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# Stomata from *npq1*, a Zeaxanthin-less Arabidopsis Mutant, Lack a Specific Response to Blue Light

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**The Arabidopsis mutant *npq1*, which cannot accumulate zeaxanthin because of a defective violaxanthin deepoxidase, was used to investigate the role of zeaxanthin in the stomatal response to blue light. Neither dark-adapted nor light-treated guard cells or mesophyll cells of the *npq1* mutant contained detectable zeaxanthin. In contrast, wild-type guard cells had a significant zeaxanthin content in the dark and accumulated large amounts of zeaxanthin when illuminated. The well-documented red light enhancement of blue light-stimulated stomatal opening, in which increasing fluence rates of background red light result in increased response to blue light, was used to probe the specific blue light response of Arabidopsis stomata. Stomata from the *npq1* mutant did not have a specific blue light response under all fluence rates of background red light tested. On the other hand, stomata from leaves of *hy4* (*cry 1*), an Arabidopsis mutant lacking blue light-dependent inhibition of hypocotyl elongation, had a typical enhancement of the blue light response by background red light. The lack of a specific blue light response in the zeaxanthin-less *npq1* mutant provides genetic evidence for the role of zeaxanthin as a blue light photoreceptor in guard cells.**

**Key words:** *Arabidopsis thaliana* — Blue light — *npq1* — Stomata — Zeaxanthin.

Stomata have a well-characterized blue light response, which has an action spectrum (maximum at 450 nm, and minor peaks at 420 and 470 nm; Karlsson 1986b) closely resembling those of other extensively studied blue light responses such as phototropism and inhibition of stem elongation (Horwitz 1994). The stomatal response to blue light is rapid and reversible, and is localized in a single cell type, the guard cell (Zeiger 1990).

Key steps in the sensory transduction of blue light in guard cells have been characterized (Kinoshita and Shimazaki 1997). Blue light activates electrogenic proton pumping at the guard cell plasma membrane, which provides a driving force for secondary ion uptake (Assmann et al. 1985, Shimazaki et al. 1986, Goh et al. 1995). Blue light also stimulates starch degradation in the guard cell chloroplast and malate synthesis in the cytosol (Ogawa 1981, Tallman and Zeiger 1988, Talbott and Zeiger 1993). The ensuing increase in osmotic potential results in water influx, an increase in turgor pressure, and stomatal opening (Assmann 1993).

Isolated guard cells and their protoplasts have typical blue light responses (Zeiger and Hepler 1977, Assmann et al. 1985, Amodeo et al. 1992), indicating that photoreception of the signal eliciting the stomatal response to blue light occurs within the guard cell proper. Work with *hy4* (*cry1*), an Arabidopsis mutant lacking blue light-dependent inhibition of hypocotyl elongation, has shown that the *HY4* (*CRY1*) gene has sequence homology to photolyases (Lin et al. 1995), suggesting that a flavin-dependent blue light-sensing mechanism mediates the inhibition of hypocotyl elongation by blue light. However, *hy4* stomata showed seemingly normal blue light responses (Lascève et al. 1999).

Recent studies have implicated the carotenoid zeaxanthin as a blue light photoreceptor in guard cells. The absorption spectrum of zeaxanthin matches the action spectrum for blue light-stimulated stomatal opening, and the zeaxanthin content of guard cells is linearly related to their blue light sensitivity (Srivastava and Zeiger 1995b, Quiñones et al. 1996). Dithiothreitol (DTT), an inhibitor of zeaxanthin formation, blocks blue light-stimulated stomatal opening in a concentration-dependent fashion (Srivastava and Zeiger 1995b). In the facultative CAM plant *Mesembryantemum crystallinum*, CAM induction is paralleled by a loss of stomatal sensitivity to blue light and a loss of the capacity for light-dependent zeaxanthin formation in the guard cell chloroplast (Tallman et al. 1997). In cultured guard cell protoplasts from *Nicotiana glauca*, DTT prevented light-dependent zeaxanthin accumulation and inhibited blue light-stimulated swelling (Taylor et al. 1998). In Pima cotton, zeaxanthin content in guard cells covaried with stomatal sensitivity to blue light and with stomatal apertures (Quiñones et al. 1998).

In this study, we analyzed the stomatal responses to blue light in *npq1* (*n*on-*p*hotochemical *q*uenching), a recently isolated Arabidopsis mutant that cannot accumulate zeaxanthin because of a defective violaxanthin de-epoxidase (Niyogi et al. 1998). The zeaxanthin content of guard cells from wild-type and *npq1* leaves was compared, and

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the stomatal responses to blue light in both genotypes were characterized. Our results showed that *npq1* stomata cannot accumulate zeaxanthin and lack a specific blue light response.

## Materials and Methods

*Materials—Arabidopsis thaliana* seedlings were grown from seeds of wild-type (Heynh. ecotype Columbia), *npq1*, and *hy4* mutants. The *npq1* mutant was in the Columbia background (Niyogi et al. 1998), while the *hy4* mutant was in the Landsberg *erecta* background. Seeds from *npq1* were a gift from Dr. K. Niyogi of the Carnegie Institution (Stanford, CA); wild-type and *hy4* seeds were obtained from the Arabidopsis Biological Resource Center (Ohio State University). Seeds were treated with dim red light and then soaked in water at 4°C for 24 h prior to planting in pots containing commercial potting soil (Sunshine Mix #1, American Horticulture Supply, Camarillo, CA, U.S.A.) in a greenhouse.

*Pigment analysis*—Leaves were collected from dark-adapted (5 h) wild-type and *npq1* plants. Epidermis from the lower surface of the leaves was detached using forceps. Preparation of uncontaminated epidermis for pigment analysis was labor intensive. Detached epidermis from 40 leaves averaging 2 $cm^2$ each were needed for each HPLC run. Detached epidermis was carefully inspected and visible, attached mesophyll fragments and veins were cut off with a razor blade. Mesophyll contaminants were further removed by sonication on ice for 30 s in a Branson Sonifier (model 250, Branson Ultrasonics Corp., Danbury, CT, U.S.A.) at a continuous duty cycle and a power setting of 7. As a further precaution, zeaxanthin analysis of illuminated guard cells was performed at a fluence rate that was below the threshold for zeaxanthin formation in mesophyll tissue. Small fragments of leaves were used for the mesophyll measurements. Samples were incubated in 20 mM KCl, 0.1 mM $CaCl_2$, and 1 mM MES-NaOH (pH 6.0), aerated with compressed air, and illuminated (Sylvania DAH 300W projector bulb, GTE Products Co., Winchester, KY, U.S.A.) or kept in darkness, at 25°C for 1 h. Pigments were extracted and analyzed by HPLC, as described previously (Zhu et al. 1998).

*Measurements of stomatal apertures*—Epidermis detached from dark-adapted leaves was incubated in 20 mM KCl, 0.1 mM $CaCl_2$, and 1 mM MES-NaOH (pH 6.0), at 25°C under red light illumination (Sylvania DAH 300-W 300PAR56/2MFL Cool Lux floodlamps, GTE Products Co., Winchester, KY, U.S.A.; Plexiglas filter, 50% cutoff at 595 nm, No. 2423, Rohm and Haas, Hayward, CA, U.S.A.) at 50, 100, and 200 $\mu$mol $m^{-2}$ $s^{-1}$ for 3 h. Additional illumination of 20 $\mu$mol $m^{-2}$ $s^{-1}$ blue (Plexiglas, 470 nm maximum, half-bandwidth 100 nm, No. 2424, Rohm and Haas) or red light was supplied during the third hour of treatment. The setup for dual illumination and constant temperature control has been described previously (Talbott and Zeiger 1993). For the measurement of stomatal response to white light, epidermis from dark-adapted leaves was illuminated with 100 $\mu$mol $m^{-2}$ $s^{-1}$ white light for 2 h in the same conditions.

Stomatal apertures were measured at the end of the incubation period using an Olympus BH-2 microscope (Olympus Corp., Lake Success, NY, U.S.A.) connected to a digital camera (JE2362A, Javelin Electronics, Torrance, CA, U.S.A.), as described previously (Talbott and Zeiger 1993, Zhu et al. 1998).

## Results

*The xanthophyll cycle of guard cells from wild-type and npq1 leaves of Arabidopsis*—The *npq1* mutant, obtained by screening mutagenized Arabidopsis plants for a lack of non-photochemical quenching in mesophyll tissue (Niyogi et al. 1998), cannot accumulate zeaxanthin because of a defective violaxanthin de-epoxidase. Pigment analysis showed that both light- and dark-treated guard cells from *npq1* contained high levels of violaxanthin and no zeaxanthin (Fig. 1). A lack of zeaxanthin in mesophyll tissue of *npq1* has been reported (Niyogi et al. 1998); the present study indicates that the genetic lesion in the violaxanthin de-epoxidase is also expressed in guard cell chloroplasts.

Mesophyll cells from wild-type Arabidopsis leaves had no detectable zeaxanthin in the dark; they began to accumulate it at fluence rates higher than 200 $\mu$mol $m^{-2}$ $s^{-1}$ (Fig. 2A). Insensitivity of the xanthophyll cycle to low light levels is typical of mesophyll chloroplasts (Fig. 2B; Bilger and Björkman 1990, Demmig-Adams and Adams 1992, Srivastava and Zeiger 1995a, Zhu et al. 1995).

For pigment analysis of guard cell chloroplasts, mesophyll contaminants need to be eliminated. Methods such as protoplast isolation (Masamoto et al. 1993) or sonication (Srivastava and Zeiger 1995a) yield purified guard cell preparations amenable to biochemical analysis (Fig. 2B), but such techniques have yet to be developed for large-scale purification of Arabidopsis guard cells. In this study,

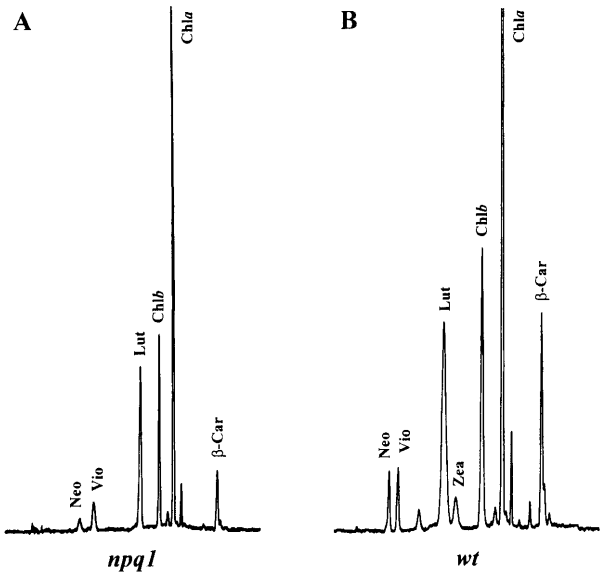


**Fig. 1** HPLC profiles of photosynthetic pigments from Arabidopsis guard cells. (A) *npq1* mutant; (B) wild type. Note the absence of the zeaxanthin peak in the *npq1* profile. Neo, neoxanthin; Vio, violaxanthin; Ant, antheraxanthin; Lut, lutein; Zea, zeaxanthin; Chl *b*, chlorophyll *b*; Chl *a*, chlorophyll *a*; *β*-car, *β*-carotene.

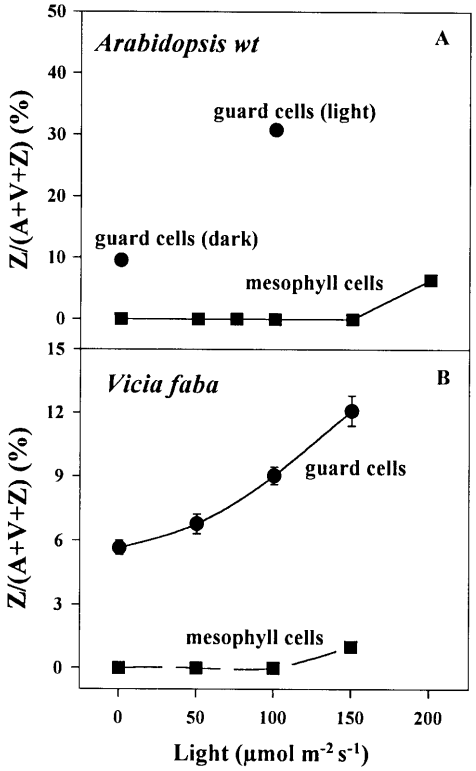


**Fig. 2** Light-induced zeaxanthin formation in guard cells and mesophyll cells. (A) Wild-type Arabidopsis; (B) *Vicia faba*. Extracted pigments were separated by HPLC (Fig. 1) and zeaxanthin content was quantified as reported previously (Zhu et al. 1998). Zeaxanthin content is plotted as a percentage of the three components of the xanthophyll cycle, violaxanthin (V), antheraxanthin (A), and zeaxanthin (Z).

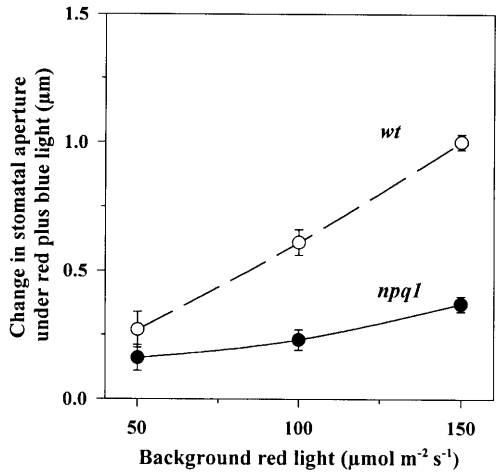


**Fig. 3** Change in stomatal aperture in response to blue light in detached epidermis of wild-type and *npq1* Arabidopsis leaves. Samples were incubated in 20 mM KCl, 0.1 mM $CaCl_2$, and 1 mM MES-NaOH (pH 6.0) for 3 h under 50, 100, or 150 $\mu$mol $m^{-2}$ $s^{-1}$ of red light. Blue light (20 $\mu$mol $m^{-2}$ $s^{-1}$) was added during the final h of incubation. Each value represents an average of three experiments (30 measurements each) ± SE. Average aperture at the start of the blue light treatment was 1.92 ± 0.02 $\mu$m for wild-type and 1.86 ± 0.09 $\mu$m for *npq1* stomata.

sonicated, detached epidermis of Arabidopsis leaves were carefully screened for mesophyll contamination and analyzed for zeaxanthin content over the range of fluence rates at which mesophyll chloroplasts were devoid of zeaxanthin. As Fig. 2A shows, dark-adapted wild-type Arabidopsis guard cells had a relatively high zeaxanthin content (approximately 10% of the total xanthophyll cycle pool). Upon illumination with 100 $\mu$mol $m^{-2}$ $s^{-1}$ light, zeaxanthin levels increased threefold to approximately 30% of the total xanthophyll cycle pool. For comparison, the zeaxanthin content of guard cell and mesophyll chloroplasts from *Vicia faba* is shown in Fig. 2B. In both Arabidopsis and Vicia, guard cell chloroplasts retained some zeaxanthin in the dark and accumulated additional zeaxanthin at low light levels. A similar high light sensitivity of the xanthophyll cycle has been found in coleoptile chloroplasts (Zhu et al. 1995), indicating that this feature is a functional property of chloroplasts specialized for sensory transduction (Zeiger and Zhu 1998). Changes in the zeaxanthin content of mesophyll and guard cells from *Vicia faba* over a broad range of fluence rates measured in a daily course have been reported (Srivastava and Zeiger 1995a).

*Stomata from npq1 lack a specific blue light response* —The blue light response of wild-type and *npq1* stomata was characterized using a dual-beam protocol designed to assay red light enhancement of blue light-stimulated opening. This protocol can separate specific blue light responses from other blue light-sensitive responses, such as photosynthesis (Schwartz and Zeiger 1984, Goh et al. 1997). Stomata in detached epidermis were illuminated at three fluence rates of background red light (50, 100, and 150 $\mu$mol $m^{-2}$ $s^{-1}$) for 3 h. A second beam of weak (20 $\mu$mol $m^{-2}$ $s^{-1}$) blue light was added to the red light background during the third h of illumination. Measured increases in apertures of wild type and *npq1* stomata over the third h of illumination are shown in Fig. 3. In wild type, stomatal apertures increased with the addition of blue light and, as shown for other species (Karlsson 1986a, Assmann 1988, Srivastava and Zeiger 1995a), the extent of blue light-stimulated opening was related to the fluence rate of background red light. Stomata from the *npq1* mutant showed little additional opening during the period of blue light illumination (Fig. 3).

In order to test if the small amount of opening seen in Fig. 3 represents a residual blue light-specific response, the photosynthetic and blue light components of the response in both genotypes were further investigated in experiments comparing opening under dim (20 $\mu$mol $m^{-2}$ $s^{-1}$) red or blue light added during the third h of treatment with 150 $\mu$mol $m^{-2}$ $s^{-1}$ red light. Addition of blue light caused

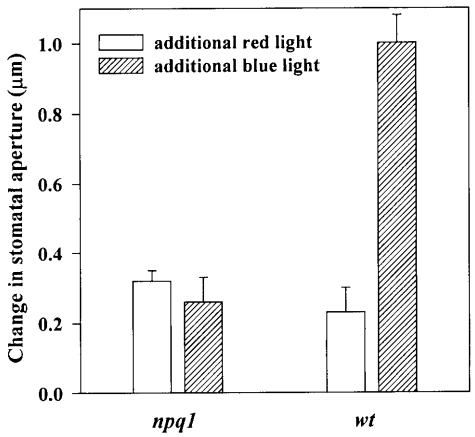


**Fig. 4** Increase in stomatal apertures in detached epidermis of wild-type and *npq1* leaves under added red or blue light. Change in stomatal apertures induced by blue or red light (20 $\mu$mol $m^{-2}$ $s^{-1}$) added to the final 1 h of a 3 h illumination with 150 $\mu$mol $m^{-2}$ $s^{-1}$ red light. Epidermis was incubated in 20 mM KCl, 0.1 mM $CaCl_2$, and 1 mM MES-NaOH (pH 6.0), and aerated with compressed air. Each value represents an average of three experiments (30 measurements each)±SE. Average aperture at the start of the blue or red light treatments was 1.63±0.08 $\mu$m for wild-type and 1.76±0.04 $\mu$m for *npq1* stomata.

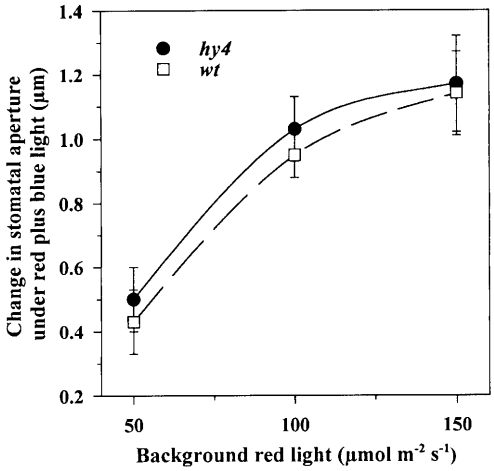


**Fig. 5** Change in stomatal apertures in response to blue light in detached epidermis of wild-type and *hy4* Arabidopsis leaves. Conditions as in Fig. 3. Each value represents an average of four experiments (30 measurements each)±SE. Average aperture at the start of the blue light treatment was 1.88±0.09 $\mu$m for wild-type and 1.89±0.09 $\mu$m for *hy4* stomata.

aperture increases in wild-type stomata that were substantially greater than those stimulated by additional red light (Fig. 4). In *npq1*, the aperture increases were small under both blue and red light, and their magnitude matched the aperture increases stimulated by red light in the wild type. Similar results were obtained using 100 and 50 $\mu$mol $m^{-2}$ $s^{-1}$ background red light (data not shown). As demonstrated in previous studies (Schwartz and Zeiger 1984, Karlsson 1986a, Assmann 1988), the large aperture increases of wild-type stomata stimulated by added blue light ensue from the activation of the specific stomatal response to blue light; this response was absent in *npq1* stomata. The reduced magnitude of the aperture increases under blue and red light in *npq1* stomata and under red light in the wild type are characteristic of photosynthesis-driven stomatal responses (Zeiger 1990). These results indicate that *npq1* stomata can respond to blue light via guard cell photosynthesis. The similar magnitude of the photosynthesis-dependent stomatal responses in wild-type and *npq1* stomata indicate that *npq1* guard cells have a seemingly normal photosynthetic response.

*Stomata from hy4 (cry 1) have a normal blue light response*—The dual beam protocol described above was also used to investigate whether stomata from the Arabidopsis mutant *hy4* (*cry1*) showed anomalies in their specific response to blue light. In contrast to *npq1*, aperture increase in *hy4* stomata stimulated by 20 $\mu$mol $m^{-2}$ $s^{-1}$ of blue light, applied in a background of 50, 100, or 150 $\mu$mol $m^{-2}$ $s^{-1}$ of red light, were indistinguishable from those of wild-type stomata (Fig. 5). These results indicate that *hy4* stomata have a seemingly normal blue light-specific response, and that the *HY4* gene product is not required for the stomatal response to blue light.

*Stomata from npq1 have a normal response to white light*—Light response curves of wild-type and *npq1* stomata from detached epidermis under white light illumination are shown in Fig. 6. Maximal apertures in both geno-

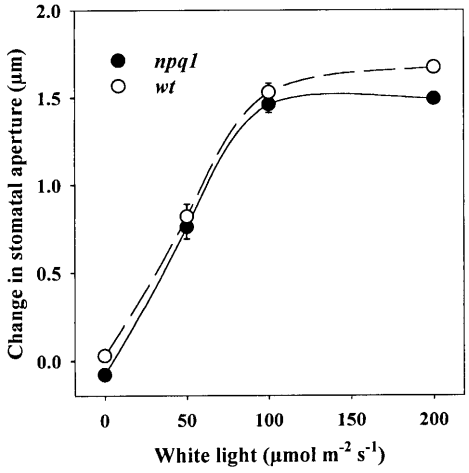


**Fig. 6** Change in stomatal aperture in detached epidermis of wild-type and *npq1* leaves under white light. Stomatal aperture values after 2 h illumination by 100 $\mu$mol $m^{-2}$ $s^{-1}$ white light. Incubation conditions as in Fig. 1. Each value represents an average of three experiments (30 measurements each)±SE. Average aperture at the start of the light treatment was 1.47±0.09 $\mu$m for wild-type and 1.53±0.02 $\mu$m for *npq1* stomata.

types were obtained at around 100 $\mu$mol $m^{-2}$ $s^{-1}$ of white light. Despite the lack of a specific blue light response in *npq1* stomata, there was little apparent difference between the stomatal responses to white light of the two genotypes.

## Discussion

The normal blue light response of *hy4* (*cry1*) stomata, tested under a stringent requirement of quantitative blue light-dependent increases in aperture as a function of increasing fluence rates of background red light (Fig. 5), confirms previous findings (Lascève et al. 1999) that a functional *CRY1* gene product is not required for the blue light response of stomata. In contrast, the inability of *npq1* stomata to respond to blue light beyond the level of the photosynthesis-dependent response (Fig. 4) provides genetic evidence for a zeaxanthin requirement in blue light-stimulated stomatal opening, and for a role of the carotenoid zeaxanthin in blue light photoreception by guard cells. Pigment analysis of *hy4* leaves in light and in darkness showed that this mutant has a normal xanthophyll cycle (X. Jin and E. Zeiger, unpublished results); thus zeaxanthin-dependent blue light responses in *hy4* stomata would be expected to operate normally.

A specific blue light response by guard cell chloroplasts (Srivastava and Zeiger 1992, Quiñones et al. 1996) provided initial evidence for a chloroplastic blue light photoreceptor in guard cells. Inhibition of the stomatal response to blue light by an inhibitor of zeaxanthin formation, DTT, singled out zeaxanthin as a putative photoreceptor (Srivastava and Zeiger 1995b). Genetic evidence for a role of zeaxanthin in guard cell photoreception was first provided by the covariance of guard cell zeaxanthin content and blue light sensitivity in stomata from segregating $F_2$ leaves of Pima cotton (Quiñones et al. 1998), and this role is confirmed in the present study using *npq1*. The *npq1* mutant of Arabidopsis is the first reported mutant lacking a specific stomatal response to blue light.

The enhancement of blue light responses by background red light has been explained by interactions between phytochrome and the blue light photoreceptor. Phytochrome has a demonstrated role in blue light responses such as phototropism and inhibition of hypocotyl elongation (Horwitz 1994, Short and Briggs 1994, Neff and Chory 1998). Phytochrome, however, has no known effect on short-term changes in stomatal apertures (Zeiger 1990), nor does it mediate the enhancement of the stomatal response to blue light by background red light (Karlsson 1988). On the other hand, a role of zeaxanthin in blue light photoreception provides a straightforward explanation for the enhancement of the stomatal response to blue light by red light (Karlsson 1986a, Assmann 1988, Srivastava and Zeiger 1995a). Zeaxanthin formation in the chloroplast has the action spectrum of photosynthesis (Yamamoto 1979), so higher fluence rates of red light result in higher zeaxanthin content in the guard cells and thus a higher blue light sensitivity (Srivastava and Zeiger 1995b, Quiñones et al. 1998).

The results with the *npq1* mutant showing that guard cell zeaxanthin is required for the stomatal response to blue light, and the close correspondence between the action spectrum for blue light-stimulated stomatal opening (Karlsson 1986b) and the absorption spectrum of zeaxanthin, implicate zeaxanthin as a chromophore mediating blue light photoreception in guard cells. Most, if not all, known photoreceptor systems are pigment proteins, and zeaxanthin is a constituent of a multi-pigment-protein complex in the antenna bed of Photosystems I and II (Demmig-Adams and Adams 1992). Guard cell chloroplasts are enriched in Photosystem II, and the light-harvesting complex IIb (LHCIIb) has been implicated as one possible site of photoreception in sensory transducing chloroplasts (Zhu et al. 1997). Isomerization of zeaxanthin by blue light, conformational changes in the associated apoprotein(s), transduction of the light signal from the chloroplast to the plasma membrane ATPase by a second messenger, stimulation of proton pumping, and stomatal opening are key putative steps in the sensory transducing cascade mediating blue light-stimulated stomatal opening (Zeiger and Zhu 1998).

The normal response of *npq1* stomata to white light is puzzling because of the apparent inconsequence of a lack of the blue light response. At saturating fluence rates, red light-stimulated stomatal opening, which depends on guard cell photosynthesis, is usually suboptimal (Zeiger 1990), pointing to an important role of the response to blue light in stomatal function. The normal stomatal response to white light in *npq1* parallels the seemingly normal growth of the mutant under natural sunlight, despite its defective photoprotection mechanisms (Niyogi et al. 1998). These observations probably reflect a redundancy of key functional properties in leaves, as evident from the fact that stomata can attain maximal apertures in either light or darkness via separate mechanisms (Schwartz and Zeiger 1984).

Both light (Srivastava and Zeiger 1995a, b) and $CO_2$ (Zhu et al. 1998) have been shown to change the zeaxanthin content of guard cells, thus underscoring a functional link between light and $CO_2$ changes in the leaf environment and the modulation of stomatal apertures. Characterization of the photochemistry of zeaxanthin under blue light excitation, its interaction with the involved apoprotein(s), and the identity of the second messenger linking the sensory transducing cascade within the chloroplast with the extrachloroplastic targets are some of the emerging questions for further investigation. The *npq1* mutant should provide a valuable experimental system for addressing these questions.

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