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The role of *SIG2* on the expression of photosynthesis genes in chloroplasts

M Hanaoka, A Nagashima, K Kanamaru, K Tanaka, H Takahashi

Laboratory of Molecular Genetics, Institute of Molecular and Cellular Biosciences, The University of Tokyo, Bunkyo-ku, Tokyo, 113-0032 Japan.
Fax: +81-3-5841-8476, E-mail: htakaha@imcbns.iam.u-tokyo.ac.jp

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Introduction

Chloroplast genes of higher plants are transcribed by at least two types of plastid RNA polymerases (for review, see Maliga, 1998; Hess and Börner, 1999). One of them is a nuclear-encoded RNA polymerase (termed NEP). NEP is a T7 bacteriophage-type, single-subunit enzyme which is related to the mitochondrial RNA polymerase (Hedtke et al., 1997), and mainly responsible for the transcription of genes encoding housekeeping proteins such as those of gene expression apparatus (Hajdukiewicz et al., 1997). Another is a plastid-encoded RNA polymerase (termed PEP), which is a eubacteria-type, multi-subunit enzyme. Photosynthesis genes including *psbA, psbD* and *rbcL* have been shown to be transcribed by PEP (Allison et al., 1996). The core subunits of PEP are encoded by genes, *rpoA*, *rpoB*, *rpoC1*, and *rpoC2* on the plastid genome. In contrast, multiple chloroplast sigma factors that are expected to recognize specific promoter sequences and initiate transcription, are encoded on the nuclear genome. It is assumed that each sigma factor is involved in transcription from different promoter sets in response to both developmental and environmental signals. However, the roles of individual sigma factor in chloroplast transcription remain to be determined.

We have identified and characterized six sigma factor genes (*SIG1* to *SIG6*) from *Arabidopsis thaliana* (Tanaka et al., 1997; Kanamaru et al., 1999; Fujiwara et al., 2000). Among them, a T-DNA insertion line of the *SIG2* gene was recently isolated. This mutant exhibits impaired chloroplast development and a consequent phenotype characterized by pale-green leaves (Shirano et al., 2000). Northern hybridization analysis was performed to examine if this mutation affects the transcriptional levels of several photosynthesis genes. However, no apparent differences in the expression of these genes were found in this mutant. In this work, we further examined transcripts of several chloroplast genes in detail. To analyze the transcripts at the promoter resolution, we carried out S1 nuclease mapping and primer extension analyses on the *Arabidopsis psbA, psbD, rbcL* and *trnE* promoters.

Materials and methods

Plasmid DNA: DNA fragments encompassing promoter region of *psbA* (from -250 to +250 with respect to the translation initiation codon, ATG), *psbD* (from -1500 to +100 of the ATG), *rbcL* (from -900 to +100 of the ATG) and *trnE* (from -441 to +41 of the mature $tRNA^{GLU}$ initiation site) were amplified by means of PCR from *Arabidopsis thaliana* DNA using gene-specific oligonucleotides as primers. The resulting DNA fragments were cloned into pBluescript SK vector using the restriction sites attached to the PCR primers. These constructs were used to prepare the DNA probes for the S1 nuclease protection analysis.

Plant materials: *Arabidopsis thaliana* seeds were sterilized with 70% (v/v) ethanol and 3% (v/v) sodium hypochlorite before sowing on MS plates containing 0.4% gelrite (TaKaRa) or Jiffy 7 (AS Jiffy Products Ltd, Norway). After stratification at 4ºC for 24 hours under dark condition, the seeds were grown at 23ºC under continuous white light for 1 to 3 weeks.

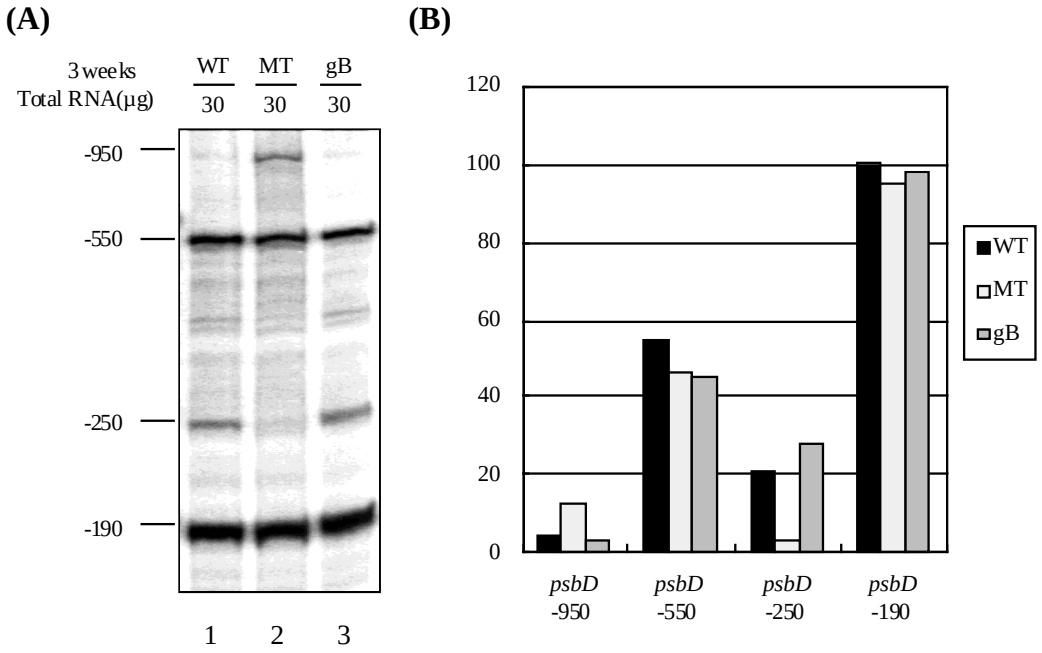

Fig. 1. Mapping of *psbD* promoters.

A. S1 mapping analysis of RNA from wild-type (WT; lane1), *sig2* mutant (MT; lane2) and genomic complemented *Arabidopsis* (gB; lane3) leaves. The numbers given on the left refer to the nucleotide position of the transcripts 5' ends relative to the ATG translation initiation codon. B. The mean relative abundance of individual transcripts, as estimated using bioimage analyzer, is plotted in the form of a bar graph.

Extraction and purification of total leaf RNA was performed with an RNA isolation kit (TRIzol reagent, Molecular Research Center Inc., U.S.A.) according to the manufacturer's protocol.

S1 nuclease protection assay: A 5'-radiolabeled DNA probe was hybridized with total RNA (5-30µg) at 37ºC for 16 h in 10 µL of hybridization buffer containing 40 mM PIPES (1,4-piperazinediethanesulfonic acid) (pH 6.4), 1 mM EDTA (pH 8.0), 0.4 M NaCl, and 80% (v/v) formamide. The solution was diluted with 100 µL of ice-cold S1 nuclease mixture containing 0.28 M NaCl, 0.05 M sodium acetate (pH 4.5), 4.5 mM $ZnSO_4$, and 500 units/mL of S1 nuclease (TaKaRa), and subsequently incubated at 20ºC for 1 h. The protected DNA fragments were analyzed on 7 M urea denaturing gel containing 5% (w/v) acrylamide (mono:bis = 19:1).

Primer extension analysis: Sequence-specific oligonucleotide was ^{32}P-end-labelled, and hybridized with total RNA (20-50µg) as described above. Primer extensions were performed according to the manufacturer's protocol using Superscript II reverse transcriptase (Gibco-BRL) at 42ºC for 1 h. Extension products were separated on 6% Long Ranger sequence gels. The 5' ends of the extension products were determined by comparison with cDNA sequences generated with the same primer using the LI-COR sequencing kit (LI-COR Inc., U.S.A.).

Results

In *Arabidopsis thaliana*, three *psbD* transcripts having the varying 5' ends locating 190, 550 and 950 nucleotides upstream from the *psbD* translational initiation codon were identified

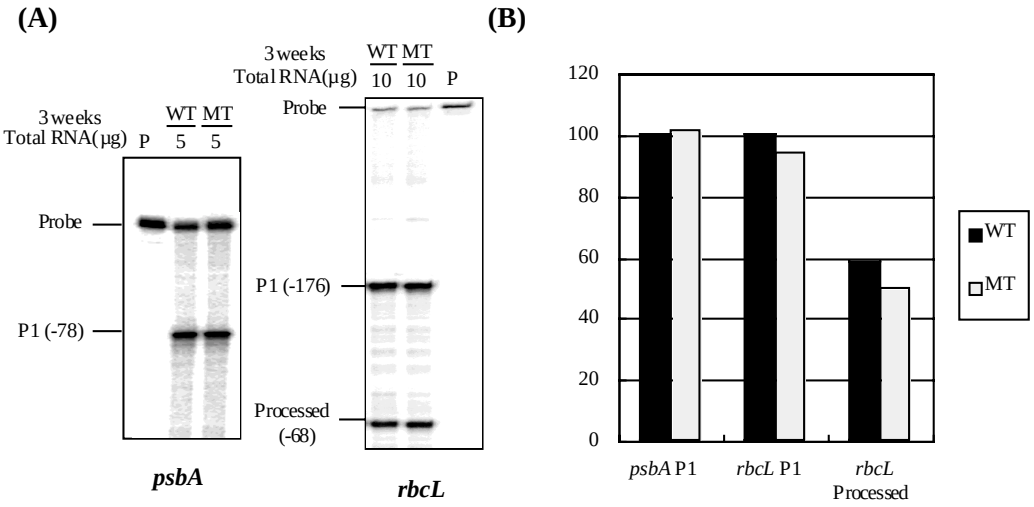

Fig. 2. Mapping of *psbA* and *rbcL* promoters.

A. S1 mapping analysis of RNA from wild-type (WT) and *sig2* mutant (MT) *Arabidopsis* leaves. The numbers given on the left refer to the nucleotide position of the transcripts 5' ends relative to the ATG translation initiation codon. The major transcript 5' end for the *rbcL* gene mapped to position -68 with respect to ATG, has been shown to result from processing of primary transcripts. B. The mean relative abundance of individual transcripts, as estimated using bioimage analyzer, is plotted in the form of a bar graph.

(Hoffer and Christopher, 1997). We initially examined the effect of the *SIG*2-lesion on the transcription from multiple chloroplast *psbD* promoters by S1 nuclease protection assays. As shown in Fig. 1A, four major transcripts were detected in the wild-type plant (Fig. 1A, lane1). Among them, the one starting at nucleotide no. -250 (*psbD* -250) was hardly detected in the *sig2*-mutant. A transcript starting from -950 was more abundant in the *sig2*-mutant (Fig. 1A, lane2). In contrast, those starting from -190 and -550 nucleotides were not affected in this mutant. We also examined the expression of two other photosynthesis genes, *psbA* and *rbcL*, by S1 nuclease protection assay. Transcription from *psbA* and *rbcL* promoters were not affected by the *SIG2* mutation (Fig. 2).

For further analysis, we established a transgenic *Arabidopsis* line, named gB, in which the genomic region of the *SIG2* gene had been introduced into the *sig2* background. Using this transgenic plant, we examined the effect of *in vivo* complementation of the *SIG2* gene by S1 mapping. As shown in Fig. 1, transcription from *psbD* –250 was completely restored up to the similar level of the wild type (Fig. 1A, lane3).

More recently, we found that accumulation of plastid-encoded $tRNA^{Glu}$ (the *trnE* gene product) was markedly reduced in the *sig2*-mutant by Northern hybridization analysis (Kanamaru et al., submitted). Here, we show the S1 mapping experiment to clarify the possible role of *SIG2* on the transcription from *trnE* promoter. As shown in Fig. 3, both

transcription from the *trnE* promoter and the consequent accumulation of $tRNA^{Glu}$, were significantly reduced in the *sig2*-mutant and restored in the gB transgenic plant (Fig. 3).

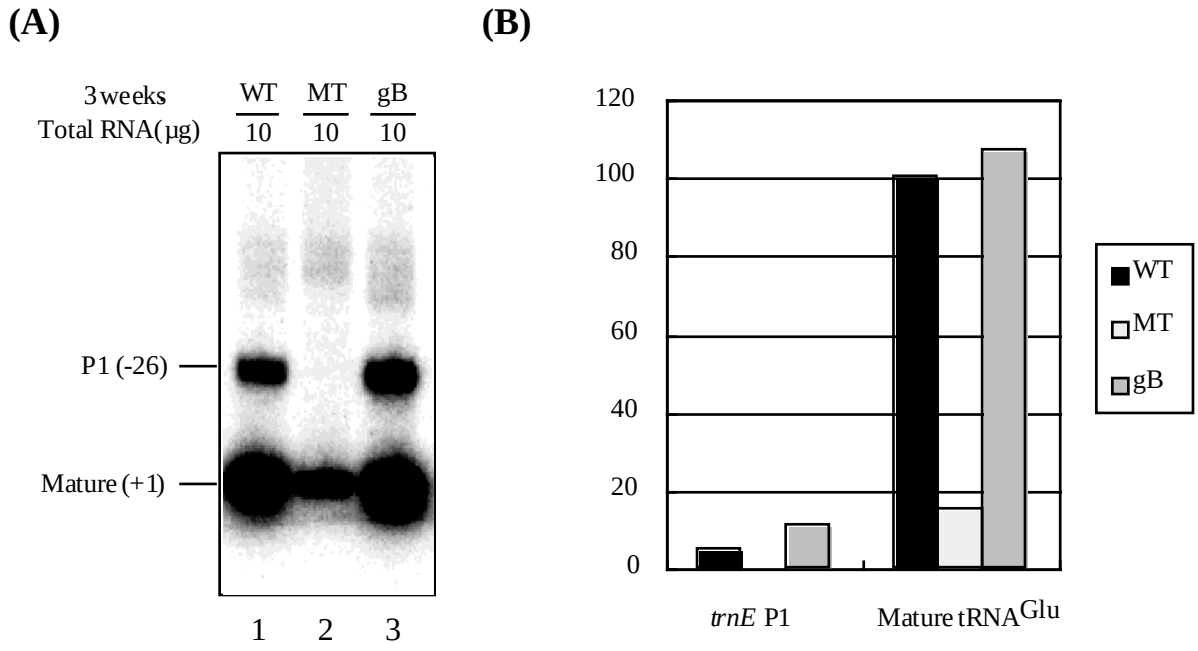

Fig. 3. Mapping of *trnE* promoter.

A. S1 mapping analysis of RNA from wild-type (WT; lane1), *sig2* mutant (MT; lane2) and genomic complemented *Arabidopsis* (gB; lane3) leaves. The numbers given on the left refer to the nucleotide position of the transcripts 5' ends relative to the mature transfer RNA initiation site. B. The mean relative abundance of individual transcripts, as estimated using bioimage analyzer, (expressed as a percentage) is plotted in the form of a bar graph.

To identify the *SIG2*-dependent promoters, we determined 5' ends of the *trnE* and *psbD* -250 transcripts by primer extension method. By comparing upstream sequences of the transcriptional start sites, the *E.coli* -like '-10' and '-35' core promoter elements, presumably recognized by PEP holoenzyme containing *SIG2*, were identified (Fig. 4).

These results suggest that *trnE* and one of multiple *psbD* promoters locating upstream of -250 (*psbD* -250) may be recognized depending on *SIG2*.

Discussion

Little is known about roles of multiple nuclear-encoded sigma factors on the selective promoter recognition of plastid-encoded genes. In this study, several lines of experimental evidence support the idea that the *Arabidopsis psbD* -250 and *trnE* promoters are positively regulated by *SIG2*. The chloroplast *psbA* and *rbcL* promoters, as well as the other *psbD* promoters (-190, -550, -950) differed from the *psbD* -250 and *trnE* promoters in the *SIG2* dependence, suggesting that such promoters are mainly recognized by sigma factor(s) other than *SIG2*.

Arabidopsis sig2 mutant exhibits a pale-green phenotype with impaired chloroplast development compared with the wild-type plants (Shirano et al., 2000). A shortage of chloroplast proteins in the *sig2* mutant caused by limited expression of several tRNA species is probably responsible for these phenotypes (Kanamaru et al., submitted).

Investigation of other *SIG2*-dependent promoters will provide further information about *SIG2*-specific promoter structure, and *in vitro* transcription assay using the recombinant SIG2 protein will be required to investigate its biochemical function in the future.

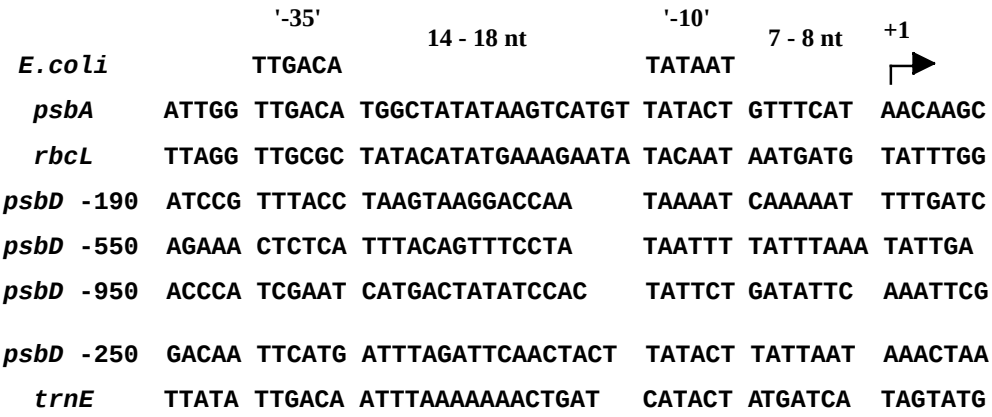

Fig. 4. Sequence comparison of *Arabidopsis psbA, psbD, rbcL* and *trnE* promoters.

The *E. coli*-like -35 and -10 promoter elements are shown. The arrow refers to the major transcription initiation site.

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