

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±2°C, 12/11 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 -80°C 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 4°C, 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 mRNA 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 (fold change)| > 2 with q-value < 0.01 was selected as simulated biological

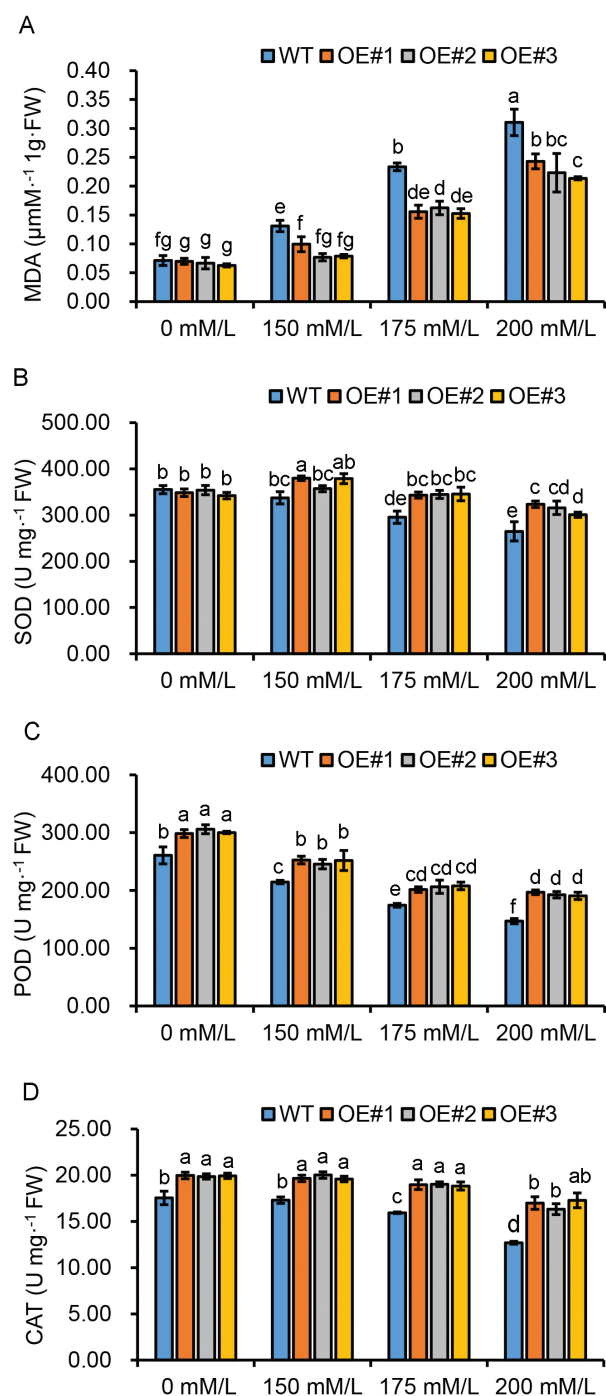


Fig. 4. Effects of salt stress on MDA, POD, SOD, and CAT activities in *ZmHSP20* overexpression tobacco lines. A, B, C, and D, the content of MDA, POD, SOD, and CAT in both maize cultivars. WT, wild-type tobacco lines; OE#1, OE#2, and OE#3 means different *ZmHSP20* overexpression tobacco lines. Histograms 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, *AtHSPA2* 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, HSPA2 binds to its promoter region to promote its transcription in a positive auto-regulatory loop. Similarly, DREB2 regulates the expression of HSPA 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

- 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**.
- YANG Y., GUO Y. Elucidating the molecular mechanisms mediating plant salt-stress responses. *New Phytol*. **217** (2), 523, **2018**.
- ALZHRANI S.M., ALARAIHD 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**.
- ZHANG H., ZHU J., GONG Z., ZHU J.-K. Abiotic stress responses in plants. *Nature Reviews Genetics*. **23** (2), 104, **2022**.
- 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**.
- 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**.
- 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**.
- 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**.
- 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**.
- 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**.
- 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**.
- 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**.
- 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**.
- 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**.
- 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**.
- 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**.
- 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**.
- 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**.
- 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**.
- 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**.
- 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**.
- 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**.
- 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., BENDAHDANE 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 nonenzymatic antioxidants 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., MARTIN 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**.