FEBS Letters 485 (2000) 178–182 FEBS 24318

Chloroplast development in *Arabidopsis thaliana* requires the nuclear-encoded transcription factor Sigma B

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Received 1 September 2000; revised 30 October 2000; accepted 30 October 2000

First published online 8 November 2000

Edited by Gunnar von Heijne

Abstract Development of plastids into chloroplasts, the organelles of photosynthesis, is triggered by light. However, little is known of the factors involved in the complex coordination of light-induced plastid gene expression, which must be directed by both nuclear and plastid genomes. We have isolated an *Arabidopsis* mutant, *abc1*, with impaired chloroplast development, which results in a pale green leaf phenotype. The mutated nuclear gene encodes a sigma factor, SigB, presumably for the eubacterial-like plastid RNA polymerase. Our results provide direct evidence that a nuclear-derived prokaryotic-like SigB protein, plays a critical role in the coordination of the two genomes for chloroplast development.

Key words: SigB; Sigma factor; RNA polymerase; Chloroplast development; *Arabidopsis thaliana*

1. Introduction

The semiautonomous plastids of plant cells exhibit various differentiated forms including the chloroplast, the organelle of photosynthesis [1]. During evolution, most of the prokaryotic genome of the ancestral cyanobacterium that gave rise to chloroplasts has been lost or transferred to the eukaryotic nuclear genome of the host cell [2]. Such prokaryotic genes that have undergone nuclear transfer have adapted to the eukaryotic transcriptional machinery for targeting their gene products to plastids [3]. The remaining 120 or so genes of the plastid genome are transcribed by mechanisms that are probably more complex than those of the cyanobacterial ancestor. Coordination between the nuclear and plastid genomes likely contributes to the diverse mechanisms of transcriptional regulation of the plastid genes. Research into such nucleus–plastid coordination is in its early stages.

Gene expression in plastids relies on at least two RNA polymerases. The nuclear gene-encoded RNA polymerase (NEP) transcribes housekeeping and many other genes, but NEP promoters are absent from most photosynthesis-related genes [3]. The plastid-encoded RNA polymerase (PEP) is essential for the light-induced conversion of proplastids to chloroplasts. PEP acts in a manner similar to that of the classical eubacterial RNA polymerase, which recognizes specific DNA elements designated −35 and −10. Targeted deletion of plastid genes that encode the core subunits of PEP-*rpoA* for α, *rpoB* for β, *rpoC1* for β′, or *rpoC2* for β″, results in photosynthetically defective plants that lack PEP activity [4]. However, the plastid genome lacks a gene for a sigma factor, an essential component of the RNA polymerase holoenzyme in eubacteria [3]. Nuclear gene-encoded sigma subunits have been identified in unicellular red algae [5]. Several genes of higher plants that encode homologs of eubacterial sigma factors have also been recently identified [6–9]. However, the role of these proteins showing homology to bacterial sigma factors was not well understood.

We now show that the nuclear gene-encoded sigma factor SigB of *Arabidopsis thaliana* plays a critical role in chloroplast development at an early stage of seedling growth, and that other sigma factors also likely contribute to this process in a somewhat redundant manner. The SigB protein and other sigma factors appear to function as a link between the nuclear genome and plastid gene expression in higher plants.

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Abbreviations: NEP, nuclear gene-encoded RNA polymerase; PEP, plastid-encoded RNA polymerase; PSII, photosystem II; PFD, photon flux density

PII: S0014-5793(00)02216-X

2. Materials and methods

2.1. Plant growth and mutant isolation

A. thaliana accession Wassilewskija was grown at 22°C with a 16-h light, 8-h dark cycle. Transgenic plants were produced on a large scale by a vacuum infiltration protocol, as described previously [10], with *Agrobacterium tumefaciens* strain GV3101 (pMP90RK) harboring the activation tagging vector pPCVICEn4HPT [11]. We isolated several mutants that exhibited the pale green leaf phenotype from ~3500 transgenic lines examined. The mutant line #3117 chosen for further study exhibited aberrant chloroplast development when examined by transmission electron microscopy, and was designated *abc1*.

2.2. Microscopy

Plant tissues were fixed with glutaraldehyde and paraformaldehyde and embedded in Quetol 812 (Nisshin EM, Tokyo, Japan) according to standard procedures. Ultrathin sections were stained with uranyl acetate, then with lead citrate, and observed with a JEM 1200EX transmission electron microscope. Plastids were also viewed with a Zeiss fluorescence microscope equipped with a CSU 10 confocal laser-scanner unit (Yokogawa, Tokyo, Japan), a digital camera (Hamamatsu, Shizuoka, Japan) and IPLab spectrum software (Vienna, Austria).

2.3. T-DNA identification and genetic characterization

Given that the T-DNA fragment used for *Arabidopsis* transformation contained the Col E1 origin and the ampicillin resistance gene [11], the sequences flanking the T-DNA in the *abc1* mutant were rescued in *Escherichia coli*. Genomic DNA was isolated from the mutant plant, digested with either *Bam*HI or *Cla*I, ligated and transformed into *E. coli* XL2-Blue. The rescued plasmids contained either a 15-kb *Bam*HI fragment or a 12-kb *Cla*I fragment. One side of the T-DNA flanking sequence was amplified from the *Bam*HI fragment by a polymerase chain reaction (PCR) with primers En4LB1 (5′-GATTTCCCGGACATGAAGCC) and AtD2-N4 (5′-CACGACGTTGTAAAACGACGTCAGCCAACCTTTGCGC), and the other side was amplified from *abc1* genomic DNA with the primers En4LB1 and AtD2-C4 (5′-GGATAACAATTTCACACAGGGTTCTATGCACAAAACATTCCA); the amplification products were then sequenced with M13forward(−29) (underlined sequence in AtD2-N4) and M13reverse (underlined sequence in AtD2-C4) primers, respectively.

The *Eco*RV genomic DNA fragment containing *sigB* was cloned into the *Sma*I site of the vector pBI101. The resulting construct was introduced into the *abc1* mutant by vacuum infiltration [10].

2.4. Fluorescence measurement by pulse amplitude-modulated fluorometry

Chlorophyll *a* fluorescence originating from photosystem II (PSII) in an intact attached leaf was measured at a controlled room temperature of 25°C with a pulse amplitude-modulated fluorometer (MINI-PAM; H. Walz, Effeltrich, Germany). The third leaf after bolting was illuminated with a low-intensity, modulated measuring beam with a photon flux density (PFD) of <0.5 μmol m^{-2} s^{-1} as well as with actinic light through a fiber-optic probe positioned at a 45° angle against the leaf. The chlorophyll fluorescence emitted from the leaf in response to excitation with the measuring beam was detected through the same fiber-optic probe. The steady-state fluorescence yield (F_s) was monitored continuously, and 1.0-s pulses of saturating light (PFD, 5000 μmol m^{-2} s^{-1}) were administered at intervals of ~300 s to determine maximal variable fluorescence (F_m'). The protocol for fluorescence measurement was similar to that described by Genty and Harbinson [12], with the exception that the measurements were performed with an attached leaf. The terminology used for fluorescence parameters is that previously recommended [13], and the relative quantum yield of PSII [Φ(PSII)] at the steady state is defined as $(F_m'-F_s)/F_m'$, or $\Delta F/F_m'$.

Fig. 1. Phenotype of the *abc1* mutant at the early stage of seedling growth. Wild-type (left) and *abc1* mutant (right) plants (about 60 plants/pot) were grown for 2 weeks.

2.5. Northern hybridization analysis

Expression levels of the major chloroplast transcripts for psbA, psbD and rbcL were analyzed as described [14].

3. Results and discussion

In a T-DNA insertion screen for mutations in chloroplast development we have isolated an *A. thaliana* mutant, designated *abc1*, that exhibits aberrant chloroplast development and a consequent phenotype characterized by pale green leaves (Fig. 1). This phenotype was also biochemically quantitated, revealing that the 4-day-old *abc1* cotyledons contain only 15% of the chlorophyll *a*/*b* content of 4-day-old wild-type cotyledons. Interestingly, although the growth of this mutant is impaired, the macroscopic morphology of this mutant appears normal in all other respects. Further characterization of this mutant by electron microscopy revealed that the developing chloroplasts in 5-day-old cotyledons of mutant plants are much smaller than the corresponding wild-type cotyledons (Fig. 2a). The thylakoid membranes and the stacked lamellar structure of grana were poorly developed, and few starch granules were apparent, suggestive of a low photosynthetic ability. In contrast, the chloroplasts from 5-day-old cotyledons of wild-type plants had substantial deposition of starch granules and the lamellar structure was far more developed. Interestingly, the size and shape of etioplasts in cotyledons grown in the dark were indistinguishable between mutant and wild-type plants (data not shown). These observations thus indicated that the phenotype of the *abc1* plants results from a mutation that affects light-induced development of chloroplasts in the early stage of seedling growth.

In contrast, the chloroplasts in 2-week-old leaves of mutant plants were nearly mature in terms of grana structure. However, they exhibit a loosened envelope, in contrast to wild-type chloroplasts where the envelope was tightly associated with the ellipsoidal content (Fig. 2b). Confocal laser-scanning microscopy of mesophyll cells in 2-week-old rosette leaves also revealed that the chloroplasts in the *abc1* cells are smaller than those of wild-type cells (Fig. 2c), consistent with the results obtained by electron microscopy. Given that the number of chloroplasts per cell appeared similar in both the mutant and wild-type plants, the pale green leaf phenotype of the 2-week-old mutant plants is likely attributable to the small chloroplast size.

The mutant phenotype segregated as a single recessive mutation and also cosegregated with the hygromycin resistance conferred by the T-DNA sequence used for gene tagging, suggesting that the mutation was caused by T-DNA insertion into the nuclear genome. We therefore isolated the T-DNA flanking sequences by plasmid rescue. The two flanking sequences of the T-DNA were subcloned into a 15-kb *Bam*HI

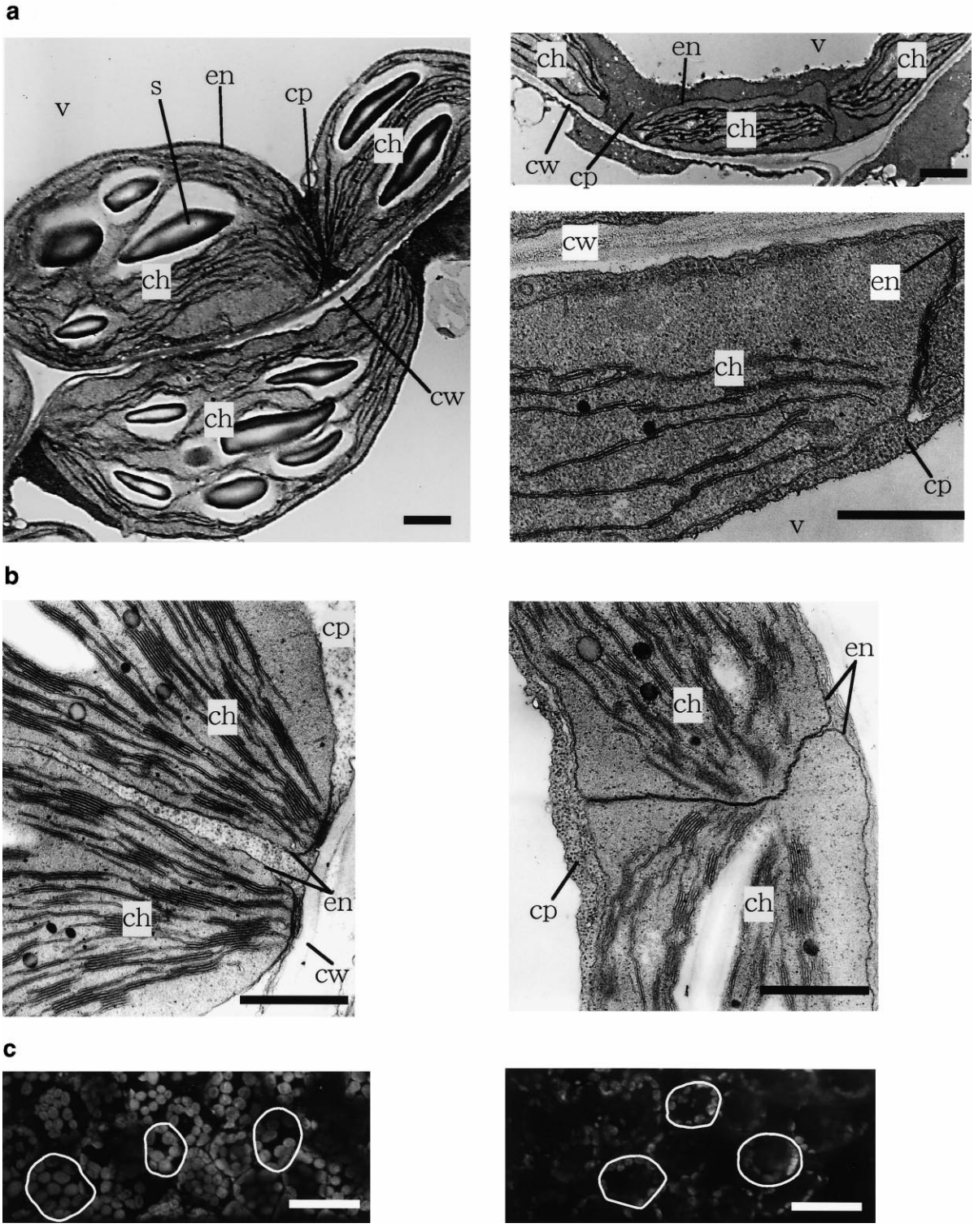

Fig. 2. (a) Cotyledon cells of 5-day-old wild-type (left) and *abc1* mutant (right) seedlings were analyzed by transmission electron microscopy. Note higher magnification in the lower panel for the *abc1* mutant. Scale bars, 1 μm. Abbreviations; ch, chloroplast; s, starch granule; cp, cytoplasm; cw, cell wall; and v, vacuole. (b) Chloroplasts of 2-week-old leaves of wild-type (left) and *abc1* mutant (right) plants. The mutant chloroplasts exhibit a loosened envelope structure. Scale bars, 200 nm. (c) Confocal laser-scanning microscopic analysis of chlorophyll fluorescence in mesophyll cells of 2-week-old leaves of wild-type (left) and *abc1* mutant (right) plants. Three cells chosen arbitrarily in each panel are outlined in white. Scale bars, 10 μm.

fragment and a 12-kb *Cla*I fragment, respectively. A physical map of the *Cla*I fragment revealed an insertion of two inverted T-DNA sequences at the targeted locus with partial deletion of one of the T-DNA fragments (Fig. 3). Sequencing of the flanking region of the *Bam*HI fragment identified the T-DNA-tagged gene as *sigB*, which encodes a putative plastid sigma factor. Amplification of the other flanking region by PCR and sequencing of the PCR product confirmed the in-

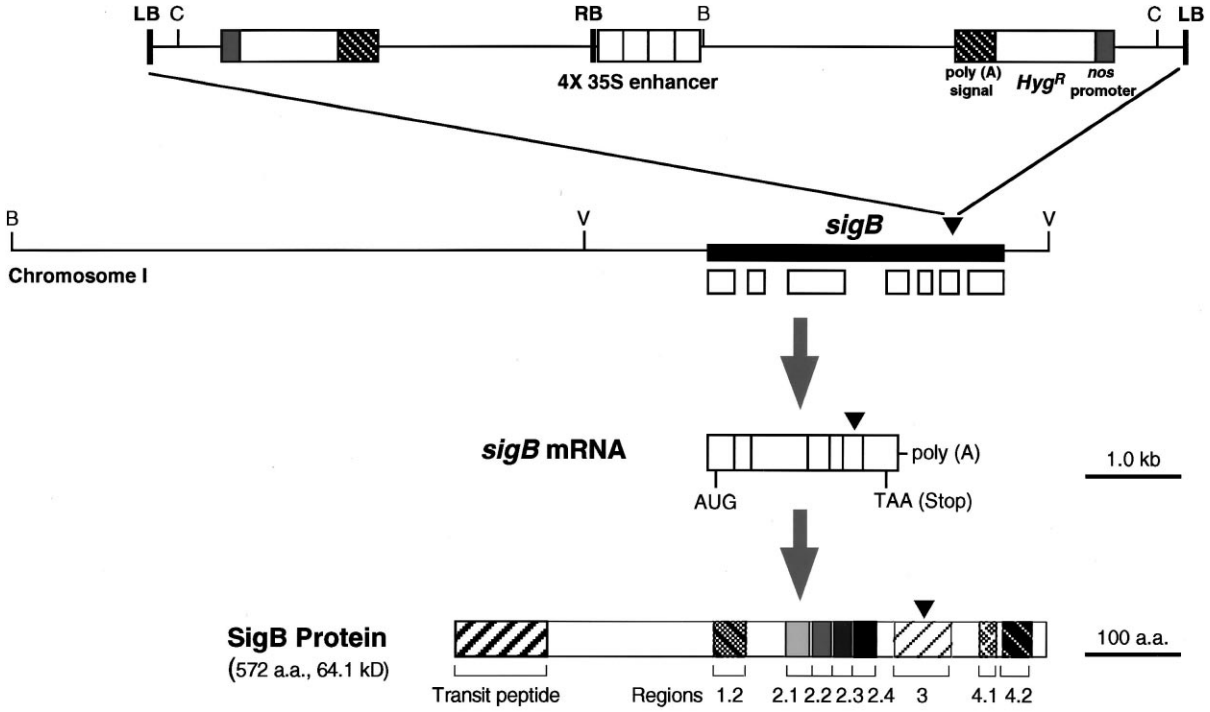

Fig. 3. Insertion of T-DNA into the *sigB* gene of *A. thaliana*. The structures of T-DNA and genomic DNA at the site of insertion, of *sigB* mRNA, and of the SigB protein are shown. The site of T-DNA insertion (exon 6 of *sigB*, encoding conserved region 3) is indicated by an arrowhead. The two inserted T-DNA elements were oriented in opposite directions, with a set of four enhancer sequences located near the center of the inserted sequence. LB, left border of T-DNA; RB, right border; C, *Cla*I; B, *Bam*HI; V, *Eco*RV; and *HygR*, hygromycin resistance gene.

sertion site and orientation of the T-DNA. To verify that the T-DNA insertion was responsible for the mutant phenotype, we isolated a 4.49-kb fragment of genomic DNA containing *sigB* from *A. thaliana* accession Columbia and introduced it into the *abc1* mutant. The transgene restored normal leaf color (data not shown). Given that the T-DNA contained a set of concatenate enhancer fragments derived from the 35*S* promoter of cauliflower mosaic virus for activation tagging, it was possible that the *abc1* mutant phenotype resulted from enhanced transcriptional activation of the aberrant *sigB* gene itself or of neighboring genes. However, the observations that heterozygous *abc1*/*ABC1* plants exhibited a normal morphology and that the wild-type *sigB* gene complemented the mutant phenotype argued against this possibility. We also introduced the full-length *sigB* cDNA under the control of the 35*S* promoter into the *abc1* mutant and obtained four transgenic lines; the resulting mature transgenic plants exhibited near normal chloroplast development (data not shown). This suggests that some level of SigB is essential for chloroplast development and further demonstrates that the *abc1* mutant phenotype was caused by loss of *sigB* function.

The SigB protein contains regions that show sequence similarity to the *E. coli* σ^{70} factor [15]. The T-DNA inserted in the *abc1* mutant into the portion (exon 6) of *sigB* that encodes region 3, resulting in a continuous reading frame that encodes an additional two amino acids, ^{457}Ser-His-Cys-Stop (Fig. 3). Thus, if expressed, the mutant gene would give rise to a truncated, chimeric protein that lacks a portion of region 3 and region 4, which are thought to be required for promoter recognition and protein–protein interaction [15]. The mutant protein, therefore, would be unable to activate transcription in a wild-type manner.

Based on the activity of sigma factors in eubacterial systems, the SigB protein likely binds to the core PEP RNA polymerase and this is consistent with recent results reported by others and ourselves. For example, we recently demonstrated import of SigB into chloroplasts with the use of transient expression assays and a chimeric fusion protein comprising the NH_2-terminal region of SigB and green fluorescent protein [16]. Additionally, PEP promoter sequences have been identified in many photosynthesis-related genes in the plastid genome [3]. PEP has also been shown to be indispensable for chloroplast development from proplastids, although it is not required for plastid maintenance [4]. The impairment in chloroplast development during the early stage of seedling

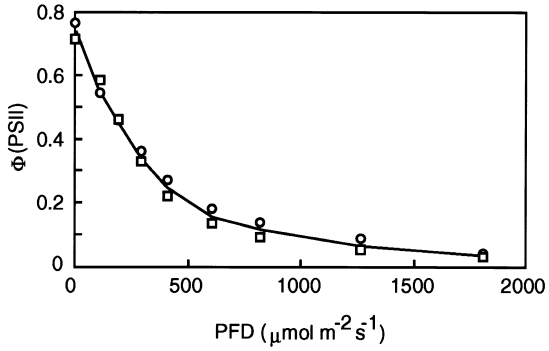

Fig. 4. Efficiency of photosynthesis in the *abc1* mutant. The relative quantum yield of PSII [Φ(PSII)] at the steady state was measured at the indicated PFDs and atmospheric partial pressures of CO_2 and O_2 of 36 Pa and 21.0 kPa, respectively. Two-week-old attached leaves of wild-type (squares) and *abc1* mutant (circles) plants were studied.

growth in the *abc1* mutant is also consistent with the high level of *sigB* expression apparent during early seedling development, as demonstrated in transgenic plants expressing the *uidA* gene under the control of the *sigB* promoter [19]. Furthermore, among transgenic *Arabidopsis* plants expressing antisense genes for five *Arabidopsis* sigma factors, *sigA* through *sigE* [16,17], only those expressing the *sigB* antisense construct exhibited a pale green leaf phenotype similar to that of the *abc1* mutant (unpublished data). Taken together, these observations indicate that the sigma factor encoded by *sigB* functions, probably through interaction with the core subunits of PEP, in the normal development of photosynthetic chloroplasts during the early stage of seedling growth.

To determine whether the *sigB* mutation affects photosynthesis, we measured the efficiency of photosynthesis with a pulse amplitude-modulated fluorometer. The quantum yield of PSII in 2-week-old leaves of mutant plants was virtually identical to that of wild-type plants under a wide range of light intensities (Fig. 4). In general, impairments of PSII or the Calvin cycle result in low quantum yields at low and high light intensities, respectively [18]. Thus, our results indicate that the efficiency of photosynthesis with regard to both electron transport by PSII and carbon fixation is normal in mutant plants at this stage of development. They also suggest that assembly of the photosynthetic apparatus is normal in the *abc1* mutant, at least during the late stages of plant development. Moreover, given that expression of photosynthesis-related genes in the plastid genome requires PEP activity [3,4], other factors appear to exist that complement the lack of SigB function in the mutant plants. The homologous genes *sigA* and *sigC* were previously identified in *Arabidopsis* [6,7], and we have recently identified *sigD*, *sigE* and *SigF* in this species [17]. Furthermore, a family of sigma gene homologs has also been found in maize [9]. The other sigma-like proteins are therefore likely candidates in the partial complementation of impairment in SigB function manifest during the early stage of *abc1* seedling growth. However, the small size of chloroplasts apparent even during the later stages of development of the *abc1* mutant suggests that the timing and level of gene expression is crucial for normal chloroplast development. The fact that *Arabidopsis* sigma factors share only $\sim$35% amino acid sequence identity suggests that each factor may target specific promoter sequences for PEP-mediated transcription, as is the case in eubacterial transcription systems [19].

To assay if the *abc1* mutation affects the transcriptional levels of the plastid-encoded major photosynthetic genes *psbA*, *psbD* and *rbcL*, Northern hybridization analysis was performed using probes specific for these genes. Total RNA was isolated from seedlings at time points between 1 and 4 days. Initial expression levels of these genes in 1-day-old wild-type seedlings is very low. However, expression increases drastically with time. No apparent differences in expression of these genes in either quantity or timing is found in the *abc1* mutant (data not shown). These results may suggest that the sigB protein is not critical for overall expression of these photosynthetic genes or other sigma factors may compensate for the sigB deficiency and induce expression of these genes. However, this data is consistent with our results showing no loss of photosynthetic ability with this mutation and suggests that these genes are not the critical sigB target(s) causing the pale green phenotype.

The link between the nuclear genome and plastid gene expression provided by sigma factors represents a unique consequence of plastid evolution in eukaryotic cells. Given that primitive red algae also possess nuclear gene-encoded sigma factors [5], this link emerged at an early stage of evolution and has been conserved in higher plants. Thus, contributing to and allowing for nuclear control of the complex transcriptional mechanisms required for regulating the light-induced development of chloroplasts and additionally, for the adaptation of the endosymbiotic genome to eukaryotic cells.

Acknowledgements: This work was supported by Special Coordination Funds for Promoting Science and Technology from the Science and Technology Agency of Japan to D.S. and K.T.; by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan to K.T. (Priority Area No. 10170207), K.K. (No. 11740434), and H.T. (No. 094460046); and by the Biodesign Research Program of RIKEN to K.T. and H.T.

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