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Plastidic RNA polymerase σ factors in *Arabidopsis*

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In plant cells, plastid DNA is transcribed by at least two types of RNA polymerase, plastid-encoded RNA polymerase (PEP) and nuclear-encoded RNA polymerase (NEP). PEP is homologous to eubacterial transcription machinery, but its regulatory subunit, sigma (σ) factor, is not encoded on the plastid DNA. We previously cloned the three nuclear-encoded sigma factor genes from *Arabidopsis thaliana* and designated them as *sigA, sigB,* and *sigC*. By means of RFLP mapping, *sigA* and *sigB* were mapped on chromosome I and *sigC* on the chromosome III. Based on comparison of the genomic structure of the three *sig* genes, intron sites in the 3′ half of the genes were shown to be identical between *sigB* and *sigC* but divergent in *sigA*, consistent with the phylogenetic relevance of the three gene products. A transient expression assay of GFP fusions in *Arabidopsis* protoplasts showed that the N-termini of all three *sig* gene products functioned as chloroplast-targeting signals. We also constructed transgenic *Arabidopsis* lines harboring the *sigA*-promoter or the *sigB*-promoter *uidA* fusion. Both the *sigA*- and *sigB*-promoters were similarly activated at cotyledons, hypocotyls, rosette leaves, cauline leaves, sepals, and siliques but not at roots, seeds, or other flower organs. In addition, the two promoters were repeatedly activated in young seedlings under continuous light, possibly in an oscillated fashion.

Key words: *Arabidopsis thaliana* — Chloroplast — Plastid-encoded RNA polymerase (PEP) — Sigma factor.

Plastids are thought to be the symbiotic resultant of a free-living prokaryote, probably an ancestral cyanobacteria. In an evolutionary process, plastids developed sophisticated metamorphoses in higher plants. They differentiate into various types of tissue- or organ-specific organelles such as chloroplasts for photosynthesis in green tissues under light conditions, etioplasts under dark conditions, amyloplasts for starch synthesis and storage in roots and seeds, and chromoplasts for carotenoid accumulation in fruits. These organelles originate from undifferentiated proplastids or from interconversion among the different plastid types. Although higher plant plastids have lost most of their genetic information, they have retained approximately 100 genes on their own genomes. These plastid genes mainly encode components for photosynthesis and elements of the transcriptional and translational apparatus (Shinozaki et al. 1986, Stern et al. 1997).

The plastid genome is transcribed by at least two distinct types of RNA polymerase (Hajdukiewicz et al. 1997, Maliga 1998). One of them is nuclear-encoded phage type RNA polymerase (NEP) (Hedke et al. 1997). NEP promoters share a loose consensus sequence, ATAGAATA/GAA, adjacent to the transcription initiation site (Hajdukiewicz et al. 1997, Liere and Maliga 1999). Another is homologous to eubacterial RNA polymerase which recognizes TTGACA (−35 consensus element) and TATAAT (−10 consensus element) in the corresponding promoter regions. The core enzyme of the plastid-encoded RNA polymerase (PEP) consists of four subunits, α, β, β', and β'' encoded by the plastid *rpoA, rpoB, rpoC1* and *rpoC2* genes, respectively. However, the PEP regulatory subunit sigma factor critical for promoter recognition is not present on the plastid genomes. Our group and another group independently identified nuclear-encoded sigma factor genes from primitive red algae, their products were localized in the chloroplast (Tanaka et al. 1996, Liu and Troxler 1996, Oikawa et al. 1998). Subsequently, we isolated three cDNA clones, named *sigA, sigB,* and *sigC*, encoding higher plant sigma factors from the bank of *Arabidopsis* Expression Sequence Tag (EST) for the first time (Tanaka et al. 1997). At present, nucleotide sequences encoding higher plant sigma factors have been identified from rice (Tozawa et al. 1998), mustard (Kestermann et al. 1998), maize (Tan and Troxler; Accession No. AF058708 and AF058709), wheat (Accession No. AJ132658 mentioned by Nakahira et al. 1998), and tobacco (Oikawa et al. unpublished). These findings suggest that the nucleus is capable of governing PEP-dependent plastid gene expression in the coordination with cell growth, metabolism, and development, mediated by control of the widespread sigma factors.

Among the plastid genes, photosynthetic genes like *rbcL, psbA*, and *psbD* possess only PEP-dependent promoters; *accD* encoding a subunit of the acetyl-CoA carboxylase is exclusively NEP-dependent, and other plastid genes including translational apparatus genes and ATP

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synthase genes are transcribed from both PEP and NEP dependent promoters (Hajdukiewicz et al. 1997). Since the loss of the plastid *rpoB* gene encoding a PEP catalytic domain does not confer lethality to tobacco cells, although it results in deficient chloroplast development (Allison et al. 1996), it is supposed that *sig* genes are also non-essential for cell viability. It is, however, still likely that sigma factors are critical for the development and the maintenance of chloroplasts in plant tissues. In this respect, the set of *Arabidopsis* sigma factors is an excellent model to address the divergency and redundancy of the expression and function of the sigma factors in higher plants. We and another group have already shown that the three *sig* genes are transcriptionally photo-inducible and green-tissue specific (Tanaka et al. 1997, Isono et al. 1997), but details of their spatial and temporal expression patterns are almost unknown.

In this paper, we mapped and sequenced the genomic DNA of the *Arabidopsis* sigma factor genes, *sigA, sigB*, and *sigC*. The intron sites in the 3′ half of the *sig* genes, encoding structurally conserved regions, are identical between *sigB* and *sigC* but are mostly divergent in *sigA*. These data corresponds to the previously reported phylogenetic distances among the three *sig* genes. We also show that all three *sig* gene products clearly possess transit peptides at their N-termini in order to target their proteins into plastids. Moreover, we indicate that at least the *sigA*- and *sigB*-promoters are activated in the same tissues and that they are activated with similar kinetics under continuous light, possibly in an oscillated fashion, during the early growth period.

Materials and Methods

Plant materials and growth conditions—Imbibed seeds of *Arabidopsis thaliana* (Columbia ecotype) were sown on Jiffy 7 (AS Jiffy Products Ltd., Norway), a fertilized peatmoss pellet covered with a fine net (42 mm in diameter), or aseptically on 0.3% gelrite plates of MS medium (Murashige and Skoog 1962) without sucrose (Wako Pure Chemical Industries Ltd., Japan). The sown seeds on plates or Jiffy-7s were placed at 4°C under dark conditions for 24 h and then cultivated at 23°C under continuous fluorescent light (50 to 70 $\mu mol\ m^{-2}\ s^{-1}$), as measured by Quantum Meter QSX-01 (Apogee Instruments, U.S.A.).

DNA and RNA techniques—Recombinant DNA techniques followed standard protocols (Sambrook et al. 1989). *Escherichia coli* strain DH5α was mainly used as the host for plasmids. Total DNA from adult *Arabidopsis* leaves was purified as previously described (Edwards et al. 1991). Restriction enzymes, modification enzymes, DNA polymerases, and kits were purchased from TaKaRa Shuzo, Japan.

Southern hybridization—Purified total DNA was digested with appropriate restriction enzymes, and each 2 μg of the digested DNA per lane was applied on 0.8% agarose gel for electrophoresis. Then the loaded DNAs were transferred onto a positively charged nylon membrane, Hybond-N+ (Amersham Pharmacia Biotech Ltd., Japan), following the standard method (Sambrook et al. 1989). ^{32}P-labeled probes specific for the three *sig* genes were prepared with the random priming method as described previously (Tanaka et al. 1997). After hybridization for 12 h at 65°C, a low stringency wash was performed twice with 6×SSC at 65°C. The hybridized filters were exposed on an X-ray film to make an autoradiogram.

Mapping of the three sig genes—Genetic mapping of three *sig* genes were carried out by the Restriction Fragment Length Polymorphism (RFLP) method using Lister & Dean's recombinant inbred (RI) lines (Lister and Dean 1993).

Screening and sequencing of genomic DNA fragments harboring the three sig genes—To obtain genomic clones covering the entire regions of *sigA, sigB*, and *sigC* genes, we screened an *Arabidopsis thaliana* genomic library in λEMBL3 (CLONTECH Laboratories Inc., U.S.A.) with a set of probes specific for each *sig* gene. Thirty to fifty positive clones for each *sig* gene were picked up from over 5×10^5 plaques. For the next selection, PCR reactions were performed with the pooled phage DNAs to confirm whether the inserted DNA covered the entire region of each *sig* gene. Finally, five to ten genomic DNA clones were assured. Among them, representative genomic DNA fragments of the three *sig* genes, 4.6 kb of *Eco*RI-*Cla*I fragment for *sigA*, 5 kb of *Eco*RI-*Eco*RV fragment for *sigB*, and 4.8 kb of *Eco*RI fragment for *sigC*, were subcloned on pBluescript and subjected to sequencing performed by LI-COR Model 4000 DNA sequencer (Li-COR Inc., U.S.A.).

Plasmids—To study the subcellular localization of the three sigma factors, we used green fluorescent protein (GFP) as the reporter (Chalfie et al. 1994, Sheen et al. 1995) by expressing translational fusions with the transit-like sequences of the *sig* gene products. cDNA fragments corresponding to the N-terminal amino acid sequences of three *sig* genes were amplified by PCR from the *Arabidopsis thaliana* Columbia cDNA library constructed by K. Yamaguchi-Shinozaki (Tanaka et al. 1997). With three sets of oligonucleotide primers, AtD1-TP-N 5′-GCGCGTCGACTAATGGCTACTGCAGCTGTT-3′ and AtD1-TP-C 5′-GCGCGAATTCCATGGGCTTCTCAGTAGAAGCAAC-3′ for *sigA*, AtD2-TP-N 5′-GGCTGTCGACGAGCATGTCTTCTTGTCT-3′ and AtD2-TP-C 5′-AAATCCATGGCCTCAGTGGAAGGACCA-3′ for *sigB*, AtD3-TP-N 5′-CATCGTCGACTCAATGGCTTCCTTCAATTC-3′ and AtD3-TP-C 5′-TGACCATGGATAGAAACGACCACCTC-3′ for *sigC*; about 270-bp of DNA fragments corresponding to 87, 88, and 89 amino acid residues were amplified and cloned into the *Sal*I-*Nco*I sites of the CaMV35S-sGFP (S65T)-*NOS* vector (Isono et al. 1997) to yield pA-TP-GFP, pB-TP-GFP, and pC-TP-GFP, respectively. To monitor *sigA*- and *sigB*-promoter activities by using β-glucuronidase (GUS) activity, we constructed *sigA*- or *sigB*-promoter *uidA* fusion on a binary vector plasmid pBI101. First, 1.44 kb of *sigA*-promoter region with 109 bp of exon 1 was amplified from *sigA* genomic DNA clone on pBluescript with a set of oligonucleotide primers, ABJ#2 5′-ATGCTTCCGGCTCGTATGTTGTG-3′ and APr-1 5′-GGCCTCTAGAGAGTGTGTGTGGCACAGACAAATCGAG-3′. Similarly, 1.15 kb of *sigB*-promoter region with 152 bp of exon 1 was amplified from *sigB* genomic DNA clone with the ABJ#2 and BPr-2 5′-GGCCGGATCCTACTTCTAATTTTCCACTTC-3′. *Hin*dIII-*Xba*I fragments of the amplified *sigA*-promoter region and *Sal*I-*Bam*HI fragments of the amplified *sigB*-promoter region were ligated into the multicloning sites on pBI101, respectively.

Transient expression method for Arabidopsis protoplasts to detect GFP signals—Protoplasts were prepared from the green tissues of *Arabidopsis thaliana* grown for two weeks on an MS agar medium with 2% sucrose under continuous light at 50 μmol

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$m^{-2} s^{-1}$. Procedures for protoplast isolation and transformation were carried out as previously described (Abel and Theologis 1994). Protoplast cells cultured for two to five days after transformation under dark conditions were collected and analyzed at ×100 magnification with a confocal laser scanning microscope TSC4D (Leica Camera AG, Germany). The fluorescent micrographs were taken with excitation at 488 nm and emissions at 530 nm for detection of GFP and at over 665 nm for detection of chlorophyll. Incorporated images from the microscopy were processed using Adobe Photoshop Ver. 3.0.

Plant transformation and establishment of transgenic Arabidopsis lines—An *Agrobacterium* strain C58 was used for *Arabidopsis* transformation by two pBI101-derived plasmids harboring either the *sigA*- or the *sigB*-promoter *uidA* fusion gene. Root tissue transformation was performed as previously reported (Valvekens et al. 1988), and the transformants were selected on MS-plates containing 50 $\mu g\ ml^{-1}$ of kanamycin. Three to five independent lines for each construct were established by cultivation over four generations for assurance of reliable GUS experiments in the absence of the antibiotics. The established lines were named Ae1G (*sigA*-promoter *uidA*) and Be1G (*sigB*-promoter *uidA*), respectively. A transgenic *Arabidopsis* line pOCA107-2, introducing *cab3*-promoter *uidA* fusion, was donated by Dr. Nobuyoshi Mochizuki (Susek et al. 1993).

Detection of GUS activity—Basically, the detection of GUS activity was followed as described by Jefferson (1987). For GUS staining, each transgenic *Arabidopsis* line was grown on MS-plates (without sucrose) or Jiffy 7s under continuous light, and one to seven day old young seedlings or 4 week old adult plants were supplied. The sample tissues were soaked in staining buffer containing 0.2 to 1 mM of 5-bromo 4-chloro 3-indoryl β-D-glucuronide (X-Gluc; Rose Scientific Ltd., U.S.A.) dissolved in X-Gluc buffer for 20 to 24 h at 37°C, followed by vacuum treatment for 10 min as described. For quantitation of the GUS activity, whole young seedlings on MS-plates were harvested at 12 h intervals and stocked at −80°C before use. The frozen plant tissues in 1.5 ml microcentrifuge tubes were thawed on ice and added to GUS extraction buffer, quickly ground with a micro homogenizer (kontes bar), sonicated for 15 s, and then centrifuged for 5 min at 4°C. The supernatants were subsequently used for fluorescent analysis of GUS activity with 4-methyl umbelliferyl β-D-glucuronide (4-MUG; Sigma-Aldrich, Japan). The fluorescent assays to determine the amount of produced 4-methyl umbelliferone were performed in triplicate with Luminescence Spectrometer LS50B (Perkin-Elmer Ltd., England), for excitation at 365 nm and emission at 455 nm. To normalize the activities, the protein concentration of each cell extract was determined with Bio-Rad Protein Assay Reagent (Bio-Rad Laboratories Inc., U.S.A.).

Results

RFLP mapping of sigA, sigB and sigC—To determine the copy number of the three *Arabidopsis sig* genes, *Xba*I, *Hin*dIII, or *Eco*RI-digested DNAs were examined by Southern hybridization with labelled full-length cDNA fragments of *sigA*, *sigB* and *sigC*, respectively (Fig. 1). The three genes were clearly detected as single bands in all lanes, indicating that the three *sig* genes are each a single copy in the *Arabidopsis* genome DNA, as shown previously (Isono et al. 1997). Then RFLP mapping of the *sig* genes on the *Arabidopsis* genome was carried out (Fig. 2).

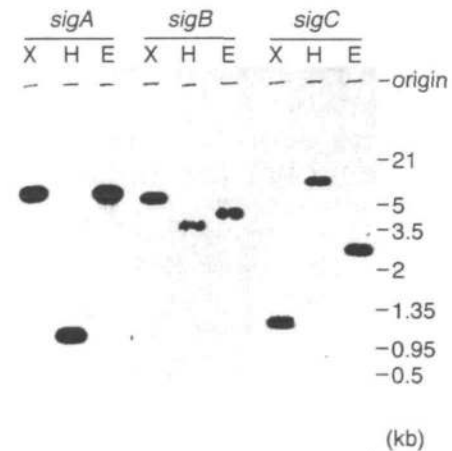

Fig. 1 Southern hybridization analysis. Southern hybridizations were performed for each *sig* gene, following low stringency washes. Specific probes of the *sig* genes were described previously (Tanaka et al. 1997). Genomic DNA was digested with *Xba*I (X), *Hin*dIII (H), and *Eco*RI (E), respectively. The molecular size (λ/*Eco*RI & *Hin*dIII) is indicated at the right side.

The *sigA* gene is located between mi424 and m315, and the *sigB* gene is located near mi372 on chromosome I. The *sigC*

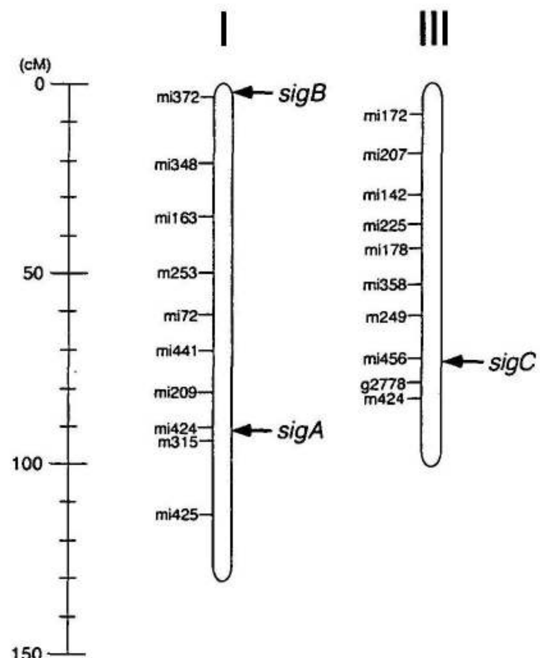

Fig. 2 Chromosomal map positions of three *sig* genes. Map positions of the three *sig* genes are indicated by arrows. The scale for chromosomes of *A. thaliana* is shown on the left in centimorgans (cM). Map positions are referenced to the November 1998 release by the Nottingham Arabidopsis Stock Center (http://genome-www.stanford.edu/Arabidopsis/ww/Nov98RImaps/index.html) of the recombinant inbred map of Lister and Dean (1993).

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gene is located between mi456 and g2778 on chromosome III. *CLAVATA2* (a regulator of meristem and organ development) and *DOC3* (dark overexpression of CAB) are close to the *sigA* locus, *DET3* (de-etiolated) and *ALB1* (albino) are close to the *sigB* locus, and *CSR1* (acetolactate synthase/multiherbicide resistant) and *EMB69* (embryo defective) are near the *sigC* locus on the *Arabidopsis* physical map. Since it is possible that any mutation in *sig* gene might lead to the phenotype of defective chloroplasts, we particularly sequenced genomic *sigB* in *det3* and *alb1* mutants. However, no critical amino acid substitutions were found in either gene (data not shown).

Isolation and sequencing of genomic DNA fragments harboring the three sig genes—We screened a commercial *Arabidopsis* genomic library to isolate genomic DNA fragments covering the entire regions of the three *sig* genes. A positive clone of each *sig* gene, if not covered entirely, was found per approximately 10,000 plaques. Finally, 4.5 to 6 kb of DNA fragments of the entire *sig* genes were subcloned on pBluescript and sequenced. Their accession numbers are AB019942 for *sigA*, AB019943 for *sigB* and AB019944 for *sigC*. Because of the progress in the *Arabidopsis* genome sequence project, the *sigB* region has also been covered in a BAC clone F22O13 (Accession No. AC003981).

Genomic structure of the sig genes—As shown in Fig. 3, the genomic *sigA, sigB,* and *sigC* genes consist of 10, 7, and 9 exons, respectively. A triplet codon for the first methionine is found in first exon in *sigB* and *sigC* and in the second exon in *sigA*. It has been proposed to assign conserved C-terminal half domains of eubacterial sigma factors from region 1.2 to 4.2 (Helmann and Chamberlin 1988). The regions are well conserved, and regions 2.4 and 4.2 are particularly important for promoter recognition (Malhotra et al. 1996). To compare the evolutionary relevance of the three genes in view of their genomic structures, positions of intron sites were arranged on aligned amino acid sequences of the *sig* gene products (Fig. 4). Four intron sites of the *sigB* and *sigC* genes in the 3′ half of the genes encoding conserved C-terminal regions are identical on the aligned amino acid sequences (a to d in Fig. 3 and filled triangles in Fig. 4), but the positions in *sigA* gene product are different, except for a site in region 2.2 (b in Fig. 3). The remaining three identical intron sites of *sigB* and *sigC* correspond to the ends of region 2.1 (a), region 2.4 (c), and region 3 (d) in the conserved C-terminal half. According to the previous phylogenetic analysis, SigA belongs a branch relatively close to cyanobacterial sigma factors (Tanaka et al. 1997). In contrast, the SigB and SigC proteins are very close to each other but branch away from SigA. Thus, these two approaches resulted in similar relationships among the three *sig* genes.

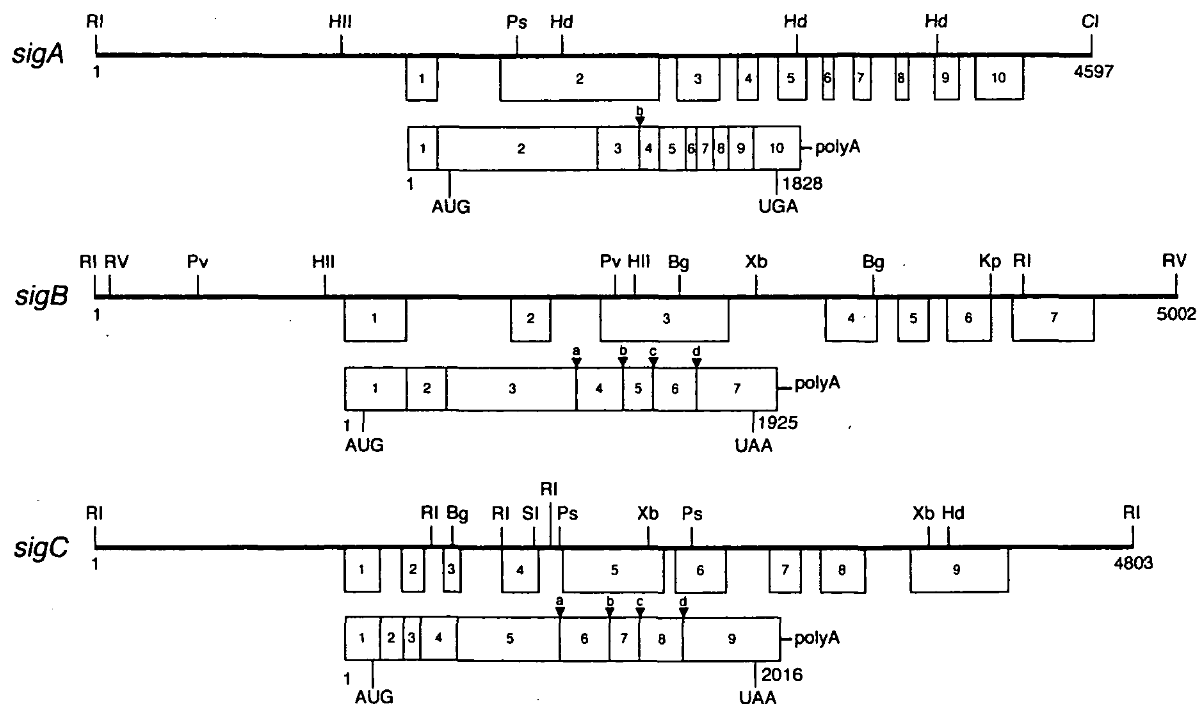

Fig. 3 Genomic structure and restriction map of three *sig* genes. The numbered boxes correspond to exons. Each pair of pictures shows genomic DNA structure (upper) and mRNA structure (lower), respectively. The filled triangles labelled with a, b, c, and d on each mRNA structure are identical intron sites in the *sig* genes (see also Fig. 4). Bg, *Bgl*II; Cl, *Cla*I; RI, *Eco*RI; RV, *Eco*RV; HII, *Hin*cII; Hd, *Hin*dIII; Kp, *Kpn*I; Ps, *Pst*I; Pv, *Pvu*II; Xb, *Xba*I.

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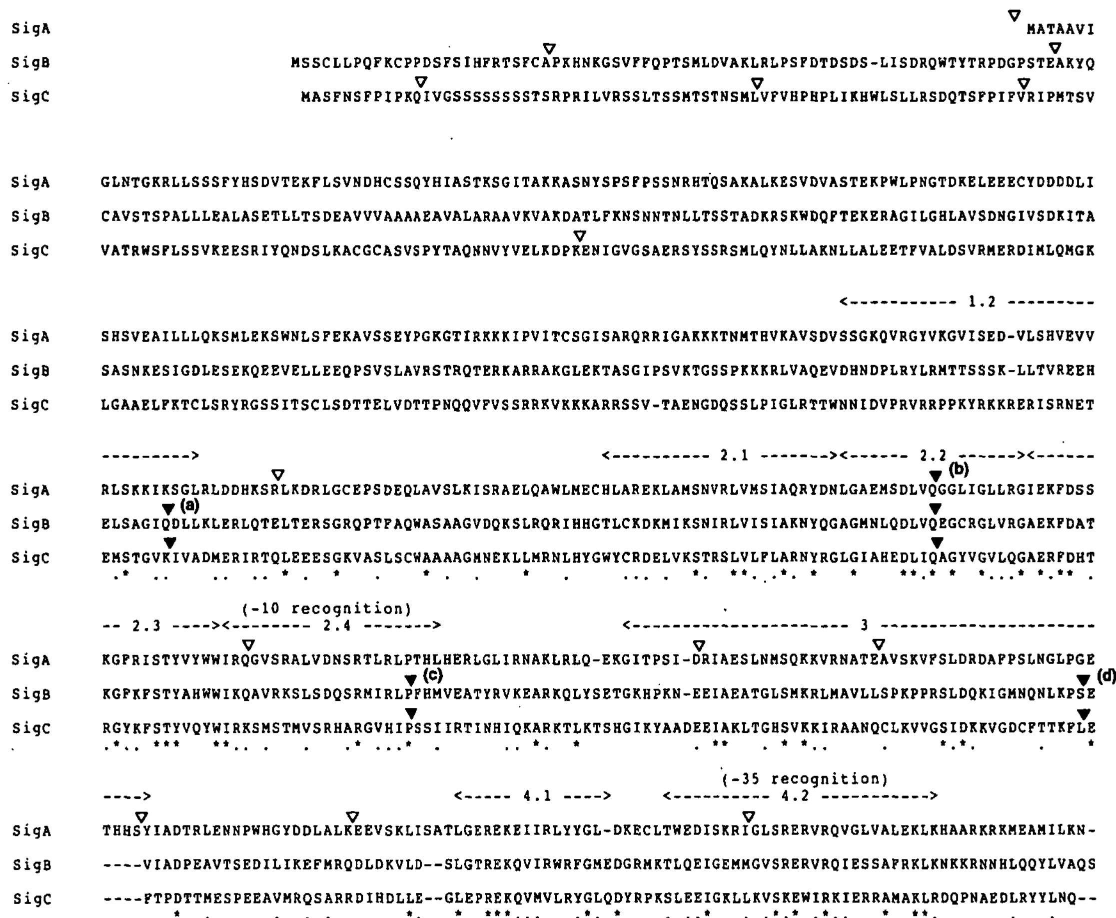

Fig. 4 Intron sites on the aligned amino acid sequences of the three *sig* gene products. Alignment of the *sig* gene products was obtained by MAlign in GENETYX (Software Development Co. Ltd., Japan). Unique intron sites are exhibited as open triangle, while identical sites in the products are shown as filled triangles labelled (a) to (d). Regions 1.2 to 4.2 are shown as dotted lines and arrows above the sequences.

Chloroplast targeting of three sig gene products—The predicted products of the three sigma factor genes contain N-terminal regions outside the conserved structures of the sigma factors that probably function as plastid-targeting transit peptides (Tanaka et al. 1997, Isono et al. 1997). The N-terminal extensions of SigA and SigC, having the typical serine- and threonine-rich sequences of a transit peptide, possess activity for chloroplast-targeting of chimeric proteins, but SigB does not (Isono et al. 1997). To investigate the subcellular localization of SigB as well as SigA and SigC, we constructed three plasmids to express the N-terminal regions of each sigma factor fused with synthetic green fluorescent protein (sGFP) under control of the *CaMV35S*-promoter. The three DNA constructs and a construct of sGFP with the transit peptide of *Arabidopsis RBCS1A* (Chiu et al. 1996) were introduced into *Arabidopsis* protoplasts by polyethylene glycol-mediated transformation (Abel and Theologis 1994). Two to five day old protoplasts after transformation were examined by confocal laser scanning microscopy (Fig. 5). Fluorescence of the sGFP could be detected clearly with very few interference effects caused by chlorophyll-autofluorescence (top panels). Fluorescence of SigA transit peptide-sGFP and SigC transit peptide-sGFP, as well as RBCS1A transit peptide-sGFP, a positive control, was found in chloroplasts with different intensities from chlorophyll signals (compare third, bottom, and second panels, respectively). SigB transit peptide-sGFP signals were also found in chloroplasts in a transformed cell (fourth panels). These indicate that the N-terminal regions of the three sigma factors function as transit peptides that target the sigma factors into plastids.

sigA and sigB expression in green tissues—To monitor promoter activities of the *sig* genes, the 5′ upstream regions of *sigA* (1.4 kb) and *sigB* (1.15 kb) were fused with the *uidA* gene on Ti-plasmid pBI101, and transgenic *Arabidopsis* lines, Ae1G and Be1G, were constructed. Both the

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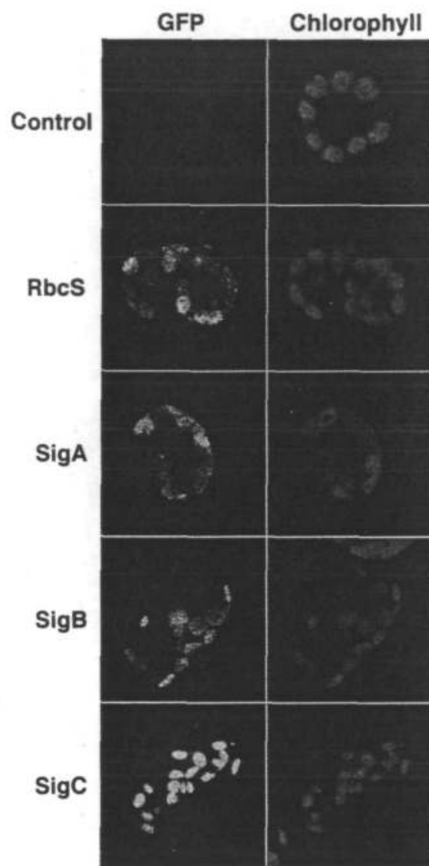

Fig. 5 Chloroplast-targeting of SigA, SigB, or SigC transit peptide-sGFP fusion protein. The fluorescent images were taken with excitation at 488 nm and emissions at 530 nm for detection of GFP (left panels) and at over 665 nm for detection of chlorophyll (right panels). The protoplasts were transformed with the plasmids encoding SigA transit peptide-sGFP (third panels), SigB transit peptide-sGFP (fourth panels), or SigC transit peptide-sGFP (bottom panels), as well as a positive control plasmid for the expression of RBCS1A transit peptide-sGFP (second panels). Top panels are negative controls without any plasmids.

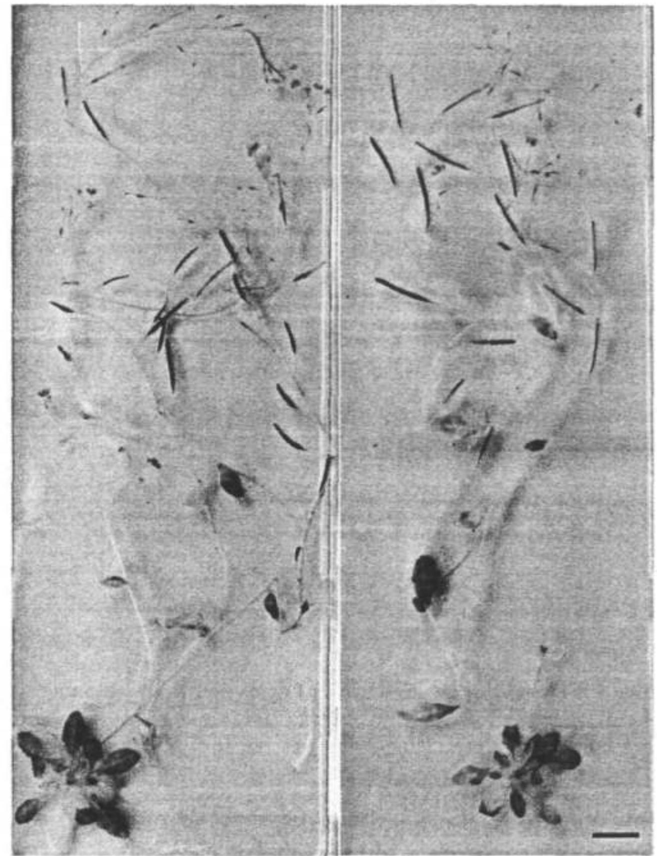

Fig. 6 Activation of *sigA*- and *sigB*-promoters in the entire plant. The four week old plants of Ae1G and Be1G were stained with X-Gluc. The left panel is staining pattern of the Ae1G, and the right is of the Be1G. The bar indicates 1 cm.

lines were expected to express a transcriptional fusion encoding β-glucuronidase proteins (GUS) with no additional amino acids at their N-termini. The established homozygous transgenic lines were first cultivated on MS-plates or peatmoss pellets under continuous light for the observation of tissue specific expression. The four week old plants were stained with X-Gluc at rosette leaves, cauline leaves, sepals, and siliques, but not at all at seeds or other flower organs (Fig. 6, 7). The mature rosette leaves and cauline leaves showed blurred or entire staining patterns (Fig. 7a to c, f to h). The distribution was random and unrelated to the age or position of the leaves. The densely-stained dots observed on adult leaves were derived from the basement cells of trichomes, although the trichomes themselves were not generally stained (Fig. 7a, g, h, i). Mature siliques were entirely stained, but smaller or immature ones were stained only at the top and the base (Fig. 7e, j). Stems were particularly well stained beneath flowers or siliques (Fig. 6, 7d, e, i, j). We also tried to construct transgenic lines to trace the *sigC*-promoter activity in the same way. However, none of the T1 transformants generated enough GUS proteins to detect any activity (data not shown).

The sigA- and sigB-promoter activities at cotyledons and the first rosette leaves—As the next experiment, we traced the early activation of the *sigA*- or *sigB*-promoter during seed germination after release from cold treatment. Imbibed seeds of the transgenic lines were placed at 4°C for 24 h under dark conditions, and then germinated at 23°C under continuous light. Most of the seed coats were torn about 24 to 36 h after the release from the cold treatment, and the cotyledons fully opened by the third day. Then, the cotyledons expanded vigorously for a week with development of chloroplasts. The first rosette leaves had mostly appeared by the sixth day.

We stained the whole seedlings of Ae1G, Be1G, and pOCA107-2 (*cab3*-promoter *uidA* fusion) every 24 h

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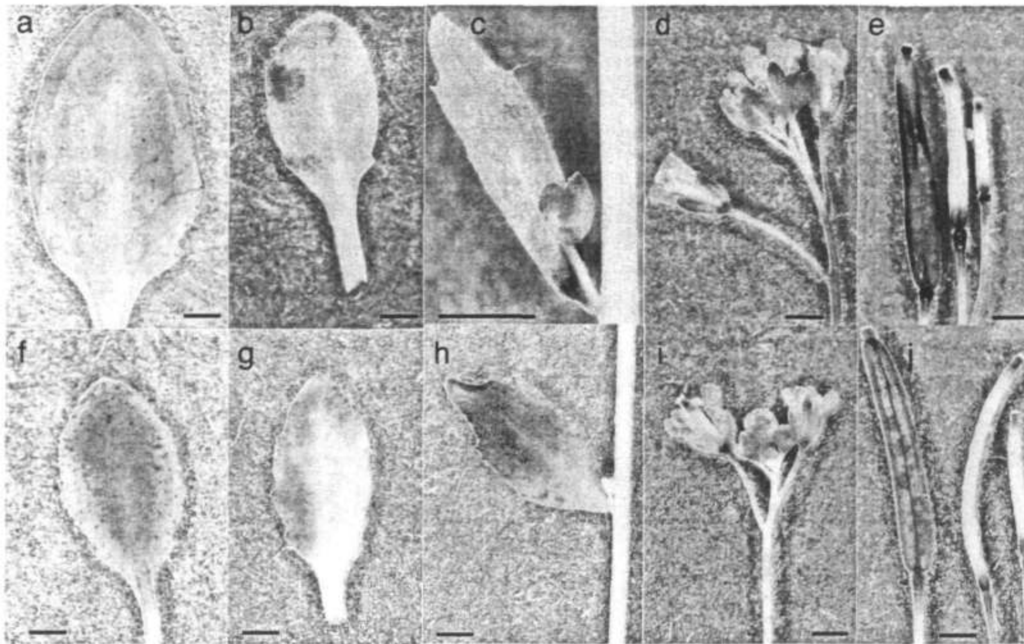

Fig. 7 Tissue specific activation of *sigA*- and *sigB*-promoters. Each tissue shown in Fig. 6 was enlarged. Upper panels (a to e) are of Ae1G, and lower panels (f to j) are of Be1G. a, b, f and g: rosette leaves. c and h: cauline leaves. d and i: flowers. e and j: siliques. The bar indicates 1 mm.

(Fig. 8). The *sigB*-promoter activity was observed at cotyledons and hypocotyls, but not at roots, as soon as 24 to 36 h after the release. This activity did not derive from any prior accumulation of the GUS proteins during embryo-

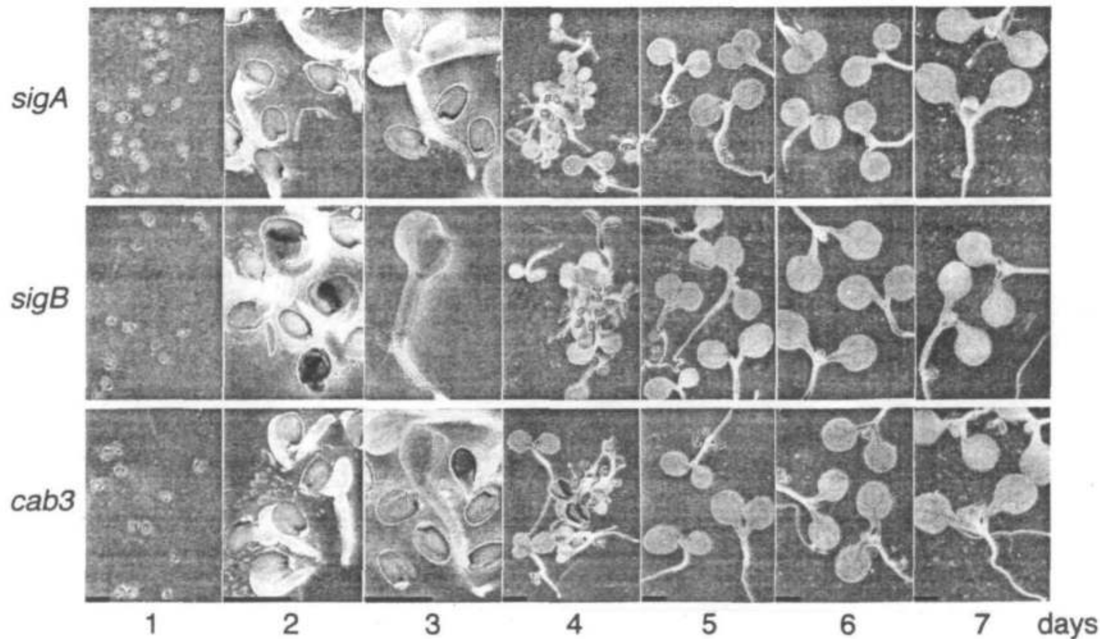

Fig. 8 Activation of *sigA*- and *sigB*-promoters in young seedlings. Young seedlings during the seven days after the release from cold treatment are shown in order. Upper panels are of Ae1G, and middle panels are of Be1G. As a control, the staining patterns of pOCA107-2 (*cab3*-promoter *uidA*) are shown in lower panels. The bar indicates 1 mm.

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genesis in the previous generation; no detectable GUS activity was found in imbibed seeds just after the release. Unlike the mosaic staining pattern of rosette leaves or cauline leaves (Fig. 7), all cotyledons were stained completely. The *sigA*- and *cab3*-promoter activities next emerged up to the third day. The emergence of *sigA*-promoter activity was seemingly delayed by at least one day from that of *sigB*-promoter activity, although the staining of the Ae1G seedlings was finally as intense as the Be1G at the fourth day. In the seven day old plants, both the *sigA*- and *sigB*-promoters were apparently activated in the first pair of rosette leaves from the marginal area inward.

Transient and repeated activation of the sigA- and sigB-promoters in young seedlings—In order to quantify the promoter activities in whole young seedlings during chloroplast biogenesis, we traced the kinetics of GUS activities in Ae1G and Be1G with every 12 h for five days after the release (Fig. 9). Consistent with the results in Fig. 8, the *sigB*-promoter activity was detected prior to the *sigA*-promoter activity. In this experiment, the first peaks of the transgenic *Arabidopsis* lines were similarly achieved at around 48 h after the release from cold treatment and remarkably diminished within next 12 to 24 h. The second peaks of the two promoter activities emerged after 84 h in the same way. Interestingly, the second peak was drastically reduced to less than 10% within the next 12 h. A

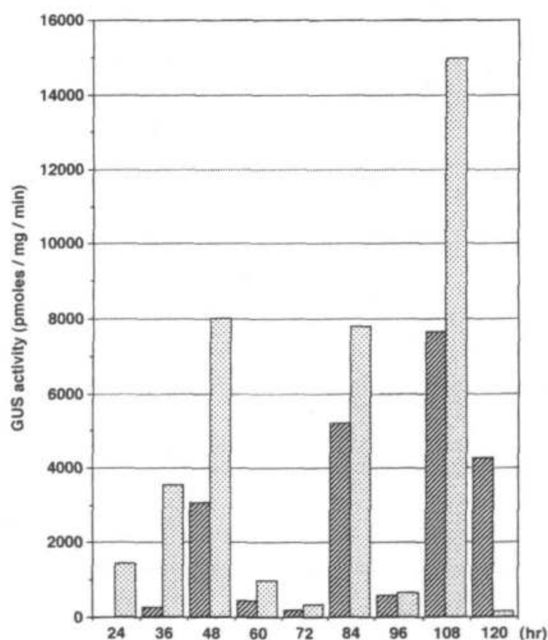

Fig. 9 Kinetics of *sigA*- and *sigB*-promoter activities in young seedlings. Young seedlings of Ae1G and Be1G were harvested and measured for GUS activity as described in Materials and Methods. Hatched bars are of Ae1G, while dotted bars are of Be1G. Each value was determined in triplicate, and the differences were within ±20%. The time of the release was taken as 0 h.

similar amplitude was revealed during the next 24 h (108 and 120 h), as if the promoter activities oscillated with a 24 h cycle. It should be noted that each value was obtained from more than 10 seedlings in populations on the same plate, not from a single seedling.

Discussion

Several genes encoding the *sigA* homolog have been already identified in higher plants, although *sigB* nor *sigC* homologs have not been reported yet. We have recently identified two novel sigma factor genes, named *sigD* (accession No. AB021119) and *sigE* (No. AB021120), encoded on chromosome V by a search through the latest *Arabidopsis* genome sequence database (Fujiwara et al. unpublished data). Thus the number of *Arabidopsis* sigma factor isoforms will be at least five and will probably increase up to the end of the genome project. We showed that *sigA* is evolutionally apart from *sigB* and *sigC* by results from not only the phylogenetic tree but also from the comparison of intron sites in the genomic sequences (Fig. 3, 4). Consistency between the two indexes is still present with the addition of the novel two sigma factor isoforms, SigD and SigE (data not shown).

We succeeded in visualizing the chloroplast-targeting of the SigB transit peptide-sGFP fusion as well as that of the SigA or SigC transit peptide-sGFP fusion (Fig. 5). Isono et al. (1997) have also shown the chloroplast targeting of SigA and SigC (They assigned to SIG2 and SIG3), but they could not confirm whether the N-terminal of SigB (SIG1) functioned as a transit peptide. Our three constructs of the GFP fusions were very similar to theirs, but we introduced the constructs into *Arabidopsis* protoplasts mediated by polyethylene glycol instead of transducing them into tobacco guard cells by bombardment. The difference might confer the SigB transit peptide-sGFP fusion with a higher efficiency of transduction or expression.

In the GUS assays of the transgenic *Arabidopsis* lines introducing *sigA*- or *sigB*-promoter *uidA* fusion, the *sigA*- and *sigB*-promoters were mainly activated in green tissues (Fig. 6 to 8). Although the data might not perfectly correspond to the actual expression pattern of the *sig* genes because of the possible effects of the inserted positions of the constructs, length of the introduced promoter regions, or elimination of introns or 3′ untranslated region, similar results for tissue specificity were also obtained from RT-PCR (Fujiwara et al. unpublished data). We don't yet have enough data to elucidate the physiological meanings of the marked expression in the sepals and siliques. However, a transgenic *Arabidopsis* line, pOCA107-2, introducing *cab3*-promoter *uidA* fusion showed a similar staining pattern (Susek et al. 1993 and data not shown). Gene expression in sepals and siliques might be a common feature of some nuclear-encoded chloroplast genes involved in pho-

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tosynthesis.

Adult rosette leaves and cauline leaves revealed the *sigA*- and *sigB*-promoter activities as entire or blurred staining patterns in the transgenic lines (Fig. 7). In contrast, all cotyledons were stained entirely in young seedlings (Fig. 8). With respect to this difference, we suggest that two physiological phases require expression and function of the sigma factors. Initially, the sigma factors are necessary for the developmental phase of chloroplasts in cotyledons and young leaves. Because cell differentiation and division of cotyledons are complete by the end of embryogenesis, what progresses in the tissue during seed germination are chloroplast development and leaf cell expansion. Therefore the *sig* gene activation is likely to occur wholly and synchronously in cotyledons, as we observed. On the other hand, chloroplast development in young rosette leaves occurs in coordination with cell differentiation (Fujie et al. 1994). This could explain why young rosette leaves tend to stain well from the marginal area inward (Fig. 8). Secondarily, the sigma factors are needed for maintenance of photosynthetic function in mature chloroplasts in response to various internal or external signals such as hormones or light. In this maintenance phase, finer arrangements of the *sig* gene expression according to need would be crucial for the function in each leaf or limited area, resulting in the mosaic phenotype of each leaf we observed in adult leaves and cauline leaves (Fig. 7).

Preliminarily, we have noticed that *sigA* and *sigB* mRNAs are most abundant isoforms of the five *Arabidopsis sig* genes (unpublished data). Perhaps the *sigA* and *sigB* gene products are the most important for PEP-dependent transcription in the *Arabidopsis* chloroplasts. We clearly showed that the *sigA*- and *sigB*-promoters were activated in a similar manner not only spatially but also temporally (Fig. 6 to 8). The only exception was that the initial emergence of the *sigA*-promoter activity occurred significantly later than that of the *sigB*-promoter activity (Fig. 8, 9). One possibility is that it is an artifact derived from positional effect or construction of the introduced fusion genes (Rose and Last 1997) or from the sensitivity of detectable GUS activity. However, when testing other independent transgenic lines introducing the same constructs, similar GUS staining patterns were observed during seed germination, and longer incubation or 20 times as much cell extract as usual still gave zero GUS activity for the *sigA*-promoter at 24 h. Another possibility is that expression of *Arabidopsis sig* genes are arranged in order and coupled with chloroplast development, particularly in cotyledons. Such programmed gene expression of sigma factors is well known in *Bacillus subtilis* during the process of endospore formation; it is named the sigma cascade (Stragier and Losick 1990). Even in a higher plant (spinach), the nuclear-encoded plastidic ribosomal protein genes are expressed in order during early chloroplast development (Bisanz-Seyer et al. 1989, Harrak et al. 1995). The earlier activation of the *sigB*-promoter might reflect the fact that the SigB protein is required as a major sigma factor for the early development of chloroplasts.

Another remarkable observation in the GUS assays was the transient activations of the *sigA*- and *sigB*-promoters during five days after release from cold treatment (Fig. 9). A possible reason for this pattern is a correlation with the temporal expression of plastid-encoded PEP component genes. Indeed, Kusumi et al. (1997) have reported that the expression of the rice *rpoB* gene occurs transiently at a limited period in early leaf development, prior to accumulation of transcripts of photosynthetic genes. To assemble the correct PEP holoenzymes in plastids when required during chloroplast development, proper provision of the regulatory subunit sigma factors from the cytoplasm into the developing organelle is probably linked with the production of core subunits encoded on the plastid genome. Coupled with the transient expression of plastidic PEP component gene(s), the nuclear-encoded regulatory factor would express in a similar manner.

An additional point we focus on is the periodicity of the *sigA*- and *sigB*-promoter activities (Fig. 9). The first amplitude of the activation surely occurred in cotyledons and hypocotyls of the young seedlings, while the second and/or the third amplitude should be attributed by the expression in young rosette leaves to some extent. We, however, postulate that the kinetics exhibit as internal circadian oscillation of the nuclear-encoded *sigA*- and *sigB*-promoters even under continuous light rather than a switching of the spatial expression from cotyledons to rosette leaves. It is worthy to note the following. First, Nakahira et al. (1998) showed that expression of a plastid-encoded photosynthetic machinery gene *psbD* is controlled at the transcriptional level by a circadian clock in wheat. Transcription of *psbD* apparently depends on PEP (Allison et al. 1996). Supporting the involvement of sigma factor(s) in the oscillation, they mentioned as a preliminary result that the wheat *sigA* mRNA exhibited circadian oscillation in coordination with *psbD* mRNA under continuous light. From an analysis of amounts of the *CAT2* mRNA in *Arabidopsis* seedlings, Zhong et al. suggested that imbibition, but neither release from cold treatment nor exposure to a light-dark cycle, can set the synchronized rhythmicity in the seed population (1998). Interestingly, the rhythmicity of the *CAT2* mRNA is not precise by the third day after the release from cold treatment, as is also the case for *sigA* and *sigB*. Moreover, it is well known that the expression of nuclear-encoded *CAB* genes for chlorophyll *a/b* binding protein are also under control of a circadian clock (Anderson and Kay 1995). These results are consistent with our idea that the *Arabidopsis sig* genes, even if not all the isoforms, have circadianic rhythmicity for the transcriptional activation.

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Eubacterial sigma factors (σ^{70}) are classified into three groups. Group 1 is the principal sigma factors; group 2 is structurally close to group 1, but non-essential for the cell viability; and group 3 is other sigma factors structurally distant from groups 1 or 2 (Lonetto et al. 1992). Based on the sequence information for *Arabidopsis sigA*, *sigB*, and *sigC*, all the gene products are intermediate between groups 1 and 2 (Tanaka et al. 1997, Isono et al. 1997). This ambiguity might somewhat reflect functional differences between higher plant sigma factors and eubacterial proteins. Supposing considerable divergency among the isoforms, the homology among the three *sig* gene products are at most 35% even in the conserved C-terminal portion from region 2.1 to region 4.2 (Tanaka et al. 1997, Isono et al. 1997). Although we show that *sigA* and *sigB* are apparently redundant rather than divergent in view of their own transcriptional activation when plants are cultivated in simple conditions under continuous light, it remains that post-transcriptional regulation, translational regulation, or response to internal/external signals would be involved in the expression of these two *sig* genes. In addition, it is not clear at all whether each of the *sig* gene products has any favorite target genes (promoter sequences) and/or timing of function. To address the nature of transcription of the *sig* genes in the function of the sigma factors in *Arabidopsis*, we need further extended studies.

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