

Genome-wide association analysis for rice salt tolerance at seedling and reproductive stages

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Abstract

Background

Salinity is one of the main adverse environmental factors severely inhibiting rice growth and decreasing grain productivity. Developing rice varieties with salt tolerance (ST) is one of the most economical approaches to cope with salinity stress. However, the valuable resources for rice breeding towards ST remain to be identified. In this study, the salt tolerance of 220 rice accessions from RDP1, representing five subpopulations, were evaluated at both seedling and reproductive stages.

Results

An apparent inconsistency was found for ST between the two stages. Through a gene-based / tightly linked genome-wide association study, a total of 214 single nucleotide polymorphisms (SNPs) related to 251 genes, significantly associated with 16 ST-related indices, were detected at both stages. Eighty-two SNPs with low frequency favorable (LFF) alleles in the population were proposed to hold high breeding potential in improving rice ST. Fifty-four rice accessions collectively containing all these LFF alleles were identified as donors of these alleles. Through the integration of meta-QTL for ST and the response patterns of differential expression genes to salt stress, thirty-one candidate genes were suggested to be involved in the regulation of rice ST.

Conclusions

In total, the present study provides valuable information for further characterizing ST-related genes and for breeding ST varieties across whole developmental stages through marker-assisted selection (MAS).

Introduction

Rice (*Oryza Sativa* L.) is one of the most important food crops feeding more than half of the world's population. It is estimated that till 2050 the world's population will reach 9 billion, which poses a great challenge for crop productivity including rice production (Tyczewska et al., 2018). Improving rice tolerance to various biotic and abiotic stresses is an important strategy to increase rice yield. Salinity stress, as one of the main adverse environmental factors, inhibits plant growth and decreases crop productivity. Globally, more than 930 million hectares of land are reportedly affected by salinity (Hu et al., 2012). In addition, irrational use and management of water and fertilizer may speed up soil salinization, which has been found in some areas especially in rice growing areas (Yamaguchi et al., 2005). Known as a glycophyte, very few rice varieties could grow on highly salinized land, and rice in seedling and reproductive stages is generally most sensitive to salt stress (Khan et al., 2008; Ali et al., 2014; Emon et al., 2015; Rohila et al., 2019). Excess salt can interfere with metabolic processes at multiple levels, resulting in a reduction of the seed germination rate, interruption of the normal growth and the ultimate grain yield losses (Khan et al., 2008; Zeng and Shannon, 2000). Developing rice varieties with built-in salt tolerance (ST) is considered as the

most promising, less resource-consuming and socially acceptable approach to cope with salinity stress and to take full advantage of marginal lands.

Rice varieties vary drastically on ST-associated characteristics, which provides the basis to develop new varieties with improved ST and high yield. In general, rice ST is a comprehensive expression of a variety of physiological responses of rice under salt stress, typically known as a quantitative trait loci (QTLs) controlled by multiple genes (Khan et al., 2008; Ganie et al., 2019). In the past two decades, dozens of ST QTLs and/or genes and their linked markers have been identified, some of which have already been successfully incorporated into commercial cultivars (Emon et al., 2015; Hossain et al., 2015; Ganie et al., 2019). A classic example is Saltol, a QTL with major effects on ST mapped on chromosome 1 (Bonilla et al., 2002), in which *SKC1* was firstly cloned as the ST-related gene encoding Na⁺-selective transporter (Ren et al., 2005). *SKC1* plays an important role in maintaining Na⁺/K⁺ homeostasis thereby contributing profoundly to ST (Mohammadi-Nejad et al., 2008; Ganie et al., 2016). Due to the minor effects, however, most of ST QTLs were in fact neither isolated nor characterized, which limited their utilization in breeding programs. Through the reverse genetic method, around 200 ST responsive genes have been identified in rice seedling stage, while efforts are apparently lacking in evaluating their breeding potential (Ganie et al., 2019). Through marker assisted selection (MAS), the *SKC1* had been incorporated into some rice varieties with apparently enhanced ST in the seedling stage. It has been well recognized that ST at seedling and reproductive stages are different (Moradi et al., 2003; Liu et al., 2019; Lei et al., 2020; Chen et al., 2020); while, only few studies have reported ST QTLs/genes at the reproductive phase so far (Hossain et al., 2015; Liu et al., 2019; Lekklar et al., 2019; Ganie et al., 2019; Theerawitaya et al., 2020). Through the linkage mapping method, Hossain et al. (2015) reported 16 QTLs affecting 6 agronomic traits plus Na⁺ content and the K⁺/Na⁺ ratio in the flag leaf under stress conditions at the reproductive stage. In recent years, genome-wide association study (GWAS) has been developed in crops to enable large-scale and high-precision identification of elite alleles and their variants widely distributed in natural rice varieties (Zhao et al., 2011; Liu et al., 2019; Lekklar et al., 2019). With respect to salt stress, Kumar et al. (2015) firstly conducted GWAS with high density SNP (single nucleotide polymorphism) markers and identified 64 SNPs significantly associated with the K⁺/Na⁺ ratio and some agronomic traits under salt stress at rice reproductive stage. Lekklar et al. (2019) investigated photosynthetic and yield-related traits of 190 Thai and Asian rice accessions exposed to salt stress at the flowering stage, and identified 448 SNPs associated with ST by GWAS. By using 708 rice accessions and high-density SNPs within genes, Liu et al. (2019) detected 2255 SNPs that were significantly associated with ST-related traits at both seedling stage in greenhouse and reproductive stage at saline fields. In total, although a lot of ST related QTLs/genes have been reported, few of them, especially for those at reproductive stage, were characterized and successfully used in breeding (Li et al., 2020; Theerawitaya et al., 2020; Nayyeripasand et al., 2021).

Besides high-density SNPs generally used in GWAS, the other advantage of GWAS is that once a natural variety population is genotyped, it can be widely used in different studies towards diverse traits. For instance, to the best of our knowledge, the rice diversity panel I (RDP1), comprised of around 420 varieties from 82 countries/regions and genotyped by high-density SNP markers, has been widely employed in studying agronomic traits, biotic and abiotic stresses (Zhao et al., 2011; Famoso et al., 2011; Norton et al.,

2014; Kang et al., 2016; McCouch et al., 2016; Chen et al., 2019; Feng et al., 2019). In the present study, the ST performance of 220 rice accessions from RDP1, representing five subpopulations (tropic japonica/TRJ, temperate japonica/TEJ, indica/IND, aus /AUS, aromatic/ARO and admixture/ADMIX), has been evaluated at both seedling and reproductive stages. By using a gene-based / tightly linked GWAS analysis as reported in Liu et al. (2019), we identified 214 SNPs related to 251 genes that are significantly associated with 16 ST-related indices at both stages. Subsequently, fifty-four rice accessions were proposed as donors with most valuable alleles in breeding and 31 promising candidate ST genes were recommended. These results provide useful information for further characterizing ST-related genes and for breeding ST varieties through MAS.

Results

Phenotypic variations at seedling and reproductive stages

At the seedling stage, we found that most accessions began to wither and turn yellow on the 4th day after salt treatment and died on the 6th day after salt treatment, while a few accessions still showed acceptable growing status on the 6th days after salt treatment (Fig. 1A, Supplementary Figure S1). According to the phenotypic distribution, we found that sRV-Chl6 and sRV-DR6 presented an apparently skewed distribution, while the rest four ST-related indices displayed a more scattered distribution (Supplementary Figure S1), demonstrating a significant variance of ST among accessions tested at the seedling stage.

At the reproductive stage, ten ST-related indices were scored (Supplementary Table S1). Na⁺ content and Na⁺/K⁺ ratio, which are commonly important indices in evaluating crop ST, clearly showed scattered distributions in the population, demonstrating varying responses to salt stress among accessions (Supplementary Figure S2). Similar trends were observed in 6 indices related to chlorophyll contents and Fv/Fm. Some accessions were severely affected by salt treatment, showing no panicle emergence, while some others exhibited apparently reduced panicle lengths (Fig. 1A). Therefore, the panicle length was measured as one of agronomical indices. We found that compared with the control, only a few accessions produced similar panicle lengths salt treatment conditions, and most had significantly shortened panicles including some without panicles (Supplementary Figure S2). The other agronomic index is seed-setting ratio (rRSD-SR), which showed a clearly scattered distribution in the population. Together, these data indicate that the accessions in this study display significant differences in the ST at both seedling and reproductive development stages.

Correlation analysis showed that almost no significant correlations were found between ST indices from seedling stage and reproductive stage (Fig. 1B). This is consistent with previous studies, in which very few ST-related QTLs were reported conferring ST at both seedling and reproductive stages (Liu et al., 2019; Hossain et al., 2015). In addition, we also conducted a multi-comparison of each ST index among 5 subpopulations, and only observed three traits that showed significant differences among some subpopulations (Supplementary Figure S3). One index was sRV-FW that significantly differed between ADMIX and TRJ

subpopulations; and the other two were rRSD-SR and rRV-Fv/Fm7, which showed significant differences between AUS and TEJ subpopulations, and between AUS subpopulation and IND and TRJ subpopulations, respectively. This implies that the five subpopulations have almost no differentiation on ST.

In order to better portray the ST performance of the accessions, we conducted PCA for all measured ST-related traits. For the seedling stage, the PCA results showed that PC1 accounted for more than 45.2% of the total variance while PC2 was responsible for 18.8% (Fig. 1C). According to the length of each index line on PCA plot, the sRV-PH presented the strongest correlation with ST, followed by sRV-FW, sRV-Chl6, sRV-DR6, sRV-DR4 and sRV-Chl4. Negative correlations between photosynthesis and survival rates under stress have been well documented (Moradi and Ismail, 2007), consistent with our data that sRV-Chl4 and sRV-Chl6 were significantly negatively correlated with the sRV-DR4 and sRV-DR6, respectively, reflected by their opposite directions on the PCA plot. For the reproductive stage, the PCA results showed that PC1 and PC2 accounted for 56.5 % and 13.5% of the total variance, respectively (Fig. 1D). Comparatively, the two agronomic traits, rRSD-SR and rRSD-PL, directly associated with grain yield, showed strong correlations with ST, while other indices presented similarly moderate contributions to ST, according to the PCA analysis. The Na⁺ content and Na⁺/K⁺ ratio, commonly used for evaluating ST in seedlings (Zelm et al, 2020), did not appear to be strongly correlated with ST at the reproductive stage. Except for the correlation between rRSD-SR and rRV-Fv/Fm7, all other correlations among 10 indices reached a statistically significant level (Fig. 1B). Both Na⁺ content and Na⁺/K⁺ ratio showed apparently negative correlations with the rRV-Chl and rRV-Fv/Fm indices, while positive correlations were identified with the two agronomic traits, rRSD-PL and rRSD-SR, indicating that a higher content of Na⁺ leads to a more severe hurt to the plants (Fig. 1B). Together, the PCA data indicate that different indices have divergent contributions to rice ST at different stages.

Genome-wide association study of ST-related indices

To elucidate the genetic variance and identify the potential gene(s) with ST at both seedling and reproductive stages, we conducted a gene-based / tightly linked GWAS for 16 ST-related indices. A total of 201332 SNPs located within genes and their flanking regions were identified, which related to 27923 unique genes (Supplementary Table S2). The average interval distance of these SNPs on genome is 1892 bp and ranges from 1543 bp to 2225 bp on different chromosomes (Chrs), showing a relatively even distribution on rice genome (Supplementary Figure S4). By using two statistical analysis models, CMLM and BLINK, we obtained a total of 252 and 508 SNPs significantly associated with ST, respectively, which covered 15 ST-related indices except for sRV-DR4 on which no significant SNP was detected (Supplementary Table S3-18). Comparatively, a total of 224 SNPs were consistently detected by both methods (Supplementary Table S19). After removing 10 SNPs that were repeatedly detected in different ST indices, 214 SNPs in total were chosen for further analysis.

A total of 117 significant SNPs were detected at the seedling stage, which were distributed on different chromosomes and corresponded to 140 genes but varied among traits (Supplementary Table S19; Fig. 2). Specifically, 32 SNPs representing 39 genes for sRV-FW were identified on Chrs 1, 2, 4, 6, 7, 8 and 12, which accounted for 8.26%- 12.31% of phenotypic variance. For sRV-DR6, 30 SNPs from 37 genes were found on the genome except for Chr 11, with a notable preference for Chr 3 and Chr 9 each harboring 7 and 9 SNPs, respectively. For sRV-Chl6, 16 SNPs belonging to 21 genes were evenly located on Chrs 1, 2, 3, 4, 6, 7, 8, 10 and 12, while the associated SNPs for sRV-Chl4 were preferentially situated on Chr 3 (5 out of 24) and Chr 10 (4 out of 24), with phenotypic variance ranging from 8.65% to 11.52%. For sRV-PH, the 18 SNPs from 21 genes were discovered on Chrs 1, 2, 3, 4, 6, 8, 9, 11 and 12, accounting for 8.79%- 10.20% of phenotypic variance. Out of the above associations, three SNPs were linked with two traits: one of them (SNP-1.33074009) was detected in both sRV-FW and sRV-PH, while the other two SNPs (SNP-4.19825069 and SNP-8.3430353) were consistently found in sRV-DR6 and sRV-Chl6, possibly due to pleiotropic effects of the SNP-carrying genes.

At the reproductive stage, a total of 97 significant SNPs corresponding to 111 genes interspersed on the genome were detected with 17 for rRV-Fv/Fm7, 17 for rRV-Na/K, 15 for rRV-Na, 14 for rRSD-SR, 10 for rRV-Chl7, 8 for rRSD-PL, 7 for rRV-Fv/Fm14, 7 for rRV-Fv/Fm21, 5 for rRV-Chl14 and 5 for rRV-Chl21, respectively (Supplementary Table S19). The phenotypic variance of these significant associations ranged from 5.62%-16.90%. Around 25% (25 out of 97) of SNPs were detected on Chr 11, including 10 for rRV-Fv/Fm7, 8 for rRV-Na/K, 6 for rRV-Na, 2 for rRSD-SR, and 1 for each of rRV-Fv/Fm14, rRV-Chl7 and rRV-Chl14; on Chr12, only 3 associations were found, 2 for rRSD-SR and 1 for rRV-Chl14. The rest ST-related signals were distributed among the other Chrs except Chr 9, each containing 5-12 SNPs. While 7 SNPs were jointly associated with Na⁺ and Na⁺/K⁺, which are highly correlated ($r = 0.94$, Fig. 1B), none of the evaluated index-associated SNPs/genes at this stage overlapped with those detected at the seedling stage, further revealing the complexity and stage specificity of rice ST.

Pyramiding effects of low-frequency favorable SNP-carrying genes

To identify favorable alleles/haplotypes with potential effects on ST improvement, we conducted a haplotype analysis for each SNP locus (Supplementary Table S19). According to the result of *t* test between the two genotypes in each SNP locus, we found that 172 SNPs related to 201 genes exhibited significant differences on corresponding ST index value, and the frequency for the favorable alleles ranged from 2.80% to 97.18% in the population (Supplementary Table S19). In particular, we are interested in those favorable alleles less than 50% in the population and consider them as low-frequency favorable (LFF) alleles, because they are not utilized in most varieties and so have a relatively high breeding value in ST improvement for most varieties. Certainly, those high-frequency favorable (HFF) alleles (>50% in the population) are also helpful to improve some varieties without them. After screening, a total of 82 LFF alleles (refer to 96 genes) with the frequency ranging from 2.80%

to 50% were shortlisted, which included 57 and 25 (67 and 29 genes) at the seedling and the reproductive stages (Supplementary Table S20), respectively.

To assess whether these LFF alleles have pyramiding effects on ST improvement, we evaluated the relations between the ST index value and the number of LFF alleles. As shown in Supplementary Figure S5 and S6, an apparently linear relationship was observed between each of 16 ST index values and the number of LFF alleles, although the degrees of slopes varied among each other that reflect the magnitude of effects. These additive effects inform that the adaptability of rice to salinity can be strengthened by combining LFF alleles that dominate ST-related indices. Based on this consideration, we then calculated the number of stage-specific LFF alleles accumulated in each accession. At the seedling stage, we found that no varieties contained more than half of the total LFF alleles and the highest number of LFF alleles accumulated in a variety was 26 (Fig. 3A). Most varieties harbored less than 10 LFF alleles and around 46% (number of 102) varieties possessed no more than 4 LFF alleles. At the reproductive stage, one variety carried the highest number of 13 LFF alleles, and up to 63% germplasms (139) contained less than 4 LFF alleles (Fig. 3B).

Considering the fact that LFF alleles are distributed in different germplasms, we selected the accessions that possessed top 15% stage-specific LFF alleles as promising germplasms at each developmental stage. At the seedling stage, 30 accessions carrying LFF alleles with the numbers ranging from 14 to 26 were identified as potential ST breed donors (Fig. 3A), which covered all 57 LFF alleles (Supplementary Table S21). Among them, most accessions contained 14 to 18 LFF alleles and 7 accessions possessed more than 20 LFF alleles. At the reproductive stage, 25 accessions carrying at least 9 LFF alleles were identified as potential ST breed donors with 3 of them possessing more than 12 LFF alleles (Fig. 3B). Together, these 25 accessions covered all 25 reproductive specific LFF alleles (Supplementary Table S22). In order to better present the ST performance of these potential donors, we projected them on the PCA plot (Fig. 3C, D). According to the directions of each ST index on the PCA plot (Fig.1 C, D), we knew that the accessions located in the right part had better ST performance. At the seedling stage, we found that except for 3 accessions, all the rest 27 varieties were distributed in the right part of PCA plot (Fig. 3C). With respect to the reproductive stage, only one variety was located at the left part of the PCA plot and over 76% (19/25) of the varieties were distributed at the bottom right section of PCA plot, suggesting that these varieties have good ST performance at the reproductive stage (Fig. 3D). Normally, rice ST acquired at the seedling stage does not sustain through the reproductive stage (Singh and Flowers, 2010). Yet, we found an accession (NSFTV72 / IR8) that featured a longer life span in ST although it did not display the best ST at each of the two stages (Fig. 3C, D). This variety accumulated 14 seedling LFF and 11 reproductive LFF alleles (Fig. 3A, B), suggesting its potential value as a donor for simultaneously transferring multiple LFF alleles from both stages in future breeding programs.

Candidate genes associated with ST-related indices

Since all the SNPs applied in the above GWAS analysis are located in or very close to the annotated genes, we would like to mine some candidate genes for ST. Out of 214 significant SNPs, we found that 39 SNPs were located in the coding regions (Supplementary Table S23). These included 17 synonymous SNPs and 22 non-synonymous SNPs resulting in either amino acid substitutions or premature stop codons. The rest 175 SNPs were in non-coding regions, which can be further subdivided into three groups: 127 in intergenic regions, 5 in 5' UTR, 7 in 3' UTR and 36 in intron (Supplementary Table S23). Altogether, these 214 significant SNPs cover 251 genes, in which 222 are annotated and 29 encoded hypothetical proteins of unknown functions (Supplementary Table S19).

To prioritize GWAS-derived SNPs/genes, we conducted a co-localization analysis with ST-related QTLs published previously. Since most of these reported QTLs data came from independent studies that were not entirely consistent with each other, we performed a meta-analysis to identify consensus QTLs with high reliability. A sum of 375 ST-related QTLs from 18 independent studies were collected for meta-analysis, which included 308 in the seedling stage, 55 in the reproductive stage and 12 in vegetative and reproductive stages (Supplementary Table S24). In meta-analysis, QTLs in vegetative and reproductive stages were all considered as reproductive ST QTL. As a result, we obtained 41 seedling meta-QTL for ST (hereafter named as sMqST) and 17 reproductive meta-QTL for ST (rMqST), which were scattered on all 12 chromosomes (Supplementary Table S25, Fig. 4A). The interval length of these sMqST and rMqST ranged from 0.054 Mb to 4.734 Mb and from 0.139 Mb to 5.614 Mb, respectively (Supplementary Table S25). The average length of sMqST was 0.789 Mb and apparently less than 2.375 Mb of rMqST (Fig. 4B). Through comparison, we found a total of 45 SNPs located in these MqST intervals (Fig. 4A).

To screen candidate genes for ST, we consulted publicly available data on differentially expressed genes (DEGs) of rice under salt stress at diverse growth stages, which offered a list of 131 DEGs that related to 116 out of 214 SNPs (Supplementary Table S26). To the best of our knowledge, only one of them, *OsPP1* (LOC_Os03g16110), encoding a protein phosphatase, has been reported as an ST regulation gene (Liao et al., 2016). Rice plants over-expressing *OsPP1a* can recover from salinity-induced oxidative damage via boosting antioxidant enzyme systems to maintain a relative redox homeostasis. SNP-3.8889557, associated with rRSD-SR on chromosome 3, was found to be located in the 3' UTR of *OsPP1*, which accounted for 14.48% of the total variation of rRSD-SR (Supplementary Table S23). Twelve accessions out of 208 (5.77%) carried the 'A' allele at this position with an average rRSD-SR of 56%, while the accessions possessing the alternative 'G' allele were more sensitive to salinity treatment, with an average rRSD-SR of 84% (Fig. 4C). In comparison, the favorable 'A' allele of *OsPP1* was able to rescue 28% losses on seed setting rate due to salt stress during the reproductive stage. This implies that mining DEG data could be a feasible strategy to screen candidate ST genes.

By comparison among 45 SNPs located within MqST intervals, we identified 31 DEGs related to 26 SNPs (Table 1). The chromosome 3 had most co-located DEGs with MqST, and no co-located DEGs and MqST were found on chromosome 5, 6 and 11 (Fig. 4A). According to the gene annotation, we found that except for 3 genes belonging to hypothetical protein or predicted protein, most of the rest 28 DEGs presented potential function in various kinds of stress resistance, such as *OsPRI1*, *OsSPL2*, *OsULT1*, *OsERF60*, and so on (Table 1). It is worth further validating the function of these candidates in ST. In addition, fifteen of these candidates were from LFF alleles, whose contributions on ST improvement required testing in practice (Supplementary Figure S7). One of the candidate genes at each stage was chosen for further analysis below.

Table 1. Details of candidate genes for salt tolerance and its co-located SNPs and MqSTs

MSU.ID	Chr	SNP	Trait	MqST	GSE.ID	LFF	Description
LOC_Os01g57240	1	SNP-1.33074009.	sRV-PH	rMqST1-1	GSE21651	yes	OsULT1, SAND domain-containing protein, Trithorax group factor, Transcriptional regulation of stress responsive genes
LOC_Os01g69830	1	SNP-1.40330641.	sRV-DR6	rMqST1-4	GSE21651	yes	OsSPL2, Squamosa promoter-binding-like protein 2; Similar to SBP-domain protein 4
LOC_Os03g22740	3	SNP-3.13130403.	sRV-DR6	rMqST3-1	GSE21651	yes	BIP102, Brassinosteroid receptor kinase-interacting protein 102; Similar to SAR DNA-binding protein-like protein
LOC_Os03g46610	3	SNP-3.26377803.	rRSD-SR	rMqST3-2	GSE21651	yes	TOGR1, DDX47, OsRH10, Thermotolerant growth required 1, ATP-dependent RNA helicase DDX47, RNA helicase 10
LOC_Os04g52550	4	SNP-4.31063518.	sRV-DR6	rMqST4-2	GSE21651	yes	OsAGO3, Protein argonaute 3-like
LOC_Os04g52560	4	SNP-4.31063518.	sRV-DR6	rMqST4-2	GSE21651	yes	FAR1 domain containing protein
LOC_Os07g14514	7	SNP-7.8262486.	sRV-FW	rMqST7-3	GSE21651	yes	Similar to OSIGBa0140C02.4 protein
LOC_Os07g15270	7	SNP-7.8805943.	sRV-FW	rMqST7-3	GSE16108,GSE21651	yes	RNA recognition motif, RNP-1 domain containing protein
LOC_Os08g04390	8	SNP-8.2146875.	sRV-FW	sMqST8-1	GSE21651	yes	OsPRI1, bHLH transcription factor, Positive regulator of iron homeostasis 1
LOC_Os08g06230	8	SNP-8.3430353.	sRV-Chl6,sRV-DR6	rMqST8-1	GSE21651,GSE6901	yes	GTP1/OBG domain containing protein
LOC_Os09g32620	9	SNP-9.19466296ct.	sRV-DR6	sMqST9-4	GSE16108,GSE21651	yes	Alcohol dehydrogenase superfamily, zinc-containing protein
LOC_Os09g32640	9	SNP-9.19466296ct.	sRV-DR6	sMqST9-4	GSE21651	yes	Similar to Quinone-oxidoreductase QR1 (Fragment)
LOC_Os10g33620	10	SNP-10.17653231.	rRSD-SR	sMqST10-2	GSE21651	yes	Ubiquitin domain containing protein
LOC_Os10g33630	10	SNP-10.17653231.	rRSD-SR	sMqST10-2	GSE21651	yes	OsABCI16, Adaptin ear-binding coat-associated protein 1 NECAP-1 family protein
LOC_Os12g06020	12	SNP-12.2783280.	sRV-FW	sMqST12-1	GSE21651	yes	Dcp1-like decapping family protein
LOC_Os01g56790	1	SNP-1.32769262.	sRV-PH	rMqST1-1	GSE21651	no	Conserved hypothetical protein
LOC_Os01g57260	1	SNP-1.33085898.	sRV-PH	rMqST1-1	GSE21651	no	Vacuolar protein sorting-associated, VPS28 family protein
LOC_Os02g45660	2	SNP-2.27769525.	sRV-PH	rMqST2-2	GSE21651	no	OsIspF, 2-C-methyl-d-erythritol 2,4-cyclodiphosphate synthase

LOC_Os03g08360	3	SNP-3.4267308.	sRV-DR6	sMqST3-1	GSE21651	no	OsON11, Fatty acid elongase (beta-ketoacyl-CoA synthase), Shoot development
LOC_Os03g08460	3	SNP-3.4343151.	sRV-DR6	sMqST3-1	GSE21651	no	OsERF60, AP2/EREBP27, OsEBP89; APETALA2/ethylene responsive factor, ERF transcription factor, Tolerance to drought and submergence stress
LOC_Os03g21950	3	SNP-3.12546468.	sRV-PH	rMqST3-1	GSE21651	no	Predicted protein
LOC_Os03g22730	3	SNP-3.13123923.	rRV-Fv/Fm7	rMqST3-1	GSE21651,GSE6901	no	BIP101, brassinosteroid receptor kinase (BR1)-interacting protein 101
LOC_Os03g44660	3	SNP-3.25139625.	rRV-Ch17	rMqST3-2	GSE21651,GSE3053	no	OsGRL7, GRX-like protein 7, glutaredoxin-like protein 7; Thioredoxin fold domain containing protein
LOC_Os03g48020	3	SNP-3.27296133.	rRV-Fv/Fm7	rMqST3-2	GSE21651	no	Conserved hypothetical protein
LOC_Os03g48030	3	SNP-3.27296133.	rRV-Fv/Fm7	rMqST3-2	GSE21651,GSE6901	no	HPP family protein
LOC_Os04g45910	4	SNP-4.27009828.	sRV-Ch16	rMqST4-2	GSE21651	no	Similar to endonuclease, polyU-specific
LOC_Os04g45920	4	SNP-4.27009828.	sRV-Ch16	rMqST4-2	GSE21651	no	OsRLCK155, Receptor-like Cytoplasmic Kinase 155
LOC_Os07g02760	7	SNP-7.1005736.	rRV-Fv/Fm14	rMqST7-1	GSE21651	no	OsFbox330, F-box domain, Skp2-like domain containing protein
LOC_Os08g06380	8	SNP-8.3544780.	sRV-DR6	rMqST8-1	GSE21651,GSE4438	no	OsCslF6, MLG (mixed-linkage glucan) synthase, Biosynthesis of MLG (cell wall polysaccharide); Similar to Cellulose synthase-like CslF6
LOC_Os09g29584	9	SNP-9.17990633.	sRV-DR6	sMqST9-4	GSE13735,GSE21651	no	OsWAK84, EGF-like calcium-binding domain containing protein
LOC_Os09g34100	9	SNP-9.20121667.	sRV-DR6	sMqST9-4	GSE21651	no	OsGLYII3, glyoxalase II-3, glyoxalase II

LOC_Os08g04390, known as *OsPRI1*, was associated with SNP-8.2146875 in the interval of sMqST8-1. In rice, *OsPRI1* is a linker between the iron-binding sensor *OsHRZ1* and the Fe-deficiency-responsive gene *OsIRO2* (Zhang et al., 2017). Under Fe deficiency, this HLH transcription factor positively regulates *OsIRO2* expression via directly binding to its promoter. Through global bioinformatics analysis, the HLH transcription factor was found to participate in salt responsive gene regulatory networks (Wang et al., 2020). Overexpression

of *OsIRO2* results in an elevation of Fe uptake and translocation in rice (Ogo et al., 2007). Being an essential mineral element for plant growth and development, Fe has also been proposed as a defensor against salinity since it can boost the production of antioxidative enzymes to mitigate salinity-induced oxidative damage (Li et al., 2016; Sharma et al., 2012; Ghasemia et al., 2014). In addition, salt stress would impose deleterious effects on Fe acquisition and distribution in plants (Li et al., 2016). Consequently, efficient absorption of Fe with the help of *OsIRO2* and its upstream regulator *OsPRI1* would have the potential to confer greater tolerance to saline-alkaline stress in rice. The accessions containing 'A' allele in the regulatory of *OsPRI1* displayed a lower sRV-FW of 52%, while only 13 accessions carried the 'G' allele that showed a better sRV-FW value (Supplementary Figure S7). This demonstrated that introducing 'G' allele of *OsPRI1* from the 13 accessions could further improve rice ST.

Another candidate gene is *OsTOGR1* (LOC_Os03g46610), encoding a DEAD-box RNA helicase co-located with rMqST3-2. At higher temperature, the intrinsic helicase activity of *OsTOGR1* increases, thereby promoting stabilization of pre-rRNA homeostasis (Wang et al., 2016). Although it is uncertain whether *OsTOGR1* directly participates in ST through regulation of RNA surveillance, its two family members, *OsPDH45* and *OsSUV3*, have been demonstrated to function in ST (Amin et al., 2012; Macovei and Tuteja, 2012; Tuteja et al., 2013). In particular, overexpression of *PDH45* confers salt tolerance to rice at both seedling and reproductive stages. SNP-3.26377803, located in the CDS region of *OsTOGR1*, contains two kinds of variations 'G' and 'T'. Thirteen accessions containing the favorable 'T' allele significantly reduced the rRSD-SR of around 25% (Supplementary Figure S7), implying its breeding potential in ST breeding program.

Discussion

Identification of genetic resources for rice breeding towards salt tolerance

Rice is relatively tolerant to salt during germination, active tillering, and maturity but is very sensitive at the early seedling stage and reproductive stage (Singh and Flowers, 2010; Ganie et al., 2019). To mitigate the adverse effects of salinity on rice, a prospective practice is to breed varieties with in-built salt tolerance at both sensitive stages and develop new rice varieties with enhanced resilience to salt stress environments. In general, ST at these two sensitive stages is not correlated to each other. Nor is there a statistically significant correlation between the ST-related indices measured at the seedling stage and the grain yield at the reproductive stage under salt stress (Moradi et al., 2003). Therefore, a comprehensive assessment of ST in rice must be based on determination of certain ST-related indices through the whole growth period, and combination of multiple ST QTLs/genes is suggested in the breeding practice. Considerable efforts have been devoted to the identification of ST QTLs/genes at each sensitive stage independently, such as the characterization of the Salto at the seedling stage (Thomson et al., 2010; Zeng et al., 2002; Mohammadi et al., 2013; Bimpong et al., 2014; Hossain et al., 2015). However, only a few attempts have been made to discover loci associated with ST across developmental stages and especially at the reproductive stage (Ganie et al., 2019; Haque et al., 2020).

In this study, we evaluated the ST of 220 rice accessions representing five subpopulations at both the seedling and the reproductive stages by assessing a combination of morphological and physiological parameters. Our results indicate that the degree of ST varies among this rice panel at both stages, while no correlation was found between ST-related indices measured at the seedling stage and the reproduce stage (Fig. 1B). Also, only few ST indices showed significant differences among subpopulations (Supplementary Figure S3). These allow us to infer that rice ST does not experience an artificial or natural selection, although apparent difference on ST related indices can be observed among varieties. This could account for the fact that not a single dominating index could directly regulate rice ST especially among various development stages. As a result, we employed PCA to determine the contributions of different indices on ST, and found that the two morphological indices (rRSD-PL and rRSD-SR) displayed apparently stronger correlations with ST than those of the other 8 indices, including Na^+ and Na^+/K^+ ratio at the reproductive stage. The rRSD-SR has been universally employed for evaluating rice ST in previous studies, while the rRSD-PL was seldom used. In future studies, the rRSD-PL could be emphasized in both ST-related genetic and breeding researches at the reproductive stage. To readily pinpoint the genes responsible for ST, a GWAS analysis was undertaken using 201k within-gene SNPs (Supplementary Table S2). A total of 117 and 97 SNPs were identified to be significantly associated with different ST-related indices at the seedling and reproductive stages (Fig. 2), respectively. However, we did not detect any overlapping SNPs at both stages, highlighting the independence of ST between these two stages and the genetic complexity.

The ultimate goal of these significant SNPs discovery is to deploy them in the breeding program to develop new salt-tolerant rice varieties or germplasm adaptable to salinity across the whole growth stages. A prospective practice is to combine diverse ST favorable alleles at both sensitive stages of rice. Comparatively, LFF alleles were on the priority list in view of their great potential in genetic improvement. Among the 214 SNPs in total, we identified 57 and 25 LFF alleles at the seedling and the reproductive stages (Supplementary Table S20), respectively. Importantly, our preliminary results indeed show that rice accessions accumulating more LFF SNPs typically display relatively superior ST to those comprising fewer LFF SNPs (Supplementary Figure S5 and S7). This means that increasing the frequency of these LFF alleles in rice population could be a feasible approach to further improve rice ST. In order to accelerate the utilization of these LFF alleles, 30 and 25 potential donors were selected at seedling and reproductive stages, respectively, which collectively covered all 82 LFF alleles and displayed a good performance on ST at both stages (Fig. 3). We also noted some deviations where accessions carrying LFF alleles similar to those of other salt-tolerant lines exhibit phenotypic salt-sensitivity. This is probably due to the complex interactions among different genes or between genes and genetic backgrounds, which results in various phenotypes although these accessions harbored similar LFF alleles. Undoubtedly, pyramiding these LFF alleles using the potential donors discovered in this study with previously identified ST loci/genes will facilitate the development of highly tolerant varieties against salinity. In addition, these identified LFF SNPs could also be converted into breeder-friendly and cost-effective markers, like Kompetitive Allele Specific Polymerase Chain Reaction (KASP) marker, for future molecular breeding.

Candidate genes involved in regulation of rice salt tolerance

The majority of the 214 SNP-carrying genes have not been studied in the context of ST. By integration of DEG data and Meta-QTL results, we obtained 31 candidate genes that could play a crucial role in tolerance against salinity (Table 1). Besides the two candidates (*OsPRI1* and *OsTOGR1*) described in the previous section, at least 4 more genes have already been reported with diverse biological functions although their contributions to ST have not been elucidated so far. Based on the prior literature, the current understanding of these 4 genes is discussed next with respect to their potential roles in ST.

LOC_Os09g34100 (SNP-9.20121667.), known as *OsGLYII3*, associated with sRV-DR6 at the seedling stage encodes a Glyoxalase II involved in the glyoxalase pathway (Yadav et al., 2007; Mustafiz et al., 2011) which is required for glutathione-based detoxification of methylglyoxal. Earlier studies have already revealed the potential role of this pathway in conferring ST of tobacco (Singla-Pareek et al., 2003; 2006): overexpression of *glyI* from *Brassica juncea* in tobacco imparts tolerance to the transgenic plants during salt stress (Veena et al., 1999); overexpression of *glyI* and *glyII* together can enhance the ST of transgenic plants to a higher extent (Singla-Pareek et al., 2003; 2006). In rice, *OsGLYII3*, together with *OsGLYI1*, were identified as salt responsive gene (Veena et al., 1999; Yadav et al., 2007), with increased transcription levels upon salt stress. Leaf segments from *OsGLYII3*-overexpressing lines could retain a larger amount of chlorophyll in the presence of salt as compared to the control (Singla-Pareek et al., 2008). Consequently, *OsGLYII3* is prominent causative gene for ST.

LOC_Os02g45660 (SNP-2.27769525.), known as *OsispF*, is associated with sRV-PH at the seedling stage and encodes a 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase, which is the fifth enzyme in the MEP pathway (Huang et al., 2018). Located in chloroplast, *OsispF* plays a pivotal role in chloroplast development. Rice plants carrying mutated forms of *OsispF* display yellow-green leaf phenotype throughout development, with significantly reduced contents of Chlorophyll and carotenoid. Relationships between photosynthesis, chloroplast and survival rates of rice under salinity have been well documented (Moradi and Ismail, 2007; Nan et al., 2020); therefore, *OsispF* could be a prominent gene for ST.

LOC_Os09g29584 (SNP-9.17990633), also named *OsWAK84*, is associated with sRV-DR6 at the seedling stage and encodes a wall-associated kinase. Wall-associated kinases in rice have been proposed to be involved in plant immunity (Li et al., 2009; Delteil et al., 2016; Harkenrider et al., 2016; Hu et al., 2017) and response to mineral toxicities (Xia et al., 2018). In contrast, there has not been a single report that reveals their relationships with rice ST. However, it was reported that an *Arabidopsis* WAK-like gene 4 (*AtWAKL4*) responded notably to salt stress, with a 5-fold increase in *AtWAKL4* transcripts after salt treatment of the seedlings, while destruction of its promoter via T-DNA insertion extenuated the adaptability of *Arabidopsis* to salt stress (Hou et al., 2005).

LOC_Os03g44660 (SNP-3.25139625), known as *OsGRL7*, is associated with rRV-Chl7 at the reproductive stage and encodes a Glutaredoxins (GRXs) family protein of GRL-type (Garg et al., 2010). Though the precise functions of GRL-type proteins in ST have not been investigated, overexpression of a CPYC-type GRX-OsGRX20 was shown to result in enhanced tolerance to salt stress (Ning et al., 2018). It was proposed that the capacity of GRXs to reduce oxidized disulfides is a decisive factor to maintain redox homeostasis under high salinity (Rouhier et al., 2005; Ning et al., 2018).

The other candidate ST genes jointly supported by DEG data and Meta-QTL results have been hardly studied in the previous literature. In summary, our findings are anticipated to add potential novel gene resource to the list of ST contributing factors. Further functional tests will be needed to validate their roles in ST.

Materials And Methods

Plant materials

A total of 220 rice accessions including 5 subpopulations from RDP1 were selected, and comprised 53 TRJ, 57 TEJ, 43 IND, 32 AUS, 2 ARO and 33 ADMIX accessions according to that reported in McCouch et al. (2016) (Supplementary Table S1). Twenty germinated seeds of each variety were placed in a 96-well PCR plate cut at the bottom and put into a container for hydroponic culture in greenhouse. At two-leaf stage, the seedlings were transferred to the IRRI nutrient solution with pH 5.5 (Xia et al., 2015). The solution was changed every three days till four-leaf stage, and then the 0.8% NaCl (136.8 mmol/L) was used for salt treatment. The nutrient solution without NaCl was used as the control.

The same rice germplasm as the seedling stage was soaked and seeded in a 32-well seedling tray. At four-leaf stage, the seedlings were transplanted into 15 L plastic buckets with soil in greenhouse. Each plastic bucket contained 5 plants, and at jointing stage 4 plants with the same growth status were retained. Then at booting stage, each barrel was treated with 0.5% NaCl (85.5 mmol/L). Each variety treated by clean water was used as the control.

Measurement of ST related traits or indices at seedling and reproductive stages

At the seedling stage, on the 2nd, 4th and 6th day after salt treatment, the second leaf from the top of the seedling was selected and the chlorophyll content of the leaf was measured by SPAD 502 chlorophyll meter. At the reproductive stage, the chlorophyll content of rice flag leaf was determined on the 0, 7th, 14th and 21st days of salt treatment. On the 6th day after salt treatment at the seedling stage, the plant height, root length and fresh weight were measured. On the 4th and 6th days after salt treatment, the number of dead seedlings under salt stress was counted, and then the death rate was calculated. 5 to 8 seedlings with the same growth status were selected as a replicate, each treatment along with the control was repeated 3 times.

At the reproductive stage, the flag leaves of rice were selected on the 7th, 14th and 21st day after salt treatment to determine the chlorophyll fluorescence parameters, respectively. After dark adaptation to 30 min, the initial fluorescence (F_o) was measured with weak measuring light, and then the maximum fluorescence (F_m) was measured with a strong flash ($5000 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, pulse time 0.7 s). The variable fluorescence (F_v) was calculated as $F_v = F_m - F_o$, as well as the maximum photochemical efficiency of photosystem II (PSII) (F_v/F_m), and the potential photochemical efficiency of PS II (F_v/F_o). After 14 days of salt treatment, the third leaf from the top of each plant was sampled and dried. The dried sample was crushed by a grinder, and 0.1 g of sample was placed in a test tube with a lid, then 10 mL of 100 mM acetic

acid was added and bathed at 90 °C for 2 h. After cooling and 5000 rpm centrifugation for 15 min, the supernatant was transferred to the 10 mL centrifuge tube. After proper dilution, the contents of Na and K were determined by atomic absorption spectrophotometer (Pin AAcle 900F, Perkin Elemer Co., Ltd). The panicle length and the number of grains per panicle of each variety were measured at the end of grain filling, and the seed setting rate was calculated. Four plants per bucket were set as a replicate. Each treatment along with the control was repeated 3 times.

According to the method of Qi et al. (2005), the ST grade of rice at seedling stage and reproductive development stages were individually classified. The relative salt damage rate (RSD) was calculated as follows: $RSD (\%) = (\text{control} - \text{salt treatment}) / \text{control} \times 100$. Additionally, the relative value (RV) of ST was calculated as follows: $RV (\%) = \text{salt stress value} / \text{control value} \times 100$. Accordingly, six ST related indices at the seedling stage were abbreviated as: sRV-PH (the RV of plant height), sRV-FW (the RV of fresh weight), sRV-DR4 (the RV of death rate on the 4th day), sRV-DR6 (the RV of death rate on the 6th day), sRV-Chl4 (the RV of chlorophyll content on the 4th day), sRV-Chl6 (the RV of chlorophyll content on the 6th day). Ten ST-related indices at the reproductive stage were abbreviated as: rRV-Na (the RV of Na⁺ content), rRV-Na/K (the RV of Na⁺/K⁺ content), rRSD-SR (the RSD of seed-setting rate), rRSD-PL (the RSD of panicle length), rRV-Chl7 (the RV of chlorophyll content on the 7th day), rRV-Chl14 (the RV of chlorophyll content on the 14th day), rRV-Chl21 (the RV of chlorophyll content on the 21st day), rRV-Fv/Fm7 (the RV of chlorophyll fluorescence parameters (Fv/Fm) on the 7th day), rRV-Fv/Fm14 (the RV of chlorophyll fluorescence parameters (Fv/Fm) on the 14th day), rRV-Fv/Fm21 (the RV of chlorophyll fluorescence parameters (Fv/Fm) on the 21st day).

Excel 2016 and R 3.6.3 were used for data processing and correlation analysis.

GWAS for rice ST indices

The publicly available 700K SNP dataset of RDP1 varieties were downloaded (<http://www.ricediversity.org/data/>) for subsequently GWAS analysis (McCouch et al, 2016). SNP filtering was carried out according to the following steps: 1) SNPs with minor allele frequency (MAF) $\geq 2\%$ and missing rate $< 25\%$ were selected; 2) based on gene functional annotations of the 'Nipponbare' genome IRGSP-1.0.46.chr.gff3 from the Rice Genome Annotation Project (<http://rice.plantbiology.msu.edu/>) and The Rice Annotation Project (<https://rapdb.dna.affrc.go.jp>), SNPs within annotated genes from 2kb upstream to 2kb downstream were extracted through SnpEff. (Cingolani et al, 2012). GWAS was performed with the software "GAPIT" and two kinds of statistical models, CMLM (Compressed Mixed Linear Model) and BLINK (Bayesian-information and Linkage-disequilibrium Iteratively Nested Keyway) were used (Zhang et al, 2010; Huang et al, 2019). A minimum Bayes factor (mBF) was applied to identify significant markers, based on the P value threshold for significance. The P (mBF) was calculated using the following formula: $mBF = -e^{P \cdot \ln(P)}$ (Goodman, 2001). Thus, the significance threshold in this study was $-\log_{10}(P) = 3.78$. Manhattan and Q-Q plots were generated with the "CMplot" package in R environment (Yin et al, 2020).

Principal component analysis

Principal component analysis (PCA) was performed using the ST-related indices with the R package “FactoMineR” and visualized with “factoextra” (<https://CRAN.R-project.org/package=FactoMineR>, <https://CRAN.R-project.org/package=factoextra>).

Differential gene expression analysis

A total of 25 rice salt-tolerant transcriptome datasets from 7 independent studies were collected from GEO database (<https://www.ncbi.nlm.nih.gov/geo/>). R package “limma” was used to mine differential expression genes (Ritchie et al, 2015). Transcripts or genes with P-values < 0.05 and absolute value of log₂ (fold change) > 1 were assigned as differentially expressed genes (DEGs).

Meta-analysis of rice ST QTLs

Information of ST QTLs at seedling and reproductive stages were collected from 18 references published from 2004 to 2020, which included QTL confidence interval, linkage molecular markers, LOD value, phenotypic variation rate (PVE%), and mapping parents (Supplementary Table S24). According to the positions of the molecular markers linked with these ST-responsive QTLs on rice reference genome of Nipponbare, an integrated physical map for all ST responsive QTLs and their linked molecular markers was obtained. Then, based on the ratio of 1 cM per 244 kb (Chen et al., 2002), this integrated physical map was further converted to a virtual genetic map, namely a consistent genetic map, which included 616 and 134 markers linked with ST QTLs at seedling and reproductive stages, respectively. This conversion is based on the assumption that the ratio of genetic to physical distance is invariant throughout the genome except that in the centromeric regions, which has been used in previous studies for meta-analysis of QTLs related to several traits (Courtois et al., 2009). According to the information of all ST responsive QTLs integrated in this consistent genetic map, the Meta-QTL analysis was performed by using the software BioMercator V4.2 (Sosnoski et al., 2012; Veyrieras et al., 2007). The method relies on a clustering algorithm based on a Gaussian mixture model and enables the determination of the probable number of clusters considered as the “true” QTLs underlying the QTLs observed in a given region. The optimal number of clusters was chosen by means of an information-based criterion, and the position and confidence interval of the meta quantitative ST loci (MqST) was then estimated.

Abbreviations

ST

salt tolerance; SNP:single nucleotide polymorphism; QTL:quantitative trait loci; MAS:marker-assisted selection; GWAS:genome-wide association study; RDP1:rice diversity panel I; TRJ:tropic japonica;

TEJ:temperate japonica; IND:indica; AUS:aus; ARO:aromatic; ADMIX:admixture; Fv/Fm:maximum photochemical efficiency of photosystem II (PSII); Fv/Fo:potential photochemical efficiency of PS II; RSD:relative salt damage rate; RV:relative value; sRV-PH:the RV of plant height; sRV-FW:the RV of fresh weight; sRV-DR4:the RV of death rate on the 4th day; sRV-DR6:the RV of death rate on the 6th day; sRV-Chl4:the RV of chlorophyll content on the 4th day; sRV-Chl6:the RV of chlorophyll content on the 6th day; rRV-Na:the RV of Na⁺ content; rRV-Na/K:the RV of Na⁺/K⁺ content; rRSD-SR:the RSD of seed-setting rate; rRSD-PL:the RSD of panicle length; rRV-Chl7:the RV of chlorophyll content on the 7th day; rRV-Chl14:the RV of chlorophyll content on the 14th day; rRV-Chl21:the RV of chlorophyll content on the 21st day; rRV-Fv/Fm7:the RV of chlorophyll fluorescence parameters (Fv/Fm) on the 7th day; rRV-Fv/Fm14:the RV of chlorophyll fluorescence parameters (Fv/Fm) on the 14th day; rRV-Fv/Fm21:the RV of chlorophyll fluorescence parameters (Fv/Fm) on the 21st day; PCA:principal component analysis; DEG:differentially expressed gene; MqST:meta quantitative ST loci; Chrs:chromosomes; CMLM:Compressed Mixed Linear Model; BLINK:Bayesian-information and Linkage-disequilibrium Iteratively Nested Keyway; LFF:low frequency favorable; HFF:high-frequency favorable; KASP:Kompetitive Allele Specific Polymerase Chain Reaction

Declarations

Ethics Approval and Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

Availability of Data and Materials

All relevant data in this study are included in the article and its supplementary files.

Competing Interests

The authors declare that no competing interests exist.

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Authors' contributions

SZ designed and supervised the works. GC, KH and JZ performed most of the experiments and analyzed the experimental data. FG, WS, QJ, JZ, ZG, ZF, ZC and XW conducted a part of experiments and analyzed part of the data. GC, KH and JZ wrote the manuscript and SZ critically commented and revised it. All authors read and approved the final manuscript.

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Figures

Fig. 1

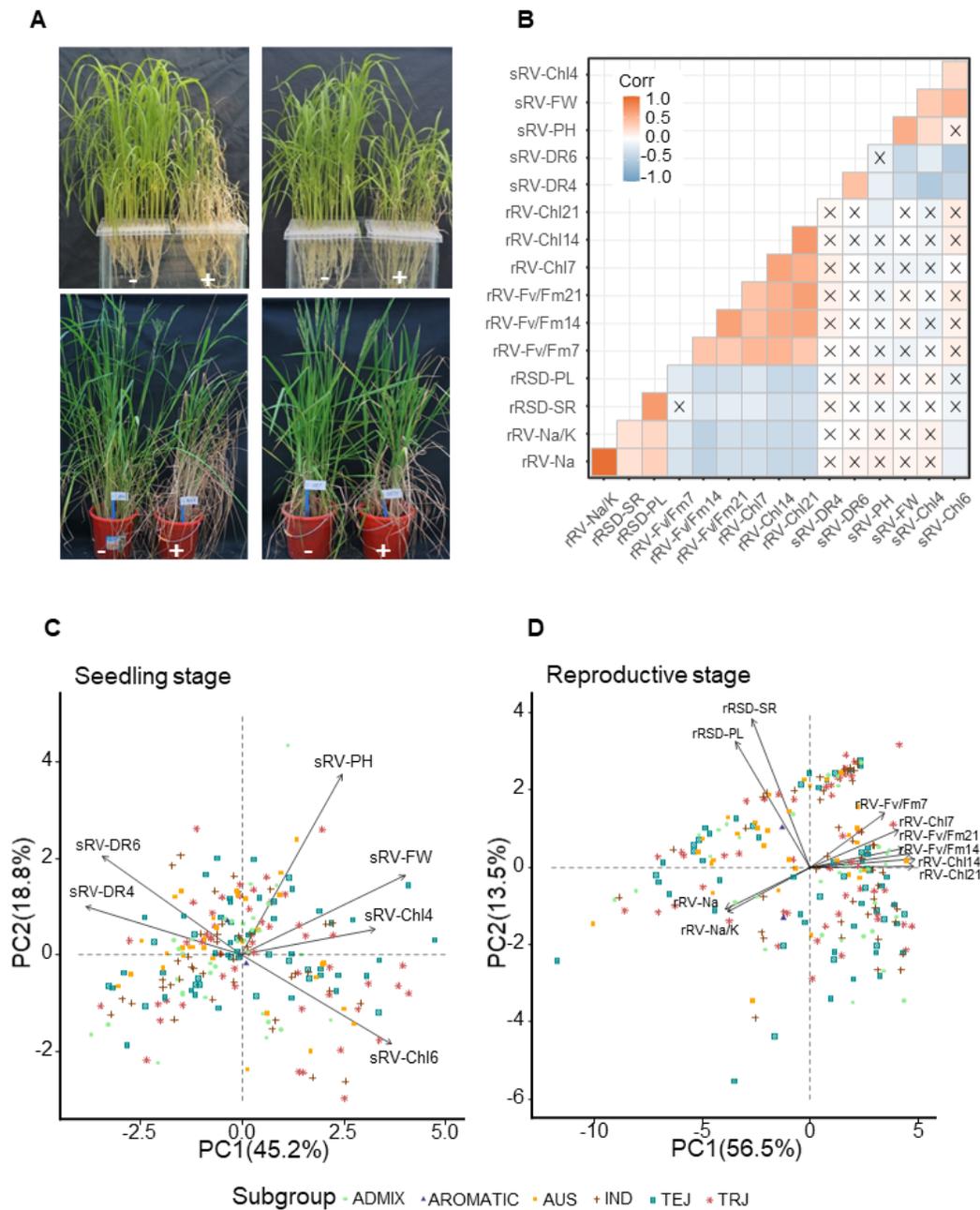


Figure 1

Phenotype and PCA analysis of indices under salt stress at seedling and reproductive stages A, Comparison of rice accessions with (+) and without (-) NaCl treatment. Pictures for seedling stages were taken at 4 days after treated with and without 0.8 % NaCl. Pictures for reproductive stages were taken at 14 days after treated with and without 0.5 % NaCl. Left and right pictures in each panel refer to different rice accessions with different tolerance to salt stress at seedling or reproductive stages, respectively. B, Pearson correlation coefficient matrix of 16 ST indices. The cross indicates that the correlations did not reach statistically

significant level at $P < 0.05$. Red and blue refer to positive and negative correlation, respectively. C and D, PCA analysis of ST indices at seedling and reproductive stages, respectively. Total of 220 accessions were plotted on PCA plot with different shapes and colors. Each arrow represents an index and the length refers to its contribution on ST. The angles captured by any of the two arrows less than 90° imply the two indices having positive correlation, otherwise represent negative correlation between the two indices.

Fig. 2

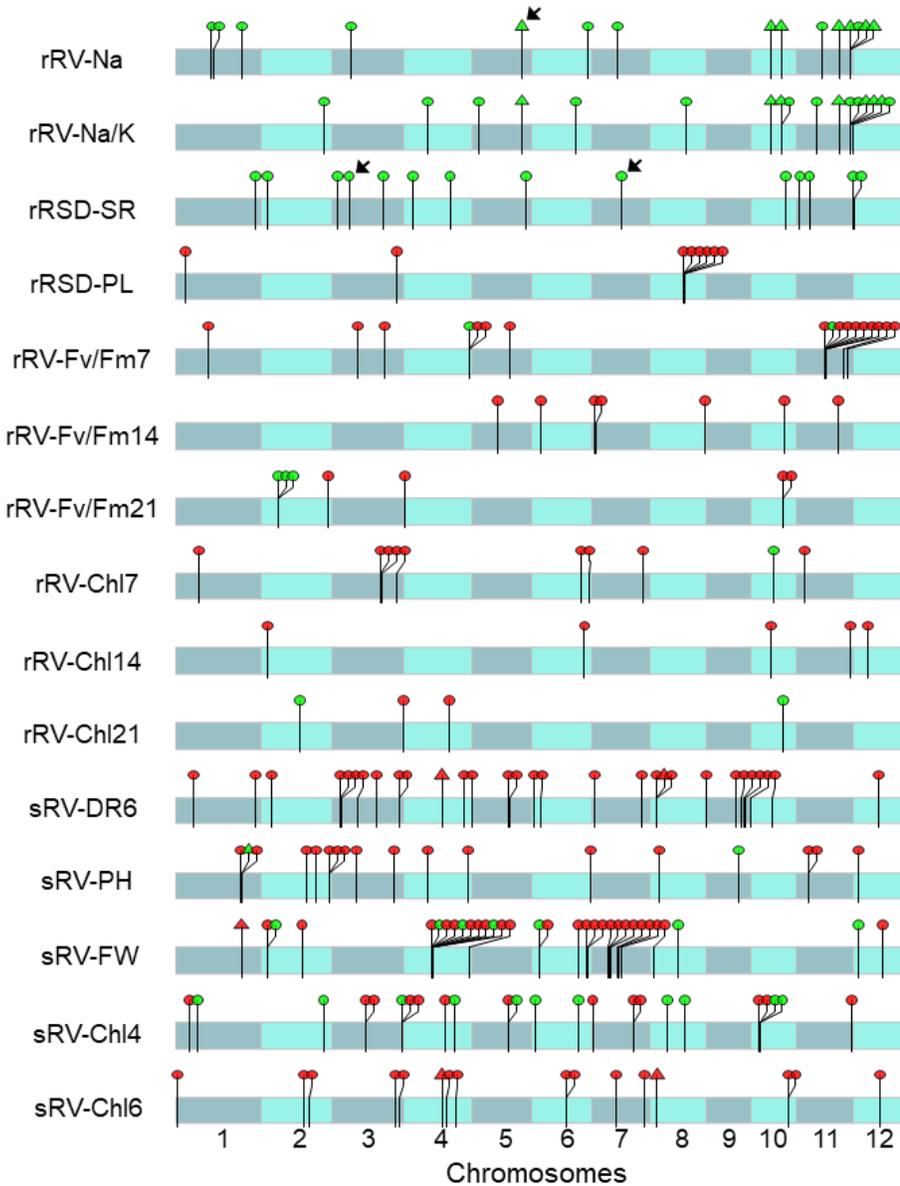


Figure 2

Distribution of significant SNPs associated with different ST indices on chromosomes. Green and red dots indicate the corresponding SNP explained phenotypic variation less than 10% and more than 10%, respectively. The arrows indicate the SNPs explain more than 14% total phenotypic variation of the corresponding ST index. Triangles indicate the SNPs associated with two different ST indices. The length of chromosome was dimensionally drawn based on the physical length of each chromosome (<http://rice.plantbiology.msu.edu>).

Fig. 3

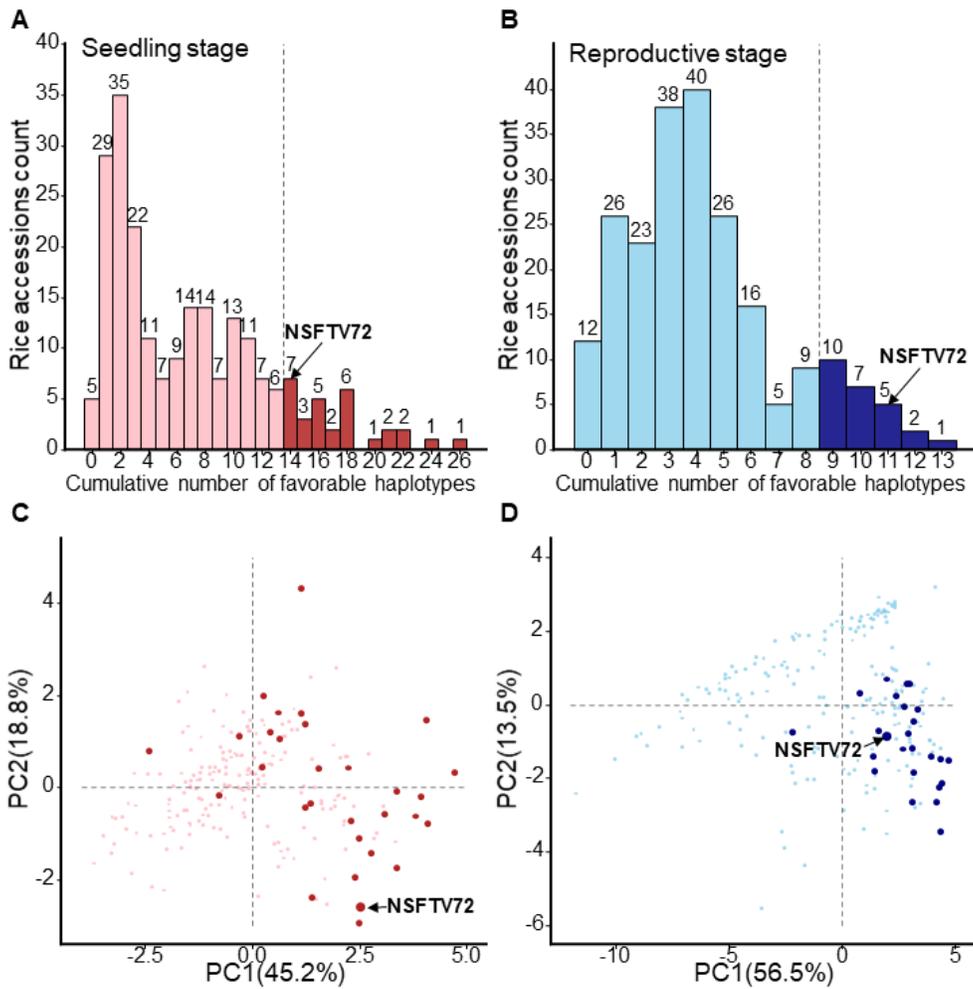


Figure 3

Distribution of accessions contained low frequency favorable alleles and the potential donor germplasms of these alleles A and B, The histograms for the count of rice accessions with cumulative number of favorable haplotypes at seedling (A) and reproductive (B) stages. The dark columns at the right of dotted line represent the top 15% accessions that carrying most low-frequency favorable (LFF) alleles at seedling and reproductive stages, respectively. Number on each column indicates the number of accessions containing the corresponding number of LFF alleles. C and D, Selected donor germplasms on the PCA plots similar to Fig 1(C, D). Dark points in C and D represent the top 15% accessions that carried most LFF alleles at seedling and reproductive stages, respectively.

Fig. 4

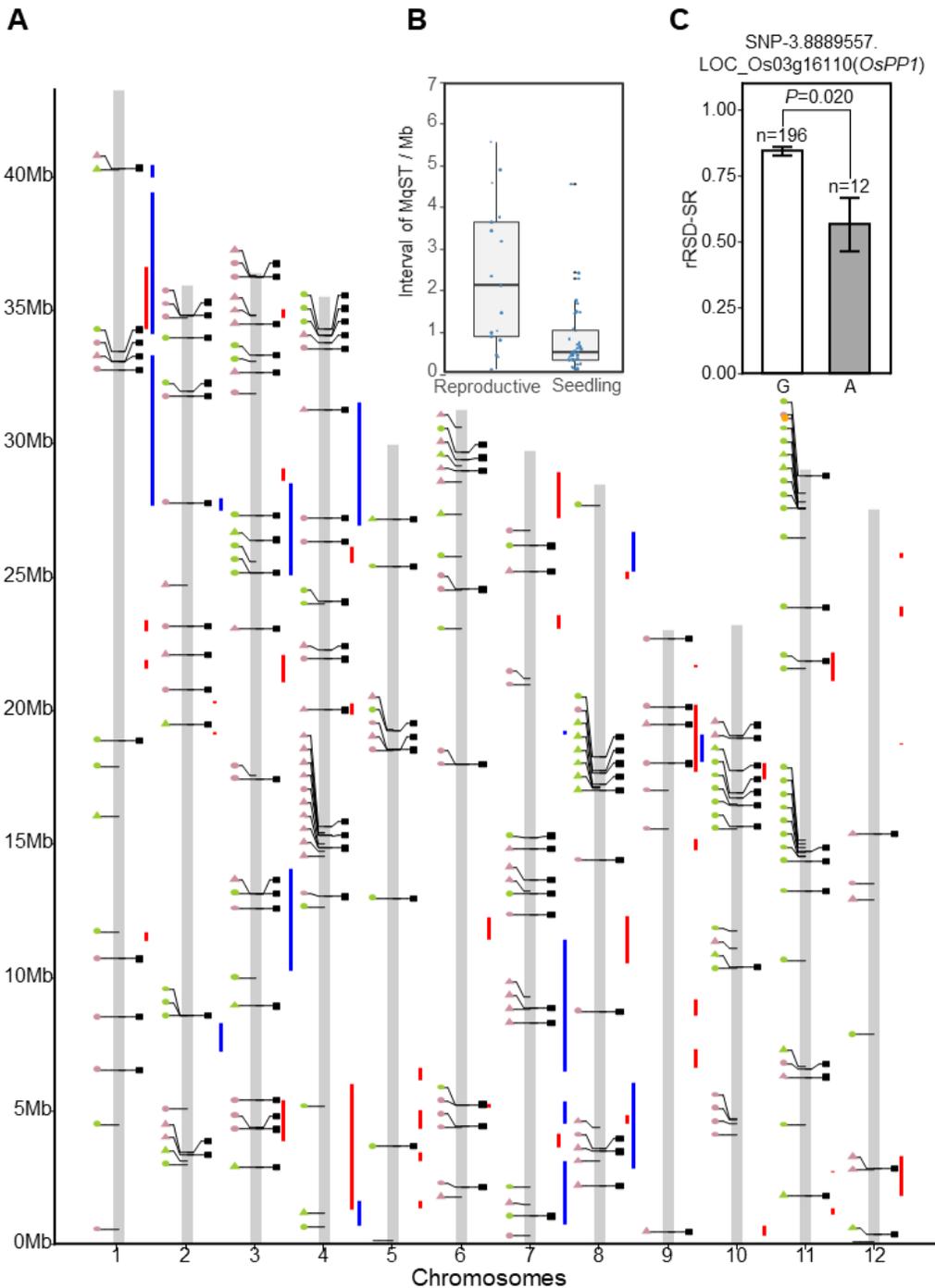


Figure 4

Comparison of the significant association SNPs, Meta-QTL and differentially expressed genes A, Integrated map for association SNPs, Meta-QTL intervals and differentially expressed genes (DEGs). Circles and triangles on the left side of each chromosome represent LFF and not LFF SNPs, respectively. Green and pink colors indicate the association SNPs detected at seedling and reproductive stages, respectively. Squares on the right side of the chromosomes represent the DEGs. Red and blue vertical lines refer to meta-QTL intervals of seedling and reproductive stages, respectively. B, Distribution of interval length of meta-QTL in reproductive stage (rMqST) and in seedling stage (sMqST). C, Haplotype analysis of the SNP-3.8889557 associated differentially expressed gene OsPP1. The n=192 and n=12 indicate the number of the accessions harbored 'G' and 'A' haplotypes, respectively. The average rRSD-SR value of the accessions containing each of the two haplotypes reached statistically significant difference at $p < 0.05$.

Supplementary Files

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