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KT/HAK/KUP potassium transporters gene family and their whole-life cycle expression profile in rice (*Oryza sativa*)

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Abstract KT/HAK/KUP potassium transporter protein-encoding genes constitute a large family in the plant kingdom. The KT/HAK/KUP family is important for various physiological processes of plant life. In this study, we identified 27 potential KT/HAK/KUP family genes in rice (*Oryza sativa*) by database searching. Analysis of these KT/HAK/KUP family members identified three conserved motifs with unknown functions, and 11–15 *trans*-membrane segments, most of which are conserved. A total of 144 putative *cis*-elements were found in the 2 kb upstream region of these genes, of which a Ca^{2+}-responsive *cis*-element, two light-responsive *cis*-elements, and a circadian-regulated *cis*-element were identified in the majority of the members, suggesting regulation of these genes by these signals. A comprehensive expression analysis of these genes was performed using data from microarrays hybridized with RNA samples of 27 tissues covering the entire life cycle from three rice genotypes, Minghui 63, Zhenshan 97, and Shanyou 63. We identified preferential expression of two *OsHAK* genes in stamen at 1 day before flowering compared with all the other tissues. *OsHAK* genes were also found to be differentially upregulated or downregulated in rice seedlings subjected to treatments with three hormones. These results would be very useful for elucidating the roles of these genes in growth, development, and stress response of the rice plant.

Keywords Potassium transporters · *Oryza sativa* · Phylogenetic analysis · *cis*-Elements · Microarray

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Introduction

Potassium (K^+) is the most abundant inorganic cation and performs important functions in various processes of plant life. Plants possess transport systems for acquisition of K^+, which are referred to as channels and transporters (Maathuis and Sanders 1994, 1997). Potassium transporters in plants can be divided into four families: the KT/HAK/KUP family, Trk/HKT family, KEA (K^+ efflux anti-porter) family, and CHX (cation/hydrogen exchanger) family. In plants, KT/HAK/KUP family genes were identified by sequence homology with K^+ uptake permease from bacteria (KUP) and high-affinity K^+ transporters (HAKs) from fungi (Quintero and Blatt 1997; Santa-Maria et al. 1997; Fu and Luan 1998; Kim et al. 1998). *KT/HAK/KUP* genes have been cloned from pepper (*Capsicum annum*) (Martinez-Cordero et al. 2004), tomato (*Lycopersicon esculentum*) (Wang et al. 2002; Nieves-Cordones et al. 2007), lotus (*Lotus japonicus*) (Desbrosses et al. 2004), grapevine (*Vitis vinifera*) (Davies et al. 2006), and seagrass (*Cymodocea nodosa*) (Garciadeblas et al. 2002). Twenty-six KT/HAK/KUP family genes in rice (*Oryza sativa*) (Amrutha et al. 2007) and 13 in *Arabidopsis thaliana* (Maser et al. 2001) have been identified based on sequence analysis.

In plants, phylogenetic analysis of 13 *KT/HAK/KUP* gene sequences from *Arabidopsis*, five from barley (*Hordeum vulgare*), and two from rice grouped these KT/HAK/KUP transporters into four clusters (Rubio et al. 2000). This was

further confirmed by phylogenetic analysis of 17 *KT/HAK/KUP* genes in rice (Banuelos et al. 2002). Most of the functionally characterized KT/HAK/KUP transporters belong to clusters I and II, while functions of clusters III and IV transporters are less known (Grabov 2007). Studies showed that most of the cluster I transporters likely have function in high-affinity K^+ uptake. For example, it was shown that expression of *AtHAK5*, a member of cluster I, becomes upregulated under K^+ starvation conditions (Ahn et al. 2004; Gierth et al. 2005; Qi et al. 2008). mRNA levels of *AtHAK5* orthologs in other plant species, such as *LeHAK5* in tomato (Wang et al. 2002) and *HvHAK1* in barley (Santa-Maria et al. 1997), were also induced under K^+ starvation. In rice, *OsHAK1*, a member of this cluster, was functionally characterized in yeast (*Saccharomyces cerevisiae*), where it mediates high-affinity K^+ uptake (Banuelos et al. 2002). It was also shown that some of this cluster transporters are also involved in Na^+ influxes, as reported in barley, *HvHAK1* (Santa-Maria et al. 1997) and in reed plants (*Phragmites australis* Trinius), *PhaHAK1* (Takahashi et al. 2007).

Most of the cluster II transporters were proposed to have a role in low-affinity K^+ transport complementing potassium channels (Senn et al. 2001; Garciadeblas et al. 2002). *OsHAK7* and *OsHAK10*, belonging to this cluster, mediate low-affinity K^+ uptake in heterologous system (Banuelos et al. 2002). Transient expression of the OsHAK10-green fluorescent protein (GFP) fusion protein in living onion epidermal cells targeted this protein to the tonoplast, strongly suggesting that this transporter locates to the tonoplast of rice cells (Banuelos et al. 2002). The putative function for tonoplast KT/HAK/KUP transporters is to maintain K^+ homeostasis under K^+-deficient conditions (Walker et al. 1996; Garciadeblas et al. 2002). Besides K^+ uptake, cluster II transporters may also involve in regulatory processes. A mutation in *AtKUP/HAK/KT2* causes short hypocotyl phenotype (Elumalai et al. 2002), while knock-out plants of *AtKUP4=AtKT3* (or *TRH1*) show tiny root hair phenotype (Rigas et al. 2001). Recently, Vicente-Agullo et al. (2004) reported that *AtKUP4=AtKT3* is required for auxin transport. Cluster II transporters may also be involved in salinity response, because expressions of *AtHAK6* and *AtHAK2* were affected by increased salt concentrations (Maathuis 2006).

KT/HAK/KUP transporters belonging to clusters III and IV have been less studied. *AtHAK11* belonging to cluster III may be involved in response to salinity (Maathuis 2006). Recently, *PhaHA5* (in cluster IV) from reed plants was predicted to localize on plasma membrane, and may act in the high-affinity K^+ uptake and could be involved in Na^+ transport (Takahashi et al. 2007).

Although the reported studies of potassium transporters are numerous, there has been no report on the coordinated expression in a global scale of the entire set of potassium transporters in any plant species. Moreover, it was suggested that regulation of potassium transporters is controlled by several signaling cascades, most of which are related to hormones (Ashley et al. 2006), indicating that phytohormones are of immense importance in the functioning of KT/HAK/KUP transporters.

Rice is a staple crop and has also become a model for plant science research in cereals because of the smallest genome among the major cereals and the availability of a range of genetic resources and genomic tools (Zhang 2007). The objective of this study was to provide a global overview of the KT/HAK/KUP family genes in the rice genome, including the identification of the entire gene family members, their phylogenetic relationship, motif compositions, protein architecture, and possible *cis*-elements. The expression profiles of the gene family in the entire rice life cycle and under different hormone treatments conditions were also analyzed using data from microarrays. Much of the analysis was also in comparison with features of this gene family in *Arabidopsis*. It is expected that such a comprehensive analysis may provide important clues for understanding the diverse roles of these KT/HAK/KUP transporters in growth and development.

Materials and methods

Data search and analyses

For obtaining sequences of the KT/HAK/KUP family genes in rice (*OsHAK*), the protein family ID PF02705 was queried in the database of The Institute of Genomic Research (TIGR) (http://www.tigr.org). All the corresponding protein sequences of the putative KT/HAK/KUP family members were downloaded and confirmed with the Pfam database (http://www.sanger.ac.uk/Software/Pfam/search.shtml). Information about the chromosomal localization, coding sequence (CDS) length, amino acid (aa) length, full-length cDNA accessions, and protein localization was obtained for each gene from TIGR and the Knowledge-Based *Oryza* Molecular Biological Encyclopedia (KOME) (http://cdna01.dna.affrc.go.jp/cDNA). The KT/HAK/KUP family genes in *Arabidopsis* were downloaded by querying the protein family ID PF02705 in the database of The *Arabidopsis* Information Resource (http://www.arabidopsis.org) and confirmed by using Pfam database (http://www.sanger.ac.uk/Software/Pfam/search.shtml).

Alignment of the protein sequences was performed using CLUSTAL_X version 1.83 (Thompson et al. 1997). A phylogenetic tree was constructed using MEGA4 (Tamura et al. 2007). Bootstrap testing was performed with 1,000 resampling. The Multiple Em (Expectation Maximization) for Motif Elicitation (MEME) program version 3.5.4 (Bailey and Elkan 1994) was used to predict the potential motifs in the putative KT/HAK/KUP family gene sequences.

Promoter sequences (2 kb upstream of the translation start codon) for all KT/HAK/KUP family members were obtained from TIGR (http://www.tigr.org/tdb) and subjected to scanning for plant *cis*-acting regulatory DNA elements (PLACE) (http://www.dna.affrc.go.jp/PLACE/signalscan.html) to identify all the plant *cis*-elements with more than 6 bp (Higo et al. 1999).

Microarray expression profile

To analyze the expression patterns, the rice KT/HAK/KUP family genes were searched against a database (http://crep.ncpgr.cn) of hybridization generated using Affymetrix whole genome arrays. In this database, hybridization was conducted using RNA samples obtained with at least two biological repeats from 39 tissues covering the entire life cycle of the plants from three genotypes of cultivated rice, Minghui 63, Zhenshan 97, and their hybrid Shanyou 63, an elite hybrid widely grown in China for more than two decades. For relevance, only hybridization data for 27 tissues were analyzed in this study: (1) calli at 15 days after subculture stage; (2) resistance calli at screening stage; (3) calli at 5 days after regeneration stage; (4) seed germination after 72 h of imbibitions stage; (5) embryo and radical after germination stage; (6) seedling at trefoil stage; (7) shoot at seedling with two tillers stage; (8) root at seedling with two tillers stage; (9) leaf, young panicle at secondary branch primordial stage; (10) sheath, young panicle at secondary branch primordial stage; (11) panicle, young panicle at secondary branch primordial stage; (12) panicle at pistil/stamen primordial differentiation stage; (13) panicle at pollen-mother cell formation stage; (14) leaf at 4–5 cm young panicle stage; (15) sheath at 4–5 cm young panicle stage; (16) panicle at 4–5 cm young panicle stage; (17) stem at 5 days before heading stage; (18) flag leaf at 5 days before heading stage; (19) stem at heading stage; (20) panicle at heading stage; (21) hull at 1 day before flowering stage; (22) stamen at 1 day before flowering stage; (23) flag leaf at 14 days after heading stage; (24) spikelet at 3 days after pollination stage; (25) endosperm at 7 days after pollination stage; (26) endosperm at 14 days after pollination stage; (27) endosperm at 21 days after pollination stage. Independent expression patterns were also analyzed to display transcript regulation of KT/HAK/KUP family genes under hormone treatments in seedling at trefoil stage of the three rice genotypes. The hormones used were naphthalene acetic acid (NAA), gibberellic acid (GA_3), and kinetin (KT).

In performing the analysis, we included only genes with 100% identity over the entire length with the entire set of the probes for each gene, which were labeled 'Present' by Affymetrix MAS 5.0 with average signal values of more than 100. Data for only one probe of each gene were used for expression analysis.

To identify the preferential expressed gene in a tissue, we selected one tissue and then compared it with all other 26 tissues by performing Student's *t* test in each genotype separately. Gene in a tissue with *P* value less than 0.05 and expression values more that twofold compared to all other tissues was considered to be expressed preferentially. Under hormone treatments conditions, genes that were upregulated or downregulated more than twofold with *P* value less than 0.05 compare to control were considered as showing differential expression. The average of biological replicates for each sample was used for consideration of twofold expression values.

RNA extraction and RT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, Shanghai, China) according to the manufacturer's instructions from the following tissues of field-growing plants of three genotypes of cultivated rice, Minghui 63, Zhenshan 97, and Shanyou 63: hull at 1 day before flowering, stamen at 1 day before flowering, flag leaf at 14 days after heading, young panicle of 4–5 cm in length, trefoil-stage seedling treated with 100 μM NAA (Sigma-Aldrich, Shanghai, China), trefoil-stage seedling treated with 100 μM GA_3 (Sigma-Aldrich, Shanghai, China), and trefoil-stage seedling treated with 100 μM KT (Sigma-Aldrich, Shanghai, China). For hormone treatments, seedlings at trefoil stage were sprayed with each hormone independently; samples were collected at 5, 15, 30, and 60 min after treatment and ground simultaneously.

Two micrograms of total RNA from each sample was reverse-transcribed in a total volume of 20 μl containing 0.5 mg oligo$(dT)_{15}$, 0.75 mM dNTPs, 10 mM DTT, and 200 units M-MLV reverse transcriptase (Promega, Beijing, China). ExTaq DNA polymerase (TaKaRa, Dalian, China) was used for PCR amplification in an ABI Thermocycler 9700 (Applied Biosciences, Foster City, CA, USA) with the following cycling profile: 94°C for 3 min, followed by 28–38 cycles (depending on the expression levels of the genes) at 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 5 min. All gene-specific primers for RT-PCR were designed based on the sequences of the respective KT/HAK/KUP family genes (Table 1). Each RT-PCR pattern was verified by triple replicate experiments, and a rice *actin* cDNA was used as an internal control.

Results

The KT/HAK/KUP potassium transporters family in rice

Search of the TIGR database obtained significant matches with 48 gene models annotated as "potassium transporter" representing 27 distinct loci (Table 2). BLAST analysis

Table 1 *OsHAK* genes primers used for RT-PCR analysis

Genes	Primers	PCR product size (bp)
OsHAK3	5′-TGTCCAAATCACCCCACT-3′ 5′-CTGTACTGCCAGACGAAGAC-3′	385
OsHAK4	5′-GACGGGTTCTACAAGTTCGT-3′ 5′-AGCAGGAACGGCACTAAGTA-3′	395
OsHAK6	5′-GATGCACTACCCACCTGTTC-3′ 5′-GGTGATGAGAACACACACGA-3′	499
OsHAK7	5′-CTATCAGCCTGGAGTTGGTG-3′ 5′-AGTGAAGTGGCCAAGATCAG-3′	502
OsHAK8	5′-TTCGTGTTCTGGACACTCAC-3′ 5′-CGATGAGTACCGTAATGCTG-3′	449
OsHAK10	5′-ACATGTCTGATGTCCCTGGT-3′ 5′-GGTAGCCGTACCTGACAATG-3′	502
OsHAK13	5′-ACTTCGAGAACCAACTGCTG-3′ 5′-CTATACCCTGTACGCCATGC-3′	500
OsHAK15	5′-GGAGGTTGGGCTCTGATAAT-3′ 5′-CTCCTCGTCAGAGTCGATGT-3′	501
OsHAK16	5′-TACACTGAATTGGTGCAAGG-3′ 5′-GGATTGGTGTAAGCATCTCC-3′	397
OsHAK17	5′-TGGTACTGTGTGTCGGTGTT-3′ 5′-CCGTCCTGTATGTTGCTGTA-3′	418
OsHAK18	5′-CAGTTCTTTCTGCGACTGGT-3′ 5′-TGCTGGCCAAAGTATAGGAG-3′	500
OsHAK20	5′-ACGTCGTGTTCACCTTCACT-3′ 5′-CCTCGTAGACGAACACCTTG-3′	510
OsHAK21	5′-TCTCCCTCATCGTCTACAGC-3′ 5′-AGAGCAGCCACAAGAGAATG-3′	501
OsHAK27	5′-ATGATCATCACAACCCTCCT-3′ 5′-CAAAGATGAGCACAGAGTGG-3′	400
Actin	5′-TATGGTCAAGGCTGGGTTCG-3′ 5′-CCATGCTCGATGGGGTACTT-3′	177

against the Pfam database showed that all of them belonged to the OsHAK family. The 27 *OsHAK* genes are distributed on eight rice chromosomes: chromosomes 1, 3, and 7 each contains four genes, and chromosomes 2, 4, 6, 8, and 9 each contains three genes.

All the predicted OsHAK proteins have a typical "k_trans" domain (PF02705), which is specific to KT/HAK/KUP potassium transporters family members. Twenty of the 27 OsHAK proteins show localization on plasma membranes by a PSORT analysis (http://psort.nibb.ac.jp/), a location that is best suited for their function as transporter. The remaining seven proteins show localization on four different organelles: three on chloroplast thylakoid membrane, two on mitochondrial inner membrane, one on vacuolar membrane (tonoplast), and the remaining one on microbody (Table 2).

The full-length cDNA sequences were compared with the corresponding genomic DNA sequences to determine the numbers and positions of exons and introns within each *OsHAK* gene (Fig. 1) by using GSDS (http://gsds.cbi.pku.edu.cn/chinese.php) (Guo et al. 2007). The coding sequences of all the *OsHAK* genes are disrupted by introns, with numbers varying from one to nine. Twelve of the 27 genes contain eight introns each. This suggests that the gene structure in rice is more divergent than *Arabidopsis*, in which the number of introns varied from five to nine (Ahn et al. 2004).

Phylogenetic analysis

Based on the joint unrooted phylogenetic tree generated from alignments of the full-length protein sequences of the KT/HAK/KUP family members in rice (27 genes) and *Arabidopsis* (13 genes) (Fig. 2), the KT/HAK/KUP family proteins fell broadly under four major clusters (I, II, III, and IV), with well-supported bootstrap values. Cluster I contains nine members (with eight members of rice and one member of *Arabidopsis*), which can be further divided into subcluster IA with four members, subcluster IB with four members, and a diverge member (*AtHAK5*). Cluster II contains 15 members (with nine members of rice and six members of *Arabidopsis*) and can be divided into subcluster IIA with seven members and subcluster IIB with eight members. Cluster III contain 12 members (with six members of rice and *Arabidopsis* each), which can be divided into subclusters IIIA and IIIB, with six members each. Cluster IV had four members all from rice, which can be further divided into subclusters IVA and IVB, with two members each.

Comparison of the phylogenetic tree with the predicted subcellular localizations of the KT/HAK/KUP family proteins indicated that the same phylogenetic grouping does not mean the same subcellular localization. For example, a proteomic analysis localized AtKUP4=AtKT3 on tonoplast (Jaquinod et al. 2007), while the three proteins OsHAK2, OsHAK3, and OsHAK7 having the highest sequence similarity with AtKUP4=AtKT3 in subcluster IIA (Fig. 2) were predicted to have different subcellular localizations: OsHAK2 and OsHAK3 on plasma membrane and OsHAK7 on microbody. In subcluster IIB, the *Arabidopsis* protein AtKUP/HAK/KT2 was located on plasma membrane (Nuhse et al. 2004), and two of the rice proteins OsHAK8 and OsHAK9 having highest similarity to AtKUP/HAK/KT2 are also predicted to localize on plasma membrane. In subcluster IIIB, two *Arabidopsis* KT/HAK/KUP transporters, AtKUP/KT5 and AtKUP/HAK/KT7, were localized on tonoplast (Jaquinod et al. 2007), whereas their close rice homologs OsHAK14 and OsHAK15 were predicted to be localized on mitochondrial inner membrane and on chloroplast thylakoid membrane, respectively. Another subcluster IIIB transporter, AtKUP/HAK/K12, was located on chloroplast membrane, but the exact localization that they either are located on envelop or are located on thylakoid membrane cannot be predicted at this stage (Kleffmann et al. 2004). High expression of *AtKUP/HAK/*

Table 2 *OsHAK* genes in rice

Gene[a]	Probeset ID[b]	Locus ID[c]	CDS[d]	aa[e]	Fl-Acc.[f]	P.L.[g]	TMS[h]	Gene reported[i]
OsHAK1	Os.11262.1.S1_a_at	Os04g32920	2,406	801	AK100669	PM	12	Banuelos et al. 2002
OsHAK2	Os.26989.1.S1_a_at	Os01g70940	2,352	783	AK070575	PM	13	
OsHAK3	Os.29851.1.S1_x_at	Os01g27170	2,427	808	AK100340	PM	14	
OsHAK4	Os.2358.1.S1_at	Os08g36340	2,094	697	AK071698	CTM.	12	
OsHAK5	Os.17158.1.S1_at	Os01g70490	2,313	770	AK241580	PM	13	
OsHAK6	Os.31355.2.S1_x_at	Os01g70660	2,247	748	AK107477	PM	13	
OsHAK7	Os.15701.1.S1_x_at	Os07g47350	2,346	781	AK100652	Mb	15	Banuelos et al. 2002
OsHAK8	OsAffx.3292.1.S1_s_at	Os03g21890	2,382	793	NF	PM	14	
OsHAK9	Os.13483.1.S1_at	Os07g48130	2,367	788	AK070738	PM	14	
OsHAK10	Os.5614.1.S1_at	Os06g42030	2,532	843	AK107132	Tonoplast	13	Banuelos et al. 2002
OsHAK11	Os.53340.1.S1_at	Os04g52390	2,376	791	AK072123	PM	13	
OsHAK12	Os.17644.1.S1_at	Os08g10550	2,382	793	AK069377	PM	11	
OsHAK13	Os.49807.1.S1_at	Os06g45940	2,337	778	AK111663	PM	13	
OsHAK14	Os.19000.1.S1_at	Os07g32530	2,580	859	AK106582	MIM.	15	
OsHAK15	Os.53894.1.S1_x_at	Os04g52120	2,604	867	AK100672	CTM.	13	
OsHAK16	Os.20541.1.S1_at	Os03g37840	2,436	811	AK066919	CTM.	11	
OsHAK17	Os.6037.2.S1_x_at	Os09g27580	2,124	707	AK068574	PM	13	
OsHAK18	Os.26972.1.S1_at	Os09g38960	2,382	793	AK065464	PM	15	
OsHAK19	NF	Os02g31910	2,229	742	AK106353	PM	12	
OsHAK20	Os.49906.1.S1_at	Os02g31940	2,244	747	AK119325	PM	12	
OsHAK21	OsAffx.3426.1.S1_at	Os03g37930	2,400	799	NF	PM	13	
OsHAK22	Os.27454.1.S1_x_at	Os07g01214	2,427	808	AK066544	MIM.	11	
OsHAK23	Os.53102.1.S1_s_at	Os09g21000	2,634	877	AK070831	PM	14	
OsHAK24	OsAffx.27660.1.S1_at	Os06g15910	2,319	772	NF	PM	15	
OsHAK25	Os.26684.1.S1_at	Os02g49760	2,316	771	AK111629	PM	14	
OsHAK26	Os.24908.1.S1_at	Os08g39950	2,220	739	AK072472	PM	13	
OsHAK27	Os.16325.1.S1_at	Os03g37830	2,436	811	AK068853	PM	13	

PM plasma membrane, *CTM* chloroplast thylakoid membrane, *MIM* mitochondrial inner membrane, *Mb* microbody, *NF* not found

[a] Systematic designation given to rice *OsHAK* genes in this study

[b] Probeset ID of *OsHAK* genes obtained from CREP (http://crep.ncpgr.cn)

[c] Locus ID of *OsHAK* genes

[d] Length of CDS in basepairs obtained from TIGR

[e] Protein length (number of amino acids) obtained from TIGR

[f] Full length cDNA accession number of *OsHAK* genes obtained from KOME

[g] Localization of OsHAK protein supported by PSORT (http://psort.nibb.ac.jp)

[h] Number of transmembrane segments posses by *OsHAK* genes, supported by Transport Classification Database (http://www.tcdb.org/analyze.php)

[i] *OsHAK* genes reported in earlier studies

KT12 was observed in young leaves when compared to old ones (Ahn et al. 2004). K^+ plays a significant role in CO_2 fixation (Pfluger and Cassier 1977). Thus, *AtKUP/HAK/KT12* may play important role in photosynthesis. However, OsHAK23 having high similarity with AtKUP/HAK/KT12 was predicted to be localized on plasma membrane. No homologous rice transporters were found to correspond to AtHAK5 (a diverge member in cluster I) and AtKUP/HAK/KT11 (subcluster IIIA); both of them were localized on plasma membrane (Qi et al. 2008; Nuhse et al. 2004). Thus, proteins with the same phylogenetic grouping by sequence similarity frequently have different subcellular localizations, suggesting different cytological functions.

Motif analysis and protein architecture

Based on the protein sequence alignment using the MEME software, three putative conserved motifs, each with 50 aa in length, were identified in the OsHAK family with E values <1.00E-30 (Fig. 3). Among these three

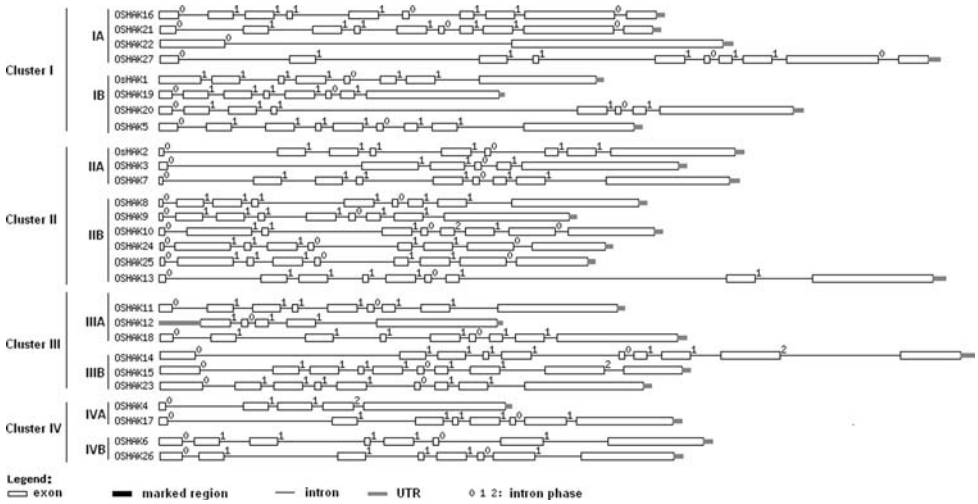

Fig. 1 The GSDS (http://gs-ds.cbi.pku.edu.cn/chinese.php) output of the *OsHAK* genes structure in rice. 0, 1, 2 intron phase indicate splicing patterns: in phase 0, splicing occurs after the third nucleotide of the first codon; in phase 1, splicing occurs after the first nucleotide of the single codon; in phase 2, splicing occurs after the second nucleotide. Subclusters IA, IB, IIA, IIB, IIIA, IIIB, IVA, and IVB correspond to the phylogenetic clusters from Fig. 2

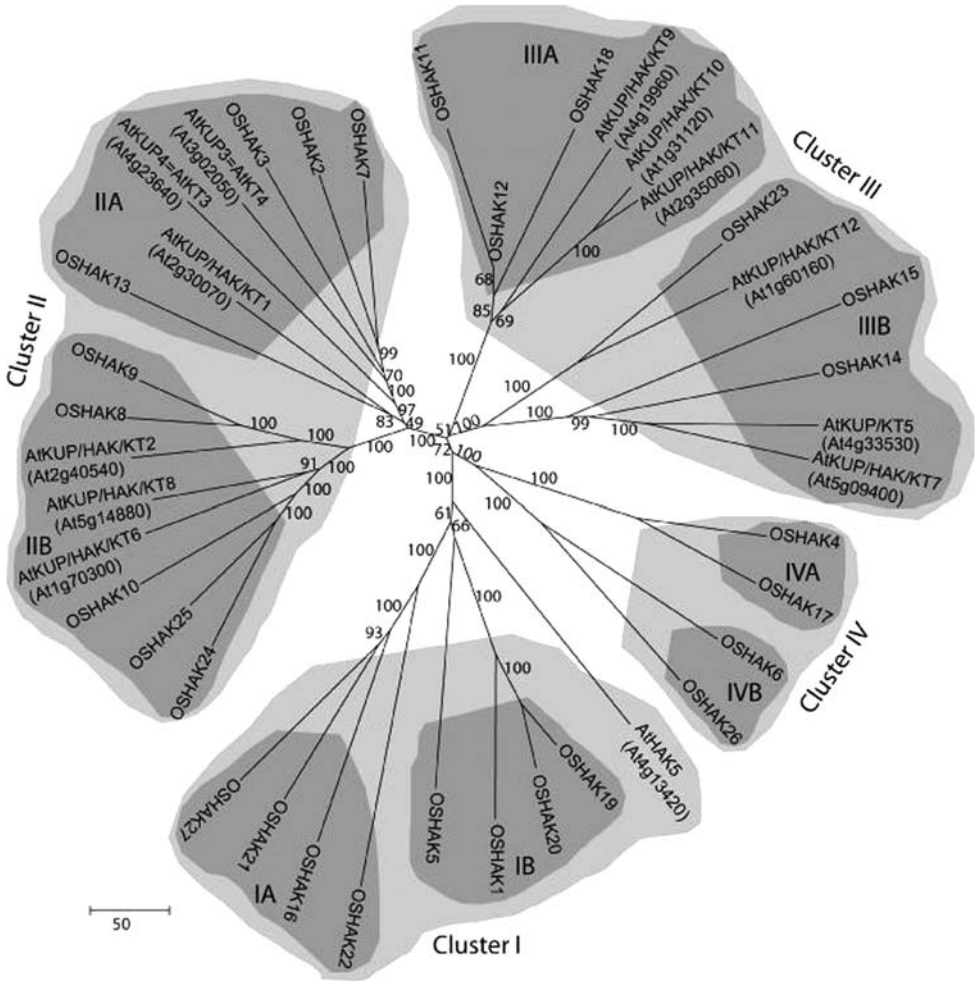

Fig. 2 Phylogenetic relationship between KT/HAK/KUP family genes in rice (*OsHAK*) and *Arabidopsis*. The joint unrooted tree was generated using MEGA4 by the neighbor-joining method. Bootstrap values from 1,000 replicates are indicated at each branch

motifs, motif 1 has six conserved amino acid residues, motif 2 has ten conserved amino acid residues, and motif 3 has 11 conserved amino acid residues exactly at the same position. All three putative conserved motifs are present in all the OsHAK family members except OsHAK22, in which only motifs 1 and 2 were identified (Fig. 4). Motif 1 appeared twice in OsHAK2 and motif 3 appeared twice in OsHAK19.

Searching of Transport Classification Database (http://www.tcdb.org/analyze.php) identified 11–15 *trans*-membrane segments (TMSs) in the KT/HAK/KUP family transporters in rice (Table 2), which were classified into 14 TMS regions by

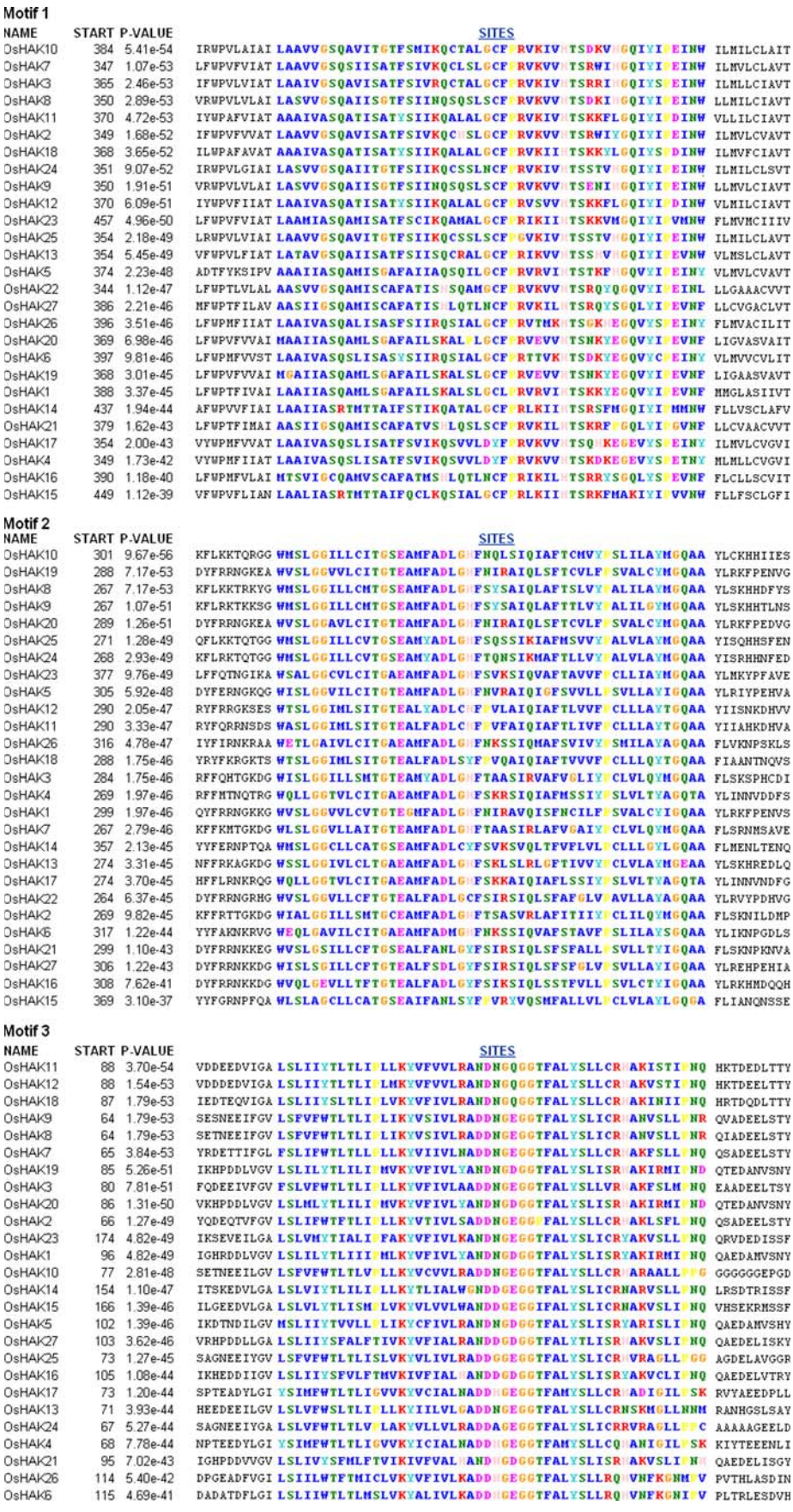

Fig. 3 Protein sequence alignment of three putative conserved motifs identified in *OsHAK* genes in rice obtained by MEME. Each of the three motifs has 50 conserved amino acids residues, as shown in color

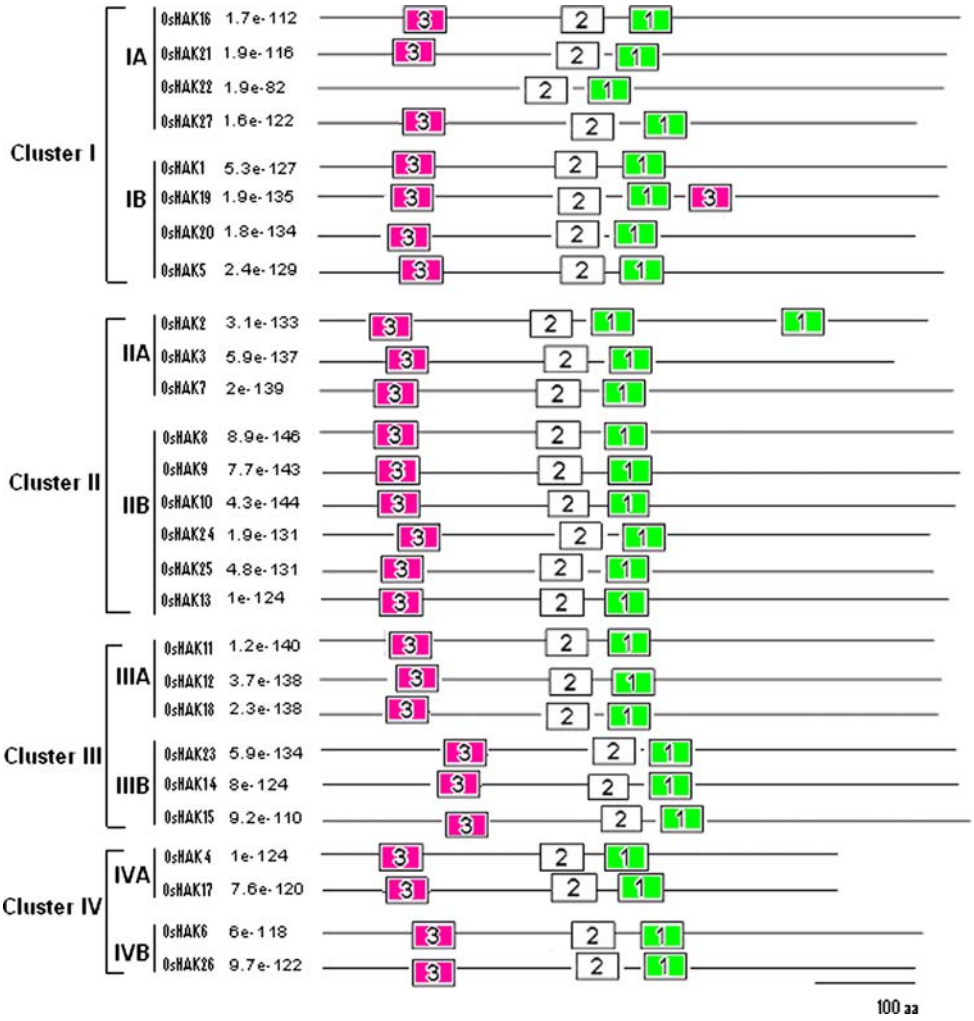

Fig. 4 Organization of putative motifs in *OsHAK* genes identified by MEME. *Numbered color boxes* represent different putative motifs. The expected values calculated by MEME are shown after the gene names. Subclusters IA, IB, IIA, IIB, IIIA, IIIB, IVA, and IVB correspond to the phylogenetic clusters in Fig. 2

protein sequence alignment (Supplementary Fig. 1). Of the 14 TMS regions, the fifth to twelfth TMS regions are highly conserved among all OsHAK transporters, indicating that these TMSs are important for the basic protein structure and function. Among the less conserved TMS regions, the 13th TMS was the most diverse, and the 14th TMS region, present at the very end of C-terminal, occurred only in five OsHAK transporters (OsHAK3, OsHAK7, OsHAK10, OsHAK14, and OsHAK24). The first TMS was missing in OsHAK3, OsHAK12, OsHAK16, and OsHAK22; the second TMS was missing in OsHAK12 and OsHAK22; the third TMS region was not found in OsHAK12, OsHAK22, and OsHAK25; fourth TMS was not found in OsHAK10.

In *Arabidopsis*, 12–15 *trans*-membrane segments (TMS) were identified in KT/HAK/KUP family transporters. Similar to rice, they can be classified into 14 TMS regions (Supplementary Fig. 2). In *Arabidopsis*, 11 (second to twelfth TMS regions) of 14 TMS regions are highly conserved among all KT/HAK/KUP family transporters. The 13th TMS region is the most diverse one among all the 14 TMS regions. Only four *Arabidopsis* KT/HAK/KUP family transporters (AtKUP3=AtKT4, AtKUP4=AtKT3, AtKT/HAK/KUP7, and AtKT/HAK/KUP12) have the 14th TMS region present at C-terminal, while only AtHAK5 does not have the first TMS region. Thus, the basic protein architecture of KT/HAK/KUP family transporters in *Arabidopsis* appears to be more conserved than rice.

cis-Element analysis

By searching the PLACE database with the 2 kb upstream regions of the 27 *OsHAK* genes as queries, a total of 144 putative *cis*-elements with more than 6 bp in length were identified (Supplementary Table S1).

Among these 144 putative *cis*-elements, 22 were found with three or more copies in at least three of the 27 OsHAK family members: ABRERATCAL, ANAERO1CONSENSUS, CARGCW8GAT, CIACADIANLELHC, CTRMCAMV35S, DPBFCOREDCDC3, EECCRCAH1, INRNTPSADB, POLASIG2, PRECONSCRHSP70A, RYREPEATLEGUMINBOX, SEBFCONSSTPR10A, SEF4MOTIFGM7S, TATABOX2, TATABOX3, TATABOXOSPAL,

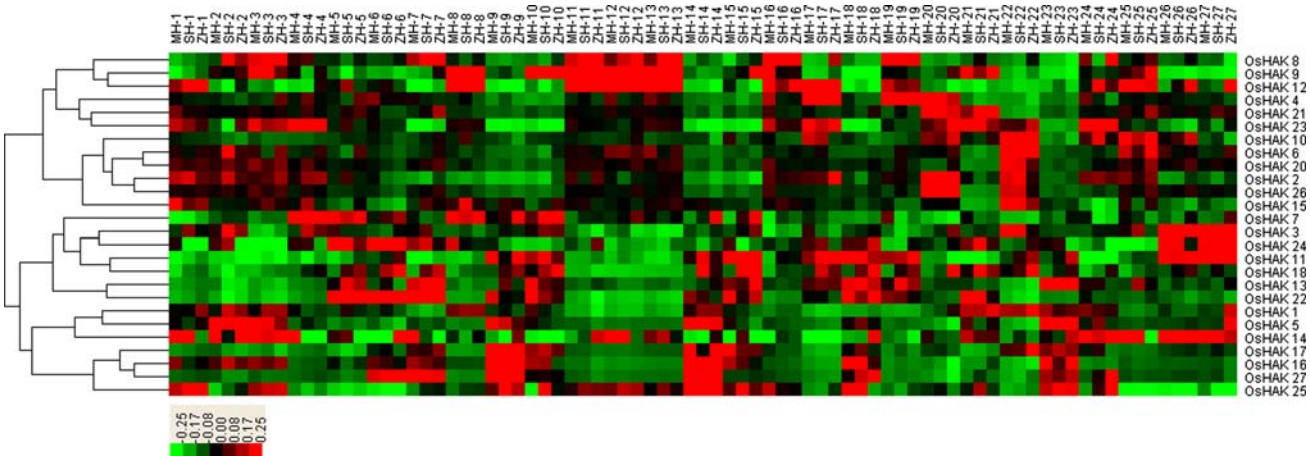

Fig. 5 Hierarchical cluster display of *OsHAK* genes in rice based on the average signal values in 27 tissues. Numbers from 1 to 27 above the cluster represent the 27 tissues covering the rice life cycle (described in 'Materials and methods'). Each tissue has three parallel displays representing Minghui 63 (MH), Shanyou 63 (SH), and Zhenshan 97 (ZH), respectively. The color scale representing average signal values is shown at the *bottom*

CANBNNAPA, MARTBOX, -300ELEMENT, TATABOX4, GAGA8HVBKN3, and GAGAGMGSA1.

ABRERATCAL, a Ca^{2+}-responsive *cis*-element found in the upstream regions of 162 Ca^{2+}-responsive upregulated genes (Kaplan et al. 2006), was present in 25 of the 27 *OsHAK* genes. Two light-responsive *cis*-elements were identified. The first, INRNTPSADB, a light-responsive transcriptional element found in the tobacco (*Nicotiana sylvestris*) *psaDb* gene (Nakamura et al. 2002), was present in 25 of the 27 *OsHAK* genes. The other, PRECONSCRHSP70A, a *cis*-element involved in induction of *HSP70A* gene in *Chlamydomonas reinhardtii* by light (von Gromoff et al. 2006), was present in 21 *OsHAK* genes. In addition, the *cis*-element CIACADIANLELHC necessary for circadian expression of light-harvesting complex protein genes (*Lhc*) in tomato (Piechulla et al. 1998) was present in 19 *OsHAK* genes.

Expression profile of KT/HAK/KUP family genes by microarrays

Probes for 26 of the 27 KT/HAK/KUP family genes could be identified in the expression database, which are listed in Table 2. Figure 5 shows a hierarchical cluster display of average signal values for the 26 *OsHAK* genes in 27 tissues covering the entire life cycle of the rice plant in three genotypes, Minghui 63, Zhenshan 97, and Shanyou 63. For these 27 tissues, two biological repeats (except tissues 5, 6, 11, 12, and 13, which have three biological repeats each with two technical repeats) were performed. Signal values of these biological repeats and their average signal values are given in Supplementary Tables S2 and S3, respectively.

Among the 26 genes analyzed in 27 tissues, all *OsHAK* genes were expressed in at least one tissue from at least one genotype. Five genes (*OsHAK2*, *OsHAK10*, *OsHAK15*, *OsHAK23*, and *OsHAK25*) showed expression in all tissues in all three genotypes. *OsHAK10* has highest expression level (>10,000) in all three cultivars in stamen at 1 day before flowering. In Minghui 63, seven genes have high expression levels (>5,000), compared with the other following genes: *OsHAK1*, *OsHAK2*, and *OsHAK15* (in stamen at 1 day before flowering), *OsHAK5* and *OsHAK16* (in flag leaf at 14 days after heading), *OsHAK23* (in hull at 1 day before flowering), and *OsHAK25* (in leaf at 4–5 cm young panicle). In Shanyou 63, five genes have high expression levels (>5,000): *OsHAK1, OsHAK2*, and *OsHAK15* (in stamen at 1 day before flowering), *OsHAK25* (in flag leaf at 14 days after heading), and *OsHAK18* (in sheath at 4–5 cm young panicle). In Zhenshan 97, six genes have high expression levels (>5,000): *OsHAK1*, *OsHAK15*, *OsHAK17*, *OsHAK18*, and *OsHAK23* (in stamen at 1 day before flowering) and *OsHAK25* (in leaf at 4–5 cm young panicle). Among the 26 genes, *OsHAK14* shows the lowest expression level (<150) in all three genotypes. From our chip data, we also found that most of the KT/HAK/KUP family genes have detectable expression in most of the studied tissues. Similar results were observed in *Arabidopsis* where many *KT/HAK/KUP* genes were expressed in roots, leaves, siliques, and flowers of plants grown under K^+-sufficient conditions (Ahn et al. 2004).

Two *OsHAK* genes (*OsHAK10* and *OsHAK15*) showed preferential expression in stamen at 1 day before flowering (tissue 22), as determined by the *t* test and the twofold difference threshold. Both of these genes show preferential

expression in Minghui 63, whereas only *OsHAK15* shows preferential expression in Shanyou 63 and *OsHAK10* in Zhenshan 97 (Fig. 6).

Plants acquire K^+ from soil through root tissues. We specifically investigated the expression of 26 *OsHAK* genes in root at seedlings with two tillers stage (Supplementary Table S3). Fourteen *OsHAK* genes (*OsHAK1*, *OsHAK2*, *OsHAK7* to *OsHAK12*, *OsHAK15*, *OsHAK16*, *OsHAK18*, and *OsHAK23* to *OsHAK25*) showed high or detectable expression simultaneously in all three genotypes, while remaining 12 genes did not show detectable expression with the threshold applied. Similarly, in *Arabidopsis*, 10 of 13 *KT/HAK/KUP* genes were expressed in root tissues (Ahn et al. 2004).

To verify the reliability of the CREP microarray data, we randomly chose five genes (*OsHAK6*, *OsHAK10*, *OsHAK13*, *OsHAK15*, and *OsHAK20*) for RT-PCR analysis using tissues of Minghui 63 (Fig. 7a), eight genes (*OsHAK3*, *OsHAK4*, *OsHAK6*, *OsHAK8*, *OsHAK10*, *OsHAK13*, *OsHAK15*, and *OsHAK20*) using tissues of Shanyou 63 (Fig. 7b), and seven genes (*OsHAK6*, *OsHAK8*, *OsHAK10*, *OsHAK15*, *OsHAK18*, *OsHAK20*, and *OsHAK21*) using Zhenshan 97 (Fig. 7c). All these RT-PCR results matched very well with DNA chip data (Fig. 7; Supplementary Table S3).

Differential expression of KT/HAK/KUP family genes with hormone treatments

The hierarchical cluster display of expression changes of the 26 genes after hormone treatments (NAA, GA_3, or KT) in the three rice genotypes was given in Fig. 8. We defined a gene as differentially upregulated or downregulated under hormone treatments if the expression level of the gene was significantly higher (more than twofold) or lower (less than twofold) than that under normal conditions in all three genotypes, in addition to being significant at the $P < 0.05$ by t test. Signal values for two biological repeats of hormonally treated tissues and their average signal values are given in Supplementary Tables S4 and S5, respectively. Five KT/HAK/KUP family genes were differentially upregulated or downregulated with hormone treatments. In Minghui 63, *OsHAK8* showed differential upregulation with NAA and KT treatments and *OsHAK27* showed differential downregulation with NAA treatments (Fig. 9a). In Shanyou 63, *OsHAK17* and *OsHAK27* showed differential downregulation with all three hormonal treatments (Fig. 9b). Similarly, in Zhenshan 97, *OsHAK7* showed differential downregulation in NAA treatments, *OsHAK16* showed differential downregulation in GA_3 and KT treatments, and *OsHAK27* showed differential downregulation

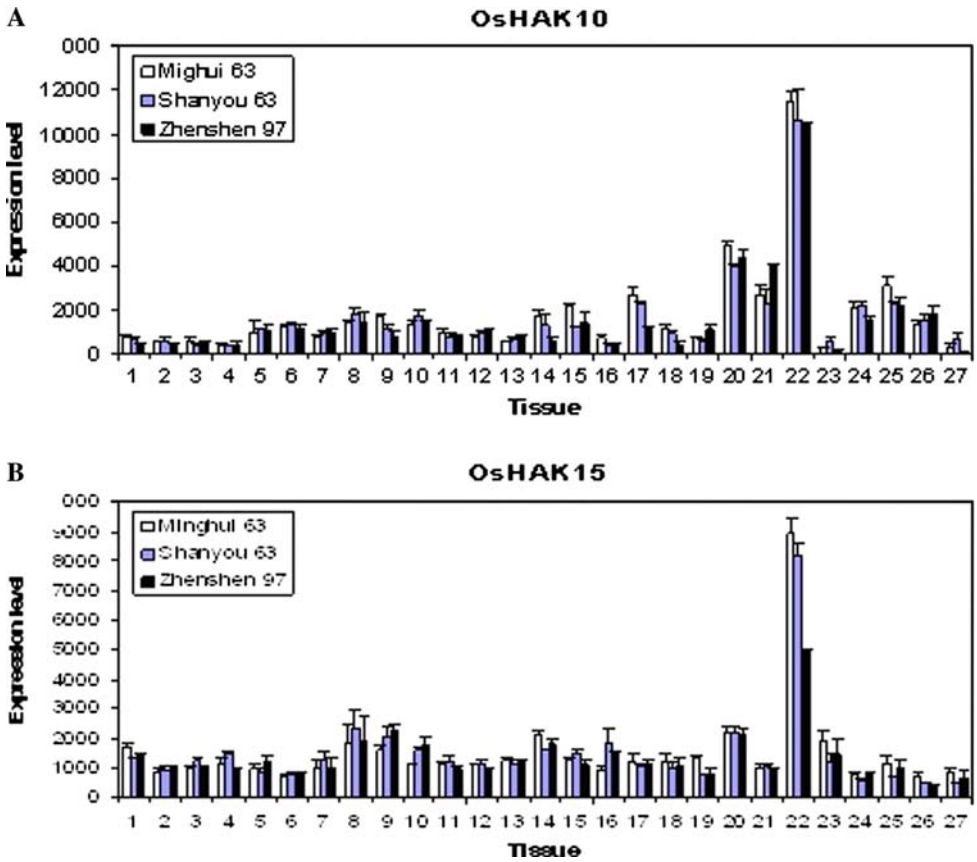

Fig. 6 The expression pattern of *OsHAK10* and *OsHAK15* genes in 27 tissues covering the rice life cycle based on average signal values in three rice cultivars, Minghui 63, Shanyou 63, and Zhenshan 97, respectively. (**a**) *OsHAK10* shows preferential expression in stamen at 1 day before flowering (tissue 22) in Minghui 63 and Zhenshan 97. (**b**) *OsHAK15* shows preferential expression in stamen at 1 day before flowering in Minghui 63 and Shanyou 63. Preferential expression was determined by performing Student's t test in each genotype separately. A gene is said to have preferential expression in a tissue, if the P value is less than 0.05 and more than twofold difference threshold in expression compared to all other 26 tissues

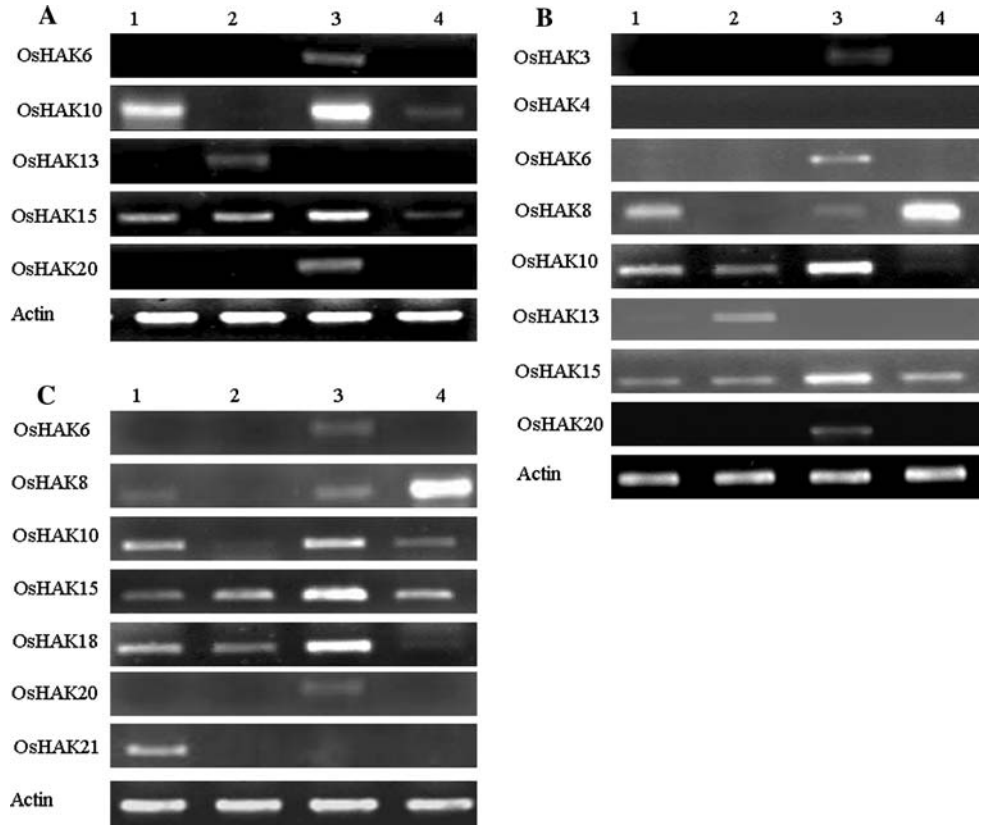

Fig. 7 RT-PCR results for four tissues of Minghui 63, Shanyou 63, and Zhenshan 97: (1) hull at 1 day before flowering; (2) flag leaf at 14 days after heading; (3) stamen at 1 day before flowering; (4) panicle at 4–5 cm young panicle stage. RT-PCR confirmed the reliability of CREP microarray data obtained for KT/HAK/KUP family genes in rice. (**a**) In Minghui 63, *OsHAK10* and *OsHAK15* show preferential expression in stamen 1 day before flowering when compared to all other tissues; *OsHAK6* and *OsHAK20* show the highest expression in stamen 1 day before flowering; *OsHAK13* shows the highest expression in flag leaf at 14 days after heading. (**b**) In Shanyou 63, *OsHAK15* show preferential expression in stamen 1 day before flowering when compared to all other tissues; *OsHAK3*, *OsHAK6*, *OsHAK10*, and *OsHAK20* show the highest expression in stamen at 1 day before flowering; *OsHAK8* shows the highest expression in panicle at 4–5 cm young panicle stage; *OsHAK13* shows the highest expression in flag leaf at 14 days after heading and *OsHAK4* do not show any expression in all the four tissues selected. (**c**) In Zhenshan 97, *OsHAK10* shows preferential expression in stamen at 1 day before flowering compared to all other tissues; *OsHAK6*, *OsHAK15*, *OsHAK18*, and *OsHAK20* show the highest expression in stamen at 1 day before flowering; *OsHAK8* shows the highest expression in panicle at 4–5 cm young panicle stage; *OsHAK21* shows the highest expression in hull at 1 day before flowering. RT-PCR was performed with the following cycling profile: 94°C for 3 min, followed by 28–35 cycles (depending on the expression levels of different genes; 28 cycles for *OsHAK15*; 35 cycles for *OsHAK8*, *OsHAK10*, *OsHAK13*, and *OsHAK18*; 38 cycles for *OsHAK3*, *OsHAK4*, *OsHAK6*, and *OsHAK21* were performed) at 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min. Rice *actin* cDNA was used as an internal control

under all three hormonal treatments (Fig. 9c). RT-PCR results matched very well with the DNA chip data (Fig. 10; Supplementary Table S5).

Discussion

KT/HAK/KUP potassium transporters family

In present study, 27 members belonging to the KT/HAK/KUP potassium transporter family in rice were identified, one more member than the previously identified 26 members (Amrutha et al. 2007), the newly identified gene being named *OsHAK27* (Table 2). This is the highest number of *KT/HAK/KUP* genes identified so far in a particular plant species, more than twice the number of *KT/HAK/KUP* genes in *Arabidopsis*. The high number of members of a gene family reflects a succession of expansion and rearrangement of the genome by extensive duplication and diversification that frequently occur during the course of evolution (Wang et al. 2007). The rice genome has undergone large-scale duplications, which has a great impact on the amplification of members of gene families in the genome. Recently, Yu et al. (2005) presented evidence for ongoing individual gene duplications in rice, which provides never-ending raw material for studying gene genesis and their functions. Twenty-seven *OsHAK* genes were located on eight rice chromosomes, and there were cases

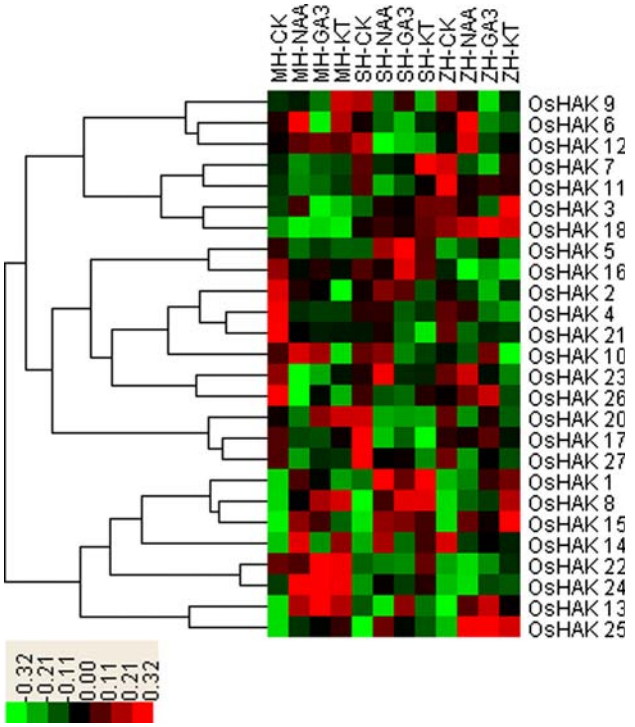

Fig. 8 Expression profile of *OsHAK* genes after NAA, GA_3, and KT treatments in three rice genotypes, Minghui 63, Shanyou 63, and Zhenshan 97. The color scale representing average signal values is shown at the *bottom*. MH-CK (Minghui 63, control), MH-NAA (Minghui 63, NAA), MH-GA_3 (Minghui 63, GA_3), MH-KT (Minghui 63, KT), SH-CK (Shanyou 63, control), SH-NAA (Shanyou 63, NAA), SH-GA_3 (Shanyou 63, GA_3), SH-KT (Shanyou, KT), ZH-CK (Zhenshan 97, control), ZH-NAA (Zhenshan 97, NAA), ZH-GA_3 (Zhenshan 97, GA_3) and ZH-KT (Zhenshan 97, KT)

where members of *OsHAK* genes were clustered together as closely related genes (Supplementary Fig. 3). These clusters suggest that these genes have evolved from regional duplications (e.g. *OsHAK19* and *OsHAK20*).

Implication of the *cis*-elements for functions of the *OsHAK* genes

cis-Elements play a key role in the regulation of gene expression. Through interaction with the corresponding *trans*-regulatory factors, the *cis*-elements can control the efficiency of the promoters and thus regulate the expression of the genes they control. Studies on *cis*-elements can provide a crucial foundation for further functional dissection of the rice *OsHAK* gene family. To date, there has not been report on the *cis*-elements of the *OsHAK* gene family members. In the current study, we found that upstream regions of most *OsHAK* gene family members contain Ca^{2+}-responsive as well as light and circadian regulatory *cis*-elements including ABRERATCAL, INRNTPSADB, PRECONSCRHSP70A, and CIACADIANLELHC. These *cis*-elements may play important roles in regulating expression of genes in response

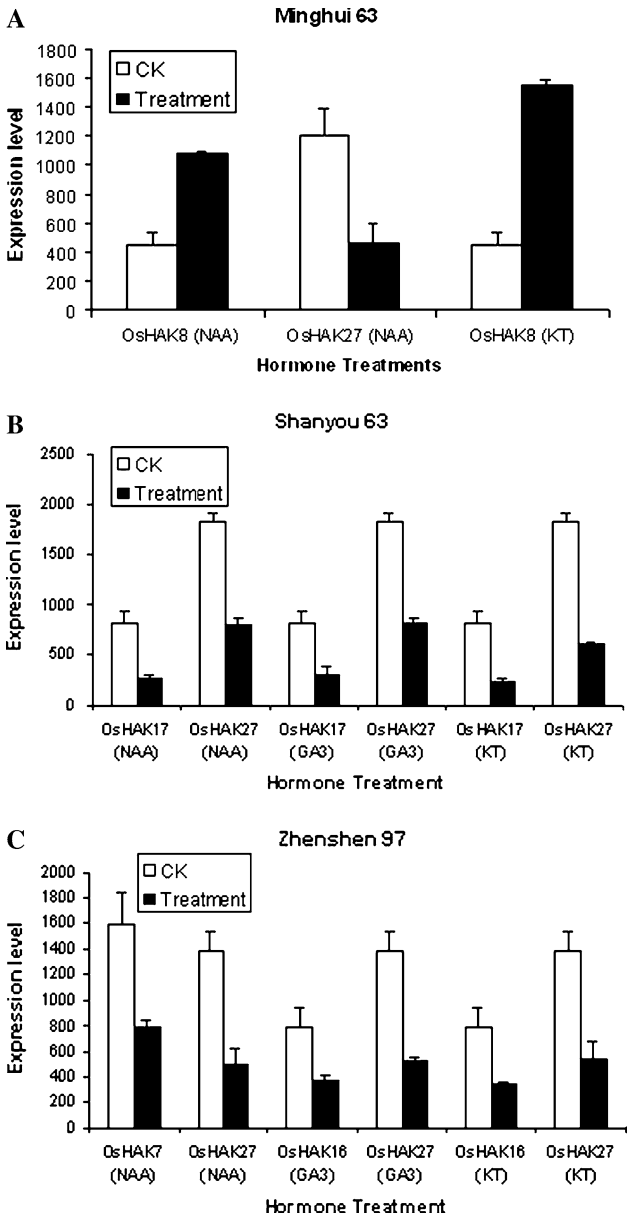

Fig. 9 Differentially upregulated or downregulated *OsHAK* genes by different hormone treatments (NAA, GA_3, or KT) in seedling at trefoil stage in three rice cultivars: (**a**) Minghui 63; (**b**) Shanyou 63; (**c**) Zhenshan 97. Differentially expressed genes under hormone stress were determined by performing Student's *t* test in each genotype separately. A gene is said to be differential, if it were upregulated or downregulated more than twofold with *P* value less than 0.05 when compared to control (CK)

to these signals. Among these *cis*-elements, the Ca^{2+}-responsive *cis*-elements, ABRERATCAL was one of the most widely distributed in the rice *OsHAK* genes. Thus, they may be associated with the key roles of Ca^{2+} as secondary messenger in plants in mediating responses to several environmental stresses that cause changes in activity of enzymes, expression of genes, and cell structure, which, together, help plants to adopt with the ever-changing environment. It was

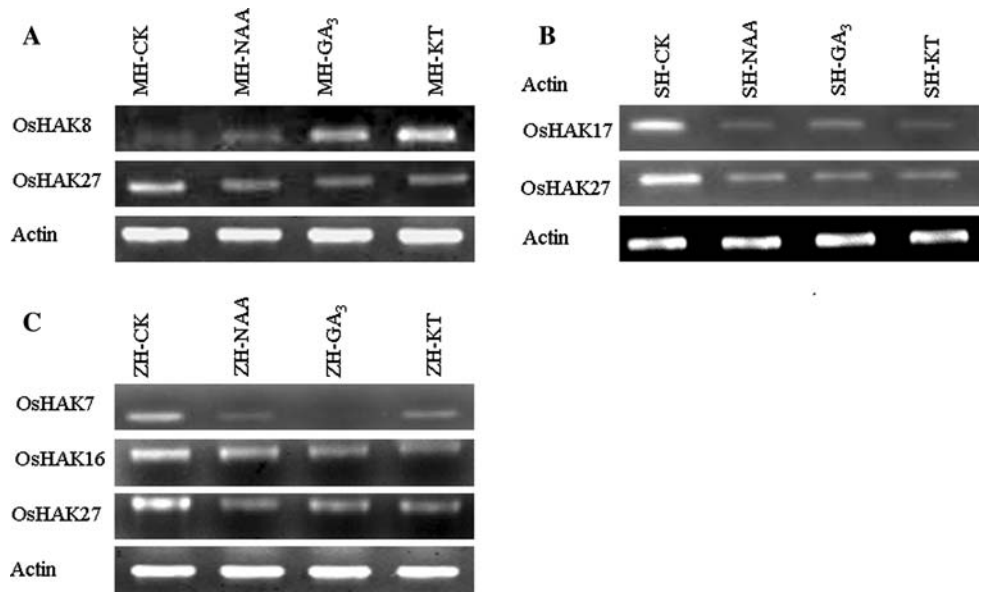

Fig. 10 RT-PCR analysis of differentially expressed genes under hormone treatments in (**a**) Minghui 63, (**b**) Shanyou 63, and (**c**) Zhenshan 97. Prior to RT-PCR, 100 μM of NAA, GA_3, or KT were sprayed on Minghui 63, Shanyou 63, and Zhenshan 97 seedlings at trefoil stage (CK, control). Rice *actin* cDNA was used as an internal control. MH-CK (Minghui 63, control), MH-NAA (Minghui 63, NAA), MH-GA_3 (Minghui 63, GA_3), MH-KT (Minghui 63, KT), SH-CK (Shanyou 63, control), SH-NAA (Shanyou 63, NAA), SH-GA_3 (Shanyou 63, GA_3), SH-KT (Shanyou, KT), ZH-CK (Zhenshan 97, control), ZH-NAA (Zhenshan 97, NAA), ZH-GA_3 (Zhenshan 97, GA_3) and ZH-KT (Zhenshan 97, KT). RT-PCR was performed with the following cycling profile: 94°C for 3 min, followed by 35 cycles for *OsHAK7* and *OsHAK8*, and 38 cycles for *OsHAK16*, *OsHAK17*, and *OsHAK21* at 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min. Rice *actin* cDNA was used as an internal control

also shown in several cases that Ca^{2+} signal is very important in the induction of gene expression by translating stress stimulus (Kaplan et al. 2006). Similar findings were observed in *Arabidopsis* among the members of KCO potassium channel family belonging to potassium transport system, where gene expression was activated by cytosolic Ca^{2+} (Czempinski et al. 1997). A Ca^{2+}-dependent deactivation of K^+ channels has also been described in mesophyll cells of *Vicia faba*, guard cells of maize, and the pulvini motor cells of *Mimosa pudica* (Fairley-Grenot and Assmann 1992; Li and Assmann 1993; Stoeckel and Takeda 1995). Thus, Ca^{2+} can activate or deactivate a gene by regulating its *cis*-elements. Similar to ABRERATCAL, two light-regulated elements, INRNTPSADB and PRECONSCRHSP70A, and a circadian-regulated element, CIACADIANLELHC, also exist in most of the rice *OsHAK* gene promoters. Fromm and Spanswick (1993), in their study on Willow (*Salix viminalis*), concluded that propagation of action potentials identified were a result of voltage- and time-dependent activities of K^+, Ca^{2+}, and Cl^- channels. Later, similar phenomenon was observed for irregular-shaped slow-wave potentials also, and these were induced by light/dark transitions (Herde et al. 1998; Stankovic and Davies 1997; Wagner et al. 1998). In this agreement, Deeken et al. (2000) also reported that potassium channel transcription in *Arabidopsis* is triggered by a local light-derived signal. In the legume *Samanea saman*, several Shakers and one KCO-2P were shown to display light and circadian control in motor cells, with large changes in transcript accumulation levels (Moshelion et al. 2002). The existence of these *cis*-elements in rice *OsHAK* genes and their likely roles in regulating gene expression suggest that *OsHAK* genes may participate in various stress responses in rice.

We also compared the cluster dendrograms obtained by average signal values from microarray in 27 rice tissues and using *cis*-elements (data not shown) of 27 *OsHAK* genes. No correlation was found between the *cis*-elements and the actual expression of genes. These deviations may arise for several reasons. First, transcription factors regulate genes by binding to *cis*-elements, and this control of gene expression depends upon factors such as tempo-spatial expression, modification, and activation by ligand binding, among others (Banerjee and Zhang 2002). Second, although we identified a large number of *cis*-elements using PLACE, there are still many *cis*-elements unidentified and missed (Higo et al. 1999). Finally, polymorphisms in *cis*-elements can result in a range of phenotypes, depending on the amount of activity that the affected motif contributes to the function of its element (Brown et al. 2007).

Preferential expression of *OsHAK* genes in stamen

Expression of genes in stamen, the male reproductive organ in plants, likely has vital functions for processes leading to

reproduction. Pollen swelling and anther dehiscence are the two most important processes leading to pollination and fertilization. In barley, the swelling of pollen occurs in a fraction of a second, and the presence of K^+ in the aperture area is responsible for rapid hydration of pollen (Rehman et al. 2004). The role of K^+ as an osmotic regulator in plants is well known (Fischer 1971; Heslop-Harrison and Heslop-Harrison 1996). K^+ is responsible for the turgidity or flaccidity of guard cells by regulating the entry or exit of water (Moore et al. 1995). Previous expression studies conducted in stamen identified genes involved in osmo-regulation that is consistent with features of stamen and/or pollen development, such as water movement associated with desiccation and dehiscence (Scott et al. 2004). The main characteristic of KT/HAK/KUP transporters is suggested to be K^+–H^+ symporters (Rodriguez-Navarro 2000). The proposed function for tonoplast KT/HAK/KUP transporter is to carry out active K^+ efflux from the vacuole to cytoplasm under K^+-limiting conditions (Walker et al. 1996), and these transporters were suggested to be involved in low-affinity K^+ uptake (Rodriguez-Navarro and Rubio 2006), while OsHAK10 is proposed to be a tonoplast transporter, possibly mediating low-affinity K^+ transport (Banuelos et al. 2002). Preferential expression of *OsHAK10* and *OsHAK15* in stamen at 1 day before flowering suggests that these genes may play important role during pollination and fertilization.

Expression analysis of *OsHAK* genes in root

Plant acquires all the mineral elements from the soil through roots. Thus, K^+ uptake from roots remains an important focus in many studies. In 1961, Epstein et al.'s pioneering work demonstrated that the potassium transport system in plants consisted of high- and low-affinity components (Epstein et al. 1961). High-affinity components work at micromolar concentration, while low-affinity components work at millimolar concentration. In current study, the high or detectable levels of expression by most of the *OsHAK* genes (14 genes) in root suggest that these genes are active in root tissues and may be involved in K^+ uptake or related functions.

In common ice (*Mesembryanthemum crystallinum*) plant, expression of two genes *McHAK1* and *McHAK4* were induced by salt stress, while *McHAK2* and *McHAK3* expressions were induced transiently under high salinity in roots (Su et al. 2002). Expression of *KT/HAK/KUP* genes also depends on developmental stages. For instance, in legume (*Lathyrus japonicus*), strong expression of *LjKUP* potassium transporter was observed in developing and growing root nodules (Desbrosses et al. 2004). It has been also demonstrated that the expression of the transcripts of KT/HAK/KUP family members in several plant species were upregulated under K^+ starve conditions (Santa-Maria et al. 1997; Kim et al. 1998; Wang et al. 2002; Ahn et al. 2004; Gierth et al. 2005; Qi et al. 2008).

Hormonal regulation of *OsHAK* genes

Plant hormones play important roles in growth and development. The commonly recognized classes of plant hormones are auxin, gibberellin, cytokinin, abscisic acid, and ethylene, among many others. Studies have shown that plant hormones regulate K^+ transport. Our findings also indicate upregulation or downregulation of transcripts of *OsHAK* genes after treatments of NAA (a member of the auxin family), GA_3 (a gibberellin), and KT (a cytokinin) in all the three rice genotypes. The first molecular studies of the hormonal regulation of K^+ transport (Van Steveninck 1976) came from findings that abscisic acid, cytokinin, and auxin strongly regulate the transcript levels of potassium channels (Philippar et al. 1999; Pilot et al. 2003). Expression of the inwardly rectifying *ZMK1* potassium channel in maize (Philippar et al. 1999) was shown to be regulated by auxin. Electrophysiological studies of maize coleoptile protoplasts showed that the transcript level of *ZMK1* was increased with the addition of auxin (Philippar et al. 1999; Thiel and Weise 1999). Together, these results suggest that the expression of KT/HAK/KUP family genes is regulated by hormones, indicating hormonal control of protein turnover in rice, which may be critical for several hormone-dependent cellular processes.

In conclusion, the KT/HAK/KUP family is important for plant growth and development. A large number of KT/HAK/KUP family genes have been predicted in rice and *Arabidopsis*. Studies have been reported about the role of the KT/HAK/KUP family genes in plants. However, functions of KT/HAK/KUP family genes in rice have not been elucidated. This study provides important clues to gain insight about the functions of *KT/HAK/KUP* genes in rice. For example, two *KT/HAK/KUP* genes (*OsHAK10* and *OsHAK15*) showed preferential expression in stamen at 1 day before flowering stage. In addition, expressions of several *KT/HAK/KUP* genes were differentially regulated under hormone treatments conditions. *cis*-Element analysis also suggested the regulation of *KT/HAK/KUP* genes under Ca^{2+}-, light- and circadian-responsive conditions. However, future research using, for example, RNAi strategy or insertion mutagenesis is required to elucidate the precise role of individual *KT/HAK/KUP* gene in rice.

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