Genome-wide identification of DNA polymorphism in salt-tolerant and sensitive rice genotypes using double digest RADseq

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Abstract

Salt-tolerant rice varietal development is necessary and the best approach to enhance crop productivity in areas with high salinity. To identify the genetic variation in stress-related genes in salt-tolerant genotypes, we carried out genotyping by sequencing (GBS) of seven rice lines with contrasting responses to salinity stress, including FL478 as a tolerant variety and IR64 as a sensitive check. The GBS reduces genomic complexity and enables us to obtain many SNP markers in the genome. A total of 7,980,092 high-quality single-end reads of 94 bp each were generated, and approximately 83% of the reads mapped to the rice reference genome (Nipponbare). We identified a total of 80,159 SNPs and 6,403 InDels among the seven rice genotypes. Forty-two percent of these SNPs were identified from the genic regions, and further, 27% were observed in the coding areas out of these genic SNPs. We used bioinformatics analysis to identify functional SNPs in the genes that changed the functions of proteins. We found variations between tolerant and sensitive genotypes by comparing the identified SNPs in coding regions. We found that 566 of the SNPs identified in the coding regions were non-synonymous (nsSNPs), affecting proteins. We identified 116 abiotic stress-related genes that contained 138 nsSNPs (between FL478 and IR64) in coding regions, thus directly affecting the proteins. Analysis indicated that these genes might contribute to the salt stress phenotype in rice varieties and the identified nsSNP markers that were useful to distinguish tolerant and sensitive genotypes. Our results revealed that the identified SNPs could be used as an essential source of genomic variability for improving and developing saline tolerant genotypes.

Introduction

Rice is an important cereal crop for human nutrition worldwide (Agarwal et al. 2016). It grows in areas where plenty of water is available with suitable temperatures (Batayeva et al. 2018). Over many decades, harsh environments had substantially reduced the yield of rice. It has been predicted that climate changes will increase the frequency of salinity, drought, high temperature, and flood (Mickelbart et al. 2015; Wang et al. 2016). Among these factors, salinity is known to inhibit plant growth, followed by excessive yield losses (Naveed et al. 2018). Soil salinity is one of the most serious threats to the productivity of this major agricultural crop, with adverse effects on all stages of plant growth (Al-Tamimi et al. 2016).

Rice often grows in coastal areas where various degrees of saline soils reduce its plant growth. Among cereals crops, rice is very sensitive to salinity. Most rice varieties can tolerate salinity up to 3 dS/m. Many breeding programs worldwide use rice lines, such as Pokkali, Nona Bokra, etc., as salt-tolerant donors (Krishnamurthy et al. 2020). These lines have helped to improve the yield of elite genotypes under salt stress conditions (Negrão et al. 2013). Many studies have used molecular markers to identify QTLs related to salt tolerance by conventional mapping to distinguish sensitive and tolerant genotypes (Ali et al. 2014; Mondal and Ganie 2014). The use of conventional markers is a laborious and time-consuming process. Rice is the first cereal crop whose genome has been sequenced. The availability of the whole-genome sequence and improvement in next-generation sequencing (NGS) provides an opportunity to explore genetic differences in salt tolerance and sensitive varieties (Negrão et al. 2013). NGS technologies have helped identify SNP markers related to QTLs or target genes (Tiwari et al. 2016). Many rice projects aimed to provide vast amounts of information on the sources of genetic variation of diverse germplasm. The Rice SNP-Seek Database identified ~ 20 million SNPs in the 3K rice genomes project by aligning with the rice reference Nipponbare genome. The outcome of this project will be helpful for the identification of novel allelic variants for crop improvement (Alexandrov et al. 2015).

NGS technologies could detect several variants in an efficient, rapid, and economical manner. These variants are significant reasons for phenotypic differences between different accessions (Ali et al. 2018). The SNPs and InDels identified have been widely used in fine mapping, QTL analysis, and marker-assisted selection. Many SNPs has facilitated fine linkage analysis and genome-wide association study (Hayashi et al. 2006). The variants within the gene coding regions could affect the coding proteins, thus changing their function positively or negatively. Therefore, the presence of variants that lead to a change in gene function(s) is essential for studying the causes of phenotypic differences (Jain et al. 2014).

Several NGS platforms have been developed and widely used for SNP discovery in recent years (Sahu et al. 2020). One straightforward approach is to prepare GBS libraries with decreasing genomic complexity to attain many SNPs and a high read coverage by using restriction enzymes, followed by restricted DNA library construction. It allows the regions flanking only the restriction sites to be sequenced, thus dramatically reducing genomic complexity and sequencing cost. Furthermore, the sequenced genomic portion is highly stable within species because restriction sites are mostly conserved across genotypes (Zhao et al. 2019). Thus, it makes the GBS procedure most suited to analyzing large numbers of SNPs within a population for genetic mapping and genomics-assisted breeding. Furthermore, by selecting appropriate restriction enzymes that have fewer cutting sites in the repetitive regions, enriched SNP coverage can be obtained for the coding regions of the genome (Chung et al. 2017). In comparison with RADseq methods, double digest Restriction site-Associated DNA sequencing (ddRADseq) allows high efficiency, flexibility, and robustness with a low cost (Peterson et al. 2012). Genetic variations in abiotic stress-related genes are already reported; however, the breeding progress using these genes is plodding. Therefore, it is essential to directly sequence rice lines selected for particular breeding objectives to identify genes and QTLs for the targeted traits, for example, salt tolerance and their applications for enhanced and accelerated breeding (Zhao et al. 2011).

We have genotyped seven rice breeding lines showing differential responses to salt stress using ddRADseq technology. The objective was to identify the loci containing SNPs associated with salt tolerance, hence differentiating tolerant genotypes from sensitive ones for salt tolerance breeding.

Materials And Methods

Rice materials

Our previous study selected seven rice lines (Tariq et al. 2019) based on their responses to salinity at the seedling stage. Of these seven lines, four were salt tolerant and three salt sensitive (Table 1).

Table 1
Salt stress responses of the rice lines used for this study

Original name	Parental cross	Designation	Given name	Source	SES scoring			Salinity tolerance
					50 mM	100 mM	150 mM	
HHZ 8-SAL9-DT1-Y1	HHZ x Phalguna (Ali et al, 2017)	GSR IR1-8-S9-D1-Y1	SAL-9	IRRI	3	5	5	Moderately tolerant
HHZ 5-SAL10-DT2-DT1	HHZ x OM1723 (Ali et al, 2017)	GSR IR1-5-S10-D2-D1	SAL-10	IRRI	3	3	3	Tolerant
HHZ 5-SAL10-DT1-DT1	HHZ x OM1723 (Ali et al, 2017)	GSR IR1-5-S10-D1-D1	SAL-11	IRRI	5	7	7	Sensitive
HHZ 11-SAL6-Y1-Y1	HHZ x IR64 (Ali et al, 2017)	GSR IR1-11-S6-Y1-Y1	SAL-12	IRRI	7	7	7	Sensitive
FL478	IR29 x Pokkali (Thomson et al, 2010)	FL478	FL478	IRRI	3	3	3	Tolerant
Pokkali	Landrace	Pokkali	Pokkali	IRRI	3	3	3	Tolerant
IR64	Came from 19 traditional rice varieties. (Mackill and Khush 2018)	IR64	IR64	IRRI	7	7	7	Sensitive
HHZ (Huanghuazhan)								

Preparation of ddRADseq library and sequencing

Genomic DNA of the seven rice lines was extracted from 14-day-old seedlings using the CTAB (cetyl trimethyl ammonium bromide) method (Tariq et al. 2019). Genomic DNA was digested with two restriction enzymes, BamHI and MluCI, for sequencing the BamHI site flanking sequences. The ddRADseq library was constructed for each line for single-ended sequencing using the Illumina HiSeq 2000 platform to generate 100-base-long reads. The FASTQ files of the reads were first trimmed to remove adapters and then the quality of the reads was checked using FastQC V0.63.

Sequence alignment and variant calling

The sequenced reads were filtered based on mapping quality ≥ 20 but the low-quality reads were filtered out with mapping quality ≤ 20 using NGS: QC Toolkit v2.3 (Patel and Jain 2012). The rice cv. Nipponbare reference genome IRGSP-1.0 pseudomolecules were downloaded from the Rice Genome Annotation Project (<http://rice.plantbiology.msu.edu/>) (Kawahara et al. 2013). The clean reads were aligned to the rice reference genome using BWA1.2.3 tool (Li and Durbin 2009) with default parameters. The Picard tool v1.126.0 (<https://broadinstitute.github.io/picard/>) was used to sort the aligned reads in the Sequence Alignment/Map (SAM) file by using SortSam function or plugin. The sorted SAM files were converted to Binary Alignment/Map (BAM) files using SAMtools v2.1 (Li et al. 2009). Variant calling was performed using Unified Genotyper of GATKTools v3.3 (DePristo et al. 2011) with a base quality $> Q30$. To minimize false-positive variants and retain high-quality variants, the variant calling file (VCF) was further filtered based on read depth > 10 and allele frequency > 0.8 using the Select Variants of GATKTools (DePristo et al. 2011). The quality of the identified SNPs and InDels was visualized using Integrative Genomics Viewer (IGV) (Robinson et al. 2011).

Variant annotation

The identified filtered variants in the rice genome were annotated using the rice7 database (https://sourceforge.net/projects/snpeff/files/databases/v4_2/) and SnpEff V4.2 tool (Cingolani et al. 2012). The locations of the SNP and InDel variants in the genic and intergenic regions were determined using the SNIPlay online tool (Dereeper et al. 2015). Based on the locations of the SNPs and InDels, the genic regions were further categorized into exons, introns, 3'UTRs, 5'UTRs, splice sites, and coding regions. The variants identified in the coding regions were further separated as synonymous (with no effect on amino acid), non-synonymous (with effects on amino acid), stop loss (the existing stop codon lost), stop gain (introduction of a new stop codon), start loss (the existing start codon lost), and start gain (introduction of a new start codon). The effect of a substituted amino acid on protein functions was determined by SIFT 4G (Vaser et al, 2016) using the rice IRGSP-1.0.23 genome build (<http://sift.bii.a-star.edu.sg/index.html>). This algorithm classified the presence of non-synonymous SNPs as tolerated (having no effect on protein function) or deleterious (having the potential to change protein function).

Identification of non-synonymous SNPs (nsSNPs) in the salt stress genes

The nsSNPs were filtered from the annotated file, retaining only those nsSNPs that were polymorphic and deleterious by function between the tolerant (FL478) and sensitive genotype (IR64). Furthermore, only those nsSNPs were kept that were specifically located in the coding regions of loci associated with salt tolerance. The functions of the identified genes were obtained from Rice Genome Annotation Project (<http://rice.plantbiology.msu.edu/>), Phytozome the Plant Genomic Resource (<http://phytozome.jgi.doe.gov/>), and uniprot.org websites.

SNP validation

The identified SNPs were validated by downloading the previously reported SNPs of Pokkali and IR64 (Jain et al. 2014) from the Rice SNP Discovery database (<http://www.nipgr.res.in/mjain.html?page=ricesnp>). The identified nsSNPs in coding regions were also validated with the available nsSNPs of FL478, Pokkali, and IR64 in the Rice SNP-Seek Database (Mansueto et al. 2017).

Genetic analysis

A distance-based neighbor-joining tree of high-quality nsSNPs was constructed using DARwin V6.0.1 (Perrier and Jacquemoud-Collet 2006). In addition, SNIPlay V4.2 generated genotyping matrices of high-quality SNPs.

Results

Responses of the seven rice lines to salt stress

The seven rice lines were further screened with three salt concentrations (i.e., 50, 100, and 150 mM) along with controls. All seven lines were evaluated as tolerant or sensitive based on the modified Standard Evaluation System (SES) score (Gregorio et al. 1997) by comparing the visual response of leaves of these genotypes with control checks under 150 mM NaCl (Tariq et al. 2019). Under this salt stress, SAL-9 (GSR IR1-8-S9-D1-Y1 = HHZ8-SAL9-DT1-Y1) and SAL-10 (GSR IR1-5-S10-D2-D1 = HHZ5-SAL10-DT2-DT1) were evaluated as tolerant because the leaves showed no sign of salt injury. In contrast, SAL-11 (GSR IR1-5-S10-D1-D1 = HHZ5-SAL10-DT1-DT1) and SAL-12 (GSR IR1-11-S6-Y1-Y1 = HHZ11-SAL6-Y1-Y1) were evaluated as sensitive because of the sign of salt injury appeared on the leaves and seedlings become dead (Table 1).

FL478 was proven to be highly tolerant of high-saline environments (Thomson et al. 2010), while IR64 is sensitive to saline conditions (Palao et al. 2013). We obtained similar results under the 150 mM NaCl stress. These results further confirmed the responses of the rice lines to salt stress. The contrasting responses of the seven rice lines to salt stress caused us to examine their DNA polymorphism at the genome level.

Genome sequencing and sequence read mapping

A total of 7,980,092 high-quality reads were obtained for the seven rice lines, with an average of 1.14 million high-quality reads per line and an average read length of 93 bp (Fig. 1). A quality check showed that 95.71% of the reads had a quality of $\geq$ Q20. Because both flanking sequences of the BamHI sites were sequenced and it was estimated that there is one BamHI site in approximately 8.5 kb of the 400-Mb rice genome, the sequence reads for a line had average coverage of 12.1x. The average coverage for SAL-9, SAL-10, SAL-11, SAL-12, FL478, Pokkali and IR64 was 13.0x, 11.5x, 11.5x, 13.0x for, 11.5x, 10.5x, and 13.0x (Table 2). Approximately 83% of the reads from each line were mapped to the rice Nipponbare reference genome, while the remaining 17% of the reads were unmapped. The average range of mapped reads was between 66% for Pokkali and 87% for SAL-9 (Fig. 1).

Table 2 Statistics of genotyping-by-sequencing results and SNP and InDel variant discovery									
Rice line	SAL-9	SAL-10	SAL-11	SAL-12	FL478	Pokkali	IR64	Total	Average
Read length (bp)	95	92	95	93	93	90	94	652	93
Mapped reads (%)	87	83	85	86	86	66	86	579	83
Read depth (x)	13.0	11.5	11.5	13.0	11.5	10.5	13.0	84	12
Raw variants	32,908	27,263	36,587	32,747	36,259	22,347	37,111	225,222	32,175
Filtered SNPs	11,417	10,532	13,803	12,038	10,151	6,757	15,461	80,159	10,146
Filtered InDels	857	865	1,115	997	778	533	1,258	6,403	915
Sequence in Mb	227.6	256.4	294.4	246.9	213.7	210.4	323.7	1,773	253.2

Distribution of SNPs and InDels in the rice genome

A total of 225,222 raw variants were identified in the seven rice lines when the rice Nipponbare genome was used as the reference. The raw variants were filtered with a minimum read depth of 10x and mapping quality of $\geq$ 30. Consequently, we obtained a total of 80,159 high-quality SNPs and 6,403 high-quality InDels (Table 2). The largest number of variants was found for chromosome 1 (9,852) and the smallest for chromosome 9 (5,328). The largest numbers of SNPs and InDels were present in IR64 (15,461 and 1,258) and SAL-11 (13,803 and 1,115), while the smallest numbers were present in Pokkali (6,757 and 533) and FL478 (10,151 and 778) (Fig. 2). The average number of variants for each chromosome was 12,366, with one variant for every 30,183 bases (Table S1). Only 186 of the SNPs were common between FL478 and IR64, and 38 were common among FL478, IR64, and Pokkali. IR64/SAL-9 and IR64/SAL-10 had 193 and 143 common SNPs, respectively, while FL478/SAL-9 and FL478/SAL-10 had 213 and 140 common SNPs, respectively. IR64/SAL-11 and IR64/SAL-12 had 228 and 193 common SNPs, respectively; however, FL478/SAL-11 and FL478/SAL-12 had 188 and 223 common SNPs, respectively (Fig. 3).

The average SNP density across the seven rice lines was 3.1/100 kb. Chromosome 10 had the highest SNP density, with average 3.7/100 kb, while chromosome 4 had the lowest SNP density, with average 2.39/100 kb, and only SAL-11 had the lowest SNP density on chromosome 12 which is 2.84/100 kb. The highest average of SNP density was observed in IR64 and SAL-11, which is 4.17/100 kb and 3.71/100 kb, while the lowest average of SNP density was observed in Pokkali and SAL-10, which is 1.82/100 kb and 2.82/100 kb (Table 3).

Table 3
SNP density among the seven rice lines and their chromosomes

Chrom.	SAL-9		SAL-10		SAL-11		SAL-12		FL478		Pokkali		IR
	SNPs	SNPs/100 kb	SNPs	SNPs/100 kb	SNPs	SNPs/100 kb	SNPs	SNPs/100 kb	SNPs	SNPs/100 kb	SNPs	SNPs/100 kb	SNPs/100 kb
1	1405	3.25	1261	2.91	1431	3.31	1347	3.11	1140	2.63	710	1.64	17
2	1146	3.19	1134	3.16	1483	4.13	1173	3.26	971	2.70	709	1.97	15
3	1208	3.32	1081	2.97	1441	3.96	1289	3.54	1066	2.93	679	1.86	16
4	789	2.22	819	2.31	1073	3.02	910	2.56	690	1.94	473	1.33	12
5	831	2.77	751	2.51	1121	3.74	905	3.02	756	2.52	594	1.98	10
6	1088	3.48	991	3.17	1305	4.18	1117	3.57	968	3.10	638	2.04	14
7	876	2.95	840	2.83	1126	3.79	994	3.35	831	2.80	572	1.93	12
8	819	2.88	747	2.63	1085	3.81	863	3.03	826	2.90	468	1.65	12
9	706	3.07	590	2.56	809	3.52	801	3.48	675	2.93	420	1.83	94
10	883	3.80	818	3.52	1021	4.40	895	3.86	726	3.13	512	2.21	12
11	930	3.20	850	2.93	1125	3.88	971	3.35	847	2.92	596	2.05	12
12	736	2.67	650	2.36	783	2.84	773	2.81	655	2.38	386	1.40	10
Total/average	11,417	3.07	10,532	2.82	13,803	3.71	12,038	3.25	10,151	2.74	6,757	1.82	15

Annotation of variants

Of the SNP and InDel variants, 35,846 were found in genic regions, while the remaining 50,140 variants were found in intergenic regions. Out of the 35,846 variants present in the genic regions, 12,850 were present in introns; 2,228 and 2,106 were present in 5'UTRs and 3'UTRs, respectively; and 18,662 were present in coding sequences (CDSs). The SNP variants in the CDSs were divided into synonymous and non-synonymous. It was found that 10,990 of the SNPs in the CDSs were non-synonymous (nsSNPs) and resulted in changed amino acids. The number of nsSNPs was higher than the synonymous SNPs (7,672) in all seven rice lines analyzed (Table 4). There were 2,101 nsSNPs and InDels common between FL478 and IR64 and 1,372 nsSNPs polymorphic between the two. A total of 566 nsSNPs in the seven rice lines had deleterious effects (SIFT score < 0.05) on protein function. The transition (Ts) frequency (112,686) was much higher than that of transversions (Tv) (47,632). The Ts/Tv ratio of the SNPs ranged from 2.2 for SAL-12 to 2.5 for IR64, with an average of 2.0 (Fig. 4).

Table 4
Distribution of SNP and InDel variants in the coding and non-coding regions of the genomes of the seven rice lines

Variant class	SAL-9	SAL-10	SAL-11	SAL-12	FL478	Pokkali	IR64	Total
5'UTR	340	307	372	328	297	197	387	2,228
3'UTR	291	295	365	346	283	175	351	2,106
Intron	1,760	1,735	2,263	1,989	1,531	1,175	2,397	12,850
Non-synonymous	1,604	1,440	1,869	1,649	1,411	992	2,025	10,990
Synonymous	1,136	1,036	1,294	1,140	980	695	1,391	7,672
Intergenic	6,987	6,444	8,551	7,388	6,267	4,547	9,956	50,140
Total	12,118	11,257	14,714	12,840	10,769	7,781	16,507	

Effects of nsSNPs on protein functions of abiotic stress-related loci

We identified 116 abiotic stress-related gene loci (between FL478 and IR64) containing a total of 138 nsSNPs in their CDSs, with 1 to 3 nsSNPs per locus, having effects on protein functions (Table S2). Among the 138 nsSNPs, 15 were highly deleterious (SIFT score = 0.0) for protein functions.

Genotyping matrices were generated on the nsSNPs. Three nsSNPs were identified in the coding region of LOC_Os02g12820 (helix-loop-helix DNA binding domain-containing protein) on chromosome 2. However, only one of them had a deleterious effect on protein function. This locus had 'A' for the nsSNP at position 6749649 for all three salt stress-sensitive lines (IR64, SAL-11, and SAL-12), while it had 'G' for the nsSNP at this position for all four salt stress-tolerant lines (FL478, Pokkali, SAL-9, and SAL-10), and the same 'G' nucleotide was present in the reference genome (Fig. 5-a). The presence of the T/C nsSNP at position 2927171 between the tolerant lines (FL478, Pokkali, SAL-9, and SAL-10) and sensitive lines (IR64, SAL-11, and SAL-12) in the "flowering locus T" gene (LOC_Os06g06300) was predicted to be deleterious and changed the function of the corresponding amino acid (Fig. 5-b). Further, the T/C nsSNP is present in all three salt stress-sensitive lines, at position 4835996 in LOC_Os10g08940 (Fig. 5-d). Moreover, we also detected a G/A nsSNP in FL478 and Pokkali (the salt stress-tolerant lines) at position 31016149 in the potassium channel protein (LOC_Os03g54100), even though the other five rice lines had no nsSNP at this

position (Fig. 5-c). These two lines (FL478 and Pokkali) had similar phenotypic expression under salt stress (Table 1). In addition, we observed a G/A nsSNP only in the salt stress-sensitive line IR64, in the “white-brown complex homolog protein 11” gene (LOC_Os12g22284), but no SNP was identified in the other six lines. This gene may be playing a role in transport and response to abiotic stress.

Genetic diversity among the seven rice lines

A phylogenetic tree was constructed using nsSNPs present in the coding regions. Figure 6 shows the genetic relationships among the seven rice lines studied. Two distinct groups were identified that corresponded to the tolerant and sensitive lines, respectively. The tolerant lines SAL-9 and SAL10 were closely related to FL478 and Pokkali, while the sensitive lines SAL-11 and SAL-12 were closely related to the sensitive line IR64 (Fig. 6).

Discussion

It is necessary to improve and develop rice varieties with a high salinity tolerance to meet the increasing demand for rice. Salinity is one of the significant abiotic stress that affects the rice production. Large tracts of agricultural lands are left unproductive because of the lack of suitable salt-tolerant rice varieties (Pundir et al. 2021). To increase rice production in these barren areas, it is essential to identify novel alleles and genes that control complex quantitative salt tolerance traits in diverse rice germplasm (Kumar et al. 2015). Several studies have identified clearly IR64 to be as sensitive (Sarhadi et al. 2012; Palao et al. 2013) and FL478 as a tolerant variety at the seedling stage (Thomson et al. 2010; Zhao et al. 2014) under high salinity stress conditions. We have sequenced and analyzed in this study the GBS data of five promising salt tolerant rice lines using IR64 and FL478 as the salt stress-sensitive and -tolerant checks, respectively.

A total of 7,980,092-base high-quality reads were obtained for the seven rice lines. Although approximately 83% of the reads were mapped to the rice Nipponbare reference genome, approximately 17% were not mapped to the reference genome. This could be largely attributed to the differences between the genomes of japonica and indica subspecies (Tang et al. 2006). Sequencing errors may be another source that caused the unmapped reads, but a high read depth of 12x showed a high quality of sequencing data, as reported earlier (Mehra et al. 2015).

Our study has shown that chromosome 1 has the most significant number of variants because it is the largest in size, while chromosome 9 has the smallest number of variants. This result is consistent with that observed in Swarna, IR64, and Pokkali (Jain et al. 2014; Rathinasabapathi et al. 2015). We have identified 80,159 high-quality SNPs, with an average of 3 SNPs/100 kb, for the seven rice lines. In contrast, an average of 1 SNP/16 kb was observed from GBS in 45 California rice cultivars (Kim and Tai 2013). The difference in SNP density could be due to their sequencing depth and genetic diversity between the genotypes studied, and Nipponbare used as the rice reference genome. The observation of the highest SNP density on chromosome 10 (3.7) and the lowest SNP density on chromosome 4 (2.39) of the seven rice lines resembled that was previously reported in Swarna (Rathinasabapathi et al. 2015). Our study shows that these rice lines have a transitional to transversal SNP ratio varying from 2.2 to 2.5, which is almost the same as that (from 2.00 to 2.53) reported in the rice genome (Hu et al. 2014; Hwang et al. 2014).

A large number of nsSNPs were identified in the CDS regions of the seven rice lines, and it has already been reported in the rice genome that the number of nsSNPs is higher than the number of synonymous SNPs (Mehra et al. 2015; Rathinasabapathi et al. 2015). However, the objective of our study was to target those nsSNPs that affect protein function, especially those associated with responses to salt stress. The amino acid residues that are necessary for the stability of proteins and biological functions are often more conserved than others. Therefore, the presence of nsSNPs in the highly conserved region of a gene might play a key role at the functional level (Arai-Kichise et al. 2011). The SIFT system uses this fact to check the effects of nsSNPs on protein function as deleterious or tolerated (Kumar et al. 2009). Deleterious SNPs affect phenotype by affecting protein synthesis. On the other hand, the SNPs in the non-coding regions could not cause changes in amino acid but might affect the splice and binding sites for transcription factor at the transcriptional level (Xu et al. 2014).

Several candidate genes have been previously identified in rice that might be involved in plant response to salt stress, such as OsNHX1, OsDREB1, Salt, OsHKT1, OsCPK17, and OsLEA5 (Wang et al. 2007; Negrão et al. 2011; Zhou et al. 2016). Our study has identified five new loci whose protein functions are affected by the presence of nsSNPs in their coding regions. We found that 35 % of the nsSNP variants are the same between FL478 and IR64. It might be because both lines are from the same rice subspecies, subsp. *indica*. One nsSNP, G/A, was identified in the first exon of the HLH gene (LOC_Os02g12820.1). The presence of 'A' in the salt stress-sensitive lines at position 6749649 of the HLH gene alters protein function by replacing alanine with valine. The functional effect of this nsSNP in the sensitive lines may down-regulate the expression of the protein under salinity stress. The nsSNP identified in the first exon of LOC_Os06g06300 at position 2927171 has resulted in amino acid substitution from valine to alanine. The finding that the gene in Pokkali has 'C' and in IR64 has 'T' at position 2927171 is the same as previously reported (<http://www.nipgr.res.in/mjain.html?page=ricesnp>). The presence of the nsSNP in the LOC_Os06g06300 gene in the salt stress-tolerant lines (FL478, Pokkali, SAL-9, and SAL-10) and its absence in the sensitive lines (IR64, SAL-11, and SAL-12) suggest that this gene may be associated with plant response to salt stress and may increase its efficiency under salt stress conditions. The LOC_Os06g06300 gene was previously reported to respond to abiotic stress (Zhang et al. 2016). A genotyping matrix showed allelic variation between the tolerant and sensitive lines at position 4835996 of the LOC_Os10g08940 gene. The presence of 'C' variant in the salt stress-sensitive lines changed the tTc codon to the tCc codon (Phe to Ser). FL478 and Pokkali that are highly tolerant of high salt stress have an nsSNP in the second exon of the TPKA gene (LOC_Os03g54100) that has changed arginine into tryptophan. Since FL478 was developed from a cross of Pokkali and IR29 (Thomson et al. 2010), we conclude that this nsSNP in the TPKA gene is conserved between FL478 and Pokkali. The analysis revealed that the conserved regions for FL478, Pokkali, SAL-9, SAL-10, SAL-11, and SAL-12 are present in the LOC_Os12g22284 locus of chromosome 12, but IR64 has an nsSNP at position 12586623. IR64 is highly sensitive to salt stress; therefore, the presence of an SNP that has changed the amino acid (Thr to Met) might be down-regulating the function of a protein. Our study reports four loci containing deleterious SNPs in coding regions. Since the deleterious SNPs influence the functions of the gene coding protein, the results provide useful information to carry out further experiments necessary to validate the predicted change in protein function. The presence of these nsSNPs in

the identified functional genes could be used in forward or reverse genetics for crop improvement. Identifying SNP markers, especially those associated with salt stress tolerance, will be helpful in marker-assisted breeding for the development of superior rice cultivars that are tolerant of salinity stress.

Declarations

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Conflicts of interest The authors declare that they have no conflict of interest.

Availability of data and material

Authors' contributions RT and MA have contributed equally to this work. RT and MA designed the research. JA developed the rice genotypes. RT conducted the salinity experiment. HBZ sequenced the genomic data. RT and MA analyzed the bioinformatic data. RT wrote the manuscript. MA, HBZ and JA revised the manuscript and improved the English writing. All the authors read and approved the final manuscript.

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Figures

Figure 1
Sequence reads and their mapping to the rice reference genome

Figure 2

Distribution of SNPs (a) and InDels (b) on the rice chromosomes.

The different color bars show the numbers of SNPs and InDels in the seven rice lines

Figure 3

Venn diagrams of specific and common SNPs in seven rice genotypes

Figure 4

Frequencies of transitional and transversal SNPs in the genomes of the seven rice lines

Figure 5

Genotyping matrices of the seven rice lines for the genes containing protein function-influenced nsSNPs, LOC_Os02g12820 (a), LOC_Os06g06300 (b), LOC_Os03g5400 (c), and LOC_Os10g08940 (d).

Red regions show the nsSNP variants and blue regions show the identical bases with respect to the reference genome. Vertical yellow dashed lines represent the position of the nsSNPs. Arrows indicate the locations of nsSNPs within the CDS that discriminate tolerant genotypes from sensitive ones

Figure 6

Phylogenetic tree showing the genetic relationships among the seven rice lines constructed based on nsSNPs.

The sensitive lines are represented by red color and the tolerant lines by green color

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