16. Test cultures were 10-ml portions of the cell-free filtrate of a producer culture, enriched with macronutrients by the addition of 1½ percent enrichment seawater I (*14*), and inoculated with the assay organism (that is, with a predecessor or successor of the dominant organism).
17. Control cultures were identical to test cultures except that the controls were autoclaved after filtration and before macronutrient addition.
18. Additional effects are considered in (*13*). The few heat-stable effects noted were consistent with bloom sequence; however, since such effects were not sought, there are probably many additional heat-stable effects which were not noted and whose effects cannot be properly considered in this study.
19. Because the allelopathic effects of a dominant species cannot occur prior to that species occurring at density, the term predecessor is limited to bloom organisms whose growth at least minimally overlaps the dominant species in question.
20. The term successor includes blooms which occurred within approximately 2 weeks of the dominant in question.
21. Because freezing sometimes produced changes in heat-labile effects, generally decreasing them, these data are considered less reliable than the others.
22. I thank L. Provasoli and G. E. Hutchinson for guidance; R. Patrick, S. Golubic, and F. Drouet for assistance with taxonomic questions; Haskins Laboratories, Yale University, and the Department of Environmental Science, Rutgers University, for support. This work was supported in part by EPA research grant RA 801387.

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Light and Stomatal Function: Blue Light Stimulates Swelling of Guard Cell Protoplasts

Abstract. *Onion guard cell protoplasts swell when illuminated with blue light. The response is a 35 to 60 percent increase in volume and is dependent on potassium ion. Epidermal cell protoplasts do not swell under the same conditions. It is postulated that a membrane-bound blue photoreceptor mediates a direct response of guard cells to light.*

Protoplasts isolated from onion guard cells by enzymatic digestion swell when illuminated with broadband blue light (peak at 410 nm). Evidence suggests that the response, which is K^+-dependent, is a manifestation of normal guard cell activities. We envision the light-stimulated mechanism as an activation of a membrane-bound electron transport chain that results in an influx of K^+ into the cell. The resulting decrease in water potential mediates an influx of water, which leads to an increase in protoplast volume.

Guard cells surround the stomatal pores on the leaf epidermis of higher plants. They control the gas and water content of the leaf by swelling and contracting, thus respectively opening and closing the stomata. Light induces opening of intact stomata (*1*, *2*). Previous studies on different species have shown that the blue, red, and far-red regions of the spectrum are effective, with blue being the most active on a quantum basis. Chlorophyll has been implicated as the photoreceptor because of the similarity between its absorbing properties and the action spectrum of stomatal opening (*1*, *2*). However, because that view fails to explain the greater sensitivity of stomata to blue light (*3*), it is possible that other pigments play a role.

The mechanism by which light affects stomatal action remains controversial. There is substantial evidence that light is sufficient (*4*, *5*) but not necessary for stomatal opening, since, in the dark, stomata open in low concentrations of CO_2 and close in high concentrations (*6*). It is most commonly accepted that all light responses are indirect and actually mediated by changes in the concentrations of CO_2 resulting from photosynthesis (*1*, *2*, *7*). However, the possibility that guard cells might have specific mechanisms that respond directly to light or that both light and CO_2 can act directly on the same receptor deserves consideration. Changes in turgor pressure that cause stomatal opening and closing are mediated by massive transport of K^+ to and from the guard cells (*7*); it thus seems possible that light, which is known to drive ion movements in several systems (*8*), might directly stimulate a similar membrane transport phenomenon in guard cells. Our findings, as well as many known features of stomatal action, seem best accounted for if light is a primary activator.

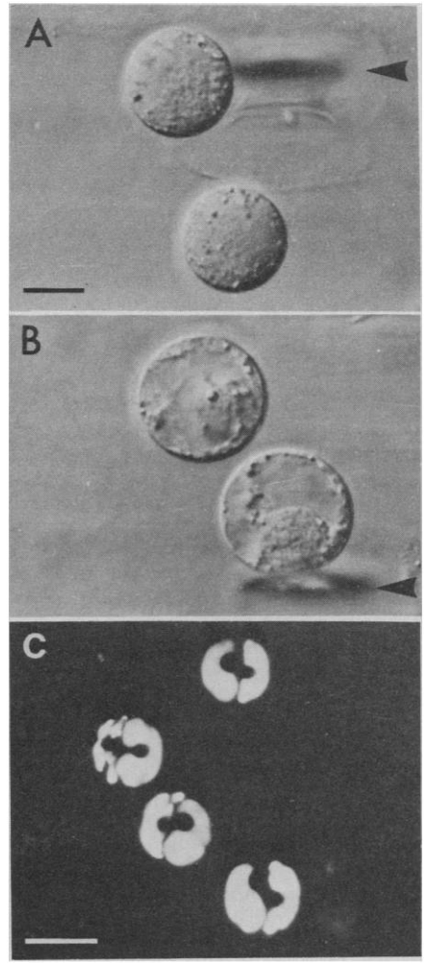

Fig. 1 (A and B). Photomicrographs of protoplasts from onion guard cells. Arrows indicate the undigested stomatal ridges that surround the pores. Scale bar, 10 μm. (A) Typical protoplasts in 0.45*M* mannitol and 0.5 m*M* $CaCl_2$, unexposed to light, showing a dense, granular cytoplasm. (B) Typical swollen protoplasts after a light treatment. A prominent vacuole occupies most of the cell volume. (C) Photomicrograph of an onion epidermal peel seen under dark-field fluorescence microscopy. Scale bar, 40 μm. An excitation filter (Reichert BG12) (peak at 410 nm) and a barrier filter (Reichert Sp3) with a 50 percent cut-off at 500 nm were used. The guard cells but not the neighboring epidermal cells fluoresce in the green. The cell wall and the nucleus are opaque.

Guard cell protoplasts are prepared in microchambers by digesting thin paradermal slices from 7- to 10-day-old onion cotyledons with 4 percent (weight/volume) Cellulysin in a 0.23*M* mannitol solution (*9*). After 8 to 14 hours of digestion, the spherical protoplasts (Fig. 1A) can easily be recognized because (i) they are smaller than epidermal or mesophyll protoplasts and lack visible chloroplasts (*10*); (ii) they display a characteristic dense, granular cytoplasm with little evidence of a vacuole; and (iii) they usually remain in the vicinity of the undigested ridge that surrounds the pore. Guard cell protoplasts kept in mannitol in the dark stream actively for several hours. However, they swell steadily and finally burst. This "dark" swelling is usually reduced by adding 0.5 m*M* $CaCl_2$ to the medium. Only preparations with protoplasts averaging less than 23 μm in diameter were used in the experiments.

A typical experiment included an overnight enzyme digestion followed by a wash with 0.3 to 0.5*M* mannitol plus 0.5 m*M* $CaCl_2$. The chambers were left in the dark for 1 to 3 hours, and a solution of 0.32*M* mannitol, 0.5 m*M* $CaCl_2$ and 30 m*M* KCl was introduced just before the measurements of protoplast diameter. Measurements were made with a split-image eyepiece (Vikers) operated at ×150 to ×375 with a green interference filter transmitting at 546 nm. Protoplasts

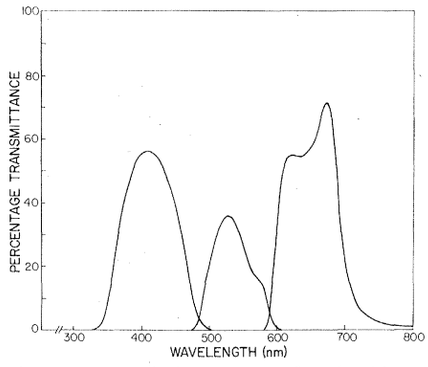

Fig. 2. Transmission spectra for the blue, green, and red filter combinations used in the light experiments, obtained in a spectrophotometer (Perkin-Elmer model 365).

were selected randomly by measuring those that fell within several defined fields. In each chamber, 40 to 80 protoplasts were measured and their diameters averaged. Immediately after a 60-minute light treatment (*11*), a second size determination was made. Transmission spectra for the filters were obtained from a spectrophotometer (Perkin-Elmer model 365) (Fig. 2). Light energies were determined with a thermopile (Eppley).

In the light, the protoplasts increase in volume over the dark controls from 35 to 60 percent (Fig. 1B and Table 1) and K^+ enhances the response substantially (Table 1). Blue was the only effective region of the spectrum. No difference was found between treatments with high- and low-intensity blue light, which suggests that the response is already saturated at 11500 erg cm^{-2} sec^{-1} (about 2.4×10^{15} quanta cm^{-2} sec^{-1}). A 50 percent increase in protoplast volume implies a 0.2*M* increase in osmotic concentration inside the cells, which is equivalent to about 5 bars. The 50 percent increase is probably a lower estimate because of the bursting of markedly swollen protoplasts.

Red light was ineffective in three experiments; in a fourth, a minor swelling above the controls was observed (*12*). We were unable, however, to reproduce the small red response and therefore conclude that blue light was the only wavelength able to generate significant changes in the water potentials of the protoplasts. The guard cell protoplasts responded to blue light only, regardless of the presence or absence of mesophyll-cell protoplasts in the microchambers (*13*).

The swelling of the protoplasts is probably a manifestation of normal guard cell activity, because (i) intact guard cells swell when the stomata open (*2*), (ii) like stomatal opening, swelling is K^+-dependent, and (iii) swelling occurs only in guard cell protoplasts and not in neighboring epidermal cell protoplasts (Table 2).

Experimental separation of the blue- and red-light stimulation of stomatal opening has been achieved in previous studies. Although blue light is effective at lower light intensities (*3, 5*) and causes more rapid responses (*14*), the red effect is always present. In contrast, onion guard cell protoplasts respond only to blue; even at very high intensities (*12*), red is ineffective. We interpret the lack of a red response as an actual physical uncoupling of the blue and blue-red responding systems. To explain the uncoupling, we reason that, in the intact leaf, the blue-red response is due to the absorbing properties of the chlorophyll in the mesophyll cells, which acts as a photoreceptor for the CO_2-related effects. A second photoreceptor, located in the membrane of the guard cell proper, could cause the enhanced stomatal response in the blue region of the spectrum (*15*). Onion guard cell protoplasts, lacking well-developed chloroplasts and devoid of any physical connections with mesophyll cells, should lack the red response and react only to blue light. Similarly, intact onion stomata in paradermal slices, which include several layers of mesophyll cells, should respond to both blue and red light, but stomata in epidermal peels should respond only to blue. This we observed (*16*).

A separate line of evidence suggesting that onion guard cells contain a blue-light-absorbing substance comes from our findings that these cells and protoplasts prepared therefrom display a characteristic, green (maximum 525 to 535 nm) autofluorescence (Fig. 1C) when irradiated with blue light (filter maximum at 410 nm). Neighboring epidermal cells lack the response. In swollen protoplasts, the fluorescence is located in the vacuole. When protoplasts burst, after the osmolarity of the medium has been lowered, the emerging vacuole retains all the fluorescence, and the rest of the cell debris appears opaque, with the sole exception of red-fluorescing chloroplasts.

The bursting of the vacuole, however, does not lead to a diffuse fluorescence; instead, smaller fluorescing vesicles are formed, which suggests that the fluorescence arises from the tonoplast (*17*). The fact that the fluorescing substance absorbs in the blue and fluoresces in the green suggests that it might be a flavin or a flavoprotein (*18*). Flavins have been implicated as photoreceptors in electron transport chains (*19*).

The specific mechanism underlying the effect of light on stomata remains unknown. In the purple membrane of *Halobacterium*, a photoreceptor, bacteriorhodopsin, extrudes protons when irradiated (*20*). A similar mechanism could exist in the stomatal complex, where a blue-light receptor, bound to the plasmalemma or tonoplast, or both, might initiate an electron flow when energized with blue light. A resulting separation of OH^- and H^+ across the membrane would then establish a charge and *p*H gradient capable of driving the uptake of K^+ (*21*). Although highly speculative, this model provides an effective way of probing the cellular nature of stomatal action.

Table 1. Swelling of guard cell protoplasts in response to light. Light intensities are given in (*11*). A vertical arrow indicates that the preparation received an additional treatment within 10 minutes after the preceding one.

Light treatment	Diameter (μm ± S.E.)	
	Initial	Final
White	21.3 ± 1.8	24.7 ± 2.1*
White (no K^+)	22.1 ± 2.1	23.1 ± 1.9†
Dark	20.0 ± 1.6	20.5 ± 1.4
↓		↓
Blue		23.1 ± 1.7*
Low blue	21.3 ± 1.7	24.1 ± 1.8*
High blue	21.1 ± 1.3	23.9 ± 1.9*
Low red	21.1 ± 1.8	21.9 ± 1.9
High red	19.4 ± 1.0	20.0 ± 1.3
↓		↓
High blue		21.9 ± 1.9*

*Final diameter significantly different ($P < .001$, two-tailed *t*-test) from the initial one. †Significantly different ($P < .01$, two-tailed *t*-test) from white light treatment.

Table 2. Swelling of guard cell and epidermal cell protoplasts in response to light. Pairs of values for individualized cells were obtained before and after a 60-minute exposure to white light in experiment 1 and blue light in experiment 2.

Source of protoplasts	*N*	Diameter (μm ± S.E.)	
		Initial	Final
Guard cell			
Exp. 1	11	22.9 ± 1.5	24.2 ± 1.7
Exp. 2	22	16.1 ± 3.3	18.9 ± 3.3*
Epidermal cell			
Exp. 1	17	47.0 ± 9.6	46.4 ± 8.7
Exp. 2	17	47.2 ± 4.7	47.3 ± 5.0

*Final diameter significantly different ($P < .01$, two-tailed *t*-test) from the initial one.

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10. Intact guard cells observed under fluorescence microscopy show small, red-fluorescing organelles that are probably chloroplasts. Onion guard cells are known to have small, poorly developed yet active chloroplasts [M. Shaw and G. A. Maclachlan, *Can. J. Bot.* **32**, 784 (1954)]. They are also distinct because they lack starch [O. V. S. Heath, *New Phytol.* **48**, 186 (1949)].
11. The light source was a 12-volt tungsten halogen lamp (Sylvania) in a lamp housing and filter holder [Reichert (Vienna, Austria) Zetopan] used with a constant voltage transformer (± 3 percent) (Sola), and a stepwise 12 volt transformer (Reichert). A heat filter (Reichert 2KG 2/g) and a 2-cm ice-cold water filter were used in all treatments. Filter combinations (all from Reichert, except when noted) and light intensity at the microchamber level (ergs per square centimeter per second) were: low-intensity blue, blue filter BG 12/h, 11,540; high-intensity blue, blue filter BG 12/h, 34,630; low-intensity red, neutral density filter NG3, red filter RG610, Wratten filter Kodak 301, 55,800; high-intensity red, red filter RG610, Wratten filter Kodak 301, 317,460; green, green filter VG 9/h and green interference filter λ = 546 mm, 19,240; white, neutral density filter NG 3, 50,500. Identical filter combinations gave different light intensities when the distance between the source and the chamber was changed. An infrared filter (Corning CS7-56), added to all filter combinations, gave infrared and heat readings which allowed us to obtain corrected values for visible light intensity. The temperature inside the microchambers, monitored with a thermocouple pair and a laboratory thermometer (Bayley Bat-4) was 22° to 24°C throughout the light exposure.
12. Protoplasts under high-intensity red light showed a 7 percent increase in volume beyond dark controls, as compared with a 38 percent increase under low-intensity blue light. The blue-red quantum ratio was 1 : 44.
13. Preparations that either include or are largely free of mesophyll-cell protoplasts can be obtained by adequate manipulation of digestion times and washing procedures (*9*).
14. T. Brogårdh, *Physiol. Plant.* **35**, 303 (1975).
15. A blue-light photoreceptor in the guard cells has been postulated (*5*, *14*).
16. Epidermal peels and paradermal slices of young onion cotyledons (*9*) were mounted in the microchambers in 0.15 and 0.23*M* mannitol, respectively. After 2 to 3 hours in the dark, 50 m*M* KCl (epidermal peels) and 30 m*M* KCl (paradermal slices) were introduced; the pore size of 30 to 50 stomatal complexes per chamber were measured with a split-image eyepiece (Vickers) under green light. A 90-minute light treatment (*11*) was given and a second pore size determination made. The results (pore size in micrometers at the beginning and end of treatment ± standard error) were, epidermal peels: blue light 6.3 ± 1.0, 7.2 ± 1.2; blue light without K^+ 6.8 ± 0.9, 6.5 ± 1.0; red light 6.5 ± 1.0, 6.4 ± 1.1; same preparation to blue light 7.1 ± 1.1; dark 6.2 ± 0.6, 6.4 ± 0.8; same preparation to blue light 7.0 ± 0.9; paradermal slices: blue light 5.6 ± 0.7, 7.5 ± 1.1; red light 4.6 ± 0.9, 5.9 ± 1.0; red without K^+ 6.2 ± 1.1, 6.4 ± 1.0; dark 4.5 ± 0.6, 4.7 ± 0.9; same preparation to blue light 7.2 ± 1.1. Each experiment was repeated once, with similar results. The results with paradermal slices agree with previous work done with onion leaves [H. Meidner, *J. Exp. Bot.* **19**, 146 (1968)]. The results with epidermal peels are small in magnitude, yet reproducible. No further attempts to increase the response were made.
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22. We thank Dr. W. Briggs and S. Britz of the Carnegie Institution of Washington for many helpful discussions and the use of equipment. We also thank S. Westrate of Scientific Instrument Corporation for generous assistance with equipment. Supported by NSF grant BMS 74-15256 to P.K.H.

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Newly Evolved Repeated DNA Sequences in Primates

Abstract. *Repeated DNA sequences in primates having identical or nearly identical members and exhibiting unusual phylogenetic specificity were analyzed. They appeared in repeated DNA sequences in each group of primates, probably within the last 10 to 15 million years, and are conserved to the same extent as unique DNA sequences. The finding allows a new approach to the construction of evolutionary trees.*

The DNA genomes of mammals and other metazoans consist of nucleotide sequences present once per haploid genome (single-copy or unique sequences) and sets of sequences that are reduplicated many times, called families of repeated sequences (*1*). Most of the DNA sequences that are in the repeated class are repeated 1000 times or more; only a small fraction of repeated DNA consists of sequences repeated less than 1000 times.

It was proposed that repeated sequences arose by the amplification of sequences previously present one or only a few times per haploid genome (*2*) and that sequences amplified in the distant evolutionary past now consist of repeated families whose members are only distantly related to one another, while sequences amplified recently consist of repeated sets whose members are mutually closely related or identical (*1*, *2*). Since repeated sequences are at a higher concentration in DNA than single-copy sequences they reanneal more rapidly (that is, at low C_0t, where C_0t is the concentration of nucleotides in moles per liter and

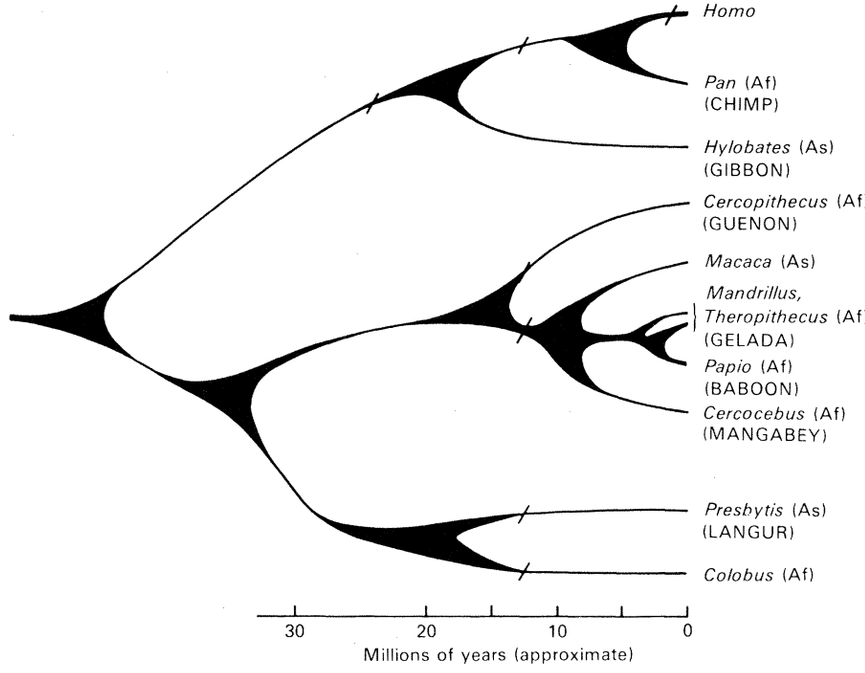

Fig. 1. Time of appearance of newly repeated DNA sequences in primates. The slash marks on the phylogenetic tree indicate the time of appearance of newly evolved repeated DNA sequences in primates, on the basis of the hybridization results of Table 1 and the unpublished experiments cited in the text.