Dissection of the Light Signal Transduction Pathways Regulating the Two *Early Light-Induced Protein* Genes in Arabidopsis

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The expression of light-regulated genes in plants is controlled by different classes of photoreceptors that act through a variety of signaling molecules. During photomorphogenesis, the early light-induced protein (*Elip*) genes are among the first to be induced. To understand the light signal transduction pathways that regulate *Elip* expression, the two *Elip* genes, *Elip1* and *Elip2*, in Arabidopsis were studied, taking advantage of the genetic tools available for studying light signaling in Arabidopsis. Using two independent quantitative reverse transcriptase-PCR techniques, we found that red, far-red, and blue lights positively regulate expression of the *Elip* genes. Phytochrome A and phytochrome B are involved in this signaling. The cryptochrome or phototropin photoreceptors are not required for blue-light induction of either *Elip* gene, suggesting the involvement of an additional, unidentified, blue-light receptor. Although the COP9 signalosome, a downstream regulator, is involved in dark repression of both *Elip*s, *Elip1* and *Elip2* show different expression patterns in the dark. The transcription factor HY5 promotes the light induction of *Elip1*, but not *Elip2*. A defect in photosystem II activity in greening of *hy5* seedlings may result from the loss of *Elip1*. Heat shock positively controlled *Elip1* and *Elip2* in a light-independent fashion. This induction is independent of HY5, indicating that heat shock and light activate transcription of the *Elip* genes through independent pathways.

Light has three main effects on plant development (for review, see Mustilli and Bowler, 1997; Batschauer, 1998). First, it is the source of energy that fuels growth through photosynthesis. Second, light is a developmental signal that modulates morphogenesis, such as de-etiolation and the transition to reproductive development. Third, light is also deleterious for plants because excess light, absorbed by the photosynthetic apparatus, promotes the formation of dangerous compounds such as active oxygen species. Because plants must quickly respond to changing and often extreme light conditions, sophisticated photosensory networks have evolved that enable plants to maximize photosynthesis while minimizing damage. One of the main mechanisms of this overall control is accomplished through regulation of gene expression.

Light is perceived in plants by a sophisticated system of photoreceptors that detect different light wavelengths. Five phytochromes mediate red and far-red light responses in Arabidopsis. Among these, phytochrome A (PhyA) is primarily responsible for the perception of constant far-red light, whereas PhyB is primarily responsible for the perception of constant red light. Three photoreceptors for blue light have been identified in Arabidopsis (for review, see Lin, 2000). Crytpochrome 1 (Cry1) is the principal blue/UV-A light receptor, modulating growth at medium and high-light intensities. Cry2 has major functions in responding to low intensities of blue light (Lin et al., 1996). Phototropin, encoded by the nonphototropic hypocotyl 1 (*NPH1*) gene, is the photoreceptor for phototropism (Liscum and Briggs, 1995). Various reports have discussed the possibility of other blue-light receptors, though their identities were enigmatic (Zeiger and Zhu, 1998; Briggs and Huala, 1999; Frechilla et al., 1999). NPL1 (NPH-like 1), a fourth blue-light receptor, recently was identified that is partly functionally redundant with NPH1, and has a major role in the chloroplast high-blue-light avoidance response (Jarillo et al., 2001; Kagawa et al., 2001; Sakai et al., 2001).

Downstream from the photoreceptors are a plethora of positive and negative regulators of light signaling (for review, see Nagy et al., 2000; Neff et al., 2000). Among these, the COP9 signalosome (CSN) is a multisubunit regulatory complex that functions through unknown mechanisms as a master repressor of photomorphogenesis in the dark (for review, see Karniol and Chamovitz, 2000). One of the targets of the CSN-mediated repression is HY5. HY5 is a basic Leu zipper transcription factor directly involved in the expression of light-inducible genes (Oyama et al., 1997; Chattopadhyay et al., 1998). No role for these receptors or signaling molecules has been reported for responses to light stress.

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Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.010270.

The effect of light on plant development is particularly evident in seedling development and the transition from growth under soil (dark) to growth above the ground (light). As photomorphogenesis is initiated, cellular and subcellular processes are initiated to allow the development of photosynthetic capable tissues. This includes chloroplast development, pigment synthesis, and assembly of the photosystems in the thylakoids. All of these processes are accomplished by and depend on the differential expression of a large number of genes. However, before a chloroplast is competent for performing photochemistry, it is saturated with photons that have no outlet, and thus form toxic compounds that can kill the developing cell. In response to this light-induced stress, plants produce photoprotective pigments such as carotenoids and xanthophylls, and protective proteins.

An example of protective proteins are the early light-induced proteins (ELIPs), nuclear-encoded thylakoid membrane proteins that are transiently expressed immediately after light stress. *Elip* transcript and protein appear considerably faster than those of other light-induced genes during the early stage of de-etiolation, and disappear before chloroplast development is completed (Grimm and Kloppstech, 1987). In mature plants, ELIP accumulation under light stress conditions correlates with the photoinactivation of photosystem II (PSII), degradation of the D1 protein, and changes in the level of pigments (Adamska et al., 1992a, 1993). ELIPs bind chlorophyll *a* and lutein and have been proposed to function as transient pigment carriers or chlorophyll exchange proteins (Adamska et al., 1999).

The regulation of ELIP expression is modulated by light and other stress signals. Blue and red light induce *Elip* transcription in etiolated plumulas of pea (*Pisum sativum*) seedlings (Adamska, 1995), whereas blue and UV-A light induce ELIP in adult tissues (Adamska et al., 1992a, 1992b). ELIP homologs from various systems have been implicated in various stress responses. For example, one of the responses to extreme dehydration of the "resurrection" plant *Craterostigma plantagineum* is the expression of the ELIP homolog *dsp*-22 (Bartels et al., 1992).

In pea (Scharnhorst et al., 1985; Kolanus et al., 1987) and tobacco (*Nicotiana tabacum*; Blecken et al., 1994), ELIP is encoded by a single gene, whereas two ELIPs was reported to exist in barley (*Hordeum vulgare*; Grimm and Kloppstech, 1987) and in Arabidopsis (Moscovici-Kadouri and Chamovitz, 1997; Heddad and Adamska, 2000). The functions of the two genes are unclear, as is the genetic regulation of *Elip* transcription.

To understand genetic mechanisms regulating light and stress control of ELIP induction, we have initiated a study of ELIP in Arabidopsis. Previous studies on *Elip* in other plants were limited to characterizing the light quality and intensity that regulate *Elip* expression, and often in dismembered leaves. Arabidopsis provides a convenient system for studying *Elip* transcription due to the large collection of characterized light-signaling mutants available. We show here that multiple photoreceptors, including a cryptic blue-light receptor, regulate *Elip* transcription, and that the two ELIP genes have differing regulation patterns, which hint at different functions.

RESULTS

Development of Semiquantitative Reverse Transcriptase (RT)-PCR Experiment Conditions

Previous studies showed that light regulation of *Elip* steady-state transcript levels is manifested at the level of transcription, allowing for a correlation between steady-state transcript levels and transcriptional control (Adamska, 1995). To study the regulation of *Elip*s in multiple genetic backgrounds under different conditions, we developed a sensitive and fast RT-PCR method for analyzing *Elip* transcript levels. We used two PCR techniques: conventional RT-PCR followed by hybridization, and the LightCycler system. *Ubiquitin10* (*Ubq10*) was used as an internal RT-PCR control because it was previously shown by RNA gel-blot analysis to be constitutively expressed in light and dark conditions (Sun and Callis, 1997). To confirm this result by RT-PCR, and to determine if the steady-state levels of *Ubq10* are affected by the developmental stage of the plant, we checked *Ubq10* levels in dark- and light-grown seedlings by both RT-PCR techniques. As seen in Figure 1, A and B, the steady-state levels of *Ubq10* in dark-grown seedlings kept in the dark or exposed to 1 h light are equal as determined by conventional RT-PCR and LightCycler RT-PCR. This endogenous mRNA standard has the advantage of serving as a control for RNA recovery and integrity, as well as for sample-to-sample variations in RT and PCR.

To avoid artifacts due to genomic DNA contamination in an RNA preparation, PCR primer pairs of *Elip1* and *Elip2* were designed around introns (see "Materials and Methods"). In addition, to avoid artifacts due to the high identity between the two *Elip* transcripts, the forward primers of *Elip1* and *Elip2* anneal to the divergent 5′ end of the genes. As shown in Figure 1C, PCR on genomic DNA and cDNA yield product sizes for *Elip1* of 414 and 330 bp, respectively, and for *Elip2*, 705 and 400 bp, respectively. In addition, RNA was treated with DNase (during total RNA preparation) prior to cDNA synthesis and the control reaction was performed in which RT is omitted (−RT; Fig. 1C). Conventional PCR was carried out for 15 cycles where the kinetics of the PCR reactions allowed quantitative analysis for all three genes, *Elip1*, *Elip2*, and *Ubq10* (Fig. 1, A and D).

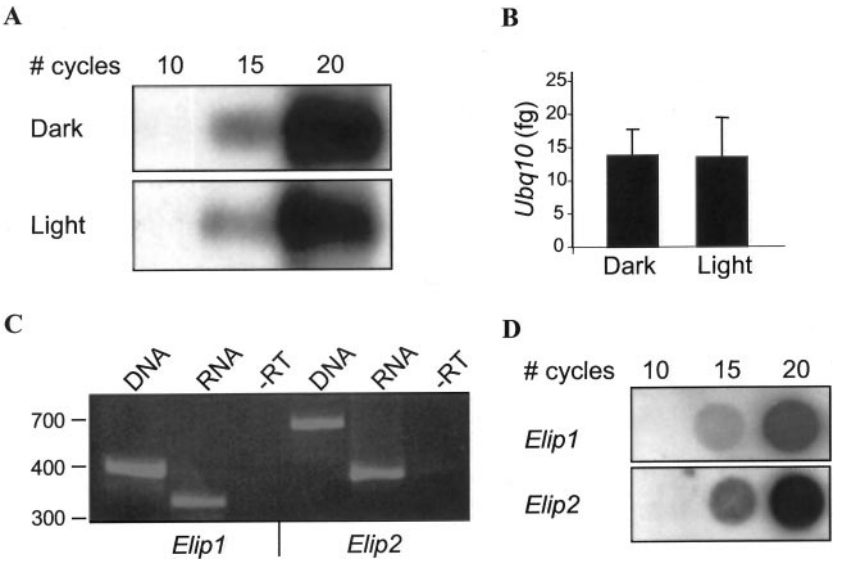

Figure 1. Development of RT-PCR experimental conditions. A and B, *Ubq10* is a constitutive control. Four-day-old Arabidopsis dark-grown wild-type seedlings were kept in the dark (Dark) or exposed to 1 h light (Light), RNA was extracted, and amounts of *Ubq10* were determined by conventional RT-PCR followed by Southern blot (A) or by the LightCycler (B). The amount of *Ubq10* PCR products as a function of polymerization cycles is shown in A. The calculated initial amount of *Ubq10* transcript is shown in B. C and D, RT-PCR controls for *Elip1* and Elip2. C, *Elip1* and *Elip2* primer pairs were used for PCR on genomic DNA and cDNA (from "Light" above) templates, and in the absence of RT (−RT). D, "Light" sample from above was used as template to determine the kinetics of PC reaction as a function of number of PCR cycles, as detected by dot blot.

Expression of *Elip* Is Mediated by Red, Far-Red, and Blue Light and by Heat Shock

To examine the regulation of *Elip* expression during photomorphgenesis in wild-type Arabidopsis seedlings, the mRNA levels of *Elip1* and *Elip2* in seedlings grown under different light qualities were determined by RT-PCR. As seen in Figure 2A, both *Elip*s are apparently not expressed, or expressed at levels below detection in 4-d-old dark-grown seedlings. Exposure of these seedlings to 1 h red, far-red, and blue light resulted in a detectable increase in transcript levels of both *Elip*s. *Elip* expression is also induced in dark-grown seedlings following heat shock at 37°C for 1 h. This indicates that *Elip* expression is induced by high irradiance response (HIR) light in etiolated seedlings exposed to 1 h light during the etioplast/chloroplast conversion, and raises the question of which photoreceptors are involved in the positive regulation of *Elip* steady-state mRNA levels.

Elip1 and *Elip2* Have Different Dark Expression Patterns

The *Elip2* cDNA was isolated from a cDNA library made from etiolated seedlings (see "Materials and Methods"). This led us to question the significance of the lack of *Elip* expression found in dark-grown seedlings (Fig. 2A; Heddad and Adamska, 2000). To further study *Elip1* and *Elip2* regulation, the experiments were repeated using the highly sensitive LightCycler method. LightCycler RT-PCR was performed with cDNA samples from 4-d-old dark-grown seedlings treated with 1 h of white light or kept in darkness. The amplification curves of this experiment are shown in Figure 2B (top). The mRNA levels of *Elip1* and *Elip2* in light-treated seedlings are similar, with fluorescence levels rising after cycle 16. However, the mRNA levels of *Elip1* and *Elip2* in dark-grown seedlings are different. The fluorescence signal of the *Elip2* transcript starts to rise after 20 cycles, whereas no *Elip1* product is detected even after 45 cycles. The melting curves analysis (Fig. 2B, bottom) of this experiment confirms that there is no *Elip1* PCR product in the dark-grown seedlings, as opposed to *Elip2* product at the same condition, or to *Elip1* and *Elip2* in the light-exposed seedlings. These data indicate that *Elip1* and *Elip2* are differentially regulated in darkness. The result further shows the advantage of using the LightCycler system in that it is highly sensitive and allows the detection of very small amounts of transcript, as compared with the conventional PCR experiments or RNA gel-blot experiments.

Phytochromes Regulate *Elip* Expression via Red and Far-Red Light

To examine the role of different phytochromes in the regulation of *Elip* expression, we used *phyA*, *phyB*, and *phyA/phyB* double mutants. Four-day-old dark-grown wild-type and mutant seedlings were kept in the dark or exposed to 1 h of red or far-red light. Total RNA was isolated and conventional PCR experiments were performed. Red and far-red lights have similar positive effects on *Elip*s in wild-type seedlings (Table I). This effect of far-red light on *Elip* mRNA was lost in the *phyA* mutant, indicating that PhyA positively regulates *Elip*s. The *phyA* mutant also showed reduced red-light induction of both *Elip*s. It is surprising that in *phyB*, the red and far-red induction of *Elip*s was not impaired. However, the absence of both phytochromes in the *phyA/phyB* mutant resulted in a loss of red and far-red induction of both *Elip*s. This suggests an essential requirement of PhyA for red-light induction of *Elip*s. Analysis with the LightCycler identified very

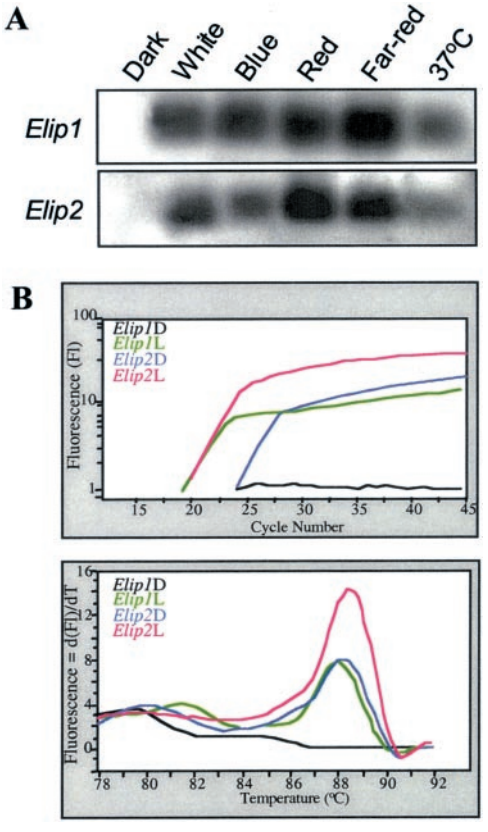

Figure 2. Effect of light on expression of *Elip*s. A, RT-PCR analysis of 4-d-old Arabidopsis dark-grown wild-type seedlings that were kept in the dark or exposed to 1 h of white, blue, red, or far-red lights, or to heat shock in the dark at 37°C. One-fifth of the PCR products were resolved in 1.7% (w/v) agarose gel and blotted onto Hybond N^+. The membrane was hybridized with DIG labeled *Elip1* and *Elip2* probes. B, RT-PCR LightCycler analysis reveals the differences between *Elip1* and *Elip2* transcript levels. Four-day-old Arabidopsis dark-grown wild-type seedlings were kept in the dark (D) or exposed to 1 h of white light (L) before total RNA extraction. One-tenth of the cDNA was amplified by the LightCycler. Top, Amplification curves of *Elip1* and *Elip2*. The diagram documents the fluorescence intensity (approximate PCR product concentration) plotted against the number of PCR cycles. Bottom, Melting curve analysis of PCR products. The diagram documents the negative derivative fluorescence plotted against temperature, where the peak therefore highlights the melting of DNA.

low levels of *Elip* transcripts in the *phyA/phyB* mutant (not shown).

Cryptochrome1, Cryptochrome2, and Phototropin Are Not Vital for the Blue-Light Induction of *Elip*

To identify the blue-light photoreceptor(s) involved in *Elip* expression, we used mutants defective in photoreceptors for blue light. Four-day-old Arabidopsis dark-grown wild type, *cry1*, *cry2*, *cry1/cry2* double mutant, *nph1*, and *phyA/phyB* double mutant seedlings were kept in the dark or exposed to 1 h of blue light before total RNA extraction. Blue light clearly results in an increase in *Elip1* levels in wild type and in the mutants (Table I), indicating that none of these photoreceptors or pairs of photoreceptors have essential roles in the transcriptional regulation of *Elip1*.

To examine the expression level of *Elip2* in these mutants, the experiments were continued using the LightCycler because conventional RT-PCR experiments yielded contradictory results (not shown). Figure 3 shows normalized initial amount of *Elip2* mRNA. Like *Elip1*, *Elip2* was induced by blue light in all mutants. However, *Elip2* transcript levels were reduced in the *cry1*, *cry2*, *cry1/cry2*, and *nph1* mutants relative to wild type, which may be indicative of a redundant function for these photoreceptors.

Downstream Regulators of *Elip* Expression

To determine the role of CSN in *Elip* regulation, RT-PCR was performed on dark-grown 4-d-old wild-type and CSN mutant *cop9* seedlings. The normalized results in Figure 4A show that although in dark-grown wild-type seedlings *Elip1* is not expressed and *Elip2* is found in very low levels, transcripts of both genes accumulate at high levels in dark-grown *cop9* seedlings. This indicates that the CSN fuctions in the repression of *Elip* expression in darkness.

To identify a potential positive regulator of *Elip* expression, we next studied the *hy5* mutant. HY5 is a light-regulated transcription factor for light-inducible genes (Oyama et al., 1997; Chattopadhyay et al., 1998). RNA was isolated from 4-d-old dark-grown *hy5* mutant seedlings that were kept in the dark or exposed to 1 h of white light. As in the wild type (Fig. 2), *Elip2* levels increase in the light in *hy5* (Fig. 4B). In contrast, *Elip1* levels do not increase in light in *hy5*. This suggests that HY5 is involved in the transcription of *Elip1* gene directly, or promotes transcription of genes that are upstream of *Elip1*. To determine if *Elip1* expression is totally silenced in *hy5*, or only in a light-dependant manner, we examined the effect of heat shock on *Elip*s in this mutant. As shown in Figure 4B, both *Elip1* and *Elip2* are induced in a light-independent fashion by heat shock in *hy5*, indicating that HY5 acts downstream in a light signal transduction pathway that positively regulates *Elip1*, but that heat shock acts through independent signaling pathways.

Microarray Analysis of *Elip1* Expression

Elip expression studies have concentrated primarily on studying *Elip* responses to singular phenomena, and until the present study, only by northern analysis. However, *Elip1* was serendipitously included in the microarray distributed through the Arabidopsis Functional Genomics Consortium. Data from the publicly available experiments can help to clarify the factors involved in *Elip* regulation and its functions in Arabidopsis. Table II presents a sum-

Table I. *Involvement of photoreceptors in the regulation of Elip1 and Elip2*

RNA was extracted from 4-d-old dark-grown seedlings that were either kept in the dark or exposed to 1 h light as indicated. RT-PCR was according to conventional procedures and quantitation was based on conventional RT-PCR analysis. The analysis for *Elip2* under blue light is shown in Figure 3. ++, RT-PCR product detected; +, some product detected (<50% of wild type); –, no RT-PCR product detected; nd, not done; w.t., wild type.

Strain	Red		Far-Red		Blue
	Elip1	*Elip2*	*Elip1*	*Elip2*	*Elip1*
w.t.	++	++	++	++	++
phyA	+	+	–	–	nd
phyB	++	++	++	++	nd
phyA/phyB	–	–	–	–	++
cry1	nd	nd	nd	nd	++
cry2	nd	nd	nd	nd	++
cry1/cry2	nd	nd	nd	nd	++
nph1	nd	nd	nd	nd	++

mary of the relevant experiments having significant results for *Elip1*. The anatomical experiments show that *Elip1* levels are higher in leaves relative to flowers, consistent with the chloroplast localization of ELIP (Kruse and Kloppstech, 1992). In addition to light, two other environmental stress situations, elevation of CO_2 and aluminum, positively effect *Elip* expression.

The phototropic stimulation experiments are compatible with our results provided above, and display a significant increase in *Elip* levels in the dark-grown wild type after exposure to 1 h blue-light illumination. In addition, the 1.03 R/G normalization ratio present in experiment number 6,619 confirms our result in Figure 2 that the Phototropin pathway has no effect on blue-light induction of *Elip1* transcription. We demonstrate this by using the phototropin photoreceptor mutant, *nph1*, whereas in the microarray experiment it was shown by analysis of *nph4*. NPH4 functions downstream of phototropin (NPH1; Harper et al., 2000).

Experiment 7,230 shows that far-red light induces *Elip1* in adult Arabidopsis plants. This information complements our result that far-red induces *Elip1* via PhyA in de-etiolation, but does not support earlier findings in green pea in which no *Elip* transcript or protein could be detected under light of 480 to 780 nm (Adamska et al., 1992a).

The Development of PSII in *hy5* Is Retarded

Because *Elip1* could not be detected during greening of etiolated *hy5* seedlings, further characterization of the *hy5* phenotype may reveal potential functions of ELIP1. ELIP was shown to play a role during the greening process (for review, see Adamska, 1997); therefore, the functional state of PSII as measured by chlorophyll fluorescence induction was studied during greening of *hy5* as compared with wild-type seedlings. Figure 5 shows the photosynthetic efficiency of 4-d-old wild-type and *hy5* dark-grown seedlings that were exposed to light for increasing periods and under different light intensities. At both 25 and 100 μmol m^{-2} s^{-1} white light, the efficiency of the photosynthetic apparatus is lower in *hy5* than in the wild type. However, the PSII efficiency values of 14-d-old light-grown wild-type and *hy5* seedlings were essentially identical, indicating that *hy5* does reach the same efficiency level as that of the wild type. These results suggest that the development of PSII activity in the mutant is temporally retarded relative to that of the wild type. The fact that this phenotype is expressed only for a limited time in the plastid development could be due to the expression of other ELIP family genes that are not affected by the mutation in *hy5*. Therefore, these results suggest that *hy5* mutant seedlings display a slower formation of PSII activity.

DISCUSSION

The work presented here addresses the question how the two *Elip* genes in Arabidopsis are regulated at the genetic level during photomorphogenesis. For this purpose, we have investigated the transcript lev-

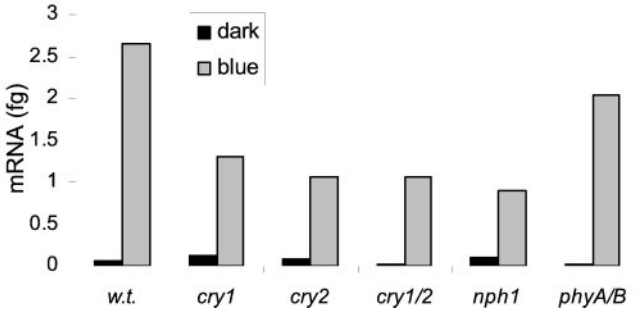

Figure 3. RT-PCR analysis of *Elip2* expression in blue-light receptor mutants. Four-day-old Arabidopsis dark-grown wild-type (w.t.), *cry1*, *cry2*, *cry1/cry2*, *nph1*, and *phyA/phyB* seedlings strains were kept in the dark or exposed to 1 h of blue light before total RNA extraction. One-tenth of the RT-reaction was amplified by the LightCycler using *Elip2* and *Ubq10* primers. The normalized initial amount of *Elip2* mRNA is shown.

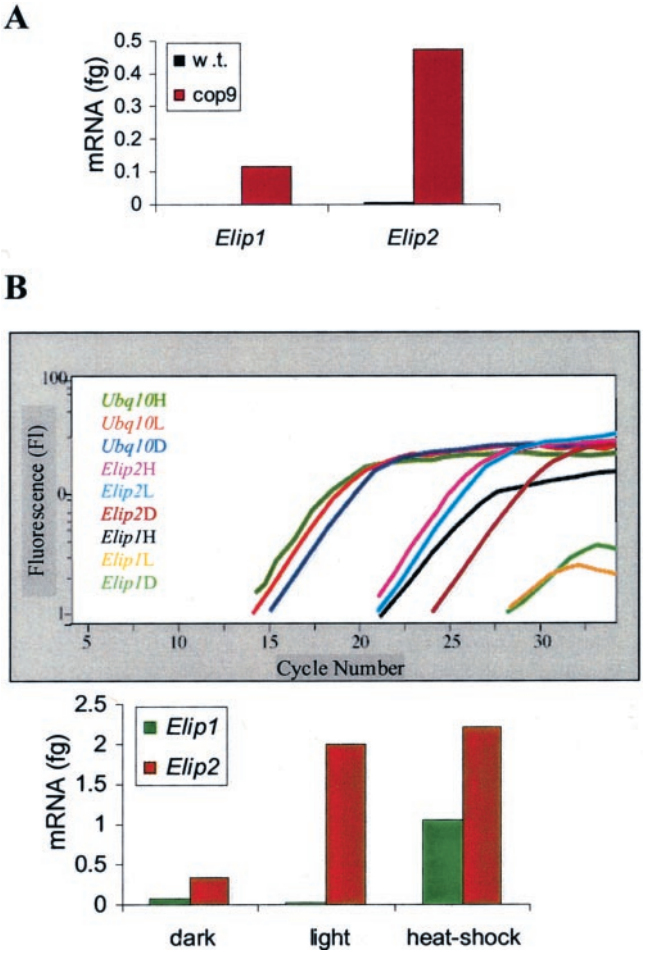

Figure 4. Analysis of *Elip* in downstream light-signaling mutants. A, RT-PCR analysis of *Elip* expression in *cop9*. RNA was isolated from 4-d-old dark-grown Arabidopsis wild-type (w.t.) and *cop9* seedlings. One-tenth of the cDNA was amplified by the LightCycler using *Elip1*, *Elip2*, and *ubq10* primers. The normalized initial amounts of *Elip1* and *Elip2* are shown. B, RT-PCR analysis of *Elip* expression in *hy5*. Four-day-old *hy5* dark-grown seedlings were kept in the dark (D) or exposed to 1 h of white light (L) or to heat shock (H) before total RNA extraction. One-tenth of the cDNA was amplified by the LightCycler. Amplification curves of *Elip1*, *Elip2*, and *Ubq10* are shown in the graph, with normalized initial amounts of *Elip1* and *Elip2* mRNA presented in the bar graph. Error bars represent SD based on three replicates of the same sample.

els of these genes under different physiological conditions and genetic backgrounds by two quantitative RT-PCR techniques. It has been shown previously that steady-state levels of *Elip* mRNA correlate to changes in transcriptional activity (Adamska, 1995). This point is further emphasized in the microarray experiment where the transcriptional inhibitor cordycepin inhibited induction of *Elip1* (Table II).

Our results that both *Elip*s can be induced in dark-grown Arabidopsis seedlings by illumination with high irradiant red, far-red, and blue lights are consistent with earlier results showing *Elip* induction in etiolated pea seedlings (Adamska et al., 1992b). The low irradiance response was not addressed here. The microarray experiments present in Table II also confirm our results for the effects of blue light, far-red light, and the phototropin pathway on *Elip1* expression. These far-red light results differ from those obtained in adult pea plants where only blue light was found to result in ELIP induction in adult plants (Adamska et al., 1992a).

The use of photoreceptor mutants provides direct evidence for the involvement of both PhyA and PhyB in *Elip* regulation. PhyA acts to induce *Elip* expression by HIR far-red light, but also has a role in perceiving HIR red light. In contrast, absence of PhyB by itself does not effect red and far-red induction of *Elip*s. In this case, PhyA and maybe the other phytochromes (PhyC–E) compensate for the lack of PhyB. However, PhyB appears to work synergistically with PhyA in controlling the red-HIR induction of *Elip*s as shown by the complete loss of red-light induction of both *Elip*s in the *phyA/phyB* double mutant. Other phenomena are also known to be under HIR control of both PhyA and PhyB in Arabidopsis, including the control of hypocotyl elongation (Quail et al., 1995; Smith 1995) and expression of chlorophyll *a/b* binding protein (*CAB*) genes (Reed et al., 1994).

The identity of the photoreceptor involved in blue-light induction of *Elip*s remains cryptic. The blue-light-induced up-regulation of *Elip*s was not silenced in *cry1/cry2* double mutants, or in the *nph1* mutants. Microarray experiment 6,619 also indicates that *Elip1* expression is not effected by at least one of the pathways regulated by the phototropin receptor. While the present manuscript was in review, a fourth blue-light receptor, NPL1, was identified (Jarillo et al., 2001; Kagawa et al., 2001). To determine if NPL1 has an essential role in mediating the blue-light induction of *Elip*s, we examined the *npl1* mutant by RT-PCR. As seen in Figure 6, both *Elip1* and *Elip2* are induced by blue-light in *npl1*. These results lead to two hypotheses: (a) The known blue-light photoreceptors have redundant functions in regulation of *Elip*s by blue light. To further test this hypothesis, *Elip* induction in an Arabidopsis quadruple mutant *cry1/cry2/nph1/npl1* would need to be analyzed. (b) A novel blue-light photoreceptor is involved in the regulation of *Elip*s. This photoreceptor would work either independently or in co-action with the four known blue-light photoreceptors. This last possibility needs further consideration because there is accumulating evidence for such a receptor. For example, the stomatal responses of light-grown *cry1*, *cry2*, *cry1/cry2*, *nph1*, and *nph1/cry1* plants did not differ from those of wild type (Lasceve et al., 1999; Eckert and Kaldenhoff, 2000). The potential role of a carotenoid derivative as a blue-light chromophore has been controversial (Palmer et al., 1996; Frechilla et al., 1999; Lasceve et al., 1999; Tlalka et al., 2001; Eckert and Kaldenhoff, 2000; Jin et al., 2001). We attempted to dissect the role of carotenoids or xanthophylls in *Elip*

Table II. *Summary of micro-array experiments with relevant Elip1 results*

Data were adapted from the Stanford Microarray Database (http://genome-www4.stanford.edu/Micro/Array/SMD). Only experiments giving reciprocal R/G results (when available as designated by two ID nos.) were chosen. R/G = 1, *Elip1* levels in the two mRNA population are equal (i.e. no induction). R/G < 1, *Elip1* is higher in the mRNA population that is marked by the green probes (G). R/G > 1, *Elip1* is higher in the mRNA population that is marked by the red probes (R). Exp. No., Experiment identification no. according to Arabidopsis Functional Genomics Consortium.

Exp. No.	Description	Experimental Conditions		R/G Normalization
		Red channel (R)	Green channel (G)	
Anatomical comparison				
11333/11375	*Elip1* is transcriptionally regulated	Untreated	Cordycepin treated	2.24
2370/2371	*Elip1* levels are higher in leaves than in flowers	Leaves	Flowers	3.91
7197/7199	*Elip1* levels are higher in leaves relative to whole plant	Leaves	Whole-plant tissue	1.26
7200/7201	*Elip1* levels are higher in flowers relative to whole plant	Whole-plant tissue	Flowers	2.05
Abiotic stress				
4649/4650	CO_2 stress positively effects *Elip1*	High CO_2	Control	1.53
3749/3743	Indole-3-acetic acid down-regulates *Elip1*	Control	Indole-3-acetic acid treated	3.37
7304/7305	Aluminum stress positively effects *Elip1*	Aluminum	Control	1.67
Light treatment				
3610	*Elip1* levels are lower in etiolated seedlings than in adult tissue	Etiolated seedlings	Leaves	0.857
6617	Blue light induces *Elip1* in etiolated seedlings	Etiolated plus blue light	Etiolated	3.925
6619	*nph4-2* does not effect blue-light induction of *Elip1* in etiolated seedlings	Wild-type etiolated seedlings exposed to blue light	*nph4-2* etiolated seedlings exposed to blue light	1.03
8266/7230	Far-red-enriched light induces *Elip1* in adult plants	Far-red-enriched light	White light	3.07

regulation through the use of the carotnoid inhibiting herbicide noflurazon (Chamovitz et al., 1991). It is unfortunate that this norflurazon treatment itself induces *Elip*s (not shown).

Both *Elip1* and *Elip2* were induced by blue light in the *phyA/phyB* double mutant. This result appears to exclude the possibility that the blue-light induction of *Elip*s is mediated by phytochrome. Other blue-light-regulated processes have also been shown to be independent of phytochrome in various systems. For example, a pea *phyA/phyB* double mutant was also recently reported to maintain normal blue-light responses, and CRY1 was shown to act independently of both PhyA and PhyB in tomato (*Lycopersicon esculentum*; Weller et al., 2001a, 2001b). However, the slight reduction in *Elip2* levels in *phyA/phyB* could indicate a possible phytochrome involvement in blue-light regulation of *Elip*s, similar to that reported for the dependence of Cry1 on phytochrome for regulating hypocotyl growth and anthocyanin accumulation (Ahmad and Cashmore, 1997).

In addition to light signals, *Elip*s are also controlled by heat shock. It was shown previously that the accumulation of *Elip* transcript in etiolated barley and pea seedlings was induced by cyclic heat shock applied for several days (Beator et al., 1992; Otto et al., 1992). However, in other experiments performed with heat-treated etiolated pea, heat shock could not induce *Elip* without involvement of short illumination (Kloppstech et al., 1991). A recent study in adult light-grown Arabidopsis plants tested the possibility that *Elip*s are induced by other environmental stresses other than light, but RNA gel-blot analysis could not detect induction of either *Elip* following heat shock (Heddad and Adamska, 2000).

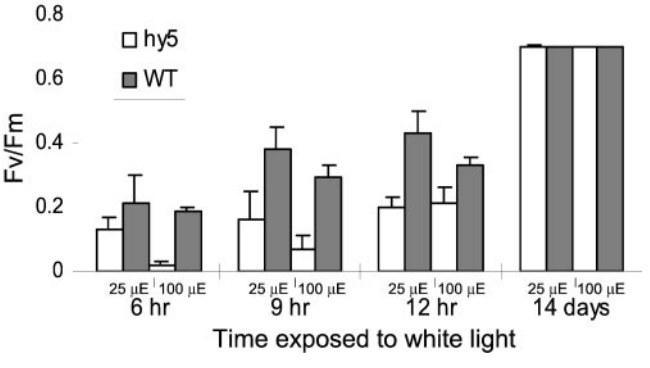

Figure 5. Development of variable chlorophyll fluorescence during greening of *hy5* and wild type. Four-day-old wild-type (WT) and *hy5* dark-grown seedlings were exposed to 25 μmol m^{-2} s^{-1} or 100 μmol m^{-2} s^{-1} white light for different periods of time. A pulse amplitude-modulated fluorimeter (PAM) was used to calculate the maximum photochemical efficiency of PSII in the dark-adapted state (F_v/F_m) parameter. Error bars represent SD based on six different measurements.

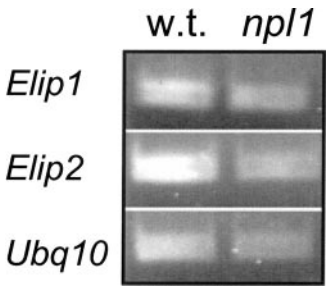

Figure 6. NPL1 is not necessary for blue-light induction of *Elip* s. Four-day-old Arabidopsis dark-grown wild-type (w.t.) and *cav1-1* (*npl-1*) seedlings strains exposed to 1 h of blue light before total RNA extraction. One-fifth of the PCR products with *Elip1*, *Elip2*, and *Ubq10* primers were resolved in 1.7% (w/v) agarose gel.

Despite the similar light and heat shock regulation patterns of *Elip1* and *Elip2*, the two genes have different accumulation patterns in the dark. *Elip2* is expressed at low levels in dark-grown seedlings, whereas no *Elip1* mRNA could be detected. This first evidence for presence of *Elip* transcript under dark conditions results from the sensitivity of the LightCycler system because no *Elip* transcript could be detected in the earlier studies performed by RNA gel blot, or in this study as shown in Figure 2, by conventional RT-PCR.

Our results demonstrate the positive role of HY5 on *Elip1* transcription during photomorphgenesis. The absence of *Elip1* mRNA in the *hy5* light-treated seedlings is not surprising because this transcription factor has been already shown to bind directly to G-box DNA sequences, well-characterized light-responsive elements in light-responsive promoters (Chattopadhyay et al., 1998). A study in transgenic pea plants had demonstrated that two light-responsive elements are involved in light-regulated expression of *Elip*. One element is similar to the GT1 binding site and the other resembles a G-box-like ACGT element (Blecken et al., 1994).

The CSN mutant, *cop9*, which mimics light growth while grown in darkness, shows high expression levels of the classic light-inducible genes such as *CAB*, *Chs*, and *PsbA* when grown in a total darkness (Wei and Deng, 1992). This study shows that the light induction of both *Elip*s is repressed in the dark by the CSN. From this result, together with the results of *hy5* experiment, it can be concluded that light signals abolish the CSN-mediated degradation of the transcription factor HY5, and thus allow it to activate directly or indirectly the transcription of *Elip1* gene. However, the reduction of *Elip2* expression in the dark must be mediated by a different mechanism because HY5 does not regulate *Elip2* expression. Based on earlier data, a working model for the signal transduction pathways that regulate *Elip* expression in Arabidopsis seedlings is suggested in Figure 7.

Our results that the dark levels of *Elip2* are higher than the dark level of *Elip1*, together with the result that HY5 is not involved in the regulation of *Elip2*, hint that the light regulation of *Elip1* occurs at the level of transcription, whereas other mechanisms may be involved in the regulation for *Elip2*. These two differences in regulation imply different function. Because *Elip1* is strictly light induced and apparently responds as a light stress protein, *Elip2* may be a "housekeeping gene" that is constantly expressed at low levels, ready to be translated under stress conditions.

The microarray data further indicate that the ELIPs are stress-related proteins. This is consistent with previous evidence that *Elip* steady-state levels are regulated by other environmental stresses. Low temperature, for example, positively regulated *Elip* transcription and stabilization (Adamska and Kloppstech, 1994). Because low temperature noticeably increases excitation accumulation of PSII, it was proposed that this cold-induction accumulation of *Elip* mRNA could be interpreted in terms of redox control of gene expression (Montane et al., 1998). In *Crateostigma plantagineum*, an ELIP-related Dsp-22 protein is induced during desiccation; in this case, abscisic acid (ABA) and light were simultaneously required for *Elip* induction (Bartels et al., 1992). In a

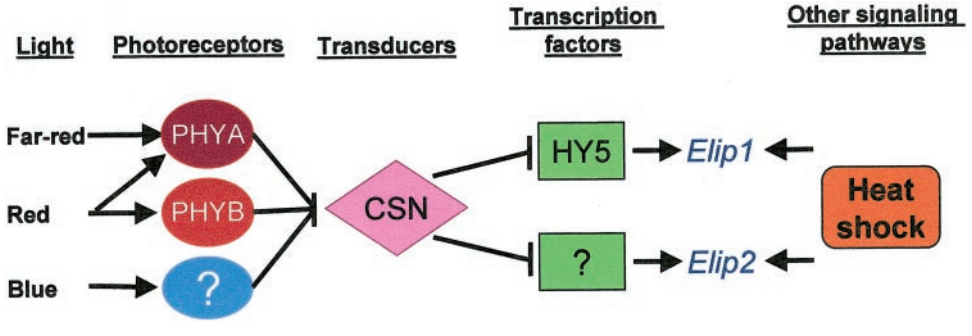

Figure 7. Working model for the signal transduction pathways that regulate the expression of the *Elip* genes in Arabidopsis seedlings. Different light qualities are sensed by at least three different photoreceptors, PhyA, PhyB, and a novel blue-light receptor, to initiate a signal transduction cascade that abolishes the repressory action of the CSN, leading to the expression of both *Elip1* and *Elip2*. The expression of *Elip1* is positively effected by HY5, whereas other transcription factors regulate *Elip2*. Heat shock stimulates the expression of *Elip* through an independent signaling pathway.

similar experiment when 6-d-old green barley plants were treated with a combination of high light and ABA, *Elip* level increased in comparison with light-treated control. However, no effect was observed with just ABA (Potter and Kloppstech, 1993). This environmental induction of *Elip1* transcription implicates additional functions that are controlled by signal transduction pathways other than those described here.

Although accumulating correlative evidence indicates that ELIPs are involved in protection of the photosynthetic apparatus, the elucidation of the physiological role of the ELIPs has been hampered by lack of a genetic system. The discovery that *hy5* lacks *Elip1* expression provides a preliminary model system to study the role of ELIP1. A defect in PSII activity displayed by *hy5* seedlings may result from the loss of *Elip1* expression in this mutant. The development of photosynthetic activity in *hy5* is retarded relative to that of the wild type. The low F_v/F_m ratio in the *hy5* seedlings seems to be due mostly to a relatively high initial (minimum) PSII fluorescence in the dark-adapted state (F_0) level. This could be due to PSII centers in which electron flow to the plastoquinone pool is partially inhibited (that is, closed PSII centers), a situation that can be induced by light stress. The fact that the F_v/F_m ratio in the mutant seedlings exposed to 100 μmol m^{-2} s^{-1}, as compared with those exposed to 20 μmol m^{-2} s^{-1}, reached lower levels supports this suggestion. However, as the time of illumination and thus of the development of the thylakoids continues, *hy5* seedlings recover from this initial light stress and the F_v/F_m ratio reaches the same values in mutant seedlings exposed to both low and high light. Following prolonged illumination, *hy5* completely recovers from the light stress and the F_v/F_m ratio is similar in the mutant to the wild type. We hypothesize that the phenotype exhibited by *hy5* during the early phase of the greening process results from a lack of *Elip1* transcription. Further study is needed to validate this hypothesis.

MATERIALS AND METHODS

Plant Materials, Growth, and Illumination Conditions

Arabidopsis seedlings were grown for 4 d on Murashhige and Skoog medium (Sigma, St. Louis) with 1% (w/v) agar, in darkness, at 22°C. Different light qualities were obtained by using a cool-white fluorescent light (100 μmol m^{-2} s^{-1}; OSRAM, Munich) with the filters (Chris James, London): blue (380–500 nm, 40 μmol m^{-2} s^{-1}), red (600–700 nm, 50 μmol m^{-2} s^{-1}), or with far-red enriched lights with a far-red filter (700–780 nm, 2 μmol m^{-2} s^{-1}). For heat shock treatment, the seedlings were incubated at 37°C in the dark. Seedlings were harvested and frozen in liquid nitrogen after 1 h of light or heat shock treatment. All subsequent manipulations were done under a green safe light.

The mutants used in this work were: *cry1-304* (Ahmad and Cashmore, 1993), *cry2-1* (Guo et al., 1998), *cry1-304/cry2-1* (Guo et al., 1998), *nph1-5* (Liscum and Briggs, 1995), *phyA* (Reed et al., 1993), *phyB* (Koornneef et al., 1980), *phyA/phyB* (Reed et al., 1993), *hy5-1* Koornneef et al., 1980), *cop9-1* (Wei and Deng, 1992), and *cav1-1* (*npl1*; Kagawa et al., 2001).

Measurement of Transcript Levels by RT-PCR

RNA Preparation and RT Reaction

Total RNA or DNA was isolated using the SV RNA isolation kit (Promega, Madison, WI) according to the manufacturer's protocol. RNA concentrations were measured using a GeneQuont spectrophotometer (Pharmacia Biotech, Uppsala), with concentration of each sample calculated from the average of six measurements. RT of total RNA was carried out using oligo(dT) as a primer. Each sample contained 1 μg of total RNA. The reaction mixture included: 500 ng of oligo(dT), 10 mM each dNTPs, 0.2 M dithiothreitol, 5× RT buffer, and 200 units of SuperScript II RT (GibcoBRL, Carlsbad, CA) in a total reaction volume of 20 μL. The reaction was incubated at 70°C for 10 min, 42°C for 50 min, and then inactivated at 70°C for 15 min.

The following primers were used for amplification by PCR: *Ubq10*, 5′-cgattactcttgaggtggag-3′ (forward) and 5′-agaccaagtgaagtgtggac-3′ (reverse); *Elip1*, 5′-gcttaaagttctgtaacctaagcg-3′ (forward) and 5′-ttaggtttcataggaggaggagg-3′ (reverse); and *Elip2*, 5′-cagtgttcgctgctccttcc-3′ (forward) and 5′-tcgatgccaacgtcaacaac-3′ (reverse). The *Elip* primers are around introns of 84 bp (*Elip1*), and 92 and 213bp (*Elip2*), yielding cDNA products of 330 bp (*Elip1*) and 400 bp (*Elip2*).

Conventional PCR Followed by Hybridization

The PCR mixture contained 0.625 μM of each oligonucleotide primer, 40 mM each dNTPs (Roche, Mannheim, Germany), 10× *Taq* polymerase buffer, 1.8 units of Supertherm DNA polymerase (Promega), and 4 μL of the RT reaction mixture (cDNA) in a total volume of 50 μL. The samples were amplified: 94°C/2 min, cycled at 94°C/1 min, 55°C/1 min, and 72°C/40 s in a PTC-100 (MJ Research, Clearwater, MN). PCR products were separated by electrophoresis through a 1.7% (w/v) agarose gel and transferred to Hybond N^+ membrane, or were loaded directly on the membrane (dot blot). The probes were labeled by random priming with "DIG High Prime" (Boehringer Mannheim) according to the manufacturer's protocol.

Membranes were prehybridized in 5× SSC (850 mM NaCl, 85 mM trisodium citrate-^2H_2O, ph 7.0), 0.1% (w/v) N-lauroylsarcosine, 0.02% (w/v) SDS, and 1% (w/v) blocking reagent for 2 h at 68°C followed by hybridization in the same solution containing denatured DIG-labeled probe at 68°C overnight. The membranes were washed to a final stringency of 0.1% (w/v) SSC and 0.1% (w/v) SDS at 68°C. Disodium 3-(4-methoxyspiro[1,2-dioxetane-3,2′-{5′-chloro}tricyclo{3.3.1.1^{3,7}}decan]4-yl) was used as a chemilumenscent detection substrate. The membranes were exposed to x-ray film to record the chemilumenscent light-signals that

were analyzed using the Scion Image software (Frederick, MD). The RT-PCR results of *Elip1* and *Elip2* were corrected according to the relative quantity of the RT-PCR product of *Ubq10* mRNA.

Real-Time PCR by LightCycler

The LightCycle System (Roche) provides simultaneous PCR amplification and product analysis. The double-stranded DNA (dsDNA) SYBR Green I stain (Wittwer et al., 1997) is included in the PCR mixture, allowing template quantification during amplification. Fluorescence is monitored once each cycle after product extension and increases above background fluorescence at a cycle number that is dependent on initial template concentration. Because this dye detects all dsDNA, including primer dimers and other undesired products, sequence confirmation for the amplified product is provided through a function termed "melting curve analysis." Melting curve analysis is performed after the amplification cycles are completed and a PCR product is formed. During this process, the temperature is slowly raised to 95°C and the fluorescence in each tube is measured every 0.2°C. As the DNA starts to denature, the SYBR Green I dye is released from the dsDNA, resulting in a decrease in fluorescence. Fluorescence data were converted into melting peaks by software that removes background fluorescence and the effect of temperature (T) on fluorescence (F), then plotted as the negative derivative of fluorescence with respect to temperature ($-dF/dT$ versus T). Each dsDNA product has is own specific melting temperature, which is defined as the temperature at which 50% becomes single stranded, and 50% remains double stranded. Because the melting curve of the products is dependent on GC content, length and sequence, specific PCR products can be distinguished from nonspecific products by their melting curves without the necessity of electrophoretic analyses. The software allows an additional step in each PCR cycle, in which the LightCycler instrument is programmed to increase the temperature before measurement. Measurement at the elevated temperature instead of measurement at the elongation temperature increases specificity. PCR product levels were recorded at the end of each cycle at 84°C, where all nonspecific products of *Elip1*, *Elip2*, and *Ubq10* primers pairs were denatured and thus not detected.

The initial amount of cDNA before the amplifiction for a particular template in the cDNA mixture was extrapolated from a standard curve with external standards. The standards run in parallel with the samples under identical PCR conditions. *Elip1* (10^{-3} to 10^{2} ng) was used as a quantification standard each experiment. This amount was corrected according to the relative amount of *Ubq10*.

The reaction mixture of the LightCycler PCR contained: 2 μL of the RT reaction mixture as a template, 4 μL of $MgCl_2$, 1× LightCycler-FastStart DNA Master SYBR Green I, and 0.5 μM each primers. The reaction condition was: 95°C/10 min (activation of the FastStart *Taq* DNA polymerase), amplification: 95°C/10 s, 62°C to 55°C/10 s, 72°C/22 s, and detection at 85°C.

Plasmids Used in This Work

The cDNA clones for *Elip1* (clone Id174P5T7) and *Elip2* (clone IdVCVCD09) were obtained through AIMS. *Elip1* was isolated from the mixed tissue cDNA library Lambda PRL2 (Newman et al., 1994). *Elip2* was isolated from a cDNA library made from 5-d-old etiolated seedlings (T. Desprez, J. Amselem, H. Chiapello, P. Rouze, M. Caboche, and H. Hofte, unpublished data).

Chlorophyll Fluorescence Measurements

Wild-type and mutant seedlings were grown on agar plates and sowed in such a way as to form clusters of several seedlings so one could measure simultaneously the fluorescence emission of at least five to seven seedlings, thus obtaining an average result. Several clusters were measured on each plate. Variable fluorescence was measured using a Pulse Modulated Fluorimeter (PAM-101, Waltz, Germany). The modulated beam (650 nm) intensity at the seedlings level was about 1 mmol m^{-2} s^{-1} at 1.5 kHz and the intensity of the saturation light pulse was 3,000 mmol m^{-2} s^{-1} for 1-s duration. The variable fluorescence, F_v, was calculated as ($F_m - F_0$). F_0 is the minimal fluorescence and was determined with the modulated beam. F_m is the maximal fluorescence and was determined with the saturation light pulse. The ratio F_v/F_m is normalized with the concentration of chlorophyll and interpreted as functional state of PSII. The plants were dark adapted for several minutes before the onset of measurements and maintained thereafter in dim-green light.

ACKNOWLEDGMENTS

We thank Dr. Blanca Shanitzki (Dyn Diagnostics, Israel) for guidance in setting up the LightCycler system; Profs. Chentao Lin (UCLA, Los Angeles), Timothy Short (Queens College, CUNY, Flushing, NY), Winslow Briggs (Carnegie Institute, Palo Alto, CA), and Kiyotaka Okada (Kyoto University, Kyoto) for providing seeds for photoreceptor mutants; Arabidopsis Biological Resource Center (Columbus, OH) for providing the cDNA clones; Dr. Nir Keren (Washington University, St. Louis) for critical reading of the manuscript; the Interdepartmental Equipment Center at the Tel Aviv University Faculty of Medicine for use of the LightCycler; and Shirley Kadouri for assistance in the early stages. This work was carried out as the Master of Science thesis of O.H.-S.

Received March 19, 2001; returned for revision June 26, 2001; accepted July 31, 2001.

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