Phosphorylation of the Parsley bZIP Transcription Factor CPRF2 Is Regulated by Light*

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The analysis of the complex network of signal transduction chains has demonstrated the importance of transcription factor activities for the control of gene expression. To understand how transcription factor activities in plants are regulated in response to light, we analyzed the common plant regulatory factor 2 (CPRF2) from parsley (Petroselinum crispum L.) that interacts with promoter elements of light-regulated genes. Here, we demonstrate that CPRF2 is a phosphoprotein in vivo and that its phosphorylation state is rapidly increased in response to light. Phosphorylation in vitro as well as in vivo occurs primarily within the C-terminal half of the factor, and is caused by a cytosolic 40-kDa protein kinase. In contrast to other plant basic leucine-zipper motif factors, phosphorylation of CPRF2 does not alter its DNA binding activity. Therefore, we discuss alternative functions of the light-dependent phosphorylation of CPRF2 including the regulation of its nucleocytoplasmic partitioning.

Light is probably the most variable environmental factor controlling plant development. To monitor light quality and quantity, plants have evolved at least three different photoreceptor systems: the red/far-red reversible phytochromes, the blue/UV-A, and the UV-B photoreceptors (1). The most well understood of these photoreceptors is the phytochrome system (2, 3).

Besides the search for appropriate mutants, other approaches have been used to understand the signal transduction mechanisms mediated by photoreceptors. (i) Characterizing the photoreceptors themselves and searching for interacting proteins, (ii) unraveling the role of signal mediators like Ca²⁺, calmodulin, cGMP, and phosphorylation events (4), and (iii) analyzing DNA-binding proteins that interact with promoter elements of light-regulated genes. As shown, for example, for chalcone synthase or chlorophyll a/b-binding protein genes, promoter elements that mediate light responsiveness frequently contain the palindromic DNA motif ACGT, that, depending on the adjacent nucleotides, is part of the so-called G-box (CACGTG) or C-box (GACGTC) sequences (5). However, G- and C-boxes are not only found in the promoters of light-regulated genes but also in promoters of genes that respond to other exogenic and endogenic stimuli such as stress, hormones, and cell cycle-related signals (6). Transcription factors containing a basic leucine-zipper motif (bZIP), as, for example, the common plant regulatory factors (CPRFs) from parsley (7–11) and G-box binding factors from Arabidopsis (12–14), were shown to bind to the G-box or the C-box, respectively, in vitro as well as in vivo and form specific homo- and heterodimers (7, 8, 12, 13). Since CPRF and G-box binding factors proteins, which have molecular masses between 35 and 45 kDa, are encoded by multigene families (15), it is difficult to define which and how many members directly act as transcription factors regulating a certain inducible gene.

The regulation of the activities of these factors in response to light is poorly understood. However, recent studies showed that the DNA binding activities of several factors of the bZIP-type are regulated by their phosphorylation state (16–18). On the other hand, it was demonstrated that bZIP proteins exist in the cytosol of dark-cultivated parsley cells (19). From these bZIPs it was shown to be CPRF2 that is localized in the cytoplasm of dark-cultivated cells and transferred to the nucleus upon irradiation (20). A detailed physiological analysis revealed that phytochrome photoreceptors induce the nuclear import of CPRF2 (20). Regulation of transcription factor activity, therefore, could be caused also by differences in the subcellular partitioning of the factors in dark- and light-grown cells.

In this study we identified a 44-kDa protein in the cytosol of dark-cultivated evacuated parsley protoplasts that in vitro is very rapidly phosphorylated in response to irradiation. In correlation with this observation we show that in vivo the phosphorylation of the 44-kDa bZIP factor CPRF2 is also rapidly enhanced by light. Since red light is most effective in inducing both phosphorylation events, we conclude that the phytochrome photoreceptor system is involved in these photoreponses. CPRF2 is phosphorylated by a cytosolic 40-kDa protein kinase at least one serine residue within its C terminus. As shown by size exclusion chromatography, CPRF2 and its kinase elute in a molecular mass range of about 300 kDa, indicating that both proteins are part of protein complexes. Since the phosphorylation of CPRF2 does not interfere with its DNA binding activity, we propose that light-induced changes in the phosphorylation state might be involved in the regulation of the nucleocytoplasmic distribution of CPRF2.

EXPERIMENTAL PROCEDURES

Isolation of Cytosolic Extracts from Cultured Parsley Cells—Protoplasts were prepared under dim-green safety light (21) from a dark-green background and were incubated with 18 U.S.C. Section 1734 solely to indicate this fact. * The work was supported in part by Deutsche Forschungsgemeinschaft Grants FR836/1 (to H. F.) and SFB388 and the Human Frontier Science Program (to E. S. and K. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: bZIP, basic leucine-zipper motif; CPRF, common plant regulatory factor; Ni-NTA, nickel nitritriacetic acid; PAGE, polyacrylamide gel electrophoresis; EMSA, electrophoretic mobility shift assay; AP, alkaline phosphatase; PVDF, polyvinylidene difluoride; p40, protein kinase with an apparent molecular mass of 40 kDa; p50, protein kinase with an apparent molecular mass of 50 kDa.
grown parsley cell culture 6 days after subcultivation. The protoplasts were evacuated and cytosolic extracts were isolated as described previously (19, 20, 22, 23).

Expression and Purification of Recombinant CPRF2—The restriction fragment encoding full-length CPRF2 was subcloned into the BamHI site of the expression vector ToxI (Qiagen) as a XhoI/HindIII fragment. The C-terminal (His), tag. Transformation of the vectors in Escherichia coli, expression and purification of the proteins on nickel nitrotriacetic acid (Ni-NTA)-agarose were performed under denaturating conditions as described in the manufacturer’s protocol (Qiagen). The purified protein was refolded by removing urea by gel filtration through NAP 5 columns (Amersham Pharmacia Biotech), mixed once with a micropette, and irradiated for 30 s on ice with a slide projector using appropriate filters and mirrors to avoid warming effects or kept in darkness (19, 21, 22). After irradiation, refolded protein was washed with 500 μl of buffer P and in frozen liquid nitrogen. After thawing, the cells were lysed in 250 μl of ice-cold buffer (50 mM Tris/HEPS, pH 7.5, 150 mM NaCl, 0.1% (v/v) Triton X-100, 1 mM benzamidine, 5 mM e-aminoacrylic acid, 2 mM phenylmethylsulfonyl fluoride, 1 μg/μl antipain, 1 μg/μl leupeptin, 1 mM 4-nitrophenyl phosphate, 1 mM sodium fluoride, 1 mM pyrophosphate), and the extracts were clarified by centrifugation. Then 5 μl either of CPRF2 antiserum or the corresponding pre-immunoserum were added and the sample incubated for 2 h on ice followed by addition of 15 μl of protein A-Sepharose (Amersham Pharmacia Biotech) and incubation for 1 h on ice. The protein A-Sepharose was added with Tris-buffered saline/Tween (TBST: 50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.1% (v/v) Tween 20) and subsequently incubated with 6M guanidine hydrochloride in buffer A for 1 h. To renature the proteins the gel was washed extensively with ice-cold 0.05% (v/v) Tween 20 buffer A (18 h). Then the gel was equilibrated in kinase assay buffer (10 mM MgCl₂, 90 μM sodium vanadate in buffer A) for 30 min. The phosphorylation reaction was performed in 10 ml of kinase assay buffer with 30 μM ATP and 60 μCi of [γ-32P]ATP for 1 h at room temperature. Afterward the gel was washed in liquid nitrogen. After thawing, the cells were lysed in 250 μl of the following mixture were prepared: 33 μCi [γ-32P]ATP and the reaction mixture was divided. One-half was incubated on ice with 0.1 unit/μl aprotin (Sigma) to hydrolyze the ATP while the other half was kept on ice. After 30 min, 10-μl aliquots of the mixtures were removed, representing ATP-free and ATP-containing cytosolic extracts and used as controls of the endogenous DNA binding activity of the transcription factors (19). The mixture was divided. One-half was incubated with 1 unit/μl aprotin (Sigma) to hydrolyze the ATP. Afterward the gel was washed in liquid nitrogen. After thawing, the cells were lysed in 250 μl of nuclear fractionation buffer (20, 33). Preparation of the radioactively labeled probes as well as experimental conditions for EMSA, electrophoretic mobility shift assay were analyzed by autoradiography. For the DNA binding activity EMSA as described above (31, 33). Preparation of the radioactively labeled probes as well as experimental conditions for EMSA, electrophoretic mobility shift assay, and treatments of evacuated protoplast extracts with alkaline phosphatase (AP) were described previously (19, 20).

RESULTS

Rapid and Light-modulated Phosphorylation of Cytosolic Proteins in Vitro—In two recent reports (11, 20) we characterized the expression and intracellular distribution of three members of the parsley CPRF transcription factor family. In our present work, we are studying the post-translational regulation of CPRF activities. Since transcription factor activities are frequently modulated by phosphorylation (34), we initially tested in vitro whether a light-induced phosphorylation of a cytosolic polypeptide occurs in the molecular mass range of about 35–45 kDa that could represent a modification of one of the CPRF proteins. For this purpose, cytosol obtained from dark-cultivated evacuated protoplasts was used. The removal of the toxic and proteolytically very active content of the vacuole from the protoplasts is necessary for obtaining functionally intact parsley protein extracts (19, 22). The cytosolic extract and experiments were performed with [γ-32P]ATP and 60 μCi of [γ-32P]ATP. The reactions were performed in buffer A. The phosphoamino acid mapping of recombinant CPRF2 wascleaved by formic acid (28). For the analysis of the phosphorylase, the purified recombinant CPRF2 was cleaved by formic acid (28). The fragments obtained upon formic cleavage of in vivo and in vivo labeled CPRF2 were separated by SDS-PAGE (29). Subsequently, the gels were dried and stained with Coomassie Brilliant Blue. Each lane of the SDS-PAGE gel contained 25 μg of recombinant CPRF2 and was stained with Coomassie Brilliant Blue. Each lane of the SDS-PAGE gel contained 25 μg of recombinant CPRF2 and was stained with Coomassie Brilliant Blue.
different molecular sizes were phosphorylated in a light-dependent manner. Of particular interest was the phosphorylation of a protein with a molecular mass of approximately 44 kDa corresponding well with that of the factor CPRF2. This protein was most strongly labeled in response to red light irradiation indicating the involvement of phytochrome photoreceptors. Since tyrosine phosphorylation is discussed to be involved in phytochrome signaling leading to the activation of the G-box-containing chalcone synthase promoter (35), the same samples used above were tested with an antibody specific for phosphotyrosine. Although several polypeptides constitutively cross-reacted with the anti-phosphotyrosine antibody, no staining of those proteins was observed that were differentially labeled in response to irradiation (Fig. 1B). These data indicate that the light-induced phosphorylation of all these proteins including the 44-kDa polypeptide occurs most likely at serine and/or threonine but not at tyrosine residues.

**Phosphorylation of CPRF2 Is Rapidly Enhanced by Light**—The importance of phosphorylation for the regulation of transcription factor activities is well studied in yeast and animals (34). Since the molecular mass of the observed phosphorylated cytosolic 44-kDa protein corresponds with that of CPRF2, we took into consideration that both proteins are identical. Therefore, we tested whether CPRF2 is a phosphoprotein in vivo and whether its phosphorylation state is changed in response to light treatment. Dark-cultivated evacuated protoplasts were in vivo labeled with $^{32}$Posphate and irradiated for 5 min with light of different wavelengths or kept in darkness. After lysis of the evacuated protoplasts, CPRF2 was isolated by immunoprecipitation and assayed for phosphorylation by autoradiography (Fig. 2A). Compared with the dark control (Fig. 2A, lane 4), irradiation with either far-red, red, or white light enhanced the incorporation of $^{32}$P into endogenous CPRF2 with red light being most effective (Fig. 2A, lanes 1–3). No signal could be detected when the pre-immunogen was used for immunoprecipitation (Fig. 2A, lane 5). These data show that irradiation rapidly increases the phosphorylation state of CPRF2. To be able to further characterize the observed phosphorylation, an in vitro assay was established using recombinant CPRF2 and cytosolic extracts. As shown in Fig. 2B, lane 1, recombinant CPRF2 was phosphorylated by cytosolic extracts leading to a strong signal after autoradiography. In contrast, no signals could be detected using cytosolic extracts without recombinant CPRF2 (Fig. 2B, lane 2).

**CPRF2 Is Phosphorylated at Serine Residues in the C-terminal Half of the Molecule**—To map the phosphorylation site of CPRF2 in vitro as well as in vivo, phosphorylated and purified recombinant as well as endogenous CPRF2 were used for formate hydrolysis. Formate treatment cleaves peptide bonds between aspartate and proline (28) and should result in the case of CPRF2, in three peptides of 6.2 kDa (corresponding to amino acid (aa) 1–56), 14.5 kDa (corresponding to aa 57–195), and 23 kDa (corresponding to aa 196–401) (Fig. 3A). After hydrolysis the fragments were separated by SDS-PAGE and the phosphorylation pattern was analyzed by autoradiography. Independently of the fact, whether CPRF2 was phosphorylated in vitro (Fig. 3B) or in vivo (Fig. 3C), the major phosphorylated peptide had an apparent molecular mass of about 25 kDa. This suggests that, on the one hand, the predominately modified amino acid residues are very likely identical within the in vivo and in vitro labeled CPRF2 and, on the other hand, are localized within the C-terminal half of CPRF2. To identify the phosphorylated amino acid, a phoshoamino acid analysis of in vitro phosphorylated recombinant CPRF2 was performed. In agreement with the results shown in Fig. 1B the phosphorylation of CPRF2 was confined exclusively to serine residues (Fig. 3D).

**Characterization of the CPRF2 Phosphorylating Activity**—The molecular properties of the CPRF2-phosphorylating serine kinase were further characterized by extending our in vitro phosphorylation approach. For this purpose, cytosol was prepared from dark-kept evacuated protoplasts. Subsequently, the cytosol was irradiated with white light and separated by size exclusion chromatography. The obtained fractions were tested for CPRF2 phosphorylating activities by addition of recombinant CPRF2 and $^{32}$P ATP. As shown in Fig. 4A, labeling of CPRF2 was mainly found in fractions peaking around a molecular mass of approximately 300 kDa. A weak CPRF2 phosphorylating activity could also be detected in fractions around the molecular mass marker of 45 kDa (Fig. 4A, lanes 8–10). These results could be interpreted that the CPRF2 phosphorylating kinase is either a very large protein or associated with other peptides in a multiprotein complex. To test these possibilities, we performed an in-gel assay. For this, recombinant CPRF2 was polymerized into the matrix of an SDS-PAGE
did not yield any detectable signals (data not shown). This demonstrates that the CPRF2 phosphorylating activities derived from substrate phosphorylation and not from autophosphorylation activity. Since the main phosphorylation activity for CPRF2 (Fig. 4A) was observed in the same high molecular weight fractions as p40 these data strongly suggest that p40 is the cytosolic CPRF2-specific serine kinase. Moreover, the appearance of p40 in a molecular mass range of about 300 kDa indicates that this kinase forms a complex with other proteins. On the other hand, those fractions containing high amounts of p50 show only very weak CPRF2 phosphorylating activities (compare Fig. 4, A with B).

The DNA Binding Activity of CPRF2 Is Not Altered by Phosphorylation—As shown previously, phosphorylation of plant bZIP-type transcription factors can modulate their DNA binding activities (16–18). We, therefore, assayed whether the phosphorylation state of CPRF2 influences its DNA binding activity in vitro. Recombinant CPRF2 was incubated with ATP-free or ATP-containing cytosol over the time period of 30 min and subjected to EMSA using a radioactively labeled G-box as DNA probe. Compared with CPRF2 incubated in ATP-free cytosol, no significant changes in the DNA binding activity of phosphorylated CPRF2 could be observed (Fig. 5A). Controls showing the weak endogenous DNA binding activity of the cytosolic extracts indicate that the signals described above derive mainly from recombinant CPRF2.

In further experiments a potential phosphorylation-dependent modulation in the DNA binding activity of endogenous CPRF2 was determined. For this purpose, total extracts were obtained from dark-kept and red light-irradiated evacuated protoplasts by the same protocol that was used for the immunoprecipitation approach. This guaranteed that CPRF2 was in a phosphorylated state especially under red light conditions (see Fig. 2A). Since red light-irradiation leads to a nuclear import of CPRF2 (20) the use of total extracts instead of cytosolic extracts avoided effects on the DNA binding activity solely due to a redistribution of the factor. The total extracts were supplemented with alkaline phosphatase to remove peptide-bound phosphate residues (19). Afterward, the samples were subjected to EMSA using a C-box as DNA probe. CPRF2 has a very high affinity to the C-box, whereas the binding activities of other CPRF proteins are low, reducing the signals of these factors in EMSA (20). The DNA/CPRF2 band was identified by addition of a CPRF2-specific antiserum to the binding reaction resulting in a supershifted DNA-CPRF2-antibody complex (Fig. 5B, lanes 7–12). Whereas additional signals deriving from yet unidentified C-box binding factors were strongly reduced, the DNA/CPRF2 complexes were not affected in response to phosphatase treatment independent whether the extracts were isolated from dark-kept or irradiated evacuated protoplasts (Fig. 5B, lanes 1–6). Although we were able to show an increase of the in vitro phosphorylation state of CPRF2 under red light conditions compared with the dark control (Fig. 2A), this increase was not observed on the level of DNA binding activity.

Cytosolic CPRF2 Is Found in a High Molecular Weight Complex—Cytoplasmic retention of transcription factors in yeast and animals is frequently achieved by a stable interaction of the factors with proteins that inhibit nuclear uptake (34, 36). To determine whether a comparable retention mechanism could exist for CPRF2 as well we tested whether endogenous CPRF2 is associated with other proteins. For this purpose, EMSA was performed with the identical cytosolic fractions described in Fig. 4. To detect CPRF2 we used a C-box as DNA probe. As shown in Fig. 6, CPRF2 depending DNA binding activity was detected in those cytosolic fractions representing the molecular mass range around 300 kDa. The appearance of
The factor in this fraction was confirmed by the use of a specific CPRF2 antiserum in supershift assays (Fig. 6B). In contrast to CPRF2-DNA-protein complexes formed by unidentified C-box binding factors peaked in cytosolic fractions of lower molecular weight ranges (Fig. 6A, CBF). Taken together, the appearance of the 44-kDa protein CPRF2 in a molecular mass range of about 300 kDa indicates that this factor is associated with other proteins.

**DISCUSSION**

In this study, we initially analyzed the light-induced phosphorylation pattern of parsley proteins in vitro. Several polypeptides could be detected that showed light quality dependent, rapid incorporation of $[^{32}P]$. During the further course of our analysis we concentrated on a 44-kDa polypeptide that was especially phosphorylated in response to red light treatment. Due to its appropriate molecular weight this protein could resemble the bZIP transcription factor CPRF2, which was shown to be localized in the cytosol of dark-cultivated parsley cells (20). To support this assumption we immunoprecipitated CPRF2 from extracts of in vivo-labeled parsley evacuolated protoplasts that were either kept in darkness or irradiated for 5 min with light of different wavelengths. Immunoprecipitated CPRF2 showed a clear $[^{32}P]$ incorporation demonstrating that it is a phosphoprotein in vivo. Furthermore, the phosphorylation state of CPRF2 could be increased in response to irradiation, with red light being most effective followed by far-red and white light. From the very similar light-modulated phosphorylation pattern we conclude that the cytosolic 44-kDa protein is most likely CPRF2. Furthermore, the efficiency of far-red and red light indicates that phytochrome photoreceptors are involved in the rapid phosphorylation of CPRF2 probably via a very low fluence response (1). The less pronounced phosphorylation of CPRF2 under white light conditions could be explained by an UV light photoreceptor system that partly inhibits the CPRF2-specific kinase activity. A similar UV light-induced inhibition of a phytochrome-triggered response was described for the flavonoid synthesis in mustard (37).Taken together, our data demonstrate for the first time a light- (e.g. phytochrome-) modulated phosphorylation of a plant transcription factor in vivo as well as in vitro.

In a series of experiments, we characterized the CPRF2-specific kinase activity in more detail. In vitro and in vivo
phosphorylation followed by formic cleavage revealed that CPRF2 is phosphorylated predominantly in its C-terminal half. A phosphoamino acid analysis of the labeled recombinant CPRF2 demonstrated that the phosphorylation is confined to serine residues. This finding is in agreement with the observation that the phosphotyrosine antibody does not cross-react with those proteins that show light-modulated in vitro phosphorylation. Since the C terminus of CPRF2 contains several serine residues, further investigations have to be performed in order to identify the modified site(s). For further characterization of the CPRF2-specific phosphorylation activity we used cytosolic gel filtration fractions for in vitro labeling experiments. It was shown that the main CPRF2-specific phosphorylation activity peaks in a high molecular mass range around 300 kDa. The same fractions were also tested by an in-gel kinase assay using recombinant CPRF2 as a substrate. In this case, two cytosolic kinases termed p40 and p50 were observed to be able to phosphorylate immobilized CPRF2. Since in those high molecular weight fractions, that show strong CPRF2 phosphorylating activities in the in vitro phosphorylation experiment (Fig. 4A), high amounts of p40 but not of p50 were detected (as demonstrated by the in-gel kinase assay; Fig. 4B), we have to conclude that p40 is most likely the protein kinase that modifies CPRF2. Furthermore, the elution of p40 in a high molecular weight range points to the fact that this kinase is part of a cytosolic multiprotein complex. Since p40 and CPRF2 co-elute during size exclusion chromatography (compare Fig. 4B with 6A), it is possible that both proteins are associated and, therefore, are part of the same complex. However, further investigations have to be performed to prove a stable interaction between CPRF2 and p40. For example, an immunoprecipitation of CPRF2 should lead to a co-precipitation of the CPRF2 phosphorylating activity in case of a stable interaction.

Those fractions in which the p50 kinase eluted during size exclusion chromatography showed very weak in vitro CPRF2 phosphorylating activities (Fig. 4). This could be due to a low molecular weight inhibitor that inactivates the kinase in the cytosol under native conditions. This inhibitor would be removed during SDS-PAGE resulting in a high CPRF2 phosphorylating activity in the in-gel kinase assay.

So far in all studies demonstrating the phosphorylation of a plant bZIP factor this modification correlated with a change in its DNA binding activity. For example, phosphorylation of G-box binding factor 1 from Arabidopsis or of H/G-box binding factor 1 in elicited soybean cells increases the DNA binding activities of these factors (16, 17). On the other hand, phosphorylation of opaque 2 from maize causes the opposite effect (18). Interestingly, dephosphorylation of bZIP proteins isolated from parsley reduced their DNA binding activities dramatically (19). However, after phosphorylation of recombinant CPRF2 neither an increase nor a decrease of the DNA binding activity could be observed. In addition, the treatment of cytosol with alkaline phosphatase had no effect on the DNA binding activity of endogenous CPRF2 independent of whether the cells were treated with red light or kept in darkness. Therefore, we conclude, first, that CPRF2 is not identical with one of the previously described parsley factors and, second, that the observed phosphorylation is not involved in the regulation of its DNA binding activity.

As demonstrated in yeast and mammalian systems, besides regulation of the DNA binding activity, phosphorylation can also modulate the transactivity and subcellular localization of transcription factors (36, 38, 39). Although we cannot totally exclude a long distance effect, a regulation of transactivity by the C-terminal phosphorylation of CPRF2 is not likely, since the proline-rich transactivation domain is localized at the far N terminus of the protein.2

In a previous report we were able to show that CPRF2 is imported from the cytoplasm into the nucleus in response to phytochrome action (20). This photoresponse correlates well with the phytochrome-enhanced phosphorylation of CPRF2 by the cytosolic p40 serine kinase described here. From this correlation we can derive an attractive working hypothesis where light activation of phytochrome photoreceptors leads to an increase in the phosphorylation state of CPRF2 and subsequently to its nuclear localization. This could be achieved either by an increase in nuclear import or by a decrease in nuclear export, or both. In this context it is highly interesting that cytosolic CPRF2 is part of a multiprotein complex. This complex may represent a kind of anchoring system that retains CPRF2 in the cytosol under dark conditions. Similar retention mechanisms are well described for several eukaryotic kinase/phosphatase retention systems (39). For the case that p40 might be a part of the CPRF2-protein complex, irradiation could result in activation of the kinase and directly to a rapid phosphorylation of CPRF2 that finally leads to its nuclear import. Further experiments are presently in progress to analyze the details of this mechanism by using a broad spectrum of photobiological, biochemical, cytological, and molecular biological approaches.

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Light-regulated Phosphorylation of CPRF2

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