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Phytochrome Signaling Mechanisms

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Phytochromes are red (R)/far-red (FR) light photoreceptors that play fundamental roles in photoperception of the light environment and the subsequent adaptation of plant growth and development. There are five distinct phytochromes in *Arabidopsis thaliana*, designated phytochrome A (phyA) to phyE. phyA is light-labile and is the primary photoreceptor responsible for mediating photomorphogenic responses in FR light, whereas phyB-phyE are light stable, and phyB is the predominant phytochrome regulating de-etiolation responses in R light. Phytochromes are synthesized in the cytosol in their inactive Pr form. Upon light irradiation, phytochromes are converted to the biologically active Pfr form, and translocate into the nucleus. phyB can enter the nucleus by itself in response to R light, whereas phyA nuclear import depends on two small plant-specific proteins FAR-RED ELONGATED HYPOCOTYL 1 (FHY1) and FHY1-LIKE (FHL). Phytochromes may function as light-regulated serine/threonine kinases, and can phosphorylate several substrates, including themselves *in vitro*. Phytochromes are phosphoproteins, and can be dephosphorylated by a few protein phosphatases. Photoactivated phytochromes rapidly change the expression of light-responsive genes by repressing the activity of CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1), an E3 ubiquitin ligase targeting several photomorphogenesis-promoting transcription factors for degradation, and by inducing rapid phosphorylation and degradation of Phytochrome-Interacting Factors (PIFs), a group of bHLH transcription factors repressing photomorphogenesis. Phytochromes are targeted by COP1 for degradation via the ubiquitin/26S proteasome pathway.

INTRODUCTION

As sessile organisms, plants have acquired a high degree of developmental plasticity to optimize their growth and reproduction in response to their ambient environment, such as light, temperature, humidity, and salinity. Plants utilize a wide range of sensory systems to perceive and transduce specific incoming environmental signals. Light is one of the key environmental signals that influences plant growth and development. In addition to being the primary energy source for plants, light also controls multiple developmental processes in the plant life cycle, including seed germination, seedling de-etiolation, leaf expansion, stem elongation, phototropism, stomata and chloroplast movement, shade avoidance, circadian rhythms, and flowering time (Deng and Quail, 1999; Wang and Deng, 2003; Jiao et al., 2007).

Plants can monitor almost all facets of light, such as direction, duration, quantity, and wavelength by using at least four major classes of photoreceptors: phytochromes (phys) primarily responsible for absorbing the red (R) and far-red (FR) wavelengths (600-750 nm), and three types of photoreceptors perceiving the blue (B)/ultraviolet-A (UV-A) region of the spectrum (320-500 nm): cryptochromes (crys), phototropins (phots), and three newly recognized LOV/F-box/Kelch-repeat proteins ZEITLUPE (ZTL), FLAVIN-BINDING KELCH REPEAT F-BOX (FKF), and LOV KELCH REPEAT PROTEIN 2 (LKP2). In addition, UV RESISTANCE LOCUS 8 (UVR8) was recently shown to be a UV-B (282-320 nm) photoreceptor (Rizzini et al., 2011). These photoreceptors perceive, interpret, and transduce light signals, via distinct intracellular signaling pathways, to modulate photoresponsive nuclear gene expression, and ultimately leading to adaptive changes at the cell and whole organism levels.

The past two decades have seen dramatic progress in molecular characterization and understanding of the photobiology and photochemistry of the phytochrome photoreceptors in higher plants. This chapter aims to highlight some of the most recent progress in elucidating the molecular, cellular and biochemical mechanisms of phytochrome signaling in Arabidopsis. Interested readers are encouraged to read the accompanying reviews on other related subjects, such as photomorphogenesis (Nemhauser and Chory, 2002), cryptochromes (Yu et al., 2010) , phototropins (Pedmale et al., 2010), and the circadian clock (McClung et al., 2002).

PLANT PHYTOCHROMES

The Discovery and Action Modes of Phytochromes

The term phytochrome, meaning “plant color”, was originally coined to describe the proteinous pigment that controls photoperiod detection and floral induction of certain short-day plants (such as cocklebur and soybean) (Garner and Allard, 1920), and

the reversible seed germination of lettuce (c.v. Grand Rapids) by R and FR light (Borthwick et al., 1952). R light promotes seed germination, whereas subsequent FR light treatment abolishes R light induction of seed germination. The germination response of lettuce seeds repeatedly treated with R/FR cycles is determined by the last light treatment. Thus R/FR photoreversibility is a characteristic feature of this response. In addition, the law of reciprocity applies to this response, i.e. the response is dependent on the total amount of photons received irrespective of the duration of light treatment.

Over the years, three action modes for phytochromes have been defined, i.e. low-fluence responses (LFRs), very-low-fluence responses (VLFRs) and high-irradiance responses (HIRs) (Table 1). The above-mentioned F/FR reversible response is characteristic of LFRs. LFRs also induce other transient responses, such as changes in ion flux, leaf movement, chloroplast rotation, and changes in gene expression (Haupt and Hader, 1994; Roux, 1994; Vince-Prue, 1994). VLFRs are activated by extremely low light intensities of different wavelengths (FR, R and B); examples include light-induced expression of the light-harvesting chlorophyll *a/b*-binding protein (*LHCB*) gene and light induction of seed germination. HIRs depend on prolonged exposure to relatively high light intensities, and are primarily responsible for the control of seedling de-etiolation (e.g. inhibition of hypocotyl elongation and promotion of cotyledon expansion) under all light qualities (Mustilli and Bowler, 1997; Casal et al., 1998; Neff et al., 2000;Table 1).

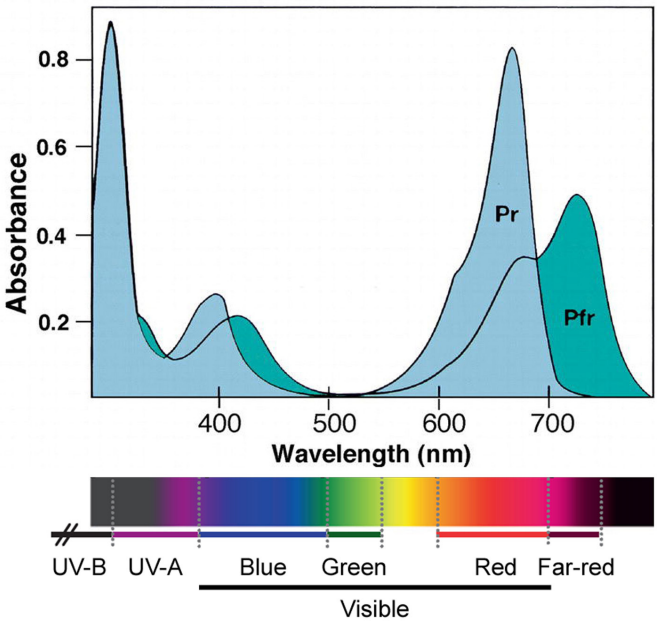

Figure 1. Absorption spectra of phytochromes.

Absorption spectra of the two forms (Pr and Pfr) of phytochromes. The Pr form absorbs maximally at 660 nm, while the Pfr form absorbs maximally at 730 nm. The visible light range of the human eye is approximately 380-700 nm. The light spectrum was adapted from Kami et al. (2010). Reprinted with permission from Elsevier.

Table 1. Diagnostic Features of Different Phytochrome Action Modes

Action Mode	Fluence Requirements	Photoreversibility	Reciprocity
VLFR	0.1 µmol/m^2 - 1 µmol/m^2	No	Yes
LFR	1 - 1000 µmol/m^2	Yes	Yes
HIR	> 1000 µmol/m^2	No	No

VLFR: very-low-fluence response;
LFR: low-fluence response;
HIR: high-irradiance response.

Chromophores and Two Reversible Forms of Phytochromes

Photoreversibility occurs because phytochromes exist as two distinct but photoreversible forms *in vivo*: the R light-absorbing form (Pr) and the FR light-absorbing form (Pfr). The Pr form absorbs maximally at 660 nm, whereas the Pfr form absorbs maximally at 730 nm (Quail, 1997a; Figure 1). The Pfr forms of phytochromes are generally considered to be the biologically active forms. It should be noted that in addition to their maximal absorptions of R and FR wavelengths, phytochromes also weakly absorb B light (Furuya and Song, 1994; Figure 1).

Phytochromes are soluble proteins and exist as homodimers. The molecular mass of the apoprotein monomer is approximately 125 kDa. Phytochrome apoproteins are synthesized in the cytosol, where they assemble autocatalytically with a linear tetrapyrrole chromophore, phytochromobillin (PΦB). The synthesis of PΦB is accomplished by a series of enzymatic reactions in the plastid that begins with 5-aminolevulinic acid (Figure 2A). The early steps in the PΦB pathway are shared with chlorophyll and heme biosynthesis. The committed step is the oxidative cleavage of heme by a ferredoxin-dependent heme oxygenase (HO) to form biliverdin IX (BV). BV is subsequently reduced to 3Z-PΦB by the enzyme PΦB synthase. Both 3Z-PΦB and its isomerized form 3E-PΦB can serve as functional precursors of the phytochrome chromophore. PΦB is then exported to the cytosol, where it binds to the newly synthesized apo-PHYs to form holo-PHYs (Terry, 1997; Figure 2A). The chromophore is attached via a thioether linkage to an invariant cysteine in a well-conserved domain among all phytochromes (see below).

The intrinsic photochemical activity of the chromophore prosthetic group allows phytochromes to convert between the two forms. Phytochromes are synthesized in the Pr form in dark-grown seedlings. It has been widely accepted that absorption of R light triggers a “Z” to “E” isomerization in the C15-C16 double bond between the C and D rings of the linear tetrapyrrole, resulting in the FR-absorbing Pfr form (Andel et al., 1996; Figure 2B). However, a recent NMR analysis showed that the A pyrrole ring around C4-C5 double bond rotates during photoconversion (Ulijasz et al., 2010). This discrepancy should be resolved in future studies. In addition, the Pr-to-Pfr transition is associated with rearrangement of the protein backbone (Figure 2B). The active Pfr form can be converted back to the inactive Pr form, either by a slow non-photoinduced reaction (dark reversion) or much faster upon absorption of FR light (Mancinelli, 1994; Quail, 1997a; Fankhauser, 2001; Figure 2B). This property allows phytochrome to function as a R/FR-dependent developmental switch.

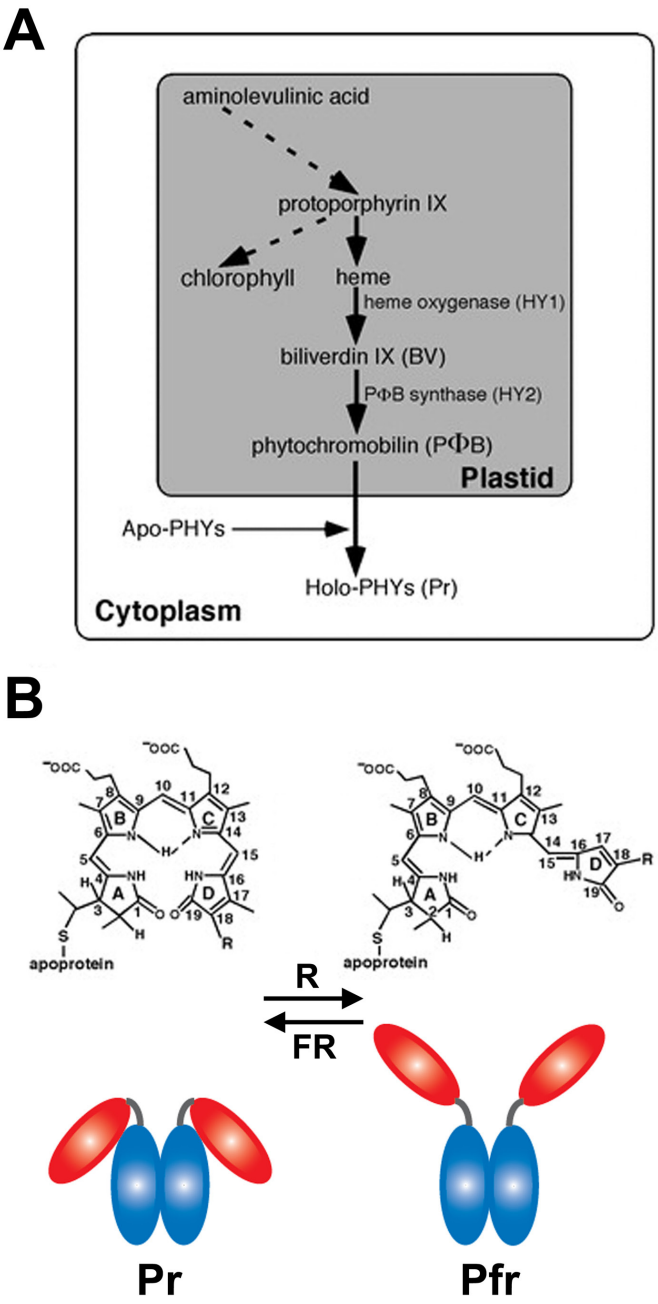

Figure 2. Arabidopsis phytochrome chromophore.

(A) The biosynthesis pathway of Arabidopsis phytochrome chromophore. Image adapted from Kohchi et al. (2001).

(B) Red (R) light triggers a "Z" to "E" isomerization in the C15-C16 double bond between the C and D rings of the linear tetrapyrrole (upper panel), which is accompanied by rearrangement of the apoprotein backbone (lower panel; adapted from Bae and Choi, 2008). This results in the photoconversion of phytochromes from the Pr form to the Pfr form. Please note that the chromophore ring A rather than D is rotated during photoconversion according to a recent NMR analysis (Ulijasz et al., 2010). The discrepancy needs to be resolved in future studies. Far-red (FR) light converts the Pfr form back to the Pr form.
Upper panel image reprinted from Bae and Choi (2008) with permission, from the Annual Review of Plant Biology, Volume 59 © 2008 by Annual Reviews (www.annualreviews.org).

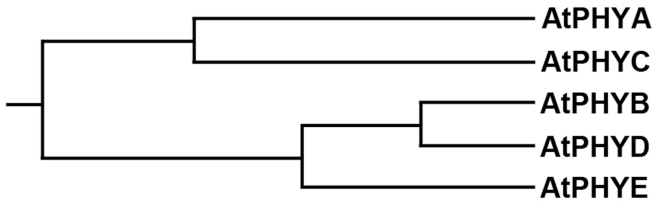

Figure 3. The phylogenetic tree of the five phytochrome species from *Arabidopsis thaliana*.

PHYB and PHYD share ~80% amino acid sequence identity, and constitute a branch of the gene family. PHYE itself, PHYA and PHYC form two other branches of the evolutionary family tree.
Image adapted from Clack et al. (1994). Reprinted with permission from Springer.

It is generally assumed that all phytochromes have the same chromophore. Arabidopsis mutants defective in the PΦB-synthetic pathway have been isolated. These mutants (*hy1* and *hy2*) have dramatically reduced levels of PΦB and consequently of functional phytochromes, and thus exhibit severely impaired photomorphogenesis (Parks and Quail, 1991). The Arabidopsis *HY1* locus encodes a heme oxygenase (AtHO1) responsible for much of PΦB synthesis in Arabidopsis (Davis et al., 1999a; Muramoto et al., 1999). Three additional *HO* genes were found in the Arabidopsis genome, designated *AtHO2* to *AtHO4* (Davis et al., 2001). The Arabidopsis *HY2* locus, likely a unique gene in the Arabidopsis genome, encodes the phytochromobilin synthase (Kohchi et al., 2001).

It should be pointed out that in addition to PΦB, phycocyanobilin (PCB), the chromophore of the light-harvesting pigment phycocyanin, can also bind phytochrome resulting in Pr and Pfr spectra that are slightly blue shifted compared with the PΦB adducts (Lagarias and Rapoport, 1980). This finding allowed the reconstitution of photoreversible phytochromes by expressing recombinant phytochrome proteins in yeast and assembling them *in vitro*.

The Phytochrome Gene Family

In *Arabidopsis thaliana*, there are five phytochromes, designated phytochrome A (phyA) to phyE. They are encoded by five distinct members of the phytochrome gene family and are classified into two groups according to their stability in light (Sharrock and Quail, 1989). phyA is a type I (light labile) phytochrome, and phyB to phyE are all type II (light stable) phytochromes. phyA is most abundant in dark-grown seedlings, whereas its level drops rapidly upon exposure to R or white (W) light. In light-grown plants, phyB is the most abundant phytochrome, whereas phyC-phyE are less abundant (Clack et al., 1994; Hirschfeld et al., 1998; Sharrock and Clack, 2002).

Sequence analysis suggests that these phytochromes can be clustered into three subfamilies: phyA/phyC, phyB/phyD, and phyE (Figure 3). Analysis of reconstituted recombinant phyA, phyB, phyC and phyE proteins revealed that they have similar but not identical spectral properties (Kunkel et al., 1996; Remberg et al., 1998; Eichenberg et al., 2000). Orthologs of Arabidopsis *PHY* genes are present in most, if not all, higher plants (Clack et al., 1994; Sharrock and Quail, 1989; Mathews and Sharrock, 1997).

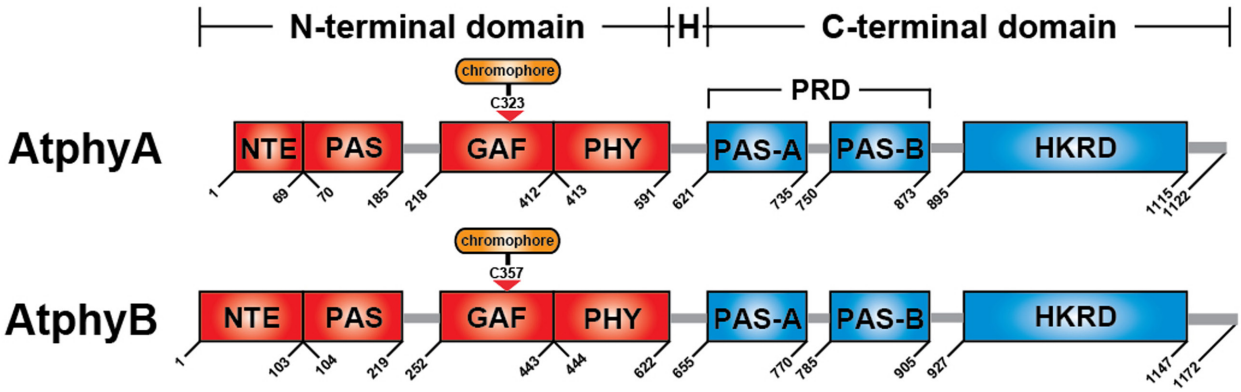

Figure 4. The domain structure of Arabidopsis phyA and phyB molecules.

H, hinge; NTE, N-terminal extension; PAS, Per (period circadian protein), Arnt (Ah receptor nuclear translocator protein), and Sim (single-minded protein); GAF, cGMP-stimulated phosphodiesterase, *Anabaena* adenylate cyclases and *Escherichia coli* FhlA; PHY, phytochrome; PRD, PAS-related domain; HKRD, histidine kinase–related domain. The chromophore is attached to a conserved cysteine residue in the GAF domain. The numbers indicate the positions of each domain.

General Structures of Phytochromes

The phytochrome molecule consists of an N-terminal domain (~ 70 kDa) and a C-terminal domain (~ 55 kDa), connected by a flexible hinge region (Figure 4). The N-terminal domain can be further divided into four consecutive subdomains: N-terminal extension (NTE), Per-Arnt-Sim (PAS), GAF, and PHY, while the C-terminal domain can also be divided into two subdomains: the PAS-related domain (PRD) containing two PAS repeats, and the histidine kinase-related domain (HKRD) (Figure 4).

Among the N-terminal subdomains, the NTE domain is uniquely present in plant phytochromes, whereas the PAS, GAF and PHY domains are also found in phytochrome-like proteins of various organisms (see below). Among the C-terminal subdomains, the PRD domain is unique to plant phytochromes, whereas the HKRD domain is also found in phytochrome-like proteins (Rockwell et al., 2006; Bae and Choi, 2008; Nagatani, 2010). The chromophore is attached to a conserved cysteine residue in the GAF domain of plant phytochromes (Figure 4). The PAS domains can be used either as platforms for protein-protein interactions, or as response modules to small ligands or changes in light conditions, oxygen levels, and redox potentials (Quail, 1997a; Neff et al., 2000). The HKRD domain lacks a critical histidine residue, and thus may be an evolutionary remnant rather than an active histidine kinase (Boylan and Quail, 1996). The putative dimerization motifs of phytochromes are also localized in the C-terminal half of the phytochrome molecules (Quail, 1997a).

A number of point mutations in the C-terminal domains of both phyA and phyB do not affect photoreversibility but eliminate the biological activity (Quail et al., 1995; Quail, 1997a), suggesting that the C-terminal domain is essential for proper downstream signaling. Consistent with this idea, a domain-swapping and deletion analysis suggested that the N terminus of phytochrome is essential for its specific photosensory properties, while the C termini of phyA and phyB are interchangeable and function as the output domains (Wagner et al., 1996). However, this notion was later challenged by the finding that the N-terminal domain of phyB, when dimerized and localized in the nucleus, confers much higher photosensitivity than the full-length phyB (Matsushita et al., 2003). These results suggest that the N-terminal domain of phyB transduces the light signal to downstream targets, whereas the C-terminal domain attenuates the activity of phyB (Matsushita et al., 2003). Similarly, the N-terminal domain of phyA also showed a partial physiological activity when dimerized and localized in the nucleus (Mateos et al., 2006). A further study demonstrated that dimers of the N-terminal 450-aa fragment of phyB (lacking the PHY domain) can still transduce the light signal upon nuclear localization (Oka et al., 2004). Therefore, these reports suggest that the N-terminal 450-aa fragment (encompassing the NTE, PAS and GAF domains) constitutes the core signaling domain of phytochrome.

PHYTOCHROME FUNCTIONS

In most instances, the roles of individual phytochromes are studied in the context of specific responses and/or developmental stages. Loss-of-function studies of monogenic phytochrome mutant and higher order mutants of various combinations combined with gain-of-function analyses are revealing the roles of individual phytochromes in regulating different aspects of plant development. It is clear now that individual phytochromes play both unique and overlapping roles throughout the life cycle of plants, regulating a range of developmental processes from seed germination to the timing of reproductive development (Table 2). Phytochrome functions in Arabidopsis development were recently reviewed by Franklin and Quail (2010), and this chapter will mainly discuss the roles of phytochromes in seed germination, seedling de-etiolation, and shade avoidance.

Table 2. Different Roles of Phytochrome Family Members in Seedling and Early Vegetative Development

Phytochrome Members	Primary Photosensory Activities	Primary Physiological Roles
phyA	VLFRs FR-HIRs	Seed germination under a broad spectrum of light conditions (UV, visible, FR); Seedling de-etiolation under FRc; promoting flowering under LD.
phyB	LFRs R-HIRs EOD-FR (R/FR ratio)	Seed germination under Rc; Seedling de-etiolation under Rc; Shade avoidance response (petiole and internode elongation, flowering).
phyC	R-HIRs	Seedling de-etiolation under Rc.
phyD	EOD-FR (R/FR ratio)	Shade avoidance response (petiole and internode elongation, flowering).
phyE	LFRs EOD-FR (R/FR ratio)	Seed germination; Shade avoidance response (petiole and internode elongation, flowering).

VLFRs: very-low-fluence responses;
LFRs: low-fluence responses;
HIRs: high-irradiance responses;
FR: far-red light;
R: red light;
FRc: continuous far-red light;
Rc: continuous red light;
LD: long day light condition;
EOD-FR: end-of-day far-red light;
R/FR ratio: red/far-red light ratio.

Seed Germination

As mentioned above, the involvement of a R/FR-reversible photoreceptor in mediating seed germination was first demonstrated by Harry Borthwick and colleagues in 1952 by studying the reversible germination of Grand Rapids lettuce seeds by R and FR treatments (Borthwick et al., 1952). The involvement of individual phytochromes in mediating Arabidopsis seed germination has been documented in many mutant studies. At least three phytochromes, i.e. phyA, phyB and phyE are involved in the control of Arabidopsis seed germination. phyA is responsible for the irreversible VLFR responses triggered by a wide variety of irradiations (ultraviolet, visible and FR light), while phyB controls the R/FR photoreversible LFRs (Reed et al., 1994; Botto et al., 1996; Shinomura et al., 1996). Seed germination can be promoted by both VLFRs and LFRs (Table 2). In addition, phyA promotes germination in continuous FR light in the HIR mode (Johnson et al., 1994; Reed et al., 1994; Hennig et al., 2002). However, phyE was also found to play a role in controlling seed germination in continuous FR light. This could be either because phyE is directly involved in the photoperception of FR light for this response, or because phyA requires phyE to mediate seed germination (Hennig et al., 2002). It is interesting that ambient temperature modulates the light-regulation of Arabidopsis seed germination, and different phytochromes display altered functional hierarchies at different temperatures (Heschel et al., 2007).

Seedling De-etiolation

Dark-grown seedlings undergo skotomorphogenesis (etiolation) and are characterized by long hypocotyls, closed cotyledons and apical hooks, and development of the proplastids into etioplasts. Light-grown seedlings undergo photomorphogenesis (de-etiolation) and are characterized by short hypocotyls, open and expanded cotyledons, and development of the proplastids into green mature chloroplasts (McNellis and Deng, 1995). Phytochromes perform a variety of overlapping functions in regulating seedling de-etiolation.

Mutants deficient in phyA display a wild-type photomorphogenic phenotype in W and R light. However, when grown in continuous FR light, *phyA* mutants display a skotomorphogenic phenotype (Figure 5), confirming that phyA is the primary photoreceptor responsible for perceiving and mediating various responses to FR light (Dehesh et al, 1993; Nagatani et al., 1993; Parks and Quail, 1993; Whitelam et al., 1993; Reed et al., 1994). Comparative transcriptional profiling of etiolated wild-type and *phyA* mutants subjected to FR light treatments revealed more than 800 phyA-regulated genes, providing the first insight into the phyA transcriptional network (Tepperman et al., 2001). It should be noted that *phyA* mutants also display elongated hypocotyls in continuous B light (Whitelam et al., 1993; Neff and Chory, 1998), suggesting that phyA also plays a pivotal role in perceiving and transducing B light.

phyB is the predominant phytochrome regulating de-etiolation in W and R light (Figure 5). However, transcriptional profiles of etiolated *phyB* mutants subjected to R light treatments did not differ dramatically from the wild-type controls (Tepperman et al., 2004). Subsequent studies showed that phyA plays a dominant role in regulating rapid gene expression responses to R light treatments (Tepperman et al., 2006). Moreover, the long hypocotyl and reduced cotyledon expansion phenotypes were enhanced in *phyA phyB* double mutants relative to *phyB* monogenic mutants in R light (Figure 5), revealing a role for phyA in responding to R light which is normally masked in the presence of phyB (Neff and Van Volkenburgh, 1994; Reed et al., 1994; Casal and Mazzella, 1998; Neff and Chory, 1998).

Mutants deficient in phyC exhibit a partial loss of sensitivity to R light, with longer hypocotyls and smaller cotyledons than the wild-type controls, indicating that phyC functions in regulating seedling de-etiolation in R light (Franklin et al., 2003a; Monte et

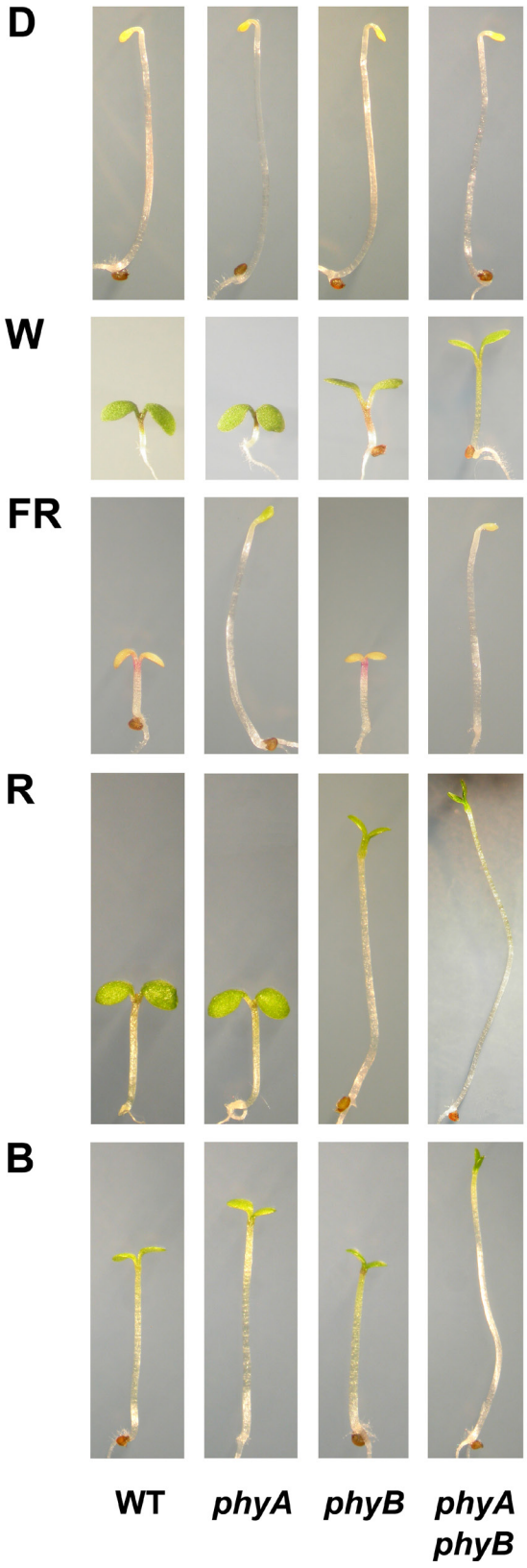

Figure 5. Phenotypes of 4-d-old wild-type (WT), *phyA*, *phyB* and *phyA phyB* mutant plants grown in darkness (D) or under continuous white (W), far-red (FR), red (R) and blue (B) light conditions.

al., 2003). However, no additive phenotype was observed in *phyB phyC* double mutants compared with their monogenic mutants, suggesting that phyC function is dependent on phyB (Monte et al., 2003). This might be explained by a recent finding that phyC does not homodimerize but rather forms heterodimers with phyB *in vivo* (Clack et al., 2009). Moreover, despite showing more sequence similarity to PHYA rather than to PHYB, PHYD and PHYE (Figure 3), phyC seems not to play a role in mediating seedling de-etiolation in FR light (Franklin et al., 2003a; Monte et al., 2003). However, the hypocotyls of *phyA phyC* double mutants are significantly longer than those of *phyC* monogenic mutants in R light (Franklin et al., 2003a; Monte et al., 2003), again confirming the contribution of phyA to seedling establishment under R light.

Although PHYD shows high sequence similarity to PHYB (Figure 3), the role of phyD in seedling de-etiolation in R seems minor, as it was reported that the Wassilewskija (Ws) ecotype of Arabidopsis contains a natural *phyD* deletion but its seedlings display only marginally longer hypocotyls in R than plants containing an introgressed *PHYD* gene (Aukerman et al., 1997). However, a synergistic relationship was observed between phyB and phyD in R, as the hypocotyls of *phyB phyD* double mutants are more than additively longer than those of each monogenic mutant (Aukerman et al., 1997). The contribution of phyE to seedling de-etiolation seems negligible, as it was shown that monogenic *phyE* mutants were indistinguishable from wild-type control plants in a variety of light conditions (Devlin et al., 1998).

As mentioned above, phytochromes also weakly absorb B light (Figure 1). In addition, phytochromes were shown to modulate cryptochrome-mediated seedling de-etiolation and phototropin-mediated phototropic curvature of Arabidopsis hypocotyls in B light (Parks et al., 1996; Ahmad and Cashmore, 1997; Hamazato et al., 1997; Janoudi et al., 1997; Casal and Mazzella, 1998; Neff and Chory, 1998). Direct physical interactions between phytochromes and cryptochromes were also reported (Ahmad et al., 1998; Mas et al., 2000). Therefore, phytochromes co-act with B/UV-A light photoreceptors to regulate seedling de-etiolation in B light.

Shade Avoidance

Plant development is regulated not only by the difference between light and darkness, but also by light quality, in particular the change of light quality due to shading by other plants. Light passed through or reflected from living vegetation is depleted in R and B wavebands, which are absorbed by chlorophyll and carotenoid pigments used for photosynthesis, leading to a reduction in the ratio of R to FR wavelengths (R:FR). This allows plants to initiate a suite of developmental responses called shade avoidance syndrome (SAS), which elevates leaves towards unfiltered daylight and enables plants to overtop competitors (reviewed in Smith and Whitelam, 1997). These responses include elongation of stems and petioles, accelerated flowering time, and increased apical dominance. The ability of plants to monitor their light environments and change their architecture provides them with a competitive strategy to survive and complete their life cycle in dense stands.

Reductions in R:FR ratio favor the conversion of phytochrome molecules to their inactive Pr form. Therefore the shade avoidance syndrome must be suppressed under high R:FR ratio conditions.

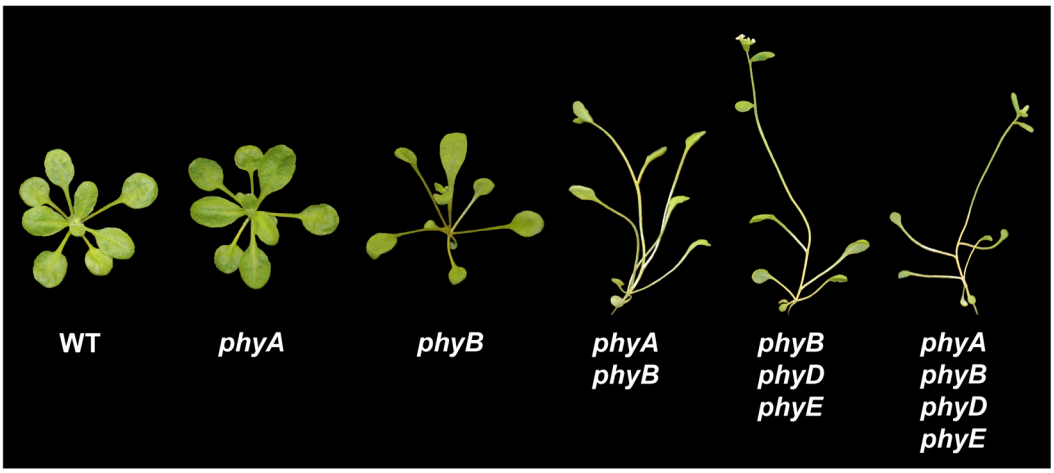

Figure 6. Phenotypes of 3-week-old wild-type (WT), *phyA*, *phyB*, *phyA phyB*, *phyB phyD phyE*, *phyA phyB phyD phyE* plants grown under white light conditions (16-h light/8-h dark).

In this sense, shade avoidance is due to the relief of suppression rather than the induction of physiological responses. phyB is the predominant suppressor of shade avoidance responses in high R:FR, as phyB-deficient plants display a constitutive shade avoidance phenotype (elongated petiole and early flowering) (Nagatani et al., 1991; Somers et al., 1991; Figure 6). The shade avoidance responses enabled by low R:FR ratios can be effectively phenocopied by end-of-day far-red (EOD-FR) treatments. This led to the discovery of the roles of phyD and phyE in shade avoidance. Although the monogenic *phyD* mutant plants have no obviously abnormal phenotype to EOD-FR, plants impaired in both phyB and phyD display significantly longer hypocotyls under either R or W light, and flower earlier than the *phyB* monogenic mutants, suggesting that phyB and phyD function redundantly in suppressing shade avoidance (Aukerman et al., 1997; Devlin et al., 1999). As with phyD, monogenic *phyE* mutants show no phenotypic alterations unless in the *phyB* mutant background, and the *phyB phyE* double mutants flower much earlier than the *phyB* monogenic mutants (Devlin et al., 1998; Franklin et al., 2003b).

As phyA is the primary photoreceptor sensing FR wavelengths, enrichment of FR in transmitted/reflected light can lead to enhanced phyA signaling in the HIR mode. Therefore, the action of phyA can substitute for the loss of phyB, phyD and phyE activity due to their conversion to the inactive Pr forms. Indeed, Arabidopsis *phyA* mutant seedlings display enhanced shade avoidance responses relative to wild-type control plants when grown in low R:FR conditions (Johnson et al., 1994; Smith et al., 1997; Salter et al., 2003). Despite the relatively close phylogenetic relationship between phyA and phyC (Figure 3), no role for phyC in mediating shade avoidance responses has been reported. This conclusion is further confirmed by the observations that *phyA phyB phyD phyE* quadruple mutants display insensitivity to reductions in R:FR ratio and EOD-FR treatments (Franklin et al., 2003b).

LIGHT-REGULATED SUBCELLULAR LOCALIZATION OF PHYTOCHROMES

As mentioned above, phytochromes are synthesized in the cytosol in their inactive Pr forms. It was widely accepted before the mid-1990s that phytochromes were cytoplasmic photoreceptors based on early biochemical and immunocytochemical studies (Nagy and Schafer, 2002). However, extensive studies conducted in the last decade have established the notion that phytochromes must enter the nucleus to trigger most light responses (Nagatani, 2004; Kevei et al., 2007; Fankhauser and Chen, 2008). Thus, light-regulated translocation of the photoreceptors from the cytoplasm into the nucleus is a key event in the phytochrome signaling cascade. Recent publications are beginning to shed light on the molecular mechanisms underlying this central control step.

Regulation of phyB Nuclear Localization

phyB nuclear accumulation is efficiently initiated by continuous R light, and to a lesser extent by continuous B light, but completely ineffective by FR light. Single pulses of R, FR and B light cannot induce phyB nuclear accumulation (Gil et al., 2000). Moreover, phyB nuclear transport by R light is reversible by FR light, a typical characteristic of LFR (Kircher et al., 1999). A similar regulation of subcellular localization was also reported for phyC, phyD and phyE (Kircher et al., 2002). It should be noted that there is a weak, but detectable level of phyB-phyE present in the nucleus of dark-grown plants (Kircher et al., 2002).

A structure-function analysis first demonstrated that the C-terminal half of phyB (amino acids 594-1172) is sufficient to localize GUS to the nucleus, suggesting that the C-terminal domain of phyB harbors a putative nuclear localization signal (NLS) (Sakamoto and Nagatani, 1996). This result was confirmed by a later study, which showed that GFP fused to the N-terminal half of phyB (amino acids 1-651) localizes to the cytoplasm, whereas GFP fused to the C-terminal half of phyB (amino acids 625-1172)

localizes to the nucleus (Matsushita et al., 2003). Subsequent further analyses of various truncations of the phyB C-terminal domain revealed that the PRD domain of phyB (amino acids 594-917) is both necessary and sufficient for nuclear localization, indicating that a putative NLS resides in this domain (Chen et al., 2005). Interestingly, this study also showed that the N-terminal photosensory GAF-PHY domains interact with the PRD domain in a light-dependent manner, thus providing a mechanistic link between light-dependent Pr/Pfr conformational alterations of phyB and the unmasking of the NLS that regulates phyB nuclear accumulation (Chen et al., 2005).

Regulation of phyA Nuclear Localization

The nuclear accumulation pattern of phyA is quite distinct from that of phyB. Firstly, all light illuminations (FR, R and B) are effective in inducing phyA nuclear translocation. A single, brief pulse of FR, R or B light induces phyA nuclear import as well (Hisada et al., 2000; Kim et al., 2000; Kircher et al., 2002). Therefore, phyA nuclear import is mediated by VLFR and HIR. Secondly, phyA nuclear translocation is very rapid (within minutes), whereas phyB nuclear import is relatively slow that takes hours (Kircher et al., 1999, Kim et al., 2000; Kircher et al., 2002). Finally, in contrast to phyB-phyE, phyA is exclusively localized in the cytosol in etiolated seedlings (Kircher et al., 2002).

FAR-RED ELONGATED HYPOCOTYL 1 (FHY1) and FHY1-LIKE (FHL). The rapid nuclear translocation of phyA, and the fact that phyA itself does not contain a typical NLS suggest the existence of an efficient transport machinery responsible for phyA nuclear import. Indeed, two small plant-specific proteins, FHY1 and FHL, have been shown to play an essential role in facilitating phyA nuclear translocation. The history of FHY1 essentially parallels the history of the molecular genetic analysis of phyA signaling pathway in Arabidopsis. The *fhy1* mutant was firstly reported in 1993, together with two other mutants, i.e. *fhy2* and *fhy3*, and all these *fhy* mutants develop elongated hypocotyls in FR light (Whitelam et al., 1993). This pioneering report showed that *FHY2* locus corresponds to *PHYA*, while the *FHY1* and *FHY3* genes were cloned as separate loci in 2001 and 2002, respectively (Desnos et al., 2001; Wang and Deng, 2002). Subsequent studies identified a FHY1-like protein, named FHL, based on its sequence homology to FHY1 (Zhou et al., 2005).

FHY1 and FHL are two small proteins (202 and 201 amino acids, respectively) in Arabidopsis that were found to have homologs in both monocot and dicot plant species (Genoud et al., 2008; Li et al., 2010). Each protein contains an NLS and a nuclear exclusion signal (NES) at their N-termini and a septin-related domain (SRD) at their C termini (Desnos et al., 2001; Zhou et al., 2005). *In vitro* binding assays showed that both proteins are capable of homo- and hetero-dimerization through their C-terminal domains (Zhou et al., 2005). The NLS and SRD motifs are functionally important, because removal of either motif disrupts the function of FHY1 (Zeidler et al., 2004).

Earlier studies involving FHY1 suggested that FHY1 is responsible for mediating a branch of phyA signaling (Barnes et al., 1996). However, microarray analysis showed that all genes affected by *phyA* mutation are also affected by *fhy1* mutation, although to a lesser degree (Wang et al., 2002). The roles of FHY1 in phyA signaling remained obscure until it was reported that FHY1/FHL physically interact with the Pfr form of phyA *in vitro* and in yeast cells through their SRD motifs, and that FHY1/FHL are required for nuclear accumulation of phyA since phyA is localized only in the cytosol of *fhy1 fhl* double mutants (Hiltbrunner et al., 2005, 2006). This conclusion was extended by a later report that the major function of FHY1/FHL is to act as adaptor proteins to chaperone photoactivated phyA into the nucleus (Genoud et al., 2008). Evidence supporting this proposed mode of action of FHY1 includes, first, sequence alignments show that the N-terminal NLS and the C-terminal phyA-interacting motifs are the only conserved motifs among all FHY1 homologs. Consistently, an artificial FHY1 consisting of a virus NLS motif and the C-terminal phyA-interacting motif of Arabidopsis FHY1 could rescue *fhy1* mutant phenotypes and colocalizes with phyA in the nucleus. Second, FHY1 becomes functionally dispensable in transgenic seedlings expressing a constitutively nuclear phyA, i.e. if phyA could enter the nucleus by itself (Genoud et al., 2008).

FHY1/FHL specifically control the subcellular localization of phyA but not phyB, because *fhy1/fhl* mutants only show long-hypocotyl phenotype in FR but not R light, and phyB nuclear import is not affected in the *fhy1* mutant (Hiltbrunner et al. 2005). As each of FHY1 and FHL has a functional monopartite NLS and NES that are indeed involved in the nuclear localization and exclusion of FHY1 (Zeidler et al., 2004), it is possible that phyA utilizes the NLS of FHY1/FHL for its nuclear transport. The fact that FHY1 homologs are widely distributed in angiosperms suggests that the mechanism uncovered in Arabidopsis may be conserved in higher plants (Genoud et al., 2008). However, interaction of phyA with FHY1/FHL alone appears to be insufficient for phyA nuclear translocation, because phyA-402, containing a missense mutation in the HKRD domain of phyA, is still capable of interacting with FHY1/FHL but does not translocate into the nucleus in R light (Muller et al., 2009).

Based on the evidence discussed above, one would assume that FHY1/FHL only function for phyA nuclear transport. However, this assumption was challenged by a recent report, which showed that FHY1/FHL might transmit phyA signals to downstream transcription factors (Yang et al., 2009). FHY1/FHL physically interact with two well-characterized transcription factors in phyA signaling network, LONG HYPOCOTYL IN FAR-RED 1 (HFR1) and LONG AFTER FAR-RED LIGHT 1 (LAF1), both *in vitro* and *in vivo*. Analysis of double and triple mutants showed that HFR1 and LAF1 independently transmit phyA signals downstream of FHY1 and FHL. Intriguingly, FHY1 was shown to mediate the assembly of a PHYA/FHY1/HFR1 signaling complex *in vitro*, suggesting that such kind of phyA signaling complexes may be assembled *in vivo* (Yang et al., 2009).

FHY3 and FAR-RED IMPAIRED RESPONSE1 (FAR1). The distinct role of FHY1/FHL in phyA nuclear accumulation suggests that any factor regulating *FHY1/FHL* transcriptionally or post-transcriptionally may indirectly affect phyA nuclear import. This notion is true, as demonstrated by the functional studies on FHY3 and FAR1. When the *FHY3* gene was cloned in 2002, sequence alignments revealed that FHY3 shares high homology with FAR1, a previously identified phyA signaling component (Hudson et al., 1999; Wang and Deng, 2002). The function of FHY3 and FAR1 was not clear at that time, but some reports showed that FHY3 positively regulates the transcript levels of *FHY1*, and that FHY3/

FAR1 proteins share substantial similarity to *Mutator*-like element (MULE) transposases and may work as transcriptional regulators (Desnos et al., 2001; Hudson et al., 2003).

The breakthrough was made in 2007, when a study unequivocally demonstrated that FHY3 and FAR1 are transposase-derived transcription factors directly binding to the *FHY1/FHL* promoters via a specific *cis*-element called the FHY3/FAR1 binding site (FBS), and activating *FHY1/FHL* gene expression (Lin et al., 2007). The N-terminal C2H2 zinc finger domains of FHY3/FAR1 are essential for DNA binding, whereas the entire C-terminal regions are required for their transcriptional activation activity (Lin et al., 2007, 2008). Thus, FHY3 and FAR1 define a new type of transposase-derived transcription factors. Moreover, phyA nuclear accumulation is abolished in the *fhy3 far1* double mutant, indicating that as the key transcriptional activators of *FHY1/FHL* expression, FHY3/FAR1 indirectly control phyA nuclear accumulation (Lin et al., 2007). This conclusion was further supported by another report that constitutively nuclear phyA could rescue the *fhy3* mutant phenotypes (Genoud et al., 2008).

The discovery of FHY3/FAR1's role in phyA signaling invites the further question of how *FHY1/FHL* expression is down-regulated by phyA signaling. A negative feedback mechanism(s) controlling *FHY1/FHL* expression should exist, as previous studies showed that *FHY1/FHL* transcript levels were rapidly down-regulated when dark-grown plants were exposed to FR light (Desnos et al., 2001; Lin et al., 2007). A recent study showed that ELONGATED HYPOCOTYL 5 (HY5), a well-characterized bZIP transcription factor involved in promoting photomorphogenesis under various light conditions, plays a major role in this process (Li et al., 2010). HY5 achieves its goal by two distinct mechanisms. The first mechanism involves steric hindrance as HY5 directly binds ACGT-containing elements (ACEs) less than 10 bp away from the FHY3/FAR1 binding sites in the *FHY1/FHL* promoters. Thus, HY5's occupation of the ACEs consequently decreases the accessibility of the *FHY1/FHL* promoters to FHY3/FAR1. The second mechanism is called "sequestration" through the physical interactions between HY5 and FHY3/FAR1, a mechanism also used in the regulation of some plant bHLH transcription factors (de Lucas et al., 2008; Feng et al., 2008; Hornitschek et al., 2009). Therefore, HY5 acts as a repressor of *FHY1/FHL* expression by modulating the transcriptional activities of FHY3 and FAR1 (Li et al., 2010; Figure 7).

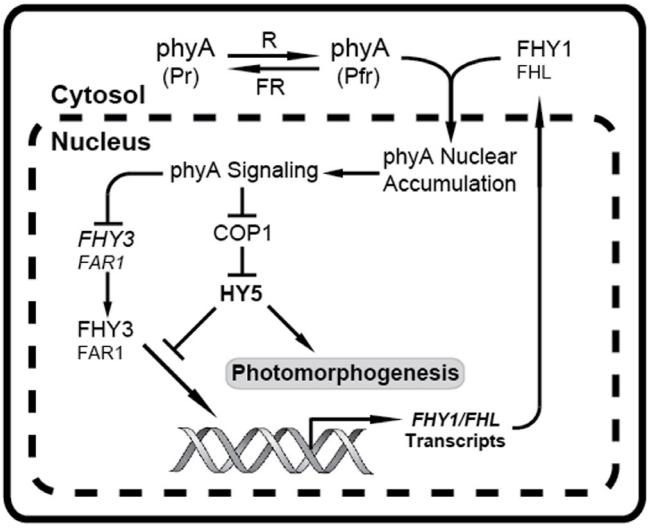

Figure 7. Control of *FHY1/FHL* expression and phyA nuclear accumulation.

FHY1 and FHL are required for phyA nuclear accumulation (Hiltbrunner et al., 2005, 2006; Genoud et al., 2008). FHY3 and FAR1 are two transposase-derived transcription factors that directly activate *FHY1/FHL* transcription, and thus indirectly regulate phyA nuclear accumulation and subsequent responses (Lin et al., 2007). phyA is localized exclusively in the cytosol in darkness in its inactive Pr form. Upon light exposure, the Pfr form of phyA is imported into the nucleus by FHY1/FHL, and thus triggers phyA signaling leading to multiple light responses, including the reduction of COP1 in the nucleus and accumulation of HY5 (Osterlund and Deng, 1998; Osterlund et al., 2000), and feedback regulation of *FHY3* and *FAR1* transcript levels (Lin et al., 2007). HY5 plays dual roles in phyA signaling: promoting photomorphogenesis, and down-regulating *FHY1/FHL* transcript levels by modulating the activities of the transcriptional activators FHY3 and FAR1 (Li et al., 2010). FHY3 and FHY1 (indicated by larger letters) are the more predominant players in the phyA signaling process compared to their respective homologs FAR1 and FHL. Pr: R-absorbing form of phyA (inactive); Pfr: FR-absorbing form of phyA (active). Arrow, positive regulation; bar, negative regulation.
Image adapted from Li et al. (2010).

Nuclear Bodies (NBs)

Upon import into the nucleus, both phyA and phyB localize to discrete subnuclear foci, called nuclear bodies or speckles. Although most studies on NBs were conducted in cells overexpressing fluorescent-protein-tagged phytochromes, native phyA and phyB have each been shown to localize to NBs by immunocytochemical studies, suggesting that the formation of NBs is not an artifact because of the overexpression of phytochromes (Hisada et al., 2000; Kircher et al., 2002). The pattern of NBs is highly dynamic and directly regulated by light quality, quantity, and periodicity, and closely correlates to phytochrome-mediated responses (Kircher et al., 2002; Chen et al., 2003). However, the precise nature of NBs is still unknown. But several phytochrome signaling components colocalize to NBs, suggesting that NBs play important roles in phytochrome signaling (Chen, 2008).

Based on the kinetics of phyB-GFP localization during the dark-to-light transition, two types of NBs have been defined for phyB. Within minutes of light exposure, small and transient phyB-GFP NBs appear (Bauer et al., 2004). Interestingly, phyB colocalizes with PIF3 in the early transient NBs, and its localization to the early NBs is PIF3-dependent *in vivo* (Bauer et al., 2004). These early transient NBs disappear after 10-15 min in the light (Kevei et al., 2007), and interestingly, the disappearance of these early NBs correlates with the light-induced and phytochrome-dependent degradation of PIF3 (Al-Sady et al., 2006), thus implying that these early phyB NBs are the sites for phyB-PIF3 interaction, and their disappearance is due to PIF3 degradation (Chen, 2008).

After the disappearance of these early phyB NBs, longer R light treatment (2-3 h) leads to the appearance of larger and more stable NBs (Nagatani, 2004; Kevei et al., 2007; Chen, 2008). Interestingly, the size and number of these NBs depend on the fluence rate of R light (Chen et al., 2003). As increasing fluence rates of R not only induced a change in the pattern of phyB NBs but also enhanced inhibition of hypocotyl elongation, it was pro-

posed that the formation of phyB NBs plays a role in the regulation of phyB-mediated signal transduction (Chen et al., 2003). The observations that PIF3 colocalizes with phyB only in the early but not late NBs indicate that the components of the early and late NBs may be different (Bauer et al., 2004).

phyA rapidly enters the nucleus in response to FR light and also forms early and late NBs (Bauer et al., 2004). PIF3 colocalizes with phyA in the early transient NBs but not late stable NBs (Bauer et al., 2004), consistent with the report that light-activated phyA also interacts with PIF3 and contributes to its degradation (Al-Sady et al., 2006). FHY1 and FHL, two phyA-interacting proteins required for phyA nuclear accumulation (see above), colocalize with phyA in the early transient NBs (Hiltbrunner et al., 2005, 2006). However, although a short R treatment also induces translocation of phyA into the nucleus, NB formation, and colocalization with PIF3, extended R light results in the complete loss of the phyA-GFP fluorescence (Bauer et al., 2004), possibly due to the photolabile nature and rapid degradation of phyA (see below).

Because both phyA and PIF3 are localized to NBs before their degradation, it has been proposed that NBs of phytochromes are sites for protein degradation (Bauer et al., 2004; Seo et al., 2004; Al-Sady et al., 2006). Recently, Chen et al. (2010) used a confocal microscopy-based screen to identify a gene, *HEMERA* (*HMR*), required for the localization of phyB-GFP to large NBs in high fluence rate of R light. Characterization of *hmr* mutants, localization of HMR protein within cells, and analysis of its biochemical function indicate that HMR is a specific and early phytochrome signaling component required for light-dependent proteolysis of phyA, PIF1, and PIF3 (Chen et al., 2010). Moreover, HMR is predicted to be structurally similar to the multiubiquitin-binding protein, RAD23, and can partially rescue yeast *rad23* mutants, thus suggesting that phytochrome nuclear bodies may serve as sites of proteolysis (Chen et al., 2010).

PHYTOCHROMES AS LIGHT-REGULATED KINASES

How do phytochromes initiate their signal transduction upon photo-activation? A long-standing but much disputed hypothesis is that phytochromes act as light-regulated kinases (Wong et al., 1986; Kim et al., 1989). This hypothesis was initially supported by the observation that purified preparations of phyA catalyzed phosphorylation of serine residues on the photoreceptor itself, i.e. autophosphorylation activity (Wong et al., 1986; Yeh and Lagarias, 1998). The discovery of phytochrome-like photoreceptors in bacteria, collectively called bacteriophytochromes (BphPs), generated further supporting evidence for such a view (Fankhauser, 2000; Vierstra and Davis, 2000). Phytochrome-like sequences were identified in the cyanobacteria *Fremeyella diplosiphon*, *Synechocystis sp. PCC6803*, the purple photosynthetic bacterium *Rhodospirillum* and non-photosynthetic bacteria such as *Deinococcus radiodurans*, *Pseudomononas putida* and *Pseudomonas aeruginosa* (Kehoe and Grossman, 1996; Hughes et al., 1997; Davis et al., 1999b; Hughes and Lamparter, 1999; Jiang et al., 1999; Wu and Lagarias, 2000). Some of them, such as Cph1 of *Synechocystis sp. PCC6803*, can bind to the plant phytochrome chromophore (phytochromobilin PΦB or phycocyanobilin PCB) autocatalytically and display R/FR absorption spectra similar to plant phytochromes (Hughes et al., 1997; Lamparter et al., 1997; Yeh et al., 1997). Further, Cph1 was shown to be a light-regulated histidine kinase. Both autophosphorylation of Cph1 and transphosphorylation of Rcp1 (the response regulator for Cph1) are inhibited by R light and stimulated by FR light (Yeh et al., 1997), suggesting that in cyanobacteria phosphorylation is an important and very early step of phytochrome signal transduction.

However, higher plant phytochromes share limited sequence similarity with Cph1 at their C-termini (Figure 8). In addition, plant phytochromes have two additional domains compared with BphPs: a serine-rich NTE region, and a PRD domain located between PHY and HKRD domains (Figures 8). Moreover, mutating several critical residues required for bacterial His kinase activity does not affect the activity of plant phytochromes, suggesting that plant phytochromes are not active His kinases (Quail, 1997b). In fact, purified recombinant plant phytochromes exhibit a serine/threonine kinase activity, suggesting that eukaryotic phytochromes are histidine kinase paralogs with serine/threonine specificity (Yeh and Lagarias, 1998). Consistent with this discovery, several *in vitro* substrates of phytochrome kinase activity were subsequently discovered, such as histone H1, PHYTOCHROME KINASE SUBSTRATE 1 (PKS1), cryptochromes, AUX/IAA proteins, and FHY1 (Wong et al., 1989; Ahmad et al., 1998; Fankhauser et al., 1999; Colon-Carmona et al., 2000; Shen et al.,

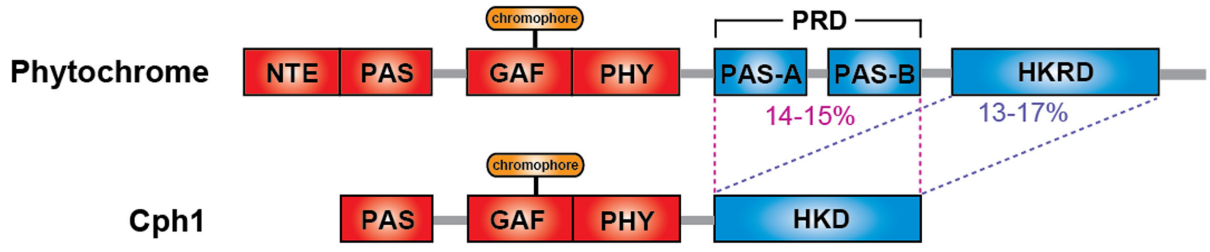

Figure 8. Structural comparison of Arabidopsis phytochromes and the bacterial phytochrome Cph1 (adapted from Yeh and Lagarias, 1998).

HKD, histidine kinase domain. The percent amino acid identities between the HKD domain of Cph1 and both PRD and HKRD domains of Arabidopsis phytochromes are indicated.
Image adapted from Yeh and Lagarias (1998). Reprinted with permission from the National Academy of Sciences.

2009). However, the kinase domain of phytochrome has not been determined. It is notable that both PRD and HKRD domains show similarities to the HKD domain of Cph1 (Yeh and Lagarias, 1998; Figure 8). In addition, to date the kinase activity has only been proven for one higher plant phytochrome: oat phyA. Although a recent report indicated that phyB shows some kinase activity *in vitro* (Phee et al., 2008), more evidence is required before a firm conclusion can be reached. Whether all phytochromes have kinase activity, and whether different phytochromes behave differently as protein kinases need to be further characterized.

The claim that higher plant phytochromes function as protein kinases invites many questions, such as, is the Pfr form more active than the Pr form? What is the biological role of this kinase activity of phytochromes in plants? Answers to these questions have just begun to be unraveled. For example, autophosphorylation of recombinant oat phyA is both chromophore and light regulated, with Pfr being more active than Pr (Yeh and Lagarias, 1998). *In vitro* kinase assays also showed that the Pfr form of phytochrome more effectively phosphorylates some substrates, such as PKS1 and CRY1 (Ahmad et al., 1998; Fankhauser et al., 1999), but for some other substrates, such as AUX/IAA proteins and FHY1, the Pr and Pfr forms showed similar kinase activities (Colon-Carmona et al., 2000; Shen et al., 2009). However, *in vivo* assays are required to verify the conclusions of these experiments. For example, FHY1 phosphorylation in Arabidopsis seedlings is solely dependent on the active Pfr form of phyA, although both Pr and Pfr forms of phyA could phosphorylate FHY1 *in vitro* (Shen et al., 2009).

Recently it was shown that oat phyA autophosphorylates two serine sites in its NTE region *in vitro* (Han et al., 2010). Mutation of these two autophosphorylation sites in transgenic Arabidopsis plants caused hypersensitive light responses, indicating an increase in phyA activity (Han et al., 2010). Consistently, the degradation of the mutant phyA was significantly slower than the wild-type phyA under light conditions, suggesting that phyA autophosphorylation plays an important role in the regulation of phytochrome signaling through the control of phyA protein stability (Han et al., 2010). Another report showed that phyA is the only photoreceptor responsible for rapid R light-dependent FHY1 phosphorylation, and interestingly, this phosphorylation is R/FR light reversible, a typical LFR mode of phytochrome action (Shen et al., 2009). Notably, phosphorylated FHY1 was shown to be a preferred substrate for ubiquitin/26S proteasome-mediated degradation, suggesting that phyA-dependent FHY1 phosphorylation in R light may serve as a biochemical mechanism to desensitize FHY1-mediated phyA signaling (Shen et al., 2005b, 2009). These examples suggest that phytochrome autophosphorylation and kinase activity may play a negative role in light signal transduction.

However, the *in vivo* functional mechanism of phytochrome kinase activity is only beginning to be understood. For example, phytochrome kinase activity is stimulated in the presence of histone H1 in a Pr-specific manner, thus it is suggested that phytochrome kinase activity is activated in the nucleus that contains cationic molecules such as histones (Yeh and Lagarias, 1998; Kim et al., 2005; Han et al., 2010). Another example showing the complexity of *in vivo* phytochrome-related kinase activity comes from the study of the PHYTOCHROME-INTERACTING FACTORS (PIFs; see below). All PIFs except PIF7 are rapidly phosphorylated and then ubiquitinated in response to light *in vivo* prior to their degradation in a phytochrome-dependent manner (Al-Sady et al., 2006; Shen et al., 2007; Shen et al., 2008). A recent study reported that both phyA and phyB mediate PIF3 phosphorylation *in vitro* (Phee et al., 2008), suggesting that phytochromes may be the protein kinases responsible for phosphorylating the PIF proteins. However, compelling evidence supporting this assumption is still lacking. Recently, it was shown that CASEIN KINASE II (CK2), a highly conserved and ubiquitous Ser/Thr kinase, directly phosphorylates PIF1 (Bu et al., 2011). Therefore, whether phytochromes (both phyA and phyB) might directly phosphorylate PIFs *in vivo*, and how CK2 functions in phytochrome-induced rapid phosphorylation of PIFs await further investigation.

PHYTOCHROME SIGNALING INTERMEDIATES

The light signals perceived by the phytochrome photoreceptors are transduced to downstream signaling intermediates, which alter the expression of target genes and ultimately lead to the modulation of the biological responses (Quail, 2002; Jiao et al., 2007). Genetic research has identified a complex and interconnecting signaling network downstream of the phytochromes, together with a considerable number of positive or negative regulators, which act either in a specific pathway (such as LAF1 and HFR1) or in all branches of phytochrome signaling pathways (such as COP1 and HY5; Figure 9).

Negative Regulators of Phytochrome Signaling

Phytochrome-Interacting Factors (PIFs). Protein-protein interactions are necessary for many signal transduction cascades. Both general screenings for phytochrome-interacting proteins and targeted protein-protein interaction studies have identified a number of phytochtome-interacting partners. Those include PIF3 and other subsequently identified PIFs (Ni et al., 1998; Leivar and Quail, 2011), PKS1 (Fankhauser et al., 1999), NDPK2 (Choi et al., 1999), cryptochromes (both CRY1 and CRY2) (Ahmad et al., 1998; Mas et al., 2000), AUX/IAA proteins (Colon-Carmona et al., 2000), FyPP (Kim et al., 2002), COP1 (Seo et al., 2004; Jang et al., 2010), PAPP5 (Ryu et al., 2005), FHY1/FHL (Hiltbrunner et al., 2005, 2006), and were summarized recently by Bae and Choi (2008). Growing evidence demonstrates that the PIF proteins, a small subset of basic helix-loop-helix (bHLH) transcription factors, play central roles in phytochrome-mediated light signaling networks (Duek and Fankhauser, 2005; Castillon et al., 2007; Leivar and Quail, 2011).

The PIF proteins belong to the 15-member Subfamily 15 of the Arabidopsis bHLH transcription factor superfamily (Bailey et al., 2003; Heim et al., 2003; Toledo-Ortiz et al., 2003). PIF3 is the foundation member of the PIF subset, initially identified in a yeast two-hybrid screen for phyB-interacting proteins (Ni et al., 1998). The second member of the PIF family, PIF4, was isolated by the convergence of both genetic and reverse-genetic approaches (Huq and Quail, 2002). Several other PIFs were then identified based on the sequence homology to PIF3, and were named PIF1, PIF5, PIF6 and PIF7 (Huq et al., 2004; Khanna et al., 2004; Oh et al., 2004; Leivar et al., 2008). As members of the bHLH superfamily transcription factors, all the PIF proteins contain a

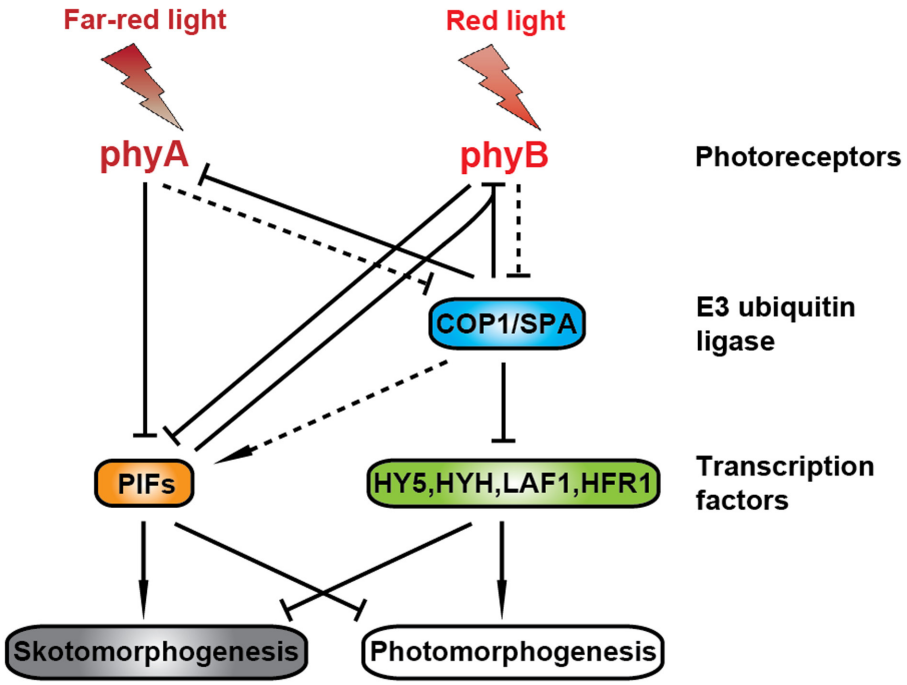

Figure 9. A simplified model of the phytochrome signaling pathway.

phyA is the primary photoreceptor responsible for perceiving and mediating various responses to FR light, whereas phyB is the predominant phytochrome regulating responses to R light. Under light conditions, these photoreceptors act to suppress two main branches of light signaling: COP1-TFs and PIFs. COP1, whose activity is repressed by phytochromes in light conditions, is an E3 ubiquitin ligase targeting several photomorphogenesis-promoting transcription factors (such as HY5, HYH, LAF1 and HFR1) for degradation. PIFs are a subset of bHLH transcription factors required for skotomorphogenesis. Photo-activated phytochromes directly interact with PIFs, resulting in PIFs' phosphorylation and degradation, while COP1 positively regulates PIFs' protein levels. Phytochromes are targeted for degradation by COP1, and PIFs contribute to the degradation of phyB by promoting COP1/phyB interaction. Arrow, positive regulation; bar, negative regulation; solid line, direct regulation; dotted line, indirect regulation.
Image adapted from Lau and Deng (2010). Reprinted with permission from Elsevier.

bHLH signature domain, consisting of a basic region (~ 15 aa) involved in DNA binding and an HLH region (~ 60 aa) involved in dimerization (Toledo-Ortiz et al., 2003; Castillon et al., 2007). The majority of the bHLH proteins bind to a *cis*-element called the E-box (CANNTG), whereas all the PIF proteins, where examined, bind specifically to a subtype of E-box, called the G-box (CACGTG) (Castillon et al., 2007; Leivar and Quail, 2011).

All PIF members contain a conserved motif in their N-termini, designated active phytochrome B-binding (APB) motif, which confers specific binding of PIFs to the biologically active Pfr form of phyB (Khanna et al., 2004; Duek and Fankhauser, 2005; Castillon et al., 2007; Leivar and Quail, 2011). However, only two PIF proteins, PIF1 and PIF3, also bind to the Pfr form of phyA, with PIF1 showing much stronger affinity for phyA than PIF3 (Ni et al., 1998; Huq et al., 2004). Accordingly, PIF1 and PIF3 each contain a motif called active phytochrome A-binding (APA) necessary for binding to phyA, but the actual sequences of these two APA motifs are not conserved (Al-Sady et al., 2006; Shen et al., 2008).

The recent finding that a quadruple mutant of PIFs, *pif1 pif3 pif4 pif5* (*pifq*), develops a *constitutively photomorphogenic* (*cop*)-like phenotype in the dark provides compelling evidence that the PIF proteins repress photomorphogenesis and promote skotomorphogenesis in etiolated seedlings (Leivar et al., 2008; Shin et al., 2009; Quail, 2011). Consistently, microarray analysis showed that the dark-grown *pifq* mutant has a gene expression pattern similar to that of R light-grown wild-type plants (Shin et al., 2009). By comparing rapidly light-responsive genes in wild-type seedlings with those responding in darkness in the *pifq* mutant, an overlapping subset of genes were identified as potential direct targets of these bHLH transcription factors (Leivar et al., 2009). Notably, transcription factor–encoding genes are highly enriched among these genes, suggesting that they may be potential primary targets of PIF transcriptional regulation.

At the same time, evidence obtained in the last decade demonstrates that one way phytochromes promote photomorphogenesis is by inducing rapid (within minutes) phosphorylation of most, if not all PIFs, upon light exposure. This subsequently leads to their ubiquitination and degradation via the ubiquitin/proteasome system (Bauer et al., 2004; Park et al., 2004; Shen et al., 2005a; Al-Sady et al., 2006; Oh et al., 2006; Nozue et al., 2007; Shen et al., 2007; Al-Sady et al., 2008; Lorrain et al., 2008; Shen et al., 2008). The interaction between phytochromes and PIFs is necessary for the light-dependent phosphorylation of PIFs, because mutant PIF1 and PIF3 proteins that abolish interactions with the Pfr forms of phyA and phyB do not undergo light-dependent phosphorylation (Al-Sady et al., 2006; Shen et al., 2008). Moreover,

upon returning light-grown plants to darkness, PIF proteins rapidly re-accumulate to high levels. Subsequent re-exposure to light once again induces rapid degradation of PIFs (Leivar and Quail, 2011), indicating that this rapid regulation of PIFs is dynamic and controlled by phytochrome LFRs. Therefore, phytochrome-induced phosphorylation and proteolysis of PIFs may represent a major biochemical mechanism of signal transfer from the photoactivated phytochromes to their interacting signaling partners in the nucleus, which rapidly alters the gene expression profiles of the genome. In addition to controlling phytochrome-regulated gene expression, PIF proteins were recently shown to also modulate phyB abundance in R light, possibly by stimulating COP1-catalyzed ubiquitination and degradation of phyB (see below) (Khanna et al., 2007; Leivar et al., 2008; Al-Sady et al., 2008; Jang et al., 2010; Figure 9).

COP/DET/FUS Proteins. Genetic screens for Arabidopsis mutants involved in light-regulated seedling development followed by biochemical analyses have identified a group of pleiotropic Constitutive Photomorphogenic/De-etiolated/Fusca (COP/DET/FUS) proteins that are central negative regulators of photomorphogenesis (Sullivan et al., 2003; Yi and Deng, 2005). This group of COP/DET/FUS proteins defines three biochemical entities: the COP1-SPA complexes, the COP9 signalosome (CSN), and the CDD complex (COP10, DDB1, and DET1), all of which are involved in proteasomal degradation of photomorphogenesis-promoting factors (Saijo et al., 2003; Serino and Deng, 2003; Yanagawa et al., 2004; Yi and Deng, 2005; Zhu et al., 2008). Interestingly, the COP1-SPA complexes and the CDD complex were recently shown to form two groups of CUL4-based E3 ligases *in vivo* (Chen et al., 2006, 2010). Therefore, these two groups of E3 ligases regulate the degradation of downstream factors to mediate light regulation of plant development (Chen et al., 2010).

COP1 is a conserved RING finger E3 ubiquitin ligase involved in multiple processes in many different organisms, including plant development and mammalian cell survival, growth, and metabolism (Yi and Deng, 2005). COP1 was first cloned and characterized in the model plant Arabidopsis as a repressor of light-regulated plant development (Deng et al., 1991, 1992; Figure 10). COP1 contains three domains: a RING finger domain in its N-terminal region, a WD40 repeat domain in its C-terminus, and a coiled-coil domain in the middle (Deng et al., 1992; Yi and Deng, 2005). COP1 has been shown to act as an E3 ligase targeting several photomorphogenesis-promoting proteins for degradation, including HY5 (Osterlund et al., 2000), HY5 HOMOLOG (HYH; Holm et al., 2002), LAF1 (Seo et al., 2003), HFR1 (Duek et al., 2004; Jang et al., 2005; Yang et al., 2005), and the phytochromes (Seo et al., 2004; Jang et al., 2010) (Figure 9). In addition, COP1 was recently shown to regulate flowering time by directly targeting transcriptional activator CONSTANS (CO) for degradation (Jang et al., 2008; Liu et al., 2008). Moreover, COP1 can interact with the substrate adaptor EARLY FLOWERING 3 (ELF3) to modulate light input signal to the circadian clock by destabilizing GIGANTEA (GI) protein (Yu et al., 2008).

SUPPRESSOR OF PHYA-105 (SPA1) was first identified as a repressor of phyA (Hoecker et al., 1998). Subsequent studies found three additional SPA1-like proteins in the Arabidopsis genome, named SPA2, SPA3, and SPA4 (Laubinger and Hoecker, 2003; Laubinger et al., 2004). Biochemical analysis demonstrated that SPA1 interacts with COP1 (Hoecker and Quail, 2001; Saijo et al., 2003; Seo et al., 2003), and interestingly, the SPA proteins can self-associate or interact with each other, forming a heterogeneous group of COP1/SPA complexes in Arabidopsis (Zhu et al., 2008). Genetic analysis showed that the four *SPA* genes are partially redundant in mediating light responses at both seedling and adult stages (Laubinger et al., 2004). Moreover, the quadruple *spa* mutant displays a phenotype similar to that of strong *cop1* alleles (Laubinger et al., 2004), consistent with the notion that the SPA proteins work in concert with COP1 in controlling photomorphogenesis.

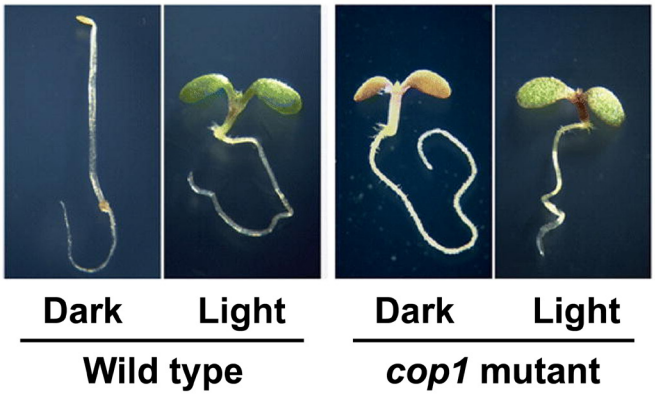

Figure 10. Dark-grown *cop1* mutant seedlings phenotypically mimic light-grown wild-type seedlings.

Empfindlicher Im Dunkelroten Licht 1 (EID1). EID1 is an F-box protein that functions as a negative regulator in phyA-specific light signaling (Buche et al., 2000; Dieterle et al., 2001). The fact that EID1 interacts with several Arabidopsis Skp1-like (ASK) proteins and Cullin1 suggests that EID1 is a component of a SCF (SKP1/Cullin1/F-box protein) ubiquitin ligase complex targeting positively acting component(s) of phyA signaling pathway to ubiquitin-dependent proteolysis (Dieterle et al., 2001; Marrocco et al., 2006). A unique feature of the *eid1* mutant is a shift in the peak of the action spectra of phyA-mediated hypocotyl elongation from FR to R part of the spectrum (Dieterle et al., 2001; Zhou et al., 2002). Although both EID1 and SPA1 function as negatively acting components in phyA-specific light signaling, mutant analysis indicated that EID1 and SPA1 have different but overlapping functions in phyA-dependent signal transduction chains (Zhou et al., 2002). Interestingly, a L946F mutation in the HKRD domain of phyA (named *phyA-402* allele) was found to suppress the hypersensitive phenotype of the *eid1-3* mutant (Muller et al., 2009). However, when *phyA-402* is introgressed into the wild-type background, only moderate phenotype was observed, indicating that the mutation mainly alters phyA functions in an EID1-dependent signaling cascade (Muller et al., 2009).

Positive Regulators of Phytochrome Signaling

HY5 and HYH. HY5, a constitutively nuclear bZIP protein, is the first known and most extensively studied transcription factor involved in promoting photomorphogenesis under a wide spectrum of wavelengths, including FR, R, B, and UV-B (Koornneef et al.,

1980; Oyama, et al., 1997; Osterlund et al., 2000; Ulm et al., 2004). It was shown that the abundance of HY5 protein is directly correlated with the extent of photomorphogenic development (Osterlund et al., 2000). Recent chromatin immunoprecipitation (ChIP)-chip studies revealed that HY5 binds directly to a large number of genomic sites, mainly at the promoter regions of annotated genes (Lee et al., 2007; Zhang et al., 2011). It seems that HY5 directly mediates both upregulation and downregulation of gene expression by light. The gene expression regulation attributable to HY5 is included largely within genes that are regulated by light and comprises ~20% of all light-regulated genes (Ma et al., 2002). Therefore, HY5 is likely to be a high hierarchical regulator of the transcriptional cascades involved in seedling photomorphogenesis (Lee et al., 2007).

COP1 is capable of directly interacting with HY5 in the nucleus through its WD40 repeat domain and targets HY5 for proteasome-mediated degradation (Ang et al., 1998; Osterlund et al., 2000). HY5 has a homolog in the Arabidopsis genome, named HYH, and interestingly, HYH was also shown to be a target of COP1 (Holm et al., 2002). HY5 and HYH physically interact with COP1 through a COP1-interaction motif (Holm et al., 2001, 2002). Consistent with the finding that COP1 forms protein complexes with the SPA proteins, SPA1 contributes to the down-regulation of HY5 abundance (Saijo et al., 2003). Multiple photoreceptors, including phytochromes and cryptochromes, promote the accumulation of HY5 under specific light conditions, possibly by reducing the nuclear abundance of COP1 (Osterlund and Deng, 1998; Osterlund et al., 2000). However, little is known as to how the light-activated photoreceptors regulate the activities of COP1, as well as other COP/DET/FUS proteins.

HFR1 and LAF1. HFR1, an atypical bHLH protein, was originally identified as a positive regulator specific to phyA signaling (Fairchild et al., 2000; Fankhauser and Chory, 2000; Soh et al., 2000). However, subsequent studies revealed that HFR1 is also a component of cry1-mediated B light signaling (Duek and Fankhauser, 2003). Thus, HFR1 may represent a point of signal integration from phyA and cry1, either as a convergence of two independent signaling pathways or as a result of interaction of phyA and cry1 at the photoreceptor molecule level (Ahmad et al., 1998). It was demonstrated that HFR1 is capable of forming homodimers as well as heterodimers with PIF3. However, in contrast to PIF3, HFR1 does not bind directly to either phyA or phyB, although the HFR1/PIF3 complex can bind preferentially to the Pfr form of both phyA and phyB (Fairchild et al., 2000). In addition, unlike the PIF proteins, HFR1 contains an atypical basic domain which might not be functional for directly binding to DNA (Fairchild et al., 2000; Heim et al., 2003). Consistent with this proposal, HFR1 was recently shown to prevent an exaggerated shade avoidance response by forming non-DNA-binding heterodimers with PIF4 and PIF5, two bHLH transcription factors directly regulating the expression of shade-responsive marker genes (Sessa et al., 2005; Lorrain et al., 2008; Hornitschek et al., 2009; Galstyan et al., 2011). Moreover, the finding that a stabilized version of HFR1 leads to a constitutively photomorphogenic phenotype in darkness (similar to that of the *pif1 pif3 pif4 pif5* quadruple mutants) suggest that HFR1 may function to sequester all these PIF proteins (Yang et al., 2003; Leivar et al., 2008; Shin et al., 2009).

LAF1 is an R2R3-MYB transcription factor with trans-activation activity, and functions as a positive component of phyA signaling (Ballesteros, et al., 2001). However, no direct target gene of LAF1 has been reported so far. The *hfr1 laf1* double mutant has an additive phenotype compared to the two single mutants, indicating that HFR1 and LAF1 regulate largely independent pathways (Jang et al., 2007). Interestingly, both HFR1 and LAF1 were found to be the targets of COP1's E3 ubiquitin ligase activity *in vitro*, and in both cases, genetic and physical interactions between HFR1/LAF1 and COP1 were also observed (Seo et al., 2003; Jang et al., 2005; Yang et al., 2005). Moreover, it was recently shown that HFR1 also physically interacts with LAF1, and this interaction stabilizes each other through inhibition of ubiquitination by COP1, thereby enhancing phyA photoresponses (Jang et al., 2007).

PHYTOCHROME CONTROL OF NUCLEAR GENE EXPRESSION

Microarray analyses conducted in the last decade revealed genome-wide gene expression profiles regulated by light. About 10% or so of the genes in the Arabidopsis genome display phytochrome-regulated changes in expression during the seedling de-etiolation transition triggered by initial exposure of etiolated seedlings to light (Tepperman et al., 2004, 2006; Quail, 2011). These genes include numerous photosynthetic genes related to the biogenesis of active chloroplasts, various auxin-, gibberellin-, cytokinin- and ethylene hormone pathway-related genes potentially mediating growth responses, and metabolic genes reflecting the transition from heterotrophic to autotrophic growth (Tepperman et al., 2004, 2006; Quail, 2011). It is believed that the changes in expression of this large number of light-responsive genes ultimately lead to various morphogenic changes during seedling de-etiolation. Significantly, among functionally classifiable early light-responsive genes responding within 1 hour of FR or R light exposure, 44% (for FR light) and 25% (for R light) encode transcription factors (Tepperman et al., 2001, 2004, 2006), suggesting that they may represent a master set of transcriptional regulators that orchestrate the expression of the downstream target genes in the phytochrome-directed transcriptional network.

Extensive studies have shed light on the mechanisms by which phytochromes regulate light-responsive gene expression. Firstly, phytochromes may promptly alter the expression of a large number of genes by inducing rapid phosphorylation and proteolysis of PIF transcription factors, as discussed above. Secondly, it is generally assumed that phytochromes rapidly inactivate the COP/DET/FUS proteins in response to light, which leads to the accumulation of photomorphogenesis-promoting transcription factors, such as HY5, HYH, LAF1 and HFR1, although the mechanisms governing this process are largely unknown (Figure 9). In addition, it has been proposed that the direct protein-protein interactions between phytochromes and COP1/SPA proteins might be responsible for the rapid, initial inactivation of COP1 activity, whereas long-term inactivation of COP1 is achieved by subsequent depletion of the molecule from the nucleus (Wang and Deng, 2003).

Moreover, it has been suggested that phytochromes might directly target light signals to the light-responsive gene promoters. phyB was shown to bind reversibly to G-box-bound PIF3 specifically upon light-triggered conversion of the photoreceptor to its biologically active Pfr form, thus suggesting a provocative model in which phytochromes may function as integral light-switchable

components of transcription regulator complexes, permitting direct targeting of light signals to target gene promoters (Martinez-Garcia et al., 2000; Quail, 2002). The direct interactions of PIF1 and PIF3 with phyA suggest that phyA could be targeted to gene promoters as well. However, conclusive evidence in favor of this model is still lacking. There is no evidence that phytochromes are associated with DNA *in vivo*, and that phytochromes indeed modulate transcription on the target gene promoters.

PHYTOCHROME SIGNALING AND THE CIRCADIAN CLOCK

The circadian clock controls many metabolic, developmental and physiological processes in a time-of-day-specific manner in both plants and animals (McClung, 2008; de Montaigu et al., 2010). Although circadian rhythms are endogenously generated, they can be modulated by external cues such as light and temperature, thus allowing plants to anticipate and adapt to daily and seasonal changes in their environment. Light signals perceived and transduced by phytochromes and cryptochromes ensure the clock is in tune with the external light/dark cycles. This process is known as entrainment. phyA, phyB, phyD, and phyE act as photoreceptors in R light input to the clock, while phyA and the cryptochromes cry1 and cry2 act as photoreceptors in B light input (Devlin and Kay, 2000). Interestingly, it was shown that phyA acts in low-intensity R light for circadian control, while phyB functions in high-intensity R light (Somers et al., 1998). In FR light, phyA is expected to be the only active photoreceptor transducing the light input to the circadian clock (Wenden et al., 2011). A recent report showed that Arabidopsis mutants deficient in all five phytochromes still displayed clock-controlled robust rhythmic oscillations of leaf position, indicating that phytochromes are not part of the core mechanism of the circadian clock and that other photoreceptors are sufficient for entrainment (Strasser et al., 2010). It should be noted that the LOV/F-box/Kelch-repeat family photoreceptors ZTL, LKP2 and FKF are also involved in the regulation of the circadian clock in Arabidopsis (Nelson et al., 2000; Somers et al., 2000; Schultz et al., 2001; Imaizumi et al., 2003; Mas et al., 2003; Somers et al., 2004; Kim et al., 2007; Demarsy and Fankhauser, 2009; Baudry et al., 2010).

The rhythm of leaf movement in the wild-type Arabidopsis plants can be reset by FR light, but this resetting was absent in the *phyA*, *fhy1* and *fhy3* mutants, suggesting that phyA signaling pathway is required for the entrainment of the circadian clock (Yanovsky et al., 2000, 2001). Interestingly, the *fhy3* mutant also shows an enhanced response to R light during seedling de-etiolation, and shows disrupted rhythmicity of central-clock and clock-output gene expression in continuous R light (Allen et al., 2006). Further, FHY3 is required for the clock resetting in response to R light pulses, suggesting that FHY3 functions in gating phytochrome signaling to the circadian clock (Allen et al., 2006). Recently, FHY3 and its homolog FAR1 were shown to bind directly to the promoter of *EARLY FLOWERING 4* (*ELF4*), a component of the central oscillator of Arabidopsis circadian clock (Li et al., 2011). Interestingly, HY5, a well-characterized bZIP transcription factor involved in promoting photomorphogenesis, and CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), two MYB-related transcription factors that are key components of the central oscillator, were also shown to bind directly to the *ELF4* gene promoter via their respective *cis*-elements. FHY3, FAR1 and HY5 activate *ELF4* expression during the day, whereas CCA1 and LHY suppress *ELF4* expression periodically at dawn. Thus, this set of light- and circadian-regulated transcription factors act directly and coordinately at the *ELF4* promoter to regulate its cyclic expression (Li et al., 2011). In addition, phyA itself is essential for the expression of *ELF4* (Khanna et al., 2003), and *ELF4* is a likely target mediating phyA-transduced FR light input to the circadian clock (Wenden et al., 2011). Moreover, phyA was shown to directly associate with FHY3 *in vivo* (Saijo et al., 2008). These data suggest that FHY3 may represent a potential molecular link directly gating the environmental light signals perceived by phytochrome to the circadian clock. It is also interesting to note that FHY3 directly binds to the promoter regions of other circadian genes such as *CCA1* and *CCA1 HIKING EXPEDITION* (*CHE*) (Li et al., 2011; Ouyang et al., 2011), while its co-regulator HY5 binds to the promoters of *CCA1*, *LHY*, *TOC1*, *ELF3* and *GI* (Lee et al., 2007). Thus, whether FHY3 could regulate other clock genes (such as *CCA1*) together with HY5 will be an intriguing question for future studies.

Another mechanism of gating the light input to the circadian clock, called the external coincidence model, has been proposed in the recent years. In Arabidopsis, *CONSTANS (CO)* plays a central role in the induction of flowering by long days (LDs) (Putterill et al., 1995; Robson et al., 2001). *CO* encodes a nuclear protein containing zinc fingers, and activates the transcription of floral regulators such as *FLOWERING LOCUS T* (*FT*) in the light. The mRNA level of *CO* is tightly controlled by the circadian clock and shows a striking temporal pattern of expression. Under LDs *CO* mRNA peaks before dusk and stays high until the following dawn, whereas under short days (SDs) *CO* mRNA peaks during the night (Suarez-Lopez et al., 2001). Under these conditions, when *CO* mRNA expression coincides with the exposure of plants to light, photoreceptors enhance the level and activity of the CO protein (Valverde et al., 2004). CO then triggers flowering by activating *FT* transcription. Genetic experiments demonstrate that photoreceptors cry1, cry2 and phyA promote flowering and stabilize the CO protein, whereas phyB delays flowering and promotes the degradation of CO (Johnson et al., 1994; Guo et al., 1998; El-Assal et al., 2001; Yanovsky and Kay, 2002; Cerdan and Chory, 2003; Searle and Coupland, 2004; Valverde et al., 2004). Therefore, the coincidence between *CO* mRNA and exposure to light (thus CO stability could be regulated post-transcriptionally by photoreceptors) is required to promote flowering.

On the other hand, several clock-related genes are involved in phytochrome signaling. For example, *GIGANTEA* (*GI*) encodes a novel protein found only in plants, and regulates flowering, circadian rhythms, and seedling photomorphogenesis under continuous R and B light (Araki and Komeda, 1993; Fowler et al., 1999; Park et al., 1999; Huq et al., 2000; Tseng et al., 2004; Mizoguchi et al., 2005; Martin-Tryon et al., 2007). Interestingly, although GI does not affect the phyA HIRs, it does affect phyA-mediated VLFRs via mechanisms that do not obviously involve its circadian functions (Oliverio et al., 2007). ELF3 is a highly conserved plant-specific nuclear protein which has been suggested to be part of the central clock oscillator and to act as a link between light and the circadian clock (Hicks et al., 1996; Zagotta et al., 1996; McWatters et al., 2000; Covington et al., 2001; Hicks et al., 2001; Liu et al., 2001; Thines and Harmon, 2010). Like *phyB*

mutants, both *gi* and *elf3* mutants display elongated hypocotyls in R light (Zagotta et al., 1996; Huq et al., 2000; Reed et al., 2000). However, the *gi* mutants are late flowering, which is in contrast with the early flowering phenotype of the *phyB* and *elf3* mutants, suggesting that ELF3 and GI play different roles or use different mechanisms in controlling hypocotyl elongation and flowering responses. Interestingly, ELF3 was shown to interact with both phyB and GI (Liu et al., 2001; Yu et al., 2008). In addition, ELF3 may act as an adaptor protein allowing COP1 to interact with GI which leads to GI degradation (Yu et al., 2008).

PHOSPHORYLATION/DEPHOSPHORYLATION AND DEGRADATION OF PHYTOCHROMES

Phosphorylation and Dephosphorylation of Phytochromes

It is well established that phytochromes are phosphoproteins, because they could be readily labeled with ^{32}P isotope *in vivo* (Wong et al., 1986; Biermann et al., 1994). The phosphorylation sites of oat phyA have been investigated since the 1980s. There are two phosphorylation sites (Ser8 and Ser18) in the NTE region, and one site (Ser599) in the hinge region of oat phyA, among which, Ser8 and Ser599 were confirmed as *in vivo* phosphorylation sites (Wong et al., 1986; McMichael and Lagarias, 1990; Lapko et al., 1997, 1999). Ser8 is constitutively phosphorylated in both the Pr and Pfr forms, whereas Ser599 phosphorylation is light dependent and is preferentially phosphorylated in the Pfr form *in vivo* (Lapko et al., 1997, 1999).

The observation that phytochromes are phosphoproteins suggests the existence of protein kinase(s) and phosphatase(s) responsible for phosphorylating and dephosphorylating phytochromes. However, despite the extensive investigations of phytochrome-interacting proteins, there is no report thus far of a protein kinase that can specifically phosphorylate phytochromes. Instead, as mentioned above, two phosphorylation sites in the NTE region of oat phyA, i.e. Ser8 and Ser18, are autophosphorylated by phyA itself *in vitro* (Han et al., 2010). Mutations of these two serines to alanines effectively abolished autophosphorylation of oat phyA *in vitro* (Han et al., 2010). However, another phosphorylation site of oat phyA, Ser599, is not autophosphorylated by phyA (Kim et al., 2004; Han et al., 2010). Therefore, the protein kinase(s) responsible for phosphorylating this site of oat phyA will be a major interest for future studies.

In contrast to the failure of identifying a phytochrome-associated kinase, there are several studies reporting the discovery of protein phosphatases that could dephosphorylate phytochromes. A phytochrome-associated protein phosphatase 2A, designated FyPP, was shown to interact with and dephosphorylate phyA (Kim et al., 2002). The transgenic Arabidopsis plants with overexpressed or suppressed FyPP levels exhibited delayed or accelerated flowering, respectively, indicating that FyPP modulates phytochrome-mediated light signaling in the timing of flowering (Kim et al., 2002). PHYTOCHROME-ASSOCIATED PROTEIN PHOSPHATASE 5 (PAPP5) was isolated from yeast two-hybrid screen using the full-length Arabidopsis phyA as bait (Ryu et al., 2005). PAPP5 interacts with both phyA and phyB, and dephosphorylates all three phosphor-serine residues of oat phyA in a Pfr-specific manner. It was shown that PAPP5 positively influences the protein stability of phytochrome and the interaction of phytochrome with a downstream signal transducer NDPK2 (Ryu et al., 2005). Another phosphatase, PHYTOCHROME-ASSOCIATED PROTEIN PHOSPHATASE 2C (PAPP2C), was recently shown to interact with both phyA and phyB and effectively dephosphorylate phytochromes *in vitro* (Phee et al., 2008).

What are the *in vivo* functional roles of phytochrome phosphorylation and dephosphorylation? Recent studies suggest two possible roles: controlling phytochrome stability, and regulating interactions of phytochromes with downstream signal transducers. In the last two decades, great efforts have been made to investigate the role of a stretch of serine residues (including Ser8 and Ser18 of oat phyA) in the first 20 amino acids of phyA proteins, and it has been well-documented that substitution of these serines to alanines, or deletion of this 20-aa region, results in an increased biological activity of phyA, suggesting that these serine residues are involved in desensitization of phyA signaling (Stockhaus et al., 1992; Jordan et al., 1996, 1997; Casal et al., 2002; Han et al., 2010). Recently it was shown that the increased activity of mutated phyA proteins is related to the increased phyA stability (Han et al., 2010). Therefore, it is likely that autophosphorylation of serine sites in the NTE of phyA controls phyA stability.

However, phosphorylation of another serine residue, Ser599 in the hinge region of oat phyA, does not affect phytochrome stability (Kim et al., 2004). Instead, it was shown that phosphorylation of Ser599 prevents the interaction of phyA with its signal transducers such as NDPK2 and PIF3, suggesting that phosphorylation of Ser599 serves as a signal modulating switch affecting protein-protein interactions between phytochrome and its signal transducers (Kim et al., 2004). Interestingly, an earlier study showed that if Ser599 was mutated (Ser599Lys), phyA autophosphorylation and phosphorylation of its substrate PKS1 were no longer regulated by light (Fankhauser et al., 1999), suggesting that Ser599 plays an important role in the regulation of phytochrome kinase activity as well.

A recent study firstly detected an *in vivo* phosphorylated form of Arabidopsis phyA protein (Saijo et al., 2008). It was shown that phosphorylated phyA preferentially associates with COP1/SPA1 complex for degradation, whereas unphosphorylated phyA predominantly associates with phyA signaling intermediates FHY1 and FHY3 for signal transduction. Interestingly, COP1 associates with unphosphorylated phyA in the absence of FHY1 or FHY3, suggesting that phyA associations with FHY1 and FHY3 protect unphosphorylated phyA from being recognized by COP1/SPA1 complex (Saijo et al., 2008). Therefore, these data suggest that light-induced phyA phosphorylation *in vivo* acts as a switch controlling differential interactions of the photoreceptor with signal propagation or attenuation machineries. However, how many phosphorylation sites contribute to produce this phosphorylated form of Arabidopsis phyA protein needs to be further characterized.

Degradation of Phytochromes

As mentioned above, phyA is a light labile phytochrome. phyA is most abundant in etiolated seedlings, but its level drops up to 100-fold after exposure to light (Clough and Vierstra, 1997;

Sharrock and Clack, 2002). The proteolysis of phyA following photoconversion from Pr to Pfr is rapid, as the Pr form has a half-life of approximately 1 week, whereas the Pfr form has a half-life of only 1-2 h (Clough and Vierstra, 1997). It was observed more than two decades ago that phyA is ubiquitinated *in vivo*, and is degraded by the ubiquitin/26S proteasome pathway (Shanklin et al., 1987; Jabben et al., 1989a, b; Clough and Vierstra, 1997).

However, the E3 ligase responsible for phyA ubiquitination had not been identified until 2004, when COP1 was shown to ubiquitinate phyA *in vitro* (Seo et al., 2004). COP1 and phyA colocalize in the nuclear bodies, and the two proteins physically interact with each other mediated by the PRD domain of phyA and the WD40 domain of COP1. Consistently, the degradation rate of phyA is decreased in *cop1* mutants (Seo et al., 2004). This finding was extended by a later report that the light signaling repressors, SPA proteins, contribute to COP1-mediated degradation of phyA, and that a COP1/SPA1 protein complex is tightly associated with phyA ubiquitination activity (Saijo et al., 2008). Consistent with the notion that phyA phosphorylation status controls its protein stability, the phosphorylated phyA form was suggested to be a preferred target for COP1-mediated degradation (Saijo et al., 2008).

Moreover, it is notable that light-induced phyA degradation still occurs in the null *cop1-5* allele, suggesting the presence of a COP1-independent phyA-degradation pathway (Saijo et al., 2008). Consistent with this assumption, it was observed long ago that a R light pulse promotes the rapid formation of phyA-containing cytosolic spots, referred to as sequestered areas of phytochormes (SAPs), which appear prior to the nuclear transport of phyA and is thought to be the place of phyA ubiquitination and degradation (Speth et al., 1986; Nagatani, 2004; Kevei et al., 2007). The cytosolic degradation of phyA is also supported by the recent reports that phyA is still degraded in the *fhy1 fhl* double mutants, in which phyA is maintained in the cytosol, although at a slower rate than in the wild-type plants (Rosler et al., 2007; Debrieux and Fankhauser, 2010).

Although the type II phytochromes (phyB-phyE in Arabidopsis) are described as “light stable”, they are slowly degraded upon irradiation with R light (Sharrock and Clack, 2002). Recently, a report showed that phyB is stable in darkness, but in R, a fraction of phyB translocates into the nucleus and becomes degraded by the ubiquitin/26S proteasome pathway (Jang et al., 2010). Nuclear phyB degradation is also mediated by COP1 E3 ligase, which preferentially interacts with the N-terminal region of phyB. Interestingly, phyB polyubiquitination by COP1 *in vitro* can be enhanced by different PIF proteins that promote COP1/phyB interaction. Consistent with these results, nuclear phyB accumulates to higher levels in *pif* and *cop1* mutants. Moreover, COP1 was shown to interact with and ubiquitinate phyC-phyE *in vitro* (Jang et al., 2010). Therefore, COP1 is an E3 ligase for all five phytochromes in Arabidopsis.

CYTOPLASMIC EVENTS OF PHYTOCHROME SIGNALING

Besides nuclear signaling events, phytochrome signaling likely entails cytoplasmic events based on the following reasons. Firstly, as mentioned above, the nuclear import of some phytochrome species (such as phyB) takes hours, and their nuclear actions obviously could not explain some rapid phytochrome responses which occur within minutes of light irradiation, such as the change in hypocotyl growth rate (Parks and Spalding, 1999). Secondly, phytochromes interact with a number of cytoplasmic proteins, such as PKS1 and NDPK2 (Choi et al., 1999; Fankhauser et al., 1999). Thirdly, genetic studies have identified several cytoplasmic proteins as signaling intermediates, such as PAT1 and FIN219 for phyA signaling (Bolle et al., 2000; Hsieh et al., 2000).

The fastest phytochrome response described so far is the cytoplasmic streaming of *Vallisneria gigantea*, which can be locally stimulated with pulsed R light and becomes measurable within 2.5 s, and is likely mediated by phyB-type phytochromes (Takagi et al., 2003). As FHY1/FHL are indispensible for phyA nuclear accumulation (see above), the cytoplasmic responses regulated by phyA were identified recently using the *fhy1 fhl* double mutant (Rosler et al., 2007). The phyA-specific cytoplasmic responses include the R-enhanced phototropism, abrogation of gravitropism in B, and the inhibition of hypocotyl elongation in B (Rosler et al., 2007). It is noteworthy that the latter two cytoplasmic responses of phyA occur in B light, which might be explained in part by a recent observation that phyA coordinates the localization and distribution of PHOT1 (Han et al., 2008).

Previous microinjection and pharmacological studies suggested the involvement of G proteins, cGMP and Ca^{2+}/calmodulin in phytochrome signaling (Bowler et al., 1994; Neuhaus et al., 1997). In particular, it was reported that transgenic Arabidopsis plants conditionally overexpressing the α subunit (encoded by *GPA1* gene) of the heterotrimeric G protein under the control of a glucocorticoid-inducible promoter exhibited a light-dependent hypersensitive response as a result of reduced hypocotyl cell elongation (Okamoto et al., 2001). However, a separate study reported that loss-of-function *gpa1* mutants display partial de-etiolation in the dark, with short hypocotyls and open apical hooks typical of light-irradiated seedlings (Ullah et al., 2001). The short hypocotyl of *gpa1* seedlings was reportedly due to a defect in cell division, but not cell elongation (Ullah et al., 2001). To resolve these discrepancies regarding GPA1 function in photomorphogenesis, Jones et al. (2003) re-evaluated the roles of the heterotrimeric G protein employing multiple alleles of *gpa1* and *agb1* (impaired in the β subunit) and their double mutants. They concluded that these mutants have wild-type sensitivity to R and FR light. In addition, there is no apparent alteration in R or FR sensitivity in transgenic plants overexpressing GPA1 or AGB1. The observed shorter hypocotyls and partially open cotyledons of *gpa1* and *agb1* mutants grown in the dark are mainly caused by a defect in cell division, rather than cell elongation (Jones et al., 2003).

Genetic studies have also provided supporting evidence for the involvement of Ca^{2+} in phytochrome signaling. The *sub1* mutant exhibits hypersensitive responses to both FR and B light (Guo et al., 2001). The *SUB1* gene was found to encode a Ca^{2+}-binding protein. Genetic interaction studies suggest that SUB1 is a component of cryptochrome signaling pathway and is a modulator of phyA signaling pathway. Further, SUB1 negatively regulates HY5, a photomorphogenesis-promoting transcription factor (Guo et al., 2001).

FUTURE ISSUES

The last two decades have seen significant progress in unraveling the signaling mechanisms of plant phytochromes. However, several important questions about phytochromes still remain to be answered:

1. We still do not understand the earliest signaling events following photo-activation. Which activities of phytochromes are sufficient to trigger light responses?
2. Further to the first question, are phytochromes light-regulated kinases? If so, which domain is the kinase domain? What is the biological role of this kinase activity in inducing light responses?
3. How do phytochromes induce the phosphorylation of PIFs? How are PIFs degraded?
4. How are nuclear bodies formed? What are their precise functions in phytochrome signaling?
5. Are phytochromes associated with the target gene promoters to directly regulate their gene expression?
6. How do phytochromes inactivate the COP1/SPA protein degradation machinery upon photo-activation?
7. How are phytochromes phosphorylated? In addition to autophosphorylation, which kinase(s) are responsible for phosphorylating phytochromes?
8. How are phytochromes degraded in the nucleus and in the cytosol?

Further investigation and ultimate elucidation of these questions and others will undoubtedly shed more light on phytochrome signaling mechanisms and photomorphogenesis in general.

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