***Lotus japonicus* LjKUP Is Induced Late During Nodule Development and Encodes a Potassium Transporter of the Plasma Membrane**

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The KUP family of potassium transporters in plants is large but poorly characterized. We isolated and characterized the first KUP transporter from a legume, LjKUP of *Lotus japonicus*. Although expressed throughout plants, LjKUP transcript levels were highest in nodules. Induction of LjKUP expression occurred late during nodule development, at a time of rapid organ expansion. A high level of LjKUP expression was maintained in mature, full-sized nodules. However, induction of LjKUP expression was independent of symbiotic nitrogen fixation (SNF), and occurred in ineffective nodules resulting from mutations in either the plant or its microsymbiont, *Mesorhizobium loti*. Heterologous expression of LjKUP in *Escherichia coli* showed that the protein is able to transport potassium. Transient expression of a GFP-LjKUP fusion protein in *Arabidopsis* cells indicated a plasma membrane location for the transporter. Taken together, the results indicate that LjKUP is a potassium transporter of the plasma membrane, which may play roles in cell expansion during nodule development and in ion homeostasis during SNF.

Additional keywords: ion transporter, real-time RT-PCR.

Symbiotic nitrogen fixation (SNF) in legumes takes place in specialized organs called nodules that develop from cortical cells of the root or stem following contact with soil rhizobia. The biological success of SNF rests upon the mutually beneficial exchange of reduced carbon from the plant for reduced nitrogen from the bacteria (Udvardi and Day 1997). Following a series of preliminary signal exchanges (Long 2001; Schultze and Kondorosi 1998; Stougaard 2000), rhizobia enter root-hair and underlying cells via an infection thread produced by redirection of cell wall synthesis (Brewin 1993; Rae et al. 1992). Rhizobia eventually are released from infection threads into cortical cells via endocytosis, which leaves the bacteria surrounded by a membrane of plant origin, called the symbiosome membrane (SM) (Verma and Hong 1996). Growth and division of rhizobia, matched by synthesis of new SM, continues until thousands of symbiosomes, each containing one or more bacteria, fill the infected cells. Rhizobia within symbiosomes eventually differentiate into nitrogen-fixing bacteroids as the physiological conditions of the developing nodule change. Nodule development also is

accompanied by a reprogramming of plant metabolism, which becomes specialized for the provision of reduced carbon and other nutrients to the bacteroids, and for the assimilation of ammonium and other nitrogen compounds from the bacteroids (Day et al. 2001; Lodwig et al. 2003; Udvardi and Day 1997).

Transporters play integral roles in nodule development and SNF (Udvardi and Day 1997). During nodule development, transporters deliver and distribute ions and metabolites for cell growth and division. This includes transporters in the plasma membrane (PM) that lines the infection thread and in the SM, which together provide nutrients to the growing population of bacteria. Within a mature, nitrogen-fixing nodule, transporters in the SM play a central role in providing energy in the form of malate to the bacteroids, and in removing the products of nitrogen fixation, including ammonium, from the symbiosomes (Tyerman et al. 1995; Udvardi et al. 1988). Transporters of the SM that have been characterized at the biochemical or biophysical level include a P-type H⁺-ATPase (Campos et al. 1996; Fedorova et al. 1999); a dicarboxylate transporter (Udvardi et al. 1988); NH₄⁺, K⁺, and Ca²⁺ channels (Roberts and Tyerman 2002; Tyerman et al. 1995); an iron transporter (LeVier et al. 1996; Moreau et al. 2002); a Ca²⁺-ATPase (Andreev et al. 1999); and one or more inorganic ion transporters (Udvardi et al. 1991). A number of genes encoding SM proteins also have been cloned: nodulin 26, an aquaporin (Rivers et al. 1997; Weaver et al. 1994); GmZIP1, a zinc transporter (Moreau et al. 2002); GmDMT1, an iron transporter (Kaiser et al. 2003); and GmSAT1 from soybean (Kaiser et al. 1998). GmSAT1 previously was reported as an ammonium transport protein (Kaiser et al. 1998); however, subsequent data indicate that this probably is not the case (Marini et al. 2000).

Relatively little is known about transporters in other plant membranes of developing or mature nodules. Two ammonium transporters of the AMT family, LjAMT1 and LjAMT2, have been cloned from *Lotus japonicus* and shown to be expressed in nodules (Salvemini et al. 2001; Simon-Rosin et al. 2003). The putative PM location of LjAMT2 indicates that this transporter may be involved in recovering ammonium lost from nodule cells by diffusion (Simon-Rosin et al. 2003). The lack of knowledge about transporters in nodules limits significantly our understanding not only of the long-distance movement of ions and metabolites into and out of nodules, but also of the intracellular transport of these and, by extension, metabolism as a whole.

Expressed-sequence-tag (EST) projects on several legume species, including soybean, *Medicago truncatula*, and *Lotus japonicus*, have uncovered many transporter genes that are ex-

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The complete LjKUP cDNA sequence data is available in the GenBank database under accession number AY359855.

pressed in nodules. In silico and experimental approaches have identified a subset of these genes that are more highly expressed in nodules than in other organs, which may play key roles in SNF (Colebatch et al. 2002; Fedorova et al. 2002; Journet et al. 2002). One of these genes, *LjKUP* from *Lotus* spp., which encodes a homolog of KUP/KT/HAK K⁺ transporters, is the focus of this article.

Potassium transporters of the KUP family are encoded by large multigene families in plants (Banuelos et al. 2002; Maser et al. 2001; Rodriguez-Navarro 2000). Although genetics studies have implicated two KUP proteins, AtKUP4/TRH1 and AtKUP2/SHY3, in elongation of root hairs and hypocotyls, respectively (Elumalai et al. 2002; Rigas et al. 2001), the physiological roles of KUP proteins remain largely unknown. The KUP family has not been studied at all in legumes. To gain insight into the possible roles of the nodule-induced KUP in *L. japonicus*, we cloned a full-length *LjKUP* cDNA and demonstrated that it encodes a functional potassium transporter by heterologous expression in *Escherichia coli*. We also measured relative transcript levels of *LjKUP* in different organs and within nodules during a development time-series. Together with data on the intracellular location of *LjKUP*, also presented here, the results indicate that KUP proteins play a wider range of roles in plants than previously suspected.

RESULTS

Isolation and sequence analysis of *LjKUP*.

Transcriptome analysis of *Lotus japonicus* uncovered several genes encoding putative transporters that were more highly expressed in nodules than in uninfected roots (Colebatch et al., 2002). One of these, LjNEST8d4, exhibited homology to the KUP family of K⁺ transporters that are present in bacteria, fungi, and plants. Subsequent sequencing of the entire LjNEST8d4 clone revealed a single, large open reading frame (ORF) with a stop near the 3'-end followed by a polyA tail. No in-frame stop codon was found at the 5'-end of the ORF, which indicated that the clone was probably not a full-length cDNA. This was confirmed by 5'-RACE, which yielded a further 163 nucleotides of up-stream sequence including three in-frame stop codons. To facilitate later functional analysis, a full-length cDNA, named *LjKUP* was physically reconstructed from the 5'-RACE and EST clones (discussed below).

The full-length *LjKUP* cDNA (GenBank accession number AY359855) is 2,451 bp long. It encodes a protein of 718 amino acids (aa) with a predicted molecular mass of 80.4 kDa and a PI of 8.77. The encoded protein is highly hydrophobic and is predicted to contain 12 transmembrane domains (Fig. 1). *LjKUP* has a long hydrophilic C-terminus (208 aa), as well as a large hydrophilic internal loop between predicted transmembrane domains 2 and 3 (63 aa). These features are common to members of the KUP family from *E. coli* (Schleyer and Bakker 1993), *Arabidopsis* spp., rice, and barley (Banuelos et al. 2002; Maser et al. 2001; Rodriguez-Navarro 2000; Rubio et al. 2000).

The amino acid sequence of *LjKUP* was compared with all *Arabidopsis* spp., rice, *E. coli*, and *Mesorhizobium loti* KUP homologs. The protein sequences aligned well between amino acids 110 and 604 of *LjKUP*, but poorly at the N- and C-terminal ends. Therefore, we utilized the central region of the multiple sequence alignment for phylogenetic analysis, which revealed five major subfamilies of KUP-related sequences in higher plants (Fig. 2). Rice contains members of all five subfamilies, whereas *Arabidopsis* spp. have members in only three (subfamilies III to V). Interestingly *LjKUP* belongs to a subfamily (I) that contains no *Arabidopsis* homolog, which appears to have diverged from the prokaryotic lineage before all other plant subfamilies.

LjKUP gene expression analysis.

Previous work showed that, although *LjKUP* is expressed in both uninfected roots and nodules of *Lotus* spp., it is much more highly expressed in the latter (Colebatch et al. 2002). To obtain a more complete picture of the pattern and relative abundance of *LjKUP* transcripts within *Lotus* plants, real-time reverse-transcription polymerase chain reaction (RT-PCR) analysis of total RNA isolated from leaves, infected and uninfected roots, and nodules was performed. Using a constitutive *Lotus* ubiquitin gene to normalize transcript levels, it was found that *LjKUP* transcript levels were approximately 40-fold higher in nodules than in roots or leaves of 7-week-old plants (Table 1). The amount of *LjKUP* transcript in entire, nodulated roots was approximately sixfold higher than in uninoculated roots of the same age, which reflected the dilution of nodule RNA by that of roots. To learn more about the regulation of *LjKUP* expression during nodulation, three mutants of *Lotus* spp. affected in nodule development were

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MNNNAHRSPNDVVVNLNVDSATPNNVTGNNKKMIKEKRDTLILAYRTLGVVFGGLVTSP - 60
LVVYPSMPLHSPTEEDYLGIYSIIFWTLTLIGVVKYANIAIKADHDHGEGGTFVAVYSLLCR - 120
HFNIGILPSKQQQQQQLGLNSMTRGTETHTWLAKLFERSVVARVLFLFVAMLGTCMLIG - 180
DGILTPAISVLSAMDGVRAPFPKVSLSVEALSAVVLIFLFLQKYGTSRVSFLFSPIMG - 240
AWTLSTPLVGIYSIIHHYPSIFKALSPHYIFRFFLRNGKSGWLLLGGIVLCITGSEAMFA - 300
DLGHFNPRSIQIAFLFTIYPSLVLTYAGQTAYLKHPNDHDDGFYKFIPKPVYWPIFTIA - 360
TLAAVASQSLISATFSVIKQSVVLDYFPRVKIIHTSHNKEGEVYSPEVNYILMVLCVAV - 420
ILIFGDGKDIGNAFGVVSVLMLLITILLTMLMIMIWRTPAILVSLYFVVFVMEGVVVS - 480
AVFTKIAEGGWIPFAISFILAFIMFGWFYGRQRKIDYEVTHKVTFERLQELLSDCSVQRV - 540
PGLCFYFTNIQDGLTPILGHYIKNMKSLHKVTIFTTLRYLLVPKVAPHERIVIKKTNLKG - 600
VYCCVIQYGYADTPTIARDDFVDQVINSLTHIQNCSDNGSFDSHIEEQVSSLEEARCS - 660
GVVHVGRKTRFYVGLNCGWFDKIMLGFYEIMHSNCRSGLPALGVSLQHRIEVGMLYEA - 718

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Fig. 1. Predicted amino acid sequence of *LjKUP*. Putative transmembrane domains are underlined. The first methionine of the shorter cDNA, LjNEST8d4, is indicated in bold and underlined. The complete *LjKUP* cDNA sequence is available from GenBank with accession number AY359855.

analyzed. The *Ljsym6-1* mutant of *Lotus* spp. is unable to develop normal nodules following inoculation but, instead, produces small bumps on the roots, which are devoid of infection threads (Schauser et al. 1998). The *Ljsym11* mutant develops pink nodules that abort and turn white relatively quickly (Schauser et al. 1998). The pink color indicates that leghemoglobin synthesis occurs, at least transiently, in these nodules. Nodule development of *Ljsym11* is blocked at a later stage than in *Ljsym6-1* (Schauser et al. 1998). Finally, the *har1-1* mutant is defective in feedback regulation of nodulation and, as a result, exhibits a hypernodulation phenotype (Szczygłowski et al. 1998; Wopereis et al. 2000). The *Har1* gene encodes a receptor-like kinase homologous to *Clavata 1* of *Arabidopsis* spp. (Krusell et al. 2002). Unlike *Ljsym6-1* and *Ljsym11*, which are unable to form effective nodules, *har1-1* nodules are able to fix nitrogen. Transcript levels of *LjKUP* did not increase significantly in *Ljsym6-1* roots after inoculation with rhizobia. In contrast, *LjKUP* expression increased in nodulated roots of both *har1-1* and *Ljsym11* (Table 1). The level of *LjKUP* transcript in nodulated roots of *har1-1* was greater than that of the wild type 7 weeks after inoculation, which probably reflected the greater number of nodules on the mutant. In contrast, *LjKUP* transcript levels in nodulated *Ljsym11* roots were not significantly different from those of the wild type.

To test whether nitrogen fixation by bacteroids influences *LjKUP* expression in nodules, an *M. loti* mutant defective in the regulatory gene, *nifA2* was utilized. The mutant elicits nodules

on *Lotus* plants but is unable to fix nitrogen (Clive Ronson, *personal communication*). *LjKUP* transcript levels in nodulated roots harboring the *nifA2*⁻ strain were similar to those harboring the wild-type rhizobia (Table 1). Thus, maximal expression of *LjKUP* during nodulation was independent of SNF.

A time-course study of *LjKUP* transcript levels in inoculated roots was performed to reveal the kinetics of gene induction during nodulation. *LjKUP* transcript levels remained low and constant for the first 7 days following inoculation with rhizobia (Fig. 3), although nodules were visible as small white bumps on the roots by day 7. By day 14, however, *LjKUP* transcript levels had increased approximately fourfold and, by day 21, transcript levels in nodulated roots were 22-fold higher than in the first week. Levels of *LjKUP* transcript did not change in uninoculated roots of control plants during this time (Fig. 3).

LjKUP functionally complements an *E. coli* potassium transport mutant.

Potassium transport activity has been demonstrated for several plant KUP homologs expressed either in *E. coli* or yeast (Banuelos et al. 2002; Elumalai et al. 2002; Fu and Luan 1998; Kim et al. 1998; Quintero and Blatt 1997; Rigas et al. 2001; Rubio et al. 2000; Santa-Maria et al. 1997; Senn et al. 2001). To test whether LjKUP transports K⁺, we expressed both full-length *LjKUP* cDNA and the partial cDNA, LjNEST8d4, in *E. coli* TK2463, which has mutations in three K⁺ transport systems, TRK, KUP, and KDP (Epstein et al.

Fig. 2. Phylogenetic tree of plant KUP sequences. The maximum likelihood tree was inferred from comparisons of the LjKUP amino acid sequence (amino acids 110 to 604) and the corresponding region of homologs from *Arabidopsis* spp. (Maser et al. 2001), rice (Banuelos et al. 2002), *Mesorhizobium loti* (MIKUP1, accession number NP_103011; MIKUP2, accession number NP_103010), and *Escherichia coli* (EcKUP, accession number NP_418203). The sequences from *E. coli* and *M. loti* were used as out-groups. Support values (percent) for the branches were derived from maximum likelihood, neighbor-joining bootstrap (1,000 replicates each), and parsimony bootstrap analysis (500 replicates), respectively. The scale bar indicates 10 changes per 100 amino acids.

1993). Although LjNEST8d4 encodes a slightly truncated LjKUP protein, the protein retains all 12 putative transmembrane domains (Fig. 1). Transcription of cDNA was driven by a lactose-inducible promoter in vector pSPORT1. Expression of both LjKUP and LjNEST8d4 in strain TK2463 increased the growth rate of the mutant in low-potassium liquid LK medium (Kim et al. 1998) supplemented with 0, 10, or 50 mM K⁺ (Fig. 4).

LjKUP is located in the PM when expressed in *Arabidopsis* cells.

Various computer programs were used to predict the intracellular location of LjKUP. Both PSORT and Protcomp predicted a PM location for LjKUP. To test this hypothesis, we determined the location of a green fluorescent protein (GFP)-LjKUP fusion protein expressed in *Arabidopsis* cells. Transient expression of the GFP-LjKUP fusion, driven by the CaMV-35S promoter, was achieved in both *Arabidopsis* leaf epidermal cells and in protoplasts of cell cultures. In both cases, GFP was detected exclusively at the periphery of cells (Fig. 5A and B). Transient expression of KCO1-GFP, a tonoplast marker (Czempinski et al. 2002), resulted in a different pattern of fluorescence: In addition to a peripheral

location, GFP typically was observed in internal membranes, which presumably lined transvacuolar strands of cytoplasm (Fig. 5C and D). KCO1-GFP also was removed from the cell periphery in places where the nucleus forced the tonoplast aside (data not shown), as described previously (Czempinski et al. 2002). The GFP-LjKUP fusion protein never was observed away from the cell periphery in turgid cells. However, in plasmolyzed cells derived from transformed protoplasts, GFP-LjKUP fluorescence surrounded the condensed or shrunken cytoplasm (Fig. 5E and F). Taken together, these data indicate a PM location of LjKUP.

DISCUSSION

LjKUP is the first legume KUP family member to be characterized. We have shown that, like other KUP proteins, LjKUP transports potassium. However, unlike any other KUP that has been localized in plant cells to date, LjKUP appears to be a PM protein. *LjKUP* transcripts were found to accumulate substantially late during nodule development, implying that the protein is important for SNF. Taken together, the results indicate that KUP proteins play a more diverse range of roles in plants than suspected previously.

Fig. 3. Kinetics of *LjKUP* induction during nodule development. Total RNA was extracted from entire root systems 0 to 21 days after inoculation with *Mesorhizobium loti*, as indicated. *LjKUP* transcript levels were normalized against those of the constitutively-expressed gene, *LjUBI* (Colebatch et al. 2002) at each time point, then expressed relative to the level of *LjKUP* transcript at time zero, as described in Table 1. Data represent mean $\pm$ standard deviation ($n = 3$).

Fig. 4. Functional complementation of *Escherichia coli* potassium transport mutant, strain TK2463. The mutant strain was transformed with pSPORT containing *LjKUP* (gray bar), *LjNEST8d4* (black bar), or the cloning vector alone (white bar). Cells were grown at 37°C in LK medium containing different concentrations of added K⁺ (indicated). Error bars represent standard deviation from mean doubling times obtained from three independent experiments.

Table 1. *LjKUP* transcript levels in different organs and genotypes^a

Lotus genotype	Organ or <i>M. loti</i> strain				
	Leaves	Nodules	Roots (with or without nodules)		
	R7A	R7A	R7A	Uninoculated	NifA [−]
Wild type	10.0 $\pm$ 1.5 (1.2)	4.8 $\pm$ 1.8 (41)	7.6 $\pm$ 2.1 (6.1)	10.2 $\pm$ 1.9 (1)	7.3 $\pm$ 3.7 (7.6)
<i>har1-1</i>	...	...	7.0 $\pm$ 2.5 (33)	12.0 $\pm$ 3.4 (1)	...
Ljsym6	...	...	11.2 $\pm$ 2.8 (0.7)	10.6 $\pm$ 1.2 (1)	...
Ljsym11	...	...	7.5 $\pm$ 0.4 (4.4)	9.7 $\pm$ 0.3 1	...

^a Plants were either uninoculated or inoculated with wild-type *Mesorhizobium loti* (R7A) or with an *nifA*[−] strain of *M. loti* (NifA[−]) and grown in sand for 7 weeks prior to harvest. C_T values for *LjKUP* obtained from real-time reverse-transcription polymerase chain reaction were normalized against those of ubiquitin (yielding $\Delta C_T \pm$ standard deviation, with at least two biological and two technical replicates in each case). The relative expression level of *LjKUP* (value in parentheses) was calculated using the formula $X_{fold} = 2^{-(\Delta C_T A - \Delta C_T B)}$, with uninoculated roots ($\Delta C_T B$) used as the reference condition in each genotype.

Developmental regulation of *LjKUP*.

Most of the *Arabidopsis KUP* genes that have been characterized, including the five genes *AtKUP1-5*, appear to be expressed more or less constitutively throughout plants, which suggests that they are involved in housekeeping functions (Elumalai et al. 2002; Fu and Luan 1998; Kim et al. 1998; Quintero and Blatt 1997; Rigas et al. 2001; Rubio et al. 2000). Massively parallel signature sequencing (MPSS) data for different *Arabidopsis* organs supports the published expression data for *AtKUP1* to *AtKUP5*, inclusive, and indicates that *AtKUP7* also is constitu-

tively expressed in all organs. Although no expression data are available for *AtKUP9* to *AtKUP11*, the MPSS database indicates that *AtKUP6* may be root specific and that *AtKUP12* is expressed in aboveground organs but not roots. Thus, the proteins encoded by these two genes may fulfill more specific roles than other KUPs. The expression pattern of *LjKUP* in *Lotus* spp. indicates that it, too, may play a more specialized biological role than other plant KUPs.

Although *LjKUP* expression was detected in all vegetative organs of *Lotus* plants by real-time RT-PCR, transcript levels

Fig. 5. Green fluorescent protein (GFP)-LjKUP is plasma membrane-located in *Arabidopsis* spp. GFP-LjKUP was expressed transiently in **A** and **B**, *Arabidopsis* leaf epidermal cells or in **E** and **F**, *Arabidopsis* cell-culture protoplast. As a control, the tonoplast marker GFP-KCO1 also was expressed transiently in **C** and **D**, *Arabidopsis* leaf epidermal cells. GFP was detected by confocal microscopy 24 to 48 h after transformation. Images of green fluorescence obtained under UV light (left panels) were overlaid onto those taken under white light (right panels) to reveal the intracellular location of GFP.

were 40-fold higher in nodules than elsewhere (Table 1). Induction of *LjKUP* expression during nodulation (Fig. 3) appeared to be under developmental control: The plant mutant *Ljsym6-1*, which is blocked at an early stage of nodule development, exhibited no increase in *LjKUP* transcript following inoculation with rhizobia (Table 1). On the other hand, high levels of *LjKUP* transcript in nodulated roots of the plant mutant *Ljsym11* inoculated with wild-type rhizobia, and of wild-type plants inoculated with an *M. loti nifA2⁻* mutant (Table 1), neither of which can fix nitrogen, indicated that *LjKUP* induction was not dependent on SNF.

The kinetics of *LjKUP* induction during nodulation (Fig. 3) revealed that the gene is activated late during nodule development, between 7 and 14 days after inoculation with rhizobia, at a time when nodule expansion is maximal. These results provide a clue to one possible physiological role of *LjKUP*, as discussed below. Sustained, high-level expression of *LjKUP* in mature, nitrogen-fixing nodules (Fig. 3; Table 1) indicates that *LjKUP* also plays an important role during SNF.

LjKUP is a potassium transporter.

Complementation of the *E. coli* potassium transport mutant, TK2463, by *LjKUP* showed that the protein from *Lotus* spp. is able to transport K^+ . Expression of *LjKUP* in the mutant reduced its doubling time significantly even with only trace amounts of K^+ in the medium (i.e., no added K^+) (Fig. 4), which indicated that the transporter has a high affinity for K^+ . Previous work on other plant KUP homologs revealed a range of affinities for K^+ among this family, including high affinity (K_m in the micromolar range; AtKUP1 [Kim et al. 1998], HvHAK1 [Santa-Maria et al. 1997], OsHAK1 [Banuelos et al. 1995], AtHAK5 [Rubio et al. 2000]), low affinity (HvHAK2 [Senn et al. 2001]), and both high and low affinities (AtKT1 [Fu and Luan 1998]).

Subcellular location of LjKUP.

As mentioned above, relatively little is known about the intracellular location of KUP proteins in plants. The rice KUP homolog OsHAK10 was targeted to the tonoplast membrane when expressed as a C-terminal GFP fusion in onion epidermal cells (Banuelos et al. 2002), suggesting that it resides in the same membrane in rice. Despite speculation that other plant KUPs may be located in the PM (Desbrosses et al. 2003; Elumalai et al. 2002; Rodriguez-Navarro 2000; Senn et al. 2001), direct evidence for a PM location has not been published previously.

Thus, the data presented here provide the first experimental evidence that some plant KUPs may reside in the PM. Although fusion of the relatively large GFP molecule to the N-terminal end of *LjKUP* could have interfered with correct targeting of the protein, it is salient to note that in silico analysis also predicted a PM location for *LjKUP*.

What is the role or roles of LjKUP in nodules?

Potassium is the most abundant cation in higher plants and is crucial for ionic homeostasis, enzyme activity, osmoregulation, and growth (Epstein et al. 1963; Epstein 1966; Kochian and Lucas 1993). Typically, K^+ concentrations in plant cells (>100 mM) are several orders of magnitude higher than those in the external medium. Transporters and channels in the PM provide the means by which cells accumulate this ion (Brownlee 2002; Zimmermann and Sentenac 1999). We have presented the first direct evidence that plant KUPs may be located in the PM (Fig. 5). Although this result should be confirmed (by immunolocalization in *Lotus* spp., for instance), it indicates that KUP transporters probably contribute to accumulation of K^+ across the PM. Consistent with this notion is

the observation that *LjKUP* and related proteins in other species are high-affinity K^+ transporters, which would enable them to access micromolar concentrations of the ion in the external medium (Fig. 3) (Banuelos et al. 1995; Kim et al. 1998; Rubio et al. 2000; Santa-Maria et al. 1997). Little is known about the mechanism of K^+ transport by plant KUPs. Consideration of data from fungi and yeast led Rodriguez-Navarro (2000) to the conclusion that plant KUPs likely function as K^+ -H⁺ symporters, which would enable them to accumulate K^+ to levels that exceed those predicted by the Nernst equation. Thus, KUPs may play an active role in ionic and osmotic homeostasis in cells.

During nodule development, root cortical and pericycle cells undergo substantial expansion (and division), which requires large amounts of K^+ , not only to maintain ion homeostasis but also to provide turgor pressure for cell growth. Massive induction of *LjKUP* gene expression during nodulation and the location of the protein in the PM indicate that *LjKUP* may play an important role in cell expansion during the latter stages of nodule development. Consistent with this idea are observations that KUP homologs in plants are essential for cell and tissue expansion. For instance, the *Arabidopsis* trh1-1 mutant, which is defective in root-hair elongation, is impaired in AtKUP4 (Rigas et al. 2001), and the shy3-1 mutant, which is defective in stem elongation, is affected in AtKUP2 (Elumalai et al. 2002). A role for KUP proteins in cell elongation also was proposed from work in cotton. Induction of a KUP gene in cotton fiber cells was associated with increased fiber osmotic and turgor potentials, which drove a rapid phase of cell elongation (Ruan et al. 2001).

Continued, high-level expression of *LjKUP* in mature nodules of *Lotus* spp. (Table 1), which, because of their determinate nature, cease to grow significantly after maturity, indicates that the protein fulfills additional or alternative functions during SNF. Obviously, K^+ transporters and channels in the PM are required throughout the life of a cell, for the reasons mentioned above. For instance, the presence of *LjKUP* in mature nodule cells may be required to maintain the ionic milieu essential for the biochemical and biophysical processes that underpin symbiotic nitrogen fixation. The amenability of *L. japonicus* to reverse genetics will enable us to test the importance of *LjKUP* in nodule development and SNF in the future.

MATERIALS AND METHODS

Biological material.

L. corniculatus cv. *japonicus* ecotype GIFU seed were scarified in liquid nitrogen (3 × 10 s), sterilized in a 2% bleach solution for 10 min, rinsed five times with sterile distilled water, and germinated in a petri dish on filter paper soaked with B&D medium (Broughton and Dilworth 1971). After germination in a phytotron (25°C, 16 h of light, 8 h of dark), 3-day-old seedlings were transferred to pots (five plants per pot) containing coarse quartz sand and inoculated with *M. loti* R7A. Inoculated plants were grown in a glasshouse with a 16-h day, 60% relative humidity, and day and night temperatures of 21 and 17°C, respectively, and watered once per day with B&D medium. Alternatively, 4-day-old seedlings were transferred to square petri dishes containing fresh B&D with 0.1 mM KNO₃ and grown in a phytotron (22°C, 16 h of light, 8 h of dark) for 3 weeks before being inoculated with *M. loti* NZP2235. Inoculated plants and uninoculated controls then were maintained under the same growth conditions until harvested.

DNA methods and constructs.

Clone LjNEST8d4 was completely sequenced, using a combination of subcloning and primer-walking, by the company

AGOWA (Berlin). To obtain the complete *LjKUP* cDNA sequence, 5' RACE was performed: Total RNA was extracted from 6- to 7-week-old nodules using an RNeasy kit (Qiagen, Hilden, Germany), and cDNA synthesis was performed with Superscript II (Invitrogen, Carlsbad, CA, U.S.A.) using the *LjKUP*-specific primer 5'-CTGGCTAGCAACAACAGCAG-3'. Amplification of the 5' end of *LjKUP* cDNA was performed with the 5' RACE PCR kit from Invitrogen following the manufacturer's instructions. The first round of PCR was performed with the abridged anchor primer from the kit and a second *LjKUP*-specific primer (GSP2: 5'-GATTCCTCCGTC-3'). Nested PCR then was performed with the universal primer and a third *LjKUP*-specific primer (GSP3: 5'-GTAAAGCGCGATGTACGAGACC-3'). The PCR program used consisted of 2 min at 95°C; 35 cycles of 95°C for 15 s, 58°C for 30 s, and 70°C for 1 min; and finally 70°C for 10 min. The resulting single PCR product was sequenced commercially by AGOWA.

To reconstruct physically the full-length *LjKUP* cDNA, the 5' end of the cDNA was PCR amplified and ligated at the internal *SpeI* site of LjNEST8d4, as follows. The 5' end of *LjKUP* was amplified from nodule cDNA using: proofreading Pfu polymerase (Stratagene, La Jolla, CA, U.S.A.); the forward primer, 5'-GGAATTCTGGGCTTCGTTTCTGTTCT-3', which added an *EcoRI* restriction site to the 5' end of the amplicon; and the reverse primer, 5'-GAGTCGACATTGAGGTTGACGA-3', which contained the unique *SpeI* site of *LjKUP*. The PCR product was separated on an agarose gel and purified using a QIAEX II Gel Extraction Kit (Qiagen). The PCR fragment and LjNEST8d4 clone in pSPORT1 were digested separately with *EcoRI*, purified, digested with *SpeI*, purified again, ligated together, and transformed into competent *E. coli* DH5 α cells. The presence of recombinant, full-length *LjKUP* cDNA in individual transformants was tested by PCR and finally confirmed by sequencing.

Construction of recombinant vector for expression of a GFP-LjKUP fusion protein in *Arabidopsis* cells was achieved using GATEWAY technology (Invitrogen). *LjKUP* cDNA was amplified using pfu polymerase, the forward primer 5'-GGAATTCTGGGCTTCGTTTCTGTTCT-3', the reverse primer 5'-CTGGCTAGCAACAACAGCAG-3', and the PCR program consisting of 94°C for 2 min, followed by 30 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 2 min. The forward and reverse primers contained the attB1 and attB2 recombination sites, respectively. The PCR product was recombined into the entry vector pSpectre (B. Trevaskis, unpublished data) using BP clonase enzyme (30 ng of insert, 150 ng of entry vector, 2 μ l of BP buffer, 1 μ l of BPclonase in 10 μ l of final volume; recombination overnight at 25°C followed by proteinase K treatment), and transformed into *E. coli* DH5 α . Entry clones containing *LjKUP* were checked by restriction analysis and by PCR, recombined with the destination vector pN-GFP (B. Trevaskis, unpublished data) (150 ng of entry clone, 150 ng of destination clone, 2 μ l of LR buffer, 4 μ l of Tris-EDTA, and 1 μ l of LRclonase in a final volume of 10 μ l; recombination for 1 h at 25°C followed by proteinase K treatment), and transformed into DH5 α . Positive recombinant clones were screened by restriction analysis, confirmed by PCR, and finally sequenced to verify the correct construction.

Phylogenetic analysis.

Phylogenetic methods were implemented using the computer program PAUP* (version 4.0b10; Sinauer Associates, Sunderland) for neighbor joining and maximum parsimony, and TreePuzzle (Schmidt et al. 2002) for maximum likelihood analysis. The BLOSUM62 model of amino acid substitutions was used for maximum likelihood inference.

Real-time RT-PCR.

Harvested plant material was frozen immediately in liquid nitrogen and stored at -70°C. Total RNA was extracted from frozen organs of *Lotus* plants using an RNeasy kit (Qiagen). Prior to cDNA synthesis, 1 to 2 μ g of total RNA was treated with RNase-free DNaseI (Sigma-Aldrich, St. Louis) to destroy genomic DNA. DNaseI-treated RNA subsequently was phenol-chloroform extracted, precipitated, and resuspended in 10 μ l of water. Reverse transcription then was performed with SuperScript II reverse transcriptase (Invitrogen). Primers to glutathione-S-transferase, which span an intron, were used to confirm absence of genomic DNA. The efficiency of cDNA synthesis was assessed by real-time PCR amplification of ubiquitin, and only those reactions that exhibited similar efficiencies (similar C_T values for ubiquitin) were analyzed further.

PCR reactions were performed in a 96-well plate with an ABI Prism 5700 Sequence Detection System (Applied Biosystems, Foster City, CA, U.S.A.), using SYBR Green to monitor double-stranded DNA synthesis. A standard PCR reaction contained approximately 4 ng of cDNA template, 12.5 μ l of Master Mix, 0.75 μ l of SYBR Green (Eurogentech, Seraing, Belgium), 7.5 pmol forward and reverse primers, and water to a final volume of 25 μ l. The following standard thermal profile was used for all PCR reactions: 50°C for 2 min; 95°C for 10 min; 40 cycles of 95°C for 15 s and 60°C for 1 min. Data were analyzed using SDS 2.0 software (Applied Biosystems). For quality control, PCR products were run on a 2% agarose gel to confirm the presence of a single amplicon. The following primers were used to amplify *LjKUP* cDNA: forward, 5'-CGTTGATTCTCGCCTACAGGAC-3'; reverse, 5'-TAAAGCGGCGATGTCACGA-3'. C_T values for *LjKUP* were normalized to levels for ubiquitin (Colebatch et al. 2002) before making comparisons between organs.

E. coli complementation studies.

E. coli TK2463 (*F*₋ *thi* *LacZ*_{amX82} *rha* Δ (*trkA*) *trkD1* Δ (*kdpFAB*)5 *enda*) was transformed with pSPORT containing *LjKUP* or *LjNEST8d4* and grown on KML media (Epstein and Kim 1971) supplemented with 50 mM KCl and ampicillin at 50 μ g/ml. For growth-curve analysis, cells from an overnight culture were washed and resuspended to an optical density at 600 nm (OD₆₀₀) of 0.005 in LK liquid media (Kim et al. 1998) supplemented with various concentrations of KCl. Growth of cultures at 37°C was monitored hourly by measuring OD₆₀₀.

Transient expression of LjKUP in *Arabidopsis* leaves.

For subcellular localization of the GFP-LjKUP fusion protein, the expression construct was introduced into epidermal cells of *Arabidopsis* leaves by particle bombardment using a BioRad PDS-1000 (BioRad, Hercules, CA, U.S.A.), as previously described (Hibbert et al. 1998). Transformation of *Arabidopsis* cell culture protoplasts also was performed using published protocols (Abel and Theologis 1998; Axelos et al. 1992). Imaging of GFP was performed using a Leica confocal scanner TCS SPII together with a Leica DM IRBE microscope (Leica, Bensheim, Germany) 24 to 48 h after bombardment (Sohlenkamp et al. 2002). KCO1-GFP was used as a tonoplast marker (Czempinski et al. 2002).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- The Center for Biological Sequence Analysis TMHMM prediction server: www.cbs.dtu.dk
- The Institute for Genetic Research (TIGR) gene indices: www.tigr.org/tdb/tgi/plant.shtml
- MPSS Arabidopsis database: mpss.ucdavis.edu/java.html
- PSORT database for the prediction of protein sorting signals and localization sites: psort.nibb.ac.jp
- Softberry bioinformatics Protcomp software: www.softberry.com