Cryptic blues: Mechanism in sight!

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Several plant responses are mediated by blue-light as also UV-A radiation. Until a few years ago, the identity of the blue/UV-A sensing photoreceptors was equivocal. In the past few years, however, the molecular genetic analysis of *Arabidopsis* mutants has greatly aided in the identification and characterization of blue/UV-A sensing cryptochrome 1 (cry1), cryptochrome 2 (cry2) and phototropin (JK224/nph1). The cry1 and cry2 receptors, like DNA photolyases, are dual chromophore proteins and may harbour both a flavin (FAD) and a pterin for primarily regulating hypocotyl growth inhibition, cotyledon expansion and flowering time, besides sensing light for entraining endogenous clocks. In contrast, JK224/nph1 anchors another flavin, FMN, and regulates phototropism. In addition to flavins and pterin, evidence is mounting in favour of a carotenoid, zeaxanthin, for regulating blue-light-induced stomatal opening. There is little information on the signalling components acting downstream to blue sensory receptors, but nph1 has an intrinsic kinase domain that on photoexcitation initiates a phosphorelay through interaction with phosphoproteins like NPH3. Although the primary mechanism of action of cry1 and cry2 is unknown, their localization in the nucleus and protein–protein interaction with phytochromes indicates that they may directly regulate changes in gene expression. The occurrence of cryptochromes in both plants and animals indicates their ubiquitous nature and a prominent role in regulating diverse responses.

TO optimize their growth and development in response to constantly changing light conditions, plants have evolved multiple sensory photoreceptors, including the red/far-red reversible phytochromes, blue/UV-A sensing cryptochromes and UV-B receptor[1]. These sensory receptors not only perceive the quality and quantity of light, they also sense its direction and duration to indicate time. Although phytochromes have been studied extensively because they control processes throughout the plant life cycle, several responses, conserved evolutionarily among lower and higher living organisms, are induced by blue-light. In higher plants, the well-documented blue-light-induced responses include phototropism, inhibition of seedling hypocotyl growth, cotyledon expansion, chloroplast development, stomatal opening, control of flowering time, entrainment of endogenous clocks and changes in gene expression[2]. Characteristically, most blue-light responses are also induced by UV-A light, requiring low incident energies, and, unlike phytochrome-induced responses, are not photoreversible. Although functional redundancy exists between blue sensing cryptochromes and phytochromes for regulating some of these responses, the action to blue-light usually becomes apparent more rapidly. There is enormous literature on the phenomenology of these blue-light-induced responses[3,4], some of which – like phototropism – have been studied for over 100 years now but, until recently, the biochemical nature of the blue-light-absorbing chromophores was debated intensely. The earliest clues came from the action spectra studies. Indeed, Galston (a contributor to this special issue), based upon the comparison of action spectrum of phototropism with the absorption spectra of known compounds, suggested nearly half-a-century ago that a flavin was the most likely chromophore. This view was also supported by experiments on light-induced absorbance changes and studies with metabolic inhibitors[5]. However, a carotenoid, zeaxanthin, has also been implicated for regulating some blue-light-induced responses[6,7].

Because of the apparent delay in the unequivocal identification of the blue-light receptor, the progress in elucidating the associated signalling pathway has been rather slow. The blue-light-induced absorbance changes (LIACs), representing flavin-mediated photoreduction of b-type cytochromes in plasma membrane fraction[4], were considered originally as one of the early steps in the transduction sequence, but its biological significance remains an enigma. Several other studies have provided correlative evidence for blue-light-induced responses and changes in redox activity[8,9]. The electrophysiological studies on young seedling hypocotyls and stomatal guard cells have also shown rapid changes in ion fluxes and membrane electrical potentials in response to blue-light[4]. Altered protein phosphorylation is a regulatory mechanism well-known in stimulus–response coupling in plants as well[10]. Based upon several physiological and biochemical studies in the late eighties, Briggs and coworkers also suggested that the blue-light-induced phosphorylation of a plasma membrane protein is involved in phototropism[4]. Despite these advances, however, the identity of the blue-light

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receptors remained a major unsolved problem in biology. The reason probably was that we were trying to evolve a unifying hypothesis in the background of diversity of responses induced by blue-light. Finally, in the recent past, the genetic potential of *Arabidopsis* has been exploited and that has led to not only the identification of multiple blue-light receptors, but also a few signalling components. In this article, the recent accomplishments in the identification of blue sensory receptors and the complexities of their response pathways have been highlighted. How these blue-light receptors crosstalk among themselves and/or with phytochromes to regulate plant development, is also addressed. The impact this family of receptors, in particular cryptochromes, has had in illuminating the endogenous timekeeping mechanism, not only in higher plants but also in moulds, insects and animals, has been covered briefly.

Cryptochromes are no longer cryptic!

The biochemical identity of the sensory pigment system responsible for blue-light responses remained elusive far too long and probably prompted Gressel[11] to coin the term 'cryptochrome', originating from the term 'cryptic', i.e. hidden (pigment). The term 'cryptochrome' has another connotation; it represents an unknown pigment in cryptogams, the lower plants that are highly sensitive to blue-light for regulating various processes. While coining the term cryptochrome, Gressel[11] probably did not have any inkling that plants have evolved a small family of blue sensory receptors and, unlike phytochromes, they may harbour different chromophores! As it would become apparent from the discussion that follows, the identity of at least four blue-light receptors is now unquestionable and discovery of more such receptors will not be surprising. However, the discovery of multiple blue-light receptors, and that too with different chromophores, has ensued a debate as to which particular subclass deserves to be designated 'cryptochrome'[12]. Although this debate may continue, here cryptochrome 1 (cry1) refers to the holoprotein, a product of the gene *CRY*1/*HY*4, anchoring the chromophores FAD and pterin.

Molecular properties of cryptochromes

Even though, historically, phototropism remains the oldest studied blue-light response, the earliest molecular studies relate to blue-light-mediated stem growth inhibition response. The recent advances in our understanding of blue-light photoreceptors have largely come about due to the discovery of mutants in *Arabidopsis*. Several long-hypocotyl (*hy*) mutants were isolated about two decades ago by Koornneef *et al.*[13], which were insensitive to red, far-red or blue-light. Though most of these EMS-mutagenized mutant strains represented lesions in phytochrome photoreceptors, at least one, *hy*4, was found to be exclusively insensitive to high irradiance blue-light for hypocotyl growth inhibition. Like some other receptor mutations, *hy*4 also showed incomplete dominance[13,14]. Realizing that the map-based cloning of *HY*4 gene may be tedious, Cashmore and coworkers, in a pioneering effort, identified a T-DNA tagged allele of *hy*4 and cloned the *HY*4 gene by marker rescue[15]. The DNA sequence analysis of *HY*4 gene (subsequently designated as *CRY*1 (ref. 16)) revealed that it encodes a protein of 681 residues, with 30% sequence identity of its N-terminal half to prokaryotic DNA photolyases, a unique class of photoactivable flavoenzymes that catalyse the repair of pyrimidine dimers in UV-damaged DNA[17]. The sequence identity of cry1 is in fact as high as 70% with the chromophore-binding domains of the photolyases. However, the two proteins differ in that the CRY1 harbours a tropomyosin-like domain as C-terminal extension, not detected in any of the photolyases and, conversely, a highly conserved tryptophan residue (W277), required essentially for binding the enzyme to damaged DNA, is conspicuously absent in CRY1 (refs 15 and 17). It is striking that at least seven of the twenty mutant *hy*4 alleles sequenced have lesions in the tropomyosin-like domain, highlighting the significance of the C-terminal extension in cry1 function.

The conservation in the chromophore-binding domains with microbial DNA photolyases indicated a dual chromphoric nature of cry1 and also provided an insight into the probable nature of its chromophores. Thus, the overexpression studies in baculovirus-insect cell system revealed that the CRY1 protein indeed binds FAD noncovalently[18]. This observation provided unflinching evidence in support of the original hypothesis proposed by Galston[5] that flavin may serve as the chromophore for blue-light perception. However, studies on the expression of the photolyase homologous region of CRY1 in *E. coli* have identified a pterin, methenyltetrahydrofolate (MTHF), as the second chromophore[19] (Figure 1).

The second cryptochrome gene, *CRY*2, was identified through screening of the cDNA library of *Arabidopsis*, employing *CRY*1 as the gene probe[20]; essentially the same gene was also isolated by Hoffman *et al.*[21] and designated as *At-PHH*1. The *CRY*2 gene encodes a protein of 619 amino acid residues with extensive similarity to cry1 in the chromophore-binding domain. However, the C-terminal extension in cry2 is smaller and bears little homology to cry1 (Figure 1). The nature of the cry2 chromophores still remains to be deciphered, although there is some evidence that it binds a flavin[20].

In consonance with the antiquity of the blue-light responses, cryptochromes appear to be ubiquitous not only in higher and lower plants but also in animal and mammalian systems[2]. Among plants, cryptochrome-like

sequences have been identified in tomato, pea and rice[2,22]. As many as five *CRY*1-like genes are represented in the genome of the fern *Adiantum capillus-veneris*, and at least three of them are expressed[23]. The *Sinapis alba* gene (*SA-PHH*1, originally designated as *SA-PHR*1) encodes a protein 89% identical to *Arabidopsis* cry2, although it lacks the C-terminal extension[21,24]. The gene *CPH*1 (ref. 25) that shares 40–50% identity with *Arabidopsis* cry2 and SA-PHH1 encodes a putative CRY protein in *Chlamydomonas*. In all these cryptochrome genes identified in diverse species, the N-terminal half has high sequence identity between species but, the C-terminal extensions not only vary in length, they share little similarity. It is thus likely that these C-terminal domains provide functional specificity, probably through interaction with downstream signalling components. Ironically, virtually nothing is known about the elements that constitute cry1 or cry2 signalling cascades. Some speculations, based upon their nuclear localization, protein–protein interaction with phytochromes, and genetic interaction studies on various mutants, have been discussed in relevant sections.

*Role of cry*1 *and cry*2 *in the de-etiolation process*

The young dark-grown seedlings of any dicot species normally display an etiolated phenotype with long hypocotyl, a tight apical hook and unexpanded cotyledons. The de-etiolation process is triggered with the photoexcitation of phytochromes and blue-light receptors, resulting almost instantly in cessation of hypocotyl growth, followed by apical hook opening and cotyledon expansion, leading eventually to more advanced photomorphogenic development[14].

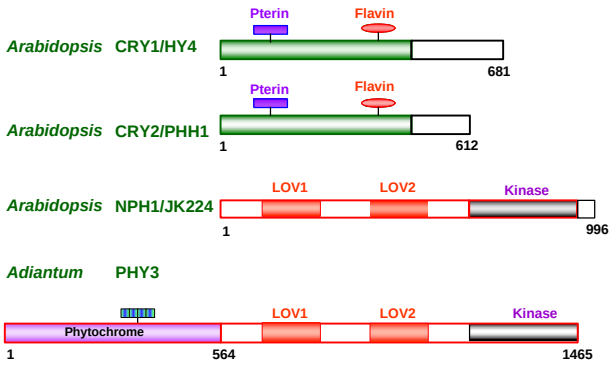

Figure 1. Schematic representation of three blue-light receptors of *Arabidopsis thaliana* and a chimeric phytochrome-cum-blue-light receptor of the fern *Adiantum capillus-veneris*. CRY1/HY4 and CRY2/PHH1 share significant homology with type I DNA photolyases and, as far as examined, they bind chromophores FAD and pterin (MTHF). NPH1/JK224 has two LOV domains towards the N-terminal, which form an adduct with FMN, and a serine/threonine kinase domain in the C-terminal part. The *Adiantum* PHY3 is large in size and shows homology to chromophore-binding region of phytochrome (1–564) and nearly entire NPH1/JK224 of *Arabidopsis*.

Thus, it is not surprising that, in addition to a long hypocotyl phenotype, the *hy*4*/cry*1 mutant also showed decreased cotyledon expansion, increased petiole elongation and leaf expansion in light-grown seedlings[26]. In contrast, the over-expression of *Arabidopsis CRY*1 in tobacco caused hypersensitivity to blue/UV-A as well as to green light, for hypocotyl growth inhibition response[16]. Subsequently, transgenic *Arabidopsis* plants over-expressing either *CRY*1 or *CRY*2 were also found to be hypersensitive to blue-light and exhibited exaggerated hypocotyl growth inhibition and cotyledon expansion, and increased anthocyanin accumulation[27,28]. However, *Arabidopsis* seedlings over-expressing *CRY*2 were more responsive to low-fluence blue-light and *CRY*1 overexpression required high-fluence blue-light for exaggerated hypocotyl growth inhibition. That the functional demarcation between cry1 and cry2 is light intensity-dependent, was further strengthened by the analysis of *cry*2 deletion mutants[29]; these mutant seedlings developed long hopocotyls and unexpanded cotyledons when grown specifically in low-fluence range blue-light. This situation is analogous to phytochromes, phyA and phyB, which respond to very weak and high energy signals, respectively[30].

Even though cryptochrome represents the first blue-light photoreceptor to be characterized in detail, the chain of events leading to physiological action remains unknown. Earlier studies have shown that a rapid and transient depolarization of epidermal cell plasma membrane precedes the blue-light-mediated stem growth inhibition in *Cucumis*[31]. Studies employing *hy*4*/cry*1 mutant and some ion channel blockers indicate that cry1, on photoexcitation by prolonged high-fluence blue-light, activates anion channels that in turn may alter the cellular water potential and retard elongation growth[32].

*cry*2 *– A sensor for photoperiodic timing*

Arabidopsis is a facultative long-day plant. The genetic interaction studies of several late-flowering mutants of *Arabidopsis* have identified at least three general pathways that promote flowering. These include the so-called long-day or photoperiodic pathway, vernalization–responsive autonomous pathway, and the gibberellin-sensitive pathway[33]. The involvement of phytochrome A and transcriptional regulators involved in phytochrome signalling, i.e. CCA1 and LHY, in the photoperiodic pathway has been demonstrated[33]. Recently, the role of cry2 in photoperiodic timing has come to light through analysis of *Arabidopsis cry*2 mutant that turned out to be allelic to a previously known late-flowering mutant, *fha*[29]; all the three *fha* alleles

have been found to be defective in *CRY*2 gene. The long-day promotion pathway in *Arabidopsis* also requires the enhanced expression of the *CONSTANS* (*CO)* gene and the expression of *CO* gene was found to be low in *cry*2*/fha* mutant. Conversely, its transcript levels were elevated in a *CRY*2 over-expressing transgenic *Arabidopsis* line[29]. Further analysis of flowering in *cry*2 and *phy*B mutants in response to various wavelengths, and its relation to *CO* gene expression, has indicated that flowering time is regulated by the antagonistic action of cry2 and phytochrome B[29].

Cellular distribution and light-sensitivity of cryptochromes

The deduced amino acid sequences show that both cry1 and cry2 are devoid of any membrane-spanning domain and thus appear to be soluble proteins[15,27,28]. However, the possibility that these proteins may associate with the membrane, for performing their functions, cannot be ruled out. The *CRY*1 transcript is detectable in young, dark-grown seedlings and all organs of mature light-grown plants of *Arabidopsis*[15], although the level varies in different tissues. Like *CRY*1, the *CRY*2 gene also expresses throughout the seedling and its mRNA levels are not affected by light. But, unlike cry1, the cry2 protein is quite unstable and its level decreases rapidly when dark-grown seedlings are irradiated with blue/UV-A or green light[28]. Whatever may be the precise mechanism of cry2 instability, it is likely that cry2 may have functional significance under conditions where light is limiting[34].

Like phytochromes A and B, it was found that both cry1 and cry2 contain putative nuclear localization signal (NLS) towards the C-terminal region[2]. The fusion proteins of CRY1 and CRY2 were thus created either with green fluorescent protein (GFP) or *b*-glucuronidase (GUS), and expression analysed in the transgenics. Unlike phytochromes, which translocate to the nucleus on photoactivation, the CRY1 and CRY2 fusion proteins, containing entire CRY proteins or only the C-terminal fragment, were detected exclusively in the nucleus, without any obvious light regulation[2,35,36]. However, like native CRY2, the abundance of CRY2 fusion protein was also down-regulated by blue-light[36]. The immunoblot analysis of proteins from various cellular fractions also showed preferential co-fractionation of CRY2 with the nucleus[35,36]. In the fern *A. capillus-veneris*, which harbours at least five cryptochromes (cry1 to cry5), the GUS–CRY fusion protein distribution analysis has revealed that only cry3 and cry4 are localized in the fern gametophyte nuclei; cry4 is nuclear localized irrespective of the light/dark conditions, whereas cry3 partitions between the nucleus and the cytoplasm in a light-dependent manner[37]. Since these sensory receptors are nuclear localized, it is likely that they do not involve elaborate downstream signalling cascade and probably themselves act as transcriptional regulators. It is also tempting to speculate that the nuclear locations of both cry1 and cry2 provide them a selective advantage (at least in terms of time kinetics) over phytochromes (whose translocation to the nucleus is light-dependent), at least for responses which are controlled by both types of sensory receptors.

Cryptochromes and phytochromes interact physically

As stated earlier, there is functional overlap between phytochromes and cryptochromes for regulating responses like hypocotyl growth inhibition, cotyledon expansion and anthocyanin biosynthesis. Whether do these two different classes of receptors crosstalk or operate via different signalling cascades, is a question that deserves consideration. The molecular analysis of several long-hypocotyl and constitutively photomorphogenic mutants of *Arabidopsis* has identified genes like *HY*5, *CCA*1, *LHY*, *PIF*3, *COP*1 and *DET*1 that encode transcriptional factors involved in controlling hypocotyl growth[38]. The epistasis analysis, employing some of the mutants defining these genes and photoreceptor mutants, has revealed that most of these factors act downstream to both phytochromes and cry1/hy4 (refs 14 and 38); cry2 has not been analysed in this regard. Moreover, the mutant *hy*5 was found to be partially impaired in both blue- and red-light-mediated hypocotyl growth inhibition[13]. While this evidence by itself is convincing that cryptochromes share at least some elements with phytochrome signalling cascade (Figure 2), the evidence that these receptors may interact physically has emerged only recently. Ahmad *et al.*[34] performed experiments with purified recombinant photoreceptors and showed that both red- and blue-light stimulate cry1 and cry2 phosphorylation *in vitro*, but only in the presence of phytochrome A, indicating that both cry1 and cry2

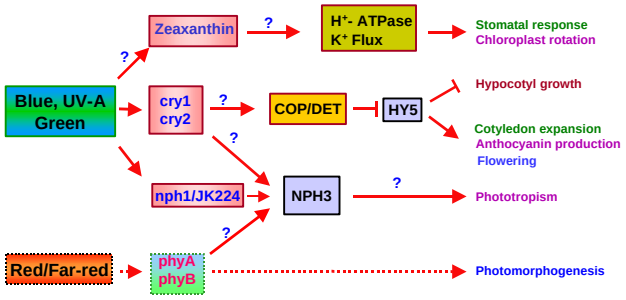

Figure 2. Schematic diagram showing the putative signalling components involved in diverse blue-light-induced responses. The crosstalk between different sensory pathways for regulating phototropism is also depicted (see text for more details).

act as substrate for phytochrome A kinase. Employing yeast two-hybrid assay, a direct physical interaction has been shown between the C-terminal domains of cry1 and phytochrome A, known to be responsible for conferring biological activity[30]. Since both cryptochromes and photoactivated phytochromes are nuclear localized, it appears that their interaction may have direct relevance to blue- and/or red/far-red-induced changes is gene expression, leading eventually to more overt morphological changes.

Possible functions of cryptochromes in entrainment of circadian clock

All living organisms possess an endogenous timekeeping mechanism called the circadian clock, to control a wide diversity of biological processes[39]. Light acts as an important environmental cue that helps in synchronizing these circadian clocks. Until few years ago, very little was known about the identity of the clock photoreceptors. To help resolve this long-standing question, Kay and coworkers[40] generated transgenic *Arabidopsis* carrying firefly luciferase gene driven by circadian clock-responsive *CAB*2 promoter and mobilized it in the genetic background of various photoreceptor or light signal transduction mutants. The analysis of these lines clearly indicated that phytochrome A and B perceive low and high intensity red light, respectively, whereas both phytochrome A and cry1 perceive and transduce low-fluence blue-light signal to the clock[40].

Since the discovery of cryptochromes and their probable role in entraining the endogenous clock in plants to light, rapid strides have been made in demonstrating a role of these blue/UV-A absorbing receptors in molds, insects, mouse and humans[41,42]. Similar to higher plants, both human and mouse have two *CRY* genes each, and *Drosophila* has one[43–45], and, as of now, human cry proteins (hcry1 and hcry2) have been shown to bind FAD and MTHF. In fact, there in evidence that the cry proteins in human and *Drosophila* may mediate circadian photoperception[45–47] and help resetting the clock by light-dependent interactions with clock proteins like TIM, as demonstrated for *Drosophila*. In contrast, there is evidence that mouse CRY proteins act as transcriptional inhibitors within the circadian feedback loop and not as photoreceptors[48]. This is based upon the observation that the light-induced expression of *PER* genes in mouse is not affected in mouse defective in both the *CRY* genes. However, both mcry1 and mcry2 promote translocation of PER proteins to the nucleus and negatively regulate the function of two other clock proteins BMAL1 and CLOCK[49]. It, therefore, appears that there is some photoreceptor other than cry1 and cry2 in mouse that stimulates phase-shift in response to light and resets the clock.

Discovery of the receptor for phototropism – From Darwin to phototropin!

Isolation and characterization of phototropism mutants

Phototropism is probably the most extensively studied response that is elicited by exposure of the young seedlings to unilateral blue/UV-A light. In fact, Charles Darwin[50] described phototropism as a phenomenon in his classic monograph *The Power of Movement in Plants*. It is ironical, however, that despite enormous literature generated on the photophysiology of the process, the identity of the photoreceptor responsible eluded us for over hundred years! Again, for this discovery too, the molecular analysis of the genetic mutants of *Arabidopsis* proved to be crucial. But, before discussing the work on *Arabidopsis*, it must be mentioned that some early work in Galston's laboratory in the 1950s demonstrated that the coleoptiles of even carotenoid-deficient mutants of maize, *viviparous* 5 (*Vp*5), and the albino barley develop significant phototropic curvature when irradiated with unilateral blue-light[51]. Vierstra and Poff[52], who treated the maize coleoptiles with a carotenoid biosynthesis inhibitor, norflurazon, drew essentially a similar conclusion, but the phototropic curvature was reduced marginally. These studies thus supported the view that it is not carotenoids, but flavins which are primarily responsible for perception of blue-light responsible for curvature development.

The work on the isolation and characterization of phototropism mutants of *Arabidopsis* was initiated by Khurana and Poff[53] with the view to not only unravel the identity of the photoreceptor, but also signalling components involved in this blue-light-regulated response. The photophysiology of phototropism is, however, somewhat complex and needs to be explained in some detail to understand the intricacies involved. The curvature that develops in young seedling hypocotyls (dark-grown) in response to low-fluence and short irradiation times is usually small and is referred as the 'first positive' curvature, and the increasing dosage of blue photons for longer duration leads to development of 'second positive' curvature with higher magnitude[14]. Since the Bunsen–Roscoe law of reciprocity is valid for only 'first positive' curvature, a strategy was designed to screen mutants in this fluence-response range. At least 35 putative mutants were obtained by screening etiolated seedlings raised from EMS-mutagenized M2 seed population[53,54]. A few of these were characterized in greater detail. The most promising among these was strain JK224, which had normal gravitropism and 'second positive' phototropism, but was specifically affected in the 'first positive' response. For this mutant, the requirement for the threshold fluence and the optimal fluence was increased by 20–30-fold in comparison

to the wild-type parent, albeit the magnitude of the response remained unaffected. Based upon these observations, Khurana and Poff[53] proposed that JK224 might represent a lesion in the photoreceptor moiety *per se* or in an early downstream signalling component. Another mutant strain, JK229, exhibited a normal 'second positive' response, except a minor shift in its fluence threshold, but it was severly defective in 'first positive' response. On the other hand, strain JK218 was impaired in phototropism *per se* and represents a null response mutant[53]. Subsequently, Briggs and coworkers isolated many more phototropism mutants in *Arabidopsis* and assigned them to four complementation groups, *nph*1 to *nph*4 (ref. 55) (*nph* for *n*on-*p*hototropic hypocotyls). On complementation analysis with previously isolated mutants, JK224 and JK218 (ref. 53) were redesignated as *nph*1–2 and *nph*3–3, respectively.

As we shall see, the molecular genetic analysis of the JK224/*nph*1 mutant has proved to be crucial for identification of the much awaited blue-light receptor for phototropism.

Molecular characteristics of the photoreceptor for phototropism

Around the same time when the JK mutants were isolated[53], Briggs and coworkers[56] demonstrated rapid blue-light-induced phosphorylation of a 120 kDa plasma membrane protein in etiolated pea seedlings. A detailed physiological analysis with regard to the occurrence of this protein in most photosensitive tissues, the fluence requirement for its phosphorylation and its response kinetics indicated that it may be involved in phototropism[4]. To substantiate the claim for the involvement of this phosphoprotein in phototropism, Briggs's group examined the phosphorylation event in JK224/*nph*1 mutant of *Arabidopsis*. Strikingly, the blue-light-dependent phosphorylation of the 120 kDa target protein was drastically reduced in JK224/*nph*1 (refs 55 and 57). These data, coupled with an earlier suggestion based upon photophysiological behaviour of JK224 (ref. 53), strengthened the possibility for this autophosphorylating protein to have a photosensory function[57]. The cloning and molecular characterization of the gene defined by the JK224/*nph*1 mutant has finally provided credibility to this assumption.

The cloning of *NPH*1 gene was facilitated by the identification of the flanking markers employing AFLP technique, followed by screening of the YAC library[58]. The *NPH*1 gene encodes a protein of 996 amino acid residues and the coding region consists of 20 exons, extending for 5.4 kb. It harbours at least three characteristic domains: a serine/threonine kinase domain towards C-terminus and two repeated domains, LOV1 and LOV2 (LOV for light, oxygen and voltage), that share similarity with diverse proteins of archaea, eubacteria and eukaryotes; the redox status of these proteins is known to be regulated by these sensory signals and probably involves a flavin moiety[58] (Figure 1). The size of the cloned *NPH*1 gene, the presence of a kinase domain and near absence of 120 kDa protein in different JK224/*nph*1 mutant alleles established beyond doubt that this gene encodes a phosphoprotein. The *NPH*1 gene from *Arabidopsis* was then expressed in a heterologous baculovirus–insect cell system and, contrary to expectation, the purified soluble protein was found to bind FMN[59], instead of FAD, which is the preferred moiety for cryptochromes and photolyases. It is remarkable that the fluorescence excitation spectrum of nph1 (containing FMN) expressed in insect cells is quite similar to the action spectrum for phototropism. In a further detailed analysis, the FMN has been shown to bind LOV domains (expressed as isolated domains in *E. coli*) in stoichiometric proportion[60]. The site-directed mutagenesis of putative FMN-binding sites of LOV1 and LOV2 domains in *Avena* nph1, has demonstrated that the formation of a stable adduct between FMN and cysteine (Cys39) is critical for photochemical activity[61]. It is striking that phytochrome chromophore also attaches autocatalytically to a highly conserved cysteine residue[30]. However, unlike phytochromes, the formation of FMN–cysteinyl adduct is itself light activated and is completely reversed in darkness. It has been proposed that this photoactivation causes a conformational change in LOV domains, leading to the activation of the C-terminal kinase domain[61]. With the conclusive evidence that nph1 is indeed the blue-light photoreceptor for phototropism, Briggs and coworkers[60] designated it as 'phototropin'.

A functional equivalent of nph1 is present in pea buds[62] and the homologues of *Arabidopsis NPH*1 gene have also been reported from *Avena sativa* (*NPH*1-1, *NPH*1-2)[12] and *Zea mays* (Gen Bank accession no. AF033263). Recently, one homologue of *NPH*1 has been cloned from indica rice by our group (Gen Bank accession no. AJ252142) and two from japonica rice[63], which are differentially photoregulated. The deduced amino acid sequences of monocot species show greater than 85% identity among themselves and 70% identity with *Arabidopsis*. The predicted monocot protein sequences are smaller in size than *Arabidopsis* but, earlier biochemical studies have also shown that the blue-light-induced phosphoprotein in species like oat, maize and wheat is smaller in size than *Arabidopsis*[64,65]. It will be interesting to examine whether the LOV domains in these nph1 proteins from diverse species selectively attach to FMN. At least for *Avena* nph1, the association of FMN has been demonstrated[60].

Chimeric photoreceptor in Adiantum

Extension of studies on the *NPH*1 gene to lower plants has brought out some surprising findings. In the fern, *A.*

capillus-veneris, a protein gene (*PHY*3) has been isolated, which encodes a chimeric protein with sequence identity (nearly 52%) to chromophore-binding domain of phytochrome A towards the N-terminus and 57% identity to entire nph1 (including both the LOV domains and the kinase domain) towards the C-terminus[66] (Figure 1). These two domains when expressed independently in heterologous expression systems, were capable of reconstituting the corresponding functional sensory receptors[60,66]. This clearly indicates that the phy3 of *Adiantum* is a dual photoreceptor that can mediate the effects of red/far-red and blue spectral regions in regulating diverse responses of this fern species.

Cellular localization of nph1 (*phototropin*)

Earlier studies, particularly on phototropism, had supported the view that blue-light receptor is membrane localized[4]. Even the extensive studies carried out by Briggs and coworkers on blue-light-induced phosphorylation of 120 kDa pea protein claimed it to be a constituent of plasma membrane fraction[4]. However, the evidence based upon the deduced amino acid sequence of NPH1 has contradicted this assumption because it is devoid of any membrane-spanning domain[58]. As mentioned in a previous section, both cry1 and cry2 are also soluble proteins. This is a situation reminiscent of phytochromes, which too, until the late 1970s, were shown to be associated with the pelletable membrane fraction. For phytochromes too, the gene isolation and characterization provided the conclusive evidence that they are of cytosolic origin[30]. However, all these sensory photoreceptors do perform certain functions that involve changes in ion fluxes or membrane polarization. It is thus imperative that these receptors, including nph1, associate with the plasma membrane either through a lipid modification or interaction with other membrane proteins. This can probably explain the earlier claims on membrane localization of these receptors, which may otherwise prove to be mere artifacts!

Phototropism signalling cascade – A phosphorelay

As is evident from the biochemical nature of phototropin, it is not unexpected that its photoexcitation will initiate a cascade of events beginning with stimulation of its kinase activity. Recently, the nph1 kinase substrate has been identified by the positional cloning of the gene defined by another phototropism mutant, i.e. *nph*3/JK218 (refs 53, 55 and 67). The predicted sequence of this 795 amino acid protein is unique to plants, and it represents a phosphoprotein with two putative protein–protein interaction domains. The NPH3 protein has been shown to interact with N-terminal domain of NPH1 in a yeast two-hybrid assay, and their interaction *in vitro* is FMN-dependent. Moreover, like NPH1, NPH3 also associates with the plasma membrane fraction, although it lacks a membrane-spanning domain. Based upon these observations, Motchoulski and Liscum[67] have proposed that NPH3 may represent a scaffold/adapter protein, similar to those involved in MAP kinase cascade[68], to which phototropin and probably other signalling proteins interact to generate a phosphorelay system (Figure 2). However, actual experimental evidence to support this presumption is awaited.

Besides the components identified directly through mutant analysis, involvement of Ca^{2+} channels in phototropism pathway has come to light recently[69]. Employing transgenic *Arabidopsis* and tobacco plants expressing aequorin (as a Ca^{2+} indicator), blue-light was shown to stimulate a transient increase in cytosolic Ca^{2+} levels. This rapid increase, however, was considerably reduced in the *nph*1 mutant though not in *cry*1 or *cry*2 mutants[69]. With the help of laser confocal microscopy and using fluorescent probes, Gehring *et al.*[70] had also shown a light-induced increase in cytosolic Ca^{2+} and lowering of cytosolic pH of cells of the shaded side of maize coleoptiles. It clearly indicates that Ca^{2+} redistribution is indeed one of the early steps involved in phototropism response cascade initiated by nph1. Whether phosphoproteins like NPH3 affect the Ca^{2+} channels to alter Ca^{2+} homeostasis will be interesting to decipher. It must be mentioned, however, that blue-light-mediated phosphorylation of nph1 is not Ca^{2+}-dependent[4].

One aspect that needs deliberation here is, whether phototropin is involved only in regulating phototropism or, like cry1 and cry2, does it regulate some other responses as well? Surprisingly, the phototropism mutants have not been analysed critically for aberrations, if any, in other blue-light-mediated responses, except that they retain normal stem growth inhibition response. Recently, however, Kaufman and coworkers have implicated the role of phototropin (nph1) in *CAB* gene expression, although it is unrelated to phototropism. In normal, wild-type *Arabidopsis* seedlings, the *CAB* gene expression is stimulated both by low-fluence and high-fluence blue-light, but *nph*1 mutant seedlings are specifically impaired in high-fluence blue response[71]. Moreover, the presence of nph1 or related proteins in primary leaf of wheat seedlings[65] and pea buds[61] also implies that phototropin may perform additional functions not suspected previously.

*Cryptochromes and phytochromes modulate phototropin (nph*1*) activity*

The studies on cryptochrome and phototropin mutants have shown that in addition to blue and UV-A radiation, wavelengths in the green region also photoactivate these

sensory receptors for eliciting diverse responses. In addition, red light has been known for long to influence, though indirectly, the magnitude of the phototropic response. The seedlings pre-irradiated with red light, usually develop an exaggerated phototropic curvature in response to unilateral blue-light[72]. The availability of various species-specific phytochrome mutants has been helpful in deciphering that phytochrome A is involved in low-fluence range and either phytochrome A or B is required for enhancement of phototropic curvature by high-fluence red light[73]. Besides the involvement of phytochromes, the *Arabidopsis* transgenics overexpressing *CRY*1 or *CRY*2 genes were found to be hypersensitive to blue-light for phototropism[74] and, in addition, the magnitude of the phototropic response of the *cry*1*cry*2 double mutant seedlings was reduced significantly, although either single mutant displayed normal phototropism[74,75]. Comparing the results obtained both with the cryptochrome and phytochrome mutants, one is tempted to conclude that phototropin is the primary photoreceptor for phototropism, whereas both cryptochromes and phytochromes may modulate its sensitivity to ultimately influence the magnitude of the response (Figure 2).

Zeaxanthin – A probable chromophore sensing blue-light for stomatal response

In addition to phototropism and stem growth inhibition, stomatal movement is another blue-light response that has been investigated in sufficient detail[76]. However, stomatal movement is not adversely affected by mutations in *CRY*1, *CRY*2 or *NPH*1 genes[12]. In recent years, Zeiger and coworkers have demonstrated a role for zeaxanthin, a chloroplastic carotenoid of the xanthophyll cycle, in blue-light-mediated responses of the guard cells and chloroplasts, in addition to phototropism[6,7]. However, the involvement of zeaxanthin, at least in the phototropic response, has been strongly contested by the Briggs' laboratory[77]. In contrast, the correlative evidence for the blue-light-mediated changes in the guard cell zeaxanthin levels and the extent of stomatal opening is convincing[6]. Moreover, the *Arabidopsis* mutant, *npq*1 (*n*on-*p*hotochemical *q*uenching 1), that is unable to convert violaxanthin to zeaxanthin[78], also shows negligible stomatal response to blue-light[79]. While these data suggest that zeaxanthin may act as the chromophore for stomatal opening, some more supportive evidence has come from the work on chloroplast movement in *Lemna trisulca*[80], where, a parallel increase between zeaxanthin and chloroplast movement was observed on irradiation with high-fluence blue-light.

As to the mechanism of stomatal opening, it is known that blue-light activates guard cell plasma membrane H^+-ATPase and the resultant hyperpolarization causes the influx of K^+ ions, eventually leading to increase in turgour of the guard cells and stomatal opening[76]. How exactly blue-light activates H^+-ATPase, remained unresolved for quite some time. But, recently, Kinoshita and Shimazaki[81] have demonstrated that blue-light activates the H^+-ATPase pump in the plasma membrane by phosphorylating its C-terminus through a serine/threonine protein kinase. Whether this phosphorylating activity itself is a property of the chromoprotein (the apoprotein attached to zeaxanthin) sensing blue-light or it constitutes a downstream component (Figure 2), will be interesting to resolve.

Conclusion and future prospects

The past decade has witnessed the emergence of multiple blue-light sensory receptors, like cry1, cry2 and phototropin, which govern specific subsets of responses in higher plants. There appears to be some functional redundancy between cry1 and cry2, at least for regulating hypocotyl growth inhibition, cotyledon expansion, anthocyanin accumulation and modulating phototropin sensitivity for phototropic response. In contrast, phototropin is mainly involved in regulating phototropism although the possibility of its role in other blue-light-mediated responses cannot be ruled out. It is intriguing that the cryptochromes like cry1, in addition to MTHF, harbour FAD as the chromophore, whereas phototropin (nph1) makes a stable adduct with FMN at a cysteine residue. What selective advantage does it provide to have FAD or FMN as the flavin chromophore and whether it has any evolutionary significance, will be interesting to decipher. In addition to crytochromes and phototropin, there is convincing evidence for the role of zeaxanthin, a carotenoid, in stomatal movement, but the existence of additional blue-light receptors is also a distinct possibility. Although much is known about the signalling cascade operative in the stomatal response, which probably is initiated by photoexcitation of zeaxanthin, very little is known about the components acting downstream to cry1 and cry2. But, their similarity with animal and microbial cryptochromes, nuclear localization and protein–protein interaction with phytochromes suggests that they may interact with the DNA, directly or indirectly, to regulate gene expression on photoexcitation with blue-light. Although there is a possibility that cryptochromes may involve the signalosome (COP9) complex in modulating gene expression, the real experimental evidence is lacking. The major challenge, however, would be to explain how the nuclear localized cry1 and cry2 initiate the early depolarization events in mediating stem growth inhibition response. At least for phototropin, two interacting partners, NPH3 and RPT2, which may participate in the phosphorelay,

have been identified through mutant analysis. The identification of more such components either by mutant analysis or through yeast two-hybrid screens will probably be the focus in the coming decade, not only for identifying components for phototropism cascade, but also for cryptochromes. This will, however, become possible only after saturation mutagenesis is achieved in the signalling pathways mediating specific blue-light responses. The completion of the *Arabidopsis* genome project in the near future will indeed facilitate cloning and help in assigning functions to the genes defined by the mutated loci.

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ACKNOWLEDGEMENTS. The work in the author's laboratory is financially supported by the Department of Biotechnology, Government of India.