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Forum Review

Phototropins: A New Family of Flavin-Binding Blue Light Receptors in Plants

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ABSTRACT

Phototropin is the designation originally assigned to a recently characterized chromoprotein that serves as a photoreceptor for phototropism. Phototropin is a light-activated autophosphorylating serine/threonine kinase that binds two flavin mononucleotide (FMN) molecules that function as blue light-absorbing chromophores. Each FMN molecule is bound in a rigid binding pocket within specialized PAS (PER-ARNT-SIM superfamily) domains, known as LOV (light, oxygen, or voltage) domains. This article reviews the detailed photobiological and biochemical characterization of the light-activated phosphorylation reaction of phototropin and follows the sequence of events leading to the cloning, sequencing, and characterization of the gene and the subsequent biochemical characterization of its encoded protein. It then considers recent biochemical and photochemical evidence that light activation of phototropin involves the formation of a cysteinyl adduct at the C(4a) position of the FMN chromophores. Adduct formation causes a major conformational change in the chromophores and a possible conformational change in the protein moiety as well. The review concludes with a brief discussion of the evidence for a second phototropin-like protein in *Arabidopsis* and rice. Possible roles for this photoreceptor are discussed. Antioxid. Redox Signal. 3, 775–788.

INTRODUCTION

A LARGE NUMBER OF RESPONSES in plants, ranging from reversible physiological changes to changes in gene expression, are activated by blue light. One of these responses is phototropism, a growth response in which the direction of growth is determined by the direction of incident blue light.

The search for a photoreceptor for phototropism and the identification of its chromophore has been long, arduous, and argumentative. A wide variety of indirect evidence has been marshaled to support a favored putative chromophore, based on the distribution of a particular pigment, the biochemical properties of a particular *in vitro* system, action spectroscopy, or even the mere incidence of a given pigment. Thus, protagonists have proposed carotenoids (58), flavins (19), pterins (18), and retinal (34) as potential blue light-absorbing chromophores. In the case of the phytochromes, isolation and characterization of the chromoprotein over 30 years ago led to the identification of a bilitriene as the chromophore for this red/far-red-reversible photoreceptor family (see 10). However, there was no such bird in hand for any blue light receptor, and the bushes were full of attractive candidates. A variety of different action spectra in the wavelength range between 300 and 500 nm merely complicated the situation (9), as some resembled the ab-

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sorption spectrum of a flavoprotein and some did not.

The discovery that blue light induces the phosphorylation of a plasma membrane-associated protein in etiolated pea seedlings (16) initiated a decade-long series of studies leading to the identification of a light-activated, autophosphorylating, flavin mononucleotide (FMN)-binding serine/threonine kinase as a photoreceptor for phototropism. This chromoprotein was recently designated phototropin (15) (later to be designated phot1). This review will first cover correlative evidence implicating phototropin in phototropism and biochemical characterization of the light-activated phosphorylation reaction. It will then describe the isolation of phototropism mutants, and the cloning and characterization of the phototropin gene and its encoded protein. Finally, it will review the biochemical and photochemical characterization of the phototropin protein and its chromophore-binding domains expressed in a heterologous system. It concludes with a brief consideration of another putative member of the phototropin family, to be designated phot2 (see below). For more general recent reviews covering blue-light responses in plants, see Ahmad (1), Batschauer (3), Briggs and Huala (8), Casal (12), Cashmore *et al*. (13), Lin (31), and Maheshwari *et al*., (35). Short and Briggs (53) reviewed some of the earlier biochemical studies, and Liscum and Stowe-Evans (33) have recently reviewed phototropism.

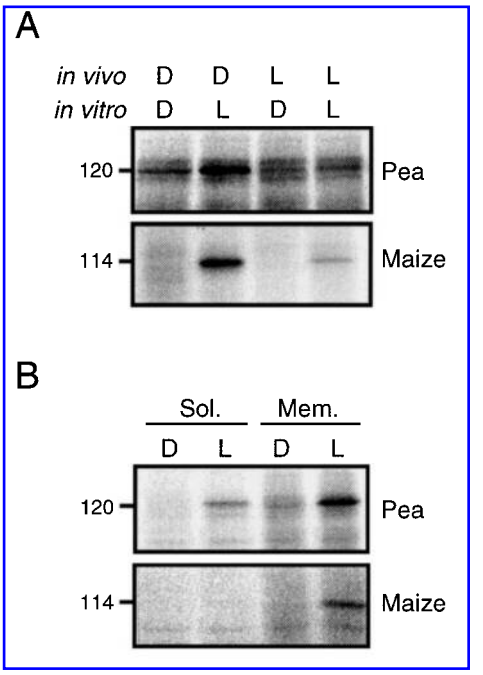

FIG. 1. Effects of light given to intact tissues or cell extracts on phototropin phosphorylation. (**A**) Effect of *in vivo* irradiation on light-inducible phosphorylation in subsequently isolated membrane fractions (pea, after 52; maize, after 22). (**B**) Effect of light treatment on tissues preincubated with ^{32}P inorganic phosphate on phosphorylation detectable in subsequently isolated soluble and membrane fractions (pea, after 54; maize, after 22). D, dark control; L, saturating blue-light treatment.

THE LIGHT-ACTIVATED PHOSPHORYLATION REACTION

When one irradiates membranes isolated from etiolated seedlings prior to adding (or in the presence of added) γ-^{32}P-labeled ATP, one obtains strong phosphorylation of a protein band between 105 and 130 kDa (pea, 52; *Arabidopsis*, 42; maize, 22, 38; oat, 48; and several additional dicot seedlings and cereal coleoptiles, 41) (Fig. 1A, lanes 1 and 2). However, if etiolated plants are irradiated with blue light immediately prior to membrane extraction, subsequent irradiation of the membranes elicits a dramatic reduction in the phosphorylation response (Fig. 1A, lanes 3 and 4). Likewise, if light-sensitive tissues are preincubated in ^{32}P inorganic phosphate to allow them to form endogenous radiolabeled ATP, subsequent blue-light irradiation of the treated tissue yields a phosphorylated protein of the same molecular size range in the membrane fraction (pea, 54; maize, 22, 38) (Fig. 1B; cf. Fig. 1A). Thus, one can activate the phosphorylation reaction either *in vivo* or *in vitro*, with the latter reaction providing a powerful tool to study the biochemical properties of the system.

CORRELATION OF LIGHT-ACTIVATED PHOSPHORYLATION WITH PHOTOTROPISM

Several lines of evidence have shown that the light-activated phosphorylation of phototropin might play a role in phototropism. First, in etiolated pea seedlings (52), and in maize (22, 39), wheat (51), and oat (47) coleoptiles, those tis-

sues most sensitive to phototropic stimulation contain the highest levels of photoactivated phosphorylation. Second, both light-activated phosphorylation *in vivo* (52) and first positive phototropism (the most sensitive phototropic response) (see 6) obey the Bunsen–Roscoe reciprocity relationship—an equal response so long as the fluence (the product of intensity × time) is constant—indicating that they are limited by first-order photochemistry. In addition, both *in vitro* (39) and *in vivo* (22) action spectra for light-activated phosphorylation were found to be consistent with the action spectrum for phototropism (2).

For several of the studies described below, it was necessary to quantify the relative amount of phosphorylation obtained *in vivo* following a particular light treatment. The extent of this response is readily determined by an indirect method. One can measure the relative phosphorylation induced by a light pulse given to intact tissue (x) by what additional residual phosphorylation can be elicited by irradiating the membranes subsequently extracted from that tissue (y). The more effective the initial light pulse, the less effective the second ($x = 1 - y$, where x is the unknown fraction of phosphorylation activated by irradiating the tissue and y is the measured fraction obtained following irradiation of the extracted membranes. The amount of phosphorylation obtained by giving saturating blue light to membranes from previously unirradiated tissue is set at 1).

It has been known for 40 years that when one irradiates seedlings with a light pulse that saturates first positive curvature, they go through a refractory period of tens of minutes before becoming fully sensitive to a second light pulse (6). By using the $x = 1 - y$ method described above, one can test whether a similar refractory period exists for *in vivo* light-induced phosphorylation. Both in maize (22, 39) and in oat (47) coleoptile tips, the photosensitive reaction is refractory immediately following a saturating blue-light pulse. Membranes or whole-cell extracts isolated following increasing dark periods after the light pulse regained photosensitivity gradually over a period of 20–30 min (maize, 39) or 60–90 min (oat, 47) at room temperature, reflecting a return to the dark unphosphorylated state. The recovery kinetics for light-driven *in vivo* phosphorylation in maize coleoptile tips following a saturating light pulse were found to be almost the same as those for recovery of phototropic sensitivity of maize coleoptiles as measured by Briggs (6) 37 years earlier (see 39).

In contrast to coleoptile tips, the more basal tissues of oat coleoptiles were found to increase dramatically in their phosphorylation capacity over dark control values following the initial refractory period. This increase may reflect a blue light-induced sensitization mechanism in these tissues, as they show a significant increase in phototropic sensitivity if they have been preirradiated with blue light (47). Pea seedlings showed a somewhat slower recovery period for phosphorylation (52) relative to maize coleoptile tips and, like coleoptile tips, did not increase their phosphorylation capacity beyond the initial dark state.

Finally, Salomon and co-workers (48, 49) demonstrated that unilateral light of certain fluences leads to a lateral gradient of phosphorylation across oat coleoptiles. These results provide the first direct demonstration of a light-induced biochemical gradient corresponding to the differential growth across a responsive plant organ.

The results cited so far suggest a tight correlation between light-activated phosphorylation and phototropism, whereby the former might be part of the mechanism leading to the latter. However, the correlation fails completely when one compares the relative photosensitivities of the two processes. Fluence–response relationships have been determined for light-activated phosphorylation both *in vivo* (with the $x = 1 - y$ method) (pea, 52, 54; maize, 39; oat, 47) and *in vitro* (pea, 54, 55; *Arabidopsis*, 42; maize, 23, 39; and oat, 46). Although considerable variation exists between species (and between methods), the threshold and saturation values all lie between one to two orders of magnitude above the threshold and saturation values for phototropism. There are several possibilities for this discrepancy. The extreme coleoptile apices, sites of maximum photosensitivity for phototropism, could be more sensitive than the more basal tissues for phosphorylation. Alternatively, the phosphorylation could be a desensitization reaction, occurring only at higher

fluences (39, 49). However, later biochemical studies suggest a more complicated model, and we will therefore defer further consideration of this discrepancy until we have reviewed these more recent studies.

SUBCELLULAR LOCALIZATION

Gallagher *et al*. (17) localized pea phototropin largely to the plasma membrane by sucrose-gradient separation of various organellar fractions. These findings were based on the co-localization of phototropin with a putative plasma membrane marker enzyme, glucan synthase II. Similarly, Hager and Brich (22) localized maize phototropin to plasma membrane fractions on sucrose gradients, based on co-localization with a different plasma membrane marker enzyme, sterol-glucosyl transferase. Subcellular localization of phototropin was also investigated by using an entirely different method, aqueous two-phase partitioning, as described by Widell and Larson (60). Given the right concentrations of polyethylene glycol and dextran T-500, this method produces an upper polyethylene glycol phase highly enriched in right-side-out plasma membrane vesicles. In all plant tissues tested by this method (pea, 55; maize, 38; wheat, 51; oat, 46), phototropin was found to be localized to the polyethylene glycol fraction.

Further studies have investigated the localization of phototropin in relation to the plasma membrane itself. Short *et al*. (55) found that increasing concentrations of the neutral detergent Triton X-100 greatly increased both dark levels and light-enhanced levels of phototropin phosphorylation, presumably by permeabilizing right-side-out vesicles and allowing radiolabeled ATP to access their interior. Hager and Brich (22) reported the same phenomenon for plasma-membrane vesicles from maize. Likewise, successive cycles of freezing and thawing, reported to produce a mixture of right-side-out and inside-out plasma-membrane vesicles (40), more than doubled the level of light-activated phototropin phosphorylation (55). From these experiments, the authors concluded that the phototropin protein was most likely localized to the inner surface of the plasma membrane. In support of this conclusion, Salomon *et al*. (46) found that, at least in microsomal membranes obtained from 5-day-old etiolated oat seedlings, a significant fraction of phototropin was protected from trypsin digestion. More recently, K. Sakamoto and W.R. Briggs (unpublished observations) have shown that a phototropin–green fluorescent protein fusion is localized to the plasma membrane in transgenic *Arabidopsis* plants. Surprisingly, phototropin was subsequently found to be a highly hydrophilic protein lacking any putative membrane-spanning domains (25). To date, the nature of its association with the plasma membrane is still not understood. Also lacking is information regarding the distribution of phototropin among different tissue types.

BIOCHEMICAL CHARACTERIZATION OF THE PHOSPHORYLATION REACTION

Phosphorylation kinetics

In all plants tested (pea, 54, 56; *Arabidopsis*, 42; maize, 22, 38), light-activated phosphorylation begins immediately on addition of ATP and reaches a maximum within ~2 min. In several cases, the level of phosphorylation declines over the next 10–20 min, although in other cases (maize, 22; *Arabidopsis*, 14) the level of phosphorylation remains unchanged for 10–20 min. The reason for this discrepancy is unclear at present, although it is likely related to differences in phosphorylation conditions. At least with pea membranes, the decline occurred whether membranes were kept in darkness after a light pulse or exposed to continuous blue light. Membrane preparations from dicots typically show some level of dark phosphorylation of phototropin (see Fig. 1A, lane 1, cf. pea and maize), and the reaction kinetics are identical to those obtained following or during a blue-light treatment (54). Similarly, the small amount of residual phosphorylation found in maize membranes extracted from coleoptile tips that had received a saturating pulse of blue light just prior to extraction follows a time

course similar to that obtained from membranes from unirradiated coleoptile tips (22).

At present, there is no clear explanation for the decline, although it may be related to intrinsic instability of the phosphorylated protein. However, inclusion of either a phosphatase inhibitor, sodium fluoride (38, 54), or the protease inhibitors, leupeptin or phenylmethylsulfonyl fluoride (38), was without effect on phosphorylation. Moreover, addition of cold ATP at any time after the phosphorylation level had reached a maximum had no influence on the subsequent course of phosphorylation (22,54), indicating a lack of phosphate turnover, at least in isolated membranes.

Phosphorylation on multiple sites

Coomassie- or silver-stained sodium dodecyl sulfate gels of both pea (52, 55) and *Arabidopsis* (32) microsomal membranes show a fairly prominent protein band corresponding to the position of the protein exhibiting light-activated phosphorylation. This band shows a significant reduction in mobility following phosphorylation, a reduction consistent with phosphorylation on multiple sites (4). Likewise, complete digestion of the excised phosphorylated band with different proteases (pea, 54, 56; oat, 46) produced multiple phosphorylated bands of different intensities, a result providing convincing evidence for phosphorylation on multiple sites. Short *et al*. (56) reported identical phosphopeptide patterns in the time course for proteolysis for pea phototropin phosphorylated in the light and in the dark, confirming the conclusion that phosphorylation in the dark occurs at the same sites as those for light-activated phosphorylation.

Various authors have observed proteins of lower molecular weight than phototropin that show light-activated phosphorylation (38, 42, 46, 51, 52). These bands appear more prominent in gels from membrane preparations from older tissues (46, 52) and may be proteolysis products of phototropin. Indeed, the proteolytic degradation pattern of these products obtained in the presence of the endopeptidase Lys C produced three of the same proteolytic fragments as did the full-length protein and produced no additional bands (46). To date, there is no evidence for involvement of phototropin in a light-activated phosphorylation cascade.

Biochemical memory for a light pulse

As mentioned above, saturating light pulses either for first positive phototropism or for *in vivo* phosphorylation are followed by a recovery period of many minutes before full light sensitivity is restored. It is still unclear whether the dark recovery for light-activated phosphorylation accompanies dephosphorylation of the existing protein or degradation of the phosphorylated protein and *de novo* synthesis of new unphosphorylated protein. Neither a phosphatase inhibitor, sodium fluoride, nor protease inhibitors affected the time course for the biochemical memory of the light signal (38). *In vitro*-irradiated membrane-associated phototropin also has a memory for the blue-light treatment. If samples are given a blue-light pulse followed by increasing dark periods, the amount of phosphorylation detected declines to the dark level over a period ranging from 10 to over 60 min (pea, 54; maize, 22, 38; oat, 46). Solubilization of the membranes with Triton X-100 significantly accelerates the dark recovery (46). The reaction is somewhat slower when samples are held on ice, but the effect is hardly dramatic (54). The lack of a strong temperature effect suggests that the dark-recovery reaction is likely to be monomolecular. Although the relaxation kinetics for the *in vivo* memory are very similar to those for the *in vitro* memory, there is a significant difference: The *in vivo* memory involves phototropin that has been phosphorylated in response to light treatment, whereas the *in vitro* memory involves the unphosphorylated form of the protein.

Three studies have tested the effect of a second light pulse following an intervening dark period and have yielded conflicting results. Palmer *et al*. (38) found only a minor enhancement of phosphorylation when they irradiated maize membranes for a second time after various dark periods following a prior irradiation, even in the presence of a protease inhibitor cocktail. By contrast, Hager *et al*. (23) found an almost complete recovery of photosensitivity of maize membranes following dark decay of the

effects of the initial light signal as did Salomon *et al*. for oat membranes (46). The only difference between these studies was the absence of detergent in the latter studies (23, 46), the probable cause of the instability. We have recently noted recovery of phosphorylation capacity in response to a second irradiation in the presence of dithiothreitol with *Arabidopsis* membranes (J.M. Christie and W.R. Briggs, unpublished results), but as detergent was absent in these experiments until just before addition of the ATP, one cannot be certain whether the loss observed by Palmer *et al*. (38) was because of the presence of Triton, the absence of reducing agent, or both. Salomon *et al*. (46) also reported that the capacity for storage of the light signal in oat membranes was lost when the membranes were solubilized with Triton X-100. We will return to the phenomenon of biochemical memory for the light pulse later in the review.

Phosphoamino acid analysis, pH sensitivity, and staurosporine sensitivity

Prior to the cloning and characterization of the phototropin gene (25) and expression of the functional protein in a heterologous system (14), numerous studies were aimed at characterizing the phosphorylation reaction. These experiments utilized crude membrane preparations, purified plasma membranes, Triton-solubilized membranes, or whole-cell extracts, mostly with similar results. The light-activated phosphorylation reaction in pea (56) and maize (38) occurred mainly on serine residues, with only a trace of phosphothreonine and no detectable phosphotyrosine. In both species, the reaction was Ca^{2+}-independent, but required Mg^{2+} (22, 38, 56) as MgATP (56).

Both pea (56) and maize (22) phototropin have a broad pH optimum near 7.5, with activity declining sharply to zero below pH 6. The pea phosphorylation reaction was less inhibited at higher pH values than was the maize reaction. This difference may simply reflect a difference in the buffers used for these experiments. As might be expected from the results reported above, the shape of the pH-dependence curve for phosphorylation of phototropin in dark-control membranes (pea, 56) was virtually identical to that for the phosphorylation induced by light. pH 7.8, the isoelectric point for pea phototropin, coincided closely with the pH optimum for phosphorylation (59). Not surprisingly, the reaction was inhibited by staurosporine, a potent inhibitor of protein kinase C and other protein kinases, with half-maximal inhibition occurring at 4 n*M* (maize, 22). Nanomolar staurosporine concentrations were also found to inhibit the phosphorylation reaction in wheat membranes (51).

Nucleoside triphosphate specificity

The reaction in pea membranes is highly ATP-specific. In competition experiments with additional cold ATP, GTP, UTP, or CTP, ATP was a strong competitor, GTP was a very weak competitor, and neither UTP nor CTP had any effect at all (56). Although the reaction in maize plasma membranes could be light-driven with radiolabeled GTP, ATP was a far more effective substrate (21). As with ATP, the GTP-dependent reaction was inhibited by staurosporine.

A putative role for flavins

Given that the action spectrum for light-activated phosphorylation resembled the absorption spectrum of a flavoprotein (22,39), Short *et al*. (54) tested several known flavin antagonists to determine their effect on the reaction. Iodide and azide ions, known to quench flavin excited states, and phenylacetic acid, a compound that forms a covalent bond with photoexcited flavins, inhibited the reaction only at high concentrations. Thus, if the chromophore is indeed a flavin, it is perhaps not readily accessible to these reagents, or the reagents themselves may in some way inactivate the system. Added FMN, flavin adenine dinucleotide (FAD), or riboflavin had no effect on the extent of the phosphorylation reaction, suggesting either that the chromophore is not a flavin at all, or that it is tightly bound and does not turn over (54). As will become clear below, the latter hypothesis has been found to be the correct one.

Redox dependence

If maize membranes had been stored frozen, subsequent light-activated phosphorylation was strongly enhanced by addition of reducing

agents such as dithiothreitol, NADH, NADPH, or ascorbate (23) prior to irradiation. Dithiothreitol added following a light pulse, however, was without effect. Dithiothreitol had little effect on the low level of phosphorylation observed in dark controls. Evidently the light reaction requires a reducing environment. These results are consistent with the findings reported above, where a second pulse of light after a dark period was found to be effective in activating phosphorylation in the presence of dithiothreitol (23), but not in its absence (38). However, Salomon *et al*. (46) obtained some recovery of photosensitivity for phosphorylation without reducing agent in oat membranes as long as they only added Triton just before adding radiolabeled ATP. It seems likely that for this system to function optimally it needs both intact membranes and a reductant to recover full sensitivity.

Using a different approach, Rüdiger and Briggs (43) investigated the effect of thiol reagents on light-activated phosphorylation in maize membranes. Iodoacetate, *N*-ethylmaleimide, and *N*-phenylmaleimide, at a concentration of 1 m*M*, all inhibited the reaction: iodoacetate, the most hydrophilic, was least effective, and *N*-phenylmaleimide, the most hydrophobic, was the most effective. Unlike the reducing reagents discussed above, the thiol reagents were equally effective when added either before or after the light pulse. Hence, the authors hypothesized that the inhibitors were interacting with at least one SH group required for the reaction. They also concluded that the SH group was not directly involved in the primary photochemistry, and was located within a hydrophobic environment. *N*-Phenylmaleimide also inhibited the photoactivation of phosphorylation in solubilized oat membranes (46).

One, two, or three proteins?

The light-activated phosphorylation reaction requires three components: a photoreceptor, a protein kinase, and a protein substrate. In principle, these components could reside on one, two, or three polypeptides. Several different biochemical studies have addressed this issue. First, the solubilized system migrates as a large complex (near 335 kDa) in nondenaturing electrophoresis, without losing photoactivity (59), a result consistent with involvement of a functional protein complex. Second, the relative quantum efficiency for photoactivation is identical whether membrane-associated or Triton-solubilized preparations are irradiated, a result consistent with a single trifunctional polypeptide (55). Third, ATP antagonists were found to interact directly with phototropin. The reagent 5′-*p*-fluorosulfonylbenzoyladenosine forms a covalent bond with ATP-binding sites (61), sites to be expected if a protein is a kinase. Using an antibody against this inhibitor, Short *et al*. (55) detected a band near 120 kDa on western blots that showed a mobility decrease similar to that observed for the protein band mentioned above when membranes were isolated from previously illuminated pea stem sections. Palmer *et al*. (38) demonstrated further that brief incubation with 5′-*p*-fluorosulfonylbenzoyladenosine prior to illumination strongly inhibits the light reaction in maize membranes, and that inhibitor binding to phototropin is prevented in the presence of excess ATP. These results are consistent with the hypothesis that the substrate protein is also a kinase and that the phosphorylation is an autophosphorylation reaction. Fourth, phototropin phosphorylation following blue-light treatment showed exactly the same time course whether carried out with membrane or Triton-solubilized preparations, again a result consistent with a single functioning protein (56). Fifth, irradiation of solubilized maize membranes prior to mixing with unirradiated solubilized pea membranes leads to phosphorylation of the pea phototropin (41), indicating cross-phosphorylation in a functional unit that may be a dimer. Similar results were obtained with membranes from several other species pairs (41). Dimerization or multimerization could explain the ~335 complex obtained on nondenaturing gel electrophoresis (59). [It should be noted that Hager (21) failed to confirm cross-phosphorylation between maize and pea phototropin.] Finally, Hager (21) showed that the phosphorylation reaction was almost as efficient at 0°C as at 30°C, a result consistent with an autophosphorylating system.

Although the above results make participation of three different polypeptides in the reaction unlikely, they do not make an airtight

case for a single autophosphorylating photoreceptor protein. This question was finally resolved by the cloning, sequencing, and characterization of the phototropin gene and a biochemical characterization of its encoded protein.

CLONING, SEQUENCING, AND CHARACTERIZATION OF THE PHOTOTROPIN 1 GENE AND ITS GENE PRODUCT

The phototropin gene

Following pioneering work from the Poff laboratory (29,30) in isolating and characterizing mutants of *Arabidopsis* impaired in their phototropic responses, the study of mutants became increasingly important in sorting out various light responses and their signal transduction pathways (see 1, 3, 8, 12, 13, 31, 35). An *Arabidopsis* mutant, JK224, previously shown by Khurana and Poff (29) to have dramatically reduced sensitivity for first positive curvature, had very little light-activated phototropin phosphorylation, establishing for the first time solid genetic evidence for participation of phototropin in phototropism (42).

Subsequently, following screening for additional *Arabidopsis* phototropism mutants and testing for complementation, Liscum and Briggs (32) identified four mutant loci involved in the signal transduction pathway for phototropism. These they designated *nph1* through *nph4* (for non-phototropic hypocotyl). JK224 turned out to be allelic to *nph1* and was designated *nph1-2*. The *nph1* mutant alleles described by Liscum and Briggs (32) all lacked light-activated phosphorylation, and lacked a 120-kDa protein that showed a light-induced mobility shift on sodium dodecyl sulfate–polyacrylamide gels. Thus, it appeared that the membrane protein phosphorylated in response to blue-light irradiation was encoded by the *NPH1* gene. (Note: the *NPH1* gene is hereafter designated *PHOT1*, any new mutant alleles as *phot1*, the encoded holoprotein as phot1, and the apoprotein as PHOT1. Already described alleles will continue to be accorded the *nph1* designation.)

Working with a fast neutron-generated *Arabidopsis* mutant, *nph1-5*, Huala *et al*. (25) finally cloned and sequenced the *PHOT1* gene. The presence of lesions in the gene in three mutant alleles, plus restoration of the phototropic sensitivity of the null mutant *nph1-5* with the wild-type gene, confirmed that the correct gene had been cloned. With the sequence at hand, one issue was immediately resolved: phot1, the encoded protein, was itself a classic serine threonine protein kinase. It has all 11 of the expected signature domains of a serine threonine kinase located in its C-terminal half and falls into the PVPK1 family of kinases within the protein kinase C group (24). Given the results of the cross-phosphorylation experiments mentioned above (41, but see 21), it is likely that phot1 can function at minimum as a homodimer.

In addition to the kinase domain, the putative phot1 protein has two PER-ARNT-SIM superfamily (PAS) domains (see 57) that are ~40% identical. As these domains are similar to domains found in a wide range of signaling proteins from archaea, eubacteria, and eukaryotes, proteins involved in detecting light, oxygen, or voltage, Huala *et al*. (25) designated them LOV domains (LOV1 and LOV2). As the NifL protein from *Azotobacter* and the aerotaxis protein from *E. coli* are both flavoproteins binding FAD, and as these two proteins had no other sequences in common with each other or with phot1, the authors hypothesized that the phot1 LOV domains could function as flavin-binding sites. In that case, phot1 was likely to be a photoreceptor.

The phot1 protein

Whereas the Huala *et al*. (25) study reduced the possible number of protein participants to two (a photoreceptor and an autophosphorylating protein kinase), a subsequent study by Christie *et al*. (14) finally resolved the photoreceptor question. When the *PHOT1* gene from *Arabidopsis* was expressed in an insect cell/*Baculovirus* system, it retained photosensitivity in the absence of any other plant proteins. The kinetics for light-activated phosphorylation of the recombinant protein and the fluence–response curve for that activation are almost identical to those of the native protein. The het-

erologously produced protein also has the property of storing the light signal in the dark, although its biochemical memory fades somewhat more slowly than that of the native photoreceptor. Rather than binding FAD, as was the case for NifL and aer, phot1 binds FMN noncovalently. The fluorescence excitation spectrum closely resembles the action spectrum for phototropism, with two peaks and a shoulder in the blue part of the spectrum, fine structure indicating that the FMN is bound in a rigid pocket, and a broad band in the UV-A. The authors concluded that phot1 undergoes blue light-induced autophosphorylation and therefore functions as the photoreceptor for phototropism.

As the phot1, NifL, and aer proteins all bound flavins, and as the only features they shared in common were LOV domains, it seemed likely, as mentioned above, that the LOV domains were the binding sites for the various flavin chromophores. Expression of constructs containing the individual phototropin LOV domains or containing both LOV1 and LOV2 (from *Arabidopsis* and oat phototropin, or the fern *Adiantum capillus-veneris* phy3) confirmed this hypothesis (15). [*Adiantum* phy3 is a phototropin homologue with phytochrome sequences at the N-terminal end (37)]. All of the purified LOV domain-containing peptides bind FMN stoichiometrically: the LOV1 and LOV2 peptides each bind a single FMN molecule, whereas the double construct binds two FMN molecules. FMN binding appears to be the same as that in the full-length insect cell/*Baculovirus* product as the absorption and fluorescence excitation spectra show the same fine structure in the blue part of the spectrum, and closely match the phototropism action spectrum. Hence, phot1 is a dual chromophoric photoreceptor: its two LOV domains each bind a single FMN molecule, and it responds to photoexcitation by autophosphorylation.

Early photochemistry and biochemistry

The LOV domains of phototropin undergo a photocycle, detectable as a fully reversible photobleaching in blue light (50). The light-minus-dark spectrum is not characteristic of a typical flavin photoreduction, but rather resembles the spectrum for a C(4a)-cysteinyl adduct (36). All LOV domains found in phot1 chromoproteins for which sequences are known contain the highly conserved amino acid sequence GRNCRFLQ (50). The cysteine at position 39 in the domain is the only cysteine in LOV2, whereas LOV1 contains a second cysteine at position 66. Modeling studies and comparison with PAS domains of known three-dimensional structure (FixL, 20; HERG, 11) suggested that the FMN might be bound in a central pocket. Site-directed mutagenesis of Cys^{39} to alanine or serine yielded chromopeptides that did not undergo a photocycle upon illumination. However, FMN binding was unaltered in the mutant chromopeptides as the absorption spectra were very similar to those of the wild-type chromopeptides (50). Hence, an early photochemical event in light sensing by the LOV domains of phototropin is light-activated formation of a cysteinyl adduct. Mutating the second cysteine in LOV1 had no dramatic effect on the photocycle reactions (50).

The crystal structure of LOV2 from *Adiantum capillus-veneris* phy3 has recently been obtained at 2.7 Å resolution and has confirmed the putative model mentioned above (16). It consists of a β-sheet core flanked by α-helices, remarkably similar to the PAS domains from FixL (20), PYP (5), and HERG (11). The core surrounds the FMN, and the cysteine SH group is $\sim$4 Å from the C(4a) position of the isoalloxazine ring of the FMN chromophore. It should be noted that despite the close structural similarity of these four peptides, the LOV domains from phototropin bind FMN, whereas the PAS domain from FixL binds heme, the PAS domain from PYP binds *p*-coumaric acid, and the PAS domain from HERG is not known to bind any ligand.

LOV1 and LOV2 are not identical in their photochemical and biochemical properties (50). LOV2 has a quantum efficiency near 0.5 for photobleaching, whereas LOV1 is far less sensitive (quantum efficiency near 0.05). Photobleached LOV1, on the other hand, decayed three times as rapidly as LOV2 to the dark state. The FMN bound to LOV1 is clearly more accessible to the aqueous environment than the FMN bound to LOV2 as the former FMN can

be released by treatment with *N*-phenylmaleimide, whereas the latter cannot. It is possible that the Cys^{39} of LOV1 provides the SH group that is accessed by *N*-phenylmaleimide in the earlier experiments by Rüdiger and Briggs (43). PAS domains not only serve to bind ligands, but can also mediate protein–protein interactions (54). Salomon and co-workers (unpublished observations) have recently shown that purified LOV1 domains from oat behave as dimers in ultracentrifugation, whereas purified LOV2 domains behave as monomers. The significance of this difference is unclear, but is further evidence that the functions of the two LOV domains in phototropin may not be identical, and that a functional unit of full-length phototropin may be a dimer formed by the LOV1 domains.

There is some evidence that the light-induced phosphorylation reaction may be hierarchical (see 7) with a pair of phosphorylations being driven by far lower fluences of light than the bulk of the phosphorylations. Thus, it is tempting to hypothesize that these highly sensitive phosphorylations, only a small fraction of the total activated by light, actually participate in inducing the physiological response, whereas the remainder serve to desensitize the system. This hypothesis may explain the apparent discrepancy between the fluence response curve for first positive phototropism and that for light-activated phosphorylation. The very sensitive phosphorylation reactions would scarcely be detectable against the majority of the less sensitive ones. Zimmerman and Briggs (62) made a detailed kinetic analysis of the fluence–response relationships for phototropism of oat coleoptiles many years ago and proposed a similar mechanism. They concluded that the only reasonable scheme to accommodate the shape of the fluence–response curve for first positive phototropism was one involving initial activation of the photoreceptor by one photon and then its inactivation by a second photon, with the second reaction having a lower quantum efficiency than the first. If this model is correct, then the more sensitive phosphorylation events would lead to activation of signal transduction, and the less sensitive bulk of phosphorylation events would lead to desensitization.

A slow dark decay of the cysteinyl adduct might account for the *in vitro* biochemical memory mentioned above. Although the reaction is relatively rapid in isolated single LOV domains (50), it is significantly slower in constructs carrying both LOV domains, with a time course similar to that for loss of the biochemical memory (M. Salomon, J.M. Christie, and W.R. Briggs, unpublished observations). Any remaining molecules with the cysteinyl adduct present could presumably still carry out phosphorylation. It is not yet clear why a reducing environment is required to permit a second light pulse to activate the phosphorylation reaction (23). The LOV domains can undergo repeated photobleaching and complete dark recovery in the absence of any external reductant and without any detectable alteration in their photosensitivity or their absorption spectra. Presumably a different part of the chromoprotein must be kept in a reduced state to maintain the capacity for light-activated phosphorylation.

It is tempting to conclude that the *in vivo* memory for a saturating light pulse may also be related to persistence of the cysteinyl adduct. However, it should be remembered that the *in vivo* memory occurs with phototropin in its phosphorylated state, whereas the *in vitro* memory just discussed occurs in the absence of phosphorylation. A full understanding of *in vivo* memory for a blue light pulse clearly awaits further experimentation.

ANOTHER PHOTOTROPIN IN *ARABIDOPSIS*

Jarillo *et al.* (26) recently reported a second member of the *PHOT1* (*NPH1*) gene family, which they designated *NPL1* for NPH1-like. Like the phot1 protein (nph1), npl1 has two LOV domains and a C-terminal serine/threonine kinase domain. The npl1 protein is somewhat smaller than phot1, and the two proteins have overall 58% identity and 67% similarity. Liscum and Briggs (32) failed to detect either first positive phototropic curvature or second positive curvature in response to low fluence rates of continuous light in the null mutant

nph1-5, although npl1 was presumably present. Hence, it was likely that npl1 mediated some other blue light response. Indeed Sakai *et al*. (44) observed strong second positive curvatures in response to high fluence-rate blue light in a *nph1* null mutant, indicating participation of a second photoreceptor in phototropism in *Arabidopsis*. Another candidate response was blue light-activated chloroplast movement for which the photoreceptor(s) was up to now unknown.

Chloroplasts are well known to change their distribution in plant cells in response to different light conditions. They will accumulate in a layer perpendicular to the direction of weak blue light to maximize light absorption for photosynthesis (accumulation response), but will move to the side walls to line up parallel to the direction of strong blue light to minimize photodamage. Kagawa *et al.* (27), utilizing a null mutant at the *NPL1* locus, have recently found that the avoidance response of chloroplasts to high fluence rates of blue light is completely lacking, although the accumulation response is normal. Sakai *et al*. (45) then showed that a *nph1 npl1* double mutant lacked both accumulation and avoidance responses. They also showed that the phototropic response that they had previously observed in a *nph1* null mutant (44) at high fluence rates of blue light was also lacking. Thus, phot1 and npl1 play roles both in phototropism and in chloroplast movement responses, with the role of nph1 predominating at low fluence rates and that of npl1 dominating at higher fluence rates.

Npl1 shares not only the major structural features of NPH1, namely two LOV domains and a C-terminal serine/threonine kinase, but also the same early photochemistry and biochemistry. Sakai *et al*. (45) have recently shown that the npl1 LOV domains bind FMN and undergo a photocycle consistent with the formation of a C(4a)-cysteinyl adduct analogous to the photocycle of the LOV domains from phot1 (see 50). Furthermore, irradiation of full-length npl1 produced in the insect cell/*Baculovirus* system shows light-activated autophosphorylation, suggesting that npl1 also functions as a photoreceptor kinase. We therefore designate this protein as phot2.

Recently, Kanagae *et al*. (28) reported two *PHOT1* (*NPH1*) homologues in rice that they designated *OsNPH1a* and *OsNPH1b*. *OsNPH1a* is most strongly expressed in coleoptiles whereas *OsNPH1b* is most strongly expressed in leaves. When dark-grown seedlings are transferred to light, *OsNPH1a* mRNA in coleoptiles declines sharply, whereas *OsNPH1b* mRNA in leaves gradually increases. The *OsNPH1a* gene is most similar to *Arabidopsis PHOT1*, whereas *OsNPH1b* is most similar to *Arabidopsis PHOT2*. It will be interesting to determine if these two photoreceptors share roles in phototropism and light-directed chloroplast movement in rice as they do in *Arabidopsis*. We will tentatively refer to the two rice proteins as phot1 and phot2.

A LOOK AHEAD

We are beginning to learn something about the early photochemistry and biochemistry of phot1 (50), but many questions remain. First, what is the precise photochemical mechanism leading to formation of the C(4a)-cysteinyl adduct? Does it involve initial formation of a flavin semiquinone, or is there some other mechanism? If semiquinone formation is involved, what is the source of the electron? Is it the cysteine itself or some other amino acid?

Second, how does the light-induced formation of the C(4a)-cysteinyl adduct result in activation of phosphorylation? Circular dichroism studies indicate that the FMN undergoes a major change in conformation on formation of the adduct (50). Indeed this light-induced conformational change is absent when the LOV2 Cys^{39} is mutated to alanine or serine, suggesting that it is a direct consequence of the adduct formation. Preliminary Fourier transform infrared spectroscopy shows a number of major vibrational changes that can be directly assigned to conformational changes within the flavin moiety. However, there are other changes that almost certainly reflect conformational changes in the protein itself (T. Swartz, R. Bogomolni, J.M. Christie, and W.R. Briggs, unpublished observations). It therefore remains to be determined how these changes can activate the kinase function.

Third, what is the role of the autophosphorylation itself? Is the phosphorylation indeed heirarchical, as postulated by Briggs (7)? Does a more sensitive phosphorylation reaction mediated by LOV2 activate signal transduction, and a less sensitive reaction mediated by LOV1 initiate desensitization? Or is the phosphorylation reaction simply involved in down-regulation of photoreceptor function? These questions must await further experimentation. The manner in which photoexcitation of phot1 activates downstream elements in the signal-transduction pathway leading to phototropic curvature remains elusive.

Fourth, is the phosphorylation of either phot1 or phot2 the initial step in a phosphorylation cascade, as is the case in so many other systems? To date, none of the authors has observed light-activated phosphorylation of any protein that is not a likely proteolytic breakdown product of phototropin itself (see 46), although the possibility of a second phosphorylation substrate cannot be rigorously excluded.

Fifth, how is the initial photochemistry and biochemistry of phot1 and phot2 transduced into two such different responses, phototropism and chloroplast movement? The first of these responses involves cell–cell communication and the transmission of a signal across many cell layers, whereas the second is strictly intracellular.

Finally, what other proteins with LOV domains might serve as blue-light photoreceptors? Even in the double mutant *nph1 npl1*, Sakai *et al*. (45) observed small curvatures induced by a fluence rate of 10 μmol m^{-2}. Hence, the list of blue-light photoreceptors is almost certainly incomplete.

Despite fairly dramatic progress in our knowledge of this new class of photoreceptors, the next few years should be exciting ones. Experimental techniques are at hand to address these and related questions. Time, patience, and careful experimentation will most certainly bring us a more complete picture of the signal transduction pathway leading from photoexcitation of phot1 and phot2 to oriented growth, and will elucidate the role of both photoreceptors in light-activated chloroplast movement and possibly the other related phenomena in higher plants.

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ABBREVIATIONS

FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; ;LOV, light, oxygen, or voltage; PAS, PER-ARNT-SIM superfamily.

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