Novel nuclear-encoded proteins interacting with a plastid sigma factor, Sig1, in *Arabidopsis thaliana*[1]

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Abstract Sigma factor binding proteins are involved in modifying the promoter preferences of the RNA polymerase in bacteria. We found the nuclear encoded protein (SibI) that is transported into chloroplasts and interacts specifically with the region 4 of Sig1 in *Arabidopsis*. SibI and its homologue, T3K9.5 are novel proteins, which are not homologous to any protein of known function. The expression of *sibI* was tissue specific, light dependent, and developmentally timed. We suggest the transcriptional regulation by sigma factor binding proteins to function in the plastids of higher plant. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Plastid RNA polymerase;
Sigma factor binding protein

1. Introduction

Transcription in plastids is mediated by at least two types of RNA polymerases [1,2]. One is nuclear-encoded phage-type RNA polymerase. The other is the multi-subunit *Escherichia coli*-type RNA polymerase termed PEP (plastid-encoded plastid RNA polymerase). PEP is responsible for the transcription of many photosynthesis-related genes. In some plants, it has been demonstrated that promoter preference of PEP was developmentally regulated [3–5]. The promoter recognition and site-specific transcription by PEP require the nuclear encoded subunit, sigma factors [6]. In *Arabidopsis*, six sigma factors (Sig1–Sig6) were annotated to have four conserved regions homologous to the functionally well-characterized four conserved regions in general bacterial sigma factors such as *E. coli* σ^{70} [7–9]. In the bacterial transcription apparatus, sigma factor binding proteins (anti-sigma factors) are involved in switching the usage of the available sigma factors or modifying their promoter preferences [10,11]. However, the regulation by sigma factor binding protein has never been found in the plastid.

Here, we firstly report the nuclear-encoded plastid sigma factor binding proteins in *Arabidopsis thaliana*, which interact with the C-terminal region 4 (R4) of Sig1 known to recognize the −35 element in σ^{70}-type promoters. The results presented

in this report suggest that the regulation by sigma factor binding proteins similar to the bacterial system exists in the plastid transcription in higher plants.

2. Materials and methods

2.1. Yeast two-hybrid screening

The cDNA corresponding to the C-terminal 89 aa of *A. thaliana* Sig1 was amplified by RT-PCR with primers Eco-sig1, 5′-CG<u>GA-ATTC</u>CGTTTGGAGAACAATCCG-3′, and Pst-sig1, 5′-GG<u>CTGC-AG</u>TCAATTCTTAAGGATCAT-3′. The amplified fragment was cloned into *Eco*RI–*Pst*I site of pAS2-1 (TRP1; Clontech) to express the GAL4 DNA binding domain (BD)–Sig1 region 4 fusion protein (pAS-sig1R4). *A. thaliana* (Columbia) MATCHMAKER cDNA library in pGAD10 (LEU2; Clontech) was screened by using pAS-sig1R4 according to the manufactures' instructions. Full-length cDNA was obtained from *A. thaliana* (Columbia) 5′-STRETCH cDNA library (Clontech) by a standard procedure.

2.2. Yeast two-hybrid assays

The cDNAs corresponding to the entire coding sequence of *sibI* and *t3k9.5* (see Section 3.1) were amplified by RT-PCR and cloned into *Nco*I–*Bam*HI site of pACT2 (Clontech) to generate plasmids, pACT-sibI and pACT-t3k9.5 designed to express AD–SibI and AD–T3K9.5 hybrid, respectively. The cDNAs corresponding to C-terminal regions of Sig2 (89 aa), Sig4 (90 aa) and Sig5 (87 aa) were amplified by RT-PCR and cloned into *Eco*RI–*Pst*I site of pAS2-1 to generate a series of plasmids: pAS-sig2R4, pAS-sig4R4 and pAS-sig5R4. These plasmids were introduced into the yeast reporter strain, CG1945, together with pACT-sibI, pACT-t3k9.5, or pTD1-1 to be tested for their reporter genes expression. As a positive control, pTD1-1 and pVA3-1 were used, which encode AD–SV40 large T antigen and BD–murine p53 fusion protein, respectively. The β-galactosidase activity was quantified according to manufacturer's instruction using CRGP substrate.

2.3. Overexpression of maltose binding protein (MBP)–SibI in E. coli

SibI was expressed as a fusion protein with MBP (MBP–SibI) in *E. coli* JM109 (pMALc2, New England Biolabs). MBP–SibI was recovered from the inclusion body or from the soluble fraction by standard methods. MBP–SibI recovered from the inclusion body was collected as the fraction precipitated by 20–30% ammonium sulfate. To recover MBP–SibI from soluble fraction, amylose column was used according to manufacturer's suggestions. The samples were stored at −20°C until use.

2.4. Glutathione-s-transferase (GST) pull-down assay

GST and GST fusion proteins with the Sig1R4 (GST–Sig1R4) and the Sig2R4 (GST–Sig2R4) were expressed in *E. coli* BL21, and immobilized on glutathione Sepharose 4B beads according to the manufacturer's instructions (pGEX-4T-1, Pharmacia Biotech).

The binding tests for MBP–SibI and MBP to the prepared beads were done by incubating each combination of the samples at 25°C for 1 h in buffer A (20 mM Tris–HCl pH 7.3, 150 mM NaCl). After complete wash by buffer A, bound proteins were eluted by a glutathione solution (50 mM glutathione, 150 mM NaCl, and 100 mM Tris–HCl, pH 8.0) and the eluates were analyzed by SDS–PAGE

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followed by silver staining (Fig. 2A) or Western analysis (Fig. 2B). For the latter, rabbit polyclonal antisera were prepared against synthetic SibI oligopeptide (CRVLHQEPFGERDSD) by Sawady Technology (Japan). Anti-SibI peptide antibody was affinity-purified with the synthetic oligopeptide. Western analysis was carried out with PVDF membranes and ECL kit (Amersham Pharmacia Biotech).

2.5. Transient expression of GFP-fusion proteins in protoplasts

The cDNA fragment corresponding to the full-length of SibI (TP-F) was amplified by PCR with primers, U-Nco: 5′-GGCCATG-GAGTCATCATCGT-3′ and D-NcoFull: 5′-GCCCATGGAATC-GATGCTTC-3′. The resulting DNA fragment was digested with NcoI and cloned into NcoI site of CaMV35S-sGFP(S65T)-nos3′ vector [8]. The plasmids expressing GFP alone (CaMV35S-sGFP(S65T)-nos3′) and GFP fused with the transit peptide of RbcS (RBCS1A, [12]) were kindly gifted by Drs. Niwa and Kobayashi (University of Shizuoka) and used as the negative and positive controls, respectively. Protoplasts isolated from rosette leaves of 4-week-old *A. thaliana* grown under 8 h photoperiods were transformed with each of the plasmids as previously described [13]. GFP fluorescence from the transformed protoplasts was observed by a conventional fluorescence microscope (OLYMPUS, IMT2-RFC). The images obtained by Cool Snap ver. 1.0.0 (Rover Inc.) were processed using Adobe Photoshop 4.0.

2.6. Northern hybridization analyses

A. thaliana (Columbia) was grown at 25°C on vermiculite for 4 weeks under 16 h photoperiods to examine the tissue specificity of *sibI* mRNA accumulation and for 7 weeks under continuous light to examine the effect of light. Flower stalks were nipped off to avoid senescence of rosette leaves. To examine the *sibI* expression in young seedlings, seeds were plated onto RM medium containing 3% sucrose [14], and grown at 25°C under 16 h photoperiods for 10 days.

Total RNAs were prepared as described previously [15]. Northern hybridization was done with 10 or 30 μg each of the total RNAs using the *AccI–ClaI* fragment of *sibI* labeled with [α-^{32}P]dCTP as a probe.

A

B

SibI   MESSSSTFLTTTSLDKKKPSPVSRKSPKQKKKTTSTNKPIKVRYISNPMR   50
          ||||||| |||| |||| |||||| ||| |||||||||||||||||
T3K9.5   MDQSSSTLLIN---QRKSSSSPTRIPPKQKRKSTTTHKPIKVRYISNPMR   47

SibI   VQTCASKFRELVQELTGQDAVDLQPEPIYSPSSDDHNLSPPAENLAPRVL   100
          | || |||||||||||||||||| |||| || ||| |||||||||||
T3K9.5   VETCPSKFRELVQELTGQDAADLPPSPTTFTAVDLHRPCESEMNLEP--L   95

SibI   HQEPFGERDSDCYEPLNAEDMFLPDQMSAGFSGFFSNGFYNVNDFGSIDSM. 151
          || ||| ||| || || ||||||| |||||||||| || |
T3K9.5   D----GEVRGEYYSPLD-EEVFNAPQMSAGLSGFFSSGFYNVNALGSIGSL. 141
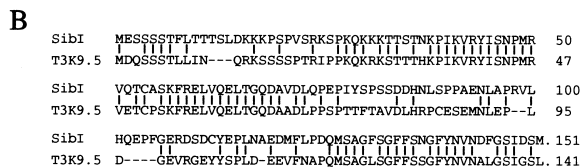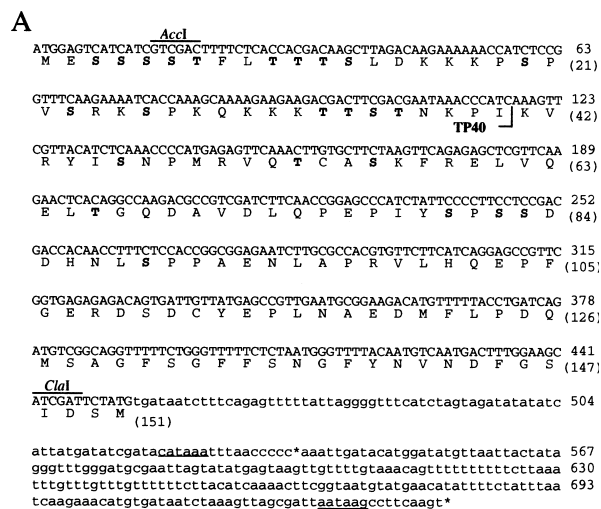
Fig. 1. A The cDNA and deduced amino acid sequences of SibI. Hydroxylated amino acid residues (S, T) which are known to be rich in chloroplast transit peptides are shown by bold letters. The putative polyadenylation signals and polyadenylation sites in the obtained cDNA clones are marked by underlines and asterisks, respectively. B Amino acid sequence alignment of SibI and its homologue, T3K9.5 (At2g41180) in *A. thaliana*.
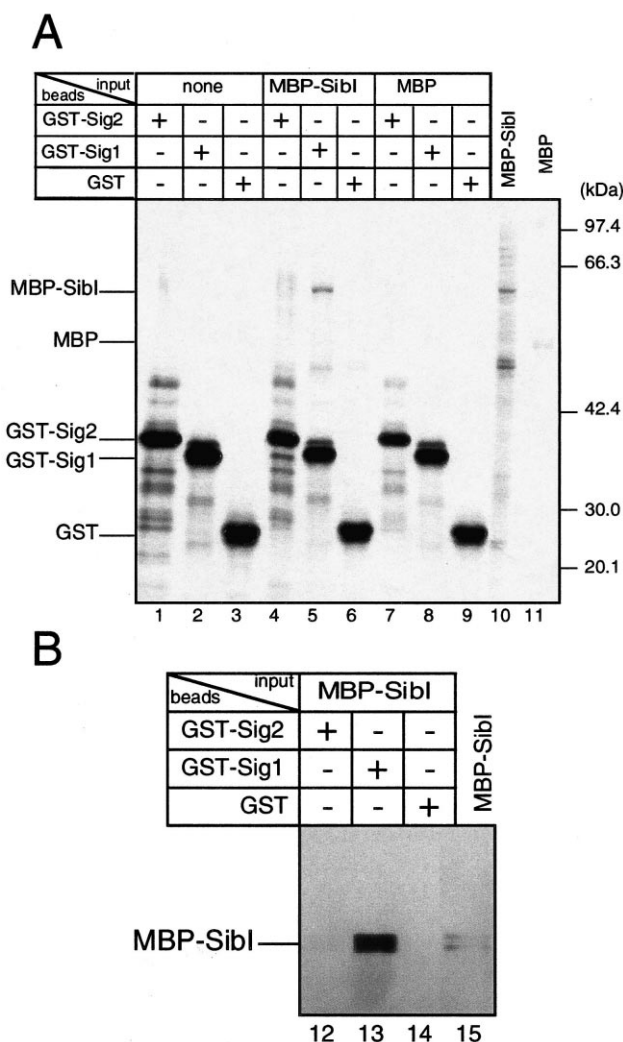

Fig. 2. Confirmation of the direct and specific interaction between SibI and Sig1R4 by GST pull-down assays. GST–Sig2R4 bound beads (lanes 1, 4, 7, 12), GST–Sig1R4 bound beads (lanes 2, 5, 8, 13) and GST bound beads (lanes 3, 6, 9) were mixed with buffer alone (lanes 1, 2, 3), MBP–SibI prepared from the inclusion body (lanes 4, 5, 6), MBP (lanes 7, 8, 9) or MBP–SibI purified with amylose column chromatography (lanes 12, 13, 14). Tightly bound proteins were eluted with glutathione solution. The eluates were fractionated by SDS–PAGE, followed by silver staining (A) or Western blotting using anti-SibI antibodies (B). The migration position of each protein is indicated on the left. The positions of the protein markers are indicated on the right.

3. Results

3.1. Identification of Sig1R4 binding proteins

Sig1 is thought as one of the most abundant sigma factors in *A. thaliana* [7]. We have searched proteins interacting with the R4 of Sig1 by yeast two-hybrid screening. Two positive clones were obtained, which encoded the same protein, but were heterogeneous in the length of their 3′-UTR (Fig. 1A). To identify the corresponding full-length cDNA, we screened the *A. thaliana* cDNA library by using the obtained cDNA fragment as a probe. The largest open reading frame in the longest cDNA encoded 151 aa residues. This protein was named SibI (sigma factor binding protein I). The *sibI* gene
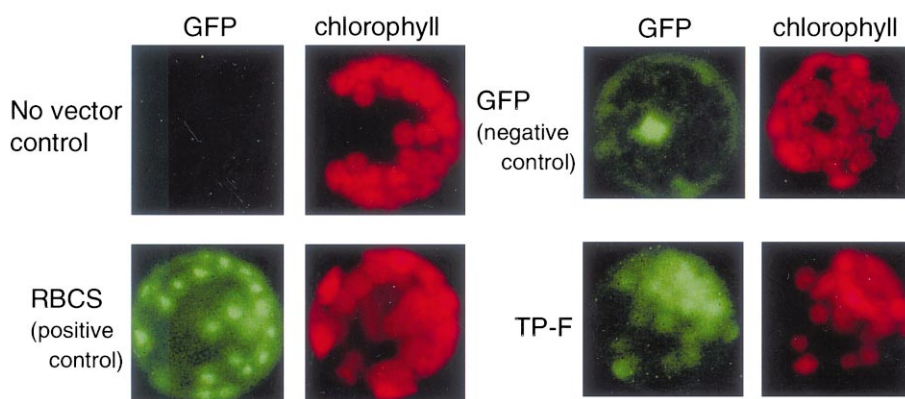
Fig. 3. Chloroplast import of SibI revealed by transient expression of GFP fusion proteins. Protoplasts were transformed with plasmids encoding GFP (CaMV35S-sGFP(S65T)-nos3′), SibI-GFP (TP-F), or RbcS transit peptide-GFP (RBCS1A). Left top panels are the negative control for GFP signal detection without any plasmids.

exists as a single copy in the genome (Southern hybridization analysis, data not shown; *Arabidopsis* genome project). Database searches showed that SibI is not homologous to any protein of known function. However, one homologue with 56.3% identity to SibI in *A. thaliana* was found (T3K9.5: GenBank accession number AC004261). Some cDNA fragments from *A. thaliana* databases (GenBank accession number AI992550) and other plant species also showed partial similarity to the SibI cDNA.

3.2. Specific binding of SibI to SigI

We tested the interaction between SibI and the Sig1R4 by GST pull-down assays. Each preparation of MBP and MBP–SibI was incubated with GST–Sig1R4 and the GST–Sig2R4 immobilized on beads. Fig. 2A shows the SDS–PAGE image of proteins eluted by glutathione solution. Lanes 1–3 are the control experiments done with the addition of neither MBP–SibI nor MBP. MBP–SibI was detected only in the eluate from the GST–Sig1R4 beads (lane 5). Comparison of the input fraction (lane 10) and the eluate (lane 5) clearly shows that MBP–SibI was selectively concentrated by the binding to the GST–Sig1R4 beads. MBP was not detected in the elution from the GST–Sig1R4 (lane 8) and MBP–SibI was not detected in the elution from the GST beads (lane 6). These indicate that binding patches are on the portions of SibI and Sig1R4 in the fusion proteins. Western analysis with anti-SibI peptide antibody confirmed that MBP–SibI specifically co-eluted with GST–Sig1R4, and little with GST–Sig2R4 (Fig. 2B). The comparison of lane 13 (the eluate) and lane 15 (input fraction of 1/10 volume) suggests that more than 50% of the loaded MBP–SibI was bound to the GST–Sig1R4

beads. Thus, the results of the pull-down assays confirmed the direct and specific interaction of SibI with the Sig1R4.

The binding specificity of SibI to Sig1R4 among plastid sigma factors in *A. thaliana* was examined by yeast two-hybrid assays (Table 1). Yeast cells co-transformed with pAS-sig1R4 and pACT-sibI (expressing AD–SibI hybrid) were histidine-autotrophic and lacZ positive. In contrast, none of baits containing R4 of Sig2, Sig4, or Sig5 activated the His and lacZ reporter gene expressions in the presence of pACT-sibI, indicating that the interaction between SibI and Sig1R4 is specific among the examined R4s of plastid sigma factors.

3.3. Chloroplast import of SibI revealed by transient expression of GFP fusion proteins

N-terminal region of SibI is positively charged and rich in hydroxylated amino acid residues (Fig. 1); this is a feature of chloroplast targeting signals, transit peptides. Considering the chloroplast localization of Sig1 in *A. thaliana* [8], it is expected that SibI functions in chloroplasts. We examined the chloroplast import of SibI by the protoplast transient expression of GFP fusion proteins. As expected, import of GFP into chloroplasts was detected in the protoplasts transformed with the TP-F and RBCS1A (positive control), but not in those transformed with CaMV35S-sGFP(S65T)-nos3′ (negative control) (Fig. 3). From these results, we concluded that SibI carries the chloroplast targeting transit signal and localizes in chloroplasts.

3.4. Tissue-specific and light-responsive expression of sibI gene

sibI mRNAs abundantly accumulated in cauline leaves, rosette leaves and roots, but not in flowers and flower stalk (Fig.

Table 1
Activation of lacZ reporter gene by the Sig1R4 binding proteins

Activation domain fusion	BD fusion				
	p53	Sig1	Sig2	Sig4	Sig5
Large T	100(+)	1.27 ± 1.07(W)	2.09 ± 0.01(W)	1.80 ± 0.38(W)	2.36 ± 1.10(W)
SibI	ND	242.75 ± 55.26(+)	1.11 ± 0.79(W)	0.52 ± 0.74(W)	1.60 ± 0.74(W)
T3K9.5	ND	89.76 ± 14.81(+)	0.54 ± 0.76(W)	0.78 ± 0.36(W)	0.98 ± 0.68(W)

The numbers provide relative lacZ activity with standard deviations for duplicate assays of each clone. The W and plus sign illustrate the colony color after freeze–thawing of the colonies and exposure to X-Gal: W indicates that the colony remained white, while '+' represents blue colonies. ND, not determined.
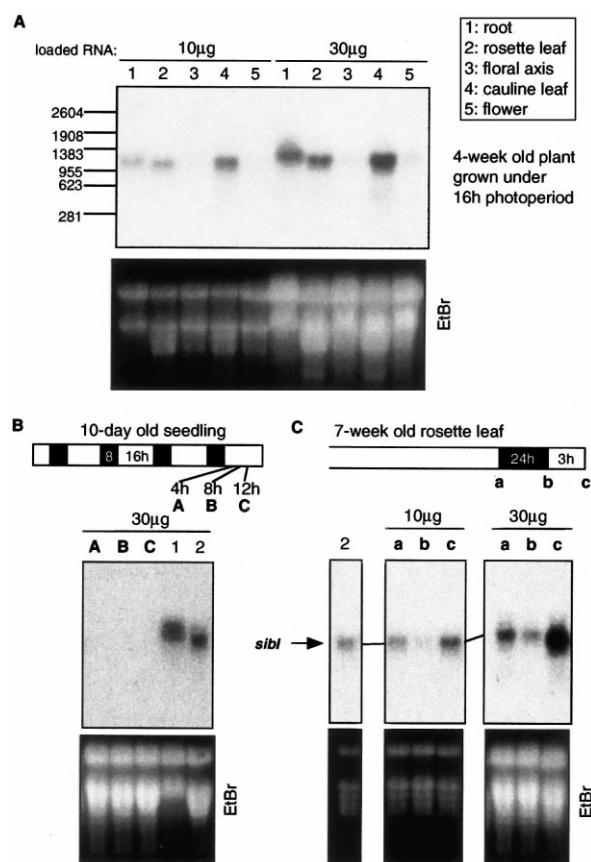
Fig. 4. Northern analyses of *sibI* transcripts (A) in different tissues of 4-week-old plants harvested 4 h after the onset of light, (B) in 10-day-old seedlings at three time points and (C) 7-week-old rosette leaves. Lanes 1 and 2 in (B) and (C) contain the same samples as in (A). The amounts of total RNAs charged are indicated on the top of lanes. Ethidium bromide staining of RNA gels shows equal loading of RNA.

4A). The size of the mRNA in roots was larger than those in other tissues, suggesting the possible existence of the tissue-specific post-transcriptional regulation. In the cotyledons and young leaves of 10 days old, *sibI* mRNA was not detected independent of light or dark (Fig. 4B). While, in older rosette leaves, the *sibI* mRNA level was clearly light-dependent as greatly reduced by the 24 h dark adaptation (lane b), and recovered by 3 h re-illumination to the level higher than that before dark adaptation (Fig. 4C).

4. Discussion

The replacement and/or the modification of the promoter recognition subunit, sigma factors, seems to be involved in the change in the promoter preference of PEP during the cell development. It has been supposed that the former is achieved by the sequential induction of each sigma factor, likely as the 'sigma cascade' in *Bacillus subtilis* [16] or the σ^{70} to σ^{S} shift during entry into the stationary phase in *E. coli* [17]. Alternatively, in mustard, the RNA polymerase associated kinase termed PTK was reported to modify the sigma factors as to alter the PEP property [18,19]. In this work, we propose additional regulation via the nuclear-encoded sigma factor binding proteins.

We focused our attention on the R4 of Sig1, because bacterial anti-σ^{70} factors and many class II activators are known to target the R4 of σ^{70}. The R4 of Sig1 is expected to be correctly folded independent of the remaining regions, based on the findings in *E. coli*, where the R4 fragment of σ^{70} expressed as a GST fusion was able to bind specifically to the -35 element in vitro [20]. We identified a novel nuclear-encoded protein, SibI, which specifically interacted with Sig1R4. As well as Sig1, SibI was imported into chloroplasts and its expression was light-dependent in mature chloroplasts. Thus, it is likely that SibI functions together with Sig1 in mature chloroplasts.

In addition to SibI, we found one homologue, T3K9.5, in *Arabidopsis* EST and genome sequences. We examined whether T3K9.5 interacts with the R4 of plastid sigma factors by yeast two-hybrid assays (Table 1). T3K9.5 as well as SibI specifically interacted with Sig1R4, and not with other sigma factors examined. SibI and T3K9.5 had no sequence similarity with the bacterial sigma factor binding proteins. Judging from a similarity-based structure prediction using non-redundant structural domain databases, SibI has no DNA BD, indicating that SibI and T3K9.5 function as a *cis*-independent transcription factor. The function of the Sig1 binding proteins may resemble that of Rsd and *E. coli* T4 bacteriophage AsiA, both of which target the R4 of σ^{70} and are categorized as anti-sigma factors. Both Rsd and AsiA are thought to play important roles in the replacement of σ^{70} (anti-sigma factor activity) and/or modification of the promoter preference. When AsiA was described as the first anti-sigma factor, it was shown that AsiA inactivates the σ^{70} in vitro to inhibit the transcription of early promoters that carries the conserved -10 and -35 elements. However, recent in vivo work demonstrated that deletion of *asiA* had no effect on the inhibition of the early promoters [21]. Another line of evidences has suggested that AsiA acts as a switching molecule that alters the promoter preference of σ^{70}. AsiA binds to the R4.2 of σ^{70} to inhibit the recognition of -35 elements, and may recruit the host RNA polymerase to the promoters having the extended -10 element (consisting of -10 element and TGn motif) [11,22]. Furthermore, AsiA acts as an activator together with another T4 encoded protein, MotA, which binds to the motA box located at the -30 region [23]. In plastid, similar promoter structures have been found for some promoters, e.g. *psbA* promoter has an functional extended -10 element [3], and *psbD* light-responsive promoter has upstream enhancer elements instead of the -35 element [15,24,25]. On the analogy with the function of Rsd and AsiA, SibI and T3K9.5 may act as anti-sigma factors and/or gene-specific transcription factors.

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