Potassium Channels in *Samanea saman* Protoplasts Controlled by Phytochrome and the Biological Clock

Hak Yong Kim, Gary G. Coté, Richard C. Crain*

Leaflet movement in legumes depends on rhythmic, light-regulated ion fluxes in opposing regions of the leaf-moving organ. In flexor and extensor protoplasts from *Samanea saman* Merrill, opening and closing of K^+ channels were rhythmic in constant darkness. When channels were open in flexor protoplasts they were closed in extensor protoplasts, and vice versa. The rhythms were shifted by a delay in the onset of constant darkness, a response typical of endogenous circadian rhythms. During the light period, the channels in flexor protoplasts were sensitive to red light that was followed by premature darkness; phytochrome was implicated as the photoreceptor.

An endogenous biological clock controls daily rhythms in the physiology and behavior of animals, plants, and eukaryotic microorganisms, ensuring that certain behaviors and physiological processes occur only at appropriate times in the cycle of day and night. Under constant environmental conditions in the laboratory, the endogenous clock maintains the physiological and behavioral rhythms with a period of ~24 hours. Daily rhythms that continue under constant conditions are termed circadian rhythms.

Many plants in the legume and oxalis families exhibit circadian rhythms of leaflet movement: Leaflets extend to face the light (open) during the day and fold together (close) at night (*1*). The rhythms continue in constant darkness, both in whole plants and in isolated leaf-moving organs (pulvini) (*2, 3*). The phase of the rhythm can be altered by exogenous signals, such as red or blue light (*4, 5*). Sensitivity of the rhythm to light is itself circadian because the magnitude of the phase shift is dependent on the time in the cycle at which light treatment occurs (*4, 5*). Light signals also have direct effects on leaflet movement: Blue light opens closed leaflets (*5*), whereas red light followed by darkness closes open leaflets (*6, 7*).

Movement of the leaflets is effected by swelling and shrinking of cells on opposite sides of the pulvinus. These changes in turgor depend on K^+ and Cl^- fluxes across the plasma membranes (*2, 8*). When extensor cells take up K^+ and Cl^- and swell, and flexor cells release K^+ and Cl^- and shrink, leaflets open. They close when the reverse ion movements and turgor changes occur. White or blue light, which induces leaflet opening, causes K^+ and Cl^- uptake by extensor cells, whereas exposure to red light followed by darkness, which favors leaflet closing, induces K^+ and Cl^- uptake by flexor cells (*6, 9*). In the pulvinus of *Samanea saman* (monkey pod tree), K^+ channels activated by hyperpolarization (*10, 11*) might carry the K^+ influx into swelling flexor and extensor cells. In response to white or blue light, inward K^+ channels are opened in extensor protoplasts and closed in flexor protoplasts (*12*). We present evidence here that the channels are also controlled by the photoreceptor pigment phytochrome and by an endogenous circadian rhythm.

The intact pulvinus is a poor system for study of the cellular response to light because extensor and flexor cells behave oppositely and because pulvini contain cells other than extensor and flexor cells (*13*). We therefore isolated protoplasts of both extensor and flexor cells from the pulvini of *S. saman* (*14*); protoplasts of both types have channels that permit K^+ influx (*10–12*). The open or closed state of these channels can be ascertained by measurement of the changes in membrane potential that occur after the extracellular K^+ concentration is increased from 20 to 200 mM (*12, 15*). The added K^+ enters and depolarizes the protoplasts only if the channels are open (*16*) (Fig. 1).

The state of the K^+ channels varied rhythmically for up to 21 hours in constant darkness (Fig. 2A) (*18*). Rhythms in flexor and extensor pulvini were one-half of a cycle out of phase with each other: At hours 4 to 8 after transfer to darkness, K^+ channels were open in flexor but closed in extensor protoplasts, whereas at hours 16 to 21 after darkness channels were open in extensor but closed in flexor protoplasts. The state of the K^+ channels in protoplasts is consistent with the K^+ fluxes that are expected on the basis of leaflet movement of intact plants in constant darkness (*3*).

To determine whether the rhythms in the state of the K^+ channels were light-sensitive, we delayed the time of transfer from light to darkness by 3 hours, which resulted in a 3-hour delay in the rhythm of K^+ channel opening and closing (Fig. 2B). The persistence of the K^+ channel rhythm for at least one cycle in constant darkness and the sensitivity of the rhythm to light indicate a circadian rhythm. The difference of one-half of a cycle between extensor and flexor rhythms demonstrates that the rhythms are not artifacts created by temporal variations in experimental manipulation.

When intact plants on a light-dark (LD) 16:8 cycle are irradiated with red light (660 nm) at hour 10 to 12 of the light period and transferred immediately to darkness, leaflet closure is accelerated (*6, 7*). Extensor and flexor protoplasts isolated and maintained under the LD 16:8 cycle were given various light treatments at hour 10 to 12 of the light period, and the state of the K^+ channels was ascertained (Table 1). Initially, K^+ channels were open in extensor protoplasts and closed in flexor protoplasts (Fig. 1 and Table 1). Irradiation with red light for 1 min followed by transfer to darkness caused K^+ channels to open in flexor and close in extensor protoplasts, as expected from the behavior of the whole plant. Transfer to darkness without previous irradiation with red light was sufficient to close the channels in extensor protoplasts but did not open the channels in flexor protoplasts. Irradiation with red light for 1 min followed by a return to normal white light had no effect on K^+ channels in either flexor or extensor protoplasts. Thus, both red light

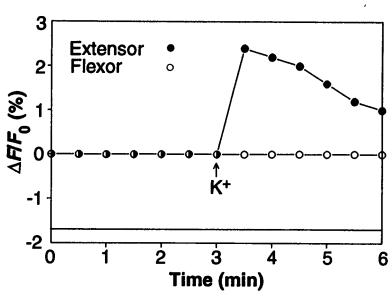

Fig. 1. Potassium ion–induced changes in membrane potential in extensor and flexor protoplasts. Extensor and flexor protoplasts were isolated in the light (*14*) and maintained in the same LD cycle as were the intact plants. After the 8-hour dark period and 8 hours of the following 16-hour light period, the protoplasts were repurified by centrifugation onto a second Histopaque (Sigma) layer and maintained in white light (*17*). The state of the K^+ channels was ascertained on the basis of K^+-induced changes in membrane potential (*15*). The external K^+ concentration was increased at the time indicated (arrow). The measured change in fluorescence (ΔF) was divided by the initial fluorescence (F_0) to normalize for variation between experiments. Data are representative of five experiments; values varied by <15% between experiments. The white bar at the bottom of the figure indicates exposure to white light.

Department of Molecular and Cell Biology, University of Connecticut, U-125, 75 North Eagleville Road, Storrs, CT 06269–3125.

*To whom correspondence should be addressed.

and transfer to darkness were required to open the K^+ channels in flexor protoplasts.

Tetraethylammonium (TEA), a K^+ channel blocker, was used to demonstrate that depolarization was mediated by K^+ channels. When 20 mM TEA was added 30 s before the addition of 200 mM K^+, depolarization decreased 73% in untreated extensor protoplasts and 78% in flexor protoplasts irradiated with red light followed by darkness (Table 1).

Many effects of red light on plants are mediated by phytochrome. Concurrent or subsequent irradiation with far-red light (~730 nm) reverses the photochemical effects of red light on phytochrome and many of the physiological effects of red light that are mediated by phytochrome. We monitored the state of K^+ channels in extensor and flexor protoplasts after simultaneous irradiation with both red light and far-red light, followed by transfer to darkness. Irradiation with far-red light reversed the effect of red light on the opening of K^+ channels in flexor protoplasts (Table 1), implicating phytochrome as the photoreceptor.

To determine whether they respond to the absence of blue or red light as darkness, protoplasts were irradiated with high-intensity red light and placed in low-intensity red or blue light instead of darkness for 3 min (*20*), and the state of the K^+ channels was assayed. Extensor protoplasts transferred to red light had closed K^+ channels, as if they had been transferred to darkness. However, when transferred to blue light they had open K^+ channels, as if they had been returned to white light (Table 1). Thus, at hour 10 to 12 of the light period, extensor protoplasts interpret blue light as light and its absence as darkness and are not responsive to red light. On the other hand, flexor protoplasts transferred to red light behaved as if they had remained in white light, exhibiting closed channels; when transferred to blue light they behaved as if they had been transferred to darkness, exhibiting open channels (Table 1). If the flexor protoplasts were transferred to blue light without irradiation first with red light, they behaved as if they had been transferred to darkness without previous irradiation with red light; that is, the channels were closed (Table 1). Thus, at hour 10 to 12 of the light period, flexor protoplasts apparently require red light stimulus followed by its absence to open K^+ channels.

Blue light affects the state of K^+ channels in both flexor and extensor protoplasts at hour 4 to 8 of the dark period, whereas red light has no effect in either type of protoplast at this time (*12*). We propose that K^+ channels in extensor protoplasts are regulated by the blue light photoreceptor rather than by phytochrome, whereas K^+ channels in flexor protoplasts are alternately responsive to the blue light photoreceptor and to phytochrome. The circadian clock may determine which photoreceptor is coupled to the K^+ channels in flexor protoplasts.

The dynamics of K^+ channel states in protoplasts that are circadian rhythmic and light-sensitive are consistent with the behavior of pulvini in intact plants. This similarity suggests that the protoplasts may be a useful system for the study of the signal transduction events by which photoreceptors and the endogenous circadian oscillator control leaflet movements.

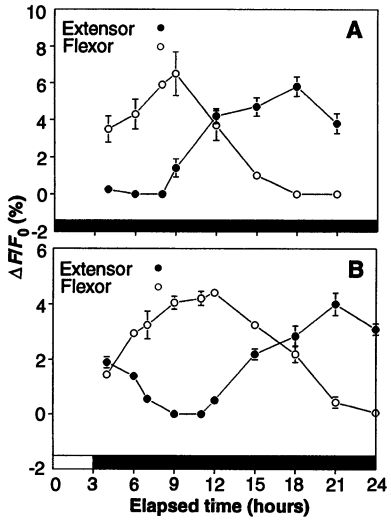

Fig. 2. Changes in the state of the K^+ channels of extensor and flexor protoplasts in constant darkness. (**A**) Extensor and flexor protoplasts were isolated in the light (*14*), transferred to darkness at the time of normal lights-out in the growth chamber (t = 0), and then maintained in constant darkness (as indicated by the black bar along the x axis). The state of the K^+ channels was ascertained (*15*) at intervals over the next 21 hours. Error bars indicate the range of values obtained over five separate experiments. (**B**) As in (A), except that protoplasts were transferred to constant darkness 3 hours after the time of normal lights-out in the growth chambers. Error bars indicate the range of values obtained over four separate experiments.

Table 1. Effects of light and darkness on the state of K^+ channels in extensor and flexor protoplasts at hours 10 to 12 of the light period. Protoplasts were isolated (*14*) and maintained as described in Fig. 1. The maximum fluorescence change measured 30 s after we increased the external K^+ concentration (*15*) is reported. Protoplasts that were initially in white light (WL) (*17*) were pulsed with red light or red light plus far-red light (*19*) and were transferred to darkness (D) or to white light, lower intensity red light, or blue light (BL) for 3 min (*20*). Other protoplasts in white light were transferred directly to darkness or to blue light without an initial red light pulse. In each case, we then added K^+ to assess the state of the K^+ channels. The abbreviation RL indicates 30 or 60 s of red light; RL/FR indicates 30 s of simultaneous red and far-red light with an additional 30 s of far-red light to ensure conversion of phytochrome to the red-absorbing form. Results represent mean ± SE, with the number of independent measurements shown in parentheses.

Treatment	Extensor protoplasts		Flexor protoplasts	
	Percent maximum $\Delta F/F_0$ (n)	K^+ channel	Percent maximum $\Delta F/F_0$ (n)	K^+ channel
WL	2.40 ± 0.195 (4)	Open	0 ± 0 (4)	Closed
WL-D	0.03 ± 0.033 (4)	Closed	0 ± 0 (4)	Closed
WL-30s, RL-D	0.23 ± 0.107 (3)	Closed	1.83 ± 0.051 (3)	Open
WL-60s, RL-D	0 ± 0 (5)	Closed	2.17 ± 0.107 (5)	Open
WL-60s, RL-WL	2.50 ± 1.207 (3)	Open	0 ± 0 (3)	Closed
WL-60s, RL–low RL	0 ± 0 (3)	Closed	0 ± 0 (3)	Closed
WL-60s, RL-BL	2.10 ± 0.0 (2)	Open	2.55 ± 0.050 (2)	Open
WL-RL/FR-D	0 ± 0 (3)	Closed	0 ± 0 (3)	Closed
WL-BL	2.35 ± 0.70 (4)	Open	0 ± 0 (3)	Closed
WL*	0.65 ± 0.131 (4)	Blocked	—	—
WL-60s, RL-D*	—	—	0.45 ± 0.065 (5)	Blocked

*In these two experiments, 20 mM TEA was added 30 s before K^+ was added.

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14. Protoplasts were isolated from separated extensor and flexor regions from open pulvini of *S. saman* Merr. grown at 26° ± 1.5°C on a cycle of 16 hours light and 8 hours dark (LD 16:8). Protoplasts were isolated as described (*12*), except that 0.5 mM dithiothreitol (DTT) replaced

1 mM ascorbic acid in all buffers.

15. Changes in the plasma membrane potential were monitored by measurement of changes in the intensity of fluorescence emission of the dye 3,3′ dipropylthiadicarbocyanine iodide [DiS-C_3-(5)], which is sensitive to membrane potential (*12*). The assay medium was 20 mM MES (pH 5.5), 370 mM sorbitol, 20 mM KCl, 1 mM $CaCl_2$, 90 mM sucrose, and 10^5 protoplasts per milliliter. The dye DiS-C_3-(5) was added to the assay medium to a final concentration of 2 μM, and the suspension was incubated at 25°C for 1 hour (in the experiments monitoring circadian rhythmicity) or for 5 min (in the experiments monitoring light sensitivity). As an antioxidant, 0.2 mM DTT was included to protect the dye during the hour-long incubations. Excitation of the dye was at 620 nm and emission was measured at 668 nm.
16. To demonstrate that the failure of added K^+ to depolarize unresponsive protoplasts did not result from a loss of membrane integrity, we incubated the protoplasts with valinomycin and 200 mM K^+. Depolarization induced by the ionophore plus K^+ demonstrated that the protoplasts were intact (*12*).
17. White light was provided with Sylvania GTE fluorescent bulbs (32 W, 4100K). Photon fluence rate, as measured with an L1-170 Quantum Meter (Li-Corp.) or an 818 series photodetector (Newport Corp.), was 20 μmol m^{-2} s^{-1}.
18. After 21 hours the protoplasts began to lose viability, preventing observations of further cycles.
19. We provided red and far-red light pulses by filtering the output of a fiber-optic lamp (Reichart Scientific Instruments, Buffalo, NY) through a heat-reflecting filter (Corion #HR750-F1-L255) and appropriate interference filters. The red filter was Oriel Optics no. 53960 and the far-red filter was Oriel Optics no. 53985. Peak transmittance was at 660 nm for red and 725 nm for far-red, with half-bandwidths of 10 nm in each case. The fluence rate was 80 μmol m^{-2} s^{-1} for red pulses and 40 μmol m^{-2} s^{-1} for far-red pulses.
20. Three-minute irradiation with white light was performed as in (*17*). Similar irradiation with red light was as in (*19*) except that the fluence rate was 3 to 4 μmol m^{-2} s^{-1}. Irradiation with high-intensity red light could not be continued beyond 60 s because the potential-sensitive dye was bleached by continued illumination, even in the absence of protoplasts. Blue light irradiation was as described previously (*12*) at a fluence rate of 3 to 4 μmol m^{-2} s^{-1}. The blue filters that were used transmitted significantly above 730 nm, in the absorbance range of the far-red–absorbing form of phytochrome. However, the blue filters were always used in combination with the heat reflecting filters, and transmittance by the combined filters was <1% at all wavelengths from 600 to 800 nm (red and far-red range).
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A Large Drop in Atmospheric $^{14}C/^{12}C$ and Reduced Melting in the Younger Dryas, Documented with ^{230}Th Ages of Corals

R. Lawrence Edwards,* J. Warren Beck, G. S. Burr, D. J. Donahue, J. M. A. Chappell, A. L. Bloom, E. R. M. Druffel, F. W. Taylor

Paired carbon-14 (^{14}C) and thorium-230(^{230}Th) ages were determined on fossil corals from the Huon Peninsula, Papua New Guinea. The ages were used to calibrate part of the ^{14}C time scale and to estimate rates of sea-level rise during the last deglaciation. An abrupt offset between the ^{14}C and ^{230}Th ages suggests that the atmospheric $^{14}C/^{12}C$ ratio dropped by 15 percent during the latter part of and after the Younger Dryas (YD). This prominent drop coincides with greatly reduced rates of sea-level rise. Reduction of melting because of cooler conditions during the YD may have caused an increase in the rate of ocean ventilation, which caused the atmospheric $^{14}C/^{12}C$ ratio to fall. The record of sea-level rise also shows that globally averaged rates of melting were relatively high at the beginning of the YD. Thus, these measurements satisfy one of the conditions required by the hypothesis that the diversion of meltwater from the Mississippi to the St. Lawrence River triggered the YD event.

Carbon-14 dating requires prior knowledge of secular variations in the atmospheric $^{14}C/^{12}C$ ratio (*1*). This ratio changes in response to shifts in the intensity of the solar or terrestrial magnetic fields, which modulate incoming cosmic rays responsible for atmospheric ^{14}C production. Changes in the atmospheric $^{14}C/^{12}C$ ratio may also result from redistribution of carbon of different isotopic compositions among the various carbon reservoirs. Detailed records of atmospheric $^{14}C/^{12}C$ have been obtained for the last 11,000 years from measurements of carbon in wood, where annual tree rings provide an independent chronology for the establishment of variations in the atmospheric $^{14}C/^{12}C$ ratio (*2–4*). However, extension of the record to earlier times has been hindered by lack of an appropriate absolute chronometer.

As early as 1965, Kaufman and Broecker (*5*) used ^{230}Th dating of carbonates for absolute time control in ^{14}C calibration. The early measurements constrained the past atmospheric $^{14}C/^{12}C$ ratio, but detailed records were not possible because alpha-counting measurements of ^{234}U and ^{230}Th were not sufficiently precise. In recent years, the development of thermal ionization mass spectrometric (TIMS) methods for the measurement of ^{234}U (*6*) and ^{230}Th (*7*) resulted in large increases in analytical precision and sensitivity. By the application of TIMS measurements to fossil corals, Edwards and colleagues (*7*) obtained ^{230}Th ages that were more precise than ^{14}C ages. This breakthrough made it possible to calibrate the ^{14}C chronometer with combined ^{230}Th age determinations and $^{14}C/^{12}C$ analyses. Fairbanks (*8*) recovered the first sequence of appropriate age for the extension of the atmospheric $^{14}C/^{12}C$ record by underwater drilling off the coast of Barbados. This sequence showed large offsets between ^{14}C and ^{230}Th ages (*9*), which suggests that the atmospheric $^{14}C/^{12}C$ ratio was high about 20,000 years ago. The subsequent drop in the atmospheric $^{14}C/^{12}C$ ratio was originally attributed to an increase in the terrestrial magnetic-field strength. However, Stuiver and co-workers (*10*) have raised questions about the Barbados data because this record disagrees with $^{14}C/^{12}C$ records from some varved lake sediments.

The same Barbados corals show a record of sea-level rise during the last deglaciation (*8*). Their depth and age relationships suggest that the rise was characterized by two meltwater pulses separated by a period of reduced melting (PRM). The timing of the PRM has implications for the cause of the Younger Dryas (YD), an interval of time during deglaciation when the climate in northern Europe returned to near-glacial conditions. However, Broecker (*11*) has questioned the record because the inferred meltwater pulses occurred at core breaks.

In this report, we present a new coral record derived from a single drill core from the Huon Peninsula, Papua New Guinea. This record has similarities to the Barbados record but also important differences, which have implications regarding the calibration of the ^{14}C time scale, the cause of a large decrease in the atmospheric $^{14}C/^{12}C$

R. L. Edwards and J. W. Beck, Minnesota Isotope Laboratory, Department of Geology and Geophysics, University of Minnesota, Minneapolis, MN 55455.
G. S. Burr and D. J. Donahue, Department of Physics, National Science Foundation Accelerator Facility for Radioisotope Analysis, University of Arizona, Tucson, AZ 85721.
J. M. A. Chappell, Department of Biogeography and Geomorphology, Research School of Pacific Studies, Australian National University, Canberra, ACT 2601, Australia.
A. L. Bloom, Department of Geological Sciences, Cornell University, 2122 Snee Hall, Ithaca, NY 14853.
E. R. M. Druffel, Department of Marine Chemistry and Geochemistry, Woods Hole Oceanographic Institution, Woods Hole, MA 02543.
F. W. Taylor, Institute for Geophysics, University of Texas at Austin, Austin, TX 78759.
*To whom correspondence should be addressed.