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An open reading frame encoding a putative haem-binding polypeptide is cotranscribed with the pea chloroplast gene for apocytochrome *f*

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Abstract

The nucleotide sequence of a 1 kbp region of pea chloroplast DNA upstream from the gene *petA* encoding apocytochrome *f* has been determined. An open reading frame of 231 codons (ORF231) encoding a putative membrane-spanning polypeptide is separated by 205 bp from the coding region of *petA*. The open reading frame is homologous to open reading frames located in a similar position with respect to *petA* in chloroplast DNA from *Marchantia polymorpha*, tobacco, rice, wheat and *Vicia faba*. The sequence around a conserved histidine residue in a putative membrane-spanning region of the polypeptide resembles sequences present in cytochrome *b* from chromaffin granules and neutrophil membranes, suggesting that the open reading frame may encode a haem-binding polypeptide, possibly a *b*-type cytochrome. Northern hybridisation analysis indicates the presence in pea chloroplasts of a complex pattern of transcripts containing ORF231. Large transcripts of 5.5 kb, 4.3 kb, 3.4 kb and 2.7 kb encode both ORF231 and apocytochrome *f*, indicating that ORF231 and *petA* are co-transcribed.

Introduction

The chloroplast genome of green plants appears to be concerned principally with the biogenesis of the photosynthetic apparatus. The determination of the complete nucleotide sequences of chloroplast DNA from a liverwort (*Marchantia polymorpha*) [30], tobacco [37] and rice [19] has provided detailed information on the potential coding capacity of the chloroplast genome. It encodes approximately 30 polypeptide components of the multisubunit complexes of ribulose 1,5-bisphosphate carboxylase, photosystems I and II, the cytochrome *bf* complex and ATP synthase. The presence of seven or more open reading frames encoding polypeptides homologous to subunits of mitochondrial NADH dehydrogenase [30, 37] suggests that it provides the genetic information for some of the subunits of a NADPH-plastoquinol oxido-reductase, which may be associated with the photosynthetic apparatus [13]. A further 60–70 chloroplast genes encode components of the chloroplast transcription and translation systems, which are present solely (?) to ensure the expression of the genes for components of the photosynthetic apparatus. The chloroplast genomes contain additional open reading frames to which no functions have yet been ascribed; it

seems highly likely that some of them will encode components of the photosynthesis or transcription-translation systems.

Although the photosynthetic apparatus of higher plants is fairly well characterised [14], the identification in the past year or so of several chloroplast genes encoding small polypeptides of photosystem II [22, 23, 29, 43, 44] and the cytochrome *bf* complex [15] indicates that further genes for photosynthesis components may be present in chloroplast DNA. Little, or nothing, is known about the constituent polypeptides of ion transport systems in the thylakoid membrane [18, 36, 38] or transporters in the chloroplast envelope [16]. Controversial components, such as the antimycin-sensitive component of cyclic electron flow [27, 28] and the low-potential form of cytochrome *b*-559 which is not associated with photosystem II [1–4], are uncharacterised genetically or at the protein level. Although the definite assignment of chloroplast genes for any of these photosynthetic functions requires a full characterisation, including amino acid sequencing, of the component polypeptides, some indication of the identity of polypeptides encoded by open reading frames may be provided by homology to proteins from other organisms.

We report that an open reading frame (ORF231) in pea chloroplast DNA cotranscribed with the *petA* gene for apocytochrome *f* encodes a putative membrane-spanning polypeptide with a short region of homology, around a conserved histidine residue, to the putative haem-binding regions of two cytochrome *b* polypeptides from chromaffin granules [32] and neutrophil membranes [31].

Materials and methods

Coupled transcription-translation

Coupled transcription-translation of the hybrid plasmid pPscB3a, containing the 3.3 kbp *Bgl* II fragment of pea chloroplast DNA in pBR322, and of restriction fragments derived from it, was carried out as described previously [47] using a cell-free lysate of *Escherichia coli* strain PR7 [7]. [^{35}S] Methionine-labelled polypeptides were separated by electrophoresis in 15% polyacrylamide gels in the presence of SDS [8] and detected by fluorography [6].

DNA sequence analysis

Restriction fragments of pPscB3a were isolated from agarose and polyacrylamide gels [11] and

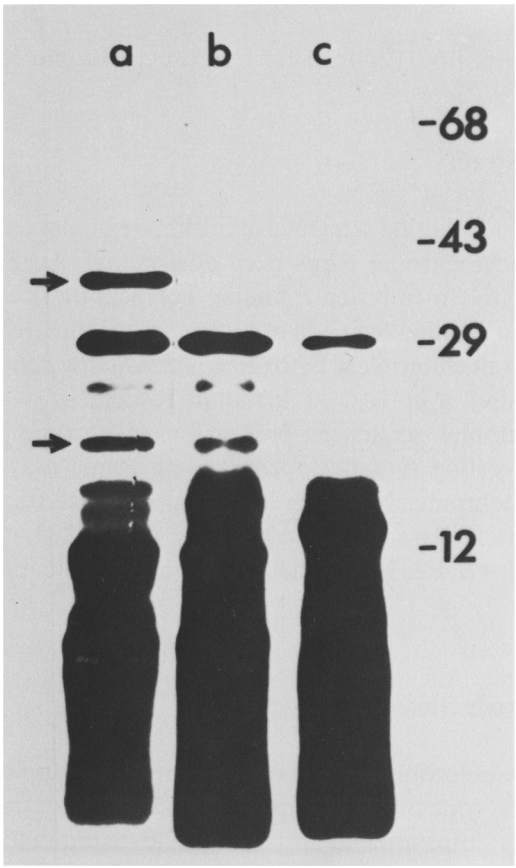

Fig. 1. Transcription-translation products of pPscB3a digested with *Bam* HI or *Hin*d III. [^{35}S] Methionine-labelled products were separated by SDS-polyacrylamide gel electrophoresis [8]. a, total products of pPscB3a; b, total products of *Bam* HI-digested pPscB3a; c, total products of *Hin*d III-digested pPscB3a. Preapocytochrome *f* (39 kDa) and the 20 kDa polypeptide are marked with arrows. Marker proteins were: bovine serum albumin (68 kDa), chicken ovalbumin (43 kDa), carbonic anhydrase (29 kDa) and horse cytochrome *c* (12 kDa).

inserted into M13mp8 and mp9 cut with 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.* [35].

RNA methods and analysis of transcripts

Total RNA was prepared from 20 g of 6-day-old pea shoots by the methods of Koller *et al.* [25] and Thompson *et al.* [40]. For northern blots, 30 μg RNA was denatured by glyoxylation [26], as described by Koller *et al.* [25]. The RNA samples were electrophoresed in 1.1% agarose gels and transferred to nitrocellulose (BA85 Schleicher and Schüll) according to Thomas [39]. Prehybridisation and hybridisation with ^{32}P-labelled probes obtained by second-strand synthesis of M13 single-stranded DNA using a pentadecamer sequencing primer [20] were carried out as described by Willey and Gray [48].

S1 nuclease analysis was carried out as described by Bird *et al.* [5] using total pea leaf RNA and 'prime-cut' probes produced by restriction enzyme digestion of double-stranded DNA synthesised from a 530 bp *Xho* I-*Hin*d III fragment inserted in M13mp8 or mp9. *Hin*d III was used to release the probe synthesised on the fragment in M13mp9, and *Eco* RI was used for the fragment in M13mp8.

Results

A 20 kDa polypeptide was detected as a product of coupled transcription-translation of a 3.3 kbp *Bgl* II fragment of pea chloroplast DNA in a cell-free system from *E. coli* during the localisation of the *petA* gene encoding apocytochrome *f* [47]. The gene for this 20 kDa polypeptide was localised by digestion of the plasmid pPscB3a, containing the 3.3 kbp *Bgl* II fragment inserted in the *Bam* HI site of pBR322, with restriction endo-

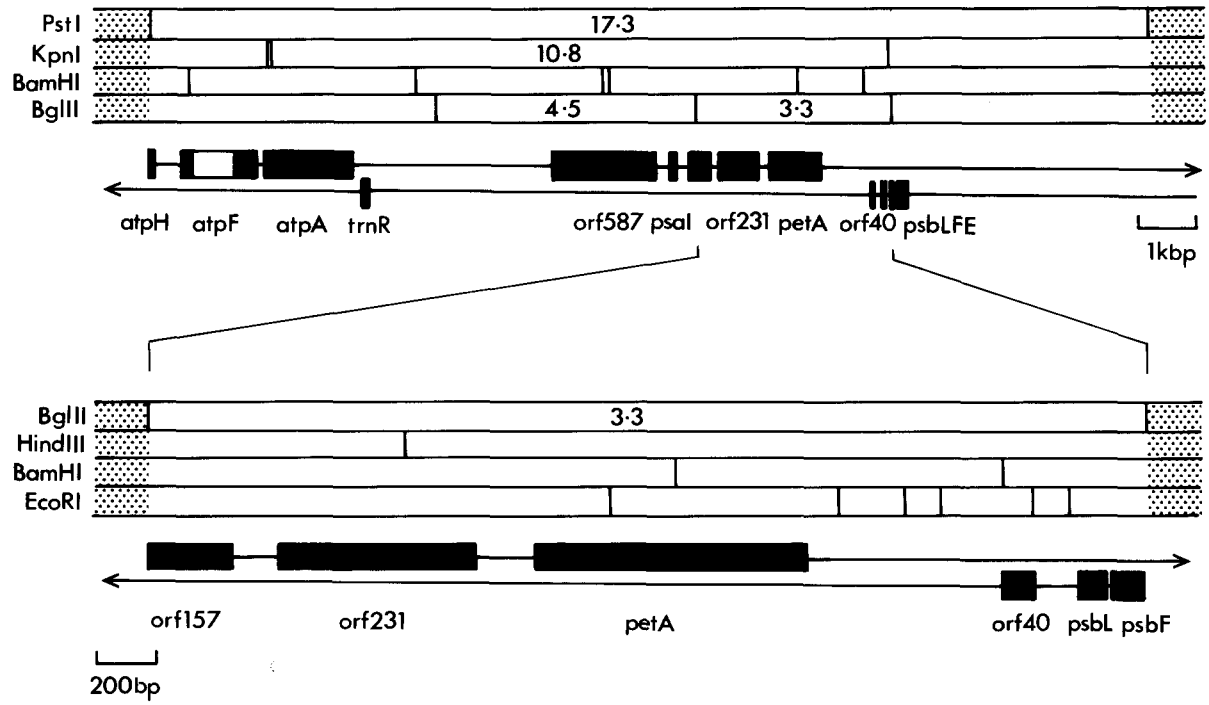

Fig. 2. Restriction map of a region of pea chloroplast DNA. The upper map shows restriction sites and genes in the 17.3 kbp *Pst* I fragment (P2) [47]. The regions between *trnR* and ORF587, and upstream of *psbE*, have not been sequenced fully and the location of genes in these regions is unknown. The lower map shows a more detailed map of the 3.3 kbp *Bgl* II fragment.

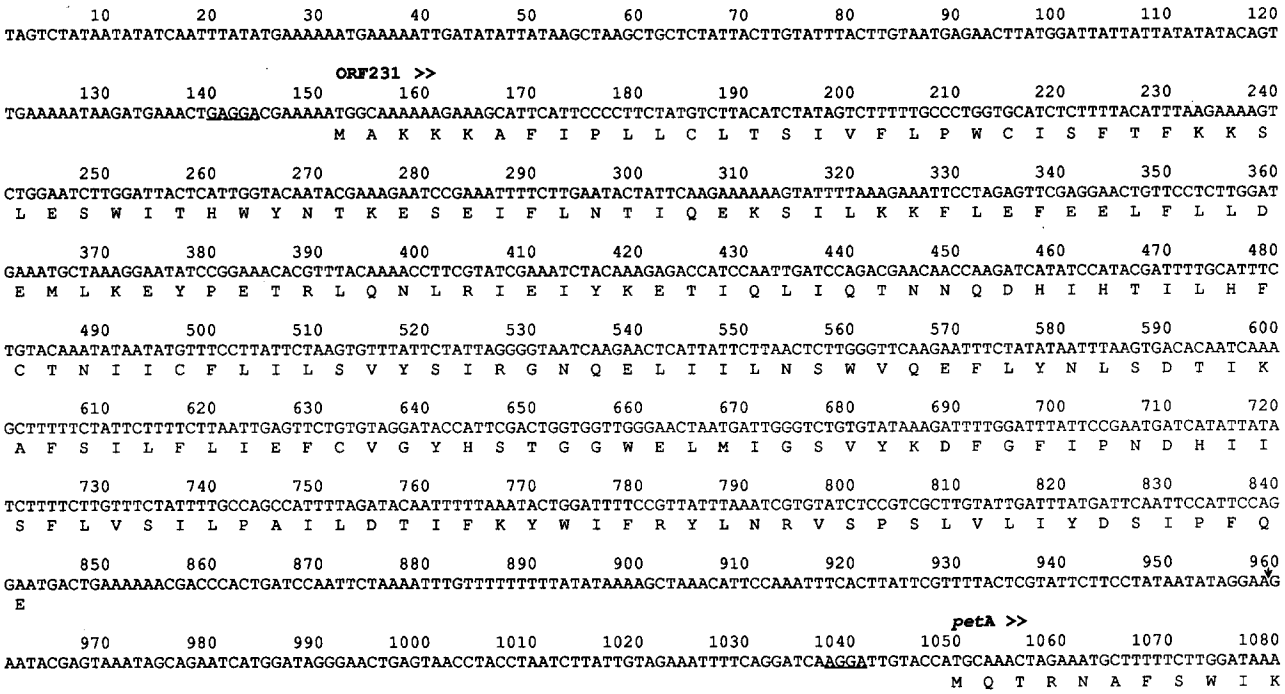

Fig. 3. Nucleotide sequence of a region of pea chloroplast DNA showing the location of ORF231. Putative ribosome-binding sites are underlined. An endonucleolytic cleavage site between ORF231 and *pet* A is marked with an arrow.

nucleases and incubation of the unseparated fragments in the *E. coli* transcription-translation system. Digestion with *Hind* III, but not *Bam* HI, resulted in the abolition of the synthesis of the 20 kDa polypeptide (Fig. 1), suggesting the presence of a *Hind* III site in or near the gene. Digestion with *Hind* III also resulted in the abolition of synthesis of the 39 kDa precursor form of the cytochrome *f* polypeptide, but it has been shown previously that the *Hind* III site is not located within *petA* [47]. A restriction map of this region of pea chloroplast DNA reveals a single *Hind* III site within the 3.3 kbp *Bgl* II fragment, approximately 450 bp upstream from the ATG translation initiation codon of *petA* (Fig. 2). The nucleotide sequence of the region upstream of *petA* was determined by the dideoxynucleotide chain-termination method on restriction fragments inserted into M13mp8 and mp9. An open reading frame of 231 codons (ORF231) is located 205 bp upstream of the ATG translation initiation codon of *petA* (Fig. 3). A putative ribosome binding site, GAGGA, is located 7–11 bp upstream from the ATG translation initiation codon of ORF231. The deduced amino acid sequence is very similar to the products of open reading frames of 229–230 codons in tobacco, rice and wheat chloroplast DNA [19, 37, Willey and Gray, unpublished], to a partial sequence derived from *Vicia faba* chloroplast DNA [24] and to the *C*-terminal region of the product of an open reading frame of 434 codons in *Marchantia* chloroplast DNA [12, 30] (Fig. 4). The pea sequence contains 68% residues identical to the tobacco protein, 56–57% residues identical to the rice and wheat proteins and 45% residues identical to the *Marchantia* protein. The pea protein shares 78% identical residues with the *Vicia* protein over the 76 amino acid residues available for comparison. Only two (His-109 and His-165) of the six histidine residues in the product of pea ORF231 are conserved in the proteins from the other species, and they appear to be located in hydrophobic regions of the protein. Hydropathy analy-

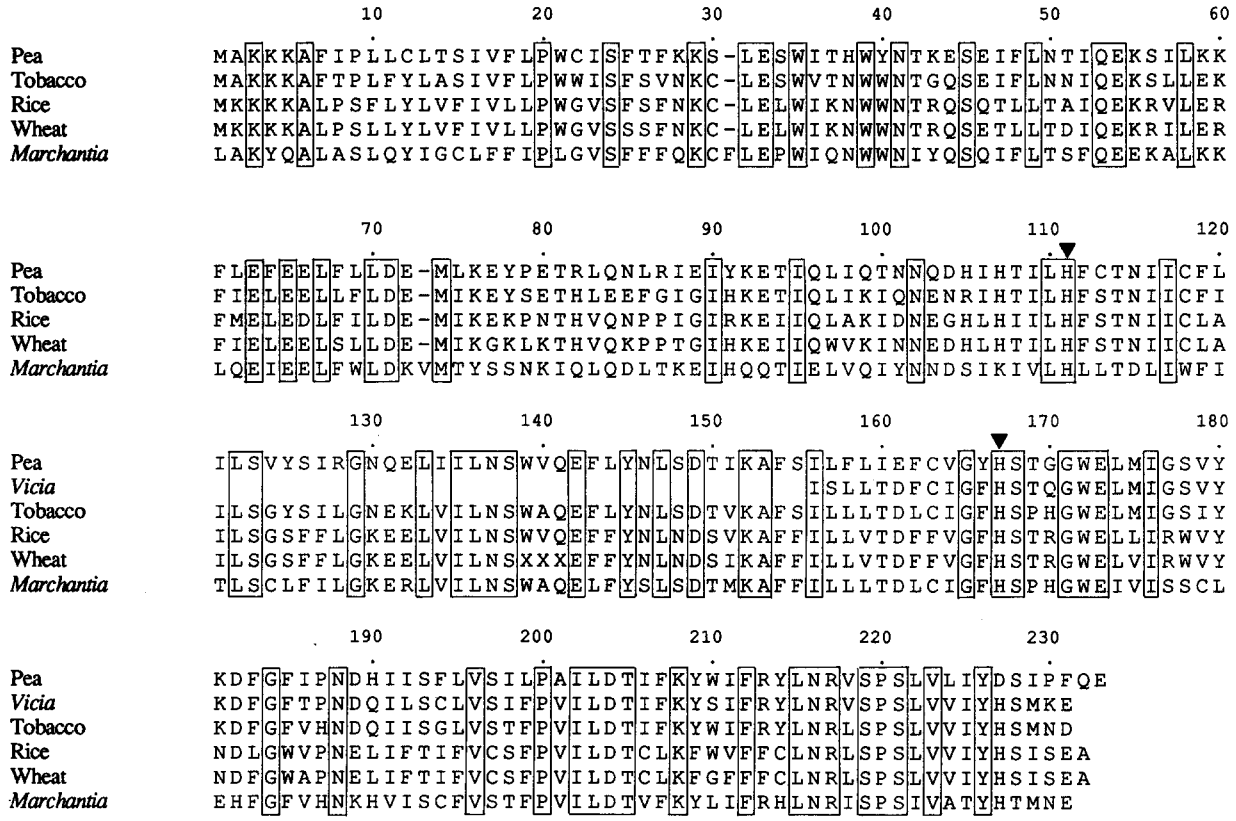

Fig. 4. Comparison of the amino acid sequence derived from ORF231 with the products of similar open reading frames from *Vicia faba* [24], tobacco [37], rice [19], wheat [Willey *et al.*, unpublished] and *Marchantia polymorpha* [12]. Identical residues are boxed. Conserved histidine residues are marked with arrowheads.

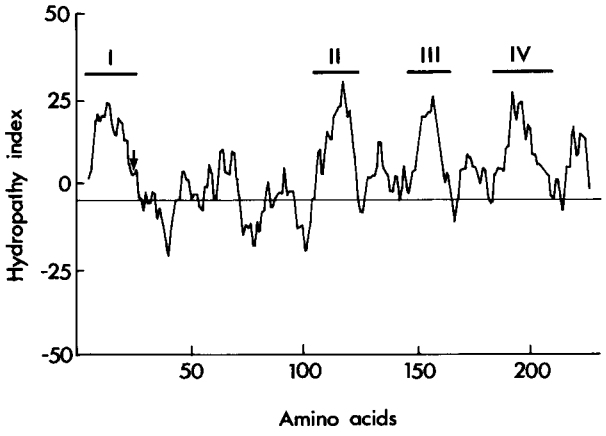

Fig. 5. Hydropathy analysis of the product of pea ORF231, using a span-length of 9 amino acid residues. Extended hydrophobic regions are marked with black bars. The putative cleavage site for signal peptidase is indicated with an arrow.

sis indicates four regions of the polypeptide which have a distinct hydrophobic character (Fig. 5). The region between residues 28 and 91 is relatively highly charged with 24 charged amino acid residues.

The ablation of preapocytochrome *f* synthesis by digestion of pPscB3a with *Hin*d III was previously taken to suggest the co-transcription of *petA* and the gene for the 20 kDa polypeptide [47]. Transcripts of ORF231 and *petA* in pea leaves were examined by hybridisation of gene-specific probes to northern blots of total pea leaf RNA, prepared by two different methods and fractionated by agarose gel electrophoresis. The 220 bp *Msp* I-*Hin*d III fragment of pea chloroplast DNA (nucleotides 379–599 in Fig. 3, corresponding to amino acid residues 77–150) was used as a probe for ORF231 and a 370 bp *Xho* I-

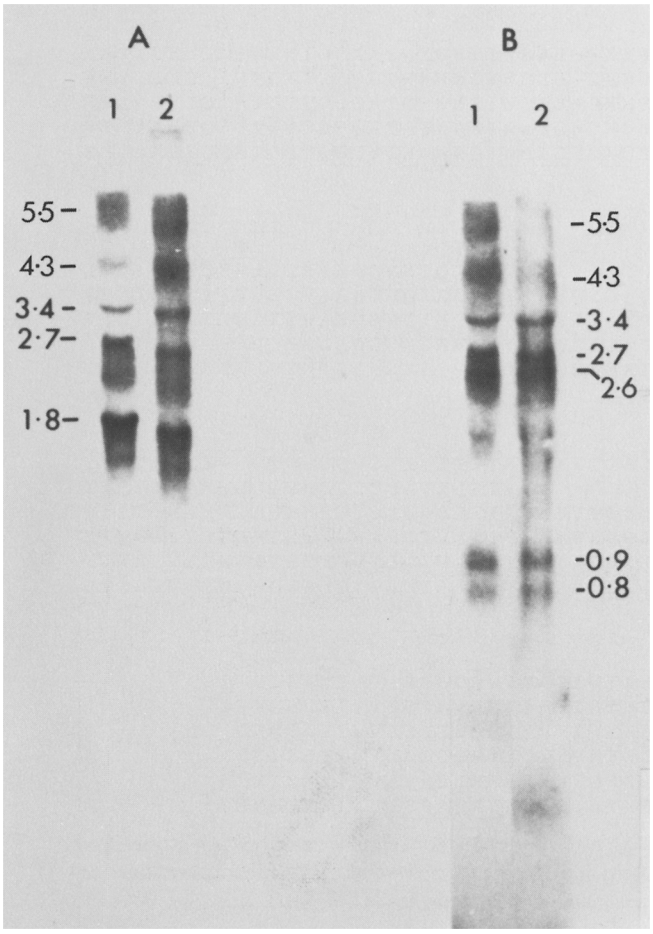

Fig. 6. Northern hybridisations of total pea leaf RNA prepared by the methods of 1, Thompson *et al.* [40], and 2, Koller *et al.* [25] and fractionated by agarose gel electrophoresis. The blots were probed with ^{32}P-labelled M13 clones containing A, the 370 bp *Bam* HI-*Xho* I fragment from *petA*, or B, the 220 bp *Hind* III-*Msp* I fragment from ORF231.

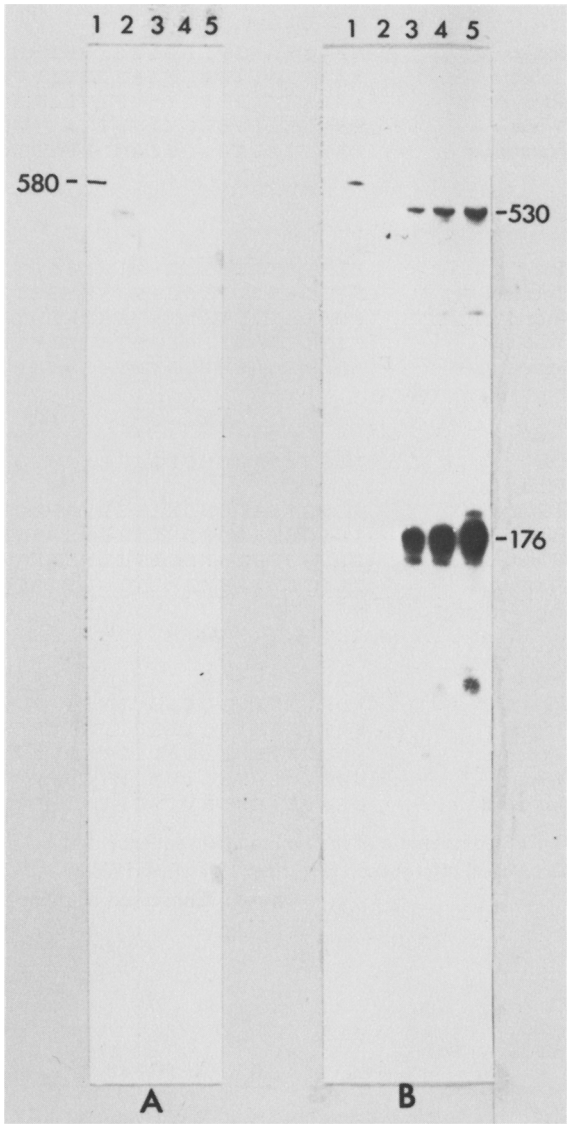

Fig. 7. S1 nuclease mapping to determine the location of the 5′ end of a transcript between ORF231 and *petA*. The 530 bp *Xho* I-*Hind* III fragment was inserted in A, M13mp8, or B, M13mp9, to generate sense and antisense 'prime-cut' single-stranded probes prepared by second-strand synthesis. The probes were hybridised with 0 μg (lanes 1 and 2), 10 μg (lane 3), 25 μg (lane 4) and 50 μg (lane 5) total pea leaf RNA. Samples 2–5 were treated with 200 units S1 nuclease for 30 min and the resistant fragments separated by electrophoresis on sequencing gels. The size of protected fragments was determined by reference to ^{32}P-labelled fragments of pBR322 cut with *Sau* 3AI.

Bam HI fragment, encoding amino acid residues 12–135 of mature cytochrome *f*, was used as a probe for *petA*. Complex patterns of hybridisation of both the ORF231 and *petA* probes were observed. The ORF231 probe hybridised with RNA species of 5.5 kb, 4.3 kb, 3.4 kb, 2.7 kb, 2.6 kb, 0.9 kb and 0.8 kb, with several areas of rather diffuse hybridisation (Fig. 6). The *petA* probe hybridised with RNA species of 5.5 kb, 4.3 kb, 3.4 kb, 2.7 kb and 1.8 kb (Fig. 6). This suggests that the larger transcripts of 5.5 kb, 4.3 kb, 3.4 kb and 2.7 kb contain the coding information of both ORF231 and *petA*. The transcripts of 0.9 kb and 0.8 kb specific for ORF231

and the 1.8 kb transcript specific for *petA* may be the result of processing the 2.7 kb transcript between ORF231 and *petA*. S1 nuclease protection experiments have shown that the 5′ end of the 1.8 kb transcript is located between ORF231 and *petA*. Pea leaf RNA protected a 176 bp fragment of a single-stranded 'prime-cut' probe containing the 530 bp *Xho* I-*Hin*d III fragment (Fig. 7). No protection from S1 nuclease digestion was obtained with the opposite strand (Fig. 7). The size of the protected fragment indicates that the 5′ end of the transcript is located near position 957 in Fig. 3. This must represent the 5′ end of the 1.8 kb transcript specific for *petA* because no smaller transcripts specific for *petA* were detected and all larger transcripts containing *petA* sequences also hybridised to the ORF231 probe indicating that their 5′ ends must be further upstream. S1 nuclease protection experiments designed to detect 3′ ends of transcripts within or downstream of the *petA* gene indicated that all transcripts containing *petA* sequences ended within the 211 bp *Eco* RI fragment downstream of the *petA* gene (data not shown). No 3′ ends of transcripts were detected within the *petA* coding sequence, indicating that all of the larger transcripts hybridising to ORF231 and *petA* probes must have their 5′ ends upstream of ORF231.

Discussion

ORF231 appears to encode a slightly acidic protein with four extended hydrophobic regions (see Fig. 5). Span II contains a region of homology to the putative haem-binding regions of the human neutrophil cytochrome *b* [31] and the cytochrome *b*-561 of bovine adrenal chromaffin granules [32] (Fig. 8). This region contains a conserved histidine residue (His-109) which is predicted to be located at the same position with respect to the membrane surface as the histidines acting as haem ligands in chloroplast cytochromes *b*-563 and *b*-559 [17, 46]. The residues surrounding this histidine residue, particularly in the *Marchantia* protein, are similar to those around the putative haem-binding histidine of the animal cytochromes

cytochrome *b*-561	107	V L H G L L H V F A	116
cytochrome *b*	92	V L H L L L S V P A	101
Marchantia	310	V L H L L T D L I W	319
tobacco	107	I L H F S T N I I C	116
wheat	107	I L H F S T N I I C	116
rice	107	I L H F S T N I I C	116
pea	107	I L H F C T N I I C	116

Fig. 8. Comparison of the region around a conserved histidine residue in the product of ORF231 with putative haem-binding regions of human neutrophil cytochrome *b* [31] and cytochrome *b*-561 of bovine adrenal chromaffin granules [32]. The corresponding sequences of the proteins from tobacco [37], rice [19], wheat [Willey and Gray, unpublished] and *Marchantia polymorpha* [12] are shown. Conserved residues are boxed.

(Fig. 8). This suggests that the ORF231 polypeptide may be a haem-binding polypeptide, possibly a *b*-type cytochrome.

There are two chloroplast cytochromes, the low-potential form of cytochrome *b*-559 [3] and cytochrome *b*-560 [34], which have not been characterised at the protein level. The existence of both of these cytochromes as native proteins is controversial. The low-potential form of cytochrome *b*-559 may be detected in freshly isolated chloroplast membranes [3, 4, 33], but it has been claimed to be an altered form of the high-potential form of cytochrome *b*-559 associated with photosystem II [9, 10]. A variety of treatments, including heat, solvents and sonication, is reported to lower the redox potential of the photosystem II-associated cytochrome *b*-559 [3, 42], but not to the redox potential of the low-potential form of cytochrome *b*-559 in freshly isolated chloroplasts [33]. The low-potential form of cytochrome *b*-559 is solubilised by digitonin treatment of thylakoid membranes, unlike the photosystem II-associated cytochrome *b*-559 [1]. It therefore seems possible that a separate and functionally distinct cytochrome *b*-559 exists in thylakoid membranes; however, the function of this cytochrome is not known. Cytochrome *b*-560 has been described only recently [34], and its function is also not known. The possibility that the ORF231 encodes the polypeptide chain of one of these cytochromes needs further investigation.

Examination of the deduced amino acid sequence of the ORF231 polypeptide suggests that

it may contain a cleavable presequence at the *N*-terminus. Application of von Heijne's rules [41] for the identification of signal sequence cleavage sites predicts cleavage between residues Ser-24 and Phe-25. This would indicate a 24-amino acid residue presequence with all the features normally ascribed to signal sequences: it contains positively charged residues (Lys-3, Lys-4 and Lys-5) near the *N*-terminus, a central hydrophobic region (residues 6–21), corresponding to hydrophobic span I in Fig. 5, and small uncharged residues (Ser-24 and Cys-22) at −1 and −3 from the cleavage site. If this region acts as a signal sequence it suggests that the *N*-terminus of the mature protein is located in the lumen of the thylakoid membrane system. The topology of the rest of the molecule is difficult to predict because of uncertainty about the number of membrane-spanning regions in the polypeptide. Although four hydrophobic regions are indicated by the hydropathy plot (Fig. 5), span I has been suggested to be part of the cleaved signal sequence and span IV appears to be too short (13 residues) to span the lipid bilayer without the inclusion of charged residues. Span II contains the histidine residue (His-109) suggested to be involved in haem binding and span III contains another conserved histidine residue (His-165) which might provide the sixth ligand for the haem Fe ion. However, span III also contains a glutamate residue (Glu-159) in the middle of the hydrophobic region, making predictions of the topology difficult. If the protein folds with two membrane-spanning regions, then both the *N*- and *C*-termini will be located in the thylakoid lumen, and the postulated haem group will be held on the lumenal side of the thylakoid membrane (Fig. 9).

The present study has demonstrated a complex pattern of transcripts from the region of pea chloroplast DNA containing ORF231 and *petA*. Woodbury *et al.* [50] have also reported complex patterns of transcripts from this region of pea chloroplast DNA. They reported transcripts as large as 6.2 kb and 5.2 kb from this region; these may correspond to the transcripts of 5.5 kb and 4.3 kb detected above. Our S1 nuclease protection experiments indicate that these transcripts

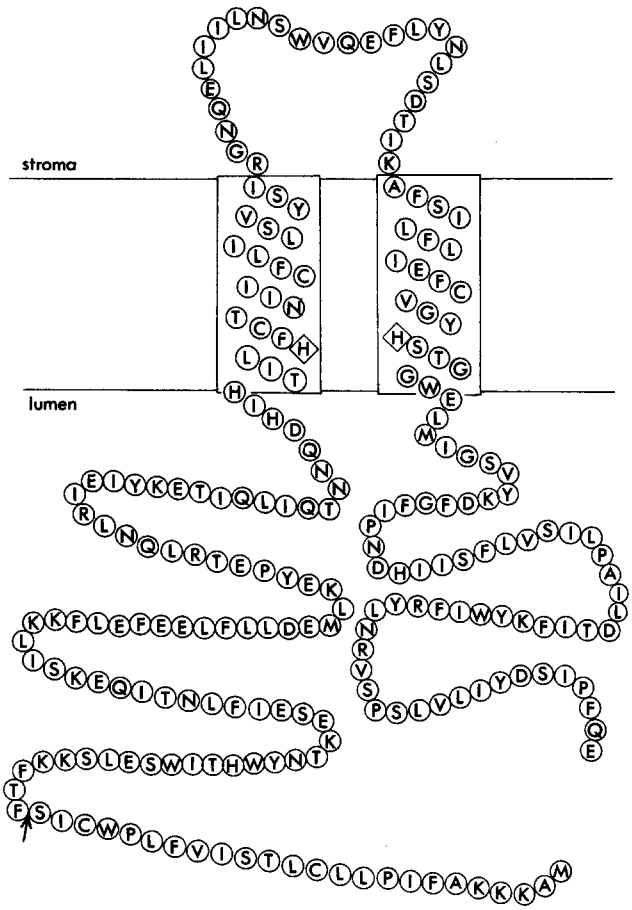

Fig. 9. Predicted topology of the product of ORF231 in the chloroplast thylakoid membrane. The conserved histidine residues are shown as diamonds. The putative cleavage site of signal peptidase is marked with an arrow.

must have their 3′ ends 400–600 bp downstream of the *petA* gene. The larger transcripts would therefore be predicted to contain sequences from ORF587 (*zpfA*), ORF40 (*psaI*), ORF157, ORF231 and *petA* (see Fig. 2). The smaller transcripts may arise by endonucleolytic processing of larger transcripts, as has been described for the *psbB-psbH-petB-petD* region [45], or by transcription initiation from additional promoters. Woodbury *et al.* [49] failed to detect transcripts initiating directly 5′ to *petA* by guanylyl transferase capping experiments *in vitro*, suggesting that processing of transcripts occurs in this region. The 5′ end of the 1.8 kb transcript of the *petA* gene was located within the sequence

AGGAAG which is present in a potential stem-loop structure between ORF231 and *petA* (see Fig. 3). This resembles the central region of the consensus sequence for endonucleolytic cleavage, UGGAAU, described by Westhoff and Herrmann [45], and detected as cleavage sites in transcripts of the pea *atpH* gene [21]. Cleavage of the 2.7 kb transcripts at this point would be predicted to produce the 1.8 kb transcript for *petA* and a 0.9 kb transcript for ORF231. A potential endonuclease recognition site, UGGAUU, is located at nucleotides 98–103 in Fig. 3, approximately 850 bp upstream. Further work is needed to define the ends of all the transcripts from this region.

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