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Validation and Fine Mapping of Quantitative Trait Loci Associated With Salinity Tolerance at Reproductive Stage on Chromosome 6 in the Rice Variety Nona Bokra

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ABSTRACT

Soil salinity is a major abiotic stress affecting rice production globally. Previously, one promising salinity-tolerant line (SL535) was identified through screening of Nona Bokra-CSSLs with a Koshihikari background. In 2016, 102 F₂ individuals derived from SL535/Koshihikari were evaluated under long-term salt stress, with salt treatment imposed from the active tillering stage to maturity to investigate salt tolerance at the reproductive stage. Three QTLs for plant dry weight, panicle number, and panicle weight were detected on chromosome 6. This genomic region was further validated in 2019 and 2020 for plant dry weight, tiller number, and Na⁺ and Cl⁻ contents using homozygous lines with or without the Nona Bokra allele in a Koshihikari background. As expected, significantly higher tiller number and lower Na⁺ and Cl⁻ contents were maintained in plants carrying the Nona Bokra allele (NIL) at the full-heading stage. In 2023, seven newly developed NILs were evaluated for agronomic traits under control and salt stress conditions. Based on their performance, the QTL for plant dry weight, qPDW6.1, was narrowed down to a 390 kb region ranging from 4.78 to 5.17 Mb on chromosome 6. Within this region, several stress-related candidate genes were predicted based on annotation information and variant effect prediction analysis. Among them, two genes, *Os06g0196700* (Auxin response factor 1) and *Os06g0199100* (P-type pentatricopeptide repeat protein), were considered the most likely candidate genes associated with salt tolerance. These findings provide a foundation for future gene cloning and functional validation studies, which may help elucidate the genetic and molecular mechanisms underlying salt tolerance at the reproductive stage.

1 | Introduction

Rice (*Oryza sativa* L.) is a major cereal crop feeding more than half of the world's population, and is cultivated in more than 100 countries (Fukagawa and Ziska 2019). Soil salinization has become a constant threat to crop production that limits crop growth and development, leading to physiological damage and ultimately threatening global food security (Balasubramaniam

et al. 2023; Kumar et al. 2022). Furthermore, extreme climatic events may trigger salinization in agricultural land, thus reducing productivity (Lesk et al. 2016; Pareek et al. 2020). Soil salinity reduces plant growth and yield from or above 4 dSm⁻¹ EC (Munns and Tester 2008; Khatun et al. 1995). In rice, it is evident that salinity affects the flowering time, tiller number, number of fertile grains, and grain yield (Fan et al. 2021). Existing irrigation practices and continuous sea water intrusion in some

Abbreviations: CSSL, chromosome Segment Substitution Line; NIL, near-isogenic line; PDW, plant dry weight; PN, panicle number; PW, panicle weight; QTL, quantitative trait loci; SNP, single nucleotide polymorphism.

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Key Points

- The present study used SL535 carrying Nona Bokra segments on chromosomes 6 and 10 in the genetic background of Koshihikari to elucidate the genetic basis of salt tolerance at the reproductive stage.
- Three quantitative trait loci for plant dry weight, panicle number, and panicle weight were detected on chromosome 6 using 102 F₂ individuals derived from SL535/Koshihikari.
- The genetic effect of the homozygous Nona Bokra allele was validated for three important physiological traits, including plant dry weight, tiller number, and Na⁺ and Cl⁻ contents in plants over two consecutive years.
- By comparing seven near-isogenic lines, one stable quantitative trait locus for plant dry weight, qPDW6.1, was narrowed down to a 390kb region on chromosome 6.
- Two genes, *Os06g0196700* (Auxin response factor 1) and *Os06g0199100* (P-type pentatricopeptide repeat protein), were predicted as potential candidate genes based on functional SNP effects estimated using the variant effect predictor.

rice-growing areas are predicted to worsen soil salinization issues. As a result, rice production and land utilization are expected to decline sharply (Gonzalez-Perez and Neuerburg 2019; Maggio et al. 2011). Considering the above factors, developing salt-tolerant rice varieties at reproductive stage or during the whole growth period is required to boost rice yield across salt-affected areas. Therefore, understanding the genetic mechanisms of salt tolerance and the subsequent development of more salinity-tolerant rice varieties have become crucial for meeting the increasing food demand (Neang et al. 2020; Chen et al. 2020).

In rice, there exist significant varietal differences as well as various tolerance levels across growth stages under short-term or long-term salt stress (Makihara et al. 1999; Xu et al. 2024; Singh et al. 2018). Saline soils usually contain high concentrations of NaCl, which is the salt that predominantly affects plant growth by inducing both osmotic and ionic stress (Munns et al. 2020; Shabala et al. 2020). NaCl also reduces the water potential of the rhizosphere zones, causing early water balance problems in the plants, leading to water shortages and reduced turgor that affects the growth of cells and plant organs (Munns 2002). Furthermore, soil salinity severely affects plant growth and crop productivity by causing Na⁺ toxicity, osmotic stress, and ionic imbalances due to the higher accumulation of Na⁺ ions in different plant parts and tissues (Basu et al. 2021). High concentrations of Cl⁻ are also toxic to plants and decrease rice grain yield (Kumar and Khare 2016), whereas Na⁺ is the primary cause of ion-specific damage in many plants such as rice (Lin and Kao 2001; Tester and Davenport 2003). In addition to Na⁺ accumulation under saline conditions, the increased uptake and accumulation of Cl⁻ can significantly reduce the total shoot nitrogen uptake (Munns and Termaat 1986). Under ionic stresses in saline

conditions, maintaining yield components has been attributed to low Na⁺ concentration and low Na⁺/K⁺ ratio in flag leaves (Hossain et al. 2015), floral parts (Abdullah et al. 2001) and panicles (Asch et al. 1999). So far, large amount of research has focused on genes involved in ion accumulation, but the breeding of salt-tolerant varieties has lagged behind.

Chromosome Segment Substitution Lines (CSSLs) are powerful genetic stock by which to identify stable QTLs and further elucidate the complex genetic basis for stress tolerance in rice. In addition, they can facilitate the production of a secondary mapping population within a short period of time and enable the performance of progeny testing by reducing the background noise. To date, a number of studies have been completed using CSSL populations to screen out salt-tolerant CSSLs and to perform QTL mapping studies in rice (Thuy et al. 2018; Mitsuya et al. 2019; Pruthi et al. 2023; Mai et al. 2021). Recently, a promising CSSL identified from Nipponbare and a wild rice CSSL population showed comprehensive salt tolerance and a higher yield, with improved transcript levels of reactive oxygen species scavenging genes (Xing et al. 2024). In rice, the QTL mapping approach has been extensively used to elucidate the molecular mechanism associated with salinity tolerance at the seedling and reproductive stages (Thomson et al. 2010; Hossain et al. 2015; Puram et al. 2017; Chattopadhyay et al. 2021; Pruthi et al. 2023; Xing et al. 2024; Mai et al. 2021; Pundir et al. 2021). However, rice varieties that are salt-tolerant at the seedling stage do not show salinity tolerance at the reproductive stage (Zeng and Shannon 2000; Hossain et al. 2015; Haque et al. 2022). Furthermore, the understanding of increased yield under salt stress during the whole growth period and respective ion content mechanisms at the reproductive stage of rice is still limited, especially regarding QTLs/genes for increasing plant dry weight (PDW), tiller number, and low Na⁺ content. Among salt tolerant donors, Pokkali, Nona Bokra, and FL478 are some of the most widely used rice cultivars that belong to the indica subspecies (Qin et al. 2020). Previously, screening of CSSLs showed that some CSSLs carrying Nona Bokra segments exhibited increased plant growth under long-term salinity stress. Therefore, it is important to identify QTLs and further narrow down the QTL region for salinity tolerance at the vegetative and reproductive stages. A CSSL population derived from the cross between Koshihikari and Nona Bokra was developed and reported previously (Takai et al. 2007). This population contains Nona Bokra segments in the genetic background of salt-sensitive Koshihikari. Previously, 44 Nona Bokra-derived CSSLs with the genetic background of Koshihikari were screened for salinity tolerance under long-term salt stress. Among them, SL535, carrying Nona Bokra introgressions on chromosomes 6 and 10, showed significantly higher PDW and grain yield compared with the salt-sensitive recurrent parent Koshihikari (Thuy et al. 2018). Given the complex genetic basis of salt tolerance at the reproductive stage, further QTL identification and characterization are required to facilitate gene discovery and the development of salt-tolerant rice varieties. Therefore, the aims of this study were as follows: (1) to identify and narrow down QTLs associated with salt tolerance at the reproductive stage by using an SL535/Koshihikari progenies; (2) to validate the detected QTLs by comparing NILs for both physiological and agronomic traits; (3) to discuss the candidate genes in the detected QTLs.

2 | Materials and Methods

2.1 | Plant Materials

SL535 and Koshihikari seeds were obtained from the Rice Genome Resource Center in Japan. The substitution line SL535 contains chromosomal segments of Nona Bokra on chromosomes 6 and 10 in the Koshihikari genetic background (Takai et al. 2007; Figure 1A). SL535 was crossed with Koshihikari to produce an F₂ population, among which 102 individuals were used for QTL analysis in 2016 (Figure 1B). In 2017, an individual plant was selected from the SL535/Koshihikari F₂ population in which the chromosomal region from Nona Bokra on chromosome 6 of SL535 was heterozygous and the other regions were fixed to Koshihikari. Then, 96 plants in which recombination had occurred in the heterozygous region were screened out from 440 plants through marker-assisted selection (MAS) and were used to further define the QTL region. In 2018, an SL535/Koshihikari F₃ line was used to produce recombinants; 87 plants in which recombination had occurred in the heterozygous region were screened from 430 plants through MAS, and the QTL region was confirmed via progeny testing. In 2019, two contrasting NILs containing homozygous alleles for either Nona Bokra or Koshihikari through self-pollination were produced and selected for physiological studies, including tiller number and ion content, with or without salinity stress. In 2020, two newly

developed NILs were selected from the screening of recombinants from the SL535/Koshihikari F₅ population using additional Indel markers. In 2023, seven recombinants between markers RM19472 and DE06-6.300 were identified from a SL535/Koshihikari F_{6;7} population derived from one heterozygous genotype with target QTL segment. They were grown under both salt and control conditions to validate and narrow down the QTL regions for PDW and yield components.

2.2 | Growth Conditions

The experiments were carried out in a vinyl house at the Faculty of Agriculture, Okayama University, Japan (34°41'05.4"N 133°54'58.1"E). In brief, seeds were surface sterilized with 0.1% Benlate fungicide and then soaked in tap water for 2 days before sowing. Uniformly germinated seeds were sown in nursery boxes (Minoru pot 220, Minoru Industrial Co. Ltd., Japan) with 220 holes. One seed was sown in each hole. All the holes were filled with nursery soil designated for rice seedlings (Kumiai Ube Ryu-joh Baido, Ube Industries Ltd., Japan). The sowing dates of each experiment were 24 May 2016, 11 June 2019, 1 June 2020, and 23 May 2023. A single seedling of 20-day-old was transferred into each 2 L pot containing paddy soil, well mixed with 8 g slow-release fertilizer (NPK 14-14-14). Pots were arranged in a randomized complete block design with a spacing of 25 cm between them and kept flooded with tap water in a water

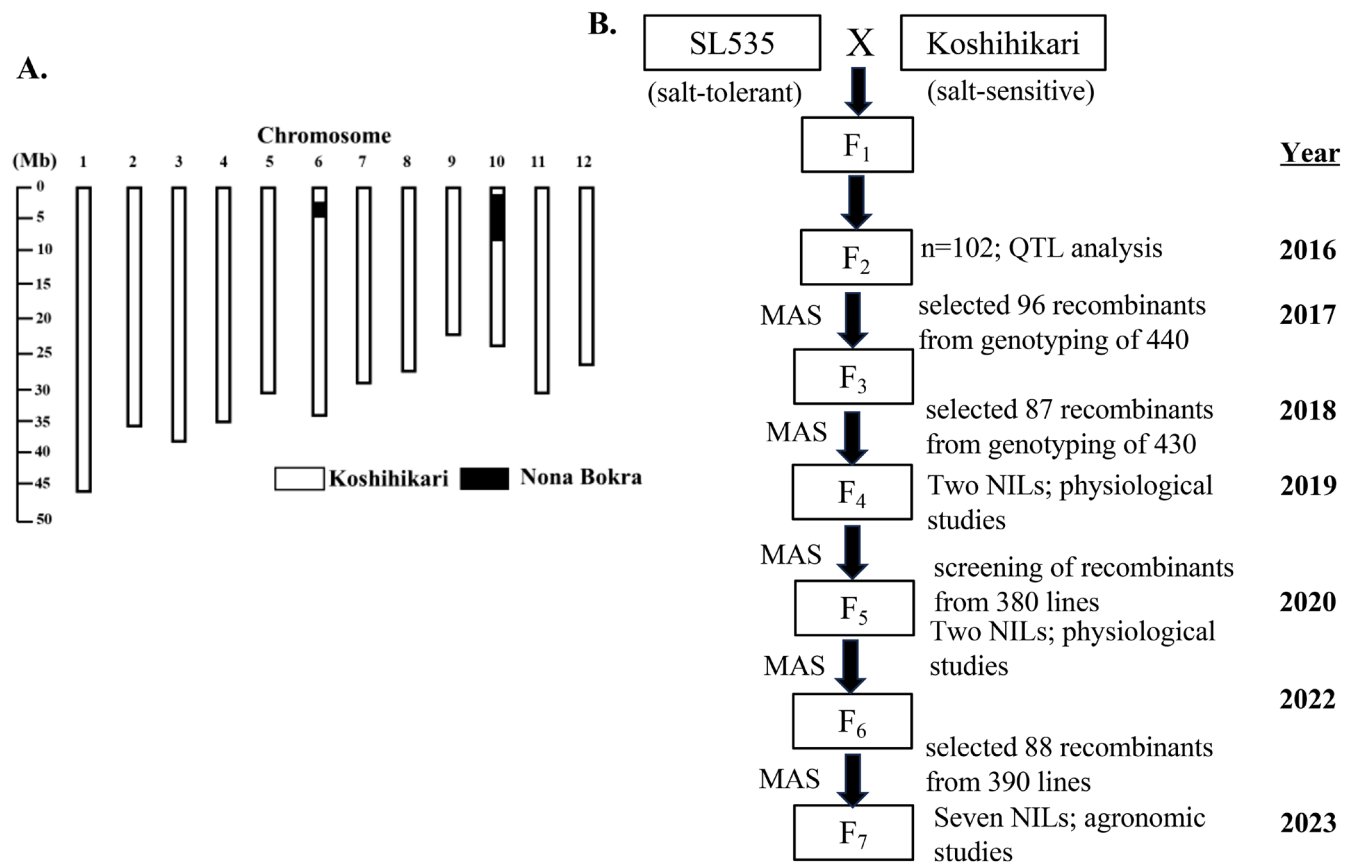


FIGURE 1 | Graphical genotype of substitution line SL535 shows chromosomal regions from Nona Bokra on chromosome 6 and chromosome 10 in the genetic background of Koshihikari (A). Black and white bars indicate chromosome segments for homozygous Nona Bokra and Koshihikari, respectively. Flowchart showing development of mapping population from the cross between salt tolerant SL535 and salt sensitive Koshihikari (B). MAS, marker-assisted selection.

pool (20 m × 1.1 m × 0.18 m). The pool was prepared on earthen ground, surrounded by wooden frames. Three layers of plastic sheets were placed on the earthen ground to prevent water leakage from the wooden pool. Before transplanting, fresh water was used to irrigate the pool. For salt stress treatment, NaCl was applied to the pool 7 days and 10 days after transplanting to induce salt stress after the successful establishment of rice seedlings. In the control condition, no NaCl was added, and normal growth conditions were ensured. In the salt treated pool, the electrical conductivity (EC) was kept at approximately 6.0–6.5 dS/m throughout the growing period starting from the active tillering and ending at the maturity stage, which was termed as long-term salt stress conditions. To ensure uniform irrigation in the pool, water was supplied through two irrigation tubes (Evaflow Type-A, Mitsui Chemicals, Tokyo, Japan) on both sides. The electrical conductivity in the pool water was recorded daily using an EC meter (2265FS, Spectrum Technologies Inc., Aurora, IL, US).

2.3 | Harvesting, Processing and Phenotypic Measurements

Plants were harvested after 40–45 days of the recorded heading date of each plant. For harvesting, each plant was cut at the stem base. Stems and leaves were packed in paper bags and oven-dried at a constant temperature of 75°C for 2 days, following which the PDW was measured. All ripened panicles were separated and packed in paper bags; these were oven-dried at 35°C for 2 days, and panicle number (PN) and panicle weight (PW) per plant were recorded. The grains were separated into unfilled and filled grains using a NaCl solution with a specific gravity of 1.06. Then, grain weight (GW) and grain filling percentage (GF) were recorded.

2.4 | Physiological Studies

To investigate physiological traits, tiller number and ion content were measured using plants grown under both salt and control stress. The tiller number of two selected NILs and two parents was counted every 2 weeks from the transplanting of seedlings to the maturity stage in 2019 and 2020. Ion contents in two selected NILs and their parents were measured in 2019. Samples were collected at three different growth stages: 2 weeks after salt treatment, full-heading stage (3 days after heading), and maturity stage. Four replicates of each line were harvested. Samples were dried at 80°C for 72 h before preparation of samples for ion measurement. Briefly, a 0.5 g sample was chopped for each replicate and extracted with 25 mL of 1% nitric acid at 80°C for 24 h. The Na⁺ and K⁺ contents in shoots were measured using a flame photometer (BWB-XP, BWB Technologies, Berkshire, UK), and the Cl[−] content in shoots was measured using a pH/ion meter (D-53, Horiba Ltd., Tokyo, Japan).

2.5 | DNA Extraction and PCR Analysis

DNA extraction, polymerase chain reaction amplification, and agarose gel electrophoresis were performed as previously described (Mai et al. 2021).

2.6 | QTL Analysis

QTL analysis was performed using QTL Cartographer 2.5 software followed by composite interval mapping with 1000 permutation tests at a 5% level of significance.

2.7 | Statistical Analysis

Each experiment consisted of at least four independent replicates. Data obtained for all measurements were expressed as mean value ± standard error (SE). The analysis of variance (ANOVA) was performed using Microsoft Excel 2022. Dunnett test was performed for phenotypic comparison using the DescTools package in R (Signorell 2024); each NIL was compared with the background parent Koshihikari.

2.8 | Prediction of Candidate Genes in the QTL Interval

To predict candidate genes, the physical locations of SSR/InDel markers flanking the major effect QTLs were compared against the Rice Annotation Project Database (accessed 14 May 2025; <https://rapdb.dna.affrc.go.jp/>). From TASUKE⁺ (<https://agrigenome.dna.affrc.go.jp/tasuke/ricegenomes/>), the candidate genes were further filtered to obtain those containing non-synonymous SNPs (missense variants) with moderate or high effects between Koshihikari and Nona Bokra. Then, the SNP effects were determined using a variant effect predictor (VEP; <https://www.plants.ensembl.org/Oryzasativa/Tools/VEP>; accessed on 14 May 2025).

3 | Results

3.1 | Frequency Distribution of F₂ Population Derived From SL535 and Koshihikari

In 2016, 102 F₂ plants derived from the cross between SL535 and Koshihikari were evaluated under long-term salt stress conditions. All of the studied phenotypic traits showed approximately normal distribution in the above population, indicating that PDW and panicle related traits are governed by polygenes or multiple QTLs (Figure 2; Table 1). Koshihikari showed lower PDW, panicle number, and panicle weight compared to SL535 by 32%, 53%, and 26%, respectively. The PDW in F₂ plants varied from 16.3 g to 76.7 g with a mean value of 43.3 g. The panicle number also showed a wide variation, ranging from 0 to 23 with a mean value of 10.2. The panicle weight varied from 0 to 7.9 g, with a mean value of 3.2 g.

3.2 | Detection of QTLs for Plant Dry Weight and Panicle Number Under Salinity Stress

In 2016, 102 F₂ plants were evaluated under long-term salt stress conditions and genotyped using 11 SSR markers, among which 2 markers covered target regions on chromosome 6 and 9 markers covered target regions on chromosome 10. During QTL analysis, three QTLs were detected on chromosome 6 for PDW, panicle

number, and panicle weight (Table 2). The PVE values of these QTLs ranged from 15.6% to 23.9% with LOD values from 3.00 to 5.31. All of these QTLs were detected on chromosome 6, and the nearest SSR marker was RM19472. However, no QTLs were found on chromosome 10 for any phenotypic traits. The source allele for increasing PDW, panicle number, and panicle weight was Nona Bokra. For fine mapping studies, additional InDel markers were developed to identify recombinants and to confirm the QTL region. In 2017, 440 SL535/Koshihikari $F_{2:3}$ individuals were genotyped to screen for 96 recombinants by using markers RM19364 and DE06-6.4409 in the genomic region between 2.29 and 6.44 Mb (Figure 3A). Similarly, a total of 430 SL535/Koshihikari $F_{3:4}$ lines were genotyped, and 87 recombinants were selected using markers RM204 and DE06-6.4409 in the genomic region between 3.17 and 6.44 Mb (Figure 3B). Additionally, 11 InDel markers were developed to genotype the recombinant individuals (Figure 3C). For the progeny test, these 87 recombinants were evaluated under long-term salt stress conditions. The genotype group homozygous Nona Bokra (BB) and L4 exhibited significantly higher PDW compared with homozygous Koshihikari (AA) (Figure 3D). In contrast, no

significant difference was observed between the AA and L1 genotype groups. The QTL for PDW was confirmed on chromosome 6 by studying the performance of recombinants, narrowing it down to a 2.4 Mb area between the markers RM19472 and DE06-6.4409.

3.3 | Validation of QTLs for Tiller Number and Ion Contents Using Two Contrasting NILs in 2019

Tiller number and ion content were also investigated under long-term salt stress conditions (Figures 4–6). To confirm the genetic effect, two NILs containing Koshihikari and Nona Bokra alleles in the genomic regions shown in Figure 4A were developed using recombinants from heterozygous plants. Between the two NILs, Line 220 contained a Nona Bokra allele while Line 211 contained a Koshihikari allele. Under control conditions, no differences were observed in tiller number among the two NILs, SL535, and the background parent Koshihikari throughout the growing period (Figure 4B). In contrast, a higher tiller number was found in Line 220 and SL535 from 28 days after

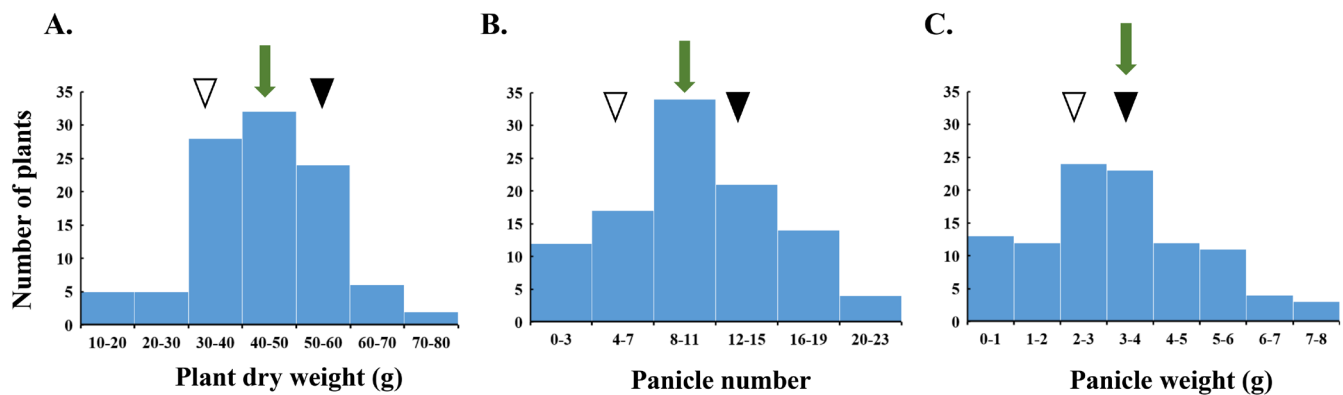


FIGURE 2 | Frequency distribution of plant dry weight (A), panicle number (B), and panicle weight (C) of F_2 population ($n=102$) developed from SL535 and Koshihikari under long-term salt stress in 2016. Black and white arrows indicate mean values for traits in SL535 and Koshihikari, respectively. Green arrow indicates corresponding phenotypic class of each phenotypic mean.

TABLE 1 | Descriptive statistics of phenotypic traits observed in the two parents and F_2 population ($n=102$) evaluated under long-term salt stress condition.

Trait name	Mean of SL535	Mean of Koshihikari	F_2 population			
			Range	Mean	Skewness	Kurtosis
Plant dry weight (g)	59.6	40.5	16.3–76.7	43.3	−0.1	3.2
Panicle number	12.7	6.0	0–23	10.2	0.1	2.7
Panicle weight (g)	3.1	2.3	0–7.9	3.2	0.3	2.7

TABLE 2 | Quantitative trait loci (QTLs) for plant dry weight and yield-related traits identified in the F_2 population ($n=102$) via composite interval mapping on chromosome 6.

QTL	Trait name	Nearest marker	Position(Mb)	LOD	AE ^a	PVE (%)
qPDW6.1	Plant dry weight	RM19472	4.07	5.11	10.64	22.7
qPN6.1	Panicle number	RM19472	4.07	4.25	7.49	18.1
qPW6.1	Panicle weight	RM19472	4.07	3.00	2.11	15.6

^aAdditive effect of the Nona Bokra allele.

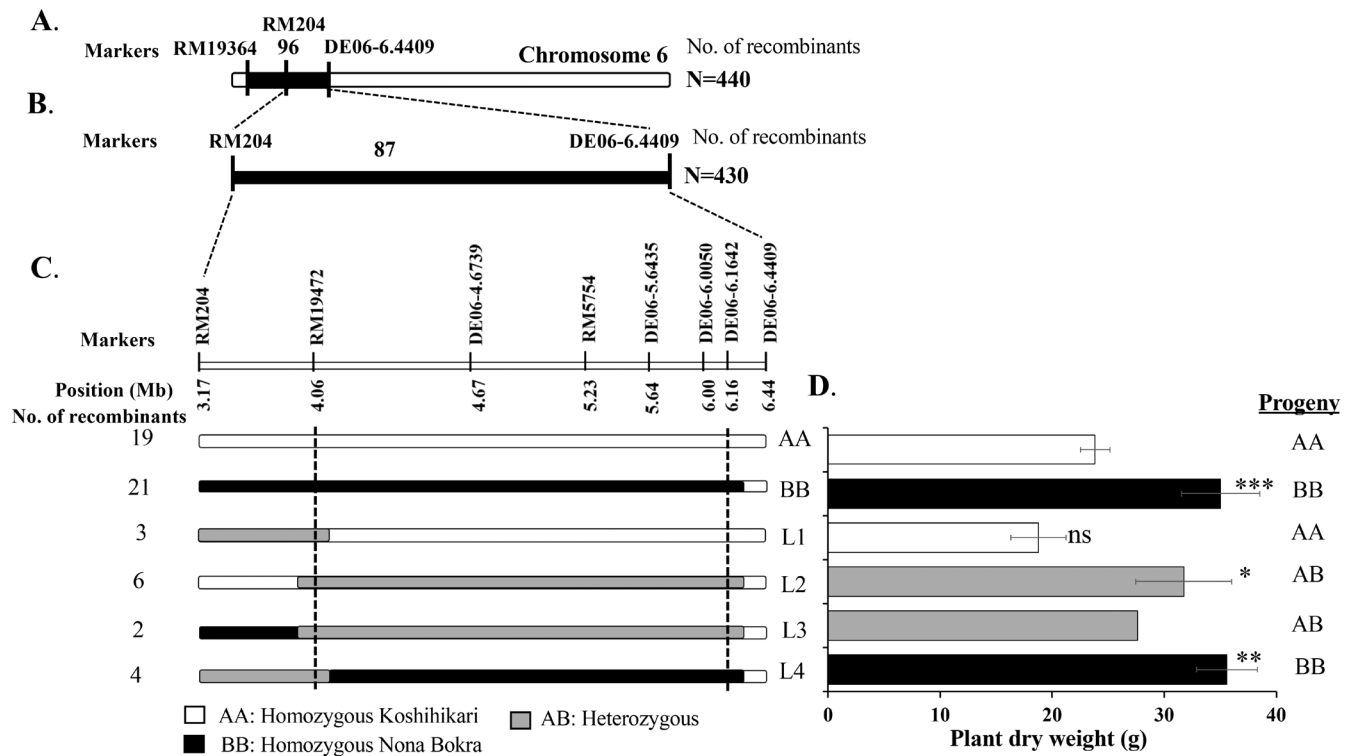


FIGURE 3 | Screening of recombinants and confirmation of QTL for plant dry weight on chromosome 6. Marker positions of the mapping markers are shown within the mapping interval used for SL535/Koshihikari $F_{2:3}$ population in 2017 (A) and $F_{3:4}$ population in 2018 (B). Graphical genotypes of the selected recombinant groups screened from 430 SL535/Koshihikari $F_{3:4}$ population (C). Progeny test of recombinants for plant dry weight under long-term salt stress. Values are mean $\pm$ standard error (D). *, **, and *** indicates a significant difference in plant dry weight relative to homozygous Koshihikari (AA) at 5%, 1%, and 0.1% level respectively via t-test. ns indicates no significant difference. White, black, and grey blocks indicate homozygous Koshihikari, Nona Bokra and heterozygous, respectively.

transplanting onward, and this trend remained consistent for the rest of the period, whereas the tiller number of Line 211 and Koshihikari began to decrease from 28 days after transplanting under saline conditions. Thus, based on the comparison of the two NILs, the average tiller number of Line 220 and SL535 was higher than that of Line 211 and the background parent Koshihikari under saline conditions (Figure 4C).

The PDW at 2 weeks after treatment, full heading stage and maturity stage were evaluated for comparison of ion contents and PDW. Additionally, the number of panicles at maturity stage was measured (Figure 5). The PDW of SL535 was significantly higher compared to the background parent Koshihikari at full heading stage under saline conditions. At maturity stage, Line 220 and SL535 showed significantly higher PDW and panicle numbers. Under control conditions, higher PDW was observed in SL535 compared with Koshihikari at full heading stage. In respect of ion content, a significantly lower Na^+ content was found in Line 220 at full-heading stage (Figure 6). In respect of Cl^- contents, a significantly lower Cl^- content was observed in Line 220 at both full-heading and maturity stages. Taken together, the presence of the Nona Bokra allele in Line 220 was associated with a higher tiller number and reduced Na^+ and Cl^- accumulation under long-term salt stress. Furthermore, the PDW, panicle number, and grain weight were used to estimate the correlation between ion contents at full-heading stage and the above agronomic traits (Figure 7). All of the phenotypic traits showed negative correlations with both Na^+ and Cl^- contents. In terms of Na^+ content, PDW, panicle number, and

grain weight showed a strong correlation, indicated by $R^2=0.794$, 0.927, and 0.935, respectively. In terms of Cl^- content, PDW, panicle number, and grain weight also showed a strong correlation, indicated by $R^2=0.940$, 0.998, and 0.841, respectively. Overall, panicle number showed the highest correlation with ion content at full-heading stage under saline conditions.

3.4 | Agronomic Performances of NILs Under Control and Salt Stress in 2020

QTLs for PDW and PN were detected and confirmed on the long arm of chromosome 6 between 4.06 and 6.30 Mb. Validation of the genetic effects via further progeny testing of the above chromosomal regions was noteworthy. To narrow down the region, two NILs were developed containing Nona Bokra or Koshihikari alleles between 4.65 and 6.30 Mb (Figure 8C). Between the two NILs, Line 45 had a Nona Bokra allele while Line 1 had a Koshihikari allele. Under control conditions, no differences in tiller number were observed between the two NILs and Koshihikari throughout the growing period (Figure 8A). In contrast, Line 45 started to show a higher tiller number from 28 days after transplanting and this continued for the rest of the period, whereas the tiller number of Line 1 and Koshihikari sharply decreased from 28 days after transplanting under salt stress conditions. Based on the comparison of the two NILs, the average tiller number of Line 45 was higher than that of Line 1 and the background parent Koshihikari

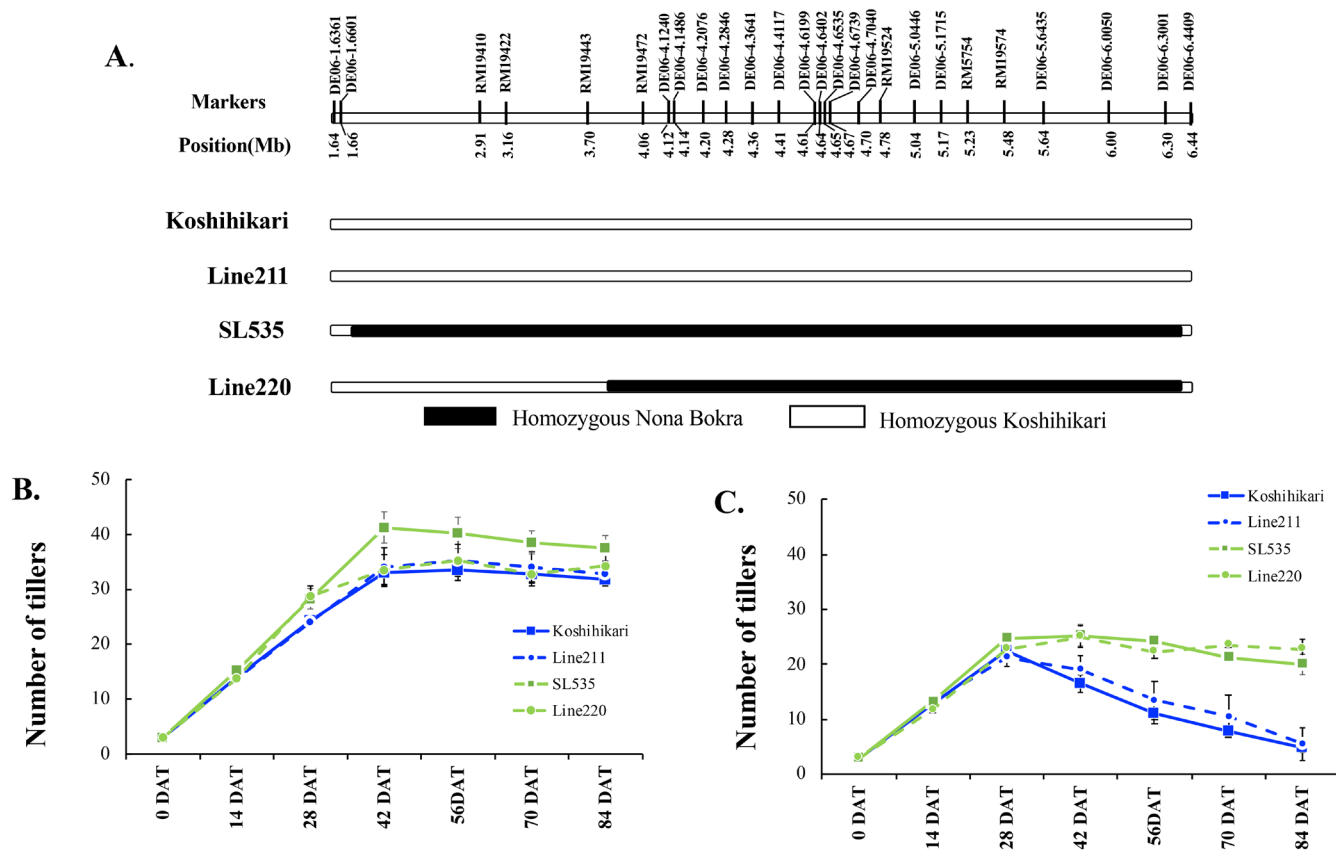


FIGURE 4 | Graphical genotypes of NILs and respective parents. Black and white blocks indicate homozygous Nona Bokra and homozygous Koshihikari, respectively (A). Line graph indicates tiller number of selected lines and their respective parents under control (B) and salt (C) conditions in 2019. DAT, days after transplanting. Values are mean $\pm$ standard error ($n = 4$).

under saline conditions (Figure 8B). Among phenotypic traits, Line 45 and SL535 showed significantly higher PDW than that of Koshihikari under saline conditions (Figure 8D). The panicle number and panicle weight were comparatively higher in Line 45 than that of the background parent Koshihikari. In respect of PDW, Line 45 showed significantly higher PDW compared to the background parent Koshihikari. Thus, the QTL for PDW was narrowed down to the ~2.2 Mb region in the marker interval between DE06-4.6402 and DE06-6.4409. As the Line 45 carrying the Nona Bokra allele in this region also showed a higher panicle number and panicle weight, the QTLs for panicle number and panicle weight were also confirmed in the above region on chromosome 6.

3.5 | Fine Mapping of QTL for Plant Dry Weight at Reproductive Stage in 2023

Based on the validation results in 2019 and 2020, a large chromosomal region ranging from 3.70 to 6.44 Mb was targeted for fine mapping studies. New Indel markers were designed based on the sequence variation of two parents, Nona Bokra and Koshihikari (Figure 9A). For fine mapping of the QTL for PDW, the SL535/Koshihikari $F_{6:7}$ population consisting of about 1000 individuals was developed, and seven recombinants between markers RM19443 and DE06-6.4409 were identified. All recombinants and homozygous lines were evaluated under both control and salt stress conditions in

2023. Under salinity stress, NIL1 and NIL4 showed significant differences in PDW compared to the background parent Koshihikari, while other recombinant lines did not show significant differences (Figure 9B). These results suggest that NIL1 and NIL4 may possess favourable alleles contributing to improved performance under salt stress and the QTL for PDW should be located in a 390 kb region between markers RM19524 and DE06-5.1715. Regarding panicle number, significant differences were observed between NIL1 and Koshihikari. However, other recombinants showed comparatively higher panicle numbers except for NIL2, which contains the Koshihikari allele. These results suggested that the QTL for panicle number might be located in the corresponding QTL interval ranging from 4.73 to 6.44 Mb. For panicle weight and grain weight per plant, NIL4 showed significant differences compared to Koshihikari, while no significant differences were observed in other recombinant lines. Under salt stress, none of the recombinants exhibited appreciable variations in grain filling percentage. However, all recombinants showed similar growth and agronomic characteristics under control conditions.

4 | Discussion

The identification of QTLs for reproductive stage salt tolerance is crucial for salinity breeding programs targeted at developing more tolerant rice cultivars for salt-affected areas. Larger

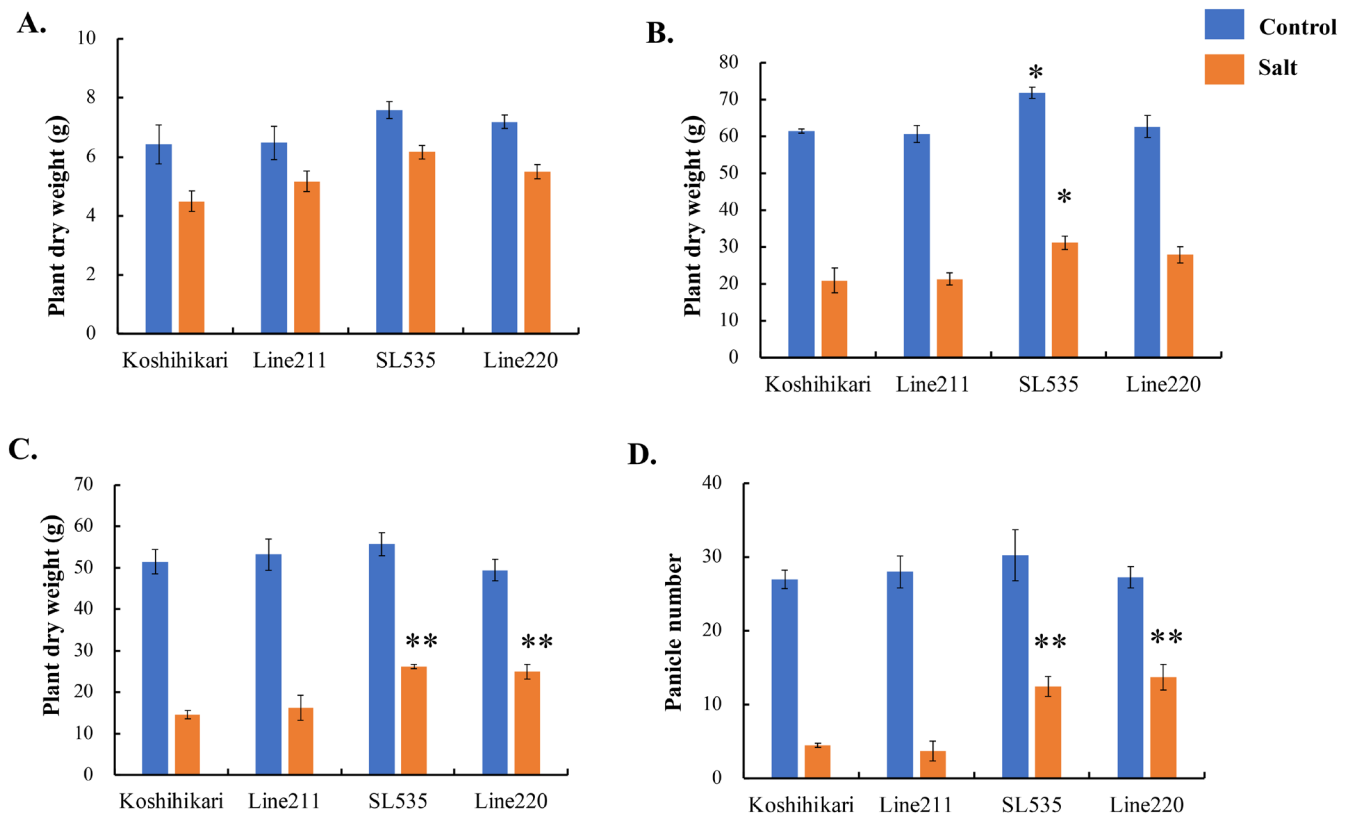


FIGURE 5 | Plant dry weight at 2 weeks after treatment (A), full heading stage (B), maturity stage (C); panicle number at maturity stage (D). Values indicate mean $\pm$ standard error ($n=4$). Significant differences for each variety from Koshihikari were evaluated using Dunnett's test. * and ** indicates significant differences at 5% and 1% level using Dunnett's test, respectively.

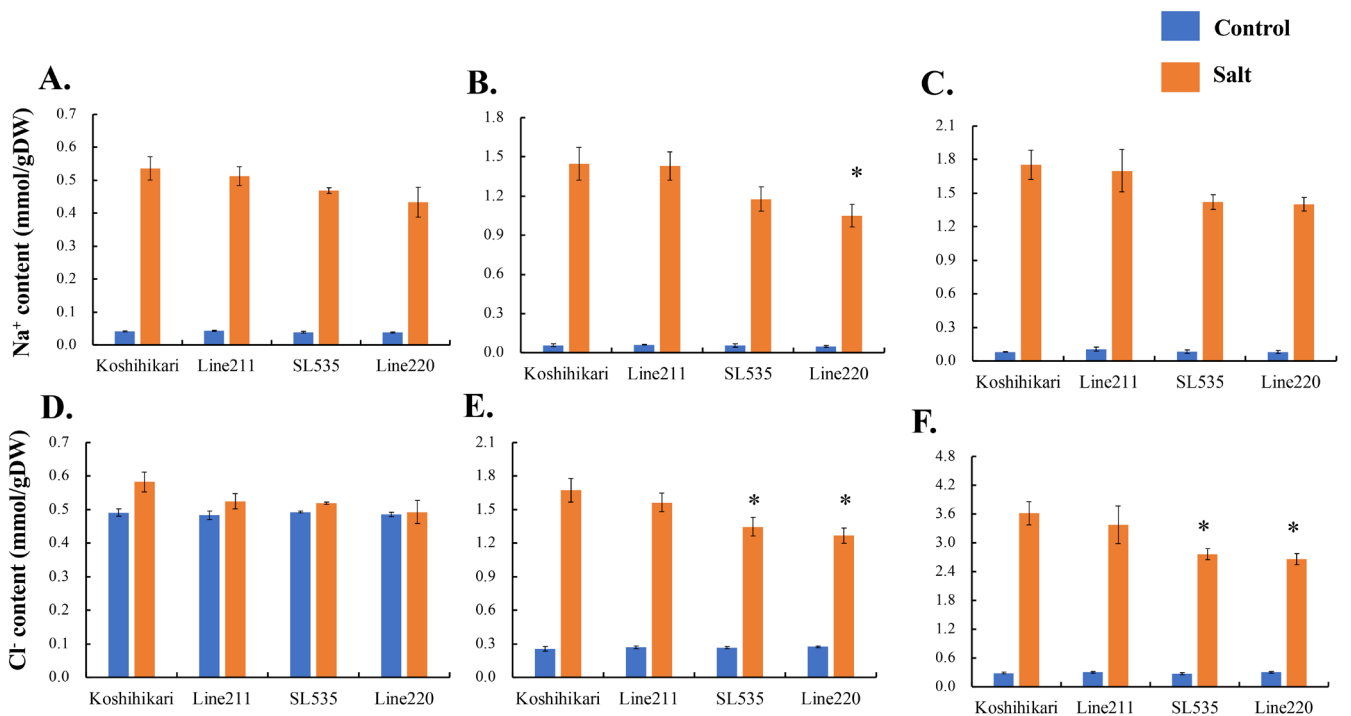


FIGURE 6 | Na⁺ and Cl⁻ contents in whole plants: 2 weeks after treatment (A, D), full-heading stage (B, E), maturity stage (C, F). Values are mean $\pm$ standard error ($n=4$). Significant differences for each variety from Koshihikari were evaluated using Dunnett's test. * and ** indicate significant difference at the 5% and 1% levels using Dunnett's test, respectively.

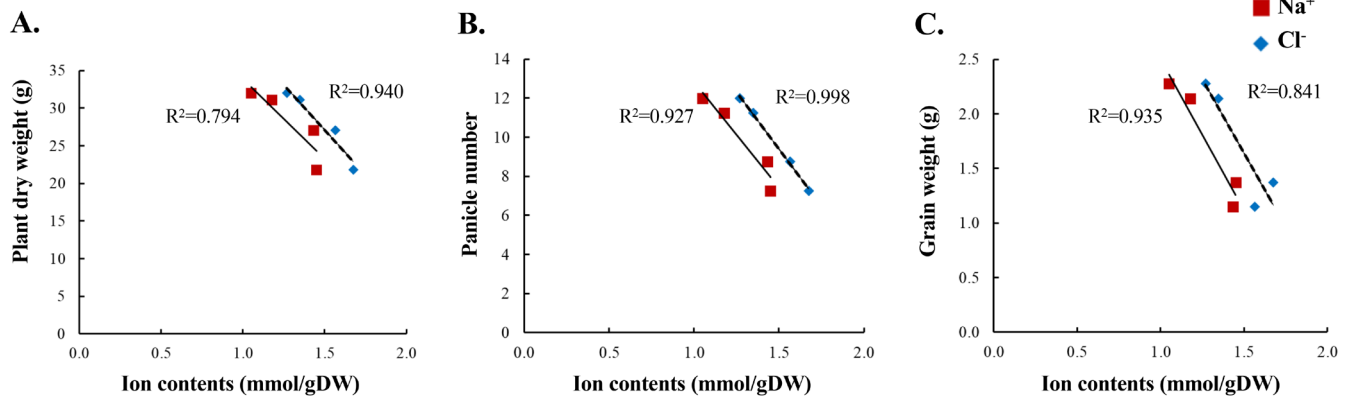


FIGURE 7 | Correlation between ion contents at full heading stage and plant dry weight (A), panicle number (B), and grain weight (C). Plants samples were taken from Line220, Line211, SL535 and Koshihikari under saline conditions. Values indicate means of four replicates.

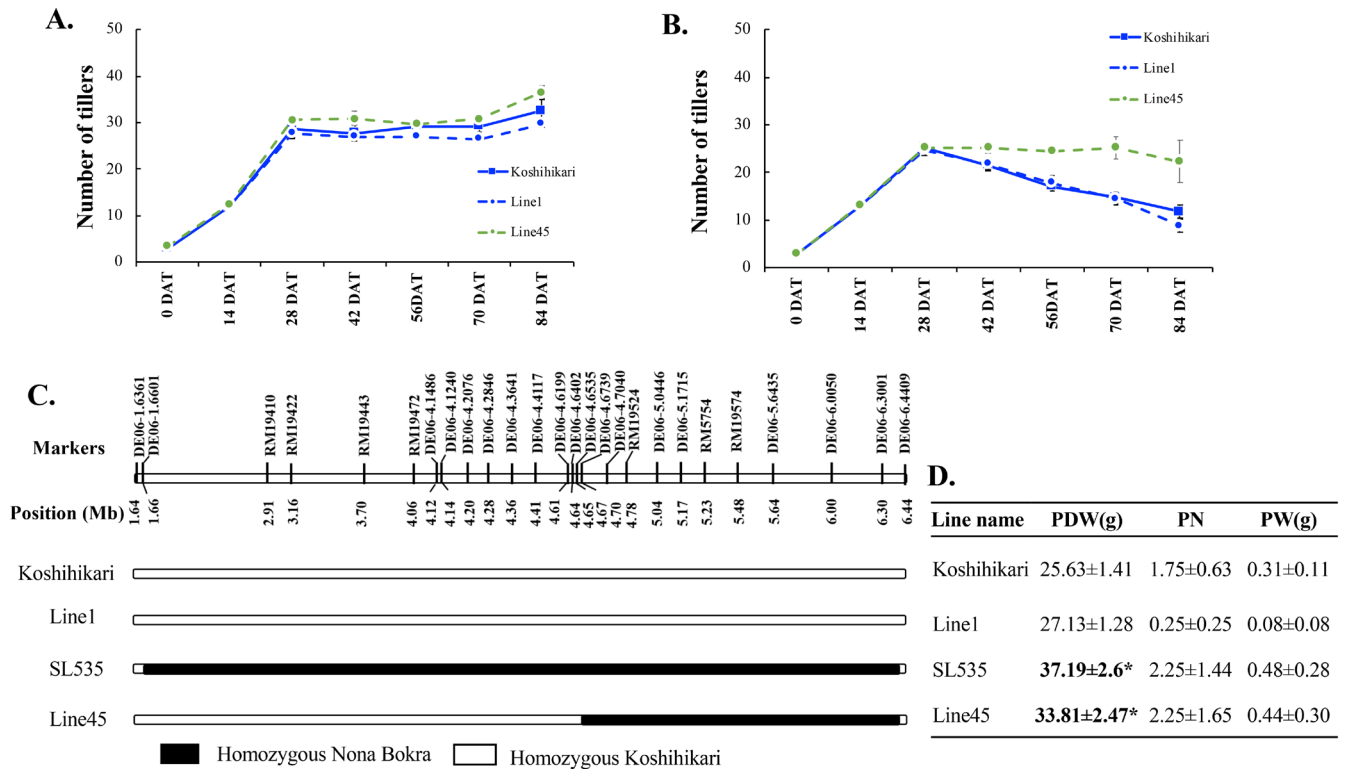


FIGURE 8 | Number of tillers in each 14 days interval under control (A) and salt (B) conditions in 2020. Graphical genotypes of NILs (C) and their agronomic traits (D) under salt stress condition in 2020. Black and white blocks indicate homozygous Nona Bokra and homozygous Koshihikari, respectively. Values are mean $\pm$ standard error ($n = 5$). * indicates a significant difference between Koshihikari and NILs at the 5% level via Dunnett's test. DAT, Days after transplanting; PDW, Plant dry weight (g); PN, Panicle number; PW, Panicle weight (g).

mapping populations greatly enhance statistical power, improve localization, and detect small-effect QTLs (Xu et al. 2017). Salinity tolerance during the reproductive stage is complex and is often controlled by numerous small- to moderate-effect loci. In most cases, the size of the mapping population varied from 138 to 232 individuals/lines (Mohammadi et al. 2013; Bimpong et al. 2014; Hossain et al. 2015; Chattopadhyay et al. 2021; Pundir et al. 2021; Pruthi et al. 2022; Mondal et al. 2022, 2025; Sugasi et al. 2024). However, some QTL mapping studies have used mapping populations at a minimal level, ranging from 70 to 112 individuals/lines and have reported promising QTLs for salt tolerance at the reproductive stage (Chai et al. 2014; Mondal et al. 2019; Mai et al. 2021; Pruthi et al. 2022). In the present

study, 102 F₂ individuals were used for initial QTL mapping, and the identified QTL was subsequently validated and narrowed using NILs. The mapping population used for QTL analysis was smaller than that used in many contemporary QTL mapping studies. Therefore, some minor-effect QTLs may have remained undetected, and the estimated QTL positions may be less precise than those obtained from larger populations. Nevertheless, the detected QTLs explained a substantial proportion of the phenotypic variation, indicating that they likely represent major genomic regions controlling salt tolerance at the reproductive stage. For fine mapping, additional SSR and InDel markers were used for genotyping, and two stable QTLs for PDW and tiller number were validated in the same chromosomal region.

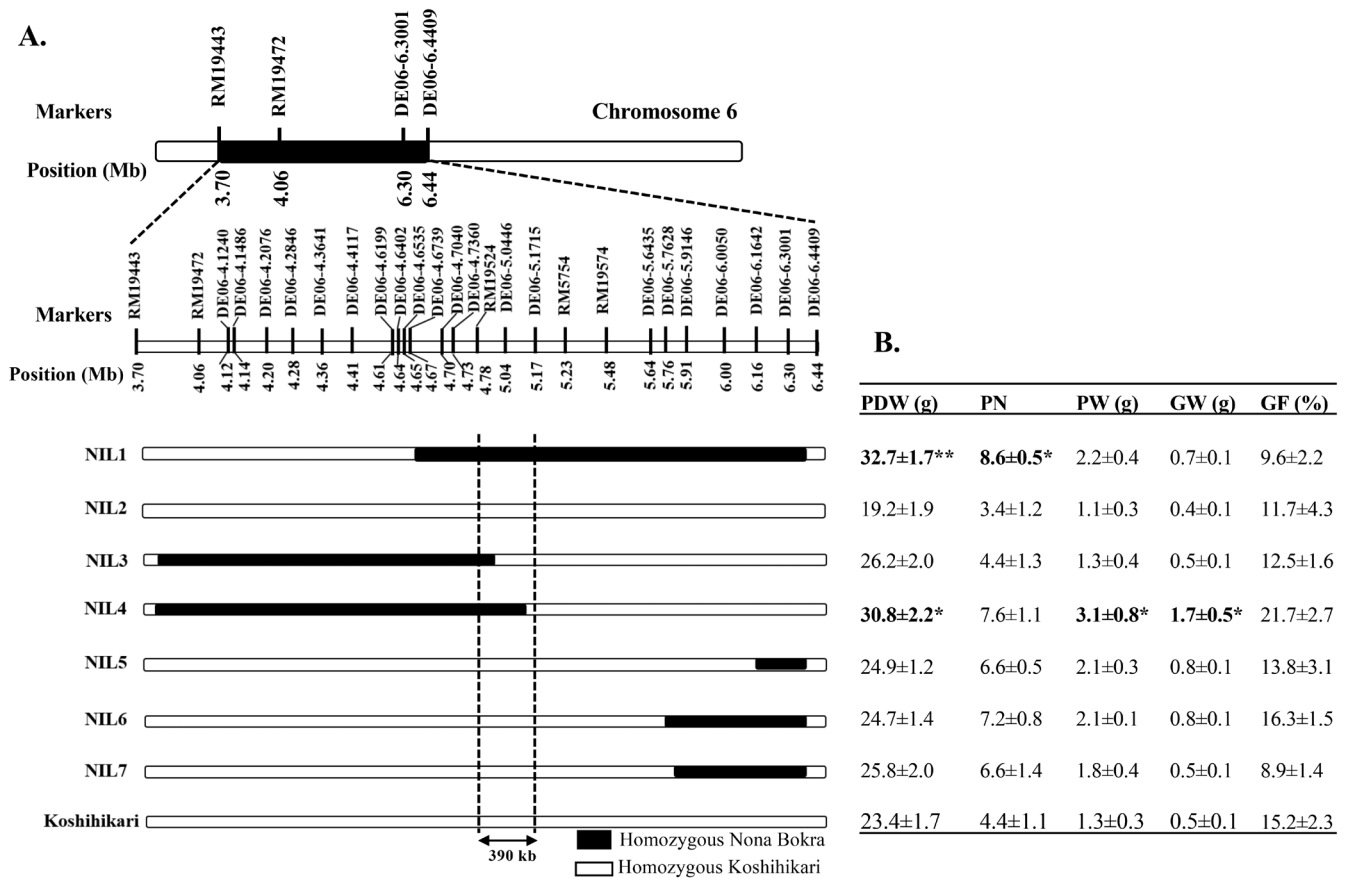


FIGURE 9 | Fine mapping of QTLs. Graphical genotypes of representative NILs (A) and their agronomic and yield-related traits under salt stress in 2023 (B). Black and white blocks represent homozygous Nona Bokra and homozygous Koshihikari, respectively. Values are mean $\pm$ standard error ($n=5$). * and ** indicates a significant difference between Koshihikari and other NILs at the 5% and 1% level via Dunnett's test, respectively. PDW, Plant dry weight (g); PN, Panicle number; PW, Panicle weight (g); GW, Grain weight per plant (g); GF, Grain filling percentage.

Finally, the QTL for PDW, qPDW6.1, was delimited to a 390kb region on chromosome 6 in the physical distance between 4.78 and 5.17Mb.

Several QTLs related to salt tolerance at the reproductive stage were identified on chromosome 6; most cases used a biparental mapping population. Previously, QTLs for panicle number, fertile spikelets, grain yield, and thousand grain weight were identified on chromosome 6 in a SADRI/FL478 F_2 population (Mohammadi et al. 2013), as well as QTLs for whole plant dry weight, Na^+/K^+ ratio in leaves, and grain yield in PS5/CSR10 F_2 lines (Pundir et al. 2021). However, these QTLs did not co-locate with the identified region in this study. Aside from that region, two QTLs for grain yield and thousand grain weight were located on the short arm of chromosome 6, overlapping with the detected genomic region (Chai et al. 2014; Bimpong et al. 2014). One QTL was located in the marker interval between RM586 and RM253, which was in the range of 3.1 to 5.4Mb, and that part of the QTL region was consistent with the detected QTL region. As these three QTLs (qPDW6.1, qPN6.1, qPDW6.1) are not co-localized with other reported QTLs, these are novel salt-tolerant QTLs at reproductive stage in rice. Additionally, the identified QTL in our study maintained a low accumulation of Na^+ and Cl^- ions under long-term salt stress, which suppressed the drying of leaves and stems, and as a result, maintained a higher panicle number and grain weight.

Another important finding of this study is that the QTL for tiller number showed stable genetic effects in two consecutive years (Figures 4 and 8). In rice, QTLs for tiller number under salt stress have been detected across chromosomes 2, 3, 6, 7, and 8 (Takehisa et al. 2004; Zang et al. 2008; Hossain et al. 2015; Mondal et al. 2022). Regarding chromosome 6, one QTL was detected in an IR64/Binam population using 99 BC₂F₈ lines (Zang et al. 2008), located in the marker interval between RM528 and RM30 near around ~26.56Mb region, which is different from the current study. Moreover, based on the performance of two contrasting NILs for tiller number and ion contents during three different growth stages, the physical region between 4.06 and 6.30 Mb was confirmed for maintaining tiller number and a low Na^+ content (Figures 4 and 6). Furthermore, the region was narrowed to ~2.2Mb in respect of the physical distance between 4.64 Mb and 6.44 Mb. In the phenotypic correlation analysis, PDW, panicle number, and grain weight showed negative correlations with ion contents (Na^+ and Cl^-). In previous studies, two co-localized QTLs for Na^+ content (qNa6.5) and Na^+/K^+ ratio (qNaK6.5) were identified in Bengal/Pokkali F_6 RILs, where alleles of the two QTLs came from salt-sensitive Bengal and had increasing effects in the concentration of Na^+ ions (De Leon et al. 2016). In respect of reproductive stage salt tolerance, QTLs for tiller number and grain yield were found in the same genetic region of chromosome 3 (Mondal et al. 2022). However, no QTLs controlling

TABLE 3 | Candidate genes that contain nonsynonymous SNPs (missense variants) located within QTL interval of chromosome 6 with predicted functional SNP effects estimated by variant effect predictor.

SNP position (bp)	Nona Bokra	Koshihikari	SNP effect	SIFT	Strand	Gene ID	Description	Gene symbol
4,849,135	G	C	gGa/gCa	0.01	-	Os06g0195600	Similar to SAC domain protein 3	OsARF16
4,926,523	T	C	cCc/cTc	0.00	+	Os06g0196700	Similar to Auxin response factor 1	
4,926,523	T	C	cCc/cTc	0.00	+			
4,930,473	C	A	Acc/Ccc	0.02	+			
5,004,677	A	G	cCc/cTc	0.01	-	Os06g0198500	RmlC-like jelly roll fold domain containing protein	OsRLCK202
5,019,547	A	C	gCg/gAg	0.03	+	Os06g0198900	LRR-receptor-like kinase (LRR-RLK) family protein	
5,029,702	T	C	Gta/Ata	0.04	-	Os06g0199100	P-type pentatricopeptide repeat (PPR) protein, chloroplast development	OsPPR11
5,034,326	T	C	Gtg/Atg	0.03	-	Os06g0199100	Conserved hypothetical protein	
5,102,539	T	C	Ctc/Ttc	0.04	+	Os06g0201000		

Abbreviation: SIFT, sorting intolerant from tolerant.

both tiller number and ion contents on chromosome 6 have been reported yet. Thus, the identified genomic region of chromosome 6 is of great importance in terms of tiller number and ion content in rice. Therefore, the above genomic region would also be valuable for identifying major candidate genes for both tiller number and ion contents in rice.

In this study, we identified and narrowed down the QTL region of chromosome 6 associated with PDW, tiller number, and ion contents under long-term salinity stress in rice. Then, we explored the QTL intervals of chromosome 6 using the Rice Annotation Project Database (<https://rapdb.dna.affrc.go.jp/>, accessed 15 May 2025) for reported genes in the QTL intervals ranging from 4.78 to 5.17 Mb. A total of 63 nonsynonymous SNPs were identified within the 48 genes located in the above QTL interval on chromosome 6. Of these, 9 SNPs were predicted to be deleterious (SIFT <0.05), indicating a potential impact on protein function through functional SNP effects (Table 3). Notably, deleterious variants were found in key candidate genes such as *Os06g0196700* and *Os06g0199100*. These genes are involved in stress responsive transcriptional regulation and chloroplast development in rice, suggesting their potential role for maintaining high PDW along with better plant growth under long-term salt stress. The gene *Os06g0196700*, known as *OsARF16*, is related to cytokinin response and Pi signalling in rice (Shen et al. 2014). Although this gene has not been reported as relating to salt tolerance, a few transcription factors from the ARF (Auxin Responsive Factor) gene family are associated with salt tolerance in rice. In respect of ARF, one transcription factor, *OsIAA18* showed a higher survival rate than that of wild-type seedlings under both salt and drought stresses (Wang et al. 2021). The candidate gene *Os06g0199100*, known as *OsPPR11*, regulates chlorophyll development in rice (Zhang et al. 2023). More importantly, one major salt tolerance gene (namely, high salt tolerant 1, *hst1*(*OsRR22*)) exists outside of our identified QTL region on chromosome 6 (Takagi et al. 2015). In previous studies, *hst1* showed a higher survival rate, increased plant biomass, low Na⁺ accumulation, and improved grain yield in rice (Rana et al. 2019). Therefore, *Os06g0196700* and *Os06g0199100* were preliminarily identified as the major candidate genes for qPDW6.1. Further gene expression analyses and functional validation studies will help clarify the roles of these candidate genes in conferring salt tolerance at the reproductive stage.

5 | Conclusions

Using a backcross mapping population from Nona Bokra substitution line SL535 and Koshihikari, three QTLs associated with PDW, panicle number, and panicle weight were identified on chromosome 6. Although the initial F₂ mapping population was relatively small, which may have limited the mapping resolution, the beneficial effects of the Nona Bokra allele were consistently validated. The Nona Bokra allele contributed to increased tiller number and reduced Na⁺ and Cl⁻ accumulation in rice. Based on the performance of NILs, the QTL for PDW was fine-mapped to a 390 kb interval between 4.78 and 5.17 Mb on chromosome 6. Among the several candidate genes located within this region, two genes, namely *Os06g0196700* (Auxin response factor 1) and *Os06g0199100* (P-type pentatricopeptide repeat protein),

were suggested as potential candidate genes based on annotated gene functions and predicted SNP effects. In the future, gene expression studies and relevant bioinformatics analysis would be helpful to identify major candidate genes and characterize their potential roles in salt tolerance and grain productivity in rice.

Author Contributions

M M Emam Ahmed: methodology, software, validation, formal analysis, investigation, data curation, writing – original draft, writing – review and editing, visualization. **Koki Maeoka:** methodology, validation, formal analysis, investigation, data curation, visualization. **Yuki Sakata:** investigation, data curation. **Asami Tomita:** methodology, validation, writing – review and editing. **Yoshihiko Hirai:** conceptualization, methodology, validation, formal analysis, resources, data curation, writing – review and editing, supervision, project administration, funding acquisition, investigation.

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Ethics Statement

The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Dataset available on request from the corresponding author.

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