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Arabidopsis Contains at Least Four Independent Blue-Light-Activated Signal Transduction Pathways[1]

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We have investigated the stomatal and phototropic responses to blue light of a number of single and double mutants at various loci that encode proteins involved in blue-light responses in Arabidopsis. The stomatal responses of light-grown mutant plants (*cry1, cry2, nph1, nph3, nph4, cry1cry2,* and *nph1cry1*) did not differ significantly from those of their wild-type counterparts. Second positive phototropic responses of etiolated mutant seedlings, *cry1, cry2, cry1cry2,* and *npq1-2,* were also similar to those of their wild-type counterparts. Although *npq1* and single and double *cry1cry2* mutants showed somewhat reduced amplitude for first positive phototropism, threshold, peak, and saturation fluence values for first positive phototropic responses of etiolated seedlings did not differ from those of wild-type seedlings. Similar to the *cry1cry2* double mutants and to *npq1-2,* a *phyAphyB* mutant showed reduced curvature but no change in the position or shape of the fluence-response curve. By contrast, the phototropism mutant *nph1-5* failed to show phototropic curvature under any of the irradiation conditions used in the present study. We conclude that the chromoproteins cry1, cry2, nph1, and the blue-light photoreceptor for the stomatal response are genetically separable. Moreover, these photoreceptors appear to activate separate signal transduction pathways.

In the past decade, studies of mutants have been of great value in delineating the functions of various photoreceptors in their independent, interdependent, and antagonistic roles in mediating the developmental and functional events of photomorphogenesis in plants (Jenkins et al., 1995; Chamovitz and Deng, 1996; Fankhouser and Chory, 1997; Quail, 1998; Whitelam et al., 1998). Although there are numerous studies of mutants at the various phytochrome loci and loci that encode components downstream from the phytochrome photoreceptors, there are fewer with mutants involving processes regulated by blue light. Because the number of known or postulated blue-light photoreceptors has climbed from zero to four (cry1, Ahmad and Cashmore, 1993; nph1, Christie et al., 1998; cry2, Hoffmann et al., 1996; Lin and Cashmore, 1996; Lin et al., 1996b; zeaxanthin, Zeiger and Zhu, 1998), it is now possible to use mutants to investigate which pathways are activated by these photoreceptors (Liscum and Hangarter, 1994; Parks et al., 1998; Briggs and Huala, 1999), as has been done with the phytochromes and their signal transduction pathways. In the present study we have used mutants in an attempt to determine which photoreceptors are involved in blue-light-induced stomatal opening and which are involved in phototropism. We have included single mutants at the *NPH1*, *CRY1*, *CRY2*, and *NPQ1* loci, the first three encoding photoreceptors and the fourth encoding an enzyme involved in zeaxanthin biosynthesis. We have also included the double mutants *nph1cry1*, *cry1cry2*, and *phyAphyB* in several experiments.

Extensive biochemical studies have been carried out on nph1, a protein associated with the plasma membrane of phototropically sensitive tissues of etiolated seedlings (Short and Briggs, 1994; Briggs and Liscum, 1996, 1997). This protein becomes rapidly and extensively phosphorylated both in vivo and in vitro upon irradiation with blue light. Biochemical and genetic evidence show that this phosphoprotein, which is a Ser/Thr kinase (Reymond et al., 1992a; Huala et al., 1997), participates early in phototropism (for review, see Short and Briggs, 1994). Recently, Liscum and Briggs (1995, 1996) described a series of mutants at four loci in Arabidopsis, designated *nph1* through *nph4* (non-phototropic hypocotyl), all impaired in their phototropic responses. In *nph1* mutants carrying alleles generated by fast-neutron bombardment (*nph1-1*, *nph1-3*, *nph1-4*, and *nph1-5*), no detectable light-induced phosphorylation was observed, and these mutants appeared to lack the target protein for phosphorylation (Liscum and Briggs, 1995; Huala et al., 1997). The *nph1-5* allele contains a deletion that removes the region coding for the C-terminal half of the gene (Huala et al., 1997). No *NPH1* mRNA is detectable in *nph1-5* (J.M. Christie and W.R. Briggs, unpublished results).

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With the exception of the weak allele *nph1-2* (mutant JK224; Khurana and Poff, 1989), which shows impaired first positive curvature but normal second positive curvature (Khurana and Poff, 1989), all known mutants at the *NPH1* locus lack first and second positive curvature of etiolated hypocotyls in response to blue light. They also lack second positive curvature of roots and hypocotyls of light-grown seedlings. Liscum and Briggs (1995) hypothesized that the *NPH1* gene encodes the photoreceptor apoprotein, a hypothesis borne out by the cloning and characterization of the gene and complementation of the *nph1-5* mutant with the wild-type gene (Huala et al., 1997). A recent study showed that NPH1 expressed in insect cells binds FMN as a chromophore and retains photosensitivity for light-induced phosphorylation similar to that of the native Arabidopsis protein and supports the hypothesis that nph1 functions as the photoreceptor for phototropism (Christie et al., 1998). The *NPH2*, *NPH3*, and *NPH4* loci encode proteins that function downstream from the nph1 protein (Liscum and Briggs, 1995, 1996).

In the past few years, the genes *HY4* and *CRY2* that encode, respectively, the apoproteins for the blue-light photoreceptors cry1 (Ahmad and Cashmore, 1993) and cry2 (Hoffmann et al., 1996; Lin et al., 1996b, 1998) in Arabidopsis have been cloned. (Note: *hy4* is the original designation for mutants at the *CRY1* locus. Subsequent isolates have been given the designation *cry1* instead of *hy4*. Following the conventions for phytochrome, the wild-type holoproteins for these photoreceptors are designated here cry1, cry2, and nph1, and the apoproteins CRY1, CRY2, and NPH1.) The cry1 protein has been partially characterized (Lin et al., 1995a, 1995b, 1996a; Malhotra et al., 1995; Lin and Cashmore, 1996); it encodes a protein with significant homology to prokaryote DNA photolyases. cry1 has two chromophores: one FAD (Lin et al., 1995a, Malhotra et al., 1995) and the other probably a pterin (Malhotra et al., 1995). The CRY2 apoprotein also has homology with prokaryotic DNA photolyases and is reported to bind at least a flavin as a chromophore (Lin et al., 1996b). Both cry1 and cry2 serve as photoreceptors mediating blue-light-induced inhibition of hypocotyl growth in Arabidopsis (Ahmad and Cashmore, 1993; Lin et al., 1995a, 1995b, 1996a, 1998), with cry2 also playing a role in flowering (Guo et al., 1998).

Recently, Ahmad et al. (1998) published evidence that a *cry1cry2* double mutant was deficient in first positive phototropic curvature and postulated a role for cry1 and cry2 in phototropism. However, both biochemical and genetic evidence indicate that at least the cry1 and nph1 proteins participate in separable signal transduction pathways (Liscum and Briggs, 1995). The *hy4-105* mutant fails to show blue-light-inducible inhibition of hypocotyl elongation but does show normal phototropism. By contrast, *nph1* null mutants show normal blue-light inhibition of hypocotyl elongation but lack any phototropic response to unilateral blue light. In addition, the *nph1hy4-105* F_1 hybrid shows both responses, and the double mutant shows neither response (Liscum and Briggs, 1995). Finally, the null mutant *hy4-2.23N* (Koornneef et al., 1980; Ahmad and Cashmore, 1993) has wild-type amounts of the plasma membrane phosphoprotein and shows wild-type levels of phosphorylation upon blue-light irradiation (Liscum and Briggs, 1995).

Another well-known response of higher plants to light is the blue-light activation of stomatal opening (Zeiger, 1983). Stomata respond to blue light at very low photon-flux densities with insufficient energy to drive stomatal opening directly (Sharkey and Ogawa, 1987). With whole plants, a blue-light pulse that was added to a strong red light background saturating for photosynthesis triggered a transient increase in stomatal opening (*Commelina communis*, Zeiger et al., 1985; wheat, Karlsson, 1986). Similar results were obtained with *C. communis* seedlings placed in darkness and CO_2-free air (Lascève et al., 1993). Thus, under saturating red light or CO_2-free air and darkness, blue light acts as a signal independent of phytochrome to promote stomatal opening; it must do so by activating a specific blue-light photoreceptor. The Arabidopsis mutant *npq1* has been shown to be defective in its violaxanthin de-epoxidase (Niyogi et al., 1998), the enzyme mediating the first step in the formation of zeaxanthin. This mutant fails to show blue-light-induced stomatal opening (Zeiger and Zhu, 1998). Thus, zeaxanthin may serve as the chromophore for a fourth blue-light-activated photoreceptor system.

Because the nph1 and cry1 pathways are known to be genetically separable, as are the cry1 and cry2 pathways, our first objective was to investigate, by studying the responses of mutants, whether any of these photoreceptors plays a role in the stomatal response to blue light. Mutants carrying lesions in genes encoding elements downstream from nph1 were also included in the study. Chory (1992) and Liscum and Hangarter (1994) mention that light-induced stomatal opening in *hy4* null mutants of Arabidopsis appears to be unimpaired, but they provide no details. To our knowledge, there is no information yet available concerning whether nph1 and/or cry2 is required for light-activated stomatal opening or, alternatively, plays a role in regulating the magnitude of the response. Our aim, therefore, was to investigate light-induced stomatal opening in available mutants at all of the *CRY* and *NPH* loci.

Our second objective was to carry out a detailed study of the phototropic responses and blue-light-induced phosphorylation of a number of different mutant alleles at the *CRY1* and *CRY2* loci. We used both single and double mutants in an effort to investigate a possible role for the cry1 and cry2 photoreceptors in these responses. Because *phyAphyB* double mutants have also shown reduced phototropism (Hangarter, 1997), a *phyAphyB* double mutant was included in this study. Phytochromes in Arabidopsis have been shown to affect the magnitude of phototropic curvature (Hangarter, 1997; Janoudi et al., 1997), without having a role in mediating detection of light direction; this could provide a model for the action of cryptochromes in phototropism. Because zeaxanthin was also hypothesized to be a photoreceptor (Quiñones and Zeiger, 1994; Quiñones et al., 1996), we included the mutant *npq1-2* in the phototropism study.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The *nph* mutants used in this study were isolated previously (Liscum and Briggs, 1995; Huala et al., 1997). The *cry1* mutants have been described: *hy4-2.23N* (Koornneef et al., 1980), *hy4-105* (Liscum and Hangarter, 1991), *hy4-B104* (Bruggemann et al., 1996), and *cry1-304* (Mockler et al., 1999). The *cry2-1* mutant has also been identified (Guo et al., 1998). With the exception of *nph3-1* and *hy4-2.23N*, which are in the Wassilewskija and Landsberg *erecta* backgrounds, respectively, all mutants are in the Columbia background.

The *nph1-5* mutant is a true null (Huala et al., 1997), and neither *cry1-304* (*hy4-304*) nor *cry2-1* produced detectable CRY1 or CRY2 apoprotein, respectively, based on western analysis (Guo et al., 1998). The *hy4-B104* mutant produced a trace of *CRY1* mRNA, detectable by northern analysis, and a trace of CRY1 apoprotein, detectable by western analysis (M.A. Olney and J.M. Christie, unpublished data). The *cry2-1* and the *hy4-B104cry2-1* mutants showed neither *CRY2* mRNA nor CRY2 apoprotein by northern or western analysis, respectively (M.A. Olney and J.M. Christie, unpublished data). The *hy4-2.23N* mutant produced a truncated transcript (Ahmad and Cashmore, 1993) and is presumably a protein null. The nature of the lesions in the mutants *hy4-105, nph3-1*, and *nph4-1* is presently unknown. Seeds for the *hy4-B104*, *cry1-304*, and *cry2-1* single mutants and the *hy4-B104cry2-1* and *cry1-304cry2-1* double mutants were kindly provided by Dr. Chentao Lin (University of California, Los Angeles). The *phyAphyB* double mutant was kindly provided by Dr. Peter H. Quail (University of California, Berkeley). The *npq1-2* mutant was the kind gift of Dr. Krishna Niyogi (University of California, Berkeley).

For investigations of light regulation of stomatal aperture, seeds were germinated and grown in sand and watered with one-half-strength Hoagland solution. The pots were placed in a growth chamber (16-h light period, 23°C, and RH 75%; 8-h dark period, 20°C, and RH 85%). For membrane preparations from etiolated seedlings to be used in the phosphorylation experiments, sterilized seeds (30% commercial bleach for 15 min followed by three rinses with sterile distilled water) were sown on moist filter lying on one-half-strength Murashige and Skoog medium in 1% agar (Sigma). After cold treatment (5°C) in darkness for 2 to 4 d and a 2-h red-light treatment to induce germination, the plates were kept horizontal, wrapped in foil, and in complete darkness for 3 d at 22°C before harvest under a dim-red light. For the phototropism studies, the sterilized seeds were placed in rows directly on one-half-strength Murashige and Skoog medium in 1% agar in square Petri plates. After the same cold and red-light treatments, the plates were placed on edge, wrapped in aluminum foil, and kept in complete darkness at 22°C for 2 or 3 d before phototropic induction. Seedling age was determined from the start of the red-light treatment.

Light Sources

For the stomatal investigations, plants were grown in growth chambers with 150-W mercury lamps (250 μmol m^{-2} s^{-1}; HQI-TS, NDL, Osram, München, Germany). To determine the effects of blue, red, or white light on stomatal opening, white light (230 μmol m^{-2} s^{-1}) was obtained from the same Osram lamps fitted with a heat-reflecting filter (Tempax 113, Schott, Wiesbaden, Germany). For blue and red light (both 60 $\pm$ 5 μmol m^{-2} s^{-1}), the same white light was filtered either through plastic filter no. 77 (blue, maximum transmittance at 465 nm) or through plastic filter no. 24 (maximum transmittance at 700 nm), respectively (R. Juliat, Paris). The emission spectra for the blue- and red-light sources are shown in Figure 1. Light for induction of phosphorylation was supplied according to the procedure described by Liscum and Briggs (1995).

Light for induction of second positive phototropic curvature was provided by a cool-white fluorescent lamp (F20T12/CW, Sylvania) filtered through one layer of blue Plexiglas (no. 2424, Rohm and Haas, Philadelphia, PA), fluence rate approximately 2 μmol m^{-2} s^{-1}. Light for induction of first positive curvature was provided by a 500-W tungsten light source (33-89-39, Bausch & Lomb, Rochester, NY) filtered through a broad wavelength band, blue-glass filter (5-60, Corning Inc., Corning, NY). For fluence-response studies of first positive curvature, the fluence rate was adjusted with neutral density filters, and exposure times varied from 1 to 100 s to cover 4 orders of magnitude of fluences. Red light for both induction of germination and testing its effects on phototropism was obtained by filtering the light from two red fluorescent lamps (F20T12/R, 20 W, Phillips, Eindhoven, The Netherlands) through Shinkolite 102 red plastic (Argo Plastics, Los Angeles, CA). The fluence rate was approximately 2 μmol m^{-2} s^{-1}.

Whole-Plant Gas-Exchange Measurements

Four- to five-week-old Arabidopsis plants (total leaf area 4–8 cm^2) were removed from the sand and inserted in the experimental chamber (Vavasseur et al., 1988). The root compartment (19°C) was supplied with an aerated one-half-strength Hoagland solution. The shoot compartment (21°C) was attached to an open-flow gas circuit (air flow, 120 L h^{-1}). At the inlet, the water-vapor pressure was held constant (1.5 kPa) and measured at the outlet with a dew-point hygrometer (model 600, EG&G, Waltham, MA). In the leaf chamber the water vapor pressure deficit was 0.66 $\pm$ 0.07 kPa. The changes in [CO_2] were quantified with an IR gas analyzer (model 225 MK3, ADC, Hoddesdon, UK).

Plants were left in the chamber for a few days before the beginning of each experiment. A single plant was used for several days, and its leaf surface area was measured daily from enlarged photographs. Because of the small size of the Arabidopsis leaves, the leaf temperature was not measured. However, under the low-light fluence rates used, we assumed, for our calculations of leaf conductances, that it remained close to air temperature. Any error introduced by this assumption would be almost constant between experiments and should not affect the conclusions.

The light responses were measured with the following procedure: After a standard dark period of 14 h at 21°C, leaf conductances were recorded during a 45-min exposure

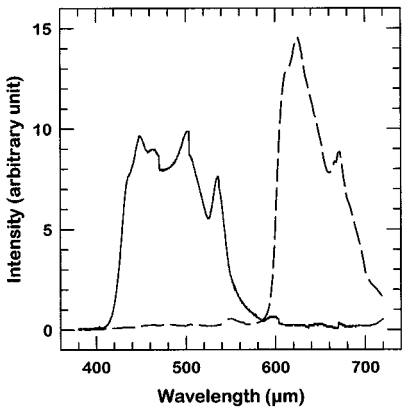

Figure 1. Spectral distribution of the blue- and red-light sources for experiments of light effects on stomatal aperture. For the blue (solid line) and red (dashed line) spectra, the fluence rate at the leaf surface was near 60 μmol m^{-2} s^{-1}.

to blue light, followed by 45 min of exposure to red light of the same fluence. A second blue-light exposure was given before returning the plants to white light. Experiments were duplicated, and the data are presented as mean leaf conductance, g (millimoles per square meter per second) and CO_2 flux (micromoles per square meter per second), both calculated for each plant.

Phototropism Studies

For induction of second positive curvature responses, seedlings were exposed to the unilateral blue-light source described above for either 6 or 24 h. The square Petri plates were kept so that the hypocotyls were oriented vertically and along the agar surface, as was the case during their germination and growth (see above). Blue light was given from one side through the overlapping sides of the Petri dish tops and bottoms. The attenuation of the blue light by the overlapping sides of the Petri dishes was included in estimating the fluence rate at the level of the seedlings. At the end of the 6- or 24-h induction, the images of the seedlings were scanned directly (ScanJet 4C/T, Hewlett-Packard) with a transparency adapter, using the software supplied (DeskScan 2 2.4, Hewlett-Packard). Curvature measurements were made directly from the printouts. All seedlings except those touching each other were measured.

For induction of first positive curvature, the lids of the Petri dishes were removed just before irradiation and the plates were fixed vertically and aligned so that the plane of the Petri dish was about 15° from the plane parallel to the light beam and there could be no shading of one seedling by another. The agar surface was kept vertical with the seedlings in the same orientation in which they had been growing. Thus, the Petri dish wall did not attenuate the light striking the seedlings save for a few closest to the wall. The technique permitted calculation of the inductive fluences with an error of less than 5%. The error introduced because the curvature was 15° out of the imaged plane was small and the same for all samples. The Petri dish lid was replaced immediately after irradiation. For induction, we first followed the multiple-pulse technique of Steinitz and Poff (1986), a procedure that amplified first positive curvature in Arabidopsis without altering threshold and peak values for the curvature response. A given total fluence was administered at 20-min intervals in five successive pulses, each one-fifth the total fluence.

Janoudi and Poff (1992) found that a pulse of red light given 2 h before a single inductive pulse of blue light strongly enhanced subsequent first positive phototropic curvature of dark-grown Arabidopsis hypocotyls. Therefore, we also used this technique with the two *cry1cry2* double mutants to determine whether they showed amplified first positive curvature under those irradiation conditions. Curvatures were imaged as they were for the second positive curvature, 120 min after the start of the first pulse. All seedlings were measured except those few that touched one another; the curvature over the apical 1 cm of the image of each hypocotyl was recorded (1 cm on the image = 0.386 cm of hypocotyl). Apart from the blue-light treatment, all manipulations were carried out in total darkness until imaging. Where required, hypocotyl-length measurements were made directly from the seedling images and then corrected for magnification by the imaging system.

Phosphorylation Studies

Phosphorylation experiments were carried out as described previously by Liscum and Briggs (1995). Quantitation was performed with a phosphor imager (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Stomatal Responses in Wild-Type and Mutant Seedlings

Figure 2 shows the results of continuous gas-exchange measurements with wild-type Columbia, wild-type Landsberg *erecta*, three null alleles at the *NPH1* locus (*nph1-1*, *nph1-4,* and *nph1-5*), one allele at the *NPH3* locus (*nph3-1*), one allele at the *NPH4* locus (*nph4-1*), two alleles at the *HY4* locus (*hy4-2.23N* and *hy4-105*), one allele at the *CRY2* locus (*cry2-1*), and *nph1-1hy4-105* and *hy4-B104cry2-1* double mutants. Despite some variability among the mutants, in every case, the initial blue-light treatment induced a strong opening response, the stomata closed under red light, a second blue-light pulse induced a second opening response, and the much higher-fluence-rate white light induced further opening.

For each of the genotypes, changes in stomatal conductance and absolute values of mean leaf stomatal conductance at the end of each irradiation treatment are given in Table I. The time courses for changes in stomatal conductance (Fig. 2) and the mean leaf stomatal conductances at the end of the irradiation periods indicate that for all the genotypes studied, blue light (60 $\pm$ 5 μmol m^{-2} s^{-1}) was far more effective than red light in promoting stomatal opening. Red light, by contrast, was unable to sustain the level of stomatal opening reached under blue light. With all genotypes, the mean leaf stomatal conductances after 45

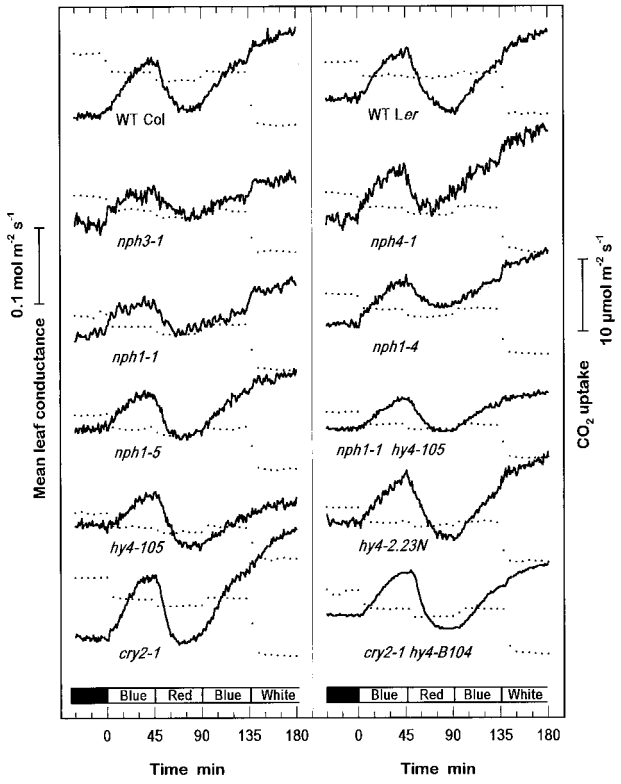

Figure 2. Changes in mean leaf stomatal conductance (solid lines) and CO_2 uptake (dashed lines). After a dark period (14 h), plants were successively irradiated with blue (60 μmol m^{-2} s^{-1}), red (60 μmol m^{-2} s^{-1}), blue (60 μmol m^{-2} s^{-1}), and then white light (230 μmol m^{-2} s^{-1}) for 45-min exposures. Each experiment was done at least twice.

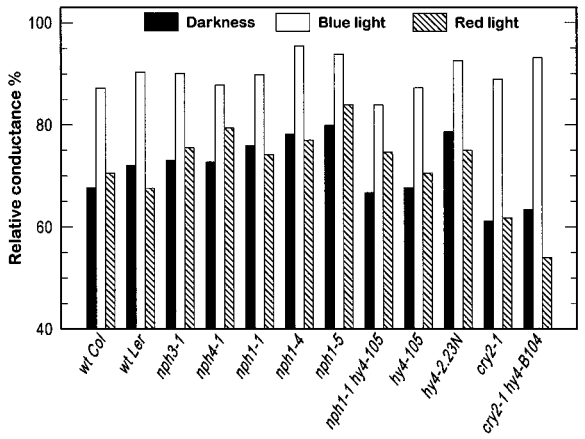

Figure 3. Relative mean conductances measured in darkness before experiments and at the end of the first blue- and red-light irradiations. Leaf conductances under the different conditions were normalized in each case to conductances measured after 45 min of white light.

min of red light treatment were similar to those measured in darkness.

The amplitude of the stomatal opening induced by white light (250 μmol m^{-2} s^{-1}) depended on the genotype (Table I). The source of this variability is unknown, but may reflect variations in leaf temperature among plants, inaccuracy in estimation of exposed leaf surface (difficult to obtain accurately without destroying the plant), or some consequence of the mutations. However, when the responses to blue or red light are plotted relative to the response to white light (Fig. 3), the blue-light responses are similar for all genotypes. Under the same fluence rates (60 μmol m^{-2} s^{-1}), the rate of CO_2 uptake was significantly lower under blue than under red light (Table II). Despite this difference, the low-fluence-rate blue light (60 μmol m^{-2} s^{-1}) induced stomatal opening similar to that induced by white light at a fluence rate (230 μmol m^{-2} s^{-1}), which had sustained approximately a 7-fold greater rate of photosynthesis. These results, consistent with those obtained with *Commelina communis* (Travis and Mansfield, 1981; Schwartz and Zeiger, 1984; Gautier et al., 1992) and fava bean (Shimazaki et al., 1986), suggest that photosynthesis does not play a major role in blue-light-induced stomatal opening in these species and that a specific blue-light-activated system must be involved.

Table I. *Mean leaf conductances, g, under darkness, or blue, red, or white light for various wild-type and mutant Arabidopsis lines*

WT Col, Wild-type Columbia; WT L*er*, Wild-type Landsberg *erecta*. Values are ±SE.

Genotype	Leaf Conductance (*g*)			
	Darkness	Blue light	Red light	White light
	mmol m^{-2} s^{-1}			
WT Col	256 ± 58	332 ± 46	279 ± 43	391 ± 42
WT L*er*	245 ± 15	307 ± 7	230 ± 22	340 ± 1
nph3-1	273 ± 8	322 ± 2	287 ± 7	342 ± 9
nph4-1	260 ± 22	327 ± 13	292 ± 22	392 ± 20
nph1-1	189 ± 19	232 ± 13	200 ± 13	262 ± 7
nph1-4	275 ± 1	332 ± 1	300 ± 3	378 ± 1
nph1-5	258 ± 12	305 ± 5	252 ± 7	338 ± 1
nph1-1 hy4-105	186 ± 17	228 ± 21	184 ± 27	239 ± 27
hy4-105	223 ± 7	263 ± 7	193 ± 3	256 ± 1
hy4-2.23N	305 ± 11	366 ± 33	285 ± 33	389 ± 20
cry2-1	183 ± 36	267 ± 37	185 ± 34	300 ± 44
cry2-1 hy4-B104	125 ± 10	184 ± 11	106 ± 8	197 ± 10

Table II. *CO_2 uptake under darkness, blue, red, or white light for various wild-type and mutant Arabidopsis lines*

WT Col, Wild-type Columbia; Wt Ler, Wild-type Landsberg *erecta*. Values are ±SE.

Genotype	CO_2			
	Darkness	Blue light	Red light	White light
	μmol m^{-2} s^{-1}			
WT Col	1.70 ± 0.94	−1.30 ± 0.93	−2.55 ± 1.37	−9.29 ± 2.12
WT Ler	0.71 ± 0.11	−1.22 ± 0.08	−1.41 ± 0.23	−6.58 ± 0.44
nph3-1	1.41 ± 0.31	−0.63 ± 0.45	−1.76 ± 0.61	−6.48 ± 0.58
nph4-1	1.68 ± 0.20	−0.32 ± 0.09	−1.31 ± 0.03	−6.38 ± 0.05
nph1-1	1.63 ± 0.18	−0.42 ± 0.01	−1.46 ± 0.17	−6.62 ± 0.87
nph1-4	0.62 ± 0.05	−1.60 ± 0.20	−2.47 ± 0.05	−7.83 ± 0.09
nph1-5	0.65 ± 0.06	−1.12 ± 0.15	−1.95 ± 0.22	−7.02 ± 0.35
nph1 hy4-105	1.02 ± 0.24	0.65 ± 0.58	−1.41 ± 0.73	−5.30 ± 1.81
hy4-105	1.76 ± 0.11	−0.22 ± 0.20	−0.91 ± 0.23	−4.53 ± 0.12
hy4-2.23N	0.57 ± 0.05	−1.42 ± 0.03	−1.88 ± 0.12	−7.16 ± 0.04
cry2-1	0.79 ± 0.07	−2.25 ± 0.44	−3.19 ± 0.77	−7.17 ± 0.71
cry2-1 hy4-B104	0.92 ± 0.13	−1.66 ± 0.27	−2.71 ± 0.31	−7.97 ± 0.91

Phototropic Responses of Wild-Type and Mutant Seedlings

Second Positive Curvature

Figure 4 shows phototropic curvatures measured after 6 and 24 h of continuous blue-light irradiation of wild-type and mutant seedlings that were 3 d old at the onset of the light treatment. All of the seedlings but the null mutant *nph1-5* showed strong second positive curvature. With the exception of the 24-h response of the *cry1-304cry2-1* double mutant, all of the mutants showed a strong phototropic response after 6 h and an even stronger response after 24 h. The results with *hy4-B104* and *cry1-304* confirmed the earlier results of Liscum and Briggs (1995) with the *hy4-105* allele.

Both phyA and phyB have been shown to play a role in determining the magnitude of the Arabidopsis second positive phototropic response (Hangarter, 1997; Janoudi et al., 1997). Development of curvature in *phyA* or *phyB* single mutants is slower than in the wild type, and *phyAphyB* double mutants show dramatically impaired curvature development during the first few hours of continuous unilateral irradiation (Hangarter, 1997). However, by 6 h the *phyAphyB* double mutant seedlings showed almost as much curvature as their wild-type counterparts (Fig. 4) and by 24 h they differed little from the wild type.

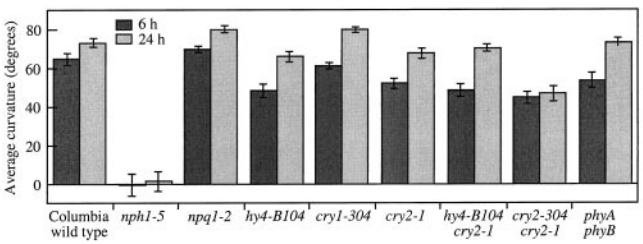

Figure 4. Second positive phototropic curvatures of wild-type and various mutant seedlings of Arabidopsis after 6 (gray bars) and 24 (black bars) h of continuous irradiation with unilateral blue light of 2 μmol m^{-2} s^{-1} (n = 18–39, except that for *npq1-2*, n = 70–97). Seedlings were 3 d old at the onset of irradiation.

First Positive Curvature

Figure 5 shows fluence-response curves covering the range from threshold to saturation for various single and double mutants at the *NPH1, NPQ1, CRY1, CRY2, PHYA*, and *PHYB* loci. All seedlings were 3 d old (±2 h) at the onset of blue-light treatment. With the exception of *nph1-5*, all of the mutants showed substantial positive curvature. Because the curvatures were smaller than the wild type in the other mutants, it was difficult to determine exact thresholds in all of the cases. However, they did not differ dramatically from that of the wild type and were between log fluence −1.0 and −0.5 μmol m^{-2}. Peak curvatures were found near the same fluence in all cases (log fluence 0–0.5 μmol m^{-2}), with saturation at log fluence 3.0 μmol m^{-2}. The curious double peak for first positive curvature first reported by Konjević et al. (1989) for Arabidopsis appears as a shoulder not only in the wild-type but also in all of the *cry*-mutant seedlings.

All of the 3-d-old *cry* mutants, both single and double, showed reduced curvatures in comparison to the wild type. This reduction may reflect either a real genetic difference between the mutants and the wild type, implicating the cry1 and cry2 photoreceptors in modulating the magnitude of the phototropic response, which is known to be the case with phyA and phyB (Hangarter, 1997; Janoudi et al., 1997), or inherent differences in growth rates from different seed lots related to different conditions during seed maturation and harvest. Therefore, we measured hypocotyl lengths of 2- and 3-d-old *cry1cry2* double mutants, *npq1-2*, and the wild type (Table III). These three mutants were chosen because *cry1cry2* double mutants failed to show first positive curvature in the Ahmad et al. (1998) study and because zeaxanthin was implicated in phototropism by the correlative studies of Quiñones and Zeiger (1994) and Quiñones et al. (1996). The results indicate that all three mutants grew more rapidly than their wild-type counterparts. Given the difference in early growth rates, we measured fluence-response curves for the 2-d-old seedlings for the *cry1cry2* double mutants and *npq1*. All three of

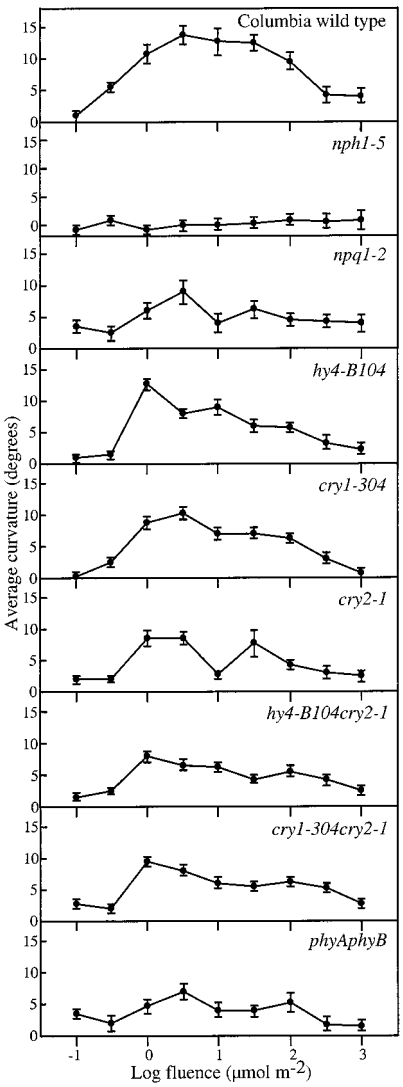

Figure 5. Fluence-response curves for first positive curvature for etiolated 3-d-old wild-type and mutant seedlings of Arabidopsis (n = 57–102; except that for *phyAphyB*, n = 32–56; for *nph1-5*, n = 29–37; and for *npq1-2*, n = 34–47). Error bars represent ±SE.

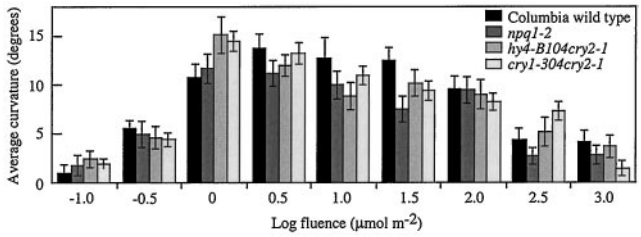

Figure 6. Fluence-response curves for first positive curvature for the etiolated 2-d-old *npql-2*, *hy4-B104cry2-1*, and *cry1-304cry2-1* seedlings (n = 36–96) and for 3-d-old etiolated wild-type seedlings (n = 57–80).

these younger mutants showed strong curvatures that were indistinguishable in magnitude from those of 3-d-old wild-type seedlings (Fig. 6). Threshold, peak, shoulder, and saturation fluence levels for the double mutants and for *npq1-2* were again the same as those for the wild type. No curvature response was detected in the shorter 2-d-old wild-type seedlings (data not shown).

Ahmad et al. (1998) failed to detect first positive curvature in response to a single pulse of blue light in the first-positive-fluence range from a *cry1cry2* double mutant. We therefore attempted to induce first positive curvature in the wild type and in the two *cry1cry2* double mutants with a single pulse instead of five multiple pulses. The fluence chosen was 3 μmol m^{-2} (log 0.5), where first positive curvature induced by multiple pulses was near its maximum (Fig. 5). Initial efforts with 3-d-old etiolated seedlings of Columbia wild type, *hy4-B104cry2-1*, and *cry1-304cry2-1* gave curvatures of only 3.9° ± 0.7°, 1.3° ± 0.7°, and 3.6° ± 0.7° (n = 91, 98, and 66), respectively. We used a 10-min pulse of red light starting 2 h before phototropic induction, a technique demonstrated by Janoudi and Poff (1992) to amplify first positive curvature strongly. In this case, the curvatures were 16.2° ± 2.6°, 11.3° ± 1.3°, and 12.1° ± 1.7° (n = 74, 72, and 69), respectively. Thus, in our experiments *cry1cry2* double mutants show first positive phototropic responses to both single and multiple blue-light pulses.

Blue-Light-Induced Autophosphorylation of nph1 in Wild-Type and Mutant Seedlings

Figure 7 shows the results of phosphorylation experiments with membrane preparations from wild-type seed-

Table III. *Lengths of hypocotyls of wild-type, cry1cry2 double mutant, and npq1 single mutant seedlings of different ages*

WT Col, Wild type, Columbia. Values are ±SE.

Genotype	2-d-Old Seedlings		3-d-Old Seedlings	
	Length	Experiment	Length	Experiment
	mm		*mm*	
WT Col	2.73 ± 0.131	1	12.11 ± 0.134	2, 3
hy4-B104cry2-1	6.97 ± 0.251	1	11.74 ± 0.211	2, 3
cry1-304cry2-1	6.74 ± 0.159	1	14.24 ± 0.258	1
WT Col	4.00 ± 0.199	4	11.03 ± 0.188	4
npq1-2	6.62 ± 0.208	4	13.38 ± 0.263	4

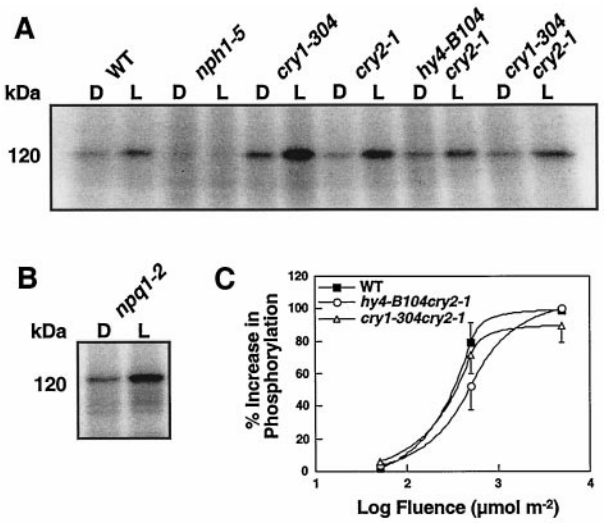

Figure 7. Light-induced phosphorylation of nph1 in etiolated seedlings from the wild-type (WT) and various mutant lines. Protein load was 20 μg per lane. A, Autoradiograms of dark-control (lanes D) membranes from the various *cry* mutants and *nph1-5* and those given saturating blue light (lanes L, $10^{3.3}$ μmol m^{-2}) immediately before phosphorylation. B, Same as A, except for *npq1-2*. C, Fluence-response curve for light-induced phosphorylation of nph1 from etiolated wild-type and *hy4-B104cry2-1* and *cry1-304cry2-1* mutant seedlings ($n = 3$).

lings and various mutants. Blue light induced a strong enhancement of phosphorylation of nph1 in wild-type membranes but no response in the null mutant *nph1-5* (Fig. 7A), confirming the results of Liscum and Briggs (1995). Saturating blue light also induced a strong enhancement of phosphorylation in membranes from both single and double mutants at the *cry* loci (Fig. 7A) and from the *npq1-2* mutant (Fig. 7B). Fluence-response curves for light-induced phosphorylation of membranes from the *hy4-B104cry2-1* and *cry1-304cry2-1* double mutants do not differ significantly from that for the wild type (Fig. 7C).

DISCUSSION

All of the mutants tested, both single and double, showed strong stomatal regulation by blue light (Fig. 2). The *nph1-1hy4-105* double mutant showed a somewhat reduced response, but its conductance in darkness was lower than that of all of the other wild-type and mutant plants; and when all of the responses were normalized to the white-light response (Fig. 3), its responses fell within the range of the other mutants and the wild type. Hence, we conclude that the action of blue light in inducing stomatal opening must be mediated by a photoreceptor and a signal transduction pathway genetically separable from the pathways mediated through cry1, cry2, or nph1—a photoreceptor probably using zeaxanthin as its chromophore. We further conclude that cry1, cry2, and nph1 do not play a major role in modulating the magnitude of the stomatal response to blue light.

With the exception of the phototropism null mutant *nph1-5*, all of the mutants tested, both single and double, showed strong second positive curvature (Fig. 4). These results support the conclusion that cry1 and cry2, either alone or together, cannot serve as the photoreceptor detecting light direction for second positive phototropism. They could modulate curvature magnitude; one of the double mutants, *cry1-304cry2-1*, showed slightly reduced curvature after 24 h of unilateral light. The result with *npq1-2* likewise indicates that zeaxanthin cannot serve to detect light direction for second positive phototropism. The only mutant failing to respond was *nph1-5*, supporting the argument that nph1 alone detects the light direction.

As was the case with second positive curvature, all of the mutants tested, except for *nph1-5*, displayed first positive curvature over the same fluence range (Fig. 5). The threshold, peak, shoulder, and saturation fluence values did not differ among the mutants and wild type. The reduced response magnitude obtained from the mutants in comparison to that of wild-type seedlings could reflect action by cry1, cry2, or zeaxanthin downstream from nph1, which appears to be the case with phyA and phyB (Fig. 5; Hangarter, 1997; Janoudi et al., 1997). On the other hand, the reduced magnitude of curvature reported here for *npq1-2* and the single and double *cry* mutants may reflect physiological differences between the seedlings arising from conditions during seed maturation and/or storage or from differences in growth rates (Table III). Seedlings of *npq1-2* and both *cry1cry2* double mutants only 2-d-old showed first positive curvature that is as strong as that of 3-d-old wild-type seedlings (Fig. 6). By contrast, 2-d-old wild-type seedlings were much shorter than 2-d-old seedlings of the double mutants or *npq1-2* (Table III) and failed to show measurable phototropic curvature (data not shown). Hence, in first positive phototropism, as with the stomatal response and second positive phototropism, there must be a separate photoreceptor and signal transduction pathway independent of zeaxanthin, cry1, or cry2 and their downstream signaling components. Again, the only mutant failing to respond was *nph1-5*, supporting the argument that only nph1 detects the light direction.

The reason that Ahmad et al. (1998) did not obtain measurable first positive curvature in their *cry1cry2* double mutant may be that their growth conditions somewhat desensitized phototropism in their seedlings. Evidence for this is suggested by the very narrow fluence range, little more than 1 order of magnitude, of their curvatures. The full fluence-response curve for first positive phototropism normally extends over at least 3 orders of magnitude (Fig. 5; Iino, 1990).

Quiñones and Zeiger (1994) reported a correlation between the level of zeaxanthin in maize coleoptiles and phototropic sensitivity and proposed that zeaxanthin was the photoreceptor for this response. Subsequently Palmer et al. (1996) showed that maize coloeptiles devoid of all carotenoids, as the result of either a genetic lesion or herbicide treatment, nevertheless showed strong second positive curvature and normal blue-light-induced phosphorylation. Horwitz and Berrocal (1997) have hypothesized that these two papers address separate phenomena. Under the appropriate fluence conditions, the curve for first positive curvature shows two peaks (Konjević et al., 1989, 1992).

Horwitz and Berrocal (1997) hypothesized that they might involve two different photoreceptors, zeaxanthin for the higher-fluence peak and some other chromophore for the lower-fluence peak. However, in the present study the mutant *nph1-5* failed to show either peak of first positive curvature, although the other mutants and the wild type showed both. Therefore, both peaks must require a functional nph1-photoreceptor holoprotein.

In vitro blue-light-activated phosphorylation of nph1 in *npq1-2* and the *cry* single and double mutants appears to be similar to that of the protein from wild-type seedlings (Fig. 7). Microsomal membranes from these mutants plus *cry1* and *cry2* single mutants showed significant increases in phosphorylation on irradiation with saturating blue light. A fluence-response curve for phosphorylation in both of the *cry1cry2* double mutants is not distinguishable from that of the wild type (Fig. 7). Ahmad et al. (1998) reported phosphorylation in wild-type membranes at fluences below those at which we have detected light-induced phosphorylation (Short and Briggs, 1990; Reymond et al., 1992b; Palmer et al., 1993; Christie et al., 1998). This discrepancy may be the consequence of differences in growth conditions. From our results, however, we conclude that neither cry1 nor cry2 serve as photoreceptors for the blue-light-induced phosphorylation of nph1.

As mentioned above, Zeiger and Zhu (1998) have shown that the Arabidopsis *npq1* mutant fails to show blue-light-induced stomatal opening. They also reported correlations between zeaxanthin content and sensitivity of the stomatal response to blue light. Their conclusion, based on these results, is that zeaxanthin functions as the photoreceptor chromophore for blue-light-induced stomatal opening. Their findings indicate that the stomatal photoreceptor contains a carotenoid chromophore rather than one involving FMN (nph1; Christie et al., 1998), or FAD and a pterin (for cry1 at least, but not known for cry2; Lin et al., 1995a, 1996b; Malhotra et al., 1995). Thus, unlike the phytochromes, which carry a bilitriene chromophore, different blue-light photoreceptors use different chromophores.

In summary, our results support the conclusion that the blue-light photoreceptors cry1, cry2, nph1, and zeaxanthin (the likely photoreceptor chromophore for blue-light-induced stomatal opening) all activate genetically separable pathways and that Arabidopsis must contain at least four different photoreceptors and signal transduction pathways. Our results also support the conclusion that only nph1 functions to detect light direction in phototropism, both first positive and second positive, although other photoreceptors may modulate subsequent differential growth response.

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