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Assessment of Natural Variation in *OsHKT1;2* Gene in Rice (*Oryza sativa*)

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Abstract: *HKT* gene family encodes Na^+ and/or K^+ transporter located on cellular membrane in plants. HKT transporter family has been proven to play a crucial role in maintaining low Na^+/K^+ ratio to help plant survive during saline condition. *OsHKT1;2* gene is a member of rice *HKT* gene family. However, the functional roles of the *OsHKT1;2* is reminded unknown. In this study, we investigated the natural variations in coding sequence of *OsHK1;2*. Firstly, the coding sequence of *OsHKT1;2* was amplified by PCR using specific primers in 13 different rice cultivars, then the PCR products were directly sequenced. The results revealed two nucleotide variations at position 343 and 1413 in eight rice cultivars. However, the predicted amino acid sequence showed three early stop codons presenting within the exon 1 in all 13 investigated rice cultivars, suggesting that *OsHKT1;2* might be a pseudogene that might not produce full-length protein.

Keywords: OsHKT1;2 rice, salt stress, natural variation.

1. Introduction

Salt stress negatively effects on plant development through two mechanisms: increasing osmotic pressure lead to root is unable to absorb water from soil and accumulating Na^+ ion to toxic concentration in cytosol [1, 2]. Therefore, one of strategies of plants to survive under salt stress is restriction of Na^+ ion to move from environment into root and further transport to photosynthetic tissues. There are three main Na^+ transporter families which involve in these processes such as HKTs, NHXs and SOS. HKT transporter family locates on cellular membrane and is thought to take an important role in maintaining low Na^+/K^+ ratio in plant cell under salt stress. AtHKT1, the only one member of HKT family in *A.thaliana*, was demonstrated to play a crucial role in controlling cytosolic Na^+ detoxification [3, 4].

In rice, 7-9 members of HKT family were identified within genome (depending on cultivars) [5] and some of them were demonstrated contributing to salt tolerance in rice. These functional genes encode for transporter with distinct transport activity and express in various tissue and/ or organs [6]. OsHKT2;1 was characterized as a Na^+/K^+ co-

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transporter, while OsHKT2;2 acts as a Na^+ uniporter and expresses specifically in root [7, 8]. HKT1;4 which expresses mainly in sheath tissue was suggested involving in compartmentation of Na^+ to xylem parenchyma to restrict the transport of Na^+ to young blades [9]. Especially, OsHKT1;5 was shown very recently to be a Na^+ transporter that maintaining K^+ homeostasis in the root under saline condition, thus increased the salt tolerance in Nona Bokra cultivar [10]. OsHKT1;2 is also belong to HKT family but studies about its function and expression are still limit.

Analyses of natural genetic polymorphism can provide insight into the mechanisms of plant adaptation to environmental conditions [11]. Rice is an important crop but generally sensitive to salt stress. Although some cultivars are very sensitive to salt stress like Nipponbare or IR28, the others are highly tolerant to saline condition such as Pokkali or Nona Bokra. The difference in salt tolerance among rice cultivars might be caused by the adaptation of plants to different environmental conditions due to natural genetic variations. SKC1/*OsHKT1;5* was identified as a quantitative trait locus to salt tolerance by cross-breed between salt sensitive Koshihikari and salt tolerant Nona Bokra [10].

In this study, we amplified the coding sequence of *OsHKT1;2* gene in 13 different rice cultivars by PCR, and then the PCR products were sequenced to searching for nucleotide polymorphisms. Furthermore, the amino acid sequence was predicted to determine how the substitutions subsequently effect on the corresponding protein.

2. Materials and method

Plant materials: Seeds of thirteen rice cultivars were provided by Vietnam National University of Agriculture (Hanoi, Vietnam), including Nipponbare, Cuom 1, Cuom 2, Tep lai, Cham, Nep Cuc, Man dang 1, Man dang 2, Cham Bien, Chiem rong, Chanh trui, IR29 and Te tep. The seedlings were grown in soil for 14 days, then the leaves were collected and stored at -80^0C for further analysis.

DNA extraction: Shoot tissue of 14-day-old seedlings of rice cultivars were flash frozen in liquid nitrogen and ground into fine powder by grinding mill (Retsch, Germany). The leaves powder was mixed well with 2% CTAB buffer and incubated at 65°C for 20 min. The mixture was cooled to room temperature before mixed with CI 24:1 (24 chloroform: 1 isoamylalcohol) then centrifuged at 14,000 rpm for 10 min at 4°C. The transparent supernatant was transferred into new 1.5mL tube, and cold isopropanol was added in ratio 2:1 for DNA precipitation at 20°C degree for 15 min. The DNA pellet was collected by centrifuged at 10,000 rpm for 5 min at 4°C and washed by 70% Ethanol to remove salt. The pellet was dried at room temperature and dissolved in TE buffer. Quality and quantity of extracted DNA was estimated by running on 1% agarose gel electrophoresis in TAE buffer.

PCR assay and sequencing of OsHKT 1;2 gene

Total DNA was used as template for PCR using 2 specific primer pairs which together completely cover the coding sequence of *OsHKT1;2* gene (Table 1). In 50-uL PCR reaction there was 2U of DreamTaq DNA polymerase (Thermo Fisher), DreamTaq Buffer 1X which contains 1.5 mM of Mg^{2+}, 0.2 mM of dNTP mixture and 0.3 µM of each primer. The PCR was performed with thermocycle consisting 95°C for 5 min of pre-denaturation, 35 cycle of (95°C for 30s, Tm for 30s, 72°C for 1.5 min.), 72°C for 5 min of prolonged extension step. 5 uL of PCR product was ran on 1% agarose gel electrophoresis in TAE buffer for 28 min under 90V. The gel was stained with Ethidium Bromide and visualized under UV illumination. PCR products were purified using GeneJET PCR Purification Kit (Thermo Fisher Scientific) and was sequenced on ABI PRISM 3730xl Genetic Analyzer (Applied Biosystems, USA) at First BASE DNA sequencing service (Singapore).

Table 1. List of specific primer pairs using to amplify *OsHKT1;2* gene

Primer name	Sequence
HKT12-1	Fw:GGCACATCTGTCTTACACCATC Rv:CTGGCCTTCAACAAGTGAAC
HKT12-2	Fw:AATCCTGCACTATGACAGAGG Rv:CTTAGCCCATTCGAGGCTCT

Sequence analysis

Nucleotide polymorphism of *OsHKT1;2* was obtained by alignment the obtained sequencing results of the gene from different rice cultivars and reference sequence in rice database using Multalin tool. The amino acid sequence was predicted from nucleotide sequence by using Expasy tool.

3. Results and discussion

3.1. Amplification of OsHKT1;2 by PCR

The DNA isolation was performed by CTAB method, and the total DNA was ran on agarose gel to check for quantity and quality (Fig. 1). The result showed that the genomic DNA had bright bands and a little of smear indicating for good quantity and quality.

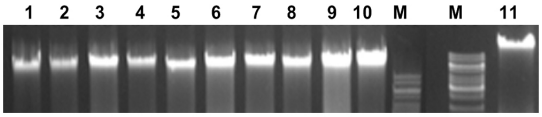


Figure 1. Gel electrophoresis of genomic DNA from rice cultivars. Lane 1-11: Nipponbare, Cuom 1, Cuom 2, Man dang 1, Man dang 2, Chiem Rong, Cham Bien, Cham, Tep Lai, Te Tep and Chanh Trui, respectively. Lane M: 1 kb marker.

Two primer pairs were designed to amplified full length of the coding region of *OsHKT1;2*. As shown in Fig. 2, PCR product was single bright sharp band with corrected size without unintended bands. Therefore, the coding regions of *OsHKT1;2* were successfully amplified in all investigated rice cultivars.

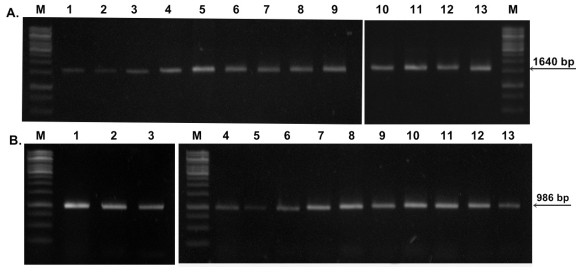
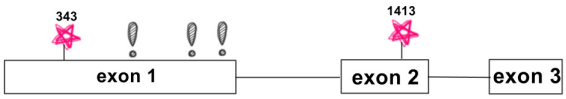


Figure 2. PCR amplification of *OsHKT1;2* using two specific primer pairs. A. PCR products using primer pair HKT1;2-1. B. PCR products using primer pair HKT1;2-2. Lane 1-13: PCR products from Nipponbare, Cuom 1, Cuom 2, Chanh Trui, Te Tep, Tep Lai, Man dang 1, Man dang 2, Chiem Rong, Cham Bien, Cham, IR29 and Nep Cuc, respectively.

3.2. Variation in nucleotide sequence of the OsHKT1;2 gene

The PCR product was purified and sent for directly sequencing. Alignment of *OsHKT1;2* coding sequences from rice cultivars and reference sequence revealed two nucleotide polymorphisms at position 343 and 116. Both of these two polymorphisms presented in 8 rice cultivar consisting of Cuom 1, Tep Lai, Cham, Man dang 1, Cham Bien, Chiem Rong, Te Tep and Man dang 2, while the other rice cultivars such as Nipponbare, Cuom 2, IR29, Chanh Trui and Nep Cuc showed no variation in *OsHKT1;2* coding sequences (Fig. 3).



Nu.po	343	1413	Cultivars
	G>A	A>G	Cuom 1, Tep Lai, Cham, Man dang 1, Cham Bien, Chiem Rong, Man dang 2, Te Tep
	G	A	Nipponbare, Nep Cuc, Cuom 2, Chanh Trui, IR29

Figure 3. Nucleotide polymorphism of *OsHKT1;2* in 13 rice cultivars and position of three early stop codons. The stars indicate for nucleotide substitutions and the exclamation marks present for early stop codons.

To further investigate about how these substitutions influence amino acid sequence, the deduced amino acid sequence was determined. The substitution at position 343 was non-synonymous which replaces Alanine by Threonine, while variation at position 1413 was synonymous. Interestingly, *OsHKT1;2* sequence from all rice cultivars contained three early stop codons within exon 1 (Fig. 3). Therefore, *OsHKT1;2* seems a pseudogene which might not be translated into full-length protein. Nevertheless the RT-qPCR result showed that *OsHKT1;2* was still be transcribed into mRNA in both shoot and root tissues but in a very low level (data not shown). Thus, it is deserved to perform analysis of gene expression at translation level to confirm if *OsHKT1;2* is really a pseudogene.

4. Conclusion

The entire coding sequence of *OsHKT1;2* gene was successfully amplified from 13 rice cultivars using PCR technique. There were two nucleotide substitutions at position 343 and 1413 in 8 rice cultivars (Cuom 1, Tep Lai, Cham, Man dang 1, Cham Bien, Chiem Rong Te Tep and Man dang 2) while the others (Nipponbare, Cuom 2, Chanh Trui, IR29 and Nep Cuc) showed no nucleotide variation compared to reference sequence. Interestingly, predicted amino acid sequence revealed three early stop codons locating within exon 1 in all 13 investigated rice cultivars indicating that *OsHKT1;2* might just be a pseudogene.

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Nghiên cứu đa hình gen *OsHKT1;2* ở cây lúa (*Oryza sativa*)

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Tóm tắt: Ở thực vật họ gen *HKTs* mã hóa cho protein màng vận chuyển ion Na^+ hoặc/và ion K^+ . Họ protein HKT được chứng minh có vai trò quan trọng trong việc duy trì tỉ lệ Na^+/K^+ thấp giúp cây trồng chống chịu với điều kiện mặn. Gen *OsHKT1;2* là một thành viên của họ gen HKT ở cây lúa. Tuy nhiên chức năng của *OsHKT1;2* vẫn chưa được làm sáng tỏ. Trong nghiên cứu này chúng tôi tiến hành nghiên cứu sự đa hình ở vùng mã hóa của gen *OsHKT1;2*. Trước tiên, vùng trình tự mã hóa của gen *OsHKT1;2* được khuếch đại bằng phương pháp PCR sử dụng cặp mồi đặc hiệu ở 13 giống lúa khác nhau, sau đó sản phẩm PCR được giải trình tự trực tiếp. Kết quả cho thấy có hai đa hình nucleotide ở vị trí 343 và 1413 xuất hiện ở 8 giống lúa nghiên cứu. Tuy nhiên, kết quả dự đoán trình tự axit amin tương ứng cho thấy sự có mặt của 3 bộ ba kết thúc sớm ở exon 1 của gen *OsHKT1;2* ở cả 13 giống lúa nghiên cứu. Như vậy, gen *OsHKT1;2* có thể chỉ là một gen giả không thể dịch mã ra một protein có chiều dài hoàn chỉnh.

Từ khóa: OsHKT1;2, lúa, stress mặn, đa hình di truyền.