Received: 7 September 2021 | Revised: 16 November 2021 | Accepted: 23 November 2021

DOI: 10.1111/ppl.13606

ECOPHYSIOLOGY, STRESS AND ADAPTATION

Physiologia Plantarum

Joint GWAS and WGCNA uncover the genetic control of calcium accumulation under salt treatment in maize seedlings

Tianhu Liang | Chunyan Qing | Peng Liu | Chaoying Zou | Guangsheng Yuan | Guangtang Pan | Yaou Shen | Langlang Ma

Key Laboratory of Biology and Genetic Improvement of Maize in Southwest Region, Maize Research Institute, Sichuan Agricultural University, Chengdu, China

Correspondence
Langlang Ma, Key Laboratory of Biology and Genetic Improvement of Maize in Southwest Region, Maize Research Institute, Sichuan Agricultural University, Chengdu 611130, China.
Email: sxyljxml@163.com

Funding information
National Nature Science Foundation of China, Grant/Award Numbers: 31871637, 32101777; Sichuan Science and Technology Program, Grant/Award Numbers: 2021JDTD0004, 2021YJ0476

Edited by: B. Huang

Abstract

Soil salinization is an important factor threatening the yield and quality of maize. Ca^{2+} plays a considerable role in regulating plant growth under salt stress. Herein, we examined the shoot Ca^{2+} concentrations, root Ca^{2+} concentrations, and transport coefficients of seedlings in an association panel composed of 305 maize inbred lines under normal and salt conditions. A genome-wide association study was conducted by using the investigated phenotypes and 46,408 single-nucleotide polymorphisms of the panel. As a result, 53 significant SNPs were specifically detected under salt treatment, and 544 genes were identified in the linkage disequilibrium regions of these SNPs. According to the expression data of the 544 genes, we carried out a weighted coexpression network analysis. Combining the enrichment analyses and functional annotations, four hub genes (*GRMZM2G051032*, *GRMZM2G004314*, *GRMZM2G421669*, and *GRMZM2G123314*) were finally determined, which were then used to evaluate the genetic variation effects by gene-based association analysis. Only *GRMZM2G123314*, which encodes a pentatricopeptide repeat protein, was significantly associated with Ca^{2+} transport and the haplotype G-CT was identified as the superior haplotype. Our study brings novel insights into the genetic and molecular mechanisms of salt stress response and contributes to the development of salt-tolerant varieties in maize.

JEL CLASSIFICATION
Ecophysiology, stress and adaptation

1 | INTRODUCTION

Soil salinization is a major environmental factor restricting plant growth and crop productivity (Hanin et al., 2016; Yang & Guo, 2018). It is estimated that, excluding arid and desert areas, 20% of the world's irrigated land is currently affected by salinization (Yamaguchi & Blumwald, 2005). Salinization affects several physiological and metabolic processes. For instance, it causes osmotic stress and ionic toxicity to plants, and reduces photosynthetic rate and stomatal conductance, and even affects starch metabolism and nitrogen fixation. Under salt stress, plants have developed a variety of coping strategies, such as stomatal regulation, ionic homeostasis, hormonal equilibrium, antioxidant activation, osmotic adjustment, and tissue water status maintenance (de Azevedo Neto et al., 2006; Hichem et al., 2009; Jafar et al., 2012; Kaya et al., 2014; Neubert et al., 2005). As one of the major cereal crops, maize plays an important role in agriculture, food, and industry (Ranum et al., 2014), but suffers from salt stress. Therefore, enhancing the salt tolerance of maize is an urgent work for maize breeders.

In the past few decades, a large number of researches have been carried out to decode the molecular mechanism of salt tolerance in plants. The HKT (high-affinity K^+ transporter) pathway is widely accepted to be involved in salt stress. Some members of the HKT family reduce the Na^+ accumulation in leaves via controlling the shoot-

root Na^+ translocation (Berthomieu et al., 2003; Munns & Tester, 2008; Ren et al., 2005). Additionally, fluctuations in plant hormones, such as auxin, ethylene, and abscisic acid (ABA), correlate with the regulation of salt stress responses (Bahieldin et al., 2016; Duan et al., 2013; Ryu & Cho, 2015).

Ca^{2+} is a central regulator in plant growth and development. Numerous pieces of evidences have revealed the importance of Ca^{2+} signaling in plant response to abiotic stimuli. The Ca^{2+} concentration in plant cytoplasm increased under high salinity, low/high temperature, phytohormone, and other abiotic or biotic stresses (Hepler, 2005; Sanders et al., 2002; Zhu, 2016). Under salt stress, plants activate various signaling pathways, including those involving Ca^{2+}, which promote sufficient cellular cascade reaction (Zhu, 2002). The salt-overly-sensitive (SOS) pathway plays a crucial role in regulating salt stress response, and it facilitates the understanding of the Ca^{2+} signatures mediated by salt stress (Chinnusamy et al., 2004; Mahajan et al., 2008). In the SOS pathway, the genes *SOS1*, *SOS2*, and *SOS3* separately encode Na^+/H^+ antiporter (NHXs), serine/threonine protein kinase, and calcium-binding protein with EF-hand motifs. Under salt stress, *SOS3* senses a surge of Ca^{2+} triggered by excessive Na^+ entering the cytoplasm (Halfter et al., 2000; Ji et al., 2013). It activates *SOS2* to form a *SOS2-SOS3* complex (Liu et al., 2000), which then activates the C-terminal region of *SOS1* by phosphorylation. The activated *SOS1* pumps Na^+ out of the cells in exchange for H^+ (Min et al., 2016; Shi et al., 2002). Other proteins also play important roles in the SOS pathway, such as MITOGEN-ACTIVATED PROTEIN KINASE 6 (MPK6) and SOS3-like CALCIUM BINDING PROTEIN 8 (SCaBP8)/CBL10, which activate SOS1 directly or indirectly (Quan et al., 2007). Under salt stress, the spikes of Ca^{2+} concentration in root cell cytoplasm activate SOS signal transduction cascade to avoid Na^+ toxicity (Liu et al., 2000).

To date, many quantitative trait loci (QTL) related to salt response have been identified in plants. In rice, *SKC1* was the first cloned QTL, which encodes an HKT-like protein and maintains Na^+/K^+ homeostasis under salt stress (Ren et al., 2005). Ren et al. (2010) reported that a QTL, *RSA1*, plays a negative role in modulating the rice seed germination and early seedling growth under salt stress. Under saline conditions, a major QTL controlling plant height was mapped using a double haploid population consisting of 240 maize individuals and 1317 single nucleotide polymorphisms (SNPs; Luo et al., 2017). Another maize salt-tolerance QTL, *ZmNC1* (*Zea mays* Na^+ *CONTENT 1*), was cloned from a recombinant inbred line population derived from the cross of Zheng58 and Chang7-2. *ZmNC1* encodes an HKT-type transporter (designated as *ZmHKT1*) and positively mediates maize salt tolerance (Zhang et al., 2018). With the advances in resequencing technology, the development of DNA markers has become more efficient and cheaper, which promotes the extensive applications of genome-wide association analysis (GWAS; Elshire et al., 2011; Xiao et al., 2017). In the past decades, GWAS has been widely used in the identification of the causal genes conferring plant salt tolerance. In *Arabidopsis*, two genes (*CYP79B2* and *HKT1*) controlling the growth and development of lateral roots were detected by GWAS under salt exposure (Julkowska et al., 2017). Using GWAS, 70 candidate genes were identified for tissue cation concentration based on 306 rice accessions (Patishtan et al., 2018). Furthermore, a total of 14 germination-associated candidate genes were identified using GWAS in alfalfa (*Medicago sativa* L.) under different levels of salt treatment. Among them, several loci were situated within the salt-tolerant QTL detected in previous studies (Yu et al., 2016).

As a regulation network analysis method, WGCNA has become a popular technique in discovering key factors related to target traits (Childs et al., 2011). Using WGCNA, two hub genes were identified and further verified to regulate the element accumulations in maize (Schaefer et al., 2018). Seven priority genes associated with seminal root length were detected in maize seedlings under drought stress by WGCNA (Guo et al., 2020). In this study, we firstly performed GWAS to identify the significant SNPs and candidate genes correlated with Ca^{2+} concentrations in an association panel consisting of 305 lines under salt stress. Subsequently, the expression values of the candidate genes were used to conduct WGCNA for excavating the hub genes. Finally, each hub genes were PCR-amplified in 70 lines and the variation loci obtained from each gene were subjected to gene-based association analysis. The objective of this study was to uncover the hub genes controlling Ca^{2+} concentrations under salt treatment and identify the favorable haplotypes of the hub genes for cultivating the maize salt-tolerant varieties by molecular marker-assisted selection (MAS) breeding.

2 | MATERIALS AND METHODS

2.1 | Plant materials and growth conditions

An association panel composed of 305 maize (*Zea mays*) inbred lines was used for the phenotypic investigation. All lines were come from the breeding program of Southwest China (Zhang et al., 2016). Each line grew under salt and control conditions as follows: seeds with uniform size were selected and soaked in 10% H_2O_2 for 15–20 min, then washed thrice with deionized water, and dipped in saturated $CaSO_4$ for 8 h to accelerate germination. Subsequently, seeds were sown in seeding trays and germinated in an artificial climate chamber, as described in our previous study (Ma et al., 2021). When the maize seedlings had three leaves, they were evenly divided into two groups (each group contained eight seedlings) and respectively cultivated in Hoagland nutrient solution (Control) and Hoagland nutrient solution supplemented with 150 mmol/L NaCl (Salt stress) (Abdel-Ghani et al., 2012). After 7 days, shoots and roots were separated and dried in an oven at 80°C for 72 h. This experiment was carried out with three biological repetitions (eight seedlings per group per repetition).

2.2 | Determination of Ca^{2+} concentrations

Approximately 0.2 g powder for each sample was digested by a microwave digestion system (MARS6). Inductively-coupled plasma mass spectrometer (ICP-MS, NexION 2000, PerkinElmer Inc.) was used to

measure the concentration of Ca^{2+}. The experiment was conducted with three biological replicates.

2.3 | Statistical analysis of phenotypes

Descriptive statistical analysis of phenotypic data was performed using SPSS 25.0 (Statistical Product and Service Solutions, Version 25.0, IBM). The difference in the phenotypic values of each trait between salt stress and control conditions was analyzed by a *t*-test. SAS 9.3 (Statistical Analysis System, Version 9.3, SAS Institute) was used to calculate the broad-sense heritability (H^2), and the calculation model was $H^2 = \sigma^2_G/\sigma^2_P$, $\sigma^2_G = ([MSG\text{-}MSE]/rep)$, $\sigma^2_P = \sigma^2_G + MSE$ (Ma et al., 2020; Pace et al., 2015). Here, σ^2_G is the genotypic variance of the association panel, σ^2_P is the phenotypic variance, MSG denotes the mean square of genotype, MSE represents the mean square of error, and rep denotes the number of independent replicates.

2.4 | Genome-wide association study

In our previous study, the 305 inbred lines were genotyped using the Maize SNP50K Bead Chip containing 56,110 SNPs based on the maize reference genome (B73 RefGen v2; Andorf et al., 2010; Zhang et al., 2016). The SNPs with missing data >20%, the minor allele frequency <0.05 or the heterozygosity >20% were removed (Ma et al., 2018). The remaining 46,408 high-quality SNP markers were finally used for GWAS. The average values among the three biological replicates for each trait were considered as the phenotypic values in GWAS. The fixed and random model Circulating Probability Unification (FarmCPU) model combines the advantages of the mixed linear model and fixed-effect model; thus, it was used to perform GWAS in this study. The FarmCPU model was executed by the FarmCPU in R package (Liu et al., 2016) in the R statistical software v4.0.3. Because a Bonferroni correction ($0.05/46408 = 1.08 \times 10^{-6}$) was too conservative, a less stringent threshold of $-\log_{10}(p)$ >4 was used to detect significant SNPs in this study (Li, Liu, et al., 2020). Moreover, all the gene models located within the linkage disequilibrium (LD) regions of the significant SNPs were extracted as potential candidate genes underlying the target traits.

2.5 | Weighted coexpression network analysis

In our previous study, two maize inbred lines L2010-3 (a salt-tolerant line) and BML1234 (a salt-sensitive line) at two-leaf stage were cultured in Hoagland's solutions (control) and Hoagland's solutions supplemented with 150 mM NaCl (salt treatment). At four stages (0, 6, 18, and 36 h), the roots of L2010-3 and BML1234 were separately collected for transcriptome sequencing (Zhang et al., 2021). The gene expression data were stored in the National Genomics Data Center (NGDC) database with the accession number CRA003872. Herein, the expression values of the candidate genes detected from GWAS were used to construct gene coexpression networks by using the WGCNA package in R software (Langfelder & Horvath, 2008). The parameters of WGCNA were set as follows: power = 5, deepSplit = 2, minModuleSize = 20, and mergeCutHeight = 0.2. Gene ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were implemented using a free online platform (http://www.omicshare.com/tools) for determining the key modules. In the key module, the identification of hub genes was based on the following principles: (1) the eigengene connectivity (KME) value >0.9; (2) the weight value (TOM) >0.2; (3) the genes obtained from the above two principles and whose functional annotations associated with salt tolerance in plant species. The gene regulatory network in the key module was drawn by the Cytoscape software (Shannon et al., 2003).

2.6 | Candidate gene association study

The hub genes identified in WGCNA were further subjected to candidate gene association study. For each gene, the gene region and its promoter sequence (upstream 2000 bp) were amplified by PCR in the 70 randomly selected lines from the association panel. The amplification sequences were assembled by the DNAMAN (version 5.2.2, Lynnon Bio-soft) and saved in Hapmap format. The variations (SNPs and InDels) were filtered with the criteria of minor allele frequency ≥0.05. The retained variations were used to detect the associations with Ca^{2+}-related traits by the GLM model in TASSEL5.0 (Pace et al., 2015). The significant threshold was set as: *P*-value <0.05/n (n denotes the number of variations). Haplotypes were calculated using the Haploview software (Luo et al., 2019). The phenotypic differences between haplotypes were analyzed using a *t*-test. The B73 genome (RefGen v4; Jiao et al., 2017) was used as the reference genome for gene functional annotations.

3 | RESULTS

3.1 | Evaluation of Ca^{2+} concentration in diverse maize germplasms

Under control and salt stress conditions, six Ca^{2+}-related traits were collected in this study. These were shoot and root Ca^{2+} concentration under control conditions (SCaC and RCaC, respectively), shoot and root Ca^{2+} concentration under salt treatment (SCaS and RCaS, respectively), and Ca^{2+} transport coefficient under control (CTC, SCaC/RCaC) or salt stress (STC, SCaS/RCaS). Among the association panel, considerable variations of the six traits were observed, with the coefficient of variation (CV) ranging from 0.78 to 1.22 (Table S1). Significant differences (*P* <0.001) in Ca^{2+} concentration in both roots and shoots were observed between control and salt stress conditions (Figure 1), indicating that the salt treatment was effective in the association panel. Under salt treatment, Ca^{2+} concentration in shoots decreased by 24.4%, whereas Ca^{2+} concentration in roots increased

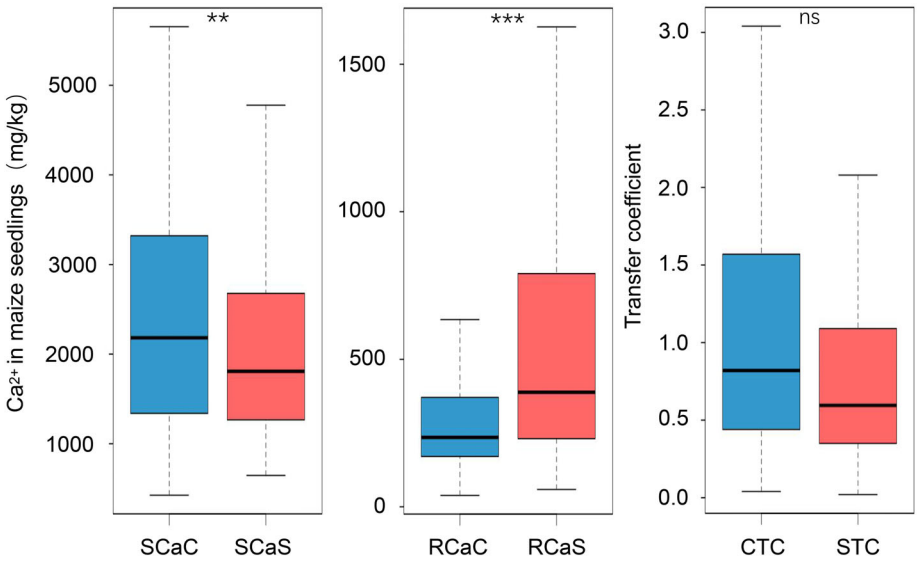

FIGURE 1 Phenotypic variations of the six Ca^{2+}-related traits. CTC, Ca^{2+} transport coefficients under control; SCaC, shoot Ca^{2+} concentration under control; SCaS, shoot Ca^{2+} concentration under salt treatment; STC, Ca^{2+} transport coefficients under salt treatment; RCaC, root Ca^{2+} concentration under control; RCaS, root Ca^{2+} concentration under salt treatment. These traits were collected in 305 lines from the association panel. The data are based on three independent biological replicates. Statistical significance was determined by two-sided *t*-test: $^{**}P$ <0.01; $^{***}P$ <0.001; ns, not significant

by 103.5%. Phenotype frequency distributions of all the six traits approximately displayed normal distributions. The heritability estimates of the six traits ranged from 0.40 to 0.83 (Table S1). Under control conditions, SCaC and CTC showed a significantly (P <0.001) positive correlation. Under salt stress, the highest positive correlation was observed between SCaS and RCaS (Table S2).

3.2 | Genome-wide association study

We performed GWAS with the FarmCPU model using 46,408 high-quality SNP markers. In control, 7, 23, and 4 SNPs were significantly associated with SCaC, RCaC, and CTC at the *P*-value threshold of 1×10^{-4}, respectively (Figure S1; Table S3). A total of 53 significant associations distributed on all 10 chromosomes were detected under salt stress, with 28 for SCaS, 13 for RCaS, and 12 for STC (Figure 2; Table S3). Among the 53 significant SNPs, 17 were located within the salt tolerance-associated QTL previously reported (Table 1). No overlaps of the significant SNPs were identified between control and salt conditions, suggesting these SNPs identified in salt treatment were specifically responsive to salt stress. Our previous study indicated that the mean LD decay was 300 kb across all chromosomes ($r^2 = 0.1$; Zhang et al., 2016). Thus, the gene models situated within 300 kb flanking regions of the significant SNPs were considered as candidate genes for target traits. Finally, we detected 401 and 544 candidate genes (B73 RefGen_v4) in the control and salt stress conditions, respectively (Tables S4 and S5). In control, 92, 264, and 45 genes were respectively found to be associated with SCaC, RCaC, and CTC (Table S4). Under salt stress, 233, 188, and 158 genes involved in SCaS, RCaS, and STC were uncovered (Table S5). Among the genes detected under salt stress, many were associated with abiotic/salt stress based on the functional annotations, such as *AC198937.4_FG005*, *GRMZM2G451792*, *GRMZM2G310144*, *GRMZM2G172053*, and *GRMZM2G340084*. Collectively, these findings demonstrate the reliability of the traits-associated SNPs detected by GWAS in the present study.

3.3 | Distribution of superior alleles in maize elite lines

As the parents of commercialized hybrid varieties, 30 elite inbred lines with excellent agronomic traits were included in our association panel. Therefore, we evaluated the utilization of the superior alleles in maize breeding. Herein, the allele associated with a higher phenotype value was considered as the superior allele for SCaS, RCaS, and STC. The percentages of superior alleles at each significant SNP ranged from 0% to 100% among the 30 elite inbred lines (Figure 3). In the 53 associated loci, the superior allele proportions at nine SNP loci (PZE-108038765, SYN8213, SYN19207, PZE-101161747, PZE-103088143, PZE-101161871, PZE-104077648, PZE-103026263, and SYN26458) were greater than 50% (Figure 3). Strikingly, PZE-108038765, SYN8213, SYN19207, and PZE-101161747 had the superior allele ratio ≥90%. The remaining 44 SNPs contained ≤30% superior alleles in the elite lines, of which five (PZE-109110551, PZE-107026578, SYN16403, PZE-105127202, and SYN14434) had no superior allele in any elite lines (Figure 3). In addition, the utilization rate of superior alleles was insufficient in the 30 elite lines with ratios ranging from 15.1% to 35.9% (Figure 3). Consequently, for breeders, more superior alleles can be integrated into these elite lines to promote the cultivation of maize salt-tolerant varieties in the future.

3.4 | Weighted coexpression network analysis

To understand the regulatory network of Ca^{2+} under salt stress, we performed a WGCNA using the expression data of the 544 genes from the transcriptome data identified in salt stress. A total of four modules (blue, brown, yellow, and turquoise) were identified, with the number of genes ranging from 22 to 163 (Figure 4). To clarify the biological meaning of gene coexpression networks, GO enrichment and KEGG pathway analyses were performed for the genes in each module. GO enrichment analysis revealed that most genes were enriched in cellular process, response to stimulus, metabolic process, catalytic

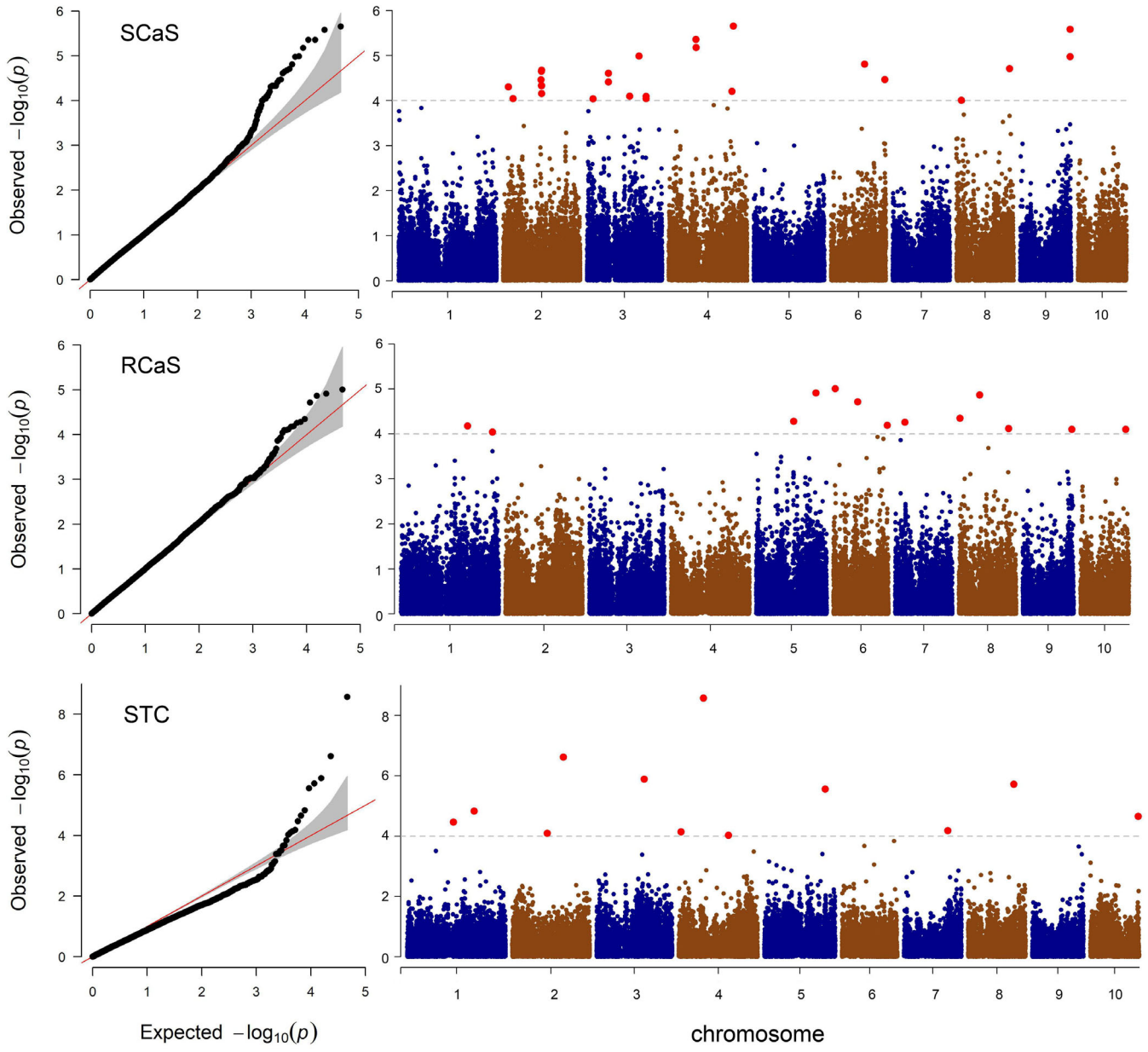

FIGURE 2 Quantile–quantile (Q–Q) and Manhattan plots from the GWAS results for salt tolerance. Q–Q and Manhattan plots for salt-tolerance phenotypes when SCaS, RCaS, and STC values were used, respectively. The dashed lines indicate the significant threshold ($P = 1 \times 10^{-4}$). The significant SNPs are shown in red

activity, membrane, and cell part (Figure 5). KEGG pathway analysis suggested that the genes in the blue, brown, yellow, and turquoise modules were significantly (P <0.05) enriched in seven (pentose phosphate pathway, amino acids biosynthesis, monobactam biosynthesis, vitamin metabolism, carbon metabolism, lysine biosynthesis, and O-glycan biosynthesis), three (ribosome, carotenoid biosynthesis, and ribosome biogenesis), two (sphingolipid metabolism and glycosphingolipid biosynthesis), and eight (fatty acid metabolism, biosynthesis of unsaturated fatty acids, fatty acid biosynthesis, cysteine and methionine metabolism, taurine metabolism, peroxisome, cutin, suberine and wax biosynthesis, and chlorophyll metabolism) pathways, respectively (Figure S2). Notably, several pathways enriched in the turquoise module were previously reported to participate in the salt stress response. For example, the *CYSTM3* (a cysteine-rich transmembrane module member), *BvM14-SAMS2* (a *S*-adenosylmethionine synthetase from sugar beet), *OsMSRA4.1* (a methionine sulfoxide reductase), and *OsPEX11* (*PEROXISOMAL BIOGENESIS FACTOR 11*) were all confirmed to regulate the growth and metabolism in *Arabidopsis*, *Oryza sativa*, and other species under salt stress (Cui et al., 2016; Guo et al., 2009; Ma et al., 2017; Xu et al., 2019). Therefore, the turquoise module was considered as the key module in this study. Combined with their functional annotations, four genes (*GRMZM2G421669*, KME = 0.976, TOM = 0.502; *GRMZM2G051032*, KME = 0.936, TOM = 0.503; *GRMZM2G004314*, KME = 0.910, TOM = 0.504; and *GRMZM2G123314*, KME = 0.904,

TABLE 1 Significant SNPs overlapping with QTL for salt tolerance identified in previous studies

SNPs	Chr	Traits	Associated QTL	Distance between SNP and QTL (Mb)	References
PZE-101161747	1	RCaS	*qSFW1s*	—	(Hoque, 2013)
			qRFW1s	—	(Hoque, 2013)
			qPFW1s	—	(Hoque, 2013)
			qSDW1s	—	(Hoque, 2013)
			qRDW1s	—	(Hoque, 2013)
PZE-101161871	1	STC	*qSFW1s*	—	(Hoque, 2013)
			qRFW1s	—	(Hoque, 2013)
			qPFW1s	—	(Hoque, 2013)
			qSDW1s	—	(Hoque, 2013)
			qRDW1s	—	(Hoque, 2013)
PZE-101116930	1	STC	*qSPH1*	—	(Luo et al., 2017)
			qPHI1	—	(Luo et al., 2017)
SYN14434	2	SCaS	*qRL2s*	—	(Hoque, 2013)
SYN2578	2	SCaS	*qRL2s*	—	(Hoque, 2013)
PZE-103055519	3	SCaS	*QSnc3*	—	(Cui et al., 2014)
PZE-103055520	3	SCaS	*QSnc3*	—	(Cui et al., 2014)
PZE-103088143	3	STC	*QTwc3*	—	(Cui et al., 2014)
PZE-104052767	4	SCaS	*qPHI4*	—	(Luo et al., 2017)
PZE-104052775	4	SCaS	*QSkn4.1*	1.02 Mb	(Cui et al., 2014)
PZE-105127202	5	RCaS	*qSPH5-1*	—	(Luo et al., 2017)
PZE-105087886	5	RCaS	*qSPH5-2*	—	(Luo et al., 2017)
	5		*qRFW5s*	—	(Hoque, 2013)
	5		*qPFW5s*	—	(Hoque, 2013)
PZE-107026578	7	RCaS	*qPFW7s*	—	(Hoque, 2013)
SYN8213	7	STC	*qPFW7s*	—	(Hoque, 2013)
PUT-163a-110541282-116	9	SCaS	*qPHI9*	0.08 Mb	(Luo et al., 2017)
PZE-110090701	10	RCaS	*qPHI10*	4.41 Mb	(Luo et al., 2017)
SYN19207	10	STC	*qPHI10*	0.56 Mb	(Luo et al., 2017)

Note: Chr, chromosome; "—" represents SNP located within the QTL; SCaS, shoot Ca^{2+} concentration under salt treatment; RCaS, root Ca^{2+} concentration under salt treatment; STC, Ca^{2+} transport coefficients under salt treatment.

TOM = 0.464) of the turquoise module were identified as the hub genes for further study (Table 2). These gene models encode respectively for a putative leucine-rich repeat receptor-like protein, sodium/hydrogen exchanger 2, a V-type proton ATPase subunit, and a putative pentatricopeptide repeat (PPR) protein, which have been verified to play crucial roles in the response to salt stress in plants (Baisakh et al., 2012; El Mahi et al., 2019; Jiang et al., 2015; Lin et al., 2020). According to the TOM values calculated by WGCNA, we constructed a gene coexpression network for the turquoise module (Figure 6).

3.5 | Candidate gene association analysis revealed the potentially functional genes

To further explore the potentially functional genes that influence salt tolerance, four hub genes (*GRMZM2G051032*, *GRMZM2G123314*, *GRMZM2G421669*, and *GRMZM2G004314*) detected in WGCNA were separately subjected to association analysis in 70 inbred lines randomly selected from the association panel. The results revealed that five significant variations were detected in the genes *GRMZM2G051032*, *GRMZM2G123314*, and *GRMZM2G421669* at a threshold of $P \leq 0.05/n$, and no significant variation was detected in *GRMZM2G004314* (Table S6). According to the significant variations, we genotyped the haplotypes for each gene. For *GRMZM2G051032*, the STC-associated marker S9_156709966 (C↔T) formed two haplotypes (C and T) in the 70 lines; however, no significant difference in STC was observed between the two haplotypes (Table S6). For *GRMZM2G123314*, four significant variations S8_15285558 (T → TGACTGA), S8_15285609 (C → CGCTG), S8_15285965 (C ↔ T), and S8_15285991 (T↔C) controlling SCaS and STC grouped the 70 inbred lines into three haplotypes (Hap 1, Hap 2, and Hap 3; Figure 7A,D; Table S6). A *t*-test indicated that the STC of Hap 2 was significantly higher than that of Hap

FIGURE 3 Heatmap of the superior allele SNP distributions in 30 maize elite inbred lines. Red and white colors represent superior and inferior alleles, respectively

1 ($P \leq 0.05$) (Figure 7C; Table S6). Therefore, the Hap 2 (G-CT) in *GRMZM2G123314* were defined as the favorable haplotype affecting STC. In addition, the two significant insertions S8_15285558 (T → TGACTGA) and S8_15285609 (C → CGCTG) were also located within *GRMZM2G421669*, which divided the 70 lines into three subgroups. However, no significant difference was detected between these subgroups in both RCaS and STC (Table S6).

4 | DISCUSSION

4.1 | Ca^{2+}-related traits were involved in the response to salt stress

Several indexes (including root length, root fresh weight, tissue water content, plant survival rate, and etc.) were usually used to explore salt

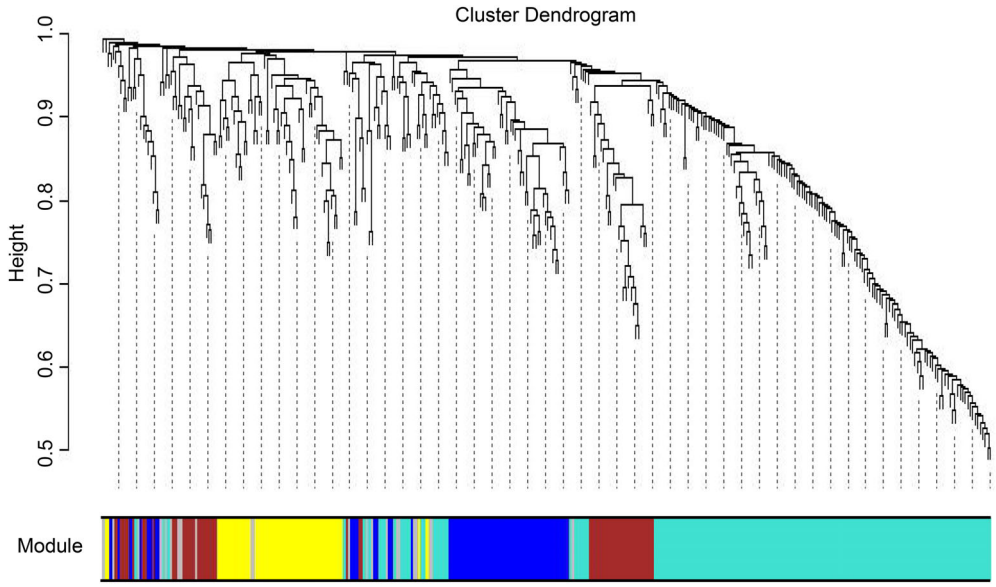

FIGURE 4 Hierarchical cluster tree. Hierarchical cluster tree showing four modules of co-expressed genes

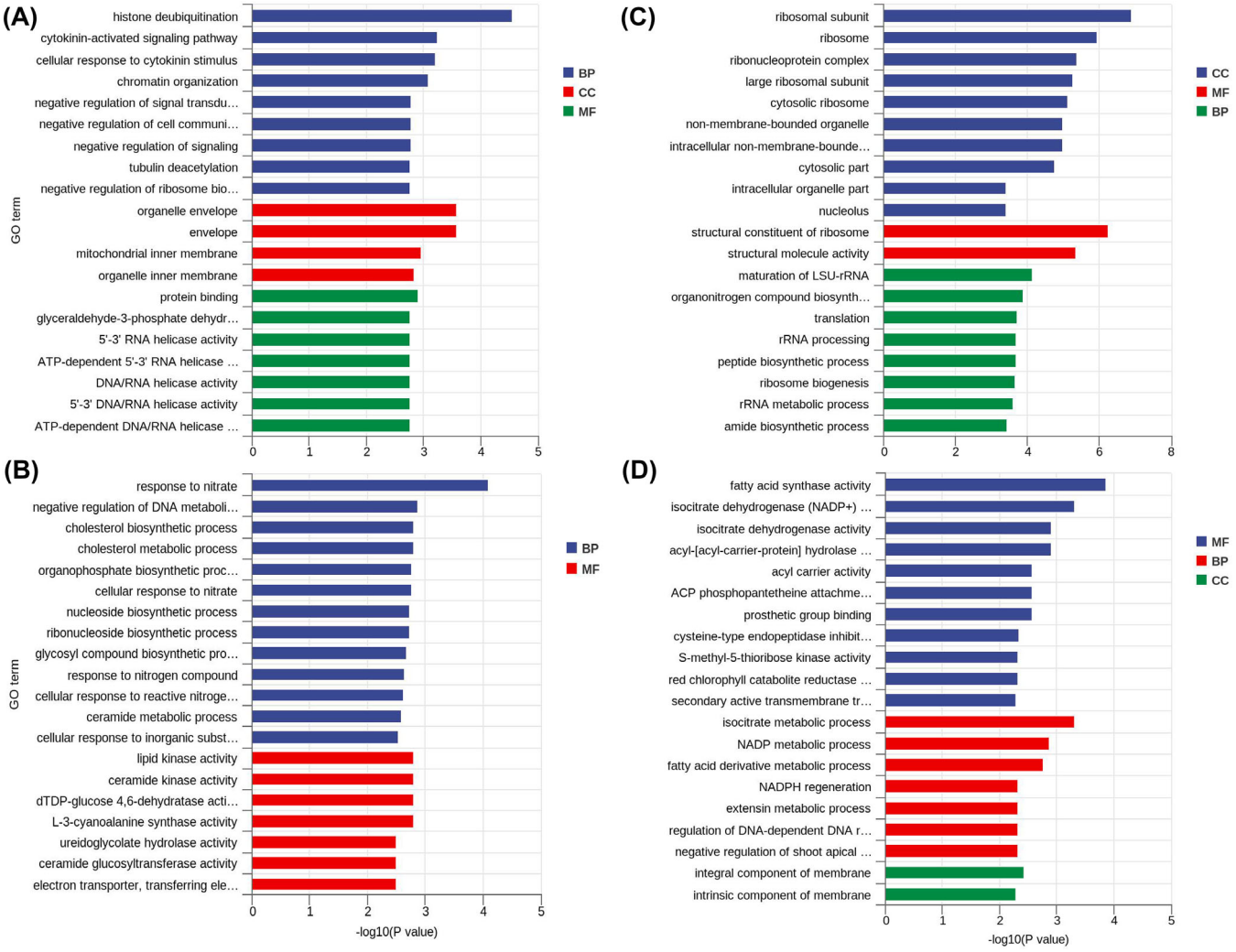

FIGURE 5 GO analysis of genes in blue (A), yellow (B), brown (C), and turquoise (D) modules. BP, biological process; CC, cellular component; MF, molecular function

TABLE 2 Hub genes identified from WGCNA

Associated SNPs	Chromosome	Position (bp)	Candidate genes	Functional annotation
PUT-163a-110,541282-116	9	153,606,814	GRMZM2G051032	Sodium/hydrogen exchanger 2
SYN15956	9	153,592,168		
PZE-103125327	3	182,714,296	GRMZM2G004314	V-type proton ATPase subunit a1
PZE-108015274	8	14,838,055	GRMZM2G421669	Putative leucine-rich repeat receptor-like protein
PZE-108015274	8	14,838,055	GRMZM2G123314	Putative pentatricopeptide repeat-containing protein

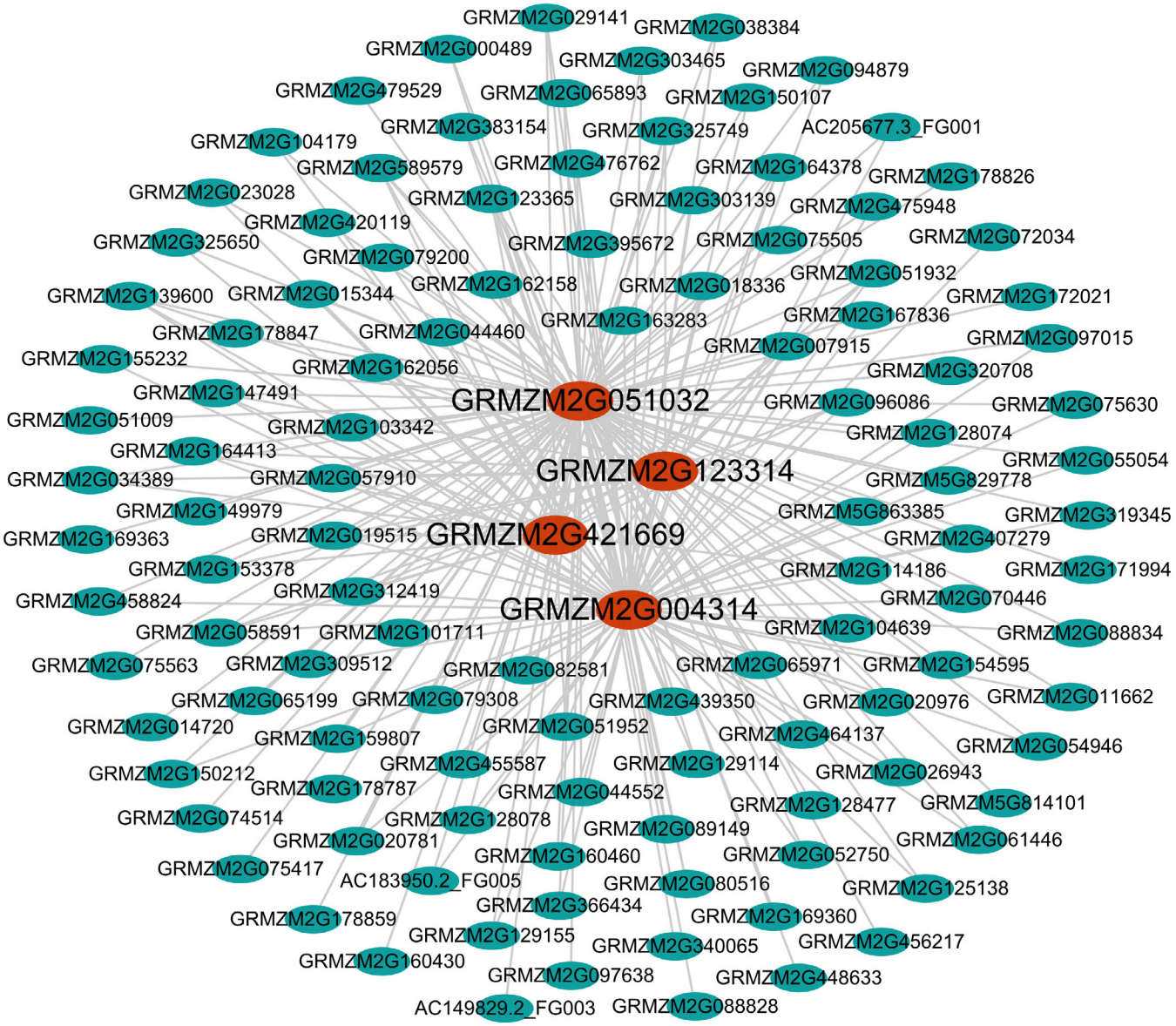

FIGURE 6 Gene coexpression network of the turquoise module. The four hub genes with higher connectivity are shown in red

tolerance in plants (Cui et al., 2014; Luo et al., 2019; Luo et al., 2021). However, all these traits are indirect manifestations of salt tolerance. As a direct assessment of plant salt tolerance, ion accumulation was also used as a related phenotype for association analysis. For example, *ZmNC2* (*Na^+ CONTENT 2*) and *ZmNSA1* (*Na^+ CONTENT UNDER SALINE-ALKALINE CONDITION*) were identified to confer the natural variation of shoot Na^+ in maize by using GWAS (Cao et al., 2020; Zhang et al., 2019). Our previous study also decoded the genetic architecture of Na^+ and K^+ content in maize seedlings and found seven SNPs controlling Na^+/K^+ in shoot, root K^+ content, and K^+ transport coefficient (Ma et al., 2021). However, few studies have attempted to demonstrate the variations of Ca^{2+} concentration in response to salt stress.

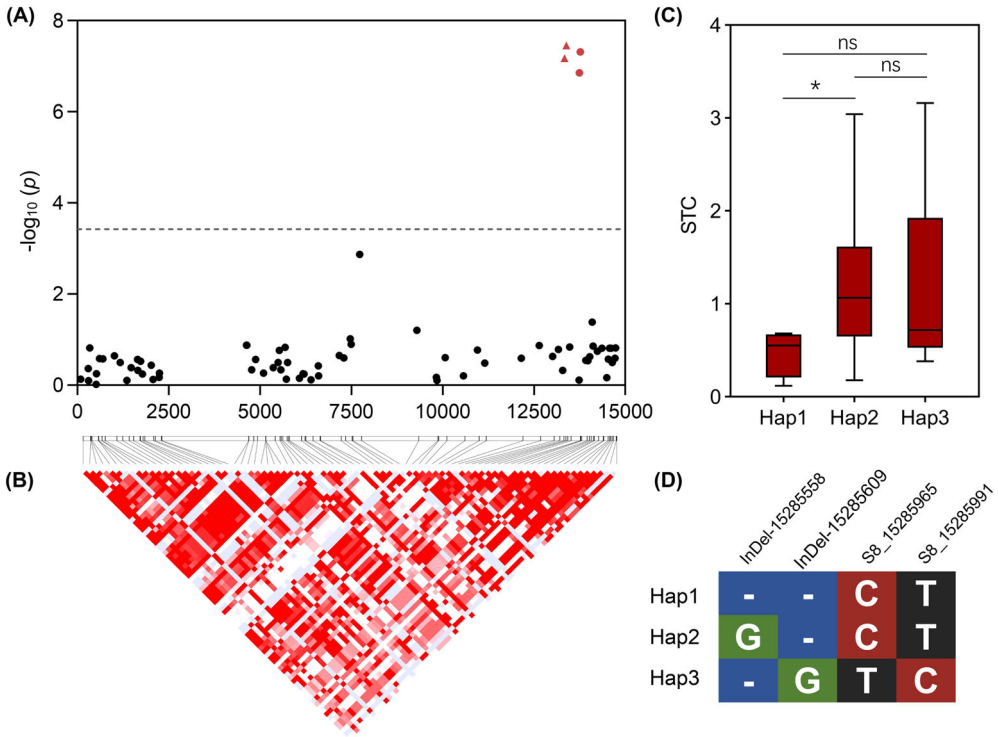

FIGURE 7 Association analysis of *GRMZM2G123314*. (A) The significant variations are shown in red, since -$\log_{10}$ (*P*) is greater than 3.21 ($P \leq 0.05/n$, n denotes the number of variations). Triangles denote InDels and dots represent SNPs. The distribution of variation loci in the gene is shown in the middle. (B) The pairwise linkage disequilibrium (LD) analysis between the markers. (C) Comparison of the STC between three haplotypes. Statistical significance was determined by two-sided *t*-test: *P <0.05. (D) Haplotypes of *GRMZM2G123314* among 70 lines. Hap, haplotype; STC, transport coefficient under salt treatment

High Na^+ concentrations will interfere with the normal growth and development of many plant species by disturbing the homeostasis of Ca^{2+} and K^+ (Cramer et al., 1987). We then explored the correlations between the six Ca^{2+}-related traits and 16 Na^+- and K^+-associated traits reported in our previous study (Ma et al., 2021). The results showed that K^+ and Ca^{2+} contents in roots under both control and salt treatment were significantly positively correlated (P <0.001; Figure S3). Similarly, the transport coefficients of K^+ and Ca^{2+} also displayed a positive correlation under the two conditions (Figure S3). Consistently, a negative correlation was found between Na^+/K^+ ratio and Ca^{2+} content in roots under both conditions (Figure S3). In the previous study, an elevated Ca^{2+} concentration has been reported to ameliorate the intracellular K^+ loss in *Arabidopsis* by regulating K^+ efflux channels under salt stress (Shabala et al., 2006). Ca^{2+} also reversed the delayed germination of *Sorghum bicolor* induced by salt stress, accompanied by a decreased Na^+/K^+ ratio. This indicates that Ca^{2+} regulates the homeostasis of Na^+ and K^+ to protect plants from salt toxicity (Mulaudzi et al., 2020). Moreover, the uptake and retention of K^+ by roots were considered important to maintain the K^+ homeostasis of salt tolerance in maize (Gao et al., 2016). These universal correlations between Ca^{2+} and K^+ contents observed in our study suggested that Ca^{2+} and K^+ participate in the response to salt stress in a synergistic way. In addition, the phenotypes of six Ca^{2+}-related traits displayed abundant variations, with CVs ranging from 0.78 to 1.22 (Table S1), and the H^2 estimates were in the range of 0.40–0.83 (Table S1). Collectively, the above findings demonstrated that the six Ca^{2+}-related traits were suitable to dissect the genetic control of maize salt tolerance by GWAS.

4.2 | Joint GWAS and WGCNA to decode genetic architecture of maize salt tolerance

GWAS has been widely used to dissect the genetic basis of complex agronomic traits, and many candidate genes controlling target traits were excavated in previous studies. Using GWAS, 18 novel candidate genes were found to correlate with head smut resistance in maize, and several of which overlapped with previous studies (Wang et al., 2012). Ma et al. (2018) identified a total of 40 candidate genes by GWAS, which were associated with the embryogenic callus regeneration ability of maize, including the previously reported gene *WOX2*. Moreover, 35 *ZmCPA* genes were identified in the recent genome-wide identification of maize cation-proton antiporter under salt stress (Kong et al., 2021). Similarly, several genes associated with salt tolerance were also detected by GWAS in maize, including *ZmHKT1*, *ZmCLCg*, and *ZmPMP3* (Luo et al., 2021; Zhang et al., 2018). In this study, we totally detected 53 significant SNPs using GWAS, 17 of which were located in the previously detected QTL for maize salt tolerance (Table 1). Based on the significant SNPs, we extracted 544 genes for further study (Table S5).

In the present study, the significant SNPs identified by GWAS were closely linked to the genes involved in transport and catabolism, signal transduction, and stimulus response. Among the candidate genes, *AC198937.4_FG005* and *GRMZM2G172053* were annotated as NAC domain proteins. NAC is a plant-specific gene family, and the N-terminal of most NAC proteins contains highly conserved DNA binding domains (Hu et al., 2006). Numerous studies showed that salt tolerance was enhanced by overexpressing NAC transcription factors in rice (Hu et al., 2006), *Arabidopsis* (Huang et al., 2015), soybean (Li,

Chen, et al., 2020), and tobacco (Liu et al., 2011). Ca^{2+} signals are decoded by SOS3-like calcium-binding proteins/calcineurin B-like proteins (SCaBPs/CBLs), which have been proved to play pivotal roles in regulating salt tolerance (Ji et al., 2013; Yang et al., 2019). Previous studies identified several Ca^{2+} sensors in higher plants, such as calmodulin (CaM), CaM-like proteins (CMLs), Ca^{2+}-dependent protein kinases (CDPKs), and calcineurin B-like proteins (CBLs) (Harper et al., 2004; McCormack et al., 2005; Reddy et al., 2011; Tang et al., 2020). Herein, we also detected several Ca^{2+} sensors, including one CaM (*GRMZM2G391364*), two CBL-interacting protein kinases (CIPKs) (*GRMZM2G052067* and *GRMZM2G051103*), and three CMLs (*GRMZM2G104523*, *GRMZM2G050553*, and *GRMZM5G827398*) (Table S5). The interactions between CBLs and CIPKs constitute a signaling network of functional diversity in response to various extracellular signals, including nutrient deprivation and abiotic stresses (Tang et al., 2020). Chen et al. identified a *zmcipk42* mutant, with a G-A variation in the coding region, leading to impaired salt tolerance in maize. However, overexpression of *ZmCIPK42* increased the tolerance to high salt stress in maize and *Arabidopsis thaliana* (Chen et al., 2021).

Although GWAS has a powerful efficiency in detecting significant SNP-trait associations, it cannot achieve an accurate identification of candidate genes. Therefore, a strategy combining GWAS and WGCNA has been used to decode the genetic basis of complex traits and explore candidate genes related to target traits at present. Using this efficient approach, Hwang et al. (2018) identified two hub genes (*GRMZM2G477685* and *GRMZM2G135536*) involved in primary root growth in maize. Similarly, in maize, a total of 168 candidate genes controlling root development and plasticity were identified under salinity, of which two hub genes, *ZmIAA1* and *ZmGRAS43*, were then validated by resequencing (Li et al., 2021). In this study, we identified four hub genes associated with salt tolerance in maize seedlings by a combination of GWAS and WGCNA. *GRMZM2G051032* was annotated as sodium/hydrogen exchanger 2. Previous studies revealed that the salt tolerance was significantly enhanced by overexpressing a sodium/proton antiporter in *Arabidopsis thaliana* and tobacco (Pehlivan et al., 2016; Wang et al., 2018; Zhang et al., 2012). Moreover, *GRMZM2G051032* was also identified by a genome-wide identification in a previous study (Kong et al., 2021). The SCaS-correlated hub gene *GRMZM2G004314* encodes a V-type proton ATPase (also known as vacuolar-type H^+-ATPase, VHA) subunit, which is an extremely conserved multi-subunit endomembrane proton pump involved in ion homeostasis and environmental stresses (Kluge et al., 2003; Ratajczak, 2000). The overexpression of an exogenous *VHA* enhanced the salt tolerance in transgenic rice (Baisakh et al., 2012) and tobacco (Wang et al., 2016). In recent years, several studies also suggested that leucine-rich repeat receptor-like kinases (LRR-RLKs) were widely associated with plant abiotic stress responses (Kang et al., 2017; Lin et al., 2020; Ouyang et al., 2010). In the present study, the hub gene *GRMZM2G421669* encodes a putative leucine-rich repeat receptor-like protein, which modulates salt tolerance in rice and *Medicago truncatula* (de Lorenzo et al., 2009). The gene model *GRMZM2G123314* linked by a significant marker (PZE-108015274) was annotated as a putative PPR protein. The PPR proteins are mostly targeted to mitochondria or chloroplasts, where they play diverse and crucial roles in plant developmental processes under abiotic stresses (Jiang et al., 2015). Zhu et al. (2014) reported that SLO2, an *Arabidopsis* PPR protein acting as mitochondrial RNA editing factor, affects the mitochondrial electron transport chain. The adult *slo2* mutants have an increased salt tolerance (Zhu et al., 2014). Mei et al. (2014) revealed that the *Arabidopsis* PPR protein SOAR1 was a negative regulator in ABA signaling. In the signal transduction responding to plant stress, ABA integrates with Ca^{2+} signal into tight signaling networks rather than independent signaling pathways (Edel & Kudla, 2016). Therefore, PPR protein may be involved in the regulation of Ca^{2+}-mediated salt stress response. The candidate gene association analysis revealed that the variations on *GRMZM2G123314* led to the difference in STC among different lines (Figure 7C). Taken together, these findings suggest that the four hub genes, especially *GRMZM2G123314*, should be considered as a priority gene for further study.

4.3 | Application of superior alleles in MAS breeding for maize salt tolerance

Our study provided the SNP markers for maize salt-tolerance breeding. By examining the utilization of superior alleles at these genetic loci across the 30 elite inbred lines, we found that nine of these SNPs contained >50% superior alleles, and four of them even exceeded 90% (PZE-108038765, 100.0%; SYN8213, 90.0%; SYN19207, 93.3%; PZE-101161747, 96.7%). This suggested that the four alleles were well maintained in the process of artificial selection. This may be due to the close linkage between these superior alleles and some crucial agronomic traits, such as yield-related traits, plant type-related traits, and other resistance-related traits. However, among the elite lines, the superior alleles in the remaining 44 SNPs were less than 30% (with an average of 10.30%). In particular, the five SNPs (PZE-109110551, PZE-107026578, SYN16403, PZE-105127202, and SYN14434) did not contain superior allele in any of the elite lines. This indicated that breeders probably have paid little attention to Ca^{2+} concentration in maize seedings under salt stress, resulting in the inefficient selection of these superior alleles in the breeding process. Additionally, for each elite line, the superior allele proportion of the 53 SNPs ranged from 15.09% to 35.85%. Therefore, future studies should focus on increasing the superior allele ratio in each line for the marker-assisted selection breeding of salt-tolerance maize varieties.

AUTHOR CONTRIBUTIONS

Langlang Ma and Yaou Shen conceived the project and designed the experiments. Tianhu Liang, Chunyan Qing, and Peng Liu performed the experiments. Tianhu Liang conducted the data analysis. Chaoying Zou and Guangsheng Yuan participated in some of the experiments. Tianhu Liang and Langlang Ma drafted the manuscript with the contribution from Yaou Shen and Guangtang Pan. All the authors reviewed and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The original sequence reads of the 28 samples used in this study were deposited in Genome Sequence Archive (GSA) of the National Genomics Data Center (NGDC) database with the accession number of CRA003872.

ORCID

Peng Liu https://orcid.org/0000-0002-5004-9983
Langlang Ma https://orcid.org/0000-0002-8520-7590

REFERENCES

Abdel-Ghani, A.H., Kumar, B., Reyes-Matamoros, J., Gonzalez-Portilla, P.J., Jansen, C., Martin, J.P.S. et al. (2012) Genotypic variation and relationships between seedling and adult plant traits in maize (*Zea mays* L.) inbred lines grown under contrasting nitrogen levels. *Euphytica*, 189(1), 123–133.

Andorf, C.M., Lawrence, C.J., Harper, L.C., Schaeffer, M.L., Campbell, D. A. & Sen, T.Z. (2010) The Locus Lookup tool at MaizeGDB: identification of genomic regions in maize by integrating sequence information with physical and genetic maps. *Bioinformatics*, 26(3), 434–436.

Bahieldin, A., Atef, A., Edris, S., Gadalla, N.O., Ali, H.M., Hassan, S.M. et al. (2016) Ethylene responsive transcription factor ERF109 retards PCD and improves salt tolerance in plant. *BMC Plant Biology*, 16(1), 216.

Baisakh, N., RamanaRao, M.V., Rajasekaran, K., Subudhi, P., Janda, J., Galbraith, D. et al. (2012) Enhanced salt stress tolerance of rice plants expressing a vacuolar H^+ -ATPase subunit c1 (SaVHAc1) gene from the halophyte grass Spartina alterniflora Loisel. *Plant Biotechnology Journal*, 10(4), 453–464.

Berthomieu, P., Conejero, G., Nublat, A., Brackenbury, W.J., Lambert, C., Savio, C. et al. (2003) Functional analysis of AtHKT1 in Arabidopsis shows that Na^+ recirculation by the phloem is crucial for salt tolerance. *The EMBO Journal*, 22(9), 2004–2014.

Cao, Y., Zhang, M., Liang, X., Li, F., Shi, Y., Yang, X. et al. (2020) Natural variation of an EF-hand Ca^{2+}-binding-protein coding gene confers saline-alkaline tolerance in maize. *Nature Communications*, 11(1), 186.

Chen, X., Chen, G., Li, J., Hao, X., Tuerxun, Z., Chang, X. et al. (2021) A maize calcineurin B-like interacting protein kinase ZmCIPK42 confers salt stress tolerance. *Physiologia Plantarum*, 171(1), 161–172.

Childs, K.L., Davidson, R.M. & Buell, C.R. (2011) Gene coexpression network analysis as a source of functional annotation for rice genes. *PLoS One*, 6(7), e22196.

Chinnusamy, V., Schumaker, K. & Zhu, J.K. (2004) Molecular genetic perspectives on cross-talk and specificity in abiotic stress signalling in plants. *Journal of Experimental Botany*, 55(395), 225–236.

Cramer, G.R., Lynch, J., Läuchli, A. & Epstein, E. (1987) Influx of Na^+, K^+, and Ca^{2+} into roots of salt-stressed cotton seedlings 1: effects of supplemental Ca^{2+}. *Plant Physiology*, 83(3), 510–516.

Cui, D., Wu, D., Somarathna, Y., Xu, C., Li, S., Li, P. et al. (2014) QTL mapping for salt tolerance based on snp markers at the seedling stage in maize (*Zea mays* L.). *Euphytica*, 203(2), 273–283.

Cui, P., Liu, H., Islam, F., Li, L., Farooq, M.A., Ruan, S. et al. (2016) OsPEX11, a peroxisomal biogenesis factor 11, contributes to salt stress tolerance in Oryza sativa. *Frontiers in Plant Science*, 7, 1357.

de Azevedo Neto, A.D., Prisco, J.T., Enéas-Filho, J., Abreu, C.E.B. & Gomes-Filho, E. (2006) Effect of salt stress on antioxidative enzymes and lipid peroxidation in leaves and roots of salt-tolerant and salt-sensitive maize genotypes. *Environmental and Experimental Botany*, 56(1), 87–94.

de Lorenzo, L., Merchan, F., Laporte, P., Thompson, R., Clarke, J., Sousa, C. et al. (2009) A novel plant leucine-rich repeat receptor kinase regulates the response of Medicago truncatula roots to salt stress. *Plant Cell*, 21(2), 668–680.

Duan, L., Dietrich, D., Ng, C.H., Chan, P.M., Bhalerao, R., Bennett, M.J. et al. (2013) Endodermal ABA signaling promotes lateral root quiescence during salt stress in Arabidopsis seedlings. *Plant Cell*, 25(1), 324–341.

Edel, K.H. & Kudla, J. (2016) Integration of calcium and ABA signaling. *Current Opinion in Plant Biology*, 33, 83–91.

El Mahi, H., Perez-Hormaeche, J., De Luca, A., Villalta, I., Espartero, J., Gamez-Arjona, F. et al. (2019) A critical role of sodium flux via the plasma membrane Na^+/H^+ exchanger SOS1 in the salt tolerance of rice. *Plant Physiology*, 180(2), 1046–1065.

Elshire, R.J., Glaubitz, J.C., Sun, Q., Poland, J.A., Kawamoto, K., Buckler, E. S. et al. (2011) A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. *PLoS One*, 6(5), e19379.

Gao, Y., Lu, Y., Wu, M., Liang, E., Li, Y., Zhang, D. et al. (2016) Ability to remove Na^+ and retain K^+ correlates with salt tolerance in two maize inbred lines seedlings. *Frontiers in Plant Science*, 7, 1716.

Guo, J., Li, C., Zhang, X., Li, Y., Zhang, D., Shi, Y. et al. (2020) Transcriptome and GWAS analyses reveal candidate gene for seminal root length of maize seedlings under drought stress. *Plant Science*, 292, 110380.

Guo, X., Wu, Y., Wang, Y., Chen, Y. & Chu, C. (2009) OsMSRA4.1 and OsMSRB1.1, two rice plastidial methionine sulfoxide reductases, are involved in abiotic stress responses. *Planta*, 230(1), 227–238.

Halfter, U., Ishitani, M. & Zhu, J. (2000) The Arabidopsis SOS2 protein kinase physically interacts with and is activated by the calcium-binding protein SOS3. *Proceedings of the National Academy of Sciences of the United States of America*, 97(7), 3735–3740.

Hanin, M., Ebel, C., Ngom, M., Laplaze, L. & Masmoudi, K. (2016) New insights on plant salt tolerance mechanisms and their potential use for breeding. *Frontiers in Plant Science*, 7, 1787.

Harper, J.F., Breton, G. & Harmon, A. (2004) Decoding Ca^{2+} signals through plant protein kinases. *Annual Review of Plant Biology*, 55, 263–288.

Hepler, P.K. (2005) Calcium: a central regulator of plant growth and development. *Plant Cell*, 17(8), 2142–2155.

Hichem, H., Mounir, D. & Naceur, E.A. (2009) Differential responses of two maize (*Zea mays* L.) varieties to salt stress: changes on polyphenols composition of foliage and oxidative damages. *Industrial Crops and Products*, 30(1), 144–151.

Hoque, M.M.I. (2013) Evaluation and mapping QTLs of maize salinity tolerance (Ph.D thesis). *Chinese Academy of Agricultural Sciences*. http://cdmd.cnki.com.cn/Article/CDMD-82101-1015520982.htm

Hu, H., Dai, M., Yao, J., Xiao, B., Li, X., Zhang, Q. et al. (2006) Overexpressing a NAM, ATAF, and CUC (NAC) transcription factor enhances drought resistance and salt tolerance in rice. *Proceedings of the National Academy of Sciences of the United States of America*, 103(35), 12987–12992.

Huang, Q., Wang, Y., Li, B., Chang, J., Chen, M., Li, K. et al. (2015) TaNAC29, a NAC transcription factor from wheat, enhances salt and drought tolerance in transgenic Arabidopsis. *BMC Plant Biology*, 15, 268.

Hwang, S.G., Kim, K.H., Lee, B.M. & Moon, J.C. (2018) Transcriptome analysis for identifying possible gene regulations during maize root emergence and formation at the initial growth stage. *Genes Genomics*, 40(7), 755–766.

Jafar, M.Z., Farooq, M., Cheema, M.A., Afzal, I., Basra, S.M.A., Wahid, M.A. et al. (2012) Improving the performance of wheat by seed priming under saline conditions. *Journal of Agronomy and Crop Science*, 198(1), 38–45.

Ji, H., Pardo, J.M., Batelli, G., Van Oosten, M.J., Bressan, R.A. & Li, X. (2013) The salt overly sensitive (SOS) pathway: established and emerging roles. *Molecular Plant*, 6(2), 275–286.

Jiang, S.C., Mei, C., Liang, S., Yu, Y.T., Lu, K., Wu, Z. et al. (2015) Crucial roles of the pentatricopeptide repeat protein SOAR1 in Arabidopsis response to drought, salt and cold stresses. *Plant Molecular Biology*, 88(4–5), 369–385.

Jiao, Y., Peluso, P., Shi, J., Liang, T., Stitzer, M.C., Wang, B. et al. (2017) Improved maize reference genome with single-molecule technologies. *Nature*, 546(7659), 524–527.

Julkowska, M.M., Koevoets, I.T., Mol, S., Hoefsloot, H., Feron, R., Tester, M.A. et al. (2017) Genetic components of root architecture remodeling in response to salt stress. *Plant Cell*, 29(12), 3198–3213.

Kang, J., Li, J., Gao, S., Tian, C. & Zha, X. (2017) Overexpression of the leucine-rich receptor-like kinase gene LRK2 increases drought tolerance and tiller number in rice. *Plant Biotechnology Journal*, 15(9), 1175–1185.

Kaya, C., Tuna, A.L. & Okant, A.M. (2014) Effect of foliar applied kinetin and indole acetic acid on maize plants grown under saline conditions. *Turkish Journal of Agriculture & Forestry*, 34(6), 529–538.

Kluge, C., Lahr, J., Hanitzsch, M., Bolte, S., Golldack, D. & Dietz, K.J. (2003) New insight into the structure and regulation of the plant vacuolar H^+-ATPase. *Journal of Bioenergetics and Biomembranes*, 35(4), 377–388.

Kong, M., Luo, M., Li, J., Feng, Z., Zhang, Y., Song, W. et al. (2021) Genome-wide identification, characterization, and expression analysis of the monovalent cation-proton antiporter superfamily in maize, and functional analysis of its role in salt tolerance. *Genomics*, 113(4), 1940–1951.

Langfelder, P. & Horvath, S. (2008) WGCNA: an R package for weighted correlation network analysis. *BMC Bioinformatics*, 9, 559.

Li, M., Chen, R., Jiang, Q., Sun, X., Zhang, H. & Hu, Z. (2020) GmNAC06, a NAC domain transcription factor enhances salt stress tolerance in soybean. *Plant Molecular Biology*, 105(3), 333–345.

Li, P., Yang, X., Wang, H., Pan, T., Wang, Y., Xu, Y. et al. (2021) Genetic control of root plasticity in response to salt stress in maize. *Theoretical and Applied Genetics*, 134(5), 1475–1492.

Li, Z., Liu, X., Xu, X., Liu, J., Sang, Z., Yu, K. et al. (2020) Favorable haplotypes and associated genes for flowering time and photoperiod sensitivity identified by comparative selective signature analysis and GWAS in temperate and tropical maize. *The Crop Journal*, 8(2), 227–242.

Lin, F., Li, S., Wang, K., Tian, H., Gao, J., Zhao, Q. et al. (2020) A leucine-rich repeat receptor-like kinase, OsSTLK, modulates salt tolerance in rice. *Plant Science*, 296, 110465.

Liu, J., Ishitani, M., Halfter, U., Kim, C.S. & Zhu, J.K. (2000) The Arabidopsis thaliana SOS2 gene encodes a protein kinase that is required for salt tolerance. *Proceedings of the National Academy of Sciences of the United States of America*, 97(7), 3730–3734.

Liu, Q., Xu, K., Zhao, L., Pan, Y., Jiang, B., Zhang, H. et al. (2011) Overexpression of a novel chrysanthemum NAC transcription factor gene enhances salt tolerance in tobacco. *Biotechnology Letters*, 33(10), 2073–2082.

Liu, X., Huang, M., Fan, B., Buckler, E.S. & Zhang, Z. (2016) Iterative usage of fixed and random effect models for powerful and efficient genome-wide association studies. *PLoS Genetics*, 12(2), e1005767.

Luo, M., Zhang, Y., Li, J., Zhang, P., Chen, K., Song, W. et al. (2021) Molecular dissection of maize seedling salt tolerance using a genome-wide association analysis method. *Plant Biotechnology Journal*, 19(10), 1937–1951.

Luo, M., Zhao, Y., Zhang, R., Xing, J., Duan, M., Li, J. et al. (2017) Mapping of a major QTL for salt tolerance of mature field-grown maize plants based on SNP markers. *BMC Plant Biology*, 17(1), 140.

Luo, X., Wang, B., Gao, S., Zhang, F., Terzaghi, W. & Dai, M. (2019) Genome-wide association study dissects the genetic bases of salt tolerance in maize seedlings. *Journal of Integrative Plant Biology*, 61(6), 658–674.

Ma, C., Wang, Y., Gu, D., Nan, J., Chen, S. & Li, H. (2017) Overexpression of S-adenosyl-l-methionine synthetase 2 from sugar beet M14 increased arabidopsis tolerance to salt and oxidative stress. *International Journal of Molecular Sciences*, 18(4), 847.

Ma, L., Liu, M., Yan, Y., Qing, C., Zhang, X., Zhang, Y. et al. (2018) Genetic dissection of maize embryonic callus regenerative capacity using multi-locus genome-wide association studies. *Frontiers in Plant Science*, 9, 561.

Ma, L., Qing, C., Frei, U., Shen, Y. & Lübberstedt, T. (2020) Association mapping for root system architecture traits under two nitrogen conditions in germplasm enhancement of maize doubled haploid lines. *The Crop Journal*, 8(2), 213–226.

Ma, L., Zhang, M., Chen, J., Qing, C., He, S., Zou, C. et al. (2021) GWAS and WGCNA uncover hub genes controlling salt tolerance in maize (*Zea mays* L.) seedlings. *Theoretical and Applied Genetics*, 134(10), 3305–3318.

Mahajan, S., Pandey, G.K. & Tuteja, N. (2008) Calcium- and salt-stress signaling in plants: shedding light on SOS pathway. *Archives of Biochemistry and Biophysics*, 471(2), 146–158.

McCormack, E., Tsai, Y.-C. & Braam, J. (2005) Handling calcium signaling: Arabidopsis CaMs and CMLs. *Trends in Plant Science*, 10(8), 383–389.

Mei, C., Jiang, S.C., Lu, Y.F., Wu, F.Q., Yu, Y.T., Liang, S. et al. (2014) Arabidopsis pentatricopeptide repeat protein SOAR1 plays a critical role in abscisic acid signalling. *Journal of Experimental Botany*, 65(18), 5317–5330.

Min, Z., Lana, S., Cuin, T.A., Xin, H., Meixue, Z., Rana, M. et al. (2016) Nax loci affect SOS1-like Na^+/H^+ exchanger expression and activity in wheat. *Journal of Experimental Botany*, 3, 835–844.

Mulaudzi, T., Hendricks, K., Mabiya, T., Muthevhuli, M., Ajayi, R.F., Mayedwa, N. et al. (2020) Calcium improves germination and growth of Sorghum bicolor seedlings under salt stress. *Plants (Basel)*, 9(6), 730.

Munns, R. & Tester, M. (2008) Mechanisms of salinity tolerance. *Annual Review of Plant Biology*, 59, 651–681.

Neubert, A.B., Zrb, C. & Schubert, S. (2005) Expression of vacuolar Na^+/H^+ antiporters (ZmNHX) and Na^+ exclusion in roots of maize (*Zea mays* L.) genotypes with improved salt resistance. In: Li, C.J. et al. (Eds.) *Plant nutrition for food security, human health and environmental protection*. Bejing, China: Tsinghua University Press, pp. 544–545.

Ouyang, S.Q., Liu, Y.F., Liu, P., Lei, G., He, S.J., Ma, B. et al. (2010) Receptor-like kinase OsSIK1 improves drought and salt stress tolerance in rice (Oryza sativa) plants. *The Plant Journal*, 62(2), 316–329.

Pace, J., Gardner, C., Romay, C., Ganapathysubramanian, B. & Lubberstedt, T. (2015) Genome-wide association analysis of seedling root development in maize (*Zea mays* L.). *BMC Genomics*, 16, 47.

Patishtan, J., Hartley, T.N., Fonseca de Carvalho, R. & Maathuis, F.J.M. (2018) Genome-wide association studies to identify rice salt-tolerance markers. *Plant, Cell & Environment*, 41(5), 970–982.

Pehlivan, N., Sun, L., Jarrett, P., Yang, X., Mishra, N., Chen, L. et al. (2016) Co-overexpressing a plasma membrane and a vacuolar membrane sodium/proton Antiporter significantly improves salt tolerance in transgenic Arabidopsis plants. *Plant & Cell Physiology*, 57(5), 1069–1084.

Quan, R., Lin, H., Mendoza, I., Zhang, Y., Cao, W., Yang, Y. et al. (2007) SCABP8/CBL10, a putative calcium sensor, interacts with the protein kinase SOS2 to protect Arabidopsis shoots from salt stress. *Plant Cell*, 19(4), 1415–1431.

Ranum, P., Pena-Rosas, J.P. & Garcia-Casal, M.N. (2014) Global maize production, utilization, and consumption. *Annals of the New York Academy of Sciences*, 1312, 105–112.

Ratajczak, R. (2000) Structure, function and regulation of the plant vacuolar H^+-translocating ATPase. *Biochimica et Biophysica Acta (BBA)—Biomembranes*, 1465(1–2), 17–36.

Reddy, A.S., Ali, G.S., Celesnik, H. & Day, I.S. (2011) Coping with stresses: roles of calcium- and calcium/calmodulin-regulated gene expression. *Plant Cell*, 23(6), 2010–2032.

Ren, Z., Zheng, Z., Chinnusamy, V., Zhu, J., Cui, X., Iida, K. et al. (2010) RAS1, a quantitative trait locus for salt tolerance and ABA sensitivity in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America*, 107(12), 5669–5674.

Ren, Z.H., Gao, J.P., Li, L.G., Cai, X.L., Huang, W., Chao, D.Y. et al. (2005) A rice quantitative trait locus for salt tolerance encodes a sodium transporter. *Nature Genetics*, 37(10), 1141–1146.

Ryu, H. & Cho, Y.-G. (2015) Plant hormones in salt stress tolerance. *Journal of Plant Biology*, 58(3), 147–155.

Sanders, D., Pelloux, J., Brownlee, C. & Harper, J.F. (2002) Calcium at the crossroads of signaling. *Plant Cell*, 14(Suppl), S401–S417.

Schaefer, R.J., Michno, J.-M., Jeffers, J., Hoekenga, O., Dilkes, B., Baxter, I. et al. (2018) Integrating Coexpression networks with GWAS to prioritize causal genes in maize. *Plant Cell*, 30(12), 2922–2942.

Shabala, S., Demidchik, V., Shabala, L., Cuin, T.A., Smith, S.J., Miller, A.J. et al. (2006) Extracellular Ca^{2+} ameliorates NaCl-induced K^+ loss from Arabidopsis root and leaf cells by controlling plasma membrane K^+ −permeable channels. *Plant Physiology*, 141(4), 1653–1665.

Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D. et al. (2003) Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Research*, 13(11), 2498–2504.

Shi, H., Quintero, F., Pardo, J. & Zhu, J. (2002) The putative plasma membrane Na^+/H^+ antiporter SOS1 controls long-distance Na^+ transport in plants. *The Plant Cell*, 14(2), 465–477.

Tang, R.J., Wang, C., Li, K. & Luan, S. (2020) The CBL-CIPK calcium signaling network: unified paradigm from 20 years of discoveries. *Trends in Plant Science*, 25(6), 604–617.

Wang, H., Ding, Q. & Wang, H. (2018) A new Na^+/H^+ antiporter gene KvNHX1 isolated from the halophyte Kosteletzkya virginica improves salt tolerance in transgenic tobacco. *Biotechnology & Biotechnological Equipment*, 32(6), 1378–1386.

Wang, J., Zhou, A., Li, Y., Li, S., Zhang, X. & Che, D. (2016) Overexpression of IrlVHA-c, a vacuolar-type H^+-ATPase c subunit gene from Iris lactea, enhances salt tolerance in tobacco. *Plant Molecular Biology Reporter*, 34(5), 877–885.

Wang, M., Yan, J., Zhao, J., Song, W., Zhang, X., Xiao, Y. et al. (2012) Genome-wide association study (GWAS) of resistance to head smut in maize. *Plant Science*, 196, 125–131.

Xiao, Y., Liu, H., Wu, L., Warburton, M. & Yan, J. (2017) Genome-wide association studies in maize: praise and stargaze. *Molecular Plant*, 10(3), 359–374.

Xu, Y., Yu, Z., Zhang, S., Wu, C., Yang, G., Yan, K. et al. (2019) CYSTM3 negatively regulates salt stress tolerance in Arabidopsis. *Plant Molecular Biology*, 99(4–5), 395–406.

Yamaguchi, T. & Blumwald, E. (2005) Developing salt-tolerant crop plants: challenges and opportunities. *Trends in Plant Science*, 10(12), 615–620.

Yang, Y. & Guo, Y. (2018) Unraveling salt stress signaling in plants. *Journal of Integrative Plant Biology*, 60(9), 796–804.

Yang, Y., Wu, Y., Ma, L., Yang, Z., Dong, Q., Li, Q. et al. (2019) The Ca^{2+} sensor SCaBP3/CBL7 modulates plasma membrane H^+-ATPase activity and promotes alkali tolerance in Arabidopsis. *Plant Cell*, 31(6), 1367–1384.

Yu, L.X., Liu, X., Boge, W. & Liu, X.P. (2016) Genome-wide association study identifies loci for salt tolerance during germination in Autotetraploid alfalfa (Medicago sativa L.) using genotyping-by-sequencing. Front. *Plant Science*, 7, 956.

Zhang, H., Liu, Y., Xu, Y., Chapman, S., Love, A.J. & Xia, T. (2012) A newly isolated Na^+/H^+ antiporter gene, DmNHX1, confers salt tolerance when expressed transiently in Nicotiana benthamiana or stably in Arabidopsis thaliana. *Plant Cell, Tissue and Organ Culture*, 110(2), 189–200.

Zhang, M., Cao, Y., Wang, Z., Wang, Z.Q., Shi, J., Liang, X. et al. (2018) A retrotransposon in an HKT1 family sodium transporter causes variation of leaf Na^+ exclusion and salt tolerance in maize. *The New Phytologist*, 217(3), 1161–1176.

Zhang, M., Liang, X., Wang, L., Cao, Y., Song, W., Shi, J. et al. (2019) A HAK family Na transporter confers natural variation of salt tolerance in maize. *Nature Plants*, 5(12), 1297–1308.

Zhang, X., Liu, P., Qing, C., Yang, C., Shen, Y. & Ma, L. (2021) Comparative transcriptome analyses of maize seedling root responses to salt stress. *PeerJ*, 9, e10765.

Zhang, X., Zhang, H., Li, L., Lan, H., Ren, Z., Liu, D. et al. (2016) Characterizing the population structure and genetic diversity of maize breeding germplasm in Southwest China using genome-wide SNP markers. *BMC Genomics*, 17, 697.

Zhu, J.K. (2002) Salt and drought stress signal transduction in plants. *Annual Review of Plant Biology*, 53, 247–273.

Zhu, J.K. (2016) Abiotic stress signaling and responses in plants. *Cell*, 167(2), 313–324.

Zhu, Q., Dugardeyn, J., Zhang, C., Mühlenbock, P., Eastmond, P.J., Valcke, R. et al. (2014) The Arabidopsis thaliana RNA editing factor SLO2, which affects the mitochondrial electron transport chain, participates in multiple stress and hormone responses. *Molecular Plant*, 7(2), 290–310.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Liang, T., Qing, C., Liu, P., Zou, C., Yuan, G., Pan, G. et al. (2022) Joint GWAS and WGCNA uncover the genetic control of calcium accumulation under salt treatment in maize seedlings. *Physiologia Plantarum*, 174(1), e13606. Available from: https://doi.org/10.1111/ppl.13606