JBC Papers in Press. Published on April 25, 2006 as Manuscript M601172200
The latest version is at http://www.jbc.org/cgi/doi/10.1074/jbc.M601172200

A bZIP TRANSCRIPTION FACTOR, G-BOX BINDING FACTOR 1, REGULATES BLUE LIGHT MEDIATED PHOTOMORPHOGENIC GROWTH IN ARABIDOPSIS*

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Running title: Seedling development by GBF1

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Several transcriptional regulators have been identified and demonstrated to play either positive or negative regulatory roles in seedling development. However, the regulatory coordination between hypocotyl elongation and cotyledon expansion during early seedling development in plants remains unknown. We report the identification of a Z-box binding factor (ZBF2) and its functional characterization in cryptochrome mediated blue light signaling. *ZBF2* encodes a G-box binding factor (GBF1), which is a basic leucine zipper transcription factor. Our DNA-protein interaction studies reveal that ZBF2/GBF1 also interacts with the Z-box light responsive element of light regulated promoters. Genetic analyses of *gbf1* mutants and overexpression studies suggest that GBF1 acts as a repressor of blue light mediated inhibition in hypocotyl elongation, however it acts as a positive regulator of cotyledon expansion during photomorphogenic growth. Furthermore, whereas GBF1 acts as a positive regulator of lateral root formation, it differentially regulates the expression of light inducible genes. Taken together, these results demonstrate that GBF1 is a unique transcriptional regulator of photomorphogenesis in blue light.

Arabidopsis seedlings are genetically defined to follow two distinct developmental pathways: skoto-morphogenesis or etiolation in the dark and photomorphogenesis or deetiolation in the light (1,2,3). In the dark, seedlings grow with elongated hypocotyls, small and closed cotyledons, and the light inducible genes are expressed either at low or below detectable levels. The presence of light inhibits hypocotyl elongation, promotes cotyledon opening and expansion, and thus results in photomorphogenesis. The light inducible genes are expressed at high level during photomorphogenic growth.

Plants are able to perceive various wavelengths of light through photoreceptors. Far-red and red light are perceived by phytochromes (phyA to phyE); whereas cryptochromes (cry1 and cry2) are involved in the perception of blue and UV-A light (4,5,6,7). Recent studies have made significant progress in the identification and functional characterization of downstream components in phytochrome signaling (1,2,6,7,8). HYH, AtPP7 and ZBF1/MYC2 have been reported as BL specific regulators of photomorphogenic growth in Arabidopsis (9,10,11). However, the connection of photo-perception to transcription in blue light still remains largely unclear (11).

Several transcription factors have been reported that are involved in early seedling development in Arabidopsis (11, 12,13,14,15,16,17,18,19,20,21,22). COP1, a repressor of photomorphogenesis in the dark, acts as an ubiquitin ligase, and it interacts with and mediates the degradation of photomorphogenesis promoting factors such as HY5, HYH, LAF1 and HFR1 in the dark (9,23,24,25,26). Recent studies have shown that COP1 interacts with SPA1, a negative regulator acting in far red light, and this interaction is critical for proteasome-mediated degradation of HY5 and LAF1 (25,27,28).

Analyses of the promoter sequences of light inducible genes including *CAB*, *RBCS* and *CHS* have led to identification of at least four commonly found light responsive elements (LREs): G, GATA, GT1, and Z-box, which have been demonstrated to be essential for light-mediated transcriptional activity (29,30,31,32,33,34,35). Several LRE specific transacting factors have been identified earlier, and in some cases the genes that encode such factors have been cloned and their functions have been investigated (31,36). A four-member gene family encoding proteins containing basic leucine zipper DNA binding domains (GBFs) have been reported (37,38). Extensive DNA-protein interaction studies have been carried out with GBFs. The light regulated modification and subcellular localization of GBFs have also been investigated. It has been proposed from these studies that the limited nuclear access may be an important control of the activities of GBFs (39). However, the in vivo functions of these genes are yet to be defined.

A Z-DNA forming sequence (ATACGTGT) is present in *CAB1* minimal promoter that is essential for light dependent developmental expression of *CAB1* gene (29). Recent studies have revealed that the Z-box containing synthetic and native promoters are responsive to phyA, phyB and cry1 photoreceptors and are under the control of downstream regulatory components such as COP1 and HY5 (33,35). To identify and clone ZBFs (Z-box binding factors), we have carried out DNA-ligand binding screening to screen an Arabidopsis cDNA expression library and have identified several such factors. One of these ZBFs, ZBF1/MYC2, has very recently been shown to be a negative regulator of blue light mediated photomorphogrnic growth. Furthermore, it has been demonstrated that ZBF1/MYC2 acts as a point of crosstalk among light, ABA and JA signaling pathways (11,40,41). We have investigated the functional relevance to light regulated gene expression and photomorphogenic growth of another ZBF (ZBF2/GBF1) in this study.

Experimental Procedures

Plant Materials and Transformations - Surface sterilized seeds were sown on Murashige and Skoog (MS) plates, kept at 4°C in darkness for 3-5 days and transferred to light at 22°C. The intensities of continuous light sources used in this study are: white light (100, 60, 30, 15, 5 and 1 μmole m^{-2} s^{-1}); blue light (40, 30, 15, 5 and 1 μmole m^{-2} s^{-1}); red light (95, 30, 15, and 5 μmole m^{-2} s^{-1}) and far-red light (90, 30, 15, and 5 μmole m^{-2} s^{-1}). Unless otherwise mentioned, the highest light intensities were used for the experiments.

The T-DNA tagged mutant lines heterozygous or homozygous for the *zbf2*/*gbf1* mutations were identified by genomic PCR analyses. Individual plants of T2 generation, obtained from a self fertilized heterozygous plant, were examined by genomic PCR using the left border specific primer LBP: 5'-GCGTGGACCGCTTGCTGCACCT-3' and *GBF1* specific primers LP13: 5'-GTGCCATAAGGCGGCATCATA-3' and RP13: 5'-TGCAAACAAACACCTTTGCATGT-3' (for *gbf1-1* mutants); and LP14: 5'-GCACCGAACCTTGGATTTCAC-3' and RP14: 5'-TTCCCATCCCCAGTTGGATCT-3' (for *gbf1-2* mutants). A segregated wild type (Col) line was used to compare the phenotypic and molecular differences with the *gbf1* mutants.

For the generation of GBF1/ZBF2 overexpessor transgenic lines, 1.15 kb fragment of *GBF1* cDNA was PCR amplified using primers 5'-GAAGATCTTGAGTAACACAAGTAAGT AGTAAGC-3' and 5'-GACTAGTAATCGTAGCTTTTGCAGCT T-3'. The PCR product was digested and cloned into BglII and SpeI site of pCAMBIA1303, a binary vector carrying the CaMV *35S* promoter. For the

complementation test, a genomic fragment containing full length *GBF1* and about 1.3 kb upstream DNA sequence was cloned into SmaI site of pBI101.2 vector. The *Agrobacterium* strain *GV3101* was transformed individually with each recombinant construct. The Arabidopsis wild type (Ws) plants (for over-expression) or *gbf1-1* mutant plants (for complementation) were transformed using *Agrobacterium* mediated vacuum infiltration method. Transgenic plants (T1) were screened on 15 μg/ml hygromycin or 20 μg/ml kanamycin containing MS plates. Several individual lines with single T-DNA locus, as determined by the segregation of hygromycin or kanamycin resistant versus sensitive ratios (3:1), were selected and homozygous transgenic plants were generated for further studies.

For the generation of *gbf1 cry1*, *gbf1 cry2* and *gbf1 phyA* double mutants, homozygous *gbf1-1* mutant plants (Col) were crossed individually with *hy4-2.23N* (Ler; 4), *cry2-1* (Col; 42) and *phyA-101* (RLD; 43) homozygous mutant lines. F2 seedlings were grown in WL (60 μmole m^{-2} s^{-1}) or FR (30 μmole m^{-2} s^{-1}) for the identification of *cry1*, *cry2* or *phyA* homozygous lines, respectively, and elongated seedlings were transferred to soil. To determine the genotype at *GBF1* locus, about 40 seedlings from each line were tested by genomic PCR. F3 progeny that are homozygous for *gbf1-1* mutant plants were further tested and designated as *gbf1 cry1*, *gbf1 cry2* and *gbf1 phyA* double mutants. Since *gbf1*, *cry1*, *cry2* and *phyA* were of different ecotype backgrounds, F2 seedlings, which were heterozygous for *cry1*, *cry2* or *phyA* mutations but homozygous wild type for *GBF1* were used as control (WT).

Transgenic Lines with Promoter-GUS Constructs and GUS Assays - The promoter-reporter constructs used in this study have been described (33,35). GUS staining (using 20-30 seedlings in each sample) and GUS activity measurements (40-50 seedlings) has been described in (34). Wild type and *gbf1* mutant plants containing the same transgene were stained for the same length of time.

Measurements of Epidermal Cell Length and Expansion - Measurements of epidermal cell length and expansion of six-day-old seedlings were essentially carried out as described (44,45).

Electrophoretic Mobility Shift (Gel shift) assays - The full length *GBF1* cDNA was cloned in pGEX4T-2 vector and *GST-GBF1* was induced using 1mM IPTG and overexpressed in *E.coli*. The overexpressed GST-GBF1 was affinity purified following manufacturer's protocol (Amersham, USA). The DNA binding assays were performed as described (13). The 189 bp DNA fragment of *CAB1* minimal promoter was cloned into pBluescript vector after PCR with primers: FP 5'CGGAATTCATAAGGATAGAGAGAT CTATTC 3' and RP 5'CGGGATCCTGAGGTTGCTATTGGCT AGTCAT 3' using genomic DNA as template. The 189 bp fragment was digested with EcoRI + BamHI, purified and 3' end labeled for using as probe for the DNA binding assays. One ng of labeled DNA was used for each binding reactions.

Northern and Immunoblot Analyses - Total RNA was extracted using the RNeasy plant minikit (Qiagen) following the manufacturer's instruction. We used a 1.15 kb full length cDNA fragment of GBF1 for probe preparation using random priming kit (Megaprime™, Amersham) following the manufacturer's instructions. The DNA fragments of *CAB*, *RBCS* and *CHS* genes were used for probes as described (11). To quantify the Northern blot data, the intensity of each band was quantified by Fluor-S-MultiImager (BioRad) and ratios of *GBF1, CAB, RBCS* or *CHS* gene versus its corresponding *18S rRNA* band were determined and plotted.

Protein extracts were prepared from wild type or *gbf1* mutant seedlings. Twenty μg total protein was used for immunoblot analysis. Proteins were separated by 8% SDS-PAGE. Pre-stained protein markers (AmershamBiosciences) were used for

molecular mass determination. The gel was stained with Coommassie Brilliant Blue R250 for visualization. For immunoblot analysis, the proteins were transferred to Hybond C-Extra (Amersham Pharmacia Biotech), blocked with 5% BSA in PBS (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 140 mM NaCl, 2.7 mM KCl) and probed with affinity-purified GBF1 polyclonal antibodies.

Chlorophyll and Anthocyanin Measurements - Chlorophyll and anthocyanin contents were measured following essentially the same protocols as described in (9).

Sequence data (GBF1) from this article have been deposited with the EMBL/GenBank data libraries under accession number: AJ843257

RESULTS

Molecular Cloning of GBF1 - We screened a cDNA expression library by DNA-ligand binding screening for the identification of ZBFs. Several genes have been identified and cloned from this screen, the products of which showed specific interactions with the Z-box (11,35). We chose one of these genes, *ZBF2* (Z-box binding factor 2), which was represented by three independent cDNA clones, for this study. 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. The same protein was earlier shown to be interacting with the G-box and designated as GBF1 (37). Therefore, hence onwards we designate this gene as *GBF1*.

GBF1 *Interacts With the Z- and G-box LREs of Light Regulated Promoters* - To further examine whether GBF1 was able to specifically interact with the Z-box, we purified glutathione S-transferase–GBF1 (GST-GBF1) fusion protein from *E. coli* and performed electrophoretic mobility shift (gel shift) assays using the Z-box DNA as probe. A high affinity DNA-protein complex was detected along with the free probe as shown in Fig. 1A (lane 3). This DNA binding activity of GBF1 was efficiently competed by 50 or 100 molar excess unlabelled Z-box DNA (Fig. 1A, lanes 4-5). Since it was earlier reported that GBF1 could interact with the G-box (Schindler et al., 1992), we also competed this binding activity with a consensus G-box (13). As shown in Fig. 1A, lanes 6-7, the G-box was able to compete more efficiently this binding activity. Whereas 50 molar excess of Z-box was unable to compete the interaction completely, the unlabelled G-box was able to do so at the same molar excess. However, GT1 failed to compete for the GBF1 binding activity even at 100 molar excess (Fig. 1A, lane 8).

To further test the relative affinity of GBF1 for the G- and Z-box LREs, we carried out similar experiments using the tetrameric G-box as probe. As shown in Fig. 1B, whereas 50 molar excess of unlabelled Z-box was unable to compete the binding activity completely, the G-box was able to efficiently compete the interaction at the same molar excess (lanes 4-7). In fact, further experiments using various amounts of unlabelled G- or Z-box revealed that whereas 40 molar excess of unlabelled G-box was able to compete the binding activity, about 70 molar excess of Z-box was required to compete the binding activity of GBF1 completely (data not shown). Taken together, these results suggest that GBF1 interacts with the Z- and G-box LREs, and the protein may have slightly more affinity towards the G-box as compare to the Z-box.

To further substantiate the interaction of GBF1 with the Z-box, we tested the ability of GBF1 to interact with the Z-box present in native *CAB1* minimal promoter. The Z-box present within the minimal promoter region of *CAB1* has been shown to be critical for light regulated expression of this gene (29). The 189 bp DNA fragment of *CAB1* was used for gel shift assays. As shown in Fig. 1C, whereas GST alone did not show any binding

activity, a strong low mobility DNA-protein complex was formed with GST-GBF1 fusion protein (lanes 2 and 3). This interaction was efficiently competed out with 80 or 120 molar excess of unlabelled Z-box (Fig. 1C, lanes 4 and 5) but not with 120 molar excess of GT1 or Zm, a mutated version of the Z-box (Fig. 1C, lane 6-7). Taken together, these results demonstrate that GBF1 specifically binds to the Z-box of native *CAB1* minimal promoter.

Isolation and Characterization of Null Mutations in GBF1 - To investigate the in vivo function of GBF1, we searched for mutants in T-DNA knockout collections (46). Two independent mutant lines with T-DNA insertion were identified and the corresponding alleles were designated as *gbf1-1* (*zbf2-1*) and *gbf1-2* (*zbf2-2*). We performed PCR genotyping analyses to determine plants that are homozygous or heterozygous for *gbf1-1* or *gbf1-2* mutations. We monitored the segregation of self-fertilized plants heterozygous for *gbf1-1*or *gbf1-2*. The segregation ratios determined by the analyses of genotyping PCR in T2 progeny suggested that a single T-DNA locus was present in each of *gbf1-1* or *gbf1-2* mutant lines. The junctions of T-DNA and *GBF1* were amplified by PCR, and the DNA sequence analyses revealed that the T-DNA was inserted in nucleotide position 80bp upstream to the start codon of *GBF1* in *gbf1-1*, and in nucleotide position 660bp downstream to the start codon of *GBF1* in *gbf1-2* mutants (Fig. 2A). Northern and immunoblot analyses were unable to detect any GBF1 mRNA or protein in *gbf1-1* or *gbf1-2* mutant backgrounds suggesting that *gbf1-1* and *gbf1-2* are likely to be null mutants (Fig. 2B-D).

Previous studies revealed that *GBF1* mRNA was present in both light and dark grown cotyledons of 5-day-old wild type seedlings (37). To quantify and expand our understanding about the pattern of expression of *GBF1* in wild type background, we carried out time course experiments. For these experiments, 5-day-old seedlings grown in constant dark or white light (WL) were transferred to WL or dark, respectively, for 0.5, 1, 2 or 4 hours, and the steady state mRNA levels were measured. About 3 fold reduction in the expression of *GBF1* was detected after 4 hours of exposure to WL as compared to dark grown seedlings (Fig. 2E, upper panel and I). In agreement with this observation, the expression of *GBF1* was increased to about 4 fold after 4 hours of exposure to dark as compared to WL grown seedlings (Fig. 2E, upper panel and I). We performed similar time course experiments to determine whether the level of GBF1 protein was also higher in dark grown seedlings. However, we could not detect any significant change at the protein level during dark to WL transitions or vice versa (Fig. 2E, lower panel). These results suggest that although the transcript level of *GBF1* varies depending on the presence or absence of WL, the protein level remains largely unaltered.

Since *GBF1* is expressed in WL, we asked whether it was expressed under various wavelengths of light including far-red light (FR), red light (RL), and blue light (BL). As shown in Fig. 2F and J, *GBF1* was expressed in all light conditions tested with maximum level of expression in FR. To further examine the light dependent expression of *GBF1*, we carried out time course experiments. For these experiments, 5-day-old seedlings grown in constant WL were transferred to various wavelengths of light for 0.5, 1, 2 or 4 hours and the steady state mRNA levels of *GBF1* were determined. As shown in Fig. 2G, the expression of *GBF1* slightly decreased or increased in RL or BL, respectively, however the expression of *GBF1* was significantly elevated after 4 hours of exposure to FR. The examination of tissue specific expression of *GBF1* in adult plants revealed that the gene was expressed in root, stem, and flower at similar levels, however about 2 fold less expression was detected in the leaf tissues (Fig. 2H and K).

gbf1 Mutants Exhibit Blue Light Specific Morphological Defects in Seedling

Development - We monitored the growth of 6-day-old *gbf1* mutant seedlings in constant dark or WL conditions. As shown in Figs. 3A and 4A-B, no morphological difference was detected between wild type and *gbf1* mutants grown in constant darkness. However, *gbf1* mutants displayed increased sensitivity to WL irradiation under various fluences and therefore, resulted in strikingly shorter hypocotyls as compared to the wild type seedlings (Figs. 3B-C and J, and 4A). The effects appeared to be more pronounced within 5 to 30 μmol m^{-2} s^{-1} fluence rates of WL. We asked whether the hypersensitive phenotype of *gbf1* was specific to a particular wavelength of light. To address this question, the growth of 6-day-old seedlings under various wavelengths of light was tested. The enhanced inhibition in hypocotyl elongation in *gbf1* was observed in constant BL, however no significant change in hypocotyl length was observed in constant RL or FR under various fluences (Figs. 3D-F and K, and 4B). Furthermore, although the hypocotyls of *gbf1* displayed hypersensitivity to WL and BL, the cotyledons were found to be less sensitive to WL and BL. Thereby, the cotyledons of *gbf1* mutants were found to be significantly smaller as compared to wild type seedlings under WL and BL grown conditions (Figs. 3G-H and L, and 4C-D). Taken together, these results suggest that GBF1 acts as a negative regulator of inhibition of hypocotyl elongation, however it functions as a positive regulator of cotyledon expansion in BL. The examination of the leaf size of wild type and *gbf1* mutant plants however revealed no significant differences (Fig. 3R). A genomic fragment containing *GBF1* and its upstream sequence of about 1.3kb was introduced into the *gbf1-1* mutants plants for complementation test. The transgenic seedlings were unable to display BL specific phenotypes suggesting that the observed phenotypes of *gbf1* mutants are due to the loss of *GBF1* functions (data not shown).

To determine whether the enhanced inhibition in hypocotyl elongation or smaller cotyledon size of *gbf1* mutants is due to the altered cell elongation or expansion, we examined the size of epidermal cells of *gbf1* mutants and compared with the wild type 6-day-old seedlings grown in BL. As shown in Figs. 3M-O and 6B, the epidermal cells of hypocotyls were significantly shorter in *gbf1* mutants as compared to wild type. Similarly, the epidermal cells of cotyledons were found to be significantly less expanded in *gbf1* mutants as compared to 6-day-old wild type seedlings (Fig. 3P-Q). These results indicate that GBF1 acts as a regulator of growth that promotes cell elongation and expansion and thus the loss of *GBF1* function mutants result in shorter hypocotyls and less expanded cotyledons during seedling development.

To investigate whether *gbf1* mutants have any additional morphological defects, we examined and compared the root growth of *gbf1* mutants with wild type plants. The *gbf1* mutant plants produced significantly less number of lateral roots as compared to wild type plants suggesting that GBF1 is essential for optimum lateral root formation (Figs. 3I, and 6D).

While propagating *gbf1* mutant plants, we observed that the *gbf1* mutation caused early flowering. Whereas long-day-grown (16h light/8h dark cycles) wild type plants start flowering after the formation of about 10-11 rosette leaves, *gbf1* mutants flower after producing about 7 to 8 rosette leaves (Fig. 6A and C). However, such effect was not detected in short-day-grown (8h light/16h dark cycles) *gbf1* mutant plants (data not shown).

Mutations in GBF1 Result in Altered Chlorophyll Accumulation - Chlorophyll and anthocyanin syntheses are two important physiological responses regulated by light. To examine whether *gbf1* mutants have altered chlorophyll or anthocyanin accumulation, we measured the chlorophyll and anthocyanin contents in *gbf1* mutant seedlings. We measured the chlorophyll content of cotyledons and normalized the chlorophyll content by cotyledon size. The chlorophyll content was found to be significantly lower in *gbf1* mutants as compared to wild type seedlings (Fig. 4E).

No difference in accumulation of anythocyanin was detected between wild type and *gbf1* mutant seedlings (data not shown).

gbf1 Mutants are Epistatic to cry1 and cry2 - To determine the involvement of photoreceptors such as cry1, cry2 and phyA in BL specific functions of GBF1, we performed epistasis analyses. We generated *gbf1 cry1*, *gbf1 cry2* and *gbf1 phyA* double mutants and examined the hypocotyl length in comparison to *cry1* (Ler), *cry2* (Col), *phyA* (RLD) and *gbf1* (Col) mutants. Measurements of hypocotyl length revealed that double mutants such as *gbf1 cry1* and *gbf1 cry2* displayed similar hypocotyl lengths as *gbf1* mutants in BL (Fig. 4F-G). However, *gbf1 phyA* double mutants exhibited hypocotyl length similar to *phyA* mutants in BL (Fig. 4H). These results suggest that GBF1 likely acts downstream to both cry1 and cry2 photoreceptors and the increased sensitivity to BL caused by the *gbf1* mutation also requires blue light perception by phyA.

GBF1 Over-expressers Display BL Specific Regulation of Hypocotyl and Cotyledon Growth in Opposite Manner- Since the loss of GBF1 function resulted in shorter hypocotyls and less expanded cotyledons, we asked whether higher level of GBF1 causes opposite effects. Several independent transgenic lines expressing *GBF1* cDNA driven by *CaMV 35S* promoter were generated for this study. We selected multiple transgenic lines segregating for a single T-DNA locus, determined by hygromycin resistance, for the production of homozygous lines and further analysis. Examination of photo-responsiveness revealed that the transgenic lines displayed significant reduction of inhibition in hypocotyl elongation in WL and BL with no visible effect in RL or FR (Fig. 5A-D, I-J and data not shown). Furthermore, cotyledons of the over-expresser transgenic lines were strikingly more expanded as compared to wild type seedlings (Fig. 5E-F and K). Determination of *GBF1* transcript and protein levels showed dramatically elevated levels of expression of this gene in over-expresser lines relative to wild type background (Fig. 5G-H). These results indicate that the altered phenotypes of the over-expresser lines observed were likely to be caused due to the elevated levels of GBF1.

We examined the length and size of epidermal cells of 6-day-old over-expresser and wild type seedlings grown in BL. The epidermal cells of hypocotyls were detected to be significantly longer in GBF1 over-expresser lines as compared to wild type seedlings (Figs. 5M-O and 6B). Similarly, the epidermal cells of cotyledons were significantly more expanded in over-expresser lines as compared to wild type seedlings (Fig. 5P-Q**)**. Taken together, these results firmly demonstrate that the bZIP protein, GBF1, is a transcriptional regulator of photomorphogenic growth that promotes cell elongation and expansion during early seedling development in Arabidopsis.

Since the loss of function mutant *GBF1* displayed less number of lateral roots as compared to wild type plants, we examined whether over-expression of GBF1 caused more lateral root formation in Arabidopsis plants. As shown in Figs. 5L and 6D, the over-expresser transgenic lines indeed formed more lateral roots as compared to wild type plants suggesting that GBF1 acts as a positive regulator of lateral root formation. Examination of flowering time of the over-expresser lines revealed that *GBF1* transgenic over-expresser lines flower significantly late after formation of 15 to 18 rosettes as compared to wild type plants (Fig. 6A and C).

GBF1 Differentially Regulates the Expression of Light Inducible Genes - The upregulation of light inducible genes such as *CAB* and *RBCS* is one of the important phenomena in photomorphogenic growth. Since GBF1 regulates the growth of hypocotyls and cotyledons in response to BL, we ask whether the bZIP transcription factor GBF1 also plays a role in light regulated gene expression. For this study,

we used 6-day-old wild type and *gbf1-1* mutant seedlings grown in constant dark or various light conditions and measured the relative steady state mRNA levels of light inducible genes. Whereas no difference in the expression of *CHS* was detected between wild type and *gbf1* mutants, the expression of *RBCS* was found to be significantly higher in *gbf1* as compared to wild type seedlings grown in BL or WL (Fig. 7A). No alteration in the expression of *CAB* was detected in WL, however the expression of the gene was significantly reduced in BL grown seedlings (Fig. 7A). To further examine the BL mediated regulation of *CAB* gene expression in *gbf1-1* mutant background, 4-day-old seedlings grown in dark were transferred to BL for 12, 24 and 48h and the transcript levels were measured. Whereas more than 7 fold induction in *CAB* gene expression was found at 24h in wild type, less than 4 fold induction was detected in *gbf1-1* mutant background (Fig. 7B and C), suggesting that the induction of *CAB* gene expression was significantly compromised in *gbf1-1* mutants. These results suggest that although GBF1 negatively regulates the expression of *RBCS*, it acts as a positive regulator of *CAB* gene expression (Fig. 7H).

To further investigate the above observation, we used two stable transgenic lines: *Z/NOS101-GUS* and *CAB1-GUS* (33,34). Both these promoter-reporter constructs were individually introduced into *gbf1-1* mutants by genetic crosses with wild type transgenic lines (35). Mutant lines homozygous for each transgene were then generated for further studies. The *Z/NOS101-GUS* transgene expressed in all the tissues in *gbf1* mutants similar to wild type seedlings in BL (Fig. 7D). Quantitative GUS activity measurements revealed that there was about 50% reduction in the activity of this promoter in *gbf1* mutants as compared to wild type background (Fig. 7F). The expression of *CAB1-GUS* transgene has been shown to be confined to the cotyledons in wild type background (35). As shown in Fig. 7E, very little expression was detected (if any) of *CAB1-GUS* transgene in the *gbf1* mutants, and the quantification GUS activity measurements revealed that the activity of the *CAB1* promoter was reduced to about 4 fold in *gbf1* mutants as compared to wild type background (Fig. 7G). Taken together, these results strongly suggest that GBF1 is required for the proper activation of the Z-box containing promoters in BL.

DISCUSSION

Although the GBF family of transcription factors has been known for more than a decade, the physiological functions of these genes remain elusive (37,38,39). Several transcription factors have been reported in light signaling that play either positive or negative regulatory role in seedling development (9,11,14,41). This study establishes GBF1 as a unique transcription factor in light signaling that plays both positive and negative regulatory roles in photomorphogenic growth and also in gene expression.

GBF1 Interacts With Both the G- and Z-box LREs - The DNA-protein interaction data in this study provide several lines of evidence that GBF1 interacts with both the Z- and G-box LREs of light regulated promoters. The competitive gel shift assays using several LREs including the Z- and G-box demonstrate that although GBF1 interacts with both Z- and G-box, the protein may have higher affinity for the G-box as compared to the Z-box LRE. The recognition of the G- and Z-box LREs by GBF1 possibly indicates that these two LREs are functionally equivalent (11) with the context to GBF1 transcription factor.

Mutations in GBF1 Result in Multiple Effects - The analysis of seedling morphology of *gbf1* mutants demonstrates that the shorter hypocotyl phenotype is restricted to BL. Therefore, although *GBF1* is expressed at various wavelengths of light, it specifically acts as a negative regulator of BL mediated inhibition of hypocotyl elongation. Our results further demonstrate that *gbf1* mutants have smaller cotyledons as

compared to wild type seedlings in blue light, and thus demonstrating a positive regulatory function of GBF1 in cotyledon expansion in a blue light specific manner. The results of epistasis analyses indicate that GBF1 acts downstream to both cry1 and cry2 photoreceptors and the increased sensitivity to BL caused by *gbf1* mutation also requires light perception by phyA. Thus GBF1 mediated inhibition is likely to play an important role in negative or positive feed back control of cryptochrome signaling, although the function of phyA is likely to be independent of GBF1. Thus, GBF1 plays a dual but opposite regulatory role in early seedling development acting downstream to both cry1 and cry2 photoreceptors (Fig. 7H).

Overexpression of GBF1 has resulted in elongated hypocotyls but more expanded cotyledons in blue light, thereby confirming the differential regulatory role of GBF1 in cotyledon and hypocotyl growth. This finding further indicates that *GBF1* transcripts may not be present at sufficiently high levels in wild type seedlings, thus may be a rate limiting factor for cotyledon expansion in blue light signaling. However, since higher level of GBF1 results in elongated hypocotyls, a fine-controlled level of GBF1 is likely to be essential for plants to obtain blue light mediated optimum photomorphogenic growth. This notion is further supported by the fact that GBF1 promotes cell elongation and expansion in hypocotyl and cotyledon, respectively.

HYH, AtPP7, SUB1 and MYC2/ZBF1 have been reported as downstream components in blue light signaling. Whereas SUB1, a Ca^{++} binding protein, functions as a negative regulator in blue and far red light signaling, AtPP7, a Ser/Thr protein phosphatase, acts as a positive regulator of blue light mediated photomorphogenic growth (10,42). HYH, a transcription factor and a close homolog of HY5, acts as a positive regulator in BL signaling (9). MYC2 is a Z-box binding transcription factor, which acts as a negative regulator in BL mediated photomorphogenic growth and is a point of crosstalk among light, ABA and JA signaling (11).

GBF1 Differentially Regulates the Expression of Light Inducible Genes - It has been shown that PIF3 exhibits opposite regulatory effects on seedling morphology and light regulated gene expression in RL and FR specific manner (22). Furthermore, the presence of parallel and branched pathways of light regulated gene expression has already been suggested (17,47,48). Analyses of light regulated gene expression in *gbf1*mutants have revealed that although GBF1 is required for the proper activation of *CAB* gene expression, it acts as a negative regulator for *RBCS* gene expression (Fig. 7H). Transgenic studies with synthetic and native promoter-reporter constructs further indicate that GBF1 is required for the proper activation of the Z-box containing promoters including *CAB1*. Extensive hetero-dimerization of bZIP proteins has been reported (37). Thus, heterodimerization of GBF1 with other bZIP proteins could be a potential mechanism in vivo to generate positive and negative regulators, which in turn may play opposite roles for light regulated gene expression and seedling development.

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FOOTNOTES

*We thank Professor Sushil Kumar, Dr. Shikha Bhatia and Sreeramaiah N. Gangappa. for critically reading and commenting on this manuscript. This work is supported by a grant from DST, Govt. of India, and the internal grant of NCPGR to S.C. C.M. and V. Y. are recipients of CSIR fellowships, Government of India.

The abbreviations used are: GBF1, G-box binding factor 1; ZBF2, Z-box binding factor 2; bZIP, basic leucine zipper; LRE, Light responsive elements; GST, glutathione S-transferase; WL, white light; D, dark; FR, far-red light; RL, red light; BL, blue light.

FIGURE LEGENDS

Fig. 1. GBF1 Interacts with the Z- and G-box of Light Responsive Promoters. **A**, Electrophoretic mobility shift assays (gel shift) using GST-GBF1 (GBF1) and the consensus dimeric Z-box LRE (35) as probe. Approximately 200 ng of recombinant protein was added (lanes 3 to 8) to the radioactively labeled Z-box. No protein was added in lane 1, and 500 ng GST protein was added in lane 2. The protein-DNA complexes were resolved on 7% native polyacrylamide gel. The triangle indicates increasing concentrations of the competitors (Comp) and the plus and minus signs indicate the presence or absence of competitors, respectively. **B**, Gel shift assays using GST-GBF1 and the consensus tetrameric G-box (13) as probe. For experimental detail see legend to Figure 1A. **C,** Gel shift assays using GST-GBF1 and the native *CAB1* minimal promoter as probe. Approximately 200 ng of recombinant protein was added (lanes 3 to 7) to radioactively labeled *CAB1* DNA fragment. For experimental detail see legend to Figure 1A.

Fig. 2. The identification of *gbf1* mutants and the expression of *GBF1* in wild type seedlings. **A**,The schematic diagram of the T-DNA insertion sites in *GBF1*. The inverted triangles show the T-DNA insertion sites. The exons and introns are shown as boxes and arrowheads, respectively. **B**, RNA gel blot analysis of *GBF1* in segregated wild type (Col) and *gbf1-1* mutant (Col) seedlings. Twenty μg of total RNA was loaded onto each lane. The 1.15 kb full length cDNA fragment of *GBF1* was used as probe. The *18S* rRNA is shown as loading control. **C,** Immunoblot of 20 μg of total protein prepared from wild type (Col) and *gbf1-1* mutants. Affinity purified GBF1 polyclonal antibodies were used as primary antibody for detection of GBF1. Coommassie stained protein gel (Total protein) is shown as loading control. **D**, Immunoblot of 20 μg of total protein prepared from wild type (Col) and *gbf1-2* mutants (Col). Affinity purified GBF1 polyclonal antibodies were used as primary antibody for detection of GBF1. The asterisk marks a cross-reacting protein band in the same blot indicating the loading control. **E**, Time course of *GBF1* transcript or protein accumulation. The upper panel: Five-day-old seedlings (Col) grown in constant dark (D) or white light (WL) were transferred to WL or D, respectively, for 0.5, 1, 2, or 4h. *GBF1* transcript of five-day-old seedlings grown in constant D or WL have been shown as 0 (zero) hour. For experimental detail see legend to Fig.2B. The lower panel: time course experiment of GBF1 protein accumulation. For experimental detail see above panel and legend to Fig.2D. **F,** Light regulated expression of *GBF1*. Six-day-old wild type (Col) seedlings grown in constant dark (D), WL, FR, RL or BL were used for RNA gel blot analyses. For the experimental detail, see to legend 2B. **G,** Expression of *GBF1* in different light qualities after WL pre-treatment. Five-day-old seedlings grown in constant WL were transferred to RL, BL, or FR for 0.5, 1, 2 or 4 hours and the steady state mRNA levels of *GBF1* were determined. For experimental detail, see to legend 2B. rRNA has been shown as loading control. **H**, Tissue specific expression of *GBF1*. Total RNA was isolated from root (R), stem (S), leaf (L) or flower (F) of 30-day-old wild type Arabidopsis plants grown in WL (16h light and 8h dark cycle). For experimental detail, see to legend 2B. **I-K**, Quantification of the Northern blot data in E (upper panel), F and I, respectively, by Fluor-S-MultiImager (BioRad)

Fig. 3. Mutation in *GBF1* results in various effects. Segregated wild type (Col) and *gbf1-1* (Col) mutants (A-I,), or *gbf1-2* (Col) mutants (J-L) are shown on the left and right, respectively. **A-F**, Six-day-old seedlings were grown in constant darkness, WL (5 μmole m^{-2} s^{-1}), WL (30 μmole m^{-2} s^{-1}), BL (30 μmole m^{-2} s^{-1}), RL (95 μmole m^{-2} s^{-1}), or FR (90 μmole m^{-2} s^{-1}), respectively. **G-H**, Cotyledons of 6-day-old seedlings grown in constant WL (30 μmole m^{-2} s^{-1}), or BL (30 μmole m^{-2} s^{-1}), respectively. **I,** Sixteen-day-old plants grown in constant WL (100 μmole m^{-2} s^{-1}). **J-K,** Six-day-old seedlings were grown in constant WL (5 μmole m^{-2} s^{-1}), or BL (30 μmole m^{-2} s^{-1}),

respectively. **L,** Cotyledons of 6-day-old seedlings grown in constant BL (30 μmole m^{-2} s^{-1}). **M-O,** Hypocotyl epidermal cells of six-day-old wild type, *gbf1-1*, *gbf1-2* seedlings, respectively, grown in constant BL (30 μmole m^{-2} s^{-1}). **P-Q,** Imprints of cotyledon epidermal cells of six-day-old wild type and *gbf1-1* seedlings, respectively, grown in constant BL (30 μmole m^{-2} s^{-1}). **R**, Sixteen-day-old wild type, *gbf1-1* and *gbf1-2* (from left to right) plants grown in WL (30 μmole m^{-2} s^{-1}).

Fig. 4. Characterization of *gbf1*mutants. About 25-30 seedlings were used for the measurement of hypocotyl length, cotyledon area or chlorophyll accumulation. The error bars indicate standard deviations. The *gbf1* mutants are in Columbia background. **A-B**, Quantification of hypocotyl length of 6-day-old wild type (Col) and *gbf1* mutant seedlings grown at various fluence rates in constant WL or BL, respectively. **C-D**, Quantification of cotyledon area of 6-day-old wild type (Col) and *gbf1* mutant seedlings grown in constant WL (30 μmole m^{-2} s^{-1}), or BL (30 μmole m^{-2} s^{-1}), respectively. **E**, Accumulation of Chlorophyll a and b in the cotyledons after normalized by cotyledon size in 6-day-old wild type (Col) and *gbf1-1* mutant seedlings. **F-H,** Hypocotyl lengths of 6-day-old wild type (WT: see Materials and methods), *gbf1*, *cry1* (Ler), *gbf1 cry1, cry2* (Col), *gbf1 cry2, phyA* (RLD), and *gbf1 phyA* seedlings grown at various fluence rates of BL.

Fig. 5. Regulation of blue light-mediated photomorphogenic growth in GBF1 over-expresser lines. In each panel from A to F, wild type (Ws) and GBF1 over-expresser seedlings (OE1 or OE2 in Ws background) are shown on the left and right, respectively. **A**, Six-day-old wild type and OE1 seedlings grown in constant WL (5 μmole m^{-2} s^{-1}). **B**, Six-day-old wild type and OE2 seedlings grown in constant WL (5 μmole m^{-2} s^{-1}). **C**, Six-day-old wild type and OE1 seedlings grown in constant BL (30 μmole m^{-2} s^{-1}). **D,** Six-day-old wild type and OE2 seedlings grown in constant BL (30 □mole m^{-2} s^{-1}). **E**, Cotyledons of 6-day-old wild type and OE1 seedlings grown in constant BL (30 □mole m^{-2} s^{-1}). **F**, Cotyledons of 6-day-old wild type and OE2 seedlings grown in constant BL (30 □mole m^{-2} s^{-1}). **G**, RNA blot analysis of *GBF1* in wild type Ws (WT), OE1 and OE2 seedlings. Twenty □g of total RNA was loaded onto each lane. The 1.15 kb full length cDNA fragment of *GBF1* was used as probe. rRNA is shown as loading control. **H,** Immunoblot of 20 □g of total protein prepared from wild type Ws (WT), OE1 and OE2 seedlings. Affinity purified GBF1 polyclonal antibodies were used for the detection of GBF1. Coommassie stained protein gel (Total protein) is shown as the loading control. **I-J**, Quantification of hypocotyl length of 6-day-old wild type Ws (WT), OE1 and OE2 seedlings grown in constant WL (5 □mole m^{-2} s^{-1}) or BL (30 □mole m^{-2} s^{-1}), respectively. About 25 seedlings were used for the measurement of hypocotyl length. The error bars indicate standard deviations. **K**, Quantification of cotyledon area of 6-day-old wild type Ws (WT), OE1 and OE2 seedlings grown in BL (30 □mole m^{-2} s^{-1}). For experimental detail see legend to Fig. 5I-J. **L,** Formation of lateral roots in sixteen-day-old wild type Ws, OE1 and OE2 plants (from left to right) grown in constant WL (80 □mole m^{-2} s^{-1}). **M-O,** Hypocotyl epidermal cells of six-day-old wild type, OE1, or OE2 seedlings, respectively, grown in constant BL (30 □mole m^{-2} s^{-1}). **P-Q,** Imprints of cotyledon epidermal cells of six-day-old wild type or OE1 seedlings, respectively, grown in constant BL (30 □mole m^{-2} s^{-1}).

Fig. 6. Characterization of *GBF1* over-expresser lines. **A,** Thirty-day-old wild type (Col), *gbf1-1*, *gbf1-2*, GBF1 over-expresser 1 (OE1) and over-expresser 2 (OE2) plants (from left to right) grown in WL (80 □mole m^{-2} s^{-1}) under 16h light/8h dark cycles. **B**, Quantification of epidermal cell lengths of hypocotyls of six-day-old wild type (Col), *gbf1-1*, *gbf1-2*, wild type (Ws), OE1 and OE2 seedlings grown in constant BL (30 □mole m^{-2} s^{-1}). **C**, Number of rosette leaves formed at the time of bolting in wild type (Col), *gbf1-1*, *gbf1-2,* wild type (Ws), OE1 and OE2 plants grown under long day conditions of 16h WL (80 □mole m^{-2} s^{-1}) and 8h dark cycle. **D,**

Quantification of the number of lateral roots formed in wild type (Col), *gbf1-1*, *gbf1-2,* OE1 and OE2 plants grown in constant WL (80 □mole m^{-2} s^{-1}) at various days (from day 10 to 15).

Fig. 7. The light regulated gene expression in *gbf1*mutants. **A**, RNA gel blot analysis of *RBCS, CAB* and *CHS* genes in segregated wild type (Col) and *gbf1-1* mutant (Col) seedlings (M) grown in constant dark (D), WL or BL conditions. Ten □g of total RNA was loaded onto each lane. *18S* rRNA has been shown as loading control. **B**, RNA gel blot analysis of *CAB* in segregated wild type (Col) and *gbf1-1* mutant seedlings. Four-day-old dark grown seedlings were transferred to BL for 12, 24 or 48 hours (h) and total RNA was extracted from each sample for RNA gel blot analysis. Ten □g of total RNA was loaded onto each lane. Six-day-old seedlings grown in dark have been shown as 0 (zero) hour. rRNA has been shown as loading control. **C**, Quantification of the data in B by Fluor-S-MultiImager (BioRad). **D-E,** Six-day-old seedlings carrying *Z/NOS101-GUS* or 12-day-old plants carrying *CAB1-GUS* transgene, respectively, were grown in BL (30 □mole m^{-2} s^{-1}) and used for GUS activity staining. In each panel wild type (Col) and *gbf1-1* mutants (Col) have been shown on the left and right side, respectively. **F-G,** GUS activities of 6-day-old constant BL (30 □mole m^{-2} s^{-1}) grown seedlings carrying *Z/NOS101-GUS* or *CAB1-GUS* transgene, respectively. The error bars indicate standard deviations. The promoter-reporter constructs are diagrammed on the top of each panel. **H,** A working model shows BL specific regulatory role of GBF1 during early seedling development in Arabidopsis.

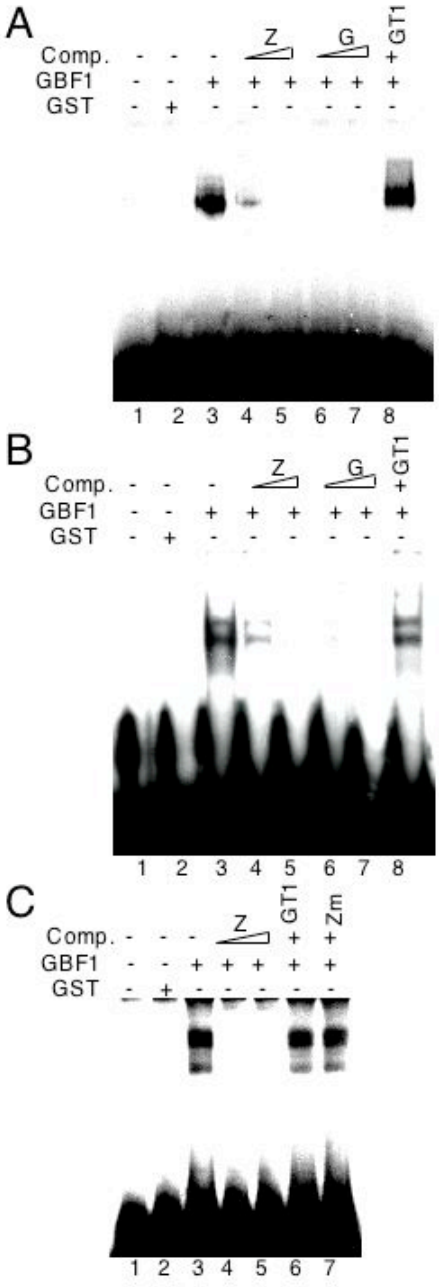


Fig.1

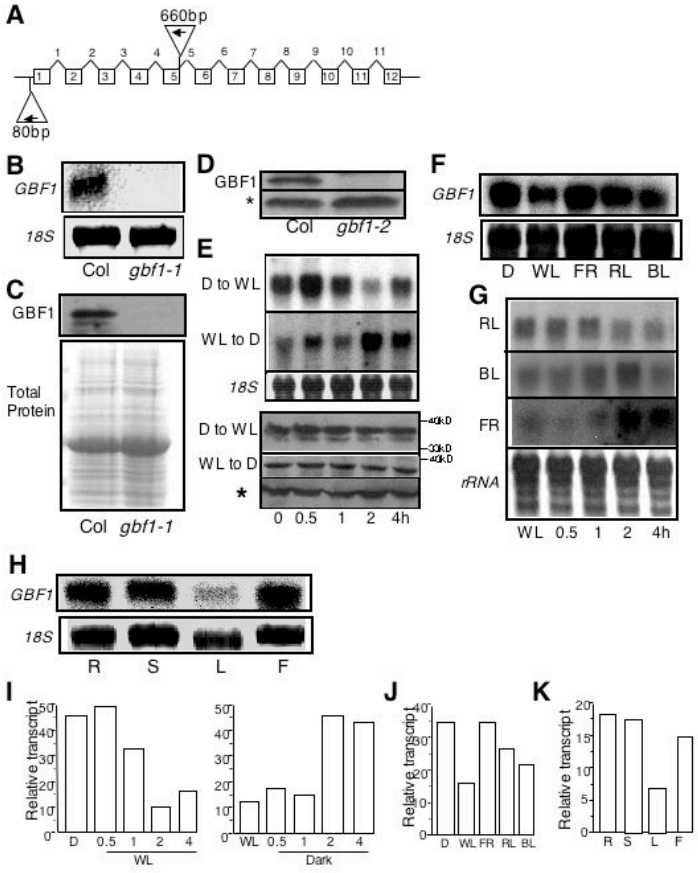
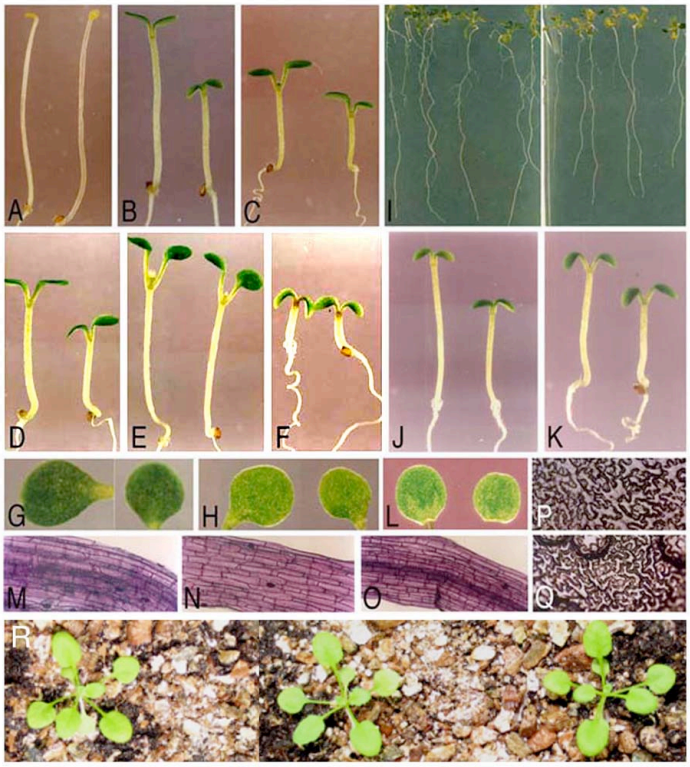


Fig. 2



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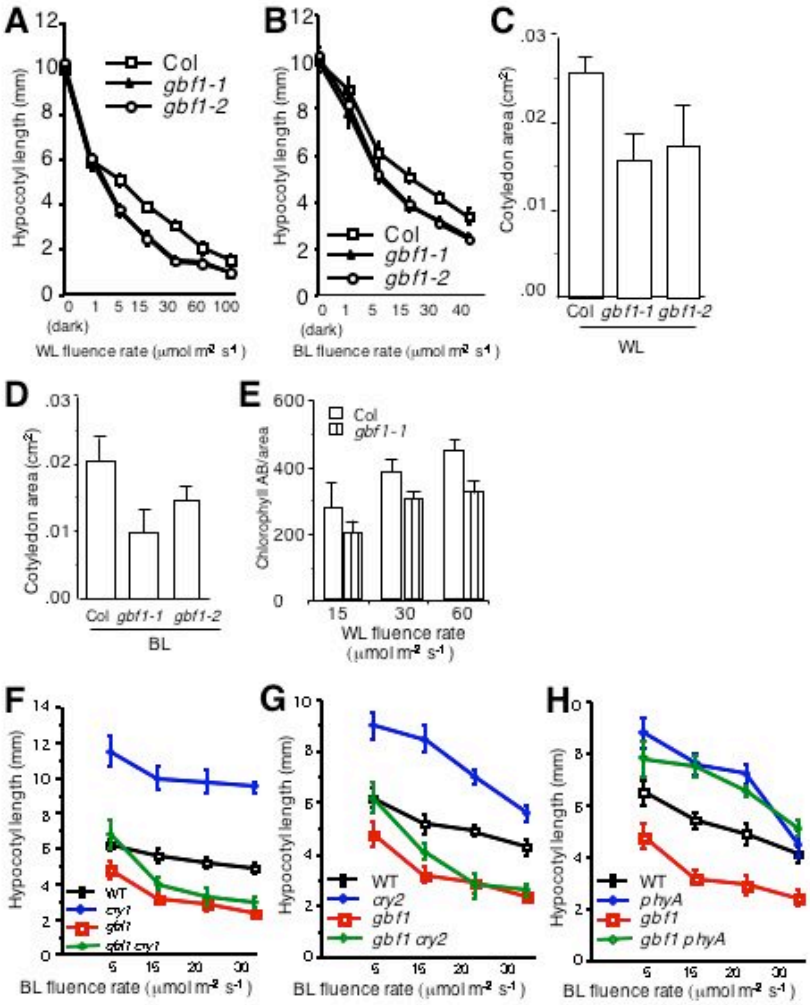


Figure 4

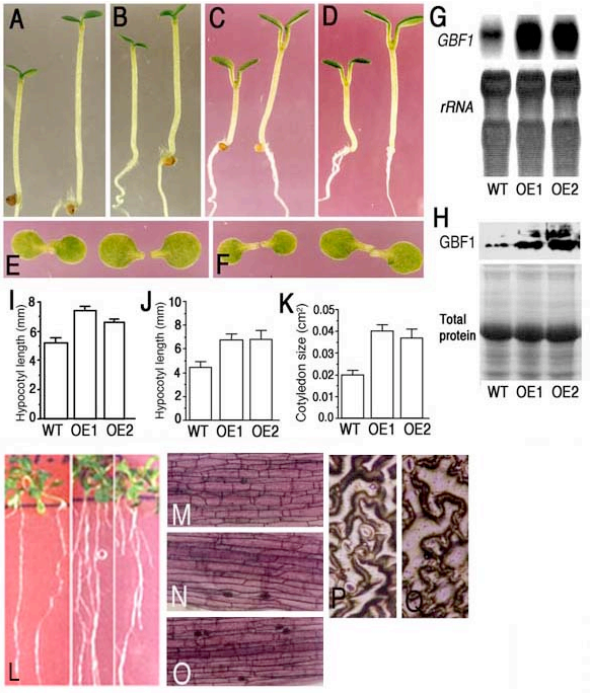
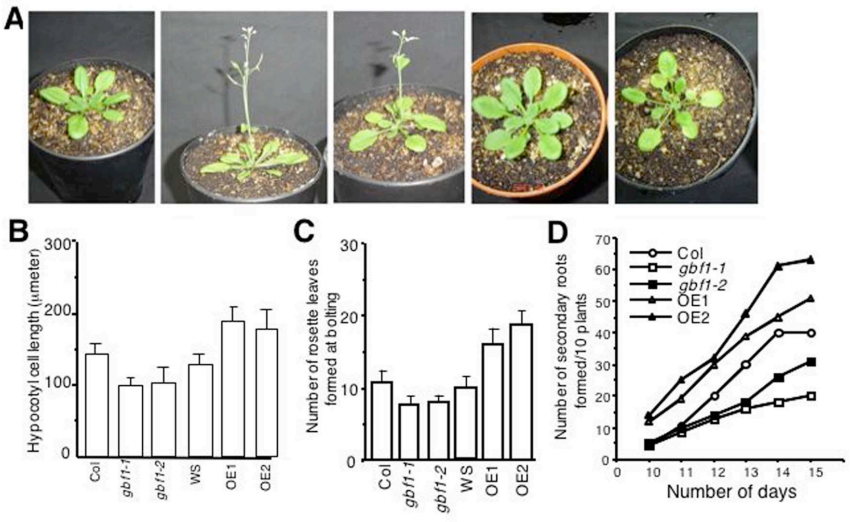


Fig. 5



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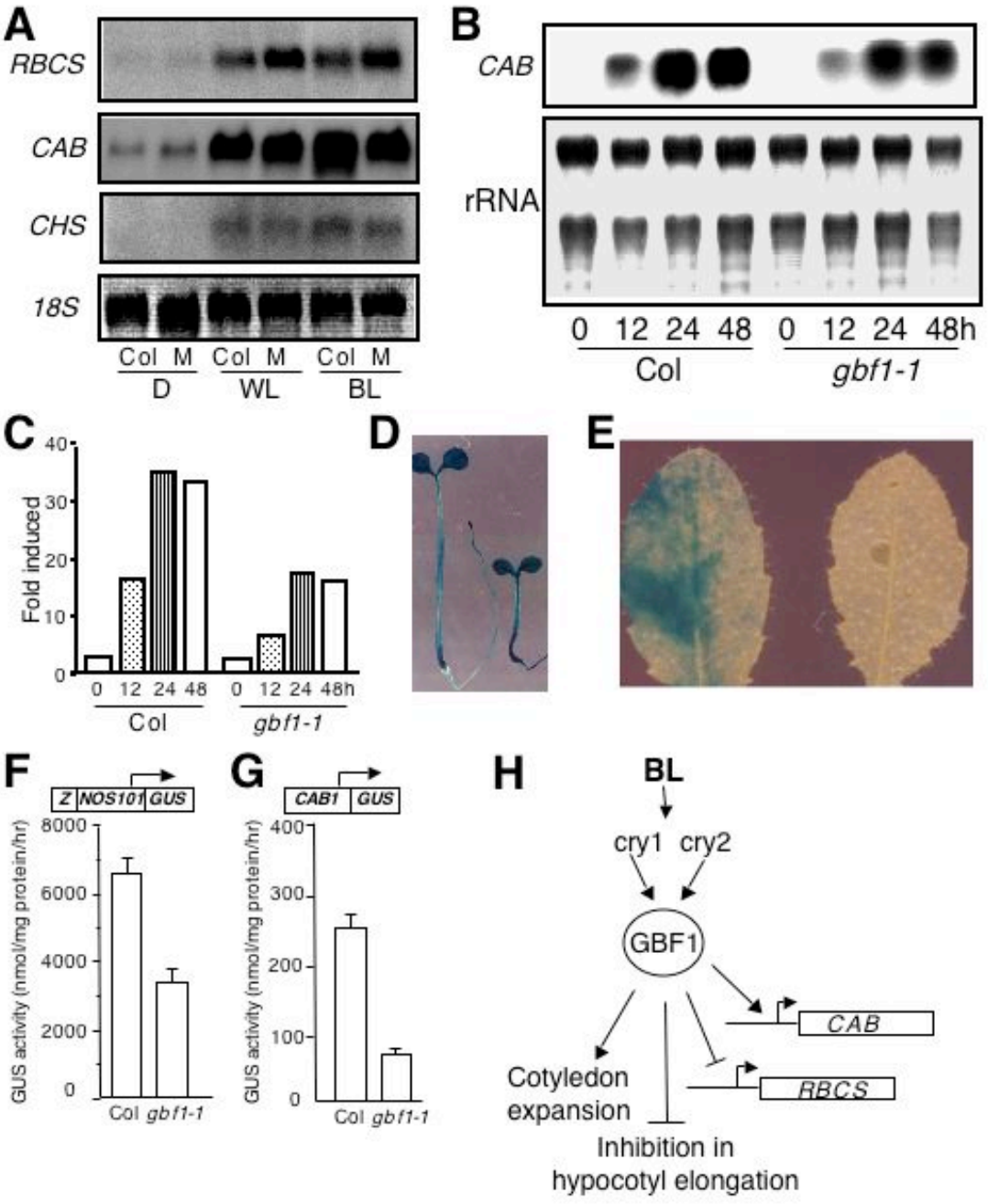

Figure 7