Volume 15 Number 13 1987 Nucleic Acids Research

Characterization of the barley chloroplast transcription units containing psaA–psaB and psbD–psbC

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Received March 9, 1987; Revised and Accepted May 22, 1987

ABSTRACT

Four plastid genes, psaA, psaB, psbD and psbC, were localized on the barley plastid genome. PsaA was adjacent to psaB in one transcription unit and psbD was adjacent to psbC in a second transcription unit. The transcription units containing psaA-psaB and psbD-psbC are separated by approximately 25 kbp on the barley plastid genome and are transcribed convergently. Transcripts hybridizing to each transcription unit were characterized by northern blot analysis, S_1 protection experiments and primer extension analysis. Two 5.3 kb transcripts hybridize to psaA-psaB. The two transcripts have a common 5' end but differ at their 3' ends by about 26 nucleotides. The transcription unit which contains psbD-psbC also includes trnS(UGA), trnG(GCC), and an open reading frame which codes for a 62 amino acid protein. Six large transcripts ranging from 5.7 kb to 1.7 kb hybridize to the psbD-psbC transcription unit as well as several RNAs of tRNA size. The large transcripts arise from three 5' ends and two clusters of 3' ends. The 3' ends map near trnG(GCC) and trnS(UGA) and could be generated by RNA processing or termination of transcription. Two of the six transcripts hybridize to psbC but not psbD suggesting that translation of psbD and psbC could occur on separate RNAs.

INTRODUCTION

The thylakoid membrane of higher plant chloroplasts contains four protein complexes which are involved in photosynthetic electron transport; Photosystem I (PSI), Photosystem II (PSII), a cytochrome b_6/f complex, and an ATP synthetase complex. All four complexes consist of several nuclear and plastid-encoded polypeptides and their associated cofactors. For example, PSII is a complex consisting of several plastid-encoded chlorophyll and/or quinone binding proteins (psbA, psbB, psbC and psbD gene products), nuclear-encoded chlorophyll a/b-binding proteins, a plastid-encoded cytochrome (cyt559) and several nuclear-encoded proteins involved in oxygen evolution (for a review see 1). PSI is composed of 8 to 12 polypeptides and is similarly complex (1). Therefore, the synthesis and assembly of protein complexes such as PSI and PSII involve coordination of nuclear and plastid gene expression as well as cofactor biosynthesis.

In dicots such as peas, plastid and leaf development are arrested at an early stage if plants are grown in darkness. In contrast, considerable plastid

and leaf development occur in dark-grown monocots such as barley (2,3,4,5,6). Plastids of dark-grown barley synthesize and accumulate most of the thylakoid membrane proteins found in mature chloroplasts, including subunits of the ATP synthetase and cytochrome b_6/f complex (7,8,9,10,11). However, proteins associated with PSI and PSII do not accumulate in dark-grown plants. The absence of of PSI and PSII proteins is due to a variety of reasons. For instance, accumulation of mRNA for the nuclear-encoded chlorophyll a/b-binding proteins of PSII requires activation of the phytochrome system by light (12). Therefore, in dark-grown plants these PSII proteins are synthesized only at very low rates. Furthermore, it has been reported that chlorophyll a/b-binding proteins are unstable in chlorophyll deficient plants such as plants grown in darkness (13, 14). Studies on plastid-encoded polypeptides have shown that the 65-68 kDa chlorophyll-apoproteins of PSI (gene products of psaA and psaB) and the 47 kDa and 43 kDa chlorophyll-apoproteins of PSII (gene products of psbB and psbC) are not detected in plastids of dark-grown barley plants despite the presence of transcripts which hybridize to the genes which encode these proteins (4,15,16, Gamble, Berends and Mullet, unpublished). The lack of the plastid-encoded chlorophyll apoproteins in dark-grown barley could be due to a lack of synthesis or rapid protein turnover (4).

In maize the psaA and psaB genes are adjacent (17,18) and a complex set of RNAs hybridizes to these genes (17). In spinach the psaA and psaB genes are also adjacent but only a single 5.7 kb RNA hybridizes to these genes (19,20, 21). A transcript of similar size hybridizes to psaA sequences in barley (4, 22). The transcription of psbD and psbC is highly complex in most higher plants. For example, the spinach psbD and psbC open reading frames overlap and at least seven RNAs hybridize to these genes (20). In barley, up to nine plastid RNAs have been reported to hybridize to a psbC probe (15). Some of the RNA heterogeneity could be due to processing of the 5' end of RNAs, as has been observed with rbcL transcripts (23). RNA heterogeneity could also be due to processing of RNA 3' ends and/or multiple sites of transcription initiation or termination. In any case, the relationship between the RNA heterogeneity reported for the transcription units containing psaA-psaB and psbD-psbC, and translation of the gene products encoded by these genes is unknown.

As a first step toward understanding the functional significance of RNA heterogeneity found in barley plastids, we have localized the psaA-psaB and psbD-psbC genes on the barley chloroplast genome, and characterized the transcripts found in plastids of dark-grown plants which hybridize to these genes.

MATERIALS AND METHODS

Reagents

^{32}P-labelled ribonucleotides and deoxyribonucleotides were purchased from New England Nuclear (NEN). Unlabelled deoxyribonucleotides were obtained from PL Biochemicals. E. coli tRNAs type XXI and salmon sperm DNA type III (Sigma Chemical Co.) were used as nucleic acid carriers. The tRNAs and salmon sperm DNA were treated as previously described (24, 25). DNA size markers (λ/HindIII and φX174/HaeIII fragments), RNA size markers, urea and agarose were purchased from Bethesda Research Laboratories, Inc. Acrylamide gels were poured with electrophoresis grade acrylamide and N-N'-methylenebisacrylamide (Eastman Kodak Co.) as previously described (25). Formaldehyde was purchased from Fisher Scientifc Co., MOPS was from Calbiochem-Behring, and glyoxal was from EM Science. The nucleic acid-grade formamide (BioRad Laboratories) was deionized prior to use according to Maniatis et al. (24). Dextran sulfate was purchased from Pharmacia. The NA-45 membranes used for DNA fragment isolations were from Schleicher and Schuell Co. Gene Screen, Gene Screen Plus and Colony Plaque Screen membranes were from New England Nuclear (NEN).

Enzymes

Restriction enzymes were purchased from Bethesda Research Laboratories, Inc., New England Biolabs, Inc., and Boehringer-Mannheim Corp. T4 DNA ligase, T4 polynucleotide kinase, DNase I, DNA polymerase I, Klenow fragment, AMV reverse transcriptase and S_1 nuclease were from Bethesda Research Laboratories, Inc. Calf intestine alkaline phosphatase was from Boehringer-Mannheim Corp.

Plant growth conditions

Barley seeds (Hordeum vulgare L. var. Morex) were planted in vermiculite, watered with full-strength Hoaglands nutrient solution, and kept in a dark, controlled environment chamber (23°C) in a light-tight room. After 4.5 days (7 days for DNA isolations), the top 3 cm of the barley seedlings were harvested for plastid isolation.

Plastid isolation

Plastids were isolated as previously described (26) on percoll gradients. When possible, all manipulations were done at 2-4°C in complete darkness. A dim, green safelight (wavelength of emission 515 to 560 nm), which does not allow conversion of protochlorophyllide to chlorophyll (27), was used only when necessary.

Plastid nucleic acid isolation

Nucleic acid was isolated from plastids by phenol extraction as previously described (23). The total nucleic acid was resuspended in TE buffer (10mM Tris-

HCl, pH 8, 0.1mM EDTA) on a per plastid basis. Small aliquots were kept at -80°C for use in northern blotting, SI nuclease protection and primer extension experiments. For cloning purposes, DNA from 7 day-old etioplasts was further purified on CsCl-EtBr gradients as described by Hallick et al (28).

Construction and screening of barley ctDNA libraries

Barley ctDNA was digested with the appropriate restriction enzyme, which was later removed by phenol:chloroform:isoamyl alcohol (25:24:1; vol:vol:vol:) extraction. The resulting fragments were cloned into pUC8 (29) and used to transform (30) bacterial host TB-1 (31). Colonies were transferred to Colony/Plaque Screen discs according to the instructions supplied by NEN. Hybridization techniques were identical to those used for the Southern blots.

Isolation of DNA restriction fragments

Plasmid DNA was digested with the appropriate restriction enzymes and the resulting fragments were separated on agarose or polyacrylamide gels. Fragments were recovered from the gel either by electroelution into a dialysis bag (24) or by electrophoresis onto NA-45 membrane (32).

Southern and northern blots

Southern probes were nick translated according to Maniatis et al(24). DNA was separated on 0.8% agarose gels and blotted onto Gene Screen Plus membranes according to NEN's instructions. Heterologous hybridizations to DNA probes were carried out at 37°C according to Maniatis et al (24). The final wash was done at 55°C. For northern blots, total nucleic acid was separated on 0.8% formaldehyde/agarose gels (24) or on 0.8% glyoxal gels (NEN's method) and blotted onto Gene Screen membranes following NEN's capillary blot procedure. Northern probes were nick-translated using a nick translation kit. Hybridizations to the RNA on the membrane were done at 42°C for a minimum of 21 hrs by NEN's dextran sulfate method. The washing procedure recommended by NEN was varied as follows. The first washes (2 x 5 min.) were done at room temperature with 2 x SSC, 0.5% SDS. Next, the membranes were washed for 2 x 30 min. at 50°C with 0.2 x SSC, 0.5% SDS. Nick-translated λ/HindIII fragments, which did not hybridize to the barley plastid nucleic acids, were used to visualize the RNA markers (data not shown).

S_1 nuclease and primer extension assays

5' end and 3' end DNA labelling was done according to Maniatis et al (24). S_1 nuclease assays were performed as previously described (23). For the primer extension assays, we followed a shortened version of the previously published procedure (23); the AMV reverse transcriptase reaction was stopped by removing

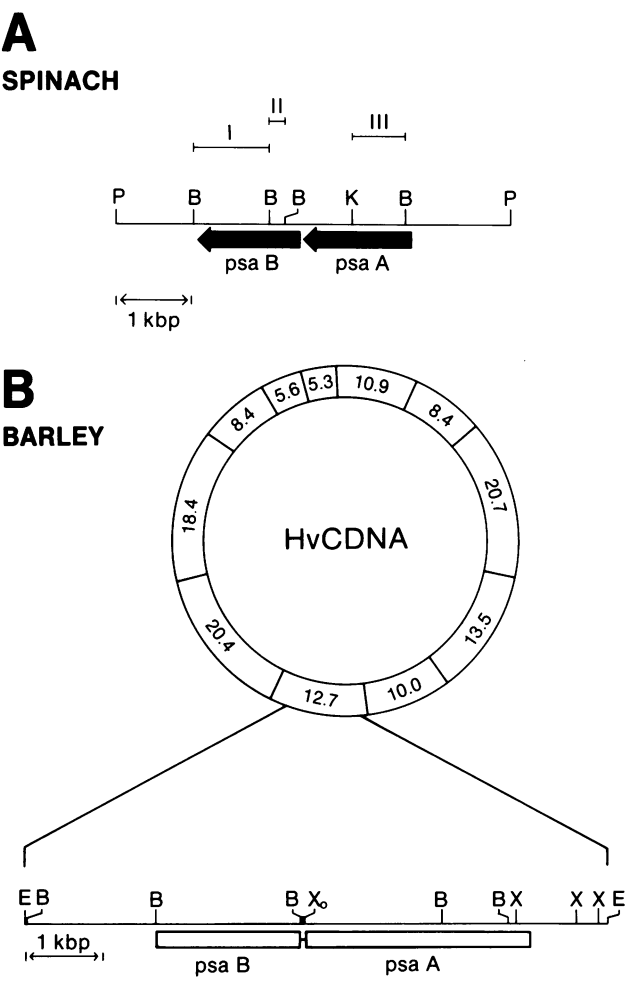

Figure 1: Restriction maps of the ctDNA regions which encode psaA and psaB in spinach (A) and barley (B). The symbols denote the following restriction sites: P-PstI; B-BamHI; K-KpnI; E-EcoRI; X-XbaI; Hf-HinfI;Xo-XhoI; and S-SalI. For clarity, not all restriction sites for a given enzyme are shown. In Figure 1A, the solid arrows indicate the location of psaA and psaB on the 8.2 kbp PstI fragment of spinach ctDNA (21). Fragments I, II and III were used for heterologous hybridizations to barley ctDNA. In the upper portion of Figure 1B, a PstI restriction map of the barley chloroplast genome is shown (39). The 7.6 kbp EcoRI fragment of pBE432-3 to which the spinach probes hybridized is enlarged in the lower portion of Figure 1B. Open boxes within the 7.6 kbp EcoRI fragment indicate DNA regions homologous to psaA or psaB.

the enzyme by phenol extraction followed by ethanol precipitation. Labelled fragments were analyzed on 8% polyacrylamide-8.3M urea gels (33).

RESULTS

A. Localization of psaA and psaB on barley ctDNA.

psaA and psaB are located on an 8.2 kbp PstI fragment of spinach ctDNA as shown in Figure 1A (19,20,21). This spinach DNA fragment which contains both genes was cloned into a pUC8 (29) plasmid (pSP8). To find the approximate location of the corresponding psaA gene on the barley chloroplast genome, fragment III from the spinach psaA coding region (Figure 1A) was nick-translated and used to screen an EcoRI library of barley ctDNA. A recombinant plasmid with a 7.6 kbp EcoRI insert (pBE432-3) hybridized to the spinach psaA sequences (data not shown).

This 7.6 kbp EcoRI fragment in turn was homologous to a 12.7 kbp PstI fragment on the barley plastid genome (Figure 1B). Further Southern blotting experiments, using spinach probes I, II and III (Figure 1A), revealed that barley plastid DNA regions homologous to psaA and psaB are adjacent to each other as shown in Figure 1B.

B. Northern analysis of psaA-psaB transcripts in barley.

In barley, psaA sequences have been reported to hybridize to a large transcript (4,22). To map this transcript, RNA was isolated from plastids of 4.5 day-old, dark-grown barley seedlings, separated on glyoxal/agarose gels, and blotted onto GeneScreen membranes. All five probes (fragments A-E, Figure 2, top panel) from the 7.6 kbp EcoRI DNA fragment hybridized to a 5.3 kb RNA, although the signal in lanes A and E of the autoradiogram was much weaker than in lanes B-D (Figure 2, lower panel). The probe in lane E hybridized to two smaller RNAs in addition to the 5.3 kb RNA. These data suggest that in barley etioplasts psaA and psaB are co-transcribed on a 5.3 kb transcript and that other transcripts of unknown origin either start or end close to psaB.

C. Determination of position and direction of psaA-psaB transcripts.

To map the 5' end of the 5.3 kb psaA-psaB transcript from barley etioplasts, an S_1 nuclease protection assay was done, using an end-labelled 1 kbp BamHI fragment as the probe (Figure 3, lane 2). This probe contained 10 bp of pUC8 vector sequence plus DNA fragment A (Figure 2). A single fragment of about 263 bp was protected by barley plastid RNA (Figure 3, lane 3). Since the pUC8 sequences would not be expected to hybridize to plastid RNA, this result localized the 5' end of an RNA at approximately 263 bp upstream of the BamHI site within the 7.6 kbp EcoRI fragment. To test for possible S_1 nuclease arti-

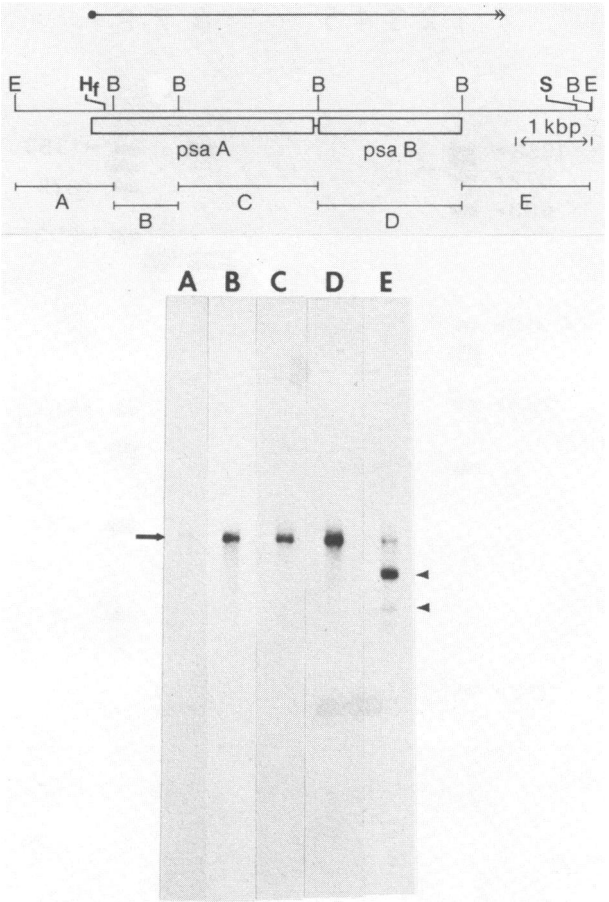

Figure 2: Northern analysis of etioplast RNA isolated from 4.5 day-old dark-grown barley seedlings. Northern probes A-E are shown below the restriction map of the 7.6 kbp EcoRI fragment (also shown in Figure 1B). Open boxes indicate the DNA regions which were homologous to psaA or psaB. Total nucleic acid in lanes A-E of the northern blot was hybridized to probes A-E, respectively. The lower portion of Figure 2 shows the corresponding autoradiograms. The small arrow on the left hand side indicates the band corresponding to the psaA-psaB transcripts. The small arrows at the right hand side indicate the other transcripts mentioned in the text. The large double arrow above the restriction map shows the position and direction of the psaA-psaB transcripts as determined by northern blotting, S_1 protection and primer extension techniques.

facts, the 5' end mapping was repeated by the primer extension method. A 113 bp HinfI-BamHI fragment, 5' end labelled at the same BamHI site within the 7.6 kbp EcoRI fragment (Figure 3, lane 5), was hybridized to barley ctRNA and extended

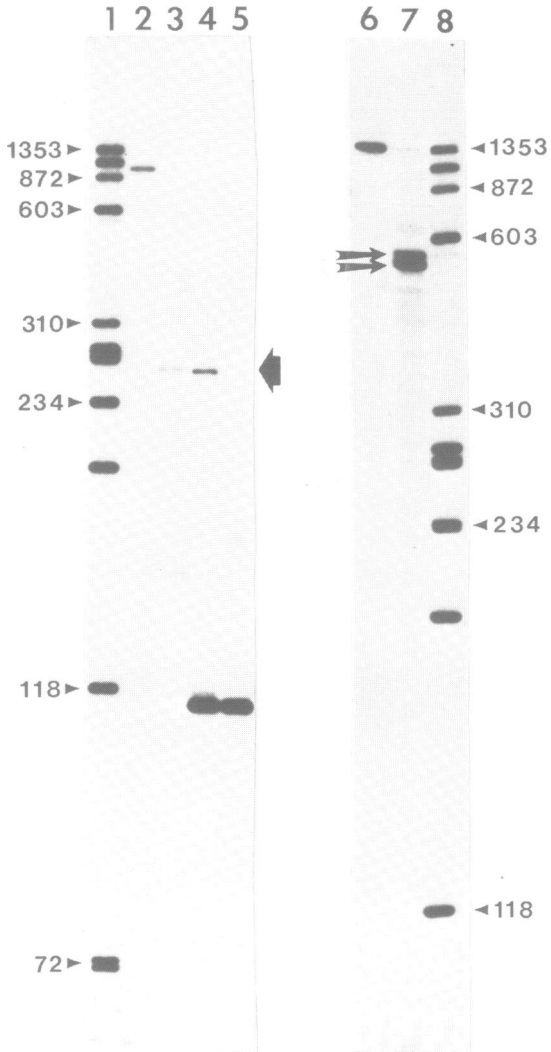

Figure 3: 5' and 3' termini of the psaA-psaB transcripts in 4.5 day-old barley etioplasts. The numbers next to lanes 1 and 8 of the autoradiograms indicate the sizes (bp) of some of the end-labeled ϕX174/HaeIII DNA fragments used as molecular weight standards. Lane 2 shows the 1 kbp BamHI-BamHI probe, which was labelled at both 5' ends. The reverse transcription probe in lane 5 (a 113 bp HinfI-BamHI fragment, 5'-end labelled at the BamHI site) was derived from the S_1 nuclease probe in lane 2. The black arrow indicates the 263 bp fragment resulting from S_1 nuclease protection (lane 3) and primer extension (lane 4) using RNA isolated from 4.5 day-old barley etioplasts. Lane 6 shows the doubly end-labelled 1.6 kbp BamHI-BamHI probe used for the 3' end determination. The open arrow shows two protected bands (548 and 522 bp) resulting from the S_1 nuclease protection assay in lane 7.

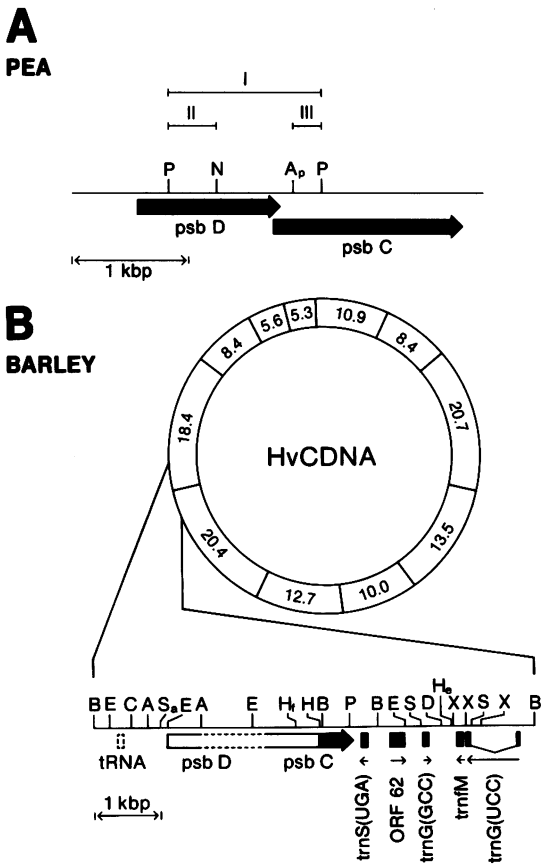

Figure 4: Restriction maps of the ctDNA regions which encode psbD and psbC in pea (A) and barley (B). The symbols denote the following restriction sites: P-PstI; N-NdeI; Ap-ApaI; B-BamHI; E-EcoRI; C-ClaI; A-AccI; Hf-HinfI; H-HindIII; S-SalI; Sa-Sau96I; D-DdeI; He-HaeIII and X-XbaI. Not all restriction sites for a given enzyme are shown. In Figure 4A the solid arrows denote the open reading frames which correspond to psbD and psbC in pea ctDNA (36,37). Fragments I, II and III (shown above the restriction map in Figure 4A) were nick-translated and used to probe a BamHI library of barley ctDNA. The 3.4 kbp BamHI fragment (of pBB531-4) which hybridized to the probes is shown in the lower portion of Figure 4B. Homology to the pea psbD and psbC sequences is indicated by the open box. The dotted line indicates the DNA region in which psbD ends and psbC starts. The small dotted box shows the approximate location of tRNA-size sequences which appeared on the northern blots in Figure 5. A 4.3 kbp HindIII fragment (of plasmid pHvC35), which starts within the 3.4 kbp BamHI fragment, has previously been sequenced by Oliver and Poulson (15). Part of this sequenced region has been included in the linear restriction map of Figure 4B; the solid arrow shows the 3' end of psbC, and the solid boxes, accompanied by the small arrows, show the location and polarity of the ORF62 and the tRNA genes downstream of psbC. The entire BamHI-BamHI DNA region shown in Figure 4B spans 6.2 kbp, and its position on a PstI restriction map of the barley chloroplast genome (39) is shown in the upper portion of Figure 4B.

by reverse transcription. The resulting fragment (Figure 3, lane 4) co-migrated with the S_1 nuclease-protected fragment (lane 3), confirming the results obtained using the S_1 protection assay.

The presence of a single 5' end upstream of psaA and the appearance of a 5.3 kb RNA which hybridizes to this DNA suggested that the corresponding 3' end would be located downstream of psaB. Therefore, a 1.6 kbp BamHI fragment (fragment E in Figure 2) was labelled at both 3' ends and used as a probe (Figure 3, lane 6) for an S_1 nuclease protection assay. Two protected bands were seen (Figure 3, lane 7) of about 548 and 522 bp. To determine the directionality of these 3' ends, the experiment was repeated with a shortened version of the same probe (a 1.55 kbp BamHI-SalI fragment), 3' end labelled only at the BamHI site closest to psaB. The same size DNA fragments were protected when this probe was used (data not shown), indicating that the 3' ends map approximately 548 and 522 bp from the BamHI site. Thus, in etiolated barley seedlings there appear to be two psaA-psaB transcripts which share the same 5' end, but differ at their 3' end by approximately 26 nucleotides.

D. Localization of psbD and psbC on barley ctDNA.

Oliver and Poulson (15) sequenced a 4.3 kbp HindIII fragment in barley ctDNA, and noted that its gene organization was very similar to a region in maize which includes 165 codons of a 473-codon open reading frame (34,35). In maize, the 473-codon open reading frame overlaps another, 353-codon open reading frame, which is highly homologous to psbD in pea. Both psbD (353 codons) (36) and psbC (473 codons) (37) have now been sequenced in pea, and the putative open reading frames overlap by 50 bp as shown in Figure 4A. Therefore, it has been inferred (15) that in barley, psbD and psbC are aligned in a similar manner, and that the 165-codon open reading frame sequenced by Oliver and Poulson (Figure 4B, dark arrow) encodes the carboxy-terminal portion of the psbC gene product.

To confirm the suggested location of psbD-psbC on barley ctDNA, a 1.1 kbp PstI probe from the pea psbD-psbC DNA region (fragment I, Figure 4A) was used to screen a BamHI clone bank of barley ctDNA. Recombinant plasmids with a 3.4 kbp BamHI insert (designated pBB531-4) hybridized to the pea psbD-psbC sequences (data not shown). A restriction map of the BamHI insert revealed that it overlapped by 63 bp with the 4.3 kbp HindIII fragment previously sequenced (15) (see Figure 4B), and the position of the psbD-psbC region on the barley plastid genome is shown in Figure 4B. Further Southern blotting experiments, using pea probes II and III, revealed that psbD and psbC may overlap in barley as well.

E. Northern analysis of psbD-psbC transcripts in barley.

In a previous study, it was found that etioplasts of 4.5 day-old dark-grown

barley synthesized a protein which co-migrated with the psbD gene product (38). In contrast, barley etioplasts did not synthesize a protein which co-migrated with the psbC gene product (38). To determine whether etioplasts contain transcripts which could encode psbD or psbC, northern analyses were performed using the nick-translated probes shown below the restriction map in Figure 5 (fragments A-L). RNA from 4.5 day-old dark-grown plastids was separated on formaldehyde/agarose gels, blotted onto GeneScreen membranes, and probed. The autoradiograms of each blot are shown below the corresponding probes in Figure 5, lanes A-L.

Fragment E, which according to the previous Southern data contains most of psbC and part of psbD, hybridized to six RNAs, which were 5.7, 4.8, 4.1, 3.3, 2.6 and 1.7 kb in length. Of these six transcripts, the 5.7 kb RNA hybridized to fragments A-J, but not to fragments K and L. This would put one end of the 5.7 kb RNA within the 0.52 kbp SalI-XbaI fragment common to probes I and J. A 4.8 kb band was seen in lanes A-G, but not in lane H. Since probe H (BamHI-EcoRI) overlaps completely with probe G (PstI-EcoRI), the 4.8 kb RNA must either start or end within the 0.44 kbp PstI-BamHI fragment. According to the restriction map, fragments A-I, which represent the DNA regions hybridizing to the 5.7 kb RNA, span only 5.4 kbp, while the regions which hybridized to the 4.8 kb RNA cover only 4.3 kbp. This suggests both the 5.7 and the 4.8 kb RNAs extend beyond the BamHI site of fragment A. Two additional RNAs were detected with probe C but not probes A or B. The longer DNA, 4.1 kb in length, exhibited the same pattern as the 5.7 kb RNA; it extended through J, but not into fragments K and L. The shorter, 3.3 kb RNA showed up as a prominent band in lanes C-F and possibly as a very faint band in lane G. It was not observed when barley plastid RNA was probed with fragment H. This suggests both the 4.8 and the 3.3 kb RNAs either start or end within the same 0.44 kbp PstI-BamHI fragment. Finally, a third set of RNAs (2.6 and 1.7 kb) was detected with probe D. Like the 5.7 and 4.1 kb RNAs, the 2.6 kb RNA hybridized to probes D-J, suggesting that the 5.7, 4.1, and 2.6 kb RNAs all have an end (5' or 3') within the 0.52 kbp SalI-XbaI fragment mentioned above. It may be worth noting that, in addition to hybridizing to the 2.6 kb transcript, probes D-J also seemed to hybridize to a 3.6 kb RNA of low abundance. However, S_1 nuclease and primer extension experiments failed to detect termini for this putative RNA species, and it is therefore not included in Figure 5. The 1.7 kb RNA, like the 4.8 and the 3.3 kb RNAs, extended through fragment G, but did not hybridize to probe H. Thus it appears that the 4.8, 3.3 and 1.7 kb RNAs all have an end within the 0.44 kbp PstI-BamHI fragment. Of the six RNAs, the four longer species (5.7, 4.8, 4.1,

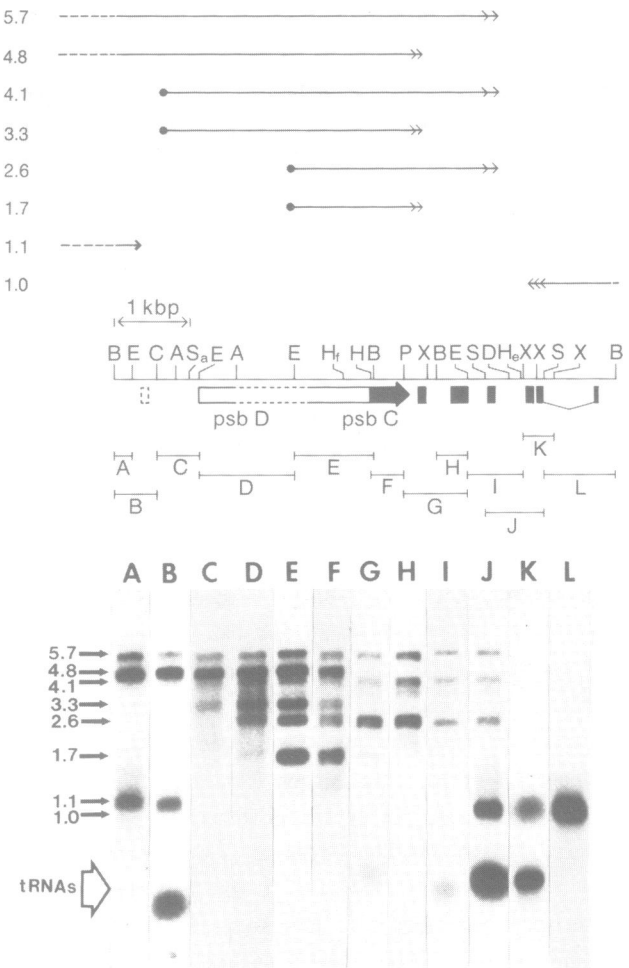

Figure 5: Northern analysis of etioplast RNA isolated from 4.5 day-old dark-grown barley seedlings. Northern probes A-L are shown below the restriction map of the psbD-psbC region, which is identical to the map in Figure 4B. Total nucleic acid in lanes A-L of the northern blot was hybridized to probes A-L, respectively. The resulting autoradiograms were aligned and are shown in the lower portion of Figure 5. The numbers next to lane A of the autoradiogram refer to the approximate RNA sizes in kb. tRNA-like transcripts are indicated by the large, open arrow. The same numbers appear above the restriction map next to the arrows which summarize the results from the northern blots, and S_1 nuclease protection and primer extension assays. The dotted lines at the beginning of the 5.7, 4.8, 1.1 and 1.0 kb RNAs indicate that the position of the 5' end was not confirmed by S_1 nuclease and reverse transcriptase techniques. The solid circles show the position of the two 5' ends belonging to the 4.1 and 3.3 kb RNAs, and the 2.6 and 1.7 kb RNAs.

and 3.3 kb) could contain both psbD and psbC, based on their lengths and approximate positions. The two smaller RNAs (2.6 and 1.7 kb) may contain just the psbC sequence.

Several other RNAs hybridized to DNA fragments surrounding the psbD and psbC genes. According to the published sequence (ref. 15, see Figures 4B and 5), probe G contains sequences from trnS(UGA), probe I contains sequences from trnG(GCC), probe J contains sequences from trnG(GCC) and trnfM, probe K contains sequences from trnfM and the 3' end of trnG(UCC), and probe L contains sequences from the 5' end of trnG(UCC). In lanes G, I, J and K several RNAs smaller than 0.1 kb hybridized to the probes. Thus these bands may represent tRNA's. In lanes J, K and L a 1.0 kb band was observed, which may represent a precursor to the glycine tRNA coded by this region, as has been suggested (15). In lane B a band similar in size to the tRNA bands in lanes G, I, J and K was observed. This may indicate the presence of one or more additional genes which encode tRNAs within the 0.32 kbp EcoRI-ClaI region of probe B (see Figure 4B). Also, a 1.1 kb RNA hybridized to DNA fragments A and B. This RNA therefore has a 3' or 5' end within the 0.57 kbp BamHI-ClaI fragment (probe B). Consequently, the 1.1 kb RNA must extend beyond the BamHI site of fragments A and B. In lanes E and F, which span 1.43 kbp, several low abundance RNAs smaller than 1.4 kb were detected. In the previous study by Oliver and Poulson (15) it was suggested that these RNAs may arise due to artifactual hybridization to rRNAs. Alternatively, they may represent RNA processing intermediates. In spinach and pea, psbC is 473 codons or 1.42 kbp long. If psbC is of a similar size in barley then it is highly unlikely that the smaller RNAs could encode all of psbC.

F. Determination of position and orientation of transcripts originating in the psbD-psbC region.

It was evident from the northern analyses that several transcripts which could encode psbD and/or psbC, originate in the 6.6 kbp BamHI-BamHI region shown in Figure 5. The termini of these transcripts were mapped using overlapping fragments covering this region. These fragments were labelled at the 5' or 3' ends and were used in S_1 nuclease protection assays on RNA isolated from plastids of 4.5 day-old dark-grown plants. 5' ends observed in S_1 nuclease protection experiments were verified by primer extension analysis; all 5' end analyses are shown in Figure 6. All 3' end mapping is shown in Figure 7. Since S_1 nuclease protection and primer extension experiments can give rise to artifacts, the relationship between an RNA terminus noted by nothern analysis, S_1 protection experiments and primer extension assays is indicated where possible. The

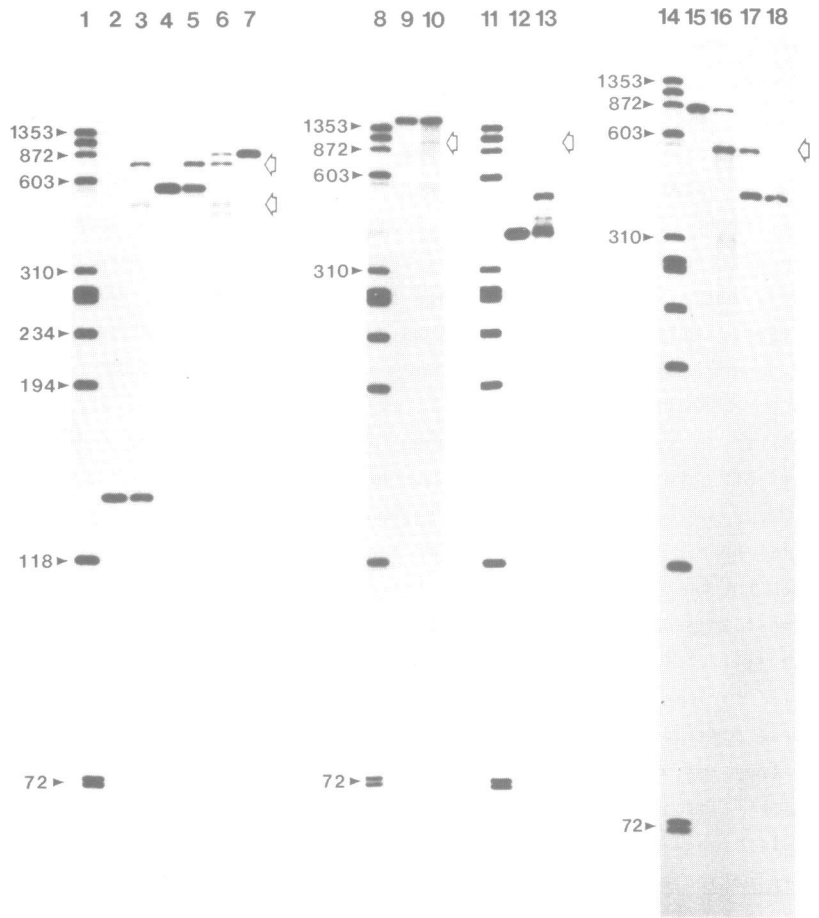

Figure 6: 5' termini of the psbD-psbC transcripts in 4.5 day-old barley etioplasts. The numbers next to lanes 1, 8 and 14 of the autoradiograms indicate the sizes (bp) of some of the end-labelled ϕX174/HaeIII fragments used as DNA molecular weight markers. The same markers were also run in lane 11. Lanes 2 and 4 show the 135 bp Sau96I-EcoRI and the 0.55 kbp ClaI-EcoRI probes (labelled at the same EcoRI site), which were used for reverse transcription in lanes 3 and 5 respectively. Lane 7 shows the corresponding 0.88 kbp EcoRI-EcoRI probe (labelled at both 5' ends) used to produce the two major S_1 nuclease-protected bands of 800 and 484 bp in lane 6, which co-migrated with the bands in lanes 3 and 5 (indicated by open arrows). Lane 9 shows the doubly-labelled 1.83 kbp AccI-HindIII probed used for S_1 nuclease protection in lane 10. The resulting 995 bp band (open arrows) was verified by primer extension in lane 13 from a 375 bp HinfI-HindIII probe labelled at the HindIII site (lane 12). Lane 16 shows the result of an S_1 nuclease protection assay with the 0.83 kbp PstI-EcoRI probe (labelled at the EcoRI site, lane 15). The same 594 bp band seen in lane 16 appeared in lane 17 (open arrow) after primer extension from the 391 bp BamHI-EcoRI fragment labelled at the EcoRI site (lane 18).

results of these experiments are presented sequentially from fragment A to fragment L in Figure 5.

A 1.1 kb RNA hybridized to DNA fragments A and B when these DNAs were used to probe northern blots (Figure 5). When probe B was 3' end labelled at the BamHI site and used in S_1 assays, DNA fragments of approximately 297 and 310 nucleotides in length were protected (Figure 7, lane 3). If these putative 3' ends correspond to the 1.1 kb RNA detected by northern analysis, the 5' end of this RNA is located approximately 750 nucleotides upstream of fragment A.

Two transcripts (4.1 and 3.3 kb) were detected with probe C but not probes A or B using northern blots. S_1 nuclease treatment of RNA hybridized to a doubly 5' end labelled 0.88 kbp EcoRI probe in this region (Figure 6, lane 7), produced three bands of approximately 800, 484, and 465 bp, indicating three possible 5' ends upstream of psbD (Figure 6, lane 6). Two minor bands of about 680 and 630 bp were also seen. A minor band of approximately 124 bp was observed occasionally but was not investigated further. Two reverse transcription experiments were done to assay for 5' ends in this same region. First, primer extension from a 0.55 kbp ClaI-EcoRI probe (Figure 6, lane 4), labelled at the EcoRI site, revealed the presence of an RNA 5' end about 800 bp from the EcoRI site (Figure 6, lane 5). The faint band at 680 bp was also observed. Second, a 135 bp Sau96I-EcoRI reverse transcription probe (Figure 6, lane 2) labelled at the same EcoRI site revealed two RNA 5' ends approximately 800 and 484 bp from the EcoRI site (Figure 6, lane 3). Again, the minor 680 bp band could be observed. The 5' end 465 nucleotides from the EcoRI site was detected in the S_1 assay shown in Figure 6 (lane 6), but was not detected in the reverse transcription assay and therefore could be transcribed from the opposite DNA strand. To test this possibility, the ClaI-EcoRI probe 5' end labeled only at the EcoRI site was used in an S_1 nuclease protection assay. DNA fragments of about 484 and 465 nucleotides were protected from S_1 nuclease (data not shown), suggesting that the 465 bp band is an S_1 nuclease artifact. The faint S_1 nuclease-resistant band of 630 bp did not match the Northern and reverse transcription data, and was therefore not investigated further.

Based on the S_1 and reverse transcription assays there appeared to be three RNAs transcribed toward psbD which had 5' ends mapping about 800 bp, 484 bp and possibly 680 bp away from the EcoRI site in fragment C. This would localize two RNA 5' ends in fragment B and one in fragment C. If this assignment were correct RNAs of at least 800 and 680 nucleotides should be observed in northern blots probed with fragment B but not with fragment A. In fact, the only difference in the northerns was the presence of RNAs of less than 0.1 kb which

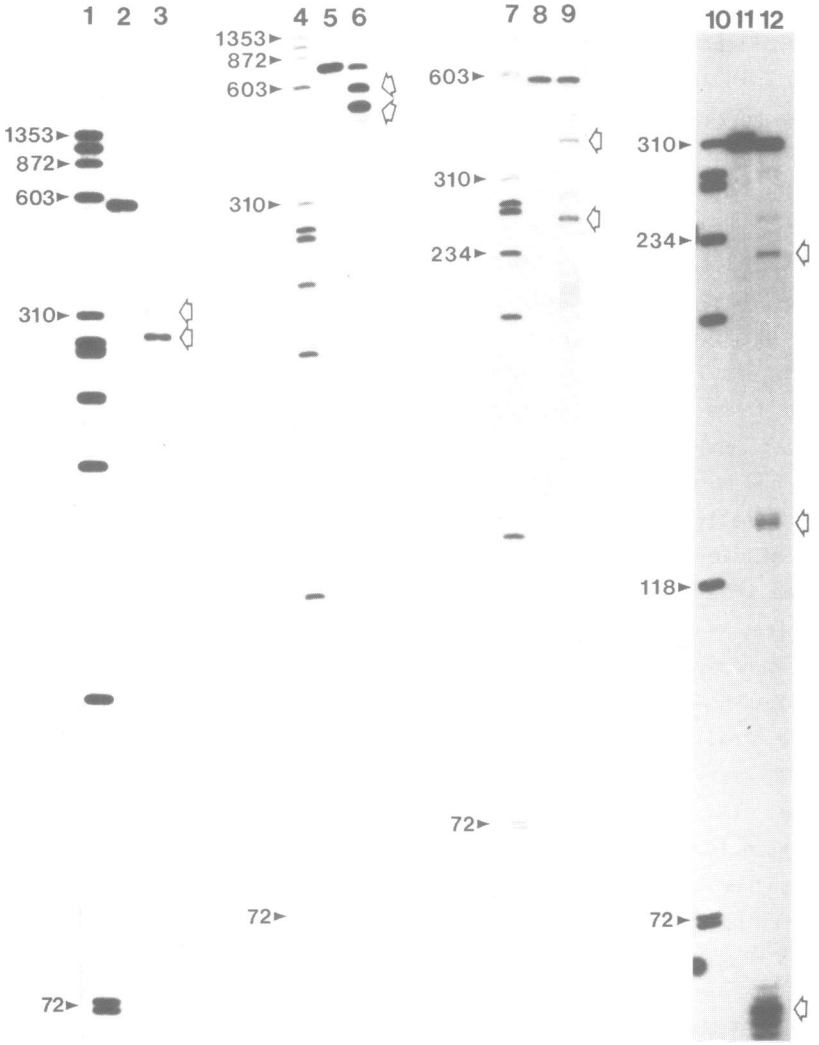

Figure 7: 3' termini of the psbD-psbC transcripts in 4.5 day-old barley etioplasts. ϕX174/HaeIII molecular weight markers were run in lanes 1, 4, 7 and 10. The open arrows next to lanes 3, 6, 9 and 12 indicate the bands which appeared after S_1 nuclease treatment. Lane 2 shows the 0.5 kbp BamHI-ClaI probe, 3' end-labelled at the BamHI site. The two corresponding protected fragments (297 bp and 310 bp) are shown in lane 3. Lane 6 has two major protected fragments which mapped to 560 and 657 bp downstream from the labelled BamHI site of the 0.74 kbp BamHI-XbaI probe (lane 5). Two fragments (395 and 263 bp) were seen in lane 9 with the 0.57 kbp EcoRI-DdeI probe labelled at the EcoRI site in lane 8. The S_1 nuclease protection assay with the 0.31 kbp HaeIII-SalI probe in lane 11 yielded three major bands 69, 123 and 214 bp upstream of the labelled SalI site. The latter two bands are surrounded by some minor bands.

hybridize to fragment B. Since the results from the northern blots, the S_1 nuclease mapping experiments and the primer extension analysis do not match in this case, the apparent 5' end signals within fragment B probably are not due to an RNA terminus. In contrast, the results from S_1 nuclease, primer extension and northern analysis all indicated the presence of an RNA 5' terminus 484 nucleotides from the EcoRI site in fragment C. The results also indicate that this position marks the 5' terminus of the 4.1 and 3.3 kb RNAs which hybridize to fragment C.

Northern analysis showed that two RNAs (2.6 and 1.7 kb) hybridized to DNA fragment D but not DNA fragments A, B or C (Figure 5). This prompted us to analyze this DNA region for 5' and 3' RNA termini. Unfortunately, analysis of this region was difficult; many putative RNA termini determined by S_1 protection assays did not coincide with those seen in primer extension assays and vice versa. For example, S_1 analysis using a 1.83 kbp AccI-HindIII probe, which overlaps fragment D, revealed the presence of several putative RNA termini (Figure 6, lane 10). Primer extension from a 375 bp HinfI-HindIII DNA, 5' end labelled at the HindIII site, also revealed several putative 5' ends (Figure 6, lane 13). However, only one minor band of approximately 995 nucleotides comigrated with a minor band protected in the S_1 protection assay (Figure 6, lanes 10 and 13). Several intense bands smaller than the 995 bp band in lane 13 may indicate steric hindrance of the reverse transcriptase due to possible secondary structures in the RNA. Since our result was consistent from experiment to experiment and with the northern analysis, we tentatively assign this putative 5' terminus to the 2.6 and 1.7 kb transcripts, although further analysis clearly is required to verify this designation.

Several clusters of 3' ends were found downstream of the psbC sequence. From the northerns it appeared that three transcripts of 4.8, 3.3, and 1.7 kb all had 3' ends within a 0.44 kbp PstI-BamHI fragment. S_1 nuclease protection with a 0.74 kbp BamHI-XbaI probe (3' end labelled at the BamHI site, Figure 7, lane 5) revealed the presence of two 3' ends about 657 and 560 bp downstream from the BamHI site (Figure 7, lane 6).

Based on the northern results we did not expect to locate 5' ends of RNAs greater than 100 bp in regions of DNA fragments G, H, or I (Figure 5). However, a putative 5' end was observed between the two 3' ends described above in DNA fragment H. This putative 5' end was located approximately 594 bp from the EcoRI site of a 0.83 kbp PstI-EcoRI probe by S_1 protection. Primer extension from a 391 bp BamHI-EcoRI probe confirmed the S_1 protection result (Figure 6, lanes 15-18). However, since the northern analysis did not verify the S_1 pro-

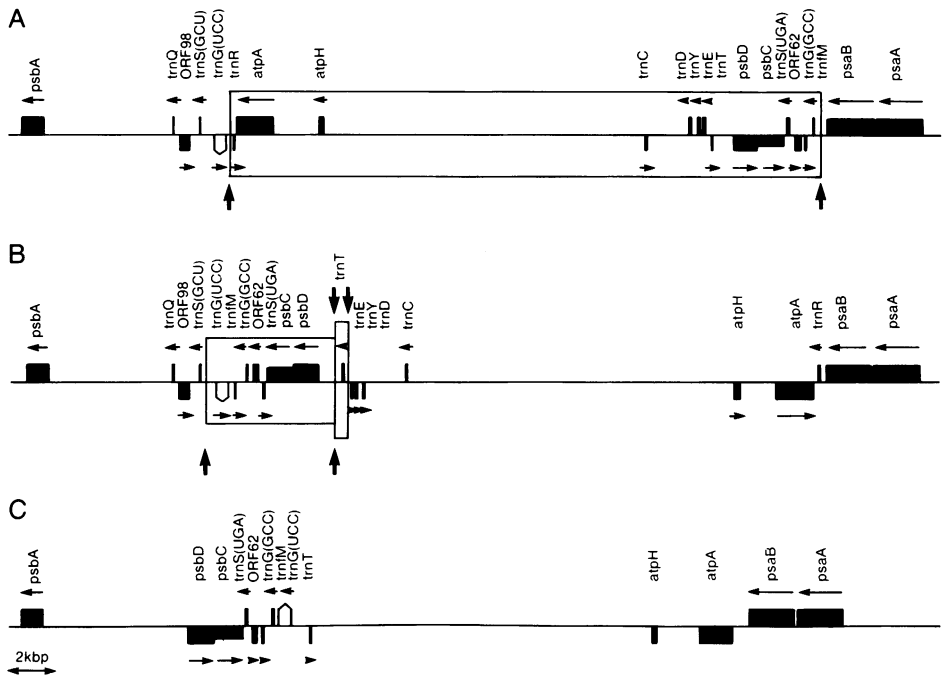

Figure 8: Possible evolutionary rearrangement scheme for the chloroplast DNA region containing psbD-psbC and psaA-psaB (42) A: map of this DNA region in spinach and/or tobacco. Solid boxes denote sequenced tRNA genes and mapped or sequenced structural genes (40 and references therein, 19,45,48-52) where known, thin horizontal arrows mark the direction of transcription. B: map of a hypothetical intermediate obtained by inversion of a 28 kbp segment of spinach/tobacco DNA marked by thick arrows and a large open box in panel A. C: map of the equivalent DNA region in barley, showing the gene organization possibly obtained by the two inversions marked by thick arrows and open boxes in panel B. Again, solid boxes denote mapped or sequenced structural genes (15,39,53 and this paper). The direction of transcription is indicated by thin horizontal arrows where known.

tection and primer extension results, the putative 5' end may be an artifact. It is possible that RNA secondary structure within the anti-sense trnS sequence within fragment H would cause such an artifact.

Northern analysis showed that three RNAs (5.7, 4.1 and 2.6 kb) hybridized to DNA fragment J but not K (Figure 5). S_1 nuclease mapping in this region using a 0.57 kbp EcoRI-DdeI probe labelled at the EcoRI site revealed the presence of two 3' termini located about 395 and 263 bp downstream of the EcoRI site (Figure 7, lanes 8 and 9). These RNA termini probably correspond to the 3' ends of the 5.7, 4.1, and 2.6 kb RNAs which terminate in this region according

to northern analysis. It should be noted that the two 3' ends described above are located near the trnG(GCC) sequence and that an RNA of tRNA size is detected in northern blots of this region (Figure 5). Therefore it is possible that 3' ends mapped in this region arise from RNA processing during tRNA synthesis.

Finally, another cluster of RNA 3' ends was detected with a 0.31 kbp HaeIII-SalI probe labelled at the SalI site (Figure 7, lane 11). The RNA termini were located about 69, 123 and 214 bp from the SalI site (Figure 7, lane 12) and this location matched one terminus of a 1.0 kb transcript thought to be a precursor of the split trnG(UCC) (15). The mapped positions of the 5.7, 4.8, 4.1, 3.3, 2.6, 1.7, 1.1 and 1.0 kb transcripts are shown in the top of Figure 5.

DISCUSSION

Location of psaA-psaB and psbD-psbC on the barley Ct genome.

The barley chloroplast genome is 135 kbp in size and has an inverted repeat of 21 kbp (39). The psaA-psaB and psbD-psbC genes were mapped in the large single copy region as shown in Figures 1B and 4B. The organization of each gene cluster in barley is similar to that in other higher plants such as pea, spinach, tobacco and maize (see Figure 8). However, the distance between the two clusters and their relative orientation varies in different plants. Three patterns have been reported. In pea chloroplast DNA, less than 5 kbp separates the psbD-psbC and psaA-psaB gene clusters, which are encoded on opposite strands and are transcribed divergently (25). In spinach and tobacco the two gene clusters are also within 5 kbp although the genes are transcribed toward each other (19, 40). In wheat, barley and maize the psbD-psbC and psaA-psaB genes are also transcribed towards each other, but are separated by approximately 25 kbp (41, 17,18,34,this paper). Our results are consistent with a DNA rearrangement scheme which has been proposed (42,43, and Figure 8). According to this scheme, a 28 kbp inversion of spinach or tobacco-like sequences, including the psbD-psbC and ORF62 genes brought the psbD-psbC and psaA-psaB gene clusters in close proximity in spinach and tobacco, in contrast to barley, maize and wheat. The resulting hypothetical intermediate is shown in Figure 8B. Quigley and Weil (42) have noted a 119 bp repeat at the trnfM-trnG(UCC) junction in wheat which suggests that this DNA region may have been involved in a rearrangement event. According to the published DNA sequence (15), this duplication is also present in barley. To explain the convergent orientation of the psbD-psbC and psaA-psaB gene clusters in barley, maize and wheat, it has been proposed (42,43) that the first inversion was followed by a second inversion, involving a 5-6 kbp DNA region around the psbD-psbC sequences (Figure 8B). Since no trnS sequence can

be found at the trnG(UCC)-trnT junction in barley (Figure 8C), the second inversion may have excluded the trnS(GCU) gene upstream of trnG(UCC) (Figure 8B). Also, our northern analysis indicates that a tRNA-size transcript originates from the DNA region upstream of psbD in 4.5 day-old, dark-grown barley. It is conceivable that a 1.1 kb barley transcript, which was shown to terminate at the putative tRNA sequence, corresponds to the ORF98 gene in tobacco (see Figure 8A). Finally, a third inversion (42) around the trnT gene in the tRNA cluster upstream of psbD in spinach and tobacco may explain its reversed transcriptional direction in wheat (43) and barley (15).

Location of transcripts which hybridize to psaA-psaB and psbD-psbC.

Northern analysis revealed the presence of a 5.3 kb transcript which hybridizes to psaA and psaB (Figure 2 and ref. 4,22). A transcript of similar size has been found to hybridize to psaA-psaB sequences in pea and spinach chloroplast RNA (21,25). In contrast, chloroplast RNAs of several sizes hybridize to psaA-psaB northern probes in maize (17). Both the psaA and psaB sequences in pea (44) and maize (18) are preceded by possible ribosome binding sites. Putative -10 and -35 promoter sequences have been noted upstream of psaA but not psaB in maize (18). S_1 nuclease analysis showed that the barley psaA-psaB RNA actually may consist of two transcripts differing by about 26 nucleotides at the 3'end. Judging from the autoradiogram in Figure 3B, the two mRNAs are present in roughly equal amounts. The functional significance of the psaA-psaB transcript heterogeneity in barley etioplasts remains to be elucidated.

A previous study by Oliver and Poulson (15) showed that up to nine plastid RNAs hybridized to a probe containing the carboxy-terminus of psbC. Our northern analysis of the psbD-psbC region revealed that at least 6 RNAs larger than 1 kb plus several RNAs smaller than 0.1 kb hybridize to barley psbD-psbC. Two of these transcripts (5.7 and 4.8 kb) had 5' ends approximately 750 bp upstream of the BamHI-BamHI DNA region investigated. These transcripts contained psbC and psbD sequences and terminated about 1175 bp and 175 bp downstream of psbC respectively. A second set of two transcripts (4.1 and 3.3 kb) had a 5' end approximately 460 bp upstream of psbD. These transcripts also contained psbD and psbC sequences and like the 5.7 and 4.8 kb transcripts terminated approximately 1175 bp and 175 bp from psbC respectively. Finally, a third set of two transcripts (2.6 and 1.7 kb) were tentatively assigned a 5' end approximately 1190 bp downstream from the start of the psbD gene, and again terminated approximately 1175 bp and 175 bp downstream of psbC respectively. At present it is not known if the 2.6 and 1.7 kb transcripts contain any psbD sequences, but it

is clear that these transcripts are potentially translatable to produce the psbC gene product without influence from translation of psbD.

The presence of multiple transcripts which hybridize to the psbD-psbC genes could arise from multiple sites of transcription initiation and termination and/or from RNA processing. Although putative promoter sequences have been noted at the 5' end of psbD in spinach (45,19) and pea (36), no experimental evidence indicates that these sequences are functional.

Two of the 5' ends identified by S_1 nuclease protection and primer extension techniques did not match the results obtained from the northern blots, and were therefore considered to be artifacts. Both 5' ends were located in DNA regions which contain genes encoding tRNAs. One of the 5' ends was located about 25 bp downstream of the 3' end assigned to the 1.1 kb transcript. This 5' end is within a 0.32 kbp EcoRI-ClaI region upstream of psbD, which hybridized to a putative tRNA sequence. The second 5' end was mapped within the trnS(UGA) gene. The trnS(UGA) gene and the psbD-psbC genes are encoded on opposite ctDNA strands. Therefore the antisense tRNA sequence is part of the longer transcripts. RNA of tRNA-size hybridized to the double-stranded probe containing the trnS(UGA) sequence, suggesting that the trnS(UGA) could be expressed. It is tempting to speculate that the sense and antisense tRNA sequences within the longer transcripts produce some secondary structure which could have caused the reverse transcriptase and S_1 nuclease artifacts. Also, the fact that all 3' end clusters mapped close to tRNAs or tRNA-size sequences may be of some significance. In HeLa cell mitochondria, for example, tRNA genes separate a majority of structural genes (for review see 46,47). After co-transcription, these tRNAs are processed from the primary transcripts to yield mRNAs and tRNAs. Processing has also been reported around anti-sense tRNA sequences (46). Alternatively, the regions of dyad symmetry around which several 3' end clusters were mapped may act as procaryotic-like transcription terminators. For instance, the first cluster of 3' ends found in the barley psbD-psbC region lies around the trnS(UGA) gene, in the proximity of an 11 bp inverted repeat (15, positions 540-550 and 558-568). Similar repeats between the psbC stop codon and the trnS(UGA) sequences have also been reported in pea (21 bp, 37) and spinach (11 bp, 19). Other regions of symmetry around which 3' end clusters were identified include the sequences between the trnG(GCC) and trnfM genes, which, like the psbC and trnS(UGA) genes, are encoded on opposite DNA strands. Similar mutually exclusive hairpin structures were first proposed in the equivalent region of wheat ctDNA (42).

Relationship between psaA-psaB, psbD-psbC transcript heterogeneity and translation.

Translation of the psaA-psaB gene products is not observed in plastids of dark-grown plants even though this paper and previous work have shown that psaA-psaB transcripts are present in these plastids (4). Northern analysis of barley RNA which hybridizes to psaA-psaB DNA sequences from dark-grown and illuminated plants showed no differences, although fine maps of the 5' and 3' ends of the RNAs from illuminated plants have not been analyzed. This indicates that psaA-psaB translation can be activated upon illumination of dark-grown plants without large changes in the RNA population.

In dark-grown barley the psbC gene product is not synthesized whereas a protein which comigrates with the psbD gene product is synthesized at high rates (38). In spinach (19,45) and pea (37) the psbD and psbC open reading frames overlap. Overlapping open reading frames suggest possible translational regulation of the two adjacent genes. Our results indicate that in dark-grown barley, the psbD gene product is always translated from transcripts which also contain psbC sequences. In contrast, the psbC gene product could be translated from transcripts which do not contain the entire psbD gene sequence. This may allow the stoichiometry of psbD and psbC gene product translation to vary in dark-grown vs illuminated plants or during plastid biogenesis. We have also noted that two new transcripts which hybridize to psbD-psbC accumulate when dark-grown plants are illuminated (Gamble, Berends and Mullet, unpublished observation). The specific nature of these transcripts will be discussed in a future publication.

ACKNOWLEDGEMENTS

We would like to thank Richard Oliver, Carsten Poulsen and Diter von Wettstein for providing us with the clone pHvC35, which contains the 4.3 kbp HindIII fragment of barley ctDNA described in the text. This research was supported by NIH grant GM 37987-01 and by the Texas Agricultural Experiment Station.

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