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Light-responsive and transcription-enhancing elements regulate the plastid *psbD* core promoter

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The *psbD* operon of higher plant plastids is regulated transcriptionally through the activity of an upstream light-responsive promoter. To identify promoter elements important for the regulation, portions of the tobacco *psbD* 5′ region were fused to the reporter gene, *uidA*, and were introduced into the tobacco plastid genome by targeted gene insertion. Examination of *uidA* mRNA accumulation in dark-adapted and light-treated transplastomic plants revealed that a 107 bp segment of *psbD* 5′ sequence was sufficient to promote light-responsive expression of the reporter gene *in vivo*. The 107 bp promoter region contains three pairs of short, repeated sequences upstream of the core promoter –10/–35 elements. Deletion of the upstream-most A-rich sequences resulted in a 5-fold decrease in reporter gene mRNA accumulation, but did not affect the light response. Additional removal of the second and third repeated elements further reduced the promoter strength ~30-fold and almost eliminated the light-dependent accumulation of *uidA* transcripts. These data indicate that the architecture of chloroplast promoters is more complex than previously assumed, and may comprise general enhancer and regulatory elements in addition to the core promoter motifs. Transcriptional regulation of *psbD* may be mediated by the chloroplast proteins which were shown to interact with the repeated sequences.

Keywords: light-response element/plastid transformation/*psbD* operon/tobacco/transcriptional enhancer element

Introduction

The *psbD* operon of tobacco plastids is composed of the genes *psbD* and *psbC*, encoding the photosystem II subunits, D2 and CP43 respectively, as well as an open reading frame, ORF62, of unknown function. In mature leaves, in contrast to most plastid genes, regulation of this operon is transcriptional, and is mediated by a promoter responding to high-irradiance blue and UV A light (Gamble and Mullet, 1989; Berends-Sexton *et al.*, 1990; Christopher and Mullet, 1994). The light response of this promoter maintains high levels of *psbD* operon transcripts in mature chloroplasts (Gamble *et al.*, 1988; Baumgartner *et al.*, 1993). Since D2 protein is subject to photo damage, it was proposed that the light-induced accumulation of *psbD* transcripts serves to maintain photosystem II activity under high light conditions (Christopher *et al.*, 1992; Christopher and Mullet, 1994).

To delimit the light-regulated *psbD* promoter, Mullet and coworkers compared sequences 5′ to the *psbD* coding region for several mono- and dicotyledonous plant species. Significant sequence conservation was found over a 90 bp region encompassing the transcription initiation sites, suggesting that the conserved sequences constituted the light-responsive promoter (termed LRP, Christopher *et al.*, 1992). Typical plastid gene promoter regions contain sequences with homology to the canonical –35 (TTGACA) and –10 (TATAAT) elements of bacterial σ70 type promoters (Hanley-Bowdoin and Chua, 1987; Gruissem and Tonkyn, 1993). Mutagenesis has demonstrated the importance of these elements for correctly initiated plastid gene transcription *in vitro* (Bradley and Gatenby, 1985; Gruissem and Zurawski, 1985) and *in vivo* (Klein *et al.*, 1992). Since only poor matches to such consensus elements were found in the conserved region of the putative *psbD* promoter, it was proposed that the region may represent a different class of plastid promoter (Christopher *et al.*, 1992). This would not be unprecedented given that non-canonical promoter elements have been described for a subset of plastid genes from the unicellular green alga *Chlamydomonas reinhardtii* (Klein *et al.*, 1992, 1994). Based on sequence similarity to the upstream regions of several light-regulated nuclear genes, it was speculated that a conserved GATA sequence found near the transcription initiation sites may be involved in the light-responsive transcription from the *psbD* 90 bp region (Christopher *et al.*, 1992). Other conserved and potentially important features of this promoter were noted, including several short, direct repeats found upstream of the initiation region.

To define light-response elements in plastid gene transcription, we have used the tobacco plastid transformation system (Svab *et al.*, 1990; Svab and Maliga, 1993; Zoubenko *et al.*, 1994; reviewed in Maliga, 1993) to characterize the LRP region of the *psbD* gene *in vivo*. We report that a 107 bp DNA sequence surrounding the transcription initiation sites directs light-responsive transcription of a fused chimeric reporter gene, and thus encompasses the LRP. Deletion experiments suggest that basal activity of the *psbD* LRP is directed by sequences which resemble –35 and –10 elements. The overall promoter strength is augmented by an upstream general enhancer sequence containing A-rich repeated sequences. The majority of the light regulation appears to be mediated by additional highly conserved, repeated sequence motifs positioned between the general enhancer element and the core promoter. Therefore, in analogy to some prokaryotic regulated promoters, the activity of weak plastid core promoters may be modulated by general enhancer elements, as well as regulatory sequences.

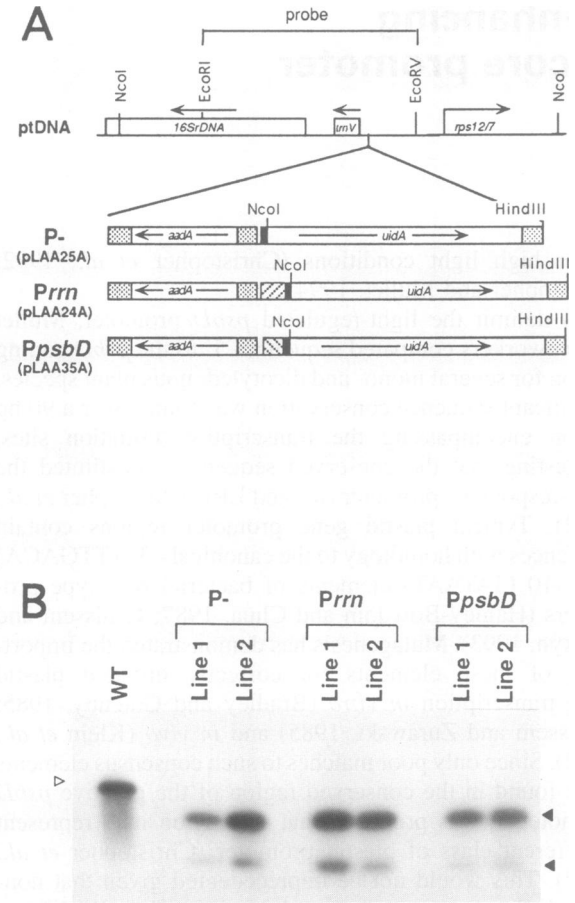

Fig. 1. (**A**) Partial map of the plastid genome (ptDNA) with the integrated transgenes from plasmids pLAA25A, pLAA24A and pLAA35A. *16S rDNA*, *trnV* and *rps12/7* are plastid genes (Shinozaki *et al.*, 1986); *aadA* codes for spectinomycin resistance, *uidA* for the GUS enzyme. The *uidA* genes are expressed from different 5′ regions (P–, P*rrn* and P*psbD*) as indicated by patterned boxes, and as described in the text. The *Eco*RI–*Eco*RV fragment of the wild-type ptDNA, which was used as a probe in DNA gel-blots, is depicted. Positions of relevant *Eco*RI, *Eco*RV, *Nco*I and *Hin*dIII restriction sites are shown. Arrows indicate direction of transcription. Not to scale. (**B**) Identification of homoplasmic plastid transformants. DNA gel-blot on total plant DNA from two plant lines transformed with each construct. DNA was digested with *Nco*I and *Hin*dIII. The probe hybridizes to a 6.4 kb fragment in wild-type ptDNAs (open arrowhead), and to 4.5 and 2.9 kb fragments in the transformed genomes (closed arrowheads). Note the absence of wild-type genomes in transformed lines.

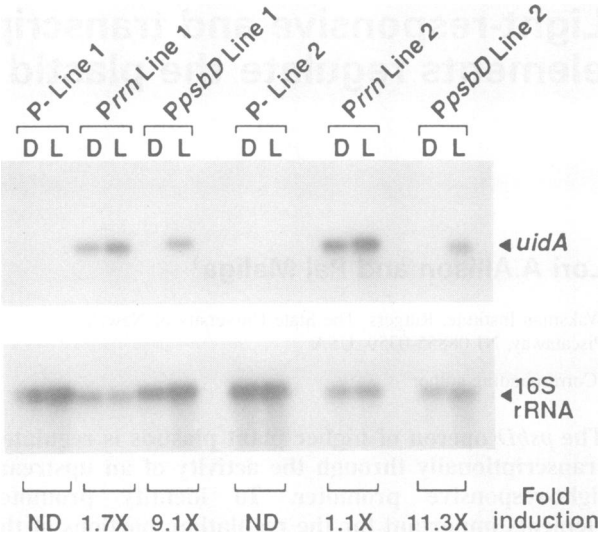

Fig. 2. Light-responsive expression of *uidA* mRNA from the 107 bp *psbD* promoter fragment. Northern analysis on total RNA from dark-adapted (D) and light-treated (L) leaves of plants transformed with *uidA* genes transcribed by: P–, no promoter; P*rrn*, the constitutive promoter of the 16S rRNA operon; P*psbD*, the *psbD* promoter. RNA loaded per lane was: P–, 2 μg; P*rrn*, 0.5 μg; P*psbD*, 2 μg. Blots were probed with a *uidA* coding region probe. Subsequently they were stripped and reprobed with a *16S rDNA* probe. Fold accumulation of the *uidA* message in the light versus the dark was quantitated using a PhosporImager, correcting for the 16S rRNA loading control. Data are shown for two independently transformed lines per reporter construct. In the absence of *uidA* signal, the fold increase for the P– construct was not determined (ND).

Results

A 107 bp sequence of the psbD upstream region directs light-responsive accumulation of uidA mRNA in transgenic plastids

To identify *psbD* sequences containing a functional LRP, a 107 bp DNA fragment containing the conserved 90 bp *psbD* region was fused with a synthetic ribosome binding site (rbs) to yield the *psbD* promoter fragment, termed P*psbD*. In plasmid pLAA35A (Figure 1A) P*psbD* is engineered upstream of the *uidA* coding region, encoding β-glucuronidase (GUS). To stabilize the mRNAs, the 3′-untranslated region of the plastid *rps16* gene (T*rps16*) was cloned downstream of the *uidA* coding region to yield the P*psbD*::*uidA*::T*rps16* chimeric gene. As controls, additional *uidA* reporter genes were constructed: the *uidA* gene in plasmid pLAA25A has no promoter region (P–), only a ribosome binding site (rbs). This construct is referred to as rbs::*uidA*::T*rps16*. The *uidA* gene in plasmid pLAA24A contains the constitutive promoter of the plastid rRNA operon (P*rrn*) driving the expression of the P*rrn*::*uidA*::T*rps16* gene. The chimeric *uidA* genes, linked to a selectable spectinomycin resistance (*aadA*) gene (Figure 1A), were introduced into the inverted repeat region of the tobacco plastid genome by the biolistic process. Transformed shoots, selected on spectinomycin-containing media, were tested for the presence of the transgene by DNA gel-blot analysis, using as probe a portion of the plastid genome insertion site. Homoplasmic transformed lines were recovered for each reporter construct, as evidenced by the presence of two hybridizing bands characteristic of transformed plastid genomes, and the absence of wild-type genome copies (Figure 1B). Two independently transformed lines for each construct were selected for further characterization.

To examine the light response of *uidA* mRNA accumulation in the transgenic plant lines, dark-adapted duplicate cuttings of each line were separated into two pools, one of which was maintained in the dark for 20 h, the other of which was subjected to constant illumination for the same time period. This light regime is sufficient to induce transcription from the endogenous tobacco *psbD* LRP (Christopher *et al.*, 1992). Total RNA from expanded leaves of the dark-adapted (D) and light-treated (L) cuttings was analyzed on RNA gel-blots, using a *uidA* coding region probe to detect reporter gene mRNAs. In Figure 2 data are shown for two independently transformed lines for each construct. In plastid transformants carrying the

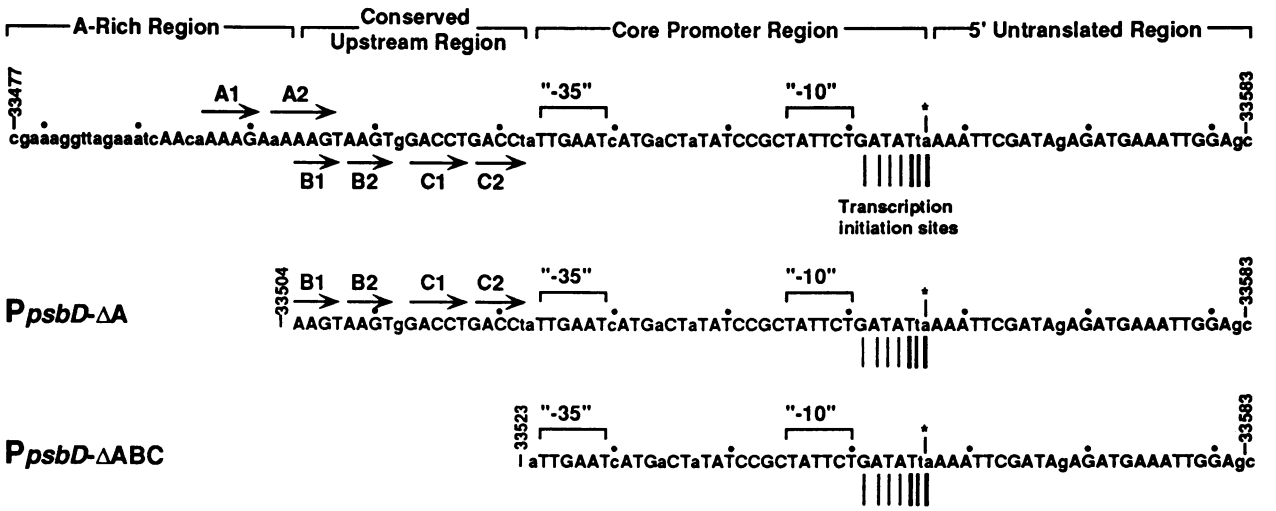

Fig. 3. Architecture of the *psbD* light-responsive promoter. DNA sequence of a 107 bp region encompassing P*psbD* (top) and the P*psbD*-ΔA and P*psbD*-ΔABC deletion derivatives. Shown are nucleotides 33 477–33 583 of the ptDNA (Shinozaki *et al.*, 1986); the conserved 90 bp *psbD* 5′ region is from nucleotides 33 492 to 33 581 (Christopher *et al.*, 1992). Capital letters depict bases of the tobacco sequence which are identical with the consensus derived from a comparison of eight plant species (Christopher *et al.*, 1992). The sequence comparison did not include the region 33 477–33 491 which is shown in lower case here. Putative –35 and –10 sequences are marked. Three pairs of short, direct repeats upstream of the core promoter motifs are underlined. Transcription initiation sites mapped previously for the endogenous *psbD* gene (Christopher *et al.*, 1992), and in this study for the chimeric constructs, are marked by bars. The thickness of the bars represents the relative intensity of the primer extension products. The single transcription initiation site mapped by Yao *et al.* (1991) is marked with an asterisk. The 107 bp fragment is divided into four regions as discussed in the text: A-rich region (33 477–33 503), conserved upstream region (33 504–33 522), core promoter region (33 524–33 557) and 5′-untranslated region (33 558–33 583).

P*psbD* reporter fusion, the steady-state levels of *uidA* mRNA were dependent on the light regime used: an increase of ~10-fold in *uidA* message levels was observed upon illumination (Figure 2; for P*psbD* compare lanes D and L). The induction was reproducible and ranged between 6- and 11-fold in different experiments. Therefore the 107 bp fragment encompassing the *psbD* light-regulated transcription initiation region was sufficient to drive light-responsive accumulation of a chimeric reporter gene *in vivo*.

In contrast, in lines transformed with the reporter gene fused to the constitutively active promoter, P*rrn*, the light regime had little effect on the steady-state levels of *uidA* mRNA: a 1- to 2-fold increase in transcript levels occurred in response to light (Figure 2; for P*rrn* compare D versus L). These data demonstrate that the light response exhibited by the P*psbD* reporter gene is not due simply to a general light-induced increase in plastid transcription. In control lines containing the promoterless reporter construct, no *uidA* transcripts were detected (Figure 2; P– lanes; Zoubenko *et al.*, 1994), indicating that there is no strong dark- or light-active endogenous plastid promoter upstream of the introduced transgene, contributing to accumulation of transgene message. It should be noted, however, that a minor amount of readthrough transcription does occur, and can be detected when the more sensitive RNase protection assay is used (see below).

The A-rich region in the psbD light-responsive promoter is required for full promoter strength

The sequence of the 107 bp light-responsive *psbD* promoter region is shown in Figure 3. We have marked putative –35 and –10 elements. Although these sequence motifs have sub-optimal spacing with respect to each other (15 bp rather than the optimal 17–19 bp), their similarity to the prokaryotic consensus motifs, as well as their strong conservation among mono- and dicotyledonous plant species, suggested to us their importance as core promoter elements. Immediately upstream of these putative core sequences are two sets of short elements each repeated once, which we refer to as the B and C repeats. These highly conserved repeat motifs were noted previously by Christopher *et al.* (1992) as sequence elements a,a′ and b,b′ respectively. Upstream of and partially overlapping the B elements are additional short direct repeats which we refer to as the A repeats. These are part of a sequence which is notably rich in A residues (16 As in 27 nucleotides). A portion of this region (positions 33 492–33 503) was included in previous sequence alignments (Christopher *et al.*, 1992) in which it was shown that the A-rich nature of the sequence is conserved in all plant species examined.

To determine whether the A-rich region is important for LRP function, a 5′ deletion was made removing all sequences upstream of the conserved B and C repeats (diagrammed in Figure 3). The promoter deletion derivative (P*psbD*-ΔA) was fused to the promoterless rbs::*uidA*::T*rps16* construct and introduced into the inverted repeat region of tobacco plastid DNA (ptDNA). Transgenic lines were generated and homoplasmy was confirmed by DNA gel-blot analysis (data not shown). The accumulation of *uidA* mRNAs was examined in leaves of two independently regenerated P*psbD*-ΔA transgenic plants (referred to as lines 1 and 2) grown in cycling light conditions (Figure 4A). In comparison with two plant lines carrying the P*psbD* construct, the steady-state levels of reporter transcript were ~5-fold lower in the P*psbD*-ΔA lines (*uidA* signal normalized to the 16S rRNA loading control). The A-rich element therefore is required for full promoter strength.

To determine the role of the A-rich element in the

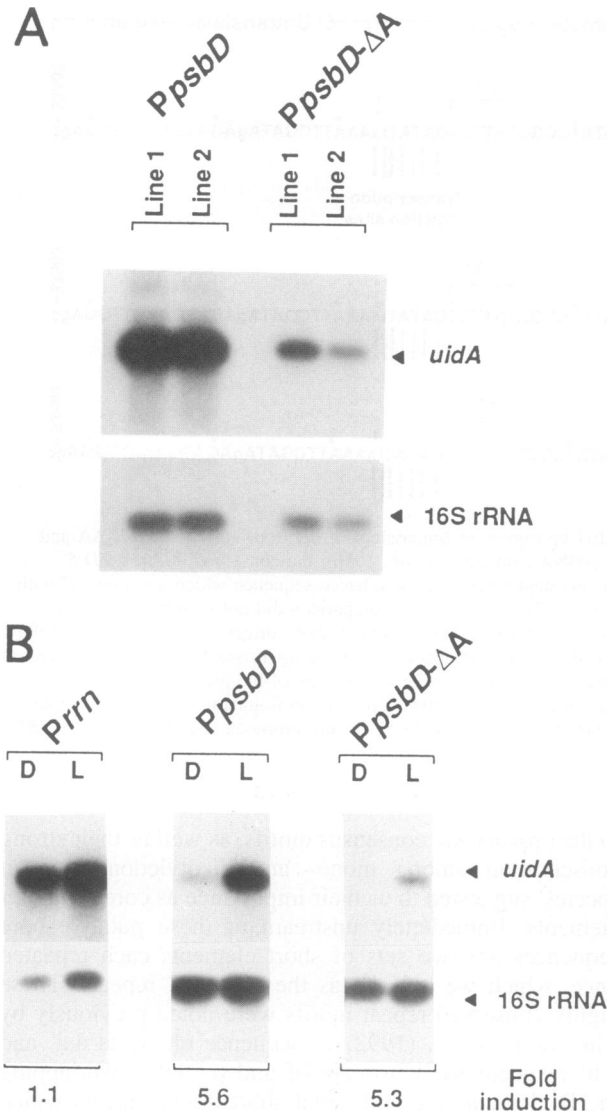

Fig. 4. Expression of *uidA* from P*psbD* and the P*psbD*-ΔA deletion derivative. (**A**) Steady-state levels of *uidA* mRNA in leaves of plants grown in cycling light (16 h light/8 h dark per cycle). Data are shown for two independently transformed lines. Two μg of total cellular RNA were loaded per lane. Blots were probed with a *uidA* coding region probe, and subsequently stripped and reprobed with a *16S rDNA* probe. (**B**) Light-dependent accumulation of *uidA* mRNA from dark-adapted (D; 3 days) and light-treated (L; 24 h) transgenic plants. RNA was loaded per lane as follows: P*rrn*, 0.5 μg; P*psbD* and P*psbD*-ΔA, 2 μg. Blots were probed with a *uidA* coding region probe, then were stripped and reprobed with the *16s rDNA* locus. Fold increase in *uidA* mRNA levels from the dark to the light was quantitated using a PhosphorImager, correcting for the 16S rRNA signal.

light response, transgenic plants were subjected to dark adaptation for 3 days, followed by continuous light treatment for 24 h. Levels of *uidA* mRNA, detected by RNA gel-blot analysis, were quantitated by normalizing to 16S rRNA abundance (Figure 4B). Although the absolute levels of *uidA* message in the dark and the light were reduced in this promoter deletion, the extent of induction by light treatment was comparable with that of the full-length P*psbD* promoter (compare P*psbD* and P*psbD*-ΔA lanes). These data suggest that the upstream A-rich sequences act to enhance promoter strength, but do not play a major role in the regulation of the promoter by light.

The conserved upstream region is required for light induction

To examine the role of the B and C repeats in *psbD* promoter function, a second promoter deletion derivative was generated (Figure 3) in which all sequences upstream of the –35 consensus element were removed. This deletion derivative (P*psbD*-ΔABC) was fused to the promoterless rbs::*uidA*::T*rps16* construct and used to generate homoplasmic transgenic plants. Transcripts from this promoter derivative were difficult to detect above background noise on RNA gel-blots. Therefore we used the more sensitive RNase protection assay to detect transcripts accumulating from this promoter. In light-grown plants, the mRNA from P*psbD*-ΔABC accumulated to ~150-fold lower levels than from P*psbD* (in Figure 5B, compare signals from lane P*psbD* L, 0.5 μg of RNA loaded, with P*psbD*-ΔABC L, 10 μg of RNA loaded). To examine the light response, transgenic plants were subjected to dark adaptation for 3 days, followed by continuous light treatment for 24 h. Quantitation of *uidA* mRNA levels indicated that the P*psbD*-ΔABC deletion derivative exhibited only a minor ~2-fold induction of reporter gene expression in response to light (Figure 5B, compare P*psbD*-ΔABC, D and L lanes). This result was reproduced with three independently transformed lines. The 2-fold induction produced by this promoter deletion upon illumination was only slightly higher than that previously measured for the control constitutive promoter transgene, P*rrn* (Figure 2). Therefore the conserved upstream region containing the B and C repeat motifs is essential for the full light response of the *psbD* promoter.

With the sensitive RNase protection assay, we detected additional *uidA*-containing transcripts, larger in size than predicted for the reporter gene (marked by stars in Figure 5B). These *uidA* mRNAs were also detectable in the plants containing the promoterless *uidA* reporter gene (Figure 5C, left panel). By primer extension, we mapped the 5′ ends of these low abundance messages to within the regulatory sequences of the selectable *aadA* gene, which was introduced into the plastid genome physically linked upstream of the *uidA* reporter constructs (diagrammed in Figure 5A). The abundance of these readthrough transcripts was proportional to the input RNA (Figure 5B), and was not regulated by the light regimes used in these experiments (Figure 5C). Therefore, we used these readthrough mRNAs as internal standards to normalize the amount of RNA present in each dark-adapted and light-treated RNA pair.

Sequences downstream of the repeat elements function as the psbD core promoter

The most severe promoter deletion (P*psbD*-ΔABC) contains only the putative –35/–10 core elements and sequences downstream (Figure 3C). To establish whether transcription from this truncated promoter initiated at the same site as the full-length promoter, the *uidA* transcript 5′ ends were mapped by primer extension. In lines carrying the P*psbD* promoter, a cluster of 5′ ends for the reporter transcript was mapped to the same region as previously reported for the endogenous *psbD* light-regulated primary transcripts in tobacco (Christopher *et al.*, 1992; Figure 6). Primer extension on *uidA* RNA derived from the P*psbD*-ΔABC promoter demonstrated that the same set of 5′ ends

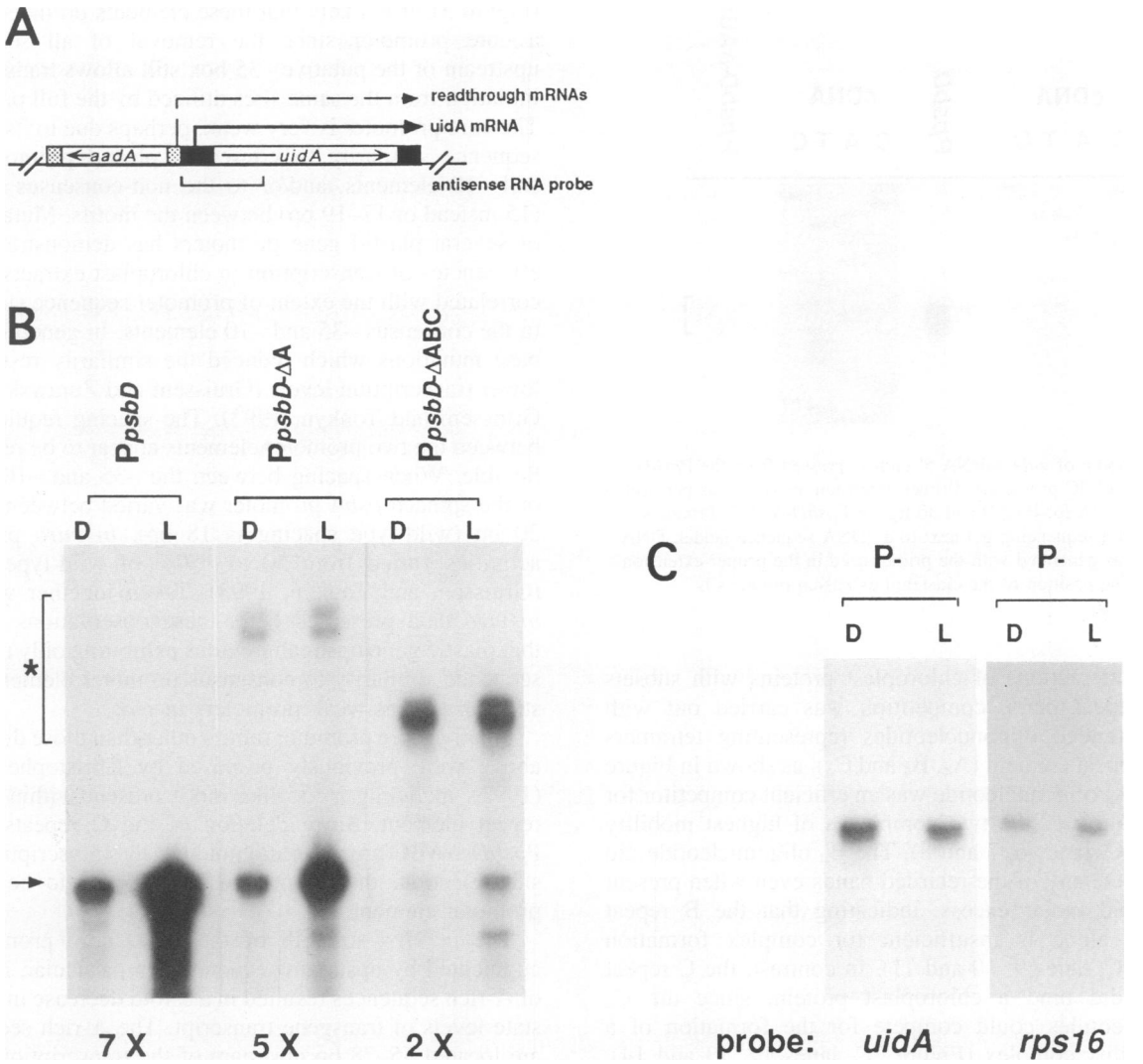

Fig. 5. RNase protection analysis of *uidA* mRNA accumulation in dark-adapted and light-treated transgenic plants. (**A**) Diagram of transcripts detected with the *uidA* probe. Shown are the linked *aadA* and *uidA* transgenes. The region of *uidA* used to generate the antisense RNA probe is bracketed. Transcripts initiating from the introduced test promoters (P*psbD*, P*psbD*-ΔA and P*psbD*-ΔABC) are depicted by a solid arrow. Readthrough transcripts initiating from within the upstream *aadA* expression sequences are indicated by a dashed arrow. (**B**) Accumulation of *uidA* mRNA (arrow) from *psbD* promoter derivatives in the leaves of dark-adapted (D; 3 days) and light-treated (L; 24 h) plants. Numbers below give the fold increase of *uidA* signal in the light versus in the dark, and are corrected for loading with the readthrough transcripts (region starred). Note that the sizes of the readthrough transcripts depend upon the size of the downstream promoter derivatives: larger deletions of P*psbD* promoter sequence result in smaller readthrough transcripts. The amounts of RNA per reaction were: P*psbD*, 0.5 μg; P*psbD*-ΔA, 5 μg; P*psbD*-ΔABC, 10 μg. Note that readthrough transcripts in the P*psbD* lanes are faint, due to the lower amounts of RNA used in these assays. (**C**) The accumulation of readthrough transcripts is not dependent on light. RNase protection assays with the *uidA* (left) and *rps16* (right) probes were carried out on dark-adapted (D; 3 days) and light-treated (L; 24 h) P– plants with 10 μg of RNA. The *rps16* probe detects transcripts from the constitutive plastid ribosomal protein gene, *rps16*, and serves as a loading control for amounts of RNA in the D and L samples.

was generated as with the P*psbD* promoter (Figure 6). These data strongly suggest that the –35/–10 homologies do indeed function as P*psbD* core promoter elements. In addition, since each P*psbD* promoter derivative generated identical *uidA* transcripts, we could rule out differential mRNA stability as the cause of reduced *uidA* mRNA accumulation with the deleted promoters.

Binding of chloroplast protein factors to the psbD promoter repeat elements

The deletion experiments demonstrated that conserved repeated sequences located immediately upstream of the *psbD* core promoter are essential for light-responsive transcription *in vivo*. If the light-responsive sequences function by interacting with transcriptional activator proteins, we should be able to detect chloroplast proteins binding to these DNA sequences. To investigate this question, a 28 bp DNA sequence containing the A, B and C repeat pairs ($A_2B_2C_2$) was used as a probe in gel mobility-shift assays (Figure 7). Incubation of the probe with tobacco chloroplast extract resulted in the formation of several complexes (Figure 7A, lane 2). The detected complexes were not due to interactions with non-specific DNA binding proteins since they could be competed by an excess of unlabeled $A_2B_2C_2$ DNA (Figure 7A lane 3), but not by an excess of a non-specific double-stranded oligonucleotide of similar length (Figure 7A, lane 4). To determine whether specific complexes were formed

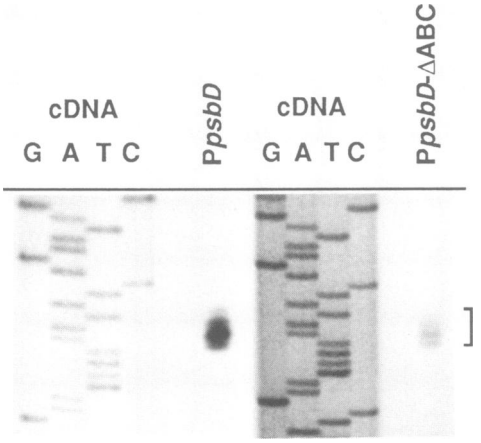

Fig. 6. Mapping of *uidA* mRNA 5′ ends expressed from the P*psbD* and P*psbD*-ΔABC promoters. Primer extension analysis was performed on 2 μg of RNA for P*psbD* and 30 μg for P*psbD*-ΔABC. Products were run on a sequencing gel next to a cDNA sequence ladder. DNA sequence was generated with the primer used in the primer extension reactions. The position of the clustered extension products is bracketed.

through interactions of chloroplast proteins with subsets of the repeat pairs, competition was carried out with double-stranded oligonucleotides representing tetramers of each repeat element (A_4, B_4 and C_4), as shown in Figure 7C. The A_4 oligonucleotide was an efficient competitor for the formation of the two complexes of highest mobility (Figure 7C, lanes 6, 7 and 8). The B_4 oligonucleotide did not compete any of the retarded bands even when present at 800-fold molar excess, indicating that the B repeat sequence alone is insufficient for complex formation (Figure 7C, lanes 9, 10 and 11). In contrast, the C repeat element did bind a chloroplast protein, since the C_4 oligonucleotides could compete for the formation of a low-mobility complex (Figure 7C, lanes 12, 13 and 14). The same low-mobility complex was partially competed by the A_4 oligonucleotide (Figure 7C, lane 8).

Discussion

This work presents the first *in vivo* analysis of a regulated plastid gene promoter from a higher plant. Our results establish that a conserved region of 5′ *psbD* operon sequence is sufficient to regulate the expression of a heterologous reporter gene in response to light. The chimeric transcripts have the same set of 5′ ends as do the light-regulated transcripts of the endogenous tobacco *psbD* operon. The 107 bp conserved region most likely encompasses the entire *psbD* light-responsive plastid gene promoter, since a larger promoter fragment, including 37 bp of additional upstream and 227 bp of additional downstream sequence, responded to light to a similar degree (data not shown). The 6- to 11-fold induction observed in this study is similar to the fold induction estimated for the endogenous *psbD* operon of rice (11.4-fold, Kapoor *et al.*, 1994) and wheat (5-fold, Kawaguchi *et al.*, 1992).

The sequence of the *psbD* promoter contains elements which loosely resemble the consensus prokaryotic-like –35 and –10 promoter motifs spaced at an appropriate distance from the major transcription initiation sites (Figure 3). It is likely that these elements do indeed form a core promoter since the removal of all sequences upstream of the putative –35 box still allows transcription initiation from the same sites utilized by the full promoter. The core promoter is very weak, perhaps due to its limited sequence similarity to consensus plastid promoter –35 and –10 elements, and/or to the non-consensus spacing (15 instead of 17–19 bp) between the motifs. Mutagenesis of several plastid gene promoters has demonstrated that efficiencies of transcription in chloroplast extracts can be correlated with the extent of promoter sequence similarity to the consensus –35 and –10 elements. In general, single base mutations which reduced the similarity resulted in lower transcription levels (Gruissem and Zurawski, 1985; Gruissem and Tonkyn, 1993). The spacing requirements between the two promoter elements appear to be relatively flexible. When spacing between the –35 and –10 motifs of the spinach *psbA* promoter was varied between 17 and 20 bp (wild-type spacing is 18 bp), *in vitro* promoter activities varied from 50 to 150% of wild-type values (Gruissem and Tonkyn, 1993). Taken together with the *in vivo* data presented here, these observations suggest that plastid gene upstream regions exhibiting only minimal sequence similarity to consensus promoter elements may still function as weak promoters *in vivo*.

Putative core promoter motifs other than those discussed above were previously proposed by Christopher *et al.* (1992), including a –35-like motif present within our C1 repeat element. Since deletion of the C repeats in the P*psbD*-ΔABC promoter did not alter the transcription start site selection, this motif does not appear to be a core promoter element.

The *in vivo* strength of the *psbD* core promoter is augmented by upstream sequences. In particular, removal of A-rich sequences resulted in a 5-fold decrease in steady-state levels of transgene transcript. The A-rich sequences are located 55–78 bp upstream of the transcription initiation site (defined by Yao *et al.*, 1989 as bp 33 557). In terms of promoter position, sequence composition, and function, they are reminiscent of the recently described third recognition element in prokaryotic promoters, the UP element (Ross *et al.*, 1993). This 20 bp (AT)-rich sequence located upstream of the core *rrnB* P1 promoter in *Escherichia coli*, increases the core promoter strength by a factor of 30 (Rao *et al.*, 1994), but is not required for specific initiation or response to nutritional conditions which regulate the promoter activity (Gourse *et al.*, 1986). The UP element functions through interactions with the C-terminal region of the α-subunit of RNA polymerase (Ross *et al.*, 1993). Specifically, RNA polymerase containing an α-subunit with a single amino acid substitution at Arg265 is unresponsive to the UP element. Interestingly, Arg is conserved at the equivalent position (Sijben-Muller *et al.*, 1986) in the predicted amino acid sequence of the *rpoA* gene from pea, wheat, spinach, tobacco, maize and liverwort (Purton and Gray, 1989). Therefore, we speculate that, at least for one plastid gene promoter, the similarity to prokaryotic promoters can be extended upstream from the –10 and –35 regions to include AT-rich UP sequences.

While promoter strength is mediated by the A-rich region, these sequences do not appear to play a major role in the *psbD* light response. Instead, the regulation by light acts mainly through the B and C repeat sequences adjacent

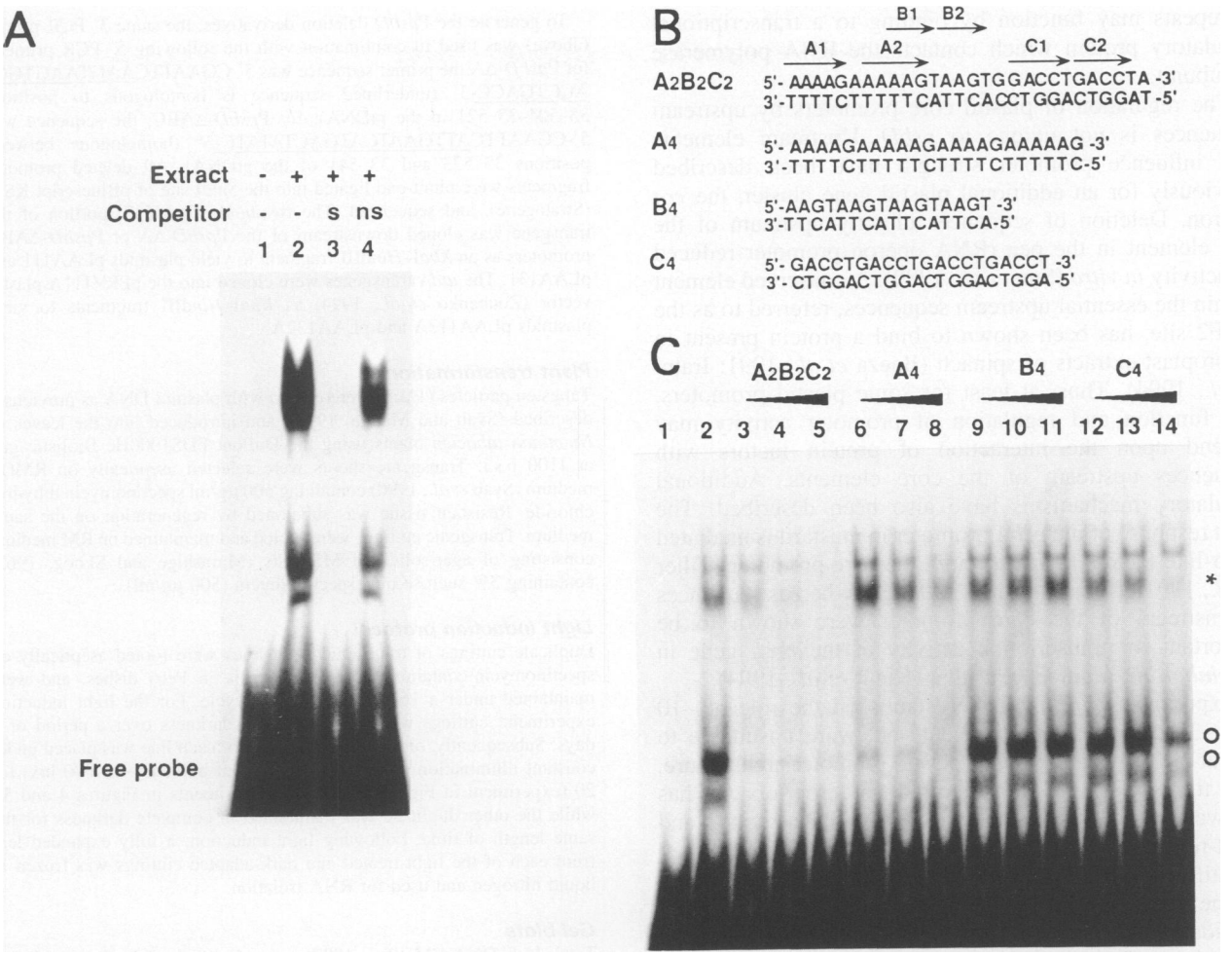

Fig. 7. The P*psbD* upstream repeat elements interact with chloroplast proteins. (**A**) Gel retardation assay with the 28 bp *psbD* upstream sequence $A_2B_2C_2$ (sequence shown in B). An end-labeled double-stranded DNA probe was incubated in the absence (lane 1) or presence (lanes 2–4) of chloroplast protein extract and run on a non-denaturing polyacrylamide gel. The reaction in lane 3 contained a 400-fold molar excess of cold $A_2B_2C_2$ competitor DNA, while that in lane 4 contained the same molar excess of a 34 bp non-specific DNA. (**B**) DNA sequence of oligonucleotides used in the gel retardation experiments. (**C**) Competition of $A_2B_2C_2$ oligonucleotides with multimers of single repeat elements in gel retardation assays. Experiments were carried out with the labeled $A_2B_2C_2$ probe in the absence (lane 2), or presence (lanes 3–14) of cold competitor oligonucleotides. For each competitor, an increasing amount of DNA was preincubated with chloroplast proteins before adding labeled $A_2B_2C_2$ DNA. Competitor amounts were: 40-fold molar excess (lanes 3, 6, 9 and 12), 200-fold molar excess (lanes 4, 7, 10 and 13), and 800-fold molar excess (lanes 5, 8, 11 and 14). Lane 1 contains labeled $A_2B_2C_2$ DNA in the absence of chloroplast extract. Complexes competed by the C_4 oligonucleotide (star), and the A_4 oligonucleotide (circles) are marked. Note that the experiments shown in A and C were carried out with different chloroplast extracts, resulting in minor variations in the relative intensities of the complexes and in their ability to compete.

to the putative –35 element. These elements are well conserved among a number of mono- and dicotyledonous plants (Christopher *et al.*, 1992). Since the repeat sequences bind to a protein(s) present in chloroplast transcription extracts, their role in promoter regulation may be directed by interactions with a transcriptional regulator protein. The binding site for such a factor may be composed of sequences comprising more than just the B and C repeats, since neither of the repeat elements alone was an efficient competitor of protein binding to the $A_2B_2C_2$ probe (Figure 7C). The existence of such regulatory proteins is supported by *in vitro* transcription experiments with the wheat *psbD* promoter (Wada *et al.*, 1994). Transcription extracts prepared from dark-grown wheat seedlings which were illuminated for 4 h exhibited a higher transcriptional activity on this promoter than extracts prepared from non-illuminated seedlings. The transcription factor(s) mediating this light-responsive activity was partially purified from light-treated seedlings. The factor(s) was in a fraction containing no RNA polymerase activity, and had different chromatographic properties from the σ-like factors purified from mustard (Tiller *et al.*, 1991; Tiller and Link, 1993a,b). It is possible that this factor is the protein species which interacts with the repeats of the *psbD* promoter.

The position of the B and C repeat elements suggests that factors binding to these elements interact with RNA polymerase positioned at the core promoter. The repeats are located immediately adjacent to the putative –35 element of the *psbD* promoter, a position analogous to the arrangement of cI binding sites in the PRM promoter of bacteriophage λ (Ptashne, 1986). Activation of transcription by cI bound at these sites is mediated by direct contact with the RNA polymerase σ subunit (Li *et al.*, 1994). Alternatively, in analogy to activation by CAP at class II prokaryotic promoters (Zhou *et al.*, 1994), the

C repeats may function by binding to a transcriptional regulatory protein which contacts the RNA polymerase α subunit.

The regulation of plastid core promoters by upstream sequences is not unique to *psbD*. Upstream elements that influence promoter strength have been described previously for an additional plastid gene cluster, the *rrn* operon. Deletion of sequences directly upstream of the –35 element in the pea rRNA operon promoter reduced its activity *in vitro* (Sun *et al.*, 1989). A conserved element within the essential upstream sequences, referred to as the CDF2 site, has been shown to bind a protein present in chloroplast extracts of spinach (Baeza *et al.*, 1991; Iratni *et al.*, 1994). Thus, at least for some plastid promoters, full function and regulation of promoter activity may depend upon the interaction of protein factors with sequences upstream of the core elements. Additional regulatory mechanisms have also been described. The light response of the *psbA* promoter in mustard is mediated by σ-like factors acting through the core promoter (Tiller *et al.*, 1991; Tiller and Link, 1993a,b), whereas sequences downstream of the core promoter were shown to be important for transcription activity of the *rbcL* gene in *C.reinhardtii*, a unicellular alga (Klein *et al.*, 1994).

Experiments *in vitro* have established the role of –10 and –35 core promoter elements but were unsuitable to reveal the full complexity of plastid promoter architecture. The transgenic approach reported here for tobacco has allowed us to establish the existence of activator and light-response elements in addition to the core promoter. Identification of these elements will facilitate the cloning of the genes for the interacting protein factors, thereby elucidating the mechanisms by which nuclear genes control plastid function.

Materials and methods

Plasmid construction

In plasmid pLAA24A (Zoubenko *et al.*, 1994), the *uidA* coding region is expressed in the P*rrn*/T*rps16* cassette (P*rrn*::*uidA*::T*rps16* gene). P*rrn* contains the strong constitutive rRNA operon promoter fused to a synthetic ribosome binding site; T*rps16* is the 3′-untranslated region of the plastid *rps16* ribosomal protein gene. In plasmid pLAA25A (Zoubenko *et al.*, 1994), the *uidA* coding region is cloned in the rbs/T*rps16* cassette. In this cassette, the rbs contains only a synthetic ribosome binding site but no promoter, therefore the *uidA* gene in plasmid pLAA25A is promoterless (rbs::*uidA*::T*rps16* construct).

In plasmid pLAA12, the *uidA* coding region is expressed in the P*psbD*/T*rps16* cassette. The LRP of the *psbD* operon was amplified from the tobacco ptDNA to include sequences from nucleotide 33 477 to 33 583 (numbering of ptDNA sequence according to Shinozaki *et al.*, 1986), flanked by engineered *Sac*I (5′) and *Xba*I (3′) restriction sites. The primers used in PCR were: 5′ PCR primer = 5′-CCAAGAGCTCCGAAAGGTTAGAAATCAAC-3′ (underlined sequences anneal from positions 33 477 to 33 495 of the *psbD* upstream region) and 3′ PCR primer = 5′-CCGTCTAGAGCTCCAATTTCATCTCTATCG-3′ (underlined nucleotides correspond to *psbD* sequence 33 583–33 563). This construct was cloned as a *Sac*I–*Xba*I fragment upstream of a *uidA* gene with the following leader sequence: 5′-GAGCTCTAGAATTCAGTTGTAGGGAGGGATCCATGG-3′ (*Xba*I–*Nco*I sites underlined). The translational initiation codon of *uidA* is included within an *Nco*I site (Zoubenko *et al.*, 1994). This site was used to link the leader sequence to the *uidA* coding region. To stabilize the mRNA, T*rps16* was cloned downstream of the *uidA* coding region utilizing the appropriate *Xba*I restriction site. The P*psbD*::*uidA*::T*rps16* gene was cloned as a *Kpn*I–*Hin*dIII fragment into the pPRV111A plastid vector (Zoubenko *et al.*, 1994) to yield plasmid pLAA35A. The promoter in this construct is referred to as P*psbD*.

To generate the P*psbD* deletion derivatives, the same 3′ PCR primer (above) was used in combination with the following 5′ PCR primers: for P*psbD*-ΔA the primer sequence was 5′-CGAATTCAAGTAAGTGGACCTGACC-3′ (underlined sequence is homologous to positions 33 504–33 521 in the ptDNA); for P*psbD*-ΔABC, the sequence was 5′-CGAATTCATTGAATCATGACTATATC-3′ (homologous between positions 33 523 and 33 541 of the ptDNA). All deleted promoter fragments were blunt-end ligated into the *Sma*I site of pBluescript KS+ (Stratagene), and sequenced. The rbs::*uidA*::T*rps16* 3′ portion of the transgene was cloned downstream of the P*psbD*-ΔA or P*psbD*-ΔABC promoters as an *Xba*I–*Hin*dIII fragment to yield plasmids pLAA111 and pLAA131. The *uidA* transgenes were cloned into the pPRV111A plastid vector (Zoubenko *et al.*, 1994) as *Kpn*I–*Hin*dIII fragments to yield plasmids pLAA112A and pLAA132A.

Plant transformation

Tungsten particles (1 μm) were coated with plasmid DNA as previously described (Svab and Maliga, 1993), and introduced into the leaves of *Nicotiana tabacum* plants using the DuPont PDS1000He Biolistic gun at 1100 p.s.i. Transgenic shoots were selected aseptically on RMOP medium (Svab *et al.*, 1990) containing 500 μg/ml spectinomycin dihydrochloride. Resistant tissue was subcloned by regeneration on the same medium. Transgenic cuttings were rooted and maintained on RM medium consisting of agar-solidified MS salts (Murashige and Skoog, 1962) containing 3% sucrose plus spectinomycin (500 μg/ml).

Light induction protocol

Duplicate cuttings of transgenic plant lines were rooted aseptically on spectinomycin-containing solid RM media in Petri dishes, and were maintained under a 16 h light/8 h dark cycle. For the light induction experiment, cuttings were placed in total darkness over a period of 3 days. Subsequently, one of the duplicates for each line was placed under constant illumination (cool white fluorescent bulbs at ~10 000 lux) for 20 (experiment in Figure 2) or 24 h (experiments in Figures 4 and 5), while the other duplicate was maintained in complete darkness for the same length of time. Following light induction, a fully expanded leaf from each of the light-treated and dark-adapted cuttings was frozen in liquid nitrogen and used for RNA isolation.

Gel-blots

Total leaf DNA (Mettler, 1987) was digested with the restriction endonucleases *Nco*I and *Hin*dIII (New England Biolabs). Restricted DNA was separated on 0.7% agarose gels and transferred to Hybond N membrane (Amersham) using the PosiBlot Transfer apparatus (Stratagene). For RNA gel-blots, total plant RNA was prepared using TRIzol (GIBCO BRL), following the manufacturer's protocol. The RNA was electrophoresed on 1% agarose/formaldehyde gels, then transferred to nylon membrane as for the DNA gel-blots. Blot hybridizations were carried out at 65°C in Rapid Hybridization Buffer (Amersham) with ^{32}P-labeled, double-stranded DNA probes generated by random-prime labeling (Boehringer Mannheim).

RNase protection assays

For the *uidA* probe used in RNase protection assays, plasmid pLAA190 was constructed to allow transcription of the *psbD* promoter and a linked *uidA* coding fragment *in vitro* with T7 RNA polymerase. Plasmid pLAA190 is a pBluescript KS+ vector (Stratagene) derivative carrying the *psbD* promoter fragment from positions 33 496 to 33 583 in the ptDNA, fused to the synthetic rbs and the first 384 bp of *uidA* coding sequence defined by the *Sna*BI restriction site in the *uidA* coding region. Upstream of the promoter fragment is a *Kpn*I (*Asp*718) restriction site. To synthesize ^{32}P-labeled antisense RNA for the partial *uidA* sequence and linked 5′ regulatory region, plasmid pLAA190 was linearized with *Asp*718 (Boehringer Mannheim) and added to a T7 polymerase Maxiscript reaction (Ambion). RNA probes were purified on denaturing polyacrylamide gels, and used in RNase protection assays with the RPAII kit (Ambion), following the manufacturer's protocol. Reaction products were run on 5% Long Ranger (AT biochem) sequencing gels and visualized by autoradiography. For the *rps16* antisense RNA probe, a 442 bp region of 5′ sequence from the *rps16* gene (nucleotides 6656–6214 in the tobacco ptDNA) was excised with *Eco*RI and *Nco*I from plasmid pJS97 (Staub and Maliga, 1994). The *Nco*I site was end-filled with T4 DNA polymerase, and the fragment was cloned into the pBluescript KS+ vector between the *Sma*I and *Eco*RI restriction sites, to create pLAA192. Plasmid pLAA192 was linearized with *Asp*718 and used to generate an antisense *rps16*-5′ probe with the Ambion T7 polymerase Maxiscript kit.

Primer extension reactions

Oligonucleotide EL22 (sequence: 5′-CCACAGTTTTCGCGATCCAG-ACTGA-3′), which anneals to the *uidA* coding region between bp 62 and 86, inclusive, was end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (New England Biolabs). Primer extensions were performed according to standard protocols (Sambrook *et al.*, 1989) using Superscript MMLV reverse transcriptase (Gibco-BRL) at 42°C. Extension products were separated on 6% Long Ranger sequencing gels. The 5′ ends of the extension products were determined by comparison with cDNA sequences generated with the same primer using the Sequenase II kit (USB).

Preparation of chloroplast protein extracts, and gel shift assays

High salt transcription extract was prepared from intact chloroplasts purified on Percoll step gradients (Orozco *et al.*, 1986). Proteins extracted in high salt from plastid membrane fractions were dialyzed against DE52 buffer (Gruissem *et al.*, 1986) then loaded on a 1.0 ml DEAE–Sephacel column equilibrated in DE52 buffer. Proteins in the column flowthrough fraction were precipitated and dissolved in DEAE buffer (Orozco *et al.*, 1986). This extract was divided into aliquots and flash-frozen.

For gel retardation assays, the $A_2B_2C_2$ oligonucleotide (Figure 7B) and its complement were end-labeled (Sambrook *et al.*, 1989), heated to 90°C for 5 min, then slowly cooled to room temperature in buffer containing 20 mM Tris pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl. Labeled probe (12.5 fmol, 2.5×10^4 c.p.m. per reaction) was incubated at room temperature for 30 min with ~2.5 μg of transcription extract in a 30 μl reaction mix (10 mM Tris pH 7.5, 40 mM NaCl, 1 mM EDTA, 4% glycerol) containing 1 μg [poly(dI–dC)–poly(dI–dC)] (Sigma). Reactions were run on 5% non-denaturing polyacrylamide gels (38:2) at room temperature in 0.5× TBE buffer, then dried and exposed to X-ray film overnight. For the competition assays with subfragments of the probe sequences, appropriate quantities (40-, 200- and 800-fold molar excess over probe concentration) of each annealed oligonucleotide and its complement were preincubated for 5 min with the chloroplast extract in reaction conditions prior to adding the labeled probe DNA. As a non-specific DNA competitor, the following oligonucleotide and its complement were used: 5′-GCTCTAGAGCGGCCGCAAAAGCACGC-CTGGTCT-3′.

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