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Light-Regulated Modification and Nuclear Translocation of Cytosolic G-Box Binding Factors in Parsley

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Functional cell-free systems may be excellent tools with which to investigate light-dependent signal transduction mechanisms in plants. By evacuolation of parsley protoplasts and subsequent silicon oil gradient centrifugation of lysed evacuolated protoplasts, we obtained a highly pure and concentrated plasma membrane-containing cytosol. Using GT- and G-box DNA elements, we were able to demonstrate a specific localization of a pool of G-box binding activity and factors (GBFs) but not one of GT-box binding activity in this cytosolic fraction. The DNA binding activity of the cytosolic GBFs is modulated in vivo as well as in vitro by light and phosphorylation/dephosphorylation activities. The regulation of cytosolic G-box binding activity by irradiation with continuous white light and phosphorylation correlates with a light-modulated transport of GBFs to the nucleus. This was shown by a GBF-antibody cotranslocation assay in permeabilized, cell-free evacuolated parsley protoplasts. We propose that a light-regulated subcellular displacement of cytosolic GBFs to the nucleus may be an important step in the signal transduction pathway coupling photoreception to light-dependent gene expression.

INTRODUCTION

The developmental changes occurring during photomorphogenesis in plants are mediated by several photoreceptor systems that respond to particular wavelengths and intensities of light; these include phytochromes, the blue/ultraviolet (UV)-A photoreceptors, and the UV-B photoreceptor (reviewed in Kendrick and Kronenberg, 1993; Ahmad and Cashmore, 1993). Phytochrome is a red/far-red-light–photoreversible chromoprotein that is synthesized in its physiologically inactive form (Pr) and converted to its active form (Pfr) by red light irradiation (Furuya, 1993). In contrast to the phytochrome system, less is known about the molecular properties of the blue/UV-A photoreceptors and the UV-B photoreceptor (Warpeha et al., 1991; Ensminger and Schäfer, 1992; Short et al., 1992; Ahmad and Cashmore, 1993).

In many photomorphogenic responses, transcriptional regulation has been demonstrated; thus, the expression of some genes is induced by irradiation (e.g., *rbcS* genes encoding the small subunit of ribulose bisphosphate carboxylase/oxygenase, *cab* genes encoding the chlorophyll *a/b* binding light-harvesting proteins, and *chs* genes encoding chalcone synthase). Other genes, however, become repressed when plants are exposed to light (these include *phyA* genes encoding phytochrome A and *pcr* genes encoding protochlorophyllide oxidoreductase) (for reviews see Tobin and Silverthorne, 1985; Quail, 1991; Thompson and White, 1991).

The study of these genes has elucidated a number of different *cis*-acting elements in their promoters that have been analyzed in great detail (reviewed in Gilmartin et al., 1990; Katagiri and Chua, 1992). In some instances, it has been shown that these elements are involved in the light responsiveness of the genes and can be occupied by protein factors. In the case of the *cabE* gene, many different factors, including the G-box binding factor (GBF), GA-1, GC-1, AT-1, and GT-1, have been shown to bind to different promoter elements, probably mediating light responsiveness through protein–protein interactions (Schindler et al., 1990, 1992a, 1992c; Dehesh et al., 1992; Gilmartin et al., 1992; Perisic and Lam, 1992). A similar picture is found in the light-regulated element of the *chs* promoter (termed unit 1) of parsley and mustard. Unit 1 is composed of two different nucleotide sequences, designated box I and box II, which mediate the functional integrity of the element and are necessary and sufficient for induction of the *chs* gene in response to continuous light of different quality (Block et al., 1990; Weisshaar et al., 1991; Frohnmeyer et al., 1992; Rocholl et al., 1994; A. Batschauer, personal communication). Box I and box II are occupied in vivo by at least two different protein factors in response to light (Schulze-Lefert et al., 1989a, 1989b; Merkle et al., 1994). Box II of unit 1 is known as the G-box and

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includes the core sequence ACGT; it is bound in vitro by GBFs from Arabidopsis and common plant regulatory factors (CPRFs) from parsley, respectively (Weisshaar et al., 1991; Schindler et al., 1992a). The isolation of genes encoding GBFs and CPRFs has demonstrated that these proteins belong to the basic leucine zipper (bZIP) class of DNA binding (transcription) factors and can form homodimers and heterodimers in vitro (Armstrong et al., 1992; Schindler et al., 1992b). The GBFs are expressed differentially in different plant tissues (Schindler et al., 1992a), and it has been reported that the expression of CPRF-1 and GBF-3 is regulated by light (Weisshaar et al., 1991; Schindler et al., 1992a).

In contrast to these recent advances, there has been little progress in the understanding of the molecular transduction events coupling the photoperception of the different photoreceptors to changes in gene expression via such DNA binding factors. Because both phytochrome and the blue/UV-A photoreceptor systems are located outside of the nucleus, cytosol-to-nucleus transduction pathways have to be postulated. As shown very recently, however, G proteins are probably involved in primary light-induced transduction events, whereas Ca^{2+}, calmodulin, and cGMP may be involved in the transfer of the primary signals to the transcription factors and *cis*-acting elements of light-regulated genes as second messengers (Lam et al., 1989; Foucroy et al., 1990; Neuhaus et al., 1993; Romero and Lam, 1993). In addition, as shown by in vitro and in vivo short-time irradiation experiments, fast changes in protein phosphorylation often precede any effects on transcriptional activity of light-regulated genes (reviewed in Ranjeva and Boudet, 1987 and in Singh and Song, 1990; Reymond et al., 1992; Short et al., 1992; Fallon et al., 1993). An in vitro modification of DNA binding activity of different (putative) plant transcription factors by phosphorylation/dephosphorylation events has been reported (Datta and Cashmore, 1989; Harrison et al., 1991; Klimczak et al., 1992; Sarokin and Chua, 1992; Sun et al., 1993). However, it has not been possible to show a causal link between the regulation of various factor activities by phosphorylation, the activation of G proteins and cGMP-dependent, Ca^{2+}-dependent, and calmodulin-dependent events, and the changes in cytosolic or nuclear phosphorylation activities.

In combination with other experimental approaches (see Chory, 1993; Neuhaus et al., 1993; Romero and Lam, 1993), plant cell-free systems developed from homogenous and stable cell suspension cultures may offer opportunities for investigating light-dependent signal transduction because one does not need to deal with the complex organization of a whole plant. The parsley suspension culture and the protoplasts and evacuolated protoplasts derived from this culture have been intensively analyzed for the light regulation of different genes, including those encoding CHS and phytochrome. Detailed studies have shown that all known photoreceptors are physiologically active in this system (Ohl et al., 1989; Frohnmeyer et al., 1992; Merkle et al., 1994; Rocholl et al., 1994; C. Poppe, B. Ehmann, H. Frohnmeyer, and E. Schäfer, manuscript submitted). We have recently established a silicon oil gradient centrifugation technique to isolate a pure and highly concentrated plasma membrane-containing cytosolic fraction from parsley protoplasts that have first been evacuolated by Percoll gradient centrifugation (Frohnmeyer et al., 1994). In this cytosolic fraction, fast and light quality–dependent changes in protein phosphorylation pattern have been observed in vitro, indicating that the photoreceptors and other requisites of early light-induced signal transduction events still exist and are functional in the cytosol (Harter et al., 1994).

To further examine the molecular mechanisms underlying the light-dependent signal transduction in parsley cells, we asked whether these events were directly coupled to putative transcription factors localized in the cytosol of evacuolated protoplasts. By using GT-box and G-box elements, we showed a specific localization of a GBF pool outside of the nucleus whose DNA binding activity could be modulated by light and by cytosolic phosphorylation/dephosphorylation activities. Furthermore, the modulation of cytosolic G-box binding activity by phosphorylation could be correlated with a light-modulated GBF translocation from the cytosol to the nucleus; this process was observable in a plant cell-free system. We have proposed a working hypothesis of light-induced signal transduction in which light-modulated nuclear transport of specific transcription factors could play a role.

RESULTS

Intracellular Distribution of G-Box and GT-Box Binding Activity

In animal and fungus systems, different molecular mechanisms coupling receptor activities to altered gene expression have been described. In addition to phosphorylation cascades extending into the nucleus and the transport of an activated protein kinase to the nuclear compartment, inducible translocation of retained transcription factors from the cytosol into the nucleus has been demonstrated previously (reviewed in Jackson, 1992; Morimoto et al., 1992; Liou and Baltimore, 1993; Parker, 1993). Because none of these mechanisms could be excluded for light-dependent signal transduction, we started our investigation by testing the cytosolic fractions for DNA binding activity by gel mobility shift assay. As DNA probes, we used two different *cis*-acting elements known to play important roles in modulating gene expression in response to light: a G-box and a GT-box sequence (Schulze-Lefert et al., 1989a, 1989b; Gilmartin et al., 1992; Perisic and Lam, 1992; Merkle et al., 1994).

Using cytosolic and nuclear preparations from dark-grown evacuolated parsley protoplasts, we obtained a G-box and GT-box binding pattern as shown in Figure 1A. Whereas almost all GT-box binding activity was found in the nucleus, G-box binding activity could be observed in both the cytosolic and the nuclear compartment. In addition, the nuclear and cytosolic G-box binding patterns were clearly different. Three

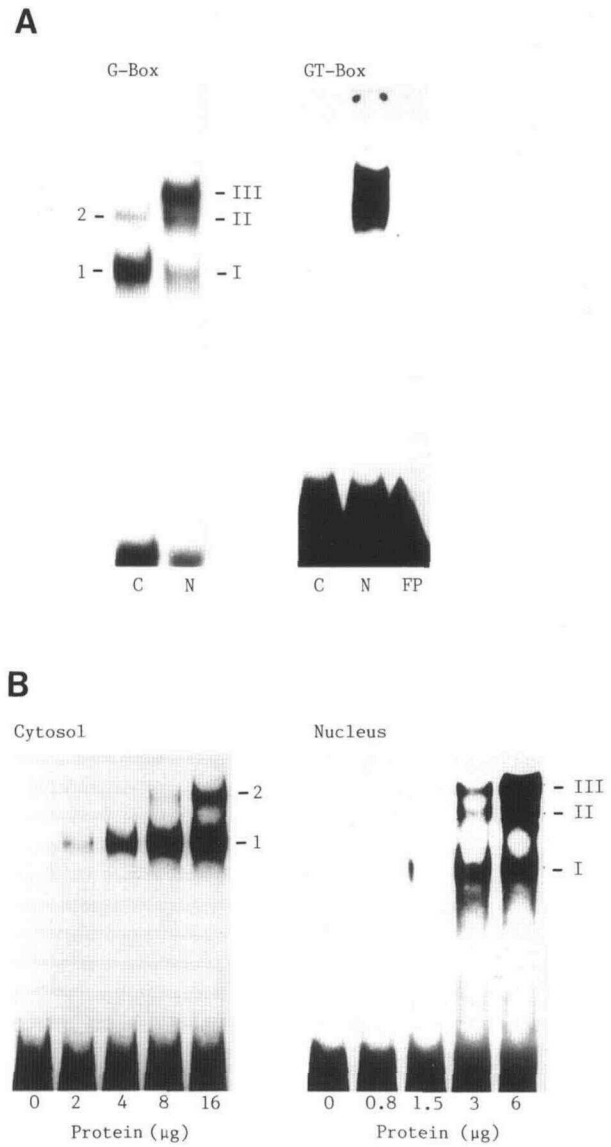

Figure 1. Subcellular Distribution of G-Box and GT-Box Binding Activities within Dark-Grown Evacuolated Parsley Protoplasts.

(A) Eight micrograms of cytosolic (C) and 4 µg of corresponding nuclear (N) proteins were tested for DNA binding activity using a tetrameric G-box (5′-ACCACGTGGC-3′)$_4$ and a tetrameric GT-box (5′-TGTGTGGTTAATATG-3′)$_4$ *EcoRI restriction fragment as gel shift probes.* A GT-box–specific DNA–protein complex can be found only in the nuclear compartment, whereas the G-box probe produces two cytosolic (C, complexes 1 and 2) and three nuclear (N, complexes I to III) DNA–protein complexes. FP, free probe.

(B) Different amounts of protein (in micrograms) of cytosolic (Cytosol) and nuclear (Nucleus) extracts from dark-grown evacuolated protoplasts were tested for G-box binding activities using the tetrameric G-box DNA fragment, demonstrating the difference in the DNA binding pattern between cytosol and nucleus. The DNA–protein complexes are indicated as in **(A)**.

The signal at the bottom of the gels represents unbound probe.

DNA–protein complexes (complexes I to III) were detected in nuclear extracts, and two were detected in the cytosolic fraction. In the cytosolic fraction, one faster migrating complex (complex 1) was dominant, with a second weaker one showing a lower electrophoretic mobility (complex 2). Although the tetrameric structure of the G-box element may in part affect the binding pattern, we never observed a third band in the cytosol isolated from dark-grown evacuolated protoplasts, even when the protein concentration was increased (Figure 1B). Whether the nuclear and cytosolic DNA–protein complexes contain the identical GBFs has yet to be elucidated. There were also differences in the specific G-box binding activities (activity per protein) of nuclear and cytosolic extracts. To obtain approximately equal amounts of retarded DNA–protein complexes, almost twice as much cytosolic as nuclear extract had to be used in the gel shift assay, indicating that there were either different concentrations of GBFs or different binding activities of the GBFs in the two fractions (Figure 1B).

Because the number of evacuolated protoplasts used per experiment was known and because we succeeded in quantifying the retarded DNA–protein complexes by laser scanning, it was possible to convert the specific G-box binding activity into the activity-per-cell unit. The result of this conversion showed that almost 50% of the detectable G-box binding activity of dark-grown evacuolated parsley protoplasts is localized in the cytosol, whereas the GT-box binding activity seems to be almost completely localized in the nucleus (Figure 2).

Using an unlabeled mutated G-box, which exhibited no DNA binding activity in vitro, together with the unlabeled G-box in

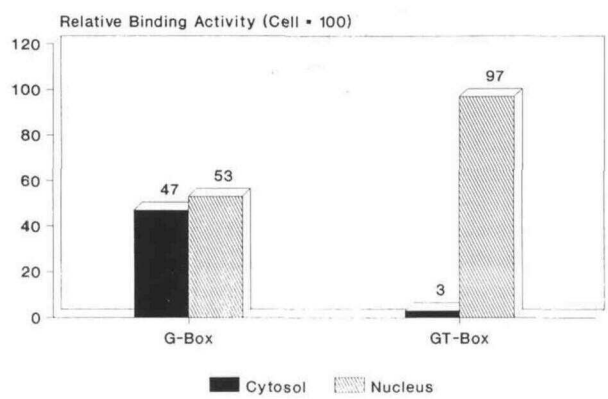

Figure 2. Quantitative Distribution of Cytosolic and Nuclear G-Box and GT-Box Binding Activities within the Cell of Dark-Grown Evacuolated Protoplasts.

The data of Figure 1 were quantified by laser scanning the retarded DNA–protein complexes and were converted into DNA binding activity per cell unit by including the number of evacuolated protoplasts used in the oil gradient centrifugation, the volume, and the protein concentration of the obtained fractions. The sum of the cytosolic and nuclear DNA binding activities was set at 100. The values of three independent experiments were quantified, and one representative result is shown.

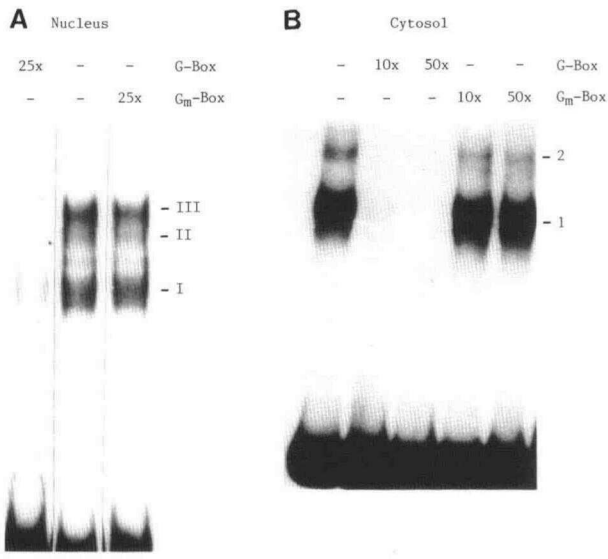

Figure 3. Sequence-Specific Competitions.

A 25- and 10-fold, respectively, molar excess of the tetrameric G-box probe (G-Box) effectively competes for the binding of nuclear and cytosolic proteins, whereas a tetrameric mutated G-box fragment (G_m-Box; sequence of [5′-ACCACTGTTC-3′]$_4$) shows no effect.
(A) Four micrograms of nuclear protein per lane were used.
(B) Ten micrograms of cytosolic protein per lane were used.
The nuclear and cytosolic DNA–protein complexes are as indicated in Figure 1. The signal at the bottom of the gels represents unbound probe.

competition assays, we showed that the cytosolic as well as the nuclear DNA binding activities are highly G-box specific (see Figures 3A and 3B). The specificity of the nuclear GT-box binding activity was also demonstrated by competition experiments (data not shown).

These results demonstrate the existence of a pool of GBFs outside of the nucleus in the cytosolic compartment of dark-grown parsley cells. Four questions are raised by these data. Do the cytosolic (and nuclear) GBFs belong to the bZIP protein family known to bind G-box elements (for example, GBFs of Arabidopsis and CPRFs of parsley)? Can light modulate the activity of cytosolic and nuclear GBFs? If so, which molecular mechanism underlies a putative light modulation? How do the cytosolic GBFs come into contact with their target promoters localized in the nucleus; in other words, is there a light-modulated translocation of GBFs from the cytosol into the nucleus?

Cytosolic and Nuclear GBFs Are Probably Members of the bZIP DNA Binding Protein Family

To address whether the cytosolic and nuclear GBFs are members of the bZIP protein family, we investigated the effects of a polyclonal antiserum raised against the bZIP protein GBF-1 from Arabidopsis (Schindler et al., 1992a, 1992b; A.R. Cashmore, personal communication) in gel shift assays. Changes in the pattern and/or the stability of retarded DNA–protein complexes should indicate that the cytosolic and nuclear GBFs belong to the bZIP protein family. As shown in Figure 4, the antiserum was capable of producing specific changes in the cytosolic and nuclear G-box binding pattern, whereas the preimmune serum had no effect. The disappearance of the DNA–protein complexes indicated that the antiserum recognized the leucine zipper and/or the basic domain of the GBFs and, therefore, inhibited their binding to the DNA (Sablowski et al., 1994). However, using the cytosolic extract, a part of the DNA–protein complex 1 still had binding activity (Figure 4A). The DNA–complex III of the nuclear extract was also very resistant to the inhibiting effect of GBF-1 antiserum (Figure 4B). These results indicated that either different classes of G-box binding factors or different modification states or members of the bZIP proteins, which are poorly recognized by the GBF-1 antiserum, are localized in the cytosolic and nuclear extracts. Taken together, these data show that the antiserum raised against GBF-1 from Arabidopsis cross-reacts with the cytosolic as well as with the nuclear GBFs, indicating that these proteins probably belong to the bZIP protein family.

Due to the cross-reaction in the gel shift experiments, we used the antiserum to examine the size of GBFs and their intracellular distribution within dark-grown evacuolated parsley protoplasts by protein gel blotting. The results obtained with the GBF-1 antiserum and preimmune serum are shown in Figures 5A and 5B. Whereas only small amounts of cross-reacting GBFs are detectable in the nuclear fraction (Figure 5B, lane 2), the larger part of the polypeptides is localized in the cytosol (Figure 5A, lane 1). The apparent molecular mass of the detected polypeptides (about 25 to 50 kD) corresponds well to the monomeric sizes of the previously characterized CPRFs and GBFs (Weisshaar et al., 1991; Schindler et al., 1992a, 1992b). The preimmune serum had no effect (Figure 5B).

Cytosolic G-Box Binding Activity Is Modulated in Vivo and in Vitro by Light

To examine the influence of light on cytosolic and nuclear G-box binding activities, we irradiated dark-grown evacuolated protoplasts with white light for varying times or kept them in darkness. After the end of the irradiation period, cytosolic and nuclear fractions were prepared, and their G-box binding activities were tested in gel mobility shift assays. Although no light-induced changes could be observed in the nuclear extracts (data not shown), irradiation of the cells clearly caused effects in the cytosolic G-box binding pattern (Figure 6A). After 1 min of white light irradiation, a general increase of G-box binding activity was visible in comparison to the dark control (Figure 6A). The increase in irradiation time to 10 and 20 min resulted in the appearance of a third DNA–protein complex (complex 3). This complex was never observed when cytosolic preparations from nonirradiated, evacuolated protoplasts were used (Figure 6A;

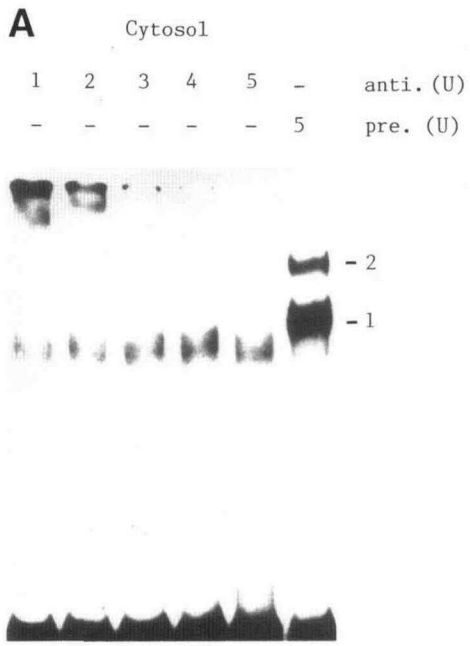

Figure 4. Use of an Antiserum Raised Against GBF-1 from Arabidopsis to Characterize the Cytosolic and Nuclear G-Box Binding Proteins of Parsley.

Different units (U) of antiserum (anti.) and preimmune serum (pre.) were preincubated with the tetrameric G-box fragment for 5 min at room temperature. Cytosolic and nuclear fractions isolated from dark-grown evacuolated protoplasts were added, and the probes were further incubated for an additional 10 min before running the gel mobility shift assay.

(A) Ten micrograms of cytosolic protein per lane were used.

(B) Four micrograms of nuclear protein per lane were used.

One unit corresponds to 0.12 μL of serum. The cytosolic and nuclear DNA–protein complexes are as indicated in Figure 1. The signal at the bottom of the gels represents the unbound probe.

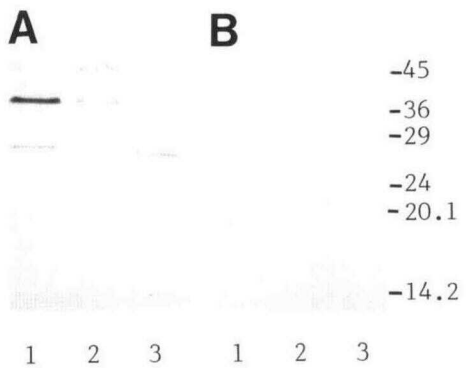

Figure 5. Protein Gel Blot Analysis Showing the Distribution of GBFs within the Cell.

Twenty-five micrograms of cytosolic protein (lanes 1), nuclear protein (lanes 2), and whole-cell extract (lanes 3) isolated from dark-grown evacuolated protoplasts were separated by SDS-PAGE, blotted, and probed with either the GBF-1 antiserum or the preimmune serum.

(A) Antiserum used previously in gel mobility shift assays (see Figure 4).

(B) Preimmune serum.

Positions of molecular mass standards are indicated at right in kilodaltons.

10 and 20 min, compare white light exposure [WL] to dark control [D]). These results demonstrate in vivo modulation of cytosolic G-box binding activity by light.

To investigate how the alterations of the cytosolic G-box binding activity may be mechanistically achieved, cytosolic fractions isolated from dark-grown evacuolated protoplasts were supplemented with an ATP-generating system and the phosphatase inhibitor sodium metavanadate; they were either irradiated with white light for 20 min or kept in darkness. As shown in Figure 6B, no changes in the cytosolic G-box binding pattern could be observed in the absence of either light or ATP, even when the gel of the ATP-containing dark control was exposed to x-ray film for a prolonged time (Figure 6B). However, light exposure together with the ATP supplementation resulted in the appearance of a third cytosolic DNA–protein complex in vitro (Figure 6B), generating the same binding pattern that is visible after in vivo irradiation of evacuolated protoplasts (compare to Figure 6A). These data suggest that the cytosolic fraction contains

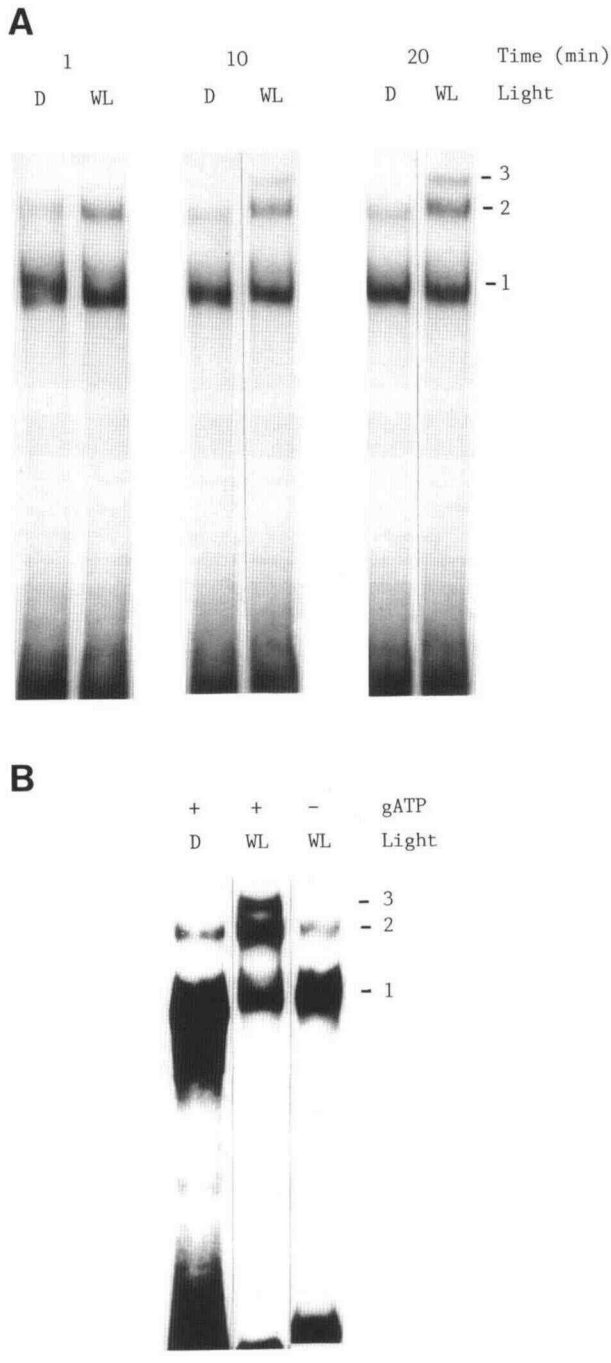

Figure 6. Light-Induced Changes in Cytosolic G-Box Binding Pattern during in Vivo Irradiation of Dark-Grown Evacuolated Protoplasts and in Vitro Irradiation of Cytosol Isolated from Dark-Grown Cells.

(A) Dark-grown evacuolated protoplasts were either irradiated for 1, 10, or 20 min with continuous white light (WL) or kept in darkness (D). Cytosolic fractions were isolated and analyzed for binding activity using the tetrameric G-box probe and 10 μg of protein.

(B) Cytosol was isolated from dark-grown evacuolated protoplasts supplemented with 10 mM sodium metavanadate as the phosphatase inhibitor and with an ATP-generating system (gATP; [+], presence; [−], absence). The probes were irradiated for 15 min with continuous white light (WL) or kept in darkness (D). Binding activity was tested using the tetrameric G-box probe and 10 μg of protein. The exposure time of the gels to the x-ray film was 24 hr (D) and 12 hr (WL), respectively. The DNA–protein complexes (1 to 3) are indicated at the right, demonstrating the in vivo and in vitro appearance of a third complex (3) in response to light.

The signal at the bottom of the gels represents unbound probe.

the functional signal transduction components connecting the photoreceptor(s) with the cytosolic GBFs. To further investigate the possible role of phosphorylation in the regulation of DNA binding activity of GBFs, we chose two different approaches.

DNA Binding Activity of GBFs Is Regulated by Cytosolic Phosphorylation/Dephosphorylation Activities

First, cytosolic and nuclear fractions isolated from dark-grown evacuolated protoplasts were incubated with immobilized alkaline phosphatase and then tested for G-box binding activity. In this assay, we obtained an almost complete loss of cytosolic and nuclear G-box binding activity (Figures 7A and 7B, lanes 2). In the probes, incubated under the same conditions but without alkaline phosphatase treatment, the G-box binding pattern remained unchanged (Figures 7A and 7B, lanes 1). Because we could use only 8 μg of cytosolic protein in these experiments, the DNA–protein complex 2 was very weak in Figure 7A. After removing the immobilized alkaline phosphatase by multiple centrifugations, we tested whether there was a kinase activity in the cytosol and/or nucleus that could restore the G-box binding activity in vitro. We added ATP (20 mM concentration) to the dephosphorylated fractions and incubated the probes for 30 min at 25°C. As shown in Figures 7A and 7B, lanes 3, only cytosolic phosphorylation activity that could partially restore the G-box binding activity was detected, indicating that the modification mechanism of GBF activity detectable with our assay system was localized outside of the nucleus.

In a second approach in our investigation of a putative phosphorylation mechanism, we tested the effects of different kinase and phosphatase inhibitors on the G-box binding pattern. For this purpose, dark-grown evacuolated protoplasts were first permeabilized in lysis solution containing the inhibitors described below. The lysate was then incubated for 5 min at 4°C under a dim-green safelight.

As shown in Figure 8A, the kinase inhibitors calmidazolium in a concentration of 10 μM, *N*-(2-[*p*-bromocinnamylamino]-ethyl)-5-isoquinolinesulfonamide in a concentration of 50 μM, and the phosphatase inhibitor okadaic acid in a concentration of 1 nM caused weak inhibitory effects in cytosolic G-box

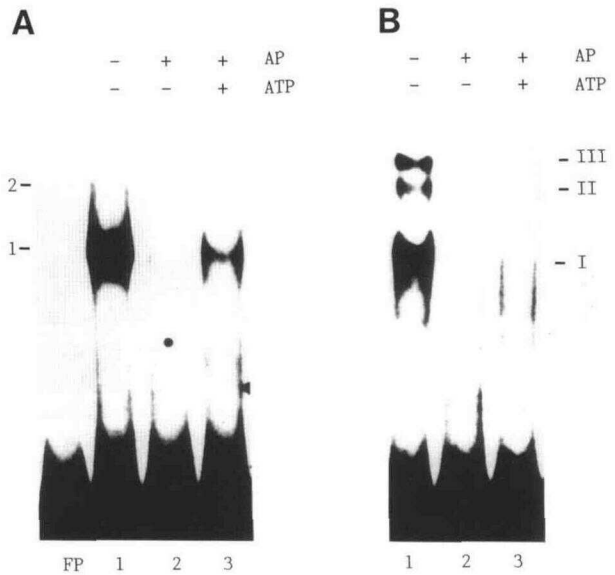

Figure 7. In Vitro Regulation of Cytosolic and Nuclear G-Box Binding Activity by Cytosolic Kinase Activity.

(A) Cytosolic fraction.
(B) Nuclear fraction.
Fractions isolated from dark-grown evacuolated protoplasts were incubated either with immobilized alkaline phosphatase (lanes 2) or without (lanes 1). After dephosphorylation, the immobilized phosphatase was removed, and the fractions were supplemented with 20 mM ATP (ATP) and incubated for 30 min (lanes 3). G-box binding activities were tested by the gel mobility shift assay using 8 μg of cytosolic protein and 4 μg of nuclear protein. DNA–protein complexes are as indicated in Figure 1. AP, immobilized alkaline phosphatase; FP, free probe; (+), presence; (–), absence. The signal at the bottom of the gels represents unbound probe.

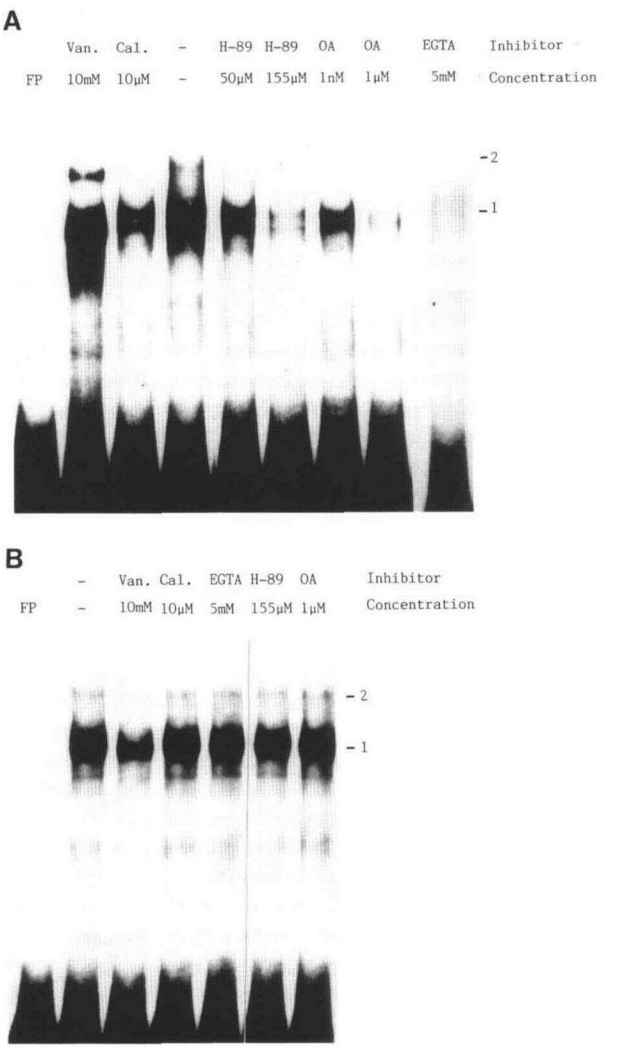

Figure 8. In Vitro Inhibition of Cytosolic G-Box Binding Activity by Kinase and Phosphatase Inhibitors.

(A) Dark-grown evacuolated protoplasts were permeabilized with lysis solution containing sodium metavanadate (Van.), calmidazolium (Cal.), *N*-(2-[*p*-bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide (H-89), okadaic acid (OA), or EGTA at the concentrations indicated. After incubation for 5 min at 4°C under a dim-green safelight, the cytosolic fractions were isolated. The G-box binding activities were tested by gel mobility shift assay using the tetrameric G-box probe and 8 μg of protein per lane.
(B) The binding activities were tested as given in **(A)**, but the inhibitors were added to the G-box probe 5 min before incubation with cytosol. The cytosolic fraction was isolated from dark-grown evacuolated protoplasts. Concentrations of the inhibitors are indicated.
DNA–protein complexes are as indicated in Figure 1. The signal at the bottom of the gels represents unbound probe. FP, free probe. (–), no inhibitor was given.

binding activity in comparison to the control extract. At higher concentrations, however, *N*-(2-[*p*-bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide (155 μM) and okadaic acid (1 μM) reduced the cytosolic G-box binding activity dramatically. A similar result was obtained with EGTA at a concentration of 5 mM. In contrast, the phosphatase inhibitor sodium metavanadate enhanced the DNA binding activity of cytosolic GBFs. *N*-(2-[*p*-Bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide at a concentration of 0.65 μM, the isoquinolinesulfonamide kinase inhibitor C-I at a concentration of 100 μM, and the kinase inhibitor K-252a at a concentration of 1 μM had no effect (data not shown). Because the cytosolic G-box binding pattern was not affected when the inhibitors were added to the binding assay before the protein extract was supplied, unspecific interactions between the inhibitors, DNA, and the GBFs can be excluded (Figure 8B). For the same reason mentioned above, the DNA–protein complex 2 is difficult to detect in Figures 8A and 8B.

The results of these experiments suggest that the modification mechanism that regulates the G-box binding activity may

be localized in the cytosolic compartment of the cell and that this mechanism consists of a complex network of different cytosolic kinases and phosphatases (for further details, see Discussion).

Cytosolic GBFs Are Translocated to the Nucleus in a Light-Regulated Manner

To examine whether the light-dependent modulation of cytosolic GBFs correlates with a light-regulated translocation of cytosolic GBFs to the nucleus, we developed a plant cell-free nuclear transport system. This system was based on dark-grown evacuolated protoplasts that were permeabilized with Triton X-100 at very low concentration (0.02%). At this concentration, the nuclear envelope and the envelopes of the organelles remain intact, whereas the plasmalemma becomes permeable (Frohnmeyer et al., 1994; Harter et al., 1994). To test for specific nuclear import of cytosolic GBFs, the permeabilized cells were supplemented with an ATP-generating system and a GBF-1 antiserum, preimmune serum, or phosphoenolpyruvate (PEP) carboxylase serum, which recognized a cytoplasmic marker protein (Budde and Chollet, 1986). Subsequently, the lysate was irradiated for 40 min with continuous white light or kept in darkness for the same period. Nuclei were then isolated, incubated with proteinase K, washed, and extracted in boiling SDS-sample buffer. The nuclear extracts were analyzed by protein gel blotting using anti–rabbit IgG-specific alkaline phosphatase–conjugated antibodies as the primary and secondary antibodies to detect the amount of the heavy chains of antibodies transported into the nucleus. Because the antiserum raised against GBF-1 recognized the cytosolic GBFs in their native form (see Figure 4A), the appearance of heavy chains of the antibody in the nuclear fraction is to be expected in the case of an import of cytosolic GBFs (GBF antibody cotranslocation assay; Tsuneoka et al., 1986; Meier and Blobel, 1992).

As shown in Figure 9, columns 1 and 3, no staining of heavy chains of the antibodies was detectable when GBF-1 preimmune serum or PEP carboxylase antiserum was tested in the transport assay, indicating that antibodies per se do not cross the nuclear envelope. In contrast, if the GBF-1 antiserum was used, antibodies appeared in the nuclear compartment (Figure 9, column 2). Furthermore, the amount of cotranslocated antibodies was modulated by the irradiation of the permeabilized cells (Figure 9, column 2). In nuclei isolated from dark-control lysate, a very small amount of cotranslocated antibodies was detectable, whereas an irradiation of the lysate with white light resulted in a clear enhancement of imported antibody. These observations suggested that cytosolic GBFs that belong to the bZIP protein family are imported to the nucleus in vitro, cotranslocating the specific antibodies. Furthermore, the accumulation of GBF antibody complexes in the nucleus was increased by light.

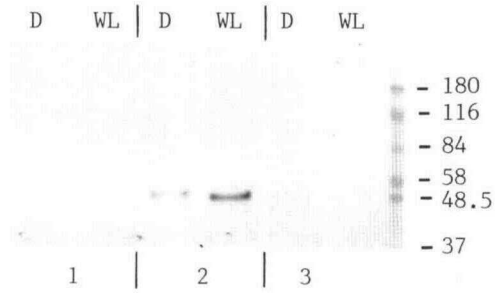

Figure 9. Light-Regulated in Vitro Cotranslocation of Cytosolic GBF–Antibody Complexes to the Nucleus.

Dark-grown evacuolated protoplasts were permeabilized in lysis solution. The permeabilized cells were supplemented with an ATP-generating system. GBF-1 preimmune serum (column 1), GBF-1 antiserum (column 2), or PEP carboxylase antiserum (column 3) was then added (amount of serum was 1 μL per 3 × 10^6 cells each). During incubation (40 min at 25°C), probes were either irradiated with continuous white light (WL) or kept in darkness (D). After irradiation, the nuclei were isolated, incubated with proteinase K (1 mg/mL) for 15 min at room temperature to remove extranuclear proteinaceous contaminations, washed, and extracted in boiling SDS-sample buffer. After separation of the proteins (20 μg of protein per lane) by SDS-PAGE, the amount of nuclear heavy chains of the different antibodies was analyzed by protein gel blotting using alkaline phosphatase–conjugated rabbit IgG-specific antibodies. The positions of molecular mass standards are indicated at right in kilodaltons.

Biochemical Characterization of the GBF-Antibody Cotransport

Although the control sera had no effect in the cotranslocation assays, we conducted additional experiments to exclude any nonspecific effects. As indicated above, the imported antibodies were resistant to digestion with proteinase K (Figure 9). However, after removal of the nuclear envelope by SDS treatment of isolated nuclei followed by incubation with proteinase K, staining of the heavy chains could no longer be observed (Figure 10A). This result indicated that the detectable GBF–antibody complexes were in fact protected from proteolytic degradation by the nuclear envelope.

Previous studies using animal systems have shown that nuclear protein import (translocation step) is inhibited by the lectin wheat germ agglutinin and is sensitive to lowered temperature and to apyrase treatment that depletes the assay system of ATP produced by the added ATP-generating system (for review, see Newmeyer, 1993). Therefore, we used these substances and incubation conditions in the plant cell-free transport system (see Figure 10B). Both wheat germ agglutinin and apyrase treatments of permeabilized cells during the irradiation period markedly reduced the amount of translocated GBF–antibody complexes as did lowering of the incubation temperature to 4°C. These results support the hypothesis that specific translocation of GBFs from the cytosol to nuclear

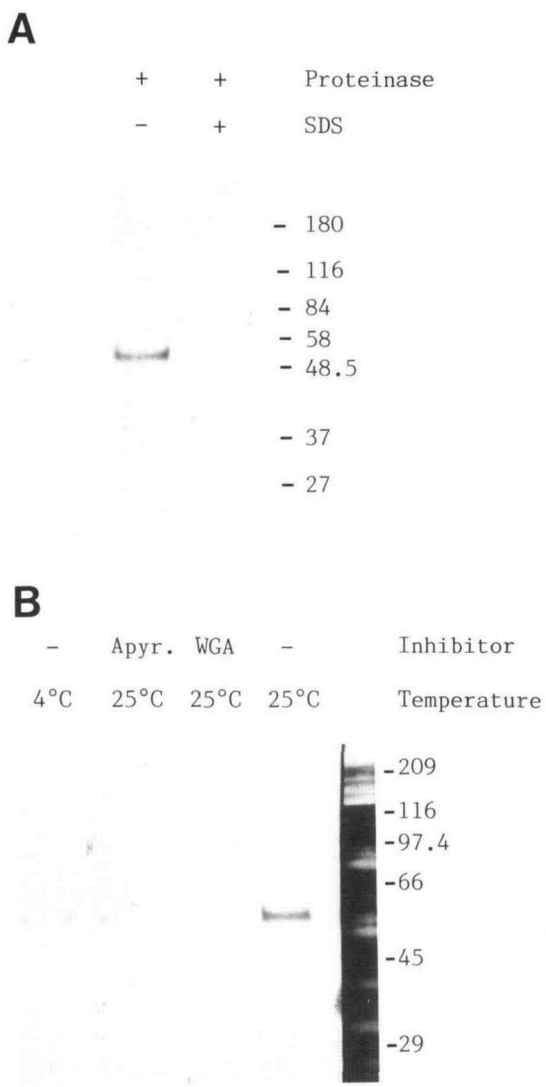

Figure 10. Biochemical Characterization of the Cytosolic GBF–Antibody Cotranslocation.

(A) Nuclei isolated after the end of the cotranslocation procedure performed under continuous white light (see Figure 9) were incubated for 15 min at room temperature either with proteinase K (Proteinase; 1 mg/mL) alone or with proteinase K (1 mg/mL) and 2.0% SDS (SDS) to destroy the nuclear envelope. The amount of nuclear heavy chains of GBF-1 antibody was analyzed as described in the legend to Figure 9.
(B) Dark-grown evacuolated protoplasts were permeabilized in lysis solution, and the lysate was supplemented with an ATP-generating system. The GBF-1 antiserum was added (amount of serum was 1 μL per 3 × 10^6 cells). In addition, either apyrase (Apyr.; 2 μg per 3 × 10^6 cells) or wheat germ agglutinin (WGA; 4 μg per 3 × 10^6 cells) was added. During incubation at 25 or 4°C, the probes were irradiated with white light. Nuclei were isolated and extracted, and the amount of nuclear GBF-1 antibody was analyzed as described in the legend to Figure 9. The positions of molecular mass standards are indicated at right in kilodaltons.
(+), presence; (−), absence.

compartment occurs in the lysate of evacuolated parsley protoplasts.

DISCUSSION

GBFs Exist in the Cytosol of Parsley Cells, Belong to the bZIP Protein Family, and Are Regulated by Light in Their Activity

Our results showed that, apart from the nuclear GBFs, there was a specific G-box binding activity in the cytosolic fraction of dark-grown parsley cells. In contrast, the GT-box binding activity was localized almost exclusively in the nucleus. Together with these results and those obtained by the protein gel blot analysis with the antiserum raised against GBF-1, these data demonstrated the localization of a GBF pool in the cytosolic compartment. The strong effects of the GBF-1 antiserum in the gel mobility shift assay showed that the nuclear as well as the cytosolic GBFs in all probability belong to the bZIP protein family (Schindler et al., 1992a, 1992b, 1992c), although we could not completely exclude that at least one other class of G-box binding factors was involved. This class could include either bZIP proteins not recognized by the GBF-1 antiserum or plant ACGT binding proteins related to the gene products of *myc* protooncogenes (discussed in Rocholl et al., 1994).

From the distinctive gel mobility shift pattern and the different resistance of the DNA–protein complexes to the GBF-1 antiserum, we concluded that either the cytosolic GBFs are not identical to the nuclear proteins or the GBFs of the two compartments are present in different modification states representing DNA–protein complexes with different electrophoretic mobility. It should be noted that the specific DNA binding activity (activity per protein) was much higher in the nuclear compartment than in the cytosol, even though the greater amount of the GBFs, as detected with the antiserum raised against GBF-1, was localized in the cytosol. Therefore, it seems highly likely that the nuclear GBFs are present in a state with a high affinity to the G-box element, whereas the cytosolic counterparts have a much lower affinity. These observations strongly suggested that the cytosolic GBFs may be the target of a biochemical mechanism localized in the cytosol and possibly regulating the cytosolic G-box binding activity in response to an exogenous or endogenous stimulus. Although strong G-box binding activity was found in the cytosol of dark-grown evacuolated protoplasts, further experiments demonstrated an in vivo as well as an in vitro modulation of the G-box binding pattern by light in the cytosolic but not in the nuclear compartment. These modulations were characterized by a relative enhancement of the DNA binding activity of the cytosolic DNA–protein complex 2 (in vitro diminishing of the activity of complex 1; see Figure 6) and by the induction of a third DNA–protein complex (complex 3) that was not present in the cytosolic extracts isolated from dark-grown cells. Furthermore, the in vitro light-induced appearance of DNA–protein complex

3 required an ATP-generating system. The comparison of the G-box binding pattern obtained with cytosol isolated under dim-green safelight with the pattern obtained after in vitro irradiation of the identical cytosol (see Figure 6) gave the impression that the appearance of DNA–protein complex 3 resulted from a modification process that transferred the cytosolic GBF activity from state 1 (DNA–protein complex 1) over state 2 (complex 2) to a state 3 (complex 3) activity. The requirement for energy equivalents to be present during this change suggested that the process may be a result of phosphorylation events, indicating that the changes in cytosolic G-box binding pattern observed in gel mobility shift assay could reflect different modification states of one cytosolic GBF rather than the appearance and disappearance of different GBF proteins. Although the photoreceptor system responsible for the changes in the cytosolic G-box binding activities remains to be elucidated, our results conclusively demonstrated that a specific pool of putative transcription factors (GBFs) is present outside of the nuclear compartment in the cytosol of plant cells and that the activity of these GBFs can be modulated by light, probably via an ATP-dependent mechanism.

Cytosolic G-Box Binding Activity Is Regulated by Cytosolic Kinase/Phosphatase Systems

During the course of our experiments, we demonstrated that a cytosolic, but not a nuclear kinase, activity could restore the G-box binding activity repressed after in vitro dephosphorylation of nuclear and cytosolic extracts by incubation with immobilized alkaline phosphatase. However, the potential complexity of the network regulation of cytosolic G-box binding activity by phosphorylation became obvious when the lysate of dark-grown cells was treated with different kinase and phosphatase inhibitors at the moment of lysis. Of the specific kinase inhibitors tested, only *N*-(2-[*p*-bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide at a concentration of 155 μM specifically inhibited the appearance of the G-box binding activity, indicating the possible participation of a casein kinase II–like activity in the regulation of cytosolic GBF activity (Chijiwa et al., 1990). The strong inhibitory effect of EGTA indicated that at least one Ca^{2+}-dependent step is also involved in the regulation of cytosolic GBFs. The effect of the release of Ca^{2+} from exogenic or endogenic sources during cell lysis may also explain the light-independent occurrence of the G-box binding activity in the cytosol isolated from dark-grown evacuolated protoplasts. Ca^{2+}-dependent mechanisms involved in light-induced regulation of gene expression in vivo were recently described for different plant and cell culture systems by Lam et al. (1989), Foucroy et al. (1990), Neuhaus et al. (1993), and Romero and Lam (1993).

The complex character of regulation of cytosolic G-box binding activity by phosphorylation/dephosphorylation mechanisms is further elucidated by the effects obtained when different phosphatase inhibitors with slightly different specifities are used. Whereas very slight changes of the G-box binding activity obtained with okadaic acid at a concentration of 1 nM excluded phosphatase 2A as the major phosphatase activity regulating the cytosolic GBFs, the involvement of phosphatase 1 is suggested by the strong inhibitory effect of okadaic acid at a concentration of 1 μM (Cohen et al., 1990). In contrast, sodium metavanadate, which has a specifity for animal phosphatase 2B to some extent (Klee et al., 1988), enhanced the DNA binding activity of the cytosolic GBFs. The opposite effects of the two different inhibitors might indicate that different phosphatases intervene in different steps of the phosphorylation/dephosphorylation events regulating the cytosolic G-box binding activity. Recently, Sheen (1993) was able to show that the inhibition of cytosolic phosphatase 1 by 1 μM okadaic acid results in a reduction of the transcriptional activity of light-regulated genes encoding the small subunit of ribulose-1,5-bisphosphate carboxylase and pyruvate orthophosphate dikinase in maize protoplasts. The inhibitor seems to influence the DNA–protein and/or protein–protein interactions at the promoters of these genes, strongly suggesting that phosphatase 1 affects the activity of transcription factors from outside of the nucleus. Although no kinase or phosphatase activity regulating the DNA binding activity of nuclear GBFs could be shown, a putative modulation of transcription activity by modification of the *trans*-activation domain of GBFs cannot be excluded.

Cytosolic GBFs Are Translocated to the Nucleus in a Light-Dependent Manner

The irradiation of permeabilized cells with white light resulted in an enhanced translocation of cytosolic GBFs to the nucleus that belong to the bZIP protein family; translocation from the cytosol to the nucleus in vitro indicated that the light-modulated and phosphorylation-dependent changes observed in the gel mobility shift assays might lead to an enhanced nuclear translocation competence of the cytosolic GBFs. We first developed an in vitro nuclear transport system (cotranslocation assay) analogous to the polypeptide–antibody cotranslocation assay that was used to analyze the nuclear transport of different mammalian and fungus nucleophilic proteins (Tsuneoka et al., 1986; Meier and Blobel, 1992). We then demonstrated that the in vitro translocation of the cytosolic GBFs was not only sensitive to light, but also to lower temperature, apyrase treatment, and incubation with wheat germ agglutinin. These results demonstrated the functional similarity of the plant nuclear transport machinery with the animal and fungal counterparts, as suggested by in vivo experiments (van der Krol and Chua, 1991; Varagona et al., 1992; Shieh et al., 1993). From these observations, we concluded that nuclear transport of a putative transcription factor was likely to be involved in one or more light-dependent signal transduction mechanisms in plant cells. In addition, the translocation observed in permeabilized cells demonstrated that the whole molecular sequence of the transduction pathway connecting the photoreceptors to the putative

transcription factor was intact and still inducible under these in vitro conditions.

As shown in Figure 9, weak translocation activity was also detectable in permeabilized cells kept in darkness. This "dark-translocation" correlated well with the observed G-box binding activity in the cytosol isolated from dark-grown evacuolated protoplasts, presumably induced in vitro by the permeabilization of the cells leading to a Ca^{2+} influx. This activity might reflect a low level of translocation competence, leading to a weak transport of GBF–antibody complexes to the nucleus. The far higher amount of translocated GBF–antibody complexes, detectable after the in vitro irradiation of the lysed cells, paralleled the light-induced appearance of DNA–protein complex 3 (modification state 3). This DNA–protein complex 3 may then reflect a modification state with a very high nuclear translocation competence. If there is a causal link between GBF translocation capacity and cytosolic G-box binding activity, we must assume a two-step mechanism of nuclear transport of cytosolic GBF reflected by different modification states represented in gel mobility shift assay by the cytosolic DNA–protein complexes 1 to 3. Whether light can induce modification state 1 in vivo by activating a Ca^{2+}-dependent kinase/phosphatase system and/or by modulating the activity of a casein kinase II remains to be determined. It is of interest that a similar two-step mechanism for protein transport to the nucleus was demonstrated or suggested for different polypeptides and transcription factors in mammalian and fungal systems (Moll et al., 1991; Rihs et al., 1991). Future research must be directed toward an investigation on how the cytosolic GBFs are retained in the cytosol, and in which way the retention is solved by irradiation via a phosphorylation/dephosphorylation pathway.

The light-regulated element of the parsley *chs* promoter, designated unit 1, is necessary and sufficient to mediate the responsiveness to continuous light of different quality (Dangl et al., 1987; Block et al., 1990; Weisshaar et al., 1991; Frohnmeyer et al., 1992, 1994; Rocholl et al., 1994; A. Batschauer, personal communication). As demonstrated by in vivo footprint experiments, the irradiation of the parsley cells with white light induced the protection of box I and the G-box (box II) of unit 1, reflecting inducible binding of putative transcription factors (GBF and box I binding protein) in response to light (Schulze-Lefert et al., 1989a, 1989b; Merkle et al., 1994; Sablowski et al., 1994). Some light-inducible binding of a GBF to box II was observable, although strong G-box binding activities are also found in the nuclear compartment of dark-control parsley cells (see Figure 1; Weisshaar et al., 1991; Armstrong et al., 1992), probably overlaying the DNA–protein complexes, which might be induced by GBFs that could be translocated into the nucleus.

By using the in vitro transcription approach with permeabilized, evacuolated protoplasts, we recently demonstrated that the light enhancement of the initiation rate of *chs* transcription is very fast (detectable within 20 min) and is not dependent on intact translation machinery. Hence, de novo synthesis of a protein factor is not an immediate part of the observed in vitro signal transduction pathway (Frohnmeyer et al., 1994). In addition, fast light quality–dependent changes in the protein phosphorylation pattern can be observed in vitro in the cytosol of evacuolated parsley protoplasts, possibly reflecting early candidates mediating the primary signal from the photoreceptors (Harter et al., 1994).

These latter observations as well as the results shown in this study can be combined into an attractive working hypothesis and can be summarized as light-regulated translocation of a specific GBF pool from the cytosol to the nucleus. This hypothesis possibly resolves the inconsistency of inducible binding of transcription factors being detectable on the background of active nuclear GBFs that are responsible for the regulation of light-independent genes. After the activation of the photoreceptors by light, the signal transduction pathway would start with the activation of G proteins (H. Frohnmeyer, unpublished data). The G proteins would then be coupled to a complex network of different, Ca^{2+}-dependent kinases and phosphatases connecting the photoreceptor signal to the GBF pool localized in the cytosol. This course of events would result in the activation of G-box binding activity in the cytosol and in the transport of the cytosolic GBFs to the nucleus. At unit 1, the incoming GBFs would interact specifically with the box I binding factor (probably preexisting in the nuclear compartment; Sablowski et al., 1994). As a defined protein complex, unit 1 binding factors would be able to mediate the induction of transcription of the *chs* gene. In this model, the specificity of light-induced gene expression, however, is at least partly transferred to a change in the subcellular localization of a specific transcription factor within the cell.

METHODS

Light Sources

Continuous white light (35 μmol m^{-2} sec^{-1}) was obtained from 40/W18 lamps (Philips, Hamburg, Germany). If not otherwise indicated, all experiments were carried out under a dim-green safelight generated as described by Schäfer (1977).

Suspension Culture, Preparation of Protoplasts, and Evacuolated Protoplasts

Protoplasts were prepared from a dark-grown parsley (*Petroselinum crispum*) cell culture 5 days after subculturing under a dim-green safelight (Dangl et al., 1987). Evacuolation of protoplasts was performed as described by Frohnmeyer et al. (1994). Evacuolated protoplasts were counted in a Fuchs–Rosenthal hemocytometer. For irradiation experiments, aliquots of 5 × 10^6 cells were transferred to Petri dishes and diluted in 5 mL of B5 medium (Dangl et al., 1987) containing 0.24 M sucrose. After irradiation, cells were collected by a short centrifugation (10,000*g* for 30 sec).

Isolation of Cytosolic and Nuclear Fractions from Evacuolated Protoplasts

Isolation of cytosol from evacuolated protoplasts was performed as described by Harter et al. (1994). In short, evacuolated protoplasts (5×10^6 cells per assay) were collected by centrifugation (10,000*g* for 30 sec at 25°C), the supernatant carefully removed, and the cells incubated on ice until use. Evacuolated protoplasts were permeabilized in ice-cold lysis solution (85 μL per 5×10^6 cells) containing 0.24 M sucrose, 0.02% Triton X-100, 5 mM benzamidine, 1 mM ε-amino caproic acid, 2 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/mL antipain, and 1 μg/mL leupeptin. The lysate (about 100 μL) was loaded on an ice-cold silicon oil gradient (Wacker-Chemie, Munich, Germany) containing a glycerol cushion (80% [v/v]) at the bottom. After centrifugation (10,000*g* for 10 min at 4°C), the cytosolic layer was carefully removed from the top of the gradient and used for experiments. For storage, the cytosol was frozen in liquid nitrogen and kept at −80°C. When kinase and phosphatase inhibitors (isoquinolinesulfonamide kinase inhibitor C-I, calmidazolium, *N*-(2-[*p*-bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide, kinase inhibitor K-252a, okadaic acid [all from Biomol, Hamburg, Germany]; sodium metavanadate [Fluka, Buchs, Switzerland]; EGTA [Roth, Karlsruhe, Germany]) were used, they were added to the lysis solution before cell permeabilization in concentrations as indicated in the legend to Figure 8. The gradients still containing the organellar and nuclear compartments were frozen in liquid nitrogen. The nuclear fraction was isolated by cutting out the pellet. The pellet was washed once in washing buffer (60 mM Hepes-KOH, pH 7.9, 12 mM mercaptoethanol, 0.12 mM EDTA, 0.24 M sucrose, 0.84 mM magnesium acetate, 1 μg/mL antipain, 1 μg/mL leupeptin). The nuclear proteins were extracted by incubation of the pellet (1 hr, 4°C, continuous shaking) in washing buffer that also contained 0.6 M KCl. The extract was cleared by ultracentrifugation (100,000*g* for 1 hr at 4°C), and the supernatant was desalted and concentrated by multiple centrifugation steps in Centricon-30 tubes (Amicon, Danvers, MA) using storage buffer (20 mM Hepes-KOH, 10 mM magnesium acetate, 50 mM potassium acetate, 12 mM mercaptoethanol, 5 mM EDTA, 25% glycerol) for equilibration. The concentrated supernatant (~50 μL) was frozen in liquid nitrogen and stored at −80°C.

Denaturating Extraction of Protein and Protein Assay

Evacuolated protoplasts were collected by a short centrifugation (10,000*g* for 30 sec at 25°C) and extracted in a small volume of boiling (95°C) SDS-sample buffer (65 mM Tris-HCl, pH 7.8, 4 M urea, 10 mM DTE, 5.0% [w/v] SDS, 0.05% [w/v] bromphenol blue) for 10 min followed by 2 min sonication. The crude extracts were clarified by 15 min of centrifugation at 20,000*g* and 25°C. Cytosolic and nuclear probes were prepared for SDS-PAGE by denaturation in one volume of boiling (95°C) SDS-sample buffer for 10 min. Total protein amounts were determined according to Harter et al. (1993).

PAGE, Protein Gel Blotting, Immunodetection, and Antisera

Protein extracts were separated by SDS-PAGE according to Schägger and von Jagow (1987). Protein gel blotting and immunodetection were performed as described by Harter et al. (1993). Preimmune serum and antiserum raised against G-box binding factor 1 (GBF-1) of Arabidopsis were a gift from A.R. Cashmore (University of Pennsylvania, Philadelphia). Antiserum raised against phosphoenolpyruvate (PEP) carboxylase of maize was obtained from R. Chollet (University of Nebraska-Lincoln; Budde and Chollet, 1986). Before use in immunodetection, the antisera were diluted 1:1000 in washing buffer as previously described (Harter et al., 1993).

Oligonucleotide Probes and Gel Mobility Shift Assay

Gel mobility probes were a tetrameric G-box according to the G-box element of the *rbcS-3A* promoter of Arabidopsis ([5′-ACCACGTGGC-3′]$_4$, Schindler and Cashmore, 1990), a mutated tetrameric G-box (G_m, [5′-ACCACTGTTC-3′]$_4$), and a tetrameric GT-box according to box II of the *rbcS-3A* promoter of pea ([5′-TGTGTGGTTAATATG-3′]$_4$, Kuhlemeier et al., 1988). The probes were synthesized and subcloned into pBluescript SK+ (Stratagene) using general cloning procedures (Maniatis et al., 1982). After EcoRI restriction digestion, the fragments were separated from the vector by ethidium bromide–agarose gel electrophoresis, electrophoretically eluted from the gel, and radiolabeled by filling the 5′ overhang with α-^{32}P-dATP (Amersham, Braunschweig, Germany) and DNA polymerase I (Klenow fragment, Amersham). Unincorporated nucleotides were removed by ammonium acetate–ethanol precipitation. For the gel mobility shift assay, a typical binding reaction mixture (15 μL) contained 7 mM Hepes, 3 mM Tris, pH 7.9, 40 mM KCl, 0.6 mM EDTA, 0.6 mM DTT, 7.5% (v/v) glycerin, 2.5 μg poly(dI-dC), 1.5 mM spermidine, 70 to 100 pg of radioactively labeled DNA (10,000 to 20,000 cpm), and 4 μg of nuclear or 8 to 10 μg of cytosolic extract. If competitor DNA or sera were used, the volume of the binding reaction mixture was kept constant by reducing the H_2O volume. After incubation for 10 min at room temperature, the reaction mixture was loaded onto a 3% polyacrylamide-agarose gel in 0.25 × Tris-borate-EDTA, which was pre-run for 1 hr at 100 V (Green et al., 1989). Electrophoresis was carried out for 2.5 hr at 200 V. Gels were fixed in 20% methanol and 10% acetic acid for 30 min, and dried and exposed overnight to x-ray film. Determination of band intensity was performed using a laser scanner (Pharmacia, Freiburg, Germany) coupled to an area integrator (Hewlett Packard, Karlsruhe, Germany).

In Vitro Dephosphorylation/Rephosphorylation Assay

For dephosphorylation assay of nuclear and cytosolic extracts, a typical reaction mixture (10 μL) contained 20 mM Hepes-KOH, pH 8.5, 10 mM KCl, 0.1 mM $MgCl_2$, 0.01 mM $ZnCl_2$, 20 μg of protein, and 0.2 units of immobilized alkaline phosphatase (Sigma), which was washed five times in 0.24 M sucrose before use. The assay was incubated for 30 min at 25°C with occasional shaking. Before using the samples in gel mobility shift and rephosphorylation assays, the alkaline phosphatase was completely removed by multiple centrifugations (three times at 1000*g* for 1 min each). For rephosphorylation assay of dephosphorylated nuclear and cytosolic proteins, a typical reaction mixture (10 μL) contained 30 mM Tris-HCl, pH 7.0, 5 mM $MgCl_2$, 0.2 mM EGTA, 0.12 mM $CaCl_2$, 20 mM ATP (Amersham), and 10 μg of dephosphorylated protein extract. The assay was incubated for 30 min at 25°C and then immediately used for the gel mobility shift assay.

In Vitro GBF-Antibody Cotranslocation Assay and Preparation of Nuclei

For the GBF–antibody cotranslocation assay, dark-grown evacuolated protoplasts were permeabilized in lysis solution (16.8 μL per 10^6 cells)

containing 0.24 M sucrose, 0.02% Triton X-100, 2 mM PMSF, 1 μg/mL antipain, and 1 μg/mL leupeptin and supplemented with the ATP-generating system (50 mM creatine-phosphate, 2 units per μL creatine-phosphate kinase [Serva, Heidelberg, Germany]). The antiserum (preimmune serum, GBF-1 antiserum, or PEP carboxylase antiserum) was then added to the concentration as indicated in Figure 9. If wheat germ agglutinin or apyrase were used in the assay, they were added to the lysate before mixing with the antiserum. The permeabilized cells (2.5×10^6 to 3.5×10^6 cells per probe) were then either irradiated with white light or kept in darkness for 40 min at 25°C. After the incubation, the lysate was mixed with 0.5 mL of ice-cold Ficoll buffer (20 mM Mes-KOH, pH 5.8, 20% [w/v] Ficoll, 20 mM potassium acetate, 0.5 mM spermidine, 0.15 mM spermine, 0.6 μM antipain, 0.6 μM leupeptin), diluted with 4 mL of ice-cold washing buffer (20 mM Mes-KOH, pH 5.8, 0.25 M sucrose, 20 mM potassium acetate, 2.0% [w/v] dextran-40, 0.5 mM spermidine, 0.15 mM spermine, 1.0 μg/mL antipain, 1.0 μg/mL leupeptin, 2 mM PMSF, 0.05% [v/v] Triton X-100), and transferred to Corex tubes. After centrifugation (2000 rpm for 5 min at 4°C), the nuclei were washed twice in 1.3 and 1.0 mL, respectively, of washing buffer. The pellet was resuspended in 100 μL of washing buffer and loaded on a gradient generated during centrifugation of 1 mL of Ficoll buffer. The suspension was centrifuged for 5 min at 2000*g* and 4°C. The sedimented nuclei were resuspended in 150 μL of proteinase K solution (1 mg/mL), incubated for 15 min at room temperature, and washed with 1 mL of washing buffer. The nuclei were extracted in a small volume of boiling (95°C) SDS-sample buffer, and the amount of nuclear heavy chains was analyzed by protein gel blotting using anti-rabbit IgG-specific, alkaline phosphatase–conjugated goat antibody (Boehringer, Mannheim, Germany).

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