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Reversal of Blue Light-Stimulated Stomatal Opening by Green Light

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Blue light-stimulated stomatal opening in detached epidermis of *Vicia faba* is reversed by green light. A 30 s green light pulse eliminated the transient opening stimulated by an immediately preceding blue light pulse. Opening was restored by a subsequent blue light pulse. An initial green light pulse did not alter the response to a subsequent blue light pulse. Reversal also occurred under continuous illumination, with or without a saturating red light background. The magnitude of the green light reversal depended on fluence rate, with full reversal observed at a green light fluence rate twice that of the blue light. Continuous green light given alone stimulated a slight stomatal opening, and had no effect on red light-stimulated opening. An action spectrum for the green light effect showed a maximum at 540 nm and minor peaks at 490 and 580 nm. This spectrum is similar to the action spectrum for blue light-stimulated stomatal opening, red-shifted by about 90 nm. The carotenoid zeaxanthin has been implicated as a photoreceptor for the stomatal blue light response. Blue/green reversibility might be explained by a pair of interconvertible zeaxanthin isomers, one absorbing in the blue and the other in the green, with the green absorbing form being the physiologically active one.

Key words: Blue light — Green light — Guard cells — Stomata — *Vicia faba* — Zeaxanthin.

Guard cells have an intrinsic blue light response that is one of the components of light-driven stomatal movement (Zeiger 1990, Tallman 1992, Assmann 1993, Kinoshita and Shimazaki 1999). Under a saturating red light background (which eliminates the photosynthesis-based response to blue light resulting from guard cell chlorophyll), blue light-stimulated stomatal opening has an action spectrum showing a maximum at 450 nm and minor peaks at 420 and 470 nm (Karlsson 1986). The chloroplastic carotenoid zeaxanthin has been implicated as the photoreceptor for this blue light response (Zeiger and Zhu 1998, Frechilla et al. 1999).

The present study shows that green light, either applied together with blue light in experiments using continuous exposures or applied as a short, bright pulse immediately following a blue light pulse, reverses blue light-stimulated stomatal opening.

Several responses to green light have been reported. For example, the action spectra for blue light-stimulated phototropism in *Arabidopsis* and lettuce extend into the green (Steinitz et al. 1985), and phytochrome-dependent green light responses have been documented (Mandoli and Briggs 1981). In guard cells, the action spectrum for stomatal opening driven by guard cell photosynthesis shows a minor sensitivity to green light (Sharkey and Ogawa 1987). All of these green light responses, however, work in the same direction as the response to blue light. The green light-dependent response reported here has the distinct characteristic of reversing the response to blue light in a manner analogous to the far red reversal of phytochrome responses. These results suggest that the reversal of blue light-stimulated stomatal opening by green light is the expression of a novel photobiological response of plant cells.

Materials and Methods

Plant material and growth conditions—Seeds of *Vicia faba* L. cv. Windsor Long Pod (Bountiful Gardens Seeds, Willits, CA, U.S.A.) were planted in pots with commercial potting mix (Sunshine mix #1, American Horticultural Supply, Camarillo, CA, U.S.A.). Plants were grown in a greenhouse under natural light, 50–75% RH, 25–30°C day/15–20°C night. Plants were watered 3 times a day with an automatic watering system and fertilised once a week (20–10–20 mix, Grow-More Research and Manufacturing Co., Gardena, CA, U.S.A.).

Preparation of detached epidermal strips and aperture measurements—Fully expanded leaflets from the second and third internodes were harvested early in the morning. Abaxial epidermis was carefully stripped by hand from the interveinal regions into 0.1 mM $CaCl_2$. The peels were then rinsed with distilled water and placed in an incubation solution containing 1 mM MES-NaOH buffer (pH 6.0), 0.1 mM $CaCl_2$, and 1 mM KCl. Initial apertures were determined from measurements of 30–40 digitised video images of stomata in epidermal peels using an Olympus BH-2 microscope connected to a Javelin JE2362A digital imaging camera. Image processing was handled with an IBM PC-based MV-1 image analysis board (Metrabyte Corp., Taunton, MA, U.S.A.) and JAVA image analysis software (Jandel Scientific, Corte Madera, CA, U.S.A.).

Opening experiments in response to light pulses—Detached peels were incubated in the solution described above under 120 $\mu mol\ m^{-2}\ s^{-1}$ background red light. Initial stomatal apertures were obtained after 50 min, at which time red light-induced opening is saturated (Talbott and Zeiger 1993). Sixty min after the onset of red light irradiation, 30 s light pulses were applied, as

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described in Results, below. Aperture measurements were obtained at 10 min intervals for an additional 40 min, at which time the stomatal response to the pulses was completed. The incubation solution was aerated with air containing 400 μl liter^{-1} CO_2 and maintained at 23°C throughout the treatment period. The set-up for red/blue illumination was as described previously (Talbott and Zeiger 1993), except that illumination with blue and green light was given using bifurcated fiber optics (Edmund Scientific, Barrington, NJ, U.S.A.). Red light was provided by a red filter (No. 2423 Plexiglas, 50% cut-off 595 nm, Rohm and Haas, Hayward, CA, U.S.A.) using Sylvania 300 W 300PAR56/2MFL Cool Lux floodlamps (GTE Products Corp., Winchester, KY, U.S.A.) as the light source. Blue and green light pulses were provided by a blue Plexiglas filter (No. 2424 Plexiglas, 470 nm maximum, half-bandwidth 100 nm, Rohm and Haas) and a green broad-band filter (50% cut-offs at 505 and 560 nm) respectively, using a xenon arc lamp (model PS1000sw-1, ILC Technology, Sunnyvale, CA, U.S.A.) as the light source. Light fluence rates were measured with a Li-Cor quantum sensor (Li-Cor Inc., Lincoln, NE, U.S.A.).

Opening experiments under continuous light—Detached peels were dark-adapted for 60 min in the incubation buffer described above. Baseline stomatal apertures were obtained at the end of this pre-treatment. Stomata were then illuminated with different light treatments for an additional 90 min interval. Final aperture measurements were obtained at the end of the light treatment. The incubation solution was aerated with air containing 400 μl liter^{-1} CO_2 and maintained at 23°C throughout the dark adaptation and light treatments. Background red light was provided as described above. Simultaneous blue and green light illumination were generated using the bifurcated fiber optics and filters described above, except that Sylvania DAH 500 W incandescent projector bulbs were used as the light source.

Action spectrum for the reversal of blue light-stimulated opening by green light—Detached epidermis was dark-adapted for 60 min in the incubation solution and baseline apertures were determined at the end of the treatment. Stomata were then exposed to continuous 10 μmol m^{-2} s^{-1} blue light as described above for continuous light experiments. An action spectrum for the reversal of blue light-stimulated stomatal opening by green light was obtained by measuring the reversal effect of added light in the 480–610 nm portion of the spectrum, tested at 10 nm intervals. At each wavelength, the inhibitory effect of 5, 10 and 20 μmol m^{-2} s^{-1} added light was determined.

Light was generated as described above for continuous light experiments, except that green light was obtained by passing light from the xenon arc lamp source through narrow bandwidth interference filters (10±2.5 nm bandwidth, Oriel Corporation, Stratford, CT, U.S.A.). The three different fluence rates of green light were obtained by varying the distance between the tip of the fiber optic and the sample.

Results

The specific stomatal response to blue light in detached epidermis can be measured without interference from blue light-stimulated guard cell photosynthesis in three different ways: in response to short blue light pulses, which activate the specific blue light response but not photosynthesis (Iino et al. 1985, Shimazaki et al. 1986); under continuous illumination with weak (<20 μmol m^{-2} s^{-1} in *Vicia*) blue light, which activates the blue light response but not guard cell photosynthesis (Travis and Mansfield 1981, Schwartz and Zeiger 1984, Talbott and Zeiger 1993); and in dual beam experiments, in which weak blue light is given in a background of red light that saturates guard cell photosynthesis (Ogawa 1981, Tallman and Zeiger 1988). We tested the reversal of blue light-stimulated stomatal opening by green light in all three conditions.

Pulse experiments—Stomata in the intact leaf open transiently in response to blue light pulses (Iino et al. 1985). The opening response continues at maximal rates for several minutes after pulse application, and stomatal apertures return to baseline levels upon completion of the response (Iino et al. 1985).

In the present study, we used the fast data acquisition capability of a computerised image system to test whether stomata in detached epidermis respond to blue light pulses. Stomata were kept under continuous red light (120 μmol m^{-2} s^{-1}), and blue light pulses were applied 60 min after the onset of the red light irradiation, a time at which the red light-dependent stomatal opening had reached steady state (Fig. 1). Exposure to a 30 s pulse of blue light under a red light background resulted in a transient increase in aperture that peaked approximately 20 min after pulse application (Fig. 1). Apertures returned to baseline levels

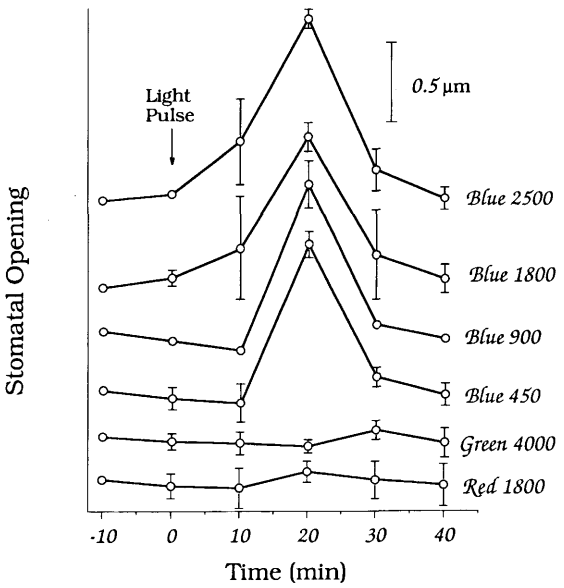

Fig. 1 Effect of 30 s blue light pulses on stomatal apertures in a red light background. Stomata were illuminated with 120 μmol m^{-2} s^{-1} continuous red light from 60 min prior to the light pulse to the end of the experiment. At time 0, 30 s light pulses of 450, 900, 1,800 or 2,500 μmol m^{-2} s^{-1} blue light were given and aperture measurements taken at 10 min intervals. Control treatments of 30 s pulses of 1,800 μmol m^{-2} s^{-1} red light or 4,000 μmol m^{-2} s^{-1} green light are also shown. Results are averages of 3–6 experiments±SD.

within 40 min of the pulse. Blue light pulses of 450 or 900 μmol m^{-2} s^{-1} applied for 30 s elicited nearly maximal aperture increases (Fig. 1). At higher fluence rates (1,800 or 2,500 μmol m^{-2} s^{-1}) apertures were only slightly greater, and the main effect of the higher doses was a faster opening time course, with progressively more opening occurring within 10 min of exposure to the pulse (Fig. 1).

Stomata responded to two consecutive 30 s pulses of 1,800 μmol m^{-2} s^{-1} blue light given 40 min apart with identical transient increases in aperture (data not shown). Pulses of 1,800 μmol m^{-2} s^{-1} red light, applied for 30 s on a background of continuous red light, did not elicit any transient opening, nor did they change baseline apertures (Fig. 1).

When a 30 s, 1,800 μmol m^{-2} s^{-1} blue light pulse, applied under 120 μmol m^{-2} s^{-1} red light background, was immediately followed by a 30 s exposure to 3,600 μmol m^{-2} s^{-1} green light, the effect of the blue light was nearly completely reversed (Fig. 2). A second 30 s exposure to 1,800 μmol m^{-2} s^{-1} blue light given immediately after the green light pulse restored the transient stomatal opening, although with slightly delayed kinetics (Fig. 2). The reversibility of the response to a blue light pulse by a subsequent green light pulse was consistently observed in 9 experiments. A typical response to a blue light pulse was seen when a 30 s pulse of 3,600 μmol m^{-2} s^{-1} green light was given prior to a 30 s pulse of 1,800 μmol m^{-2} s^{-1} blue light (data not shown).

Green light pulses of up to 4,000 μmol m^{-2} s^{-1} applied in a red light background did not have any effect on baseline apertures (Fig. 1). Since baseline apertures under the red light background are steady-state apertures, they would be expected to change in response to the bright green pulses if green light activated a specific photoresponse regulating stomatal apertures.

Continuous illumination experiments with weak blue light—Typical increase in stomatal aperture after 90 min of continuous exposure to 10 μmol m^{-2} s^{-1} blue light averaged 1.8 μm over baseline aperture (Fig. 3, inset). Simultaneous exposure to an equal fluence rate of green light reduced stomatal opening by an average of 47±9% (Fig. 3). Exposure to 20 μmol m^{-2} s^{-1} green light, or twice the blue light fluence rate, completely reversed the stomatal response to blue light (107±11% reduction, Fig. 3). Similar results were obtained in experiments using 20 μmol m^{-2} s^{-1} blue light. Under 30 and 40 μmol m^{-2} s^{-1} blue light, twice the fluence rate of green light only partially reversed the blue light-stimulated opening (data not shown). The inability of green light to fully reverse blue light-stimulated opening at these higher fluence rates could be accounted for by the fact that at rates above 20 μmol m^{-2} s^{-1}, blue light absorption by chlorophyll activates stomatal opening driven by guard cell photosynthesis (Schwartz and Zeiger 1984).

Continuous illumination experiments with blue light in a background of saturating red light—Green light also reversed the stomatal response to blue light under a saturating red light background (Fig. 4). As reported in previous studies (Schwartz and Zeiger 1984, Talbott and Zeiger

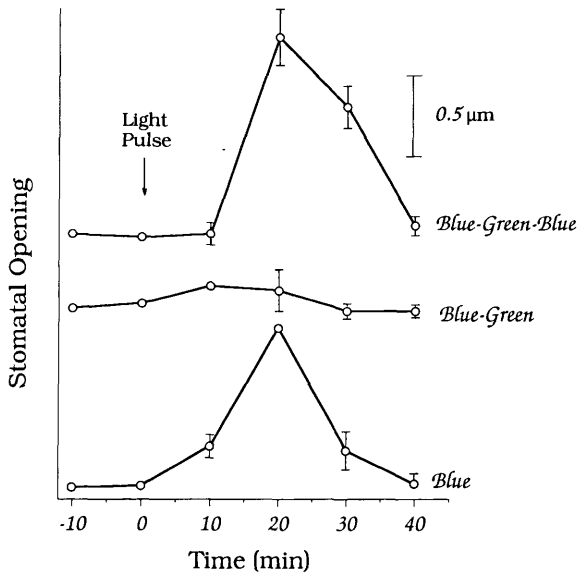

Fig. 2 Blue/green reversibility of stomatal opening in pulse experiments. Stomata were illuminated with continuous 120 μmol m^{-2} s^{-1} red light as in Fig. 1. At time 0, stomata were given a 30 s pulse of 1,800 μmol m^{-2} s^{-1} blue light, a 30 s pulse of blue light followed immediately by a 30 s pulse of 3,600 μmol m^{-2} s^{-1} green light, or a 30 s pulse of blue light followed by a 30 s pulse of green light followed immediately by another 30 s pulse of 1,800 μmol m^{-2} s^{-1} blue light. Results are the average of 4-9 experiments±SD.

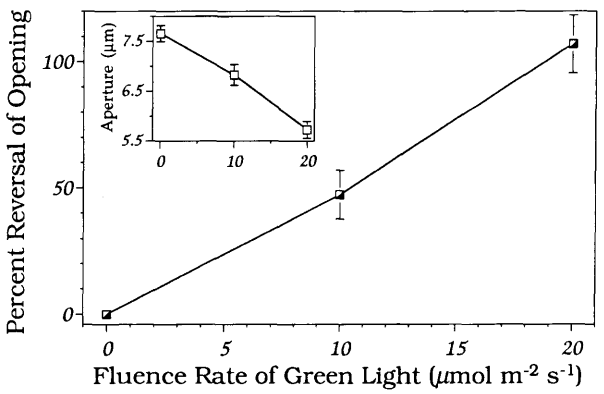

Fig. 3 Green light reversal of blue light-stimulated opening in continuous light experiments. Stomata were illuminated with continuous 10 μmol m^{-2} s^{-1} blue light for 90 min. The effect on final aperture of 0, 10 and 20 μmol m^{-2} s^{-1} added green light, given concurrently with the blue light irradiation, are shown as the percent reduction of blue light opening. Results are the average of 3 experiments±SD. Inset: average final aperture of the 3 experiments±SD. Initial apertures averaged 5.9±0.2 μm.

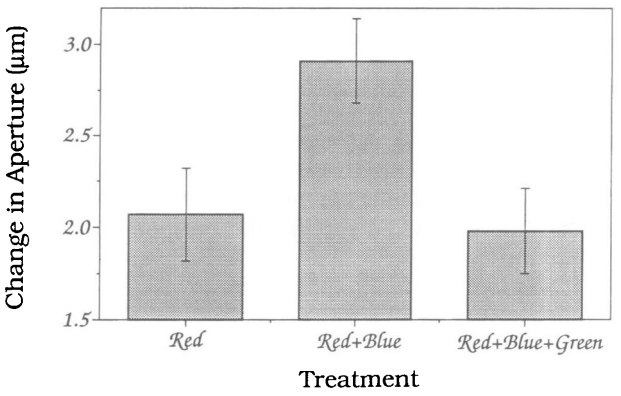

Fig. 4 Green light reversal of blue light-stimulated stomatal opening under a saturating red light background. Isolated stomata were dark-adapted for 60 min to obtain steady-state baseline aperture and then illuminated with continuous 120 μmol m^{-2} s^{-1} red light; 120 μmol m^{-2} s^{-1} red light and 10 μmol m^{-2} s^{-1} blue light; or 120 μmol m^{-2} s^{-1} red light, 10 blue μmol m^{-2} s^{-1} light and 20 μmol m^{-2} s^{-1} green light. Average change in aperture after 90 min of illumination ± SE of the measurement is shown for 3 experiments. Initial apertures averaged 4.7 ± 0.1 μm.

1993), addition of 10 μmol m^{-2} s^{-1} blue light to a 120 μmol m^{-2} s^{-1} red light background caused a 20% increase in aperture over that under red light alone (average aperture increase of 2.9 ± 0.2 μm). However, if 20 μmol m^{-2} s^{-1} of green light was added to the red and blue light irradiation, the average aperture increase was indistinguishable from that under red light alone (2.1 ± 0.2 vs. 2.0 ± 0.2 μm).

Additional experiments tested the effect of green light on stomatal opening in the absence of blue light. Green light at 120 μmol m^{-2} s^{-1} stimulated only a slight opening (0.3 ± 0.2 μm), which contrasted with the 1.6 ± 0.1 μm opening stimulated by 120 μmol m^{-2} s^{-1} red light (Fig. 5). Addition of 120 μmol m^{-2} s^{-1} green light to 120 μmol m^{-2} s^{-1} red light illumination had little, if any, effect. The small opening measured in response to green light alone is consistent with the minor green light component of the action spectrum for photosynthesis-dependent stomatal opening (Sharkey and Ogawa 1987). A photosynthesis-mediated, green light-stimulated opening would also explain the minimal effect of green light in the presence of saturating red light. On the other hand, the lack of any closing effect of green light on red light-stimulated opening

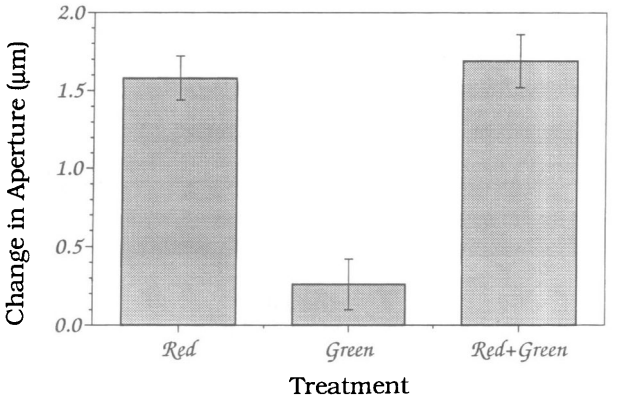

Fig. 5 Effect of green light on red light-stimulated opening. After a 60 min dark pre-treatment, stomata were illuminated for 90 min with 120 μmol m^{-2} s^{-1} red light; 120 μmol m^{-2} s^{-1} green light; or 120 μmol m^{-2} s^{-1} red light and 120 μmol m^{-2} s^{-1} green light. Results of 3 experiments are shown as in Fig. 4. Initial apertures averaged 5.1 ± 0.1 μm.

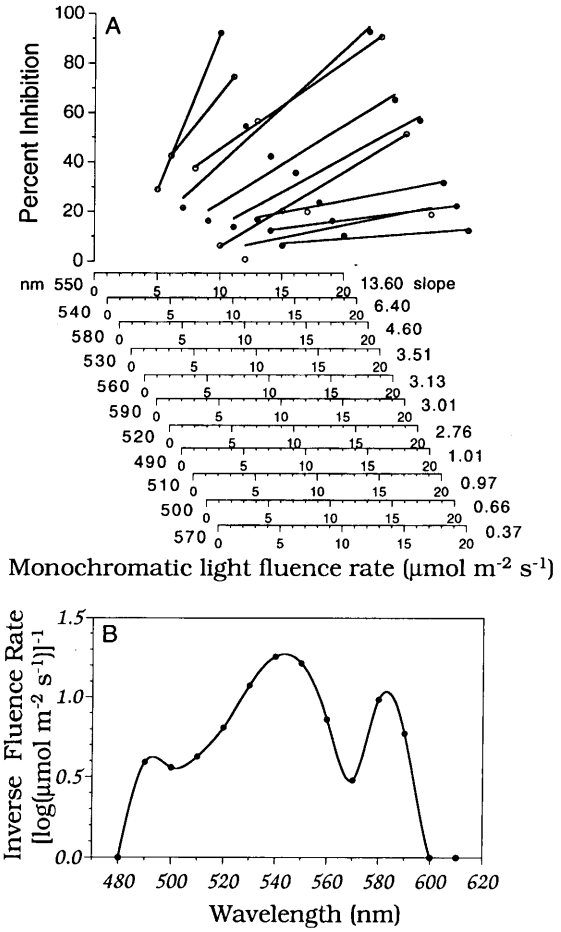

Fig. 6 Action spectrum for the inhibition of blue light-stimulated stomatal opening by green light. (A) Stomata in detached epidermis were illuminated with continuous 10 μmol m^{-2} s^{-1} blue light. Fluence rate response curves for the inhibitory effect of added monochromatic light were determined at 10 nm intervals from 480–610 nm by testing 3 different fluence rates (2 fluence rates for 540 and 550 nm), as in Fig. 3. The points shown are the average of 3 experiments each. The abscissas, presented in order of decreasing slope, are shifted for each wavelength, with the value of the slope from the linear regression indicated next to each abcissa. (B) Action spectrum obtained by calculating the fluence rate necessary to obtain 50% inhibition of opening using the linear regressions from the data shown in 6A.

(Fig. 5) and the reversal of blue light-stimulated opening by green light under a red light background (Fig. 4) indicate that the closing effect of green light is expressed only when the blue light response has been activated.

Action spectrum for the reversal of blue light-stimulated stomatal opening by green light—An action spectrum for the reversal of blue light-stimulated stomatal opening by green light was obtained in 90 min experiments measuring the inhibition of blue light-stimulated stomatal opening by monochromatic light given at 10 nm increments from 470–620 nm. Blue light was applied as described for the continuous light experiments, above. The reversal effect of each wavelength was tested at 5, 10 and 20 μmol m^{-2} s^{-1} light (Fig. 6A). The slope of the fluence rate/reversal relationship was used to calculate the fluence rate required for 50% inhibition of the opening caused by 10 μmol m^{-2} s^{-1} blue light. These values were used to construct the action spectrum shown in Figure 6B. The spectrum shows a maximum at 540 nm and secondary peaks at 490 and 580 nm.

Discussion

The green light response—Green light reversed blue light-stimulated opening of stomata in detached epidermis when given simultaneously with continuous blue light, with or without a saturating red light background. Continuous green light stimulated a slight stomatal opening when given alone, and had no detectable effect on red light-stimulated opening when applied together with photosynthetically saturating red light. Green light pulses reversed the opening stimulated by a blue light pulse, and the blue light response was restored if a second blue light pulse was given after the green pulse. An initial green light pulse did not alter the response to a subsequent blue light pulse. The extent of the reversal of blue light-stimulated opening by green light depended on fluence rate; complete reversal was observed when the green light dose was twice the blue light dose.

Photoreception—The stomatal response to blue light has been studied for several decades (Meidner 1987). Advances in the last few years have identified zeaxanthin as a blue light photoreceptor in the guard cell chloroplast (Zeiger and Zhu 1998, Frechilla et al. 1999). The reversal of blue light-stimulated stomatal opening by green light appears to be another key feature of the photobiology of guard cells.

The action spectra for blue light-stimulated phototropism and for inhibition of stem elongation in *Arabidopsis* and lettuce extend into the green (Steinitz et al. 1985, Lin et al. 1996, Liscum and Briggs 1996), and a semiquinone form of a flavin has been proposed as a possible photoreceptor for green light (Lin et al. 1995). These green light responses differ from the reversal of blue light-stimulated opening by green light reported here in two major ways. First, the response to green light in *Arabidopsis* and lettuce works in the same direction as the blue light response, whereas green light is antagonistic to blue light in stomatal opening (Fig. 2, 3). Second, responses to green light in *Arabidopsis* and lettuce can be observed in the absence of blue light, but blue light is required for the expression of the antagonistic effect of green light in guard cells. Thus, the mechanisms underlying these green light responses appear markedly different.

Two guard cell photoreceptors, chlorophyll and phytochrome, are known to respond to green light. Chlorophylls absorb in the green, and the action spectrum for photosynthesis-dependent stomatal opening shows minor activity in the green region of the spectrum (Sharkey and Ogawa 1987). The slight opening observed under continuous green light, and the lack of any green light effect on steady-state apertures measured under saturating red light, can thus be explained on the basis of known properties of photosynthesis-dependent opening. In contrast, the reversal of blue light-stimulated opening by green light cannot be explained as a photosynthetic response, because in that case red light should have been significantly more effective than green. Furthermore, the action spectrum for the green light response (Fig. 6) is drastically different from typical spectra for photosynthesis.

Phytochrome absorbs in the green both in its red and far red forms, and modulation of blue light responses by green light has been explained as a phytochrome response (Mandoli and Briggs 1981, Steinitz et al. 1985). Guard cells contain phytochrome, but studies aimed at characterising interactions between phytochrome and the stomatal response to blue light gave negative results (Karlsson 1988). Furthermore, the action spectrum for the green reversal of the stomatal response to blue light (Fig. 6B) gives no indication of phytochrome action.

Reversibility by green light of a high irradiance response to far red light has been reported for leaf movements in *Albizzia* and flowering in a long-day plant (Tanada 1997). The putative green light photoreceptor has been postulated to be chemically distinct but functionally related to phytochrome. Although photobiological homologies between this far red/green reversibility and the blue/green reversibility reported here should not be ruled out, their markedly different spectral sensitivities suggest that they are different responses.

Zeaxanthin isomerization as the photochemical basis of blue/green reversibility?—The action spectrum for the reversal of blue light-stimulated stomatal opening by green light shows peaks at 490, 540 (maximum) and 580 nm. The 540 nm maximum is red-shifted from the 450 nm spectrum maximum for the blue light-stimulated opening by about 90 nm. The interpeak distance of the red-shifted spectrum shows a broadening that is characteristic of spectral shifts from polyene chromophores.

Zeaxanthin has an absorption spectrum that matches the action spectrum for blue light-stimulated stomatal opening (Quiñones et al. 1996), and isomerizations are the dominant photochemical reactions of carotenoids. Large spectral shifts caused by isomerization of protein-bound polyenes are well characterised (Spudich and Bogomolni 1988).

The kinetics of the stomatal response to blue light pulses in the intact leaf has been explained by two interconvertible forms of a photoreceptor (Iino et al. 1985). In that study, the response to two consecutive, 30 s blue light pulses separated by varying time intervals was used to characterise the time constants of the blue light response. The time constant for the blue light-dependent reaction was relatively fast, whereas the one for the opposite, thermal reaction was an order of magnitude slower (Iino et al. 1985). The reversal of the response to a blue pulse by a green pulse, and the restoration of the response by a subsequent blue pulse, could be explained by the conversion of the blue light-absorbing form of the photoreceptor into a green-absorbing form, which can revert to the blue light-absorbing form slowly in a thermal reaction, or with a much faster time constant in response to green light. Such photochemical characteristics are reminiscent of the well-known red/far red reversibility of phytochrome (Vierstra and Quail 1983).

Isomers of zeaxanthin and other carotenoids in organic solvents have absorption spectra that typically do not extend beyond 500 nm (Molnar and Szabolcs 1993). However, photoisomerization of a protein-bound xanthophyll, astaxanthin, has been shown to cause the main carotenoid absorbtion band to be red shifted by about 100 nm (Britton et al. 1997). It is therefore possible that photoisomerization of zeaxanthin within a protein environment could shift the absorbtion of the chromophore to the green and thus explain the action spectrum for the green light-reversal shown in Fig. 6. Some spectroscopic aspects of this working hypothesis are currently under investigation.

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