Blue Light Activates a Specific Protein Kinase in Higher Plants

Philippe Reymond, Timothy W. Short, and Winslow R. Briggs
Carnegie Institution of Washington, Department of Plant Biology, Stanford, California 94305

ABSTRACT

Blue light mediates the phosphorylation of a membrane protein in seedlings from several plant species. When crude microsomal membrane proteins from dark-grown pea (Pisum sativum L.), sunflower (Helianthus annuus L.), zucchini (Cucurbita pepo L.), Arabidopsis (Arabidopsis thaliana L.), or tomato (Lycopersicon esculentum L.) stem segments, or from maize (Zea mays L.), barley (Hordeum vulgare L.), oat (Avena sativa L.), wheat (Triticum aestivum L.), or sorghum (Sorghum bicolor L.) coleoptiles are illuminated and incubated in vitro with [γ-32P]ATP, a protein of apparent molecular mass from 114 to 130 kD is rapidly phosphorylated. Hence, this system is probably ubiquitous in higher plants. Solubilized maize membranes exposed to blue light and added to unirradiated solubilized maize membranes show a higher level of phosphorylation of the light-affected protein than irradiated membrane proteins alone, suggesting that an unirradiated substrate is phosphorylated by a light-activated kinase. This finding is further demonstrated with membrane proteins from two different species, where the phosphorylated proteins are of different sizes and, hence, unambiguously distinguishable on gel electrophoresis. When solubilized membrane proteins from one species are irradiated and added to unirradiated membrane proteins from another species, the unirradiated protein becomes phosphorylated. These experiments indicate that the irradiated fraction can store the light signal for subsequent phosphorylation in the dark. They also support the hypothesis that light activates a specific kinase and that the systems share a close functional homology among different higher plants.

Light provides plants with many different kinds of information about their environment. This information is integrated by sensory systems and used to optimize a variety of physiological and morphological processes. Different classes of responses are defined according to the region of the light spectrum that activates them. The effects of red light on photomorphogenesis are well documented and the photoreceptor phytochrome has been isolated and extensively studied (see ref. 5). Blue light also regulates numerous processes in higher plants, including phototropism, growth inhibition, stomatal opening, and several enzymic reactions (13, 14). Although photoreceptors involved in blue light responses have not yet been identified, a number of studies based on action spectra suggest that flavoproteins are the most probable candidates (8, 20); however, other photoactive molecules such as carotenoids or pterins should not be dismissed (2, 7, 9, 19). In addition, very little is known about the transduction chain between the photoexcitation of the receptor and the physiological response.

Protein phosphorylation is a ubiquitous regulatory mechanism involved in signal transduction (1). Evidences for its involvement in plants have been reviewed recently (3, 11), and it is established that protein phosphorylation can be influenced by light (see ref. 4). Gallagher et al. (6), studying the phosphorylation of membrane proteins extracted from etiolated pea epicotyls, discovered that light was affecting the phosphorylation of a 120-kD protein associated with the plasma membrane. Subsequently, Short et al. (16, 17) showed that in vitro irradiation induced a strong enhancement of the phosphorylation of a 120-kD protein in pea and a 114-kD protein in maize (10, 15). The characteristics of the reaction (localization, kinetics, fluence requirement) indicated that this could be an early step in the transduction chain for phototropism (16). The same conclusion was strongly supported by a study with Arabidopsis mutants, where a mutant with altered phototropic sensitivity showed a dramatic reduction of the blue light-induced phosphorylation of a membrane protein near 124 kD (12). In addition, Short et al. (18) observed that the reaction could be driven either in vivo or in vitro and was blocked by flavin antagonists. It was hypothesized that irradiation with blue light was either (a) activating a specific kinase or (b) exposing sites on the substrate polypeptide for phosphorylation by a constitutively active kinase.

The purpose of the present study was to investigate the effect of blue light on the phosphorylation of a membrane protein in several monocot and dicot species. Since this phosphorylation has been postulated to be an early step in the transduction of the phototropic stimulus, and since phototropism is a general phenomenon in higher plants, one would expect the reaction to be widely represented in divergent species. The other aim of this work was to test the hypothesis that blue light is inducing the phosphorylation of the membrane protein by activating a specific kinase.

---

1 This work was supported in part by National Science Foundation grants DGBB-8-19137 and IBM-91168392 to W.R.B., a National Science Foundation predoctoral fellowship to T.W.S., and postdoctoral fellowship grants from the Swiss National Foundation for Scientific Research and the Philip Morris company to P.R. This is Carnegie Institution of Washington Department of Plant Biology Publication No. 1132.

2 Present address: Institut de Biologie et Physiologie Végétale, Batiment de Biologie, Université de Lausanne 1015 Lausanne, Switzerland.

3 Present address: Plant Gene Expression Center, U.S. Department of Agriculture, 800 Buchanan Street, Albany, CA 94710.
MATERIALS AND METHODS

Chemicals
Sucrose (protease free) was obtained from Boehringer. BAPTA* was from Molecular Probes, Inc. Mops was from Research Organics (Cleveland, OH). Murishige and Skoog salts were obtained from Gibco Laboratories. Agar was from Difco Laboratories. [y-32P]ATP was purchased from Amer- sham. All other chemicals were standard enzyme grade from Sigma Chemical Co.

Plant Material
Seedlings of pea (Pisum sativum L. cv Alaska), sunflower (Helianthus annuus L. cv Mam. Grey Stripe), and zucchini (Cucurbita pepo L. cv) were grown in total darkness for 7 d as described (6), except that seeds were allowed to imbibe and were grown with one-quarter strength Hoagland solution. Stem sections (8–10 mm) from the rapidly growing region were harvested under dim red light (approximately 0.5 μmol m-2 s-1). Each sample normally consisted of 100 sections. Seedlings of maize (Zea mays L. Northrup King hybrid pX9540), oat (Avena sativa L. Garry), wheat (Triticum aestivum L. cv Yamhill), barley (Hordeum vulgare L. cv CM 72), and sorghum (Sorghum bicolor L. cv Redland × Greenleaf) were grown as above except that 5-mm coleoptile tips (100/sample) were harvested after 5 d. Seedlings of Arabidopsis (Arabidopsis thaliana L. cv Columbia) and tomato (Lycopersicon esculentum L. cv UC82) were grown as follows. Seeds (25 mg/plate) were surface sterilized by shaking for 10 min with 30% commercial bleach (final concentration of sodium hypochlorite 1.58%), rinsed with sterile water, shaken a second time with 30% bleach, and rinsed four times with double-distilled water. Seeds were then resuspended in a slurry of 1% Bactoagar and spread onto filter paper that had been placed over 30 mL of solidified 1% agar containing standard Murishige and Skoog nutrient medium plus 3% sucrose in 25 × 200 mm Petri plates. After 7 d of growth in darkness at 24°C (±1°C), filter papers with attached seedlings were lifted from the agar, and hypocotyls with cotyle- dons still present were harvested with curved scissors. Each sample consisted of 10 plates.

Light Sources and Irradiations
Safelight conditions, light sources, and protocols for irradiation were described elsewhere (16, 18). For in vivo and in vitro irradiations, a 1-min pulse of blue light was used to yield a fluence of approximately 1013 μmol m-2, known to saturate the phosphorylation of the pea, maize, and Arabidopsis light-affected proteins (10, 12, 16).

Isolation of Membrane Fractions
Crude microsomal membrane fractions were prepared as described (16, 18) with minor modifications. Harvested tissues were ground in a chilled mortar containing 4.5 mL of homogenization buffer (25 mM Mops, 0.25 mM sucrose, 5 mM BAPTA, 0.1 mM MgCl2, 8 mM L-cysteine, 120 mM N-methyl-d-glucamine, pH 7.8). The homogenate was filtered through 20-μm mesh nylon cloth and centrifuged for 10 min at 9,700g. The supernatant was then centrifuged for 30 min at 100,000g, and the microsomal membrane pellet was resuspended in 300 μL of resuspension medium (0.25 mM sucrose, 4 mM KNO3, 5 mM K2PO4, brought to pH 7.8 with H2SO4) and mixed in a Potter-Elvehjem tissue homogenizer. Membranes were stored at −80°C prior to phosphorylation. Aliquots were removed for Lowry protein assays as described by Short and Briggs (16). All manipulations were carried out in a cold room at 4°C under dim red light (approximately 0.5 μmol m-2 s-1).

In Vitro Phosphorylation and Protein Separation and Analysis
Procedures for the in vitro phosphorylation (18) and the separation and analysis of proteins by SDS-PAGE and autoradiography (6) have been described previously. Unless otherwise specified, 200 μg of crude microsomal membrane proteins were used for the phosphorylation reaction after solubilization in 0.5% Triton X-100. Relative levels of phosphorylation in the SDS-PAGE gel bands were quantitated with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

RESULTS
Phosphorylation of a Membrane Protein from Different Species
Microsomal membrane fractions were prepared from shoots or coleoptiles of 10 different species of higher plants. Harvested segments were either kept in the dark or exposed to 1032 μmol m-2 of blue light in vivo. After extraction and solubilization, the proteins were kept in the dark or irradiated in vitro with the same dose of light prior to phosphorylation with [γ-32P]ATP. Results obtained with sunflower and oat seedlings are shown in Figure 1. Blue light induced the phosphorylation of a membrane protein in both species. Their patterns of phosphorylation are very similar, but the light-affected proteins are of different molecular masses. In sunflower, when membrane proteins extracted from dark-grown seedlings were not irradiated in vitro, a protein of approximately 130 kD shows a relatively low level of phosphorylation (lane 1). However, if these proteins are irradiated in vitro, a strong increase in in vitro phosphorylation is subsequently observed (lane 2). Similarly, membrane proteins isolated from blue light-exposed stem sections exhibit a low signal when kept in the dark (lane 3) and an enhancement of phosphorylation when irradiated in vitro (lane 4), but in both cases, the levels of phosphorylation are reduced compared to the protein from plants kept in the dark. In addition, a protein near 84 kD shows a weak in vitro enhancement of phosphorylation (lanes 1 and 2) and is barely detectable in membrane proteins isolated from irradiated stem sections (lanes 3 and 4). Results are essentially identical with oat coleoptiles (Fig. 1), except that the molecular mass of the light-responsive protein is approximately 114 kD, and no other protein is detectably affected by light.

* Abbreviation: BAPTA, 1,2-bis(2-aminophenoxy)ethane N,N,N', N'-tetraacetic acid.
flower, tomato). The absolute level of phosphorylation varies considerably from species to species.

**In Vitro Phosphorylation of a Maize Membrane Protein**

The phosphorylation reaction was carried out in *Z. mays* with different membrane protein concentrations (Fig. 3). The level of phosphorylation of the 114-kD protein rises linearly with increasing amounts of irradiated membrane proteins. However, when decreasing amounts of unirradiated membrane proteins are added to increasing amounts of irradiated membrane proteins just before the incubation with [γ-<sup>32</sup>P]-ATP, so that the total amount of membrane proteins is always 200 μg, the intensity of the phosphorylated band is higher than that obtained with irradiated membrane proteins alone. This result suggests that a kinase from irradiated membrane proteins may not only be phosphorylating its own substrate, but also some substrate from the unirradiated membrane proteins. To test whether this enhancement of phosphorylation was caused by some unrelated thermostable element present in the unirradiated membrane proteins that simply increased the efficiency or extent of phosphorylation of the 114 kD protein in the irradiated fraction, we boiled the unirradiated membrane proteins (100°C, 2 min) before adding them to the irradiated ones. No increase of phosphorylation over the control was obtained with boiled proteins (Fig. 3). Hence, participation by a thermostable element from the unirradiated protein fraction in additional phosphorylation of the irradiated fraction is ruled out.

In a similar experiment, we exposed a fixed amount of maize membrane proteins (100 μg) to 10<sup>−12</sup> μmol m<sup>−2</sup> of blue light and then added increasing amounts of unirradiated proteins prior to in vitro phosphorylation (Fig. 4). The enhancement of phosphorylation of the 114-kD protein increases almost linearly as greater amounts of unirradiated

**Figure 1.** Effect of blue light given in vitro or in vivo on the phosphorylation of solubilized membrane proteins isolated from sunflower hypocotyls and oat coleoptiles. Autoradiograph image following SDS-PAGE. Lane 1 one each gel, Etiolated segments, proteins kept in darkness; lane 2, etiolated segments, proteins exposed to blue light; lane 3, segments irradiated with blue light prior to membrane isolation, but proteins kept in darkness; lane 4, same as lane 3, but proteins exposed to blue light. All light treatments: 10<sup>−3</sup> μmol m<sup>−2</sup>, Dk, Dark; Lt, light.

**Figure 2.** Effect of blue light given in vitro on the phosphorylation of membrane proteins isolated from several monocot or dicot species. Membranes were extracted from the growing region of etiolated coleoptiles or shoots, solubilized, and treated with blue light (10<sup>−12</sup> μmol m<sup>−2</sup>) prior to phosphorylation. The light-affected proteins are distributed between the two arrows. For comparison, only the irradiated samples are presented. Autoradiograph image following SDS-PAGE.

**Figure 3.** Phosphorylation of a 114-kD maize protein following blue-light irradiation. Different concentrations of solubilized maize membrane proteins were exposed to 10<sup>−12</sup> μmol m<sup>−2</sup> of blue light and added to either unirradiated proteins (●) or unirradiated boiled proteins (▲) just prior to phosphorylation, so that the total amount of protein was always 200 μg. The control (○) consisted of increasing the amount of irradiated proteins only. Dk, Dark; Lt, light. The (●) represent two separate experiments.
membrane proteins are added to the sample. When the amount of unirradiated membrane proteins is twice the amount of the irradiated ones—the highest concentration tested—the increase of the signal reaches about 70% over the dark control.

Phosphorylation Between Species

In the above experiments, we could not rule out the possibility that some thermolabile element of the protein preparation added in the dark contributed to the enhancement of the phosphorylation of proteins in the irradiated fraction. To address this possibility, we studied the simultaneous phosphorylation of membrane proteins from two species, pea and maize, where the responsive proteins are of distinct molecular mass (see Fig. 2) and, hence, can be unambiguously separated on gel electrophoresis. We reasoned that if a kinase from one species were activated, we might see phosphorylation of the appropriate protein from the unirradiated species, provided that there was some interaction between the kinase and substrate from these two widely divergent plants. Thus, a given amount of maize proteins was irradiated with $10^{13}$ mol m$^{-2}$ of blue light and then added to an equivalent amount of unirradiated pea proteins prior to the phosphorylation reaction. As seen in Figure 5, the 120-kD pea protein shows a strong increase in phosphorylation (left lane) over the dark control (central lane), the intensity of the signal being nearly as high as when the pea proteins are themselves irradiated (right lane). The 114-kD maize protein shows a normal level of phosphorylation (left lane). Conversely, when pea proteins are exposed to light and then added to unirradiated maize proteins, the maize protein exhibits a small but detectable enhancement of phosphorylation (right lane) over the dark control (middle lane). Again, the irradiated pea protein shows a normal level of phosphorylation (right lane). The same result is obtained with zucchini and maize proteins (Fig. 5). As a control experiment, irradiated membrane proteins from one species were mixed with boiled membrane proteins from the other species and showed no induced phosphorylation of the unirradiated protein (data not shown).

The same kind of experiment was investigated between several different species and gave similar results: a blue light-activated system from one species is able to phosphorylate specifically a substrate from an unirradiated species (Table I). This phenomenon is observed within dicots or between a monocot and a dicot species. It could not be tested within monocots because the light-affected proteins of these species have the same apparent molecular mass and, hence, are indistinguishable by gel electrophoresis. Quantitation of the phosphorylation cross-reaction (Table I) yields distinct patterns of interactions. Activated maize membrane proteins induce the phosphorylation of the unirradiated protein in all dicots tested with an activity ranging approximately from 40 (Arabidopsis) to 250% (tomato) of the levels obtained by direct irradiation of the dicot membranes themselves. With pea, for instance, the activated maize system fully phosphorylates the unirradiated pea protein to a level similar to that seen with irradiated pea membranes. On the other hand, all activated dicots systems have only a weak effect on the phosphorylation of the maize or the oat protein (between 7 and 19% of the level obtained by irradiation of the coleoptile membranes themselves).

DISCUSSION

The blue light-mediated phosphorylation of a membrane protein is found in a broad array of plant species, with a molecular mass ranging from approximately 114 to 130 kD (Fig. 2). Each species shows a phosphorylation pattern very similar to that of sunflower (dicot) or oat (monocot) as presented in Figure 1. These results correspond closely to what has been reported for pea (16), Arabidopsis (12), and maize (10, 15). However, the absolute level of phosphorylation varies from species to species. This variation might be ex-
Table 1. Phosphorylation between Species

Membrane proteins from one species were irradiated with a saturating dose of blue light (10^3.3 μmol m⁻²) and then added to unirradiated membrane proteins from another species prior to the phosphorylation reaction with [γ-³²P]ATP. The level of phosphorylation of the unirradiated protein was quantitated. Values (±s.e.) are expressed as percent of the level obtained with irradiated membrane proteins only. The number of experiments (n) is shown in parentheses.

<table>
<thead>
<tr>
<th>Irradiated Species</th>
<th>Unirradiated Species</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Maize</td>
</tr>
<tr>
<td>Maize</td>
<td>—</td>
</tr>
<tr>
<td>Oat</td>
<td>—</td>
</tr>
<tr>
<td>Sunflower</td>
<td>11.7 (2)</td>
</tr>
<tr>
<td>Tomato</td>
<td>7.2 (2)</td>
</tr>
<tr>
<td>Arabidopsis</td>
<td>19.6 (2)</td>
</tr>
<tr>
<td>Zucchini</td>
<td>6.7 (2)</td>
</tr>
<tr>
<td>Pea</td>
<td>8.8 ± 1.7 (7)</td>
</tr>
</tbody>
</table>

—, Not measurable.  
ND, Not determined.

explained by differences in the efficiency of the reaction or in the concentration of active molecules. Because three types of organs are compared (hypocotyls with or without cotyledons, epicotyls, coleoptiles), these variations might also be due to morphological differences. It has been shown in pea that the strongest phosphorylation of the light-affected protein occurs in the growing region and progressively decreases in specific activity along the shoot axis (16). In all monocot species tested, the protein has the same apparent molecular mass, but these plants belong to the same family and other more divergent monocots should be tested. In dicots, two species belonging to the Solanaceae have a phosphorylated band of different apparent molecular mass (tomato and tobacco, data not shown). Although distributed within a relatively small mass range, this diversity suggests that some structural differences may exist at the protein level, but without major consequence to this particular photoresponse. Although we cannot be certain that the difference in molecular mass is not the consequence of proteolysis, it seems unlikely since addition of protease inhibitors PMSF (1 mm) and leupeptin (1 μg/ml) to the homogenization medium or phosphorylation buffer did not change the apparent molecular mass of the light-affected protein in pea or maize preparations, and had no measurable effect on the level of phosphorylation (T.W. Short, J.M. Palmer, personal communication).

In most species, blue light induces the phosphorylation of only one specific protein. In addition, a light-affected protein of approximately 84 kD is present in sunflower (Fig. 1). A similar result was described in pea (16) for a protein also near 84 kD that was found mainly in more basal, nongrowing parts of the epicotyl. Whether these lower molecular mass proteins are related to the major photosensitive protein—possibly as breakdown products—or are unique and play some different role remains to be determined.

The level of in vitro phosphorylation in dark controls is always higher in dicot than in monocot species, where it is barely detectable (see Fig. 1 for example). This phosphorylation in the absence of any light has been suggested to be an artifact of the extraction procedure (18), in which case the monocot system seems somewhat less labile during extraction. We do not at present understand these differences and await isolation of the major components of the reaction for further analysis.

Since it was postulated that this process could be an early step in the transduction chain for phototropism (12, 16, 17), it is important to know if this reaction is widely represented. As shown in Figure 2, the blue light-mediated phosphorylation of a membrane protein described here may well be ubiquitous in higher plants. We do not know whether this system is also present in organisms other than higher plants, but preliminary studies in fungi did not uncover any similar response (T.W. Short, E.D. Lipson, unpublished results).

Regarding the mode of action of light on the phosphorylation reaction, the results obtained by Short et al. (18) were consistent with either of two hypotheses: (a) irradiation in some way exposed sites for phosphorylation, or (b) light stimulated a kinase activity. We tested the second hypothesis by mixing irradiated with unirradiated maize membrane proteins (Figs. 3 and 4). The fact that the light signal is retained for at least 10 min (18) allowed us to mix the membrane fractions just prior to adding [γ-³²P]ATP without losing the capacity for phosphorylation. In this experiment, we observe that a significant amount of the unirradiated substrate protein is phosphorylated. This result suggests that a kinase from the irradiated sample not only phosphorylates its own homologous substrate, but is also able to phosphorylate some heterologous substrate in the unirradiated sample. In that case, the result could represent the amplification of a signal, since each activated kinase could be phosphorylating more than one substrate and, hence, could increase the number of affected substrate molecules. However, we cannot eliminate the possibility that in addition to this activation, light also exposes sites to phosphorylation or excites a photoreceptor moity that can then activate the specific kinases in each species. Furthermore, we do not know whether the in vitro reaction has the same characteristics of cross-reactivity as the in vivo system.

Although a control experiment showed that the addition of unirradiated boiled membrane proteins to irradiated membrane proteins produced no increase of phosphorylation (Fig. 3), there was a possibility that some thermolabile element of the unirradiated membrane proteins could stimulate the re-
action in the already irradiated fraction, contrary to the hypothesis that light was activating the kinase. The cross-species experiments eliminate this possibility because the light-affected proteins are of different molecular mass, and, hence, the effect of protein from one species on the protein from the other is clearly distinguishable. The results unambiguously show that irradiated protein from one species leads to the phosphorylation of unirradiated protein from the other species (Fig. 5). This species cross-reactivity is found between several plant pairs (Fig. 5; Table I) and indicates (a) that the light signal is stored by the system, (b) that the kinase specifically phosphorylates only one single protein in each species, and (c) that the systems must share a strong functional homology to permit direct interaction between widely divergent plants. We cannot be certain that the homology is at the level of the substrates such that they can respond to kinases from different species, or at the level of the kinases, such that they can be photocatalyzed by photoreceptor moieties from different species.

Quantitation of these interspecific experiments shows that the reaction between irradiated maize or oat proteins and dicot species is much more effective in inducing the phosphorylation of the protein from the unirradiated species than the opposite (Table I). Several explanations may account formally for this result. First, the protein from the monocot species might be less accessible for interspecific phosphorylation than that of the dicot. Second, the coleoptile kinase might have broader specificity than the dicot kinase. Third, the concentration of active molecules might differ among species. If, for instance, the concentration of the blue light-activated kinase is 10 times higher in maize than in pea (per μg of protein), then irradiated maize membrane proteins would be much more effective in phosphorylating the pea protein. Finally, the kinetics of the phosphorylation reaction might not be similar. Short and Briggs (16) have found that the phosphorylation reaction in pea reaches its maximum after 2 min. A much faster reaction would allow the maize kinase to phosphorylate more substrates in the same period of time. However, recent data indicate that both species may have similar kinetic properties with respect to this phosphorylation reaction (J.M. Palmer, personal communication).

We demonstrate here that intra- and interspecific phosphorylation can occur in vitro, but we do not know what the significance of this finding is in vivo. We currently have no information about the physical relationship of any one photoreceptor-kinase-substrate moiety with respect to any other in the same cell, and whether intermolecular phosphorylation is possible. It has been shown that the light-affected protein is localized to the plasma membrane in pea (6) and maize (10), and that pea plasma membranes can be dissolved in detergent and retain their photoactivity without loss of efficiency (18). These results indicate that the components necessary for activity must either be tightly associated at the plasma membrane level or be combined as a single polypeptide with multiple functions in its native form. It might then be conceivable that upon activation of the kinase, one or more substrates located in the close vicinity of the kinase become phosphorylated in vivo.

From the results presented above, we conclude that the blue light-mediated phosphorylation of a membrane protein—probably a plasma membrane protein—is ubiquitous in plants. This finding reinforces the hypothesis postulated in other studies that the reaction might play an early role in the transduction chain for phototropism. Furthermore, light leads, at minimum, to the activation of a specific protein kinase. The components of this reaction must then have strong similarities among plants to permit the sort of direct interaction observed between divergent species. Full understanding of the system, however, must await the purification of all necessary elements as well as the molecular analysis of the genes involved in this response to blue light.

ACKNOWLEDGMENTS

The authors are deeply thankful to Ms. Ann McKillop for her excellent technical assistance, and to Drs. Julie M. Palmer and Katherine M. F. Warpeha for their careful reading of the manuscript.

LITERATURE CITED


