Genome-wide identification, characterization and expression analysis of *HAK* genes and their role in responding to potassium deficiency and abiotic stress in *Medicago truncatula*

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Background. HAK (high-affinity K^+)/ KUP (K^+uptake)/ KT (K^+transporter) (HAK) family is the largest potassium transporter family, playing a vital role in potassium uptake, plant growth, plant biotic and abiotic stress responses. Although *HAK* family members have been characterized and functionally investigated in many species, study of these genes in *Medicago truncatula* (*M. truncatula*) is still poorly known.

Methods. In this study, a screen of *M. truncatula HAK* family members (*MtHAKs*) was conducted. The identification, phylogenetic analysis and prediction of conserved motifs of *MtHAKs* were extensively explored. Moreover, expression levels *of MtHAK s*under potassium deficiency, drought, and salt stresses were assayed by quantitative real-time PCR (RT-qPCR).

Results. Here, a total of 20 *MtHAK* family members were identified and classified into three clustersbased on phylogenetic relationships. Conserved motif analyses showed that allMtHAK proteins except for MtHAK10 contained the highly conserved K^+ transport domain (GVVYGDLGTSPLY). The RT-qPCR analysis showed that several *MtHAK* genes in roots were expressed in abiotic stress-responsive manners. In particular, transcript abundance of *MtHAK15*, *MtHAK17* and *MtHAK18* was strongly and specifically up-regulated in *M. truncatula* roots under potassium deficiency, drought, and salt stress conditions, implying that these genes are candidates for high-affinity K^+ uptake and have essential roles in drought and salt tolerance.

Discussions. Collectively, these results not only provide the first genetic description and evolutionary relationships of the potassium transporter family in *M. truncatul*a, but also provide potential genes responding to potassium deficiency and abiotic stresses, laying the foundation for molecular breeding of stress-resistant legume crops in the future.

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Abstract

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Discussions. Collectively, these results not only provide the first genetic description and evolutionary relationships of the K^+ transporter family in *M. truncatul*a, but also provide potential genes responding to K^+ deficiency and abiotic stresses, laying the foundation for molecular breeding of stress-resistant legume crops in the future.

Introduction

Potassium (K^+) is an essential macronutrient to drive various plant physiological functions, such as the maintenance of electrical charge balance and the transport of nitrate and sugars (Li et al. 2018). Owing to the limited K^+ resource, plants have evolved a series of K^+ transport systems to mediate K^+ uptake and transport (Amrutha et al. 2007; Ashley et al. 2005; Gierth et al. 2005; Very et al. 2014). In plants, K^+ transporters were classified into four major families: HAK (high-affinity K^+)/KUP (K^+ up-take)/KT (K^+ transporter), Trk/HKT, CHX (cation/hydrogen exchanger), and efflux antiporters KEA (K^+ efflux antiporter) (Gupta et al. 2008). Among them, the HAK/KUP/KT (HAK) family constitutes the largest K^+ transporter family that is responsible for K^+ transport and ubiquitously presents in plant genomes with varied numbers, such as 13 genes present in *Arabidopsis*, 27 in maize, and 27 in rice (Ahn et al. 2004; Corratge-Faillie et al. 2010; Gupta et al. 2008; Rubio et al. 2000; Zhang et al. 2012).

HAK genes have been found to play key roles in plant development and stress-related responses. For instance, *AtKUP4/TRH1* (*Tiny Root Hairs 1*) maintains polar localization of AtPIN1 and auxin homeostasis and maxima in the root apex, which promotes root gravitropism response and root hair elongation(Rigas et al. 2001; Rigas et al. 2012; Vicente-Agullo et al. 2004). VvKUP2 (Vitis vinifera) promotes the expansion of berry epidermal cells (Davies et al. 2006). *AtKUP2/SHY3* (*Short Hypocotyl 3*) mediates K^+-dependent cell expansion in growing

tissues, and *shy3-1* mutant plants have shorter hypocotyls, smaller leaves, and shorter flowering stems compared to the wild type (Elumalai et al. 2002b). *Arabidopsis Kup2/6/8* triple mutants display obviously larger plant bodies, suggesting their roles as negative regulators during the turgor pressure–dependent growth (Osakabe et al. 2013). *Arabidopsis* root meristem activity are maintained by *KUP9* through regulating the K^+ level and auxin homeostasis upon low K^+ level (Zhang et al. 2020). *HAK5* promotes the expression of *INTEGRIN-LINKED KINASE1* (*ILK1*) to positively regulate innate plant immunity and abiotic stress response in *Arabidopsis* (Brauer et al. 2016). The expression of rice *OsHAK1* is induced obviously under K^+ deficiency stress, OsHAK1 overexpression plants display enhanced salt and drought tolerance (Chen et al. 2015; Chen et al. 2017; Chen et al. 2018). In addition, constitutive overexpression or mutation analysis of *OsHAK5*, *OsHAK21* and *OsHAK16* indicated their role in K^+homeostasis and salt tolerance (Feng et al. 2019; Horie et al. 2011; Shen et al. 2015). HvHAK1 confers salt and drought tolerance in barley by enhancing leaf mesophyll H^+ homoeostasis and improving K^+ nutrition (Feng et al. 2020; Mangano et al. 2008).

In plants, the HAK family is the homolog of bacterial K^+ transporter KUP and fungal K^+ transporter HAK (Bañuelos et al. 1995; Schleyer & Bakker 1993; Very et al. 2014). Based on the hydropathy profiles, the plant HAK proteins have been predicted to have 10–14 transmembrane (TM) domains, including a conserved K^+ transport domain (GVVYGDLGTSPLY) (A & B 2007; Alonso & Rodríguez-Navarro 2000). Analysis of mutation assay revealed that the role of K^+ transport capacity is determined by the 8th TM domain and the C-terminus of HAKs (Alonso & Rodríguez-Navarro 2000; Gomez-Porras et al. 2012; Mangano et al. 2008). Based on phylogenetic analysis, HAK family genes were generally classified into four clusters (I-IV) (Bañuelos et al. 1995; Gupta et al. 2008). HAK family members exhibit considerable diversity in subcellular localizations, including the plasma membrane, tonoplast, endoplasmic reticulum and other endomembranes (Osakabe et al. 2013; Rigas et al. 2012). Expression analysis revealed that many members of the HAK family are expressed in root hairs and root tip cells, implying the functions of HAK family members in K^+ uptake (Ahn et al. 2004; Elumalai et al. 2002b; Qin et al. 2019; Yang et al. 2014). Indeed, several HAK family members have been shown recently to participate in K^+ uptake and translocation in a few model species, such as *Arabidopsis*, rice, barley, maize, and tomato (Very et al. 2014).

M. truncatula has been a model system for studying legume genetics and its relatively small genome size facilitates the study for nodule symbiosis (Young et al. 2011). Despite the functional importance of the HAK genes, surprisingly little is known about these family members in *M. truncatula*. In this study, we performed comprehensive genome-wide analyses of *M. truncatula HAK* family genes including phylogenetic relationships, chromosome distributions, gene duplications, gene structures, *cis*-acting regulatory elements and expression pattern in response to K^+ deficiency and abiotic stress. These results uncover the structures and expression patterns of 20 *MtHAKs* genes, laying the foundation for future functional analysis of *HAK* genes in *M. truncatula*.

Materials & Methods

Identification and sequence analysis of *MtHAKs*

MtHAKs sequences were obtained from *Medicago truncatula* genome databases (http://www.medicagohapmap.org/). The amino acid sequences of *Arabidopsis* (TAIR, http://www.arabidopsis.org/) and rice (TIGR, http://rice.tigr.org) HAKs were used as the reference sequences searching predicted homolog sequences in *M. truncatula* by HMMER3.0 software (http://hmmer.org/), subsequently screened the genes using a threshold of less than 1E-100 E-value (full sequence and best 1 domain). Candidate protein members were verified by SMART databases (http://smart.embl-heidelberg.de/) and NCBI-Conserved Domain data base (CDD, https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) (Zhao et al. 2021), while the proteins with shorter amino acid length (<400 aa), and containing incomplete K^+ transporter domains were discarded. The longest gene was chosen for further analysis if it had alternative splicing variants. Subcellular localization of MtHAK proteins was predicted using WOLF PSORT software (https://www.genscript.com/wolf-psort.html) and TMHMM Server 2.0 online tool was used for prediction of protein transmembrane helices (http://www.cbs.dtu.dk/services/TMHMM-2.0/).

Construction of MtHAKs phylogenetic tree

HAK protein sequences of *Arabidopsis* and rice were retrieved from the NCBI database (https://www.ncbi.nlm.nih.gov) (Table S1) and multiple sequence alignment was conducted through ClustalW program (Version 2.1; http://www.clustal.org/). MEGA7.0 was used to

construct the phylogenetic tree with Neighbor-Joining method and the bootstrap replicates are up to 1000 (Liu et al. 2019; Liu et al. 2020).

Gene structure and conserved motif analysis

Gene structure and conserved motifs were visualization by TBtools software (Chen et al. 2020). The conserved and identified motifs of protein sequences were predicted through the MEME program (Version 5.1.1), the maximum protein motif number was set as 10, and the other parameters were set as default (http://meme-suite.org/tools/meme) (Bailey et al. 2009).

Chromosomal location and synteny analysis

MtHAK Chromosomal location was illustrated by the circos diagram through annotating genes to specific chromosomal location in their genome sequences using software TBtools. These syntentic analysis were carried out by using the MCScanX with gene duplication parameters.

Analysis of *cis*-Acting regulatory elements in *MtHAKs* promoter regions

Putative *cis*-acting regulatory elements were analyzed using the PlantCARE software online (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). The 2.0 kb promoter sequences that located in the upstream of the transcription starting site in each *MtHAK* were extracted from the *M. truncatula* genome database.

Analysis of microarray expression profile

Expression profiles by microarray data of *MtHAKs* in roots, vegetative bud, stem, petiole, leaf, flower, pods, and seeds and responses to abiotic stress were obtained from the MtGEA (Benedito et al. 2008). When a gene corresponding to multiple probes, maximum value of the probe was selected for the subsequent analysis. The normalized microarray data were used to create the heatmap through the software TBtools, basing on the mean value of each gene expression in all analyzed organs. The expression patterns of *MtHAKs* response to salt, drought, and cold stresses were obtained from the NCBI under GEO accession number GSE136739 (Song et al. 2017). Expression abundance of each *MtHAK* gene was represented by fragments per kilobase million (FPKM). The relative expression levels upon stresses were calculated by comparing with the control samples (0 h). The clustered heatmap was exhibited using the software TBtools and based on their relative expression.

Stress treatment and qRT-PCR

For K^+ deficiency stress treatment, two-week old seedlings were incubated in 1/2 Hoagland nutrient medium without K^+ for 0 (control), 1, 6, 12, 24, and 48 h, respectively. For salt stress

treatment, two-week old seedlings were incubated in 1/2 Hoagland nutrient medium with 300 mM NaCl for 0, 1, 6, 12, 24, and 48 h, respectively. For drought stress treatment, two-week old seedlings were incubated in 1/2 Hoagland nutrient medium with 18% PEG6000 for 0, 1, 6, 12, 24, and 48 h, respectively. The root samples were cut, then immediately frozen in liquid nitrogen and stored at − 80 °C until use. The qRT-PCR analysis was performed in quadruplicate for each of the biological replicates. The relative expression level was calculated according $2^{-\Delta\Delta Ct}$ analysis method (Liu et al. 2019). The expression levels of control samples (0 h) were normalized to 1. The *MtActin* gene was used as the internal control. Standard deviations are indicated by error bars and the significant differences are indicated with ‘‘*’’ ($P < 0:05$).

Results

Identification of HAK members in *M. truncatula*

To identify *M. truncatula HAK* genes, a genome-wide searches were conducted using the HMMER3.0 Software (http://hmmer.org/) basing on the *M. truncatula* genome sequences with *Arabidopsis* and rice *HAK* genes as subjected queries. Then, a total of 20 nucleotide sequences with a typical canonical K^+ transporter domain (Pfam accession no. PF02705) were identified using Pfam and SMART databases, which were entitled as *MtHAK1* to *MtHAK20* depending on their chromosomal positions (Table 1). Detailed information of the 20 *HAK* genes was listed in Table S1. The protein transmembrane segments (TMS) ranged from number of 10 to 13, with the most common number of 12–13 TMS (70%). All the examined HAK proteins were predicted mainly being localized in the plasma membrane by a PSORT analysis (http://www.psort.org). The protein length of the 20 identified HAK proteins ranged from 619 amino acids (aa) (MtHAK3) to 856 aa (MtHAK2) with an average number of 778 aa, and their relative molecular weights (MW) varied from 69.03 kDa (MtHAK3) to 95.67 kDa (MtHAK2). The isoelectric points (pI) ranged from 5.44 (MtHAK8) to 9.39 (MtHAK19).

HAKs phylogenetic relationship among *M. truncatula*, *Arabidopsis* and rice

To analyze the evolutionary relationships of the MtHAK proteins, phylogenetic analyses of 60 *HAK* amino acid sequences, including 20 in *M. truncatula*, 13 in *Arabidopsis*, and 27 in rice, were performed to constructed a phylogenetic tree by neighbor-joining method. According to the evolutionary tree, all *HAK* members were classified into four major groups: Groups I- IV. MtHAK proteins were classified into three clusters (from I to III), with MtHAK6, -14, -15, -17,

and -18 in cluster I, MtHAK4, -7, -9, -10, -11, -12, -13, -19, and -20 in cluster II and MtHAK1, -2, -3, -17, -5, and -8 in cluster III (Figure 1). All members in group IV belong to rice. The most abundant members existed in Cluster II in *M. truncatula*, comprising 45% among all MtHAKs. The phylogenetic tree showed that MtHAKs were mostly closely related to *Arabidopsis* KUPs than those of rice HAKs, indicating that MtHAKs might have conserved function in evolution with *Arabidopsis* KUPs. All MtHAKs in cluster I were distributed together with the already-identified AtHAK5, suggesting that they may play crucial roles in K^+ uptake from a low-K^+ level soil (Lara et al. 2020). Among members in cluster II, MtHAK4 shared high sequence identity with AtKUP2 (Elumalai et al. 2002) and MtHAK19 shared high sequence identity with AtKUP4 (Rigas et al. 2001; Vicente-Agullo et al. 2004), implying they are likely to be involved in plant development processes. In addition, among cluster III, MtHAK1 and MtHAK8 clustered together with AtKUP7 (Han et al. 2016), suggesting the role of MtHAK7 in K^+ acquisition and translocation under low K^+ concentration.

Gene structure and motif composition of *MtHAK* genes

MtHAK proteins were listed in order based on the phylogenetic analysis (Figure 2A), which were consistent with the results in figure 1. Closely related members share similar exon/intron structures which is related to their biological function. Gene structures of the *MtHAKs* were harbored based on the arrangement of sequence in the untranslated region, exon, and intron using software TBTools. As shown in Figure 2B, the exons number of *MtHAK* genes varied from 8 to 10, with the longest exon in the last one except for *MtHAK2*, which is consistent with previous reported *HAKs* (He et al. 2012; Hyun et al. 2014). In addition, most of the *MtHAKs* in the same cluster shared high exon-intron structure similarity (Figure 2B).

To study the structural features, conserved protein motifs of MtHAKs were analyzed by MEME program. In total, conserved protein motifs varying from number 29 to 50 aa. in lengths were identified and named as motifs 1-10. Conserved protein motif information was shown in Table S2. The highly conserved K^+ transport domain (GVVYGDLGTSPLY), included in motif 9, existed in all MtHAK proteins except for MtHAK10 (Figure2, Table S2). Motifs 1, 2, 3, 4, 5, 6, 7, 8, and 10 were almost evenly distributed with possessing a feature domain of K^+ transporter (Figure 2C, Table S2) in all MtHAK proteins. Together, the motifs of conserved K^+ transporter and similarities of gene structure in the same cluster implied the closing function among these HAK members.

Chromosomal distribution and synteny analysis of *MtHAK* genes

All identified *MtHAK* genes were mapped onto chromosomes from the *M.truncatula* genome database to identify and locate their chromosomal distribution. Results showed that *MtHAKs* were distributed on seven of the eight chromosomes, with chromosome 8 containing the highest number of six *MtHAK* genes (Figure 3). Five *MtHAK* genes were located on chromosome 5, three on chromosome 2, two on chromosome 4 and 6, one on chromosome 3 and 7, and no gene was allocated on chromosome 1 (Figure 3). These results indicated that *MtHAKs* were scattered randomly onto different chromosome locations.

Synteny analysis was further performed between *M. truncatula* and *Arabidopsis* to verify the evolutionary relationships and history of *MtHAK*s. Seven collinear gene pairs in total were found between *M. truncatul*a and *Arabidopsis* in the dataset (Figure 3 and Table S3), indicating that these identified genes might have already existed before protein structure divergence, further implying strong phylogenetic relationship. In addition, only one gene pair (*MtHAK2*/*MtHAK5*) exist as paralogs in *M. truncatula*.

Analysis of *cis*-Acting elements in the promoter region of *MtHAK* genes

To further investigate gene function and regulation mechanism of *MtHAKs*, 2 kb upstream regions of the translation start site of the 20 *MtHAK* genes were analyzed using the PlantCARE database. A total of 73 putative *cis*-elements were identified in the *MtHAK* promoters based on functional annotation, and the *cis*-elements of various major types was shown in figure 4 and table S4. *Cis*-elements responding to different plant hormones such as auxin (TGA-element and AuxRE-core), gibberellin (GARE-motif and P-box), MeJA (TGACG-motif and CGTCA-motif), ethylene (ERE-box), ABA (ABRE), and salicylic acid (TCA-element) were identified in the promoter regions of all *MtHAKs* genes except *MtHAK20*, suggesting that *MtHAKs* expression may be regulated by different phytohormones. Elements responsive to abiotic stress were also found in all *MtHAKs* promoter region except *MtHAK20*, such as the stress-responsive element STRE, ARE, WRE3, WUN-motif, MBS, LTR, DRE-core and DRE1 and TC-rich repeats. Additionally, zein metabolism regulation element (O2-site), endosperm expression element (GCN4-motif and AACA-motif), palisade mesophyll cells element (HD-Zip 1), meristem expression element (CAT-box and CCGTCC-motif),and seed regulation element RY-element were also enriched in the promoter of *MtHAKs* except *MtHAK20*. Among *MtHAK20*, light-responsive elements were identified in abundance (Table S4).

Spatial expression profiles of *MtHAK* genes

To gain further insights into the potential biological function of *MtHAK* genes, publicly available microarray data of the *Medicago truncatula* Gene Expression Atlas (MtGEA, https://mtgea.noble.org/v3/) were used to investigate the temporal and spatial expression pattern of *MtHAKs*. *MtHAK4* showed relatively high expression in all tissues, while the expression of *MtHAK18* in all tissue was low (Table S5). Notably, *MtHAK6* and *MtHAK16* were expressed preferentially in the roots, implicating their role in K^+ acquisition from the soil (Figure 5, Table S5).

MtHAK2 and *MtHAK3* both from cluster III exhibited similar expression patterns and relatively high expression in leaves. *MtHAK13* was exclusively and highly expressed in floral organs, and *MtHAK8* was only highly expressed in immature seeds (Figure 5, Table S5). Interestingly, *MtHAK5* and *MtHAK12* exhibited highly and gradually increased expression patterns during reproductive stages and peaked at 24 days after pollination (DAP), while *MtHAK15* was specifically highly expressed in immature seeds (10 DAP) and the expression pattern gradually decreased along with the seed maturation. The spatial and temporal expression profiles suggested functional diversity of *MtHAK* genes in Medicago development.

Expression patterns of *MtHAK* genes under K^+ deficiency

Due to the major function of *HAK* family in K^+ transport, we investigated the expression profiles of *MtHAK* genes in the roots upon K^+ deficiency conditions by qRT-PCR. As shown in figure 6, among the examined 20 genes, we obtained 8 genes that showed upregulated expression pattern after K^+ deficiency treatment. *MtHAK6*, *MtHAK7* and *MtHAK17* transcripts were slightly increased and finally peaked at 48 h. *MtHAK15* and *MtHAK18* showed nearly the same expression pattern at the five time points, respectively. *MtHAK9*, *MtHAK10* and *MtHAK11* transcripts were strongly up-regulated at 6 h, arriving at peak value at 12 h and 24 h, and fell back at 48 h. The results suggested that the these *MtHAK* genes were K^+ deficiency responsive. It is noteworthy that *MtHAK6* were highly and specifically expressed in Medicago roots and significantly upregulated in response to K^+ deficiency.

Expression patterns of *MtHAK* genes under salt and drought stresses

Several *HAK* genes have been reported to participate in abiotic stresses (Elumalai et al. 2002; Vicente-Agullo et al. 2004) (Chen et al. 2015; Shen et al. 2015) . To verify this hypothesis, the expression profiles of 8 K^+ deficiency responding genes were evaluated by qRT-PCR under

salt and drought treatments. The results revealed that all examined 8 genes could be induced by salt and drought stresses to different degrees (Figure 7 and 8).

For the salt treatment, the expression profile of *MtHAK* genes was determined in Medicago roots at different times (0 h, 1 h, 6 h, 12 h, 24 h, and 48 h) under salt treatment (300 mM NaCl in nutrient solution). The results showed that *MtHAK7*, *MtHAK9*, *MtHAK15*, *MtHAK17* and *MtHAK18* exhibited appreciably up-regulated expression levels in Medicago roots. Interestingly, *MtHAK7* and *MtHAK18* were quickly and continuously upregulated from 1 h and subsequently increased at 48h (Figure 7).

Additionally, the expression profiles of *MtHAK* genes in Medicago roots were assayed under drought treatment simulated by 18% PEG6000 at different times (0 h, 1 h, 6 h, 12 h, 24 h, and 48 h). Under drought treatment, all selected genes except for *MtHAK9* were upregulated, albeit to different levels at different times (Figure 8). In particular, *MtHAK10*, *MtHAK15,* and *MtHAK18* responded rapidly to dehydration at 1 h. Comparatively, *MtHAK17* was moderately upregulated from 6 h to 48h. *MtHAK6* and *MtHAK7* exhibited highly induced expression at 24 h.

Interestingly, we found that *MtHAK15*, *MtHAK17,* and *MtHAK18* were strongly upregulated by salt and drought stresses. The expression level of *MtHAK18* increased rapidly at 1h than control under salt and drought treatment (Figure 7 and 8).

Discussion

HAK family genes play key roles in catalyzing K^+ acquisition and uptake, as well as in plant growth, development, and abiotic stress response (Osakabe et al. 2013; Zhao et al. 2016). Although comprehensive genome-wide analysis of the *HAK* gene family has been widely reported in various plants, studies of the *HAK* gene family in the model legume *M. truncatula* are still lacking (Ahn et al. 2004; Gupta et al. 2008; Zhang et al. 2012). The release of the *M. truncatula* genome information makes it possible to systematically characterize and identify the *HAK* genes. In this study, a total of 20 *HAK* genes were identified in *M. truncatula.* We characterized their genetic structures as well as their expression patterns in different tissues or during stress responses.

The 20 identified *HAK* members were classified into three clusters (cluster I to III) based on the evolutionary relationships, which is consistent the with previous classification of *Arabidopsis* (Figure 1)(Rubio et al. 2000). Phylogenetic analysis of HAK proteins revealed that MtHAKs

share high similarity with AtHAKs compared to OsHAKs (Figure 1), suggesting that MtHAKs may have similar functions with *Arabidopsis* AtHAKs. Gene structure analysis showed that *MtHAK* genes contained 8 to 10 exons, with the last exon in the gene structure exhibited the maximum length except for *MtHAK2*, which is consistent with the previously reported exon-intron structure of *HAKs* (He et al. 2012; Hyun et al. 2014). Conserved protein motif analysis indicated that all the identified MtHAKs had at least five typical K^+ transporter motifs.

The gene tissue specific expression patterns probably reflect their function and potential biological roles in plants. Approximately 10 of 13 *Arabidopsis AtHAK* genes are strongly expressed in the root organ (Ahn et al. 2004). *MtHAK6* were preferentially highly expressed in the roots, and belonged to the same clades of *AtHAK5* in the phylogenetic tree, which expressed in roots and mediated high-affinity root K^+ uptake (Lara et al. 2020), implicating their role in K^+ acquisition from the soil (Figure 5, Table S5). *MtHAK16* showed high similarity to *AtKUP12*, which were relatively specific expressed in root hair (Ahn et al. 2004). These results may help clarify the biological function of *Arabidopsis* orthologue *MtHAK* genes in K^+ acquisition in *M. truncatula*.

Some plant *HAK* genes were proved to participate in plant growth and development. For instance, mutation of A*tKUP4/TRH1* impaired root gravitropism response and root hair elongation (Rigas et al. 2001; Rigas et al. 2012; Vicente-Agullo et al. 2004). Knockout of *AtKT2/KUP2* caused shorter hypocotyl length, small rosette leaves, and short flowering stem phenotype (Elumalai et al. 2002). *MtHAK13* was exclusively and highly expressed in floral organs, while showed low expression level in other tissues, suggesting its critical role in floral development. *MtHAK5*, *MtHAK8*, *MtHAK12*, and *MtHAK15* were specifically and highly expressed in reproductive stages, implying their roles in facilitating seed maturation and maintaining fertility. The different tissues expression pattern of *MtHAK* genes indicate their diverse functions in plants.

Under K^+ deficiency conditions, plants maintain K^+ homeostasis in the cytoplasm through absorbing K^+ through HAKs, and K^+ transporter genes represent a major mechanism upon low-K^+ stress by transcriptional regulation. AtHAK5 and AtKT1 are two essential transporters mediating high-affinity K^+ uptake in the roots of *Arabidopsis*, and the double-mutant roots was insufficient to sustain plant growth (Lara et al. 2020). ZmHAK5 was characterized as a high-affinity K^+ transporter in maize (Qin et al. 2019). The expression of OsHAK1 and OsHAK5 was

significantly upregulated in roots under low- K^+ conditions, maintaining K^+ uptake and translocation from root to shoot (Chen et al. 2015; Chen et al. 2017; Chen et al. 2018; Yang et al. 2014). In our study, K^+ deficiency upregulated eight *MtHAK*s transcript expression in the roots, especially *MtHAK10* and *MtHAK11* (Figure 6). *MtHAK6* was preferentially highly expressed in the roots, and increased under K^+ deficiency stress (Figure 6). Therefore, we anticipate that several *HAK* genes could increase the capacity of K^+ absorption upon K^+ deficiency.

Previous studies reported that *HAK* genes were crucial to regulate water potential and turgor pressure during osmotic adjustment. These genes also positively regulated stress responses in plants through controlling the balance of K^+ influx and efflux, such as *OsHAK1* transcript expression elevates in the rice roots after K^+-starved condition and positively regulates the response to salt and drought tolerance (Chen et al. 2015; Chen et al. 2017; Chen et al. 2018). Consistent with the results above, in our study, for example, *MtHAK7*, *MtHAK9*, *MtHAK15*, *MtHAK17* and *MtHAK18* exhibited appreciably up-regulated expression levels in Medicago roots under salt tolerance (Figure 7). Interestingly, many *cis*-acting elements related to phytohormone, plant growth and development, abiotic stress response were extensively distributed in the promoter regions of *MtHAKs* (Figure 4). ABREs are elements that response to drought stress (Sah et al. 2016). *MtHAK* genes that possess ABRE elements in promoters and response to drought stress further imply that *MtHAKs* participate in drought responses (Figure 4 and 6). Moreover, qRT-PCR analysis showed that most selected *MtHAK* genes were clearly upregulated after drought stress. Notably, *MtHAK6*, which was preferentially highly expressed in the roots, was significantly upregulated upon drought stresses (Figure 8). In particular, transcript abundance of *MtHAK15*, *MtHAK17* and *MtHAK18* was strongly and specifically up-regulated in *M. truncatula* roots under K^+ deficiency, salt, and drought stress conditions, implying that these genes are candidates for high-affinity K^+ uptake and have essential roles in salt and drought tolerance.

Conclusions

A total of 20 MtHAK protein sequences were identified and characterized from *M. truncatula* which were grouped into three clusters base on phylogenetic analysis. Chromosome location, conserved protein motif and gene structure analyses of all the *M. truncatula HAK* genes were performed. The *cis*-acting elements regulating plant growth and development, or responsive

372 to phytohormone and abiotic stress were predicted in the *MtHAKs* promoter regions. Gene
373 expression analysis assay revealed that *MtHAKs* exhibited different expressed patterns in various
374 tissues using the publicly available RNA-seq data. In addition, eight upregulated expression
375 genes showed different expression pattern after K^+ deficiency treatment. The analysis of
376 expression patterns under potassium deficiency, drought, and salt stress suggested that these
377 genes are candidates for high-affinity K^+ uptake and have essential roles in drought and salt
378 tolerance. These results provide the first genetic description of the K^+ transporter family in *M.*
379 *truncatula*, laying the foundation for molecular breeding of stress-resistant legume crops in the
380 future.
381

382 ## Acknowledgements
383 We would like to thank the reviewers for their comments and suggestion.
384

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Table 1(on next page)

CharacteristicsofMtHAKgenes in *M. truncatula*

Gene name	Gene ID	No. of aa	MW (kDa)	pI	TMS	Subcellular localization
MtHAK1	Medtr2g008820.1	849	94.74	5.66	11	PM
MtHAK2	Medtr2g438150.1	856	95.67	8.26	13	PM
MtHAK3	Medtr2g438160.1	619	69.03	9.28	13	PM
MtHAK4	Medtr3g094090.1	794	89.3	7.24	13	PM
MtHAK5	Medtr4g094660.1	787	88	8.08	13	PM
MtHAK6	Medtr4g099260.1	815	90.92	8.74	11	PM
MtHAK7	Medtr5g034500.1	782	87.25	8.24	13	PM
MtHAK8	Medtr5g070670.1	849	95.05	5.44	12	PM
MtHAK9	Medtr5g071630.1	725	81.46	6.63	12	PM
MtHAK10	Medtr5g071827.1	666	74.26	7.22	10	PM
MtHAK11	Medtr5g071860.1	754	84.24	7.02	11	PM
MtHAK12	Medtr6g007697.1	776	87.1	7.71	13	PM
MtHAK13	Medtr6g033165.1	819	91.56	8.71	12	PM
MtHAK14	Medtr7g108480.1	773	87.24	7.77	12	PM
MtHAK15	Medtr8g022130.1	766	85.45	7.77	12	PM
MtHAK16	Medtr8g063840.1	840	93.11	6.51	12	PM
MtHAK17	Medtr8g063900.1	745	83.49	8.45	11	PM
MtHAK18	Medtr8g088200.1	782	87.17	9.03	10	PM
MtHAK19	Medtr8g099090.1	792	88.52	9.39	12	PM
MtHAK20	Medtr8g107510.1	782	86.83	8.32	12	PM

1 aa, amino acid; MW, molecular weight; pI, isoelectric points; TMS, transmembrane segments;

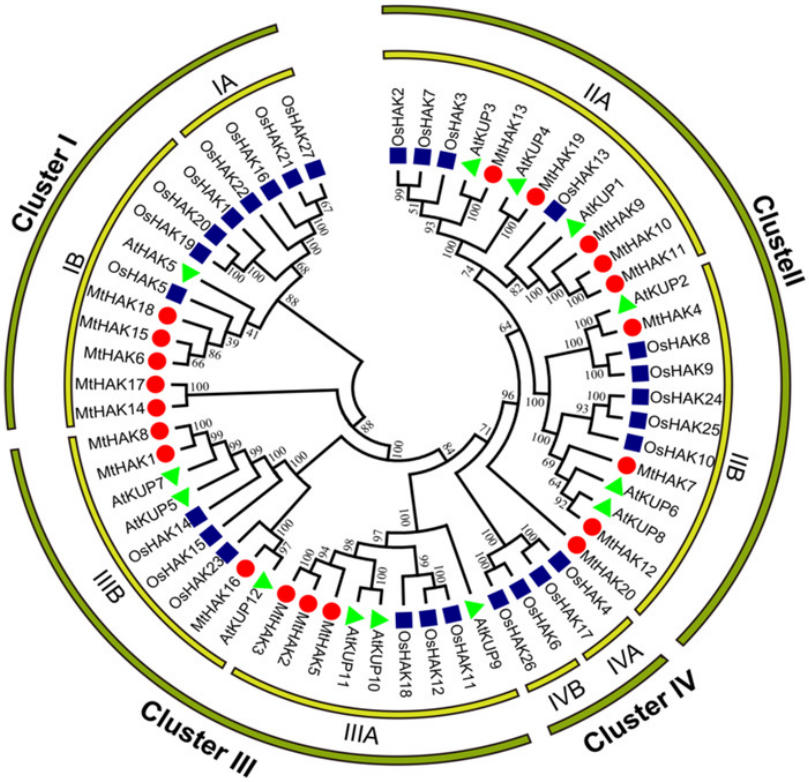
2 PM, plasma membrane.

3

Figure 1

Phylogenetic analysis of HAK proteins in *M. truncatula* (red circle), *A. thaliana* (green triangle), and *O. sativa* (blue square).

The tree was constructed using MEGA7.0 software by the Neighbor-Joining method. The numbers next to the branch showed the 1000 bootstrap replicates expressed in percentage.

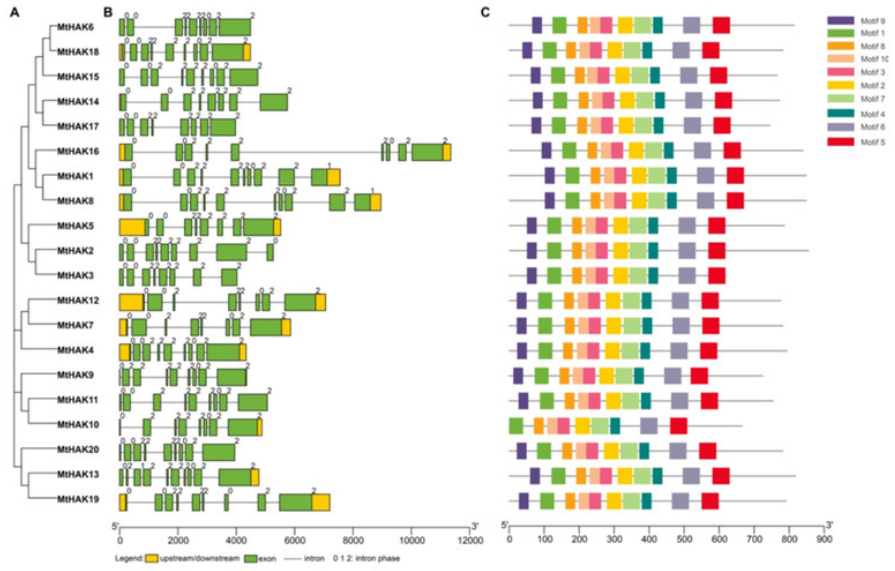

Cluster I
IA
IB
Cluster III
IIIA
IIIB
ClusterII
IIA
IIB
Cluster IV
IVA
IVB
OsHAK27
OsHAK21
OsHAK16
OsHAK22
OsHAK1
OsHAK20
OsHAK19
AtHAK5
OsHAK5
MtHAK18
MtHAK15
MtHAK6
MtHAK17
MtHAK14
MtHAK8
MtHAK1
AtKUP7
AtKUP5
OsHAK14
OsHAK15
OsHAK23
MtHAK16
AtKUP12
MtHAK3
MtHAK2
MtHAK5
AtKUP11
AtKUP10
OsHAK18
OsHAK12
OsHAK11
AtKUP9
OsHAK26
OsHAK6
OsHAK17
OsHAK4
MtHAK20
MtHAK12
AtKUP8
AtKUP6
MtHAK7
OsHAK10
OsHAK25
OsHAK24
OsHAK9
OsHAK8
MtHAK4
AtKUP2
MtHAK11
MtHAK10
AtKUP1
OsHAK13
MtHAK19
AtKUP4
MtHAK13
AtKUP3
OsHAK3
OsHAK7
OsHAK2

1

Figure 2

Phylogenetictree, gene structure and conserved motifs of HAKs in *M. truncatula*.

(A) Phylogenetic tree of MtHAK proteins. (B) Exon–introns tructure distribution. (C) Conserved protein motifs.


A
B
C
MtHAK6
MtHAK18
MtHAK15
MtHAK14
MtHAK17
MtHAK16
MtHAK1
MtHAK8
MtHAK5
MtHAK2
MtHAK3
MtHAK12
MtHAK7
MtHAK4
MtHAK9
MtHAK11
MtHAK10
MtHAK20
MtHAK13
MtHAK19
5'
3'
0
2000
4000
6000
8000
10000
12000
Legend: upstream/downstream exon intron 0 1 2: intron phase
Motif 9
Motif 1
Motif 8
Motif 10
Motif 3
Motif 2
Motif 7
Motif 4
Motif 6
Motif 5
0
100
200
300
400
500
600
700
800
900

1

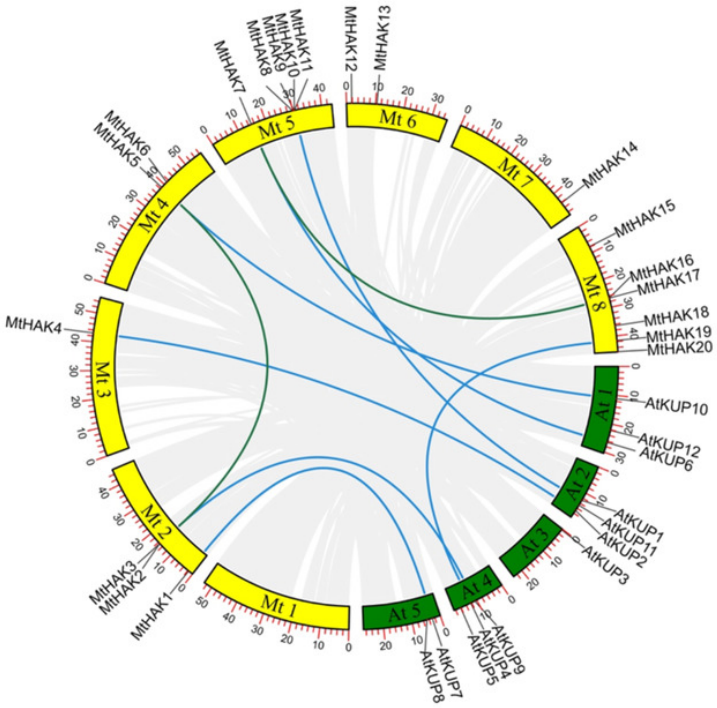
2

Figure 3

The synteny analysis of *MtHAKs*was displayed between *M. truncatula*and *Arabidopsis*genomes.

The *M. truncatula* and *Arabidopsis*chromosomes were represented by yellow and green boxes, respectively. Blue lines indicate the collinear relationship of *MtHAKs*between *M. truncatula*and *Arabidopsis*, and green lines indicate*MtHAK*gene pairs.

1


Mt 1
Mt 2
Mt 3
Mt 4
Mt 5
Mt 6
Mt 7
Mt 8
At 1
At 2
At 3
At 4
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MtHAK2
MtHAK3
MtHAK4
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MtHAK6
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MtHAK16
MtHAK17
MtHAK18
MtHAK19
MtHAK20
AtKUP10
AtKUP12
AtKUP6
AtKUP1
AtKUP11
AtKUP2
AtKUP3
AtKUP9
AtKUP4
AtKUP5
AtKUP7
AtKUP8

2

Figure 4

Cis-acting regulatory elementsanalysis in the promoter region of *MtHAK*genes.

Depending on functional annotation, the elements were classifiedinto three main categories: phytohormone responsive, abiotic stresses, and plant growth and development. The frequency of elements in the promoter region was represented by the numbers and the depth of red colure.

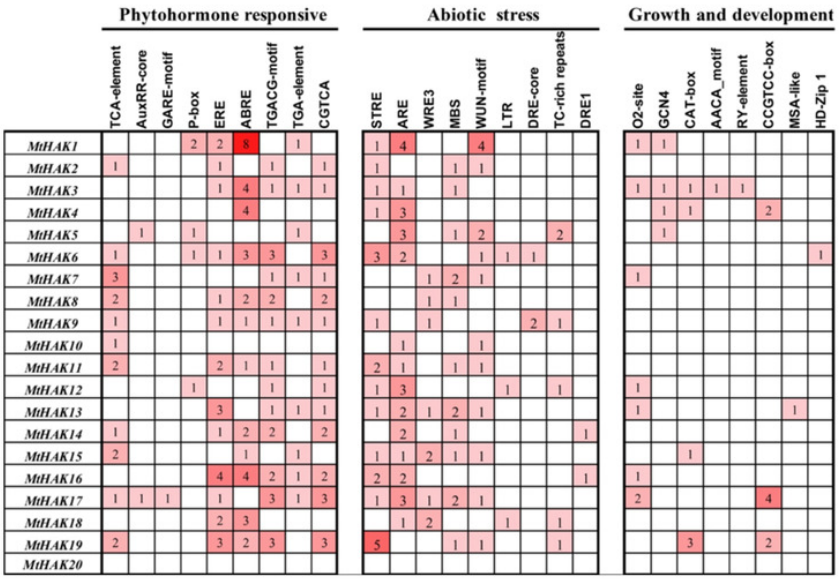

Phytohormone responsive
Abiotic stress
Growth and development
TCA-element
AuxRR-core
GARE-motif
P-box
ERE
ABRE
TGACG-motif
TGA-element
CGTCA
STRE
ARE
WRE3
MBS
WUN-motif
LTR
DRE-core
TC-rich repeats
DRE1
O2-site
GCN4
CAT-box
AACA_motif
RY-element
CCGTCC-box
MSA-like
HD-Zip 1
MtHAK1
MtHAK2
MtHAK3
MtHAK4
MtHAK5
MtHAK6
MtHAK7
MtHAK8
MtHAK9
MtHAK10
MtHAK11
MtHAK12
MtHAK13
MtHAK14
MtHAK15
MtHAK16
MtHAK17
MtHAK18
MtHAK19
MtHAK20

Figure 5

Expression patterns of *MtHAK*genes in different developmental tissues.

The microarray data were normalized based on the mean value of each gene in all analyzed organs. The heat map was portrayed by the relative expressions after log_2transformed.

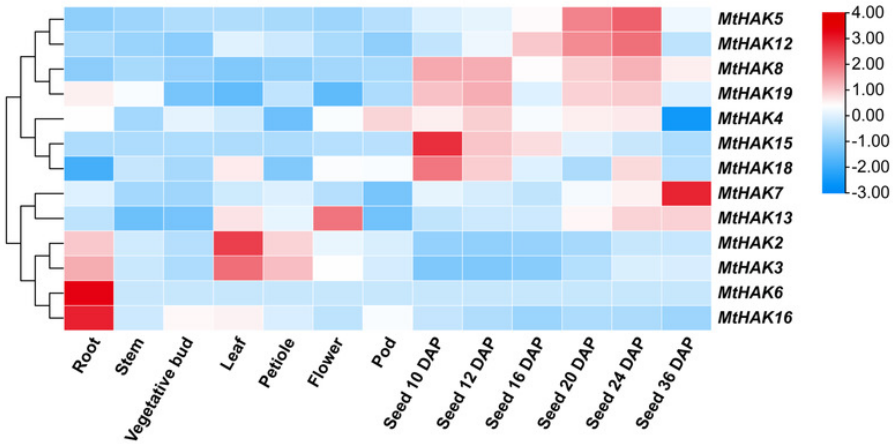

MtHAK5
MtHAK12
MtHAK8
MtHAK19
MtHAK4
MtHAK15
MtHAK18
MtHAK7
MtHAK13
MtHAK2
MtHAK3
MtHAK6
MtHAK16
4.00
3.00
2.00
1.00
0.00
-1.00
-2.00
-3.00
Root
Stem
Vegetative bud
Leaf
Petiole
Flower
Pod
Seed 10 DAP
Seed 12 DAP
Seed 16 DAP
Seed 20 DAP
Seed 24 DAP
Seed 36 DAP

Figure 6

Relative expression of *MtHAK* genes in response to K^+ deficiency treatment.

Two-week-old seedlings were placed in K^+ deficiency conditions for 0, 1, 6, 12, 24, and 48 h. Mean values and standard errors were calculated from three biological replicates. * indicate significant difference between K^+deficiency and control at $p < 0.05$.

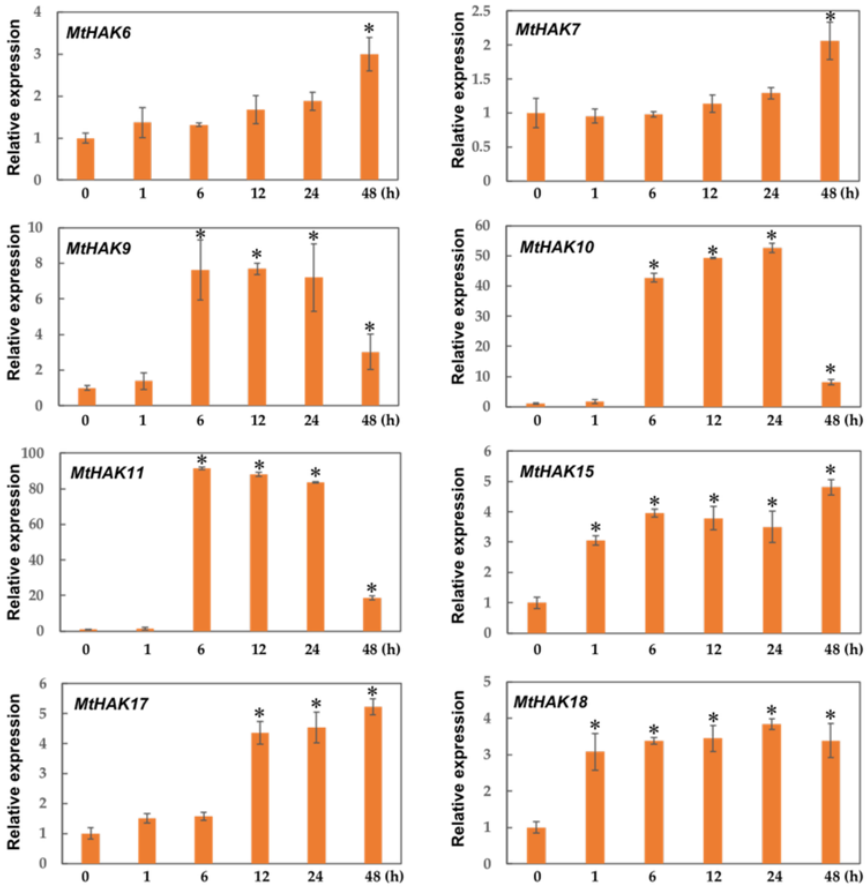

MtHAK6
MtHAK7
MtHAK9
MtHAK10
MtHAK11
MtHAK15
MtHAK17
MtHAK18
Relative expression
0 1 6 12 24 48 (h)

Figure 7

Relative expression of MtHAK genes in response to salt stress.

Two-week-old seedlings were treated with 300 mM NaCl for 0, 1, 6, 12, 24, and 48 h. Mean values and standard errors were calculated from three biological replicates. *indicate significant difference between salt and control at $p < 0.05$.

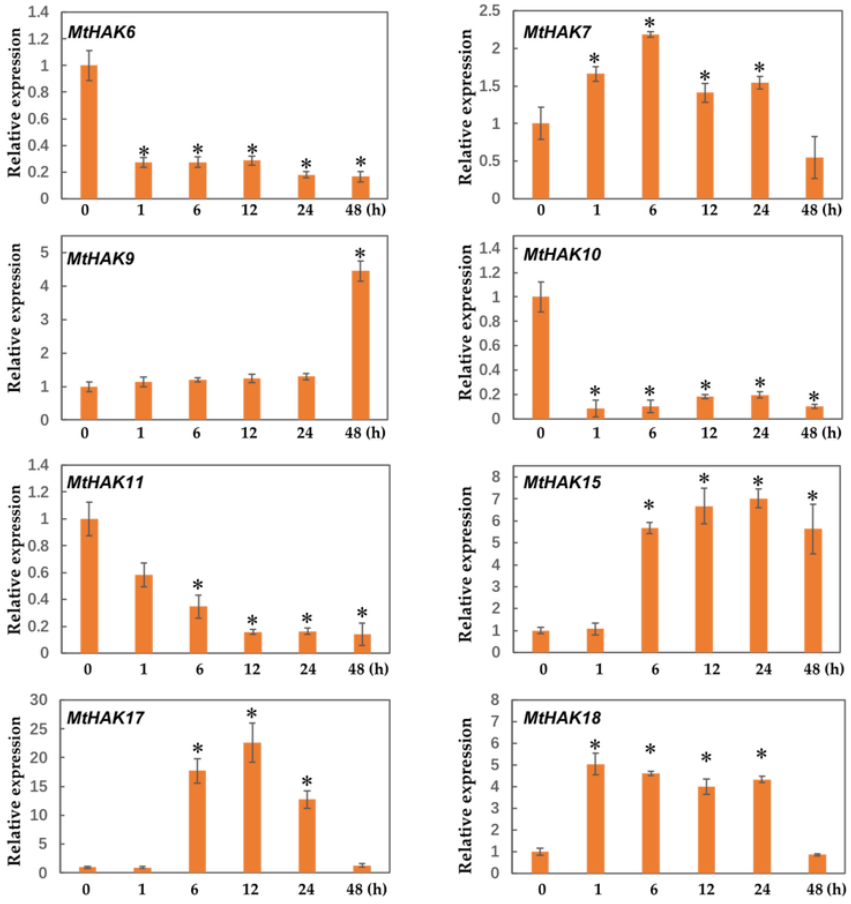
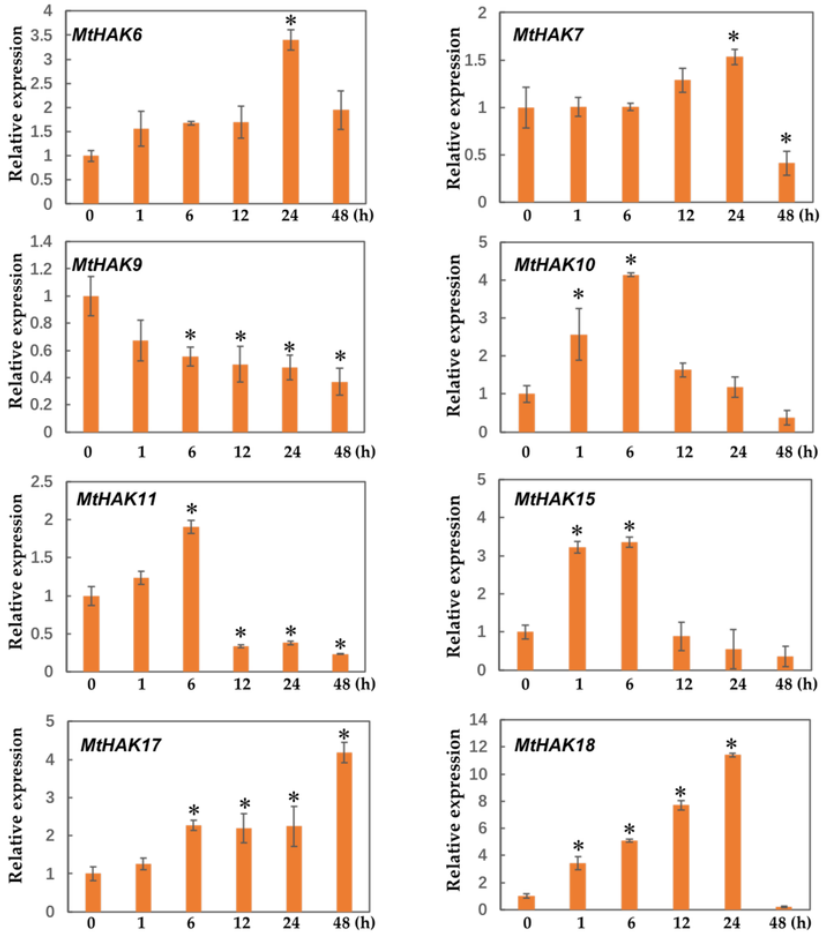

MtHAK6
MtHAK7
MtHAK9
MtHAK10
MtHAK11
MtHAK15
MtHAK17
MtHAK18
Relative expression
0
1
6
12
24
48 (h)

Figure 8

Relative expression of MtHAK genes in response to drought stress.

Two-week-old seedlings were treated with 18% PEG6000 for 0, 1, 6, 12, 24, and 48 h. Mean values and standard errors were calculated from three biological replicates. *indicate significant diference between drought and control at $p < 0.05$.


MtHAK6
MtHAK7
MtHAK9
MtHAK10
MtHAK11
MtHAK15
MtHAK17
MtHAK18
Relative expression
0 1 6 12 24 48 (h)