Chapter 5: Identification of a Z-box binding factor (ZBF1) and its molecular characterization
5.1 Introduction

Regulation of transcription of specific genes is one of the important mechanisms by which light regulates plant growth and development (Tobin and Kehoe, 1994; Terzaghi and Cahsmore, 1995; Millar and Kay, 1996). Some of these genes, such as nuclear-encoded photosynthesis related genes for chlorophyll a/b binding proteins (CAB) and ribulose 1,5-bisphosphate carboxylase small subunit (RBCS), are induced by light. On the other hand, some genes, such as PHYA, NADPH-protochlorophyllide reductase and asparagine synthase are down-regulated by light (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).

DNA-protein interaction studies have identified several trans-acting factors that interact with specific LREs such as G, GATA or GT1. However, no Z-box binding factor has been reported thus far. Three putative Z-DNA forming sequences (ATACGTGT), the Z-box, have been reported to be present in the Arabidopsis CAB1 promoter. Deletion analyses of this promoter have suggested that the Z-box, which is present in the light regulated minimal promoter region, is essential for the light dependent developmental expression of the CAB1 gene (Ha and An, 1988). However, although G, GATA and GT1 LREs have been studied in detail, corresponding information about Z-box LRE is yet not available (Gilmartin et al., 1992; Foster et al., 1994; Tobin and Kehoe, 1994; Menkens et al., 1995; Terzaghi and Cashmore, 1995; Puente et al., 1996; Chattopadhyay et al., 1998a). In the previous chapter, we have demonstrated how the synthetic and native Z-box containing promoters are regulated by various photoreceptors such as phyA, phyB and cry1 of light signaling pathways. We have also investigated the role of two downstream components, SPA1 and RED1, in the regulation of various Z-box containing promoters. In this chapter, we show the presence of Z-box specific DNA binding activity in light grown Arabidopsis seedlings, identify a Z-box binding factor (ZBF1) and demonstrate its in vitro molecular functions.
5.2 Results

5.2.1 The Z-box binding factor (ZBF) activity is present in *Arabidopsis*

Deletion analyses of the *CAB1* promoter have suggested that the Z-box is essential for light activation of this promoter (Ha and An, 1988). However, no trans-acting factor has been reported so far that specifically interacts with the Z-box. Therefore, to determine if there is any protein factor present that specifically interacts with the Z-box, we first carried out modified South-Western blot analysis. We prepared the nuclear extracts of 12 days old constant light grown seedlings and performed DNA binding assays with the Z-box DNA. The protein-DNA complex was cut out from the native gel and the extracted protein was loaded on to SDS PAGE with nuclear extracts as control. This was blotted on to the nylon membrane and probed with Z-box DNA. As shown in Figure 1, a protein of about 40 kD was detected suggesting Z-box binding activity is present in *Arabidopsis* light grown seedling.

To further determine if there is any protein factor present that specifically interacts with the Z-box, we used whole cell extracts of *Arabidopsis* seedlings and dimeric Z-box DNA (2Z) as a probe in gel shift assays. As shown in Figure 2, a strong low mobility DNA-protein complex (shifted band) could be detected along with the free probe (Figure 2, lane 2). This shifted band was further intensified at the same position when twice the amount of whole cell extract was used (Figure 2, lane 3). Furthermore, whereas a 50 or 100 molar excess of unlabeled 2Z DNA competed out the binding activity (Figure 2, lanes 4 and 5), no competition was observed with a 100 molar excess of 4GT1 or 4G-box (Figure 2, lanes 6 and 7), suggesting that the interaction was specific to Z-box.

To confirm the above results, we used mutated versions of the Z-box for gel shift assays. A 25 base pair DNA fragment containing three base pair substitutions in the Z-box (Zm-box) was used for gel shift assays (Figure 3A). While no DNA-protein complex was detected (Figure 3B, lane 2) with labeled Zm-box, a clear DNA-protein complex was formed with the labeled 2Z using the same extract (Figure 3B, lane 4). This complex was competed out by a 50 or 100 molar excess of unlabeled 2Z, but could not be competed out by a 100 molar excess of unlabeled Zm-box (Figure 3B, lanes 5, 6 and 7). These
Figure 1. Southwestern blot using nuclear extracts from light grown Seedlings (crude) and proteins purified (purified) from the DNA-protein complex formed. The Z-box was used as a probe.
Figure 2. Electrophoretic mobility shift (gel shift) analysis using the whole cell extracts of 12 days old constant light grown seedlings and 42 base pair Z-box DNA as probe. No protein extract was added in lane 1. When 4 μg of extract was added in lane 2, 8 μg of whole cell extract was added in lanes 3 to lane 7. The amounts of competitors added in lanes 4, 5, 6 and 7 were 50 ng 2Z, 100 ng 2Z, 100 ng 4GT1 and 100ng 4G, respectively. The increasing concentration of 2Z unlabelled DNA is shown by triangle. Plus and minus signs indicate the presence and absence, respectively, of whole cell extracts (Pro. Ext.) or competitors (COMP).
Figure 3. Identification of Z-box specific DNA-binding activity. 

A, DNA sequences of Z-box and its mutated version (Zm). B, Electrophoretic mobility shift (gel shift) analysis using the whole cell extract and 42 base pair Z-box DNA (lane 3 to 7) or 25 base pair Zm-box DNA (lane 1 and 2) as probe. No whole cell extract was added in lanes 1 and 3. Eight μg of whole cell extract was added in lanes 2, and 4-7. The amount of competitors added in lanes 5, 6 and 7 were 50ng 2Z, 100ng 2Z, 100ng Zm, respectively.
results together conclude the presence of a DNA-binding activity of Z-box binding factor (ZBF) that specifically interacts with the Z-box.

5.2.2 DNA-Ligand binding screening leads to molecular cloning of Z-box binding factors

In our laboratory, a DNA-ligand binding screening was set up to identify and clone ZBF(s). About $2 \times 10^6$ clones of a cDNA expression library were screened, made of 5-day-old constant white light grown seedlings, using a dimeric Z-box LRE as probe. Thus far, three genes have been identified and cloned from this screening, the products of which showed specific interactions with the Z-box. One such gene, ZBF1, represented by four independent cDNA clones, was selected here for further study. The coding sequence of ZBF1 cDNA isolated from the ligand binding screening (Accession no. AJ843256) appeared to be a full length cDNA (At1g32640). It codes for a protein of 623 amino acids (predicted molecular mass of 68 kD) with a basic helix loop helix (bHLH) domain (Figure 4 and 5). Previously, the same protein was identified from ligand binding screenings by two independent groups and designated as RAP1 (de Pater et al., 1997), and AtMYC2 (Abe et al., 1997, 2003).

Studies with RAP1 revealed that the protein interacted with the G-box (CACNTG) motif in pea lectin promoter (de Pater et al., 1997). On the other hand, studies with AtMYC2 demonstrated that the protein interacted with the CACATG sequence, a dehydration-responsive cis-acting element in rd22 promoter. Furthermore, recent studies have demonstrated that AtMYC2 is a transcriptional activator in ABA signaling pathways (Abe et al., 2003). Very recently, AtMYC2 (JIN1) has been shown to play a regulatory role in jasmonate-regulated defense responses (Boter et al., 2004; Lorenzo et al., 2004). In general, the bHLH proteins are demonstrated to be interacting with hexameric DNA sequence referred to as E-box (CANNTG). Recently, depending on the phylogenetic analysis, bHLH proteins have been divided into four monophyletic groups (Ledent and Vervoort, 2001). One such group binds to ACGTG core sequence, which is included in the Z-box (ATACGTGT).
Figure 4. DNA and deduced amino acid sequences of ZBF1.
Query: 1 MTDYRLQPTMNLTDDNASMMEAFMSSSDSDLWPPASATTATTATTETTPAMEIPAQ 60

Sbjct: 1 MTDYRLQPTMNLTDDNASMMEAFMSSSDSDLWPPASATTATTATTETTPAMEIPAQ 60

Query: 61 AGFNQETLQQRLOALIEGETHEGWYAIIFWQPSYDFSGASVLGWGDGYKGEEDKANPRR 120

Sbjct: 61 AGFNQETLQQRLOALIEGETHEGWYAIIFWQPSYDFSGASVLGWGDGYKGEEDKANP RR 120

Query: 121 SSSPPFSTPADQEYRKLSELNLISGGVAPSDDAVDEEVTDEWFLVSMTQSFACGA 180

Sbjct: 121 SSSPPFSTPADQEYRKLSELNLISGGVAPSDDAVDEEVTDEWFLVSMTQSFACGA 180

Query: 181 GLAGKAFATGNAVWSGDQLSGSGCERAKQQGFGMHTICAPSANGVVEVGSTEPIRQ 240

Sbjct: 181 GLAGKAFATGNAVWSGDQLSGSGCERAKQQGFGMHTICAPSANGVVEVGSTEPIRQ 240

Query: 241 SSDLINKVRLFNDGGAGDLQSLGNWNLDPDQGENDPSMINDPITPGSNEPNGAPSS 300

Sbjct: 241 SSDLINKVRLFNDGGAGDLQSLGNWNLDPDQGENDPSMINDPITPGSNEPNGAPSS 300

Query: 301 SSQLFSKSIQFENGSSSTITENPNLDPSTPSVPVHSQTQPKNFNTSFRELFSTSSSTLVK 360

Sbjct: 301 SSQLFSKSIQFENGSSSTITENPNLDPSTPSVPVHSQTQPKNFNTSFRELFSTSSSTLVK 360

Query: 361 PRSEILNFGDEGKRSGNPDPSSYSQTQFENKRRKRSMLNEDKVLSGDKTAGESDHS 420

Sbjct: 361 PRSEILNFGDEGKRSGNPDPSSYSQTQFENKRRKRSMLNEDKVLSGDKTAGESDHS 420

Query: 421 DLEASVKEVAKEKRKPGRGRKPSNGREEPLNHVEAQRQREKLMQRFYALRAVVPNVSK 480

Sbjct: 421 DLEASVKEVAKEKRKPGRGRKPSNGREEPLNHVEAQRQREKLMQRFYALRAVVPNVSK 480
Figure 5. ZBF1 codes for AtMYC2 bHLH protein (At1g32640.1). A, The homology between ZBF1 and MYC2 has been shown. The bHLH domain (447-496aa) is shown as bold letters, whereas the NLS domain (433-445aa) is marked by underline. B, Schematic diagram of the ZBF1 protein showing bHLH domain.
5.2.3 ZBF1 interacts with the Z-box LRE commonly found in minimal light responsive promoters

ZBF1 was overexpressed and purified as a glutathione S-transferase (GST) fusion protein from E. coli. As shown in Figure 6, ZBF1 was cloned in SalI+NotI site of pGEX4T-2 and the over-expressed protein was purified from E. coli (Figure 7). We used this purified glutathione S-transferase–ZBF1 (GST-ZBF1) fusion protein and a dimeric Z-box DNA as a probe in gel shift assays. A high affinity DNA-protein complex was detected along with the free probe as shown in Figure 8 (lane 3). Whereas this DNA binding activity was competed out with 50 or 100 molar excess of unlabelled Z-box DNA (Figure 8, lanes 4 and 5), no competition was observed with 100 molar excess of GT1 or Zm, a mutated version of the Z-box (Figure 8, lanes 6 and 7).

Next, the ability of ZBF1 to interact with the Z-box of native light regulated CAB1 minimal promoter was tested. A 189bp light responsive minimal promoter region of Arabidopsis CAB1 was used for gel shift assays (Figure 9A). As shown in Figure 9B, whereas GST alone did not show any binding activity, a strong low mobility DNA-protein complex was formed with GST-ZBF1 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 9B, lanes 4 and 5) but not with 100 molar excess of GT1 or Zm (Figure 9B, lane 6, 7). Taken together, these results suggest that ZBF1 specifically interacts with the Z-box LRE.

5.2.4 ZBF1 is expressed in both dark and light grown seedlings

To determine the light mediated regulation of expression of ZBF1 during early seedling development, 6-day-old constant dark (D) or various light grown seedlings including red light (RL), far red light (FR) and blue light (BL) were used for RNA gel blot analysis. As shown in Figure 10A and C, ZBF1 is expressed in the dark and in all light conditions tested. The level of expression was found to be almost similar in various light and dark grown conditions with slightly lower level in FR (Figure 10C). These results suggest that ZBF1 is expressed constitutively in dark and light grown Arabidopsis seedlings. Next, the tissue specific expression of ZBF1 in 16h light and 8h dark cycle
Figure 6. Cloning of ZBF1 in pGEX4T-2 in protein expression vector. 
A, vector map showing the restriction sites used for cloning ZBF1 in pGEX4T-2 vector. B, Restriction digestion of recombinant clones with border enzymes Sall and NotI (2kb fragment) and with internal enzyme Sacl (0.7kb fragment). M stands for the molecular weight marker: from top to bottom:21, 5, 3, 2, 1.8, 1.5, 1.3, 0.95, 0.83, 0.56 kb.
Figure 7. Overexpression and purification of ZBF1. A, BL21 cells transformed with pGEX4T-2-ZBF1 and induced with 1mM IPTG for 2, 3, 4 hours at 30°C. Lane 1, 3, 5 are uninduced and 2, 4, 6 are induced. B, Purification of GST-ZBF1 protein using glutathione sepharose beads, 2-10 are the different fractions collected and ran on to SDS PAGE and stained with Coomassie.
Figure 8. DNA binding assays using the purified GST-ZBF1 fusion protein and 2Z DNA as a probe. Lane 1 no protein added, Lane 2 1µg GST protein. One µg of purified GST-ZBF1 protein was added in lanes 3-7. The amount of competitors (Comp) added in lane no. 4, 5, 6 and 7 are 50ng 2Z, 100ng 2Z, 100ng GT1 and 100ng Zm, respectively. The increasing concentrations of unlabelled 2Z DNA used is indicated by the triangle.
Figure 9. Identification of Z-box specific binding activity in CAB1 minimal promoter region. The 189 bp fragment of light responsive minimal CAB1 promoter (A) with Z-box shown in bold letters, was tested for the binding with fusion protein GST-ZBF1. B, lane no.1 and 2 contain no protein or one μg GST as controls. About One μg of purified GST-ZBF1 was added to lane no.3-7. The amount of unlabelled DNA used as competitors (Comp) was 50ng 2Z, 100ng 2Z, 100ng GT1, 100ng Zm in lane no.4,5,6,7. The increasing concentration of unlabelled 2Z DNA is shown by triangle. Asterisk indicate a spurious band present in all lanes in figure B.
Figure 10. Expression pattern of ZBF1. A, Expression of ZBF1 under different wavelengths of light. Five day old seedlings grown under constant dark (D), white light (WL), red light (RL), far-red light (FR) and blue light (BL) were used for RNA isolation. About 10 μg of total RNA was used and probed with 1.9kb ZBF1 cDNA. B, Tissue specific expression of ZBF1. RNA was isolated from leaf, stem, root and flower of one month old plants. Ten μg of total RNA was used for northern blot analysis. C and D, Quantification of the data by Flour-S-Multi Imager (Biorad) of A and B, respectively.
grown *Arabidopsis* plants was examined. It was found that *ZBF1* was predominantly expressed in stem with very little expression in other organs of the adult *Arabidopsis* plants (Figure 10B and D).

### 5.2.5 *ZBF1* is constitutively localized in the nucleus

It has been demonstrated that the light dependent shuttling of COPI, a negative regulator of photomorphogenesis, between cytosol and nucleus is crucial for photomorphogenic growth in *Arabidopsis* (Osterlund et al. 1998; Deng and Quail., 1999; Stacey et al., 1999). *ZBF1* has a nuclear localization signal (de Pater et al., 1997) and very recently it has been demonstrated by transient expression analysis in tobacco BY2 cells that this protein is localized in the nucleus (Lorenzo et al., 2004). To further determine whether the nuclear localization of *ZBF1* is light dependent, we examined the subcellular localization of *ZBF1*-GUS fusion protein in a transient assay system in onion epidermal cells. The *uidA* gene, which encodes β-glucuronidase (GUS), was fused in frame to the coding sequence of *ZBF1*, and the expression of the fusion gene was driven by *CaMV 35S* promoter (Figure 11). As shown in Figure 12, whereas *ZBF1*-GUS protein was present in the nucleus under constant dark and WL conditions (Figure 12A-D), GUS protein alone was detected throughout the cytoplasm (Figure 12E-F). These results confirm the nuclear localization of *ZBF1* as observed by Lorenzo et al., 2004. Our results further demonstrate that nuclear localization of *ZBF1* is constitutive and is independent of light stimuli.
Figure 11. Cloning of ZBF1 with NLS domain. A, Colony PCR of putative positive clones to screen for the positive ones (2, 4, 7, 8, 10, 12, 14) for the construct pCambia-NLS. B, The 1.8kb fragment of ZBF1 with nuclear localization signal (NLS) was cloned between BgIII and Spel restriction sites in pCambia1303 vector. Molecular marker: 10, 8, 6, 5, 4, 3, 2, 1.5, 1.0, 0.5kb.
Figure 12. Nuclear localization studies of ZBF1. ZBF1 protein is constitutively localized in the nucleus when transiently expressed in onion epidermal cells. Onion epidermal peels were bombarded with constructs for the expression of either GUS reporter only or GUS-ZBF1. The peels were then incubated in darkness or light for 48 hours before GUS staining. GUS activity for GUS-ZBF1 is present in nucleus both in light and dark (A and C). Gus activity for the GUS reporter is present in both cytoplasm and nucleus under light and dark conditions (E). B, D and F show the positions of nucleus in the cell by DAPI staining. The arrows indicate the nuclei.
5.3 Discussion

Gel shift assays were carried out to determine if there was any trans-acting factor present that could specifically interact with the Z-box. A strong DNA-binding activity was detected when the wild type version of the Z-box was used. Competitive gel shift experiments with Z-box, mutated version of the Z-box and other LREs clearly demonstrated that the DNA-binding activity was specific to the Z-box.

ZBF1 was identified in a ligand binding screening using Z-box LRE as a probe. Our results with DNA-protein interaction studies provide several lines of evidence that ZBF1 recognizes the Z-box LRE of light regulated promoters. Modified South-Western blot analysis have indicated the presence of 40kD protein specific to Z-box. ZBF1 is a 68kD protein, which is not being detected in our experiment. It is likely due to less sensitivity of the method, and higher amount of total or purified protein is required for the detection of ZBF1. It is worth to mention here that a bZIP protein (ZBF2) of 38kD has also been identified in the ligand binding screening. Previous DNA-protein interaction studies demonstrated that ZBF1 interacted with the CACATG sequence, a dehydration-responsive cis-acting element in rd22 promoter (Abe et al., 1997). The bHLH proteins have commonly been shown to interact with hexameric DNA sequence known as “E-box” (CANNTG). Among the four different classes of bHLH proteins (Ledent and Vervoort, 2001), one class of bHLH protein interacts with ACGTG core sequence that is present in the Z-box sequence (ATACGTGT).

It has been shown that the expression of ZBF1 (AtMYC) is induced at a higher level by jasmonic acid and ABA, however the gene is expressed constitutively in various tissues such as flowers, siliques, stems, leaves and roots of normally grown (without any hormone) Arabidopsis plants, with maximum level of expression in siliques and stems (Abe et al., 1997; Lorenzo et al., 2004). On the other hand de Pater et al., have shown that the expression of RAP1 (ZBF1) is expressed at the highest levels in roots and stem in normally grown Arabidopsis plants. Partly in agreement with these observations, it has been found that ZBF1 is predominantly expressed in stem with very little expression in other organs of the adult plant (Figure 10B and D). The apparent discrepancy in organ specific expression levels could be attributed to the growth conditions such as light
intensities used or ecotypes. In this study, constant white light (100 μmole/sec/m²) grown plants were used for the analyses of transcript levels.

It has been demonstrated earlier that HY5, the first genetically defined transcription factor in light signaling pathways, is expressed constitutively under all light conditions and is constitutively localized in the nucleus under both dark and light conditions (Oyama et al., 1997; Chattopadhyay et al., 1998a). Furthermore it was shown that COP1 and HY5 physically interact with each other (Ang et al., 1998). All these studies lead to a hypothesis that COP1 interacts with HY5 in the dark and thus prevents HY5 from interacting with it promoters resulting in skotomorphogenesis in the dark. However, in the presence of light, COP1 is out of the nucleus and thus HY5 is free to interact with the G-box containing promoters to bring photomorphogenic development. Recent studies have revealed that COP1 acts as an ubiquitin ligase and helps in the degradation of photomorphogenesis promoting HY5, HYH, and LAF1 transcription factors. Our studies with subcellular localization of ZBF1 clearly demonstrate that this protein is constitutively present in the nucleus both in dark and light conditions. Although the molecular characterization of ZBF1 including specific interactions with the Z-box LRE, expression and subcellular localization patterns indicate that it may have a role in light mediated gene regulation, it needs further investigation to determine the in vivo function of ZBF1 in light regulated seedling development and gene expression, if any.