S24-019

Regulation of plastid gene transcription by sigma factor binding protein

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Keywords: sigma, transcription, sibI, chloroplast, PEP.

Introduction

Plastid sigma factors have four conserved regions homologous to those of bacterial class I and II sigma factors including *E.coli* σ^{70}. The function of each region of *E.coli* σ^{70} has been detailed (Lonetto et al., 1992). The region 2 is divided into five subregions. The region 2.4 is responsible for the recognition of the -10 element and the region 2.5 recognizes the TGn motif found just upstream of the -10 element in some promoters. The region 4 is also divided into two subregions, and the region 4.2 recognizes the -35 element. In *E.coli*, many proteins interacting with the region 4 of σ^{70} have been reported, e.g., Class II activators and anti-σ^{70} factors. We expected the presence of the protein factors that selectively interact with each of the plastid sigma factors as to regulate their functions and decided to identify the factors which specifically interact with the region 4 of *Arabidopsis thaliana* Sig1 (At Sig1).

Materials and methods

Y*east* ***two-hybrid screening.*** The cDNA corresponding to the C-terminal 89 amino acids of At Sig1, which covers the region 4, was amplified by RT-PCR with primers; Eco-sigA 5'-CGGAATTCCGTTTGGAGAACAATCCG-3' and Pst-sigA 5'-GGCTGCAGTCAATTCTTAAGGATCAT-3'. The amplified fragment was cloned into *Eco* RI-*Pst* I site of pAS2-1 (TRP1; Clontech) so as to achieve an in-frame fusion with the GAL4 DNA-binding domain (pAS-sig1R4). *A.thariana* (Columbia) MATCHMAKER cDNA Library in pGAD10 (LEU2; Clontech) was screened by using pAS-sig1R4 according to the manufactures' instructions. Two positive clones were obtained from about 2 x 10^6 clones, and they encoded *sibI*. The *sibI* sequence has been deposited in GenBank (accession number AF224762)

Transcriptional run-on assay with transformed protoplasts. The transformed protoplasts were suspended in CSB (0.3M sorbitol, 50mM Hepes-NaOH, pH7.6, 2mM EDTA) at a chlorophyll concentration of 2.5 mg/ml. 40 µl of the protoplast suspension was directly added to 160 µl of a run-on mixture (37.5mM Hepes-NaOH, pH 7.9, 12.5mM MgCl2, 50mM KCl, 25µg/ml heparin, 625µM each of ATP, CTP, and GTP, 62.5µM UTP, 200µCi [α-^{32}P]UTP, 240U RNase inhibitor) as to simultaneously disrupt the protoplasts and chloroplasts. After

incubation for 10 min at 25˚C, labeled transcripts were purified and hybridized to a membrane dot-blotted with the CDSs of plastid genes. The plastid DNAs prepared by PCR were *psbD*: 925bp (78-1002), *rbcL*: 944bp (104-1047), *rrn16*: 923bp (363-1285), *psbA*: 918bp (106-1023), *psbB*: 930bp (554-1483), *psaA*: 963bp (433-1395), and *rpoB*: 949bp (1795-2743). The numbers in the parentheses indicate the positions from translation initiation sites. The final wash condition was 0.1x SSC, 0.1% SDS at 50˚C for 30 min. The image was obtained by BAS2000.

Results

We searched proteins interacting with the region 4 of a plastid sigma factor, At Sig1, by the GAL4-based yeast two-hybrid screening. Two positive clones were obtained, both of which carried the cDNAs encoding the same amino acid sequence. The corresponding full-length cDNAs were obtained by screening the *A.thaliana* cDNA library by using as a probe the cDNA fragment obtained. The largest open reading frame in the longest cDNA encoded 151 amino acid residues, and we called this protein SibI (Sigma factor binding protein I). Database searches showed that SibI is not homologous to any proteins of known function, but we found one homologue which had 56.3% identity to SibI in *A.thaliana* (T3K9.5 genbank accession no. AC004261).

We examined the interactions of SibI with the region 4 of four *Arabidopsis* sigma factors (Sig1, Sig2, Sig4, and Sig5) by yeast two-hybrid assays. No interaction was found between SibI and the region 4 of the tested sigma factors except for Sig1 (data not shown). Furthermore, GST-pull-down assays demonstrated that SibI directly interacted with the region 4 of Sig1 (data not shown).

The N-terminal region of SibI was rich in hydroxylated amino acid residues; this is a feature of chloroplast targeting signals, transit peptides. PSORT (a program for protein sorting, http://psort.nibb.ac.jp) predicted a putative nuclear localization signal within this N-terminal region. To examine the chloroplast import of SibI, we made a plasmid to express SibI fused with the synthetic green fluorescent protein (SibI-GFP) under the control of *CaMV35S*-promoter. Protoplasts prepared from *A.thaliana* rosette leaves were transformed with the plasmid by polyethylene glycol method (Abel and Theologis, 1994), and the location of GFP fluorescence was examined. Import of GFP signals into chloroplasts was detected in the protoplasts transformed with the SibI-GFP expressing plasmid (Fig. 1). However, we noticed that the proportion of protoplasts that showed the GFP signals in chloroplasts varied depending on the preparation of the transformed protoplasts. In addition, GFP signals were observed in or around the nucleus more often than the GFP alone (data not shown). There is a possibility that the plastid targeting of SibI is controlled by cellular conditions. The subcellular distribution of the endogenous SibI under various conditions remains to be investigated.

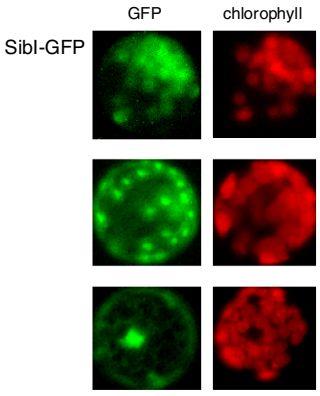

Figure 1. Chloroplast import of SibI revealed by the transient expression of SibI-GFP fusion protein. Green channel shows the GFP signal in a transformed protoplast cell. Red channel shows the chlorophyll fluorescence from chloroplasts. RBCS is the transit peptide of RbcS fused with GFP, and serves as the positive control for the chloroplast import, while GFP alone is the negative control. Images were taken by conventional fluorescent microscope.

To clarify the function of SibI in the plastid gene transcription, we established an assay system by utilising protoplasts. Protoplasts prepared from rosette leaves were transformed with a plasmid that was made to express the entire coding sequence of SibI under the control of *CaMV35S*-promoter. Transformed protoplasts were incubated for 12h, and submitted to a transcriptional run-on assay. The typical results obtained by two independent experiments are shown in Fig. 2. Tremendous up-regulation of the *psbD* transcription, together with a strong enhancement of the *psbA* transcription was found in SibI expressed protoplasts (Fig. 2A). The transcriptions of other plastid genes were not affected by the transient expression of SibI. In another case shown in Fig. 2B, the effect of SibI expression was limited compared with the results shown in Fig. 2A, but the transcriptional activation caused by SibI was specific for the *psbA* and *psbD* genes. The transcription pattern in non-transformed protoplasts in Fig. 2B was different from that in Fig. 2A, suggesting the difference of the transcription apparatus in the two series of protoplasts prepared from independently grown plants. In some cases, *sibI* expression did not have any effect on the transcription rates of the examined genes (data not shown). Thus, the degree of SibI induced *psbD* and *psbA* transcription varied depending on the protoplast preparations, but repeated assays on a given protoplast preparation led to almost identical results.

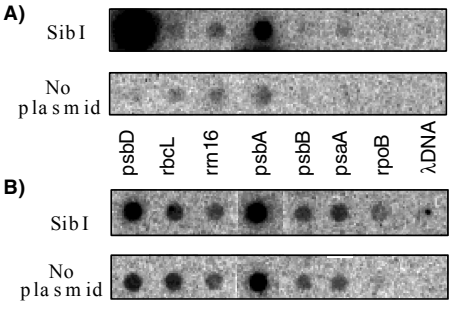

Figure 2. The up-regulation of *psbD* and *psbA* transcription in transformed protoplasts expressing SibI. λDNA is a negative control for the signal detection. "No plasmid" is the result using protoplasts treated as the same way but the transforming plasmid was not added. The extent of the enhancement was different depending on the protoplast preparation (compare A and B)

Discussion

We found that the transient expression of SibI in leaf protoplasts specifically enhanced *psbD* and *psbA* transcription. Both *psbD* and *psbA* promoters exhibit the –35 element independent transcription (Satoh et al. 1999; Nakahira et al., 1998; Kim et al., 1999). Although the molecular mechanism of SibI function remains to be investigated in detail, it might be the plastidial counterpart of AsiA or Rsd. In *E.coli*, T4 bacteriophage AsiA (anti-sigma A) protein binds to the region 4.2 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) (Severinova et al., 1998). Interestingly, the *psbA* promoter has an extended -10 element including well conserved –10 element (Satoh et al., 1999). Furthermore, AsiA acts as an activator together with another T4 encoded protein, MotA, which binds to the motA box located at the -30 region (Ouhammonch et al., 1995). The *psbD* light responsive promoter has enhancer elements just upstream of its core promoter region. These facts suggest that the similar mechanisms of transcriptional regulation function in chloroplasts and bacteria, although no similarity is found between AsiA and SibI. Otherwise, SibI may have anti-sigma factor activity and replace Sig1 in the plastid RNA polymerase with another sigma factor that recognizes more strongly the *psbD* and *psbA* promoters than Sig1 does. It is unlikely that SibI-Sig1 interaction is the sole system to support *psbA* and *psbD* transcription, since we recently found that both *psbA* and *psbD* transcription were activated by the transient expression of Sig5 (Tsunoyama et al., in preparation). Detailed expression analyses of plastid sigma factors and transcription factors associated with *psbA* and *psbD* promoters are necessary to understand the unique transcriptonal regulation of these genes.

Acknowledgments

We thank Dr. Y. Niwa and Dr. K. Kobayashi (University of Shizuoka) for sGFP(S65T) vectors.

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