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Original Article

Identification of significant SNPs for yield-related salt tolerant traits in rice through genome-wide association analysis

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Abstract

Rice salt tolerance is highly anticipated to meet global demand in response to decreasing farmland and soil salinization. Therefore, dissecting the genetic loci controlling salt tolerance in rice for improving productivity is of utmost importance. Here, we evaluated six salt-tolerance-related traits of a biparental mapping population comprising 280 F2 rice individuals (Oryza sativa L.) at the seedling and reproductive stages. We performed a genome-wide association study (GWAS) to identify marker-trait associations under artificially induced salt stress using the 1K RICA chip (Agriplex Genomics, Cedar Avenue, Suite 250, Cleveland, 011444106, USA). We have identified 8 single nucleotide polymorphisms (SNPs) representing eight genomic regions on chromosomes 5, 8, 9, and 10. These were significantly associated with the six salt-tolerance-related traits, no. of tillers per plant (TPP), effective tillers per plant (ETP), spikelet fertility percentage (SFP), field grain number (FGN), grain length breadth ratio (LBR) and thousand-grain weight (TGW). FGN has two significant SNPs (SNP0758 and SNP0759) on Chromosome 9, whereas SFP on chromosomes 8 and 12 (SNP1127 and SNP0966, respectively). Similarly, for TPP (SNP0796), a significant SNP was detected on chromosome 10, and for ETP (SNP0414) on chromosome 5. Two significant SNPs were found in chromosome 12 for LBR (SNP0920) and TGW (SNP0976). Based on all loci, we screened 3 possible candidate genes in chromosomes 8, 9, and 12 between the genomic region of SNP0920 and SNP1127 under salt stress. Interestingly, these genes were involved in protein coding, none of which was previously reported as being involved in plant salt tolerance. Further, the genetic relationship between the mapping population and population structure was classified by STRUCTURE v 2.3. Genotypes with ≥ 80% of shared ancestry were explained into two major clusters (I and II), and < 80% of shared ancestry were categorized as admixtures. An unrooted alpha was developed by TASSEL 5.0, dividing the genotypes into three major groups where 97 individuals were in Cluster 1, cluster 2 consisted of 93 individuals, and the remaining Cluster 3 included 90 individuals. A kinship matrix developed from 860 SNPs indicated group formation and more substantial relatedness among the genotypes with a red zone. Our findings provide valuable information for enhancing the understanding of complicated salt tolerance mechanisms in rice seedlings and the identified candidates potentially used for breeding salt-tolerant genotypes.

Keywords: Rice, Salinity, Population structure, GWAS, SNPs, Candidate genes

1. Introduction

Global warming-induced climate change has dramatically reduced crop production over the years. The adverse weather that may create salinity, drought, heat, or submergence becomes a great danger to growing cereals, especially rice, one of the oldest staple foods consumed by half of the world's population. Among them, salinity stress is one of the most alarming environmental changes affecting more than 800 million hectares of land, including approximately one-third of the total rice cultivating area of the world [1, 2]. Salinity induces the accumulation of NaCl, KCl, MgCl2, Na2SO4, MgSO4, CaSO4, and Na2CO3 in the soil, whereas Na+ and Cl- are the major injury-causing ions for crops [3]. Salt stress interferes with stomatal closure reducing photosynthesis, retardation of shoot elongation, and leaf tip burning [4-6] at the earlier

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stage and finally causing ionic stress by accumulating Na+ ions through transpiration stream to aerial parts of plants that leads to plant growth inhibition [7-9]. Rice is a more salt-sensitive cereal than maize, wheat, or barley. Rice is affected by Salinity from seed germination to tillering as well as maturity, but severely at the seedling stage and its reproductive stage that determines its yield. It loses 12% yield for each dSm-1 when it crosses the EC of 3 dSm-1, and the reduction may reach more than 50% when the EC comes to 6 dSm-1 [8,10,11].

Salinity tolerance is a polygenic character, and plants must modify their physiological and metabolic mechanisms, like maintaining good Na+/K+ homeostasis to withstand sodic soils [12]. But tolerance towards salt is highly affected by the environment. Several quantitative trait loci (QTL) have been identified at seed germination [13, 14] and seedling stages [15-17]. However, only two genes named SKC1 [18] and qSE3 [19] were cloned and characterized. For the reproductive stage, also plenty of QTLs were identified for example,16 QTLs were reported by [20], 35 QTLs by Mohammadi et al. [21] and 64 SNPs by Kumar et al. [22]. But no genes have been cloned for this stage yet [23].

Therefore, next-generation sequencing of high-density SNPs to study genome-wide association (GWAS) based on linkage disequilibrium mapping can be the best tool to identify new genes for salinity tolerance [24]. Their suitable allelic variants identification can be used wisely to generate the salinity-tolerant genotypes of rice. The genomic region of different traits strongly related to salt tolerance, like Na/K ratio, fresh and dry weight ratio, etc., can be identified by association mapping ([8, 25]. Hence, our study used F2 progenies of a cross between salt-tolerant and salt-susceptible variety to identify significant SNPs for salt-tolerant traits and their physical mapping on the chromosome. Overall our study provided the SNPs linked with salt tolerance that can be used in future breeding programs for generating climate-smart salt-tolerant rice.

2. Materials and Methods

F2 progenies (280 in number) derived from a cross between salt susceptible mega variety BRRI Dhan 28 (Bangladesh) and a salt-tolerant line IR59418-7B-21-3 (developed by IRRI: IR 10198-66-2//IR 50404/AT 401) were selected as a base material of the experiment.

2.1. Phenotypic evaluation and statistical analysis

The experiment was conducted at the Department of Genetics and Plant Breeding field laboratory, BSMRAU, Gazipur, following the modified methods of Gregorio et al. [26] with three replications explaining RCBD design. PVC tubes 40cm long and 15cm diameter were sealed with a net cloth and filled with soil collected from rice fields and sun-dried, and mixed with cow dung. Experimental soil was prepared with a standard dose of fertilizer used in Bangladesh [27]. The tubes were placed in a box measuring 56 cm in length, 38 cm in breadth, and 36 cm in height, allowing the plants to grow semi-aerobically throughout the duration of the experiment. There was no flooded irrigation; instead, water was maintained at a level 4 cm below the plant roots. Sea water was diluted (4ds/m) to apply at 21 days old seedlings that was increased to 6ds/m and practiced throughout the vegetative stage following the interval of 15 days from the day of the first application. A salinity level of 8ds/m was maintained up to maturity. A polyethylene shade was used to avoid rainfall and was removed at an interval to maintain temperature and humidity. At maturity, 280 F_2 plants were used to record the data on tillers per plant (TPP), effective tillers per plant (ETP), spikelet fertility percentage (SFP), field grain number (FGN), grain length breadth ratio (LBR), thousand-grain weight (TGW) and grain weight per plant (GWP). Analysis of variance for individual and box plot analysis was calculated using STAR software (IRRI), whereas the frequency distribution of the traits was explained using Excel.

The phenotypic and genotypic coefficient of variation was calculated using phenotypic (δ^2p) as well as genotypic (δ^2g) variances [28].

$$\delta^2 p = \frac{MSg}{r}, \delta_2 g = MSp - \frac{MSe}{r} \; and \; \delta_2 e = \frac{Mse}{r}$$

Where MS_p = mean squares of phenotypes
MS_g = mean squares of genotypes
MS_e = mean of error
r= number of replications

The phenotypic coefficient of variation (PCV) and genotypic co-efficient of variation (GCV) were recorded according to Singh and Chaudhury [29] using the sample mean as follows:

$$PCV\ (\%) = \frac{\sqrt{\delta^2 p}}{x} \times 100$$

$$GCV\ (\%) = \frac{\sqrt{\delta^2 g}}{x} \times 100$$

Here,
δ^2p = phenotypic variance
δ^2g = genotypic variance
x = sample mean.

According to Falconer $\frac{\delta^2 g}{\delta^2 p}$30], broad sense heritability (h^2) was estimated as h^2 = and Genetic advance (GA) as $k \times \frac{\delta^2 p}{\mu} \times 100$ [31], where k is a standardized selection differential constant (2.063). A bubble map was illustrated between GA% and broad sense heritability (h^2) using Excel. Correlation between the traits was revealed using the R program, and the spider map was exemplified through Excel.

2.2. DNA isolation and SNP calling

Young leaves were crashed, and isolated total genomic DNA was using the CTAB (Cetyl Trimethyl Ammonium Bromide) method ([32], and a nano-drop spectrophotometer was used to quantify the extracted DNA (NanoDropTM 2000/2000 c, Thermo Fisher Scientific, DE, United States). Genotyping for single-nucleotide polymorphism (SNP) of DNA of each sample was conducted using RICA 1K RICA chip in Agriplex genomics, Cedar Avenue, Suite 250, Cleveland, 011444106, USA.

2.3. Population Structure Analysis and LD

The distribution of 860 polymorphic SNP over the 12 chromosomes for 280 genotypes was analyzed in STRUCTURE v2.3.4 (http://pritchardlab.stanford.edu/structure.html) using a Bayesian Model of ADMIXTURE [33] with 10 independent runs with 100000 burn-ins and MCMC pe-

riod for each of K was set to 100000. The optimum number of sub-populations was derived using STRUCTURE HARVESTER [34]. TASSEL v5.0 [35] was utilized to confirm the cluster created through STRUCTURE by drawing an NJ tree and calculating PCA. Linkage Disequilibrium (LD) for the entire genome was calculated using 860 SNPs using TASSEL v5.0 with the squared Pearson correlation coefficient (r^2). A scatter plot was illustrated as LD between adjacent markers and chromosome distance (Kb), whereas the significance threshold was r^2=0.1.

2.4. Genome-Wide Association Analysis

The BLUP values obtained from the six traits were used as phenotypic information in GWAS. A total of 860 SNPs were found after filtering with >10% missing data points, and MAF< 5% were discarded. The Mixed Linear Model (MLM)- based on the Kinship matrix and MLM based on both K-matrix and Q-matrix, were used to find the association between SNPs and traits. Markers with -log10(P-value) ≥ 2.92 were recorded as significant following the Bonferroni-Holm Correction testing [36]. After detecting the significant SNPs, several candidate genes were identified using the position of those SNPs from the Gramene website.

3. Results

3.1. Phenotyping Variability and Correlation Analysis for Population

Analysis of variance (ANOVA) showed a significant difference among the genotypes for all the traits except LBR (Supplementary Table 1). Boxplot analysis implied that most genotypes were near the mean value for TPP, LBR, FGN, and TGW traits but dispersed for SFP and ETP (Supplementary Table 2 and Fig 1). The trait TPP and LBR showed a normal frequency distribution curve, whether

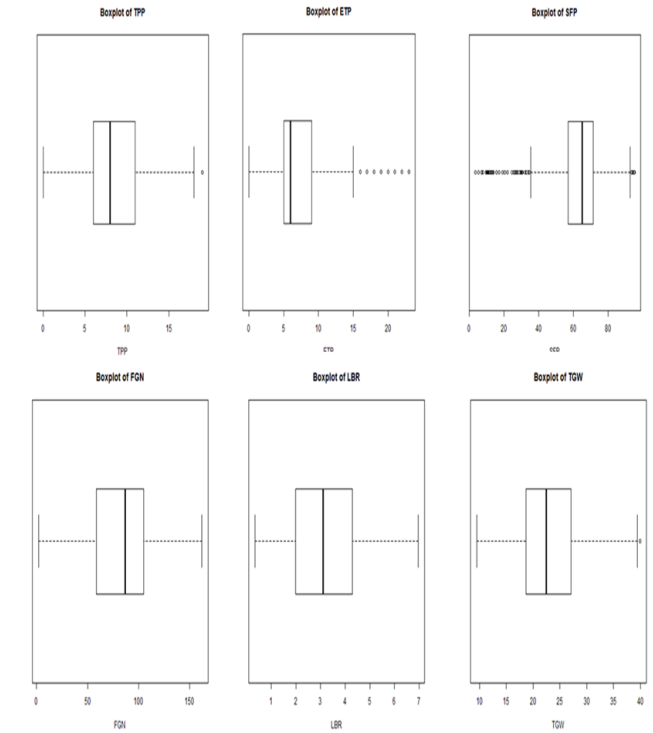

Fig. 1. Boxplot showing mean performance of the genotypes for tillers per plant (TPP), effective tillers per plant (ETP), spikelet fertility percentage (SFP), field grain number (FGN), grain length breadth ratio (LBR), and thousand-grain weight (TGW).

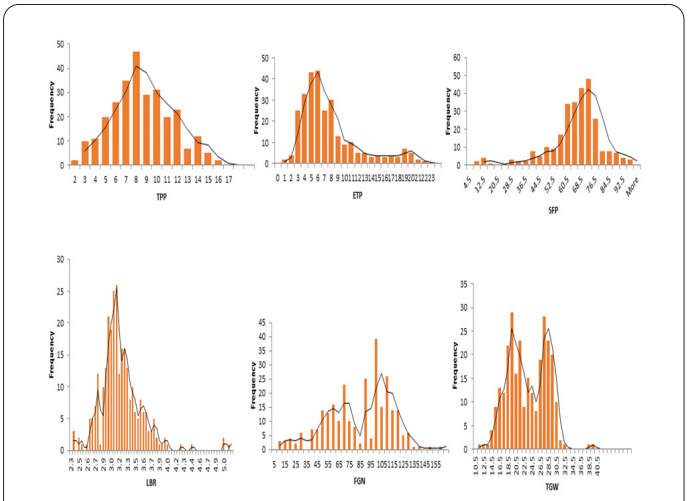

Fig. 2. Frequency distribution of 280 F_2 genotypes for tillers per plant (TPP), effective tillers per plant (ETP), spikelet fertility percentage (SFP), field grain number (FGN), grain length breadth ratio (LBR), and thousand-grain weight (TGW).

SFP had a negatively skewed curve and ETP had a positively skewed distribution over the 280 F_2 genotypes (Fig 2). The Salinity affects the grain filling and weight widely between susceptible and tolerable ones that resulted from a bi-modal distribution for the traits FGN and TGW. GCV, PCV, broad sense heritability, and GA were estimated for seven traits among 280 F2 genotypes. There is no significant difference between GCV and PCV for SFP and TGW traits, whereas a minimum difference was found for ETP (Fig 3a). Besides these, a substantial difference between GCV and PCV (Supplementary Table 2) was found in TPP (37.9 and 46.26 respectively) and LBR (4.36 and 19.83 respectively) that, indicates the effect of environment on them. According to the classification of Robinson et al. [37], the highest percentage of heritability (>60%) was determined in all the traits, i.e., TPP (67.11%), ETP (95.18%), SFP (98.82%) and TGW (97.27%) except LBR that showed the lowest (<30%) broad sense heritability. High heritability and genetic advance (GA) will help predict the reliable selection and the chances of recovering on their transgressive segregants [38]. Among the 6 traits, the highest GA% and high heritability were only found for ETP along with TPP, which estimates a moderate value (~65%) (Fig 3b). The strongest significant and positive correlation (0.68) of spikelet fertility percentages (SFP) was known with filled grain number (FGP), as expected (Figure 1c). However, due to the salinity effect at the grain-filling stage, the thousand-grain weight negatively correlated with all the other traits that were expected to be positive. Grain weight per plant (g) ranged from 0.13 to 5.02 g (Suplementary Table 2).

3.2. Physical Mapping, Structure, and PCA Analysis

A total of 280 F_2 individuals, along with parents, were genotyped using 1K RICA rice chip. After SNP filtering, 860 high-quality SNPs were physically mapped across 12 chromosomes. In contrast, the heterozygous locus was counted as 62644 with the proportion of heterozygous as 0.26015 and an average minor allele frequency of 0.23716 (Supplementary Table 3). The genetic relationship between the mapping population and population structure was classified by STRUCTURE v 2.3.4 [39]. The highest

peak of K was estimated as 2 from STRUCTURE Harvester, which was used to estimate the clusters of accessions (Fig 4a). Genotypes with ≥ 80% of shared ancestry were explained into two major clusters (I and II), and < 80% of shared ancestry were categorized as admixtures (Fig 4b). An unrooted alpha (Fig 4c) was developed by TASSEL 5.0, which also divided the genotypes into three major groups. Where 97 individuals were in Cluster 1, Cluster 2 consisted of 93 individuals, and Cluster 3 included 90 individuals. However, these three clusters were again torn apart into different subgroups. Principle component analysis also supported the clustering above and suggested that PC1 accounted for the maximum variation for six traits related to the other two components (Fig 4d and Supplementary Table 4). A kinship matrix developed from 860 SNPs indicated the formation of a group and more substantial relatedness among the genotypes with the red zone (Fig 5).

3.3. Linkage disequilibrium and GWAS analysis

Evaluation of yield-related traits like tiller per plant (TPP), effective tiller per plant (ETP), spikelet fertility percentage (SFP), field grain number (FGN), length breadth ratio (LBR), and thousand-grain weight (TGW) under salt stress were considered for genome-wide studies using 1K Rica SNP derived and filtered markers whereas the BLUP value obtained from 280 genotypes were considered for association mapping analysis using Mixed Linear Model (MLM) was done by TASSEL V5.3.1 software. Linkage disequilibrium was estimated as r2 and considered significant only when the P value is less than 0.01. Fig 6 illustrates the SNP distribution on chromosome 12 according to r2 and p-value. The darker red shade indicated highly linked SNPs, ensuring their inheritance (Fig 5). The range of r2 was from 0.354 to 0.584 throughout the chromosome (Supplementary Table 5). Manhattan plots indicated the most significant associations ((log-(p-value)> 2.92) generated from GWAS (Fig 7b). The QQ plot (Fig 7a) also scanned the normal distribution of phenotypic traits. The Manhattan plot illustrated 8 significant SNPs considering the p>0.001, whereas two SNPs were related to FGN and SFP and each SNP to TPP, ETP, LBR, and TGW (Fig 6b, Supplementary Table 7). For FGN, the two significant SNPs with high probability (SNP0758 and SNP0759) were in Chromosome 9, whereas for SFP, on chromosomes 8 and 12 (SNP1127 and SNP0966, respectively). One significant SNP was detected on chromosomes 10 and 5 for TPP (SNP0796) and ETP (SNP0414) traits, while two significant SNPs were found in chromosome 12 for LBR (SNP0920) and TGW (SNP0976), respectively

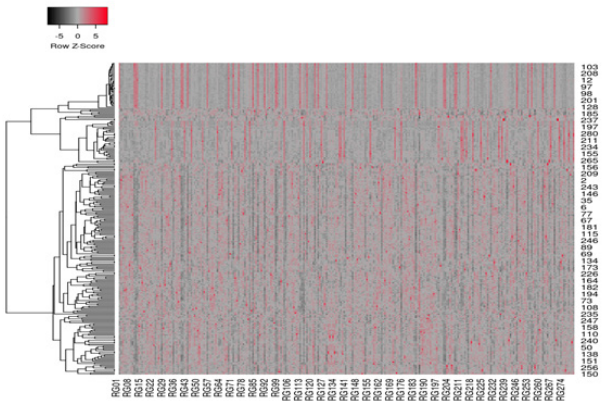

Fig. 5. The kinship matrix developed from 860 SNPs formed groups and relatedness among the genotypes.

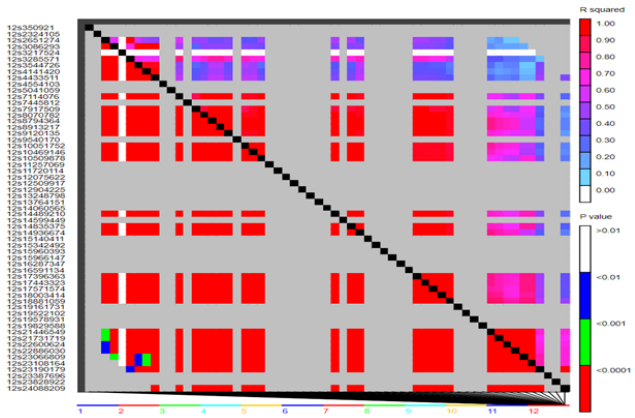

Fig. 6. Distribution of SNPs on chromosome 12 according to r2 and p-value.

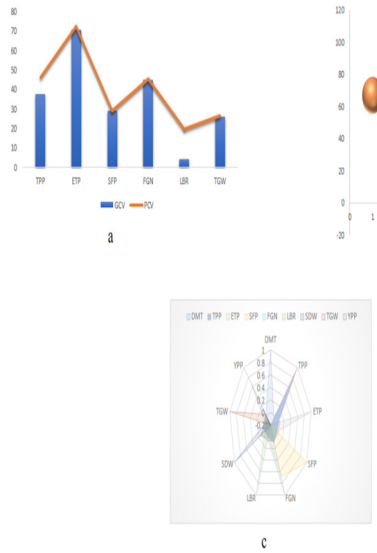

Fig. 3. GCV, PCV (a), broad sense heritability (b), and GA (c) among 280 F_2 genotypes for six traits tillers per plant (TPP), effective tillers per plant (ETP), spikelet fertility percentage (SFP), field grain number (FGN), grain length breadth ratio (LBR) and thousand-grain weight (TGW).

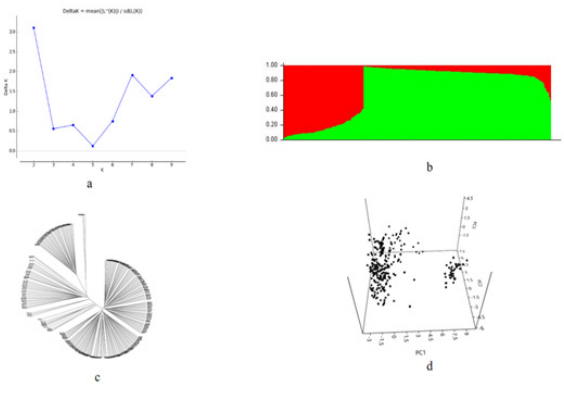

Fig. 4. Genetic relationship among the mapping population and population structure (a) clusters of accessions, (b) shared ancestry category, (c) relatedness of the genotypes based on SNP marker data by alpha (rooted) matrix, and (d) principle component analysis for six traits.

Table 1. Positions of Genes across the significant SNPs.

Trait	Marker	Chr	Position	Gene	Location	Gene type	Base pairs
SFP	SNP1127	8	27133000	Os08g0542200	8: 27130095-27133316	Protein coding gene	2789
FGN	SNP0759	9	21348882	Os09g0542100	21347264-21350949	Protein coding gene	1840
LBR	SNP0920	12	4554103	Os12g0190100	4551091-4556193	Protein coding gene	1926

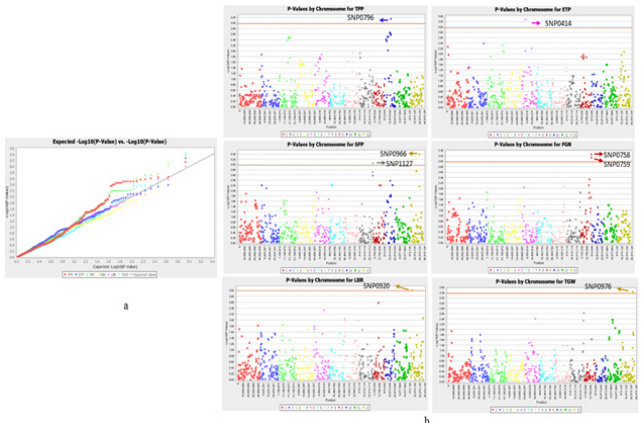

Fig. 7. (a) Q/Q plots of association mapping for the studied traits using the GLM model, (b) Manhattan plot showing significant association for the traits tillers per plant (TPP), effective tillers per plant (ETP), spikelet fertility percentage (SFP), field grain number (FGN), grain length breadth ratio (LBR) and thousand-grain weight (TGW) with the SNP molecular data.

(Supplementary Table 6). From the Gramene Browser, 8 putative genes were identified in chromosomes 8, 9, and 12 between the genomic regions of SNP0920 and SNP1127. Among them, three genes were identified as protein-coding genes for SFP, FGN, and LBR traits (Table 1).

4. Discussion

Accumulation of soluble salts in the soil, termed Salinity, is now occupying about 20% of total agricultural land as well as 50% of the cropland worldwide [40]. It is estimated that salinity causes approximately US$11 billion in global losses each year, with projections indicating that this figure may continue to rise over time [41]. Rice is one of the most consumable staple foods that feed billions worldwide and a susceptible one compared to maize, wheat, or barley. Saline soil can affect the rice at different growth stages, from seed germination to reproduction. Rice production reduces by 2% for each ds/m when the soil salinity crosses 3ds/m, a significant loss for coastal farmers. It's needed to develop new genotypes that can withstand the saline soil and give an expected yield to the farmers. Conventional breeding is a time-consuming and challenging process, as Salinity is controlled by more than one gene. So, the most reliable and convenient solution is to understand the plant's genetic makeup through GWAS, a new generation approach to finding genes across the chromosome. As a prerequisite of GWAS that needed wide genetic variation [42], we took 280 F2 genotypes for genotyping and phenotyping. Though recombinant inbred lines (RIL) or near-isogenic lines (NIL) are preferable to study genome-wide association, researchers also reported using other populations rather than these two [43, 44]. We continued the Salinity till the reproductive stage. Phenotypic trait analysis is the critical factor in getting a good result in combination with genotypic data. Frequency distribution was normal for TPP and LBR, whereas it skewed for SFP and ETP. Bi-modal distribution was reported for FGN and TGW. Haque et al. [45] also reported bi-modal distribution for FGN and FGW traits in transgressive segregants of Horkuch and IR29 due to salinity stress at the reproductive stage. Though all characteristics were found with higher heritability percentages, the two traits directly related to yield, i.e., ETP and TPP both were spotted with higher heritability and GA%, indicating their maximum probability to pass on their transgressive segregants [38]. This result is supported by earlier findings on rice by Lipi et al., [46], Laxuman et al. [47], and Khatun et al. [48]. STRUCTURE analysis divided the F2 mapping population into three major groups based on their genetic relationship, and the Neighbour-joining tree drawn by TASSEL 5.0 and PCA also supported the clusters. The low Fst value ranged from 0.011 to 0.196, resembling the value found by Mogga et al. [49] and Oloka et al. [50]. An association map was drawn from BLUP values using the most suitable Mixed Linear Model that ensures minimizing false positives created by population structures [3]. Manhattan plot painted the eight significant SNPs with log-(p-value)> 2.92 generated from GWAS. Two significant SNPs were found for the most critical trait for grain yield, i.e., FGN (SNP 0758 and SNP 0759) and SFP (SNP 1127 and SNP 0966).

While this study presents significant insights into SNP associations with salt-tolerance traits, certain limitations should be considered. First, the sample size of 280 F2 individuals, while adequate for initial GWAS mapping, may limit the statistical power to detect smaller effect loci, and expanding the population or using multi-generational analysis could enhance the robustness of the findings. Additionally, salt tolerance was evaluated under controlled, artificially induced salt stress, which may not fully capture the environmental complexity that plants encounter under field conditions. Environmental factors such as temperature, soil pH, and natural variation in salinity could influence trait expression and SNP associations. Thus, further studies across diverse field environments would be beneficial to validate the applicability of these SNPs under real-world conditions. Lastly, while population structure analysis provided clustering insights within the F2 population, it would be advantageous to evaluate the identified SNPs in other genetic backgrounds to determine their potential use in broader rice breeding programs. These limitations underscore the need for future research to validate our findings in more genetically diverse populations and under variable environmental conditions.

Our study's identification of significant SNPs associated with salt tolerance traits in rice aligns with previous research efforts, yet it introduces unique insights into salt tolerance mechanisms. Prior studies have identified SNPs linked to salt tolerance traits like spikelet fertility and grain

weight, often implicating candidate genes related to stress response pathways [51, 52 53]. Unlike these studies, our findings highlight novel SNPs on chromosomes 5, 8, 9, and 12, with specific relevance to yield-related traits such as effective tillers per plant and grain length-to-breadth ratio. Notably, our study identified candidate genes in the region between SNP0920 and SNP1127, which have protein-coding functions yet are unreported for their involvement in salt tolerance. This divergence suggests potentially unexplored genetic pathways that could influence rice's response to salinity stress. Additionally, our population structure analysis, identifying two primary genetic clusters, complements similar findings [54, 55)], affirming a genetic basis for salt tolerance traits. Overall, our results contribute valuable genomic markers and candidate genes that could be incorporated into breeding programs aimed at developing salt-tolerant rice varieties, thereby supporting sustainable rice production under saline conditions.

As Quantitative trait Loci (QTLs) control plant tolerance against Salinity, numerous QTLs, including a major one named "Saltol," were identified, cloned, and transferred to the improved varieties to make them salt-tolerant at their seedling stage [56]. We got 860 SNPs through the GWAS study at the reproductive stage, and 42 were encoded earlier for several genes responsible for different tolerable traits [57, 58, 59] also reported Three SNPs for grain filling were reported by Lekklar et al. [8] on chromosomes, but they didn't confirm any major QTL. We got three protein-coding putative genes, i.e., Os08g0542200 at 2905bp upstream of SNP1127 on chromosome 8, Os09g0542100 at 1618bp upstream of SNP0759 on chromosome and Os12g0190100 at 3012bp upstream of SNP0920 on chromosome 12 that was responsible for SFP, FGN, and LBR traits respectively. Haque et al. [45] reported two significant QTLs for FGN (qFGN.10@58.5) and SFP (qSF.10@59), but both are in chromosome 10. So further study could lead to identifying major QTLs that can reveal these genes as novel ones. Through this experiment data, GWAS of the F_2 population revealed associated significant SNPs with salt-tolerant traits that could be used for future breeding programs.

However, this study has identified significant SNPs associated with salt-tolerance-related traits and several potential candidate genes, further research is needed to confirm their functional roles. Future work could focus on functional validation of these candidate genes through gene-editing techniques such as CRISPR-Cas9 or RNA interference (RNAi) to establish their precise role in salt tolerance. Additionally, exploring the expression patterns of these genes under varying levels of salt stress across different developmental stages could provide insights into their regulatory mechanisms. Environmental conditions, such as soil pH, temperature, and moisture, can significantly impact gene expression and trait manifestation; thus, testing these genes under diverse environments will be essential to ensure stability and efficacy of salt tolerance traits. Such studies could pave the way for the development of climate-resilient, salt-tolerant rice varieties, which are crucial for sustainable rice production under adverse conditions.

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Conflicts of Interest
The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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