

Purification, characterisation and cDNA cloning of a type 2 (7 kDa) lipid transfer protein from *Triticum durum*[☆]

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Abstract

A type 2 non-specific lipid transfer protein (ns-LTP2) was isolated from wheat (*Triticum durum* Desf.) endosperm and the primary structure of the protein was fully determined. The protein is basic with a predicted pI of 8.4 and a molecular mass of 6980 Da. It contains 67 amino acids including eight cysteine residues. Oligonucleotide probes covering part of the protein sequence were designed to isolate the corresponding cDNA that contains an open reading frame of 96 amino acids, including a 29 amino acid signal peptide. The *T. durum* ns-LTP2 showed 37.3–77.6% identity with other ns-LTP2 and is most closely related to the barley and rice sequences. PCR carried out on wheat genomic DNA indicated that the gene encoding this ns-LTP2 does not contain introns. Within developing seeds, the level of *ltp7.1* gene transcripts detected by RT-PCR remained broadly similar from the cellular division phase to dry seed. This contrast with the expression pattern of the *ltp9.1* gene, the transcripts of which started to accumulate during the filling phase. During germination, the level of *ltp7.1* gene transcripts decreased more rapidly than those of the *ltp9.1* gene. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Wheat; Lipid transfer protein; cDNA sequence; Protein purification; Gene expression

1. Introduction

Plant non-specific lipid transfer proteins (ns-LTPs) are widely distributed and form a superfamily of related proteins, which is subdivided into two families: the 9 kDa LTPs or type 1 ns-LTPs (ns-LTP1) and the 7 kDa LTPs or type 2 ns-LTPs (ns-LTP2). The ns-LTP1 have been studied in greater depth than the ns-LTP2. By definition, ns-LTPs can mediate in vitro the transfer of phospholipids from liposomal donor membranes to mitochondrial acceptor membranes [1]. However, their in planta functions have not yet been elucidated and remain controversial, the most commonly argued hypotheses being a role in the transport of cutin

monomers [2,3] and in plant defence mechanisms [4,5] for ns-LTP1. The expression pattern of ns-LTP2 genes is different, suggesting other functions for the corresponding ns-LTP2 that may be good markers of differentiation process [6,7]. There are more than 100 sequences of plant ns-LTPs in databases, but only a few have been isolated from plant, most of the ns-LTPs having been identified on the basis of sequence homology, sequences that were deduced from cDNA clones or genes. The first ns-LTP1 was isolated from maize seedlings [8], and since then ns-LTP1 have been isolated from spinach [1], ragi [9], castor bean [10], barley [11], rice [12], wheat [13,14], sunflower [15], radish [4], grapevine [16], carrot [17], broccoli [18], rape seedlings [19], onion [20] and peach [21]. Also, in vitro lipid transfer or binding has been demonstrated for a limited number of proteins. The presence of both ns-LTP1 and ns-LTP2 has been demonstrated in maize [22] and castor bean [23] seedlings. To our knowledge, no ns-LTP2 has been purified and fully characterised from plants and all the known ns-LTP2 primary structures

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were deduced from cDNA clones or genes. Indeed, genes encoding ns-LTP2 have been isolated from rice [24], *Vigna unguiculata* [25], *Zinnia elegans* [7,26], barley [27,28] and *Brassica rapa* [29].

In wheat, a ns-LTP1 has been isolated from *Triticum durum* [13] and *T. aestivum* [14] and a previous paper describes the isolation of a cDNA clone encoding a *T. durum* ns-LTP1 [30]. This paper describes the purification and determination of the primary structure of a *T. durum* ns-LTP2 and the isolation of the corresponding cDNA clone.

2. Methods

2.1. Plant material

T. durum Desf. (cv. Chandur) seeds were provided by GIE Blé dur (France) and semolina (77% purification yield) was prepared on a semi-industrial semolina production mill. *T. durum* (cv. Néodur) was grown outdoors (experimental station of Melgueil, INRA, Montpellier). Heads of wheat were tagged on the day of flowering (i.e., when anthers first started to emerge) and were collected at various stages after anthesis. Whole ears were immediately frozen in liquid nitrogen and stored at -70°C until needed. Germination was carried out as described by Labhilili et al. [31]. Restriction enzymes, modification enzymes and synthetic oligonucleotides were supplied by Appligene and Eurogentec. All reagents were of analytical grade for biochemical uses.

2.2. Wheat LTP purification

Eight hundred grams *T. durum* semolina were extracted for 15 min with 2 l of extraction buffer containing 100 mM Tris, 4 mM cysteine chloride, 1 mM EDTA and 8 mM mercaptoethanol, adjusted to pH 7.0. All steps of the purification protocol were carried out at 4°C . The extract was centrifuged for 15 min at $15000 \times g$ and the supernatant was adjusted to have pH 5.1 and centrifuged a second time in the same conditions to give the 'crude extract' fraction. pH was then adjusted to 7.0 and proteins precipitated overnight with ammonium sulphate (52 g/100 ml supernatant). Proteins were pelleted by centrifugation (30 min, $15000 \times g$), solubilised in a minimal volume of buffer A containing 10 mM Tris-HCl pH 7.0, 8 mM mercaptoethanol and 3 mM sodium azide, dialysed against buffer A for at least 5 h and centrifuged for 10 min at $15000 \times g$ to give the 'ammonium sulphate' fraction. This fraction was applied to a G-75 Sephadex (Pharmacia) column (5×85 cm) and proteins eluted with buffer A (flow rate: 90 ml/min). Lipid transfer activity of fractions was monitored as described below and active

fractions were pooled to give the 'Sephadex' fraction that was then loaded onto a carboxymethyl sepharose (CM 6B; Pharmacia) column (2.6×27 cm) and proteins eluted with a linear gradient of 0–250 mM NaCl. The wheat ns-LTP2 was purified by a final chromatography on a G50 Sephadex (Pharmacia) column (2.6×75 cm) equilibrated with buffer A.

2.3. Lipid transfer activity

Assessment of phospholipid transfer activity was carried out on dialysed protein fractions using [^3H] phosphatidylcholine/cholesteryl [^{14}C] oleate-labelled liposomes as donor and mitochondria as acceptor membranes as described by Kader et al. [1]. The increase in $^3\text{H}/^{14}\text{C}$ ratio was used to follow transfer activity, with cholesteryl [^{14}C] oleate assumed to be a non-exchange tracer. Results are expressed as the percentage of the liposomal phosphatidylcholine recovered in the mitochondria as described by Douady et al. [8]. One unit of transfer activity is defined as the amount of protein needed to transfer 1 nmol of phospholipid per min at 30°C from liposomes to mitochondria.

2.4. SDS-PAGE analysis

Reduction of proteins was performed by incubation in boiling water for 5 min in the presence of 2-mercaptoethanol. Reduced proteins were separated on 7.5–15% acrylamide gradient gels using 25 mM Tris, 130 mM glycine, 3 mM SDS buffer adjusted to pH 8.3 then stained with Coomassie brilliant blue.

2.5. N-terminal sequence analysis and primary structure determination

The N-terminal amino acid sequence of the wheat purified ns-LTP2 was determined by automatic Edman degradation in a 470 protein sequencer (Applied Biosystems) with on-line detection of the stepwise liberated PHT amino acid derivatives in a 120 A analyser (Applied Biosystems). For reduction, the vacuum dried protein (100 nmol) was incubated in the dark for 4 h at 20°C in 380 mM Tris-HCl (pH 8.8), 3 M guanidium chloride and 10 mM mercaptoethanol. Subsequently, 4-vinyl-pyridine was added and the thiol blocking reaction allowed to take place for 90 min at 20°C in the dark. The alkylation reaction was stopped by adjusting the pH to below 5.0. The reduced-alkylated protein was separated from the reagents by reverse phase chromatography on an Aquapore RP300 column ($7 \mu\text{m}$; 30×0.46 cm; Brownlee) previously equilibrated with solvent I (water containing 1.15% trifluoroacetic acid). Following a 7 min wash with solvent I, a 30 min linear gradient from 0 to 100% solvent II (1% trifluoroacetic acid, 50% acetonitrile, 25% isopropanol, 25% H_2O) in

solvent I was applied. The flow rate was 1 ml/min. Peptides from the digestion with V8 staphylococcus endopeptidase were separated on a Nucleosyl 5 C18 column (5 μ m; 25 $\times$ 0.46 cm; Société Française de Chromatographie) in the same conditions. Peptides from the digestion with trypsin were separated on the Nucleosyl 5 C18 column by a 40 min linear gradient from 0 to 80% solvent II in solvent I following a 4 min wash with solvent I.

2.6. Screening of the wheat cDNA library

The construction of a cDNA library from *T. durum* Desf. (cv. Agathé) mid-maturation seeds has been previously described by Gautier et al. [32]. Colonies were transferred to Hybond C extra membrane (Amersham) according to the manufacturer's instructions and hybridised with a mix of synthetic oligonucleotides, 5'-TG(TC)TT(TC)TG(TC)CA(AG)TA(TC)GC-3', derived from the CFCQYA sequence (residues 34–38) of the wheat ns-LTP2. Oligonucleotides were labelled at their 5'-end with γ -³²P ATP by T4 polynucleotide kinase. Hybridisation conditions were as described by Gautier et al. [32]. Hybridised filters were washed at room temperature twice with 2 $\times$ SSPE, 0.1% (w/v) SDS for 15 min; twice with 2 $\times$ SSPE for 15 min and then exposed to X-ray film at –70 °C with two intensifying screens.

2.7. DNA sequencing and analysis

The sequence of pTd18 was determined on both strands using the Applied Biosystems Taq Dye Primer Cycle Sequencing and the Taq Dye Deoxy Terminator Cycle Sequencing kits (PE Applied Biosystems). Samples were electrophoresed, detected and analysed on an Applied Biosystems 370A DNA sequencer (PE Applied Biosystems). Nucleotide and amino acid sequence analyses were performed with the IntelliGenetics PC/GENE programme. Database search was carried out using BLAST [33], under default parameter settings, and sequences were retrieved from the database using the BLAST interface on the World Wide Web.

2.8. RNA extraction and RT-PCR

Total RNA was extracted from *T. durum* seeds at different days after flowering (4, 13, 20, 27, 34 and 48 DAF) and germination (0, 1 and 3 DAG) by a hot phenol method as described by Verwoerd et al. [34] and modified by Chevalier et al. [35]. To eliminate contaminating DNA, RNA samples were treated (1 h at 37 °C) with RNase-free DNase (Promega) in presence of ribonuclease inhibitor (Rnasin, Promega). RNA samples were quantified with the RiboGreen™ RNA quantitation kit (Molecular Probes). Total RNA (1 μ g) was

reverse transcribed using 20 pmol oligo-(dT) and M-MLV reverse transcriptase (Advantage RT for PCR, Clontech) under conditions specified by the supplier. PCR was carried out on a 1/25 sample of RT products according to the manufacturer's directions (AdvanTaq Plus PCR, Clontech). The amplification was for a total of 30 cycles, which was within the logarithmic range of amplification. The sense-strand primers were LTP7.1P2 (5'-CGTGAGAGTGTCTCAGACTATAGCT-3') or LTP9.1P2 (5'-TGGGCGACGATCCGTCAAGCTG-3'; EMBL accession number X63669) and the antisense-strand primers LTP7.1M1 (5'-TTCATTCCAGTACCAGCAAAGTC-3') or LTP9.1M2 (5'-AAGTCCATAAACACTGGGAGTG-3'). To normalise results, PCR was carried out with glyceraldehyde 3-phosphate dehydrogenase primers (GAPDHP1 5'-TAACTGCCTTGCTCCTCTTGCT-3' and GAPDHM1 5'-GTTTCCCTCAGACTCCTCCTT-3') deduced from a wheat gene (EMBL accession number AF251217). Control reactions without reverse transcription were included to ensure that PCR products were not the results of amplification of contaminating DNA. The PCR products were analysed by running 1/5 sample of reaction on a 2% agarose gel.

3. Results

3.1. Purification to homogeneity of a *T. durum* ns-LTP2

In preliminary experiments, the 'crude extract' fraction obtained from semolina showed significant transfer of phosphatidylcholine from liposomes to mitochondria, indicating that lipid transfer proteins had been extracted. Subsequently, a ns-LTP2 was purified from a 'crude extract' fraction prepared from 800 g of semolina by a four-step process including ammonium sulphate insolubilisation, gel filtration, anion-exchange chromatography and a final gel filtration. Lipid transfer activity was monitored at each step of the purification process to identify fractions that contained active proteins, and the purification of the wheat ns-LTP2 was followed by SDS-PAGE analysis. Insolubilisation with 80% ammonium sulphate allowed the elimination of the majority of the wheat extracted proteins (88%) and recovery of 69% of the transfer activity (Table 1). The ammonium sulphate fraction was then loaded onto a G75 Sephadex column and two peaks of activity were detected (data not shown), the main one containing mainly low molecular weight proteins (5–15 kDa) (Fig. 1(C), lane 1). Fractions of this peak were pooled (Sephadex fraction) and separated by cation-exchange chromatography on a carboxymethyl sepharose column as shown in Fig. 1(A). After the unbound proteins were

washed off, a NaCl gradient was applied. Two peaks of transfer activity (I and II) were identified, the highest level of lipid transfer activity being shown by peak I eluting at approximately 100 mM NaCl: this peak was analysed by SDS-PAGE. A main band, the apparent molecular mass of which was 6 kDa, plus minor bands of higher molecular mass, were present in the three fractions (10 ml each) composing peak I (Fig. 1(C), lanes 2–4). After pooling, these fractions were further purified by gel filtration on a G50 Sephadex column. Almost all lipid transfer activity was restricted to peak 2 (Fig. 1(B)) and the central fractions were found by SDS-PAGE analysis to give a single band of approximately 6 kDa, indicating that the protein had been purified to homogeneity (Fig. 1(C), lane 5). Differences between calculated and apparent molecular mass of proteins as determined by SDS-PAGE have been observed previously. Purity of the wheat ns-LTP2 preparation was further confirmed by sequence determination. Starting with 800 g of semolina, this purification process yielded 12 mg of homogeneous ns-LTP2. The overall purification yield was 3% and the wheat ns-LTP2 was purified 57 times (Table 1). The decrease in specific activity observed at the last step of the purification process may be explained by the elimination of compounds interfering with lipid transfer activity.

3.2. Primary structure of the *T. durum* ns-LTP2

The strategy used to obtain the primary structure of the *T. durum* ns-LTP2 is shown in Fig. 2. Direct N-terminal sequencing of the protein yielded the first 26 amino acid residues. The amino acid sequence up to residue 67 was determined by sequencing fragments released by V8 protease and trypsin proteolysis. A microheterogeneity was shown at position 58 that was found to be either a threonine or a glutamine residue depending on the fragment analysed. The 67 residue protein had a molecular mass of 6980 Da and a predicted *pI* of 8.4. This protein contained the typical eight cysteine residues the position of which is highly conserved in all plant ns-LTPs.

Table 1
Purification of a wheat ns-LTP2 from *T. durum* semolina

Fractions	Protein (mg)	Total activity	Specific activity	Yield (%)	Purification factor
Crude extract	22 050	7276	0.3	100	
Ammonium sulphate	2565	4873	1.9	69	6
Sephadex ^a	333	2031	6.1	28	18
Peak 1 CM	53	1834	34.6	25	105
Peak 2 G50	12	233	18.8	3	57

The steps of purification are described in Section 2. Data are from 800 g semolina. Total activity is expressed as nmol of phosphatidylcholine transferred per hour and specific activity as nmol of phosphatidylcholine transferred per mg protein per hour.

^a Pooled active fractions from Sephadex G75.

3.3. Isolation and characterisation of a cDNA clone encoding a *T. durum* ns-LTP2

A *T. durum* cDNA library constructed with poly (A)⁺ RNA isolated from mid-maturation seeds [32] was screened to identify sequences encoding wheat ns-LTP2. A mix of synthetic oligonucleotides deduced from the CFCQYA sequence (residues 34–38 of the *T. durum* isolated ns-LTP2) was used as a probe. After several cycles of screening one clone, pTd18, showing a strong hybridisation signal was isolated and fully characterised.

The nucleotide sequence of pTd18 and the deduced primary structure of the *T. durum* ns-LTP2 are shown in Fig. 3. pTd18 contains an uninterrupted reading frame of 288 bp that encodes a polypeptide of 96 residues. There are three in frame ATG sequences in the 5' region of pTd18; however, only the ATG at position 56 fits the Kozak consensus sequence (AXXATGGC) for the start point for translation [36]. Therefore, this ATG was assumed to be the initiation codon and the presence of an in frame stop codon 6 bp upstream strengthened this hypothesis. Residues 30–96 correspond to the primary structure of the ns-LTP2 purified from wheat semolina and, according to the (–3, –1) rule reported by von Heijne [37], a potential cleavage site was found between position 29 and 30, giving ACQA as N-terminal. Indeed, residues 1–29 conform to the characteristics of a signal peptide indicating that the *T. durum* ns-LTP2 is synthesised as a preprotein. The deduced mature protein contains 67 amino acids and shows 100% identity to the primary structure of the wheat isolated protein, which has threonine as residue 58.

3.4. Comparison of plant ns-LTP2

The overall sequence identity between the ns-LTP2 of the different plant species, aligned in Fig. 4, ranges from 77.6 to 37.3%. Cereal ns-LTP2 are highly conserved with wheat LTP having 77.6% identity and 83.6% similarity with a barley ns-LTP2 [27], and 62.7% identity and 79.1% similarity with the rice ns-LTP2 [24].

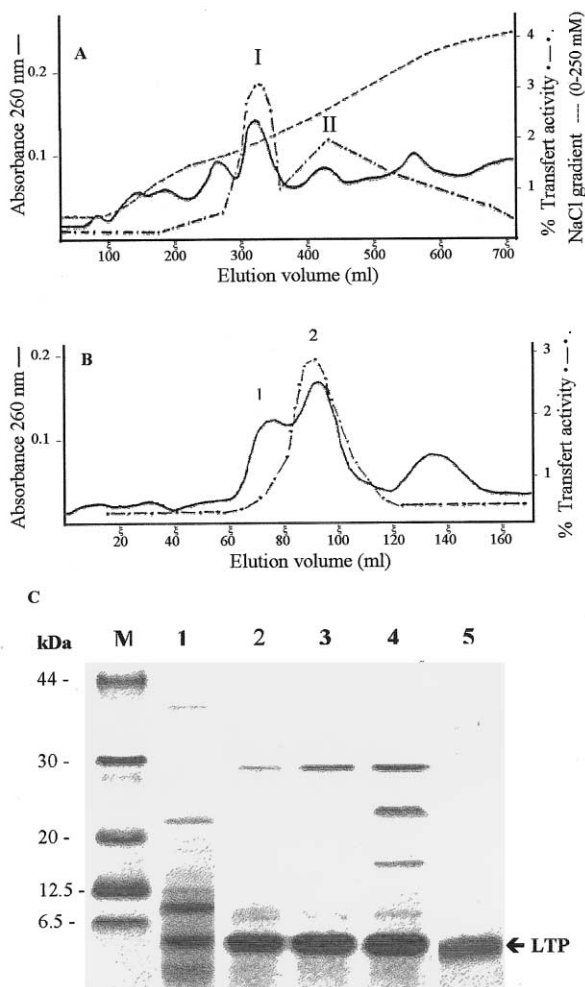


Fig. 1. Purification of a *T. durum* ns-LTP2. (A) Cation-exchange chromatography on carboxymethyl 6B sepharose. Active fraction I was eluted around 100-mM NaCl in buffer A. (B) Gel filtration on G50 Sephadex. Fraction I from the cation-exchange separation was loaded on G50 Sephadex and eluted with buffer A. Peak 2 contains the purified ns-LTP2. Lipid transfer activity (one unit of transfer activity is defined as the amount of protein needed to transfer 1 nmol of phospholipid per min at 30 °C from liposomes to mitochondria) is expressed as a percentage and was determined for 10 ml fractions. (C) SDS-PAGE analysis of LTP fractions, Coomassie stained gel: lane M, molecular mass standards (3–43 kDa prestained markers, Life Technologies); lane 1, Sephadex active fraction loaded on carboxymethyl 6B sepharose; lanes 2–4, fractions 30–32 eluted by a NaCl gradient from carboxymethyl 6B sepharose; lane 5, purified ns-LTP2 after gel filtration on G50 Sephadex (peak 2).

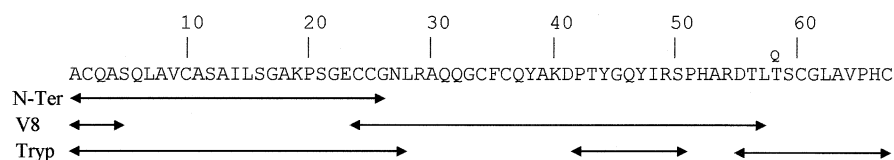


Fig. 2. Determination of the primary structure of a *T. durum* ns-LTP2. N-terminal sequencing of undigested protein, as well as tryptic and V8 fragment sequences was carried out on reduced and alkylated LTP. Double-headed arrows indicate sequence data obtained from various fragments that were not all fully sequenced.

There is also a high similarity of sequences between *Z. elegans* and *Kleimia odora* (EMBL accession number L33791) ns-LTP2, the two *Z. elegans* ns-LTP2 [7,26] showing 98.6% identity. A dendrogram (data not shown) obtained with the CLUSTAL V program indicated that the barley LTP (*Hv3*) expressed in roots is more closely related to LTP dicot sequences that are also expressed in vegetative tissues. In contrast, the closely related wheat, rice and barley (*Hv1* and *Hv2*) genes are all expressed in seeds.

In addition to the conserved position of the eight cysteine residues, the position of 11 residues (Leu7, Ala13, Pro20, Pro42, Pro51 and Pro65, Gln31, Tyr38 and Tyr47, Lys40 and Gly61; *T. durum* ns-LTP2 numbering) is highly conserved. The position of these four proline residues (Pro20, Pro42, Pro51 and Pro65; *T. durum* ns-LTP2 numbering) is conserved in all plant ns-LTP2, except for an *Arabidopsis thaliana* protein, which has a glutamine residue at position 51. The position of two tyrosine residues (Tyr38 and Tyr47; *T. durum* ns-LTP2 numbering) is also conserved except in rice and *Brassica* sequences that contain a phenylalanine residue at position 38.

3.5. Accumulation of *ltl* transcripts during wheat seed development

During seed development, the steady-state level of transcripts of *ltl7.1* gene did not show major variation between 4 and 48 DAF as indicated by analysis of RT-PCR products (Fig. 5(B)). On the contrary, the level of *ltl7.1* gene transcripts started to decrease after 24 h of germination. This compares with transcripts of *ltl9.1* gene encoding the wheat 9 kDa LTP [30] which started to accumulate between 13 and 20 DAF and remained present in the dry seed. A slight decrease was observed after 72 h of germination (Fig. 5(C)). The level of transcripts of the *GAPDH* gene indicated even amounts of template present in all samples (Fig. 5(A)). No PCR products were obtained in negative controls in which no reverse transcription step had been carried out (not shown). Assuming that the amount of RT-PCR products is an indicator of the quantity of transcripts, transcripts of *ltl7.1* gene are less abundant than those of *ltl9.1* gene.

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AGACTCCATTCCCATCACACACACGGACACACCTGCAAGAGCGAGAGCGTGAACC 55
ATGGCGGGCATGATGAAGAAGCAGGTCGTGACGGCGCTGATGTTGGCGCTGGTGGTGTGTCGGCGCGCGCCGGGCGGGGCGCGCGCGCG 145
M A G M M K K Q V V T A L M L A L V V L A A A P G G A R A A 30
TGCCAGGCGTCGACGTGGCGGTGTGCGCGTCGGCGATCCTCTCCGGGGCCAAGCCGAGCGGCGAGTGCTGCGGCAACCTGCGGGCGCAG 235
C Q A S Q L A V C A S A I L S G A K P S G E C C G N L R A Q 60
CAGGGGTGCTTCTGCCAGTACGCCAAGGACCCACCTACGGGCAGTACATCCGAGCCCCACGCGCGCGACACCTCACCTCCTGCGGC 325
Q G C F C Q Y A K D P T Y G Q Y I R S P H A R D T L T S C G 90
CTCGCGTCCCGCACTGCTAGGCGCGGCGAGGCCCTGGCGCATATGGCTAGCTAGCTGCGCGCGTGAGAGTGTGAGCTATAGCTAGCC 415
L A V P H C * 96
CTAGCTGAGTGCGTACGTGTTGCGTCCATGAACGCATCTTCTGCTAAACTACTATGTGGTTCGACTTTGCTGGTACTGGAATGAATAAA 505
ATGTTGCGTGATGATCTGCGCGCGCCAGGTTNCTTCTGGCTGCTGCTGCTGGTAAAAA 559
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Fig. 3. Nucleotide sequence of the cDNA clone pTd18 and deduced primary structure of the precursor of a *T. durum* ns-LTP2. The putative signal peptide is underlined. The stop codon is marked with a star and the putative polyadenylation signal double underlined.

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      1
      ↓
Td      MAGMMKKQVVTALMLLVLAAAPGGARAACQASQLAVCASAILSGAKPSGECCCGNLRAQQGCFC
Hv1     MAMAMGMAMRKEAAVAVMMVMVVTLAAGADAGAGAACEPAQLAVCASAILGGTKPSGECCCGNLRAQQGCCLC
Hv2     MAMRKTKELLLAMMLLVLVAARAAPCEVGQLTVCMPAITTGAKPSGACCCANLGAQQGCFC
Hv3     MKYLAQLVVALAFSAVVLVAPAGAEAATCNALQLTPCAGAIMGNAAPTASCCSKMREQQPCMC
Os      MMRKLAVLVAVAMVAACGGGVGVGAGASCNAGQLTVCAAAIAGGARPTAACCCSLRAQQGCFC
Br      ILLTLFPAPNEAADTNVEAACDPKQLQPCLAITGGGQPSGDCCAKLKEQQPCLC
Ze1     MRSITYTSLYAAAILMMILAGSQTTMAVTCQVTQLAPCASAISSSSPSSQCCAKIKEQKPCLC
Ze2     MRSITYTSLYAAAILMMILAGSQTTMAVTCLVTQLAPCASAISSSSPSSQCCAKIKEQKPCLC
Ko      SAIYIVALLVMIVAGSKVATAATCSVTELMPCSSAFTSSAAPTACCTKLKEQSPCLC
Vu      MTMKMKMMSVVCVVVVALFLIDVGPVAEAVTCNPTELSSCVPAITGGSKPSTCCSKLKVQEPCLC
At      MKFTKLLFVASVMIVMSPLAPTRATVVGWGIEEKAACIVTNLMSCLPAILKGSQPPAYCCEMLKEQQSCLC
      *      *      *      *
Td      QYAKDPTYGQYIRSPHARDTLQSCGLAVPHC-
Hv1     QYVKDPNYGHYVSSPHARDTLNLCGIPVPHC-
Hv2     QYAKDPALGRYITSPHARDTLLLSCGLAVPRC-
Hv3     QYARDPNLKQYVDSFNGKKVMAACKVPVPSC-
Os      QFAKDPYGRYVNNPNARKTVSSCGIALPTCH
Br      GFSKNPFAFYIISPNSRKVLTACGIPYPSC-
Ze1     QYMKNPSLKAYVSSPNAKKVANACGVPIPKC-
Ze2     QYMKNPSLKAYVSSPNAKKVANACGVPIPKC-
Ko      GYLKNPTLKQYITNPNAKKVTSTCGVPIPNC-
Vu      NYIKNPSLKQYVNSPGAKKVLSNCGVTYPNC
At      GYIKSPTFGHYVIPQNAHKLLAACGILYPKC
      * * * * *
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Fig. 4. Alignment of precursors of plant ns-LTP2. *T. durum*, Td (this work); *Hordeum vulgare*, Hv1 [27], Hv2 [28] and Hv3 [48]; *Oryza sativa*, Os [24]; *B. rapa*, Br [29]; *Z. elegans*, Ze1 [7] and Ze2 [26]; *K. odora*, Ko (EMBL accession number L33791); *V. unguiculata*, Vu [25]; and *A. thaliana*, At (EMBL accession number AL137080). The N-terminal end of the *T. durum* ns-LTP2 isolated from wheat is indicated by an arrowhead. The cysteine residues are in bold and amino acids highly conserved in mature protein sequences are indicated with ★.

4. Discussion

In this study, we report the purification and characterisation of a *T. durum* ns-LTP2 and isolation of the corresponding cDNA clone. Although the presence of ns-LTP2 has been reported in maize [22] and castor bean [23], to our knowledge the wheat ns-LTP2 is the only one that has been purified from plants. All other ns-LTP2 primary structures have been deduced from cDNA clone or gene sequences. Moreover, our results show that ns-LTP2 are able to transfer phospholipids between two membranes in vitro.

The primary structure of the wheat ns-LTP2 contains the eight cysteine residues the positions of which are conserved in ns-LTPs. The positions of other amino acids that are conserved (Leu7, Ala13, Pr20, Pro42, Pro51 and Pro65, Gln31, Tyr38 and Tyr47, Lys40 and Gly61; *T. durum* ns-LTP2 numbering) could play an important role in maintaining the tertiary structure and function of ns-LTP2. The three-dimensional structures of wheat [38], maize [39], barley [40] and rice [41] ns-LTP1 have been determined, but no ns-LTP2 three-dimensional structures are known. NMR structure analysis is currently being undertaken on the *T. durum*

ns-LTP2 synthesised in *Pichia pastoris* which was shown to be a very efficient system for producing wheat ns-LTP1 [42]. Structure/function relationship studies are now a major goal for future work on wheat ns-LTP2 in comparison with ns-LTP1. An important issue for comparative structure analysis is the identification of certain ns-LTPs as panallergen [43–46] or pollen allergen [29].

A microheterogeneity was identified when the ns-LTP2 was sequenced; either a threonine or a glutamine residue was found at position 58 suggesting that *T. durum* may contain two ns-LTP2 isoforms. These two ns-LTP2 sequences were either derived from closely related genes or, if the genes are located on the same homologous chromosome of genomes A and B, it is possible that they are alleles of the same gene. The protein encoded by the pTd18 cDNA corresponds to one of these two ns-LTP2 isoforms. On the other hand, there is a total conservation of the primary structure of the *T. durum* ns-LTP2 deduced from clone pTd18 and a ns-LTP2 sequence deduced from a PCR product amplified from *T. aestivum* genomic DNA (data not shown). This indicates that this wheat ns-LTP2 gene does not contain introns, a characteristic of the two ns-LTP2 genes isolated [24,47]. As already known for ns-LTPs, the *T. durum* ns-LTP2 is synthesised as a preprotein whose signal peptide cleavage site has been validated by sequencing of the wheat isolated protein.

During wheat seed development, transcripts of *ltp7.1* and *ltp9.1* genes accumulated differently: *ltp7.1* gene transcripts were present from the cellular division phase to the dry seed, while *ltp9.1* gene transcripts started to accumulate at the time of the grain filling phase. During germination, the level of *ltp7.1* gene transcripts decreased more rapidly than that of *ltp9.1* gene transcripts. A rice [24] and a barley [27] *ltp2* gene are also expressed in developing seeds, although the rice *ltp2*

gene expression that is seed-specific under normal developmental conditions is inducible in vegetative tissues under abiotic stress conditions [24]. In barley, three cDNA clones [27,28,48] and one gene [47] encoding ns-LTP2 have been isolated. Gene expression patterns vary greatly, ranging from aleurone-specific expression in developing grain [27,47], to nucellar-cell-specific expression in ovary [28], to inducible expression by potassium deprivation in roots [48]. In *Z. elegans*, two cDNA clones encoding nearly identical ns-LTP2 have been isolated from mesophyll cells [7,26]. Both genes are expressed in the cell differentiation process in association with trachery element differentiation, but at different stages [6,7]. In barley, the aleurone-specific *ltp2* gene is also expressed very early after the onset of aleurone cell differentiation [47]. In *V. unguiculata*, transcripts of a ns-LTP2 gene accumulated during nodule development in the *Rhizobium*-nodule symbiosis [25]. All these data underline the large spectrum of ns-*ltp2* gene expression patterns and consequently different biological functions of the corresponding proteins can be hypothesised.

Other cDNA clones encoding ns-LTP2 have been recently isolated from wheat, indicating that they belong to a multigene family, the characterisation and expression pattern of which are under study; the biological role of ns-LTP in developing seed remains to be elucidated.

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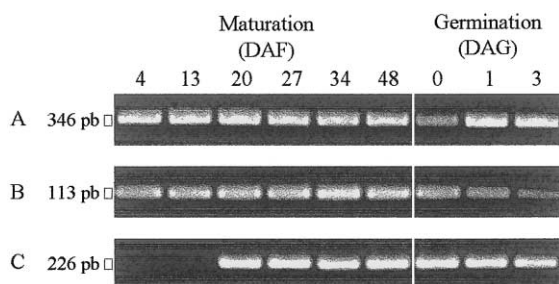


Fig. 5. Steady-state level of *ltp7.1* and *ltp9.1* gene transcripts during wheat seed development. RT-PCRs were performed on total RNA extracted at different days after flowering (4–48 DAF) and germination (0–3 DAG). Each lane contains equivalent loading of PCR products (1/5) obtained after initial reverse transcription of wheat seed RNA and electrophoresed on 2% (w/v) agarose gels. The numbers to the left of the figure indicate the size of the amplicons in bp. (A) Wheat *GAPDH* gene transcripts. (B) Wheat *ltp7.1* gene transcripts. (C) Wheat *ltp9.1* gene transcripts.

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