Binding Proteins to Phytochrome A in Etiolated Pea Seedlings

Takashi Shimada 1, Mitsue Miyao-Tokutomi 2 and Satoru Tokutomi 1

1 Research Institute for Advanced Science and Technology, University of Osaka Prefecture, Sakai, Osaka, 599-8231 Japan
2 Laboratory of Photosynthesis, National Institute for Agrobiological Resources (NIAR), Kannondai, Tsukuba, 305-8602 Japan

In order to detect and characterize a putative receptor(s) for a signal from PhyA, proteins that bind to purified pea PhyA were searched for in the crude extract of etiolated pea seedlings with affinity chromatography. PhyA was coupled to the column substrate either in P R form (P R column) or in red-irradiated form (P FR column). The coupled PhyA of both columns retains its spectral reversibility between P R and P FR, although their peptide mapping by trypsin digestion suggests that the C-terminal half of PhyA in the P FR column is partially fixed in P R structure. 15 polypeptides were detected reproducibly in the elution from the P FR column by silver-staining of SDS-PAGE. These 15 polypeptides may form two complexes judging from their elution profiles. Of the 15 polypeptides, the 6 major polypeptides have approximate mol wt of 80, 55, 53, 46, 40 and 35 kDa. On the other hand, only a trace amount of protein, which mainly consists of the 46 kDa species, was eluted from P R column, indicating the presence of P FR-specific BPs in the crude extract of etiolated pea seedlings. Of the 6 major polypeptides, the 40 kDa species binds to the PhyA in a photoreversible manner.

Key words: Affinity chromatography — Binding protein — Phytochrome — Phototransformation — Pisum sativum — Signal transduction.

Plants require sensing systems for the intensity, direction, quality (color) and periodicity of light since they depend on photosynthesis and are non-motile. Of the photoreceptors in these systems, phytochrome (Kendrick and Kronenberg 1994) is the best characterized and important. Phytochrome has two stable absorption forms named P R and P FR, that are interconvertible with each other by R and FR irradiation, respectively, and acts as a light-sensitive molecular switch regulating a variety of morphogenetic responses, such as, germination, greening and flowering.

A prototype phytochrome possibly appeared in the history of life when cyanobacterium-like microorganisms acquired photosynthetic ability (Kehoe and Grossman 1996). In higher plants, at least five genes that code phychrome (phytochromes A–E) have been identified so far (Quail 1994). The functions of the major two members, PhyA and PhyB (Furuya 1989), have been well studied using mutants and transformants of Arabidopsis (Quail et al. 1995). PhyA is abundant in etiolated tissues, however, decreases drastically under light illumination since its gene expression is down-regulated by light (Lissemore and Quail 1988) and its P R is unstable (Somers et al. 1991). On the other hand, population of PhyB is constant in between etiolated and green tissues since its gene expression is light-insensitive (Sharrock and Quail 1989) and its P FR is relatively stable (Somers et al. 1991). PhyA is responsible for the so-called VLFR and the FR-HIR, while PhyB mediates the LFR (Shinomura et al. 1996, Frankhauser and Chory 1997).

PhyA, the best characterized species, is a homodimeric chromoprotein in a four-leaved shape (Tokutomi et al. 1989) with a monomer molecular weight of 124 kDa. A monomer binds a linear tetrapyrrolic chromophore named phytochromobilin (Rüdiger and Thümmler 1991). Absorption of quanta by its π-electron induces photochemical reactions of the chromophore, possibly such as cis-trans isomerization (Fodor et al. 1990) accompanied by proton migration (Mizutani et al. 1991). These reactions alter the conformation of the protein moiety, which may trigger biochemical reactions to transfer signals to the next component(s) as has been shown in the photoreception of rhodopsin (Stryer 1991). After cascades of biochemical reactions, enzymatic activities or expression of target genes may be regulated and physiological responses will be expressed. Protein kinase (Schneider-Poetsch 1991, Yen et al. 1997), Ca-calmodulin and G-protein (Miller et al. 1994) have been proposed to be involved in the signal transduction cascade.

In spite of many efforts to elucidate the molecular mechanism of signal transduction, little is known about primary processes in signal transduction from phytochromes to the downstream intermediates in higher plants (Wagner et al. 1997). In order to understand the molecular mechanism of this process, we have started to detect and characterize the protein(s) interacting with PhyA and concerning to the reception of transmitted signal from...
PhyA. For this purpose, we tried to detect BP to pea PhyA using PhyA-coupled affinity column chromatography. In this paper, we report the detection of the BPs and discuss its significance.

Materials and Methods

Preparation of pea large PhyA—Pea large PhyA, which lacks an N-terminal 6-kDa polypeptide (114 kDa on a monomer basis), was used in this study. In the following, PhyA indicates the pea large PhyA unless otherwise specified. PhyA was prepared from 7-day-old etiolated seedlings of Alaska pea (Pisum sativum cv. Alaska) under a dim green safety light at 4°C as described in Tokutomi et al. 1988. The purity was more than 98% judging from the densitometry of the silver staining of the gel (see the left lane 1 in Fig. 2). The specific absorbance ratio (SAR, A^282/A^280) of PhyA was calculated using the extinction coefficient of 1.2 × 10^4 (Yamamoto, K.T., personal communication).

Preparation of PhyA-coupled affinity column—2 mg of PhyA was coupled to each of prepacked HiTrap NHS-activated columns (bed vol. 1 ml, Pharmacia Biotech) according to the manufacturer’s instructions. One group of columns were prepared from PhyA in Pr under a green safety light and was referred to as the Pr column. The other columns, from R-irradiated PhyA under R irradiation and as the Pfr column. In the preparation of the latter column, PhyA solution in a transparent syringe was preirradiated for 5 min with R light to convert Pr to Pfr, injected into the column and left for 4 h to complete the coupling reaction, during which time the column was irradiated with R light to prevent dark reversion of Pfr to Pr. Uncoupled PhyA was quantitated by measuring the absorption spectrum of the column eluate and was found to be less than 5% in all the coupling reactions. Residual reactive groups in the column matrix were deactivated as described in the instructions.

Absorption difference spectra of coupled PhyA—The column matrix was removed from the Pr and the Pfr columns, and suspended in a solution of 25 mM HEPES and 1 mM Na2EDTA (pH 7.8). Absorption difference spectra were measured in a quartz cuvett (1 × 1 × 5 cm) by a Hitachi 557 spectrophotometer.

Peptide-mapping of coupled PhyA—50 μl of column matrix was removed from the Pr and the Pfr columns, washed and suspended in 230 μl of a 100 mM potassium phosphate and 1 mM Na2EDTA (pH 8.3) solution. 1 μg of modified trypsin (Promega) was added to each suspension and incubated at room temperature under either R or FR irradiation. Digestion by the trypsin was followed by sampling 50 μl of the supernatant of the suspensions at 0, 1, 2 and 3 min after the initiation of the incubation. Sampled solutions were immediately mixed with 50 μl of SDS-PAGE sample solution, boiled for 2 min and used for SDS-PAGE.

Detection of BP to coupled PhyA—7-day-old etiolated seedlings of the Alaska pea (0.2–0.23 kg) were homogenized in a Waring blender. The homogenate was centrifuged (8,000 rpm × 15 min) to remove insoluble materials. Acidic materials were precipitated by the addition of polyethyleneimine (final concentration, 0.02%). The supernatant was collected, dialyzed against a 25 mM Tris (pH 7.8) solution containing 1 mM Na2EDTA and 28 mM 2-ME, and then ultracentrifuged at 30,000 rpm for 60 min.

The supernatant was recovered as a crude extract from tissues. 300 ml of the crude extract was applied to the Pr or Pfr columns, which had been equilibrated with the above 25 mM Tris buffer, at a flow rate of 1 ml min^-1 using the FPLC system (Pharmacia Biotech). After washing with the buffer, proteins bound to the column were eluted by a linear gradient of KCl from 0 to 1.5 M at the same flow rate with a fraction size of 0.5 ml. The absorption of the eluate was monitored at 280 nm. The eluted fractions were dialyzed against 0.5 mM Tris buffer (pH 9.5), and then freeze-dried. Each freeze-dried sample was dissolved in 50 μl of water plus 50 μl of SDS-PAGE sample buffer, boiled for 2 min and used for SDS-PAGE.

In the experiments with Pfr columns, they were preirradiated with R light for 5 min to assure the PhyA to be in Pfr. The light was kept on during the loading of the extract onto the column and the elution of BP.

Since the crude extract contains a variety of proteolytic enzymes, a new column was used for each BP experiment to avoid the effect of degradation of the coupled PhyA by these enzymes. All the procedures were performed at 4°C under dim green safety light.

Photoreversibility of binding to coupled PhyA—A Pr column was irradiated with R light for 10 min to convert Pr to Pfr. 300 ml of the crude extract (see the above section) was applied to the R-irradiated column, and it was washed with 15 ml of the 25 mM Tris buffer under R irradiation. Then, the column was irradiated with FR for 15 min to reverse Pfr to Pr. Released BP by the FR irradiation was eluted with 15 ml of the 25 mM Tris buffer and the remaining BP was eluted with the linear gradient of KCl from 0–1.5 M under the FR irradiation. Both the eluates were collected and made into SDS-PAGE samples as described above. FPLC was operated at the flow rate of 1 ml min^-1 with a fraction size of 0.5 ml. All the procedures were performed at 4°C under green safety light.

SDS-PAGE—SDS-PAGE was carried out after the method of Laemmli 1970. The bands in the gel were detected with Coomassie Brilliant Blue or a silver staining, and western-blotting by polyclonal rabbit anti-pea phytochrome antibody. The pattern and density of the stained bands were analyzed on a microcomputer using 1D Image Analysis Software (Kodak Digital Science).

Light source—Dim green safety light was provided by either 40 W fluorescent tubes (Toshiba FL 40S W) or portable incandescent lamps wrapped with two layers of dark green and one layer of Italian blue plastic films (4421C and 4515C, Nakagawa Chemical Co.). R light was supplied with Toshiba fluorescence tubes (FL 205 BR) filtered through one layer of red acrylic plates (Mitsubishi Rayon, Acrylight). FR light, with the tubes (FL 20S FR) through dark red acrylic plates (Sumitomo Chemicals, Sumipeo).

Results

Spectral properties of PhyA coupled to column matrix—Absorption difference spectra of the PhyA coupled to NHS-activated Sepharose were measured in the suspension samples (Fig. 1). A(ΔA) of the PhyA were calculated as 0.40 and 0.39 for the Pr and the Pfr columns, respectively. The values are comparable to 0.47 of the non-coupled PhyA in the suspension of deactivated Sepharose matrix. Furthermore, both the couplings induced little shift in the absorption maximum of both Pr and Pfr.
Binding proteins to phytochrome

Fig. 1 Absorption difference spectra of PhyA coupled to P_R (thick line) and P_FR (middle thick line) columns, and uncoupled PhyA in the suspension of deactivated column matrix (thin line), obtained by subtracting the absorption spectrum of a R-irradiated sample from that of the R- and then FR-irradiated sample. \( \Delta(\Delta A) \) is defined as the absorption difference between absorption at the P_R and the P_FR peaks of an absorption difference spectrum. The absorption difference spectra are normalized so that the absorption at P_R peak in P_R form of each sample equals to 0.70.

Peptide map of PhyA coupled to column matrix—

Molecular conformation of the PhyA coupled to the columns was studied by peptide-mapping. Fig. 2 shows the banding patterns of trypsin-digested PhyA coupled to NHS-activated Sepharose for each of four digestion times (0, 1, 2, and 3 min).

PhyA in P_R column shows only two major bands with a mol wt of 59 and 25 kDa after digestion under FR (Fig. 2, P_R FR). The former polypeptide is so-called small phytochrome corresponding to the N-terminal chromophoric domain and the latter probably the 25-kDa chromophore-containing fragment (Yamamoto 1990). Tryptic digestion of PhyA in an aqueous solution also produces two major bands (data not shown), the small phytochrome and the 39-kDa chromophore-bearing fragment which undergoes a phototransformation between P_R and a bleached form (Yamamoto 1990). Under R, many bands appear in the mol wt region from 39 to 59 kDa besides the two (Fig. 2, P_R R). PhyA in an aqueous solution also shows these bands after digestion under R (data not shown), which have been proved to come from the C-terminal non-chromophoric domain (Yamamoto and Tokutomi 1989). Proteolytic digestion of intact oat (Grimm et al. 1988) and pea (Manabe and Nakazawa 1997) phytochromes in P_FR in solutions have been also shown to produce polypeptides from the C-terminal non-chromophoric domains. These indicate that, in P_R column, molecular conformation of the coupled PhyA can be changeable between P_R and P_FR in a way similar to that of PhyA in aqueous media although its molecular conforma-

Fig. 2 Effect of R and FR (indicated lower) irradiation on digestion by trypsin of PhyA coupled to P_R and P_FR (indicated upper) columns. Silver-staining of a 10.5% SDS-polyacrylamide gel. 0, 1, 2 and 3 are the reaction times in minutes. PhyA indicates purified pea large phytochrome. Arrows in the left side show the position of the mol wt of 200, 116, 97, 66, 45 and 31 kDa, respectively.
tions are not identical to those in aqueous solutions.

In P_{FR} column, the digestion of PhyA under R produces fragments similar to those of P_{R} column under R, indicating that PhyA in P_{FR} column is coupled to the column matrix in a molecular conformation similar to that of P_{R} column under R (compare Fig. 2, R between P_{R} and P_{FR}). PhyA in P_{FR} column also retains the ability of conformational change by FR irradiation to a large extent judging from the prominent difference in the band patterns at 3 min after digestion under between R and FR (Fig. 2, P_{FR}). However, they exhibit extra bands to those of P_{R} column under FR in the mol wt range less than 25 kDa and that from 39 to 59 kDa (compare the patterns under FR between P_{R} and P_{FR} in Fig. 2). These extra bands are characteristic to the digestion of PhyA in both the P_{R} and P_{FR} columns under R, namely in P_{FR} form, indicating that the PhyA is not completely transformed to the conformation of the PhyA of the P_{R} column under FR, i.e. P_{R} form. Furthermore, PhyA in P_{FR} column is more resistant to the digestion and the N-terminal 59-kDa peptide (small phytochrome) seems more difficult to be released than those of P_{R} column under both R and FR.

Detection of BP to PhyA coupled to column matrix—Fig. 3 shows elution profiles of proteins from a P_{R} column and a P_{FR} column monitored by A_{280}. Only a trace peak is observed in the elution from the P_{R} column, while a large elution peak was observed from the P_{FR} column at around a KCl concentration of 380 mM, indicating the presence of the proteins that had been bound to PhyA in the P_{FR} column. Silver staining detected six major bands (80, 55 doublet, 53, 46, 40 and 35 kDa), 3 medium bands (60, 33 and 30 kDa) and 6 minor bands (115, 78, 67, 41, 38 and 36.5 kDa) (Fig. 4) in the elution from the P_{FR} column. These bands were detected reproducibly with 4 different P_{FR} columns. Three of the above bands (55, 53 and 46 kDa)
Fig. 6  Elution profiles of proteins bound to coupled PhyA in a R-irradiated Pr column under FR irradiation without (0-30 ml) and with (30-60 ml) KCl monitored by A280. Dotted line indicates the concentration of KCl used for the elution. Fraction size is 0.5 ml and the first fraction corresponds to the volume 0-0.5 ml.

were also observed faintly in the elution from the Pr column, where the 46 kDa band is the main component (data not shown). Densitometric analyses on the 6 major and the 3 medium bands show that the elution species can be classified into two groups, one eluting at KCl concentration of 350 mM and the other at 450 mM (Fig. 5), suggesting that the BPs form two different complexes. Since PhyA is a dimeric molecule (Tokutomi et al. 1988), PhyA coupled in a molecular structure exposing its contact sites may bind endogenous PhyA or its degraded products that may contaminate the eluates. This possibility, however, can be excluded as far as the thick bands are concerned since no band was detected with Western blotting.

Photoreversibility of binding to coupled PhyA—Since PhyA coupled to the Pr column preserves photoreversibility with respect to both spectrophotometry and molecular conformation, the Pr column was used for studying the photoreversibility of the binding of the BPs to coupled PhyA. The chromatogram in Fig. 6 shows that about one-fourth of the bound protein to a Pr column under R irradiation is released on FR irradiation before the start of the KCl gradient and that the remaining BPs are eluted at a KCl concentration of around 380 mM. An analysis of the proteins in the first peak shows that the peak consists of a marked 40 kDa band with six faint bands, 60, 55, 46, 35, 33 and 30 kDa (Fig. 7). The presence of the first peak demonstrates the photoreversible binding of these BPs. The second peak has a similar band pattern of polypeptides (Fig. 8) to that of the Pr column shown in Fig. 4, although the densities and elution profiles of bands differ slightly and two minor bands with the mol wt of 38 and 36.5 kDa are missing.

Discussion

Molecular topology of PhyA coupled to column matrix—Since the succimide group is known to react with free amino groups, PhyA is probably fixed to the gel matrix at multiple amino acid residues. However, between the NHS group and the gel matrix of the NHS-activated Sepharose, is a long hydrocarbon chain, and thus fixation of ligands may be rather loose. The PhyA in both the columns retain the spectral photoreversibility (Fig. 1). It has been known that the N-terminal 59-kDa chromophoric domain is responsible for this spectral photoreversibility (Yamamoto 1990). The preservation of the spectral photoreversibility indicates that the N-terminal domain is
not fixed tightly to the matrix to affect the photochemical reaction of the chromophore in both P_R and P_FR columns.

The peptide mapping suggests that the PhyA in the P_R column has a molecular conformation similar to but not identical to its intact form and that the conformation is changeable between P_R and P_FR as in aqueous media (Fig. 2, P_R). However, in the P_FR column, the digestion pattern under FR differs from that of P_R column under FR, which has the bands characteristic to the P_FR in both the P_R and P_FR columns and in aqueous media, indicating that the protein moiety of the PhyA loses partially its transformability. Since these additional bands observable in the mol wt region from 39 to 59 kDa derive from the C-terminal domain of P_FR, the PhyA in the P_FR column appears to be partially fixed in the P_FR conformation probably due to rather tight attachment to the column matrix in the C-terminal non-chromophoric domain. Futhermore, the absence of the N-terminal 59-kDa peptide band in the P_FR column suggests that PhyA is attached to the matrix in a conformation that inhibits trypsin to approach to the cleavage site between the N-terminal and the C-terminal domains and/or that the N-terminal domain attaches to the matrix with an extra bonding specific to P_FR that impedes its dissociation from the gel matrix.

Presence of BP to coupled PhyA—The present results clearly indicate the presence of BPs to PhyA in the crude extract of the etiolated pea seedlings although the PhyA is attached to a gel matrix. Most of the BPs discriminate the different molecular conformations between P_R and P_FR and bind specifically to PhyA in P_FR columns (Fig. 3). These BPs bind also to PhyA in the R-irradiated P_R column (Fig. 8), indicating that molecular conformations of PhyA are similar in between P_FR columns and R-irradiated P_R columns as far as the binding sites to these BPs are concerned. Based on the observation that the P_FR and P_R columns adsorb much and little amount of BPs, respectively, and that the P_R columns preserving spectrophotometrical and molecular topological transformabilities similar to those of PhyA in aqueous media, come to bind the BPs by R irradiation, it can be said that these BPs recognize the different molecular structure between P_R and P_FR and that they bind to the P_FR specifically, at least on column matrix.

Since the PhyA in P_R columns retains partially its intact molecular conformation both in P_R and P_FR (Fig. 2), some of the interactions between these BPs and the PhyA in P_FR may reproduce their in vivo interactions with phytochrome A and one or more of the BPs might be involved in some catalytic pathways in vivo. Phytochrome A is known to have a PEST sequence (Roger et al. 1986) and to be degraded rapidly after conversion to P_FR via ubiquitin-proteasome degradation pathway of proteins (Shanklin et al. 1987), therefore, some of the BPs might have some roles in this pathway. Futhermore, one or more of the BP might be concerned to the reception of signal transmitted from phytochrome A. The used PhyA lacks an N-terminal 6-kDa polypeptide, which has been reported to be necessary for the expression of FR-HIR (Boylan et al. 1994). Thus, if the BPs have something to do with light-signal transduction, VLFR, rather than FR-HIR, may be a candidate for the involvement.

Multiple component of BP—The multiple components of the BP (Fig. 4, 8) may be ascribed partly to degradation of intact BPs during the experimental procedures since the absence of the protease inhibitors results in an increase in the number of bands, especially with a smaller mol wt. However, the degradation can not completely explain the multi-bands of BP. The present results suggest that the BPs form two different complexes (Fig. 5). It has been reported that a large complex of proteins, the COP family, is involved in the light-signal transduction pathway (Staub and Deng 1996), although the involvement is at the downstream of the signal transduction pathway and that their functions are still unclear. Several recent studies have shown that multi-protein complexes are involved in the transduction of biological signal (for example, see Maniatis 1997). Thus, the involvement of protein complexes in the light-signal transduction from phytochrome A is probable.

 Stoichiometry of binding—Total amount of BPs eluted from the columns are estimated from their total A_{280} using the relationships that A_{280} of 1% (W/V) solution of bovine serum albumin is 6.6. The ratio (W/W) of the total BP eluted from the column to the coupled PhyA to the column substrate (2 mg) ranges from 0.56 (the elution peak from the Pfr column in Fig. 3) to 0.87 (sum of the two elution peaks in Fig. 6). If the average mol wt of the BPs is assumed to be 53 kDa in consideration of their SDS-PAGE patterns (Fig. 4, 5), the molecular ratio of a BP (monomer) to a PhyA (dimer) can be calculated as from 2.3 to 3.6. The ratios can be reasonably understood since it gives a 1 to 1 stoichiometry of association between PhyA and the representative BP if this forms a dimer or a trimer.

 Photoreversibility of binding—Since PhyA in the P_R column binds only a trace amount of BP and retains its photoreversibility of the molecular conformation, the BP bound to the R-irradiated P_R column is expected to dissociate on FR irradiation. However, only a portion is released from the column by FR irradiation (Fig. 6). It has been shown that VLFR is mediated by phytochrome A, however, shows no reversibility by FR (Shinomura et al. 1996). Since purified phytochrome A itself can be transformed photoreversibly between P_R and P_FR, the absence of the photoreversibility of VLFR should be ascribed to its signal transduction system. The observed photoreversibility of the binding, in turn, might suggest an involvement of the BPs in a signal transduction system of VLFR. BPs may form a complex(es) through intermolecular allosteric...
interactions to bind to PhyA in \(P_{FR}\) form in the \(P_R\) column which may stabilize the PhyA in \(P_{FR}\) form and may prohibit its conversion back to \(P_R\). In the present results, only the 40 kDa species dissociates partly on transformation back to \(P_R\). This can be possibly explained as follows. Among the BPs, the 40 kDa species may interact with PhyA directly to recognize the conformational changes induced by the phototransformation from \(P_R\) to \(P_{FR}\). Some 40 kDa BPs may form incomplete BP complexes which are unable to prohibit the photoconversion of the PhyA from \(P_{FR}\) to \(P_R\). These 40 kDa BPs may dissociate on the conversion.

**Significance of BP**—Recently, a phosphotransfer from a phytochrome-like protein to a CheY-like protein of a cyanobacterium *Synechocystis* sp. PCC 6803 has been demonstrated and the involvement of this phosphotransfer in the light-signal transduction has been proposed (Yen et al. 1997). However, in higher plants, despite many efforts to understand the molecular mechanism of phytochrome signal transduction, the primary events in the pathway remain unknown. There are three major strategies for defining the putative signal receptor of phytochrome. These are the isolation of the receptor genes from plants with mutations related to signal transduction and by the two-hybrid method, and the identification of the receptor itself by biochemical methods.

The use of *Arabidopsis* mutants is the most popular among the three. COP (Deng and Quail 1992), DET (Chory et al. 1994), and SHY (Kim et al. 1996), that behave as if they receive a light signal in the dark, and HYS (Koornneef et al. 1980) and FHY (Whitelam et al. 1993) that respond to light signals abnormally, have been isolated. Some of these mutant genes have been cloned and their molecular properties have been studied. However, all the mutant genes are thought to be located at middle or later part of the signal transduction pathway and most of their functions are unknown. Recently, RED mutant has been isolated to clone a putative signal receptor from phytochrome B (Wagner et al. 1997), which is the only mutant possibly related to the initial processes in the signal transduction. There are no studies that have reported success with the second method so far.

Concerning the third biochemical approach, there are several interesting reports. Proteins that carry protein kinase activity have been copurified with oat phytochrome (Wong et al. 1989) or immunoprecipitated by anti-phytochrome antibody in maize extracts (Biermann et al. 1994). Furthermore, TCP-related molecular chaperone has been copurified with oat phytochrome (Mummert). However, these reports are claimed by two research groups (Gus-Mayer et al. 1994, Parker et al. 1995). The mol wt of these proteins is reported to be about 60 kDa, however, the present results show only a minor band in the corresponding mol wt region. The present study provides the concrete evidence for the presence of proteins that interact with phytochrome A. One of these proteins may hopefully be involved in the primary process in the signal transduction from phytochrome A. The present results may provide some clue that will help to unravel the light-signal transduction mechanism.

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Binding proteins to phytochrome


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