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ORIGINAL ARTICLE

Foxtail millet SiHAK1 excites extreme high-affinity K^+ uptake to maintain K^+ homeostasis under low K^+ or salt stress

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Abstract

Key message **This is the first evidence that SiHAK1 acts as a K^+ transporter and is modulated by internal and external K^+, which expands our understanding of the significant physiological roles of large HAK/KUP/KT transporters in crops.**

Abstract Crop genomes have shown the richness of K^+ transporters in HAK/KUP/KT (High Affinity K^+/K^+ Uptake Proteins/K^+ Transporter) family, and much progress have been achieved toward understanding the diverse roles of K^+ uptake and translocation, and abiotic stresses resistance in this family. The HAK/KUP/KT family has increasingly been recognized to be at a pivotal status in the mediation of K^+ translocation and long-term transport; however, our understanding of the molecular mechanisms remains limited. Foxtail millet is an ideal plant for studying long-distance potassium (K) transport because of its small diploid genome and better adaptability to arid lands. Here, we identified 29 putative HAK/KUP/KT proteins from the *Setaria italica* genome database. These genes were distributed in seven chromosomes of foxtail millet and divided into five clusters. *SiHAK1* exhibited widespread expression in various tissues and significant up-regulation in the shoots under low K condition. SiHAK1 was localized in the cell membrane and low K elicited SiHAK1-meidated high-affinity K^+ uptake activity in Cy162 yeast cells and *Arabidopsis athak5* mutants. The transport activity of SiHAK1 was coordinately modulated by external K^+ supply and internal K^+ content in the cell under low K and high salt environment. Our findings reveal the K uptake mechanisms of SiHAK1 and indicated that it may be involved in the mediation of K homeostasis in *S. italica* under K^+-deficiency and salt stress.

Keywords *Setaria italica* · HAK/KUP/KT transporter · High-affinity K^+ transporter · K^+ homeostasis · Salt tolerance

Introduction

Potassium (K) is an essential nutrient for plants and is the primary cation in the cell cytoplasm, playing a significant role in many basic physiological functions, such as the maintenance of membrane electrical balance, osmoregulation, and abiotic stress adaptation, as well as acting as an activator of a number of enzymes (Anschütz et al. 2014). However, large agricultural areas of the world are reported to be deficient in K supply, including 3/4 of paddy soils in China. Soils with low K are often sandy, waterlogged, saline, or acidic, and K has become a limiting element for crop production (Goulding and Loveland 1986). In K-deficient crops, photosynthesis is impaired, and sugars accumulate in the leaves, which affects both the yield and quality. Otherwise, high K concentrations in crops are often considered as an "insurance strategy" to enable the plant to better survive in environmental stresses (Zörb et al. 2014). Therefore, the

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plant K^+ transport system, particularly transporters mediating K^+ uptake across a wide spectrum of external K concentrations, functions irreplaceable status of ion homeostasis.

The HAK/KUP/KT (high affinity K^+/K^+ uptake proteins/K^+ transporter) transporters have been wildly accepted as K^+ transporters across membranes in bacteria, fungi, and plants, but not in animals. The genes of this family are present in all plant genomes, and their ubiquitous distribution in plants differs from the other kingdoms which reflects the important roles for the life of plant (Véry et al. 2014). However, our understanding of this family is very fragmentary due to the limitations in electrophysiological experiments, which prevent detailed biophysical analyses. Many well-characterized transporters belong to Cluster I, owning the same feature that highly up-regulated in the roots upon K^+ starvation and involved in high-affinity K^+ uptake from the soil under low K^+ condition (< 1 mM), including HvHAK1, AtHAK5, OsHAK1, OsHAK5, ThHAK1, SlHAK5, CaHAK1, and CcHAK1 (Chen et al. 2015; Alemán et al. 2009; Gierth et al. 2005; Martínez-Cordero et al. 2004; Nieves-Cordones et al. 2007; Ruiz-Lau et al. 2016; Santa-María et al. 1997; Yang et al. 2014). Crop genomes have revealed the richness of HAK/KUP/KT family compared with dicotyledonous species, such as *Arabidopsis*, especially in the Cluster I subfamily (Nieves-Cordones et al. 2016). Many recent advances in the Cluster I proteins of rice have been reported and have revealed the significance of the HAK/KUP/KT family in crops, particularly in abiotic stress adaptation. OsHAK1 contributes about 50–55% K^+ uptake under K^+-starved condition (0.05–0.1 mM) and critically mediates drought and salt resistance (Chen et al. 2015, 2017). OsHAK1, OsHAK5, and OsHAK21 coordinately maintain Na^+/K^+ homeostasis under salt stress as well as the membrane potential of root cells (Chen et al. 2015; Shen et al. 2015; Yang et al. 2014). Some HAK/KUP/KT proteins also function at high K^+ levels, such as KUP2, KUP3, KUP4 in *Arabidopsis*, and HvHAK2 in barley (Quintero and Blatt 1997; Rubio et al. 2000; Senn et al. 2001). Sensing changes in external K^+ supply or internal K^+ concentration is a key step for the regulation of K^+ homeostasis in plants (Adams and Shin 2014). However, no particular HAKKUP/KT type transporter has been found to dynamically coordinate K^+ supply and internal K^+ content in cells for the mediation of K^+ hemostasis, particularly under abiotic stresses.

The increase in published plant genomes has facilitated greater interest in studying functional genes in plant species other than *Arabidopsis* and rice. Foxtail millet (*Setaria italica* L.), an important gramineous cereal food crop with good nutritional value, was originated in Northern China and has been cultivated over 8000 years (Diao et al. 2014; Doust et al. 2009). It is being promoted as a novel species for functional gene studies because of its small diploid genome, short life cycle, self-pollination, small adult stature, and prolific seed production (Bennetzen et al. 2012; Jia et al. 2013). Furthermore, foxtail millet is also an ideal model plant for studying stress-tolerance mechanisms, as it is more adapted to barren and arid lands than other typical cereal crops, particularly with regards to efficient nutrient absorption and utilization in these environments.

In this study, we initially identified 29 putative HAK/KUP/KT family genes from the *S. italica* genome database and well characterized one member of this family. We discovered that SiHAK1 facilitates extreme high-affinity K^+ uptake activity under low K^+ condition and salt stress. Interestingly, the transport activity was regulated by external K^+ supply and internal K^+ content. Our results significantly uncovered a new mechanism in the HAK/KUP/KT family and provide potential insights into molecular breeding applications in other crops.

Materials and methods

Isolation of HAK/KUP/KT genes from the *S. italica* genome database

Protein sequences of HAK/KUP/KT family genes in *Arabidopsis* and rice were obtained from the NCBI website. These sequences were used as a BLAST query in the *S. italica* genome to identify putative homologues. Nucleic acid and amino acid sequences of the identified putative HAK/KUP/KT members in *S. italica* were obtained from https://phytozome.jgi.doe.gov/pz/portal.html. A phylogenetic tree was constructed in MEGA5.0 software using candidate HAK/KUP/KT proteins in *S. italica* and identified members in *Arabidopsis* and rice.

Analysis of the chromosomal location, gene structure, and expression patterns of HAK/KUP/KT genes in *S. italica*

The HAK/KUP/KT genes in *S. italica* were BLASTed in the foxtail millet genome in Phytozome to retrieve the respective genes, transcripts, and coding sequences (CDS). Furthermore, chromosomal locations, including the chromosome number, position of the gene start, and end, and the gene orientation, were also identified. The HAK/KUP/KT genes were then plotted into the respective chromosomes of foxtail millet in an ascending order of physical position (bp), from the short arm telomere to the long arm telomere. A physical map was constructed using previously described methods (Yang et al. 2009). Gene Structure Display Server (http://gsds.cbi.pku.edu.cn) was used to predict the exon–intron positioning of the genes. The expression data were downloaded from *S. italica* genome database, and a heatmap was constructed using online software (http://www.biocloud.net).

Plant material and growth conditions

Seeds of foxtail millet (*S. italica* cv. Yugu18) were used in this study. The seeds were grown in slots filled with Hoagland's nutrient solution. The plants were grown in a green chamber with 24 °C with a 16 h/8 h (day/night) photoperiod and 60–70% relative humidity.

Seeds of *Arabidopsis* were plated on MP (medium lacking potassium using for plant culture) medium with 0.5% sucrose and 0.8% agar for a week at 21–22 °C with a 16 h/8 h (day/night) photoperiod and 60–70% relative humidity.

HAK/KUP/KT genes isolation from *S. italica* and expression pattern analysis

Total RNA of Yugu18 was isolated from the roots and shoots of 10-day-old seedlings grown on soil using TRIzol reagent, following which first-strand cDNA was prepared with PrimeScript™ RT Master Mix (Takara, Shiga, Japan). The full-length *SiHAK1* and *SiHAK2* genes were amplified by PCR using specific primers. The primers used are listed in Supplemental Table 1. To evaluate the expression patterns under abiotic stresses, RNA samples were collected from foxtail millet grown in slots filled with Hoagland's nutrient solution for 10 days as a control, and then transferred to MP medium (–K) or Hoagland's buffer with 350 mM sodium chloride (NaCl) or with 10% polyethylene glycol (PEG) 6000 for 6 h. Real-time PCR was performed using a Bio-Rad CFX96 (Hercules, CA, USA). Foxtail millet 18S was used as the endogenous reference. The primers used are listed in Supplemental Table 1.

SiHAK1 over-expression transgenic lines in *Arabidopsis athak5*

SiHAK1 was constructed into the vector pBASTA, with green fluorescent protein (GFP) expression driven by the CaMV 35S promoter present in the parent plasmid pBI121 (Cutler et al. 2000) using Gateway technology, and then transformed into *Arabidopsis athak5* plants using the floral dip method. Transgenic T2 seeds were used for the phenotype analysis. Seeds from the wild type, *athak5*, and two different transgenic lines overexpressing *SiHAK1* on an *athak5* background (*SiHAK1-1* and *SiHAK1-2*) were planted in a seed germination pouch with liquid MP medium containing different concentrations of potassium chloride (KCl) (Hirsch et al. 1998). The seed germination pouches were placed vertically and photographed after 6 days.

Subcellular localization of SiHAK1

The *SiHAK1* over-expression transgenic T1 generation seeds of *athak5* were germinated and grown for 4 days at 21–22 °C under dark condition. The leaf epidermal cells of the etiolated seedlings were used for microscopic observation. FM-4-64 was used to stain the cell plasma membrane. These 4-day seedlings were immersed in 20 ug/mL FM-4-64 buffer for 1 min and then washed with water twice. Confocal images were captured using Zeiss Live 5 equipment. The fluorescence signals were excited at 488 nm for GFP and 561 for the FM-4-64 dye.

Functional complementation of SiHAK1 in Cy162 Yeast strain

SiHAK1 and *SiHAK2* were amplified with special primers and digested with the BglII/EcorI or XbaI/Ecor I restriction enzymes, respectively, and ligated into the yeast expression vector pYPGE15 (Brunelli and Pall 1993). For functional complementation experiments, pYPGE15, pYPGE15-*SiHAK1*, pYPGE15-*SiHAK2*, pYPGE15-*HvHAK1*, pYPGE15-*AtHAK5*, and pYPGE15-*OsHAK5* were transformed into yeast (*Saccharomyces cerevisiae*) strain CY162 *trk1*△*trk2*△ (Anderson et al. 1992) and B31 *ena1-4*△ *nha1*△ (Bañuelos et al. 1998).

Yeast complementation assays at low K^+ were performed in solid AP-U (arginine phosphate medium lacking Uracil) medium, as described previously (Mangano et al. 2008), and supplemented with concentrations of K^+ ranging from 0.1 to 10 mM, and in the absence or presence of various concentrations of NaCl (50–750 mM). For the growth curve, the yeast strains (transformed with pYPGE15, pYPGE15-*SiHAK1*, and pYPEG15-*SiHAK2*) were grown in liquid SD-U (minimal Synthetic Defined base with -Ura dropout supplement) medium at 30 °C overnight and then transferred to liquid AP-U medium supplemented with different concentrations of K^+ (0.05 or 5 mM) with the same initial OD_{600} of about 0.1. The shaker was adjusted to 200 rpm, and the OD_{600} of the strains was measured every 3 h for three consecutive days of growth. This experiment was repeated in triplicate.

K^+ depletion and K^+ (Na^+) content measurements in the Cy162 yeast strain

For K^+ depletion experiments, yeast cells were grown overnight at 30 °C in liquid SD-U medium and then then transferred to liquid AP-U medium for about 4 h for K starvation. The cells were then suspended in 10 mM 2-(N-morpholino) ethanesulfonic acid (MES) supplemented with 2% glucose and adjusted to pH 6 with $Ca(OH)_2$. At time zero, the indicated concentrations of KCl were added to the medium and the samples were collected at intervals over a 2-h period.

For the measurement of K^+ (Na^+) content in the yeast strains, cells were grown at 30 °C on AP-U medium with various external K^+ (0.1–10 mM K^+) or Na^+ (50–750 mM) concentrations. The cells were suspended in pre-cooled

sterile water, adjusted to OD_{600} 0.3, and then repeatedly heated and frozen. K^+ was identified and quantified by atomic emission spectrophotometry using a Perkin-Elmer Model 2380 spectrophotometer (Fraile-Escanciano et al. 2010).

Results

Identification of homologous HAK/KUP/KT genes in *S. italica*

To explore the HAK/KUP/KT family genes in *S. italica*, BLAST was performed in the Phytozome genome database using the HAK/KUP/KT members of 27 proteins in rice and 12 proteins in *Arabidopsis* (Ahn et al. 2004; Bañuelos et al. 2002). Ultimately, 29 genes were identified as putative HAK/KUP/KT family members in *S. italica*, and were accordingly named *SiHAK1-29* based on the rice HAK/KUP/KT genes (Table 1). The protein lengths of the HAK/KUP/KT transporters in *S. italica* ranged from 711 aa to 894 aa and were predicted to contain 10–14 trans-membrane regions. The TargetP (http://www.cbs.dtu.dk/services/TargetP/) tool predicted that most of these proteins were localized in the plasma membrane.

To analyze the phylogenetic relationships, a phylogenetic tree was constructed based on the alignment of the 29 proteins in *S. italica* and the HAK/KUP/KT proteins in *Arabidopsis* and rice. A recent study identified a total of 913 HAK/KUP/KT sequences in 46 plant genomes, and these genes can be clearly divided into 5 major groups (Nieves-Cordones et al. 2016). The 29 HAK/KUP/KT members contained all the 5 clusters, 11 in Cluster I, 9 in Cluster II, 3 in Cluster III, 3 in Cluter IV, and 3 in cluster V (Fig. 1a). Of all the crop genomes, *S. italic*a contains the highest numbers of proteins in Cluster I while in rice there are eight, in maize there are nine, and in barley there are five (Nieves-Cordones et al. 2016).

Physical mapping of the identified HAK/KUP/KT family genes onto the *S. italica* genome illustrated the distribution of these genes across the chromosomes (Fig. 1b). These genes were present in chromosomes 1, 2, 4, 5, 6, 7, and 9 with a maximum of seven HAK/KUP/KT genes on chromosome 2 and 7. *SiHAK22* could not be mapped due to the lack of complete annotation of *Setaria italica* genome sequence. Tandem duplication events contributed to two of these HAK/KUP/KT proteins, *SiHAK1, SiHAK19, SiHAK28*, and *SiHAK29* were located in one tandem repeat, while *SiHAK16, SiHAK4*, and *SiHAK23* were located in the other. Interestingly, the two more HAK/KUP/KT proteins in *Setaria italica* than rice, namely *SiHAK28* and *SiHAK29*, constituted expansion genes of *SiHAK1* generated via tandem repeat events and were highly similar in terms of sequence and gene structure (Figs. 1b, 2a). Gene structure analysis showed the variable distribution of introns and exons in the HAK/KUP/KT genes (Fig. 2a). The results showed that there were no changes in exon/intron structure in each group of tandem repeat genes, though individual introns in different genes varied obviously in length. Through comparisons, these genes in the tandem repeats represented two typical exon/intron structures of HAK/KUP/KT genes, which indicated that tandem and segmental duplication events dominated the expansion of HAK genes in *S. italica* (Figs. 1b, 2a).

Table 1 The list of 29 identified HAK/KUP/KT proteins in *Setaria italica*

Gene	Protein length	Protein ID	TMS[a]	PL[b]
SiHAK1	774	Si009421m.g	12	Plasma membrane
SiHAK2	784	Si028991m.g	13	Plasma membrane
SiHAK3	774	Si006705m.g	12	Mitochondrial membrane
SiHAK4	801	Si034309m.g	12	Plasma membrane
SiHAK5	775	Si000392m.g	11	Plasma membrane
SiHAK6	735	Si004956m.g	10	Plasma membrane
SiHAK7	787	Si000375m.g	12	Mitochondrial membrane
SiHAK8	805	Si034304m.g	12	Plasma membrane
SiHAK9	788	Si028982m.g	12	Plasma membrane
SiHAK10	816	Si005871m.g	10	Plasma membrane
SiHAK11	804	Si009378m.g	13	Plasma membrane
SiHAK12	789	Si015735m.g	14	Plasma membrane
SiHAK13	856	Si028913m.g	12	Plasma membrane
SiHAK14	853	Si028916m.g	12	Plasma membrane
SiHAK15	850	Si009581m.g	12	Plasma membrane
SiHAK16	811	Si034290m.g	11	Plasma membrane
SiHAK17	711	Si029077m.g	11	Mitochondrial membrane
SiHAK18	788	Si028983m.g	14	Plasma membrane
SiHAK19	777	Si012185m.g	12	Plasma membrane
SiHAK20	731	Si020215m.g	12	Plasma membrane
SiHAK21	805	Si034300m.g	12	Plasma membrane
SiHAK22	894	Si020681m.g	11	Plasma membrane
SiHAK23	805	Si034303m.g	11	Plasma membrane
SiHAK24	777	Si005895m.g	13	Plasma membrane
SiHAK25	774	Si016378m.g	13	Mitochondrial membrane
SiHAK26	740	Si015728m.g	11	Plasma membrane
SiHAK27	814	Si004208m.g	10	Plasma membrane
SiHAK28	791	Si012684m.g	11	Plasma membrane
SiHAK29	762	Si009457m.g	10	Plasma membrane

[a]TMS: number of trans-membrane segments posses

[b]Localization of SiHAKs protein supported by Target (http://www.cbs.dtu.dk/services/TargetP/)

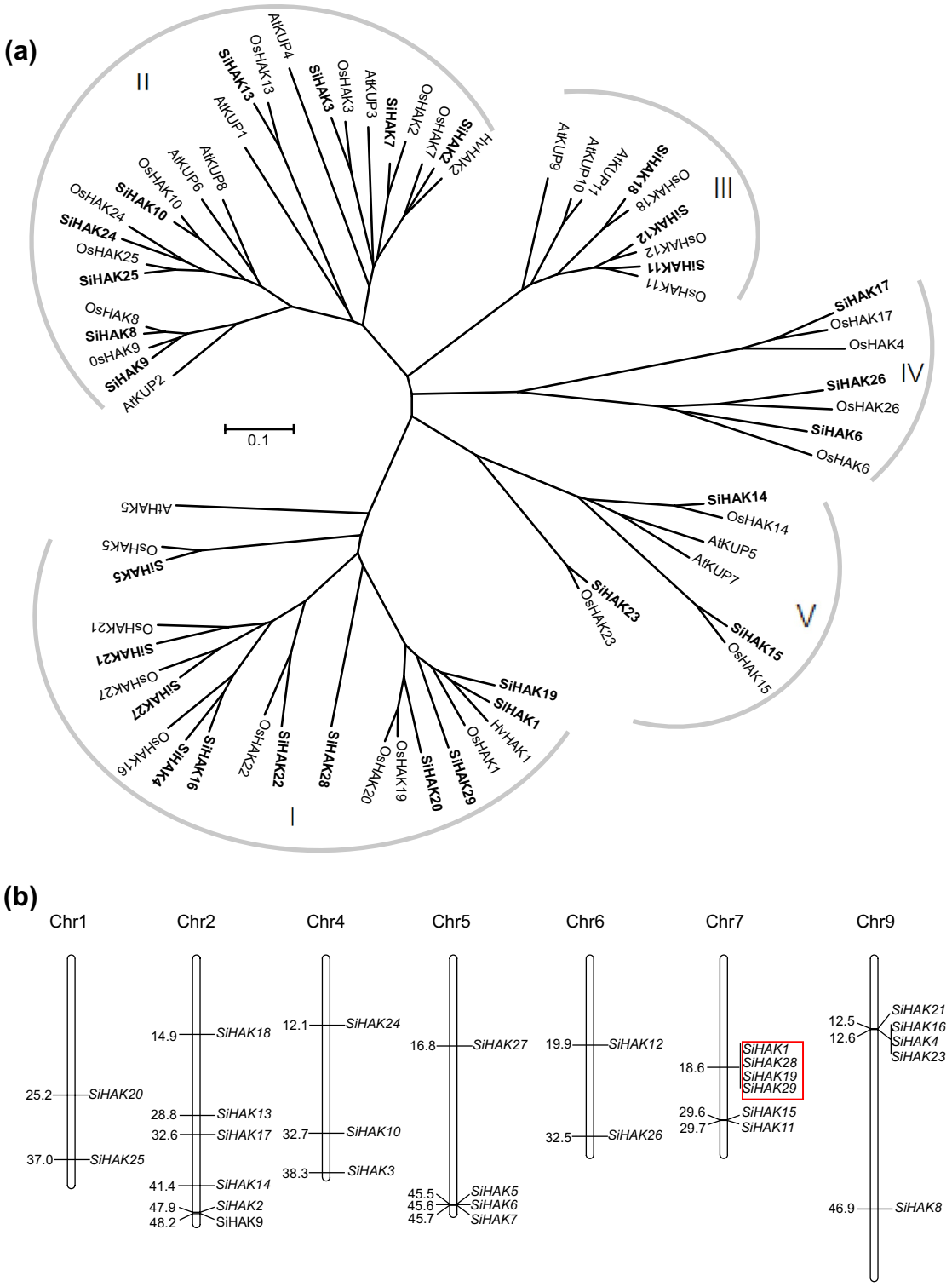
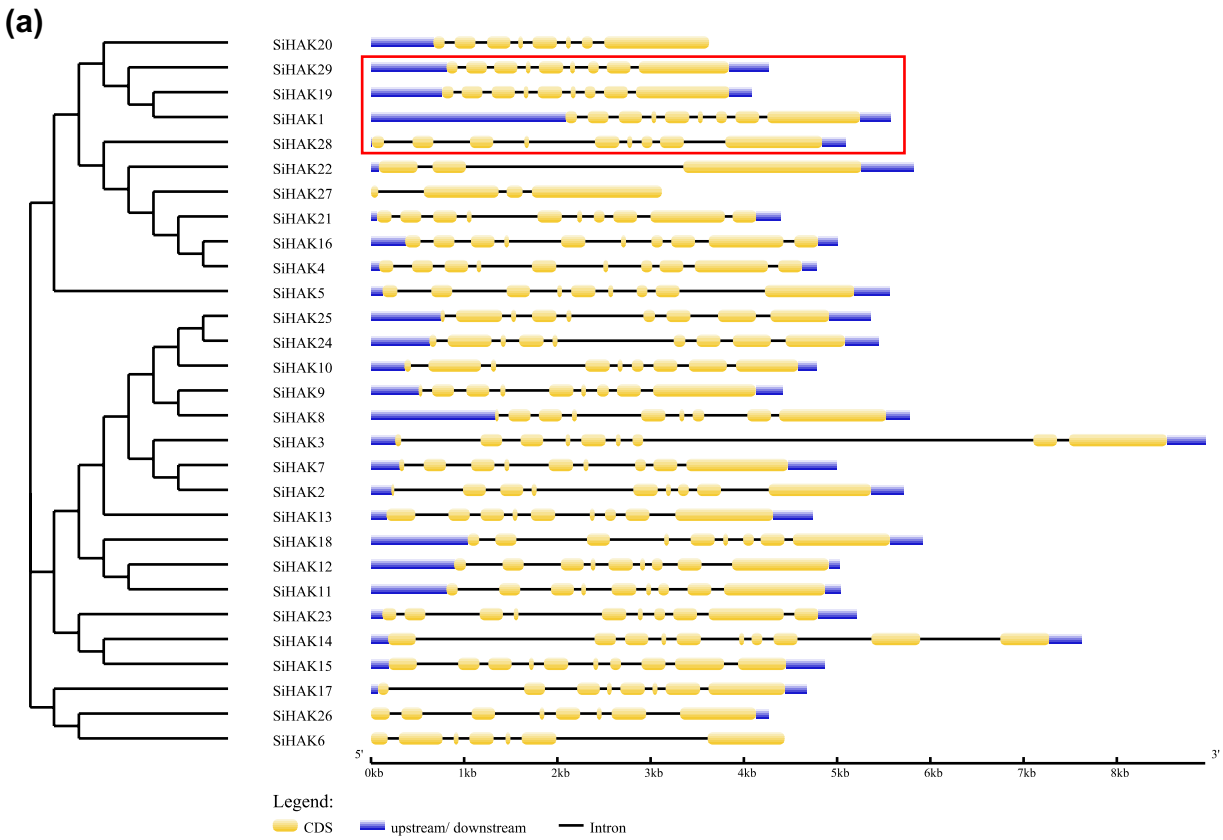
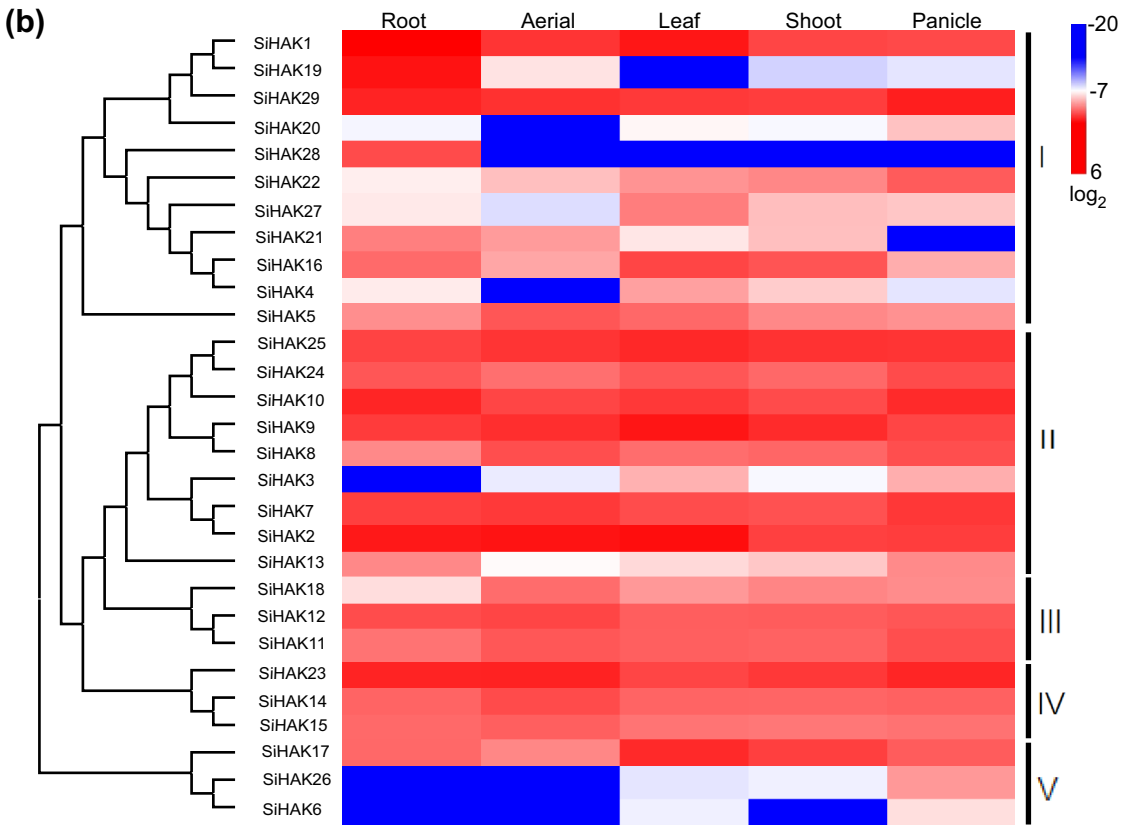


Fig. 1 Identification of 29 HAK/KUP/KT proteins in the *S. italica* genome. **a** Phylogenetic relationships between HAK/KUP/KT proteins in *S. italica* and model plants. The phylogenetic tree was generated with MEGA5.0 software using the Neighbor-Joining method. The scale indicates the genetic distance. *S. italica* transporters are shown in black letters. **b** A physical map of HAK/KUP/KT transporters on the chromosomes of *S. italica* genome. The number on the right side of the bar donates the identified genes of the HAK/KUP/KT transporters and the left side indicates the physical position of the map in mega base pairs (Mb). The SiHAK1 tandem duplicated genes are highlighted in a red box. (Color figure online)


(a)
SiHAK20
SiHAK29
SiHAK19
SiHAK1
SiHAK28
SiHAK22
SiHAK27
SiHAK21
SiHAK16
SiHAK4
SiHAK5
SiHAK25
SiHAK24
SiHAK10
SiHAK9
SiHAK8
SiHAK3
SiHAK7
SiHAK2
SiHAK13
SiHAK18
SiHAK12
SiHAK11
SiHAK23
SiHAK14
SiHAK15
SiHAK17
SiHAK26
SiHAK6
5'
3'
0kb
1kb
2kb
3kb
4kb
5kb
6kb
7kb
8kb
Legend:
CDS
upstream/ downstream
Intron


(b)
Root
Aerial
Leaf
Shoot
Panicle
SiHAK1
SiHAK19
SiHAK29
SiHAK20
SiHAK28
SiHAK22
SiHAK27
SiHAK21
SiHAK16
SiHAK4
SiHAK5
SiHAK25
SiHAK24
SiHAK10
SiHAK9
SiHAK8
SiHAK3
SiHAK7
SiHAK2
SiHAK13
SiHAK18
SiHAK12
SiHAK11
SiHAK23
SiHAK14
SiHAK15
SiHAK17
SiHAK26
SiHAK6
I
II
III
IV
V
-20
-7
6
log2

◂**Fig. 2** The gene structures and expression patterns of HAK/KUP/KT genes. **a** The phylogenetic tree was constructed with HAK/KUP/KT proteins in foxtail millet (left). The intron–exon positions of the respective members are shown on the right. The four tandem duplicated genes, including *SiHAK1*, are highlighted with a red box. **b** The phylogenetic tree was constructed with HAK/KUP/KT proteins in foxtail millet on the left. Transcription overview of expression pattern in all *Setaria italica* HAK/KUP/KT proteins with a $\log_2$-fold change on the right. (Color figure online)

The gene expression data of these 29 proteins in different tissues under normal growth conditions were downloaded from the Phytozome website. As shown in Fig. 2b, most of the genes in Cluster I, except *SiHAK1* and *SiHAK29*, were lowly expressed which may be a prerequisite for induction. The Cluster II genes in *S. italica* were relatively highly expressed, with extensive expression observed in all tissues. Cluster II proteins are widely believed to function under high K^+ condition and do not respond to low K^+ stimuli, such as HvHAK2 (Senn et al. 2001). As only few studies have focused on Clusters III, IV, and V, we thus cannot conclude any useful information from this expression pattern overview.

SiHAK1 isolation and expression pattern analysis

Large amounts of required K^+ are taken up from the soil and upwardly translocated for redistribution in the different tissues. About 40–90% of root-acquired K^+ will be retranslocated and cycled (Lu et al. 2005). Interestingly, the expression database (Fig. 2b) showed widespread expression patterns in the Cluster I genes of HAK/KUP/KT family, especially *SiHAK1* and *SiHAK29*. We designed specific primers to isolate the *SiHAK* genes from the extracted RNA of 10-d-old Yugu18 seedlings. Ultimately, *SiHAK1* and *SiHAK2* were isolated through sequencing. In addition, *SiHAK1* could also be obtained from the roots under normal conditions, which is in accordance with the expression patterns in the *S. italica* database (Supplemental Fig. S1). Real-time PCR assays were performed to investigate the induced expression patterns in the shoots of *SiHAK1* under various abiotic stresses. The results revealed that *SiHAK1* expression was obviously enhanced by low K^+ conditions (about 12 times) and slightly up-regulated by PEG and salt treatment (about 3–4 times). However, the expression of *SiHAK2* showed less sensitivity to low K stimuli (about three times) and no response to PEG and salt (Fig. 3a).

As the subcellular localization of a transporter is critical for its functioning, we examined subcellular localization using a GFP-fusion approach. We developed the *SiHAK1::GFP* fusion construct under the control of the 35S promoter and transformed it into *Arabidopsis athak5* mutant. The transcription of *SiHAK1* in the transgenic lines was detected in the *athak5* mutant (Fig. 3b). We observed GFP fluorescence on the epidermal cells of leaves of 4-day-old seedings of the SiHAK1::GFP transgenic lines and found that the fluorescence was particularly strong on the plasma membrane and also coincided well with FM4-64 (Fig. 3c).

SiHAK1 is a high-affinity K^+ transporter and is modulated by K supply and internal K content

To study the function of the SiHAK1 transporter, we introduced *SiHAK1* and *SiHAK2* into the CY162 *trk1*△*trk2*△ yeast strain, which is a perfect system for studying the function of HAK/KUP/KT proteins (Anderson et al. 1992). Drop serial dilutions of each strain were cultured on agar plates in AP-U medium with various K^+ added and images were captured after 4 days of growth. Only the *SiHAK1* expression strains could restore the growth of Cy162 under low K^+ condition (< 1 mM), while the growth of the *SiHAK1, SiHAK2*, and empty vector transformants were similar in the presence of 1 mM K^+ (Fig. 4a). Interestingly, when the external K^+ content increased to 5 mM, Cy162-*SiHAK2* demonstrated perfect growth compared with the empty vector transformants, which indicated that SiHAK2 facilitated K^+ uptake in a high K environment. However, the *SiHAK1* transformants did not demonstrate better growth with increasing K^+ content compared with the empty vector transformants, but maintained a similar growth trend with low K supply (Fig. 4a). Growth curves of the cells in liquid AP-U medium at different concentrations of K^+ further demonstrated the growth capacity of the *SiHAK1* and *SiHAK2* transformants. At 50 μM K^+, only the strain expressing *SiHAK1* grew, while at 5 mM K^+, the strain expressing *SiHAK2* exhibited strong growth compared with the empty vector transformants, whereas the *SiHAK1* strains exhibited low growth capacity with the OD_{600} only reaching 1.5 (Fig. 4b). To further assess the relationship between Cy162 growth and K^+ uptake, the K^+ content in these yeast strains cultured in different K conditions was measured. We found that the cells expressing *SiHAK1* maintained a stable K^+ concentration under AP medium with the addition of 100, 300, 600 μM, 1, 5, 10, or 50 mM external K^+, even exhibited efficient K^+ uptake under extremely low K^+ conditions (AP medium without K^+ added). In contrast, the growth of *SiHAK2* or the empty vector transformants gradually displayed improved growth as the external K^+ content increased (Fig. 4c). In the kinetic study of the medium K^+ depletion, Cy162 transformed with *SiHAK1* yeast could deplete the external K^+ in the medium (35 uM), while no such depletion was observed in the Cy162-*SiHAK2* strains and empty vector strain (Fig. 4d). All the yeast results revealed that SiHAK1 confers K^+ transportation. Particularly, except for its regulation by external K^+ content, SiHAK1 can also be modulated by internal K in the cell and strictly controls the K^+ content in cells.

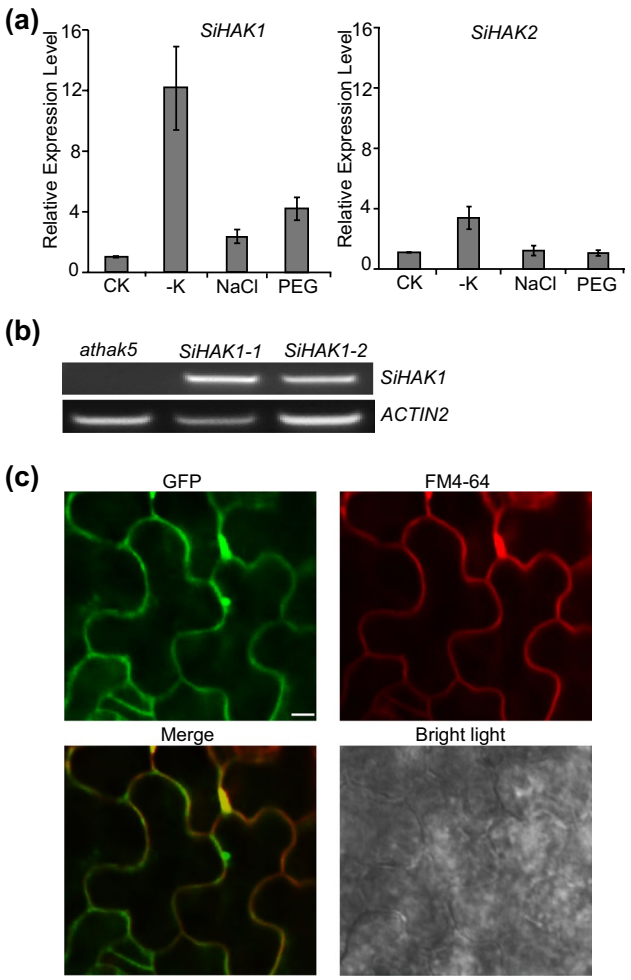


Fig. 3 Induced expression pattern of *SiHAK1* in the shoots and the subcellular localization of SiHAK1. **a** Real-time PCR analysis of the mRNA level of *SiHAK1* and *SiHAK2* in the shoot tissue under different abiotic stresses. The RNA samples were extracted from 10-day-old of seedlings that had been cultured in Hoogland's medium (CK), then transferred to MP (–K), 350 mM NaCl (NaCl), or 10% PEG6000 (PEG) solution for 6 h. **b** RT-PCR analysis of the *SiHAK1* mRNA level in *athak5* and two *SiHAK1* over-expression transgenic lines (*SiHAK1-1, SiHAK1-2*). The total RNA samples were extracted from the roots of 7-day-old *Arabidopsis* seedlings growing on MP medium. **c** Leaf epidermal cells in transgenic *Arabidopsis* plants expressing *35S-SiHAK1*::GFP. In the images, the GFP signal (green) is shown on the top left; the plasma membrane stained with FM4-64 (red) is on the top right; a merge (of green and red) is on the bottom left; the bright field microscope images is shown on the bottom right. Scale bar = 20 μm. (Color figure online)

To further examine the K^+-dependent growth phenotype on SiHAK1, we generated *SiHAK1* over-expression transgenic lines in the *Arabidopsis athak5* mutant (Rubio et al. 2008) under the control of CaMV *35S* promoter. We selected two homozygous lines to assess the growth of plants subjected to low K^+ condition compared with the wild type and *athak5*. When grown at extremely low K^+ condition (only MP medium), the *athak5* mutant plants exhibited severe growth defects, and the expression of *SiHAK1* obviously rescued the phenotype of *athak5* and even showed stronger growth compared with the wild type (Fig. 5a). The phenotype of *athak5* gradually became indistinguishable from the wild type with increasing K^+ content. The root length and fresh weight of *athak5* were significantly reduced under 0.1 mM K^+ content in the medium compared with the wild type, however, the *SiHAK1* over-expression lines exhibited a rescued *athak5* phenotype (Fig. 5b, c). This result further confirmed that SiHAK1 acts as a high-affinity K^+ uptake transporter under extremely low K^+ condition.

SiHAK1 exhibits extreme Na^+ insensitivity and high-affinity K^+ uptake activity under salt treatment

We found that the growth of Cy162-*SiHAK1* and Cy162-*SiHAK2* was inhibited when NH_4^+ and Cs^+ were added (Supplemental Fig. 2). However, the growth of the *SiHAK1* transformants was insensitive to Na^+, and these transformants were even capable of growing in an external 750 mM NaCl environment at 100 μM K^+ (Fig. 6a). Interestingly, under 10 mM K^+, the growth of *SiHAK2* and the empty vector transformants strains showed inhibited growth under high external Na^+. However, compared with the Cy162-*SiHAK2* strains, the strains expressing *SiHAK1* exhibited improved growth with increasing external Na^+ concentrations in contrast to their inferior growth observed under 10 mM K^+ condition (Fig. 6b).

High salinity affects plants in two phases, osmotic stress, and ion imbalance (Roy et al. 2014). To identify how SiHAK1 mediates salt resistance, drop complementation assays were performed in different osmotic AP-U buffers adjusted by sorbitol. We added 1 or 1.5 M sorbitol to AP-U medium with 100 μM K^+ which was adjusted to a similar osmotic pressure with the addition of 500 or 750 mM NaCl (Fig. 6c). However, the *SiHAK1* transformants showed similar growth under osmotic stress and normal conditions. Therefore, the maintenance of K^+/Na^+ homeostasis is the primary strategy of SiHAK1 in salt resistance. This phenomenon implied that SiHAK1 might function as a K^+/Na^+ antiporter, simultaneously mediating K^+ uptake and Na^+ exclusion. To test this hypothesis, we expressed SiHAK1 in the B31 *ena1-4*△ *nha1*△ yeast strain, in which Na^+ export pumps are disrupted. However, SiHAK1 still strongly enhanced the growth of the strains under low K^+ condition, but did not affect the Na^+ sensitivity of B31 strain (Fig. 6d). Therefore, the mediation of salt tolerance by SiHAK1 was due to its K uptake and capability to substantially increase K absorption. Maintaining K^+ uptake rates at high external Na^+ is crucial for K^+/Na^+ homeostasis and salt tolerance (Munns and Tester 2008; Cheng et al. 2015). In the presence of high Na^+ concentrations, the low K^+ induction gene

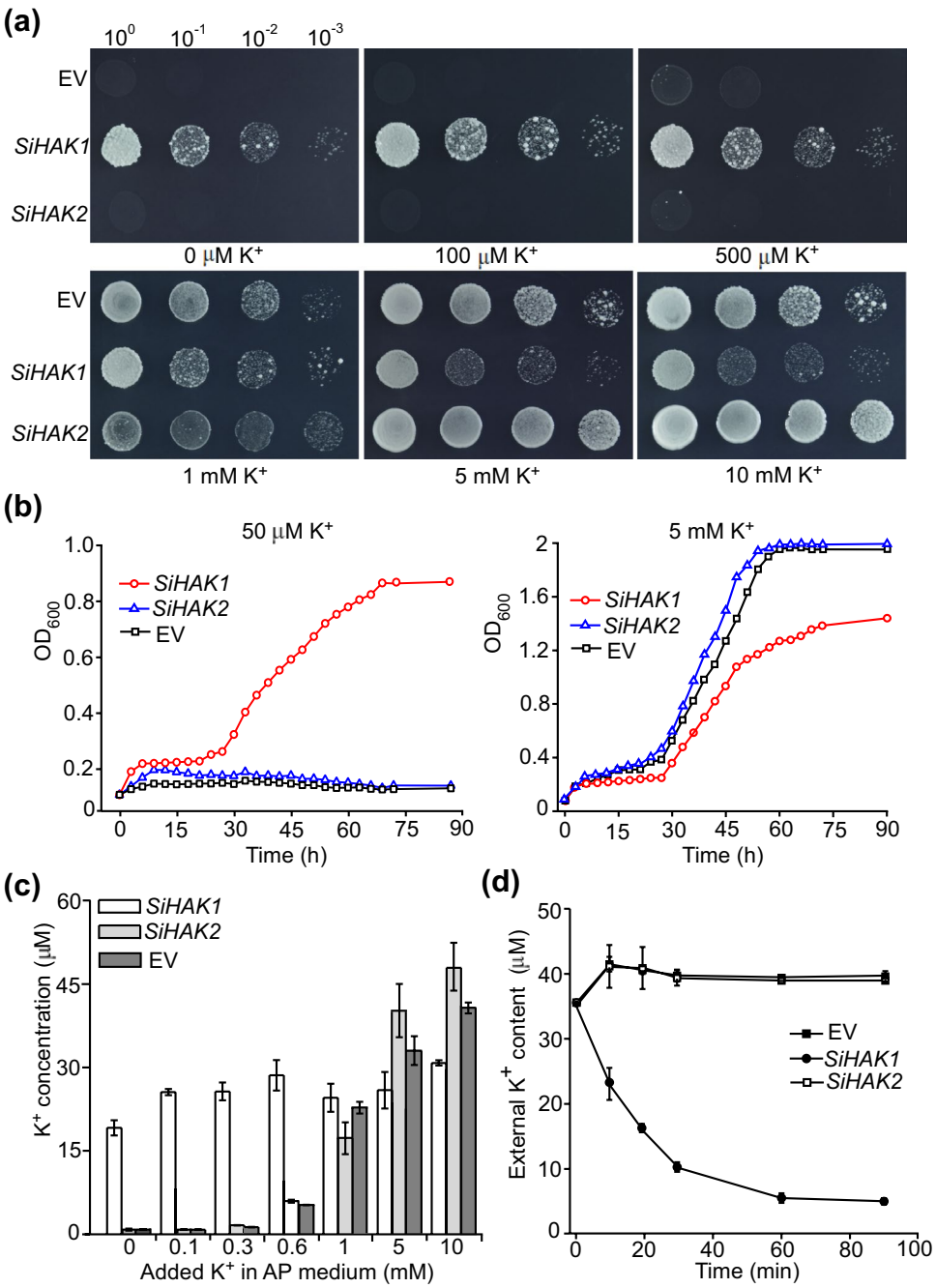


Fig. 4 *SiHAK1* and *SiHAK2* complementation assays in yeast cells deficient in the Trk1 and Trk2 K^+ uptake system. **a** Growth of the mutant Cy162 strains in solid AP-U medium with various concentrations of K^+. The Cy162 strains were transformed with the empty vector pYPGE15 (EV) or with pYPGE15-*SiHAK1* (*SiHAK1*) and pYPGE15-*SiHAK2* (*SiHAK2*). Drop serial dilutions of each strain were cultured on agar plates. **b** Growth curves of the Cy162 strains transformed with empty vector, *SiHAK1* or *SiHAK2* in liquid AP-U medium with 50 μM K^+ or 5 mM K^+ added. **c** Measurement of K^+ content in Cy162 strains transformed with empty vector, pYPGE15-*SiHAK1* or *SiHAK2* in solid AP-U with various concentrations of K^+. The yeast cells were suspended in pre-cooled water, adjusted to $OD_{600}=0.3$, and then ruptured. The K^+ concentration in the buffer was measured. The figures show the data of a representative experiment of three independent repetitions. The data represent the mean ± standard deviation (SD). **d** K^+ depletion experiment in the presence of 35 μM K^+ in AP-U medium. The Cy162 strains transformed with empty vector, pYPEG15-*SiHAK2*, or pYPEG15-*SiHAK1* were subjected to K^+ starvation for 4 h prior to the beginning of the experiment. The K^+ content in the buffer was measured at intervals over a 2 h period. Three independent experiments were carried out and the data represent the mean ± SD

SiHAK1 was also up-regulated, which indicated a pivotal role in the maintenance of K^+ upward translocation and plant growth under high salt conditions (Fig. 3a).

AtHAK5 plays important role in the maintenance of high affinity K^+ uptake and plant growth in the presence of high Na^+ and biomass accumulation in the *Arabidopsis* wild type is significantly higher than in *athak5* (Nieves-Cordones et al. 2010). The expression of *SiHAK1* in *athak5* rescued the salt-sensitive phenotype *athak5* and even enhanced salt tolerance compared with the wild type under NaCl treatment in low K^+ condition (Fig. 6e, f). The results further confirmed that SiHAK1 demonstrates extreme Na^+-insensitive high-affinity K^+ uptake, therefore improving salt tolerance in plants.

The capability of SiHAK1 to mediate high-affinity K^+ uptake is stronger than the reported Cluster I transporters of the HAK/KUP/KT family

To compare the high-affinity K^+ uptake activity of SiHAK1 with other Cluster I proteins, we isolated some typical reported Cluster I genes and transformed them into pYPEG15 vector, including *HvHAK1, AtHAK5*, and

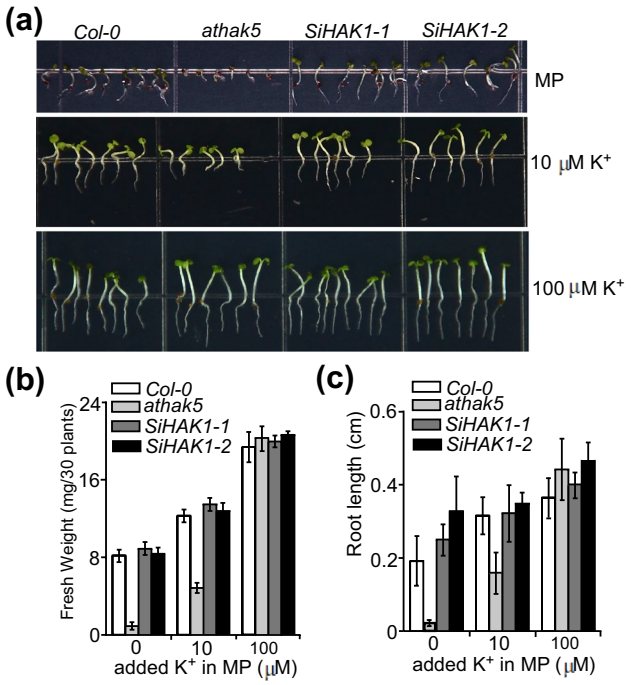


Fig. 5 The rescued phenotype of the over-expression of *SiHAK1* in *Arabidopsis* mutant *athak5*. **a** The wild type, *athak5*, and two *35S-SiHAK1* transgenic lines in *athak5* (*SiHAK1-1* and *SiHAK1-2*) were grown for 7 days on liquid MP medium with various K^+ added. **b** Fresh weight of the plants in **a**. Each bar represents the mean fresh weight ($n=3$) of 30 seedlings. The data represent the mean ± SD. **c** Root length of the plants in **a**. Each bar represents the mean root length of 40 seedlings from three independent experiments. The data represent the mean ± SD

OsHAK5. We used drop complementation assays in AP-U medium supplemented with various concentrations of K^+ added and the images were captured after only 3 days of growth. The *SiHAK1* transformants demonstrated the best growth among the four yeast stains, particularly in an extremely low K^+ environment (Fig. 7a). To compare the salt tolerance between the *SiHAK1* and *HvHAK1* transformants, we performed drop complementation assays at different external Na^+ conditions under 100 μM K^+. The *HvHAK1* transformants could tolerate up to 200 mM NaCl, while the *SiHAK1* transformants maintained perfect growth under 500 mM NaCl treatment (Fig. 7b). To examine the salt tolerance mechanism of SiHAK1 in detail, the K^+ and Na^+ contents in the strains were measured. The quantities of K^+ uptake in the *SiHAK1* transformants were critically higher than the *HvHAK1* expression strains; however, the Na^+ contents were highly similar between them (Fig. 7c). All these findings indicated that SiHAK1 facilitated better and more efficient high-affinity K^+ uptake in comparison with the reported HAK/KUP/KT transporters, especially in mediating K homeostasis under low K^+ condition or salt stress.

Discussion

The homeostasis and root-to-shoot translocation of K^+ determine nutrient balance, growth, and stress tolerance (Ahmad and Maathuis 2014). The plant genome contains large number of HAK/KUP/KT transporters that exhibit the diverse roles in K^+ uptake, translocation, salt tolerance, and osmotic regulation (Li et al. 2017). Significant recent progress in HAK/KUP/KT transporters has revealed the important role of this family for plant. OsHAK1 is the best characterized transporter of these HAK/KUP/KT members in crop species, and exhibits critical K^+ uptake during abiotic stresses, including low K, high salt, and drought stresses (Chen et al. 2015, 2017). In fact, HAK1-type transporters are distributed in all sequenced crop species, and the similarity of these HAK1 proteins reaches 88% (Supplemental Fig. 3). In our research, we scanned the HAK/KUP/KT transporters in foxtail millet and revealed the important physiological functions of SiHAK1 which exhibited many distinctive characteristics.

Tandem repeats of SiHAK1 genes in chromosome 7

Segmental duplications, tandem duplications, and random translocations contributed to the species-specific expansion of the rice and maize HAK/KUP/KT family following the split of the monocots and dicots (Yang et al. 2009; Zhang et al. 2012). This phenomenon was also observed in *S. italic*a, particularly the tandem duplication event was occurred during the expansion of *SiHAK1* genes which did not occur in other crops (Fig. 1b). These four tandem duplication genes are clustered together in the phylogenetic tree and exhibit similar gene structures (Fig. 2a). Based on our understanding of *OsHAK1, SiHAK1* and its different tandem duplication genes may be related to the strong adaption of foxtail millet in barren and arid regions.

Highly regulated by low K environment in the whole plant

Cluster I genes of the HAK/KUP/KT family in different species have all been reported to be highly up-regulated in the roots upon K starvation and other abiotic stresses (Chen et al. 2015; Fernando et al. 2009; Gierth et al. 2005; Martínez-Cordero et al. 2004; Nieves-Cordones et al. 2007; Ruiz-Lau et al. 2016; Santa-María et al. 1997; Yang et al. 2014). However, reports on Cluster I genes in other tissues are few. In addition to the roots, *OsHAK1* and *HvHAK1* show low expression levels in other tissues, regardless of the abiotic conditions being normal or stressful. Although *OsHAK5* and *OsHAK21* can are detectable in the shoots, they are only weakly induced by low K or salt stress compared with the

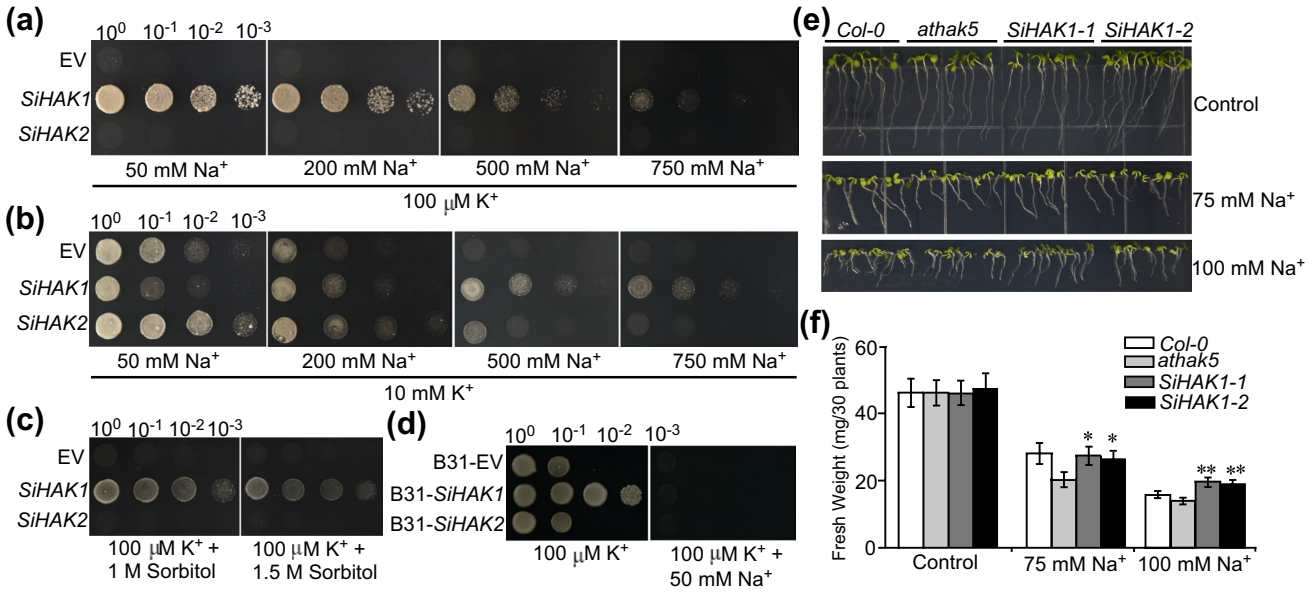


Fig. 6 Salt tolerance of the *SiHAK1* complementation assays in Cy162 strains. **a, b** Growth of the Cy162 strains in solid AP-U medium with 100 μM K^+/10 mM K^+ and various concentrations of Na^+. The Cy162 strains were transformed with empty vector pYPGE15 (EV), pYPGE15-*SiHAK1* (*SiHAK1*), or pYPGE15-*SiHAK2* (*SiHAK2*). Drop serial dilutions of each strain were cultured on agar plates. **c** The growth of Cy162 strains transformed with the empty vector pYPGE15 (EV), pYPGE15-*SiHAK1* (*SiHAK1*), or pYPGE15-*SiHAK2* (*SiHAK2*) in solid AP-U medium with various sorbitol contents under 100 μM K^+. **d** The growth of B31 strains transformed with the empty vector pYPGE15 (EV), pYPGE15-*SiHAK1* (*SiHAK1*), or pYPGE15-*SiHAK2* (*SiHAK2*) in solid AP-U medium without or with 50 mM Na^+ under 100 μM K^+. **e** The wild type, *athak5*, and two *35S-SiHAK1* transgenic lines in *athak5* (*SiHAK1-1* and *SiHAK1-2*) grown for 7 days on solid MP medium with various contents of Na^+ under 100 μM K^+. **f** Fresh weight of 7-day-old wild type, *athak5, SiHAK1-1*, and *SiHAK1-2* seedlings in **e** were measured. Each bar represents the mean fresh weight ($n=3$) of 30 seedlings. The data represent the mean ± SD, and Student's *t* test was used to identify significant differences at $P<0.05$ (*) and $P<0.01$ (**)

roots (Chen et al. 2015; Fulgenzi et al. 2008; Gierth et al. 2005; Shen et al. 2015). *SiHAK1* showed a relatively high expression level in both the roots and the shoots under normal conditions (Supplemental Fig. 1), and was highly upregulated by a low K environment in the shoots (Fig. 3a). *SiHAK1* was widely expressed in various tissues, including the roots, shoots, leaves, and panicles (Fig. 2b). Its expression pattern is likely to be tightly related to its physiological function. It would be interesting to investigate the function and regulation of HAK/KUP/KT transporters in specific tissues and at specific development stages in plants.

Extreme efficient high-affinity K^+ uptake

All our research on the function of SiHAK1 ultimately focused on its extreme high-affinity K^+ uptake activity under either low K^+ or high salt conditions. Compared with the transport activity of reported HAK/KUP/KT proteins in Cy162 yeast strains, SiHAK1 exhibited stronger transport activity under extremely low K conditions (Fig. 7a) which implied that an important high-affinity K^+ transporter has been discovered. Under a high salt environment, K^+/Na^+ homeostasis is crucial for ion balance, development, and growth (Munns and Tester 2008). In rice, OsHAK1 dominates Na^+-sensitive high affinity K^+ uptake (Chen et al. 2015), and the By2 cell expressing *OsHAK5* enhances the accumulation of K^+ but has no effect on Na^+ (Horie et al. 2011). The high-affinity K^+ transporter in the regulation K^+/Na^+ homeostasis under salt stress may be linked to altered membrane potential, which might allow transporters to mediate K^+ uptake (Nieves-Cordones et al. 2008). The highly strong salt resistance of the Cy162-*SiHAK1* strains and the sensitivity to Na^+ of B31-*SiHAK1* indicated that the salt tolerance is due to the efficient absorption of K^+, but is not related to Na^+ (Figs. 6d, 7b, c). Therefore, the strong K^+ uptake by SiHAK1 in extremely low K conditions can critically enhance abiotic stress resistance for the plant, which may be a consequence of the adaptation of *S. italica* to arid environments.

Modulated by not only K^+ supply but also internal K^+ content

The Cy162-*SiHAK1* strains exhibited poor growth under a high K^+ environment, but demonstrated better growth when high concentrations of Na^+ were added (Fig. 6a, b).

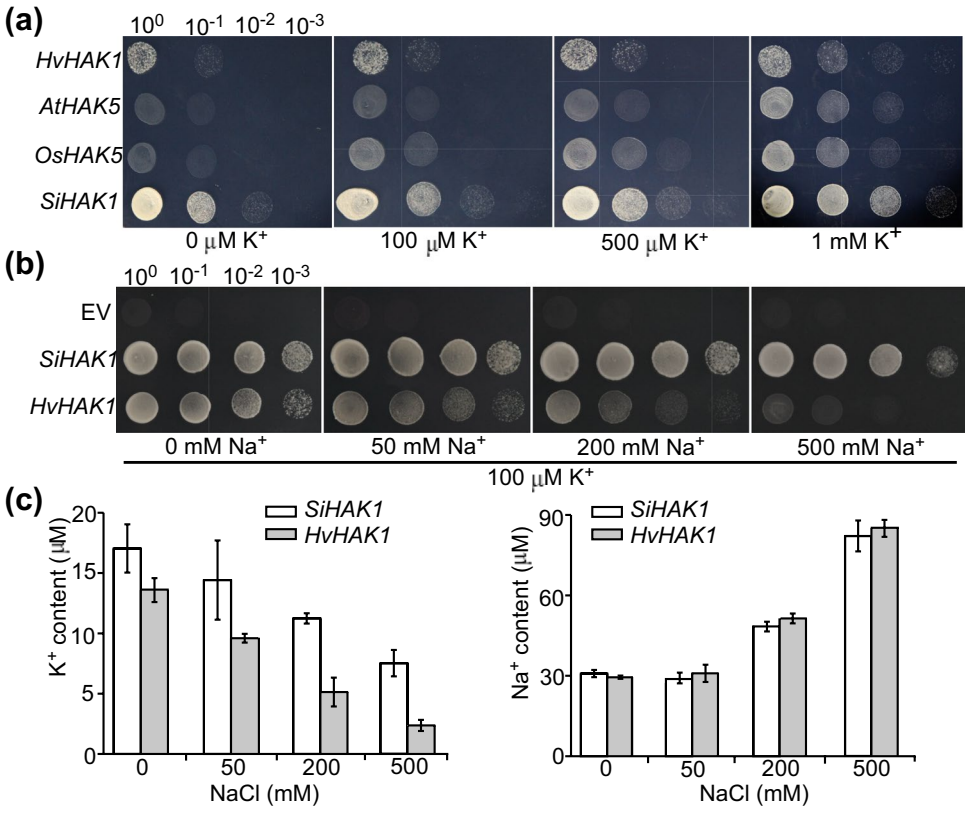


Fig. 7 Stronger K uptake activity of SiHAK1 under low K and salt stress compared with typical Cluster I HAK genes. **a** The growth of Cy162 strains transformed with pYPGE15-*HvHAK1* (*HvHAK1*), pYPGE15-*AtHAK5* (*AtHAK5*), pYPGE15-*OsHAK5* (*OsHAK5*), and pYPGE15-*SiHAK1* (*SiHAK1*) in solid AP-U medium with various concentrations of K^+. **b** The growth of Cy162 strains transformed with the empty vector, pYPEG15-*SiHAK1* or pYPEG15-*HvHAK1* in solid AP-U medium with various concentrations of Na^+ under 100 μM K^+. **c** The K^+ and Na^+ contents in each strain in **b** were measured. Figures show the data of a representative experiment of three independent repetitions. The data represent the mean ± SD

This result significantly indicates that SiHAK1 is strongly mediated by internal K^+ content in the cell. The salt resistance relied on the triggering of the high-affinity K^+ transport activity of SiHAK1 as a result of decreased K content under high Na^+ conditions. This phenomenon has not been observed in other HAK/KUP/KT proteins. OsHAK1 was found to function in K uptake at both low and high K supply in yeast strain (Chen et al. 2015). *AtHAK5, HvHAK1*, and *CcHAK1* exhibited high-affinity K^+ uptake under low K supply in yeast cells, but transformants of these genes show the same growth with empty vector transformants under high K supply (> 1 mM) in yeast mutant stains (Alemán et al. 2014; Ruiz-Lau et al. 2016; Senn et al. 2001). OsHAK5 functions in K uptake at concentrations ranging between 0.05 μM and 10 mM (Yang et al. 2014). This novel discovery of internal K^+ content modulating SiHAK1 extends our knowledge of the function of high-affinity transporters, however, the exact regulatory mechanisms and physiological functions require further elucidation in future research, particularly including the further evaluation of the *SiHAK1* mutant in *S. italica*.

Author contribution statement HZ and RL conceived and designed the experiments. WX, WY, and HZ performed the experiments. HZ, RL, and WX analyzed the data. LY, LL, and JW contributed reagents/materials/analysis tools. HZ, WX, and RL wrote the manuscript. All the authors read and approved the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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