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Molecular cloning and expression analysis of a gene encoding KUP/HAK/KT-type potassium uptake transporter from *Cryptomeria japonica*

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Authors' contributions

Y.H. designed the study and drafted the manuscript. Y.H. and Y.K. carried out gene isolation, sequence analysis, and gene expression analysis. Y.H., K.N., and N.U. participated in complementation tests. N.U. and K.N. helped to draft the manuscript. All authors read and approved the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

Key message

The molecular mechanism of potassium ion transport across membranes in conifers is poorly known. We isolated and analyzed a gene encoding a potassium transporter from the conifer *Cryptomeria japonica*.

Abstract

Abstract Potassium ion (K^+) is an essential and the most abundant intracellular cation in plants. The roles of K^+ in various aspects of plant life are closely linked to its transport across biological membranes such as the plasma membrane and the tonoplast, which is mediated by membrane-bound transport proteins known as transporters and channels. Information on the molecular basis of K^+ membrane transport in trees, especially in conifers, is currently limited. In this study, we isolated one complementary DNA, *CjKUP1*, which is homologous to known plant K^+ transporters, from *Cryptomeria japonica*. Complementation tests using an *Escherichia coli* mutant, which is deficient in K^+ uptake activity, was conducted to examine the K^+ uptake function of the protein encoded by *CjKUP1*. Transformation of the K^+-uptake-deficient mutant with *CjKUP1* complemented the deficiency of this mutant. This result indicates that CjKUP1 has a function of K^+ uptake. The expression levels of *CjKUP1* in male strobili were markedly higher from late September to early October than in other periods. The expression levels in male and female strobili were higher than those in other organs such as needles, inner bark, differentiating xylem, and roots. These results indicate that CjKUP1 is mainly involved in K^+ membrane transport in the cells of reproductive organs of *C. japonica* trees, especially in male strobili during pollen differentiation.

Keywords: *Cryptomeria japonica*, Male strobilus, Membrane transport, Potassium, Transporter

Introduction

Potassium ion (K^+) is an essential nutrient for plants, and the most abundant cation in plant cells. It plays a crucial role in various aspects of plant life at the cellular and whole-plant levels, such as enzyme activation, cell expansion, osmoregulation, stomatal movements, and stress tolerance (Marschner 2012; Wang et al. 2013). Trees show strong K^+ demand in wood formation via cambial activity (Dünisch and Bauch 1994a, b; Kuhn et al. 1997; Dünisch et al. 1998; Wind et al. 2004). These processes involve K^+ movement across the biological membranes of the cell, e.g., K^+ uptake into the cell across the plasma membrane and K^+ storage in or retrieval from the vacuole across the tonoplast. At the molecular level, these movements are mediated by membrane proteins known as transporters and channels (Mäser et al. 2001; Very and Sentenac 2003; Ashley et al. 2006; Szczerba et al. 2009). Elucidating the molecular basis of K^+ membrane transport in trees is important to obtain a better understanding of the growth, reproduction, and environmental adaptation of trees.

The gene encoding K^+ uptake permease (Kup) was first identified from *Escherichia coli* (Bossemeyer et al. 1989) and further characterization of Kup has been performed (Schleyer and Bakker 1993; Sato et al. 2014). Kup is also known as high-affinity K^+ transporter (HAK) or K^+ transporter (KT) for homologous proteins that have been isolated in other organisms (Mäser et al. 2001). To date, plant genes encoding KUP/HAK/KT-type transporters have been cloned from a variety of angiosperm species including model plants (Gierth and Mäser 2007; Grabov 2007; Szczerba et al. 2009). *Arabidopsis thaliana* and rice (*Oryza sativa*) possess 13 and 27 *KUP/HAK/KT* genes, respectively, in their completely sequenced genomes (Mäser et al. 2001; Yang et al. 2009). KUP/HAK/KT transporters have been proposed to be expressed in nearly all plant tissues and localize in the plasma membrane or the tonoplast of the cell (Bañuelos et al. 2002; Su et al. 2002; Ahn et al. 2004; Gupta et al. 2008). These transporters have been shown to mediate K^+ uptake across the membrane, and their contributions to plant

physiology have also been discussed (Shabara 2003; Ashley et al. 2006; Gierth and Mäser 2007; Grabov 2007; Szczerba et al. 2009). The present knowledge of *KUP/HAK/KT* genes has mostly been gained in herbaceous angiosperms, whereas limited information is available on these genes in trees, especially in conifers. Concerning *Populus* spp., one *KUP* (*PtKUP1*) gene has been cloned (Langer et al. 2002), and 31 *HAK* (*PtHAK*) genes have been identified using genome-wide sequence analysis (He et al. 2013). The function and expression profile of *PtKUP1* have been studied (Langer et al. 2002; Escalante-Pérez et al. 2009), but little is known about those of *PtHAK* genes. In conifers, identification and cloning of *KUP/HAK/KT* genes have rarely been undertaken.

Cryptomeria japonica is a tall evergreen conifer distributed throughout Japan and in areas of China. It is one of the most important forestry species in Japan because of its straight stem, rapid growth rate, excellent wood quality, and strong rot resistance (Ohba 1993). On the other hand, *C. japonica* is anemophilous and its pollen causes pollinosis, which is becoming a serious health problem in Japan. Related to its economic importance and allergenicity, the collection of expressed sequence tags (ESTs) and the construction of complementary DNA (cDNA) libraries from various tissues of *C. japonica* have been conducted, and an EST sequence database for this species (ForestGEN; http://forestgen.ffpri.affrc.go.jp/en/index.html) has been constructed (Tsumura et al. 1997; Ujino-Ihara et al. 2000; 2003; 2005; Futamura et al. 2006; 2008; Yoshida et al. 2007). Thus, *C. japonica* is likely to be a suitable conifer for discovery and functional investigations of novel genes.

In this study, a cDNA encoding a KUP/HAK/KT transporter (CjKUP1) was isolated from *C. japonica*. The K^+ uptake function of CjKUP1 was examined by complementation tests using an *E. coli* mutant deficient in K^+ uptake. In addition, the expression levels of *CjKUP1* in different organs of *C. japonica* trees was measured using quantitative real-time reverse

transcription (RT)-polymerase chain reaction (PCR), and changes in its expression during male strobilus development and expression differences among organs were examined.

Materials and methods

Plant materials

Samples of needles, inner bark, differentiating xylem, male strobili, and female strobili were collected from approximately 50-year-old *C. japonica* trees (mean height 24.5 m; mean diameter at breast height 39.7 cm) growing in the field of the Faculty of Agriculture, Shinshu University, Japan. Current-year needles, inner bark, and differentiating xylem were collected in late June 2010 when the radial growth of trees approaches its peak (Funada et al. 1990). Female strobili were collected in the pollination period in early April 2010. Male strobili were collected at 1- to 4-week intervals from the pollen mother cell stage in late August 2010 until just before pollen release in late February 2011. Samples of roots were collected from 3-year-old *C. japonica* saplings (mean height 74.4 cm; mean basal diameter 1.2 cm) planted in pots and grown in the field in late May 2010. The collected samples were stored at −80°C for further study.

Gene isolation and sequence analysis

ESTs of *C. japonica* homologous to known K^+ transporters were searched in the ForestGEN database and databases at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/). An EST homologous to the amino (N)-terminal region of the known KUP/HAK/KT transporters (accession number: ForestGEN, Cj.12720; GenBank BY878266) and that homologous to the carboxyl (C)-terminal region of the transporters (accession number: ForestGEN, Cj.19298; GenBank BY897118) were identified. Based on the sequence of these ESTs, the following primers were designed for amplification of *C.*

japonica cDNA containing the full-length open reading frame (ORF) of a K^+ transporter gene: KUP-F1 (5′-GTA ACA AAG CAT TAT AGA CAG CCA GAG-3′) and KUP-R1 (5′-CCT CAT GTC CAT CTC CAG ATC-3′).

Total RNA was extracted from each sample using a CTAB method (Chang et al. 1993). The target cDNAs were generated by RT-PCR using the SuperScript Ⅲ One-Step RT-PCR System with Platinum Taq High Fidelity (Life Technologies, Carlsbad, CA, USA). Reaction mixtures of 50 μl were prepared in accordance with the manufacturer's protocol, and RT-PCR reactions were conducted using a Piko themal cycler (Thermo Fisher Scientific, Waltham, MA, USA) with the following conditions: reverse transcription for 30 min at 55°C, followed by initial denaturation for 3 min at 94°C, 40 cycles of denaturation for 30 s at 94°C, annealing at 55°C for 30 s, and extension for 2 min 30 s at 68°C, and final extension for 5 min at 68°C. The Cj.12720 (BY878266) and Cj.19298 (BY897118) ESTs derive from a full-length cDNA library of *C. japonica* male strobili. Therefore, total RNA extracted from male strobilus samples was used for RT-PCR. Amplified products were subcloned into the pCR2.1-TOPO vector using the TOPO TA Cloning Kit (Life Technologies) and then transformed into *E. coli* DH5α competent cells (Toyobo, Osaka, Japan) for sequencing.

Sequencing was conducted using the BigDye Terminator v3.1 kit (Life Technologies) on an ABI 3730XL DNA analyzer (Life Technologies). The ORF was predicted and sequence similarities were investigated using the ORF finder and BLAST servers at the NCBI. Multiple sequence alignment was performed using the ClustalW algorithm in the BioEdit software (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and a phylogenetic tree was generated using the neighbor-joining method (Saitou and Nei 1987) with the MEGA software (http://www.megasoftware.net). A hypothetical model for the membrane topology of the protein encoded by the amplified cDNA was built using the TMHMM server (http://www.cbs.dtu.dk/services/TMHMM/).

Complementation tests in *E. coli*

The *E. coli* LB2003 strain (*F-, thi, lacZ, gal, rha,* Δ*kdpFABC5, trkΔ1, ΔtrkA*), which lacks three types of K^+ uptake systems, namely Trk, Kup, and Kdp (Stumpe and Bakker 1997), was used for complementation tests. The full-length ORF of *CjKUP1* was amplified by PCR using PrimeSTAR HS DNA Polymerase (Takara Bio), the forward primer with the *Bam*HI site (KUP-F2, 5′-AAG GAG GAT CCA ATA TGG ATC ACA GTG CAG-3′), the reverse primer with the *Pst*I site (KUP-R2, 5′-ACC TAC TGC AGT GCA CTG AAC TCT AGA TTT C-3′), and the pCR2.1-TOPO vector containing the *CjKUP1* insert as a template. Reaction mixtures of 20 μl were prepared in accordance with the manufacturer's protocol and PCR reactions were conducted using a Piko themal cycler with the following cycling profile: 1 cycle at 94°C for 3 min, followed by 35 cycles at 94°C for 30 s, at 55°C for 30 s, and at 72°C for 2 min 30 s, with a final extension at 72°C for 5 min. Amplified fragments were digested with *Bam*HI and *Pst*I (Roche Diagnostics, Basel, Switzerland), and inserted into the corresponding sites of the pPAB404 vector (Buurman et al. 1995) using Ligation high Ver.2 (Toyobo). The resulting vector was transformed into the *E. coli* LB2003 strain, and the transformants were then tested for their ability to grow in solid medium containing 46 mM Na_2HPO_4, 23 mM NaH_2PO_4, 8 mM $(NH_4)_2SO_4$, 0.4 mM $MgSO_4$, 6 μM $FeSO_4$, 1 μg/ml thiamine hydrochloride, 1% glucose, and 1% agarose in the presence of 30 μg/ml ampicillin, 0.2 mM isopropyl β-D-1-thiogalactopyranoside, and different concentrations of K^+ adjusted with KCl (Epstein and Kim 1971; Nakamura et al. 1996). The LB2003 cells expressing an *A. thaliana* K^+ channel, KAT1 (Uozumi et al. 1998), were used as a positive control. As a negative control, the LB2003 cells transformed with the empty pPAB404 vector were used. All transformants were grown at 30°C for 2 days.

The effect of the cations, Ca^{2+} and Cs^+, on the growth complementation produced by CjKUP1 expression was examined as described elsewhere (Kim et al. 1998; Fairbairn et al.

2000; Sun et al. 2006). LB2003 cells expressing CjKUP1 or the empty vector were grown overnight at 37°C in KML medium (1% tryptone, 0.5% yeast extract, and 50 mM KCl) containing 50 μg/ml ampicillin. The overnight cultures were added to the medium containing a low level (approximately 2 mM) of K^+ (1% tryptone, 0.2% yeast extract, and 100 mM mannitol) and different concentrations of Ca^{2+} or Cs^+ to a starting optical density at 600 nm (OD_{600}) of 0.05. The cultures were grown at 37°C under the presence of 50 μg/ml ampicillin, and their OD_{600} was measured 9 h later. Concentrations of Ca^{2+} and Cs^+ were varied by supplementation from sterile stock solutions of the appropriate chloride salt, and OD_{600} was measured using a spectrophotometer (GeneQuant100; GE Healthcare, Little Chalfont, UK).

Analysis of gene expression using quantitative real-time RT-PCR

First-strand cDNAs were synthesized from 1 μg of each total RNA sample from various organs using the PrimeScript RT Reagent Kit with gDNA Eraser (Takara Bio, Shiga, Japan) in accordance with the manufacturer's instructions. Synthesized cDNAs were diluted 1:5 with nuclease-free water. Primers for real-time PCR were designed using the FastPCR software (http://primerdigital.com/fastpcr.html). The sequences of primers specific for *CjKUP1* were KUP-F3 (5′-AAC CGT GTA GAG GCT GTG TAT G-3′) and KUP-R3 (5′-ATG GAG GAC CTA CAG TTG TTC TG-3′). Primers designed based on the sequence of the cluster Cj.4622 in the ForestGEN database were employed as an internal control: ACT-F1 (5′-TTG GCG CTG AGC GAT TC-3′) and ACT-R1 (5′-TCC CAA TTA GAG ATG GCT GGA A-3′). The cDNA fragments amplified by these primers are homologous to actins constitutively expressed in *A. thaliana* and poplar (Szyroki et al. 2001; Brunner et al. 2004). Primers were evaluated via non-quantitative PCR, and primer combinations that yielded only one clear amplification product of expected length were used for real-time PCR.

Reaction mixtures of 20 μl containing 1× SYBR Premix Ex Taq (Takara Bio), 0.25 μM

of each primer, and 2 μl diluted cDNA were prepared. Subsequently, real-time PCR reactions were conducted using a Thermal Cycler Dice Real-Time System (TP800; Takara Bio) with the following conditions: initial denaturation at 95°C for 30 s, 45 cycles of the sequence of denaturation at 95°C for 5 s and annealing/elongation at 60°C for 30 s, followed by a dissociation curve. The data were analyzed using Thermal Cycler Dice Real-Time System software (Takara Bio), and relative expression levels of *CjKUP1* were determined by a comparative C_T method (Livak and Schmittgen 2001) in accordance with the manual provided with the software. All quantifications were normalized on the basis of the amount of cDNA fragments amplified by ACT-F1 and ACT-R1 primers. We performed six technical replicates for each of two biological replicates per organ or sampling date.

Results

Isolation and sequence analysis of *CjKUP1* cDNA

One *C. japonica* cDNA, *CjKUP1* (GenBank accession number AB915694), was amplified by RT-PCR using primers designed from ESTs homologous to the known KUP/HAK/KT-type transporters and a total RNA template prepared from male strobili. The *CjKUP1* cDNA was predicted to contain an ORF of 2244 base pairs that encodes a protein of 748 amino acids with a calculated molecular mass of 83.3 kDa. A sequence alignment of CjKUP1 with previously identified KUP/HAK/KT transporters in *A. thaliana* and rice at the amino acid level is shown in Fig. 1a. The translated sequence of the *CjKUP1* cDNA was homologous to KUP/HAK/KT transporters derived from various angiosperm species, including *A. thaliana* and rice. CjKUP1 contained all amino acid residues conserved in all bacterial, fungal, and plant KUP/HAK/KT transporters and most amino acid residues conserved in only plants (Rodríguez-Navarro 2000; Rubio et al. 2000). The TMHMM server predicted that CjKUP1 contained 12 transmembrane segments and had the N-terminus, the C-terminus, and a long

loop between the second and third transmembrane segments inside the membrane (Fig. 1b). This is consistent with the membrane topology of *E. coli* Kup (Sato et al. 2014). According to the phylogenetic tree representing relationships among CjKUP1 and KUP/HAK/KT transporters from *A. thaliana* and rice (Fig. 2), CjKUP1 was highly homologous to the transporters belonging to cluster Ⅳ among the four clusters in the KUP/HAK/KT transporter family described by Rubio et al. (2000), and showed the highest homology to OsHAK26, with 51% identity and 69% similarity.

Functional complementation of K^+ uptake-deficient *E. coli* mutant

The K^+ uptake-deficient *E. coli* LB2003 strain has been applied for functional assay of the eukaryotic K^+ uptake system (Uozumi et al. 1998; Uozumi 2001; Bañuelos et al. 2002; Ahn et al. 2004). The LB2003 cells containing the empty pPAB404 vector (negative control) grew in the medium supplemented with a high level (30 mM) of K^+ (KCl), but did not grow in the medium supplemented with a low level (5 mM) of K^+ (Fig. 3a). The LB2003 cells expressing KAT1 (positive control) grew both in the medium supplemented with high K^+ and that with low K^+ (Fig. 3a). The LB2003 cells expressing CjKUP1 were also capable of growing in the medium supplemented with low K^+ (Fig. 3a). The effect of Ca^{2+} and Cs^+ on the growth of LB2003 cells expressing CjKUP1 was then tested (Fig. 3b). The cells were able to grow on the medium containing low K^+ (approximately 2 mM) and lacking Ca^{2+} ($CaCl_2$) and Cs^+ (CsCl). Cell growth was strongly inhibited by 1, 5, and 10 mM Ca^{2+}. Growth was also susceptible to inhibition by Cs^+, especially by 5 and 10 mM Cs^+. The growth of LB2003 cells containing the empty pPAB404 vector was poor, irrespective of the concentration of Ca^{2+} and Cs^+ in the medium.

Expression pattern of *CjKUP1* determined by real-time RT-PCR

Figure 4 shows the seasonal changes in relative expression level of *CjKUP1* in male strobili of *C. japonica* trees from late August 2010 to late February 2011. The expression of *CjKUP1* showed a marked increase on September 25th. High expression of *CjKUP1* was also detected in the male strobilus samples collected on October 2nd. The expression levels on September 25th and October 2nd were considerably and significantly higher than those observed on the other sampling dates. From October 18th the expression level remained low until the following February.

Figure 5 shows the relative expression level of *CjKUP1* in various organs of *C. japonica* trees. The expression level of *CjKUP1* in the samples of male strobili collected on October 2nd was markedly higher than that of all other organs. The expression levels in the needle, inner bark, differentiating xylem, and root samples were equivalent to or lower than those of male strobili collected on February 22nd. The expression level in the samples of female strobili was higher than that of male strobili collected on February 22nd and that of other organs, although it was lower than that of male strobili collected on October 2nd.

Discussion

The sequence of the predicted protein encoded by the *CjKUP1* cDNA showed high homology with KUP/HAK/KT transporters derived from other plant species and contained amino acid residues conserved in such transporters across bacteria, fungi, and plants. Genes encoding KUP/HAK/KT transporters are present in all plant genomes that have been studied so far, whereas they have not been found in Protista and Animalia (Grabov 2007). The results of this study indicate that a conifer, *C. japonica*, also possesses a *KUP/HAK/KT* gene. According to the ARAMEMNON database (Schwacke et al. 2003), all KUP/HAK/KT transporters from *A. thaliana* and rice have between 10 and 14 transmembrane segments and the N-terminus inside

the membrane, except for OsHAK3. These transporters are also considered to have a long loop between the second and third transmembrane segments inside the membrane and a long tail in the C-terminus (Gierth and Mäser 2007). The predicted membrane topology of CjKUP1 is consistent with the topology predicted for AtKUP and OsHAK transporters.

KUP/HAK/KT transporters have been classified into four clusters based on their amino acid sequences (Rubio et al. 2000). The most-studied members of the KUP/HAK/KT family belong to the two largest groups, clusters I and Ⅱ (Bañuelos et al. 2002). All plant species that have been studied so far possess members of clusters I or Ⅱ. Genes encoding transporters of cluster Ⅲ have been found only in *A. thaliana* and rice. Both *A. thaliana* and rice have six members of this cluster (Gupta et al. 2008). Cluster IV is the smallest cluster and contains four rice transporters and is absent in *A. thaliana* (Gupta et al. 2008). Besides rice, one gene encoding a transporter belonging to cluster Ⅳ (PhaHAK5) was cloned from *Phragmites australis* (Takahashi et al. 2007). The results of the present study showed that CjKUP1 belonged to cluster Ⅳ and suggested that *C. japonica* possessed the smallest cluster of KUP/HAK/KT transporters.

A heterologous expression system in *E. coli* has been developed as a powerful tool with which to investigate the structure and function of eukaryotic K^+ membrane transport proteins (Uozumi et al. 1998; Uozumi 2001). In particular, elucidation of the function of KUP/HAK/KT transporters in *E. coli* cells is useful because yeast mutant and *Xenopus* oocyte expression systems are often unable to be applied for evaluation of their K^+ uptake activity (Kim et al. 1998). The K^+ uptake function of plant KUP/HAK/KT transporters has been investigated using the *E. coli* expression system (Kim et al. 1998; Bañuelos et al. 2002; Garciadeblas et al. 2002; Langer et al. 2002; Ahn et al. 2004; Davies et al. 2006). The *E. coli* LB2003 strain, which contains mutations in three kinds of K^+ uptake systems, requires 25 mM K^+ for half-maximal cell growth (Epstein and Kim 1971). The LB2003 strain expressing

CjKUP1 was able to grow in K^+-limited (5 mM) medium similar to that expressing KAT1 as a positive control (Uozumi et al. 1998). This indicates that CjKUP1 was correctly integrated into the *E. coli* inner membrane and complements the defect in K^+ uptake function of this mutant. We therefore concluded that CjKUP1 functions as a K^+ uptake system in the *E. coli* plasma membrane. Growth of the LB2003 strain expressing CjKUP1 was strongly inhibited by Ca^{2+} and Cs^+. Langer et al. (2002) demonstrated that the growth of the strain expressing PtKUP1 from poplar is inhibited by Ca^{2+} and Cs^+, which suggests that PtKUP1 is sensitive to these cations. Fu and Luan (1998) and Kim et al. (1998) reported that K^+ uptake mediated by AtKUP1 is inhibited by Cs^+. It seems likely that CjKUP1 is also sensitive to these K^+ transporter cation inhibitors. Most of the functionally characterized KUP/HAK/KT transporters belong to clusters I and Ⅱ, whereas the functions of the transporters belonging to clusters Ⅲ and Ⅳ are less well known (Grabov 2007). Concerning the transporters of cluster Ⅳ, Takahashi et al. (2007) suggested that PhaHAK5 was located in the plasma membrane and functioned as a K^+ uptake transporter, but four OsHAKs have not been functionally characterized yet. The present study suggests that CjKUP1, which belongs to cluster Ⅳ, is a K^+ uptake transporter sensitive to Ca^{2+} and Cs^+.

Alignment of amino acid sequences of eukaryotic KUP/HAK/KT transporters indicates they all bear a common structure and, although they do not show extensive conserved regions, high numbers of amino acid residues are conserved in exactly the same position in all transporters (Rodríguez-Navarro 2000). One of the sequence characteristics of KUP/HAK/KT transporters is a characteristic in their first transmembrane segment. In this segment, there is a motif for which the different sequences vary only slightly from the consensus **GD**LGT**SPLY** sequence (the amino acids conserved in all sequences are in bold). Sequence analysis of the transporters belonging to clusters I and Ⅱ has revealed that there is a cluster-specific pattern in this consensus sequence. The sequence of the segment in cluster I is GDXGTSPLY, whereas

that of cluster Ⅱ is GDLS(T/I)SPLY (Rodríguez-Navarro 2000; Senn et al. 2001). Senn et al. (2001) demonstrated that substitutions of several amino acid residues in the sequence GDX**GT**SPLY (substituted amino acids are in bold) of a barley (*Hordeum vulgare*) cluster I transporter, HvHAK1, to the residues characteristic of cluster Ⅱ (sequence GDX**SI**SPLY) reduced K^+ transport capacity of HvHAK1. These authors suggested that these conserved amino acids in the first transmembrane segment of cluster I transporters are specifically required for HvHAK1 function. A recent study on *E. coli* Kup showed that the aspartate in the first transmembrane segment is crucial for its K^+ uptake function (Sato et al. 2014). To date, no region involved in ion transport has been identified in cluster Ⅳ transporters. The translated sequence for *CjKUP1* contained the sequence GDLGTSPLY in the first putative transmembrane segment. This sequence is similar to the consensus sequence of cluster I. It is possible that amino acids in this sequence are involved in the K^+ transport function of CjKUP1.

In *C. japonica*, primordia of male strobili are formed in the axes of small branches from late June to late August. Meiosis of pollen mother cells in microsporangia begins in September and tetrads consisting of similar-sized microspores are formed in October. Individual microspores separate from the tetrads and form free spores from late October to late November. Mature pollen grains are formed in December, and male strobili then remain in an arrested state of development until the following spring when the pollen grains are disseminated (Nagao et al. 1989; Hosoo et al. 2005; Ueuma et al. 2009). The expression of *CjKUP1* in male strobili greatly increased in late September and remained high until early October. These results suggest that *CjKUP1* expression in male strobili increases in the periods of meiosis and microspore separation during pollen differentiation.

Previous studies have shown that *KUP/HAK/KT* genes are expressed throughout the plant body, including in flowers, leaves, and stems. However, these studies also show that the

expression patterns of individual *KUP/HAK/KT* genes in the plant body are varied (Kim et al. 1998; Su et al. 2002; Ahn et al. 2004; Gupta et al. 2008; Alemán et al. 2011). Ahn et al. (2004) investigated the expression patterns of each *AtKUP* gene in various organs of *A. thaliana*. The expression levels of many *AtKUP* genes were higher in the aboveground parts of the plant. *AtKUP2* to *AtKUP4* were highly expressed in developing siliques, whereas *AtKUP6*, *AtKUP8* to *AtKUP10*, and *AtKUP12* were highly expressed in leaves. In flowers, *AtKUP2* and *AtKUP8* were more highly expressed than other *AtKUP* genes. The expression of *AtKUP2* in flowers and developing siliques was higher than that in other parts of the plant. In the common ice plant (*Mesembryanthemum crystallinum*), *McHAK1* and *McHAK2* expression is apparent in reproductive tissues, whereas *McHAK3* is specifically expressed in roots (Su et al. 2002). In rice, some *OsHAK* genes show higher expression levels in stamens or panicles compared with other tissues (Gupta et al. 2008). Conversely, poplar *PtKUP1* is constitutively expressed at low levels (Langer et al. 2002; Escalante-Pérez et al. 2009). In the present study, the expression levels of *CjKUP1* in male and female strobili, especially in male strobili from late September to early October, were higher than those in other organs. This result suggests that the expression of *CjKUP1* is different among organs or developmental stages rather than being constitutive.

Some KUP/HAK/KT transporters play important roles in plant physiology and development. Transporters of cluster I, including *A. thaliana* AtHAK5, characterized so far are likely to play an important role in K^+ acquisition, particularly when K^+ availability is low (Bañuelos et al. 2002; Rodríguez-Navarro and Rubio 2006). The physiological functions of cluster Ⅱ transporters are probably quite diverse and their role in potassium nutrition is poorly defined. In *A. thaliana*, AtKUP2 is known to be involved in regulation of turgor-driven cell expansion in the shoot (Elumalai et al. 2002) and AtKUP4 is putatively required for root hair development and gravitropic responses (Rigas et al. 2001; Vicente-Agullo et al. 2004). The

role of a cluster Ⅱ transporter in turgor-dependent growth has been demonstrated in rapidly expanding cotton fibers (Ruan et al. 2001). Transporters of cluster Ⅲ and Ⅳ have been less intensively studied. AtKUP11 (a member of cluster Ⅲ) may be involved in plant responses to salinity (Maathuis 2006). The present results indicate that CjKUP1, a member of cluster Ⅳ, is mainly involved in K^+ membrane transport in the cells of reproductive organs.

In conclusion, one gene encoding a KUP/HAK/KT-type transporter (CjKUP1) was isolated from a conifer, *C. japonica*. Complementation of a K^+ uptake-deficient *E. coli* mutant demonstrated that the function of CjKUP1 is associated with K^+ uptake. CjKUP1 is likely to be mainly involved in K^+ membrane transport in the cells of reproductive organs, especially in the cells of male strobili during pollen differentiation. Precise information on the molecular mechanism of K^+ membrane transport in trees is important to understand their growth, reproduction, and environmental adaptation. The information gained in the present study is expected to contribute to effective conservation and management of *C. japonica* in the future.

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Figure captions

Fig. 1 Structure of CjKUP1. **a** Alignment of the predicted amino acid sequence of CjKUP1 with the amino acid sequences of rice OsHAK26 and *Arabidopsis thaliana* AtKUP1. Amino acid residues shaded in black are conserved in all bacterial, fungal, and plant KUP/HAK/KT transporters, and grey shading denotes amino acid residues conserved in plant KUP/HAK/KT transporters (Rodríguez-Navarro 2000; Rubio et al. 2000). Putative transmembrane segments are underlined. The GenBank accession numbers of CjKUP1, OsHAK26, and AtKUP1 sequences are AB915694, AK072472, and AF029876, respectively. **b** Proposed topological model of CjKUP1 constructed with the TMHMM server

Fig. 2 Phylogenetic tree for translated sequences of *KUP/HAK/KT* genes. The alignment comprised sequences for CjKUP1, *A. thaliana* AtKUP, and rice OsHAK transporters. The tree was constructed using the neighbor-joining algorithm with the MEGA software. The scale bar corresponds to the branch length and shows a distance of 10 changes per 100 amino acid positions. Bootstrap values from 1,000 replicates are indicated at each branch. The GenBank accession numbers are as follows: *AtKUP2*, AF012657; *AtKUP3*, AF207621; *AtKUP4*, AJ296155; *AtKUP9*, AL021637; *AtKUP10*, AK229208; *AtHAK5*, AF129478; *OsHAK2*, AK070575; *OsHAK5*, AK241580; *OsHAK6*, AK107477; *OsHAK9*, AK070738; *OsHAK11*, AK072123; *OsHAK13*, AK111663; *OsHAK16*, AK066919; *OsHAK18*, AK065464; *OsHAK19*, AK106353; *OsHAK25*, AK111629; *OsHAK27*, AK068853. The numbers of *CjKUP1*, *AtKUP1*, and *OsHAK26* are shown in the caption of Fig. 1

Fig. 3 Complementation of *Escherichia coli* LB2003 strain, which lacks K^+ uptake systems, by CjKUP1. **a** Growth of LB2003 cells transformed with the pPAB404 vector carrying cDNA of *CjKUP1* or an *A. thaliana* K^+ channel (*KAT1*; positive control) and those transformed with

the empty vector (negative control) on solid medium supplemented with 5 or 30 mM KCl. **b** Effect of Ca^{2+} and Cs^{+} on the growth of LB2003 cells expressing CjKUP1 or the empty vector in liquid media containing approximately 2 mM K^{+} and various concentrations of Ca^{2+} (CaCl) or Cs^{+} (CsCl). Optical density at 600 nm (OD_{600}) was measured after culture for 9 h from a starting OD_{600} of 0.05. The growth in medium lacking CaCl and CsCl was used as a control (C in the graph). The error bars represent the standard deviations of the mean (n = 6). Asterisks indicate a significant difference when compared with the control (Steel test, P < 0.05)

Fig. 4 Expression pattern of *CjKUP1* during male strobilus development in *Cryptomeria japonica* trees as determined by real-time reverse transcription (RT)-polymerase chain reaction (PCR). The expression levels of *CjKUP1* were normalized to those of actin, an endogenous control. The error bars represent the standard deviation of the mean (n = 12). Different letters indicate a significant difference in expression level among sampling dates (Steel–Dwass test, P < 0.01)

Fig. 5 Relative expression level of *CjKUP1* in various organs of *C. japonica* trees as determined by real-time RT-PCR. N, needle; B, inner bark; X, differentiating xylem; M_O, male strobili on October 2nd, 2010; M_F, male strobili on February 22nd, 2011; F, female strobili; R, root. The expression levels were normalized to those of actin. The error bars represent the standard deviation of the mean (n = 12). Different letters indicate a significant difference in expression level among organs (Steel–Dwass test, P < 0.01)

a

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CjKUP1   -----MDHS---AEEGGVLETIRVQ----EDGQCSSNQVPKSSSDE------KSIADAYLIQPTKAKIFGTCETILLAYQTLGVVYGDLG 72
OsHAK26  MEYHHRPHSPPPSDDDVVVIQMNAAAIAAVDEWSSTNEVDDAAAGKGGGLTRRTFSQAYKMKHRTPLEFTWRQVALLSFQSLGVVYGDLG 90
AtKUP1   -----MNQSPSLIEQG-----------------------------------------------ISQQHLKTLSCANVLTLAYQSLGVIYGDLS 41

CjKUP1   TSPLYTYSS-----IAIENPEEKDLLGILSMIFWTLTMIALVKYVFIVIRADDHGEGGTFALYSLLCRYVNVGQMAGSQFKRLESDSRLR 157
OsHAK26  TSPLYVFSS-----ISLDDPGEADFVGILSIILWTFTMICLVKYVFIVLKADDHGEGGTFALYSLLRQHVNFKGNMPVPVTHLASDINLK 175
AtKUP1   TSPLYVYKTTFSGKLSLHEDDE-EIFGVFSFIFWTFTLIALFKYVFIVLSADDNGEGGTFALYSLLCRYAKLSILP----NHQEMDEKLS 126

CjKUP1   YFSENHG--KNLKSKTKQLLENSPILQRVLLIFVVIGTCMVLGDGALTPAISVLSAVQGIQSKGGNLNQDAVVGISAVILLLLFLVQRFG 245
OsHAK26  FHSKKR----ILTSKLLKFLEQSTKWQAVITYIVLAGTCMVLGDGALTPAISVLSAVQGIQSRSSSITQAHVVLLSVIILFILFFFQKHG 261
AtKUP1   TYATGSPGETRQSAAVKSFFEKHPKSQKCLLLFVLLGTCMAIGDSVLTPTISVLSAVSGVKLKIPNLHENYVVIIACIILVAIFSVQRYG 216

CjKUP1   TGKVSFMFSPVMIIWFITNALIGIYNIIKHYPAAFKGLNPYYIVYFFQKQQKGGWVLLGGVVLCITGAEAMFADLGDFNKRSIQIAFTAL 335
OsHAK26  TSKVSFTFSPIMILWFTFVAFIGLYNIIKHYPPILKAVSPHYIIIYFIRNKRAAWETLGAIVLCITGAEAMFADLGHFNKSSIQMAFSVI 351
AtKUP1   THRVAFIFAPISTAWLLSISSIGVYNTIKWNPRIVSALSPVYMYKFLRSTGVEGWVSLGGVVLSITGVETMFADLGHFSSLSIKVAFSFF 306

CjKUP1   VYPALIITYTGQAAYLIKNPADMKNAFYASIPNEVYWPMFIIATLSAIVASQALISASFSIIKQSVALGCFPRVKMVHTSKKNEGRIYSP 425
OsHAK26  VYPSMILAYAGQAAFLVKNPSKLSTTFYSSTPEPLFWPMFIIATLAAIVASQALISASFSIIRQSIALGCFPRVTMKHTSGKHEGQVYSP 441
AtKUP1   VYPCLILAYMGEAAFLSKHHEDIQQSFYKAIPEPVFWPVFIVATFAAVVGSQAVISATFSIISQCCALDCFPRVKIIHTSSKIHGQIYIP 396

CjKUP1   EVNYVLMVICLAIVVGFKGGPEIGNAYGVAVIGVMFITTCLVSLVMLLVWNMHFLFILPFLIIFGFIEGVYLSAVLNKVPQGGWVPFAVS 515
OsHAK26  EINYFLMVACILITVGFKGGPEIGQAFGVAVIFVMLFTTNLMTVVMLIIWESNIALASLFFVFFFSIEGIYMTSLMNKILQGGWVPFAIT 531
AtKUP1   EVNWMLMCLCLAVTIGLRDTNMMGHAYGLAVTSVMLVTTCLMTLVMTIVWKQRIITVLAFVVFFGSIELLYFSSCVYKVPEGGWIPILLS 486

CjKUP1   IFFLIIMYSWNYGRQIKYEHLTQRKLSTEDLHMLIAS-VRSRTPGVCFLCSDLIYGVPPILRHYVKNVGSLHEILIILTIRIVPVTTVLL 604
OsHAK26  AFFLIITLSWTYGRSKKGEYELANVMEREEFIKTVT--TRSRVPGVCIFCTDMMNGIPPIVRHYVQHVASLRELMVFVTIRVLPVRTVLP 619
AtKUP1   LTFMAVMYIWNYGTTKKHEFDVENKVSMDRIVSLGPSIGMVRVPGIGLVYSNLVTGVPAVFGHFVTNLPAFHKILVFVCVKSVQVPYVGE 576

CjKUP1   EERFLVGKLGPK--GVYRCLAQYGYNDVTPSVEGEDFLTQVTKSIKYLIINEELKDSINDHPRNPMARDKNLEAEFEELQQLELANRVEA 692
OsHAK26  EERFIIDKLEPV--GVYRCIVQYGYMDN-HNMEGDDYVASVIASLKEIAENDD------------------------EILVLDSALINGS 682
AtKUP1   EERFVISRVGPKEYGMFRSVVRYGYRDV--PREMYDFESRLVSAIVEFVETEPGLEE---------EEMSSVRRKKEECMEIMEAKEAGV 655

CjKUP1   VYVLGKTILRTGIMRGFFS-VIIMDIYRFLQNNCRSSISTMKVPPDQLLQVGMVYEI 748
OsHAK26  TFVLGRTIIKMGTRHNCLKRFFINNLYRFLQKNFRSNMSSLKINPGKTLQVGMLYEI 739
AtKUP1   AYILGHSYAKAKQSSSVLKKLAVNVVFAFMSTNCRGTDVVLNVPHTSLLEVGMVYYV 712
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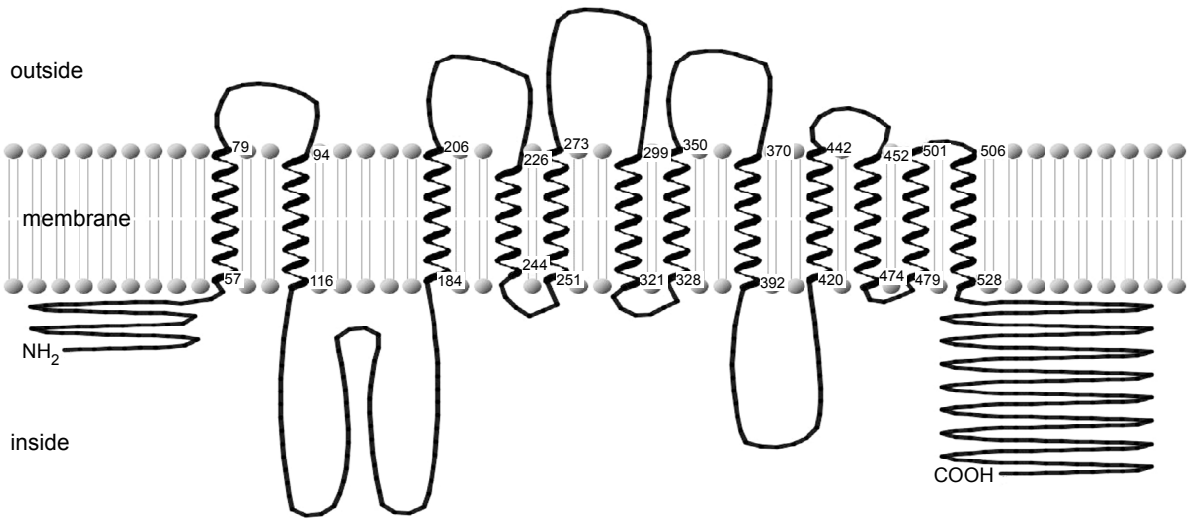
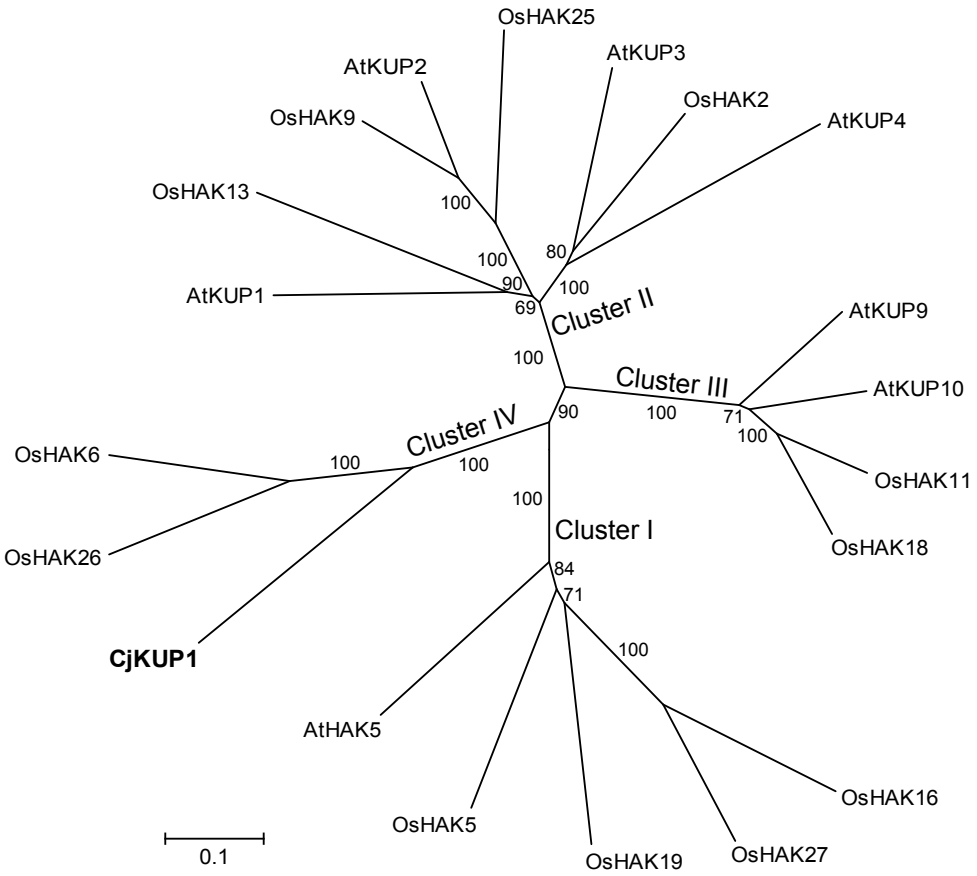
b



Hosoo et al. Fig. 2



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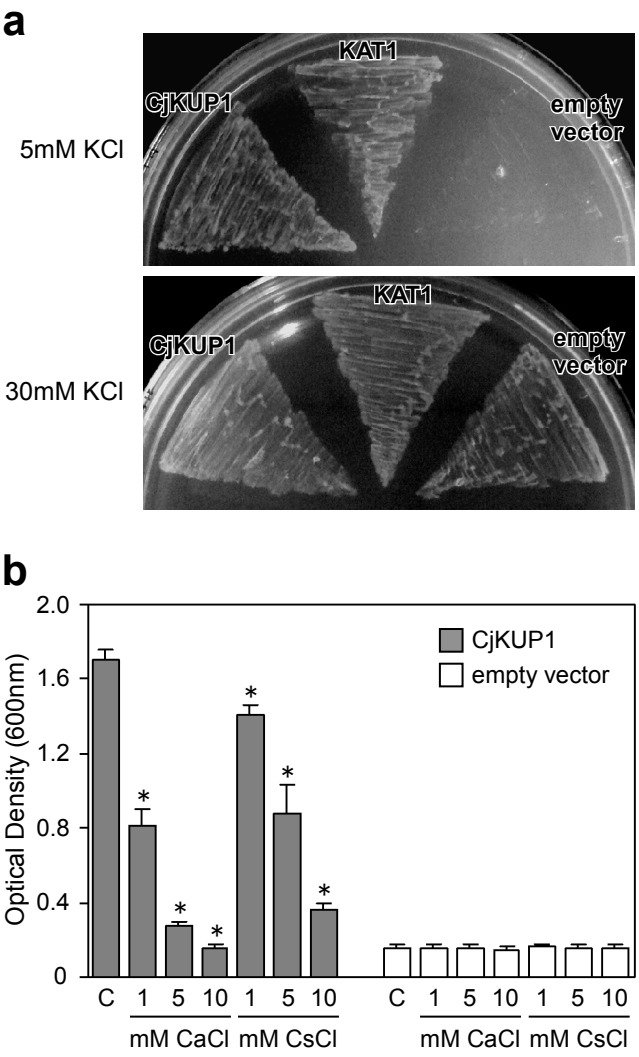
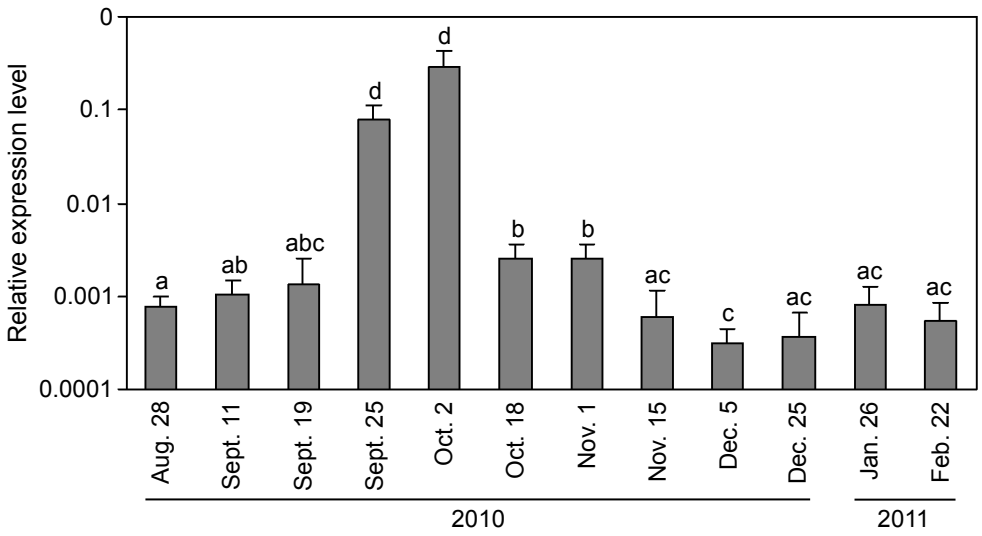


Hosoo et al. Fig. 4



Hosoo et al. Fig. 5