Review

Novel light-activated protein kinases as key regulators of plant growth and development[§]

S C MAHESHWARI[†], J P KHURANA* and S K SOPORY

International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi 110 067, India
**Department of Plant Molecular Biology, University of Delhi South Campus, New Delhi 110 021, India*

[†]*Corresponding author (Fax, 91-11-6162316; Email, maheshwarisc@hotmail.com).*

Plants have evolved highly sensitive sensory photoreceptor systems to regulate various aspects of their growth and development. Many responses such as seed germination, flowering and dormancy are controlled by red and far-red regions of the solar spectrum through the phytochrome family of photoreceptors. However, several other responses such as stem growth inhibition, phototropism and opening of stomata are controlled by blue and/or ultraviolet light absorbing photoreceptors called cryptochromes and phototropin.

Despite their central role in plant biology, the mode of action of these photoreceptors has been shrouded in mystery. Even the biochemical isolation of a photoreceptor, as in the case of phytochrome was accomplished decades ago, did not help in elucidating the mechanism of action. Nevertheless, due to advances in recombinant DNA technology, generation of extensive databanks and the capability to predict function by base sequence analysis, a breakthrough has now come about. It is clear that certain phytochromes, at least in the cyanobacteria and algae which represent the simplest plants, are hybrid photoreceptor-cum-kinases. These novel kinases utilize captured photons rather than conventional ligands to trigger conformational change and in consequence enzyme activity. The kinases apparently, then, cause phosphorylation of many other types of target molecules, leading eventually to various developmental changes. There is suggestive evidence that in higher plants, too, at least some phytochromes may operate as kinases.

As compared to work on phytochromes, the blue light photoreceptors have begun to be studied only recently. However, the exciting discovery has been made of at least one photoactive kinase that is critically required for phototropism.

This article summarizes the above discoveries from the perspective of general biology.

1. Introduction

If one compares the mode of development of plants with that of animals, one striking fact which is immediately apparent is that light exercises a profound effect on plant growth and development. This should not be surprising because rooted as plants are, they have had to develop special mechanisms to withstand and survive the vagaries of nature and the constantly changing environment – through the daily cycle as also the seasons. Moreover, in order to time and control various processes, such as breaking of dormancy of seeds and their germination, resumption of bud growth, extension of hypocotyls and stems, phototropism of seedling shoots, expansion of leaves, and onset of flowering, plants had to evolve mechanisms to sense not only the quality and quantity of light, but even its direction. As can be readily appreciated, these effects of light are quite distinct from those in photosynthesis. In the control of plant growth and development, light operates catalytically (the role is an

Keywords. *Arabidopsis* (mutants); cryptochromes; photomorphogenesis; phototropism; phytochromes; protein kinase; sensory photoreceptors

[§]Dedicated to the memory of Drs Harry Borthwick, Sterling Hendricks and James Bonner whose classical studies paved the way for modern researches on mechanism of action of plant photoreceptors and whom the senior author was previleged to know.

informational one), whereas in photosynthesis the energy of photons is converted stoichiometrically into chemical energy. Also, whereas photosynthesis largely relies on chlorophylls and carotenoids, plants utilize a distinct set of sensory photoreceptors for regulating their development. The developmental effects mentioned above are due largely to phytochromes [absorbing red (R) and far-red (FR) light]. On the other hand, cryptochromes [absorbing blue (B) and ultraviolet (UV) light] mediate such responses as phototropism and opening of stomata. In certain phenomena, like control of extension growth of hypocotyls and stems, entrainment of circadian clock, and even flowering in certain families (such as the Cruciferae to which *Arabidopsis* belongs), both phytochromes and cryptochromes play a co-operative role (see Kendrick and Kronenberg 1994; Guo *et al* 1998; Somers *et al* 1998).

How does this catalytic control operate in plants? This is what we wish to address in this article. Although we shall briefly review the major developments leading to the identification of various photoreceptors, the focus here will be on recent studies that paved the way for the discovery and involvement of an entirely new class of protein kinases which appear to be regulated by light. The novelty is that photoreceptors themselves may have kinase activity as well.

2. Discovery of R, FR and B/UV absorbing photoreceptors

2.1 *Discovery of phytochromes*

2.1a *Action spectra and the revelation of R/FR effects*: The studies that eventually led to the discovery of phytochrome in fact began in 1920s when the concept of photoperiodism was enunciated by Garner and Allard. Then, a decade later, the observation was made by Hamner and Bonner that in short-day plants a brief light pulse given during the critical night period completely nullified its flower-inducing effect. These findings led in the fifties to detailed action spectra studies by Borthwick, Hendricks and their co-workers at United States Department of Agriculture (USDA) in Beltsville, USA. A pronounced and striking effect of the R light region of the spectrum was confirmed, but it was observed simultaneously that the FR light given immediately following red irradiation, reversed the inductive effect of R light, and had the same effect as darkness over a longer period (some hours). This led them to propose the involvement of a special pigment, now called phytochrome, that exists in two photointerconvertible forms, Pr and Pfr, and regulates plant development as shown in figure 1 (for an excellent treatment of the classical researches and references see Sage 1992).

Although there is as yet no real proof, the Pfr form has long been considered the active form because not only most of the classical effects of light are brought about by R light and thus by the Pfr form, but several estimates of photoconversion show that even as little as one-hundredth of the total population of Pr molecules converted to Pfr can initiate a particular effect. Since in ordinary sunlight, in nature, there is far more R than FR, it is thought that the direction of net photoconversion at the end of day is from Pr to Pfr. The reverse would then happen at night and it appears that the relative length of day and night is reflected in the changing Pfr/Pr ratio which in turn regulates plant development.

2.1b *Purification and chemistry of phytochrome*: During the sixties and seventies, phytochrome was purified from several plants and photoreversibility of this pigment was demonstrated by many workers not only *in vivo* but also *in vitro*. As shown in figure 2A, the Pr and Pfr forms of phytochrome have maximum absorption at 666 and 730 nm, respectively. Since chlorophyll pigments strongly interfere in such assays, almost all these studies utilized dark-grown, etiolated seedlings. The phytochrome molecules are of a rather large size – the polypeptide is of a molecular mass of about 124 kDa (see Quail 1997a and other reviews in Smith 1997), but they exist *in vivo* as dimers with an open-chain tetrapyrrole chromophore attached to each monomer in the N-terminal part (see figure 2B). The chromophore undergoes a cis-trans type of conformational change upon conversion of Pr into Pfr (figure 3). Progress in unravelling the mechanism of action of phytochrome, however, was very slow until in the last decade when the advent of the recombinant DNA techniques allowed rapid strides to be made. Through cloning and gene sequencing techniques, we now know the deduced amino acid sequences of phytochrome molecules of various plants. Transformation techniques have allowed phytochrome genes to be transferred from one plant into another – either intact or after various manipulations such as deletion of a part of the gene. These studies show that even though the N-terminal half of the molecule is adequate for phototransformation, the biological activity resides largely in the C-terminal half (figure 4; for further details see Quail 1997a).

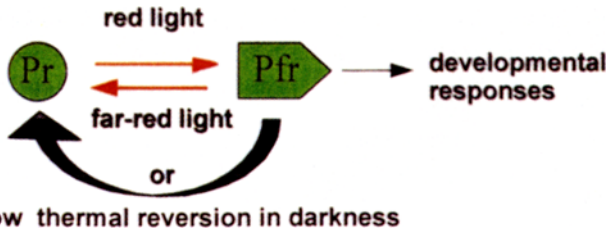

Figure 1. The scheme of phytochrome photoconversions and action.

2.1c *Multiple phytochromes*: One specially interesting outcome of the use of recombinant DNA technology and analysis of genomic libraries of several plants is that there are multiple phytochrome genes in higher plants as against only one or two in the lower plants (see Mathews and Sharrock 1997). In *Arabidopsis thaliana*, five different phytochromes have been characterised: PhyA, PhyB, PhyC, PhyD and PhyE. The N-terminal region is by and large similar among them as judged from the deduced amino acid sequences, but the C-terminal ends are somewhat variable (for a review with emphasis on evolution, see Pepper 1998). To summarize, there is a family of phytochromes rather than just one or two as was generally believed till the early eighties.

2.2 *Discovery of cryptochrome*

2.2a *Some developmental effects are solely due to B/UV light*: As mentioned earlier, certain phenomena like phototropism and opening of stomata are regulated specifically by B light. At this point, one can ask: What is the nature of the B light absorbing photoreceptor? Since phytochrome also absorbs B light to some extent, it has always been somewhat problematic to distinguish whether a particular response is due to phytochrome or a B light photoreceptor. However, a lot has been learnt since Darwin made the original observation of plants bending towards light way back in 1881. Many action spectra studies have been made – not only of phototropism but

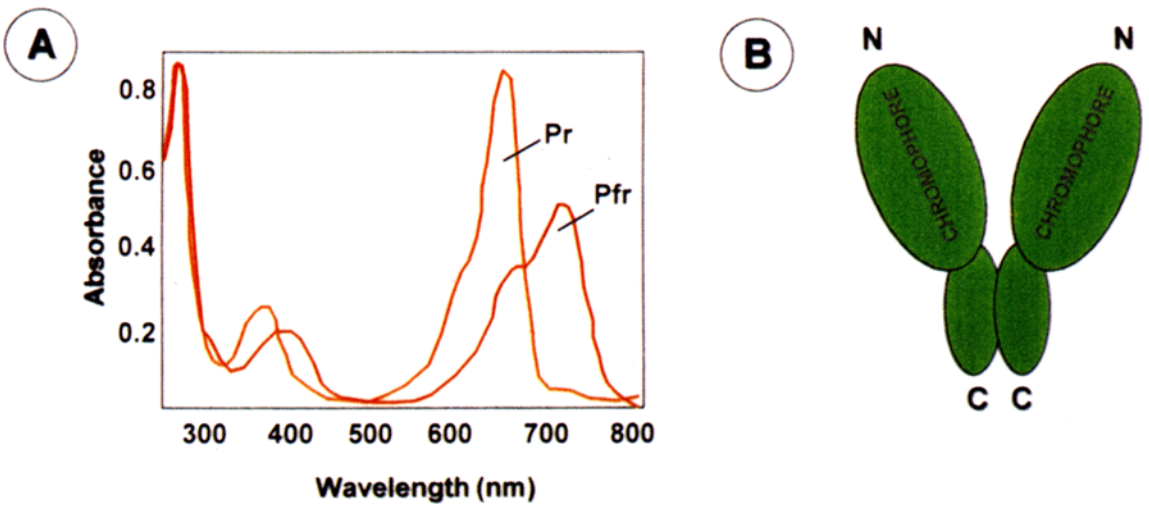

Figure 2. (**A**) The absorption spectra of the Pr and Pfr forms of phytochrome. (**B**) Model of the phytochrome dimeric molecule.

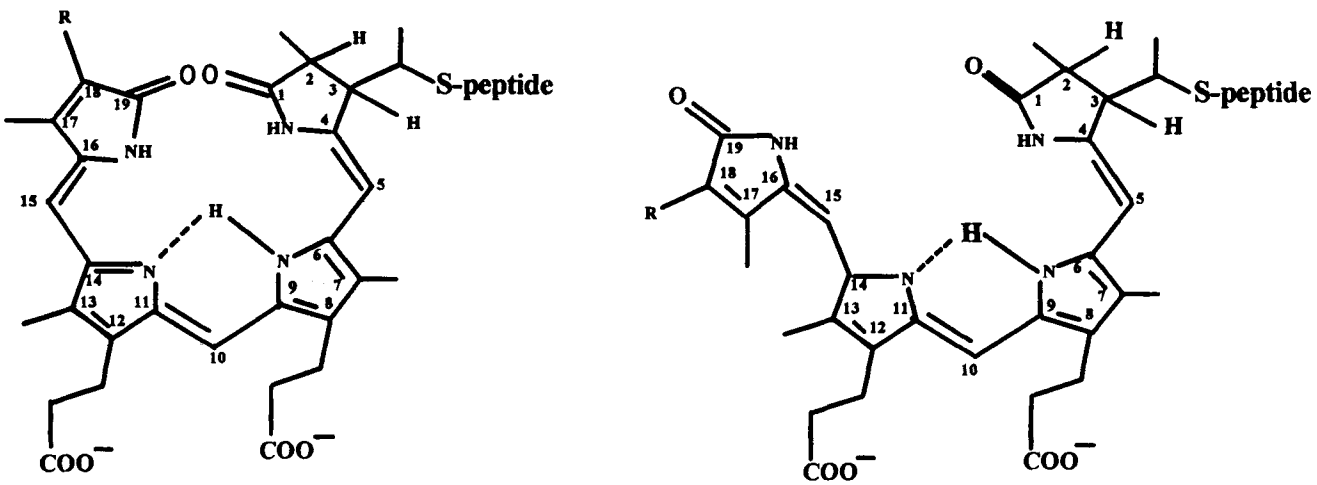

Figure 3. The proposed structure of chromophore. On the left is the structure in Pr and on the right in Pfr form.

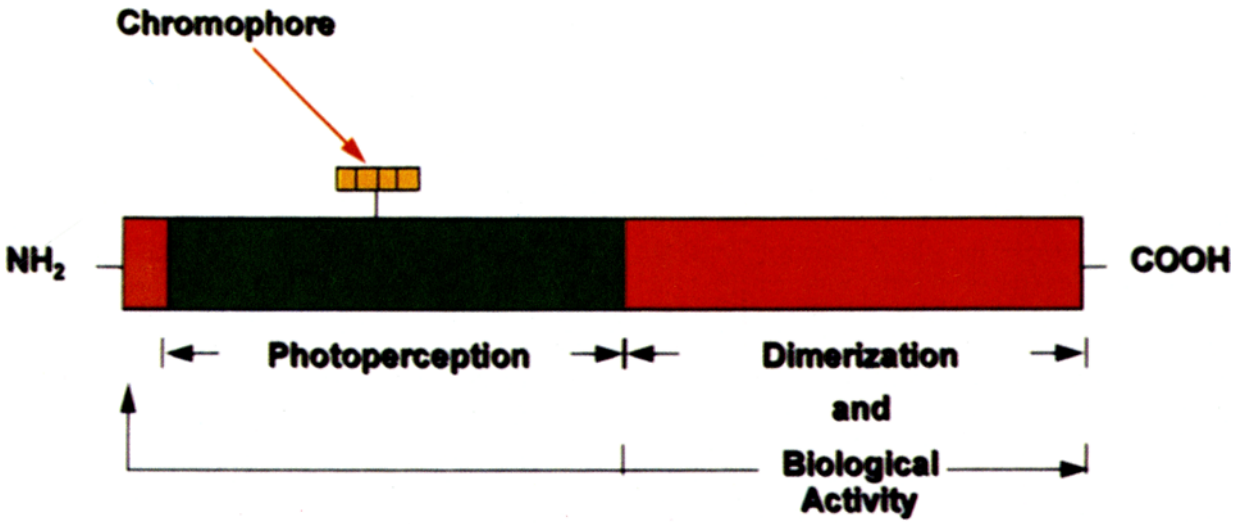

Figure 4. The general structure and functional domains in a typical phytochrome molecule.

of stomatal opening, hypocotyl growth, and other responses – and it is clear that some of these are brought about by B light, either specifically or quite independent of R or FR light, providing firm and unequivocal evidence that a distinct B light photoreceptor must exist (figure 5). Evidence has now come for a family of B light photoreceptors, named as cryptochromes, which may to some extent absorb UV light too (see Cashmore 1997; Khurana *et al* 1998). The name cryptochrome was coined in the seventies because of the cryptic (hidden) nature of the pigment responsible for eliciting B light-mediated responses.

The earliest biochemical study of light absorption by the tissue active in phototropism (oat coleoptile tips) was made in mid-thirties by Wald and DuBuy (1934) who proposed that the chromophore was probably a carotenoid. But in 1949 Galston proposed that a flavin could be the photoreceptor (figure 5). This was based upon the finding that riboflavin sensitized the photooxidation of the plant growth hormone, auxin, IAA, in crude plant extracts, with the action spectrum for this *in vitro* reaction closely matching that of phototropism. The flavin proposal was attractive because it was quite consistent with the Went-Cholodny hypothesis of asymmetric distribution of auxin bringing about bending of coleoptiles due to unilateral light (enhanced destruction of IAA on illuminated side would cause lowering of auxin level and inhibition of growth). In reality, however, there has been considerable controversy all these years whether a carotene or a flavin is the candidate chromophore. The controversy has just begun to clear up now.

2.2b *Work on Arabidopsis mutants and the discovery of CRY1 and CRY2 photoreceptors*: The use of molecular genetic tools has brought about a revolution in our

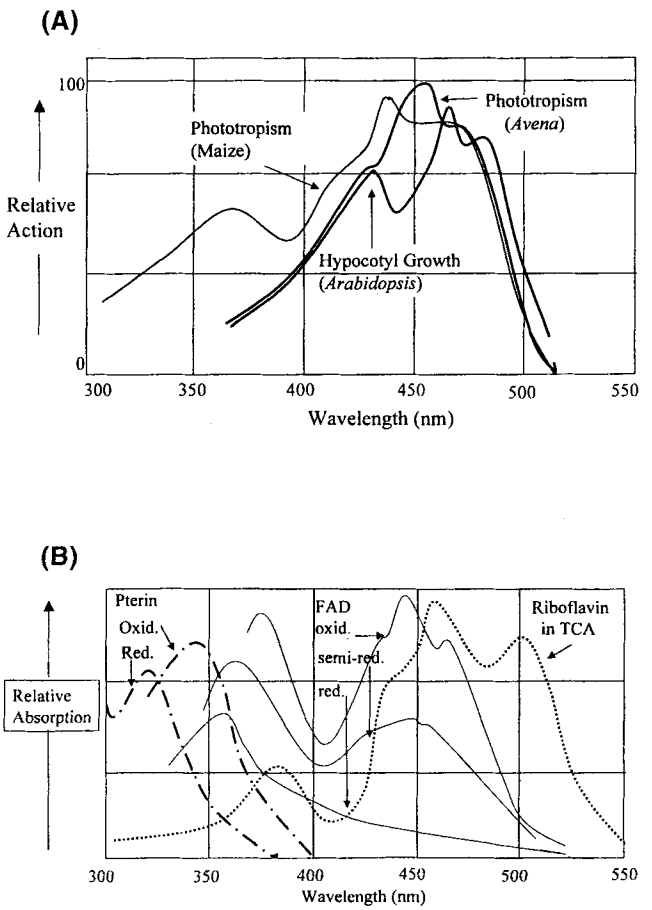

Figure 5. Comparison of action spectra (**A**) of a few typical blue light mediated responses with absorption spectra (**B**) of pterins and flavins (the spectra of both the reduced and oxidized forms are given). The spectrum of riboflavin on the basis of which the original proposal of a flavin-binding blue light photoreceptor was made is also shown.

understanding of B light perception and action mechanism. The advances are to a large extent the result of use of *A. thaliana* as a model plant. The decisive role was played by mutants lacking one or the other well-established blue light responses. Work on obtaining phototropism mutants of *Arabidopsis* was begun by Khurana and Poff (1989). These mutants, called JK mutants, lacked the phototropic response either partially or totally. Similar work was later undertaken by Briggs and coworkers (Liscum and Briggs 1995; see also Briggs and Liscum 1997).

While the groups of Poff and Briggs concentrated on phototropism, Cashmore and coworkers analysed mutants of *Arabidopsis* impaired in B light-induced suppression of stem growth extension. A long-hypocotyl mutant, called *hy*4 (originally isolated by Koornneef *et al* 1980), found to be insensitive to B light for hypocotyl growth inhibition, was employed by Cashmore's group.

The work on *hy*4 mutant will be discussed first in some detail since definitive identification of the first B light photoreceptor, CRY1, was done employing this mutant (Ahmad and Cashmore 1993). To isolate the photoreceptor gene, a T-DNA tagged mutant was employed which was allelic to the original EMS-induced *hy4* mutant. The gene was then identified and cloned by marker rescue and found to encode a 75·8 kDa protein of 681 amino acids. The most crucial evidence relating to the identity of chromophores associated with the CRY1 apoprotein has come by sequence homology search in protein databases. The gene has turned out to be extraordinarily interesting as a long stretch of the encoded product – of about 500 amino acids – at the N-terminus has high sequence identity to microbial DNA photolyases that have been known for some time to carry out light-dependent cleavage of cyclobutane ring between pyrimidine dimers formed by UV light and thus repair the damaged DNA (the real plant DNA photolyase is coded by a different gene; Batschauer 1993). In fact, in the eighties two types of DNA photolyases had been cloned from several organisms (see Sancar 1994). The homology of CRY1 protein is higher with the long-wavelength photolyase which is known to bind both a flavin and a deazaflavin. However, a fusion protein harbouring photolyase-like domain of CRY1 when expressed in *Escherichia coli* binds a pterin, characteristic of short-wavelength photolyases (Malhotra *et al* 1995); a diagrammatic representation of the structure of CRY1 photoreceptor is shown in figure 6. Whether the native CRY1 protein of *Arabidopsis* does indeed bind a pterin remains to be verified. Nonetheless, current thinking is that although flavin is primarily responsible for the photoreceptor action, pterin (or any other second chromophore) serves as an antenna (extending light absorption in the near UV region) and passes on energy of captured photons to the flavin (figure 7), much like LHC or CAB complexes pass excitation energy to reaction centre chlorophylls of PSI and PSII complexes during photosynthesis.

The use of *CRY* gene probe has allowed the search for similar genes not only in *Arabidopsis* but also in other plants, e.g., the crucifer *Sinapis alba*, where the presence of both a flavin and a pterin chromophore has been confirmed (Malhotra *et al* 1995). It appears that CRY represents a family of blue absorbing photoreceptors with highly conserved N-termini, but displaying variations at the C-terminal end (as is the case with phytochromes). In *Arabidopsis* itself, a second gene (*CRY*2) has been discovered encoding a protein whose N-terminal region has high homology with that of CRY1, but such is not the case with the C-terminus (figure 6; Lin *et al* 1996). It appears to have an overlapping function in B light responses, although certain differences (CRY2 protein is very light labile) are indicative of the essentiality of both genes for a plant (Ahmad *et al* 1998a).

2.2c *Photoreceptors for phototropism*: Since cryptochromes were found to mediate several B light-dependent responses, it became imperative to find out if they do mediate phototropic response as well. Initial experiments by Cashmore and coworkers provided answer in the affirmative because although each *cry* mutation by itself does not affect phototropism, in the *cry1/cry2* double mutant, the response is severely inhibited (Ahmad *et al* 1998b). Moreover, the transgenic plants overexpressing CRY1 and CRY2 show accentuated phototropic response. But, as it will become apparent from the later discussion, the subject is in a state of rapid flux and, in fact, there is now evidence for yet another photoreceptor involved in phototropism.

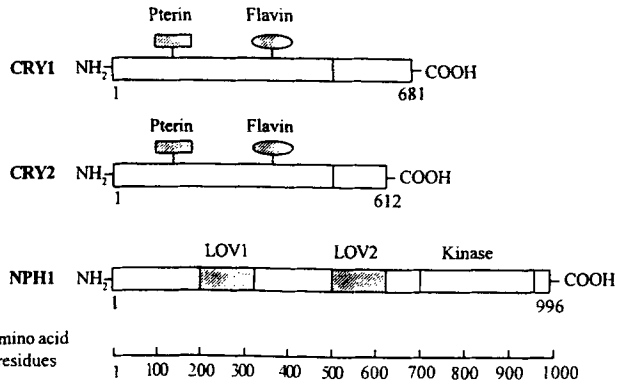

Figure 6. Comparison of structures of CRY1, CRY2 and NPH1 proteins which are key elements in blue light perception and signal transduction.

2.3 *Chimeric sensory photoreceptors*

Besides existence of multiple phytochromes and cryptochromes in diverse species with an independent identity, evidence has recently emerged supporting the existence of an as yet unexpected class of *chimeric* blue and R/FR receptors. A detailed characterization of a novel phytochrome from the fern *Adiantum* has shown that its N-terminal end is like true phytochromes, but the C-terminus is almost identical to the full-length NPH1 homologue (which includes the unique LOV1 and LOV2 domains), suggesting that this phytochrome can mediate actions brought about by both red and blue light (Nozue *et al* 1998). Another tantalizing finding is the reported existence of phytochromes in such organisms as the purple bacteria, and myxomycetes (slime moulds). The phytochrome of the purple bacterium, *Rhodospirillium centenum*, is again extraordinary in the sense that although it is very much like conventional phytochromes, at least in the central region where the bilin chromophore is normally attached and the histidine-like C-terminal region as in the blue-green algae, an additional N-terminus sequence bears a covalently attached p-hydroxy-cinnamic acid moiety which absorbs blue light instead of red light (Jiang *et al* 1999)! This is yet another example of chimeric molecules, where functions of the two photoreceptors are combined in one molecule.

3. Mechanism of action of photoreceptors

How do these photoreceptors work, is the question that has long engaged the attention of a large number of investigators. To obtain clues, many efforts have been made to determine, specially, the intracellular localization of phytochrome. In algae as also in many other plants, the orientation of the chloroplast is controlled by phytochrome to enable it to intercept light optimally. Haupt and coworkers (see Haupt 1970) studied the effect of microbeam irradiations on chloroplast orientation of the green alga *Mougeotia*. They found that irradiation of the peripheral regions of the cell, rather than of the chloroplast itself, was critical for the response. However, if polarized R or FR light was used, it was observed that the polarizer had to be turned by an angle of 90° in order to achieve R/FR reversibility effects. This lead to the conclusion that the phytochrome molecules are intimately associated with a membrane (probably the plasma membrane), and they specifically reorient when converted from one form to the other while still associated with the membrane. Similar conclusions have been reached by a study of phototropism in mosses where the positive response of the aerial protonemal tips is critically dependent on the plane of polarization of the R or FR light beams (see Hartmann and Weber 1990; Hughes and Hartmann 1999). Yet, unfortunately, compelling as all

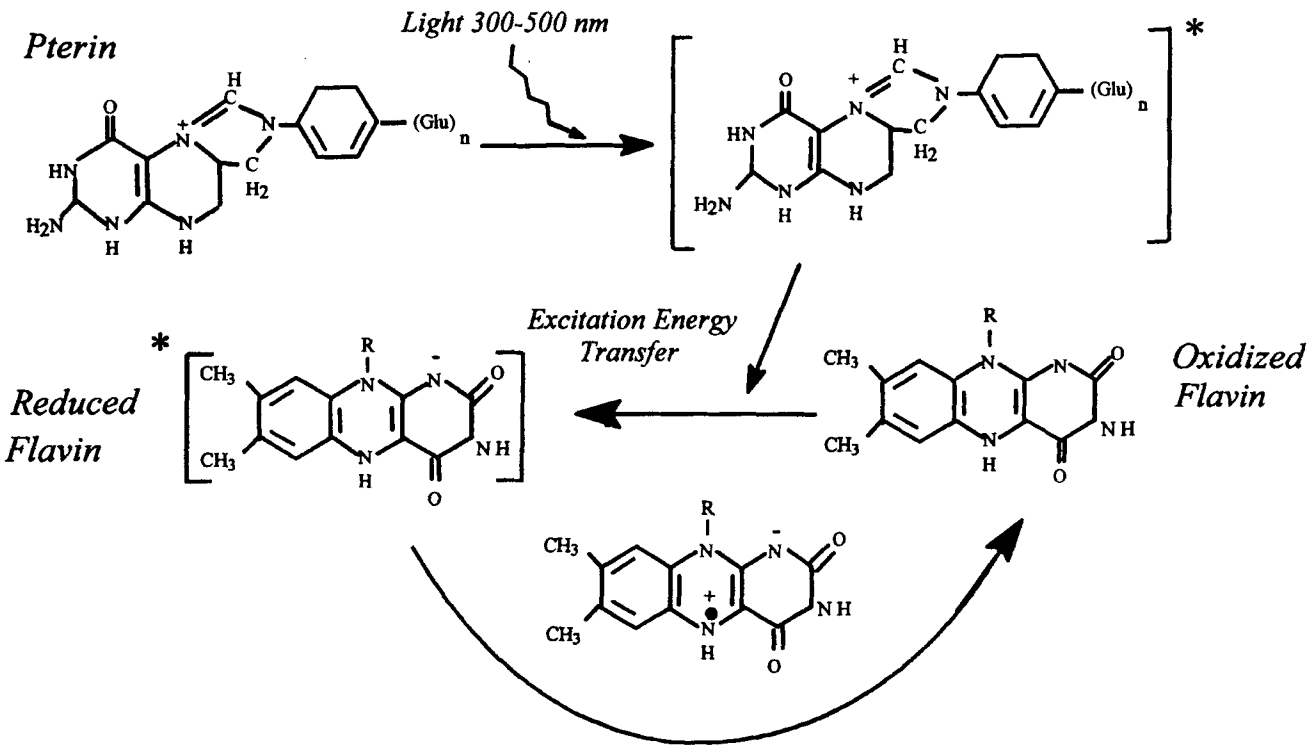

Figure 7. The structure of pterins and flavins and the possible manner in which photons captured by these chromophores may initiate physiological action.

these results are indicative of a membrane localization, they are in sharp contrast with those obtained by investigators who have employed biochemical or histochemical approaches in different systems and suggested phytochrome to be distributed largely in the cytoplasm, although sometimes also in organelles such as mitochondria, chloroplasts and nuclei (see Pratt 1994; Sakamoto and Nagatani 1996; Kircher *et al* 1999). A general conclusion has therefore been difficult to draw concerning the biochemical basis of these responses.

However, the advances in the field of general biochemistry have had a major impact in unravelling the mechanism of action of both R/FR and B light photoreceptors. Through the work in the last two decades, it is now known that protein kinases are among the key regulatory molecules in a living cell (see Sopory and Munshi 1998). A protein kinase phosphorylates a protein changing its conformation and works in close conjunction with a protein phosphatase which removes the phosphate group. The kinases are now known to be involved in such diverse activities as action of hormones (through receptor signalling), transport of ions and molecules across membranes, modulation of enzyme activity and regulation of gene activity. Certain target molecules may have multiple sites of phosphorylation: for example, the CTD tail of RNA polymerase II can have as many as 50 phosphate groups attached to it. Further, the protein kinases, which are of several kinds, are themselves affected by a number of ligands and second messengers like Ca^{2+}, cAMP, cGMP and DAG. They can be broadly classified into several superfamilies and groups. One superfamily embraces cAMP, cGMP, Ca^{2+} and calmodulin-activated protein kinases. Another superfamily is made up of the so-called MAP kinases and yet another comprises the receptor kinases. An interesting recent development is that at least some members of the phytochrome family are indeed protein kinases. In case of cryptochromes, again, there is a distinct possibility that certain members of the family may work by associating physically with a kinase or be kinases themselves. These kinases are novel in that instead of chemicals or ions (such as Ca^{2+} and cAMP), a photon plays the role of a 'ligand'. The recent developments providing convincing evidence for this are discussed below.

3.1 *Phytochrome as a protein kinase*

3.1a *Involvement of Ca^{2+} and cGMP in phytochrome action*: Since there are several phytochromes in higher plants and despite a strong probability there is as yet no real proof that all phytochromes must be kinases, we must mention briefly other findings and proposals that have been made to explain phytochrome action. Thus, one proposal that has long existed in the literature is that the phytochrome may control membrane permeability, for example, transport of specific ions, through modulation of activity of ion channels. Leaf movement in legumes is controlled by osmotic pressure and turgor changes in pulvinal cells, which itself is under control of phytochrome. The ion which has received the maximum attention is Ca^{2+}, in view of its well-established role as a second messenger in animals. Much work has been done on protoplasts isolated from dark-grown seedlings and the R light-induced influx of Ca^{2+} ions and efflux in response to FR is among the most rapid effects known of phytochrome transformation (see Roux 1994; Mehta *et al* 1993). In leaf pulvinal cells, the bulk movement is of K^+ ion (see Sage 1992) but even this may be primarily controlled by Ca^{2+} ions, as Ca^{2+}-regulated K^+ channels are now well-established in living organisms (see Aidley and Stanfield 1996). In addition to Ca^{2+}, which may generally work through calmodulin, there is also evidence for cGMP as a mediator of certain phytochrome-controlled responses (see Bowler and Chua 1994).

However, the question that still remains unanswered is how does phytochrome alter levels of cGMP or Ca^{2+}. One possibility is that phytochrome may interact with a G-protein, which could then activate an enzyme such as guanyl cyclase or phospholipase C. Considering the example of phospholipase C, the hydrolyzed products of its action, IP_3 and DAG, can then bring about a variety of effects including not only increase of cytosolic Ca^{2+} but also activation of protein kinases (Sopory and Chandok 1996). As to influx of Ca^{2+} from outside the cell, the mechanism is not yet known, but the interaction of phytochrome with a G-protein could in turn open a Ca^{2+} channel. However, no phytochrome-specific G-protein has been found yet and, thus, alternative mechanisms for light-regulated protein kinases deserve serious consideration.

3.1b *Proposal of light-regulated protein kinases*: That the phytochrome has some role to play in regulating phosphorylation came to light, first through work on pea in Roux's laboratory (Datta *et al* 1985), who showed that R irradiation caused phosphorylation of three nuclear proteins. This was followed by another report by Otto and Schaeffer (1988) who also found rapid changes in phosphorylation induced by R light of certain proteins in *Avena coleoptiles*. In both these studies, the effect of R was reversed by FR, implying that some protein kinase activity is closely associated with phytochrome transformations.

But, more interestingly, claims of a phosphorylation activity associated with phytochrome itself and possibly leading to autophosphorylation of phytochrome and resultant protein kinase activity came from Lagarias and co-workers (Wong *et al* 1986, 1989; Wong and Lagarias 1989). The work was undertaken consequent to the findings that phytochrome itself is a phosphoprotein containing ~0·5 mol P per dimer (Hunt and

Pratt 1980) and there is a cluster of 8 serines at the N-terminus. However, subsequent work by others cast considerable doubt on the idea, even though association of protein kinase activity with semi-purified preparations of phytochrome was confirmed. Groups of both Song (Kim *et al* 1989) and Rudiger (Grimm *et al* 1989) reported that protein kinase activity decreased with increasing purification of phytochrome, implying that the results were a consequence of a contaminating protein kinase.

Crucial for reviving the idea of phytochrome having intrinsic kinase activity have been the contributions of Schneider-Poetsch, Thummler and their associates in Germany. The first group cloned and sequenced phytochrome genes of several lower plants (liverworts and mosses) employing PCR generated probes (Schneider-Poetsch and Braun 1991; Schneider-Poetsch *et al* 1991). What struck these workers is the close similarity of the C-termini of many of the phytochromes with the sensor-regulator domains that are part of the so-called "two-component" signalling systems that had been found earlier in many prokaryotes like *E. coli*, *Salmonella*, *Klebsiella* and *Rhizobium* (figure 8). The second group found that the C-terminus of phytochrome of one moss, *Ceratodon purpureus*, had similarity with the serine/threonine type of kinases (Thummler *et al* 1992). But, to appreciate better the emergence of this novel concept in higher plants, let us first consider signalling in bacteria.

3.1c *The bacterial two-component signalling system*: It will be beyond the scope of this article to go into the details of bacterial signal transduction (for recent reviews see Alex and Simon 1994; Wurgler-Murphy and Saito 1997; Chang and Stewart 1998). However, in brief, typically in bacteria there is a pair of sensor and response-regulator modules which, together, are responsible for sensing changes in environment (such as osmotic potential, chemoattractants) and responding appropriately to the signal through a battery of protein kinases analogous to the MAP kinase cascade. Basically, the sensing mechanisms are similar to what have been discovered in mammals and other higher eukaryotes. However, in contrast with the serine/threonine and tyrosine superfamilies of kinases, in bacteria the critical amino acids are histidine and aspartate. The sensor module has an "input" domain at the N-terminal end and a "transmitter" domain at the C-terminus. In the response regulator, on the other hand, there is a "receiver" domain at the N-terminal end and an "output" domain at the C-terminal end. Although considerable variations exist in the input domain (the system is geared to receive signals of many different kinds) and to some extent in the output domains (of the response regulator), there is a high degree of conservation in the transmitter and receiver domains. On sensing the signal (not all details of sensing mechanisms are however known yet), the histidine in the transmitter domain undergoes autophosphorylation and the energy-rich phosphate group is transferred immediately onto an aspartate in the receiver module of the response regulator, setting in motion a cascade of phosphorylations including those of transcription factors until gene activity is altered or induced (for example to synthesize more aquaporins in response to osmotic stress).

3.1d *More about lower plant phytochromes*: In phytochromes of lower plants it is the region spanning the last 250 amino acids at the C-terminal end which has a motif almost similar to that in the transmitter modules (figure 9). The N-terminal end bearing the chromophore must then pass the signal to the transmitter. In terms of direct experimental evidence, the idea of phytochrome

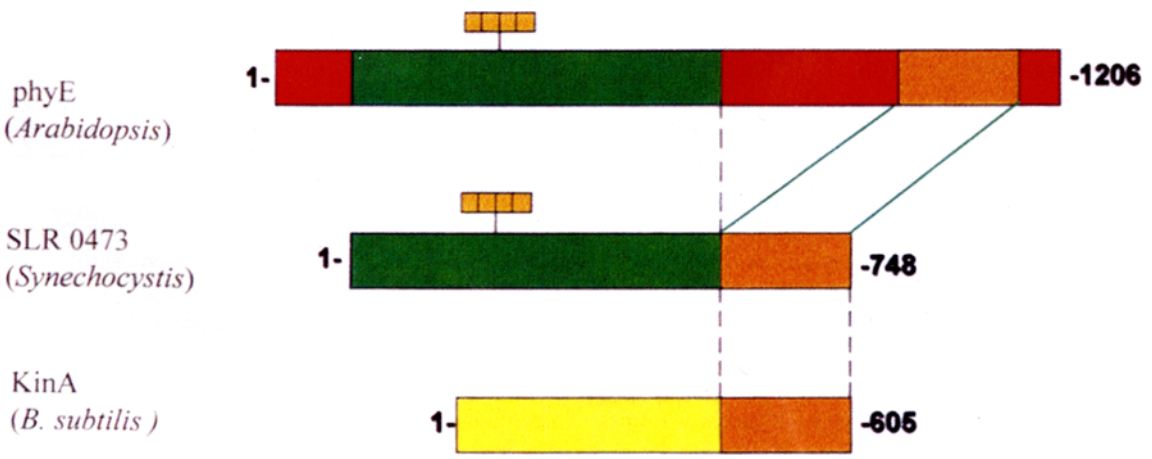

Figure 8. Comparison of phytochromes of a higher plant and an alga with bacterial histidine kinase. Dotted lines mark the regions of homology.

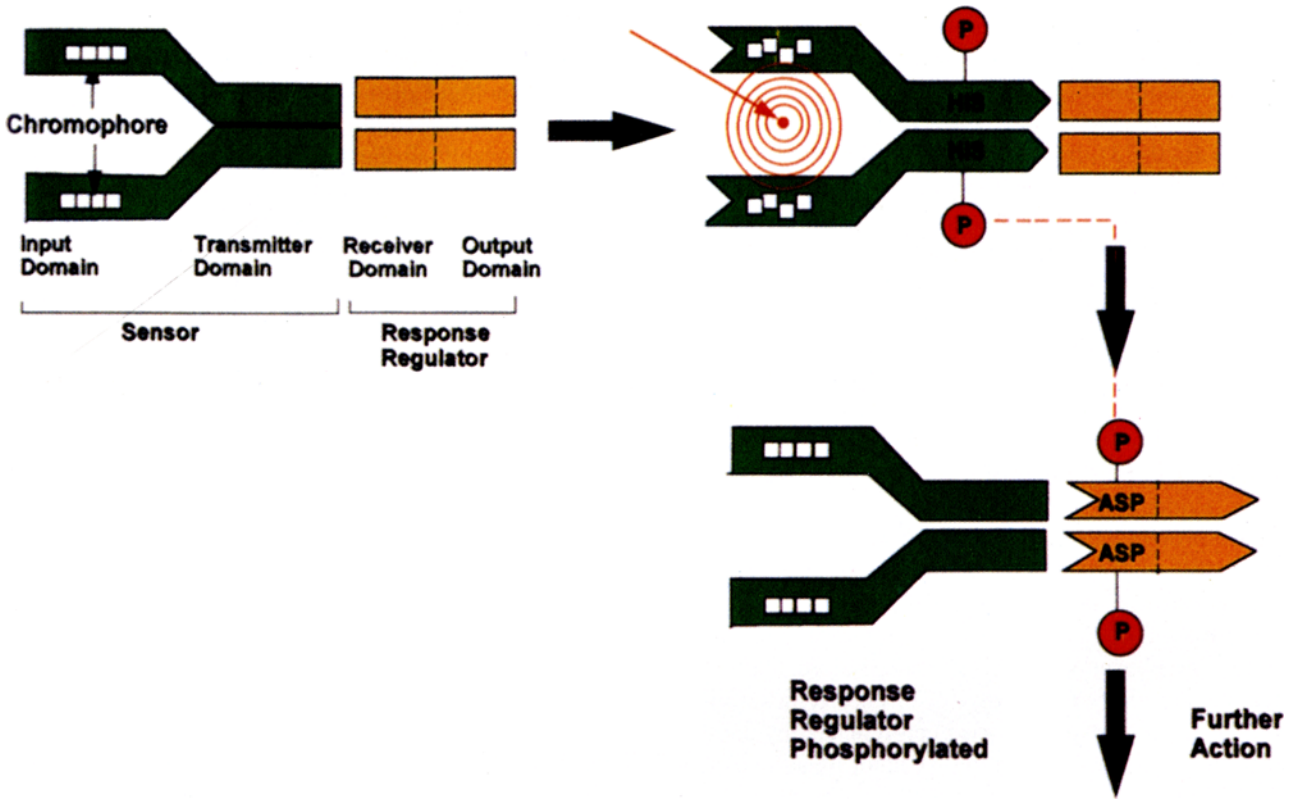

Figure 9. Diagrammatic representation of the mode of autophosphorylation of phytochrome. Phytochrome in its native form is a dimer and absorption of light leads to a slight alteration in chromophore structure which in turn causes a functionally more significant conformational change in the protein leading ultimately to the phosphorylation of a response regulator.

being a light-activated protein kinase received strong support by the work of Thummler *et al* (1992) who isolated a 140 kDa phytochrome from a moss, *Ceratodon purpureus*, and found considerable homology of the C-terminal end with conserved domains of many members of the serine/threonine/tyrosine protein kinase family (although no membrane spanning domains have been found in any phytochrome, *Ceratodon* phytochrome is closest to a tyrosine protein kinase of *Dictyostelium*)*. Further, this group has not only shown autophosphorylation of the 140 kDa phytochrome but even claimed *in vitro* R/FR reversible phosphorylation of several *Ceratodon* protonemata proteins, the target of phosphorylation being serine or threonine residues (Algarra *et al* 1995; Thummler *et al* 1995).

Since the proposal was first made by groups of Lagarias in eighties and then again by Schneider-Poetsch and Thummler at the beginning of this decade (references cited above), several years had gone by, but by and large there was considerable scepticism still persisting. However, in the last couple of years, compelling evidence has come that phytochrome is indeed a light-regulated kinase (for some other recent reviews see Allen and Matthijs 1997; Elich and Chory 1997; Quail 1997b; Reed 1998). Most crucial has been the work on two cyanobacteria, *Fremyella* and *Synechocystis*. The first to be investigated was *Fremyella* which is known for the phenomenon of chromatic adaptation, where in R light the organism largely synthesizes phycocyanin turning it bluish-green, but in green light it accumulates phycoerythrin turning it red (Chiang *et al* 1992). These pigments are involved in photosynthesis and the alga ensures that photosynthesis is driven maximally by synthesizing the right type of pigment under a particular light regime. The gene encoding a 74 kDa polypeptide responsible for sensing the light quality for chromatic adaptation was cloned by Kehoe and Grossman (1996) and was identified by its ability to complement mutant strains defective in adapting to spectral changes. Strikingly, the N-terminus of the putative polypeptide coded by this gene is similar to the chromophore domain at the N-terminus of phytochrome whereas the C-terminus has a

*It has to be noted, however, that this phytochrome may represent a minor species since Lamparter *et al* (1995), who have also studied this species, reported that the sequence of the predominant phytochrome is somewhat different.

high degree of homology to the histidine kinase domain of two-component sensor kinases.

The discovery of a similar protein, believed to be a true phytochrome, in *Synechocystis* has now come as a reward of genome sequencing study completed recently (Hughes *et al* 1997; Lamparter *et al* 1997; Yeh *et al* 1997). An ORF (SLR 0473) has been found that codes for a phytochrome and whose C-terminus is similar to a histidine kinase. Subsequently, phytochromes from both the cyanobacteria have been purified and R/FR reversibility shown *in vitro*. Since in *Synechocystis* gene manipulation techniques are well-developed, even the essentiality of histidine has been demonstrated – it has been found that its substitution by another amino acid by mutation results in loss of auto-phosphorylating activity (Yeh *et al* 1997).

What is specially interesting in *Synechocystis* is that there is evidence for the existence of even a response regulator protein whose ORF is only 10 nucleotides away from the phytochrome ORF. Both the phytochrome and the response regulator have been recently purified after overexpression in *E. coli*, and it is remarkable that the sensor can phosphorylate the response regulator by irradiation *in vitro* (Yeh *et al* 1997). Further, a response regulator in which aspartate has been replaced by another amino acid – through a mutation – cannot be so phosphorylated. However, it is intriguing that autophosphorylation of the histidine and subsequent transfer of the phosphoryl group to the aspartate is stimulated by FR rather than by R.

3.1e *The present scenario in higher plants and some speculations*: To summarize, the long-standing mystery of how phytochromes work has begun to be unravelled. To be able to respond to environment, plants evolved long ago a novel light-activated protein kinase where instead of ions like Ca^{2+} or small molecules such as cyclic nucleotides, photons serve as the 'ligands' for regulation of its activity (for a model of phytochrome action, see figure 9). In the meantime, the general idea of two-component "sensor-transmitter and response-regulator" system functioning in plants has received strong support also from work on mode of action of plant hormones – according to recent studies, ethylene and cytokinins also work through such a system (Kakimoto 1996; see Wurgler-Murphy and Saito 1997; Chang and Stewart 1998). Yet, to extend to higher plants the concepts developed from studies on simpler organisms and to really prove the idea of phytochrome being a light-activated protein kinase, a great deal of biochemical work will still be necessary. Since higher plants have been worked on for nearly five decades, whereas cyanobacteria and lower plants have been investigated only recently, this statement may sound rather strange. Yet, the difficulties and reluctance in accepting the kinase proposal arise for two reasons. Firstly, most phytochromes in higher plants do not have a histidine residue in the conserved region and in one that has – PhyA – its substitution by another amino acid does not make any difference. Secondly, the natural substrate that may be phosphorylated by phytochrome is not known and the homologue of the response regulator has yet to be identified and characterized.

However, with further work and some receptivity to new ideas a solution to our understanding of action of phytochromes of higher plants may not be far. Thus, one needs to bear in mind that in recent years new classes of kinases have been discovered which do not fit into any of the well-established categories (e.g., see Ryazanov *et al* 1997). It is entirely likely that in higher plants, instead of histidine, a serine is phosphorylated which too can be in a novel and a unique site. From work in Song's laboratory (Lapko *et al* 1997), it appears that Ser-7 in the cluster of serines at the N-terminus is the one which may be phosphorylated in *Avena* by R light, though other sites, such as in the hinge region that joins the N-terminal and C-terminal regions have also been implicated (Wong *et al* 1989; McMichael and Lagarias 1990). In a more recent study, Lapko *et al* (1999) have determined more precisely the location of phosphorylated serines. Apart from Ser-7, Ser-598 is phosphorylated and it now seems certain that R light causes the phosphorylation of Ser-598 more specifically, the other serines being phosphorylated equally in both dark- and light-grown seedlings.

The most significant and recent studies in this context are of Yeh and Lagarias (1998), who have expressed the recombinant phytochrome gene of *Avena* and the alga *Mesotaenium* in *E. coli*. The apophytochrome has then been assayed for autophosphorylation as well as kinase activity, the latter using histone H1 and the Rcp1 cyanobacterial response regulator protein. The experiments demonstrate that not only autophosphorylation does occur – the label being predominantly in serine – but it is clearly dependent both on an attached chromophore and on light. R light elicited a higher response than did FR (supporting the long held belief of Pfr being the active form). Phosphorylation of histone and the cyanobacterial response regulator protein also occurs although as for phytochrome the predominant amino acid residue where transfer of phosphate occurs appears to be serine (or threonine) because the use of wild type or the mutated version of response regulator protein shows that the presence or absence of aspartate makes no difference.

As higher plant phytochromes might have evolved from lower ancestral forms, the question arises as to how exactly has this been accomplished. Although the experiments with *Avena* and *Mesotaenium* do not provide any information on the location of the phosphorylatable serine residue(s), amino acid sequence comparison of previously published phytochrome B of *A. thaliana* with the cyanobacterial phytochromes shows that not only the C-terminal sequence but even the inserted sequence of approximately 250 amino acids bears a homology with the

histidine kinase region. Since genetic studies have shown earlier that missense mutations in the inserted region are far more deleterious than in the original C-terminal sequence, the lesson is that in higher plants it is the inserted sequence that has acquired the key biological role and is responsible for a change for the specificity of the phosphorylatable residue from histidine to serine.

To identify proteins that interact with C-terminal domain of phytochrome, which harbors serine–threonine kinase activity, various laboratories have conducted yeast two-hybrid screens. One of the first reported is PIF3 (phytochrome interacting factor) found in *Arabidopsis* by Quail's group (Ni *et al* 1998), which has turned out to be a novel basic helix–loop–helix protein that is nuclear localized and interacts with C-terminal fragments of both phytochrome A and B. It has been further shown that PIF3 may be a primary signalling partner of phytochrome B which translocates to the nucleus under the influence of red light (Ni *et al* 1999). More recently, another nuclear protein, FAR1 (identified through a *far*-red-impaired response mutant), has been found in the same plant and appears to be specifically involved in phytochrome A signalling (Hudson *et al* 1999). The FAR1 protein does not show homology to any known protein in the database but it contains a predicted nuclear localization signal and is targeted to the nucleus in transient transfection assays. Phosphorylation of neither PIF3 nor FAR1 has been reported. However, in the laboratories of Chory and Lagarias (Fankhauser *et al* 1999), another protein, PKS1 (protein kinase substrate) has been discovered in *Arabidopsis* which does undergo phosphorylation at the serine and to some extent threonine residues. Both autophosphorylation of phytochrome and phosphorylation of PKS1 occur more heavily under R light. The important question now is as to what is the relationship of PKS1 with other molecules like PIF3. Genetic engineering experiments indicate that PKS1 is a negative regulator of phytochrome B signalling. An attractive idea has now been proposed that PKS1 may bind phytochrome B and sequester the latter in the cytoplasm. On exposure with R light, however, the complex may dissociate allowing phytochrome to enter the nucleus and control gene expression through interaction with nuclear proteins like PIF3.

Finally, while most investigators have been checking for a kinase function, it may well be that certain photoreceptors could activate protein phosphatases exercising a negative control on regulation much in the same manner as ABA, ethylene and cytokinin do (in humans a CRY photoreceptor has been found to activate a phosphatase; see Zhao and Sancar 1997). Even for phytochrome, it has been reported that removal of serines at the N-terminus – as found by use of transgenes after deliberate deletions – augments R-mediated responses (Stockhaus *et al* 1992; Jordan *et al* 1997), although one has yet to determine whether dephosphorylation does occur *in vivo* and, if so, what is its mechanism. In contrast with the algae and cryptogams, the higher plants seem to have approximately half-a-dozen phytochromes and certain differences may well exist in regard to the mode of action of a particular photoreceptor.

3.2 *Cryptochromes may also modulate kinase activity*

3.2a *Remarks on other related investigations*: Before we discuss more recent developments on how do cryptochromes work, some comment on earlier physiological studies would be in order. Although compared to R/FR, rather limited investigations have been done on B-mediated effects, similar changes in ion fluxes as in response to phytochrome have been observed for B light. Indeed, biopotential changes have been known for more than two decades (Hartmann 1975) and, in recent years, employing patch-clamp technique, B-induced opening of anion channels has been shown to occur within seconds (Noh and Spalding 1998). Proton extrusion has been also shown by a number of workers (e.g., Shimazaki *et al* 1986) in guard cell protoplasts of certain plants. Coming to CRY1 and CRY2, till date, the mechanism of action of either of these B light photoreceptors is not known with certainty. The C-terminal end of CRY1 has a tropomyosin-like domain (figure 6). CRY2 resembles CRY1 but has C-terminal end that is shorter by about 50 amino acids (Ahmad and Cashmore 1996; Ahmad *et al* 1998a, b). Nevertheless, the notion that light-activated protein kinases are key regulators of plant development has also received strong support recently from work on phototropism (Huala *et al* 1997; Christie *et al* 1998).

3.2b *More about mutants of phototropism – the JK224 mutant*: The pioneering work of Poff's laboratory (Khurana and Poff 1989; Khurana *et al* 1989; Konjevic *et al* 1992) relating to non-phototropic mutants was briefly mentioned earlier. But this and subsequent contributions need to be covered in greater detail. Basically, these workers obtained two types of mutants: (i) the "null" mutant that seemed to lack phototropic activity totally towards unilateral blue radiation, and (ii) mutants in which phototropic activity was altered partially. Strain JK218 represented a mutant of the first kind, whereas JK224 was a mutant of the second category (figure 10). Since the lesion in JK224 caused a shift in the fluence requirement of the first positive curvature, without affecting the magnitude of the response, it was speculated by Khurana and Poff (1989) that it could well represent a defect in the photoreceptor itself. The exciting finding was made a few years ago in the Briggs laboratory, employing the wild-type *Arabidopsis* and the JK224 mutant that whereas a 120 kDa protein in the plasma membrane fraction from the hypocotyl of wild-type plants underwent phosphorylation by BL irradiation

not only *in vivo* but also *in vitro*, this ability in the JK224 mutant had been considerably impaired (figure 11; Reymond *et al* 1992b). The earlier work in the Briggs laboratory had shown that a protein in the same molecular weight range was also phosphorylated in pea membranes (Short and Briggs 1990, 1994; Short *et al* 1992, 1994). Subsequently, such work has also been extended to a number of other plants, including tomato, zucchini, maize, barley and wheat (Reymond *et al* 1992a; Palmer *et al* 1993; Sharma *et al* 1997). It has been widely believed that this protein was either the BL photoreceptor itself or positioned close – perhaps immediately next – to the photoreceptor in the phototropism-phototransduction chain.

One specially noteworthy observation of Briggs and co-workers was that *in vitro* phosphorylation occurred even after Triton-solubilization of the plasma membrane fraction. Further, the addition of *irradiated* membrane fraction to non-irradiated fraction brought about a higher degree of phosphorylation than with irradiated membranes alone indicating that not only the 120 kDa protein underwent autophosphorylation, but phosphorylation was caused of similar proteins – not irradiated – indicating

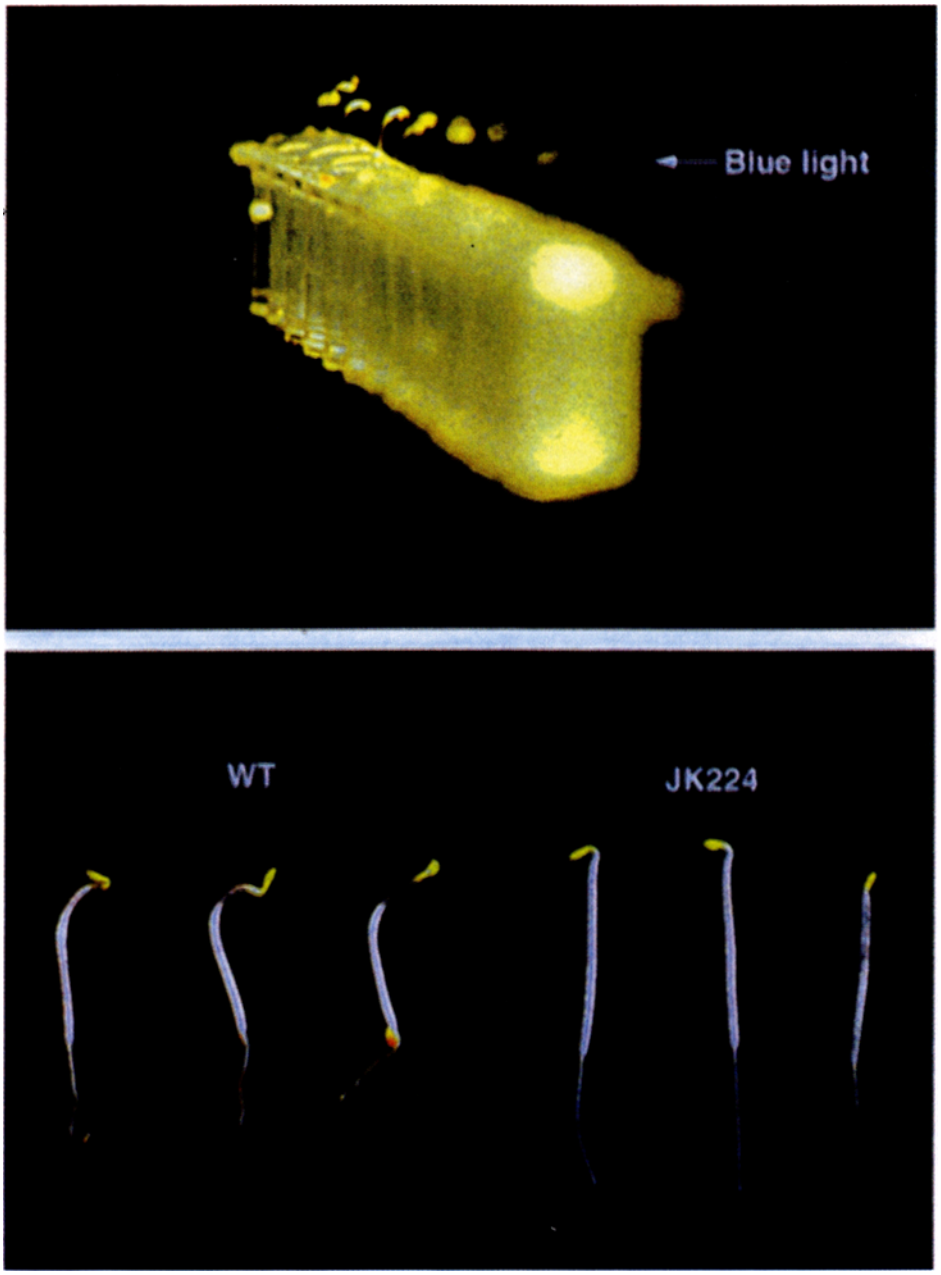

Figure 10. A set-up to monitor phototropic response in *A. thaliana* and a comparison of the response of the JK224 mutant with that of the wild type seedlings.

that the 120 kDa protein was a kinase capable of phosphorylating *other* substrates. TLC analysis of hydrolyzed substrate protein has demonstrated that the phosphorylated amino acid is serine and perhaps multiple residues are phosphorylated (Short *et al* 1994).

3.2c *Cloning of NPH1 gene by use of a mutant allelic to JK224*: Very recently, the gene coding for the putative kinase has been cloned employing a new series of non-phototropic mutants, named *nph* that Briggs and coworkers have generated in *Arabidopsis* by bombardment with fast neutrons (Liscum and Briggs 1995; Huala *et al* 1997). Among these, *nph*1 is allelic to JK224 and lacks the functional 120 kDa protein as is the case in JK224. Mapping studies have shown that *NPH*1 gene is located on chromosome III. Since the gene was located within only 26 centimorgans of an already mapped gene (*GL*1), using flanking DNA markers (obtained by AFLP), an *Arabidopsis* YAC library has been screened and the *NPH*1 gene cloned by chromosome walking. The gene apparently codes for a protein of 996 amino acid residues, as deduced from the cDNA sequence (but analysis of the genomic clone shows that the actual gene extends to 5·4 kb and has as many as 20 introns). Comparison of the cDNA sequence with GenBank sequences and other databases shows that the end corresponding to the C-terminus of the coded protein is similar to that of a serine-threonine protein kinase (figure 6). With 11 sequence motifs typical of protein kinases, the kinase falls in the PVPK family, the first member of which was cloned from *Phaseolus vulgaris* by Lamb and coworkers (Lawton *et al* 1989). As Huala *et al* (1997) note, interestingly, *PHY*3 gene from the fern *Adiantum capillus* also is similar to *NPH*1 but, unlike *NPH*1, it codes for a product that has a phytochrome-like domain at the N-terminal end.

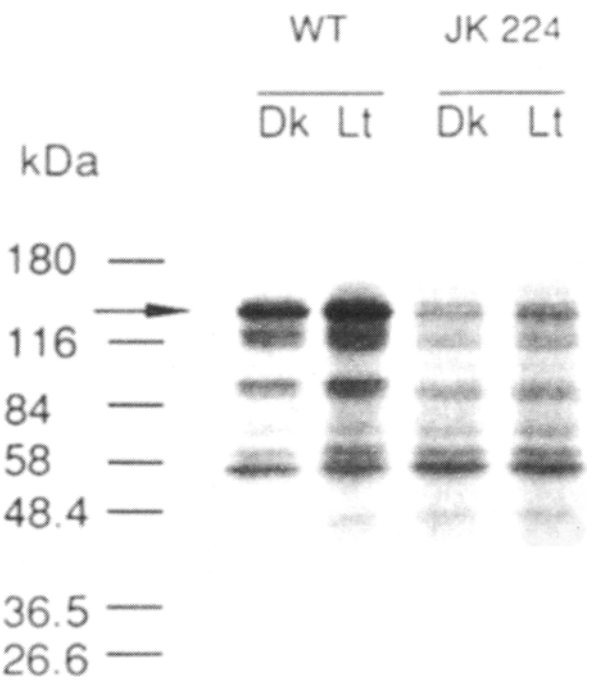

Figure 11. Profiles of phosphorylated proteins in wild type seedlings with those of the JK224 mutant to show the normally phosphorylated 120 kDa protein band in the former upon blue light irradiation (adapted from Reymond *et al* 1992b).

3.2d *Does NPH1 code for a light-regulated kinase*?: Does NPH1 protein itself bind a chromophore or does it operate in conjunction with CRY1 or CRY2 photoreceptors, in a manner such that the initial conformational change in the photoreceptor leads to the activation of latent kinase activity residing in another molecule? A study in Cashmore's laboratory supported the latter idea. It was shown that although in either single mutant there was not much effect on phototropism, in the *cry*1/*cry*2 double mutant, there is neither any phototropic response nor phosphorylation of the 120 kDa protein (Ahmad *et al* 1998b). This implied that the energy of a photon captured by the pterin or the flavin moiety in CRY1 or CRY2 is transferred to the kinase bringing about conformational change in it. The proposal was also consistent with another interesting finding made earlier by the Briggs laboratory (Huala *et al* 1997), of the existence of two special so-called LOV domains (light, oxygen, and voltage sensitive), at the N-terminal end of NPH1, and which have been known to bind flavins as also sense redox and voltage changes in proteins of several organisms (figure 6). Clearly, the existence of a flavin binding domain in the 120 kDa NPH1 protein could facilitate intermolecular transfer of signal between the two molecules, one of which could be considered primarily a photoreceptor and the other primarily a kinase. Such domains have turned out to be important for the functioning of several types of proteins such as Bat (bacteriorhodopsin) in *Halobacterium*, WSC-1 (white collar protein involved in resetting of the circadian rhythm by light) in *Neurospora crassa*, as also Aer (for aerotaxis signalling) in *E. coli* and ELK, the voltage sensitive potassium channel subunit in *Drosophila melanogaster* (see Huala *et al* 1997).

The latest results of the Briggs laboratory provide strong experimental support for the existence of a flavin moiety attached to NPH1. However, they go an important step further and their observations are in one sense contradictory to the conclusions drawn by Cashmore's group since they prove that the NPH1 protein, like phytochrome, is not merely a kinase but also, itself, a primary photoreceptor for phototropism. The clinching evidence has come from detailed physiological and biochemical studies – made possible by overexpression of the recombinant NPH1 protein in a baculovirus/insect cell system (Christie *et al* 1998). Spectral analysis and thin layer chromatography have led to the identification of flavin mononucleotide (FMN) as the chromophore. The fluorescence excitation spectrum of the protein is in fact

remarkably similar to the action spectrum of phototropism, although unlike the tetrapyrrole chromophore – in phytochromes – FMN attaches to NPH1 non-covalently and the recombinant protein apparently picks it up from the insect cells. Nevertheless, the most striking observation is that the recombinant protein is heavily phosphorylated *in vitro* in response to brief irradiation with B light. Very recently, the photoreceptor coded by *NPH1* gene has been renamed 'phototropin' (Christie *et al* 1999).

To summarize the above findings, there is now convincing evidence that the B light receptor for phototropism is indeed a hybrid photoreceptor-cum-kinase. Further, since the *cry1/cry2* double mutant continues to display the "second positive" phototropic response (in contrast to the stronger alleles of *nph1* mutant which show neither the "first" nor the "second" positive curvature), the view is now gaining ground that, whereas NPH1 is the primary photoreceptor for phototropism, the cryptochromes may modulate or accentuate the phototropic response in a way analogous to phytochrome (Janoudi *et al* 1997).

4. Concluding remarks

Since Darwin reported the effect of light on phototropism and Garner and Allard on photoperiodic control of flowering, we have come a long way towards the understanding of light effects on plant growth and development. The secrets of nature are being revealed rapidly with the combined application of genetic and molecular biological approaches. To summarize the wisdom gleaned in a capsule form: a milestone has been reached by the discovery of a new class of "hybrid" protein kinases which have attached chromophores and which are activated by the capture of photons.

Nonetheless, many challenges still lie ahead. There is a multiplicity of phytochromes and cryptochromes. It seems logical that they may function through distinct and somewhat different transduction pathways that need to be dissected and identified. Neither phytochromes nor cryptochromes have any membrane spanning domain, yet they control several membrane associated phenomena which is a paradox. Also, whereas kinase (or phosphatase) activity explains the modulation of activities of cytosolic proteins or of transcription factors, the rapid and apparently immediate effect on ion transport across membranes is not so easily explained. One wonders whether these photoreceptors/kinases can affect ion-channel activity directly. Another action of light is seen on localization of critical macromolecules and in particular their distribution between cytoplasm and nucleus. For example, phytochrome B which has been found to have nuclear localization signals, translocates from the cytoplasm to the nucleus after exposure to R light. The precise mechanism of such directional movement also needs to be resolved.

To conclude, while discovery of light-regulated kinases (and phosphatases) constitutes a landmark achievement in plant biology, and this reflects the mode of action of certain R/FR as also B light absorbing photoreceptors, revelation of other parallel modes for effective utilization of the full range of the light spectrum is awaited.

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