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Three Protein Kinases from the Etiolated Oat Seedlings Phosphorylate Oat Phytochrome A *In Vitro*

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Phosphorylation of phytochrome may play important functional roles to control plant photomorphogenesis. Many attempts have failed to identify the protein kinase that phosphorylates phytochrome *in vivo*. It has been reported that a polycation-stimulated protein kinase activity was associated with the purified phytochrome. However, it is not known if the kinase activity is an intrinsic property of phytochrome or whether it comes from a contaminant of the purified phytochrome. In the present study, three protein kinases that phosphorylate phytochrome have been identified from etiolated oat seedlings. A polycation-stimulated protein kinase that had very similar enzymatic properties with that associated with the purified phytochrome was identified in the cytosolic extract. It phosphorylated several contaminant proteins in the kinase preparation as well as phytochrome and had a broad substrate specificity. A CK II-type protein kinase phosphorylated phytochrome and the exogenously added casein. It is likely that this kinase may not be a feasible candidate for the kinase phosphorylating phytochrome *in vivo* since the content of the kinase seemed to well exceed the content of phytochrome in the etiolated oat seedlings. Another protein kinase that had unique enzymatic properties phosphorylated phytochrome very specifically and seemed to be present in a small quantity in the etiolated seedlings. It is expected that one of three kinases may be responsible for the phytochrome phosphorylation *in vivo*.

Keywords: Casein kinase, Oat, Phosphorylation, Phytochrome A, Protein kinase.

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Introduction

Phytochrome plays a central role in plant growth and development controlled by red and far-red lights (Furuya, 1993). It has been extensively studied during the last two decades for its molecular properties and physiological responses. Recently, the main interest in phytochrome studies is its signal transduction mechanism to evoke physiological responses.

Protein phosphorylation is a good tool for transmitting the environmental signals to target sites of cells to control the physiological activity of cells (Marks, 1996). Its activity can be sophistically controlled by intricate interplay between protein kinases and phosphoprotein phosphatases. Protein phosphorylation is also believed to be implicated in phytochrome signal perception and transduction, since red and far-red lights changed the phosphorylation states of several cytosolic and nuclear proteins in plants (Park and Chae, 1989; Romero *et al.*, 1991; Schafer *et al.*, 1997). In addition, the phytochrome has been known to be a phosphoprotein which is of interest in relation to its physiological function (Hunt and Pratt, 1980; Lapko *et al.*, 1997). The intact phytochrome contains 0.35–0.5 mol of phosphate per molecule of phytochrome even though the exact nature of the phospho-amino acid is not identified (Lapko *et al.*, 1996). A receptor phosphorylation is a common event in signal transduction, as can be seen in rhodopsin phosphorylation, β-adrenergic receptor phosphorylation, and receptor tyrosine kinases (Short and Briggs, 1990; Ron *et al.*, 1994). However, the nature and functional roles of phytochrome phosphorylation remain to be elucidated.

Phytochrome phosphorylation has been extensively studied by mammalian protein kinases to probe the phosphorylation-induced conformational changes of phytochrome (McMicheal and Lagarias, 1990). Two mammalian protein kinases, PKA and PKC, phosphorylate serine residues near the N-terminal segment (Ser-17) as well as at the hinge region (Ser-598) of oat phytochrome

(McMicheal and Lagarias, 1990). Although the phosphorylation by PKA did not induce significant changes in the secondary structure of phytochrome, it subtly affected the topographic and electrostatic status near the phosphorylation sites. The phosphorylation also changed the accessability of proteases to the proximity of the phosphorylation sites (Lapko *et al.*, 1996). It implies that phytochrome phosphorylation may have an important function in its action mechanism since the removal of the phytochrome N-terminus exhibits hyperactivity of phytochrome action (Jordan *et al.*, 1996).

It has been reported that phytochrome has a polycation-stimulated protein kinase activity (Wong *et al.*, 1986). Since the intrinsic kinase activity of phytochrome has a strong implication to phytochrome function, many research interests have been focused on the phytochrome-associated kinase (Grim *et al.*, 1989; Kim *et al.*, 1989; Wong and Lagarias, 1989; Bierman *et al.*, 1994). However, it is still an unanswered question as to whether phytochromes from higher plants have protein kinase activity.

To clarify the nature and function of phytochrome phosphorylation *in vivo*, the first step may be to identify a protein kinase that specifically phosphorylates phytochrome. We have screened protein kinases that phosphorylates phytochrome A *in vitro* from the extracts of etiolated oat seedlings and report here three protein kinases that phosphorylated oat phytochrome with distinctively different enzymatic properties.

Materials and Methods

Materials Oats (*Avena sativa* L. CV. Garry) were purchased from Maine Potato Growers Inc. (Presque Isle, USA). Hydroxyapatite (HA) was prepared as described previously (Kim *et al.*, 1989). [γ-^{32}P] ATP and [γ-^{32}P] GTP were obtained from Amersham (Aylesbury, Buckinghamshire, England). 8-azido [α-^{32}P] ATP was from ICN Pharmaceuticals Inc. (Irvine, USA). Polyacrylamide gel materials and cellophane membrane backing were obtained from Bio-Rad (Richmond, USA). The other chemicals were purchased from Sigma Chemical Co. (St. Louis, USA) and Boehringer-Mannheim Biochemicals (Indianapolis, USA).

Phytochrome A preparation Intact 124 kDa phytochrome A (phytochrome) was purified in the Pfr form according to the method of Lapko and Song (1995) except that the gel filtration step was omitted. Oat seedlings that were grown in the dark for 4 d were harvested, after irradiation with red light (Sylvania gold F40/GO lamp) for 5 min, and extracted with an extraction buffer [100 mM Tris-Cl (pH 8.3), 140 mM $(NH_4)_2SO_4$, 10 mM Na_2-EDTA, 40% (v/v) ethylene glycol, 20 mM β-mercaptoethanol, 20 mM $Na_2S_2O_5$, 4 mM PMSF]. Phytochrome was purified to a specific absorbance ratio (A_{660}/A_{280}) of ca. 0.6 from the extract by two ammonium sulfate precipitation steps followed by ammonium sulfate back extraction. All procedures were performed in a cold chamber of 4°C or in the ice bath under red light. Buffers and other solutions were prepared freshly before use.

Extraction and fractionation of protein kinases One kilogram of the etiolated oat seedlings was extracted with 1 L of a kinase extraction buffer [100 mM Tris-Cl (pH 8.3), 100 mM KCl, 2 mM Na_2-EDTA, 5% (v/v) glycerol, 14 mM β-mercaptoethanol, 20 mM $Na_2S_2O_5$, 4 mM PMSF]. The extract was precipitated with 0.1% (v/v) polyethyleneimine (pH 7.8) followed by 60% $(NH_4)_2SO_4$. The pellet was dissolved in buffer A [20 mM Tris-Cl (pH 7.2), 1 mM EDTA, 14 mM β-mercaptoethanol] and applied on a HA column (5.3 × 9 cm). Proteins were eluted from the column with a stepwise gradient of 100 mM and 300 mM potassium phosphate buffer [300 mM KPB, 300 mM potassium phosphate (pH 7.8), 1 mM EDTA, 14 mM β-mercaptoethanol]. The kinase fraction that phosphorylated phytochrome was pooled, applied on the phenyl Sepharose-CL 4B column (3.8 × 7 cm) equilibrated with buffer A. The column was washed with 300 mM KPB and the bound proteins were eluted with 300 ml buffer A containing 75% ethylene glycol and then applied to a DEAE-Cellulose column (1.9 × 6.5 cm) equilibrated with buffer A containing 75% ethylene glycol. After washing the column with buffer A, the bound proteins were eluted with a stepwise gradient of 0.1, 0.2, and 0.3 M NaCl. The unbound fraction to the DEAE-Cellulose column was readsorbed to a carboxymethyl (CM)-Cellulose column (1.9 × 6.5 cm) equilibrated with buffer A containing 75% ethylene glycol. Proteins were eluted with a stepwise gradient of 0.05, 0.15, 0.25, and 0.35 M NaCl dissolved in buffer A. Each fraction from the columns was assayed for kinase activity using the purified phytochrome as a substrate.

Assay of kinase activity Kinase activity was assayed basically by the same method as described by Kim *et al.* (1989). The assay mixture in a total volume of 60 μl contained 15 μl of a 4-fold stock solution of kinase assay buffer [20 mm Tris-Cl (pH 7.4), 5 mM $MgSO_4$, 4 mM Na_2-EDTA, 5% ethylene glycol], 20 μl of kinase solution, 10 μl of substrate, 10 μl of ATP (final concentration: 10 μM ATP, 2 μCi [γ-^{32}P] ATP), and 5 μl of effector solution of an appropriate concentration. The absorbance of phytochrome at 660 nm used for the substrate was 0.2. The reaction was initiated by adding ATP solution to the reaction mixture and terminated by adding SDS-PAGE sample buffer, after incubation at 28°C for 20 min. The reaction mixture was then subjected to SDS-PAGE followed by autoradiography for the activity measurement. Autoradiography was performed by layering the dried gel on Kodak X-Omat AR film with a DuPont Cronex intensifying screen for an appropriate period followed by development with Kodak products. For a quantitative measurement of ^{32}P-incorporation, the phytochrome band was cut from the dried gel and subjected to liquid scintillation counting as previously described by Kim *et al.* (1989).

Photoaffinity labeling with 8-azido [α-^{32}P] ATP Thirty microliters of the kinase fraction was mixed with 30 μl of the kinase assay buffer containing 2 μCi 8-azido [α-^{32}P] ATP and 10 μM ATP. After incubation in an ice bath for 1 min, the reaction mixture was irradiated for 1 min with a UV lamp (254 nm, model UVGL-25) at a distance of 3 cm from the bottom of the reaction tube in the ice. The reaction was performed in a dark room and quenched by the addition of 10 mM dithiothreitol (DTT) as final concentration. The reaction mixture was electrophoresed on an SDS gel and subjected to autoradiography (Douglas and Lehman, 1987).

Polyacrylamide gel electrophoresis Protein samples were electrophoresed on polyacrylamide gel in the presence of SDS as described by Laemmli (1970). The electrophoresed gel was stained with Coomassie brilliant blue R-250 or silver nitrate (Meril *et al.*, 1981), and dried onto Cellophane membrane backing (Bio-Red, Model 583 drying supports).

Estimation of protein concentration Protein concentration was determined spectrophotometrically by the absorbance at 280 nm or by the method of Bradford (1976).

Results

It is well known that the partially purified phytochrome possesses kinase activity that phosphorylates itself, but the highly purified phytochrome lost the kinase activity (Kim *et al.*, 1989). The present phytochrome preparation had a major band of 124 kDa phytochrome on the SDS-PAGE and a specific absorbance ratio (A_{660}/A_{280}) of ca. 0.6. Although the present preparation was partially purified, it did not possess the endogenous protein kinase activity under the present assay conditions. It may indicate that the major contaminant band of 60 kDa that could be removed by gel filtration with Bio-Gel A-1.5m was not involved in the kinase activity.

The endogenous kinase activity that phosphorylated phytochrome was observed only when the assay mixture was incubated for a prolonged period of time (more than 30 min). If it retained an endogenous kinase activity, the phytochrome-phosphorylating activity was measured by the ^{32}P incorporation into phytochrome over a control that did not contain kinase fractions. During the isolation of the phytochrome-phosphorylating kinases, it was not possible to measure the purity indices of the kinases (since there were so many kinase activities) until each kinase was separated by the ion exchange chromatographies. In HA column chromatography, protein kinase activities were eluated in all column fractions, that is, from the washing, 100 mM KPB eluate, and 300 mM KPB eluate. However, the kinase activity that phosphorylated phytochrome was eluted only in the 300 mM KPB eluate (data not shown). Phytochrome was completely eluted off the column in the 100 mM KPB eluate, as expected from the previous results (Kim *et al.*, 1989).

When the 300 mM KPB fraction was applied on the DEAE-Cellulose column, a small portion of the kinase activity was retained in the column and more than half of the activity passed through the column. In the eluate from the DEAE-Cellulose column, the phytochrome-phosphorylating kinase activity appeared in the 0.2 M NaCl eluate (designated as DE I). The DE I fraction had a lot of protein bands on the SDS-PAGE (Fig. 1). In order to identify a putative protein kinase from the protein bands, an ATP-binding site on the proteins was probed by photoaffinity labeling with 8-azido [α-^{32}P] ATP. However, no band had been labeled, indicating that the phytochrome-phosphorylating kinase was a minor component of the DE I fraction. The unbound fraction to the DEAE-Cellulose was readsorbed to a cation-exchanger, CM-Cellulose column. All the phytochrome-phosphorylating kinase activity was bound to the column, while some kinase activity passed through the column. When eluated with a NaCl stepwise gradient, the phytochrome kinase activity was eluted in two fractions; 0.15 M NaCl fraction (designated as CM I) and 0.25 M NaCl fraction (designated as CM II). These two kinase fractions also had many protein bands on the 8% SDS gel (Fig. 1). In an attempt to identify ATP-binding sites, a band of ca. 75 kDa in the CM I fraction and a band of ca. 38 kDa in CM II were labeled with 8-azido [α-^{32}P]

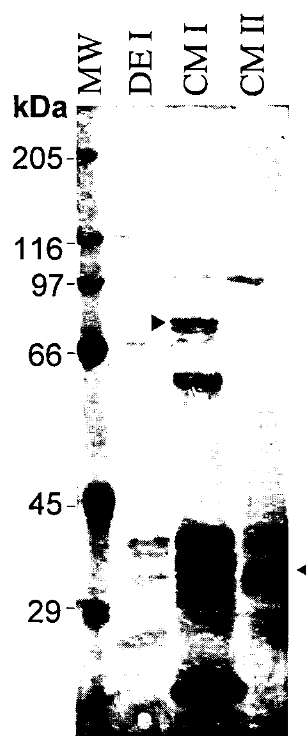

Fig. 1. SDS gel electrophoretic patterns of the DEAE-Cellulose and CM-Cellulose column fractions that had kinase activity phosphorylating phytochrome. The active kinase pool of HA column (300 mM KPB eluate) was applied on a DEAE-Cellulose column equilibrated with buffer A (20 mm Tris-Cl, pH 7.2, 1 mM EDTA, and 14 mM β-mercaptoethanol) containing 75% ethylene glycol. The bound proteins were fractionated with a step-wise gradient of 0.1 M, 0.2 M (DE I) and 0.3 M NaCl dissolved in the buffer A. The unbound fraction to the DEAE-Cellulose was applied on a CM-Cellulose column equilibrated with buffer A containing 75% ethylene glycol. After washing the column with buffer A, the bound proteins were eluted with a step-wise gradient of 0.05 M, 0.15 M (CM I), 0.25 M (CM II), and 0.35 M NaCl dissolved in the equilibrium buffer. The fractions having the phytochrome-phosphorylating kinase activity [0.2 M NaCl eluate (DE I) of the DEAE-Cellulose, 0.1 M NaCl (CM I) and 0.2 M NaCl (CM II) eluates of the CM-Cellulose] were electrophoresed on an 8% SDS-PAGE. ▶ indicates the band labeled with 8-azido [α-^{32}P] ATP.

ATP (Fig. 1). The presence of the ATP-binding site suggested that these two bands may be responsible for the kinase activities. In particular, the CM II fraction was relatively pure with a major band of 38 kDa which was labeled with 8-azido [α-^{32}P].

The enzymatic properties of the three kinases were analyzed by chemicals that have inhibitory and stimulatory effects on the protein kinase activity (Fig. 2). The three kinases responded in different manners to the effectors that had been used to classify the protein kinases into families. Polylysine and histones are polycationic reagents that stimulate polycation-dependent protein kinases in plants. cAMP is a stimulant that is specific to PKA. Ca^{2+} has a stimulatory effect on PKC and CDPK. Heparin is a strong specific inhibitor to casein kinases (Barik and Banerhee, 1992; Miyata and Yahara, 1992). The kinase activity of the DE I fraction (Fig. 2C) was strongly activated by histone IIIS, moderately activated by polylysine, and slightly by Ca^{2+}. It was strongly inhibited by heparin and pyrophosphate. This enzymatic property indicates that the DE I fraction has enzymatic properties of a polycation-stimulated protein kinase. Two kinase activities from the CM-Cellulose column were inhibited by heparin, polylysine, and histone IIIS, but were not appreciably affected by cAMP and Ca^{2+} (Figs. 2A and 2B). However, the inhibitory effects of heparin and polylysine were much more pronounced with the CM II fraction (Fig. 2B) than with the CM I fraction (Fig. 2A); 42 μg/ml of polylysine and 20 μg/ml of heparin completely inhibited the activity of the former, but not appreciably the latter. The enzymatic property of the CM II kinase fraction indicated that it had a typical enzymatic property of casein kinase (Barik and Banerhee, 1992; Miyata and Yahara, 1992). The three phytochrome-phosphorylating kinases had distinct differences in their substrate specificities. The DE I kinase effectively phosphorylated histone IIIS, whereas it moderately affected phytochrome and casein (Fig. 3). It also phosphorylated many endogenous contaminant proteins in the kinase assay mixture. The differences between the CM I and CM II kinase fractions were more pronounced with the substrate specificities. Although the CM I fraction had many contaminants on the SDS gel, it phosphorylated only phytochrome in the kinase assay mixture (Fig. 3). It did not phosphorylate histone IIIS or casein when both were added as a substrate. The CM II kinase fraction effectively phosphorylated casein but not histone IIIS.

These results indicate that at least three protein kinases are present in the etiolated oat seedlings that phosphorylate phytochrome *in vitro*.

Discussion

Three kinases that phosphorylate oat phytochrome A *in vitro* are apparently different species from each other since they had distinct characteristics in substrate specificities, enzymatic properties, and binding properties to ion exchangers.

Both the inhibitory effect of heparin and the substrate specificity indicate that the CM II kinase belongs to a casein kinase family (Barik and Banerhee, 1992; Miyata and

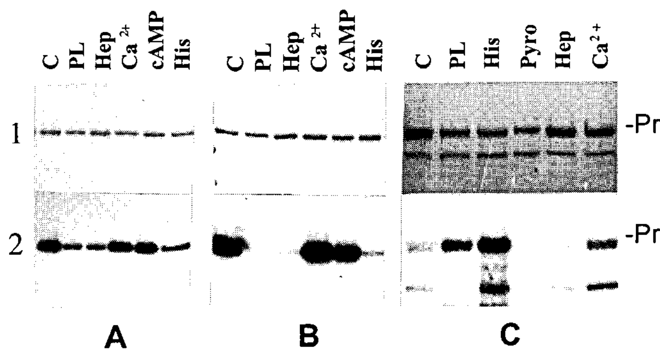

Fig. 2. Effects of kinase effectors on the phytochrome-phosphorylating activity of the kinase fractions from the ion-exchangers. The phytochrome-phosphorylating activity of the CM I (A), CM II (B), and DE I (C) fractions in Fig. 1 were assayed in the presence of several effectors as described in the Materials and Methods. The final concentrations used were: 42 μg/ml of polylysine (PL), 200 μg/ml of heparin (Hep), 4 μg/ml of histone IIIS (His), 60 μM of cAMP, 10 μM of Ca^{2+}, and 3 μg/ml of pyrophosphate (Pyro). The kinase assay mixtures were electrophoresed on 8% SDS gels (1). The gels were dried and subjected to autoradiography (2) for the measurement of the phytochrome-phosphorylating activity. C; a control without effectors. Pr; the position of the phytochrome band.

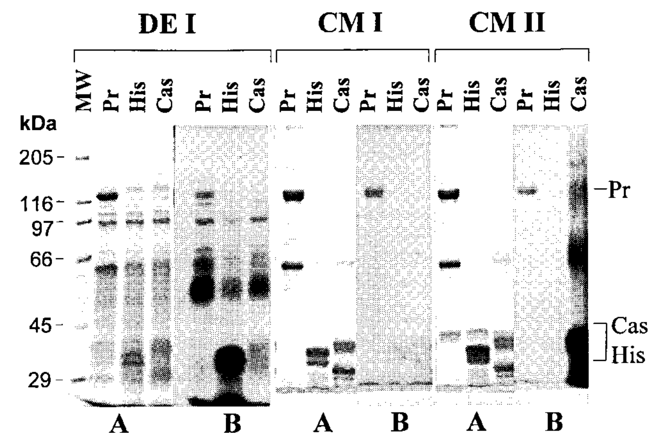

Fig. 3. Phosphorylation of artificial substrates by the phytochrome-phosphorylating kinases. Phytochrome (Pr), histone IIIS (His), or casein (Cas) used as a substrate was incubated with the kinase assay mixture containing the DE I, CM I, and CM II kinase fractions in Fig. 1. The reaction mixtures were electrophoresed on 8% SDS gels (A) and subjected to autoradiography (B). Pr, Cas, and His on the right column indicate the band positions of phytochrome, and casein histone IIIS, respectively.

Yahara, 1992). Although the CM I kinase activity was slightly inhibited by heparin, it did not phosphorylate casein at all, which makes it difficult to assign the CM I kinase to any kinase family. The CM I kinase does not belong to highly conserved protein kinases that are universal in eukaryotes. Taken together with the results of kinase effectors and substrate specificity, it is likely that the DE I kinase fraction is a polycation-stimulated protein kinase. It is very interesting that the polycation-stimulated protein kinase of the present study had very similar enzymatic properties with the phytochrome-associated protein kinase. Both kinases are strongly activated by polycations such as polylysine and histones III-S, and strongly inhibited by pyrophosphate (Wong *et al.*, 1986; 1989).

It has still been an open argument as to whether the phytochrome-phosphorylating kinase activity comes from phytochrome itself or from a contaminant protein in the purified phytochrome. Since Wong *et al.* (1986) proposed that oat phytochrome may have an intrinsic kinase activity, several evidences have supported the idea. Oat phytochrome A has been shown to possess an ATP binding site by photoaffinity labeling of phytochrome with 8-azido ATP (Wong and Lagarias, 1989). An immunoprecipitated maize phytochrome which was washed with both salt and detergent also had autophosphorylating kinase activity (Bierman *et al.*, 1994). In addition, it has also recently been reported that a gene of *Ceratodon purpureus* phytochrome (phyCer) has a kinase domain (Thummler *et al.*, 1997) and cyanobacterial phytochrome (Cph 1) is a light-regulated histidine kinase (Yeh *et al.*, 1997).

On the other hand, it had been reported that the highly purified phytochrome had no kinase activity (Grim *et al.*, 1989; Kim *et al.*,1989), suggesting that phytochrome was phosphorylated by a co-purifying contaminant kinase. The present study has shown that a protein kinase which had similar enzymatic properties with the phytochrome-associated kinase was separable from phytochrome. Phytochrome was eluated from the HA column in the 100 mM KPB eluate, while the polycation-stimulated kinase in the 300 mM KPB eluate. In addition, a kinase preparation that was free of phytochrome phosphorylated phytochrome with the same enzymatic properties of the phytochrome-associated kinase (Wong *et al.*, 1989).

This suggests the possibility that the phytochrome-associated kinase activity may originate from a co-purifying protein kinase. The polycation-stimulated protein kinase, in general, phosphorylates casein. However, the DE I kinase did not phosphorylate casein, indicating that it is a unique plant protein kinase. These unique properties of the DE I kinase suggest that it may be involved in the early stage of signaling, such as phytochrome light perception. On the other hand, it is likely that the kinase was nonspecific in phosphorylating phytochrome and had broad substrate specificities.

The CM II kinase could be classified into casein kinase (CK II), in terms of its inhibitory and stimulatory properties by the kinase effectors. It also phosphorylated casein very effectively. The etiolated oat seedlings seemed to contain a larger amount of this enzyme than the other two kinases. A major band of 38 kDa in the CM II kinase fraction seemed likely to be the kinase, since it had an ATP binding site when tested by photoaffinity labeling with 8-azido [α-^{32}P] ATP. It is not likely that the kinase phosphorylates phytochrome *in vivo*, because its quantity may exceed phytochrome contents in the etiolated oat seedlings. The CM I kinase fraction still had a lot of protein impurities. In contrast to the other kinases, however, it was highly specific in phosphorylating phytochrome. It could be a candidate for a phytochrome-phosphorylating kinase, since this kinase was specific in phosphorylation of phytochrome and seemed to be present in a very small quantity in the etiolated oat seedlings.

All three protein kinases phosphorylate phytochrome *in vitro* but phytochrome may not be a natural substrate *in vivo* for the three kinases. It was rather surprising that the etiolated oat seedlings contained at least three kinases that phosphorylated phytochrome. Within the cells, one protein kinase may function in phosphorylating phytochromes that elicit physiological responses.

In order to identify the phytochrome-phosphorylating kinase and to elucidate the role of phytochrome phosphorylation in light signal transduction, we are attempting to purify the protein kinases that phosphorylate phytochrome.

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