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Molecular Characterization of the PEND Protein, a Novel bZIP Protein Present in the Envelope Membrane That Is the Site of Nucleoid Replication in Developing Plastids

Naoki Sato,[a,1] Kimihisa Ohshima,[b] Ai Watanabe,[a] Niji Ohta,[c] Yoshitaka Nishiyama,[d] Jacques Joyard,[e] and Roland Douce[e]

[a] Department of Biochemistry and Molecular Biology, Faculty of Science, Saitama University, Urawa 338, Japan
[b] Saitama Prefectural Shim-Misato Water Filtration Plant, 1 Minami-hasunuma, Misato 341, Japan
[c] School of Human Science, Waseda University, Mikajima, Tokorozawa 359, Japan
[d] Department of Regulation Biology, National Institute for Basic Biology, Okazaki 444, Japan
[e] Physiologie Cellulaire Végétale, URA 576 CEA/CNRS/Université Joseph Fourier, Département de Biologie Moléculaire et Structurale, CEA-Grenoble 38054, Grenoble-cedex 9, France

Plastid nucleoids are known to bind to the envelope membrane in developing chloroplasts. Here, plastid DNA is extensively replicated. We previously detected a DNA binding protein in the inner envelope membranes of developing plastids in pea and named it PEND (for plastid envelope DNA binding) protein. In this study, we report on the structure and molecular characterization of a cDNA for the PEND protein. As a result of screening cDNA libraries in λgt11 with one of the target sequences of the PEND protein as a probe, we obtained a clone (PD2) for a novel DNA binding protein consisting of 633 amino acid residues. Analysis of the N-terminal sequence of the purified PEND protein indicated that the transit peptide is just 16 residues long. The PEND protein was detected specifically in the plastid envelope membrane of young unopened leaf buds by immunoblot analysis. The PEND protein consists of a basic region plus zipper region, an unprecedented sextuple repeat region, and a putative membrane-spanning region. The basic region with a zipper region seems to have diverged from that of other plant transcription factors. In addition, the PEND protein could be a distant homolog of the *trans*-Golgi network integral membrane proteins. The PEND protein is therefore a novel type of DNA binding protein that binds to the membrane as an intrinsic membrane protein.

INTRODUCTION

Chloroplast DNA (cpDNA) or plastid DNA contains genes that are involved not only in photosynthesis but also in gene expression (transcription and translation) and some aspects of plastid metabolism (reviewed in Mullet, 1988; Sugiura, 1992). In plastids, cpDNA exists as a large protein–DNA complex that is called "plastid nucleoid" or "plastid nucleus" (Kuroiwa, 1991). Previous studies have suggested that each nucleoid particle contains several to >10 copies of cpDNA (Kuroiwa, 1991). Although the replication and transcription of cpDNA are likely to be regulated by the organization of plastid nucleoids, little is known about the relationship between the structure and function of nucleoids. In this regard, we (Sato et al., 1993, 1997) as well as others (Miyamura et al., 1986; Sodmergen et al., 1989) have pointed out that the morphology of plastid nucleoids is subject to dynamic changes during the development of plastids. The proplastid contains a single nucleoid, which is located at the center of the plastid. In the developing plastid, nucleoids are extensively replicated and localized in the periphery of the plastid. A study using electron microscopy (Herrmann and Kowallik, 1970) clearly demonstrated binding of cpDNA to the envelope membrane of developing plastids. In the mature chloroplast, plastid nucleoids are located within the plastid as small particles. During leaf senescence, the number of plastid nucleoids as well as the copy number of cpDNA decrease.

We are interested in the binding of plastid nucleoids to the envelope membrane in the developing plastid. Several lines of evidence strongly suggest that this binding is likely to be related to the replication and segregation of cpDNA. Studies from the Mullet group (DuBell and Mullet, 1995a, 1995b) among others clearly show that the activities of replication and transcription are highest in the developing plastids (immature chloroplasts) in unexpanded pea leaves. This stage corresponds to or slightly precedes the stage at which plastids

[1] To whom correspondence should be addressed. E-mail naokisat@molbiol.saitama-u.ac.jp; fax 81-48-858-3384.

divide actively (Sato et al., 1997). At the same stage, plastid nucleoids are present at the periphery of plastids and appear to bind to the envelope membrane (Sato et al., 1993, 1997). We previously found a DNA binding protein in the envelope membranes of developing plastids that may anchor plastid nucleoids (Sato et al., 1993). This protein, designated PEND (for plastid envelope DNA binding protein), migrates as a 130-kD polypeptide during lithium dodecyl sulfate (LDS)–PAGE and binds to several regions of cpDNA, such as the downstream half of the *petA* gene (for apocytochrome *f*), two regions within the *rpoC2* gene (for the β″ subunit of RNA polymerase), and a region that includes the *psbM* gene (for a photosystem II polypeptide) (Sato et al., 1993, 1995). This DNA binding protein, as detected by DNA binding blot analysis, was localized to the inner envelope membrane of developing plastids from unopened leaf buds of 6-day-old pea seedlings. These findings suggest that the PEND protein is involved in the binding of plastid nucleoids to the envelope membrane, which in turn might affect the processes of replication, segregation, and/or transcription of cpDNA. Detailed properties and the structure of the PEND protein have yet to be determined.

Here, we report the cloning of the cDNA for the PEND protein. The strategy consisted of two lines of experimentation. In one line of work, we tried to purify the PEND protein from developing plastids of young pea leaves. We tested various chromatographic procedures, but the very hydrophobic nature of PEND protein implied the use of high concentrations of detergents, which made chromatographic separation very difficult. Nevertheless, we were able to purify the PEND protein by using an affinity technique with magnetic beads. In another line of work, we tried to obtain cDNA clones for the PEND protein by DNA binding screening of cDNA expression libraries. We obtained three clones of DNA binding proteins, and one of them, designated PD2, was found to encode the PEND protein. We determined that the PD2 cDNA encoded PEND by direct comparison of the N-terminal amino acid sequence of the PEND protein with the deduced amino acid sequence of PD2. The PEND protein's structural features are characterized. We also report on unprecedented distant homology of the PEND protein with *trans*-Golgi network (TGN) proteins.

RESULTS

Purification of the PEND Protein

In previous reports (Sato et al., 1993, 1995), we found four regions of plastid DNA that have an affinity for the PEND protein. They are a 0.4-kb region of the *psbM* gene, a 0.57-kb region downstream of the *petA* gene, and a 1.2-kb region within the *rpoC2* gene containing two binding sites. Among these, probe 1 (previously called probe I) containing a 0.4-kb region of the *psbM* gene is highly specific for the PEND protein, whereas the three other DNA regions have an affinity for several other DNA proteins in the plastid extract as well. Therefore, we decided to use probe 1 in both the purification of the PEND protein and for DNA binding screening.

First, we attempted to purify the PEND protein from envelope membrane preparations; however, purification was difficult because (1) the DNA binding activity of the PEND protein decreased rapidly during the preparation and storage of envelope membrane and (2) the total amount of the PEND protein within the envelope membranes as well as the yield of envelope membrane from young developing pea plastids were extremely limited. Instead, we used intact developing plastids as the starting material. Plastids corresponding to 500 mg of protein (obtained from ~600 g fresh weight of leaf buds of 6-day-old seedlings) were solubilized with 2% (34 mM) Nonidet P-40, a nonionic detergent that had been used in the preparation of nucleoids from plastids (Nemoto et al., 1988). We found that 0.1% (1.7 mM) Nonidet P-40 was sufficient for the solubilization of plastids but that higher concentrations of the detergent were necessary for maintaining the PEND protein in a solubilized form. In addition, the use of 0.1% Nonidet P-40 or octylglucoside resulted in the loss of the PEND protein during column chromatography. Therefore, 2% Nonidet P-40 was used in the initial solubilization; thereafter, 0.5% (8.4 mM) Nonidet P-40 was added throughout the purification of the PEND protein.

This high concentration of detergent, on the other hand, notably lowered the resolution during the column chromatography. We tested various chromatographic resins, but the purification was not quite successful because the DNA binding activity rapidly disappeared during repeated chromatography. Therefore, preserving the activity of the PEND protein as well as efficiently separating this protein were prerequisites for successful purification of the PEND protein. Freezing at −75°C resulted in a loss of 90% activity. We found that storage of the protein in a medium containing 1 to 2% Nonidet P-40 and 33% glycerol at −15°C without freezing were the best possible storage conditions. Under these conditions, the DNA binding activity of the PEND protein was stable for at least 6 months.

The most important part of PEND protein purification was affinity purification with magnetic particles that were coated with a specific DNA probe. To this end, streptavidin-coated magnetic particles that are commercially available as Dynabeads M-280 Streptavidin were conjugated with biotin-labeled probe 1 and used as a DNA affinity matrix. This technique was unaffected by the presence of detergents. In addition, the best result was obtained with high concentrations of detergents that prevented nonspecific binding of proteins to Dynabeads. Figure 1A shows a representative purification of the PEND protein with DNA–Dynabeads. The input to the affinity purification was an effluent from anion exchange column chromatography on a Q cartridge, but this fraction contained many proteins (Figure 1A, lane 1). The binding was performed in the presence of 0.05 M NaCl; after

extensive washing with a buffer containing 0.05 M NaCl, the PEND protein was eluted with 0.3 M NaCl (Figure 1A, lane 3). Further washing with 1 M NaCl recovered only a small amount of additional PEND protein (Figure 1A, lane 4). This purification was remarkably efficient, resulting in a preparation that contained the PEND protein as a major protein.

DNA binding activity of the purified PEND protein in the preparation was confirmed by DNA binding blotting (Figure 1B). The 175-kD DNA binding protein that we also found in the previous study did not bind to the DNA affinity matrix under our experimental conditions. The polypeptide at ~70 kD observed in the 0.3 M eluate seemed to be a contaminant from an abundant polypeptide in the plastid extract. Figure 1 demonstrates that although the 70-kD polypeptide had an affinity for the DNA affinity matrix, it was not detected by DNA binding blotting and therefore had no relationship to the PEND protein.

The PEND protein was further purified and concentrated by anion exchange column chromatography on a Resource Q column in the presence of 4 M urea (results not shown). The PEND protein was eluted at ~0.3 M NaCl. The purified PEND protein was separated by LDS-PAGE and transferred to a polyvinylidene difluoride membrane. The N-terminal amino acid sequence was then determined. The resulting sequence was AKKNDSQERKxRIR (where x is an unidentified residue). No sequence in databases was similar to this N-terminal sequence.

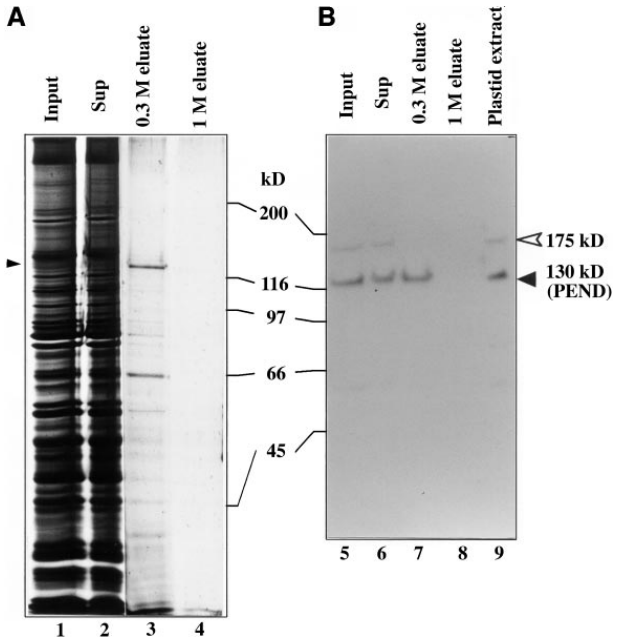

Figure 1. Affinity Purification of the PEND Protein.

Plastids from 6-day-old seedlings were lysed with 2% Nonidet P-40, and the supernatant was fractionated by anion exchange column chromatography on an Econo-Pac Q cartridge. The high-salt fraction (0.5 to 0.8 M NaCl) was used as an input (lanes 1 and 5) for DNA–Dynabeads affinity purification. Sup (lanes 2 and 6) is the supernatant that did not bind to the DNA–Dynabeads. Eluate (0.3 M; lanes 3 and 7) is the eluate with 0.3 M Dynabeads buffer. Eluate (1 M; lanes 4 and 8) is the eluate with 1 M Dynabeads buffer. In lanes 1 to 8, a 10-μL aliquot of protein fractions was loaded per lane. The numbers between panels indicate molecular mass markers.

(A) Analysis of protein by LDS-PAGE. A 10% gel with a low concentration (0.13%) of *N*,*N*′-methylene-*bis*(acrylamide) was used. After electrophoresis, the gel was stained with silver nitrate. Arrowhead, the PEND protein.

(B) DNA binding blotting analysis of the PEND protein. Lane 9 is the plastid extract (50 μg protein), which was used in the purification. Open arrowhead, 175-kD protein; filled arrowhead, the PEND protein.

DNA Binding Screening

DNA binding screening of cDNA expression libraries was also performed using probe 1. We used various cDNA libraries in λgt11 that had been made from poly(A)$^+$ RNA from 5- or 7-day-old leaf buds of pea grown in either light or darkness. We obtained three partial cDNA clones for DNA binding domains, which were named PD1, PD2, and PD3 (PD stands for plastid DNA binding). The localization of the PD1 and PD3 proteins to the plastid nucleoids was documented in a preliminary report (Sato et al., 1995). The *PD1* and *PD3* genes were also found to be expressed in very young leaves, especially in etiolated leaves (Sato et al., 1995; Shinada and Sato, 1997). All of these clones were partial cDNA clones, and the initial PD2 clone was only 0.4 kb long. We obtained full-length cDNAs for each of these by screening λgt10 libraries. Finally, PD2, which was obtained from a cDNA library of 5-day-old light-grown seedlings, was found to encode an amino acid sequence identical to that which we had determined for the purified PEND protein (Figure 2).

The PD2 cDNA clone is 2206 residues long and encodes an open reading frame of 633 amino acid residues. The cDNA contains a poly(A) tail consisting of several tens of adenine residues. As shown in Figure 3A, the protein encoded by the PD2 cDNA presents a series of interesting features. First, the N-terminal sequence that had been determined for the purified PEND protein corresponds to the sequence beginning at residue 17. This result as well as those provided below clearly indicate that PD2 is a cDNA clone for the PEND protein. It also suggests that there is a short transit peptide that may allow the transport of the PEND protein to the plastid inner envelope membrane. Second, the N-terminal region is highly rich in basic amino acid residues. This region is followed by a zipper region. This zipper region contains valine residues rather than leucine residues at many heptad points. Interestingly, the zipper region was found in the original partial cDNA clone (0.4 kb) that had been obtained by DNA binding screening (see above). Therefore, the PEND protein is likely to be a bZIP (basic region plus zipper region) DNA binding protein.

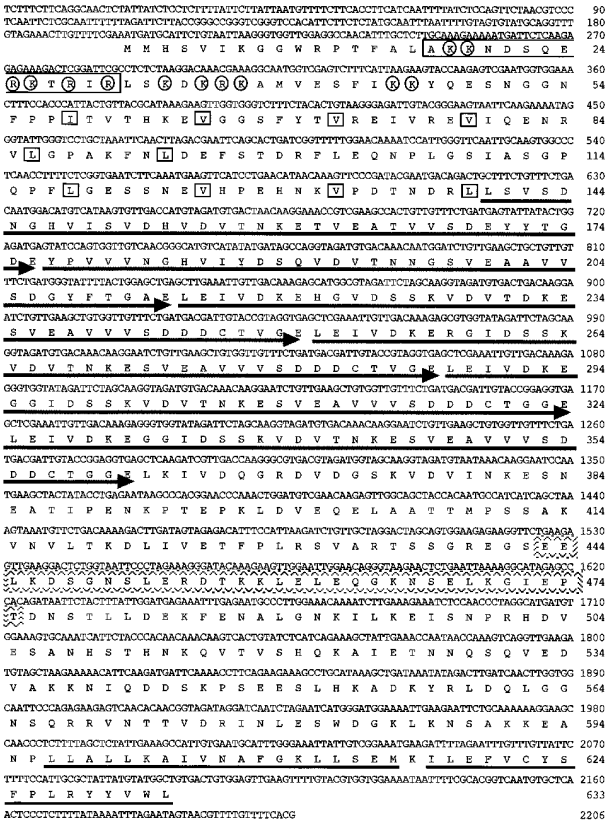

Figure 2. Nucleotide Sequence of a cDNA for the PEND Protein (PD2) and the Deduced Amino Acid Sequence.

We have not determined which of the first two methionine codons is the correct initiation codon. The boxed amino acid sequence indicates the N-terminal sequence of the purified PEND protein. Circled residues are basic amino acid residues present in the basic region of the bZIP DNA binding domain. The boxed residues are large hydrophobic residues that are thought to form the zipper of the bZIP domain. Six large arrows indicate a sextuple repeat. The box with wavy lines indicates a putative coiled-coil region. Underlined sequences are putative membrane-spanning regions. This nucleotide sequence has EMBL, GenBank, and DDBJ accession number X98740.

Another interesting feature was observed in the middle of the PEND protein sequence. Here, we found a large repeat region containing six complete motifs and one partial motif consisting of 37 amino acid residues. This sextuple repeat is unique to the PEND protein. Finally, there is a putative coiled-coil region and a C-terminal hydrophobic region (Figure 3B). This last region was determined to be membrane spanning by using TopPred II software (Claros and von Heijne, 1994). Each motif of the sextuple repeats is a composite of hydrophobic and hydrophilic regions (Figure 3B); therefore, this repeat might also be involved in the binding of the PEND protein to the membrane, although further study is required before the exact membrane binding site can be identified. The PEND protein is predicted to be an acidic protein with a calculated pI of 4.56.

Hybridization Analysis

Figure 4A shows the results of DNA gel blot hybridization analysis performed using a partial cDNA probe containing the N-terminal DNA binding region of the PEND protein. Only a single hybridization band was detected for each of the digests when HincII or SacI was used. Both restriction enzymes cut the cDNA in the repeat region. These results suggest that there is only a single copy of the *PEND* gene in the haploid genome of pea.

The same probe was used to detect the transcript of the *PEND* gene (Figure 4B). A single transcript of 2.4 kb was detected in the RNA gel blot hybridization. This size is reasonable, because the full-length cDNA contained 2206 nucleotides with an additional poly(A) tail. The *PEND* transcript was detected in leaf buds on the sixth day during growth in the dark (Figure 4B, lane 1), but the highest level of the transcript was found in the leaf buds of light-grown seedlings of an identical age (Figure 4B, lane 2). During subsequent growth in the light, the level of the transcript decreased rapidly, and no transcript was detected in the leaf buds of the seedlings on days 14 and 20. These results are consistent with the previous observation that the level of the PEND protein, as detected by its DNA binding activity, was maximal in

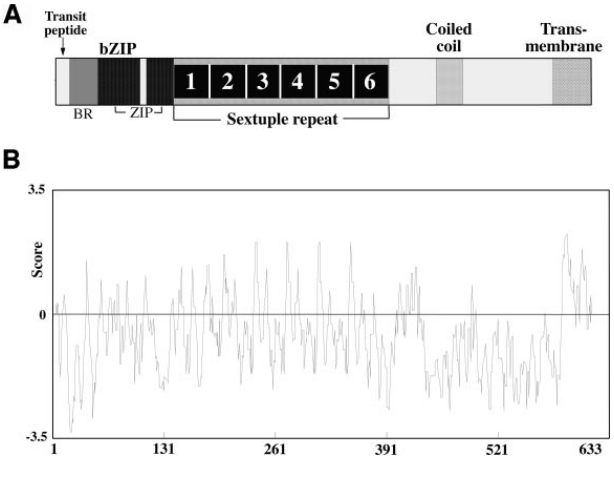

Figure 3. Structural Features of the PEND Protein.

(A) Schematic model for the PEND protein. BR, basic region.
(B) Hydropathy plot of the PEND protein. This plot was generated with the GENETYX software package (Software Developing Company, Tokyo, Japan) with the Kyte-Doolittle parameter set and a window size of 7. Positive scores indicate hydrophobicity.

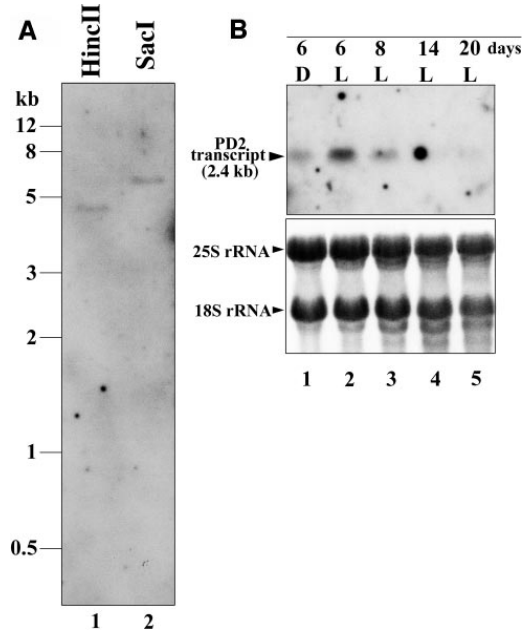

Figure 4. Hybridization Analysis of the *PEND* Gene.

(A) DNA gel blot hybridization analysis of genomic DNA of pea. Genomic DNA (16 μg per lane) was digested with HincII or SacI and then separated on a 0.8% agarose gel. After transfer to a positively charged nylon membrane (Hybond N+; Amersham), the blot was probed with the digoxigenin-labeled partial PD2 probe. Numbers at left indicate molecular length markers.

(B) RNA gel blot hybridization analysis of total cellular RNA of pea seedlings. Total RNA (9 μg per lane) was glyoxylated and analyzed in duplicate on 1.0% agarose gels. After transfer to a nylon membrane (Biodyne A; Pall Corp., Glen Cove, NY), one piece of the blot was probed with the digoxigenin-labeled partial PD2 probe (top); another piece of the blot was stained with methylene blue to visualize the rRNA (bottom). Lane 1, leaf buds of dark-grown (D) 6-day-old seedlings; lane 2, leaf buds of light-grown (L) 6-day-old seedlings; lane 3, developing leaves of light-grown 8-day-old seedlings; lane 4, mature leaves of light-grown 14-day-old seedlings; and lane 5, senescing leaves of light-grown 20-day-old seedlings.

the envelope membranes from young plastids of pea seedlings at the age of 5 or 6 days (Sato et al., 1993).

Production of PD2 Fusion Proteins in *Escherichia coli*

The DNA binding domain of PD2 cDNA (PD2zip) was fused to glutathione *S*-transferase (GST), and the fusion protein GST–PD2zip was expressed in *E. coli* cells. Only one of the several different constructs that we tried was successfully expressed in *E. coli*, and this fusion protein was purified by affinity chromatography with a glutathione–Sepharose 4B column. The fusion protein was highly pure in terms of protein in the eluate with glutathione, but it was copurified with *E. coli* DNA. This DNA was removed by gel filtration in the presence of 0.5 M NaCl (results not shown). The fusion protein was eluted as a 160-kD protein under these conditions. Because the molecular mass of the fusion protein is calculated to be 45 kD, the fusion protein is likely to be an oligomer consisting of three or four subunits; this would explain the apparent molecular mass of 160 kD that was obtained by gel filtration. This point is currently being studied.

The purified GST–PD2zip protein was used to immunize mice. The antisera thus obtained were used to probe the PEND protein in immunoblots (Figure 5). Figure 5A shows results of the expression of a full-length PD2 cDNA with a short hexahistidine tag in *E. coli.* The expression of full-length PD2, which was low and unstable, was possible when plasmid pRSET-A, having the T7 promoter in conjunction with the M13 helper phage harboring the gene for T7 RNA polymerase, was used. Despite this elaborated expression system, the protein product of the full-length PD2 cDNA was barely detectable when Coomassie blue was used to stain the gel. Nevertheless, it was clearly detected by immunoblotting with the antibodies raised against GST–PD2zip. A 140-kD polypeptide was detected in the induced cells. This value is consistent with the apparent mobility of the PEND protein (Figure 5A, lane 6), which migrated as a 130-kD polypeptide. In contrast, both values are quite different from the predicted molecular mass of 70 kD of the PD2 protein. The 130-kD band could represent a stable dimer of the PD2 polypeptide, which was not dissociated during sample preparation and electrophoresis with LDS and DTT. In fact, we never observed any 70-kD polypeptide having the immunological and functional properties of the PEND protein, even when various experimental conditions (e.g., solubilization with SDS and DTT at 100°C) were used for the preparation of samples for gel electrophoresis. Therefore, the 130-kD band is unlikely to be a dimer of 70-kD polypeptide. More likely, it is a monomer of the PEND protein that behaves atypically during gel electrophoresis. This very unusual retardation in migration is most likely due to the hydrophobic nature of the PEND protein.

Localization of the PEND Protein to the Plastid Envelope Membrane

Figures 5B and 5C present a series of experiments designed to localize the PEND protein within plastids and analyze whether cell nuclei extracts contain such a protein. The PEND protein was not detected in the cell nuclei preparations (Figure 5B, lane 5), although a nonspecific smear was detected below the interface of the separation gel and the stacking gel. This might have been due to the electrostatic binding of the antibodies to the DNA on the blot. Plastids from 6-day-old pea seedlings were solubilized with 2% Nonidet P-40, and nucleoids (Figure 5B, lane 6) and the post-nucleoid supernatant (Figure 5B, lane 7) were then analyzed.

A major part of the PEND protein was solubilized from the plastids and recovered in the supernatant. Nevertheless, only a minor part of the PEND protein was recovered with nucleoids. This result seems to contradict our proposal that the PEND protein anchors plastid nucleoids to the envelope membrane (Sato et al., 1993). If we consider this problem in terms of partition of the protein between detergent micelles and nucleoids, one explanation is that the binding of the PEND protein with nucleoids is not stable in the presence of the high (2%) concentrations of detergent that were used for the initial solubilization. Indeed, this hypothesis is consistent with our previous observation that high concentrations of the detergent are necessary to keep the PEND protein in a solubilized form (see above).

Next, we analyzed the PEND protein in various plastid fractions that had been prepared without the use of detergent (Figure 5C). In good agreement with our previous results obtained with DNA binding blotting (Sato et al., 1993),

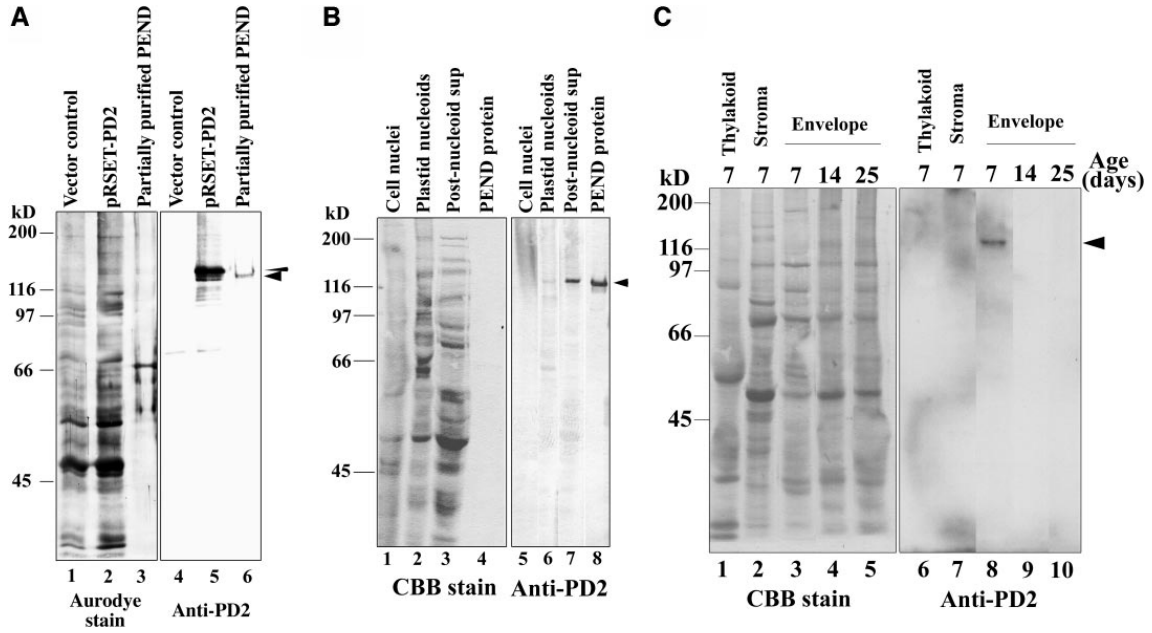

Figure 5. Immunoblot Analysis of the PEND Protein.

(A) Production of a full-length polypeptide of PD2 polypeptide. The complete open reading frame of the PD2 cDNA was fused to a short leader sequence encoding a polypeptide containing a hexahistidine tag in pRSET-A and then expressed in *E. coli* XL-1 Blue cells by infection with M13mp18/T7. The fusion protein was purified on a Ni-affinity column under denaturing conditions. Lanes 1 and 4 contain column eluate obtained in the experiment with a vector control with pRSET-A; lanes 2 and 5, column eluate obtained in the experiment with pRSET-PD2; and lanes 3 and 6, the partially purified PEND protein that was obtained by DNA–Dynabeads affinity purification. A 5-μL aliquot of various fractions was loaded per lane. Shown at left are the results of staining with Aurodye, a high-sensitivity colloidal gold staining reagent (Amersham). Note that the PEND protein stains less intensely with Aurodye than with silver nitrate (Figure 1). We do not know why. Shown at right are the results of immunodetection with a polyclonal antiserum raised against GST–PD2zip. Bold arrowhead, the PEND protein; thin arrowhead, full-length PD2 recombinant protein. Numbers at left indicate molecular mass markers.

(B) Plastid localization of the PEND protein. Ten micrograms of protein per lane, except in lanes 4 and 8, was analyzed by LDS-PAGE. At left is staining with Coomassie blue (CBB); at right is immunodetection with a polyclonal antiserum raised against GST–PD2zip (Anti-PD2). Lanes 1 and 5 contain cell nuclei from leaf buds of 6-day-old seedlings; lanes 2 and 6, plastid nucleoids from leaf buds of 6-day-old seedlings; lanes 3 and 7, supernatant (sup) of plastid extract that was obtained after the sedimentation of nucleoids; and lanes 4 and 8, partially purified PEND protein that was obtained by DNA–Dynabeads affinity purification. The precise quantity of the PEND protein was not determined ($<$0.1 μg per lane) because of its extremely low concentration. Note that the plastids were solubilized with Nonidet P-40 to obtain nucleoids and supernatant in this experiment. The bands of histones, major proteins in the cell nuclei, are located at the bottom of the gel and were not shown in the Coomassie blue staining of the membrane. Arrowhead, the PEND protein. Numbers at left indicate molecular mass markers.

(C) Localization of the PEND protein to the plastid envelope membrane. Ten micrograms of protein per lane was analyzed by LDS-PAGE. At left is staining with Coomassie blue; at right is immunodetection with a polyclonal antiserum raised against GST–PD2zip. Lanes 1 and 6, thylakoid membranes; lanes 2 and 7, stroma fraction; lanes 3 and 8, envelope membranes from leaf buds of 7-day-old seedlings; lanes 4 and 9, envelope membranes from mature leaves of 14-day-old seedlings; and lanes 5 and 10, envelope membranes from senescing leaves of 25-day-old seedlings. Note that no detergent was used to fractionate membranes in this experiment. Arrowhead, the PEND protein. Numbers at left indicate molecular mass markers.

we observed that the PEND protein was specifically detected in the envelope membranes of young plastids (Figure 5C, lane 8). A single band was immunostained with the antibody raised against GST–PD2zip. This protein was not detected in the thylakoid membrane (Figure 5C, lane 6) or in the stroma (Figure 5C, lane 7) of young plastids. In addition, the PEND protein was no longer detected in the envelope membranes of mature (Figure 5C, lane 9, 14 days,) or senescing (Figure 5C, lane 10, 25 days) chloroplasts.

DNA Binding Activity of GST–PD2zip and the PEND Protein

The DNA binding activity of the fusion protein GST–PD2zip was analyzed and compared with the activity of the purified PEND protein (Figure 6). The GST–PD2zip fusion protein is identical to the protein used in making antibodies and contains only the bZIP region of the PD2 sequence. This construct (from 1 to 96 amino acid residues) contains only the basic region and the first half of the zipper region. The construct with the entire bZIP region (from 1 to 147 amino acid residues) was not expressed efficiently. As stated above, probe 1, which is 414 bp long, was found to be highly specific to the PEND protein. In this analysis, we used probe 1 and several partial fragments (L, M, R, LM, and MR) of probe 1 (Figure 6A) as DNA probes. All of these DNA fragments were prepared by polymerase chain reaction (PCR) and end-labeled using polynucleotide kinase.

Figure 6B shows results with GST–PD2zip. The fusion protein showed affinity for probes M (Figure 6B, lane 14), LM (Figure 6B, lane 1), and MR (Figure 6B, lane 5). Probes L and R also bound to the fusion protein in the absence of competitors (Figure 6B, lanes 10 and 16), but this binding was abolished by calf thymus DNA and poly(dA-dT) (Figure 6B, lanes 11 and 17). In contrast, probe M had a strong affinity for the fusion protein even in the presence of these competitors (Figure 6B, lane 14). The binding of fusion protein GST–PD2zip with probes LM and MR were efficiently reduced by the addition of unlabeled probe M (Figure 6B, lanes 3 and 6). These results suggest that region M contains the DNA binding site of GST–PD2zip. Essentially similar results were obtained with the purified active PEND protein, that is, the PEND protein obtained in the experiment shown in Figure 1, which contained a small amount of contaminants.

Homology with TGN Proteins

Searches for homologous sequences in various databases did not uncover sequences that were significantly similar to the PEND protein, with the exception of a GS-box binding factor 1 (GenBank accession number X91138), which shares 42% amino acid sequence identity with the PEND protein in the bZIP region (residues 17 to 140). But the similarity of this protein with the PEND protein was limited to the first zipper region (data not shown). Furthermore, the expressed sequence tag databases of Arabidopsis and rice did not contain any homologs of the PEND protein. This might be explained by the difficulty we experienced when cloning the PEND cDNA. However, we found that the PEND protein has some similarity with mammalian TGN proteins (Figure 7). The TGN proteins lack both the transit peptide and the basic region of the PEND protein; however, the sextuple repeat was conserved in an imperfect form. Repeats 2, 3, 5, and 6 are relatively better conserved in TGN proteins. The coiled-coil and the C-terminal regions were also similar between the PEND protein and TGN proteins, although the conservation among TGN proteins was remarkable in the C-terminal region. These results might suggest that the PEND protein and TGN proteins could be distant homologs, although their function in the cell is quite different now. We would suggest that the PEND protein is an ancestral form of TGN proteins, because the TGN proteins contain remnants of the sextuple repeat, which is found in the PEND protein. Because no homolog of the PEND protein was found either in the genome of cyanobacterium *Synechocystis* sp strain PCC6803 (Kaneko et al., 1996) or in the genome of any other prokaryotic organism that has been sequenced, the origin of the PEND and TGN proteins remains to be determined.

DISCUSSION

Identification of the cDNA for the PEND Protein

The data presented here demonstrate that PD2 is a cDNA for the PEND protein. The criteria for such an identification are as follows. First, the N-terminal sequence of the purified PEND protein matches the deduced sequence of the PD2 cDNA perfectly. Second, the antibody raised against the GST–PD2zip protein reacted with the purified PEND protein. In plastids, only envelope membranes from young plastids were immunostained by the antibody, and no other protein in the plastids or cell nuclei reacted with the antibody. Third, both the GST–PD2zip fusion protein and the PEND protein showed affinity for the same central part (region M) of a DNA probe (probe 1; see Figure 6). Fourth, the level of the *PD2* transcript was maximal in the light-grown young seedlings, which is consistent with the abundance of the PEND protein that had been determined by DNA binding blotting (Sato et al., 1993).

A major problem in the identification of the PEND cDNA pertains to its actual size. We have shown that the PEND protein behaves as a 130-kD polypeptide in the LDS-PAGE system, whereas the PD2 cDNA encodes a 70-kD polypeptide. The PD2 cDNA was determined to be full length, because the transcript length (2.4 kb) is similar to the length of the cDNA clone (2206 bp). The open reading frame that we found in PD2 is considered to be the longest among all of the possible ones. There is an in-frame stop codon just upstream of the initiator methionine, although which of the two

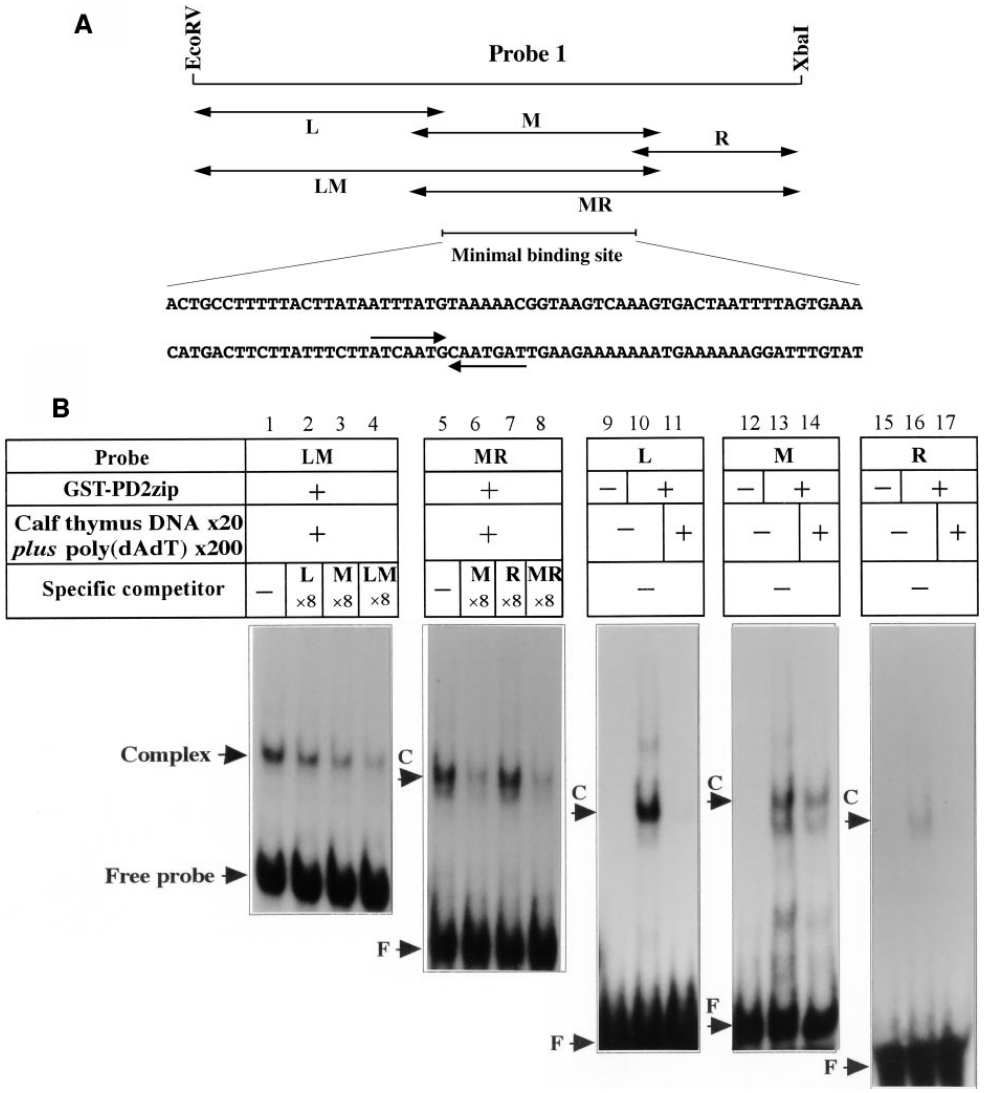

Figure 6. DNA Binding Activity of the PEND Protein.

(A) Enlarged map of binding site 1. L, M, R, LM, and MR are DNA probes that were used in the binding experiments shown in **(B)**. The nucleotide sequence of the minimal binding site for the PEND protein that was deduced from the results of experiments shown in **(B)** is shown below. An inverted repeat is indicated by arrows.

(B) DNA binding activity of the bZIP domain of PD2. End-labeled DNA probes were mixed with GST–PD2zip and then analyzed after nondenaturing gel electrophoresis. Signals were detected by autoradiography. Unlabeled competitors were added as indicated. x8 indicates eightfold excess over the probe. Plus and minus signs indicate presence and absence of the particular component, respectively. C and F stand for complex and free probe, respectively.

consecutive methionine codons is used for the initiation remains to be determined. Because we found a poly(A) tail at the 3′ end of the cDNA, there is no possibility that the open reading frame extends farther downstream. Therefore, we consider that the open reading frame of 633 (or 632, depending on the initiation codon) amino acid residues corresponds to the precursor to the PEND protein. Evidence supporting this conclusion is that the full-length PD2 polypeptide with a short hexahistidine tag, which was produced in *E. coli* cells, also migrated as a 140-kD polypeptide when subjected to LDS-PAGE. The slightly larger size of the recombinant PD2 protein is accounted for by the presence of

the transit peptide (16 residues) and the hexahistidine tag (36 residues). For the moment, we have no clear explanation for the unusual electrophoretic mobility of the PEND protein and of the full-length PD2 polypeptide. Because the GST–PD2zip protein migrated normally when subjected to SDS-PAGE, the bZIP region may not be the cause of unusual mobility. We are currently making various partial polypeptides of PD2 to determine the reason for the unusual electrophoretic behavior of the PEND protein.

The results of N-terminal sequencing of the PEND protein suggest that this protein is synthesized as a slightly larger precursor, which is then processed to the mature form. The putative transit peptide for import into the plastid is estimated to consist of 16 or 15 amino acid residues. Most of the transit peptides of known proteins of the inner envelope membrane, such as the phosphate translocator, are longer (~70 residues) than that of the PEND protein, but the transit peptide of the 37-kD inner envelope membrane protein (Dreses-Werringloer et al., 1991) contains only 21 amino acid residues. A close comparison of the putative transit peptide of the PEND protein and the transit peptide of the 37-kD protein suggests a certain similarity between these two sequences. The helical wheel representation of these two peptides (data not shown) suggests that both can form similar amphiphilic helical structures, with hydrophobic residues on one side and positively charged residues on the other. We are currently trying to determine definitively the presence of transit peptide that serves as a signal for chloroplast import. A serious drawback has been that it is not possible to express the entire polypeptide of the PEND protein in *E. coli* cells, except for the one with a histidine tag, possibly because of the toxicity of this protein to *E. coli* cells. The toxicity is probably caused by the binding of the PEND protein to the *E. coli* genome. It is even quite difficult to construct a plasmid clone that contains intact the entire open reading frame of the PEND protein. The plasmid becomes very unstable with this cDNA insert. Because of these problems, we have been unable to produce an intact PEND protein in vitro that can be used for import experiments. However, we could use an in vitro–coupled transcription–translation product of a PCR-generated cDNA. We could also attempt to transform plant cells with a fusion gene that encodes a fusion protein consisting of the transit peptide of the PEND protein and a reporter, such as β-glucuronidase or the green fluorescent protein.

Molecular Features and DNA Binding of the PEND Protein

The PEND protein is a membrane-bound DNA binding protein in the plastid. This is significant because most other known

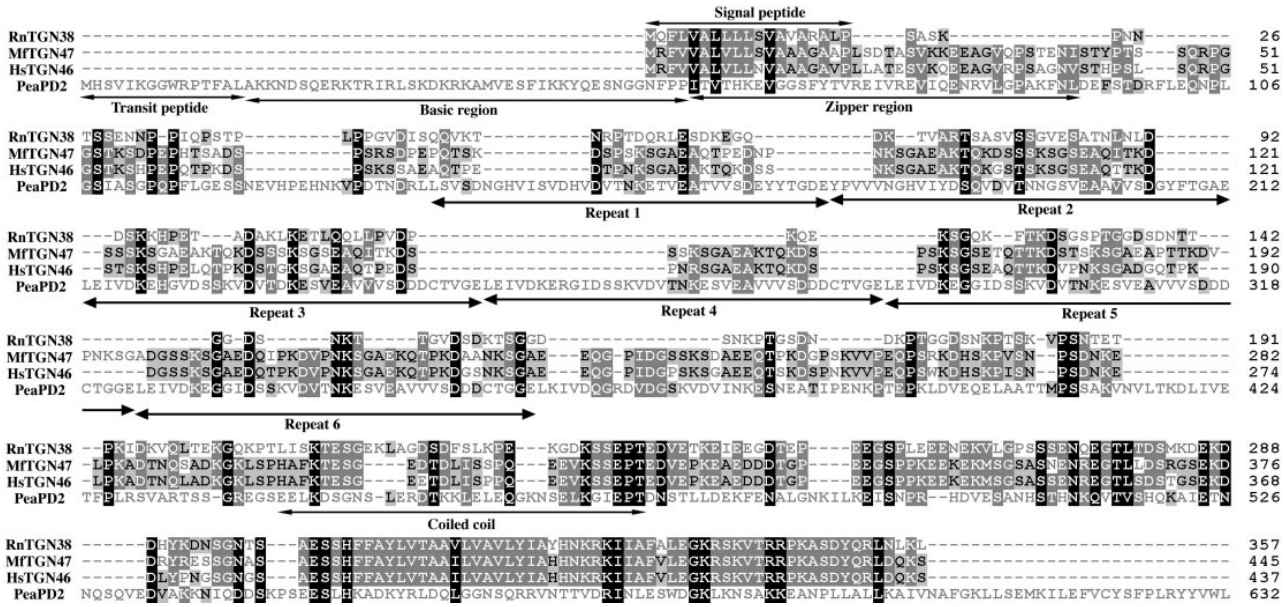

Figure 7. Alignment of the PEND Protein with TGN Proteins.

The amino acid sequences were aligned with Clustal W software, version 1.6 (Thompson et al., 1994), and the resulting alignment was shaded and modified by drawing software. The residues that are conserved in all of the sequences are shown by white letters on a black background. The residues that are conserved in three sequences are shown by white letters on a gray background. The residues that are conserved in two sequences are shown by black letters on a gray background. Other residues are shown in gray. Dashes indicate gaps inserted to improve alignment.

DNA binding proteins are soluble proteins, chromosomal proteins, or extrinsic membrane proteins. The PEND protein is, in contrast, an integral membrane protein of the inner envelope membrane and is only solubilized with a high concentration of detergent (see Results). Bacterial DNA binding proteins, such as SeqA and DnaA, that are known to bind to the membrane may not be integral membrane proteins because they are purified as soluble proteins (Slater et al., 1995; Garner and Crooke, 1996).

In this context, it is interesting that the PEND protein was found to be a distant homolog of mammalian TGN proteins (Figure 7). These proteins are also integral membrane proteins, but they are located in the *trans*-cisternae of the Golgi network of mammalian cells. The TGN proteins lack the transit peptide for plastid targeting and the basic region of the DNA binding motif of the PEND protein, but the remaining sequence features are conserved between the TGN proteins and the PEND protein. Although the TGN is involved in the processing of glycoproteins that are to be transported to the final destination, such as plasma membrane or lysosome, no definite function has been attributed to TGN proteins. Currently, no homolog of either the PEND protein or TGN proteins has been identified in prokaryotes. A detailed analysis of the bacterial genome sequences might reveal an origin of these proteins.

Another interesting point is that the PEND protein binds to the target DNA with its bZIP motif. Various bZIP transcription factors have been isolated in plants (reviewed in Menkens et al., 1995), and these bZIP domains were found to be highly conserved. However, the bZIP domain of the PEND protein has diverged from the bZIP domains of plant transcription factors. The presence of two zipper regions that are separated by $\sim$20 amino acid residues might suggest that the DNA binding domain of the PEND protein is a new type of bZIP domain. We are interested in determining the function of this new type of bZIP domain, but several attempts to express the entire bZIP domain in *E. coli* cells have not been successful (see Results). The results of this study, however, indicate that the partial bZIP domain, which includes the basic region and the first zipper region, can bind to the target DNA. The second zipper region is likely to enhance protein–protein interactions, thereby ensuring stable binding of the PEND protein to DNA. This hypothesis will be tested in future experiments.

Four sites of cpDNA have been identified as target sites of the PEND protein (Sato et al., 1995), and we have now identified a 124-bp sequence as a minimal binding site (Figure 6A). We attempted to identify a common sequence motif(s) in these target sites, but to date we have been unable to detect significant consensus sequences. We are also attempting to determine the canonical target sequence by using a technique that has cycles for selecting target DNA and amplifying by PCR. The binding site might not be as small as a hexanucleotide or an octanucleotide, which are both known as binding sites of various transcription factors.

How does the PEND protein bind to the cpDNA, which is tightly complexed with various proteins within the nucleoid? A hypothesis based on the structures of the PEND protein and some nucleoid proteins is that the bindings of the PEND protein and the nucleoid proteins to the cpDNA are not mutually exclusive. The bZIP domain, such as the one in the GCN4, a yeast transcription factor, is known to bind to the major groove of double-stranded DNA (Keller et al., 1995). The PEND protein is also likely to bind to the major groove of the cpDNA. The structures of the cDNA clones for DNA binding proteins (PD1 and PD3) of plastid nucleoid (Sato et al., 1995; Shinada and Sato, 1997) indicate that both of these contained multiple AT hook motifs that are known to bind to the minor groove of AT-rich DNA (Reeves and Nissen, 1990). It is therefore possible that the PEND protein and these AT hook proteins bind to the same target sequence of cpDNA from opposite sides.

Probable Functions of the PEND Protein

What is the function(s) of the PEND protein? In a previous paper (Sato et al., 1993), we proposed three possible functions of the PEND protein. They are related to replication, segregation, and transcription. A clue to the probable functions of the PEND protein might be in the characteristics of *PEND* gene expression. We have extensive data on the expression of this gene. The results of RNA gel blot hybridization clearly indicate that the *PEND* gene is expressed predominantly in young developing leaves of 6-day-old seedlings that are greening in the light. The results of immunoblot and DNA binding blot analysis also showed that the PEND protein can be detected only in the envelope membranes of young developing chloroplasts. The previous results of fractionation studies clearly indicate that the PEND protein is localized in the inner envelope membrane (Sato et al., 1993). These results are all consistent with each other and suggest that the PEND protein is synthesized only in the young developing leaves. At this stage, plastids are rapidly developing to become chloroplasts. The results of DuBell and Mullet (1995a, 1995b) indicate that both the replication and the transcription of cpDNA are maximal in developing chloroplasts from pea seedlings at 6 days of age. Our microscopy results also indicate that plastid nucleoids are present near the envelope membrane in the plastids of pea leaves at 5 days of age (Sato et al., 1997). We used the RNA and plastids from pea seedlings at 6 days of age in this study because it was almost impossible to prepare plastids from 5-day-old seedlings, which are very small and rather resistant to homogenization. Nevertheless, the *PEND* gene is likely to be expressed in the seedlings at 5 days of age, because the cDNA for the PEND protein was isolated from a library constructed with the RNA from 5-day-old seedlings.

It is also interesting that in wheat, topoisomerase II was found to be localized in the periphery of developing plastids (Marrison and Leech, 1992). This enzyme is in general needed to decatenate daughter DNA molecules after replication. Marrison and Leech (1992) showed that plastid nu-

cleoids were colocalized with topoisomerase II in the periphery of plastids; in mature chloroplasts, nucleoids and topoisomerase II were found at different sites within the chloroplasts. It seems that all of the machinery of replication and transcription is localized in the periphery of plastids near the envelope membrane in developing plastids. All of these data suggest that the PEND protein is present in the inner envelope membrane of plastids when the plastid nucleoids are attached to the envelope membrane and when the cpDNA is most actively replicated and transcribed. The PEND protein is therefore likely to be involved in the binding of the nucleoids to the envelope membrane in these plastids and possibly in providing a solid support for replication and transcription, as suggested for the nuclear matrix (reviewed in Razin et al., 1995).

We also envision a broader role for the binding of the plastid nucleoid to the envelope membrane. For instance, recent observations indicate that envelope membranes could be involved in some steps of chloroplast mRNA translation, at least at some stages of plastid development. First, the stabilizing factors of some specific chloroplast mRNAs encoding thylakoid membrane proteins were detected only in a yellow membrane fraction deriving from Chlamydomonas chloroplasts and corresponding most likely to envelope membranes (Zerges and Rochaix, 1998). In contrast, these membrane-bound RNA binding proteins were not found in the stroma or associated with the thylakoid membrane (which is the final localization of the translated product). Second, unpublished observations (N. Rolland, L. Janosi, M.A. Block, M. Shuda, E. Teyssier, C. Miège, C. Cheniclet, J.P. Carde, A. Kaji, and J. Joyard, submitted manuscript) have demonstrated that a ribosome recycling factor homolog (cpRRFH) is present in chloroplasts and that a fraction of it could be associated with the envelope membranes. Such a protein potentially could be involved in the chloroplast protein translation machinery. The association of the chloroplast mRNA stabilizing factors, the cpRRFH, and the PEND protein could therefore be consistent with a functional association of plastid nucleoids with the inner envelope membrane.

METHODS

Plant Materials

Two cultivars of *Pisum sativum* were used. Cultivar Douce Provence was used to prepare plastid fractions (thylakoid, stroma, and envelope), according to the method of Douce and Joyard (1982), as well as to prepare cDNA libraries. Cultivar Alaska was used to prepare cell nuclei, plastid nucleoids, and the postnucleoid supernatant, to purify the PEND protein, and to prepare DNA and RNA for hybridization analysis. The seeds were allowed to imbibe tap water overnight and were sown on moist vermiculite. They were covered with aluminum foil until seedlings emerged on day 4. Seedlings were then grown in the light ($\sim$10,000 lux or 200 μE m^{-2} sec^{-1}) at $\sim$25°C. In the RNA gel blot experiment shown in Figure 4B, seedlings that were grown in complete darkness were also used.

DNA Binding Blotting

Various fractions of plastids and the purified PEND protein were analyzed by DNA binding blotting, as described previously (Sato et al., 1993). Probe 1 (identical to probe I in Sato et al. [1993]) was used in most experiments.

Purification of the PEND Protein

All manipulations were done at 4°C or on crushed ice unless otherwise specified. Intact plastids ($\sim$500 mg of protein obtained from $\sim$600 g fresh weight of leaf buds of 6-day-old seedlings) that were suspended in 100 mL of extraction buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM 2-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride [PMSF]) supplemented with a tablet of Complete (a mixture of protease inhibitors; Boehringer Mannheim) were lysed in 2% Nonidet P-40 (Wako Pure Chemicals, Osaka, Japan) with gentle stirring for 20 min. Insoluble material was then removed by centrifugation at 2000*g* for 5 min and subsequent filtration through a cellulose acetate membrane filter (nominal aperture of 3 μm). Otherwise, the postnucleoid supernatant that had been stored with 33% glycerol at −75°C was used (Sato et al., 1997).

The supernatant was diluted with an equal volume of extraction buffer. NaCl was added to a final concentration of 50 mM. The solution was then passed through a set of anion exchange columns (two 5-mL Econo-Pac Q cartridges connected in series; Bio-Rad) that had been equilibrated with 50 mM column buffer (50 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM 2-mercaptoethanol, 0.1 mM PMSF, 0.5% Nonidet P-40, and 10% glycerol). After washing with 50 mM column buffer, bound proteins were eluted with a linear gradient from 50 mM to 1 M NaCl over a 30-min period. The eluate with 0.5 to 0.8 M NaCl (normally 12 mL) was pooled and dialyzed against 0 M Dynabeads buffer (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM PMSF, 5 mM 2-mercaptoethanol, 0.5% Nonidet P-40, and 20% glycerol) without glycerol (see below). Glycerol was then added to the dialysate to a final concentration of 20% (3 mL), and the mixture was subjected to DNA–Dynabeads affinity purification.

DNA–Dynabeads Affinity Purification

DNA probe 1, which had been cloned in pBluescript SK+ (Stragene, La Jolla, CA), was amplified by polymerase chain reaction (PCR) with a T7 primer and a biotinylated T3 primer. Biotinylated DNA probe 1 ($\sim$15 μg) was mixed with 1.5 mL of a 10% suspension of Dynabeads M-280 Streptavidin (Dynal, Oslo, Norway), which had been washed with and suspended in 50 mM Dynabeads buffer (50 mM NaCl in 0 M Dynabeads buffer). After gentle mixing for 2 hr, Dynabeads particles were collected by centrifugation followed by magnetic separation, as described by the manufacturer. The DNA–Dynabeads conjugate was blocked in 0.4 mM biotin and 0.2% BSA in 50 mM Dynabeads buffer and then washed with 50 mM Dynabeads buffer and 1 M Dynabeads buffer (1 M NaCl in 0 M Dynabeads buffer) followed by 50 mM Dynabeads buffer.

To each 15 mL (after addition of glycerol) of the dialyzed eluate from anion exchange column chromatography was added 1 mL of a 10% suspension of DNA–Dynabeads conjugate and 30 μL of 0.1 M PMSF, which was mixed gently for 2 hr. The supernatant was then removed, and the DNA–Dynabeads particles were washed three times with 50 mM Dynabeads buffer. The bound proteins were eluted three times with 200 μL of 0.3 M Dynabeads buffer (0.3 M NaCl in 0 M Dynabeads buffer) and subsequently three times with 200 μL of 1 M Dynabeads buffer. The supernatant was subjected to an identical purification procedure two more times to increase the yield of the PEND protein. In most of the analyses described in this report, this preparation of the PEND protein was used, because further purification of the PEND protein under denaturing conditions resulted in a loss of activity.

The PEND protein was finally purified on a 1-mL Resource Q column (Pharmacia Biotech) under denaturing conditions. The buffer contained 4 M urea, 20 mM Tris-HCl, 0.1 mM EDTA, 1 mM 2-mercaptoethanol, 15% glycerol, 0.5% Nonidet P-40, and additional NaCl, as specified below. The column was eluted at a flow rate of 1 mL/min with a gradient of NaCl from 50 to 500 mM in 20 min and then flushed with 1 M NaCl. The eluate at 14 to 15 min was pooled and dialyzed against a 0 M column buffer and then concentrated by ultrafiltration with Ultrafree C3 (Millipore, Bedford, MA). The protein was analyzed by lithium dodecyl sulfate (LDS)–PAGE and then transferred to a polyvinylidene difluoride membrane. The protein band, which was located by staining with Coomassie Brilliant Blue R 250, was subjected to a gas phase amino acid sequencer (model 492; ABI, Foster City, CA).

DNA Binding Screening and Cloning of the Full-Length cDNA

Poly(A)$^+$ RNA was obtained from leaf buds of 5-day-old pea seedlings (cv Douce Provence), as described previously (Sato, 1988). Poly(A)$^+$ RNA (larger than ~2 kb) was obtained by fractionation on urea–agarose gel and transfer to poly(U) paper (Orgenics, Yavne, Israel), as described previously (Sato et al., 1986). cDNA was synthesized with a Time Saver cDNA synthesis kit (Pharmacia Biotech) and then inserted into λgt11 vector (Promega). These cDNA libraries (~2×10^6 independent clones) were screened by DNA binding blotting with radiolabeled probe 1. cDNA libraries that had been made from various plant materials were also tested. Three positive clones were obtained. The clone PD2 was obtained from the library described above, and this partial cDNA was inserted into pBluescript SK+ and sequenced. A full-length cDNA clone for PD2 cDNA was obtained by screening cDNA libraries in λgt10. The cDNA clone was finally inserted in pBluescript SK+ and sequenced using standard techniques.

Hybridization Analysis

The probe used for hybridization analysis was the partial cDNA obtained by DNA binding screening. The clone corresponds to a sequence from positions 232 to 665 in the sequence of full-length PD2 (Figure 2). DNA gel blot hybridization analysis was done with pea genomic DNA, as described previously (Sato, 1988). Total RNA from leaf buds or leaves of seedlings at various stages of growth was obtained by phenol extraction, as described previously (Sato and Furuya, 1985). RNA gel blot hybridization analysis was performed with a digoxigenin-labeled probe prepared by PCR with digoxigenin-labeled dTTP (Sato, 1995).

Preparation of PD2 Fusion Proteins

The basic region plus zipper region (bZIP) of the PD2 cDNA (PD2zip, nucleotides 227 to 642) was amplified by PCR with primers PD22F (5′-ATGGATCCTGGAGGCCAACATTTGCTCTTGCA-3′, added restriction site underlined) and PD21R (5′-TACTCGAGACATGTCCATTGTCAGAAACAGAAAGCAG-3′) and then digested with BamHI (in the PD22F primer) and EcoRI (at nucleotide 482). The resulting DNA fragment was inserted into pGEX-4T-1 (Pharmacia Biotech) and then transformed into *Escherichia coli* XL-1 Blue cells (pGEX-PD2zip). The overexpression and purification with glutathione–Sepharose 4B (Pharmacia Biotech) of the GST–PD2zip fusion protein was performed according to the manufacturer's directions. The fusion protein that was eluted from the column was further purified by gel filtration with a Superdex 200HR column (Pharmacia Biotech) that had been equilibrated with 0.5 M NaCl and 0.05 M sodium phosphate, pH 7.0. We also tried to insert the whole PCR product after digestion with BamHI and XhoI, but we could not obtain a transformant, possibly because of the toxicity of the product.

The entire open reading frame of the PD2 cDNA was amplified by PCR with primers PD2F (5′-ATGGATCCCATTCTGTAATTAAGGGTGGTTGG-3′) and PD2R (5′-CGAAGCTTCCACAGTCACAGCCATACATAATA-3′) and then digested with BamHI and HindIII. The resulting DNA fragment was inserted into pRSET-A (Invitrogen, San Diego, CA) and transformed into *E. coli* XL-1 Blue (pRSET-PD2). Overexpression and purification of the fusion protein under denaturing conditions were performed according to the manufacturer's instructions (Invitrogen).

Immunoblot Analysis

Polyclonal antisera against the fusion protein GST–PD2zip was raised in mice. Immunoblot analysis was performed essentially as described for DNA binding analysis until the step of transfer to membrane. Reactions with antibodies were performed essentially as described previously (Sato, 1995). Alkaline phosphatase activity was located with either a set of chromogenic substrates (nitro blue tetrazolium and bromochloroindolyl phosphate; Promega) or a chemiluminescent substrate (CDP-Star; Boehringer Mannheim). Plastid nucleoids were prepared by the method of Nemoto et al. (1988), as modified by Sato et al. (1997). Cell nuclei for comparison were prepared from 6-day-old seedlings, according to the method of Watson and Thompson (1986).

Gel Mobility Shift Analysis

A radiolabeled DNA probe and protein as well as unlabeled competitor DNA (calf thymus DNA [Sigma] and poly[dA-dT] [Pharmacia Biotech]) were mixed in a 10-μL reaction mixture containing 60 mM KCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10% glycerol, and 0.01% bromophenol blue. After standing on ice for 30 min, the mixture was loaded on a nondenaturing 5% polyacrylamide gel (80 mm high × 84 mm wide), which had been preelectrophoresed for 2 hr. The buffer for gel electrophoresis contained 6.7 mM Tris, 10 mM acetic acid, and 0.1 mM EDTA, pH 8.0. After electrophoresis at 15 V/cm for ~1 hr, the gel was dried and autoradiographed.

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