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GENETIC DIVERSITY, POPULATION STRUCTURE, AND ASSOCIATION MAPPING USING SSR MARKERS IN COTTON (*G. HIRSUTUM* L.) RECOMBINANT INBRED LINES UNDER SALT STRESS CONDITIONS

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SUMMARY

Salt stress is a major abiotic factor that considerably negatively affects crop growth, development, and ultimately, yield at the global scale. Upland cotton (*Gossypium hirsutum* L.), being moderately salt tolerant, provides a suitable model for dissecting salt-responsive traits. This study evaluated the cotton recombinant inbred line (RIL) population under salt stress conditions at the early growth stage. The conduct of association mapping used the general linear (GLM) and mixed linear (MLM) models, as implemented in TASSEL software. The identification of significant marker–trait associations succeeded, with the marker BNL3977 found to be significantly associated with fresh plant weight and fresh shoot weight. Meanwhile, the marker BNL2655 showed an association with total plant length, total root length, and leaf number under salt stress conditions. Genomic positions of these markers in the TM-1 reference genome had flanking sequences extracted and analyzed using the AUGUSTUS gene prediction tool, which identified putative coding regions. These predicted gene sequences underwent comparison against the NCBI database using BLAST, revealing candidate genes potentially involved in salt responses. Notably, several genes were distinct, including *GDSL*, *GhGLIP*, *receptor-like protein kinase 5*, *GhUGT80B1*, *SRK2*, *GRP*, *ACD11*, *GhCSL*, *CBSX5*, *STY46*, and *CRF1*, which play vital roles within salt stress conditions.

Keywords: Upland cotton (*G. hirsutum* L.), recombinant inbred lines, salt stress conditions, mapping, marker-trait associations, growth traits, candidate genes

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Key findings: The results provide valuable insights into the genetic mechanism underlying salt tolerance in upland cotton (*G. hirsutum* L.). Similarly, they offer promising targets for marker-assisted selection (MAS) in breeding programs aimed at developing salt-tolerant cultivars. These results can be beneficial in developing salt-tolerant cotton cultivars in Uzbekistan.

INTRODUCTION

High salt concentration adversely affects diverse crops across the globe, resulting in diminished plant harvests. The salt concentration may be ascribable to the excessive accumulation of water-soluble salts in the soil (Peng *et al.*, 2016). The salt stress conditions negatively influence plant growth, geographic distribution, and the crop's productivity (Taghizadeh *et al.*, 2018). The water-soluble ions that lead to soil salinity include potassium (K^+), magnesium (Mg^{2+}), calcium (Ca^{2+}), chloride (Cl^-), sodium (Na^+), carbonate (CO_3^{2-}), and bicarbonate (HCO_3^-) ions (Paul and Lade, 2014). The soils with the high sodium and chloride ion concentrations modify cellular metabolic processes and inhibit plant growth and development and, eventually, reduce yield (Liu *et al.*, 2017). Therefore, implementing a strategic approach and developing effective solutions to alleviate soil salinity is crucial.

These molecular approaches facilitate the acceleration of targeted speed breeding. Using natural saline field conditions is considerable in developing new salt-tolerant cotton cultivars (Normamatov *et al.*, 2023). Among molecular markers, SSR markers are crucial in analyzing plant cultivars, conducting genetic research, tracking trait inheritance across the next generation, and analyzing trait variation. With extensive polymorphism and exceptional precision, microsatellite markers have reached wide usage in various crop plants (Hasnaoui *et al.*, 2012). Genetic diversity at the molecular level plays a vital role in breeding programs.

Genetic modifications require extensive analyses to identify the function and quantity of genes (Lavanya *et al.*, 2008). Genetic diversity, population structure, and association mapping succeeded in implementation through DNA fingerprinting approaches (Agrama *et al.*, 2007). Several previous studies have

investigated and determined the genetic variations and phylogenetic relationships in various crop plants utilizing polymorphic SSR markers (Norbekov *et al.*, 2024). At the Center of Genomics and Bioinformatics, Academy of Sciences, Uzbekistan, a distinctive upland cotton (*Gossypium hirsutum* L.) germplasm, comprising diverse cotton cultivars and advanced lines, have attained collection from different countries worldwide.

The said germplasm collection is an essential biomaterial for molecular research and a valuable genetic resource for cotton breeding. Identifying the genetic purity of cotton cultivars and evaluating genetic relationships is a crucial fundamental goal. Genetic makeup and diversity of cotton RIL genotypes are of paramount importance for effectively regulating gene function and generating novel cultivars withstanding abiotic stressors (D'Agostino *et al.*, 2018). The molecular research enables the identification of cotton-tolerant genotypes and the optimization of genetic resources, thereby supporting the development of high-productivity and salt-stress-tolerant cotton cultivars.

Therefore, based on the above discussion, the following study aimed to a) evaluate the cotton phenotypic traits under salt stress, b) determine the genetic diversity in cotton RIL population, and c) identify putative candidate genes in cotton RIL related to salt stress conditions. These results will be effective for marker-assisted selection and molecular-genetic approaches, together with the transfer of targeted phenotypic cotton traits.

MATERIALS AND METHODS

Genetic material and experimental design

In this research, the genetic resources employed a recombinant inbred line (RIL) population of upland cotton (*G. hirsutum* L.).

This population resulted from biparental crosses between the elite cultivar Namangan-77 and salt-tolerant cotton line KK-1795. Furthermore, the local cotton cultivar An-Boyovut-2 was a choice for use as a salt stress-resistant control. The RIL population, as developed through the single-seed descent method, came from segregated individual hybrid plants of the F₂ generation (F₇₋₈). The experiment layout was in a randomized complete block design with three replications. Phenotypic traits assessment proceeded under three distinct NaCl levels (T₀ = 0 mM-control, T₁ = 100 mM, and T₂ = 200 mM) under controlled laboratory conditions.

Phenotypic traits measurement

At the third true-leaf stage of cotton's growth and development phase, an assessment of morpho-biological traits commenced. The various traits evaluated include total plant length (TPL), total shoot length (TShL), total root length (TRL), fresh plant weight (FPW), fresh shoot weight (FShW), fresh root weight (FRW), dry shoot weight (DShW), dry root weight (DRW), and leaf number (LN). The study followed the methodology of Zhang *et al.* (2011). The measurement of chlorophyll content (ChlC) continued using an SPAD-502 in the top three leaves (Ling *et al.*, 2011). After a 15-day salt treatment, seedling collection and weighing ensued, with the data recorded using a digital precision analytical balance (model FA2204). After recording all the data, the maintenance of samples was within a drying oven at 80 °C for two days.

DNA extraction and genotyping by SSR primers

The plant's DNA extraction transpired from young leaves of each cotton plant by the CTAB method (Paterson *et al.*, 1993). The SSR microsatellite markers tended to have high polymorphism, reproducibility, and co-dominance compared with other DNA marker types in molecular breeding. The SSR markers have also been effective in genetic diversity analysis and genetic mapping on cotton (Santosh *et al.*, 2022). DNA samples of the

cotton plants gained amplification by polymerase chain reaction (PCR) based on a previously optimized method (Sahu *et al.*, 2012). The PCR occurred under the following conditions: initial denaturation phase (5 min, 94 °C), followed by 35 cycles of 94 °C (30 s), 52 °C–58 °C (30 s), and final step extension at 72 °C (5 min). Amplicons' separation employed horizontal gel electrophoresis using a 3.5% high-resolution agarose gel with 5 µl of ethidium bromide (1 mg/ml) added for visualization of the products. The Gene Ruler 25 bp DNA Ladder (Thermo Scientific) size standard served to determine the size of the PCR fragments. The DNA fragments entailed visualization using the Molecular Imager ChemiDoc XRS+ gel doc system (Bio-Rad). Genotyping continued by analyzing the gels in the Gene Analyzer 19.1 software.

Data analysis

The phenotypic data assessment of the cotton plants grown under different levels of salt stress used the analysis of variance (ANOVA) in OriginPro 2024 software. Population structure analysis proceeded using STRUCTURE 2.3.4 software, based on a Bayesian clustering model utilizing an 'admixture model.' For association mapping, applying both MLM and GLM models in the TASSEL 5.2.80 program ensued using SSR markers filtered by the minor allele threshold (MAF ≥ 5%). The sequences flanking the SSR marker sequences associated with QTLs for salt stress tolerance sustained analysis *in silico* using the AUGUSTUS 3.3.3 program to identify the putative coding sequences. In this way, the predicted candidate genes identified incurred annotation using comparative analysis (BlastN, BlastX, and BlastP) with sequences in the NCBI database.

RESULTS AND DISCUSSION

Phenotypic diversity analysis

In the RIL population of upland cotton (*G. hirsutum* L.), investigations of the key traits associated with salt stress tolerance were

Table 1. Analysis of variance of the phenotypic traits in upland cotton under salt stress conditions.

| Traits | T0 (control) | | | T1 (100 mM NaCl) | | | T2 (200 mM NaCl) | | | | | |
|--------|--------------|-------|---------|------------------|---------|---------|------------------|--------|---------|----|---------|--------|
| | Cluster | SS | F Value | Prob>F | Cluster | SS | F Value | Prob>F | Cluster | SS | F Value | Prob>F |
| TPL | 0.76 | 32.70 | <0.0001 | 1.16 | 67.04 | <0.0001 | 0.65 | 38.57 | <0.0001 | | | |
| TShL | 0.73 | 29.58 | <0.0001 | 0.76 | 37.53 | <0.0001 | 0.46 | 22.07 | <0.0001 | | | |
| TRL | 0.54 | 19.15 | <0.0001 | 1.02 | 46.03 | <0.0001 | 0.70 | 26.34 | <0.0001 | | | |
| FPW | 0.72 | 34.14 | <0.0001 | 0.65 | 30.48 | <0.0001 | 0.40 | 23.12 | <0.0001 | | | |
| FShW | 0.49 | 23.75 | <0.0001 | 0.58 | 38.75 | <0.0001 | 0.46 | 23.06 | <0.0001 | | | |
| FRW | 0.70 | 37.76 | <0.0001 | 0.20 | 8.66 | <0.0001 | 0.48 | 19.44 | <0.0001 | | | |
| DShW | 0.47 | 42.71 | <0.0001 | 0.51 | 21.58 | <0.0001 | 0.30 | 14.65 | <0.0001 | | | |
| DRW | 1.09 | 53.31 | <0.0001 | 0.09 | 3.28 | <0.05 | 0.35 | 23.45 | <0.0001 | | | |
| LN | 0.55 | 18.07 | <0.0001 | 0.57 | 23.28 | <0.0001 | 0.06 | 2.91 | <0.05 | | | |
| ChIC | 0.29 | 6.34 | <0.001 | 0.34 | 14.65 | <0.0001 | 0.09 | 1.85 | 0.14374 | | | |

Total plant length (TPL), Total shoot length (TShL), Total root length (TRL), Fresh plant weight (FPW), Fresh shoot weight (FShW), Fresh root weight (FRW), Dry shoot weight (DShW), Dry root weight (DRW), Leaf number (LN), and Chlorophyll content (ChIC).

successful. These traits included TPL, TRL, TShL, FPW, FShW, FRW, DShW, DRW, LN, and ChIC. All the traits' assessment relied on the phenotypic evaluation of cotton plants grown with varying salt stress levels under controlled conditions. The ANOVA revealed high genetic diversity among the RIL population (Table 1). The TPL trait exhibited a significant ($P < 0.001$) difference. However, the highest variation occurred under the T1 (100 mM NaCl) condition ($F = 67.04$). The T1 salt stress condition significantly affected the TPL compared with the T0 (0 mM NaCl) ($F = 32.7$) and T2 (200 mM NaCl) ($F = 38.5$) conditions. The most significant differences in the trait TRL were evident under the T1 condition ($F = 46.03$, $P < 0.0001$), while under the T2 condition ($F = 26.34$, $P < 0.0001$), the salt stress led to a reduction in root length. Under T2 salt stress conditions, the F-value declined significantly, demonstrating that salt stress reduced the trait FPW. Overall, the analysis revealed salt stress remarkably influenced the cotton key traits, notably inducing variations in plant growth, biomass accumulation, and leaf number.

Genetic diversity and population structure

DNA extraction from each cotton genotype sought to identify the SSR markers associated with salt stress in the RIL population. The PCR results enabled the detection of genetic markers and facilitated the association

mapping. In total, 41 polymorphic markers (5.1%) out of 851 SSR primer pairs surfaced between the cotton parental genotypes of the RIL population. Although the CIR and Gh SSR marker sets succeeded in amplification, however, no polymorphism resulted in parental genotypes and RIL population samples. Using specific SSR markers, the obtained PCR products served to analyze the segregation of cotton paternal and maternal alleles within the RIL population. Based on such markers, association mapping has reached extensive application in cotton and other crops worldwide to identify the heritable genetic loci associated with resistance to biotic and abiotic stress factors (Abdurakhmonov *et al.*, 2008). At the preliminary stage, the population structure determination used the STRUCTURE software. The analysis further revealed the RIL population incurred division into several genetically distinct clusters (Figure 1). The identified groups showed the levels of genetic variability within the population and also explored the inheritance patterns among the cotton recombinant inbred lines. The results attained interpretations based on the genetic characteristics of each cluster.

Based on the results of the population structure analysis, the supplementary information appears in Table 2. The determination of the optimal number of clusters in the population used the statistical parameter ΔK , calculated according to the proposed method of Evanno *et al.* (2005).

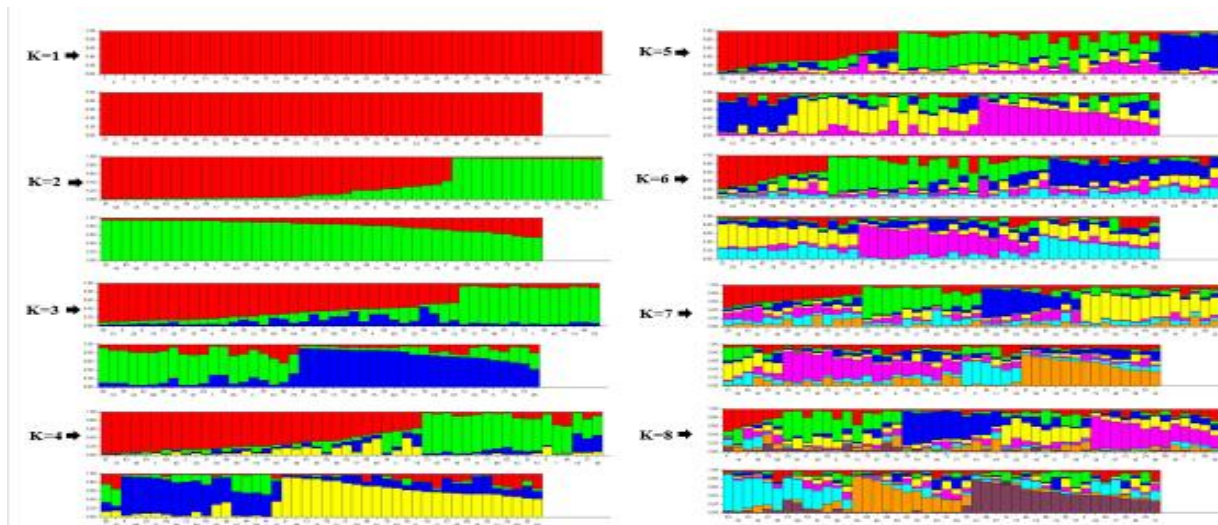


Figure 1. Population structure analysis of the RIL population of *G. hirsutum* L. using the STRUCTURE program with K values from 1 to 8. Each vertical bar represents an individual RIL, and the colored segments indicate the proportion of its genome assigned to each genetic cluster.

Table 2. The highest ΔK value in the LnP (D) analysis was notable at $K = 3$, indicating the optimal number of genetic clusters in upland cotton.

| K | Reps | Mean Ln P(K) | SD Ln P(K) | Ln _I (K) | Ln _{II} (K) | Delta K (ΔK) |
|---|------|--------------|------------|---------------------|----------------------|------------------------|
| 1 | 10 | -3002.91 | 0.9183 | - | - | - |
| 2 | 10 | -2895.79 | 16.5534 | 107.12 | 20.61 | 1.245058 |
| 3 | 10 | -2809.28 | 17.5156 | 86.51 | 93.03 | 5.311256 |
| 4 | 10 | -2815.8 | 215.9567 | -6.52 | 202.02 | 0.935465 |
| 5 | 10 | -3024.34 | 774.0781 | -208.54 | 444.79 | 0.574606 |
| 6 | 10 | -2788.09 | 160.8114 | 236.25 | 464.09 | 2.885927 |
| 7 | 10 | -3015.93 | 618.9231 | -227.84 | 185.55 | 0.299795 |
| 8 | 10 | -3058.22 | 768.7981 | - | - | - |

During the modeling of the population structure, ΔK values ranged from 1 to 8, corresponding to the presumed number of genetic groups in the cotton RIL population. The mean log-likelihood ($\text{LnP}[K]$) reflects the logarithmic probability of the population's structure at a given number of clusters. The first derivative of the log-likelihood $\text{Ln}(\text{DIK})$ revealed how long the probability varies as K increases. However, the ΔK values significantly varied across different numbers of clusters (Table 3). Past studies explored the highest ΔK values and enunciated the most optimal number of clusters within the structured population (Pritchard *et al.*, 2000).

The genetic diversity and distribution in the RIL population illustrated the underlying population structure identified in this study (Figure 2). In Figure 2A, the STRUCTURE bar plot ($\Delta K = 3$) showed that individuals grouped into three major genetic clusters, each represented by a distinct color (red, green, and blue). In Figure 2B, three separate histograms (B1, B2, and B3) were available, each one representing the F_{st} distribution. The statistical analysis of F_{st} index represented $F_{st1} = 0.599$, $F_{st2} = 0.560$, and $F_{st3} = 0.289$ in Figures 2B1, 2B2, and 2B3, respectively. In addition, a cluster tree plot construction (C) ensued, with each point corresponded to cotton genotypes.

Table 3. SSR markers related to salinity tolerance in the *G. hirsutum* L. population.

| Traits | Markers | GLM | | MLM | | MO. |
|--------|-------------|----------|----------------|----------|----------------|-----|
| | | p-value | r ² | p-value | r ² | |
| FPW | BNL3977_130 | 5.19E-05 | 0.16386 | 1.11E-04 | 0.15063 | 44 |
| FShW | BNL3977_130 | 1.21E-04 | 0.14914 | 2.21E-04 | 0.13851 | 44 |
| LN | BNL2655_110 | 6.97E-04 | 0.11806 | 6.98E-04 | 0.11805 | 72 |
| TPL | BNL2655_110 | 4.65E-04 | 0.12529 | 4.66E-04 | 0.12529 | 57 |
| TRL | BNL2655_110 | 2.65E-04 | 0.13529 | 2.65E-04 | 0.13528 | 57 |

Fresh plant weight (FPW), Fresh shoot weight (FShW), Leaf number (LN), Total plant length (TPL), and Total root length (TRL).

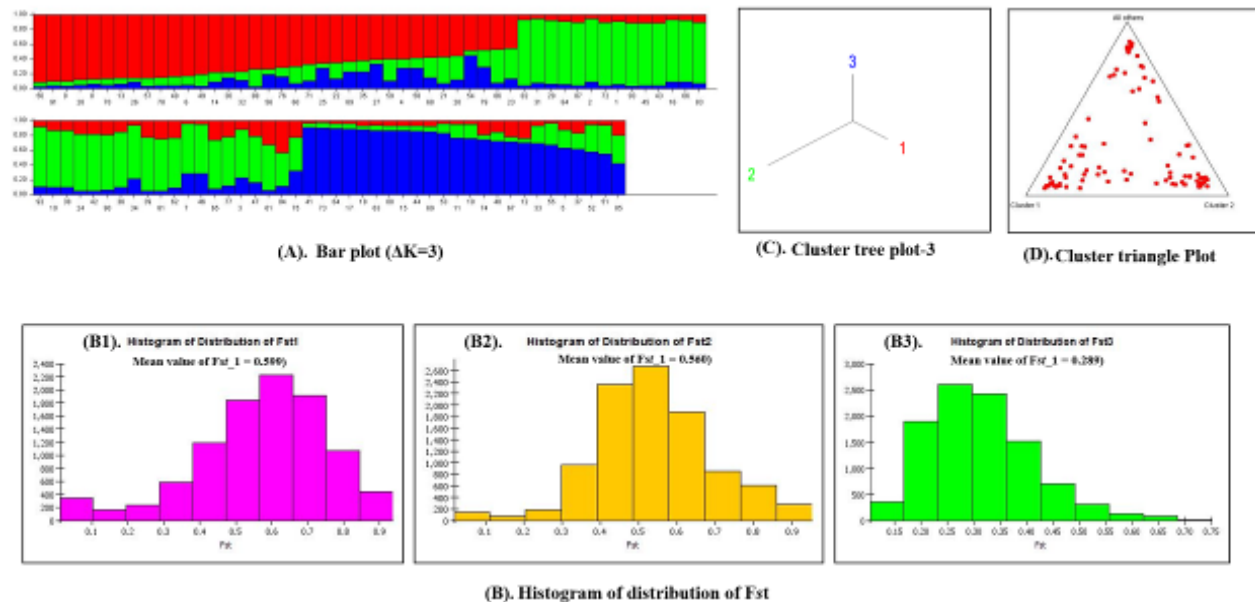


Figure 2. A) Bar plot of population structure at $\Delta K = 3$; B) Histograms of F_{st} values for each cluster: B1 – Cluster 1, B2 – Cluster 2, and B3 – Cluster 3; C) Cluster tree showing genetic relationships; and D) Triangular plot depicts cluster distribution and membership and admixture in upland cotton.

This dendrogram visually evaluates multidimensional datasets and highlighted the genetic differences across the clusters. Concurrently, the cluster triangle plot (D) also succeeded its generation. This plot revealed the genetic affinity between the clusters. These plots are commonly useful in genetic research and have a remarkable role in the assessment of population genetic diversity and similarity.

Genetic association analysis of SSR markers with salt tolerance traits

Association mapping (AM) progressed using both the general linear (GLM) and mixed linear

(MLM) models in the TASSEL 5.2.80 software package. The analysis included 41 polymorphic SSR markers, in which identifying 66 alleles emerged after filtering based on the minor allele frequency (MAF). A matrix containing the phenotypic evaluation scores related to salt tolerance helped to identify the different associations with the traits of interest. Figure 3 showed the relationship between genotypic and phenotypic traits. According to the GLM analysis, the 110-bp allele of marker BNL2655 showed $-\log_{10}(P) = 3.4$ for plant height, 3.6 for root length, and 3.2 for leaf number. Moreover, the 125-bp allele of marker BNL3977 expressed $-\log_{10}(P) = 4.3$ for fresh

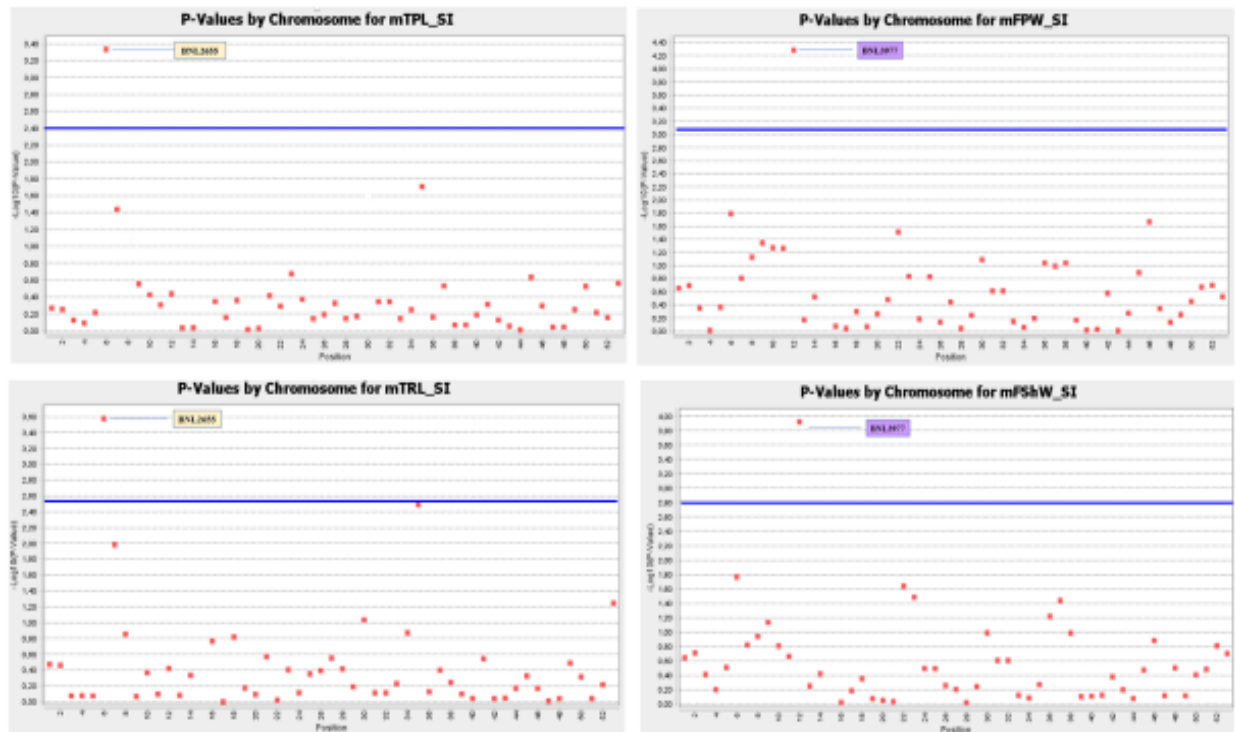


Figure 3. Association mapping of salt stress tolerance in the *G. hirsutum* RIL population using SSR markers based on both GLM and MLM models.

plant weight and 3.9 for fresh shoot weight. Based on the assessed significant results, the BNL2655 and BNL3977 SSR markers appeared to have considerable association with different phenotypic traits in evaluated cotton genotypes.

According to the GLM analysis, the results showed a considerable ($P \leq 0.001$) association of the BNL3977-130 allele with the FPW trait ($P = 5.19\text{E-}05$) (Table 3). Additionally, the said allele expressed association with the FShW ($P = 1.21\text{E-}05$, $P \leq 0.001$). The analysis also confirmed a significant connection of the BNL2655-110 allele with several other traits measured under salt stress conditions at the early seedling stage, i.e., LN (number of leaves) – $P = 6.97\text{E-}05$ ($P \leq 0.001$), TPL (total plant length) – $P = 4.65\text{E-}05$ ($P \leq 0.001$), and TRL (total root length) – $P = 2.65\text{E-}05$ ($P \leq 0.001$). The outcomes obtained from the MLM model analysis also displayed the same pattern of associations.

Based on the whole-genome sequence of the TM-1 cotton line, determining the marker loci positions identified through association analysis was successful. For each locus, adjacent genomic regions reached selection as potentially harboring genes involved in salt stress tolerance. These regions received analysis using the AUGUSTUS 3.3.3 program (Stanke *et al.*, 2006), which further allowed for the prediction of putative coding sequences, including exons, introns, and gene loci. The predicted sequences subsequently underwent BLAST analysis against the NCBI database to assess sequence homology as well as to identify the likely functions of the predicted genes (Schäffer *et al.*, 2001). The said approach enabled the identification of candidate genes potentially involved in molecular response to the salt stress conditions in cotton. In this study, 10 morphological traits of upland cotton (*G. hirsutum* L.) entailed evaluation at the early growth stage, with single marker traits' association (SMTA)

Table 4. Putative candidate genes and corresponding proteins within QTL regions associated with salt stress tolerance in upland cotton, identified through NCBI-BLAST analysis of SSR marker sequences.

| No. | SSR markers | Genes | Putative protein function | References |
|-----|-------------|--------------------------------|---|-----------------------------------|
| 1 | BNL3977 | GDSL | Esterase/lipase, involved in lipid metabolism under salt stress | (De-Almeida <i>et al.</i> , 2024) |
| | | GhGLIP | Glycosyltransferase/DSL lipase/hydrolase Cell wall remodeling under abiotic stress | (Ma <i>et al.</i> , 2018) |
| | | Receptor-like protein kinase 5 | Stress signal perception and transduction | (Zhao <i>et al.</i> , 2013) |
| | | GhUGT80B1 | Gh sterol 3- β -glucosyltransferase Cell membrane stability under stress | (Mishra <i>et al.</i> , 2015) |
| | | SRK2 | Serine/threonine-protein kinase ABA signaling pathway under salt stress | (Liu <i>et al.</i> , 2017) |
| | | GRP (Glycine rich protein) | Cell wall structural protein Drought and salt tolerance-like | (Czolpinska and Rurek, 2018) |
| 2 | BNL2655 | ACD11 | Accelerated Cell Death 11 homolog protein Involved in PCD and defense under stress | (Li <i>et al.</i> , 2020) |
| | | GhCSL1/4 | Cellulose synthase under salt stress condition | (Li <i>et al.</i> , 2017) |
| | | CBSX5 | CBS domain-protein ion homeostasis | (Yoo <i>et al.</i> , 2011) |
| | | STY46 | Serine/threonine-protein kinase ABA regulated under salt stress | (Dong <i>et al.</i> , 2020) |
| | | CRF1 | Cytokinin-responsive factor ethylene signaling in salt stress | (Xie <i>et al.</i> , 2019) |

analyzed using SSR markers. Both GLM and MLM approaches' employment identified significant marker-trait associations. In the presented research, the MLM model analysis effectively minimized false positives and negatives by incorporating population structure and kinship matrix as covariates, thereby filtering out many of the spurious associations identified by GLM. Associations between polymorphic markers and the cotton traits related to salt and drought stress tolerance were detectable using both models (Jia *et al.*, 2014).

In the concerned study, 11 candidate genes also gained identification within two regions corresponding to putative QTLs that harbor the detected marker alleles. The identified putative genes using in-silico analysis of the alleles succeeded in mapping to the whole-genome sequence of the cotton line TM-1 (Table 4). The results indicated the BNL2655 marker had a significant association with the cotton traits TPL, TRL, and LN, while the BNL3977 marker showed a linkage to the traits FPW and FShW under salinity stress conditions. Putative coding elements were predictable within the investigated QTL regions using the

AUGUSTUS program, with their functional annotation performed through sequence homology search in NCBI databases. The detected genes, including *GDSL*, *GhGLIP*, *receptor-like protein kinase 5*, *GhUGT80B1*, *SRK2*, *GRP*, *ACD11*, *GhCSL*, *CBSX5*, *STY46*, and *CRF1*, play pivotal roles in conferring cotton tolerance to salt stress conditions at the molecular level.

Abiotic stress triggers a complex cascade of phenotypic, physiological, biochemical, and molecular changes that negatively affect plant growth and development as well as the final productivity. For instance, overexpression of *GhGLIP* in *Arabidopsis* plants caused enhanced seed development, including seedling length and fresh weight (Ma *et al.*, 2018). The *UGT80B1* gene participates in the biosynthesis of sterol glucoside, found crucial for maintaining cell membrane structure. The *STY46* gene contributes to stress response by regulating carbon utilization in plant leaves, thereby enhancing plant growth, development, and survival (Dong *et al.*, 2020). Li *et al.* (2020) identified that the *ACD11* protein reached considerable reduction via the *XBAT35.2*

protein with abiotic stress tolerance in *Arabidopsis*. As a result, the plant expedited enhanced tolerance to salt and drought stresses. These genes may potentially be participants in protective mechanisms contributing to plant responses under salt stress conditions.

CONCLUSIONS

The genetic diversity within the RIL population of upland cotton (*G. hirsutum* L.) succeeded in consistent confirmation using STRUCTURE software based on ΔK grouping, log-likelihood values, fixation index, cluster tree, and triangular plotting. Furthermore, association analysis using both GLM and MLM models identified significant marker-trait associations, with the marker BNL3977 linked to the cotton traits fresh plant weight and fresh shoot weight. However, the BNL2655 showed an association with the traits of total plant length, total root length, and leaf number. The study results will be helpful in developing new cotton cultivars with enhanced salt tolerance through the successfully established MAS program in Uzbekistan.

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