Phototropin 1 and dim-blue light modulate the red light de-etiolation response

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Light signals regulate seedling morphological changes during de-etiolation through the coordinated actions of multiple light-sensing pathways. Previously we have shown that red-light-induced hypocotyl growth inhibition can be reversed by addition of dim blue light through the action of phototropin 1 (phot1). Here we further examine the fluence-rate relationships of this blue light effect in short-term (hours) and long-term (days) hypocotyl growth assays. The red stem-growth inhibition and blue promotion is a low-fluence rate response, and blue light delays or attenuates both the red light and far-red light responses. These de-etiolation responses include blue light reversal of red or far-red induced apical hook opening. This response also requires phot1. Cryptochromes (cry1 and cry2) are activated by higher blue light fluence-rates and override phot1's influence on hypocotyl growth promotion. Exogenous application of auxin transport inhibitor naphthylphthalamic acid abolished the blue light stem growth promotion in both hypocotyl growth and hook opening. Results from the genetic tests of this blue light effect in auxin transporter mutants, as well as *phytochrome kinase substrate* mutants indicated that *aux1* may play a role in blue light reversal of red light response. Together, the phot1-mediated adjustment of phytochrome-regulated photomorphogenic events is most robust in dim blue light conditions and is likely modulated by auxin transport through its transporters.

Introduction

In the developing seedling the transition from a dark growth program to a light growth program includes a well described set of physiological changes. The process of photomorphogenesis is typified by hypocotyl/stem growth inhibition, apical hook opening, chloroplast development, cotyledon expansion and other responses that are finely tuned to match the ambient light environment. Light quality, quantity, direction, and duration are all the key parameters in guiding this transition. Blue (B), red (R), and far-red (FR) light induce the most conspicuous changes. These alterations begin with photons activating specific photoreceptors, the cryptochromes (crys) and phototropins (phots) sensing B and UV-A light, and the phytochromes (phys), responsive to R and FR light. The different light signals initiate specific transduction events that work independently, cooperatively, or even counteractively to modulate photomorphogenic events.[1-3]

There is substantial evidence of genetic, physical and biochemical interaction between photosensors during developmental transitions. The phys are required for cry-mediated stem inhibition induced by B light.[1,4,5] Similarly, B light-induced phototropic responses likely require coordinated actions of several photoreceptors.[3,6] The degree of phot-mediated phototropic bending at least partially depends on the phy-antagonizing effect on hypocotyl agravitropism.[7-9] Additionally, crys also work together with phots to modulate phototropism according to the fluence rate of B light.[6] On the other hand, R or FR light-induced hypocotyl growth inhibition is most likely an event solely mediated by phys.[2,10] Addition of high fluence rate B to R or FR light background generally enhances this inhibition through crys. These are examples where light inputs are complementary, additive or synergistic, otherwise known as "coaction."

While B light responses typically advance photomorphogenesis, in rare cases it has been shown that phototropin directs processes that retard development. When dim B light (0.1 μmol/m^{2}s) is delivered simultaneously with R light (<70 μmol/m^{2}s), the R light-induced hypocotyl inhibition reverses and the stem growth rate is enhanced.[11,12] Genetic tests indicate that this effect is mediated by phot1 and requires the presence of NPH3.[12] These findings present special cases in plant development where coaction between multiple photosensory systems guides seedling acclimation to the light environment.

This study examines the phot1-mediated B light reversal of phytochrome responses in greater detail. Fluence-rate / response and time course experiments detail the interactions between phototropin 1 and other sensory systems with respect to hypocotyl growth rates and hook opening. Pharmacological and genetic tests show that the B light response is likely mediated by redundant transporters. These tests provide additional information

about interaction between low-fluence-rate B light and opposition of R-light-mediated events during early photomorphogenic development.

Results

Dim B light opposes red-induced hypocotyl growth inhibition and apical hook opening

A previous report showed that dim B light could attenuate R or FR light induced hypocotyl growth rate inhibition when applied simultaneously.[12] The present study expands these findings to further characterize B and R/FR light interaction during photomorphogenic development. Apical hook opening and chlorophyll biosynthesis are 2 events associated with R light induced de-etiolation. **Figure 1A** shows that addition of dim B light to the background of R light can eliminate hypocotyl growth inhibition. The simultaneous change in hook opening was also affected. Seedlings treated with R light for 180 min possessed an average hook angle of 67.6 ($\pm$9.5) degrees. However, when a dim B light (0.1 μmol/m^{2}s) was co-irradiated with R light, the hook angle was only 28.1 ($\pm$6.3) degrees after 180 min (**Fig. 1B**). Eventually, the apical hook fully opened with no visible differences in both experimental light after 24 h (data not shown). The same effect on the apical hook opening was also observed when B light was delivered with FR light (**Fig. 1C**). No differences in chlorophyll accumulation kinetics were observed (**Fig. S1**).

Blue light fluence rate/response in the modulation of red de-etiolation responses

The results from **Figure 1** show that a R light response may be negated by a dim B light pulse. To characterize the fluence-rate/response relationship of this phenomenon, the R light grown seedlings were treated with co-illumination of B light at different fluence rates. The results in **Figure 2** show that a fluence rate of 1.5 μmol/m^{2}s B light applied in conjunction with the R treatment was sufficient to partially reverse the R-light-mediated growth inhibition (**Fig. 2A**). Increasing B light fluence rate to 10 μmol/m^{2}s enhanced R-light-induced stem inhibition. Fluence rate-response experiments on hook opening show that increasing B light fluence rate to 1.5 μmol/m^{2}s also delayed the hook opening relative to R light alone. However, the higher fluence rate of B light ($\geq$10 μmol/m^{2}s) accelerated the R light-induced hook opening process (**Fig. 2B**).

The roles of crys and phot1 in the B-light-mediated adjustment of hook opening response

The phot1 receptor has been shown to mediate the dim B reversal of R/FR light-induced hypocotyl inhibition. The R-light-induced hook opening kinetics were also examined in the *phot1* mutant background. The results in **Figure 3A and B** showed that slight delays in the onset of hook opening, and slower opening in FR, were found between R/FR light and R/FR light plus dim B light treated mutant seedlings. The cry receptors are responsible for higher-fluence rate seedling responses to B
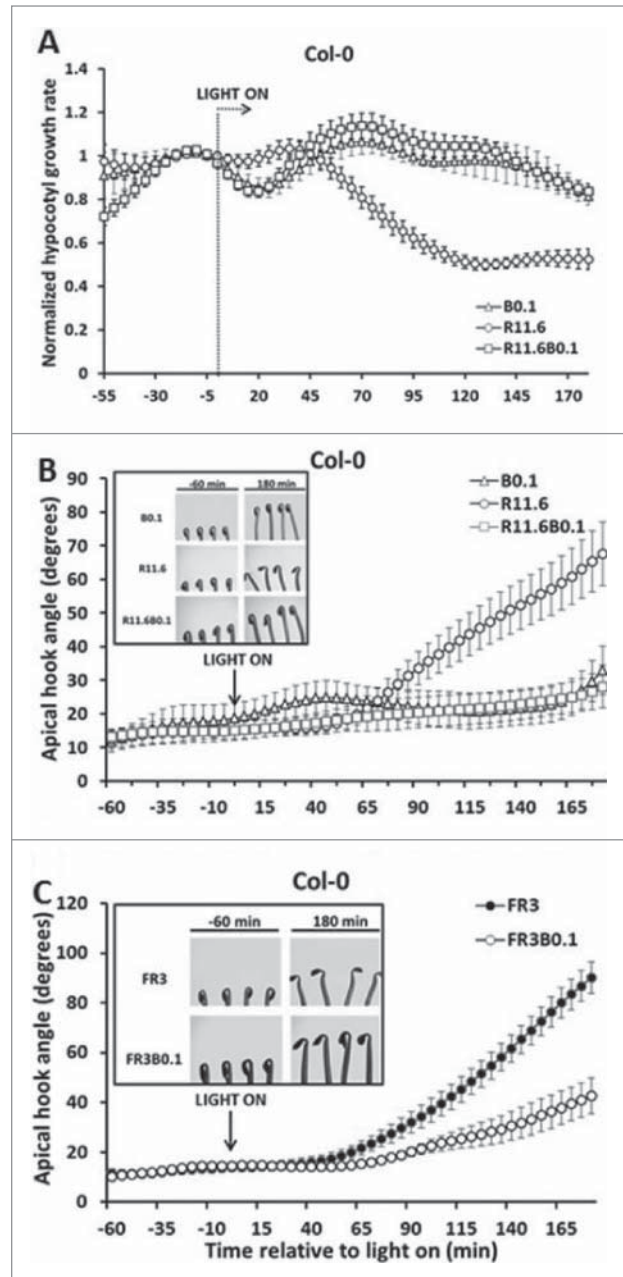

Figure 1. Dim B light attenuates R light-induced hypocotyl inhibition and hook opening. R11.6 = R light, 11.6 μmol/m^{2}s, B0.1 = B light, 0.1 μmol/m^{2}s. Error bars represent SEM (8$\leq$n$\leq$12). (**A**) B light attenuates R light-induced hypocotyl growth inhition; (**B**) B light attenuates R light-induced apical hook opening. (**C**) B light attenuates FR light-induced apical hook opening.

light. Tests in *cry1* and *cry2* mutants show that R-light-induced hook opening by addition of high B ($\geq$10 μmol/m^{2}s) is largely impaired in mutant seedlings (**Fig. 3C**).

B reversal of R light-induced responses is blocked by NPA

The fact that very low fluence-rate B light can negate or delay R-light-induced stem growth inhibition and apical hook opening lead us to speculate that auxin transport might play a role in this

response. To test this hypothesis, we included the polar auxin transport inhibitor *N*-1-Naphthylphthalamic Acid (NPA) into the media. The same light treatments were applied to seedlings grown on media containing different concentrations of NPA. The results in **Figure S2A** showed that at an NPA concentration of 0.5 μM, the absolute hypocotyl growth rate kinetics were similar between NPA treated and untreated seedlings. However, NPA B light negation of the R light response was reduced at this NPA concentration (**Fig. S2A**). When the NPA concentration was increased to 1 μM, the B light effect was completely absent, and the R-induced hypocotyl growth inhibition was slightly enhanced (**Fig. 4A**). NPA

Figure 2. B light fluence rate response in R light-induced hypocotyl inhibition and hook opening. (**A**) Increasing B light fluence rate reduced the amplitude of B-negation of R light stem response in the short-term assay; (**B**) R light stimulates apical hook opening (dark circles), addition of dim B light delays R induced hook opening (open circles). Increasing B light fluence rate to 1 μmol/m²s still attenuates R light-induced hook opening (light gray circles), but 10 μmol/m²s B light enhanced R light-induced hook opening process (dark gray circles).

delayed and slowed hook opening kinetics in response to R light at 2 different concentrations (1 and 5 μM) in our short-term assays (**Fig. S2B** and **Fig. 4B**). No effect of dim B light on the R light response was observed. Taken together, these results indicate that polar auxin transport may play a role in B-light attenuation of R light-mediated de-etiolation events.

Genetic tests of B light mechanism

The directional flow of phytohormone auxin from cell to cell is mediated by specific auxin transporters. These transporters facilitate auxin molecules in (influx carriers) and out (efflux carriers) of a cell depending on external and internal signals. Some of these transporters, such as PINs, ABC transporters, and AUX/LAX have been indicated to modulate plant tropism response.[14-19] Thus, we postulated that one or several of these transporters might mediate the B light negation of the R light response. To test this hypothesis, we measured stem growth and hook opening kinetics in several PIN transporter mutants (*pin1*, *pin3*, *pin4*, *and pin7*), one ABC transporter mutant (*abcb19/pgp19*), and one auxin influx transporter mutant (*aux1*). These mutants were chosen because of their cognate gene expression patterns in the hook/hypocotyl area.[19-21] Meanwhile the auxin response factor mutant *arf7/nph4* was also included for the test based on its role in phototropism response.[22,23]

Generally, the transporter mutants maintained a normal or near normal B light negation effect to R light. Specifically, *pin1*, *pin3* and *pin7* mutant seedlings responded to R light or the addition of dim B light like wild-type seedlings (**Fig. S3A, B, and C**). The *pin4* mutant seedlings showed a reduction in the amplitude of the B light negation of R light-induced hypocotyl inhibition (**Fig. 5A**). The *abcb19* mutant seedlings were tested at a higher R light fluence rate (50 μmol/m²s) in order to obtain robust stem inhibition. While the kinetics of hypocotyl growth inhibition were affected in *abcb19* mutants, a B light effect was still observed (**Fig. S3D**). The *aux1* mutant seedlings showed an enhanced R-light-induced hypocotyl inhibition response, but the B-light-negation of R-light-induced hypocotyl response is still observed
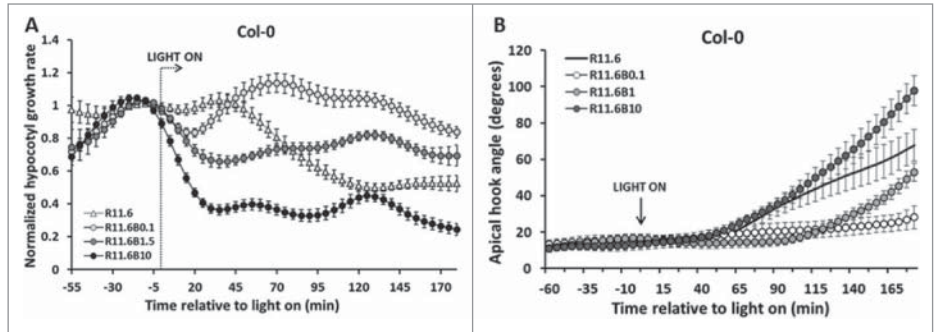
(**Fig. 5C**). The auxin response factor mutant *arf7/nph4* showed a greatly reduced R light hypocotyl inhibition response (also at 50 μmol/m²s). However, the addition of dim B light did partially negate the limited R light hypocotyl inhibition observed in *nph4* (**Fig. 5E**).

When apical hook opening was assessed, the *pin4* mutant showed slightly delayed opening kinetics compared with wild-type plants in response to R light, and B light appears to reverse this trend (**Fig. 5B**). The *arf7/nph4* mutants consistently presented a slightly open initial apical hook angle in darkness compared to wild-type seedlings (**Fig. 5F**). Interestingly, none of *aux1*, and *arf7/nph4* mutants showed the B light attenuation of R light induced hook opening response (**Fig. 5B, D, and E**). These findings seem to be consistent with the results from NPA treatment tests, indicating that normal auxin transport or reponse is important for the development of the B negation of R responses.

Genetic tests of *pks* mutants

PKS protein family has been reported to serve as the signaling component in both phy signaling pathway and phototropism. It was therefore of interest to test the role of individual PKS proteins, if any, in the phot1-mediated B light negation response to R light. In order to obtain a robust R-light stem inhibition response, a higher R-light fluence rate (50 μmol/m²s) was used. All the *pks* mutants (*pks1*, *pks2*, *pks4*) showed approximately wild-type hypocotyl growth rate kinetics under higher R conditions, and all responded normally to the B light treatment (**Fig. S4**).

Discussion

Phytochrome is a classic example of how two wavelengths of light can work in opposition to tailor physiological resposnes to the ambient environment. Such push-pull interactions allow for tailored responses, especially at critical developmental transitions,
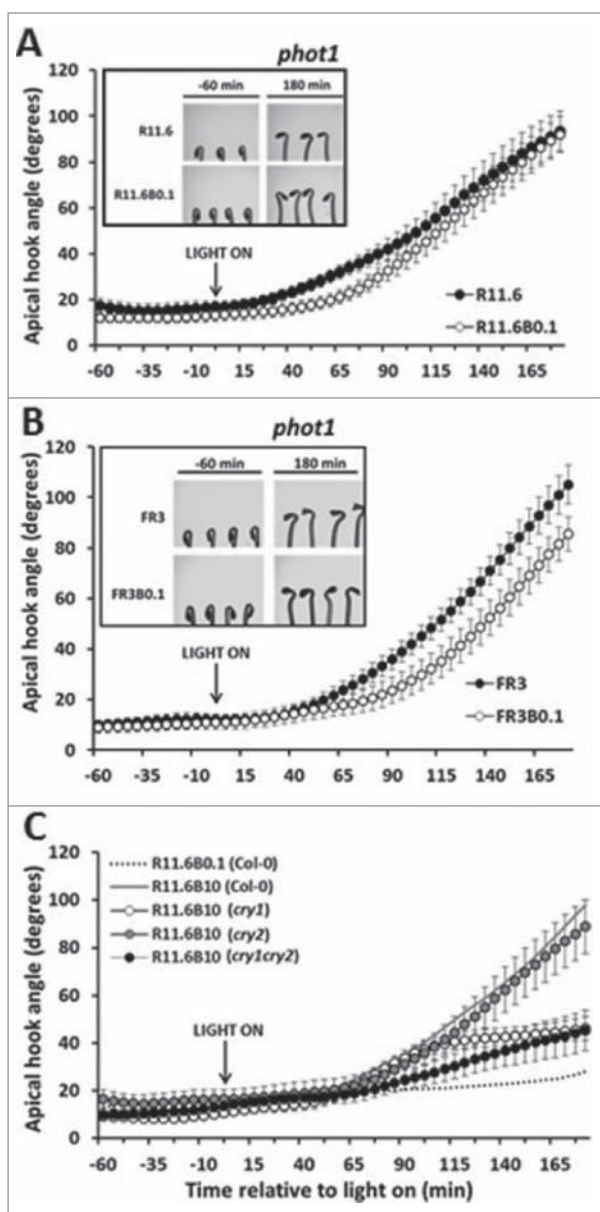
Figure 3. Roles of phot1 and cry in the blue attenuation of red induced hook opening. The dim B light attenuation of R light (**A**) or FR light (**B**)-induced hook opening response was absent in *phot1* mutant background. Inset: R/FR or R/FR plus B light treated seedlings at the beginning (−60 min relative to light on) and the end (180 min) of one representative short-term experiment; (**C**) The enhanced R light-induced hook opening by higher fluence rate B light was normal in *cry2* (gray circles) mutant, but was partially impaired in *cry1* (open circles) and *cry1-cry2* (dark circles) mutants.

such as the switch from etiolated to autotrophic growth. Here we expand on previous findings by Wang et al.[12] that show that B light can either enhance or negate a R light response, depending on fluence rate. Dim B light signals eliminate the R light response through phot1 action, while higher fluence rates override this inhibition through the cryptochromes.

Early light-induced morphological and physiological changes enable a young seedling to prepare for autotrophic growth. This

transition is mediated by multiple photo-sensing systems that couple the light environment to changes in gene expression and physiological response. The phy receptor family (phyA-E) mainly responds to R and FR wavebands. In the initial stage of R light-induced hypocotyl growth inhibition, phyA mediates the fast growth inhibition response which happens within 1-3 hrs after R light onset.[10,12] The phyB receptor contributes after hours of illumination for a persistent effect. Addition of B light to R light background activates 2 sets of B light photosenory systems, the crys and the phots.

The cry-mediated B light response generally accelerates/enhances the process of R-light-induced early photomorphogenic events. Such cry effects are most robust under high (>10 μmol m^{-2} s^{-1}) fluence rate of B light and similar results are seen in this work (**Fig. 2A and 3C**). Tests of *cry* mutants for the hook opening response suggest that cry1 plays the major role in stimulating hook opening process. This observation is inconsistent with an earlier report that indicates cry1 does not affect hook opening kinetics under high B light irradiation,[13] likely because the present work was performed with simultaneous R and B light treatment.[1,4,5]

In contrast, in the R light environment the phot1 receptor mediates two opposing responses based on B light fluence rate. In dim B light alone, phot1 mediates rapid growth inhibition within minutes of treatment.[24] However, the same treatment in the presence of R light opposes stem growth inhibition.[12] The addition of dim B light slows R-light mediated hook opening. One possible explanation is that dim B light provided from above acts as a phototropic guide, causing the plant to extend toward the light without guidance from a side-oriented signal. At higher fluence rates the inhibitory effects of blue light overcome the upward phototropic movement.

Auxin regulates tropic response. Disruption of auxin transport by the auxin transport inhibitor NPA suppresses both phototropic and gravitropic responses.[14,25,26] The maintenance of apical hook requires the presence of auxin gradient at the concave side of the hook, and application of NPA eliminates this auxin gradient and stimulates hook opening of dark grown seedlings.[21] Additionally, the hypocotyl elongation of light-grown *Arabidopsis* seedlings also requires auxin transport.[25] The fact that NPA can suppress both B-light-mediated attenuation of R-light-induced hypocotyl inhibition and hook opening in our experiments indicates a possible involvement of auxin transport in such responses.

R light not only regulates auxin biosynthesis, but also affects its transport, content, and distribution.[27] The phot1-mediated negation of R-light-induced hypocotyl and hook responses is not likely mediated by phot1 antagonizing phy-regulated gene expression, but instead, might be a result of phot1 modulation of auxin transporter localization and activity within a cell.[12] In the latter scenario, phot1 and its signal transducer NPH3 may modify the cellular distribution of PIN3 and PIN2 in hypocotyl and root, respectively.[15,28] Mutation of *PIN3* gene reduces the hypocotyl curvature toward continuous unilateral white light illumination.[14,15] However, a recent study suggests that PIN3 and PIN7 mediate the pulse-induced first positive phototropic response, but not the continuous-light-induced second positive

phototropic response when the seedlings were placed vertically on the media.[18] Similarly, mutations in *ABCB19* lead to the abnormal localization of PIN1 transporter, which ultimately enhances hypocotyl phototropism.[29] It has been shown that phot1 inhibits ABCB19 activity by direct phosphorylation of this protein in a HeLa cell system.[17] Reduction of ABCB19 transporter activity suppresses basipetal auxin transport, resulting in more auxin channeled to lateral directions through PIN3 depending on light directions.[17]

It was of interest to determine if any of these transporters affected the B light hypocotyl and hook responses in the R environment. The *pin1*, *pin3*, *pin7*, and *abcb19* showed normal B-light-negation of the R light hypocotyl inhibition response. Only *pin4* showed slight reductions in the amplitude of the response. This result agrees with previous findings,[18] and indicates likely redundant roles in the response. The *aux1* was originally discovered as root agravitropic mutant, and its role in unilateral BL-induced hypocotyl phototropism is subtle when ARF7/NPH4 is functional.[19,30,31] Meanwhile, AUX1 has also been suggested to facilitate auxin transport from the source (leaf) to the sink (root) in *Arabidopsis* seedlings.[32] The enhanced R light-induced hypocotyl inhibition in *aux1* mutant indicates AUX1 may antagonize R light hypocotyl response by maintaining a proper auxin concentration in hypocotyl cells. B light might not be able to fully attenuate the enhanced R light inhibition to the wild-type level under such scenario.

The ARF7/NPH4 has been shown to have altered phototropic and gravitropic responses, and thus may directly function in hypocotyl differential growth by modulating auxin responsiveness.[22,23,33] The reduced R light (50 μmol/m²s) hypocotyl inhibition in *nph4* mutant indicates NPH4 may partly mediate the initial R light hypocotyl inhibition growth. Even under reduced hypocotyl inhibition conditions, B light can still oppose this response (**Fig. 5E**). The *nph4* mutant showed larger initial apical hook angle
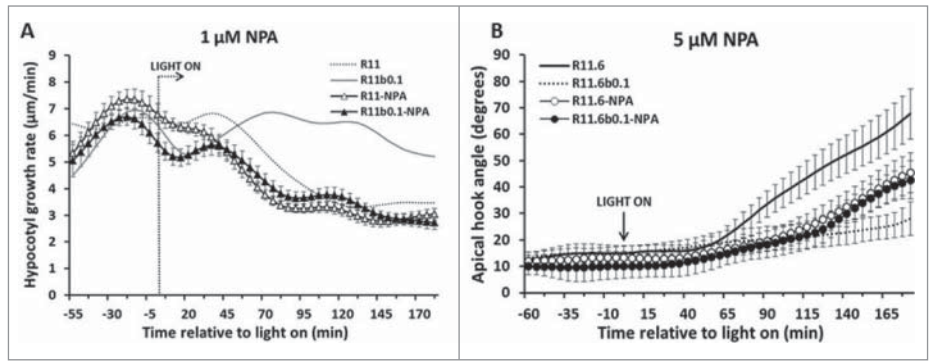

Figure 4. B light attenuation of R-mediated hypocotyl and hook responses require normal auxin transportation. (**A**) The B light negation of R-light-induced hypocotyl growth inhibition is fully suppressed by 1 μM NPA; (**B**) The R-light-induced hook opening process was abolished when treated with 5 μM NPA. In panel (**A**), open triangles represent the absolute hypocotyl growth rate under R light (11.6 μmol/m²s) conditions; dark triangles represent the hypocotyl growth rate under R light plus dim B light (0.1μmol/m²s) conditions. In panel (**B**), open circles represent the hook opening kinetics under R light conditions; dark circles represent the hook opening kinetics under R light plus dim B light conditions. Wild-type Col-0 in response to R light (dotted line) and R light plus dim B light (gray line) without NPA treatment were included for comparison in all panels.
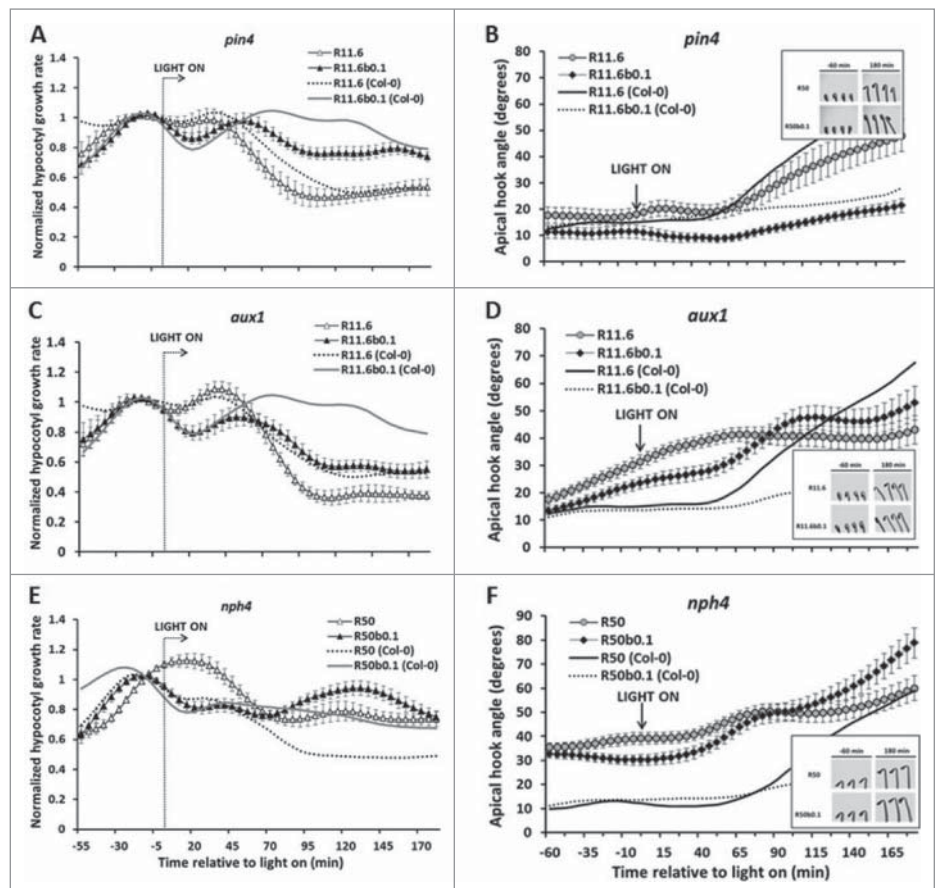

Figure 5. Genetic tests of the dim B light reversal of R light response in auxin transport and response mutants. The dim B light negation of R light-induced hypocotyl growth inhibition and hook opening responses were tested in *pin4* (**A and B**), *aux1* (**C and D**), and *arf7/nph4* (**E and F**). Wild-type Col-0 in response to R light (dotted line) and R light plus dim B light (gray line) were included for comparison.

compared with wild-type plant, indicating its important role in maintaining folded apical hook, which is consistent with previous report.[33] It is likely that some of the auxin responsive genes that regulated by ARF7/NPH4 are mediating the B and R light hook opening response at the end point. On the other hand, the normal auxin transport may also be important for the B light negation of hook opening response. Results from pharmacological and genetic tests support such hypothesis.

Several studies have demonstrated that the PKS proteins serve as a molecular link between phy- and phot- meidated responses.[34,35] Therefore, we tested the hypothesis that the B light-attenuation of R light hypocotyl response was mediated by one of the PKS proteins. Results reveal no significant differences between mutant and wild-type seedlings. Although these data do not support for the hypothesis, it does not exclude the possibility that multiple PKS proteins might redundantly participate in this response.

Materials and Methods

Plant materials

The *Arabidopsis thaliana* (hereafter *Arabidopsis*) photoreceptor mutants tested in this study are identical to those previously assessed [12]: *cry1-304*, *cry2-1*, *phot1-3* (*nph1-3*). The *nph3-6* mutant seeds were obtained from Dr. Mannie Liscum. The *pks1-1*, *pks2-1*, and *pks4-1* single mutant seeds were obtained from Dr. Christian Fankhauser. The *pin3-5* (CS9364), *pin4-3* (CS9368), *pin7-2* (CS9366), and *arf7/nph4* (CS24607) were ordered from the Arabidopsis Biological Resource Center (ABRC) at Ohio State University (Columbus, OH). The *abcb19-3* mutant seeds were provided by Dr. Edgar Spalding. The *pin1* (CS86744) and *aux1-7* (CS3074) mutant seeds are gifts from Dr. Bala Rathinasabapathi. All genotypes tested in this study are in Columbia (Col-0) background, which was used as wild-type.

Light sources and treatments

Light treatments were generated using narrow-bandwidth LED arrays (Light Emitting Computers, Victoria, BC Canada). The peak wavelengths of B light, R light, and FR light are 470, 660, and 730 nm. Light fluence rates were measured using a LI-COR LI-250 photometer with a PAR sensor (LI-COR, Lincoln, NE).

Time course hypocotyl growth and hook opening kinetics

Arabidopsis seeds were surface-sterilized by covering them with 95% ethanol on blotting paper in a laminar flow hood. Upon drying, the seeds were distributed in Petri dishes on minimal media (1mM KCl and 1mM $CaCl_2$) solidified with 1% Difco agar (Beckton, Dickinson and Co, Sparks, MD). The plates were covered in foil and then stratified for 48-72 h at 4°C. The seeds were then treated with 2 h of fluorescent white light (16 μmol/m^2s) at 23°C to synchronize germination. Seedlings were grown in absolute darkness at 23°C for 40-48 h. Newly-emerged etiolated seedlings were used for imaging, and images were acquired in a 5-min-interval and analyzed according to the method described.[13] For the auxin transport inhibition experiments, germinating seedlings grown on minimal media without NPA were transferred onto minimal media supplemented with NPA (Sigma-Aldrich, St. Louis, MO) at different concentrations. Seedlings were acclimated 40–60 min in darkness on NPA before light experiments.

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