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Detection and characterization of a plastid envelope DNA-binding protein which may anchor plastid nucleoids

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Chloroplast DNA (cpDNA) binds to the envelope membrane of actively dividing chloroplasts (plastids) in young pea leaves. South-western blotting was used to identify and characterize the protein involved in the binding of cpDNA to the envelope membrane. A 130 kDa protein in the inner chloroplast (plastid) envelope membrane binds specific sequences within the cpDNA. These included a 0.41 kbp sequence located upstream of the *psaAB* gene, a 0.57 kbp sequence located downstream of the *petA* gene and a 1.2 kbp sequence located within the *rpoC2* gene. The protein was detected in the envelope membrane of young pea leaves in which the cpDNA had been located by fluorescence microscopy at the chloroplast periphery, whereas it was undetectable in mature leaves. We therefore propose that the 130 kDa protein is involved in the binding of cpDNA to the envelope membrane, and named it plastid envelope DNA-binding protein.

Key words: chloroplast DNA/chloroplast development/pea/plastid envelope DNA-binding protein

Introduction

Since the replicon model was proposed by Jacob *et al.* (1963), the binding of DNA to membranes has been studied in various prokaryotes and eukaryotes. The best studied prokaryotes are *Escherichia coli* (Ogden *et al.*, 1988; Hwang and Kornberg, 1990; Landoulsi *et al.*, 1990) and *Bacillus subtilis* (Firshein, 1989). In eukaryotic cells, reversible association of chromosomal DNA to the nuclear envelope membrane has been demonstrated (Gerace and Burke, 1988; Pfaller *et al.*, 1991). The binding of mitochondrial DNA to the mitochondrial membranes has also been extensively studied (Nass, 1969; Shearman and Kalf, 1977; Kawano and Kuroiwa, 1985; Echeverria *et al.*, 1991).

Chloroplast DNA (cpDNA) is also known to bind to membranes (for a review, see Possingham and Lawrence, 1983), but the situation is complicated by the presence of two types of membrane in the chloroplast, namely the envelope membrane and the thylakoid membrane. Many electron microscopy studies have shown that cpDNA binds to the thylakoid membrane (Woodcock and Fernandez-Moran, 1968; Rose and Possingham, 1976; Liu and Rose, 1992), while Herrmann and Kowallik (1970) presented images of serial sections of young plastids of *Beta vulgaris* that showed binding of cpDNA to the envelope membrane. Observation of chloroplasts stained with DAPI (4',6-diamidino-2-phenylindole) using fluorescence microscopy provides a convenient way of locating cpDNA within the chloroplast. In some algae, the cpDNA is located in the stromal periphery (Coleman, 1979; Kuroiwa *et al.*, 1981), while in mature chloroplasts of higher plants, cpDNA is dispersed within the chloroplast (Kuroiwa *et al.*, 1981). Detailed studies in wheat (Miyamura *et al.*, 1986) and rice (Sodmergen *et al.*, 1989) have revealed that the amount, morphology and location of cpDNA are surprisingly dynamic: the cpDNA, which is concentrated at the center of the proplastid at the beginning of germination, is extensively amplified and becomes attached to the envelope membrane in young plastids. Then, as the chloroplast matures, the cpDNA becomes dispersed as small nucleoids within the stroma. The cpDNA seems to be attached to the thylakoid membrane in mature chloroplasts. The binding of cpDNA to the envelope appears to be involved in the amplification (replication and segregation) of cpDNA, while the binding of cpDNA to the thylakoid membrane appears to be involved in the expression of plastid genes that are necessary for photosynthesis.

Identification of the proteins involved in the binding of cpDNA to both types of membrane is an initial step towards elucidating the role of the binding of cpDNA to membranes. The present study aims at detecting and identifying the proteins that are involved in the binding of cpDNA to the envelope membrane. We have chosen pea leaves as the plant material, since pea is practically the only plant that can be used to obtain reasonable yields of envelope membranes from plants of different ages.

Results

Fluorescence microscopy

Protoplasts that were liberated from unopened leaves of young pea seedlings (6 or 7 days old) or from mature leaves of 14 day old plants were fixed and stained with DAPI and then examined by fluorescence microscopy (Figure 1). Chloroplast nucleoids (cp-nucleoids), which were seen as bright bluish white particles, were abundant and located mostly at the chloroplast periphery in young chloroplasts (Figure 1A, C and D). At this stage, the chloroplasts looked pale red, indicating that the chloroplasts contained only a small amount of chlorophyll. In mature leaves of 14 day old plants, however, chloroplast grana that emitted strong red fluorescence were highly developed, and the cp-nucleoids were dispersed as small fluorescent particles within interthylakoidal stroma (Figure 1B and E). Very few cp-nucleoids appeared near the envelope membrane. These results are essentially consistent with the results obtained in

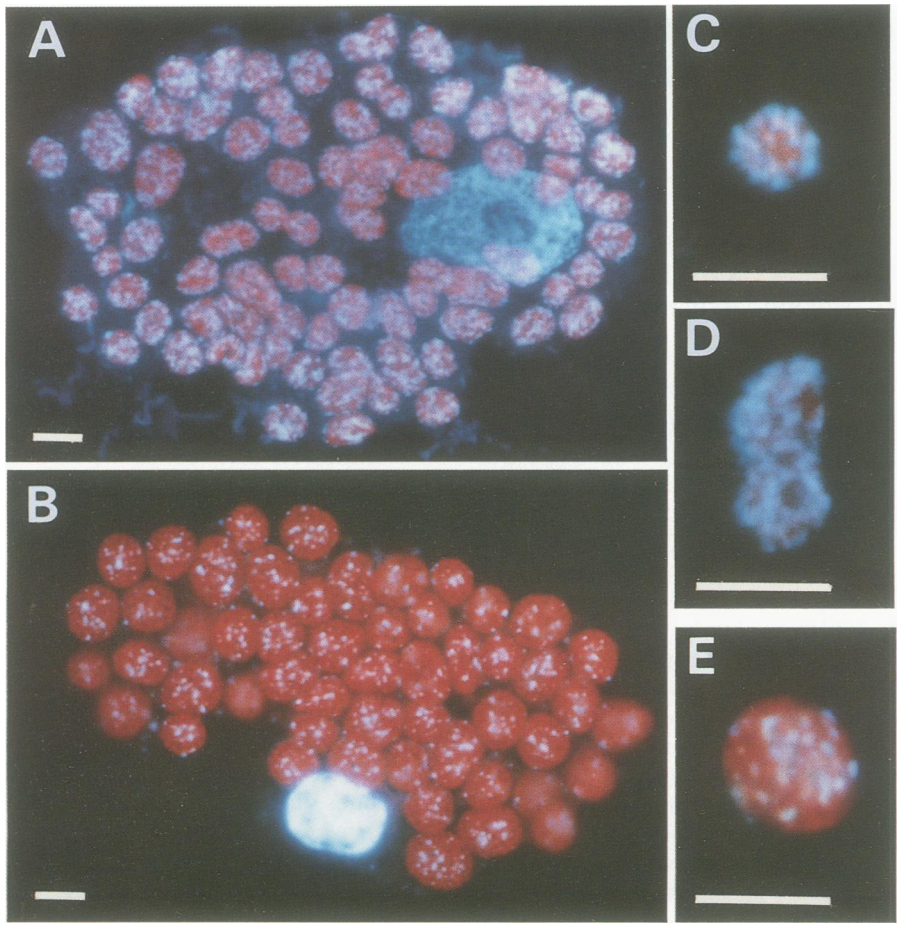

Fig. 1. Visual localization of plastid nucleoids in young and mature leaves of pea. Protoplasts were stained with DAPI and then examined by fluorescence microscopy after squashing. **A**, **C** and **D**, 6 day old plant; **B** and **E**, 14 day old plant. C–E are enlarged pictures of liberated single chloroplasts. Bars represent 5 μm.

wheat (Miyamura *et al.*, 1986) and rice (Sodmergen *et al.*, 1989). It should be noted that a large proportion of plastids in the young leaves appeared to be in the process of division (Figure 1A). In these dividing plastids, some of the cp-nucleoids are often aligned along a spiral (Figure 1D). It seems likely that the attachment of cp-nucleoids to the envelope membrane is a way of achieving a uniform distribution of cp-nucleoids along the surface of the chloroplast envelope and of ensuring an even partition of cp-nucleoids to daughter chloroplasts during division of the chloroplast.

South-western blotting

We made use of South-western blotting (Bowen *et al.*, 1980) to detect DNA-binding proteins in various fractions of chloroplasts from 7 day old pea leaves (Figure 2). In the initial experiments, purified cpDNA that had been cut with restriction endonucleases and end-labelled with ^{32}P by filling-in was used as a probe. The addition of calf thymus DNA to the binding mixture greatly reduced non-specific binding (compare Figure 2a and b). A 175 kDa band appeared in both the stroma fraction (lane S) and the envelope membrane fraction (lane E) (Figure 2b). Since the intensity of this band in the envelope membrane fraction diminished considerably after washing of the envelope membrane by centrifugation (not shown), the presence of the 175 kDa protein in the envelope membrane fraction was judged to

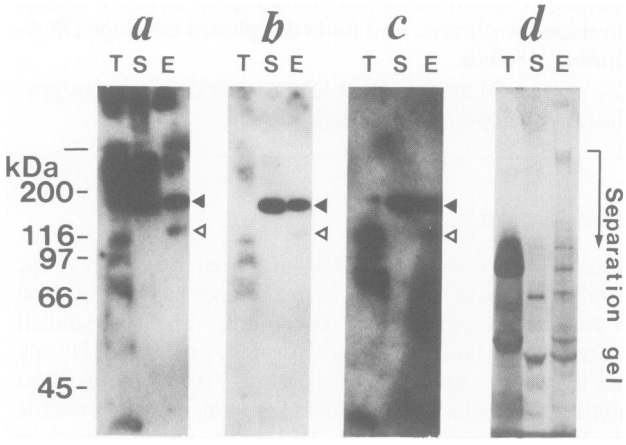

Fig. 2. South-western detection of DNA-binding proteins in various fractions of pea chloroplasts under different binding conditions. A mixture of *Bam*HI-digested cpDNA and *Hind*III-digested cpDNA was end-labelled by filling-in and used as a probe. Lane T, thylakoid membrane fraction (30 μg protein); lane S, stroma fraction (5 μg protein); lane E, envelope membrane fraction (10 μg protein). **a**, Calf-thymus DNA was omitted from the complete binding buffer (see Materials and methods); **b**, complete binding buffer; **c**, EDTA was replaced by 1 mM $ZnCl_2$ in the complete binding buffer. In each experiment, washing was done with the binding buffer that was used during the binding reaction and that did not contain radiolabelled probe. The bands corresponding to the 175 kDa and the 130 kDa proteins are indicated by closed and open arrowheads, respectively. **d**, Staining with Aurodye.

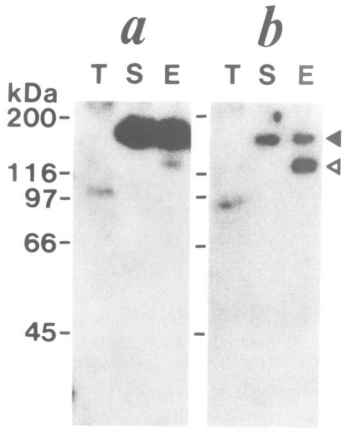

Fig. 3. Specific binding of the 3.9 kbp *Xho*I fragment for a 130 kDa DNA-binding protein in the envelope membrane fraction. Experimental conditions were similar to those in Figure 2b except for the probes used: **a**, total cpDNA (a mixture of *Bam*HI-digested and *Hin*dIII-digested) was used as the probe (this is therefore a repetition of the experiment shown in Figure 2b; **b**, a 3.9 kbp *Xho*I fragment of cpDNA (probe A in Figure 6) that was cloned in pUC12 and excised from the plasmid was used as the probe. The bands corresponding to the 175 and 130 kDa proteins are indicated by closed and open arrowheads, respectively.

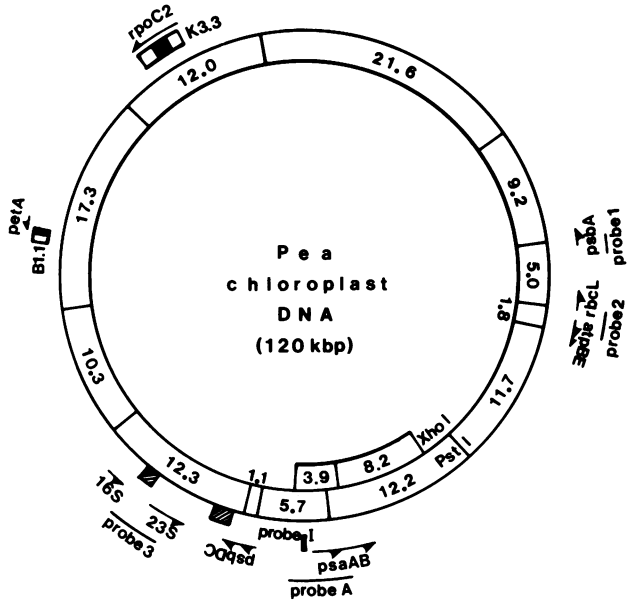

Fig. 4. A *Pst*I restriction map of pea cpDNA. This figure was adapted from the transcription map in Woodbury *et al.* (1988). Each number indicates the size of *Pst*I fragment in kbp. Two *Xho*I fragments are also shown. Location of appropriate genes and the probes tested in South-western analysis (see Materials and methods) are indicated by arrows and bars, respectively. The location of D-loops (Kolodner and Tewari, 1975; Meeker *et al.*, 1988) is shown by dashed boxes. Regions that were shown to have affinity for the 130 kDa protein in the first mapping are indicated by open boxes (K3.3, B1.1). The dark part within each of these boxes shows the protein-binding site determined in a detailed mapping (see text).

be due principally to the contamination of this fraction with stroma or to the weak peripheral binding of the protein to the envelope membrane. A faint band at 130 kDa was also detected in the envelope membrane fraction (Figure 2b, lane E). This band was found in neither the stroma nor the thylakoid fraction. The latter fraction contained various proteins that had affinity for the cpDNA probe, especially

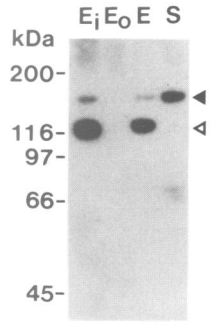

Fig. 5. Localization of the 130 kDa DNA-binding protein in the inner envelope membrane. Probe A (Figure 6) was used for the South-western analysis. Lane E_i, inner envelope membrane fraction; lane E_o, outer envelope membrane fraction; lane E, total envelope membrane fraction; lane S, stroma fraction. In each case, 10 μg protein was loaded per lane. The bands corresponding to the 175 and 130 kDa proteins are indicated by closed and open arrowheads, respectively.

in the presence of zinc ions (Figure 2b and c, lane T). In the rest of this report, we will concentrate on the DNA-binding proteins in the envelope membrane, and therefore only results that used EDTA (without zinc ions) in the binding mixture will be shown. No other bands appeared in the envelope membrane fraction when $MgCl_2$ or $CaCl_2$ was included in the binding buffer. These salts did, however, decrease significantly the intensity of the bands at 175 and 130 kDa (results not shown).

We have found that a 3.9 kbp *Xho*I fragment of cpDNA (probe A; see Figures 4 and 6) binds preferentially to the 130 kDa protein (Figure 3). This DNA fragment contained the *trnD* gene as well as a 5′ half of the *psaAB* operon and its upstream region (Figure 4). The binding of this DNA fragment to the 175 kDa protein was less marked. This suggests that the 130 kDa protein recognizes specific sequences of cpDNA. It is not clear if the 175 kDa protein recognizes specific sequences. The 130 kDa protein was detected in the inner envelope membrane fraction but not in the outer envelope membrane fraction (Figure 5).

Sites of cpDNA that bind to the 130 kDa protein

We have tested various cloned cpDNA fragments for their affinity for the 130 kDa protein. Neither the promoter region of the *psbA* gene (probe 1 in Figure 4) nor that of the *rbcL* and *atpBE* operon (probe 2 in Figure 4) nor the D-loop region located within the 16S rRNA gene (probe 3 in Figure 4) had affinity for the 130 kDa protein in the envelope membrane (not shown).

The site within the 3.9 kbp *Xho*I fragment that binds to the 130 kDa protein was localized by using end-labelled DNA fragments of various subclones. A series of South-western analyses both with and without competitor DNA (summarized in Figure 6) showed that a 0.41 kbp DNA fragment I had a very high affinity to the 130 kDa protein. Examples of the binding and competition experiments are shown in Figure 7. The binding of fragment I to the protein was not affected by the addition of non-radioactive fragments E or H as competitors (Figure 7, right half), whereas the binding of fragment D, which includes the sequence of fragment I, was markedly reduced by the addition of non-radioactive fragment I (Figure 7, left half). These results clearly indicate that DNA fragment I contains a binding site for the 130 kDa protein. Fragment I had not been sequenced before the present study, despite its location between long

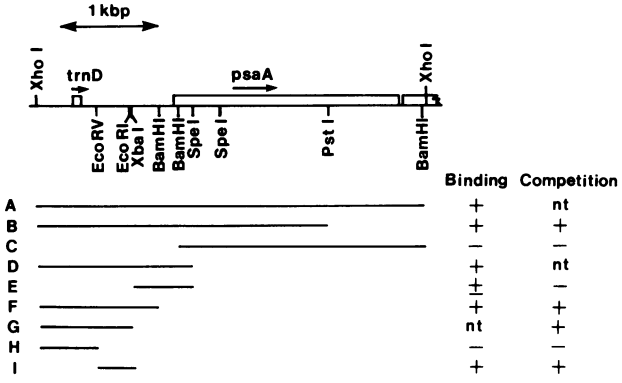

Fig. 6. Determination of the site in the pea cpDNA that binds to the 130 kDa DNA-binding protein. This figure shows a summary of South-western analysis using different DNA fragments (A–I) as probes. Key to binding column: +, probe bound to the 130 kDa protein; −, no significant binding; 'nt', not tested. The competition experiments were done with a radioactive probe and ~10-fold molar excess of a non-radioactive competitor [probe A with competitors B and C, probe D for competitors E, F, G, H and I, and probe I for competitors E and H (see Figure 7)].

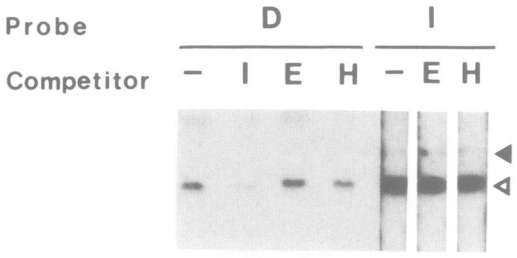

Fig. 7. South-western analyses showing that fragment I is the binding site for the 130 kDa protein. **Left**, probe was fragment D (Figure 6) and competitors were fragments I, E or H. **Right**, probe was fragment I (Figure 6) and competitors were fragments E or H. In eash case, ~7-fold molar excess of competitor was used. The bands corresponding to the 175 and 130 kDa proteins are indicated by closed and open arrowheads, respectively.

stretches of sequenced tRNA genes (Rasmussen *et al.*, 1984) and the *psaAB* gene (Lehmbeck *et al.*, 1986). Figure 8 shows its nucleotide sequence. A short open reading frame encoding the *psbM* gene (Ikeuchi *et al.*, 1989) was detected in the middle of the sequence. There are long stretches of A or T residues in the latter half of the sequence. The nucleotides which interact with the 130 kDa protein have not yet been determined precisely.

We also attempted by PCR to identify all the sites of cpDNA that have affinity for the 130 kDa protein. A DNA library was constructed by inserting a mixture of partial *Sau*3AI digests of cpDNA into the *Bam*HI site of pBluescriptII SK+. The inserts were cut out with *Bss*HII, 3′-end-labelled and used as probes in South-western blotting. The bound DNA was then eluted from the blotting membrane and amplified by PCR. The product was used as the probe in the second South-western analysis. After five rounds of binding and amplification, the PCR product was used as a probe in Southern analysis of a set of cloned cpDNA (Figure 9A). The PCR product hybridized strongly to the 12.0 and 17.3 kbp *Pst*I fragments. Less intense hybridization was found with the 5.7 kbp *Pst*I fragment and the 3.9 kbp *Xho*I fragment (both contain fragment I in Figure 6), as well as with 12.3 and 9.2 kbp *Pst*I fragments. Further mapping

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GATATCATCGAGATCCTATTTATACCATTGAATTTAGTGACGAAAGATTTTTCATTTCTA  60
------(EcoRV)

TGGGATTAAATCCCGAAGTATTTATTAGAAATTAAAGAGAAAGAAAACATAGAGATTATG 120
                                                    (psbM) Met

GAAGTAAATATTCTCGCATTTATAGCTACTGCACTCTTCATTCTAGTTCCTACTGCCTTT 180
GluValAsnIleLeuAlaPheIleAlaThrAlaLeuPheIleLeuValProThrAlaPhe

TTACTTATAATTTATGTAAAAACGGTAAGTCAAAGTGACTAATTTTAGTGAAACATGACT 240
LeuLeuIleIleTyrValLysThrValSerGlnSerAsp***

TCTTATTTCTTATCAATGCAATGATTGAAGAAAAAAATGAAAAAAGGATTTGTATTTCGG 300

GTCTTTGTTTGAATCTTTGTTTTAAATAAAATGATCAGACTACATAACAAAAAGGATCTT 360

CGACAACCCCCAAAAAAGTATATTTCTATATTTCCTATCGAATTGAATTCTAGA       414
                                    (EcoRI)------
                                        (XbaI)------
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Fig. 8. Nucleotide sequence of the fragment I. Both strands of DNA were sequenced by the chemical cleavage method (Maxam and Gilbert, 1980). This sequence is located between and links the nucleotide sequences of *trn*D (Rasmussen *et al.*, 1984) and *psa*AB (Lehmbeck *et al.*, 1986). This nucleotide sequence has been registered in the EMBL/GenBank/DDBJ databases under accession number D12535.

of the cloned cpDNAs (Figure 9C) showed that the strong hybridization was due to a 3.3 kbp *Kpn*I fragment (K3.3 in Figure 4) within the 12.0 kbp *Pst*I fragment that contains the internal sequence of the *rpoC2* gene (Cozens and Walker, 1986) and a 1.1 kbp *Bam*HI fragment (B1.1 in Figure 4) within the 17.3 kbp *Pst*I fragment that contains the downstream half of the *petA* gene (Willey *et al.*, 1984; Willey and Gray, 1989). These regions were further mapped to a 1.2 kbp *Eco*T14I fragment of the *rpoC2* gene and a 0.57 kbp *Bam*HI–*Eco*RI fragment of the *petA* gene, respectively (Figure 9D). These results suggest that several distinct sites of cpDNA (see Figure 4) have affinity for the 130 kDa protein. The 1.2 kbp *Eco*T14I fragment and the 0.57 kbp *Bam*HI–*Eco*RI fragment contained highly AT-rich regions as did fragment I, but whether these AT-rich regions are involved in the binding to the 130 kDa protein awaits detailed sequence analysis of the PCR products.

Developmental change in the content of the 130 kDa protein

The 130 kDa protein was clearly and reproducibly detected in the envelope membranes from 7 day old plants, while it was virtually undetectable in mature leaves of 14 or 20 day old plants (Figure 10). This result is consistent with the results of fluorescence microscopy studies which showed that cp-nucleoids are attached to the envelope membrane in young plants but not in mature plants (Figure 1).

Discussion

The results presented here show that the envelope membrane of young chloroplast contains a DNA-binding protein which recognizes specific sequences of cpDNA. This DNA-binding protein (apparent molecular mass 130 kDa) is likely to be involved in the binding of cp-nucleoids to the envelope membrane in young pea leaves, so we have called it plastid envelope DNA-binding (PEND) protein.

Major arguments for this proposal are the following. (i) PEND protein recognizes and binds to short specific DNA sequences of the pea cpDNA. The binding is not abolished by the addition of either calf thymus DNA or flanking cpDNA sequences as competitors. This specificity of binding suggests that PEND protein binds the cpDNA by interaction with these specific sequences. (ii) Most of the cp-nucleoids appear to be attached to the envelope membrane in young chloroplasts but not in mature chloroplasts. PEND protein is present in the envelope membrane of young chloroplasts

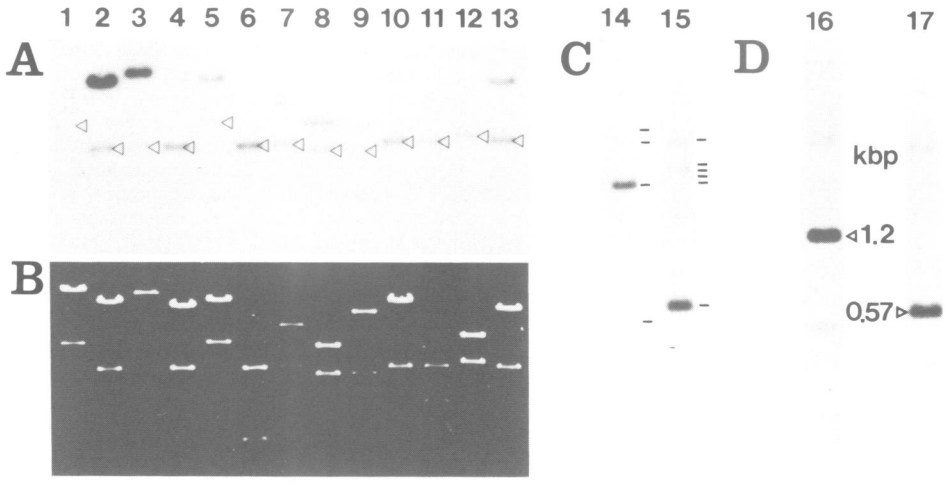

Fig. 9. Mapping of the sites that have affinity for the 130 kDa protein. The DNA fragments that bound to the 130 kDa protein in the South-western analysis were amplified by PCR and then used to probe blots of cpDNA clones. **A**, Autoradiogram of a Southern blot; **B**, ethidium bromide-stained gel; **C**, autoradiogram of a Southern blot; **D**, autoradiogram of a Southern blot for fine mapping. Lanes 1–7 and 10–13 are *Pst*I digests of clones of *Pst*I cpDNA fragments with sizes of 21.6, 12.0, 17.3, 10.3, 12.3, 1.1, 5.7, 11.7, 1.8, 5.0 and 9.2 kbp, respectively (see Figure 4 and Materials and methods). Lanes 8 and 9 are *Xho*I digests of clones of *Xho*I cpDNA fragments with sizes of 3.9 and 8.2 kbp, respectively. Lane 14, *Kpn*I digest of a cloned 12.0 kbp *Pst*I fragment; lane 15, *Bam*HI digest of a cloned 17.3 kbp *Pst*I fragment; lane 16, double digest with *Kpn*I and *Eco*T14I of the 12.0 kbp *Pst*I clone; lane 17, double digest with *Bam*HI and *Eco*RI of the 17.3 kbp *Pst*I clone. The arrowheads in (A) indicate the bands of vectors whose linker sequences weakly hybridized with the PCR primers. In (C), the position of DNA fragments that were detected by ethidium bromide staining are indicated on the right of each lane. Concentration of agarose gel was 0.8% in (A), (B) and (C), and 1.3% in (D).

but not in the envelope membrane of mature chloroplasts. This temporal coincidence of the occurrence of PEND protein and the binding of cpDNA to the envelope membrane (see Figure 1) suggests a role of PEND protein in the attachment of cp-nucleoids to the envelope membrane. (iii) The location of PEND protein in the inner envelope membrane is consistent with the supposed function of this protein in the binding of cp-nucleoids to the envelope membrane.

Various soluble proteins that are capable of binding DNA have been found in chloroplasts. Some of them bind DNA almost non-specifically (Crevel *et al.*, 1989; Nemoto *et al.*, 1990; Baumgartner and Mullet, 1991) and are supposed to play a structural role in packing cpDNA within cp-nucleoids (Nemoto *et al.*, 1989). Sequence-specific DNA-binding proteins that bind to the promoter regions of chloroplast genes (Lam *et al.*, 1988; Baeza *et al.*, 1991; Khanna *et al.*, 1992) or to the replication origin (Wu *et al.*, 1989) have also been described. These proteins are supposed to be soluble and, therefore, clearly different from PEND protein.

Since some of the subunits of RNA polymerase (Lerbs *et al.*, 1983; Zaitlin *et al.*, 1989; Rajasekhar *et al.*, 1991) have been shown to have affinity for DNA, we suspected that PEND protein might be a subunit of RNA polymerase, which is eventually associated with the envelope membrane; we therefore tested if some of the subunits of spinach chloroplast RNA polymerase (gift from Dr Job, University of Marseille) react with cpDNA in our system. No binding of cpDNA to any of the subunits of the RNA polymerase was detected in South-western analysis (unpublished result). PEND protein is therefore distinct from RNA polymerase. These arguments clearly indicate that PEND protein is a new protein which is different from the DNA-binding proteins described before.

PEND protein recognizes several distinct sites within the cpDNA molecule. Until now, three of the sites have been identified. These sites were located within or near the *psbM*,

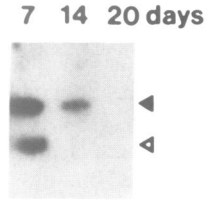

Fig. 10. South-western detection of DNA-binding proteins in envelope membranes (20 μg protein) from plants of different age. In this experiment, envelope membranes were washed with 10 mM Tris, 1 mM EDTA (pH 8.0) by ultracentrifugation. Probe A (Figure 6) was used. The bands of the 175 kDa and the 130 kDa proteins are indicated by closed and open arrowheads, respectively.

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psbM          172 ACTGCCTTTTTACTTATAATTTATGTAAAAACGGT  206
petA 3'(c) 1703 ACTCCATTTTTATTGATAAATTCTGTAAAGACCAT 1669
petA 3'(c) 1663 CTTTCTAGTTTTTTTATACTATATTTAGAGAATTC 1629
rpoC2         993 AGGGGATTAGCGATTATTCAATATTTAATGAAATC 1027
rpoC2        1806 TAAAAGTTGTCAATTATATTCTTTATGGAAATGGA 1840
rpoC2(c)     1044 GAATGATCCGTACATATGATTTCATTAAATATTGA 1010
rpoC2(c)     2046 GGTTCATTTCTTTTTCTAATATATGAAGTATTCGA 2012

consensus              Tt  t  TTATa T T T TARa A
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Fig. 11. Sequence similarities found in the DNA regions that have affinity for the 130 kDa protein. Homologous sequences were searched by a computer program provided by Software Developing Company (Tokyo). The consensus bases in upper case are identical in all or six of the sequences, while the bases in lower case are identical in only five. Sources of nucleotide sequences: 3′ region of *petA*, Willey and Gray (1989); *rpoC2*, Cozens and Walker (1986).

rpoC2 and *petA* genes. All of these sequences are highly AT-rich, as are the nuclear scaffold-associated DNA regions (SARs) (Gasser and Laemmli, 1986; Slatter *et al.*, 1991), but the 'A-box' and 'T-box' (Gasser and Laemmli, 1986) which have been frequently found in the SAR are not found in the recognition sequences of PEND protein. Instead, a computer search picked up sequences with homologies in these regions (Figure 11). The sequences of *psbM* (coordinates 172–206, coding region) and *petA* (coordinates

1703−1669, 3′ non-coding region) are highly homologous, while other sequences shown in Figure 11 showed less extensive homology. The higher yield of PCR amplification of the sequences related to *rpoC2* and *petA* over that of the sequences related to *psbM* (Figure 9A) could be explained by the higher affinity for the 130 kDa protein of the former two sequences, but the sequence comparison in Figure 11 does not support this view: firstly, the sequence homology in the region listed in Figure 11 was quite high between *psbM* (172−206) and *petA* (1703−1669); secondly, no other significant homology was found between the *rpoC2* and *petA* genes. The high yield of amplification of the *rpoC2* and *petA* genes might be due to the presence of multiple sites that are recognized by PEND protein, and/or to the efficient amplification caused by unknown reasons. A more detailed analysis of the site(s) of cpDNA that interacts with PEND protein awaits extensive analysis of the PCR products.

A possible function of PEND protein is to anchor cpDNA during replication. In support of this, PEND protein is present in the envelope membrane of young chloroplasts, which are active in division. Nevertheless, the biological meaning of the anchoring of cpDNA during its replication is still unclear, although the role of the attachment of the bacterial genome to the membrane during the initiation of replication has been suggested recently (Ogden *et al.*, 1988; Landoulsi *et al.*, 1990). A role in equal partition of cpDNA copies to daughter chloroplasts is also probable.

The importance of the chloroplast (plastid) envelope in the metabolism of lipids and carotenoids, in the transport of metabolites and ions and in the transport of nuclear-encoded chloroplast proteins has been well documented (for a review, see Joyard *et al.*, 1991). We now suggest that the plastid envelope binds the plastid genome, although the probable function of this binding in the regulation of replication and transcription of plastid genome is still unclear. The chloroplast envelope membrane is an interface between the cytoplasm and the interior of the plastid, and thus the binding of cpDNA to the envelope membrane could be a key step in the transmission to the plastid of cellular signals that trigger a new round of replication or transcription of the plastid genome, which is necessary for the development and differentiation of plastid into chloroplast. We believe that the discovery of PEND protein will open a new field of research on the structure and function of plastid envelope membranes.

Materials and methods

Plant materials

Seeds of dwarf pea (*Pisum sativum* cv. Douce Provence) were sown on moist vermiculite and grown for 7, 14 or 20 days in a growth room with a light period of 13 h and a dark period of 11 h. The light intensity was ~20 000 lux at the level of the plants. The temperature was 28°C during the light period and 22°C during the dark period.

Fluorescence microscopy

Protoplasts were liberated from leaves, fixed in 0.5% glutaraldehyde and stained with 1.0% DAPI, and then squashed between a microscope slide and a cover slip. They were observed with a fluorescence microscope (Olympus, Tokyo) using a UV excitation beam (mostly at 334 and 365 nm). Details of sample preparation and microscopy have been described previously (Miyamura *et al.*, 1986; Sodmergen *et al.*, 1989).

Isolation of envelope membrane

Intact chloroplasts were isolated according to the method of Douce and Joyard (1982) using a Percoll gradient. Chloroplasts (20−50 mg chlorophyll) were suspended in 20 ml of 1.2 M sucrose in basal buffer [10 mM HEPES−KOH (pH 7.5), 1 mM EDTA] and then frozen at −80°C for at least 2 h. The chloroplasts were then thawed at room temperature. The concentration of sucrose was adjusted to 1.3 M by adding 2.0 M sucrose in basal buffer. 1.2 M sucrose in basal buffer and 0.4 M sucrose in basal buffer were overlayed on the suspension of chloroplasts at the bottom of SW27 centrifugation tubes. The tubes were centrifuged at 112 000 *g* for 5 h at 4°C. Crude envelope membranes at the interface of the 0.4 M and 1.2 M layers were recovered. This flotation technique was effective in removing thylakoid contamination. The membranes were diluted with basal buffer and then purified through a discontinuous gradient of sucrose consisting of 0.4 M/1.0 M/1.2 M (at 112 000 *g* for 1 h). The yellow band (envelope membrane) was recovered, diluted with basal buffer and finally pelleted by centrifugation at 112 000 *g* for 1 h.

To prepare stroma and thylakoid fractions, chloroplasts were broken by hypotonic treatment and then layered over 0.6 and 0.93 M solutions of sucrose in basal buffer. The tubes were centrifuged at 87 000 *g* for 1 h. The uppermost layer (0 M sucrose) was recovered as stromal fraction, while the precipitate was recovered as crude thylakoid fraction. The thylakoids were resuspended in 0.3 M sucrose and sedimented at 3000 *g* for 10 min. This fraction also contained a large amount of rRNA and chloroplast DNA.

Outer and inner envelope membranes were prepared according to the method described by Keegstra and Yousif (1986) using a continuous sucrose density gradient from 0.5 to 1.2 M.

Preparation of cpDNA

Chloroplast DNA was isolated from intact chloroplasts prepared from 7 day old pea seedlings as described previously (Ohta *et al.*, 1991).

South-western blotting

Fractions of chloroplasts (thylakoid, stroma or envelope) were electrophoresed in a 10% polyacrylamide gel (0.13% BIS) containing 0.1% lithium dodecyl sulfate according to Piccioni *et al.* (1982). Separated proteins were then transferred to two sheets of nitrocellulose membrane (Advantec, Tokyo) in 0.025 M Tris, 0.192 M glycine for 2 h at 0.4 A using a Mini-transblot apparatus (Bio-Rad, Richmond, CA). The second membrane and part of the first membrane corresponding to the marker lanes were washed extensively in phosphate-buffered saline and then stained with colloidal gold (Aurodye, Amersham) according to the protocol provided by the supplier. The remaining part of the first membrane was blocked in 5% skim milk (Difco), 10 mM HEPES−KOH (pH 7.7) for 1 h on ice, and then incubated with end-labelled DNA probes (2 fmol cpDNA/ml or 100 fmol cloned DNA/ml, at a specific radioactivity of $\sim 1\times10^6$ d.p.m./μg) dissolved in binding buffer [0.25% skim milk, 10 mM HEPES−KOH (pH 7.7), 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol] containing 1 μg/ml calf thymus DNA (Boehringer-Mannheim) at room temperature for 2 h. In certain experiments, EDTA was replaced by 1 mM $ZnCl_2$ in binding buffer to examine the effects of zinc ions. The membranes were then washed in binding buffer four times for 15 min each. Each piece of membrane was sealed in a thin polypropylene bag and then autoradiographed for 1−5 days at −80°C. A mixture of *Bam*HI-digested cpDNA and *Hin*dIII-digested cpDNA was used as a probe representing total cpDNA. A cloned 3.9 kbp *Xho*I fragment (probe A, see Figures 4 and 6) was obtained from Dr J.D.Palmer (Indiana University). The DNA insert was purified by agarose gel electrophoresis and then labelled by filling-in with T4 DNA polymerase and [^{32}P]dCTP. Other DNA fragments (Figure 4) that were tested in South-western analysis were: probe 1, a 1.2 kbp *Bam*HI fragment containing the promoter and the first half of the coding region of the *psbA* gene; probe 2, a 1.8 kbp *Pst*I fragment containing the overlapping promoter regions of the *rbcL* and *atpB* genes, which was excised from the plasmid with flanking linker sequences; probe 3, a 3.3 kbp *Bss*HII fragment containing one of the D-loop regions.

Probes and competitors

The probes and competitors used in detailed analysis of the protein binding site within the 3.9 kbp *Xho*I fragment (Figure 6) were as follows: A, 3.9 kbp *Xho*I fragment; B, 2.9 kbp *Xho*I−*Pst*I (plus linker sequence of pBluescript to *Xho*I site) excised from 5.7 kbp *Pst*I clone; C, 2.5 kbp *Bam*HI fragment excised from 3.9 kbp *Xho*I clone; D, 1.6 kbp *Xho*I−*Spe*I fragment excised from 3.9 kbp *Xho*I clone; E, 0.65 kbp *Xba*I−*Spe*I (plus linker sequence to *Xba*I site) excised from pXhSp1 (fragment D cloned in pBluescript SK+) and subcloned in pBluescript; F, 1.25 kbp *Xho*I−*Bam*HI fragment with a *Bam*HI−*Xho*I linker sequence was excised from pXhSp2 which was constructed by cloning fragment D with a linker sequence from *Pst*I−*Xho*I in pBluescript SK+; G, 0.95 kbp *Eco*RI fragment excised from pXhSp2 (used as a competitor); H, 0.65 kbp *Xho*I−*Eco*RV fragment with a linker sequence beginning from an *Eco*RV site to a *Xho*I site was excised from pXhSp2, subcloned in the *Eco*RV site of pBluescript SK+ and excised

with *Ava*I; I, 0.41 kbp *Eco*RV–*Xba*I fragment excised from pXhSp2 was subcloned in pBluescript SK+ and excised with *Xho*I and *Xba*I.

DNA amplification

A mixture of partial *Sau*3AI digests of cpDNA was inserted into the *Bam*HI site of pBluescript II (SK+), and then the inserts were excised by *Bss*HII with linker sequences. This mixture was 3′ end-labelled and allowed to bind to the protein blot as described above. After localization of the band by autoradiography, the membrane corresponding to the 130 kDa protein band was cut out and incubated in 10 mM Tris–HCl, 1 mM EDTA, 1% Sarkosyl, 0.2 mg/ml proteinase K at 37°C for 30 min. The eluted DNA (100 μl) was treated with phenol–chloroform and then passed through a spun column (cDNA spun column, Pharmacia, Uppsala) equilibrated with 10 mM Tris–HCl, 1 mM EDTA, 50 mM KCl. An aliquot (10 μl) was used to make a standard PCR cocktail (Perkin-Elmer-Cetus, 100 μl) with the SK and KS 17mer primers (Stratagene, La Jolla, CA). 30 cycles of PCR were performed, each consisting of 94°C for 1 min, 45°C for 2 min, and 72°C for 3 min.

The DNA was treated with phenol–chloroform and recovered by ethanol precipitation, and then used to probe a DNA blot of cpDNA clones. The clones used were: 21.6 and 12.3 kbp *Pst*I fragments in pBR322 (gift of J.D.Palmer); 3.9 and 8.2 kbp *Xho*I fragments in pUC12 (gift of J.D.Palmer); 5.0 kbp *Pst*I fragment in pUC118 (Ohta *et al.*, 1991); 12.0, 17.3, 10.3, 1.1, 5.7, 11.7, 1.8 and 9.2 kbp *Pst*I fragments in pBluescript (SK+) (Ohta *et al.*, 1991). The hybridization conditions were similar to those described previously (Ohta *et al.*, 1991).

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