Phytochromes as light-modulated protein kinases

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Many phytochrome responses in plants are induced by red light and inhibited by far-red light. To explain the biochemical basis of these observations, it was speculated that plant phytochromes are light-regulated enzymes more than 40 years ago. The search for such an enzymatic activity has a long and rather tumultuous history. Biochemical data in the late 1980s had suggested that oat phytochrome might be a light-regulated protein kinase. The topic was the subject of intense debate, but solid experimental data backing the kinase model has been published recently. Two lines of research played a key role in this finding: the production of biologically active highly purified recombinant phytochrome and the discovery of phytochromes in prokaryotes. This review discusses the key steps of this discovery, and suggests some hypotheses for the role of protein kinase activity in photomorphogenesis.

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Prokaryotic phytochromes suggest a biochemical mechanism for light signalling

Phytochromes are a class of red/far-red light-absorbing photoreceptors. They are found as soluble homodimers with each protein covalently attached to a linear tetrapyrrole chromophore. They are composed of two domains separated by a small hinge region. The amino-terminal half is necessary and sufficient for chromophore binding and normal spectral properties. The carboxy-terminal domain is essential for light signalling and can be regarded as the output domain. Red light converts the red-absorbing phytochrome (Pr, \( \lambda_{\text{max}} 660 \text{ nm} \)) to a far-red-absorbing form (Pfr, \( \lambda_{\text{max}} 730 \text{ nm} \)). The presence of Pfr correlates with a large number of physiological and gene expression responses. Because many of these responses are reversed by FR light (which converts Pfr back to Pr), Pfr is thought to be the active form for many phytochrome mediated responses. The discovery of phytochromes in prokaryotes had two important consequences: it suggested an evolutionary history for this class of photoreceptors that was believed to be unique to plants, and it revived an old hypothesis concerning the potential protein kinase activity associated with plant phytochromes. Davis and Vierstra cover prokaryotic phytochromes more thoroughly in the review in this same issue. The impact of these prokaryotic phytochromes on our knowledge of phytochrome signalling is emphasized in this first section.

Certain cyanobacteria can regulate the composition of the light harvesting complexes affording them an adaptive advantage as they can optimize photosynthesis depending on the light conditions. These light-regulated changes in the phycobilisome composition are known as complementary chromatic adaptation (CCA). The study of CCA in the cyanobacterium *Fremyella diplosiphon* allowed for the identification of RcaE, a potential photoreceptor controlling the biosynthesis of phycobilisome components. The amino terminal portion of the protein shows homology to the plant phytochrome chromophore attachment domain, or bilin lyase domain (BLD) defined by Wu and Lagarias. This domain represents a subset of the GAF domain which has been described previously. The carboxy terminal portion of RcaE has the signature of a typical bacterial histidine kinase domain (HKD) (see Figure 1). This was the first report of a non-plant protein with a BLD domain. This paper also suggests protein phosphorylation as the primary biochemical mechanism by which information from the light surroundings could be relayed into the signalling cascade.

Around the same time the Kazusa sequencing project of the cyanobacterium *Synechocystis sp. PCC6803* uncovered another prokaryotic protein with striking similarities to plant phytochromes. Again the amino-terminus looks
very similar to a BLD and the carboxy-terminus has all the motifs of a HKD. This prokaryotic phytochrome could be produced in *E. coli* and purified rather easily.\textsuperscript{8} Recombinant *Synechocystis* phytochrome binds to phytochromobilin (the plant phytochrome chromophore, \(\text{P} \Phi \text{B}\)) or phycocyanobilin (PCB) autocatalytically and displays absorption spectra with photoreversible red (Pr) or far-red (Pfr) absorption maxima typical of plant phytochromes.\textsuperscript{8–10} Moreover this putative cyanobacterial photoreceptor coined Cph1 (Cyanobacterial phytochrome 1) by the Lagarias group, was shown to be a light-regulated histidine kinase.\textsuperscript{10} Both autophosphorylation of Cph1 and transphosphorylation of Rcp1 (response regulator for cyanobacterial phytochrome—a small response regulator encoded in the same operon as Cph1) are inhibited by red light and stimulated by far-red light.\textsuperscript{10} This study suggests very strongly that in cyanobacteria phosphorylation is an important and very early step of phytochrome signal transduction. It is interesting to note that the His kinase activity of Cph1 is inhibited by red light and stimulated by far-red light; whereas most (but not all) phytochrome-mediated responses in plants are stimulated by red and inhibited by far-red light. The crucial question concerning Cph1 is its role in *vivo*. In the absence of a function for Cph1 it will be difficult to address the relevance of the protein kinase activity in light signalling. However, deletion of the *plpA* gene in *Synechocystis*, encoding another protein containing a BLD domain, compromises the balance between the two photosystems.\textsuperscript{11} Further structure function analysis of the PlpA protein and/or the discovery for an *in vivo* function for Cph1 gene should improve our understanding of phytochrome mediated light signalling in cyanobacteria.

More recently a phytochrome-related photoreceptor Ppr was described in the purple photosynthetic bacterium *Rhodospirillum centenum*.\textsuperscript{12} This protein is very similar to RcaE and Cph1 but it has a PYP (photoactive yellow protein) domain at the amino-terminus followed by the BLD and the HKD (see Figure 1).\textsuperscript{12} Recombinant Ppr reconstituted with p-hydroxycinnamic acid (the chromophore of PYP) displays spectral properties quite similar to those of PYP from *Ectothiorhodospira halophila*;\textsuperscript{12} reconstitution with PCB or \(\text{P} \Phi \text{B}\) have not been reported. However, this gene encodes a protein that can potentially bind to two distinct chromophores. Interestingly Ppr is also a light-regulated histidine kinase, again implicating light-regulated phosphorylation at the heart of prokaryotic phytochrome signalling. In this case blue light which is absorbed by p-hydroxycinnamic acid bound Ppr inhibits the protein kinase activity. This light-regulation of the kinase activity is chromophore dependent. Deletion of this gene demonstrates a role for Ppr in blue light induced chalcone synthase expression.\textsuperscript{12} It is noteworthy that chalcone synthase is also under phytochrome regulation in plants.

The latest members of the growing prokaryotic phytochrome family have been discovered in non-photosynthetic bacteria such as *Deinococcus radiodurans* and *Pseudomonas aeruginosa*.\textsuperscript{13} The *Deinococcus* phytochrome reconstituted with PCB or \(\text{P} \Phi \text{B}\) has the typical phytochrome absorption spectra despite the lack of the conserved cysteine essential for chromophore binding in
phytochromes.\textsuperscript{13} (Please consult the accompanying review by Davis and Vierstra for further details.) This phytochrome is implicated in light-induced biosynthesis of carotenoids which may protect the bacteria from visible light.\textsuperscript{13} Here again a response regulator is encoded in the same operon as the photoreceptor strongly suggesting His kinase activity in phytochrome-mediated signalling in \textit{Deinococcus}.

Although it is still in its infancy, the study of phytochrome mediated signalling in prokaryotes certainly provides us with compelling evidence that in certain bacteria light signals are perceived by phytochrome-like molecules which relay this information via a His kinase signalling cascade. However, many questions remain to be answered: What is the actual phytochrome chromophore in those bacteria? What are the targets of the His kinase cascade and how does light-regulated phosphorylation affect their activity? In some systems we do not know which light responses are controlled by those photoreceptors. The ease and speed with which some of these prokaryotes can be manipulated promise great progress in the near future.

\textbf{Plant phytochromes prefer Ser and Thr}

In addition to the light-sensing domain (BLD) and the carboxy-terminal output domain (histidine kinase related domain, HKRD) plant phytochromes also have an amino-terminal extension (NTE) of about 100 amino acids (see Figure 1). The carboxy-terminal portion of plant phytochromes appears to be the result of the duplication of a bacterial HKRD.\textsuperscript{14} The first of those domains also contains two PAS repeats. These modules have been found in a wide variety of organisms and play important signalling roles in response to small ligands, changes in light conditions, oxygen levels or redox potential.\textsuperscript{15} Interestingly PAS domains are also highly related to PYP, and they have almost identical protein folds.\textsuperscript{16} It therefore looks as if plant phytochromes have reshuffled all the protein domains present in Ppr from the purple bacterium \textit{Rhodospirillum centenum} (see Figure 1). Genetic and molecular studies have shown that the PAS repeats are very important for phytochrome function. The majority of missense mutations in \textit{Arabidopsis} phytochromes cluster in this part of the protein affecting the non-photo-induced reversion of Pfr to Pr and/or interaction of phytochrome with signalling components.\textsuperscript{3,17–19}

In 1992, Schneider-Poetsch recognized that the very C-terminus of plant phytochromes show modest but significant similarity to bacterial histidine kinases.\textsuperscript{20} The recent discovery of phytochromes in prokaryotes confirms that this homology is meaningful. However, several critical residues required for activity in the majority of bacterial sensor kinases are not conserved in all plant phytochromes.\textsuperscript{21} Moreover, mutating some of the remaining critical residues for His kinase activity did not affect the activity of such mutant phytochromes when expressed in plants.\textsuperscript{21} It therefore appears that plant phytochromes are not functionally active His kinases. The key step for finding a biochemical activity associated with plant phytochromes was to develop a reliable system for recombinant expression. Following upon their biochemical characterization of cyanobacterial Cph1 the group of Clark Lagarias went on to tackle this old question for plant phytochromes. They developed recombinant systems to express and purify plant phytochromes in yeast.\textsuperscript{22,23} Purified oat phyA expressed in \textit{S. cerevisiae} and \textit{Mesotaenium caldarium} phytochrome expressed in \textit{P. pastoris} have the expected spectroscopic properties and display protein kinase activity.\textsuperscript{14} However, unlike their cyanobacterial counterparts they auto-phosphorylate on Ser/Thr rather than His/Asp.\textsuperscript{14} Both phytochromes autophosphorylate in a light and chromophore regulated manner. To test if this is an intramolecular property of the photoreceptor, autophosphorylation of oat phyA was performed with varying protein concentrations. The data show that this is a concentration independent reaction, strongly suggesting an intramolecular mechanism.\textsuperscript{14} However, as phytochromes are dimers in solution, one monomer could phosphorylate the other. Recombinant phytochromes purified from two different sources (\textit{S. cerevisiae} or \textit{P. pastoris}) have very similar biochemical properties to oat phytochrome purified from the plant.\textsuperscript{14,24} These are very strong indications that this is an intrinsic property of plant phytochromes and not an artefact due to co-purification of another protein kinase. Another indirect evidence for the relevance of this activity is the finding that oat phyA is a phosphoprotein \textit{in vivo}, and the phosphorylation state \textit{in planta} is also light-regulated suggesting autophosphorylation.\textsuperscript{25} Phytochromes are not the first eukaryotic Ser/Thr kinases with His kinase ancestry, but much remains to be done to characterize these unusual enzymes.\textsuperscript{26} The ATP binding site has not been determined and the protein kinase domain has not been mapped. The \textit{in vitro} protein kinase activity is rather weak compared with protein kinases from the Ser/Thr super-family, but it may be that the best possible substrates and assay conditions have not yet been found. It should be pointed out that the specific \textit{32P}-incorporation for oat phyA and Cph1 are very similar (J. Clark Lagarias personal communication). At this point in time the kinase activity has only been proven for one higher plant phytochrome: oat phyA. It will be interesting to see if different phytochromes playing distinct roles in...
the plant (such as phyA and phyB for example) behave differently as protein kinases.

In vitro kinase assays have identified other substrates of phytochrome. Of particular interest are the cryptochrome blue light receptors cry1 and cry2, which interact with phyA in vitro and are substrates for the phyA protein kinase activity. Moreover, cry1 phosphorylation is stimulated by red light in vivo.27 These results are particularly noteworthy in view of the large body of photobiological evidence suggesting an interaction between phytochrome and the blue light receptors.28,29 PKS1 (phytochrome kinase substrate), like the cryptochromes, interacts with the C-terminus of phytochromes.30 PKS1 phosphorylation, like phyA autophosphorylation, is light-regulated in vitro. Moreover phosphorylation of PKS1 in vivo is stimulated by red light, suggesting that phytochrome is the protein kinase.30 Gain of function studies suggest that PKS1 acts as a negative regulator of phytochrome function in vivo.30 This study suggests a role for protein phosphorylation in phytochrome-mediated signalling in Arabidopsis thaliana.

A role for phosphorylation during plant photomorphogenesis?

The previous paragraphs reviewed the data showing that plant phytochromes display Ser/Thr protein kinase activity and discussed a number of substrates of this activity. Red light-induced protein phosphorylation has been well documented in plants, but the kinases responsible for this activity have remained elusive.31–34 Phytochrome is probably one of those kinases, but one important remaining question is: What is the importance of this biochemical activity during plant photomorphogenesis? Because the protein kinase activity of phytochromes is light regulated, it could be the primary mechanism by which changing light conditions modulate plant development. However, in eukaryotic cells, super-imposed on the light-regulated kinase activity, light also affects the subcellular localization of phytochromes. These two processes could be linked but at this point in time this is pure speculation. (For a detailed review of the light-regulated nuclear import of plant phytochromes please read the review by Nagy et al. in this issue.)

Oat phytochrome A autophosphorylates in vitro and is a phosphoprotein in vivo but the role of this post-transcriptional modification is still unknown.14,25 Interestingly, two oat phyA in vivo phosphorylation sites have been determined: Ser7 and Ser599. Ser599 is phosphorylated in a light-dependent fashion, suggesting that phosphorylation of this residue results from autophosphorylation or from phosphorylation by another phytochrome (Figure 2).25 Ser599 is also a major phospho-acceptor site identified in in vitro phosphorylation studies with mammalian cAMP- and cGMP-dependent protein kinases.24,35 It should be pointed out that a mutant phyA, where Ser599 was replaced by a lysine, no longer displays light-regulated autophosphorylation and transphosphorylation onto PKS1 in vitro.30 The importance of this residue in vivo remains to be determined. The other mapped phosphoacceptor site is Ser7 but this residue is constitutively phosphorylated.25 This site is likely to be the same site of autophosphorylation reported earlier for purified oat phyA.35 It is interesting that phosphorylation of this portion of the protein has been implicated in down-regulation of phyA signalling (Figure 2).36

A number of hypotheses for a role for phyA autophosphorylation could be brought forward. Degradation of phyA is light-dependent and requires selective recognition and ubiquitination of Pfr only.37 Phytochrome-dependent ubiquitination is a well-described mechanism for regulated proteolysis.38 As phyA autophosphorylates in a light-dependent fashion and is degraded only after conversion to Pfr, these two events might be linked. Alternatively light-modulated phosphorylation could lead to a modulation of the subcellular localization of phytochrome. Here again there are precedents for proteins with nuclear localization sites that are phosphorylation dependent.39 It must however be pointed out that translocation from the cytoplasm into the nucleus is a rather slow process (Nagy et al., in this issue). Unmasking of a nuclear localization site (NLS) after light-modulated autophosphorylation of phytochrome should be a very rapid process. There might therefore not be a simple link between these two events.

Alternatively light-modulated phosphorylation of phytochrome substrates such as PKS1 could also affect nuclear import of phytochromes. PKS1 is a constitutively cytoplasmic protein, which inhibits phytochrome signalling when it is overexpressed.30 It has therefore been postulated that PKS1 could be a cytoplasmic retention factor for phytochromes, very similar to the mode of action of IKB which inhibits NFKB nuclear import.40 The cytoplasmic retention of phytochromes by PKS1 could also be modulated by light-regulated phosphorylation.

Another potential target of phytochrome phosphorylation is NDPK2 (nucleoside diphosphate kinase 2). Red light stimulates phosphorylation of NDPK2 in vivo suggesting that this protein may also be a substrate for phytochrome kinase activity.41,42 Like other substrates of phytochrome’s kinase activity such as cry1, cry2 and PKS1, NDPK2 has been found as a phytochrome-interacting protein in a yeast two-hybrid
Figure 2. The model explains how light-modulated phytochrome protein kinase activity might affect light signalling. Light-regulated phytochrome phosphorylation could affect the stability of the photoreceptor in the case of phyA, the subcellular localization of phytochromes, and/or their ability to interact with signalling components. Light-modulated phytochrome protein kinase activity could modulate the activity of signalling intermediates in a light-regulated fashion. The amino acid numbers correspond to oat phyA.

Moreover Pfr (the more active form of the protein kinase) stimulates the enzymatic activity of NDPK2. This may or may not be an effect of NDPK2 phosphorylation since Pfr also binds NDPK2 better than PrA. \textsuperscript{19} ndpk2 mutants have altered responses to both red and far-red light, suggesting that this regulator interacts \textit{in vivo} with both phyA and phyB. The mechanism of action for NDPK2 in plants is not known. Studies in other organisms implicate this enzyme in many developmental processes.\textsuperscript{19, 42, 43}

Interestingly earlier studies have suggested that phytochrome signalling involves phosphorylation of nuclear proteins.\textsuperscript{31} This is particularly interesting in view of the latest developments in phytochrome signalling. Phytochrome is translocated into the nucleus in response to light and a number of proteins genetically or molecularly identified as early components of phytochrome signalling are also nuclear proteins (reviewed by Peter Quail in this issue).\textsuperscript{44} It will be interesting to test if some of those proteins are substrates of phytochrome kinase and how this post-transcriptional modification might affect their function.

Of particular interest is PIF3, a phytochrome signalling component identified both genetically and in a yeast two-hybrid screen for phytochrome interacting proteins.\textsuperscript{18, 45} phyB binds to DNA-bound PIF3 in a light-dependent fashion.\textsuperscript{46} Light-induced conformation-specific binding of a photoreceptor to a transcription factor is a very attractive model for phytochrome signalling.\textsuperscript{47} The activity of a number of transcription factors is regulated by phosphorylation.\textsuperscript{48} It will be important to test if PIF3 is modified in response to light, and if phytochrome is a PIF3 kinase directly modulating the activity of this transcription factor. Modulation of the activity of circadian clock-associated 1 (CCA1) by phosphorylation, another DNA binding protein acting downstream of phytochromes, has been reported.\textsuperscript{49} However, CK2 rather than phytochrome appears to be the protein kinase mediating CCA1 phosphorylation.\textsuperscript{50}

In prokaryotic systems the search for targets of phytochrome kinase substrate is facilitated by the gene organization into operons. Response regulators, encoded in the same operon as the sensor kinases, can easily be identified in the case of cyanobacteria Cph1 or Deinococcus BphP, for example. Plants also encode numerous histidine kinases, histidine kinase-related proteins (phytochromes) and response regulators.\textsuperscript{51} By analogy with prokaryotic phytochrome signalling, it is tempting to speculate that among this quite large class of proteins there are phytochrome kinase substrates. There is no molecular or genetic data indicating that this is the case, but our knowledge of the role of plant response regulators is still very limited.\textsuperscript{51}

How does light modulate phytochrome-mediated signalling in plants? Currently the most popular hypothesis is through changes in subcellular localization (Nagy \textit{et al}.)

### Figure 2

- **Pr**
  - Ser7
  - Cys322
  - Ser599
  - Protein kinase activity
  - Desensitization
  - Subcellular localization?
  - Stability?

- **Pfr**
  - Ser7
  - Cys322
  - Ser599
  - Protein kinase activity
  - Subcellular localization?
  - Stability?
  - Light-regulated kinase activity?
  - Protein-protein interaction?

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**Figure 2.** The model explains how light-modulated phytochrome protein kinase activity might affect light signalling. Light-regulated phytochrome phosphorylation could affect the stability of the photoreceptor in the case of phyA, the subcellular localization of phytochromes, and/or their ability to interact with signalling components. Light-modulated phytochrome protein kinase activity could modulate the activity of signalling intermediates in a light-regulated fashion. The amino acid numbers correspond to oat phyA.
in this issue). However, it is worth pointing out that the light-induced Pr to Pfr transformation is very rapid, but phyB only accumulates in the nucleus after several hours; PfrB must therefore be present in the cytoplasm. In the case of phyA, nuclear translocation is more rapid. Another element in favour of a cytoplasmic site of action for phytochromes is the identification of cytoplasmic phytochrome signalling components such as NDPK2, PKS1 and PAT1. Many phytochrome responses such as membrane depolarization or changes in hypocotyl growth rates occur within minutes of irradiation with light. It is therefore likely that the nucleus is not the only site of action of phytochromes. One could therefore imagine that phytochrome-mediated phosphorylation would be a mode of action for very rapidly induced phytochrome responses. In contrast, more long-term commitments requiring gene regulation would rely on light-modulated import of phytochrome into the nucleus. Those two modes of action are of course not mutually exclusive and a combination of both probably occurs. As already pointed out earlier in this discussion, nuclear import of phytochrome might be regulated by phosphorylation, and phytochrome could phosphorylate both cytoplasmic and nuclear proteins, since PKS1 is cytoplasmic and the cryptochromes are nuclear. Some of these ideas are schematically represented in Figure 2.

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