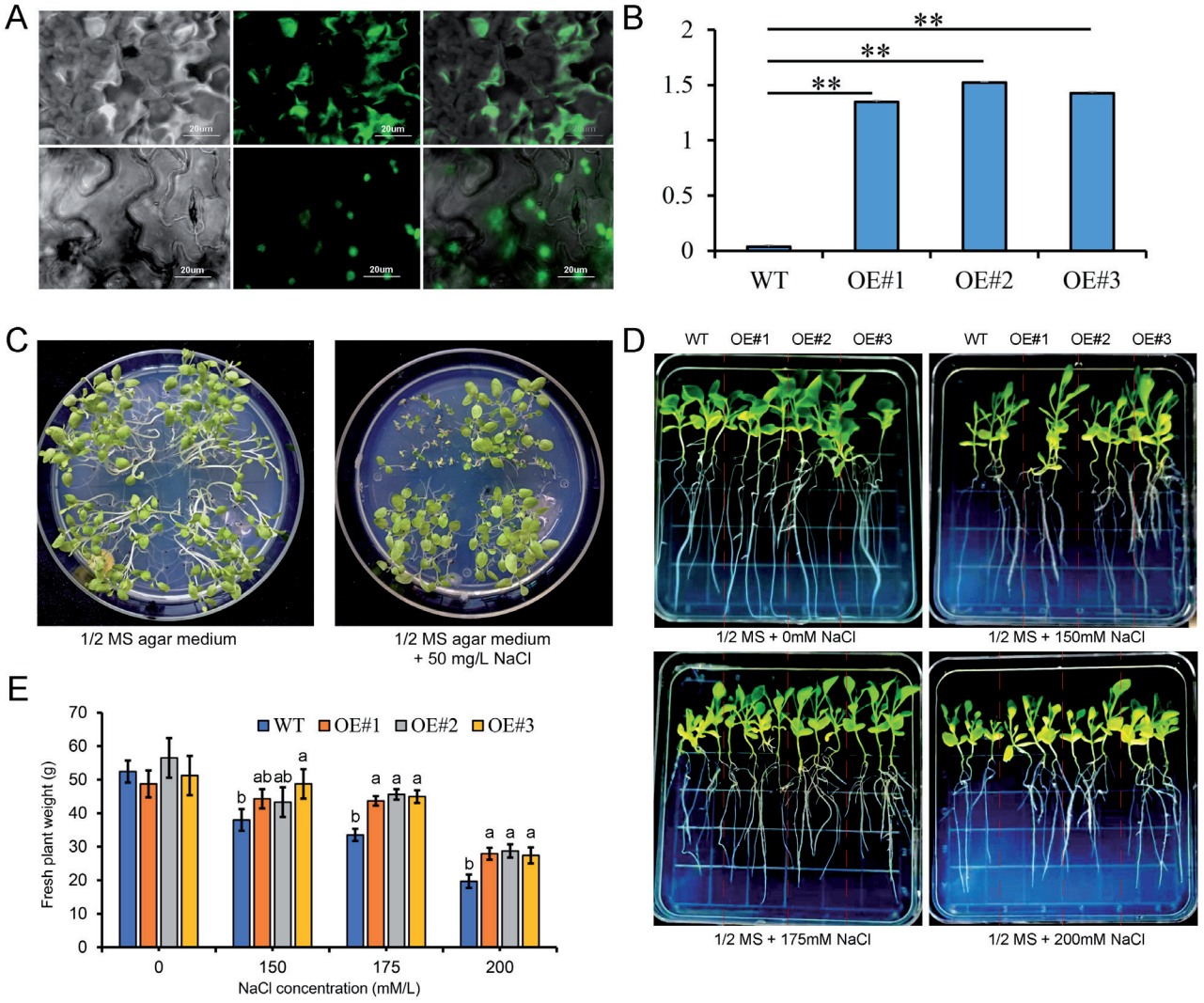

Fig. 3. Salt stress on ZmHSP20 overexpressing tobacco seedlings.
A, Subcellular localization. B, RT-qPCR results of *ZmHSP20* in tobacco lines. C, The germination rate in the WT and *ZmHSP20* tobacco seeds. D, The root length of *ZmHSP20* overexpressing seedlings and the WT under both saline and non-saline conditions. E, Fresh plant weight of WT and the *ZmHSP20* overexpression seedlings under different salt content stress; Histograms on top of which the same letter appears represent means that are not statistically different ($p>0.05$); different letters identify means that are significantly different ($p<0.05$).

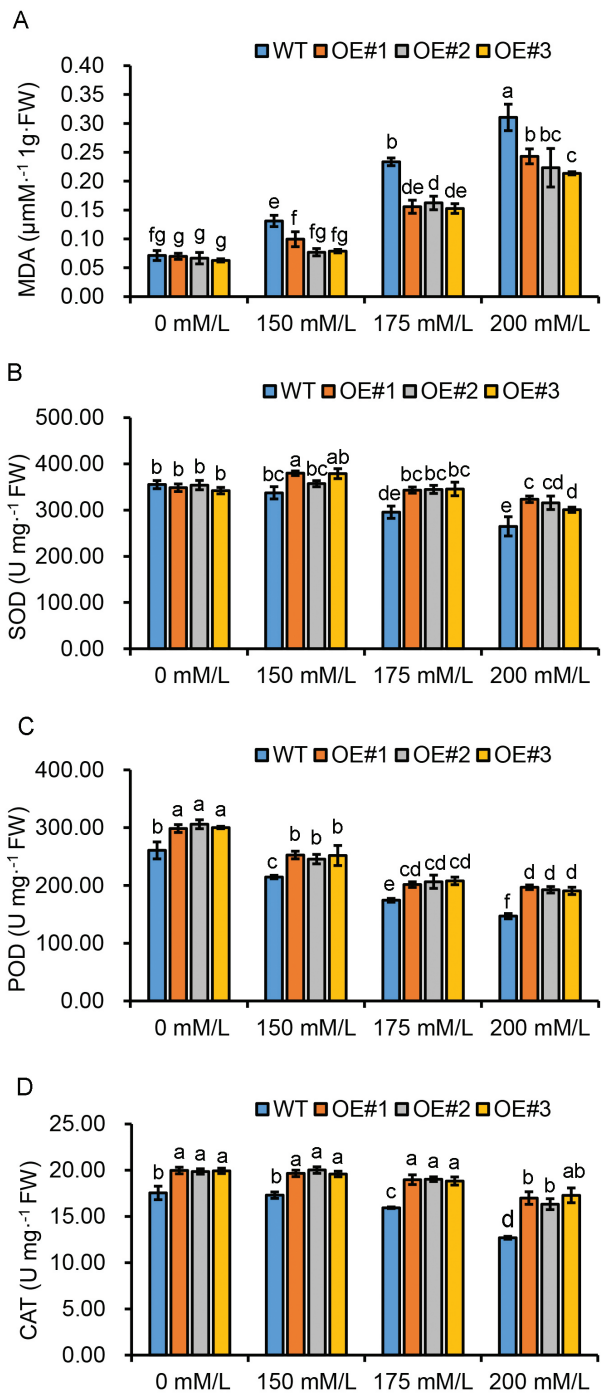

Fig. 4. Effects of salt stress on MDA, POD, SOD, and CAT activities in *ZmHSP20* overexpression tobacco lines.
A, B, C, and D, the content of MDA, POD, SOD, and CAT in both maize cultivars. WT, wild-type tobacco lines; OE#1, OE#2, and OE#3 means different *ZmHSP20* overexpression tobacco lines. Histograms on top of which the same letter appears represent means that are not statistically different ($p>0.05$); different letters identify means that are significantly different ($p<0.05$).

HSP17.8 enhances the survival rate and promotes the growth of Arabidopsis roots under salt stress [29]. In the present study, we found that salinity upregulated the expression of *ZmHSP20* in Tol but not in Sens.

Salt stress can induce oxidative stress bursts by increasing the production of ROS [30]. Meanwhile, oxidative stress induces the expression of HSPs [31]. As a heat-response protein, *AtHSFA2* overexpression enhances heat and oxidative stress tolerance in *Arabidopsis thaliana* [32]. In addition to tobacco's own *NtHSP20*, the high expression of *ZmHSP20* enhanced the salt tolerance of transgenic tobacco. Also, the MDA activity was higher in tobacco overexpressing *ZmHSP20*, implying alleviation of oxidative stress. Hence, it can be hypothesized that salt stress induces oxidative stress, but tobaccos cautioned themselves against related damages by increasing the expression of *ZmHSP20*. In addition, we found that overexpression of *ZmHSP20* increased the activity of antioxidant enzymes, consistent with previous reports [13, 33, 34]. Studies have also shown that HSP gene expression positively correlated with the activities of (oxidative stress-related enzymes) protective enzymes. Over-expression of HSP17.8 enhances the SOD activity in Arabidopsis, whereas HSP16.9 increases the activities of POD, CAT, and SOD in tobacco [35]. Post-transcriptional modification, such as alternative splicing, also regulates the expression of HSFs. Under heat stress, HSFA2 binds to its promoter region to promote its transcription in a positive auto-regulatory loop. Similarly, DREB2 regulates the expression of HSFA under stress, which regulates the expression of stress-related genes in many plants [36]. Similarly, miRNAs also play a vital role in the stress response by down-regulating the expression of stress-related genes. The transcription of certain miRNAs such as miR159, miR319, miR395, and miR402 are over-transcribed under drought, cold, salinity, hormone, and nutrient deficiency stresses [37]. Also, miR398 negatively regulates the expression of CSD1, CSD2, and CCS, increasing the expression of SOD in Arabidopsis [35]. So, the changes of miRNA cannot be ignored and should be included in our next step study.

In this study, the overexpression of *ZmHSP20* could effectively increase the activities of SOD, POD, and CAT, whether these effects occur as the consequence of ZmHSP20 impacting a single pathway or multiple pathways continues to be investigated. Although this study has obtained a large amount of data to prove that the *ZmHSP20* gene has a certain salt tolerance effect, there are still shortcomings. Further validation is required through overexpression and interference experiments in maize in the later stage.

Conclusions

Here, we identified *ZmHSP20* gene in maize seedlings that were highly expressed when subjected to salt stress. *ZmHSP20* could alleviate the oxidative stress that is induced by salt stress. Also, *ZmHSP20* might act as a crossing role of different biological processes related to resistance to stress. Overexpressing of *ZmHSP20* increased the resistance of tobacco against salt stress, and

enhanced the SOD, POD and CAT activity. In brief, our study establishes a basic foundation for further research on the function of *ZmHSP20*, and reports a preliminary exploration of the role of *ZmHSP20* resistance to salt stress. Further investigations are required to reveal the mechanism by which *ZmHSP20* regulates resistance against salt stress in maize and tobacco.

Acknowledgments

This work was supported by the Science and Technology Development Plan Project of Jilin (20220202010NC).

Data Availability Statement

Informed consent was obtained from all subjects involved in the study. All the data and code used in this study can be requested by email to the corresponding author Yi-Chen Su. Email: suliu1111@163.com.

Conflicts of Interest

The authors declare no conflict of interest.

Reference

1. MONTES-OSUNA N., GÓMEZ-LAMA CABANÁS C., VALVERDE-CORREDOR A., LEGARDA G., PRIETO P., MERCADO-BLANCO J. Evaluation of Indigenous Olive Biocontrol Rhizobacteria as Protectants against Drought and Salt Stress. Microorganisms. **9** (6), 1209, **2021**.
2. YANG Y., GUO Y. Elucidating the molecular mechanisms mediating plant salt-stress responses. New Phytol. **217** (2), 523, **2018**.
3. ALZAHRANI S.M., ALARAIDH I.A., KHAN M.A., MIGDADI H.M., ALGHAMDI S.S., ALSAHLI A.A. Identification and Characterization of Salt-Responsive MicroRNAs in Vicia faba by High-Throughput Sequencing. Genes (Basel). **10** (4), 303, **2019**.
4. ZHANG H., ZHU J., GONG Z., ZHU J.-K. Abiotic stress responses in plants. Nature Reviews Genetics. **23** (2), 104, **2022**.
5. ZHANG Y., LV Y., JAHAN N., CHEN G., REN D., GUO L. Sensing of Abiotic Stress and Ionic Stress Responses in Plants. International Journal of Molecular Sciences. **19** (11), 3298, **2018**.
6. YU Z., DUAN X., LUO L., DAI S., DING Z., XIA G. How Plant Hormones Mediate Salt Stress Responses. Trends in Plant Science. **25** (11), 1117, **2020**.
7. ARIF Y., SINGH P., SIDDIQUI H., BAJGUZ A., HAYAT S. Salinity induced physiological and biochemical changes in plants: An omic approach towards salt stress tolerance. Plant Physiology and Biochemistry. **156**, 64, **2020**.
8. GUO L.-M., LI J., HE J., LIU H., ZHANG H.-M.A class I cytosolic HSP20 of rice enhances heat and salt tolerance in different organisms. Scientific Reports. **10** (1), 1383, **2020**.
9. WANG X., ZHANG H., SHAO L.-Y., YAN X., PENG H., OUYANG J.-X., LI S.-B. Expression and function analysis of a rice OsHSP40 gene under salt stress. Genes & Genomics. **41** (2), 175, **2019**.
10. NING M., TANG F., ZHANG Q., ZHAO X., YANG L., CAI W., SHAN C. The quality of Gold Queen Hami melons stored under different temperatures. Scientia Horticulturae. **243**, 140, **2019**.
11. ZHANG X., LIU P., QING C., YANG C., SHEN Y., MA L. Comparative transcriptome analyses of maize seedling root responses to salt stress. PeerJ. **9**, e10765, **2021**.
12. CHEN F., JI X., ZHUANG Z., PENG Y. Integrated Transcriptome and Proteome Analyses of Maize Inbred lines in Response to Salt Stress. Agronomy. **12** (5), 1053, **2022**.
13. UL HAQ S., KHAN A., ALI M., KHATTAK A.M., GAI W.-X., ZHANG H.-X., WEI A.-M., GONG Z.-H. Heat Shock Proteins: Dynamic Biomolecules to Counter Plant Biotic and Abiotic Stresses. International Journal of Molecular Sciences. **20** (21), 5321, **2019**.
14. CHEN F., FANG P., PENG Y., ZENG W., ZHAO X., DING Y., ZHUANG Z., GAO Q., REN B. Comparative Proteomics of Salt-Tolerant and Salt-Sensitive Maize Inbred Lines to Reveal the Molecular Mechanism of Salt Tolerance. International Journal of Molecular Sciences. **20** (19), 4725, **2019**.
15. SUN Y., XU J., MIAO X., LIN X., LIU W., REN H. Effects of exogenous silicon on maize seed germination and seedling growth. Scientific Reports. **11** (1), 1014, **2021**.
16. RAHMAN M.M., MOSTOFA M.G., DAS A.K., ANIK T.R., KEYA S.S., AHSAN S.M., KHAN M.A.R., AHMED M., RAHMAN M.A., HOSSAIN M.M., TRAN L.P. Ethanol Positively Modulates Photosynthetic Traits, Antioxidant Defense and Osmoprotectant Levels to Enhance Drought Acclimatization in Soybean. Antioxidants (Basel). **11** (3), 516, **2022**.
17. LIANG Y., LI X., ZHANG D., GAO B., YANG H., WANG Y., GUAN K., WOOD A. J. ScDREB8, a novel A-5 type of DREB gene in the desert moss Syntrichia caninervis, confers salt tolerance to Arabidopsis. Plant Physiol Biochem. **120**, 242, **2017**.
18. WEI M., LI X., YANG R., LI L., WANG Z., WANG X., SHA A. Novel Insights Into Genetic Responses for Waterlogging Stress in Two Local Wheat Cultivars in Yangtze River Basin. Frontiers in Genetics. **12**, 681680, **2021**.
19. KHALIL-UR-REHMAN M., SUN L., LI C.-X., FAHEEM M., WANG W., TAO J.-M. Comparative RNA-seq based transcriptomic analysis of bud dormancy in grape. BMC Plant Biology. **17** (1), 18, **2017**.
20. LIAN C., LI Q., YAO K., ZHANG Y., MENG S., YIN W., XIA X. Populus trichocarpa PtNF-YA9, A Multifunctional Transcription Factor, Regulates Seed Germination, Abiotic Stress, Plant Growth and Development in Arabidopsis. Frontiers in Plant Science. **9**, 954, **2018**.
21. ZHANG J., LIU D., ZHU D., LIU N., YAN Y. Endoplasmic Reticulum Subproteome Analysis Reveals Underlying Defense Mechanisms of Wheat Seedling Leaves under Salt Stress. International Journal of Molecular Sciences. **22** (9), 4840, **2021**.
22. MA J., RUKH G., RUAN Z., XIE X., YE Z., LIU D. Effects of Hypoxia Stress on Growth, Root Respiration, and Metabolism of Phyllostachys praecox. Life (Basel). **12** (6), 808, **2022**.
23. ARABZADEH N., KHAVARI-NEJAD R.A. Effect of drought stress on superoxide dismutase activity in two

species of Haloxylon aphyllum and Haloxylon persicum. Pakistan Journal of Biological Sciences. **16** (8), 351, **2013**.
24. WANG X., ZHANG H., SHAO L.Y., YAN X., PENG H., OUYANG J.X., LI S.B. Expression and function analysis of a rice OsHSP40 gene under salt stress. Genes Genomics. **41** (2), 175, **2019**.
25. ZOU J., LIU C., LIU A., ZOU D., CHEN X. Overexpression of OsHsp17.0 and OsHsp23.7 enhances drought and salt tolerance in rice. Journal of Plant Physiology. **169** (6), 628, **2012**.
26. YU X., ZHAO Y., WANG Y., ZOU Z., LI F. Maize ZmHSP90 plays a role in acclimation to salt stress. PeerJ. **11**, e15819, **2023**.
27. DO J.-M., KIM H.-J., SHIN S.-Y., PARK S.-I., KIM J.-J., YOON H.-S. OsHSP 17.9, a Small Heat Shock Protein, Confers Improved Productivity and Tolerance to High Temperature and Salinity in a Natural Paddy Field in Transgenic Rice Plants. Agriculture. **13** (5), 931, **2023**.
28. UL HAQ S., KHAN A., ALI M., KHATTAK A.M., GAI W.X., ZHANG H.X., WEI A.M., GONG Z.H. Heat Shock Proteins: Dynamic Biomolecules to Counter Plant Biotic and Abiotic Stresses. International Journal of Molecular Sciences. **20** (21), 5321, **2019**.
29. LI J., ZHANG J., JIA H., LI Y., XU X., WANG L., LU M. The Populus trichocarpa PtHSP17.8 involved in heat and salt stress tolerances. Plant Cell Reports. **35** (8), 1587, **2016**.
30. CHOUDHURY F.K., RIVERO R.M., BLUMWALD E., MITTLER R. Reactive oxygen species, abiotic stress, and stress combination. Plant Journal. **90** (5), 856, **2017**.
31. KUMAR R.R., RAI G.K., KOTA S., WATTS A., SAKHARE A., KUMAR S., GOSWAMI S., KAPOOR N., BABU P., MISHRA G.P., KUMAR S.N., CHINNUSAMY V., PRAVEEN S. Fascinating Dynamics of Silicon in alleviation of heat stress Induced oxidative damage in plants. Plant Growth Regulation. **100** (2), 321, **2023**.
32. JACOB P., HIRT H., BENDAHMANE A. The heat-shock protein/chaperone network and multiple stress resistance. Plant Biotechnology Journal. **15** (4), 405, **2017**.
33. ERGIN S., GüLEN H., KESICI M., TURHAN E., IPEK A., KöKSAL N. Effects of high temperature stress on enzymatic and nonenzymaticantioxidants and proteins in strawberry plants. Turkish Journal of Agriculture and Forestry. **40** (6), 908, **2016**.
34. HAO HAI-BO H.J.-C., WANG Q., JUAN J.-X., XIAO T.-T., SONG X.-X., CHEN H., ZHANG J.-J. Effects of heat stress on the differential expression of antioxidant enzymes and heat shock protein genes of Agaricus bisporus. Mycosystema. **40** (3), 616, **2021**.
35. DRIEDONKS N., XU J., PETERS J.L., PARK S., RIEU I. Multi-Level Interactions Between Heat Shock Factors, Heat Shock Proteins, and the Redox System Regulate Acclimation to Heat. Frontiers in Plant Science. **6**, 999, **2015**.
36. LALOUM T., MARTíN G., DUQUE P. Alternative Splicing Control of Abiotic Stress Responses. Trends in Plant Science. **23** (2), 140, **2018**.
37. ISLAM W., WAHEED A., NAVEED H., ZENG F. MicroRNAs Mediated Plant Responses to Salt Stress. Cells. **11** (18), 2806, **2022**.