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Phospholipid Transfer Protein: Full-length cDNA and Amino Acid Sequence in Maize

AMINO ACID SEQUENCE HOMOLOGIES BETWEEN PLANT PHOSPHOLIPID TRANSFER PROTEINS*

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We have determined the primary structure of a phospholipid transfer protein (PLTP) isolated from maize seeds. This protein consists of 93 amino acids and shows internal homology originating in the repetition of (do)decapeptides.

By using antibodies against maize PLTP, we have isolated from a cDNA library one positive clone (6B6) which corresponds to the incomplete nucleotide sequence. Another cDNA clone (9C2) was obtained by screening a size-selected library with 6B6. Clone 9C2 (822 base pairs) corresponds to the full-length cDNA of the phospholipid-transfer protein whose mRNA contains 0.8 kilobase. Southern blot analysis shows that the maize genome may contain several PLTP genes. In addition, the deduced amino acid sequence of clone 9C2 reveals the presence of a signal peptide. The significance of this signal peptide (27 amino acids) might be related to the function of the phospholipid-transfer protein.

The amino acid sequence of maize PLTP was compared to those isolated from spinach leaves or castor bean seeds which exhibit physicochemical properties close to those of the maize protein. A high homology was observed between the three sequences. Three domains can be distinguished: a highly charged central core (around 40–60), a very hydrophobic N-terminal sequence characteristic of polypeptide-membrane interaction, and a hydrophilic C terminus. A model for plant phospholipid-transfer proteins is proposed in which the phospholipid molecule is embedded within the protein with its polar moiety interacting with the central hydrophilic core of the protein, whereas the N-terminal region plunges within the membrane in the transfer process.

The intracellular transport of phospholipids is thought to require the participation of phospholipid-transfer proteins (PLTP)[1] mainly found in the cytosol. Phospholipid-transfer proteins presumably play important roles in the biogenesis and renewal of membranes as well as in the transport of hydrophobic compounds (1–5). These proteins have been purified to homogeneity from various eucaryotic cells: animals (6–9), plants (10–12), yeasts (13–14), and from a procaryotic cell (15). The primary structure of various PLTPs has been determined: beef-liver phosphatidylcholine transfer protein specific for phosphatidylcholine (16), nonspecific lipid transfer protein (nsLTP) from beef liver (17), nsLTP isolated from rat liver (also called "sterol-carrier protein 2") (18), and PLTPs purified from spinach leaves (19) and castor bean seeds (20).

Considerable information about the *in vitro* ability of PLTPs to transfer phospholipids is available (1–5). However, no clear *in vivo* demonstration of the role of PLTPs has been given up to now. One way of determining this role is to establish a correlation between membrane biogenesis and PLTP biosynthesis. The synthesis of this protein has only recently been studied by immunoprecipitation of *in vitro* translation products for a nsLTP in rat adrenocortical cells (21). No cDNA or genomic DNA coding for these proteins are available from either animal or plant systems.

PLTPs are abundant in plant organs such as seeds or leaves. Highly purified PLTPs have been obtained from maize seedlings, spinach leaves, or castor bean endosperm (10–12). In the present work, we have chosen as a model the PLTP isolated from maize seeds since (i) maize PLTP has been isolated to homogeneity (11), (ii) specific polyclonal antibodies are available (22), and (iii) mRNAs for PLTP might be relatively abundant in maize coleoptiles (23). This led us to

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J04176.

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[1] The abbreviations used are: PLTP, phospholipid-transfer protein; nsLTP, nonspecific lipid transfer protein; HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate; PTH, phenylthiohydantoin.

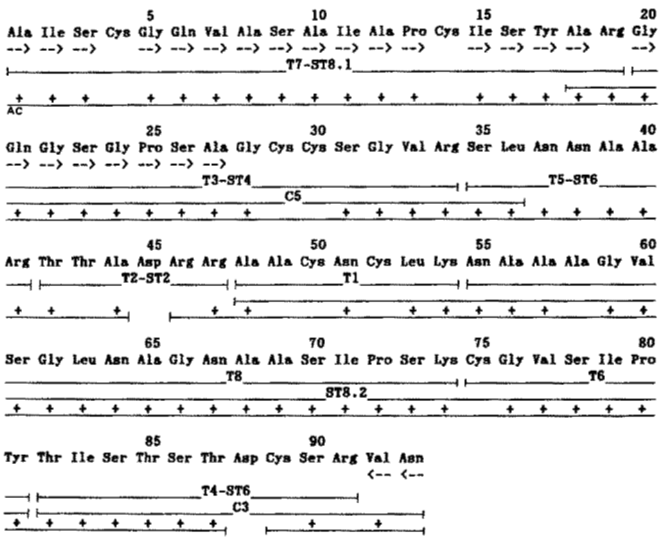

FIG. 1. **Amino acid sequence of maize phospholipid-transfer protein.** *T*, tryptic peptides; *ST*, tryptic peptides obtained after protein succinylation; *C*, chymotryptic peptides; *AC*, acetic acid cleavage of the protein; – –>, automatic Edman degradation; <– –, carboxypeptidase A sequencing; +, residues identified in the mixture of peptides obtained through acetic acid cleavage.

establish the primary structure of maize PLTP and to search for cDNA clones corresponding to this protein.

We report here the complete amino acid sequence of maize PLTP and the isolation and characterization of a full-length cDNA coding for this protein. This gave the first opportunity to compare the structural properties of various plant proteins having a common functional role of transferring phospholipids. This comparison clearly revealed structural homologies between these proteins and led to the proposal of a model for their mode of action. To our knowledge, this is the first isolation of a cDNA coding for a protein of this family.

MATERIALS AND METHODS[2]

RESULTS

Amino Acid Sequence of Maize PLTP—Fig. 1 shows the complete sequence of the maize PLTP derived from the alignment of the peptides obtained through different hydrolytic procedures. Unlike what has been reported by Takishima *et al.* (20) on castor bean PLTP, no microheterogeneity has been observed in the amino acid derivative analysis of maize PLTP. The very low number of aromatic amino acids has to be noticed (no tryptophan, no phenylalanine, and only two tyrosines); the same proportion was found in the other plant lipid-transfer proteins. The absence of histidine, methionine, and glutamic acid, noted in the maize PLTP, is more or less constant among these proteins.

Internal sequence homologies within the maize PLTP have been revealed using DIAGON with a percent score of 122 resulting from the regular repetition of a common consensus sequence of 10–12 residues (Fig. 2). Point mutations are frequent from one peptide to another, revealing the evolution degree of the maize PLTP.

The Isolation of Positive Clones—A cDNA library was prepared from 7-day-old coleoptiles E41 (double hybrid line). From 600 recombinant clones screened with anti-PLTP antibodies, one positive clone was isolated (6B6). This clone contains an insert of 677 base pairs. Sequencing of the cDNA insert revealed that the deduced amino acid sequence corresponds to the partial amino acid sequence of purified PLTP (data not shown). Therefore the large *Pst*I fragment of this clone was used to screen a new library prepared from size-selected cDNA synthesized from 7-day-old coleoptiles W64A (pure inbred line) mRNAs in the hope of detecting the full-length cDNA.

From 1000 recombinant colonies screened, four positive clones were obtained. The inserts were isolated and their size determined. The longest one (822 nucleotides) was characterized (9C2). Its partial restriction map is given in Fig. 3.

cDNA Sequence—The nucleotide sequence of 9C2 was determined as indicated in Fig. 3 and is presented in Fig. 4. The length of the cDNA is 822 nucleotides, excluding the poly(dG) and the poly(dC) at the 5′ end and 3′ end, respectively. This result is in agreement with the Northern analysis (Fig. 5*A*). The first AUG codon (position 102) presents the consensus sequence of an initiator codon (42). This codon is followed by a large open reading frame of 360 nucleotides which corresponds to 120 amino acid residues with a calculated molecular mass of 11,635. The open reading frame ends with a TGA terminator codon at position 462 followed by 358 nucleotides, an unusually long 3′ noncoding sequence (43). A perfect consensus polyadenylation sequence is located in the middle of the 3′ noncoding region. This part of the sequence contains several small repeated sequences such as: CCCG, TTTG, and TTTA. The codon usage for the gene encoding PLTP is shown in Table I. There is a strong preference for C + G (93%) in the third position. The coding region contains 72.3% of (G + C) in contrast with the 5′ (63%) and 3′ noncoding regions (48.7%). The fact that A and U are almost totally excluded from the third position of the codons has also been noted for other maize proteins such as glutelin-2 (30), gluthathione-*S*-

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AGCTGCCATTGCCATCTACTGACCTGTGACCTCCGCATTCCCAAACAGCC      50

CGAGCGAGCGAGCTAGCAGAGCGGCGGCAGGCACCTCCCTCCTCAAGGAAC   101

ATG GCC CGC ACG CAC CAC TTG GCA GTA GTG GCG ACC GCC    140
Met Ala Arg Thr Gln Gln Leu Ala Val Val Ala Thr Ala     13

GTG GTC GCC TTG GTG CTG CTG GCG GCG GCG ACC TCG GAG    179
Val Val Ala Leu Val Leu Leu Ala Ala Ala Thr Ser Glu     26

GCG GCC ATC AGC TGC GCG CAG GTG GCG TCG GCC ATC GCG    218
Ala Ala Ile Ser Cys Gly Gln Val Ala Ser Ala Ile Ala     39

CCC TGC ATC TCC TAC GCG CGC GGC CAG GGC TCG GGG CCC    257
Pro Cys Ile Ser Tyr Ala Arg Gly Gln Gly Ser Gly Pro     52

TCC GCC GGC TGC TGC ACC GGC GTC AGG AGC GTC AAC AAC    296
Ser Ala Gly Cys Cys Ser Gly Val Arg Ser Leu Asn Asn     65

GCC GCC CGC ACC ACC GCC GAC CGC CGC GCC GCC TGC AAC    335
Ala Ala Arg Thr Thr Ala Asp Arg Arg Ala Ala Cys Asn     78

TGC CTC AAG AAC GCC GCC CGC GGC GTC AGC GGC CTC AAC    374
Cys Leu Lys Asn Ala Ala Arg Gly Val Ser Gly Leu Asn     91

GCC GGT AAC GCC GCC AGC ATC CCC TCC AAG TGC GGC GTC    413
Ala Gly Asn Ala Ala Ser Ile Pro Ser Lys Cys Gly Val    104

AGC ATC CCC TAC ACC ATC AGC ACC TCC ACC GAC TGC TCC    452
Ser Ile Pro Tyr Thr Ile Ser Thr Ser Thr Asp Cys Ser    117

AGG GTG AAC TGA ACCCTAAACGACGACGACGGCGCCGGCGGCCGCCT    499
Arg Val Asn  -                                         120

GAAGACGATCCATATCTATCCCGCCGTCGTCGGCGCCCCGCGGATCGGACCG   550

TCATGACCATATCTACCTACGCATGATCACTACTTGAATGTCTACTACTGC    601

TACGTACTACTACTGAATAAAAAACCTCTCCGCCGCACATCGTCCTTGTGTT   652

TGTGTGTGTGAGAGAGAGAGGAGACGGAGAGCCGGCCATGTCCTTTTGGCT   703

GTTTGTTTGGTTTATTTTTGAGTTCATGCGGTCTTTATTTATTTCCATGTA   754

CGCTGCTTCCTCGTACCCACTTTAAATTTGTACCGATGCATGCATGTTCTT    805

GATGATACAAAAAAAAA                                      822
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FIG. 4. **Nucleotide and corresponding amino acid sequences of clone 9C2.** The signal peptide sequence is *underlined*. The putative polyadenylation signal in the 3′ noncoding region is *underlined*. The coding sequence is in **bold characters.**

[2] Portions of this paper (including "Materials and Methods," Figs. 2, 3, 5, 6, 8, and 9, and Tables I and II) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

transferase (44), and histones H3 and H4 (45). This is also true for barley amylase (46), but not for maize zein (47) where A represents 30% of the third position and the other bases are equally represented.

Southern Blot Analysis—To study the gene organization, genomic DNA from W64A was digested with *Eco*RI for which there is no site in the cDNA. The products were analyzed by Southern blot (Fig. 5*B*). Four DNA fragments hybridized with the 6B6 probe. The hybridization pattern suggests that the maize genome may contain several PLTP genes.

Comparison of the Primary Structure of Plant PLTPs—In Table II are gathered some data calculated from the amino acid sequence of the maize PLTP and their comparison to those of spinach and castor bean PLTPs. The maize protein molecular mass is the closest to the spinach protein one; this is mainly due to the comparable mean molecular mass of the residues, particularly low in these proteins. All these proteins are basic as indicated by their isoelectric points which are respectively 8.97, 9.36, and 8.56 for the maize, spinach, and castor bean PLTPs.

Fig. 6 exhibits the alignment matrices obtained with the program DIAGON run with a percent score of 125. It shows that PLTPs are very homologous to each other, except in the region 23–27, and that the maize PLTP is closest to the spinach protein than to the castor bean one. Besides, it shows the internal peptide repeats of PLTPs. The precise alignment of the sequences is shown in Fig. 7: a deletion of 2 residues is necessary at positions 25 and 26 for aligning the spinach protein to the maize protein, and a single residue shift toward the C-terminal end is needed to align the castor bean protein with the others. The percent match between the aligned sequences (51.6, 47.8, 41.3, and 32.3% for the comparisons of maize *versus* spinach, maize *versus* castor bean, spinach *versus* castor bean, and of all sequences, respectively) and the homology scores (−260, −253, and −212 for the comparisons of maize *versus* spinach, maize *versus* castor bean, and spinach *versus* castor bean, respectively) indicate the very high homology between these proteins. Most of the alterations correspond to conservative replacements which occur all along the polypeptide chain, with the exception of the two regions of lower homology. One of these regions is located at residues 21–30, 22–23, and 21–31 for the comparisons of maize *versus* spinach, maize *versus* castor bean, and spinach *versus* castor bean, respectively. The other region is located at residues 59–67 and 58–67 for the comparisons of maize *versus* castor bean and spinach *versus* castor bean, respectively. The conservation and the regularity of the positions of cysteinyl residues and of charged amino acids have to be noted.

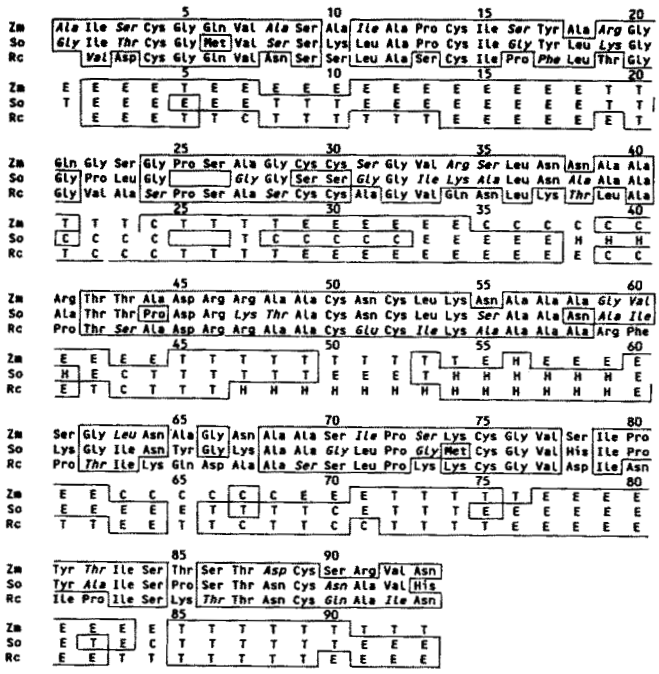

FIG. 7. **Comparison of the primary and deduced secondary structures of phospholipid-transfer proteins.** *Zm*, maize; *So*, spinach; *Rc*, castor bean. *Boxes* emphasize homologies, including the replacements by structurally equivalent amino acids (*printed in italics*). The secondary structure has been calculated with all decision constants equal to zero, except for α-helix (decision constant = 100); *H*, α-helix; *E*, extended structure; *T*, β-turn; *C*, aperiodic structure.

Secondary Structure Prediction of Plant PLTPs—Fig. 7 also shows the comparison of the predicted secondary structures, obtained with a decision constant of 100 for α-helix prediction (48). This value arises from the fact that the PLTPs are predicted as "all β" proteins, due to the high percentage of β-structure compared to other ones (Table II). In these conditions, the maize PLTP can be characterized by the highest proportion in extended structure and the lowest one in α-helix. From Fig. 7, one can observe that N- and C-terminal ends are all predicted in extended structure joined by turns and that the central core of the molecules, although exhibiting the strongest sequence homology, is predicted in various conformations, which suggests the occurrence of aperiodic structures in this portion. Besides, except in the central part of the molecules, turns occur regularly, like cysteinyl residues, every 10–12 residues.

Hydropathy and Flexibility—The hydropathy profiles of the three PLTPs are presented in Fig. 8*A*. These diagrams are roughly similar to each others and are symmetrical around a center situated at residues 40–42. The identical patterns are: (i) the high values of the hydropathy index which reach 1.81, 1.33, and 1.86 around the residue 13 for maize, spinach, and castor bean PLTPs, respectively; these values are characteristic of protein-membrane interaction; (ii) a very hydrophilic region near the position 43, in the central core of the sequence, surrounded by two hydrophobic areas; (iii) a hydrophobic C-terminal region ending with a hydrophilic C terminus. With exception of the N-terminal highly hydrophobic portion, all the other hydrophobic areas are characterized by hydropathy indices ranging about 1.2–1.3, typical values of the interior of proteins.

The flexibility profiles of the various PLTPs are shown in Fig. 8*B*. As the hydropathy profiles, they are all roughly comparable to each other. The three identical regions which can be identified through this parameter correspond to positions 11–25, 40–60, and the C-terminal end after residue 72. These regions are those which share in common a similar hydropathy profile.

The comparison of the various structural parameters of the maize PLTP (Fig. 9) reveals the presence of characteristic regions which are very conservative in the other plant PLTPs (Fig. 7): (i) the highly charged portion at positions 45–47; (ii) the position of cysteinyl residues, regularly distributed along the sequence like the positively charged residues, in a manner symmetrical around positions 40–42; (iii) the regular appearance of turns (positions 9, 28, 47, 74, and 84); (iv) the β-structure at the N terminus up to residue 19, between positions 30 and 39 and at the C-terminal end; (v) concerning the hydropathy and flexibility profiles, the conservative regions correspond to positions 11–25, 40–60, and the C-terminal end after residue 72.

Relationships between Structure and Function of PLTPs—Considering that the function of maize PLTP is to extract a

phospholipid molecule from a membrane bilayer and to transfer it through a polar medium, we have tentatively drawn a schematic model which is consistent with the data presented in this paper. The major assumption is that the maize PLTP is designed in such a way that it forms a sort of cage around the hydrophobic moiety of the phospholipid while the charged phosphate group is directly interacting with the central highly charged core of the protein (residues 46–47). Considering the dimensions, it is worth recalling that the size of a whole phospholipid molecule is about 38 Å long, *i.e.* the same length than that of a β-strand of 11 residues (the fatty acid chain itself corresponds to an 8-residue β-strand). Thus, the 10–12 amino acid peptides in β-pleated sheet, separated from each other by charged residues and β-turns, may be disposed all along the phospholipid hydrophobic moiety. The size of the interior of the cage formed by the β-strands is compatible with the width of a phospholipid molecule. On the other hand, the charges allow the complex formed by the protein and the phospholipid to be soluble.

Fig. 10 represents such a model, taking into account most of the parameters described in this paper. One can observe the proximity of the cysteinyl residues which could form disulfide bridges between polypeptide strands. As regards the possible interaction of the PLTP with the membrane, one can observe that the N-terminal β-sheet is characterized by a hydropathy index around 1.85 and that this is the only part of the molecule exhibiting such a high value. This is consistent with a polypeptide chain interacting with a membrane and not with the hydrophobic interior of a protein (49). It is likely that the N-terminal region should be able to plunge into the lipid bilayer, facilitating the interaction of the phosphate with the lysyl residues in position 46–47. On the other hand, the C-terminal end is quite polar and may contribute to extract the protein-phospholipid complex from the membrane. The flexibility of the molecule around positions 22 and 45 (the presumed phosphate interaction site) and at the C terminus would allow the embedding of the N-terminal end within the membrane bilayer, leaving the charged 20–45 loop in the cytoplasm, resulting in the phospholipid extraction from the bilayer.

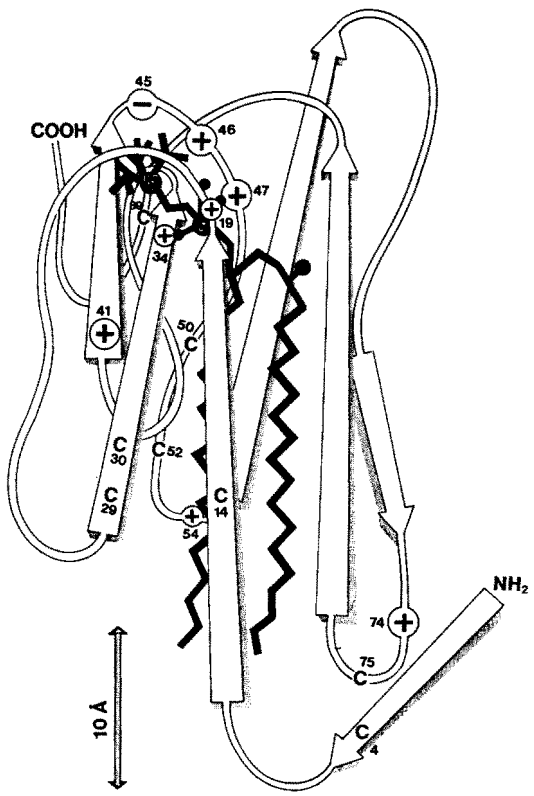

FIG. 10. **Tentative model of the interaction of a phospholipid molecule with a phospholipid-transfer protein.** The phospholipid molecule is *drawn in black. Arrows* indicate β-structure strands. *C*, cysteine; +, positive charge; −, negative charge.

DISCUSSION

PLTPs have been purified from various cells (1–5). Their amino acid sequences have been established, but so far no nucleotide sequence has been available. In the present work, we have isolated and characterized the first cDNA clones for a phospholipid-transfer protein. One of these clones corresponds to the full-length sequence of maize PLTP.

The amino acid sequence of maize protein presents a high homology with the sequence of PLTP from spinach leaves (19) and castor bean endosperm (20). This homology could be linked to their common function of transferring phospholipids. It is worthwhile to notice that these PLTPs are characterized by (do)decapeptides in tandem repeats which have nevertheless evolved from each other. The regular repetition of peptide units within the plant PLTPs proteins strongly suggests that they have originated through the duplication of a common ancestral gene. The constancy of internal repetitions results in an apparent symmetry of the PLTP molecules. The comparison of several structural parameters of the PLTPs reveals some common properties, like the positively charged residues in the central part of the molecule, the position of the cysteinyl residues, or the hydropathy and flexibility profiles.

Considering this information about the primary structure of plant PLTPs, we have drawn a model consistent with the data, but still speculative. In agreement with this hypothesis, it has been shown that a phosphatidylcholine molecule is bound to the beef liver phosphatidylcholine-transfer protein (1, 50), whereas phosphatidylinositol and phosphatidylcholine are bound, but not simultaneously, to the same hydrophobic site of the phosphatidylinositol-specific protein from beef brain (phosphatidylinositol-transfer protein) (51). A model for the acyl-binding sites of phosphatidylcholine-transfer protein from beef liver has been proposed recently (52). According to this model, the sn1 and sn2 acyl chain sites are distinct, the sn2 site being wider than the sn1 site. These conclusions are based on studies on the binding of different species of pyrenyl-phosphatidylcholine. Binding of phospholipids on rat liver nsLTP has been demonstrated only recently by using a fluorescent-labeled phospholipid (53). Plant PLTPs are also able to bind, but more weakly than animal proteins, phosphatidylcholine. This has been shown with maize (54) or spinach (10) proteins. The involvement of electrostatic charges in the binding of phospholipid molecule to the protein can be correlated with the inhibiting effect of cations on the activity of maize or spinach proteins (10, 54). This also could explain the inability of these proteins to transfer cholesteryl-oleate (10, 54). The model presented in this paper permits the hypothesis of a probable mechanism of the PLTP function which acts as a kind of "detergent mechanism." Besides crystallographic data, it has to be supported by other experimental results, mainly the positioning of disulfide bridges and the study of the conformation changes provoked by membrane-PLTP interactions.

When the amino acid sequence of PLTP deduced from the nucleotide sequence is compared with the sequence of the purified protein, the only difference observed is the presence of an extra peptide of 27 amino acids at N terminus. This

fragment presents the characteristics of signal peptides.

The physiological significance of this observation could be related to the interactions, already described, of these proteins and intracellular membranes. Using immunochemical techniques, it has been shown that mitochondria isolated from maize coleoptiles (55) or rat liver (56) contain bound PLTPs. Indeed, the presence of a signal peptide shows that this protein can be bound to membranes or stretch across the membrane although the major part is recovered in the cytosol. Given the presence of the signal peptide, it would be interesting to study the localization of the synthesis of PLTPs. It is of interest to note that high molecular mass precursors of nsLTP have been found in rat tissues (21). Immunoprecipitation of rat adrenal nsLTP, synthesized in a cell-free translation system, revealed a band corresponding to 14.4 kDa; this value is higher than that of the mature protein which is 11.3 kDa, suggesting the presence of a signal peptide (21). From these data, it is concluded that this nsLTP undergoes post-translational processing leading to the mature protein.

In conclusion, the availability of cDNA corresponding to maize PLTP opens new perspectives for the study of the processing of PLTP precursor and the isolation of the PLTP gene(s). This cDNA could be used as a probe for studies on the variations of the levels of PLTP mRNA in plant tissues. Future investigations will involve site-directed mutagenesis and transformation of plants in order to establish the physiological role of PLTPs in plant cells. It should be also interesting to understand why these cytosolic proteins are synthesized with a signal peptide, usually characteristic of exported proteins.

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Supplementary material to

PHOSPHOLIPID-TRANSFER PROTEIN
FULL-LENGTH cDNA AND AMINO ACID SEQUENCE IN MAIZE

AMINO ACID SEQUENCE HOMOLOGIES BETWEEN PLANT PHOSPHOLIPID-TRANSFER PROTEINS

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MATERIALS AND METHODS

Materials - Restriction enzymes were purchased from New England Biolabs, Boehringer Mannheim and Appligene. E.coli DNA polymerase I (Klenow fragment) was from Amersham and T4 polynucleotide kinase was obtained from NEN Research Products. Bacterial alkaline phosphatase was from Appligene and avian myeloblastosis virus (AMV) reverse transcriptase was purchased from Promega Biotec. (α-^{32}P) ATP (111 TBq/mmol), (γ-^{32}P) dCTP (111 TBq/mmol) and ^{125}I protein A (1,11 GBq/mg) were from Amersham. CNBr-activated Sepharose 4B was from Pharmacia. The nitrocellulose papers HATF-0.45 um and BA 85 were respectively from Millipore and Schleicher and Schuell. Hybond N was from Amersham.

Purification of maize PLTP - Maize PLTP was isolated from maize seeds (INRA 508) as previously described (11). It was desalted by gel filtration on Sephadex G-25, equilibrated and eluted with 3.5M acetic acid.

Hydrolyses and separation of peptides - Enzymatic and chemical cleavages of the protein as well as the fractionation of peptides by HPLC were done as described elsewhere (19).

Amino acid analysis - The amino acid analyses were conducted following the PICO-TAG method adapted from Heinrikson and Meredith (24) . The amino acid derivatives were separated with a Nova-Pak standard column at 32°C using a 25 min linear gradient of 5 to 45 % of B buffer (A buffer := 7.5 mM sodium acetate pH 5.9 ; B buffer : 60 mM sodium acetate pH 5.9, 60 % acetonitrile) with a flow rate of 1.3 ml/min.

Amino acid sequencing - Automated Edman degradation of the whole protein and of peptides was performed using an Applied Biosystems 470A sequenator and its dedicated PTH HPLC-analyzer model 120A with reagents and methods of the manufacturer. The N-terminal sequence of the whole protein was conducted at the 0.2 nanomole level, whereas peptide sequencing was completed to end with 0.5 to 1 nanomole. Neither Cys nor cysteic acid could be identified with the HPLC system used ; Cys 4 and Cys 14 were determined by sequencing of the S-carboxymethylated protein and other Cys identified by amino acid analysis of succinylated trypsin peptides prepared following Bouillon et al (19). C-terminal sequencing was conducted on performic acid denaturated protein at 100 nmol level according to Ambler (25) . The C-terminal amino acids, splitted by bovine pancreas carboxypeptidase A (Boehringer), were analyzed with a Biotronik 5001 apparatus using lithium citrate buffers.

mRNA preparation and construction of cDNA libraries - Maize seedlings (Zea mays c.v. W 64A and E41) come from a pure imbred line (W64A) and a double hybrid line (E41). The seedlings were germinated for 7 days in the dark at 27°C as previously described (26). Poly(A)+ RNAs were extracted from coleoptiles and were purified by two successive passages through an oligo dT cellulose chromatographic column (27).

First and second cDNA strands were synthesized as described by Gubler and Hoffman (28). A first cDNA library was constructed from E41 by inserting total cDNA into the vector pBR322 at the Pst I site by dG/dC tailing (29) and using E. coli HB 101 as a host. In addition, a second library was prepared by the same technique (28) with size-selected cDNAs (0.5-2.5 Kb) obtained from W64A.

Library screening - To isolate cDNA clones encoding PLTP, the first library was screened with anti-PLTP antibodies. The antibodies were prepared as previously described (22). To remove the nonspecific antibodies against E. coli, the total proteins of E. coli were fixed onto a CNBr-activated Sepharose 4B according to the indications of the manufacturer. The complex (400 µl) was mixed to PLTP antibodies (900 µl) with phenylmethyl-sulfonyl fluoride (12 µM) and apoprotinin (7.5 uM), shaken for 2 h at room temperature and then filtered through glass fibers. Recombinant clones were screened using the method described by Prat et al. (30). One positive clone was identified (6B6) and was further investigated.

The size-selected library was screened with the large Pst I fragment from the 6B6 insert (the restriction map of 6B6, not shown, is quite similar to that of 9C2). This probe was radiolabelled by random priming according to Feinberg and Vogelstein (31), and used to hybridize the colonies (32). Several positive clones were obtained and the longest one (9C2) was further investigated.

Subcloning and sequencing of cDNA - Plasmid DNAs were isolated using the method of lysis by boiling (33). Inserts from recombinant plasmids were purified by agarose gel electrophoresis (33). Excised cDNA inserts or appropriate restriction fragments were then subcloned into M13mp10, M13mp11 M13mp18 or M13mp19 (34). The nucleotide sequence was determined using the dideoxy chain termination method of Sanger et al. (35). The sequence was totally established on both strands.

Northern Blot Analysis - Total cellular RNA (10 µg) was heated at 65°C, fractionated by electrophoresis through an 1.5 % agarose gel containing formaldehyde (7%), then transferred to HATF-0.45 µm paper (33). Prehybridization was carried out as described previously (36). Hybridization was carried out overnight at 50°C using the same conditions as for prehybridization, it was performed in the presence of the large Pst I fragment isolated from the 6B6 insert labelled by random priming (31). Following hybridization the membrane was washed in 0.3 X SSC, 0.1 % SDS at 65°C and autoradiographed at -80°C using intensifying screens.

Southern Blot Analysis - For Southern blot analysis, DNA was prepared from maize seedlings (W64A) and digested with EcoR I (33). Aliquots (8 µg) were electrophoresed on 0.8% agarose gels and transferred to nylon membranes (Hybond N) as described by the manufacturer. The filters were hybridized with the plasmid (6B6) labelled by nick translation (33) to a specific activity of 10^8cpm/µg. Hybridization was carried out overnight at 62°C in 5 X SSC, 0.5% SDS, 5 X Denhardt, 100 µg/ml of calf thymus DNA, 2.5 mM EDTA and 50 mM NaH_2PO_4 pH 7.5. The filters were washed at 62°C three times for 30 min in 3 X SSC and 0.1% SDS, and once in 0.3 X SSC and autoradiographed.

Computer programs and analysis - All nucleotide and amino acid sequencing data were analyzed using CITI 2 data base (Paris) and Micro Genie (Beckman).

Sequence homology determination- Homologies between lipid-transfer proteins were revealed using the DIAGON algorithm of Staden (37) and alignments performed using the treatment of Kanehisa (38) with a window width of 10 to 2 uplets. Homologies were also investigated using the Kanehisa's algorithm with the following libraries : NBRF (release 46) and EMBL (release 10) available at the French data base BISANCE. All computations were done using the similarity matrix of M.O. Dayhoff.

Structural parameters computation- Secondary structure predictions were established using the algorithm of Garnier et al. (39) ; hydropathy profiles were done with the programme of Kyte and Doolittle (40) and flexibility diagrams obtained with the method of Karplus and Schulz (41).

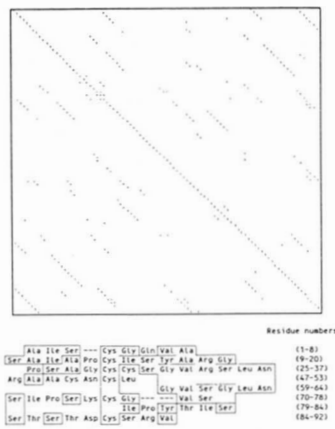

Fig. 2. Internal homologies within the maize phospholipid-transfer protein

The DIAGON plot was calculated using a percent score of 122. The homologies of the tandem repeats are emphasized by boxes.

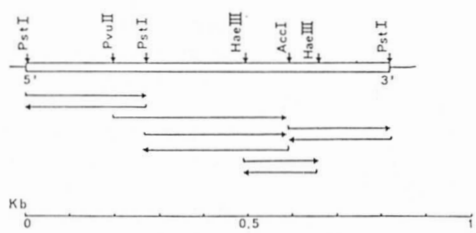

Fig.3
Partial restriction map and sequencing strategy of 9C2 cDNA.

The restriction cleavage map was determined by single or double digestion of 9C2 with various restriction enzymes. The partial restriction map shows only the sites used in the present study. The arrows indicate the directions and approximate lengths of the sequences determinated. The sequence was determined on both strands by the dideoxy chain termination procedure (35).

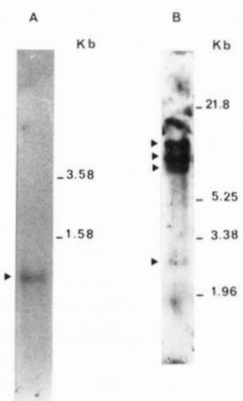

Fig.5
Northern and Southern blot analyses.

For Northern blot analysis (A), 10 µg of total cellular RNA from maize coleoptiles (W64A) were used. Hybridization was carried out with the large Pst I insert of 6B6 labelled by random priming. The arrow indicates the mRNA to which the probe has hybridized. The size markers indicated on the right side of the figure correspond to 18S (1.92 Kb) and 25S (3.58 Kb) ribosomal RNAs.
For Southern blot analysis (B), 10 µg of maize genomic DNA (W64A) was digested with EcoR I. Hybridization was carried out with the 6B6 plasmid labelled by nick translation. The arrows indicate the DNA fragments to which the probe has hybridized. The size of markers are indicated on the right side of the figure.

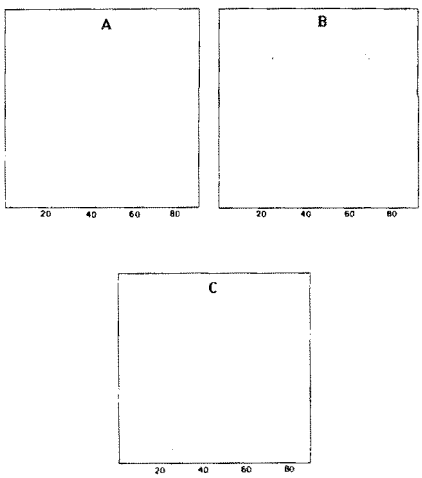

Fig.6. Comparison of the sequences of phospholipid-transfer proteins from maize, spinach and castor bean using DIAGON diagrams

The percent score used was 125.
A : horizontal sequence : maize ; vertical sequence : spinach
B : horizontal sequence : maize ; vertical sequence : castor bean
C : horizontal sequence : castor bean ; vertical sequence : spinach.

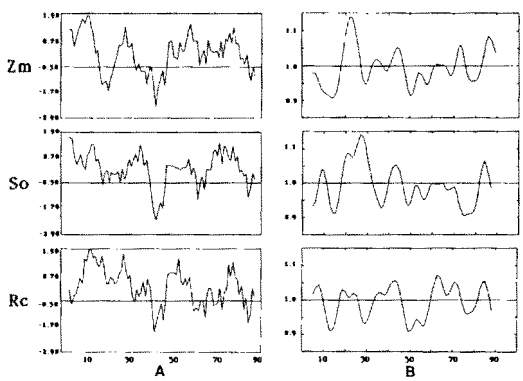

Fig. 8. Hydropathy and flexibility of PLTPs

A : hydropathy ; B : flexibility ; Zm : maize ; So : spinach ; Rc : castor bean.

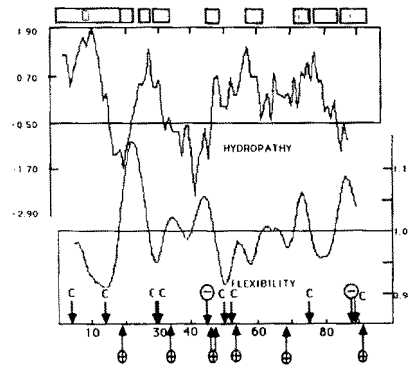

Fig. 9. Structural parameters of the maize phospholipid - transfer protein

▮▮▮ : extended beta structure ; ▒▒ : beta turn

\+ : positive charge ; − : negative charge ; C :cysteinyl residue

Table I
Codon usage for the gene encoding maize PLTP including peptide signal.

Amino acid	Codon	N°of codons	Amino acid	Codon	N°of codon	Amino acid	codon	N°of codon
Leu	UUA	0	Ile	AUC	3	Ser	UCU	0
	UUG	2		AUC	6		UCC	5
	CUU	0		AUA	0		UCA	0
	CUC	5					UCG	1
	CUA	0	Val	GUU	0		AGU	0
	CUG	2		GUC	5		AGC	2
				GUA	1			
Thr	ACU	0		GUG	6	Pro	CCU	0
	ACC	7					CCC	6
	ACA	0	Tyr	UAU	0		CCA	0
	ACG	1		UAC	2		CCG	0
Ala	GCU	0	Gln	CAA	1	Asn	AAU	0
	GCC	10		CAG	3		AAC	7
	GCA	1						
	GCG	8	Cys	UGU	0	Lys	AAA	0
				UGC	8		AAG	2
Asp	GAU	0						
	GAC	2	Arg	CGU	0	Gly	GGU	1
				CGC	5		GGC	7
Glu	GAA	0		CGA	0		GGA	0
	GAG	1		CGG	0		GGG	2
				AGA	0			
Met	AUG	1		AGG	2	End	UAA	0
							UAG	1
His	CAU	0	Phe	UUU	0		UGA	0
	CAC	0		UUC	0	Trp	UGG	3

Table II : Comparison of physicochemical parameters derived from the sequence of phospholipid-transfer proteins.

	Residue number	Mm	MMm	pHi	% H	% E	% T	% C
Maize	93	9054	97.3	8.97	1	48.4	36.6	14
Spinach	91	8833	97.1	9.36	11	45	30.8	13.2
Castor bean	92	9313	101.2	8.56	14.1	37	38	10.9

Secondary structure proportions have been calculated with all decision constants equal to zero, except for alpha-helix (decison constant=0.0).
MMm : mean molecular mass of a residue ; pHi : calculated isoelectric point ; %H : percent alpha-helix ; % E : percent extended structure ; % T : percent beta-turn ; % C : percent aperiodic structure.