

RESEARCH ARTICLES

Calmodulin7 Plays an Important Role as Transcriptional Regulator in *Arabidopsis* Seedling Development^W

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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 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 (Corneliusen 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 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 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 Hypocotyl 5 (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

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gene expression (Neuhaus et al., 1993, 1997; Bowler et al., 1994). All these studies suggest that Ca^{2+} /CaM is involved in light-mediated seedling development and gene expression. However, the molecular and physiological function of CaM or structurally related Ca^{2+} binding protein, which interprets and specifically transduces the information into appropriate cellular responses, remains largely unknown (Veitja, 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 high 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 overexpresser 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 in the dark, the expression was strikingly elevated in transgenic seedlings. To determine the light-controlled expression of the *CAB* or *RBCS* gene, 5-d-old dark-grown seedlings were transferred to WL for various time points. The level of expression was further elevated in transgenic seedlings compared with wild-type background (Figure 3B). Taken together, these results provide evidence that CAM7 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 coimmunoprecipitated with

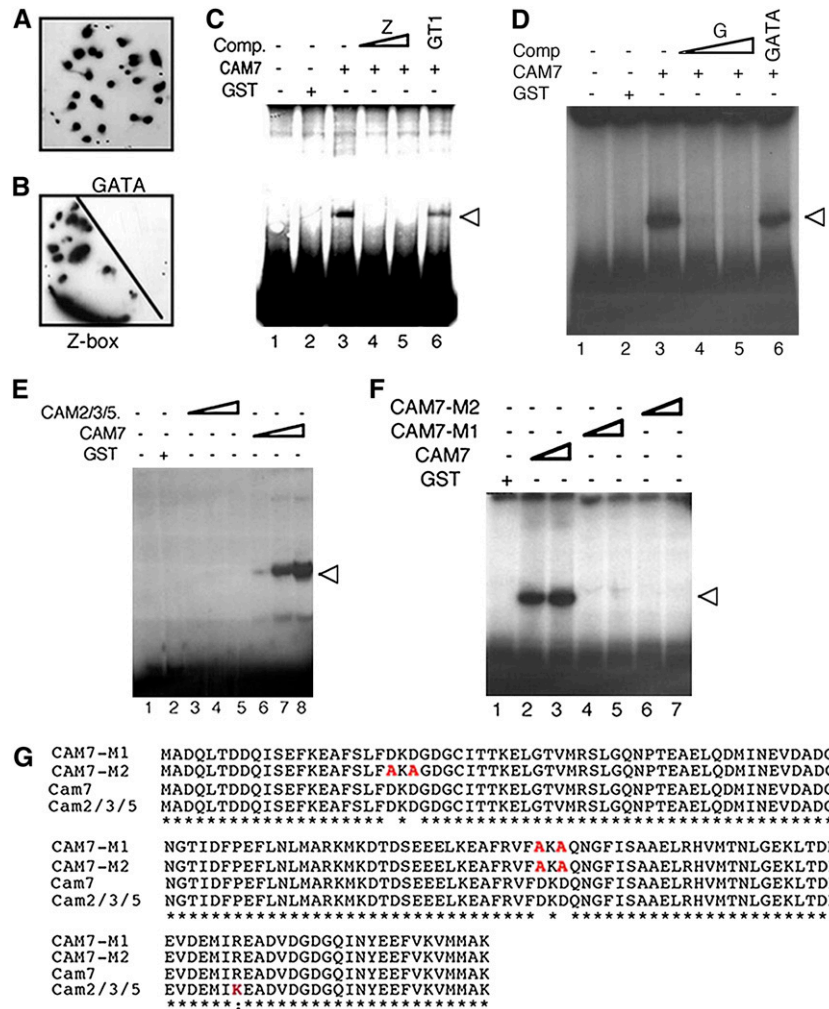


Figure 1. CAM7 Binds to the Essential Z-/G-Box of *CAB1* or *RBCS-1A* Minimal Promoter.

(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 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.

(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 μ g, and 3 μ g (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.

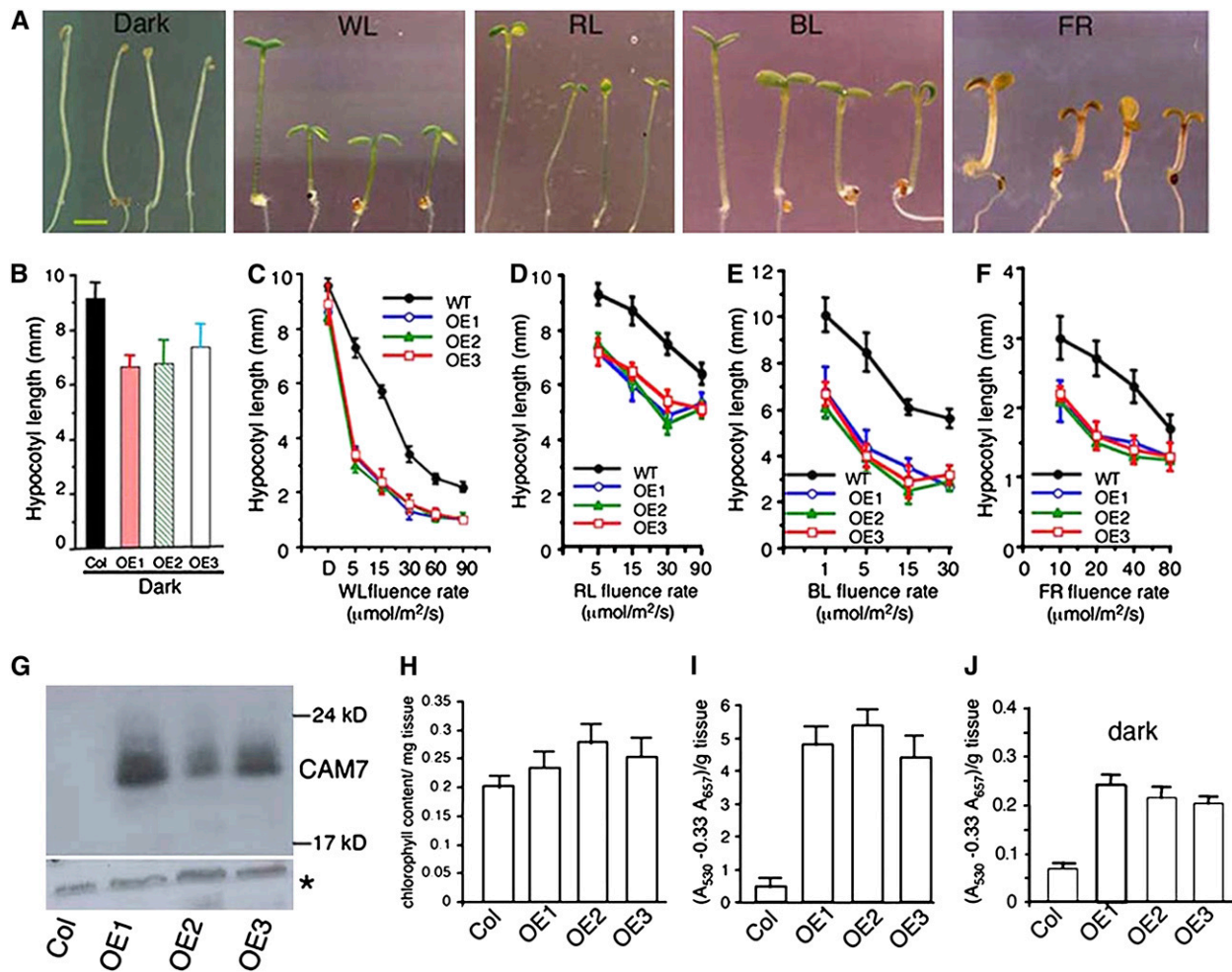


Figure 2. CAM7 Promotes Photomorphogenic Growth.

(A) The visible phenotypes of the seedlings grown in constant dark, WL ($15 \mu\text{mol}/\text{m}^2/\text{s}$), RL ($30 \mu\text{mol}/\text{m}^2/\text{s}$), BL ($20 \mu\text{mol}/\text{m}^2/\text{s}$), or FR ($40 \mu\text{mol}/\text{m}^2/\text{s}$) 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 ($30 \mu\text{mol}/\text{m}^2/\text{s}$) is shown.

(I) and (J) Accumulation of anthocyanin in 6-d-old wild-type or transgenic seedlings grown in WL ($30 \mu\text{mol}/\text{m}^2/\text{s}$) 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

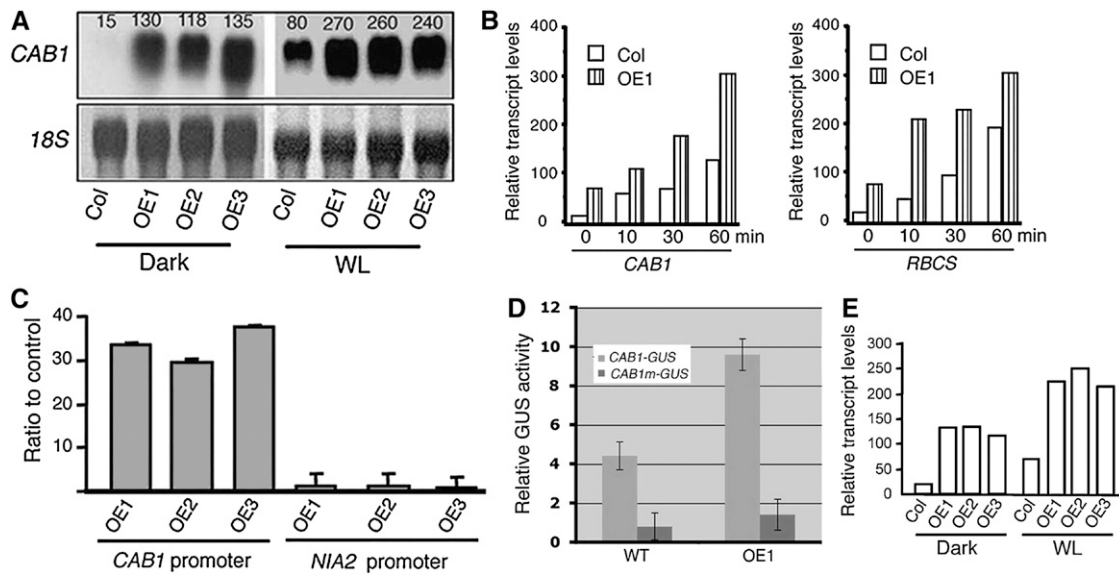


Figure 3. CAM7 Interacts with *CAB1* Promoter in Vivo and Promotes Light-Regulated Gene Expression.

(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 ($30 \mu\text{mol}/\text{m}^2/\text{s}$). 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-Multimager (Bio-Rad), and ratios of *CAB1* versus its corresponding rRNA band were determined and plotted (Fluor-S-Multimager; 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 ($30 \mu\text{mol}/\text{m}^2/\text{s}$) for various time points.

(C) ChIP assays of *CAB1* promoter from OE1, OE2, or OE3 transgenic seedlings 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 overexpresser transgenic seedlings to nontransgenic control plants.

(D) Expression of *CAB1-GUS* or *CAB1m-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 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

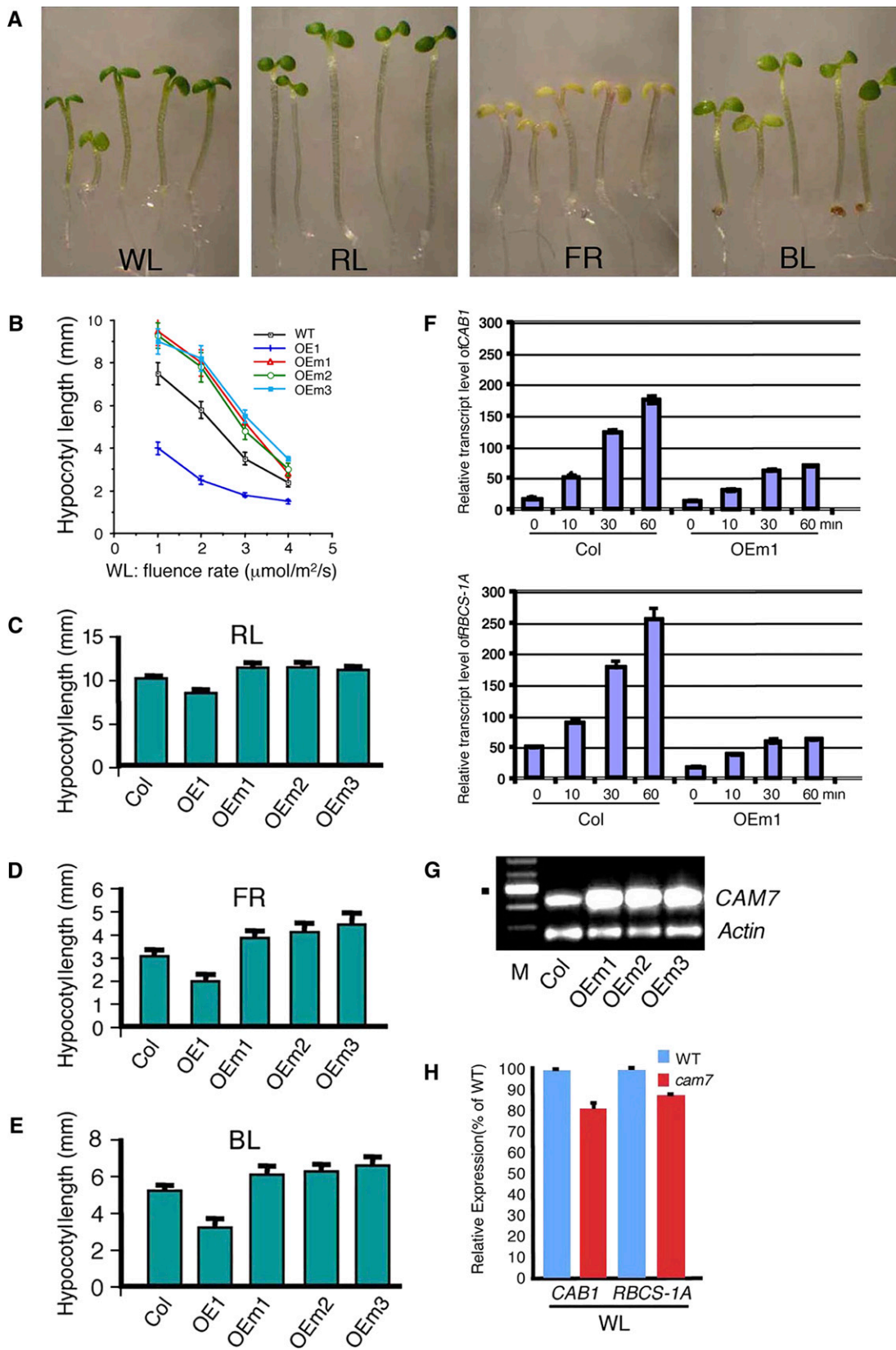


Figure 4. Overexpression of CAM7-M2 Results in Suppression of Photomorphogenic Growth Irrespective of Light Qualities.

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* overexpresser transgenic seedlings for immunoblot analysis. The accumulation of CAM7 protein was significantly reduced in WL

Figure 4. (continued).

- (A)** Visible phenotypes of 6-d-old wild-type (Col), and transgenic seedlings grown in various light conditions. In each panel, wild-type (Col), OE1, OEm1, OEm2, and OEm3 seedlings are shown from left to right, respectively.
- (B) to (E)** Quantification of hypocotyl length of 6-d-old seedlings grown in WL, RL (60 $\mu\text{mol}/\text{m}^2/\text{s}$), FR (40 $\mu\text{mol}/\text{m}^2/\text{s}$), or BL (20 $\mu\text{mol}/\text{m}^2/\text{s}$). For each measurement of hypocotyl length, 25 to 30 seedlings were used. The error bars indicate SD. All the samples were significantly different from the wild type at each light condition ($P < 0.01$; $n = 3$).
- (F)** The abundance of *CAB1* and *RBCS-1A* transcripts in total RNA from wild-type (Col) and CAM7-M2 overexpresser transgenic seedlings (OEm1) grown in dark (0) for 5 d and then transferred to white light (30 $\mu\text{mol}/\text{m}^2/\text{s}$) for various time points was determined by quantitative real-time PCR, and the transcript levels were normalized to the level of *ACTIN2* transcript abundance. Error bars represent SD ($P < 0.01$ between Col versus OEm1 at each time point exposed to light; $n = 3$).
- (G)** RT-PCR results (using *CAM7*-specific primers) show the level of expression of *CAM7-M2* in overexpresser transgenic lines (OEm1, OEm2, and OEm3) or in the corresponding wild-type (Col) background. Actin band shows the loading control. M indicates molecular weight markers (100-bp ladder), and the dot shows a DNA fragment of 500 bp.
- (H)** Real-time quantitative PCR results show the relative expression of *CAB1* and *RBCS-1A* in 6-d-old wild-type versus *cam7* mutant seedlings ($P < 0.03$ between the wild type versus *cam7* mutants; $n = 4$) grown in WL (30 $\mu\text{mol}/\text{m}^2/\text{s}$).

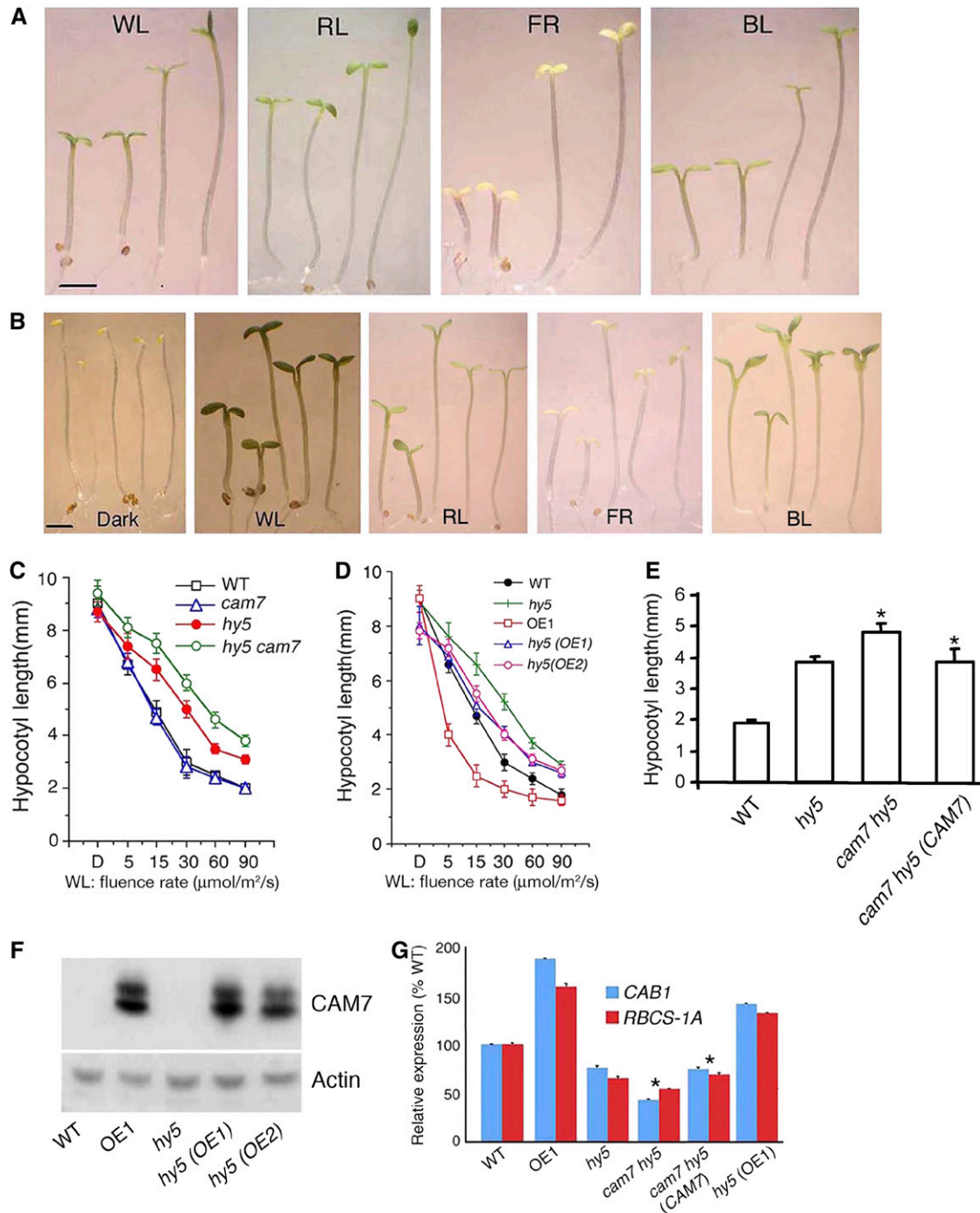


Figure 5. The Elongated Hypocotyl Phenotype of *hy5* Is Enhanced in *cam7 hy5* Double Mutants.

(A) The visible phenotypes of 6-d-old *cam7 hy5* double mutant seedlings grown in WL (30 $\mu\text{mol}/\text{m}^2/\text{s}$), RL (60 $\mu\text{mol}/\text{m}^2/\text{s}$), FR (40 $\mu\text{mol}/\text{m}^2/\text{s}$), or BL (20 $\mu\text{mol}/\text{m}^2/\text{s}$) 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 $\mu\text{mol}/\text{m}^2/\text{s}$), RL (60 $\mu\text{mol}/\text{m}^2/\text{s}$), FR (40 $\mu\text{mol}/\text{m}^2/\text{s}$), or BL (20 $\mu\text{mol}/\text{m}^2/\text{s}$) 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 $\mu\text{mol}/\text{m}^2/\text{s}$, it was further reduced at 15 or 30 $\mu\text{mol}/\text{m}^2/\text{s}$ and strikingly reduced at 60 $\mu\text{mol}/\text{m}^2/\text{s}$ 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 Ca^{2+} 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 although 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 Ca^{2+} -responsive element (CACGTG[T/C/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 Ca^{2+} flux plays important regulatory functions in sensing dark–light transition of *Arabidopsis* seedlings (Sai and Johnson, 2002). Furthermore, the role of Ca^{2+} /CaM in phytochrome signaling has been postulated, and the potential connection between light and Ca^{2+} /CaM signaling has started emerging, especially with the identification and functional characterization of SUB1, a Ca^{2+} binding protein operative 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 Ca^{2+} 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 Ca^{2+} or other divalent cation in CAM7-mediated *Arabidopsis* seedling development remains to be elucidated. For example, recent studies have suggested that Mg^{2+} may structurally bridge the DREAM protein to DNA, whereas Ca^{2+} -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 ZBF1/MYC2 and ZBF2/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

Figure 5. (continued).

(E) Quantification of hypocotyl length of 6-d-old seedlings grown in WL (60 $\mu\text{mol}/\text{m}^2/\text{s}$). Approximately 25 to 30 seedlings were used for the measurement of hypocotyl length. The error bars indicate SD ($P < 0.01$, between *cam7 hy5* versus *cam7 hy5* [CAM7] as marked by asterisks; $n = 3$).
(F) Immunoblot (using c-Myc antibodies) of 20 μg of total protein prepared from wild-type, OE1, *hy5*, and *hy5* (OE1), and *hy5* (OE2) seedlings. The actin bands indicate approximate equal loading.
(G) Real-time quantitative PCR results ($P < 0.01$; between *cam7 hy5* versus *cam7 hy5* [CAM7] as marked by asterisks; $n = 3$) show the relative expression of *CAB1* and *RBCS-1A* in 6-d-old seedlings grown in WL (30 $\mu\text{mol}/\text{m}^2/\text{s}$).

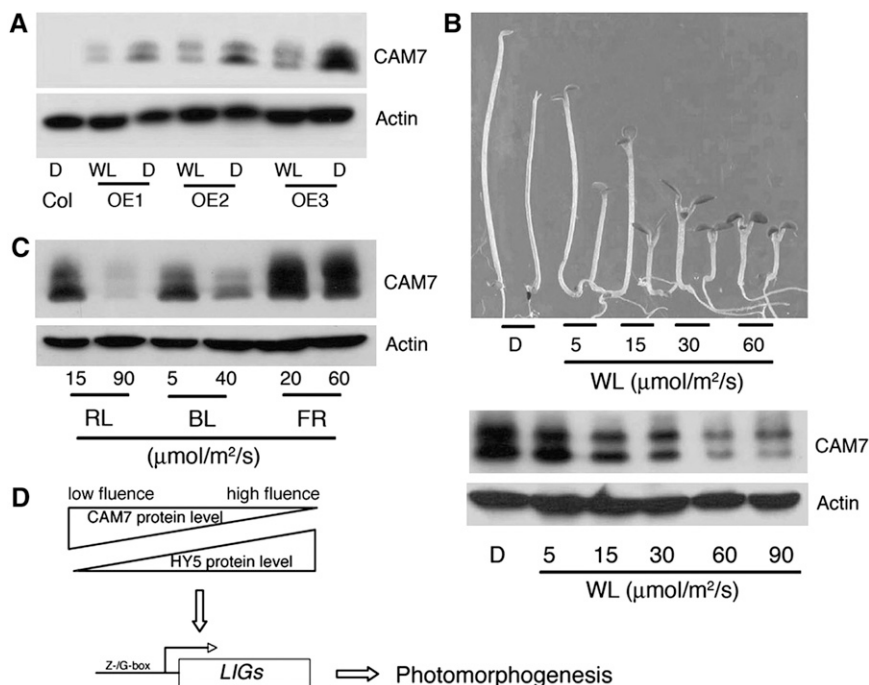


Figure 6. The Accumulation of CAM7 Protein Is Altered in Dark and Light.

(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 overexpresser 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.

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, Ca^{2+} /CaM-mediated signaling has been shown to be involved in brassinosteroid biosynthesis and auxin signaling pathways (Yang and Poovaiah, 2000; Du and Poovaiah, 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

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'-GCGTGGACCGCTTGCTGCAACT-3'; and CAM7-specific primers LP15, 5'-GACCATCTCCTCTCCGTCTTTGTCGAA-3', and RP15, 5'-CGAATGTGTTTCGTT TACAGTTCA-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 CAM7 in *cam7-1* (salk_074336) and *cam7-2* (Flag_397A10), respectively. The hypocotyl length measurement data were analyzed by one-way analysis of variance using a post-hoc Dunnett test. All analyses were performed using the SPSS15.0 program.

For the generation of overexpresser transgenic lines, a 571-bp fragment of cDNA was PCR amplified using primers with *c-Myc* sequence: FP, 5'-CATGCCATGGCAATGAACATCTCAGAGTTCAAGGAGGCTT-3'; RP2A, 5'-GGAGATTAGCTTTTGTTCACCGTTCAAATCTTCTTCAGAAA-TCAAATTTTGTTCACCGTCGAGCTTAGCCATCATGACTTTGACAAACT-3'; RP2B, 5'-GACTAGTACCGTCGAGTCCGTTCAAGTCTTCTCTGAGAT-TAATTTTGTTCACGTTCAAGTCTTCTCCTCGGAGATTAGCTTTTGTTCAC-CGTTCAAAT-3'. The PCR products were digested and cloned into the *NcoI* and *SpeI* sites of pCAMBIA1303, and the transgenic lines were generated as described by Mallappa et al. (2006).

The *cam7 hy5* double mutants were constructed by genetic crosses using *hy5-ks50* (Wassilewskija) and *cam7-1* (Col) single mutants following similar methods described by Yadav et al. (2005). A segregated wild-type line of the T2 generation was used as a control to compare the phenotypic and molecular differences. The *hy5* transgenic lines containing the 35S promoter-CAM7-*c-Myc* transgene were constructed by genetic crosses between *hy5-KS50* and OE1 or OE2 transgenic plants containing the 35S promoter-CAM7-*c-Myc* transgene. For the complementation test, a 2.1-kb fragment containing CAM7 and its 1.5-kb upstream promoter region was cloned into the *NcoI* and *SpeI* sites of pCAMBIA1303, and the transgenic lines were generated as described by Mallappa et al. (2006).

Chlorophyll and Anthocyanin Measurements

Chlorophyll and anthocyanin levels were measured following protocols as described by Holm et al. (2002). Briefly, seedlings were collected into microcentrifuge tubes, weighed, and crushed by a pestle in 700 μ L of chilled 80% acetone. Cellular debris was removed by centrifugation at 4°C, and the supernatant containing chlorophyll was collected into a fresh microcentrifuge tube, and volume was made up to 1 mL. Then the absorbance was measured at the wavelengths of 645 and 663 nm. The total chlorophyll content was calculated with the following formula: $(20.2 \times A_{645}) - (8.02 \times A_{663}) = \mu\text{g/g}$ of fresh tissue weight.

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 *CAB1m* promoter has been described by Yadav et al. (2002).

RNA Gel Blot Analysis

For RNA gel blots, total RNA was extracted using the RNeasy plant minikit (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-Multimager (Bio-Rad) and ratios of the gene versus its corresponding rRNA band were determined and plotted (Fluor-S-Multimager; 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 pGEX4T-2 vector, and GST-CAM7 was induced using 1 mM isopropylthio- β -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 μ L 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 μ L for every 100 μ L 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 μ L 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 Hybond C-Extra (Fermentas) at 100 mA for 2 h in transfer buffer (7.56 g Tris, 47 g glycine, and 20% methanol in 2.5 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).

The ChIP assays were essentially performed as described (He et al., 2005). The sequence of primer pairs (resulting products of ~500 bp) used were as follows: *NIA2*-PROMO-FP, 5'-CTATACATGTTCCGAGACG-3'; *NIA2*-PROMO-RP, 5'-AGTATCGTGCCGAATCACACG-3'; *CAB1*-PROMO-FP, 5'-GGTTTACATTGATGCTCTCAGGATTC-3'; *CAB1*-PROMO-RP, 5'-CGTGGTTAATGGCTCGCACTTCGC-3'.

Real-Time Quantitative PCR

Total RNA was isolated using the RNeasy plant minikit (Qiagen) extraction kit according to the manufacturer's protocol. cDNA was synthesized from 1 μ g of the total RNA using RT-AMV reverse transcriptase (Roche). Real-time PCR was performed using Light Cycler faststart DNA Master^{plus} SYBR Green1 (Roche). Values were normalized with the amplification of Actin as a constitutively expressed internal control. Primers used were as follows: *CAB1*-FP, 5'-CCCATTCTTGGCTTACAACAAC-3'; *CAB1*-RP, 5'-TCG-GGGTCAGCTGAAAGTCCG-3'; *RBCS-1A*-FP, 5'-GAGTCACACAAAGA-GTAAAGAAG-3'; *RBCS-1A*-RP, 5'-CTTAGCCAATTCGGAATCGGT-3'.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under accession numbers AM422556 (ZBF3/CAM7) and At1g37130 (*NIA2*).

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.

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