Identification of candidate genes involved in salt stress response at germination and seedling stages by QTL mapping in upland cotton

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Abstract

Salinity is a major abiotic stress at critical stages of seed germination and seedling establishment. Germination rate (GR) and field emergence rate (FER) are the key traits that determine the basic number of plants stand under field conditions. To explore molecular mechanisms in upland cotton under salt stress, a population of 177 recombinant inbred lines, and their parents were evaluated for seed germination traits (GP, germination potential; GR, FW, fresh weight; DW, dry weight; GL, germinable length) and seedling traits (FER, SH, seedling height; NL, number of main stem leaves) in 2016–2018. Based on the linkage map contained 2,859 single nucleotide polymorphism and simple sequence repeat markers, traits under salt stress (E1) and normal conditions (E2), and in the converted relative index (R-value) dataset of 3 years’ trials were used to map quantitative trait loci (QTL). A total of 3 QTL and 2 clusters were detected as salt-tolerant QTL. Three QTL (qGR-Chr4-3, qFER-Chr12-3, and qFER-Chr15-1) were detected under salt stress conditions and R-value dataset, which explained variance of phenotype 9.62–13.67%, 4.2–4.72%, 4.75–8.96%, respectively. Two clusters (Loci-Chr4-2 and Loci-Chr5-4) harboring the QTL for 4 germination traits (GR, FER, GL, and NL) and 6 seedling traits (GR, FER, DW, FW, SH, and NL) were detected related under salt stress. A total of 691 genes were found in the candidate QTL or clusters. Among them, 4 genes (Gh_A04G1106, Gh_A05G3246, Gh_A05G3177, and Gh_A05G3266) showed expression differences between salt-sensitive and -tolerant lines under salt stress conditions, and were assigned as candidate genes in response to salt stress. The consistent salt-tolerance QTL identified in both germination and seedling stages will facilitate novel insights into effective utilization of cotton genetic resources.

Keywords: salt stress; seed germination; seedling emergence; quantitative trait loci; upland cotton

Introduction

High salinity is one of the major abiotic stresses limiting plants growth and decreasing the yield (Parida and Das 2005; Ismail and Horie 2017). Salinity affects 1 billion hectares of arid and semi-arid areas globally, and soil salinity will become progressively more severe due to climatic changes, excessive irrigation, and excessive fertilization (Han et al. 2015).

Salt stress inhibits osmotic and ionic homeostasis at a cellular level, further inhibits photosynthesis and reduces cellular energy, and eventually results in redox imbalance during seed imbibition, germination, and root elongation (Liang et al. 2018). Many factors can affect the salt tolerance evaluation of plants, including the phase of plant growth, the duration of exposure to salinity, the salt concentration, the plant species, and the method of evaluation (Higbie et al. 2010). Plants at germination, emergence, and seedling stages are more susceptible to salt stress than other stages (He et al. 2019). The reduced germination potential (GP), fresh mass, and vigor index lead to delayed germination and fewer germinated seeds (Ahmad et al. 2002). At seedling stage, root is the first organ exposed to salt stress (Galvan-Ampudia et al. 2013; Robbins et al. 2014). Salinity generally disrupts the cell cycle activity at the root meristems, resulting in reduced root development and afterwards plant growth in cotton (West et al. 2004). To evaluate the performance and tolerance in upland cotton plants under salt stress, germination, and seedling characteristics were the most viable criteria (Ahmad et al. 2002). Most common salt tolerance traits used in the quantitative trait loci (QTL) mapping under salt stress include GP, germination rate (GR), and root growth-related traits (shoot length, root length, root fresh weight, and root dry weight) (Du et al. 2016; Diouf et al. 2017; Yuan et al. 2019; Sikder et al. 2020). Traits such as plant height, shoot dry weight, and root dry weight are sensitive to a salinity of 150 mM NaCl at the seedling stage of 5–6 leaves in cotton (Du et al. 2016).

Upland cotton (Gossypium hirsutum L., AADD, 2n = 52), an important cash crop, is considered as a pioneer crop in saline-alkali
land. The release of genome data of upland cotton (Li et al. 2015; Zhang et al. 2015; Hu et al. 2019; Wang et al. 2019; Yang et al. 2019; Chen et al. 2020; Huang et al. 2020) has greatly facilitated researches to develop high-density linkage map construction. Different strategies were used to construct genetic maps (Jamsheed et al. 2016; Zhang et al. 2016). Currently, array-based single nucleotide polymorphism (SNP) markers are the effective genotyping method in cotton due to their low cost and limited reliance on computational resources (Jia et al. 2016). A newly released genetic map harbored 3,009 polymorphic SNP markers and covered a total genetic distance of 4,983.73 cM with an average marker interval of 1.66 cM (Zhu et al. 2021). Furthermore, a high-density linkage map containing 6,187 bin markers spanning 4,478.98 cM with an average marker interval of 0.72 cM has been constructed (Guo et al. 2020).

QTL mapping is an effective strategy for quantitative trait research, and has been widely used in agriculture to map a set of traits, such as salt tolerance (Ashraf and Foolad 2013). To date, numerous QTL for seed germination and seedling growth under salt stress conditions have been identified. A total of 8 clusters controlled at least 2 salt-tolerance traits located on chromosomes (Chr) A02, A06, A12, D03, D06, D08, and D13 were identified using a F2:3 population of 277 lines derived from an interspecific cross between 2 upland cotton accessions CCR135 and NH. In these clusters, 12 genes were highly expressed in response to salt treatment (Diouf et al. 2017). Moreover, 11 consistent QTL have been identified in 8 chromosomes with qRL-Chr16-1 of root length, explaining the variance of phenotype ranged from 11.97% to 18.44% (Oluoch et al. 2016). Furthermore, 13 QTL controlled dry root weight on 9 chromosomes have been detected under salt stress treatment in an introgressed recombinant inbred line (RIL) population of upland cotton (Abdelrahem et al. 2018). The QTL qSat-A04-1 associated with salt tolerance in the seed germination stage has been characterized via using an RIL upland cotton population of 577 lines (Gu et al. 2021). Several important SNPs responding to salt stress at seedling stage have also been identified in upland cotton, for instance, 23 SNPs, which represented 7 genomic regions in Chr A01, A10, D02, D08, D09, D10, and D11, have been found to be associated with the relative survival rate (RSR) and salt tolerance level (STL) by using a natural population comprising 713 upland cotton accessions CCR135 and NH. Of which, 6 genes (Gh_D10G1888, Gh_D02G0060, Gh_D09G0943, Gh_D10G1821, Gh_D09G0958, and Gh_A10G1756) related to salt tolerance were validated (Gu et al. 2021). In addition, a total of 4 genes (GhIPF3A, GhSAG29, GhTZF4, and GhTZF4e) located on chromosome D01 were assigned as candidate genes associated with relative GR under salt stress conditions (Sun et al. 2019).

Seedling number is critical for population construction in commercial production all over the world. However, few studies have been reported on the effects of salt stress at the seedling stage in upland cotton under natural saline conditions. In the present study, 8 phenotypic traits related to salt tolerance were evaluated and data were collected from 177 RILs derived from a cotton hybrid “Xinza 1” (Shang et al. 2015, 2016). The genetic linkage map consisting of 2,859 bins was used to analyze salt tolerance-related QTL (Guo et al. 2021). The objective of this research was to identify candidate genes in response to salt stress from the QTL and provide valuable insights into the mechanisms underlying salt stress. The findings of this study provide valuable insights in developing salt-tolerant cultivars and screening salt stress-responsive genes.

Materials and methods

Plant materials

The RIL population derived from a hybrid “Xinza 1” (GX1135 × GX100-2) was employed by single seed descent method in upland cotton (Shang et al. 2016), including 177 lines. The control set included GX1135, “Xinza 1” F1, GX100-2, and a commercial hybrid “Ruiza 816” used as competition control (Ma et al. 2020; Guo et al. 2022). A total of 177 RILs of F16–F18 generations were assigned in the field salt stress experiment. One hundred forty-four RILs of F16 generations were used in the indoor germination experiment (Supplementary Table 1).

Indoor seed germination test and phenotypic evaluation

The salt tolerance experiment was carried out under 150 mM NaCl and control conditions, respectively, and both with a 12–12 h day/night photoperiod, corresponding to a temperature of 28°C ± 1°C. A total of 300 full and robust tufted seeds were selected for the salt tolerance experiment, of which 50 seeds for each repeat were soaked for 12 h. For salt stress treatment, the seeds were soaked in 150 mM NaCl solution for 12 h, then wrapped in the filter paper and irrigated with 150 mM NaCl solution to maintain water levels (2L) at germination stage. Under normal conditions, deionized water was used during the test period. The germination test was carried out by using the method of filter paper roll germination. A replicated complete design with 3 biological replicates was adopted in salt stress or normal conditions.

To analyze the traits, we performed indoor germination experiments in which cotton seeds were sown enrolling within the filter papers. On the third day after sowing, we investigated the trait GP (%). On day 7 after sowing, we investigated the traits GR (%), germinable length (GL, cm), fresh weight (FW, g), and dry weight (DW, g).

Field arrangement and traits evaluation

Two field trials under salt and control conditions were conducted in 2017, 2018, and 2019 in Quzhou Experimental Station of China Agricultural University, Handan City, Hebei Province (36°7′8″N, 114°92′E).

Two independent field trials were arranged in neighboring fields following a randomized complete block design with 2 replications each trial on 2017 May 2 (spring of 2017, 2017t1), 2017 August 22 (summer of 2017, 2017t2), 2018 April 29 (spring of 2018, 2018t1), and 2019 May 6 (spring of 2019, 2019t1), and 2019 July 6 (summer of 2019, 2019t2). For the field salt tolerance experiments, field emergence rate (FER), seedling height (SH), and number of main stem leaves (NL) were determined at the seedling stage. The FER was investigated on 2017 May 16, 2017 August 31, 2018 May 17, 2019 May 28, 2019 July 30, respectively. The SH and NL traits were investigated on 2019 June 17 and 2019 August 16.

A total of 362 plots with 2 rows (22 individuals each row) were conducted, respectively. Two repeats of 177 RILs (F16–F18) were planted together with 2 control sets (GX1135, GX100-2, “Xinza 1,” and “Ruiza 816”). Every 2-row plot was 80 and 60 cm row spacing with plot length of 2.4 m, following 0.7 m pavement apart for field experiments.

For salt stress treatment, shallow saline groundwater with the concentration of 5 g/L (85 mM) saline was used to irrigate the field twice before sowing in 2017 and 2018 (Guo et al. 2021), and shallow saline groundwater with the concentration of 7 g/L (120 mM) saline was used to irrigate the field in 2019. For the control treatment, regular irrigation was performed when needed. Field
management followed the local standard field practices. The normal irrigation treatment included regular irrigation consistent with the standard agronomic practices for the conditions in that year.

**Soil samples collected and soil property measurement**

Soil samples were collected from the depth of 0 to 20 and 20 to 40 cm after sowing. To cover the experiment area, sampling points were chosen every 15 m from north to south in the experiment field. Three soil samples collected for each sample site were mixed into 1 sample for soil quality determination (Guo et al. 2021). Soil saturated paste extracts (1:2 by weight) were prepared to measure the electrical conductivity (EC) and total content of water-soluble salt (ρ) of the samples (Rhoades 1996; Shang et al. 2016). Three independent repeats were detected for each sample.

**Genetic linkage map construction**

Linkage map analysis was conducted using Join Map 4.0. The adjacent markers from the same parent were recorded as 1 bin (Xie et al. 2010). A total of 2,859 bins which including 330 simple sequence repeat (SSR) markers and 2,529 SNPs were distributed on the new linkage map (Supplementary Fig. 2). The genetic map covered 2,133.53 cM of cotton genome with an average interval of 0.785 cM (Guo et al. 2021). Among the 26 linkage groups, the average bin spacing was 5.979 cM, and the range of interval on 26 chromosomes was 3.647–10.361 cM, and only 1 gap larger than 10 cM existed on Chr D05.

**Data analysis and QTL mapping**

Three datasets of (1) salt stress conditions (E1); (2) normal conditions (E2); and (3) the converted relative index (R-value) were used in the present study. The original data of 8 salt-tolerant-related traits were obtained from the trials under E1 and E2, respectively. The R-value dataset of 8 salt-related traits were calculated using the following formula: (phenotypic effects value under E1/phenotypic effects value under E2) × 100%. To estimate phenotypic traits across multiple environments, the phenotypic values of the accessions from multiple environments were estimated via best linear unbiased prediction based on a linear model fitted with the lme4 R package (Technow et al. 2012). Analysis of variance (ANOVA) was conducted for all traits on a linear model fitted with the lme4 R package (Technow et al. 2012). The parents of XZ1 (female GX1135 and male GX100-2) were used in the present study. The original data of 8 salt-tolerant-resistant-trait datasets were obtained in the trials under E1 and E2, respectively. The R-value dataset of 8 salt-related traits were calculated using the following formula: (phenotypic effects value under E1/phenotypic effects value under E2) × 100%. To estimate phenotypic traits across multiple environments, the phenotypic values of the accessions from multiple environments were estimated via best linear unbiased prediction based on a linear model fitted with the lme4 R package (Technow et al. 2012). Analysis of variance (ANOVA) was conducted for all traits separately for estimating variance components for evaluation of the significance of genotypes and environmental effects and their interactions in the RIL population. SD, coefficients of variation, and ANOVA, and correlation analyses were estimated using the software SPSS (Version 21.0, Chicago). The broad-sense heritability percentage, $h^2_b$, was calculated for each trait using the formula (Singh et al. 2017):

$$h^2_b = \frac{\sigma^2_G}{\sigma^2_G + \sigma^2_{G\times E} + \sigma^2_E} \times 100\%.$$  

With $\sigma^2_G$ is the genotypic variance; $\sigma^2_{G\times E}$ is genotype × environment interaction; $\sigma^2_E$ is phenotypic variance (PV); $n_e$ is the number of environments, and $n_i$ is the number of replications for each environment.

QTL mapping and the genetic effect values at single-locus level were conducted by QTL Cartographer software (Version 2.5) using composite interval mapping (CIM) method (Wang et al. 2007). We set parameter in the confidence interval of 95% with the CIM method for QTL mapping. The threshold of LOD values was estimated after 1,000 permutations tests to declare a significant QTL with a significance level of $P < 0.05$, whereas QTL in another trial with LOD of at least 2.0 was considered as common QTL (Liang et al. 2013; Shang et al. 2016).

**Gene function annotation and enrichment analysis**

Genes located in the confidence intervals of the candidate QTL were fetched from the CottonGen (https://www.cottongen.org) by using their positions of flanking markers in G. hirsutum TM-1 genome (Zhang et al. 2015) and considered as the candidate genes. Gene ontology (GO) enrichment and KEGG pathway analysis were carried out for all candidate genes. The GO enrichment was performed on BMK Cloud platform (www.biocloud.net).

**RNA sequencing and analysis**

The parents of XZ1 (female GX1135 and male GX100-2) were used for RNA-seq analysis. Seeds were germinated in the sand and then the identical seedlings were transferred into modified 1/2 Hoagland solution. The seedlings were planted in the 1/2 Hoagland solution containing 150 mM NaCl at the 3-leaf stage (Guo et al. 2015). Plants were grown at 28°C/20°C, and a photoperiod of 14 h light/10 h dark. Roots of these seedlings were sampled at 1, 3, 12, and 48 h after salt stress treatment, and frozen with liquid nitrogen and stored at −80°C for use.

Total RNA was extracted from the roots of GX1135 and GX100-2 seedlings using a modified cetyltrimethyl ammonium bromide method (Zhao et al. 2012), which were treated with 150 mM NaCl solution for 0, 1, 3, 12, and 48 h, respectively, and with 3 biological replicates. RNA-seq libraries were sequenced on Illumina Hiseq 2500 platform (Biomarker Technology Corporation, Beijing, China).

The clean reads of each library were aligned to the reference genome using TopHat software (Version 2.0.1). Gene expression levels were calculated by Cufflinks (Version 2.2.1) of fragments per kilobase of exon per million mapped reads (FPKM) value (Trapnell et al. 2012). Differentially expressed genes (DEGs) were detected using DESeq (Wang et al. 2010) with F-value <0.01 and fold change (salt treated cv. Oh) >2, which was performed on the BMK Cloud platform (www.biocloud.net).

**RNA extraction and gene expression validation**

Total RNA was isolated from the leaf of salt-tolerant lines (T), and salt-sensitive lines (S) under salt stress conditions and normal conditions (Zhao et al. 2012), respectively. To validate the potential function in salt stress response, the expression patterns of candidate genes were verified with qRT-PCR using RNA of leaf in salt-tolerant lines and salt-sensitive lines. Gene relative expression level was calculated with $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001). Primers for the qRT-PCR analysis were listed in Supplementary Table 2. Three independent replicates were performed for each sample. GhUBQ7 gene was used as a reference gene.

**Results**

**Phenotypic performance under salt stress condition and normal condition**

To ensure the effectiveness of salt stress in the field trial, the EC and ρ of soil samples in the experimental area were measured (Supplementary Table 3). The average ρ of soil samples in the
depth of 0–20 cm collected during the sowing period in 2018 were identified as mild-soil salinization (1.67 g/kg) in normal condition and moderate soil salinization (2.08 g/kg) in saline soil, respectively. The average p of soil in 20–40 cm depth in 2018 was slightly salinized (0.98 g/kg) under E2 and slightly salinized (1.66 g/kg) under E1. According to the grading standard of saline soil based on EC, the soil samples collected in the depth of 0–20 cm (0.88–0.95 g/kg) was nonsaline soil, while the highly saline soil (4.37 g/kg, 2019t2) or moderately salinized soil (3.97 µs/cm, 2019t1) was collected in the depth of 20–40 cm. The results indicate that there was a difference in salt concentration between the 2 experiments after saline irrigation.

Salt stress significantly inhibited the germination and seedling formation of cotton in both fields and laboratory experiments (Supplementary Figs. 3–6). In most cases, the difference between the 2 parents was significant under E2, but not significant under E1. The phenotypic of male parent GX100-2 is better than that of female parent GX1135. The transgressive inheritance. The absolute values of kurtosis and skewness for most traits were <1, except for the kurtosis values of FW and DW were >10.

The ANOVA results revealed significant differences between the year (Y), environment (E), and Y * E interaction for all 5 traits (FER, GP, SH, and NL), and significant genetic effect only existed for FER and NL. The broad-sense heritability of the traits ranged from 14.72 (DW) to 39.69 (FER) (Table 1).

**Correlation analysis among salt-tolerant related traits**

Correlation analysis was performed using the phenotypic values of salt-tolerant traits under E1 and E2, as well as R-value (Table 2, Supplementary Table 5). Under E2, GR was significantly positively correlated with FER in 2018 but was not correlated with FER in 2017 and 2019. Under E1, the correlation was not significant between GR and FER in 3 years’ trials (Table 2). NL was significantly positively correlated with SH under E1 and E2 (Supplementary Table 5). No significant correlation was detected for other traits (Table 3 and Fig. 1). A total of 12 stable QTL were detected, of which 2 QTL (qFER-Chr5-1, qFER-Chr10-1) were detected in E1 and E2, 7 QTL (qFER-Chr13-1, qFER-Chr15-1, qGP-Chr15-2, qGP-Chr19-1, qGR-Chr4-3, and qSH-Chr19-1) were detected in E1 and R-value, and 2 QTL (qFER-Chr5-1, qFER-Chr10-1) were detected in E2 and R-value, respectively. A total of 4 QTL were detected in both E1 and R-value at the germination stage with 3 QTL (qGP-Chr1-1, qGP-Chr15-2, and qGP-Chr19-1) for GR and 1 (qGR-Chr4-3) for GR.

A total of 5 QTL, which explained the PV of 4.04–8.95%, were detected in the field trials at least 2 years with 4 (qFER-Chr2-1, qFER-Chr4-2, qFER-Chr10-1, and qFER-Chr14-3) for E1 and 1 (qNL-Chr5-3) for NL, respectively. Four of the stable QTL, qFER-Chr3-2, qGP-Chr15-2, qGP-Chr19-1, and qGR-Chr4-3, explaining more than 10% of the PV (5.34–17.34%) in at least 1 environment were identified as major QTL. Under E1, the number of QTL received favorable alleles from the female GX1135 (19) was more than that from the male GX100-2 (17). However, the number of QTL received favorable alleles contributed by the female GX1135 (20) was less than that from the male GX100-2 (28) under E2. We also detected many QTL (125) in 1 environment (Supplementary Table 6). These major and stable QTL were used to explore salt tolerance genes in upland cotton.

### Table 1. ANOVA and broad-sense heritability for 8 salt-tolerant traits in RIL population.

| Trait | Source | Df | Mean Sq | F value | Pr > F | h²B (%) |
|-------|--------|----|---------|---------|--------|----------|
| FER   | Y      | 3  | 227,788 | 2,489.58 | c      | 39.69    |
|       | E      | 1  | 2,168   | 23.69    | c      |          |
|       | G      | 176| 383     | 4.191    | c      |          |
| Y*E   | 3      | 33,134 | 362.135 | c      |        |
| Y*G   | 528    | 184 | 2.01    |         |        |
| E*G   | 176    | 97  | 1.063   |         |        |
| Y*E*G | 480    | 88  | 0.96    |         |        |
| SH    | Y      | 2  | 3,268   | 149.608  | c      | 26.20    |
|       | E      | 1  | 47,231  | 2,161.974| c      |          |
|       | G      | 176| 25      | 1.137    | c      |          |
| Y*E   | 1      | 3,108 | 142.269 | c      |        |
| Y*G   | 176    | 32  | 1.462   |         |        |
| E*G   | 176    | 20  | 0.894   |         |        |
| Y*E*G | 113    | 35  | 1.593   |         |        |
| NL    | Y      | 2  | 751.1   | 1,194.86 | c      | 18.42    |
|       | E      | 1  | 928.8   | 1,477.521| c      |          |
|       | G      | 176| 0.8     | 1.243    | c      |          |
| Y*E   | 2      | 182.6 | 290.532 | c      |        |
| Y*G   | 352    | 0.7 | 1.193   |         |        |
| E*G   | 176    | 0.4 | 0.687   |         |        |
| Y*E*G | 286    | 0.5 | 0.835   |         |        |
| GP    | E      | 1  | 288,349 | 465.576  | c      | 18.00    |
|       | G      | 148| 1,196   | 1.932    | c      |          |
|       | F*G   | 148| 459     | 0.742    | c      |          |
| GR    | E      | 1  | 44,371  | 89.048   | c      | 24.17    |
|       | G      | 148| 1,386   | 2.782    | c      |          |
|       | F*G   | 148| 336     | 0.675    | c      |          |
| FW    | E      | 1  | 9.668   | 651.928  | c      | 34.02    |
|       | G      | 148| 0.021   | 1.428    | b      |          |
|       | F*G   | 148| 0.013   | 0.863    | a      |          |
| DW    | E      | 1  | 0.0019927 | 9.738   | b      | 14.72    |
|       | G      | 148| 0.0003959 | 1.935   | c      |          |
|       | F*G   | 148| 0.0001581 | 0.773   | c      |          |
| SL    | E      | 1  | 33,116  | 11,025.41| c      | 18.31    |
|       | G      | 148| 23      | 7.683    | c      |          |
|       | F*G   | 148| 21      | 6.844    | c      |          |

a,b,c Signiﬁcant at P = 0.05, P = 0.01, and P < 0.001, respectively.

| Field emergence rate; SH, seedling height; NL, number of main stem leaves; GP, germination potential; GR, germination rate; FW, fresh weight; DW, dry weight; GL, germinal length; E, environment (salt stress conditions and normal conditions); G, genotype; h²B, broad-sense heritability.
### Table 2.
Correlation analyses for FER and GR traits under salt stress conditions, normal conditions, and -value.

| Environment | Trait   | Year 1 | Year 2 | Year 3 | Year 4 | Year 5 | Year 6 | Year 7 | Year 8 | Year 9 | Year 10 | Year 11 | Year 12 | Year 13 | Year 14 |
|-------------|---------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| E1          | FER     |         |        |        |        |        |        |        |        |        |        |        |        |        |        |
| E1          | GR      |         |        |        |        |        |        |        |        |        |        |        |        |        |        |
| E2          | G-value |         |        |        |        |        |        |        |        |        |        |        |        |        |        |

There was a significant correlation at 0.05 level (bilateral). The critical value for correlation coefficients at probabilities of 0.05 and 0.01 are 0.145 and 0.190, respectively.

#### Pleiotropic effects

A QTL cluster is defined as a QTL region on a chromosome that contains many QTL associated with different traits (Rong et al. 2007). Here, totally, 25 clusters distributed on 16 chromosomes showed pleiotropic effects involving 74 QTL (Table 4, Supplementary Table 7). Among them, 4 clusters were identified on Chr5, 3 were detected on Chr4 and Chr15 each, and 2 were detected on Chr1 and Chr24 each; only 1 was detected on the other 11 chromosomes, respectively.

A total of 4 clusters (Loci-Chr4-1, Loci-Chr4-2, Loci-Chr5-3, Loci-Chr5-4) harbor both FER and GR traits. Among them, QTL (qFER-Chr4-1) with favorable alleles contributed by the GX100-2 were detected in R-value in 2017, and qGR-Chr4-1 with favorable alleles contributed by the GX1135 detected in R-value was involved in Loci-Chr4-1. While the second, Loci-Chr4-2 contains 6 QTL, of which 2 QTL controlled FER (qFER-Chr4-2, qFER-Chr4-3), which were detected in E1 in 2017 and 2018, and 2 controlled GR (qGR-Chr4-2, qGR-Chr4-3), which were detected in E1 and R-value, and the region also controlled NL (qNL-Chr4-1) and GL (qGL-Chr4-2). The third, QTL (qFER-Chr5-3) controlled FER on Loci-Chr5-3 was detected in E2 in 2018 and QTL qGR-Chr5-1 that controlled GR detected in E1, which received favorable alleles contributed by the GX100-2. The fourth, 6 QTL were detected on Loci-Chr5-4, of which, qFER-Chr5-4, affecting FER was detected in E1 and E2 in 2018, and qGR-Chr5-2 was detected in E1. This cluster also controlled DW, FW, SH, and NL, and the favorable alleles in the QTL originated from the male parent GX100-2.

Only 1 cluster (Loci-Chr19-1) controlled GP and GR in the present study including 1 QTL (qGP-Chr19-1) detected in E1 and R-value, and another (qGR-Chr19-1) detected in E2. The favorable alleles in these 2 QTL were contributed by GX1135. Six clusters (Loci-Chr2-1, Loci-Chr3-1, Loci-Chr4-3, Loci-Chr5-2, Loci-Chr15-2, Loci-Chr15-3) were detected controlling FER and GP.

#### Identification of candidate genes in response to salt stress

A total of 4 clusters (Loci-Chr4-1, Loci-Chr4-2, Loci-Chr5-3, Loci-Chr5-4) controlling FER and GR were detected. Two clusters (Loci-Chr4-2 and Loci-Chr5-4) explaining PV of >10% in at least 1 experiment were selected for further analysis. The cluster (Loci-Chr4-2) includes 6 QTL with qFER-Chr4-2 controlling FER in E1 across 2 years (2017 and 2018). 2 QTL (qGR-Chr4-2, qGR-Chr4-3) for GR explained 9.63–13.67% of PV in R-value and E1 and 3 QTL controlling GP, GL, and NL. The favorable alleles of these QTL were contributed by GX1135 except qGR-Chr19-1. The confidence interval of Loci-Chr4-2 was overlapped between the marker bin 457 and bin 479, which was corresponding to S9,365,196–61,232,513 bp in Chr A04, including 137 genes (GA04G1024–GA04G1110). Among them, there are 116 annotated genes and 21 genes with uncharacterized protein information. Loci-Chr5-4 included 6 QTL, of which the QTL qFER-Chr5-4 controlled FER explained 4.71% (E1) and 5.50% (E2) of PV, respectively, and 1 QTL (qGR-Chr5-2) controlled GR explained 5.50% of PV were detected in E1, the loci also controlled NL, SH, FW, and DW. The favorable alleles of these QTL were contributed by GX100-2. The confidence interval of Loci-Chr5-4 was overlapped between the marker bin 633 and bin 645, which was corresponding to 83,534,339–88,001,753 bp in Chr A05. The physical distance of this interval is 4,467,414 bp harboring 175 genes (GA05G3184–GA05G3358).

We annotated these genes by GO and classified the GO terms. These genes within these 4 clusters were divided into 52 subgroups, belonging to 3 major categories including cellular
Table 3. Major QTL or stable QTL for salt-tolerant related traits.

| Trait       | QTL                 | Year | Flanking marker | Physical interval | Under E1 | Under E2 | R-value |
|-------------|---------------------|------|----------------|-------------------|----------|----------|---------|
|             |                     |      | L              | R                 | LOD      | A        | Var%    |         | LOD      | A        | Var%    |         |
| FER (%)     | gFER-Chr2-1*        | 2017t1 | bin159         | bin160            | 17,151,118 | 18,031,248 | 3.02    | 1.48    | 6.03    |
|             |                     | 2017t1 | bin161         | bin162            | 27,475,788 | 24,856,482 | 2.51    | 1.21    | 4.51    |
|             | gFER-Chr3-2         | 2019t1 | bin166         | bin167            | 40,699,263 | NBR10014   | 2.62    | 2.93    | 4.83    |
|             |                     | 2019t1 | bin248         | bin249            | 4,056,547 | 4,699,558  | 3.86    | -3.36   | 8.04    | 8.7      | -6.53   | 17.34   |
|             | gFER-Chr4-2*        | 2017t1 | bin468         | bin469            | 203,840    | 171,548    | 3.24    | 1.93    | 6.53    |
|             |                     | 2018  | bin478         | bin479            | 60,980,959 | 61,232,513 | 4.7     | 3.01    | 8.95    |
|             | gFER-Chr5-1         | 2019t2 | bin556         | bin557            | NAU1042    | 21,063,321 | 3.13    | 11.45   | 6.67    |
|             |                     | 2018  | bin632         | bin633            | 83,151,316 | 83,550,175 | 2.6     | -2.32   | 5.5     |
|             | gFER-Chr5-4         | 2019t1 | bin1052        | bin1053           | 84,715,085 | 84,316,379 | 2.8     | -2.19   | 4.71    |
|             |                     | 2018  | bin1057        | bin1058           | 14,338,049 | 20,743,965 | 3.37    | 2.94    | 6.94    |
|             | gFER-Chr12-3        | 2018  | bin1305        | bin1306           | 7,544,817  | 33,791,740 | 2.01    | 4.57    | 4.04    |
|             |                     | 2018  | bin1311        | bin1312           | 63,877,295 | 26,826     | 2.61    | 2.19    | 4.72    |
|             | gFER-Chr14-2        | 2017t1 | bin1527        | bin1528           | 16,178,195 | 24,117,598 | 5.26    | -2.09   | 11.36   |
|             |                     | 2018  | bin1538        | bin1539           | 30,930,147 | 42,055,147 | 2.93    | -2.36   | 5.64    |
|             | gFER-Chr14-3*       | 2017t1 | bin1540        | bin1541           | 45,210,599 | 54,668,176 | 3.3     | -1.71   | 7.71    |
|             |                     | 2017t1 | bin1571        | bin1572           | 985,874    | SWU13909   | 4.57    | -1.71   | 8.96    |
| GP (%)      | gGP-Chr1            | 2018  | bin4           | bin4              | 5,253,419  | 5,253,419  | 3.46    | 3.4     | 7.98    |
|             |                     | 2018  | bin5           | bin6              | 5,275,311  | 5,383,055  | 3.23    | 2.97    | 7.68    |
|             | gGP-Chr19-1         | 2018  | bin1650        | bin1651           | 37,570,122 | 37,527,797 | 2.29    | -2.44   | 5.34    |
|             |                     | 2018  | bin2063        | bin2064           | 13,292,260 | 12,935,942 | 6.8     | 3.42    | 9.85    |
| GR (%)      | gGR-Chr4-2          | 2018  | bin457         | bin458            | 59,365,198 | 59,380,549 | 6.5     | 3.55    | 16.17   |
|             |                     | 2018  | bin462         | bin463            | 60,287,107 | 60,207,332 | 6.18    | 4.05    | 13.67   |
|             | gGR-Chr4-3          | 2018  | bin467         | bin468            | 193,255    | 203,840    | 4.31    | 3.09    | 9.62    |
| NL (%)      | gNL-Chr5-3*         | 2018  | bin2416        | bin2417           | NAU3966    | 37,287,704 | 4.81    | -3.53   | 10.4    |
|             |                     | 2018  | bin642         | bin643            | 85,089,802 | SWU20917   | 5.25    | -0.21   | 11.22   |
| SH (cm)     | gNH-Chr10-2         | 2017t1 | bin664         | bin665            | 87,061,817 | 88,001,753 | 7.01    | -0.2    | 13.99   |
|             | gSH-Chr5-1          | 2017t1 | bin1115        | bin1116           | NAU3540    | SWU20501b  | 4.77    | 0.11    | 10.25   |
|             |                     | 2018  | bin554         | bin555            | NAU3530    | NAU828     | 6.45    | -2.15   | 21.03   |
|             | gSH-Chr5-2          | 2018  | bin750         | bin751            | 66,154,600 | 77,314,184 | 6.12    | 1.31    | 11.38   |
|             |                     | 2018  | bin2054        | bin2055           | 8,848,052  | 8,858,304  | 2.2     | -1.92   | 4.57    |
|             | gSH-Chr5-4          | 2018  | bin2054        | bin2055           | 8,848,052  | 8,858,304  | 3.21    | -1.02   | 6.78    |

FER, Field emergence rate; GP, germination potential; GR, germination rate; NL, Number of main stem leaves; SH, seedling height; FW, fresh weight; DW, dry weight; GL, germinial length. 2017t1, spring of 2017; 2017t2, summer of 2017; 2019t1, spring of 2019; 2019t2, summer of 2019. E1, salt stress conditions; E2, normal conditions; R-value, the converted relative index dataset. A, additive effect. Var%, phenotypic variation explained by a single locus QTL (%). Figures underlined referred to the common QTL detected on 2 datasets in the present study. QTL noted by ** were referred to common QTL detected on 2 datasets. Bold fonts referred to stable QTL that explained phenotypic variation >10%.

To explore the candidate genes related to salt tolerance, RNA-seq analysis was performed using RNA extracted from the root of GX1135 and GX100-2 at different time points (1, 3, 12, and 48h) after salt treatment. After differential expression analysis (P < 0.01, absolute value fold change ≥2) of the 2 parents at different sampling points, 5,131 DEGs were detected (Supplementary Table 9).

QTL identified in both salt stress conditions and R-value can be used as salt-tolerance-related QTL. Two clusters (Cluster-Chr4-2, Cluster-Chr5-4) controlled 3 or more traits, and 3 QTL (qFER-Chr12-3, qFER-Chr15-1, qGR-Chr4-3) detected in at least 2 datasets were selected for further analysis. A total of 370 genes were identified in candidate QTL, of which 334, 32, and 4 genes were identified within Loci-Chr4-2 and Loci-Chr5-4, 5 genes (Gh_A04G1053, Gh_A05G3291, Gh_A05G3266, Gh_A05G3257, Gh_A04G1106) were upregulated in GX100-2 and downregulated in GX1135.
after salt stress treatment, and 4 (Gh_A04G1046, Gh_A05G3246, Gh_A04G1036, Gh_A05G3177) showed opposite expression patterns (Fig. 2a; Supplementary Table 10). The confidence interval of qFER-Chr12-3 was overlapped between marker bin 1305 and bin 1312, corresponding to the reference genome of upland cotton TM-1 7,544,817–33,791,740 bp in Chr A12, the physical distance of this interval is 26,246,923 bp, harboring 334 genes (Gh_A12G0392–Gh_A12G0725). We found that 7 DEGs within the QTL qFER-Chr12-3 presented different expression patterns after 150 mM NaCl treatment. A total of 4 genes (Gh_A12G0415, Gh_A12G0615, Gh_A12G0437, Gh_A12G0468) were upregulated in GX100-2 and downregulated at all time points in GX1135 after salt stress treatment, and 3 (Gh_A12G0499, Gh_A12G0501, Gh_A12G0495) showed opposite expression patterns after salt stress (Fig. 2b; Supplementary Table 10).

To validate the potential function of candidate genes in salt stress response, the expression patterns of 16 genes were verified with qRT-PCR in 2 salt-tolerance lines (T) and 2 salt-sensitive lines (S) at germination stages after salt stress (Fig. 3). The expression of 5 genes (Gh_A12G0415, Gh_A12G0615, Gh_A12G0437, Gh_A12G0499, Gh_A12G0495) were not detectable in salt-tolerance and -sensitive lines. Three genes (Gh_A04G1106, Gh_A05G3246, Gh_A05G3177) were significantly downregulated in salt-tolerant lines and no significant difference or upregulated in salt-sensitive lines under salt stress conditions. The gene (Gh_A05G3266) encode zinc finger BED domain-containing protein which was downregulated in 2 salt-sensitive lines while upregulated in salt-tolerant lines under salt stress conditions. The other 7 genes showed the same expression pattern in salt-tolerance and salt-sensitive lines after salt stress treatment.

**Discussion**

Salt tolerance is a genetically complex trait controlled by many minor-effect genes (Flowers 2004). With the rapid development of SNP arrays and next-generation sequencing technology, the efficiency of QTL mapping applied in cotton salt tolerance was greatly improved. Little research has been reported toward determining the effect of salt stress at the seedling stage in upland cotton, especially under field conditions. In the present study, 5 salt-
tolerant related traits (GP, GR, FW, DW, and GL) at germinating stage and 3 (FER, SH, and NL) at seedling stage were evaluated for salt-tolerance, and then QTL mapping was performed to identify genetic loci related to salt tolerance. Then RNA-seq analysis was used to identify genes within the QTL intervals that are responsive to salt stress treatments.

**Comprehensive analysis of salt tolerance at germination and seedling stages**

Plants are more sensitive to salt stress at germination and seedling stages than all the other stages (Ahmad et al. 2002). Most researches about salt tolerance evaluation were performed in laboratory experiments, such as paper roll systems (Yasir et al. 2019), hydroponics (Oluoch et al. 2016), and pot experiments (Wang et al. 2020) at germination or seedling stage. These methods allow a rapid, accurate, and high-throughput analysis of salt-tolerance at an early growth stage; however, it is difficult to evaluate the actual growth pattern of plants under field salt stress environment. In the present study, an RIL population was used for salt tolerance QTL mapping in both controlled indoor environment and field conditions, which could evaluate the salt tolerance of cotton at germination and seedling stages comprehensively.

Salinity has a strong effect on seed germination, causing reduced GR and affecting seedling morphogenesis in cotton. Previous studies have demonstrated that 150 mM NaCl results in a significant difference in the germination and growth of cotton between the salt-tolerant and salt-sensitive cotton cultivars (Zhang et al. 2011; Yasir et al. 2019).

It is reported that salt tolerance of the cotton seedlings can be effectively identified at 0.3% NaCl content (3g/kg), and 2 salt-

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### Table 4. Pleiotropic loci for 3 salt-tolerant related traits (FER, GP, and GR).

| Cluster | QTL | Year | Flanking marker | Physical interval | Under E1 | Under E2 | R-value |
|---------|-----|------|-----------------|-------------------|----------|----------|---------|
| Loci-Chr4-1 | qGL-Chr4-1 | 2018 | bin384 to bin385 | 7,167,948 to 7,183,670 | 3.75 | 3.65 | 9.59 |
| Loci-Chr4-1 | qSH-Chr4-1 | 2019 | bin385 to bin386 | 7,183,670 to 7,206,099 | 2.56 | 1.02 | 7.50 |
| Loci-Chr4-1 | qFER-Chr4-1 | 2018 | bin403 to bin404 | 24,618,966 | 2.89 | 5.72 | 6.41 |
| Loci-Chr4-2 | qGR-Chr4-1 | 2018 | bin459 to bin460 | 57,566,299 | 2.11 | 2.20 | 4.88 |
| Loci-Chr4-2 | qNL-Chr4-1 | 2017 | bin457 to bin458 | 59,380,549 | 2.09 | 0.07 | 4.34 |
| Loci-Chr4-2 | qGR-Chr4-2 | 2018 | bin462 to bin463 | 60,287,107 to 60,207,332 | 6.18 | 4.05 | 13.67 |
| Loci-Chr5-3 | qFER-Chr5-3 | 2019 | bin611 to bin612 | 52,903,480 to 54,596,538 | 4.24 | 2.87 | 8.19 |
| Loci-Chr5-3 | qNL-Chr5-2 | 2019 | bin619 to bin620 | 78,964,923 to 78,233,305 | 2.54 | 0.15 | 5.23 |
| Loci-Chr5-3 | qGR-Chr5-1 | 2018 | bin623 to bin624 | 79,979,525 to 80,357,464 | 3.11 | 2.75 | 6.53 |
| Loci-Chr5-3 | qGR-Chr5-2 | 2018 | bin633 to bin634 | 83,550,175 to 83,534,339 | 2.60 | 2.51 | 5.00 |
| Loci-Chr5-4 | qFER-Chr5-4 | 2016 | bin465 to bin466 | 60,406,140 | 3.36 | 0.41 | 8.82 |
| Loci-Chr5-4 | qGW-Chr5-1 | 2018 | bin635 to bin636 | 83,593,530 to 84,429,725 | 2.93 | 0.00 | 8.06 |
| Loci-Chr5-4 | qFW-Chr5-1 | 2018 | bin638 to bin639 | 84,715,085 to 84,416,379 | 2.78 | 0.01 | 7.83 |
| Loci-Chr5-5 | qSH-Chr5-3 | 2019 | bin640 to bin641 | 84,995,410 to 85,222,043 | 4.42 | 1.13 | 8.50 |
| Loci-Chr5-5 | qNL-Chr5-3* | 2019 | bin642 to bin643 | 85,089,802 to 86,001,753 | 7.01 | 0.20 | 13.99 |
| Loci-Chr19-1 | qGP-Chr19-1 | 2018 | bin2062 to bin2063 | 13,292,260 to 12,935,942 | 3.68 | 4.29 | 8.52 |
| Loci-Chr19-1 | qGR-Chr19-1 | 2018 | bin2063 to bin2064 | 12,935,942 to 13,782,236 | 6.50 | 5.35 | 16.17 |
| Loci-Chr19-2 | qSH-Chr19-2 | 2019 | bin2067 to bin2068 | 19,114,235 to 19,022,070 | 3.54 | 2.45 | 7.72 |
| Loci-Chr19-2 | qGR-Chr19-1 | 2018 | bin2069 to bin2070 | 20,025,366 to 19,397,657 | 3.76 | 2.91 | 8.97 |
| Loci-Chr2-1 | qFER-Chr2-1* | 2017 | bin159 to bin160 | 17,151,118 to 18,031,248 | 3.02 | 1.48 | 6.03 |

E1, salt stress conditions; E2, normal conditions; R-value, the converted relative index dataset; 2017t1, spring of 2017; 2017t2, summer of 2017; 2019t1, spring of 2019; 2019t2, summer of 2019. Var, phenotypic variation explained by a single locus QTL (%). Figures underlined referred to the common QTL detected on 2 datasets in the same year. QTL noted by *” referred to common QTL detected on 2 datasets. Bold fonts referred to stable QTL that explained phenotypic variation >10%.
tolerant traits including the RSR and STL were selected to evaluate the salt tolerance of upland cotton (Sun et al. 2018). We found that the total content of water-soluble salt of soil sampling in the saline soil was <3 g/kg in 2017 and 2018, while more than 3 g/kg in 2019 (Guo et al. 2021) (Supplementary Table 3). However, identification of cotton salt tolerance in the field is rarely reported. In the field experiment of spring in 2019, FER was <10% in saline soil and the relative FER was the lowest (Supplementary Figs. 1, 3, 4 and Supplementary Table 4). The phenotypic repeatability of field experiments is inevitably affected by changed environments. Salt tolerance of upland cotton could be effectively detected when the concentration of soil salt concentration was 3 g/kg in the field (Guo et al. 2021). In the critical period of seedling formation, appropriate field management is critical for preventing the low FER due to the water deficiency. Although the salt concentration in field experiments is difficult to control, we found that repeated experiments in the same plot of saline soil over multiple years can effectively reduce experimental errors.

Salt stress causes both primary and secondary effects in crops. Primary effects include both osmotic and ion-toxicity effects on cells, whereas secondary effects such as oxidative stress, which were damaged to cellular components, and metabolic dysfunction (Zelm et al. 2020). In our study, the RIL population and their parents were exposed to salt stress at seedling stage under field saline soil and strictly controlled indoor salt treatment conditions. According to the classification standard of saline soil, the soil sample collected from the saline soil belongs to moderately or highly salinized soil, and the soil sample of normal condition belongs to nonsalinized or lightly salinized soil (Supplementary Table 3). It is shown that there was a significant difference in these salt tolerance traits between salt stress and normal conditions in 3-year trials. Under salt stress conditions, the GP, GR, and FER were significantly decreased in contrast to the control (Supplementary Figs. 4 and 5). The GP, GR, and FER of GX100-2 were higher than that in GX1135 under salt stress conditions, which indicated that GX100-2 was more tolerant to salt stress. It has been reported that salinity was negatively correlated with GP, GR, root length, shoot length, fresh root weight, and fresh shoot weight (Wan et al. 2018). Many traits related to salt tolerance are used for QTL mapping in cotton, such as GP and GR (Du et al. 2016, Diouf et al. 2017), RSR (Sun et al. 2018), shoot height (Oluoch et al. 2016), FW and dry weight of seedlings, MDA (malondialdehyde), EC, chlorophyll content (Diouf et al. 2017), fiber quality, yield,
and its components (Zhu et al. 2020). Therefore, the mining of key genes that control seed germination and seedling establishment under salt stress conditions to elucidate their underlying molecular mechanisms is an urgent and important objective in salt tolerance breeding in cotton.

**Stable QTL underlying salt tolerance traits**

The QTL explained PV more than 10% detected in at least 1 environment was defined as a major QTL. Three QTL (qGP-Chr1-1, qGP-Chr15-2, qGP-Chr19-1) controlled GP, 2 (qGR-Chr4-3, qGR-Chr22-1) controlled GR, and 2 (qFER-Chr12-3, qFER-Chr15-1) controlled FER that were detected in both E1 and R-value (Table 3). Three QTL (qFER-Chr2-1, qFER-Chr3-2, qFER-Chr5-4) controlled FER were detected in both E1 and E2, 2 QTL (qFER-Chr5-1, qFER-Chr10-1) controlled FER were detected in E2 and R-value (Table 3). One QTL (qGR-c7) for GR located on Chr7 in upland cotton has been submitted in the Cotton QTLdb database (http://www.cottonqltdb.org:8081). In the present study, qFER-Chr7-1 explained 4.86% of PV was detected in R-value, while no QTL was detected in the indoor germination experiment. Sun et al. (2019) reported that there are 2 SNPs (i02237Gh, i02243Gh) on Chr D01 (Chr14) that were predicted to be stable genetic loci associated with relative GR under salt stress using GWAS analysis. In the present study, 4 QTL controlling FER located on Chr D01. Among them, 3 were detected under normal condition, and 1 QTL (qFER-Chr14-4) was detected in R-value. The results of these 2 studies are consistent, which indicated that the QTL mapping under salt stress in the field is repeatable. There are 23 SNPs located on Chr A01, A10, D02, D08, D09, D10, and D11 that were significantly associated with the 2 salt-tolerant related traits, RSR and STL detected by GWAS using 713 upland cotton accessions at the seedling stage (Sun et al. 2019). In the present study, we found that QTL for FER were detected on these 7 chromosomes, while only 2 QTL (qGR-Chr22-1, qGR-Chr22-2) for GR were detected on Chr D09. Olwoch et al. (2015) reported that 2 QTL (qSh-Chr15-1, qSh-Chr24-1) for SH (shoot height), 1 QTL (qSfw-Chr15-1) for SFW (shoot FW), and 3 QTL (qBFu-Chr15-1, qBFu-Chr15-1, qBFu-Chr26-1) for RFW (root FW) were detected in both salt stress conditions and normal conditions. In the present study, 1 QTL for SH explaining PV of 5.13% was detected under normal conditions (Table 5). This is consistent with previous research that salt stress-related QTL may be aggregated on specific chromosomes although the salt stress treatments were different.

Salt tolerance is a complex quantitative trait that is controlled by many small-effect genes. A QTL cluster is defined as a densely populated QTL region on a chromosome that contains many QTL associated with different traits (Rong et al. 2007). In total, 25 clusters distributed on 16 chromosomes showed pleiotropic effects (Table 4, Supplementary Table 7). Germination and seedling stages are more sensitive to salt stress than other stages in cotton development (Foolad and Jones 1993). The GR and FER are critical features to evaluate the seedling quality and viability under salt stress treatment. In the present study, 4 clusters (Loci-Chr4-1, Loci-Chr4-2, Loci-Chr5-3, Loci-Chr5-4) influencing FER and GR were identified (Table 4). Loci-Chr4-2, which was detected in E1 and R-value, controlled 5 salt-tolerant related traits. Of which, 1 QTL controlled FER (qFER-Chr4-2) was detected on E1 in 2017 and 2018, and 2 QTL controlled GR (qGR-Chr4-2, qGR-Chr4-3) were major QTL, of which qGR-Chr4-2 was detected in R-value and qGR-Chr4-3 was detected in E1, and the cluster also controls GP, GL, and NL. The favorable alleles in QTL included in Loci-Chr4-2 were originated from the female GX113S except for the QTL (qNL-Chr4-1) (Table 4). These QTL might be the main effect QTL controlling the salt tolerance of upland cotton. The marker interval of bin 457-bin 466 may serve as an important target site for marker-assisted selection in improving salt tolerance.

Previous studies have shown that genes responsible for salt tolerance in the allotetraploid cotton genome were mainly derived from the Dt subgenome (Oluoch et al. 2016; Diouf et al. 2017). In the present study, 4 clusters on Chr 5 (Loci-Chr5-1, Loci-Chr5-2, Loci-Chr5-3, and Loci-Chr5-4) affected FER were detected in our study with 2 (Loci-Chr5-3, Loci-Chr5-4) controlling FER and GR, and 1 (Loci-Chr5-2) controlling FER and GP (Table 4). The favorable alleles in QTL included in these 4 clusters on Chr 5 were contributed by the male parent GX100-2 except qNL-Chr5-2 and qFER-Chr5-1. The results suggest that the clusters detected on Chr 5 (A05) may play a positive role in salt stress response.

We mapped a major QTL (qSH-Chr2-2) controlling SH located on Chr 5 controlling 21.03% of PV under E1 (Table 3). Similarly, the QTL had been reported as qPH-chr19-1 of plant height in previous research (Chen et al. 2006). An increasing number of studies have focused on the roles of transcription factors in response to salt stress in cotton (Yuan et al. 2019). In the present study, GX113S was more tolerant to salt stress than GZH113S (Supplementary Figs. 4 and 5). The gene Gh_A05G3246, encoding calcium-dependent protein kinase (CDPK), was significantly downregulated in salt-tolerant lines after salt treatment, which may act as a negative regulator in salt stress response (Fig. 3). CDPKs have been reported to play important roles in response to salt stress in plants, and overexpressed CDPK2 in potato promoted ROS scavenging, chlorophyll stability, and the induction of stress-responsive genes conferring tolerance to salinity (Grossi et al. 2022). Silencing of 4 CDPKs (GhCPK8, GhCPK38, GhCPK54, and GhCPK55) severely decreased the basal tolerance to salt stress in cotton (Gao et al. 2018). The zinc finger proteins are involved in responsiveness to stress, but the relationship between zinc finger BED domain-containing protein and salt stress has not been reported. In the present study, the gene (Gh_A05G3266) encoding zinc finger BED domain-containing protein was increased in salt-tolerant lines after salt treatment, which may act as a negative regulator in salt stress response (Fig. 3). CDPKs have been reported to play important roles in response to salt stress in plants, and overexpressed CDPK2 in potato promoted ROS scavenging, chlorophyll stability, and the induction of stress-responsive genes conferring tolerance to salinity (Grossi et al. 2022). Silencing of 4 CDPKs (GhCPK8, GhCPK38, GhCPK54, and GhCPK55) severely decreased the basal tolerance to salt stress in cotton (Gao et al. 2018). The zinc finger proteins are involved in responsiveness to stress, but the relationship between zinc finger BED domain-containing protein and salt stress has not been reported. In the present study, the gene (Gh_A05G3266) encoding zinc finger BED domain-containing protein was increased in salt-sensitive lines after salt treatment.

High concentration of salt stress can cause leaf senescence. The senescence marker genes, SEN4 (senescence 4) and SAG12 (senescence-associated gene 12), were increased rapidly after salt stress treatment (Sakuraba et al. 2018). In our study, senescence-related genes (7 GhSAG39 genes) were enriched within Loci-Chr5-4. These genes may be involved in leaf senescence induced by salt stress. Further expression analysis and gene function studies are needed to validation. Because of limitations related to population type or size, further pinpointing the candidate interval based on the RIL population will be difficult. We plan to perform additional work involving QTL fine mapping and functional verification of salt stress-related genes in future studies.
Data availability
The frequency distributions of all traits are shown in Supplementary Fig. 1. The locations of QTL are shown in Supplementary Fig. 2. The phenotype of cotton under salt stress condition and normal condition are shown in Supplementary Figs. 3–6. The GO and KEGG annotation of genes within Loci-Chr4-2 and Loci-Chr5-4 are shown in Supplementary Figs. 7 and 8. The RILs used to map QTL in indoor germination study was given in Supplementary Table 1. The total content of water-soluble salt of soil in the field was in Supplementary Table 3. Supplementary Table 4 contains the descriptive statistical analysis for all traits. Supplementary Table 5 contains the correlation analyses for all traits. Supplementary Tables 6 and 7 contain single locus QTL and pleiotropic loci detected in our research, respectively. Supplementary Table 8 contains annotation of genes within Loci-Chr4-2 and Loci-Chr5-4. The FPKM value of DEGs is given in Supplementary Table 9. Supplementary Table 10 contains annotation of candidate salt stress-related genes. All raw sequences and processed data of RNA-seq have been deposited in NCBI’s GEO database under the accession number GSE186533 (The private link: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE186533, enter token azyljoswhjeyjvuto the box). The phenotype data and the genotype data used for QTL mapping can be found in Supplementary Tables 11 and 12, respectively. Supplementary Table 13 shows the information of SNP or SSR markers contained in the linkage map. Supplemental material is available at G3 online.

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Conflicts of interest
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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