Heterologous expression of *MiHAK14* enhances plant tolerance to K⁺ deficiency and salinity stresses in *Arabidopsis*

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Research Article

Keywords: Mango, Potassium, KT/HAK/KUP family transporter, Ion homeostasis, Stress tolerance

Posted Date: January 13th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1214241/v1>

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Abstract

As one of the most abundant ions in cells, potassium (K^+) is closely related to plant growth and development and contributes to plant tolerance to various abiotic stresses. However molecular mechanisms towards K^+ uptake and transport are unclear in tropic fruit trees. In this study, 18 KT/HAK/KUP family genes (*MiHAKs*) were isolated and characterized in mango. Results showed that *MiHAKs* were unevenly expressed in distinct tissues and were differentially responded to K^+ depletion, PEG, and NaCl stresses in roots, in which K^+ depletion and PEG treatment significantly enhanced while NaCl treatment mainly reduced responsive *MiHAK* genes. In particular, *MiHAK14* was the most abundant KT/HAK/KUP family gene in mango, especially in roots. Functional complementation in *TK2420* mutant revealed that *MiHAK14* could uptake external K^+ . Moreover, overexpression of *MiHAK14* in *Arabidopsis* enhanced plant tolerance to K^+ depletion and NaCl stresses with strengthened K^+ nutritional status and ROS scavenging ability. This study provides molecular basis for further functional studies of KT/HAK/KUP transporters in tropic fruit trees, and favorably demonstrates the essentiality of K^+ homeostasis in plant tolerance to abiotic stresses, including K^+ deficiency and NaCl stress.

Introduction

Plants suffering from undesired abiotic stresses can produce excess internal reactive oxygen species (ROS), such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($\cdot OH^-$), which adversely elicit oxidative damage on cellular structures and metabolism (Song et al., 2014a; Song et al., 2014b; Sandalio and Romero-Puertas, 2015; Huang et al., 2016; Zhang et al., 2021). High concentrations of sodium (Na^+) can badly inhibit plant growth and development that further affect the fruit yield and quality (Munns and Tester, 2008; Isayenkov and Maathuis, 2019; van Zelm et al., 2020). In particular, plants can scavenge oxidative stress via the antioxidant defense system that depending on the activity of antioxidant enzymes, including superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX), which favorably enhanced plant tolerance to adverse environmental stresses (Miller et al., 2010; Huang et al., 2013; Huang et al., 2016; Zhang et al., 2021).

As one of the most important cations in plant cells, potassium (K^+) contributes to many crucial physiological and metabolic processes, and K^+ deficiency negatively impaired photosynthesis, chlorophyll synthesis, and chloroplast ultrastructure (Lebaudy et al. 2007; Song et al., 2014a; Song et al., 2015b; Wang and Wu, 2015; Gao et al., 2021). Therefore, plants have to uptake sufficient amount of K^+ from the soil to maintain normal growth and development. Notably, K^+ transporters acquiring K^+ , catalyzing K^+ uptake across a wide range of external K^+ concentrations, and maintaining internal ion homeostasis (Epstein et al. 1963; Lebaudy et al. 2007; Cherel et al. 2014; Wang and Wu, 2015). In plants, there are 4 different kinds of K^+ transporters, including KT/HAK/KUP, KEA, CHX and Trk/HKT (Véry and Sentenac 2003; Lebaudy et al. 2007; He et al. 2012; Wang and Wu, 2015), that play important roles in improving plant tolerance to adverse environmental stresses, such as salinity (Mian et al. 2011; Upadhyay

et al. 2012; Bose et al. 2014; Song et al. 2014a), drought (Li et al. 2011; Song et al. 2014b; Chen et al. 2017) and heavy metal (Song et al., 2015a; Ahmad et al. 2016).

In orchards, K⁺ fertilizer application favorably improved tree growth, flowering, fruit quality and yield (Hartz et al. 2005; Yamasaki and Yano 2009; Lester et al. 2010; Song et al., 2016a). However, molecular mechanisms underlying K⁺ uptake and utilization in fruit trees are largely rare, especially of tropical crops. Mango is one of the world's most important tropical fruits and its genome has been successfully sequenced (Tharanathan et al., 2006; Wang et al., 2020). In this study, we isolated 18 KT/HAK/KUP family genes (*MiHAKs*) from 'Guire 82' diploid mango, analyzed their expression profiles under abiotic stresses, including K⁺ depletion, PEG and NaCl treatment, and determined the K⁺ uptake function of MiKUP14 transporter.

Materials And Methods

Plant materials and growth conditions

Five-year-old 'Guire 82' mango grown in the Chinese Academy of Tropical Agricultural Sciences (Haikou, China) were used throughout the whole study. Samples of annual new leaves (April 1st), new phloem tissues (April 1st), new roots (April 1st), full blooming flowers (April 1st), young fruits (May 1st), and mature fruits (August 1st) were collected in 2019 and frozen immediately in liquid nitrogen for further tissue specific expression analysis.

For abiotic stress treatments, one-year-old 'Guire 82' grafted seedlings with similar growth status were transferred to aerated plastic containers, supplied with 1/2MS solution that containing 1 mmol·L⁻¹ K⁺ (control conditions). For K⁺ depletion (–K) treatment, K⁺ was omitted from 1/2MS medium by adding equal molar Na⁺ (Song et al., 2015c, Gao et al., 2021). For NaCl treatment (+NaCl), seedlings were grown in 1/2MS nutrient solution containing 200 mmol·L⁻¹ NaCl. For PEG treatment (+PEG), seedlings were grown in 1/2MS nutrient solution supplied with 15% (m/v) polyethylene glycol (PEG6000). The nutrient solution was changed every other day.

Physiological analysis

5-d-old *Arabidopsis* seedlings after germination were exposed to K⁺ depletion or 100 mmol·L⁻¹ NaCl on 1/2MS solid plates for 7 days before physiological analysis. Thirty seedlings were collected and weighed to obtain the total fresh weight. The roots were scanned with an Epson Rhizo scanner (2004b), and the total root length and lateral root number were analyzed using the Epson WinRHIZO software. Seedlings were dried in an oven at 105 °C for 30 min and baked at 70 °C for 48 h to obtain the total dry weight. Dried samples were further digested with the concentrated HNO₃–HClO₄ method, and the concentration of K⁺ and Na⁺ was determined by ICP-AES systems (IRIS Advantage, Thermo Electron, Waltham, MA, USA). The H₂O₂ concentration and antioxidant enzyme activities of SOD, POD, CAT, and APX were

quantified using commercial detection kits (Nanjing Jiancheng Bioengineering Institute, China), as described by Song et al. (2014a, 2014b, 2015a).

Identification of *MiHAK* genes in mango

To obtain putative KT/HAK/KUP family genes in mango, BLAST searches were carried out against the Mango Genome Data Resource (Wang et al., 2020), by taking the full-length of 13 *Arabidopsis* KT/HAK/KUP protein sequences as reference (Mäser et al., 2011). To confirm the presence of K⁺ transporter (PF02705, K03549) domains, the amino acid sequences of putative mango KT/HAK/KUP proteins were verified via the Pfam (<http://pfam.xfam.org>) and InterProScan 4.8 (<http://www.ebi.ac.uk/Tools/pfa/iprscan/>) online servers.

Phylogenetic tree analysis

Plant KT/HAK/KUP family transporter homologs are obtained from mango, *Arabidopsis*, rice, poplar, peach, pear, purple osier, and strawberry (Gupta et al., 2008, Mäser et al., 2011, Song et al., 2015b, Li et al., 2018, Liang et al., 2020, Gao et al., 2021). The phylogenetic tree of was constructed using the MEGA 13.0 with the Maximum Likelihood method.

Quantitative real-time PCR (qRT-PCR)

qRT-PCR was carried out on 7500 Real Time PCR System (Applied Biosystems, New York, USA), using SYBR Green fluorescence labelling kit (TaKaRa, Kyoto, Japan). The *MiActin* gene of mango was used as the internal control. To calculate the starting template concentration and PCR efficiency of each sample, the linear regression of the log (fluorescence) per cycle number data was used as described previously (Ramackers et al. 2003). Relative expression levels of the target genes were presented after normalization to the internal control from four independent biological replicates. For abiotic stress treatments, the Ct values were calculated using a log₂ scale, and the heatmap was constructed using Heml software.

Functional verification of *MiHAK14* in bacterial *TK2420* mutant

Functional determination of *MiHAK14* in *Escherichia coli* (*E. coli*) *TK2420* mutant was employed as described by Song et al. (2015b, 2016a) and Gao et al. (2021). The recombinant plasmid of pPAB404-*MiHAK14* was constructed by cloning the CDS of *MiHAK14* into pPAB404 vector (Biovector, Beijing, China), using the forward primer of 5'-GAGAGAGCTCATGAAGCCATCACAAGAAT-3' (*Sac* I was underlined), and reverse primer of 5'-GCGACCCGGGTTAGACGTAGTAAATCATGC-3' (*Sma* I was underlined). The recombinant plasmid was transformed into the *TK2420* mutant, which was disabled in three K⁺ uptake systems (Kdp⁻ Kup⁻, Trk⁻) that can not grow on KML medium when the K⁺ concentration was less than 1 mmol·L⁻¹ (Epstein et al., 1993, Song et al., 2015b, Song et al., 2016a, Gao et al., 2021). Functional verification was detected on KML plates, supplemented with 0.2 mmol·L⁻¹ KCl, 0.2 mmol·L⁻¹ KCl plus 0.5 mmol·L⁻¹ IPTG, and 10 mmol·L⁻¹ KCl, respectively. Arginine was used to

adjust the medium to pH 7.5. *E. coli* cells were grown on 10 mmol·L⁻¹ K⁺ plates at 37°C for 16 h and 0.2 mmol·L⁻¹ K⁺ plates at 37°C for 48 h.

Generation of transgenic *Arabidopsis* overexpressing the *MiKUP14* gene

The CDS of *MiKUP14* was subcloned into the binary expression vector pBH (Biovector, Beijing, China) using the forward primer of 5'-GCCGAGCTCATGAAGCCATCACAAGAAT-3' (*Sac*I was underlined) and the reverse primer of 5'-GCGTCTAGATTAGACGTAGTAAATCATGC-3' (*Xba*I was underlined). The recombinant expression vector was introduced into *Agrobacterium tumefaciens* EHA 105, and then transformed the *Arabidopsis* Col-0 ecotype host using the floral dip method. Independent T1 transgenic lines were obtained by screening kanamycin-resistant regenerated *Arabidopsis* seedlings and further verified by PCR of a 2262 bp product of *MiKUP14*.

Statistical and graphical analysis

Data were statistically analyzed using the SPSS 13.0 software (SPSS, Chicago, Illinois, USA). Details are presented in figure legends. Graphs were produced using Origin 8.0 software.

Results

Isolation of putativeKT/HAK/KUP family genes from mango

By BLAST searching of the Mango Genome Data Resources (Wang et al., 2020), 18 putative strawberry *MiHAK* genes were identified, which were originally entitled as *MiHAK1* to *MiHAK18*. Protein domain verification demonstrated that all of them possess the K⁺ transporter domain (PF02705, K03549). Detailed information about these *MiHAK* genes, including gene ID, gene location, coding sequence (CDS) length, and peptide length, were listed in Table 1. Physical and chemical properties of *MiHAK* proteins were listed in supplemental Table 1. All *MiHAK* genes were distributed on 10 distinct chromosomes, in which 3 genes on Chr3, Chr8 and Chr10 chromosomes, respectively, and 2 genes on Chr9 and Chr19 chromosome, and 1 gene on each of the other 5 chromosomes. The amino acid sequences of *MiHAK* proteins shared an overall identity of 55% (Supplemental Figure 1). Interestingly, 9 (*MiHAK1*, 6, 7, 8, 11, 2, 3, 5 and 16) and 4 (*MiHAK4*, 5, 14 and 18) *MiHAK* transporters in mango exhibited identical tertiary structures, respectively, while *MiHAK9* and *MiHAK17* exhibited quite distinct tertiary structures (Fig. 1).

Phylogenetic tree construction showed that the KT/HAK/KUP family proteins of known higher plants were classified into 4 groups (I-IV), including 3, 6, 6 and 3 members, respectively (Fig. 2). Notably, KT/HAK/KUP homologs from Rosaceae (peach, pear and strawberry) or Salicaceae (poplar and purple osier) may have the closest genetic relationship, and mango *MiHAK* transporters (like *MiHAK3*, 6, 7, 8, 9, 12, 14, 16, and 17) are prone to be closely clustered with poplar and purple osier homologs (Fig. 2).

Expression profiles and response of *MiHAK* genes to abiotic stresses

qRT-PCR investigation showed that *MiHAK* genes were unevenly expressed in different 'Guire 82' tissues or organs under control conditions (Fig. 3). The maximum expression of 9 genes (*MiHAK1*, 5, 9, 10, 11, 13, 14, 17, 18) was observed in roots and 4 genes (*MiHAK3*, 6, 8 and 15) in leaves, more than that of the other tissues, while the maximum expression of *MiHAK4* and *MiHAK12* was observed in phloem tissues and full-bloom flowers, respectively. In particular, *MiHAK14* was the most abundant KT/HAK/KUP family gene throughout the whole plant of mango, especially in roots. Both *MiHAK7* and *MiHAK16* were not detected in all tested mango tissues. Although expression level was relatively low, *MiKUP2* was evenly expressed among different tissues (Fig. 3).

To investigate the role of *MiHAK* genes in maintaining K⁺ homeostasis in mango, we analyzed the expression profiles of *MiHAK* genes in roots under different abiotic stresses. Results showed that expression of *MiHAKs* were differentially responded to K⁺ deficiency, PEG and NaCl treatment in mango roots (Fig. 4). In details, 10 (*MiHAK1*, 3, 4, 6, 8, 9, 12, 13, 17 and 18) and 8 (*MiHAK1*, 2, 3, 5, 8, 9, 12, 17 and 18) genes were responsive to K⁺ deficiency and PEG treatment, respectively, which were significantly increased. Expression of 8 genes were sensitive to NaCl treatment, in which 6 genes (*MiHAK1*, 3, 5, 9, 12, 17 and 18) were decreased while *MiHAK11* and *MiHAK14* were significantly enhanced (Fig. 4). Notably, *MiHAK1*, 3, 12, 17 and 18 were the most sensitive genes, whose expression was easily affected under all tested treatments, whereas expression of *MiHAK7*, 10, 15 and 16 were quite steady that had no response to any treatment.

Functional analysis of *MiHAK14* in bacterial TK2420 mutant

As the most abundant KT/HAK/KUP family gene in mango, the maximum expression of *MiHAK14* was observed in roots that had no response to external K⁺ variation. We further verified its function in the *E. coli* TK2420 mutant. Heterologous expression of *MiHAK14* restored the normal growth of TK2420 mutants on KML medium that supplied with 0.2 mmol·L⁻¹ K⁺ and 0.5 mmol·L⁻¹ IPTG (Fig. 5), while TK2420 mutants harboring the empty vector could not grow (Fig. 5). Without IPTG induction, both the wild type and TK2420 mutants hardly grew on KML medium that containing 0.2 mmol·L⁻¹ KCl, but grew well under 10 mmol·L⁻¹ KCl conditions. These findings implies that *MiHAK14* is a functional K⁺ transporter that can uptake or utilize external K⁺.

Heterologous expression of *MiHAK14* in *Arabidopsis*

The CDS of *MiKUP14* was subcloned into the binary expression vector pBH (Fig. 6A). Putative T1 generation transgenic *Arabidopsis* seedlings were verified using semi-quantitative RT-PCR for the presence of a 2.2 kb fragment of *MiHAK14*, which was expressed throughout the whole plant of #1, #2, and #3 transgenic seedlings (Fig. 6B). And the T2 generation of #1 transgenic *Arabidopsis* lines were randomly selected for subsequent studies.

Under control conditions, there was no difference in growth status between WT and #1 transgenic lines, and the total fresh weight, dry weight, root length, and lateral root number was non-significantly changed (Table 2). Under both K⁺ depletion and NaCl treatment, #1 transgenic lines exhibited better growth status, either shoots or roots, compared to WT seedlings (Fig. 6C). Under K⁺ depletion treatment, the total fresh weight, dry weight, root length, and lateral root number of #1 transgenic lines was increased by 78.82%, 79.41%, 127.65%, and 138.78%, respectively (Table 2). Under NaCl treatment, the total fresh weight, dry weight, root length, and lateral root number of #1 transgenic lines was increased by 772.84%, 45.19%, 65.42%, and 55.93%, respectively (Table 2). There was no difference in tissue K⁺ concentration under control conditions, and both K⁺ depletion and NaCl treatment significantly reduced the K⁺ concentration in all tested seedlings. In particular, there was an increase of K⁺ concentration in #1 transgenic lines under both K⁺ depletion and NaCl treatment, at the whole plant level (Table 2). Although there was no difference between WT and #1 transgenic lines under control conditions, tissue Na⁺ concentration was significantly increased under NaCl treatment but changed little under K⁺ depletion, and Na⁺ concentration was significantly lower in #1 transgenic lines than WT seedlings under NaCl treatment (Table 2).

Further antioxidant enzyme activity analysis showed that the activities of SOD, POD, CAT and APX were significantly increased in both WT and transgenic *Arabidopsis* seedlings, respectively, under both K⁺ depletion and NaCl treatments for 7 days. In particular, higher activities of endogenous SOD and POD were observed in #1 transgenic lines under K⁺ depletion, but no difference was detected in CAT and APX activities between WT and #1 transgenic seedlings. While the activities of SOD, POD, CAT and APX were all significantly higher in #1 transgenic lines under NaCl treatment (Table 2). Simultaneously, the endogenous H₂O₂ content was significantly enhanced under either K⁺ depletion or NaCl treatment, and the H₂O₂ content in #1 transgenic lines was significantly lower than that of WT seedlings (Table 2).

Discussion

In plants, KT/HAK/KUP family transporters play crucial roles in K⁺ uptake, transport and ion homeostasis (Véry and Sentenac 2003; Cherel et al. 2014; Wang and Wu, 2015). However, molecular mechanisms of K⁺ uptake and transport in tropical crops are largely unclear. In this study, 18 KT/HAK/KUP family genes were isolated and characterized in tropical fruit mango. In terms of woody crops, the number of KT/HAK/KUP family genes in mango (18) is larger than that of peach (16), but less than that of poplar (31) and pear (21), which may reflect the fact that the genome size of mango (393 Mbp) is larger than that of peach (230 Mbp) but smaller than that of poplar (520 Mbp) pear (512 Mbp) (He et al. 2012; Song et al. 2015a; Li et al., 2018; Wang et al., 2020; Gao et al., 2021).

The amino acid identity of 18 MiHAK proteins is relatively high (55%), and all MiHAK proteins are hydrophobic proteins that possessed 9-14 TM domains but had no signal peptide (Supplemental Table 1), which is in line with previously reported plant KT/HAK/KUP transporters (Véry and Sentenac, 2003; Song et al., 2015 GMR). In particular, KT/HAK/KUP transporters from Rosaceae plants or Salicaceae plants are closely clustered together, implying that functional domains of KT/HAK/KUP transporters are

highly conserved among Rosaceae and Salicaceae plants (Fig. 2). Notably, the phylogenetic distance of KT/HAK/KUP orthologs between Mango and Salicaceae plants are closer than that of *Arabidopsis*, rice and Rosaceae plants, implying that the biological role of KT/HAK/KUP transporters are likely to be similar among Mango and Salicaceae plants.

In mango roots, *MiHAKs* were differentially affected by K⁺ depletion, PEG and NaCl stresses (Fig. 4). Both K⁺ depletion and PEG treatment enhanced while NaCl treatment mainly reduced responsive *MiHAK* genes, which is in accord with previous reports in rice (Gupta et al. 2008), alligatorweed (Song and Su, 2013; Song et al., 2014a), peach (Song et al., 2014b; Song et al., 2015a), pear (Li et al., 2018), and strawberry (Gao et al., 2021). In particular, *MiHAK14* was the most abundant expressed gene in all tested tissues and functional determination in *TK2420* mutants revealed that *MiHAK14* could uptake external K⁺ (Fig. 5), indicating that this gene may be a functional K⁺ transporter in mango. More convincingly, overexpression of *MiHAK14* favorably improves plant tolerance to K⁺ depletion and NaCl stress in transgenic *Arabidopsis* seedlings (Fig. 6C and Table 2). Nonetheless, the observed K⁺ uptake or utilization capacity of *MiHAK14* in bacteria can be replicated in transgenic *Arabidopsis* seedlings.

In agriculture, K⁺ deficiency and high salinity are two major limiting environment factors to plant growth and development. Favorably, K⁺ contributes to crop production and plant tolerance to various abiotic stresses (Mian et al. 2011; Li et al. 2011; Upadhyay et al. 2012; Bose et al. 2014; Song et al. 2014a; Song et al. 2014b; Chen et al. 2017). In this present study, both K⁺ depletion and NaCl treatment definitely suppressed the growth of *Arabidopsis* plants, embodied in impaired biomass, root elongation and K⁺ accumulation, which are consistent with previous reports (Mian et al., 2011; Upadhyay et al., 2012; Song et al., 2014a). Interestingly, overexpression of *MiHAK14* in *Arabidopsis* actively strengthened plant performance under both K⁺ depletion and NaCl treatment (Fig. 6C). Possibly, undesired K⁺ depletion or NaCl stress may stimulate root development in transgenic seedlings, which in turn strengthened the roots K⁺ uptake and transport into the shoots. More convincingly, #1 transgenic lines exhibited higher tissue K⁺ accumulation (Table 2), which may allow plants to maintain indispensable K⁺-dependent activities or metabolic processes that further contributes to cope with adverse K⁺ depletion or NaCl stress. Although NaCl stress significantly decreased the tissue K⁺ concentration and increased tissue Na⁺ accumulation, tissue Na⁺ concentration was significantly lower in #1 transgenic lines than WT seedlings under NaCl stress, implying that overexpression of *MiHAK14* in *Arabidopsis* indeed altered the K⁺/Na⁺ ratio in a beneficial way to maintain the ion homeostasis under NaCl stress. Again, these findings support the proposition that sufficient cytosolic K⁺ and high K⁺/Na⁺ ratio is crucial to protect plant cells from detrimental Na⁺ toxicity (Demidchik et al., 2010; Upadhyay et al., 2012; Song et al., 2014a).

Excess internal ROS are prone to be produced when plants suffering from undesired abiotic stresses. In particular, Na⁺ will be rapidly accumulated in the cytosol along with salt-induced H₂O₂ ROS accumulation under salinity stresses (Foyer and Noctor, 2005; Song et al., 2014a). In this study, both K⁺ depletion and NaCl treatment adversely enhanced H₂O₂ accumulation and antioxidant enzyme activities of SOD, POD,

CAT, and APX in *Arabidopsis* seedlings, which are also found in previous studies (Mian et al., 2011; Upadhyay et al., 2012; Song et al., 2014a). Remarkably, the SOD level was indeed higher in transgenic *Arabidopsis* lines under both K⁺ depletion and NaCl treatment, which again support the proposition that higher SOD accumulation in salt-tolerant plants are required for the rapid production of endogenous H₂O₂ (Ellouzi et al., 2011; Bose et al., 2014; Song et al., 2014a). Simultaneously, the POD activity was higher in transgenic lines than that of WT seedlings under either K⁺ depletion or NaCl treatment, and the CAT and APX activity was higher in transgenic lines under NaCl treatment but changed little K⁺ depletion (Table 2). These findings may partially explain the decreased endogenous H₂O₂ accumulation in transgenic *Arabidopsis* lines, which also support the proposition that enzymatic antioxidants are involved in scavenging the internal H₂O₂ (Ellouzi et al., 2011; Bose et al., 2014; Song et al., 2014 a).

Nonetheless, overexpression of mango *MiHAK14* favorably strengthened plant tolerance to K⁺ depletion and NaCl stresses along with enhanced K⁺ homeostasis and ROS scavenging capacity in transgenic *Arabidopsis* seedlings.

Declarations

Conflict of interest

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

Acknowledgments

This work was financially supported by grants from the National Key R&D Program of China (2019YFD1000500), the National Natural Science Foundations of China (31501743), and the Agricultural Variety Improvement Project of Shandong Province (2019LZGC009).

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Tables

Table 1 Information of *MiHAK* genes

Gene	Locus No.	Location	Chromosome distribution	CDS (bp)	No. of amino acids
<i>MiHAK1</i>	mango030880	GWHABLA00000010 21632010..21637980 +	Chr10	2382	793
<i>MiHAK2</i>	mango028864	GWHABLA00000009 11553467..11558763 +	Chr9	2253	750
<i>MiHAK3</i>	mango026133	GWHABLA00000008 8908203..8914877 +	Chr8	2346	781
<i>MiHAK4</i>	mango012532	GWHABLA00000019 12228952..12231935 +	Chr19	2415	804
<i>MiHAK5</i>	mango012876	GWHABLA00000019 14476232..14480796 +	Chr19	2382	793
<i>MiHAK6</i>	mango002054	GWHABLA00000012 5730389..5735750 -	Chr12	2061	686
<i>MiHAK7</i>	mango018636	GWHABLA00000003 8317369..8322318 +	Chr3	2355	784
<i>MiHAK8</i>	mango018946	GWHABLA00000003 10292697..10298166 +	Chr3	2328	775
<i>MiHAK9</i>	mango022688	GWHABLA00000005 16077113..16080055 -	Chr5	1188	395
<i>MiHAK10</i>	mango015093	GWHABLA00000020 14855060..14865795 +	Chr20	2556	851
<i>MiHAK11</i>	mango030881	GWHABLA00000010 21641213..21645118 +	Chr10	1923	640
<i>MiHAK12</i>	mango027991	GWHABLA00000009 886000..892031 -	Chr9	2343	780
<i>MiHAK13</i>	mango026132	GWHABLA00000008 8888520..8894496 +	Chr8	2514	837
<i>MiHAK14</i>	mango004405	GWHABLA00000014 12151192..12156002 +	Chr14	2262	753
<i>MiHAK15</i>	mango017775	GWHABLA00000003 3449413..3466390 +	Chr3	2340	779
<i>MiHAK16</i>	mango025913	GWHABLA00000008 4144182..4150692 +	Chr8	1734	577
<i>MiHAK17</i>	mango030671	GWHABLA00000010 18761768..18766723 -	Chr10	2223	740
<i>MiHAK18</i>	mango035076	GWHABLA00000013 17423678..17427336 +	Chr13	2214	737

Table 2. Physiological analyses of transgenic *Arabidopsis* seedlings under K⁺ depletion and NaCl treatments.^a

Treatments	Control		K ⁺ depletion		NaCl treatment	
	WT	#1 lines	WT	#1 lines	WT	#1 lines
Total fresh weight (g)	23.80 ± 2.42	24.97 ± 2.75	9.87 ± 1.02	17.65 ± 2.21 **	11.45 ± 1.24	19.79 ± 2.07 **
Dry weight (g)	2.28 ± 0.32	2.34 ± 0.26	1.02 ± 0.12	1.83 ± 0.21 **	1.35 ± 0.15	1.96 ± 0.22 **
Total root length (cm)	35.67 ± 3.98	37.29 ± 4.93	17.32 ± 2.34	39.43 ± 4.25 **	20.42 ± 3.56	33.78 ± 4.32 **
Lateral root number	145 ± 12	149 ± 15	49 ± 6	117 ± 16 **	59 ± 7	92 ± 9 **
K ⁺ concentration (g•kg ⁻¹ DW)	36.46 ± 2.34	47.43 ± 3.21*	13.45 ± 1.44	18.63 ± 1.52 **	22.43 ± 2.21	30.63 ± 2.16 **
Na ⁺ concentration (g•kg ⁻¹ DW)	9.06 ± 0.74	8.72 ± 0.71	8.74 ± 0.91	8.59 ± 0.89	27.35 ± 2.17	15.43 ± 1.09 **
SOD activity (U• mg ⁻¹ protein)	10.78 ± 1.08	11.76 ± 1.23	26.34 ± 2.74	36.13 ± 3.25 **	40.68 ± 4.15	52.13 ± 4.78 **
POD activity (U• mg ⁻¹ protein)	2.10 ± 0.23	2.25 ± 0.29	5.35 ± 0.27	6.43 ± 0.39 *	5.36 ± 0.37	7.09 ± 0.44 **
CAT activity (U• mg ⁻¹ protein)	0.59 ± 0.07	0.71 ± 0.08	1.74 ± 0.21	2.13 ± 0.27	2.64 ± 0.32	3.84 ± 0.43 **
APX activity (U• mg ⁻¹ protein)	1.25 ± 0.13	1.19 ± 0.14	2.06 ± 0.23	2.19 ± 0.35	4.09 ± 0.31	5.89 ± 0.43 **
H ₂ O ₂ content (mmol•kg ⁻¹ FW)	1.62 ± 0.15	1.54 ± 0.13	3.22 ± 0.29	1.95 ± 0.21 **	4.92 ± 0.35	3.21 ± 0.28 **

^a 5-day-old *Arabidopsis* seedlings were exposed to K⁺ depletion or 100 mmol•L⁻¹ NaCl treatment, based on 1/2 MS liquid solution, for 7 days before examination. Data are given as the means ± SE (n=36), from 3 sets of experiments using each 12 samples. Asterisks indicate statistical differences between #1 transgenic lines and WT seedlings (0.01 < *P < 0.05, **P < 0.01, independent samples *t* test).

Figures

Figure 1

Tertiary structure prediction of mango MiHAK family transporters

Figure 2

Phylogenetic tree of the known KT/HAK/KUP family proteins. A phylogenetic tree was constructed by multiple alignment of KT/HAK/KUP family proteins from mango, *Arabidopsis*, rice, poplar, peach, pear, purple osier, and strawberry (Gupta et al., 2008, Mäser et al., 2011, Song et al., 2015a, Li et al., 2018, Liang et al., 2020, Gao et al., 2021) using MEGA13.0 software with the Maximum Likelihood method.

Figure 3

Tissue-specific expression analysis of *MiHAK* genes. Samples of annual new leaves (April 1st), phloems (April 1st), roots (April 1st), full blooming flowers (April 1st), young fruits (May 1st), and mature fruits (August 1st) were collected from 5-year-old 'Guire 82' mango in 2019, and frozen immediately in liquid nitrogen for qRT-PCR analysis.

Figure 4

Expression changes of *MiHAK* genes under K^+ depletion, PEG and NaCl treatment. one-year-old 'Guire 82' grafted seedlings with similar growth status were transferred to aerated plastic containers, supplied with 1/2MS solution containing $1 \text{ mmol} \cdot \text{L}^{-1} K^+$ (control conditions). For K^+ depletion ($-K$), K^+ was omitted from the 1/2MS medium by adding equal molar Na^+ . For PEG treatment (+PEG), seedlings were grown in the 1/2MS nutrient solution supplied with 15 % (m/v) polyethylene glycol (PEG6000). For NaCl treatment (+NaCl), seedlings were grown in the 1/2MS nutrient solution containing $200 \text{ mmol} \cdot \text{L}^{-1} NaCl$. The nutrient solution was changed every other day.

Figure 5

Functional determination of *MiHAK14* in bacterial mutant. *E. coli* TK2420 strains containing the pPAB404-*MiHAK14* recombinant plasmid or pPAB404 empty vector were grown in KML media (pH 7.5), supplemented with $0.2 \text{ mmol} \cdot \text{L}^{-1} KCl$, $0.2 \text{ mmol} \cdot \text{L}^{-1} KCl$ plus $0.5 \text{ mmol} \cdot \text{L}^{-1} IPTG$, and $10 \text{ mmol} \cdot \text{L}^{-1} KCl$,

respectively. Arginine was used to adjust the medium to pH 7.5. *E. coli* cells were grown on 10 mmol·L⁻¹ K⁺ plates at 37°C for 16 h and on 0.2 mmol·L⁻¹ K⁺ plates 72 h.

Figure 6

Generation of transgenic *Arabidopsis* overexpressing the *MiKUP14* gene. (A) Schematic of plasmid construct pHBI that contains the CDS of *MiHAK14* driven by the CaMV35S promoter. (B) PCR verification of *MiHAK14* gene in WT and T1 generation transgenic *Arabidopsis* lines. (C) Growth of *Arabidopsis* lines overexpressing *MiHAK14* under K⁺ depletion and NaCl treatment. 5-d-old *Arabidopsis* seedlings were exposed to K⁺ depletion or 100 mmol·L⁻¹ NaCl on 1/2MS solid plates for 7 days before analysis.

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