



**ARTICLE**

# The Potassium Transporter AtKUP12 Enhances Tolerance to Salt Stress through the Maintenance of the $K^+/Na^+$ Ratio in *Arabidopsis*

Hua Zhang<sup>#</sup>, Zhongmin Yang<sup>#</sup>, Xilong You, Youqiang Heng and Yan Wang<sup>\*</sup>

Xinjiang Key Laboratory of Biological Resources and Genetic Engineering, College of Life Science and Technology, Xinjiang University, Urumqi, 830046, China

<sup>\*</sup>Corresponding Author: Yan Wang. Email: wangyanxju@126.com

<sup>#</sup>These authors have contributed equally to this work

Received: 03 September 2020 Accepted: 25 October 2020

## ABSTRACT

Potassium ( $K^+$ ) is a necessary nutrient for plant growth and crop production. The  $K^+$  transporter plays crucial roles in the absorption and transport of  $K^+$  in plants. Most  $K^+$  transporters in *Arabidopsis* have been reported, but AtKUP12, which is a member of the KT/KUP/HAK family, has not yet been the subject of relevant in-depth research. In the present study, we demonstrated that AtKUP12 plays a crucial role in  $K^+$  uptake in *Arabidopsis* under 100  $\mu$ M low- $K^+$  and 125 mM salt stress conditions. AtKUP12 transcripts were induced by  $K^+$  deficiency and salt stress. We analyzed the  $K^+$  uptake of AtKUP12 using the  $K^+$  uptake-deficient yeast R5421 and *Arabidopsis* mutant *atkup12*. Transformation with AtKUP12 rescued the growth defect of mutant yeast and *atkup12* mutant plants at the low- $K^+$  concentration, which suggested that AtKUP12 might be involved in high-affinity  $K^+$  uptake in low- $K^+$  environments. In comparison to the wild-type (WT) and *atkup12*-AtKUP12 complementation lines, *atkup12* showed a dramatic reduction in potassium concentration,  $K^+/Na^+$  ratio, and root and shoot growth on 12-day-old seedlings under the salt conditions; however, there was no significant difference between the complementation and WT lines. Taken together, these results demonstrate that AtKUP12 might participate in salt tolerance in *Arabidopsis* through  $K^+$  uptake and  $K^+/Na^+$  homeostasis.

## KEYWORDS

AtKUP12; *atkup12* mutant;  $K^+$  uptake;  $K^+/Na^+$  homeostasis; salt tolerance

## 1 Introduction

Potassium ( $K^+$ ) is an essential and the most abundant macroelement that plays several critical roles in plant growth and productivity [1] and accounts for approximately 2% to 10% of the dry weight in plants [2]. In contrast to the relatively high and stable  $K^+$  concentration (approximately 100–150 mM) in the cytoplasm of plant cells, the  $K^+$  concentration in soil solution is relatively low and highly variable, ranging from 0.01–1 mM [3]. The ability of plants to absorb and utilize  $K^+$  is relatively limited; thus, increasing the efficiency of  $K^+$  absorption and transport by means of molecular biology and genetics is an important method to alleviate the shortage of  $K^+$  resources for cash crops [4]. For example, overexpression of *ZmHAK5* promotes  $K^+$  uptake and seedling growth in maize under low  $K^+$  levels [5].



Additionally, the expression of *OsHAK5* in rice increases  $K^+$  acquisition and transport from roots to shoots facing low external  $K^+$  [6]. Plants possess a series of transport systems that play key roles in  $K^+$  absorption, redistribution and ion homeostasis [7], such as the Shaker, TPK and Kir-like  $K^+$  channel families [8] and the KT/HAK/KUP, Trk/HKT, KEA and CHX  $K^+$  transporter families [9]. Among them, KT/HAK/KUP is the largest family and is widely distributed in bacteria, fungi and plants but absent in animal cells [10]. Generally,  $K^+$  channels conduct low-affinity  $K^+$  uptake under higher  $K^+$  conditions, whereas  $K^+$  transporters perform high-affinity  $K^+$  uptake under lower  $K^+$  conditions [11]. At present, 13 and 27 members of the KT/HAK/KUP family genes have been identified in *Arabidopsis* and rice (*Oryza sativa* L.), respectively [12,13]. KT/HAK/KUP  $K^+$  transporters play versatile roles in  $K^+$  uptake and transport,  $K^+$  homeostasis, salt tolerance, osmotic regulation, and the morphogenesis of root and phenotype in plants [14]. The genes of this family can be divided into four distinct clusters (I–IV) by their amino acid sequence homology [15]. There is only one member in the *Arabidopsis* cluster I family, and the other 12 members are mainly distributed in cluster II and cluster III. In rice, 8 members are distributed in cluster I, 15 members are distributed in cluster II and cluster III, and 4 members are distributed in cluster IV [16]. Cluster I transporters such as AtHAK5, HvHAK1 and OsHAK1 may play key roles in high-affinity  $K^+$  uptake when external  $K^+$  is low [6,15,17,18], while cluster II transporters such as AtKT2/KUP2 and AtKT3/AtTRH1 from *Arabidopsis thaliana* and OsHAK7 and OsHAK10 from rice are involved in low-affinity  $K^+$  transport [15,19]. The representative members of cluster III and IV are AtKUP12 and OsHAK11, respectively. Cluster III members may function in maintaining  $K^+/Na^+$  homeostasis [15], and little research has been conducted regarding their functions [9]. The results of phylogenetic analysis showed that cluster II and cluster III members are highly conserved [16], and they may be the ancestors of the KT/HAK/KUP transporter family. Cluster II and III members are crucial in completing plant life activities, while those of cluster I and cluster IV may play a positive role in adapting to the environment by regulating plant  $K^+$  concentration [20].

$K^+$  deficiency leads to a decrease in total root length (TRL) and root length density (RLD) in the last stage of wheat growth [21]. KT/HAK/KUP transporters play significant roles not only in absorption and transport of  $K^+$  but also morphogenesis of the root and shoot in plants [17,22,23]. Mutation of *AtKUP2* (*shy3-1*) decreases cell expansion in shoots, resulting in a dwarf phenotype in *Arabidopsis*, and tiny root hairs were observed in the knockout mutation of *AtKUP4/TRH1* [19]. Rubén Tenorio-Berrio et al. investigated a significant reduction in embryo and cotyledon size of *Arabidopsis hak/kt12*, *kup4*, and *kup9* mutants relative to wild-type *Arabidopsis*, and *AtHAK/KT12* and *AtKUP4* were regulated by auxin IAA and IAM [24]. Knockout of *OsHAK5* not only decreased  $K^+$  uptake by roots and  $K^+$  upward transport from roots to shoots with low external  $K^+$  [6], but also significantly reduced tillering number irrespective of  $K^+$  status [14]. The destruction of *OsHAK1* dramatically reduced the  $K^+$  concentration in both roots and shoots, and severely stunted the growth and development of rice, resulting in delayed grain filling and decreased grain yields [25]. AtKUP2, AtKUP6, and AtKUP8 are three kinds of  $K^+$  efflux transporters. Triple mutation *atkup2/6/8* had a greater effect on cell expansion, which indicated that these transporters were involved in the negative regulation of turgor pressure-dependent growth [26]. Under the low- $K^+$  level, the *athak5* mutant displayed a shorter primary root length than wild-type plants. In maize, the loss of function of *ZmHAK5* reduced  $K^+$  uptake and inhibited seedling growth, while opposite results were present in the overexpression lines [5].

The tolerance of plants to salt stress is shown in the adaptation to  $Na^+$  toxicity and the maintenance of their own  $K^+$  nutrition [27]. Maintaining the  $K^+$  uptake ratio at a high level of exogenous  $Na^+$  is essential for  $K^+/Na^+$  homeostasis and salt tolerance [11]. There is increasing evidence of the involvement of KT/HAK/KUP members in  $K^+$  absorption, transport, and regulation of plant salt tolerance in *Arabidopsis* [6]. OsHAK5 and OsHAK1 may play a pivotal role in salt tolerance because high-salt treatment temporarily induces their expression in some rice organs [6,24]. Expression of *OsHAK5* in tobacco BY2 cells

improves salt tolerance by accumulating more  $K^+$  without affecting the  $Na^+$  concentration [28]. Knockout of *OsHAK5* or *OsHAK21* results in a decrease in the  $K^+/Na^+$  ratio in tissues, and mutants are more sensitive than wild-type plants to salt [6,29]. AtHAK5 also plays a key role in maintaining high-affinity  $K^+$  absorption and regulating plant growth under salt conditions [30].

In previous studies, we cloned and obtained the *HcKUP12* gene from a suppression subtractive library of the halophyte *Halostachys caspica*, and found that it responded positively to salt stress [31]. Also, sequence homology comparison revealed that its closest homology is *AtKUP12*. We speculated that *AtKUP12* might also participate in response to salt stress. *Arabidopsis thaliana* is a model organism; thus, studies examining the  $K^+$  absorption, transport and salt tolerance of the KT/HAK/KUP family in *Arabidopsis* can provide a reference for the study of homologous gene functions in other species. Previous studies have found that *atkup12* mutant plants display a sensitive phenotype under abiotic stress such as low  $K^+$ , salt and oxidation [32]. However, the physiological functions of *AtKUP12* in  $K^+$  uptake and salt tolerance of plants remain unclear and warrant further investigation.

In this study, we investigated the expression patterns of *AtKUP12* in *Arabidopsis* under low- $K^+$  and salt stress conditions. We studied the  $K^+$ -uptake activity of *AtKUP12* using  $K^+$  uptake-deficient yeast CY162 and *atkup12* mutants. We measured the root length, fresh weight and ion concentration of wild-type (WT), *atkup12* mutant and *atkup12-AtKUP12* complementation lines of *Arabidopsis* under low- $K^+$  or salt stress conditions. The expression of *AtKUP12* completely rescued the low- $K^+$  and salt-sensitive phenotype of *atkup12* mutant. Overall, we demonstrated that *AtKUP12* participated in  $K^+$  uptake, and then improved the plant salt tolerance by maintaining the  $K^+/Na^+$  ratio in *Arabidopsis*.

## 2 Materials and Methods

### 2.1 Plant Materials, Growth Conditions and Stress Treatments

*Arabidopsis thaliana* ecotype Columbia (Col) was used as the WT plant. We obtained *Arabidopsis atkup12* mutant (SALK\_083613.47.35.x) seeds from the *Arabidopsis* Biological Resource Center (<http://www.Arabidopsis.org/abrc/>), Ohio State University, USA.

Seeds were stratified after surface sterilization at 4°C in the dark for 3 days. For potassium treatments, seeds were sown on low- $K^+$  (LK, 10, 100, 200  $\mu$ M KCl) or high- $K^+$  (HK, 1000  $\mu$ M KCl) Murashige and Skoog (MS) medium (pH 5.8) supplemented with 1.5% (w/v) sucrose and solidified with 0.7% (w/v) agar to grow for 12 days under conditions of 22°C, 16 h light/8 h dark and a relative humidity of 70%. LK medium was modified from MS medium [33]. For salt stress, seeds were sown on MS medium supplemented with 0, 100 and 125 mM NaCl and grown for 12 days under the above conditions.

### 2.2 Expression Analysis of *AtKUP12*

Sterile two-week-old seedlings were subjected to stress treatments by removal to filter paper supplemented with 100 mM NaCl and 0.5  $\mu$ M low- $K^+$ , and the seedlings were harvested at 0, 1, 6, 12 and 24 h, respectively. Total RNA was extracted from different samples with the RNA Plant Plus Reagent Kit (Tiangen, China) following the manufacturer's instructions. First-strand cDNA was synthesized with M-MLV reverse transcriptase (TaKaRa, China). Quantitative real-time PCR analysis was performed with SYBR<sup>®</sup> Select Master Mix (Applied Biosystems, USA) under the following cycling conditions: 50°C for 2 min, 95°C for 2 min, 95°C for 15 s and 40 cycles at 60°C for 1 min. The  $\beta$ -*Actin* gene of *Arabidopsis* was used as the internal control. The relative gene expression was calculated by using the  $2^{-\Delta\Delta CT}$  method [34]. The primer pairs RqAtKUP12P1 and RqAtKUP12P2 for *AtKUP12* and qAtactinP1 and qAtactinP2 for  $\beta$ -*Actin* were listed in Tab. S1. *AtCOR15A* acts as a marker gene for abiotic stresses [35,36]. Each PCR was carried out for three biological replicates; each corresponded to three technological repetitions of a separate experiment.

### 2.3 Functional Complementation Assay of *AtKUP12* in Yeast

*AtKUP12* was cloned into the pYES2.0 vector, which was linearized by double digestion with *Kpn* I and *Xba* I. The vectors pYES2.0-*AtKUP12* and pYES2.0 were then transformed into K<sup>+</sup> uptake-deficient mutants of yeast strain CY162 (MAT $\alpha$ ,  $\Delta$ trk1, trk2::pCK64, his3, leu2, ura3, trp1 and ade2) [37]. The transformant was selected on SD-agar plates without uracil contained additional KCl to a final K<sup>+</sup> concentration of 100 mM. Yeast strain CY162-pYES2.0 was employed as a negative control, and yeast strain CY162-pYES2.0-*AtAKT1* (gifted by Dr Yue Shen, Nanjing Agricultural University) was used as a positive control. Subsequent growth assays for yeast were performed under low-K<sup>+</sup> (LK, 10, 20, 30 mM KCl) or high-K<sup>+</sup> (HK, 100 mM KCl) concentrations. Three independent clones per construct were used and analyzed in all growth tests on solid medium incubated at 30°C for 2 days.

### 2.4 Generation of the *atkup12-AtKUP12* Complementation Line

Based on the TAIR database (<http://www.Arabidopsis.org>), the open reading frame of *AtKUP12* (GenBank: NM\_104706) was amplified. The PCR product was digested with *Kpn* I and *Pst* I and ligated into the pCAMBIA1301-1 expression vector, with the CaMV35S promoter added at multiple cloning sites. The construct was introduced into *Agrobacterium tumefaciens* strain GV3101 and then transformed into the *atkup12* mutant using the floral-dip method [38]. The transgenic lines were selected with 30  $\mu$ g mL<sup>-1</sup> hygromycin and identified by PCR. Homozygous T3 plants were selected for further analysis.

### 2.5 Assays for Root Length, Fresh Weight, and Mineral Determination

Under low-K<sup>+</sup> or salt stress conditions, the seeds germinated and grew for 12 days. Primary root length was measured using ImageJ software, and the fresh weight of seedlings was recorded. The measurement of K<sup>+</sup> and Na<sup>+</sup> concentration followed previously described procedures [39]. *Arabidopsis* seedlings were rinsed in distilled water to wash away possible surface contamination of Na<sup>+</sup> and K<sup>+</sup> from the culture solution and soaked. The seedlings were dried in an oven at 105°C for 30 min, and then baked at 70°C for 3 days. The dried materials (ca 50 mg) were extracted in 20 mL of boiling water for 2 h. Na<sup>+</sup> and K<sup>+</sup> concentration was determined using ICP-AES systems (IRIS Advantage, Thermo Electron, Waltham, Massachusetts, USA). The experiment was performed four times. The phenotypic results were from one representative experiment, and the presented data were based on four biological replicates.

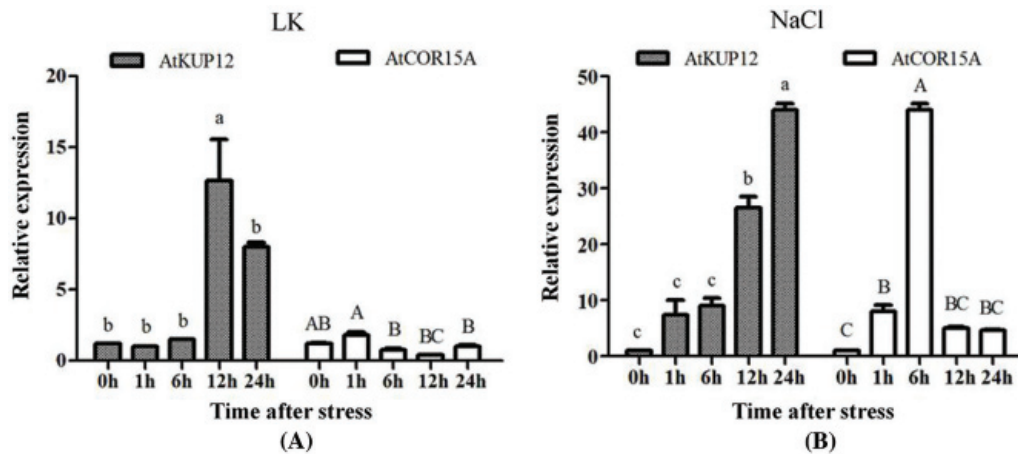
### 2.6 Statistical Analysis

Statistical differences were determined by one-way ANOVA and Tukey's multiple comparison tests using GraphPad Prism 5 software (San Diego, CA, USA). The means were considered to be significantly different at  $p < 0.05$  or  $p < 0.01$ .

## 3 Results

### 3.1 Expression Analyses of *AtKUP12*

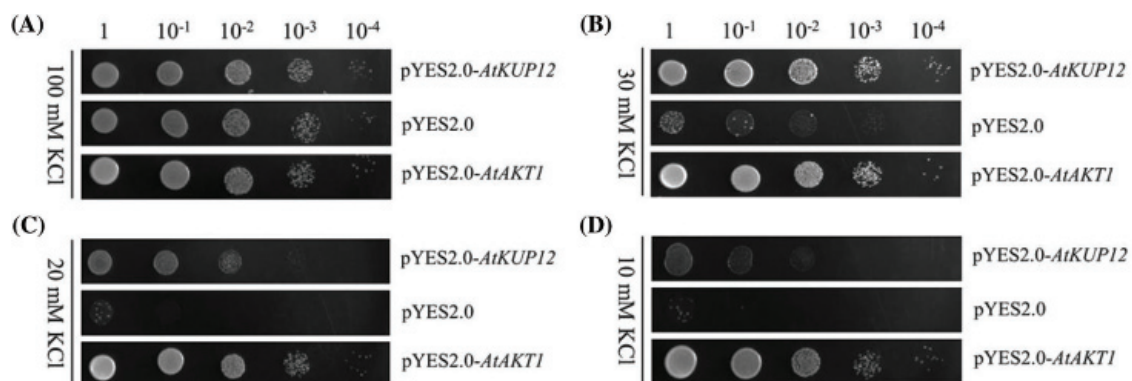
Expression levels of *AtKUP12* under conditions of low-K<sup>+</sup> and NaCl stresses were detected by qRT-PCR. The *Arabidopsis AtCOR15A* gene is upregulated at the transcriptional level under adverse stresses, such as high salt, ABA, cold, and drought [35,36]. Therefore, we used *AtCOR15A* gene as a marker gene. *AtCOR15A* was indeed upregulated under adverse conditions, except low-K<sup>+</sup> (Fig. 1). *AtKUP12* gene was induced by low-K<sup>+</sup> stress, reaching its peak after 12 h, and then it gradually declined until 24 h of stress (Fig. 1A). Under NaCl stress, the *AtKUP12* was continuously upregulated, peaking at 24 h by 30-fold (Fig. 1B). These results indicated that the expression of *AtKUP12* was stimulated by stress conditions, such as low-K<sup>+</sup> and NaCl treatments, and may play significant roles in the response of *Arabidopsis* to adverse environmental conditions.



**Figure 1:** *AtKUP12* expression levels under different conditions: Expression of *AtKUP12* in *Arabidopsis* under 100  $\mu\text{M}$  low- $\text{K}^+$  (A) or 100 mM NaCl (B). Letters denote significantly different groups identified by Tukey's test ( $p < 0.05$ ). Data the means  $\pm$  SE ( $n = 3$ )

### 3.2 Function of *AtKUP12* in $\text{K}^+$ Uptake in Yeast Cells

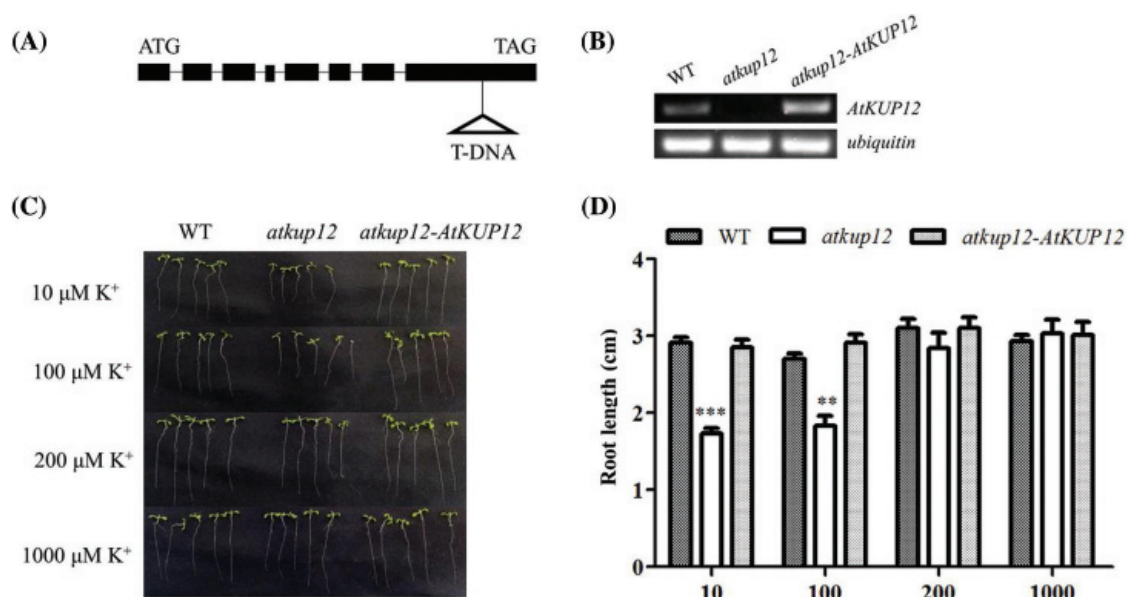
To investigate the  $\text{K}^+$ -uptake activity of *AtKUP12*, we performed complementation tests using the mutant yeast strain CY162. We used *AtAKT1* as a positive control, which mediated  $\text{K}^+$  uptake from solutions containing 10  $\mu\text{M}$   $\text{K}^+$  [40], and we used empty pYES2.0 vector as a negative control. Then, we performed yeast growth assays on SD-Ura medium containing 10, 20, 30 and 100 mM  $\text{K}^+$ . We found no remarkable differences among transformants when they were grown on SD-Ura medium containing 100 mM KCl (Fig. 2A). When the concentration of  $\text{K}^+$  in SD-Ura medium was decreased to 30 mM, the growth of the yeast strain CY162 transformed with the empty pYES2.0 vector severely inhibited, but there was no visible difference between the yeasts transformed with *AtAKT1* and the vector harboring *AtKUP12* (Fig. 2B). When the concentration of  $\text{K}^+$  in SD-Ura medium was decreased to 20 mM and 10 mM, *AtKUP12* partially rescued the growth defect of mutant yeast CY162 (Figs. 2C and 2D). These results indicated that *AtKUP12* conferred significant  $\text{K}^+$  uptake and growth of yeasts at low- $\text{K}^+$  concentrations.



**Figure 2:** Functional complementation of *AtKUP12* for  $\text{K}^+$  uptake. Growth status of  $\text{K}^+$  uptake-deficient yeast mutant CY162 cells expressing *AtKUP12*, empty pYES2.0 vector (negative control) and *AtAKT1* (positive control) on SD-Ura medium containing different  $\text{K}^+$  concentrations

### 3.3 Functional Characterization of *AtKUP12* in *Arabidopsis*

To test whether *AtKUP12* has physiological roles in response to the low- $K^+$  stress (10, 100, 200  $\mu$ M KCl), the *atkup12* mutant At1g60160 (SALK\_083613) was used in this study. The T-DNA insertion site was located at the eighth exon of the *AtKUP12* gene (Fig. 3A). Subsequently, we generated complementation lines for *atkup12*. As shown in Figs. 3B–3D, the primary root length of the *atkup12* mutant was significantly shorter than that of WT plants when grown on low- $K^+$  medium (10 and 100  $\mu$ M  $K^+$ ), while the complementation line (*atkup12-AtKUP12*) completely rescued the low- $K^+$  sensitive phenotype of the *atkup12* mutant. Along with the increased  $K^+$  concentrations (200 and 1000  $\mu$ M  $K^+$ ), the low- $K^+$  sensitive phenotype of *atkup12* disappeared (Figs. 3B–3D). These results indicated that the low- $K^+$  sensitive phenotype of *atkup12* was due to the disruption of *AtKUP12*.

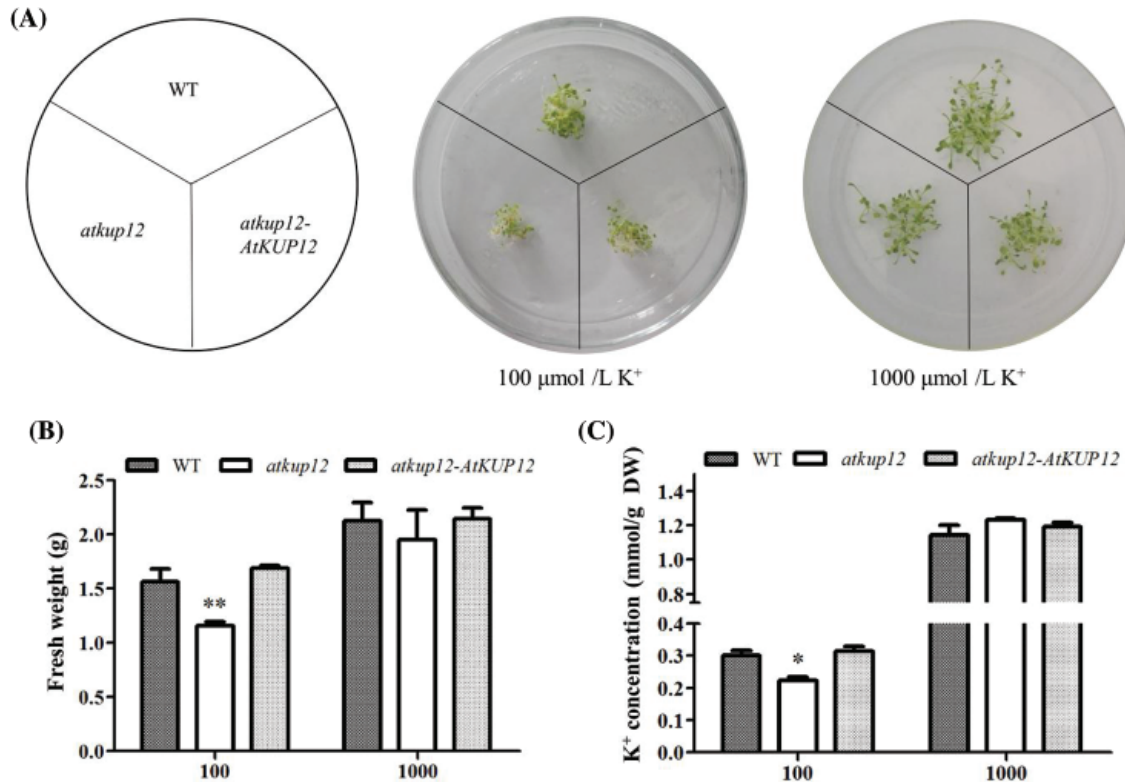


**Figure 3:** Expression of *AtKUP12* rescued the low- $K^+$ -sensitive phenotype of the *Arabidopsis atkup12* mutant. (A) Schematic diagram of the *AtKUP12* gene. Black boxes indicate exons, and lines represent introns. The T-DNA insertion site in the *atkup12* mutant is shown by a triangle. (B) RT-PCR verification of *AtKUP12* expression in the WT, *atkup12* mutant and complementation lines. Ubiquitin was used as an internal control. (C) Phenotypic comparison among WT, *atkup12*, and *atkup12-AtKUP12* complementation lines under different  $K^+$  concentrations. Seeds were germinated and grown on low- $K^+$  (10, 100 and 200  $\mu$ M) or  $K^+$ -sufficient (1000  $\mu$ M) medium for 12 days. (D) The primary root length among the plant materials is shown in (C). Asterisks indicate significantly different groups identified by Tukey's test ( $p < 0.05$ ). Data means  $\pm$  SE ( $n = 4$ ) of 5 seedlings

### 3.4 Loss of Function of *AtKUP12* Inhibits Seedling Growth and Reduces $K^+$ Uptake

Under the 100  $\mu$ M low- $K^+$  condition, the primary root length of the *atkup12* mutant was significantly shorter than that of the WT and *atkup12-AtKUP12* complementation lines. Therefore, we selected 100  $\mu$ M  $K^+$  as the low- $K^+$  stress for the determination of fresh weight and  $K^+$  concentration. Under sufficient  $K^+$  conditions (1000  $\mu$ M  $K^+$ ), for 12 days, we observed no substantial difference in either fresh weight or  $K^+$  concentrations among the plant materials (Figs. 4A–4C). However, under low- $K^+$  stress, the *atkup12* mutant shoots were smaller, and the fresh weight and  $K^+$  concentration were much lower than those of WT plants, while the fresh weight and  $K^+$  concentration in the complementation line recovered,

which was similar to WT plants (Figs. 4A–4C). These results implied that AtKUP12 might function in  $K^+$  uptake in *Arabidopsis* plants.



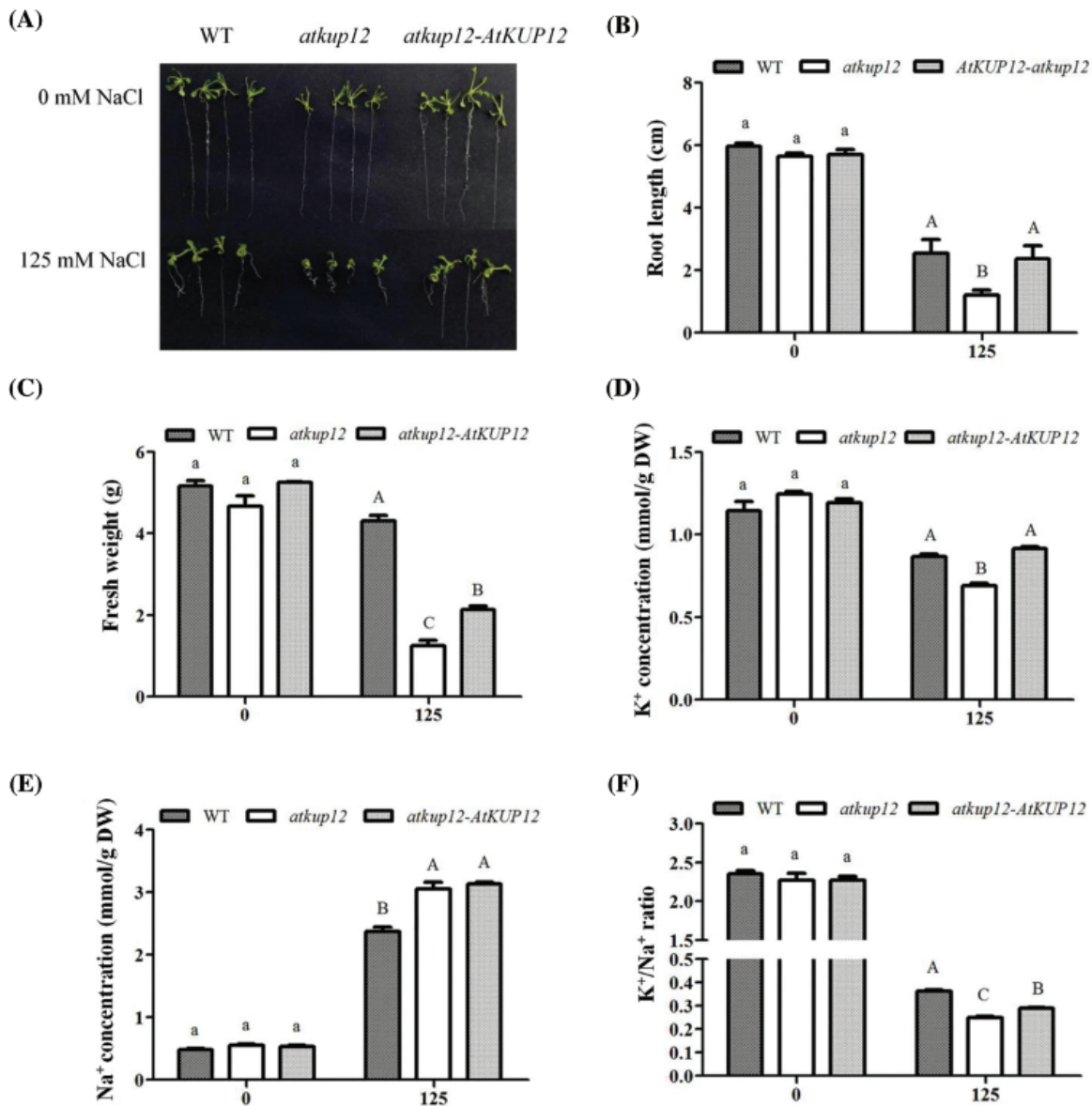
**Figure 4:** Effects of *AtKUP12* disruption on growth and  $K^+$  concentration under low- $K^+$  stress. Seeds germinated on medium containing 100 and 1000  $\mu M$   $K^+$  for 12 days growth. (A) Growth phenotype of shoots. (B) Fresh weight. (C)  $K^+$  concentration. Asterisks indicate significant differences from the WT at  $p < 0.05$  by the Student's *t*-test. Data are means  $\pm$  SE ( $n = 4$ ) of 60 seedlings

### 3.5 Effect of Disruption of *AtKUP12* on Growth Tolerance to Salt, $K^+$ Uptake and $K^+/Na^+$ Homeostasis

To confirm that the loss of function of *AtKUP12* was responsible for the salt-sensitive phenotype, seeds of the WT, *atkup12* and *atkup12-AtKUP12* complementation lines were germinated and grown on medium containing 125 mM NaCl for 12 days. As shown in Fig. 5, the phenotypes among the plant materials, including the WT, *atkup12* and complementation lines, did not obviously differ under control conditions. However, the primary root length of *atkup12* was inhibited to a greater extent than that of the WT under 125 mM NaCl stress, and the complementation lines almost rescued the salt-sensitive phenotype of *atkup12* mutants (Figs. 5A and 5B). We also observed and measured the primary root length of different plants under 100 mM NaCl stress, but there was no difference among the plant materials (Figs. S1A and S1B).

In addition, we measured the fresh weight and further determined the  $K^+$  and  $Na^+$  concentrations of diverse plant materials (Figs. 5C–5E). Under control conditions, there was no obvious difference in fresh weight,  $K^+$  and  $Na^+$  concentrations, and  $K^+/Na^+$  ratio among various plants (Figs. 5C–5F). Under salt stress conditions, the *atkup12* mutant showed a reduced fresh weight that was statistically significant, while the fresh weight of the complementation line was partly resumed (Fig. 5C). *atkup12* showed lower  $K^+$  and greater  $Na^+$  concentrations than WT plants, while  $K^+$  concentration in the complementation line

was restored to levels close to the corresponding WT values. However, the  $\text{Na}^+$  concentration was significantly higher in the complementation line than in the WT (Figs. 5D and 5E). Furthermore, the calculated  $\text{K}^+/\text{Na}^+$  ratio in *atkup12* was significantly lower than WT and partly restored in the complementation line (Fig. 5F). These data suggest that *AtKUP12* may function in  $\text{K}^+$  uptake and maintenance of the  $\text{K}^+/\text{Na}^+$  ratio under salt stress, which is crucial for salt tolerance in plants.



**Figure 5:** Complementation lines rescue the salt-sensitive phenotype of *atkup12* mutants. (A) Phenotypic comparison among WT, *atkup12*, and *atkup12-AtKUP12* complementation line under either salt stress (125 mM NaCl) or not (0 mM NaCl). Seeds germinated and grew on medium containing 0 and 125 mM NaCl for 12 days. Primary root length (B), fresh weight (C),  $\text{K}^+$  concentration (D),  $\text{Na}^+$  concentration (E) and  $\text{K}^+/\text{Na}^+$  ratio (F) are shown. Letters denote significantly different groups identified by Tukey's test ( $p < 0.05$ ). Data are means  $\pm$  SE ( $n = 4$ ) of 60 seedlings



#### 4 Discussion

The importance of  $K^+$  is self-evident.  $K^+$  transporters play crucial roles in  $K^+$  absorption, transport and maintenance of  $K^+$  homeostasis under  $K^+$  deficient environments to ensure the normal growth of plants under abiotic stress [41]. At present,  $K^+$  transporter family genes responding to salt, drought and hormone stress have been found in a variety of plants, such as the *Arabidopsis AtHAK5*, *AtHKT1*, and *AtKUP6* genes, tomato *LeHAK5* gene, rice *OsHAK21* gene, and pear *PbHAK1*, *PbHAK7* and *PbHAK10* genes, which are significantly enhanced by adverse stresses [26,29,42–44].

*Arabidopsis AtCOR15A* responds positively to adverse stresses such as high salt, ABA, cold, and drought; thus, it is often used as a marker gene of *Arabidopsis* under the above abiotic stresses [35,36]. In this experiment, *Arabidopsis AtCOR15A* was employed as a positive control under stress treatment. The expression of *AtKUP12* under different stress treatments showed that *AtKUP12* responded positively to abiotic stresses of salt and low- $K^+$  treatment. The expression patterns of the *AtKUP12* gene under NaCl stress was continuously upregulated, reaching a maximum value at 24 h, and the expression increased by 30 fold, compared with the control. The above experimental results show that the *AtKUP12* had the strongest response to salt stress.

As a single cell eukaryote, yeast is often used for functional verification and analysis of genes because of its short culture cycle and easy genetic transformation. The CY162 yeast strain is a type of  $K^+$  uptake-deficient yeast cell caused by the deletion and insertion of *Trk1* and *Trk2*; thus, it has become a model material for studying  $K^+$  transport and related proteins [6,29]. The inward-rectifier  $K^+$  channel of the shaker family, *AtAKT1* in *Arabidopsis*, is involved in  $K^+$  uptake at both high- and low-affinity ranges [40]. The major regulation of the *AtAKT1* activity has been described at the protein level; *AtAKT1* activity is modulated by the CBL1/9–CIPK23 pathway under low- $K^+$  supply conditions [45]. Here, we used *AtAKT1* as a positive control. The heterologous expression of *OsHAK21* in CY162 yeast has shown that *OsHAK21* can mediate specific  $K^+$  transport under low- $K^+$  [6]. Han et al. also indicated that *AtKUP7* has  $K^+$  transport activity by using the yeast mutant but that the transformed  $K^+$ -deficient yeast strain can only grow at a concentration of more than 500  $\mu$ M, suggesting that *AtKUP7* is a low-affinity  $K^+$  transporter [46]. Qin et al. found that the heterologous expression of maize *ZmHAK5* and *ZmHAK1* in CY162 yeast can mediate  $K^+$  uptake under a low- $K^+$  condition, but *ZmHAK1*  $K^+$  transport activity is weaker than *ZmHAK5* [5]. Similarly, our results showed that the heterologous expression of *AtKUP12* in the  $K^+$  uptake-deficient yeast CY162 could mediate  $K^+$  uptake under low- $K^+$  conditions, indicating that *AtKUP12* in *Arabidopsis* participates in high-affinity  $K^+$  uptake and transport.

*Arabidopsis AtHAK5* is a high-affinity  $K^+$  uptake transporter gene; after the destruction of its alleles, the *athak5* mutant showed a certain growth inhibition under a low- $K^+$  condition [17]. However, the heterologous expression of rice *OsHAK21* and maize *ZmHAK5* transporters completely restored the defect of *athak5* mutants [5,23]. A complementation line of the *AtKUP7* gene in *Arabidopsis* restored the growth inhibition of the *atkup7* mutant under low- $K^+$  conditions [46]. Our results also showed that the *atkup12-AtKUP12* complementation line alleviated the growth inhibition of *atkup12* under low- $K^+$  stress (Figs. 3 and 4). Under 100  $\mu$ M low- $K^+$  treatment, the fresh weight of *atkup12* mutant was significantly lower than that of WT, but there was no significant difference between the complementation and WT lines, suggesting that the *AtKUP12* gene might regulate plant growth through  $K^+$  uptake. It should be noted that both *atkup12* and *athak5* mutants [5,17] affected the root length of plants under low- $K^+$  stress, while *atkup7* mutants exhibited chlorosis symptoms compared with WT plants [46], which may be related to the specific functions of different transporters. The  $K^+$  concentration testing showed that the  $K^+$  concentration in the complementation line resumed, which was similar to the  $K^+$  concentration in WT plants under low- $K^+$  conditions. This finding indicated that *AtKUP12* conferred significant  $K^+$  uptake capacity and improved seedling growth and root development in *Arabidopsis* at low- $K^+$  concentrations.

The  $K^+/Na^+$  ratio in cytoplasm is critical for the salt tolerance of plants. Knockout of *OsHAK5* or *OsHAK21* leads to a decreased  $K^+/Na^+$  ratio in rice tissues and to salt sensitivity, while overexpression of *OsHAK5* significantly improves the salt tolerance of transgenic rice [6,29]. In addition, some transporters, such as HvHAK1 and PhaHAK5, are also able to function in low-affinity  $Na^+$  uptake in the presence of NaCl [47,48]. In this study, under salt stress conditions, the *atkup12* mutant also showed salt-sensitivity, and the *atkup12-AtKUP12* complementation line significantly alleviated the inhibition of root length and fresh weight. Under 125 mM NaCl treatment, the  $K^+$  concentration of the *atkup12* mutant was significantly lower than that of WT and complementation lines, while the  $Na^+$  concentration of *atkup12* and the complementation line was significantly higher than that of WT line. This might be due to the similarity in physicochemical properties between  $Na^+$  and  $K^+$ , resulting in competition between  $Na^+$  and  $K^+$  [49]. However, the  $K^+/Na^+$  ratio of the *atkup12* mutant was significantly lower than that of WT, while the  $K^+/Na^+$  ratio in the complementation line was partially restored under salt stress. Therefore, we speculate that AtKUP12 is an important KT/HAK/KUP family member that improves the salt tolerance of plants by  $K^+$  uptake and the  $K^+/Na^+$  ratio balance.

## 5 Conclusions

From the results presented in this study, we demonstrated that a  $K^+$  transport gene *AtKUP12* was induced by low- $K^+$  and NaCl stresses, while AtKUP12 participated in  $K^+$  acquisition and the growth of the yeast mutant strain CY162 at low- $K^+$  concentrations. In addition, AtKUP12 also promoted plant growth, enhanced the  $K^+$  concentration under low- $K^+$  conditions, and enhanced the salt tolerance of plants by maintenance of the  $K^+/Na^+$  ratio. These results may facilitate further investigation of the function and mechanism underlying  $K^+$  uptake and utilization of AtKUP12 under low- $K^+$  and salt stress conditions.

**Acknowledgement:** We gratefully thank Dr Jianhua Yang and Shen Zhang for revising the manuscript.

**Funding Statement:** This work was supported by the National Natural Science Foundation of China [Grant No. 31860061], Opening of Key Laboratory of Autonomous Region [Grant No. 2017D04026] and Tianshan Youth Program [Grant No. 2019Q013].

**Conflicts of Interest:** The authors declare that they have no conflicts of interest to report regarding the present study.

## References

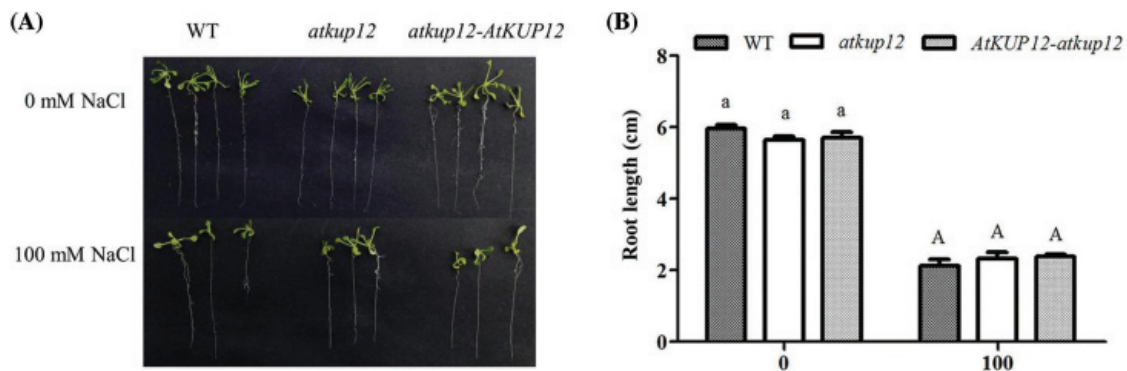
1. Li, L. G., Kim, B. G., Cheong, Y. H., Pandey, G. K., Luan, S. (2006). A  $Ca^{2+}$  signaling pathway regulates a  $K^+$  channel for low-K response in *Arabidopsis*. *Proceedings of the National Academy of Sciences of the United States of America*, 103(33), 12625–12630. DOI 10.1073/pnas.0605129103.
2. Watanabe, T., Broadley, M. R., Jansen, S., White, P. J., Takada, J. et al. (2007). Evolutionary control of leaf element composition in plants. *New Phytologist*, 174(3), 516–523. DOI 10.1111/j.1469-8137.2007.02078.x.
3. White, P. J. (2013). Improving potassium acquisition and utilisation by crop plants. *Journal of Plant Nutrition and Soil Science*, 176(3), 305–316. DOI 10.1002/jpln.201200121.
4. Shen, X. Y., Jiang, C. R., Wang, Z. L. (2011). Optimization of the method for screening *Arabidopsis* low- $K^+$  tolerant mutants. *Journal of Henan Agricultural Sciences*, 40(3), 56–58. DOI 10.15933/j.cnki.1004-3268.2011.03.001.
5. Qin, Y. J., Wu, W. H., Wang, Y. (2019). ZmHAK5 and ZmHAK1 function in  $K^+$  uptake and distribution in maize under low  $K^+$  conditions. *Journal of Integrative Plant Biology*, 61(6), 691–705. DOI 10.1111/jipb.12756.
6. Yang, T. Y., Zhang, S., Hu, Y. B., Wu, F. C., Hu, Q. D. et al. (2014). The role of a potassium transporter OsHAK5 in potassium acquisition and transport from roots to shoots in rice at low potassium supply levels. *Plant Physiology*, 166(2), 945–959. DOI 10.1104/pp.114.246520.

7. Wang, Y., Jiang, G. Q., Han, Y. N., Liu, M. M. (2013). Effects of salt, alkali and salt–alkali mixed stresses on seed germination of the halophyte *Salsola ferganica* (Chenopodiaceae). *Acta Ecologica Sinica*, 33(6), 354–360. DOI 10.1016/j.chnaes.2013.09.010.
8. Lebaudy, A., Véry, A. A., Sentenac, H. (2007). K<sup>+</sup> channel activity in plants: Genes, regulations and functions. *FEBS Letters*, 581(12), 2357–2366. DOI 10.1016/j.febslet.2007.03.058.
9. Alexander, G. (2007). Plant KT/KUP/HAK potassium transporters: Single family multiple functions. *Annals of Botany*, 99(6), 1035–1041. DOI 10.1093/aob/mcm066.
10. Corratgé-Faillie, C., Jabnourne, M., Zimmermann, S., Véry, A. A., Fizames, C. et al. (2010). Potassium and sodium transport in non-animal cells: The Trk/Ktr/HKT transporter family. *Cellular and Molecular Life Sciences*, 67(15), 2511–2532. DOI 10.1007/s00018-010-0317-7.
11. Cheng, X. Y., Liu, X. D., Mao, W. W., Zhang, X. R., Chen, S. L. et al. (2018). Genome-wide identification and analysis of HAK/KUP/KT potassium transporters gene family in wheat (*Triticum aestivum* L.). *International Journal of Molecular Sciences*, 19(12), 3969–3990. DOI 10.3390/ijms19123969.
12. Yang, Z. F., Gao, Q. S., Sun, C. S., Li, W. J., Gu, S. L. et al. (2009). Molecular evolution and functional divergence of HAK potassium transporter gene family in rice (*Oryza sativa* L.). *Journal of Genetics and Genomics*, 36(3), 161–172. DOI 10.1016/S1673-8527(08)60103-4.
13. Mäser, P., Thomine, S., Schroeder, J. I., Ward, J. M., Hirschi, K. et al. (2001). Phylogenetic relationships within cation transporter families of Arabidopsis. *Plant Physiology*, 126(4), 1646–1667. DOI 10.1104/pp.126.4.1646.
14. Li, W. H., Xu, G. H., Alli, A., Yu, L. (2018). Plant HAK/KUP/KT K<sup>+</sup> transporters: Function and regulation. *Seminars in Cell & Developmental Biology*, 74, 133–141. DOI 10.1016/j.semcd.2017.07.009.
15. Okada, T., Nakayama, H., Shinmyo, A., Yoshida, K. (2008). Expression of *OsHAK* genes encoding potassium ion transporters in rice. *Plant Biotechnology*, 25(3), 241–245. DOI 10.5511/plantbiotechnology.25.241.
16. Véry, A. A., Nieves-Cordones, M., Daly, M., Khan, I., Fizames, C. et al. (2014). Molecular biology of K<sup>+</sup> transport across the plant cell membrane: What do we learn from comparison between plant species. *Journal of Plant Physiology*, 171(9), 748–769. DOI 10.1016/j.jplph.2014.01.011.
17. Pyo, Y. J., Gierth, M., Schroeder, J. I., Cho, M. H. (2010). High-affinity K<sup>+</sup> transport in *Arabidopsis*: AtHAK5 and AKT1 are vital for seedling establishment and postgermination growth under low-potassium conditions. *Plant Physiology*, 153(2), 863–875. DOI 10.1104/pp.110.154369.
18. Fulgenzi, F. R., Peralta, M. L., Mangano, S., Danna, C. H., Vallejo, A. J. et al. (2008). The ionic environment controls the contribution of the barley HvHAK1 transporter to potassium acquisition. *Plant Physiology*, 147(1), 252–262. DOI 10.1104/pp.107.114546.
19. Ahn, S. J., Shin, R., Schachtman, D. P. (2004). Expression of *KT/KUP* genes in *Arabidopsis* and the role of root hairs in K<sup>+</sup> uptake. *Plant Physiology*, 134(3), 1135–1145. DOI 10.1104/pp.103.034660.
20. Li, X. W., You, X. L., Wang, Y. (2019). Research progress of HAK/KUP/KT potassium transporter family in plant response to salt stress. *Plant Science Journal*, 37(1), 101–108. DOI 10.11913/PSJ.2095-0837.2019.10101.
21. Roshani, G. A., Narayanasamy, G. (2010). Effects of potassium on temporal growth of root and shoot of wheat and its uptake in different soils. *International Journal of Plant Production*, 4(1), 25–32.
22. Han, M. (2015). *Functional analysis of potassium transporter KUP7 in Arabidopsis responses low-K<sup>+</sup> stress* (Ph.D. Thesis). China Agricultural University, Beijing.
23. Shen, Y. (2015). *Functional analyses of potassium transporter OsHAK21 and channel OsKx in response to salt stress in rice* (Ph.D. Thesis). Nanjing Agricultural University, Nanjing.
24. Tenorio-Berrio, R., Pérez-Alonso, M. M., Vicente-Carbajosa, J., Martín-Torres, L., Dreyer, I. et al. (2018). Identification of two auxin-regulated potassium transporters involved in seed maturation. *International Journal of Molecular Sciences*, 19(7), 2132–2148. DOI 10.3390/ijms19072132.
25. Chen, G., Hu, Q. D., Luo, L., Yang, T. Y., Zhang, S. et al. (2015). Rice potassium transporter OsHAK1 is essential for maintaining potassium-mediated growth and functions in salt tolerance over low and high potassium concentration ranges. *Plant, Cell & Environment*, 38(12), 2747–2765. DOI 10.1111/pce.12585.

26. Osakabe, Y., Arinaga, N., Umezawa, T., Katsura, S., Nagamachi, K. et al. (2013). Osmotic stress responses and plant growth controlled by potassium transporters in *Arabidopsis*. *Plant Cell*, 25(2), 609–624. DOI 10.1105/tpc.112.105700.
27. Zhang, H. W., Xiao, W., Yu, W. W., Jiang, Y., Li, R. F. (2020). Halophytic *Hordeum brevisubulatum* HbHAK1 facilitates potassium retention and contributes to salt tolerance. *International Journal of Molecular Sciences*, 21(15), 5292–5306. DOI 10.3390/ijms21155292.
28. Horie, T., Sugawara, M., Okada, T., Taira, K., Kaothien-Nakayama, P. et al. (2011). Rice sodium-insensitive potassium transporter, OsHAK5, confers increased salt tolerance in tobacco BY2 cells. *Journal of Bioscience and Bioengineering*, 111(3), 346–356. DOI 10.1016/j.jbiosc.2010.10.014.
29. Shen, Y., Shen, L., Shen, Z. X., Jing, W., Ge, H. L. et al. (2015). The potassium transporter OsHAK21 functions in the maintenance of ion homeostasis and tolerance to salt stress in rice. *Plant, Cell & Environment*, 38(12), 2766–2779. DOI 10.1111/pce.12586.
30. Nieves-Cordones, M., Aleman, F., Martinez, V., Rubio, F. (2010). The *Arabidopsis thaliana* HAK5 K<sup>+</sup> transporter is required for plant growth and K<sup>+</sup> acquisition from low K<sup>+</sup> solutions under saline conditions. *Molecular Plant*, 3(2), 326–333. DOI 10.1093/mp/ssp102.
31. Yang, Z. M., Wang, Y. (2015). Cloning of potassium transporter gene (*HcKUP12*) from *Halostachys capsica* and its expression profile under salt stress. *Plant Science Journal*, 33(4), 499–506. DOI 10.3969/j.issn.1004-311X.2014.03.0074.
32. You, X. L., Yang, Z. M., Wang, Y. (2017). Identification of potassium transporter mutant *atkup12* from *Arabidopsis* and sensitivity analysis to abiotic stresses. *Biotechnology*, 27(5), 497–504. DOI 10.16519/j.cnki.1004-311x.2017.05.0079.
33. Liu, L. L., Ren, H. M., Chen, L. Q., Wang, Y., Wu, W. H. (2013). A protein kinase, calcineurin B-like protein-interacting protein Kinase9, interacts with calcium sensor calcineurin B-like Protein3 and regulates K<sup>+</sup> homeostasis under low-K<sup>+</sup> stress in *Arabidopsis*. *Plant Physiology*, 161(1), 266–277. DOI 10.1104/pp.112.206896.
34. Livak, K. J., Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2<sup>-ΔΔCT</sup> Method. *Methods*, 25(4), 402–408. DOI 10.1006/meth.2001.1262.
35. Ishitani, M., Xiong, L. M., Lee, H., Stevenson, B., Zhu, J. K. (1998). *HOS1*, a genetic locus involved in cold-responsive gene expression in *Arabidopsis*. *Plant Cell*, 10(7), 1151–1161. DOI 10.1105/tpc.10.7.1151.
36. Lim, G. H., Zhang, X., Chung, M. S., Lee, D. J., Woo, Y. M. et al. (2010). A putative novel transcription factor, AtSKIP, is involved in abscisic acid signalling and confers salt and osmotic tolerance in *Arabidopsis*. *New Phytologist*, 185(1), 103–113. DOI 10.1111/j.1469-8137.2009.03032.x.
37. Anderson, J. A., Huprikar, S. S., Kochian, L. V., Lucas, W. J., Gaber, R. F. (1992). Functional expression of a probable *Arabidopsis thaliana* potassium channel in *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences*, 89(9), 3736–3740. DOI 10.1073/pnas.89.9.3736.
38. Clough, S. J., Bent, A. F. (1998). Floral dip: A simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant Journal*, 16(6), 735–743. DOI 10.1046/j.1365-313x.1998.00343.x.
39. Matsushita, N., Matoh, T. (1991). Characterization of Na<sup>+</sup> exclusion mechanisms of salt-tolerant reed plants in comparison with salt-sensitive rice plants. *Physiologia Plantarum*, 83(1), 170–176. DOI 10.1111/j.1399-3054.1991.tb01298.x.
40. Nieves-Cordones, M., Martínez, V., Benito, B., Rubio, F. (2016). Comparison between *Arabidopsis* and rice for main pathways of K<sup>+</sup> and Na<sup>+</sup> uptake by roots. *Frontiers in Plant Science*, 7(ra43), 992–1005. DOI 10.3389/fpls.2016.00992.
41. Shabala, S., Pottosin, I. (2014). Regulation of potassium transport in plants under hostile conditions: Implications for abiotic and biotic stress tolerance. *Physiologia Plantarum*, 151(3), 12165–12187. DOI 10.1111/pp1.12165.
42. Sun, Y. L., Kong, X. P., Li, C. L., Liu, Y. X., Ding, Z. J. (2015). Potassium retention under salt stress is associated with natural variation in salinity tolerance among *Arabidopsis* accessions. *PLoS One*, 10(5), e0124032. DOI 10.1371/journal.pone.0124032.

43. Bacha, H., Ródenas, R., López-Gómez, E., García-Legaz, M. F., Nieves-Cordones, M. et al. (2015). High  $\text{Ca}^{2+}$  reverts the repression of high-affinity  $\text{K}^+$  uptake produced by  $\text{Na}^+$  in *Solanum lycopersicum* L. (var. microtom) plants. *Journal of Plant Physiology*, *180*, 72–79. DOI 10.1016/j.jplph.2015.03.014.
44. Li, Y., Peng, L. R., Xie, C. Y., Shi, X. Q., Dong, C. X. et al. (2018). Genome-wide identification, characterization, and expression analyses of the HAK/KUP/KT potassium transporter gene family reveals their involvement in  $\text{K}^+$  deficient and abiotic stress responses in pear rootstock seedlings. *Plant Growth Regulation*, *85*(2), 187–198. DOI 10.1007/s10725-018-0382-8.
45. Xu, J., Li, H. D., Chen, L. Q., Wang, Y., Liu, L. L. et al. (2006). A protein kinase, interacting with two calcineurin B-like proteins, regulates  $\text{K}^+$  transporter AKT1 in *Arabidopsis*. *Cell*, *125*(7), 1347–1360. DOI 10.1016/j.cell.2006.06.011.
46. Han, M., Wu, W., Wu, W. H., Wang, Y. (2016). Potassium transporter KUP7 is involved in  $\text{K}^+$  acquisition and translocation in *Arabidopsis* root under  $\text{K}^+$ -limited conditions. *Molecular Plant*, *9*(3), 437–446. DOI 10.1016/j.molp.2016.01.012.
47. Santa-Maria, G. E., Rubio, F., Dubcovsky, J., Rodríguez-Navarro, A. (1998). The HAK1 gene of barley is a member of a large gene family and encodes a high-affinity potassium transporter. *Plant Cell*, *9*(12), 2281–2289. DOI 10.1105/tpc.9.12.2281.
48. Takahashi, R., Nishio, T., Ichizen, N., Takano, T. (2007). High-affinity  $\text{K}^+$  transporter PhaHAK5 is expressed only in salt-sensitive reed plants and shows  $\text{Na}^+$  permeability under  $\text{NaCl}$  stress. *Plant Cell Reports*, *26*(9), 1673–1679. DOI 10.1007/s00299-007-0364-1.
49. Ashraf, M., Ali, Q. (2007). Relative membrane permeability and activities of some antioxidant enzymes as the key determinants of salt tolerance in canola (*Brassica napus* L.). *Environmental and Experimental Botany*, *63*(1–3), 266–273. DOI 10.1016/j.envexpbot.2007.11.008.

## Appendix



**Figure S1:** Phenotype test of *atkup12* mutants and the *atkup12-AtKUP12* complementation lines on medium containing 100 mM NaCl. (A) Phenotypic comparison among WT, *atkup12*, and *atkup12-AtKUP12* complementation lines under 100 mM NaCl stress. Seeds germinated and grew on medium for 12 days. (B) Primary root length. Letters denote significantly different groups identified by Tukey's test ( $p < 0.05$ ). Data are means  $\pm$  SE ( $n = 4$ )

**Table S1:** Primer sequences used in this study

Primer name	Primer sequence (5'-3')
RqAtKUP12P1	GAAGTAGTGACCGGAGATGGGTTGA
RqAtKUP12P2	TGTGACCATGAGAACCTGCAATTTC
qAtactinP1	CCAAAGGCCAACAGAGAGAAGAT
qAtactinP2	TGAGACACACCATCACCAGAA