CHAPTER 4:

Identification of a Z-box binding factor (ZBF3) and its functional characterization in *Arabidopsis* seedling development
4.1. Introduction:

Calcium is a crucial regulator of growth and development in plants. The calcium is used as a second messenger in higher organisms and there are special and magnitudinal aspects of calcium signals that trigger specific responses via CaM. CaMs have a common structural motif, EF-hand, a helix-loop-helix structure, which is known to perform its regulatory function by modulating the activity of specific CaM binding proteins. CaM regulated basic-helix-loop-helix (bHLH) family of transcription factors has been reported, where CaM inhibits the DNA-protein interactions by competing with the DNA binding domains of bHLH proteins (Corneliussen et al., 1994). Recent studies have shown that some proteins with EF-hands have the ability to directly interact with DNA in a Ca ++ dependent manner. The human DRE-antagonist modulator (DREAM) has four EF-hands and specifically interacts with DRE element, which is prevented by Ca ++ (Carrion et al., 1999). In another example of, URE3-BP an *E. histolytica* protein having two EF hands with no canonical DNA binding motif binds to URE3 DNA motif in Ca ++ dependent and sequence specific manner which can modulate the gene expression both in positive and negative manner (Gilchrist et al., 2001). Calcium fluxes in the amount of intracellular ions are important signals and determine gene expression in eukaryotes. Calcium decreases the affinity of recombinant URE3-BP for DNA as measured by EMSA. Relatively high concentrations of calcium were required to block the binding of DNA by URE3-BP in comparison with DREAM (100-500 μM versus 5-10 μM).

CaM is ubiquitous in eukaryotes, and is a highly conserved Ca ++ binding protein that plays multiple regulatory functions responding to a wide variety of stimuli (Berridge et al., 2000; Hepler et al., 2005). *Arabidopsis* genome contains seven CAM genes that encode four protein isoforms such as CAM1 and CAM4; CAM2, CAM3 and CAM5; CAM6; and CAM7. CAM7 is the most consensuses among all the members of the family. Whereas CAM1/CAM4 differs from CAM7 by four amino acids, CAM2/3/5 and CAM6 differ from CAM7 by a single amino acid substitution (Cormack et al., 2005). Several nuclear factors have been reported whose DNA specific interactions are modulate by three isoforms of CaM such as CaM1/CaM4, CaM2/3/5 and CaM6 in a calcium concentration dependent manner (Szymanski et al., 1996). However, similar information on CaM7, the only member of
the gene family that contains two perfect helix loop helix motifs, is not yet available to our knowledge.

Various studies indicate the involvement of Ca\(^{++}\)/CaM in multiple signaling pathways in plants (Szymanski et al., 1996; Braam et al., 1990; Knight et al., 1991). The homeostasis of Ca\(^{++}\) has been shown to be associated with blue/UV-A light induced gene expression (Long and Jenkins, 1998). A recent genetic study using SUB1 has suggested the possible involvement of local Ca\(^{++}\) concentration change in cryptochrome-mediated blue light signaling. The SUB1 protein contains two EFhands and has been shown to be a low affinity calcium binding protein (Guo et al., 2001). Biochemical and pharmacological studies in soybean cell culture have shown to induce the accumulation of \(CAB\) mRNA in the absence of light (Lam et al., 1989). Microinjections of cells of a phyA mutant with Ca\(^{++}\) and CaM induced the expression of light inducible genes (Neuhaus et al., 1993; Bowler et al., 1994). These studies have further revealed three branched pathways of light-induced gene expression in light signaling (Bowler et al., 1994; Neuhaus et al., 1993, 1997), where in one of these pathways, CaM has been shown to be involved in the regulation \(CAB\) gene expression (Neuhaus et al., 1997). Although the physiological functions and the mechanism of action of CaMs remain unknown, these studies suggest that Ca\(^{++}\)/CaM is involved in the regulation of gene expression in light signaling pathways. However, the molecular and physiological function of CaM or structurally related Ca\(^{++}\) binding protein, which interprets and specifically transduce the information into appropriate cellular responses, remains unknown.

CaMs are not only highly related in their encoded protein but also in their overall expression patterns. However, evidence of distinct organ or tissue specific expression requires more detail cell and or tissue specific analysis. The \(CAMs\) are expressed during all developmental stages (Cormack et al., 2005); the \(CAMs\) encoding identical proteins (\(CAM2/CAM3/CAM5\) and \(CAM1/CAM4\) are clustered together by Geneinvestigator, indicating that the closest paralogs share the most similar expression during development. It has been shown through Geneinvestigator compilation of \(Arabidopsis\) microarray experiments that \(CAM7\) (At3g43810) expression is highest during early developmental stages. There is a gradual decrease in expression till 29 days of growth and after 45 days of adult stage there is no expression of \(CAM7\) detected (Cormack et al., 2005). The documented difference
among the CAMs is that CAM2, also known as TCH1, is the only CAM whose expression is upregulated by touch (Lee et al., 2005).

Trichomes are thought to be important to protect the plant against predators, water loss and UV irradiation (Johnson et al., 1975; Mauricio and Rausher, 1997). A large number of mutants were isolated that show defects in Trichome development (Marks et al., 1997; Hulskamp et al., 1999; Larkin et al., 2003). The TRANSPARENT TESTA GLABRA1 (TTG1) locus regulates trichome differentiation and anthocyanin biosynthesis in Arabidopsis encodes a WD40 repeat protein. GL3 is a bHLH protein that interacts with TTG1; single gl3 mutation only affects the trichome pathway with a modest trichome number reduction (Esch et al., 2003). However the overexpression GL3 protein suppresses the trichome defect of the pleiotropic ttg1 mutation. EGL3 encodes a bHLH Transcription factor 1, EGL3 deficient plant in gl3 mutant lead to totally glabrous plant (Zhang et al., 2003). The protein is functionally redundant with GL3 and TT8 and interacts with TTG1. Mutation in EGL3 locus causes reduced trichomes, anthocyanin and seed coat mucilage and abnormally patterned stomata. In Arabidopsis, ZWICHEL was identified by genetic screens for altered trichome morphology, zwi mutants are affected in trichome stalk expansion and (Bouché et al., 2002) only branching, but not in other aspects of plant development. The ZWICHEL protein is a kinesin-like CaMBP (AtKCBP).

Light signaling regulates plant growth and development through transcriptional regulatory networks (Tobin and Kehoe, 1994; Terzaghi and Cahsmore, 1995; Millar and Kay, 1996). The coordinated activation and repression of specific downstream genes such as chlorophyll a/b binding proteins (CAB) and ribulose 1,5-bisphosphate carboxylase small subunit (RBCS), and chalcone synthase (CHS) which are induced by light whereas some genes, such as PHYA, NADPH-protochlorophyllide reductase and asparagine synthase which are down-regulated by light plays important role in light mediated development (Silverthorne and Tobin, 1987; Ha and An, 1988; Donald and Cashmore, 1990; Gilmartin et al., 1990; Sun and Tobin, 1990; Quail, 1991). The light responsive elements: G, GATA, GT1, and Z-box, which commonly occur in light regulated promoters, have been demonstrated to be essential for light-controlled transcriptional activity (Tobin and Kehoe, 1994; Terzaghi and Cashmore, 1995; Millar and Kay, 1996). The CAB minimal promoter contains a Z-DNA forming sequence (ATACGTGT) that is essential for light.
dependent developmental expression of \textit{CAB1} gene (Ha and An, 1988). Recent studies have revealed that the Z-box containing synthetic and native promoters are responsive to photoreceptors and are under the control of downstream light regulatory components, COP1 and HY5. It was earlier shown by DNA protein interaction studies that Z-box binding activity is present in \textit{Arabidopsis} (Yadav et al., 2002). Recent studies have identified and functionally characterized two Z-box binding factors: ZBF1/MYC2 and ZBF2/GBF1 in \textit{Arabidopsis} seedling development (Yadav et al., 2005; Mallappa et al., 2006). We performed DNA ligand binding screening to screen an \textit{Arabidopsis} cDNA expression library for Z-box interacting factors. In this chapter, we demonstrate in \textit{vitro} and \textit{in vivo} molecular functions of a Z-box binding factor 3 (ZBF3/CAM7) in light mediated seedling development.
4.2. Results

4.2.1. Molecular cloning of ZBF3/CAM7

A DNA-ligand binding screen was performed to identify and clone Z-box binding factors (ZBFs). Around $2 \times 10^6$ clones of cDNA expression library (in λZAPII vector) from 5-day old constant white light grown seedlings, were screened using a dimeric Z-box LRE as probe. Few putative positive clones in primary screen was selected for secondary screening. In secondary screen, phage particles were plated from individual primary stocks and probed with the Z-box. Eleven secondary putative positive clones were identified and tested individually in subsequent tertiary screen. Four clones obtained in tertiary screen were clonally pure, a stage at which all plaques blotted to membrane are positive (Figure 4.1B.a-b). Once the clonal purity was obtained, we tested the specificity of interaction of these gene products using other LREs along with the Z-box. In this specificity test, phages from 12.1-phage stock were used to infect with *E. coli* XL1 blue and plated. After lifting those plaques on to IPTG soaked membrane, one half of it was probed with Z-box and the other half with GT1 or GATA (Figure 4.1B.c-d). As shown in (Figure 4.1B.c-d), the binding activity of the clones tested was detected in case of Z-box probe but not with GATA-box or GT1 LRE. These results indicate that the gene product has specificity to interact with the Z-box and not with other LREs like GATA or GT1. After confirming specificity of the clone, plasmid rescue was carried out using a filamentous phage, EX-ASSIST and *E. coli* SOLAR strain (Stratagene protocol). After plasmid rescue, the cDNAs were sequenced. Out of four clones obtained three encoded CAM7 protein and the fourth one encoded ZBF1. Since ZBF1 was already investigated earlier (Yadav et al., 2005), we decided to investigate the genetic and molecular functions of CAM7 (clone no. 12.1: Z-box binding factor 3: ZBF3). All three cDNAs of CAM7/ZBF3 obtained from the ligand binding screen were turn out to be truncated.

4.2.2. ZBF3 codes for Calmodulin7

The clone 12.1 encodes for a protein of 142 amino acids with a predicted molecular mass of about 15.62 kD. CaM proteins have a common structural motif, 4 EF hands, a helix loop helix structure; and each EF hand is known to bind a calcium ion. The full length cDNA of CAM7 encodes protein of 149 amino acids with molecular mass of 16 kD (Figure 4.1C). Therefore, we cloned the full length cDNA
A

Light responsive elements (LREs)

GT1: TGTGTGGTTAATATG
G: TGACACGTGGCA
GATA:AAGATAAAGATT
Z: CGTATACGTGTCAC

CAB1 minimal promoter

ATAAGGATAGAGAGATCTATCTGATACGTGTCACGTC
ATGAGGTGCGGTCTGCCCAATCCATGAAAGCCACCTAG
ATATCTAAACACATATCAATTGCGAATCTGCAAGTG
CGAGCCATTAACACGTAAAGCACAACACAAACACTAAAC
GCCAAAAAATGTATAGGCAATAGCAACCTCA

B

Figure 4.1. CAM7/ZBF3 is the Z/ G-box binding protein
(A) The DNA sequence of various LREs used in this study. (B) Identification of CAM7/ZBF3 in ligand binding (Southwestern) screen. (a-b) the blotted membrane (plaques of the tertiary screen) was probed with the radioactively labeled Z-box and G-box light responsive element (LRE) respectively (c-d) the specificity of interaction of CAM7/ZBF3 to the Z-box in Southwestern analysis. The blotted membrane was cut into the halves and probed with the Z-box or GT1 LRE and Z-box or GATA LRE respectively. (C) Schematic diagram of ZBF3/CAM7 protein showing four EF hands. (D) Semiquantitative RT-PCR of full length of CAM7 transcript (531bp) amplified from six day old seedlings (1) and from leaf of adult plant (2), where M indicates molecular marker and * denotes DNA fragment of 500bp.
4.2.3. ZBF3/CAM7 interacts with the Z-box and G-box LRE found in minimal light responsive promoter

To re-examine the result we obtained in ligand binding screening, we carried out electrophoretic mobility shift assays (EMSAs) using radiolabelled Z-box DNA. ZBF3 was cloned in BamH1-Xho1 restriction site of pGEX4T-2 and the overexpressed protein (41 kDa) was purified from E. coli. We used this purified glutathione S-transferase–ZBF3 (GST-ZBF3) fusion protein and a dimeric Z-box DNA as a probe in gel shift assays. GST-ZBF3/CAM7 interacted with the Z-box and this binding activity was competed out by excess of unlabelled Z-box (50 and 100 mM) DNA. However, 100 mM of unlabelled GT1, another LRE, was unable to compete out the binding activity of CAM7 to the Z-box (Figure 4.2A).

We tested, an 189 bp native light regulated CAB1 minimal promoter that contains a Z-box for gel shift assays. As shown in (Figure 4.2B), GST alone did not show any binding activity, a strong low mobility DNA-protein complex was formed with GST-ZBF3/CAM7 fusion protein (lanes 2 and 3). This DNA-protein complex was efficiently competed out with 50 and 100 molar excess of unlabelled Z-box (Figure 4.2B, lanes 4 and 5) but not with 100 molar excess of GT1. These results suggest that ZBF3/CAM7 specifically interacts with the Z-box LRE of CAB1 minimal promoter.

We then tested the ability of ZBF3/CAM7 to interact with the G-box, which is known to be functionally equivalent to Z-box of native light regulated RBCS minimal promoter. We performed gel shift assay using purified GST-ZBF3 fusion protein and consensus tetrameric (26 bp) G-box LRE as probe. GST-CAM7 interacted with G-box and the binding activity was competed out by 50 mM excess unlabelled G-box, whereas 50 mM excess of GATA was not able to compete the binding activity (Figure 4.3A).

To further substantiate the above result, we used a 196 bp minimal promoter fragment of RBCS-IA for gel shift assay that has one GT1 and two GATA LREs, in addition to the G-box. A strong band of DNA-protein complex was detected, which was competed out with 50 and 100 mM excess of G-box but not with 100 mM.
Figure 4.2. GST-CAM7 specifically binds to the essential Z-box of *CAB1* minimal promoter

(A) Approximately 200ng of recombinant protein was added (lanes 3-6) to radioactively labelled Z-box. About 500ng GST protein was added in lane 2. The protein-DNA complexes were resolved on 10% native polyacrylamide gel. The triangle indicates increasing concentration of the Z-box (50 and 100 molar excess) in lanes 4 and 5, respectively, as competitors (Comp). 100 molar excess GT1 was added in lane 6. The plus and minus signs indicate the presence or absence of competitors, respectively. The star indicates a spurious band present in all the lanes. (B) EMSAs showing GST-CAM7 specifically binds to the Z-box of *CAB1* minimal promoter. For experimental details see Figure 4.2(A) legend. The arrow indicates DNA-Protein complexes.
Figure 4.3. GST-CAM7/ZBF3 protein specifically binds G-box and the G-box containing 196 bp *RBCS-1A* minimal promoter

(A) Approximately 200 ng of recombinant protein was added (lanes 3 to 6) to radioactively labelled G-box fragment. About 500 ng GST protein was added in lane 2. The protein-DNA complexes were resolved on 10% native polyacrylamide gel. The triangle indicates increasing concentration of unlabelled G-box (50 and 100 molar excess) in lanes 4 and 5, respectively, as competitors (Comp). 100 molar excess GATA was added in lane 6. The plus and minus signs indicate the presence or absence of competitors, respectively. (B) Approximately 200 ng of recombinant protein was added (lanes 3 to 6) to radioactively labelled 196 bp *RBCS minimal* promoter. The triangle indicates increasing concentration of unlabelled G-box (50 and 100 molar excess) in lanes 4 and 5, respectively, as competitors (Comp). 100 molar excess GATA was added in lane 6. The arrow indicates DNA-protein complex (shifted band).
GATA. Taken together, these results suggest that ZBF3/CAM7 interacts with both Z- and G-box LREs of light regulated promoter (Figure 4.3B).

4.2.4. Ca\(^{++}\) inhibits the interaction of CAM7 with the \textit{CABI} minimal promoter

To explore the role of Ca\(^{++}\) in binding affinity of CAM7 to \textit{CABI} promoter interaction, we carried out EMSAs using various concentrations of Ca\(^{++}\). Increasing concentrations (170, 340, 510, 680 mM) of \(\text{CaCl}_2\) were added in lanes 4 to 7 and 680 mM of \(\text{MnCl}_2\) and \(\text{CuCl}_2\) was added in lanes 8 and 9, respectively. These experiments were carried out in two ways: either \(\text{CaCl}_2\) was added to the binding reactions at the beginning or, after incubation with probe in the binding reaction for 20 minutes. While added at the beginning of the binding reactions approximately 510 mM or higher concentrations of Ca\(^{++}\) could compete out the binding ability of CAM7 to \textit{CABI} promoter (Figure 4.4A). However, while added after 20 minutes, even 680 mM of \(\text{CaCl}_2\) was unable to compete out the binding activity of CAM7 to \textit{CABI} promoter (Figure 4.4B). These results suggest that higher concentrations of Ca\(^{++}\) can inhibit the DNA-binding ability of CAM7. However, once the Protein-DNA complex is formed, similar higher concentration of Ca\(^{++}\) is unable to efficiently compete the binding activity of CAM7 to the Z-box.

4.2.5. CAM7, but not CAM2/3/5, interacts with the \textit{CABI} minimal promoter

CAMs have highly conserved amino acid sequences, and the amino acid sequence of CAM2/3/5 isoform differs from CAM7 by only a single amino acid substitution (Figures 4.5 and 4.6). To determine whether CAM2/3/5 was also able to interact with the Z-box \textit{CAM3} cDNA was amplified by RT-PCR and cloned in BamH1-Xho1 restriction sites of pGEX4T-2. We performed EMSAs using \textit{E.coli} purified GST-CAM2/3/5 fusion protein and \textit{CABI} minimal promoter as probe. Approximately 200 ng, 1 mg and 3 mg (lanes 3 to 5) of GST-CAM2/3/5, and 100ng, 200 ng and 300 ng (lanes 6 to 8) of GST-CAM7 were added to radioactively labeled \textit{CABI} minimal promoter. However, no DNA-protein complex was detected, suggesting that CAM2/3/5 was unable to interact with the \textit{CABI} promoter (Figure 4.7A).
Figure 4.4. Ca$^{++}$ inhibits the interaction of CAM7 with the *CAB1* minimal promoter

(A) Approximately 200ng of GST-CAM7 protein was added (lanes 3 to 9) to *CAB1* minimal DNA fragment. Increasing concentrations (170, 340, 510 and 680 mM) of CaCl$_2$ was added in the reaction buffer in lanes 4 to 7, respectively. 680 mM of MnCl$_2$ and CuCl$_2$ was added in lanes 8 and 9, respectively. (B) GSA with GST-CAM7 incubated with *CAB1* minimal for 15 min and then increasing concentration of CaCl$_2$ was added in the reaction buffer. For other experimental details see legend 4.4(A).
Figure 4.5. Amino acids sequence of site directed mutagenesis products of CAM7 with amino acids substitution in EF hands 1 (CAM7-M1) and in EF hands 1 and 3 (CAM7-M2) are shown. The substituted amino acids are marked in red color. The identical amino acids are marked as star and the similar amino acids are marked as colons.
Figure 4.6. The four CaM isoforms encoded by seven *Arabidopsis CAM* genes are aligned with mouse CaM amino acids. The regions corresponding to the E helices, Ca++ binding loops and the F helices are indicated by the black, grey and black bars respectively. The consensus sequences for these regions are indicated beneath the relevant sequences. "E" stands for glutamic acid, 'h' for hydrophobic aminoacids, '*' for any aminoacids. Amino acids sequence identities are shaded yellow.

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Figure 4.7. Mutations in one or few amino acids in CAM7 causes complete abolition of DNA binding ability

(A) CAM7, but not CAM2/3/5 with closest amino acid sequences to CAM7, interacts with *CAB1* minimal promoter. Approximately 200ng, 1mg and 3 mg (lanes 3 to 5) of GST-CAM235 and 100ng, 200ng and 300ng (lanes 6 to 8) of GST-CAM7 recombinant protein were added to radioactively labelled *CAB1* minimal promoter. (B) EMSA showing CAM7-M1 and CAM7-M2 are unable to the *CAB1* minimal promoter. Approximately 200, 300, 200, 500, 200 and 500ng (lanes 2-7, respectively) of recombinant proteins were added to radioactively labeled 189bp *CAB1* DNA fragment.
4.2.6. Amino acid substitution in CAM7 abolishes its DNA binding ability

To further test this observation, we generated mutated versions of CAM7, CAM7-M1 (two amino acids substituted in third EF- hands) and CAM7-M2 (two amino acids substituted in first and third EF- hands) by site directed mutagenesis (Figure 4.5). These mutated cDNAs were cloned in pGEX4T2 in BamH1 and Xho1 restriction site. 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. None of these mutated versions of CAM7 was able to interact with the CAB1 minimal promoter (Figure 4.7B). These results further support the notion that although four Arabidopsis CaM isoforms are highly similar, one or two amino acid changes may contribute to select the target specifically of calmodulin proteins.

4.2.7. Light Controls Sub-cellular Redistribution of CAM7

CaM can perceive the cytosolic Ca++ changes and transduce into altered target activity (Hepler et al., 2005). Since CAM7 binds to the light regulated promoters, we asked whether CAM7 is indeed a nuclear protein and also whether its sub-cellular localization is controlled by light. To address these questions, we generated transgenic Arabidopsis plants expressing either GUS alone or CAM7-GUS driven by CaMV 35S promoter. (Figure 4.8A). To determine the GUS staining pattern, after obtaining, homozygous transgenic lines, we used hypocotyls cells of 5-day-old transgenic seedlings grown in constant dark or WL and observed under Fluorescent microscope. In the dark grown seedlings, the GUS staining was detected exclusively in the nucleus in all hypocotyls cells (Figure 4.8B). However, the corresponding hypocotyls cells of constant light-growth seedlings consistently showed GUS stain in the cytosol as well as in the nucleus (Figure 4.8F). These results indicate that light controls redistribution of CAM7 protein between the nucleus and cytosol. To monitor the effect of dark-light transitions on the sub-cellular localization of GUS-CAM7, we transferred 4-day-old dark or WL grown seedlings to WL or darkness, respectively, and then stained at various time points. When the dark grown seedlings were transferred to WL, the GUS stain was predominantly visible in the nucleus up to 30 minutes of exposure (Figure 4.8C). However, the weak cytosolic GUS stain, which was detectable at 30 minutes of exposure to WL, became more intense and spread throughout the cell in almost all hypocotyls cells when exposed to WL for 60 minutes or longer time period (Figure 4.8D). When 4-day-old WL grown seedlings were
Figure 4.8. Sub-cellular Localization of CAM7 in Hypocotyls of Transgenic Seedlings

In each panel from (A) to (D) and (F) to (H), the hypocotyls of transgenic seedlings were stained for GUS (left panels), and for DNA using DAPI to identify nuclei (right panels). The arrow heads indicate nuclei. (A) Hypocotyl cells of dark grown seedlings containing GUS transgene alone. (B) Hypocotyl cells of dark grown seedlings containing CAM7-GUS transgene. (C) Hypocotyl cells of 5-day-old constant dark grown seedlings containing CAM7-GUS transgene transferred to WL (30 μmol/m²/s) for 30 minutes. (D) Hypocotyl cells of 5-day-old constant dark grown seedlings containing CAM7-GUS transgene transferred to WL for 1 hour. (E) Visible phenotypes of 6-day-old wild type, transgenic lines containing GUS-transgene alone (GUS), or two independent transgenic lines containing CAM7-GUS transgene (CAM7-GUS (1) and CAM7-GUS (2)) grown in constant WL (30 μmol/m²/s). Quantification of hypocotyl length of seedlings shown in the left panel. (F) Hypocotyl cells of 5-day-old constant WL grown seedlings containing CAM7-GUS transgene. (G) Hypocotyl cells of 5-day-old constant WL (30 μmol/m²/s) grown seedlings containing CAM7-GUS transgene transferred to dark for 12 hours. (H) Hypocotyl cells of 5-day-old constant WL grown seedlings transferred to dark for 48 hours.
transferred to darkness, even after 48 hours of incubation in the darkness, although the nuclear stain became more intense, the GUS-stain was also clearly visible in the cytosol (Figure 4.8G-H). Taken together, these results demonstrate that light-induced translocation of CAM7 from nucleus to cytosol is strikingly faster than translocation from cytosol to nucleus induced by darkness.

4.2.8. CAM7 is expressed at various wavelengths of light

To study tissue specific expression of CAM7 at seedling stage, 1.1 kb CAM7 upstream promoter region from the start codon was fused in correct orientation to GUS in pBI101.2 vector between SalI and BamHI restriction sites. We generated several stable homozygous transgenic lines and examined tissue specific expression of CAM7 during early seedling development. CAM7 was predominantly expressed in dark and at various wavelength of light grown seedling. GUS stain was visible predominantly in cotyledons and weak stain was detected in hypocotyls of six day old seedlings (Figure 4.9).

4.2.9. CAM7 is expressed in all parts of adult plants

To determine the tissue specific expression pattern of CAM7 at the adult stage, we used the same transgenic lines as stated above. Tissue specific expression studies show that CAM7 is expressed in flowers, specifically in anthers, stigma and it was also found to be expressed in the siliques. Semiquantitative RT-PCR reveal that CAM7 is expressed in all major organ of adult plant, including root, stem, leaves and flower. Expression in root however was found to be significantly less as compared to other organs (Figure 4.10).

4.2.10. Overexpression of CAM7 in Arabidopsis transgenic plants leads to hyperphotomorphogenic growth

Since CAM7 binds to the Z-/G-box of light inducible minimal promoters, we investigated the physiological function of CAM7 in light mediated seedling development. We generated two construct, in which three copies c-MYC were tagged either at 5' or 3'end of CAM7 cDNA which give rise to c-Myc attached to either C- or N-terminal end of CAM7 protein respectively. For the generation of overexresser c-MYC tagged CAM7 transgenic lines, 571 bp fragment of cDNA was PCR amplified using primers with cMyc sequence. The PCR product were digested and cloned into
Figure 4.9. CAM7 is expressed under all light conditions
Six day old seedling carrying CAM7 promoter-GUS transgene were grown in dark or at various wavelengths of light including RL (60 µmol/m²/s), WL (30 µmol/m²/s) FR (60 µmol/m²/s), and BL (30 µmol/m²/s) respectively, and used for GUS activity staining.
Figure 4.10. Tissue specific expression of CAM7

(A) Gus activity staining of various organs of transgenic Arabidopsis plants containing CAM7 promo-GUS transgene. (B) Semiquantative RT-PCR results obtained using WT plants shows the expression of CAM7 in root, stem, leaf and flower.
NcoI and SpeI site of pCAMBIA1303, a binary vector carrying the CaMV 35S promoter. Semiquatative RT-PCR and immunoblot analyses confirmed the increased levels of CAM7 transcript and protein in overexpression backgrounds. (Figure 4.11).

Examination of the growth of these transgenic seedlings overexpressing CAM7-cMyc in dark and white light conditions revealed that overexpression of CAM7 led to hyperphotomorphogenic growth at various intensities of white light (Figure 4.12A-C). Additionally, the transgenic seedlings displayed photomorphogenic growth with short hypocotyls and partly opened cotyledons without apical hooks in the darkness (Figure 4.12A-B). To further investigate, whether the hyperphotomorphogenic phenotype is dependent on specific wavelength of light; we examined the growth of six-day-old transgenic seedling at various wavelength of light. We found that the CAM7 overexpression caused increased photomorphogenesis in (red light (RL), blue light (BL), and far-red light (FR) at various fluences (Figure 4.13A-B). The increased inhibition of hypocotyls elongation was found to be more significant at lower fluence rates of light. These results indicate that CAM7 acts as a positive regulator of photomorphogenic growth at various wavelength of light.

Since overexpression of CAM7 results in hyperphotomorphogenic growth, the effect of higher level accumulation of CAM7-GUS on seedling morphology was analyzed. Consistent with the observation described in (Figure 4.12) the six day old transgenic seedlings over-expressing CAM7-GUS exhibited enhanced inhibition in hypocotyls elongation (Figure 4.8E).

4.2.11. Overexpression of CAM7 leads to increased trichome number in emerging leaves

While examining the morphology of the overexpresser transgenic seedlings, it was observed that the transgenic seedling had significantly higher number of trichomes in the emerging true leaves as compared to wild type (Figure 4.14A). The function of several genes including ZW1, EGL3 and TTG1 has been implicated in trichome development. We therefore examined the expression levels of these genes in CAM7 over expresser lines by semiquantative RT-PCR. The expression of these genes were dramatically elevated in all transgenic lines compared to wild type (Figure 4.14B).
Figure 4.11. CAM7- cMyc overexpression in transgenic plants

(A) Semi-quantitative PCR (RT-PCR) results show the level of expression of CAM7 in overexpresser transgenic lines and in corresponding wild type (Col) background using CAM7 specific primers. M indicates molecular weight markers (100 base pair ladder), and the dot shows a DNA fragment of 500 base pair. (B) Immunoblot (using anti c-Myc antibodies, Sigma) of 20 µg of total protein prepared from wild type (Col) and overexpresser transgenic plants. The star in the bottom panel shows a cross reacting band in the same gel as loading control.
Figure 4.12. Overexpression of CAM7 leads to hyperphotomorphogenic growth

(A) The morphology of the seedlings grown in D (dark), and WL (15 μmol/m²/s). In each panel, six-day old wild type (Col) and CAM7 overexpresser transgenic seedlings (OE1, OE2 and OE3 in Col background) are shown from left to right, respectively. (B) and (C) Quantification of hypocotyl length of six-day old seedlings grown in constant dark or at various fluences of WL. About 25-30 seedlings were used for the measurement of hypocotyl length. The error bars indicate standard deviations.
Figure 4.13. CAM7 overexpressing causes hyperphotomorphogenic growth under all wavelength of light

(A) The morphology of the seedlings grown in RL (red light: 30 μmol/m²/s), BL (blue light: μmol/m²/s), or FR (far-red light: 40 μmol/m²/s) are shown, respectively. (B) Quantification of hypocotyls length of six-day-old seedlings grown at various fluences of RL, BL or FR. About 25-30 seedlings were used for the measurement of hypocotyls length. The error bars indicate standard deviations.
Figure 4.14. Overexpression of CAM7 caused increase trichome number in emerging true leaves

(A) Increased trichome number in OE1, OE2 and OE3 compared to WT.

(B) Semiquantative RT-PCR showing increased expression of genes essential for trichome development in CAM7 overexpressing transgenic seedlings. M indicates 100 bp DNA ladder and the dot represent a DNA fragment of 500bp in the gel.
4.2.12. Overexpression of CAM7 results in a higher level of chlorophyll and anthocyanin accumulation

The accumulation of anthocyanin and chlorophyll are two important physiological responses of photomorphogenic growth. Measurement of chlorophyll and anthocyanin contents showed that six-day-old transgenic seedling had significantly higher level of accumulation of chlorophyll and anthocyanin than wild type (Figure 4.15A-B). Furthermore, the level of anthocyanin was found to be drastically elevated in six-day-old transgenic seedlings in dark as compared to wild type plants (Figure 4.15C).

4.2.13. CAM7 positively regulates the expression of light inducible genes

We examined the steady state mRNA levels of light inducible genes like \(CAB\) and \(RBCS\) in six day old seedlings grown in constant white light or in darkness. Enhanced expression of \(CAB\) gene was detected in transgenic seedlings compared to wild type background in light. Whereas very little expression of \(CAB\) gene was detected in wild type background in dark, the expression of \(CAB\) gene was drastically elevated in transgenic seedlings (Figure 4.16). To study the kinetics of expression of these light regulated genes 5-day-old dark grown seedling where transferred to white light for different time interval. It was found that rate of expression of \(CAB1\) and \(RBCS\) genes were fast but the amount of transcript was not significantly elevated during the transfer from dark to light, this can be due to high level of transcript level already present in the dark (Figure 4.17).

4.2.14. CAM7 directly interacts to \(CAB1\) promoter in vivo

We performed chromatin immunoprecipitation (ChIP) experiments to determine whether CAM7 interacts with \(CAB1\) minimal promoter in vivo. The CAM7-cMyc fusion protein in transgenic plants was immunoprecipitated by antibody to cMyc protein. The genomic DNA fragments that coimmunoprecipitated with the CAM7-cMyc were analyzed by quantitative real time PCR. The quantification of these results revealed that the amount of DNA fragments of \(CAB1\) promoter coimmunoprecipitated from the transgenic background was about 30-35 fold more than that precipitated from the non-transgenic seedlings, and about 10-fold higher than the \(NIA2\) promoter (At1g37130), which does not contain any Z-/G-box.
Figure 4.15. Accumulation of chlorophyll and anthocyanin is increased in CAM7 OE transgenic lines
(A) The level of chlorophyll a/b content in six day old transgenic seedlings grown in WL (60 µmol/m²/s) is shown. (B) and (C) Accumulation of anthocyanin in wild type and transgenic seedlings grown in WL (60 µmol/m²/s) and in dark respectively. About 25-30 seedlings were used for the measurement of chlorophyll or anthocyanin accumulation. The error bars indicate standard deviations.
Figure 4.16. CAM7 promotes \textit{CAB1} gene expression in constant dark and white light

(A) The RNA gel blot shows \textit{CAB} gene expression in 6-day-old seedlings of wild type (Col) and CAM7 overexpression transgenic seedlings (OE1, OE2 and OE3) grown in dark or white light (WL: 60 \(\mu\text{mol/m}^2/\text{s}\)). 10 \(\mu\text{g}\) of total RNA was loaded in each lane. 18S rRNA has been shown as loading control. (B) The relative mRNA levels of \textit{CAB} as shown in (A). The intensity of each band was quantified by Fluor-S-Multilmager (BioRad) and ratios of \textit{CAB} versus its corresponding 18S rRNA band were determined and plotted.
Figure 4.17. Light mediated induction of \textit{CAB} and \textit{RBCS} gene expression in CAM7 overexpresser transgenic seedlings

(A) The RNA gel blot shows \textit{CAB} and \textit{RBCS} gene expression in wild type (Col) and CAM7 overexpresser transgenic seedlings (OE1) grown in dark (0) for 5 days and then transferred to white light (60 μmol/m²/s) for various time points 10 μg of total RNA was loaded onto each lane. rRNA has been shown as loading control. (B) The relative mRNA levels of \textit{CAB} and \textit{RBCS} as shown in (A). The intensity of each band was quantified by Fluor-S-Multilimeter (BioRad) and ratios of \textit{CAB} or \textit{RBCS} versus its corresponding 18S rRNA band were determined and plotted.
LRE (Figure 4.18). These results provide direct evidence that CAM7 interacts with 
*CAB1* minimal promoter *in vivo*.

### 4.2.15. Overexpression of CAM7-M2 results in dominant negative effects

We investigated the physiological function of the mutated version of CAM7 
protein, CAM7-M2, which lost the DNA binding ability due to amino acid 
substitutions in the EF-hand. To explore the role of CAM7-M2 in *Arabidopsis*, we 
cloned *CAM7-M2* cDNA in pCAMBIA binary vector in between Neo1 and Spe1 
restriction site and generated transgenic lines by stable transformation method. We 
obtained several homozygous transgenic lines overexpressing CAM7-M2 transgene, 
which was confirmed by semiquantitative RT-PCR (Figure 4.19A). We examined the 
growth of the transgenic seedlings (OEm1, OEm2 or OEm3) over-expressing CAM7-
M2 in dark and WL condition (Figure 4.19B-C). We found although the transgenic 
lines did not show any altered morphology in the dark, the seedling displayed 
elongated hypocotyls in WL. We further observed elongated hypocotyl phenotype of 
transgenic seedlings while grown at various wavelength of light. The elongated 
hypocotyls phenotype conferred by the introduction of CAM7-M2 could be 
attributable to dominant-negative interference of the light-signaling pathway by 
CAM7-M2 protein (Figure 4.20A-B).

### 4.2.16. Overexpression of CAM7-M2 results in reduced expression of light 
regulated genes

We were further interested to know whether CAM7-M2 overexpression can 
affect the expression of light regulated genes. We transferred 5-day-old dark grown 
seedling to white light for various time points and harvested the same age seedling for 
total RNA isolation. The rate of light-mediated induction of *CAB* and *RBCS* genes 
was dramatically reduced in OEm1 transgenic seedlings as compared to wild type 
(Figure 4.21).

### 4.2.17. Hyperphotomorphogenic phenotype of CAM7 OE is compensated with 
increased Ca$$^+$$

Calcium ions caused inhibition of DNA-binding affinity of CAM7, as revealed 
by in vitro DNA-protein interaction studies. We ask whether increase in calcium 
concentration can cause some affect on morphology of overexpression lines of CAM7
Figure 4.18. CAM7 interacts with *CAB1* minimal promoter in vivo.

Chromatin immunoprecipitation (ChiP) assays of *CAB1* promoter from OE1, OE2 or OE3 transgenic seedlings using antibodies to c-Myc. **(A)** Results are presented as the ratio of the amount of DNA immunoprecipitated from overexpresser transgenic seedlings to nontransgenic control plants. The light inducible *NIA2* promoter, which does not contain any Z or G-box was used as an internal control. Quantification of the result was done by Real Time PCR using SYBR green dye. **(B)** PCR reaction was run on agarose gel after various cycle depending upon the target for amplification. (a) Primer specific for *CAB1* promoter used for amplification (16 cycle) from immunoprecipitate (lppt) (b) *NIA2* promoter specific primers used for amplification (35 cycles) from lppt used as internal control. (c) *CAB1* promoter was amplified for 25 cycle from supernatant (Input).
Figure 4.19. Dominant negative effects of overexpression of CAM7-M2 in transgenic plants

(A) RT-PCR results show the level of expression of CAM7-M2 in overexpressor transgenic lines of CAM7-M2 and in corresponding wild type (Col) background. Actin was used as control. M indicates the DNA size marker, where the dot represent 500bp position in the gel. (B) In each panel, six-day-old wild type (Col), OE1 and CAM7-M2 overexpressor transgenic seedlings (OEm1, OEm2 and OEm3 in Col background) are shown from left to right, respectively. (C) Hypocotyl length measurement of WT (Col), OE1 and CAM7-M2 overexpression in cdark and cWL of six day old seedlings are shown.
Figure 4.20. Dominant negative effects of overexpression of CAM7-M2 in transgenic plants

(A) Morphology of six day old seedlings grown in constant red light (60 µmol/m²/s) far-red light (40 µmol/m²/s) and Blue light (20 µmol/m²/s) respectively.

(B) Quantification of hypocotyl length of six day old seedling grown in constant RL (60 µmol/m²/s), FR (40 µmol/m²/s), or BL (20 µmol/m²/s).
Figure 4.21. Light mediated induction of *CAB* and *RBCS* gene expression in CAM7-M2 overexpresser transgenic seedlings

(A) The RNA gel blot shows *CAB* and *RBCS* gene expression in wild type (Col) and CAM7M2 overexpresser transgenic seedlings (OE1) grown in dark (0) for 5 days and then transferred to white light (60 μmol/m²/s) for various time points. 10 μg of total RNA was loaded onto each lane. rRNA has been shown as loading control. (B) The relative mRNA levels of *CAB* and *RBCS* as shown in (A) the intensity of each band was quantified by Fluor-S-Multilmager (BioRad) and ratios of *CAB* or *RBCS* verses its corresponding 18S rRNA band were determined and plotted.
and CAM7-M2. To examine any such role of Ca\(^{++}\), six day old seedling were grown with increasing concentration of CaCl\(_2\) in growth medium in white light (30 \(\mu\text{mol/m}^2/\text{sec}\)). Hypocotyl length measurement of transgenic seedling overexpressing CAM7 suggested that with increase in Ca\(^{++}\) concentration the inhibition of hypocotyls length was decreased. At 1056 \(\mu\text{M}\) of calcium chloride in the growth medium, the WL mediated enhanced inhibition of hypocotyls elongation in CAM7 overexpresser transgenic lines was almost suppressed. However, no such effect of exogenously added Ca\(^{++}\) was observed in CAM7-M2 overexpresser transgenic lines (Figure 4.22). Hypocotyl length was compared to control in which seedlings were grown without additional calcium chloride in the growth medium. Although we were unable to measure the amount of Ca\(^{++}\) in take by the plant at each increasing Ca\(^{++}\) concentration, these preliminary data suggest that overexpression of CAM7 probably titrate out Ca\(^{++}\) and thereby change the homeostasis of Ca\(^{++}\) in the cell and resulting in altered seedling morphology.
Figure 4.22. Hyperphotomorphogenic phenotype of OE lines is compensated with increased Ca^{++}

Hypocotyl measurements of six day old seedlings of WT (Col), OEM2, OE1 and OE2 grown at cWL (30 μmol/m²/s) with increasing concentration of 66, 132, 264, 528, 1058 micromolar CaCl₂ in the growth medium. Control represent the same seedling grown without additional CaCl₂ in the growth medium.
4.3. Discussion

Competitive gel shift experiments with Z-box, mutated version of the Z-box and other LREs in earlier report clearly demonstrated that the DNA-binding activity specific to the Z-box is present in *Arabidopsis thaliana*. (Yadav et al., 2002). DNA-ligand binding screening have identified three ZBFs (Z box binding factors), two of these factors ZBF1/MYC2 and ZBF2/GBF1, have very recently been shown to be involved in cryptochrome mediated blue light signaling. Whereas ZBF1/MYC2 acts as a negative regulator of photomorphogenic growth, ZBF2/GBF1 acts as both negative as well as positive regulator of photomorphogenesis.

We have identified and cloned a third Z-box binding factor (ZBF3) in this study that specifically interacts with the Z-box LRE. ZBF3 turns out to be a calmodulin protein, CAM7, with four EF hands (At3G43810). DNA-protein interaction studies further reveal that ZBF3 also interacts with the G-box LRE of light regulated promoters. This result is consistent with the earlier findings that ZBF1/MYC2 or ZBF2/GBF1 interacts with the G-box LRE as well (Yadav et al., 2005; Mallappa et al., 2006). Therefore, collectively we conclude that the Z-box and G-box are functionally equivalent with context to at least all three Z-box binding factors known till date. CaM has not been shown earlier to be a DNA binding protein in eukaryotes. Although the primary amino acid sequences are widely different, various DNA binding domains including leucine zipper, zinc fingers and helix-turn-helix have α-helices with a relatively conserved orientation, which can recognize the major groove of DNA. The topology of EF-hand motif of CaM is almost identical to helix-turn-helix DNA binding domain.

The Z-box (ATACGTGT) or G-box (CACGTG) recognized by CAM7 has very close or identical sequences to the recently identified Ca"++ responsive element, (CACGTG [T/C/G]) (Kaplan et al., 2006). DNA-protein interaction studies with increasing Ca"++ have demonstrated that higher concentrations of Ca"++ inhibit binding ability of CAM7 to CAB minimal promoter. Similar to in the case of CAM7, the human DREAM that has four consensus EF-hands specifically binds to DRE element, and Ca"++ prevents DREAM to bind to DRE element (Carrion et al., 1999).

The examination of amino acid sequences of all four sub-groups of *Arabidopsis* CaM family reveal that all CAM proteins, expect CAM7, have at least one amino acid substitution in the EF-hand (Cormack 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 similar amino acid sequences, substitution of one or two amino acids in EF-hand may contribute to select target specificity. A detail NMR (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 that how a single amino acid substitution alters the target specificity of CAM7.

The role of Ca ++ in phytochrome signaling has long been postulated, and the potential connection between light and Ca ++ signaling has started reemerging, especially with the identification and functional characterization of SUB1, a Ca ++ binding protein operative in both cryptochrome and phytochrome-mediated light signaling (Guo et al., 2001). The data in this study collectively provide evidence that a bonafied Ca ++ sensor, CAM7, acts as a transcriptional regulator and promotes photomorphogenic growth and light-regulated gene expression. The enhanced inhibition of hypocotyl elongation of transgenic seedlings appeared to be more prominent at lower fluences of various wavelengths of light. Accumulation of chlorophyll and anthocyanin are two important physiological response, and the overexpression lines accumulated significantly higher anthocyanin and chlorophyll than wild type in light grown seedling. The accumulation of anthocyanin, which is negligible in dark grown wild type seedling, was found to be drastically higher in dark grown overexresser transgenic lines.

The expression of *CHS* is significantly increased in the overexpression lines in the light and dark condition, and in addition the increased expression of *TTG1* which is known to regulate trichome development and anthocyanin accumulation in *Arabidopsis*, may act in additive manner leading to increased accumulation of anthocyanin in CAM7 overexpression lines. The expression of light regulated gene such as *CAB1* and *RBCS* was elevated in transgenic seedlings in dark as well as in light compared to wild type. However, the rate of induction of *CAB* and *RBCS* gene expression in the transgenic lines was not dramatically elevated compared to wild type background apparently due to already higher-level of expression of the gene in transgenic seedlings in dark. Chromatin immunoprecipitation (ChiP) studies using the transgenic line overexpressing CAM7-cMyc further provides the direct evidence that CAM7 interacts with *CAB1* minimal promoter *in vivo*. 

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Light induced shuttling of negative regulator such as COP1 between nucleus and cytoplasm has been considered to be an important regulatory mechanism of light-mediated seedling development (von Arnim and Deng, 1994; Jiao et al., 2007). On the other hand, a positive regulator of photomorphogenesis, HY5, is constitutively present in the nucleus both in dark and light conditions (Chattopadhyay et al., 1998a). Recently, TFII-I, a widely expressed transcription factor in human, has been shown to have an unanticipated function in the cytoplasm as a negative regulator of agonist-induced Ca^{++} entry (Caraveo et al., 2006). In this study, the light-regulated partitioning of photomorphogenesis promoting factor, CAM7, indicates that although CAM7 is exclusively present in the nucleus, it is also present in the cytosol in light grown seedlings. The rate of translocation of CAM7 protein from nucleus to cytosol, induced by light, is dramatically faster than dark induced translocation from cytosol to nucleus.

CAM7 is involved in multiple developmental processes, besides its role in light signal transduction. This is relevant in the overexpression lines of CAM7, which caused increased trichome number in the transgenic line compared to wild type and the level of regulatory gene, crucial for trichome development are upregulated in the CAM7 overexpression lines. The tissue specific expression studies during early seedling development demonstrated that this gene is expressed in dark as well as in various wavelength of light. Earlier reports by RNA gel blot and RT-PCR experiments revealed that CAM7 is expressed in leaves, flowers and siliques (Zielinski et al., 2002). Here we are able to further support this observation by RT-PCR analysis, which confirms CAM7 expression in stem, leaves, flower and root. Promoter-GUS analysis showed CAM7 is expressed in flowering organs like stamens, anthers and pistil. We were not able to detect the expression in leaves and roots in adult promoter-GUS transgenic lines. This could be either due to 1kb promoter region that lacks specific regions or cis-elements required for CAM7 expression in leaves and roots or since the detection system by GUS stain is not equally sensitive to RT-PCR.

We investigated the physiological function of the mutated version of CAM7 protein by overexpressing it in WT Arabidopsis plants. The overexpression of mutated version of CAM7 in wild type plant cause dominant negative effect with elongated hypocotyl at various wavelength of light. Studying of kinetics of light regulated gene expression we found that expression of CAB and RBCS genes are compromised in the transgenic line overexpressing mutated CAM7 protein compared
to wild type. Thus, in addition to CAM7 protein, CAM7/M2 is able to titrate out at least one more photomorphogenesis promoting factor \textit{in vivo} and thereby, causing dominant negative effect exhibited by reduce photomorphogenesis in light. The alternate possibility of cosuppression of the endogenous CAM7 gene expression caused by over-expression of CAM7-M2 seems to be less likely since null allele of \textit{cam7} mutants do not display any altered photomorphogenic growth. However, it could be possible that overexpression of CAM7-M2 cosuppresses endogenous CAM7 gene expression and one or more additional genes of 7 member gene family of \textit{CAM} or 50-member gene family of \textit{CML} (CaM like) (Cormack et al., 2005). In either case, further study on identification and functional characterization of such gene(s) is required to test such possibility.

Interestingly, we observed that with increase in calcium concentration, the hyperphomorphogenic phenotype in the CAM7 overexpression lines was compensated although there was no such effect on the CAM7-M2 overexpresser transgenic lines or wild type. Thus in accordance with \textit{in vitro} Z-/G-box-binding data, it could be envisioned that higher concentration of Ca$^{++}$ in the cell may exert inhibitory effect on transcriptional regulation of CAM7 and thereby photomorphogenic growth and light regulated gene expression.