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Production in *Escherichia coli* and site-directed mutagenesis of a 9-kDa nonspecific lipid transfer protein from wheat

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The sequence encoding a wheat (*Triticum durum*) nonspecific lipid transfer protein of 9 kDa (nsLTP1) was inserted into an *Escherichia coli* expression vector, pET3b. The recombinant protein that was expressed accumulated in insoluble cytoplasmic inclusion bodies and was purified and refolded from them. In comparison with the corresponding protein isolated from wheat kernel, the refolded recombinant protein exhibits a methionine extension at its N-terminus but has the same structure and activity as demonstrated by CD, lipid binding and lipid transfer assays. Using the same expression system, four mutants with H5Q, Y16A, Q45R and Y79A replacements were produced and characterized. No significant changes in structure or activity were found for three of the mutants. By contrast, lipid binding experiments with the Y79A mutant did not show any increase of tyrosine fluorescence as observed with the wild-type nsLTP1. Comparison of the two tyrosine mutants suggested that Tyr79 is the residue involved in this phenomenon and thus is located close to the lipid binding site as expected from three-dimensional structure data.

Keywords: lipid transfer protein; mutagenesis; recombinant protein; structure/ function relationships; wheat.

Lipid transfer proteins (LTPs) are a family of proteins that is found in yeasts, bacteria, animals and plants [1–3]. LTPs are able to transfer lipids between membranes *in vitro* and show either a narrow specificity against the transferred lipid or are nonspecific (ns). In plants, only the nonspecific class of LTPs has been studied; such proteins have been shown to bind fatty acids and acyl-coenzyme A also [4–6]. A large number of cDNA clones coding these nsLTPs has been isolated [7–10] and comparison of their primary structure shows a high degree of similarity; they are low molecular mass, basic proteins synthesized with a signal peptide and show a common pattern of eight cysteines that are engaged in disulphide bridges [11–13]. However, their biological role remains unknown; their subcellular location suggest that they may be involved in cutin biosynthesis [14,15], pathogen-defence reactions [16–18] and/or response to environmental changes [19,20]. Therefore, it appears that they could play a role in a number of different biological functions through their ability to bind and/or carry lipophilic compounds. In addition, it was found that barley nsLTP is involved in the formation of beer foam, thus opening interesting applications in food technology [21].

In wheat, nsLTPs of 7 and 9 kDa have been extracted from kernels [22]. A cDNA clone encoding a *Triticum durum* 9 kDa nsLTP (nsLTP1) has been characterized [23] and its deduced primary structure is identical to that of a nsLTP purified from *T. aestivum* [12]. Structural analysis of the *T. aestivum* nsLTP1, investigated by infrared and Raman spectroscopy, indicated that this protein is composed mainly of α-helices stabilized by four disulphide bridges [12]. The three-dimensional structure, obtained by modelling from multidimensional ^{1}H-NMR spectroscopy data, showed that the wheat nsLTP1 is composed of a bundle of four α-helices packed against a C-terminal fragment forming a nonstandard loop [24]. Furthermore, a stereo view of the hydrophobic side chains has revealed a cavity in which the acyl part of a phospholipid can be inserted.

In order to obtain sufficient material to pursue structural and functional studies, and to carry out site-directed mutagenesis for the structure/function relationship analysis, the wheat nsLTP1 was produced in *Escherichia coli*. This report describes the cloning of the sequence encoding the wheat nsLTP1 into a bacterial expression vector, the construction of four mutants of the protein and the purification and characterization of the recombinant wild-type and mutant proteins.

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Abbreviations: LTP, lipid transfer protein; lysoPC, 1-palmitoyl-L-α-lysophosphatidylcholine; ns, nonspecific; Pyr-PC, 3-palmitoyl-2-(1pyrenedecanoyl)-Lα-phosphatidylcholine; Pyr-PG, 3-palmitoyl-2-(1pyrenedecanoyl)-Lα-phosphatidylglycerol.

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MATERIALS AND METHODS

Materials

T. aestivum (var. Etoile de Choisy) seeds were kindly provided by G. Branlard (INRA, Clermont-Ferrand, France). Restriction enzymes, modifying enzymes, *Taq* DNA polymerase, and the synthesized oligonucleotides used as PCR primers and for mutagenesis were from Appligene; dNTPs were from Pharmacia; Geneclean DNA purification kit was from Bio 101; AmpliTaq Fluorescent Dye Deoxy-Terminator Cycle Sequencing kit was from Perkin-Elmer; Benzon Nuclease was from Merck; bicinchoninic acid assay kit was from Pierce; nitrocellulose membrane was from Schleicher & Schuell ; prestained low molecular weight (43–3 kDa) protein markers were from

Bethesda Research Laboratories; 3-palmitoyl-2-(1pyrenedecanoyl)-L-α-phosphatidylcholine (Pyr-PC) and 3-palmitoyl-2-(1pyrenedecanoyl)-L-α-phosphatidylglycerol (Pyr-PG) were from Molecular Probes ; and phosphatase alkaline labelled goat anti-(rabbit IgG) and other reagents used were from Sigma.

Bacterial strains and vectors

The plasmid pTd4.90 has been described previously [23]. The plasmid pUC119 [25] and *E. coli* strain DH5α (Bethesda Research Laboratories) were used for the cloning of the sequence encoding the mature wild-type nsLTP1. The cloning vector M13mp18 (Pharmacia) and the *E. coli* strains TG1 and RZ1032 were used for the mutagenesis experiments. *E. coli* strain BL21(DE3)pLysS and the expression vector pET3b [26] used for the expression of wild-type and mutant nsLTPs were provided by F.W. Studier (Brookhaven National Laboratory, Upton, NY, USA).

Purification of the wheat 9-kDa nsLTP

Wheat nsLTP1 was isolated and purified from *T. aestivum* seeds as described previously [12].

Cloning methods

Standard methods for endonuclease analysis of plasmid DNA and DNA cloning were used [27]. Plasmid DNA was purified by the method of Stephen *et al*. [28].

Cloning of the sequence encoding the wheat mature wild-type nsLTP1 in the expression vector

Plasmid pTd4.90 was used as a template in PCR mutagenesis to create an *Nde*I restriction site 5′ to the sequence encoding the N-terminus of the mature nsLTP1 and a *Bam*HI site 3′ to the stop codon of the coding sequence. The DNA thermocycler was a GeneAmp PCR System 9600 (Perkin-Elmer). The reaction mixture contained, in a final volume of 100 μL, 50 ng plasmid DNA, 1 μM of each primer, 250 mM of each dNTP, 1 U *Taq* DNA polymerase, and 1/10 volume of 10X reaction buffer. Twenty cycles were carried out, each consisting of a denaturation step at 94 °C for 1 min, an annealing step at 55 °C for 1.5 min and an extension step at 72 °C for 2 min. The amplified DNA was then treated with the Klenow fragment of DNA polymerase I and phosphorylated by T4 polynucleotide kinase according to the manufacturer's instructions. The phosphorylated fragment was ligated into the dephosphorylated plasmid pUC119 digested with *Sma*I. The resulting vector (pUC119-LTP) was used to transform *E. coli* strain DH5α as described by Hanahan [29]. The plasmid (pUC119-LTP) was then digested with *Nde*I and *Bam*HI and the insert corresponding to the coding sequence of the wheat mature nsLTP1 was purified by electrophoresis through agarose followed by absorption to glass powder (Geneclean). The purified fragment was ligated into the *Nde*I–*Bam*HI digested pET3b to form pET-LTP that was then used to transform *E. coli* BL21(DE3)pLysS.

Site-directed mutagenesis of nsLTP1

The *Xba*I–*Bam*HI insert excised from pET-LTP was subcloned into the M13mp18 cloning vector digested by the same restriction enzymes and used to transfect *E. coli* TG1 as described by Sambrook *et al*. [27]. DNA from hybrid phage was then used to infect *E. coli* RZ1032 to collect uracil-containing single-stranded template for mutagenesis as described by Kunkel *et al*. [30]. Four different oligonucleotides were used as primers to produce four independent mutants of the wheat nsLTP1. After T4 kinase phosphorylation, oligonucleotides were annealed to the uracil-containing template and primer extensions were carried out with T7 polymerase and T4 DNA ligase. Products of the reactions were used to transform *E. coli* TG1. Mutated phage DNA were selected by sequencing and the *Xba*I–*Bam*HI inserts were then reintroduced into the expression vector pET3b .

DNA sequencing

All of the constructs were controlled by sequencing according to the method of Sanger *et al*. [31] using the AmpliTaq Fluorescent Dye Deoxy-Terminator Cycle Sequencing kit. Sequencing was performed on double-stranded DNA with M13 or T7 primers. Samples were electrophoresed and detected on a 370-A DNA sequencer (Perkin-Elmer).

Expression and purification of recombinant proteins

E. coli BL21(DE3)pLysS containing a recombinant expression vector was grown overnight at 37 °C in Luria–Bertani medium with 0.1 $mg \cdot mL^{-1}$ ampicillin and 0.025 $mg \cdot mL^{-1}$ chloramphenicol. Cells were diluted 1 : 60, allowed to grow until absorbance at 600 nm reached 0.5 and then induced by the addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.1 mM in the culture medium. After 3 h of induction, bacteria were harvested by centrifugation and stored at −20 °C. For recombinant protein purification, cells were resuspended in 1 : 20 of the culture volume of lysis buffer (50 mM Tris/HCl, pH 7.8, 1 mM EDTA) and broken, after two freeze-thaw cycles, by passing four times through a French Press at 35 MPa. The resulting lysate was incubated 30 min at 37 °C with 12 $U \cdot mL^{-1}$ Benzon Nuclease to digest the nucleic acids; the bacterial lysate was centrifuged for 10 min at 13 500 ***g***. The pellet, containing the inclusion bodies, was resuspended in three lysate volumes of washing buffer (50 mM Tris/HCl, pH 7.8, 0.1 M KCl, 10 mM EDTA), kept at room temperature for 10 min, and centrifuged for 5 min as above. The washing step was repeated twice with buffer A (50 mM Tris/HCl, pH 7.8, 0.1 M KCl, 1 mM EDTA) and the pellet was resuspended, with constant stirring, in buffer A containing 2% 2-mercaptoethanol and 7.5 M urea, at a protein concentration of 1 $mg \cdot mL^{-1}$. After 1 h, particulate material was removed by centrifugation as described before and one volume of the supernatant was diluted with 10 vol. of buffer A and stirred for 1 h. Denaturing agents were then removed by dialysis against buffer A for 12 h and water for 24 h using Spectrapor dialysis tubing (Spectrum, 3000 M_r cut-off). After dialysis, samples were centrifuged at 12 000 ***g*** for 2 h, concentrated by ultrafiltration using a Microsep 3K (Filtron) and lyophilized.

Analytical methods

Protein concentration was determined by the bicinchoninic acid assay [32] with BSA as a standard and according to the manufacturer's instructions. Protein analysis was by 15% SDS/PAGE as described by Laemmli [33]. Gels were stained with Coomassie brilliant blue R250. The precise molecular masses of the recombinant nsLTPs (dissolved in 10% formic acid) were obtained using a Perkin-Elmer Sciex model API I single-quadrupole mass spectrometer equipped with a pneumatically assisted electrospray ion source. Positive multi charged peptide

ions were generated by spraying the sample solution through a 75-μm internal diameter fused silica capillary held at 5.5 kV. The samples were delivered to the spayer by a syringe infusion pump at a flow rate of 2 $\mu L \cdot mL^{-1}$.

Immunodetection

After SDS/PAGE, gels were soaked in the transfer buffer (25 mM Tris, 192 mM glycine, 30% methanol, pH 8.4) and electrotransferred at 0.8 $mA \cdot cm^{-2}$ for 50 min with a semi-dry blotting apparatus Novablot (Pharmacia) onto a 0.1-μm nitrocellulose membrane, as described by Towbin *et al*. [34]. The immunoblot was saturated in Tris/NaCl buffer (20 mM Tris/HCl, pH 8.0, 150 mM NaCl) with 3% gelatin and incubated for 3 h with a 1 : 1000 dilution of rabbit antiserum against wheat-purified nsLTP1 in the same buffer but with 1% gelatin. The membrane was washed once in Tris/NaCl and twice in Tris/NaCl, 0.05% Tween-20 (10 min each) and incubated for 30 min in a 1 : 5000 dilution of alkaline phosphatase-labelled goat anti-rabbit IgG. Detection was achieved with the chromogenic substrates, 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium salt, according to the manufacturer's instructions.

CD

The far ultra violet CD spectra (185–260 nm) of the proteins were recorded with a Jobin Yvon Mark V dichrograph at a scan speed of 20 $nm \cdot min^{-1}$. According to the protein concentration (0.15 $mg \cdot mL^{-1}$) quartz cells with 1 mm path width, maintained at 25 °C with a circulating water bath, were used. The spectra were corrected for the solvent and expressed as mean residue ellipticity ($deg \cdot cm^{-2} \cdot dmol^{-1)}$). Results presented are the average of five measurements. The percentage α-helix content was estimated according to the method of Chen *et al*. [35].

Fluorescence lipid transfer assay

Transfer activity was determined by measuring the transfer of pyr-PC or pyr-PG, due to the addition of a nsLTP, from quenched donor vesicles (50% Pyr-PC/50% Pyr-PG) to unquenched acceptor vesicles made of egg yolk phosphatidylcholine as described previously [36].

Lipid binding activity

Lipid binding was probed by following the intrinsic fluorescence spectroscopy of tyrosine residues of the wheat nsLTP after the addition of increasing amount of 1-palmitoyl-L-α-lysophosphatidylcholine (lysoPC) as described by Gomar *et al*. [37]. The dissociation constants (K_d) were determined by fitting experimental curves assuming one binding site [37].

RESULTS

Construction of an expression vector carrying the sequence encoding the wheat nsLTP1 and production of the protein in *E. coli*

For the production of the wheat nsLTP1, an inducible expression system using the expression vector pET3b [26] was chosen to avoid problems due to possible toxicity of the recombinant protein. In wheat, nsLTP1 is synthesized as a preprotein with a signal peptide that is cleaved to give the mature protein [23], a processing that does not take place in bacteria. Therefore, PCR mutagenesis of the plasmid pTd 4.90 [23] was used both to delete the sequence encoding this signal peptide, and to introduce restriction enzyme recognition sites to allow the sequence encoding the wheat mature nsLTP1 to be cloned into the expression vector pET3b. The 5′ primer (5′-ATA*CATATG*ATCGACTGCGGCCACGTTG-3′) used in PCR corresponded to the sequence encoding the N-terminus of the mature protein (first 18 nucleotides) downstream of an *Nde*I site (in italics) that contains an initiation codon. The 3′ primer (5′-ATA*GGATCC*ATTACACCCTGCTGCAGTCG-3′) annealed to the sequence encoding the C-terminus of the protein and contained a *Bam*HI site (in italics) next to the stop codon (Fig. 1). The 291-bp PCR fragment was first subcloned into pUC119 and sequenced in order to check that no mutations had been introduced into the sequence encoding the wheat mature nsLTP1 during amplification. Then, the *Nde*I–*Bam*HI insert from pUC119-LTP was ligated to the expression vector pET3b digested with the same

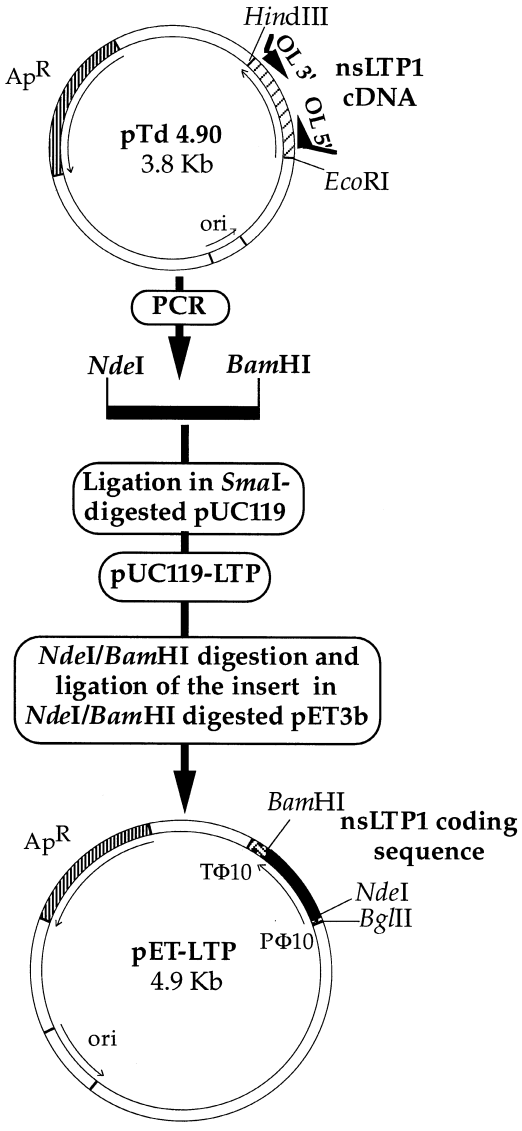

Fig. 1. Construction of the recombinant expression vector pET-LTP. pTd4.90 [23] was used as a template for PCR with the two oligonucleotides OL5′ and OL3′. Nucleotides added to the 5′ end of OL5′ and OL3′ primers that are not homologous to the cDNA are represented by the raised 5′ end. The PCR fragment was cloned into the pUC119 vector and the *Nde*I–*Bam*HI insert of the resulting pUC119-LTP vector inserted into the expression vector pET3b. Key restriction enzyme sites are indicated.

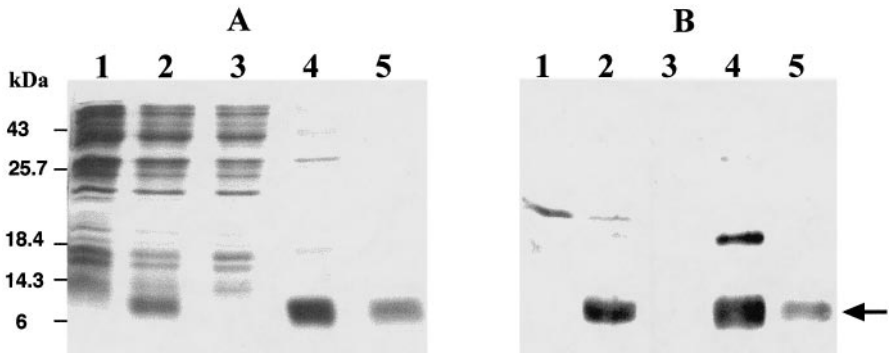

Fig. 2. SDS/PAGE and immunoblot analysis of wheat nsLTP1 production. Lane 1, total cell proteins from uninduced *E. coli* BL21(DE3) pLysS containing pET-LTP; lane 2, total cell proteins from induced bacteria; lane 3, supernatant from centrifugation of total proteins from induced bacteria; lane 4, pellet from centrifugation of total proteins from induced bacteria; lane 5, wheat-extracted nsLTP1. The arrow indicates the nsLTP. Molecular markers are indicated on the left. (A) SDS/PAGE after Coomassie blue staining. (B). Immunodetection after incubation with the wheat nsLTP1 antiserum.

enzymes. The resulting plasmid, pET-LTP, was used to transform *E. coli* BL21(DE3)pLysS.

Bacteria containing the expression vector (pET-LTP) were grown at 37 °C in Luria–Bertani medium and induced for 3 h with isopropyl-β-D-thiogalactopyranoside. The bacterial pellets were harvested, disrupted directly in the gel loading buffer and the proteins were analysed by SDS/PAGE under reducing conditions. After Coomassie blue staining of the gel, a protein with the same electrophoretic mobility as the wheat-extracted nsLTP1 was detected in induced bacteria carrying the pET-LTP plasmid but was absent from uninduced control bacteria (Fig. 2A). Western blot analysis of the same gel confirmed that this recombinant protein corresponded to the wheat 9-kDa nsLTP1 (Fig. 2B).

It is known that recombinant proteins overproduced in bacteria often form inclusion bodies in which the protein is in an insoluble form [38]. To determine the solubility of the recombinant protein, the bacterial lysate from induced bacteria was centrifuged to separate soluble from insoluble cytoplasmic proteins. The resulting supernatant and pellet were analysed by SDS/PAGE (Fig. 2A). The recombinant protein was found in the pellet indicating that the protein was insoluble in the *E. coli* cytoplasm. This result was further confirmed by Western blotting (Fig. 2B). An additional band from inclusion bodies was detected with the wheat nsLTP1 antiserum and corresponds to a dimeric form of the LTP that is sometimes observed on SDS/PAGE depending on loading.

Purification and characterization of wild-type recombinant nsLTP1

Because the wheat nsLTP1 was produced as an insoluble form in *E. coli*, a denaturation and renaturation protocol was performed in order to study the structure and function of the protein. Inclusion bodies, in which the recombinant protein was relatively pure (Fig. 2A), were treated as described in Materials and methods. This procedure yielded ≈50 mg of the wheat nsLTP1 from 1 L of bacterial culture. The recombinant protein was analysed by ion spray MS and CD and compared to the nsLTP1 isolated from wheat. MS analysis gave an average molecular mass of 9730.85 ± 1.25 Da for the recombinant nsLTP1 and 9600.58 ± 0.9 Da for the nsLTP1 extracted from wheat (Table 1). This difference was attributed to the nonexcision of the initiation methionine in the recombinant protein. Indeed, an ATG codon was introduced at the 5′ end of the coding sequence of the wheat mature nsLTP1 in the expression construct and it is known that the *E. coli* methionyl-aminopeptidase is not able to remove the methionine if the following amino acid is an isoleucine [39,40] as observed in the wheat nsLTP1. Experimental data were also compared to calculated data deduced from the primary structure of the recombinant or the wheat-extracted nsLTP1 (Table 1) and showed that all of the cysteines of the recombinant protein were involved in disulphide bonds as expected for the wheat nsLTP1 [12]. The CD spectra of the wild-type recombinant and the wheat-extracted nsLTP1 were compared and found to be identical (data not shown). Therefore, the recombinant protein had refolded correctly following the purification and renaturation process used, and this expression system could be used to produce mutants of the wheat nsLTP1.

Construction and production of nsLTP1 mutants in *E. coli*

Because the biological function of plant nsLTPs is probably related to their capacity to bind and/or transport amphiphilic lipids, these are the activities to consider in the study of structure/function relationships. Therefore, four independent mutations were introduced into the sequence encoding the protein. Choice of mutations took into account the protein structure data and comparison of sequence and activity of other nsLTPs from cereal seeds (Fig. 3). Indeed, the structure of maize [13], barley [41] and rice [42] nsLTPs were found to share the same overall pattern as the wheat protein. However, maize nsLTP appeared to be the most active in lipid transfer [43,44] and did not show the same affinity as the wheat protein towards lysolecithins with fatty acid chains of different lengths [44]. The two aromatic tyrosine residues (at positions 16 and 79) of the wheat nsLTP1 were individually replaced by alanine because they are conserved in all of the four nsLTP sequences and could

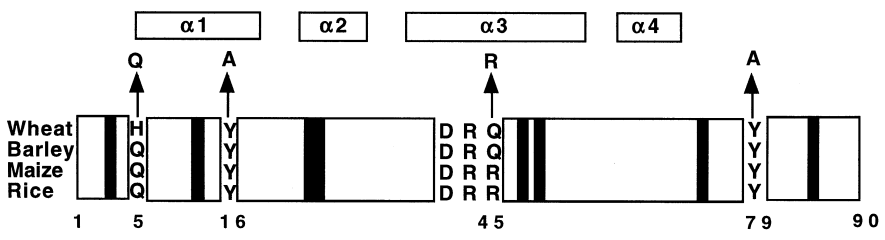

Fig. 3. Position of the substituted amino acids in mutants of the wheat nsLTP1. Only the amino acids discussed in the text are shown (numbering is according to the wheat nsLTP1). Barley [7], maize [8], and rice [49], nsLTP sequences are also indicated for comparison and were aligned to maximize similarities. Position of the conserved cysteines (3, 13, 27, 28, 48, 50, 73, 87) are marked by black boxes. Location of the four α-helices (5–18; 26–36; 41–57; 63–69) are according to NMR data [24].

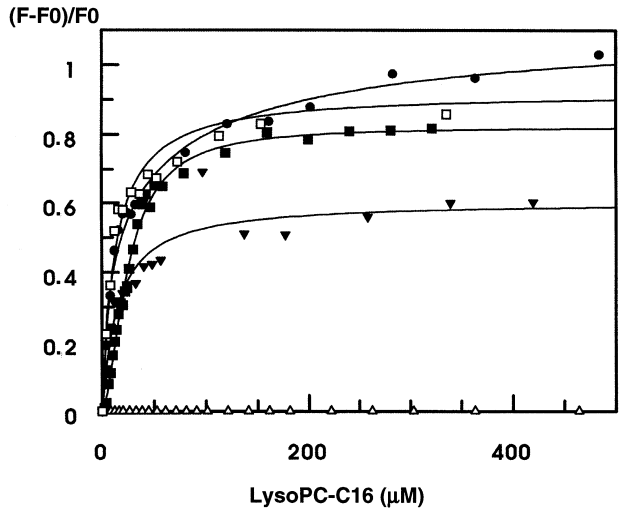

Fig. 4. Lipid binding activity of the wild-type and wheat nsLTP1 mutants. The lipid binding activity was measured by the increase of nsLTP1 fluorescence following addition of lysoPC as described by Gomar *et al*. [37]. Wild-type nsLTP1 (■); His5Gln mutant nsLTP1 (▼); Tyr16Ala mutant nsLTP1 (●); Gln45Arg mutant nsLTP1 (□); Tyr79Ala mutant nsLTP1 (△).

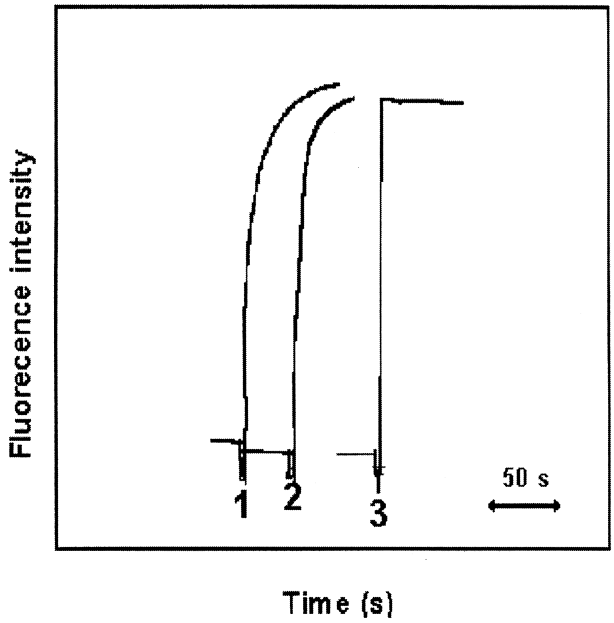

Fig. 5. Binding of Pyr-PG to the wild-type wheat nsLTP and the Tyr79Ala mutant. Conditions of the assay were the same as for lipid transfer activity measurement without addition of acceptor liposomes. Increase of fluorescence intensity of Pyr-PG was observed following the addition of wild-type LTP (1) or Tyr79Ala mutant (2) to quenched donor vesicles. Total fluorescence is released following the addition of 2% Triton X100 (3).

be involved in lipid binding [24]. The histidine at position 5, that was only found in the wheat nsLTP1 sequence, was changed to glutamine in one of the mutants to recover the consensus sequence of the cereal nsLTPs. The glutamine in the sequence Asp-Arg-Gln (residues 43–45) that was found in wheat and barley nsLTPs was replaced by the sequence Asp-Arg-Arg found in the maize protein (the most active in lipid transfer). It is interesting to note that these last two mutations also changed the net charge of the protein. For site-directed mutagenesis of the wheat nsLTP1 coding sequence, M13 subcloning of the *Xba*I–*Bam*HI insert from pET-LTP and the method described by Kunkel *et al*. [30] were used. An oligonucleotide carrying the desired mutation was annealed *in vitro* to the uracilated single-strand template and used as an elongation primer to produce the corresponding mutated sequence. Four independent mutants of the wheat nsLTP1 called His5Gln, Tyr16Ala, Gln45Arg and Tyr79Ala were obtained using, respectively, the following oligonucleotides: 5′-CTGCGGCCA*A*GTTGACAGC-3′, 5′-CT GCCTGAGC*GC*CGTTCAGG-3′, 5′-GAGCGATCGCC*G*AA GCGCTT, 5′-CAACCTCCCA*GC*CACCATCA-3′ (position of mismatches are given in italic). Sequences encoding the four mutants of the wheat nsLTP1 were all introduced into the pET vector and the corresponding plasmids were used to transform *E. coli* BL21(DE3)pLysS. Induction of the bacteria carrying the mutated sequences with isopropyl-β-D-thiogalactopyranoside led to the production of the recombinant mutants in inclusion bodies (data not shown) as found for the wild-type recombinant protein. Thus, the same purification/renaturation protocol was used to recover the proteins in the soluble; 20–50 mg protein·L^{-1} bacteria culture was obtained depending on the mutation.

Characterization of mutants of the wheat nsLTP1

Presence of the mutations in the primary structure of the four recombinant LTPs was first confirmed by MS analysis. Experimental data were compared to calculated data (Table 1) and were shown to be consistent with the amino acid substitutions made. Furthermore, these analysis confirmed the formation of four disulphide bonds in the renatured proteins. In order to analyse structure modification due to the mutation, the CD spectra of the recombinant proteins were recorded and compared to that of the wild-type nsLTP1. Table 2 shows that none of the amino acid substitutions led to significant changes in the percentage of α-helices except the Tyr79Ala mutation that displayed a slight decrease in helix content.

Lipid transfer and binding activity of the nsLTPs

To test the lipid transfer activity of the recombinant proteins a fluorescent lipid transfer assay was used as described by Van

Table 1. MS analysis of nsLTPs and comparison with the data calculated from primary structure.

Proteins	Experimental data (Da)	Calculated data (no disulphide bonds; Da)	Calculated data (four disulphide bonds; Da)
Wheat-isolated nsLTP1	9600.58 ± 0.9	9606.84	9598.84
Recombinant wild-type LTP1	9730.85 ± 1.25	9738.03	9730.03
His5Gln mutant LTP1	9723.25 ± 1.15	9729.02	9721.02
Tyr16Ala mutant LTP1	9639.05 ± 0.95	9645.93	9637.93
Gln45Arg mutant LTP1	9759.15 ± 1.11	9766.09	9758.09
Tyr79Ala mutant LTP1	9639.15 ± 2.25	9645.93	9637.93

Table 2. Percentage α-helix content and lipid transfer and binding activities of the wheat-isolated nsLTP and recombinant nsLTPs.

Proteins	α−helix (%)	Lipid transfer activity (nmol pyrene−phospholipids·min^{-1}mg^{-1})	Lipid binding nsLTP K_d (μM)
Wheat-isolated nsLTP1	29	0.18	28.9 ± 1.6
Recombinant wild-type LTP1	28	0.07	25.1 ± 0.7
His5Gln mutant LTP1	29	0.07	20 ± 4.0
Tyr16Ala mutant LTP1	27	0.05	30 ± 8.0
Gln45Arg mutant LTP1	28	0.18	16.3 ± 3.1
Tyr79Ala mutant LTP1	24	0.14	–

Paridon *et al*. [36]. Transfer activity of the protein was determined by measuring the transfer of pyr-PC or pyr-PG from quenched donor vesicles (Pyr-PC/Pyr-PG, 1 : 1) to unlabelled acceptor vesicles of egg yolk phosphatidylcholine as a function of time. Table 2 summarizes the results of lipid transfer activities of the proteins compared with the wheat-extracted nsLTP1 used as a control. Experimental values are in accordance with activities determined with another method [22] and are in the same order indicating that the mutations chosen did not lead to important changes in the lipid transfer activity.

By contrast, lipid binding experiments revealed important differences between the wild-type and one of the nsLTP1 mutants (Fig. 4). Lipid binding was probed by intrinsic fluorescence spectroscopy measurement of the nsLTP following the addition of increasing amount of lysoPC according to Gomar *et al*. [37]. The increase of fluorescence intensity upon lipid binding is likely to originate from the fluorescence emission of the phenolic side chains of tyrosine residues present, respectively, in the H1 helix and the C-terminal end of the wheat wild-type nsLTP1; however, the specific contribution of each fluorophore could not be determined [37]. Analysis of the lipid binding of the two tyrosine mutants allowed the separation of the effect of each tyrosine. Fig. 4 illustrates the relative increase of fluorescence intensity of all the recombinant nsLTP1 as a function of lysoPC concentration and shows that no increase in fluorescence was displayed by the Tyr79Ala mutant in the presence of lipids. Other nsLTP1 mutants showed the same curve as the wild-type protein with the exception of the His5Gln mutant, which shows a maximum fluorescence intensity increase lower than the wild-type protein. Furthermore, curve fitting was carried out with a mathematical model assuming one binding site and allowed calculation of the dissociation constants. As shown in Table 2, all of the recombinant nsLTPs display dissociation constants in the same order of magnitude except the Tyr79Ala mutant for which the dissociation constant could not be determined. Thus, only this mutant appeared to be affected in the lysoPC binding indicating either absence of lipid binding or that Tyr79 was the only aromatic residue involved in the observed fluorescence increase. As the Tyr79Ala mutant was capable of transferring phospholipids, the latter hypothesis was highly probable. Thus, the lipid binding capability of the Tyr79Ala mutant was tested in lipid transfer assay conditions but without the addition of the acceptor liposomes (Fig. 5). The observed fluorescence intensity increase from quenched Pyr-PG donor liposomes confirmed the ability of the Tyr79Ala mutant to bind phospholipids.

DISCUSSION

The present results are part of a study of the wheat 9-kDa nsLTPs. We found that the wheat 9-kDa nsLTP1 is synthesized efficiently in *E. coli* and thus should be a convenient source for preparing large amounts of the wild-type or mutated protein needed for biochemical studies. However, it accumulates in the cytoplasm in an insoluble form. Neither changing the expression conditions nor coproduction of the GroES and GroEL chaperonins had allowed the recovery of the recombinant LTP in the soluble fraction ([45] and unpublished data). Recently however, it was shown that *Pichia pastoris* could be used as an efficient system to secrete a wheat LTP as a soluble protein [46].

Our results show that the recombinant LTP produced in *E. coli* can be easily solubilized and refolded from inclusion bodies. Furthermore, a sufficient quantity of recombinant nsLTP1 (50 mg·L^{-1} bacterial culture) could be recovered for structural and functional studies. Recombinant nsLTP1 retains the initiation methionine that has been included in the construction for expression in *E. coli* but displays the same structure as the wheat-extracted nsLTP1 as observed by CD and three-dimensional NMR spectroscopy (P. Sodano, CNRS-Orléans, France, unpublished data). The recombinant protein also shows lipid transfer and binding activities in the same range as the wheat-extracted protein. Thus, even if the recombinant protein has to be renatured the expression system used here appears to be more efficient than the production of a maltose binding protein fusion [47]. Indeed, the fusion protein has been shown to be soluble and to display lipid transfer activity but the wheat nsLTP1 could not be recovered after cleavage with factor Xa.

Our data show that the four mutants of the wheat nsLTP1 (His5Gln, Tyr16Ala, Gln45Arg, Tyr79Ala) have the same overall structure as the protein isolated from wheat kernel allowing a comparative study of their activities. Their lipid transfer activities do not appear to be affected by the mutations introduced in the nsLTP1 sequence. However, lipid binding was shown to be significantly perturbed by the replacement of Tyr79 with an alanine residue. Comparison of the cereal nsLTP sequences has already shown the high conservation of the two Tyr residues and it has been suggested they could play an important role in the function of the proteins. Tyr79, belonging to the C-terminal fragment has been located at the extremity of the hydrophobic cavity and is supposed to be perturbed by the lipid binding [24]. This residue is thought to be one of the key residue in the interaction with the palmitoyl moiety of the ligand as described in maize and barley nsLTPs [13,41] and it is possible that this residue plays a function as a molecular cover of the hydrophobic cavity in the uncomplexed form as proposed by Heinemann *et al*. [41]. Furthermore, the flexibility of the C-terminal region together with location of this Tyr residue near the extremity of the hydrophobic cavity suggests a possible role in binding and subsequent release of the lipid [13,48]. Indeed it has been shown by X-ray analysis of the maize nsLTP-fatty acid complex that the fatty-acid carboxyl group is involved in hydrogen bonds with the hydroxyl of Tyr81 (Tyr79 in wheat) and two water molecules [13]. Lerche *et al*. [48] also found the Tyr79 from barley nsLTP to be one of the key residues involved in the interaction with the fatty-acid moiety of the palmitoylCoA ligand. Data obtained from lipid binding assays with the Tyr79Ala mutant of the wheat nsLTP1 indicate either absence

of lysoPC binding with this mutant or that the Tyr79 is the residue responsible for the fluorescence intensity increase observed with the wild-type nsLTP1 and, as a consequence, is the residue perturbed by lipid binding. The second hypothesis is retained because the Tyr79Ala mutant is active in lipid transfer activity and we conclude that Tyr79 is in close proximity to the lipid binding site as expected from three-dimensional structure data. Furthermore, lipid binding of the Tyr79Ala mutant has been confirmed by a lipid transfer assay in which the acceptor liposomes were omitted. Because wheat nsLTP1 is capable of forming well-defined complexes with lipids in solution [44] study of the equivalent Tyr79Ala mutant complex is of interest. The Tyr16 located at the end of the H1 helix seems, however, to be exposed to the solvent. The other two mutants, His5Gln and Gln45Arg, do not show any significant differences in lipid transfer or binding activities in comparison with the wheat-isolated nsLTP1 and therefore His5 and Gln45 do not appear to be key residues for the lipid/protein interactions. However, even if lysoPC affinity of the His5Gln mutant is found to be identical to that of the wild-type nsLTP1 (the dissociation constants are of the same order of magnitude) it seems that the charge difference due to the mutation leads to a change in the Tyr79 environment that could explain the lower fluorescence intensity level observed for this mutant in Fig. 4.

Whereas all of the cereal nsLTPs show a similar global fold, packing of the hydrophobic side chains is different; therefore the size of the hydrophobic cavity appears to vary and could be important to the ability of these proteins to bind and transfer lipids. Thus, the use of a number of lipidic compounds and the introduction of other mutations in the cavity is required to analyse further the lipid/protein interactions.

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REFERENCES

1. Tai, S.P. & Kaplan, S. (1985) Phospholipid transfer in microorganisms. *Chem. Phys. Lipids* **38**, 41–50.
2. Wirtz, K.W.A. & Gadella, T.W.J. (1990) Properties and mode of action of specific and non specific phospholipid transfer proteins. *Experientia* **46**, 592–599.
3. Kader, J.C. (1997) Lipid transfer proteins: a puzzling family of plant proteins. *Trends Plant Sci.* **2**, 66–70.
4. Rickers, J., Spener, F. & Kader, J.C. (1985) A phospholipid transfer protein that binds long-chain fatty acids. *FEBS Lett.* **180**, 29–32.
5. Tsuboi, S., Osafune, T., Tsugeki, R., Nishimura, M. & Yamada, M. (1992) Nonspecific lipid transfer protein in castor bean cotyledon cells: subcellular localization and a possible role in lipid metabolism. *J. Biochem.* **11**, 500–508.
6. Ostergaard, J., Vergnolle, C., Schoentgen, F. & Kader, J.C. (1993) Acyl-binding /lipid-transfer proteins from rape seedlings, a novel category of proteins interacting with lipids. *Biochem. Biophys. Acta* **1170**, 109–117.
7. Mundy, J. & Rogers, J.C. (1986) Selective expression of a probable amylase/protease inhibitor in barley aleurone cells: comparaison to the barley amylase/subtilisin inhibitor. *Planta* **169**, 51–63.
8. Tchang, F., This, P., Stiefel, V., Arondel, V., Morch, M.D., Pages, M., Puidomenech, P., Grellet, F., Delseny, M., Bouillon, P., Huet, J.C., Guerbette, F., Beauvais-Cante, F., Duranton, H., Pernollet, J.C. & Kader, J.C. (1988) Phospholipid transfer protein: Full-length cDNA and amino acid sequence in maize; amino acid homologies between plant phospholipid transfer proteins. *J. Biol. Chem.* **263**, 16849–16855.
9. Bernhard, W.R., Thoma, S., Botella, J. & Somerville, C.R. (1991) Isolation of a cDNA clone for spinach lipid transfer protein and evidence that the protein is synthesized by the secretory pathway. *Plant Physiol.* **95**, 164–170.
10. Tsuboi, S., Suga, T., Takishima, K., Mamiya, G., Matsui, K., Ozeki, Y. & Yamada, M. (1991) Organ-specific occurence and expression of the isoforms of nsLTP in castor bean seedlings and molecular cloning of a full-length cDNA for a cotyledon-specific isoform. *J. Biochem.* **110**, 823–831.
11. Takishima, K., Watanabe, S., Yamada, M., Suga, T. & Mamiya, G. (1988) Amino acid sequences of two nonspecific lipid-transfer proteins from germinated castor bean. *Eur. J. Biochem.* **166**, 387–391.
12. Désormeaux, A., Blochet, J.E., Pezolet, M. & Marion, D. (1992) Amino acid sequence of a non specific wheat phospholipid transfer protein and its conformation as revealed by infrared and Raman spectroscopy. Role of disulfide bridges and phospholipids in the stabilization of the α-helix structure. *Biochim. Biophys. Acta* **1121**, 137–152.
13. Shin, D.H., Lee, J.Y., Hwang, K.Y., Kim, K.K. & Suh, S.W. (1995) High resolution crystal structure of the non-specific lipid-transfer protein from maize seedlings. *Structure* **3**, 189–199.
14. Sterck, P., Booij, H., Schellekens, G.A., Van Kammen, A. & De Vries, S. (1991) Cell-specific expression of the carrot EP2 lipid transfer protein gene. *Plant Cell* **3**, 907–921.
15. Pyee, J., Hongshi, Y. & Kolattukudy, P.E. (1994) Identification of a lipid transfer protein as the major protein in the surface wax of broccoli *(Brassica oleracea)* leaves. *Arch. Biochem. Biophys.* **311**, 460–468.
16. Cammue, B.P.A., Thevissen, K., Hendricks, M., Eggermont, K., Goderis, I.J., Proost, P., Van Damme, J., Osborn, R.W., Guerbette, F., Kader, J.C. & Broekaert, W.F. (1995) A potent antimicrobial protein from onion seeds showing sequence homology to plant lipid transfer proteins. *Plant Physiol.* **109**, 445–455.
17. Garcia-Olmedo, F., Molina, A., Segura, A. & Moreno, M. (1995) The defensive role of nonspecific lipid-transfer proteins in plants. *Trends Microbiol.* **3**, 72–74.
18. Molina, A. & Garcia-Olmedo, G. (1997) Enhanced tolerance to bacterial pathogens caused by the transgenic expression of barley lipid transfer protein LTP2. *Plant J.* **12**, 669–675.
19. Torres-Schumann, S., Godoy, J.A. & Pintor-Toro, J.A. (1992) A probable lipid transfer protein gene is induced by NaCl in stems of tomato plants. *Plant Mol. Biol.* **18**, 749–757.
20. Soufleri, I.A., Vergnolle, C., Miginiac, E. & Kader, J.C. (1996) Germination-specific lipid transfer protein cDNAs in *Brassica napus* L. *Planta* **199**, 229–237.
21. Sorensen, S.B., Bech, L.M., Muldbjerg, M., Beenfeldt, T. & Breddam, K. (1993) Barley lipid transfer protein 1 is involved in beer foam formation. *MBAA Technical Q.* **30**, 136–145.
22. Monnet, F.P. (1990) *Caractérisation d'une protéine de fixation de lipides du blé dur, purification, séquençage, ADN complémentaire; relations aux protéines végétales de transfert de lipides et aux inhibiteurs d'amylases/trypsine des céréales.* PhD Thesis. University of Montpellier, France.
23. Dieryck, W., Gautier, M.F., Lullien, V. & Joudrier, P. (1992) Nucleotide sequence of a cDNA encoding a lipid transfer protein from wheat (*Triticum durum* Desf.). *Plant Mol. Biol.* **19**, 707–709.
24. Gincel, E., Simorre, J.P., Caille, A., Marion, D., Ptak, M. & Vovelle, F. (1994) Three-dimensional structure in solution of a wheat lipid transfer protein from multidimensional ^{1}H- NMR data. *Eur. J. Biochem.* **226**, 413–422.
25. Vieira, J. & Messing, J. (1987) Production of single-stranded plasmid DNA. *Methods Enzymol.* **123**, 3–11.
26. Rosenberg, A.H., Lade, B.N., Chui, D., Lin, S., Dunn, J.J. & Studier, F.W. (1987) Vectors for selective expression of cloned DNAs by T7 RNA polymerase. *Gene* **56**, 125–135.
27. Sambrook, J., Fritsch, E.F. & Maniatis, T. (1989) *Molecular Cloning. a Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, New York.

28. Stephen, C.J., Jones, C. & Schofield, J.P. (1990) A rapid method for isolating high quality plasmid DNA suitable for DNA sequencing. *Nucleic Acids Res.* **18**, 7463–7464.
29. Hanahan, D. (1985) Technique for transformation of *E. coli*. *In DNA Cloning: a Practical Approach*, **Vol. 1** (Glover, D.M., ed.), pp. 109–135, IRL Press, Oxford.
30. Kunkel, T.A., Roberts, J.D. & Zakour, R.A. (1987) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Meth Enzymol.* **154**, 367–382.
31. Sanger, F., Nicklen, S. & Coulson, A.R. (1977) DNA sequencing with chain terminating inhibitors. *Proc. Natl Acad. Sci. USA* **74**, 5463–5467.
32. Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. & Klenk, D.C. (1985) Measurement of protein using the bicinchoninic acid. *Anal. Biochem.* **150**, 76–85.
33. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the bacteriophage T4. *Nature* **227**, 680–685.
34. Towbin, H., Staehelin, T. & Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl Acad. Sci. USA* **76**, 4350–4354.
35. Chen, Y.H., Yang, J.T. & Chau, K.H. (1974) Determination of the helix and β form of proteins in aqueous solution by circular dichroism, *Biochemistry* **13**, 3350–3359.
36. Van Paridon, P.A., Gadela, T.W.J. & Wirtz, K.W.A. (1988) The effect of polyphosphoinositides and phosphatidic acid on the phosphatidylinositol transfer protein from bovine brain: a kinetic study. *Biochim. Biophys. Acta* **943**, 76–86.
37. Gomar, J., Petit, M.C., Sodano, P., Sy, D., Marion, D., Kader, J.C., Vovelle, F. & Ptak, M. (1996) Solution structure and lipid binding of a non specific lipid transfer protein extracted from maize seeds. *Protein Sci.* **5**, 565–577.
38. Marston, F.A.O. (1986) The purification of eukaryotic polypeptides synthesized in *Escherichia coli*. *Biochem. J.* **240**, 1–12.
39. Ben-Bassat, A., Bauer, K., Chang, S.Y., Myambo, K., Boosman, A. & Chang, S. (1987) Processing of the initiation methionine from proteins: properties of the *Escherichia coli* methionine aminopeptidase and its gene structure. *J. Bacteriol.* **169**, 751–757.
40. Hirel, P.H., Schmitter, J.M., Dessen, P., Fayat, G. & Blanquet, S. (1989) Extent of N-terminal methionine excision from *Escherichia coli* proteins is governed by the side chain length of the penultimate amino acid. *Proc. Natl Acad. USA* **86**, 8247–8251.
41. Heinemann, B., Andersen, K.V., Nielsen, P.R., Bech, L.M. & Poulsen, F.M. (1996) Structure in solution of a four-helix lipid binding protein. *Protein Sci.* **5**, 13–23.
42. Lee, J.Y., Min, K., Cha, H., Shin, D.H., Hwang, K.Y. & Suh, S.W. (1998) Rice non-specific lipid transfer protein: the 1.6 Å crystal structure in the unliganded state reveals a small hydrophobic cavity. *J. Mol Biol.* **276**, 437–448.
43. Breu, V., Guerbette, F., Kader, J.C., Kannangara, C.G., Svensson, B. & Von Wettstein-Knowles, P. (1989) A 10 kD barley basic protein transfers phosphatidylcholine from liposomes to mitochondria. *Carlsberg Res. Commun.* **54**, 81–84.
44. Petit, M.C., Sodano, P., Marion, D. & Ptak, M. (1994) Two-dimensional 1H-NMR studies of maize lipid-transfer protein. *Eur J. Biochem.* **22**, 1047–1054.
45. Lullien-Pellerin, V., Ihorai, T., Devaux, C., Marion, D., Ptak, M., Joudrier, P. & Gautier, M.F. (1998) Site-directed mutagenesis of wheat 9 kDa lipid transfer protein. *In Plant Protein from European Crops: Food and Non Food Applications* (Guegen, J. & Popineau, Y., eds), pp. 88–93, Springer-Verlag, Berlin.
46. Klein, C., de Lamotte-Guéry, F., Gautier, F., Moulin, G., Boze, H., Joudrier, P. & Gautier, M.F. (1998) High-level secretion of a wheat lipid transfer protein in Pichia pastoris. *Prot. Exp. Purif.* **13**, 73–82.
47. Dieryck, W., Lullien-Pellerin, V., Marion, D., Joudrier, P. & Gautier, M.F. (1995) Purification and activity of a wheat 9-kDa lipid transfer protein expressed in *Escherichia coli* as a fusion with the maltose binding protein. *Prot. Exp. Purif.* **6**, 597–603.
48. Lerche, M.H., Kragelund, B.B., Bech, L.M. & Poulsen, F.M. (1997) Barley lipid transfer protein complexed with palmitoyl CoA. *Structure* **5**, 291–306.
49. Yu, Y.G., Chung, C.H., Fowler, A. & Suh, S.W. (1988) Amino acid sequence of a probable amylase/protease inhibitor from rice seeds. *Arch. Biochem. Biophys.* **265**, 466–475.