# *Arabidopsis* transcriptional regulation by light stress via hydrogen peroxide-dependent and -independent pathways

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## Abstract

***Background*: High (intense) light stress causes the formation of oxygen radicals in chloroplasts and has the potential to damage them. However, plants are able to respond to this stress and protect the chloroplasts by various means, including transcriptional regulation in the nucleus. Although the corresponding signalling pathway is largely unknown, the high light response in the expression of the *Arabidopsis APX2* gene is reported to be mediated by hydrogen peroxide.**

***Results*: We characterized light stress signalling by analysing expression profiles of another high light-inducible gene of *Arabidopsis*, *ELIP2*, with the aid of an *ELIP2* promoter-luciferase gene fusion. The established *ELIP2::LUC* transgenic *Arabidopsis* showed activation by high light, but not by hydrogen peroxide. On the other hand, the native *ELIP2* gene as well as the *APX2* gene was activated by the hydrogen peroxide. The activation of *ELIP2::LUC* by intense light was not inhibited by K252a but by okadaic acid.**

***Conclusion*: The light stress signalling from the chloroplast to the nucleus is revealed to be mediated through at least two pathways: both hydrogen peroxide-dependent and –independent. The latter pathway is thought to be mediated by the protein phosphatase 2A/1 activity that is suppressed by okadaic acid.**

## Introduction

The primary step in photosynthesis is the capture of light energy by chlorophylls in the chloroplasts. The absorbed light energy is utilized to excite electrons in the pigments, transforming the light energy into electrochemical energy. The excited energy is then transferred to the reaction centres of the photosystems, and flows into the electron transport system in the thylakoid membrane according to the redox potential. However, when the photosynthetic apparatus is irradiated with an unmanageable amount of light, which often occurs under natural conditions, some of electrons leak from the excited chlorophylls, resulting in the generation of oxygen and lipid radicals. These radicals are also generated by the overflow of electrons from the electron transport system (Asada 1996; Niyogi 1999). They damage proteins, lipids, pigments, DNA and all other chloroplast components (Asada 1996). In extreme conditions, such as in plants sensitized to high (intense) light by carotenoid depletion, this random activity of the radicals can lead to the loss of all recognizable internal structures of the chloroplast and ultimately to its destruction (Yamamoto *et al.* 2000). However, plants have developed several strategies to protect the chloroplast from high light. These include protection of the reaction centres by a reduction of antenna size, activation of the light energy dissipation system by changing carotenoid composition (xanthophyll cycle), development of radical scavengers, and activation of repair and new synthesis in the photosystems (Asada 1996; Niyogi 1999). A lack of some of these responses caused by genetic mutations or by inhibitor treatments actually sensitizes the plant to high light (Niyogi 1999).

High light treatment causes an alteration in gene expression in the nucleus. While genes encoding chlorophyll *a/b*-binding light harvesting proteins are shut down by high light (Oelmüller 1989; Taylor 1989), stress-related genes, including genes for oxygen radical scavengers, are activated (Karpinski *et al.* 1997). Analysis of the expression of an *Arabidopsis* ascorbate

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peroxidase gene, *APX2*, revealed that the high light activation of *APX2* expression is mediated by hydrogen peroxide, a derivative of oxygen radicals (Karpinski *et al.* 1999). This work revealed one example of high light signalling. However, besides this pioneering work by Karpinski *et al.* (1999), the nature of the high light signalling pathway is largely unknown. For example, the receptor of hydrogen peroxide, as well as the mediators of the signal for gene expression of *APX2*, has not been identified. Furthermore, it is not known if all the high light responsive genes are controlled in the same manner as *APX2*.

Early Light Inducible Protein (ELIP) was first identified as a protein that was transiently induced in the very early stages of the greening process in etiolated pea seedlings (a 24 000-$M_r$ precursor protein (Meyer & Kloppstech 1984)). ELIP is a stress-related protein and belongs to the *CAB* superfamily (Adamska 1997; Jansson 1999). *ELIP* is found in a wide range of organisms, from photosynthetic bacteria to higher plants (Adamska 1997). The protein product of *ELIP* locates in the thylakoid membrane (Grimm & Kloppstech 1987; Meyer & Kloppstech 1984), and has recently been reported to bind to chlorophylls with low affinity (Adamska *et al.* 1999). Expression of *ELIP* is induced by high light stress in a wide range of organisms, and in some plants other stresses also activate its expression (Adamska 1997). Although the precise function of the *ELIP* product is not well understood, a closely related protein to ELIP, PsbS, was found to be necessary for nonphotochemical quenching to dissipate the excess light energy absorbed by chlorophylls (Li *et al.* 2000). Other *ELIP*-related genes in *Synechocystis* PCC6803, *HliA-D*, have recently been reported to be necessary for growth under intense light conditions (He *et al.* 2001). These functions fit with the high light-inducible nature of their gene expression.

Because *ELIP* expression is induced by high light, we have decided to focus on the analysis of *ELIP* expression in order to elucidate the molecular machinery of high light signal transduction in *Arabidopsis*. Utilization of an *ELIP* promoter-luciferase reporter fusion allowed the separation of the transcriptional regulation driven by the utilized promoter from the complex regulation of *ELIP2* expression that was found. Our initial characterization of the high light signalling using the reporter system revealed the presence of multiple pathways for high light signalling from the chloroplasts to the nucleus, one of which was mediated by protein phosphatases 2A and/or 1.

## Results

### Establishment of *ELIP2::LUC* transgenic *Arabidopsis*

There are two *Arabidopsis ELIP* genes, *ELIP1* and *ELIP2* (Heddad & Adamska 2000; Moscovici-Kadouri & Chamovitz 1997). At the time of our database search, only *ELIP2* on the ATFCA1 contig (GenBank accession no. Z97336) had been sequenced completely by the *Arabidopsis* genome-sequencing project. Therefore, we decided to clone the promoter region of this gene. The promoter region of *ELIP2*, −1907 to −2 bp relative to the translation start site, was fused to the firefly luciferase reporter gene (Millar *et al.* 1992) and introduced into *Arabidopsis* plants by *Agrobacterium*-mediated stable transformation. Treatment of the prepared $T_2$ seedlings with strong light (400 W/m$^2$) for 3 h resulted in a 10–100-fold induction of the *in vivo* luciferase activity of all of the six lines examined. There was a variation in the basal expression levels (data not shown). One line containing the T-DNA at a single locus, YA210-62, which will be referred as the *ELIP2::LUC* line in this report, was used to establish $T_3$ homozygous lines for further analysis.

### High light response of *ELIP2::LUC* expression

Figure 1 shows the spectrum of the high light used in this study. In order to avoid the effects of UV and heat, light of wavelength shorter than 400 nm and longer than 700 nm was removed with glass filters. Using such a light source, the effect of strong light on *ELIP2::LUC* was analysed. As shown in Fig. 2A, treatment for 3 h with strong light (150 W/m$^2$) activated the expression of *ELIP2::LUC*, whereas luciferase genes under the

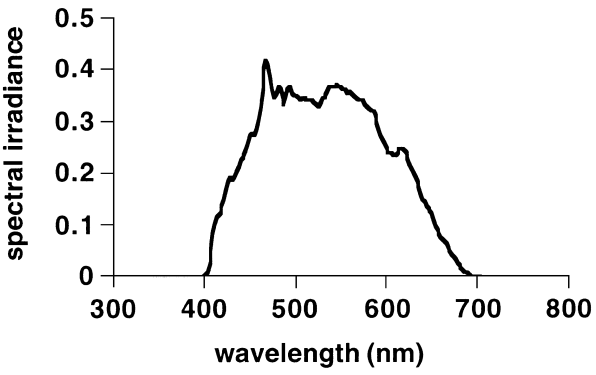


**Figure 1** Radiation spectrum of the high light used in this study.

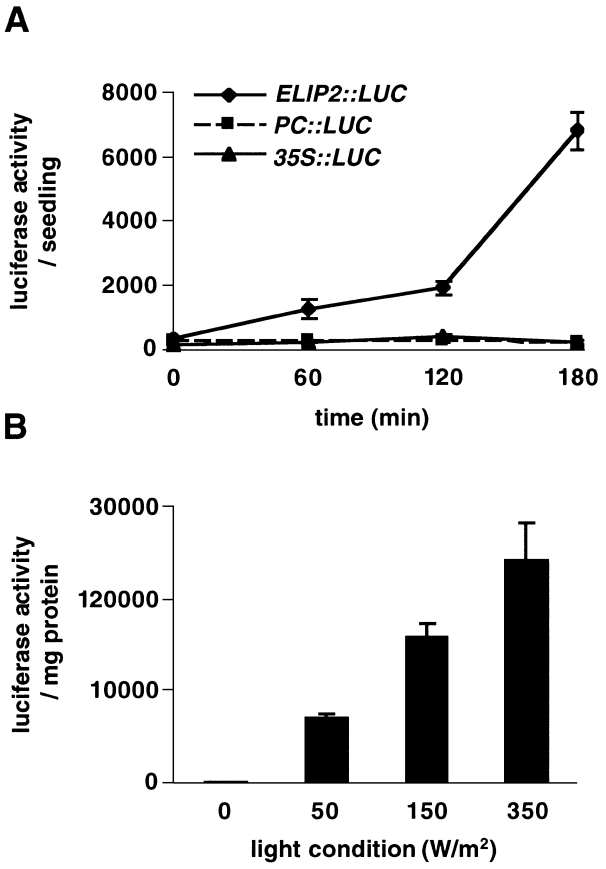


**Figure 2** (A) Time course of high light induction of *ELIP2::LUC* expression. *In vivo* luciferase activity after treatment with high light (150 W/m$^2$) for the indicated time periods. For the control constructs, results of plastocyanin promoter-luciferase fusion (*PC::LUC*) and a constitutive 35S promoter-luciferase fusion (*35S::LUC*) are also shown. (B) Dose response of *ELIP2::LUC* expression. Seedlings grown under low light (6 W/m$^2$) were treated for 3 h with the indicated intensity of light and subjected to *in vitro* luciferase assay. Averages and standard deviations are shown.

control of either the plastocyanin promoter [*PC::LUC* (Dijkwel *et al.* 1996)] or the CaMV 35S promoter [*35S::LUC* (Dijkwel *et al.* 1996)] were not affected by the same treatment. Therefore, activation of *ELIP2::LUC* expression by high light should represent the activity of the *ELIP2* promoter. This analysis revealed that high light activation of *ELIP2* expression (Heddad & Adamska 2000) is controlled by transcriptional regulation acting at the promoter region in the *ELIP2::LUC*. Figure 2B shows that an increase in light intensity up to 350 W/m$^2$, resulted in an increase in reporter gene activity, revealing light dose-dependence of *ELIP2::LUC* expression.

## Response of the endogenous ELIP2 gene to high light

Quantitative RT-PCR analysis was carried out to show that the *ELIP2::LUC* expression pattern reflected that of the endogenous *ELIP2* gene (Fig. 3). The time course of *ELIP2* accumulation (Fig. 3A) was similar to that of *ELIP2::LUC* expression (Fig. 2A). Therefore, *ELIP2::LUC* is a good representation of the expression of the internal *ELIP2* gene. In the case of the light-dose-response, an increase of light intensity up to

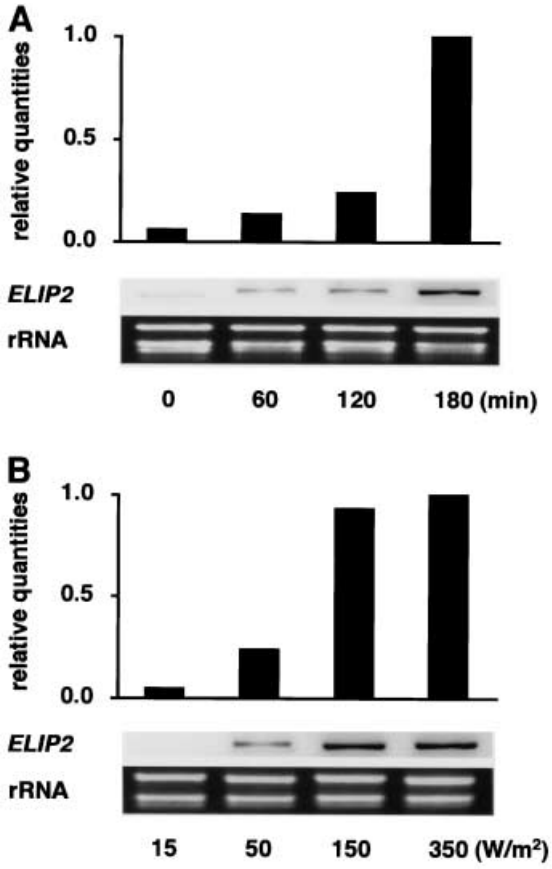


**Figure 3** Quantitative RT-PCR analysis of the authentic *ELIP2* gene. Fluorescence image of Vistra Green staining of the RT-PCR products after 27 cycles (*ELIP2*) and EtBr stained image of the RNA template (rRNA) used for the assays. Also shown are the quantitative result of the RT-PCR products after 23, 25 and 27 cycles with the aid of standard curves (graph). (A) Wild-type seedlings were treated with strong light (150 W/m$^2$) for the indicated periods and mRNA accumulation of *ELIP2* was quantified. (B) Wild-type seedlings were treated for 3 h with strong light at the intensity indicated and the mRNA accumulation was quantified. Both results shown here were confirmed by Northern analysis (data not shown).

350 W/m$^2$ led to an increase of *ELIP2* mRNA accumulation, which was also seen in the *ELIP2::LUC* expression. Although the effect of increasing the light intensity from 50 W/m$^2$ to 150 W/m$^2$ appeared to be more pronounced in the accumulation of mRNA than in the expression of the reporter expression, the overall expression of *ELIP2::LUC* accurately represents the expression of the endogenous *ELIP2* gene.

## Effect of other stresses on *ELIP2::LUC* expression

Although the source of light used for the strong light treatment was depleted of wavelengths higher than 700 nm, which are the major source of heat, it is not theoretically possible to completely remove the effect of heat and the resultant dehydration. To eliminate the possibility of these being involved in the activation of *ELIP2::LUC* expression, we directly examined the effect of heat and dehydration on its expression. As shown in Table 1, drought stress did not affect the expression of *ELIP2::LUC*, nor that of *PC::LUC* or *35S::LUC*. Heat stress, however, caused a reduction in *ELIP2::LUC* expression, but this was not observed in *PC::LUC* or *35S::LUC*. These results show that the activation of *ELIP2::LUC* by the high light treatment is not a result of accompanying heat or drought stress.

## Effect of hydrogen peroxide on ELIP2 expression

High light stress causes the activation of a set of genes involved in oxygen radical scavenging (Karpinski *et al.* 1997). Among these genes is the *Arabidopsis* ascorbate peroxidase gene (*APX2*), and expression profiles of this gene were studied in further detail. The high light response of *APX2* was found to be mediated by hydrogen peroxide, which is produced by the photo-oxidation induced by high light (Karpinski *et al.* 1999). Next we examined effect of hydrogen peroxide on *ELIP2* expression by RT-PCR (Fig. 4A). As shown in Fig. 4B, 2 h after the treatment *APX2* was partially activated by hydrogen peroxide (Fig. 4B, $-H_2O_2$-HL vs. $+H_2O_2$-HL) and the response to brief high light treatment for 30 min was enhanced by hydrogen peroxide ($-H_2O_2$+HL vs. $+H_2O_2$+HL). This is

**Table 1** Response of *ELIP2::LUC* to drought and heat stresses. Averages and standard deviations of *in vivo* luciferase assays expressed in luciferase activity/seedling are shown

| | No treatment | Drought | Heat |
|---|---|---|---|
| *ELIP2::LUC* | 398.5 ± 37.2 | 342.5 ± 32.7 | 87.8 ± 14.9 |
| *PC::LUC* | 337.5 ± 37.1 | 390.6 ± 50.6 | 354.5 ± 36.0 |
| *35S::LUC* | 184.8 ± 39.7 | 275.4 ± 41.0 | 227.4 ± 29.2 |

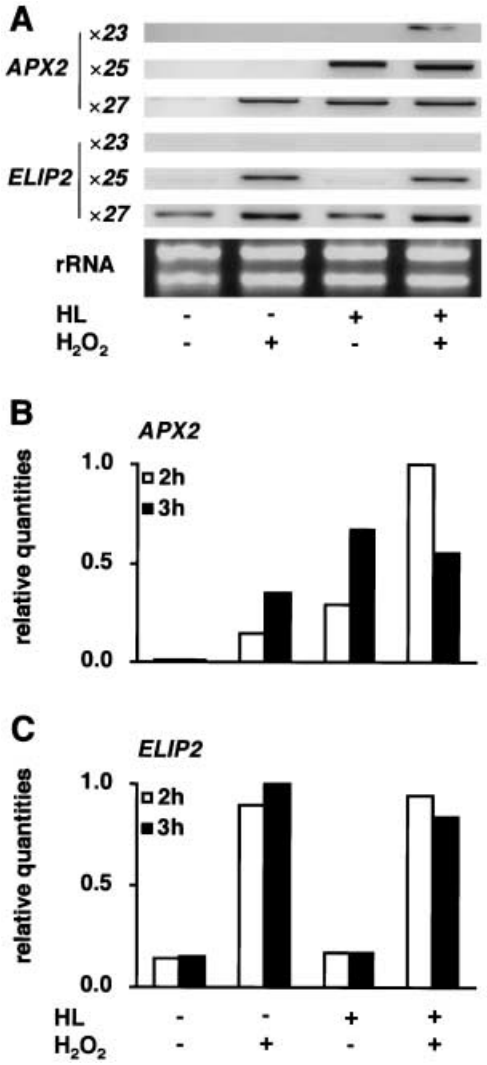


**Figure 4** Activation of *ELIP2* expression by hydrogen peroxide. Results of a quantitative RT-PCR are shown. Wild-type seedlings were treated with hydrogen peroxide ($H_2O_2$+) or water ($H_2O_2$−) in combination with brief strong light treatment for 30 min (HL+). (A) Response after 2 h. Fluorescence image of Vistra Green staining of the RT-PCR products (*APX2* and *ELIP2*) after 23, 25 and 27 cycles and EtBr stained image of the RNA template (rRNA) used for the assays. (B) and (C) Quantitative results of the RT-PCR products (*APX2* and *ELIP2*) with the aid of standard curves. Responses of 2 h and 3 h after the treatment are shown.

consistent with a previous report (Karpinski *et al.* 1999). At 3 h the response to hydrogen peroxide alone was developed further, but the response disappeared under brief high light treatment (Fig. 4B). Analysis of *ELIP2* expression revealed that it was also activated by hydrogen peroxide (Fig. 4C). Brief high light treatment did not affect its expression. This analysis revealed that the expression of *APX2* and *ELIP2* are controlled by a shared machinery, in which hydrogen peroxide plays a role in the signal transduction. The *APX2::LUC* fusion gene is also reported to be activated by hydrogen peroxide treatment (Karpinski *et al.* 1999).

When we tried to confirm the activation by hydrogen peroxide using *ELIP2::LUC*, we were surprised to find that it did not activate *ELIP2::LUC* expression, whereas *APX2::LUC* showed activation by the same treatment (Fig. 5). This null response of *ELIP2::LUC* to hydrogen peroxide was reproducibly observed in *in vitro* luciferase analyses, as shown in Fig. 5 (data not shown), as well as in *in vivo* analyses (data not shown). Therefore, we concluded that the *ELIP2* promoter used in this study does not respond to the hydrogen peroxide signal, although the same promoter does respond to the high light signal (Fig. 2). In conclusion, our functional analysis of the *ELIP2* promoter revealed a novel, hydrogen peroxide-independent pathway for high light response.

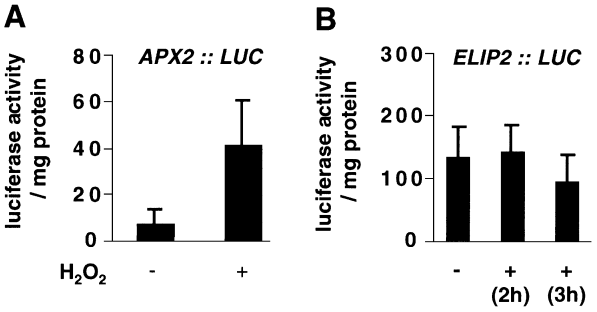


**Figure 5** No activation of *ELIP2::LUC* expression by hydrogen peroxide. (A) *APX2::LUC* transgenic line was treated with water ($H_2O_2^-$) or hydrogen peroxide ($H_2O_2^+$), incubated for 2 h, and harvested for *in vitro* luciferase assay. (B) *ELIP2::LUC* line was subjected to the same assay. Incubation time after hydrogen peroxide was 2 h and 3 h as indicated in the Figure. Averages of the activities and the corresponding standard deviations are shown.

## Analysis of the hydrogen peroxide-independent pathway using *ELIP2::LUC*

We further characterized the signalling pathway for *ELIP2::LUC* activation by high light, which is a hydrogen peroxide-independent pathway. We decided to analyse the effect of protein phosphorylation. K252a is a broad range inhibitor of protein kinases (Hidaka & Kobayashi 1992). When *ELIP2::LUC* was treated with 100 nM K252a, the activation by high light was not affected (Fig. 6A).Treatment with K252a at a higher concentration (300 nM) arrested growth just after germination (data not shown). As *PC::LUC* expression was activated by K252a (data not shown), it was apparent that the treatment was enough to introduce K252a into plant cells. We then examined the involvement of protein phosphatases in high light signalling. Okadaic acid is a specific inhibitor of protein phosphatase 2A and 1, and inhibits the type 2A with 100-fold higher sensitivity than the type 1 (Cohen 1989). As shown in Fig. 6A, treatment with okadaic acid cancelled the high light activation of *ELIP2::LUC*. Because its effect on the *35S::LUC* expression was negligible, okadaic acid does not generally reduce the luciferase reporter activity, but the effect is high light signalling-specific. This analysis revealed that the high light signalling to *ELIP2::LUC* expression is mediated by protein phosphatase 2A/1 activity which is inhibited by okadaic acid. Interestingly, addition of K252a to okadaic acid-treated *ELIP2::LUC* plants partially suppressed the inhibition of okadaic acid on the high light

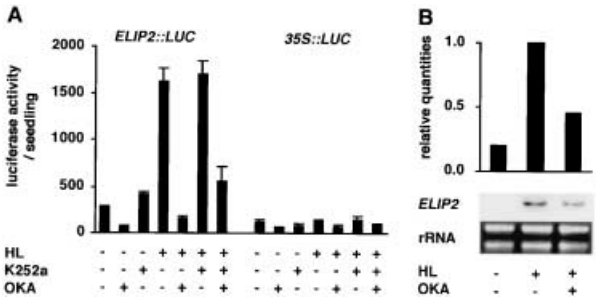


**Figure 6** Effect of okadaic acid and K252a on the high light response of *ELIP2::LUC* expression. (A) Response of *ELIP2::LUC*. Seedlings were treated with K252a and/or okadaic acid (OKA), and the response to high light was monitored by *in vivo* luciferase assays. Averages and standard deviations are shown. (B) Response of the internal *ELIP2* gene. Wild-type seedlings were treated with okadaic acid (OKA) and the response to high light was determined by quantitative RT-PCR. Fluorescence image of Vistra Green staining of the RT-PCR products after 23 cycles (*ELIP2*) and EtBr stained image of the RNA template (rRNA) used for the assays are shown. Also shown are the quantitative results of the RT-PCR products after 23, 25 and 27 cycles with the aid of standard curves (graph).

response. As K252a inhibits protein phosphorylation, this result is consistent with the finding of dephosphorylation-mediated positive signalling for *ELIP2::LUC* activation.

Figure 6B shows the effect of okadaic acid on internal *ELIP2* expression as determined by RT-PCR. The figure shows that high light-induced accumulation of the *ELIP2* transcript was also inhibited by okadaic acid. This analysis indicates that protein phosphatase-mediated transcriptional regulation is actually reflected in the accumulation profile of the native *ELIP2* transcript.

### Effect of chloroplast destruction on *ELIP2::LUC* expression

There are two possibilities for how high light affects the transcriptional regulation of genes. The first possibility is recognition by an high light sensor that is activated independently from the light stress in the chloroplast. The second possibility is that the light stress itself triggers the high light signalling. In order to separate these two possibilities, we induced damage to chloroplasts by feeding inhibitors instead of treating with strong light. Norflurazon is an inhibitor of carotenoid biosynthesis and its target enzyme is phytoene desaturase (Chamovitz *et al.* 1991). Norflurazon-treated plants, depleted of carotenoids, are highly sensitized to light and easily undergo photo-oxidative damage in their chloroplasts under normal light conditions (Oelmüller 1989). As shown in Fig. 7A, treatment with norflurazon activated *ELIP2::LUC* expression, whereas the effect on *PC::LUC* and *35S::LUC* was negligible. The light-dependent nature of *ELIP2::LUC* activation by norflurazon (Fig. 7A) strongly suggests that the target site of norflurazon in this assay should be the chloroplasts. Analysis of *ELIP2* mRNA by Northern hybridization revealed the activation of *ELIP2* gene expression by norflurazon treatment (data not shown), which is consistent with the response of *ELIP2::LUC*. Tagetin is a plastid-specific inhibitor of RNA polymerase (Kapoor *et al.* 1997). Similar to norflurazon, Tagetin also specifically activated *ELIP2::LUC* expression (Fig. 7B). Therefore, *ELIP2* was found to be activated by chloroplast destruction without treatment with strong light. These results suggest that the high light signalling to *ELIP2* is triggered by light stress itself in the chloroplasts and not by an independent photoreceptor.

When the characteristics of the signalling to *ELIP2::LUC* induced by norflurazon-activated chloroplast destruction were compared with those of the high light signalling seen in the pharmacological analysis shown in Fig. 6, we found no difference between the two

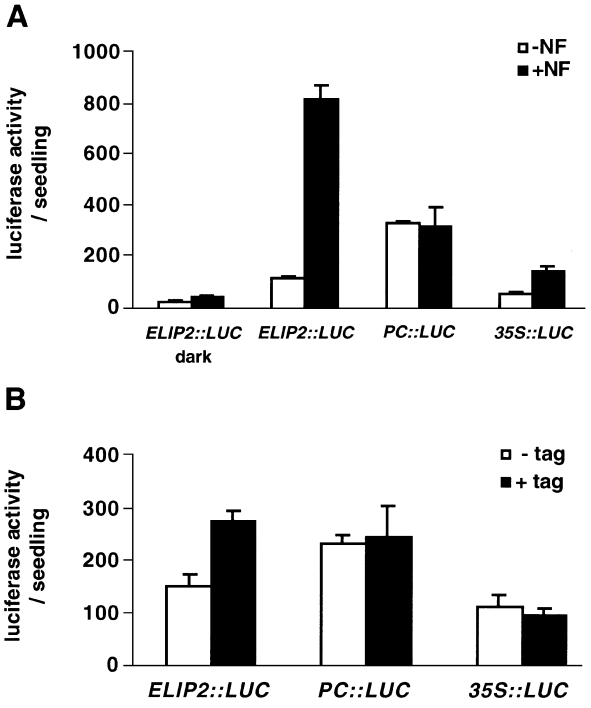


**Figure 7** Effect of chloroplast destruction on *ELIP2::LUC* expression. Unless otherwise mentioned, the seedlings were grown under continuous low light condition (6 $W/m^2$) or in the dark. Response of *ELIP2::LUC* to norflurazon (A, NF) and Tagetin (B, tag) was determined by *in vivo* luciferase assays. Destruction of chloroplasts by norflurazon (NF), a carotenoid biosynthesis inhibitor, and Tagetin (tag), a chloroplast-specific inhibitor of RNA polymerase, resulted in significant activation of *ELIP2::LUC*, while *PC::LUC* or *35S::LUC* expression were not affected by the same treatments.

experimental systems. As shown in Fig. 8, the norflurazon activated *ELIP2::LUC* expression (Fig. 8, −NF−K252a−OKA vs. +NF−K252a−OKA), and okadaic acid inhibited the activation (Fig. 8, +NF−K252a−OKA vs. +NF−K252a+OKA). K252a did not inhibit the NF-activation (+NF−K252a−OKA vs. +NF+K252a−OKA), but rather compensated for the effect of okadaic acid (+NF−K252a+OKA vs. +NF+K252a+OKA). These results revealed the characteristics of norflurazon-activation of *ELIP2::LUC* are the same as those of the high light-activation as shown in Fig. 6. This analysis confirms the idea that the norflurazon treatment activates the high light signalling.

## Discussion

In this report, we have established an *ELIP2* promoter-luciferase reporter system in *Arabidopsis* in order to

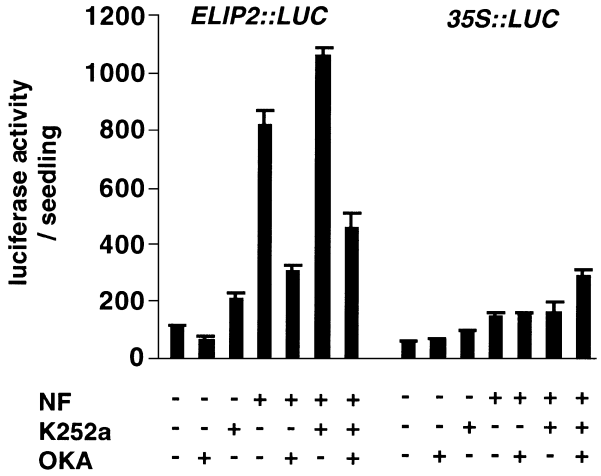


**Figure 8** Suppression of signalling of norflurazon-dependent chloroplast destruction to *ELIP2::LUC* by okadaic acid. Seedlings were treated with K252a and/or okadaic acid (OKA), and the response to norflurazon (NF) were determined by *in vivo* luciferase. The response to norflurazon was inhibited by okadaic acid, as in the case of intense light response.

analyse transcriptional regulation in the nucleus by high light signalling. The specific activation of luciferase activity by high light stress allows us to critically dissect the high light signalling.

## Specific activation of *Arabidopsis ELIP2* expression by high light

*ELIP*, a subfamily of the chlorophyll *a/b*-binding protein superfamily, is present in higher plants as well as in photosynthetic bacteria (Adamska 1997; Jansson 1999). Although *ELIP* expression seems to be induced by high light treatment in most species (Adamska 1997), in some plants the *ELIP* gene is activated by other stresses, such as UV (Adamska *et al.* 1992), heat shock (Beator *et al.* 1992), drought (Bartels *et al.* 1992), or cold stress (Shimosaka *et al.* 1999). A detailed examination of *ELIP2::LUC* expression revealed that the activation of *ELIP2* expression by high light is not due to a secondary effect of the treatment, such as heat or drought, for the following reasons. Firstly, *ELIP2::LUC* was not activated by heat or drought stresses (Table 2). Our results are consistent with the expression profile of *ELIP2* (Heddad & Adamska 2000). Secondly, when treated with norflurazon to induce high light-sensitized conditions, the response was observed under low light conditions that should be free from heat and drought stresses (Fig. 7). Taking these results into consideration, it can be concluded that *ELIP2* expression is activated by the high light stress associated with chlorophyll destabilization, and is not due to other secondary stresses. Heddad & Adamska (2000) also report that *ELIP2* is not activated by cold stress or wounding, which is consistent with our observation of *ELIP2::LUC* expression (data not shown). Therefore, activation of the *Arabidopsis ELIP2* appears to be extremely high light stress-specific. *ELIP* expression is also reported to be regulated by circadian rhythm (Kloppstech 1985). However, *ELIP2::LUC* did not show any circadian oscillation in a preliminary experiment (T. Kondo, personal communication).

Non-treated seedlings with high light express some *ELIP2* mRNA (Fig. 4A, HL-, $H_2O_2$-). This is also true in *ELIP2::LUC* expression (e.g. Table 1). The spatial and temporal expression profiles of *ELIP2::LUC* during development under normal conditions are under investigation (Y.Y. Yamamoto, M. Kimura & M. Matsui, unpublished results).

## *ELIP2* expression and *ELIP2::LUC* expression

Isolation and analysis of the functional *ELIP2* promoter was used to dissect high light signal transduction and revealed two independent pathways: hydrogen peroxide-dependent and -independent. Both signalling pathways control *ELIP2* expression. However, the promoter is not involved in the response to the former pathway and therefore the corresponding *cis*-regulatory elements for the hydrogen peroxide-dependent pathway should locate downstream of the promoter in either the transcribed region or in the 3′ nontranscribed region. These data suggest either the presence of a hydrogen peroxide-responsive enhancer at the 3′ nontranscribed region or regulation of the *ELIP2* mRNA stability by hydrogen peroxide. Although there are reports of post-translational regulation of *ELIP* (Adamska *et al.* 1993, 1996), control of mRNA stability of *ELIP* is not known. Further study would be necessary to address this possibility.

## The signal transduction for the high light stress response

Figure 9 summarizes the signal transduction pathways for the high light response. Irradiation of leaves with high light results in photo-oxydation caused by electron leaking from excited chlorophylls ($^1$Chl), which leads to accidental $O_2^-$ production in the chloroplasts (Niyogi 1999). Photo-oxidation is also triggered

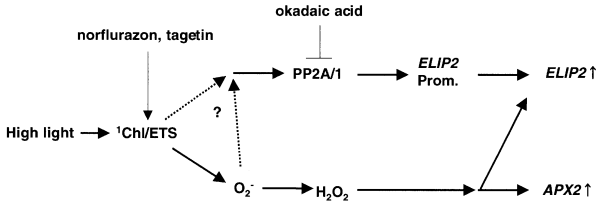


**Figure 9** High light signalling of *Arabidopsis*. High light treatment causes chlorophylls ($^1$Chl) to become excited and out of control. As a result, the excited electrons of chlorophylls or electrons from the electron transport system (ETS in the Figure) is nonenzymatically transferred to oxygen molecules which results in the production of oxygen radicals, including $O_2^-$. An oxygen radical scavenger, superoxide dismutase, which is not shown in the figure, transforms $O_2^-$ into hydrogen peroxide ($H_2O_2$), and which is metabolized further by peroxidases and catalases. At the same time, the accumulation of $H_2O_2$ triggers a signal for the activation of *APX2* and *ELIP2* expression. In addition, *ELIP2* activation by strong light is transduced by a hydrogen peroxide-independent pathway which is mediated by protein phosphatase(s) 2A/1. The *ELIP2* promoter receives the signal only from the latter (upper) pathway.

by norflurazon, and possibly, Tagetin (Oelmüller 1989). $O_2^-$ is then enzymatically catalysed by a set of oxygen radical scavengers and an intermediate molecule, hydrogen peroxide, mediates in the activation of *ELIP2* and *APX2* expression (Karpinski *et al.* 1999). Furthermore, *ELIP2* is also activated by the hydrogen peroxide-independent pathway through transcriptional regulation (*ELIP2* Prom. in Fig. 9). This branching of the signalling is further supported by the fact that *ELIP2::LUC* activation by high light is cell-autonomous (Y.Y. Yamamoto, M. Kimura & M. Matsui, unpublished results) while *APX2::LUC* activation, which is hydrogen peroxide-dependent, is reported to be mediated by cell-to-cell communication (Karpinski *et al.* 1999). Because hydrogen peroxide is also a signal for the plant defence response (Bolwell 1999), there could be crosstalk between the high light response and the defence response. The pathway for *ELIP2* promoter activation is mediated by protein phosphatase type 2A and/or 1 (PP2A/1), which are inhibited by okadaic acid (Figs 5 and 7). This is the first report to show the involvement of a protein phosphatase for transcriptional regulation of nuclear genes mediated by strong light. PP2A/1 activity is also known to be necessary for the gene expression of maize rbcS and C4ppdk1 genes, which are activated by light (Sheen 1993). Therefore, *Arabidopsis* light signalling is also expected to include PP2A/1 activity as an essential component. The relationship between light signalling and high light signalling is not clear. Because PP2A as well as PP1 constitute multigene families in the *Arabidopsis* genome, it would seem likely that these two signallings are mediated through distinct PP2A/1 proteins. The completely sequenced *Arabidopsis* genome (The Arabidopsis Genome Initiative, 2001) contains 21 genes for PP2A, and 12 genes for PP1 (Y.Y. Yamamoto & K. Kimura, unpublished results). Reverse genetic analysis would be necessary in order to specify which gene copies are involved in these signalling pathways.

### Application of *ELIP2::LUC Arabidopsis*

This system provides a powerful tool for the molecular genetic analysis of the high light signalling pathway because the luciferase reporter gene monitors the response in a nondestructive manner. The specific response of *ELIP2::LUC* allows a pinpoint analysis of the intense light signalling. The *ELIP2::LUC* line has been mutagenized by introducing activation tagging T-DNAs (Hayashi *et al.* 1992) and the *En-I* transposon (Arts *et al.* 1995), as well as by EMS mutagenesis. Genetic screening of these mutagenized lines has allowed the isolation of a number of mutants that have altered expression profiles of *ELIP2::LUC* (M. Kimura & Y.Y. Yamamoto, unpublished results). A molecular genetic analysis using these mutants would be useful for understanding the hydrogen peroxide-independent light stress signalling pathway.

## Experimental procedures

### Light source

For strong light production, light from a 1000 W xenon lamp (Ushio, Tokyo) was filtered through a cold glass filter and a condenser lens. Illumination area and direction was controlled with a zoom lens and a mirror. Light flux and the spectrum were measured with a light-meter (LI1800; LI-COR Inc.), respectively. The xenon lamp was turned on for at least 30 min before starting experiments to stabilize its output. The light flux was measured each time and adjusted appropriately by tuning the zoom lens and/or placing white copy paper(s) on the plates to reduce the light intensity.

### Plant growth and high light treatment

Seeds of *Arabidopsis thaliana* were surface sterilized and plated on GM medium (Valvekens *et al.* 1988) supplemented with 1.0% sucrose and 0.8% Bactoagar (Difco, Detroit). After 2–5 days of vernalization, the plates were transferred to a growth chamber and grown for 8 days at 22 °C under continuous low light

 

conditions (6 W/m$^2$). Just before the treatment with strong light, seedlings were sprayed with sterile water. During the intense light treatment, plates were covered with a sheet of cellophane to avoid desiccation of the seedlings. The treatment was carried out at 22 °C and, during the treatment, the temperature of the irradiated media remained lower than 23.0 °C. For the desiccation treatment, seedlings grown in GM plates were uncovered for 3 h at a light intensity of 6 W/m$^2$. Heat treatment was carried out at 37 °C for 3 h.

## Construction of *ELIP2::LUC*

Unless otherwise mentioned, all the molecular biological methods were performed according to Sambrook *et al.* (1989). Two *ELIP2* (Heddad & Adamska 2000)-specific primers (5′-CGC GTC GAC ATA ATA TTT ATT TAT TTA GTG ATT C-3′) and (5′-CGC GTC GAC TGA TTA GGT TTT CTA AAA GCC GA-3′), were used for PCR to amplify the promoter region of *ELIP2* from −2074 to −2 bp relative to the translation start site. The template was genomic DNA from *Arabidopsis* Columbia. The PCR product was digested with *Sal*I and inserted into the *Sal*I site of p6GLUC (Aoyama & Chua 1997). The resultant plasmid was digested with *Sca*I/*Pvu*II and the fragment containing the *ELIP2::LUC* fusion with a T3A polyA signal (Aoyama & Chua 1997) was blunt-ended and inserted into the *Sma*I site of pUC119. The resultant plasmid, yy211, was then digested with *Hin*dIII and the fragment containing *ELIP2::LUC* with a T3A terminator was inserted into the *Hin*dIII site of a binary vector, SLJ755I5 (http://www.jic.bbsrc.ac.uk/Sainsbury-Laboratory/jonathan-jones/plasmid-list/plasmid.htm) to make yy210. The final construct contained a BASTA marker gene and *ELIP2::LUC* with T3A terminator within the T-DNA region. The *ELIP2* promoter region of yy210 starts from −1907 to −2 bp relative to the translation start site of the *ELIP2* gene of the FCA1 contig (GenBank accession no. Z97336).

## Transformation of *Arabidopsis*

yy210 was introduced into *Agrobacterium tumefaciens* GV3101 pMP90 (Koncz *et al.* 1994) by triparental mating using the *E. coli* helper strain HB101pRK2013 (Walkerpeach & Velten 1994). An *Agrobacterium* clone with no plasmid rearrangements was identified by restriction digestion analysis (data not shown) and used to transform *Arabidopsis thaliana* L*er* by vacuum infiltration (Bechtold *et al.* 1993). Preparation of *35S::LUC* and *PC::LUC* was described by Dijkwel *et al.* (1996) and *APX::LUC* by Karpinski *et al.* (1999), respectively.

## *In vivo* luciferase assay

One day before the high light illumination, 5 mM luciferin (Promega, Tokyo) containing 0.1% Triton X-100 was sprayed on to seedlings grown on a medium to remove any pre-existing luciferase (Millar *et al.* 1992). Next day, after high light treatment, the seedlings were sprayed again with 5 mM luciferin, 0.1% Triton X-100, and kept in the dark for 5 min to quench the delayed chlorophyll fluorescence. Luminescence caused by the luciferase reporter gene was measured sequentially 10 times with a 1.0 min exposure time using the Argus 50 VIM-CCD camera system (Hamamatsu Photonics, Hamamatsu, Japan). As the *in vivo* luminescence was stable between 10 min and 50 min after spraying (data not shown), image files starting 14 min after spraying were utilized for quantitative analysis in typical experiments. In early experiments, in order to assay $T_2$ seedlings of independent transgenic lines, the luminescence of 15 individual seedlings from each line, all placed in a glass vial, was measured using a scintillation counter. (Tri-carb2000, Packard Japan, Tokyo) instead of the Argus 50.

## *In vitro* luciferase assay

The aerial parts of 8-day-old seedlings were treated with or without intense light for 3 h and then harvested, homogenized, and subjected to *in vitro* luciferase assays, as described elsewhere (Yamamoto & Deng 1998).

## *In vivo* pharmacological analysis

For the inhibitor treatments, seeds were placed on GM medium containing 1.0% sucrose, 0.8% Bactoagar, supplemented with 100 nM okadaic acid (Sigma, Tokyo), 100 nM K252a (Sigma), 600 nM Tagetin (Epicentre, Madison) and 100 nM norflurazon (Yamamoto *et al.* 2000). Because the norflurazon, okadaic acid and K252a were dissolved in ethanol, the corresponding amount of ethanol was added to the media for the control experiments (1.27% for experiments in Fig. 6A, 0.8% for Figs 6B, and 1.35% for Fig. 8). Seeds were germinated on media described above, grown at 22 °C under constant light conditions (6 W/m$^2$) for 4 days, sprayed with 5 mM luciferin containing 0.1% Triton X-100, and then subjected to an *in vivo* luciferase assay the next day. For the hydrogen peroxide treatments, 3.0% (w/w) hydrogen peroxide was sprayed on to seedlings, and for the control experiments, water was sprayed in the same manner as hydrogen peroxide treatment.

## RNA analysis

The aerial parts of 8-day-old seedlings were treated with or without strong light, harvested and total RNA was extracted (Yamamoto *et al.* 1995). The amount of *ELIP2* and *APX2* mRNA was determined by quantitative RT-PCR (Sambrook *et al.* 1989). The primers used for *APX2* amplification are described by Karpinski *et al.* (1997), and the primers for *ELIP2* are (5′-TAT TGA CTA CAC GCA ACA TCA GAA-3′) and (5′-GTT TTC TCC CTT TGA TAA CTC CAT-3′). Equal amounts of total RNA (500 ng) were subjected to RT-PCR analysis using Superscript II reverse transcriptase (LifeTechnology, Tokyo) (Sambrook *et al.* 1989). The products of RT-PCR were: 483 bp for the *ELIP2* genomic fragment and the unspliced transcript; 278 bp for the mature *ELIP2* transcript

1908 bp for the *APX2* genomic fragment and the unspliced transcript; and 740 bp for the mature *APX2* transcript. After 23, 25, 27 and 30 cycles, samples were collected from the PCR, stained with Vistra Green (Amersham Pharmacia Biotech, Tokyo) and separated by agarose gel electrophoresis. Each band was then quantified by a fluorescence scanner (FluoroImager SI, Amersham Pharmacia Biotech). A series of diluted RNA samples with the highest accumulation in an experiment, determined by preliminary experiments, were also subjected to the same analysis and the amounts of the transcripts were determined, based on the individual standard curves (data not shown).

To make a probe for Northern analysis, the *ELIP2*-specific region, from −28 to +73 bp relative to the translation start site, was amplified by PCR. *ELIP2* cDNA, isolated from an *Arabidopsis* cDNA library (Seki *et al*. 1998) was used as a template with the following primers (5′-GGA ATT CAG TGT GAG TAA TTT AGG CGT CGT T-3′) and (5′-GGA TCC TAA TAC GAC TCA CTA TAG GGA GGA GAA GAG TTG GTT TGT GTT TCT GA-3′), which contains a T7 promoter. The PCR product was used as a template to produce a [$^{32}$P]-labelled riboprobe. The *ELIP2* riboprobe and an 18S rDNA probe were used to detect the corresponding mRNA species by Northern hybridization (Yamamoto *et al*. 1998).

## Acknowledgements

We would like to thank Dr S. Smeekens (University of Utrecht) for providing the *35S::LUC* and *PC::LUC* transgenic *Arabidopsis*, Dr S. Karpinski (Swedish University of Agricultural Sciences) for the *APX2::LUC* transgenic *Arabidopsis*, Dr J.D.G. Jones (John Innes Centre) for the SLJ755I5 vector, Dr K. Sekino (SDS Biotech) for norflurazon, and Dr R. Oelmülar (Friedrich-Schiller-Universität Jena) for information about a method for okadaic acid application to seedlings. We also acknowledge Dr T. Kondo (Nagoya University) for circadian assay of *ELIP2::LUC*, Ms K. Harrison and Mr M. Smoker (John Innes Centre) for advice on *Agrobacterium* transformation, and Ms Y. Sekine for technical assistance with *Arabidopsis* transformation. Y.Y.Y. was a Special Postdoctoral Researcher of RIKEN, and supported by a Grant-in-Aid for the Encouragement of Young Scientists from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

## References

Adamska, I. (1997) ELIPs – Light-induced stress proteins. *Physiol. Plant*. **100**, 794–805.

Adamska, I., Kloppstech, K. & Ohad, I. (1992) UV light stress induces the synthesis of the early light-inducible protein and prevents its degradation. *J. Biol. Chem*. **267**, 24732–24737.

Adamska, I., Kloppstech, K. & Ohad, I. (1993) Early light-inducible protein in pea is stable during light stress but is degraded during recovery at low light intensity. *J. Biol. Chem*. **268**, 5438–5444.

Adamska, I., Lindahl, M., Roobol-Boza, M. & Andersson, B. (1996) Degradation of the light-stress protein is mediated by an ATP-independent, serine-type protease under low-light conditions. *Eur. J. Biochem*. **236**, 591–599.

Adamska, I., Roobol-Boza, M., Lindahl, M. & Andersson, B. (1999) Isolation of pigment-binding early light-inducible proteins from pea. *Eur. J. Biochem*. **260**, 453–460.

Aoyama, T. & Chua, N.-H. (1997) A glucocorticoid-mediated transcriptional induction system in transgenic plants. *Plant J*. **11**, 605–612.

Arts, M.G.M., Corzaan, P., Stiekema, W.J. & Pereira, A. (1995) A two-element *Enhancer-Inhibitor* transposon system in *Arabidopsis thaliana*. *Mol. Gen. Genet*. **247**, 555–564.

Asada, K. (1996) Radical production and scavenging in the chloroplasts. In: *Phytosynthesis and the Environment* (ed. N.R. Baker), Vol. 5, pp. 123–150. Dordrecht: Kluwer Academic Publishers.

Bartels, D., Hanke, C., Schneider, K., Michel, D. & Salamini, F. (1992) A desiccation-related *Elip*-like gene from the resurrection plant *Craterostigma plantagineum* is regulated by light and ABA. *EMBO J*. **11**, 2771–2778.

Beator, J., Pötter, E. & Kloppstech, K. (1992) The effect of heat shock on morphogenesis in barley. *Plant Physiol*. **100**, 1780–1786.

Bechtold, N., Ellis, J. & Pelletier, G. (1993) *In planta Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. *C. R. Acad. Sci*. **316**, 1194–1199.

Bolwell, G.P. (1999) Role of active oxygen species and NO in plant defense responses. *Curr. Opin. Plant Biol*. **2**, 287–294.

Chamovitz, D.A., Pecker, I. & Hirschberg, J. (1991) The molecular basis of resistance to the herbicide norflurazon. *Plant Mol. Biol*. **16**, 967–974.

Cohen, P. (1989) The structure and regulation of protein phosphatases. *Annu. Rev. Biochem*. **58**, 453–508.

Dijkwel, P.P., Kock, P.A.M., Bezemer, R., Weisbeek, P.J. & Smeekens, S.C.M. (1996) Sucrose represses the developmentally controlled transient activation of the plastocyanin gene in *Arabidopsis* thaliana seedlings. *Plant Physiol*. **110**, 455–463.

Grimm, B. & Kloppstech, K. (1987) The early light-inducible proteins of barley. Characterization of two families of 2-h-specific nuclear-coded chloroplast proteins. *Eur. J. Biochem*. **167**, 493–499.

Hayashi, H., Czaja, I., Lubenow, H., Schell, J. & Walden, R. (1992) Activation of a plant gene by T-DNA tagging: auxin-independent growth *in vivo*. *Science* **258**, 1350–1353.

He, Q., Dolganov, N., Björkman, O. & Grossman, A.R. (2001) The High Light-inducible polypeptides in *Synechocystis* PCC6803: expression and function in high light. *J. Biol. Chem*. **276**, 306–314.

Heddad, M. & Adamska, I. (2000) Light stress-regulated two-helix proteins in *Arabidopsis thaliana* related to the chlorophyll *a/b*-binding gene family. *Proc. Natl. Acad. Sci. USA* **97**, 3741–3746.

Hidaka, H. & Kobayashi, R. (1992) Pharmacology of protein kinase inhibitors. *Annu. Rev. Pharmacol. Toxicol*. **32**, 377–397.

Jansson, S. (1999) A guide to the *Lhc* genes and their relatives in *Arabidopsis*. *Trends Plant Sci*. **4**, 236–240.

Kapoor, S., Suzuki, J.Y. & Sugiura, M. (1997) Identification and

 

functional significance of a new class of non-consensus-type plastid promoters. *Plant J.* **11**, 327–337.

Karpinski, S., Escobar, C., Karpinska, B., Creissen, G. & Mullineaux, P.M. (1997) Photosynthetic electron transport regulates the expression of cytosolic ascorbate peroxidase genes in Arabidopsis during excess light stress. *Plant Cell* **9**, 627–640.

Karpinski, S., Reynolds, H., Karpinska, B., Wingsle, G., Creissen, G. & Mullineaux, P. (1999) Systemic signaling and acclimation in response to excess excitation energy in *Arabidopsis*. *Science* **284**, 654–657.

Kloppstech, K. (1985) Diurnal and circadian rhythmicity if the expression of light-induced plant nuclear messenger RNAs. *Planta* **165**, 502–506.

Koncz, C., Martini, N., Szabados, L., *et al.* (1994) Specialized vectors for gene tagging and expression studies. In: *Plant Molecular Biology Manual* (eds S.B. Gelvin & R.A. Schilperoot), pp. B2: 1–22. Dordrecht: Kluwer Academic Publishers.

Li, X.-P., Björkman, O., Shih, C., *et al.* (2000) A pigment-binding protein essential for regulation of phtosynthetic light harvesting. *Nature* **403**, 391–395.

Meyer, G. & Kloppstech, K. (1984) A rapidly light-induced chloroplast protein with a high turnover coded for by pea nuclear DNA. *Eur. J. Biochem.* **138**, 201–207.

Millar, A.J., Short, S.R., Hiratsuka, K., Chua, N.-H. & Kay, S.A. (1992) Firefly luciferase as a reporter of regulated gene expression in higher plants. *Plant Mol. Biol. Rep.* **10**, 324–337.

Moscovici-Kadouri, S. & Chamovitz, D.A. (1997) Characterization of a cDNA encoding the Early Light-Inducible Protein (ELIP). *Plant Physiol.* **115**, 1287.

Niyogi, K.K. (1999) Photoprotection revisited. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **50**, 333–359.

Oelmüller, R. (1989) Photooxidative destruction of chloroplasts and its effect on nuclear gene expression and extraplastidic enzyme levels. *Photochem. Photobiol.* **49**, 229–239.

Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Seki, M., Carninci, P., Nishiyama, Y., Hayashizaki, Y. & Shinozaki, K. (1998) High-efficiency cloning of Arabidopsis full-length cDNA by biotinylated CAP trapper. *Plant J.* **15**, 707–720.

Sheen, J. (1993) Protein phosphatase activity is required for light-inducible gene expression in maize. *EMBO J.* **12**, 3497–3505.

Shimosaka, E., Sasanuma, T. & Handa, H. (1999) A wheat cold-regulated cDNA encoding an early light-inducible protein (ELIP): its structure, expression and chromosomal location. *Plant Cell Physiol.* **40**, 319–325.

Taylor, W.C. (1989) Regulatory interactions between nuclear and plastid genomes. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **40**, 211–233.

The Arabidopsis Genome Initiative (2001) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature* **408**, 796–815.

Valvekens, D., Van Montagu, M. & Van Lijsebettens, M. (1988) *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. *Proc. Natl. Acad. Sci. USA* **85**, 5536–5540.

Walkerpeach, C.R. & Velten, J. (1994) *Agrobacterium*-mediated gene transfer to plant cells: cointegrate and binary vector systems. In: *Plant Molecular Biology Manual* (eds S.B. Gelvin & R.A. Schilperoot), 2nd edn, pp. B1: 1–19. Dordrecht: Kluwer Academic Publishers.

Yamamoto, Y.Y. & Deng, X.-W. (1998) A new vector set for GAL4-dependent transactivation assay in plants. *Plant Biotech.* **15**, 217–220.

Yamamoto, Y.Y., Matsui, M., Ang, L.-H. & Deng, X.-W. (1998) Role of COP1 interactive protein in mediating light-regulated gene expression in Arabidopsis. *Plant Cell* **10**, 1083–1094.

Yamamoto, Y.Y., Nakamura, M., Kondo, Y., Tsuji, H. & Obokata, J. (1995) Early light-response of *psaD*, *psaE* amd *psaH* gene families of photosystem I in *Nicotiana sylvestris*: *psaD* has an isoform of very quick response. *Plant Cell Physiol.* **36**, 727–732.

Yamamoto, Y.Y., Puente, P. & Deng, X.-W. (2000) An *Arabidopsis* cotyledon-specific albino locus: a possible role in 16S rRNA maturation. *Plant Cell Physiol.* **41**, 68–76.

*Received:* 31 January 2001
*Accepted:* 11 April 2001