

Bifunctional lipid-transfer: fatty acid-binding proteins in plants

Vincent Arondel, Chantal Vergnolle, Françoise Tchang and Jean-Claude Kader
*Laboratoire de Physiologie Cellulaire (U.R.A. 1180 C.N.R.S.), Université Pierre et Marie Curie,
4 place Jussieu, 75005 Paris, France*

Key words: lipid, transfer, protein, fatty acid, binding, plants

Summary

A cytosolic protein, able to facilitate intermembrane movements of phospholipids *in vitro*, has been purified to homogeneity from sunflower seedlings. This protein, which has the properties of a lipid-transfer protein (LTP), is also able to bind oleoyl-CoA, as shown by FPLC chromatography. This finding, in addition to previous observations suggesting that a lipid-transfer protein from spinach leaves can bind oleic acid and that oat seedlings contain a fatty acid-binding protein with similar features than lipid transfer proteins, provides a clear demonstration that plant cells contain bifunctional fatty acid/lipid transfer proteins. These proteins can play an active role in fatty acid metabolism which involves movements of oleyl-CoA between intracellular membranes.

Abbreviations: FABP – Fatty Acid-Binding Proteins, LTP – Lipid-Transfer Protein, PC – Phosphatidylcholine, PI – Phosphatidylinositol, PE – Phosphatidylethanolamine, pI – Isoelectric point

Introduction

Plant cells contain proteins able to facilitate *in vitro* intermembrane movements of phospholipids [1]. These proteins, called lipid transfer proteins (LTP), have been purified to homogeneity from various plant tissues like spinach leaves [2], maize [3] or castor bean [4] seedlings. Plant LTP have similar properties, like molecular mass (about 9 kDa), basic pI (around 9) and a broad non specificity for phospholipids since phosphatidylcholine (PC), phosphatidylinositol (PI) and phosphatidylethanolamine (PE) are transferred from liposomes (donor membranes) and mitochondria (receptor membranes). These plant LTP have properties roughly similar to those of nonspecific phospholipid transfer proteins from animal tissues like bovine liver which are able to transfer phospholipids as well as sterols [5, 6]. In addition to these LTPs, proteins able to bind oleoyl-CoA and oleic

acid (fatty acid-binding proteins; FABP) have been isolated from oat seedlings [7]. The experiments were based on the incubation of the protein with the fatty acids and then a separation either by the dextran-coated charcoal method or by isoelectric focusing. The fact that this protein has similar properties than plant LTPs led to study the eventual fatty acid binding by spinach LTP. The isoelectric focusing technique indicated that a binding of oleic acid indeed occurs on spinach LTP [8].

In order to provide decisive arguments in favour of this double property of transferring and binding lipids, it was tempting to study oil-forming seeds, like those of sunflower, which have intense fatty acid metabolism linked to the synthesis of triacylglycerols. Starting from germinating sunflower seeds, a pure LTP was isolated, having properties roughly similar to those of other plant LTP. When assayed with oleoyl-CoA, a binding to the sunflower protein was observed as indicated by FPLC chro-

matography of the fatty acid/protein complex. The present work demonstrates that bifunctional lipid transfer/fatty acid binding proteins are present in sunflower seedlings. At the light of these results and the previous observations on LTP from spinach and FABP from oat, it is concluded that plant cells contain bifunctional fatty acid-binding/lipid-transfer proteins.

Materials and methods

Materials

Sunflower seeds (*Helianthus annuus* L.c.v. rodeo) were obtained either from experimental farms of CETIOM (Paris). Cholesteryl (1-¹⁴C) oleate was purchased from Amersham. ³H phosphatidylcholine was prepared as previously indicated [9].

Preparation of the protein extracts

Sunflower seeds (400 g) were grown on filter paper at 30° C in the dark for four days. All the following manipulations were carried out at 4° C. Seedlings were ground in 500 ml of 0.1 M Tris-HCl (pH 7.5), 0.4 M sucrose, 6 mM Na₄ EDTA, 1 mM cysteine, 9 mM β-mercaptoethanol. After readjusting the pH to 7.5, the homogenate was filtered through a double layer of cheesecloth and centrifuged at 8,000 g for 30 min. After lowering its pH to 5.1, the supernatant was centrifuged. The pH of the supernatant was then readjusted to 7.5 and proteins were allowed to precipitate by adding 52 g of (NH₄)₂ SO₄ per 100 ml. The mixture was stirred overnight and then centrifuged at 8,000 g for 30 min. The pellets were resuspended in a minimal volume of 10 mM sodium phosphate (pH 7.2), 10% glycerol, 8 mM β-mercaptoethanol, 3 mM NaN₃ and the resulting suspension was dialyzed for 5 hours against 20 volumes of the same buffer in order to eliminate (NH₄)₂ SO₄. After lowering the pH to 5.1, the dialyzate was centrifuged at 8,000 g for 30 min to remove insoluble material. The supernatant, after pH adjustment at 7.2, was used as the protein extract for further purifications.

Transfer assay

Transfer assays were carried out as previously described [2]: protein extracts were incubated with 3PC (transferable lipid) and ¹⁴C cholesteryl oleate (non transferable lipid) liposomes and with purified maize mitochondria at 30° C for 30 min. The mitochondrial pellets were recovered by centrifugation, resuspended in 2% Triton X-100 and the radioactivity measured in a liquid scintillation counter. The transfer activity of the protein extract was expressed as nmol of PC transferred to mitochondria.

Binding assay

The protein fractions were incubated with 100 nmol of oleoyl-CoA (specific radioactivity: 2 GBq · mmol⁻¹; Amersham) for 30 min at +4° C. The mixture was then chromatographed on a FPLC MonoS column (Pharmacia).

Electrophoresis

Protein extracts were analyzed by acrylamide-SDS gel electrophoresis using gel slabs made from a 7.5–15% acrylamide gradient. Gels were coloured using either Coomassie blue or AgNO₃ [2, 3].

Results

Purification of the sunflower protein

The operations used to purify the protein are summarized in Table 1.

Step 1. The protein extract obtained after ammonium sulphate precipitation is able to transfer phosphatidylcholine from liposomes to mitochondria with a specific activity of 0.08 nmol mg protein⁻¹ min⁻¹.

Step 2. The protein extract was loaded on a Sephadex G75 column equilibrated with 10 mM sodium

phosphate (pH 7.2), 10% glycerol, 3 mM sodium azide and 8 mM β -mercaptoethanol (Buffer A). The active fractions (78–100) were pooled; they correspond to low molecular-mass proteins (Fig. 1A).

Step 3. The active fractions were applied to a DEAE-Trisacryl column equilibrated with buffer A in order to remove the acidic fractions which have a negligible activity. The effluent was directly passed through a CM-Sepharose column equilibrated with the same buffer. Bound proteins were eluted using a linear 0.01–0.25 M phosphate gradient in buffer A. Two majors peaks of activity were observed, CM1 and CM2, which correspond to a salt concentration respectively of 160 and 200 mM (Fig. 1B). The specific activity of the CM2 peak is equal to $2.88 \text{ nmol mg}^{-1} \text{ min}^{-1}$; a similar value was found for the CM1 peak (data not shown).

Step 4. The purification of the protein was achieved by FPLC. CM2 fraction was chromatographed on a MonoS column (Pharmacia) equilibrated with Buffer B (Buffer A minus glycerol) (Fig. 1C). Proteins were eluted with a 0–0.5 M NaCl gradient in the same buffer. The chromatogram obtained from CM2 showed a major peak corresponding to a salt concentration of 0.23 M. Fractions 22 and 23 were pooled and used as a purified protein having a specific activity of $10.56 \text{ nmol mg}^{-1} \text{ min}^{-1}$. A 132-fold purification was noted.

Properties of the sunflower protein

The analysis by SDS-PAGE of CM2 fraction showed a single band with an apparent molecular mass of 9 kDa. The same band was detected in the CM1 peak, but with other additional bands; for this reason, further purifications were made on only the CM2 peak. The value of 9 kDa is in good agreement with those obtained from the other plant LTP [1], but it differs from animal nonspecific proteins which have higher molecular masses [5, 6]. It is to be noted that this protein is detected as a distinct band in electrophoregrams of the total extract suggesting that the protein is abundant in sunflower seedlings. This can be correlated to the fact that maize LTP represents 2 to 4% of the total soluble proteins as determined by immunochemical methods [10, 11].

The observation that sunflower protein is bound on CM Sepharose indicates a high pI. This was confirmed by a chromatofocusing technique which indicated a pI near 9 (data not shown). This is in agreement with the high pI found in the case of maize, spinach and castor bean proteins although some acidic proteins, but less abundant, have been detected in these plants [1].

Interestingly, the transfer activity is associated with a double peak of activity (CM1 and CM2). The existence of several peaks of activity in separations on CM column has also been showed during the isolation of maize and spinach LTP. That suggests the existence of different isoforms, which could have the same molecular mass and slightly different pI.

Table 1. Purification procedure of sunflower protein. The different steps of purification of lipid transfer protein from sunflower seedlings is described in Materials and methods. The various steps correspond respectively to the protein extract after dialysis (step 1), to the pooled active fractions from Sephadex G75 column (step 2), the active fraction from CM-Sepharose column (isoform CM2) (step 3) and the final pure protein from the MonoS FPLC column (step 4). The specific activity is expressed as nmol of phosphatidylcholine transferred from liposomes to mitochondria per mg of protein per min.

Steps	Protein mg · ml ⁻¹	Volume ml	Total Protein mg	Specific activity nmol · mg ⁻¹ · min ⁻¹	Purification
Crude extract (step 1)	44	120	5280	0.08	1
Sephadex G75 (step 2)	1.5	315	472	1.06	13.25
CM2 (step 3)	0.36	149	53.6	2.88	37
MonoS (step 4)	0.32	4	1.3	10.56	132

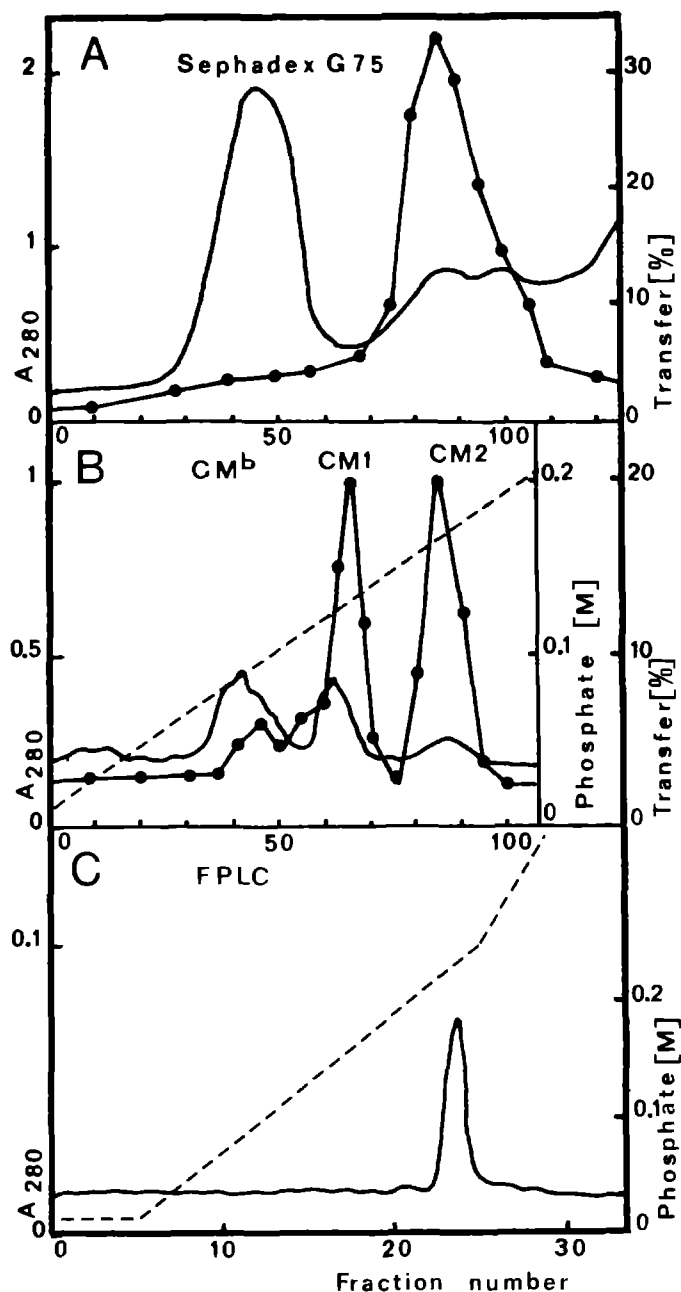


Fig. 1. Elution pattern of sunflower proteins. A: Chromatography on Sephadex G-75. The protein extract (5 g) was loaded on a 5 × 70 cm column equilibrated with 10 mM sodium phosphate (pH 7.2), 10% glycerol, 3 mM sodium azide and 8 mM β-mercaptoethanol. The flow rate was 80 ml h⁻¹ and 15 ml fractions were collected. The absorbance was measured at 280 nm and the transfer activity was expressed as a percentage of phosphatidylcholine transferred from liposomes to mitochondria using 3 ml of each fraction. Fractions 78–100 were pooled. B: CM-Sepharose. The unbound effluent from DEAE-Trisacryl (step 3) were applied to a 2.6 × 25 cm column equilibrated with the same buffer indicated in A. Elution was effected using a linear gradient formed by 11 of the equilibration buffer and 11 of the same one made up to 250 mM sodium phosphate. The flow rate was 60 ml h⁻¹ and 25 ml fractions were collected. The salt concentration was determined by measuring their conductivity. Absorbance at 280 nm and transfer activity are indicated as in A. Fractions 65–85 (CM1) and 86–95 (CM2) were pooled separately. C: FPLC on MonoS. 1.3 mg of the CM2 fraction were applied to a MonoS column (Pharmacia) equilibrated with 10 mM sodium phosphate (pH 7.2), 3 mM sodium azide, 8 mM β-mercaptoethanol. Proteins were eluted using a 0.5 M NaCl gradient prepared in the same buffer. The flow rate was 60 ml h⁻¹ and 1 ml fractions were collected. Absorbance was monitored at 280 nm. The shape of the gradient is indicated by a discontinuous line. Fractions 22 and 23 were pooled.

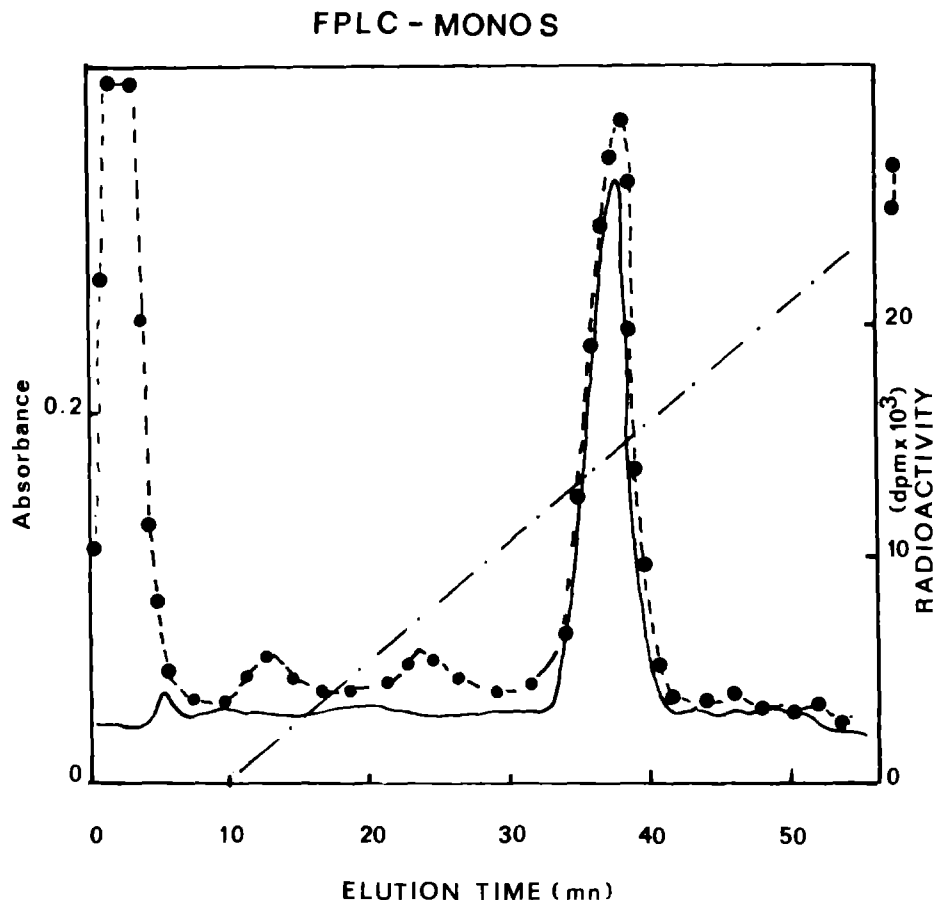


Fig. 2. Binding of oleoyl-CoA on the sunflower protein. After incubation of 0.9 mg of CM2 fraction with 100 nmol of ^{14}C -oleoyl-CoA (see Materials and methods), the mixture was separated by chromatography on a MonoS column in the experimental conditions indicated in Fig. 1C. The radioactivity (circles) is expressed as dpm per ml of fraction.

Binding of oleoyl-CoA

When the CM2 fraction was incubated with oleoyl-CoA, a radioactive peak, corresponding to the protein, was detected after separation of the incubation mixture by FPLC on a Mono S column (Fig. 2). Around 40% of the total ^{14}C label was recovered in the protein peak.

NH₂-terminal sequence

A NH₂-terminal sequence comprising 24 residues has been determined for the CM2 fraction (Fig. 3). The most important feature of this sequence is its hydrophobic character, especially between resid-

ues 6–17. The property of this part of sequence seems to be common to other plant LTP (maize, spinach or castor bean) [12].

Discussion and conclusions

The present work establishes that a protein highly purified from an oil-forming seed, is not only able to transfer phospholipids between membranes but also to bind oleoyl-CoA. We propose that this protein belongs to a category of bifunctional proteins having the ability to bind and transfer lipids.

The sunflower protein, isolated in this work, shares with plant LTPs common properties: same molecular mass and around 9 kDa and high pI.

The determination of the NH₂-terminal sequence of the sunflower protein confirmed its homology with other plant LTPs. A comparison between those sequences shows a high homology; the percent match is respectively of 29.2%, 33.3% and 20.8% with spinach, castor bean and spinach proteins which have, respectively, in total 91, 92 and 93 residues. Interestingly, the positions of cysteine residues (3 and 13 in sunflower and castor bean; 4 and 14 in maize and spinach) are conserved. The other cysteines, found in a total number of 8, in maize, castor bean and maize, are also located in the same positions in the LTPs [12–15]. It is supposed that these cysteines play an important role in the mode of action of the protein. In contrast, only one cysteine residue was detected in rat liver FABP [16].

The observation that sunflower protein binds oleoyl-CoA leads to the conclusion that this pro-

Interestingly, a protein with a molecular mass of 8.7 kDa, able to bind fatty acids, has been purified from oat seedlings [7]. This protein has a pI of either of 8.4 (when the protein is delipidated) or 4.8 (with the lipidated protein). In these experiments, the binding assay was based on the isolation of protein-fatty acid complex by isoelectric focusing. The same assay was carried out with spinach LTP, indicating that this protein has the ability to bind fatty acids [8].

The properties of plant FABP-like proteins are different from those of animal FABP. The molecular mass of these latter proteins vary from 12 to 14 kDa while their pI vary from 4.3 to 9.3, the rat liver FABP exhibiting a pI around 5.2 to 6.9 [16, 18]. Isoforms of animal FABP have been found, as for plant FABP. Animal FABP, like plant FABP/LTP, are abundant proteins, accounting for 4 to 5%

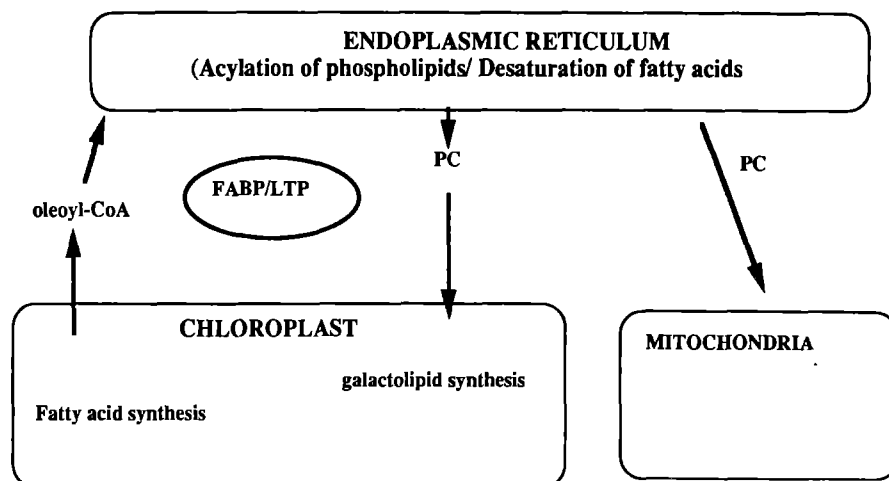


Fig. 4. Scheme indicating the cooperation between organelles (endoplasmic reticulum and chloroplast) for the biosynthesis of galactolipids in plant cells.

of cytosolic proteins. Based on SDS-PAGE of crude extracts, sunflower protein seems to be abundant. A precise quantitation has been made by ELISA for maize LTP, indicating that this protein represents 2 to 4% of the total protein extract. If it is assumed that all plant LTP have a fatty acid binding ability, it can be concluded that, as animal FABP, plant FABP are abundant in the cytosol.

One major question about plant FABP concerns their *in vivo* role. As for animal cells, it can be assumed that plant FABP i/ facilitate the intracellular transport of fatty acids in the cytosol; ii/ regulate the intracellular pool of fatty acids and acyl CoA esters by storing these compounds since FABP are abundant; iii/ exert a protecting effect by avoiding large concentrations of fatty acids and acyl-CoA esters in the cytosol.

Another possible involvement of FABP, specific for plant cells, could be their participation to lipid metabolism. It is accepted now that the site of fatty acid synthesis in plants is the chloroplast (or the plastid) [19]. The fatty acids synthesized are exported in the form of oleoyl-CoA which is transported to the endoplasmic reticulum and then used for the synthesis of polyunsaturated fatty acids (Fig. 4). The polyunsaturated fatty acids (linoleic or linolenic acids) are then transported to the plastid, esterified to phospholipid molecules, and used for the synthesis of galactolipids. Alternatively, in

oil-forming seeds, phosphatidylcholine can be used as an acyl donor for triacylglycerol synthesis [19]. A dual role for the lipid transporting proteins is thus plausible.

For future investigations, it will be necessary to provide decisive arguments in favour of the *in vivo* role of plant FABP/LTP. One possibility is to follow the fluctuations of the amount of proteins during various physiological processes like germination of seeds or greening of leaves. Another way is to use the site-directed mutagenesis approach to determine precisely the site of interaction of fatty acids. This could be made with maize LTP since a full-length cDNA has been isolated and characterized [12]. The study of the expression of this cDNA in yeast and *E. coli* is thus envisaged. For long-term research, the use of the anti-sense mRNA approach or the preparation of transgenic plants can provide decisive informations about the role of FABP/LTP in plant cells.

Acknowledgements

The authors are much indebted to Mr. PIERRE and ARNAUD from CETIOM for providing sunflower seeds and to Mrs SEGUI (INSA, Toulouse) for amino acid analysis.

References

1. Kader JC: Lipid-binding proteins in plants. *Chem Phys Lipids*. 38: 51–62, 1985
2. Kader JC, Julienne M, Vergnolle C: Purification and characterization of a spinach leaf protein capable of transferring phospholipids from liposomes to mitochondria or chloroplasts. *Eur J Biochem* 139: 411–416, 1984
3. Douady D, Guerbette F, Kader JC: Purification of phospholipid transfer protein from maize seeds using a two-step chromatographic procedure. *Physiol Vég* 23: 373–380, 1985
4. Watanabe S, Yamada M: Purification and characterization of a non-specific lipid transfer protein from germinated castor bean endosperms which transfers phospholipids and galactolipids. *Biochim Biophys Acta* 876: 116–123, 1986
5. Wirtz KWA: Phospholipid transfer proteins. In: PC Jost and OH Griffith (eds) *Lipid-Protein Interactions*. Elsevier, Amsterdam, 1982, pp 151–223
6. Kader JC, Douady D, Mazliak P: Phospholipid transfer proteins. In: JN Hawthorne and GB Ansell (eds) *Phospholipids, a comprehensive treatise*, Elsevier, Amsterdam, 1982, pp 279–311
7. Rickers J, Tober I, Spener F: Purification and binding characteristics of a basic fatty acid binding protein from *Avena sativa* seedlings. *Biochim Biophys Acta* 794: 313–319, 1984
8. Rickers J, Spener F, Kader JC: A phospholipid transfer protein that binds long-chain fatty acids. *FEBS Lett*. 180: 29–32, 1985
9. Douady D, Grosbois M, Guerbette F, Kader JC: Purification of basic phospholipid transfer protein from maize seedlings. *Biochim Biophys Acta* 710: 143–153, 1982
10. Douady D, Grosbois M, Guerbette F, Kader JC: Phospholipid transfer protein from maize seedlings is partly membrane-bound. *Plant Sci* 45: 151–156, 1986
11. Grosbois M, Guerbette F, Douady D, Kader JC: Enzyme immunoassay of a plant phospholipid transfer protein. *Biochim Biophys Acta*. 917: 162–168, 1987
12. Tchang F, This P, Stiefel V, Arondel V, Morch MD, Pages M, Puigdomenech P, Grellet F, Delseny M, Bouillon P, Huet JC, Guerbette F, Beauvais-Cante F, Duranton H, Pernollet JC, Kader JC: Phospholipid transfer protein: full length cDNA and amino acid sequence in maize. *J Biol Chem* 263: 16849–16855, 1988
13. Takishima K, Watanabe S, Yamada M, Mamiya G: The amino acid sequence of the nonspecific lipid transfer protein from germinated castor bean endosperm. *Biochim Biophys Acta* 870: 248–250, 1986
14. Takishima K, Watanabe S, Yamada M, Suga T, Mamiya G: Amino acid sequences of two nonspecific lipid transfer proteins from germinated castor bean. *Eur J Biochem* 177: 241–249, 1988
15. Bernhard WR, Somerville CR: Coidentity of putative amylase inhibitors from barley and finger millet with phospholipid transfer proteins inferred from amino acid sequence homology. *Arch Biochem Biophys* 269: 695–697, 1989
16. Glatz JFC, Veerkamp JH: Intracellular fatty acid-binding proteins. *Int J Biochem* 17: 13–22, 1985
17. Pastuszyn A, Noland BJ, Bazan JF, Fletterick RJ, Scallen TJ: Primary sequence and structural analysis of sterol carrier protein 2 from rat liver: Homology with immunoglobulins. *J Biol Chem* 262: 1319–13227, 1987
18. Bass NM: The cellular fatty acid binding proteins: Aspects of structure, regulation and function. *Int Rev Cytol* 111: 143–184, 1988
19. Harwood JL: Fatty acid metabolism. *Annu Rev Plant Physiol Plant Mol Biol* 39: 101–138, 1988

Address for offprints: J.C. Kader, Laboratoire de Physiologie Cellulaire, TOUR 53 E3, 4 place Jussieu, 75005 Paris, France