Photocontrol of Dark Circadian Rhythms in Stomata of *Phaseolus vulgaris* L.

M. GEOFFREY HOLMES*1 AND WILLIAM H. KLEIN
Smithsonian Environmental Research Center, 12441 Parklawn Drive, Rockville, Maryland 20852

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**ABSTRACT**

Stomatal diffusion resistance in primary leaves of *Phaseolus vulgaris* L., which had been grown in light:dark cycles followed a marked circadian rhythm when the plants were transferred to continuous darkness. Reentrainment of the rhythm required more than one inductive change in photoperiod. The phasing of the rhythm of dark stomatal opening was controlled primarily by the light-on (dawn) signal, whereas the rhythm of dark closure was related to the light-off (dusk) signal. The evidence points to a dual control of the circadian clock in which a product of photosynthesis plays a major role. No evidence for phytochrome involvement in the phasing of the rhythm was found. An influence of phytochrome on the amplitude of the stomatal rhythm was observed in which removal of phytochrome-far-red absorbing form caused rapid damping.

It is several decades since circadian changes in stomatal aperture in continuous darkness were first reported (23). Stälfelt (26) described a detailed study in which he observed a circadian rhythm in DD in both stomatal aperture and in the width of the guard cells of *Vicia faba* and *Stellaria media*. This rhythmic increase in cell width in the few h preceding the time at which dawn usually started was interpreted as indicating a daily increase in turgor in preparation for the light-mediated opening response. Studies of the relative effectiveness of the light-on signal, the light-off signal, and photoperiod, on stomatal movement in DD have led to various conclusions. In many respects, these differences can be ascribed to the variety of experimental approaches and to the restricted range of treatments. Following up experiments of Schwabe (24), Mansfield and Heath (14–16) demonstrated that the phase of onset of dark stomatal opening, the phase of maximal dark opening, and the phase of maximal opening in response to light, were all shifted by the timing of the end of the photoperiod in *Xanthium pennsylvanicum* (now *X. strumarium*). Conversely, Kana and Miller (11) interpreted their observations with *V. faba* as indicating that the phase of maximal stomatal opening in DD correlates with dawn (light-on signal), and that neither dusk (light-off signal) nor photoperiod were effective in determining phase. Studies with *Tradescantia virginiana* had previously led Martin and Meidner (18, 19) to conclude that the light-off signal controls the phasing of both maximal closure and opening in DD, but that the light-on signal determines only the time of maximal stomatal closure. Heath's (7) elaborate studies with *Commelina communis* led him to conclude that the light-on and light-off signals were approximately equally effective in phase-shifting the time of both maximal closure and maximal opening of stomata in DD; the duration of previous light or darkness had no effect.

With the exception of one brief study (11), these ostensibly confounding observations have been based on the apparent phase shift induced by one—or in the case of Heath (7), two—changes in light cycle. It is therefore impossible to determine whether the treatments resulted in a true rephasing of the rhythm or whether they represented transient perturbations; a direct effect of the treatment on the endogenous pacemaker is demonstrated only if it changes the steady-state phase (21, 27). We therefore undertook a study of light-induced phase-shifting of dark stomatal rhythms in which entrained rhythms were analyzed. The objective of this study was to determine the relative effectiveness on potential dawn (light-on), dusk (light-off), and photoperiod signals of the phasing of both stomatal opening and stomatal closure in DD in *Phaseolus vulgaris* L.

**MATERIALS AND METHODS**

**Plant Material.** *Phaseolus vulgaris* L., cv Provider (Meyer Seed Co., Baltimore MD) was grown from seed in vermiculite over an approximately 10 mm deep layer of gravel in individual 50 mm diameter perforated plastic cups at 25°C and 60% RH. After sowing in tap water, 0.5 strength Formula A1 Purdue nutriculture medium (28) was used for subsequent irrigation. The plastic cups were placed in a constant depth of 10 mm of temperature preequilibrated nutriculture medium to avoid exogenous influences of irrigation on the stomatal rhythm. Measurements were started 14 to 16 d after sowing, at which stage the first trifoliate leaves were less than 5 mm long.

**Rhythm Entrainment.** The basic (reference) 12:12 h L:D photoperiod was from 04:00 to 16:00 EST and advances or delays in the times of light-on and light-off were created about the reference photoperiod. Light was provided by a bank of Sylvania (Danvers, MA) F48T12/D/VHO “Daylight” fluorescent lamps which produced 100 μmol m⁻² s⁻¹ (400–700 nm) except where otherwise stated.

**Stomatal Resistance in DD.** Plants were transferred to an adjacent growth cabinet approximately 30 min before the onset of DD. This cabinet was identical in all respects to those used for entrainment or rephasing, but contained the porometer sample cups. The three cups were attached to the primary leaves of three plants and the recording equipment was reset.

The measurement system is reported in detail elsewhere (9). Briefly, three Li-Cor (Lincoln, NE) LI-700 transient porometers were fitted with modified sample cups which enabled *in situ* irradiation of the leaf, reduced long-term damage, provided a better seal, and avoided indirect effects of the porometer on the rhythm. The mV outputs from the porometer were connected to

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1 Present address: M. G. Holmes, (Botany School, University of Cambridge, Downing Street, Cambridge CB2 3EA, U.K.)

2 Abbreviations: DD, continuous darkness; EST, eastern standard time; FR, far-red light; L:D, light:dark; R, red light; WL, white light.
a computer-based acquisition and control system consisting of an Apple IIe microcomputer, Cyborg model 91A ISAAC system controller with I-130 interface, I-140A preamplifier, card cage and associated hardware. The software provided either simultaneous or alternate acquisition of data from the three porometers, and an error-handling routine for the occasional spurious reading which is a characteristic of these porometers (for details, see Ref. 9). Alternate 5 min measurements of porometer transit times were recorded, reference-calibrated, and stored on diskette. This provided four mean values (with se) of diffusion resistance every h. Between 9 and 18 individual recordings (i.e. replicates with different plants) were made for each treatment.

Onset of opening is defined as the time at which the last peak of diffusion resistance is achieved before the onset of consistent stomatal opening. Maximum opening (see Fig. 4) is the time of the first minimum in stomatal resistance after consistent stomatal opening. Onset of closure is the intercept of the slope of closure and the previous lowest value of diffusion resistance.

**Photosynthesis and Respiration.** Net photosynthesis and respiration were measured with an automated differential IR gas analyzer (Analytical Developments Co., Hoddesdon, Herts., England) operating on an open circuit and equipped with a purpose-built polycarbonate cuvette (volume 19.8 ml) which enclosed an 840 mm² area of both the adaxial and abaxial leaf surfaces. Ambient CO₂ concentrations of about 400 μl L⁻¹ were used for measurements and CO₂ standards were used for calibration. Data acquisition and system control were similar to that described for the porometers.

**Radiation Sources.** Red and FR (for Fig. 9, Tables I and II) were obtained by filtering the output from Sylvania F48T12/236/VHO and F48T12/232/VHO fluorescent lamps, respectively, through two layers of Roscolene (Rosco Laboratories, Port Chester, NY) 823 plastic filter. Blue (Table II) was derived from Sylvania F48T12/246/VHO fluorescent lamps wrapped in two layers of Roscolene 863 plastic film. A 50 mm band at the end of all lamps was covered with black tape to absorb radiated IR from the cathodes.

For photosynthesis measurements, the spectral characteristics of the fluorescent lamp WL source was simulated by filtering the output of a xenon arc source through one 3 mm Schott (Mainz, FRG) KG4 heat-absorbing filter and two Corion 705 nm cut-off (longer wavelength blocking) filters. Spectrally neutral layers of Al gauze were used to attenuate the radiation.

Radiation sources were measured with an EG & G Gamma Scientific (San Diego, CA) computer-controlled C-9 spectroradiometer system which was fitted with a double holographic grating monochromator providing a half-power bandwidth of 3 nm and scanned in 2 nm steps. The system was calibrated against a tertiary standard traceable to the National Bureau of Standards. Periodic checks of the WL used for rhythm entrainment were made with a cross-referenced Li-Cor LI-185B meter and LI-190SB quantum sensor.

**RESULTS**

Stomatal diffusion resistance of plants which have been grown in 12:12 h L:D cycles follows a circadian rhythm when the plants are placed in DD at the end of the photoperiod (Fig. 1). Two cycles of stomatal closure (increasing diffusion resistance) and stomatal opening (decreasing diffusion resistance) were usually observed. The mean period length was 24.24 h between onset of opening acrophases and 24.78 h between onset of closure acrophases. The rhythm damped rapidly after two cycles and three or more cycles were observed in only 27% of the observations. These circadian oscillations are considered to be phased by the L:D cycles alone because inverting the L:D cycles resulted in a 12 h shift in the phase of the rhythm. Also, possible effects of daily changes in the ambient CO₂ concentration in the building were excluded because no detectable difference was observed between experiments run during the working week and those run at weekends.

**Kinetics of Entrainment.** To ensure that the effect of all light treatments was a true reentrainment of the rhythm, the kinetics of reentrainment were studied during five consecutive inductive cycles following transference of the plants from 12 h to 6 h photoperiods. Plants which had been grown under 4:00 to 16:00 EST photoperiods were transferred to either 10:00 to 16:00, or 4:00 to 10:00 photoperiods, representing a 6 h delay in light-on and a 6 h advance in light-off. These experiments demonstrated that one inductive treatment does not produce a full reentrainment in the rhythm of either stomatal opening (Fig. 2A) or closure (Fig. 2B). In subsequent experiments, we have therefore taken the approach of studying the effects of entraining the plants from sowing to a particular L:D cycle.

**Light-on versus Light-off.** To determine the contributions of the potential light-on, light-off and photoperiod signals on free-running rhythms in stomatal aperture in DD, plants were sown and grown in each of the tested L:D cycles (Fig. 3) until 14 to 16 d old, then transferred to DD at the beginning of the entrained dark period for measurement of cyclic stomatal resistance changes. This provided information on the phase relationship of fully entrained plants to the various irradiation treatments. One
set of treatments consisted of advancing or delaying the time of light-on in 3 h steps, thereby producing 15, 18, and 21 h, or 9, 6, and 3 h photoperiods, respectively. In the other set of treatments, the time of light-off was either advanced or delayed in 3 h steps, producing 9, 6, and 3 h, or 15, 18, and 21 h photoperiods, respectively. The effects of these treatments on the phasing of stomatal opening (Fig. 4) and closure (Fig. 5) in DD were monitored.

**Dark Stomatal Opening.** In photoperiods which were shorter than 12 h, entrained plants showed a 1:1 relationship between the delay in the time of light-on and the delay in the phase of stomatal opening in DD (Fig. 4A).

In photoperiods of 15 h or longer, both the light-on (Fig. 4A) and light-off (Fig. 4B) signals influence the phasing of stomatal opening in DD, but direct effects on the clock are partially confounded by indirect effects of the last light treatment which could be detected by referring to the second cycle of stomatal opening in DD. Indirect effects are exhibited because the stomata require about 6 h to complete closure in DD at the end of the photoperiod (cf. Fig. 1) before dark opening can occur. In the case of the 18 h and 21 h photoperiods, the about 6 h requirement for initial dark closure precludes the physical ability of the stomata to exhibit more than about a 3 h advance in the onset of opening. This conclusion is supported by the observation that the phase of maximal opening (which takes place several h later than the onset and is therefore not restricted by early light-on signals) is advanced substantially in response to advances in the time of light-on (Fig. 4A).

The apparent delay in stomatal opening under long photoperiods caused by the delay in the time of light-off (Fig. 4B) cannot be ascribed to a direct effect on the stomatal clock. As with the extreme advances in the time of light-on described above, the stomatal mechanism requires a period of time to close after light-off and an artificial delay in phase is imposed before the onset of opening can occur. Again, a closer approximation of the dark opening rhythm is given by the phase of maximal opening (Fig. 4B).

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**Fig. 3.** Schematic description of the procedure used to compare the relative effectiveness of the light-on and light-off signals on the phase of rhythmic stomatal opening and closure in DD. Plants were grown for 14 to 16 d in the appropriate L:D regime before diffusion resistance measurements commenced at the start of the entrained dark period. The dark period was then extended to incorporate at least the first two acrophases of stomatal closure and opening.

**Fig. 4.** Comparison of the effectiveness of the light-on (A) and light-off (B) signals in shifting the phase of the onset of dark stomatal opening (●); × = phase shift in time of maximal opening. Vertical bars indicate twice the se.
Dark Stomatal Closure. In response to photoperiods of less than 12 h, both the time of light-on (Fig. 5A) and the time of light-off (Fig. 5B) influence the timing of the onset of stomatal closure in DD. The most marked effect on dark closure was the response under long (>12 h) photoperiods in which a 1:1 relationship was observed between the time of light-off and the time of dark stomatal closure (Fig. 5B). By contrast, the time of light-on had no influence on dark closure in long photoperiods (Fig. 5A). The requirement for a period of dark closure at the end of the last photoperiod did not influence the cyclic DD response; this was confirmed by referring to the second cycle of closure in DD (data not shown).

Duration of Dark Stomatal Opening. A linear correlation exists between duration of stomatal opening (i.e., onset of opening to onset of closure) in subsequent DD and photoperiod in plants entrained to photoperiods between 3 and 15 h in length (Fig. 6A). The plateau for 18 h and 21 h photoperiods is caused by the indirect light treatment effects described for stomatal opening in Figure 4A. When these are accounted for by relating stomatal opening to the phase in the second cycle in DD, an approximately linear relationship between duration of opening and all photoperiods between 3 and 21 h in length is observed (Fig. 6A).

If photosynthesis is not involved in the phasing of the dark stomatal rhythm, then the rate of photosynthesis will have no influence on the duration of dark stomatal opening for any given photoperiod. This was tested for plants sown and grown under 6 h photoperiods receiving 200 instead of 100 μmol m$^{-2}$ s$^{-1}$ WL (400–700 nm). Net daily CO$_2$ uptake under 200 μmol m$^{-2}$ s$^{-1}$ WL in 6 h photoperiods was equivalent to 10.3 h under 100 μmol m$^{-2}$ s$^{-1}$ WL (Fig. 6B). The effect of the daily 200 μmol m$^{-2}$ s$^{-1}$ light treatment was to increase the duration of dark stomatal opening in proportion to the increase in daily CO$_2$ uptake (● in Fig. 6A). An influence of photosynthesis on the phasing of the dark stomatal rhythm is clearly inferred.

Respiration. As energy cycling was considered to be a possible basis for circadian rhythmicity, we attempted to measure dark stomatal movements in photosynthetically incompetent plants treated with Norflurazon. This herbicide inhibits carotenoid biosynthesis, thereby allowing Chl photodestruction (1) and preventing daily photosynthesis. The plants exhibited no detectable rhythm in stomatal movement in DD (Fig. 7). Also, there was no detectable rhythm in respiration in DD (data not shown). This contrasts with normal green plants which showed a marked circadian rhythm in CO$_2$ output in DD (Fig. 8). The possibility that this rhythm was an artificial product of daily ambient changes in CO$_2$ concentration perfusing through the plant from outside the cuvette was excluded because inverting the 12:12 h L:D period resulted in a 12 h shift in the rhythm.

![Fig. 5. Comparison of the effectiveness of the light-on (A) and light-off (B) signals in shifting the phase of the onset of dark stomatal closure. Vertical bars indicate twice the se.](image1)

![Fig. 6. A. Duration of dark stomatal opening (onset of opening to onset of closure) as a function of photoperiod and net daily CO$_2$ uptake in entrained plants grown under standard 100 μmol m$^{-2}$ s$^{-1}$ WL (○). O, corrected duration of opening (onset of opening to onset of closure in second cycle in DD) which allows for interference of light treatment with the true dark rhythm. ●, Entrained plants grown under 6 h photoperiods, but 200 μmol m$^{-2}$ s$^{-1}$ WL and plotted as a function of net daily CO$_2$ uptake, not photoperiod (for details, see text). Error bars are twice the root mean square of the se of the times of onset of opening and onset of closure. B. Photosynthetic CO$_2$ uptake as a function of fluence rate (400–700 nm) in a primary leaf of P. vulgaris L. A sample measurement is depicted for a single leaf, taken over an approximately 4 h period near the middle of an entrained 6 h photoperiod.](image2)
Phytochrome Involvement. Exhaustive attempts to photo-induce a phase shift in the rhythm of stomatal opening or closure which could be ascribed to phytochrome were unsuccessful. Hourly 5 min pulses with R light (30 μmol m⁻² s⁻¹) were perceived as darkness when given as a substitute for WL in either the first, or last, 6 h of the standard 12 h photoperiod. Phytochrome did, however, modulate the amplitude of the rhythm by determining the extent of opening in DD. Removal of Pfr with 5 min FR radiation at the end of the standard 12 h photoperiod resulted in initially faster stomatal closure but no effect on the extent of closure (Fig. 9). Although there was no effect on the time of opening, the magnitude of opening in DD was markedly reduced following end-of-d treatment with FR. The response was FR/R reversible (Table I). A notable feature of FR-treated plants was a much faster damping of the rhythm in extended DD.

Table II compares the effect of a single cycle of either monochromatic blue or R in substituting for WL during the first 8 h of a 12 h photoperiod (cf. effect of single cycle in Fig. 2). The substantial delay in the onset of dark stomatal opening caused by an 8 h delay in the time of light-on is largely substituted for by both blue and R radiation. These observations do not support the concept that phytochrome is the responsible photoreceptor because the quantum effectiveness of blue for phytochrome photoconversion is approximately 50-fold less than R (22). The possibility that the phytochrome high irradiance reaction is responsible is excluded because this reaction has never been proven to exist in green plants (10) using the argument put forward by Hartmann (6) for dark-grown seedlings. On the other hand, the spectral response to blue and R is compatible with the action spectra of photosynthesis (4) and photorespiration (5).
DISCUSSION

Three paired factors point to the conclusion that the rhythm of dark stomatal opening in *P. vulgaris* is controlled primarily by the light-on signal and that the rhythm of dark stomatal closure is related to the light-off signal. First, 1:1 rephasing (i.e. a 1 h phase shift in response to a 1 h shift in signal) is only observed between a delay in light-on and stomatal opening (Fig. 4A), and between a delay in light-off and stomatal closure (Fig. 5B). Second, phase advances in stomatal opening are only observed in response to advances in light-on (Fig. 4A), and advances in closure only in response to light-off (Fig. 5B). Third, only light-off signals can have no effect on opening (Fig. 4B), and only light-on signals can have no effect on closure (Fig. 5A).

Thus, although stomatal opening and stomatal closure in DD must be operationally linked, the evidence points to a dual control of the clock which controls the circadian rhythm. On the basis that energy cycling may act as a basic oscillator in circadian rhythm (2, 3, 20), one possibility is that the light-on signal triggers the clock for dark stomatal opening via the onset of photosynthesis, while the light-off signal triggers the clock for dark stomatal closure via the cessation of photosynthesis. Referring to the data in Figure 6, a more likely possibility is that the light-on signal may trigger the clock and an integrated product of photosynthesis (e.g. photosynthetic rate x time) may determine the phasing of the onset of dark closure. Although phytochrome has been proposed as being the photoreceptor responsible for entraining stomatal circadian rhythms (12, 13, 17, 25), R/FR photoreversibility has never been demonstrated. Our extensive attempts to demonstrate phytochrome involvement either by substitution of 6 h of the WL period with hourly 5 min R light pulses (which were potentially FR reversible), or by end-of-day R/FR photoreversibility, were unsuccessful. Phytochrome effects on the amplitude of the rhythm were observed, but these can be explained on the basis of phytochrome-modulated transmembrane ion transport (8) and do not infer interaction with the circadian clock.

Substantial circumstantial evidence points to Chl as being the photoreceptor responsible for light-mediated phase control of stomatal opening and closure in DD. First, there is a strong correlation between photoperiod (or daily net CO₂ fixation) and phase shift (Fig. 6). Second, increasing the fluence rate during the photoperiod results in a phase shift in proportion to the increase in daily photosynthesis (Fig. 6). Third, photosynthetically incompetent plants do not exhibit a stomatal rhythm in DD (Fig. 7). Fourth, the spectral sensitivity of the photoreceptor responsible for phase-shifting is representative of Chl (Table II). Conclusive evidence may be provided by an action spectrum for the light-mediated response.

LITERATURE CITED


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