Two small open reading frames are co-transcribed with the pea chloroplast genes for the polypeptides of cytochrome b-559

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Summary. The genes encoding the 9 kDa and 4 kDa polypeptides of cytochrome b-559 have been located in pea chloroplast DNA by coupled transcription-translation of cloned restriction fragments of chloroplast DNA in a cell-free extract of Escherichia coli and by nucleotide sequence analysis. The genes (psbE and psbF) are located approximately 1.0 kbp downstream of the gene for cytochrome f and are transcribed in the opposite direction, similar to the arrangement in the chloroplast genomes of other higher plants. Nucleotide sequence analysis of this region revealed four open reading frames encoding hydrophobic proteins of 83 (psbE), 39 (psbF), 38 and 40 amino acid residues, which are co-transcribed as a single major RNA of 1.1 kb. The 5' and 3' ends of this RNA have been located by primer extension and S 1 nuclease mapping. The 5' end of the RNA is located 140 bp upstream of the initiating ATG codon of psbE and is preceded by typical chloroplast promoter sequences. The 3' end of the RNA is located approximately 515 bp downstream of the TAA stop codon of psbF close to a stable stem-loop structure.

Key words: Chloroplast DNA – Cytochrome b-559 – Open reading frame – Transcription

Introduction

Cytochrome b-559 is an integral part of photosystem II in higher plants and is present in all oxygen-evolving photosystem II preparations (Berthold et al. 1981; Nanba and Satoh 1987). Photooxidation of cytochrome b-559 occurs under conditions when oxygen evolution is inhibited (Floyd et al. 1971; Velthuys 1981) and it has been suggested that cytochrome b-559 may act as an electron donor to P680 under these conditions, thus preventing oxidative damage to the chloroplast (Bendall 1982). Cytochrome b-559 can also be photoreduced in a reaction inhibited by herbicides acting at the Qb site suggesting that cytochrome b-559 is reduced by the plastoquinone pool (Tsujimoto and Arnon 1985). The possibility that cytochrome b-559 acts to cycle electrons around photosystem II has been coupled to a suggestion that it acts as a proton pump (Butler and Matsuda 1983). However the physiological function of cytochrome b-559, particularly its role in oxygen evolution, remains obscure.

Cytochrome b-559 preparations contain apparently stoichiometric amounts of two polypeptides of 9 kDa and 5 kDa, whose N-terminal amino acid sequences have been determined (Widger et al. 1984, 1985). These polypeptides are the products of two genes, psbE and psbF, which were first located in the spinach chloroplast genome (Herrmann et al. 1984). A similar arrangement of the genes was subsequently reported for a wide range of photosynthetic organisms including wheat (Hird et al. 1986), Oenothera and tobacco (Carrillo et al. 1986), Marchantia polymorpha (Ohya- ma et al. 1986), barley (Krupinska and Berry-Lowe 1988), Synechocystis 6803 (Pakrasi et al. 1988), Cyanophora paradoxa (Cantrell and Bryant 1988) and Euglena gracilis (Cushman et al. 1988). Each of the two gene products contains a single histidine residue located in a hydrophobic region of the polypeptide, which has been suggested to span the lipid bilayer (Herrmann et al. 1984). The haem in spinach cytochrome b-559 is coordinated to two histidine residues (Babcock et al. 1985) indicating that the native protein must consist of at least two polypeptides. Herrmann et al. (1984) put forward homodimeric and heterodimeric models for the structure of cytochrome b-559, but these models have not been investigated further.

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In spinach the two genes for polypeptides of cytochrome b-559 are transcribed into a single 1.1 kb RNA species (Westhoff et al. 1985). This is a relatively large transcript for two chloroplast genes whose coding sequences account for less than 400 bp. We have characterised the transcription of the region of pea chloroplast DNA containing the cytochrome b-559 genes and have determined the transcription initiation and termination sites. In pea the 1.1 kb transcript contains two small open reading frames in addition to the coding information for the 9 kDa and 4 kDa polypeptides of cytochrome b-559.

Materials and methods

Reagents. L-[35S]methionine and α[32P]dATP were obtained from Amersham International plc (Amersham, UK). Protein A-Sepharose was obtained from Sigma London Chemical Co. Ltd. (Poole, UK). Restriction endonucleases BglII, PstI, SacI and XhoI were obtained from Amersham International plc, ASp718 was from BRL (Greenock, UK) and RsFl from New England Biolabs (Bishops Stortford, UK). Reverse transcriptase was obtained from Roche Diagnostics (England Biolabs (Bishops Stortford, UK). Reverse transcriptase was obtained from Sigma London Chemical Co. Ltd. (Poole, UK). Protein A-Sepharose was obtained from Amersham International plc (Amersham, UK). Protein A-Sepharose was obtained from Sigma London Chemical Co. Ltd. (Poole, UK). Nuclease from BRL. T4 DNA ligase and S1 nuclease from Anglian Biotechnology Ltd (Colchester, UK) and S1 nuclease from E. coli. DNA ligase and E. coli DNA polymerase large fragment were obtained from Dr. T. Hunt, Biochemistry Department, Cambridge University.

Coupled transcription-translation. Coupled transcription-translation of the hybrid plasmid pPScP2 containing the 17.3 kbp PstI restriction fragment (P2) of pea chloroplast DNA was carried out as described previously (Willey et al. 1983) using a cell-free lysate from E. coli strain PR7 (Bottomley and Whitfeld 1979). [35S]Methionine-labelled products were identified by immunoprecipitation with antibodies to barley cytochrome b-559 (Koenig and Moiler 1982) and protein A-Sepharose (Howe et al. 1982). Polypeptides were analysed by electrophoresis in 15% polyacrylamide gels in the presence of SDS (Laemmli 1974) followed by fluorography (Bonner and Laskey 1974).

DNA sequence analysis. Restriction fragments of pPScP2 were isolated from agarose and polyacrylamide gels (Dretzen et al. 1981) and inserted into M13t130, tgl31, mp18 and mp19 cut with the appropriate restriction enzymes. Isolation of single-stranded DNA and sequencing by the dideoxynucleotide chain termination method were carried out as described by Sanger et al. (1980).

RNA methods and analysis of transcripts. Total RNA was prepared from 20 g of 7-day-old pea shoots as described by Koller et al. (1982). For Northern blots 30 µg RNA was denatured by glyoxalisation (McMaster and Carmichael 1977) as described by Koller et al. (1982). The RNA samples were electrophoresed in 1.1% agarose gels and transferred to nitrocellulose (BA85 Schleicher and Schull) according to Thomas (1980). The filters were prehybridised at 42°C for 16 h in 80% formamide, 0.4 M NaCl, 1 mM EDTA, 10 mM PIPES pH 6.4. The samples were denatured at 95°C for 3 min and the hybridisations were washed with four changes of 2X SSC, 0.1% SDS at 50°C for 1 h total and with two changes of 0.1X SSC, 0.1% SDS at 50°C for 15 min each.

Primer extension. The single-stranded clone containing the 4.3 kb BglII-PstI fragment from pPScP2 inserted in M13mp19 was labelled by second-strand synthesis and the BglII-SacI subfragment was isolated using the "prime-cut" method (Biggin et al. 1984). The labelled probe was ethanol precipitated with 10 μg and 25 μg total pea leaf RNA and the dried pellets were dissolved in 15 μl of formamide annealing buffer, 50% formamide, 0.5 M NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA (Biggin et al. 1984). The samples were denatured at 95°C for 3 min and annealed at 50°C for 1 h then at 42°C overnight. The DNA-RNA hybrids were ethanol precipitated and the dried pellets were dissolved in 20 µl of reverse transcriptase buffer (50 mM Tris-HCl pH 8.3, 6 mM MgCl₂, 40 mM KCl, 1 mM each dNTP) with 10 units of reverse transcriptase and incubated at 42°C for 45 min. Following phenol/chloroform extraction and ethanol precipitation, the dried pellets were dissolved in 4 µl 10 mM Tris-HCl, 1 mM EDTA pH 8.0 and 4 µl formamide with 0.06% bromophenol blue and 0.06% xylene cyanol FF. The samples were denatured by heating at 95°C for 3 min and analysed by electrophoresis on 6% acrylamide/8 M urea sequencing gels.

S1 nuclease analysis. The 1.1 kb ASp718-Ndel restriction fragment was labelled at the 5' ends using the Klenow fragment of DNA polymerase and α[32P]dATP (Maniatis et al. 1982) and a strand-specific probe was generated by digestion of the labelled fragment with XbaI. The labelled probe was ethanol precipitated with 10 μg and 25 μg total pea leaf RNA and the dried pellets dissolved in 20 μl of 80% formamide, 0.4 M NaCl, 1 mM EDTA, 10 mM PIPES pH 6.4. The samples were denatured at 95°C for 3 min and the hybridisations were washed with four changes of 0.1X SSC, 0.1% SDS at 50°C then at 42°C for 16 h. The hybrids were digested with S1 nuclease (120 units) for 30 min at 37°C as described by Bird et al. (1985) and the samples analysed on 6% acrylamide/8 M urea sequencing gels.

Results

Localisation and nucleotide sequence

The gene for the 9 kDa polypeptide of cytochrome b-559 was localised to the 17.3 kbp PstI (P2) fragment of pea chloroplast DNA by coupled transcription-translation in a cell-free extract of E. coli. The plasmid pPScP2, containing the 17.3 kbp PstI fragment in pBR322, directed the synthesis of a 9 kDa polypeptide which could be immunoprecipitated by antibodies to barley cytochrome b-559 (Fig. 1). This indicated that the gene for the 9 kDa polypeptide is located in the same region of the chloroplast genome as the gene for cytochrome f (Willey et al. 1983), as has been shown previously for spinach (Herrmann et al. 1984) and wheat (Hird et al. 1986). In these plants the genes for the 9 kDa and 4 kDa polypeptides are located approximately 1 kbp downstream of the gene for cytochrome f. Nucleotide sequencing of this region of pea chloroplast DNA, using the strategy shown in Fig. 2, revealed the presence of four open reading frames. The nucleotide sequence was measured with four changes of 2X SSC, 0.1% SDS at 25°C for 1 h total and with two changes of 0.1X SSC, 0.1% SDS at 50°C for 15 min each.