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# Phytochrome and Blue Light-Mediated Stomatal Opening in the Orchid, *Paphiopedilum*

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**Guard cells of the orchid genus, *Paphiopedilum* have been reported to lack developed chloroplasts and detectable chlorophyll *a* autofluorescence. *Paphiopedilum* stomata lack a photosynthesis-dependent opening response but have a blue light-specific opening. The present study found that low fluence rate green and red light elicited stomatal opening in *Paphiopedilum* and this opening was reversed by far red light, indicating the presence of a phytochrome-mediated opening response. Phytochrome-dependent, red light-stimulated opening was largest under low fluence rates and decreased to near zero as fluence rate increased. A recently discovered green light reversibility of blue light-specific stomatal opening was used to probe the properties of the blue light response in *Paphiopedilum* stomata. Blue light-stimulated opening was completely reversed by green light in the presence of far red light. Red light enhanced the blue light response of *Paphiopedilum* guard cells when given as a pretreatment or together with blue light. Analysis of guard cell pigments showed that guard cells have small amounts of chlorophyll *a* and *b*, zeaxanthin, violaxanthin, antheraxanthin and lutein. Zeaxanthin content increased in response to blue light or ascorbate and declined in the dark or under illumination in the presence of dithiothreitol, indicating the presence of an active xanthophyll cycle. Thus *Paphiopedilum* stomata possess both a blue light-mediated opening response with characteristics similar to species with normal chloroplast development and a novel phytochrome-mediated opening response.**

**Keywords**: Blue light — *Paphiopedilum* — Phytochrome — Stomata — Zeaxanthin.

## Introduction

The stomatal response to light is regulated by two major photoreceptor systems, photosynthesis in the guard cell chloroplast and a specific blue light response (Schwartz and Zeiger 1984, Tallman 1992, Assmann 1993). The operation of a third photoreceptor, phytochrome, has been reported (Roth-Bejerano and Itai 1987), but these measurements remain controversial because they are complicated by photosynthetic responses stimulated by red light and have been hard to replicate (Karlsson 1988). *Paphiopedilum* is the only reported genus with guard cells containing undeveloped chloroplasts (Nelson and Mayo 1975, Rutter and Willmer 1979, D'Amelio and Zeiger 1988). A lack of detectable chlorophyll autofluorescence suggested that *Paphiopedilum* stomata should respond to blue light but not red light, as verified experimentally (Zeiger et al. 1983).

Blue light-specific stomatal opening has a number of distinct characteristics. The action spectrum shows a maximum at 450 nm and subsidiary peaks at 420 and 470 nm (Karlsson 1986b). Low fluence rates of blue light (1–10 μmol $m^{-2}$ $s^{-1}$) are effective in stimulating opening (Schwartz and Zeiger 1984). Red light enhances the amount of stomatal opening resulting from a given dose of blue light (Karlsson 1986a). Most recently, blue light-stimulated opening has been found to be reversible by green light (Frechilla et al. 2000). The stomatal response to pulses of blue and green light is determined by the order of the pulses, in a manner analogous to the red/far red reversibility of phytochrome responses. The action spectrum for the green reversal resembles the three-peaked action spectrum for blue light-stimulated opening, red-shifted by about 90 nm (Frechilla et al. 2000).

The blue light photoreceptor in guard cells awaits definitive identification. Work with Arabidopsis mutants has identified two gene products, phot1 and phot2 (formerly nph1 and npl1), that are involved in blue light-stimulated phototropism (Huala et al. 1997) and chloroplast movement (Kagawa et al. 2001). Recently, the phot1/phot2 double mutant has been shown to lack blue light-stimulated stomatal opening (Kinoshita et al. 2001). The phot1 and phot2 proteins are serine/threonine protein kinases (Huala et al. 1997) and have been implicated in blue light-stimulated increases in cytosolic calcium (Baum et al. 1999). Both activities have been implicated in one aspect of blue light-stimulated stomatal opening, the initiation of proton pumping at the plasma membrane (Kinoshita and Shimazaki 1999, Shimazaki et al. 1999). Phot1 and Phot2 have been proposed as blue light photoreceptors on the basis of their having flavin mononucleotide binding sites and, when bound to a flavin under in vitro conditions, an absorption spectrum resembling the action spectrum for blue light responses (Christie et al. 1999).

Other studies have implicated the carotenoid, zeaxanthin, as a blue light photoreceptor of guard cells (Srivastava and Zeiger 1995a, Taylor et al. 1998, Frechilla et al. 1999). The

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absorption spectrum of zeaxanthin also matches the action spectrum for blue light-stimulated stomatal opening (Quiñones and Zeiger 1994) and the zeaxanthin content of guard cells has been quantitatively related to blue light-stimulated apertures (Srivastava and Zeiger 1995b). The Arabidopsis mutant, *npq1*, which has a defective zeaxanthin de-epoxidase and cannot accumulate zeaxanthin, also lacks red light enhancement of blue-light specific stomatal opening (Frechilla et al. 1999).

The two proposed photoreceptors would differ in location and mechanism of operation. The serine/threonin kinases with flavin binding regions would most likely be localized in the cytoplasm and operate via redox reactions in the LOV domains of the protein (Huala et al. 1997). Zeaxanthin is a component of the xanthophyll cycle found in chloroplasts and, in common with all carotenoids, undergoes photoisomerization reactions. Zeaxanthin content is dependent on lumen pH, with more acid pH stimulating zeaxanthin accumulation (Yamamoto 1979). The properties of zeaxanthin thus readily explain two of the characteristics of blue light-stimulated opening. Red light is effective in lowering lumen pH and thus increases zeaxanthin content and sensitivity to blue light (Quiñones and Zeiger 1994). Photoisomerization of carotenoids in a protein environment results in shifts in absorption spectra approximating those seen in the blue green reversibility phenomenon (Britton et al. 1997).

Blue light-stimulated opening in *Paphiopedilum* offers an opportunity to increase our understanding of blue light photoperception. A cytoplasmic-localized flavin photoreceptor would likely be unaffected by the undeveloped nature of these orchid chloroplasts. However, the presumed lack of chloroplast pigments and photosynthetic electron transport in *Paphiopedilum* stomata would suggest that both the green light reversal and red light enhancement of blue light-stimulated opening would be impaired. The study reported here investigated the green reversibility and red light enhancement of blue light-stimulated stomatal opening in *Paphiopedilum*, the pigment composition of *Paphiopedilum* guard cells and the properties of a novel red and green light-stimulated opening response found in the course of this work.

## Results

Fluorescence microscopy is commonly used as a diagnostic tool for the presence of chlorophyll in guard cell chloroplasts. As reported for other *Paphiopedilum* species and their hybrids (Rutter and Willmer 1979, Zeiger et al. 1983, D'Amelio and Zeiger 1988), stomata of *P. makuli* and *P. darling* had no detectable chlorophyll autofluorescence (data not shown).

### *Blue and green light-stimulated stomatal opening in Paphiopedilum*

As reported for *P. harrisianum* (Zeiger et al. 1983), stomata from *P. makuli* and *P. darling* showed robust opening in response to low fluence rate blue light. A 3 h exposure to 5 μmol $m^{-2}$ $s^{-1}$ blue light increased 1.5 μm baseline apertures to 4 μm (Fig. 1).

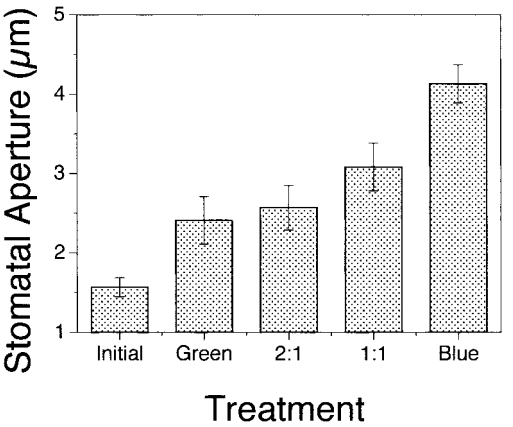


**Fig. 1** Green light reversal of blue light-stimulated stomatal opening in *Paphiopedilum*. Aperture values given are for: before light treatment (Initial); after 3 h illumination with continuous 10 μmol $m^{-2}$ $s^{-1}$ green light (Green); 10 μmol $m^{-2}$ $s^{-1}$ green and 5 μmol $m^{-2}$ $s^{-1}$ blue light (2 : 1); 5 μmol $m^{-2}$ $s^{-1}$ green and 5 μmol $m^{-2}$ $s^{-1}$ blue light (1 : 1); and 5 μmol $m^{-2}$ $s^{-1}$ blue light (Blue). Results are the average of three experiments ± SE of the measurement.

In the course of testing green light reversibility of blue light-stimulated opening, it was found that 3 h of 10 μmol $m^{-2}$ $s^{-1}$ of green light stimulated net aperture increases of 0.8 μm in *Paphiopedilum* (Fig. 1). This opening represents 33% of the net increase stimulated by 5 μmol $m^{-2}$ $s^{-1}$ of blue light. In contrast, *Vicia* stomata illuminated with green light showed an insignificant, 0.2 μm increase in aperture (Frechilla et al. 2000).

Reversal of blue light-stimulated opening by green light in *Paphiopedilum* was fluence dependent, but a 2 : 1 green to blue light ratio failed to elicit full reversal (Fig. 1). In *Vicia faba* and all other species tested, full reversal of blue light-stimulated opening was observed at fluence rates of green light twice that of blue light (Frechilla et al. 2000, Talbott et al. 2002). Higher fluence rates of green light failed to elicit full reversal in *Paphiopedilum* (data not shown). The magnitude of the blue light-stimulated opening that could not be reversed by green light was comparable to the opening stimulated by green light alone (Fig. 1), suggesting the presence of both a blue light-specific and novel green light-mediated opening mechanisms in *Paphiopedilum* stomata.

### *Evidence for phytochrome-mediated stomatal opening in Paphiopedilum*

The green light-stimulated opening of *Paphiopedilum* stomata is clearly more pronounced than the photosynthesis-dependent, green light-stimulated opening reported for other species (Talbott et al. 2002). Phytochrome has been shown to mediate green light-dependent photoresponses in several sys-

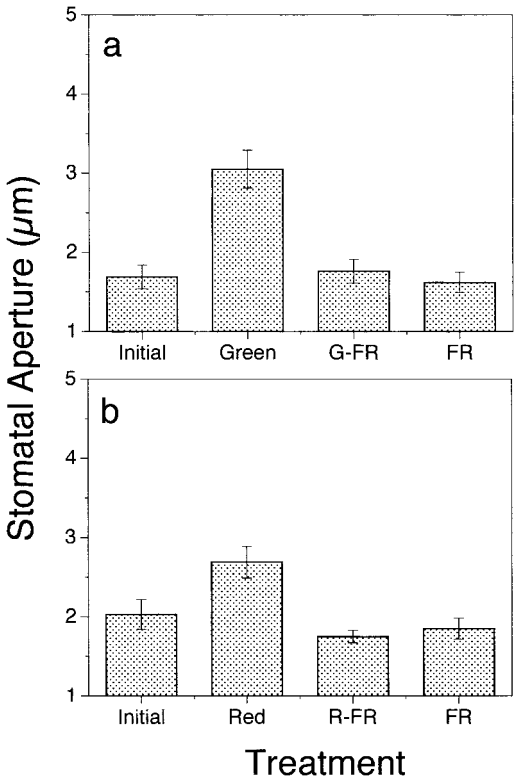


**Fig. 2** Reversal of low fluence rate green and red light-stimulated opening in *Paphiopedilum* by far red light. (a) Aperture values after a 3 h illumination with continuous 10 μmol $m^{-2}$ $s^{-1}$ green light (Green), or a combination of 10 μmol $m^{-2}$ $s^{-1}$ green and 180 μmol $m^{-2}$ $s^{-1}$ far red light (G-FR). (b) Aperture values after a 3 h illumination with continuous 10 μmol $m^{-2}$ $s^{-1}$ red light (Red), or a combination of 10 μmol $m^{-2}$ $s^{-1}$ red and 180 μmol $m^{-2}$ $s^{-1}$ far red light (R-FR). Initial aperture values and aperture values after a 3 h treatment with 180 μmol $m^{-2}$ $s^{-1}$ far red light (FR) are given in both panels. Results are the average of three experiments ± SE of the measurement.

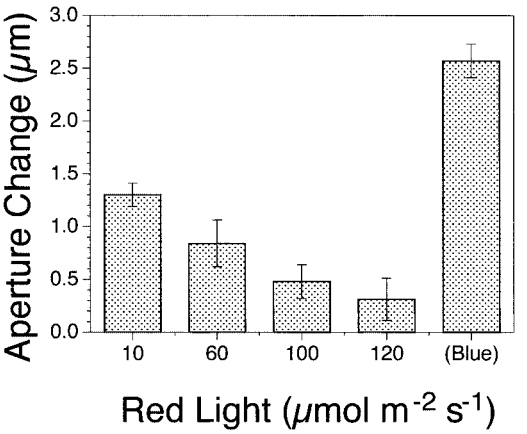


**Fig. 3** Effect of increasing fluence rates of red light on stomatal aperture in *Paphiopedilum*. Change in aperture from initial values after a 3 h illumination are shown for four fluence rates of red light. Aperture change after a 3 h illumination with 5 μmol $m^{-2}$ $s^{-1}$ blue light (Blue) is shown for comparison. Results are the average of four experiments ± SE of the measurement.

tems (Mandoli and Briggs 1981, Steinitz et al. 1985), suggesting that the green light-dependent opening observed in *Paphiopedilum* stomata could be mediated by phytochrome. We investigated that possibility in experiments in which *Paphiopedilum* stomata in epidermal strips were simultaneously illuminated with green and far red light (Fig. 2). Far red light fully reversed the green light-stimulated opening but had no effect on stomatal apertures when applied alone.

A role of phytochrome in green light-stimulated opening appears inconsistent with results from previous studies showing that *Paphiopedilum* stomata fail to respond to red light (Zeiger et al. 1983). However, the fluence rates of red light applied in the previous study were in the range commonly used to stimulate photosynthesis-dependent stomatal opening (Schwartz and Zeiger 1984), which are several-fold higher than the fluence rates of green light used in the present study. We investigated the possibility that responses to low fluence rates of red light were similar to those elicited by green light. The results showed that 10 μmol $m^{-2}$ $s^{-1}$ of red light stimulated stomatal opening of the same magnitude as low fluence rate green light and that this red light-stimulated opening was reversed by far red light (Fig. 2).

A detailed study of the dose-response relationship of red light-stimulated stomatal opening in *Paphiopedilum* showed that the lowest fluence rate of red light tested, 10 μmol $m^{-2}$ $s^{-1}$, stimulated the largest apertures, whereas increasingly higher fluence rates gave progressively smaller responses. Opening was negligible when red light was applied at 120 μmol $m^{-2}$ $s^{-1}$ (Fig. 3).

*Characteristics of blue light-stimulated opening in Paphiopedilum*

The inhibition of phytochrome-mediated, green light-stimulated opening with far red light made it possible to re-examine the blue–green reversal of stomatal opening in *Paphiopedilum*. When illuminated with a 2 : 1 ratio of green to blue light in the presence of far red light, *Paphiopedilum* stomata failed to open beyond initial aperture values (Fig. 4). Far red light had no effect on blue light-stimulated opening. The blue light-specific component of opening thus appears to be fully reversible by green light, in common with all other species examined (Frechilla et al. 2000, Talbott et al. 2002).

A stimulatory effect of background red light is a basic property of the blue light response of stomata (Schwartz and Zeiger 1984, Karlsson 1986a). We investigated whether the blue light response of *Paphiopedilum* stomata has the same characteristic in experiments measuring stomatal opening in response to 5 μmol $m^{-2}$ $s^{-1}$ of blue light under increasing fluence rates of background red light. Far red light was applied throughout the dose response curve to eliminate the red light-

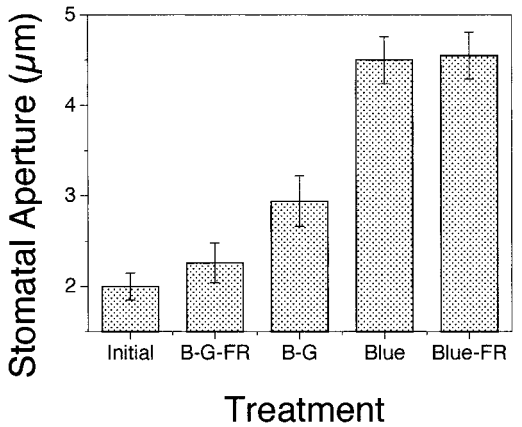


**Fig. 4** Effect of far red light on the green reversal of blue light-stimulated opening in *Paphiopedilum*. Aperture values given are for: before light treatment (Initial); after 3 h illumination with continuous 5 μmol $m^{-2}$ $s^{-1}$ blue light plus 10 μmol $m^{-2}$ $s^{-1}$ green light plus 180 μmol $m^{-2}$ $s^{-1}$ far red light (B-G-FR); 5 μmol $m^{-2}$ $s^{-1}$ blue light plus 10 μmol $m^{-2}$ $s^{-1}$ green light (B-G); 5 μmol $m^{-2}$ $s^{-1}$ blue light (Blue), or 5 μmol $m^{-2}$ $s^{-1}$ blue light plus 180 μmol $m^{-2}$ $s^{-1}$ far red light (Blue-FR). Results are the average of five experiments ± SE of the measurement.

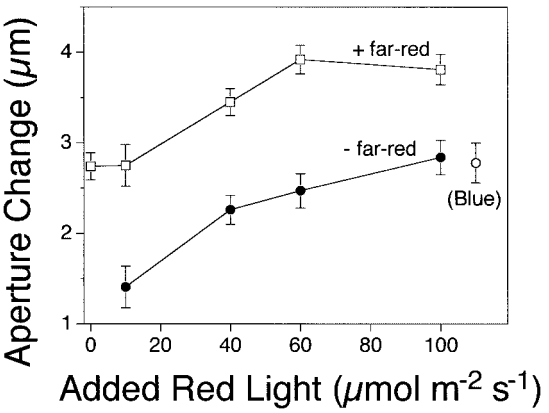


**Fig. 5** Enhancement of blue light-stimulated opening in *Paphiopedilum* by increasing fluence rates of red light. Stomata were given increasing fluence rates of red light together with constant 5 μmol $m^{-2}$ $s^{-1}$ blue in the presence (open square) or absence (closed circle) of 180 μmol $m^{-2}$ $s^{-1}$ far red light. Aperture change resulting from 5 μmol $m^{-2}$ $s^{-1}$ blue light alone (Blue) is given for comparison. Changes in aperture values after 3 h are shown for each treatment. Results are the average of three experiments ± SE of the measurement.

mediated phytochrome responses. Results showed that red light enhanced blue light-stimulated opening, with the response saturating at around 60 μmol $m^{-2}$ $s^{-1}$ (Fig. 5).

The red light enhancement of the stomatal response to blue light was also observed in experiments in which increasing fluence rates of background red light were given in the absence of far red light. Final aperture values increased in proportion to increasing fluence rate of red light, but aperture values were lower than those measured in response to blue light alone (Fig. 5). This suggests that the phytochrome and blue light sensory transduction pathways interact, and that the phytochrome response negatively modulates the blue light response.

Additional experiments tested the enhancement of the blue light response by red light pre-treatments of increasing duration. Stomata in detached epidermis were pre-treated with 120 μmol $m^{-2}$ $s^{-1}$ red light for 0.5, 1 and 1.5 h and then incubated under 5 μmol $m^{-2}$ $s^{-1}$ blue light for 3 h. Apertures increased as a function of the red light pre-treatment in a dose dependent fashion (Fig. 6).

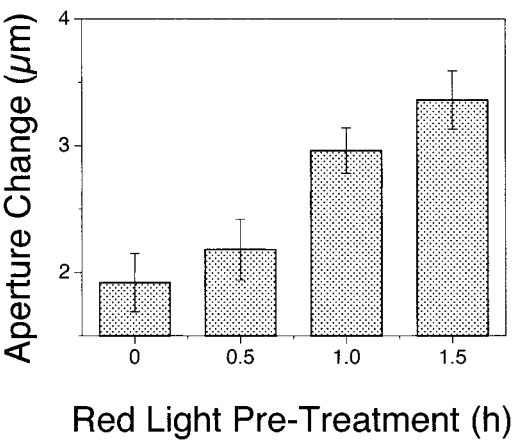


**Fig. 6** Enhancement of blue light-stimulated opening in *Paphiopedilum* by a pretreatment with red light. Stomata in epidermal peels were pre-treated with 120 μmol $m^{-2}$ $s^{-1}$ red light for 0, 0.5, 1.0 or 1.5 h and then treated with 5 μmol $m^{-2}$ $s^{-1}$ blue light for an additional 3 h. Change in aperture values after the blue light treatment are shown for each pretreatment. Results are the average of four experiments ± SE of the measurement.

*Paphiopedilum guard cells have pigmented plastids and a functional xanthophyll cycle*

The HPLC pigment profile of mesophyll tissue from greenhouse-grown *Paphiopedilum* plants has a normal complement of photosynthetic pigments including violaxanthin, antheraxanthin, lutein, zeaxanthin, chlorophyll *a* and *b*, and β-carotene (Fig. 7). *Paphiopedilum* leaves appear to operate a normal xanthophyll cycle, since a 6 h dark adaptation decreased zeaxanthin content to about 10% of the total xanthophyll cycle pool (data not shown).

Sonicated, abaxial epidermis from *Paphiopedilum* leaves had intact guard cells and broken epidermal cells. When extensively washed as described in Material and Methods, the sonicated epidermis showed no detectable contamination from mesophyll chloroplasts. An HPLC analysis of pigments from light-treated, sonicated abaxial epidermis from *Paphiopedilum* showed a different pigment composition from that of mesophyll tissue (Fig. 8). The chromatogram profile showed several unknown peaks, small lutein, violaxanthin, antheraxanthin

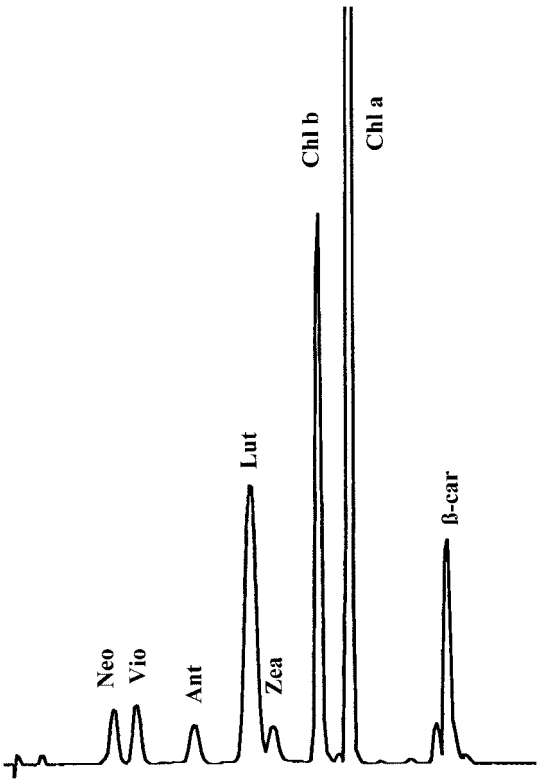


**Fig. 7** HPLC profile of photosynthetic pigments from greenhouse-grown *Pahiopedilum* leaves. Neo, neoxanthin; Vio, violaxanthin; Ant, antheraxanthin; Lut, lutein; Zea, zeaxanthin; Chl a, chlorophyll *a*; Chl b, chlorophyll *b*; β-car, β-carotene.

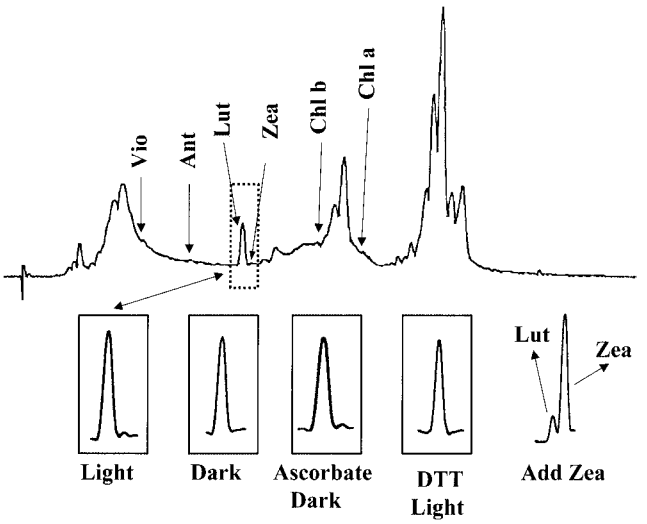


**Fig. 8** Typical HPLC profile of light-treated epidermis of *Pahiopedilum*. The location of the violaxanthin (Vio), lutein (Lut), and zeaxanthin (Zea) peaks were identified using pigment standards. The lower boxes show the lutein and zeaxanthin peaks in epidermis dark-adapted for 1.5 h; treated for 1.5 h with 150 μmol $m^{-2}$ $s^{-1}$ blue light; treated with 100 mM ascorbate in the dark; or treated with 150 μmol $m^{-2}$ $s^{-1}$ blue light in the presence of 10 mM DTT. A profile of this region of the chromatogram after the addition of a zeaxanthin standard is shown for peak identification.

and zeaxanthin peaks, and indications of chlorophylls *a* and *b*. Lutein content was estimated at $1.58\times10^{-7}$ nmol per guard cell.

*Paphiopedilum* guard cells appear to have a functional xanthophyll cycle. After a 90 min treatment with 150 μmol $m^{-2}$ $s^{-1}$ blue light, zeaxanthin content was approximately $4\times10^{-9}$ nmol per guard cell. Comparable samples maintained in the dark showed no detectable zeaxanthin. Addition of the inhibitor of zeaxanthin formation, dithiothreitol (DTT), to light-treated samples prevented zeaxanthin accumulation, while addition of ascorbate stimulated zeaxanthin formation in the dark (Fig. 8 insets).

## Discussion

Stomata of the orchid genus *Paphiopedilum* have proven valuable for the study of guard cell chloroplast function (Nelson and Mayo 1975, Rutter and Willmer 1979, Zeiger et al. 1985, Assmann and Zeiger 1985, Assmann 1988, D'Amelio and Zeiger 1988). *Paphiopedilum* stomata open in response to blue light but lack a photosynthesis-dependent, red light response, and are relatively insensitive to ambient $CO_2$ concentrations. Because of their lack of red-fluorescing chloroplasts, *Paphiopedilum* guard cells have been used for studies with fluorescing pH indicators (Gehring et al. 1998). Guard cell plastids in *Paphiopedilum* have abundant inner membranes but are devoid of thylakoids (D'Amelio and Zeiger 1988).

*Phytochrome-mediated opening in Paphiopedilum*

Green light-stimulated opening in *Paphiopedilum* is unusually large, as compared to that of other species (Sharkey and Ogawa 1987, Frechilla et al. 2000, Talbott et al. 2002). The action spectrum for the blue light response does not extend into the green region of the spectrum (Karlsson 1986b) and green light is poorly absorbed by photosynthetic pigments (Ogawa 1981). The relatively high green light response of *Paphiopedilum* stomata thus suggests that a third photoreceptor system is active in these stomata. Phytochrome photoequilibrium can be altered by green light, and green light-dependent, phytochrome-mediated inhibition of stem elongation and phototropism has been documented (Mandoli and Briggs 1981, Steinitz et al. 1985). The reversal of green light-stimulated opening by far red light (Fig. 2) implicates phytochrome as the photoreceptor mediating the green light-stimulated opening in *Paphiopedilum* stomata.

Thus far, *Paphiopedilum* stomata are the only ones found to have a phytochrome-mediated opening under green light, with all other species tested (*Vicia faba*, *Commelina communis*, *Pisum sativum*, *Nicotiana glauca*, *N. tabacum*, *Arabidopsis thaliana*, *Allium cepa* and *Hordeum vulgare*) lacking a significant green light-stimulated opening (Frechilla et al. 2000,

Talbott et al. 2002). Far red-reversible, green light-stimulated opening appears to be an optimal probe for phytochrome-mediated stomatal responses in species with normal guard cell chloroplasts, because it obviates simultaneous activation of the photosynthesis-dependent opening response.

Since both the $P_r$ and $P_{fr}$ forms of phytochrome absorb more effectively in the red than in the green, a role of phytochrome in green light-stimulated opening seems inconsistent with the reported insensitivity of *Paphiopedilum* stomata to high fluence rates of red light (Zeiger et al. 1983). However, detailed investigation of the red light response of *Paphiopedilum* stomata showed that they open in response to low fluence rates of red light, and that this opening is far-red reversible (Fig. 2). Unexpectedly, a dose-response analysis of the red light-stimulated opening showed that 10 μmol $m^{-2}$ $s^{-1}$ red light elicited the largest opening, with apertures becoming progressively smaller at higher fluence rates (Fig. 3). Opening was negligible at high fluence rates of red light, as reported previously (Zeiger et al. 1983).

The inverse dose-relation of the stomatal response to red light in *Paphiopedilum* could have functional implications. *Paphiopedilum* is a shade plant and stomatal conductance is very low under most environmental conditions (Williams et al. 1983, Assmann and Zeiger 1985, Assmann 1988). Given the low apertures elicited by the phytochrome system, and the partial inhibition of blue light-stimulated opening by red light, the phytochrome response could function as a negative regulatory mechanism reducing stomatal apertures under super-optimal irradiances with high red/far red ratios (Fig. 5). A detailed analysis of far-red, red and blue responses and their interactions is needed for a thorough understanding of the function of phytochrome in *Paphiopedilum* stomata. Many interactions between blue light and phytochrome have been reported (Mazzella et al. 2001). Further studies should clarify whether a phytochrome-dependent regulatory control of stomatal movements can only be found in the absence of photosynthesis-dependent stomatal opening, as in *Paphiopedilum*, or whether it is also present in other species with typical chloroplasts.

*Characteristics of blue light-stimulated opening in Paphiopedilum*

Under continuous illumination, blue light-stimulated stomatal opening in several species is fully reversed by green light applied at twice the fluence rate of blue light (Frechilla et al. 2000, Talbott et al. 2002). In contrast, blue light-stimulated stomatal opening in *Paphiopedilum* is only partially reversed by green light (Fig. 1), even when applied at fluence rates substantially higher than the 2 : 1 ratio that suffices for full reversal in other species. The fact that the residual opening seen under a 2 : 1 ratio of green to blue light equals the opening seen under green light alone (Fig. 1) suggests that two different responses can be attributed to green light: the reversal of blue light-stimulated opening and the stimulation of phytochrome-dependent opening. This hypothesis is supported by the fact that green light completely reversed blue light-stimulated opening when applied under far red light, which would reverse the phytochrome-mediated component of opening (Fig. 4). Thus the same basic mechanism underlying blue/green reversibility of stomatal opening present in all other species tested also appears to operate in *Paphiopedilum*.

The enhancement of the blue light response of stomata by red light is a second basic property of blue light-stimulated opening (Karlsson 1986a). An increase in blue light-stimulated stomatal conductance in *Paphiopedilum* by background red light has been reported (Assmann 1988). Results of the present study show a dose-dependent enhancement of the blue light response by red light in the presence or absence of far red light, and in response to red light pre-treatments.

Red light has been postulated to enhance the blue light sensitivity of stomata by increasing baseline apertures (Karlsson 1986a), and by increasing the energy supply ensuing from photosynthetic activity of the guard cell chloroplast (Ogawa et al. 1978). The observed enhancement of the blue light response by red light in *Paphiopedilum* is inconsistent with both of these postulates because increasing fluence rates of red light decrease stomatal apertures (Fig. 3), and *Paphiopedilum* guard cells are devoid of sufficient photosynthetic capacity to significantly increase their energy pool.

*Paphiopedilum guard cells operate the xanthophyll cycle*

Pigment analysis showed that *Paphiopedilum* guard cells contain violaxanthin, antheraxanthin, lutein, zeaxanthin, and chlorophyll *a* and *b*. The significantly different chromatographic profile obtained from mesophyll cells strongly argues against the presence of contamination from mesophyll cells in the guard cell preparations. Plastids from dark-grown coleoptiles have a xanthophyll composition similar to that of dark-adapted *Paphiopedilum* guard cells (Quiñones and Zeiger 1994, Zhu et al. 1997).

Extracts from light-treated guard cells contained zeaxanthin, but no zeaxanthin was found in dark-treated samples (Fig. 8). DTT, an inhibitor of violaxanthin de-epoxidase (Yamamoto and Kamite 1972, Srivastava and Zeiger 1995a), blocks the accumulation of zeaxanthin in the light, while ascorbate, a promoter of zeaxanthin epoxidation, stimulates zeaxanthin formation in the dark (Fig. 8). These results indicate that *Paphiopedilum* guard cells have a functional xanthophyll cycle and that, as in species with normal guard cell chloroplasts, zeaxanthin accumulates in the light and decreases in the dark. While lacking organized grana, *Paphiopedilum* plastids contain a membranous peripheral reticulum (D'Amelio and Zeiger 1988). *Paphiopedilum* plastids thus appear to have rudimentary pigmentation and pigment function rather than being devoid of function as has been previously assumed. The operation of the xanthophyll cycle in *Paphiopedilum* guard cell plastids may thus be analogous to the reported xanthophyll cycle activity in etiolated coleoptile chloroplasts (Quiñones and Zeiger 1994, Zhu et al. 1997).

*Implications for identification of the stomatal blue light-photoreceptor*

Results of this study thus show that the blue light response of *Paphiopedilum* stomata share two key properties of blue light opening found in species containing normal guard cell chloroplasts: blue/green reversibility and red light enhancement. This result leads to the conclusion that fully developed chloroplasts are not essential for blue light-stimulated opening. On the other hand, *Paphiopedilum* chloroplasts do contain a rudimentary complement of photosynthetic pigments and are able to operate an active xanthophyll cycle; characteristics reminiscent of the chloroplasts of etiolated coleoptiles (Zhu et al. 1997). Xanthophyll cycling therefore remains a viable mechanism for explaining the green reversibility and red light enhancement properties of blue light-specific opening.

## Materials and Methods

*Plant material and growth conditions*

In previous studies of orchid stomata (Zeiger et al. 1983, D'Amelio and Zeiger 1988) it was found that mottled leaves were the only ones from which detached epidermis could be readily obtained. For this reason, *P. harrissianum* was used in a previous study of stomatal opening in epidermal peels (Zeiger et al. 1983). Several orchid growers queried were unable to supply us with sufficient *P. harrissianum* plants for the present study. Instead, two other *Paphiopedilum* hybrids with mottled leaves were used: *P. makuli* {*Maudiae* (*callosum* × *lawrenceanum*) × *sukhakulii*}, and *P. darling* {Madame Martinet (*callosum* × *delenatii*) × *lawrenceanum*}. All plants were supplied by The Orchid Bench, Fort Bragg, CA, U.S.A. No difference between the stomatal responses of the two hybrids was detected. Plants were shipped in pots containing a bark mixture and were kept in a greenhouse under a 50% shade cloth. A misting system was used to maintain 80–90% relative humidity, and also supplied water to the bark mixture. Plants were fertilized 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*

Abaxial epidermis was carefully stripped by hand and placed into an incubation solution containing 0.1 mM $CaCl_2$ and 70 mM KCl. Detached peels were sonicated for 3 s at 50% power (Branson model 250, Branson Ultrasonics Corp., Danbury, CT., U.S.A.) to remove air from the stomatal pores, and then dark-adapted for 60 min, after which baseline stomatal apertures were measured. The epidermal strips were then transferred to 3 ml dishes containing the same incubation medium and illuminated with different light treatments for 3 h. Final aperture measurements were obtained at the end of the light treatment. The incubation solution was aerated and maintained at 23°C throughout the dark adaptation and light treatments.

Average apertures were determined from measurements of 30 digitized 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.).

A trifurcated fiber optic was used for illumination (Oriel Instruments, Stratford, CT, U.S.A.). Monochromatic blue (450 nm), red (640 nm) and green (540 nm) light was provided by interference filters (10±2.5 nm bandwidth, Oriel Instruments). Dolen-Jenner fiber optic illuminators (Edmund Scientific, Barrington, NJ, U.S.A.) were used as light sources for red and green light. A xenon arc lamp (ILC Technology, Sunnyvale, CA, U.S.A.) was used as the source for blue light. Far red light was supplied by LEDs having an emission maximum at 730 nm (on loan from Dr. John Sager, Kennedy Space Center, U.S.A.). Light fluence rates were measured with a Li-Cor quantum sensor for visible light or a Li-Cor pyranometer for far red light (Li-Cor Inc., Lincoln, NE, U.S.A.).

*Analysis of guard cell pigments*

Detached leaves were kept in the dark for 4 h. Abaxial epidermis was remove by hand under dim room light and incubated in Petri dishes containing 1.0 mM KCl, 0.1 mM $CaCl_2$, and 1.0 mM MES/NaOH buffer, pH 6.8. The peels were incubated 90 min in darkness ±100 mM ascorbate or in 150 µmol $m^{-2}$ $s^{-1}$ blue light ±20 mM DTT. All solutions were aerated and kept at 23°C. At the end of each treatment, the epidermal peels were sonicated on ice for 30 s at a 50% power setting and 90% duty cycle, and then washed thoroughly on a metal mesh. Sonication and washing was repeated four times prior to pigment extraction to eliminate any contamination from mesophyll chloroplasts. The sonicated peels were carefully examined by fluorescence microscopy to check for mesophyll contamination. No contamination could be detected.

Pigments from cleaned epidermal peels were extracted under dim room light. The epidermis was placed in a mortar with a solution of 2 : 1 acetone : hexane saturated with $NaHCO_3$ and 500 mg anhydrous $Na_2SO_4$, then ground thoroughly on ice. One ml of 0.2 mM KCl was then added and the mixture was centrifuged at 1,500×*g* for 3 min. The upper solvent phase of the extract was collected, evaporated in vacuo, and immediately analyzed for pigment composition using a Rainin 81–20 system (Rainin Instrument Co., Woburn, MA, U.S.A.) equipped with UV detector and two Sperisorb ODS-1 5µ columns (Alltech Associates, Deerfield, IL, U.S.A.) connected in series. Pigments were eluted for 5 min with a 72 : 8 : 3 acetonitrile : methanol : 0.1 mM Tris-HCl buffer, pH 8.0, followed by a 15 min linear gradient to 100% 4 : 1 methanol : hexane solution (2 ml $min^{-1}$ flow rate). Pigments were detected at 440 nm. Violaxanthin, antheraxanthin, lutein, zeaxanthin, chlorophyll *a*, and chlorophyll *b* peaks were identified by addition of known standards to orchid mesophyll and guard cell extracts.

Response factors obtained from known standards were used to quantify lutein and zeaxanthin content of epidermal extracts. Results, expressed on a per guard cell basis, were calculated from the weight of the epidermal sample using empirically obtained data for stomatal density and weight/unit area.

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