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Integrating IRRI standardized phenotyping and genome-wide association analysis to decipher salinity tolerance and yield responses in rice

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Abstract Soil salinization poses a severe threat to rice production, and the reproductive growth is particularly sensitive to salt stress. Although the Standard Evaluation System (SES) developed by the International Rice Research Institute (IRRI) provides a rapid visual scoring system for salinity tolerance screening, the relationship between SES scores, final yield components, and their underlying genetic basis remains insufficiently understood. In this study, a population of 118 lines from diverse origins was employed for field experiments under salt stress. Salinity tolerance of the materials was classified according to the IRRI-SES standard, key agronomic traits were measured at maturity and quantitative trait loci (QTL) associated with salt tolerance were identified via genome-wide association analysis (GWAS). The 118 lines were categorized into five SES classes, and their agronomic traits were closely correlated with SES scores. Highly significant differences ($p < 0.01$) were observed between SES 3 (tolerant) and SES 5 (moderately tolerant) groups for plant height and panicle length. while significant differences ($p < 0.01$) were detected between SES 5 and SES 7 (sensitive) groups for thousand-grain weight. Correlation analysis further indicated that plant height, panicle length, and thousand-grain weight were highly sensitive to salt stress, and subsequent genetic analysis revealed the loci governing these traits. Collectively, our findings demonstrate that SES scoring provides a useful framework for evaluating salt stress responses and predicting variation in agronomic performance among rice genotypes. By integrating standardized phenotypic evaluation with genetic analysis, this approach facilitates the identification of candidate genomic regions associated with salt tolerance, thereby providing valuable molecular markers

and genetic resources for breeding salt-tolerant and high-yielding rice varieties.

Keywords: salt tolerance, salinity stress, natural population, agronomic traits, genome-wide association study (GWAS)

Introduction

Soil salinization has become an increasingly serious threat to global agricultural production in recent years. According to recent reports from the food and agriculture organization (FAO), approximately 1.381 billion hectares of land worldwide were affected by salinity in 2024, accounting for 10.7% of Earth's land surface, while nearly 20% of irrigated land is directly threatened by the saline conditions (Lan et al. 2025; Siyao Dai et al. 2025; Cheng et al. 2025). As a staple food crop for nearly half of the world's population, rice (*Oryza sativa* L.) is particularly sensitive to saline stress (Wang et al. 2025; Riaz et al. 2019). Previous studies have shown that salt stress during the reproductive stage can reduce grain yield by approximately 50%, depending on genotypes and stress intensity (Aninbon et al. 2026; Zheng et al. 2023). Therefore, developing salt tolerant rice cultivars is essential for ensuring global food security and promoting the sustainable utilization of saline soils (Joie Ramos et al. 2026).

Rice responds to salt stress through coordinated morphological, physiological, biochemical, genetic and agronomic adaptations. Morphologically, salt stress significantly inhibits plant height, tiller production, and biomass accumulation (Li et al. 2026; Qin et al. 2020). Moreover, salt sensitive genotypes exhibit substantially greater reductions in these growth parameters than salt-tolerant genotypes (Ye et al. 2021; Liu et al. 2022). At the physiological and biochemical levels, salt stress disrupts cellular osmotic homeostasis and induces ion toxicity. Excessive accumulation of Na^+ and Cl^- ions disrupts ionic balance, suppresses the K^+ and Ca^{2+} uptake, and

promotes reactive oxygen species (ROS) accumulation, thereby impairing enzymatic activities and photosynthetic performance (Lindberg and Premkumar 2024); (Sackey et al. 2025a). To alleviate osmotic stress, rice accumulates compatible solutes such as soluble sugars, and proline (Mmbando and Massawe 2026; Sackey et al. 2025b), which help maintain cellular osmotic balance (Nounjan et al. 2024). Simultaneously, plant activate the enzymatic antioxidant defense system including superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR) to scavenge excessive ROS and mitigate oxidative damage (Ikram et al. 2025).

At the genetic level, salt tolerance in rice is a complex quantitative trait controlled by multiple loci distributed across the genome (Singh et al. 2021b; Zhang et al. 2026; Kim et al. 2025). Individual quantitative trait loci (QTLs) may explain less than 5% to more than 40% of the observed phenotypic variation (Singh et al. 2021a). Among the major genes identified to date, *OsHKT1;5* plays a key role by mediating Na⁺ unloading from the xylem, thereby maintaining Na⁺/K⁺ homeostasis in aerial tissues and enhancing the salt tolerance (Choi et al. 2025; Zhang et al. 2025a; Kim et al. 2025). In addition, transcription factors including *OsZIP23*, *OsNAC3*, and *OsMYB6*, have been demonstrated to regulate the rice responses to salt stress by modulating ROS scavenging, ion transport, and osmotic adjustments (Xiang et al. 2008; Ikram et al. 2025); (Singh et al. 2021a; Negi and Kumar 2025). Agronomically, the reproductive stage is considered one of the most salt-sensitive developmental stages in rice. Previous studies have reported that salt stress reduces grain yield, with considerably greater yield losses observed in salt-sensitive cultivars (Pandit et al. 2024; Li et al. 2023; Aninbon et al. 2026; Santanoo et al. 2025).

The International Rice Research Institute (IRRI) developed the standard evaluation system (SES) for rapid phenotypic assessment of salt tolerance in rice. Although SES is traditionally applied at the seedling stage, the injury descriptors can also provide useful visual indicators of salinity responses during late developmental stages. In this study, SES scoring was applied at the mature stage to integrate visual stress symptoms with final yield-related agronomic traits. The SES classifies salt injury responses using a nine-point scale ranging from 1 to 9, with higher scores indicating increasing injury severity (Hidayah et al. 2022). A score of 1 indicates normal growth without visible symptoms and represents highly salt tolerant plants. A score of 3 denotes slight leaf tip whitening and indicates tolerant plants. A score of 5 represents leaf rolling and moderate injury. A score of 7 indicates severe damage characterized by extensive leaf drying and high salt sensitivity, whereas a score of 9 corresponds to plants that are almost entirely dead or dying, and exhibits extreme susceptibility. This standardized scoring system enables rapid, reproducible, and large-scale phenotypic screening under controlled conditions (Singh et al. 2021a). Moreover, SES scores have been shown to be negatively correlated with grain yield under salt stress. Increasing SES scores are associated with reductions in plant height, seed setting rate, and effective tiller number (Mondal et al. 2022; Sejuti Mondal et al. 2025; Xu et al. 2024). Consequently, SES has become an efficient tool for preliminary screening of salt tolerance in rice germplasm. Nevertheless, because the scoring system is primarily based on visual symptoms, its relatively coarse classification should ideally be complemented by quantitative agronomic measurements and detailed phenotypic assessments of plant structures to achieve a more comprehensive evaluation (Zeng et al. 2002).

Beyond refining phenotypic evaluation, elucidating the genetic architecture underlying

these agronomic responses is essential for improving breeding efficiency. Advances in molecular genetics and high-throughput genotyping have already accelerated the identification and mapping of the salt-tolerance related QTLs, providing a valuable resource for rice improvement. To date, approximately 935 QTLs associated with salt tolerance have been identified in rice, providing a valuable genetic resources for marker assisted selection (MAS) (Padmavathi et al. 2024; Singh et al. 2021a). Among these, the *Saltol* locus explains approximately 43-70% of phenotypic variation in seedling-stage salinity tolerance and has been successfully introgressed into several elite rice cultivars (Akhlasur Rahman et al. 2024; Yadav et al. 2020). Similarly, introgression of *Saltol* into the elite rice cultivar Pusa Basmati-1 significantly enhanced seedling stage salt tolerance (Kumar et al. 2018). Beyond *Saltol*, three major reproductive stage QTLs, *qGY3.1*, *qNFS3.1*, and *qPT3.1* have been identified as being associated with grain yield, seed setting rate, and effective panicle number under salt stress (Mondal et al. 2022). More recently, the application of high-density single nucleotide polymorphisms (SNP) markers and genomic selection has enabled more accurate foreground and background selection, facilitating the pyramiding of favorable QTLs to enhance salt tolerance while improving the breeding efficiency and shortening breeding cycles (Mohanavel et al. 2024; Marè et al. 2023; Muthu et al. 2020; Akhlasur Rahman et al. 2024).

Despite considerable progress in understanding rice salt tolerance, several knowledge gaps remain. In particular, the relationship between seedling-stage salt stress responses and reproductive stage yield performance within specific genetic backgrounds remains poorly understood. In addition, salt-tolerance QTLs that provide enhanced tolerance without compromising yield have not yet been fully characterized (Singh et al. 2021a; Aninbon et al.

2026; Fan et al. 2021). Most previous studies have focused either on seedling survival in biparental populations or on yield performance during the reproductive stage, with limited efforts to integrate early-stage stress responses with agronomic performance at maturity. Furthermore, although IRRI SES provides a standardized framework for evaluating salt tolerance, its predictive value for field performance and agronomic traits require further validation (Singh et al. 2021a). The hypothesis was that visual SES scores are predictive of reproductive stage agronomic performance and that integrating phenotypic evaluation with genome wide association analysis would facilitate the identification of candidate loci (QTLs) associated with salt tolerance. Therefore, this study utilized 118 natural lines to evaluate salt tolerance with three specific objectives: First, to evaluate the relationship between visual SES scores for key agronomic traits under field salt stress; Second, to quantify the genotypic variation in salt-responsive agronomic and yield related traits; Third, to identify candidate genomic regions associated with salt tolerance through genome wide association analysis. By integrating standardized phenotyping with genetic analysis, this work aims to identify candidate genomic regions and provide potential molecular resources for breeding rice cultivars adapted to saline environments.

Materials and methods

Plant material and experimental design

A population of 118 natural population was used in this study. The field experiment was conducted at the Wensu Rice Experimental Station, Institute of Crop, Xinjiang Academy of Agricultural Sciences, Xinjiang China (41.3015°N, 80.1462°E). Seeds were sown on 5 April

2025, and seedlings were transplanted 30 days after sowing (5 May 2025). Two treatments were established: salt stress and control (freshwater irrigation). For phenotypic comparison, one salt-tolerant check variety and one salt-sensitive check variety were included.

The evaluation pools were filled with uniformly mixed, salt-free soil (electrical conductivity $< 1 \text{ dS m}^{-1}$). Freshwater (electrical conductivity $< 1 \text{ mS cm}^{-1}$, pH 6.5–7.5) was used for irrigation and ponding. Basal fertilizer was applied 40 kg/ha of compound fertilizer with a 12:5:11 N-P₂O₅-K₂O ratio. 3–5 days before transplanting. Seeds were sown and seedlings raised at the appropriate time.

For seed preparation, the seeds of all tested germplasm were placed in an oven at 28°C for 36 hours, then treated with 2.5% (v/v) NaClO solution for 30 minutes, rinsed three times with distilled water, and subsequently soaked and germinated under controlled conditions. The germinated seeds were sown and managed following conventional nursery practices.

Uniform, healthy seedlings at the 3-4 leaf stage were transplanted into evaluation pools as single seedling per hill, with a spacing of 20 cm×10 cm between plants within rows. Each line was planted in three rows with 10 plants per row and each row considered as replication and arranged in a randomized complete block design. Management practices for both salt-stress and control (freshwater) groups were strictly identical, including pest, disease, and weed control, following standard field management procedures; all corresponding operations were completed on the same day.

After transplanting, the seedlings were allowed to recover for 5–7 days. Subsequently, saline water was uniformly applied to the evaluation pools. A portable conductivity meter was

used to measure to monitor electrical conductivity at no fewer than five points in each pool. Freshwater and saline water were mixed to maintain the electrical conductivity of the water layer at 6 mS cm⁻¹ at 25°C), with a water depth of 3–5 cm. The salinity and conductivity of the water layer were monitored daily, and adjustments were made by adding freshwater or saline water as needed to keep the conductivity at 6 mS/cm, with a variation maintained within ± 1 mS cm⁻¹. The control group received freshwater irrigation, while all other management conditions were kept the same.

Phenotypic evaluation

Standard evaluation system (SES) for salt tolerance

Rice salt tolerance was evaluated according to SES developed by IRRI. Based on the severity of salt injury symptoms, plants were classified into five tolerance levels corresponding to SES scores 1, 3, 5, 7, and 9. An SES score of 1 indicates normal growth without visible symptoms and represents highly salt tolerant plants. A score of 3 is characterized by slight leaf discoloration, chlorosis, whitening, or rolling under salt stress, accompanied by a moderate reduction in tillering, and represents tolerant plants. Plants assigned a score of 5 exhibits obvious growth inhibition, extensive leaf discoloration or whitening and rolling, and reduced tiller number, representing moderately tolerant genotypes. A score of 7 indicates severe growth inhibition, extensive leaf desiccation, and partial plant mortality, reflecting salt-sensitive genotypes. Plants with a score of 9 are almost entirely dead or dying and are classified as highly sensitive to salt stress (Table 1). SES scores were recorded at maturity stage (124 Days after sowing). SES scores were subsequently used as categorical indicators comparison of agronomic traits and genetic analysis.

Table 1. Standard Evaluation System (SES) of the International Rice Research Institute (IRRI) for visual assessment of salt injury in rice.

SCALE (Salt injury)		
Score	Description	Tolerance Classification
1	Growth and tillering nearly normal	Highly tolerant
3	Growth nearly normal but there is some reduction in tillering and some leaves discolored/whitish and rolled	Tolerant
5	Growth and tillering reduced; most leaves discolored/whitish and rolled; only a few elongating	Moderately tolerant
7	Growth completely ceases; most leaves dry; some plants dying	Sensitive
9	Almost all plants dead or dying	Highly sensitive

Note: Scores range from 1 to 9 with increasing scores indicating progressively greater damage.

Field imaging

Field images were acquired at the maturity stage using a Canon EOS 700D digital camera. Photographs were taken during two fixed time periods each day (07:00-09:00 and 16:30-18:30) to minimize the variation caused by changing light conditions. For each line, both overhead and sideview images were captured to document canopy architecture and individual plant morphology under salt stress.

Measurement of agronomic traits

Agronomic traits were measured at physiological maturity. Three representative plants were randomly selected from each experimental unit for trait measurement. The following traits were measured:

- (i) plant height (cm) was measured from the soil surface to the tip of the tallest panicle, excluding awns.
- (ii) panicle weight (g) After the rice seeds are fully mature, cut the panicle intact at the

panicle neck and weigh it on an electronic balance.

- (iii) Leaf area (cm²) was estimated based on flag leaf length and maximum width of the flag leaf from the main culm using the following equation:

Leaf area = Length × Width × 0.75 (A correction coefficient for flag leaf shape in rice, converting the rectangular product to actual leaf area)(Liu et al. 2023)

- (iv) effective panicle number was determined by counting fully emerged panicles from the flag leaf sheath. The average number of effective panicles per plant was calculated.

- (v) Seed setting rate (%) was calculated by manually threshing grains from each panicle and classifying them as filled or unfilled grains. The seed setting rate was calculated as:

Seed setting rate (%) = (Number of filled grains/Total number of grains) × 100

- (vi) Thousand grain weight (g) was determined using fully filled grains collected from the three sampled plants. After thorough mixing, grains were divided into three subsamples of 200 grains each, grain weight was measured and converted to thousand-grain weight.

DNA extraction, genotyping, and GWAS analysis

DNA extraction

Genomic DNA was extracted from fresh leaf tissues using the cetyltrimethylammonium bromide (CTAB)-based method. Approximately 2 g of fresh leaf tissues were frozen in the liquid nitrogen and grinded into a fine powder. Samples were mixed with preheated CTAB extraction buffer containing 2% CTAB, 20 mM EDTA (pH: 8.0), 1.4 M NaCl, and 100 mM Tris-HCL (pH: 8.0), followed by incubation for 30 min. DNA was purified by chloroform extraction and precipitated with 0.8 volumes of isopropanol. The DNA pellet was washed twice with 99% ethanol, air dried, and re-suspended in 100 µL of nuclease free water. DNA

concentration and purity were determined using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA). Samples containing at least 3 µg of genomic DNA were used for sequencing library construction.

Whole genome sequencing

Sequencing libraries with insert sizes of approximately 400-500 bp were constructed and sequenced on the Illumina HiSeq 2000 platform (Illumina, San Diego, CA) to generate 90 bp paired end reads. Raw sequencing data were processed by removing adapter sequences and low-quality reads (defined as reads with 50% of the bases having scores ≤ 5.0). Library construction, sequencing, and initial data processing were performed by BGI (Shenzhen, China).

Genotype imputation and genetic analysis

Genotype imputation was performed using Beagle software (version 3.3.2). The imputed genotype dataset was converted into BED format using PLINK (version 1.9) for downstream analysis. The SNP dataset for genome-wide association study (GWAS) was obtained from previous publication (Liu et al. 2021) based on whole-genome resequencing of 118 rice accessions from the MiniCore Collection. High-quality SNPs (1,889,946) were selected for GWAS by removing SNPs with minor allele frequency (MAF) $< 5\%$ prior to analysis. Genome-wide association analysis was performed using GEMMA (version 0.941) based on a linear mixed model (LMM), with the estimated kinship matrix incorporated as a random effect to account for relatedness among individuals. Population structure was controlled by including the first four principal components as covariates.

Genome wide significance thresholds were determined using 200 phenotypes permutations.

For each permutation, phenotypic values were randomly shuffled and analyzed independently. The lowest p -value obtained from each permutation was recorded and the upper 5% quantile of these values were used as the genome wide significance threshold. Manhattan plots and quantile-quantile (Q-Q) plots were generated using R software.

Results

Phenotypic classification of salt tolerance in natural population

The 118 lines were phenotypically evaluated under salt stress according to the IRRI's SES (Supplemental Table S1). Based on the severity of leaf chlorosis, leaf desiccation, and overall plant damage, the lines were classified into 5 salt tolerance levels: highly tolerant, tolerant, moderately tolerant, sensitive, and highly sensitive. These five classes correspond to IRRI SES scores of 1, 3, 5, 7, and 9, respectively. Among the 118 lines, 1 was classified as highly tolerant, 43 as tolerant (36.44% of the total), 70 as moderately tolerant (59.32% of the total), 3 as sensitive (2.54% of the total) and 1 as highly sensitive. The majority of lines exhibited intermediate response (SES 3-5), whereas extremely tolerant and highly sensitive phenotypes were rare. To illustrate the phenotypic variation among SES categories, one representative line from each category was selected for imaging (Figure 1).

The highly tolerant line 25H121 maintained green, fully expanded leaves with no apparent inhibition of plant growth under salt stress. The tolerant line 25H135 exhibited slight whitening at the leaf tips, indicating mild salt injury. The moderately tolerant line 25H23 displayed leaf rolling accompanied by approximately one-sixth leaf senescence. The sensitive line 25H91 exhibited extensive leaf desiccation and partial plant mortality after salt treatment. In contrast, the highly sensitive line 25H57 showed severe growth inhibition, with only four of the thirty

plants surviving the stress treatment, and surviving plants retained only a few green leaves (Figure 1).

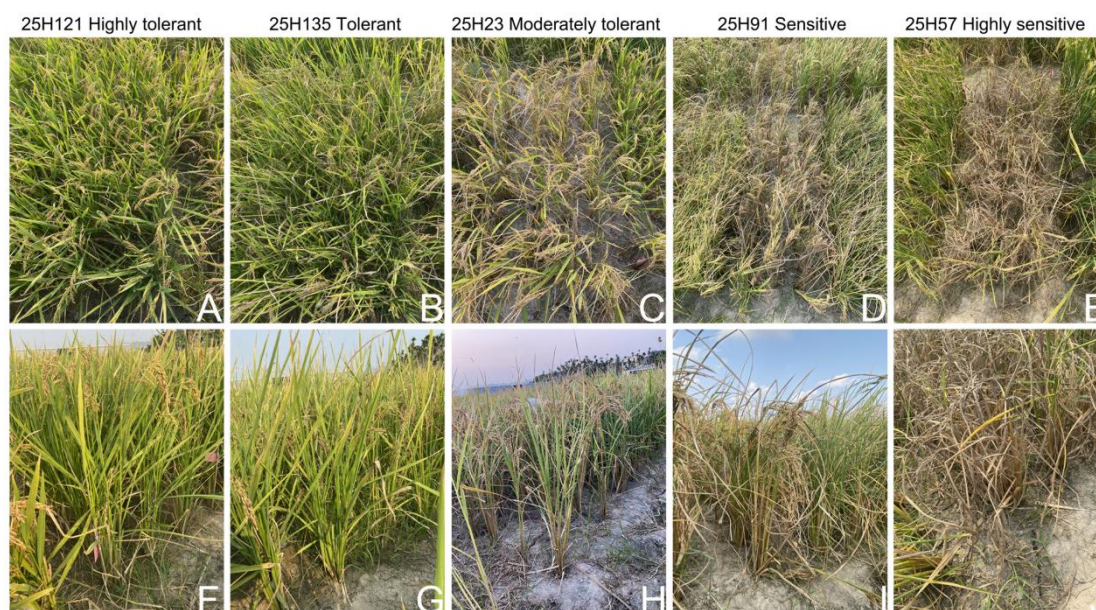


Figure 1. Representative phenotypes of rice lines exhibiting different levels of salt tolerance based on the IRRI's standard evaluation system (SES). Highly tolerant (A, F), tolerant (B, G), moderately tolerant (C, H), sensitive (D, I), highly sensitive (E, J).

Comparison of agronomic traits among different salt tolerance groups

To evaluate the effects of salt stress on agronomic performance, lines were grouped according to SES categories into three groups: SES 3 (tolerant), SES 5 (moderately tolerant), and SES 7 (sensitive). Because the SES 1 (highly tolerant) and SES 9 (highly sensitive) each contained only one line, statistical analysis was not performed. Pairwise comparisons were subsequently performed to determine whether plant height, thousand grain weight, seed setting rate, panicle length, and effective number of panicles per plant significantly differed among these groups (Table 2). Among agronomic traits evaluated, plant height and panicle length showed significant differences between the SES 3 and SES 5 groups. The mean plant height of

SES 5 group (94.02 cm) was significantly greater than that of SES 3 group (85.01 cm) ($p = 0.005$). Similarly, the average panicle length of SES 5 group (19.41 cm) was significantly greater than that of SES 3 group (17.12 cm) ($p = 0.002$). No significant differences in either trait were observed between the SES 7 group and other two groups. For thousand-grain weight, the SES 5 group (23.67 g) exhibited a significantly higher value than the SES 7 group (19.31 g) ($p = 0.033$). Although the difference between SES 3 and SES 7 groups was not statistically significant, a decreasing trend was observed ($p = 0.068$). No significant differences were detected among the three SES groups for seed setting rate or effective number of panicles per plant (Table 2).

Table 2. Comparison of agronomic traits among rice lines classified according to IRRI SES categories.

Agronomic metrics	Group 1 (score)	Group 2 (score)	Group 1 average	Group 2 average	P value	significance
Plant height (cm)	3	5	85.007	94.019	0.005	**
Plant height (cm)	3	7	85.007	84.033	0.920	NS
Plant height (cm)	5	7	94.019	84.033	0.299	NS
Thousand grain weight (g)	3	5	23.206	23.668	0.428	NS
Thousand grain weight (g)	3	7	23.206	19.307	0.068	NS
Thousand grain weight (g)	5	7	23.668	19.307	0.033	*
Seed setting rate (%)	3	5	75.515	78.833	0.174	NS
Seed setting rate (%)	3	7	75.515	74.143	0.781	NS
Seed setting rate (%)	5	7	78.833	74.143	0.326	NS
Panicle length (cm)	3	5	17.721	19.405	0.002	**
Panicle length (cm)	3	7	17.721	18.263	0.742	NS
Panicle length (cm)	5	7	19.405	18.263	0.483	NS
Effective panicles ($\times 10^4/\text{hm}^2$)	3	5	184.54	190.93	0.558	NS
Effective panicles ($\times 10^4/\text{hm}^2$)	3	7	184.54	204.45	0.576	NS
Effective panicles ($\times 10^4/\text{hm}^2$)	5	7	190.93	204.45	0.411	NS

Note: * indicates significant difference; ** indicates highly significant difference; NS indicates no significant difference.

Distribution of agronomic traits across salt tolerance classes

To further examine the relationship between SES categories and agronomic traits, the distribution of each trait was visualized using boxplots (Figure 2). For plant height most lines in SES 3 group ranged from 75-105 cm, whereas SES 5 lines were ranged between 85 and 105 cm. The three SES 7 lines showed a wider observed range, with two individuals measuring approximately 90 cm and one individual measuring 66 cm. For thousand-grain weight, the majority of SES 3 lines ranged from 22 to 27 g, whereas SES 5 lines were concentrated between 22 and 24 g. All three SES 7 lines showed TGW below 22 g. For panicle length, the SES 3 group was primarily distributed between 16 to 19 cm, whereas the SES 5 group ranged from 18 to 21 cm. The SES 7 group exhibited a wider distribution spanning approximately 15-22 cm. Overall, these distributions illustrate clear phenotypic variation among tolerance classes, particularly for plant height, panicle length and thousand-grain weight.

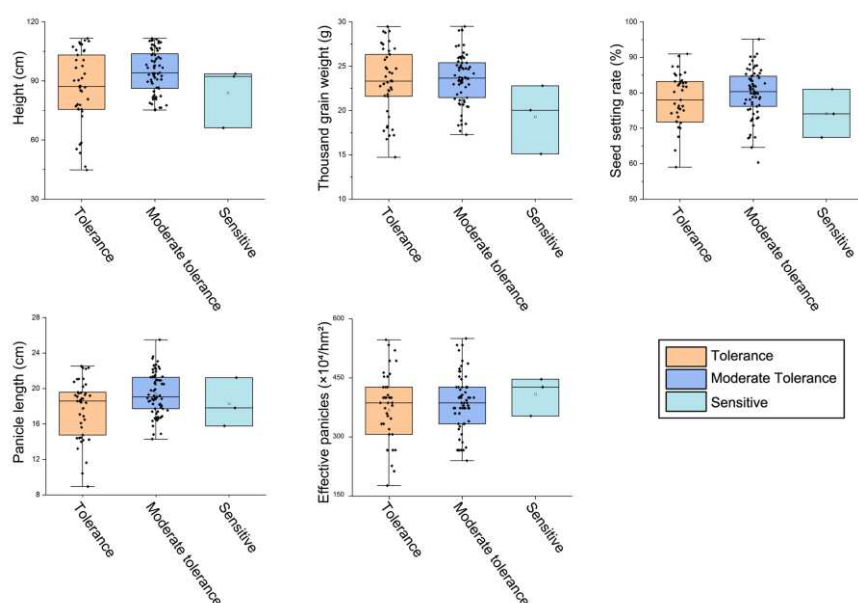


Figure 2. Distribution of major agronomic traits among rice lines, classified into different salt tolerance groups according to IRRI SES.

Boxplots show the distribution of plant height (cm), thousand grain weight (g), seed setting

rate (%), panicle length (cm), and effective panicles per hm across three salt tolerance classes [SES 3: tolerant, n=43 (36.44% of the total); SES 5: moderately tolerant, n=70 (59.32% of the total); SES 7: sensitive, n=3 (2.54% of the total)]. The boxes represent the interquartile range (IQR) with the median indicated by the horizontal line; whiskers extend to the minimum and maximum values within $1.5 \times \text{IQR}$. Significant differences between groups were determined by pairwise comparisons: $**p < 0.01$, $*p < 0.05$. Data for SES 7 are presented descriptively due to the limited sample size (n=3). SES: Standard Evaluation System.

Phenotypic variation and diversity of agronomic traits in natural populations

Under uniform salt stress conditions, the 118 lines showed varying degrees of phenotypic variation for five agronomic traits (Table 3). The highest coefficient of variation (CV) was observed for panicle weight at 41.32%, indicating significant phenotypic variation among genotypes for this trait under salt stress. Effective panicle number showed the second highest coefficient of variation (31.47%), indicating considerable variation among genotypes under salt stress. Plant height and seed-setting rate showed CV values of 18.57% and 16.06%, respectively, indicating moderate phenotypic variation among natural population lines during vegetative and reproductive growth stages. Although the CVs for thousand-grain weight (15.83%) and panicle length (14.89%) were relatively lower compared to other traits, they still reflected substantial variation among the lines.

Table 3. Descriptive statistics of agronomic traits in the natural population lines under saline stress, data represents the population statistics of 118 lines.

Agronomic metrics	Average	Standard deviation	Coefficient of variation
Plant height (cm)	91.0833945	16.91444978	18.57028922

Seed setting rate (%)	0.769660599	0.123585724	16.05717174
Thousand grain weight (g)	23.39953394	3.703249938	15.82616965
Panicle length (cm)	18.85944954	2.808344324	14.89091353
Effective panicles ($\times 10^4/\text{hm}^2$)	372.86267895	117.345044775	31.47138382
Panicle weight (g)	41.42697248	17.11606764	41.31624063

Note: Mean values and standard deviation were calculated based on the mean of replicates within each line; CV (coefficient of variation) = (standard deviation / mean) $\times$ 100%. All values are rounded to two decimal places.

Relationships between SES scores and agronomic traits

To further evaluate the relationship between IRRI SES-based salt tolerance classification and agronomic performance, natural populations were grouped into three levels according to their stress scores: tolerant (SES 3), moderately tolerant (SES 5), and sensitive (SES 7). Spearman's rank correlation coefficient (ρ) between SES scores and individual agronomic traits were calculated for each line. Subsequently, correlation coefficients were summarized within each tolerance group to assess the consistency and directionality of trait responses under saline stress. For each trait, the mean ρ value, standard deviation, median and proportion of significantly positive, significantly negative and non-significant correlations were calculated (Table 4). Based on the observed response patterns agronomic traits were classified into three functional categories: highly stress sensitive traits, stress stable traits and stress responsive traits.

Highly stress sensitive traits

Plant height, panicle length and panicle weight exhibited strong sensitivity to saline stress and were classified as highly stress sensitive traits. In the SES 3 (tolerant) group, these traits showed predominantly negative correlation with SES scores, with 77.3%, 68.2%, and 40.9% of

lines displaying negative correlations for plant height, panicle length and panicle weight, respectively. The mean ρ values for these traits were consistently negative. In the SES 5 (moderately tolerant) group the proportion of significantly negative correlations decreased considerably and for panicle length, a proportion of lines (28.6%), even exhibited significant positive correlations. Most correlations in the group were non-significant, indicating reduced sensitivity of these traits to stress intensity. In contrast, in the SES 7 (sensitive) group, all three traits showed uniformly stronger negative correlations with SES scores (100% significantly negative correlations), indicating severe growth suppression under high stress intensity. Among these traits, plant height exhibited the strongest and most consistent response across all tolerant levels, suggesting that it is the most reliable indicator for distinguishing salt tolerant and salt sensitive genotypes.

Table 4. Spearman's rank correlation coefficients (ρ) between agronomic traits and SES scores within each tolerance group.

SES 3						
Agronomic metrics	Average ρ	Standard error	Median	Negative association(%)	Positive association(%)	Not significant(%)
Plant height	-0.237310371	0.137492097	-0.269899559	0.772727273	0	0.227272727
Seed setting rate	-0.097834545	0.104278974	-0.141073249	0.068181818	0.022727273	0.909090909
Panicle length	-0.211027387	0.097893041	-0.233202843	0.681818182	0	0.318181818
Panicle weight	-0.15111267	0.100709391	-0.169120435	0.409090909	0	0.590909091
Effective panicles ($\times 10^4/\text{hm}^2$)	-0.078350698	0.086001272	-0.070346497	0	0	1
Thousand grain weight	-0.05566688	0.056637482	-0.079536978	0.113636364	0.022727273	0.863636364
SES 5						
Agronomic metrics	Average ρ	Standard error	Median	Negative association(%)	Positive association(%)	Not significant(%)
Plant height	0.082406987	0.05380472	0.063840897	0	0.057142857	0.942857143
Seed setting rate	0.184959811	0.108436718	0.149532654	0.014285714	0.285714286	0.7
Panicle length	0.145544606	0.096917108	0.11203654	0	0.285714286	0.714285714
Panicle weight	0.003838434	0.091614806	0.007800873	0.042857143	0	0.957142857
Effective panicles	0.202870163	0.093369007	0.209334171	0	0	1

($\times 10^4/\text{hm}^2$)						
Thousand grain weight	-0.084616053	0.101109335	-0.107465379	0.014285714	0.914285714	0.071428571
SES 7						
Agronomic metrics	Average p	Standard error	Median	Negative association(%)	Positive association(%)	Not significant(%)
Plant height	-0.839631753	0.05001266	-0.839631753	1	0	0
Seed setting rate	-0.47120073	0.04389501	-0.47120073	1	0	0
Panicle length	-0.757389125	0.320233704	-0.938652205	1	0	0
Panicle weight	-0.828157446	0.058241917	-0.829631751	1	0	0
Effective panicles ($\times 10^4/\text{hm}^2$)	-0.198799831	0.104240707	-0.198799831	0.5	0	0.5
Thousand grain weight	-0.9308974	0.079369002	-0.970725343	1	0	0

Identification and validation of salt-tolerance-associated loci via GWAS

To identify genetic loci associated with salt tolerance in the natural population, a genome-wide association study (GWAS) was conducted using the salt tolerance data as the trait. The analysis results are shown in Figure 3, where the y-axis represents the $-\log_{10}(p)$ values of each SNP (criteria not mentioned in methodology for SNP filtering), the x-axis indicates chromosome numbers, and the suggested significance threshold line is marked.

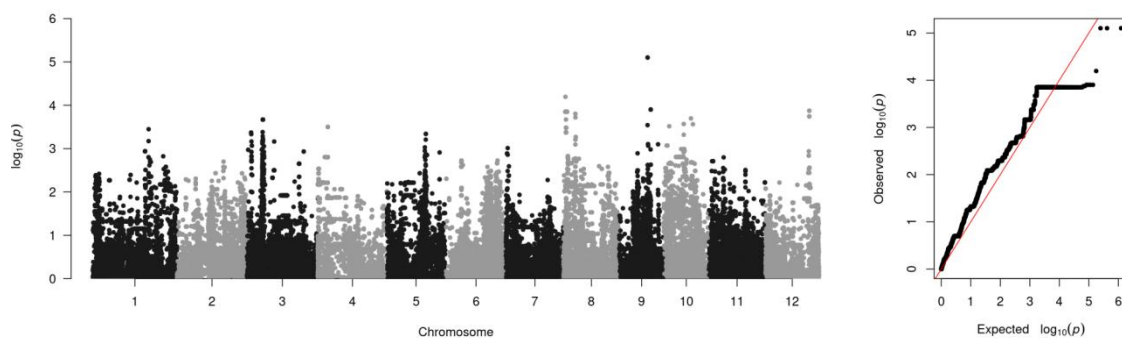


Figure 3. QTL mapping result of survival rate from GWAS.

The Manhattan plot shows multiple SNP peaks above the threshold line across the entire genome, distributed on chromosomes 1, 3, 4, 5, 8, 9, 10, and 12 (Supplemental Table S2).

Among these, significant peaks on chromosomes 4, 9, and 10 did not yield stable candidate regions after LD interval refinement, and thus were excluded from further analysis.

To further analyze the gene composition within intervals harboring significant association signals, this study identified six intervals based on LD blocks surrounding each significant peak (Figure 4). These intervals are located on chromosome 1 (26.80–28.74 Mb), chromosome 3 (8.12–8.24 Mb and 2.06–2.24 Mb), chromosome 8 (0.81–0.92 Mb and 5.96–6.18 Mb), and chromosome 12 (22.31–22.74 Mb). The number of specific SNPs in these six intervals is shown in Table 5. As indicated, chromosome 8 has the highest SNP density (447 SNPs), followed by chromosome 3 (45 SNPs), while fewer SNPs are distributed across the remaining intervals.

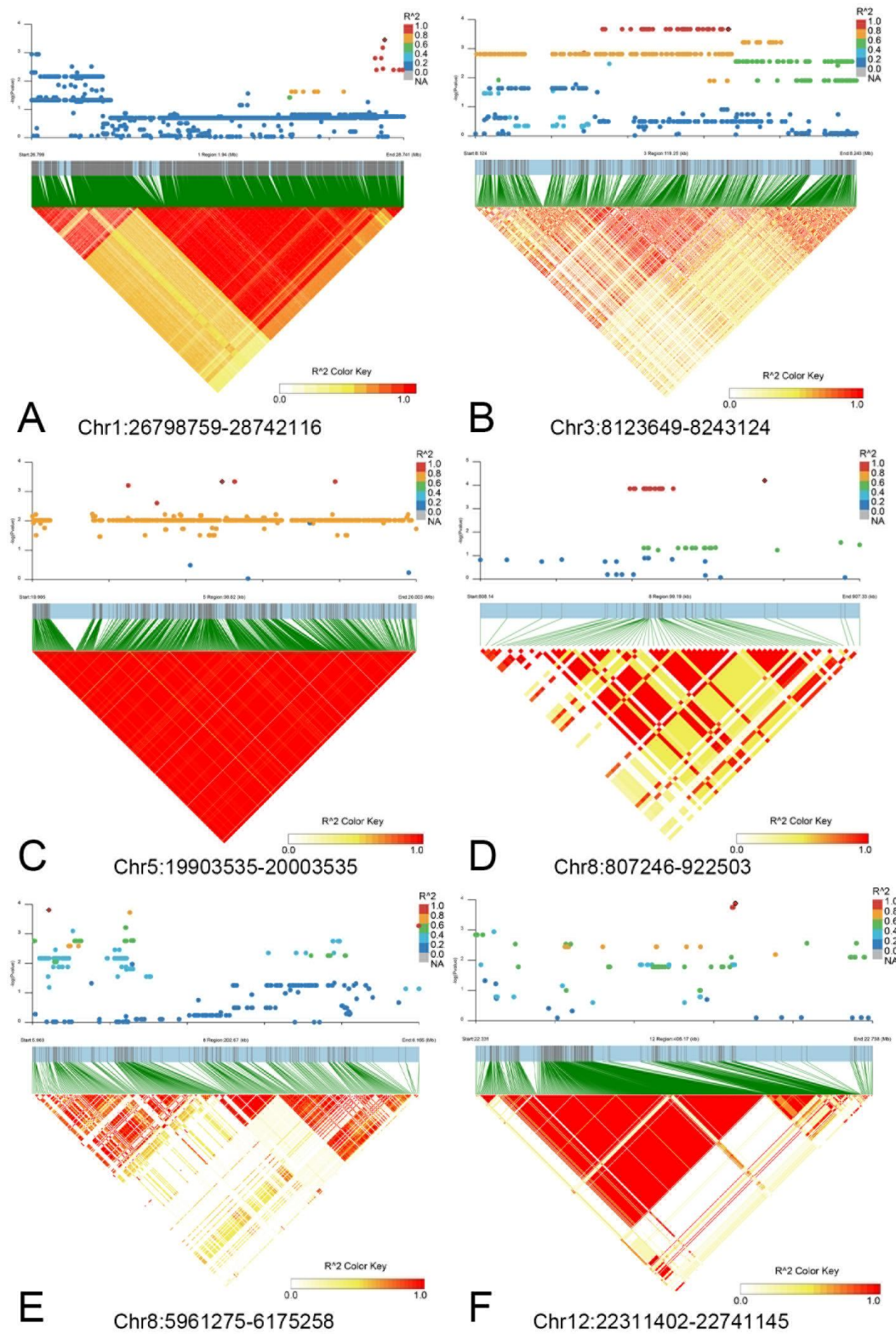


Figure 4. Significant GWAS Loci with Local LD Block Structures

Table 5. Distribution of the top 500 peaks across chromosomes.

Chromosome	Interval start (bp)	Stop coordinate (bp)	Interval length (bp)	SNP count
Chr1	26798759	28742116	200001	1
Chr3	8123649	8243124	119476	45
Chr8	807246	922503	778896	447
Chr8	5961275	6175258	193984	2
Chr12	22311402	22741145	42155	4
Chr12	22338722	22594987	256266	1

Distribution of loci associated with salt tolerance (survival rate) across different IRRI grades

Among the six LD intervals, five contained genes with clear functional annotations, distributed on chromosomes 1, 3, 8, and 12 (notably, only one of the two intervals on chromosome 12 harbored annotated genes). These five intervals were therefore included in the candidate gene analysis, yielding a total of 16 candidate genes across four chromosomes (Table 6; Figure 5). Among them, *OsCSLD4*, *PSSI*, *OsCML7*, *OsAHL1*, *OsPLAIII α* , *OsPEAMT1*, *OsSIRH2-23*, and *OsGAPC3* have been reported to be related to salt tolerance. Additionally, *OsPRI0a* and *PBZ1* are associated with rice disease resistance (Tun et al. 2023; Williamson-Benavides and Dhingra 2021). *OsSWN2* is linked to plant height and gibberellin biosynthesis (Guha et al. 2024). *DCET1* is involved in male reproductive development in rice (Khan et al. 2021a), *OsATX1* encodes a copper chaperone protein (Zhang et al. 2018b), *OsTPR3* participates in ABA signaling pathways and transcriptional regulation (Tang et al. 2016), *OsRAVII* is involved in drought stress response and flowering regulation (Zhang et al. 2025b) and *OsHT* primarily functions in amino acid transport (LIU et al. 2005).

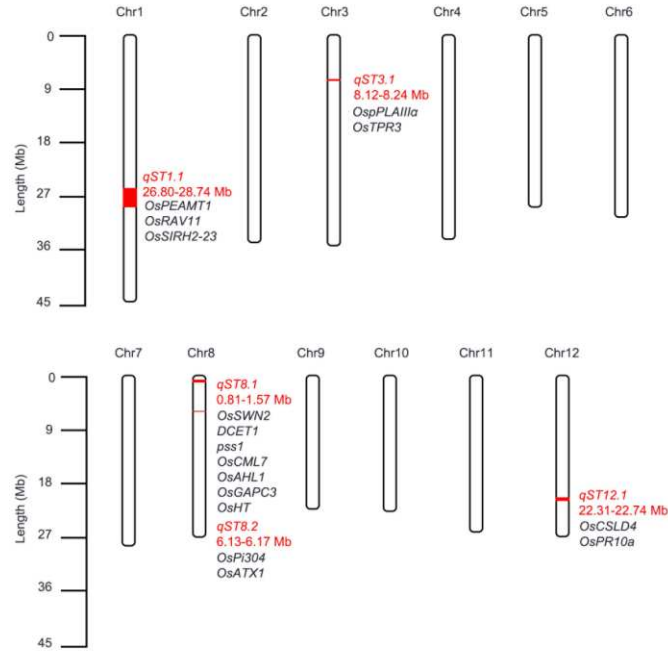


Figure 5. Genomic distribution of salt tolerance QTLs and candidate genes. The figure shows the physical positions of five QTL intervals (qST1.1, qST3.1, qST8.1, qST8.2, and qST12.1) on chromosomes 1, 3, 8, and 12. Red bars represent QTL intervals, with interval lengths in megabases (Mb) indicated. Candidate genes annotated within each interval are listed alongside the corresponding QTL.

Table 6. Candidate genes identified within significant QTL regions associated with salt tolerance.

Gene ID	Gene name	Chromosome	Nearest SNP ID	Reference
Os12g0555500	<i>OsPR10a</i>	Chr12	12m22597141	Not previously reported
Os08g0115800	<i>OsSWN2</i>	Chr8	8m858589	Not previously reported
Os08g0116400	<i>DCET1</i>	Chr8	8m882503	Not previously reported
Os12g0555600	<i>OsCSLD4</i>	Chr12	12m22597300	(Zhao et al. 2022a)
Os08g0117000	<i>pss1</i>	Chr8	8m882503	(Xiao et al. 2021)
Os08g0117400	<i>OsCML7</i>	Chr8	8m882503	(Chinpongpanich et al. 2012)
Os08g0118000	<i>OsAHL1</i>	Chr8	8m882503	(Zhou et al. 2016b)
Os08g0205150	<i>OsPi304</i>	Chr8	8m5971275	Not previously reported
Os08g0205400	<i>OsATX1</i>	Chr8	8m5971275	Not previously reported
Os03g0254700	<i>OsTPR3</i>	Chr3	3m8164454	Not previously reported
Os03g0254400	<i>OsPLAIIIa</i>	Chr3	3m8163649	(Liu et al. 2015)
Os01g0695100	<i>OsPEAMT1</i>	Chr1	1m28642116	(Yu et al. 2025)
Os01g0693400	<i>OsRAV11</i>	Chr1	1m28642116	Not previously reported
Os01g0692700	<i>OsSIRH2-23</i>	Chr1	1m28642116	(Kim et al. 2022)
Os08g0126300	<i>OsGAPC3</i>	Chr8	8m1533998	(Zhang et al. 2011)
Os08g0127100	<i>OsHT</i>	Chr8	8m1561779	Not previously reported

Genotype validation of known salt-tolerance-related genes/QTLs.

In addition to the 16 candidate genes identified through whole-genome screening, this study also conducted targeted detection of eight previously reported salt-tolerance-related genes/QTLs (*SKC1*, *OsWRKY53*, *STG5*, *OsMADS56*, *qSE3*, *GS3*, *OsARF18*). To evaluate the effects of these known loci within the population, genotyping was performed for each line, and survival rates corresponding to different genotypes were calculated across various salt tolerance levels (Supplemental Table S3). Since *OsMADS56* showed a uniform "salt-tolerant, long-grain, early-flowering" phenotype across all lines in the three tolerance groups, and *qSE3* exhibited a consistent "non-tolerant" phenotype in all lines, neither locus displayed genotype segregation and thus was excluded from comparative analysis within the groups.

Based on survival rates across different levels, the average survival rate for the SES 3 group was 51.29%, for the SES 5 group it was 61.39%, and for the SES 7 group it was 42.22%. Below is an analysis of survival rates at each locus across different tolerance levels.

OsWRKY53: In the SES 3 group, there was no significant difference in survival rate between "salt-tolerant" and "non-tolerant" lines (50.98% vs 51.59%), but in the SES 5 group, the survival rate of "salt-tolerant" lines was higher than that of "non-tolerant" lines (65.46% vs 59.60%), although this difference was not statistically significant. *STG5*: In SES 3, the survival rate of "salt-tolerant" lines was significantly higher than that of "non-tolerant" lines ($p < 0.05$), but no significant difference was observed in SES 5 (SES 3: 62.54% vs 49.37%; SES 5: 63.78% vs 61.02%). In SES 7, all lines were classified as "non-tolerant."

GS3: The survival rates of lines with the "larger grain, heat-tolerant, alkali-tolerant, salt-tolerant" genotype were consistently higher across all SESs compared to those with the "smaller

grain, heat-sensitive, alkali-sensitive, salt-sensitive" genotype (SES 3: 64.66% vs 32.42%; SES 5: 62.27% vs 60.49%; SES 7: 18.3% vs 54.67%). In SES 3, the survival rate of the former was significantly higher than that of the latter, whereas in SES 7, the opposite was true. For *OsARF18*, no significant differences were observed between "salt-tolerant" and "non-tolerant" lines across any tolerance level (SES 3: 51.04% vs 51.69%; SES 5: 62.02% vs 60.53%).

Finally, when the number of salt-tolerance alleles at the four loci—*OsWRKY53*, *STG5*, *GS3*, and *OsARF18* were summed (Figure 6), the average survival rate of lines with ≥ 3 salt-tolerance loci in the SES 3 was 61.77%, significantly higher than that of lines with ≤ 2 loci (46.43%). In the SES 5, the average survival rate of lines with ≥ 3 salt-tolerance loci was 66.04%, which was not significantly different from that of lines with ≤ 2 loci (59.55%). In the SES 7, the accumulated number of salt-tolerance loci for three lines was 1 (25H84), 2 (25H91), and 1 (25H125), with corresponding survival rates of 44.44%, 18.33%, and 63.89%.

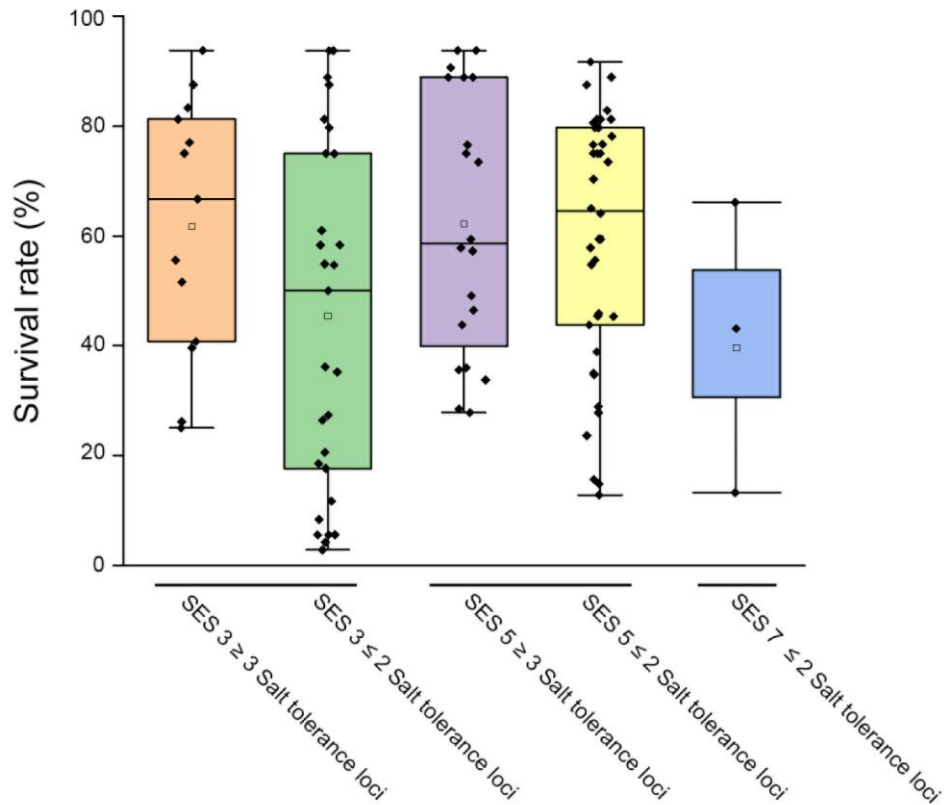


Figure 6. Cumulative effect of salt tolerance loci on survival rate across three SES classes, lines were grouped according to the number of salt tolerance loci they carried within each SES.

Discussion

The differences in the degree of variation among traits under salt stress, suggest that certain traits are more responsive to salinity (Shi et al. 2017). This pattern of variation indicates that genetic diversity is not uniformly distributed across all agronomic traits, but more concentrated on which directly related to yield components (panicle weight and effective panicles), while panicle length and thousand-grain weight remain relatively conserved. This phenotypic variation pattern suggests that panicle weight and effective panicles are promising traits for further genetic dissection, as their higher CVs imply greater potential for detecting associated loci. The diverse origins of these natural lines may have contributed to the observed phenotypic variation, with salinity stress amplifying genetic differences among them.. This provides valuable material for subsequent studies on QTL mapping, genome-wide association analysis, and extreme phenotype analysis. Meanwhile, the low coefficients of variation for thousand-grain weight and panicle length do not imply a lack of genetic control, rather, they suggest that expression of these traits is influenced by major-effect genes or environmental factors, resulting in little segregation within the population. From a breeding perspective, such stable traits help maintain rice yield under salt stress, but offer limited scope for improvement through conventional selection, necessitating molecular marker-assisted screening or introduction of novel germplasm resources (Zhang et al. 2021; Kong et al. 2021; C et al. 2022).

The differential degrees of trait variation observed under salt stress highlight traits that are most responsive to salinity, which were subsequently subjected to genetic analysis.. Salt

tolerance is a complex quantitative trait, reproductive organ development and vegetative growth were may be regulated by distinct genetic mechanisms(Liang et al. 2026; Quan et al. 2021). In this study, significant differences were observed in plant height, thousand-grain weight, and panicle length among lines classified by IRRI tolerance levels, suggesting that salt-tolerant genes controlling vegetative and reproductive growth may exhibit multiple effects, as well as reflecting the genetic diversity within the population(Mohammadi et al. 2013). For breeding, identifying extreme tolerant and sensitive genotypes can be directly utilized as parental lines for targeted improvement(Theerakulpisut 2024).

Phenotypic Effects of Agronomic Traits under Salt Stress

It should be noted that the SES 7 group contained only three lines, therefore, the following discussion focuses primarily on the comparison between SES 3 and SES 5, with SES 7 serving as a reference due to its limited sample size. The IRRI SES classifies rice lines into different salt tolerance levels based on leaf phenotypes (chlorosis, curling, and wilting). Its core principle relies on leaf symptoms to reflect plant responses to stress. By analyzing 118 lines, this study elucidates the nonlinear relationship between morphological changes and yield-contributing factors, providing a foundation for better understanding how salt stress affects crop growth and productivity.

Plant height and panicle length may be the most sensitive to salt stress and could serve as effective early-warning indicators. In this study, highly significant differences ($p < 0.01$) were observed in plant height and panicle length between SES 3 and SES 5, with plant height showing the highest coefficient of variation ($CV = 16.65\%$). This trade-off between growth and defence may account for the significantly greater plant height observed in the SES 5 compared

to the SES 3(Wu et al. 2025). Salt-tolerant (SES 3) tend to prioritize defence responses under salt stress, allocating more resources to osmotic adjustment and oxidative damage repair at the expense of vegetative growth, whereas moderately tolerant (SES 5) are able to maintain relatively higher growth rates (Quan et al. 2025). In addition, since the plants used in this study are natural lines, the observed difference might also reflect differences in genetic background among lines of diverse origins, the SES 5 group may be enriched for lines carrying 'growth-promoting' alleles that sustain plant height under stress..In contrast, significant differences were detected only between SES 5 and SES 7 for thousand grain weight ($p = 0.033$), whereas no significant differences were found in seed setting rate and effective panicles across all groups. This suggests that nutritional indicators show detectable changes even under mild stress conditions, as plants reduce leaf area to minimize transpirational water loss(Dell'Acqua et al. 2025). In contrast, yield-related traits only undergo substantial changes under stronger stress, likely due to plants mobilizing reserve substances (e.g., sucrose, starch, soluble sugars, proteins) to mitigate salt damage(Sami et al. 2016). This threshold difference may reflect the plant's resource allocation strategy under salt stress, suppressing non-essential processes to conserve resources, and only affecting reproductive growth when stress exceeds tolerance limits(Singh et al. 2021a; Yuan et al. 2020). Notably, no significant differences were observed in seed setting rate and effective panicles among the SESs, indicating that these two traits possess strong buffering capacity against salt stress and may be regulated by multiple factors(Song et al. 2021). However, this inference requires further validation through dynamic growth analysis. In addition, it can also be concluded from this study that the associations between SESs and yield components are specific. Significant differences among SESs in plant height, panicle length,

and thousand-grain weight were observed when comparing different levels of salt stress tolerance, indicating a temporal lag between leaf stress symptoms, suppressed vegetative growth, and yield loss. Therefore, in evaluating salt tolerance, SES classification should be combined with key yield indicators(Song et al. 2021). For salt-tolerant materials, morphological traits can reflect plant responses to stress, whereas for sensitive materials, thousand-grain weight provides a more direct indication of stress impact on yield.

The observed differences in plant height, panicle length, and thousand-grain weight across SES groups suggest that these traits are under differential genetic control, which we further investigated through QTL analysis.

Genetic architecture of salt tolerance

To clarify the genetic basis underlying the above phenotypic differences, this study identified five QTL intervals located on chromosomes 1, 3, 8, and 12. Within the same intervals, both known salt-tolerance genes and those with unclear functions coexist. On the short arm of chromosome 8 (0.81–0.92 Mb), three salt-related genes-*OsCML7* (calmodulin-like protein) (Ru et al. 2025; Chinpongpanich et al. 2012), *pss1* (involved in ROS accumulation during reproductive stage), and *OsAHL1* (AT-hook nuclear localization protein)(Jacob et al. 2017; Zhou et al. 2016a)are present. This region also includes *OsSWN2* (Zhang et al. 2024; Lee et al. 2020), *DCET1* (regulating callose biosynthesis in rice anthers and pollen exine formation) (Khan et al. 2021b), *OsATXI* (encoding a copper chaperone involved in heavy metal stress response)(Zhang et al. 2018a; Xu et al. 2026), and OsPi304 (induced by cold stress) (Yang et al. 2018), Although the functions of these genes are well defined, their potential roles in rice salt tolerance remain to be verified.

In chromosome 12, *OsCSLD4* has been clearly associated with rice salt tolerance (Zhao et al. 2022a; Pandit et al. 2024; Ma et al. 2026b). While *OsPRI0a* has not been directly linked to salt tolerance. However, other homologous members of the *PRI0* family have been shown to participate in salt tolerance regulation (Ersong et al. 2021; Wu et al. 2016), suggesting that this gene may also contribute to salt tolerance in rice. In the chromosome 1, although *OsPEAMT1* does not directly respond to salt stress, its catalytic product, phosphocholine (P-Cho), enhances plant salt tolerance (Yu et al. 2025). In chromosome 3, the transcription level of *OspPLAIII* α increases under salt stress (Liu et al. 2015), while the function of *OsTPR3* (a member of the rice TPL/TPR transcriptional co-repressor family) (Tang et al. 2016; Chengfang et al. 2026) in salt tolerance remains to be verified. Although these genes have not yet been reported to be involved in salt tolerance, they participate in biological processes related to plant responses to abiotic stress. Therefore, they are worthy of further exploration in subsequent studies. Additionally, although no candidate genes were annotated on chromosome 5. However, this chromosome harbors *STG5*, a major-effect gene for salt tolerance (Wei et al. 2024; Dong and Lin 2024; Ma et al. 2026a), which can serve as a key region for subsequent fine mapping.

Among the genes that have been confirmed to confer salt tolerance, *OspPLAIII* α , *OsCSLD4*, and *OsGAPC3* require further validation. Overexpression of *OsCSLD4* not only enhances rice salt tolerance but also increases grain weight (Zhao et al. 2022b). In contrast, overexpression of *OspPLAIII* α reduces hundred-grain weight by 40% and shortens grain length (Liu et al. 2015). *OsGAPC3* exerts pleiotropic effects on rice quality, increasing protein content while also leading to higher chalkiness in rice grains (Peng et al. 2024). These loci show potential as targets for gene pyramiding breeding. However, their practical value for breeding

can only be confirmed after functional validation, which remains a limitation of the current study. Future work involving transgenic or gene-editing approaches will be necessary to verify the causal roles of these genes before they can be effectively utilized in breeding programs.

Furthermore, the salt-tolerance candidate genes identified in this study, such as *OsSWN2* and *DCET1* can be prioritized for functional validation through targeted CRISPR/Cas9 editing. These efforts will help elucidate the molecular mechanisms underlying salt tolerance in this population and provide directly applicable functional markers for marker-assisted breeding.

Ethics approval and consent to participate

This study does not involve human or animal subjects, and therefore no ethics approval or consent to participate was required.

Consent for publication

All corresponding authors and relevant personnel have consented to the publication of this article.

Availability of data and material

The original contributions presented in the study are included in the article/Supplementary Files, further inquiries can be directed to the corresponding author/s.

Competing interests

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.

Authors' contributions

WQ and YJ designed the experiment; BL and WQ contributed equally to this work and wrote the manuscript; BL, MH, and WH contributed to data interpretation and manuscript

writing; BL and WH designed the figures and tables; CJ, YJ, WX, KM, and ZY participated in the processing of experimental materials; WS and MH revised the manuscript.

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