RESEARCH ARTICLES

Calmodulin7 Plays an Important Role as Transcriptional Regulator in Arabidopsis Seedling Development\footnote{Online version contains Web-only data. www.plantcell.org/cgi/doi/10.1105/tpc.107.057612}

Ritu Kushwaha, Aparna Singh, and Sudip Chattopadhyay\footnote{Address correspondence to sudipchatto@yahoo.com. The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Sudip Chattopadhyay (sudipchatto@yahoo.com).}
National Institute for Plant Genome Research, Aruna Asaf Ali Marg, New Delhi 110067, India

Although calmodulin (CaM) is known to play multiple regulatory roles in eukaryotes, its direct function as transcriptional regulator is unknown. Furthermore, the physiological functions of CaM are largely unknown in plants. Here, we show that one of the four Arabidopsis thaliana CaM isoforms, CAM7, is a transcriptional regulator that directly interacts with the promoters of light-inducible genes and promotes photomorphogenesis. CAM7 overexpression causes hyperphotomorphogenic growth and an increase in the expression of light-inducible genes. Mutations in CAM7 produce no visible effects on photomorphogenic growth, indicating likely redundant gene functions. However, cam7 mutants display reduced expression of light-inducible genes, and cam7 hy5 double mutants show an enhancement of the hy5 phenotype. Moreover, overexpression of CAM7 can partly suppress the hy5 phenotype, indicating that the two factors work together to control light-induced seedling development. The mutational and transgenic studies, together with physiological analyses, illustrate the concerted function of CAM7 and HY5 basic leucine zipper transcription factor in Arabidopsis seedling development.

INTRODUCTION

Calmodulin (CaM) is ubiquitous in eukaryotes and is a highly conserved Ca$^{2+}$ binding protein that plays multiple regulatory functions responding to a wide variety of stimuli (Berridge et al., 2000; Hepler, 2005). CaM has a common helix-loop-helix structure, the EF-hand, which is known to perform its regulatory function by modulating the activity of specific CaM binding proteins. CaM regulation of basic-helix-loop-helix transcription factors has been reported, where CaM inhibits the DNA-protein interactions by competing with the DNA binding domains of the basic-helix-loop-helix proteins (Cornellussen et al., 1994). Interestingly, recent studies have shown that some proteins with EF-hands have the ability to directly interact with DNA. For example, the human DRE antagonist modulator (DREAM) has four EF-hands and specifically interacts with the DNA DRE element (Carrion et al., 1999; Gilchrist et al., 2001; Craig et al., 2002). Various studies have shown that Ca$^{2+}$/CaM is involved in multiple signaling pathways in plants (Miller and Sanders, 1987; Braam and Davis, 1990; Knight et al., 1991; Szymanski et al., 1996; Yang and Poovaiah, 2002; Yoo et al., 2004). The Arabidopsis thaliana genome contains seven CAM genes that encode only four protein isoforms: CAM1/CAM4, CAM2/CAM3/CAM5, CAM6, and CAM7. The CAM7 protein sequence shows the most similarity to consensus among all the members of the family, but all the CAM isoforms are very highly conserved. CAM1/CAM4 differs from CAM7 by four amino acids, whereas CAM2/3/5 and CAM6 differ from CAM7 by a single amino acid substitution (McCormack et al., 2005).

Arabidopsis seedlings grow with two distinct developmental patterns in the presence and absence of light (Nagy and Schaefer, 2002; Chen et al., 2004; Huq and Quail, 2005). The dark-grown seedlings exhibit elongated hypocotyls and closed cotyledons with apical hooks, designated as skotomorphogenic growth. When exposed to light, seedlings grow with a short hypocotyl and open and expanded cotyledons, known as photomorphogenic growth. The expression of light-inducible genes, which remains suppressed in the dark, is strongly induced during photomorphogenesis. A complex molecular network operates to sense the dark-light transitions and regulate the seedling morphology and gene expression accordingly (Jiao et al., 2007). The basic leucine zipper transcription factor, Long Hypocotyl5 (HY5), plays an important role in the transition from skotomorphogenesis to photomorphogenesis. The loss-of-function mutants of HY5 display partial photomorphogenic growth at various wavelengths of light with reduced expression of light-regulated genes (Oyama et al., 1997; Ang et al., 1998; Chattopadhyay et al., 1998). The abundance of HY5 protein has been correlated with the extent of photomorphogenic growth (Osterlund et al., 2000). Recently, genome-wide promoter target studies have revealed that there are >3000 chromosomal sites in the Arabidopsis genome that have putative HY5 binding targets (Lee et al., 2007)

The homeostasis of Ca$^{2+}$ has been shown to be associated with blue/UV-A light-inducited gene expression (Long and Jenkins, 1998). A recent genetic study using SHORT UNDER BLUE LIGHT1 (SUB1) has suggested the possible involvement of local Ca$^{2+}$ concentration change in phytochrome- and cryptochrome-mediated light signaling (Guo et al., 2001). Biochemical and pharmacological studies have revealed three branched pathways of light-induced gene expression. In one of these pathways, CaM has been shown to be involved in the regulation CAB
gene expression (Neuhaus et al., 1993, 1997; Bowler et al., 1994). All these studies suggest that Ca²⁺/CaM is involved in light-mediated seedling development and gene expression. However, the molecular and physiological function of CaM or structurally related Ca²⁺ binding protein, which interprets and specifically transduces the information into appropriate cellular responses, remains largely unknown (Veitia, 2005).

The activity of a CAB1 minimal promoter containing an essential Z-box light-responsive element (LRE) is controlled by HY5 (Yadav et al., 2002). Recently, two Z-box binding transcription factors, ZBF1/MYC2 and ZBF2/GBF1, have been identified from a ligand binding screen and shown to function in cryptochrome-mediated blue light signaling (Yadav et al., 2005; Mallappa et al., 2006). In this study, we demonstrate the functional relevance of ZBF3, encoding CAM7, in light-mediated seedling development and gene expression.

RESULTS

CAM7 Specifically Binds to the Z-/G-Box of Light-Regulated Promoters

We had identified and cloned ZBF3/CAM7, which was represented by three independent cDNA clones in a ligand binding screen (Yadav et al., 2005). The DNA binding analyses, which examined binding of labeled probe DNA sequences to proteins immobilized on nylon membranes, revealed that ZBF3/CAM7 was able to specifically bind to the Z-box LRE (Figures 1A and 1B). To further examine the results obtained from these analyses, we performed electrophoretic mobility shift assays (EMSAs) using CAB1 minimal promoter containing an essential Z-box and purified glutathione S-transferase-CAM7 (GST-CAM7) fusion protein. As shown in Figure 1C, GST-CAM7 was able to bind to the Z-box of CAB1 minimal promoter. Excess unlabeled Z-box DNA, but not a nonspecific competitor (GT1 LRE; Chattopadhyay et al., 1998), was able to compete for the binding activity of GST-CAM7. Since recent studies have suggested that the Z- and G-box LREs are functionally equivalent (Yadav et al., 2005; Mallappa et al., 2006), we also investigated the binding ability of CAM7 to the G-box. As shown in Figure 1D, CAM7 could specifically bind to the essential G-box of RBCS-1A minimal promoter. Taken together, these results suggest that CAM7 specifically binds to the Z-/G-box of light-regulated CAB1 and RBCS-1A minimal promoters.

It has been postulated that substitution of amino acid in the EF-hand could contribute to select the target specificity of CaM (McCormack et al., 2005). CaMs have highly conserved amino acid sequences, and the amino acid sequence of the CaM2/3/5 isoform differs from CaM7 by a single amino acid substitution (Figure 1G). To determine whether CaM2/3/5 was also able to interact with the Z-box, EMSAs were performed using purified GST-CAM3 fusion protein and CAB1 minimal promoter as probe. However, no DNA–protein complex was detected; thereby, these results suggest that CaM2/3/5 is unable to bind to the CAB1 minimal promoter (Figure 1E). To further test this observation, we generated mutated versions of CAM7, CAM7-M1 and CAM7-M2, by site-directed mutagenesis. Whereas two Asp residues of CAM7 were substituted by Ala in CAM7-M1, four Asp residues were substituted by Ala in CAM7-M2 protein (Figure 1G). We used purified GST-CAM7-M1 or GST-CAM7-M2 fusion proteins in EMSAs (see Supplemental Figure 1 online). None of these mutated versions of CAM7 was also able to bind to the CAB1 minimal promoter (Figure 1F). Taken together, these results suggest that CAM7 is likely to be a unique member of the CAM gene family that directly binds to the Z-/G-box of light-regulated promoters.

Overexpression of CAM7 Leads to Hyperphotomorphogenic Growth Irrespective of Light Qualities

To investigate the physiological function of CAM7 in light-mediated seedling development, 27 Arabidopsis transgenic lines overexpressing CAM7 fused to three copies of c-Myc epitope were generated. The c-Myc epitope was fused to either the C- or N-terminal end of the CAM7 protein, and the proteins showed higher levels of accumulation in the transgenic lines (Figure 2G). The transgenic seedlings exhibited short hypocotyl phenotype at various wavelengths of light, including red (RL), far-red (FR), and blue light (BL) (Figure 2A). Measurements of hypocotyl length revealed that the enhanced inhibition of hypocotyl elongation was more evident at lower fluence rates especially in RL- or FR-grown seedlings (Figures 2C to 2F). Strikingly, the transgenic seedlings displayed a weak photomorphogenic growth with shorter hypocotyl and partly opened cotyledons without apical hooks in the darkness (Figures 2A and 2B). The overexpressor transgenic seedlings also showed higher levels of chlorophyll in light and of anthocyanin in both dark and light growth conditions (Figures 2H to 2J). Taken together, these results suggest that overexpression of CAM7 induces a partial photomorphogenic development in the dark and also promotes photomorphogenic growth in various wavelengths of light.

CAM7 Interacts with CAB1 Minimal Promoter in Vivo and Promotes Light-Induced Gene Expression

To determine whether CAM7 was able to promote the transcriptional activity of light-regulated genes, we performed RNA gel blot analysis using transgenic seedlings grown in constant dark or light. The expression of CAB was strongly elevated in transgenic seedlings compared with the wild type in white light (WL) (Figures 3A and 3E). Whereas very little expression, as expected, of CAB was detected in wild-type background (Figure 3B). Taken together, these results suggest that CAB acts as a positive regulator of CAB and RBCS gene expression.

We performed chromatin immunoprecipitation (ChIP) experiments to determine whether CAM7 binds to CAB1 minimal promoter in vivo. The CAM7-c-Myc fusion protein in transgenic plants was immunoprecipitated by antibody to c-Myc. The genomic DNA fragments that communoprecipitated with
Calmodulin7 Plays an Important Role as Transcriptional Regulator in Arabidopsis Seedling Development

Ritu Kushwaha, Aparna Singh, and Sudip Chattopadhyay
National Institute for Plant Genome Research, Aruna Asaf Ali Marg, New Delhi 110067, India

Although calmodulin (CaM) is known to play multiple regulatory roles in eukaryotes, its direct function as transcriptional regulator is unknown. Furthermore, the physiological functions of CaM are largely unknown in plants. Here, we show that one of the four Arabidopsis thaliana CaM isoforms, CAM7, is a transcriptional regulator that directly interacts with the promoters of light-inducible genes and promotes photomorphogenesis. CAM7 overexpression causes hyperphotomorphogenic growth and an increase in the expression of light-inducible genes. Mutations in CAM7 produce no visible effects on one of the four ways of light-induced gene expression. Mutations in CAM7 can partly suppress the hy5 phenotype, indicating that the two factors work together to control light-induced seedling development. The mutational and transgenic studies, together with physiological analyses, illustrate the concerted function of CAM7 and HY5 basic leucine zipper transcription factor in Arabidopsis seedling development.

INTRODUCTION

Calmodulin (CaM) is ubiquitous in eukaryotes and is a highly conserved Ca2+ binding protein that plays multiple regulatory functions responding to a wide variety of stimuli (Berridge et al., 2000; Hepler, 2005). CaM has a common helix-loop-helix structure, the EF-hand, which is known to perform its regulatory function by modulating the activity of specific CaM binding proteins. CaM regulation of basic-helix-loop-helix transcription factors has been reported, where CaM inhibits the DNA-protein interactions by competing with the DNA binding domains of the basic-helix-loop-helix proteins (Cornelissen et al., 1994). Interestingly, recent studies have shown that some proteins with EF-hands have the ability to directly interact with DNA. For example, the human DRE antagonist modulator (DREAM) has four EF-hands and specifically interacts with the DNA DRE element (Carrión et al., 1999; Gilchrist et al., 2001; Craig et al., 2002). Various studies have shown that Ca2+/CaM is involved in multiple signaling pathways in plants (Miller and Sanders, 1987; Braam and Davis, 1990; Knight et al., 1991; Szymanski et al., 1996; Yang and Poovaiah, 2002; Yoo et al., 2004). The Arabidopsis thaliana genome contains seven CaM genes that encode only four protein isoforms: CAM1/CAM4, CAM2/CAM3/CAM5, CAM6, and CAM7. The CAM7 protein sequence shows the most similarity to consensus among all the members of the family, but all the CAM isoforms are highly conserved. CAM1/CAM4 differs from CAM7 by four amino acids, whereas CAM2/3/5 and CAM6 differ from CAM7 by a single amino acid substitution (McCormack et al., 2005).

Arabidopsis seedlings grow with two distinct developmental patterns in the presence and absence of light (Nag and Schafer, 2002; Chen et al., 2004; Huq and Quail, 2008). The dark-grown seedlings exhibit elongated hypocotyls and closed cotyledons with apical hooks, designated as skotomorphogenic growth. When exposed to light, seedlings grow with a short hypocotyl and open and expanded cotyledons, known as photomorphogenic growth. The expression of light-inducible genes, which remains suppressed in the dark, is strongly induced during photomorphogenesis. A complex molecular network operates to sense the dark-light transitions and regulate the seedling morphology and gene expression accordingly (Jiao et al., 2007). The basic leucine zipper transcription factor, Long Hypocotyl5 (HY5), plays an important role in the transition from skotomorphogenesis to photomorphogenesis. The loss-of-function mutants of HY5 display partial photomorphogenic growth at various wavelengths of light with reduced expression of light-regulated genes (Oyama et al., 1997; Ang et al., 1998; Chattopadhyay et al., 1998). The abundance of HY5 protein has been correlated with the extent of photomorphogenic growth (Osterlund et al., 2000). Recently, genome-wide promoter target studies have revealed that there are >3000 chromosomal sites in the Arabidopsis genome that have putative HY5 binding targets (Lee et al., 2007).

The homeostasis of Ca2+ has been shown to be associated with blue/UV-A light-induced gene expression (Long and Jenkins, 1998). A recent genetic study using SHORT UNDER BLUE LIGHT1 (SUB1) has suggested the possible involvement of local Ca2+ concentration change in phytochrome- and cryptochrome-mediated light signaling (Guo et al., 2001). Biochemical and pharmacological studies have revealed three branched pathways of light-induced gene expression. In one of these pathways, CaM has been shown to be involved in the regulation CAB
**Figure 1. CAM7 Promotes Photomorphogenesis**

(A) Identification of CAM7 in a ligand binding (protein/DNA gel blot) screen. The blotted nylon membrane (containing protein-expressing plaques from the tertiary screen for proteins that bind to the Z-box) was probed with the radioactively labeled Z-box LRE (Yadav et al., 2005).

(B) The specificity of interaction of CAM7 to the Z-box. The blotted nylon membrane was cut into two halves and probed with the Z-box or GATA LRE (Yadav et al., 2002).

(C) EMSAs showing GST-CAM7 (CAM7) protein specifically binds to the essential G-box of 196-bp RBCS-1A minimal promoter (Chattopadhyay et al., 1998). For experimental detail, see (C). In this case, the unlabeled competitor DNA is G-box LRE.

(D) EMSA showing GST-CAM7 (CAM7) protein specifically binds to the essential Z-box of 189-bp CAB1 minimal promoter (Yadav et al., 2005). Approximately 200 ng of recombinant protein was added (lanes 3 to 6) to radioactively labeled CAB1 promoter fragment. Approximately 500 ng GST protein was added in lane 2. The triangle indicates the increased amount of unlabeled Z-box DNA added (50 and 100 molar excess in lanes 4 and 5, respectively) to the reaction as competitor (Comp.). In lane 6, 100 molar excess GT1 LRE (Yadav et al., 2002) was added. The presence of CAM7 or GST protein is indicated by plus signs in their respective rows. The minus signs indicate the absence of competitors CAM7 or GST. The arrowhead indicates the protein-DNA complex.

(E) EMSA of CAM2/3/5 to the CAB1 minimal promoter. Approximately 200 ng, 1 μg, and 3 μg (lanes 3 to 5), and 100, 200, and 300 ng (lanes 6 to 8) of recombinant proteins were added to radioactively labeled CAB1 minimal promoter. For experimental detail, see (C).

(F) EMSAs showing that CAM7, but not CAM7-M1 and CAM7-M2, is able to bind to CAB1 minimal promoter. Approximately 200, 300, 200, 500, 200, and 500 ng (lanes 2 to 7, respectively) of recombinant proteins were added to radioactively labeled CAB1 minimal promoter. For experimental detail, see (C).

(G) Amino acid sequences of CAM7, CAM2/3/5, and site-directed mutagenesis products of CAM7 (CAM7-M1 and CAM7-M2) are shown. The amino acid substitutions are shown in red.
CAM7–c-Myc were analyzed by real-time quantitative PCR. The analyses of these data revealed that the amount of DNA fragment of CAB1 promoter communoprecipitated from the transgenic seedlings was >30-fold higher than that precipitated from the nontransgenic seedlings, and ~10-fold higher than the NIA2 promoter, which is induced by light but does not contain any Z/-G-box LRE (Figure 3C). These results demonstrate that CAM7 binds to the CAB1 minimal promoter in vivo.

To determine whether CAM7 binding to the Z-box is required for the in vivo activation of CAB1 promoter, we used CAB1 minimal promoter containing either wild type or mutated Z-box fused to the β-glucuronidase reporter gene construct (CAB1 promoter-GUS or CAB1m promoter-GUS). We examined the activity of these promoters in transiently transformed protoplasts made from wild-type or CAM7 overexpressor transgenic plants (OE1). The activity of CAB1 promoter was increased by more than twofold in OE1 compared with wild-type background, confirming that CAM7 promotes CAB1-GUS expression. By contrast, overexpression of CAM7 was unable to activate the CAB1m promoter in vivo (Figure 3D). Together, the above results
Figure 2. CAM7 Promotes Photomorphogenic Growth.

(A) The visible phenotypes of the seedlings grown in constant dark, WL (15 μmol/m²/s), RL (30 μmol/m²/s), BL (20 μmol/m²/s), or FR (40 μmol/m²/s) are shown. In each panel, 6-d-old wild-type (Columbia [Col]) and CAM7 overexpresser transgenic seedlings (OE1, OE2, and OE3 in Col background) are shown from left to right, respectively. OE1 and OE2 contain CAM7 with c-Myc tagged at the N-terminal end, whereas OE3 contains CAM7 with c-Myc tagged at the C-terminal end. Bar = 1 mm.

(B) to (F) Quantification of hypocotyl length of 6-d-old seedlings grown in constant dark or at various fluences of WL, RL, BL, or FR. Approximately 25 to 30 seedlings were used for the measurement of hypocotyl length. The error bars indicate SD.

(G) Immunoblot (using anti-c-Myc antibodies) of 20 μg of total protein prepared from wild-type (Col) or overexpresser transgenic plants. The asterisk in the bottom panel shows a cross-reacting band in the same gel as loading control.

(H) The level of total chlorophyll content in 6-d-old wild-type (Col) or transgenic seedlings grown in WL (30 μmol/m²/s) is shown.

(I) and (J) Accumulation of anthocyanin in 6-d-old wild-type or transgenic seedlings grown in WL (30 μmol/m²/s) or dark, respectively. Approximately 30 to 40 seedlings were used for the measurement of chlorophyll or anthocyanin accumulation. The error bars indicate SD.

CAM7-c-Myc were analyzed by real-time quantitative PCR. The analyses of these data revealed that the amount of DNA fragment of CAB1 promoter coimmunoprecipitated from the transgenic seedlings was >30-fold higher than that precipitated from the nontransgenic seedlings, and ~10-fold higher than the NIA2 promoter, which is induced by light but does not contain any Z-/G-box LRE (Figure 3C). These results demonstrate that CAM7 binds to the CAB1 minimal promoter in vivo.

To determine whether CAM7 binding to the Z-box is required for the in vivo activation of CAB1 promoter, we used CAB1 minimal promoter containing either wild type or mutated Z-box fused to the β-glucuronidase reporter gene construct (CAB1 promoter-GUS or CAB1m promoter-GUS). We examined the activity of these promoters in transiently transformed protoplasts made from wild-type or CAM7 overexpresser transgenic plants (OE1). The activity of CAB1 promoter was increased by more than twofold in OE1 compared with wild-type background, confirming that CAM7 promotes CAB1-GUS expression. By contrast, overexpression of CAM7 was unable to activate the CAB1m promoter in vivo (Figure 3D). Together, the above results.
Figure 1. CAM7 Binds to the Essential Z/G-Box of CAB1 or RBCS-1A Minimal Promoter.

(A) Identification of CAM7 in a ligand binding (protein/DNA gel blot) screen. The blotted nylon membrane (containing protein-expressing plaques from the tertiary screen for proteins that bind to the Z-box) was probed with the radioactively labeled Z-box LRE (Yadav et al., 2005).

(B) The specificity of interaction of CAM7 to the Z-box. The blotted nylon membrane was cut into two halves and probed with the Z-box or GATA LRE (Yadav et al., 2002).

(C) EMSAs showing GST-CAM7 (CAM7) specifically binds to the Z-box of 189-bp CAB1 minimal promoter (Yadav et al., 2005). Approximately 200 ng of recombinant protein was added (lanes 3 to 6) to radioactively labeled CAB1 promoter fragment. Approximately 500 ng GST protein was added in lane 2. The triangle indicates the increased amount of unlabelled Z-box DNA added (50 and 100 molar excess in lanes 4 and 5, respectively) to the reaction as competitor (Comp.). In lane 6, 100 molar excess G1 LRE (Yadav et al., 2002) was added. The presence of CAM7 or GST protein is indicated by plus signs in their respective rows. The minus signs indicate the absence of competitors CAM7 or GST. The arrowhead indicates the protein-DNA complex.

(D) EMSA showing GST-CAM7 (CAM7) protein specifically binds to the essential G-box of 196-bp RBCS-1A minimal promoter (Chattopadhyay et al., 1998). For experimental detail, see (C). In this case, the unlabeled competitor DNA is G-box LRE.

(E) EMSA of CAM2/3/5 to the CAB1 minimal promoter. Approximately 200 ng, 1 µg, and 3 µg (lanes 3 to 5), and 100, 200, and 300 ng (lanes 6 to 8) of recombinant proteins were added to radioactively labeled CAB1 minimal promoter. For experimental detail, see (C).

(F) EMSAs showing that CAM7, but not CAM7-M1 and CAM7-M2, is able to bind to CAB1 minimal promoter. Approximately 200, 300, 200, 500, 200, and 500 ng (lanes 2 to 7, respectively) of recombinant proteins were added to radioactively labeled CAB1 minimal promoter. For experimental detail, see (C).

(G) Amino acid sequences of CAM7, CAM2/3/5, and site-directed mutagenesis products of CAM7 (CAM7-M1 and CAM7-M2) are shown. The amino acid substitutions are shown in red.
To investigate the transgenic seedlings displayed elongated hypocotyls in 4E). The elongated hypocotyl phenotype conferred red by the overexpression of CAM7 (Figure 4A). The elongated hypocotyl phenotype conferred red by the overexpression of CAM7.

The RNA gel blot results (quantified as described above) show light-mediated induction of CAB1 and RBCS gene expression in wild-type (Col) and OE1 transgenic seedlings grown in dark (0) for 5 d and then transferred to WL (30 μmol/m²/s) for various time points.

Overexpression of CAM7-M2 Confers Possible Dominant-Negative Effects

To determine whether overexpression of CAM7-M2 leads to similar effects on light-regulated gene expression, we performed quantitative real-time PCR experiments of CAB1 and RBCS-1A genes. The rate of light-mediated induction of CAB1 and RBCS-1A genes was significantly reduced in transgenic seedlings compared with wild-type background (Figure 4F).

Loss-of-Function Mutants of CAM7 Have Reduced Expression of Light-Inducible Genes

To determine whether loss of CAM7 function would lead to reduced photomorphogenesis, we searched for mutants in T-DNA knockout collections (Alonso et al., 2003). We identified two such T-DNA insertion knockout lines (cam7-1 and cam7-2) and performed PCR genotyping analyses to identify plants homozygous or heterozygous for a cam7 mutation (see Supplemental Figure 2 online). The segregation ratios of self-fertilized plants homozygous for cam7, determined by the genotyping PCR on T2 progeny, suggested that a single T-DNA locus was present in each of the cam7 mutant lines. The junctions of T-DNA and CAM7 were amplified by PCR, and the DNA sequence analyses revealed that the T-DNA was inserted in nucleotide position 225.
Figure 4. Overexpression of CAM7-M2 Results in Suppression of Photomorphogenic Growth Irrespective of Light Qualities.
Figure 4. Overexpression of CAM7-M2 Results in Suppression of Photomorphogenic Growth Irrespective of Light Qualities.
Figure 3. CAM7 Interacts with CAB1 Promoter in Vivo and Promotes Light-Regulated Gene Expression.

(A) The RNA gel blot shows the level of CAB1 gene expression in 6-d-old wild-type (Col) and CAM7 overexpresser transgenic seedlings (OE1, OE2, and OE3) grown in dark or WL (30 μmol/m²/s). Ten micrograms of total RNA was loaded onto each lane. 18S rRNA has been shown as loading control. The numbers indicate the relative mRNA levels. To quantify the RNA gel blot data, the intensity of each band was quantified by the Fluor-S-Multilmager (Bio-Rad), and ratios of CAB1 versus its corresponding rRNA band were determined and plotted (Fluor-S-Multilmager; Bio-Rad).

(B) The RNA gel blot results (quantified as described above) show light-mediated induction of CAB1 and RBCS gene expression in wild-type (Col) and OE1 transgenic seedlings grown in dark (0) for 5 d and then transferred to WL (30 μmol/m²/s) for various time points.

(C) ChiP assays of CAB1 promoter from OE1, OE2, or OE3 transgenic seedings using antibodies to c-Myc. The light-inducible NIA2 promoter fragment, which does not contain any Z- or G-box, was used as a control. Results of real-time quantitative PCR are presented as the ratio of the amount of DNA immunoprecipitated from overexpressor transgenic seedings to nontransgenic control plants.

(D) Expression of CAM1-GUS or CAM1m-GUS reporter gene (reflected by GUS activities) relative to the 35S-GUS internal control in Arabidopsis protoplasts made from wild-type or CAM7 overexpresser (OE1) plants. Error bars indicate SE (n = 5). The experiment was repeated three times.

(E) Normalized graph of the data in (A) (quantified as described in [A]).

demonstrate that CAM7 acts as a transcriptional activator of CAB1 promoter in vivo and the Z-box is essential for such activation mediated by CAM7 protein.

Overexpression of CAM7-M2 Confers Possible Dominant-Negative Effects

To investigate the physiological function of the mutated version of CAM7 protein, which lost DNA binding ability due to amino acid substitutions (Figure 1G), we constructed a series of 32 transgenic lines overexpressing CAM7-M2 (Figure 4G). Examination of 2- to 6-d-old transgenic seedlings did not show any altered morphology in the dark. However, 6-d-old WL-grown transgenic seedlings displayed a longer hypocotyl compared with the corresponding wild type (Figure 4A). Furthermore, the transgenic seedlings displayed elongated hypocotyls in all light conditions tested compared with the corresponding wild type (Figure 4A). The measurements of hypocotyl length revealed a significant reduction (P value < 0.01; n = 3) in light-mediated inhibition of hypocotyl elongation in transgenic seedlings compared with the wild type in different light conditions (Figures 4B to 4E). The elongated hypocotyl phenotype conferred by the overexpression of CAM7-M2 could be attributable to dominant-negative interference of the light signaling pathways by CAM7-M2 protein. To determine whether overexpression of CAM7-M2 leads to similar effects on light-regulated gene expression, we performed quantitative real-time PCR experiments of CAB1 and RBCS-1A genes. The rate of light-mediated induction of CAB1 and RBCS-1A genes was significantly reduced in transgenic seedlings compared with wild-type background (Figure 4F).

Loss-of-Function Mutants of CAM7 Have Reduced Expression of Light-Inducible Genes

To determine whether loss of CAM7 function would lead to reduced photomorphogenesis, we searched for mutants in T-DNA knockout collections (Alonso et al., 2003). We identified two such T-DNA insertion knockout lines (cam7-1 and cam7-2) and performed PCR genotyping analyses to identify plants homozygous or heterozygous for a cam7 mutation (see Supplemental Figure 2 online). The segregation ratios of self-fertilized plants heterozygous for cam7, determined by the genotyping PCR on T2 progeny, suggested that a single T-DNA locus was present in each of the cam7 mutant lines. The junctions of T-DNA and CAM7 were amplified by PCR, and the DNA sequence analyses revealed that the T-DNA was inserted in nucleotide position 225...
and 113 bp upstream to the ATG codon of CAM7 in cam7-1 and cam7-2 mutants, respectively (see Supplemental Figure 2A online). RT-PCR analyses were unable to detect any CAM7 mRNA in either of the cam7 mutant lines (see Supplemental Figure 2B online).

When the growth of cam7 mutant seedlings was examined in dark and in various light conditions, cam7 mutants grew normally in the dark and at various wavelengths of light tested, showing no sign of altered photomorphogenic growth (Figures 5A and 5C). These results indicate that CAM7-mediated inhibition of hypocotyl elongation is functionally redundant. However, the level of CAB1 and RBCS-1A expression was compromised in cam7 mutants (P value < 0.03; n = 4), thereby suggesting that CAM7 is required for the optimum expression of CAB1 and RBCS-1A genes (Figure 4H).

**CAM7 and HY5 Function in an Independent and Interdependent Manner to Promote Photomorphogenesis**

HY5 is thus far the only known transcription factor in light signaling that promotes photomorphogenesis in RL, FR, and BL. Since higher-level accumulation of CAM7 also leads to hyperphotomorphogenic growth irrespective of light qualities, we asked whether HY5 and CAM7 are functionally interrelated. We constructed cam7 hy5 double mutants and examined the genetic interactions between cam7 and hy5. Similar to hy5 or cam7 single mutants, cam7 hy5 double mutants did not show any altered growth in the dark. However, the characteristic long hypocotyl phenotype of hy5 in WL irradiation was further enhanced in cam7 hy5 double mutants, exhibiting a super tall phenotype (Figure 5A). Furthermore, as shown in Figure 5A, cam7 hy5 double mutants also displayed reduced sensitivity in RL, FR, and BL compared with hy5 single mutants. Measurements revealed that the hypocotyl length of cam7 hy5 double mutants was strikingly increased compared with hy5 or cam7 alone, indicating a synergistic effect of cam7 and hy5 mutations on hypocotyl length irrespective of light qualities (Figure 5C; see Supplemental Figures 3A to 3C online). The expression of light-regulated genes has been shown to be downregulated in hy5 mutants (Ang et al., 1998; Chattopadhyay et al., 1998). When tested, the level of CAB1 and RBCS-1A gene expression was found to be further reduced in cam7 hy5 double mutants compared with the cam7 or hy5 mutant background (Figure 5G). A genomic fragment containing CAM7 and 1.5 kb of its upstream sequence was introduced into the cam7 hy5 double mutant plants for a complementation test. The transgenic seedlings did not display the super-tall phenotype, and the expression of light-regulated genes was also restored to hy5 mutant levels (Figures 5E and 5G). These results confirm that the observed super-tall phenotype of cam7 hy5 double mutants was caused by the additional loss of CAM7 function.

To further test this observation, we introduced the 35S-CAM7-c-Myc transgene from the overexpresser transgenic lines (OE1 and OE2) into hy5 mutant background by genetic crosses. The higher level of CAM7 protein in hy5 transgenic seedlings was indeed able to suppress the elongated hypocotyl phenotype of hy5 (Figures 5B, 5D, and 5F). When examined under various wavelengths of light, the hy5 phenotype was significantly suppressed in transgenic hy5 seedlings grown in RL, FR, and BL (Figure 5B; see Supplemental Figures 3D to 3F online). Furthermore, similar to OE1, hy5 transgenic seedlings also displayed partial photomorphogenic growth with slightly reduced hypocotyl length in the darkness. However, unlike OE1, the cotyledons remained closed with apical hooks in hy5 transgenic seedlings in the darkness (Figures 5B, dark, and 5D). The lower-level expression of light-inducible genes, such as CAB1 and RBCS-1A, in hy5 mutants was also restored in hy5 transgenic seedlings (Figure 5G). Taken together, these results suggest that CAM7 and HY5 function in an independent and interdependent manner to promote photomorphogenic growth and light-regulated gene expression.

**The Accumulation of CAM7 Protein Is Dependent on Light Intensity**

Since abundance of HY5 protein has been correlated with the extent of photomorphogenic growth, we asked whether CAM7 protein also accumulated in a similar fashion correlating with photomorphogenic growth. To address this question, we first used 6-d-old constant dark- or WL-grown CAM7-c-Myc over-expresser transgenic seedlings for immunoblot analysis. The accumulation of CAM7 protein was significantly reduced in WL...
Figure 5. The Elongated Hypocotyl Phenotype of hy5 Is Enhanced in cam7 hy5 Double Mutants.

(A) The visible phenotypes of 6-d-old cam7 hy5 double mutant seedlings grown in WL (30 μmol/m²/s), RL (60 μmol/m²/s), FR (40 μmol/m²/s), or BL (20 μmol/m²/s) are shown. In each panel, wild-type (segregated wild type in F2), cam7-1, hy5, and cam7-1 hy5 seedlings are shown from left to right. Bar = 1 mm.

(B) The visible phenotype of 6-d-old hy5 transgenic seedlings (hy5 [OE1] and hy5 [OE2]), containing 35S promoter-c-Myc-CAM7 transgene, grown in dark, WL (30 μmol/m²/s), RL (60 μmol/m²/s), FR (40 μmol/m²/s), or BL (20 μmol/m²/s) is shown. In each panel, wild-type, OE1, hy5, hy5 (OE1), and hy5 (OE2) seedlings are shown from left to right. Bar = 1 mm.

(C) and (D) Quantification of hypocotyl length in dark (D on x-axis label) or various fluence rates of WL. Approximately 25 to 30 seedlings were used for the measurement of hypocotyl length. The error bars indicate SD.
compared with dark-grown seedlings (Figure 6A), which is contrary to the accumulation pattern of HY5 protein under similar conditions.

We then examined whether the reduced accumulation of CAM7 protein in WL was dependent on light intensity. As shown in Figure 6B (bottom panel), the accumulation of CAM7 protein decreased with increase in fluence rates of WL in overexpresser transgenic lines (OE1). Whereas the level of accumulation of CAM7 protein was slightly reduced at 5 μmol/m²/s, it was further reduced at 15 or 30 μmol/m²/s and strikingly reduced at 60 μmol/m²/s or higher fluence rates of WL. The enhanced inhibition in hypocotyl elongation, caused by higher level of CAM7 protein, was also gradually reduced with higher fluence rates of WL, suggesting a likely correlation between the level of CAM7 protein and the extent of hyperphotomorphogenic growth of the transgenic seedlings. To determine whether WL-mediated reduction of CAM7 protein is wavelength specific, we examined the level of CAM7 protein in 6-d-old seedlings grown at low or relatively high intensities of RL, FR, and BL. As shown in Figure 6C, similar to WL, CAM7 protein was strikingly reduced at higher fluence rates of RL and BL, although the accumulation of CAM7 was largely maintained at higher fluence rates of FR.

DISCUSSION

The primary structures of CaMs are highly similar in plants and animals with respect to their Ca²⁺ binding loops and E and F helices. The topology of the EF-hand motif of CaM is similar to the helix-turn-helix DNA binding domain of various transcription factors that can recognize the major groove of DNA. Examination of amino acid sequences of all four subgroups of Arabidopsis CaM family reveals that all CaM proteins, except CAM7, have at least one amino acid substitution compared with CAM7 (McCormack et al., 2005). The binding of CAM7, but not CAM2/3/5, CAM7-M1, or CAM7-M2, to the Z/G-box of light-regulated promoters supports the notion that four Arabidopsis CaM isoforms have very similar amino acid sequences, substitution of amino acids in the EF-hand region may contribute to select target specificity. Consistent with this notion, the human DREAM, which has four consensus EF-hands, specifically binds to the DRE element (Carrion et al., 1999). It is worth mentioning here that the Z-box (ATACGTGT) and G-box (CACGTG) motifs recognized by CAM7 in this study have very similar (or identical in the case of the G-box) sequence to the recently identified Ca²⁺-responsive element (CACGTG[T/CA/G]) (Kaplan et al., 2006). A detailed nuclear magnetic resonance study using various isoforms and mutated versions of CAM7 in the presence or absence of Z-/G-box would address the question of how amino acid substitution alters the target specificity of CAM7.

It has been shown that change in Ca²⁺ flux plays important regulatory functions in sensing dark–light transition of Arabidopsis seedlings (Sai and Johnson, 2002). Furthermore, the role of Ca²⁺/CaM in phytochrome signaling has been postulated, and the potential connection between light and Ca²⁺/CaM signaling has started emerging, especially with the identification and functional characterization of SUB1, a Ca²⁺-binding protein operable in both cryptochrome- and phytochrome-mediated light signaling (Guo et al., 2001). Recent studies have also shown the involvement of phototropins in blue light–mediated Ca²⁺ and H⁺ fluxes (Babourina et al., 2002). The data in this study collectively provide evidence that CAM7 acts as a transcriptional regulator and promotes photomorphogenic growth and light-regulated gene expression. However, the possible role of Ca²⁺ or other divalent cation in CAM7-mediated Arabidopsis seedling development remains to be elucidated. For example, recent studies have suggested that Mg²⁺ may structurally bridge the DREAM protein to DNA, whereas Ca²⁺-induced dimerization of DREAM disrupts DREAM–DNA interactions (Osawa et al., 2005).

It has been predicted that, similar to other proteins that have interacting protein partners, mutations in CaM might result in dominant-negative effects (Veitia, 2005). Recent protein microarray analysis data also support such prediction (Popescu et al., 2007). The elongated hypocotyl phenotype and reduced expression of light-regulated genes conferred by the overexpression of CAM7-M2 could be attributed to dominant-negative interference of the light signaling pathways by CAM7-M2 protein. The alternate possibility of cosuppression of the endogenous CAM7 gene expression caused by overexpression of CAM7-M2 seems to be less likely since the cam7 mutants do not display any altered photomorphogenic growth. Furthermore, cam7 cam3 double mutants also do not display any altered morphology. However, it could be possible that overexpression of CAM7-M2 cosuppresses endogenous CAM7 gene expression and one or more additional genes of the seven-member gene family of CAM (except CAM3) or the 50-member gene family of CML (Cam-like) (McCormack et al., 2005). In either case, further study on identification and functional characterization of such genes is required to test the possibility.

HY5 is considered to be an important signal integration point of major branches downstream to all known photoreceptors (Jiao et al., 2007). The Z- and G-box have been shown to be functionally equivalent in the context of 2BF1/MYC2 and 2BF2/GBF1 transcription factors (Yadav et al., 2005; Mallappa et al., 2006). Recently, genome-wide promoter target studies using ChIP-chip analysis have revealed that the Z- and G-box sequences are
enriched in the promoter region of HY5 target genes (Lee et al., 2007). Therefore, it is possible that CAM7 and HY5 regulate the expression of a common set of downstream genes in light signaling and have partially overlapping functions in light-dependent development. Although mutations in CAM7 do not cause any visible morphological defects, the expression of light-regulated genes is downregulated in cam7 mutants (Figure 4H). It could be envisioned that a coactivator (possibly one of the other CAMs or CAM-like proteins) may recognize both CAM7 and HY5 proteins, which are already bound to the respective promoter elements. In the absence of CAM7, the coactivator and HY5 interaction might be sufficient (omitting the requirement for DNA binding of CAM7) to promote photomorphogenic growth, thus making CAM7 protein functionally redundant. However, under this condition (in the absence of CAM7), the expression of the light-regulated genes is moderately downregulated. Alternatively, functional redundancy of CAM7 may be due to the overlapping functions of light and another signaling pathway working via CAM7 protein. For example, Ca2+/Cam-mediated signaling has been shown to be involved in brassinosteroid biosynthesis and auxin signaling pathways (Yang and Poovaia, 2000; Du and Poovaia, 2005). HY5 has also been shown to act as a regulatory protein in auxin signaling (Sibout et al., 2006). Recent studies have revealed that seedlings that are deficient in gibberellin synthesis or signaling exhibit photomorphogenic growth in the darkness. Furthermore, these studies have shown that HY5 is a point of crosstalk between light and jasmonic acid signaling pathways (Alabadi et al., 2004, 2008).

This study reveals that CAM7 protein accumulates at higher levels in dark or at lower intensity of WL, which directly correlates with its physiological functions under such conditions (Figures 2 and 6). Under certain growth conditions, two bands of CAM7 were detected in protein gel blot analyses, one of which might be a posttranslationally modified form of the protein. HY5 accumulates at a lower level at lower intensities of WL, and the level of HY5 protein increases with exposure to higher intensity of WL (Osterlund et al., 2000). The overexpression of full-length or truncated HY5 is unable to promote photomorphogenic growth or derepression of light-regulated genes in the darkness (Ang et al., 1998). On the other hand, CAM7 overexpresser transgenic seedlings not only display partial photomorphogenic growth in the dark, but the light-regulated genes are also expressed under the similar conditions. Overexpression of CAM7 in hy5 transgenic lines not only partially suppresses the hy5 phenotype in light, it

Figure 6. The Accumulation of CAM7 Protein Is Altered in Dark and Light.

(A) Six-day-old wild-type (Col) or OE1 transgenic seedlings (OE1, OE2, and OE3) grown in constant dark (D) or WL were used for immunoblot analyses of CAM7 protein (using c-Myc polyclonal antibodies). The actin bands (probed by anti-actin monoclonal antibodies) indicate approximate equal loading. (B) Top panel: The visible phenotypes of seedlings grown in constant dark or various fluences of WL are shown. Six-day-old wild-type (Col) and CAM7 overexpressor transgenic seedlings (OE1) are shown alternatively from left to right. Bottom panel: The level of CAM7 protein in OE1 transgenic seedlings as described in the top panel. The actin bands indicate approximate equal loading. (C) Six-day-old OE1 transgenic seedlings grown at various wavelengths of light and at different fluence rates were used for immunoblot detection of CAM7 protein. The actin bands indicate approximate equal loading. (D) A schematic model for CAM7- and HY5-mediated regulatory pathways. The triangles indicate light intensity-dependent gradual decrease or increase of CAM7 or HY5 protein, respectively. LIGS, light-inducible genes.

The Plant Cell
also promotes photomorphogenic growth in the darkness (Figure 5B). Furthermore, accumulation of CAM7 in hy5 transgenic lines fully restored the expression of light-regulated genes (Figure 5G). Collectively, this study demonstrates that CAM7 acts as a positive regulator of photomorphogenic growth and light-regulated gene expression and highlights the concerted function of CAM7 and HY5 in Arabidopsis seedling development (Figure 6D).

**METHODS**

**Transgenic Plants and Mutants**

Plant growth and light conditions were as described by Yadav et al. (2005). The segregation ratios of self-fertilized plants heterozygous for cam7-1 or cam7-2 determined by the analyses of genotyping PCR (left border-specific primer LBP, 5'-GGTGACCGCCTGGCTGCAACT-3'; and CAM7-specific primers LP15, 5'-GACCATCCTCTCCGTTT-GTGAAC-3'; and RP15, 5'-GACCATTGTTCTGGTACAAGTCA-3') in T2 progeny suggested that a single T-DNA locus was present in cam7-1 or cam7-2 mutant lines. The junctions of T-DNA and CAM7 were amplified by PCR, and the DNA sequence analyses revealed that the T-DNA was inserted at 225 and 113 bp upstream to ATG of length (20.2-nt) GTCGAA-3', and Site-specific primer LBP: 5'-GCGTGGACCGCTGGCTGCAACT-3'.

**RNA Gel Blot Analysis**

For RNA gel blots, total RNA was extracted using the RNasey plant mini kit (Qiagen) following the manufacturer’s instructions. The DNA fragment of CAB1 or RBCS gene was used as probe as described (Yadav et al., 2005) using a random priming kit (Megaprime; Amersham). To quantify the RNA gel blot data, the intensity of each band was quantified by the Fluor-S-Multilager (Bio-Rad) and ratios of the gene versus its corresponding rRNA band were determined and plotted (Fluor-S-Multilager; Bio-Rad).

**Protein Analysis**

The ligand binding screen (DNA binding to filter-immobilized protein) was performed as described (Yadav et al., 2005). For EMSAs, CAM7 cDNA was cloned in pGEX-4T-2 vector, and GST-CAM7 was induced using 1 mM isopropyl-β-D-galactoside and overexpressed in Escherichia coli. The overexpressed GST-CAM7 was affinity purified following the manufacturer’s protocol (GE). GST-CAM3, GST-CAM7-M1, or GST-CAM7-M2 proteins were also purified similarly. EMSAs were performed as described (Mallappa et al., 2006). Protein gel blot analysis was performed using the Super Signal West Pico chemiluminescent substrate kit (Pierce) following the instructions as described in the user’s manual. Protein extracts were prepared from wild-type or transgenic seedlings. The seedlings (100 mg) were frozen in liquid nitrogen and ground in 300 μL of grinding buffer (400 mM sucrose, 50 mM Tris-Cl, pH 7.5, 10% glycerol, and 2.5 mM EDTA), and PMSF was added (0.5 μL for every 100 μL of grinding buffer). The protein extract was transferred to a fresh microcentrifuge tube and centrifuged at 5000 rpm for 5 min to pellet down the debris. The supernatant was transferred to a fresh tube, and an aliquot of 5 μL was taken out in a separate tube for the estimation of protein by Bradford assay. Proteins were separated by 10% SDS-PAGE. Prestained protein markers (GE) were used for molecular mass determination. The samples were then transferred to Hybrid C-Extra (Fermentas) at 100 mA for 2 h in transfer buffer (7.5 g Tris, 47 g glycine, and 20% methanol in 2 liters) in a mini blot protein gel apparatus (GE). The membrane was blocked with 5% milk in PBS (10 mM Na2HPO4, 1.8 mM KH2PO4, 140 mM NaCl, and 2.7 mM KCl) and probed with c-Myc polyclonal antibodies or anti-Actin monoclonal antibodies (Sigma-Aldrich).

**ChIP assays**

The ChIP assays were essentially performed as described by He et al. (2006). The sequence of primer pairs (resulting products of ~500 bp) used were as follows: NA2-PR2-00, 5'-CTATACCGTCTGGCAGAC-3'; NA2-PR2-00, 5'-AGTATCGTGCCGAATCACAGC-3'; CAMB1-PROMO-FP, 5'-GGTTTACATTGACCTCCTAGGATATTCTT-3'; CAMB1-PROMO-RP, 5'-CCTGGAATTGCTGCCACTTCC-3';

**Quantitative PCR**

Total RNA was isolated using the RNasey plant mini kit (Qiagen) extraction kit according to the manufacturer’s protocol. cDNA was synthesized from 1 μg of the total RNA using RT-AMV reverse transcriptase (Roche). Real-time PCR was performed using Light Cycler faststart DNA Master*Green I (Roche). Values were normalized with the amplification of Actin as a constitutively expressed internal control. Primers used were as follows: CAB1-FP, 5'-CCCATTTTCTGCTTGAACAC-3'; CAB1-RP, 5'-CCTGGGATGCTGAGCC-3'; RCBS-1A-FP, 5'-GAGTCATCAGACAAAG-TGAAAGG-3'; RCBS-1A-RP, 5'-CTTACCGTCTGGCAGAC-3';

**Arabidopsis thaliana Protoplast Transfection Assays**

Arabidopsis protoplasts were isolated and transfection assays were performed following the methods described by Wang et al. (2005). The mutated Z-box used in CAM7 promoter has been described by Yadav et al. (2002).
Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Comparison of Quality of Various Purified Proteins Used for EMSAs.

Supplemental Figure 2. Identification of cam7 Mutants.

Supplemental Figure 3. CAM7 and HY5 Promote Photomorphogenic Growth at Various Wavelengths of Light.

ACKNOWLEDGMENTS

We thank Asis Datta, Sudhir K. Sopory, Sunil Mukherjee, and Ashis K. Nandi for critically reading and commenting on the manuscript. This work is supported by a research grant (Ramanna Fellowship) from the Department of Science and Technology, Government of India to S.C. R.K. and A.S. are recipients of fellowships from the University Grants Commission and the Council of Scientific and Industrial Research, respectively, of the Government of India.

Received December 16, 2007; revised June 15, 2008; accepted June 27, 2008; published July 11, 2008.

REFERENCES


...


GBF1, a Transcription Factor of Blue Light Signaling in Arabidopsis, Is Degraded in the Dark by a Proteasome-mediated Pathway Independent of COP1 and SPA1

Chandrashekar Mallappa, Aparna Singh, Hathi Ram, and Sudip Chattopadhyay

From the National Institute for Plant Genome Research, Laboratory 101, Aruna Asaf Ali Marg, New Delhi 110067, India

Arabidopsis GBF1/ZBF2 is a bZIP transcription factor that plays dual but opposite regulatory roles in cryptochrome-mediated blue light signaling. Here, we show the genetic and molecular interrelation of GBF1 with two well-characterized negative regulators of light signaling, COP1 and SPA1, in photomorphogenic growth and light-regulated gene expression. Our results further reveal that GBF1 protein is less abundant in the dark-grown seedlings and is degraded by a proteasome-mediated pathway independent of COP1 and SPA1. Furthermore, COP1 physically interacts with GBF1 and is required for the optimum accumulation of GBF1 protein in light-grown seedlings. Taken together, this study provides a mechanistic view of concerted function of three important regulators in Arabidopsis seedling development.

Light is an important environmental factor for plant growth and development (1–3). Three distinct families of photoreceptors are involved in perception of various wavelengths of light: far-red and red light by phytochromes (phyA to phyE), blue and UV-A light by cryptochromes (cry1 to cry3) and phototropins (phot1 and phot2) (3–6). Recent studies demonstrate that, by altering the subcellular localization patterns, light controls the activity of photoreceptors. The cytosolic phytochromes are translocated into the nucleus upon light-mediated activation. Whereas cry2 is constitutively localized in the nucleus, cry1 is nuclear in the dark and is mostly cytoplasmic in the presence of light. cry3 has recently been shown to be transported into the chloroplast and mitochondria. Phototropins are mostly associated with the plasma membrane, and at least some fraction of phot1 is released into the cytoplasm upon light-mediated activation (7–10).

Significant progress has been made in recent years in understanding the functions of various downstream components in phytochrome signaling (1–3). However, similar information in cryptochrome-mediated blue light signaling is largely unknown (3, 5). Several regulatory proteins, including HY5, HYH, AtPP7, HFR1, SUB1, HRB1, OBP3, MYC2/ZBF1, and GBF1/ZBF2, have been reported to function in cryptochrome-mediated blue light signaling (11–19). Among these regulatory proteins, HYH, AtPP7, MYC2, and GBF1 are specifically involved in blue light-mediated photomorphogenic growth.

Photomorphogenesis is associated with several physiological responses that include: opening of apical hooks, expansion of cotyledons, inhibition of hypocotyl elongation, far-red light controlled blocking of greening, and accumulation of chlorophyll and anthocyanin (20–23). The expression of about one-third of the total genes in Arabidopsis is altered during the shift from skotomorphogenic to photomorphogenic growth (24, 25). COP1 acts as an ubiquitin ligase and helps to degrade the photomorphogenesis-promoting factors such as HY5, HYH, LAF1, and HFR1 in the dark (15, 26–30). The cop1 mutant seedlings show photomorphogenic growth in darkness, hypersensitive responses to light and less lateral roots as compared with wild-type plants (31, 32). Recent studies have shown that COP1 interacts with SPA1, and this interaction modulates the proteasome-mediated degradation of HY5, HFR1, and LAF1 (33–37). SPA1 was originally isolated as a negative regulator of far-red light signaling. The spa1 mutants are hypersensitive to far-red light, red light, and blue light (33, 38, 39).

A group of bZIP transcription factors (GBFs) has been isolated that can specifically interact with the G-box, one of the four light-responsive elements commonly found in the light-regulated promoters (40–45). The subcellular localization studies indicate that the light-controlled nuclear translocation is one of the important mechanisms for the activities of GBFs (46). However, the specific functions of most of these genes in vivo have yet to be defined. Recently, GBF1/ZBF2 has been identified in a Southwestern screen using the Z-box light-responsive element as ligand (19). The investigation of physiological functions of GBF1 has revealed that it functions in cryptochrome-mediated blue light signaling and plays a dual but opposite regulatory role in Arabidopsis seedling development (19). In this study, we show the functional relationships of GBF1 with two well-characterized negative regulators of light signaling, COP1 and SPA1, in Arabidopsis seedling development.

EXPERIMENTAL PROCEDURES

Plant Materials, Growth Conditions, and Generation of Double Mutants—Surface-sterilized seeds of Arabidopsis thaliana were sown on MS plates, stratified at 4°C in darkness for 3–5 days, and then grown in light for 3 days. The plantlets were then transferred to a controlled environment growth chamber. All the experiments were repeated two to three times. The results are representative of the experiments performed in the growth chamber.

© 2008 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the USA.
days, and transferred to specific light conditions at 22 °C. The Arabidopsis growth conditions and the intensities of continuous light sources used in this study have been described in a previous study (18). For the generation of double mutants, such as gbf1-1 cop1-4, gbf1-1 cop1-6, or gbf1-1 spa1-1 homozygous gbf1-1 mutant plants were crossed individually with cop1-4, cop1-6, or spa1-1 homozygous mutant lines. In F2 generation, seedlings were grown in WL (60 mmol m⁻² s⁻¹) for the identification of cop1-4 or cop1-6 homozygous lines, or FR (40 mmol m⁻² s⁻¹) for spa1-1 homozygous lines, and seedlings with typical short hypocotyl phenotype of cop1 mutants were selected and transferred to soil. For spa1, seedlings were kept in WL for 2 days and then short hypocotyl phenotype spa1 mutants were transferred to soil. To determine the genotype at gbf1 locus, ~40 seedlings from each line were tested by genomic PCR. F3 progeny that are homozygous for gbf1-1 mutant plants were further examined and considered as gbf1 cop1-4, gbf1 cop1-6, or gbf1 spa1 double mutants. Blocking of greening phenotype of cop1 and gbf1 cop1 was done as described before (47). Root growth and flowering phenotype experiments were carried out essentially as described (19). FR-mediated blocking of greening phenotype was carried out as described (33).

For 26 S proteasome inhibitors (MG132, MG115, and N-acetyl-leucyl-leucyl-norleucinal) and DMSO treatments, the seedlings were grown under required light conditions (as mentioned in the figure legends) and then seedlings were collected by forceps and incubated in liquid MS medium (Sigma) containing proteasome inhibitor (50 μM dissolved in DMSO) or containing 0.1% DMSO under required conditions as indicated in text for 12 h. At the end of the incubation, the seedlings were thoroughly washed with liquid MS medium three times to remove residual proteasome inhibitor or DMSO, and the seedlings were either incubated in liquid MS medium for different time points as indicated in text or frozen in liquid nitrogen for total protein extraction.

Affinity Purification of GBF1 Antibody—The antibody of GBF1 was raised against N-terminal GBF1 protein (from 1 to 172 amino acids) by Bangalore Genet, Bangalore, India. The steps of affinity purification of antibodies are given below.

Ligand coupling: 2–3 mg of 6× His-AN-ZBF2 protein was dialyzed against 13% polyethylene glycol 8000 in coupling buffer (0.2 mM NaHCO₃, 0.5 mM NaCl, pH 8.3) to concentrate to 1 mL. The top cap of the NHS-HP column was removed, and a drop of ice-cold 1 mM HCl was added to avoid air bubbles. A Hi-Trap adaptor was connected to the top of the column. Iso-propanol in the column was washed with ice-cold 1 mM HCl with a flow rate of 1 mL/min. Immediately afterward, 1 mL of ligand solution was injected into the column, and the column was left to stand for 4 h at 4 °C. Any excess active groups not coupled to the ligand were deactivated and washed out by washing with Buffer A (0.5 M ethanolamine, 0.5 M NaCl, pH 8.3) and Buffer B (0.1 M sodium acetate, 0.5 M NaCl, pH 4.0). Finally, 2 mL of phosphate-buffered saline buffer with neutral pH was injected.

Antibody purification: A blank run was performed before the experiment to ensure loosely bound ligand is washed off. The column was prepared by washing with 3 mL of start buffer (phosphate-buffered saline) and 3 mL of elution buffer (100 mM glycine, pH 2.5). The column was equilibrated with 10 column volumes of start buffer. Antibody sample was adjusted to the composition of start buffer. This was done by diluting the sample with start buffer. The sample was filtered through a 0.45 μm filter before it was applied to the column. Antibody sample was applied using a syringe fitted to luer adaptor. A flow rate of 0.2–1 mL/min was maintained. The column was incubated for 4–6 h in a cold room. Then the column was washed with 5–10 column volumes of start buffer, until no material was left in the effluent. Antibody was eluted with 1–3 mL of elution buffer. Column was re-equilibrated by washing with 5–10 column volumes of start buffer. Column is stored in storage buffer (0.05 M sodium phosphate buffer, 0.1% sodium azide, pH 7.0).

Total Protein Extraction from Arabidopsis—The seedlings (100 mg) were frozen in liquid nitrogen and ground in 300 μL of grinding buffer (400 mM sucrose, 50 mM Tris-Cl, pH 7.5, 10% glycercin, 2.5 mM EDTA), and phenylmethylsulfonyl fluoride was added (0.5 μL for every 100 μL of grinding buffer). The protein extract was transferred to fresh microcentrifuge tube and centrifuged at 5000 rpm for 5 min to pellet down the debris. The supernatant was transferred to a fresh tube, and an aliquot of 5 μL was taken out in a separate tube for the estimation of protein by Bradford assay. To the rest of the protein extract, appropriate volume of SDS sample buffer (200 mM Tris-Cl, pH 6.8, 400 mM dithiothreitol, 4% SDS, 0.025% Bromphenol Blue, 20% glycerol), i.e. 1/4th of grinding buffer = volume of 4× sample buffer, was added and boiled for 5 min before loading on SDS-PAGE.

Western Blot Analysis—Western blotting was performed using the SuperSignal West Pico chemiluminescent substrate kit (Pierce) and following the instructions as described in the user’s manual provided by the manufacturer. The samples were then run on SDS-PAGE gel and transferred to Hybond ECL nitrocellulose membrane (GE Healthcare) at 100 mA for 2 h in transfer buffer (Tris (7.56 g), glycine (47 g), 20% methanol in 2.5 liters) in Mini blot protein gel apparatus (GE Healthcare). 40 μg of total protein was used for Western blot analysis. The membrane was stained with Ponceau-S to confirm the protein transfer and washed with sterile milli-Q water. The membrane was then incubated for 1 h in 2 mL of blocking buffer (5% nonfat dry milk in Tris-buffered saline and 0.05% Tween 20) at room temperature with shaking. The blocking reagent was removed, and the affinity-purified primary antibody diluted (1:300 to 1:500) in 15 mL of blocking buffer with 0.05% Tween 20 was added and incubated for 2 h with shaking at room temperature. The membrane was then washed with 15 mL of wash buffer (Tris-buffered saline and 0.05% Tween 20) for thrice, 5 min each. The secondary antibody, conjugated with horseradish peroxidase diluted (1:20,000) in 15 mL of blocking buffer with 0.05% Tween 20, was added and incubated for 1 h with shaking at room temperature. The membrane was washed with 15 mL of wash buffer for five times at room temperature. The working solution of substrate was prepared by mixing peroxide solution and luminol/enhancer solution in 1:1 ratio, and the blot was incubated in that working solution for 5 min in darkness. The blot was then removed from the working solution and covered with plastic wrap in a cassette and exposed to x-ray film for different times.
**RESULTS**

The Photomorphogenic Growth of *cop1* Mutants in Dark Is Enhanced in *gbf1* *cop1* Double Mutants—Acting as an E3 ubiquitin ligase, *COP1* helps to degrade or stabilize several regulatory proteins in darkness and thereby suppresses photomorphogenesis. The *cop1* mutants display photomorphogenic growth in the dark and hypersensitive responses to various wavelengths of light. We ask whether the BL-specific transcription factor, GBF1, which plays both positive and negative regulatory roles in light signaling, is functionally related to *COP1*. Among the various alleles of *cop1* mutants identified and characterized, *cop1-4* and *cop1-6* mutants are relatively weak alleles, and *cop1-6* mutants display shorter hypocotyl than *cop1-4* in the darkness. Because the null alleles of *cop1* are seedling-lethal, we chose *cop1-4* and *cop1-6* mutants for our studies. Whereas *cop1-4* encodes a truncated *COP1* terminat at amino acid 282, *cop1-6* allele contains an in-frame five-amino acid insertion between codons 301 and 302.

To determine the genetic interactions between *COP1* and *GBF1*, we constructed *gbf1-1 cop1-4* and *gbf1-1 cop1-6* double mutants and investigated the morphology of the double mutants in darkness and various light conditions. Although *gbf1* seedlings did not exhibit any altered morphology in darkness, *gbf1* *cop1* double mutants exhibited shorter hypocotyl than *cop1* mutants, suggesting that *gbf1* and *cop1* function synergistically in repressing photomorphogenic growth in the darkness (Fig. 1A). We then examined the growth of 6-day-old *gbf1* *cop1* double mutants under various light conditions, including red light (RL), far-red light (FR), and blue light (BL). The *gbf1* *cop1* double mutants were significantly shorter than *cop1* or *gbf1* single mutants in BL (Fig. 1B), indicating an additive effect of *gbf1* and *cop1* mutations on BL-mediated inhibition of hypocotyl elongation. However, in WL (white light), RL, or FR, *gbf1* *cop1* seedlings were morphologically indistinguishable from *cop1* single mutants, suggesting that *cop1* is epistatic to *gbf1* in WL-, RL-, or FR-mediated inhibition of hypocotyl elongation (Fig. 1, C–E).

Although GBF1 plays a negative regulatory role in BL-mediated inhibition of hypocotyl elongation, it acts as a positive regulator of cotyledon expansion. The cotyledons of *gbf1* mutants are less expanded compared with corresponding wild-type seedlings (19). The BL-grown *cop1-4* mutant seedlings have more expanded cotyledons than wild type, however this effect is less prominent in *cop1-6* mutant seedlings. Examination of the cotyledon size revealed that *gbf1* *cop1* double mutants have cotyledon size similar to wild-type seedlings suggesting that GBF1 and *COP1* function antagonistically in regulating the BL-mediated cotyledon expansion (Fig. 1F).

The Fusca and Blocking of Greening Phenotypes of *cop1* Are Further Enhanced in *gbf1* *cop1* Double Mutants—The *cop1* mutants display dark purple color fusca phenotype due to high level accumulation of anthocyanin, however such effects are not visible in *gbf1* mutants. The number of fusca phenotype, when examined, was dramatically increased in *gbf1* *cop1* double mutants as compared with *cop1* single mutants. Although the effect was only visible in *gbf1* *cop1-6* double mutants at lower fluence rates of WL, both *gbf1* *cop1-4* and *gbf1* *cop1-6* double

**Interplay of GBF1, COP1, and SPA1**

The actin band probed with anti-actin (Catalog no. A0A80, Sigma) antibodies was used as a loading control.

**Protein-Protein Interaction Studies—In vitro binding assay:** GST and GST-GBF1 fusion proteins were expressed in *Escherichia coli* strain (BL21/DE3) and purified using glutathione-agarose beads (GE Healthcare) as described (18). About 5 mg of GST or GST-GBF1 was bound to the glutathione-Sepharose 4B beads by incubating for 2 h at 4 °C. Beads were washed and incubated with COPI-6His (5 mg) for overnight at 4 °C. Beads were washed thrice with the binding buffer, boiled with the loading dye, and loaded onto the SDS-PAGE. The blot was probed with anti-COP1 antibodies. To generate constructs for yeast two-hybrid assays, full-length GBF1 and HY5 were cloned into pGADT7 vector with ECORI-BamHI and NdeI-Clal restriction sites, respectively, to produce translational fusions with the activation domain. To generate DBD-COP1, an EcoR1-Pst1 PCR fragment of full-length COP1 was cloned into the corresponding sites of the vector pGBK7T (Clontech) to produce translational fusion with DNA binding domain. The constructs were transformed into Yeast strain AH109 according to the Clontech protocol. Expression of AD-GBF1 and AD-HY5 fusion proteins were examined by hemagglutinin and DBD-COP1 by c-Myc antibodies. The protein-protein interactions were examined by β-galactosidase assays. The relative β-galactosidase activities were calculated according to Clontech instructions. *In vitro* The coimmunoprecipitation experiment was as follows: ~5 mg of anti-GST antibodies was bound to protein A-agarose beads by incubating for 6 h at 4 °C. After washing the beads bound to the antibodies, GST-GBF1 and COPI-6His proteins were added and incubated for overnight at 4 °C in a 250-ml reaction volume. Beads (pellet) were washed thrice with the binding buffer, boiled with the loading dye, and loaded separately along with the supernatant onto SDS-PAGE. The blot was probed with anti-COP1 antibodies.

**Chlorophyll and Anthocyanin Measurements—** Chlorophyll and anthocyanin levels were measured following protocols as described in a previous study (15).

**Quantitative Real-time PCR Analyses—** Wild-type and different mutant seedlings were grown under various conditions. Total RNA was extracted from seedlings at different time points using an RNAeasy Plant Mini Kit (Qiagen), and cDNA were synthesized from total RNA using a Titan Master mixes (Roche Applied Science) following the manufacturer’s instructions. Real-time PCR analyses of gene expression were carried out by using LightCycler® FastStart DNA MasterPLUS SYBR Green 1 (Roche Applied Science) and LightCycler® 2.0 system (Roche Applied Science). Primers used for PCR amplification of *CAB1* were 5'-TTGCTGTTCTTGCCCTTGTTCTTTGG-3' and 5'-TCTTGGGATCGCTGAGAATCTCC-3'; primers for *RBCS-IA* were 5'-GAATTCCAAAAGATGTGAAGAAGAAG3' and 5'-CTTACCACTTGCGAATGCTG-3'; primers for *ADHI* were 5'-CTTACACGAGAAATATCGTCGAGACGAAGG-3' and 5'-GGATCTCAGAGGATGTTCTTCTCT-3'; primers for *ACTIN2* were 5'-GCTTCCTAAAAAGCTTCTCTTCT-3' and 5'-GCTCGTATGCAAGCACAAGC-3'. The transcript levels are normalized to the level of *ACTIN2* transcript abundance.

[35774 JOURNAL OF BIOLOGICAL CHEMISTRY](http://www.jbc.org) VOLUME 283 • NUMBER 51 • DECEMBER 19, 2008
mutants displayed higher percentage of fusca phenotype at higher intensity of WL. (Fig. 2, A and B). Consistent with this observation, the quantification of anthocyanin levels revealed an increase in anthocyanin accumulation in gbf1 cop1 double mutants as compared with cop1 alone (Fig. 2C).

The dark-grown cop1 mutants are sensitive to high intensity light, and some of them do not turn green when transferred to light. The COP1-mediated blocking of greening phenotype becomes more intense with longer incubation in the darkness (47). We examined the blocking of greening effects in gbf1 cop1 double mutants. A lower percentage of gbf1 cop1 double mutants than cop1 alone were able to turn green when 5-day-old dark-grown seedlings were transferred to light (Fig. 2D), suggesting that gbf1 can enhance the blocking of greening phenotype of cop1. Examination of chlorophyll contents reveals that the chlorophyll content of gbf1 cop1 double mutants was lower than either of the single mutants (Fig. 2E).

The Altered Root Growth and Flowering Time of cop1 Mutants Are Enhanced by Additional Loss of Function of GBF1—Both cop1 and gbf1 plants show less number of lateral roots compared with wild-type plants, although the effect is more pronounced in cop1 mutants. To determine the genetic relationships between cop1 and gbf1 mutant plants in lateral root formation, we examined the root growth of 16-day-old gbf1 cop1 double mutants and compared with cop1 or gbf1 single mutants. As shown in Fig. 3A, gbf1 cop1 double mutants developed fewer lateral roots compared with gbf1 or cop1 single mutants. There was hardly any lateral root visible in gbf1 cop1-6 double mutants up to 16 days. Mutations in GBF1 or COP1 result in early flowering under long day conditions. Examination of flowering time in gbf1 cop1 double mutants revealed that the early flowering phenotype of gbf1 is significantly (p < 0.02) enhanced in gbf1 cop1 double mutant background under long day conditions (Fig. 3B).

GBF1 and SPA1 Act in an Independent and Interdependent Manner in BL-mediated Photomorphogenic Growth—The light-specific negative regulator, SPA1, has been shown to be functionally associated with COP1 in degradation of photomorphogenesis-promoting factors in the dark. The loss-of-function mutants of SPA1 do not show any morphological defects in the darkness; however, spa1 mutants show hypersensitive response to far-red, red, and blue light. Three alleles of
spa1 mutant were originally identified, which showed similar morphological defects. Among these, in spa1-1 mutants, SPA1 carries a single base pair substitution resulting in a stop codon, and thereby produces a 848-amino acid truncated protein. Because analyses of gbf1 cop1 double mutants reveal functional interrelation between GBF1 and COP1, we ask whether GBF1 is also functionally connected to SPA1.

To examine possible genetic interactions between gbf1 and spa1, we constructed gbf1-1 spa1-1 double mutants and examined their growth in darkness and various light conditions. The dark-grown gbf1 spa1 double mutant seedlings showed wild-type phenotype, similar to gbf1 or spa1 single mutants. In WL and BL, the enhanced inhibition of hypocotyl elongation caused by gbf1 mutations was found to be similar to spa1. The gbf1 spa1 double mutants displayed hypocotyl length similar to gbf1 or spa1 single mutants, suggesting that gbf1 and spa1 act independently in WL- and BL-mediated inhibition of hypocotyl elongation (Fig. 4, A and C). However, under RL or FR, gbf1 spa1 double mutants showed similar hypocotyl lengths to spa1, suggesting spa1 is epistatic to gbf1 in RL- or FR-mediated inhibition of hypocotyl elongation (Fig. 4, B and D). Similar to cop1 mutants, spa1 mutants also display more expanded cotyledons in BL. The cotyledon size of gbf1 spa1 double mutants was found to be similar to spa1 in BL, suggesting that spa1 is epistatic to gbf1 in BL-mediated cotyledon expansion (Fig. 4E).

Genetic Interactions between GBF1 and SPA1 Modulate Physiological Responses and Root Growth—When grown in RL and FR, spa1 mutants accumulate higher levels of anthocyanin than wild-type seedlings. Although gbf1 mutants do not display significant difference in anthocyanin accumulation as compared with wild type, the anthocyanin content of gbf1 spa1 double mutants was drastically increased as compared with corresponding single mutants in BL (Fig. 5A). On the other hand, accumulation of anthocyanin in gbf1 spa1 double mutants was found to be similar to spa1 alone in WL and FR (Fig. 5, B and C). Pre-exposure to FR of Arabidopsis seedlings prevent greening when seedlings are subsequently exposed to WL. To determine the FR-mediated blocking of greening effect in gbf1 spa1 double mutants, we grew the seedlings in FR for 3 days and then transferred them to white light. Whereas gbf1 or spa1 mutant seedlings showed 20–80% of pale green phenotype with no visible...
bleaching effects, >40% gbfl spa1 double mutants were completely bleached out (Fig. 5D). However, because gbfl and spa1 mutants are in different ecotype backgrounds, the slight differences in bleaching effects may be attributed to such background differences. Measurement of chlorophyll contents showed dramatic reduction in accumulation of chlorophyll in gbfl spa1 double mutants as compare with gbfl or spa1 single mutants (Fig. 5F).

To determine whether mutation in SPA1 could modulate the lateral root formation in gbfl, we examined the root growth of gbfl spa1 double mutants. Examination of root growth of 16-day-old adult plants revealed that spa1 single mutants also produced less lateral roots similar to gbfl mutants, and the effect was more severe in spa1 mutants than gbfl (Fig. 5F). The number of lateral roots formed in gbfl spa1 double mutants was found to be between the number of lateral roots formed in gbfl or spa1 single mutants at 16-day-old plants, suggesting that GBF1 and SPA1 function antagonistically in controlling the lateral root formation (Fig. 5F).

The Modulation of Light-inducible Gene Expression by GBF1, COP1, and SPA1—The light-mediated induction of CAB and RBCS gene expression is differentially regulated by GBF1. Whereas GBF1 acts as a positive regulator of CAB, it acts as a negative regulator of RBCS gene expression. To determine the effect of genetic interactions between gbfl and cop1 or spa1 mutants on light-regulated gene expression, we monitored the induction of CAB1 and RBCS-1A genes in BL by quantitative real-time PCR. For this experiment, 5-day-old dark-grown seedlings were exposed to BL for 8 h. In dark-grown seedlings, the expression of CAB1 was found to be significantly higher in cop1 or spa1 mutants as compared with gbfl or wild-type backgrounds. Similar higher level expression was detected in corresponding double mutants (Fig. 6A). Consistent with the earlier results (19), the BL-mediated induction of CAB1 was significantly reduced in gbfl mutants as compared with wild type (Fig. 6A). The higher level induction of CAB1 in cop1 mutants was also reduced in gbfl cop1 double mutants after 8-h exposure to BL, suggesting that gbfl and cop1 may function antagonistically in regulating the expression of CAB1. On the other hand, the level of induction of CAB1 was significantly elevated in spa1 mutants as compared with wild type and remained about the same in gbfl spa1 double mutants with no detectable effect of
Interplay of GBF1, COP1, and SPA1

gbf1 mutation (Fig. 6A). These results demonstrate that SPA1 acts as a negative regulator of CAB1 expression and spa1 is epistatic to gbf1 in BL-mediated induction of CAB1 expression.

The level of expression of RBCS-IA was found to be higher in cop1 and also in gbf1 cop1 double mutants in dark-grown seedlings (Fig. 6B). However, no elevated level of expression of RBCS-IA was detected either in spa1 or gbf1 spa1 double mutants in darkness. The light-induced expression of RBCS-IA was significantly elevated in gbf1 cop1 double mutants as compared with gbf1 or cop1 single mutants, suggesting an additive function of GBF1 and COP1 in the regulation of RBCS-IA gene expression in BL. Similar additive function of GBF1 and SPA1 was also detected in BL-mediated induction of RBCS-IA gene expression (Fig. 6B).

GBF1 Accumulates at a Lower Level in Dark-grown Seedlings—The stability of the regulatory proteins plays an important role in light-mediated seedling development. Earlier studies have shown that GBF1 mRNA was present at higher level in darkness as compared with WL-grown seedlings. Furthermore, it has also been shown that the accumulation of GBF1 protein remains at the similar levels in darkness and lower intensity of WL. To further test and expand our understanding about the pattern of accumulation of GBF1 protein in wild-type background, we grew the seedlings in darkness or at various fluence rates of WL and performed immunoblot analyses. It is worth mentioning here that, although the affinity-purified antibody to GBF1 is sufficiently specific to monitor the level of GBF1 protein, it cross-reacted with a protein band (also present in gbf1 null mutant background) that migrates just below GBF1. Furthermore, to examine whether the affinity-purified antibody of GBF1 used in this study was able to cross-react with GBF2 or GBF3 proteins, we performed Western blot analyses using purified GST-GBF1, GST-GBF2, and GST-GBF3 proteins. However, no cross-reactivity was detected (Fig. 7E). As shown in Fig. 7A, whereas the GBF1 protein accumulated to a lower level in darkness or at lower intensity of WL, the level of GBF1 protein increased at higher fluence rates of WL. To determine kinetics of accumulation of GBF1 protein, we transferred 4-day-old dark-grown seedlings to WL for various time points. GBF1 protein was detectable at lower levels in dark-grown seedlings, and the level of accumulation of the protein increased with longer exposure to WL (Fig. 7B). These results collectively suggest that GBF1 protein accumulates to a lower level in darkness and at lower intensity of WL, however it accumulates at higher levels at higher fluence rates of WL.

Because GBF1 is a BL-specific transcription factor, we wanted to determine whether the light-mediated accumulation of GBF1 protein is specific to a particular wavelength of light. To address this question, seedlings grown in the constant darkness or various wavelengths of light were used. As shown in Fig. 7C, although slightly increased levels of accumulation of GBF1 protein were detected in RL and FR, the level of accumulation was found to be maximum in WL and BL. To further test these results, we transferred dark-grown seedlings to RL, FR, or BL for various time points and monitored the GBF1 protein level. Similar to WL, GBF1 protein accumulated at higher levels in seedlings exposed to BL (Fig. 7D). Whereas exposure to RL showed a weak increase in the level of accumulation of GBF1 protein, there was hardly any increased level of accumulation of the protein in FR (Fig. 7D). Taken together, these results suggest that light-mediated accumulation of GBF1 is more prominent in BL and WL.

Degradation of GBF1 in Dark Is Independent of COP1 and SPA1—To determine the possible roles of COP1 or SPA1 in the accumulation of GBF1 protein, we examined the level of GBF1 in cop1 or spa1 mutant backgrounds in dark- and light-grown conditions. As shown in Fig. 8A, the level of GBF1 protein was similar in cop1, spa1, and wild-type seedlings in darkness, suggesting that COP1 or SPA1 are not involved in the reduced stability of GBF1 protein in the darkness. In contrast, the level of GBF1 protein was significantly reduced in WL-grown cop1 or spa1 mutants as compared with wild-type seedlings (Fig. 8B). However, the effect of cop1 or spa1 mutations was not seen at lower fluence rates of WL (Fig. 8B). Because the accumulation of GBF1 was found to be higher in BL similar to WL, we examined whether COP1 and SPA1 were also required for the stability of GBF1 in BL. We performed immunoblot experiments using seedlings grown in BL. As shown is Fig. 8C, whereas there was a slight reduction (if any) in the level of GBF1 protein in spa1 mutants, a drastic reduction in the accumulation of GBF1 was detected in cop1 mutant backgrounds in BL. The GBF1 protein levels did not alter in hy5 and hyh mutant backgrounds.
Interstitial of GFB1, COP1, and SPA1

5-day-old dark-grown wild-type or cop1 mutant seedlings were treated with MG132 or mock treated with 0.1% DMSO for 12 h. The seedlings were then washed in the dark and incubated in darkness for different time points. Total protein was extracted and subjected to immunoblot analysis. As shown in Fig. 9 (B–D), significantly higher levels of GFB1 protein were detected up to 6 h after the treatment of MG132 as compared with DMSO treatment both in wild-type and cop1 mutants. Furthermore, the level of GFB1 protein declined similarly at 12 h either in wild-type or cop1 mutant background. These results demonstrate that GFB1 protein is subject to 26S proteasome-mediated proteolysis in dark-grown seedlings. These results, taken together with the results in Fig. 8A, further demonstrate that the 26S proteasome-mediated degradation of GFB1 in the dark is indeed independent of COP1.

To determine whether lower level accumulation of GFB1 protein at lower fluence rates of WL is not due to 26S proteasome-mediated proteolysis.

**GFB1 Physically Interacts with COP1**—To determine whether the requirement of COP1 for the optimum level of accumulation of GFB1 protein is through direct physical interactions between these two proteins, we carried out protein-protein interaction studies. To examine such possible physical interactions, first an in vitro binding experiment was performed. As shown in Fig. 10A, the amount of COP1 retained by GST-GFB1 beads were significantly higher than the background level of COP1 retained by the control GST beads. Because equal amounts of GST-GFB1 or GST were used in the binding experiments, these results indicate a direct protein-protein interaction between GFB1 and COP1 in vitro. To further substantiate the observed in vitro COP1 and GFB1 interaction, a yeast two-hybrid protein-protein interaction assay was carried out. As shown in Fig. 10B, chimeric fusion protein of the GAL4 activation domain and GFB1 (AD-GFB1) or HY5 (AD-HY5; used as a control) activated transcription of the lacZ reporter gene in the presence of GAL4 DNA-binding domain-COP1 fusion protein (BD-COP1) but not with GAL4 DNA binding domain (BD) alone, suggesting a direct protein-protein

---

**FIGURE 8. Accumulation of GFB1 protein in cop1 and spa1 mutant backgrounds.** Immunoblots of anti-actin (Actin) is shown below as loading control. The arrow indicates GFB1 protein band. A, immunoblot using 6-day-old constant dark-grown wild-type and various mutant seedlings. B, immunoblot using 6-day-old constant WL (5 or 100 μmol m⁻² s⁻¹) grown wild-type (Col) and various mutant seedlings. C–E, immunoblot using constant BL, FR, or RL-grown wild-type (Col) and various mutant seedlings, respectively.
interaction between COP1 and GBF1 in yeast cells. Finally, we performed an in vitro communoprecipitation experiment to further substantiate the physical interaction between GBF1 and COP1 proteins. As shown in Fig. 10C, GBF1 interacted with COP1 in an in vitro interaction assays using recombinant proteins. Approximately 11% of the added COP1 protein was communoprecipitated with GST-GBF1, whereas <0.8% of the added COP1 was able to bind to GST alone. Taken together, these results demonstrate that GBF1 can physically interact with COP1.

**DISCUSSION**

In this study, we establish the genetic and molecular relationships of a BL-specific transcription factor, GBF1, with two well-characterized negative regulators of light signaling, COP1, and SPA1 in photomorphogenic growth and light-regulated gene expression. This study further demonstrates that GBF1 protein is less abundant in the dark-grown seedlings and is degraded by a proteasome-mediated pathway independent of COP1 and SPA1. On the other hand, COP1 physically interacts with GBF1 and is required for the optimum accumulation of GBF1 protein in light-grown seedlings (Fig. 10D).

**The Integrated Functions of GBF1, COP1, and SPA1 in Photomorphogenic Growth and Light-regulated Gene Expression**—The double mutant analyses reveal that GBF1 and COP1 function redundantly in the dark to suppress photomorphogenic growth. The enhanced inhibition of hypocotyl elongation displayed by gbf1 mutants in WL requires functional COP1, however not in BL (Fig. 1). Thus, whereas GBF1 requires COP1 function in darkness to suppress photomorphogenic growth, these two proteins are likely to function in parallel pathways and in an additive manner to suppress photomorphogenic growth in BL. GBF1 and SPA1 appear to act as independent negative regulators in WL-mediated inhibition of hypocotyl elongation (Fig. 4). GBF1 acts as a positive regulator of cotyledon expansion and the expanded cotyledon phenotype of cop1-4 mutants was completely suppressed in gbf1 cop1 double mutants in BL. Therefore, GBF1 and COP1 act antagonistically in BL-mediated cotyledon expansion. On the other hand, gbf1 spa1 double mutants exhibit similar cotyledon expansion as spa1 single mutants suggesting that the positive regulatory function of GBF1 requires functional SPA1.

The differential regulation of GBF1 in CABI and RBSCS-1A gene expression is almost nullified in gbf1 cop1 and gbf1 spa1 double mutants, because both the genes are expressed at higher levels in double mutant backgrounds (Fig. 6). However, the light-mediated enhanced expression of CABI in cop1 mutants was significantly reduced in gbf1 cop1 double mutants suggesting that COP1 and GBF1 might play antagonistic roles in the regulation of CABI gene expression. The expression of CABI was deregressed in the dark in spa1 mutants, however no such effect of spa1 mutation was detected on RBSCS-1A gene expression. The higher level expression of RBSCS-1A in cop1 or gbf1 mutants was further enhanced in gbf1 cop1 and gbf1 spa1 double mutants. Therefore, GBF1 functions in an additive manner with COP1 or SPA1, and thus likely acts in parallel pathways to regulate the expression of RBSCS-1A.

**Accumulation of GBF1 Depends on the Presence of COP1 and SPA1 in Light-grown Seedlings**—Light-regulated shuttling of COP1 between the nucleus and cytoplasm is an important regulatory mechanism of light-mediated seedling development (3, 27). COP1 degrades several photomorphogenesis promoting factors in the dark to suppress photomorphogenic growth in the darkness. SPA1 is an associated factor of COP1 for the degradation of some of these regulatory proteins such as HY5, LAF1, and HFR1 (28, 29, 33–36). On the contrary, COP1 positively regulates PIF3 accumulation in darkness (48).

Our results demonstrate that, whereas COP1 and SPA1 are not associated with the stability of GBF1 protein in the dark, functional COP1 protein is required to maintain the stability of GBF1 protein in WL and BL. This study further demonstrates that GBF1 physically interacts with COP1, suggesting a direct role of COP1 in maintaining the stability of GBF1 protein. GBF1 plays both negative and positive regulatory roles in Arabidopsis seedling development. Higher level accumulation of GBF1 results in elongated hypocotyls, however more expanded cotyledons (19). Therefore, a fine-controlled level of GBF1 is likely to be essential to obtain light-mediated optimum photo-
morphogenic growth. Because GBF1 protein accumulates at higher levels with higher fluence rates of WL in a COP1-dependent manner, it could be envisioned that light-induced translocation of COP1 between nucleus and cytosol might be a potential mechanism to control the level of GBF1 protein and thereby its function in photomorphogenesis.

The mechanism of differential regulatory function of GBF1, COP1, and SPA1 might be that they differentially degrade or stabilize the proteins with positive or negative regulatory functions (for example HY5 and PIF3) and thereby differentially regulate the expression of the target genes. GBF1, on the other hand, might function either as a transcriptional activator or repressor, depending on the promoter determinants of the target genes. Alternatively, it could be envisioned that hetero-dimerization of GBF1 with other bZIP proteins (such as HY5 or HYH) might be a potential mechanism to generate positive and negative regulators, which in turn play positive or negative regulatory roles in signaling cascades. In either case, further study is required to test the possibility.

REFERENCES

**Interplay of GBF1, COP1, and SPA1**