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In Vitro Analysis of Light-Induced Transcription in the Wheat *psb*D/C Gene Cluster Using Plastid Extracts from Dark-Grown and Short-Term-Illuminated Seedlings[1]

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We describe a plastid in vitro transcription system that reflects characteristic features of the light-regulated transcription observed in vivo. Multiple transcripts of the wheat (*Triticum aestivum*) *psb*D/C gene cluster comprise six distinct 5′ ends including four transcription initiation sites designated as D/C-1 through D/C-4. Transcripts from one particular site, D/C-3, were found to be conspicuously enhanced in abundance after 4 h of illumination in vivo. The plastid extract prepared from 5-d-old dark-grown wheat seedlings was capable of transcribing from the D/C-2 and D/C-4 sites in vitro but had almost no transcription activity from the light-responsive D/C-3 site (the D/C-1 site was not examined). The plastid extract from 4-h-illuminated seedlings initiated transcription from the light-responsive site (D/C-3). Transcription from the D/C-2 and D/C-4 sites was not enhanced by using the extract from 4-h-illuminated seedlings, indicative of specific activation of the light-responsive promoter on the D/C-3 site by the extract from 4-h-illuminated seedlings. The plastid extract from 4-h-illuminated seedlings was divided into two fractions on a heparin-Sepharose column, into which the light-induced component(s) responsible for activation of the D/C-3 promoter and RNA polymerase were separated. The fraction containing the component(s) activating the D/C-3 promoter induced the transcription activity from the D/C-3 site in the plastid extract from dark-grown seedlings. It is concluded that the plastid extract from 4-h-illuminated seedlings contains some light-regulatory component(s) that activate specifically the light-responsive promoter.

Genetic and biochemical analyses of light-induced chloroplast biogenesis in higher plants have yielded novel information on the controls of organelle gene expression and the communication between different genomes in plant cells (Chory and Peto, 1990; Deng et al., 1992; Tiller and Link, 1993b). Exposure of dark-grown plants to light results in a dramatic increase in the synthesis and accumulation of plastid proteins (Mullet, 1988). In many cases, plastid mRNAs encoding photosynthesis-related proteins accumulate to relatively high levels in dark-grown plants, suggesting that post-transcriptional and/or translational processes are dominant mechanisms for regulating plastid gene expression (Mullet, 1988; Gruissem, 1989). However, much evidence has supported the possible involvement of transcriptional regulation in light-induced plastid gene expression.

Transcriptional activities (Klein and Mullet, 1990; Sexton et al., 1990a; Klein, 1991; Schrubar et al., 1991; Kawaguchi et al., 1992; Baumgartner et al., 1993) and levels of the transcripts of some chloroplast genes (Rodermel and Bogorad, 1985; Klein and Mullet, 1987; Gamble et al., 1988; Kawaguchi et al., 1992; Baumgartner et al., 1993) are affected by light. For example, run-on transcription experiments with barley and wheat (*Triticum aestivum*) have shown that transcription of *psb*A, the gene encoding the PSII "D1" protein, is differentially enhanced by illumination (Klein and Mullet, 1990; Kawaguchi et al., 1992). Some particular transcripts from the *psb*D/C operon among several transcripts within the operon have also been observed to be light induced during light-induced greening of dark-grown seedlings of several monocotyledonous and dicotyledonous plant species (Christopher et al., 1992; Kawaguchi et al., 1992). Similar observations have been made with the *pet*E operon of maize (Haley and Bogorad, 1990) and the *psb*B operon of wheat (Kawaguchi et al., 1992). Thus, although posttranscriptional control may be of importance in the light-dependent regulation of plastid gene expression, it is also evident that some chloroplast genes are regulated at the level of transcription.

Studies on the transcriptional control of nuclear genome expression have been greatly advanced by the isolation of various transcriptional regulatory factors from cell lysates (Gilmartin et al., 1990; Thompson and White, 1991). Concerning organelle gene expression, it has been revealed by using plastid extracts that the chloroplast transcription apparatus has many features in common with that of prokaryotes, such as the use of prokaryotic-type promoter elements (Link, 1984; Bradley and Gatenby, 1985; Gruissem and Zurawski, 1985a, 1985b; Igloi and Kössel, 1992). However, there is only limited information about the light-regulatory mechanisms of plastid gene transcription.

As illustrated in Figure 1, the wheat *psb*D/C gene cluster, which is differentially expressed in vivo during light-induced plastid development (Christopher et al., 1992; Kawaguchi et al., 1992), has four distinct promoters (Kawaguchi et al., 1994). Three of these operate in the dark, but the other one (D/C-3 in Fig. 1) responds to light, producing two light-induced transcripts. Similar results were previously obtained

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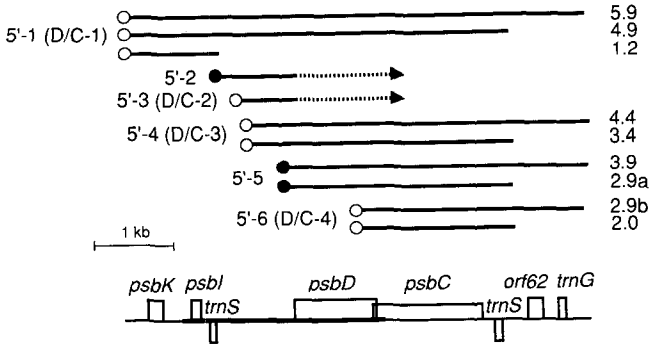

Figure 1. Gene (lower) and transcript (upper) map of the wheat *psb*D/C gene cluster. Genes are designated by open boxes. *psb*I, *trn*S, *psb*D, and a part of *psb*C are positioned based on the wheat chloroplast DNA sequence (bold line). Other genes in the map are positioned based on the chloroplast genome map of rice (Hiratsuka et al., 1989). Solid lines represent the transcripts that have been visualized by northern hybridization analysis (Kawaguchi et al., 1994). The site of the 5′ end and length of each transcript were determined by combination of the results of northern hybridization with seven different probes and primer extension using six probes as primers. The 3′ end of each transcript was not determined, but estimated from the site of the 5′ end and the length of each transcript. S1 nuclease protection assays gave signals at the positions designated as 5′-2 and 5′-3, but the transcript mapping to these ends was not detected in northern hybridization analysis. Open circles represent transcriptional initiation sites determined by in vitro capping assay (Kawaguchi et al., 1994). Solid circles indicate putative processing sites. Numbers to the left and to the right of each transcript indicate the name of 5′ end and size (in kb) of the transcript, respectively. Four transcription initiation sites are designated as D/C-1 through D/C-4.

in barley (Sexton et al., 1990a). This light-responsive promoter was also recently reported to be conserved in chloroplast DNAs from several diverse higher plant species, including wheat (Christopher et al., 1992). To elucidate molecular mechanisms responsible for light-induced transcription from the D/C-3 promoter, we isolated transcriptionally active plastid extracts from dark-grown and short-term-illuminated wheat seedlings. The extract prepared from 4-h-illuminated seedlings was capable of transcribing in vitro from the light-responsive site (D/C-3), but the extract from dark-grown seedlings was not. Here we present evidence suggesting that certain light-regulatory component(s) that selectively activate the D/C-3 promoter are produced or converted into an active form during 4 h of illumination of dark-grown seedlings.

MATERIALS AND METHODS

Plasmid DNA

Recombinant plasmid pWB15 was prepared by inserting a 3.3-kb *Bam*HI DNA fragment (B15) of the wheat *psb*D/C gene cluster (Courtice et al., 1985; Howe et al., 1988) into pUC19. An 866-bp *Eco*RI fragment of pWB15, containing a portion of the upstream region of the *psb*D gene (−1161 to −295 relative to the ATG codon of *psb*D) was subcloned into pUC18 in the same direction as transcription of the *lacZ* gene to give plasmid pW18E866 (Fig. 2). An 1145-bp *Eco*RI-*Sac*I fragment of pWB15 (−989 to +156 relative to the ATG codon of *psb*C) was also inserted into pUC18 in the same manner as pW18E866 to give plasmid pW18ES1145 (Fig. 2). These constructs were used as templates for in vitro transcription experiments (template pW18E866 and template pW18ES1145).

Plant Growth and Plastid Isolation

Wheat (*Triticum aestivum*) seeds were germinated and grown in a dark chamber at 25°C. After 5 d, a portion of the dark-grown wheat seedlings was transferred to illumination with a fluorescent light (Mitsubishi FL40SD/38) at 200 μmol m^{-2} s^{-1} and kept there for 4 h. Both unilluminated and 4-h-illuminated seedlings were harvested quickly and immediately cooled on ice. Intact plastids were isolated from both types of the seedling by centrifugation through 10 to 80% Percoll linear gradients (Kawaguchi et al., 1992) and quantitated by a microscope using a hemacytometer. Mature chloroplasts were also isolated by the same procedure from 5-d-old light-grown seedlings. All manipulations were performed at 4°C in the dark (under dim green light) for dark-grown seedlings or under room light for 4-h-illuminated and light-grown seedlings.

Preparation of High-Salt Extracts from Plastids

Transcriptionally active plastid extracts were prepared according to Orozco et al. (1985). The intact plastids were lysed in a hypotonic buffer and the membrane fraction was collected by centrifugation. The resulting pellet was dispersed in a high-salt buffer containing 20 mM Hepes-KOH (pH 8.0), 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 mM benzamidine, 5 mM ε-amino-*n*-caproic acid, 15% (v/v) glyc-

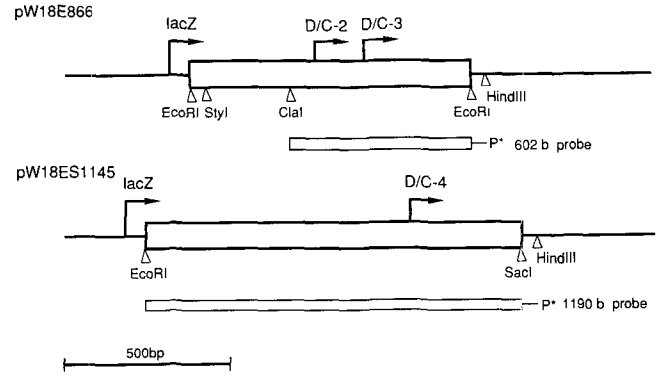

Figure 2. Schematic representations of the truncated *psb*D and *psb*C constructs used for analysis of in vitro transcription. The 866-bp *Eco*RI fragment of wheat chloroplast DNA containing putative promoter regions of the D/C-2 and D/C-3 sites was inserted into pUC18 to give pW18E866. The 1145-bp *Eco*RI-*Sac*I fragment containing the D/C-4 promoter was inserted into pUC18 to give pW18ES1145. The arrows show the direction and sites of transcription initiation. The boxed regions indicate wheat chloroplast DNA sequences and the single lines indicate vector sequences. The 602-base *Cla*I-*Hin*dIII probe and 1190-base *Eco*RI-*Hin*dIII probe used for S1 protection analysis of the in vitro synthesized RNAs are shown below the figures of their respective plasmids.

erol, and 1 M NaCl. The protein components extracted in the solution were precipitated with 50% ammonium sulfate and resuspended in DEAE buffer (1 mL for extract from 1×10^{10} plastids) consisting of 50 mM Tricine-KOH (pH 8.0), 50 mM KCl, 0.5 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 1 mM benzamidine, 5 mM ϵ-amino-*n*-caproic acid, and 5% (v/v) glycerol. The resulting solution was dialyzed against the DEAE buffer overnight at 4°C, frozen in liquid nitrogen, and stored at −80°C prior to use.

In Vitro Transcription

The extracts from 1.6×10^8 plastids and 800 ng of supercoiled template DNA were incubated in 40 μL of reaction mixture consisting of 12 mM Hepes-KOH (pH 8.0), 20 mM Tricine-KOH (pH 8.0), 60 mM KCl, 10 mM $MgCl_2$, 0.2 mM EDTA, 2% (v/v) glycerol, 10.2 mM DTT, 0.4 mM benzamidine, 2 mM ϵ-amino-*n*-caproic acid, 0.2 mM PMSF, 1.0 unit/μL of RNase inhibitor (Takara), 0.5 mM CTP, 0.5 mM GTP, 0.05 mM ATP, and 0.05 mM UTP at 30°C for 60 min. Then, 160 μL of a solution containing protease K (Merck) was added to the mixture to stop the reaction (500 μg/mL, final concentration) and incubated at 37°C for a further 30 min. After protease K digestion, the solution was subjected to phenol-chloroform extraction and ethanol precipitation. The RNAs were purified from the nucleic acid mixture by treatment with RQ-1 DNase (Promega), followed by phenol-chloroform extraction and ethanol precipitation.

In Vitro RNA Processing

As shown in Figure 3, an 866-bp *Eco*RI restriction DNA fragment spanning three 5′ ends of *psb*D/C (5′-2, 5′-3 [D/C-2] and 5′-4 [D/C-3]) was cloned into the polylinker region of pSP73 plasmid vector (pW73E866). The plasmids were cleaved at a vector restriction site (*Eco*RV or *Nde*I) located downstream of the insert fragment and submitted to transcription (Fig. 3). The transcription was carried out using the T7 RNA polymerase promoter located upstream of the insert in the linearized plasmid in the presence of [α-^{32}P]GTP. After the DNA templates were digested by treatment with RQ-1 DNase, the in vitro synthesized RNAs (2000 cpm/reaction) were incubated in the plastid extract prepared from 4-h-illuminated seedlings at 30°C for 60 min under the same conditions as the in vitro transcription experiments except that template DNA was excluded. Following incubation, the resulting RNAs were isolated and analyzed by electrophoresis and autoradiography.

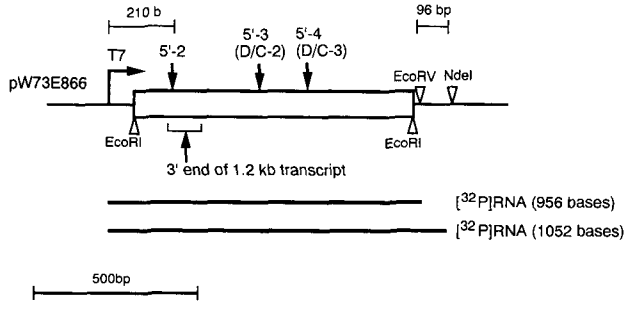

Figure 3. Schematic representations of the synthesized RNAs used for in vitro processing experiments in the plastid extract. The 866-bp *Eco*RI fragment of wheat chloroplast DNA containing 5′-2, 5′-3 (D/C-2), and 5′-4 (D/C-3) was inserted into pSP73 to give pW73E866. The boxed region indicates the wheat chloroplast DNA sequence, and the single line indicates the vector sequence. The vertical arrows show the sites of 5′-2, D/C-2, and D/C-3. The possible range of the 3′ end of the 1.2-kb transcript is shown by the horizontal bracket. The 956- and 1052-base RNAs used for in vitro processing were synthesized using the T7 RNA polymerase promoter on the templates pW73E866 linearized at the *Eco*RV and *Nde*I sites, respectively.

S1 Nuclease Protection Experiments

A 602-bp *Cla*I-*Hin*dIII fragment from pW18E866 and an 1190-bp *Eco*RI-*Hin*dIII fragment from pW18ES1145, which contain 51-bp and 41-bp vector sequences, respectively, following each plastid DNA sequence, were 5′-end labeled at the *Hin*dIII site within the vector sequence using polynucleotide kinase and [γ-^{32}P]ATP. An 834-bp *Sty*I-*Eco*RI fragment from pW18E866 was also 5′-end labeled at the *Eco*RI site within the plastid DNA sequence. The resulting radiolabeled *Cla*I-*Hin*dIII and *Eco*RI-*Hin*dIII DNA fragments were used as probes for S1 nuclease protection assays of the in vitro synthesized RNAs. The radiolabeled *Sty*I-*Eco*RI DNA fragment was used for the assay of the mRNAs accumulated in vivo in the isolated plastids. Each ^{32}P probe (20,000 cpm per assay) was mixed with the in vitro synthesized RNAs or the RNAs isolated from the plastids and co-precipitated by the addition of ethanol. The resulting pellet was resuspended in a buffer containing 80% formamide, 40 mM Pipes (pH 6.4), 1 mM EDTA, and 0.4 M NaCl, denatured at 85°C for 10 min, and incubated at 37°C overnight for hybridization. Then the solution was diluted with 100 μL of S1 buffer (0.28 M NaCl, 0.05 M sodium acetate [pH 4.5], 4.5 mM $ZnSO_4$, and 200 units/mL of S1 nuclease) and incubated at 20°C for 1 h. S1-protected products were phenol-chloroform extracted, ethanol precipitated, and analyzed on a 6% polyacrylamide-7 M urea sequencing gel.

Separation of Possible Light-Responsive Transcription-Regulatory Factor(s) and RNA Polymerase

Plastids (8.9×10^9) from 4-h-illuminated wheat seedlings were lysed in a buffer (2.7 mL) containing 50 mM Tris-HCl (pH 7.6), 4 mM EDTA, 40 mM 2-mercaptoethanol, 25% (v/v) glycerol, 50 μg/mL PMSF, 1 mM benzamidine, and 1.0% (v/v) Triton X-100 (Tiller et al., 1991). Adjusting the concentration of $(NH_4)_2SO_4$ to 0.1 M, the lysate was applied to a 3-mL column of heparin-Sepharose CL-6B (Pharmacia) equilibrated with a buffer [50 mM Tris-HCl (pH 7.6), 0.1 mM EDTA, 5 mM 2-mercaptoethanol, 10% (v/v) glycerol, 50 g/mL PMSF, 1 mM benzamidine, 0.1% (v/v) Triton X-100, and 0.1 M $(NH_4)_2SO_4$]. The flow through (3.0 mL) was collected as fraction A. After extensive washing with the equilibration buffer, the absorbed materials were eluted with the buffer additionally containing 0.7 M $(NH_4)_2SO_4$. The whole fraction eluted with 0.7 M $(NH_4)_2SO_4$ was collected as fraction B (1.0 mL). Both fractions A and B were dialyzed against a buffer

containing 50 mM Tris-HCl (pH 7.6), 0.1 mM EDTA, 5 mM 2-mercaptoethanol, 10% (v/v) glycerol, 0.1% (v/v) Triton X-100, and 0.01 M $(NH_4)_2SO_4$ and stored at −80°C for further analysis. In vitro transcription assays of each fraction and/or a combination of these fractions were carried out as described above.

RESULTS

In wheat plastids, the *psb*D and *psb*C genes, which encode the D2 and CP-43 polypeptides in PSII, respectively, overlap by 50 bp (Gray, 1987). Based on the homologous chloroplast genome maps of barley (Sexton et al., 1990b) and rice (Hiratsuka et al., 1989), two PSII genes (*psb*I and *psb*K) are predicted to be located upstream of the wheat *psb*D gene (Fig. 1). From this gene cluster at least nine distinct transcripts ranging from 1.2 to 5.9 kb are produced that accumulate in etioplasts of dark-grown seedlings (Fig. 1) (Kawaguchi et al., 1992). Upon illumination, two novel transcripts of 4.4 and 3.4 kb accumulate (Kawaguchi et al., 1992). All of these transcripts share six different 5′ ends, four of which have been suggested to be transcription initiation sites by in vitro capping analysis (Kawaguchi et al., 1994). These sites are designated as D/C-1 to D/C-4 in Figure 1. The two light-induced transcripts share a common 5′ end, D/C-3, located 610 bp upstream of the *psb*D translation initiation codon.

In Vitro Transcription of RNA from the *psb*D/C Gene Cluster

To elucidate the molecular mechanisms underlying the light induction of transcription from the *psb*D/C gene cluster, we prepared crude plastid extracts from dark-grown wheat seedlings and 4-h-illuminated seedlings and examined their in vitro transcription activities for three transcriptional initiation sites, D/C-2, D/C-3, and D/C-4. Figure 2 shows the structure of the templates used for these analyses. The plasmid pW18E866 contains an 866-bp *Eco*RI restriction fragment of wheat chloroplast DNA spanning the entire putative promoter regions of D/C-3 and D/C-2. The plasmid pW18ES1145 bears an 1145-bp *Eco*RI-*Sac*I fragment of wheat chloroplast DNA that contains the putative promoter region of D/C-4. The latter promoter is located on the 3′ coding region of *psb*D and produces *psb*C gene-specific transcripts. After the in vitro transcription reactions, the amounts of each transcript were assessed by S1 nuclease protection experiments using the *Cla*I-*Hin*dIII probe or the *Eco*RI-*Hin*dIII probe, both of which were designed not to give a signal from the endogenous chloroplast RNA or DNA, if they exist (see Fig. 2). We found previously from run-on transcription experiments that the overall in vivo transcription rate of the *psb*D/C gene cluster was transiently enhanced about 5-fold during the first 2 to 6 h after transfer to the light and then declined to a level lower than that before illumination (Kawaguchi et al., 1992). Taking into account these findings and those from barley (Sexton et al., 1990a), the effect of illumination was examined mainly by using the plastid extract prepared from 4-h-illuminated seedlings rather than the chloroplast extract prepared from light-grown seedlings.

In Figure 4, the RNAs synthesized in vitro in the plastid extracts prepared from 5-d-old dark-grown seedlings and 4-h-illuminated seedlings using template pW18E866 are shown (Fig. 4A) and compared with the RNAs accumulated in vivo in the corresponding plastids (Fig. 4B). S1 protection analysis of the in vitro synthesized RNAs from template pW18E866 in the plastid extract prepared from dark-grown seedlings yielded a major signal (522 bases) indicative of the initiation of transcription at the D/C-2 site (Fig. 4A, lanes 1 and 2). The full-length (602 bases) S1-protected fragment detected as another major signal in lanes 1 through 3 may be the result of transcription from the *lac*Z promoter in the template, since the signal intensities of their bands are significantly stronger than those of the corresponding bands in the control experiments without template or without extract shown in lanes 4 through 7. A significant difference in the intensities of these two signals detected at 522 and 602 bases was not observed between the two different plastid extracts from dark-grown and 4-h-illuminated seedlings. However, a significant difference was observed in the intensity of the signal corresponding to the transcript of 370 bases. The signal intensity at 370 bases was greatly enhanced by using the extract from 4-h-illuminated seedlings (Fig. 4A, lane 2). The size of the labeled band corresponds to the size of the transcript that is expected to initiate at the D/C-3 site. Control reactions without template (Fig. 4A, lanes 4 and 5) or without extract (Fig. 4A, lane 6) gave no signal except for the full-length signal that may be due to probe reannealing, supporting the hypothesis that these signals are indeed the results of specific transcription from the template DNA.

The D/C-2 site on the template DNA was recognized as a transcriptional initiation site by *Escherichia coli* RNA polymerase holoenzyme, but transcripts mapping to the D/C-3 site were significantly less abundant (Fig. 4A, lane 3), suggesting the requirement of a plastid-specific factor(s) for activation of the D/C-3 promoter. Two weak signals detected at 450 and 310 bases (indicated by arrowheads in Fig. 4A) in both extracts were also detected when *E. coli* RNA polymerase was used, suggesting that prokaryotic promoter-like sequences may act as weak promoters in in vitro transcription.

Figure 4B shows the results of S1 protection analysis carried out using the ^{32}P-labeled *Sty*I-*Eco*RI DNA fragment probe for the mRNAs that accumulate in vivo in the plastids isolated from dark-grown (lane 1) and 4-h-illuminated seedlings (lane 2). In both cases, six S1-protected fragments were detected. The sizes of four of these fragments correspond to the sizes expected for the undigested probe and the S1-protected fragments that are produced from the mRNAs mapping to 5′-2 (750 bases), 5′-3 (D/C-2, 471 bases), and 5′-4 (D/C-3, 319 bases). Two minor fragments may be due to insufficient digestion by S1 nuclease. The mRNAs mapping to the D/C-3 site increased greatly in abundance in the plastids isolated from 4-h-illuminated seedlings. In contrast, the mRNAs mapping to the D/C-2 site were slightly decreased by 4 h of illumination (compare Fig. 4B, lanes 1 and 2). These results are consistent with the results of the in vitro transcription. It should be mentioned, however, that the in vivo level of the mRNAs mapping to the D/C-2 site in the plastids isolated from 4-h-illuminated seedlings appears to be significantly lower than the level of the mRNAs mapping to the D/C-3 site. This behavior cannot be explained by the results of the

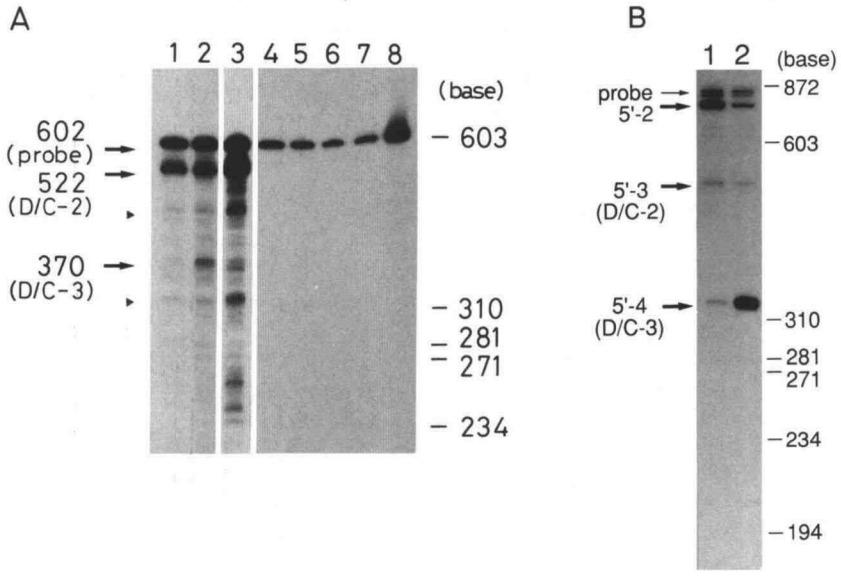

Figure 4. Comparison of in vitro synthesis of the RNAs from D/C-2 and D/C-3 sites in the plastid extracts and in vivo accumulation of the mRNAs mapping to the corresponding sites. A, S1 nuclease protection analysis was carried out for the transcripts synthesized in plastid high-salt extract from wheat seedlings in the presence of template pW18E866. Lanes 1 and 2 show the results with the extracts from dark-grown and 4-h-illuminated seedlings, respectively. Lane 3 shows the result with *E. coli* RNA polymerase holoenzyme (0.5 unit/40 μL). Lanes 4 and 5 are control reactions where no template DNA was added to the extracts from dark-grown and 4-h-illuminated seedlings, respectively. Lane 6 is a control reaction without extract. Probe alone before (approximately 1000 cpm) and after treatment with S1 nuclease are also presented, in lanes 8 and 7, respectively. The sizes of the transcripts synthesized in vitro are indicated on the left and the sites of transcription initiation are in parentheses. The full-length (602 bases) bands correspond to the undigested probe. Numbers to the right indicate the sizes of φX174-*Hae*III mol wt markers. B, S1 nuclease protection analysis was carried out for the mRNAs accumulated in vivo in the plastids isolated from the dark-grown and 4-h-illuminated seedlings. Lanes 1 and 2 show the results with the plastids isolated from the dark-grown and 4-h-illuminated seedlings, respectively. 5′-2, 5′-3 (D/C-2), and 5′-4 (D/C-3) are indicated by thick arrows and undigested probe by the thin arrow. Numbers to the right indicate the size of φX174-*Hae*III mol wt markers.

in vitro transcription and may be due to the difference in the stability of these two mRNAs in vivo. The level of the mRNAs mapping to 5′-2 significantly decreased in the plastids isolated from 4-h-illuminated seedlings compared with that in the plastids from dark-grown seedlings.

Figure 5 shows the results of in vitro transcription from template pW18ES1145 in plastid extracts from dark-grown, 4-h-illuminated, and light-grown seedlings. The level of the transcripts mapping to the D/C-4 site (the 397-base signal) was similar between the plastid extracts from dark-grown and 4-h-illuminated seedlings (Fig. 5, lanes 1 and 2, respectively). In contrast, the signal obtained using the extract from the mature chloroplasts seems to be considerably weaker than that from the other two extracts (Fig. 5, lane 3). These results are consistent with the accumulation of the transcripts from the D/C-4 site observed in vivo (Kawaguchi et al., 1992b). *E. coli* RNA polymerase holoenzyme can also initiate transcription at the D/C-4 site. Control reactions in the plastid extract from 4-h-illuminated seedlings without template DNA (Fig. 5, lane 5) and without the plastid extract (Fig. 5, lane 6) gave no signal.

In Vitro RNA Processing in the Plastid Extract

To confirm that the fragments giving the signals at the positions corresponding to the D/C-2 and D/C-3 sites in Figure 4A are the primary transcripts rather than the products of processing, we examined in vitro processing in the plastid extract of the RNAs synthesized using the *psb*C/D templates. In Figure 6, the results obtained with the 956-base RNAs synthesized from the template pW73E866 linearized at the *Eco*RV site (see Fig. 3) are shown. In addition to the signal of the synthesized RNAs of 956 bases, two distinct and a few weak signals are detected on the autoradiograph. The arrowheads on the right side of lane 2 in Figure 6 show the positions of the expected fragments, if the synthesized RNAs of 956 bases were processed at the 5′-2, 5′-3 (D/C-2), and 5′-4 (D/C-3) sites. One of the distinct signals was located at the position corresponding to the fragment processed at the 5′-2 site. When we run the gel out longer to expand the high mol wt region, the band at the position of the 5′-2 site in lane 2 was clearly observed to be different in length from the slightly larger band in lane 1. The other distinct band was a 210-base fragment. However, any signal exhibiting production of fragments with 5′ ends at the D/C-2 and D/C-3 sites was not detected. These results strongly suggest that the synthesized RNAs are processed in vitro at the 5′-2 site in the plastid extract prepared from 4-h-illuminated seedlings, but not at the D/C-2 and D/C-3 sites. These results are consistent with the results of in vitro capping experiments demonstrating that D/C-2 and D/C-3 are transcription initiation sites and 5′-2

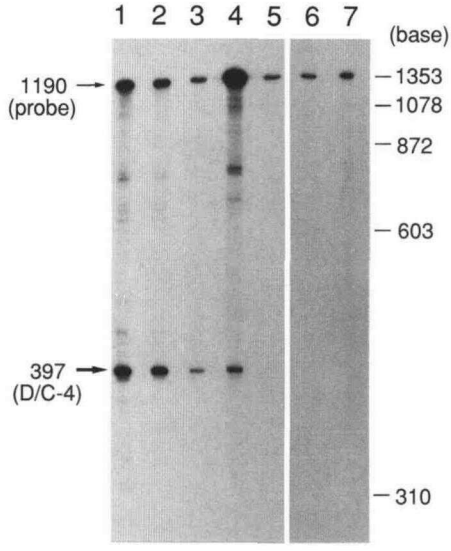

Figure 5. S1 nuclease protection analysis of the transcripts synthesized in plastid high-salt extracts from wheat seedlings. In vitro transcription was carried out using template pW18ES1145. Lanes 1 and 2 show the results with the extracts from dark-grown and 4-h-illuminated seedlings, respectively. Lane 3 shows the result with the extract from mature chloroplasts isolated from 5-d light-grown seedlings. Lane 4 shows the result with *E. coli* RNA polymerase holoenzyme (0.5 unit/40 μL). Lanes 5 and 6 are control reactions without template in the presence of the extract from 4-h-illuminated seedlings and without the extract, respectively. Probe alone (approximately 1000 cpm) is also presented, in lane 7.

is a processing site (Sexton et al., 1990a; Kawaguchi et al., 1994).

To identify the origin of the 210-base fragment, we carried out similar experiments using the 1052-base RNAs that were synthesized from template pW73E866 linearized at the *Nde*I site (see Fig. 3). Even when the 1052-base RNAs, which bear an additional 96-base vector sequence at the 3′ terminal end, were used, a labeled 210-base fragment was produced (data not shown). This fact indicates that the RNA fragment of 210 bases is not produced from the 3′ end of the synthesized RNAs. A length of 210 bases is slightly longer than that expected for the 3′- end fragment of the synthesized RNAs processed at the 5′-2 site (199 bases). As shown in Figure 1, a transcript of 1.2 kb accumulates in the plastids from both dark-grown and 4-h-illuminated seedlings. If the 210-base fragment arises from processing the 210 bases downstream from the 5′ end of the synthesized RNA, the processing site should be 1030 bases upstream of the initiating ATG of *psb*D gene. As shown in Figure 3, this site is coincident with the position of the 3′ end of the 1.2-kb transcript (within experimental error). Consequently, it is not unreasonable to suppose that the 210-base fragment arises from a processing event that determines the 3′ end of the 1.2-kb transcript.

Separation of Light-Responsive Transcription Factor from RNA Polymerase

As a first step to isolate the light-induced factor(s) that activate the D/C-3 promoter from the plastid extract of 4-h-illuminated seedlings, we tried to separate the factor(s) from RNA polymerase using a heparin-Sepharose column. Intact plastids from 4-h-illuminated seedlings were disrupted by treatment with Triton X-100. The lysates were subjected to a heparin-Sepharose column and divided into two fractions containing the nonabsorbed components (fraction A) and absorbed components (fraction B). The in vitro transcriptional activities of both fractions were then examined. In Figure 7, the results obtained with template pW18E866 and with fraction A, fraction B, or both are shown. Addition of fraction A to the reaction mixture for in vitro transcription did not give any significant signal except for the full-length signal (602 bases) that may due to probe reannealing (Fig. 7, lane 1). In contrast, fraction B produced transcripts from the D/C-2 site (522 bases) and *lacZ* promoter located in the vector sequence (602 bases) (Fig. 7, lane 2). This indicates that the plastid RNA polymerase is present in fraction B, presumably together with a σ-like factor(s), consistent with results previously reported (Eisermann et al., 1990; Tiller and Link, 1993a). Interestingly, however, transcription from the D/C-3 site was not observed by the addition of fraction B alone to the reaction mixture. Transcription from the D/C-3 site was recovered by the simultaneous addition of fraction A and fraction B (Fig. 7, lane 3). These results indicate that light-regulated transcription factor(s) were recovered in the fraction containing the nonabsorbed components by heparin-Sepharose (fraction A) as substantially separated from the plastid RNA polymerase. In lane 1 of Figure 7, extremely weak signals are shown to be detectable at the positions of D/C-2

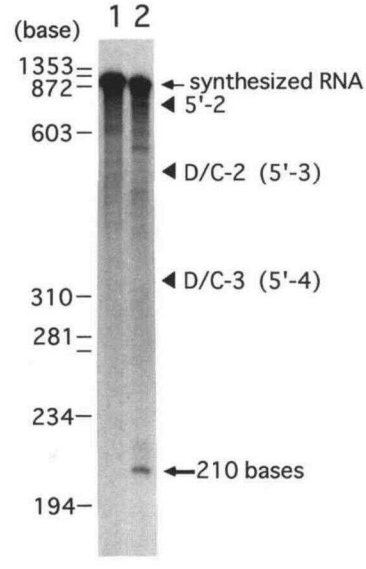

Figure 6. In vitro RNA processing in the plastid extract. The 956-base RNAs synthesized from the linearized template (pW73E866) at the *Eco*RV site were incubated in the mixture used for in vitro transcription with the plastid high-salt extract from 4-h-illuminated seedlings (lane 2) or without extract (lane 1). The arrowheads on the right side of lane 2 show the positions of the fragments expected if the 956-base RNAs were processed at the 5′-2, D/C-2, and D/C-3 sites. Numbers to the right indicate the sizes of φX174-*Hae*III mol wt markers.

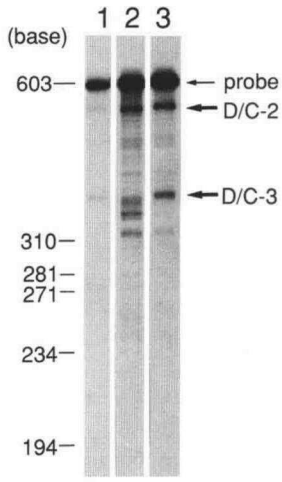

Figure 7. In vitro transcription activities of fractions A and B from the 4-h-illuminated plastid lysate separated by a heparin-Sepharose column. In vitro transcription was carried out using plasmid pW18E866 as a template. The results with 8 μL of fraction A (lane 1), 8 μL of fraction B (lane 2), and 8 μL each of fraction A and fraction B (lane 3) in 40 μL of in vitro transcription reaction mixture are shown. The signals corresponding to transcription from the D/C-2 and D/C-3 sites are designated by thick arrows. Numbers to the left indicate the sizes of φX174-*Hae*III mol wt markers.

and D/C-3. These signals may be a result of contamination of fraction A with very small amounts of the plastid RNA polymerase.

To confirm the existence of the factor(s) in fraction A, the ability of fraction A to induce the transcriptional activity from the D/C-3 site in the extract from the plastids of dark-grown seedlings was examined. In this experiment, the lysate prepared by Triton X-100 treatment of the plastids from dark-grown seedlings was used instead of the high-salt extract from the plastids. Figure 8 shows the results obtained with template pW18E866. Comparison of lanes 2 and 3 clearly demonstrates that fraction A from the plastids of 4-h-illuminated seedlings induces the transcriptional activity from the D/C-3 site in the extract from dark-grown seedlings.

DISCUSSION

It has been shown that light specifically affects transcription from one particular promoter among the multiple promoters present in the *psb*D/C gene cluster (Sexton et al., 1990a; Christopher et al., 1992; Kawaguchi et al., 1994). It was reported that this behavior is conserved in dicots and monocots (Christopher et al., 1992) and the promoter is affected by blue light (Gamble and Mullet, 1989). These findings may indicate the existence of specific plastid transcriptional regulators that selectively activate transcription from particular chloroplast promoters during light-induced development.

A number of chloroplast DNA-binding proteins (Lerbs et al., 1983; Reiss and Link, 1985; Bülow et al., 1987; Lam et al., 1988; Zaitlin et al., 1989; Eisermann et al., 1990; Baeza et al., 1991; Khanna et al., 1991) and σ-like factors (Surzycki and Shellenbarger, 1976; Bülow and Link, 1988; Lerbs et al., 1988; Tiller et al., 1991) have been reported. Some of these proteins are expected to be involved in the transcriptional regulation of plastid genes. Indeed, polypeptide compositions of the DNA-protein complexes isolated from the plastids of mustard seedlings were found to vary depending on the stage of plastid development (Reiss and Link, 1985). The level of the plastid DNA-binding factor 1 in bundle-sheath extracts was reported to be 3-fold higher than in mesophyll cell extracts in maize (Lam et al., 1988). The proteins binding to the promoter of *psb*A in the plastids of mustard seedlings were suggested to be different between chloroplasts and etioplasts (Eisermann et al., 1990). Thus, the protein components that interact with the promoter region of plastid genes appear to vary depending on the stage of plastid development and the cell type.

In this work, we observed that among three transcriptional initiation sites (D/C-2 through D/C-4) in the *psb*D/C gene cluster, in vitro transcription from the D/C-3 site is selectively enhanced in the plastid extract prepared from 4-h-illuminated wheat seedlings compared with that from dark-grown seedlings. This behavior well reflects the feature of in vivo light-regulated transcription from the *psb*D/C gene cluster (Sexton et al., 1990a; Kawaguchi et al., 1994), indicating that transcriptional regulation is actually involved in light-induced variations of the *psb*D/C gene transcripts.

Mutational analyses using chloroplast in vitro transcription systems have suggested that prokaryotic "−35" and "−10" sequences are required for the transcription of many plastid genes (Link, 1984; Bradley and Gatenby, 1985; Gruissem and Zurawski, 1985a, 1985b). Upstream of the transcription initiation sites at D/C-2 and D/C-4 in the wheat *psb*D/C gene cluster, DNA sequences similar to the prokaryotic promoter

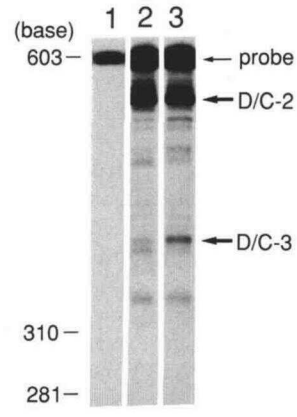

Figure 8. Induction of the transcriptional activity from the D/C-3 site in the plastid lysate from dark-grown seedlings by the addition of fraction A. In vitro transcription was carried out using plasmid pW18E866 as a template. Lanes 1 and 2 show the results with 12 μL of fraction A from the plastid lysate of 4-h-illuminated seedlings and 4 μL of the plastid lysate from dark-grown seedlings, respectively, in 40 μL of in vitro transcription reaction mixture. Lane 3 shows the results of the simultaneous addition of 4 μL of the plastid lysate of dark-grown seedlings and 12 μL of fraction A. The signals corresponding to the transcriptions from the D/C-2 and D/C-3 sites are designated by the thick arrows. Numbers to the left indicate the sizes of φX174-*Hae*III mol wt markers.

motifs are present. These motifs are also present in several other positions of the plastid DNA sequences in the templates, some of which are weakly but significantly recognized as promoters in in vitro transcription by the plastid extracts. The activities of in vitro transcription from the D/C-2, D/C-4, and other prokaryotic promoter-like motifs in the templates are independent of the plastid extracts used, and these promoter-like motifs, including the D/C-2 and D/C-4 promoters, were also recognized by *E. coli* RNA polymerase holoenzyme.

In contrast, the *psb*D/C-3 promoter was pointed out to be unusual in DNA sequence at the −35 and −10 regions and in producing microheterogeneity on the 5′ end of the transcripts (Sexton et al., 1990a). Transcription from the D/C-3 site was scarcely observed in the plastid extracts from dark-grown seedlings and was not initiated by *E. coli* RNA polymerase. Judging from the present results, transcription from the D/C-3 site requires additional factor(s) that may be induced at an early stage of light-induced development. In barley, the primer extension signals for the 5′ end of the light-induced transcripts in the *psb*D/C gene cluster were reported to be scattered over 25 nucleotides (Sexton et al., 1990a). This 5′ end microheterogeneity was suggested to be a conserved feature of light-induced *psb*D/C transcripts among some monocot plants, including wheat, and dicot plants, such as tobacco and spinach (Christopher et al., 1992). In our previous work with wheat, the primer extension signal for the D/C-3 site exhibited a tailing over 8 to 10 bases, although the ladder structure was not observed (Kawaguchi et al., 1994). Although the relationship between the usage of the unusual promoter and the 5′ end microheterogeneity of the transcripts has not been clarified yet, these features of the D/C-3 site may be correlated with a requirement of additional factor(s) for initiation of transcription.

Two possible mechanisms underlying the initiation of transcription from the D/C-3 site may be considered. The first possibility is the light induction or activation of transcriptional regulatory factor(s) that specifically activate the promoter at the D/C-3 site or release the specific depression of the D/C-3 promoter activity in the plastids of unilluminated seedlings. In either case, such light-induced regulatory factor(s) should be collected in fraction A, because fraction A (no RNA polymerase activity) induces transcriptional activity from the D/C-3 site in the plastid extract from dark-grown seedlings. At the present stage, the regulatory factor(s) have not yet been identified, but they might be sequence-specific DNA binding proteins, similar to the *trans* factors suggested to be involved in the transcriptional regulation of nuclear genes coding for plastid proteins (Gilmartin et al., 1990; Thompson and White, 1991).

The other possibility is that there is light-induced modulation of RNA polymerase through the modification of σ-like factors. In prokaryotes, different σ factors are used for transcription of various classes of genes (Helman and Chamberlin, 1988). In mustard, RNA polymerase preparations from chloroplasts and etioplasts are functionally distinct with respect to salt requirements for DNA binding, affinity to the *psb*A promoter, and the stability of the core enzyme σ-like factor complexes, although they are structurally similar to each other (Tiller and Link, 1993a). Recently, it has been demonstrated that reversible phosphorylation (etioplast) and dephosphorylation (chloroplast) of both the core enzyme and σ-like factors determines the plastid-type-specific properties of the RNA polymerase (Tiller and Link, 1993b). In the case of mustard, the σ-like factors dissociated from the plastid RNA polymerase holoenzyme tightly bind to heparin-Sepharose and are eluted only by very high salt [1.0–1.3 M $(NH_4)_2SO_4$] (Tiller and Link, 1993a). This is in contrast to the case of the light-induced regulatory factor(s) collected in fraction A as nonbinding component(s) on heparin-Sepharose columns. Therefore, if the regulatory factor(s) in fraction A are a kind of σ-like factor, they should be very different from the σ-like factors found in mustard in their affinity to heparin-Sepharose. Although we cannot distinguish between these two possibilities at this stage, we succeeded in showing the existence of a factor(s) in the plastid extract from 4-h-illuminated wheat seedlings and in separating this factor from the plastid RNA polymerase. Isolation and characterization of the factor(s) will greatly help to give insight into a mechanism of the light-regulatory transcription of plastid genes.

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