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Close correspondence between the action spectra for the blue light responses of the guard cell and coleoptile chloroplasts, and the spectra for blue light-dependent stomatal opening and coleoptile phototropism

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ABSTRACT **Fluorescence spectroscopy was used to characterize blue light responses from chloroplasts of adaxial guard cells from Pima cotton (*Gossypium barbadense*) and coleoptile tips from corn (*Zea mays*). The chloroplast response to blue light was quantified by measurements of the blue light-induced enhancement of a red light-stimulated quenching of chlorophyll *a* fluorescence. In adaxial (upper) guard cells, low fluence rates of blue light applied under saturating fluence rates of red light enhanced the red light-stimulated fluorescence quenching by up to 50%. In contrast, added blue light did not alter the red light-stimulated quenching from abaxial (lower) guard cells. This response pattern paralleled the blue light sensitivity of stomatal opening in the two leaf surfaces. An action spectrum for the blue light-induced enhancement of the red light-stimulated quenching showed a major peak at 450 nm and two minor peaks at 420 and 470 nm. This spectrum matched closely an action spectrum for blue light-stimulated stomatal opening. Coleoptile chloroplasts also showed an enhancement by blue light of red light-stimulated quenching. The action spectrum of this response, showing a major peak at 450 nm, a minor peak at 470 nm, and a shoulder at 430 nm, closely matched an action spectrum for blue light-stimulated coleoptile phototropism. Both action spectra match the absorption spectrum of zeaxanthin, a chloroplastic carotenoid recently implicated in blue light photoreception of both guard cells and coleoptiles. The remarkable similarity between the action spectra for the blue light responses of guard cells and coleoptile chloroplasts and the spectra for blue light-stimulated stomatal opening and phototropism, coupled to the recently reported evidence on a role of zeaxanthin in blue light photoreception, indicates that the guard cell and coleoptile chloroplasts specialize in sensory transduction.**

Blue light responses in plants are primarily associated with photomorphogenesis and plant movements (1). Both flavins and carotenoids have been suggested as blue light photoreceptors but the involved chromophore(s) is yet to be identified. Recent work in our laboratory has singled-out the carotenoid zeaxanthin as a putative blue light photoreceptor in both corn coleoptiles (2) and *Vicia* guard cells (3, 4).

Blue light-stimulated stomatal opening is a well-characterized blue light response, usually probed under blue light applied on a background of saturating red light (3, 5, 6). The response has an action spectrum showing a major peak at 450 nm and minor peaks at 420 and 470 nm (5). Light-dependent stomatal opening has a second, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU)-sensitive component, that is usually probed under red light and has the action spectrum of photosynthesis (6).

A reported DCMU- and carbonylcyanide *m*-chlorophenylhydrazone (CCCP)-sensitive stimulation of a H^+-ATPase at the guard cell plasma membrane by red light implicates the guard cell chloroplast in the sensory transduction of stomatal opening mediated by photosynthetic active radiation (7). The characterization of a blue light response of the guard cell chloroplast (8) and of a role of the chloroplastic pigment zeaxanthin in blue light-stimulated stomatal opening (3, 4) further implicates the guard cell chloroplast in the sensory transduction of blue light.

Blue light-stimulated coleoptile phototropism is a classical blue light response that has an action spectrum showing major and minor peaks at 450 and 470 nm, respectively, and a shoulder at 430 nm (9). The close correspondence between the action spectra for blue light-stimulated stomatal opening and phototropism suggests that the two blue light responses might share early steps in the sensory transduction process. Coleoptile chloroplasts have been proposed as a possible site of photoreception for phototropism in 1960 (10) but this concept was abandoned with the emergence of the flavin hypothesis of blue light photoreception (11). Recent work showing that zeaxanthin content in coleoptile tips is tightly correlated with coleoptile sensitivity to blue light (2) point to a role of the coleoptile chloroplast in blue light-stimulated coleoptile phototropism.

In the present study, we used fluorescence spectroscopy to further our understanding of the blue light responses of the guard cell and the coleoptile chloroplast. Obtained results show that the red light-stimulated, chlorophyll *a* fluorescence quenching from adaxial (upper) guard cells from cotton leaves is enhanced by low fluence rates of blue light, while abaxial (lower) guard cells are devoid of this response. This differential blue light sensitivity of adaxial and abaxial guard cells parallels the blue light sensitivity of stomatal opening in the two leaf surfaces (12). An obtained action spectrum for the blue light-induced enhancement of the fluorescence quenching closely matches the action spectrum for blue light-stimulated stomatal opening. Corn coleoptile chloroplasts also show the red light-stimulated fluorescence quenching and an enhancement of this quenching by blue light. The action spectrum for this blue light response closely matches the action spectrum for blue light-stimulated coleoptile phototropism.

MATERIALS AND METHODS

Plant Material. Cotton plants (*Gossypium barbadense* L., Pima 32) were grown in a greenhouse. The seeds were planted in 7.5-dm^3 pots with garden soil (Armstrong Potting Soil, Armstrong Garden Centers, Glendora, CA), and the plants

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were grown under natural sunlight (1800–2000 $\mu mol \cdot m^{-2} \cdot s^{-1}$). At noon, air temperature was 30 ± 3°C and relative humidity was *ca*. 50%. Plants were irrigated daily and fertilized weekly (Spoonit: 18% N, 20% P, 16% K, 0.225% Fe).

Corn seeds (*Zea mays* L., Pioneer hybrid 3362) were soaked in tap water for 24 h and planted between two layers of garden soil in metal or plastic trays. The trays were placed in a greenhouse under full sunlight (1800–2000 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Air temperature was 30 ± 3°C and the humidity was *ca*. 50% at noon. All trays were watered daily. Light-grown coleoptiles were harvested 5 days after sowing. Coleoptile tips, 1.5 mm long, were used for chlorophyll *a* fluorescence measurements. The tips were devoid of any leaf tissue.

Measurement of Chlorophyll *a* Fluorescence Quenching. The blue light-induced enhancement of the red light-stimulated quenching of chlorophyll *a* fluorescence was used to quantify the blue light response of guard cell and coleoptile chloroplasts. Guard cell preparations were obtained from adaxial (upper) and abaxial (lower) epidermal strips isolated from Pima 32 cotton leaves as described by Lu *et al.* (12). Briefly, adaxial and abaxial epidermis were isolated and cleaned enzymatically from contaminant mesophyll and epidermal cell chloroplasts. The purified preparations contained intact, abaxial or adaxial guard cells. For the fluorescence measurements, guard cell suspensions containing 1 μg of chlorophyll [determined according to Marker (13)] were incubated in 0.25 M mannitol/1 mM $CaCl_2$, pH 6. Batches of 10 coleoptile tips placed on top of a water-soaked filter paper were used for fluorescence measurements of coleoptile chloroplasts.

Guard cell and coleoptile preparations were dark-adapted for several minutes (>10) prior to the fluorescence measurements, and the samples were irradiated with actinic light for 40 s to determine the slope of the fluorescence quenching. Chlorophyll *a* fluorescence was measured with a PAM 100 modulated chlorophyll fluorometer (Walz, Effeltrich, Germany). Modulated fluorometers use a weak modulated light source to induce chlorophyll fluorescence and measure this fluorescence at the frequency and phase of the modulated light. This approach makes it possible to measure fluorescence in the presence of actinic light of any intensity and wavelength. The fluorometer was linked to a personal computer equipped with a data acquisition system. A four-armed fiber optic, aimed at the sample cuvette, was connected to the modulated light source, the detector, and two actinic light sources. A Kodak 4000 slide projector equipped with a WKO EXR halogen lamp and a Schott RG-6 filter (Duryea, PA) was used to obtain actinic red light. Monochromatic blue lights of different wavelengths were provided by a set of Ealing interference filters (South Natick, MA) used with a Dolan–Jenner high-intensity fiber illuminator (Woburn, MA). Fluence rates were measured with a Li-Cor LI-185B quantum meter (Lincoln, NE).

Data Processing and Curve Fitting. Linear regressions (Fig. 3 *A* and *B*) and curve fitting (Fig. 3 *C* and *D*) were obtained by processing the data with SigmaPlot (Jaendel Scientific, Corte Madera, CA).

RESULTS

Enhancement of the Red Light-Stimulated Quenching of Chlorophyll *a* Fluorescence by Blue Light in Pima Cotton Guard Cells. Typical fluorescence transients from dark-adapted guard cell chloroplasts (8, 14) show a maximum followed by a decline or quenching ensuing from a decrease in the fluorescence yield (see *Inset* of Fig. 1). This quenching primarily reflects photochemical and nonphotochemical de-excitation of photosystem II (15).

We analyzed the effect of blue light on the red light-induced fluorescence quenching from adaxial and abaxial guard cells from Pima cotton by comparing the rates of quenching ($-dv/dt$) induced by saturating red light with those induced by simultaneous excitation with red and blue light. Blue light induced a substantial increase in the quenching rate from adaxial guard cell chloroplasts (Fig. 1*A*), ranging from 20% under 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of added blue light to 50% at saturation (200–300 $\mu mol \cdot m^{-2} \cdot s^{-1}$). In contrast, added blue light caused only a slight increase in the quenching rate from abaxial guard cell chloroplasts (Fig. 1*A*). When the same red light background was supplemented with additional red light, quenching rates from both adaxial and abaxial cells changed only slightly (Fig. 1*B*), indicating that the enhancement of the red light-induced chlorophyll *a* fluorescence quenching of adaxial guard cell chloroplasts is blue light-specific.

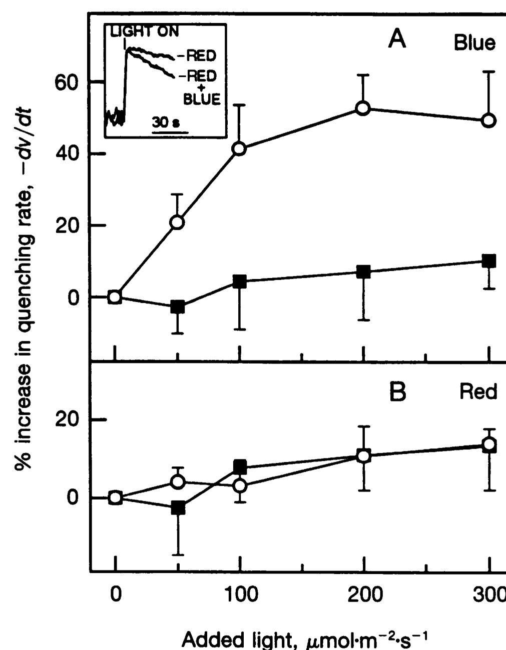

FIG. 1. Fluence rate dependence of the blue light-induced enhancement of the red light-stimulated chlorophyll *a* fluorescence quenching of adaxial and abaxial guard cells from Pima cotton. Quenching rates from adaxial (○) or abaxial (■) epidermal strips were first measured using red light (500 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and then using the same red light plus additional blue (*A*) or red (*B*) light of fluence rates ranging between 50 and 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The enhancement of the quenching rate induced by the added light is expressed as % increase. Values are the average of six experiments ± SD. (*Inset*) Typical chlorophyll *a* fluorescence transients (in arbitrary units of fluorescence intensity) from adaxial guard cells illuminated with red or red plus blue light.

We also compared the rate of fluorescence quenching from adaxial and abaxial guard cell chloroplasts induced by a broad range of fluence rates of actinic red or blue light. Fig. 2 shows that the quenching rates of abaxial guard cell chloroplasts were similar under both light qualities, whereas adaxial guard cell chloroplasts had slower quenching rates under red light and faster quenching rates under blue light. The differential responses to red and blue light were observed at all fluence rates tested, indicating that the cells were responding to light quality and not to light quantity.

It is of interest that the increasing quenching rate of adaxial guard cells as a function of fluence rate of blue light seemed biphasic (Fig. 2*B*). Blue light-stimulated stomatal opening in the intact leaf has similar characteristics (16), and it has been interpreted on the basis of two different components. At low fluence rates, blue light stimulates an intrinsic blue light response, whereas at high fluence rates it stimulates a chlorophyll-mediated response, which can also be observed under red light illumination. The effect of light quality on quenching rates of adaxial and abaxial guard cells suggests a similar mechanism.

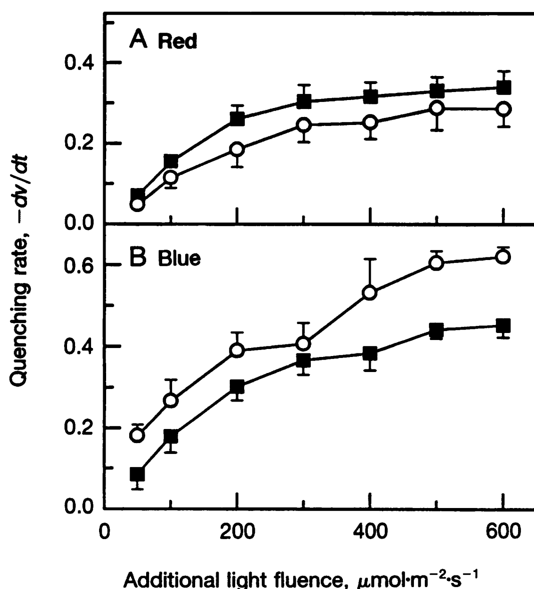

FIG. 2. Chlorophyll *a* fluorescence quenching rates of adaxial and abaxial guard cells from Pima cotton. Samples of adaxial (○) and abaxial (■) epidermal strips were illuminated with different fluence rates of red (*A*) or blue (*B*) light. Values are the average of six measurements ± SD.

Fluorescence transients from mesophyll tissue of cotton leaves showed the fine structure reported for other species (17), and red light-induced quenching rates were unaltered by addition of blue light over a broad range of fluence rates (data not shown). These observations indicate that cotton mesophyll chloroplasts lack the blue light response seen in adaxial guard cell chloroplasts from this species (Fig. 1*A*) and in abaxial guard cell chloroplasts from *Vicia* (8).

Action Spectrum for the Blue Light Response of Adaxial Guard Cell Chloroplasts. We obtained an action spectrum for the blue light-induced enhancement of the red light-stimulated chlorophyll *a* fluorescence quenching from adaxial guard cells by measuring the increase in quenching induced by low fluence rate, monochromatic blue light added to background, saturating red light (Fig. 3*A*). The slope of the fluence rate–response traces was used to calculate the fluence rate of each wavelength required to obtain a criterion response (the maximum response obtained with 450-nm light). The values obtained for each wavelength were used to calculate the action spectrum shown in Fig. 3*C*. The spectrum has a major peak at 450 nm and minor peaks at 470 and 420 nm. This fine structure is strikingly similar to the action spectrum for blue light-stimulated stomatal opening (ref. 5; Fig. 3*C*) and to the absorption spectra of carotenoids isolated from guard cells (18).

Enhancement of the Red Light-Induced Fluorescence Quenching by Blue Light in Corn Coleoptiles. Light-grown corn coleoptiles have functional chloroplasts (19) and show chlorophyll *a* fluorescence transients similar to those obtained from guard cell chloroplasts (data not shown). We investigated whether blue light can enhance the red light-stimulated fluorescence quenching from coleoptile chloroplasts, in a response homologous to that observed with guard cell chloroplasts. For these experiments, we applied different fluence rates of blue light to 1.5-mm coleoptile tips exposed to a background of saturating red light. The results showed that blue light enhanced the red light-stimulated quenching rate by up to 20% (Fig. 4). In contrast, addition of red light to saturating red light background had no effect on the quenching rate (Fig. 4). This indicates that the enhancement of the red light-induced chlorophyll *a* fluorescence quenching of corn coleoptile chloroplasts was, as in guard cell chloroplasts, blue light-specific.

Action Spectrum for the Blue Light Response of Corn Coleoptile Chloroplasts. We obtained an action spectrum for

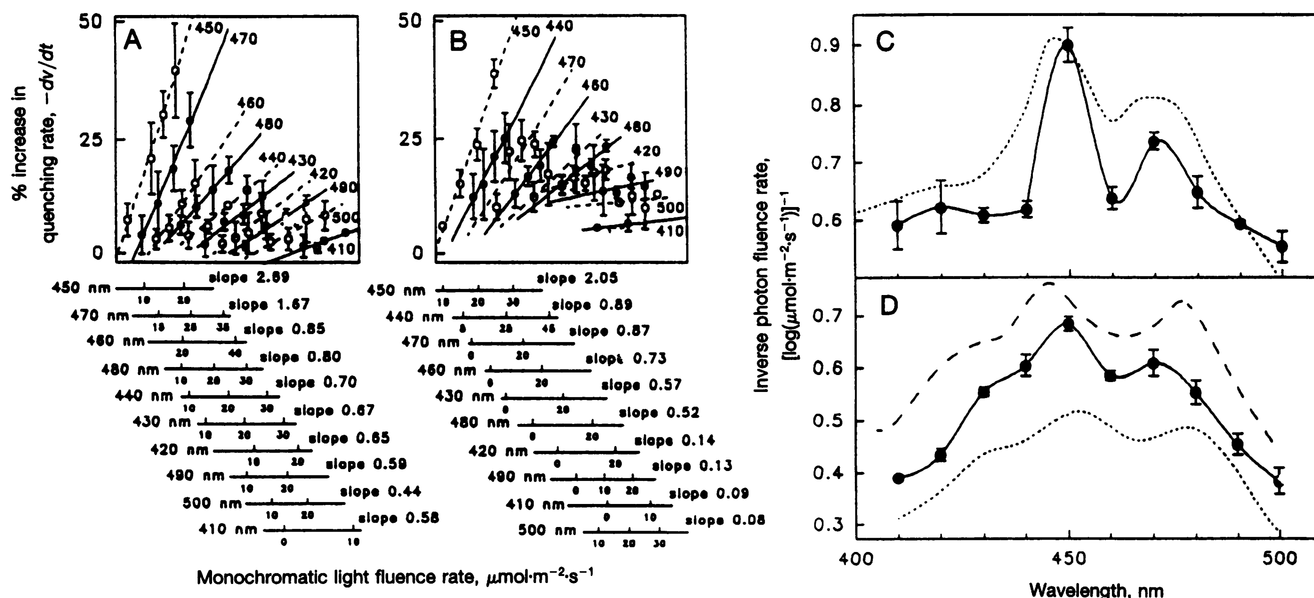

FIG. 3. Fluence rate–response curves and action spectra for the blue light-induced enhancement of the red light-stimulated chlorophyll *a* fluorescence quenching of adaxial guard cells from Pima cotton and light-grown corn coleoptiles. Adaxial epidermal strips from cotton (*A*) or corn coleoptile tips (*B*) were illuminated with a background of 500 μmol·m^{-2}·s^{-1} of red light plus monochromatic lights between 400 and 500 nm. Four different fluence rates (3–25 μmol·m^{-2}·s^{-1}) were tested for each wavelength. (*A* and *B*) The abscissas are shifted for each wavelength and, next to them, the slopes for the linear regressions are indicated. Values are the average of three measurements ± SD. (*C*) Calculated action spectrum for the blue light-stimulated enhancement of the red light-induced chlorophyll *a* fluorescence quenching of adaxial guard cells from cotton (—). The action spectrum for the blue light-stimulated stomatal opening (······) from Karlsson (5) is shown for comparison. (*D*) Calculated action spectrum for the blue light-stimulated enhancement of the red light-induced chlorophyll *a* fluorescence quenching of corn coleoptiles (—). The action spectrum for the phototropic curvature of oat coleoptiles (———) from Shropshire and Withrow (9) and the absorption spectrum of zeaxanthin (······) are added for comparison. (*C* and *D*) The inverse logarithms of the fluence rates needed to obtain the maximum response (obtained from *A* and *B*) were plotted against the wavelength. Vertical bars represent the average SD for the different fluence rates used at each wavelength.

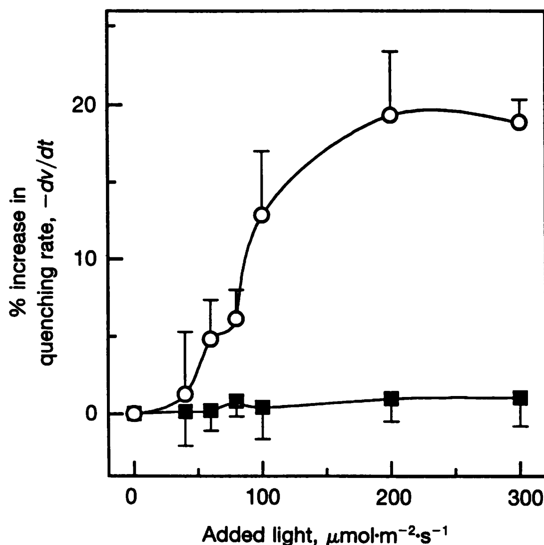

FIG. 4. Blue light-induced enhancement of the red light-stimulated chlorophyll *a* fluorescence quenching in light-grown corn coleoptiles. Ten 1.5-mm coleoptile tips were illuminated with a background of saturating red light (500 μmol·m^{-2}·s^{-1}) plus additional blue (○) or red (■) light of fluence rates between 40 and 300 μmol·m^{-2}·s^{-1}. The enhancement of quenching rate induced by the added light is expressed as % increase. Values are the average of three experiments ± SD.

the blue light-induced enhancement of the red light-stimulated quenching from corn coleoptiles using the same method employed with guard cell chloroplasts (Fig. 3*B* and *D*). The obtained spectrum has a major peak at 450 nm, a minor peak at 470 nm, and a shoulder at 430 nm (Fig. 3*D*). This fine structure closely matches the action spectrum for the phototropic response of oat coleoptiles (9) and the absorption spectrum of the carotenoid zeaxanthin, a putative photoreceptor for phototropism in corn coleoptiles (ref. 2; Fig. 3*D*).

DISCUSSION

Chlorophyll *a* fluorescence, an effective tool for the study of *in vivo* photosynthesis, has been extensively used in recent work on photochemical and nonphotochemical quenching (20).

Chlorophyll fluorescence studies have also been important for the characterization of the photosynthetic properties of the guard cell chloroplast (ref. 14 and references therein) and for the discovery of an intrinsic blue light response of these organelles (8). The blue light sensitivity of the fluorescence quenching from adaxial guard cells from cotton leaves (Figs. 1 and 2) closely resembles the blue light response of isolated guard cell chloroplasts from *Vicia* (8). In both preparations, low fluence rates of blue light added to saturating red light enhanced the quenching rates observed with red light alone, and quenching rates observed under blue light were faster than those measured under red light over a broad range of fluence rates. These properties indicate that this blue light response of the guard cell chloroplast is independent from intrinsic light reactions of photosynthesis.

The differential blue light sensitivity of the fluorescence quenching from adaxial and abaxial guard cells parallels their contrasting pigment content and the differential blue light sensitivity of blue light-stimulated stomatal opening in the two leaf surfaces (12). These contrasting properties of adaxial and abaxial guard cells and their chloroplasts probably reflect the drastically different light environments in which the two guard cell types develop and function. Further studies on the biochemical and molecular properties of adaxial and abaxial guard cell chloroplasts should prove valuable for the elucidation of the role of these organelles in acclimations and adaptations of guard cells to different light environments.

The action spectrum for the blue light response of the guard cell chloroplast (Fig. 3*C*) is similar to reported spectra for many blue light responses (11), including blue light-stimulated stomatal opening (5). The close correspondence between the action spectrum for the blue light response of the guard cell chloroplast and the spectrum for blue light-stimulated stomatal opening provides further evidence for a role of the guard cell chloroplast in the stomatal response to blue light.

The transduction of photosynthetic active radiation by the guard cell chloroplast into proton pumping at the guard cell plasma membrane implicates a second messenger carrying the signal from the chloroplast to the membrane-bound H^+-ATPase (7, 21). Photosynthetic intermediates (7), calcium (22, 23), and diacylglycerol (21) are putative candidates for second messengers. The vanadate sensitivity of blue light-stimulated proton pumping also implicates a H^+-ATPase at the guard cell plasma membrane (24). A role of the guard cell chloroplast in the sensory transduction of both photosynthetic active radiation and blue light suggests that the two photosensory pathways could share key transducing components. Calcium has been implicated as a second messenger in the transduction of blue light-stimulated proton pumping (25) and in the regulation of the proton-pumping ATPase at the guard cell plasma membrane.

The implication of zeaxanthin as a blue light photoreceptor for phototropism (2) has rekindled interest in coleoptile chloroplasts. A recent study has characterized photosynthetic properties of corn coleoptile chloroplasts which contrast with those from corn mesophyll and are strikingly similar to the properties of guard cell chloroplasts (19). These properties include high rates of photosystem II-dependent oxygen evolution, low rates of carbon fixation, a large starch content, and a xanthophyll cycle which is highly sensitive to low fluence rates of incident radiation. Results from the present study show that, like their guard cell counterparts, coleoptile chloroplasts also have a red light-stimulated fluorescence quenching that is enhanced by low fluence rates of blue light (Fig. 4). The close similarity between the action spectra for the blue light responses of the coleoptile and guard cell chloroplast and between these spectra and the action spectra for blue light-stimulated stomatal opening and phototropism (Fig. 3) strengthens the concept of a role of these chloroplasts in the sensory transduction of blue light responses.

The sensory transduction process mediating light-stimulated phototropism involves changes in hormone distribution and flow, and asymmetric growth (26). Regulation by proton pumping of a pH gradient across the plasma membrane of coleoptile cells is a central feature of the chemiosmotic model of auxin transport (27). By analogy with the guard cell system, the coleoptile chloroplast could transduce the sensing of light gradients in the lighted and shaded sides of the coleoptile into a modulation of proton pumping and concomitant changes in pH gradients leading to asymmetric hormone distribution and bending.

The 400- to 500-nm region of the action spectra for the blue light responses of the guard cell and coleoptile chloroplasts (Fig. 3) shows the three-peak signature typical of action spectra for phototropism, inhibition of stem elongation, and blue light-stimulated stomatal opening (5, 9, 11). At room temperature, absorption spectra of oxidized flavins and flavoproteins show a 450-nm maximum but are consistently devoid of any fine structure in the 400- to 500-nm region (28). Spectra of reduced flavins often show shoulders at around 420 and 470 nm but are also devoid of discrete peaks. On the other hand, the fine structure of the spectra for blue light-stimulated phototropism and stomatal opening, and for the chloroplastic response to blue light in both guard cells and coleoptiles is

typical of room temperature absorption spectra of carotenoids, including zeaxanthin (Fig. 3).

It is of further interest that the most conspicuous difference between the action spectra for stomatal opening and phototropism, the minor peak and the shoulder, respectively, seen in the 410- to 430-nm region, is conserved in the action spectra for the blue light responses for the guard cell and coleoptile chloroplasts (Fig. 3). This conserved fine structure, which might reflect binding properties of the chromophore, presumably zeaxanthin, to its apoprotein, provides further evidence for a functional link between the blue light response of the coleoptile and guard cell chloroplasts and the blue light responses of the intact systems.

Recently reported work implicating zeaxanthin as a blue light photoreceptor in guard cells and coleoptiles (2–4) has provided quantitative evidence for a specific chromophore functioning as a photoreceptor in two well-defined blue light responses. The striking similarity between the spectral properties of the guard cell and coleoptile chloroplast reported here points to a common role of these organelles in the sensory transduction of blue light.

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