A GWAS approach to find SNPs associated with salt removal in rice leaf sheath

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- **Background and Aims** The ability for salt removal at the leaf sheath level is considered to be one of the major mechanisms associated with salt tolerance in rice. Thus, understanding the genetic control of the salt removal capacity in leaf sheaths will help improve the molecular breeding of salt-tolerant rice varieties and speed up future varietal development to increase productivity in salt-affected areas. We report a genome-wide association study (GWAS) conducted to find single nucleotide polymorphisms (SNPs) associated with salt removal in leaf sheaths of rice.
- **Methods** In this study, 296 accessions of a rice (*Oryza sativa*) diversity panel were used to identify salt removal-related traits and conduct GWAS using 36 901 SNPs. The sheath:blade ratio of Na⁺ and Cl⁻ concentrations was used to determine the salt removal ability in leaf sheaths. Candidate genes were further narrowed via Gene Ontology and RNA-seq analysis to those whose putative function was likely to be associated with salt transport and were up-regulated in response to salt stress.
- **Key results** For the association signals of the Na⁺ sheath:blade ratio, significant SNPs were found only in the *indica* sub-population on chromosome 5. Within candidate genes found in the GWAS study, five genes were upregulated and eight genes were downregulated in the internal leaf sheath tissues in the presence of salt stress.
- **Conclusions** These GWAS data imply that rice accessions in the *indica* variety group are the main source of genes and alleles associated with Na⁺ removal in leaf sheaths of rice under salt stress.

Key words: Salt removal ability, leaf sheath, genome-wide association study, single nucleotide polymorphism, rice, RNA-seq, salt stress.

INTRODUCTION

Rice (*Oryza sativa* L.), which is cultivated in 114 countries and eaten by 3 billion people across the globe, is one of the least salt-tolerant species among major cereal crops (Bouman *et al.*, 2007; Munns and Tester, 2008; Virmani and Ilyas-Ahmed, 2008). Rice is susceptible to salt stress with a low threshold electrical conductivity of 3 dS m⁻¹, equivalent to 30 mM NaCl (Maas and Hoffman, 1977; Lutts *et al.*, 1995; Pitman and Läuchli, 2002). Saline soil, carrying mostly Na⁺ and Cl⁻ ions, is a severe abiotic stress commonly affecting rice plants (Lutts *et al.*, 1995), and elevated salt is harmful to the main metabolic processes in all developmental stages of rice, which significantly limits rice productivity worldwide (Garcia *et al.*, 1997; Zeng and Shannon, 2000; Tester and Davenport, 2003; Munns and Tester, 2008; Horie *et al.*, 2012; Todaka *et al.*, 2012). The world population is estimated to reach 9 billion people by 2050, so improving salt tolerance in rice crops is a major solution to increase productivity and maintain global food supply (Turrall *et al.*, 2011). Therefore, it is necessary to enhance our current understanding of the genetic mechanisms of salt tolerance to develop more salt-tolerant rice varieties through breeding programmes to maximize rice yield in salt-affected areas.

Over time, salt stress increases concentrations of Na⁺ and Cl⁻ to toxic levels and causes premature leaf senescence, stunting and death of rice seedlings (Munns, 2002; Tester and Davenport, 2003; Horie *et al.*, 2012; Todaka *et al.*, 2012). Hence, the removal of Na⁺ and Cl⁻ from leaves is crucial for rice salt tolerance. In rice, the maintenance of low Na⁺ and Cl⁻ concentrations in active photosynthetic tissues, especially leaf blades, has been shown to increase salt tolerance in rice (Zhu, 2003; Pardo *et al.*, 2006; Munns and Tester, 2008; Zhang *et al.*, 2016).

There are two stages of the removal of Na⁺ and Cl⁻ in rice plants to decrease transport to the leaf blade (Ren *et al.*, 2005; Shi *et al.*, 2013). For the first stage of salt removal, known as ‘salt removal by the root’, Na⁺ is unloaded from xylem vessels to xylem parenchyma cells mediated by OsHKT1;5 (Ren *et al.*, 2005), whereas removal of Cl⁻ from the xylem vessels may be controlled by Cl⁻-permeable NPF2.4 and SLAC1 proteins (Li *et al.*, 2016; Qiu *et al.*, 2016). The second stage of salt removal is the removal of Na⁺ and Cl⁻ in the leaf sheath (Cotsaftis *et al.*, 2012; Kobayashi *et al.*, 2017; Neang *et al.*, 2019). In the rice leaf sheath, Na⁺ and Cl⁻ are preferentially removed from xylem vessels and then transported to the fundamental parenchyma cells (Neang *et al.*, 2019). Moreover, it

has been demonstrated that Na⁺-selective HKT1 transporter proteins such as OsHKT1;5 in rice (Kobayashi *et al.*, 2017) and TmHKT1;4 in durum wheat (Lindsay *et al.*, 2004; James *et al.*, 2006) unload Na⁺ from xylem vessels into xylem parenchyma cells in the leaf sheath, whereas there are no reports on Cl⁻ transporters excluding Cl⁻ in the leaf sheath.

The ability to remove Na⁺ and Cl⁻ in the leaf sheath is evaluated by comparing the concentrations of Na⁺ and Cl⁻ in leaf sheaths and leaf blades, expressed as a ratio of Na⁺ and Cl⁻ concentration between leaf sheaths and leaf blades (James *et al.*, 2006, 2011; Neang *et al.*, 2019). In addition, there is a genotypic variation of the Na⁺ sheath:blade ratio between salt-tolerant and salt-sensitive rice genotypes (Neang *et al.*, 2019), which suggests corresponding genes/proteins associated with the variation of Na⁺ removal ability in the leaf sheath of rice. However, the complete understanding of the genetic control of Na⁺ and Cl⁻ removal ability in leaf sheaths is still obscure.

To understand the molecular mechanism of salt tolerance in rice, a quantitative trait locus (QTL) mapping approach has been used for rice. A number of QTLs have been identified in relation to rice salt tolerance traits (Koyama *et al.*, 2001; Lin *et al.*, 2004; Yao *et al.*, 2005; Lee *et al.*, 2007; Mohammadi-Nejad *et al.*, 2008; Sabouri and Sabouri, 2008; Ammar *et al.*, 2009; Pandit *et al.*, 2010; Thomson *et al.*, 2010; Islam *et al.*, 2011; Cheng *et al.*, 2012; Hu *et al.*, 2012; Ghomi *et al.*, 2013; Mohammadi *et al.*, 2013; Hossain *et al.*, 2015; Tiwari *et al.*, 2016; Chen *et al.*, 2020; Jahan *et al.*, 2020). Among many QTLs that have been identified in the rice genome, *Saltol* and *SKC1* on chromosome 1 are well-characterized QTLs, that are known to regulate the shoot Na⁺:K⁺ ratio under salt stress (Bonilla *et al.*, 2002; Lin *et al.*, 2004; Thomson *et al.*, 2010).

To date, genome-wide association studies (GWAS) have identified important loci controlling variation in salt tolerance among rice germplasms, which offers larger mapping resolution and the ability to evaluate greater allelic diversity compared with the QTL method (Yu and Buckler, 2006; Myles *et al.*, 2009; Pandit *et al.*, 2010; Negrão *et al.*, 2011). Regarding salt tolerance mechanisms, the application of GWAS revealed a large number of SNPs and candidate genes associated with rice salt tolerance-related traits such as stress susceptibility indices of the vigour index, germination time, Na⁺ and K⁺ contents, net photosynthetic rate, grain yield, yield components, seedling length ratio, and fresh and dry weight ratios in different growth stages from germination to flowering stages under saline conditions (Shi *et al.*, 2017; Patishtan *et al.*, 2018; Lekklar *et al.*, 2019; An *et al.*, 2020). However, neither QTLs nor GWAS have been applied for the analysis of genetic sources of salt removal ability in leaf sheaths, which is determined by the sheath:blade ratio of the Na⁺ and Cl⁻ concentration.

In the current study, we performed GWAS for salt removal ability in leaf sheath using the sheath:blade ratio of Na⁺ and Cl⁻ under salt stress conditions and 36 901 single nucleotide polymorphisms (SNPs) in 296 accessions of a rice diversity panel. The rice panel consists of *indica*, *aus*, *tropical japonica*, *temperate japonica*, *aromatic* and *admixed* sub-populations (Zhao *et al.*, 2011). The aim of this study was to conduct GWAS to find associated SNPs and candidate genes responsible for salt removal ability in rice leaf sheaths. To narrow down the candidate genes, we hypothesized that the genes associated with the salt removal ability in the leaf sheath may be upregulated by salt

treatment and we selected genes whose expression levels were altered by salinity. Additionally, we determined Na⁺ and Cl⁻ concentrations in leaf blades and the standard evaluation score (SES), an indicator of salt tolerance, to evaluate the contribution of salt removal ability in leaf sheath to lowering Na⁺ and Cl⁻ in the leaf blade and whole-plant salt tolerance at the seedling stage.

MATERIALS AND METHODS

Plant materials and growth conditions

We used a rice diversity panel that consisted of 296 varieties (Supplementary data Table S1), which were collected from many countries, representing all the major rice-growing countries across the world (Zhao *et al.*, 2011). The rice panel is composed of six major sub-populations of rice: 70 *indica*, 49 *aus*, 61 *temperate japonica*, 61 *tropical japonica*, 11 *aromatic* and 44 highly *admixed* accessions. For check varieties, FL 478 was used as a salt-tolerant check and NSIC Rc 222 was used as a salt-sensitive check. These rice accessions were requested from the International Rice Gene Bank at the International Rice Research Institute (IRRI), in the Philippines. Plant cultivation was conducted in a greenhouse at the IRRI using a randomized complete block design with three replications. Seeds were incubated for 5 d at 50 °C to break dormancy. Seeds were then placed in Petri dishes with moistened filter paper and incubated at 30 °C for 48 h to germinate. Pre-germinated seeds were sown onto holes (two seeds per hole) on a Styrofoam seedling float, suspended on distilled water in 10 L plastic trays for 3 d. Each plastic tray had 96 holes, with four holes used for one variety. Twenty-two varieties and two check varieties were grown per tray using 14 plastic trays per replicate except for the final tray which had ten varieties and two check varieties (Supplementary data Fig. S1). The floats were then transferred to Yoshida hydroponic solution (Yoshida *et al.* 1976) and plants were further grown until the sixth leaves of each variety were fully expanded. After the complete development of the sixth leaves, NaCl was added to the culture solution to bring its electrical conductivity (EC) to 12 dS m⁻¹ (approx. 100 mM NaCl) using an EC meter (HI-9835, Hanna Instruments) and plants were grown for a further 7 d. The pH of the culture solution was adjusted daily to pH 5.0 by adding either KOH or HCl (Yoshida *et al.*, 1976), and the solution was renewed every 7 d.

Phenotype data analyses

Seven days after the initiation of salt treatment, the sixth leaves of all rice accessions (two plants per variety, six in total from three replications) were harvested and rinsed with distilled water twice. Samples were then dried at 70 °C for >48 h, separated into leaf blades and leaf sheaths, and used for the measurement of the concentrations of Na⁺ and Cl⁻ according to Neang *et al.* (2019). Na⁺ and Cl⁻ concentrations are presented on a unit dry weight basis. The sheath:blade ratio of Na⁺ and Cl⁻ was calculated according to the formula: concentration in leaf sheath/concentration in leaf blade, as described in Neang *et al.* (2019). Data from two plants from each variety were used to calculate the average for each replication. The average of each variety was calculated using the average of all three replications and then used for GWAS analysis.

Standard evaluation score for salt tolerance

Salt tolerance screening of the rice diversity panel was done at the IRRI phytotron facility with day/night temperatures controlled at 29/21 °C and 70 % relative humidity. For check varieties, we used FL 478 as the salt-tolerant check and IR 29 as the salt-sensitive check. Plants were hydroponically grown as described above. Salt treatment was done by salinizing the hydroponic solution to EC = 12 dS m⁻¹ as described above. After 2 weeks in EC 12 dS m⁻¹, the visible reactions of plants to salt stress were evaluated using the SES for salt tolerance (where 1 = very tolerant, and 9 = very susceptible) (IRRI, 1996).

Genome-wide association study

The GWAS was performed on the Na⁺ sheath:blade ratio and Na⁺ and Cl⁻ concentrations in leaf blades of 296 diverse rice accessions using a linear-mixed model implemented in the efficient mixed-model association (EMMA) by the R package of the Genome Association and Prediction Integrated Tool package (GAPIT) (Zhang *et al.*, 2010; Lipka *et al.*, 2012). For the genotypic dataset, a 44 K SNP chip was used for the association with phenotypic traits, which can be found on www.ricediversity.org/44kgwas. SNP calling was performed using the program ALCHEMY as described in Wright *et al.*, 2010. A total of 36 901 high-density SNPs that showed a call rate >70 % and a minor allele frequency >0.01 were selected to use for all analyses. SNPs that showed a low call rate and allele frequency across all samples were discarded from the dataset (Zhao *et al.*, 2011). The genome-wide significance threshold was determined using the strict Bonferroni correction method (P -value = 0.01/number of SNPs) (Benjamini and Hochberg, 1995).

Identification and analysis of candidate genes

To determine candidate genes that are associated with salt removal ability in leaf sheaths, bioinformatic servers such as the IRRI Galaxy (<http://galaxy.irri.org/>) and the Rice Genome Annotation Project (<http://rice.plantbiology.msu.edu>), and SNP data (the most significant SNPs with the lowest P -value) were used. Significantly associated SNPs from the *indica* sub-population for the Na⁺ sheath:blade ratio were selected for candidate gene analysis. Candidate genes were searched within 200 kb genomic regions (Zhao *et al.*, 2011; Patishtan *et al.*, 2018) of selected significant SNPs using IRRI Galaxy. Then, the classification and annotation of candidate genes was done using the Rice Genome Annotation Project. Among all the candidate genes, the genes categorized in expressed protein, hypothetical protein, retrotransposon and transposon were discarded.

RNA-seq analysis

For RNA-seq analysis, a salt-tolerant variety FL 478 whose Na⁺ sheath:blade ratio (9.9) was close to the average of all 296 rice varieties (9.0) was used. FL 478 was hydroponically grown using Yoshida solution (Yoshida *et al.*, 1976) in a growth

chamber with a light/dark cycle of 12/12 h at 30/25 °C and 70 % relative humidity as described in Neang *et al.* (2019). After 2 weeks, the seedlings were treated with 50 mM NaCl for 2 d followed by 100 mM NaCl for 1 d (3 d in total). The fifth leaf sheaths of FL 478 treated or not with NaCl were used to make cross-sections. Then, central and peripheral parts of the leaf sheath were immediately dissected from each cross-section under a stereo microscope. After that, the central and peripheral parts of leaf sheaths were collected and frozen in liquid nitrogen. Total RNA was extracted using an RNeasy Plant mini kit (Qiagen). The quality of RNA was determined using an Agilent Bioanalyzer 2100 system (Agilent Technologies) and all samples showed an RNA integrity number (RIN) >8.5. After processing RNA samples using a TruSeq stranded mRNA sample prep kit (Illumina), a cDNA library was prepared using a TruSeq stranded mRNA library prep kit (Illumina). Single-end 50 bp reads were generated with the Illumina Novaseq 6000. The sequences were mapped to the IRGSP-1.0 reference genome sequence using STAR v2.6.0a. Duplicated reads were checked using Picard v2.18.25. The expression level of each transcript was expressed as the reads per kilobase per million reads (RPKM) value, which was calculated based on the number of mapped reads. The data have been deposited into the DDBJ Sequence Read Archive (DRA) (accession no. DRA009377).

Statistical analysis

A randomized complete block design with three replications was used. The analysis of variance (ANOVA) and multiple comparisons using general linear hypothesis test (GLHT) were performed for phenotypic comparison between sub-populations using R software. Relationships among phenotypic traits were analysed using Pearson's correlation coefficient.

RESULTS

Phenotype variation for salt removal-related traits among rice accessions

Table 1 shows the phenotype variations in the rice diversity panel under NaCl-treated conditions. The Na⁺ and Cl⁻ sheath:blade ratios, indicators of the salt removal ability in leaf sheaths, showed a large phenotypic variation among rice accessions. The Na⁺ sheath:blade ratio ranged from 1.90 to 50.20, while the Cl⁻ sheath:blade ratio ranged from 0.83 to 10.93. Regarding the SES which indicates the whole-plant salt tolerance, the score varied from 2.30 (highly salt tolerant) to 8.11 (highly salt susceptible) (Table 1).

Relationships of all phenotypic traits under saline conditions

To examine the contribution of salt removal ability in the leaf sheath to the salt accumulation in the leaf blade and salt tolerance, Pearson correlation analysis was conducted across all the rice diversity accessions (Table 2). The Na⁺ sheath:blade ratio showed significantly negative correlations with Na⁺ of the leaf blade and a positive correlation with the SES of salt tolerance.

The Cl^- sheath:blade ratio showed significantly negative correlations with Cl^- concentration in the leaf blade but no correlation with the SES of salt tolerance. The Na^+ sheath:blade ratio and Cl^- sheath:blade ratio showed a significantly positive correlation. The Na^+ and Cl^- concentration in leaf blades showed significantly positive correlations with the SES of salt tolerance.

Phenotypic distribution and GWAS for the Na^+ sheath:blade ratio

Comparisons between each of the sub-populations showed a significant difference for the Na^+ sheath:blade ratio (Fig. 1B). In the rice diversity panel, *aromatic* and *aus* accessions had the longest mean Na^+ sheath:blade ratio, *indica* and *temperate japonica* accessions had an intermediate Na^+ sheath:blade ratio, and *admixed* and *tropical japonica* accessions had the shortest (Fig. 1B).

When the rice diversity panel was analysed as a whole, no significant associated SNPs were observed in any chromosomes (Supplementary data Fig. S2). When rice sub-populations were analysed individually, significant associated SNPs were seen in the *indica* sub-population on chromosome 5 (Fig. 2) but not in other sub-populations (Supplementary data Fig. S2). The region that showed significantly strong association in *indica* sub-population contained 11 SNPs (Supplementary data Table S2), which were used to identify candidate genes responsible for the Na^+ sheath:blade ratio.

Phenotypic distribution and GWAS for the Cl^- sheath:blade ratio, and Na^+ and Cl^- concentrations in leaf blades

With regards to the Cl^- sheath:blade ratio, a small variation among rice genotypes and sub-populations was observed compared with the Na^+ sheath:blade ratio (Fig. 1). Furthermore, all

sub-populations showed comparable mean Cl^- sheath:blade ratios (Fig. 1D). Therefore, GWAS on the Cl^- sheath:blade ratio was not analysed due to the small genetic variation of the Cl^- sheath:blade ratio among rice accessions and sub-populations.

Next, since the concentrations of Na^+ and Cl^- in leaf blades significantly correlated with the SES of salt tolerance (Table 2), we performed GWAS analysis of these two parameters as salt tolerance-related traits. The Na^+ concentration in leaf blades showed genetic variation among rice genotypes and sub-populations (Supplementary data Fig. S3A, B). The *tropical japonica* sub-population had the highest mean Na^+ concentration in leaf blades followed by the *admixed* sub-population compared with other sub-populations (Supplementary data Fig. S3B). The *aus*, *aromatic*, *indica* and *temperate japonica* sub-populations showed the lowest mean Na^+ concentration in leaf blades (Supplementary data Fig. S3B). However, no significant associated SNPs for Na^+ concentration in leaf blades were observed in any chromosomes when analysing using the whole rice diversity panel as well as individual rice sub-populations (Supplementary data Fig. S4).

For the Cl^- concentration in leaf blades, genetic variation was shown among rice genotypes and sub-populations (Supplementary data Fig. S3C, D). The *tropical japonica* sub-population showed the highest level among all sub-populations, while other sub-populations showed a comparable level of Cl^- concentration in leaf blades (Supplementary data Fig. S3D). There were no significant associated SNPs detected regarding Cl^- concentration in leaf blades (Supplementary data Fig. S5).

Candidate genes associated with salt removal ability

Candidate genes were identified using SNP data derived from GWAS results and bioinformatic servers (IRRI Galaxy and Rice Genome Annotation Project). In the *indica* sub-population, 25 candidate genes derived from many functional classes were determined in the region of significant SNPs associated with the Na^+ sheath:blade ratio (Table 3).

TABLE 1. All phenotypic traits related to salt removal ability used for GWAS analysis

Trait name	Min	Max
Na^+ concentration in leaf sheaths (mg g^{-1} d. wt)	1.51	41.77
Cl^- concentration in leaf sheaths (mg g^{-1} d. wt)	22.26	162.76
Na^+ concentration in leaf blades (mg g^{-1} d. wt)	0.17	18.82
Cl^- concentration in leaf blades (mg g^{-1} d. wt)	14.80	110.05
Na^+ sheath:blade ratio	1.90	50.20
Cl^- sheath:blade ratio	0.83	10.93
SES of salinity tolerance	2.30	8.11

Up- and downregulated genes in the central and peripheral parts of the internal leaf sheath under salinity

To explore the candidate genes associated with salt distribution in the internal tissues of the leaf sheath, RNA-seq analysis was performed to determine the expression level of candidate genes in the regions associated with Na^+ removal in the leaf sheath in the central and peripheral parts of the leaf sheath. The

TABLE 2. Phenotypic correlations of all salt removal-related traits in all rice varieties under salt stress

Traits	$[\text{Na}^+]$ leaf blades	Na^+ sheath:blade ratio	$[\text{Cl}^-]$ leaf blades	Cl^- sheath:blade ratio	SES
$[\text{Na}^+]$ leaf blades	1.000				
Na^+ sheath:blade ratio	-0.819*	1.000			
$[\text{Cl}^-]$ leaf blades	0.762*	-0.638*	1.000		
Cl^- sheath:blade ratio	-0.089	0.251*	-0.384*	1.000	
SES	0.268*	-0.195*	0.148†	-0.096	1.000

Abbreviations: $[\text{Na}^+]$, Na^+ concentration; $[\text{Cl}^-]$, Cl^- concentration.

*Correlation is significant at the 0.01 level (two-tailed).

†Correlation is significant at the 0.05 level (two-tailed).

FIG. 1. Phenotype distribution for the Na⁺ and Cl⁻ sheath:blade ratio. (A) Histogram of the Na⁺ sheath:blade ratio in all rice accessions. (B) Boxplot showing the differences in the Na⁺ sheath:blade ratio among sub-populations. (C) Histogram of the Cl⁻ sheath:blade ratio in all rice accessions. (D) Boxplot showing the differences in the Cl⁻ sheath:blade ratio among sub-populations. Different letters indicate significant differences among sub-populations at $P < 0.05$ [multiple comparisons using general linear hypothesis test (GLHT)].

FIG. 2. Genome-wide association for the Na⁺ sheath:blade ratio. Manhattan plot (A) and quantile-quantile plot (B) showing the association signal of GWAS analysis using the Na⁺ sheath:blade ratio of the *indica* sub-population as phenotypic data. The x-axis indicates the SNP positions across the 12 rice chromosomes. The y-axis shows the P -value for the association test at each locus on a log scale. The horizontal green line represents the significance threshold. Coloured dots indicate SNPs.

TABLE 3. List of candidate genes associated with the Na⁺ sheath:blade ratio in the indica sub-population

GO name	Locus	Putative function
Binding	LOC_Os05g25180	Zinc finger, C3HC4-type family protein, expressed
Catalytic activity	LOC_Os05g23880	Lipoxygenase, putative, expressed
	LOC_Os05g24500	Terpene synthase, N-terminal domain-containing protein, expressed
	LOC_Os05g24880	Dehydrogenase, putative, expressed
Endoplasmic reticulum	LOC_Os05g24770	Reticulon domain-containing protein, putative, expressed
Enzyme regulator activity	LOC_Os05g23860	Rab GDP dissociation inhibitor alpha, putative, expressed
Hydrolase activity	LOC_Os05g23830	Endo-1,4-beta-xylanase, putative, expressed
	LOC_Os05g23924	Glycosyl hydrolase family 10 protein, putative, expressed
	LOC_Os05g24550	Papain family cysteine protease domain-containing protein, expressed
Intracellular	LOC_Os05g24970	LSM domain-containing protein, expressed
Kinase activity	LOC_Os05g24010	Receptor protein kinase-like, putative, expressed
Post-embryonic development	LOC_Os05g25060	PPR repeat domain-containing protein, putative, expressed
Protein binding	LOC_Os05g24780	OsCML21 – calmodulin-related calcium sensor protein, expressed
Protein metabolic process	LOC_Os05g23740	DnaK family protein, putative, expressed
Response to stress	LOC_Os05g23990	Rp3 protein, putative, expressed
Sequence-specific DNA-binding transcription factor activity	LOC_Os05g23780	OsMADS70 – MADS-box family gene with M-alpha type-box, expressed
	LOC_Os05g23910	Histone-like transcription factor and archaeal histone, putative, expressed
	LOC_Os05g25260	AP2 domain-containing protein, expressed
Transferase activity	LOC_Os05g23950	TRAF-type zinc finger family protein, expressed
	LOC_Os05g24660	IPP transferase, putative, expressed
	LOC_Os05g25150	Transferase family protein, putative, expressed
Translation factor activity, nucleic acid binding	LOC_Os05g23800	RNA recognition motif-containing protein, putative, expressed
	LOC_Os05g23940	Transcription initiation factor IIB, putative, expressed
Transport	LOC_Os05g24601	Coatomer subunit delta, putative, expressed
Transporter activity	LOC_Os05g25194	Metal cation transporter, putative, expressed

results demonstrated that, of the 25 candidate genes in the region of significant SNPs associated with the Na⁺ sheath:blade ratio, five genes were upregulated and eight were downregulated in either the central or the peripheral regions of the internal leaf sheath under saline conditions (Table 4).

DISCUSSION

Variations in the salt removal in the leaf sheath among rice genotypes

To our knowledge, this is the first report showing variation in the Na⁺ and Cl⁻ removal ability in rice leaf sheath from diverse rice backgrounds consisting of all variety groups of *O. sativa*. The rice diversity panel consisting of 296 rice accessions showed a wide range of Na⁺ sheath:blade ratios (Fig. 1A, B), indicating a wide variation in the Na⁺ removal ability in leaf sheaths. In addition, correlation analysis results indicated that Na⁺ removal ability in leaf sheaths may contribute to lowering Na⁺ accumulation in leaf blades and salt tolerance at the whole-plant level (Table 2). Therefore, it is suggested that Na⁺ removal in the leaf sheath is a crucial trait to make rice tolerant to salt stress by reducing Na⁺ concentrations in the leaf blade at the seedling stage. This is well matched to the result of Platten *et al.* (2013) showing that salt tolerance is well correlated with leaf Na⁺ concentration across 115 rice accessions including *O. sativa* and *O. glaberrima*. Additionally, in the rice diversity panel, *aromatic* and *aus* accessions showed the highest Na⁺ sheath:blade ratio, whereas *admixed* and *tropical japonica* accessions showed the lowest (Fig. 1B). Platten *et al.* (2013) reported that the *aromatic* allele group had the highest salt exclusion in roots followed by the *aus* allele groups, whereas *japonica* had the least. From this it can be inferred that the *aromatic* and *aus* sub-variety groups might have a higher tolerance to salt stress.

In contrast, the rice diversity panel showed relatively small variation in the Cl⁻ sheath:blade ratio as most were in a small range of 0–3 (Fig. 1C, D), while most Na⁺ sheath:blade ratios ranged between 0 and 35 (Fig. 1A, B). In addition, all sub-populations showed similar mean Cl⁻ sheath:blade ratios (Fig. 1D), suggesting a low genetic variation of Cl⁻ removal in the leaf sheath in the rice diversity panel, in comparison with Na⁺ removal ability. In the correlation analysis, the Cl⁻ sheath:blade ratio negatively correlated with the Cl⁻ concentration in the leaf blade, whereas it showed no correlation with the SES (Table 2). This result indicates that Cl⁻ removal in the leaf sheath contributes to lowering Cl⁻ accumulation in leaf blades but not to the salt tolerance at the seedling stage. In rice, Na⁺ ions primarily cause ion-specific damage more than Cl⁻ (Lin and Kao, 2001; Tester and Davenport, 2003), therefore, Na⁺ removal ability in the leaf sheath correlates with salt tolerance more than Cl⁻ removal ability.

Association mapping

In this study, GWAS succeeded in detecting 11 significantly associated SNPs with Na⁺ removal ability in the leaf sheath from the *indica* sub-population (Supplementary data Table S2). The positions of SNPs associated with the Na⁺ sheath:blade ratio in this study have not yet been reported in any publications of QTLs and GWAS for salt tolerance in rice.

Candidate genes in the regions associated with salt removal-related traits

Excluding genes encoding unknown, expressed, hypothetical, retrotransposon and transposon proteins, 25 genes were detected in the significantly associated regions with Na⁺ removal

TABLE 4. Candidate genes associated with the Na⁺ sheath:blade ratio in the indica sub-population, which were up- and down-regulated in the central and peripheral parts of the internal leaf sheath under salinity

GO Name	Locus	Putative function	Expression level (RPKM)			
			Central part		Peripheral part	
			Control	Saline	Control	Saline
Genes upregulated under salinity						
Enzyme regulator activity	LOC_Os05g23860	Rab GDP dissociation inhibitor alpha, putative, expressed	39.0	79.9*	32.6	68.0*
Hydrolase activity	LOC_Os05g23924	Glycosyl hydrolase family 10 protein, putative, expressed	0.0	0.1*	0.0	0.9*
Protein metabolic process	LOC_Os05g23740	DnaK family protein, putative, expressed	54.9	138.6	84.7	215.9*
Sequence-specific DNA binding transcription factor activity	LOC_Os05g23910	Histone-like transcription factor and archaeal histone, putative, expressed	0.0	4.3*	0.0	19.5*
Transport	LOC_Os05g24601	Coatomer subunit delta, putative, expressed	50.3	53.2	29.1	39.4*
Genes downregulated under salinity						
Binding	LOC_Os05g25180	Zinc finger, C3HC4-type family protein, expressed	27.3	22.9*	24.2	22.3
Catalytic activity	LOC_Os05g23880	Lipoxygenase, putative, expressed	3.9	2.6	6.7	4.5*
	LOC_Os05g24880	Dehydrogenase, putative, expressed	69.7	36.4*	61.2	38.7*
Hydrolase activity	LOC_Os05g24550	Papain family cysteine protease domain-containing protein, expressed	198.9	102.6*	198.6	81.8*
Intracellular	LOC_Os05g24970	LSM domain-containing protein, expressed	73.4	56.6*	68.5	61.8
Kinase activity	LOC_Os05g24010	Receptor protein kinase-like, putative, expressed	26.8	14.9*	22.0	10.2*
Protein binding	LOC_Os05g24780	OsCML21 – calmodulin-related calcium sensor protein, expressed	1.7	0.0*	1.3	0.1*
Response to stress	LOC_Os05g23990	Rp3 protein, putative, expressed	9.9	5.7*	6.9	3.0*

Abbreviation: RPKM, reads per kilobase million.

*Significant difference at $P < 0.05$ among conditions.

ability in the leaf sheath (Table 3). Gene Ontology (GO) analysis showed that these candidate genes were derived from many functional categories such as transport, enzymes, kinase activity, protein binding and many others, as shown in Table 3. Among these genes, we hypothesized that transporter genes that directly transport Na⁺, and calcium signalling-related genes and transcription factor genes that indirectly regulate the transport of Na⁺, may be involved in controlling genotypic variation in the removal ability of Na⁺ in the leaf sheath. In addition, we hypothesized that the involved genes might be upregulated or at least not downregulated in the peripheral and central parts of the leaf sheath in response to salt treatment. This was because the Na⁺ removal ability in rice leaf sheath is upregulated by salinity in the salt-tolerant rice variety FL 478 (Neang *et al.*, 2019). Also, the leaf sheath unloads Na⁺ from xylem vessels in the vasculature in the peripheral part, then preferentially transports Na⁺ from the peripheral part to the central part, sequestering this ion into the fundamental parenchyma cells in the central part (Neang *et al.*, 2019). Therefore, we speculated that the upregulated genes in the peripheral parts might be related to Na⁺ unloading, and upregulated genes in the central parts might be involved in Na⁺ sequestration into the fundamental parenchyma cells under salinity.

There was one gene for Na⁺ removal ability in the leaf sheath in the GO classification of ‘transporter activity’ (Table 3). For Na⁺ removal ability, the metal cation transporter (LOC_Os05g25194) was a potential candidate gene (Table 3). This metal cation transporter gene is homologous to genes encoding ZIP (Zn-regulated transporter, Iron-regulated transporter-like Protein) proteins which are involved in the transport of metals such as Zn, Cu, Cd, Fe and Mn (Liu *et al.*, 2019). There is no direct evidence so far as to whether plant ZIP proteins permeate sodium ions, but NaCl inhibits Zn uptake of rice OsZIP3 protein (Ramesh *et al.*, 2003), which indicates the possibility of sodium ion transport by ZIP proteins. However, the expression of this metal cation transporter gene did not respond to NaCl treatment.

A calmodulin-like protein-encoding gene (*OsCML21*; LOC_Os05g24780) was detected in the associated regions with Na⁺ removal ability in leaf sheaths (Table 3). *CML* genes are involved in calcium signalling under salinity (Magnan *et al.*, 2008). Calcium signalling regulates sodium homeostasis under salinity via the SOS (salt overly sensitive) pathway (Manishankar *et al.*, 2018). In addition, salt stress increases cytosolic calcium concentrations, which is important to decrease Na⁺ content in shoots (Rahman *et al.*, 2016). Therefore,

this gene may be a potential candidate for further investigation regarding its involvement in Na⁺ removal ability in leaf sheaths. However, *OsCML21* was downregulated in the central and peripheral parts of the internal leaf sheath under salinity (Table 4), indicating that this gene may negatively regulate or not be related to the salt removal ability of the leaf sheath.

Moreover, there were three candidate genes for Na⁺ removal ability in leaf sheath in the GO classification of ‘sequence-specific DNA binding transcription factor activity’ (Table 3). Within the GO classification of transcription factor activity, one gene (histone-like transcription factor and archaeal histone) for Na⁺ removal ability in the leaf sheath was upregulated (Table 4), suggesting its involvement in salt removal in the leaf sheath under saline conditions. Transcription factor genes regulate stress-responsive downstream genes and play a role in response to abiotic stress in a number of plant species, including salinity and drought stress in rice plants (Amir Hossain *et al.*, 2010; Ding *et al.*, 2014; Wang *et al.*, 2015; Baillo *et al.*, 2019). Also, transcription factors are involved in regulating sodium homeostasis under salinity (Hichri *et al.*, 2017). Hence, this upregulated transcription factor gene is worth further investigation regarding its involvement in regulating genotypic variation in Na⁺ removal ability in the leaf sheath.

Conclusions

This study successfully discovered associated SNPs and candidate genes that are closely related to the variation of salt removal in the rice leaf sheath, which is an important trait to reduce salt ions in the active leaf blade, under salt stress conditions. The rice diversity accessions consisting of all sub-populations of *O. sativa* showed wide phenotypic variations based on ion sheath:blade ratios and the SES under salt stress. In the rice panel, *aromatic* and *aus* accessions, compared with other sub-variety groups, seemed to have better ability to remove Na⁺ from the leaf blade and sequester Na⁺ in the leaf sheath, and all sub-variety groups might have a similar ability to remove Cl⁻ in the leaf sheath. GWAS was applied to identify association signals for salt removal ability in leaf sheaths, which has not been done previously. Our GWAS data implied that rice accessions in the *indica* variety group are the major source of genes or alleles that mediate Na⁺ removal in leaf sheaths of rice. RNA-seq performance confirmed the expression level of candidate genes found in the GWAS study and revealed five upregulated and eight downregulated genes in the internal leaf sheath tissues in the presence of salt stress. Furthermore, these findings from GWAS and RNA-seq have provided numerous SNPs and candidate genes that are useful for future studies to explore the deeper role of each candidate gene through transgenic techniques and mutation. This research may enhance our understanding of the molecular mechanism of salt removal ability in the rice leaf sheath under salt stress to improve salt tolerance in rice.

SUPPLEMENTARY DATA

Supplementary data are available online at <https://academic.oup.com/aob> and consist of the following. Figure S1: arrangement of

the experiment. Figure S2: Manhattan plots and quantile–quantile plots showing the association signals of GWAS analysis using the Na⁺ sheath:blade ratio as phenotypic data. Figure S3: phenotype distribution for Na⁺ and Cl⁻ concentrations in leaf blades. Figure S4: Manhattan plots and quantile–quantile plots showing the association signals of GWAS analysis using Na⁺ concentration in leaf blades of all rice varieties and individual sub-populations as phenotypic data. Figure S5: Manhattan plots and quantile–quantile plots showing the association signals of GWAS analysis using Cl⁻ concentration in leaf blades of all rice varieties and individual sub-populations as phenotypic data. Table S1: list of the 296 rice accessions used in this study. Table S2: significant associated SNPs for the Na⁺ sheath:blade ratio.

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