# Diurnal and Circadian Regulation of Putative Potassium Channels in a Leaf Moving Organ[1]

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In a search for potassium channels involved in light- and clock-regulated leaf movements, we cloned four putative K channel genes from the leaf-moving organs, pulvini, of the legume *Samanea saman*. The *S. saman* SPOCK1 is homologous to KCO1, an Arabidopsis two-pore-domain K channel, the *S. saman* SPORK1 is similar to SKOR and GORK, Arabidopsis outward-rectifying *Shaker*-like K channels, and the *S. saman* SPICK1 and SPICK2 are homologous to AKT2, a weakly-inward-rectifying *Shaker*-like Arabidopsis K channel. All four *S. saman* sequences possess the universal K-channel-specific pore signature, TXXTXGYG, strongly suggesting a role in transmembrane $K^+$ transport. The four *S. saman* genes had different expression patterns within four leaf parts: 'extensor' and 'flexor' (the motor tissues), the leaf blades (mainly mesophyll), and the vascular bundle ('rachis'). Based on northern blot analysis, their transcript level was correlated with the rhythmic leaf movements: (a) all four genes were regulated diurnally (*Spick2*, *Spork1*, and *Spock1* in extensor and flexor, *Spick1* in extensor and rachis); (b) *Spork1* and *Spock1* rhythms were inverted upon the inversion of the day-night cycle; and (c) in extensor and/or flexor, the expression of *Spork1*, *Spick1*, and *Spick2* was also under a circadian control. These findings parallel the circadian rhythm shown to govern the resting membrane $K^+$ permeability in extensor and flexor protoplasts and the susceptibility of this permeability to light stimulation (Kim et al., 1993). Thus, *Samanea* pulvinar motor cells are the first described system combining light and circadian regulation of K channels at the level of transcript and membrane transport.

The *Samanea saman* leaf movement is regulated by alternations of light and dark and the endogenous biological clock (reviewed by Satter and Galston, 1981). These movements, carried out through the volume changes of cells in the leaf motor organs, pulvini (Fig. 1, $P_{II}$ and $P_{III}$), are accompanied by and depend on transcellular $K^+$ fluxes via K channels (Moran et al., 1988; reviewed by Satter et al., 1988). Both light (Lowen and Satter, 1989; Kim et al., 1992; Suh et al., 2000) and the circadian clock (Kim et al., 1993) have been shown to regulate the K permeability of the motor cell membranes. Two types of K channels, characterized in electrophysiological experiments in the plasma membrane of the motor cells, $K_D$ (depolarization-activated K) channels and $K_H$ (hyperpolarization-activated K) channels, most likely serve as conduits for the efflux and the influx, respectively, of $K^+$ during the cell volume changes (Moran et al., 1988; Moran and Satter, 1989; Moran, 1990; Moran et al., 1990; Yu et al., 2001). Based on this premise, we expect these channels to be regulated by light and the biological clock. To resolve the details of their regulation, we set out to clone the pulvinar K channel genes. Here we report that diurnal changes in illumination regulate the level of transcript of four *S. saman* K channel gene orthologs, and that three of them are also under the control of the circadian clock.

## RESULTS

To identify the molecules constituting the K channels in the *S. saman* motor cells, we screened a cDNA library constructed from the *S. saman* motor organs (Becker et al., 1998; Moshelion et al., 1998, 2001), using probes based on known plant K channel genes. We used the Arabidopsis *Kco1* (a 2-P-domain family member; Czempinski et al., 1997) and *Skor* (a Shaker family member; Gaymard et al., 1998) to probe for outward-rectifying K channels. Additional probes were based on two inward-rectifying K channel genes of the *Shaker* family, the Arabidopsis *Kat1* (Anderson et al., 1992) and an *Akt2* (Cao et al., 1995)-like gene from *Vicia faba*, *Vfk1* (accession no. X10579).

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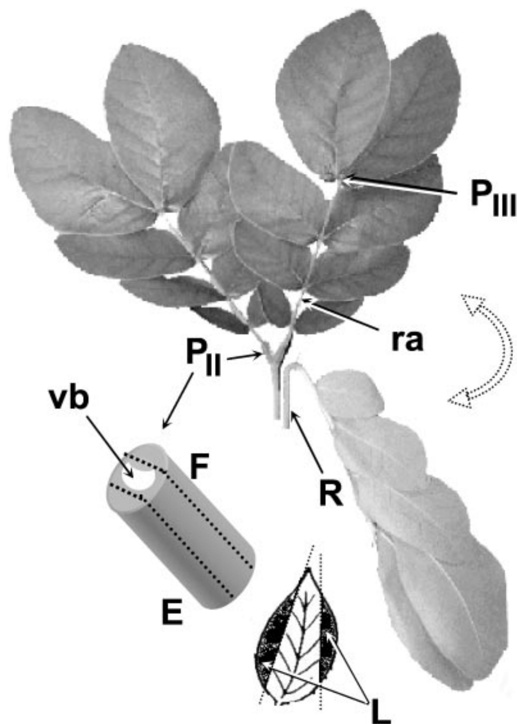


**Figure 1.** Schematic representation of the top part of the leaf of *S. saman*, displaying movement (semicircular arrow) between the open and the folded positions (dark gray and light gray, respectively). $P_{II}$ and $P_{III}$, Secondary and tertiary pulvini, respectively; L (blackened areas), leaf blades with the larger veins excluded; R, rachis; ra, rachilla; E, F, and vb, extensor, flexor, and the vascular bundle regions of the secondary pulvinus shown schematically, enlarged. The dotted lines indicate planes of E and F excision.

In consecutive screens, we isolated two new K channel cDNAs using the *Vfk1* probe, which we named *Spick1* and *Spick2* (for ***S****amanea* ***P****ulvini* ***I****nward-rectifying* ***C****hannel for* ***K***$^+$ no. 1 and no. 2; accession nos. AF099095 and AF145272, respectively). In addition, we isolated one *Skor* ortholog, *Spork1* (for ***S****amanea* ***P****ulvini* ***O****utward-****R****ectifying* ***K*** Channel no. 1; accession no. AJ299019) and one *Kco1* ortholog, *Spock1* (for ***S****amanea* ***P****ulvini* ***O****utward-rectifying* ***C****hannel for* ***K***$^+$ no. 1; accession no. AF099096). A *Kat1* ortholog was not detected in the pulvinar library.

### Sequence Comparison

Sequence comparison of the predicted polypeptides using BLAST indicated that SPICK1 and SPICK2 belong to the AKT2 channel subfamily, SPORK1 belongs to the outward-rectifying SKOR and GORK subfamily—both members of the *Shaker* family—and SPOCK1 is related to the 2-P-domain KCO1 channel family, separate from plant *Shaker*-like K channels. A high degree of overall identity, 59% to 66%, exists between the close orthologs (Table I).

Arranging the predicted amino acid sequences of the new genes on a hydropathy scale yielded a pattern of seven hydrophobic domains, resembling those of their homologs: six transmembrane domains (S1–S6) and a pore-like domain (P) in SPICK1, SPICK2, and SPORK1, and four transmembrane domains (S1–S4) and two pore-like domains (P1–P2) in SPOCK1 (Fig. 2A).

The high degree of homology is illustrated in the comparisons of the different channels' pore regions and their flanking transmembrane segments: S5-P-S6 in the *Shaker* family, and S1-P1-S2 in the *Kco1* family (Fig. 2B). Notably, all of the *S. saman* putative K channels share the universal K channel pore motif, TXXTXGYG, found not only in all of the known functional Arabidopsis K$^+$-selective channels, but also in K$^+$-selective channels of procaryotes (bacteria: Doyle et al., 1998; cyanobacteria, Synechocystis: Chen et al., 1999) and viruses (Plugge et al., 2000). Additionally, motif sequence analysis reveals conserved regulatory domains in the C terminus of the *Shaker*-like *S. saman* 'K channels', similar to those in their respective Arabidopsis orthologs: a putative cyclic-nucleotide-binding site and ankyrin-like motifs (SPICK1, SPICK2, and SPORK1), as well as the Ca$^{2+}$-binding 'EF hands' (SPOCK1).

### Temporal Regulation of K Channel Expression

All four *S. saman* K channel transcripts in the pulvinar tissues were detected by northern blot analysis and the individual channel transcript levels were examined during the different stages of leaf movement.

**Table I.** *Sequence comparisons between S. saman K channels and Arabidopsis K channels*

Numbers, overall identity (and overall homology). Comparisons between members of the same subfamilies are in bold.

| *S. saman* | Arabidopsis | | | | | |
|---|---|---|---|---|---|---|
| | KAT1 | AKT1 | AKT2 | SKOR | GORK | KCO1 |
| SPICK1 | 33 (49) | 39 (57) | **64 (78)** | 27 (50) | 25 (47) | 7 (16) |
| SPICK2 | 32 (48) | 34 (54) | **59 (75)** | 26 (48) | 25 (47) | 6 (16) |
| SPORK1 | 22 (38) | 25 (44) | 27 (45) | **66 (81)** | **61 (77)** | 6 (15) |
| SPOCK1 | 6 (17) | 6 (15) | 6 (16) | 6 (15) | 6 (15) | **56 (71)** |

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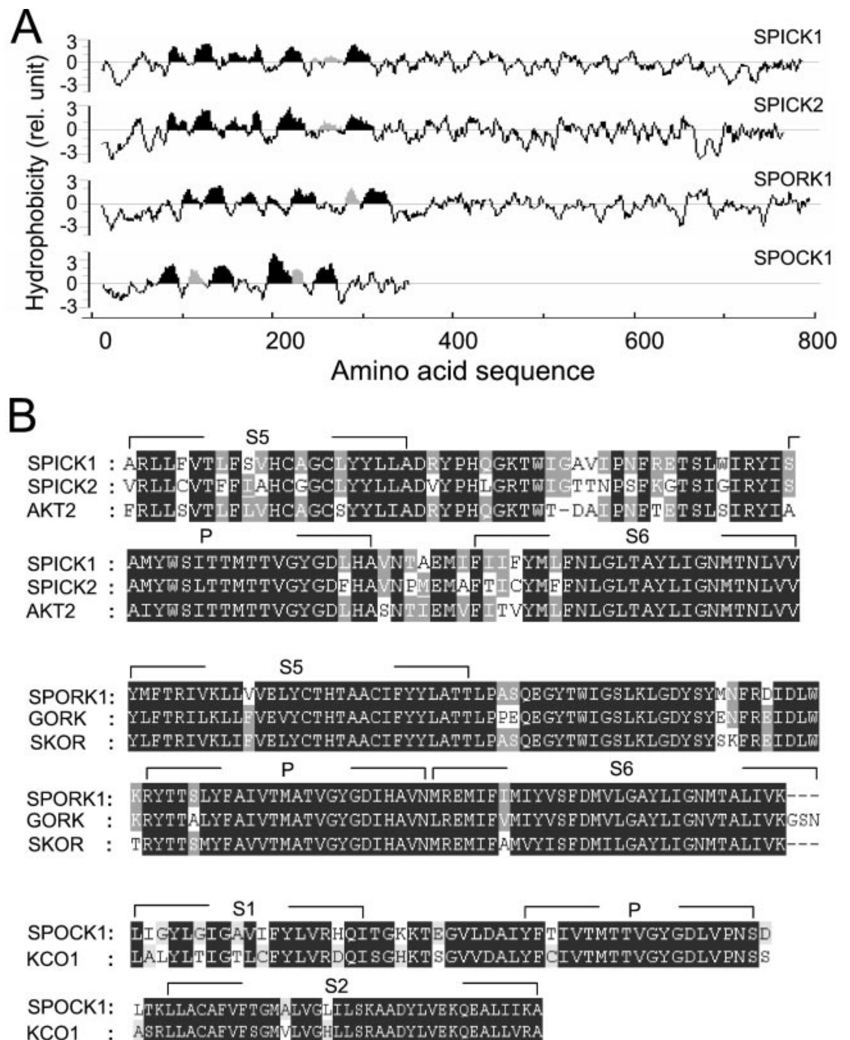


**Figure 2.** Sequence analysis of the *S. saman* putative K channels. A, Hydropathy plot of the predicted amino acid sequences of the *S. saman* K channels. Lines, Kyte and Doolittle hydrophobicity values using an 11-amino acid window. Black, Predicted transmembrane domains. Gray, Predicted pore domains. B, Alignment of predicted amino acid sequences of the most conserved parts in the *S. saman*—the pore region (P) and its flanking transmembrane segments (S)—of *S. saman* K channels with their respective homologs from Arabidopsis (SPICK1, SPICK2 versus AKT2; SPORK1 versus SKOR and GORK; and SPOCK1 versus KCO1). Identical and highly similar amino acids are labeled with black boxes, and less similar ones are labeled with gray boxes (see "Materials and Methods"). TXXTVGYGD represents the core of the pore domain and is the most conserved sequence among the plant K channels.

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### Leaf Angle

In the conditions of our experiments, the *S. saman* leaf movements were synchronized to the daily alternations of light and dark as already reported before (reviewed by Satter and Galston, 1981). In a plant exposed to inverted light/dark cycles, the leaf closing-opening periodicity readjusted to the new illumination regime within 2 to 3 d. When exposed to continuing darkness for 4 d, the rhythmic leaf movement continued in close correlation with the subjective day and night timing (for review, see Satter and Galston, 1981, and see below).

### Diurnal Transcript Regulation

*Spork1* and *Spock1* mRNA signals in total RNA appeared the strongest, whereas *Spick2* signals appeared the weakest. The transcript level of all of the K channels was strongly light dependent, as seen from the significant peaks of each of the gene's transcripts (Figs. 3 and 4, D/L), and, in *Spork1* and *Spock1*, from the inversion of the timing of this peak following the inversion of the light/dark illumination regime (Fig. 3, Dfl-INV). Three of the putative K channels genes—*Spick2*, *Spork1*, and *Spock1*—were diurnally regulated only in the motor tissues, E and F, and the fourth gene—*Spick1*—in the rachis, as well as in E. None of the genes was regulated diurnally in the leaf blades, which do not participate in the movement (Fig. 4, D/L, asterisks). In E and F, the transcript level of *Spick2*, *Spork1* and *Spock1* peaked in the morning, but the *Spick1* transcript level peaked about midnight in E and in the evening in the rachis, remaining invariant in the F.

### Circadian Transcript Regulation

In three of the genes, the rhythmic control of gene expression continued during constant darkness (D\D treatment), only in the motor tissues: When assayed on the 2nd and the 3rd d in D/D (after the first Light-On signal was omitted), the *Spork1* transcript peaked in the subjective (*s*-) morning in both E and F, *Spick2* transcript peaked also in the *s*-morning, but

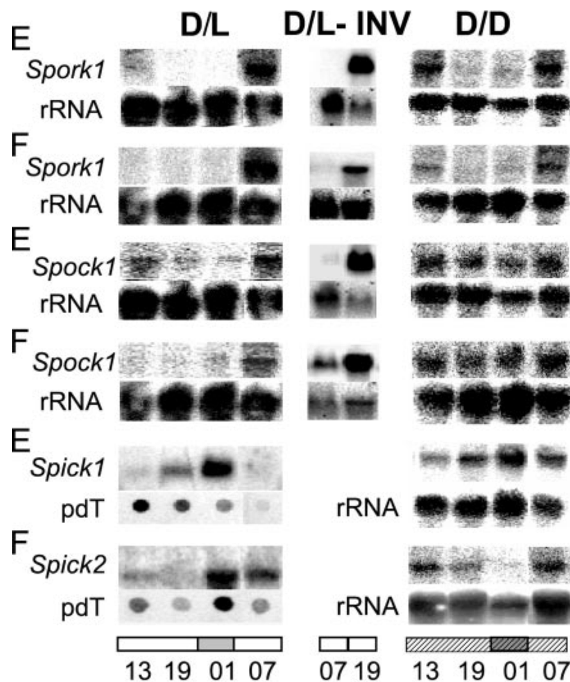


**Figure 3.** Rhythmic variation of *S. saman* K channels expression levels in the motor tissues. All panels, except *Spick1* and *Spick2*: D/L, phosphor-imager scans of total RNA northern blots from E or F parts of the secondary pulvini, using the channel cDNA as a homologous probe and a probe to ribosomal RNA 18S (rRNA). *Spick1* and *Spick2*: D/L, scans of autoradiograms of mRNA northern blots, probed with the channel cDNA (top) and poly-deoxy-Thymidine (pdT; bottom). Numbers at the bottom are abbreviations of the time of sampling: noon (13), evening (19), night (01), morning (07). D/L, Diurnal alternations of dark and light (on: 5 AM; off: 9 PM); D/L-INV, dark-light illumination inverted (on: 5 PM; off: 9 AM); leaves were harvested 7 d after the inversion. D/D, continuous darkness; measurements between h 39 and 58 after lights went off at the end of a normal day. White bars, light; gray bars, dark; hatched bars, subjective day; hatched gray bars, subjective night. Note that, while the mRNA levels of all four channels fluctuated in E and/or F during D/L, and, in Spork1 and Spock1, also after D/L inversion, *Spock1* mRNA level did not fluctuate during continuous darkness (D/D).

only in F, and *Spick1* peaked about *s*-midnight, only in E. The mRNA level of *Spock1* became constant at all times during the 2nd and the 3rd d in D/D (Figs. 3 and 4A, D/D).

The transcript level fluctuations of spick1, spick2, and spork1 were subsequently examined also in the whole pulvinus during the 3rd and the 4th d of D/D (Fig. 4B, whole pulvinus). The mRNA signal from the whole pulvinus is expected to represent the most prominent signals from the E, F, and rachis pooled together. Thus, as expected, the transcript level of *Spick1* in the whole pulvinus peaked at *s*-midnight (Fig. 4B, crosses), as it did during the preceding cycle in the separate E samples (Fig. 4A, *Spick1*, D/D, asterisk). The peaks of *Skor1* and *Spick2* transcript levels appear shifted by roughly 4 h in the whole pulvinus (Fig. 4B, squares and triangles) relative to the *s*-morning timing of their most prominent peak during the preceding cycle in the separated tissues (Fig. 4A, *Spick2* and *Spork1*, D/D, asteriscs). *Spock1* transcript level—already invariant during the fist cycle of D/D—was not examined during the following cycle in the whole pulvinus.

## DISCUSSION

### The Mechanism of Leaf Movement

During the morning hours, swelling E cells in the bottom (abaxial) section of the secondary pulvinus, lift up the unfolding top part of the compound leaf of *S. saman* (Fig. 1), about 100-fold heavier than the mass of the E tissue itself. This is aided by the concomitant shrinking of F in the top section of the pulvinus. F cells, swelling during the afternoon hours, push this leaf part down, aided by the gravitational pull and the concomitant shrinking of the E. These movements, resulting from motor cell volume changes, are powered by osmotic forces due mainly to the accumulation of $K^+$ and $Cl^-$ in the swelling cells and concomitant loss of these ions from the shrinking cells (Satter and Galston, 1971; Satter et al., 1974).

### The Hypothesized Role of K Channels in Leaf Movements

The periodic, light- and clock-regulated, massive fluxes of $K^+$ across the plasma membrane of the motor cells are very likely mediated via the AKT2-like $K_H$ channels (influx, Cao et al., 1995; Ketchum and Slayman, 1996; Yu et al., 2001) and via the SKOR-like $K_D$ channels (efflux, Moran et al., 1988; Gaymard et al., 1998). For the time being, we have refrained from suggesting a function for SPOCK1, since, like the outward-rectifying Arabidopsis KCO1, it has EF hands and therefore is likely to be regulated by $Ca^{2+}$ (Czempinski et al., 1997), whereas *S. saman* $K_D$ channels are largely $Ca^{2+}$ independent (Moshelion and Moran, 2000).

If the activity of $K_D$ and $K_H$ channels is rate limiting for $K^+$ fluxes, then light and clock are expected to regulate $K^+$ fluxes by regulating overall K channel activity. Thus, $K_H$ channel activity is expected to be elevated during the swelling process: E in the morning, F in the afternoon. Conversely, $K_D$ channel activity is expected to be elevated during the process of shrinking: E in the afternoon, F in the morning. Furthermore, if SPICK1 and SPICK2 represent the $K_H$ channel, while SPORK1 represents the $K_D$ channel, it is not entirely surprising that the transcripts of *Spick1*, *Spick2*, and *Spork1* in the motor cells undergo diurnal and circadian regulation. Our model does not attribute specific regulation to K channels in the other tissues, leaf and rachis, since $K^+$ exchanges have been shown to occur between the F and E parts, rather than between the motor tissues and the vas-

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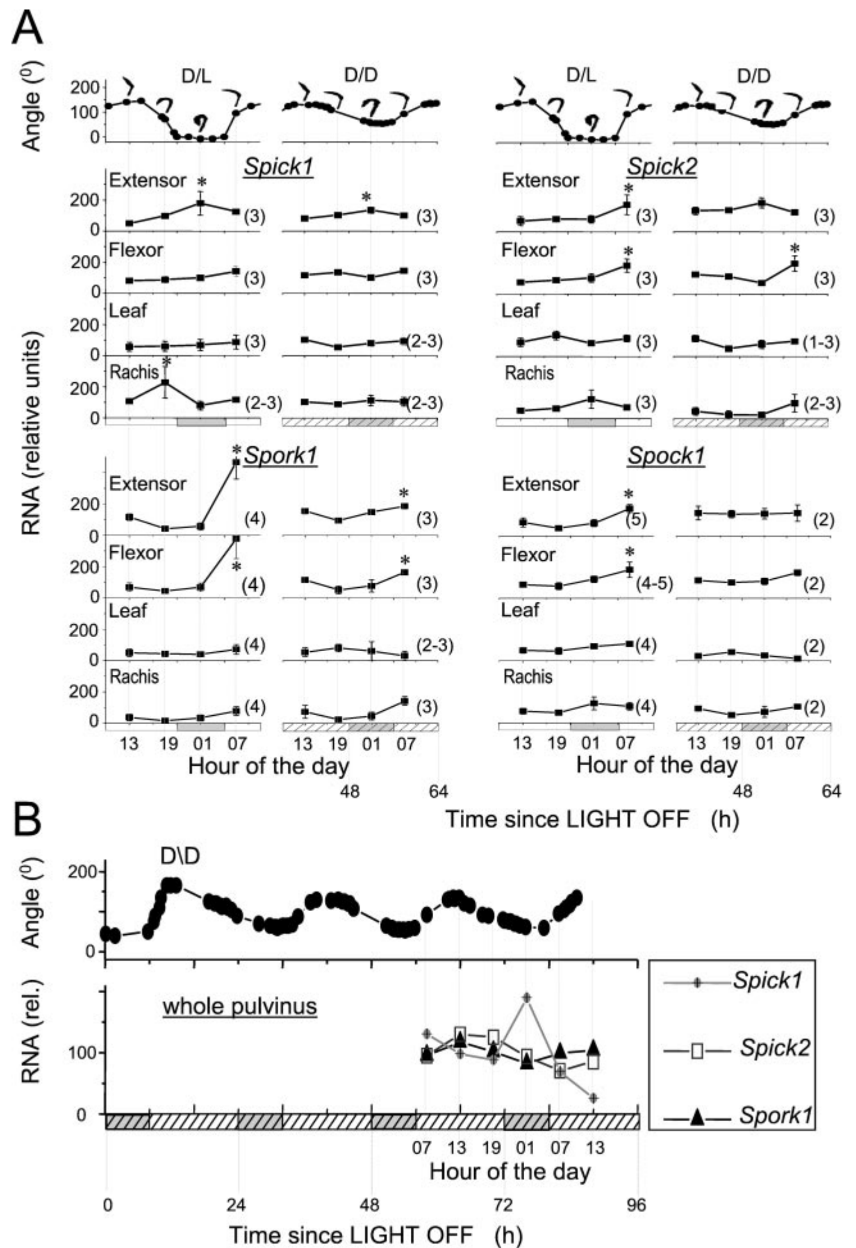

**Figure 4.** Temporal pattern of expression of *S. saman* K channel genes. A, top, An angle between rachis and terminal rachilla in an intact, tree-attached leaf (see Fig. 1). D/L, dark/light alternations; D/D, continuous darkness. Inset, Pulvinus angles illustrated. All other panels show normalized transcript levels of the individual genes (indicated) in the different tissues (indicated, as in Fig. 1) during D/L or during D/D. Symbols (±SE; number of repeats are in parentheses) are mean transcript levels in various leaf parts. Where not seen, the error is smaller than the symbol (except in *Spick2* leaf, 07 h: a single sample). *, Transcript levels significantly higher than in (at least one) other sample(s) in the same tissue. See also a summary in Table II. Horizontal bars: white, day; black, night; hatched, subjective day; hatched gray, subjective night. Abscissa, Abbreviated hours (as in Fig. 3) and, beneath, in the 2nd line, time count in D/D starting with the last lights off signal at the end of a normal day. B, Whole pulvinus: an additional (single) series of normalized mRNA signals obtained from a whole pulvinus total RNA probed sequentially with three channel probes (see "Materials and Methods" for details).

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cular bundle (Campbell et al., 1981; supported by findings in the pulvini of *Phaseolus vulgaris*, Irving et al., 1997).

### Diurnal Transcript Rhythms versus Anticipated K Channel Function

The midnight or the morning peaks of *Spick1* and *Spick2* transcripts (respectively) in E (Fig. 4A, D/L) could lead to increased subsequent abundance of the candidate $K_H$ channel protein (SPICK1 or SPICK2) and, in turn, increased $K_H$ channel activity. This would be in accord with the presumed requirement for increased permeability to $K^+$ during the morning swelling of E cells. Similarly, the morning peak of *Spork1* transcript in F (Fig. 4A, D/L) would be in accord with the presumed requirement for increased permeability to $K^+$ during the morning shrinking of F cells, realized through the increased abundance and activity of the $K_D$ channel, i.e. its candidate protein, SPORK1.

In other cases, an additional (but not improbable) several-hour delay is required to relate the expected increased afternoon activity of $K_H$ channels (SPICK2) in F cells to the morning peak of *Spick2* in F (Fig. 4A, D/L). A similar delay is required to relate the morning peak of *Spork1* in E (Fig. 4A, D/L) to the expected afternoon activity of $K_D$ channels (SPORK1) in these cells.

### Circadian Transcript Rhythms Versus Anticipated K Channel Function

Similar relations may be expected between the circadian rhythms of the K channel transcripts and plasma membrane permeability for $K^+$, mediated by the corresponding $K_H$ or $K_D$ channels, or their respective candidate proteins. This includes the *s*-midnight peak of *Spick1* transcript in E (presumed to lead to morning $K_H$ channel/SPICK1 activity), or the *s*-morning peak of *Spork1* transcript in F (presumed to lead to morning $K_D$ channel/SPORK1 activity; Fig. 4A, D/D). This includes also, albeit with a larger delay, the relation between the *s*-morning transcript peak of *Spick2* and the anticipated afternoon $K_H$ channel (SPICK2) activity in F and the *s*-morning transcript peak of *Spork1* and the anticipated afternoon $K_D$ channel (SPORK1) activity in E.

An additional cycle of transcript levels of *Spick1*, *Spick2*, and *Spork1* was observed in total RNA sampled from the whole pulvinus between h 58 to 87 of D/D (Fig. 4B, D/D). In particular, the *Spick1* transcript level in D/D in the whole pulvinus continued fluctuating with the same phase as that during the preceding cycle in the separate E (Fig, 4A, D/D). This resembles SsAQP2 transcript level fluctuations in D/D in the same whole pulvinus (Moshelion et al., 2001).

### Circadian $K^+$ Permeability Rhythm versus Anticipated K Channel Function

The rhythmic changes in the permeability of the plasma membrane to $K^+$ were assayed in continuous darkness (employing a membrane-potential-reporting fluorescent dye, Kim et al., 1993) and were attributed to $K_H$ channels. These changes appear shifted, particularly in the F, relative to the predictions based on the hypothesized role of $K_H$ channels in pulvinar movements: In Es, $K^+$ permeability increases throughout the morning and early afternoon; in Fs, it increases throughout the night. This apparent shift, however, may be due to the protoplast preparation procedure.

### Functional Expression

Our trials to express functionally the *S. saman* K channels in frog (*Xenopus laevis*) oocytes (*Spork1, Spick1*, and *Spick2*), the insect SF9-cell line (*Spock1, Spick1, and Spick2*), and the mammalian HEK-293T cell line (*Spick1*) failed. The difficulty to express plant K channels functionally in various heterologous expression systems is increasingly recognized (e.g. Szabo et al., 2000), as is the difficulty to express some animal K channels (Salinas et al., 1997a). In some of the cases the underlying reason is the failure of the system to direct the channels to the plasma membrane, where the function is tested (Salinas et al., 1997a). Moreover, a few "electrically silent" K channel homologs have been identified in animal cells and presumed to function as modulators of other channels in heteromeric complexes (Hugnot et al., 1996; Post et al., 1996; Salinas et al., 1997b; Kramer et al., 1998). Either of these could be the explanation for the present lack of success in expressing functionally the *S. saman* clones as homomeric units.

## CONCLUSION

The following findings, together, are a very strong indication that the protein products of the K channel genes fulfill important roles in the pulvinar movements. (a) The *S. saman* clones are very close to their orthologs in Arabidopsis with a proven K channel function (Table I). (b) They have been cloned from a cDNA library prepared from the pulvinar tissues. Furthermore, (c) our recent patch-clamp studies revealed inward-rectifying K channels in the plasma membrane of protoplasts isolated from both E and F tissues of *S. saman*, which resemble AKT2 channels, rather than KAT1 channels, in their susceptibility to proton block (Marten et al., 1999; Yu et al., 2001). This is in accord with the abundance of the *Akt2* orthologs, *Spick1* and *Spick2*, and the failure to detect *Kat1*, in the pulvinar motor tissues. (d) The rhythmic regulation of their transcript level in the motor organ, pulvinus, is correlated, on one hand, with the rhythmic changes in the leaf angle (Figs. 3 and 4; Table II), and, on the

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**Table II.** *Transcript level peaks*

Summary of the ANOVA of transcript levels of *S. saman* K channel genes in the different tissues, under diurnal (◑) or circadian (◷) regulation. Numbers, the hour of the day (or the subjective day) of the significantly highest expression level of the gene (indicated also by asterisks in Fig. 4A). n, Insignificant difference between the expression levels.

| K Channel | Tissue Regulation Mode | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Extensor | | Flexor | | Rachis | | Leaf | |
| | ◑ | ◷ | ◑ | ◷ | ◑ | ◷ | ◑ | ◷ |
| *Spick1* | 01 | 01 | n | n | 13 | n | n | n |
| *Spick2* | 07 | n | 07 | 07 | n | n | n | n |
| *Spork1* | 07 | 07 | 07 | 07 | n | n | n | n |
| *Spock1* | 07 | n | 07 | n | n | n | n | n |

other hand, with the rhythmic changes in $K^+$ permeability of the motor cells (Kim et al., 1993). The detection and quantification of these K channel proteins, to test our hypothesis that they too are regulated by light and the biological clock, awaits the availability of appropriate antibodies or transgenic plants.

## MATERIALS AND METHODS

### Plant Material and Leaf Movement

*Samanea saman* L. (Jacq.) Merr. trees were grown in a greenhouse under an 8-h dark/16-h light (D/L) regime and temperature of 35°C ± 5°C/23°C ± 4°C, with light intensity of 300 to 700 $\mu$mol $m^{-2}$ $s^{-1}$. For the determination of transcript level rhythmicity, trees were transferred to a growth chamber with humidity of 77% ± 5% (±SD) during light/dark alterations and 80% ± 3% (±SD) during constant dark, and a constant temperature of 28°C ± 1°C. The same D/L regime was continued for 3 to 5 d until leaf harvest (light intensity was 50 to 100 $\mu$mol $m^{-2}$ $s^{-1}$, depending on the exact location in the chamber), or, after 3 d of accommodation in the chamber, the lights were turned off and leaves were sampled between h 39 and 58 (and in one series, also between h 58 and 87) of constant darkness using green "safe" light (Suh et al., 2000). Leaf parts were harvested directly into liquid nitrogen. Leaf angles were measured in sequential frames of a digital video movie of an undetached, intact moving leaf. During the video recording, flashes of the same green safe light were used for exposures in the dark (Fig. 4).

### cDNA Library Construction and Screening

Secondary pulvini of *S. saman*, harvested at 7 AM, 1 PM, and 7 PM from greenhouse-kept plants, were pooled prior to isolation of total RNA as described by Logemann et al. (1987). poly(A)$^+$ RNA was purified using oligo(dT)-coated particles (Dyna beads, Dynal Biotech GmbH, Hamburg, Germany). cDNA was synthesized with the λ Zap II System (Stratagene, Amsterdam) and cloned into λ phages. All steps were performed according to the manufacturers' protocol. The amplified cDNA library was screened by plaque hybridization using radioactive labeled probes: *Kco1* (Czempinski et al., 1997), *Skor* (Gaymard et al., 1998), and the *Vicia faba Akt2* (Cao et al., 1995)-like gene (accession no. X10579). In all cases we labeled the full open reading frame of the gene. The positively identified plaques were excised from agar plates, and cDNA-containing plasmids were isolated by in vivo excision. Subsequent sequence analysis revealed different cDNA clones with complete open reading frames and high homologies to known plants' K channels (see results).

DNA and protein sequences data were analyzed using Mac DNASIS (Hitachi, Yokahama, Japan). Multiple sequence alignments were performed using ClustalW 1.6 and edited with Genedoc (http://www.psc.edu/biomed/genedoc).

### Northern Blots and Quantification of mRNA Levels

For the study of the rhythmicity of the in planta expression, a series of four consecutive samples were collected at 6-h intervals[3] from four different leaf parts (the two motor tissues: E and F from the secondary pulvini, leaflet blades without the midrib/middle veins (L) and parts of rachis (R) below the terminal secondary pulvini, including the central portion of the pulvinus remaining after excision of E and F, i.e. mainly the vascular bundle. Each such 16-sample series is considered a repeat. One additional D/D series consisted of RNA extracted from a whole, undivided pulvinus between h 58 to 87 of D/D. The D/L samples of all genes consist each of one repeat of mRNA blots and two (*Spick1* and *Spick2*) or three (*Spock1* and *Spork1*) repeats of total RNA blots. The D/D series consist each of three repeats of total RNA blots (except *Spock1*, which has two repeats). Northern-blot experiments were performed according to standard protocols (Sambrook et al., 1989) with cDNA probes labeled (separately) using the "Ready to go" kit (Amersham Biosciences UK Ltd., Buckinghamshire, UK). The signals from the mRNA series were quantified by densitometry of autoradiograms and normalized to dot blots of the mRNA samples, using $^{32}$P end-labeled poly(dT) as a probe. The total RNA series were digitized directly by a phosphor-imager and normalized to 18S ribosomal RNA from the same samples. Prior to the normalization, the densitometry procedure included, in each case (a) using a fixed-size area for measuring the densities of each band, (b) using the same fixed-size area for measuring the densities of the background in the same lane above and below (at a fixed distance from, but not immediately adjacent to) each band, and (c) subtraction of the mean background value from the band density value. The 16 samples of each series (and the six samples from the single whole pulvinus series) were then expressed as percentage of the mean level of channel mRNA (each in their own) series. The corresponding percent values of all the repeated series were subsequently averaged over each separate time point.

### Statistics

Data are presented as mean ± SE, unless otherwise indicated. The criterion for rejecting the null hypothesis (that the compared values do not differ) was $P$ <0.05, unless specified otherwise.

To analyze the rhythmicity of mRNA levels simultaneously for all individual (already normalized, but not yet averaged) samples, we used two-way ANOVA as implemented in the program JMP (SAS Institute Inc., Cary, NC).

[3] Since roughly 100 leaves were needed for approximately 1 g each of F and E tissues, each harvest lasted about 1 to 2 h. Thus, although 6 h separated between starts of sampling, the net intervening periods lasted only between 4 and 5 h. The RNA samples are displayed versus the time of the middle of each such harvest period.

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