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The DNA Binding Properties of the Parsley bZIP Transcription Factor CPRF4a Are Regulated by Light*

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The common plant regulatory factors (CPRFs) from parsley are transcription factors with a basic leucine zipper motif that bind to *cis*-regulatory elements frequently found in promoters of light-regulated genes. Recent studies have revealed that certain CPRF proteins are regulated in response to light by changes in their expression level and in their intracellular localization. Here, we describe an additional mechanism contributing to the light-dependent regulation of CPRF proteins. We show that the DNA binding activity of the factor CPRF4a is modulated in a phosphorylation-dependent manner and that cytosolic components are involved in the regulation of this process. Moreover, we have identified a cytosolic kinase responsible for CPRF4a phosphorylation. Modification of recombinant CPRF4a by this kinase, however, is insufficient to cause a full activation of the factor, suggesting that additional modifications are required. Furthermore, we demonstrate that the DNA binding activity of the factor is modified upon light treatment. The results of additional irradiation experiments suggest that this photoresponse is controlled by different photoreceptor systems. We discuss the possible role of CPRF4a in light signal transduction as well as the emerging regulatory network controlling CPRF activities in parsley.

Light is probably the most important environmental stimulus for plants, controlling central developmental processes such as germination, deetiolation, and the transition from the vegetative to the reproductive phase (1). To monitor differences in the quality, intensity, and direction of light, plants have evolved different photoreceptor systems that control the expression of an enormous number of genes (2). The analysis of some of these light-controlled genes has revealed several *cis*-acting elements that are involved in mediating light responsiveness. One of these elements, the so-called G-box, is a hexameric DNA-motif (CACGTG) that is frequently found not only in the promoters of light-regulated genes but also in promoters that respond to other environmental or endogenous stimuli, such as hormones, stress, or cell cycle-related signals (3). Screening of expression libraries with G-box probes has led to the identification of several G-box binding proteins that belong to the family of basic leucine zipper motif (bZIP)[1] transcription factors, including the common plant regulatory factors (CPRFs) from parsley and the G-box binding factors (GBFs) from *Arabidopsis* (5–7). The bZIP motif is frequently found in eukaryotic transcription factors and mediates sequence specific DNA binding, as well the formation of either homo- or heterodimers (8).

The results of previous studies suggest that several of the identified G-box binding proteins are involved in light signal transduction. For example, we recently reported that one member of the CPRF family, CPRF2, is exclusively localized in the cytosol in the dark and that light treatment causes an almost complete import of the factor into the nucleus (9). In contrast, the factor CPRF1 is constitutively localized in the nucleus, whereas CPRF4a is found in the nucleus as well as in the cytosol under all conditions tested (9). The molecular mechanisms leading to the regulation of the intracellular distribution of CPRF2 are not fully understood. However, we recently described that CPRF2 is phosphorylated *in vivo* in a light-dependent manner (10). Because phosphorylation reactions are frequently involved in the regulation of the intracellular distribution of transcription factors of yeast and animals (11, 12), the phosphorylation of CPRF2 might play a role in triggering the nuclear import of the factor. This idea is supported by the fact that the import and the phosphorylation of CPRF2 are both strongly influenced by red light treatment, pointing to an involvement of the red light sensing phytochrome photoreceptors (10).

In this report, we describe the role of phosphorylation in the regulation of the parsley transcription factor CPRF4a. We demonstrate that the DNA binding activity of the factor is modulated in a phosphorylation-dependent manner and that cytosolic components are involved in this process. In agreement with this result, we have identified a cytosolic CPRF4a-kinase. Phosphorylation of recombinant CPRF4a by this kinase, however, is not sufficient to cause a full activation of the factor, suggesting that additional modifications are required. Moreover, we found that light treatment of dark-grown parsley cells leads to an increased DNA binding activity of CPRF4a. This effect is accompanied by a change of the CPRF4a-specific DNA binding pattern observed in an electrophoretic mobility shift assay (EMSA). The results of further irradiation experiments point to an involvement of different photoreceptor systems in the regulation of this process. We discuss the possible role of CPRF4a in light signal transduction, as well as the different mechanisms that contribute to the regulation of CPRF transcription factors.

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[1] The abbreviations used are: bZIP, basic leucine zipper motif; CPRF, common plant regulatory factor; GBF, G-box binding factor; PAGE, polyacrylamide gel electrophoresis; EMSA, electrophoretic mobility shift assay.

This paper is available on line at http://www.jbc.org

EXPERIMENTAL PROCEDURES

Isolation of Cytosolic and Nuclear Extracts from Cultured Parsley Cells—Cytosolic and nuclear extracts were isolated from a dark-grown parsley cell culture 6 days after subcultivation as described previously (9, 13–15). For irradiation experiments, evacuolated protoplasts were irradiated for 20 min with white light, red light, or far-red light or kept in darkness (13). Preparation of cytosolic extracts from irradiated evacuolated protoplasts was done in green safety light (13).

Expression and Purification of Recombinant CPRFs—Restriction fragments encoding full-length CPRF1, CPRF2, and CPRF4a were subcloned into the *Bam*HI sites of the vectors pQE70 (CPRF2) or pQE30 (CPRF1 and CPRF4a) to produce fusion proteins with C-terminal (CPRF2) or N-terminal (CPRF1 and CPRF4a) histidine tags. Transformation of the vectors in *Escherichia coli* and expression and purification of the proteins on nickel nitrilotriacetic acid-agarose were performed under denaturing conditions, as described in the manufacturer's protocol (Qiagen). The purified proteins were refolded by removing urea by gel filtration through NAP 5 columns (Amersham Pharmacia Biotech) against 25 mM Tris/HCl, pH 7.8, 100 mM NaCl, and 1 mM dithiothreitol. The protein content of the eluate was determined after centrifugation (1 h at 100,000 × *g* and 4 °C) using a method that is based on Coomassie Blue G-250 (16). 2 μg of the recombinant proteins were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12% (w/v) acrylamide gel (17). Subsequently, the proteins were stained with Coomassie Blue R-250.

In Vitro Phosphorylation of Recombinant CPRFs—2 μg of the recombinant CPRFs were mixed in a total volume of 20 μl with 50 μg of cytosolic protein or 20 μg of nuclear protein and 1⁄10 volume of 0.3 M Tris/HCl, pH 7.4, 50 mM $MgCl_2$, 1.2 mM $CaCl_2$. 1 min after addition of 5 μCi of [γ-^{32}P]ATP, the reactions were stopped with 500 μl of 6 M guanidine hydrochloride, 0.1 M NaH_2PO_4, 0.01 M Tris, pH 8.0. The histidine-tagged proteins were isolated on nickel nitrilotriacetic acid-agarose and eluted with 50 μl of 8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris, pH 6.3, and 100 mM EDTA. The eluates were subjected to SDS-PAGE using a 12% (w/v) acrylamide gel (17). Subsequently, the gels were silver-stained (18), dried, and analyzed by autoradiography.

For kinase inhibitor experiments, the reaction mixtures containing recombinant CPRF4a and cytosolic protein were supplemented with one of the following substances (final concentrations are indicated): Me_2SO (0.1% (v/v)), K252a (100 nM), H-89 (50 μM), staurosporine (200 nM), chelerythrine chloride (10 μM), genistein (50 μM), KN62 (10 μM), and hypericin (5 μM). All kinase inhibitors were purchased from Calbiochem and diluted in Me_2SO. The total concentration of Me_2SO did not exceed 0.1% (v/v). For activation of hypericin, the samples were irradiated for 10 min with white light prior to addition of [γ-^{32}P]ATP. The labeling reactions, as well as the purification of the recombinant proteins and their analysis, were performed as described above.

Electrophoretic Mobility Shift Assays, Supershift Assays, and Phosphatase Treatment—For EMSA, either a monomeric G-box probe (5′-AATTCTCCCTTATTCCACGTGGCCATCCGG-3′) or a tetrameric G-box probe (5′-(ACCACGTGGC)$_4$-3′) was used (G-box core sequences are underlined). For the mutated tetrameric G-box probe, the core sequence of the second G-box repeat was changed from CACGTG to CACTGT. Preparation of the radioactively labeled probes and experimental conditions for EMSA were described previously (9, 13). For supershift assays the binding reaction mixtures containing either cytosolic or nuclear protein in a total volume of 15 μl were incubated for 10 min on ice with 1 μl of the CPRF antisera or the corresponding preimmunosera prior to EMSA. The CPRF antisera used in this study have been described previously (9). Treatment of protein extracts with alkaline phosphatase was performed as follows: 0.5 μl of 4.2 units/μl alkaline phosphatase (Sigma, P-7915) was added to 50 μg of cytosolic or 20 μg of nuclear protein in a total volume of 10 μl and incubated for 10 min at room temperature. Identical samples were supplemented with 0.5 μl of the phosphatase storage buffer (50% (v/v) glycerol, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, and 30 mM triethanolamine, pH 7.6) for control reactions. Subsequently, the DNA probe was added, and the samples were subjected to EMSA. The experimental conditions for the dephosphorylation of cytosolic extracts with immobilized alkaline phosphatase and for the rephosphorylation of dephosphorylated cytosolic protein have been described previously (13).

DNA Binding Activity of Recombinant CPRF4a after Phosphorylation—The phosphorylation kinetics of recombinant CPRF4a was performed as described previously (10).

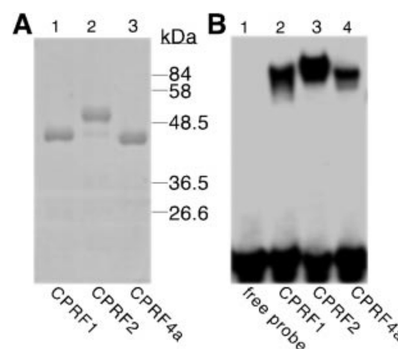

FIG. 1. **Purification and characterization of the recombinant CPRFs.** *A,* after purification, 2 μg of recombinant CPRFs (as indicated) were analyzed by SDS-PAGE and Coomassie Blue staining. The position of protein molecular mass markers in kilodaltons are indicated on the *right*. *B,* after the folding procedure, the recombinant CPRF proteins were subjected to EMSA to test their G-box binding activities. A monomeric G-box probe was used for this assay. In *lanes 2–4,* 200 ng of the individual factors were tested. In *lane 1,* no protein was added (*free probe*).

RESULTS

Expression and Characterization of Recombinant CPRF Proteins—To allow for a comparative analysis of different CPRF transcription factors, three members of the CPRF family (*e.g.* CPRF1, CPRF2, and CPRF4a) were expressed in *E. coli* as fusion proteins with terminal histidine tags. The use of histidine tags allowed a purification of the recombinant proteins by affinity chromatography. Under all conditions tested, overexpression of the CPRFs resulted in an almost complete incorporation of the recombinant proteins into inclusion bodies. Therefore, inclusion bodies were isolated, and the recombinant proteins were solubilized by treatment with a solution containing 6 M guanidinium hydrochloride. Subsequently, purification of the proteins was performed in the presence of 8 M urea using nickel nitrilotriacetic acid-agarose. Fig. 1*A* shows the purified proteins after SDS-PAGE and Coomassie staining. Whereas the CPRF1 and CPRF4a preparations were highly pure (Fig. 1*A, lanes 1* and *3*), the CPRF2 preparation contained a minor contaminant of a lower apparent molecular weight (Fig. 1*A, lane 2*). Western blotting analysis using specific CPRF2 and histidine tag antibodies revealed that this protein is a C-terminal CPRF2 fragment (data not shown). Folding of the purified CPRF proteins was performed by removing urea by gel filtration. For all CPRFs, the protein yield after the folding procedure was about 50%. The factors were subjected to EMSA to test their DNA binding activities. As shown in Fig. 1*B,* all CPRFs were capable of binding a G-box containing probe. This result is in agreement with previous reports showing G-box binding activity to be a characteristic feature of native CPRF proteins (4, 5, 19).

Cytosolic and Nuclear Kinase Activities for CPRF Proteins—In yeast and in animals, the important role of phosphorylation in the regulation of transcription factor activities has been described in detail (12). Because we were recently able to report a light-dependent phosphorylation of the factor CPRF2 (10), we addressed the question of whether phosphorylation events are involved in the regulation of other CPRF proteins as well. A prerequisite for such a regulatory mechanism is the existence of specific kinases for the individual factors. Therefore, we initiated our study by analyzing compartment specific CPRF phosphorylation activities. For this purpose, we mixed the recombinant bZIP factors with cytosolic and nuclear extracts, which were obtained from evacuolated parsley protoplasts. These subcellular extracts were chosen due to our previous results showing that CPRFs are localized in the nucleus

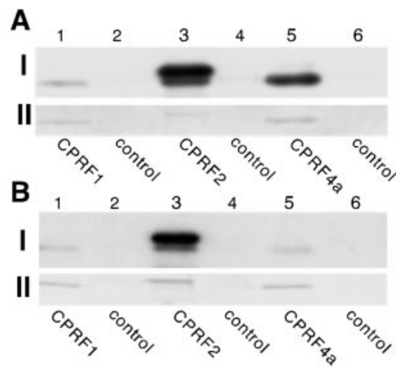

FIG. 2. **Cytosolic and nuclear phosphorylation activities for the recombinant CPRFs.** 2 μg of the recombinant proteins were mixed with cytosolic (*A*) or nuclear (*B*) extracts. The phosphorylation reaction and the purification of the factors were performed as outlined in the text. After SDS-PAGE, the purified proteins were analyzed by autoradiography (*panel I*) and silver staining (*panel II*).

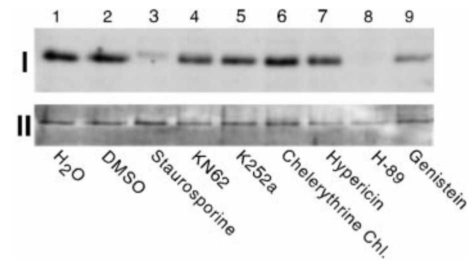

FIG. 3. **Inhibition of the cytosolic CPRF4a kinase activity.** 2 μg of recombinant CPRF4a were mixed with 50 μg of cytosolic protein and 1 μl of Me_2SO (*DMSO*)-diluted kinase inhibitors as indicated (*lanes 3–9*). As controls, identical samples were supplemented with either 1 μl of H_2O (*lane 1*) or Me_2SO (*DMSO*) (*lane 2*) to a final concentration of 0.1% (v/v). The phosphorylation reaction and the purification of the factors were performed as outlined in the text. After SDS-PAGE, the purified proteins were analyzed by autoradiography (*panel I*) and silver staining (*panel II*).

as well as in the cytosol (9). After [γ-^{32}P]ATP was added, the reaction mixtures were incubated for 1 min at room temperature. Subsequently, the recombinant factors were purified on nickel nitrilotriacetic acid-agarose under denaturing conditions and subjected to SDS-PAGE. Silver staining of the gels showed that the recombinant proteins were recovered in similar amounts (Fig. 2, *A* and *B, panel II*). Thus, the signals obtained after autoradiography (Fig. 2, *A* and *B, panel I*) were compared directly. CPRF1 was only marginally phosphorylated in the presence of cytosolic extracts (Fig. 2*A, lane 1*) and nuclear extracts (Fig. 2*B, lane 1*), whereas CPRF2 was strongly labeled in the presence of both extracts (Fig. 2, *A* and *B, lane 3*). In contrast, CPRF4a was strongly phosphorylated by cytosolic extracts (Fig. 2*A, lane 5*) but very weakly by nuclear extracts (Fig. 2*B, lane 5*). Neither cytosolic nor nuclear extracts showed detectable signals in the autoradiogram in the absence of purified recombinant CPRFs (Fig. 2, *A* and *B, lanes 2, 4,* and *6*). Taken together, our results show different and, in the case of CPRF4a, compartment-specific kinase activities for the individual CPRF proteins.

Characterization of the Cytosolic CPRF4a Kinase Activity by Inhibitor Studies—In a previous report, we studied the phosphorylation activity for CPRF2 in detail, showing that this factor is modified in its C-terminal half by a 40-kDa serine kinase in a light-dependent reaction (10). To gain a better understanding of the mechanisms regulating CPRF activities, we focused our analysis on the compartment-specific kinase activity for CPRF4a. We performed *in vitro* phosphorylation experiments with cytosolic extracts as described above in the presence of different kinase inhibitors to characterize the CPRF4a specific kinase in more detail. The results of these experiments (Fig. 3) revealed that the CPRF4a kinase is strongly inhibited by staurosporine (*lane 3*) and H-89 (*lane 8*), whereas other substances caused no or only very weak effects. Staurosporine is a general inhibitor of serine/threonine kinases (20), suggesting that CPRF4a is modified on either serine or threonine residues, or both. H-89 was originally designed to specifically inhibit protein kinase A in nanomolar concentrations (20). However, higher concentrations of H-89 (50 μM was used in Fig. 3) affect other types of kinases as well (20). Interestingly, H-89 in micromolar concentrations strongly inhibits the G-box binding activity of bZIP-like proteins that are localized in the cytosol of evacuolated parsley protoplasts (13). Because a pool of CPRF4a factors is found in the cytosol (9) and the cytosolic phosphorylation of CPRF4a is strongly decreased by H-89 treatment, we next tested whether phosphorylation may contribute to the regulation of its DNA binding activity.

The DNA Binding Activity of CPRF4a Is Controlled by Phosphorylation—To study the possible effect of phosphorylation on the activity of CPRF4a, we isolated nuclear and cytosolic extracts to perform DNA binding studies with endogenous CPRF4a. Fig. 4 shows the results of EMSA experiments in which the extracts were tested using a monomeric G-box probe. The signals deriving from CPRF4a were identified by addition of a specific CPRF4a antiserum to the binding reactions, resulting in the reduction of the CPRF4a-signals in cytosolic as well as nuclear extracts, with a concomitant appearance of supershifted DNA-CPRF4a antibody complexes (Fig. 4, *A* and *B, lane 5*). No effects were observed when the corresponding preimmunoserum was added (Fig. 4, *A* and *B, lane 6*). To remove peptide-bound phosphate residues, we added alkaline phosphatase to cytosolic as well as nuclear extracts prior to EMSA (Fig. 4, *A* and *B, lane 3*). This treatment resulted in a strong decrease of the CPRF4a-specific signals. In contrast, mock treatment caused no effects, indicating that the reduction of the DNA binding activity of CPRF4a is not due to a modification of the reaction conditions (Fig. 4, *A* and *B, lane 4*). Taken together, these results suggest that the DNA binding activity of CPRF4a is controlled in a phosphorylation-dependent manner.

We next tested whether the cytosolic kinase activity for CPRF4a is sufficient for an activation of the factor. For this purpose, we mixed recombinant CPRF4a with ATP-containing or ATP-free cytosol and incubated the reaction mixtures over a time period of 30 min. After different incubation times, aliquots of the reaction mixtures were removed, and the samples were subjected to EMSA using a monomeric G-box probe (Fig. 5). Whereas the DNA binding activity of CPRF4 incubated in ATP-free cytosol remained unchanged (Fig. 5, *–ATP*), we observed a gradual up-shift as well as a weak increase of the signals deriving from CPRF4a incubated in ATP-containing cytosol during the course of the experiment (Fig. 5, *+ATP*). Interestingly, under the identical experimental conditions, no change in the DNA binding activity of recombinant CPRF2 was found (10). Controls displaying the weak endogenous DNA binding activity of the cytosolic extracts indicate that the signals described above derived mainly from recombinant proteins (Fig. 5, *cytosol*).

In contrast to the strong decrease of the DNA binding activity of endogenous CPRF4a after phosphatase treatment (Fig. 4), the effects shown in Fig. 5 were relatively weak. We have demonstrated that recombinant CPRF4a is phosphorylated in the presence of ATP-containing cytosol (Fig. 2). Therefore, the results shown in Fig. 5 suggest that phosphorylation of recombinant CPRF4a by the cytosolic kinase activity is insufficient to cause a full activation of the factor (see under "Discussion" for further details).

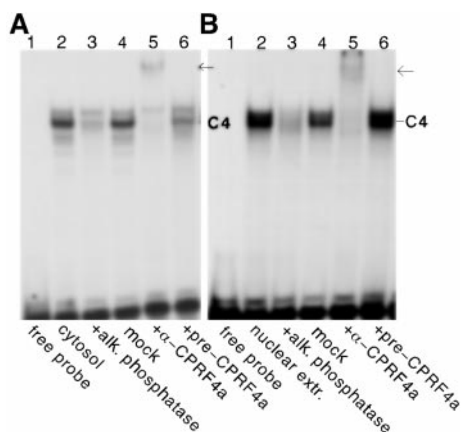

FIG. 4. **The DNA binding activity of CPRF4a is reduced after phosphatase treatment.** 50 μg of cytosolic protein (*A*) and 20 μg of protein from a nuclear extract (*B*) were subjected to EMSA using a monomeric G-box probe. The extracts were either treated with alkaline phosphatase prior to EMSA (*lane 3*) or mock-treated (*lane 4*). CPRF4a-specific signals were identified by addition of a specific CPRF4a polyclonal serum (*lane 5*) or the corresponding preimmunoserum (*lane 6*). The signals deriving from CPRF4a are indicated (*C4*). Supershifts are marked with an *arrow*. In *lane 1,* no protein was added (*free probe*), and in *lane 2,* the extracts were tested without further treatment.

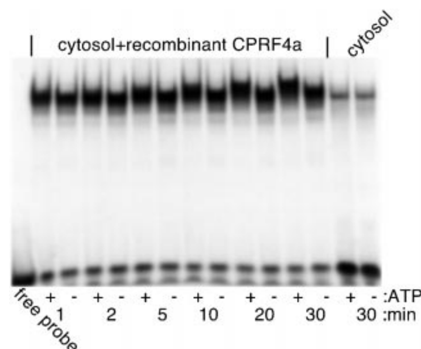

FIG. 5. **Analysis of the DNA binding activity of recombinant CPRF4a after phosphorylation.** Recombinant CPRF4a was mixed with ATP-containing cytosol (+*ATP*) or ATP-free cytosol (–*ATP*). Reactions were stopped after 1, 2, 5, 10, 20, or 30 min as indicated. As a control, endogenous DNA binding activities of ATP-free and ATP-containing cytosol are also shown (*cytosol*). The samples were tested by EMSA using a monomeric G-box probe.

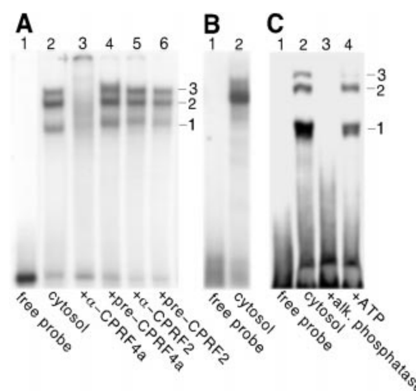

FIG. 6. **Identification of CPRF4a in cytosolic extracts.** *A,* 20 μg of cytosolic protein were tested in EMSA using a tetrameric G-box probe. The three signals deriving from untreated cytosolic extracts (*lane 2*) are numbered (*1–3*) on the right. To identify CPRF-specific signals, 1 μl of either CPRF4a antiserum (*lane 3*) or CPRF2 antiserum (*lane 5*), as well as of the corresponding preimmunosera (*lanes 4* and *6*), was added to the binding mixtures prior to EMSA. An *arrow* indicates the supershifted CPRF4a-DNA complex in lane 3. In *lane 1,* no protein was added (*free probe*). *B,* 20 μg of cytosolic protein were tested in EMSA using a tetrameric probe in which the second of the G-box repeats was disrupted by nucleotide exchanges within the core sequence. No protein was added in *lane 1* (*free probe*). *C,* 20 μg of cytosolic protein were treated with immobilized alkaline phosphatase (*lanes 3–4*) or mock-treated (*lane 2*). Subsequently, the immobilized phosphatase was removed by centrifugation. The samples were then either supplemented with ATP (*lane 4*) or mock-treated (*lanes 2* and *3*) and, after a 30-min incubation step, subjected to EMSA. The three signals deriving from cytosolic extracts are numbered (*1–3*) on the *right*. In *lane 1,* no protein was added (*free probe*).

CPRF4a Is Identical to the Previously Described Cytosolic bZIP-like Factors—As mentioned above, cytosolic bZIP-like proteins from parsley have been described in a previous report in which they were identified by their ability to bind to a probe containing four G-box elements (tetrameric G-box probe). The results of several experiments presented in this study and elsewhere strongly suggested that these factors are identical with CPRF4a: (i) CPRF4a and the unknown factors contribute very strongly to the overall G-box binding activity. (ii) Both CPRF4a and the unknown factors are localized in the cytosol and in the nucleus, displaying a comparable intracellular distribution (9, 13). (iii) The unknown factors were classified as bZIP-like by their interaction with antibodies raised against the *Arabidopsis* bZIP factor GBF1 (13). Likewise, GBF1 antiserum strongly cross-reacts with endogenous CPRF4a.[2] To test whether these factors are, in fact, identical with CPRF4a, we performed supershift experiments with cytosolic extracts using the tetrameric G-box probe (Fig. 6*A*). In agreement with previous results (13), we observed the formation of three distinct protein-DNA complexes (Fig. 6*A, lane 2*). The addition of the specific CPRF4a antiserum to the binding reaction mixtures caused a strong decrease of all three signals as well as a supershifted band (Fig. 6*A, lane 3*). In contrast, neither addition of a specific CPRF2 serum nor the corresponding preimmunosera caused any effects (Fig. 6*A, lanes 4–6*). These results indicate that CPRF4a is involved in the formation of all three bands.

The appearance of three distinct signals deriving from CPRF4a activity can be readily explained by different amounts of the factor that are bound to the tetrameric G-box probe. This would lead to DNA-protein complexes of different sizes that would be differentially retarded in the gel. To test this possibility, we used a tetrameric probe for EMSA in which one of the binding-sites was disrupted by nucleotide changes within the G-box core sequence (Fig. 6*B*). In accordance with our hypothesis, we observed a decrease in the number of signals compared with the nonmutated probe (Fig. 6*A*), suggesting that the number of factors that can bind to the probe is reduced.

As shown in Fig. 4, we have demonstrated that the DNA binding activity of CPRF4a is reduced after phosphatase treatment. To confirm that the three signals observed in Fig. 6*A* are indeed derived from CPRF4a, we tested whether these signals are affected by dephosphorylation as well. For this purpose, cytosol was treated with alkaline phosphatase that was immobilized on Sepharose beads. Prior to EMSA, the immobilized phosphatase was removed by centrifugation. As shown in Fig. 6*C, lane 3,* the formation of all three signals was abolished by phosphatase treatment. This result supports our conclusion that all three signals derive from CPRF4a-activity.

After Dephosphorylation, the DNA Binding Activity of CPRF4a Can Be Restored in an ATP-dependent Reaction—As shown in Figs. 4 and 6*C,* the DNA binding activity of CPRF4a is reduced after phosphatase treatment suggesting that phosphorylation is crucial for the regulation of the factor. This idea was confirmed by an experiment, in which ATP was added to alkaline phosphatase-treated cytosol, from which the immobi-

[2] F. Wellmer, unpublished observation.

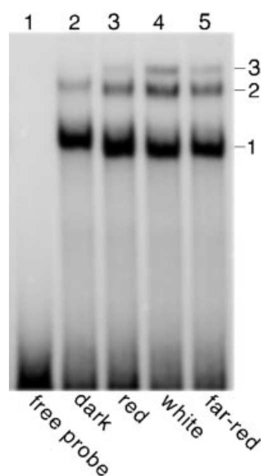

FIG. 7. **Light-dependent modification of the DNA binding properties of CPRF4a.** Dark-grown evacuolated protoplasts were either irradiated for 20 min with red light (*lane 3*), white light (*lane 4*), or far-red light (*lane 5*) or kept in darkness (*lane 2*). Subsequently, cytosolic extracts were isolated, and 10 μg of protein were analyzed by EMSA using a tetrameric G-box probe. In *lane 1,* no protein was added (*free probe*). The three signals deriving from cytosolic extracts after irradiation are numbered (*1–3*) on the *left*.

lized enzyme had been removed by centrifugation. This treatment caused an almost complete restoration of the DNA binding activity of CPRF4a reflected by the reappearance of all three signals in EMSA (Fig. 6*C, lane 4*; compare with *lane 2*), whereas in an ATP-free control reaction, no effects were observed (Fig. 6*C, lane 3*). This result indicates that ATP-dependent processes are involved in the regulation of the DNA binding activity of endogenous CPRF4a. Furthermore, it demonstrates that cytosolic components are involved in the regulation of the factor.

The DNA Binding Properties of CPRF4a Are Influenced by Light—Interestingly, the uppermost of the three signals that derive from CPRF4a activity was never detected in cytosolic extracts that were isolated from cells kept in darkness (13). Further experiments revealed that light induces the formation of this signal in an ATP-dependent reaction (13). To determine the photoreceptor systems that are involved in the regulation of this process, we irradiated dark-kept evacuolated protoplasts with different light qualities and subsequently isolated cytosolic extracts. These extracts were tested in EMSA using the tetrameric G-box probe (Fig. 7). Whereas white light strongly induced the formation of the third, uppermost signal (Fig. 7, *lane 4*), such a DNA-CPRF4a complex was not observed in extracts isolated from dark-kept protoplasts (Fig. 7, *lane 2*). Additionally, the signal of the middle band was enhanced. In comparison to white light treatment, irradiation with either red or far-red light caused similar but significantly weaker effects (Fig. 7, *lanes 3* and *5*). In total, light treatment resulted in an enhancement of the overall G-box binding activity of cytosolic CPRF4a and in the formation of an additional signal that might reflect a different modification state of the factor (see under "Discussion").

The effects of red light treatment and of far-red light treatment indicate an involvement of the red light sensing phytochrome photoreceptors in the control of CPRF4a activity (1). However, white light had a stronger effect on the DNA binding activity of the factor than red light alone, suggesting that photoreceptor systems in addition to phytochromes contribute to the regulation of CPRF4a activity.

DISCUSSION

In a previous report, we have studied the regulation of bZIP-like factors from parsley that are localized in the cytosol as well as in the nucleus (13). Here, we were able to show that these factors are identical with CPRF4a (Fig. 6). This finding allows us to integrate the previously reported data (13) with the results presented in this study.

We have demonstrated that the DNA binding activity of endogenous CPRF4a is affected by the kinase inhibitor H-89 as well as by two different phosphatase inhibitors (13). These results implied that a kinase/phosphatase system contributes to the regulation of the factor. In agreement with this idea, the DNA binding activity of endogenous CPRF4a is strongly reduced by dephosphorylation (Figs. 4 and 6*C*) and the G-box binding activity of cytosolic CPRF4a after dephosphorylation can be restored in an ATP-dependent reaction (Fig. 6C). Taken together, the results presented in this study and elsewhere (13) indicate the important role of phosphorylation in the regulation of CPRF4a.

As mentioned above, we were able to show that the DNA binding activity of cytosolic CPRF4a after dephosphorylation can be restored in an ATP-dependent reaction (Fig. 6C). Interestingly, in a similar experiment, the G-box binding activity of nuclear localized CPRF4a could not be restored (13). These results imply that cytosolic components are necessary for the activation of the factor. The identification of a cytosolic CPRF4a-specific kinase (Fig. 2), as well as the phosphorylation-dependent regulation of the DNA binding activity of CPRF4a, suggested that the kinase might directly activate the factor by changing its phosphorylation state. In accordance with this idea, we found similar effects of the kinase inhibitor H-89 on the activity of the CPRF4a-kinase (Fig. 3) and on the DNA binding activity of the factor (see above). However, compared with the nonphosphorylated factor, the DNA binding activity of recombinant CPRF4a changes only weakly after phosphorylation (Fig. 5). We conclude, therefore, that the phosphorylation of CPRF4a by the cytosolic kinase is not sufficient for a full activation of the factor and that additional modifications are therefore required. In this scenario, recombinant CPRF4a was not fully activated by ATP-containing cytosol because these additional modifications require either noncytosolic components (*e.g.* membrane proteins) and/or an intact cell structure. Accordingly, the ATP-dependent restoration of the DNA binding activity of cytosolic CPRF4a after dephosphorylation (Fig. 6*C*) would indicate that the dephosphorylation process does not affect the additional modifications that are required for a full activation of the factor.

CPRF4a contains a conserved nuclear localization sequence (5). However, a large pool of CPRF4a was found in the cytosol under all conditions tested (9). Therefore, it has been proposed that CPRF4a is partly retained in the cytosol by an unknown mechanism (9). Intracellular distributions similar to those observed for CPRF4a have been described for the bZIP factors GBF1 from *Arabidopsis* and for G/HBF1 from soybean (21, 22). Interestingly, the DNA binding activities of both factors are regulated by phosphorylation. Whereas GBF1 is phosphorylated by a nuclear casein kinase II (23, 24), G/HBF1 is rapidly phosphorylated in elicited soybean cells by a cytosolic kinase, leading to an enhancement of its DNA binding activity *in vitro* (22). Dephosphorylation of G/HBF1 leads to a changed immunoreactivity of the factor, pointing to a major conformational change within the protein (22). It has been proposed that this conformational change leads to an unmasking of the nuclear localization signal of G/HBF1 allowing its nuclear import (22). An altered conformation might also explain the additional CPRF4a signal that appeared upon irradiation. Our results suggest that the appearance of this band is due to an increased number of factors that are bound to the tetrameric G-box probe (Fig. 6). This effect cannot be solely explained by an increase of

the overall DNA binding activity of the cytosolic CPRF4a pool after irradiation because even high amounts of protein from dark-kept cytosolic extracts do not cause the formation of this band (13). An altered conformation, however, could facilitate binding to the different G-boxes of the tetrameric probe that are in close proximity by reducing steric hindrance between the factors.

The appearance of the additional CPRF4a signal in cytosolic extracts is not only light-dependent but also ATP-dependent (13). Furthermore, this effect is accompanied by an increase of the DNA binding activity of CPRF4a (Fig. 7). As a working hypothesis, we therefore suggest that the kinase/phosphatase system that is involved in controlling the DNA binding activity of CPRF4a is also involved in its light-dependent modification.

Interestingly, a G-box binding pattern that is similar to that of cytosolic CPRF4a after irradiation has been observed for nuclear localized CPRF4a (13). Therefore, a modification of CPRF4a might be a prerequisite for nuclear localization. Moreover, it has been demonstrated by cotranslocation assays using GBF1 antibodies that white light treatment resulted in an enhanced translocation of cytosolic bZIP-like factors to the nucleus (13). Because GBF1 antibodies strongly cross-react with CPRF4a,[2] this result could indicate a light-induced nuclear import of CPRF4a. However, we cannot rule out the possibility that this effect is due to cross-reactions of the GBF1 antibodies with cytosolic bZIP factors others than CPRF4a. In supershift experiments, we failed to detect a major change of the cytosolic CPRF4a pool after light treatment (9). However, CPRF4a accumulates very rapidly upon irradiation (5). This newly synthesized protein could replace those factors that are transported to the nucleus, leading to a relatively constant number of proteins in the cytosolic CPRF4a pool.

The results of our irradiation experiments suggest that different photoreceptor systems contribute to the regulation of CPRF4a. The effect of red and far-red light on the DNA binding properties of CPRF4a implies the involvement of the red light sensing phytochrome photoreceptors (1, 2). However, compared with irradiation with white light, red light caused only minor effects. This result can be readily explained by the involvement of a second photoreceptor system that acts with phytochromes in an additive manner. Good candidates for such receptors are the UV-A/blue light sensing cryptochromes, which, like the phytochromes, play an important role in the development of plants (25). This idea is supported by the fact that irradiation with UV and blue light causes a rapid accumulation of CPRF4a protein (5). However, further experiments have to be performed to confirm a role of cryptochromes in the regulation of CPRF4a.

The important role of G-boxes in light-dependent gene regulation has been confirmed recently by the identification of the G-box binding factors HY5 and PIF3 from *Arabidopsis* (26–28). Reduced levels of these factors cause a decrease in the photoresponsiveness of their putative target genes (29). Interestingly, it has been shown that these factors are not capable of inducing all light-regulated genes that contain functional G-boxes in their promoters, indicating that additional factors are required (29). The results of this study suggest that CPRF4a could be such an additional G-box binding factor. A putative target gene for CPRF4a is the chalcone synthase gene from parsley that is induced by UV light. It has been recently suggested that a CPRF1-containing bZIP heterodimer is involved in the regulation of this gene (30). Because CPRF4a can form heterodimers with CPRF1 (5), it is a likely candidate for being the partner of CPRF1 in chalcone synthase regulation. In addition to its contribution in the formation of heterodimer complexes, CPRF4a might also compete with other G-box binding proteins (*e.g.* HY5 or PIF3) for binding sites and thereby may fine-tune the activities of these factors. It is remarkable that other CPRF factors that are regulated in response to light are controlled by mechanisms that are different to that of CPRF4a. For example, light treatment does not alter the DNA binding properties of CPRF2 (10). However, although CPRF2 is absent from the nucleus in the dark (9), light treatment leads to a nuclear import of the factor. These results imply that an activation of CPRF2 is achieved by its intracellular redistribution (9). Furthermore, in contrast to CPRF4a, CPRF2 is efficiently modified in response to red light, whereas other light qualities have only minor effects (9, 10). These differences in light responsiveness and in the mechanisms regulating their activities suggest that CPRF2 and CPRF4a contribute to different aspects of light signal transduction and thereby act in a nonredundant manner.

REFERENCES

1. Kendrick, R. E., and Kronenberg, G. H. M., eds. (1994) *Photomorphogenesis in Plants*, Kluwer, Dordrecht, Netherlands
2. Batschauer, A. (1998) *Planta* **206,** 479–492
3. Menkens, A. E., Schindler, U., and Cashmore, A. R. (1995) *Trends Biochem. Sci.* **20,** 506–510
4. Weisshaar, B., Armstrong, G. A., Block, A., da Costa e Silva, O., and Hahlbrock, K. (1991) *EMBO J.* **10,** 1777–1786
5. Kircher, S., Ledger, S., Hayashi, H., Weisshaar, B., Schäfer, E., and Frohnmeyer, H. (1998) *Mol. Gen. Genet.* **257,** 595–605
6. Schindler, U., Menkens, A. E., Beckmann, H., Ecker, J. R., and Cashmore, A. R. (1992) *EMBO J.* **11,** 1261–1273
7. Menkens, A. E., and Cashmore, A. R. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 2522–2526
8. Liu, L., White, M. J., and MacRae, T. H. (1999) *Eur. J. Biochem.* **262,** 247–257
9. Kircher, S., Wellmer, F., Nick, P., Rügner, A., Schäfer, E., and Harter, K. (1999) *J. Cell Biol.* **144,** 201–211
10. Wellmer, F., Kircher, S., Rügner, A., Frohnmeyer, H., Schäfer, E., and Harter, K. (1999) *J. Biol. Chem.* **274,** 29476–29482
11. Jans. D. A., and Hübner, S. (1996) *Physiol. Rev.* **76,** 651–685
12. Hunter, T., and Karin, M. (1992) *Cell* **70,** 375–387
13. Harter, K., Kircher, S., Frohnmeyer, H., Krenz, M., Nagy, F., and Schäfer, E. (1994) *Plant Cell* **6,** 545–559
14. Harter, K., Frohnmeyer, H., Kircher, S., Kunkel, T., Mühlbauer, S., and Schäfer, E. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 5038–5042
15. Frohnmeyer, H., Hahlbrock, K., and Schäfer, E. (1994) *Plant J.* **5,** 437–449
16. Sedmark, J. J., and Grossberg, S. E. (1977) *Anal. Biochem.* **79,** 544–552
17. Laemmli, U. K. (1970) *Nature* **227,** 680–685
18. Heukeshoven, J., and Dernick, R. (1985) *Electrophoresis* **6,** 103–112
19. Armstrong, G. A., Weisshaar, B., and Hahlbrock, K. (1992) *Plant Cell* **4,** 525–537
20. Hidaka, H., and Kobayashi, R. (1993) in *Protein Phosphorylation: A Practical Approach* (Hardie, D. G., ed.) IRL Press, New York
21. Terzaghi, W. B., Bertekap, R. L., and Cashmore, A. R. (1997) *Plant J.* **11,** 967–982
22. Dröge-Laser, W., Kaiser, A., Lindsay, W. P., Halkier, B. A., Loake, G. J., Doerner, P., Dixon, R. A., and Lamb, C. (1997) *EMBO J.* **16,** 726–738
23. Klimczak, L. J., Schindler, U., and Cashmore, A. R. (1992) *Plant Cell* **4,** 87–98
24. Klimczak, L. J., Collinge, M. A., Farini, D., Giuliano, G., Walker, J. C., and Cashmore, A. R. (1995) *Plant Cell* **7,** 105–115
25. Lin, C. (2000) *Trends Plant Sci.* **5,** 337–342
26. Oyama, T., Shimura, Y., and Okada, K. (1997) *Genes Dev.* **11,** 2983–2995
27. Chattopadhyay, S., Ang, L.-H., Puente, P., Deng, X.-W., and Wei, N. (1998) *Plant Cell* **10,** 673–684
28. Ni, M., Tepperman, J. M., and Quail, P. H. (1998) *Cell* **95,** 657–667
29. Marinez-Garcia, J. F., Huq, E., and Quail, P. H. (2000) *Science* **288,** 859–863
30. Sprenger-Haussels, M., and Weisshaar, B. (2000) *Plant J.* **22,** 1–8