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Molecular genetic analysis of chloroplast gene promoters dependent on SIG2, a nucleus-encoded sigma factor for the plastid-encoded RNA polymerase, in *Arabidopsis thaliana*

Mitsumasa Hanaoka, Kengo Kanamaru, Hideo Takahashi and Kan Tanaka*

Laboratory of Molecular Genetics, Institute of Molecular and Cellular Biosciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan

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ABSTRACT

Most photosynthesis-related genes in mature chloroplasts are transcribed by a eubacterial-type RNA polymerase (PEP) whose core subunits are encoded by the plastid genome. It has been shown previously that six putative nuclear genes (*SIG1* to *SIG6*) encode promoter-specificity factors for PEP in *Arabidopsis thaliana*, and we isolated a T-DNA insertion line of *SIG2* (*sig2-1* mutant) that manifests aberrant chloroplast development. With the use of S1 nuclease protection and primer extension analyses, we have now characterized the SIG2-dependent chloroplast promoters in *A.thaliana*. The amounts of transcripts derived from one of the multiple *psbD* promoters (*psbD* –256) and from the promoters of two tRNA genes (*trnE*-UUC and *trnV*-UAC) were markedly and specifically decreased in the *sig2-1* mutant. The abundance of these transcripts was restored to wild-type levels by introduction into the mutant of a *SIG2* transgene. The recombinant SIG2 protein mixed with *Escherichia coli* core RNA polymerase could bind to a DNA fragment that contains the SIG2-dependent *psbD* –256, *trnE*-UUC or *trnV*-UAC promoter. Sequences similar to those of the –35 and –10 promoter elements of *E.coli* were identified in the regions of the SIG2-dependent chloroplast genes upstream of the transcription initiation sites.

INTRODUCTION

Chloroplasts are semiautonomous, photosynthetic organelles with their own genome. The genetic system operative in chloroplasts is similar to that of eubacteria, especially that of cyanobacteria, supporting the notion that extant chloroplasts of plant cells originated from a symbiotic event involving ancient oxygen-generating photosynthetic bacteria (cyanobacteria). The chloroplast genome in most higher plants comprises a double-stranded, circular DNA molecule with a size of ~150 kb. To date, approximately 120 genes of the chloroplast genome have been identified as encoding four rRNAs, 30 tRNAs and about 80 proteins, all of which are required for transcription, translation and chloroplast functions such as photosynthesis (1).

Chloroplast genes of higher plants are transcribed by at least two types of plastid RNA polymerase (2,3): one is a nucleus-encoded plastid RNA polymerase (NEP), a T3-T7 bacteriophage-type, single-subunit enzyme (4,5) that predominantly mediates the transcription of housekeeping genes such as those for components of the gene expression machinery (6); the other is a plastid-encoded plastid RNA polymerase (PEP), which is a eubacterial-type, multi-subunit enzyme. Photosynthesis-related genes, such as *psbA*, *psbD* and *rbcL*, which encode D1 and D2 subunits of the photosystem II complex and the large subunit of ribulose-1,5-bisphosphate carboxylase-oxygenase, respectively, are transcribed by PEP (7,8).

Although the genes for core subunits of PEP (*rpoA*, *rpoB*, *rpoC1* and *rpoC2*) are located in the plastid genome, the promoter-specificity (sigma) factors of this enzyme appear to be encoded by nuclear genes (9). The first description of a nuclear gene encoding a chloroplast RNA polymerase sigma factor in red algae (10,11), was followed by the identification of several nuclear genes encoding putative PEP sigma factors in higher plants, including *Arabidopsis thaliana* (12–14), *Nicotiana tabacum* (15) and *Oryza sativa* (16). To date, six genes encoding putative PEP sigma factors (*SIG1* to *SIG6*) have been identified and characterized in *A.thaliana* (12–14, 17,18). A T-DNA insertion line of *SIG2* (*sig2-1*) exhibits a pale-green leaf phenotype presumably due to impaired chloroplast development (19) (Fig. 1). Northern blot analysis did not reveal any overall reduction in the abundance of mRNAs derived from photosynthesis-related genes in this mutant. The amounts of some of the proteins encoded by these genes, however, were markedly reduced. Further analysis indicated that the abundance of a specific class of chloroplast tRNAs is reduced in the *sig2-1* mutant. It was thus concluded that this defect in tRNA expression is responsible for the impaired chloroplast development and function in this mutant (18).

To identify the promoter elements recognized by the PEP-SIG2 holoenzyme, we have now characterized chloroplast

*To whom correspondence should be addressed. Tel: +81 3 5841 7825; Fax: +81 3 5841 8476; Email: kntanaka@iam.u-tokyo.ac.jp

transcripts specifically affected by the *sig2-1* mutation with the use of S1 nuclease mapping and primer extension analyses. Transcription from one of the multiple *psbD* promoters and two tRNA gene promoters was markedly and specifically impaired in the *sig2-1* mutant. Alignment of the sequences upstream of the corresponding transcription initiation sites revealed that SIG2-dependent promoters in *A.thaliana* share structural similarity with *Escherichia coli* –10 and –35 core promoter elements.

MATERIALS AND METHODS

Plant materials

Seeds of *A.thaliana* WS (Wassilewskija), the *sig2-1* mutant (18) and the *sig2-1*-complemented transgenic strain gB (18) were sterilized with 70% ethanol and 3% sodium hypochlorite before sowing on MS plates containing 0.4% Gelrite (Wako) or Jiffy 7 (AS Jiffy Products). After stratification at 4°C for 24 h in the dark, the seeds were grown at 23°C under continuous white light for 3 weeks. Total RNA was extracted and purified from leaves with the use of an RNA isolation kit (TRIzol reagent, Molecular Research Center).

Plasmid DNA for S1 probes

Standard recombinant DNA techniques were performed basically as described (20). DNA fragments encompassing the promoter regions of *psbA* (nucleotides –250 to +250 relative to the translation initiation codon), *psbD* (–1500 to +100 relative to the translation initiation codon), *rbcL* (–900 to +100 relative to the translation initiation codon), *trnE*-UUC (–441 to +41 relative to the 5′ end of the mature $tRNA^{Glu}$), *trnY*-GUA (–250 to +50 relative to the 5′ end of the mature $tRNA^{Tyr}$), *trnD*-GUC (–350 to +50 relative to the 5′ end of the mature $tRNA^{Asp}$), *trnV*-UAC (–461 to +39 relative to the 5′ end of the mature $tRNA^{Val}$), *trnM*-CAU (–441 to +41 relative to the 5′ end of the mature $tRNA^{Met}$) or *trnfM*-CAU (–441 to +41 relative to the 5′ end of the mature $tRNA^{fMet}$) were amplified by the polymerase chain reaction from *A.thaliana* genomic DNA with following primers: *psbA*, 5′-GGTGGATCCTTCATATGATTTGGAAAAA-3′ and 5′-GAGAAGCTTGAATAATGGCACCGGAAAT-3′; *psbD*, 5′-GGTGGATCCAAAATACCCCGTTAAGTAA-3′ and 5′-GAGAAGCTTACCAACCTACAAAAACGAA-3′; *rbcL*, 5′-GTACTGCAGGTACCGGACCAATGATTTG-3′ and 5′-GAGAAGCTTAAGATATCAGTATCCTTGGT-3′; *trnE*-UUC, 5′-GCGCTGCAGTTCCCTAATTAGAAGATGGAT-3′ and 5′-GACAAGCTTCCTTGAAAGAGAGATGTCCTG-3′; *trnY*-GUA, 5′-GGCTGCAGTTCTATAGAAAAAAGAAAAAAC-3′ and 5′-GCAAGCTTTTGCCAACGAATTTACAGTCCG-3′; *trnD*-GUC, 5′-GGCTGCAGGACTTTACTTTTTTCTTTATTT-3′ and 5′-ATAAGCTTCAGCTTCCGCCTTGACAGGGCG-3′; *trnV*-UAC, 5′-GGCTGCAGAGACAATTGAGGCTAATCTAGC-3′ and 5′-GCAAGCTTGTGTAAACGAGGTGCTCTACCT-3′; *trnM*-CAU, 5′-GCGCTGCAGATTGAGTAGACTGGGTATTCA-3′ and 5′-GGCAAGCTTGCCGTATGAAAGCAATACTCT-3′; or *trnfM*-CAU, 5′-GTCCTGCAGATAGGGAGAAGAAGAGGCAAA-3′ and 5′-GACAAGCTTGGTTATGAGCCTTGCGAGCTA-3′.

The amplified DNA fragments were cloned into the pBluescript SK+ vector (Stratagene) with the use of restriction sites attached to the PCR primers (underlined). The resulting constructs were used to prepare the DNA probes for S1 nuclease protection analysis.

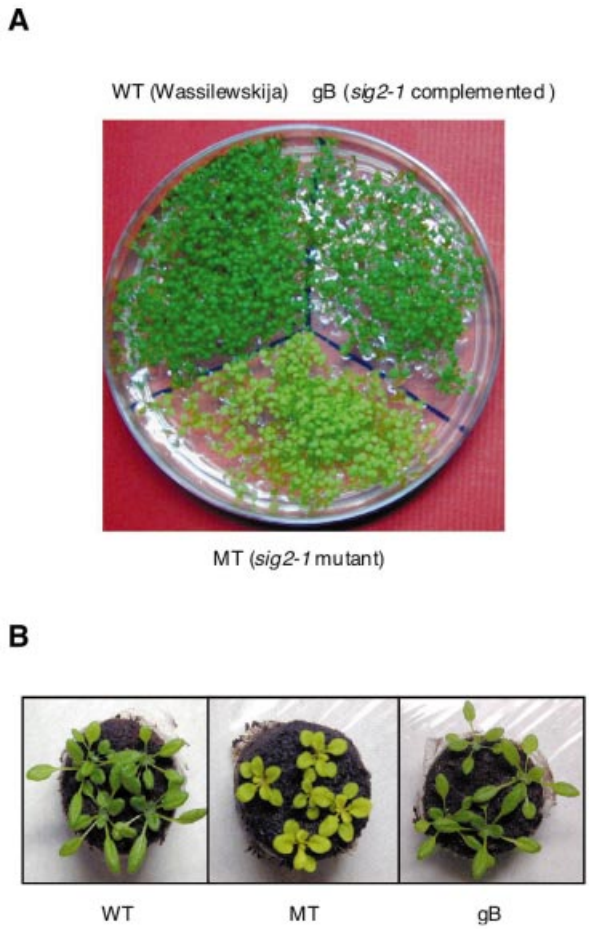

Figure 1. Pale-green phenotype of the *sig2-1* mutant of *A.thaliana* during seedling growth. The wild-type (Wassilewskija, WT), *sig2-1* mutant (MT), and *sig2-1* mutant complemented with the entire genomic region of *SIG2* (gB) were grown under continuous light at 23°C either for 1 week on an MS-Gelrite plate (**A**) or for 3 weeks on Jiffy 7 (**B**).

S1 nuclease protection assay

DNA probes labeled at their 5′ ends with ^{32}P were hybridized with total RNA (5–30 µg) at 37°C for 16 h in 10 µl of hybridization buffer containing 40 mM PIPES–NaOH (pH 6.4), 1 mM EDTA, 0.4 M NaCl and 80% formamide. The hybridization mixture was then diluted with 100 µl of ice-cold S1 nuclease mixture containing 280 mM NaCl, 50 mM sodium acetate (pH 4.5), 4.5 mM $ZnSO_4$ and S1 nuclease (500 U/ml) (TaKaRa). After incubation for 1 h at 20°C, the protected DNA probes were detected by electrophoresis through 5% polyacrylamide gels containing 7 M urea followed by analysis with a BAS1000 image analyzer (Fujix).

Primer extension analysis

Sequence-specific oligonucleotides were end-labeled with ^{32}P and hybridized with total RNA (25 µg) as described for S1 nuclease protection analysis. Primer extension was performed

for 1 h at 42°C with Superscript II reverse transcriptase (Invitrogen). Extension products were separated on 5% Long Ranger sequencing gels (BMA). The 5′ ends of the extension products were determined by comparison with cDNA sequences generated from the same primers with the use of a LI-COR sequencing kit (EPICENTRE). The primer sequences were as follows: *psbD* –256 and *psbD* –186, 5′-TTCAGGGCGCTCAAATTCTATCATTTGTTT-3′; *psbD* –541, 5′-GTTGACGGGTTGAAGCAAAAAGGGAACTTT-3′; *rbcL*, 5′-GAGAAGCTTAAGATATCAGTATCCTTGGT-3′; *trnE*-UUC, 5′-GACAAGCTTCCTTGAAAGAGAGAT-GTCCTG-3′; and *trnV*-UAC, 5′-GCAAGCTTGTGTAAAC-GAGGTGCTCTACCT-3′.

In vitro capping and RNase protection assay

Total leaf RNA from wild-type seedlings was capped by guanylyltransferase (Ambion) as described (21). Labeled RNA was hybridized with an *in vitro* transcribed antisense RNA probe for the *psbD* gene and subjected to the ribonuclease protection assay (22) using RNase cocktail (Ambion). The protected RNA was electrophoresed through 5% polyacrylamide gels containing 7 M urea and analyzed with a BAS1000 image analyzer (Fujix). To prepare the protecting RNA, the *psbD* –256 upstream region (nucleotide position from –501 to –220 relative to the ATG translation initiation site) was amplified with PCR using primers 5′-GGCGGATCCCATAAGGGCATGTACATATAG-3′ and 5′-GGCAAGCTTGGATCAACTCAATTTGTTTCT-3′. The amplified product was digested with BamHI–HindIII and cloned into pBluescript SK+ vector (Stratagene). For generating antisense RNA probe, the resulting plasmid was linearized with BamHI and transcribed using T7 Ribomax kit (Promega) following the manufacturer's protocol.

Expression and purification of recombinant SIG2 protein

The *SIG2* cDNA fragment (13) was digested with BamHI and ligated in frame into pET-15b vector (Novagen), resulting in pET-SIG2 with six histidine residues at the N-terminus of partial SIG2 protein (amino acids 187–572). Following overexpression in the BL21 strain, the fusion protein was purified on a Ni-NTA agarose column (Qiagen) according to the manufacturer's protocol.

Gel mobility shift assay

The recombinant sigma factor protein (30 ng) was mixed with 50 fmol ^{32}P-labeled DNA probe, 3 µg poly [dI–dC] and 100 ng purified *E.coli* core RNA polymerase (23) in 50 µl solution of 10 mM Tris–HCl (pH 8.0), 50 mM KCl, 1 mM EDTA, 5 mM $MgCl_2$ and 5 mM DTT. The mixture was incubated at 30°C for 30 min, and DNA–protein complexes were separated on a native 4% polyacrylamide gel followed by analysis with a BAS1000 image analyzer (Fujix). To prepare the probe DNA, the *psbD* –256, *trnE*-UUC and *trnV*-UAC upstream regions were amplified with PCR and labeled with T4 polynucleotide kinase (TaKaRa). The primer sequences were as follows: *psbD* –256, 5′-GGCGGATCCCATAAGGGCATGTACATA-TAG-3′ and 5′-GGCAAGCTTGGATCAACTCAATTTGTT-TCT-3′; *trnE*-UUC, 5′-GCGCTGCAGTTCCCTAATTAG-AAGATGGAT-3′ and 5′-GACAAGCTTCCTTGAAAGA-GAGATGTCCTG-3′; and *trnV*-UAC, 5′-GGCTGCAGAGA-CAATTGAGGCTAATCTAGC-3′ and 5′-GCAAGCTTGTG-TAAACGAGGTGCTCTACCT-3′.

RESULTS

Transcription from one of the multiple *psbD* promoters is specifically reduced in the *sig2-1* mutant

Three *psbD* transcripts with 5′ ends located 190, 550 and 950 nt upstream from the translation initiation codon have been identified in *A.thaliana* (24). We initially examined the effect of the *sig2-1* mutation on transcription from the multiple *psbD* promoters with the use of S1 nuclease protection analysis. Three major transcripts were detected in the wild-type plant, one of which, that starting at nucleotide –256 (the nucleotide position corresponding to the 5′ end of each transcript was determined by primer extension analysis as described below), was barely detectable in the *sig2-1* mutant (Fig. 2A). In contrast, those starting at nucleotides –186 and –541 were not affected by the mutation. A transcript starting at nucleotide –946 was more abundant in the mutant than in the wild-type. The *sig2-1* mutation thus appeared to result in a specific reduction in transcription from the *psbD* –256 promoter. We also examined the expression of two other photosynthesis-related genes, *psbA* and *rbcL*, by S1 nuclease protection analysis. Neither transcription from the *psbA* and *rbcL* promoters nor the abundance of a putative processed transcript of *rbcL* (*rbcL* –70) was affected by the *sig2-1* mutation (Fig. 2B).

Accumulation of specific plastid-encoded tRNAs is SIG2 dependent

With the use of northern blot analysis, we previously showed that the abundance of transcripts of four tRNA genes (*trnE*-UUC, *trnD*-GUC, *trnM*-CAU and *trnV*-UAC) was reduced in the *sig2-1* mutant (18). To clarify the role of SIG2 in transcription of these genes, we first analyzed *trnE*-UUC transcripts by the S1 nuclease protection assay. Analysis of total RNA from wild-type leaves revealed two transcripts: a major transcript that corresponded to the mature glutamate tRNA ($tRNA^{Glu}$), and a minor transcript that had a 5′ end located 26 bp upstream from that of the mature $tRNA^{Glu}$ and was likely the precursor (Fig. 3A). The abundance of both of these RNA molecules was markedly reduced in the *sig2-1* mutant. The amounts of the primary and mature transcripts derived from *trnV*-UAC were also substantially reduced in the *sig2-1* mutant (Fig. 3B).

We then analyzed several other tRNA genes (*trnY*-GUA, *trnD*-GUC, *trnM*-CAU and *trnfM*-CAU). For *trnY*-GUA, *trnD*-GUC and *trnM*-CAU, S1 mapping analysis identified only one major transcript, corresponding to the mature tRNA ($tRNA^{Tyr}$, $tRNA^{Asp}$ and $tRNA^{Met}$, respectively). The abundance of each tRNA was slightly reduced in the *sig2-1* mutant compared with that in the wild-type at 3 weeks of age (Fig. 3C). We also detected only one major transcript of *trnfM*-CAU, again corresponding to the mature tRNA ($tRNA^{fMet}$), but the amount of this transcript did not differ between mutant and wild-type plants.

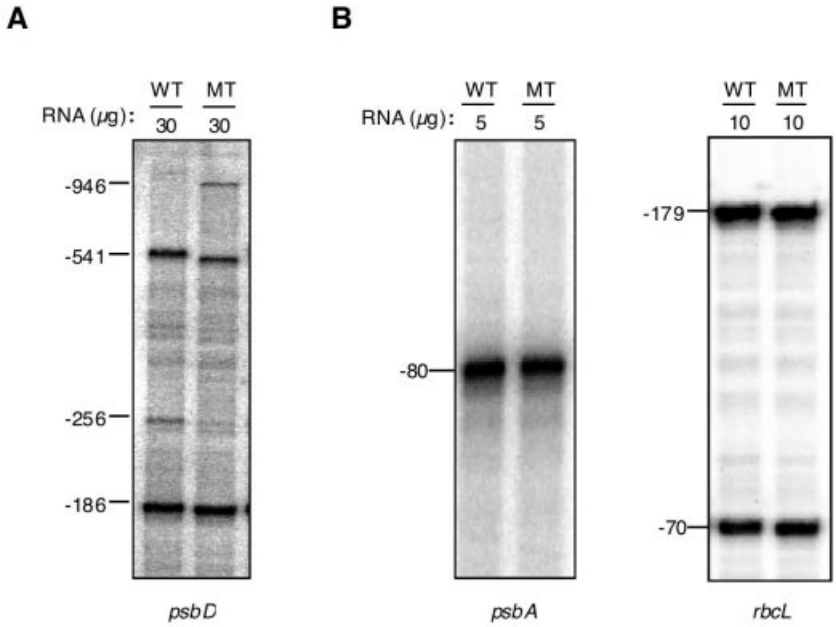

Figure 2. Transcript mapping of photosynthesis-related genes in *A.thaliana*. Transcripts of *psbD* (**A**) or of *psbA* and *rbcL* (**B**) were analyzed by the S1 nuclease protection assay with the indicated amounts of total RNA from wild-type (WT) and *sig2-1* mutant (MT) leaves. The nucleotide positions of the 5′ ends of transcripts relative to the ATG translation initiation site were determined by primer extension analysis and are indicated on the left.

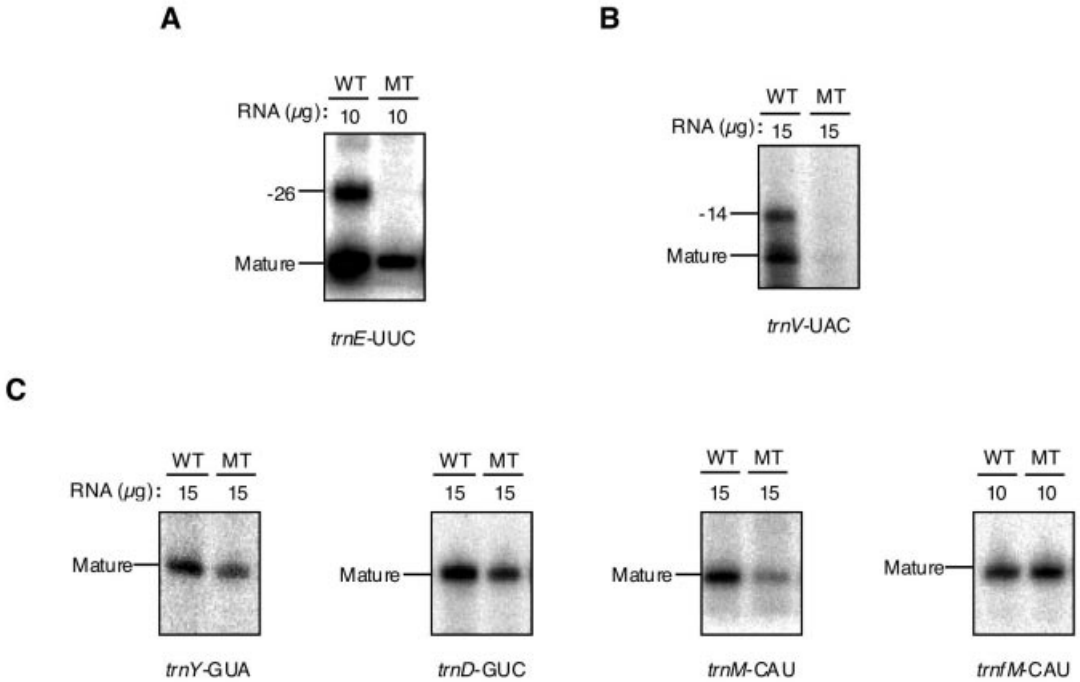

Figure 3. Transcript mapping of plastid tRNA genes in *A.thaliana*. Transcripts of *trnE*-UUC (**A**), *trnV*-UAC (**B**) or *trnY*-GUA, *trnD*-GUC, *trnM*-CAU and *trnfM*-CAU (**C**) were analyzed by the S1 nuclease protection assay with the indicated amounts of total RNA from wild-type (WT) and *sig2-1* mutant (MT) leaves. The nucleotide positions of the 5′ ends of transcripts relative to those of the mature tRNAs are indicated on the left.

Mapping of the 5′ ends of the chloroplast transcripts by primer extension

Our S1 nuclease protection analysis identified three transcripts (*psbD* –256, *trnE*-UUC and *trnV*-UAC) whose abundance was substantially reduced in the *sig2-1* mutant. We mapped the precise positions of the transcription initiation sites of these RNA species by primer extension analysis. The 5′ ends of the transcripts so determined were consistent with the results obtained with the S1 assays (Fig. 4A–C); moreover, the

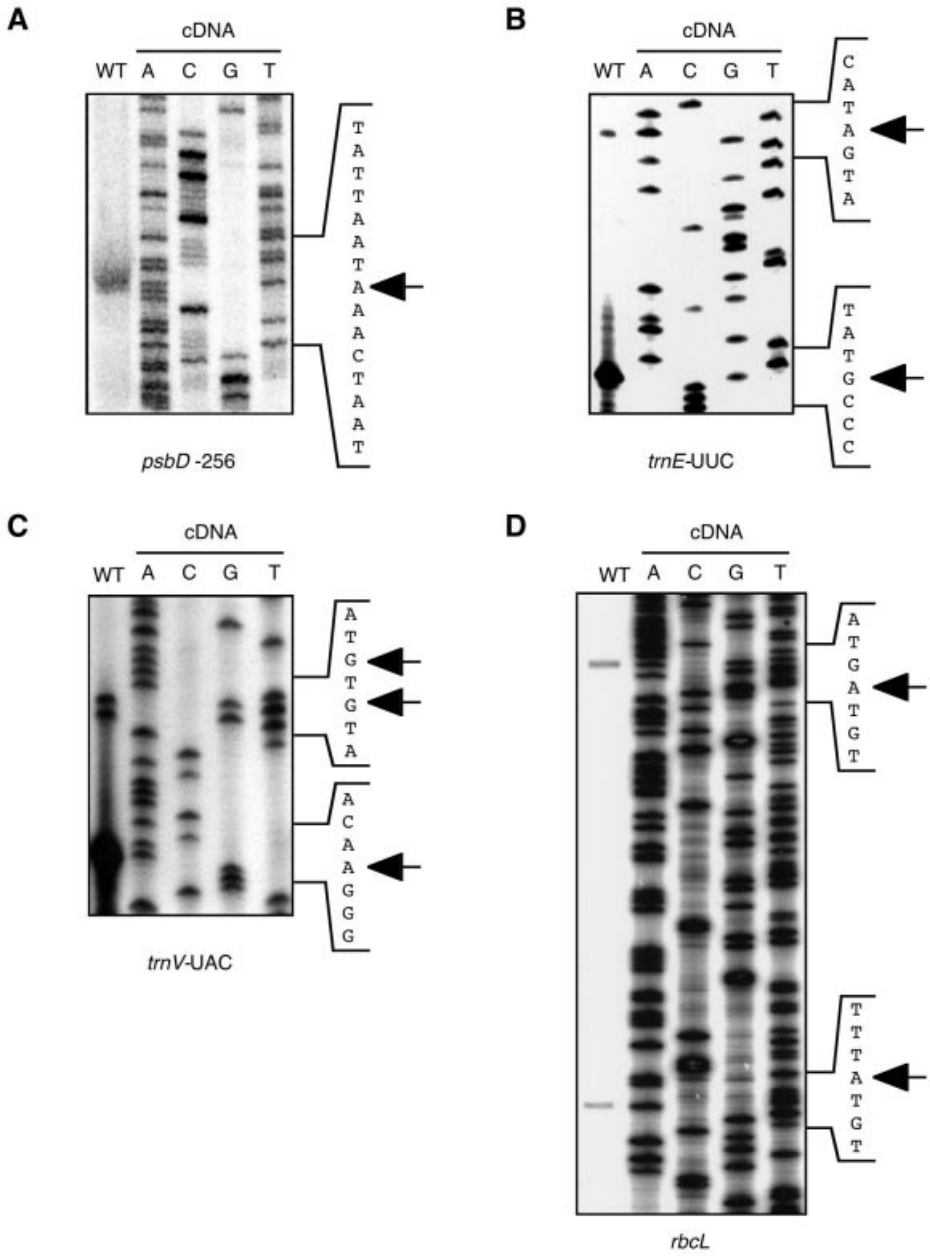

Figure 4. High-resolution mapping of the 5′ ends of SIG2-dependent (A–C) and SIG2-independent (D) transcripts. The *psbD* –256 (**A**), *trnE*-UUC (**B**), *trnV*-UAC (**C**) and *rbcL* (**D**) transcripts were analyzed by primer extension analysis with 25 µg of total RNA from wild-type (WT) leaves. The cDNA sequences (A, C, G and T) obtained with the same primers are also shown. Arrows indicate the positions of the 5′ ends of each transcript that correspond to the bands shown in Figures 2 and 3.

signals obtained with the *sig2-1* mutant were weaker than those obtained with the wild-type (data not shown). The 5′ ends of *Arabidopsis psbA* (25) and *psbD* –946 (24) were identified previously. We mapped the 5′ ends of *psbD* –190, *psbD* –550 and *rbcL* transcripts more precisely as *psbD* –186, *psbD* –541 (data not shown) and *rbcL* –179 (Fig. 4D).

The 5′ end of *psbD* –256 is of a primary transcript

In Figure 2A, the amount of a transcript from *psbD* –256 was specifically decreased in the *sig2-1* mutant. In order to verify whether this transcript is derived from RNA processing or the actual 5′ end of a primary transcript, we analyzed this transcript by *in vitro* capping and RNase protection assay. A protected 37-nt transcript that corresponds to the 5′ end of the *psbD* –256 transcript was clearly detected (Fig. 5). This result demonstrates that the 5′ end of *Arabidopsis psbD* –256 is a site of a primary transcript, not a processing product.

DNA binding affinity of the recombinant SIG2 protein to SIG2-dependent chloroplast promoters

We identified three SIG2-dependent transcripts (*psbD* –256, *trnE*-UUC and *trnV*-UAC) by means of S1 mapping analysis. To further confirm the role of SIG2 in recognition of these promoters, we used gel-shift DNA binding assays. The

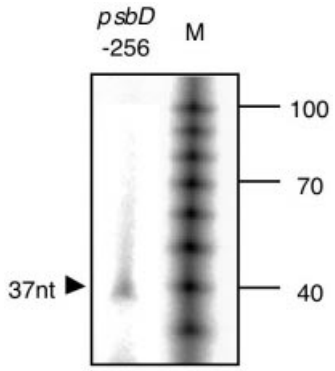

Figure 5. Ribonuclease protection assay for the *in vitro* capped primary transcript from *psbD* –256. Fifty micrograms of total RNA from wild-type leaves was capped by guanylyltransferase and [α-^{32}P]GTP, followed by RNase protection assay using *in vitro* transcribed antisence RNA for the *psbD* gene as a probe. Note that the probe protects a 37-nt fragment for the transcript from *psbD* –256. Lane M shows RNA marker of the sizes indicated at the right.

heterologous system was based on previous reports demonstrating that the recombinant chloroplast sigma factor(s) mixed with *E.coli* core RNA polymerase could efficiently bind to the promoter DNA (26,27). As shown in Figure 6, a shifted band corresponding to a DNA–protein complex was detected (lanes 3) when the recombinant SIG2 protein and *E.coli* core RNA polymerase was incubated with a DNA fragment containing the promoter region of *psbD* –256 (left panel), *trnE*-UUC (middle panel) or *trnV*-UAC (right panel). This binding signal was markedly reduced in the presence of excess unlabeled promoter DNA as a competitor (lanes 4). None of the control reactions consisting of the probe DNA alone (lanes 1), or the probe DNA and the recombinant SIG2 protein (lanes 2), gave a shifted band of the promoter-SIG2 complex. No shifted band was also detected when the DNA probe was mixed with *E.coli* core RNA polymerase alone (lane C). These observations strongly suggest that these three promoters are recognized by PEP-SIG2 holoenzyme.

Marked homology of *Arabidopsis* SIG2-dependent promoters upstream of the transcription initiation sites

We next compared the nucleotide sequences of genes upstream of the mapped 5′ ends of transcripts (Fig. 7). The consensus sequence of sigma70-type promoters in *E.coli* comprises TTGACA (–35 element) and TATAAT (–10 element) with a 17–19-bp spacer (28,29). We identified –35 element-like and –10 element-like sequences upstream of the transcription initiation sites of the SIG2-dependent *psbD* –256, *trnE*-UUC and *trnV*-UAC promoters as well as of those of the promoters of *psbA*, *rbcL* and *psbD* –946. The –10 element was partially conserved between *psbD* –256 (TAT-ACT), *trnE*-UUC (TACTAT) and *trnV*-UAC (TAAGAT) promoters, whereas the –35 element was identical (TTGACA) in the two tRNA gene promoters. In addition, the conserved pentanucleotide element [A(A/T)TTA] in the spacer region was found only in three SIG2-dependent promoters.

In vivo complementation of SIG2-dependent transcription defects by a *SIG2* transgene

We established previously a transgenic *Arabidopsis* line, designated gB, by introducing the *SIG2* genomic region into the *sig2-1* background (18). Normal leaf color was restored in the transgenic plants (Fig. 1). We examined the effect of *in vivo* complementation of the *sig2-1* mutation on SIG2-dependent transcription by S1 mapping. The marked reduction in transcription from the *psbD* –256 promoter observed in the *sig2-1* mutant was no longer apparent in the gB transgenic line, which showed a transcript abundance similar to that of

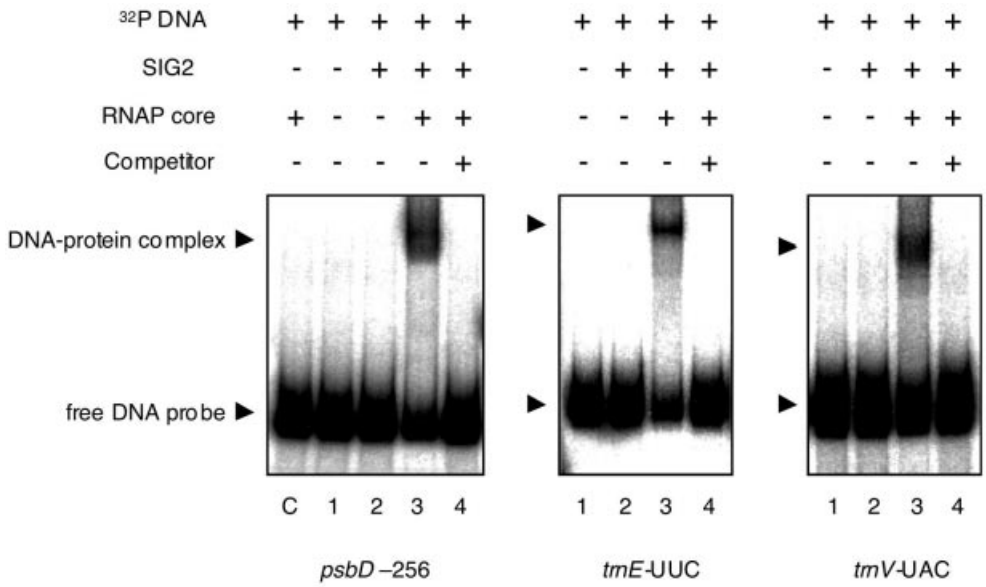

Figure 6. Gel-shift DNA binding assay with the recombinant SIG2 protein. The SIG2 protein was incubated with a ^{32}P-labeled DNA fragment containing *psbD* –256 (left), *trnE*-UUC (middle), *trnV*-UAC (right) promoter region in the absence (lane 2) or presence (lane 3) of *E.coli* core RNA polymerase. Labeled DNA alone (lane 1), and a DNA probe with core RNA polymerase (lane C) were also shown as controls. Competition experiments were carried out as in lane 3 with a 100-fold excess of unlabeled each promoter fragment (lane 4).

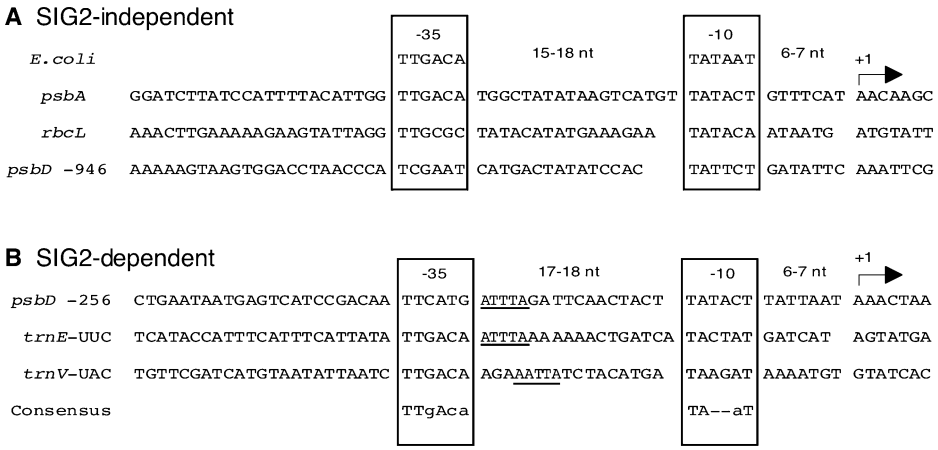

Figure 7. Alignment of nucleotide sequences of the regions upstream of the transcription initiation sites of SIG2-independent (**A**) and SIG2-dependent (**B**) *Arabidopsis* gene promoters. The arrows indicate the position corresponding to the 5′ end of each major transcript. The *E.coli*-like –35 and –10 promoter elements are boxed, and the underlined regions refer to conserved promoter elements specific to the three SIG2-dependent promoters. The consensus sequence motifs for the –35-like and –10-like elements of the SIG2-dependent promoters are also indicated in (B); upper case and lower case letters denote nucleotides that are completely or partially conserved among the three promoters.

the wild-type (Fig. 8A). The gB plant also did not exhibit the marked decreases in the abundance of both the primary and mature transcripts of *trnE*-UUC and *trnV*-UAC observed in the *sig2-1* mutant (Fig. 8B). These results thus support the notion that the *psbD* –256, *trnE*-UUC and *trnV*-UAC promoters are recognized by PEP in a SIG2-dependent manner.

DISCUSSION

Little is known of the roles of the multiple nucleus-encoded sigma factors in selective recognition of the promoters of plastid-encoded genes. Our study has now provided several lines of experimental evidence indicating that the *Arabidopsis psbD* –256, *trnE*-UUC and *trnV*-UAC promoters are transcribed in a SIG2-dependent manner. Sequence analysis of the regions flanking the transcription initiation sites of these genes also revealed that they contain promoter elements (–35-like and –10-like elements) similar to those required for transcription in *E.coli*. *In vivo* complementation demonstrated that the marked decreases in the amounts of the corresponding transcripts apparent in the *sig2-1* mutant were corrected by the introduction of a *SIG2* transgene. Moreover, DNA fragments containing the promoter region of these genes could be specifically bound to the recombinant SIG2 protein with *E.coli* core RNA polymerase. These findings as well as the result by *in vitro* capping experiment strongly indicate that the *psbD* –256 mRNA is a primary transcript generated from a SIG2-dependent promoter rather than a product of RNA processing.

A *psbD* transcript initiated at nucleotide position –946 has also been identified and shown to be derived from a blue light-responsive promoter (BLRP) (24,30,31). This transcript was more abundant in the *sig2-1* mutant than in the wild-type, possibly because transcription from the *psbD* BLRP depends on a sigma factor whose ratio of association with the PEP core enzyme is increased in the *sig2-1* mutant, or because of compensatory mechanisms among sigma factors or

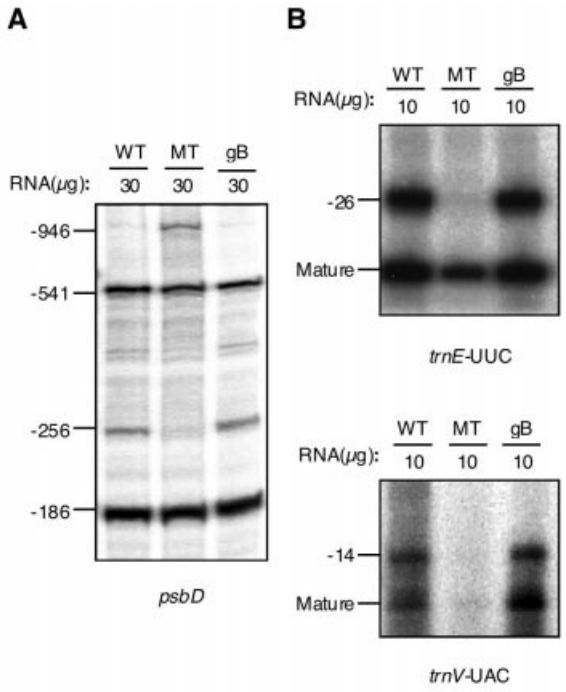

Figure 8. *In vivo* complementation of SIG2-dependent transcriptional defects. The *psbD* (**A**) as well as *trnE*-UUC and *trnV*-UAC (**B**) transcripts were analyzed by S1 nuclease protection assays with the indicated amounts of total RNA from wild-type (WT), *sig2-1* mutant (MT) and gB transgenic leaves. The nucleotide positions of the 5′ ends of transcripts relative to the ATG translation initiation site or to the 5′ end of the mature tRNA are indicated on the left.

gene-specific DNA-binding proteins such as PTF1, a transcription factor for the AGT box in the *psbD* BLRP (32). Very recently, blue light dependency of the *SIG5* expression was reported and suggested to be involved in the recognition of the *psbD* BLRP in *A.thaliana* (33). Our results therefore now indicate that at least two different sigma factors mediate

transcription from the multiple *psbD* promoters in *A.thaliana*. In maize, complementary expression of two sigma factors has also been suggested to support the differential expression of plastid genes (34).

We have also identified the 5′ ends of two *Arabidopsis rbcL* transcripts. The transcript whose 5′ end mapped to position –70 relative to the translation initiation site has been shown previously to be generated by RNA processing in other plants (35–37). The nucleotide sequence of *A.thaliana rbcL* upstream of the position (–179) corresponding to the 5′ end of the other transcript is highly homologous to that of functional *rbcL* promoters in other plants (data not shown). The abundance of neither of the *rbcL* transcripts nor that of *psbA* mRNA was affected by the *sig2-1* mutation, suggesting that the corresponding promoters are recognized by sigma factors other than SIG2. This conclusion is supported by the results of heterologous *in vitro* transcription assays showing that SIG1 contributes to transcription from *psbA* and *rbcL* promoters in mustard and *Arabidopsis* (26,38).

We performed S1 mapping and gel mobility shift analyses and showed that SIG2-dependent promoter was located upstream of the *trnE* gene. The *trnE*, *trnY* and *trnD* genes are tandemly arranged on the plastid genome (39) and are co-transcribed in tobacco chloroplasts (40). We have now shown that the accumulation of all three encoded tRNAs is SIG2 dependent. These observations may indicate that SIG2 mediates transcription of the *trnE-trnY-trnD* operon from the promoter upstream of *trnE* in *Arabidopsis*.

The tRNAs encoded by *trnV*-UAC and *trnM*-CAU also accumulated in a manner dependent on SIG2. Although both tRNA genes share conserved upstream sequences (18), we detected a precursor only for *trnV*. Given that no genes have been assigned nearby *trnM* (39), transcripts derived from the upstream region of this gene may be processed immediately to the mature $tRNA^{Met}$. A similar explanation may underlie our failure to detect a precursor of the SIG2-independent *trnfM*-CAU gene; although it is located downstream of the *psaA-psaB-rps14* operon, co-transcripts extending to this gene were not detected in tobacco (41).

The sequences of the SIG2-dependent promoters of *psbD* –256, *trnE*-UUC and *trnV*-UAC are distinct but share structural similarities to *E.coli*-type –35 (TTGACA) and –10 (TATAAT) core promoter elements. The –35 elements of the *trnE*-UUC and *trnV*-UAC promoters are identical to that of the sigma70 consensus sequence (TTGACA) whereas that of *psbD* –256 (TTCATG) is less conserved, possibly explaining the differential dependence of these genes on SIG2.

In the chloroplast of plants, the $tRNA^{Glu}$ is known to be required for translation of plastid genes as well as for synthesis of ALA, the precursor of tetrapyrrole compounds (42,43). Thus, these observations might explain in higher plant species that SIG2-dependent expression of plastid tRNA genes plays quite important roles in coupling of protein and chlorophyll biosynthesis for normal chloroplast development.

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