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In silico approach to investigate the potential *HKT* gene responsive to salt stress in rice

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Abstract

Rice is frequently subjected to various environmental stresses, resulting in significant production losses, with drought and salinity are the leading causes of plant damage globally. This study aims to characterize and understand the function of rice high-affinity potassium transporters (*HKTs*) genes in response to salinity stress. Initially, the genome-wide analysis was undertaken to reveal the evolutionarily conserved function of the *OsHKT* in higher plants. To investigate the transcription level of *OsHKT* during the vegetative and reproductive stages, two microarray datasets (GSE19024 and GSE3053) were analyzed, and salt-treated samples were subsequently evaluated using real-time PCR. Differentially expressed genes (*DEGs*) were identified from microarray datasets (GSE41650 and GSE14403), followed by constructing a *DEG* network that highlighted interaction partners of the *OsHKTs*. Genome mining of rice revealed 9 *HKT* genes, namely *OsHKT1;1–1;5* and *OsHKT2;1–2;4*. These genes exhibited a well-conserved domain structure called *TrkH*. Comprehensive phylogenetic and motif analyses clustered genes encoding *HKT* proteins into seven monophyletic groups, and the motifs were relatively conserved. Ka/Ks ratios indicated a high degree of purifying selection during evolutionary time. Gene ontology findings suggested the involvement of *OsHKT* in stress response. Besides, several CRE motifs in the promoter regions of *OsHKT* have demonstrated their potential roles in abiotic stress responses. Furthermore, we analyzed the top 250 significant *DEGs* from the two datasets (p-value < 0.05; fold two change ≥ 1 or ≤ − 1) to evaluate the relationship among the *DEGs* and *HKTs*. Three co-expressed *OsHKT* genes were discovered to be upregulated in seedlings under salinity treatment, including *OsP5CS2*, *OsHAK1*, and *OsNHX2*, whereas *OsP5CS1* and *OsHAK27* were downregulated. The transcripts of *OsHKT* were found to be differentially expressed in a tissue-specific manner. Analysis of microarray datasets validated by real-time PCR shows that *OsHKT1;5* had a higher expression level, followed by *OsHKT1;1*, *OsHKT1;3*, and *OsHKT2;1* after salinity treatment. In addition, several micro-RNA targets in rice *HKT* genes regulate their expression in response to stress. This study paves the way for future investigation on genes and miRNA-target interaction in plants under environmental stresses, offering potential strategies to enhance stress tolerance in crops via targeted ion transport modification.

Keywords HKT genes, Ion transport, Abiotic stress, Expression profile, Rice

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Introduction

Rice (*Oryza sativa* L.) is a staple food for nearly half the world's population, especially in tropical Latin America and most Asian countries (Shankar et al. 2016). Over the past decade, abiotic stresses have increased significantly due to environmental changes, land debasement, and declining water quality (Wassmann et al. 2009). Among these, soil salinity is one of the most devastating environmental stresses, causing significant reductions in cultivated land area, crop productivity, and quality (Shahbaz and Ashraf 2013). More than 800 million hectares of land are affected by salt, making up ~7% of the total land area (Munns et al. 2006). High salinity is estimated to affect 20% and 33% of total cultivated and irrigated agricultural lands worldwide. By 2050, it's projected that over 50% of agricultural land will be salinised due to low precipitation, high surface evaporation, weathering of native rocks, irrigation with saline water, and poor cultural practices (Jamil et al. 2011). Notably, most crops, especially rice, are salt-sensitive, prompting extensive research studies to uncover the mechanism of salt tolerance in crop species (Sytar et al. 2017).

Soil salinization hinders plant growth and development by increasing the concentration of sodium (Na^+) and chloride (Cl^-) ions in the soil, which disrupts seed germination, reproductive development, and vegetative growth (Munns and Tester 2008; Guo et al. 2015; Guo et al. 2018; Kamran et al. 2019). Under salt-affected areas, osmotic stress triggers physiological changes in plants, such as stomatal closure, increase in leaf temperature, inhibit photosynthesis (Awlia et al. 2016), and adverse effects on root architecture and cell wall properties (Geng et al. 2013; Julkowska et al. 2014; Feng et al. 2018). Excessive accumulation of Cl^- ions can hinder photosynthesis, protein synthesis, and other essential enzyme activities (Yamaguchi et al. 2013; Hasanuzzaman et al. 2018), and ultimately affects premature leaf senescence and cell death in plants due to its toxicity (Munns et al. 2006; Munns and Tester 2008; Roy et al. 2014). Despite being toxic at high concentrations, Na^+ plays a role in osmoregulation and is a substitute for potassium (K^+) under low K^+ conditions due to their similar physiochemical properties. The use of Na^+, notwithstanding, requires tight control over K^+ and Na^+ uptake, transport, and compartmentalization, which becomes crucial in states of high Na^+ concentration in plant vascular tissue (Flowers 1985; Hasegawa et al. 2000; Mühling and Läuchli 2002). Maintaining the K^+/Na^+ ratio is fundamental for plant longevity, emphasizing the importance of Na^+ transport, water direct, and signaling atoms under salt stress exposure (Hilker and Schmülling 2019; Wang et al. 2019).

Maintaining the Na^+/K^+ ratio in the cytosol for metabolic processes and salinity tolerance in plants is crucial because Na^+ can disrupt the K^+ balance in plants (Assaha et al. 2017). Several types of Na^+ transporters have been reported to play critical roles in Na^+ homeostasis during salinity. These, include the sodium-hydrogen antiporter (*NHX*) involved in vacuolar sequestration of Na^+, salt overly sensitive (*SOS*) responsible for root avoidance of toxic concentration, the non-specific cation channel (*NSCC*) which provides the main pathway for Na^+ uptake and translocation into the root at high NaCl concentrations, and high-affinity potassium transporter (*HKT*) that aids in the removal of Na^+ from the cell (Demidchik and Maathuis 2007; Quan et al. 2018; Yang and Guo 2018; Arabbeigi et al. 2019; Bernstein 2019). In addition, the presence of K^+-transporting membrane proteins, such as *AKT/KAT*-type channels, *HKT*-type transporters, and *HAK/AT/KUP*-like transporters has also been observed to participate in low and/or high affinity K^+ uptake systems of rice (Mäser et al. 2001; Golldack et al. 2002).

High-affinity potassium transporters (*HKTs*) are membrane proteins that play a vital role in facilitating cation transport across the plasma membranes of plant cells (Waters et al. 2013) and are also crucial in managing salt tolerance and mitigating the effects of salinity on plants. *HKTs* prevent the entry of Na^+ particles into shoot tissues by eliminating Na^+ from the xylem and regulating Na^+ and K^+ levels in parenchyma cells (James et al. 2006). The high-affinity K^+-update system was first discovered in wheat *HKT1* protein with some others encoded as Na^+ uniporters (Uozumi et al. 2000) and Na^+ and K^+ co-transporters (Schachtman and Schroeder 1994). *HKT* gene family has also been widely found in eudicotyledon and monocotyledon plant species, such as Arabidopsis (Maser et al. 2002), barley (Haro et al. 2005), eucalyptus (Liu et al. 2001), grapevine (Jabnoune et al. 2009), ice plant (Su et al. 2003), rice (Horie et al. 2001; Garciadeblás et al. 2003), and wheat (Schachtman and Schroeder 1994). *HKT* is divided into two subfamilies. *HKT* subfamily 1 is a Na^+ transporter, while HKT subfamily 2 is merely found in monocotyledon plants and is responsible for transporting K^+ and Na^+ across the cells. According to a phylogenetic study (Platten et al. 2006), both *HKT* subfamilies have a distinct conserved amino acid residue in the first pore loop of the amino acid sequence, with subfamily 1 members containing the amino acid Ser-Gly-Gly-Gly, while serine is replaced by glycine in subfamily 2 members, which designated as Gly-Gly-Gly-Gly (Maser et al. 2002; Garciadeblás et al. 2003). While the presence of glycine permits the transport of Na^+ and K^+ depending on the external ion concentration, the presence of serine favors the transport of Na^+ over other cations.

Despite the critical role of *OsHKT* as Na^+ and K^+ transporters under salinity stress, there is a need for more comprehensive studies. In this study, we investigated the functions and evolutionary relationship of *OsHKT* gene family members in rice with eudicot plants by performing in silico analysis on the publicly available sequenced genome. Our findings on gene structure, chromosomal localization and duplication pattern, promoter analysis, expression patterns, miRNA pattern, and co-expression networks suggest that *OsHKT* genes are involved in ion homeostasis through Na^+ and K^+ transport in response to the salinity. Our results offer a valuable resource for functional studies of the *OsHKT* gene to mitigate abiotic stress problems in rice.

Materials and methods

Identification of *OsHKT* family members in rice
To obtain the potential candidate *HKT* amino acid sequences in rice, the Hidden Markov Model (HMM) profiles of the conserved *HKT* domain (PF02386) were downloaded from Pfam (http://pfam.sanger.ac.uk/) (Mistry et al. 2021). HMM profiles are powerful probabilistic models designed to capture the evolutionary variations in a group of related sequences. The BLASTP search using the HMM profile was carried out to scan the protein database on the MSU Rice Genome Annotation Project (MSU-RGAP) (rice.plantbiology.msu.edu), Phytozome (https://phytozome.jgi.doe.gov) (Goodstein et al. 2012), Ensemble Genomes (https://plants.ensembl.org/Oryza_sativa/Info/Index) (Monaco et al. 2014) with the parameters of an E-value threshold of -1 and the BLOSUM62 comparison matrix. Also, *HKT* genes were retrieved from the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov) (Sayers et al. 2021) using the keyword search 'HKT'. The list of *HKT* genes was then integrated, followed by the removal of redundant genes. The Online CD-search tool of NCBI (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), Pfam (http://pfam.sanger.ac.uk), and Simple Modular Architecture Tool (SMART) (http://smart.embl-heidelberg.de/smart/batch.pl) (Letunic and Bork 2018) was used to reconfirm the class of predicted *HKT* proteins.

Protein features analysis of *OsHKT* family in rice
The physic-chemical properties of *OsHKT* protein, including the number of amino acids, molecular weight (Da), isoelectric point (pI), instability index, and grand average of hydropathicity (GRAVY), were predicted using the ProtParam tool in the ExPASy database (https://web.expasy.org/protparam/) (Gasteiger et al. 2005). Subsequently, the transmembrane structure of the *OsHKT* protein was predicted using DeepTMHMM (https://dtu.biolib.com/DeepTMHMM) (Hallgren et al. 2022).

Phylogenetic analysis
In this study, two phylogenetic trees were constructed among the *HKT* gene family members in (i) monocotyledons (rice, wheat, and maize) and (ii) eudicotyledons (Arabidopsis, grape, and tomato). The amino acid sequences of *HKT* proteins, namely *OsHKT* (rice), *TaHKT* (wheat), *ZmHKT* (maize), *AtHKT* (Arabidopsis), *VvHKT* (grape), and *SlHKT* (tomato) were downloaded from Phytozome v13, Ensemble Plants, and NCBI. Multiple sequence alignments for each analysis were performed using the ClustalX2.0 (Li et al. 2015) tool with the default parameters. The phylogenetic tree analysis was then constructed using MEGA X software with the Maximum Likelihood (ML) method, and the bootstrap value was set to 1000 replicates with a complete deletion mode (Kumar et al. 2018).

Gene structure and conserved motifs analysis
We used the Gene Structure Display Server 2.0 (http://gsds.gao-lab.org) to visualize the exon–intron arrangement of the *OsHKT* gene by aligning the genomic DNA with the corresponding cDNA sequences (Hu et al. 2015). To further support the evolutionary relationship, conserved motifs in the *OsHKT* protein sequences were identified using the MEME suite version 5.3.3 (https://meme-suite.org/meme/) (Bailey et al. 2009). The parameters used for motif discovery were as follows: site distribution models = zoops, number of motifs = 10, width of motifs > 6 and < 50, and sites of motif > 2 and < 600. The function of each motif was then searched against the CD-search tool of the NCBI database with a default E-value cutoff of 0.01 (Marchler-Bauer et al. 2017). MyHits (https://myhits.sib.swiss/) was also used to annotate the motif sequence for functional prediction.

Chromosomal localization and gene duplication analysis
To illustrate the gene locations on the rice chromosome, the chromosomal positions of *OsHKT* genes were acquired from the phytozome and mapped using TBtools software (Chen et al. 2020a). Two or more genes located on the same chromosome represent the possibility of tandem duplication, whereas genes on different chromosomes indicate segmental duplication (Zhu et al. 2014; Nasim et al. 2016). Therefore, the tandem and segmental duplications of the *OsHKT* gene were observed based on their locations in the chromosome. To further calculate the evolutionary time of the *OsHKT* gene family, the non-synonymous (d_N or Ka) and synonymous (d_s or Ks) values were calculated using PAL2NAL (Suyama et al. 2006). The duplication time of the gene pairs was estimated using the formula of the synonymous mutation rate of substitution per synonymous site per year

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as follows: $T = Ks/2x$, $(x = 6.56 \times 10^{-9})$, where, T = time of divergence, Ks is the synonymous substitution per synonymous site, and x is the mean rate of synonymous substitution (Yuan et al. 2015). The d_s/d_N ratio was used to detect the selective pressure on the *HKT* genes and by aligning the DNA-coding sequence of the *HKT* genes in rice to identify site-specific positive or purifying selection by the Selecton Server (Stern et al. 2007).

Subcellular localization, transcription factor binding sites (TFbs) and *cis*-regulatory elements (CREs) analysis, and gene regulation of *OsHKT* family in rice

To predict the subcellular localization of the *OsHKT* protein, the protein sequences were blasted against eukaryote protein sequences in the CELLO2GO webserver with an E-value of 0.001 (http://cello.life.nctu.edu.tw/cello2go/) (Yu et al. 2014). The 1.5 kb upstream of the genomic sequences were retrieved from the Phytozome to identify the promoter regions of the *OsHKT*. Furthermore, the transcription binding sites were predicted using 1.5 kb genomic sequences as input data and searched against a multiple promoter analysis database, PlantPAN 2.0 (http://plantpan2.itps.ncku.edu.tw/) (Chow et al. 2016) and CREs using PlantCARE. The PlantRegMap (http://plantregmap.gao-lab.org) was utilized to retrieve gene regulation information containing interaction between transcription factors that regulate the *OsHKT* gene (Tian et al. 2019). The interactions between the TF and *OsHKT* genes were then visualized by using Cytoscape v3.8.2 (Shannon 2003).

Genome-wide expression analysis of the *OsHKT* family in rice

The expression datasets for the *OsHKT* gene family in 22 tissues for the indica rice variety Minghui 63 were extracted from the Affymetrix rice microarray data in the Collection of Rice Expression Profiles (CREP) database under accession number GSE19024 (Wang et al. 2010). For salinity treatment, we used the microarray dataset GSE3053 from NCBI GEO, which includes salt-tolerant FL478 and salt-sensitive IR29 genotypes (Walia et al. 2005). The strongest signal was used using multiple probe sets for a single gene. GEO expression datasets and the treatment $\log_2$ fold change dataset were normalized using a gene-wise normalization combination technique. To cluster the expression data of *OsHKT* under salinity and tissues, we generated the heatmap using TBtools software (Chen et al. 2020a).

Array data collection acquisition and identification of DEGs

This study retrieved two sets of microarray series containing expression profiles from the GEO database (Clough and Barrett 2016). The keywords "salinity" and "rice" were selected to search GEO datasets for related gene expression profiles. GSE41650 consists of 27 samples, nine of which are control (7-day-old seedlings without treatment) and 18 are salinity (7-day-old seedlings with salinity treatment). GSE14403, on the other hand, contains 23 samples, including 11 untreated root and 12 salt-treated root samples as control and salinity, respectively (Cotsaftis et al. 2011). Both datasets were obtained using the platform GPL2025 [Rice] Affymetrix Rice Genome Array.

To examine the differentially expressed genes (*DEGs*), the online statistical tool GEO2R was utilized (Barrett et al. 2012). The GEO2R inbuilt methods, such as the T-test and Benjamini and Hochberg (false discovery rate), were applied to calculate the p-value and false discovery rate (FDR) determining the DEGs between control and salinity group (Aubert et al., 2004). The principal criteria of |log (fold change)| > 1 and $p < 0.05$ were applied to identify significant DEGs from the dataset. The DEGs were considered upregulated if the logFC ≥ 1 and downregulated if the logFC ≤ − 1.

Establishment of *OsHKT* protein networks and gene ontology (GO) annotation

A protein–protein interaction (PPI) network of differentially expressed *HKTs* was constructed using the Cytoscape String App (Doncheva et al. 2019). A confidence score ≥ 0.4 was employed to retrieve the PPI information of statistically significant *DEGs* from the STRING database. The PPIN of *HKTs* was then visualized using Cytoscape software v3.7.1 (Shannon 2003). To annotate the *OsHKT* genes, all the protein sequences were blasted against eukaryote protein sequences in the CELLO2GO webserver with an E-value of 0.001 (Yu et al. 2014). The results were then categorized into biological processes, molecular functions, and cellular components.

miRNA target site prediction of *OsHKT* proteins in rice

First, mature miRNA was obtained from the PmiREN website (https://www.pmiren.com/) to identify the *OsHKT* gene family's target locations in rice (Guo et al. 2022). Next, we used the web server program PsRNA (https://www.zhaolab.org/psRNATarget/) with the default settings to search the CDSs of the *OsHKT* genes against mature miRNAs (Dai and Zhao 2011). Cytoscape was used to build the networks connecting the anticipated miRNAs (Shannon 2003).

Plant materials and treatment

Mature seeds of pokkali and IR64 were used for expression analysis. Seeds were then sterilized with 5% sodium hypochlorite solution for 10 min and rinsed with distilled water 5–6 times. Next, sterile seeds were submerged in

deionized water at 30 °C for two days before being placed in a growth chamber and incubated for 24 h at 28 °C. The seedlings were grown in hydroponic solution for 21 days according to IRRI protocol (Yoshida et al. 1976). The uniform 21-day-old seedlings were imposed to 100, 150, and 200 mM NaCl with control. Tissues were collected immediately for control and after 24h NaCl treatments for RNA isolation.

RNA isolation, cDNA synthesis and qRT-PCR

According to the manufacturer's instructions, total RNA was extracted from each genotype using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, USA). Total RNA was tested for purity and integrity with a Nanodrop. The RNA sample was taken at an A260/280 ratio of 1.8–2.0 and an A260/230 ratio of 2.0–2.2, and it has been kept at −80 °C until further usage. Following the manufacturer's instructions, the first strand of cDNA was synthesized with a HiScriptIII First Strand cDNA Synthesis Kit (Vazyme Biotech, China). For two minutes at 42 °C, 100 ng of total RNA was combined with 2 μL of 5×gDNA Mix wiper and RNase-free sterile water. The cDNA synthesis mixture contains 4 μL of RNAse-free sterile water, 1 μL of Oligo (dT) 20 VN, 1 μL of Random hexamers, and 2 μL of 10×RT Mix. The mixture was incubated at 37 °C (15 min) and 85 °C for 5 s. The resultant cDNA products are frozen at −80 °C until needed. THUNDERBIRD® SYBR® qPCR mix (TOYOBO, Japan) was used to perform qPCR amplification on cDNA aliquots of 3 μL in 20 μL reaction volumes with gene-specific primer and actin as an internal control (Supplementary Table 1) in 96-well plate Applied Biosystems 7500 Fast Real-Time PCR system. The $2^{-\Delta\Delta CT}$ method was used to analyze the relative expression of genes (Livak and Schmittgen 2001).

Statistical analysis

The statistical analysis was conducted utilising analysis of variance (ANOVA), and the means were compared using the Least Significant Difference (LSD) at a significance level of $P \leq 0.05$, employing the R program. The steps taken to analyze the *HKT* family members in rice are depicted in Fig. 1.

Results

Genome-wide identification of *HKT* family proteins in rice

In our study, nine *HKT* genes were identified in rice, namely *OsHKT1;1*, *OsHKT1;2*, *OsHKT1;3*, *OsHKT1;4*, *OsHKT1;5*, *OsHKT2;1*, *OsHKT2;2*, *OsHKT2;3*, and *OsHKT2;4*. Among them, *OsHKT1;2* and *OsHKT2;2* are known as pseudogenes in *Oryza sativa* Nipponbare (Horie et al. 2001). The *HKT* family members in rice consist of a highly conserved domain structure called *TrkH*, a cation transport protein domain responsible for actively transporting sodium ions into the cell. Figure 2 illustrates the presence of the *TrkH* conserved protein motif within the *HKT* family in rice.
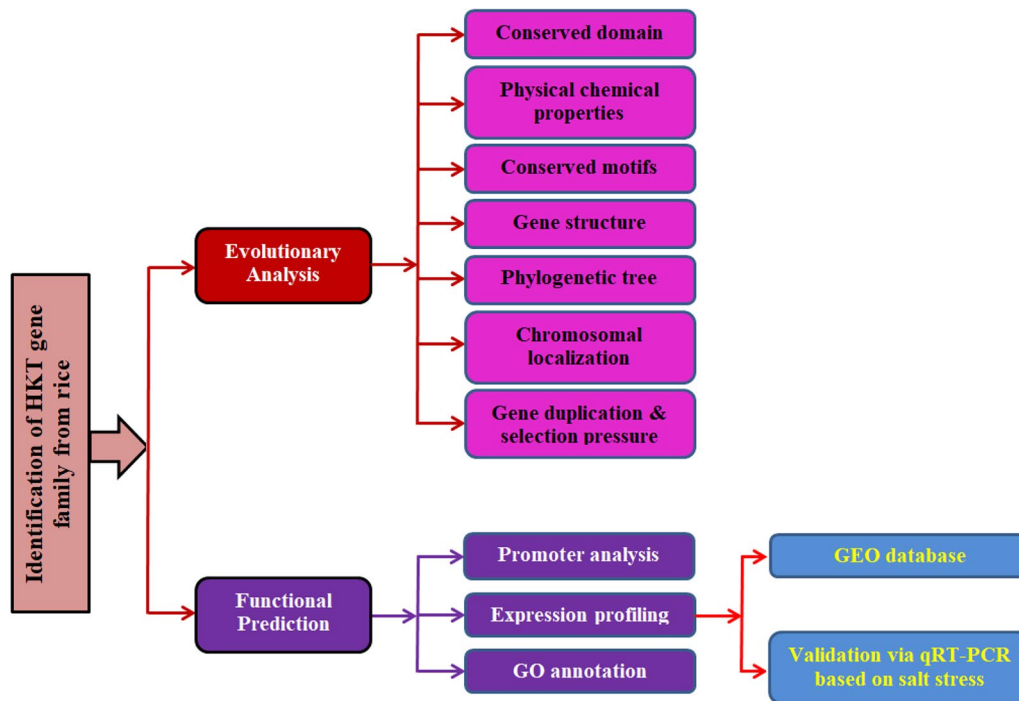

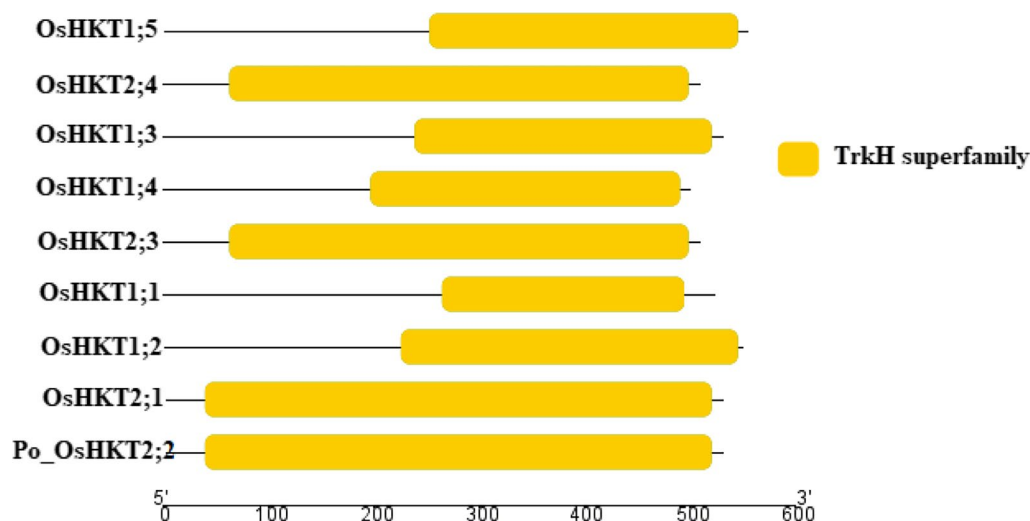**Fig. 1** Schematic representation of the steps taken to analyze the *HKT* family proteins in rice

Fig. 2 The structure of the *TrkH* domain of *HKT* family proteins in *Oryza sativa*

The *OsHKT* family genes exhibit significant variations in the size and properties of the encoded proteins (Table 1). We predicted *OsHKT1;5* to be the longest *HKT* protein, with 554 aa, whereas *OsHKT1;4* has the shortest length of amino acids, with 500 aa. A wide range of predicted molecular weights was found among *OsHKT* genes, ranging from 54.24 kDa to 60.22 kDa, and an isoelectric point (pI) ranging from 8.74 to 9.49. Minor differences in molecular weight and theoretical isoelectric point are observed among *HKT* proteins, suggesting subtle differences in physical and chemical characteristics in rice. The grand average of hydropathicity (GRAVY) values of positive and negative residues indicates a protein's hydrophobicity and hydrophilicity, respectively. All the *HKT* proteins in rice showed positive GRAVY values, indicating that *OsHKT* proteins were hydrophobic. *OsHKT* genes, namely *OsHKT1;1, OsHKT1;3, OsHKT2;1,* and *OsHKT2;2* was predicted to be stable proteins based on the cut-off instability index < 40, while the *OsHKT1;4, OsHKT1;5, OsHKT2;3* and *OsHKT2;4* was unstable with an instability index > 40 as shown in Table 1. Protein sequence similarity indicated that *OsHKT2;1* and *Po_OsHKT2;2* showed the highest levels of protein sequence similarity (82.58%), whereas *OsHKT1;1* and *OsHKT1;4* showed the least protein sequence similarity (31.84%) (Supplementary Table 2). In addition, *OsHKT* family proteins were found to be in the plasma membrane and comprise an equal number of transmembrane helices, as shown in Table 1.

Phylogenetic analysis and identification of conserved motifs and gene structure of *OsHKT* family proteins
A phylogenetic tree was constructed to gain insights into the evolutionary relationship among the *OsHKT* genes (Fig. 3A). The constructed phylogenetic tree is composed of two major monophyletic branches, comprising four paralogous pairs of *OsHKT* such as *OsHKT1;1-OsHKT1;2,* *OsHKT1;4-OsHKT1;5,* *OsHKT2;1-Po_OsHKT2;2* and *OsHKT2;3-OsHKT2;4.* This reflects the highly conserved nature of the *OsHKT* family gene, particularly among the *OsHKT1* and *OsHKT2* groups. Also, MEME analysis discovered ten distinct motifs in the *OsHKT* protein sequences, with motif lengths ranging from 24 to 50 amino acids (Fig. 3B). Nine out of ten motifs appeared in all the *OsHKT* proteins, suggesting that both *OsHKT1* and *OsHKT2* are relatively conserved; however, one motif was discovered to be uniquely present in all genes within the monophyletic group of *OsHKT2*, indicating *OsHKT2* to have a distinct function compared to *OsHKT1*.

To infer the function of each motif, we further annotated the motifs using motif scan and CD-search tools. Motifs 1–7 and 9 were mainly annotated as *TrkH* (cation transport protein). Several site-specific motifs were also detected among the motifs. For instance, Motif 1 is associated with the N-glycosylation site and the protein kinase C phosphorylation site. Motif 4, on the other hand, is related to the N-myristoylation site, and Motif 5 is associated with the cAMP- and cGMP-dependent protein kinase phosphorylation sites. Motif 7 is related to

Table 1 Characteristics of Rice *HKT* family gene

Transcript ID	Gene name	Chr.no	Location Start–End	Strand	CDS (bp)	Protein length (aa)	Molecular weight (Da)	pI	Gravy	Insta-bility index	Intro/Exons no	SL	Transme-mbrane topology
LOC_Os04g51820.1	*OsHKT1;1*	4	30724244–30727084	r	1572	523	58872.94	9.17	0.283	39.52	1:02	PM	8
LOC_Os02g07830.1	*OsHKT1;3*	2	4103333–4105657	f	1596	531	59304.53	9.41	0.28	26.94	2:03	PM	8
LOC_Os04g51830.1	*OsHKT1;4*	4	30734183–30739334	r	1503	500	54239.3	8.96	0.38	43.9	2:03	PM	8
LOC_Os01g20160.1	*OsHKT1;5*	1	11458955–11463442	r	1665	554	60137.11	8.52	0.378	40.24	2:03	PM	8
LOC_Os06g48810.1	*OsHKT2;1*	6	29538934–29541219	r	1593	530	59295.08	9.43	0.506	37.45	2:03	PM	8
LOC_Os01g34850.1	*OsHKT2;3*	1	19242042–19243853	f	1530	509	56374.38	9.13	0.472	46.89	2:03	PM	8
LOC_Os06g48800.1	*OsHKT2;4*	6	29534805–29536553	r	1530	509	56116.72	8.74	0.443	45.79	2:03	PM	8
BAB61791.1 (Protein acc. no.)	*Po_OsHKT2;2*	–	–	–	1593	530	59151.84	9.49	0.448	37.56	0:01	PM	8
AJ506745* (Nucleo. acc. no.)	*OsHKT1;2*	–	–	–	1659	549	61990.90	9.60	0.254	38.21	2:03	PM	8

* Nucleotide sequence was used to translate protein sequence. r indicates reverse, f indicates forward, bp indicates base pair, aa indicates amino acids, Da indicates Dalton, and pI indicates protein isoelectric point. Subcellular localization (SL) predictions: PM indicates plasma membrane
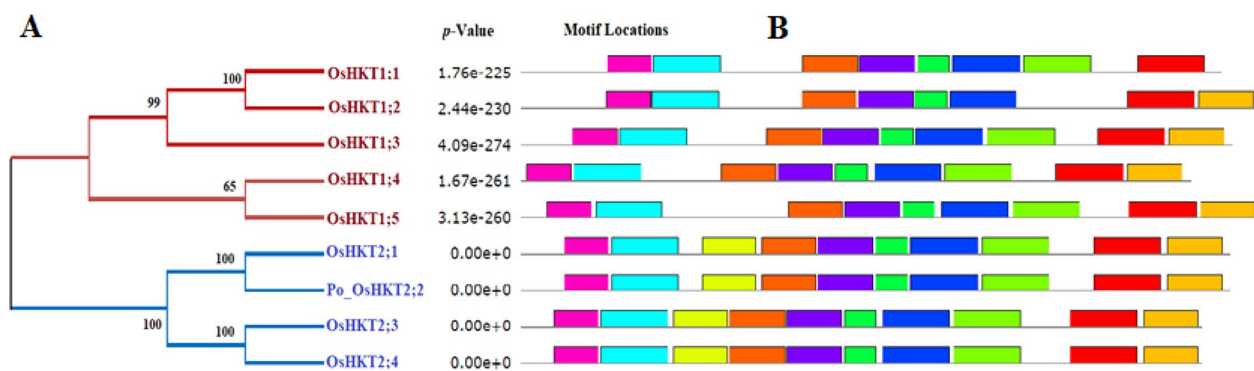
Fig. 3 Phylogenetic relationship (**A**) and schematic representation of the conserved motifs (**B**) of *HKT* family proteins in *Oryza sativa*

the tyrosine kinase phosphorylation site, and Motif 8 is associated with the casein kinase II phosphorylation site. Motif 9 is also annotated for the protein kinase C phosphorylation site, and motif 10 is related to the cAMP- and cGMP-dependent protein kinase phosphorylation sites and the casein kinase II phosphorylation sites. The absence of motif 10 in *OsHKT1* family genes could be attributed to the diversification and loss of specific sequences during the evolution of rice. This phenomenon might have resulted in distinct functions between the *OsHKT1* and *OsHKT2* groups.

The diversification and arrangement of gene structures have had a significant impact on the evolution of gene families. Figure 4 depicts detailed information on introns, exons, and untranslated regions of *OsHKT* genes. Exons are the coding regions that code for amino acids and are separated by noncoding regions called introns. Introns play essential roles in various cellular processes, including genomic recombination, which can lead to gene rearrangements and contribute to the evolution of genes and species. The monophyletic group of *OsHKT1* genes

is composed of 2–3 exons and separated by 1–2 introns. The paralogous pair of *OsHKT1;4-OsHKT1;5* have the same number of exons and introns, while *OsHKT1;1* and *OsHKT1;3* have a variable number of introns, with 1 and 2 introns, respectively. Two introns were identified for the *OsHKT2* genes. The variable numbers of introns in *OsHKT* members indicated the possibility of loss and gain of exons during evolution. This may explain the functional variations among members despite being grouped in a similar phylogenetic clade.

Chromosomal localization, gene duplication and detection of selection

In this study, *HKT* genes were mapped on rice chromosomes. Specifically, two genes (*OsHKT1;5* and *OsHKT2;3*) were found to be located on chromosome 1, followed by one gene (*OsHKT1;3*) on chromosome 2, two genes (*OsHKT1;1* and *OsHKT1;4*) on chromosome 4, and two genes (*OsHKT2;1* and *OsHKT2;4*) on chromosome 6, as demonstrated in Fig. 5. The study provides valuable information about the genomic distribution of the
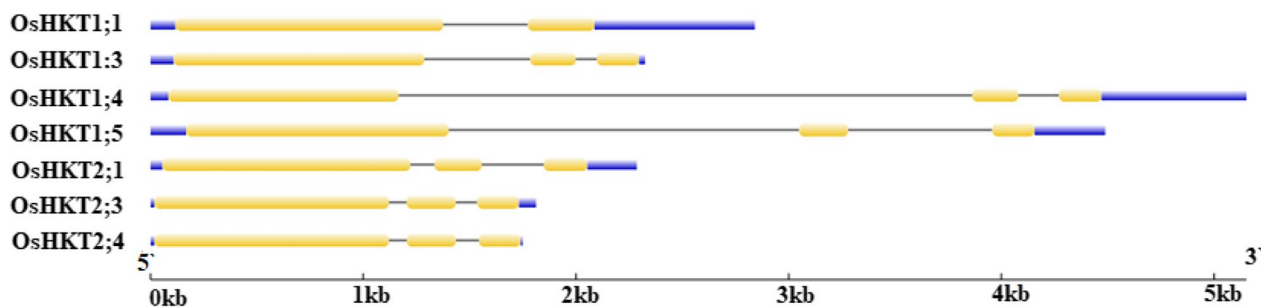
Legend:

▬ CDS ▬ upstream/downstream — Intron

Fig. 4 Gene structure representation *HKT* family genes in *Oryza sativa*. Yellow boxes symbolize exons, and black lines denote introns. Blue boxes indicated the untranslated regions (UTRs) and exons-introns sizes estimated using the scale at its bottom (Available genomic sequence was used to draw the gene structure)
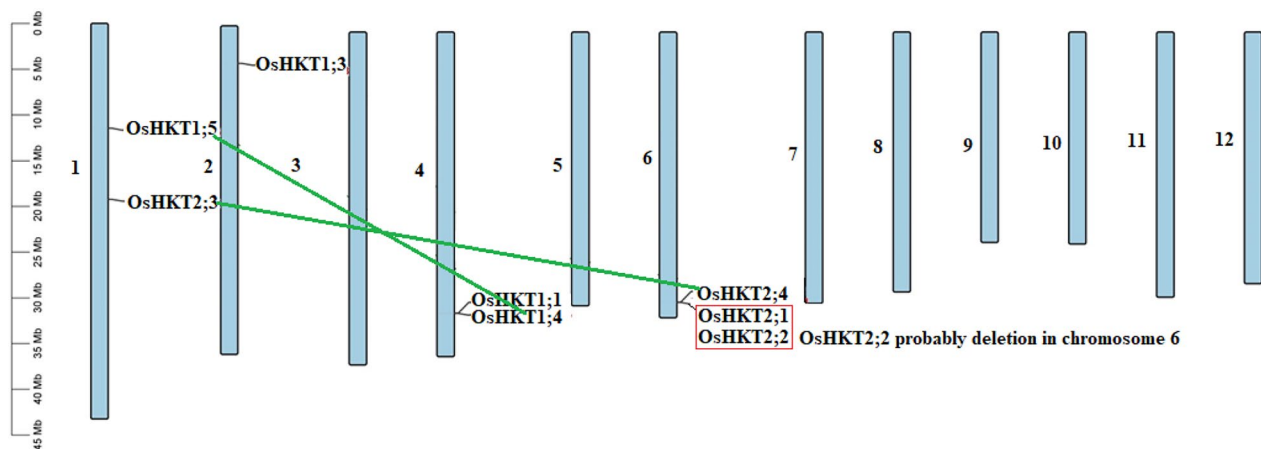
Fig. 5 Chromosomal map and duplication event coordinates of *HKT* genes that are paralogous in *Oryza sativa*. The lines indicate the two pairs of paralogous genes presented in duplicated blocks, representing segmental duplication

HKT genes. For example, two or more *OsHKT* genes on the same chromosome may occur due to tandem duplication events, while genes on different chromosomes suggest the possibility of segmental duplication (Nasim et al. 2016; Zhu et al. 2014). To further understand the evolutionary mechanism of *OsHKT* genes, we found that two gene pairs (*OsHKT1;4/OsHKT1;5* and *OsHKT2;3/OsHKT2;4*) were the results of segmental duplications, implying the possible expansion events of the *HKT* gene family in rice (Fig. 5). The selection pressure on rice *HKT* genes during their evolutionary process was evaluated to support this hypothesis. The non-synonymous (d_N or Ka) and synonymous (d_s or Ks) substitution rates, as well as the Ka/Ks ratio and the approximate date of duplication using the Ks values, were calculated (Table 2). The Ks value of two pairs of segmented duplicates (*OsHKT1;4/ OsHKT1;5* and *OsHKT2;3/OsHKT2;4*) ranges from 0.0688 Mya to 27.0803 Mya. Meanwhile, we discovered that the duplication times for segmental duplicates range from 5.2439 Mya to 2064.0473 Mya. The Ka/Ks values for segmental duplication were less than 1 (0.0168 to 0.5131), indicating *OsHKT* genes have been subjected to intense purifying selective pressure.

Comparative analysis of rice *HKT* family genes with Wheat, Maize, Arabidopsis, Tomato and Grape

To see how the *HKT* family genes in rice and other monocots and eudicots have changed over time, a maximum likelihood phylogenetic tree was made from full-length sequences of amino acids (Fig. 6). Based on our phylogenetic analysis, nine *OsHKTs*, seven *TaHKTs*, three *ZmHKTs*, one *AtHKTs*, two *SlHKTs,* and six *VvHKTs* were clustered into seven monophyletic groups I-VII (Fig. 6). Our evolutionary study also supports the 7-classification of the *HKT* gene family in rice and other organisms based on the conservation of their *TrKH* domain structure. The *OsHKT* genes were discovered to be clustered with other plant *HKT* genes, except for groups III and VII. Two members of the rice (*OsHKT1;1,* and *OsHKT1;2*) proteins belonged to group I. *OsHKT1;4* clustered with one wheat (*TaCS2A02G430600*) and one maize (*Zm00008a006337*), and *OsHKT1;3* clustered with two wheat (*TaCS6D02G144500* and *TaCS7B02G182600*) *HKT* proteins in group II and IV, respectively. Group V consists of one rice (*OsHKT1;5*) one maize (*Zm0008a011700*) and two wheat (*TaCS4B02G370800* and *TaCS7D02G361300*) *HKT* proteins. Two *OsHKTs* (*OsHKT2;3* and *OsHKT2;4*)

Table 2 Duplicated paralogous *HKT* gene pairs and their duplication time in *Oryza sativa*

| No	Paralogous pair	Ka	Ks	Ka/Ks	Duplicated type	Purify selection	Time (Mya[*])
1	*OsHKT1;4/OsHKT1;5*	0.4538	27.080	0.0168	Segmental	Yes	2064.05
2	*OsHKT2;3/ OsHKT2;4*	0.0353	0.068	0.5131	Segmental	Yes	5.2439
3	*OsHKT2;1/ Po_OsHKT2;2*	0.0515	0.116	0.4417	Tandem	Yes	8.8872
Orthologous pair							
1	*OsHKT1;5/Zm00008a011700 (68.13% sequence similarity and have two introns)*	0.20	1.83	0.11	–	Yes	139.36

[*] Mya: Million years ago
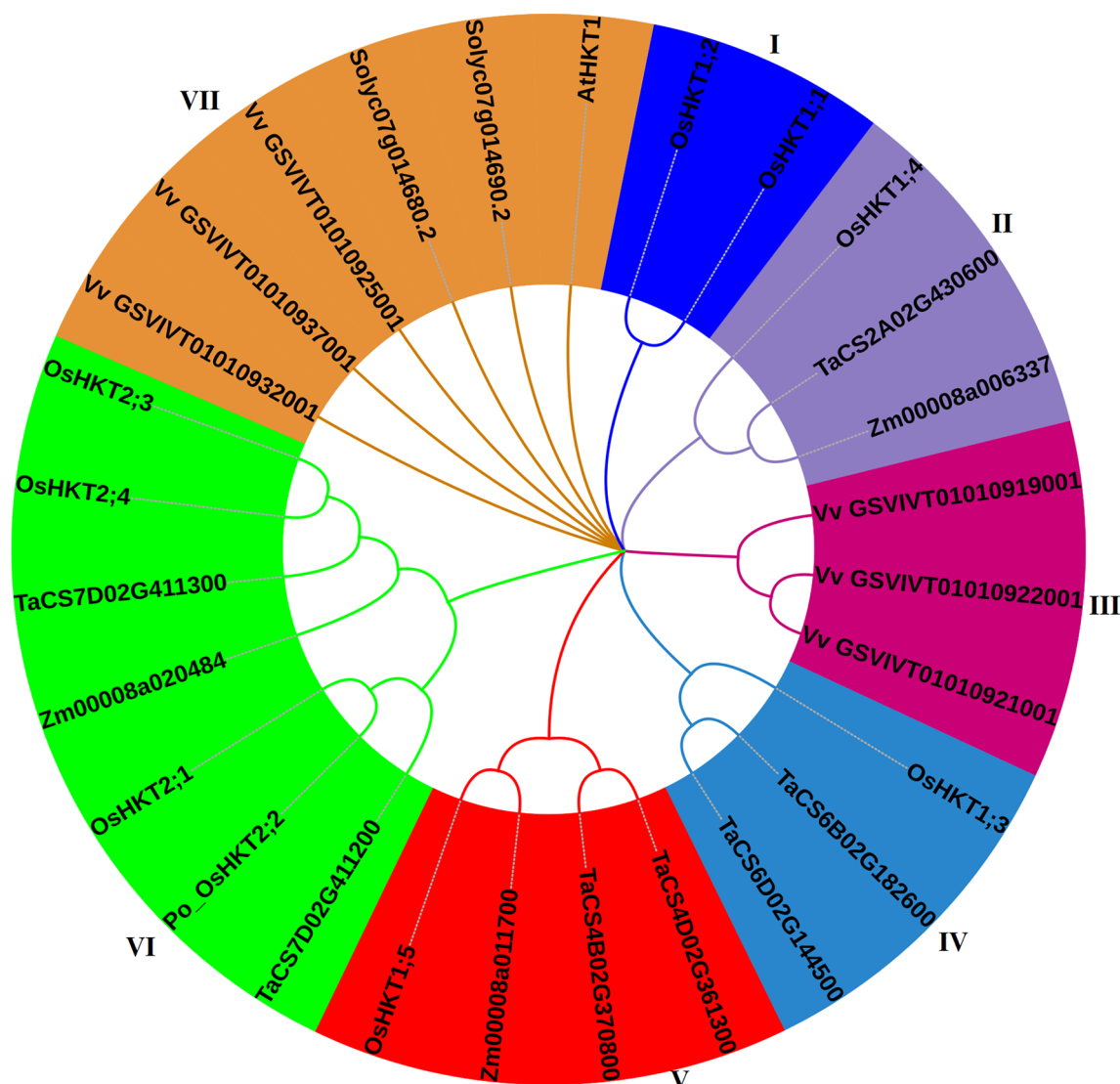
Fig. 6 Phylogenetic trees of full-length *HKT* proteins in rice, wheat, maize, Arabidopsis, tomato, and grape. Group V indicates an orthologous pair of rice *HKT* genes

were found to be clustered in a similar monophyletic group VI, together with *TaHKT* (*TaCS7D02G411300*) and *ZmHKT* (*Zm0008a020484*). On the other hand, group VI also comprised two rice (*OsHKT2;1* and *Po_OsHKT2;2*) and one wheat *HKT* (*TaCS7D02G411200)* protein. Group VI has the most significant number of *HKT* genes. From phylogenetic analysis, *OsHKT* genes were highly conserved among monocots and dicots, especially with *TaHKT* and *ZmHKT* proteins. Several paralogous genes were also clustered in the same monophyletic group, such as *OsHKT1;1/OsHKT1;2* in group I, *OsHKT2;1/Po_OsHKT2;2* and *OsHKT2;3/OsHKT2;4* in group VI, two sets of wheat *TaHKT* in group V (*TaCS4B02G370800/TaCS7D02G361300*),

IV (*TaCS6D02G144500/TaCS7B02G182600*) and one set of grape *VvHKT* (*VvGSVIVT01010921001/ VvGS-VIVT01010922001*) in group III, indicating species-specific duplication events of *HKT* genes. The orthologous gene pair *OsHKT1;5/Zm00008a011700* was also identified between rice and maize in group V with 68.13% sequence similarity (Table 2). Further, the divergence of the orthologs between rice and maize *HKT* genes was investigated by calculating the Ka/Ks ratio. The result indicated that the Ka/Ks ratio of the orthologous gene pair was less than 1 (0.11), revealing purifying selection. The orthologous gene *OsHKT1;5/Zm00008a011700* exhibits a conserved gene organization, as it shares the same number of introns. Additionally, the orthologous

gene also showed a Ks value of less than 2.0, indicating a higher association with segmental duplication (Table 2). Overall, this study revealed that the early rice *HKT* gene duplication event was observed in maize as compared to other plant species.

Analysis of putative TFbs of rice HKT family

The *cis*-acting regulatory elements (*CREs*) play a significant role in regulating the expression of genes in response to stress, light, and growth. To understand the interaction between transcription factors and binding sites of *OsHKT* genes, we predicted 1.5 kb upstream regions using plant promoter databases, PlantPAN 2.0 and Plant-CARE. Further, PlantRegMap was used to retrieve transcription factor information that regulates the *OsHKT* genes. In our study, we discovered nine important binding sites, including WRKY, bHLH, bZIP, MYB, AP2/ERF, GATA, B3, Dof, and C2H2 that were highly distributed

in all the promoter regions of *OsHKT* genes (Fig. 7). The AP2/ERFbs responsive elements were highly abundant in the *OsHKT* gene promoters, followed by B3, GATA, and bZIP. The highest number of binding sites was found in *OsHKT1;5*, while the lowest was found in *OsHKT2;4* (Fig. 8A). To better understand the regulatory mechanisms of *OsHKT* genes, the CREs were predicted using the PlantCare databases. A large number of CREs were found in the promoter region of *OsHKT* genes that are known as light-responsive elements such as GT1-motif, as-1, G-box, and TCCC-motif; hormone-responsive elements (CGTCA/TGACG-motif, ERE, ABRE, TCA and GARE); environmental stress-responsive elements (ARE, LTR, MBS, TC-rich repeats, W-box, DRE, STRE, MYB, and MYC) and plant growth and development-related elements (HD-Zip, AT-rich, CAT box, O2 site, and AAGAA-motif). Detailed information on the *CREs* is presented in Figs. 8B and 9.
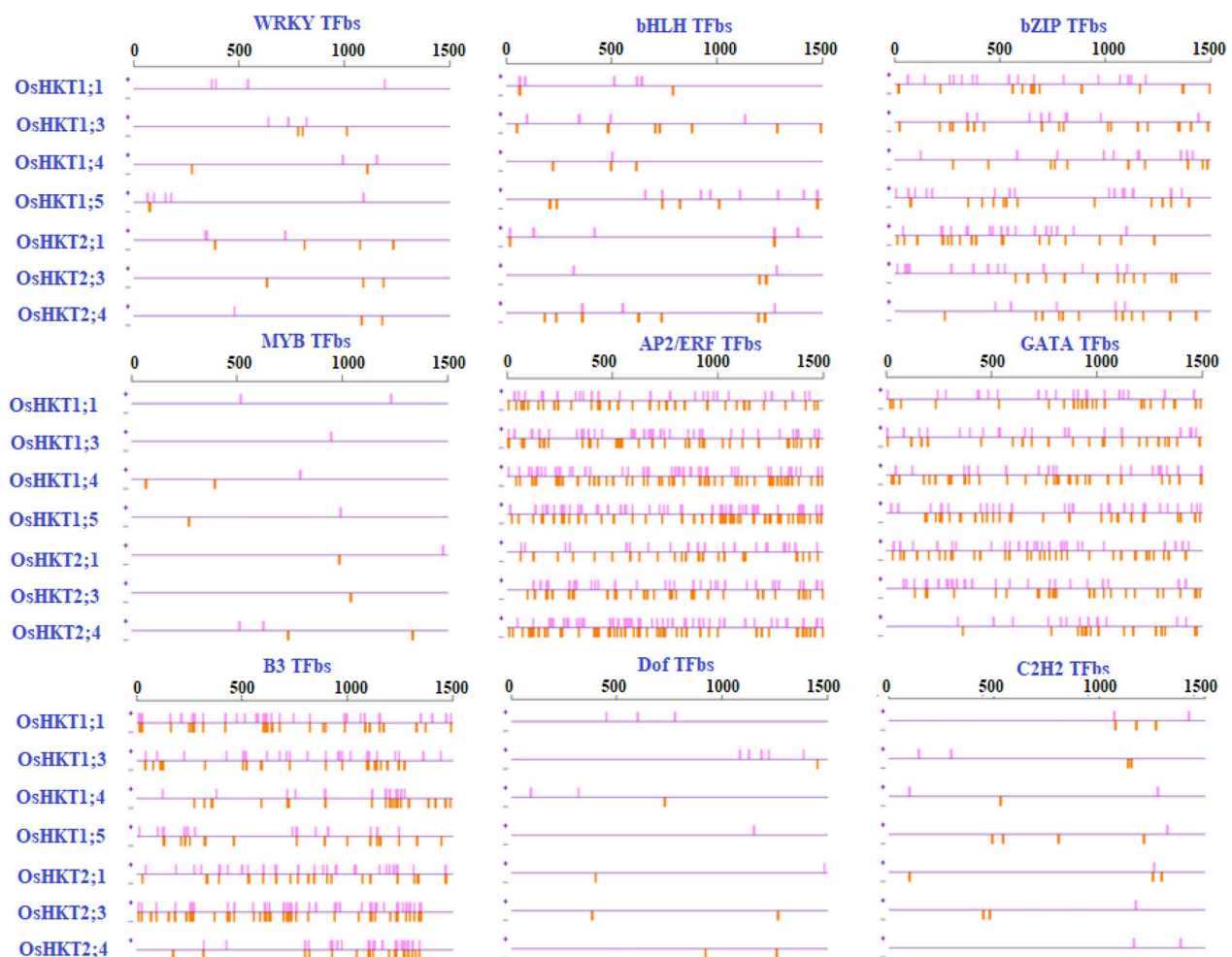

Fig. 7 Distribution of nine TFbs in rice *HKT* gene promoter regions. The pink and brown bars represent putative WRKY, bHLH, bZIP, MYB, AP2/ERF, GATA, B3, Dof, and C2H2 binding sites on the positive and negative strands of DNA, respectively
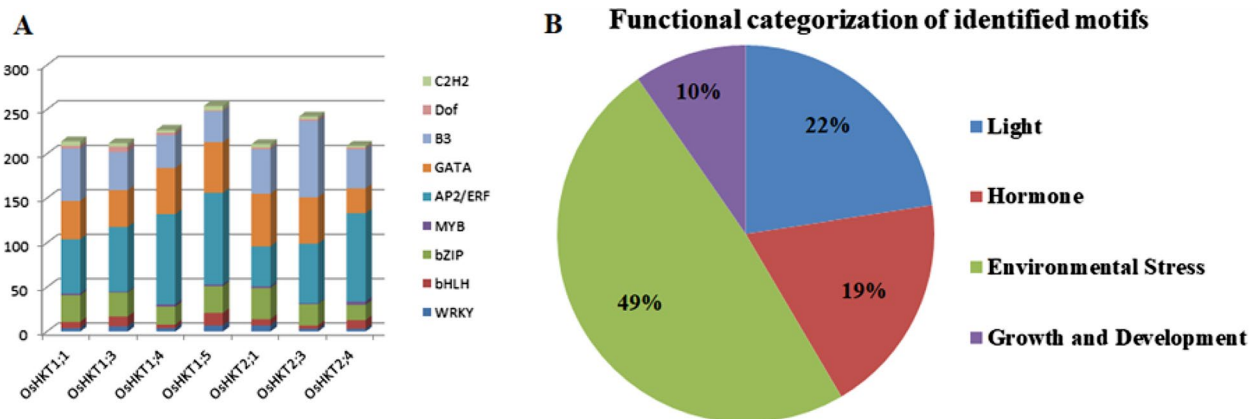
Fig. 8 **A** Distribution of TFbs and **B** Functional categorization of identified motifs in *OsHKT* gene promoter regions
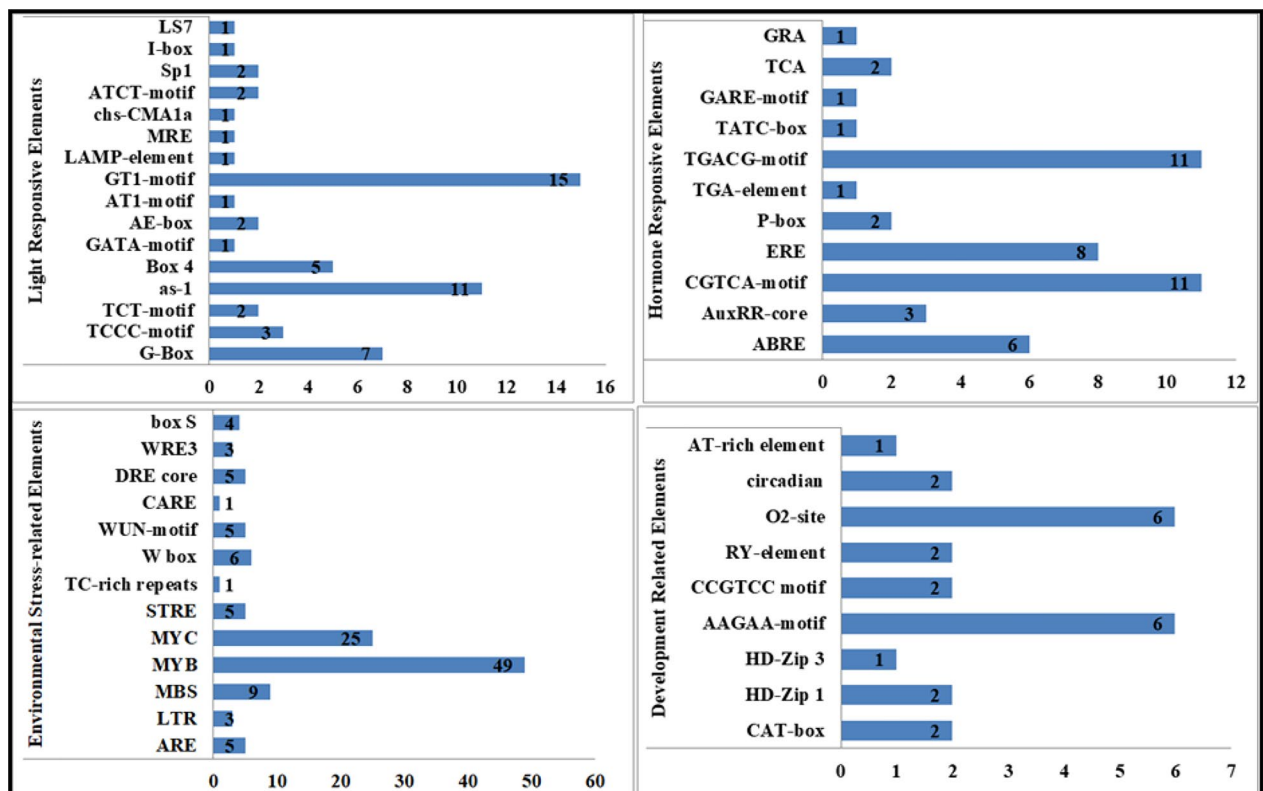

Fig. 9 Frequency of annotated motifs and their roles in response to light, hormones, stress, and developments in 1.5 kbp upstream regions of *OsHKT* genes

Furthermore, 24 transcription factors (TFs) were discovered to regulate the *OsHKT* genes (Fig. 10). One C2H2 zinc finger protein type TF regulates *OsHKT1;1*; four Myb and SBP type TFs regulate *OsHKT1;3*; six B3, Dof, and trihelix family type TFs regulate *OsHKT1;4*; two B3 domain containing RAV and trihelix family type TFs regulate *OsHKT1;5*, ten Dof, C2H2, HD-ZIP and Myb family type TFs regulate *OsHKT2;1*; and six ARF, ERF, B3, and Dof family type TFs regulate both *OsHKT2;3* and *OsHKT2;4* genes in rice.

Expression pattern of HKT genes in rice
To better understand *OsHKT* genes' response across the whole rice life cycle, we analyzed the expression patterns
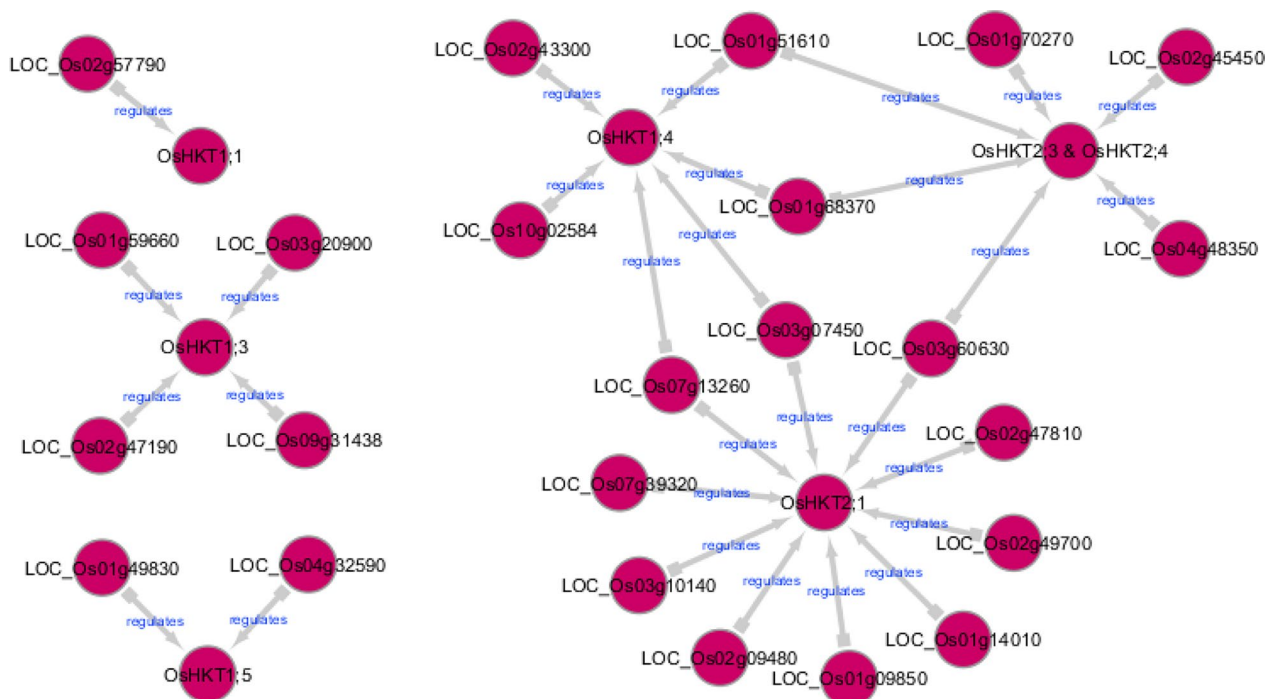
Fig. 10 Transcription Factors (TFs) regulate the *OsHKT* genes in rice

at 22 tissue-specific and developmental stages of the indica cultivar Minghui 63 using Affymetrix rice microarray data (Supplementary Table 3). The expression levels of *OsHKT* genes could be divided into two groups (Fig. 11A). Group I consists of two genes (*OsHKT1;1* and *OsHKT1;3*) that have shown higher transcript accumulations, whereas *OsHKT1;1* has the highest expression level in the entire rice life cycle and *OsHKT1;3* has a high expression level in seedlings, shoots, leaves, sheaths and stamen. On the other hand, five genes belonged to group II, namely *OsHKT1;5, OsHKT2;1, OsHKT2;4, OsHKT1;4* and *OsHKT2;3. OsHKT1;5* has shown high expression levels in both vegetative and reproductive stages, such as seedlings, roots, stems, panicles, and spikelets. Other genes from group II exhibited low expression signals.

For the salinity treatment, the microarray data were analyzed to examine the responsiveness of *OsHKT* genes to salt stress. Two well-characterized salt-tolerant FL478 and salt-sensitive IR29 genotypes were used, with untreated seedlings serving as a control during the vegetative stage. Two major categories can be distinguished between the levels of *OsHKT* gene expression (Fig. 11B). Supplementary Table 4 displays the relative fold-change in *OsHKT* gene expression in response to salt treatment. Three *OsHKT* genes from group I (*OsHKT1;5, OsHKT1;1,* and *OsHKT1;3*) exhibited increased expression in the salt-tolerant FL478 and salt-sensitive IR29 genotypes under salinity stress. In group II, *OsHKT2;1* displayed

higher expression in both FL478 and IR29 genotypes, while the remaining genes (*OsHKT1;4, OsHKT2;3,* and *OsHKT2;4*) showed moderate expression in FL478 and lower expression in IR29 genotypes.

Real-time PCR was used to obtain further verification of the *OsHKT* gene expression pattern under salt stress (Fig. 12). Seven *HKT* genes were found to be strongly expressed in the salt-tolerant cv. Pokkali's roots and shoots, except *OsHKT2;4*. However, shoot *OsHKT1;1, OsHKT1;3, OsHKT2;1,* and *OsHKT2;4* greater expression was found in pokkali. Meanwhile, the most substantial upregulation of *OsHKT1;4, OsHKT1;5,* and *OsHKT2;3* was found in pokkali root after 24 h of 100 mM, 150 mM, and 200 mM salt treatment, respectively. On the other hand, salt-sensitive IR64 plant exhibited a significant decrease in the expression of all *OsHKT* family genes in both the root and shoot regions, except for *OsHKT2;4*. Interestingly, *OsHKT2;4* displayed an increase in expression specifically in the roots region after 24 h. Remarkably, our findings strongly suggested *OsHKT1;5, OsHKT1;1, OsHKT1;3, OsHKT2;1,* and *OsHKT2;3* as critical genes responsible for rice salinity tolerance.

Identification of *DEGs* and protein network of *OsHKT*
The GEO2R tool was used to find differentially expressed genes (*DEGs*) in salt stress from two gene expression datasets, GSE14403 and GSE41650. The *DEGs* with |log2FC|>1 and |log2FC|<-1 and p<0.05 were
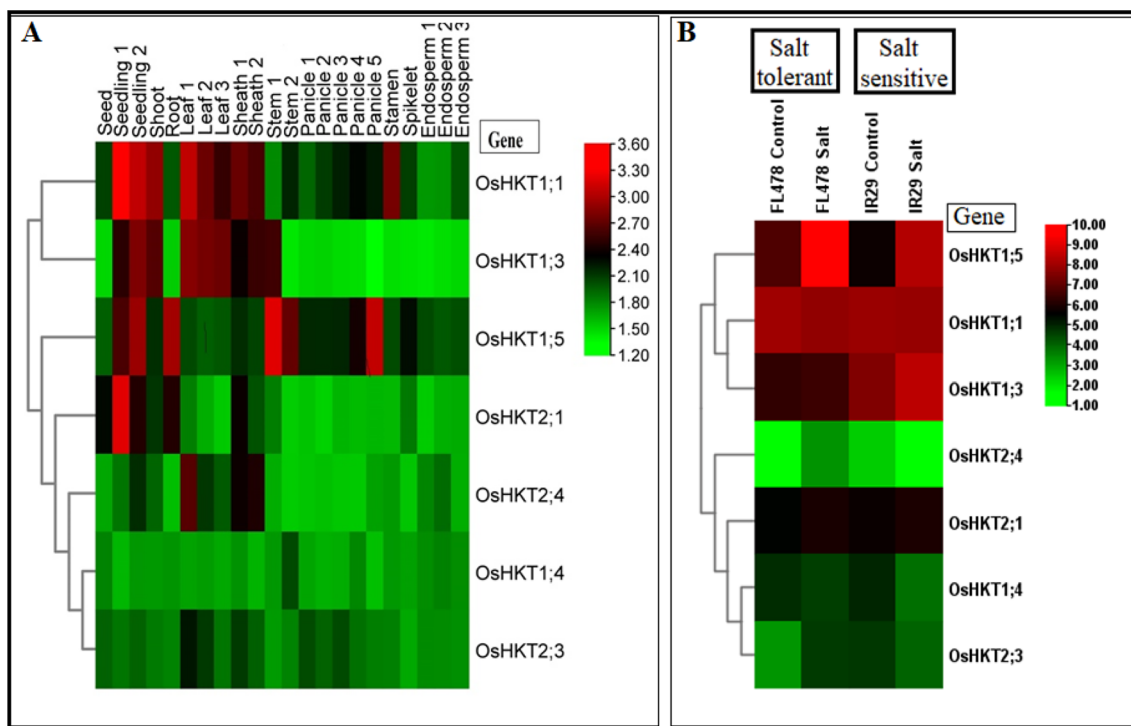
Fig. 11 Hierarchical clusters show expression patterns of *OsHKT* genes during the entire life cycle of rice (**A**) and under salinity (**B**). The color bar at the right represents the $\log_2$ expression values: red, black, and green indicate high, medium, and low expression, respectively

considered statistically significant, as demonstrated in Fig. 13A, B. From the *DEG*-based PPI network, a total of nine interactions from the GSE14403 and eight interactions from the GSE41650 datasets were discovered as demonstrated, in Fig. 13C, D, respectively. Among them, we found significant upregulation of *OsHKT1* (also known as *OsHKT2;1*) to interact with pyrroline-5-carboxylate synthetase 2 (*P5CS2*), rice potassium transporter 1 (*OsHAK1*) and rice Na^+/H^+ antiporters (*OsNHX2*). On the other hand, a downregulated *OsHKT4,* also called *OsHKT1;1,* significantly interacted with other downregulated *DEGs*, such as rice pyrroline-5-carboxylate synthetase 2 (*OsP5CS2),* pyrroline-5-carboxylate synthetase 1 (*OsP5CS1),* potassium transporter 27 (*OsHAK27),* and peroxidase 90 (*prx90*). This finding suggests that *OsHKT1* and *OsHKT4* play a significant role in regulating the concurrent expression of several genes under salt stress.

Functional GO annotation

The GO annotation analysis was conducted to describe the participation of *OsHKT* genes in the biological process and other functional relevance (Fig. 14). The GO annotation analysis demonstrated that the *OsHKT* genes were involved in transmembrane transporter activity at their molecular levels, and most of the *OsHKT* genes

were found to be in the plasma membrane and nucleus, indicating their importance in cellular functioning activities. The *OsHKT* genes also play a crucial role in various biological functions, including response to stress, ion transport, and homeostatic processes.

miRNA target site prediction of OsHKT family in rice

MicroRNAs (miRNAs) regulate the expression of specific genes by cleaving mRNA or preventing its translation into proteins. Following conserved domain sequence (CDS) identification, miRNA binding sites were discovered within the *OsHKT* genes. We discovered that the *OsHKT* gene family is targeted by 101 mature miRNAs (Fig. 15, Supplementary Table 5). Some miRNAs have several target sites inside a single gene and across many genes. Osa-miR11339, Osa-miR11343, and Osa-miR2275 have 15, 4, and 3 target sites in *OsHKT1;1*, respectively. Osa-miR5819, Osa-miR444, and Osa-miR5148 have 3 target sites in *OsHKT1;4*, *OsHKT2;3*, and *OsHKT2;4,* respectively. One Osa-miRN2268 has two target sites in *OsHKT1;5*. On the other hand, Osa-miR1846 has 4–5 target sites in different rice *HKT* genes, such as *OsHKT1;4, OsHKT2;1, OsHKT2;2, OsHKT2;3,* and *OsHKT2;4.* Osa-miRN45 has two target sites in different genes, such as *OsHKT1;3, OsHKT2;3, and OsHKT2;4.* Two miRNAs, Osa-miR5150 and Osa-miRN2366, share similar target sites in multiple genes.
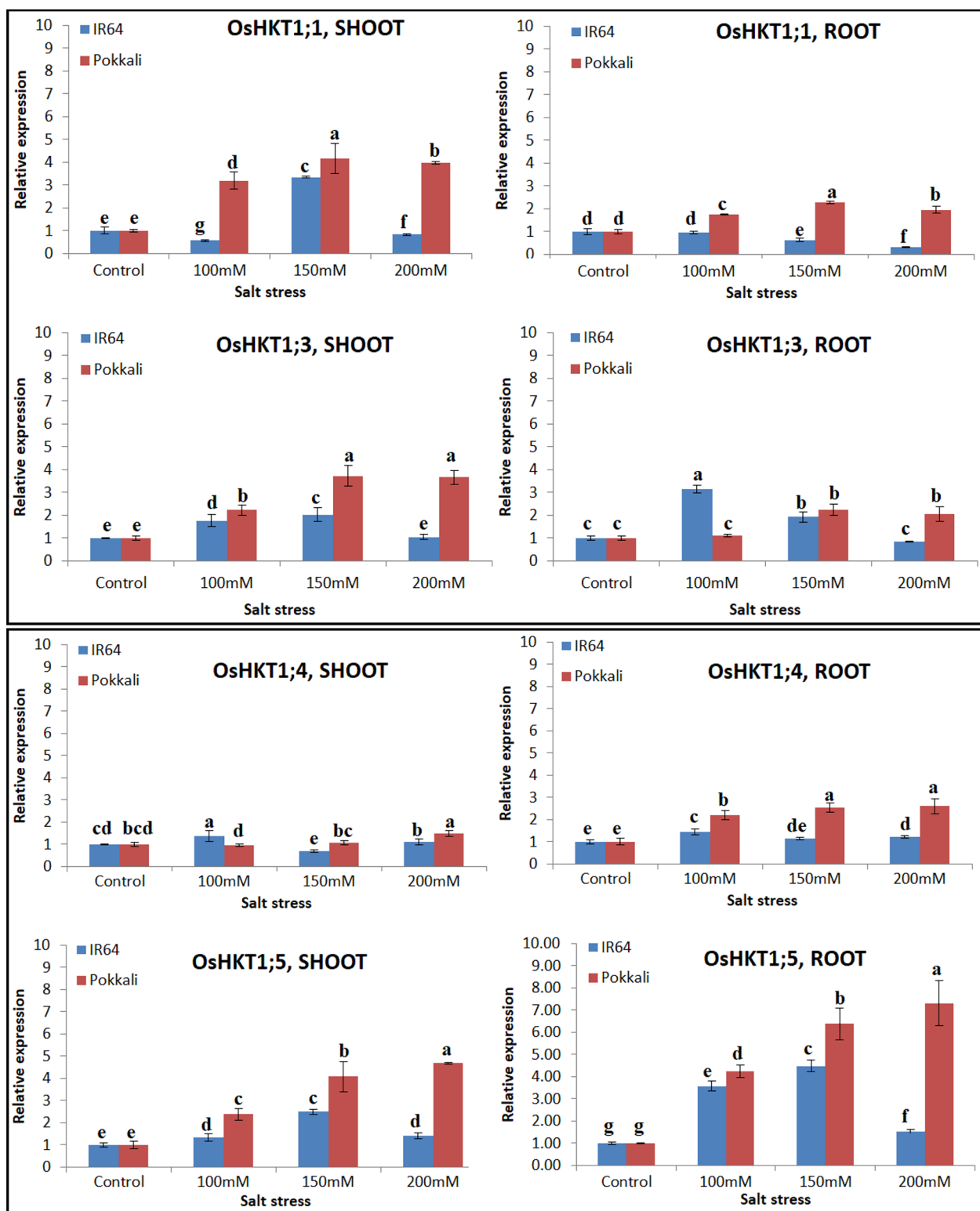
Fig. 12 qRT-PCR analysis of rice *HKT* genes from shoot and root in salt-tolerant pokkali and salt-sensitive IR64 after 24 h. Statistical significance was determined using ANOVA at the p < 0.05 level. Letters at the top of the bar indicates significant differences. The data points represent the mean-standard deviation of three replicates
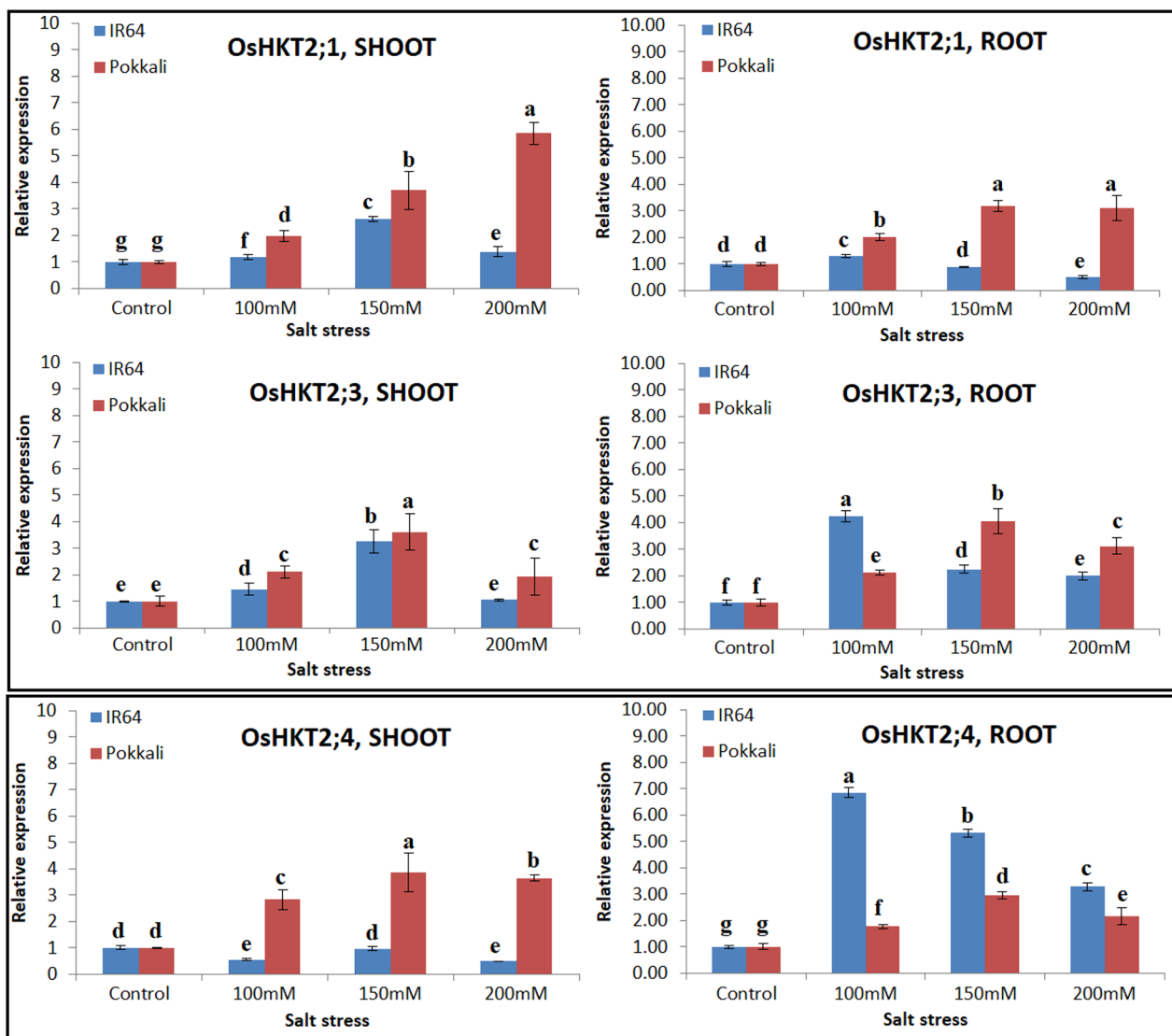
Fig. 12 continued

These genes include *OsHKT1;1, OsHKT1;3, OsHKT2;1,* and *OsHKT2;2*. There are three miRNAs with target sites in both the *OsHKT2;1* and *OsHKT2;2* genes: Osa-miRN2260, Osa-miR1861, and Osa-miRN2309. These findings reveal the interactions between Osa-miR11339, Osa-miR11343, Osa-miR2275, Osa-miR5819, Osa-miR444, and Osa-miR5148 with other miRNA families, demonstrating the interplay between miRNAs. These interactions might impact the expression levels of *OsHKT* due to miRNA manipulation.

Discussions

Salinity and drought are two of the most common abiotic stresses that plants frequently encounter and cause a negative impact on their growth, development, and production due to ion toxicity and physiological drought (Munns and Tester 2008; Tang et al. 2016). High-affinity potassium transporter (*HKT*) family proteins are anticipated to play an essential role in plant salt stress tolerance. *HKTs* were first identified as high-affinity potassium (K^+) transporters and were proven to transport sodium (Na^+)
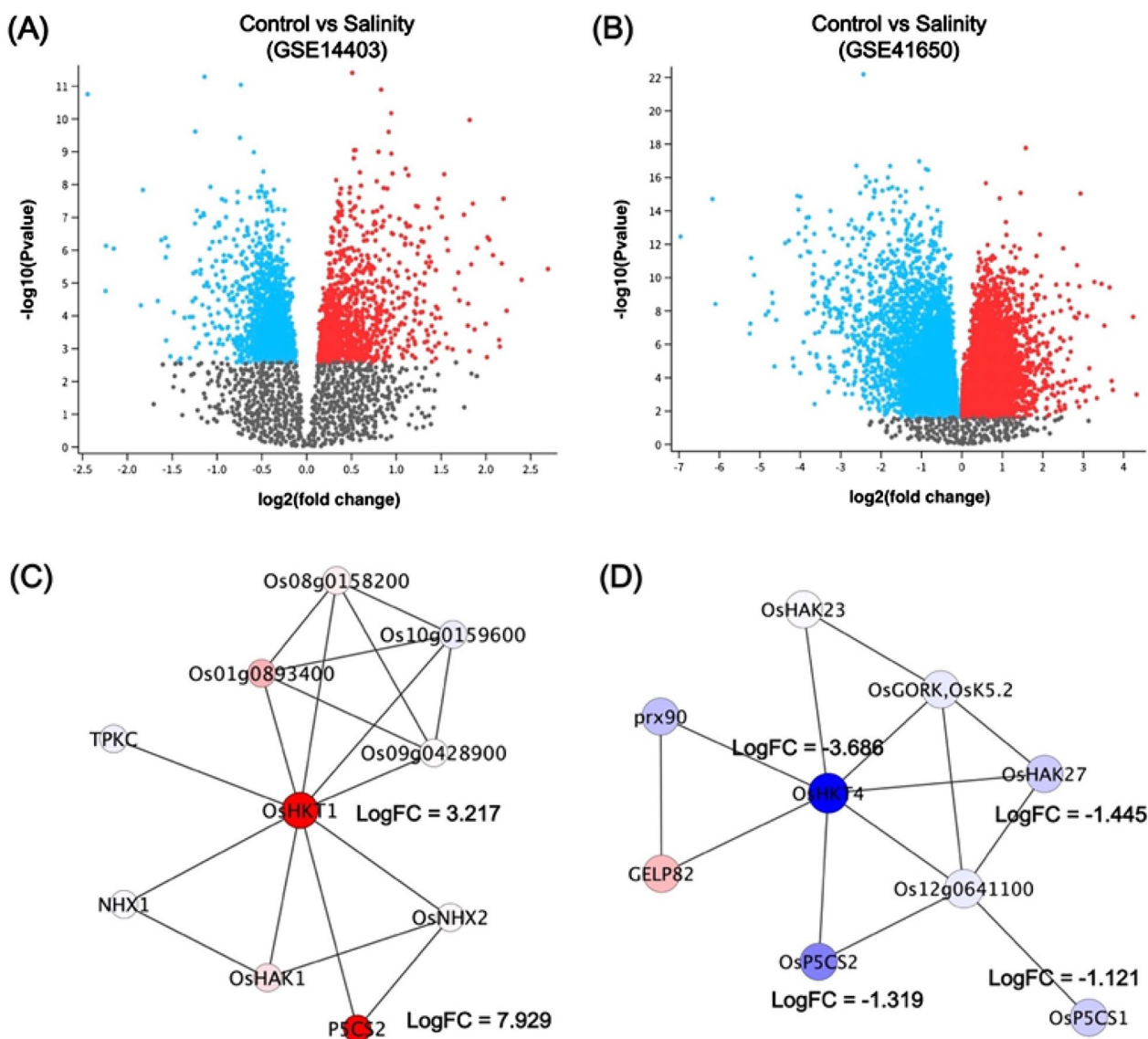
Fig. 13 Visualization of *DEGs* volcano plots using GEO2R and network establishment of the HKT protein network. **A** and **B** compared the DEGs between control and salinity from the dataset. The genes upregulated in the array are on the right panel, and downregulated ones are on the left panel of the plot. **C** and **D** PPI networks show the interaction of *DEGs* from the GSE14403 and GSE41650 datasets. The nodes and edges are retrieved from the STRING and visualized Cytoscape software. Red nodes represent up-regulated *DEGs*, and blue nodes represent down-regulated *DEGs*. *OsHKT1* and *OsHKT4* revised name are *OsHKT2;1* and *OsHKT1;1* respectively
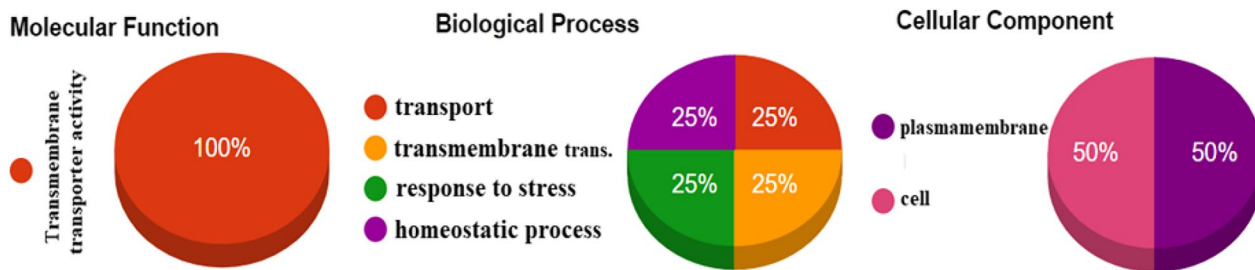

Fig. 14 Classification of *HKT* proteins in rice based on their molecular function, biological process, and cellular component
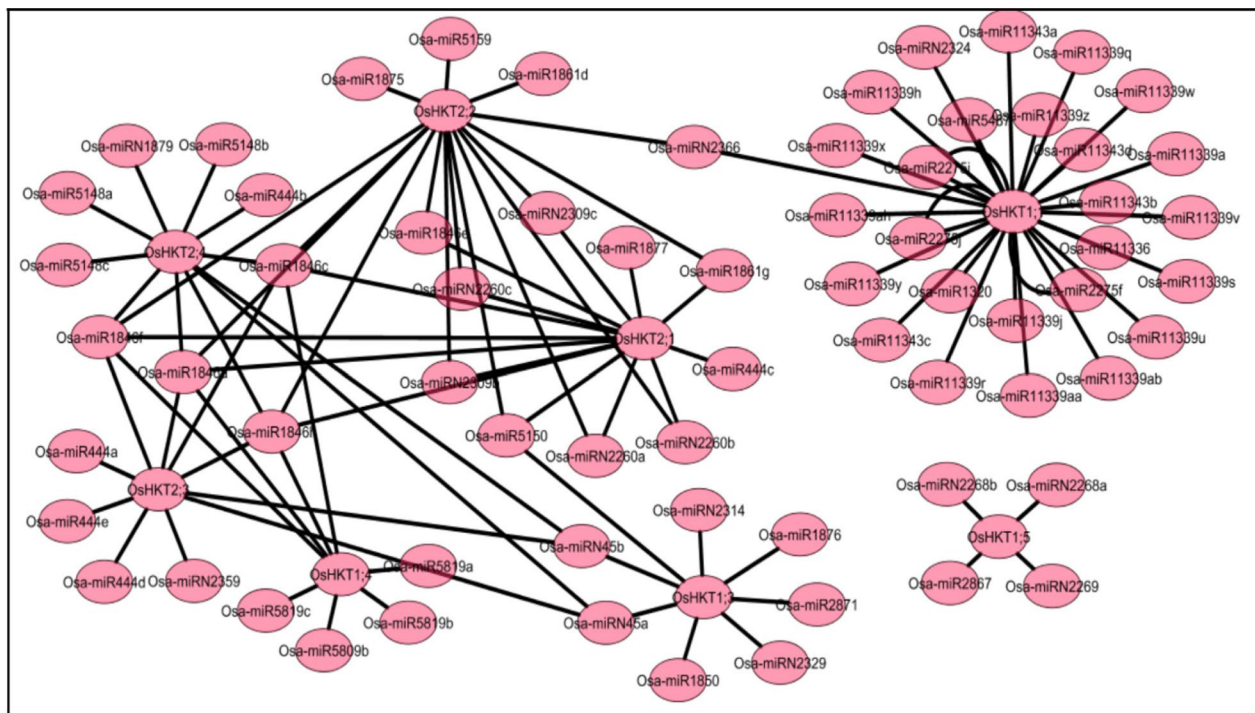
Fig. 15 The regulatory network between putative miRNAs and *OsHKT* genes

via channels with other cations (Horie et al. 2009). *HKTs* are also responsible for Na^+ and K^+ transport as well as Na^+-K^+ homeostasis in plants during plant development, making them potential goals for the development of salt tolerance in crops. Most of the research on the functional study of *HKT* family genes has focused on yeast and model plants such as Arabidopsis, as well as crops like wheat, maize, sorghum, barley, and eucalyptus (Liu et al. 2001; Rus et al. 2001; Haro et al. 2005; Munns et al. 2012; Ren et al. 2015; Li et al. 2019). However, a more thorough investigation needs to be into *HKT* family genes in rice.

In this study, we identified nine *HKT* genes, whereas eight are functional depending on the japonica and indica cultivars from the rice genome database. Based on the conserved domain search, we confirmed that *TrkH* is conserved in all the *HKT* genes (Fig. 2). The TrkH domain is a hydrophobic membrane protein vital in controlling Na^+ and K^+ movement in higher plants, contributing to enhanced salinity tolerance (Horie et al. 2009; Su et al. 2015). All *OsHKT* proteins have similar physiological properties, comprising equal transmembrane helices, and are mainly localized in the plasma membrane (Table 1). These findings indicate that *HKT* proteins have a close evolutionary connection with plants during biological evolution. The paralogous pairs of *OsHKT* genes found in subfamily 1 and 2 of the monophyletic tree also indicate that each subfamily's *HKT* genes undergo

the same evolutionary process and serve the same functions, and potentially retain plant resistance to salt stress (Maser et al. 2002; Li et al. 2019). Furthermore, ten distinct motifs were discovered, and most of the motifs appeared in rice *HKT* proteins (Fig. 3B), suggesting that they are relatively conserved and have a strong evolutionary relationship (Singh et al. 2002). *HKT* genes have three exons and two introns (Fig. 4), demonstrating even more clearly that *HKT* genes in plants have been evolutionary conserved because the exon–intron arrangement has been utilized as supporting proof for developmental relationships between genes (Koralewski and Krutovsky 2011). In addition, gene duplication events are one of the critical factors that could provide a profound explanation for gene family expansion in plants (Moore and Purugganan 2005). The chromosomal location offers valuable information about tandem and segmental duplications of a specific family gene. Two or more genes located on the same chromosome reveal the possibility of tandem duplication, while genes situated on different chromosomes indicate segmental duplication events (Zhu et al. 2014; Nasim et al. 2016). Notably, two pairs of paralogous *OsHKT* genes (*OsHKT1;4/OsHKT1;5* and *OsHKT2;3/OsHKT2;4*) are segmentally duplicated, including *OsHKT2;1/Po_OsHKT2;2* was discovered to be tandemly duplicated due to gene distributions on the same chromosome (Fig. 5). To understand functional sites and

functional protein alterations, selective pressure investigations are generally required as they reveal selective benefits for changing amino acid sequences in the protein (Morgan et al. 2010). The Ka/Ks value < 1 indicates the purifying selection, while the Ka/Ks ratio > 1 proposes the probability of a positive selection (Yang and Bielawski 2000; Bowers et al. 2003). Based on the Ka/Ks values for both segmental and tandem, they indicated that rice *HKT* genes have undergone intense purifying selection pressure (Table 2). The phylogenetic tree (Fig. 6) indicated that rice *HKT* proteins were related to monocot and dicot; however, they were more closely related to wheat and maize *HKT* proteins. The highly conserved cluster of *HKT* genes provided evidence that they perform similar functions rather than being the result of a series of evolutionary events (Zhang et al. 2013). One pair of orthologous *OsHKT* genes with maize (*OsHKT1;5/Zm00008a011700*) revealed the purifying selection and showed the same intron numbers, which means conserved gene organization (Table 2). These findings suggest that the orthologous pair arose from the common inherited genes that existed before the divergence of the monocots and dicots. It is also mentioned that purifying selection played a vital role in the evolution of the *HKT* genes in other crop species (Zhang et al. 2019).

Transcription factors are pivotal in regulating the plant's response to abiotic stress by modulating the gene expression (Lindemose et al. 2013). Regulatory elements are crucial for detecting gene expression patterns, as regulatory elements control the expression of many genes through distinct binding sites (Mariño-Ramírez et al. 2009). WRKY, bHLH, bZIP, MYB, AP2/ERF, GATA, B3, Dof, and C2H2 type important binding sites that were highly distributed in all the promoter regions of *OsHKT* genes. TFs like WRKY, NAC, bHLH, bZIP, MYB, and AP2/ERF play vital roles in the responses to abiotic and biotic stress in many plant species (Lindemose et al. 2013; Das et al. 2019). Moreover, B3, a plant-specific transcription factor, has a variety of roles in the growth and development of plants (Peng and Weselake 2013), GATA is involved in light responsiveness (Behringer and Schwechheimer 2015), C2H2 type transcription factor plays a diverse role in plant growth and development as well response to stress (Yin et al. 2020) and Dof transcription factor also participates in many plant development stages and the response to different environmental stressors (Khan et al. 2021). The *OsHKT* genes possess a high abundance of stress-responsive cis-regulatory elements (CREs) such as MYB, MYC, MBS, W-box, ARE, STRE and DRE core. The expression of MBS, which is a binding site for MYB TF, changes in many plants when exposed to salt, suggesting that these plants are responding to salt stress (Hua et al. 2006). Moreover, members of

this TF also influence the abscisic acid (ABA), polyethylene glycol (PEG), and SA-signalling pathways, which confer resistance to abiotic stresses (Ambawat et al. 2013). TGACG, CGTCA-motif, ERE, and ABRE were also abundant in the rice *HKT* promoter region. The next most common type of cis-acting elements were those that regulated growth and development, hormones, and light. The GT1-motif, as1, G-box, TCCC, and Box-4 are more frequently present in rice *HKT* promoters that were responsive to light. On the other hand, TGACG and CGTCA-motif were responsive to methyl jasmonate, ERE was responsive to ethylene, ABRE was responsive to abscisic acid (ABA), TCA was responsive to salicylic acid, TGA was responsive to auxins and P-box and GARE-motif were responsive to gibberellin. Plant hormones and other signalling pathways are important for adequate and integrated stress responses (Ryu and Cho 2015). ABA, methyl jasmonate, and ethylene have been suggested as factors governing adaptive responses to abiotic stimuli, while auxin, salicylic acid, and gibberellin are essential in growth and development. The presence of TGACG and CGTCA motifs suggests that methyl jasmonate may be involved in the regulation of the *OsHKT* gene. In line with this, a significant concentration of methyl jasmonate has been found in salt-tolerant rice cultivars (Kang et al. 2005). ERE-containing genes are regulated in the context of ethylene. It has been proposed that ethylene controls salt-responsive gene expression under salt stress (Verma et al. 2016). Most abiotic stress-responsive gene promoter regions comprise two cis-regulating elements, namely ABA-responsive elements (ABRE) and dehydration-responsive elements (DRE), both of which contain the core sequences ACGTGG/TC and TACCGA CAT or A/GCCGAC, respectively (Kobayashi et al. 2004; Yamaguchi-Shinozaki and Shinozaki 2006) and the ABRE motif participates in the ABA-dependent gene expression under high-stress situations (Finkelstein 2013). According to previous research, there are interactions between ABA and methyl jasmonate at the MYC2 transcription factor, which is implicated in the control of gene expression under salt stress (Moons et al. 1997). Thus, cis-elements pertaining to ABA, ethylene, and methyl jasmonate suggest that these hormones have roles in *OsHKTs* in response to abiotic stresses. Various elements, such as W-box, WUN-motif, LTR, and TC-rich repeat also present in *OsHKT* promoter regions, are implicated in the salt stress response and defence (Gao et al. 2010; Li et al. 2013; Manimaran et al. 2017). According to promoter analysis, *OsHKTs* may respond to environmental stress and stimuli to regulate plant growth and development.

On the other hand, we have discovered that a wide range of transcription factors control *OsHKT* gene

expression. These include C2H2 zinc finger protein, Myb transcription factor, Dof zinc finger domain-containing protein, DNA binding domain-containing protein, B3 domain-containing RAV and trihelix family-type protein, ARF, and dehydration-responsive transcription factors. Recent findings have reported that the expression of *OsHKT1;1* has been positively regulated by *OsMYBc*. It binds to specific conserved DNA regions in the *OsHKT1;1* promoter, modulating Na^+ concentration and preventing sodium toxicity in leaf blades (Wang et al. 2015). Knocking out *OsMYBc* also led to a decrease in the salt-induced expression of *OsHKT1;1*, and modifications in specific promoter regions resulted in reduced *OsHKT1;1* promoter activity. To increase the expression of *OsHKT1;5*, the stable complex of *OsSUVH7*, *OsMYB106,* and *OsBAG4* binds to the promoter of *OsHKT1;5* (Wang et al. 2020). It has been shown that the bHLH transcription factor *OsbHLH035* controls the expression of the genes *OsHKT1;3* and *OsHKT1;5*. These findings indicate that *OsbHLH035* positively influences the expression of *OsHKT1;3* and *OsHKT1;5* (Chen et al. 2018). Chen and his team identified a popular TF, PalERF109, as a positive regulator of the *PalHKT1* gene expression (Chen et al. 2020b). In Arabidopsis, several TFs, such as *AtbZIP24*, *ARR1*, *ARR12*, and *ABI4*, have been found to regulate *AtHKT1;1* expression (Yang et al. 2009; Mason et al. 2010; Shkolnik-Inbar et al. 2013). The observation indicated that *AtbZIP24, ARR1,* and *ARR12* are negative regulators of *AtHKT1;1* gene expression. Additionally, ABI4 TF also negatively regulates *AtHKT1;1* gene expression, and the involvement of the abscisic acid signal transduction pathway in salt responses in Arabidopsis suggests that *OsHKT1;5* may play a similar function in rice. The GT factors are a family of transcription factors found exclusively in plants and share a common DNA-binding trihelix domain. GT elements have A/T-rich core sequences and are highly degenerate cis-elements. *OsGTγ-1, OsGTγ-2,* and *OsGTγ-3* genes were upregulated in response to high salinity and other abiotic stimuli, suggesting a role for this subfamily transcriptional regulation of stress responses. At the vegetative stage, transgenic rice with an overexpression of *OsGTγ-1* demonstrated an improvement in their salt tolerance (Fang et al. 2010). On the other hand, DREB TFs, which mainly bind with C-repeat/DRE (A/GCCGAC), influence the expression of several cold or drought-inducible genes in an ABA-independent route, enhancing plant abiotic stress tolerance (Chen et al. 2008). Thus, these TFs have roles in *OsHKTs* in response to abiotic stresses.

In our study, *OsHKT1;1* and *OsHKT1;5* had higher expression levels in both vegetative and reproductive tissues. On the other hand, *OsHKT1;3*, *OsHKT2;1*, and *OsHKT2;4* had higher expression in the vegetative stage, while *OsHKT1;4* had lower expression in the vegetative stage (Fig. 11A). Also, both salt-tolerant and salt-sensitive genotypes have higher expression of *OsHKT1;5, OsHKT1;1, OsHKT1;3*, and *OsHKT2;1* (Fig. 11B). Our real-time PCR results have confirmed that *OsHKT1;5, OsHKT1;1, OsHKT1;3, OsHKT2;1,* and *OsHKT2;3* are crucial genes responsible for rice salinity tolerance (Fig. 12). The previous study on *OsHKT1;4* also reported lower expression in a vegetative stage during stress (Suzuki et al. 2016). A rice *QTL*, *SKC1*, corresponded to *OsHKT1;5* and maintained K^+ ion homeostasis under salt stress (Ren et al. 2005), and *OsHKT1;5* mutants also showed Na^+ exclusion and protected leaf blades under salt stress (Kobayashi et al. 2017). *OsHKT1;1* and *OsHKT1;4* contribute to Na^+ exclusion from leaf blades under salt stress (Cotsaftis et al. 2012; Wang et al. 2015; Suzuki et al. 2016). *OsHKT2;1/2* is also involved in Na^+ and K^+ co-transport under high salt concentrations and has been reported to maintain an appropriate ionic balance in Nona bokra (Oomen et al. 2012). The previous report also suggested that *HKT* genes play a vital role in response to salt stress in many plant species like wheat (Munns et al. 2012; Schachtman and Schroeder 1994), Arabidopsis (Sunarpi et al. 2005), maize (Ren et al. 2015) and barley (Mian et al. 2011). Thus, *HKT* genes in rice may be excellent candidate genes for accelerating transgenic research for salinity stress management in plant growth and development.

Proteins rarely function alone. A protein's activity can be activated, inhibited, or otherwise regulated through its interactions with other proteins or biological components. So far, no study has been disclosed identifying interaction partners for any *HKT* protein. The protein–protein interaction exhibited exciting facts about the substantial contribution of *OsHKT* to numerous physiological functions (Fig. 13). Our findings show that rice *HKT* genes (*OsHKT1;1* and *OsHKT2;1*) interact with the *P5CS*, which participates in salt stress tolerance and plays a vital role in proline biosynthesis (Zhang et al. 2014; Funck et al. 2020). Likewise, the *OsHKT* gene interacted with *NHX1* and *NHX2*, a sodium/hydrogen exchanger-related protein that plays a central role during plant exposure to K^+ deficiency and high salinity (Fukuda et al. 2011; Barragán et al. 2012; Teng et al. 2017); *HAK1, HAK23,* and *HAK27* are related to high-affinity potassium transporters that also transport rubidium. Furthermore, *Os01g0893400* is a putative BTB and TAZ domain protein, and *TPKC* is an inward-rectifying potassium channel family protein that is known to be important in plant growth and development (Bhattacharjee et al. 2016; Wang et al. 2018). Interestingly, a small heat shock family protein, *Hsp20/alpha*-crystallin family protein, also interacts with rice *HKT* genes. Small HSPs are hypothesized

to act as chaperones, protecting their targets against denaturation and aggregation when organisms are exposed to diverse biotic and abiotic stimuli. A recent study indicated that *OsHSP20* exhibits molecular chaperone functions in vitro*,* and overexpression has been shown to improve heat and salt stress tolerance in *E. coli*, *P. pastoris*, and transgenic rice plants. It was also found that the N-terminal part of *OsHSP20* is tightly linked to both in vivo stress tolerance and in vitro chaperone activity (Guo et al. 2020). Our results highlighted the significance of *HKT* transporters in rice salinity tolerance via their interactions with other proteins.

Plant cellular responses to abiotic stresses such as salinity, cold, and dehydration were revealed to be regulated by microRNA. Several miRNAs target genes that are actively involved in gene regulation or their associated transcription factors in response to stress. MiRNAs may play an essential role in reactions triggered by stress (Cheng and Long 2007; Sunkar et al. 2008). Fifteen Osa-miR11339 (bona fide mRNAs from rice) found in rice *HKT* genes represent lipid metabolisms in rice by targeting the terpene synthase gene (*LOC_Os07g11790*) (Baldrich et al. 2015) and also revealing the role in protein and starch metabolisms during grain filling under high day time temperature (HDT) stress (Payne et al. 2023). Four Osa-miR11343 genes found in rice *HKT* genes are involved in biotic stress by targeting the MLO domain-containing protein (*LOC_Os10g39520*) in rice. One novel Osa-miR5819 has three target sites found in rice *HKT* gene-targeted *CPuORF*-containing *bZIP38* TF and lipid transfer protein (LTPL118) subject to translational control via regulation by sucrose (Baldrich et al. 2015). Sucrose is a signal molecule that is involved in the activation of plant defense mechanisms. Another miR444 found in the rice *HKT* gene specifically targets the MADS-box transcription factors, which play an essential role in the HDT-induced caryopsis development, (Payne et al. 2023). Evidence has also shown that heat stress has been demonstrated to induce an upregulation of miR444 in maize (He et al. 2019). Interestingly, we found two microRNAs, Osa-miR1846 and Osa-miRN45, have several target sites in rice *HKT* genes. In both CDT and HDT, Osa-miR1846 was found to be strongly expressed during grain filling in the spikelets and its targeting of a heat shock factor (HSF) (Kushawaha et al. 2021). HSFs may cause chalkiness by increasing the expression of heat shock proteins (Kaneko et al. 2016). Therefore, it is probable that increased amounts of Osa-miR1846 in the Cypress inhibit this HSF, resulting in less chalkiness. On the other hand, Osa-miRN45 has a particular function to play throughout the differentiation process at the time of grain filling in rice (Peng et al. 2013). Together, these findings lay the groundwork for future genetic studies of *OsHKT* genes and facilitate the breeding of novel rice cultivars.

Conclusions

HKT family proteins are anticipated to be essential in plant salt stress tolerance. We extensively analyzed the *HKT* gene family in rice, both bioinformatically and functionally. This in silico investigation highlighted possible biological and molecular functions of the *OsHKT* genes in rice development and stress response. Phylogenetic and structural evaluations revealed that *TrkH* domains were highly significant for their respective roles. The rice *HKT* genes demonstrated purifying selection on chromosomes. Identification of cis-regulatory elements revealed their function in abiotic stress tolerance. Several transcription factors also modulate *OsHKT* gene expression to prevent salt toxicity in rice. *OsHKT* genes were found to be more active in roots and leaves under salt stress, suggesting they regulate rice plant growth, as revealed by tissue-specific expression studies. Our findings could help choose or target candidate genes for functional validation via molecular cloning in response to high salinity stress tolerance to improve crop plants.

Supplementary Information

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Supplementary material 1.

Availability of data and materials
Data is provided in the Additional files.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
All authors have read and agreed to the publication of the manuscript.

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