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Investigation of polymorphisms of exon 1 region of *Os*HKT1;5 gene in high yielding rice

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This study was carried out to analyze the polymorphic region of exon 1 of OsHKT1;5 of 22 high yielding rice varieties that are salt tolerant. The result of the phenotypic screening for salinity stress was done using the standard protocol of the International Rice Research Institute which recorded that all rice varieties developed very well under control condition, 85.38% were quite tolerant to salinity treatment of 4‰, 68.18% were moderately tolerant to salinity treatment of 8‰ and 36.36% were moderately tolerant to salinity treatment of 10‰, similar to Pokalli. The result of sequencing exon 1 region of OsHKT1;5 gene recorded five Single Nucleotide Polymorphisms (SNP) markers. Five nucleotide substitutions in coding sequence of OsHKT1;5 were found at the positions: 382, 418, 484, 551 and 994. All five non-synonymous nucleotide substitutions caused changes in amino acids (D51N, P63A, V86I, R107H and H255D).

Key words: High yielding rice, OsHKT1;5, single nucleotide polymorphisms (SNP) makers.

INTRODUCTION

In recent years, the Mekong Delta has been identified as one of the most seriously affected delta by climate change, where salinity intrusion has caused serious influence to agricultural production of the whole region (IPCC, 2007; ADB, 2009). The complicated development of drought and salinity intrusion in early 2016 is considered the most serious in the past 100 years, causing severe influence on rice production in the Mekong Delta provinces (Ministry of Science and Technology, 2016). High salt concentration affects seed germination, plant growth and crop productivity (Hussain et al., 2017), in which, rice is one of the salinity sensitive species (Maas and Hoffman, 1977; Hussain et al., 2017). According to Turan et al. (2012), in salinity environment, excess Na^+ leads to the loss of ionic homeostasis. Potassium acts as a coenzyme for many cytoplasmic enzymes, but when excess Na^+ is present in rhizosphere, it competes for K^+ particularly at low affinity K^+ channels, leading to low K^+/Na^+ ratio in cytoplasm. Genetic engineering of genes for antiporter or ion channels have been successful in generation of salt tolerant plants by maintaining higher K^+/Na^+ ratio. In addition, Na^+ in reproductive organs prevents photosynthesis and transports starch, therefore reducing rice yields (Hussain

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et al., 2017).

The capability of salinity tolerance in rice has been reported as an extreme complex mechanism controlled by many genes (Reddy et al., 2017). Many plant membrane transport channels play a major role in stress resisting mechanisms, especially Na^+ and K^+ channels related to salinity tolerance (Hamamoto et al., 2015). Studies on ion balance in plants have shown that protein HKT (High-Affnity K + Transporter) plays an important role in ion balance in the cells. This gene family includes many genes such as: *Os*HKT1;2, *Os*HKT2;3, *Os*HKT1;3, *Os*HKT1;1, *Os*HKT1;4, *Os*HKT2;1, and *Os*HKT1;5 (Waters et al., 2013; Hamamoto et al., 2015). In particular, *Os*HKT1; 5 plays the role of encoding ion Na^+ transport proteins from roots to shoots and control shoot Na^+concentration in rice (Ren et al., 2005) as the key mechanism to salinity tolerance of rice. In another study, a novel function of *Os*HKT1;5 was reported in mediating Na^+ exclusion in the phloem to prevent Na^+ transfer to young leaf blades (Kobayashi et al., 2017). The *Os*HKT1;5 is about 4,487 bp length in which coding region takes 1,665 bp, consisting of 3 exon regions with their length of 1,235 bp for exon 1, 231 bp for exon 2, and 199 bp for exon 3 (www.rapdb.dna.affrc.go.jp). Therefore, the exon 1 was advantagious for sequencing and also containing enough information to detect SNPs. This study focused on polymorphic sequence analysis of exon 1 of gene *Os*HKT1;5 in high yielding rices showing changes in protein structure and finding out the role of salt tolerance mechanism in helping breeding of rice varieties in the current period.

MATERIALS AND METHODS

Rice materials

Seeds of 22 rice cultivars were provided by The Cuu Long Delta Rice Research Institute (CanTho, Vietnam), including 20 high yielding rice cultivars, Pokkali was used as salinity tolerant control and IR28 used as salinity sensitive control.

Phenotypic screening for salinity stress

In this study, 22 rice cultivars were used for salinity screening at the seedling stage following International Rice Research Institute (IRRI) standard protocol using Yoshida nutrient solution (Yoshida et al., 1976). The seedlings allowed to grow to a height of 1.5 to 2.0 cm, were placed in the boxes on the meshed foam sheet and then placed in a rectangular plastic container filled with 10 L of Yoshida nutrient solution. After 3 days, removed the weakest seedling, keeping three seedlings per hole. The experiment was arranged in a completely randomized design (CRD) with 3 replications at four NaCl concentrations of 0, 4, 8 and 10‰ (1 M of NaCl containing 58.44 g/L, while 4, 8, and 10‰ consisting of 4, 8, and 10 g/L NaCl, so they become 68, 136, and 171 mM, respectively). Nutrient solution was replaced at every 8 day intervals and the pH was maintained at 5 daily. The modified standard evaluation score (SES) of IRRI was used to evaluate the visual symptoms of salt toxicity when IR28 cultivar is completely dead (Table 1).

DNA extraction

Young leaves of 10-days old seedlings of rice cultivars (15 to 20 cm) were used for genomic DNA extraction using the cetyltrimethylammonium bromide (CTAB) method (Rogers and Bendich, 1988).

Primer designing and amplification of *Os*HKT1;5

The exon 1 region of gene *Os*HKT1;5 in Nipponbare cultivar was downloaded from the Rice Anotation Project website (www.rapdb.dna.affrc.go.jp), and used to design primer pairs by Primer3Plus software. Total genomic DNA of 22 genotypes was used as template for PCR using the designed primer pairs, including F-5'GGACCTGATCTTCACGTCGG3' and R-5'GAGCACCATCTCACCGGAG3'. Amplifier product length was 1000 bp.

The mixture for PCR reaction consisted of genomic DNA 50 ng, 1X master PCR buffer kit containing dNTPs and $MgCl_2$, 0.3 µM primers, 1 unit of Taq Polymerase. The PCR was performed in thermocycle with the following steps: 95°C for 2 min of pre-denaturation, 30 cycles (94°C for 30 s, Tm 58°C for 30 s, 72°C for 45 s) and 72°C for 5 min of prolonged extension step. PCR product was run on 2% agarose gel electrophoresis in TBE buffer 1X for 45 min under 140 V. DNA bands were visualized by safe view staining and took gel pictures with Biorad UV gel camera. PCR products that showed only one bright single band on gel were sequenced. Sequencing was done by PhuSa Biochemical Co., Ltd, Can Tho City.

Sequencing the whole exon 1 of OsHKT1;5

From the *Os*HKT1;5 sequence data of rice cultivars, gene sequences were analyzed using Bioedit for SNPs detection. From SNPs, amino acid sequences in the corresponding protein were inferred and the differences compared among rice cultivars.

RESULTS AND DISCUSSION

Phenotypic screening of rice cultivars at seedling stage for salinity tolerance

Twenty two (22) cultivars of rice seedling were used for screening salinity tolerance. The result recorded in Table 2 and Figure 1 shows that all the rice varieties grew very well in the control condition (0‰) and were also quite tolerant to salinity treatment at 4‰ (19/22 cultivars accounted for 85.38% of the total of cultivars). Pokkali, OM20, OM396, OM429, OM6976, OM5629, OM8108, OM10252, OM355, OM2514, OM9921, OM2517, OM11735, OM8959, and OM6677 were found to be moderately tolerant to salinity treatment of 8‰ (15/22 cultivars accounted for 68.18% of the total of cultivars). There were seven cultivars, namely, OM20, OM396, OM429, OM6976, OM5629, OM8108 and OM10252 that were moderately tolerant similar to Pokalli in salinity treatment of 10‰ (accounted for 36.36% of the total of cultivars); there were three cultivars namely OM6677, OM18 and OM6162 highly susceptible to salinity treatment of 10‰ (accounted for 18.18% of the total of

Table 1. Modified standard evaluation score (SES) of visual salt injury at seedling stage (IRRI, 1997).

Score	Observation	Response category
1	Normal growth, no leaf symptoms	Highly tolerant
3	Nearly normal growth, but leaf tips of few leaves whitish and rolled	Tolerant
5	Growth severely retarded, most leaves rolled; only a few are elongating	Moderately tolerant
7	Complete cessation of growth; most leaves dry; some plants dying	Susceptible
9	Almost all plants dead or dying	Highly susceptible

Table 2. Modified standard evaluation score (SES) of visual salt injury at seedling stage of twenty two modern cultivars.

Cultivar	SES Scoring		
	4‰	8‰	10‰
Pokkali	3	5	5
OM20	3	5	5
OM396	3	5	5
OM429	3	5	5
OM6976	3	5	5
OM5629	3	5	5
OM8108	3	5	5
OM10252	3	5	5
OM355	3	5	7
OM2514	3	5	7
OM9921	3	5	7
OM2517	3	5	7
OM11735	3	5	7
OM8959	3	5	7
OM6677	3	5	9
OM8017	3	7	7
OM9916	3	7	7
OM576	5	7	7
OM9915	5	7	7
OM18	3	9	9
OM6162	3	9	9
IR28	9	9	9

cultivars), similar to IR28.

This result was similar to that of Quynh-Hoa et al. (2016) where they recorded that OM6677 was highly susceptible and also similar to that of Nguyen (2012), whereby testing cultivars of AS996, ST20, IR50404, OM6677, and OM6377, they showed that OM6976 developed very well under salt stress among the rice cultivars. Besides, through salinity treatments of 4, 8, and 10‰, the salinity damage increased gradually through phenotypic observation in the surveyed rice varieties.

Analysis of exon 1 region of *Os*HKT1;5 gene in rice cultivars

The leaves of 10 to 12 days rice seedlings were collected for genomic DNA extraction. The extracted DNA was used as template for the amplification of the exon 1 region of *Os*HKT1;5 gene in the PCR assay. As shown in Figure 2, the PCR products showed only one bright single band on gel, which was specific and with the same size across the rice cultivars, without unintended bands. Thus, the exon 1 region of *Os*HKT1;5 gene was successfully amplified in all the investigated rice cultivars.

Amplified DNA fragments were purified and the exon 1 region of *Os*HKT1;5 gene of 22 high yielding rice cultivars sequenced in this research. The stable signal area was recorded as 938 nucleotides from nucleotide 230 to nucleotide 1168. This nucleotide region is used to analyze SNP markers. The result of sequencing exon 1 region of *Os*HKT1;5 allowed the detection of five nucleotide substitutions at positions: 382, 418, 484, 551, and 994 when compared with the reference sequence in the database (Nipponbare allele). Among the twenty two high yielding rice cultivars, six cultivars recorded three SNP markers. Among these six cultivars, Pokalli and OM18 have three SNP markers at position C418G (C is replaced by G at nucleotide position 418), G551A, C994G; OM6677, OM9921, OM2517, and OM8108 have three same SNP markers at position G382A, C418G, and C994G. Seven cultivars that recorded one SNP marker at position C994G were OM20, OM576, OM396, OM429, OM5629, OM10252, and IR28. Interestingly, IR28 and Nipponbare were used as controls for sensitive cultivars (Table 3).

All the five nucleotide substitutions were non-synonymous substitutions. Five non-synonymous substitutions mean that nucleotide substitutions lead to amino acid substitutions were D51N (Aspartic acid was replaced by Asparagine at position 51), P63A (Proline was replaced by Alanine at position 63), V86I (Valine was replaced by Isoleucine at position 86), R107H (Arginine was replaced by Histidine at position 107), and H255D (Histidine was replaced by Aspartic acid at position 255). It can be seen, in this research, that nucleotide substitutions in the exon 1 region of *Os*HKT1;5 gene lead to amino acid substitutions in twenty two rice cultivars when compared with Nipponbare cultivar.

Some studies about SNPs of OsHKT1;5 gene were reported: Mishra et al. (2016) carried out the experiment on 299 wild rice accessions collected from different agro-climatic regions of India under salt stress condition. Of these, 95 representative accessions were sequenced for

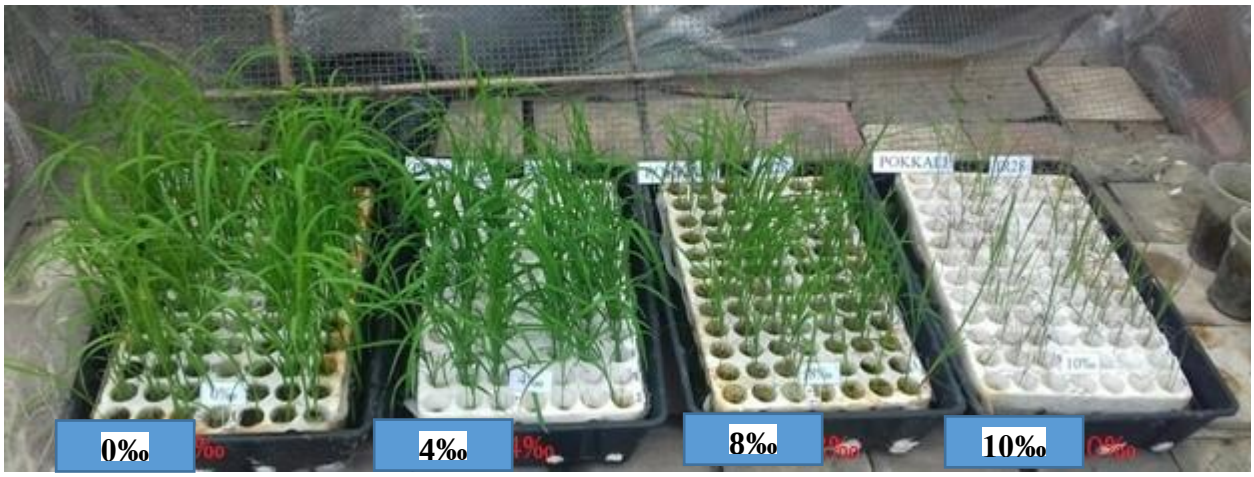

Figure 1. Phenotypic screening of 22 rice cultivars in 0, 4, 8, and 10‰ of NaCl after 16 days.

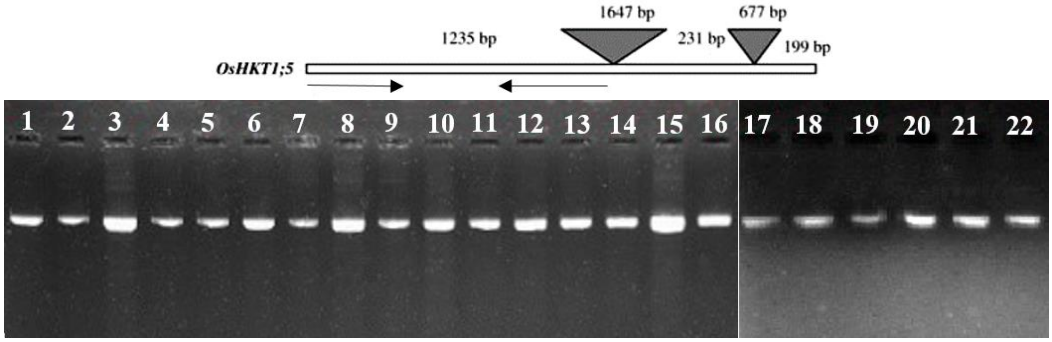

Figure 2. PCR products the exon 1 region of *Os*HKT1;5 gene. 1. OM2514; 2. OM20; 3. OM8959; 4. OM6162; 5. OM6677; 6. OM576; 7. OM9921; 8. OM11735; 9. OM25; 10. OM35517; 11. Pokkali; 12. IR28; 13. OM355; 14. OM429; 15. OM5629; 16. OM10252; 17. OM9915; 18. OM9916; 19. OM817; 20. OM8108; 21. OM18; 22.OM6976.

Table 3. Polymorphism in exon 1 region of *Os*HKT1;5 gene.

Nipponbare	Nucleotide position	Amino acid substitution					High yielding rice cultivars
		D51N	P63A	V86I	R107H	H255D	
G	382	-	G	-	A	G	Pokalli, OM18
C	418	A	G	-	-	-	OM2514, OM8959, OM11735, OM355, OM9915
G	484	-	-	-	-	G	OM20, OM576, OM396, OM429, OM5629, OM10252, IR28
G	551	-	G	-	-	G	OM6162
C	994	A	G	-	-	G	OM6677, OM9921, OM2517, OM8108
-	-	-	G	-	-	-	OM9916
-	-	-	-	A	-	G	OM8017, OM6976

members of HKT ion transporter family genes (the whole OsHKT1;5 gene). The results for *Os*HKT1;5 gene has 45 NSPs (8 from coding and 37 from non-coding). In addition, the *Os*HKT1;5 gene of Godawee (a Sri Lankan traditional rice variety known for its salinity tolerance) was sequenced, and 122 SNPs were found (Sanjeewa et al., 2017). In the current study, five SNPs were found in exon 1 of OsHKT1;5 gene leading to five amino acid substitutions of D51N, P63A, V86I, R107H and H255D. It seems to be less polymorphism, but in coding region

only. Similarly, Ren et al. (2005) found six SNPs in the OsHKT1;5 coding region that led to four amino acid changes, namely P140A, R184H, H332D and L395V. Negrão et al. (2013) found three nonsynonymous SNPs in OsHKT1;5 coding region leading to three amino acids change consinting of T67K, P140A and R184H, in which two residue differences between 'Nipponbare' and IR29 (includes IR64) were observed, specifically D129N and P140A.

With the present data, as reported by Quynh-Hoa et al. (2016) and Do et al. (2016), it is quite difficult to point out the relationship between nucleotide polymorphism in the exon 1 region of *Os*HKT1 gene and the salt tolerance level of the testing rice cultivars. It might be helpful to explore the nucleotide polymorphism in *Os*HKT1;5 gene, which plays role in the regulation of gene expression.

Conclusion

The results of the phenotypic screening for salinity stress, among 22 high yielding rice varieties, recorded that nineteen cultivars were quite tolerant in salinity treatment at 4‰, fifteen cultivars were moderately tolerant in salinity treatment of 8‰ and only eight cultivars were moderately tolerant similar to Pokalli in salinity treatment of 10‰. Besides, through salinity treatments of 4, 8, and 10‰, the salinity damage increased gradually through phenotypic observation in the surveyed rice varieties. The exon 1 region of *Os*HKT1;5 gene was successfully amplified in all the investigated rice cultivars. The results of sequencing exon 1 region of *Os*HKT1;5 gene recorded five SNP markers at positions: 382, 418, 484, 551, 994. All five non-synonymous nucleotide substitutions caused changes in amino acids (D51N, P63A, V86I, R107H, and H255D).

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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