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ORIGINAL ARTICLE

Genome-wide identification and expression analysis of HAK/KUP/KT potassium transporter provides insights into genes involved in responding to potassium deficiency and salt stress in pepper (*Capsicum annuum* L.)

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Abstract

In plants, the HAK/KUP/KT family is the largest group of potassium transporters, and it plays an important role in mineral element absorption, plant growth, environmental stress adaptation, and symbiosis. Although these important genes have been investigated in many plant species, limited information is currently available on the *HAK/KUP/KT* genes for Pepper (*Capsicum annuum* L.). In the present study, a total of 20 *CaHAK* genes were identified from the pepper genome and the *CaHAK* genes were numbered 1 – 20 based on phylogenetic analysis. For the genes and their corresponding proteins, the physicochemical properties, phylogenetic relationship, chromosomal distribution, gene structure, conserved motifs, gene duplication events, and expression patterns were analyzed. Phylogenetic analysis divided *CaHAK* genes into four cluster (I–IV) based on their structural features and the topology of the phylogenetic tree. Purifying selection played a crucial role in the evolution of *CaHAK* genes, while whole-genome triplication contributed to the expansion of the *CaHAK* gene family. The expression patterns showed that CaHAK proteins exhibited functional divergence in terms of plant K^+ uptake and salt stress response. In particular, transcript abundance of *CaHAK3* and *CaHAK7* was strongly and specifically up-regulated in pepper roots under low K^+ or high salinity conditions, suggesting that these genes are candidates for high-affinity K^+ uptake transporters and may play crucial roles in the maintenance of the Na^+/K^+ balance during salt stress in pepper. In summary, the results not only provided the important information on the characteristics and evolutionary relationships of CaHAKs, but also provided potential genes responding to potassium deficiency and salt stress.

Keywords Potassium transporter · *HAK/KUP/KT* gene family · Evolution · Gene expression profiles · *Capsicum annuum* L.

Introduction

Potassium (K^+) is an essential macronutrient, required for plant growth and development, and constituting 2% to 10% of plant dry matter (Leigh and Wyn Jones, 1984; Amrutha et al. 2007). As the most abundant cation in plant cells, K^+ plays crucial roles in plant physiological processes, such as photosynthesis, transmembrane transport, enzyme activation, and response to stress (Leigh and Wyn Jones, 1984; Wang and Wu 2013; Luan et al. 2017). Due to heavy leaching losses, chemical fixation, and the low diffusion rate in the soil, the availability of K^+ for plant roots is limited over large areas of agricultural land worldwide (Maathuis 2009; Rengel and Damon 2008).

As the K^+ concentration in the rhizosphere rarely exceeds the range 0.01 – 1 mM, plants depend on a variety of K^+

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absorption systems to mediate K^+ uptake under these conditions (Luan et al. 2009; Maathuis 2009; White 2013). It has been well recognized that there are two K^+ uptake systems operating in roots, namely a low-affinity transport system (LATS), operating via K^+ channels under high external K^+ concentrations (> 0.5 mM), and a high-affinity transport system (HATS), operating via K^+ transporters, which function at low external K^+ concentrations (< 0.2 mM) (Epstein et al. 1963; Ashley et al. 2006; Chérel et al. 2014; Li et al. 2018). In plants, genes encoding K^+ transporters are grouped into four major families: KT (K^+ Transporter)/HAK (high-affinity K^+ transporter)/KUP (K^+ uptake permease), Trk (transport of K^+)/HKT (high-affinity K^+/Na^+ transporter), KEA (K^+ exchange antiporters), and CHX (cation/H^+ exchanger) (Uozumi et al. 2000; Cellier et al. 2004; Aranda-Sicilia et al. 2016; Wang and Wu 2017). The HAK/KUP/KT group is the largest family and plays crucial roles in K^+ uptake under different potassium conditions (Ahn et al. 2004; Li et al. 2018). In plants, the HAK/KUP/KT family comprises the majority of the plant K^+ transporters identified so far and has been divided into four major clusters (I–IV) (Grabov 2007; Wang and Wu 2013). Multiple transporters in cluster I, such as HvHAK1, the first plant HAK/KUP/KT transporter member cloned from barley, AtHAK5 in *Arabidopsis*, and OsHAK1/OsHAK5 in rice, have high-affinity K^+-uptake capacity and are up-regulated in response to low K^+ conditions (Schachtman and Schroeder 1994; Santa-María et al. 1997; Gierth et al. 2005; Yang et al. 2014; Feng et al. 2021). OsHAK1 played a key role in the maintenance of K^+ homeostasis and positively regulates tolerance to salt and drought stress (Chen et al. 2015). It was recently reported that OsHAK5 could impact on tiller number and development of roots by regulating an ATP-dependent auxin transporter in rice (Yang et al. 2020a, b). Members of cluster II of HAK/KUP/KT seem to be involved in plant development processes (Elumalai et al. 2002; Yang et al. 2009; Osakabe et al. 2013). In *Arabidopsis*, AtKUP4/TRH1 affects the growth of root hair tips by establishing auxin gradients in the root apex (Templalexis et al. 2021). The expression of *AtKUP6* was induced by drought stress and involved in plant cell osmoregulation. Interestingly, AtKUP2, AtKUP6, and AtKUP8 function as efflux K^+ transporters, and a triple knockout mutation of *atkup2/6/8* significantly increased root cell size and decreased the sensitivity of guard cells and lateral root cells to abscisic acid (ABA) (Elumalai et al. 2002; Osakabe et al. 2013). In addition to the abiotic stress-responsive *HAK* genes, an increasing number of mutualistic symbiosis-induced *HAK* genes belonging to the cluster II HAK/KUP/KT family have been identified, and their functions have been shown to be associated with the mycorrhizal K^+-uptake pathway (Desbrosses et al. 2004; Guether et al. 2009; Liu et al. 2019). Members of cluster III may maintain the balance of K^+/Na^+ (Okada et al. 2008). The potential functions and physiological roles of members of cluster IV had less reports than those in the other clusters (Grabov 2007; Han et al. 2016). Besides, the transcriptional regulation of HAK/KUP/KT transporters had also been reported. The OsAKT1 channel is modulated by the Calcineurin B-Like protein 1 (CBL1)–CBL-Interacting Protein Kinase 23 (CIPK23) complex (Li et al. 2014). The R2R3-type MYB transcription factor gene (AtMYB77) positively regulates the expression of AtHAK5 and enhanced K^+ acquisition under low K^+ stress (Feng et al. 2021) in Arabidopsis. Recently, Song et al. demonstrate that an endoplasmic reticulum-localized OsCYB5-2 protein can binding to OsHAK21, and then promoting OsHAK21-mediated K^+ uptake and enhance salt tolerance in rice (Song et al. 2021).

Over the past two decades, the functional characterization of the HAK/KUP/KT family focused mainly on rice and *Arabidopsis*, in particular the members from the cluster I subfamily, such as AtHAK5, OsHAK1, OsHAK5 and OsHAK21 (Gierth et al. 2005; Yang et al. 2014; Chen et al. 2015; Shen et al. 2015). With the availability of whole complete genome sequences, a series of plant HAK/KUP/KT genes have been identified and characterized in a number of plant species, including 13, 27, 21, and 56 *HAK/KUP/KT* genes in *Arabidopsis thaliana*, rice (*Oryza sativa*), tomato (*Solanum lycopersicum* L.) and wheat (*Triticum aestivum* L.), respectively (Ahn et al. 2004; Yang et al. 2009; Hyun et al. 2014; Cheng et al. 2018).

Pepper (*Capsicum annuum* L.) is an economically important vegetable and spice plant of the Solanaceae family, is a rich source of vitamins, nutrients, and capsaicin, and is widely cultivated throughout the world (Lee et al. 2004; Moscone et al. 2007). In addition to promoting the growth of pepper, supplementary potassium can enhance the dry matter and chlorophyll concentrations under high salt treatments (Wamser et al. 2017; Kaya et al. 2020). As pepper is a salt-sensitive vegetable crop, maintaining high K^+/Na^+ ratios in the tissues is important in achieving salt tolerance. Whereas, HAK/KUP/KT transporter gene of pepper was poorly studied. *CaHAK1* was cloned and its expression was inhibited by NH_4^+ when the exogenous K^+ supply was interrupted was demonstrated, which indicated that CaHAK1 might play a crucial role in K^+ uptake and tolerance to Na^+ (Martınez-Cordero 2004; Martinez-Cordero et al. 2005). *CcHAK1* gene, which is a high-affinity K^+ transporter gene from habanero pepper, was induced by K^+ starvation in roots and was not inhibited in the NaCl stress. Furthermore, K^+ transporter activity of CcHAK1 in yeast is inhibited by external millimolar concentrations of NH_4^+ and Cs^+, but not inhibited by Na^+ (Ruiz-Lau et al. 2016).

Here, we identified putative *HAK/KUP/KT* transporter gene family members were not identified in pepper genome. In this study, we will identify 20 CaHAKs in genome-wide, then took the phylogenetic analysis of HAK/KUP/KT relationships among different plant species, provide conserved

motifs in the corresponding proteins, as well as the features of gene structures, chromosomal locations, and characteristics of *cis*-regulatory elements in promoter regions. Then investigated the evolution and domestication of the *CaHAK* family members. In addition, the expression profiles of *CaHAK* family members in response to K^+ deficiency or salt stress will catch to select the candidate genes involving in responding to K^+ deficiency or salt stress. As a result, this study can provide a valuable information for further functional characterization of *CaHAK* genes and show insight to genetic improvement of potassium-utilization efficiency in the pepper breeding.

Methods

Plant materials and stress treatments

Pepper cultivar *Zunla-1* was selected in this study, for its wide cultivation in Southern China and high-quality genome sequence. Seeds of pepper were surface-sterilized and germinated in a growth chamber with a photoperiod and temperature regime of 16 h light at 28 °C and 8 h dark at 20 °C. The seedlings were then transplanted to sterilized quartz sand for a 3-week culture period, meantime they were irrigated with full strength nutrient solution containing the following: 1 mM NH_4^+, 4 mM NO_3^-, 2 mM K^+, 1 mM phosphate (Pi), 0.75 mM Ca^{2+}, 0.5 mM Mg^{2+}, 0.25 mM Cl^-, 0.5 mM SO_4^{2-}, 20 µM Fe^{2+}, 9 µM Mn^{2+}, 46 µM BO_3^{3+}, 8 µM Zn^{2+}, 3 µM Cu^{2+}, and 0.03 µM MoO_4^{2+}. After 3 weeks, the seedlings were then transferred to a hydroponic culture system in nutrient media to achieve different stress treatments. For the K^+-deficiency treatment, 21-day-old pepper plants were cultured in the full strength nutrient medium solution containing 0.2 mM K^+, with the pH adjusted to 5.5 with NaOH (Liu et al. 2019). Under the salt-stress treatment, 21-day-old pepper plants were cultured with full-strength nutrient medium containing 2 mM K^+ and 200 mM NaCl (Liu et al. 2016a, b). The salt-stress treatment sample were harvested at 24 h after the treatments. Control plants for both treatments were cultured in the full-strength medium containing 2 mM K^+. After each treatment was completed, the roots and leaves of the pepper plants (three replicates per treatment, three seedlings per replicate) were harvested, rinsed, and patted dry, then immediately snap-frozen in liquid nitrogen and stored at − 80 °C for subsequent RNA isolation.

Identification of the *CaHAK* gene family in pepper

To obtain all the *HAK*/*KUP*/*KT* family genes from the pepper genome, the *Arabidopsis* genes from this family were each queried in the pepper genome sequence downloaded from the NCBI genome database (https://www.ncbi.nlm.nih.gov/genome/?term=Capsicum+annuum+L). Each sequence was selected as a candidate HAK/KUP/KT protein with an e-value < 10^{-10} and a query of greater than 50%. All the candidate gene sequences obtained were submitted to the Pfam (http://pfam.xfam.org/) and SMART (http://smart.embl-heidelberg.de/) databases for further confirmative analysis (Finn et al. 2014; Letunic et al. 2020). Ultimately, a total of 20 *HAK*/*KUP*/*KT* genes were identified and named as *CaHAK1-20* based on the corresponding orthologous genes from tomato. Physicochemical properties of the pepper *HAK* transporter genes, including gene name, ID, protein length, isoelectric point, predicted protein molecular weight, grand average of hydropathicity (GRAVY), and predicted protein subcellular localization were searched for using the ProtParam tool (http://web.expasy.org/protparam/) (Artimo et al. 2012). The subcellular locations of the CaHAK proteins were predicted by SOFTBERRY (http://linux1.softberry.com/) and Cell-PLoc 2.0 (http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc-2/).

The full-length protein sequences encoded by *HAK*/*KUP*/*KT* from *C. annuum* (CaHAKs), *O. sativa* (OsHAKs) (Gupta et al. 2008), *A. thaliana* (AtHAK/KUP/KTs) (Ahn et al. 2004), *C. reinhardtii* (CrHAKs), *P. patens* (PpHAKs), *V. vinifera L. (*VvHAKs*)* (Nieves-Cordones et al. 2016), *S. melongena* (SmHAKs) and *S. lycopersicum* (SlHAKs) (Hyun et al. 2014; Liu et al. 2019) were determined using the program Clustal X (v1.8) with default parameters (gap opening penalty = 10, gap extension penalty = 0.2, protein weight matrix = Gonnet), and a phylogenetic tree was constructed by the Neighbor-Joining (NJ) method model with the MEGA7 software (Saito et al. 2013). The bootstrap parameter was set at 1,000 replicates (Kumar et al. 2016).

To further investigate the evolution of the HAK/KUP/KT family genes from algae to angiosperm like dicotyledons *C. annuum*, we identified HAK/KUP/KT genes in one algae (*C. reinhardtii*); one lycopodiophyta (*S. moellendorffii*); one early angiosperm (*A. trichopoda*); two bryophytes (*M. polymorpha*, *P. patens*); three monocotyledons (*O. sativa*, *Z. mays*, *S. bicolor*); five dicotyledons (*A. thaliana*, *C. annuum*, *S. lycopersicum*, *S. tuberosum*, *V. vinifera*), and count the numbers of HAK genes in four major clusters, then constructed the phylogenetic tree with MEGA7.0 using the NJ method (Saito et al. 2013) and bootstrap parameter with 1,000 replicates (Kumar et al. 2016).

Conserved motif recognition and gene structure analysis

The cDNAs and their corresponding DNA sequences from the *CaHAK* gene family were obtained from the genome database. Exon/intron structure analysis was carried out by comparing each cDNA sequence with the corresponding genomic DNA

sequence. The 2,000-bp genomic DNA sequence upstream of each *CaHAK* gene was selected for promoter analysis from the pepper genome database. Then, the PlantCARE database was used to analyze the *cis*-regulatory elements in the promoters (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/search_CARE.html). Eventually, the *cis*-regulatory elements associated with stress response were selected to draw the distribution map in the promoter via TBtools software (Chen et al. 2020). The NCBICD (Conserved Domain) search was used for protein domain analysis (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi). The conserved motifs of the pepper CaHAK family proteins were identified using the MEME tool (http://meme-suite.org/tools/meme) (Bailey et al. 2009). The TMHHM Serve 2.0 tool (http://www.cbs.dtu.dk/services/TMHMM/) was used to predict the transmembrane (TM) domains of the CaHAK proteins.

Chromosome location and syntenic analysis

The localization information of the *CaHAK* gene family on the pepper chromosomes was obtained from the pepper GFF (general feature format) genome files. Then, TBtools was used to construct the chromosome localization map of the *CaHAK* gene. The orthologous genetic relationship among *HAK* genes from pepper, *Arabidopsis*, rice, grape, potato, eggplant and tomato was identified using the MCScanX program. The advanced Circos tool was used to visualize the syntenic relationship between different species. Nonsynonymous (Ks), synonymous (Ka), and Ka/Ks values for each CaHAK gene pairs were calculated using the TBtools V 1.802 simple Ka/Ks calculator. The Ks value was used to estimate the divergence time of CaHAK duplicated gene pairs ($T = \mathrm{Ks}/2R$ Mya, Millions of years), where T refers to divergence time and R is the rate of synonymous substitutions ($R = 1.5 \times 10^{-8}$) (Edlund et al. 2004).

Transcriptome profiling analysis

To further explore the potential functions of the 20 *CaHAK* genes, the expression patterns of these members were analyzed in different tissues, namely roots, stems, leaves, flowers, bud tissues, and nine developmental stages of fruit, using public RNA-sequence data from pepper cultivar *Zunla-1*(Qin et al. 2014). The relative expression was expressed with fragments per kilobase of transcript per million mapped reads (FPKM) (Table S4) and showed with heat map. The heat map was drawn using TBtools v1.086.

Total RNA extraction and quantitative real-time PCR analysis

Total RNA was extracted from 100 mg fresh weight of pepper leaves and roots. The total RNA (approximately 1 µg) from the root sample was used to synthesize cDNA using a reverse transcription kit (Takara, Dalian, China). Using the synthesized cDNAs as templates, quantitative real-time PCR (qPCR) was performed in three technical replicates for each sample, using the SYBR Premix Ex Taq kit (Takara, Japan). The primer pairs used for *CaHAK* genes are listed in Table S1. All primers for RT-PCR were designed using Primer 5.0 software, and a melting curve was performed to check for gene-specific amplification (Fig. S1). The relative transcript abundance of each target gene was $\log_2$-normalized against *β-Actin* transcript level (Ma et al. 2019). Any changes in expression levels of *CaHAK* genes were quantified using the $2^{-\Delta\Delta Ct}$ method (Li et al. 2009).

The RNA sequence data for tissue-specific expression profiling of *CaHAK*s (including roots, stems, leaves, buds, flowers, and the nine fruit developmental stages), were downloaded from the GEO Datasets (https://www.ncbi.nlm.nih.gov/gds/?term=GSE45037) (Qin et al. 2014). The relative expression levels of *CaHAKs* were analyzed by the fragments per kilobase per million reads (FPKM) values. Heatmaps of these genes were generated by TBtools v1.086 (Chen et al. 2020).

Statistical analysis

The experiment data were analyzed by ANONA (IBM SPSS Statistics 25.0), followed by Tukey's test ($^{*}P < 0.05$, $^{**}P < 0.01$). The obtained data were processed by GraphPad Prism 8.0.1.

Results

Genome-wide identification of *CaHAK* gene family members in pepper

We identified 20 putative *HAK/KUP/KT* genes in pepper genome basing on the BLAST search results (Table S2). These putative genes were named *CaHAK1* to *20*, and all of them encoded proteins with, in most cases, similar physicochemical properties. Information about all the putative genes was listed in Table S2, including gene name, ID, protein length, isoelectric point, predicted protein molecular weight, grand average of hydropathicity (GRAVY), and predicted protein subcellular localization. The lengths of the 20 HAK/KUP/KT putative proteins ranged from 713 to 848 amino acids, the molecular weights and isoelectric points of these proteins varied from 79.2 to 94.4 kDa and from 6.04 to 9.34, respectively (Table S2). GRAVY analysis showed that all the proteins were hydrophobic, while the predicted subcellular locations of the CaHAK proteins were all at the plasma membrane (PM), the results of both properties suggested

that the protein functions were to maintain K^+ homeostasis in pepper.

Phylogenetic analyses of *HAK/KUP/KT* family genes

To reveal the genetic relationship among the *CaHAK* genes, a total of 144 plant HAK protein sequences from eight species, namely *Capsicum annuum* L, *Solanum lycopersicum*, *Solanum tuberosum*, *Vitis vinifera*, *Oryza sativa*, *Arabidopsis*, and *Physcomitrella patens*, *Chlamydomonas reinhardtii*, were aligned using the Clustal X program, and an unrooted tree was constructed using the Neighbor-Joining method using MEGA7 software. According to the phylogenetic tree, *CaHAK* family genes could be divided into four major clusters (I–IV) (Fig. 1). In cluster I, the six *CaHAK* members, *CaHAK1*, *CaHAK5*, *CaHAK9*, *CaHAK13*, *CaHAK15*, and *CaHAK16* belong to cluster IA, and it is worth noting that cluster IB contained only members from rice. In cluster II, the eight *CaHAK* members, namely *CaHAK2*, *CaHAK3*, *CaHAK4*, *CaHAK6*, *CaHAK7*, *CaHAK8*, *CaHAK10*, and *CaHAK17*, were classified into three sub-clusters (cluster IIA, IIB, and IIC). In cluster III, CaHAK family contained five members and was composed of sub-cluster IIIA (*CaHAK18* and *CaHAK19*) and III B (*CaHAK11*, *CaHAK12*, and *CaHAK20*). Cluster IV contained only one pepper gene, *CaHAK14*. Notably, several orthologous CaHAK members were found in tomato genome, indicated that a whole-genome triplication contributed to the expansion of the *CaHAK* gene family.

To further investigate the evolution of the HAK/KUP/KT family genes, we identified 223 HAK/KUP/KT genes in 14 species from algae, lycopodiophyta, early angiosperm, bryophytes, monocotyledons to dicotyledons. The counts of different cluster of HAK/KUP/KT genes are shown in Fig. 2. The number of HAK/KT/KUP family gene of algae and bryophyta was less than that in higher plants. Interestingly, the cluster IV was the largest subfamily both in algae and bryophyta species, indicated that the cluster IV began to expand during the plant terrestrialization. In addition, the numbers of cluster II and III family gene were highly conserved in magnoliophyte and mesangiospermae, respectively.

Chromosomal position and duplication analysis of *CaHAK* genes

The chromosomal locations of the *CaHAK* genes were mapped from the pepper genome database, using TBtools. As shown in Fig. S2, apart from *CaHAK7*, which was located on a scaffold chromosome (NW_015960857.1), all the *CaHAK* genes were mapped onto 9 of the 12 pepper chromosomes. Chromosome 3 carried the most *CaHAK* genes (five). Chromosome 2 carried four *CaHAK* genes, followed by chromosomes 5, 9, and 11 (two *CaHAK* genes on each chromosome), and chromosomes 1, 6, 8, and 12, each carrying a single *CaHAK* gene. A similar random

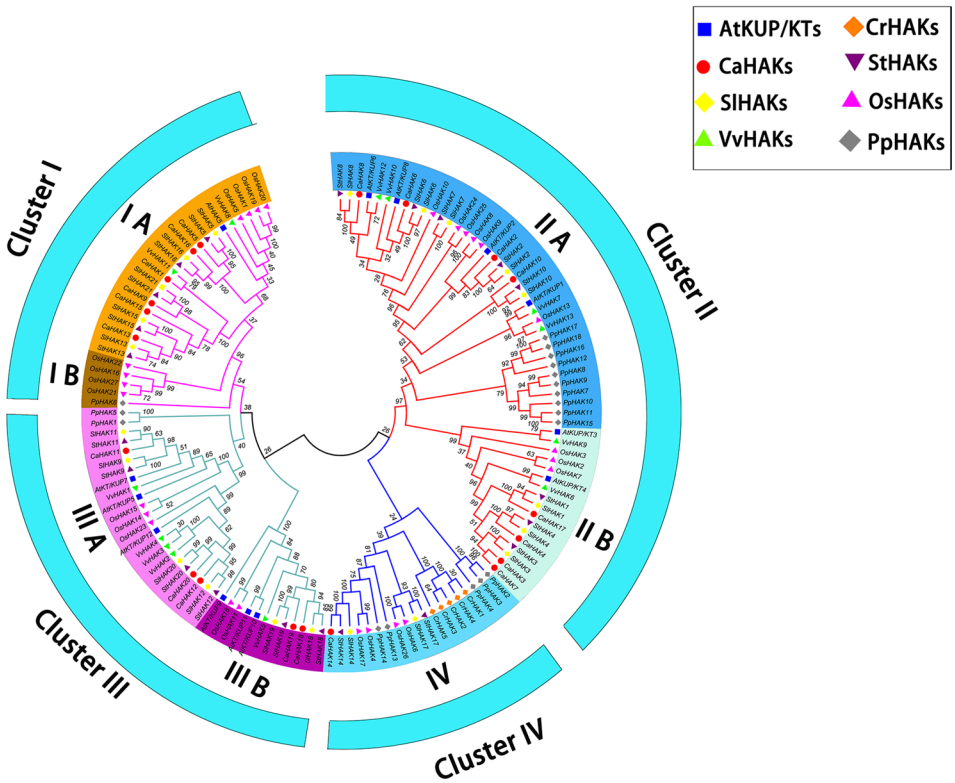

Fig. 1 Phylogenetic analysis of CaHAK proteins from *Capsicum annuum* L. with orthologs from *Arabidopsis thaliana*, *Oryza sativa*, *Physcomitrella patens*, *Chlamydomonas reinhardtii, Solanum lycopersicum*, *Solanum tuberosum*, and *Vitis vinifera*. Different colors indicate different clusters. The phylogenetic tree was constructed by the Neighbor-Joining method, using MEGA7 software and 1,000 bootstrap replicates

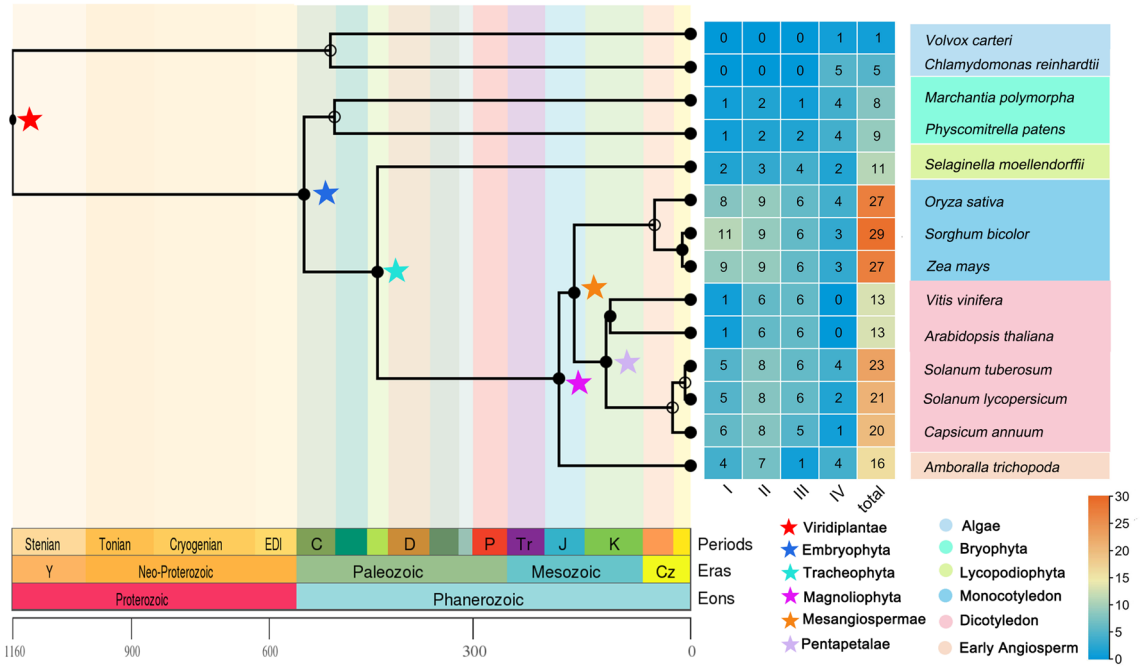

Fig. 2 Phylogeny and diversity of *HAK/KT/KUP* genes in 14 species. The species tree of 14 selected species was constructed using the online software TIMETREE (http://timetree.org/). The number of *HAK/KT/KUP* family gene was visualized by TBtools v1.086. The different color stars and circles represent plant species

distribution model of *HAK/KUP/KT* gene families was also observed in *Arabidopsis* and pears (Ahn et al. 2004; Wang et al. 2018).

In addition, the duplication modes of the *CaHAK* genes were analyzed in pepper, using TBtools. The results identified three segmentally duplicated gene pairs (*CaHAK6*/*CaHAK8*, *CaHAK15*/*CaHAK2*, and *CaHAK4*/*CaHAK18*), and one tandem duplication type gene pair (*CaHAK13*/*CaHAK15*) (Table 1). The frequencies of non-synonymous (Ka) and synonymous (Ks) substitutions and the corresponding ratio (Ka/Ks) can be used to estimate the evolutionary date and the direction of the subsequent selection pressure on these genes (Qiao et al. 2015; Wang et al. 2018). In the present study, the Ka/Ks ratios of these duplicated *CaHAK*s gene pairs were calculated. The results showed that all of the Ka/Ks ratios were < 0.5, indicating that purifying (negative) selection played the main force driving for the evolution of *HAK* genes. The time of divergence of segmentally/tandem *CaHAK* gene pairs was also estimated. According to the results, the divergence times of duplicated genes occurred between 2.81 and 8.29 million years ago (mya).

To further understand the phylogenetic relationship among pepper *CaHAK* genes, the collinear relationships with five other species were constructed, including four dicotyledonous plants (*Arabidopsis*, grape, eggplant and tomato) and one monocotyledonous plant (rice) (Fig. 3). In total, 27 syntenic gene pairs of *CaHAK*s with orthologs of the other four species were found in the pepper genome. The *CaHAK*s genes had eight syntenic gene pairs with tomato (Table S3), nine syntenic gene pairs with eggplant, six syntenic gene pairs with grape, and two syntenic gene pairs with

Table 1 The *Ka* and *Ks* values of duplicated *CaHAK* gene pairs

Duplicated gene pairs	Duplication type	*Ka*	*Ks*	*Ka/Ks*	Time (mya)	Purifyingselection
CaHAK2 vs CaHAK4	Segmental	0.42	1.96	0.22	6.54	Yes
CaHAK6 vs CaHAK8	Segmental	0.37	2.26	0.16	7.54	Yes
CaHAK14 vs CaHAK16	Segmental	0.56	2.49	0.22	8.29	Yes
CaHAK11 vs CaHAK17	Segmental	0.69	2.06	0.33	6.88	Yes
CaHAK1 vs CaHAK9	Tandem	0.17	0.84	0.17	2.81	Yes

Ka, non-synonymous substitution rate; *Ks*, synonymous substitution rate; mya, million years ago

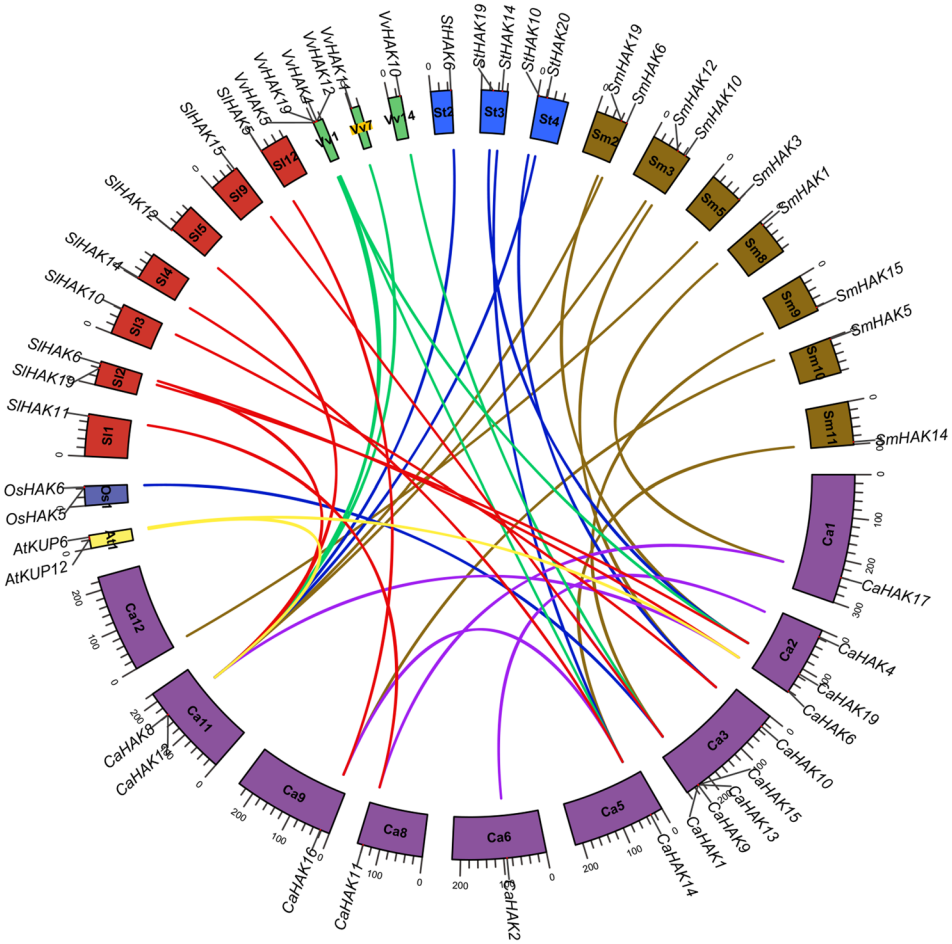

Fig. 3 Syntenic analysis of the *HAK*/*KUP*/*KT* family genes. The lines in different colors inside the circle indicate collinearity relationships among the genes from *Capsicum annuum* and *Arabidopsis thaliana*, *Oryza sativa*, *Solanum lycopersicum*, and *Vitis vinifera*. The duplications between *HAK*s were identified using TBtools software

each of *A. thaliana* and rice. Additionally, the numbers and chromosomal positions of the *HAK*/*KUP*/*KT* family genes in pepper were closely aligned with those of tomato and eggplant, indicating that *HAK*/*KUP*/*KT* genes of solanaceous species were highly conserved during evolutionary divergence.

Gene structure of *HAK*/*KUP*/*KT* family genes

To gain insights into the structural features of *CaHAK*s, the exon/intron structures of 20 *CaHAK*s were analyzed by comparing the corresponding coding sequences (CDS) and genomic sequences (Hu et al. 2015). As shown in Fig. 4, most *HAK*/*KUP*/*KT* genes in pepper contain 8 – 12 exons, and highly conserved exon/intron structures could be observed in the *CaHAK*s belonging to the same cluster. Notably, most of the exons common to all of the *CaHAK*s were 225 bp, 248 bp, 50 bp (or 53 bp), and 260 bp in length, a finding which is consistent with previous results from tomato and pear, indicating that this gene structure was evolutionarily conserved among these species (Liu et al. 2019; Wang et al. 2018). Interestingly, some of the *CaHAK* genes either lacked one intron (*CaHAK6* and *CaHAK8*) or had gained one intron (*CaHAK3*, *CaHAK10*, and *CaHAK16*).

In general, the function of individual CaHAK proteins was reflected in their protein structure. All of the CaHAK proteins contained the K^+ transporter domain (PF02705), which indicated that all 20 *CaHAK* genes could perform similar K^+ acquisition functions. The distribution of 10 conserved motifs in the CaHAK proteins was analyzed, and all 20 CaHAK proteins contained at least eight typical motifs associated with K^+ potassium acquisition, including motifs 2, 3, 4, 6, 7, 8, 9, and 10 (Fig. 4). Although most of the motifs were present and conserved in all 20 CaHAK proteins, some of the motifs were lost from individual CaHAK proteins, such as motif 16, 12 was absent from CaHAK15 and CaHAK10, respectively. This phenomenon had also been reported in *HAK*/*KUP*/*KT* family genes from other species, such as pear and wheat (Cheng et al. 2018; Wang et al. 2018).

Motif analysis of HAK/KUP/KT proteins

To further investigate the protein domains and conserved motifs of CaHAKs, the protein sequences were submitted

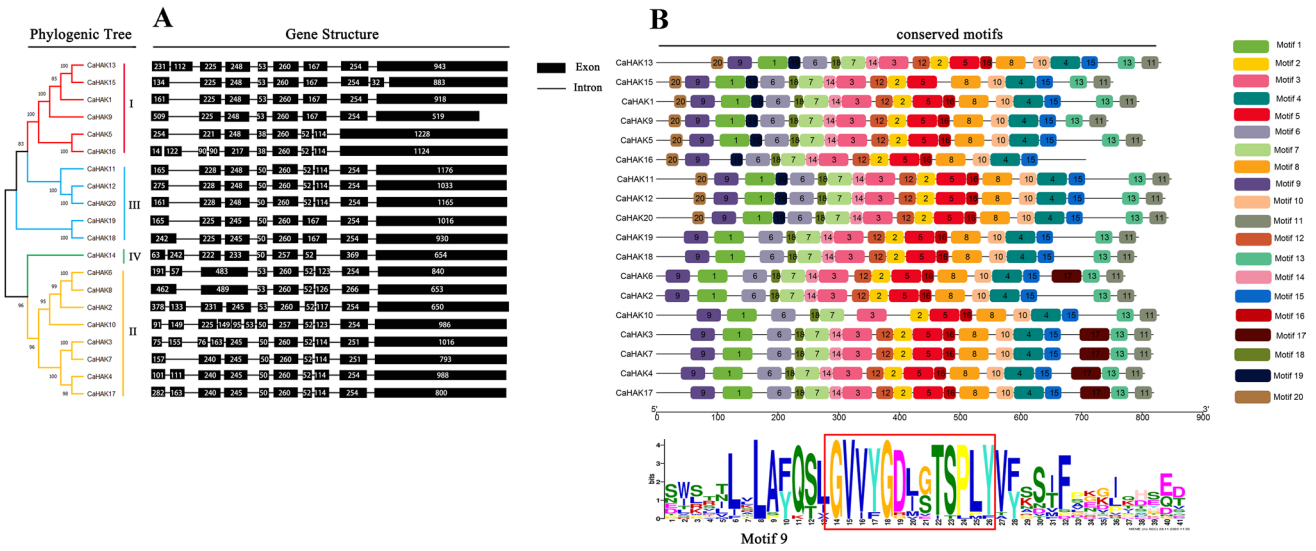

Fig. 4 Gene structure characters of *CaHAK* genes from pepper and the architecture of the conserved motifs in CaHAK proteins. **A** Gene structures of *CaHAK* genes were based on phylogenetic relationship. Black boxes represent exons and lines represent introns. **B** Conserved protein motifs. Conserved motifs of CaHAK proteins were identified by the MEME tool and are shown in different-colored boxes

to the online program MEME and NCBI conserved domain database (Bailey et al. 2009). A subsequent conserved domain analysis also confirmed all of the predicted CaHAK genes. As shown in Fig. S3, there are four types of members for "K_trans superfamily" (cl15781). The conserved domains named as "K_trans", "PLN00149", "PLN00151" were identified in CaHAK14, CaHAK8, and three members of cluster I (CaHAK11, CaHAK12, and CaHAK20), respectively. The other 15 CaHAK members harbor conserved domain of "K_trans superfamily". This result is consistent with that encoded by the reported pear and barley *HAK* gene family (Wang et al. 2018; Cai et al. 2021). In total, 20 conserved motifs were identified in putative CaHAK proteins (Fig. S4). Generally, the 20 motifs were relatively highly conserved among CaHAK family members, a finding which was consistent with the exon/intron structures. It was worth noting that a highly conserved K-transport domain (GVVYGDLGTSPLY) (named motif 9) existed in all CaHAK proteins. In addition, motif 19 only existed in CaHAKs from clusters I and III, whereas motif 17 could only be found in members of cluster II. Based on the TMME2.0 program, it was predicted that the HAK/KUP/KT proteins in pepper had 10–14 transmembrane (TM) domains (Fig. S5), a finding which was similar to previous results from other higher plants (Li et al. 2017; Yang et al. 2020a, b).

Analysis of putative *cis*-regulatory elements in the *CaHAK* promoters

To better understand the expression differences and transcriptional regulation of *CaHAK*s, PlantCARE database was used to identify *cis*-regulatory elements (CREs) in the promoter regions. A total of seventeen types of CREs were identified (Fig. 4). Among the CREs responding to phytohormones, the numbers of abscisic acid- (35) and methyl jasmonate- (30) responsive elements were the highest. The auxin-responsive element was observed in the promoters of only three *CaHAK*s. A total of 13 *CaHAK*s contained the MYB-binding site, with eight *CaHAK* genes containing the MYBHv1-binding site (Fig. 4). Many CREs responding to different stresses, such as defense, anaerobiosis and low temperature, were identified among the *CaHAK*s. Of these, the anaerobic induction response element was the most common. There were also CREs involved in plant growth, including endosperm expression and "zein" metabolism regulatory elements, which were identified in the promoters of three and six *CaHAK* members, respectively (Fig. 5). Interestingly, as the most important environmental factor, light responsiveness was observed in the promoters of almost all *CaHAK*s, except for *CaHAK16*.

Tissue-specific expression patterns of *CaHAK*s

Each of the *CaHAK* genes was expressed in at least one pepper tissue, with 12 *CaHAK* genes exhibiting expression in all the tissues tested in this study (Fig. 6). The heat map of the *CaHAK*s showed that *CaHAK4*, *CaHAK6*, *CaHAK18*, and *CaHAK19* had high levels of constitutive expression in all the tissues analyzed, whereas the expression levels of *CaHAK1* and *CaHAK16* showed much lower levels of expression than those shown by other members, suggesting functional divergence of *CaHAK* genes in different tissues

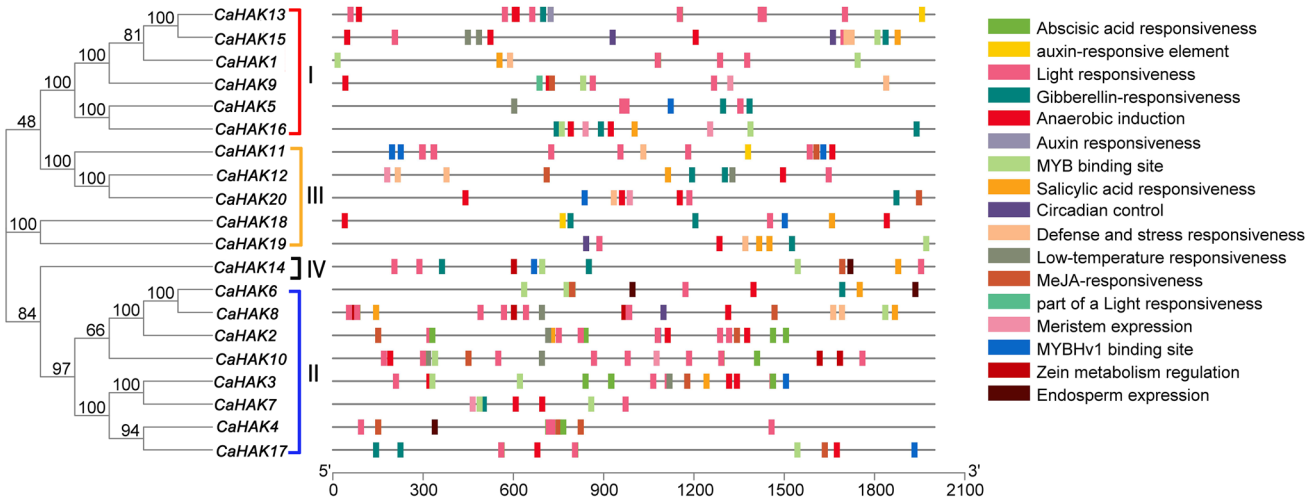

Fig. 5 *Cis*-regulatory elements (CREs) in the promoter regions of *CaHAK* genes. Promoter sequences (− 2000 bp) of twenty *CaHAK* genes were analyzed using PlantCARE. Different CREs are represented by boxes of different colors

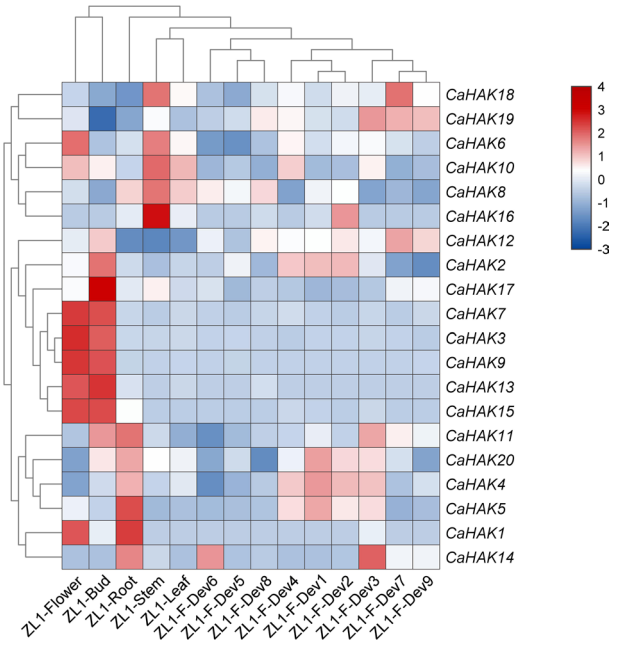

Fig. 6 Expression patterns of 20 *CaHAK* genes in different tissues and stages of fruit development of pepper. The transcript abundance data were processed by $\log_2$ normalization. The red and white colors represent the highest and lowest relative expression levels, respectively

of pepper. Notably, five *CaHAKs* (*CaHAK3*, *7*, *9*, *13*, and *15*) exhibited higher expression levels in flowers and flower buds than in other organs. As an indispensable mineral for higher plant, K^+ plays a key role in pollen germination and tube growth via maintaining osmotic pressure. Similarly, OsHAK1, 19 and 20 clustered with CaHAK9, 13 and 15, also showed high expression levels in anthers and played an important role in receptor-like kinase mediates K^+ homeostasis in pollen tube growth and integrity. This result indicating that these genes with high levels in flower might be involved in flower and bud development via K-mediated turgor pressure. As with the orthologs *AtHAK5* and *OsHAK5*, *CaHAK5* showed high expression levels in roots, implying that *CaHAK5* might function particularly with respect to K^+ acquisition by roots from the soil. In addition, *CaHAK8* and *CaHAK10* showed their highest expression levels in stem tissues. Based on the high expression level of *CaHAK1* and *CaHAK5* genes in pepper roots, we speculated that these genes might potentially play a crucial role in uptake or transport of K^+/Na^+ nutrition.

Expression analysis of pepper *HAK/KUP/KT* genes in response to K^+-deficiency and salt stresses

To investigate the transcriptional response of pepper plant to low potassium conditions, the expression profiles of *CaHAK*s in leaves and roots at 7 days after exposing to low K^+ stress were studied. Among the 20 *CaHAK* genes, nine genes (*CaHAK9*, *CaHAK10*, *CaHAK12*, *CaHAK13*, *CaHAK14*, *CaHAK15*, *CaHAK16*, *CaHAK17*, and *CaHAK18*) were not expressed under the experimental conditions. The expressions of *CaHAK1*, *CaHAK3*, *CaHAK5*, and *CaHAK7* were upregulated in response to low K^+ conditions (Fig. 7). The transcript abundance of *CaHAK5* was significantly higher than that exhibited by the other *CaHAK*s. It is noteworthy that the expression levels of *CaHAK3* and *CaHAK7* were significantly upregulated in pepper roots in response to K^+ deficiency. Hence, *CaHAK3* and *CaHAK7* were selected as candidates of high-affinity K^+ uptake transporters in pepper plants.

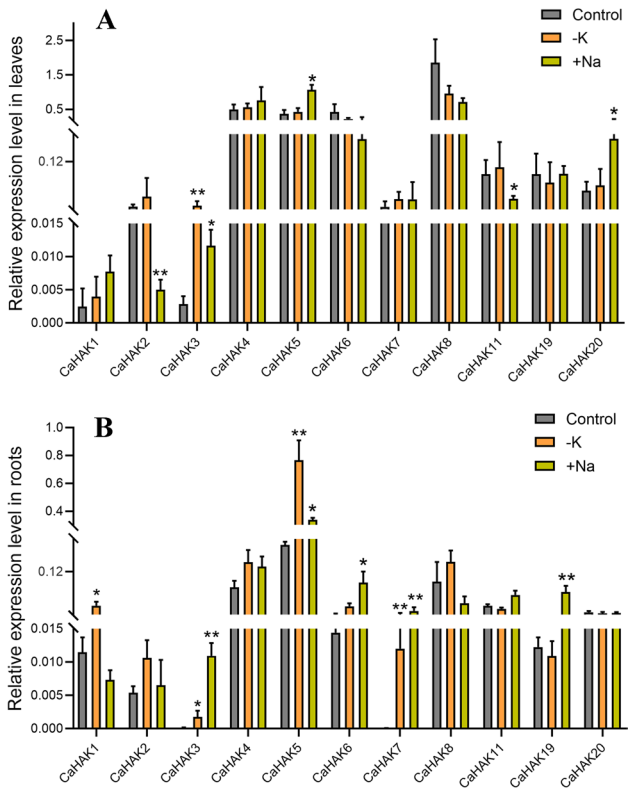

Fig. 7 Expression levels of *CaHAK* genes in response to K^+ deficiency (− K) or salt stress (+Na). The relative transcript levels of *CaHAK* genes in pepper leaves and roots were determined by quantitative real-time PCR (RT-PCR). Error bars represent standard error (SE) ($n=3$). The asterisks indicate significant differences from the corresponding control ($^{*}P<0.05$, $^{**}P<0.01$), using analysis of variance (ANOVA)

To further determine how the expressions of *CaHAK*s were affected by salt stress, the expression profile of *CaHAK*s was determined in pepper plants after 24-h salt treatment (200 mM Na^+). The results showed that most of the *CaHAK*s examined exhibited appreciably up-regulated expression levels in pepper roots, such as *CaHAK3*, *CaHAK5*, *CaHAK6*, *CaHAK7*, and *CaHAK19*, in responding to slat stress (Fig. 7). While, *CaHAK2* and *CaHAK11* showed down-regulated expression levels in pepper leaves under salt stress conditions.

Discussion

As one of the three essential plant macronutrients, K^+ plays crucial roles in multiple physiological and biochemical processes (Anschutz et al. 2014). The HAK/KUP/KT family of genes has been reported to be involved in K^+ uptake under different K^+ supply conditions and in the presence of salt or drought stress, in species, such as *Arabidopsis*, rice, maize, wheat, and tea plants (Cheng et al. 2018; Yang et al. 2009; Yang et al. 2020a, b; Zhang et al. 2012). Still, there is limited information of the molecular mechanisms of potassium uptake in pepper. The completion of whole-genome sequencing in pepper allowed us to comprehensively investigate the regulation and structure of *HAK*/*KUP*/*KT* family genes in pepper (Qin et al. 2014).

In the present study, a total of 20 HAK/KUP/KT family genes were identified from the pepper genome, using BLASTN searches. The HAK/KUP/KT genes were distributed among four major clusters (I ~ IV) (Fig. 1). Additionally, *CaHAK* numbers from clusters II, III, and IV were distributed randomly among the subgroupings, indicating that the monocot/dicot split occurred before the significant duplication events that gave rise to clusters II, III and IV. In the current work, the number of exons in pepper *HAK* genes ranged from 8 to 12 (Fig. 4), a number which is highly conserved with respect to the orthologous *HAK* genes from rice and tomato (Hyun et al. 2014; Yang et al. 2009). Furthermore, the position and length of exons were also highly conserved in pepper and other plant species, such as tomato and pear plants (Hyun et al. 2014; Wang et al. 2018). Interestingly, *CaHAK10* contains 12 exons, which is the largest exon number among the pepper HAK/KUP/KT genes, showing marked similarities with the tomato gene ortholog *SlHAK10*, which is the arbuscular mycorrhiza-induced K^+ transporter gene (Liu et al. 2019). The conservation of gene structure allowed us to predict the potential functions of the pepper HAK/KUP/KT family genes.

Gene duplication contributes significantly to the expansion of gene numbers in plant species and leads to gene diversification, driving the evolution of paralogous genes (Maher et al. 2006). The duplication mechanisms include whole-genome duplication (WGD), segmental and tandem duplication, and rearrangements at the gene and chromosomal levels (Edger and Pires 2009; Jiao et al. 2011). In Chinese white pear, segmental duplication events played a more important role than did tandem duplication events in the expansion of the *PbrHAK* gene family (Wang et al. 2018). Likewise, the expansion of the *HAK*/*KUP*/*KT* family genes was driven primarily by segmental duplication events in poplar (He et al. 2012). This result showed that segmental and tandem duplication events both played vital roles in the evolution of *CaHAK* genes, with the segmental duplication type (representing 80% of duplication events) being more important than the tandem duplication type (20%), suggesting that the evolution of potassium absorption mainly evolved as a result of segmental duplication events, the genes involved showing high levels of conservation in pepper (Table 1). The selection pressure associated with duplicated gene pairs can be divided into three types, namely purifying ($Ka/Ks<1$), positive ($Ka/Ks>1$), and neutral selection ($Ka/Ks=1$) (Yang 2007). In the present study, the *Ka/Ks* ratios of all the duplicated *CaHAK* gene

pairs were < 0.5, suggesting that purifying selection may play the crucial role in adaptation to environmental changes during *CaHAK* gene evolutionary history. It should be noted that the time of divergence of tandem duplicated gene pairs (*CaHAK1*/*CaHAK9*) was less than for segmental duplication events. This finding is similar to previous observations, such as with the fatty acid desaturase (FAD) enzyme family genes in wheat (Hajiahmadi et al. 2020).

Syntenic analysis of *HAK*/*KUP*/*KT* family genes were performed to assess the evolutionary relationship of the *CaHAK* genes in four plant families, namely the Gramineae (*O. sativa*), the Brassicaceae (*A. thaliana*), the Vitaceae (*V. vinifera)*, and the Solanaceae (tomato and eggplant). The results showed that the *CaHAK* genes had eight and nine syntenic gene pairs with tomato and eggplant, respectively, which was more than with rice (two pairs) grape (six pairs) or *Arabidopsis* (two pairs) (Fig. 3, Table S4). Qin et al. (2014) showed that the pepper diverged from tomato ~ 36 mya, and that approximately 38-Mb of genomic sequences of pepper was aligned to tomato, with 14% nucleotide divergence (Qin et al. 2014). There were more collinear gene pairs between pepper and tomato than between pepper and the other species, consistent with the fact that both pepper and tomato are members of the Solanaceae family.

The transcriptional and post-transcriptional regulation of genes encoding K^+ transporters or channels are two common mechanisms in plants for achieving increased fitness to K^+-deficiency conditions (Li et al. 2018; Wang and Wu 2013, 2017). Previous studies revealed that the *HAK* genes in cluster I and cluster V were mainly induced by potassium deficiency. For example, the expression of *AtHAK5*, *AtKUP7*, *OsHAK1*, *OsHAK5*, and *HvHAK1* was significantly upregulated in roots under low-K^+ conditions, maintaining K^+ uptake and translocation from root to shoot (Chen et al. 2015; Gierth et al. 2005; Han et al. 2016; Santa-María et al. 1997; Yang et al. 2014). The *AtHAK5* T-NDA insertion mutant plants showed a lower K^+ accumulation in roots compared to wild-type plants under K^+-deficient conditions. The K^+ content in shoots but not in roots of *atkup7* mutant plants exhibited a significantly decreased compared with those in the wild-type plants (Gierth et al. 2005; Han et al. 2016). Recent study revealed that the *atkup9* mutant exhibited a short-root phenotype growth under low-K conditions. In addition, the Cs^+ accumulation was significantly increased in the *atkup*9 mutant plants, indicated that AtKUP9 played an important role in root growth and Cs^+ homeostasis in *Arabidopsis*. In this study, the expression of *CaHAK1*/*CaHAK5* (cluster I) and *CaHAK3*/*CaHAK7* (cluster II) was upregulated following exposure to low-K^+ stress, implying that these genes are involved in mediating K^+ uptake and transport in pepper under K^+-deficiency conditions. Interestingly, the *CaHAK3* and *CaHAK7* genes showed similar expression patterns in response to either K^+ deficiency or salt stress in pepper roots. As the two pepper *HAK*/*KUP*/*KT* paralogs, *CaHAK3* and *CaHAK7*, showed a high degree of overlapping expression, this finding strongly implied genetic redundancy with the two genes.

In higher plant species, maintaining K^+ uptake and the cellular K^+/Na^+ ratio is essential for salt tolerance (Cuin et al. 2003; Maathuis 2006; Shabala & Cuin 2008). It has been repeatedly documented that *HAK*/*KUP*/*KT* members are upregulated in response to salt stress. In *Arabidopsis*, transcript abundances of *AtKUP6* and *AtKUP11* were significantly upregulated in response to salt stress (Maathuis 2006). The *OsHAK5* and *OsHAK21* transcript levels were also upregulated under high Na^+ conditions, playing crucial roles in salt stress tolerance by maintaining the K^+/Na^+ homeostasis in plant cells (Shen et al. 2015; Yang et al. 2014). In pepper, the expression level of *CaHAK5* and *CaHAK11* was significantly upregulated by salt stress, whereas *CaHAK1* expression was rapidly downregulated under high-Na^+ condition. These results implied that the *CaHAK* genes played a role in maintaining the K^+/Na^+ homeostasis in pepper under salt stress.

Conclusion

In the present study, a total of 20 *CaHAK* genes were identified in pepper (*Capsicum annuum* L). These genes were classified into four clusters (I–IV), based on the topology of the phylogenetic tree and on structural feature analysis. Exon/intron distribution and comparisons of domains/motifs were conserved in each cluster. Promoter analysis revealed associated with plant growth and development, stress-responsive and phytohormone elements in the *CaHAK*s promoter regions, indicating functional roles of CaHAKs in response to different stresses and developmental stages. *CaHAK* genes, except *CaHAK7*, were distributed on 9 of the 12 pepper chromosomes. According to the analysis of collinearity, the whole-genome triplication might contribute to the expansion of the *CaHAK* gene family. Moreover, the results of the *Ka/Ks* ratio revealed that tandem/segmental duplications have contributed to the expansion of the *CaHAK* family genes, and purifying selection played the key role in the divergence of the CaHAK genes in pepper. High expression of CaHAKs showed that *CaHAK3*, *CaHAK7*, *CaHAK9*, *CaHAK13*, and *CaHAK15* in flower and buds, implied their potential functions in flower and buds development, even fruit setting. The analysis of expression patterns under low K^+ and high Na^+ conditions suggested that CaHAKs exhibited functional divergence in the processes of plant K^+ uptake and salt stress tolerance. Taken together, this study provided insights into the HAK-mediated low K^+ response of pepper at the transcriptional level and identified

candidate genes that may be exploited to improve crop tolerance to abiotic stresses.

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Author contributions JRZ, GHQ, and JJL planned and designed the research; JRZ, JJL, CYL and JYL performed the experiments; XLL, JRZ and JZ analyzed the data; GHQ, and JJL wrote the manuscript.

Declarations

Conflict of interest The authors declare that they have no competing interests.

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