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RESEARCH ARTICLE

PtHAK5, a candidate for mediating high-affinity K^+ uptake in the halophytic grass, *Puccinellia tenuiflora*

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Abstract *Puccinellia tenuiflora* is a typical salt-excluding halophytic grass with strong salt-tolerance, which enhances tolerance by restricting Na^+ influx as well as having a strong selectivity for K^+ over Na^+. The HAK5 K^+ transporters generally modulate effective K^+ acquisition in plants, especially under low K^+ condition. In this study, *PtHAK5* from *P. tenuiflora* was isolated by RT-PCR and characterized using yeast complementation. The results showed PtHAK5 consisted of 784 amino acids and shared over 80% homology with the identified high-affinity K^+ transporter HAK5 from other higher plants. The expression of *PtHAK5* rescued the K^+-uptake-defective phenotype of yeast strain CY162. In conclusion, PtHAK5 is a candidate for mediating high-affinity K^+ uptake under low K^+ conditions.

Keywords K^+ uptake, PtHAK5, *Puccinellia tenuiflora*, yeast complementation

1 Introduction

As a necessary, abundant and valuable mineral element, potassium is indispensable to growth and of plants, contributing to activation of a substantial number of enzymes, and other major physiological and biochemical processes[1]. Among all the cationic components of the cell, K^+ is the most abundant[2,3] which accounts for 8% of plant dry weight[4]. Despite potassium being an abundant element in the earth's crust, only small quantities are present in the soil solution to be absorbed by plants. In general, since plants rapidly absorb nutrients, the actual concentration of K^+ at the root surface of a plant is lower than it in the bulk soil solution, usually down to the micromolar range[5]. To maintain adequate K^+ concentrations under low K^+ conditions, plants have complex mechanisms for obtaining K^+ from soil. Previous studies have revealed that the KT/KUP/HAK family has an irreplaceable role in K^+ transport in plants[6]. The members of the KT/KUP/HAK family were initially cloned from *Arabidopsis thaliana* and *Hordeum vulgare* (barley). They have since been found in a substantial number of other plant species and are considered essential to maintaining K^+ homeostasis in plants[5]. The members of the KT/KUP/HAK family are classified into four clusters (I–IV)[7]. Members of cluster I mediate high-affinity K^+ transport, such as AtHAK5, OsHAK1 and OsHAK5[8]. The sequences and functions of proteins in cluster II vary widely, e.g., AtKUP1 mediates high- and low-affinity K^+ transport[9], while other members merely mediate low-affinity K^+ transport, e.g., HvHAK2[10] and CnHAK1[11]. Also, OsHAK2 preferentially mediates Na^+ transport rather than K^+ transport[12]. At present, the proteins of cluster III and IV are not well characterized[13]. Nieves-Cordones et al.[14] assumed that inward-rectifying K^+ channel AKT1 and high-affinity K^+ transporter mediate K^+ uptake synergistically under low K^+ conditions (100 $\mu mol \cdot L^{-1}$). However, it is import to recognize that HAK5 is the only system that participates in K^+ uptake when the concentration of external K^+ is exceedingly low (10 $\mu mol \cdot L^{-1}$). Yang et al.[8] also conclude that OsHAK5 is of immense importance in K^+ acquisition by roots faced with low external K^+ and in upward K^+ transport from roots to shoots in K^+-deficient rice plants. Therefore, members of HAKs are of great importance in K^+ uptake.

Puccinellia tenuiflora is an excellent halophytic grass with strong salt-tolerance widely distributed in the saline-alkali soil of northern regions of China[15,16]. As a typical salt-excluding halophytic grass, *P. tenuiflora* maintains higher K^+ content and a higher K^+/Na^+ ratio in shoots to enhance salt tolerance under salinity[15–17]. However, information about the molecular mechanism underlying

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K^+ transport in *P. tenuiflora* is scarce.

We cloned *PtHAK5* from *P. tenuiflora* by reverse transcription-polymerase chain reaction (RT-PCR) and then undertook a preliminarily demonstration of the function of PtHAK5 in mediating K^+ uptake using expression test in yeast.

2 Materials and methods

2.1 Plant materials, growth conditions and treatments

Seeds of *P. tenuiflora* were collected from Yuzhong Campus of Lanzhou University, China. Surface-sterilized plump seeds were germinated in the dark on hydroponic culture wire gauze covered evenly with tissue paper in rectangular trays filled with distilled water. The distilled water was replaced with modified Hoagland nutrient solution (5 mmol·L^{-1} KNO_3, 1.5 mmol·L^{-1} $Ca(NO_3)_2·4H_2O$, 0.5 mmol·L^{-1} $NH_4H_2PO_4$, 0.5 mmol·L^{-1} Fe-citrate, 0.25 mmol·L^{-1} $MgSO_4·7H_2O$, 92 μmol·L^{-1} H_3BO_3, 18 μmol·L^{-1} $MnCl_2·4H_2O$, 1.6 μmol·L^{-1} $ZnSO_4·7H_2O$, 0.7 μmol·L^{-1} $(NH_4)_6Mo_7O_{24}·4H_2O$, 0.6 μmol·L^{-1} $CuSO_4·5H_2O$) and renewed every 3 d after germination was consistent. Seedlings were cultivated in a greenhouse with day and night temperatures of 28±2°C and 23±2°C, 16L:8D h photoperiod with flux density about 600 μmol·m^{-2}·s^{-1} and 60%–80% RH. The treatment solutions were changed once per day to maintain a constant ion concentration.

2.2 Bacteria and yeast strains

Escherichia coli DH5α competent cells were purchased from TransGen Biotech (Beijing, China). Functional complementation assays were carried out with K^+ transporter-deficient (TRK1 and TRK2) *Saccharomyces cerevisiae* CY162 (*MATa, Δtrk1, trk2*:: *pCK64, his3, leu2, ura3, trp1, ade2)*[18], in which growth is repressed when the K^+ concentration is below 7 mmol·L^{-1}[19] and G19 (*MATa, his3, leu2, ura3, trp1, ade2, ena1*:: *HIS3*:: *ena4*), which has disrupted *ENA1-4* genes encoding Na^+ export pumps[20].

2.3 Cloning the 3′-and 5′-end fragments of *PtHAK5* gene

Four-week-old seedlings were submitted to K starvation treatment for 7 d and subsequently irrigated with modified Hoagland nutrient solution containing 0.01 mmol·L^{-1} KCl for 6 h. Total RNA was extracted from roots of treated seedlings using the TRIzol reagent (Sangon, Biotech Co., Ltd, Shanghai, China) according to the manufacturer's instructions. The first strand cDNA of 5′- and 3′-end fragments were synthesized from high purity total RNA following the manufacturer's protocol for the 5′ RACE Kit (catalog nos. 634923 and 634924) and the 3′ RACE Kit (catalog no. L1500-01), respectively. 5′- and 3′-end fragment-specific primers were designed using DNAMAN 6.0 (Lynnon Biosoft, Qc, Canada) and Primer 5.0 software (Premier Biosoft International, Palo Alto, CA, USA), respectively. The 5′-end exon and nested fragments were amplified using 5′-end exon specific primer P1 (Table 1) and nested specific primer P2 (Table 1) paired with 5′-end primer and 5′-end nested primer provided in the GeneRacer™ kit (Invitrogen™, USA), respectively. Similarly, the 3′-end exon and nested fragments were amplified using 3′-end exon specific primer P3 (Table 1) and nested specific primer P4 (Table 1) paired with 3′-end primer and 3′-end nested primer, respectively. PCR amplification was performed using 50 μL PCR with the following cycling profile: initial DNA denaturation at 98°C for 30 s, followed by 30 cycles of amplification (denaturation at 98°C for 10 s; primer annealing at 58°C for 5′-end and 60°C for 3′-end) for 30 s and primer extension at 72°C for 40 s, and a final extension at 72°C for 10 min. All gene-specific primers for RT-PCR were designed based on the sequences of the *PtHAK5* fragment obtained from *P. tenuiflora*. RT-PCR products were detected and purified from 1.2% agarose gel electrophoresis and subsequently ligated into pMD19-T (simple) vector, followed by transformation of *E. coli* DH5α. Then PCR products were screened by blue and white spots in LB liquid medium containing ampicillin (100 μg·mL^{-1}), confirmed by agarose gel electrophoresis, and the positive clones were selected and sequenced by Sangon (Shanghai, China).

Table 1 Primers used in this work

Primers	Sequences
P1	GTTGTTTGACACTTTGTTCTTGAGC
P2	AAATCGCTGAACAAGAAAGAGGAC
P3	TCGTGTCATCCACACTTCAAAAAC
P4	GCAATATAGAAACAAACGAACGGTTCC
P5	TGCTCTAGAATGGCTGAGCCTCTGAA
P6	GGACCCGGGCTAGATCTCATATGTCAT

2.4 Sequence and phylogenetic analysis

The sequences of *PtHAK5* gene fragment were used for BLAST search on the NCBI[21] against sequences from GeneBank[22] and the predicted amino acid sequence were obtained. The translation and open reading frame (ORF) of *PtHAK5* gene sequence was analyzed by the DNAMAN 6.0 software. Sequence alignments with homologous amino acid sequence were performed using BioEdit software (Tom Hall, Ibis Biosciences, Carlsbad, CA). Prediction of transmembrane regions of TMHMM amino acids was accomplished with online biology platform[23]. The phylogenetic tree was constructed by the MEGA 6.0[24] software using the maximum-likelihood method.

2.5 Plasmid construction

The cDNA fragment containing the ORF of *PtHAK5* was verified using ORF Finder software[25]. Upstream specific primer P5 (Table 1) (containing restriction site *Xba*I) and downstream specific primers P6 (Table 1) (containing *Sma*I restriction site) were designed at the beginning and end by Primer 5.0 and DNAMAN program, respectively. The ORF of *PtHAK5* was amplified by RT-PCR under the following conditions: 98°C for 30 s; 30 cycles of 98°C for 10 s, 55°C for 30 s and 72°C for 40 s; and a final extension at 72°C for 10 min. Constructing *PtHAK5* vectors after the sequences of the *PtHAK5* ORF were verified and calibrated. The resulting products were ligated into a yeast expression vector p416 GPD with ampicillin resistance[26] using T4DNA restriction endonuclease, transferred to *E. coli* DH5α and positive clones identified by ampicillin, obtained by PCR and sent to Sangon (Shanghai, China) for sequencing. Matched vectors p416-*PtHAK5*, p416-*AtAKT1* and p416-*AtHKT1*;*1* were used as positive controls.

2.6 Yeast complementation

Yeast transformations with p416-*PtHAK5*, p416-*AtAKT1* and p416-*AtHKT1*;*1* were accomplished using LiAc as described by Chen et al.[27]. Positive monoclonal strains were selected in liquid uracil-free selective medium (0.67% w/v yeast nitrogen base without amino acids, 2% w/v dropout supplement without uracil, 20 $g \cdot L^{-1}$ glucose and 100 $mmol \cdot L^{-1}$ KCl). Yeast cells were plated on medium using 10-fold serial dilutions from OD_{600} = 0.5 to 0.5×10^{-3}. The CY162 strain transformed with plasmids and without an insert were cultured in arginine-phosphate (AP) medium (0.77 $g \cdot L^{-1}$ dropout supplement without uracil, 10 $mmol \cdot L^{-1}$ L-arginine, 2% w/v glucose, 0.2 $mmol \cdot L^{-1}$ $CaCl_2$, 2 $mmol \cdot L^{-1}$ $MgSO_4$, 8 $mmol \cdot L^{-1}$ H_3PO_4, plus vitamins and trace elements, and 2% w/v agar powder, pH 6.5)[28] containing different concentrations of K^+ (0.1, 2, 10 or 100 $mmol \cdot L^{-1}$) in the order of dilution from low to high. The G19 strain transformed with plasmids and without an insert were suspended in liquid AP medium with added K^+ (1 $mmol \cdot L^{-1}$) and supplemented with various concentrations of Na^+ (0, 25, 50 or 75 $mmol \cdot L^{-1}$). Yeast cells were incubated at 28°C for 48 h.

3 Results

3.1 Isolation and characterization of PtHAK5

The full-length sequence of *PtHAK5* (Fig. 1) was 2880 bp with a 5′-untranslated region (UTR) of 111 nucleotides, and a predicted ORF of 2355 nucleotides encoding 784 amino acids and a 3′-UTR of 414 nucleotides. The predicted amino acid sequence has 11 highly conserved transmembrane regions (Fig. 2). We designated this gene as *PtHAK5*, i.e., *HAK5* gene from *P. tenuiflora*.

The result of multiple sequence alignment indicated that PtHAK5 shared high similarity with other HAK sequences previously characterized in higher plants (Fig. 3). The amino acid sequence identity to *OstHAK5* from *Oryza sativa* and *ZmHAK5* from *Zea mays* was 86.1% and 81.9%, respectively. *PtHAK5* also shared homology with *SlHAK5* from *Solanum lycopersicum* (56.2%) and *AtHAK5* from *A. thaliana* (54.7%) (Fig. 4). The phylogenetic tree results revealed that PtHAK5 belonged to a subcluster of the KT/HAK/KUP family known as cluster I, in the same clade as OsHAK5. Thus, PtHAK5 and OsHAK5 have a close evolutionary relationship (Fig. 5).

3.2 PtHAK5 mediates K^+ uptake in yeast cells

CY162 is a K^+-uptake-deficient yeast mutant deficient in the two K^+ transporters, TRK1 and TRK2[18]. *Arabidopsis* AtAKT1 was able to complement the growth of yeast trk1Δ trk2Δ mutant under low K^+ concentration by restoring the K^+ uptake capacity of the yeast cells[29]. To ascertain whether PtHAK5 is involved in K^+ uptake, we expressed *PtHAK5* and *AtAKT1*, *AtHKT1*;*1* (as positive control) in CY162 (Fig. 6). All of the yeast transformants grew adequately and uniformly on the AP medium with high concentration of K^+ (10 or 100 $mmol \cdot L^{-1}$). However, CY162 transformed with empty p416 GPD and p416 *AtHKT1*;*1* did not grow on the 2 $mmol \cdot L^{-1}$ K^+ and low-K^+ 0.1 $mmol \cdot L^{-1}$ medium after incubation for 48 h. Thus, the expression of *PtHAK5* as well as *AtAKT1* permitted CY162 cells to grow (Fig. 6) but p416-PtHAK5 transformants gave significantly weaker growth than p416-AtAKT1 transformants. In short, both p416-PtHAK5 and p416-AtAKT1 could rescue the growth defect of CY162, and mediate K^+ uptake under low-K^+ conditions. The lower ability of *PtHAK5* to promote growth suggests that it may be less effective at restoring K^+ uptake than AtAKT1.

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1       GTGATTCGCAGTGATTCGAGCTCGGTACCCGGGGATCCTCTAGAGATTAAGCAGTGGTATCAACGCAGAGTACATGGGGAGGCCCAGGAA
91      AGATATCATCCTACCTCAAGGATGGCTGAGCCTCTGAAAACAAGCAGCAATGGAGTTGCTGAAGGGGATTCAAACTCTGCATTTGCATCG
1                                M  A  E  P  L  K  T  S  S  N  G  V  A  E  G  D  S  N  S  A  F  A  S
181     GAGACGACACCATCACCGCCAAGGAGGCTGCAGAGGTTCGATTCGCTCCACACCGAAGCTGGAATGATTCCGGGCGGCCGAAGCCATGCA
24       E  T  T  P  S  P  P  R  R  L  Q  R  F  D  S  L  H  T  E  A  G  M  I  P  G  G  R  S  H  A
271     GCCAAGGTCGGCTGGGTCACGACACTACACTTGGCACTTCAGAGCCTGGGTGTGGTTTATGGGGACATGGGAACTTCTCCCCTGTATGTG
54       A  K  V  G  W  V  T  T  L  H  L  A  L  Q  S  L  G  V  V  Y  G  D  M  G  T  S  P  L  Y  V
361     TTCTCCAGCACATTTACTGGCGGGATCAAGGACACGAATGACATCCTTGGTGTAATGTCTCTGATTATCTACACCGTGGCTCTCCTTCCA
84       F  S  S  T  F  T  G  G  I  K  D  T  N  D  I  L  G  V  M  S  L  I  I  Y  T  V  A  L  L  P
451     TTGCTGAAATATTGTTTCATTGTTTTGAGAGCTAACGACAACGGTGACGGCGGAACATTTGCACTTTATTCCTTGATATCTCGGTATGCA
114      L  L  K  Y  C  F  I  V  L  R  A  N  D  N  G  D  G  G  T  F  A  L  Y  S  L  I  S  R  Y  A
541     AGGATTAGCTTGATACCTAATCAGCAGGCTGAAGATGCGACGGTCTCTCACTACAAGTTAGAGTCCCCTTCGAATCGTGTGAAGCGAGCT
144      R  I  S  L  I  P  N  Q  Q  A  E  D  A  T  V  S  H  Y  K  L  E  S  P  S  N  R  V  K  R  A
631     CATTGGATTAAGGAAAAGATGGAAAACAGCCCAAACTTTAAGGTCATACTTTTCCTGGTGACAATTCTAGCAACATCAATGGTTATTGGT
174      H  W  I  K  E  K  M  E  N  S  P  N  F  K  V  I  L  F  L  V  T  I  L  A  T  S  M  V  I  G
721     GATGGTGTGCTAACTCCATGTATCTCAGTGCTTAGCGCAGTTGGGGGAATCAAGGAATCAGCAAAGTCCTTGACTCAAGAGCAGATTGCT
204      D  G  V  L  T  P  C  I  S  V  L  S  A  V  G  G  I  K  E  S  A  K  S  L  T  Q  E  Q  I  A
811     GGCATCGCAATCGCCATTTTGATCGTCCTCTTTCTTGTTCAGCGATTTGGCACAGACAAAGTTGGTTACACGTTTGGCCCAGTAATCTTG
234      G  I  A  I  A  I  L  I  V  L  F  L  V  Q  R  F  G  T  D  K  V  G  Y  T  F  G  P  V  I  L
901     ACATGGTTCATCTTAATTGGTGGTATTGGAATTTATAATCTGATCAAACATGATATCGGAATTCTGAAAGCATTCAACCCCAGATATATC
264      T  W  F  I  L  I  G  G  I  G  I  Y  N  L  I  K  H  D  I  G  I  L  K  A  F  N  P  R  Y  I
991     GTGGACTACTTCCAAAGAAATGGGAAGGAGGGATGGATTTCGCTTGGAGGTGTTGTTTTGTGCATTACAGGAACTGAAGCAATGTTTGCC
294      V  D  Y  F  Q  R  N  G  K  E  G  W  I  S  L  G  G  V  V  L  C  I  T  G  T  E  A  M  F  A
1081    GACCTTGGTCATTTCAATGTGAGAGCAATTCAGATTGGCTTTTCTGTAACTCTGCTCCCATCAGTGCTACTGGCTTACATGGGACAAGCT
324      D  L  G  H  F  N  V  R  A  I  Q  I  G  F  S  V  T  L  L  P  S  V  L  L  A  Y  M  G  Q  A
1171    GCATATCTTCGCATCTACCCTGAAAATGTTGCTGATACATTCTACAAATCAATCCCAGGTCCATTATATTGGCCAACATTTGTGGTGGCT
354      A  Y  L  R  I  Y  P  E  N  V  A  D  T  F  Y  K  S  I  P  G  P  L  Y  W  P  T  F  V  V  A
1261    GTGGCTGCTGCTATAATTGCAAGCCAAGCCATGATATCTGGTGCCTTTGCAATCATTGCTCAGTCCCAAATTCTTGGTTGCTTTCCACGG
384      V  A  A  A  I  I  A  S  Q  A  M  I  S  G  A  F  A  I  I  A  Q  S  Q  I  L  G  C  F  P  R
1351    GTTCGTGTCATCCACACTTCAAAAACGTTTCATGGGCAAGTCTACATCCCGGAGATCAACTATGCATTAATGATTTTATGTGTAGCCGTG
414      V  R  V  I  H  T  S  K  T  F  H  G  Q  V  Y  I  P  E  I  N  Y  A  L  M  I  L  C  V  A  V
1441    ACAGCTTTTTTCCAAACTACAGAGAAGATCGGCAATGCATATGGTATCGCTGTCGTCTTTGTGATGTTCATAACAACACTTCTAGTGACA
444      T  A  F  F  Q  T  T  E  K  I  G  N  A  Y  G  I  A  V  V  F  V  M  F  I  T  T  L  L  V  T
1531    CTGGTAATGGCCATGATATGGAAGACAAGTCTTCTGTGGATTGCACTCTTTCCAGTAATATTCGGTGGTGCAGAGCTCATGTACTTATCA
574      L  V  M  A  M  I  W  K  T  S  L  L  W  I  A  L  F  P  V  I  F  G  G  A  E  L  M  Y  L  S
1621    TCAGCGTTCTACAAATTTACAGAAGGTGGCTACTTGCCACTAGGTTTTGCAGCAATTTTGATGTTTATAATGGGCACATGGCACTATGTT
604      S  A  F  Y  K  F  T  E  G  G  Y  L  P  L  G  F  A  A  I  L  M  F  I  M  G  T  W  H  Y  V
1711    CATGTTCACCGGTACAAATATGAGCTCAAGAACAAAGTGTCAAACAACTATGTGGCAGAGTTGGCAATAAGGAGGAATCTCGCTAGGCTG
634      H  V  H  R  Y  K  Y  E  L  K  N  K  V  S  N  N  Y  V  A  E  L  A  I  R  R  N  L  A  R  L
1801    CCAGGAATAGGTGTTCTGTACTCTGAGCTTGTGCAAGGGATCCCACCCATACTTCCTCATTTGGTAGAGAAAGTACCATCCATACATTCA
664      P  G  I  G  V  L  Y  S  E  L  V  Q  G  I  P  P  I  L  P  H  L  V  E  K  V  P  S  I  H  S
1891    GTTCTTGTGATTATCTCAATAAAGTACTTACCAATCAGCAATATAGAAACAAACGAACGGTTCCTCTTCAGATATGTGGAGCCAAGAGAA
694      V  L  V  I  I  S  I  K  Y  L  P  I  S  N  I  E  T  N  E  R  F  L  F  R  Y  V  E  P  R  E
1981    TACAGGGTATTCCGATGTGTGGTGCGCTATGGCTACAACAATAAAGTAGAAGATCCAAGAGAGTTCGAGAACTTGCTCATCGGGAACTTG
724      Y  R  V  F  R  C  V  V  R  Y  G  Y  N  N  K  V  E  D  P  R  E  F  E  N  L  L  I  G  N  L
2071    AAGCAATTCATCCATGAAGAATCATTCTACAGCCAAAGTAGCCATTCCCTTGGAGGAGAAGATAACTCAACTGAAGAGTCAGGAAATGCA
754      K  Q  F  I  H  E  E  S  F  Y  S  Q  S  S  H  S  L  G  G  E  D  N  S  T  E  E  S  G  N  A
2161    CTGGAGCCTTCCATTGAAGTTCAAGATACAGGGTTGCCGAAAAGTTTCATAGATGGAATCCATGCTGGTCCACCAAATGGGTGCATGGAT
784      L  E  P  S  I  E  V  Q  D  T  G  L  P  K  S  F  I  D  G  I  H  A  G  P  P  N  G  C  M  D
2251    GAGATAGAGTTAATTGAGAGAGGGATGGAGGATGGTGTTGTCCATCTGTTAGGAGAAACTAACGTGGTGGCAGAGCAAAATGCCGGGTTA
814      E  I  E  L  I  E  R  G  M  E  D  G  V  V  H  L  L  G  E  T  N  V  V  A  E  Q  N  A  G  L
2341    GTGAAGAAAATAATAGTTGACTACGCCTATAATTTCATGAGGAAGAACTTCAGGCAACCAGAGAAGATCACATGCGTCCCTCATAACAGA
844      V  K  K  I  I  V  D  Y  A  Y  N  F  M  R  K  N  F  R  Q  P  E  K  I  T  C  V  P  H  N  R
2431    CTTCTGAGGGTGGGAATGACATATGAGATCTAGATAGATGCTCAGCAGATTGATCAGATTGACACCATAATCAGTTTTTTTAAGTGTCAA
874      L  L  R  V  G  M  T  Y  E  I  *
2521    GTGCAGAAAGCATATGCAGAGGAAGGCGGTGACCACATAACCAAGCCTCCCTGACTGAAGAGCAGAGGATGGACAGATTTTACGCGTACA
2611    AGCACGGAACAACGAGAGTACATCCAATGGTTATGGAAATTCTACCACATAAGTGTGTGTAGCAATAACTATGCATCACAGAGTGTGTTA
2701    TTTCTACAAAGTGTGTATATGTAGATTGGCTGTGTCCAGCTTTTGTTCGTAGCAATATGTATGCATCTTTTTTTTTCCGTAGTATATACA
2791    ATTGTACACAAAGAGTGTAACCTGTCGGCAAAAGGCAAACGACGAAAAAATATGACTTTGGAGATTCTAAAAAAAAAAAAAAAAAAAAAA
2881    AGT
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Fig. 1 The nucleic acid sequence and encoded amino acid sequence of *PtHAK5* in *Puccinellia tenuiflora*. The full-length *PtHAK5* sequence was used for BLAST search on the NCBI platform with sequences acquired from GenBank. The 2880 bp contained a 5′-untranslated region of 111 bp nucleotides, a predicted ORF of 2355 bp nucleotides encoding 784 amino acids and a 3′-untranslated region with 414 bp nucleotides.

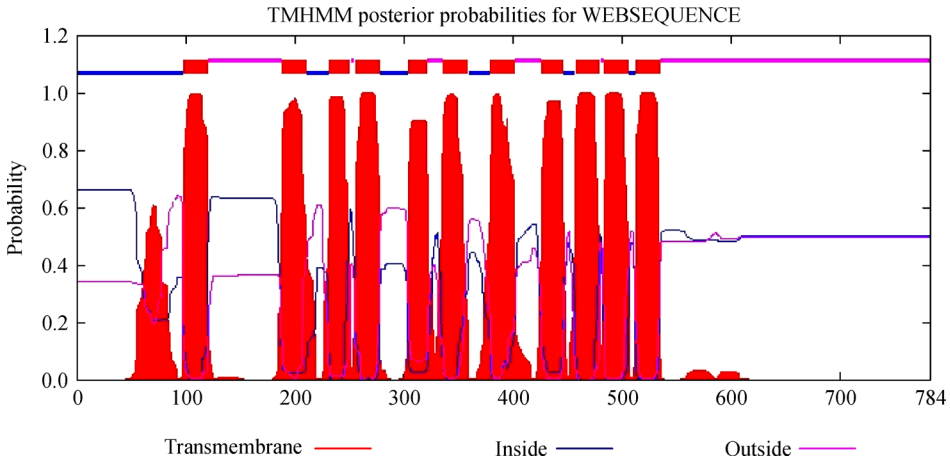

Fig. 2 Transmembrane domain prediction for *PtHAK5* from *Puccinellia tenuiflora*. There are 11 highly conservative transmembrane domains predicted by TMHMM online biology platform. The abscissa indicates the amino acid site and contains a total of 784 amino acids.

3.3 PtHAK5 does not participated in Na^+ transport in yeast cells

The Na^+ transport activity of PtHAK5 was tested by transforming empty p416 GPD vector, p416-PtHAK5 and p416-AtAKT1, p416-AtHKT1;1 into a yeast mutant strain G19 (Fig. 7) which displays higher sensitivity to Na^+ than the wild-type as a result of disruptions in genes *ENA1* to *ENA4* encoding Na^+-extruding ATPase[20]. Since AtHKT1;1 conferred increased Na^+ sensitivity on G19 by mediating Na^+ uptake[30], we used AtHKT1;1 as a positive control for analyzing Na^+ uptake. All the yeast cells grew equally well on the Na^+-free control AP medium (Fig. 7). With the increasing Na^+ concentration, the growth of G19 strains transformed with p416-PtHAK5 and empty p416 GPD vector were slightly weakened under high-Na^+ conditions (50 or 75 mmol·L^{-1}), but their growth was almost uniform. These observations suggest that the expression of *PtHAK5* does not enhance the Na^+ sensitivity of yeast mutant G19, which is deficient in Na^+-extrusion.

4 Discussion

4.1 PtHAK5 encodes a high-affinity K^+ transporter in *Puccinellia tenuiflora*

The KT/KUP/HAK family includes 27 transporters from *O. sativa* and 13 transporters from *A. thaliana*[7,19]. In this study, the phylogenetic analysis of PtHAK5 and 40 proteins of the KT/KUP/HAK family from *A. thaliana* and *O. sativa* showed that PtHAK5 and OsHAK5 fall within the same clade, suggesting that PtHAK5 may have a similar function to OsHAK5. Su et al.[30] reported that the proteins from *A. thaliana* and *O. sativa* in this family have 10–14 transmembrane domains and a longer cytoplasmic loop structure between the second and third transmembrane domains. In this study, we found that *PtHAK5* also contains 11 transmembrane regions (Fig. 2). This result is consistent with the report of Su et al.[31]. Currently it is thought that the sequences of the genes in the KT/KUP/HAK family do not have a completely consistent conserved domains, but overall they have relatively conservative amino acid sequences. The characteristic feature of HAK transporters has been the presence of the consensus motif, GVVYGDLGTSPLY, found in all members of this family[32]. In present study, the results showed that PtHAK5 also contains the very similar amino acid sequence, GVVYGDMGTSPLY (Fig. 3). This evidence indicates that *PtHAK5* is a member of the KT/KUP/HAK family, and thus might encode a high-affinity K^+ transporter.

4.2 PtHAK5 has a potential role in mediating K^+ uptake in *Puccinellia tenuiflora*

The function of proteins in the KT/KUP/HAK family has in most cases been identified by heterologous expression. Some transporters in cluster I, such as AtHAK5, HvHAK1, OsHAK1 and OsHAK5, mediate high-affinity K^+ uptake in yeast as well as *E. coli*[3,33]. The members of the HAK family are all crucial in absorption of K^+ in plants. In *A. thaliana*, the expression of *AtHAK5* was upregulated by K^+ starvation. Under low levels of K^+ supply, the biomass accumulation of wild type yeast is much higher than the *athak5* mutant, which implies that AtHAK5 is a major contributor to K acquisition in roots growing under low external K^+[34]. HAK also responds to salinity and expression of *OsHAK5* in *Nicotiana tabacum* (tobacco) BY2 cells improved salt tolerance. Furthermore, the concentration of Na^+ remained steady while the cells accumulated a

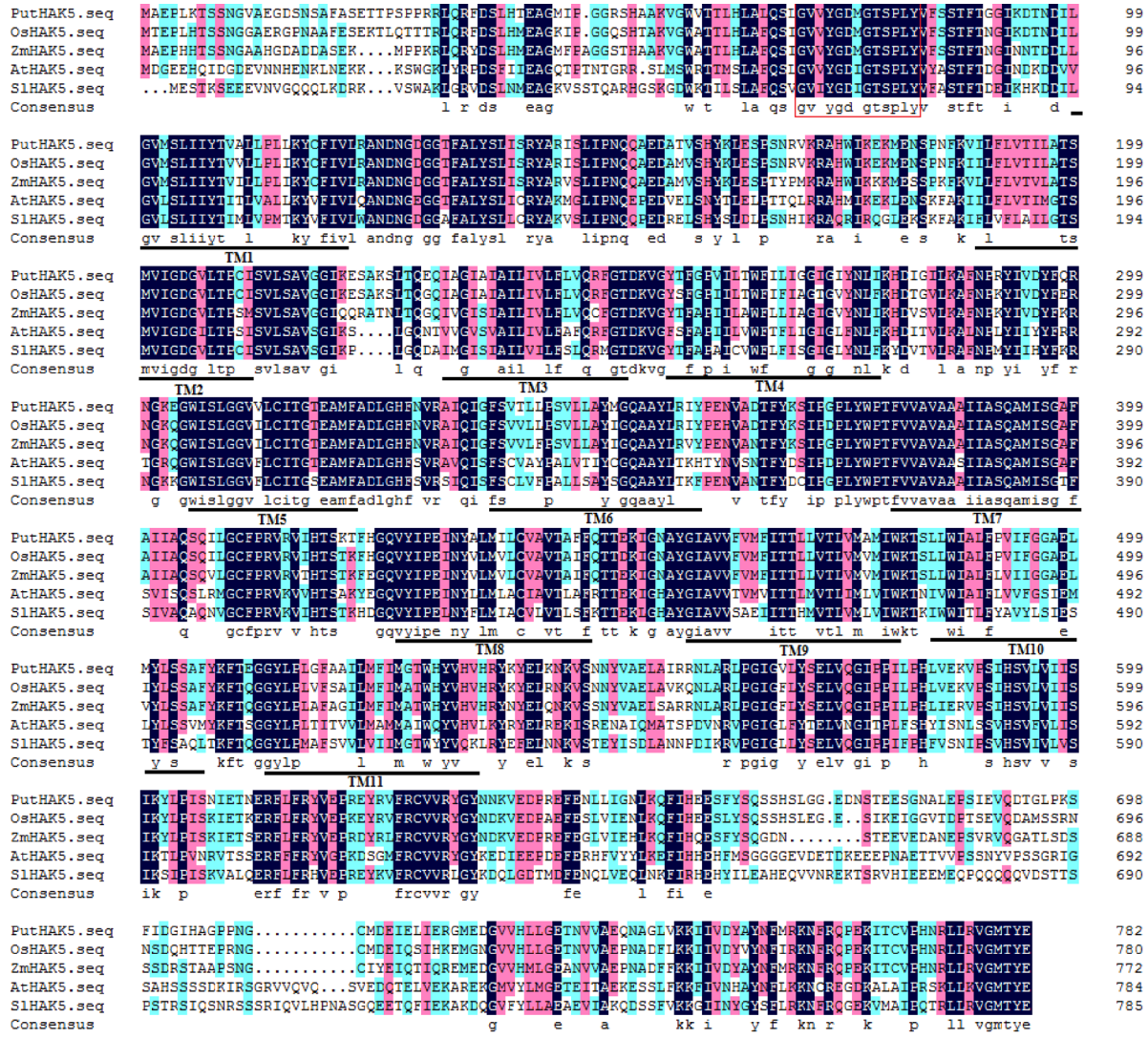

Fig. 3 Sequence alignment of *PtHAK5* and HAK5-like proteins from higher plants. Sources of HAK5 and their GenBank accession numbers were as follows: OsHAK5 (*Oryza sativa*, BAD87321.1), ZmHAK5 (*Zea mays*, AHH35045.1), SlHAK5 (*Solanum lycopersicum*, ABF22603.1), AtHAK5 (*Arabidopsis thaliana*, AT4G13420). The sequences were aligned with DNAMAN 6.0 software. The 11 putative transmembrane domains (TM1–TM11) are underlined.

large amount of K^+[12]. In ice plant (*Mesembryanthemum crystallinum*), *HAK* gene expression increased transiently under high salt conditions[31]. Yang et al.[8] also found that under normal K^+ conditions, the expression of *OsHAK5* in rice was markedly upregulated by high concentrations of NaCl in the rhizosphere, and OsHAK5 mediates accumulation of K^+ in *E. coli* and BY2 cells under salt treatment[12]. Thus, PtHAK5 could be of great significance in salt tolerance by participating in the maintenance of K^+ nutrition in *P. tenuiflora* under salinity. This suggestion is supported by the fact that expression of *PtHAK5* rescued the K^+-uptake-defective phenotype of yeast strain CY162 under low-K^+ medium (Fig. 6). Therefore, PtHAK5 is a potential candidate for mediating high-affinity K^+ uptake.

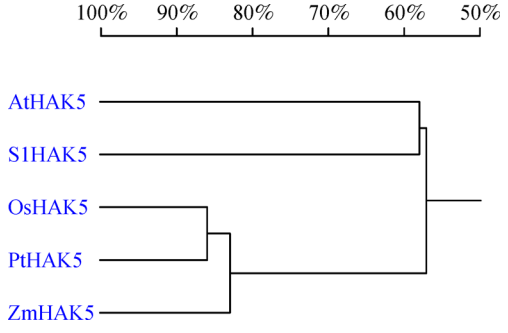

Fig. 4 Phylogenetic tree analysis of PtHAK5 amino acid sequences. AtHAK5 from *Arabidopsis thaliana*, SlHAK5 from *Solanum lycopersicum*, OsHAK5 from *Oryza sativa*, ZmHAK5 from *Zea mays*.

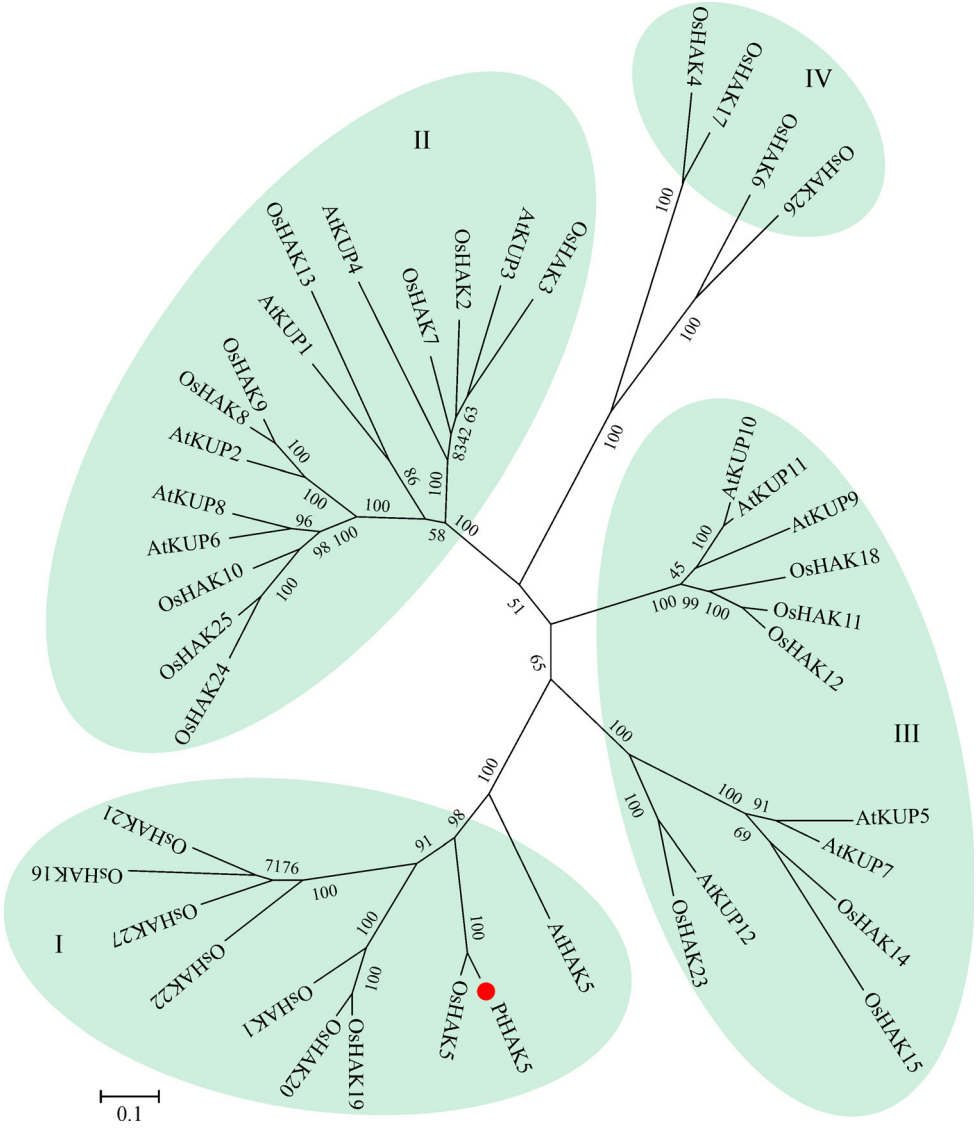

Fig. 5 Phylogenetic tree analysis of PtHAK5 and HAK/KUP/KT family members from *Arabidopsis thaliana* and *Oryza sativa* (rice). The phylogenetic tree was generated by MEGA 6.0 software using the maximum-likelihood method. Sources of K^+ transporter amino acid sequence and their GenBank accession numbers are as follows: in *A. thaliana*, AtKUP1 (AT2G30070), AtKUP2 (AT2G40540), AtKUP3 (AT3G02050), AtKUP4 (AT4G23640), AtKUP5 (AT4G33530), AtKUP6 (AT1G70300), AtKUP7 (AT5G09400), AtKUP8 (AT5G14880), AtKUP9 (AT4G19960), AtKUP10 (AT1G31120), AtKUP11 (AT2G35060), AtKUP12 (AT1G60160), AtHAK5 (AT4G13420); and in *O. sativa*, OsHAK1 (NC_008397), OsHAK2 (BAF07234), OsHAK3 (AJ427974), OsHAK4 (AK071698), OsHAK5 (AP003272), OsHAK6 (NP_001045298), OsHAK7 (AJ427976), OsHAK8 (Q8VXB5), OsHAK9 (AJ427978), OsHAK10 (AJ427979), OsHAK11 (AJ427980), OsHAK12 (AJ427981), OsHAK13 (BAF20244), OsHAK14 (AJ427983), OsHAK15 (AJ427984), OsHAK16 (BAF12444), OsHAK17 (AJ427975), OsHAK18 (NP_001063938), OsHAK19 (BAF08880), OsHAK20 (BAF08882), OsHAK21 (BAH92235), OsHAK22 (EEC81370), OsHAK23 (BAF24960), OsHAK24 (BAF19271), OsHAK25 (EEC73938), OsHAK26 (BAF24119), OsHAK27 (BAF12443).

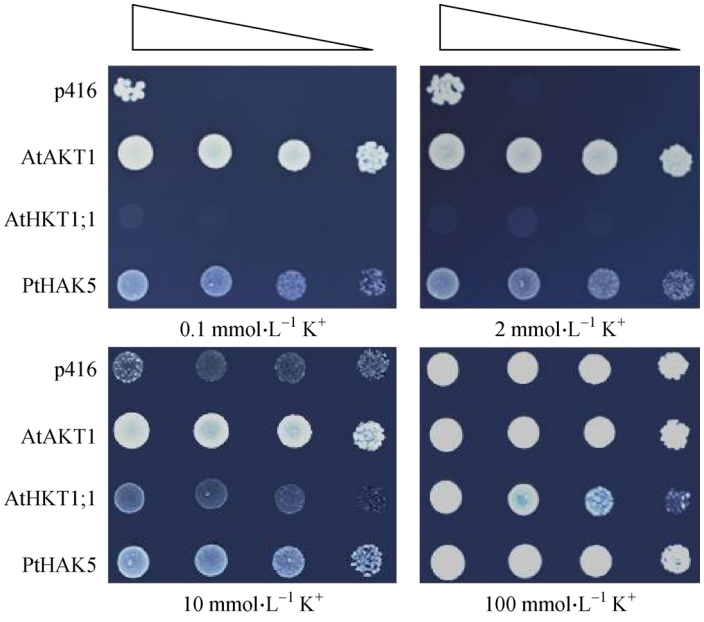

Fig. 6 Complementation of the K^+ uptake-deficient *Saccharomyces cerevisiae* mutant strain CY162 by expressing *PtHAK5, AtAKT1, AtHKT1*;*1* and empty vector p416 GPD. Each yeast cell was plated on minimal AP medium containing four concentrations of K^+ (0.1, 2, 10 and 100 mmol·L^{-1}) at 10-fold serial dilutions from OD_{600} = 0.6 to 0.6 × 10^{-3}. AP medium with 100 mmol·L^{-1} K^+ was used as a control, and CY162 expressing AtAKT1, AtHKT1;1 and p416 GPD were used as positive and negative controls, respectively. The triangles indicate that the cell concentration was diluted 10-fold from left to right.

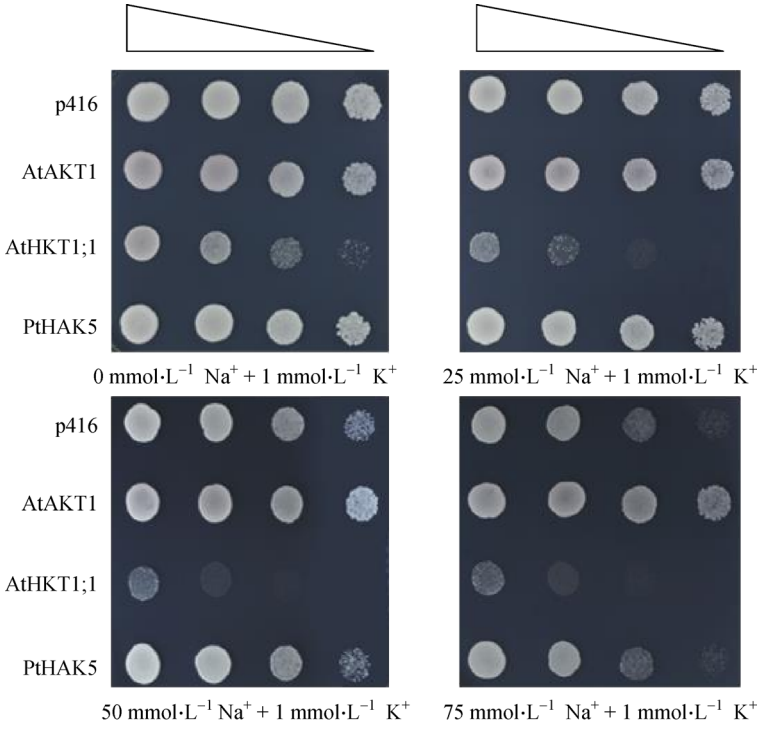

Fig. 7 Na^+-induced growth-inhibition of *Saccharomyces cerevisiae* mutant strain G19 expressing *PtHAK5, AtAKT1, At HKT1*;*1* and empty vector p416 G19. Yeast cells were plated on minimal AP medium containing 1 mmol·L^{-1} K^+ and various concentrations of Na^+ (0, 25, 50 and 75 mmol·L^{-1}) at 10-fold serial dilutions from OD_{600} = 0.6 to 0.6 × 10^{-3}. AP medium without Na^+ was used as a control, and G19 expressing AtHKT1;1, AtAKT1 and p416 GPD were used as positive and negative controls, respectively. The triangles indicate that the cell concentration was diluted 10-fold from left to right.

5 Conclusions

The *PtHAK5* gene of *P. tenuiflora* was grouped in cluster I of the KT/KUP/HAK family, which encodes high-affinity K^+ transporters, and is a potential candidate for mediating high-affinity K^+ uptake under low K^+ concentrations. It is concluded that it is likely to be crucial for salt tolerance of *P. tenuiflora* by facilitating efficient K^+ uptake under saline conditions.

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Compliance with ethics guidelines Haili Yang, Weidan Zhang, Weiwei Chai, Wenying Wang, Li Gao, Jing Zhang, Yongping Wang, and Suo-Min Wang declare they have no conflicts of interest or financial conflicts to disclose.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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