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ANALYSIS OF NATURAL VARIATION IN *OsHKT1;1* GENE SEQUENCE AND GENE EXPRESSION IN RELATION TO SALINITY IN RICE (*Oryza sativa* L.)

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ABSTRACT

Rice is a main food crop, but sensitive to salinity. The high affinity K^+ transporters (HKTs) have been proven to be important to salt tolerance in plants. In the current study, the natural variations in gene sequence of *OsHKT1;1* were investigated for uncovering the potential allelic variants in salt tolerance in rice. Sequence analysis of *OsHKT1;1* gene was conducted in the coding and promoter regions of all investigated rice cultivars. There were nine SNPs in the coding sequences and ten SNPs in the promoter sequences. Amongst nine SNPs found in the coding sequences, four were nonsynonymous (C89T, C280T, T536C, A773G) leading to four substituted amino acids P30L, L94F, F179S, and N258S. *In silico* analysis revealed no potential effects of the substituted amino acids to protein structure, but caused changes in post-translational modifications. In the promoter sequences, eight out of ten SNPs caused to five additions and six deletions of the cis-regulatory elements, in which some of them were shown to be involved in stress responses. *OsHKT1;1* gene expression was analyzed in the roots and leaves in response to different salt concentrations. While the expression of *OsHKT1;1* was induced in the leaves at the beginning of salt treatment (day 1 and day 2), it was decreased or unchanged in the roots.

Keywords: *OsHKT1;1*, salinity, rice, polymorphism.

INTRODUCTION

Salinity is one major environmental problem that reduces agricultural productivity. Salt stress causes serious problems to plants by combining both ionic stress and osmotic stress. High accumulation of toxic elements, such as Na^+, leads to toxic to the cell. Therefore, regulation of Na^+ concentration in the cell plays a key role for survival of plants under saline condition (Tester and Davenport, 2003; Almeida *et al.*, 2017).

High affinity K^+ transporters (HKTs) are an essential determinant for salt tolerance in plants. Members of HKTs are proposed to protect the leaves from overaccumulation of Na^+ by mediating xylem Na^+ unloading in the roots, phloem Na^+ loading in the shoots (Berthomieu *et al.*, 2003; Sunarpi *et al.*, 2005; Ren *et al.*, 2005; Byrt *et al.*, 2007; Davenport *et al.*, 2007; Almeida *et al.*, 2017), and by enhancing exclusion of Na^+ ion from leaf blades during salt stress (Wang *et al.*, 2015; Kobayashi *et al.*, 2017). Overexpression of *AtHKT1;1* enhances salt tolerance in rice, and *GmHKT1;4*-overexpressed transgenic tobacco showed improvement of salinity tolerance (Plett *et al.*, 2010; Chen *et al.*, 2014). Cucumber grafted with salt tolerant pumpkin as rootstock showed growth enhancement under salt stress (Sun *et al.*, 2018).

The HKT protein family can be divided in two classes based on structure and transport characteristics. Class 1 HKT transporter has a serine in the first pore domain, forming an S-G-G-G motif, and shows Na^+-selective transport. Whereas, class 2 members possess a glycine with ~~an~~ G-G-G-G motif, and generally exhibit Na^+ - K^+ co-transport (Mäser *et al*, 2002; Platten *et al.*, 2006). Rice has nine HKT genes, consisting of *OsHKT1;1, OsHKT1;2, OsHKT1;3, OsHKT1;4*, *OsHKT1;5, OsHKT2;1, OsHKT2;2, OsHKT2;3,* and *OsHKT2;4*. *OsHKT1;2* is a pseudo gene in which the stop codon appears inside the gene and *OsHKT2;2* is not found in Nipponbare cultivar (Platten *et al.*, 2006). The rice class 1 members, except OsHKT1;2, show permeable to Na^+ only (Ren *et al.*, 2005; Jabnoune *et al.*, 2009; Suzuki *et al.*, 2016); while class 2 displays diverse transport properties. OsHKT2;1 is either Na^+-K^+ symport or Na^+ uniport depending on concentration of external K^+ and Na^+ (Garciadeblas *et al.*, 2003; Horie *et al.*, 2007; Jabnoune *et al.*, 2009). OsHKT2;2 shows Na^+/K^+ co-transport, while OsHKT2;4 exhibits strong K^+ permeability with ability to also transport Mg^{2+} and Ca^{2+} (Yao *et al.*, 2010; Horie *et al.*, 2011). The rice HKT genes are found to diversely express (Almeida *et al.*, 2013 and Almeida *et al.*, 2017 and reference herein). Some members of the rice HKT transporter family are involved in the salt tolerance mechanism, such as OsHKT1;1, OsHKT1;4, OsHKT1;5 (Ren *et al.*, 2005; Cotsaftis *et al.*, 2012; Wang *et al.*, 2015; Suzuki *et al.*, 2016; Campbell *et al.*, 2017; Kobayashi *et al.*, 2017).

OsHKT1;1 belongs to class 1 of rice HKT family. OsHKT1;1 has been proven to play important roles in salt tolerance of rice by enhancing exclusion of Na^+ ion from leaf blades. The regulation of Na^+ ion exclusion from the plant shoot potentially via xylem-to-phloem transfer mechanism (Wang *et al*., 2015). The *OsHKT1;1* gene was found to be highly expressed in the vascular tissue of the shoot than those of the root (Jabnoune *et al*., 2009; Wang *et al*., 2015). And *OsHKT1;1* gene was well expressed at the seedling stage (Campbell *et al*., 2017). Upon salt stress, *OsHKT1;1* expression was increased 3- to 5-fold in the shoots, but not in the roots (Wang *et al*., 2015). *OsHKT1;1* is the causal gene in the RNC4 QTL controlling the root Na^+ content and Na^+ to K^+ ratio (Campbell *et al*., 2017).

Rice (*Oryza sativa)* is a main food crop worldwide, but sensitive to salinity. Improving salt tolerance in rice is crucial. Previous works have demonstrated the importance of allelic variations in the sequences of ion transporter genes to salt tolerance in plants. A clear example showing the important role of the allelic variations to salt tolerance found in *OsHKT1;5* gene. The nucleotide substitution in the coding sequence that lead the V395L substitution in OsHKT1;5 transporter of the salt tolerant Nona Bokra cultivar compared to the salt sensitive Koshihikari cultivar enhanced Na^+ transport activity (Ren *et al*., 2005; Cotsaftis *et al*., 2012). In other findings, three nucleotide polymorphisms within *OsHKT1;1* gene were proposed to associate with modified Na^+ accumulation in the rice root (Campbell *et al*., 2017), and two nucleotide substitutions in *ZmHKT1;5* gene sequence were proven to associated with salt tolerance in maize (Jiang *et al*., 2018).

In the current study, the natural variations in the *OsHKT1;1* gene sequence were investigated in different rice cultivars. Both the coding sequence and the regulatory upstream sequence of the gene were subjected for studying, and the detected nucleotide polymorphisms were further analyzed to evaluate potential influence on protein characteristics and transcriptional/ translational regulation. In addition, *OsHKT1;1* gene expression in response to different salt concentrations was studied in two contrasting rice cultivars.

MATERIALS AND METHODS

Rice materials and stress treatment: Seeds of nine rice cultivars, including Pokkali, Nipponbare, Cuom-2, Chanh-Trui, IR29, Nuoc-Man-2, Cham-Bien, Chiem-Rong, Nuoc-Man-1 were kindly provided by Vietnam National University of Agriculture (Hanoi, Vietnam). Most of the cultivars were found in the coastal area of Vietnam. Pokkali was used as salt tolerant reference cultivar, while Nipponbare and IR29 were considered as salt sensitive reference cultivars. Plant cultivation and salt treatment were performed as described previously in Do *et al.* (2018).

Salt tolerance evaluation: Rice cultivars were evaluated for salt tolerance using modified standard evaluation score (SES) which is based on visual salt injury of plants at seedling stage (Gregorio *et al.*, 1997; Bado *et al.*, 2016). Three plants per cultivar was used.

Extraction of genomic DNA: The genomic DNA was extracted from the leaf materials using the CTAB method as described previously in Do *et al.*, 2018.

***OsHKT1;1* gene amplification using PCR technique:** *OsHKT1;1* upstream sequence and gene sequence were amplified from DNA template by PCR using specific primers, following by direct sequencing. The list of PCR primers is shown in Table 1 and primer binding sites in the cds and promoter sequences are indicated in Supplementary Fig. S1.

Each PCR mixture contained genomic DNA (about 50 ng), Dream Taq polymerase buffer (1×), MgCl2 (1.5 mmol/L), Dream Taq polymerase (1 U), primers (0.4 µmol/L), and dNTPs (0.2 mmol/L). Thermal cycle condition for each PCR reaction was conducted as following: 95°C for 5 min, 35 cycles of denature at 95°C for 30 s, annealing for 30 s and extension at 72°C for 2 min, and 72°C for 5 min. After purification the products of PCR were sequenced by the First BASE DNA sequencing service (Singapore). The obtained sequences were sent to the GenBank database. The accession numbers for gene sequences containing the coding sequences are: MK541575 (Nipponbare), MK541577 (Nuoc-Man-2), MK541576 (Nuoc-Man-1), MK541582 (Chanh-Trui), MK541578 (Cuom-2), MK541580 (Pokkali), MK541579 (IR29), MK541581 (Chiem-Rong), and MK541583 (Cham-Bien). The accession numbers for the upstream regions of *OsHKT1;1* gene are: MK541584 (Nipponbare), MK541585 (Nuoc-Man-2), MK541591 (Nuoc-Man-1), MK541586 (Cuom-2), MK541590 (Chanh-Trui), MK541587 (Pokkali), MK541588 (IR29), MK541592 (Chiem-Rong), and MK541589 (Cham-Bien).

Sequence analysis: To analyze the nucleotide sequences Multalin webserver (Corpet, 1988) and Bioedit (Hall, 1999) were used. The amino acid sequences were predicted and analyzed by using the Expasy webserver (http://web.expasy.org/translate/). The sequences of *OsHKT1;1* of different rice cultivars were compared to Nipponbare cultivar's sequence.

Table 1. Primers used for PCR amplification of *OsHKT1;1.*

	Primer name	Sequence				Amplicon size (bp)
Primer for gene sequence	CdsHKT1.1-1	FW	5'-	GGCAGCGTTACACAAGTACCA-3'	-3'	775
		RV	5'-	ACCAACACAGCGCAAGGATT-3'	-3'	
	CdsHKT1.1-2	FW	5'-	GATGCTAATGGGAGGAGAGGTG	-3'	784
		RV	5'-	TTCACCGGCTTGTCTTGTAT	-3'	
	CdsHKT1.1-3	FW	5'-	CCTGGGGAGACTGGCTACAA	-3'	680
		RV	5'-	TGAAGCATCGGAAGGAAGGTA	-3'	
	CdsHKT1.1-4	FW	5'-	TGCAGCTTGCTAGGTTTTATCG	-3'	570
		RV	5'-	TGCTGGTTCAGTGGTTCTTAGTG	-3'	
	CdsHKT1.1-5	FW	5'-	CTGATAACCAGCCCTTGACAG	-3'	688
		RV	5'-	CAATCGTGATTCGGGGTCAATC	-3'	
Primer for Upstream sequence	ProHKT1.1-1	FW	5'-	GGGTGAATTAGACATAACCATAGGA	-3'	706
		RV	5'-	ACTACGCGTTGGTTTGTGC	-3'	
	ProHKT1.1-2	FW	5'-	AGTCCATAGAAAGCAAGCGG	-3'	721
		RV	5'-	TACTCCGAAAAGGACTCGTGG	-3'	
	ProHKT1.1-3	FW	5'-	TGTAGCGACTTCCTTGACTGC	-3'	578
		RV	5'-	GACAGGAACCCACAGAGATGC	-3'	

Examination of putative changes at protein level: Several bioinformatics tools were used to determine consequent influences of the nucleotide substitutions on variant protein properties. Transmembrane protein structure prediction based on PSIPRED web server (http://bioinf.cs.ucl.ac.uk/psipred/) and UniProt database (https://www.uniprot.org/). The 3D structures of the proteins were predicted by I-TASSER program (Zhang *et al.*, 2008). The UCSF Chimera v1.13.1 was used to visualize the 3-D protein structure (Pettersen *et al.*, 2004). To analyze the protein putative post-translational modifications, the PlantPhos was applied for phosphorylation site prediction (Lee *et al.*, 2011), a web-based server http://csb.cse.yzu.edu.tw/UbiSite/ was used for ubiquitination site analysis, and SUMOplot™ (http://www.abgent.com/sumoplot) was applied to analyze the possible SUMOylation sites.

Analysis of putative cis- regulatory elements present in *OsHKT1;1* promoter: The PLACE database ~~were~~ was used to analyze the putative cis-regulatory elements present in the 1.45-kb upstream region from start codon of the gene (Higo *et al.*, 1999).

***OsHKT1;1 g*ene expression by real-time PCR:** The leaves and roots of salt-treated and control seedlings were collected at day 1, 2, 3, and 7 of salt treatments. The RNA isolation and synthesis of cDNA were performed as described in Do *et al.,* 2018.

The *OsHKT1;1* gene expression was analyzed by real-time PCR technique using the following primers: FW 5'- TGCAGTCGTCATGTACCTTCC -3', RW: 5'-CGGTAAAATTCCTCCACAGTGC -3'. PCR mixture included 1 µL of diluted cDNA, 0.6 µL of primer mix (10µM), 10 µL of SYBR Green Master Mix 2X (Luminaris Higreen low ROX qPCR master mix, Thermo Fisher Scientific) in a 20 µL reaction. The PCR reaction was run as: 95^0C for 10 min, then 40 cycles of (95^0C for 15s, 60^0C for 1 min) in ABI Fast 7500 System (Applied Biosystems). Actin1 was used as reference gene (Caldana *et. al.,* 2007) with primers FW: 5'-CTCCCCCATGCTATCCTTCG-3', RW: 5'-TGAATGAGTAACCACGCTCCG-3'. The specificity of the PCR reaction was assessed by analyzing melting curve and agarose gel electrophoresis. The expression levels of *OsHKT1;1* gene were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Two biological replicates and two technical replicates were performed.

RESULTS

Variation in salinity tolerance among rice cultivars: The nine rice cultivars were cultivated using hydroponic culture system and were treated with salt stress (100mM NaCl) at the seedling stage. As described in study of Gregorio *et al.* (1997) the scoring of visual injury symptom at seedling stage is the reliable parameter for distinguish the tolerant, moderate, and susceptible groups in rice. In our previous study, the seven out of nine rice cultivars were classified into different salt tolerant levels based on the leaf scoring (Do *et al.*, 2018). In which, Pokkali, Chanh-Trui, Nuoc-Man-1, Cuom-2 were considered as salt tolerant; Nuoc-Man-2, Chiem-Rong were classified moderate; and Nipponbare was defined as sensitive. In this study, two rice cultivars, IR29 and Cham-Bien, were included. The scoring of Cham-Bien was 3.0 and IR29 was 9.0. Thus, Cham-Bien was

classified as tolerant and IR29 was considered highly susceptible.

Variations in the *OsHKT1;1* coding sequence: In order to identify the polymorphisms in the *OsHKT1;1* gene sequence, the gene was firstly amplified by the PCR technique using different primer pairs (Table 1). After sequencing, only the coding sequence of the gene was further analyzed. Using Nipponbare culvitivar gene sequence as reference, sequencing analysis could allow to identify nine nucleotide substitutions in the *OsHKT1;1* coding sequence (Fig 1). The alignment of protein sequences is shown in Supplementary Fig. S2.

Amongst nine detected SNPs, four were non-synonymous (C89T, C280T, T536C, A773G) and other five were synonymous substitutions (T189C, T225C, A321G, A885C, C912T). The non-synonymous C89T, C280T, T536C, A773G caused the amino acid changes, including P30L, L94F, F179S, N258S, respectively (Table 2).

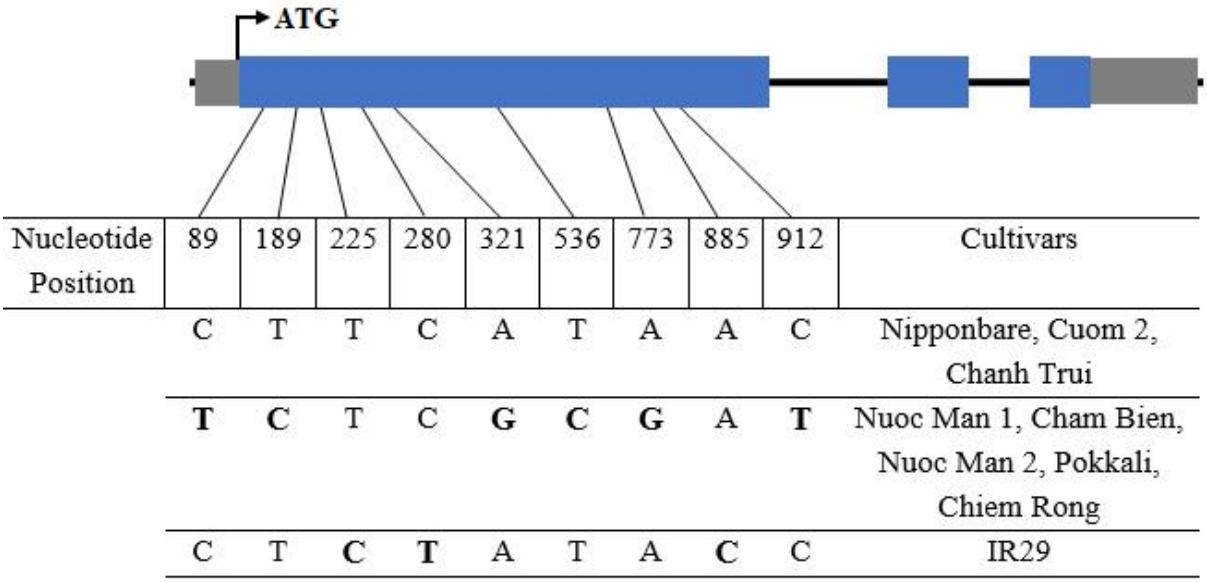

Figure 1. Allelic polymorphisms in the *OsHKT1;1* coding region. Bold letters indicate the nucleotide polymorphisms.

Table 2. Putative effect of nucleotide polymorphisms present in the *OsHKT1;1* coding region.

Position	Polymorphic type		Effect on post-translational modification	Cultivars
89	C/T	Non-Synonymous (**P30L**)		Chiem-Rong, Cham-Bien, Nuoc-Man-2, Pokkali, Nuoc-Man-1
189	T/C	Synonymous		
225	T/C	Synonymous		IR29
280	C/T	Non-Synonymous (**L94F**)	Sumoylation at 92K	
321	A/G	Synonymous		
536	T/C	Non-Synonymous (**F179S**)	Phosphorylation at 179S, 181S	Chiem-Rong, Cham-Bien, Nuoc-Man-2, Pokkali, Nuoc-Man-1
773	A/G	Non-Synonymous (**N258S**)	Phosphorylation at 254S Ubiquitination at K255	
885	A/C	Synonymous		IR29
912	C/T	Synonymous		Chiem-Rong, Cham-Bien, Nuoc-Man-2, Pokkali, Nuoc-Man-1

To elucidate the putative influence of nucleotide variations on protein characteristics, the structural model of OsHKT1;1 protein was predicted and the positions of these non-synonymous substitutions on protein domains were analyzed. As shown in Fig 2B, the OsHKT1;1 3D model has the presence of three glycine (Gly 275, Gly

399, and Gly 501) and one serine (Ser 119), forming a selectivity filter pore. OsHKT1;1 possesses eight transmembrane domains (TMDs) with four pore loops A, B, C, and D (Fig 2A). The substituted amino acid P30L is located in the N-ternimal cytosolic region, L94F and N258S lay in the outer loops between TMD 1 and TMD2 and between TMD 3 and TMD 4, respectively, and F179S is positioned in inner loop between TMD 2 and TMD 3 (Fig 2). Relied on the predicted structure, these substitutions unlikely interfere the Na^+ transport activity of the variant OsHKT1;1 transporter.

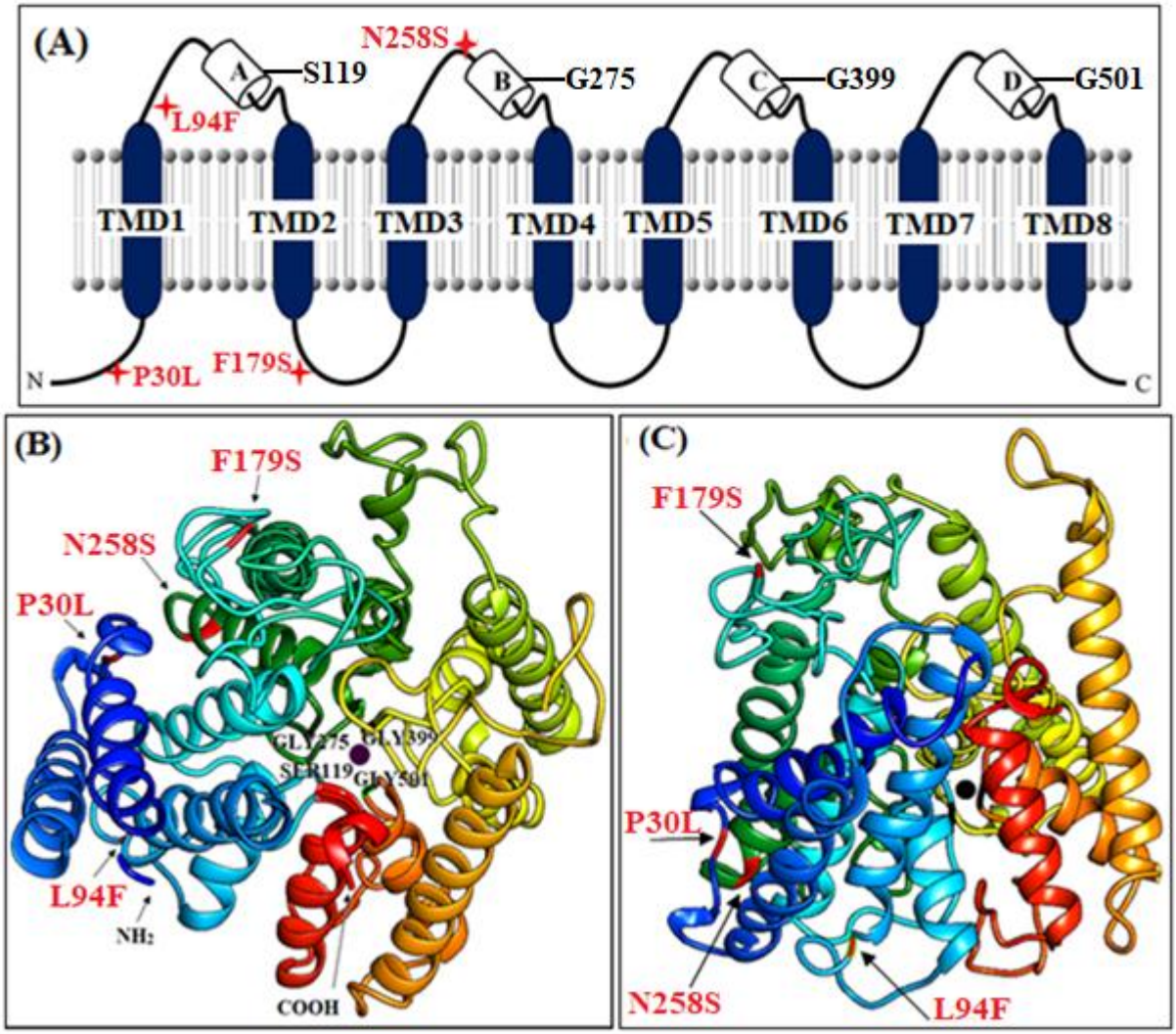

Figure 2. Modelling of OsHKT1;1 protein structure. A: The 2D model with motif of four transmembrane–pore loop–transmembrane domain. Asterisks indicate positions of substituted amino acids. B: 3D model of the OsHKT1;1 transporter from the top/from the side. The Ser-Gly-Gly-Gly motif forms the ion filter pore. The black dot at center of the pore represents Na^+ ion. C: Visualization from the side of protein model, showing positions of four amino acid variants.

Additionally, the consequent effects of the amino acids on putative post-translational modifications, such as ubiquitination, SUMOylation and phosphorylation of OsHKT1;1 protein were analyzed. The substitutions of phenylalanine to serine (F179S), and asparagine to serine (N258S) provided two serine residues which were predicted to be phosphorylated. The substitutions of phenylalanine to serine (F179S) increased the probability of phosphorylation at 181-serine (Table 2). The N258S substitution also increased the probability of ubiquitination at 255-lysine residue. And the substitution of leucine to phenylalanine (L94F) increased the SUMOyl probability of 92-lysine residue. The putative phosphorylation and ubiquitination were found in cultivars Chiem-Rong, Cham-Bien, Nuoc-Man-2, Pokkali, Nuoc-Man-1; while the predicted SUMOylated

Table 3. Polymorphisms in the *OsHKT1;1* upstream sequence

Position	Type of Polymorphism	Cis-regulatory element	Number of change	Functions	References	Cultivars
-1412	**T/C**	GT1CONSENSUS	-1	Light responsive element	Le Gourrierec *et al.*, 1999	Chiem-Rong, Nuoc-Man-1, Nuoc-Man-2, Cham-Bien, Pokkali.
-1353	**G/A**	ARE1	-1	Antioxidant responsive element	Rushmore et al., 1991	
		BIHD1OS	+1	Disease resistance responsive element	Luo *et al.*, 2005	
		WRKY71OS	+1	GA-responsive element	Zhang *et al.*, 2004	
-1271	**A/G**	LECPLEACS2	-1	Elicitor responsive element	Matarasso *et al.*, 2005	
-1132	G/T					
-505	**C/T**	SURECOREATSULTR11	+1	Core of sulfur responsive element	Maruyama *et al.*, 2005	Cuom-2
		POLASIG3	+1	Plant polyadenylation signal	Joshi (1987)	
-373	**A/T**	LECPLEACS2	-1	Elicitor responsive element	Matarasso *et al.*, 2005	
-372	**T/A**	ROOTMOTIFTAPOX1	-2	Root-specific cis-element	Elmayan *et al.*, 1995	
-183	A/C					Chiem-Rong, Nuoc-Man-1, Nuoc-Man-2, Cham-Bien, Pokkali, IR29.
-132	**G/A**	DOFCOREZM	+1	Dof proteins binding site, response to some stresses	Yanagisawa *et al.*, 1999; Corrales *et al.*, 2014	Nuoc-Man-1, Nuoc-Man-2, Cham-Bien, Cuom-2.
-57	**A/G**	CACTFTPPCA1	-1	Mesophyll-specific Cis-element	Gowik *et al.*, 2004	Chiem-Rong, Nuoc-Man-1, Nuoc-Man-2, Cham-Bien, Pokkali.

L94F was observed in only cultivar IR29 (Table 2). If these predicted amino acid residues are indeed sites for post-translational modifications, they might alter the functions of the variant OsHKT1;1 transporter.

Variations in the *OsHKT1;1* upstream region: The *OsHKT1;1* upstream sequence was amplified using PCR technique. Different primer pairs were designed to amplify three amplicons which together covering the 1.45 kb upstream sequence of *OsHKT1;1* gene. To elucidate the nucleotide variation in this regulatory region of *OsHKT1;1* gene, the amplified DNA fragments were then sequenced. By comparing the nucleotide sequences, ten nucleotide substitutions (T-1412C, G-1353A, A-1271G, G-1132T, C-505T, A-373T, T-372A, A-183C, G-132A, A-57G) were identified (Table 3). The alignment of nucleotide sequences is shown in Supplementary Fig.S3. Since the nucleotide substitutions can cause changes in cis-regulatory elements, which in turn affect the regulatory function of promoter, the putative cis-regulatory elements in *OsHKT1;1* promoter were predicted using PLACE database. The result showed that 8 out of 10 nucleotide substitutions led to cis-regulotary elements change (Table 3). Amongst them, some of the changed cis- regularoty elements are involved in stress responses, such as SURECOREATSULTR11, GT1CONSENSUS, DOFCOREZM, and WRKY71OS. SURECOREATSULTR11 is responsive to sulfur deficiency conditions in Arabidopsis (Maruyama-Nakashita *et al.*, 2005); GT1CONSENSUS is known as cis-regulatory elements responsive for light regulation (Le Gourrierec *et al.*, 1999). WRKY71OS is a binding site of transcriptional repressor WRKY71 involved in the gibberellin signaling in rice (Zhang *et al.*, 2004). DOFCOREZM with the motif "AAAGT" sequences is a binding site of Dof protein which play roles in plant abiotic stress responses (Yanagisawa *et al.*, 1999, Corrales *et al.*, 2014). The cis-element, DOFCOREZM, which is responsive to salt stress is represented in salt-tolerant cultivars, such as Nuoc-Man-2, Chiem-Rong, Nuoc-Man-1, Cham-Bien, and Pokkali. The full list of cis-regulatory elements found in the *OsHKT1;1* upstream sequence is shown in Supplementary Table 1.

Gene expression analysis under salinity conditions: The expression level of *OsHKT1;1* gene in response to different salt stresses (50 mM and 100 mM NaCl) was investigated by using quantitative real-time RT-PCR technique. As shown in Fig 3, *OsHKT1;1* gene expression was generally not changed under salt stress in the Nipponbare roots (Fig 3A), but decreased in the Pokkali roots (Fig 3B). In the leaves, an increase of gene expression was observed at day 1 and day 2 in both cultivars (Fig 3C, D), but prolongation of the salt treatment did not induce the expression of *OsHKT1;1.* Generally, the expression of *OsHKT1;1* was higher in the leaf than in the shoot, which was observed in both cultivars; and the expression of the gene was higher in Pokkali cultivar compared to that in Nipponbare cultivar.

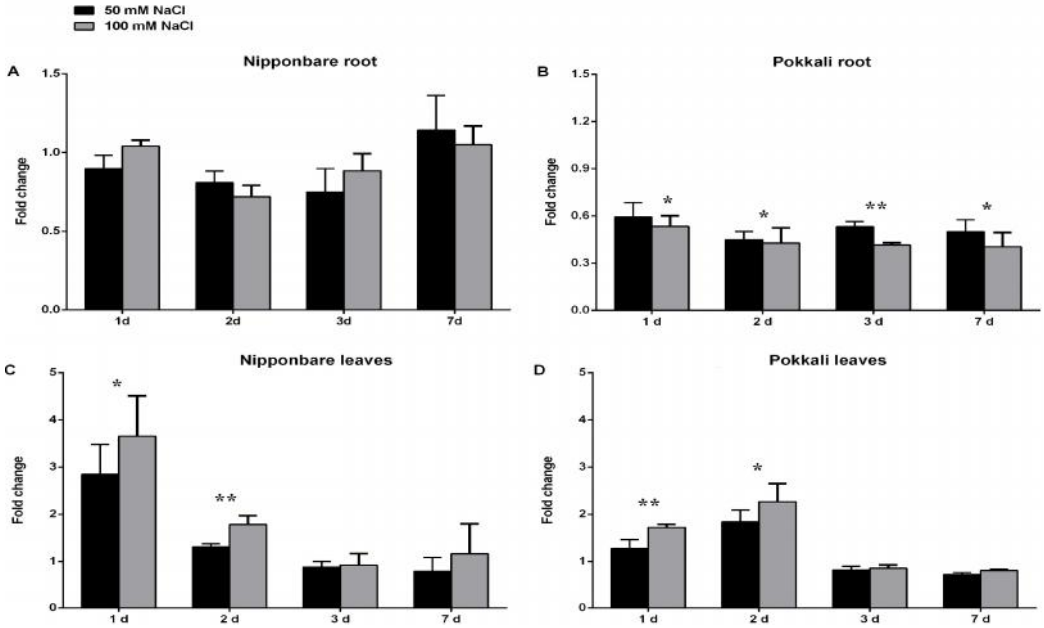

Figure 3. *OsHKT1;1* expression profile under different salt conditions. The expression level of *OsHKT1;1* gene was computed with the expression level of actin 1. Fold changes were calculated by using ΔΔCt method (Livak and Schmittgen, 2001). Values represent the mean and standard deviation of two biological replicates with two technical replicates per biological experiment (n=4). The asterisk indicates the significant difference; in which one asterisk shows significantly differences between salt and control conditions, two asterisks means significantly differences between salt and control conditions as well as between two salt conditions.

DISCUSSION

Though rice is an important food crop worldwide, it is particularly sensitive to soil salinity. Exploring natural genetic resources to find novel alleles for salt tolerance improvement of crop plants is of importance. In the current work, the natural variation in the sequence and the expression of *OsHKT1;1* gene was investigated. We could identify in total nine nucleotide variations in the coding sequence and ten ones in the promoter sequence of the *OsHKT1;1* (Fig 1, Table 3).

In the coding sequence, four nonsynonymous SNPs were detected, including C89T, C280T, T536C, A773G, which caused the amino acid changes; P30L, L94F, F179S, and N258S, respectively (Table 2). The three substituted amino acids (P30L, F179S, N258S) were observed in the salt-tolerant cultivars Cham-Bien, Chiem-Rong, Nuoc-Man-1, Pokkali, Nuoc-Man-2, while L94F was found in the salt-sensitive cultivar IR29. In order to predict the putative effects of these allelic variants, the protein structure model of OsHKT1;1 was conducted. Based on molecular models of OsHKT1;1, the P30L is located in the N-ternimal cytosolic region, L94F and N258S are laid in the outer loops and F179S is positioned in inner loop (Fig 2). The selective filter pore with ~~an~~ S-G-G-G motif was found with presence of three 3 glycine residues (Gly 275, Gly 399, and Gly 501) and one serine residue (Ser 119). This predicted S-G-G-G motif of OsHKT1;1 is in agreement with report of high selectivity for Na^+ of OsHKT1;1 transporter (Jabnoune *et al.*, 2009). Because four amino acid variants are not located in or close to the filter pore of OsHKT1;1 transporter, they are unlikely to have a direct influence in Na^+ transport of the variant OsHKT1;1 protein However, in the previous study of Campbell *et al.* (2017), the three amino acid variants (P30L, F179S, N258S) were described to associate with modified Na^+ accumulation in the roots and responsible for higher Na^+ transport activity. We next thought that the effect of these variants on the OsHKT1;1 transporter's functions might be at post-translational regulations. Thus, some post-translational modifications were analyzed using available bioinformatics tools. The results showed that the F179S, N258S alterations could affect the putative phosphorylation of serine residues at position 179 and 258 of OsHKT1;1 protein. The F179S substitution not only was predicted to be phosphorylated serine 179, but also increased the probability of phosphorylation of serine 181. Furthermore, N258S substitution was predicted to enhance the probability of ubiquitination of lysine residue at position 255 and L94F alteration increased the sumoylation probability of lysine 92 (Table 2). It is known that post-translational modifications are involved in the process of activation or suppression of the protein's activity (Friso and Wijk, 2015; Barber and Rinehart, 2018). The post-translational modifications are important factors that can be regulated to influence salinity tolerance in plants (Roy *et al.*, 2014). Salt overly sensitive 1 (SOS1), an Na^+/H^+ antiporter, is activated through a protein phosphorylation process by SCaBP8–SOS2 (Quan *et al.*, 2007) or by SOS2-SOS3 complex (Qiu *et al.*, 2002; Quintero *et al.*, 2011). SOS1 functions in Na^+ extrusion and hence plays roles in plant salt tolerance (Shi *et al.*, 2000; Olías *et al.*, 2009). The NHX antiporters are also the key determinants of salt tolerance in plants, which are involved in the Na^+ sequestration into vacuole, thereby maintaining the ion homeostasis (Rodríguez-Rosales *et al.*, 2009). Phospho-proteomic studies of rice plasma membrane and vacuolar membrane indicated that the NHXs are regulated by phosphorylation (Whiteman *et al.*, 2008). In the study of Negrão *et al.* (2013), a substitution of serine 477 to asparagine (S477N) in OsNHX1 caused loss of a putative phosphorylation site, and therefore may affect the regulation of this transporter, consequently interfering its function. The reversible conjugation of the SUMO to target protein (sumoylation) may attenuate degradation of target protein (Miura *et. al.*, 2007). Srivastava *et. al.* (2016) reported that "the control of SUMOylation by OsOTS1 affects salt tolerance in rice". The post-translational regulations ~~is~~ are of complexity, allowing the organism to smoothly response to environmental changes. For example, in yeast, nitrate transporter (YNT1) transports nitrate and nitrite into the cell. But, adding glutamine to cells grown in nitrate causes a rapid loss of YNT1 activity. This phenomenon is due to the post-translational regulation. When suppling the cells with low nitrate concentration, YNT1 is phosphorylated, preventing YNT1 delivery to vacuole and also decreases ubiquitination. Thus, YNT1 accumulates at plasma membrane, allowing yeast to efficiently use nitrate. When the cells are transferred to medium with glutamine, YNT1 is dephosphorylated, and ubiquitinated. It is then delivered to vacuole for degradation (Navarro *et al.*, 2006; Navarro *et al.*, 2008). Although there are very few experimental reports about the post-translational regulation of HKT- transporter members, this is an interesting possibility to consider. In the current study, the two nonsynonymous (F179S, N258S) of OsHKT1;1 caused the post-translational modifications (phosphorylation, ubiquitination) were observed in salt-tolerant cultivars Cham-Bien, Chiem-Rong, Nuoc-Man-1, Pokkali, Nuoc-Man-2; while the nonsynonymous L94F leads to sumoylation modification which was found only in salt-sensitive cultivar IR29. We assume that the post-translational modifications might play a role in regulation of OsHKT1;1 activity, the difference in post-translational regulation might explain the formation of salt-tolerant or salt-intolerant phenotypes. In addition, one possibility for the difference in salt tolerance of rice plants is the expression level of *OsHKT1;1* gene. Therefore, we next

examined the relative expression of *OsHKT1;1* gene under different salinity conditions using two contrasting rice genotypes, Pokkali and Nipponbare.

In our study, the expression of *OsHKT1;1* was upregulated upon salt stress in the leaves of both rice cultivars, but not in the roots. In the roots, *OsHKT1;1* expression level was decreased in Pokkali, but remained unchanged in Nipponbare (Fig. 3). In the previous work, *OsHKT1;1* gene expression was reported to induce 3- to 5- fold by salt stress in the shoots, but not in the roots of Nipponbare cultivar (Wang *et al.*, 2015). Thus, the expression change pattern of *OsHKT1;1* observed in Nipponbare cultivar in our study is in agreement with the results of Wang *et al.*, 2015. However, Garciatablas *et al.*, 2003 reported that the *OsHKT1;1* (*OsHKT4*) expression was marginally changed upon stress conditions. The differences in expression pattern of *OsHKT1;1* in the work of Garciatablas *et al.* (2003) might come from the experimental design where a combination of different concentrations of K^+ and Na^+ was used instead of only Na^+ as in our study. OsHKT1;1 is known to functions in regulating Na^+ exclusion from the shoot of rice (Wang *et al.*, 2015). Thus, the upregulation of *OsHKT1;1* expression in the leaves under salt stress of two rice cultivars might help to exclude the Na^+ from the leaves in response to salt treatment. If indeed this transporter mainly functions in the shoot tissue of rice plants, the regulation of OsHKT1;1 at transcriptional level might be not different between Pokkali and Nipponbare in response to salt stress as the expression pattern in the shoots of both cultivars was similar. Upon salt stress, both rice cultivars showed upregulation of *OsHKT1;1* gene expression in the shoots.

The upstream sequence of the gene with presence of cis- regulatory elements plays the key roles in regulating gene expression (Mariño-Ramírez *et al.*, 2009). In the upstream region of *OsHKT1;1* gene, we could detect in total ten nucleotide substitutions, in which eight caused change in cis-regulatory elements. Amongst these changed cis-elements, some are stress responsive elements, including SURECOREATSULTR11 (sulfur responsive), GT1CONSENSUS (light responsive), DOFCOREZM (salt responsive), and WRKY71OS (GA responsive). The addition of one salt-stress responsive cis-element, DOFCOREZM, was observed in salt-tolerant cultivars, such as Chiem-Rong, Nuoc-Man-1, Nuoc-Man-2, Cham-Bien, and Pokkali (Table 3). However, the DOFCOREZM cis-element present in high number (16) at promoter region of *OsHKT1;1* gene (Supplementary Table 1). Thus, it might unlikely that one addition in this cis-element influences gene expression under salt stress, but further experiment should be performed to validate.

In conclusion, we could detect nine SNPs in the coding sequence, and ten SNPs in the upstream sequence of the *OsHKT1;1* gene. *In silico* analysis showed that the SNPs in the coding sequence caused no effect on protein structure, but affected on post-translational modifications of protein. The SNPs in the upstream sequence of the gene led to changes in several cis-regulatory elements, in which some are involved in abiotic stress response. *OsHKT1;1* gene expression in response to different salt stress was upregulated in the leaves, but not in the roots.

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