

Article

Genetic Dissection and Fine-Mapping of QTL for Salinity Tolerance at the Reproductive Stage on Chromosome 2 of Rice Variety Nona Bokra

Farjana Rauf ¹ , Tran Thi Thu Hien ², Nguyen Thi Thu Thuy ², Asami Tomita ¹  and Yoshihiko Hirai ^{1,*} 

¹ Graduate School of Environmental, Life, Natural Science and Technology, Okayama University, 1-1-1 Tsushima-naka, Kita-ku, Okayama 700-8530, Japan; farjanasst@bau.edu.bd (F.R.); asamitomita@okayama-u.ac.jp (A.T.)

² Graduate School of Environmental and Life Science, Okayama University, 1-1-1 Tsushima-naka, Kita-ku, Okayama 700-8530, Japan; p9i425v2@s.okayama-u.ac.jp (T.T.T.H.); thuynt@nafoods.com (N.T.T.T.)

* Correspondence: yhirai@okayama-u.ac.jp; Tel.: +81-86-251-8316

Abstract

Soil salinity is a major constraint limiting rice productivity, particularly at the reproductive stage. To elucidate the genetic basis of reproductive stage salinity tolerance, this study validated and fine-mapped quantitative trait loci (QTL) for yield-related traits using a salinity-tolerant line SL506, identified through screening of Nona Bokra–CSSLs in a Koshihikari background. In 2016, the F₂ population derived from SL506/Koshihikari was evaluated under long-term salt stress; three QTLs associated with plant dry weight, panicle number, and grain weight were detected on chromosome 2. In 2023, validation analysis using F₃ individuals confirmed the presence of these QTLs. Subsequent fine-mapping using F₄ near-isogenic lines (NILs) delimited the QTL to a 1.7 Mb interval and high-resolution mapping using an F₅ recombinant population progressively refined it to a 473 kb genomic region containing 69 annotated genes. Variant Effect Predictor analysis identified 15 deleterious nonsynonymous variants (SIFT < 0.05) in six candidate genes. Based on annotated gene functions, predicted variant effects, and their membership in stress-related gene families, OsPP2C24, OsFbox102, and OsWAK14 were suggested as the most promising candidate genes underlying qPDW2. These findings provide insights into the genetic basis of reproductive-stage salinity tolerance from valuable resources for the future improvement of salt tolerance and yield stability in rice.

Keywords: rice (*Oryza sativa*); reproductive stage; salinity stress; quality trait loci (QTL); fine mapping



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1. Introduction

Rice (*Oryza sativa*) is one of the most important staple crops globally and a major source of food for more than half the global population. However, rice productivity is increasingly threatened by soil salinity, particularly in irrigated and coastal agricultural areas. Salinity affects approximately 20% of irrigated rice-growing areas [1,2] and is expected to intensify due to climate change, rising sea level, and poor irrigation practices. Salt stress severely disrupts plant growth, ion homeostasis, and metabolic processes, leading to significant yield losses [3–5], especially when stress occurs during the reproductive stage of plant development, which is highly sensitive to salinity stress [1]. Therefore, improving salt tolerance in rice has become a major priority for sustaining global food security.

Rice exhibits stage-specific responses to salinity, demonstrating comparatively higher tolerance during germination, active tillering, and grain filling while being highly sensitive at the early seedling stage and, most critically, the reproductive stage [6]. Thus, enhancing our understanding of and improving salt tolerance in rice across different growth stages is critical for the efficient utilization of saline soils [7]. Among the various agronomic traits used to evaluate salt tolerance, plant dry weight (PDW) or biomass yield under a long growth period is an efficient criterion of plant performance under stress conditions [8,9]. PDW or biomass reflects the cumulative effects of salt stress on photosynthesis [9,10], mineral nutrient uptake [11], and assimilate partitioning [10] throughout the growth period, thereby providing an integrated measure of plant productivity and tolerance. Consequently, identifying genetic loci associated with improved biomass accumulation under saline conditions can significantly contribute to the development of salt-tolerant rice varieties.

Salt tolerance in rice is a complex quantitative trait governed by multiple genes and is strongly influenced by environmental interactions [12]. Quantitative trait locus (QTL) mapping provides a powerful framework for unraveling the genetic architecture underlying salinity tolerance in rice [13,14]. The application of genotyping based on single-nucleotide polymorphism (SNP) enables detailed dissection of salt stress responses by facilitating the detection of genomic regions associated with tolerance, particularly when using well-characterized tolerant donor lines [15–17]. Several quantitative trait loci (QTLs) associated with salinity tolerance during the reproductive stage have been identified across different rice chromosomes [17–21], contributing to traits such as ion homeostasis, biomass accumulation, and yield stability under salt stress conditions. However, the fine mapping of these QTLs, which is essential for their effective deployment in breeding programs, remains relatively incomplete.

Chromosome segment substitution lines (CSSLs) provide a powerful genetic resource for validating and refining QTL regions because each line carries defined chromosomal segments from a donor parent in the genetic background of a recurrent parent [22–24]. The use of CSSLs enables the detection of QTL effects with reduced background noise and allows for more precise localization of genomic regions associated with complex traits [22,24,25]. Additionally, segregating generations derived from CSSLs can further enhance mapping resolution by generating recombination events within introgressed segments [22]. In 2018, Thuy et al. [25] evaluated 44 CSSLs derived from the salt-tolerant donor Nona Bokra in the genetic background of the susceptible cultivar Koshihikari to identify lines with enhanced salinity tolerance. Initial evaluation of 44 CSSLs under both control and saline (6 dS m^{-1}) conditions showed that SL506 did not differ significantly from Koshihikari under control conditions, whereas it exhibited improved performance under salinity, including significantly higher grain weight. Subsequent detailed evaluation of five selected CSSLs further confirmed that SL506 maintained superior agronomic performance under salt stress, with significantly higher plant dry weight, panicle number, and grain weight at maturity compared with Koshihikari, while no significant differences were observed under control conditions [25]. This finding suggested that the Nona Bokra substituted region on chromosome 2 of SL506 might be responsible for the enhanced performance of SL506 under salt stress conditions. Based on these findings, we selected SL506 to develop segregating populations targeting the QTL associated with reproductive-stage salinity tolerance. In addition, we combined phenotypic evaluation with molecular marker analysis using near-isogenic and recombinant populations to progressively delimit the target QTL to a narrow genomic interval through high-resolution mapping. This fine-scale resolution is essential to reduce the number of candidate genes within the region, thereby improving the efficiency of identifying the most likely candidate genes underlying the trait. We further evaluate sequence variation between parents within the refined interval to prioritize candidate genes

carrying potentially functional polymorphisms. Together, this approach provides a strong foundation for downstream gene validation and for the development of robust molecular markers to support salinity tolerance breeding in rice.

2. Materials and Methods

2.1. Plant Materials

SL506 and Koshihikari seeds were obtained from the Rice Genome Resource Center in Japan. SL506 contains chromosomal segments of Nona Bokra on chromosome 2 in the genetic background of Koshihikari [26] (Figure S1A). SL506 was crossed with Koshihikari to produce an F₂ population. In 2016, 80 individuals were phenotyped under salt stress and genotyped using 17 simple sequence repeat (SSR) markers in the substituted region of chromosome 2 and used for QTL analysis (Figure S1B). To validate the QTL region in 2023, 690 plants were genotyped in the F₃ generation using two flanking markers, and 102 plants were screened for recombination in the heterozygous region for phenotypic evaluation. Then, 11 more SSR and insertion-deletion (InDel) markers were added between the flanking markers to further define the QTL region. To narrow down the region for QTLs in 2023, 14 near-isogenic lines (NILs) of the F₄ population were grown and genotyped using 14 SSR markers, and three NILs were selected for phenotypic evaluation under control and salt stress conditions. To fine-map the QTL region in 2024, five lines were identified from the SL506/Koshihikari F₅ population. From these five lines, a mapping population of 580 plants was developed (Figure S1B), and similar recombinants were grouped together to fine-map the QTL region related to PDW at maturity.

2.2. Growing Conditions and Salt Treatment

Pot experiments were conducted during the 2016, 2023, and 2024 growing seasons at the experimental field of the Faculty of Agriculture, Okayama University, Japan (34°41′05.4″ N 133°54′58.1″ E). Before sowing, seeds were surface-disinfected with 0.1% Benlate fungicide and soaked in tap water for 48 h. Evenly germinated seeds were then sown in plastic nursery trays (Minoru Pot 220, Minoru Industrial Co. Ltd., Okayama, Japan) consisting of 220 cells (16 mm diameter × 25 mm depth) filled with commercial nursery medium (Kumiai Ube Ryu-Joh Baido; Ube Industries Ltd., Tokyo, Japan). Sowing was performed on 20 May 2016; 16 May 2023; and 24 May 2024. After 20 days, seedlings were transplanted into 2-L plastic pots (No. 5.5; Kaneya Co. Ltd., Aichi, Japan) filled with paddy soil thoroughly mixed with 10 g of slow-release fertilizer (LPS140E-80; N-P-K = 14–14–14). The pots were arranged at 25 cm intervals and placed in a water pool (20 m × 1.1 m × 0.18 m) located under a two-side-open vinyl house, where standing tap water was maintained throughout the growth period. For salinity treatment, sodium chloride (NaCl) was added seven and 10 days after transplanting to establish saline conditions with an electrical conductivity (EC) of approximately 6 dS m^{−1}, corresponding to a NaCl concentration of approximately 50 mM. Control plants were grown without NaCl addition. Moreover, irrigation was supplied through a drip tube system (Evaflow Type-A; Mitsui Chemicals, Tokyo, Japan), and electrical conductivity was monitored using an EC meter (2265FS; Spectrum Technologies Inc., Aurora, IL, USA). The electrical conductivity levels were maintained within the target range until plant maturity.

2.3. Phenotypic Evaluation

Salt tolerance was evaluated by harvesting plants 40–45 days after the individual heading date under both control and salinity conditions. At harvest, the plants were cut at the basal node. Leaves and stems were placed in paper bags and oven-dried at 80 °C for 72 h, after which the PDW was determined. Panicles were collected separately and counted

before being dried at 35 °C for 24 h and weighed to obtain panicle weight (PW). The grains were subsequently separated into filled and unfilled fractions using a NaCl solution with a specific gravity of 1.06, and the grain-filling percentage (GFP) was calculated. In 2016, five phenotypic traits—PDW, panicle number (PN), GFP, grain weight (GW), and 1000-GW—were recorded using 80 F₂ plants for QTL analysis. In 2023, six morphological traits, namely, PDW, PW, PN, GW, GFP, and 1000-GW, were recorded for 102 plants and three NILs. In 2024, three morphological traits, namely, PDW, PW, and PN, were recorded in the recombinants.

2.4. DNA Extraction and Polymerase Chain Reaction Analysis

Genomic DNA extraction and polymerase chain reaction (PCR) amplification were conducted according to a previously reported protocol [27]. Leaf tissues collected from the F₂, F₃, F₄, and F₅ populations, as well as from both parental lines, were used for DNA isolation using an SDS/NaCl-based extraction buffer. The extracted DNA was subsequently genotyped using polymorphic SSR and InDel markers. PCRs were performed in a total volume of 10 µL, comprising 1.5 µL of genomic DNA, 3.0 µL of Milli-Q water, 5.0 µL of Taq DNA polymerase master mix, and 0.25 µL each of the forward and reverse primers (10 µM). Amplification was carried out in a thermal cycler (TP600; Takara Bio Inc., Shiga, Japan) under the following conditions: initial denaturation at 95 °C for 2 min; 35 cycles of denaturation at 95 °C for 20 s, annealing at 50 °C for 20 s, and extension at 72 °C for 30 s; followed by a final extension at 72 °C for 2 min. The reactions were then held at 15 °C. PCR products were stained with RedSafe™ nucleic acid stain (iNtRON Biotechnology, Seongnam, Korea) and resolved by electrophoresis on 2% agarose gels. DNA fragments were visualized under UV illumination, and the alleles were scored manually.

2.5. QTL Mapping

QTLs were identified using composite interval mapping implemented in QTL Cartographer version 2.5. The significance threshold for QTL detection was determined by 1000 permutation tests at a 5% probability level. For each detected QTL, additive and dominance effects, as well as the proportion of phenotypic variance explained (R^2), were estimated based on the corresponding peak logarithm of odds (LOD) score.

2.6. Statistical Analysis

All experiments were conducted with a minimum of four independent replicates. Measured data were presented as mean values $\pm$ standard error (SE). Analysis of variance (ANOVA) was performed using Microsoft Excel (2022). For phenotypic comparisons, Tukey's multiple comparison test and a standard Dunnett's test were applied using the DescTools package in R 4.5.2, with each NIL evaluated relative to the recurrent parent Koshihikari.

2.7. Prediction of Candidate Genes in the QTL Interval

The genes annotated in the QTL region were predicted from the Rice Annotation Project Database (RAP-DB; <https://rapdb.dna.naro.go.jp>; accessed on 29 January 2026). Genes located within the corresponding QTL intervals were further examined using TASUKE+ (<https://agrigenome.dna.affrc.go.jp/tasuke/ricegenomes/>; accessed on 21–27 February 2026) to identify sequence polymorphisms between the parental cultivars Koshihikari and Nona Bokra. Candidate genes were filtered by selecting those harboring nonsynonymous SNPs (missense variants) predicted to have moderate or high effects on protein function. The functional consequences of identified SNPs were subsequently assessed using the Variant Effect Predictor (VEP) implemented in En-

sembl Plants (https://plants.ensembl.org/Oryza_sativa/Tools/VEP; accessed between 24 and 28 February 2026).

3. Results

3.1. Evaluation of Phenotypic Characteristics in the F_2 Population

The F_2 population derived from the cross between SL506 and Koshihikari showed significant phenotypic variations in the measured traits under long-term salt stress. Superiority in trait values for PDW, PN, and GW was observed in the tolerant line SL506 compared to the susceptible parent Koshihikari, indicating contrasting genetic backgrounds for traits related to salt tolerance (Table 1).

Table 1. Mean performance of parents and F_2 population for different parameters screened for salt tolerance.

Parameters	Parental Lines		F_2 Population			
	Koshihikari	SL506	Mean	Range	Skewness	Kurtosis
PDW	10.17	24.68	18.42 ± 0.76	3.18–40.01	0.48	0.35
PN	5.00	16.80	10.07 ± 0.57	1.00–28.00	0.72	0.72
GW	1.16	5.04	4.83 ± 0.34	0.58–13.86	1.08	1.04
1000-GW	15.50	14.74	16.51 ± 0.11	14.05–18.97	0.08	−0.14
GFP	25.74	31.99	48.44 ± 1.15	19.34–72.53	−0.29	0.25

PDW: plant dry weight at maturity (g/plant), PN: panicle number (no./plant), GW: grain yield (g/plant), 1000-GW: 1000 grain weight, GFP: grain filling percentage.

In the F_2 population, phenotypic traits exhibited continuous frequency distributions with intermediate population means, confirming their quantitative inheritance (Figure 1). PDW and PN displayed wide phenotypic ranges, with individuals exceeding both parental values, demonstrating transgressive segregation (Table 1). This pattern suggests that favorable alleles contributed by both parents recombine in the segregating population. The distributions of PDW and PN were moderately skewed toward the tolerant parent (SL506), indicating the possible involvement of major-effect QTLs with directional additive effects. Conversely, GW exhibited a near-normal distribution with limited transgression, suggesting polygenic control with relatively small individual genetic effects. The skewness and kurtosis values for all traits were within acceptable limits, supporting the suitability of the population for QTL analysis.

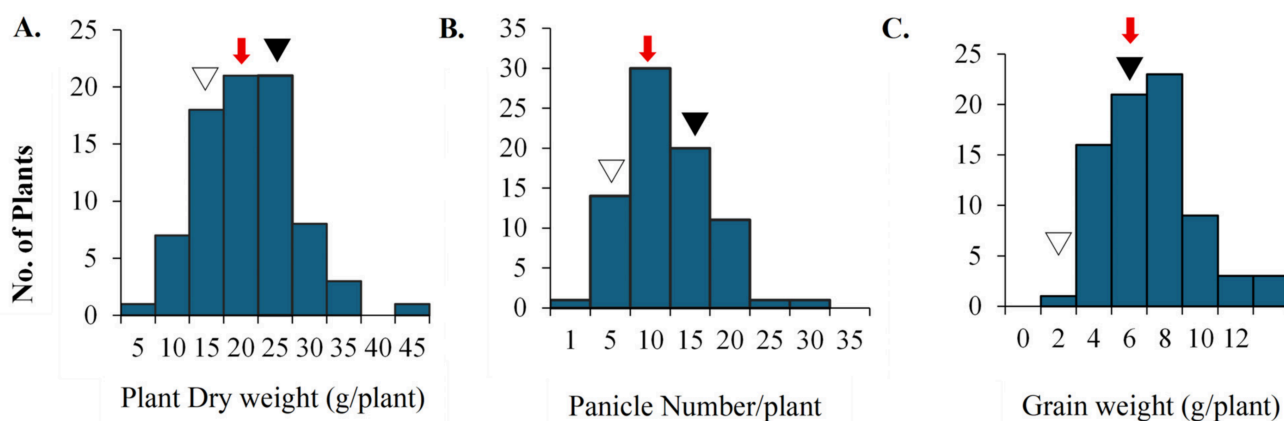


Figure 1. Frequency distribution of PDW (A), PN (B), and GW (C) in the F_2 population (n = 80) derived from Koshihikari and SL506 under long-term salt stress. Black and white arrows indicate the mean values of SL506 and Koshihikari, respectively, while the red arrow denotes the phenotypic class corresponding to the population mean.

Pearson's correlation analysis revealed strong positive associations between PDW, PN, and GW ($r = 0.83\text{--}0.90$, $p \leq 0.001$), indicating close interdependence among the major yield components under saline conditions (Table 2). In contrast, the weight of a thousand grains showed weak and non-significant correlations with other traits, suggesting independent genetic control. The grain filling percentage exhibited a moderate positive correlation with GW ($r = 0.35$, $p \leq 0.01$), implying a partial contribution to yield formation.

Table 2. Pearson correlation matrix of traits measured in response to salt stress in F₂ population.

Trait	PDW	PN	GW	1000-GW	GFP
PN	0.899 ***				
GW	0.828 ***	0.895 ***			
1000-GW	0.057	0.033	0.176		
GFP	0.055	0.175	0.351 **	0.171	1

PDW: plant dry weight at maturity, PN: panicle number, GW: grain yield, 1000-GW: 1000 grain weight, GFP: grain filling percentage, ** and *** indicate significance at the 1% and 0.1% levels, respectively.

3.2. Detection of QTLs for Salt Tolerance

In 2016, 80 SL506/Koshihikari F₂ plants were evaluated under salt stress conditions and genotyped using 17 SSR markers in the substituted region of chromosome 2. Composite interval mapping detected three QTLs on chromosome 2 for all measured phenotypic traits (Table 3).

Table 3. Quantitative trait loci (QTLs) for plant dry weight and yield-related traits identified in the F₂ population ($n = 80$) via composite interval mapping on chromosome 2 in 2016.

QTL	Trait	Position	LOD Score	Additive Effect	Phenotypic Variances (R ²)
1	Plant dry weight (qPDW2)	15.9–26.24 Mb	5.45	−3.82	21.97
2	Panicle number (qPN2)	15.9–24.02 Mb	3.57	−2.36	15.48
3	Grain weight (qGW2)	15.9–24.02 Mb	3.76	−1.42	15.20

The QTLs detected in this study explained 15–22% of the phenotypic variance, with LOD values ranging from 3.57 to 5.45. The additive effect of all QTLs was derived from the tolerant parent, Nona Bokra. A QTL was detected for DW, qPDW2, in the interval between RM13178 and RM13688 on chromosome 2, with an LOD value of 5.45, explaining 21.97% of the phenotypic variance. Moreover, two QTLs were detected for PN and GW, qPN2 and qGW2, with LOD values of 3.57 and 3.76, respectively. These QTLs accounted for 15.4% and 15.2% of the phenotypic variance and were mapped between markers RM13178 and RM13593. To validate the QTL region in 2023, 690 SL506/Koshihikari F₃ individuals were genotyped, and 102 recombinants were selected using newly developed flanking markers in the genomic region. Further, to cover the target region, 13 molecular markers were used to genotype the recombinant individuals. The 102 recombinants were evaluated under long-term salt stress conditions. After combining the genotypic and phenotypic data, the QTL region was validated and narrowed down between markers DE02-20.1262 and DE02-26.1599 in the F₃ population.

3.3. Validation of QTLs for Salt Tolerance

As QTL for PDW were detected on chromosome 2 in two consecutive generations, narrowing down and validating these regions was noteworthy. Using 11 polymorphic SSR and InDel markers, we screened for recombinants from the SL506/Koshihikari F₄ population. As Nona Bokra alleles had positive effects on these QTLs, we selected three NILs carrying different introgressed Nona Bokra regions in the background of Koshihikari alleles in the QTL region (Figure 2).

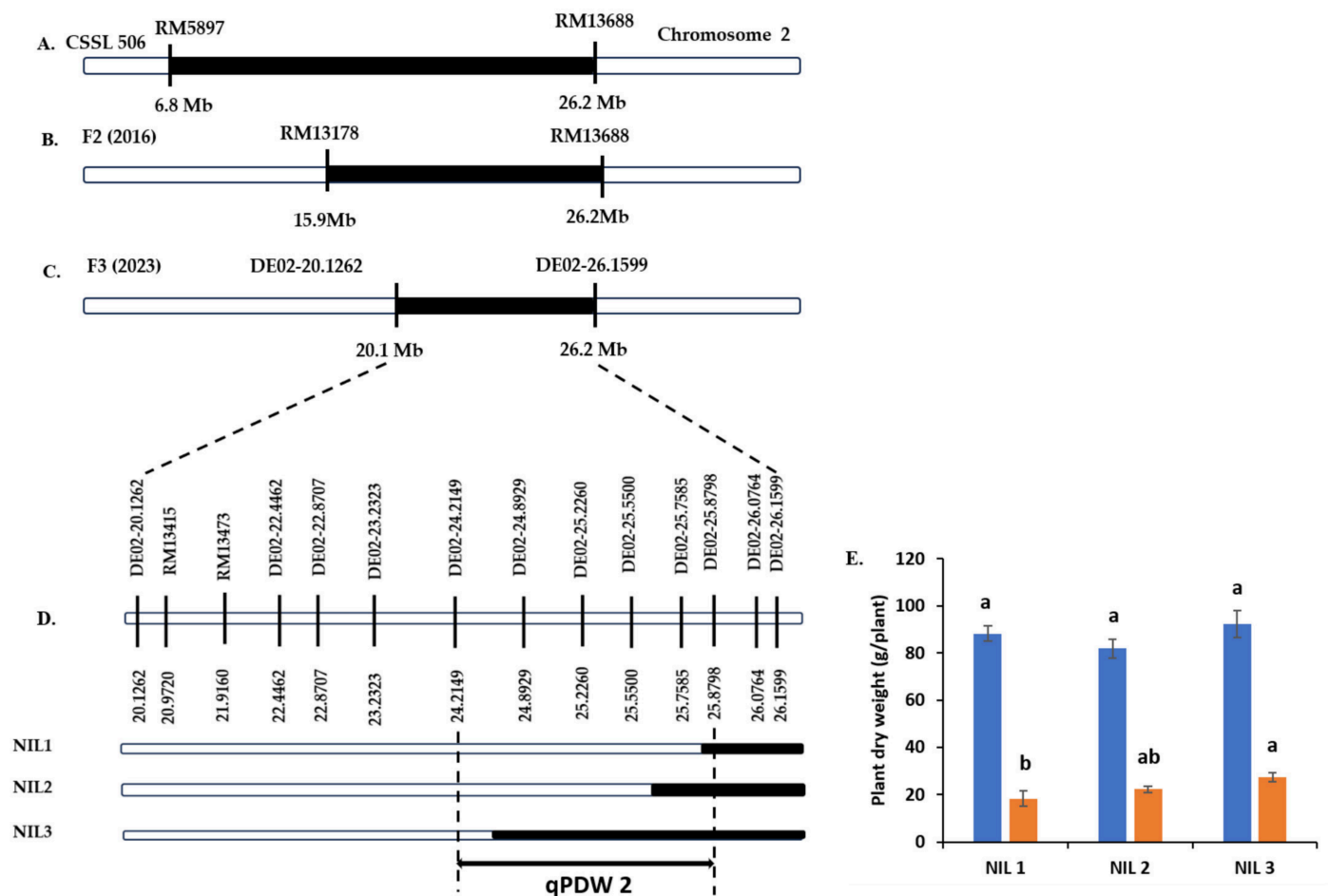


Figure 2. (A) Nona Bokra substituted region in Chromosome 2 of CSSL 506. (B) QTL-identified region in the F₂ population in 2016; (C) QTL-identified region in the F₃ population in 2023. (D) Graphical presentation of NILs grown in 2023. Fine mapping of qPDW on chromosome 2. Locus qPDW was narrowed down to a 1.2 Mb interval defined by markers DE02-24.2149 and DE02-25.8798. (E) Mean values of PDW of the NILs under long-term salt stress in 2023. Blue and orange bars indicate the mean values for the control and salt conditions, respectively. Error bars represent $\pm$ standard error (SE) of biological replicates (n = 5). Bars sharing the same lowercase letter within the control treatment are not significantly different according to Tukey's multiple comparison test ($p < 0.05$). Bars sharing the same lowercase letter within the salt treatment are not significantly different according to Tukey's multiple comparison test ($p < 0.05$).

NILs were evaluated for traits related to previously identified QTLs under both control and long-term salt stress conditions. Phenotypic evaluation of the NILs revealed variation among the lines carrying introgressions from the tolerant parent under salt stress conditions, where no significant difference was observed under control conditions. NIL3 exhibited the highest mean value for PDW, whereas NIL1 and NIL2, which also contained introgressed segments from the tolerant parent within the target chromosomal region, exhibited comparatively lower mean values (Figure 2). To further interpret the genetic basis of the observed variation, introgression patterns among the NILs were examined. NIL1, NIL2, and NIL3 shared overlapping chromosomal segments derived from the tolerant parent but differed in the extent of introgressed regions. Based on these differences, the QTL region was delimited to the marker interval between DE02-24.2149 and DE02-25.8798 present in NIL3. This inference was further supported by single-marker analysis, which revealed a consistent association between markers within this region and trait values across evaluated lines.

3.4. Fine-Mapping of QTL for PDW for Salt Tolerance

Based on the results above, fine-mapping was carried out for qPDW2, whose estimated and maximum substituted lengths were between DE02-24.2149 and DE02-25.8798, respectively (Figure 3). For fine mapping, a population of 580 individuals was developed from the SL506/Koshihikari F₅ generation, and nine groups of recombinants were selected, of which seven groups had different tolerant parent introgressed regions in the background of homozygous Koshihikari, while the other two groups had homozygous Koshihikari and SL506 in the target QTL region.

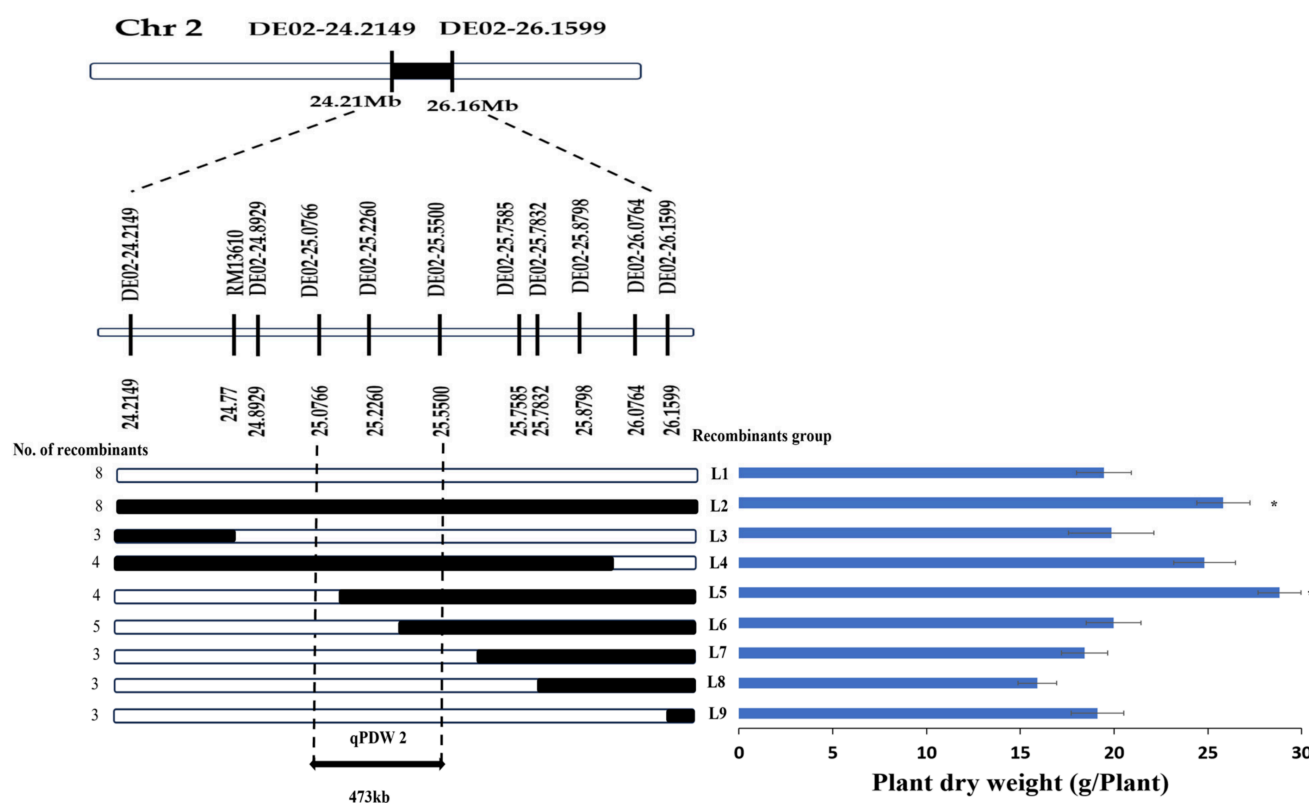


Figure 3. Fine mapping of qPDW on chromosome 2 in 2024. Locus qPDW was narrowed down to a 473 kb interval defined by markers DE02-25.0766 and DE02-25.5500. The bar graph represents the mean value of the PDW of recombinant groups and vertical bars are standard errors of the mean. * Indicates significant at the 5% level of probability compared to the homozygous Koshihikari group L1.

When the PDW of one group significantly differed from that of the homozygous Koshihikari group L1, a QTL was detected in the substituted segments of that group. When multiple substitution segments in other groups with the target trait overlapped, the QTL was localized to the overlapping region [28]. Based on substitution mapping, the major QTL region on chromosome 2, qPDW2, was delimited to an estimated interval of 473 kb between markers DE02-25.0766 and DE02-25.5500 (Figure 3).

3.5. Prediction of Candidate Genes of qPDW2

Gene annotation using the Rice Annotation Project Database (<https://rapdb.dna.naro.go.jp>; accessed on 29 January 2026) identified 69 predicted genes (Table S1). Among these, 43 genes possessed annotated biological functions, while the remaining genes were classified as hypothetical, conserved proteins, or proteins with unknown functions. To further identify potential candidate genes, sequence polymorphisms between the parental lines were analyzed. SNP analysis detected 192 nonsynonymous (missense) variants within

the genes located in the QTL interval. Functional prediction using the Sorting Intolerant From Tolerant (SIFT) algorithm indicated that multiple variants had low SIFT scores (<0.05), suggesting potentially deleterious effects on protein function. Based on the presence of nonsynonymous SNPs and predicted functional effects, a subset of genes was identified as possible candidate genes within the QTL interval (Table 4). These genes included those encoding F-box domain-containing proteins, wall-associated kinase family proteins, protein kinase catalytic domain-containing proteins, and protein phosphatase 2C family proteins.

Table 4. Candidate genes that contain nonsynonymous SNPs (missense variants) located within the QTL interval of chromosome 2 with predicted functional SNP effects estimated by the variant effect predictor.

SNP Position	Koshihikari	Nona Bokra	Variant Consequence	SIFT ^a	Strand	Gene ID	Description	Gene Symbol
25192275	C	T	tCg/tTg	0	+	Os02g0630000	F-box domain, cyclin-like domain-containing protein	OsFbox 102
25340151	G	T	Gat/Tat	0.04	+	Os02g0632500	Arf GTPase-activating protein family	
25350479	T	A	Agg/Tgg	0	—	Os02g0632800	Wall-associated kinase, positive regulation of rice blast resistance	OsWAK14
25350851	C	A	Gct/Tct	0.04	—			
25350856	G	C	tCt/tGt	0.02	—			
25350934	G	C,T	tCg/tGg	0.03	—			
25351363	G	A	aCc/aTc	0	—			
25351453	G	C	cCc/cGc	0.05	—			
25351545	G	C	tgC/tgG	0.02	—			
25351580	C	T	Gtg/Atg	0.04	—			
25376039	T	A	Aat/Tat	0	—	Os02g0633066	Protein kinase, catalytic domain-containing protein	
25415049	T	G	Aac/Cac	0.02	—	Os02g0633900	Similar to T-cell activation protein phosphatase 2C-like protein	OsPP2C 24
25415193	C	T	Gcg/Acg	0.04	—			
25415295	T	C	Gcg/Acg	0.03	—			
25488345	G	T	tGc/tTc	0	+	Os02g0635600	Tyrosine protein kinase domain-containing protein	

^a SIFT: Sorting Intolerant From Tolerant.

4. Discussion

The use of indica–japonica introgression in CSSL is a powerful strategy to enhance rice yield potential due to substantial phenotypic variation and the opportunity to exploit inter-subspecific heterosis [25]. In this context, Thuy et al. [25] evaluated 44 CSSLs derived from the salt-tolerant donor Nona Bokra in the genetic background of the susceptible cultivar Koshihikari and identified multiple lines with improved salinity tolerance. Notably, CSSL 506, harboring a Nona Bokra-derived segment on chromosome 2, exhibited enhanced PDW and grain weight compared to Koshihikari under salt stress. This line also maintained ionic conditions by limiting the accumulation of toxic ions, particularly Na⁺ and Cl[−]. Previous findings demonstrated that CSSL 506 accumulated lower levels of Na⁺ and Cl[−] in the whole plant at 29 days after treatment, at the heading stage, and at maturity compared with Koshihikari, with the reduction being especially significant at maturity [25]. But no significant difference was observed for all the studied parameters under the control condition. Therefore, it is likely that the substituted Nona Bokra chromosomal region in SL506 contributes to reduced toxic ion accumulation, thereby helping sustain plant growth and grain yield under salt stress conditions. Further QTL analysis using F₂ and F₃ populations derived from SL506/Koshihikari detected loci associated with yield-related traits. Subsequent substitution mapping with NILs and recombinants refined the QTL controlling PDW to a 473 kb interval on chromosome 2.

Reproductive-stage QTLs are critical determinants of plant survival and final yield under salinity stress, often exerting a greater impact than seedling-stage tolerance to salinity stress. Despite their importance, studies focusing on salinity tolerance during the reproductive stage remain comparatively limited [18]. In 2016, QTL analysis using 80 individuals of the SL506/Koshihikari F_2 population identified three QTLs (qPDW2, qPN2, and qGW2) within the 15.9–26.2 Mb interval associated with plant dry weight (PDW) at maturity, panicle number (PN), and grain weight (GW) (Table 3). In the subsequent F_3 population, which was composed of 102 individuals, the previously identified QTLs were validated in 2023. In previous years, genome-wide QTL mapping generally used population sizes that ranged from 138–232 individuals to ensure sufficient statistical power and mapping resolution [17,19,21,29–34]. However, smaller populations consisting of 70–112 individuals have also been successfully used to identify major-effect QTLs or validate previously reported genomic regions [27,33,35,36]. In this study, the F_2 ($n = 80$) and F_3 ($n = 102$) population sizes were considered appropriate based on recombinants, as the QTL analysis was confined to a predefined interval on chromosome 2 rather than a whole-genome QTL scan. Therefore, moderate-sized segregating populations were used for validating major-effect QTLs, whereas larger populations are mainly required for detecting minor-effect loci and improving mapping resolution.

In previous years, eight meta-QTLs derived from 81 QTLs have been reported on chromosome 2 for different traits under salinity stress during the seedling and reproductive stages [37]. However, these meta-QTLs did not overlap with the region on chromosome 2 studied here. Previously, 140 F_2 populations derived from PS5, which is saline-sensitive, and CSR10, a saline-tolerant rice variety, were evaluated for salinity tolerance at the reproductive stage for 18 different morphological, biochemical, and physiological traits. Moreover, 39 QTLs on chromosomes 1–12 were identified, along with a QTL for the number of productive tillers on chromosome 2 between the 21.84–25.8 Mb region [16]. More recently, Sugasi et al. [21] used recombinant inbred lines (RILs) to identify QTLs for salinity tolerance at the reproductive stage and identified 18 QTLs on chromosomes 1, 2, 6, 10, 11, and 12. However, fine-mapping studies on these QTLs or specific traits have not yet been fully executed. The QTL region identified in this study on chromosome 2 (25.08–25.55 Mb) partially co-localizes with previously reported QTLs for PDW (25.47–27.45 Mb) and PN (25.47–29.71 Mb) [27]. Although the genomic intervals were not completely identical, an overlapping region detected across studies suggests that this chromosomal segment may represent a stable locus contributing to salinity tolerance across different genetic backgrounds and agronomic traits. In contrast to the broader QTL intervals previously reported (approximately 2–4 Mb), the present study delimited the target locus to a narrower 473 kb region, thereby improving the positional resolution within the overlapping segment and substantially reducing the candidate genomic space for downstream candidate gene identification and functional validation.

Grain yield is a complex quantitative trait strongly influenced by environmental factors, as widely observed in cereal crops. Direct selection based solely on yield is often inefficient and unreliable. In this context, the association between yield and its component traits, along with stress-related indices, is critically important for developing effective salt-tolerant varieties. Three near-isogenic lines (NILs) evaluated under salinity stress in 2023 delimited qPDW2 to a 1.2 Mb interval between the markers RM13571 and DE02-26.1599. (Figure 2). Subsequent use of an F_5 -generation high-resolution mapping population and substitution analysis of recombinant groups further refined the qPDW2 locus to a 473 kb region between the markers DE02-25.0766 and DE02-25.5500 (Figure 3). Although QTLs associated with GW were detected in the F_2 and F_3 populations under salt stress, these effects were not consistently observed in the subsequent F_4 NIL and F_5 recombinant populations.

Environmental factors may have substantially contributed to the unstable expression of GWs across generations. Variations in temperature, humidity, and salinity intensity during the grain-filling period, together with genotype $\times$ environment interactions, could have masked or altered the phenotypic effects of these QTLs in the subsequent generations. Furthermore, differences in heading date [38] and exposure to high temperatures, which disrupt pollen viability and spikelet fertility [39], may also have influenced grain development and produced sterile panicles, thereby affecting the expression of GW-related loci. Conversely, QTLs associated with PDW were consistently retained throughout the NIL and the recombinant-based fine-mapping process, indicating the presence of a more stable and major-effect locus on chromosome 2. The persistence of this QTL across generations highlights its robustness, suggesting that maintaining biomass under salinity stress may be a more genetically stable indicator of tolerance than GW.

Gene annotation identified 69 predicted genes within the QTL region, of which 43 have been assigned biological functions, while the remaining 26 genes have been assigned hypothetical or uncharacterised functional status (Table S1). To prioritize potential candidate genes underlying the observed variation in salinity tolerance, sequence polymorphisms between the parental cultivars, Koshihikari and Nona Bokra, were examined. Among the identified variants, non-synonymous SNPs causing amino acid substitutions were considered particularly relevant because they have the potential to alter protein structure and function [40]. SIFT predicts whether these amino acid substitutions are deleterious, enabling the prioritization of candidate genes that are more likely to contribute to phenotypic variation in the target trait [40]. In total, 15 deleterious nonsynonymous SNPs (SIFT < 0.05) were identified in six candidate genes, indicating their potential functional importance (Table 4). Among these candidates, Os02g0633900 (OsPP2C24), Os02g0635600 (OsFbox102), and Os02g0632800 (OsWAK14) encode proteins belonging to the protein phosphatase 2C (PP2C), F-box, and wall-associated kinase (WAK) families, respectively. These gene families have been widely implicated in plant responses to environmental stresses [41–49]. Members of the rice PP2C family function as important regulators of ABA signalling and several PP2C genes, including OsPP108, OsPP2C09, OsPP18, OsBIPP2C1, and OsPP2C68, have been reported to regulate tolerance to salinity and other abiotic stresses through ABA-dependent signalling pathways [41–46,50,51]. Likewise, F-box proteins regulate selective protein degradation through the SCF ubiquitin ligase complex and participate in diverse stress-response pathways, while WAK proteins function as receptor-like kinases involved in extracellular signal perception and stress signalling [47–49,52]. Previous studies have demonstrated that certain rice WAK and Fbox family members, such as OsWAK112 and OsFBX257, contribute to salt stress responses [48,49]. Although the specific functions of OsPP2C24, OsFbox102, and OsWAK14 in salinity tolerance have not yet been experimentally demonstrated, the identification of deleterious nonsynonymous variants in these genes, together with their localization within the fine-mapped qPDW2 interval and their membership in gene families previously associated with abiotic stress responses, suggests that they represent promising candidate genes underlying the QTL. Further functional analyses, including gene expression profiling and reverse genetic approaches, will be required to determine their precise roles in reproductive-stage salinity tolerance in rice.

5. Conclusions

This study represents a significant advancement in elucidating the genetic architecture of salinity tolerance in rice by validating and fine-mapping QTLs associated with reproductive-stage traits under salt stress. Utilizing a CSSL derived from a cross between the salt-sensitive cultivar Koshihikari and the salt-tolerant donor Nona Bokra, the study systematically confirmed the stability of key loci on chromosome 2 governing biomass

accumulation across multiple generations. Through fine-mapping with NILs and high-resolution mapping with recombinant populations, the QTL controlling PDW was precisely delimited to a 473 kb genomic interval, encompassing 69 annotated genes. Variant Effect Predictor analysis further prioritized six candidate genes carrying deleterious nonsynonymous variants, among which OsPP2C24, OsFbox102, and OsWAK14 emerged as the most promising candidates because they belong to the PP2C, F-box, and wall-associated kinase (WAK) gene families, respectively, which have previously been implicated in plant abiotic stress responses. These findings provide a valuable foundation for future functional validation of candidate genes and support the development of molecular breeding strategies to improve reproductive-stage salinity tolerance and yield stability in rice.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/agronomy16161518/s1>.

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Abbreviations

The following abbreviations are used in this manuscript:

qPDW	Plant dry weight QTL
qPN	Panicle number QTL
qGW	Grain weight QTL
NIL	Near isogenic lines
CSSL	Chromosome segment substitution line
QTL	Quantitative trait loci
PDW	Plant dry weight
PN	Panicle number
PW	Panicle weight
GW	Grain weight
GFP	Grain filling percentage
SNP	Single Nucleotide Polymorphism

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