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Research Article

eQTL regulates *OsHAK5* in enhancing potassium content under salt stress in rice

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Abstract
To alleviate salt stress in rice, the role of potassium in countering the ill effects of sodium is gaining importance. Towards this, OsHAK5 a member of the high-affinity potassium transporter family was known for enhancing the intracellular potassium under reduced/low K^+ availability during salt stress. The full-length cDNA of *OsHAK5* was cloned and sequenced from the roots of *Pokkali* and cv. IR64 however, no nucleotide polymorphism detected. Interestingly, the expression of *OsHAK5* in roots of salt-tolerant landrace *Pokkali* and salt susceptible cv. IR64 revealed a significant upregulation in the former under low K^+ ($1/4^{th}$) condition. Subsequent analysis of the amino acid sequence of CBL-interacting protein kinase (OsCIPK23), the upstream regulator of *OsHAK5* revealed two INDELs and one of them turned out to be functionally relevant. The *OsCIPK23* variant from salt-tolerant *Pokkali* should be favorably regulating the expression of the downstream *OsHAK5* leading to an increased K^+ uptake under salt stress.

Keywords: Rice, HAK, eQTL, Potassium, Salt stress

INTRODUCTION

Rice, the staple food crop was classified as salt sensitive (Maas, 1986). Salinity is a major abiotic stress particularly in rice affecting its growth and productivity (Rekha *et al*., 2018; Amaravel *et al*., 2019; Govindaraju and Balakrishanan, 2002). It is the outcome of an excess of dissolved salts like chlorides and sulfates of sodium, magnesium, and calcium in soil and irrigation water (Bernstein, 1975; Wali *et al*., 2021). In India, salt-affected land accounts for around 6.73 Mha and was estimated to bring down global rice production by 50% (NRSA, 2008). Rice breeders had been continuously attempting to improve the level of salt tolerance by targeting major QTLs such as SALTOL which was primarily responsible for sodium exclusion from the root xylem parenchyma (Ren *et al.,* 2005).

Alternatively, an enhanced potassium uptake appears promising for maintaining Na/K homeostasis, considered a key salt-tolerant trait (Shabala and Cuin, 2008; Shabala and Pottosin, 2010). Potassium, the most abundant inorganic cation plays a key role in the plant development and osmoregulation process (Talbott and Zeiger, 1996; Pandey and Mahiwal, 2020). The potassium uptake from the soil environment and its transport within the plant necessitates the role of membrane-bound transporters and channels (Maathuis and Sanders, 1994; Maathuis *et al*., 1997). These transporters and channels were further grouped into four multigene families (i.e) high-affinity K^+/Na^+ transporter (HKT), K^+ uptake permease (KT/KUP/HAK), K^+ exchange antiporters (KEA), and cation/H^+ exchangers (CHX transporters) of which HAK transporter

family plays a major role in increasing the K^+ uptake and thereby enhancing salinity tolerance in plants (Zhang *et al*., 2012). Studies show that the majority of HAK transporters respond to K^+ limiting saline environments and help in increasing the intracellular K^+ content thereby improving salt tolerance in rice (Li *et al*., 2023).

Salt stress manifests via changes in Ca^{2+} levels which in turn transduces a signaling scheme involving the Ca^{2+}binding calcineurin B-like proteins (CBLs; Batistic and Kudla, 2009), the CBL interacting protein kinases (CIPKs; Sanyal *et al.,* 2020), the Ca^{2+} dependent protein kinases (CDPK; Asano *et al*., 2005) leading to the regulation of ion transporters in cytosol. CIPKs are a family of serine/threonine kinases to which CBLs can interact and activate the downstream genes involved in plant growth, development, and cellular processes (Liu *et al*., 2013; Mao *et al*., 2023). In rice, *CBL1/9* located on chromosomes 10 and 1, respectively, and *OsCIPK23* on chromosome 7 leads to activation of *OsHAK5* and *OsAKT1* located on chromosome 1(Ragel *et al*., 2015; Li *et al*., 2006).

Expression QTL (eQTL) indicates the possible location of variation in DNA sequence that may result in an observable variation in the abundance of mRNA transcript. The eQTLs can be classified into *cis* and *trans* eQTLs based on the position of genetic variation. In *cis* eQTL, the variation can be within the gene or nearby whereas, *trans* eQTL can be located outside the gene location (Druka *et al*., 2010). In a study by Holloway and Li (2010), such genetic variations can help in understanding the regulation of key upstream or downstream components and their stress response. In this study, we have attempted to characterize the genetic variations in *OsCIPK23* for its role in regulating the expression of an associated downstream transporter, *OsHAK5* concerning potassium uptake and salt tolerance.

MATERIALS AND METHODS

Plant materials and growth conditions: Two rice genotypes were chosen for the study viz., *Pokkali* a well-known salt tolerant landrace widely deployed in rice breeding programs and cv. IR64 is an elite mega rice but salt susceptible. The above genotypes were raised hydroponically in a Yoshida nutrient medium (pH 4.5; Yoshida, 1976) in a greenhouse maintained at 30 °C (± 3 °C). Individual genotypes were raised in two-inch plastic net cups filled with perlite for anchorage and assembled on a polystyrene sheet. The entire setup was placed on a plastic container filled with Yoshida nutrient medium. The experiment included two treatments *viz*.,

a) Yoshida nutrient medium
b) Yoshida nutrient medium + 150 mM NaCl

Three biological replicates were maintained for each of the treatments. Salt stress was imposed at 55 DAS and continued for two weeks. The potassium content in the roots of control and salt-stressed plants was analyzed.

Estimation of potassium content in roots via flame photometry: The root samples from three biological replicates were grounded using liquid nitrogen. One gram of the powdered root sample was used for K^+ analysis. The samples were digested overnight in 15 ml of triple acid *viz*., nitric acid: sulphuric acid: perchloric acid in a 9:2:1 ratio in a 250 ml conical flask. The partially digested samples were placed in a sand bath till the solution turned clear. The digested mixture was filtered through Whatman no.1 filter paper and finally made up the volume to 100 ml using a volumetric flask (Overman and Davis 1947). Five standards ranging from 20 to 100 ppm were prepared using KCl and the concentration of potassium in the samples was expressed in ppm.

Total RNA extraction and first-strand cDNA synthesis: Total RNA from partially differentiated adventitious roots of *Pokkali* and cv. IR64 from salt-stressed treatment was extracted using TRI Reagent (Sigma-Aldrich) - chloroform-isopropanol (Chomczynski and Sacchi, 1987). Approximately, 200 mg of the root tissue was finely grounded with liquid nitrogen and extracted in 1ml of TRI reagent for five minutes and centrifuged at 12000 rpm for 10 min at 4˚C. The supernatant was transferred into a fresh 2 ml microfuge tube containing an equal volume of chloroform, shaken vigorously for 10-15 sec, and centrifuged at 12000 rpm for 10 min at 4˚C. The upper aqueous phase containing the total RNA was transferred into a new 1.5 ml tube to which 0.6 volume of isopropanol was added. The samples were incubated at -20 ˚C overnight and the total RNA was pelleted after centrifugation at 12000 rpm for 10 min and washed the pellet with 100 µl of 75% ethanol prepared using nuclease-free water. The pellet was finally air-dried and dissolved in 25-30 µl nuclease-free water. Downstream processing includes DNaseI treatment (#EN0521, Thermo Scientific, USA) to remove any contaminating genomic DNA during the preparation. The synthesis of first strand cDNA was carried out using Revert Aid first strand cDNA synthesis kit (#K1622, Thermo Scientific, USA) as per manufacturer guidelines.

PCR-based cloning of full length *OsHAK5* cDNA: Nucleotide sequence of *OsHAK5* (GenBank NM_001409013.1) from *Oryza sativa* ssp. japonica cv. *Nipponbare* was used as a reference to design PCR primers for cloning the full-length cDNA as well as catering to real-time PCR applications. For full length cDNA cloning, the following primers F: 5'- TCGGCCTGAGCAAACATTC- 3' and R: 5'-CATCACCATCAGTCTAGATCTC-3' were used in combination with High fidelity Q5 DNA polymerase (#M0492S; NEB, UK). A total volume of 20 µl reaction mixture containing 10 µl of Q5 DNA polymerase master mix (2X), 1 µl each of forward and reverse primers, 7 µl of nuclease-free water, and 1µlof cDNA were used. The reaction was set up in an Eppendorf master cycler system given the following conditions, initial denaturation at 98 ˚C for 3 min, cycle denaturation at 98 ˚C for 15 sec, annealing at 54.8 ˚C for 30 sec, extension at 72 ˚C for 2

min and 10 sec and final extension at 72 ˚C for 5 min, for 35 cycles. The expected amplicon was 2390 bp in length as per the *OsHAK5* reference sequence, the PCR product was purified using a Macherey-Nagel PCR clean-up kit (#NC0389462; Thermo Scientific, USA) and proceeded for ligation.

The purified cDNA of *OsHAK5* from both the genotypes were cloned into an intermediary cloning vector pJET1.2 (#K1231; Thermo Scientific JET PCR cloning kit). The ligation reaction comprised 10 µl of PCR product, 1 µl of pJET1.2 cloning vector, 5 µl of 2X reaction buffer (Thermo Scientific, USA), 3 µl nuclease-free water, and 1 µl T4 DNA ligase (#EL0011; Thermo Scientific, USA). The ligated product was transformed into *E. coli DH5α* competent cells and plated on LB medium containing ampicillin (100 mg/ml). The transformant colonies were screened by colony PCR technique using *OsHAK5* gene-specific primers to identify the recombinants and further characterized by sequencing.

Gene expression analysis of *OsHAK5* in rice roots: In an independent experiment, rice genotypes *Pokkali* and cv. IR64 were grown hydroponically in Yoshida nutrient medium (pH 4.5; Yoshida, 1976) in greenhouse conditions and subjected to salt stress at 55 DAS.

a) Yoshida nutrient medium
b) Yoshida nutrient medium + 150 mM NaCl
c) Yoshida nutrient medium with 1/4K
d) Yoshida nutrient medium with 1/4K + 150 mM NaCl

In the case of 1/4th potassium, the amount of K_2SO_4 was reduced accordingly retaining other nutrients as per the prescriptions of Yoshida medium.

Total RNA was extracted and cDNAs were synthesized from the roots of all four treatments with three biological replicates as per the protocol described above and the cDNA concentration was adjusted to 100 ng/µl. The real-time PCR primers for *OsHAK5* and the reference gene *Polyubiquitin* (*OsUbi*; XM_026026381.1) were designed using the Primer3Plus tool (https://www.primer3plus.com/index.html) (**Table 1**). The primers were designed to have a GC content of 50 % and T_m at 60 ˚C. Real-time PCR profile includes initial denaturation at 96 ˚C for 20 sec, denaturation at 96 ˚C for 5 sec, annealing at 60 ˚C for 10 sec, and extension at 72 ˚C for 15 sec and 40 cycles. Melt curve analysis was done between 65 ˚C and 95 ˚C.

The reaction mix consisted of 5 µl of *Sso Advanced Universal SYBR Green Supermix* (BioRad; cat no. #1725271), 0.5 µl of forward and reverse primers @ 2 pico moles, 3 µl of nuclease-free water and 1µl of cDNA (200 ng), the total reaction volume was made up to 10 µl. Real-time amplification was performed in the BioRad CFX connect PCR machine. The relative gene expression was calculated following the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Analysis of nucleotide polymorphisms: The nucleotide and derived amino acid sequences of *OsHAK5* from *Pokkali* and cv. IR64 was aligned globally using EBI-EMBL Align (www.ebi.ac.uk/Tools/). The coding sequence (CDS) of *OsCIPK23* from rice genotypes *Pokkali*, *Nonabokra,* and cv. IR64 was retrieved from the RFGB database (https://www.rmbreeding.cn/) and *Nipponbare* was retrieved from NCBI GenBank (www.ncbi.nlm.nih.gov/genbank/). Multiple sequence alignment (MSA) was performed with derived amino acid sequences of *OsCIPK23* using EBI-EMBL ClustalW2 (https://www.ebi.ac.uk/Tools/msa/clustalw2/). The functional motifs on the *OsCIPK23* protein were predicted using the ExPASy ScanProsite tool (http://prosite.expasy.org).

RESULTS AND DISCUSSION

On expected lines, under 150 mM NaCl stress, a 47-ppm increase in root potassium content was observed in salt-tolerant rice cultivar *Pokkali* whereas, a decline in the K^+ content by 23 ppm was observed in cv. IR64 (**Fig.1**). Of recent, there is increasing evidence emphasizing the role of an enhanced intracellular potassium content in response to salt stress more significantly in salt-tolerant rice genotypes thus shifting the Na/K ratio in favor of salt tolerance (Shabala and Cuin, 2008). Towards this, the role of high-affinity potassium transporters (HAKs) in regulating the potassium concentration under salt stress assumes importance. The rice genome (cv. *Nipponbare*) was annotated to harbor 27 *OsHAK* genes and only a few members of the *OsHAK* family were characterized for their role in regulating Na/K homeostasis under salt stress viz., *OsHAK1*, *OsHAK5,* and *OsHAK16* (Chen *et al*., 2015; Yang *et al*., 2014; Feng *et al*., 2019). Among them, *OsHAK5* plays the role as a Na^+-insensitive K^+ transporter in regulating the intracellular potassium content under salt stress despite a high level of extracellular sodium concentration. Further, transgenic plants over-expressing *OsHAK5* resulted in a lowered Na/K ratio under salt stress

Table 1. qRT-PCR primers used for gene expression analysis

Gene	Primer sequence	Amplicon length
qUBI92	qUBI-F: 5'- AGGATATGATGGTGCGAAGGC- 3'	92 bp
	qUBI-R: 5'- GCCATTTGAATTCCGCTTTGC- 3'	
qHAK5	qHAK5-F: 5'- AGGACAAATTGCTGGCATCG- 3'	75 bp
	qHAK5-R: 5'-ACTTTGTCCGTGCCAAAACG- 3'	

while, *OsHAK5* knock-out showed an increased Na/K resulting in salt susceptibility (Yang *et al.,* 2014). Earlier, it was demonstrated that the *OsHAK5* transporter was localized on the plasma membrane in roots and capable of overcoming the physiological symptoms during plant growth under low K^+ conditions through an increased K^+ uptake (Horie *et al*., 2011).

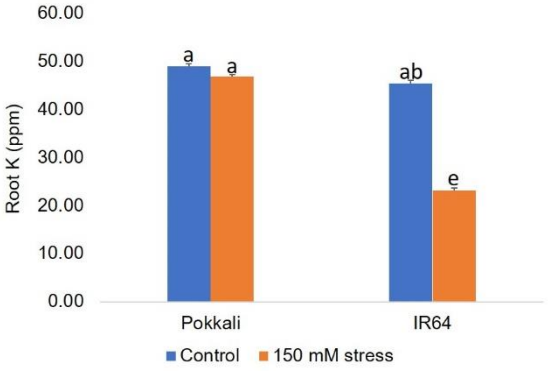

Fig. 1 Potassium content in roots of *Pokkali* and cv. IR64 under control and 150 mM NaCl stress

Each bar represents an average K^+ content in roots from three biological replicates. Values are mean ±SE (n=3); superscript 'a' is the best treatment and 'e' is the poorest treatment.

Molecular studies established the role of a major regulatory protein calcineurin B-like protein 9 and 1 (CBL1/CBL9), calcineurin B-like interacting protein kinases 23 (CIPK23) and high-affinity potassium transporter 5 (HAK5) in potassium uptake under salt stress in *Arabidopsis* and rice. The importance of the protein, CBL1/CBL9-CIPK23 complex in regulating K^+ uptake under low-K^+ conditions was stated by Xu *et al*. (2006). Under low-K^+ conditions, CBL1/CBL9 gets activated by cytosolic Ca^{2+} and interacts with CBL-interacting protein kinase 23 (CIPK23), which in turn phosphorylates HAK5 leading to an enhanced K^+ uptake under low-K^+ (Xu *et al*., 2006; Li *et al*., 2006; Ragel *et al*., 2015). These studies underscore the importance of *OsHAK5* as a probable candidate for enhancing cellular potassium concentrations under salt stress (Okada *et al*., 2018; Horie *et al*., 2011; Yang *et al*., 2014).

Towards exploring the prospects of *OsHAK5* in the rice improvement program, the full length cDNAs of *OsHAK5* from two contrasting rice genotypes *Pokkali* and cv. IR64 were successfully cloned and characterized by DNA sequencing. Rice landrace *Pokkali* is an internationally acclaimed reference genotype for salt tolerance and rice cultivar IR64 is a high-yielding mega rice variety but extremely susceptible to salt stress. The cDNA sequences of both genotypes *Pokkali* and cv. IR64, respectively were found to be equal in length at 2390 bp. This includes coding sequence (CDS) of 2346 bp plus partial sequences of the 5' and 3' UTR regions (**Fig. 2**). Pairwise analysis of the *OsHAK5* nucleotide sequences from *Pokkali* and cv. IR64 was performed to discriminate the alleles based on the nucleotide polymorphisms that existed between them. However, the coding sequence of *OsHAK5* of both genotypes showed no nucleotide polymorphisms between them.

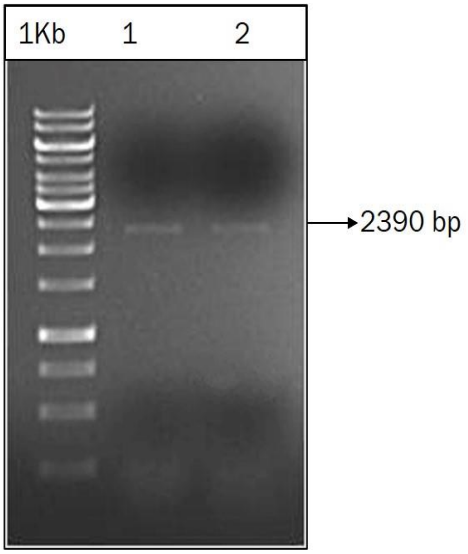

Fig. 2 PCR amplification of *OsHAK5* cDNA from *Pokkali* and cv. IR64

Lanes 1 and 2 are *Pokkali* and cv. IR64, respectively. The *OsHAK5* of size 2390 bp was amplified from both genotypes *Pokkali* and cv. IR64

Earlier studies on *OsHAK* transporters revealed that among the *HAK* family, *OsHAK5* was induced under low K^+ conditions (Okada *et al*., 2018; Uchiyama *et al.,* 2022). Additionally, Rubio *et al*. (2014) stated that a low level of intracellular K^+ concentration is required for the activation of *HAK5*-mediated high-affinity K^+ uptake in the roots of *A. thaliana*. In the present study, the differential upregulation of *OsHAK5* between *Pokkali* and cv. ASD16 under normal and low ($1/4^{th}$) K^+ concentrations were analyzed. Interestingly, the expression of *OsHAK5* was significantly upregulated under 150 mM NaCl stress in the roots of *Pokkali* under $1/4^{th}$ potassium concentration with a fold change (FC 1.63) as compared to the normal concentration (FC 1.25). Contrastingly, in rice cv. IR64, the upregulation of potassium under salt stress was significantly minimal as evident from the fold change values of 0.238 and 0.184 under normal and $1/4^{th}$ potassium concentrations, respectively **(Fig. 3**). This demonstrates the differential regulation of *OsHAK5* between the salt-tolerant and susceptible rice genotypes and its role in enhancing potassium uptake in response to 150 mM NaCl salt stress only in salt tolerant rice genotype *Pokkali* as compared to salt susceptible rice cv. IR64.

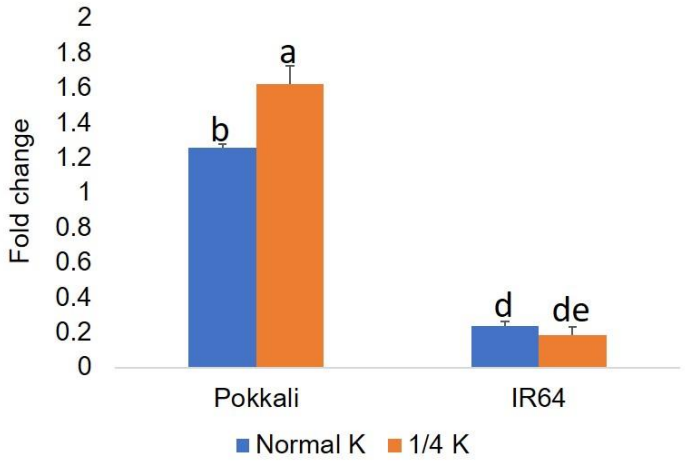

Fig. 3. Relative expression of *OsHAK5* in the roots of *Pokkali* and cv. IR64 between control and 150 mM NaCl stress at normal and low (1/4th) potassium concentrations

Each bar represents an average fold change from three biological replicates. Values are mean ±SE (n=3); superscript 'a' is the best treatment and 'e' is the poorest treatment.

The regulation of *OsHAK5* under salt stress will be a cascading process beginning with the induction of calcineurin B-like proteins (*OsCBL1/9*) and their interaction selectively with the protein kinase (*OsCIPK23*) resulting in the formation of an activated transcription complex (OsCBL1/9-OsCIPK23) which in turn bind to the promoter of the downstream *OsHAK5* gene (**Fig. 4**).

In the absence of any nucleotide polymorphisms in the CDS of *OsHAK5* between *Pokkali* and cv. IR64, we next explored the nucleotide variations in the coding sequence (CDS) of the immediate upstream regulator *OsCIPK23*. Multiple sequence alignment (MSA) based on the derived amino acid sequences of *OsCIPK23* from two salt tolerant landraces (*Pokkali* and *Nonabokra*) and two highly salt susceptible genotypes (*Nipponbare* and cv. IR64) exhibited INDELs at two different positions between the contrasting groups. Accordingly, deletion of glutamic acid (E) at 78 th position was observed only among the highly salt tolerant landraces *Pokkali* and *Nonabokra* whereas, insertion of glutamine (Q) at 334th position was found in the highly salt susceptible genotypes *Nipponbare* and cv. IR64. Both the groups however exhibited the same number of amino acids, length wise (**Fig. 5**).

Fig. 4. OsCBL - OsCIPK interaction in the activation of the *OsHAK5* gene in rice

Further, functional motifs on OsCIPK23 protein predicted using ExPASy Scan Prosite revealed that the N-terminal amino acids ranging between 13 and 267 folded into a kinase domain and the INDEL at 78th position fell within in this region. An activation loop with conserved serine, threonine, and tyrosine residues was located within the N-terminal kinase domain and these conserved residues act as the phosphorylation site for the activation of CIPKs (Guo *et al*., 2001; Gong *et al*., 2002). And, the second INDEL at 334th position fell close to the NAF domain that plays a role in interacting with the activated OsCBL1/9 (**Fig. 6**).

All CIPKs generally had a highly conserved autoinhibitory motif called NAF in the C-terminus. The NAF accounts for the highly conserved asparagine-alanine-phenylalanine residues within the 21 amino acid stretch of length (Sanyal *et al*., 2016; Guo *et al.,* 2001). The intramolecular interaction between the NAF motif and kinase domain generally inhibits the kinase activity of CIPKs and keeps them inactive. The increased cytosolic Ca^{2+} levels due to stress or other factors will be sensed by the CBLs. The Ca^{2+} bound and activated CBL in turn binds to the NAF motif of CIPK and enables the kinase free to bind with the downstream ion transporters (Gong *et al*., 2002). The INDELs as observed in *OsCIPK23* could lead to distinct structural changes and functional differences between the allelic variants of the salt-tolerant (*Pokkali* and *Nonabokra*) and susceptible rice genotypes

(cv. IR64 and *Nipponbare*). As a consequence, the expression of *OsHAK5* gets differentially regulated and in turn, does the K^+ uptake under salt stress (**Fig. 7**). This is a convincing example of an expression quantitative trait (eQTL) at work in regulating the K^+ uptake process under salt stress in rice.

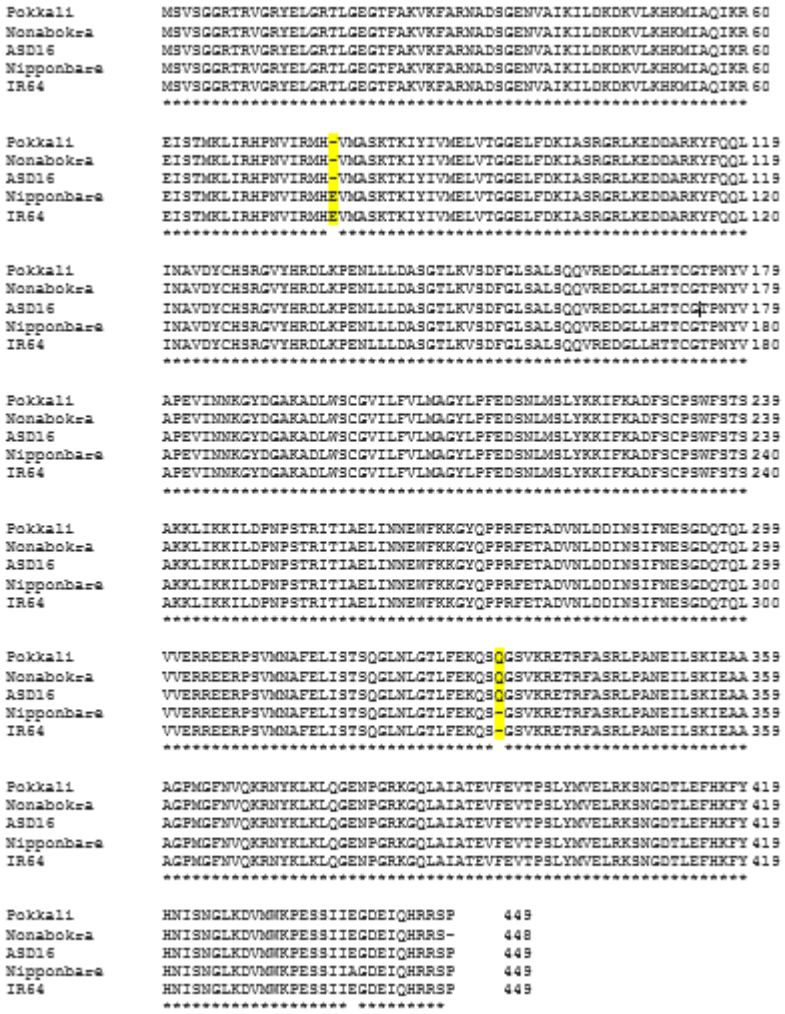

Fig. 5 Multiple sequence alignment of amino acid sequence of *OsCIPK23* from salt tolerant (*Pokkali* and *Nonabokra*) and salt susceptible (cv. IR64 and *Nipponbare*) genotypes

(Highlighted yellow bars denote the INDELs that differentiate the alleles between selected genotypes)

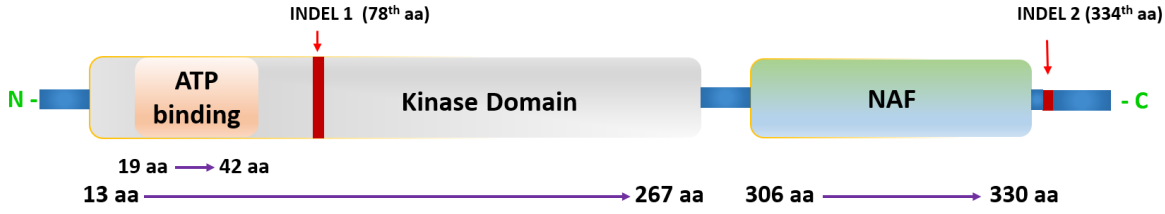

Fig. 6. Functional motifs predicted on OsCIPK23 protein

(Red bars represent the position of INDEL 1 and 2, respectively; the 'Lilac' arrow represents the length of each motif)

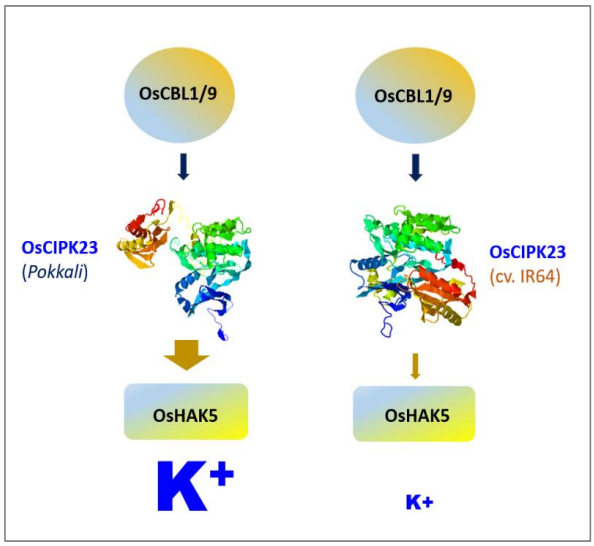

Fig. 7. Allelic variants of OsCIPK23 differentially regulate the expression of *OsHAK5* between *Pokkali* and cv. IR64

The functionally superior structural variant of *OsCIPK23* as in *Pokkali* will enhance the expression of *OsHAK5* leading to an increased K^+ uptake and in turn salt tolerance.

The present investigation looks forward to characterize the role of a specific high-affinity potassium transporter gene *OsHAK5* and its regulation between salt tolerant and susceptible genotypes. Here, we propose an association between the allelic variants of *OsCIPK23* and the expression levels of *OsHAK5*. Thus, the differential expression of *OsHAK5* under salt stress is regulated by the eQTL in *trans*. The genetic variations in *OsCIPK23* pave the way for developing allele-specific PCR primers and facilitate targeted introgression of the eQTL governing enhanced potassium uptake under salt stress through molecular breeding.

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