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LZF1, a HY5-regulated transcriptional factor, functions in Arabidopsis de-etiolation

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Summary

We surveyed differential gene expression patterns during early photomorphogenesis in both wild-type and mutant Arabidopsis defective in HY5, an influential positive regulator of the responses of gene expression to a light stimulus, to identify light-responsive genes whose expression was HY5 dependent. These gene-expression data identified light-regulated zinc finger protein 1 (*LZF1*), a gene encoding a previously uncharacterized C2C2-CO B-box transcriptional regulator. HY5 has positive *trans*-activating activity toward *LZF1* and binding affinity to *LZF1* promoter *in vivo*. HY5 is needed but not sufficient for the induction of *LZF1* expression. Anthocyanin content is significantly diminished in *lzf1* under far red, which is the most efficient light for the induction of *LZF1*. The expression of *PAP1*/*MYB75* is elevated in plants overexpressing *LZF1*, which leads to the hyperaccumulation of anthocyanin in transgenic Arabidopsis. The transition from etioplast to chloroplast and the accumulation of chlorophyll were notably compromised in the *lzf1* mutant. We provide molecular evidence that LZF1 influences chloroplast biogenesis and function via regulating genes encoding chloroplast proteins. In the absence of HY5, mutation of *LZF1* leads to further reduced light sensitivity for light-regulated inhibition of hypocotyl elongation and anthocyanin and chlorophyll accumulation. Our data indicate that LZF1 is a positive regulator functioning in Arabidopsis de-etiolation.

Keywords: light, HY5, LZF1, chlorophyll, anthocyanin, Arabidopsis.

Introduction

Plants, unable to flee from their habitat, have developed distinct strategies to cope with an ever-changing environment and to achieve optimal growth and development. Light is the most conspicuous environmental stimulus for plants. The proper interpretation of light signals allows plants to emerge from the soil, escape from canopy shade, bloom at a specific time of the year and, finally, control senescence.

Several classes of plant photoreceptors sensing and integrating light signals from the ambient environment include cryptochromes (cry1, -2), phototropins (phot1, -2) and FKF1 proteins for responding to UV-A/blue light, and phytochromes (phyA–phyE) for detecting red/far-red light (reviews see Chen *et al.*, 2004; Franklin and Whitelam, 2004; Lin and Shalitin, 2003; Quail, 2002; Schepens *et al.*, 2004). Recent biological studies have provided unequivocal evidence for the light-regulated partitioning of cry1 and phyA–phyE between the cytoplasm and nucleus (Guo *et al.*, 1999; Kircher *et al.*, 2002; Sakamoto and Nagatani, 1996; Yamaguchi *et al.*, 1999; Yang *et al.*, 2000). These findings have significantly changed our previous conception about the subcellular locations at which the photoreceptor molecules act. Moreover, genetic screens have yielded many Arabidopsis mutants with aberrant photo-

morphogenic phenotypes (for reviews see Chen *et al.*, 2004; Franklin and Whitelam, 2004; Sullivan and Deng, 2003; Wang and Deng, 2003). Like phytochromes and cryptochromes, the protein products of many of the genes identified in mutant screens are localized within the nucleus. These include COPs (von Arnim and Deng, 1994; Staub *et al.*, 1996), HY5 (Chattopadhyay *et al.*, 1998), PIF3/4 (Huq and Quail, 2002; Ni *et al.*, 1998), SPA1 (Hoecker *et al.*, 1999), the FAR1/FHY3/FRS family (Hudson *et al.*, 1999; Lin and Wang, 2004; Wang and Deng, 2002), GI (Huq *et al.*, 2000), HFR1/RSF1 (Fairchild *et al.*, 2000), ELF3 (Liu *et al.*, 2001), LAF1 (Ballesteros *et al.*, 2001), EID1 (Dieterle *et al.*, 2001) and FHY1 (Zeidler *et al.*, 2004). The fact that many of these are transcriptional regulators implies the existence of a regulatory network for fine-tuning the transcriptional response to changes in the quality and quantity of light. Indeed, in microarray experiments, hundreds of genes show differential expression patterns upon light treatment in wild-type Arabidopsis and different photomorphogenic mutants, which reflects the complexity of the responses of plants to this environmental stimulus (Jiao *et al.*, 2003; Ma *et al.*, 2001, 2002, 2003; Tepperman *et al.*, 2001, 2004; Thum *et al.*, 2004; Wang *et al.*, 2002).

HY5, a basic leucine zipper (bZIP) transcription factor necessary for responses to a broad spectrum of wavelengths of light, 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.*, 1998; Koornneef *et al.*, 1980). Arabidopsis plants defective in HY5 show aberrant light-mediated phenotypes, including an elongated hypocotyl, reduced chlorophyll/anthocyanin accumulation and reduced chloroplast development in greening hypocotyls (Holm *et al.*, 2002; Oyama *et al.*, 1997). HY5 is believed to be one of the central modulators for the coordination of light signals and the regulation of appropriate gene expression (Quail, 2002; Sullivan and Deng, 2003). HY5 also integrates both light and hormone signaling pathways (Cluis *et al.*, 2004; Vandenbussche *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). However, for most light-modulated genes, the sequence of steps linking HY5 to changes in gene expression is still largely unknown. Whether other transcriptional regulator(s) is/are involved in amplifying the HY5-mediated light signaling pathway remains to be addressed.

In this study, we identified a new transcriptional regulator gene, light-regulated zinc finger protein 1 (*LZF1*), whose induction is both regulated by light and dependent on HY5. Our experiments indicate that LZF1 acts synergistically with HY5 in regulating hypocotyl elongation during photomorphogenesis. Our data also suggest that LZF1 modulates early chloroplast development and anthocyanin accumulation. The molecular mechanism by which LZF1 contributes to these two biological processes is discussed.

Results

The early light-responsive expression of LZF1 *depends largely on HY5*

Target genes of the transcription factor HY5 were revealed by a global survey of genes differentially expressed at an early photomorphogenic phase between wild-type Arabidopsis and the *hy5-1* (*hy5* hereafter) mutant when grown in white light. A 12 000-element cDNA microarray, equivalent to approximately 7500 unique genes, was used in this gene expression profiling experiment (Wu *et al.*, 2001). The gene expression kinetics were monitored in a time course over the first 6 h of seedling photomorphogenesis. Cluster analysis (Eisen *et al.*, 1998) was applied to identify groups of genes with similar expression patterns over the time course. One group of genes was upregulated in the wild type but not in *hy5* plants during the time period examined, which suggests that these genes are regulated by HY5 (Figure 1a). [The expression data for all three replicates are presented to show their high reproducibility. Gene expression data corresponding to Figure 1(a) are listed in Table S1.] This group of genes includes those responsible for the biosynthesis of anthocyanin (chalcone synthase and chalcone flavonone isomerase) and chlorophyll (chlorophyll *a*/*b*-binding family protein/ELIP1). Also in this cluster was a previously uncharacterized gene encoding a putative zinc-finger-type transcription factor, *LZF1* (*At1g78600*), which we hypothesized acts downstream of HY5 to modulate the responses of gene expression to light. The remaining experiments were designed to evaluate this supposition.

The light responsiveness of *LZF1* expression during the first 6 h of light exposure was confirmed by northern blot analyses (Figure 1b). Real-time RT-PCR analysis was employed to confirm that the light-regulated expression of *LZF1* largely depended on HY5 (Figure 1c). The light-regulated expression of *LZF1* was significantly diminished in *hy5* mutants. Moreover, the gene expression pattern of *LZF1* coincided well with that for *HY5* among various plant tissues, developmental stages and stress responses on query of the Genevestigator expression database (Zimmermann *et al.*, 2004). We also noticed a residual expression of *LZF1* in the *hy5* mutant, especially after the seedlings had been exposed to light for 6 h or more (Figure 1c). Additional transcriptional regulator(s) might compensate for the induction of *LZF1* in the absence of HY5. The additive light phenotype of the *hy5lzf1* double mutant also supports this hypothesis (see below).

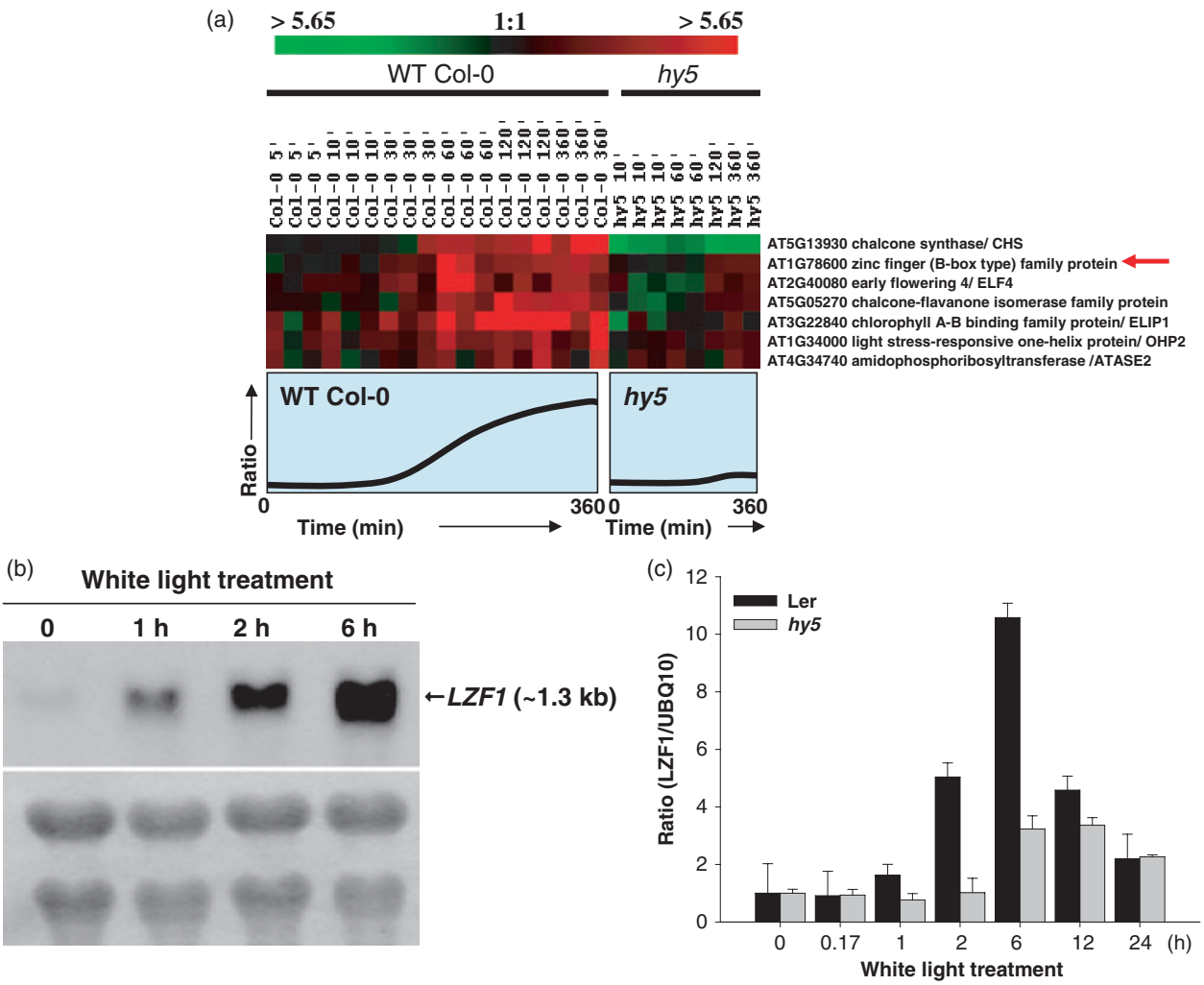

Figure 1. The light-inducible expression of *LZF1* is HY5 dependent.
(a) Light-induced expression of *LZF1* is compromised in the *hy5* mutant. Only genes with net intensities ≥500 that were flagged as well measured and those that showed differential expression patterns between Col-0 and *hy5* were included in the cluster analyses. Expression data were collected from samples treated with white (W) light for the minutes indicated. Data from all available biological replicates were used in the cluster analyses. The degree of expression change was color coded, with red and green representing up and downregulation, respectively. *LZF1* is indicated by the red arrow. The expression trend of this group of genes was also plotted for better visualization of the expression differences between Col-0 and *hy5* plants.
(b) Northern blot confirmation of *LZF1* microarray expression data. The RNA samples were isolated from 4-day-old etiolated seedlings (0 min) and etiolated seedlings exposed to W light for various times. The methylene blue-stained image was used to show equal loading of the total RNA samples.
(c) Real-time quantitative RT-PCR was used to monitor the expression of *LZF1* in both L*er* and *hy5* etiolated seedlings exposed to W light for the indicated times. The expression value of *UBQ10* in each sample was used as an internal control. The *LZF1* expression level in etiolated seedlings (0 min) was set to 1. The expression level of *LZF1* is presented as the amount of increase at each time point relative to etiolated seedlings and represented as 'ratio'. The means and standard deviations were calculated from three replicates and plotted.

LZF1 *responds primarily to FR light during photomorphogenesis*

We next investigated whether the expression of *LZF1* depends on the light quality. The expression behavior of both *HY5* and *LZF1* under far-red (FR), red (R) and blue (B) light was examined in a time course. As shown in Figure 2, *HY5* was upregulated under all light regimes tested, which agrees with a previous report that HY5 protein stably accumulates under R, FR and B light (Osterlund *et al.*, 2000). However, the expression of *LZF1* was primarily responsive to FR and only modestly induced by R or B light. Thus, HY5 might be necessary but not sufficient for the induction of *LZF1* in response to a light stimulus. FR is probably the major light signal for the HY5-dependent induction of *LZF1*.

HY5 regulates LZF1 *expression via directly binding to the* LZF1 *promoter*

To monitor the effect of HY5 protein on the regulation of *LZF1* gene expression, we created an effector–reporter construct pair for use in an Arabidopsis protoplast transient

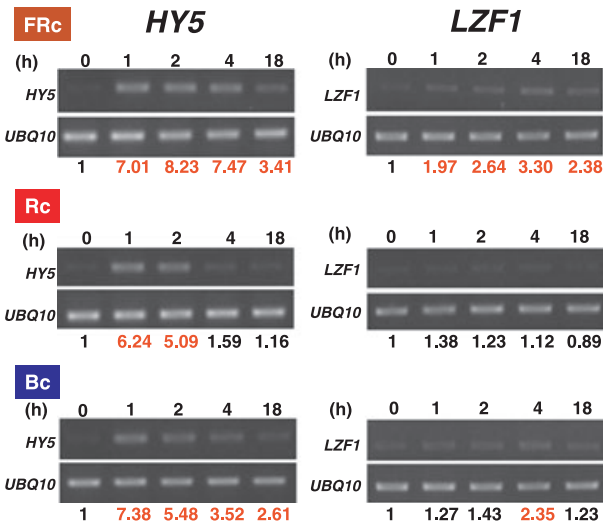

Figure 2. LZF1 is primarily responsive to far-red light.
Reverse transcriptase-PCR was used to monitor the expression of both *HY5* and *LZF1* in Col-0 etiolated seedlings exposed to continuous far red (FRc), continuous red (Rc) or continuous blue (Bc) light for the indicated times. The expression of *HY5* and *LZF1* was normalized to that of *UBQ10*. The expression level in etiolated seedlings (time 0) was set to 1. Induction values ≥1.97 relative to time 0 are marked in red.

assay. As shown in Figure 3(a), the expression of the luciferase gene driven by the *LZF1* promoter was significantly enhanced when co-expressed with *HY5* in Arabidopsis protoplasts, which indicates that HY5 has positive *trans*-activating activity towards *LZF1 in vivo*. Previous research indicated that the HY5 protein has strong affinity to the G-box (CACGTG) and ACE box (ACGT-containing element; Chattopadhyay *et al.*, 1998; Shin *et al.*, 2007) present in the promoter region of genes responding to a light signal. When the 1-kb promoter region of *LZF1* was analyzed, we found a perfect G-box in *LZF1-C* and ACE boxes in *LZF1-A*, *LZF1-B* and *LZF1-D* (Figure 3b). Thus, HY5 could regulate the expression of *LZF1* by directly binding to the *LZF1* promoter. Interestingly, *LZF1* was found to be a potential target of HY5 in a recent study adopting a chromatin immunoprecipitation (ChIP)-chip approach (see Table 1 in Lee *et al.*, 2007). To test this finding, we performed ChIP experiments to examine whether HY5 can bind to the promoter of *LZF1 in vivo* in transgenic plants expressing myc-tagged HY5 by ChIP-quantitative qPCR. The amplification of fragment *LZF1-E* was included as an off-promoter control. Results showed that HY5 has the highest affinity toward *LZF1-B* in plants grown under either continuous FR or exposed to 4 h of FR (Figure 3b). Far-red treatment was chosen because it is the most efficient light for inducing the expression of *LZF1* (Figure 2). The binding was enriched only in transgenic plants expressing myc-HY5 but not in those expressing myc-GFP, which indicates that the binding of HY5 to the LZF1 promoter is specific *in vivo* (Figure 3b). We have also observed that HY5 has *in vitro* binding affinity only to the

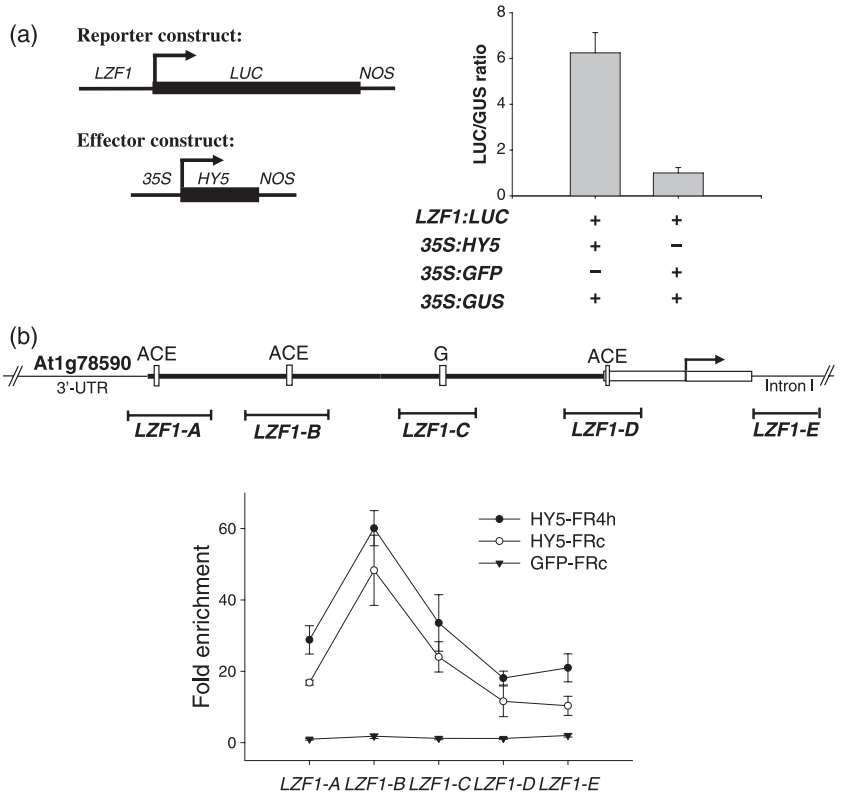

Figure 3. HY5 transactivates the expression of *LZF1* and specifically binds to *LZF1* promoter.
(a) The expression of luciferase driven by the *LZF1* promoter was used to determine the effect of HY5 on the expression of *LZF1* in Arabidopsis protoplasts. The effector–reporter constructs are *35S:HY5* and *LZF1:LUC*, respectively. The activation of the reporter gene is presented by arbitrary values representing the LUC/GUS ratio (*Y*-axis). *35S*:GUS was used as an internal normalization control by co-transfection. *35S:GFP* was used as a negative effector control.
(b) Fragments *LZF1-A* to *E* used for chromatin immunoprecipitation (ChIP)-quantitative qPCR were marked with their relative location in the *LZF1* promoter. G and ACE boxes are also marked. The HY5 binding affinity toward *LZF1* promoter fragments (*LZF1-A* to *E*) was determined by qPCR. The relative abundance of fragments LZF1-A to E in immunoprecipitated DNA from HY5-FR4h, HY5-FRc and GFP-FRc seedlings was normalized against input DNA from each corresponding sample. The fold enrichment was calculated by raising the abundance of LZF1-B in GFP-FRc to 1.

wild-type ACE element but not to mutated ACE elements in *LZF1-B* (data not shown). This implies that HY5 can bind to the ACE element of *LZF1-B in vivo* for the upregulation of *LZF1*.

Taken together, the findings suggest that HY5 regulates the expression of *LZF1* by directly binding to the *LZF1* promoter. We also hypothesize that LZF1 functions downstream of HY5 in conveying the light signal.

Expression characteristics of LZF1

Tissue-specific expression of *LZF1* was examined by both real-time RT-PCR and by searching the Massively Parallel Signature Sequencing (MPSS; http://mpss.udel.edu/at/) database. The transcripts of *LZF1* were ubiquitously present in Arabidopsis, with slightly higher accumulation in flowers and roots, which suggests that LZF1 may contribute broadly to growth and development in Arabidopsis. Interestingly, *LZF1* was also upregulated following salicylic acid treatment (data not shown), which implies that LZF1 may also participate in light-dependent salicylic acid signaling in plants (Genoud *et al.*, 2002).

LZF1 is distantly related to the CONSTANS B-box proteins

LZF1 is present as a single-copy gene encoding a protein containing two B-box motifs at its N-terminus. BLASTP analyses with this N-terminal domain (amino acids 1–104) revealed another 30 B-box-containing proteins sharing significant sequence similarity to LZF1, with an *E*-value of 3×10^{-5}. These 31 proteins largely overlapped with the previously annotated CO and CO-like (COL) proteins in The Arabidopsis Information Resources (TAIR; http://www.arabidopsis.org/) database and with a C2C2-CO-like family in the Arabidopsis Gene Regulatory Information Server (AGRIS; http://arabidopsis.med.ohio-state.edu/AtTFDB/). Multiple sequence alignment was conducted with these 31 B-box-containing zinc finger proteins (Figure S1a; Appendix S2) and used for phylogenetic analysis. Results in Figure S1(b) indicate that these 31 proteins can be further divided into five distinct clades. Among them, clades I, II and III consist of the CONSTANS (CO) family (CO and COL1–16) described previously (Griffiths *et al.*, 2003; Robson *et al.*, 2001). The protein COL-3 has been documented to be a positive regulator of the R light signaling pathway (Datta *et al.*, 2006). LZF1 is in clade IV, with 99% bootstrap support. Except for STO and STH, proteins in this clade are, for the most part, uncharacterized. STO complements the salt-sensitive phenotype of the yeast *can* mutant (Lippuner *et al.*, 1996), and was recently characterized to function as a negative regulator in phytochrome and B-light signaling pathways (Indorf *et al.*, 2007). STH is a homolog of STO, and both proteins interact with COP1 in a yeast two-hybrid system (Holm *et al.*, 2001).

To better visualize the protein architecture of LZF1 and its relation with CO and COLs, we investigated representative domains for proteins within each clade, illustrated in Figure S1c). Although LZF1 is related to the CO family, the homology is limited to the N-terminal B-box motifs. The CO family members also share a conserved C-terminal CCT (CO, COL, TOC1) domain. Previous research indicated that both B-box motifs and the CCT domain are important for promoting flowering under long-day conditions by COs (Robson *et al.*, 2001). Because the characteristic CCT domain is absent in LZF1, LZF1 might have a biochemical function distinct from CO.

Arabidopsis mutants with altered LZF1 expression

Available Arabidopsis mutants with T-DNA inserted in exons of LZF1 were obtained from the Arabidopsis Biological Resource Center (*lzf1-1*, SALK_105367, inserted in the second exon and *lzf1-2*, SALK_021776, inserted in the 3′ untranslated region; Figure S2a; Appendix S2). Homozygous *lzf1-1* and *lzf1-2* plants were obtained and confirmed by PCR-based genotyping (data not shown). Transgenic plants overexpressing *LZF1* (*LZF1-ox*) were also constructed by introducing a *35S:LZF1* construct into Arabidopsis via *Agrobacterium tumefaciens*-mediated transformation (Figure S2b). Northern blot analyses indicated that *lzf1-2* still has a substantial number of residual *LZF1* transcripts covering the open reading frame (Figure S2a,d) and thus was not further characterized. *lzf1-1* (abbreviated as *lzf1* hereafter) was confirmed to be a null mutant allele by both RT-PCR (Figure S2c) and northern blot analyses (Figure S2d). As shown in Figure S2(c), levels of *LZF1* transcript were significantly higher in the selected overexpression lines.

LZF1 is a positive regulator in the light signaling pathway

We compared the light-induced inhibition of hypocotyl elongation in wild-type, *lzf1* and *LZF1-ox* seedlings. Although *lzf1* has a hypocotyl length similar to that of wild-type Arabidopsis, *LZF1-ox* exhibits a hypersensitive response to R light (Figure S3a). This phenotype was observed at all fluences tested (Figure S3b). Since R alone could not effectively induce the expression of *LZF1* in wild-type Arabidopsis (Figure 2), the hypersensitive response of *LZF1-ox* under R light is likely to be due to its exaggerated expression under this light. The FR-light responsiveness of *lzf1* and *LZF1-ox,* however, was similar to that in wild-type Arabidopsis (Figure S3a) under all light fluences tested (Figure S3b). Similar to the wild type and *phyB* and *hy5* mutants, the *lzf1* mutant exhibited normal FR-mediated blocking of the greening phenotype (Figure S3c). This finding is consistent with the lack of contribution of *HY5* to this process (Barnes *et al.*, 1996) and that *LZF1* functions downstream of *HY5* under FR light (Figures 1 and 2).

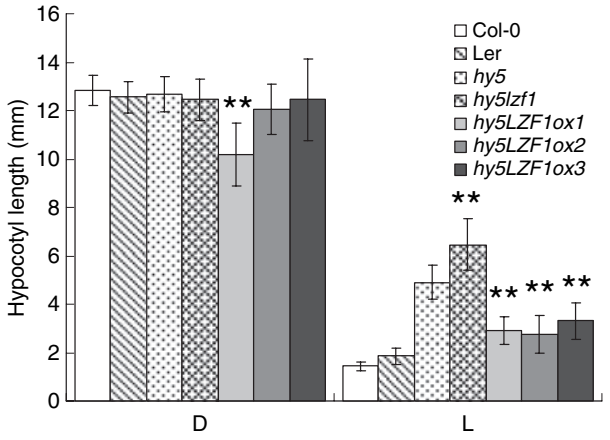

Figure 4. LZF1 acts synergistically with HY5 in the light-regulated inhibition of hypocotyl elongation.
The hypocotyl lengths were measured for 4-day-old etiolated or white-light-grown Col-0, L*er*, *hy5*, *hy5lzf1* and 3 *hy5LZF1ox* lines (*hy5LZF1ox1*, *hy5LZF1ox2* and *hy5LZF1ox3*). **The hypocotyl length of *hy5lzf1* or *hy5LZF1ox* plants are significantly different from those of *hy5* plants ($P < 0.01$, Student's *t*-test; $n = 10–20$).

Since HY5 regulates a wide range of light-responsive genes, some of these genes may function redundantly to LZF1. To test this hypothesis, we generated the *hy5lzf1* double mutant and constructed transgenic *hy5* lines overexpressing *LZF1* (*hy5LZF1-ox*) to further evaluate the involvement of LZF1 in light-regulated inhibition of hypocotyl growth. Results showed an additive light hyposensitivity of the *hy5lzf1* double mutant (Figure 4), which indicates a possible synergistic impact of HY5 and LZF1 in the regulation of the hypocotyl elongation process. This phenotype was observed for all fluences tested under R and FR light (Figure S4a,b). The residual expression of *LZF1* in the *hy5* mutant (Figure 1c) might account for the relatively smaller light hyposensitivity of this mutant when compared with the *hy5lzf1* double mutant. This finding indicates that LZF1 contributes to the inhibition of hypocotyl elongation during photomorphogenesis. The overexpression of *LZF1* in the *hy5* mutant also partially rescued its long hypocotyl phenotype (Figure 4). These data support LZF1 as a positive regulator in the light signaling pathway.

LZF1 *regulates anthocyanin accumulation*

HY5 regulates anthocyanin accumulation in Arabidopsis (Holm *et al.*, 2002). Thus, we examined whether *LZF1* contributes to this HY5-regulated process. *LZF1-ox* seedlings accumulated significantly more anthocyanin than wild-type plants did under continuous FR, B and R light (Figure 5a–c). In contrast, the amount of anthocyanin in *lzf1* was significantly lower than that in the wild type under FR (Figure 5a) and white (W) light (data not shown). Similar results were seen in mature plants grown under long days and transferred to continuous light (data not shown). The reduced anthocyanin accumulation in *lzf1* under FR could be rescued by introducing LZF1 back to the *lzf1* mutant Arabidopsis (*lzf1*/*LZF1* in Figure 5a). These results indicate that the lost function of *LZF1* is indeed responsible for the defect in anthocyanin accumulation in *lzf1* plants. The additive light hyposensitivity in the *hy5lzf1* mutant was further strengthened by its significantly lower accumulation of anthocyanin under B and R light (Figure 5b,c). The overexpression of *LZF1* in the *hy5* mutant also restored the anthocyanin to the level in wild-type plants under FR and B (Figure 5a,b).

Because of the presence of putative DNA-binding B-boxes in the N-terminus, LZF1 probably acts as a transcriptional regulator modulating gene expression in response to a light signal. Thus, we sought to identify candidate target genes regulated by this transcription regulator. *MYB75*/*PAP1* was found to be among the genes significantly upregulated in *LZF1-ox* plants when compared with wild-type plants (Figure 5d and Table S2). These results were further confirmed via RT-PCR analyses (Figure 5e). Previously, overexpression of *MYB75*/*PAP1* resulted in accumulation of excess anthocyanin (Borevitz *et al.*, 2000). Our results indicated that LZF1 could regulate the anthocyanin accumulation, possibly via modulation of the expression of *MYB75*/*PAP1*.

Figure 5(d) lists genes whose expression patterns are significantly altered in *LZF1-ox* plants. We also sought to examine the expression behaviors of these genes upon light treatment in the Arabidopsis early photomorphogenic stage (by Genevestigator). Genes upregulated in *LZF1-ox* plants showed a positive light responsiveness, whereas the expression of LZF1-downregulated genes was repressed by light (Figure 5d). Similar patterns of light responsiveness were obtained for genes regulated by LZF1 in the de-etiolation process (see below and Figure S5a,b). This finding further supports LZF1 as a positive regulator for conveying the light signal.

Plastid development and the accumulation of chlorophyll are regulated by LZF1

HY5 is involved in chloroplast development and the accumulation of chlorophyll (Holm *et al.*, 2002; Oyama *et al.*, 1997). We therefore examined whether LZF1 is involved in this HY5-mediated process. *lzf1* plants accumulated less chlorophyll than wild-type seedlings under FR, B and R light (Figure 6a–c), whereas more chlorophyll was found in *LZF1-ox* plants under FR and R light (Figure 6a,c). In 6-day-old or mature plants grown under W light, *lzf1* plants accumulated less chlorophyll than wild-type plants, with more chlorophyll found in *LZF1-ox* plants (data not shown). Significantly less chlorophyll was accumulated in *hy5* mutant – a phe-

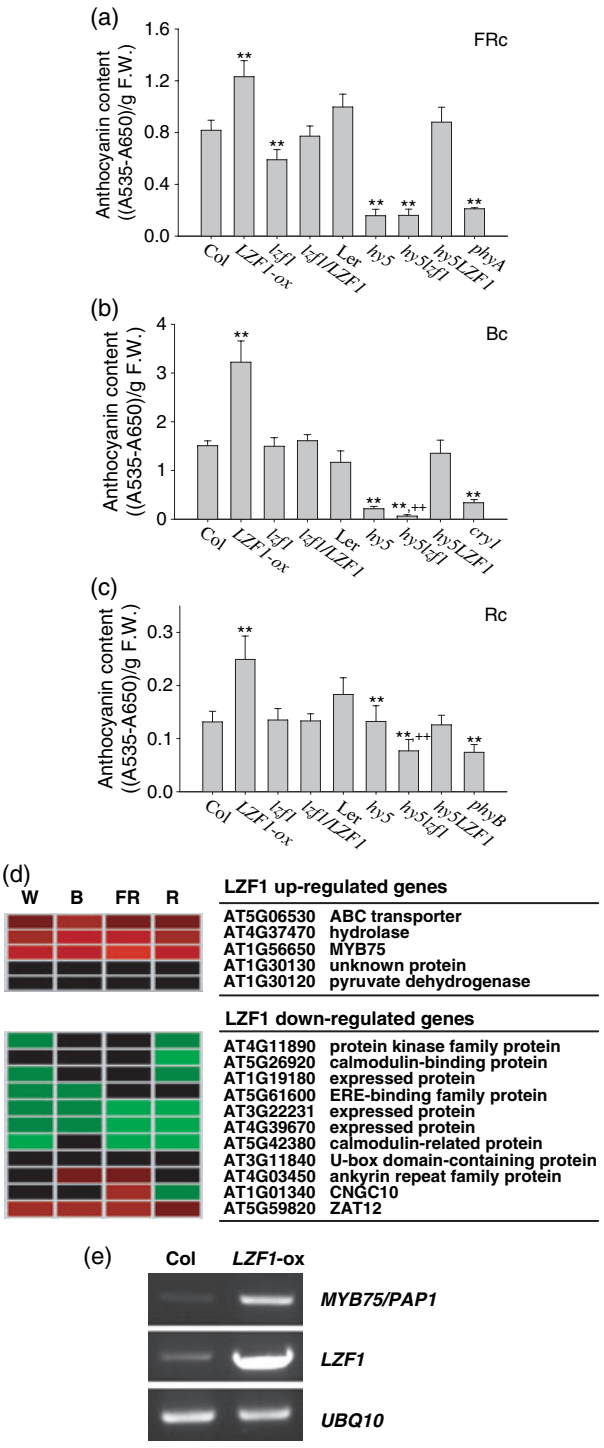

Figure 5. LZF1 activates *MYB75/PAP1* for the accumulation of anthocyanin.
The anthocyanin contents were measured for 5-day-old Col-0, *LZF1-ox* (line 3 in Figure S2), *lzf1*, *lzf1/LZF1*, L*er*, *hy5*, *hy5lzf1* and *hy5LZF1* (ox3 in Figure 4) grown under continuous far-red (FRc) (a), blue (Bc) (b) and red (Rc) light (c). F.W., fresh weight. **Data points significantly different from their corresponding control ($P < 0.01$, Student's *t*-test; $n = 4–7$). $^{++}$Indicates the anthocyanin contents are significantly different ($P < 0.01$) between *hy5* and *hy5lzf1*.
(d) The expression patterns of genes upregulated (greater than or equal to twofold) or downregulated (≤0.5-fold) in 3-week-old *LZF1-ox* rosette leaves relative to Col-0 (Table S2). The expression of these genes under white (W), blue (B), far-red (FR) and red (R) light was retrieved from Genevestigator and color coded (red, upregulated; green, downregulated; black, unchanged). Data were originally published in AtGenExpress: NASCArrays-124 and reassigned and averaged from three replicates of seedlings treated with both 45-min and 4-h W light (109_22;_23;_24;_46; 47;_48), B light (109_13;_14;_15;_37;_38;_39) and FR light (109_4;_5;_6;_28;_29;_30), R (109_10;_11;_12;_34;_35;_36) and non-treated etiolated 4-day-old seedlings (109_1;_2;_3;_25;_26;_27).
(e) Semi-quantitative RT-PCR analyses of *LZF1* and *MYB75/PAP1* in Col-0 and *LZF1-ox* plants. *UBQ10* was used as a control.

notype which could be released by overexpressing *LZF1* in *hy5* mutant (Figure 6a–c).

We also observed that the development of etioplasts into functional chloroplasts was significantly delayed in *lzf1* cotyledons. When 4-day-old etiolated wild-type seedlings were illuminated with 0, 4, and 24 h of W light, the etioplasts gradually developed into mature and functional chloroplasts

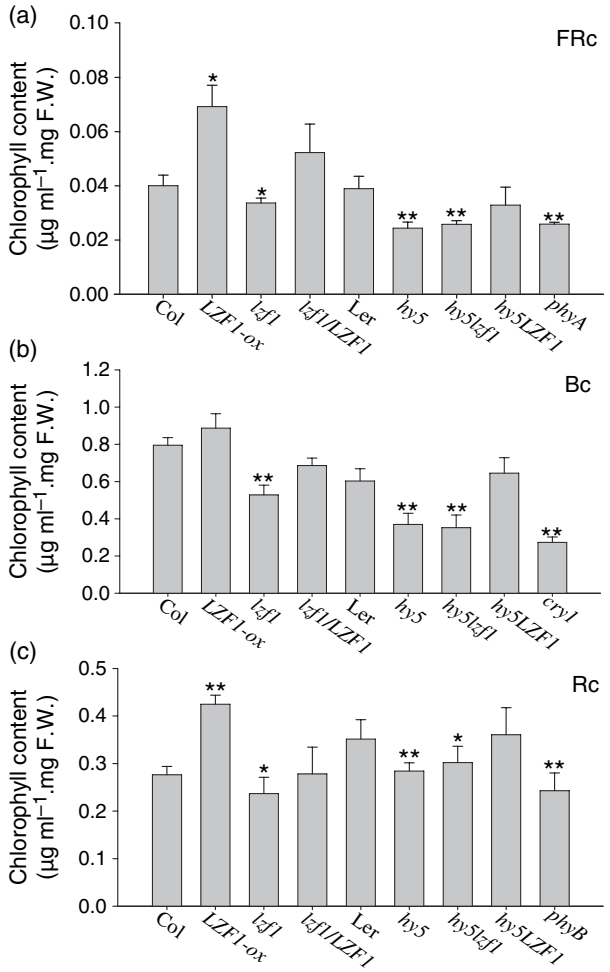

Figure 6. LZF1 is a positive regulator modulating chlorophyll accumulation.
The chlorophyll contents of 6-day-old Col-0, *LZF1-ox*, *lzf1*, *lzf1/LZF1*, L*er*, *hy5*, *hy5lzf1* and *hy5LZF1* (ox3 in Figure 4) grown under continuous far red (FRc) (a), blue (Bc) (b) and red (Rc) light (c). F.W., fresh weight. ** and * represent chlorophyll contents significantly different from their corresponding control, Col-0 or L*er* ($P < 0.01$, 0.05, respectively, Student's *t*-test; $n = 6$).

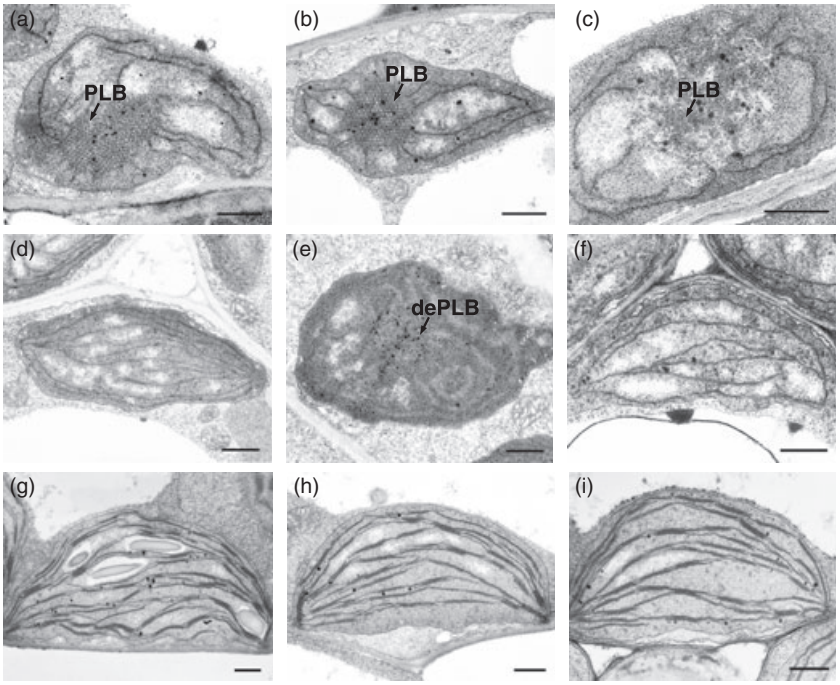

Figure 7. *lzf1* seedling exhibits delayed chloroplast development.
The transformation of etioplasts into chloroplasts in Col-0, *lzf1* and *lzf1/LZF1* was monitored in cotyledons of seedlings grown in the dark for 4 days and transferred to continuous white light (Wc) for the times indicated.
(a), (b) and (c) Col-0, *lzf1* and *lzf1/LZF1* in Wc for 0 h.
(d), (e) and (f) Col-0, *lzf1* and *lzf1/LZF1* in Wc for 4 h.
(g), (h) and (i) Col-0, *lzf1* and *lzf1/LZF1* in Wc for 24 h.
Bar size 0.5 μm. PLB, prolamellar body; dePLB, deformed PLB.

with well-developed grana and visible starch granules (Figure 7a,d,g). No significant differences could be observed in etioplasts from cotyledons of wild-type, *lzf1* and *lzf1/LZF1* plants (Figure 7a–c). In the wild type, the prolamellar body (PLB) in plastids had undergone morphogenesis to form flattened thylakoid membranes after 4 h of light treatment (Figure 7d), whereas in *lzf1*, only deformed PLBs were observed (Figure 7e). Also, electron-dense bodies and fragmented membrane structures were observed in the matrix of all *lzf1* chloroplasts examined under a 4-h light treatment (Figure 7e). Thylakoid membrane stacking was evident in *lzf1* after 8 h of light (data not shown), but the number of thylakoid membranes per granum in *lzf1* was still notably lower than that in wild-type plastids even after 24 h of light treatment (Figure 7g,h). Taken together, these results imply that the formation of functional chloroplasts is delayed in *lzf1*. The defect in early plastid development in *lzf1* was successfully rescued by a complementation assay (Figure 7c,f,i). We thus conclude that LZF1 contributes to early plastid development.

Transcriptomic comparison was performed to reveal genes differentially expressed in wild-type and *lzf1* seedlings at their early chloroplast developmental stages (4 h of light treatment; Figure S5a,b and Table S3). A total of 1117 genes showed at least twofold induction in wild-type seedlings after 4 h of light treatment and were thus designated as 'light induced'. Among them, 42 were designated as 'LZF1-induced' genes by their reduced light responsiveness in *lzf1* (less than two-thirds of the response observed in wild-type seedlings).

Figure 8(a) shows gene ontology (GO) assignments for genes induced by LZF1 by light and genes of the whole Arabidopsis genome. To determine whether LZF1 preferentially induces genes of certain GOs, two-tailed *P*-values for GO enrichment of LZF1-induced genes were computed against light-induced (Figure 8b) and whole-genome genes (Figure 8c) with use of Fisher's exact test (http://www.matforsk.no/ola/fisher.htm; Agresti, 1992). In both comparisons, genes encoding 'cytosol-localized' proteins are underrepresented in the population of genes induced by LZF1, as supported by their significant *P* values (Figure 8b,c). In contrast, the LZF1-induced genes encoding 'chloroplast-localized' proteins are over-represented (Figure 8b,c). This result echoes our previous results that Arabidopsis seedlings lacking functional LZF1 exhibit delayed chloroplast development (Figure 7).

Discussion

Characteristics of LZF1 *gene expression*

We have identified a C2C2-zinc finger transcription regulator, *LZF1*, whose expression was regulated by a light signal in a HY5-dependent manner (Figure 1). Previous gene-expression profiling experiments have shown that *HY5* is induced transiently by light (Tepperman *et al.*, 2001, 2004). The induction of *HY5* peaks 1 h after the light treatment and then lessens. The expression of *LZF1* is also transiently increased following exposure to light but at a slower pace than that of *HY5,* with peak expression occurring at 2–6 h.

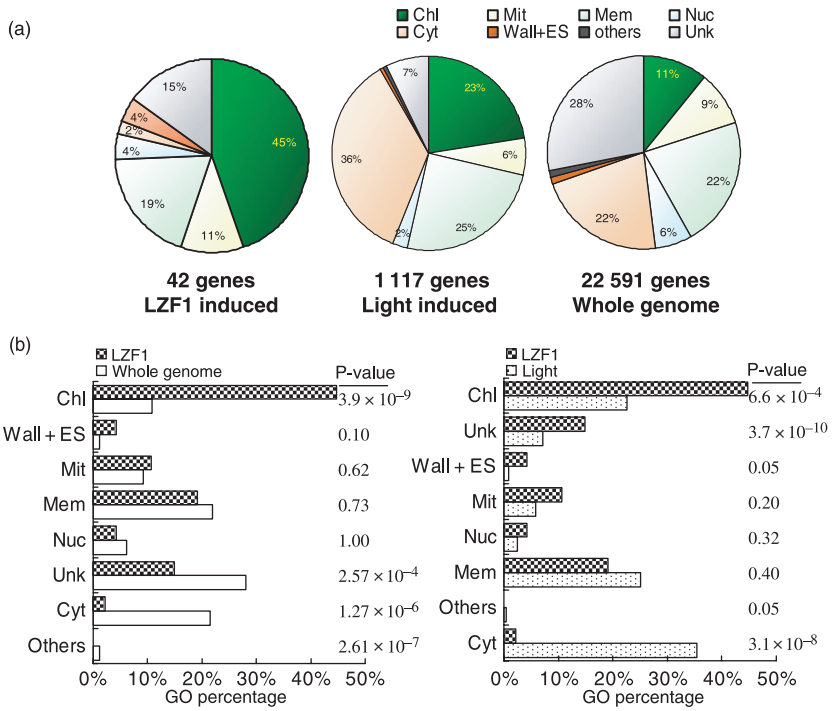

Figure 8. LZF1 predominantly upregulates genes encoding chloroplast proteins.
(a) The 42 LZF1-induced, 1117 light-induced and the whole genome of 22,591 Arabidopsis genes are shown in pie charts with percentages calculated on the basis of GO: subcellular localization. Eight categories are chloroplast (Chl), mitochondria (Mit), membrane (Mem), nucleus (Nuc), cytosol (Cyt), wall and extracellular space (Wall + ES), others and unknown (Unk). The distribution of gene ontology (GO) percentage among LZF1-induced (shaded), whole genome (empty) and light-induced (dotted) genes is shown. *P* values indicating the probabilities of differential GO distribution for the LZF1-induced genes to that of the whole genome (b) or light-induced genes (c) were calculated by Fisher's exact test. The transcriptome analysis was performed with 4-day-old seedlings grown in the darkness with or without 4-h light treatment.

After 24 h of treatment with light, the expression of *LZF1* was indistinguishable between the wild type and *hy5* mutant (Figure 1). The residual expression of *LZF1* in the *hy5* mutant at later time points of light treatment implies that additional factors may substitute for HY5, to a lesser extent, to trigger the expression of LZF1 (Figure 1c). This possibility was further supported by the synergistic effect of HY5 and LZF1 in the regulation of hypocotyl elongation during photomorphogenesis (Figure 4). Furthermore, the expression of *LZF1* in response to light treatment was not compromised in a recent transcriptomic data analysis of *hy5-215* (Sibout *et al.*, 2006). *LZF1* could be responding to the liquid-culture conditions in a light- and HY5-independent manner. Indeed, in addition to *LZF1* being a light-responsive gene, a search of the Genevestigator database revealed that it also positively responds to various biotic and abiotic stresses (data not shown).

Many studies have reported the global gene expression patterns of both wild-type Arabidopsis and photomorphogenic mutant seedlings exposed to different light conditions. A search of these expression data revealed that the expression of *LZF1* was repressed when seedlings were transferred to the dark (Ma *et al.*, 2001), which is consistent with our observation that *LZF1* is a light-inducible gene. Also, the expression of *LZF1* is elevated in the photomorphogenic mutants *cop1-8*, *cop10-1* and *det1-6* under both light and dark conditions (Ma *et al.*, 2003). In the dark, HY5 is recruited by COP1 and targeted to the 26S proteasome for selective protein degradation, but light signals abrogate this targeted degradation by modulating the nuclear abundance of COP1 (Osterlund *et al.*, 2000). DET1 is part of the CUL4-based E3 ligase complex contributing to diverse developmental processes, including photomorphogenesis, in Arabidopsis (Bernhardt *et al.*, 2006; Chen *et al.*, 2006). Recent data indicated that this CUL4-based E3 ligase positively regulates COP1-mediated degradation of HY5 protein (Chen *et al.*, 2006). Taken together, the results indicate that although the transcript level of *HY5* is not altered, the HY5 protein is predicted to be stable in these mutants. The induction of *LZF1* in these mutants might be due to increased levels of HY5 protein.

It is also interesting to note that *LZF1* preferentially responds to FR light and HY5 binds to the promoter of *LZF1* under FR light (Figures 2 and 3b). Results of data mining of gene expression data also support this observation. For example, *LZF1* transcripts were elevated under FR (Tepperman *et al.*, 2001), very low UV-B (Ulm *et al.*, 2004) and W light (this study). However, R or B light treatment did not evidently alter *LZF1* expression (Jiao *et al.*, 2003; Tepperman *et al.*, 2004). The induction of *LZF1* was severely reduced in the *phyA* mutant under FR light, probably due to lack of *HY5* expression in the *phyA* mutant (Tepperman *et al.*, 2001). *HY5* was still R-light responsive in the *phyB* mutant. However, R light could not effectively induce the expression of *LZF1* in either wild-type Arabidopsis or the *phyB* mutant (Tepperman *et al.*, 2004). These results imply that HY5 is necessary but not sufficient for the light-responsive expression of *LZF1*. The simplest explanation is that an additional factor(s) is required to act in concert with HY5 to induce *LZF1* and that this presumed second factor is

more available or functional under FR light. Alternatively, the HY5 protein may be modified and function differently under different light qualities or intensities.

Molecular signatures of the LZF1 protein

Among the 31 known C2C2-CO B-box proteins in Arabidopsis, 17 have both B-box motifs and a CCT domain. Both B-box motifs and the CCT domain are crucial for the full function of CO (Robson *et al.*, 2001). That the B-box motifs and the CCT domain might act independently has been proposed, because proteins with only one of the two domains exist in Arabidopsis. For instance, TOC1 has only the CCT domain (Strayer *et al.*, 2000), and the other 14 proteins listed in Figure S1 have only B-box motifs. Among the 14 proteins lacking a CCT domain, STO is involved in salt tolerance (Lippuner *et al.*, 1996) and has recently been characterized to function as a negative regulator in phytochrome and blue-light signaling pathways (Indorf *et al.*, 2007). This current work adds new information about the biological roles of another member of this class.

B-box proteins are prevalent in many organisms and participate in diverse fundamental biological processes, including transcriptional regulation. Previous reports suggested that B-box motifs can bind one zinc ion per B-box motif and are involved in protein–protein interactions (for review see Torok and Etkin, 2001). Thus, it will be of great interest to characterize the interacting partner(s) of LZF1 to elucidate other components of the HY5/LZF1 light signaling pathway.

Biological functions of LZF1 protein

Our results indicate that, in the background of *hy5*, the further introduction of LZF1 mutation would lead to additive light hyposensitivity in light-regulated inhibition of hypocotyl growth (Figure 4) and anthocyanin accumulation (Figure 5) in the *hy5lzf1* double mutant. These results imply that although HY5 is the major transcriptional regulator directing the light-dependent expression of *LZF1*, the residual LZF1 in the *hy5* mutant also exerts functions in conveying a light signal in these biological processes.

Although the alteration of hypocotyl length is modest in *lzf1* when compared with that of wild-type plants, the contribution of LZF1 in the light-induced inhibition of hypocotyl elongation was better measured in the *hy5lzf1* double mutant. In the presence of HY5, the loss of *LZF1* could possibly be compensated by other light- and HY5-regulated genes, which may contribute redundantly or in parallel with LZF1 to regulate the inhibition of hypocotyl elongation. This redundancy may also explain why previous genetic screens for light-sensing mutants based on hypocotyl length did not uncover *lzf1* mutants. Overexpression of *LZF1* in the *hy5* mutant partially rescued the long hypocotyl phenotype (Figure 4) but restored the wild-type level of anthocyanin and chlorophyll (Figures 5 and 6), which supports that LZF1 is a positive regulator in the light signaling pathway.

Anthocyanin content is significantly diminished in *lzf1* under FR which is the most efficient light for the induction of *LZF1* (Figures 5a). This finding suggests LZF1 regulates the process of anthocyanin accumulation. Reduced anthocyanin is also observed in *hy5lzf1* under B and R light (Figures 5b,c). This suggests that LZF1 plays a complementary role to HY5 in this process under B light. The upregulation of *MYB75*/*PAP1* by LZF1 probably explains the function of LZF1 in controlling the anthocyanin accumulation (Figure 5d,e).

lzf1 plants showed reduced chlorophyll accumulation under all light conditions tested (Figure 6a–c). Transcriptomic analyses of *lzf1* plants revealed that, in the early photomorphogenic stage, LZF1 preferentially regulates genes encoding chloroplast proteins, a subset of genes responsive to light signals (Figure 8, Figure S5a,b). Among the 42 LZF1-induced and 33 LZF1-repressed genes, 17 and 20 genes, respectively, are evidently up- or downregulated by HY5 as revealed by comparing the transcriptome data of etiolated L*er* seedlings and the *hy5* mutant exposed to 4 h of W light (Table S4; CJC and SHW, unpublished data). It is interesting to note that, of the 42 LZF1-induced and 33 LZF1-repressed genes, only 33 and 26 genes, respectively, are also light responsive in ecotype L*er* at the time point examined. Our transcriptome analyses indicate only 65% of light upregulated and 70% light downregulated genes are in common between L*er* and Col-0 ecotypes, possibly reflecting gene expression differences among ecotypes. These genes might have different light-responsive kinetics and might be regulated by light at earlier or later time points not examined in our current study.

Genes induced by LZF1 include those involving in the regulation of chloroplast redox potential. These are proteins of the ferredoxin (At4g14890) and thioredoxin families [At1g43560; ATY2 (Collin *et al.*, 2004)] and oxidoreductase (At1g15140) (Table S4). The thioredoxins could be involved in regulating the activities of enzymes in multiple biochemical pathways in chloroplasts (Gelhaye *et al.*, 2005). Also, the translation of light-regulated chloroplast proteins could be activated by the changes in redox potential in *Chlamydomonas reinhardtii* (Danon and Mayfield, 1991, 1994). Indeed, ferredoxin and thioredoxin are required for the active translation of chloroplast *psbA* mRNA *in vivo* (Danon and Mayfield, 1994). LZF1 could relay the light signal for chloroplast biogenesis/function by activating, at least in part, the thioredoxin–ferredoxin cascade, which subsequently activates both the translation of chloroplast mRNAs and the activity of enzymes in the Calvin cycle. A parallel observation for the involvement of LZF1 in modulating the translation of chloroplast mRNAs comes from the LZF1-induced expression of

genes related to the translation machinery in chloroplasts. These include genes encoding two aminoacyl-tRNA synthetase class I (W and Y) family proteins (At2g25840 and At3g02660; (Berg *et al.*, 2005; Duchene *et al.*, 2005) and RNase Z (At2g04530) (Table S4). Among them, nuclear RNase Z is required for tRNA maturation in plants (Kruszka *et al.*, 2003). Taken together, the results suggest that LZF1 functions in early chloroplast development by coordinating the light-regulated translational activation of chloroplast mRNAs.

In conclusion, our study uncovered a new member of a light-signaling network, LZF1, which functions downstream of HY5 and conveys the light signal for the inhibition of hypocotyl elongation, anthocyanin and chlorophyll accumulation and chloroplast development. These functions could be contributed by the transcriptional regulatory activity of LZF1, which alters the expression patterns of dozens of genes. Future characterization of the relation between LZF1 and these genes will expand our current knowledge of light-signaling networks.

Experimental procedures

Plant materials and growth conditions

Two T-DNA insertion lines (Alonso *et al.*, 2003) SALK_105367 (*lzf1-1*) or SALK_021776 (*lzf1-2*), *hy5-1* (Koornneef *et al.*, 1980), *phyA-201* and *phyB-5* seeds were obtained from the Arabidopsis Biological Resource Center. *hy5-1* and *lzf1-1* were crossed to generate the *hy5lzf1* double mutant used in this study. More than five independent *lzf1*/*LZF1* lines were constructed by introducing *35S:LZF1* into *lzf1-1*, and the expression was confirmed by RT-PCR (data not shown). One representative *lzf1*/*LZF1* line C5 was used in this study. Three independent *hy5*/*LZF1* lines were constructed by introducing *35S:LZF1* into *hy5-1*, and the expression was confirmed by RT-PCR (data not shown). Seeds of *Arabidopsis thaliana* Col-0, Landsberg (*erecta*) (L*er*-0), mutants and transgenic plants were germinated either on half-strength MS medium with 0.8% agar or directly in soil and placed at 4°C for 4 days to synchronize the germination. For light treatments, etiolated seedlings were grown in the dark for 4 days at 22°C and then treated with continuous light (100 μmol m^{-2} sec^{-1}) for 5 min to 6 h for microarray experiments, 10 min to 24 h for real-time PCR, or 1–18 h for RT-PCR. For gene expression examination under different qualities of light, 4-day-old etiolated seedlings were illuminated with 18 μmol m^{-2} sec^{-1} of R light, 1.13 W m^{-2} of FR light or 5 μmol m^{-2} sec^{-1} of B light for the times indicated before RNA extraction. For isolation of protoplasts from rosette leaves, Col-0 plants were grown under a 12-h/12-h light/dark photoperiod at 22°C for approximately 3–4 weeks.

Constructs

Sequences for all primers used in this study are listed in the Supplementary Primer Table (Appendix S1). The expression vector smGFP (Davis and Vierstra, 1998) was kindly provided by I. Hwang (Pohang University of Science and Technology, Korea). To prepare the *LZF1-His6* construct, the *LZF1* coding region was amplified with *Xba*I-pLZF1-F and pLZF1-His6-*Sst*I-R.

pCAMBIA1390 (CSIRO, http://www.csiro.au/) was used for generating transgenic Arabidopsis overexpressing *LZF1*. The *LZF1* coding region was amplified with the primers AflIII-pLZF1-F and pLZF1-*Xba*I-R1. For generating complementation *lzf1*/*LZF1* lines, the *35S:LZF1-his6* fragment in smGFP (as described above) was subcloned into pCAMBIA1390.

pBI221 (Clontech, http://www.clontech.com/) and pJD301 (Promega, http://www.promega.com/) were used for constructs of *p35S:HY5-His6* and *pLZF1:LUC*. To prepare the *35S:HY5-His6* fusion construct, the *HY5* coding region was amplified by PCR with the primers *Xba*I-pHY5-F and His-Stop-*Sac*I from a plasmid harboring *HY5* cDNA. For pLZF1:LUC, the 1-kb *LZF1* promoter region upstream of translational start site was amplified with the primers *Bam*HI-pLZF-F and pLZF-*Nco*I-R from Arabidopsis genomic DNA.

All constructs used in this study were confirmed by sequencing.

Protoplast isolation and transient expression

The mesophyll protoplasts were isolated and transfected as described previously (Sheen, 2001; http://genetics.mgh.harvard.edu/sheenweb/protocols_reg.html). For effector (*p35S:HY5-His6*) and reporter (*pLZF1:LUC*) experiments, 1.5×10^5 protoplasts were co-transfected with 10 μg of each construct in each experiment. pBI221 (*35S:*GUS) was used as an internal control for activity calibration in each transfection experiment. The transfected protoplasts were incubated at room temperature for 20 h and harvested for activity assays. Three independent replicates were performed for each sample. Levels of LUC luminescence were measured using an EG&G Berthold Lumat LB9507 luminometer (Berthold Technologies, http://www.bertholdtech.com/). A DynaQuant200 fluorometer (Hoefer, Inc.; http://www.hoeferinc.com) was used to quantify GUS activity.

Chromatin immunoprecipitation-quantitative real time PCR (ChIP-qPCR) assay

ChIP was performed as described (Shin *et al.*, 2007). Briefly, 4-day-old seedlings of the myc-tagged HY5 line were grown under continuous FR or darkness then exposed to 4 h of FR light. The myc-tagged GFP seedlings were used as a control. The isolated chromatin was sonicated to approximately 200–400 bp and immunoprecipitated with anti-myc antibody. The immunoprecipitated DNA were dissolved in 250 μl TE (10 mM Tris, pH 8.0, 1 mM EDTA). Quantitative PCR analysis involved 5 μl of this DNA as a template and primers, pLZF1-AF and pLZF1-AR, pLZF1-BF and pLZF1-BR, pLZF1-CF and pLZF1-CR, pLZF1-DF and pLZF1-DR, pLZF1-EF and pLZF1-ER, respectively, to give rise to fragments LZF1-A to E in Figure 3(b) (primers are listed in the Supplemental Primer Table, Appendix S1). Means and standard deviations were calculated from three replicates and plotted.

RNA isolation

Arabidopsis total RNA was isolated as described previously (Chang *et al.*, 1993) with minor modification. Plant tissues were frozen and ground in liquid nitrogen and extracted by vortexing with eight volumes of extraction buffer [2% hexadecyltrimethylammonium bromide, 2% polyvinylpyrrolidone K 30, 100 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS)–HCl, pH 8.0, 25 mM EDTA, 2.0 M NaCl, 0.5 g l^{-1} spermidine, 2% 2-mercaptoethanol] prewarmed at 65°C. The homogenate was then extracted twice with an equal volume of chloroform:isoamyl alcohol (24:1)

by vortexing and centrifugation for 10 min at 12 000 ***g***. One-quarter volume of 10 M LiCl was then added to the aqueous phase for selective precipitation of RNA molecules. After overnight incubation at 4°C, the RNA pellet was harvested by centrifugation at 12 000 ***g*** for 20 min, washed with 75% ethanol (EtOH) and dissolved in 200 μl RNase-free water. The quality of RNA samples was assessed with use of the Agilent Bioanalyzer2100 (Agilent, http://www.home.agilent.com/) and the Agilent RNA 6000 Nano Assay Kit.

Real-time quantitative RT-PCR

Samples were collected from Arabidopsis L*er* or *hy5* mutant seedlings treated with light for 0 min to 24 h. Total RNA was isolated as described above and quantified by use of a spectrophotometer (U-2001; Hitachi, http://www.hitachi.com/) or Bioanalyzer2100. Complementary DNA was synthesized from 5 μg of total RNA with use of SuperScript II reverse transcriptase (Invitrogen, http://www.invitrogen.com/) and poly T primer. All primers were designed by Primer Express (Applied Biosystems, http://www.appliedbiosystems.com/). A total of 50 μl real-time PCR reaction contained the following: primers (the concentration and ratio of the two primers were determined experimentally as suggested by the manufacturer and listed below), 5 μl cDNA (equivalent to ~10 ng of mRNA) and 25 μl SYBR Green PCR Master Mix (Applied Biosystems). Real-time PCR involved the use of the ABI Prism 7000 Sequence Detection System (Applied Biosystems) with programs recommended by the manufacturer (2 min at 50°C, 10 min at 95°C and 40 cycles of 95°C for 15 sec and 60°C for 1 min). Three independent replicates were performed for each sample. The comparative C_T method was used to determine the relative amount of gene expression, with the expression of *UBQ10* used as an internal control. The primers used for *LZF1* were pLZF-ABI-1-F and pLZF-ABI-2-R at a ratio of 18:1; for Col-0, *UBQ10* pUBQ10-ABI-1-F and pUBQ10-ABI-2-R at a ratio of 3:9; and for L*er*, *UBQ10* pUBQ10-ABI-1-F and ABI-Ler-pUBQ-2-R at a ratio of 3:9.

RT-PCR

A total of 1 μg total RNA was used in a 20 μ l reverse transcription reaction. In brief, RNA was mixed with 25 ng Oligo-dT-V (A/C/G) primer, incubated at 65°C for 10 min and then placed on ice. The reaction was initiated by bringing the components to the final concentration of 500 μM dNTP (Amersham Biosciences, http://www5.amershambiosciences.com/), 10 mM DTT, Superscript II reverse transcriptase (7.5 U), and 1 × buffer supplied (Invitrogen). After incubation at 42°C for 1 h, one-tenth of the sample (~1 ng cDNA) was used as a PCR template. The abundance of *UBQ10* cDNA was used as an internal control and amplified with the primers pUBQ10-F and pUBQ10-R. Primers pLZF1-F and pLZF1-R, pHY5-F and pHY5-R, pMYB75-F and pMYB75-R were used for *LZF1*, *HY5* and *MYB75* cDNA amplifications, respectively.

Measurement of hypocotyl length

Wild-type and mutant Arabidopsis seedlings were germinated under dark, 16-h light/8-h dark of W (100 μmol m^{-2} sec^{-1}), continuous R (Rc; 18 μmol m^{-2} sec^{-1}) or continuous FR (FRc) light (1.13 W m^{-2}) for 4 days. The fluence rate was measured with use of an LI-250 radiometer (Li-Cor, http://www.licor.com/). The hypocotyl length was measured with NIH imaging software Image J, version 1.34.

Anthocyanin quantification

Plants were sowed on a 1/2 MS, 1% sucrose agar plate and grown under FRc (1 W m^{-2}), Bc (30 μmol m^{-2} sec^{-1}) and Rc light (30 μmol m^{-2} sec^{-1}) for 5 days. The extraction of anthocyanin was performed as previously described (Lange *et al.*, 1971). In brief, anthocyanin was extracted from approximately 50–200 seedlings with 1% HCl, 18% propanol by immersion in boiling water for 90 sec. Extracts were centrifuged for 15 min at 13,000 ***g*** prior to spectroscopy measurement by use of the automated microplate spectrophotometer Bio-Tek PowerWave X340 (Bio-Tek Instruments, Inc., http://www.biotek.com/). The anthocyanin content was determined by use of the equation $A_{535\text{-corrected}} = A_{535} - A_{650}$ and normalized to fresh weight (Montgomery *et al.*, 1999). The results were averages of five to eight replicates and were examined by Student's *t*-test.

Chlorophyll quantification

Plants were grown under FRc (1 W m^{-2}), Bc (10 μmol m^{-2} sec^{-1}) and Rc light (10 μmol m^{-2} sec^{-1}) for 6 days. Chlorophyll was extracted from approximately 50–200 seedlings directly into 100% dimethyl formamide at the ratio of 2–7% (w/v). The extracts were stored in the dark for 24 h at 4°C prior to spectroscopy examination by use of a spectrophotometer (U-2001; Hitachi). Chlorophyll content was determined by use of the equation $Ct = Ca + Cb = 7.04A_{664} + 20.27A_{647}$ (Moran and Porath, 1980). Results were from six independent measurements and were assessed by Student's *t*-test.

DNA microarray fabrication and hybridization and data analyses. The DNA elements for the DNA microarrays were amplified as part of the Arabidopsis Functional Genomics Consortium (AFGC). The DNA solutions were printed on CMT-GAPS2-coated glass slides (Corning, http://www.corning.com/) with use of the OmniGrid 100 microarrayer (GeneMachines; http://www.genomicsolutions.com). After printing, slides were baked at 80°C for 6 h and blocked (0.1 mg ml^{-1} BSA, 3× SSC, 0.1% SDS) before hybridization.

The RNA isolated from Arabidopsis seedlings was first amplified with use of the MessageAmp™ II aRNA Amplification Kit (Ambion Inc., http://www.ambion.com/) and labeled with 0.4 mM aminoallyl-dUTP (Sigma, http://www.sigmaaldrich.com/) by reverse transcription with 0.075 μg μl^{-1} random primer. Then, the monofunctional NHS-esters of Cy3 and Cy5 (Amersham Biosciences) were coupled separately to aminoallyl-dUTP labeled control and experimental cDNA. Detailed methods were as described in Lin and Wu (2004). The hybridization signals for each DNA element were acquired with the use of an Axon GenePix 4000B scanner and analyzed with use of GenePix 4.0 (Axon Instruments, Inc., http://www.axon.com/). Expression data for Figure 1 can be viewed and retrieved through the Stanford Microarray Database (SMD; Gollub *et al.*, 2003; http://genome-www5.stanford.edu/). Experiment IDs are 11384, 11385, 14743, 14746, 14747, 14749, 14751, 14753-54, 14757-58, 14760, 14762, 14765, 14776-77, 14779-82, 14807-12. Genes showing four-fold up or downregulation in at least 2 of these 26 expression data sets were selected for clustering analyses. Figure 1(a) represented a tight cluster of genes upregulated by light in wild-type but not in *hy5* seedlings.

Gene expression data from the cDNA microarrays were analyzed as described previously (Lin and Wu, 2004). The gene expression data were imported into GeneSpring 7.2 (Silicon Genetics, http://www.silicongenetics.com/) and normalized with 'LOWESS Normalization' and 'PerChip: normalized to 50th percentile'. Genes with net intensities in the Cy5 or Cy3 channels of <350 (500 for Figure 1)

intensity units, for the up- or downregulated genes, respectively, were removed because these are often poorly measured. Statistical analyses to examine the reproducibility of the triplicate data were performed with the use of SAM (Tusher *et al.*, 2001). Only genes that passed the criteria of a false discovery rate of <1% were selected for further analyses.

Affymetrix ATH1 genome array hybridization and data analyses

ATH1 genome array hybridization was performed with the use of the Arabidopsis ATH1 Genome Array (Affymetrix Inc., http://www.affymetrix.com/), which contains >22 810 probe sets representing approximately 22 591 gene sequences on a single array. Total RNA from 4-day-old etiolated or 4-h light-treated wild-type and *lzf1* seedlings was isolated as described above. The fluorescent cRNA was labeled and used to hybridize the ATH1 Genome Array as suggested by the manufacturer. The results were quantified and analyzed with use of the MicroArray Suite 5.0 software (Affymetrix Inc.).

Gene expression data for Affymetrix ATH1 were analyzed as described previously (Lin and Wu, 2004). The average intensity of all probe sets of each chip was scaled to 500 so that the hybridization intensity of all chips was equivalent. 'Set measurements <0.01–0.01', 'Per Chip: Normalize to 50th percentile' and 'Per Gene: Normalize to control mean' were applied for data normalization when Affymetrix data files were imported into GeneSpring 7.2 (Silicon Genetics) for further analyses. Genes marked as 'absent' in all the chips analyzed were removed from further data analyses. Statistical analyses to examine the reproducibility of the triplicate data involved use of SAM (Tusher *et al.*, 2001). Only genes that passed the criteria of a false discovery rate <1% were selected for further analyses. Genes considered up or downregulated had ratio values ≥2 or ≤0.5, respectively, and intensity values >50. Only genes that reduced the light induction and repression >1.5-fold in *lzf1* compared with the wild type were considered LZF1-dependent light-responsive genes. Subcellular localization gene ontology (GO) annotations applied in Figure 8 and Table S3 were adopted from TAIR (ATH_GO_GOSLIM.20061014; released 14 October 2006).

Transmission electron microscopy

Four-day-old etiolated seedlings of wild type, *lzf1* or *lzf1/LZF1* were transferred to W light for 4, 8 and 24 h before cotyledons were fixed in 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.0, at room temperature for 4 h. After three 20-min rinses, the samples were post-fixed in 1% OsO_4 in the same buffer for 4 h at room temperature followed by three buffer rinses. Samples were dehydrated in an acetone series, embedded in Spurr's resin, and sectioned with a Leica Reichert Ultracut S or Leica EM UC6 ultramicrotome (Leica Microsystems GmbH, http://www.leica-microsystems.com/). The ultrathin sections (70–90 nm) were stained with uranyl acetate and lead citrate. A Philips CM 100 TEM (FEI Company; http://www.feicompany.com) at 80 kV was used for viewing. At least three seedlings and more than 30 individual chloroplasts for each time point and each plant genotype were examined.

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Supplementary Material

The following supplementary material is available for this article online:

Figure S1. LZF1 is a member of the C2C2 B-box protein family.

Figure S2. Molecular characterization of *lzf1* and *LZF1-ox* Arabidopsis.

Figure S3. Phenotypic analyses of *lzf1* mutant and transgenic plants overexpressing *LZF1*.

Figure S4. *hy5lzf1* seedlings show increased light hyposensitivity in light-regulated inhibition of hypocotyl elongation under continuous red and far-red light.

Figure S5. Genes differentially expressed between wild-type and *lzf1* mutant during the early photomorphogenic stage.

Table S1. Expression data for genes differentially regulated between wild-type Col-0 and *hy5* mutant.

Table S2. Expression data for genes differentially regulated between wild-type Col-0 and *LZF1-ox* transgenic plant.

Table S3. Expression data for genes differentially regulated between wild-type Col-0 and *lzf1* mutant.

Table S4. Expression data of LZF1-responsive genes in Col, L*er*, *lzf1* and *hy5* seedlings treated with 4-h white light.

Appendix S1. Supplementary Primer Table: list of primers described in Experimental procedures.

Appendix S2. Supplementary methods: experimental procedures used for Supplementary Figures S1 and S2.

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