A DExD/H box RNA helicase is important for K$^+$ deprivation responses and tolerance in *Arabidopsis thaliana*

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The molecular mechanism for sensing and transducing the stress signals initiated by K$^+$ deprivation in plants remains unknown. Here, we found that the expression of *AtHELPS*, an *Arabidopsis* DExD/H box RNA helicase gene, was induced by low-K$^+$, zeatin and cold treatments, and downregulated by high-K$^+$ stress. To further investigate the expression pattern of *AtHELPS*, *pAtHELPS::GUS* transgenic plants were generated. Histochemical staining indicated that *AtHELPS* is mainly expressed in the young seedlings and vascular tissues of leaves and roots. Using both *helps* mutants and overexpression lines, we observed that, in the low-K$^+$ condition, *AtHELPS* affected *Arabidopsis* seed germination and plant weight. Interestingly, the mRNA levels of *AKT1*, *CBL1/9* and *CIPK23* in the *helps* mutants were much higher than in the overexpression lines under low-K$^+$ stress. Moreover, under low-K$^+$ stress, the *helps* mutants displayed increased K$^+$ influx, whereas the overexpression line of *AtHELPS* had a lower flux rate in the roots by the noninvasive micro-test technique. Taken together, these results provide information for the functional analysis of plant DEVH box RNA helicases, and suggest that *AtHELPS*, as an important negative regulator, plays a role in K$^+$ deprivation stress.

Introduction

Soil nutrients are essential for plant growth and metabolism. Plant roots acquire nutrients from soil, and have developed adaptive mechanisms to ensure nutrient acquisition despite varying nutritional conditions in soil [1]. K$^+$ concentrations in soil usually range from 0.04% to 3%, but the worldwide distribution of K$^+$ is inconsistent [2]. In the tropics and subtropics, one-quarter of the soil has been threatened because of a lack of K$^+$ [3]. K$^+$ is essential for plants, and is required in large quantities. Under low-K$^+$ stress, most plants show K$^+$ deficiency symptoms, typically leaf chlorosis and subsequent inhibition of plant growth and development [4]. As K$^+$ availability in soil may vary considerably, depending on environmental and soil conditions, plants must be able to adjust to changing K$^+$ concentrations. When plants are deprived of K$^+$, the roots activate some important adaptive mechanisms for the uptake of K$^+$ that help support plant growth and survival. To ensure an adequate supply of K$^+$, plants have a number of redundant mechanisms for K$^+$ acquisition and translocation [5–7]. In the past decade, several high-affinity K$^+$ transporters, such as AKT1, the HKT family, and the KT/KUP/HAK family, were identified in different plant species [8–11]. Recent studies have provided direct evidence that, in *Arabidopsis*, mediation of K$^+$ uptake at low K$^+$ concentrations via AKT1 requires interaction with CIPK23 and CBL1/9 [12,13]. However, little is known about how plant cells sense and respond to changes in the K$^+$ concentrations encountered in their environment [14,15].

Helicases belong to a class of molecular motor proteins in yeast, animals, and plants, and they are

Abbreviations

ABA, abscisic acid; FW, fresh weight; GUS, β-glucuronidase; NMT, noninvasive micro-test technique.

divided into three superfamilies. RNA helicases use energy derived from the hydrolysis of a nucleotide triphosphate to unwind dsRNAs [16]. The majority of RNA helicases belong to the superfamily 2 subclass, which is characterized by sequence homology within a helicase domain consisting of eight or nine conserved amino acid motifs. Superfamily 2 consists of three subfamilies, known as DEAD, DEAH, and DExH/D, on the basis of variations within a common DEAD (Asp-Glu-Ala-Asp) motif [17–19]. RNA helicases have been shown to be involved in every step of RNA metabolism, including nuclear transcription, pre-mRNA splicing, ribosome biogenesis, nucleocytoplasmic transport, translation, RNA decay, and organellar gene expression [16,17,20]. Given their multiple functions in cellular RNA metabolism, it is not surprising that RNA helicases are also involved in responses to abiotic stress.

Recently, an *Arabidopsis* DEAD box RNA helicase, LOS4, was shown to be involved in responses to low temperature, high temperatures, and abscisic acid (ABA) [21,22]. Another two DEAD box RNA helicases, STRS1 and STRS2, were shown to improve *Arabidopsis* responses to multiple abiotic stresses, such as salt, osmotic stress, heat stress, and ABA [23]. These investigations indicate that DEAD box RNA helicases may play an important role in building resistance to abiotic stress during plant growth and development. For plant DExH box helicase, however, *Arabidopsis* CAF/DICER-LIKE 1 has been shown to be critical for the biogenesis of microRNAs and plant development [24,25]. *Arabidopsis* TEBICHI was shown to be required for regulating cell division and differentiation in meristems [26], and ISE2, localized in cytoplasmic granules, was shown to be involved in plasmodesmata function during embryogenesis in *Arabidopsis* [27]. Although DEAD or DEAH box RNA helicases have been shown to participate in cold, salt and osmotic stresses [21–23], whether DExH box RNA helicases are involved in plant responses to abiotic stresses remain to be addressed.

In this study, we identified and characterized an *Arabidopsis* DEVH box RNA helicase named AtHELPS. The transcripts of *AtHELPS* in *Arabidopsis* were affected by multiple treatments, including low K^+, zeatin, and cold. By using wild-type, *helps* mutant and overexpression lines of *Arabidopsis*, we demonstrated that, in the low-K^+ condition, AtHELPS inhibited *Arabidopsis* seed germination via decreased K^+ influx into roots. Importantly, the expression of *AKT1*, *CBL1*, *CBL9* and *CIPK23* was regulated by AtHELPS under low-K^+ stress. To our knowledge, this is the first report of a plant DEVH box RNA helicase regulating K^+

deprivation tolerance. This study provides a valuable reference for future research in this area.

Results

AtHELPS is a putative DExD∕H box RNA helicase

To study the function of the DExD∕H box RNA helicase in plant stress responses, we identified a putative DEVH box cDNA sequence (*AtHELPS*) in *Arabidopsis thaliana*. The full-length *AtHELPS* contains 4175 nucleotides, and is predicted to encode a protein of 1347 amino acids with an estimated molecular mass of 151 kDa (Fig. 1A). Database searches revealed that the protein possesses eight conserved motifs: I, Ia, Ib, II, III, IV, V, and VI. They are conserved in other DExD∕H box helicases, on the basis of their highly conserved residues Asp-Glu-x-His (where x can be any amino acid) in motif II (Fig. 1A).

To determine the function of *AtHELPS* in stress tolerance, both mutant and overexpression lines were generated. One knockdown allele, designated *helps*, was identified with the use of SALK *Arabidopsis* T-DNA insertion mutant collections (SALK_118579). A gene map showing the T-DNA position is shown in Fig. 1B. PCR analysis and sequencing were used to verify the T-DNA insertion site. The *AtHELPS* transcript was still detectable in mutant plants, albeit at 26% of the wild-type level, indicating that *AtHELPS* was knocked down but not knocked out in *helps* mutants (Fig. 1C). Additionally, to generate *AtHELPS*-overexpressing lines, Col-0 plants were transformed with a *35S::AtHELPS* construct. Homozygous transformant seedlings were screened with kanamycin selection, increased *AtHELPS* transcript accumulation was further confirmed by real time (PCR RT-PCR), and the line with highest expression in the T_3 generation, OE6, was selected for further analysis (Fig. 1C).

Spatiotemporal expression pattern of *AtHELPS* in *Arabidopsis*

To reveal the expression pattern of *AtHELPS* in *Arabidopsis*, total RNA was extracted from shoots and roots at three different developmental stages (5 days old, 2 weeks old, and 6 weeks old) and then used for real-time quantitative PCR analysis. The results showed that the expression levels of *AtHELPS* in shoots and roots of 5-day-old seedlings were almost identical. However, for both 2-week-old (juvenile phase) and 6-week-old (flowering phase) plants, *AtHELPS* was expressed much more in roots than in shoots (Fig. 2A).
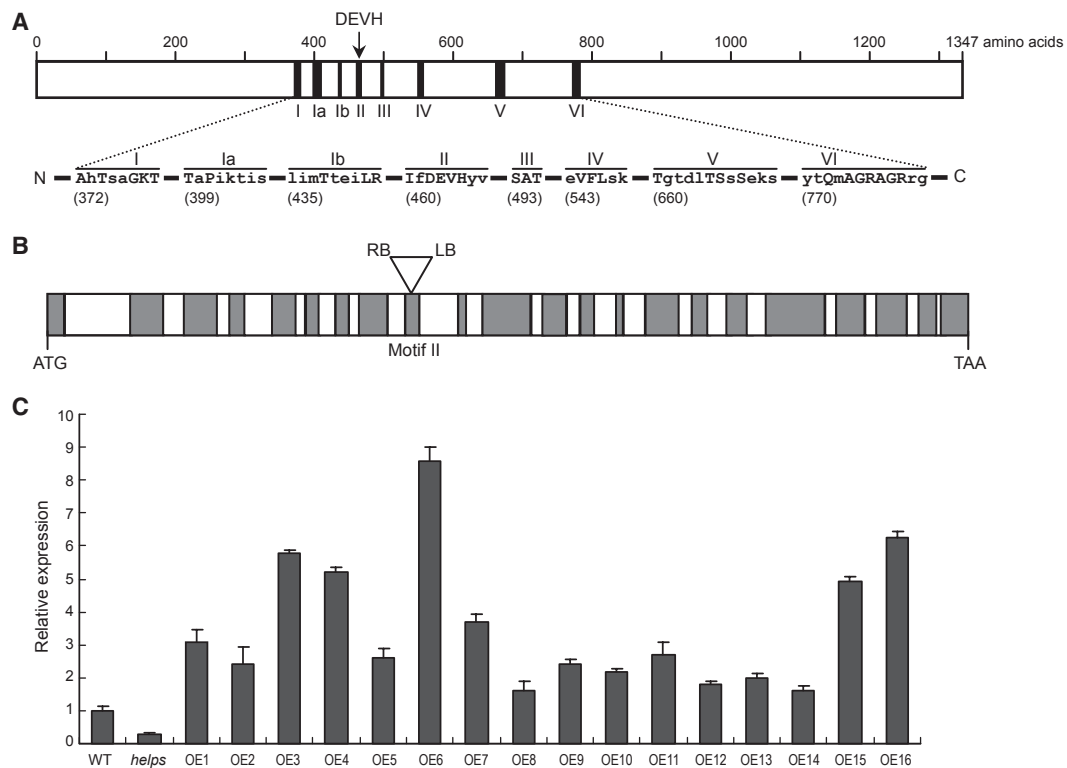
Fig. 1. Characterization and expression analysis of the T-DNA insertion for the *helps* mutant and OE lines of *AtHELPS*. (A) The conserved motifs of DExD∕H-box RNA helicases in AtHELPS. Numbers represent the amino acid position of the AtHELPS protein sequence. Black boxes represent I, Ia, Ib, II, III, IV, V, and VI. The arrow marks the highly conserved residues Asp-Glu-Val-His in motif II. The detailed scheme of the conserved motifs in AtHELPS is shown on the underside. The amino acids in capitals and in lower case demonstrate high sequence identity and sequence similarity, respectively. Numbers in parentheses represent the amino acid position of the first residue in each motif. (B) Scheme of the *AtHELPS* gene. Black boxes represent exons and blank boxes represent introns. The position and orientation of the T-DNA insertion is depicted. LB, left border sequence; RB, right border sequence. (C) Real-time PCR analysis of *helps* mutants and 16 independent OE lines. Gene expression was normalized to the wild-type expression level, which was assigned a value of 1. Standard errors are shown as bars above the columns.

In order to investigate the detailed expression pattern of *AtHELPS*, the promoter sequence was cloned and fused to the β-glucuronidase (GUS) reporter gene and introduced into *Arabidopsis* to generate *pAtHELPS::GUS* transgenic plants. Histochemical GUS staining suggested that *AtHELPS* is mainly expressed in young seedlings and vascular tissues of leaves, such as the midrib of the cotyledon, the hypocotyl, and the root vasculature (Fig. 2E). When the plants were 2 weeks old, the GUS staining in the vascular tissues of leaves was only slightly detectable, and GUS still remained mostly in the stem and root vasculature (Fig. 2F). For 6-week old plants, the expression of *AtHELPS* in the vascular tissues of leaves disappeared; it was detected only in the roots (Fig. 2G). Furthermore, quantitative GUS activity assay of the 2-week-old plants also revealed that AtHELPS displayed nearly 5-fold higher GUS activity in roots than in shoots, which is consistent with the histochemical

GUS staining data and quantitative real-time PCR analysis (Fig. 2B). Taken together, these results imply that *AtHELPS* might play a role in nutrient regulation, such as ion transport, in plants.

Expression of *AtHELPS* is regulated by low and high K⁺

To obtain clues about the molecular mechanisms of the regulation of *AtHELPS* expression, we first performed GENEVESTIGATOR analysis (http://www.genevestigator.ethz.ch/). The results showed that the expression of *AtHELPS* might be involved in responses to multiple abiotic stresses. To determine whether the expression of *AtHELPS* is modulated by low/high K^+, high salt, drought, cold, heat, or several plant hormones, we performed quantitative real-time PCR analysis with total RNA extracted from 2-week-old wild-type seedlings under different treatment conditions. As shown in
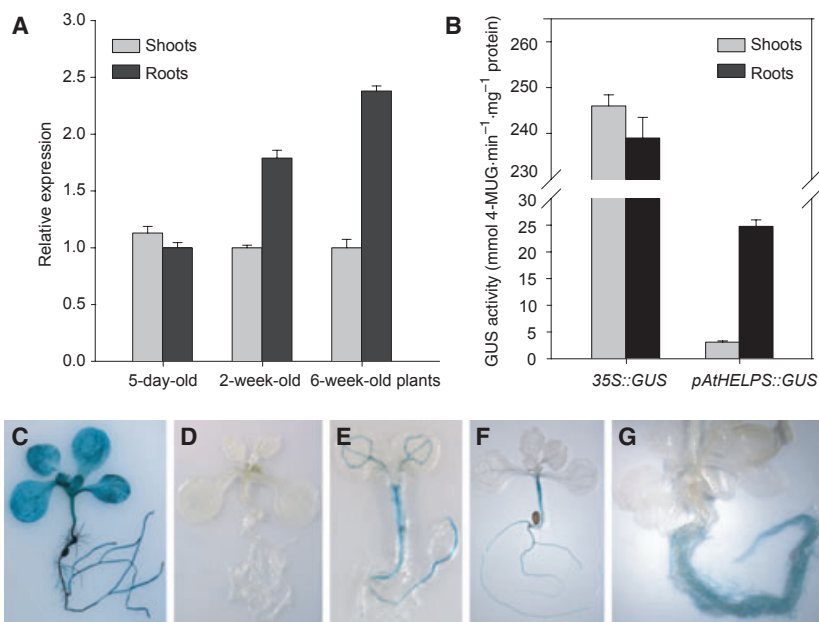
Fig. 2. Temporal and spatial expression of *AtHELPS*. (A) The relative expression of the *AtHELPS* gene in shoots and roots at different developmental stages, as revealed by real-time quantitative PCR analysis. (B) GUS activities from shoots and roots of the 2-week-old *pAtHELPS:GUS* and *35S::GUS* transgenic seedlings are shown. The average GUS activity was obtained from at least five independent transformants, and each assay was repeated three times. Standard errors are shown as bars above the columns. (C, D) GUS localization in the 2-week-old *35S::GUS* (C) and empty-vector transgenic seedlings (D) as controls. (E, F, G) GUS localization in the 5-day-old, 2-week-old and 6-week-old *pAtHELPS:GUS* transgenic seedlings, respectively.

Figs 3 and S1, the *AtHELPS* transcript was upregulated by 100 μM K^+, 2 mM CsCl, zeatin and cold treatments, and downregulated by 100 mM K^+ and 200 mM NaCl treatments. Moreover, detailed analysis indicated that the expression of *AtHELPS* gradually increased from 3 to 72 h under low-K^+ treatment, and decreased under high-K^+ treatment (Fig. 3A,B). These results suggest that the DEVH box RNA helicase AtHELPS might be involved in K^+ stress responses in *Arabidopsis*.

The *helps* mutants exhibit enhanced tolerance to K^+ deprivation stress

To understand the biological function of *AtHELPS*, we performed phenotype analysis using *helps* mutant, the overexpression line OE6, and wild-type *Arabidopsis*. The results showed that both seedlings and adults from the *helps* mutant and OE6 lines exhibited no morphological or developmental differences from wild-type *Arabidopsis* when grown under normal conditions (Fig. 4D). In addition, the percentages of *helps* mutant and OE6 seeds that germinated on Murashige and Skoog plates in the absence of stress were also identical to the number of the wild-type seeds that germinated. However, the number of *helps* mutant seeds that germinated in a medium containing 100 μM K^+

(low K^+) at only 2 days after stratification was about 20% and 28%, respectively, higher than the number of wild-type and OE6 seeds that germinated. By 7 days after stratification, *helps* mutant seeds exhibited 78% germination, whereas wild-type seeds showed ∼ 65% germination, and OE6 seeds showed only 55% germination (Fig. 4A). In addition, all mutant plants grew faster than both wild-type and OE6 plants under low-K^+ stress (Fig. 4E). Quantification of fresh weight (FW) at 7 days after germination demonstrated that mutant seedlings were 39.5% and 59.4% larger than wild-type and OE6 seedlings, respectively (Fig. 4B).

AtHELPS regulates the expression of K^+ transporter genes

To gain insight into the molecular basis of *AtHELPS* responses to low-K^+ stress, we next examined the expression of the genes encoding the well-characterized plant K^+ transporters and their upstream regulators, including *AKT1*, *CBL1*, *CBL9*, and *CIPK23* [13,28–31]. The real-time quantitative PCR analysis revealed that, in the low-K^+ condition, the expression of *AKT1*, *CBL1/9* and *CIPK23* in the three kinds of seedling was differentially induced (Fig. 5). The expression levels of *AKT1*, *CBL1/9* and *CIPK23* in the *helps* mutants were
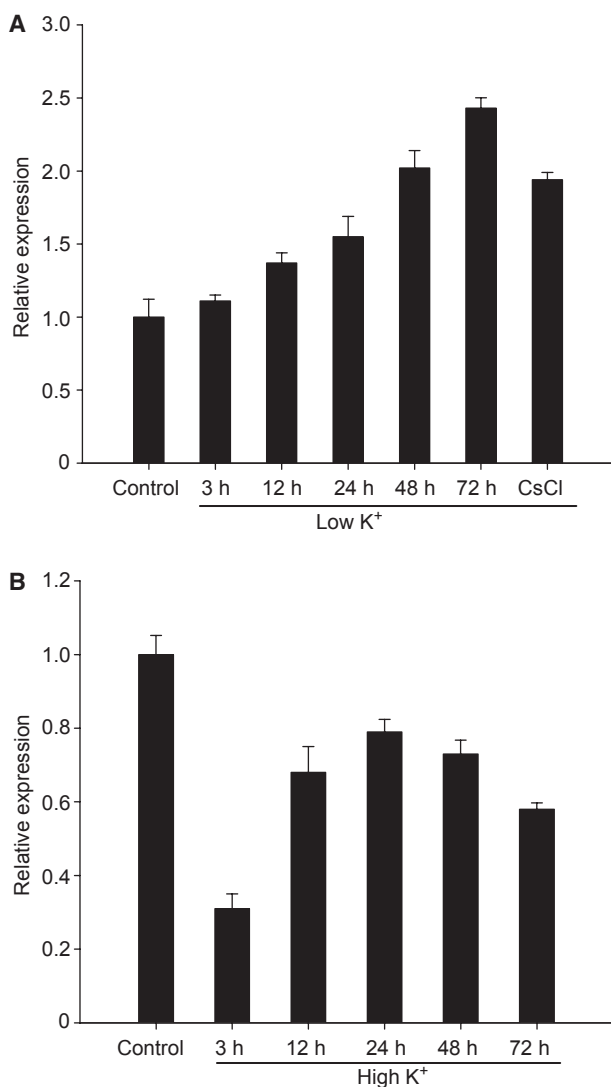
A

B

Fig. 3. Relative expression level of *AtHELPS* in the 2-week-old wide-type *Arabidopsis* seedlings after treatment with low K^+ (100 μM K^+), CsCl (2 mM) and high K^+ (100 mM K^+). (A, B) Expression pattern of *AtHELPS* after treatment with low K^+, CsCl and high K^+ at different time intervals (3, 12, 24, 48, and 72 h), as revealed by real-time quantitative PCR analysis. Gene expression was normalized to the wild type unstressed expression level, which was assigned a value of 1. Data represent the average of three independent experiments ± standard deviation. Standard errors are shown as bars above the columns.

consistently higher than those in the wild-type and OE6 plants after low-K^+ stress treatment. Moreover, the expression levels of the above genes in OE6 plants were lowest under low-K^+ stress but were higher in the normal growth condition. These results suggest that At-HELPS may play an important role in regulating the expression of *AKT1*, *CBL1/9* and *CIPK23* in *Arabidopsis* plants under low-K^+ stress.

Net K^+ flux increased in the *helps* mutant roots under low-K^+ stress

For plants, K^+ efflux and influx systems are very important for cellular ion relationships in natural conditions. Increasing influx, decreasing efflux or both can maximize K^+ uptake to maintain K^+ homeostasis in plants [32,33]. Using the noninvasive micro-test technique (NMT), we measured steady flux profiles of K^+ in the root meristem zone (100 μm from the root tip) of 7 day old *Arabidopsis* wild-type, *helps* mutant and OE6 plants, respectively (Fig. S3). The results indicated that, under normal growth conditions, the net K^+ efflux in the meristem zones of *Arabidopsis* roots were not significantly different among the three genotypes (Fig. 6A). Under K^+ deprivation, however, the net K^+ influx in all three kinds of plants was differentially induced. It is noteworthy that, in the *helps* mutant, a significant induced K^+ influx response was measured from root meristem zones (205 ± 20 pmol·cm^{-2}·s^{-1}), whereas wild-type and OE6 roots showed much smaller low-K^+ stress-induced K^+ influx (60–100 and 110–150 pmol·cm^{-2}·s^{-1}, respectively). Moreover, the root K^+ influx in the meristem zones showed an invariable pattern, with a stable level increase after 3 days of low-K^+ stress. In comparison, the *helps* mutant showed greater K^+ influx than wild-type and OE6 plants over the recording period ($\sim$ 5 min) (Fig. 6B). This finding suggests that AtHELPS might be involved in regulating K^+ flux under K^+ deprivation via the K^+ ion transport.

Discussion

RNA helicases catalyse the unwinding of duplex RNA by utilizing nucleoside triphosphates as the energy source, and they have become a focus of interest in recent years because of their participation in different cellular processes [34–36]. In *Arabidopsis*, more than 120 members of the RNA helicase family can be predicted from the TAIR database (http://www.arabidopsis.org/), and about 40 genes encode a DExD/H box RNA helicase. Recently, *ISE2* was shown to encode a putative DEVH box RNA helicase, which was involved in plasmodesmata function during embryogenesis in *Arabidopsis* [27]. As a DECH box RNA helicase, CAF/DICER-LIKE 1 was shown to be critical for the biogenesis of microRNAs and *Arabidopsis* development [24,25]. *Arabidopsis* TEBICHI, containing an N-terminal DELH box RNA helicase domain and a C-terminal DNA polymerase I domain, was shown to be required for the regulation of cell division and differentiation in meristems [26]. To our knowledge, although the DExD/H box RNA helicases have been intensively
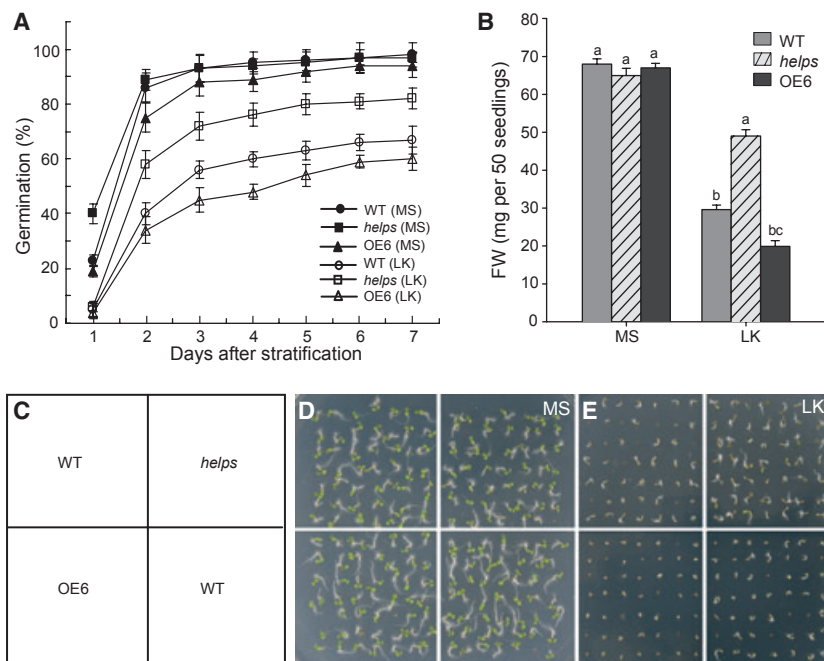
Fig. 4. Phenotype analysis of three different genotypes under low-K+ stress. (A) Percentage of germination of wild-type (WT), *helps* mutant and OE lines on normal Murashige and Skoog (MS) plates and in a medium containing 100 μM K+ (LK). Each data point was repeated three times. (B) FW of the 7-day-old wild-type, *helps* mutant and OE seedlings on normal MS plates and in a medium containing 100 μM K+. Standard errors are shown as bars above the columns. The columns labeled with different letters are significantly different at $P < 0.05$. (C) Diagram of the genotypes used. (D, E) Seed germination of wide-type, *helps* mutant and OE lines on normal MS plates and in a medium containing 100 μM K+, respectively. Photographs were taken on the fifth day after stratification.
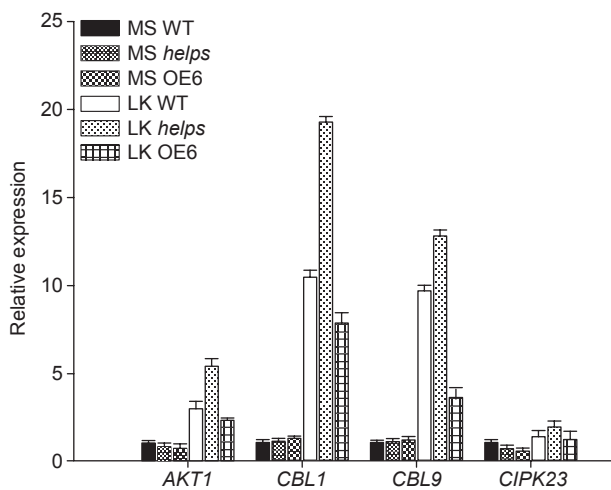

Fig. 5. Relative expression levels of K+ transporters and their upstream regulators in the three different genotypes. The expression levels of *AKT1*, *CBL1*, *CBL9* and *CIPK23* in the 2-week-old wide-type, *helps* mutant and OE line seedlings on normal Murashige and Skoog (MS) plates and in a medium containing 100 μM K+ (LK). Gene expression was normalized to the wild-type unstressed expression level, which was assigned a value of 1. Data represent the average of three independent experiments ± standard deviation. Standard errors are shown as bars above the columns.

studied in animals and yeast [37–39], only a few DExD∕H members were identified in plants and revealed to be involved in the regulation of plant growth and development. Obviously, the biological functions of most other DExD∕H box RNA helicases need to be investigated.

In this study, we characterized a new DExD∕H box RNA helicase, AtHELPS, which showed a unique expression pattern and response to abiotic stress as compared with the known *Arabidopsis* DExD∕H members. The *AtHELPS* promoter::*GUS* and quantitative real-time PCR analysis indicated that *AtHELPS* is mainly expressed in the vascular tissues, such as the midrib of the cotyledon, the hypocotyl, and the root vasculature (Fig. 2E), and is upregulated by 100 μM K+ (low-K+ stress) and downregulated by 100 mM K+ (high-K+ stress) (Fig. 3). The different expression patterns found for DEVH box RNA helicases might mirror their diverse functions. Our results imply that AtHELPS might be involved in regulating nutrient transport, especially ion transport, in *Arabidopsis*. Several studies have reported that the members of the other subfamily of RNA helicases, such as the DEAD box helicases LOS4, STRS1, and STRS2, play a role
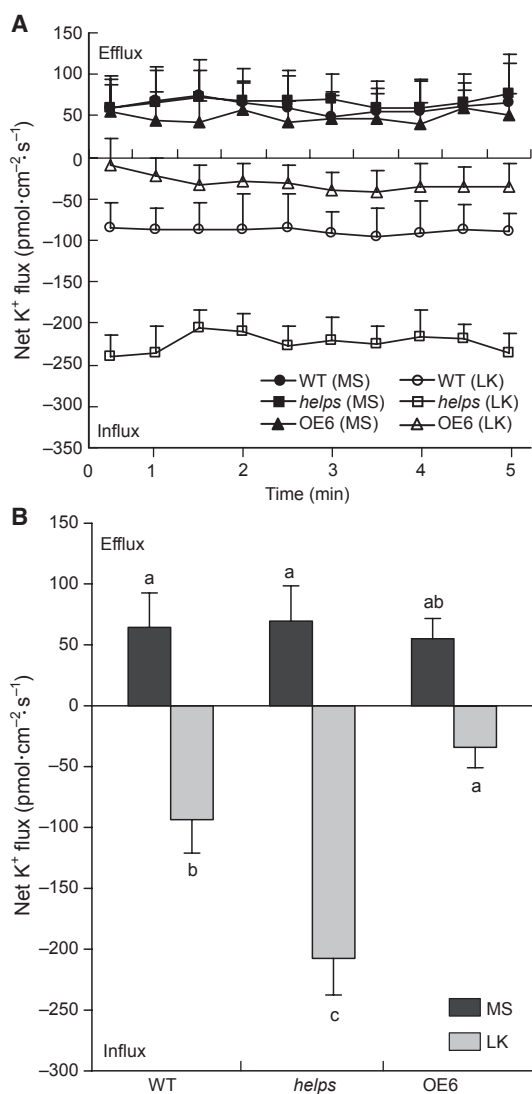
Fig. 6. Effects of low-K$^+$ stress on the steady flux profile of K$^+$ in the root meristem zone of *Arabidopsis*. (A) Effect on K$^+$ flux (positive ion flux indicates influx; negative ion flux indicates efflux) measured on 7-day-old wide-type, *helps* mutant and OE line seedlings on normal Murashige and Skoog (MS) plates and in a medium containing 100 μM K$^+$ (LK). The steady-state flux profile of K$^+$ was examined by continuous flux recording (5–10 min). Each point indicates mean ± standard error (when larger than the symbol) for the same time interval (15 data points per minute averaged) from different plant genotypes (n = 5–7). Standard errors are shown as bars above the curves. (B) The mean flux values during the measuring periods are shown in the panels. Standard errors are shown as bars above the columns. The columns labeled with different letters are significantly different at $P < 0.05$.

in freezing, salt and drought stress tolerances in *Arabidopsis* as negative regulators [22,23]. As a DEVH box RNA helicase, AtHELPS might also function as a regulator in plant stress tolerance.

K$^+$ is a crucial nutrient, and is acquired from soil by roots for plant growth and development. Recently, great progress in determining the molecular mechanism of the regulation of K$^+$ uptake in plants has been made [10,11,40]. *AKT1* was first reported to be expressed in roots and involved directly in the mineral nutrition of *Arabidopsis* [29,30,41]. Two calcineurin B-like proteins, CBL1 and CBL9, were then identified as calcium sensors in the differential regulation of abiotic stress responses, and in the ABA signaling and stress-induced ABA biosynthetic pathways, respectively, in *Arabidopsis* [42–44]. Further studies revealed that CBL1 and CBL9 functioned in *Arabidopsis* as the upstream regulators of the Ser/Thr protein kinase CIPK23, and that CIPK23 phosphorylated the K$^+$ transporter AKT1, and then enhanced K$^+$ uptake. These studies suggested that an AKT1-mediated and CBL/CIPK-regulated K$^+$ uptake pathway in higher plants played a crucial role in K$^+$ uptake, particularly under K$^+$-deficient conditions [12,13]. Generally, the K$^+$ transport system in plants is considered to consist of low-affinity channels and high-affinity transporters [30,45,46]. Although many components of different plant species have already been identified, such as KAT1, AtKCO1, AtHKT1, and HAK1/5 [6,47–49], it is assumed that a number of genes involved in regulating K$^+$ uptake and K$^+$ transport remain unknown.

Our results revealed that the expression of *AtHELPS* was upregulated by low-K$^+$ stress and downregulated by high-K$^+$ stress in *Arabidopsis* seedlings (Fig. 3). The seed germination percentage and seedling FW of the *helps* mutants were higher than those of wide-type and OE6 plants in the low-K$^+$ condition, whereas no differences were observed among the three genotypes under normal-K$^+$ or high-K$^+$ treatment (Fig. 4). To gain insights into the molecular mechanisms of *AtHELPS* responses to low-K$^+$ stress, we examined the expression of a number of genes responsible for encoding K$^+$ transporters and channels in *Arabidopsis*. Interestingly, the expression levels of *AKT1*, *CBL1/9* and *CIPK23* in the *helps* mutants were consistently higher than those in wild-type and OE6 plants after low-K$^+$ stress treatment (Fig. 5). AtHELPS did not affect the expression of other transporter and channel genes, such as *AtKCO1*, *SKOR*, and *AtCNGC1* (Fig. S2). We thus suggest that the DEVH box RNA helicase AtHELPS might be involved in the regulation of the AKT1-mediated and CBL/CIPK-regulated K$^+$ uptake pathway under low-K$^+$ stress.

Recently, noninvasive ion-selective microelectrode ion flux measurements have become a useful tool in physiological research on plants [50–53]. In this study,

we applied this technique to clarify genotype differences of K^+ flux profiles from root meristem zones of *Arabidopsis*. The net K^+-induced influx in *helps* mutants was greater than that of wild-type and OE6 seedlings when they were exposed to K^+ deprivation (Fig. 6), suggesting that AtHELPS might be involved in regulating K^+ uptake in *Arabidopsis* roots via high-affinity transporters such as AKT1. When *helps* mutants were exposed to low-K^+ stress conditions, the greater induction of *AKT1* expression at the transcriptional level might have resulted in an increase in K^+ uptake or net K^+-induced influx. Taking the findings together, this study not only identifies a new DExH box RNA helicase that responds to abiotic stress, but also provides information about how RNA helicase acts as a negative regulator in K^+ deprivation signaling pathways in *Arabidopsis*. However, the precise mechanism of the regulation between AtHELPS and K^+ deprivation in plants remains to be elucidated. Besides, zeatin and cold treatments also increased the accumulation of *AtHELPS* mRNA in seedlings (Fig. S1), suggesting that additional roles of AtHELPS might exist in *Arabidopsis*.

Experimental procedures

Plant material

A. thaliana (Col-0) seeds were surface-sterilized and sown on Murashige and Skoog plates. Seeds were stratified at 4 °C for 2 days, and then transferred to 22 °C for 2 weeks. Col-0 was used as the wild type, and was the genetic background for transgenic plants. *Helps* (SALK_118579, At3g46960) was isolated from a pool of T-DNA insertion lines (SIGnAL, Salk Institute Genomic Analysis Laboratory, La Jolla, CA, USA). One-month-old plants were grown under a 16-h light/8-h dark photoperiod at 22 °C with cool white light (120 mmol·photons·m^{-2}·s^{-1}), and used for transformation. For different stresses, 2-week-old seedlings were transferred to blotting paper without stress treatment, or saturated with 100 μM KCl, 2 mM CsCl, 100 mM KCl, 20 μM zeatin (4 °C), 200 mM NaCl, 10 μM indole 3-acetic acid, 10 μM 6-benzylaminopurine, 50 μM ABA, and 100 μM gibberellin, respectively, at different time intervals, such as 1, 3, 6, 12, 24, 48, and 72 h. According to previous studies [54–56], excessive Cs^+ (exceeding 200 μM) in the rhizosphere could induce K^+ starvation in plants, and Cs^+ was also used as a control to imitate low-K^+ stress in our experiments. Seedlings grown on filter papers soaked with water were used as the control. All of these treatments were carried out under a growth regime of 16-h light/8-h darkness at 22 °C, unless otherwise specified. For RNA extraction, the whole plants were frozen and stored in liquid nitrogen immediately after harvest [57].

Arabidopsis transformation

Using the pBI121 binary vector [58], the *AtHELPS promoter::GUS* and *35S::AtHELPS* expression cassettes were generated by removing the 35S promoter and the GUS gene, respectively. The vectors were introduced into *Agrobacteriun tumefaciens* strain GV3101, and the wild-type *Arabidopsis* plants were transformed by floral dipping [59]. The transgenic plants were screened on Murashige and Skoog medium containing 50 μg·mL^{-1} kanamycin. T1 transgenic *Arabidopsis* plants were identified by semiquantitative real-time PCR and quantitative real-time PCR to amplify the *AtHELPS* gene, with the specific primers shown in Table S1. The corresponding T_2 transgenic seedlings that segregated at a ratio of 3 : 1 (resistant/sensitive) were selected for propagation of T_3 individuals, which were used for further analysis.

Histochemical GUS staining

AtHELPS and its putative promoter sequence were acquired from the TAIR database (http://www.arabidopsis.org/). We used a length of 1403 bp in this study. Primers for amplifying the promoter sequence are shown in Table S1. The *pAtHELPS:GUS* recombinant construct was transformed into *Ag. tumefaciens* (GV3101), and then introduced into *Arabidopsis* by the floral dip method [59]. Histochemical localization of GUS activities in the transgenic seedlings or different tissues was determined after the transgenic plants had been incubated overnight at 37 °C in 1 mg·mL^{-1} 5-bromo-4-chloro-3-indolyl-glucuronic acid, 5 mM potassium ferrocyanide, 0.03% Triton X-100, and 0.1 M sodium phosphate buffer (pH 7.0). The tissues were then cleaned with 70% ethanol. The cleaned tissues were observed, and photographs were taken with a stereoscope. For examination of the detailed GUS staining, the tissues were observed with a bright-field microscope and photographed. These GUS staining data were representative of at least five independent transgenic lines for each construct.

Protein extraction and fluorometric GUS assay

Plant protein extraction and assay for GUS activity were performed as previously described [60]. The protein concentration of the extract was determined with a nanodrop instrument. Fluorescence was measured with a Microplate Spectrofluorometer. For analysis of GUS activity in different tissues, the data were obtained by subtracting the background 4-methyiumbelliferyl glucuronide of the transgenic plants. The average GUS activity was obtained from at least five independent transformants, and each assay was repeated three times.

RNA extraction

For RNA isolation, the plant tissues were harvested separately, frozen in liquid nitrogen, and stored at −80 °C until

use. Total RNA was isolated from different *A. thaliana* seedlings with Trizol reagent (Invitrogen, Carlsbad, CA, USA).

Quantitative real-time PCR analysis

Total RNA was extracted with Trizol reagent from different tissues of *Arabidopsis*. Contaminated DNA was removed with RNase-free DNase I. First-strand cDNA synthesis was performed with 4 μg of RNA, using oligo(dT) primer and the Qiagen one-step real-time PCR kit. Primers for amplifying *AtHELPS* and the other genes were designed according to the sequences downloaded from the TAIR database (http://www.arabidopsis.org/). The real-time PCR experiment had been carried out at least three times under identical conditions, with actin as an internal control. Details of primers are shown in Table S1.

Measurement of net K⁺ flux with the NMT

The net flux of K^+ was measured noninvasively by Xuyue-Sci. & Tech. Co. (Beijing) (http://www.xuyue.net), with the NMT (BIO-IM, Younger USA LLC, Amherst, MA, USA), as previously described [61]. The concentration gradients of the target ions were measured by moving the ion-selective microelectrode between two positions close to the plant material in a preset excursion with a distance of 20 μm, a whole cycle being completed in 5.25 s.

Prepulled and silanized glass micropipettes (2–4-μm aperture, XYPG120-2; Xuyue) were first filled with a back-filling solution (K^+: 100 mM KCl) to a length of ∼ 1 cm from the tip. The micropipettes were then front-filled with approximately 180-μm columns of selective liquid ion exchange cocktails (K^+, Sigma, 60031; Sigma-Aldrich, St Louis, MO, USA). Ion-selective electrodes were calibrated prior to flux measurements with different concentrations of K^+ buffer (0.05, 0.1, and 0.5 mM).

Only electrodes with Nernstian slopes of > 50 mV per decade were used in our study. Ion flux was calculated by Fick's law of diffusion:

$$J = -D(\mathrm{d}c/\mathrm{d}x)$$

where J represents the ion flux in the x-direction, $\mathrm{d}c/\mathrm{d}x$ is the ion concentration gradient, $\mathrm{d}x$ is 20 μm in our experiments, which is the distance of microelectrode movement between a near point and far point, and D is the ion diffusion coefficient (1.96×10^{-5} cm²·s⁻¹ at 25 °C) in a particular medium. Data and image acquisition, preliminary processing, control of the electrode positioner and stepper-motor-controlled fine focus of the microscope stage were performed with IMFLUX software [62].

Data analysis

Ionic fluxes were calculated with MAGEFLUX, developed by Y. Xu (http://xuyue.net/mageflux).

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Supporting information

The following supplementary material is available:

Fig. S1. Relative expression levels of *AtHELPS* in *Arabidopsis* after treatment with multiple abiotic stresses.

Fig. S2. Expression of K^+ transporters and channels among *helps* mutant, OE line and wild-type *Arabidopsis*.

Fig. S3. The root meristem zone (100 μm from the root tip) of *Arabidopsis* was used to measure the steady flux profile of K^+.

Table S1. Primers for amplifying the full-length cDNA, promoter and the length of other sequences used in this study.

This supplementary material can be found in the online version of this article.

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