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Identification of a key locus, *qRL8.1*, associated with root length traits during seed germination under salt stress via a genome-wide association study in rice

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Abstract

Background Salt stress is a major abiotic constraint limiting rice (*Oryza sativa* L.) production worldwide, particularly in saline-affected regions. Improving salt tolerance at the seed germination stage is crucial for increasing stand establishment and yield stability, especially under direct seeding conditions. Identifying loci associated with salt-tolerant germination and characterizing key candidate genes offers valuable insights for breeding strategies.

Results We evaluated the salt tolerance of 406 drought-resistant rice accessions at the germination stage under 0, 100, 150, and 200 mM NaCl conditions. Four germination-related traits—germination potential (GP), relative germination potential (RGP), root length (RL), and relative root length (RRL)—were measured. Significant phenotypic variation was observed, with GP, RGP, RL, and RRL sharply decreasing as the NaCl concentration increased. Using a genome-wide association study (GWAS) with 65,069 high-quality SNPs, we identified 27 significantly associated loci. Among these genes, 9 colocalized with known QTLs/genes, and 18 were identified as novel. The key locus qRL8.1, identified under 200 mM NaCl stress, contained multiple closely linked SNPs and strongly associated with RL and RRL. Expression analyses of candidate genes within *qRL8.1* indicated that *LOC_Os08g41790* (encoding a phosphatidylinositol/uridine kinase family protein) and *LOC_Os08g42080* (encoding a peroxidase precursor) were both highly expressed in roots and strongly induced by salt stress. Haplotype analysis revealed that favorable alleles of these genes are associated with improved seed germination and root elongation under salt stress conditions. Several elite varieties carrying superior haplotypes of both genes were identified, providing valuable genetic resources for breeding salt-tolerant rice cultivars.

Conclusions This study identified multiple loci conferring salt tolerance at the germination stage, with *qRL8.1* emerging as a key locus. Two candidate genes, *LOC_Os08g41790* and *LOC_Os08g42080*, were significantly associated with increased salt tolerance. The elite haplotypes and varieties identified here can be directly utilized in rice breeding

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programs. These findings increase our understanding of the genetic mechanisms underlying salt tolerance during early seedling establishment and offer new avenues for developing salt-resistant rice varieties.

Keywords Salt tolerance, Genome-wide association study (GWAS), Rice, Germination potential, *qRL8.1*, Haplotypic analysis

Introduction

Rice (*Oryza sativa* L.) is a staple food crop for approximately 4 billion people worldwide [1]. However, its growth is highly susceptible to abiotic stresses, with salinity recognized as a major constraint. Approximately 6% of the global land area and 20% of irrigated land are affected by salinity [2, 3]. Rice is particularly sensitive to salt stress compared with many other crops [4–6]. Seed germination is the initial stage of the rice life cycle and is critical for stand establishment. Improving salt tolerance at this early stage is especially important in direct-seed systems, where rapid and uniform germination ensures better crop performance [7].

Seed vigor is a complex trait controlled by multiple genes and is associated with seed germination, seedling emergence, growth, storage capacity, and stress tolerance [8, 9]. Previous studies reported that *qSE3* promotes the uptake of K^+ and Na^+ during rice seed germination, induces the expression of abscisic acid (ABA) biosynthesis and signaling pathway genes, and inhibits reactive oxygen species (ROS) accumulation in seeds, thereby enhancing the germination of salt tolerance [7]. The transcription factor *OsBZIP23*, a positive regulator of the ABA signaling pathway, activates the downstream target gene *PER1A*, and the peroxidase *PER1A* enhances seed vigor by scavenging ROS in seeds [10, 11]. Mutation of *PFPβ* alters rice grain quality by significantly reducing total starch and amylose contents while increasing fat and protein contents, leading to abnormal development of seed radicles and coleoptiles and significantly lowering germination rates [12]. The overexpression of *HDT701* in transgenic rice reduced resistance to ABA, salt stress, and osmotic stress during seed germination, delayed germination, and was associated with reduced histone H4 acetylation and downregulated expression of GA biosynthesis genes [13]. The late embryogenesis-abundant protein *OsLEA5* interacts with *ZFP36*, enhancing *ZFP36* activation of the *OsAPX1* promoter. Together, these genes regulate ABA-inhibited seed germination by modulating *OsAPX1* expression [14]. However, many key genes related to salt tolerance germination remain to be elucidated.

Genome-wide association studies (GWASs) have emerged as powerful tools for dissecting complex traits and identifying superior alleles in natural populations [15–17]. Several GWASs on salt tolerance in rice have identified numerous SNPs associated with Na^+/K^+ ratios and other salt tolerance-related traits [18–19]. Using high-density SNPs, GWASs can provide insights into the genetic basis of salt tolerance at the germination stage.

In this study, we used a drought-resistant core population of 406 rice accessions to evaluate the germination potential (GP), relative germination potential (RGP), root length (RL), and relative root length (RRL) under various NaCl concentrations (0, 100, 150, and 200 mM). A GWAS using 65,069 SNPs was performed to identify key loci associated with salt tolerance during seed germination. Notably, *qRL8.1* was identified as a major locus for root growth under severe salt stress. Expression and haplotype analyses suggested that *LOC_Os08g41790* and *LOC_Os08g42080* within *qRL8.1* are potential key genes governing salt tolerance. These findings provide valuable targets for marker-assisted breeding and the improvement of salt-resistant rice cultivars.

Materials and methods

Planting materials

The experiment utilized a natural population of 406 rice accessions derived from the core germplasm of water-saving and drought-resistant rice maintained at the Shanghai Agrobiological Gene Center (SAGC). Among these, 215 accessions were previously identified as drought-resistant, demonstrating their adaptive differentiation in response to drought conditions [20]. The average germination potential (GP) of the 406 rice accessions under H_2O conditions exceeded 95%. All the accessions were cultivated at the Zhuanghang Comprehensive Experimental Station of the Shanghai Academy of Agricultural Sciences in Fengxian District, Shanghai. Field management followed local standard practices. All the seeds were harvested at maturity and dried at 42 °C for 7 days to break seed dormancy.

Evaluation of seed salt tolerance

Surface sterilization of 30 healthy grains from each rice accession was performed via 0.5% sodium hypochlorite solution for 15 min, followed by rinsing three times with sterile distilled water. The seeds were evenly distributed in 9 cm diameter Petri dishes lined with filter paper. Four different concentrations of NaCl solution (0, 100, 150, and 200 mmol/L) were added (10 mL per dish) for treatment, with each treatment replicated three times biologically. The dishes were incubated in a growth chamber under controlled conditions (30 °C, 12-hour light/dark cycle; GXZ multiprogram incubator, Ningbo Jiangnan Instrument Factory). The NaCl solutions were replaced

daily to maintain stable concentrations. Germination was monitored daily, with the final count taken at the experimental endpoint. Germination was visually defined as the emergence of the radicle through the hull, extending at least 2 mm. Seedling establishment was defined as the stage when the root length equaled the seed length, and the shoot length reached half the seed length [21]. The germination potential (GP) and relative germination potential (RGP) were recorded on day 4, whereas the root length (RL) and relative root length (RRL) were assessed on day 7. The calculation formulas are as follows: GP = (number of normally germinated seeds on day n/total number of seeds) ×100, RGP = (GP under treatment/GP under control) × 100, RRL = (RL under treatment/RL under control) × 100.

Genome-wide association study

The 406 rice accessions in our study include 215 accessions that were previously sequenced using the Rice60K SNP array, following the established workflow. This workflow included quality control with Fastp, mapping to the MSU v.6.1 reference genome using BWA, and variant calling with GATK [20]. The remaining 210 accessions were newly sequenced following the same workflow, ensuring consistent data quality and variant identification across all 406 samples. SNP data were filtered via BCFtools with the criteria of a minor allele frequency (MAF) ≥ 0.05 and a missing rate < 10%. After filtering, a total of 65,069 high-quality SNPs were obtained for further analysis. Genome-wide association studies (GWASs) were conducted via the mixed linear model (MLM) in Tier 5. On the basis of the study, the significance threshold for all indices was set at $P \leq 1.0e\text{-}5$, which corresponds to the red horizontal line at $-\log_{10}(P) \geq 5$ on the Manhattan plot [22]. The GWAS results for GP, RGP, RL, and RRL were visualized as Manhattan plots and quantile–quantile plots via the R package CMplot. SNPs with a $\log_{10}P$ value exceeding the threshold and having a physical distance of < 100 kb from adjacent SNP clusters were integrated into a single locus. These loci were named *qGP*, *qRGP*, *qRL*, and *qRRL* (Table 1).

Candidate gene analysis of *qRL8.1*

To identify candidate genes associated with the *qRL8.1* locus, we first examined approximately ± 100 kb flanking regions of the lead SNPs that exhibited significant associations [23]. By integrating colocalization evidence from previously reported QTL intervals, we refined this initial range and defined a final candidate genomic interval of approximately 247.6 kb. Within this region, we extracted all annotated genes and predicted gene models from the rice reference genome (MSU v6.1). We then screened for SNPs in these genes that were located either within their promoter regions (approximately 3 kb upstream of the transcription start site) or within exonic regions likely to cause nonsynonymous substitutions. We subsequently cross-referenced these candidate genes with expression data from the Rice RNA Database (https://plantrnadb.com/ricerna/), focusing on those showing preferential expression in root tissues and pronounced induction under salt stress [24]. Genes meeting these expression criteria were further investigated via haplotype analysis and functional prediction to confirm their potential roles in conferring salt tolerance at the *qRL8.1* locus.

Haplotypic classification and phenotypic association analysis of *LOC_Os08g41790*, *LOC_Os08g41890*, *LOC_Os08g42030*, and *LOC_Os08g42080*

For the genes *LOC_Os08g41790*, *LOC_Os08g41890*, *LOC_Os08g42030*, and *LOC_Os08g42080*, haplotype classification was based on all SNPs with a minor allele frequency (MAF) greater than 0.05. The functional regions considered for this analysis included the 5' flanking sequence (within 3 kb of the first ATG codon) and the coding sequence (CDS) of the target genes, as described in a previous study [25].To ensure robustness in the comparison, at least five of the studied accessions containing the investigated genes were included in the haplotype analysis [26].

On the basis of the haplotypes of *LOC_Os08g41790* and *LOC_Os08g42030*, we conducted a phenotypic comparison across all varieties to identify the favorable haplotypes that may enhance seed germination under salt stress. This analysis aimed to pinpoint specific combinations of haplotypes from these genes that could contribute to improved salt tolerance and seed germination performance under stress conditions.

Statistical analysis

The experimental data were analyzed via Excel 2021. Significant differences were assessed via Student's t test or Fisher's least significant difference (LSD) test at the 5% and 1% probability levels, respectively.

Results

Identification of salt tolerance phenotypes in 406 seeds of a drought-tolerant core population

A total of 406 drought-resistant core seed samples were assessed for their salinity tolerance in 2017, with a focus on four key germination-related traits: germination potential (GP), relative germination potential (RGP), root length (RL), and relative root length (RRL) under three NaCl concentrations (100 mM, 150 mM, and 200 mM) (Table S1). Phenotypic statistical analysis revealed significant variation in these traits across different salt stress conditions, as illustrated by the boxplot distributions (Fig. 1).

Table 1 Significant QTLs associated with seed germination potential and root length traits under different NaCl salinity conditions in rice and their SNP characteristics

QTL	Trait	Conditions	SNP	Position	Chr.	*P* value	MAF	PVE (%)	Known QTLs/Genes
qGP1.1	germination potential	CK	F0125348059TC	25,348,059	1	8.52208E-06	0.45890411	3.10	*OsDfr* [27]
	germination potential	CK	R0125352354TC	25,352,354	1	8.52208E-06	0.45890411	3.10	
	germination potential	CK	R0125364640AC	25,364,640	1	8.52208E-06	0.45890411	3.10	
qGP1.2	germination potential	CK	R0134864883AC	34,864,883	1	2.04499E-08	0.05342466	15.20	
	germination potential	CK	F0135078972AG	35,078,972	1	5.94335E-07	0.07945205	13.29	
	germination potential	CK	F0135092748GT	35,092,748	1	1.22225E-06	0.07808219	12.29	
qGP1.3	germination potential	CK	R0138401805AG	38,401,805	1	2.27509E-08	0.4630137	3.22	*sd1*,*qSD1-2* [28]
qGP3.1	germination potential	CK	F0302540464AC	2,540,464	3	9.23826E-06	0.4739726	0.47	*OsTIP1;1* [29]
qGP4.1	germination potential	CK	F0405046058GA	5,046,058	4	2.01777E-06	0.09315068	13.99	
qGP5.1	germination potential	CK	F0507568644AG	7,568,644	5	1.08391E-08	0.22876712	30.78	
qGP7.1	germination potential	CK	F0705499267AC	5,499,267	7	3.34504E-07	0.47671233	0.23	*OsZDS* [30]
	germination potential	CK	F0705539122TG	5,539,122	7	3.34504E-07	0.47671233	0.23	
	germination potential	CK	F0705600721TC	5,600,721	7	3.34504E-07	0.47671233	0.23	
	germination potential	CK	F0705622686TG	5,622,686	7	1.02156E-09	0.47123288	0.00	
qGP3.2	germination potential	100 M	F0325976416CT	25,976,416	3	8.10421E-06	0.30461538	2.58	
qGP4.2	germination potential	100 M	F0424434950GA	24,434,950	4	4.13942E-06	0.13846154	3.44	
	germination potential	100 M	R0424449947CT	24,449,947	4	4.13942E-06	0.13846154	3.44	
qGP11.1	germination potential	100 M	R1116828278TC	16,828,278	11	6.82073E-06	0.42307692	2.46	
qGP2.1	germination potential	150 M	F0204103432TC	4,103,432	2	5.58455E-07	0.17610063	6.65	*OsHKT1;3*; *OsHKT6* [31]
qGP2.2	germination potential	150 M	R0211071031CT	11,071,031	2	9.71526E-07	0.08490566	7.51	*OsCBL8* [32]
qGP2.3	germination potential	150 M	F0211345927CA	11,345,927	2	6.48062E-06	0.07389937	7.18	*OsLKRT1*; *dice2* [33]
	germination potential	150 M	F0211462811AG	11,462,811	2	2.39147E-06	0.07861635	7.45	

Table 1 (continued)

QTL	Trait	Conditions	SNP	Position	Chr.	*P* value	MAF	PVE (%)	Known QTLs/Genes
qGP3.3	germination potential	150 M	F0301272979AT	1,272,979	3	2.30182E-06	0.18553459	18.43	*OsUBC2*, *TAD1* [34]
	germination potential	150 M	F0301273177TG	1,273,177	3	2.30182E-06	0.18553459	18.43	
	germination potential	150 M	R0301273835AC	1,273,835	3	2.30182E-06	0.18553459	18.43	
	germination potential	150 M	F0301273896AG	1,273,896	3	1.87203E-06	0.32704403	4.80	
	germination potential	150 M	F0301275139TG	1,275,139	3	1.87203E-06	0.32704403	4.80	
	germination potential	150 M	R0301275355GA	1,275,355	3	2.30182E-06	0.18553459	18.43	
	germination potential	150 M	F0301275842CT	1,275,842	3	2.30182E-06	0.18553459	18.43	
qRGP1.1	Relative germination potential	100 M	R0125208043CA	25,208,043	1	4.12264E-06	0.49839744	30.88	
qRGP3.1	Relative germination potential	100 M	F0302540464AC	2,540,464	3	1.17773E-06	0.5	27.78	*OsTIP1;1* [29]
qRGP3.2	Relative germination potential	100 M	F0305380169AC	5,380,169	3	3.82475E-07	0.49198718	30.14	
qRGP6.1	Relative germination potential	100 M	F0625120125TG	25,120,125	6	5.01351E-06	0.49038462	29.01	
	Relative germination potential	100 M	R0625131528CT	25,131,528	6	5.01351E-06	0.49038462	29.01	
qRGP7.1	Relative germination potential	100 M	F0705499267AC	5,499,267	7	6.75055E-11	0.5	31.44	
	Relative germination potential	100 M	F0705539122TG	5,539,122	7	6.75055E-11	0.5	31.44	
	Relative germination potential	100 M	F0705600721TC	5,600,721	7	6.75055E-11	0.5	20.40	
	Relative germination potential	100 M	F0705622686TG	5,622,686	7	8.79311E-13	0.49358974	9.91	
qRGP8.1	Relative germination potential	100 M	F0803950653AG	3,950,653	8	4.35247E-06	0.4775641	29.16	
	Relative germination potential	100 M	F0803951903TG	3,951,903	8	4.35247E-06	0.4775641	29.16	
	Relative germination potential	100 M	F0803954101TC	3,954,101	8	4.35247E-06	0.4775641	29.16	
qRGP8.2	Relative germination potential	100 M	F0804277375TC	4,277,375	8	3.77925E-06	0.49839744	29.34	
	Relative germination potential	100 M	F0804299685GT	4,299,685	8	1.30985E-06	0.49358974	29.83	
	Relative germination potential	100 M	F0804303807TC	4,303,807	8	3.77925E-06	0.49839744	29.34	
	Relative germination potential	100 M	F0804328783AG	4,328,783	8	5.07739E-07	0.49519231	30.04	
qRGP11.1	Relative germination potential	100 M	F1101399015TC	1,399,015	11	2.63996E-09	0.49198718	29.05	
qRGP2.1	Relative germination potential	150 M	F0204103432TC	4,103,432	2	2.03975E-07	0.17207792	6.52	*OsHKT1;3*; *OsHKT6* [31]
qRGP2.2	Relative germination potential	150 M	R0211071031CT	11,071,031	2	6.58274E-07	0.08116883	1.44	*OsCBL8* [32]
qRGP2.3	Relative germination potential	150 M	F0211345927CA	11,345,927	2	8.59917E-07	0.07305195	7.27	*OsLKRT1*; *dice2* [33]
	Relative germination potential	150 M	F0211462811AG	11,462,811	2	2.21032E-07	0.07792208	7.49	

Table 1 (continued)

QTL	Trait	Conditions	SNP	Position	Chr.	*P* value	MAF	PVE (%)	Known QTLs/Genes
qRL3.1	Root length	200 M	F0301414105AG	1,414,105	3	1.96315E-09	0.48742138	1.71	*OsSIZ1* [35]
qRL3.2	Root length	200 M	F0303778853TC	3,778,853	3	9.93724E-06	0.47641509	1.94	
qRL4.1	Root length	200 M	R0405093297TG	5,093,297	4	8.62243E-06	0.05660377	4.98	
qRL8.1	Root length	200 M	F0826286488TC	26,286,488	8	6.79926E-06	0.48742138	1.59	
	Root length	200 M	F0826314588GA	26,314,588	8	6.79926E-06	0.48742138	1.59	
	Root length	200 M	F0826333229AC	26,333,229	8	6.79926E-06	0.48742138	1.59	
	Root length	200 M	F0826387587AG	26,387,587	8	1.47167E-08	0.49056604	1.83	
	Root length	200 M	R0826404280CT	26,404,280	8	1.47167E-08	0.49056604	1.83	
	Root length	200 M	F0826457211AG	26,457,211	8	1.65474E-09	0.49371069	1.84	
	Root length	200 M	F0826464749TC	26,464,749	8	1.65474E-09	0.49371069	1.84	
	Root length	200 M	F0826493502AT	26,493,502	8	1.65474E-09	0.49371069	1.84	
	Root length	200 M	F0826496416TC	26,496,416	8	1.65474E-09	0.49371069	1.84	
	Root length	200 M	F0826498163AT	26,498,163	8	1.65474E-09	0.49371069	1.84	
	Root length	200 M	F0826500370TC	26,500,370	8	1.65474E-09	0.49371069	2.15	
	Root length	200 M	F0826503556TG	26,503,556	8	1.65474E-09	0.49371069	1.84	
	Root length	200 M	F0826504823TC	26,504,823	8	1.65474E-09	0.49371069	1.84	
qRL10.1	Root length	200 M	F1022634041AG	22,634,041	10	1.0659E-07	0.47484277	2.09	
qRL11.1	Root length	200 M	F1106062992TC	6,062,992	11	3.457E-06	0.48113208	2.15	
	Root length	200 M	F1106221348CG	6,221,348	11	2.3804E-06	0.49842767	1.90	
qRRL3.1	Relative root length	200 M	F0301414105AG	1,414,105	3	9.21737E-09	0.48571429	4.41	*OsSIZ1* [35]
qRRL8.1	Relative root length	200 M	F0826387587AG	26,387,587	8	3.14971E-07	0.48888889	4.38	
	Relative root length	200 M	R0826404280CT	26,404,280	8	3.14971E-07	0.48888889	4.38	
	Relative root length	200 M	F0826457211AG	26,457,211	8	7.61201E-08	0.49206349	4.33	
	Relative root length	200 M	F0826464749TC	26,464,749	8	7.61201E-08	0.49206349	4.33	
	Relative root length	200 M	F0826493502AT	26,493,502	8	7.61201E-08	0.49206349	4.33	
	Relative root length	200 M	F0826496416TC	26,496,416	8	7.61201E-08	0.49206349	4.33	
	Relative root length	200 M	F0826498163AT	26,498,163	8	7.61201E-08	0.49206349	4.33	
	Relative root length	200 M	F0826500370TC	26,500,370	8	7.61201E-08	0.49206349	4.94	
	Relative root length	200 M	F0826503556TG	26,503,556	8	7.61201E-08	0.49206349	4.33	
	Relative root length	200 M	F0826504823TC	26,504,823	8	7.61201E-08	0.49206349	4.33	
qRRL10.1	Relative root length	200 M	F1022634041AG	22,634,041	10	3.25508E-07	0.47301587	4.83	
qRRL11.1	Relative root length	200 M	F1106062992TC	6,062,992	11	4.37242E-06	0.47936508	4.94	
	Relative root length	200 M	F1106221348CG	6,221,348	11	4.50904E-06	0.5	4.47	

Notes: Conditions: CK = 0 mM NaCl, 100 M = 100 mM NaCl, 150 M = 150 mM NaCl, 200 M = 200 mM NaCl; Traits: GP = Germination Potential, RGP = Relative Germination Potential, RL = Root Length, RRL = Relative Root Length; MAF: Minor Allele Frequency; PVE: Phenotypic Variance Explained

Under normal conditions (CK), the average GP was remarkably high at 96.42% ± 0.41, ranging from 56.67 to 100%, indicating that most of the seeds retained excellent germination capacity. However, as the salinity increased, the GP declined sharply. At 100 mM NaCl, the mean GP decreased to 64.81% ± 1.70 (range: 6.67–100%); at 150 mM NaCl, it further decreased to 50.19% ± 1.87 (range: 0–100%); and at 200 mM NaCl, it plummeted to 12.56% ± 0.98 (range: 0–80%). The skewness and kurtosis values indicated a shift in the distribution of GPs, with a positive skew at 200 mM NaCl, suggesting that only a few seeds maintained high germination potential under severe stress. A similar trend was observed for RGP. Under 100 mM NaCl, the mean RGP was 66.78% ± 1.70 (range: 6.67–100%), whereas it decreased further under higher salinity conditions: 51.54% ± 1.89% at 150 mM NaCl and 12.87% ± 0.87% at 200 mM NaCl. These changes were reflected in the increasing asymmetry in the data, as indicated by the skewness values, under stronger salt treatments. Root traits showed even more drastic decreases. Under normal conditions, the average RL was 8.63 cm ± 0.12 (range: 5.03 cm to 15.62 cm), but it decreased to 4.44 cm ± 0.08 under 100 mM NaCl (range: 2.00–7.92 cm), 1.65 cm ± 0.04 under 150 mM NaCl (range: 0.57–3.90 cm), and only 0.55 cm ± 0.03 under 200 mM NaCl (range: 0–2.79 cm). Similarly, the RRL decreased from 52.18% ± 0.77 under normal conditions to 19.69% ± 0.46 and 6.73% ± 0.33 under 100 mM and 200 mM NaCl, respectively. The exceptionally high skewness (10.05) for RRL under 200 mM NaCl highlighted the significant reduction in root growth across most samples.

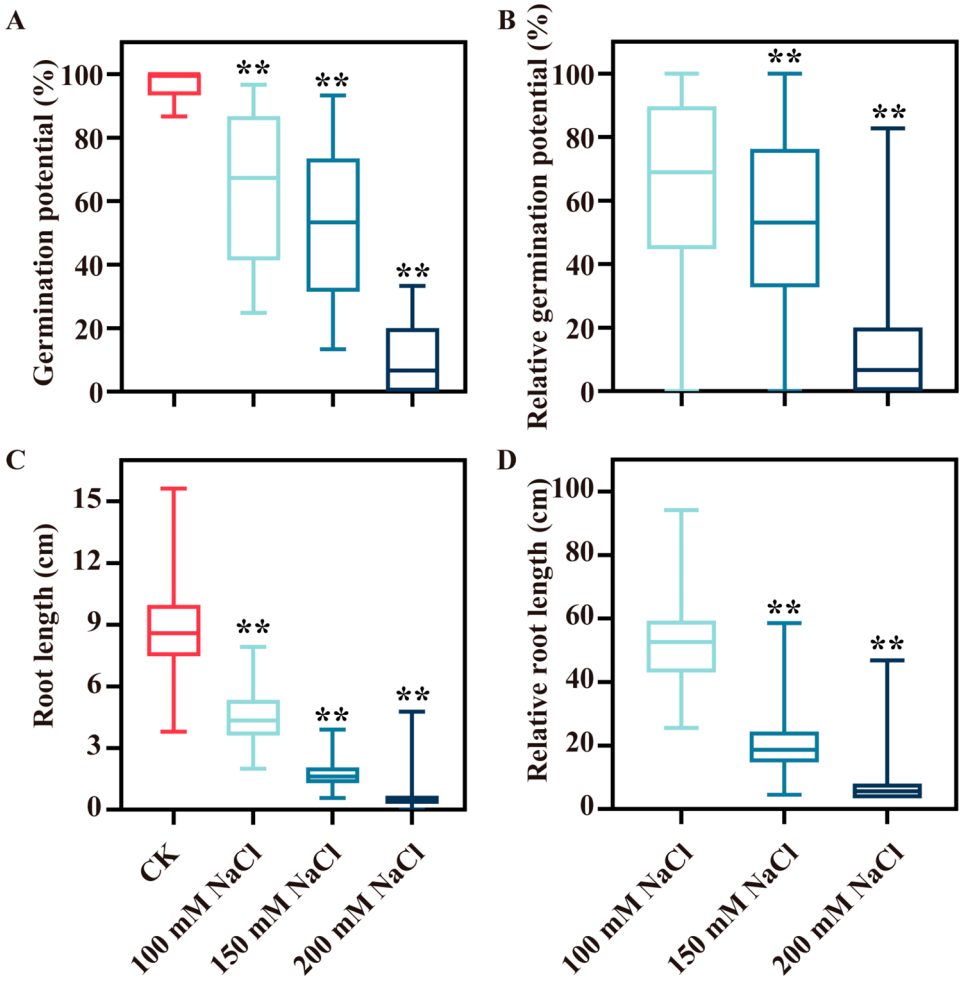

Fig. 1 Phenotypic variation of 406 natural rice populations under salt stress conditions. Box plots represent the distribution of four phenotypic traits under control (CK), 100 mM NaCl, 150 mM NaCl, and 200 mM NaCl conditions: (**A**) Germination potential. (**B**) Relative germination potential. (**C**) Root length. (**D**) Relative root length. The data are the means ± SDs ($n = 406$). Significant differences between conditions were determined by Student's t test and are indicated by ** ($P < 0.01$)

These results emphasize the severe impact of salt stress on seed germination and root growth, with some accessions showing resilience at moderate salinity (100 mM NaCl) and most being highly inhibited at relatively high NaCl concentrations. This broad phenotypic variation across traits provides a solid foundation for identifying and selecting seeds with enhanced salt tolerance for future breeding programs.

Identification and characterization of loci associated with salinity tolerance during germination

To identify loci associated with salt tolerance during seed germination, we performed a genome-wide association study (GWAS) on the four traits of GP, RGP, RL, and RRL under 100 mM, 150 mM, and 200 mM NaCl treatment conditions via Tassel 5.0 (Figs. 2, 3, S1, S2). A total of 27 loci were found to be significantly associated with these traits under salt stress. Nine loci colocalized with previously reported QTLs or genes, whereas the remaining 18 loci represent potentially new loci identified in this study. Notably, eight loci colocalized across both the GP and RGP traits and the RL and RRL traits. These loci included *qGP2.1*, *qGP2.2*, *qGP2.3*, *qGP3.1*, *qRL3.1*, *qRL8.1*, *qRL10.1*, and *qRL11.1* (Table 1).

Interestingly, *qGP2.1* colocalizes with the low-affinity sodium ion transporter *OsHKT1;3*, which was previously associated with salt tolerance [31]. The *qGP2.2* locus colocalizes with *OsCBL8*, a gene encoding the calcium-binding subunit of calcineurin B, which positively regulates salt tolerance in rice [32]. The *qGP2.3* locus contains the *OsLKRT1* gene, which is known to influence seed root length and hydrogen peroxide levels [33]. Additionally, the aquaporin *OsPIP1;1*, which has been shown to regulate the seed germination rate, was found to be located in the *qGP2.3* region, where it is induced by salt stress, gibberellin, and abscisic acid [29]. The *qRL3.1*

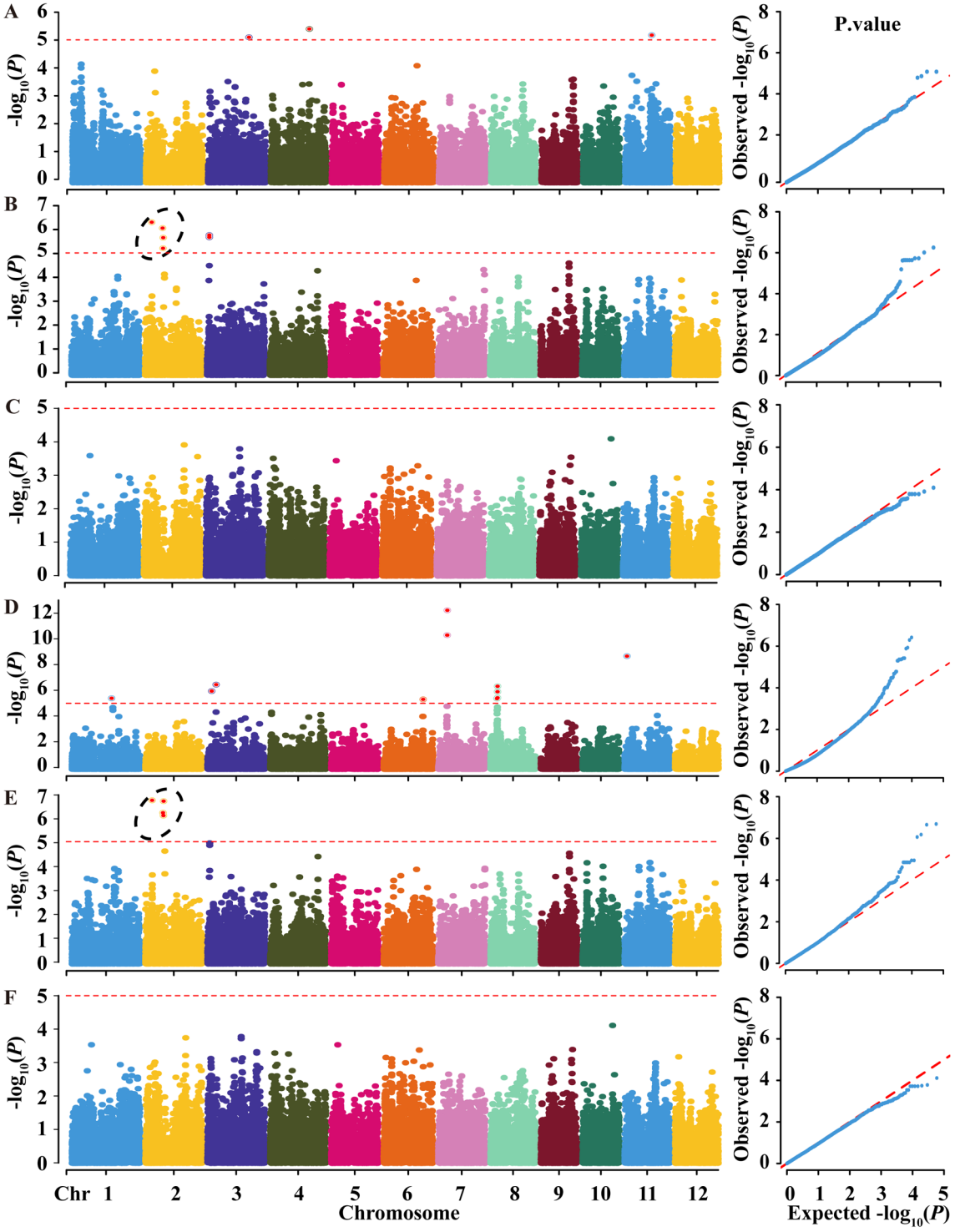

Fig. 2 Manhattan plots showing significant SNP associations for rice germination potential and relative germination potential under different salt stress conditions. (**A–C**) Germination potential under 100, 150, and 200 mM NaCl conditions on the fourth day. (**D–F**) Relative germination potential under 100, 150, and 200 mM NaCl conditions on the fourth day. The horizontal red dashed line represents the genome-wide significance threshold ($P < 10^{-5}$), highlighting significant SNPs. Notable significant SNPs colocalized on chromosome 2 are highlighted with black dashed circles. The Q–Q plots on the right illustrate the observed versus expected – log10(P) distribution for each condition, confirming the reliability of the association analysis

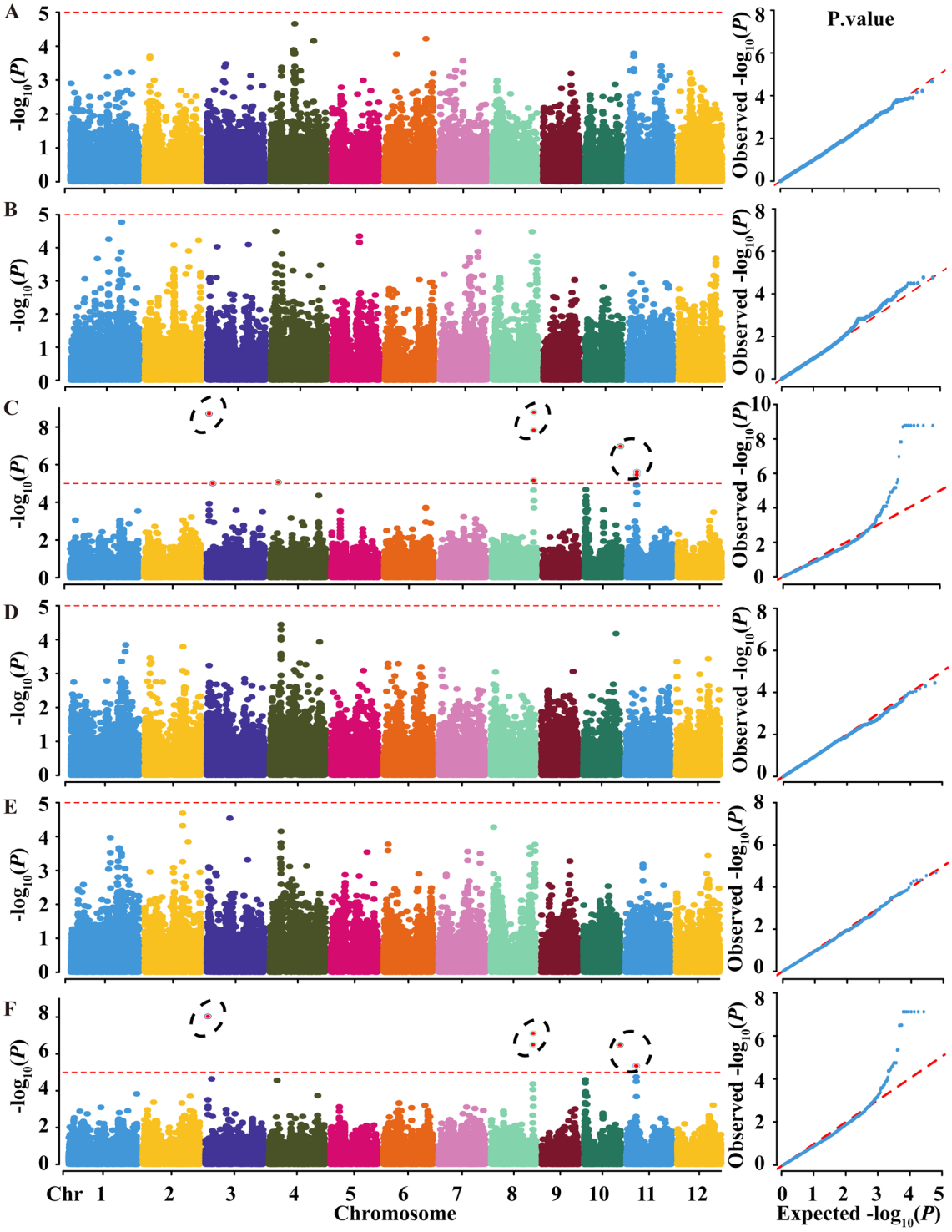

Fig. 3 Manhattan plots showing significant SNP associations for rice root length and relative root length under different salt stress conditions. (**A–C**) Root length under 100, 150, and 200 mM NaCl conditions on the seventh day. (**D–F**) Relative root length under 100, 150, and 200 mM NaCl conditions on the seventh day. The horizontal red dashed line represents the genome-wide significance threshold ($P < 10^{-5}$), highlighting significant SNPs. Notably, significant SNPs colocalized on chromosomes 3, 8, and 11 are highlighted with black dashed circles. The quantile–quantile plots on the right illustrate the observed versus expected – $\log_{10}(P)$ distribution for each condition, confirming the reliability of the association analysis

locus harbors the gene *OsSIZ1*, which enhances resistance to various abiotic stresses when expressed heterologously in rice [35].

For the loci *qRL8.1*, *qRL10.1*, and *qRL11.1*, no salt tolerance-related genes have been reported to date. However, *qRL8.1* stands out because of the presence of several closely clustered SNPs and a particularly high P value of 1.65474E-09, indicating its potential importance in salt tolerance. As a result, further investigation of *qRL8.1* is warranted to better understand its role in regulating salt tolerance.

Expression analysis of candidate genes in the *qRL8.1* locus

To identify the target genes controlling seed germination under salt stress at the *qRL8.1* locus, we analyzed all the candidate genes within the 247.612 kb region of *qRL8.1* via the Japonica rice reference genome (http://rice.plantbiology.msu.edu). A total of 32 candidate genes were id entified in this region, including 3 genes encoding transposons, 7 genes encoding expressed proteins, and 17 unreported genes. Additionally, 5 genes that have been cloned and reported in previous studies were also found, namely, *OsMCP* (*LOC_Os08g41830*), *OsNPP1* (*LOC_Os08g41880*), *qGW8* (*LOC_Os08g41940*), *OsMADS7* (*LOC_Os08g41950*), and *OsGSAT* (*LOC_Os08g41990*) (Table S2). These genes may play significant roles in regulating seed germination and salt tolerance in rice under salt stress.

We further explored the expression patterns of these candidate genes across various rice tissues via the Rice RNA Database (https://plantrnadb.com/ricerna/). Among the genes analyzed, *LOC_Os08g41790*, *LOC_Os08g41890*, *LOC_Os08g42030*, and *LOC_Os08g42080* presented the highest expression levels in the root tissue. Moreover, these genes were significantly induced by salt stress, distinguishing them from other genes within the *qRL8.1* region. Given these findings, we propose that these four genes are potential target genes for the *qRL8.1* locus and may play crucial roles in regulating root growth and salt tolerance (Fig. 4 and S3).

Haplotypic analysis of candidate genes at the *qRL8.1* locus

Building on previous analyses, we conducted haplotypic analysis of four key candidate genes—*LOC_Os08g41790*,

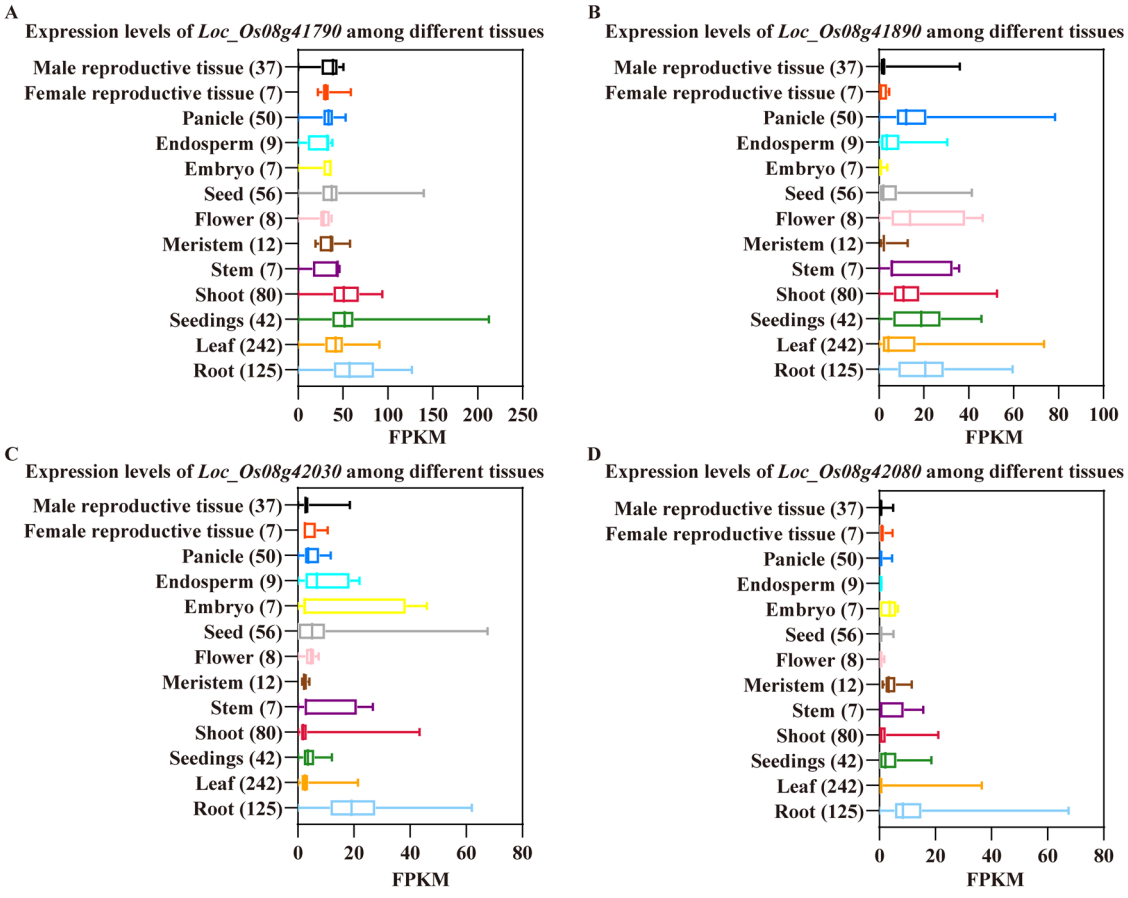

Fig. 4 Expression of genes within the *qRL8.1* locus in different rice tissues. The expression levels of genes within the *qRL8.1* locus are shown across various rice tissues, with sample sizes indicated in parentheses. Different colors represent distinct tissues. A-D correspond to the genes *LOC_Os08g41790*, *LOC_Os08g41890*, *LOC_Os08g42030*, and *LOC_Os08g42080*, respectively

LOC_Os08g41890, *LOC_Os08g42030*, and *LOC_Os08g42080*—to assess whether their haplotypes influence seed germination under salt stress. We analyzed chip data from the drought-resistant core population, which revealed that, with the exception of *LOC_Os08g42080*, the promoters of the other three genes contained SNPs that could affect their response to salt stress. Notably, a SNP in the coding region of LOC_Os08g41790 resulted in a nonsynonymous mutation, changing leucine (Leu) to serine (Ser) (Fig. 5A).

To explore the functional significance of these SNPs, we performed a detailed haplotypic analysis based on significant SNPs in the three candidate genes (Fig. 5B-G). For *LOC_Os08g41790*, the haplotype Hap3 presented longer root length and relative root length than did Hap1 (AC). The primary difference between Hap1 and Hap3 occurred at a SNP in the promoter region, suggesting that this SNP plays a regulatory role in gene expression. The promoter of *LOC_Os08g42030* contains a SNP, but this variation did not significantly impact the traits of the population, indicating that *LOC_Os08g42030* is unlikely to be the target gene for *qRL8.1*.

For *LOC_Os08g42080*, two SNPs in the promoter region led to the formation of two distinct haplotypes. Hap1 (AT) presented significantly greater root length and relative root length under salt stress than did Hap2 (CC). These findings support the hypothesis that *LOC_Os08g41790* and *LOC_Os08g42080* are potential target genes for the *qRL8.1* locus, contributing to the observed variation in salt tolerance across the population.

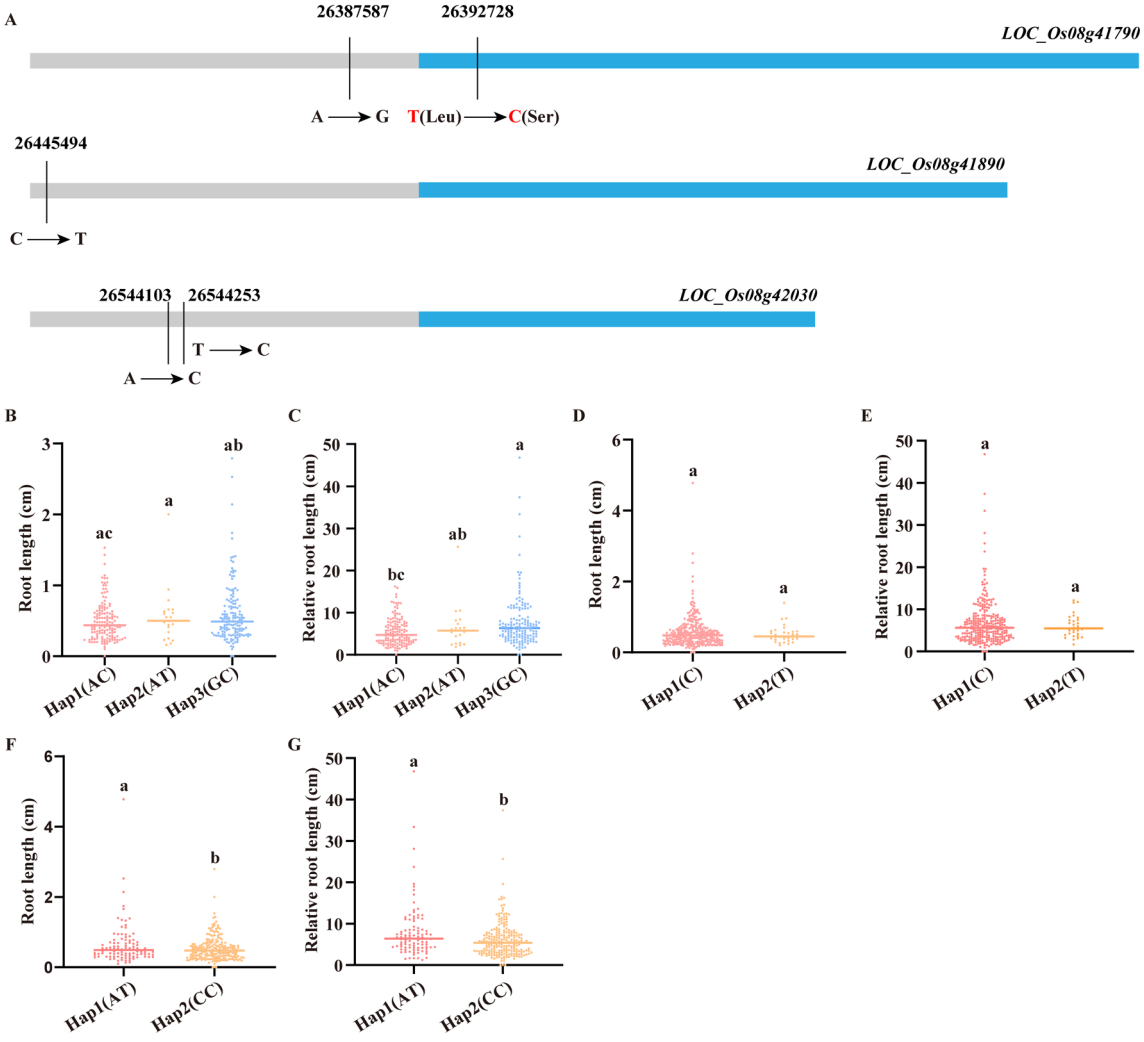

Fig. 5 Haplotypic analysis of candidate genes within the *qRL8.1* locus. **A**. Information on important SNPs within the candidate genes. The gray and blue bars represent the promoter regions and coding sequences (CDSs) of the candidate genes, respectively. Red text indicates mutated bases or amino acids. **B–G**. Root length and relative root length of different haplotypes of three candidate genes, *LOC_Os08g41790* (**B–C**), *LOC_Os08g41890* (**D–E**), and *LOC_Os08g42030* (**F–G**), under 200 mM NaCl stress. Different letters indicate significant differences at the 0.05 level

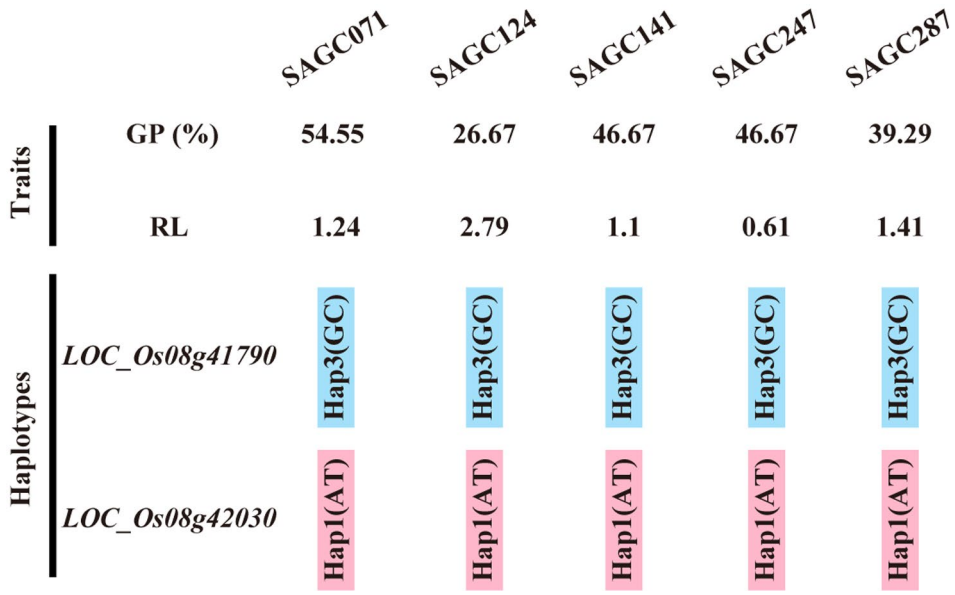

Fig. 6 Identification of salt-tolerant rice varieties with favorable haplotypes from *LOC_Os08g41790* and *LOC_Os08g42030* under 200 mM NaCl stress. The top 10 salt-tolerant rice varieties were identified on the basis of their germination potential and root length under 200 mM NaCl stress. Five of these varieties carry the beneficial haplotype Hap3 from *LOC_Os08g41790* and Hap1 (AT) from *LOC_Os08g42030*, demonstrating enhanced salt tolerance

Screening for salt-tolerant materials on the basis of the recombination of superior haplotypes from *LOC_Os08g41790* and *LOC_Os08g42030*

Using seed germination potential and root length as key phenotypic traits under 200 mM NaCl stress, we identified the top 10 most salt-tolerant varieties from a pool of 406 accessions. A total of 17 materials were selected, with 5 varieties carrying the favorable haplotype Hap3 from the *LOC_Os08g41790* gene and Hap1 (AT) from the *LOC_Os08g42080* gene (Fig. 6). These varieties exhibited excellent performance in terms of either germination potential or root length under salt stress, highlighting their potential as elite candidates for rice salt tolerance breeding. The presence of these beneficial haplotypes in the selected accessions indicates their promising role in enhancing salt tolerance in rice breeding programs.

These elite varieties, identified on the basis of their favorable haplotypes, represent valuable resources for the development of salt-tolerant rice cultivars. The use of these superior haplotypes in breeding programs could significantly improve salt tolerance, providing a foundation for more resilient rice varieties in regions affected by soil salinity.

Discussion

Critical role of *qRL8.1* in enhancing salt tolerance

The identification of *qRL8.1* as a significant locus associated with root length and relative root length under salt stress underscores its pivotal role in conferring salt tolerance. The candidate genes *LOC_Os08g41790* and *LOC_Os08g42080* within this locus presented high expression levels in root tissues and were significantly induced under salt stress conditions. *LOC_Os08g41790* encodes a phosphoinositol/uridine kinase family protein, which is implicated in signal transduction and membrane stability [36, 37]. These proteins are crucial for maintaining cellular integrity and facilitating adaptive responses to abiotic stresses.

LOC_Os08g42080, encoding a peroxidase precursor, likely plays a role in reactive oxygen species (ROS) scavenging, thereby mitigating oxidative damage induced by salt stress [37, 38]. The coordinated action of these genes suggests a mechanism in which increased signal transduction and ROS detoxification contribute to improved root growth and overall plant resilience under saline conditions. Previous studies have highlighted the importance of ROS management and ion homeostasis in salt tolerance, reinforcing the relevance of these candidate genes [18, 39].

Haplotype analysis and its implications for rice breeding

The haplotype analysis revealed that specific combinations of favorable haplotypes of *LOC_Os08g41790* (Hap3) and *LOC_Os08g42080* (Hap1 [AT]) significantly increased the germination potential and root length under 200 mM NaCl stress. This finding demonstrates the utility of haplotype-based selection in breeding programs aimed at improving salt tolerance. By leveraging these favorable haplotypes through marker-assisted selection (MAS) or genomic selection (GS), breeders can efficiently incorporate salt tolerance traits into elite rice

varieties, thereby accelerating the development of resilient cultivars [40].

Moreover, the identification of elite varieties carrying these beneficial haplotypes validates the practical applicability of the GWAS findings. These varieties can serve as valuable genetic resources for future breeding endeavors, ensuring that advantageous alleles are effectively utilized to increase salt tolerance in diverse genetic backgrounds.

Necessity for functional validation and future research directions

While GWAS and haplotype analyses revealed strong associations between *qRL8.1* and salt tolerance traits, functional validation of the candidate genes is imperative to elucidate their precise roles. Future studies should employ gene editing techniques such as CRISPR-Cas9 to create knockout and overexpression lines for *LOC_Os08g41790* and *LOC_Os08g42080*, thereby confirming their contributions to salt tolerance [41]. Additionally, transcriptomic and metabolomic analyses under salt stress conditions can offer deeper insights into the regulatory networks and metabolic pathways influenced by these genes.

Furthermore, investigating the interaction of *qRL8.1* with other identified loci (e.g., *qGP2.1* and *qGP2.2*) will enhance our understanding of the polygenic nature of salt tolerance. Such integrative approaches can reveal synergistic effects and epistatic interactions that contribute to the complex trait of salt tolerance [42].

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

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Author contributions

Conceptualization: L.L., and X.Y.; Writing—original draft preparation: P.Z.and G.L..; Investigation: P.Z.; Resources: D.K.; Data curation: P.Z.; Methodology: D.K. All authors have read and agreed to the published version of the manuscript.

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Data availability

All the data generated or analyzed during this study are included in this published article and provided within the manuscript or supplementary information files.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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