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Update on Photobiology

Prokaryotes and Phytochrome. The Connection to Chromophores and Signaling[1]

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Prokaryotic systems have been important in phytochrome studies on several different levels. Bilins from cyanobacterial phycobiliproteins have allowed the production of recombinant holophytochrome and have provided insights into the attachment and functioning of the chromophore, while the recent discovery of functional phytochromes in the cyanobacterium *Synechocystis* and other prokaryotes has catalyzed work in the field. *Synechocystis* phytochrome is useful experimentally and, by making the modular structure and potential biochemical functions of phytochromes clearer, has provided an improved focus and new viewpoints for research.

Some of the earliest studies of photobiology concerned phenomena in cyanobacteria: the complementary chromatic adaptation (CCA) of photosynthetic pigments to the light environment was first described in Engelmann's laboratory in Berlin a century ago. Numerous other effects such as phototaxis, photoperiodism, cell division, and differentiation are also regulated by light in cyanobacteria. Plant plastids probably evolved from endosymbiotic cyanobacteria whose genes gradually moved to the host nucleus. There is thus every reason to expect evolutionary relationships between photoperception systems in cyanobacteria and plants. Detailed information about cyanobacterial photoreceptors was lacking, however, until genomic sequencing revealed a cyanobacterial phytochrome: ironically, the ease with which molecular methods can be used in prokaryotes has now turned the tables, with the cyanobacterial model providing a wealth of new ideas about the origins of phytochrome and its mode of action. Here we review the different ways in which cyanobacteria and other prokaryotes have contributed to research into plant photomorphogenesis and the phytochrome system (for review, see Elich and Chory, 1997; Quail, 1997a; Pepper, 1998).

Phytochrome is an ubiquitous plant photoreceptor that was first characterized in the late 50s in relation to its peculiar photochromic behavior in red and far-red light (Butler et al., 1959). Phytochromes carry an open-chain tetrapyrrole (bilin) chromophore, which the apoprotein autocatalytically attaches to a conserved C residue (#380 in our alignment[2]) via a Schiff base (Lagarias and Lagarias, 1989). In darkness, this autoassembly produces the red-light-absorbing form Pr ($\lambda_{max} \approx 660$ nm). In red light this is photoisomerized to another form, Pfr, which absorbs maximally in far-red light ($\lambda_{max} \approx 730$ nm). In far-red light, Pfr is in turn converted back to Pr. Both forms are thermodynamically stable and can be interconverted by any number of photocycles. Because even tiny amounts of Pfr have major physiological effects, it is generally accepted that this is the active form of phytochrome, while Pr seems to be physiologically inactive. In plants, phytochromes control a variety of developmental processes such as seed germination, stem elongation, construction of the photosynthetic apparatus, chloroplast movements, shade avoidance, and photoperiodic induction of flowering. In lower plants they are also involved in sensing light direction.

CYANOBACTERIA AND PLANTS CONTAIN FIVE DIFFERENT BILIN CHROMOPHORES

Based on the first spectral measurements, it was correctly argued that the phytochrome chromophore might be a bilin similar to those of phycobiliproteins in cyanobacteria and red algae (Butler et al., 1959). Phycobiliproteins bear four types of bilin, namely phycocyanobilin (PCB), the chromophore of phycocyanin (PC, which is thus generally the most abundant), phycoerythrobilin (PEB), phycoviolobilin (PVB), and phycourobilin (PUB) (see Fig. 1). Apophytochromes autoassemble with PCB to form holophytochrome photoreceptors—a useful feature, as PCB can be prepared rather easily from commercially available cyanobacteria, enabling the preparation of functional phytochromes by recombinant methods (Wahleithner et al., 1991). However, in all land-plant phytochromes examined so far, another member of the family, phytochromobilin (PΦB), is the natural chromophore (Rüdiger and Thümmler, 1994). Phytochrome chromophores undergo a characteristic Z → E isomerization around the C15═C16 double bond between rings C and D during Pr → Pfr conversion. PEB also assembles with apophytochrome in vitro, but the product

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[2] Residues numbered with a # refer to an alignment available at http://www.plantphysiol.org/cgi/content/full/121/4/1059/DC2 and http://www.biologie.fu-berlin.de/phytochrome/align2x.htm. Other numbering refers to the specific gene product involved (start codon = 1).

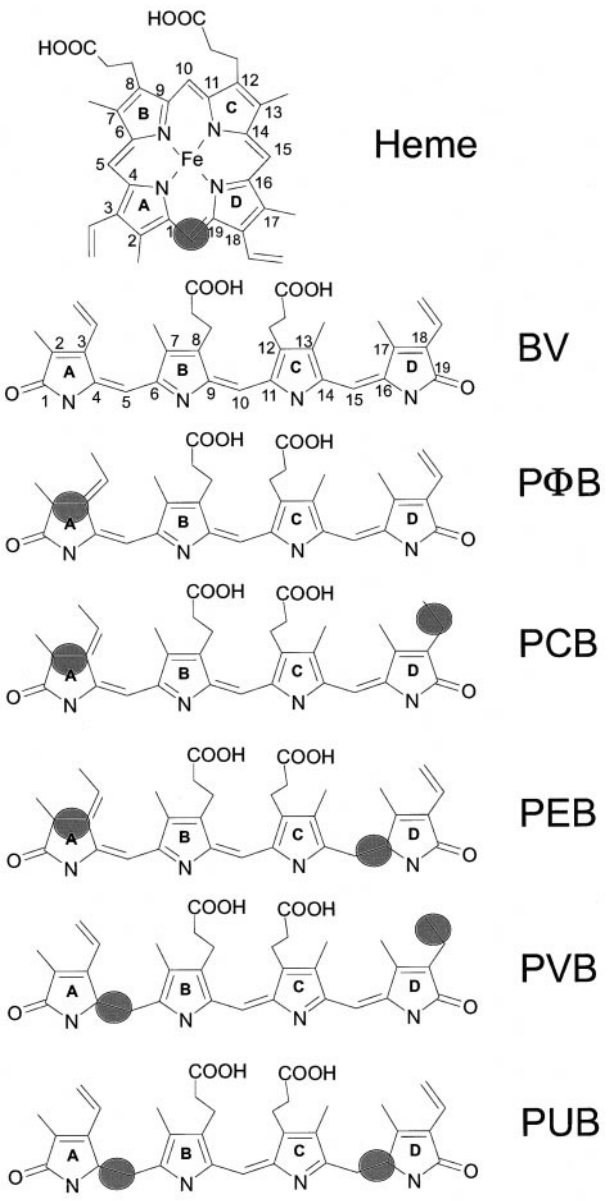

Figure 1. Structure of heme and the natural bilins biliverdin (BV), PΦB, PCB, PEB, PVB, and PUB. Heme oxygenase converts heme to bilivirdin by cleaving between rings A and D at the positions marked. Differences in the other bilins with respect to bilivirdin are also indicated.

is not photochromic because the C15-C16 bond is saturated (Li and Lagarias, 1992). The five bilins (PCB, PΦB, PVB, PEB, and PUB) above differ only in single double bonds and are derived from biliverdin, the first open-chain tetrapyrrole in this biosynthetic pathway (Fig. 1).

ONLY THE FIRST GENE FOR THE BILIN SYNTHETIC PATHWAY IS KNOWN

In seed plants, the enzymes for bilin synthesis are located in the plastids but are nuclear encoded. The pathway begins with the opening of the heme tetrapyrrole ring between pyrroles A and D by heme oxygenase to form the linear tetrapyrrole (bilin) biliverdin IXα. Genes for this enzyme are known from the cyanobacterium *Synechocystis* PCC6803 (Cornejo et al., 1998), from the plastome of red algae, and from the genomes of Arabidopsis and several animals. In Arabidopsis heme oxygenase is encoded by the nuclear gene *HY1* (Muramoto et al., 1999). The *ptr116* phototropic mutant of the moss *Ceratodon* can be rescued by exogenous biliverdin by microinjecting cells with mammalian heme oxygenase enzyme or by overexpressing *HY1*, showing that all three functionally complement the defective *ptr116* gene (Brücker et al., 1999).

The subsequent steps are not fully understood biochemically, nor have any further associated genes been described, although the Arabidopsis HY2 locus is a likely candidate. Further sequences from the *Synechocystis* genome must also be involved in the production of PCB and other bilins.

CYANOBACTERIAL MODELS FOR PHYTOCHROME

Early physiological studies indicated that cyanobacteria might harbor useful information about the phytochrome system. Action spectroscopy revealed photoreversible effects analogous to those of plant phytochrome but with the interesting distinction that, while in plants the responses are maximally induced by red light and reverted by far-red light, most photoreversible effects in cyanobacteria respond to red light ($\lambda_{max} \approx 650$ nm) and green light ($\lambda_{max} \approx 520$ nm) (Vogelman and Scheibe, 1978).

The most intensely studied effect here is CCA. Unlike plants, cyanobacteria possess phycobilisome structures that funnel energy into the photosynthetic system. The principle accessory pigments involved are the blue-green (red-absorbing) PC and allophycocyanin and the red (blue-green-absorbing) phycoerythrin (PE). Some species are able to use CCA to adjust the ratio of these pigments according to environmental conditions. In green light PE dominates, whereas in red light PC dominates. In this way, the λ_{max} of photosynthesis is shifted to the λ_{max} of the light environment (Gaidukov, 1902).

When a series of green ($\approx$ 540 nm) and red ($\approx$ 650 nm) pulses was given to a culture of the cyanobacterium *Fremyella diplosiphon* and the culture kept in darkness, the last light pulse determined the dominant accessory pigment formed (Vogelman and Scheibe, 1978). This kind of photoreversibility points to a photoreceptor with photochromic properties, an unusual spectral feature that allowed plant phytochrome to be isolated and characterized. However, this approach was less successful in cyanobacteria, principally because, unlike angiosperms, they do not etiolate. Although a photoreversible pigment showing difference maxima at 520 and 650 nm has been described (Scheibe, 1972), it was not characterized further.

Later, the α-subunit of the minor phycobilisome component phycoerythrocyanin (PEC) was shown to be photochromic—but with difference maxima at 500 and 570 nm. The physiological role of PEC is unknown, although it might have a role as a photoreceptor as well as acting as a photosynthesis antenna. The PECα chromophore is PVB,

which undergoes a Z → E photoisomerization analogous to phytochrome (Zhao et al., 1995). Moreover, the PECα and PCα sequences are approximately 65% identical and the molecules have similar three-dimensional structures—yet only the former is photochromic. While one might therefore suppose that the differences between these biliproteins could provide a master key to unlock the secrets of photochromicity at the atomic level, whether this key would fit phytochrome is questionable. First, there is no sequence homology between phycobiliproteins and phytochrome. Second, isolated phycobiliproteins show far stronger fluorescence than phytochrome, implying that their chromophores are much more tightly held and/or have a very different photochemistry. Third, relative to the dark state, the conformational changes in PECα are associated with a blue shift, whereas a red shift is seen with phytochrome.

Interestingly, although phycobiliprotein apoproteins are generally capable of autocatalytically attaching bilin chromophores in vitro, it has been shown that specific lyases mediate the assembly in vivo, accelerating the reaction and ensuring correct bilin attachment. Phytochrome autoassembles in vitro too, but whether a discrete phytochrome bilin lyase exists is simply not known.

TWO-COMPONENT SIGNALING ENTERS THE FRAY

A quite different line of investigation also connects plant phytochromes with prokaryotic systems. Schneider-Poetsch et al. (1991) drew attention to a significant amphiphilic sequence similarity between the phytochrome C terminus and the transmitter module of bacterial sensory His protein kinases (HPKs). HPKs are a group of proteins responsible for the first step in the so-called two-component signal transduction pathways (see Fig. 2A) that provide the prokaryotic cell with its capacity for perception and response.

The activity of each HPK is regulated by an associated sensory module whose conformation changes in response to an environmental signal such as an interaction with a specific ion or molecule. The HPK is a dimer and, upon sensor activation, each subunit phosphorylates the other at a conserved H target residue ($H_{\#995}$) within the transmitter module. The phosphate is then transmitted to a conserved D residue in the receiver module on the second component of the transduction system, the response regulator. This then does as its name suggests, either by activating transcription of specific genes itself or by interacting with other proteins to bring about specific physiological changes in the cell appropriate to the environmental signal. These two-component signal transduction systems seem to be the primary regulatory connections between prokaryotic metabolism and the environment.

Schneider-Poetsch's suggestion that phytochrome might represent a plant sensory HPK was enhanced by the discovery of eukaryotic HPK homologs SLN1 in yeast and ETR1 in plants a year or so later, and indeed the idea that phytochrome might be a light-dependent kinase was nothing new. A seductive aspect was that it provided phytochrome with its long-sought reaction partner: This should be a response regulator homolog. Many were not convinced by the HPK/phytochrome homology, however, and there was one very large problem: although all of the functional subdomains characteristic of HPK transmitters are recognizable in phytochromes, the all-important $H_{\#995}$ target residue itself is poorly conserved (see alignment). It seemed that the affair was over when Boylan and Quail (1996) showed that even in phytochromes in which $H_{\#995}$ was conserved, it could be mutated without noticeable physiological effect.

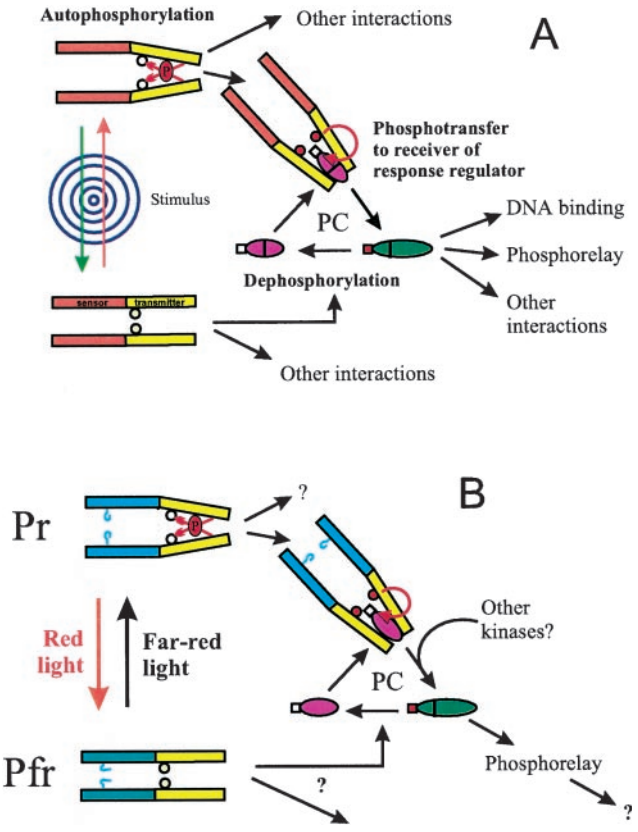

Figure 2. Two-component signal transduction. A, Basic scheme. The HPK dimer is activated by conformation changes induced by stimuli perceived by the sensor module, usually N-terminal. The transmitter module of each subunit then transfers a phosphate (red dot) from ATP to a conserved His residue (circle) of the other subunit. The phosphate group is transferred to a conserved Asp residue (square) of the cognate response regulator, which is thereby activated. An autophosphatase activity returns the response regulator to its inactive state in the phosphorylation cycle (PC)—the rate of hydrolysis is sometimes regulated by interactions with the "inactive" form of the same HPK or with another molecule. B, Cph1/Rcp1 system. Pr, The ground state of the phytochrome in darkness or far-red light is the active HPK; irradiation with red light converts the molecule to Pfr, in which the HPK activity of the transmitter module is inhibited. Following autophosphorylation at $H538_{\#995}$, Pr transfers the phosphate to the response regulator Rcp1. The biochemical functions of Pfr and the response regulator are unknown.

POSSIBLE PHOTORECEPTORS IN CYANOBACTERIA

Work with the cyanobacterial CCA perception system, however, continued independently. Complementation

methods were used to clone genes involved in the regulation of chromatic adaptation in *F. diplosiphon*. RcaE (Kehoe and Grossman, 1996) encodes a 74-kD polypeptide with an approximately 150-residue portion toward the N terminus showing homologies to several regions around the chromophore-binding domain of plant phytochromes, as well as C-terminal motifs typical of transmitter modules. Intriguingly, RcaE also bears two subdomains (T2L and R2L, $T105_{\#265}$-$L129_{\#289}$ and $R241_{\#436}$-$L268_{\#463}$, see alignment) showing homology to the plant ethylene receptor ETR1. Although the biochemistry has yet to be demonstrated, RcaE probably phosphorylates the response regulator RcaF, which in turn phosphorylates RcaC. The latter bears a transmitter- and two receiver-like modules, as well as a DNA-binding motif thought to mediate differential transcription of the PC and PE gene complexes. In the similar cyanobacterium *Calothrix*, RcaD and RcaA act as phosphorylation-dependent activators of the PC and PE gene clusters, respectively.

The 155-kD conceptual gene product of *PlpA* (Wilde et al., 1997; sll1124 in CyanoBase) in *Synechocystis* PCC6803 also shows regions of similarity to phytochromes (hence the name Plp for phytochrome-like protein) and two-component modules (see alignment). Indeed, BLAST searches show that RcaE has approximately 25% amino acid identity (40% similarity) to PlpA, although the latter has a long N-terminal extension. This particular *Synechocystis* strain does not show CCA. It does, however, change the stochiometry between PS1 and PS2 according to the irradiance and spectral distribution of the light environment: In $plpA^-$ knockouts the balance between the photosystems is disturbed. Furthermore, in contrast to the wild type, the $plpA^-$ mutant cannot grow photoautotrophically in blue light.

Although both PlpA and RcaE are clearly important in cyanobacterial photoperception, it has proven difficult to demonstrate that they are photoreceptors. The sensory function could be fulfilled by a separate molecule, as in many two-component systems. At least on the basis of homology to the phytochrome N terminus, there is little reason to expect either *RcaE* or *PlpA* gene products to be bona fide phytochromes: If one assumes that the chromophore is thioether-linked to a C residue, as in phycobiliproteins and plant phytochromes, the alignment in this region is constrained to $C198_{\#380}$ and $C784_{\#380}$ for RcaE and PlpA, respectively. The surrounding subdomain is quite different from that seen in phytochromes, where it is well conserved and changes generally lead to a complete loss of function. However, although difficulties with overexpressing *PlpA* and *RcaE* in *Escherichia coli* have hampered in vitro studies, it has now been reported that both do seem to be capable of attaching bilins (A. Wilde, T. Börner, D. Kehoe, and A. Grossman, unpublished data).

A PROKARYOTIC PHYTOCHROME CREATES EXCITEMENT IN THE FIELD

Quite separately, the entire 3.57-Mbp chromosome of *Synechocystis* PCC6803 was sequenced in a singularly efficient project at the Kazusa Institute in Japan (CyanoBase: http://www.kazusa.or.jp), providing the scientific community with a wealth of valuable new data. Among the 3,168 open reading frames identified, a phytochrome-like sequence (slr0473) was recognized (Kaneko et al., 1995; Hughes et al., 1996). The N-terminal moiety showed patchy but unmistakable similarity to phytochromes, including the all-important chromophore binding region, whereas the 30-kD C-terminal moiety was clearly homologous to typical two-component transmitter modules with characteristically conserved H-, N-, G1-, F-, and G2-boxes (see alignment and Fig. 3).

The question nevertheless remained: is it a phytochrome? The 85-kD gene product was further analyzed simultaneously by Lagarias's group at University of California-Davis and by our laboratory (Hughes et al., 1997; Lamparter et al., 1997; Yeh et al., 1997). The apoprotein overexpressed in *E. coli* autocatalytically attached PCB chromophore in vitro to form a blue-green photochromic pigment, clearly establishing that it encodes a bona fide cyanobacterial phytochrome, Cph1.

The discovery of this phytochrome caused an immediate paradigm shift in the field. Schneider-Poetsch's suggestion that the unknown mechanism of primary signal transduction could be related to the well-established two-component system in bacteria suddenly became a very hot topic. Moreover, the utility of a prokaryotic phytochrome system in biochemical and molecular-genetic studies opens new experimental possibilities. In particular, the efficiency with which highly soluble recombinant phytochrome can be prepared from *E. coli* overexpressors offers fresh hope that the three-dimensional structure of this class of photoreceptors could be resolved via NMR and x-ray diffraction analysis of phytochrome crystals.

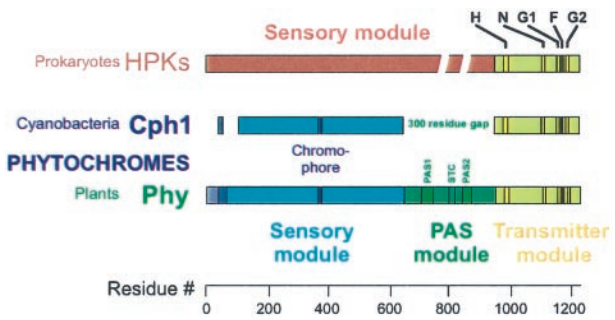

Figure 3. *Synechocystis* phytochrome (Cph1) in relation to plant phytochromes and sensory His protein kinases (HPK's). Residue numbering is that from the alignment (http://www.plantphysiol.org/cgi/content/full/121/4/1059/DC2 and http:/www.biologie.fu-berlin.de/phytochrome/align2x.htm). The N-terminal bilin-bearing sensor module (blue) is recognizable in all phytochromes, while the C-terminal transmitter module (yellow) is common to phytochromes and most HPKs of the two-component type. The sensory modules of other HPKs (brown) are different and are sometimes carried on separate polypeptides. A PAS module (green), important for plant phytochrome signal transduction, is absent from Cph1. The chromophore attachment site, two PAS repeats, the signal transduction core (STC or Quail box), and the H-, N-, G1-, F-, and G2-boxes are highly conserved. Plant B-type phytochromes generally have an N-terminal extension, otherwise the N terminus of all phytochromes is Ser/Thr rich.

Cph1 IN VITRO

Cph1 has been subjected to a range of optical and biophysical studies in our laboratory (Lamparter et al., 1997) and in those of our colleagues. Recombinant apoprotein (Cph1°) is expressed remarkably efficiently in *E. coli*, accumulating to approximately 30% of cytosolic protein. Moreover, equipped with a C-terminal oligohistidine tag, it can be purified almost to homogeneity in a single Ni^{2+}-affinity chromatographic step. *E. coli* does not support bilin synthesis, and thus autoassembly does not occur in vivo. If Cph1° is added to PCB in vitro, however, a dramatic blue to blue-green color change occurs within seconds. This results from two processes. Initially, recombinant holoprotein (Cph1*) is formed as Pr, whereby the PCB red absorbance peak at 610 nm is shifted to 658 nm as the helical form of the free bilin becomes unwound in the protein environment. Thereafter, if observed in daylight, the Pr is photoconverted to Pfr, whose absorbance peak is shifted even further to 702 nm.

Both photochromic forms are quite stable in darkness. The yield of pure Cph1* is routinely about 20 mg per liter of culture. It can be concentrated to above 15 mg/mL quite easily, satisfying an important further precondition for many biophysical and physicochemical studies, including crystallization. Like other HPKs and plant phytochromes, Cph1* behaves as a dimer in solution. As one would expect for a photoreceptor, the extinction coefficient of Cph1* is very high (approximately 100 $mM^{-1}\ cm^{-1}$ for Pr at λ_{max}) and the quantum conversion efficiency is about 0.16 in both directions—values similar to those for plant phytochromes. Cph1° can also be assembled with other chromophores: The PΦB adduct shows a red shift of about 15 nm for both Pr and Pfr, as seen with plant phytochromes. PEB adducts cannot photoconvert because of their missing C15═C16 double bond (see Fig. 1); the quantum energy is released as fluorescence and their absorbance maximum is blue-shifted to 579 nm.

Fourier-transform Raman-resonance (FTRR) and flash photolysis (Remberg et al., 1997), low-temperature fluorescence (Sineshchekov et al., 1998), and Fourier-transform IR absorbance (FTIR, H. Förstendorf and F. Siebert, unpublished data) spectroscopic methods have also been used. Despite the considerable differences in the peptide sequences, Cph1* shows remarkably similar physicochemical properties to those of B-type phytochromes. FTRR is a sensitive probe for the status of the chromophore in biliproteins, revealing in this case many similarities between the chromophores of native oat phytochrome A and the equivalent PΦB adduct of Cph1*. For both phytochromes, spectral differences between the Pr and the Pfr form reflect the Z → E isomerization of the chromophore and changes in its hydrogen bonding with the protein. Moreover, as in plant phytochromes, subtle differences between the PCB and the PΦB adduct of Cph1* can be attributed to the ring D side chain (vinyl group for PΦB versus ethyl group for PCB).

FTRR also indicated different torsions around methine bridges within the chromophore and differences in chromophore/protein interactions between Cph1* and oat phytochrome. As for other phytochromes, the formation of intermediates during Pr → Pfr photoconversion of Cph1* was readily observed by flash photolysis and fast spectroscopy. The first photoproduct detected (lumi-R) of Cph1* appeared substantially more quickly than that for plant phytochromes and was followed by a novel intermediate whose kinetics were delayed almost 2-fold by ^2H exchange, implying that a protonation/deprotonation is involved at this point. FTIR difference spectra also indicate ^2H effects, and a photoreversible pH shift (J. Hughes and J. van Thor, unpublished data) seems to confirm that proton extrusion accompanies Pfr formation.

Fluorescence measurements at low temperature address the photoconversion from a different point of view. Whereas at ambient temperature phytochrome fluorescence yields are very low, these rise dramatically upon cooling; Pr → Pfr photoconversion is inhibited, although photoconversion into intermediate forms is sometimes possible. For plant PHYA at 70 K, up to 50% of the Pr can convert into lumi-R, whereas this conversion is not possible for plant PHYB. PCB and PΦB Cph1* adducts are also unable to form lumi-R at this temperature, implying that Cph1 is more related to PHYB than to PHYA photochemically. Different activation barriers for the photoreaction are thought to explain the differences between phytochrome types. The only intermediate photoproduct found after allowing the temperature to rise seemed to be rather different from the lumi-R of plant phytochromes.

Cph1* IS A LIGHT-DEPENDENT HIS PROTEIN KINASE

The Lagarias group (Yeh et al., 1997) analyzed the biochemistry of Cph1* regarding its apparent homology to two-component systems (see Fig. 2B). Cph1* autophosphorylates at the expected $H538_{\#995}$, but it was a great surprise that the active kinase was not Pfr but Pr. This flew in the face of most plant physiological data, which implied that Pfr was the active form. There was more to come, however.

Unlike eukaryotes, prokaryotes often group biochemically related genes together in a single cistron, thereby keeping the job of coordinating expression simple while obligingly providing the scientist with clues to unknown biochemical associations. While biochemists had long sought the primary reaction partner(s) for plant phytochrome, the likely reaction partner for *Synechocystis* phytochrome was clear from the Kazusa chromosome map. Fifteen bases downstream of the *Cph1* stop codon begins a short open reading frame, slr0474, unmistakably coding for a 17-kD response regulator of the two-component type (Lamparter et al., 1997). Yeh et al. (1997) overexpressed this gene in yeast and demonstrated that the autophosphorylated Pr form of Cph1* promptly transmitted its phosphate to the expected Asp residue D68 of the putative response regulator. slr0474 was thus the first primary reaction partner for phytochrome to be identified and was named response regulator for cyanobacterial phytochrome, Rcp1. Here again, Pr was more active than Pfr. All of the known enzymatic activities of phytochrome (bilin ligase, His autokinase, His-Asp transphosphorylase, and, as we shall see, Ser/Thr kinase) were first described by the Lagarias laboratory.

Although many response regulators are DNA-binding proteins and act as transcriptional activators, the *Synechocystis* model is not quite so simple. Rcp1 has no DNA-binding motifs and thus presumably acts as an intermediate phosphocarrier in a more complex relay. This is also seen in other two-component systems such as Spo and Rca. The epithet refers to the transmitter module of the kinase and the receiver module of the response regulator, whereas the transduction system as a whole can be considerably more extensive, with pathways converging and branching to form a sophisticated control network. So with what does Rcp1 interact? Unfortunately, in *Synechocystis* only *Cph1* and *Rcp1* are co-transcribed, the flanking genes being read in the opposite direction. As the next partner cannot simply be deduced from the genome map, finding the rest of the transduction chain will prove more difficult.

Cph1 IN VIVO REMAINS A MYSTERY

Although *Synechocystis* certainly contains abundant PCB, the native chromophore of Cph1 is not known. CCA in other cyanobacteria shows well-separated maxima in the blue-green and red regions, whereas the absorbance maxima of PCB and PΦB adducts of Cph1* are poorly separated and are at longer wavelengths (see above). If a Cph1 homolog is indeed the photoreceptor for CCA, then the blue shift might result from the use of a different chromophore. Alternatively, a quite separate photoreceptor might be involved. Measuring difference spectra in extracts of *Synechocystis* was unsuccessful because of masking pigments, even when using PC$^-$ deletion mutants. In an attempt to overcome this, homologous recombination was used to replace the wild-type chromosomal gene with a sequence extended to provide an oligohistidine-tagged translation product similar to that in the *E. coli* overexpression clones, thereby allowing the photoreceptor to be purified by affinity methods. However, despite this technology, the extracts have yielded only tiny amounts of the modified native Cph1, indicating a very low expression level. Interestingly, the purified fraction showed not only the expected red/far-red difference spectrum with maxima around 650 and 700 nm, but also a red/green difference with maxima at 530 and 650 nm, close to the maxima for CCA. Whether the red/green reversible signal relates to a co-purified protein or directly to Cph1 remains to be determined (T. Lamparter, A. Wilde, and T. Hübschmann, unpublished data).

Ironically, despite all the studies of Cph1 in vitro, its physiological function is unknown: What aspect of the light environment does it perceive and what response does it mediate? This gap in our knowledge is all the more surprising because the efficient homologous recombination available in *Synechocystis* allows knockout mutants to be created with some ease. Indeed, both Cph1$^-$ and Cph1$^-$/Rcp1$^-$ knockouts have been generated in several labs, but an associated phenotype has yet to be found (D. Scanlan, A. Wilde, and T. Börner, unpublished data). Perhaps the effects are masked by another photoreceptor system, or the Cph1-Rcp1 pathway might lead to a physiological dead end in this particular strain. One might expect Cph1 to regulate CCA—but, unfortunately, PCC6803 and most other strains of *Synechocystis* lack PE entirely and thus could not show CCA even if they wanted to. However, *Synechocystis* PCC6701 shows classical CCA, carrying a PE gene cluster closely homologous to that in *Fremyella* and replacing PC with PE in blue-green light. The Cph1 homolog in CCA-active *Calothrix* has also now been cloned (N. Tandeau de Marsac, unpublished data). It will be interesting to see if a *cph1*$^-$ knockout in one of the CCA-active types shows the Rca$^-$ phenotype.

OTHER PROKARYOTIC PHYTOCHROMES HAVE ALSO BEEN IDENTIFIED

Several other *Synechocystis* genes also show similarities to phytochromes. It seems that the true homolog of *RcaE* is not *PlpA* (sll1124), as implied above, but, rather, is represented in Cyanobase by two pseudogenes separated by a transposon (sll11473–sll1475); in other PCC6803 cultures the *RcaE* homolog is intact (A. Wilde, unpublished data). sll0821 is also intriguing as it shows two regions with homology to the phytochrome chromophore subdomain, both of which bind PCB in vitro (S.-H. Wu and J.C. Lagarias, unpublished data).

Even further removed from plant phytochromes are the *BphP* (bacterial phytochrome photoreceptor) genes recently found on the chromosomes of *Deinococcus radiodurans* and *Pseudomonas aeruginosa*. The former autoassembles with bilin chromophores in vitro to yield a phytochrome-like red/far-red light photochromic product (R. Vierstra and S. Davis, unpublished data). This result is surprising because, although a region resembling the phytochrome chromophore subdomain is apparent, residue #380 is M rather than C. While we assume that the chromophore attachment site of Cph1 is $C259_{\#380}$, this has yet to be demonstrated chemically. (See also "Note Added in Proof")

FROM PROKARYOTES TO PLANT PHYTOCHROME

Most of the above discussion concerns phytochrome in prokaryotes, but how does that help the plant physiologist? Most importantly, it provides conceptual links. First, the alignment of Cph1 to plant phytochromes and HPKs provided a new and clearer view of phytochrome molecular architecture. Second, while the initial cloning of phytochrome was a great technical achievement in itself, the sequence did not provide us with beguiling homologies to molecules of known function. Cph1 provides a link not only to bacterial two-component systems, but also to several other eukaryotic homologs including SLN1, DHKA and DHKB, ETR1, and CKI1, all thought to take part in phosphorelay-mediated signaling. This rejuvenated the idea that phytochromes might be light-dependent protein kinases.

PLANT PHYTOCHROMES POSSESS A PAS MODULE INVOLVED IN SIGNAL TRANSDUCTION

Cph1 alignments revealed an additional approximately 300-residue module peculiar to plant phytochromes,

placed between the sensory and transmitter modules (probably between $A497_{\#648}$ and $L498_{\#954}$, see alignment and Fig. 3). The module is also missing from the *Deinococcus* and *Pseudomonas* homologs. This region of the plant phytochrome sequence had already aroused interest since it contains a repeated motif (#709-#751 and #846-#888) related to the PAS[3] domain family (Jones and Edgerton, 1994; Lagarias et al., 1995). It would seem that this PAS module was added to a Cph1-like progenitor early in plant evolution, perhaps even before eukaryotes arose, bringing with it a set of biochemical features probably including a new signaling mechanism.

PAS domains (see Taylor and Zhulin, 1999) are found in diverse proteins throughout the living world; particularly interesting is the apparent PAS homology of the bacterial photoreceptor PYP (photoactive yellow protein) (Lagarias et al., 1995). PAS domains often bind ligands and are involved in protein-protein interactions including signal transduction. There is ambivalent evidence that the PAS repeats $S599_{\#675}$ to $L683_{\#766}$ and $L685_{\#768}$ to $R815_{\#901}$ are involved in the dimerization of phytochrome A (Edgerton and Jones, 1993; Quail, 1997b). Furthermore, random mutagenesis studies indicate that the PAS module is crucial to the plant phytochrome signaling mechanism (Quail et al., 1995). Yeast two-hybrid studies identified several phytochrome interacting factors that seem likely to bind to the PAS module. One of these is involved in phytochrome signaling in vivo, is nuclear localized, and even possesses a DNA-binding domain (Ni et al., 1998; Halliday et al., 1999), offering a remarkably—if not deceptively—simple picture of plant phytochrome action, given that newly formed Pfr migrates to the nucleus (Sakamoto and Nagatani, 1996).

Although the plant phytochrome PAS module is missing from Cph1, a Hidden Markov model (http://coot.embl-heidelberg.de/SMART/) detects PAS-domain-related structures in Cph1, RcaE, and PlpA at different positions. A weak but significant similarity between HPK modules and the PAS domain has also been pointed out (Yeh and Lagarias, 1998), providing the latest twist to an unfinished story.

KINASE AND KINASE-RELATED FUNCTIONS?

As far as kinase function is concerned, the conceptual framework is not simple. As we have seen, athough the $H538_{\#995}$ target in Cph1 and its homologs in other sensory HPKs in both prokaryotes and eukaryotes is essential for autokinase and phosphorelay function, the homologous residue in plant phytochromes is neither conserved nor functional. While it is nevertheless possible that plant phytochromes could act as HPKs (for example, some PHYAs show an H-box-like [L/V][A/P]SHELQ[Q/H]$AL_{\#961\text{-}\#970}$ motif at the PAS/transmitter module boundary) and response-regulator homologs certainly exist in plants, we emphasize that there is no evidence that any plant phytochrome functions as an HPK. Nevertheless, as we shall see, the HPK transmitter domain seems to be very much involved in signal transduction.

The two-component paradigm might help us to understand plant phytochrome function independently of the prokaryotic kinase action; the H-box is by no means the best conserved of the two-component transmitter subdomains in plant phytochromes. The structures might have been retained for some purpose other than autophosphorylation and phosphotransfer. The unusual architecture of the chemotaxis HPK CheA suggests two possibilities.

First, the three-dimensional structure of CheA shows a relict H-box in the conventional position, which, along with downstream residues, comprises the $K290_{\#981}$ to $R354_{\#1055}$ dimerization site (Bilwes et al., 1999). All HPKs seem to form stable dimers with submicromolar dissociation constants as a result of subunit binding in this region. Plant phytochromes are also dimers but the domains involved are uncertain (see Quail, 1997b). The CheA dimerization domain is made up of two antiparallel, highly amphiphilic α-helices with hydrophobic residues exposed on the subunit surface; PHD (http://www.embl-heidelberg.de/predictprotein) predicts that this region in plant phytochromes is also largely helical with an amphiphilic pattern.

Second, recent studies (U. Sweere and K. Harter, unpublished data) in Arabidopsis indicate that the N-terminal 100-residue fragment of phytochrome B binds the response regulator homolog ARR4, whereas the equivalent phytochrome A fragment does not. B-type phytochromes generally bear a characteristic N-terminal extension (#1–#37, see alignment) relative to other family members, so it is possible that the extension mediates the interaction. This would be analogous to the unconventional H-target subdomain at the N terminus of CheA, although little sequence homology is apparent and there is no evidence that the phytochrome is involved in a phosphotransfer. The system seems to connect to a two-component system involved in hormone signaling.

Missing a conserved $H_{\#995}$-target residue in plant phytochromes, it was suggested that the perfectly conserved $Y_{\#991}$ nearby might have taken over the acceptor function (Schneider-Poetsch et al., 1991). Tyr and Ser/Thr protein kinases (YPKs and S/TPKs, respectively) work differently from HPKs; after autophosphorylation, rather than donating their own single phosphates, they phosphorylate their substrates with phosphate groups from free ATP[4]. There are, however, precedents for protein kinases showing a different substrate specificity from that implied by their primary structure. Moreover, immunological methods suggest a light-regulated Y phosphorylation of oat PHYA (Sommer et al., 1996). Although the residue involved is not

[3] Originally the PAS domain referred to the entire region including both repeats, but as it now seems that the repeated region in PAS can also appear alone, we refer here to a "single-copy" PAS domain.

[4] This is significant in signal transduction. Although an HPK activated by its sensor module might be able to carry out autophosphorylation and phosphotransfer many times, amplifying the initial signal (gain > 1), the rest of the prokaryotic phosphorelay does not amplify (gain < 1). Eukaryotes commonly employ Y and S/T protein kinases in a cascade, providing strong amplification (gain $\gg 1$). Interestingly, the eukaryotic two-component HPK systems SLN1 and ETR1 both connect to such cascades.

known, motifs around $Y_{\#319}$ and $Y_{\#1055}$ in various phytochromes resemble the phosphotyrosine-binding site of SH2 domains. While this appears to be the sum of current evidence for phytochrome YPK function, the possible relationship between two-component systems and YPK/Ras GTPase signaling (Stock and Lukat, 1991) should encourage a careful search for related mechanisms in the case of phytochrome.

PLANT PHYTOCHROME IS A DIFFERENT KIND OF KINASE

Even before the sequence of oat PHYA was published, Quail and co-workers drew attention to its peculiarly S/T-rich N terminus as a possible kinase substrate. This was perhaps born of necessity as it was the only feature of the sequence that hinted at a function. While phytochrome N-terminal sequences are not well conserved, S and T residues predominate—also in Cph1. Indeed, $S8_{\#45}$ in oat PHYA is phosphorylated; however, the physiological significance of this is unclear, as the level of phosphorylation is similar for Pr and Pfr (Lapko et al., 1997). Interestingly, mutation of the N-terminal Ser residues in PHYA increases rather than decreases its physiological potency in transgenic plants, so the phospho-Ser modification might serve to attenuate phytochrome action, analogously to arrestin-mediated quenching of rhodopsin (Elich and Chory, 1997). In the case of phytochrome it is uncertain whether this is an autophosphorylation event or whether a separate kinase is involved.

The idea that phytochrome might be a S/T kinase bothered biochemists for many years, but recent evidence using recombinant systems in vitro and in vivo indicates that plant phytochromes can indeed autophosphorylate S/T residues and phosphorylate other proteins, including Rcp1, in a light-dependent manner (Yeh and Lagarias, 1998; Fankhauser et al., 1999; Lapko et al., 1999). Major differences from the Cph1 system should be made clear, however. First, plant Pfr becomes more strongly labeled than Pr, implying that the assembled sensory module in its ground state represses the autokinase activity—the opposite of Cph1*. Second, although plant phytochrome phosphorylated the Rcp1 response regulator in vitro, the target was not the D68 used by Cph1*. As histones too were effective substrates, the relevance of this observation might be called into question. However, it seems that the phytochrome kinase substrate PKS1 is phosphorylated by Pfr both in vitro and in vivo, with overexpression leading to repression of phytochrome action. Third, rather than $H538_{\#995}$, one or more unknown S/T residue(s) in plant phytochrome are autophosphorylated. $S599_{\#675}$ of oat PHYA—within the PAS module but N-terminal of the first repeat—shows Pfr-enhanced phosphorylation in vivo and would thus seem to be an obvious candidate. However, it is not conserved and the $S599K_{\#675}$ mutant still autophosphorylates and phosphorylates PKS1—although light regulation is lost. In relation to domain function it is interesting to note that, while it is probably not a functional HPK, the plant phytochrome transmitter module alone is sufficient for PSK1 binding.

Three-dimensional structural studies with kinases and their allies are already advanced (for example, Bilwes et al., 1999), providing useful background information regarding possible functions in phytochrome. Most of the residues directly responsible for ATP binding in HPKs and gyrases ($GXG_{\#1166\text{-}\#1168}$, $GLGL_{\#1196\text{-}\#1199}$ and $G_{\#1213}$) are well conserved in phytochromes–interestingly deviant are $N_{\#1118}$ and $D_{\#1168}$. We look forward to the day when phytochrome will contribute to studies of kinase function in general.

Pr VERSUS Pfr

For the plant physiologist, perhaps the most intriguing aspect of Cph1 is that Pr is the active kinase, while in plants Pr is thought to be inactive, Pfr being the "active form of phytochrome". The red-light-induced formation of tiny amounts of Pfr from the Pr pool in the cytoplasm of imbibed seeds or dark-grown seedlings leads to the profound physiological changes associated with germination or deetiolation. Physiological responses do correlate quite well with the Pfr concentration of PHYA in etiolated tissues, although in green tissues this is less certain because spectroscopic measurements are hampered by strong chlorophyll fluorescence and the approximately 100-fold lower amounts of phytochrome. Genetic studies seem to have settled the issue, however, as *phy*$^-$ mutants phenocopy Pr.

There is also an attitude problem. For the physicist, photoreceptors are in their ground state in darkness and are excited by light—thus the ground state of Cph1 is Pr and the excited state is Pfr. But for the Cph1 biochemist, the active kinase is Pr—mirroring the behavior of the *Rhizobium* HPK oxygen sensor, FixL, in which ligand binding represses kinase activity. For the biologist, on the other hand, prolonged darkness is equivalent to starvation for a photosynthetic organism—"ground state" is hardly an appropriate description.

As discussed above, there is no reason to suppose that plant phytochromes act as HPKs—even if that was the original function of Pr. On the other hand, it seems now that plant phytochromes act as Pfr-active S/T protein kinases. This leaves a question open: What is the biochemical function of Pfr in cyanobacteria? Once again, the two-component paradigm provides possible answers. Many HPKs are known to be bi-functional, phosphorylating or promoting de-phosphorylation of the response regulator according to their conformation as determined by the sensor module. Both activities are important as they allow the transduction system to differentiate rather than integrate the input signals from the sensor module, a principle that also applies to eukaryotic G-protein-coupled signaling, as in the rhodopsin/arrestin system. The photochromic nature of Cph1 offers the attractive possibility that the excited Pfr form might play the opposite role to that of Pr, promoting the de-phosphorylation of Rcp1. Of course, cyanobacterial Pfr might have a quite separate biochemical activity or it may simply be inactive. Whatever the role of Pfr in Cph1 is, it might be retained in plant phytochromes.

CONCLUSIONS AND OUTLOOK

As we have discussed, photochromic detection systems and chromophores associated with the cyanobacterial phycobilisome have made crucial contributions to the study of phytochrome at the molecular and conceptual levels. The unexpected discovery of phytochromes in other prokaryotes both answers and poses many questions. Phytochrome apparently appeared before eukaryotes, evolving over vast tracts of time and under changing selection pressures to glean and then transmit pertinent information about the light environment to allow the organism to respond appropriately. Perhaps most surprising, then, is the clarity of the homologies between prokaryotic and plant phytochromes. The origin of the PAS module involved in plant phytochrome signaling can also be traced to prokaryotes. Thus, a variety of prokaryotic models are acting catalytically in studies of phytochrome, the active sites being the modes of signal transduction and the photochromic mechanism itself. The reaction products should prove most interesting.

NOTE ADDED IN PROOF

Since this review was submitted, Jiang et al. (Z.Y. Jiang, L.R. Swen, B.G. Rushing, S. Devanathan, G. Tollin, C.E. Bauer [1999] Science **285:** 406–409) have reported a photoreceptor, Ppr, in the purple photosynthetic bacterium *Rhodospirillum centenum* showing homology to HPKs and phytochromes but, like Cph1, missing the PAS module. An N-terminal extension resembles PYP and, like PYP, the apoprotein binds *p*-hydrocinnamic acid. The reconstituted holoprotein is a functional HPK whose autokinase activity is inhibited by blue light. Also, BphP from *Deinococcus* has now been shown to attach PCB at $H260_{\#381}$ — adjacent to $C_{\#380}$ (M in PphP), the traditional binding site (S. Davis and R. Vierstra, unpublished data). Interestingly, $H_{\#381}$ is conserved in all phytochromes (see alignment).

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