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Divergent mRNA transcription in the chloroplast *psbB* operon

Takayuki Kohchi[1], Tadashi Yoshida, Tohru Komano[1] and Kanji Ohyama

Research Center for Cell and Tissue Culture and [1]Department of Agricultural Chemistry, Faculty of Agriculture, Kyoto University, Kyoto 606, Japan

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The genes *psbB, psbH, petB* and *petD* for the components in photosystem II and the cytochrome *b6/f* complex are clustered and co-transcribed in liverwort *Marchantia polymorpha* chloroplasts. On the opposite DNA strand in the spacer region between the genes *psbB* and *psbH*, we deduced an open reading frame consisting of 43 sense codons, and designated it as the ORF43 gene. The ORF43 gene was actively transcribed in liverwort chloroplasts. The ORF43 transcripts were entirely complementary to a part of the primary transcripts of the *psbB* operon. Heterogeneous Northern hybridization showed that the mRNA transcripts for the ORF43 gene increased with greening in pea seedlings. This is the first demonstration of divergent overlapping transcription in chloroplasts.

Key words: antisense RNA/chloroplast *psbB* operon/light induction/liverwort/*Marchantia polymorpha*/ORF43

Introduction

Chloroplasts have their own genetic system (Whitfeld and Bottomley, 1983; Ohyama *et al.*, 1986; Shinozaki *et al.*, 1986; Ozeki *et al.*, 1987). The entire gene organization of the chloroplast genome has been elucidated for a liverwort, *Marchantia polymorpha* (Ohyama *et al.*, 1986) and for a tobacco, *Nicotiana tabacum* (Shinozaki *et al.*, 1986) by the determination of complete nucleotide sequences. Most functionally related genes are clustered as seen in prokaryotic operons. For instance, the clusters of chloroplast ribosomal protein genes have similar orders to the S10 (Zurawski and Zurawski, 1985), *spc* (Cerretti *et al.*, 1983), α (Bedwell *et al.*, 1985) and *str* (Post and Nomura, 1980) operons in *Escherichia coli*. The H^+-ATPase genes in chloroplasts are also clustered, as are the *unc* operon in *E.coli* and the gene clusters in cyanobacteria (Cozens *et al.*, 1986). The genes for the components of photosystems and the photoelectron transfer complex, such as *psaA-B, psbD-C* and *psbE-F* in chloroplasts, are also clustered. One typical gene cluster in spinach, *psbB* operon, has been well-characterized (Westhoff *et al.*, 1983), and this gene organization is well conserved in chloroplasts of land-plants (Courtice *et al.*, 1985; Ohyama *et al.*, 1986; Shinozaki *et al.*, 1986; Rock *et al.*, 1987).

Genes on the same DNA strand have been found to overlap in several genomes such as those of coliphages ϕX174 and G4 (Sanger *et al.*, 1977; Godson *et al.*, 1978) and the virus SV40 (Fiers *et al.*, 1978). The bovine mitochondrial genes A6L and ATPase-6 also overlap by 40 nt (Fearnley and Walker, 1986). In chloroplasts, except for liverwort (Ohyama *et al.*, 1986) and pea (Zurawski *et al.*, 1986), the stop codon of the gene encoding the β subunit of H^+-ATPase, *atpB*, overlaps the initiation codon for the ϵ subunit, *atpE* (Krebbers *et al.*, 1982; Zurawski *et al.*, 1982). In these genes, the overlapping patterns can be seen either in a single transcript for multiple genes or within two transcripts of the same DNA strand. On the other hand, few genomes have been found to have divergently overlapped genes, which are encoded on both DNA strands in the same region. RNA I and RNA II involved in ColE1 plasmid replication have complementary sequences that control the function of the other sequence (Tomizawa *et al.*, 1981; Tomizawa and Itoh, 1981). The O protein gene in the early transcripts from the right promoter in bacteriophage λ overlapped the *oop*RNA coding sequence in the complementary DNA strand (Schwarz *et al.*, 1978). Schwarz *et al.* (1981) have reported the presence of overlapping transcripts in the *in vitro* transcription system of maize chloroplasts. The liverwort ORF43 gene was located on the complementary DNA strand between *psbB* and *psbH* genes in the *psb*B operon. The genes in the *psbB* operon were co-transcribed as a single transcription unit. Recently Tanaka *et al.* (1987) observed a transcription initiation site upstream from the *psbB* gene, but did not detect a transcription initiation site in the spacer region between *psbB* and *psbH* genes. However, the ORF43 gene on the complementary DNA strand to the *psbB* operon was actively transcribed in the liverwort chloroplasts *in vivo*. This is the first report to demonstrate divergently overlapping transcription in chloroplasts.

Results

Gene organization of the chloroplast psbB gene cluster and ORF43 loci

The *psbB, psbH, petB* and *petD* genes encode the 51-kd chlorophyll *a* binding protein (Morris and Herrmann, 1984), the 10-kd photosystem II phosphoprotein (Westhoff *et al.*, 1986; Hird *et al.*, 1986), cytochrome *b*6, and subunit IV of the cytochrome *b6/f* complex (Westhoff *et al.*, 1983) respectively. The order and orientation of these genes are well conserved among chloroplast genomes in spinach (Westhoff *et al.*, 1983), pea (Courtice *et al.*, 1985), tobacco (Shinozaki *et al.*, 1986), maize (Rock *et al.*, 1987) and liverwort (Ohyama *et al.*, 1986). These genes grouped together have been designated as the *psbB* operon (Westhoff *et al.*, 1986). No gene has been mapped in the spacer region between *psbB* and *psbH* genes; nevertheless, there is a fairly large spacer region. The results of overall DNA sequence analyses of liverwort chloroplasts, such as the GC content, codon usage pattern (extremely high AU preference for the third letter in the codons), and secondary structure of nucleotide sequence, suggest that there are two possible reading frames between *psbB* and *psbH* (Figure 1). They are designated as ORF35 and ORF43, and consist of 35 and

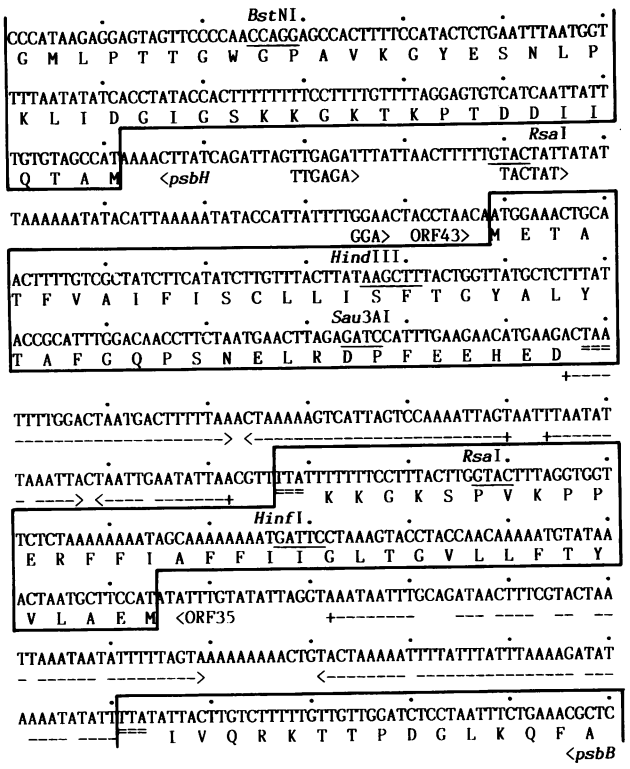

Fig. 1. Nucleotide sequence and information deduced between the *psbH* and *psbB* genes. Deduced amino acid sequences are shown under the nucleotide sequence by one-letter symbols. Double underlines indicate termination codons. Possible stem structures are shown by arrows under the sequence. Promoter and ribosome binding sequences are shown under the nucleotide sequence with arrowheads.

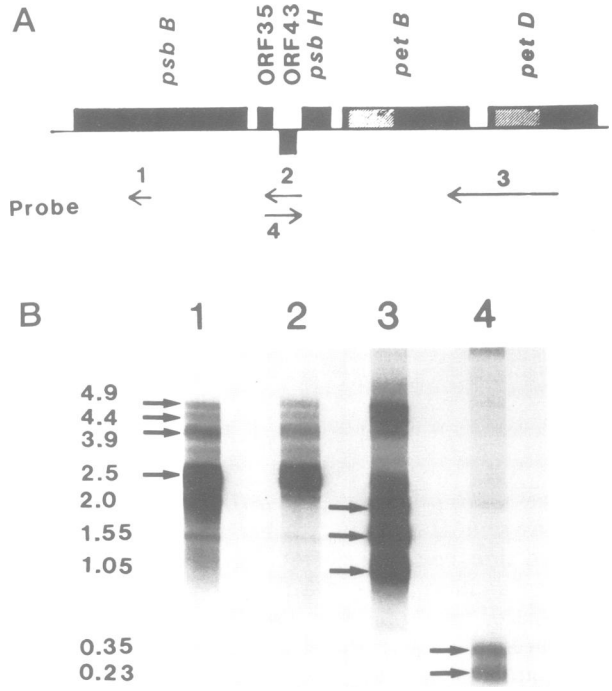

Fig. 2. Northern blot hybridization of the liverwort chloroplast RNA with specific riboprobes. (**A**) Gene organization of liverwort chloroplast *psbB* operon and ORF43 gene. (**B**) Northern hybridization analysis. **Lane 1**, *psbB* probe containing an internal portion in the *psbB* coding sequence; **lane 2**, anti-RNA probe of ORF43 containing *Rsa*I-generated fragment (298 bp); **lane 3**, *petB/D* probe covering from the *petB* second exon to the *petD* second exon; **lane 4**, ORF43 probe containing *Rsa*I-generated fragment (298 bp) in the opposite direction from the probe in **lane 2**. Numbers (kb) indicate the size of various transcripts (see also Figure 4).

43 amino acid residues respectively. Suprisingly, ORF43 was on the DNA strand opposite to that of the *psbB* operon. Thus, the nucleotide sequence between the *psbB* and *psbH* genes in the liverwort chloroplast genome is efficiently filled with genetic information such as reading frames, stem−loop structures and promoter elements.

Transcription of the ORF43 gene and psbB operon in liverwort chloroplasts

We have performed systematic Northern hybridization to detect the transcripts of the ORF43 gene and *psbB* operon. We prepared strand-specific hybridization riboprobes for *psbB* (*Eco*RI−*Pst*I fragment), anti-ORF43 (*Rsa*I−*Rsa*I fragment) and *petB/D* (*Rsa*I−*Rsa*I fragment) in the *psbB* operon, and for ORF43 (*Rsa*I−*Rsa*I fragment) encoded on the complementary DNA strand of the *psbB* operon (Figure 2). We detected a primary transcript (4.9 kb; lanes 1−3, Figure 2, and see also band a in Figure 4) for the *psbB* operon. The size of the primary transcript was in good agreement with the results of our S1 protection analyses done to identify the initiation and termination sites of the primary transcript in the *psbB* operon (unpublished data). The presence of introns in the *petB* and *petD* genes (Fukuzawa *et al.*, 1987) and various processing sites gave complicated hybridization patterns in the transcripts of the *psbB* operon (see also Figure 4). The hybridization patterns of *psbB, petB* and *petD* in liverwort were similar to those reported for other plant species (Westhoff *et al.*, 1983; Morris and Herrmann, 1984; Rock *et al.*, 1987; Westhoff *et al.*, 1986). On the other hand, Northern hybridization with a strand-specific probe for the ORF43 gene gave a simple hybridization pattern consisting of two bands (230 and 350 nt long; lane 4, Figure 2). The DNA region containing ORF43 is unique in the chloroplast genome. This was confirmed by the homology analysis of the entire chloroplast sequence which excluded the possibility that the transcripts for ORF43 originate from another locus of the chloroplast genome. These sizes are long enough to encode a polypeptide with 43 amino acids. S1 protection analysis of ORF43 mRNA showed a single initiation site and two termination sites (Figure 3). The initiation site was 47 nt upstream from the first AUG codon of ORF43 (panel A, Figure 3). The termination sites were 50 and 170 nt downstream of the termination codon (arrows in panel B, Figure 3). A signal 50 nt downstream was at the root of a long stem−loop structure, and another signal 170 nt downstream was within the ORF35 gene with the opposite orientation. To locate the overlapping region of the ORF43 transcript in processed mRNA for the *psbH* gene, S1 nuclease protection analysis was performed with 5′ end-labeled DNA fragment covering *psbH* and upstream (panel C, Figure 3). There were four signals derived from the different lengths of the 5′ leader sequences of the *psbH* gene. The nearest major signal from the translational initiation codon was ~50 nt upstream (arrow in panel C, Figure 3)

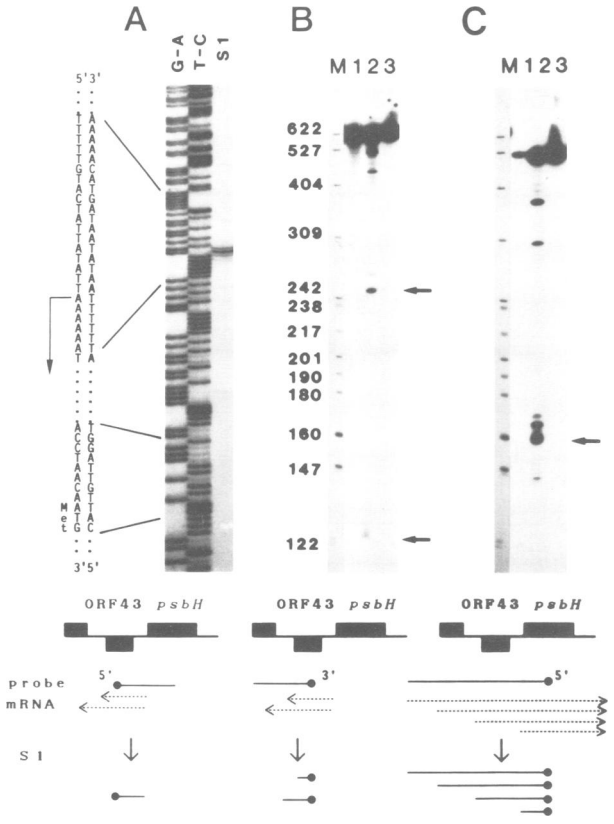

Fig. 3. S1 nuclease protection analysis of ORF43 transcripts and the 5′ terminus of *psbH* mRNA. (**A**) 5′ End of ORF43 transcript. The 5′labeled *Sau*3AI−*Bst*NI fragment (310 bp) covering ORF43 and *psbH* was used as a hybridization probe. **Lanes G−A** and **T−C** indicate the chemical cleavage ladders specific to the respective bases; **lane** S1 is for S1 nuclease treatment with chloroplast RNA. The arrow shows the initiation site and the direction of the transcript. (**B**) 3′ Ends of ORF43 transcripts. The 3′ end-labeled *Hin*dIII−*Taq*I fragment covering ORF43 and *psbB* was used as a probe. **Lane M**, the size standard of the pBR322-*Hpa*II digest; **lane 1**, S1 nuclease treatment without chloroplast RNA; **lane 2**, S1 nuclease treatment with chloroplast RNA; **lane 3**, probe prepared without S1 nuclease treatment. Arrows indicate major signals. (**C**) 5′ End of mRNA processed for the *psbH*. The 5′ end-labeled *Bst*NI−*Hin*fI fragment was used as a probe. **Lanes M**, **1**, **2** and **3** are the same as in (B). Arrows indicate a signal for major processed *psbH* mRNA.

and overlapped divergently by a few nucleotides with the 5′ leader sequences of the ORF43 mRNA.

To locate the primary transcription initiation sites in the *psbB* operon and ORF43 gene on the complementary DNA strand, *in vitro* chloroplast RNA capping and hybridization experiments were performed (Figure 4). The primary transcript (4.9 kb; band a, Figure 4) was not observed in the autoradiogram, probably because of less efficient transfer of large capped molecules from membrane to membrane. There were two slightly capped molecules corresponding to precursor transcripts; the mRNA in which the first intron in *petB* gene was spliced (4.4 kb; band b, Figure 4) and the mRNA in which both introns in *petB* and *petD* genes were spliced out (3.9 kb; band c, Figure 4). This observation also coincides with that of the ordered splicing of the introns in the maize *psbB* operon (Rock *et al.*, 1987). Major capped molecules corresponded to the mRNA encoding for *psbB* to *psbH* (2.5 kb; band d, Figure 4) and the mRNA encoding for *psbB* and ORF35 (2.0 kb; band e, Figure 4). These results indicate that rapid processing of the primary transcript occurred at both the 3′ flanking region of ORF35 gene and the 3′ flanking region of the *psbH* gene (Fukuzawa *et al.*, 1987). This fact coincides with the presence of monocistronic mRNA for the *psbH* gene (Westhoff *et al.*, 1986). A significant capped molecule (1.7 kb; band f, Figure 4) containing only the *psbB* coding region was also observed. A significant capped molecule in the intron and coding region for the *petD* gene (1.0 kb; band h, Figure 4) was not expected. We could not understand the reason why it was capped although a similar capped signal was also suggested in the *petD* intron of the maize *psbB* operon (Rock *et al.*, 1987). Shorter capped mRNA for the *psbB* gene was also detected (1.3 kb; band g, Figure 4). This may be due to the degraded product of bands d, e and f. A wide band corresponding to a length of 1.4 kb is due to the contaminated mRNA for *psbA* protein, which is the most abundant *in vitro* capped RNA in chloroplasts. Two different capped RNA molecules were also observed in the ORF43 region (bands i and j, Figure 4). These signals were in good agreement in size to the two bands in ORF43 Northern blot analysis (Figure 2) and S1 protection analysis (Figure 3). These capping experiments, therefore, demonstrated two transcription initiation sites: one for the *psbB* operon and one for the ORF43 gene.

Light induction of ORF43 transcripts

We used an RNA preparation from pea seedlings in Northern hybridization to evaluate the light induction of ORF43 mRNA because of the efficient light induction of mRNA in pea seedlings (Sasaki *et al.*, 1985) and the conserved organization of the pea *psbB* operon (Courtice *et al.*, 1985). We detected a single species of ORF43 mRNA in pea by Northern hybridization with a liverwort ORF43 probe, although liverwort ORF43 mRNAs were composed of two heterogeneous RNAs in terms of their 3′ termini. The amount of ORF43 mRNA that accumulated as the period of illumination increased is shown in Figure 5. We could not detect mRNA for ORF43 in pea seedlings grown in the dark. The transcript for ORF43 gene is accumulated under light illumination in chloroplasts.

Discussion

Comparison of the genes between psbB and psbH with those in other plants

The nucleotide sequence between *psbB* and *psbH* has been determined in spinach (Morris and Herrmann, 1984; Westhoff *et al.*, 1986), in tobacco (Shinozaki *et al.*, 1986), in maize (Rock *et al.*, 1987), and partially in wheat (Hird *et al.*, 1986). We located the genes homologous to liverwort ORF43 and ORF35 from their DNA sequences. The amino acid sequences in liverwort ORF43 and ORF35 are compared to those of the ORFs from other species in Figure 6. The amino acid sequence of liverwort ORF35 had high homology (85.7%) with that of tobacco and maize, although the reading frame in tobacco is composed of 34 amino acid residues and that in maize of 33 amino acid residues. The amino acid sequence of liverwort ORF43 showed 86.0, 86.0 and 83.7% homology with the reading frames deduced from the DNA sequences in spinach (Morris and Herrmann, 1984), tobacco (Shinozaki *et al.*, 1986) and maize (Rock *et al.*, 1987) respectively, although the corresponding frame

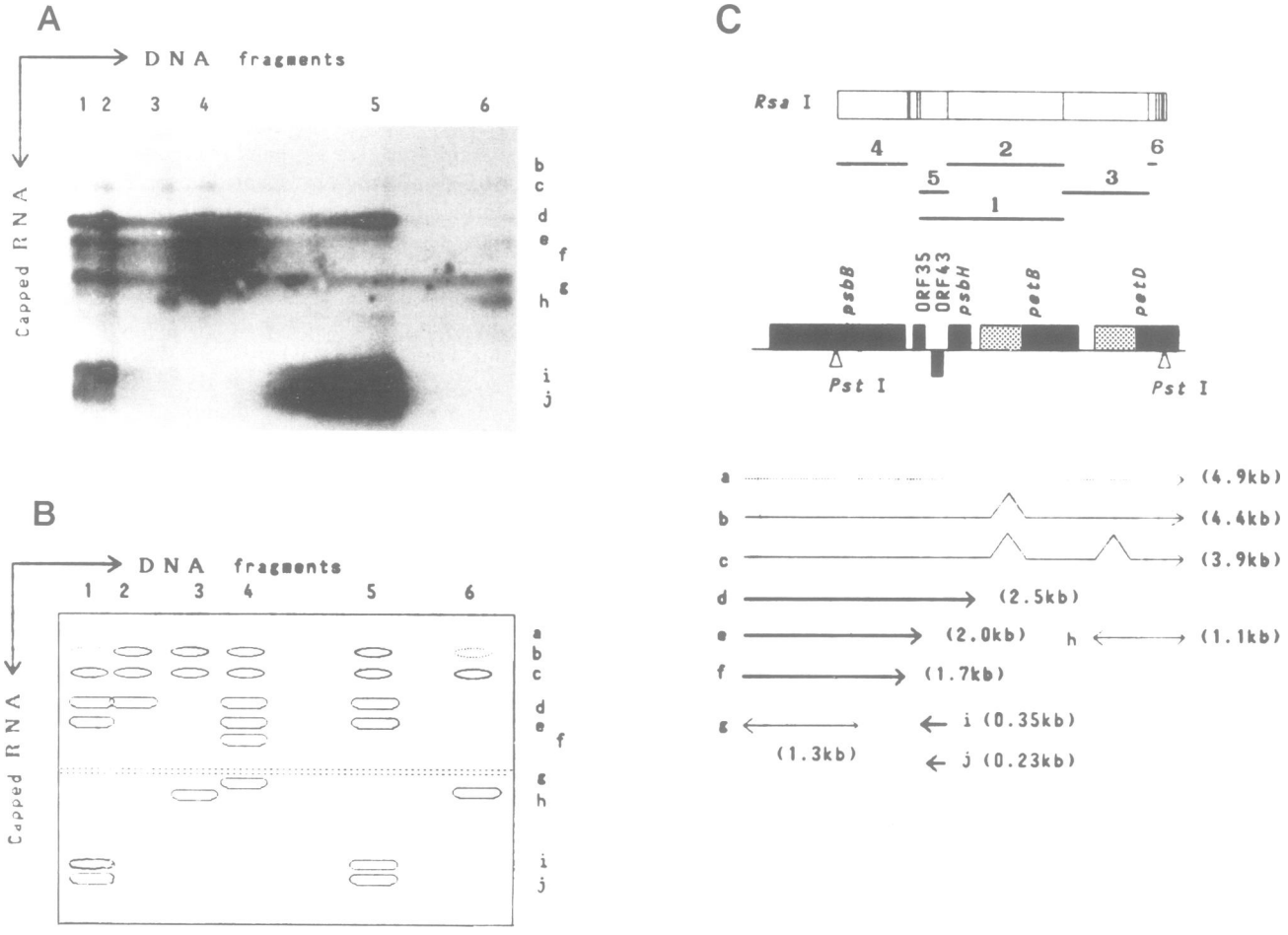

Fig. 4. Southern-cross hybridization of *in vitro* capped transcripts for *psbB* operon and ORF43. (**A**) An autoradiogram. DNA fragments were run from left to right in agarose gel and blotted onto the membrane. The *in vitro* capped chloroplast RNA was run from top to bottom and blotted on the membrane. (**B**) A schematic presentation of the autoradiogram. (**C**) Physical map, gene organization and alignment of the detected transcripts. The *Pst*I-eleventh fragment of liverwort chloroplast DNA covering the *psbB* gene to the *petD* gene was digested by *Rsa*I. The resulting fragments except the small fragments are shown. **1**, the DNA fragment for the ORF35 gene to *psbH* gene; **2**, from *psbH* to *petB*; **3**, from *petB* to *petD*; **4**, *psbB*; **5**, from ORF35 to ORF43; **6**, *petD*. See the text for the explanation of the bands (a–i).

in tobacco had an additional in-frame methionine codon in the upstream region. The liverwort ORF43 locus had a high degree of sequence homology with that of spinach, tobacco and maize only when one of the six reading frames was taken. The liverwort ORF35 locus gave similar results. These obervations suggest the high reliability of small open reading frames.

The nucleotide sequences between ORF43 and *psbH* are compared in Figure 7. The transcription initiation site in liverwort ORF43 was identified by S1 nuclease protection analysis. *In vitro* capping experiments for the ORF43 gene also showed that there was a primary transcription initiation site in the region of the opposite DNA strand of the *psbB* operon. The promoter elements (−35 and −10 regions) for ORF43 gene were well conserved among green plants. The stem−loop structure could be constructed on the mRNA for ORF43 as shown in Figure 8A. This structure may correspond to the termination signal for ORF43 transcription. On the other hand, the precursor mRNA for the *psbB* operon also form one stem−loop strucure ($-\Delta G$ = 23.0 kcal) between ORF35 and the *psbB*, and two structures ($-\Delta G$ = 38.3 kcal; $-\Delta G$ = 9.24 kcal) between

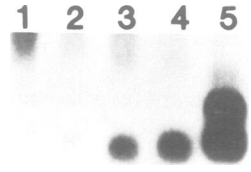

Fig. 5. Light induction of pea transcripts homologous to liverwort ORF43. Pea total RNA was extracted from pea seedlings that had been illuminated for different amounts of time after 7 days growth in the dark (Sasaki *et al.*, 1985). **Lane 1**, 0 h; **lane 2**, 24 h; **lane 3**, 48 h; **lane 4**, 72 h; **lane 5**, liverwort chloroplast RNA (0.5 μg). Liverwort ORF43 probe was used for Northern hybridization (see lane 4, Figure 3).

ORF35 and *psbH*. These structures may correspond to the processing signals for the primary transcripts.

Possible function of divergent transcripts in an operon

There have been reports indicating that gene expression in plastids of higher plants can be efficiently controlled by the

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ORF43

LV       METATFVAIFISCLLISFTGYALYTAFGQPSNELRDPFEEHED 43
SP       :::::L::::::G::V::::::::::::::QQ::::::::G: 43
TB (MIR):::::L::::::G::V::::::::::::::QQ::::::::G: 43(46)
MZ       :::::L:::S::G::V::::::::::::::QQ::::::::G: 43

ORF35

LV       MEALVYTFLLVGTLGIIFFAIFFREPPKVPSKGKK 35
TB       ::::::::::::S:::::::::::::::::::T:KN 34
MZ       ::::::::::::S:::::::::::::::::::T:K  33
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Fig. 6. Comparison of amino acid sequences deduced from the nucleotide sequence of liverwort ORF43 and ORF35 with those of the corresponding ORFs from the following species: SP, spinach (Morris and Herrmann, 1984; Westhoff *et al.*, 1986); TB, tobacco (Shinozaki *et al.* 1986); MZ, maize (Rock *et al.*, 1987). The numbers of amino acid residues are shown at the end of the sequences.

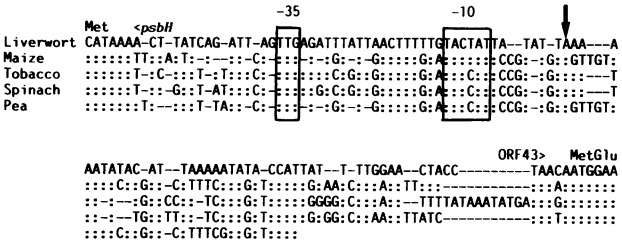

Fig. 7. Comparison of nucleotide sequences of the 5′ flanking region of ORF43. Liverwort nucleotide sequence upstream of ORF43 is compared with that of other plants (Shinozaki *et al.*, 1986; Rock *et al.*, 1987; Westhoff *et al.*, 1986; Hird *et al.*, 1986). Promoter sequences (−10 and −35 regions) are boxed. The arrow indicates the initiation site of ORF43 mRNA in liverwort.

RNA stability at the post-transcriptional level as a result of environmental changes and/or developmental processes (Mullet and Klein, 1987; Deng and Gruissem, 1987). Our experimental results obtained from the liverwort cultured cells grown photomixotrophically under continuous illumination (Katoh, 1983) imply that gene expression in the *psbB* operon, consisting of two deferentially expressed groups of genes for components of photosystem II and cytochrome *b/f* complex, is regulated by divergently overlapped transcription as well as developmental stages.

Divergently overlapping transcripts within an operon have several effects on gene expression during either transcription or translation. The complementary RNA is a highly specific inhibitor that helps to regulate plasmid replication (Tomizawa *et al.*, 1981; Tomizawa and Itoh, 1981; Rosen *et al.*, 1981) and bacterial or phage gene expression (Mizuno *et al.*, 1984; Green *et al.*, 1986). In chloroplasts, the expression of the *psbB* gene has been said to be light inducible (Westhoff *et al.*, 1983). In our experiments, the pea chloroplast ORF43 gene was also found to be actively transcribed with light. On the other hand, mRNA for the *psbH, petB* and *petD* genes has been reported to be present in etioplasts in the dark (Westhoff *et al.*, 1983, 1986). *In vitro* capping experiments showed that there was a single transcriptional initiation site upstream from the *psbB* gene in the *psbB* operon (Figure 4). These observations suggest the presence of controlled mRNA processing or premature transcription termination for gene expression in the *psbB* operon.

There are several explanations for the regulation of mRNA

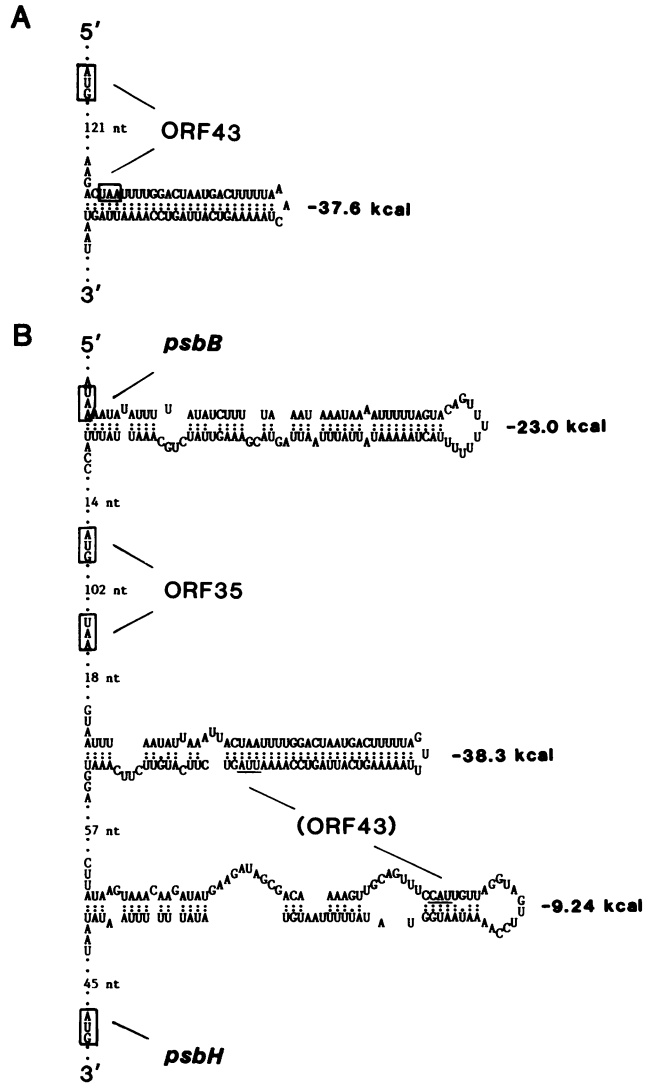

Fig. 8. Possible secondary structures in the spacer regions of ORF43 mRNA (**A**) and precursor mRNA for *psbB* operon (**B**). Base pairings are shown by one dot for GU pairing and two dots for GC and AU pairings. Computer analysis was done by IDEAS-SEQL program (Kanehisa, 1982) with parameters (G_{max}, −5.0; L_{wid}, 80; LH_{max}, 25; LI_{max}, 15; LB_{max}, 10; *LEN*, 100). The stem−loop structure with the highest free energy was shown when the overlapped secondary structure can be formed. Numbers indicate free energy ($-\Delta G$).

processing. RNA is generally transcribed unidirectional from dsDNA by RNA polymerase. When different messages are encoded on both strands in the same region, RNA polymerase molecules face each other in simultaneous transcription, resulting in the inhibition of mRNA synthesis. However, this possibility is unlikely because of the high copy number of the genome in chloroplasts. Another possibility is that the ORF43 mRNA acts as a ρ-independent terminator (Yanofsky, 1981) for premature termination in the *psbB* operon because light-inducible transcripts for ORF43 form dsRNA structure with the primary transcripts of the *psbB* operon. The transcription of the following *psbH, petB* and *petD* genes will then be repressed during illumination. Consequently, this explanation coincides with the fact that amounts of transcripts for these genes (*psbH, petB* and *petD*) do not change under light illumination. Finally, translational regulation by anti-sense RNA is also one function of mRNA

interfering complementary (mic) RNA (Mizuno *et al.*, 1984). The primary transcripts of the *psbB* operon may have the function of micRNA for ORF43 gene expression. The transcripts of the *psbB* operon are processed to make monocistronic mRNA for the *psbH* gene and two dicistronic mRNAs for the *psbB*−ORF35 and *petB*−*petD* genes as translatable mRNA (Westhoff *et al.*, 1986; Rock *et al.*, 1987; Fukuzawa *et al.*, 1987; and this paper). This fact suggests that the ORF43 mRNA is present as a translatable mRNA, probably because of rapid RNA degradation of antisense RNA. On the other hand, our experimental results, showing that the 5′ end of mature mRNA for *psbH* overlapped the 5′ end of ORF43 mRNA by only a few nucleotides, indicate that the ORF43 mRNA may not function as micRNA for monocistronic mRNA of *psbH*. There is no evidence for micRNA regulation in chloroplasts.

Materials and methods

Isolation of chloroplast RNA

Chloroplast RNA was prepared and purified by repeated Sarkosyl-phenol extraction, LiCl precipitation and ethanol precipitation from a suspension of cultured cells of a liverwort, *M.polymorpha* (Ohyama *et al.*, 1982; Yamano *et al.*, 1984). Total RNA of the pea *Pisum sativum* var. Alaska from the seedlings illuminated for a certain time was provided by Dr Y.Sasaki (Sasaki *et al.*, 1985).

Preparation of DNA fragment

Plasmid pMP710 consisted of *Pst*I eleventh fragment of liverwort chloroplast DNA and a cloning vector pUC18 (K.Ohyama *et al.*, in preparation). Restriction DNA fragments were run on 0.7−1.2% preparative agarose gels or 3.5−8% polyacrylamide gels and eluted from the gels electrophoretically (Maniatis *et al.*, 1982).

Labeling of nucleic acids

For probes of S1 nuclease protection analysis, the 5′ ends of restricted DNA fragments were labeled with [γ-^{32}P]ATP (5000 Ci/mM, Amersham) by polynucleotide kinase (Takara Shuzo). The 3′ ends of restricted DNA fragments were labeled with [α-^{32}P]dCTP (3000 Ci/mM, Amersham) by Klenow fragment of DNA polymerase I (Takara Shuzo). The labeled ds-DNA fragments were digested with a restriction endonuclease. Either the 5′ or the 3′ end-labeled DNA fragments were separated electrophoretically. For the hybridization probe, *in vitro* ^{32}P-labeled transcripts from pSP64 vector (Melton *et al.*, 1984) containing appropriate chloroplast DNA fragments were used.

Filter hybridization

Liverwort chloroplast RNA or total pea RNA (10 μg/lane) was dissolved in 20 mM 3-(*N*-morpholino)propanesulfonic acid (Mops) buffer (pH 7.0) containing 1 mM EDTA, 2.2 M formaldehyde and 50% formamide, heated at 65°C for 5 min and quenched on ice. Electrophoresis was then done on 1% agarose gel in 20 mM Mops buffer containing 1 mM EDTA and 2.2 M formaldehyde. Fractionated RNA was blotted to membrane filters (Zeta probe, Bio-Rad Laboratories) by capillary action with 20 × SSC buffer and fixed by being baked at 80°C for 2 h *in vacuo*. Prehybridization was done at 45°C for 4 h in 6 × SSC buffer containing 50% formamide, 0.5% SDS, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% BSA and 200 μg/ml heat-denatured calf thymus DNA. Hybridization was performed at 45°C overnight in the same buffer. The membrane filters were then washed with 2 × SSC buffer containing 0.1% SDS at room temperature, with 1 × SSC buffer containing 0.1% SDS at 65°C, and again at 65°C with 0.1 × SSC buffer containing 0.1% SDS. Autoradiography was done with exposures of from 1 h to 3 days on X-ray films (RX, Fuji Photo Film Co., Ltd).

S1 nuclear protection analysis

S1 nuclease protection analysis involved the procedure of Berk and Sharp (1977) with a slight modification. DNA probes were labeled and purified as described above. For hybridization, 30 μg chloroplast RNA preparations were incubated with labeled probes in 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (Pipes) buffer (pH 6.4) containing 80% formamide, 0.4 M NaCl and 1 mM EDTA for 30 min at 75°C and then for 4 h at 20°C. The mixture was diluted (1:10) with 30 mM NaOAc buffer (pH 4.6) containing 250 mM NaCl and 4 mM $ZnSO_4$, and incubated with S1 nuclease (1000 U/ml, Takara Shuzo) at 20°C for 30 min. After repetitive phenol extraction and iso-propanol precipitation, the samples were electrophoresed on 6% polyacrylamide gels containing 50% urea together with size markers (^{32}P-labeled *Hpa*II-generated pBR322 DNA fragments) and with chemically cleaved sequence ladders (Maxam and Gilbert, 1977).

In vitro capping analysis of chloroplast RNA in vivo

The conditions of the *in vitro* capping reaction were generally as described by Monroy *et al.* (1978) and Strittmatter *et al.* (1985). First, 100 μg of liverwort chloroplast RNA was capped at the triphosphate-bearing 5′ ends in 100 μl of reaction mixture containing 50 mM Tris−HCl, pH 7.9, 1.25 mM $MgCl_2$, 6 mM KCl, 2.5 mM DTT, 200 μCi [α-^{32}P]GTP (400 Ci/mM, Amersham), and 8 U guanylyltransferase (Bethesda Research Laboratories) for 120 min at 37°C. The reaction was stopped by the addition of 5 μl of 10% SDS followed by repeated phenol/chloroform extraction. The mixture was precipitated by ethanol with ammonium acetate two times to remove free radioactive GTP.

The 'Southern Cross' hybridization method (Potter and Dressler, 1986) was modified. The labeled RNA was separated on agarose gel and blotted on a nylon membrane (Gene Screen, New England Nuclear Corp.) as described above. On the other hand, non-radioactive DNA fragments containing the *psbB*, ORF43, *psbH* and *petB/D* regions were generated by *Rsa*I digestion of the *Pst*I-eleventh fragments of chloroplast DNA (see Figure 4), separated on agarose gel and blotted onto Zete-probe membrane in the usual way. The membrane was baked for 2 h at 80°C and prehybridized with hybridization buffer containing 50% formamide for 16 h at 45°C. The radioactive membrane and non-radioactive membrane sandwich was then constructed, heated for 20 min at 80°C and hybridized for 16 h at 42°C as descrbed by Potter and Dressler (1986). The recipient membrane was washed for 4 × 5 min at room temperature in 2 × SSC, 0.1% SDS, and then for 2 × 40 min at 48°C in 1 × SSC, 0.1% SDS, after which the membrane was dried and autoradiographed.

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