A phytochrome–phototropin light signaling complex at the plasma membrane

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Phytochromes are red/far-red photochromic photoreceptors central to regulating plant development. Although they are known to enter the nucleus upon light activation and, once there, regulate transcription, this is not the complete picture. Various phytochrome effects are manifested much too rapidly to derive from changes in gene expression, whereas others seem to occur without phytochrome entering the nucleus. Phytochromes also guide directional responses to light, excluding a genetic signaling route and implying instead plasma membrane association and a direct cytoplasmic signal. However, to date, no such association has been demonstrated. Here we report that a phytochrome subpopulation indeed associates physically with another photoreceptor, phototropin, at the plasma membrane. Yeast two-hybrid methods using functional photoreceptor molecules showed that the phytochrome steering growth direction in *Physcomitrella protonemata* binds several phototropins specifically in the photoactivated Pfr state. Split-YFP studies *in planta* showed that the interaction occurs exclusively at the plasma membrane. Coimmunoprecipitation experiments provided independent confirmation of *in vivo* phy-phot binding. Consistent with this interaction being associated with a cellular signal, we found that phytochrome-mediated tropic responses are impaired in *Physcomitrella phot⁻* mutants. Split-YFP revealed a similar interaction between *Arabidopsis* phytochrome A and phototropin 1 at the plasma membrane. These associations additionally provide a functional explanation for the evolution of neochrome photoreceptors. Our results imply that the elusive phytochrome cytoplasmic signal arises through binding and coaction with phototropin at the plasma membrane.

The breakthrough in understanding plant phytochrome action was the discovery that, upon light activation, the photoreceptor enters the nucleus and there orchestrates major changes in the transcriptional pattern through interactions with transcriptional regulators including PIF family members (1). In the case of phyA, the predominant phytochrome in etiolated cells, the Pr ground state is cytoplasmic, whereas photoconversion to the Pfr state leads to rapid FHY1-mediated nuclear transport. phyB, predominant in light-grown tissue, is thought to act similarly, although the translocation mechanism is much slower and not associated with FHY1 (2–4). Pfr regulation of transcriptional regulators involves phosphorylation, but although the C-terminal domain resembles that of prokaryotic histidine kinases, it seems to be unnecessary for phytochrome signaling (5, 6). Instead, the critical interaction of phyB with PIF3 appears to involve the PAS-GAF cleft in the N-terminal photosensory module (7, 8).

Various phytochrome-mediated effects cannot, however, derive from such a system (4). Several occur within minutes or even seconds, far too fast for gene regulation. In *Arabidopsis fhl⁻/fhy1⁻* mutants, light-dependent phyA nuclear translocation is lost along with most but not all phyA-mediated responses, implying that a cytoplasmic signal also exists (9). In fern and moss protonemal filaments, tip cells show phototropic bending toward red light (R), an effect that is reversible by far-red light (FR). In these groups and also in certain algae, light-induced chloroplast movement too is a R effect reversible by FR. None of these responses can derive from gene regulation, not only because they are too fast but also because they require that directional

information from the stimulus is transmitted to the response (which is, of course, impossible for a transcription/translation mechanism). Interestingly, these vectorial responses show strong action dichroism and can even be steered by the polarization of the light stimulus. Wolfgang Haupt evolved an elegant model able to explain all these effects on the basis of phytochromes attached anisotropically to the plasma membrane and showing a rotation of the transition dipole moment between Pr and Pfr (10–12). To date, the only direct evidence for phytochrome association with the plasma membrane derives from a fluorescence correlation microscopy study in *Ceratodon* that implied that phytochrome assembled with phycoerythrobilin was less mobile at the cell periphery than in the cytoplasm (13). In general, however, phytochromes are not considered to be membrane-associated, Pr at least being freely cytosolic. This paradox was partly solved in cases involving ferns (14) and algae (15) with the discovery that the photoreceptor responsible comprised a phytochrome sensory module fused to a phototropin blue light (B) receptor. This chimeric molecule was named neochrome. As higher plant phototropins are known to be membrane-associated (16, 17), neochromes might show this property too, thereby providing for the predicted action dichroism in accordance with the Haupt model. Neochromes are, however, absent from mosses and higher plants. Indeed, targeted knockout in the moss *Physcomitrella patens* showed that a canonical phytochrome Pp.phy4 was responsible for vectorial responses including phototropism, polarotropism, and chloroplast relocation (18). We were thus interested in how this and perhaps higher plant phytochromes might provide an appropriate signal within the cytoplasm.

Results

Our initial approach was to try to identify Pp.phy4 holophytochrome interacting partners in yeast. We established both Pp.phy4 and *Arabidopsis* phytochrome A (At.phyA) as functional, full-length, holoprotein bait molecules fused to the DNA-binding domain (BD) for yeast two-hybrid (Y2H) experiments (Fig. 1A). Both phytochromes showed light-independent homodimerization (Fig. 1B and Fig. S1A) using equivalent prey constructs carrying the activator domain (AD), whereas At.phyA and FHY1 (19) hybrids interacted exclusively in R, the effect being reversed by FR and only seen if the cells were fed with phycocyanobilin (PCB) (Fig. 1B and C and Fig. S1A). These positive controls indicated that correctly folded, photochromically functional holophytochrome was present in the yeast cells. We then screened a *Physcomitrella* full-length cDNA prey library with N-terminal ADs using Pp.phy4 bait hybrids with the BD attached either to the C or N terminus (Pp.phy4:BD and BD:Pp.phy4, respectively). Several prey molecules bound the latter R/FR reversibly, indicating that these bait constructs were

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Fig. 1. Yeast two-hybrid studies reveal Pfr-enhanced phytochrome–phototropin interaction. Various hybrid constructs (**A**, green and yellow bars indicate HA- and MYC-tags, respectively) were assayed for growth (**B**) and reporter activity (**C**) following different light treatments as described in Materials and Methods. Full-length holophytochrome bait constructs with N- or C-terminal BDs were expressed together with different prey constructs in PCB-fed yeast. In **B**, 2×10^5 AH109 cells were spotted onto QSD medium. In **C**, β -galactosidase activity of Y187 cultures was measured using the Miller assay with ONPG substrate.

also functional. Additionally, we found that all four *Physcomitrella* phototropins (Pp.photA1, -A2, -B1, and -B2) showed phytochrome state-dependent interactions with BD:Pp.phy4 according to both quantitative growth on appropriate dropout media (Fig. 1B) and β -galactosidase assays (Fig. 1C). AD:Pp.photA1 in particular showed moderate interaction even in darkness but in this as in the cases of the other three phototropins, interaction was enhanced three- to fivefold in pulsed R, effects that were fully reversed by subsequent FR pulses. No specific effects of B were apparent. Although the isolated Pp.phy4 sensory module showed no interaction with Pp.phot, constitutive binding was seen for the C terminus (Fig. S1D and E). These data in yeast pointed to a direct, Pfr-enhanced binding of phytochrome to all four phototropins.

Although phototropins lack obvious features that might lead to a direct association with cell membranes, fluorescent protein (FP)-tagging experiments in *Arabidopsis* have shown that they are indeed membrane associated (16, 17). Additionally, light-dependent phototropin internalization has been observed; this effect was suppressed by pretreatment with R (20). To date, no studies of phototropin localization in *Physcomitrella* have been reported. We therefore constructed hybrid proteins in which an FP was attached to the N terminus of each *Physcomitrella* phototropin and examined the cellular localization in *Physcomitrella* filaments. This study showed that, as expected, all phototropins are exclusively associated with the plasma membrane (Fig. 2A and Fig. S2; for comparison, Fig. S3 shows plasma membrane, cytoplasmic, and nuclear markers). Similar localization was seen in onion epidermis cells. However, we did not observe dissociation from the plasma membrane upon irradiation as reported for *Arabidopsis* (20).

Surprisingly, in contrast to phytochromes in higher plants, it was reported earlier that *Physcomitrella* phytochromes 1–4 were cytoplasmic, showing no tendency to enter the nucleus upon Pfr formation (21). Using the same conventional hybrid configuration (Pp.phy4:FP), we observed a weak fluorescence signal in the nucleus with no particular enhancement following light pretreatments (Fig. 2B and Fig. S4). In the context of the results obtained from the Y2H experiments (Fig. 1), we constructed FP:Pp.phy4 hybrids for *in planta* studies. Interestingly, with this configuration nuclear localization was never observed, the hybrid being exclusively cytoplasmic (Fig. 2C and Fig. S4). Pp.phy4 with split-YFP moieties attached N-terminally (YFP_N:Pp.phy4 and YFP_C:Pp.phy4) showed equivalent distributions, additionally indicating homodimerization and thus correct folding (Fig. S5). As in the case of higher plant phytochromes, there was no obvious enhanced fluorescence at the plasma membrane.

We then used split-YFP methods to detect whether a subpopulation of Pp.phy4 nevertheless interacts with phototropin, as seen in Y2H studies (Fig. 1). Indeed, YFP_C:Pp.phy4 bound YFP_N:Pp.phot hybrids in both *Physcomitrella* (Fig. 2D and Fig. S6) and onion (Fig. 2E and Fig. S6) exclusively at the plasma membrane. These signals were much weaker than those from FP:Pp.phy4 and split-YFP:Pp.phy4 homodimers in the cytoplasm (Fig. 2C and Figs. S4 and S5). At the highest resolution accessible, the split-YFP signal seemed restricted to distinct tiny areas at or near the plasma membrane (Fig. 2E and Fig. S6E).

We confirmed phytochrome–phototropin interaction independently by coimmunoprecipitation (coIP). HA:Pp.phy4 and MYC:Pp.photA1 or -B1 overproduced in *Nicotina benthamiana* were liberated from the membrane fraction by mild detergent treatment and pulled down together from the supernatant with anti-HA antibody. Immunoblots probed with anti-MYC antibody clearly showed coIP of both phototropins with Pp.phy4 (Fig. 2G). Experiments carried out in darkness following saturating light pulses given either *in vivo* 16 h before extraction or *in vitro* before the pull-down provided no evidence for Pr/Pfr-dependent association (Fig. S7).

We reasoned that if the Pp.phy4–Pp.phot interaction is related to tropic reactions to light, these might be impaired not only in *Pp.phy4*[−] (18) but also in phototropin mutants. Indeed, using

Fig. 2. *Physcomitrella* phytochrome and phototropin localization and interaction in planta, phytochrome–phototropin coimmunoprecipitation, and genetic evidence for phototropin involvement in phytochrome signaling. (A–C) Localization of phototropins with CFP:Pp.phot constructs (A) and of Pp.phy4 with Pp.phy4:CFP (B) and GFP:Pp.phy4 (C) in *Physcomitrella* following different light pretreatments. Split-YFP showing interaction of Pp.phy4 with phototropins at the plasma membrane in *Physcomitrella* filament cells after 15 min R (D) and in onion epidermis cells (E, right at high magnification; scale bar: 10 μ m) showing YFP_C:Pp.phy4 with YFP_N:Pp.photB2 interaction in discrete peripheral areas. *Arabidopsis* phytochrome A–phototropin 1 interaction at the plasma membrane in onion epidermal cells revealed by split-YFP after 15-min R pretreatment (F, YFP_N:At.phot1 with YFP_C:At.phyA). (Scale bars: 50 μ m.) Anti-HA antibody immunoprecipitation (IP) of HA:Pp.phy4 with MYC:Pp.photA1 or B1 following *Agrobacterium*-mediated coexpression in *Nicotiana benthamiana* (G), detected with anti-HA (Upper, HA:Pp.phy4 as blue arrows) and anti-MYC (Lower, MYC:Pp.photA1 or B1 as yellow arrows). Negative controls were simulated IPs without anti-HA (-AB) and extracts from untransformed leaves (WT). All procedures carried out in white light (plant growth and protein extraction) or R (IP). Pp.phy4-mediated steering of directional growth in *Physcomitrella* wild type and *phy4*⁻ or multiple *phot*⁻ knockout filament tip cells (H). R-induced phototropism (dark gray) and polarotropy (light gray). Equivalent phototropism experiments with B induced no significant bending (white). SEs are shown.

multiple *Pp.phot*[−] knockouts (22), both photo- and polarotropic responses in R were compromised (Fig. 2H). In particular, *photA2*[−]/*B1*[−]/*B2*[−] and *photA2*[−]/*B1*[−] approximately phenocopied *Pp.phy4*[−], whereas the behavior of *photB1*[−]/*B2*[−] more resembled that of the wild type, implying that the interaction with *photA2* predominates in these tropic responses, but that the other family members capable of interacting with *Pp.phy4* can also contribute to the response. Despite the involvement of a B photoreceptor, no phototropic reaction to B was seen, as expected.

We considered that the *Physcomitrella* phytochrome–phototropin interaction described above and perhaps its cytoplasmic signaling route might be conserved in higher plants. In onion, we observed translocation of At.phyA from the cytoplasm to the nucleus in light using hybrids with the FP conventionally attached to the C terminus (At.phyA:FP; Fig. S8). We estimate that ~75% of the fluorescence signal was restricted to the nucleus after 1 h of FR irradiation, whereas no significant signal was apparent in the nucleus in the dark controls. Similar behavior was apparent for N-terminal FP hybrids (FP:At.phyA; Fig. S8). In *Physcomitrella*, At.phyA showed a marked tendency to enter the nucleus in light in the case of At.phyA:FP but not FP:At.phyA (Fig. S9), resembling the pattern seen for *Pp.phy4*. At.phyB, on the other hand, showed weak light-dependent nuclear localization irrespective of the positioning of the FP tag in both onion and *Physcomitrella* (Figs. S10 and S11). Equivalent split-YFP experiments with YFP_N:At.phyA and YFP_C:At.phyA (Fig. S5) showed similar light-dependent distributions and additionally confirmed homodimerization, implying that the hybrids were correctly folded and photochromically functional. Similarly to our findings for *Physcomitrella*, YFP_C:At.phyA with YFP_N:At.phot1 yielded a fluorescence signal exclusively at the plasma membrane (Fig. 2F). On the other hand, Y2H investigations analogous to those for *Physcomitrella* (Fig. 1) gave no evidence for direct binding of At.phyA to At.phot1 or -2, despite the stringent At.phyA–At.FHY1–positive control.

Discussion

Phytochrome Localization. A major step forward in understanding phytochrome biology came with the discovery that both phyA and phyB are translocated from the cytoplasm to the nucleus upon formation of the physiologically active Pfr state (2, 23–25), providing a phenomenological basis for phytochrome-mediated regulation of the transcriptome (26–29). However, whereas FHL/FHY1-mediated nuclear translocation of phyA in *Arabidopsis* is rapid, most having been shuttled into the nucleus within an hour of irradiation, the effect is much less pronounced in the case of phyB, most of which remains in the cytoplasm even after 2 h of irradiation (3). Furthermore, the mechanism underlying phyB nuclear localization is unclear, and certainly does not involve the FHL/FHY1 system. phyC, -D, and -E, on the other hand, were reported to be constitutively nuclear localized (30). These studies exploited transgenic phytochromes carrying FP tags at the C terminus. Here, we show that light-dependent translocation of phyA into the nucleus in higher plant cells occurs when the FP tag is attached either to the N or the C terminus (Fig. S8), indicating *in planta* functionality in both cases.

Remarkably little is known about phytochrome-mediated effects of light on gene expression in lower plants. Surprisingly, earlier studies showed that *Physcomitrella* phytochromes remained cytoplasmic irrespective of light treatments (21). Our data confirm these findings (Fig. 2C and Fig. S4). It is thus remarkable that At.phyA:FP in *Physcomitrella* filament cells showed similar Pfr-dependent nuclear translocation to that seen in higher plant cells (Fig. S9), all of the more so because no FHY1 homolog is immediately obvious in the *Physcomitrella* genome. Detailed searches for FHY1-related functional elements in the *Physcomitrella* genome (31) revealed XP_001772282.1 as a potential candidate, however.

Physcomitrella Phototropins Are Associated with the Plasma Membrane. Light-directed growth (phototropism) in lower plants occurs in response to R and is mediated by phytochrome, phototropism in higher plants occurs in response to B and is mediated by phototropin photoreceptors. Although phototropins show no features characteristic of membrane proteins, they are plasma membrane-associated in higher plants and also *Chlamydomonas* (16, 17, 32, 33). Here we show that all four *Physcomitrella* phototropins are associated with the plasma membrane (Fig. 2A and Fig. S2). Detailed studies of At.phot1 in *Arabidopsis* revealed a B-dependent dissociation from the plasma membrane which could be ameliorated by pretreatment with R via phyA (20). However, we failed to observe a similar dissociation effect of B in the case of *Physcomitrella* phototropins. Phototropin association with the plasma membrane is probably mediated by one or more anchor proteins, either bona fide plasma membrane proteins or partners otherwise fixed at the periphery of the cell. None of the phototropin partners identified to date are likely candidates.

A Subpopulation of Physcomitrella Phytochrome 4 Binds Directly to Phototropin at the Plasma Membrane. Phytochrome 4 is the photoreceptor predominantly responsible for vectorial light sensing and response in *Physcomitrella* (18), a paradoxical situation if phytochromes were to act via transcriptional regulation alone (4, 12). Using Y2H methods (Fig. 1) we describe the binding of full-length, photochemically active holophytochrome 4 from *Physcomitrella* to phototropins. Characteristically, apophytochromes autoassemble with specific bilins to form the functional holoprotein photoreceptor. Full-length phytochromes produced in PCB-fed yeast are obviously able to carry out this process, as only then would they be able to show R/FR reversible binding to prey molecules. In the case of the *Pp.phy4* and phototropins, interaction is enhanced in continuous or pulsed R, the latter effect being reversed by subsequent FR pulses, clear evidence of Pfr-enhanced binding. This interaction is likely to be autonomous, as additional factors are probably missing in yeast.

We show bimolecular fluorescence complementation signals at the plasma membrane in both onion epidermis and *Physcomitrella* filament cells coexpressing *Pp.phy4* and *Pp.phot* constructs carrying N-terminal split-YFP tags (Fig. 2D and E and Fig. S6). The split-YFP signal was much weaker than that seen from FP: *Pp.phy4* constructs, which did not themselves show appreciably enhanced fluorescence at the plasma membrane (Fig. 2B and C and Fig. S4), implying that only a small proportion of the phytochrome interacts with phototropin. We do not know whether this represents a dynamic equilibrium with the phytochrome population in the cytoplasm or a particular subpopulation of the phytochrome molecules.

Although the phytochrome–phototropin interaction involves a membrane protein complex that would thus not be accessible to coIP methods, we were able to confirm the interaction via coIP by liberating the complex from the plasma membrane by mild detergent treatment before pull-down (Fig. 2G). The success of these experiments implies that phytochrome–phototropin binding is more stable with regard to the detergent treatment than is the association of the complex with the plasma membrane. We found no evidence for light effects on the coIP signal (Fig. S7).

Phytochrome Cytoplasmic Signal Requires Phototropin. Targeted knockout studies in *Physcomitrella* implied that chloroplast B avoidance is primarily mediated by *photA2* although other family members also contribute to the response (22). However, chloroplast movement is also steered by phytochrome. Interestingly, these workers found that the latter response to R was defective particularly in the *photA2*[−] null mutant. Using the same phototropin knockout lines, we show here that phytochrome-mediated R photo- and polarotropism too are dependent particularly upon *Pp.photA2* (Fig. 2H).

Kasahara and coworkers (22) formulated an explicit model to explain light-induced chloroplast movement based on signal

transfer from phytochrome to phototropin and thence to the actin and myosin cytoskeleton. Phytochrome-mediated phototropism in moss protonemata was shown to involve rearrangements of actin microfilaments (34, 35), consistent with their model and our findings.

Although the coaction implied by these physiological studies does not necessarily require that the photoreceptors interact directly, the fact that they do so at the plasma membrane provides not only a plausible physical basis for the coaction but one entirely consistent with Haupt's proposal to explain both polaro- and phototropic bending of protonemata and chloroplast movements in lower plants (10–12) whereby phytochrome molecules are considered to be anchored anisotropically at the plasma membrane with the transition dipole moment parallel to the cell surface in the Pr ground state. Upon formation of the physiologically active Pfr state in R, the dipole for FR becomes perpendicular to the surface. On this basis, both dichroic effects and fluence rate gradients within the cell can be correlated with local concentrations of Pfr. The weakness of this model was that, up to now, phytochromes were considered to be cytosolic rather than associated with the plasma membrane. The data presented here seem to resolve this problem in the case of mosses. On the other hand, in certain algal and fern groups, neochrome photoreceptors have evolved to fulfill this function (14, 15, 36). Neochromes comprise an N-terminal phytochrome sensory module attached to an almost complete phototropin moiety. We propose that the phytochrome–phototropin binding we describe here has been genetically consolidated in the form of neochromes.

Phytochrome–Phototropin Interaction in Higher Plants. We show that *Arabidopsis* phyA and phot1 interact at the plasma membrane in onion epidermis cells (Fig. 2F). At first sight, this observation might seem to contradict the notion of cytosolic Pr and nuclear translocation of Pfr, however, both localization and physiological studies indicate that a proportion of phyA always remains in the cytoplasm irrespective of the light conditions (2–4). Our findings imply that only a small fraction of the phytochrome population is associated with phototropin, explaining also why no signal enrichment at the plasma membrane is apparent from FP:At.phyA constructs (Figs. S8 and S9). Our data are therefore consistent with and provide possible explanations of previous findings. For example, involvement of phytochrome in tropic responses is well established, R enhancing B-induced phototropism in the hypocotyl (37) by suppressing light-induced internalization of phot1 via phyA (20). The recent finding that NPH3 acts as an E3 ubiquitin ligase possibly involved in clathrin-mediated vesicle formation fits well into this scheme (38). Interestingly, R enhancement of B-induced phototropism is retained in the *fhl1/fhy1*[−] mutant in which phyA remains in the cytoplasm after Pfr formation (9). However, we see no reason to exclude an additional effect of phyA-mediated changes in gene expression. It was reported recently that nuclear phyA Pfr promotes whereas the *fhl1/fhy1*[−] mutation delays phototropism (39), but this does not call the existence of a cytoplasmic signaling route into question. We propose that phyA–phot1 binding at the plasma membrane we report is associated with the enhancement effect of phyA Pfr on phototropism, perhaps representing an evolved role of the analogous interaction in lower plants revealed in *Physcomitrella*. We do not yet know whether higher plant phytochromes other than phyA associate with phototropin, nor do we know whether phot1 is involved alone or with phot2.

In contrast to our Y2H findings for *Physcomitrella* (Fig. 1) and the situation in *planta* (Fig. 2F), interaction between *Arabidopsis* phyA and phot1 was not apparent in Y2H studies, perhaps because, in higher plants, a larger molecular complex is involved comprising components missing in yeast. One putative candidate is PKS1, which (i) binds phyA, phot1 and its signaling partner NPH3, (ii) is associated with the plasma membrane, (iii) is necessary for both B-induced phototropism in the shoot (40, 41) and R-induced root phototropism (42), and (iv) is absent from lower plants (31, 43).

Phytochrome Cytoplasmic Signaling. It was recently reported that phytochrome down-regulates protochlorophyllide oxidoreductase A (PORA) at the translational level via cytoplasmic Pfr (44). Although the PNT1 putative cap-binding protein involved appears cytosolic, the location of the phy–PNT1 interaction was not studied. We do not yet know if these findings are physiologically related to ours, but it is clear that both are distinct from phytochrome-regulated transcription. The phytochrome–phototropin complex we describe is likely to include a still unknown membrane anchor protein as well as further components associated with signal transfer. At least in the case of protonemata this is likely to connect physically to an actin microfilament organizer to preserve the vectorial information. Various predictions of the Haupt model can be tested on the basis of the phytochrome–phototropin interaction. It will also be interesting to investigate the physiological significance of the apparently related interaction seen in higher plants especially in view of the diverse roles played by phototropins 1 and 2.

Materials and Methods

Cloning. For Y2H studies, N-terminally fused FHY1, phytochrome, and phototropin bait and prey plasmids were generated in pGBKT7 and pGADT7 (Clontech), respectively. A C-terminal At.PHYA:BD hybrid was created by blunt cloning into pBAC, a derivative of pBRIDGE (Clontech). cDNAs from *Physcomitrella* PHY4, PHOT1, PHOT2, PHOTB1, and PHOTB2 and *Arabidopsis* PHYA and PHOT1 were cloned into Gateway entry vectors (Invitrogen). Expression clones for N- and C-terminal FP tags were generated by LR-clonase-mediated recombination into appropriate destination vectors (45). N-terminal fusion constructs for split YFP-based in vivo interaction assays were generated in pSAT destination vectors (46). Expression constructs for coIP studies were generated as follows. The Pp.PHY4 entry clone was LR-recombined with pGWB415 (47) yielding HA:PHY4_pGWB415. PHOT1_pDONR221 and PHOTB1_pDONR221 were obtained by BP-recombination of CFP:PHOT1 and -B1 constructs with donor vector pDONR221 (Invitrogen), respectively, followed by LR reaction with pGWB421 yielding MYC:PHOT1_pGWB421 and MYC:PHOTB1_pGWB421.

Y2H Interaction Assays. PCB was obtained by methanolysis of soluble proteins from *Spirulina* as described (48). Yeast was transformed as described (49, 50). For quantitative growth assays, 2×10^5 AH109 cells were spotted on -W/-L/-H/-A quadruple synthetic dropout (QSD) selective media containing 30 μ M PCB and 3-AT (0.5 mM phyA:BD; 2.5 mM BD:phy4). Plates were incubated for 5 d in 0.7 μ mol m^{−2} s^{−1} R (660-nm LEDs), 3 μ mol m^{−2} s^{−1} FR (740-nm LEDs), 0.7 μ mol m^{−2} s^{−1} B (465-nm LEDs), or darkness. β -galactosidase assays were carried out according to the Yeast Protocols Handbook (Clontech) using yeast strain Y187. Cultures were grown in -W/-L DSD media supplemented with 20 μ M PCB in the dark. Overnight cultures were diluted to OD 1, and 1.4 mL was inoculated into 5.6 mL of YPDA expansion cultures with 20 μ M PCB. Light pulses were given as follows: R, 5 min at 20 μ mol m^{−2} s^{−1}; FR, 10 min at 20 μ mol m^{−2} s^{−1}; B, 5 min at 12 μ mol m^{−2} s^{−1}. Cultures were incubated for further 3 h in darkness, and Miller assays using 2-nitrophenyl- β -D-galactopyranoside (ONPG) were performed in 540 nm dim green safelight.

Biolistic Transfection and Cytological Studies. *Physcomitrella* filaments were grown on solid BCE225 medium for 5 d followed by 5-h equilibration on 0.2 M mannitol to reduce turgor. Biolistic transfection was carried out as described (51), using gold particles (1.6 μ m, Inbio Gold) for *Physcomitrella* cells and tungsten particles (1.1 μ m, Bio-Rad) for onion epidermal cells. After transfection, cells were kept in darkness for 2–3 d before preirradiation as appropriate (1 h of R, FR, or B, each at 5 μ mol m^{−2} s^{−1}) immediately before observation/documentation. For split-YFP-based in vivo interaction studies, transfected material was kept in darkness for ~3 d, then preirradiated in R (660-nm LEDs) for 15 min at 35 μ mol m^{−2} s^{−1} before observation (see *SI Materials and Methods* for microscopy details).

Coimmunoprecipitation of Pp.phy4 and Pp.photA1 and -B1. 35S-driven HA:Pp.PHY4 and MYC:Pp.PHOT1 or B1 were coexpressed together with P19 in *Nicotiana bethamiana* leaves following *Agrobacterium* infiltration. After 5- to 7-d, 16-h white light (~100 μ mol m^{−2} s^{−1})/8-h dark cycle, proteins were extracted in white light by grinding in buffer (100 mM Tris-HCl, pH 8.3/5 mM EDTA/0.1% β -mercaptoethanol with Complete protease inhibitor mixture; Roche) on ice; the membrane fraction was pelleted and resuspended in the same buffer containing 0.3% Triton X-100 to solubilize associated proteins. Following clarification, monoclonal anti-HA antibody (Sigma) was added to

an aliquot of the supernatant and mixed for 40 min at 4 °C in R. Subsequently, protein G-coupled magnetic beads (NEB) were added and mixed further. The beads were then washed in the same buffer, and the captured proteins analyzed by SDS/PAGE/immunoblotting with anti-HA and anti-MYC monoclonal antibodies according to standard procedures. Similar procedures omitting the pull-down antibody or using untransformed leaf tissue constituted negative controls. Light effects were checked in vivo by pre-treating the leaf material with FR followed by 16 h darkness before extraction and colP in darkness and in vitro by irradiating conventional extract with R and FR before colP in darkness.

Photo- and Polarotropism in *Physcomitrella*. The *Pp.phy4* knockout line was as described (18). Double and triple phototropin knockout lines were kindly donated by Masahiro Kasahara (22). Phototropism of negatively gravitropically grown *Physcomitrella* protonemata was investigated essentially as described (18) using R and B LED arrays (λ_{max} : 660 and 465 nm, respectively; FWHM: 20 nm; Roithner) at 1 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Polarotropism was assessed

using *Physcomitrella* protonemal filaments grown horizontally under polarized R at 30 $\text{nmol m}^{-2} \text{s}^{-1}$. After 7 d, the plane of polarization was rotated by 45° and growth continued for a further 24 h.

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