CHAPTER 4

Genetic interactions between ZBF2/GBF1 and HY5 in Arabidopsis seedling development
4.1. Introduction

Several transcription factors have been reported that are involved in early seedling development in Arabidopsis (Oyama et al., 1997; Chattopadhyay et al., 1998b; Ni et al., 1998; Fairchild et al., 2000; Soh et al., 2000; Spiegelman et al., 2000; Ballesteros et al., 2001; Huq and Quail, 2002; Kim et al., 2003a; Yadav et al., 2005). A group of bZIP transcription factors, GBF1, GBF2 and GBF3 has been isolated that can specifically interact with the G-box, one of the four light-responsive elements commonly found in the light regulated promoters (Schindler et al., 1992b; Foster et al., 1994; Menkens and Cashmore, 1994; Terzaghi and Cashmore, 1995; Puente et al., 1996; Chattopadhyay et al., 1998a). AtbZIP16 and AtbZIP68, two more bZIP factors of G-family has also been established recently as G-box binding factors (Shen et al., 2008a). Besides their basic and leucine zipper domains, GBFs contain three conserved proline rich domains at the N-terminal, which have been shown to have transcriptional activation potential (Jakoby et al., 2002). GARP transcriptional activators have been reported to interact with the proline rich activation domain shared by GBFs (Tamai et al., 2002). However, the specific functions of most of these genes in vivo have yet to be defined.

In our previous studies, GBF1/ZBF2 has been identified in a Southwestern screen using the Z-box light-responsive element as ligand. One of the cDNAs of ZBF2 isolated from the ligand binding screen appeared to be a full-length cDNA (At1g36730). It codes for a protein of 315 amino acids with a basic leucine zipper (bZIP) DNA binding domain. Previous studies revealed that GBF1 mRNA was present in both light and dark grown cotyledons of 5-day-old wild-type seedlings and GBF1 mRNA being present at slightly lower levels in light grown leaf tissue (Schindler et al., 1992). Dark to light transition decreased the GBF1 transcript level in seedlings; however, light to dark transition increased the accumulation of GBF1 transcript (Mallappa et al., 2006). Totally reverse regulation was found for the GBF1 protein. GBF1 protein accumulated to a lower level in darkness or at lower intensity of WL, and the level of GBF1 protein increased at higher fluence rates of WL. In dark, GBF1
has been found degraded via a proteasome-mediated pathway independent to COP1 (Mallappa et al., 2008).

The investigation of physiological functions of GBF1 has revealed that it functions in cryptochrome mediated blue light signaling and plays a dual but opposite regulatory role in Arabidopsis seedling development. \textit{gbf1} mutant seedlings show an increased inhibition of hypocotyls elongation and reduced expansion of cotyledons when grown in light (Mallappa et al., 2006). GBF1 has been reported to bind to the G-box of \textit{RBCS-1A} or Z-box of \textit{CAB1} promoter (Smykowski et al., 2010; Giuliano et al., 1988; Schindler et al., 1992a; Mallappa et al., 2006). We have shown earlier that GBF1 acts as a positive regulator as well as a negative regulator for induction of \textit{CAB1} and \textit{RBCS1A} gene expression, respectively (Mallappa et al., 2006).

\textit{HY5} is a constitutively nuclear localized basic leucine zipper (bZIP) transcription factor and is necessary for responses to a broad spectrum of wavelengths of light. It acts as a positive regulator in photomorphogenesis by affecting the expression of downstream genes in response to a light signal (Ang et al., 1998; Chattopadhyay et al., 1998b). Arabidopsis plants defective in \textit{HY5} show aberrant light mediated phenotypes, including an elongated hypocotyl, reduced chlorophyll/anthocyanin accumulation and reduced chloroplast development in greening hypocotyls (Somers et al., 1991; Oyama et al., 1997; Holm et al., 2002). \textit{HY5} mRNA accumulates in all tissues examined such as root, hypocotyl, cotyledon, leaf, stem and floral organs. \textit{HY5} mRNA accumulates in wild type seedlings to an extent approximately two times greater in the light than in darkness indicating that the expression of \textit{HY5} gene is induced by light. Both \textit{COP1} and \textit{DET1} genes repress the expression of \textit{HY5} gene in roots (Oyama et al., 1997). \textit{HY5} protein is constitutively nuclear localized regardless of light conditions and cell types (Chattopadhyay et al., 1998).

\textit{HY5} is believed to be one of the central modulators for the coordination of light signals and the regulation of appropriate gene expression (Quail, 2002a; Sullivan and Deng, 2003). \textit{HY5} interacts directly with the G-box in the promoters of light inducible genes to mediate light controlled transcriptional
activity (Chattopadhyay et al., 1998b). Recently, it has been found to bind many other promoter sites in Arabidopsis genome (Lee et al., 2007). Furthermore, in the absence of HY5, the expression of hundreds of genes is affected by UV-B or blue light (Holm et al., 2002; Ulm et al., 2004).

HYH, another important b-ZIP factor has been reported as an exclusive positive regulator of BL-mediated signal transduction. It has been found to interact with COP1, which results in degradation of HYH in dark. hyh mutant seedlings show slight longer hypocotyl when grown at medium fluence of blue light. Chlorophyll content is slight higher in mutants than the wild type, however anthocyanin content seems to be lower, but in either way more than the hy5 mutant. hyh mutants are of early flowering type as like hy5 mutant.

cDNA from this locus (AT3G17610) encodes a 149 amino-acid protein with a predicted molecular mass of 16.9kD, containing a bZIP domain in C-terminal half. HYH shows 49% overall aminoacid identity with HY5. The highest level of identity is found in the DNA binding domain where only three amino acids are different. Although the N-terminal half of the proteins are less conserved, a sequence motif previously identified in HY5 as a COP1 interacting motif and a casein kinase2 phosphorylation site are conserved in HYH (Holms et.al. 2002). Transcription of HYH is found to be dramatically higher in light grown seedlings in compared with seedlings grown in darkness. HYH is transcribed at a higher rate in R and BL. HYH mRNA accumulation is greatly reduced at FRL. HYH is nuclear protein and colocalizes with COP1 in living plant cells (Holm et al., 2002).

In this chapter, we have investigated the genetic and physiological interrelations between ZBF2/GBF1 and HY5 in Arabidopsis seedling development.
Figure 1. The functional relationships of *gbf1* and *hy5* for inhibition of hypocotyl elongation in WL.

Fluence response curve showing hypocotyl length of segregated WT, *gbf1*, *gbf1 hy5*, and *hy5* seedlings grown in different fluence rates of WL for 6 days. Experiments were performed thrice with similar results. Graph depicts data from representative experiment. Error bars represent standard deviations (n≥30).
Figure 2. Altered functional relationships between \textit{gfb1} and \textit{hy5} at various intensities of WL.

(A)-(F) Representative pictures of 6-days-old seedlings from left Segregated WT, \textit{gfb1}, \textit{gfb1 hy5} and \textit{hy5} seedlings grown in constant dark (A), WL 5\textmu mol/m\textsuperscript{2}/s (B), WL 15\textmu mol/m\textsuperscript{2}/s (C), WL 30\textmu mol/m\textsuperscript{2}/s (D), WL 60\textmu mol/m\textsuperscript{2}/s (E), WL 90\textmu mol/m\textsuperscript{2}/s (F).
4.2. Results

4.2.1. The photomorphogenic growth of \textit{gfb1} is altered by additional mutation in \textit{HY5} in light quality and quantity dependent manner

GBF1 plays a negative regulatory role in light mediated inhibition of hypocotyl elongation and is a major player in cBL mediated photomorphogenic growth (Mallappa et al., 2006). Although, HY5 exhibits a positive regulatory role for photomorphogenic growth at various wavelengths of light (Ang and Deng., 1994). To determine the genetic interaction between GBF1 and HY5, we constructed \textit{gfb1 hy5} double mutant and investigated the morphology of the seedlings in dark and light conditions. While examining the growth of 6-day-old \textit{gfb1 hy5} double mutants at various intensities of WL, it was observed that the inhibition of hypocotyl elongation in \textit{gfb1 hy5} seedlings was similar to \textit{gfb1} at lower fluence rates (Figure1 and 2B). However at moderate light intensities of WL, \textit{gfb1 hy5} displayed hypocotyl length similar to WT (Figure1, 2C and D). Furthermore, at higher fluence rates of WL, \textit{gfb1 hy5} mutants showed hypophotomorphogenic growth similar to \textit{hy5} (Figure1, 2E and F). These results revealed altered functional relationship between GBF1 and HY5 depending upon the light intensities. \textit{gfb1} is epistatic to \textit{hy5} at lower fluences, however they work antagonistically at moderate fluence rates of WL nullifying each other’s effect on hypocotyls growth. At higher fluence rates, \textit{hy5} seems to be epistatic to \textit{gfb1} in regulation of hypocotyls growth. It is worth mentioning here that earlier studies have revealed more prominent morphological defects of \textit{gfb1} at lower fluence rates, whereas \textit{hy5} mutants display the strong phenotype at higher fluence rates of WL (Osterlund et al., 2000b; Mallappa et al., 2006).

Interestingly, the light intensity dependent genetic interaction on growth in WL between \textit{gfb1} and \textit{hy5} was not observed in BL. As shown in Figure 3A and B, \textit{gfb1 hy5} seedlings showed hypocotyl length in between \textit{gfb1} and \textit{hy5} single mutants in BL, suggesting that \textit{gfb1} and \textit{hy5} are antagonistic to each other in light mediated inhibition of hypocotyl elongation in BL. In darkness, \textit{gfb1}, \textit{hy5} and \textit{gfb1 hy5} exhibited similar skotomorphogenic growth as wild type seedlings (Figure 2A).
Figure 3. GBF1 and HY5 work antagonistically in BL-mediated inhibition of hypocotyl elongation.

(A) Fluence response curve showing hypocotyl length of segregated WT, gbf1, gbf1 hy5, hy5 grown under different fluence rates of BL for 6 days. Experiments were performed thrice with similar results. Data has been shown from representative experiment. Error bars represent standard deviations (n≥30).

(B) Representative pictures of 6-day-old seedlings, from left segregated WT, gbf1, gbf1 hy5, hy5 grown in BL 15µmol/m²/s.
Figure 4. Physiological responses of *gfb1 hy5*.

(A)-(B) Quantitative representation of chlorophyll A and B content in 3-day-old segregated WT, *gfb1*, *hy5*, *gfb1 hy5* seedlings grown in WL 90μmol/m²/s (A), and in BL 45μmol/m²/s (B).

(C)-(D) Quantitative representation of anthocyanin accumulation in 3-day-old segregated WT, *gfb1*, *hy5*, *gfb1 hy5* seedlings grown in WL 90μmol/m²/s (C), and in BL 45μmol/m²/s (D). Experiments were performed thrice with similar results. Data has been shown from representative experiment. Error bars represent standard deviations (n≥3).
4.2.2. Genetic interactions between GBF1 and HY5 modulate physiological responses in early seedling development

The hy5 mutants accumulate lower level of anthocyanin and chlorophyll than the wild type seedlings (Holm et al., 2002). To determine the genetic interactions between GBF1 and HY5 for anthocyanin accumulation, we examined anthocyanin level in 3-day-old seedlings grown in cWL and cBL conditions. gbf1 seedlings accumulated higher level of anthocyanin compared to WT and the anthocyanin content in gbf1 hy5 was similar to hy5 seedlings in either light conditions (Figure 4C and D). These results suggest that hy5 is epistatic to gbf1 for anthocyanin accumulation.

Examination of chlorophyll content revealed that gbf1 and hy5 mutants accumulate less chlorophyll, however gbf1 hy5 double mutants displayed chlorophyll accumulation reaching to the level as in wild type seedlings in WL (Figure 4A). Whereas gbf1 mutants showed slightly lower level of chlorophyll accumulation than wild type, the chlorophyll accumulation was drastically reduced in hy5 mutant in BL. Measurements of chlorophyll contents in gbf1 hy5 double mutants revealed a similar level of chlorophyll accumulation as hy5 (Figure 4B). These results indicate that although GBF1 and HY5 act antagonistically in WL, HY5 works downstream to GBF1 in BL-mediated accumulation of chlorophyll.

4.2.3. GBF1 and HY5 work antagonistically to regulate light regulated gene expression

The light mediated induction of CAB1 and RBCS-1A gene expression is differentially regulated by GBF1 (Mallappa et al., 2006). We have shown earlier that GBF1 acts as a positive regulator of CAB1, however negatively regulates RBCS-1A gene expression respectively (Mallappa et al., 2008). A recent study strongly puts HY5 as a positive regulator for induction of both CAB1 and RBCS-1A gene expression (Lee et al., 2007). To determine the effect of genetic and physical interactions between GBF1 and HY5 on light regulated modulation of CAB1 and RBCS-1A gene expression, we performed Northern blot analyses for both the transcripts using cBL grown 6-day-old seedlings. At
Figure 5. Additional \textit{hy5} mutation in \textit{gbf1}, reverts back the light mediated gene expression to WT level.

\textbf{(A)-(B)} Transcript level of \textit{CAB1} in 6-day-old Col, WS, \textit{gbf1}, \textit{hy5}, \textit{gbf1 hy5} seedlings grown in BL 20\textmu mol/m^2/s \textbf{(A)}, densitometry \textbf{(B)}.

\textbf{(C)-(D)} Transcript level of \textit{RBCS-1A} in cotyledons of 6-day-old Col, WS, \textit{gbf1}, \textit{hy5}, \textit{gbf1 hy5} seedlings grown in BL 20\textmu mol/m^2/s \textbf{(C)}, densitometry \textbf{(D)}.

Error bars in graph show the standard deviations.
Figure 6. *gbf1* is antagonistic to *hy5* in controlling the root growth.

(A) Root growth of 12-day-old WL 90μmol/m²/s grown seedlings. From left Segregated WT, *gbf1*, *gbf1 hy5*, *hy5*.

(B) Graph depicting number of lateral root primordias. Error bars represent standard deviation (n>30). The experiments were repeated thrice with similar results and data from the representative experiment have been shown here.
the moderate fluence rates of cBL, *CAB1* expression in *gpf1* and *hy5* was lower than the wild type, whereas the expression of *CAB1* in *gpf1 hy5* double mutant was found to be similar to that of wild type background (Figure 5A and B). We further examined the expression of *RBCS-1A* in cotyledons of the seedlings. We found an elevated expression of *RBCS-1A* in *gpf1* line comparative to wild type supporting our earlier observation. The *hy5* mutants showed a similar expression like wild type, which was consistent to a previous study (Ang and Deng, 1994). Expression of *RBCS-1A* in *gpf1 hy5* seedlings was in between that of both the wild type ecotypes (Figure 5C and D). These results indicate that *GBF1* and *HY5* interact antagonistically to regulate the expression of *CAB1* and *RBCS-1A*.

4.2.4. *GBF1* is antagonistic to *HY5* in lateral root formation

To determine whether mutation in *HY5* could modulate the lateral root formation in *gpf1*, we examined the root growth of adult plants of *gpf1 hy5* double mutants. Although 12-days-old plants of *gpf1* showed less lateral roots, *hy5* mutant plants have been found to grow with more number of lateral roots, consistent to previous reports. Instead, *gpf1 hy5* adult plants showed less pronounced effect of mutation at *HY5* locus, showing the number of lateral roots in between both the parents (Figure 6A and B). These results suggest that *GBF1* works antagonistically with *HY5* in regulation of lateral roots formation.
4.3. Discussion

Photomorphogenesis consists of different morphological changes in seedling development such as opening of apical hook, expansion of cotyledons, inhibition of hypocotyl elongation and chloroplast biogenesis (Nagatani et al., 1993; Whitelam et al., 1993; Neff et al., 2000). These morphological changes are likely to be the result of differential gene expression at the onset of light signal (Ma et al., 2001). This in turn is regulated by different transcription factors, combinatorially regulating the gene expression in concert with the quality and quantity of light and cis-box elements present in the light regulated promoters (Jiao et al., 2007).

In our study, we found that *gfb1* seedlings show shorter hypocotyl length comparative to wild type in cWL at all the light intensities tested and *hy5* show hypophotomorphogenic growth consistent to previous studies. *gfb1 hy5* was found to grow with altered photomorphogenic growth comparative to both the parents, depending upon the light intensities. At lower intensity of WL, *gfb1 hy5* showed the hypocotyl length similar to *gfb1*, putting GBF1 functionally downstream to HY5, however, moderate light intensities promoted antagonistic interaction of both the factors, working in a parallel pathway. At higher intensities, hypocotyl length of *gfb1 hy5* increased closer to that of *hy5* seedlings. This altered morphogenic response of *gfb1 hy5* could be attributed to altered interaction of both the molecules at transcriptional and post translational level. In a recent chromatin immunoprecipitation study, GBF1 upstream region was found to be present in the immunoprecipitates with αHA antibody from 35S-HA:HY5 transgenic lines (Lee et al., 2007), which indicates that HY5 binds to the promoter of GBF1, and therefore these in vivo studies suggest that GBF1 may act downstream to HY5. On another hand, antagonistic interaction between *gfb1* and *hy5* indicate that they act antagonistically in parallel pathways. At higher intensities of white light, long hypocotyl phenotype of *gfb1 hy5* reveals the increasing essentiality of functional HY5. Interestingly, consistent with these observations, earlier studies have revealed that whereas GBF1 plays the major role at lower fluences, HY5 predominantly functions at higher fluences of white light in Arabidopsis seedling development. It could be
envisioned that wild type plants growing in ambient environment might be able to differentiate between the incident light intensities through different interactions at genetic and molecular level.

In blue light mediated inhibition of hypocotyl elongation in \textit{gbf1 hy5} was found to be largely outcome of the antagonistic interaction of \textit{gbf1} and \textit{hy5}. Intensity dependent altered functional relationship between the two was not seen in blue light. Probably cryptochromes transduce the light signal to these two downstream components in two parallel pathways and favour preferably the interaction between the two, which proves to be antagonistic between a positive (HY5) and a negative (GBF1) regulator of the light mediated inhibition of hypocotyl elongation. Furthermore, blue light mediated induction of light regulated gene expression was also found to be regulated antagonistically by GBF1 and HY5 as \textit{gbf1 hy5} mutant seedlings restored the expression level of \textit{CAB1} and \textit{RBCS1A} similar to wild type. These findings further strengthened our hypothesis of heterodimerization of GBF1 and HY5 and their combinatorial regulation of light regulated promoters.

Interestingly, we found \textit{hy5} working epistatic to \textit{gbf1} for some of the physiological responses. 3-days-old white as well as blue light grown seedlings of \textit{gbf1 hy5} showed anthocyanin accumulation similar to that of \textit{hy5} seedlings, which was at least 7-10 fold less comparative to wild type. However, \textit{gbf1} mutant seedlings exhibited 2-3 times higher accumulation of anthocyanin. Besides these, chlorophyll accumulation in \textit{gbf1 hy5} seedlings was found to be antagonistically regulated by GBF1 and HY5, when grown in WL. Although in blue light conditions, chlorophyll accumulated in \textit{gbf1 hy5} double mutant at very low level similar to \textit{hy5} seedlings. This part of study indicates that defects in anthocyanin accumulation of \textit{gbf1} seedlings is dependent upon presence of functional HY5 in both phytochromes and cryptochromes mediated signaling. For chlorophyll accumulation both the molecules act interdependently in white light as well as blue light conditions. These interactions could be mediated indirectly by other factors.

In this study we have reached to the conclusion that GBF1 and HY5 function antagonistically in regulation of lateral root formation. The lateral root
initiation is the phenomenon, which could also be resultant of different hormone signals, and other factors might as well be involved. It is reasonable to suspect that such interactions are involved in integrating signals from different light signalling branches and other factors, such as temperature, salinity, sugar, pathogens and hormones.