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OsHKT1;3 gene sequence polymorphisms and expression profile in rice (*Oryza sativa* L.)

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Rice is sensitive to salt stress, but its sensitivity varies among genotypes, indicating natural variation in regulatory mechanisms and genetic makeup. High-affinity potassium transporters (HKTs) that transport cations across membranes play important roles in stress responses of plants. In this study, the gene sequence polymorphisms and expression level of *OsHKT1;3* which is a member of the rice *HKT* gene family was assessed. Sequence analysis indicated 5 single nucleotide polymorphisms (SNPs) in the coding sequence; 4 nucleotide substitutions, one nucleotide deletion and one nucleotide insertion in the promoter region of *OsHKT1;3* gene. Among 5 SNPs in the coding sequence, one was non-synonymous (C598G) which caused the change in amino acid L200V and 4 were synonymous substitutions (A798C, G2083A, T2101C, C2122T). The substituted amino acid L200V was predicted to locate in the third transmembrane segment of OsHKT1;3 protein. In the promoter region, 3 nucleotide substitutions at position -879, -453, and -202 caused the change in cis-elements with 8 deletions and 3 additions. Expression levels of *OsHKT1;3* were analyzed in the leaves and the roots under 2 different salt concentrations and showed a tendency of reduction in most of the conditions.

Keywords: *OsHKT1;3*, salt stress, rice, polymorphism.

INTRODUCTION

Salt stress is a severe abiotic stress reducing the productivity of crop plants. Salinity affects plant growth by both osmotic and ionic stresses (Zhu, 2002; Tester and Davenport, 2003; Bressan et al., 2009). To overcome with the toxicity of elevated Na^+ level, plants developed different mechanisms to regulate the Na^+ content by minimizing Na^+ influx into cells, maximizing Na^+ efflux out of the cells, and promoting Na^+ sequestration into the vacuole. These activities are mediated by specific transporters (Chinnusamy et al., 2005).

High-affinity potassium transporters (HKTs) are plant-specific proteins that transport cations across membranes (Almeida et al., 2013). The *HKT* gene family is segregated into 2 sub-classes. Class 1 consists of Na^+-selective transporters having a serine at

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the first pore domain, whilst members of class 2 have a glycine at this position (with an exception of OsHKT2;1) and comprise transporters permeable to Na^+ and K^+ (Platten et al., 2006; Hauser and Horie, 2010). The gene number of class I differs amongst plants (Garciadeblas et al., 2003; Huang et al., 2008). Though arabidopsis has only one *AtHKT1;1* gene, rice contains 4 to 5 depending on cultivar, including *OsHKT1;1, OsHKT1;2, OsHKT1;3, OsHKT1;4* and *OsHKT1;5* (Garciadeblas et al., 2003; Platten et al., 2006). Some members of the class I play roles in salt tolerance of the plants by retrieving Na^+ from the xylem sap and preventing Na^+ to accumulate in the shoots (Berthomieu et al., 2003; Byrt et al., 2007; Sunarpi et al., 2005; Ren et al., 2005; Sun et al., 2018), and by excluding Na^+ from leaf blades (Cotsaftis et al., 2012; Wang et al., 2015; Suzuki et al., 2016; Kobayashi et al., 2017).

Natural genetic polymorphisms are proven to contribute to stress tolerance in plants. Hence, investigation of these natural variations helps to illustrate the underlying mechanisms of stress responses (Baxter et al., 2010; Brady et al., 2005; Rus et al., 2006). In the report of Ren et al. (2005) by using a population derived from the salt-tolerant cultivar Nona Bokra and the salt-sensitive cultivar Koshihikari, they could identify *OsHKT1;5* as the candidate gene for salt QTL and the allelic variation of the Nona Bokra potentially contributed to an increase in Na^+ transport activity. Later, Cotsaftis et al. (2012) proved that the V395L substitution present in OsHKT1;5 transporter protein of Nona Bokra could be responsible for the change in Na^+ transport activity. Additionally, in the study of Negrão et al. (2013) other two substitutions in OsHKT1;5 were shown to be significantly associated with salt-tolerance related traits. By analyzing genetic variation of *OsHKT2;1* gene in a collection of 49 rice cultivars, Oomen et al. (2012) identified in total nine SNPs, but no considerable effect on transport properties was found. However, a new rice HKT *OsHKT2;2/1*gene was identified in the highly salt-tolerant cultivar Nona Bokra (Oomen et al., 2012). Recently, using a rice diversity panel for genome-wide association mapping, Campbell et al. (2017) detected three non-synonymous variants within *OsHKT1;1* gene associated with altered Na^+ accumulation in the root. Using the same approach of association mapping, Jiang et al. (2018) could detect 2 SNPs present in the coding region of *ZmHKT1;5* which were significantly associated with salt tolerance in maize.

OsHKT1;3 is a member of class I of HKT gene family in rice. This gene is expressed in both the roots and the leaves and encodes protein which transports selectively Na^+ (Jabnoune et al., 2009). Till now, to our knowledge, there is only one study on the nucleotide polymorphisms of *OsHKT1;3* gene sequence which focused on wild rice (Mishra et al., 2016). Hence, in this study, variations in the *OsHKT1;3* gene sequence including the promoter region using different rice genotypes was analyzed. The polymorphisms present in the coding and the promoter sequences were further analyzed *in silico* to elucidate the potential effect on either protein properties or transcriptional regulatory via cis-regulatory elements, respectively. The expression profile of *OsHKT1;3* gene in the roots and the leaves under different salt conditions were analyzed using real-time RT-PCR.

MATERIALS AND METHODS

Plant cultivation and salt treatment

Seeds of 7 rice (*Oryza sativa* L.) cultivars, consisting of Nipponbare, Chiem Rong, Nuoc Man 1, Nuoc Man 2, Cuom 2, Chanh Trui, and Pokkali, were kindly supplied by Vietnam National University of Agriculture (Hanoi, Vietnam). The seeds were germinated for 4 days. Then, the rice seedlings were grown in Yoshida solution (Yoshida, 1976) and placed either in a greenhouse for phenotyping or in a growth chamber (12 h days with 500 µE m^{-2} s^{-1} at 26°C and 12 h night at 22°C) for gene expression analysis. The growth media were renewed every week. After 14 days of normal growth, the media were replaced for media with the appropriate salt concentrations (0, 50, and 100 mM NaCl). Stress treatment was performed for 7 days at 50 and 100 mM NaCl in a gene expression experiment, and for 14 days at 100 mM NaCl in phenotyping experiment.

Evaluation of salt tolerance

The leaf scoring was performed for salt-treated plants based on modified standard evaluation score (SES) of visual injury symptom at seedling stage of rice as described in Gregorio et al. (1997) and Bado et al. (2016).

Total DNA extraction from the leaves

The DNA extraction was carried out using the CTAB method. About 200 mg leaf powder was thoroughly mixed with 500 µL CTAB buffer. After incubating at 65°C for 20 min, 500 µL CI (chloroform: isoamylalcohol) was added. The collected supernatant was mixed with cold isopropanol for 15 min. After centrifugation, the DNA pellet was collected and washed with 70% ethanol. Then, the DNA pellet was left to dry at room temperature. The DNA was dissolved in Tris-EDTA buffer and stored at -20°C for further usage.

Amplification of entire *OsHKT1;3* gene by PCR

The 1942-bp promoter fragment and 2465-bp fragment covering entire gene sequence of *OsHKT1;3* were amplified separately from genomic DNA material by PCR technique. Primers used for amplifying the *OsHKT1;3* coding sequence are cds-FW (5′-CACCACTAACTCTTTGATGCTGA-3′) and cds-RW (5′-GCTAAGCTCGAATCTGTCGC-3′); and for amplifying the *OsHKT1;3* promoter region are Pro-FW (5′-TCGTCTAAAGGATGGCAATGA -3′) and Pro-RW (5′-CAGCAAAGGAGATCAGGGCAA-3'). The PCR reaction contained DNA (20 to 50 ng), dNTPs mixture (0.2 mmol/L), $MgCl_2$ (1.5 mmol/L), primers (0.4 µmol/L), Dream Taq polymerase (1 U), and Dream Taq polymerase buffer (1×). The thermal cycle of PCR reaction was 95°C for 5 min, 35 cycles of 95°C for 30 s, 58°C (cds

primers)/59°C (promoter primers) for 30 s and 72°C for 2 min, and 72°C for 5 min. Then, 5 µL of PCR products were run on 1% agarose gel. After purified using GeneJET PCR Purification Kit (Thermo Fisher Scientific), the PCR products were sent to the First BASE DNA sequencing service (Singapore) for sequencing. The sequences were submitted to GenBank database under accession numbers MH727499 for Nipponbare cds, MH727500 for Nuoc Man 2 cds, MH727501 for Chiem Rong cds, MH727502 for Nuoc Man 1 cds, MH727503 for Chanh Trui cds, MH727504 for Cuom 2 cds, MH727505 for Pokkali cds, MH727492 for Nipponbare upstream region, MH727493 for Chiem Rong upstream region, MH727494 for Nuoc Man 1 upstream region, MH727495 for Nuoc Man 2 upstream region, MH727496 for Cuom 2 upstream region, MH727498 for Chanh Trui upstream region, and MH727497 for Pokkali upstream region.

Sequence analysis

Bioedit (Hall, 1999), Multalin webserver (Corpet, 1988), and Expasy web server (http://web.expasy.org/translate/) were used to analyzed sequences. The coding and promoter sequences of *OsHKT1;3* of all cultivars were compared to those of Nipponbare.

Construction of 3D model of OsHKT1;3 protein

The PHYRE2 program (Kelley et al., 2015) and SWISS-MODEL (http://swissmodel.expasy.org; Biasini et al., 2014) were used to predict the 3D model of OsHKT1;3 protein. The discovery studio 4.5 visualizer was used to visualize the protein structure.

Prediction of putative cis-regulatory elements in the promoter regions of *OsHKT1;3*

Nipponbare 2-kb upstream sequence from the start codon of *OsHKT1;3* gene was taken from the MSU rice genome annotation database (http://rice.plantbiology.msu.edu/; Kawahara et al., 2013) (this region was re-sequenced in this study also). The putative cis-elements present in the promoter regions of Nipponbare and other cultivars were predicted by using PLACE database (Higo et al., 1999; http://www.dna.affrc.go.jp/PLACE/).

RNA isolation and first-strand cDNA synthesis

The leaves and the roots of both control and salt-stressed seedlings were harvested at days 1, 3, and 7 of salt treatments. The leaf/root materials of different plants per cultivar and treatment were homogenized using a ball mill (Mixer Mill MM 400, Retsch, Germany) and equally pooled. Total RNA was extracted using GeneJET Plant RNA purification Kit (Thermo Fisher Scientific, USA). RNA concentration was examined photometrically using NanoDrop ND-1000 UV-Vis spectrophotometer (Nanodrop Technologies, Wilmington, DE). The genomic DNA was removed by using DNase I (Thermo Fisher Scientific, USA) and the absence of DNA was checked by PCR for amplification of OsHKT2;1 intron sequence (FW: 5'-ATCATCAGGTGTGTTCCTCTCTC-3', RW: 5'-CATTGGCTTGATGCCCAGTGT-3'). 1 µg of purified RNA was used to transcribe into cDNA using Revert-Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA).

Quantitative real-time PCR for gene expression analysis

The transcript levels of *OsHKT1;3* gene in different samples were quantified by real-time PCR analysis using specific primers for *OsHKT1;3* (FW: 5'- TTTGCATCACAGAACGGGAC-3', RW: 5'-TCCATATGCACTGACGACTTC-3'). The reference gene, *Actin1*, was used to normalize variance in the amount of input cDNA. Each real-time PCR reaction contained 1 µL of diluted cDNA, 10 µL of SYBR Green Master Mix 2X (Luminaris Higreen low ROX qPCR master mix, Thermo Fisher Scientific, USA), 0.6 µL of primer mix (10 µM) in a total reaction volume of 20 µL. The thermal cycle of PCR was performed as: 95°C for 10 min, 40 cycles of (95°C for 15s, 60°C for 1 min) in 96-well optical reaction plates employing ABI Fast 7500 System (Applied Biosystems, Foster City, CA). The relative mRNA levels of *OsHKT1;3* gene (described as fold change) in different samples were computed using the $2^{-\Delta\Delta Ct}$ method as described previously by Livak and Schmittgen (2001).

RESULTS

Classification of salt tolerance

The rice plants were subjected to salt stress at the vegetative stage using hydroponic culture. The modified standard evaluation score (SES) of visual injury symptom at seedling stage has been proven to be the reliable parameter for discriminating amongst the susceptible, the tolerant, and the moderate groups (Gregorio et al., 1997). Thus, in this study, modified SES was used for classification of salt tolerance of rice. As shown in Table 1, the cultivars Pokkali, Chanh Trui, Cuom 2, and Nuoc Man 1 were classified as salt-tolerant; while Chiem Rong and Nuoc Man 2 were moderate. Nipponbare was sensitive.

Polymorphisms in the coding sequence of *OsHKT1;3* gene

The *OsHKT1;3* gene sequence is 2325 bp in length with 3 exons and 2 introns. To investigate the genetic variation in the *OsHKT1;3* coding sequence, the entire gene sequence of *OsHKT1;3* was amplified by PCR using specific primers. The amplified DNA fragments were further sequenced allowing the detection of five SNPs in the coding sequence (Figure 1). Among 5 SNPs in the coding sequence, one was non-synonymous (C598G) and 4 were synonymous substitutions (A798C, G2083A, T2101C, C2122T). These variants were found in Chanh Trui, and Nuoc Man 1.

The non-synonymous C598G led to the amino acid change of L200V. To elucidate the putative effect of the non-synonymous (C598G) on protein structure, the 3D molecular model of OsHKT1;3 transporter was predicted and the position of substituted amino acid L200V on protein domains was analyzed. The 3D models of OsHKT1;3 shows the presence of 3 glycine residues (Gly247, Gly371, and Gly471) and one serine residue (Ser93) forming a selectivity filter (Figure 2B). The substituted amino acid (L200V) locates in the third transmembrane segment of OsHKT1;3 (Figure 2A and C). It was concluded that this substitution unlikely interferes with the capacity of Na^+ transport of the variant

Table 1. Salinity score of rice plants under salt treatment.

Cultivar	Salinity score	Reaction to salinity
Pokkali	2.3	Tolerant
Chanh Trui	2.3	Tolerant
Cuom 2	3.7	Tolerant
Nuoc Man 1	3.7	Tolerant
Chiem Rong	4.3	Moderate
Nuoc Man 2	5.7	Moderate
Nipponbare	7.7	Susceptible

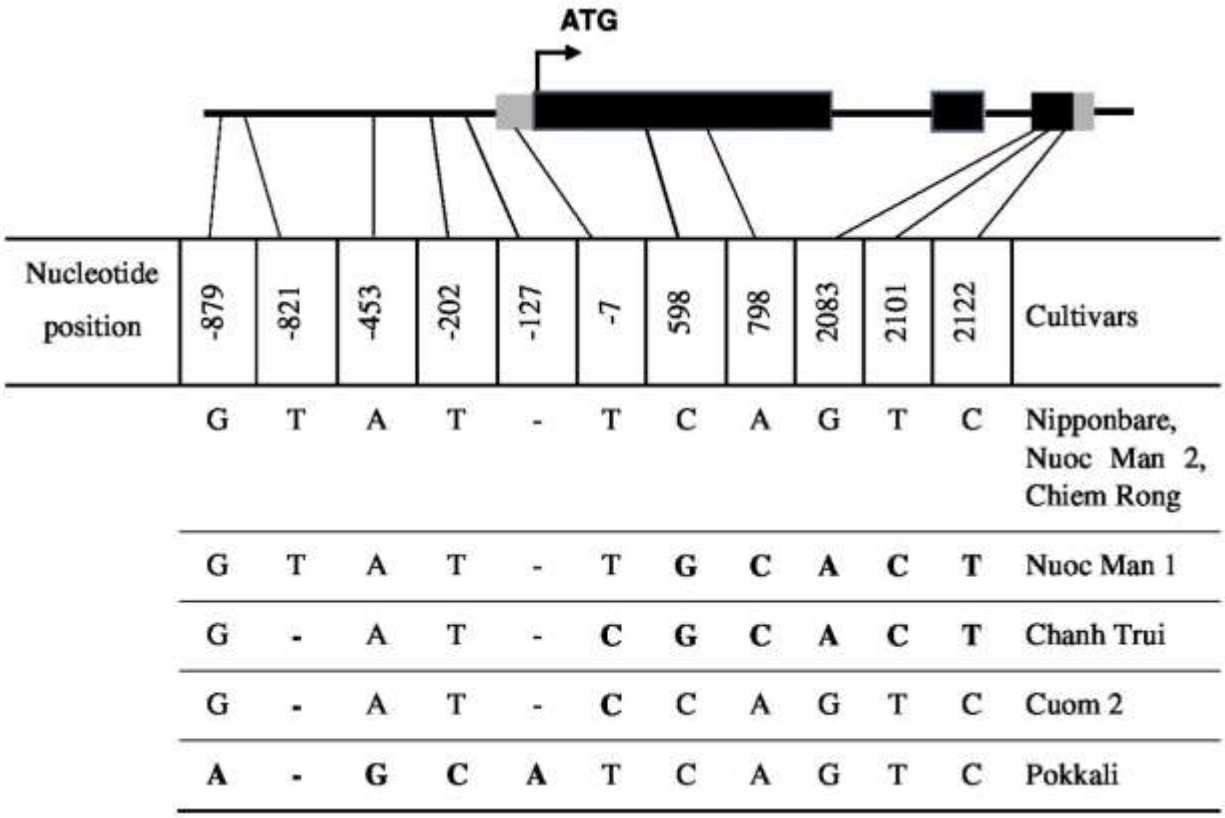

Figure 1. Nucleotide polymorphisms in the *OsHKT1;3* coding sequence and upstream region. The *OsHKT1;3* coding and upstream sequences of different cultivars were compared to those of Nipponbare. Letters in bold indicate the polymorphisms.

OsHKT1;3 transporter.

OsHKT1;3 gene upstream region polymorphism

The 1942-kb upstream region from the start codon of *OsHKT1;3* was amplified by PCR following direct sequencing. By comparing the obtained sequences, 4 nucleotide substitutions (T-7C, T-202C, A-453G, and G-879A), one deletion of T at position -821, and one addition of A at position -127 in the upstream sequence of *OsHKT1;3* gene were identified (Figure 1). To dissect the consequent effect of polymorphisms on transcriptional regulatory functions of the promoter, the cis-elements were predicted using PLACE database. It has been shown that the nucleotide substitution at position -202 and -453 caused deletions of 3 cis-elements, consisting of 2 CAATBOX1 and one RAV1AAT (Table 2); while nucleotide substitution at position -879 caused replacement of 5 cis-elements by other 3 cis-elements involving to stress responses (Table 2). The changes in cis-elements occurred in only cultivar Pokkali.

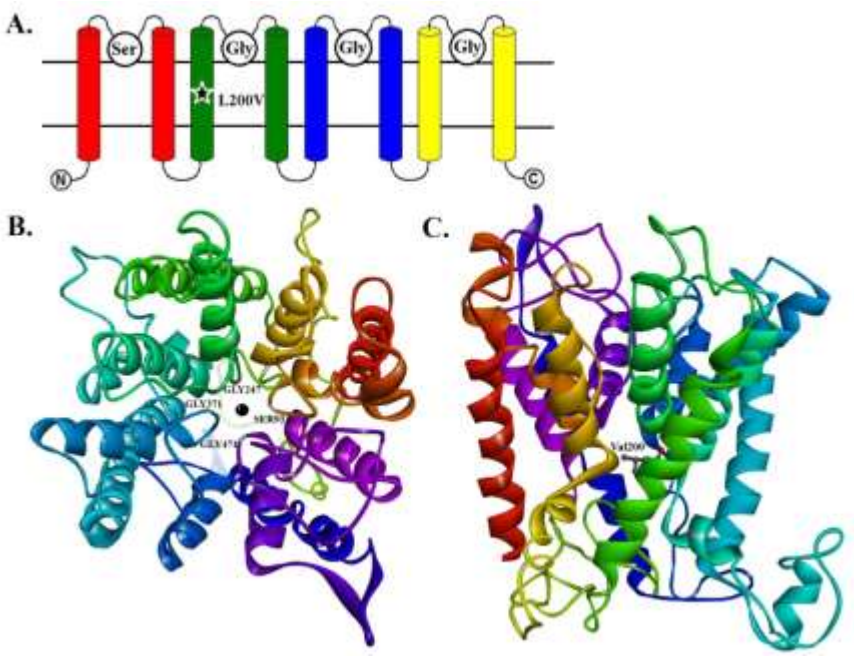

Figure 2. Molecular model of the OsHKT1;3 transporter. A: Schematic representation of 4 transmembrane–pore loop–transmembrane domain model. The location of changed amino acid is marked with a star. B: Visualization of OsHKT1;3 protein model from the top. The ion filter pore is formed by 4 amino acid residues, including Ser, Gly, Gly, and Gly. The black dot located at the pore center is indicated for Na^+ ion. C: Visualization of OsHKT1;3 protein model from the side with an indication of the L200V polymorphism site.

Expression profile of *OsHKT1;3* gene

Expression analysis of *OsHKT1;3* gene was carried out using Real time RT-PCR in 2 rice cultivars, namely Pokkali and Nippponbare, under control and salinity conditions (50 and 100 mM NaCl) to determine the different responses in expression level of *OsHKT1;3* gene. As shown in Figure 3, the expression of *OsHKT1;3* gene was decreased in both leaf and root samples of both rice cultivars. The reduction in expression of *OsHKT1;3* was more pronounced in the roots than in the leaves.

DISCUSSION

Rice (*O. sativa* L.) is an important crop, but its productivity has been limited by salinity. Investigation of novel genes/alleles for salt tolerance in rice is of necessity. In this study, the natural allelic variation in sequence and expression of rice *OsHKT1;3* encoding the Na^+- selective transporter belonging to HKT family using rice cultivars varying in salt-tolerant levels was explored (Table 1). In total, eleven nucleotide variations were identified in the gene sequence and the upstream region of the *OsHKT1;3* (Figure 1).

In the coding sequence of the gene, one non-synonymous substitution (C598G) was detected, leading to an amino acid substitution (L200V). It has been previously reported that SNPs in the coding sequence of *HKT* genes affect the functions of transporters and associated with salt-tolerant traits (Rubio et al., 1995; Ren et al., 2005; Baxter et al., 2010; Ali et al., 2016; Mishra et al., 2016; Campbell et al., 2017; Jiang et al., 2018). Therefore, the 3D models of OsHKT1;3 was predicted to elucidate the putative effect of the C598G polymorphism on functions of the variant transporter. In the predicted protein model, 4 amino acid residues, including 3 glycine and one serine, which form a selectivity filter were identified (Figure 2B). That structure determines the Na^+-selective transport property of OsHKT1;3, which agrees with the finding of the highly selective Na^+ transporter of OsHKT1;3 reported by Jabnoune et al. (2009). The substituted amino acid L200V locates in the third transmembrane domain (Figure 2A and C), and is unlikely to interfere with Na^+ transport in the variant transport.

The upstream region of the gene, where the cis-elements present, plays important roles in controlling the expression of the gene. Identification of cis-elements may elucidate expression patterns of the gene (Mariño-Ramírez et al., 2009). Thus, variation in the cis-element

Table 2. Polymorphisms in the upstream region of *OsHKT1;3* and putative cis-elements.

Position	Polymorphic type	Cis-element	Change in number	Sequence (5'-3')	Function of cis-element	References*
-879	G/A	CAATBOX1	-1	CAAT	Responsible for tissue specific promoter activity	Shirsat et al. (1989)
		WBOXATNPR1	-1	TTGAC	Salicylic acid (SA)-induced element	Yu et al. (2001)
		WBOXHVISO1	-1	TGACT	Sugar-responsive elements	Sun et al. (2003)
		WBOXNTERF3	-1	TGACY	Wounding-responsive elements	Nishiuchi et al. (2004)
		WRKY71OS	-1	TGAC	GA-responsive elements	Zhang et al. (2004)
		MYB2AT	+1	TAACTG	Responsive to water stress	Urano et al. (1993)
		MYB2CONSENSUSAT	+1	YAACKG	MYB recognition site involved in dehydration responsiveness	Abe et al. (2003)
		MYBCORE	+1	CNGTTR	Binding site of MYB proteins involved in the regulation of dehydration-responsive genes and flavonoid synthesis genes	Urano et al. (1993)
-453	A/G	CAATBOX1	-1	CAAT	Responsible for tissue specific promoter activity	Shirsat et al. (1989)
-202	T/C	CAATBOX1	-1	CAAT	Responsible for tissue specific promoter activity	Shirsat et al. (1989)
		RAV1AAT	-1	CAACA	The binding site of transcription factor RAV1	Kagaya et al. (1999)

*The reference for function of cis-element.

number and pattern in the upstream region of the rice cultivars can have a decisive impact on *OsHKT1;3* gene expression, and thereby on a response of plants to salt stress. In the current study, in total 4 nucleotide substitutions (T-7C, T-202C, A-453G, and G-879A), one deletion of T occurred at site -821, and one addition of A at position -127 were detected in the upstream sequence of *OsHKT1;3* gene (Figure 1). Further *in silico* analysis revealed that the nucleotide substitution at position -202 and -453 caused deletions of 3 cis-elements, consisting of 2 CAATBOX1 and one RAV1AAT (Table 2). The cis-element CAATBOX1 determines tissue-specific activity of the promoter (Shirsat et al., 1989), while RAV1AAT is the binding site of the transcription factor RAV1 (Kagaya et al., 1999). The nucleotide substitution at position -879 caused replacement of 5 cis-elements, including CAATBOX1, WBOXATNPR1, WBOXHVISO1, WBOXNTERF3, and WRKY71OS, by other 3 cis-elements, consisting of MYB2AT, MYB2CONSENSUSAT, and MYBCORE (Table 2). The 3 replacing cis-elements (MYB2AT, MYB2CONSENSUSAT, and MYBCORE) are involved in water stress responses (Abe et al., 2003; Urao et al., 1993); while the 5 replaced cis-elements CAATBOX1, WBOXATNPR1, WBOXHVISO1, WBOXNTERF3, and WRKY71OS have different roles. CAATBOX1 is responsible for tissue-specific promoter activity (Shirsat et al., 1989); while WBOXATNPR1, WBOXHVISO1, WBOXNTERF3, and WRKY71OS are responsible for salicylic acid, sugar, wounding and gibberellin, respectively (Yu et al*.,* 2001; Sun et al., 2003; Nishiuchi et al*.,* 2004; Zhang et al., 2004). These cis-element changes might affect the expression pattern of *OsHKT1;3* gene in response to stress conditions.

Since the polymorphisms in the upstream region of *OsHKT1;3* gene that lead to the changes in cis-elements occurred only in salt-tolerant cultivar Pokkali, then the difference in gene expression response to salt stress of 2 contrasting rice cultivars, Pokkali and Nipponbare were next examined. The results showed that *OsHKT1;3* gene expression was decreased in both leaves and roots samples of both rice cultivars (Figure 3). In the previous study, the *OsHKT1;3* gene was found to express in both the roots and the mature leaves (Jabnoune et al., 2009; Abdulhussein et al., 2018). The expression of *OsHKT1;3* gene in the roots and the leaves was not changed upon the different growth conditions (Jabnoune et al., 2009).

OsHKT1;3 is found to be located in the Golgi membrane, not in the plasma membrane (Rosas-Santiago et al., 2015). OsHKT1;3 can mediate both inward and outward currents but displays weak inward rectification in *Xenopus* oocytes (Jabnoune et al., 2009). Thus, it might be that the reduced expression of *OsHKT1;3* under salinity conditions help decrease the transport of Na^+ from

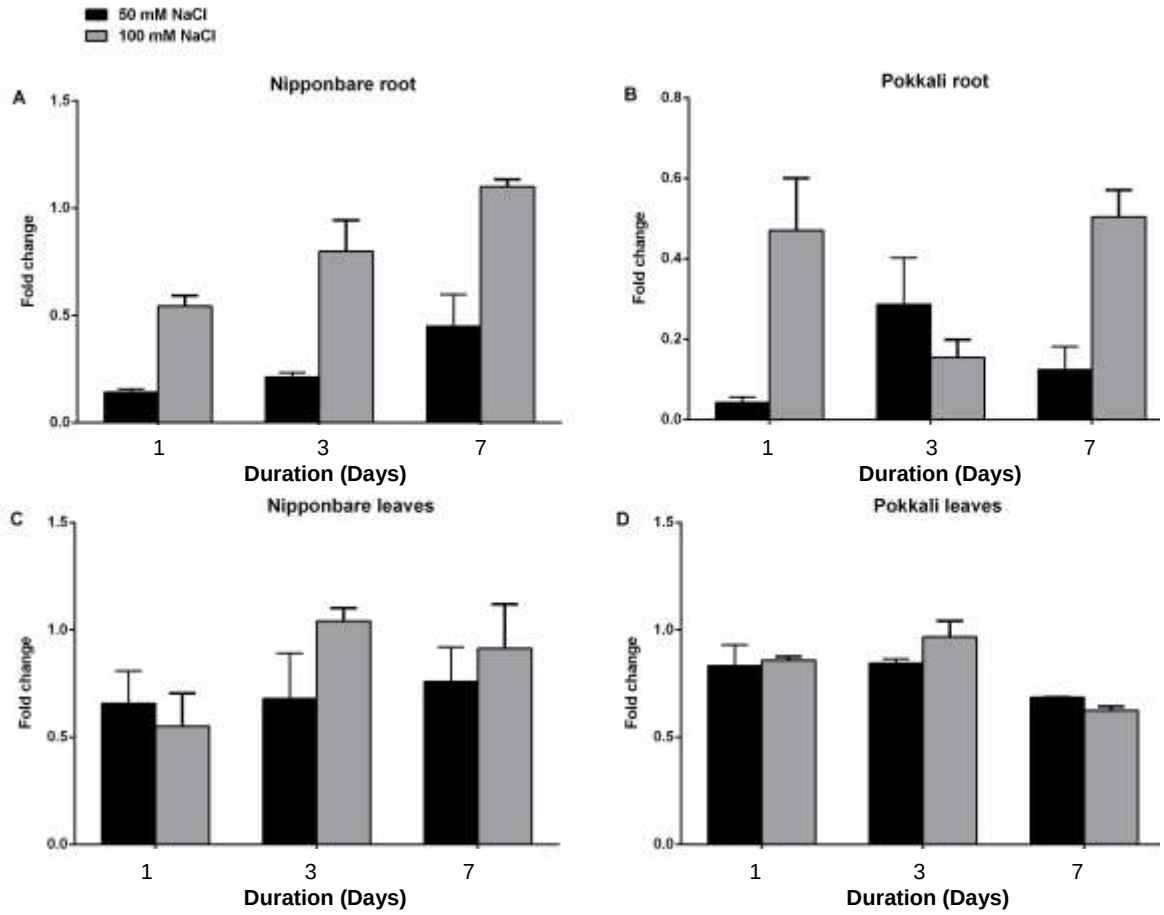

Figure 3. Expression of *OsHKT1;3* gene under control and salinity conditions in two contrasting rice cultivars. The expression of *OsHKT1;3* gene in different samples was normalized with the expression of rice actin 1, the internal control gene. Fold changes in *OsHKT1;3* expression in different rice genotypes at different time points were calculated to corresponding control using ΔΔCt method (Livak and Schmittgen, 2001). Values represent the mean and standard deviation of 3 technical replica.

Golgi to the cytoplasm, which in turn protects the cell from the toxicity of accumulated Na^+ in the cytoplasm. This hypothesis is supported by the findings of Rosas-Santiago et al. (2015) that the yeast is more susceptible to Na^+ when the *OsHKT1;3* is expressed.

In conclusion, polymorphisms were detected in the coding and promoter sequences of *OsHKT1;3* gene of the salt-tolerant cultivars (Pokkali, Chanh Trui, Cuom 2, and Nuoc Man 1), but not of the moderate cultivars (Chiem Rong, Nuoc Man 2) and sensitive cultivar Nipponbare. Amongst those polymorphisms, SNP C598G caused the change in amino acid L200V in tolerant cultivars Chanh Trui and Nuoc Man 1; and SNP G-879A led to the addition of several water stress related cis-elements in the tolerant cultivar Pokkali which showed more reduction in *OsHKT1;3* expression level in the roots than that of the sensitive cultivar Nipponbare. Thus, it shall be useful to further characterize these 2 SNPs to demonstrate their decisive roles in salt tolerance. Site-directed mutagenesis study using modern techniques such as CRISPR-Cas9 would help resolve whether SNP C598G affects transport activity of protein or SNP G-879A alters gene expression level, which subsequently clarifies the roles of these 2 SNPs to plant stress tolerance. Rice plants responded to salinity by reducing the expression of *OsHKT1;3* gene.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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