Phytochrome: A serine kinase illuminates the nucleus!

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Plants detect ambient light environment for their optimal growth and survival using a battery of photoreceptors, of which phytochrome is the most prominent. Phytochrome acts as a serine kinase and can autophosphorylate itself or transphosphorylate its partner proteins. The exposure of light to plants triggers translocation of phytochrome from the cytosol to the nucleus where it interacts with several proteins, including transcriptional factors. It is now believed that many photoresponses in plants may be mediated by direct regulation of transcription by phytochrome in conjunction with transcription factors.

PLANTS being sessile organisms need stringent mechanisms to overcome changes in their ambient environment. To optimize their survival, the higher plants have evolved several mechanisms to sense environmental changes. Among these, the mechanism to sense light is the most elegant. Plants can detect almost all facets of light such as direction, duration, spectral quality, quantity, solar angle, etc. This feat is achieved by plants by evolving specialized photoreceptor molecules that allow them to sense light throughout the visible spectrum. The examination of spectral sensitivity of different plant photoresponses has revealed that these responses are basically elicited using three specific wavebands of the solar spectrum: UV-B (280–320 nm), UV-A/blue (320–500 nm), and red (R)/far-red light (FR) (600–750 nm)[1]. The extensive studies conducted on the physiology of these photoresponses have divulged that each of these wavebands is sensed by a specific group of photoreceptor(s) (Table 1).

Many of the photoresponses elicited by the red/far-red waveband show a unique feature, i.e. the reversibility of light action. Several photoresponses such as red-light-induced seed germination can be reversed, if followed immediately by a far-red irradiation. This characteristic photoreversibility of response aided researchers to purify and characterize the responsible photoreceptor molecule – phytochrome. In fact, for several years, phytochrome was the only plant photoreceptor that had been purified and characterized. In the plant kingdom, phytochrome is ubiquitously present in all species[2], and in cyanobacteria[3]. The distribution of phytochrome has now expanded beyond photosynthetic organisms with the discovery of phytochrome-like proteins in the eubacteria *Deinococcus radiodurans* and *Pseudomonas aeruginosa*[4]. Phytochrome acts as a light-regulatory switch throughout the plant life cycle, right from seed germination to vegetative and reproductive development till senescence. It also enables the perception of shade and detection of neighbouring plants.

The discovery of phytochrome in the early fifties was soon followed by several hypotheses about its likely mode of action in regulating photomorphogenesis. In many cases, the investigators sought a strict correlation with the photoreceptor level and the final photoresponses, and responses deviating from this were termed as 'phytochrome paradoxes'. However, the results obtained in the past decade using a combination of physiology, genetics and molecular biology have led to a totally new perspective in our understanding of the mode of phytochrome action in higher plants. The present review summarizes the current status of the mode of action of phytochrome.

Molecular and physiological properties of phytochrome

The realization that phytochrome accumulates in bulk in dark-grown seedlings allowed its purification with relative ease due to lack of photosynthetic pigments in the seedlings. Based on various physiological evidences, it was predicted that phytochrome exists *in vivo* in two photoreversible forms[5]. The purification of phytochrome confirmed this view and showed that in dark-grown plants, phytochrome is present in the Pr form. On exposure to red light, the Pr form is converted to the Pfr form, which is considered as the biologically active form. The Pfr form on absorption of far-red light is converted back to the Pr form. This photoconversion of phytochrome is correlated with the change in absorption maxima of these two forms: the purified phytochrome in the Pr form is blue in colour and absorbs maximally at 666 nm, whereas the Pfr form is olive-green in colour and absorbs maximally at 730 nm[6].

Phytochrome is a chromoprotein similar to algal biliproteins, with a molecular mass of ~120 kDa. The

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Table 1. Typical photoresponses regulated by different photoreceptors

Wavelength (nm)	Photoreceptor	Molecular properties	Functions
600–750	Phytochrome(s)	Serine kinase with linear tetrapyrrole as chromophore	Photoperiodism, seed germination, shade avoidance
	Cryptochrome(s)	Flavoprotein, resembles microbial DNA photolyase	Photoperiodism, hypocotyl elongation
320–500	Phototropin	Serine/threonine kinase with flavin as chromophore	Phototropism
	Rpt	Novel protein with putative NLS and phosphorylation site	Phototropism
280–320	UV-B photoreceptor	Not known	Flavonoid biosynthesis

open-chain tetrapyrrole chromophore called as phytochromobilin (PB) is linked to the protein moiety through a cysteine residue[6]. The synthesis of phytochromobilin uses heme as the first step, which is converted by the action of heme oxygenase[7,8] to biliverdin IX. The biliverdin IX is converted to 3E-phytochromobilin by PB synthase, and finally an isomerization forms 3Z-phytochromobilin.

The phytochrome molecule exists as a dimer *in vivo* with each monomer having a single chromophore. The photoreversibility of the molecule can be attributed to *cis–trans* isomerization of the chromophore, which also causes changes in the protein conformation generating the Pr and Pfr forms[6]. Physiological and biochemical analyses of light- and dark-grown plants suggested the existence of two different pools of phytochrome species: a light-labile species predominantly present in dark-grown plants and a light-stable species characteristic of light-grown plants. Physiological studies led to the recognition that many of the photoresponses, though elicited by red or far-red light, lack photoreversibility. Additionally, several photoresponses in plants can be mediated by extremely low fluence of light, whereas many other photoresponses require continuous irradiation with high fluence red or far-red light. The diversity of the photoresponses elicited by phytochrome led to the speculation that the variation is due to existence of multiple phytochrome species[9].

The approaches using newer tools and molecular-genetic analyses of photoresponses confirmed the presence of multiple species of phytochrome. These studies first conducted in *Arabidopsis* revealed that phytochrome is encoded by a small multigene family. In this plant at least five phytochrome genes (*PHY)* have been identified, namely *PHYA, PHYB, PHYC, PHYD* and *PHYE* encoding distinct apoproteins[10,11]. Among these, the *PHYA* gene encodes a phytochrome species, which accumulates predominantly in etiolated seedlings, and is rapidly downregulated on exposure to light. The remaining phytochrome species are relatively more stable in light and therefore are predominantly present in lightgrown mature plants. Based on biochemical and physiological properties, these phytochrome species can be grouped in two types[12]. The type 1 (light-labile) is found in etiolated seedlings and is phytochrome A that accumulates in bulk in the seedlings. The red-light exposure triggers decline of phytochrome A by inhibiting its transcription, and degradation of its mRNA. In addition, the Pfr form of phytochrome A protein is also quickly degraded by ubiquitination. The remaining phytochrome species, i.e. phytochrome B to phytochrome E constitute the type 2 (light-stable) phytochrome, which is the predominant pool of phytochrome in green plants. Unlike type 1, the type 2 phytochromes are more stable and their levels do not decline on light exposure.

Since type 2 phytochrome species are present in very low amounts in plants, these have not been purified to homogeneity using conventional biochemical techniques, except phytochrome B. The phytochrome B, purified from transgenic *Arabidopsis*, showed spectral properties similar to phytochrome A[13]. Although plants synthesize and use phytochromobilin (PB) as chromophore *in vivo*, phytochrome apoprotein can also covalently bind and use phycocyanobilin (PCB) as chromophore[14]. This finding has allowed the constitution of photoreversible phytochromes by expressing recombinant phytochrome proteins in yeast and assembling them *in vitro.* A recent study has shown that the yeast-assembled phytochrome C has 661/725 nm and phytochrome E has 670/724 nm as the red/far-red absorption maxima[15]. The spectral characteristics of phytochromes C and E were found to be very different from those of phytochromes A and B.

Role of different phytochrome species in plant development

The realization that different photoresponses have different requirements for light, followed by discovery of multiple phytochrome species indicated that these phytochrome species may have distinct and overlapping function in plant development[16,17]. The mutant studies showed that phytochrome A is essential for de-etiolation of seedlings in far-red light and mediates

photoresponses to very low fluences of blue, red and far-red light. Nevertheless, phytochrome B is the major phytochrome species regulating many of red/far-red light reversible responses, whereas phytochrome D and E probably aid phytochrome B action. This is evident because the function of phytochrome D and E can be seen only in phytochrome B mutant. It also illustrates redundancy between phytochromes B, D and E, with phytochrome B being the main regulatory molecule[18–20]. The genetic analysis of *Arabidopsis* phytochrome mutants has shown that the major phytochrome is phytochrome B. The mutations in *PHYB* gene have substantial effects on *Arabidopsis* development throughout the life cycle[21]. So far, no phytochrome C mutant has been isolated; nevertheless, the overexpression of phytochrome C in transgenic plants suggests a role in primary leaf expansion[22]. The analysis of photomorphogenic mutants has highlighted a complex network of interactions not only among phytochromes, but also with other photoreceptors such as cryptochromes and phototropin.

Is Pr the active form of phytochrome?

The proposal that the Pr form of phytochrome may also be the biologically active form had been made several times with equally vehement rejections by others. Since in dark-grown plants phytochrome accumulates in the Pr form, it is presumed to be biologically inactive. At the same time, the realization that several of phytochrome A mediated responses can be induced by far-red light is inconsistent with the idea that Pfr is the active form, at least for phytochrome A. Now a possible explanation has emerged from the finding that the Pr form of phytochrome A after undergoing photo-cycling between the Pr and Pfr forms, shows greater autophosphorylation activity. In contrast, the non-cycled Pr form, which is present in dark-grown tissue, shows less amount of autophosphorylation[23]. It is likely that this actively cycled Pr species may be responsible for far-red mediated effects of phytochrome A[23]. This notion is supported by recent experiments of Shinomura *et al.*[24] demonstrating that brief cyclic pulses of far-red light with three-minute intervals inhibit hypocotyl elongation to the same extent as continuous far-red light. Since the response is reversible by red light and inducible by far-red light, it is most likely mediated by a form other than Pfr. In all likelihood, this active form is the Pr form of phytochrome that was generated by far-red pulses. It is expected that more experiments on this line would resolve this issue.

Phytochrome is a light-regulated kinase

Soon after the discovery of phytochrome, the Beltsville group proposed that it might be an enzyme[25]. However, the above group quickly abandoned this view and favoured the idea that phytochrome may act by regulating membrane properties[26]. The former view was rekindled by Lagarias who proposed that purified phytochrome has a protein kinase activity[27]. But controversy erupted again when this finding was quickly contested by other researchers who could not find a kinase activity in phytochrome preparations or could separate both the activities biochemically[28]. The analysis of phytochrome A amino acid sequence also did not show any motif for a typical protein kinase[28a].

However, comparison of gene sequences of phytochrome species with other known proteins led to the proposal that phytochrome C has motifs similar to bacterial sensor proteins of the 'two-component' regulatory system[29]. Bacteria constantly regulate their physiology and behaviour to respond and adapt to external environment and a typical 'two-component' regulatory system consists of a sensor protein and a response regulator protein[30]. The sensor protein detects a change in the external environment and communicates this information to the response regulator protein, which in turn either controls the expression of specific genes or initiates other appropriate cellular functions to respond to environmental stimuli. The communication between the sensor protein and the response regulator protein occurs via phosphorylation–dephosphorylation steps. It is well known that these sensor proteins act as histidine kinases, autophosphorylating themselves. The phosphate group is then transferred to a regulator molecule, leading to a cascade of events that modulate gene expression. This finding led to an intensive re-examination of phytochrome sequence to deduce the putative motifs for kinase activity.

It is believed that phytochrome has evolved from the biliproteins present in cyanobacteria. The sequencing of the complete genome of *Synechocystis*, a unicellular colony-forming cyanobacterium[31], facilitated examination of the presence of a phytochrome-like open reading frame in this cyanobacterium. The phytochrome-like *Cph1* gene from *Synechocystis PCC6803* was discovered by homology search of the above database. The deduced amino acid sequence of *Cph1* gene product showed several similarities with higher plant phytochrome, particularly towards the C-terminal domain, which has a histidine kinase motif. Later *in vitro* studies showed that the Cph1 holoprotein can indeed act as a R/FR-dependent histidine autokinase and transphosphorylate an aspartate residue of Rcp1 regulator molecule, which is encoded 15 bases downstream of *Cph1* gene on the *Synechocystis* chromosome. Both autophosphorylation and transphosphorylation activities were higher in the Pr form than in the Pfr form[3,32]. This finding lent strong support to the view that phytochrome protein may have autokinase and phosphotransferase activities, characteristics of transmitter molecules.

The finding mentioned above stimulated a fresh search for kinase activity in higher plant phytochromes. The new results suggest that these phytochromes also function as kinases. Their C-terminal signalling ends consist of two repeats of a domain called the PAS and another histidine kinase-related domain, which bears weak similarity to the histidine kinase domain of cph1. Studies on site-directed mutagenesis of higher plant phytochrome genes have revealed that the phytochrome molecule consists of two major parts: a highly conserved photosensory domain at the N-terminus and a C-terminal domain, which plays a key role in onward transmission of the light signal. However, the higher plant phytochromes do not show a histidine kinase motif at their C-terminus, similar to the cyanobacterium Cph1 protein. Nevertheless, purified or recombinant oat phytochrome A can autophosphorylate as well as phosphorylate histone H1 (ref. 23). It can also phosphorylate the cph1 substrate. However, it is autophosphorylated on serines rather than on a histidine, which is the case for cyanobacterial phytochrome. Likewise, it phosphorylates serines or threonines on the substrates rather than any aspartate residue. Based on biochemical analysis of recombinant phytochromes, it was suggested that during the course of evolution the cyanobacterial phytochrome has been modified, yielding a higher plant phytochrome species that functions as a serine/threonine kinase. In case of higher plants, phytochrome autophosphorylation is somewhat higher in the Pfr form. The cycling of phytochrome between Pr and Pfr increases the autophosphorylation activity of Pr. It has also been found that phytochrome A has at least two *in vivo* phosphorylation sites and in light grown plants one of these sites is preferentially phosphorylated.

Phytochrome A and B migrate to nucleus on activation by light

The experiments on phytochrome localization using the technique of subcellular fractionation always favoured the view that it is predominantly located in the cytosol of the plant. In contrast physiological studies on *Mougeotia* supported the view that phytochrome is either bound or is in close proximity of the membrane[33], whereas Arthur Galston[34] detected phytochrome in pea nuclei by microspectrophotometry. These subcellular locations were subsequently not favoured as potential sites for phytochrome localization and phytochrome was believed to be a cytosolic protein. However, the notion that phytochromes reside in the cytosol turned out to be only half-true because, when activated by light, phytochromes do accumulate inside the nucleus. The recent advances in gene technology have permitted a re-examination of the question of phytochrome localization by using fusion proteins. The study of intracellular localization of phytochrome B by fusing it to GUS reporter protein showed the presence of phytochrome B in the nucleus[35]. These results indicated that the C-terminal region of phytochrome B possesses a functional nuclear localization signal. That phytochrome B indeed moves to the nucleus was confirmed by immunoblot analysis of isolated nuclei. Since the level of phytochrome B is higher in the nucleus of light-grown plants than in the nucleus of dark-grown plants, it indicates that the migration of phytochrome B to the nucleus is light dependent. The dark-adaptation of plants reduces the level of phytochrome B in the nucleus, confirming that nuclear localization of phytochrome requires light[36,37]. In fact, the phytochrome B level in the nucleus isolated from dark-grown plants is below the detection limit; however, a brief pulse of red light triggers accumulation of phytochrome B in the nucleus. Since far-red light causes a decrease in the level of phytochrome B from the nucleus, this process appears to be autoregulated by phytochrome. This is further evident from the observation that a mutated phytochrome B lacking a chromophore-binding site, which is needed for photoreversibility, failed to enter the nucleus[37]. These fusion proteins were biologically active as transgenic plants showed enhanced light responses. However, the accumulation of phytochrome B in the nucleus was very slow and required several hours after transfer from dark to light.

Similarly, phytochrome A too is localized to the nucleus on light exposure. In accordance with the molecular properties of phytochrome A, its nuclear localization was promoted by very low fluences of either red light or by far-red light, a characteristic of physiological responses regulated by phytochrome A[37]. Interestingly, phytochrome A fusion protein was detected in the nucleus after about 15 min compared to phytochrome B that takes several hours to accumulate in the nucleus[36,37]. Thus, both phytochrome species move to the nucleus in their active Pfr form, and this nuclear migration may be linked to their capacity to regulate light-mediated gene expression in the plants.

Phytochrome activation involves associations with partner proteins

Protein–protein interactions are necessary for many signal transduction cascades. It is reasonable to expect that phytochrome also interacts with some partner protein(s) to pass on information about light environment in the cells. The presence of kinase activity in phytochrome and its migration to the nucleus suggests that, on activation, phytochrome may phosphorylate proteins and/or it may also take a partner to the nucleus. Such interacting partners for phytochrome were searched using yeast two-hybrid screens with sequences from signalling regions of phytochrome molecules as baits. The phyto-

chrome interacting factor 3 (PIF3), a basic helix–loop–helix protein, was isolated using the entire C-terminal domain of phytochrome B as bait. PIF3 interacts *in vitro* with C-terminal domains of both phytochromes A and B[38]. The binding of phytochrome B shows red/far-red reversibility, with phytochrome B binding to PIF3 only in its active Pfr form[39]. This reversible binding is also consistent with the fact that the phytochrome B is involved in regulating red/far-red light reversible responses in plants. Therefore, PIF3 qualifies as a potential candidate for being a direct messenger of phytochrome B signals. The proof that PIF3 protein indeed plays some role in phytochrome signalling came from the finding that *Arabidopsis poc1* mutant showing altered red-light responses lacks this protein[40]. The PIF3–GUS fusion protein in onion epidermal cells is present in the nucleus, both in light and dark, indicating that it is a constitutive nuclear protein. It is likely that the PIF3 protein might be a transcription factor. The transgenic plants over-expressing *PIF3* gene in the sense direction showed only a little increase in sensitivity to red light, whereas antisense plants showed a long hypocotyl phenotype under both red and far-red light.

Similar to PIF3, using a portion of the second repeat of the cryptic sensor kinase domain of phytochrome A as bait in yeast two-hybrid screen, another protein PKS1 (phytochrome kinase substrate1) protein was discovered[41]. This protein too interacts with both phytochromes A and B, similar to PIF3. Although PKS1 is present *in vivo* in a phosphorylated form, red light exposure to plants further stimulates the magnitude of PKS1 phosphorylation. It is also phosphorylated *in vitro* by phytochrome A, which is slightly higher with the Pfr form of phytochrome A. In contrast to PIF3, transgenic plants over-expressing PKS1 showed elongated hypocotyls in red light, but antisense plants had a phenotype like the wild type. It has been proposed that PKS1 may negatively regulate phytochrome B responses. While it is suggested that phytochrome B, acting as a kinase may phosphorylate PKS1, its role in phytochrome signalling is unclear. The PKS1 protein is localized in the cytoplasm and not in the nucleus. It is possible that PKS1 interacts with phytochrome B in the cytoplasm, but gets dissociated when it migrates to the nucleus.

A third partner for phytochrome was discovered by Song's group, a nucleoside diphosphate kinase 2 (NDPK2), which performs many functions in eukaryotes such as suppression of tumours in animal and human cells. This protein too was screened from *Arabidopsis* using yeast two-hybrid screening. The study of localization of NDPK2 in tobacco stomatal cells shows NDPK2–GFP fusion protein in both cytosol and nucleus. The activity of NDPK2 kinase is increased *in vitro* in the presence of purified phytochrome A by red light, but not by far-red light[42]. However, this evidence indicates that NDPK2 is most likely a positive regulator of phytochrome B signalling. A role for NDPK2 in the phytochrome signal transduction is evident also from the observation that T-DNA insertion in an intron of the *NDPK2* gene results in a defect in red-light-mediated processes such as cotyledon opening and greening.

In addition to interacting with proteins involved in the signal transduction cascade, phytochrome also interacts with another photoreceptor cryptochrome1, which detects blue light. The recombinant cryptochrome1 could be *in vitro* phosphorylated by recombinant oat phytochrome A. The magnitude of phosphorylation of cryptochrome1 by phytochrome A was higher in red light or blue light than in the dark[43]. Regarding *in vivo* phosphorylation of cryptochrome1, it was higher in red light and was reversible by far-red light. The physiological significance of phytochrome-dependent phosphorylation of cryptochrome1 remains to be established.

Phytochrome regulates gene expression by binding to a transcription factor complex

One of the attractive proposals for phytochrome action envisages regulation of gene expression by direct interaction of phytochrome with the transcription system (Figure 1). A detailed investigation into the function of PIF3 has indeed demonstrated the existence of such a mechanism in *Arabidopsis*. The transgenic plants expressing the *PIF3* gene in the antisense direction showed reduction in light-inducible expression of a subset of light-inducible genes. Since PIF3 has the motif of a transcription factor, it is likely that this reduction results due to retardation of transcription arising from depletion of phytochrome B–PIF3 complex. In a recent paper, Martinez-Garcia *et al.*[44], have made an important breakthrough in understanding the mechanism by which the phytochrome-perceived signal is transduced to the nucleus to modulate gene expression. They showed that PIF3, which is a basic helix–loop–helix transcription factor, specifically binds to various light-regulated promoters containing a G-box DNA-sequence motif[44]. Phytochrome B binds to this G-box-bound PIF3, particularly after red-light-mediated formation of biologically active Pfr form. Importantly, this interaction is photoreversible and phytochrome dissociates upon FR-induced conversion of phytochrome to the inactive Pr form. These results support the view that phytochrome may function as an integral part of a transcription regulating complex, which can be turned on or off depending upon light availability. This provides a very rapid signalling mechanism where phytochrome itself binds to protein–DNA complexes, to regulate target gene expression. Such a mechanism would allow plants to continuously monitor their light environment and react to changes in light availability by concomitant changes in light-regulated gene expression.

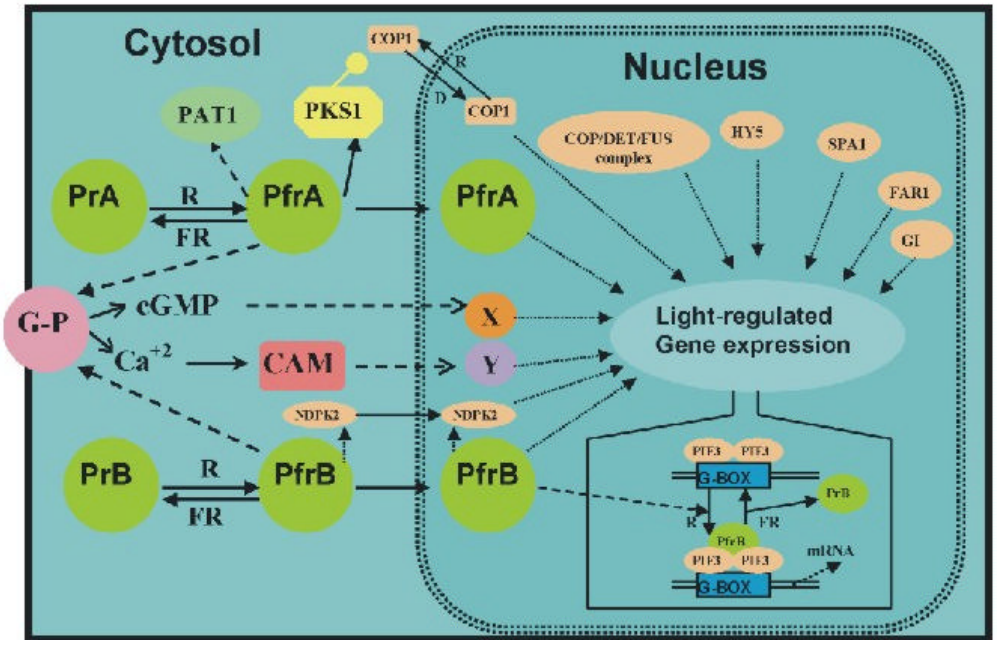

Figure 1. Sketch illustrating the possible pathways of light-regulated gene expression modulated by phytochromes. The red light (R) alters the conformation of the Pr form to the Pfr form and triggers migration of the Pfr form of phytochrome A (PfrA) or phytochrome B(PfrB) from cytosol to the nucleus. The light-induced formation of Pfr is also accompanied by autophosphorylation of phytochrome and transphosphorylation of PKS1 in the cytosol. The formation of Pfr form also triggers, through yet unknown steps, activation of heterotrimeric G-proteins (G-P) and leads to changes in the levels of cGMP and Ca^{2+}. These second messengers may then set a signal chain activating hypothetical nuclear proteins (X and Y), which may regulate transcription. In the nucleus, phytochrome A and B may regulate expression of light-regulated genes by directly or indirectly interacting with nuclear proteins such as SPA1, FAR1, GI and PIF3. The light may also control transcription regulated by transcription factors such as HY5, by modulating the level of COP1. In darkness, the COP1 with help from COP/DET/FUS complex may regulate the HY5 level by proteolysis. The exposure of light may inactivate the COP1 protein and also induce its translocation from the nucleus to the cytosol and dissociation of the COP/DET/FUS proteosome complex. The expanded inset inside the nucleus shows that phytochrome B (PfrB) imported in the nucleus, binds to PIF3, a nuclear transcription factor, bound to G-box motifs in light-sensitive promoters. The PfrB–PIF3 complex then activates/represses transcription by modulating the transcription machinery. The far-red light reverts phytochrome B to the PrB form, triggering its dissociation from PIF3 complex, leading to inhibition/enhancement of transcription of light-regulated genes. The dashed lines indicate that additional steps in the signal transmission are yet to be discovered.

Phytochrome activation causes redistribution of proteins between nucleus and cytoplasm

One of the ways by which phytochrome regulates photomorphogenesis appears to be the modulation of the subcellular distribution of key regulatory factors. In dark-grown suspension culture cells of parsley, CPRF2 – a member of the common promoter-binding transcription factor family (CPRF) – is almost exclusively in the cytosol. The N-terminus of the CPRF2 protein has two domains responsible for its retention in the cytosol[45]. However, the cytosolic retention of the protein is abolished by red light treatment, which promotes its nuclear translocation in red–far red reversible manner. The CPRF2 protein is a phosphoprotein *in vivo*, which is phosphorylated on the C-terminal half by a cytosolic protein serine kinase. The phosphorylation of CPRF2 does not alter its DNA-binding activity; so it may not be related to transcription, but may have a role in nucleocytoplasmic partitioning[46].

One of the best characterized examples is light-regulated distribution of COP1 protein in *Arabidopsis* during photomorphogenesis between nucleus and cytosol. Although phytochrome itself migrates to the nucleus after activation, it also regulates the level of certain proteins in the nucleus, which in turn may allow further modulation of light-mediated gene transcription. The analysis of *Arabidopsis* mutants which had a light-grown phenotype in darkness, has led to the identification of a novel regulator protein COP1 (ref. 47). Since the mutant seedlings displayed constitutive photomorphogenesis and the mutation was recessive in nature, it indicated that COP1 might act as a negative regulator of photomorphogenesis. COP1 protein shows a typical bipartite NLS and three other structural motifs, viz. an N-terminal RING-finger, a coiled-coil domain and a C-terminal WD-40 repeat region[48]. These three domains are involved characteristically in protein–protein interactions required for COP1 function. Interestingly, COP1 also possesses a cytoplasmic-

localization sequence (CLS), which is perhaps needed for moving COP1 out of a nucleus on exposure to light[48].

Transgenic seedlings over-expressing full-length COP1 protein show an etiolated phenotype in different light conditions, substantiating the notion that COP1 is involved in repressing photomorphogenesis and acts downstream of photoreceptors[49]. Similar to phytochrome, COP1 also shows accumulation to a high level in nuclei of hypocotyl cells, but this high level is seen only in dark-grown seedlings[47]. In light-grown plants, the COP1 level in nuclei is very much reduced. Light appears to cause a massive redistribution of COP1 between the nucleus and cytosol. The search for the partners of COP1 protein has led to identification of additional components of light-signalling pathway. In the cytosol of plants, COP1 interacts with a cytoskeleton-localized protein CIP1, which perhaps acts as a cytoplasmic anchoring mechanism for regulating partitioning of COP1 between nucleus and cytosol[50].

Studies on factors regulating nuclear localization of COP1 show presence of a complex network involving several proteins. Apparently, interaction among them is responsible for nuclear accumulation of COP1. The constitutively photomorphogenic phenotype similar to *cop1* mutant is seen for at least ten other mutants which are mutated at different loci, such as *det1*, *fus4–fus*6, *fus11*, *fus12*, *cop8–cop10*, and *cop16* (ref. 51). The essentially similar phenotype of these mutants suggests that these are constituents of a network that functions co-operatively in regulating photomorphogenesis. In fact, an examination of GUS–COP1 localization in this group of *cop*/*det*/*fus* mutants has shown that the dark-grown seedlings do not accumulate GUS–COP1 fusion protein in the hypocotyl nuclei[52]. This reinforces the view that this network of genes in the wild type has a role in nuclear localization of COP1. Based on these observations, it has been proposed that light might be regulating the nuclear level of COP1 by controlling the activity of several COP/DET/FUS proteins, which may exist as part of a large protein complex. In fact, a multisubunit COP9 complex, renamed as signalosome and localized within the nucleus contains many of the COP/DET/FUS proteins as its constituents[53]. The COP9 complex has eight core subunits that exhibit sequence similarity to non-ATPase subunits of the 19S regulatory particle of the 26S proteasome. It is now believed that *COP/DET/FUS* genes operate by repressing the default pathway of photomorphogenesis in darkness. The *COP/DET/FUS* gene family seems to be evolutionarily conserved and its homologues are present even in mammals[53]. Based on this homology some of the COP/FUS proteins have been recently renamed as CSN (COP9 *sign*alosome), with new names such as CSN1 (COP11, FUS6), CSN4 (COP8, FUS4), and CSN7 (FUS5) and CSN8 (COP9)[53].

In the nucleus of plant cell, the COP1 protein is distinctly associated with other nuclear proteins. Interestingly, the first nuclear target identified for COP1 represents a protein, the loss of which gives a phenotype opposite to that of *cop1* in *Arabidopsis*. The *hy5* mutation in *Arabidopsis* is epistatic to *cop1* mutation, and displays a phenotype showing etiolated seedlings in light with elongated hypocotyls. The cloning of *HY5* gene showed that it encodes a bZIP-type transcription factor[54], which is involved in light-mediated transcription regulation by the promoters containing the G-box[54]. The HY5 protein acts as a positive regulator of photomorphogenesis and suppresses *cop1* mutations. The recent studies show that the COP1 negatively regulates HY5 activity. The direct interaction between COP1 and HY5 was observed both in yeast two-hybrid assays and *in vitro* binding assays. The co-localization studies have provided evidence for an *in vivo* interaction between COP1 and HY5. In onion epidermal cells, COP1 and HY5 colocalize to specific nuclear spots, apparently in close contact with each other, as evident by the transfer of fluorescence resonance energy between them[55]. The abundance of HY5 in the nucleus is directly correlated with the extent of photomorphogenic development, and that the COP1–HY5 interaction specifically targets HY5 for proteasome-mediated degradation in the nucleus[56]. These results show that photomorphogenesis of plants is facilitated by light-dependent stabilization of a transcription factor that is rapidly degraded in darkness. It also shows that in photomorphogenesis too, similar to cell division and circadian rhythms, gene regulation may be governed by proteolysis.

Most likely, the suppression of photomorphogenesis by COP1 in the dark involves its interaction with multiple transcription factors to repress gene expression. This view is supported by the identification of CIP7, a transcriptional regulator, as another protein that interacts with COP1. The transgenic plants having *CIP7* gene in antisense direction show reduced expression of several light-inducible genes. Since the expression of CIP7 is upregulated by light, it may be a downstream target of COP1 (ref. 57). In essence, the accumulation of COP1 in the nucleus is supported by the action of *COP/DET/FUS* gene products. In the nuclei of dark-grown plants, COP1 interacts with transcription factors and inhibits photomorphogenesis. Exposure to light reduces the abundance of COP1 in the nucleus and relieves the inhibitory effect on transcription factors regulating photoresponses.

Novel proteins involved in phytochrome signal transduction pathways

Many putative intermediates of phytochrome signal pathways have been recently identified by designing

genetic screens for light insensitive/hypersensitive phenotypes. Several mutants such as *fhy1, fhy3, spa1, fin2, pat1* and *far1* have been identified as being disrupted in phytochrome A signal transduction pathway, of which *spa1*, *far1* and *pat1* have been characterized at the molecular level[58–60]. The *PAT1* gene encodes a cytoplasmic protein of 490 amino acids with sequence homologies to the plant-specific GRAS regulatory protein family. The *PAT1* gene is localized in the cytoplasm and acts as a positive component downstream of phytochrome A signal transduction pathway[60]. The SPA1 is a suppressor of a weak phytochrome A mutant, encodes a nuclear protein, possibly a transcription factor that functions as a negatively acting factor at an early step in phytochrome A-specific signal transduction. The cloning of the *SPA1* gene showed that it encodes a WD (tryptophan–aspartic acid)-repeat protein that also shares sequence similarity with protein kinases[58]. Likewise, FAR1 protein also contains a nuclear localization signal and is targeted to the nucleus in transient transfection assays[59]. A class of mutants specifically affected in red-light signalling, such as *red1, pef2* and *pef3*, has also been isolated in *Arabidopsis*[61,62], which shows phenotype similar to phytochrome B mutants. The analysis of the molecular nature of these genes in future would aid in our understanding of phytochrome B signalling.

Second messengers may mediate phytochrome responses

The multiplicity in the phytochrome-regulated responses signifies that this diversity results from operation of multiple signal transduction pathways triggered by the phytochrome. One attractive proposal is that phytochrome activation modulates the levels of certain secondary messengers in the plants. The second messenger concept was developed after the discovery that cyclic adenosine 3′,5′-monophosphate (cAMP) is an intracellular mediator of glycogenolyic hormones in the liver. In recent years, enough evidence has accumulated to support regulation of photoresponses via a set of second messengers.

Phytochrome-deficient mutants, such as tomato *aurea* (*au*) mutant, display a phenotype showing elongated pale-green seedlings[63]. These seedlings also lack anthocyanin and have poorly developed chloroplasts. Gunther Neuhaus and colleagues could successfully restore the photomorphogenic responses in seedlings of *aurea* mutant by microinjection of oat phytochrome A into single subepidermal hypocotyl cells[64,65]. The restoration of photomorphogenesis by microinjection of phytochrome provided a convenient assay for finding components of the phytochrome signalling pathway.

One of the early components in the signal transduction chain appears to be a G-protein, as evident from the observation that inhibitors of heterotrimeric G-protein completely blocked the action of injected phytochrome. This view is strengthened by emulation of phytochrome-mediated responses in *au* cells by injecting GDP-*g*-S which activates G-proteins. Beyond the G-protein, the signalling splits into two parallel and/or interacting pathways: (i) the calcium/calmodulin pathway controlling chloroplast development; (ii) the cGMP pathway controlling anthocyanin induction. These pathways show a reciprocal control and have a crosstalk among them such as shut-off of the photosynthetic complex synthesis by high levels of cGMP and switch-off of the anthocyanin pathway by high levels of calcium[64,65].

The most ubiquitous second messenger is the Ca^{2+} ion, which on activation is translocated from sequestration sites in the cell or from outside into the cytoplasm where free Ca^{2+} concentration is very low. In maize leaf protoplast, red light stimulated calcium uptake within two minutes of light exposure and was reversed by far-red light. Additionally, 5-hydroxytryptamine also enhanced calcium uptake in dark, indicating a role for phosphoinositides in light-regulated responses[66]. Besides regulating calcium uptake and distribution, phytochrome may also control phosphoinositide metabolism in plant cells. There are additional steps in signal transmission such as phosphorylation of proteins by protein kinase C, which in turn regulates gene expression of nitrate reductase[67]. Additionally, the metabolite sucrose also appears to play an important role in regulating phytochrome-controlled responses[68].

Interaction between plant hormones and phytochrome

It is logical to assume that phytochrome which regulates several processes in plant development, and plant hormones which control and coordinate several plant development responses, must interact closely at least at a few points. For example, many phytochrome mutants show elongated seedlings under specific light regimes. It is known that plant hormones also regulate stem elongation, suggesting a cross-talk between the two pathways. The evidence in favour of such interaction has come again from analysis of hormonal levels in photomorphogenic mutants. The dark-grown seedlings of brassinosteroid mutants of *Arabidopsis* show the phenotype of light-regulated plants, with short hypocotyls and expanded cotyledons as if seedlings are growing in light[69]. The adult phenotype of these mutant dwarf plants with short stems and petioles, dark-green leaves and delayed senescence is essentially the opposite of phytochrome B mutants[70]. Apparently, these two processes appear to be unlinked, as light does not seem to regulate the brassinosteroid biosynthetic genes. Recently, a link has been found between phytochrome B and brassinosteroids by isolation of an extragenic,

dominant, gain-of-function suppressor of a phyB missense mutation called BAS1-D, which codes for a cytochrome P450 catalysing the inactivation/degradation of brassinosteroids[71]. The *bas1-D phyB-4* double mutant has no detectable brassinolide (BL), the most active BR. The *bas1-D* suppresses a *phyB*-null allele, but not a *phyA*-null mutation. Though it is still not known how light regulates BAS1 activity, a link between photoreceptors and brassinosteroid metabolism is apparent[71].

A few of the photomorphogenic mutants show a phenotype similar to phytohormone mutants, for example, the *spindly* mutant defective in gibberellin (GA) signalling resembles phytochrome B mutants with long stems, pale leaves and early flowering[72]. Phytochromes can regulate the transcription of GA biosynthesis genes[72]. Similarly, a role for auxins in photomorphogenesis is indicated by the study of *hy5* mutation, which results in seedlings with long hypocotyls under light. The *HY5* gene encodes a bZIP transcription factor[54] that is involved in auxin signalling. A link between auxin signalling and photomorphogenesis is provided by cloning of *SHY2* gene. The *shy2* mutant suppresses the long-hypocotyl phenotype of *hy2* mutant[73] and this dominant mutation resides in the auxin-induced gene *IAA3*, indicating a link between light and auxin in regulation of hypocotyl elongation[74]. The search for a suppressor of COP1 led to identification and characterization of a new light regulated gene, *FIN219*, that represents a novel locus whose mutation results in a long hypocotyl only in far-red light. *FIN219* plays a role in phytochrome A mediated FR inactivation of COP1 and in promoting photomorphogenesis[75]. The sequence of *FIN219* gene is highly similar to a family of proteins defined by the soybean early auxin-inducible gene *GH3*. Similar to *GH3*, the expression of *FIN219* is rapidly inducible by auxin. This finding indicates a molecular cross-talk between auxin response and light regulation, suggesting that by regulating *FIN219* expression, auxin may in turn play a role in light regulation of development. Such an interaction between phytochrome and auxin signalling is also seen for the negative regulator ATHB-2, a transcription factor upregulated by far-red light. The transgenic plants over-expressing ATHB-2 confer a shade avoidance phenotype, which is partly due to interference with auxin transport[76].

Phytochrome regulation of flowering

The observation that daylength regulates flowering in many angiosperms led to the discovery of phytochrome. However, regulation of flowering by phytochrome is part of a complex regulatory pathway, which has two components – light quality and the circadian clock. The perception of light and onset of dusk and dawn is mediated by phytochromes and by cryptochromes, blue-light-absorbing photoreceptors of plants. Phytochrome regulation of flowering has been extensively studied in *Arabidopsis*, and recently many of the components regulating flowering have been identified[77,78]. A key link between the phytochrome and regulation of flowering genes is provided by the *GIGANTEA* gene. *GIGANTEA (GI*) is involved in control of flowering in response to daylength, the *gi* mutation causes late flowering under long days and makes the plant insensitive to daylength. The *GI* gene encodes a large protein, which based on homology to other proteins was predicted to be a membrane protein, since it contains five membrane-spanning domains in the N-terminus. The C–terminus of GI is hydrophilic and is not homologous to any known protein[79,80].

Ever since the discovery of phytochrome one has sought an answer to how phytochrome regulates floral initiation? The finding that both phytochrome and cryptochrome, which act as light sensors for photoperiodic light detection, can enter the nucleus invokes the possibility that these photoreceptors may directly regulate expression of flowering genes. The analysis of a mutant that shows reduced seedling de-etiolation under red light provided evidence that phytochrome regulates flowering by direct regulation of some of the key genes. Cloning of the above mutant locus revealed that the mutated gene is the same as the *GIGANTEA* gene involved in control of flowering time. Using GUS–GI and GFP–GI fusion proteins, Huq *et al.*[81] have showed that GI is constitutively targeted to the nucleus in transient transfection assays. Moreover, the observation of green fluorescence in nucleus showed distribution of GFP–GI protein throughout the nucleoplasm. Interestingly, even though based on homology to other membrane proteins, it was predicted that GI would be a plasma membrane-localized protein, the above finding shows that it is a nucleoplasmically localized protein. This finding, though in conformity with its role in phytochrome B signalling, also adds a new dimension that photoregulation of flowering may be mediated by regulation of nuclear proteins.

Perspective

Finally, one may ask what use we can make of the information about phytochrome regulation in plant development. It may be still too early to answer this question as more has to be learned about phytochrome and its interaction with other regulators present in the plants. However, the analyses of transgenic plants over-expressing phytochrome have highlighted a few of the potential applications. For example, transgenic tobacco plants, which over-express oat phytochrome A show improved harvest index[82] by alleviating the shade-avoidance response in a densely planted plot. In potato,

over-expression of *Arabidopsis* phytochrome B improved photosynthetic performance and increased life span, leading to higher yield of tubers[83]. However, these approaches can have their own pitfalls; for example, though the number of tubers is increased in potato, the tubers were smaller than in the wild type[83]. It is hoped that information on phytochrome and its interacting partners and light-regulated genes would aid in our knowledge about plant development. This information would be helpful to regulate the crop yield in conjunction with other regulatory systems of plants.

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