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Light-responsive elements of the tobacco PSI-D gene are located both upstream and within the transcribed region

Yoshiharu Y. Yamamoto[1,†], Yukiko Kondo[1,‡], Akira Kato[2], Hideo Tsuji[3] and Junichi Obokata[1,*]
[1]*Division of Bioscience, Graduate School of Environmental Earth Science, Hokkaido University, Sapporo 060, Japan,*
[2]*Hokkaido National Agricultural Experimental Station, Sapporo 062, Japan, and*
[3]*Department of Biology, Kobe Women's University, Suma, Kobe 654, Japan*

Summary

***psaDb* is a nuclear gene encoding the ferredoxin-binding subunit of photosystem I in *Nicotiana sylvestris*. The organization of the light-responsive *cis*-elements of *psaDb* was studied using transgenic tobacco plants. Three types of *psaDb* chimeric constructs were created: (1) a 5′ upstream fragment of *psaDb* transcriptionally fused with the β-glucuronidase (*GUS*) gene, and a series of its 5′ deletion derivatives, (2) the transcribed region of *psaDb* driven by the cauliflower mosaic virus (CaMV) 35S promoter, and (3) the 5′ terminal 35 bases (the entire leader, +1 to +23, and the initiation codon context, +24 to +35) of the *psaDb* mRNA translationally fused with a *GUS* reporter gene under the operation of the CaMV 35S promoter. Light-responsiveness of these fusions in transgenic plants was examined by GUS assay and primer extension analysis. The results indicate that the light-responsive elements (LRE) of *psaDb* are located both upstream (–170 to +24) and within (+1 to +861) the transcribed region. The internal LRE is utilized in etiolated seedlings but not in green leaves. The leader and initiation codon context construct (+1 to +35) did not show any light-response under the conditions tested. Therefore, it is likely that a combination of the upstream and internal LREs generates the complex light-responsive and tissue-specific regulation of this gene. This study also revealed that *psaDb* has adjacent activator (–267 to –254) and repressor (–253 to –234) regions for basal transcriptional activity; the former contains the ACGT binding motif recognized by many plant bZIP proteins, and the latter has the R3 decamer motif found in several photosystem I-related genes.**

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*For correspondence (fax +81 11 706 5291; e-mail jo@bio.hokudai.ac.jp).
†Present address: Department of Biology, Yale University, New Haven, CT 06520–8104, USA.
‡Present address: Tokita Seed Co. Ltd. Nakagawa, Omiya, Saitama 330, Japan.

Introduction

A variety of plant nuclear genes are activated in response to light, and generally their activation is thought to occur at the level of transcription (Batschauer *et al.*, 1994; Terzaghi and Cashmore, 1995; Thompson and White, 1991). Extensive studies of the genes for the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbcS*) and chlorophyll *a/b* binding proteins (*cab*) have provided a model in which the light-responsive *cis*-elements (LRE) located upstream of the transcriptional start site confer light-responsive transcriptional activation in conjunction with *trans*-acting factors which are linked to the signal transduction pathways of photoreceptors (Batschauer *et al.*, 1994; Terzaghi and Cashmore, 1995; Thompson and White, 1991; Tobin and Kehoe, 1994). However, there have been several examples in which gene activation appears to occur differently from the above model. For example, in *Petunia rbcS* genes, the transcriptional activator sequence appears to be located downstream of the coding region (Dean *et al.*, 1989). Light-induction of *Amaranthus rbcS* occurs at translational steps but not at the mRNA level (Berry *et al.*, 1988, 1990). The pea ferredoxin gene, *Fed-1*, which is not interrupted by introns, has a light-responsive element within the coding region (Dickey *et al.*, 1992, 1994; Elliott *et al.*, 1989a; Gallo-Meagher *et al.*, 1992). We do not know whether these instances are exceptions, since relatively little attention has been paid to the regions downstream of the transcription start site.

Recent studies of the phytochrome signal transduction pathways suggest that the genes activated by these pathways are classified into three groups: (1) genes for anthocyanin biosynthesis, (2) genes for photosystem II (PSII), light-harvesting chlorophyll *a/b* binding proteins (*cab*), ATP synthase and the small subunit of ribulose-1,5- bisphosphate carboxylase/oxygenase (*rbcS*), and (3) genes for photosystem I (PSI), ferredoxin, plastocyanin and cytochrome b_6/f complex (Bowler *et al.*, 1994a,b; Neuhaus *et al.*,1993). Differentiation between the second and the third groups seems to allow plants to change the stoichiometry of PSI and PSII in response to different light conditions to ensure high photosynthetic efficiency (Bowler *et al.*, 1994a). Numerous studies have been performed for the second group of the genes, including *rbcS* and *cab*, while relatively little is known about the third group. It is possible that the position, sequence and function of the *cis*-LREs are different between the second group and PSI-related genes.

PSI consists of at least 13 subunits whose genes are denoted *psaA* to *psaL* and *psaN*, and mediates light-driven

electron transfer from plastocyanin to ferredoxin (Bryant, 1992; Golbeck, 1992; Obokata *et al.*, 1993). The internal LRE of pea *Fed-1* is responsible for the major part of this gene's light-response, and requires a translatable reading frame (Dickey *et al.*, 1994). In other words, mRNAs of this gene seem to have light-regulated stability which is linked to its translation. In the cases of the pea plastocyanin gene (*petE*) and spinach *psaD*, transcriptional fusions of their 5′ upstream sequences to *GUS* (β-glucuronidase) reporter genes did not cause the expected levels of light activation in transgenic tobacco, implying that these genes might have internal or downstream LREs (Flieger *et al.*, 1994; Pwee and Gray, 1993).

Tobacco is one of the most suitable plant materials for studying the PSI nuclear genes, since, in tobacco, organization of the PSI genes and their products is well characterized (Nakamura and Obokata, 1994; Obokata *et al.*, 1993, 1994; Yamamoto *et al.*, 1993) and transgenic plant analysis can be performed in a homologous host–transgene system. *psaDb* (Yamamoto *et al.*,1993) encodes an isomer of the ferredoxin-binding subunit (PSI-D subunit) of the PSI complex in *Nicotiana sylvestris*, a diploid progenitor of *N. tabacum* with an amphidiploid genome (Obokata *et al.*, 1990; Yamamoto *et al.*,1993). The light-induced accumulation of *psaDb* mRNA as well as its translation product is very quick in comparison with those of the other PSI genes (Yamamoto *et al.*, 1995a). Furthermore, etiolated seedlings contain a detectable amount of the *psaDb* mRNA, although its translation product cannot be detected until the seedlings are illuminated, indicating that the translational and/or post-translational step of *psaDb* expression requires light-activation (Yamamoto *et al.*, 1995a). The 5′ untranslated leader sequence of *psaDb* contains an unique translational enhancer (Yamamoto *et al.*, 1995b), raising the question of whether this element modulates the translational activity of *psaDb* in response to light.

In this study, we examined the organization of the light-responsive *cis*-elements (LRE) of *psaDb* in a transgenic tobacco system. We found that this gene has both upstream and internal LREs, the internal LRE functions in a tissue-specific manner, and that the translational enhancer within the 5′ leader does not behave as an LRE.

Results

Construction of chimeric genes

In order to elucidate the organization and arrangement of the light-responsive *cis*-elements of *psaDb*, we first made three types of *psaDb* chimeric constructs: *psaDb*::*GUS*, CaMV::*psaDb*, and CaMV::*psaDb–GUS*′ (Figure 1). The *psaDb*::*GUS* construct is a transcriptional fusion of the 5′ upstream region of *psaDb* (–1728 to +24, relative to the transcriptional start site) and the *GUS* reporter gene, flanked 3′ by a NOS (nopalin synthase gene) terminator. In this construct, the *psaDb* sequence was extended 24 bases downstream of the transcription start site in order to ensure precise transcriptional initiation. The second construct, CaMV::*psaDb*, was designed to detect any possible LREs within the transcribed region. This construct carries the *psaDb* sequence +1 to +861 under the operation of the light-insensitive cauliflower mosaic virus (CaMV) 35S promoter. The third construct, CaMV::*psaDb–GUS*′, was made to examine the possible light-responses mediated by the 5′ leader (+1 to +23) and initiation codon context (+24 to +35). This construct contains the *psaDb* sequence +1 to +35 translationally fused with a *GUS* coding region. The 5′ leader sequence of *psaDb* contains a novel type of translational enhancer element (Yamamoto *et al.*, 1995b). If the activity of this element is modulated by light, the CaMV::*psaDb–GUS*′ transgene should exhibit light-response.

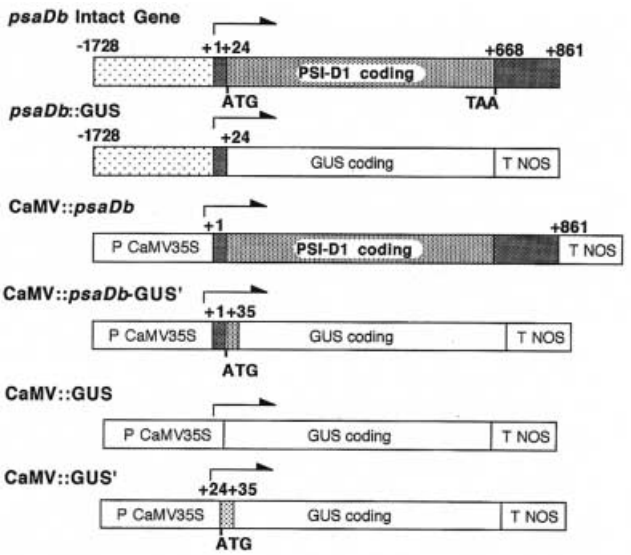


Figure 1. Construction of the chimeric genes.
psaDb encodes the PSI-D1 protein of photosystem I complex (Yamamoto *et al.*, 1993). Marked areas are derived from *psaDb*. The transcription start site of *psaDb* is denoted +1.

To assess the light-responses of the above three fusion genes, we prepared two types of *GUS* control genes, namely CaMV::*GUS* and CaMV::*GUS*′ (Figure 1). CaMV::*GUS* is derived from pBI121(Jefferson *et al.*, 1987), and the encoded *GUS* protein is identical with that of *psaDb*::*GUS*. CaMV::*GUS*′ was specifically designed for studying CaMV::*psaDb–GUS*′, and both encode an identical reporter protein, *GUS*′, which has extra 12 amino acids at its N-terminus (Yamamoto *et al.*, 1995b). The initiation codon context of CaMV::*GUS*′ is derived from *psaDb* (+24 to +35), but, by introducing two base substitutions, its characteristic palindromes were destroyed (Yamamoto *et al.*, 1995b).

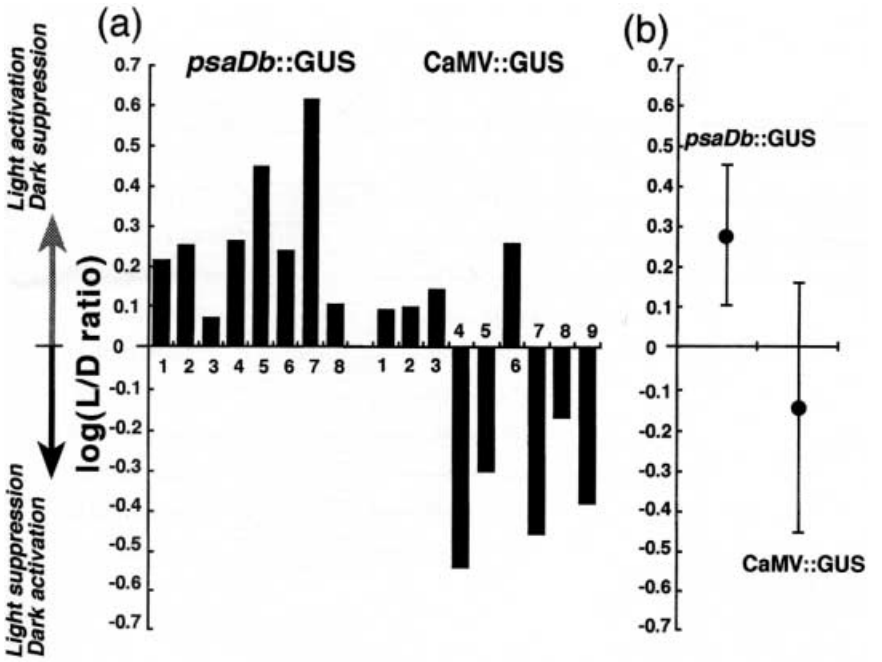


Figure 2. Light/dark response of *psaDb::GUS* and CaMV::*GUS* constructs in transgenic M_1 seedlings.
(a) Ratio of the *GUS* activity of light-grown seedlings to that of dark-grown ones for individual transgenic lines. The M_1 seedlings were grown in the light or dark for 6 days.
(b) Average values and standard deviations of the values from (a).

All these fusion constructs were introduced, via *Agrobacterium*, into *N. tabacum* var. SR1, and regenerated transgenic plants (M_0 generation) and their seeds (M_1 generation) were used for the following studies. *N. sylvestris,* from which *psaDb* was isolated (Yamamoto *et al.*, 1993), was not used for the transgenic host plants for the following reasons: (1) cultivar SR1 of *N. tabacum* flowers earlier than *N. sylvestris*, hence is more suitable for studies with the M_1 generation; (2) through the process of Ti-mediated transformation and regeneration, *N. sylvestris* seems to produce genetic variations with altered phenotypes much more frequently than *N. tabacum,* which is not a suitable characteristic for the transgenic host plants in this study (Yamamoto and Obokata, unpublished results); (3) *N.sylvestris* is a diploid progenitor of *N.tabacum* with an amphidiploid genome (Obokata *et al.*, 1990), hence, transgenic *N. tabacum* is expected to have the molecular machinery necessary for the proper regulated expression of *psaDb.*

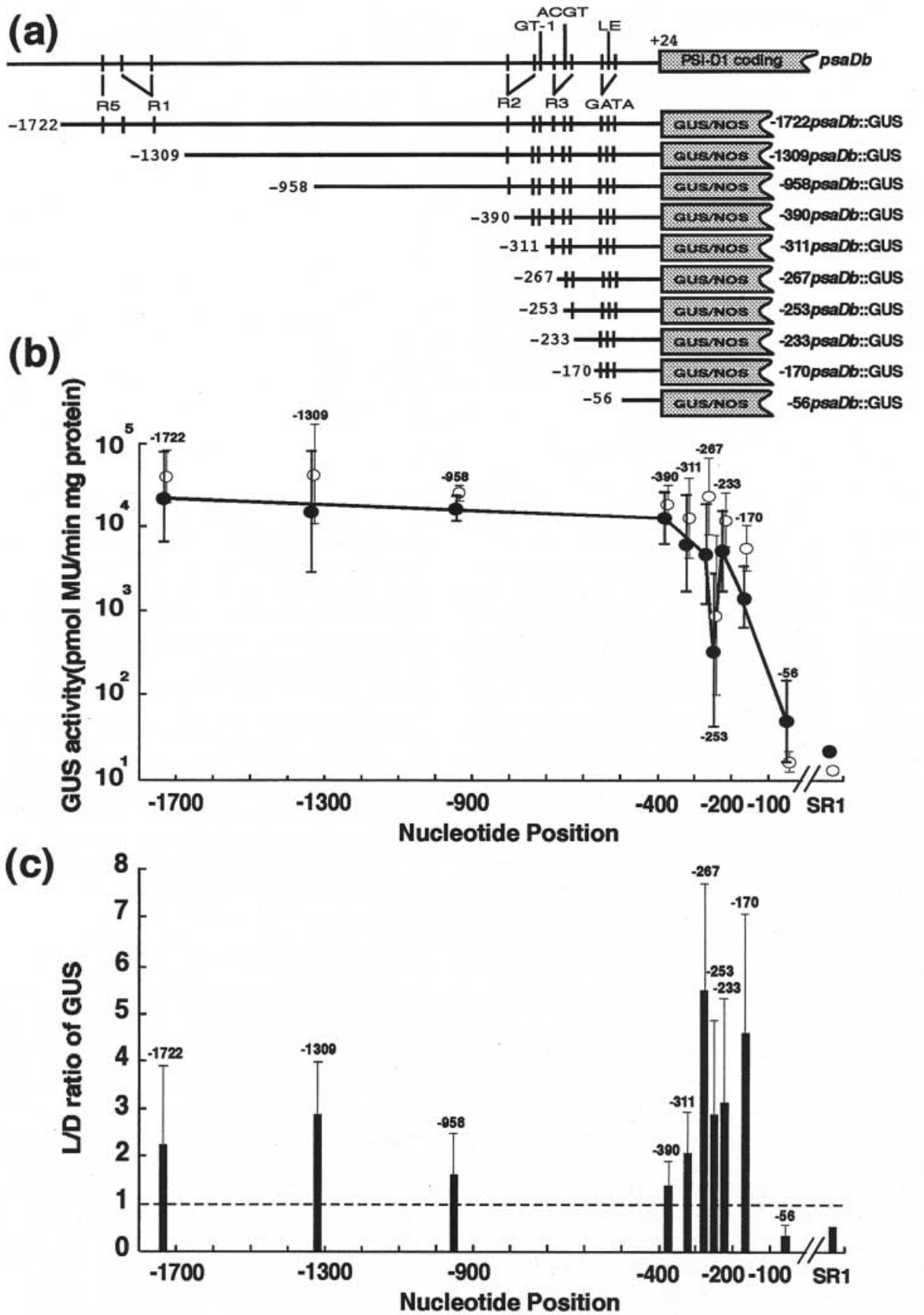
The psaDb *promoter is light-responsive in transgenic seedlings*

To examine the light-responsiveness of the *psaDb* promoter, we studied the expression of *psaDb::GUS* in transgenic M_1 seedlings, in comparison with CaMV::*GUS*. The seeds harvested from each transgenic M_0 plant were sown on two agar plates, and they were each placed in the dark or light for 6 days. From the GUS activities determined for the resulting seedlings, we obtained the light/dark ratio of the enzymatic activity for individual transgenic lines, and plotted them on a logarithmic scale as shown in Figure 2(a). A logarithmic scale is suitable for displaying activation and suppression of the same proportion. Figure 2(b) represents the average value for each construct, indicating that the light treatment enhanced the *psaDb::GUS* expression about twofold (log 1.9 = 0.28), although the CaMV::*GUS* expression was weakly suppressed (log 0.7 = –0.15). As the CaMV 35S promoter is almost light-insensitive (Fluhr and Chua, 1986; Lam and Chua 1990; Morelli *et al.*, 1985), this result indicates that the *psaDb* fragment from –1728 to +24 confers light-enhanced transcription to the *GUS* reporter gene. We designated the light-responsive *cis*-acting element(s) located in this region as an upstream LRE(s). The weak light suppression of the CaMV::*GUS* fusion seems to be caused by the light-induced destabilization of *GUS* mRNA as referred to in the discussion.

5′ deletion analysis of the psaDb *promoter*

psaDb has several characteristic motifs upstream of its transcription start site (Figure 3a). To examine whether these motifs take part in the upstream LRE, we made a series of 5′ promoter deletion mutants of *psaDb::GUS* (Figure 3a), and analyzed their expression in transgenic M_1 seedlings.

Figure 3(b) represents the GUS activities of the seedlings grown in the dark or in the light for 6 days. The 5′ deletions from –1722 to –390 had little effect on the dark and light GUS activity levels, but these significantly decreased as the deletion proceeded downstream of –390, indicating that the main regulatory elements of the *psaDb* promoter lie downstream of this site. A deletion from –267 to –253 reduced the GUS activity of the dark-grown seedlings 14-


(a)
ACGT
GT-1
LE
+24
PSI-D1 coding
psaDb
R5
R1
R2
R3
GATA
-1722
-1309
-958
-390
-311
-267
-253
-233
-170
-56
GUS/NOS
-1722psaDb::GUS
-1309psaDb::GUS
-958psaDb::GUS
-390psaDb::GUS
-311psaDb::GUS
-267psaDb::GUS
-253psaDb::GUS
-233psaDb::GUS
-170psaDb::GUS
-56psaDb::GUS
(b)
GUS activity(pmol MU/min mg protein)
Nucleotide Position
-1700
-1300
-900
-400
-200
-100
SR1
(c)
L/D ratio of GUS
Nucleotide Position

fold, but this reduction was restored by the next deletion to –233. The GUS activities of the light-grown seedlings showed essentially the same tendency as those of the dark-grown seedlings. This result indicates that activating and repressing elements for basal transcription are located in these 14 bp (–267 to –254) and 20 bp (–253 to –234) regions, respectively. The former region contains the ACGT motif recognized by many plant bZIP proteins (Foster *et al.*, 1994), while the latter includes the R3 motif common to several PSI-related genes (Nakamura and Obokata, 1995; Obokata *et al.*, 1992; Yamamoto *et al.*, 1993). Transformants harboring –170*psaDb*::*GUS* still exhibited GUS activity of one-tenth of –1722*psaDb*::*GUS*, however, the next deletion to –56 reduced the GUS activity almost to the background level (Figure 3b). This indicates that the *psaDb* fragment truncated at –56 is no longer transcriptionally active; hence, another transcriptional activator element must be present between –170 and –56. Because this final deletion almost abolished the basal transcription, we could not clarify whether this –56 fragment still contains a LRE or not.

Figure 3(c) shows the light/dark responses of the deletion mutants. The magnitude of the light response was highest in –267*psaDb*::*GUS*, with a light/dark GUS activity ratio of 5.6 ± 2.3. Since the light/dark ratio of –311*psaDb*::*GUS* was only 2.1 ± 0.9, the nucleotide sequence between –311 and –267 must suppress the light activation. The response of –267*psaDb*::*GUS* was then reduced to 2.9 ± 2.1 by the next 14 bp deletion to –253. Hence, this 14 bp region (–267 to –254), which contains the ACGT motif, seems to be required for efficient light/dark response as well as to maintain high basal transcriptional activity, as described above. Because –170*psaDb*::*GUS* still demonstrated high light-responsiveness with a light/dark ratio of 4.7 ± 2.6, we conclude that at least one light-responsive element lies downstream of –170. These above results are summarized in Figure 4.

Transcribed region of psaDb *responds to light in etiolated seedlings but not in green leaves*

Primer extension profiles of *psaDb* mRNA were compared among *N. sylvestris* (seedlings), *N. tabacum* (green leaves), and transgenic *N. tabacum* carrying the CaMV::*psaDb* fusion (M_0 green leaves) (Figure 5a). Since *N. tabacum* has an intrinsic *psaDb* gene derived from the ancestral *N. sylvestris* genome (Obokata *et al.*, 1993), the *psaDb*-specific primer detected the native *psaDb* mRNAs both in *N. tabacum* and *N. sylvestris* (Figure 5a, arrowheads). In addition to these bands, the transgenic plant showed a strong signal of larger size derived from the CaMV::*psaDb* transgene (Figure 5a, arrow). Even in the presence of this strong signal, intrinsic *psaDb* mRNA showed a light/dark response (Figure 5a, arrowheads in the lanes of CaMV::*psaDb*, D and L) similar to that in *N. tabacum* without the transgene (Figure 5a), suggesting that the light-responsive of *psaDb* operates normally in the presence of the CaMV::*psaDb* transgene.

Figure 5(b) shows the effect of light on the *psaDb* message level in etiolated M_1 seedlings. In all the transgenic lines examined here, light-illumination for 8 h led to the accumulation of CaMV::*psaDb* mRNA (Figure 5b, arrow) as well as the intrinsic *psaDb* mRNA (Figure 5b, arrowheads). Quantitative analysis of the radioactivity of each band revealed that the CaMV::*psaDb* mRNA level increased twofold during this light treatment (Table 1). This result shows that *psaDb* has a light-responsive element within the transcribed region, between +1 and +861. We refer to this element as an internal LRE.

The *psaDb* expression is induced by light not only in etiolated seedlings but also in dark-adapted green leaves, and their induction profiles are quite similar (Yamamoto *et al.*, 1995a). We next examined whether the internal LRE responds to light in dark-adapted leaves as well. To our surprise, the internal LRE did not operate in green leaves. Transgenic M_0 plants were grown in Magenta culture boxes under continuous light illumination, and resultant plants with leaves of 3–6 cm were placed in the dark for 4 days, followed by re-exposure to light for 8 h. During this re-illumination process, the CaMV::*psaDb* mRNA level did not change at all (Table 1), although the intrinsic *psaDb* mRNA level increased sixfold (data not shown).

The 5′ leader and translation initiation context of psaDb *are not involved in light-response*

Transgenic M_1 seedlings carrying the CaMV::*psaDb–GUS′* and CaMV::*GUS′* constructs were grown in the light or dark for 6 days, and then the light/dark GUS activity ratio

Figure 3. 5′ deletion analysis of the *psaDb*::*GUS* construct.
The transcription start site of *psaDb* is denoted +1.
(a) Schematic illustration of the 5′ deletion mutants of *psaDb*::*GUS* in comparison with intact *psaDb*. This gene has several characteristic motifs upstream of the transcription start site: the GT-1 box (GGTTAA) (Green *et al.*, 1988), ACGT bZIP binding motif (Foster *et al.*, 1994), GATA motif (Castresana *et al.*, 1987), and R1, R2, R3 and R5 sequences commonly found in PSI-related genes (Obokata *et al.*, 1992). LE indicates a putative common light-regulatory sequence as determined by Grob and Stüber through a computer search (Grob and Stüber 1987).
(b) *GUS* activities of the transgenic M_1 seedlings grown in the dark (●) and the light (○) for 6 days. SR1 indicates untransformed plants. The mean and standard deviations of 5–8 transgenic lines are shown for each construct.
(c) The light-response of the 5′ deletion derivatives of *psaDb*::*GUS*. The GUS activities of the 6-day-old light-grown M_1 seedlings were divided by those of dark-grown seedlings. The mean average and standard deviations of 5–8 transgenic lines were calculated for each construct.

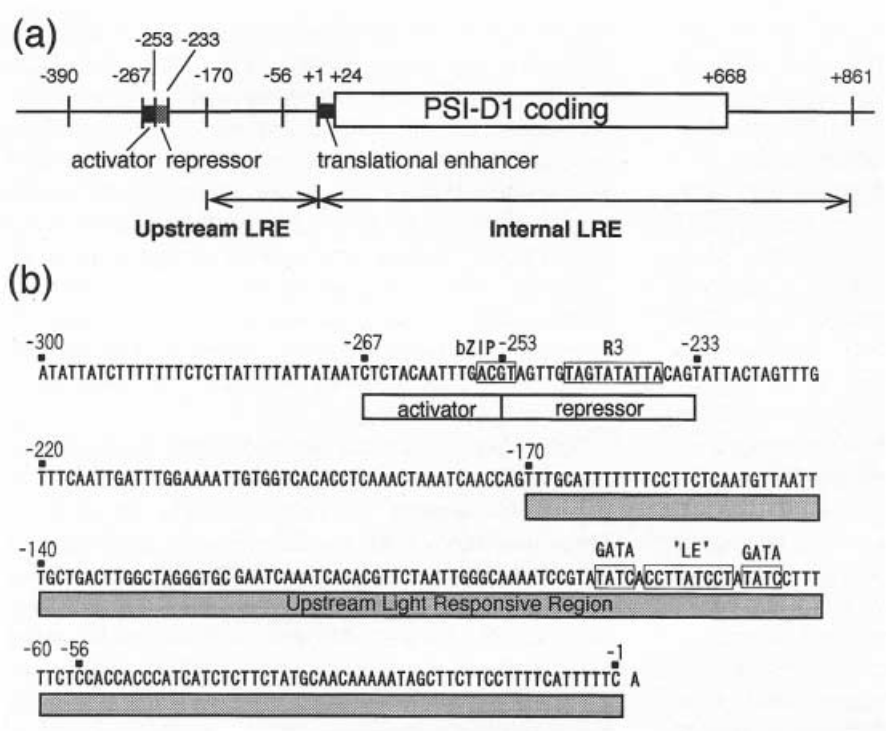


Figure 4. Arrangement of the *cis*-regulatory elements in the *psaDb* gene. Transcription start site is denoted +1.
(a) Summarized illustration of *psaDb*. The activator, repressor, upstream LRE and internal LRE are noted. The translational enhancer element has been described previously (Yamamoto *et al.*, 1995b).
(b) 5′ flanking sequence of the transcribed region. The ACGT bZIP binding sequence (Foster *et al.*, 1994), R3 motif (Nakamura and Obokata, 1995; Obokata *et al.*, 1992), GATA motif (Castresana *et al.*, 1987), and the putative light regulatory sequence (Grob and Stüber 1987) are boxed.

was determined for the individual transgenic lines. As shown in Figure 6, no significant difference in light-response was found between CaMV::*psaDb–GUS*′ and CaMV::*GUS*′. This indicates that the inserted *psaDb* sequence of CaMV::*psaDb–GUS*′, which includes the entire 5′ leader and initial four codons, does not confer any light-response to the *GUS*′ reporter gene.

Discussion

This study was undertaken to elucidate the organization of the light-responsive *cis*-regulatory elements (LRE) in the *psaDb* gene, and revealed that they are located both upstream and within the transcribed region, as summarized in Figure 4(a).

Upstream light-responsive elements for transcriptional regulation

Transgenic plants carrying *psaDb*::*GUS* or its 5′ deletion derivatives disclosed that *psaDb* has upstream LREs conferring transcriptional regulation (Figures 2, 3 and 4a). However, the magnitude of their light-responses appeared to be smaller than that of the intact *psaDb* gene (Yamamoto *et al.*, 1995a). This discrepancy was caused, at least in part, for the following reasons. The first is that the light-response of *psaDb* is mediated by not only the upstream LRE but also the internal LRE. Hence, the upstream LRE alone is not sufficient for inducing the full light-response. The second is that *GUS* mRNA is destabilized by light in transgenic tobacco plants, causing the light-dependent suppression of reporter activity (Dickey *et al.*, 1992; Yamamoto and Obokata, unpublished results). This would have diminished the apparent magnitude of the light activation in this study.

The 5′ deletion analysis revealed three regions of particular interest as summarized in Figure 4: the 14 bp activator region between –267 and –254, the 20 bp repressor region from –253 to –234 , and the sequence downstream of –170 which confers both basal and light-responsive transcription to the *GUS* reporter gene. The activator region is required not only for high transcriptional activity but also for an efficient light/dark response (Figure 3), and contains the ACGT motif (Figure 4b) recognized by many plant bZIP DNA binding proteins (Foster *et al.*, 1994). The TGACGT hexamer in this activator region matches the binding sequence of TGA1, a plant transcriptional activator protein (Katagiri *et al.*, 1990; Schindler *et al.*, 1992; Yamazaki *et al.*, 1990). The adjacent 20 bp repressor region from –253 to –234 contains the R3 decamer motif (Figure 4b) that is found in several PSI-related genes at similar distances from the ATG initiator (Obokata *et al.*, 1992). Thus, the ACGT motif and the R3 motif are good candidates for the target sites of transcriptional activator and repressor protein(s), respectively. Because these 14 bp activator and 20 bp repressor regions are close to each other (Figure 4), there is a possibility that they interact to form a functional 'unit' involved in the the regulation of *psaDb* (Figure 3). In

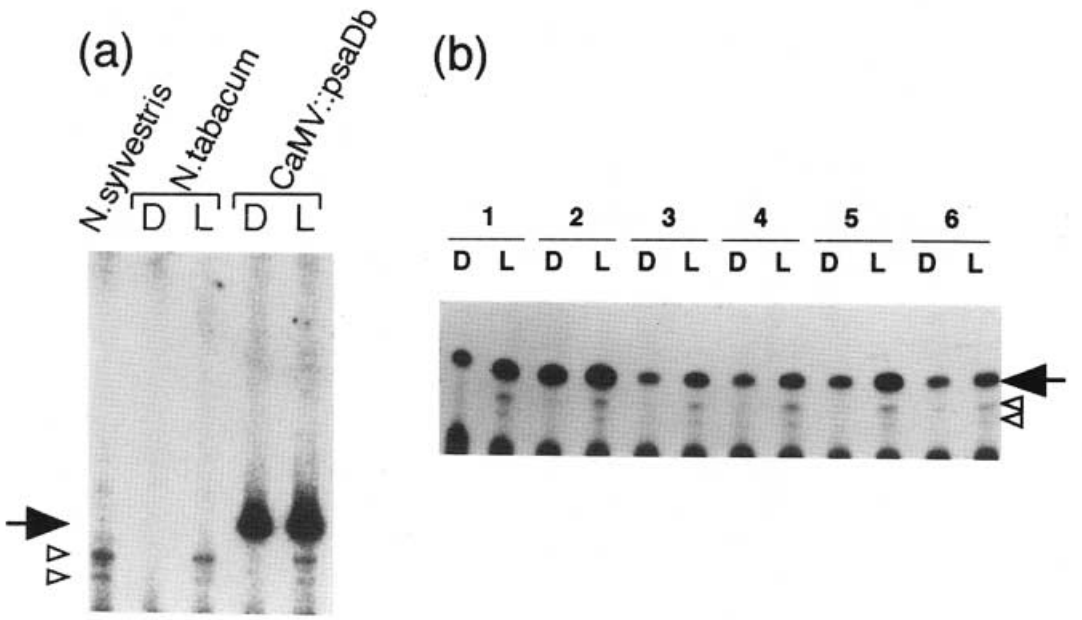


Figure 5. Light response of the CaMV::*psaDb* transgene.
(a) Primer extension profiles of the *psaDb* message are compared among RNAs from *N. sylvestris*, *N. tabacum*, and transgenic *N. tabacum* (M_0 generation). D and L denote dark-adapted mature plants and those after 8 h re-illumination, respectively. *N. sylvestris* RNA was isolated from greening seedlings. The solid arrow indicates the signal from the CaMV::*psaDb* transgene, while open arrow heads indicate inherent *psaDb* gene expression.
(b) Primer extension profiles of *psaDb* message in the transgenic M_1 seedlings. RNAs were isolated from the 6-day-old etiolated seedlings before (D) and after (L) light treatment for 8 h.

Table 1. Light/dark ratio of the *psaDb* mRNA level derived from the CaMV::*psaDb* transgene in tobacco plants

	Etiolated seedlings	Mature plants
	2.5	1.7
	1.6	0.4
	1.7	1.0
	1.9	1.0
	3.0	1.3
	1.6	
Mean ± SD	2.1 ± 0.5	1.1 ± 0.4

Levels of transgene mRNA in 6-day-old etiolated seedlings were measured for individual transgenic lines, before and after 8 h white light exposure. Dark-adapted mature plants of the M_0 generation were treated similarly. The mean and standard deviations are indicated.

the case of chalcone synthase genes, a light-responsive *cis*-element, termed Unit 1, is composed of two elements, Box I (possibly recognized by *myb*-type proteins) and Box II (G box recognized by bZIP proteins) (Kaiser *et al.*, 1995; Rocholl *et al.*, 1994).

The results of 5′ deletion analysis also indicate that at least one upstream LRE is located between −170 and +24. Since deletion to −56 almost abolished the transcriptional activity of the transgene (Figure 3b), we could not determine the position of this LRE relative to −56. Consequently, we presume that the DNA fragment from −170 to −1 should carry at least one LRE (Figure 4). This 170 bp region contains a CCTTATCAT sequence (labelled 'LE' in Figure 4b), which was predicted to be a common light-regulatory element following a computer search (Grob and Stüber, 1987), and tandemly repeated GATA motifs (Figure 4b) which are conserved among several *cab* and *rbcS* genes (Batschauer *et al.*, 1994; Castresana *et al.*,1987; Terzaghi and Cashmore, 1995). Therefore, the LREs in this region (−170 to +24) may share common properties with the *cab*/*rbcS* group genes characteristically activated by calcium/calmodulin (Neuhaus *et al.*, 1993; see introduction), although we currently have no data to indicate that the GATA LE unit of this gene (Figure 4b) functions as a LRE. Our gain-of-function experiments using a −88 to −57 fragment covering the entire GATA LE unit did not show any light-response (data not shown).

Internal light-responsive element

The most notable finding in this study is that *psaDb* has an internal light-responsive element between +1 and +861. This 861 bp sequence is composed of three parts, including a 5′ leader of 23 bp, a protein coding region of 645 bp (+24 to +668) without introns (Yamamoto *et al.*, 1993), and a 193 bp sequence downstream of the termination codon (Figure 4a). Since the 5′ region from +1 to +35, which includes the entire 5′ leader and initiation codon context, did not confer any light-responsiveness to the

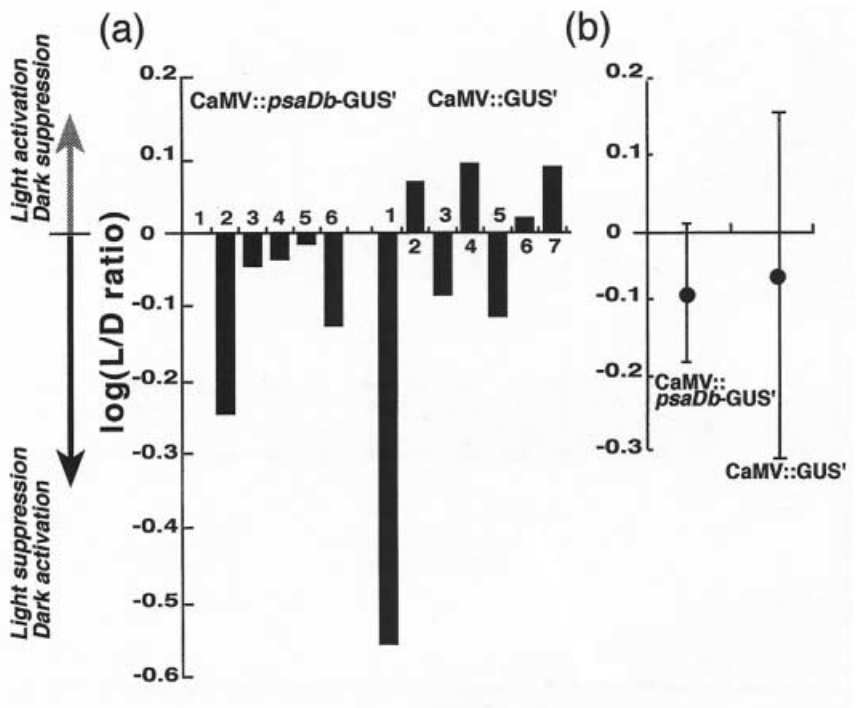


Figure 6. Light/dark response of the CaMV::*psaDb*::*GUS*' and CaMV::*GUS*' constructs.
(a) Light/dark GUS activity ratios of the transgenic seedlings. The M_1 seedlings were grown in the light or dark for 6 days, then GUS activity was determined for individual transgenic lines. (b) Average values and standard deviations of the values in (a).

GUS reporter gene (Figure 6), the internal LRE is likely to be located downstream.

The pea ferredoxin gene, *Fed-1*, is a known unique case of an internal light-responsive element that is located within the protein-coding region, and this element is thought to affect mRNA stability (Dickey *et al.*, 1992, 1994). *psaDb* and *Fed-1* have several similarities: both genes carry internal LREs, do not have introns (Elliott *et al.*, 1989b; Yamamoto *et al.*, 1993), respond to light very quickly (Kaufman *et al.*, 1986; Yamamoto *et al.*, 1995a), and have upstream LREs (Gallo-Meagher *et al.*,1992). What is more interesting is that the protein encoded by *psaDb* is the docking site of ferredoxin on the stromal surface of PSI (Bryant, 1992; Golbeck, 1992; Yamamoto *et al.*, 1993). Hence, it is not surprising that *psaDb* and *Fed-1* share similar regulatory mechanisms, and the internal LRE of *psaDb* may also function as a light-dependent mRNA stabilizer. A recent study suggests that light-responsive stabilization of the *Fed-1* message requires a translatable reading frame (Dickey *et al.*, 1994). However, the possibility still remains that the *psaDb* internal LRE modulates transcription rather than mRNA stability. Further analysis is required to clarify this point.

Despite the many similarities between *psaDb* and *Fed-1*, we should note that their internal LREs exhibited one clear difference: the internal LRE of *psaDb* exerts its function not in green leaves but in etiolated seedlings (Table 1), while that of *Fed-1* plays a more prominent role in green leaves than in etiolated seedlings (Gallo-Meagher *et al.*, 1992). The molecular mechanism and biological significance concerning this difference deserve further attention.

Translational and post-translational regulation

psaDb mRNA accumulates in etiolated seedlings, although its translation product is not detected until greening proceeds under light illumination (Yamamoto *et al.*, 1995a). This indicates that translation or a post-translational event(s) are arrested in etiolated seedlings. How does light turn on one or both of these processes? The 5′ leader of *psaDb* contains a unique translational enhancer element which has characteristic motifs in common with the 5′ leader of the *Arabidopsis* ferredoxin gene (Yamamoto *et al.*, 1995b). In this study, we examined the hypothesis that translation initiation of *psaDb* mRNA is light-dependent by virtue of this translational enhancer or some initiation codon context. However, as shown in Figure 6, the 5′ leader or initiation codon context of *psaDb* did not cause any light activation of the translational fusion gene, indicating that the above hypothesis of translation initiation is unlikely. It might be possible that the light-dependent translational control occurs at an elongation step rather than translation initiation, but we have no data to support this point. The above-mentioned arrest in etiolated seedlings can also be explained by another possible mechanism: the *psaDb* message might be translated even in etiolated seedlings, but its translation products may be rapidly degraded when unable to assemble into a stable PSI complex. This explanation seems simple and more likely, since many chloroplast proteins of multi-protein complexes are known to be degraded when unable to assemble (Gruissem and Tonkyn, 1993). The physiological role of the translational enhancer in the *psaDb* leader (Figure 4a; Yamamoto *et al.*, 1995) remains unclear.

Combinatorial control by the upstream and internal cis*-elements*

The internal LRE of *psaDb* is, to our knowledge, the first light regulatory element whose function has been detected in etiolated seedlings but not in green leaves. Light-induction of *psaDb* mRNA occurs by two steps in both etiolated and green leaves: one is a very quick response within 1–2 h after light exposure, and the other is a slower response with a lag period of 1–2 h that is commonly observed for the nuclear-encoded PSI genes (Yamamoto *et al.*,1995a). As the internal LRE does not work in green leaves (Table 1), it is unlikely that this element causes the quick response. In greening of etiolated seedlings, the mRNA level of the intrinsic *psaDb* gene increases about sevenfold in 6 h (Yamamoto *et al.*, 1995a), while the mRNA derived from CaMV::*psaDb* increases only twofold even in 8 h (Table 1). Hence, the contribution of the internal LRE accounts for only a part of the total light-response even in etiolated tissues. The combination of the upstream and internal LREs seems necessary to fulfil the total magnitude of the light response, as well as to generate complex tissue-specific and light-responsive expression of this gene. Further analysis of these LREs should shed light on the molecular diversity of photo-induction mechanisms in plant nuclear genes.

Experimental procedures

Construction of chimeric genes

psaDb::GUS *and its 5′ deletion mutants.* The *Fok*I fragment of yaDG20 (Yamamoto *et al.*, 1993), which contains the promoter and 5′ flanking region of *psaDb* (–2074 to +24 relative to the transcription start site), was blunt-ended and subcloned into the *Bam*HI site of pBI221 (Jefferson *et al.*, 1987) . The resultant construct, CaMV-*psaDb*::*GUS*, was cut with *Pst*I and *Xba*I, and digested with exonuclease III (Ausubel *et al.*, 1987) to obtain the *psaDb*::*GUS* construct as well as its 5′ deletion derivatives. From the resultant plasmids, *Hin*dIII–*Eco*RI fragments containing the *psaDb* fusions were subcloned into a binary vector pBI101 (Jefferson *et al.*, 1987).

CaMV::psaDb. The transcribed region of *psaDb* (+1 to +861) was amplified by PCR (Ausubel *et al.*, 1987) with specific primers containing an *Xba*I site, and cloned into pBluescript II vector (Stratagene). After checking the sequence of the generated clone, the insert was subcloned into the *Xba*I site of pBI121G$^-$, a derivative of pBI121 in which a *Sma*I–*Sac*I fragment containing the GUS coding region has been deleted.

CaMV::psaDb–GUS′ *and CaMV*::GUS′. These fusion genes were constructed as described previously by Yamamoto *et al.*, (1995b).

Tobacco transformation

Binary vectors containing the chimeric genes were introduced into *Agrobacterium tumefaciens* LBA4404, and used for the transformation of tobacco (*Nicotiana tabacum* cv. Petite Havana SR1) as previously described (Yamamoto *et al.*, 1995b). Regenerated M_0 plants were grown in a greenhouse and allowed to self. Because the insides of sheaths are usually germ-free, we carefully harvested M_1 seeds and sowed them on agar plates without surface sterilization. No contamination by fungi or bacteria was observed in these plates.

Light treatment of the transgenic tobacco seedlings and GUS assay

About 50–100 seeds of the M_1 generation from an independent transformant were sown on 0.8% agar plates containing half-strength MS medium and 100 μg ml^{-1} kanamycin. These plates were initially placed under white fluorescent light for 12 h in order to promote germination, and then kept in darkness or under the continuous white fluorescent light (70 μE m^{-2} sec^{-1}) for 6 days. After that, liquid N_2 was poured directly onto the Petri plates, and portions of the seedlings not embedded in the agar were harvested. GUS activity was determined as described previously by Yamamoto *et al.* (1995b).

Light treatment of CaMV::psaDb *transformants and RNA analysis*

Regenerated M_0 plants carrying CaMV::*psaDb* were cultured in Magenta culture boxes (Sigma) under continuous light. Plants with leaves of 3–6 cm were dark-adapted for 4 days, then re-exposed to white fluorescent light (70 μE m^{-2} sec^{-1}) for 8 h. Leaves were harvested before and after the 8 h light treatment, frozen in liquid N_2, and subjected to RNA extraction by a modified AGPC method (Yamamoto *et al.*, 1995a). Handling in the dark was carried out quickly under green safe light from fluorescent tubes (Toshiba FLR20G/A) wrapped with 12 layers of green plastic film. For the analysis of M_1 seedlings, 50–200 seeds of the M_1 generation harvested from independent transformants were sown on 0.8% agar plates containing half-strength MS medium and 100 μg ml^{-1} kanamycin. These plates were initially placed under white fluorescent light for 12 h in order to promote germination, then kept in darkness for 6 days. Some of these etiolated seedlings were illuminated for 8 h under fluorescent tubes (70 μE m^{-2} sec^{-1}). Before and after this 8 h light treatment, liquid N_2 was directly poured onto the Petri plates, and portions of the seedlings not embedded in the agar were subjected to RNA extraction. Primer extension analysis was carried out as described previously by Yamamoto *et al.* (1995b), and radioactivity of each band was analyzed using a Fujix BAS2000 (Fuji Photo Inc., Japan).

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References

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1987) *Current Protocols in Molecular Biology.* New York: John Wiley.

Batschauer, A., Gilmartin, P.M., Nagy, F. and Schafer, E. (1994) The molecular biology of photoregulated genes In *Photomorphogensis in Plants*, 2nd Edn (Kendrick, R.E. and Kronenberg, G.M.H., eds). Dordrecht: Kluwer Academic Publishers, pp. 559–599.

Berry, J.O., Carr, J.P. and Klessig, D.F. (1988) mRNA encoding ribulose-1,5-bisphosphate carboxylase remain bound to polysomes but are not translated in amaranth seedlings transferred to darkness. *Proc. Natl Acad. Sci. USA*, **85**, 4190–4194.

Berry, J.O., Breiding, D.E. and Klessig, D.F. (1990) Light-mediated control of translational initiation of ribulose-1,5-bisphosphate carboxylase in Amaranth cotyledons. *Plant Cell*, **2**, 795–803.

Bowler, C., Neuhaus, G., Yamagata, H. and Chua, N.-H. (1994a) Cyclic GMP and calcium mediate phytochrome phototransduction. *Cell*, **77**, 73–81.

Bowler, C., Yamagata, H., Neuhaus, G. and Chua, N.-H. (1994b) Phytochrome signal transduction pathways are regulated by reciprocal control mechanisms. *Genes Devel.* **8**, 2188–2202.

Bryant, D.A. (1992) Molecular biology of photosystem I. In *The Photosystems: Structure, Function and Molecular Biology* (Barber, J., ed.). Amsterdam: Elsevier Science Publishers, pp. 501–549.

Castresana, C., Staneloni, R., Malik, V.S. and Cashmore, A.R. (1987) Molecular characterization of two clusters of genes encoding the type I CAB polypeptides of PSII in *Nicotiana plumbaginifolia*. *Plant Mol. Biol.* **10**, 117–126.

Dean, C., Favreau, M., Bond-Nutter, D., Bedbrook, J. and Dunsmuir, P. (1989) Sequences downstream of translation start regulate quantitative expression of two petunia *rbcS* genes. *Plant Cell*, **1**, 201–208.

Dickey, L.F., Gallo-Meagher, M. and Thompson, W.F. (1992) Light regulatory sequences are located within the 5′ portion of the *Fed-1* message sequence. *EMBO J.* **11**, 2311–2317.

Dickey, L.F., Nguyen, T.-T., Allen, G.C. and Thompson, W.F. (1994) Light modulation of ferredoxin mRNA abundance requires an open reading frame. *Plant Cell*, **6**, 1171–1176.

Elliott, R.C., Dickey, L.F., White, M.J. and Thompson, W.F. (1989a) *cis*-acting elements for light regulation of pea ferredoxin I gene expression are located within transcribed sequences. *Plant Cell*, **1**, 691–698.

Elliott, R.C., Pedersen, T.J., Fristensky, B., White, M.J., Dickey, L.F. and Thompson, W.F. (1989b) Characterization of a single copy gene encoding ferredoxin I from pea. *Plant Cell*, **1**, 681–690.

Flieger, K., Wicke, A., Herrmann, R.G. and Oelmüller, R. (1994) Promoter and leader sequences of the spinach PsaD and PsaF genes direct an opposite light-response in tobacco cotyledons: PsaD sequences downstream of the ATG codon are required for a positive light-response. *Plant J.* **6**, 359–368.

Fluhr, R. and Chua, N.-H. (1986) Developmental regulation of two genes encoding ribulose-bisphosphate carboxylase small subunit in pea and transgenic petunia plants: phytochrome response and blue-light induction. *Proc. Natl Acad. Sci. USA*, **83**, 2358–2362.

Foster, R., Izawa, T. and Chua, N.-H. (1994) Plant bZIP proteins gathered at ACGT elements. *FASEB J.* **8**, 192–200.

Gallo-Meagher, M., Sowinski, D.A., Elliott, R.C. and Thompson, W.F. (1992) Both internal and external regulatory elements control expression of the pea *Fed-1* gene in transgenic tobacco seedlings. *Plant Cell*, **4**, 389–395.

Golbeck, J.H. (1992) Structure and function of photosystem I. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **43**, 293–324.

Green, P.J., Yong, M-H., Cuozzo, M., Kano-Murakami, Y., Silverstein, P. and Chua, N.-H. (1988) Binding site requirements for pea nuclear protein factor GT-1 correlate with sequences required for light-dependent transcriptional activation of the *rbcS*-3A gene. *EMBO J.* **7**, 4035–4044.

Grob, U. and Stüber, K. (1987) Discrimination of phytochrome dependent light inducible from non-light inducible plant genes. Prediction of a common light-responsive element (LRE) in phytochrome dependent light inducible plant genes. *Nucl. Acids Res.* **15**, 9957–9973.

Gruissem, W. and Tonkyn, J.C. (1993) Control mechanisms of plastid gene expression. *Crit. Rev. Plant Sci.* **12**, 19–55.

Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. (1987) *GUS* fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* **6**, 3901–3907.

Kaiser, T., Emmler, K., Kretsch, T., Weisshaar, B., Schäfer, E. and Batschauer, A. (1995) Promoter elements of the mustard *CHS1* gene are sufficient for light regulation in transgenic plants. *Plant Mol. Biol.* **28**, 219–229.

Katagiri, F., Lam, E. and Chua, N.-H. (1990) Two tobacco DNA-binding proteins with homology to the nuclear factor CREB. *Nature*, **340**, 727–730.

Kaufman, L.S., Roberts, L.L., Briggs, W.R. and Thompson, W.F. (1986) Phytochrome control of specific mRNA levels in developing pea buds. *Plant Physiol.* **81**, 1033–1038.

Lam, E. and Chua, N.-H. (1990) GT-1 binding site confers light-responsive expression in transgenic tobacco. *Science*, **248**, 471–474.

Morelli, G., Nagy, F., Fraley, R.T., Rogers, S.G. and Chua, N.-H. (1985) A short conserved sequence is involved in the light-inducibility of a gene encoding ribulose 1,5-bisphosphate carboxylase small subunit of pea. *Nature*, **315**, 200–204.

Nakamura, M. and Obokata, J. (1994) Organization of the *psaH* gene family of photosystem I in *Nicotiana sylvestris*. *Plant Cell Physiol.* **35**, 297–302.

Nakamura, M. and Obokata, J. (1995) Tobacco nuclear genes for photosystem I subunits, *psaD*, *psaE* and *psaH* share an octamer motif bound with nuclear proteins. *Plant Cell Physiol.* **36**, 1393–1397.

Neuhaus, G., Bowler, C., Kern, R. and Chua, N.-H. (1993) Calcium/calmodulin-dependent and -independent phytochrome signal transduction pathways. *Cell*, **73**, 937–952.

Obokata, J., Mikami, K., Hayashida, N. and Sugiura, M. (1990) Polymorphism of photosystem I subunit caused by alloploidy in *Nicotiana*. *Plant Physiol.* **92**, 273–275.

Obokata, J., Yamamoto, Y., Kubota, T. and Nakamura, M. (1992) Structure of the nuclear genes coding for photosystem I subunits in *Nicotiana sylvestris*. In *Research in Photosynthesis* III (Murata, N., ed.) Dordrecht: Kluwer Academic Publishers, pp. 367–370.

Obokata, J., Mikami, K., Hayashida, N., Nakamura, M. and Sugiura, M. (1993) Molecular heterogeneity of photosystem I: *psaD*, *psaE*, *psaF*, *psaH* and *psaL* are all present in isoforms in *Nicotiana* spp. *Plant Physiol.* **102**, 1259–1267.

Obokata, J., Mikami, K., Yamamoto, Y. and Hayashida, N. (1994) Microheterogeneity of PSI-E subunit of photosystem I in *Nicotiana sylvestris*. *Plant Cell Physiol.* **35**, 203–209.

Pwee, K.-H. and Gray, J.C. (1993) The pea plastocyanin promoter directs cell-specific but not full light-regulated expression in transgenic tobacco plants. *Plant J.* **3**, 437–449.

Rocholl, M., Talke-Messerer, C., Kaiser, T. and Batschauer, A. (1994) Unit 1 of the mustard chalcone synthase promoter is sufficient to mediate light-responses from different photoreceptors. *Plant Sci.* **97**, 189–198.

Schindler, U., Beckmann, H. and Cashmore, A.R. (1992) TGA1 and G-box binding factors: two distinct classes of Arabidopsis leucine zipper proteins compete for the G-box-like element TGACGTGG. *Plant Cell*, **4**, 1309–1319.

Terzaghi, W.B. and Cashmore, A.R. (1995) Light-regulated transcription. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **46,** 445–474.

Thompson, W.F. and White, M.J. (1991) Physiological and molecular studies of light-regulated nuclear genes in higher plants. *Annu. Rev. Plant. Physiol. Plant. Mol. Biol.* **42,** 423–466.

Tobin, E.M. and Kehoe, D.M. (1994) Phytochrome regulated gene expression. *Semin. Cell Biol.* **5,** 335–346.

Yamamoto, Y., Tsuji, H. and Obokata, J. (1993) Structure and expression of a nuclear gene for the PSI-D subunit of photosystem I in *Nicotiana sylvestris. Plant Mol. Biol.* **22,** 985–994.

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

Yamamoto, Y.Y., Tsuji, H. and Obokata, J. (1995b) 5′ leader of a photosystem I gene in *Nicotiana sylvestris, psaDb,* contains a translational enhancer. *J. Biol. Chem.* **270,** 12466–12470.

Yamazaki, K., Katagiri, F., Imaseki, H. and Chua, N.-H. (1990) TGA1a, a tobacco DNA-binding protein, increases the rate of initiation in a plant *in vitro* transcription system. *Proc. Natl Acad. Sci. USA,* **87,** 7035–7039.