Volume 311, number 2, 119–123 FEBS 11640 October 1992

Molecular cloning of a cDNA clone for tobacco lipid transfer protein and expression of the functional protein in *Escherichia coli*

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Received 21 August 1992

A cDNA clone encoding a lipid transfer protein (LTP) was isolated from tobacco by screening a library with a PCR-amplified spinach LTP gene. DNA sequence analysis showed a large open reading frame (344 bp) encoding a polypeptide of 114 amino acids. The first 23 amino acids of the deduced protein have the characteristics of a signal peptide for protein secretion or targeting into dense microbody-like vesicles. The cDNA clone was then inserted into an expression vector, pMAL, and expressed in *E. coli* as a fusion with the maltose binding protein (MBP). The MBP–LTP fusion protein was purified to homogeneity and subjected to factor Xa cleavage to yield the LTP domain. A lipid transfer assay demonstrated that the resulting LTP was functional. The availability of the expression system in *E. coli* will facilitate the elucidation of in vivo function(s) of plant LTPs.

Lipid transfer protein; cDNA; DNA expression; *Escherichia coli*; Tobacco

1. INTRODUCTION

Lipid transfer proteins (LTPs) catalyze transfer of lipids between membranes in vitro [1]. These proteins have been purified from various organisms: animals [2,3], yeast [4,5], plants [6–8] and bacteria [9]. In higher plants, both specific (sp-) and non-specific (ns-) LTPs have been reported, and the latter LTPs are all basic soluble proteins with a low molecular mass of approximately 9 kDa [1,10]. Although LTPs had been thought to be cytosolic and shuttle lipids intracellularly from organelle to organelle, such as between chloroplasts and mitochondria, analyses of several cDNA clones of nsLTPs revealed that they are synthesized as precursors containing N-terminal signal peptides, suggesting LTPs would be secreted [11–14] or targeted to dense microbody-like vesicles [15]. Considerable information about the localizations of nsLTPs [10,13,15,16] and the pattern of their expression, as well as the primary structures of LTP genes, has become available, but in vivo physiological functions of plant LTPs have remained controversial. To elucidate the role of LTPs, we have analyzed a full-length cDNA clone for nsLTP from tobacco. The major reasons why we have chosen tobacco are as follows: (i) tobacco is easy to manipulate genetically, permitting analysis of the in vivo function(s) of LTPs; (ii) a cDNA library for mRNA from the flower organs, where the transcripts of LTP genes should be abundant [13], was available in our laboratory; (iii) in our tobacco cell suspension system, we have found that LTPs are secreted in the medium after elicitor treatment, and therefore, we can develop this study to analyze the expression pattern of LTP gene under specific stress conditions.

We report here the analysis of a full-length cDNA of a tobacco LTP (TobLTP) and the expression of a functional TobLTP in *E. coli*. To our knowledge, this is the first demonstration of the expression of a LTP in *E. coli*.

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2. MATERIALS AND METHODS

2.1. *Materials*

A λgt10 cDNA library from mRNA of tobacco flower organs (*Nicotiana tabacum* L. cv. Bright Yellow 4) was constructed by a conventional method. The library was originally made for purposes of analysis of flower-specific gene expression (manuscript in preparation). Restriction enzymes and other DNA modifying enzymes were purchased from Takara Shuzo (Kyoto, Japan) unless otherwise stated. The protein fusion and purification system, using pMAL vectors, was imported from New England Biolabs (USA). [^{3}H]Phosphatidylcholine (1,850 GBq/mmol) and cholesteryl-[1-^{14}C]oleate (2.07 GBq/mmol) were from NEN and Amersham, respectively. Egg yolk phosphatidylcholine was obtained from Sigma. The other reagents used in the experiments were from Wako Pure Chemical (Tokyo, Japan). Oligonucleotides used as PCR primers were synthesized by the phosphoamidite method on an Applied Biosystems 380A model.

2.2. *PCR amplification of spinach LTP gene*

Nucleic acids were extracted in buffer (25 mM Tris-HCl, pH 7.5, 25 mM $MgCl_2$, 25 mM KCl, 1% SDS) from spinach leaf tissue by a conventional phenol extraction method, and then DNA was separated by LiCl precipitation. Based on the cDNA sequence of spinach LTP gene [12], the PCR primers were designed; the first-strand primer,

CCGAATTCAATATGGACGCCACACATACC; the second-strand primer, CCAAGCTTATGGCTAGCTCCGCTGTTATC. The PCR reaction mixture (50 μl) contained 67 mM Tris-HCl, pH 8.8, 16.6 mM $(NH_4)_2SO_4$, 6.7 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 200 mM dNTPs, 1 μg of each primer and 5 U *Tth* DNA polymerase (Toyobo, Japan), and was subjected to a 25 cycle program of 92°C for 1.5 min, 42°C for 2 min and 72°C for 3 min. The PCR product was cloned in pUC119 and used as a probe for screening.

2.3. *Screening of the TobLTP gene*

Plaque lifting and hybridization were performed according to the methods recommended by the supplier of the nylon membrane (Biodyne A, Pall). The λgt10 library was screened with the PCR-amplified spinach LTP DNA. The probe was synthesized by the random primer DNA labelling kit from Takara, Japan. The membranes were prehybridized for 2.5 h at 65°C in 6 × SSC, 5 × Denhardt's solution, 0.5% SDS and 50 μg/ml denatured salmon sperm DNA. The hybridization was carried out in the same solution at 65°C overnight with the probe described above. The membranes were washed in 2 × SSC containing 0.1% SDS at room temperature twice at 30 min intervals. The cDNA inserts obtained from positive plaques were then subcloned into a plasmid vector (pUC119).

2.4. *Determination and analysis of nucleotide sequence for the TobLTP gene*

The nucleotide sequences of the two cDNA inserts were determined by the dideoxy chain-termination method [17]. For the DNA sequence analysis, three computer programs, Microgenie (Beckman), DNASIS (Hitachi) and GeneWorks (Intelligenetics) were employed.

2.5. *Expression of TobLTP in* E. coli

The cDNA of TobLTP was inserted between *Eco*RI and *Hin*dIII sites in pMAL-cRi to produce pMAL-Tob, so that it was in the same translational reading frame as the vector's *mal*E gene, resulting in a MBP–TobLTP fusion protein. Expression of the fusion protein was performed essentially as described in the instruction for the pMAL vectors with some modifications. *E. coli* JM 83 was transformed with pMAL-Tob. The transformant was grown at 30°C for 8 h in 5 ml of 2XYT and inoculated to 1 l of rich medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl). After addition of IPTG (0.1 mM), incubation was carried out at 23°C overnight. Cells were harvested and suspended in lysis buffer (10 mM phosphate, pH 7.0, 0.5 M NaCl, 0.25% Tween20, 10 mM 2-mercaptoethanol, 10 mM EDTA). The *E. coli* cells in the suspension were lysed by two consecutive passes through a French press at 1,500 kg/cm² and centrifuged at 9,000 × *g* for 30 min at 4°C. The resulting supernatant was loaded on the amylose resin affinity column equilibrated with buffer A (10 mM phosphate, pH 7.2, 0.5 M NaCl, 1 mM EDTA, 0.25% Tween 20). After washing with 3 column vols. of buffer A followed by 5 column vols. of buffer A without Tween, the fusion protein was eluted with buffer A containing 10 mM maltose. The sample was then dialyzed against factor Xa buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2 mM $CaCl_2$), and concentrated to about 1 mg/ml in an Amicon Centricon. To separate the TobLTP domain from MBP, factor Xa cleavage was carried out [18].

2.6. *Lipid transfer assay*

The assay was essentially according to Watanabe and Yamada [6] except that [^{3}H]phosphatidylcholine (PC) and cholesteryl-[1-^{14}C]oleate were used. A stock of liposomes for 30 reactions was prepared from 4.5 μmol egg yolk PC, [^{3}H]PC (1,500,000 dpm) and cholesteryl-[1-^{14}C]oleate (600,000 dpm). Liposomes (100 μl) were incubated with potato mitochondria (1 mg protein) in the presence or absence of the samples in a total volume of 500 μl. After 30 min incubation at 30°C, the reaction mixture was centrifuged at 15,000 × *g* for 10 min. The mitochondrial pellets were then suspended in 750 μl 1% Triton X-100. After 5 ml of Aquasol-2 (NEN) was added to the mixture, the radioactivities of ^{3}H and ^{14}C were measured using a liquid scintillation counter. The counts of ^{14}C represent the cross-contamination.

3. RESULTS AND DISCUSSION

The PCR method was used to obtain a cDNA clone of a spinach LTP [12], which was eventually used as a probe to screen a λgt10 cDNA library derived from tobacco flower poly(A)$^+$ RNA. First-strand cDNA synthesis was primed by a single 29-mer oligonucleotide complementary to the domain 45 bases upstream of the 3′ end of the coding region to avoid the sequence with a high content of G+C. The sequence for the second-strand primer (29-mer) is from the 5′ end of the coding region, including the N-terminal signal peptide. Both first-strand and second-strand primers were designed to contain an *Eco*RI and a *Hin*dIII site at their extremities, respectively. The PCR-amplified DNA of a spinach LTP was cloned in pUC119, and the DNA sequence was confirmed. Out of 10,000 recombinant plaques screened, 15 positive plaques were obtained.

Two inserts with a size of approximately 700 bp were subcloned into the *Eco*RI site of pUC119 and sequenced. The two inserts were found to be identical, and the complete nucleotide sequence with the deduced amino acid sequence is shown in Fig. 1. The cDNA was 679 bp long. The presumed initiation AUG codon is followed by a large open reading frame (position 94–438) encoding a polypeptide of 114 amino acids with a calculated molecular weight of 11,416 kDa. The TobLTP shares about 50% homology at the nucleotide level and 65% homology at the amino acid level with spinach LTP. The deduced polypeptide is thought to contain the N-terminal preprotein of 23 amino acids by comparison with other mature LTPs [11–15,19,20].

The hydropathy profiles of the tobacco, tomato and spinach pro-LTPs are shown in Fig. 2. The identical patterns may reflect the conserved secondary structural

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   1 GGAACATGGAAATATTTGTAGTATTATACCCTTCACTACTTCGAATCCTGACACTACACT   60
  61 CTATTGTTTTATCTTCCCCCAATTTTTTAAGAATGGAAATGGTAGGTAAGATTGCATGCT  120
                                 M  E  M  V  G  K  I  A  C
 121 TTGTGGTTTTGTGCATGGTGGTGGTTGCACCCCATGCAGAGGCACTGAGCTGCGGCCAAG  180
     F  V  V  L  C  M  V  V  V  A  P  H  A  E  A  L  S  C  G  Q
 181 TTCAGTCCGGCTTGGCTCCTTGTCTCCCTTATTTGCAGGGCCGCCGCCCTTTGCGGACGT  240
     V  Q  S  G  L  A  P  C  L  P  Y  L  Q  G  R  G  P  L  C  S
 241 GTTCTGGTGGTGTTAAAGGTCTGTTGGGTGCAGCCAAGTCCCTATCTGACCGCAACACTG  300
     C  C  G  G  V  K  G  L  L  G  A  A  K  S  L  S  D  R  K  T
 301 CATGCACTTGCCTGAAATCGGCTGCTAATGCTATTAAGGGTATTGATATGGGCAAAGCCG  360
     A  C  T  C  L  K  S  A  A  N  A  I  K  G  I  D  M  G  K  A
 361 CTGGTCTCCCTGGTGCTTGTGGTGTCAACATTCCTTACAAGATCAGCCCCTCCACAGACT  420
     A  G  L  P  G  A  C  G  V  N  I  P  Y  K  I  S  P  S  T  D
 421 GCTCTAAGGTCGAGTAAGTTTGNTGAAAGNTCAGATTTTCACCGCACTCTTTGGCAGTAG  480
     C  S  K  V  Q  *
 481 TGCAATCATGATCAGGAGCAGAATATAATAAGAATATGGATATCGAGTTATTTGATCCAG  540
 541 CTTATGGATCCATTTCTATTTTGTGTGTTATCTCTTCTTTTTTCTGTCTTTCGAGTA   600
 601 ATANTCGAGTCTTGTATGAAACTTTGGTCGATGGTTGTATTATAATCCAATCTTGATATT  660
 661 TAAAAAAAAAAAAAAAAAA
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Fig. 1. Nucleotide sequence and deduced amino acid sequence of the TobLTP cDNA. The signal peptide sequence and the putative 3′ polyadenylation signal are underlined.

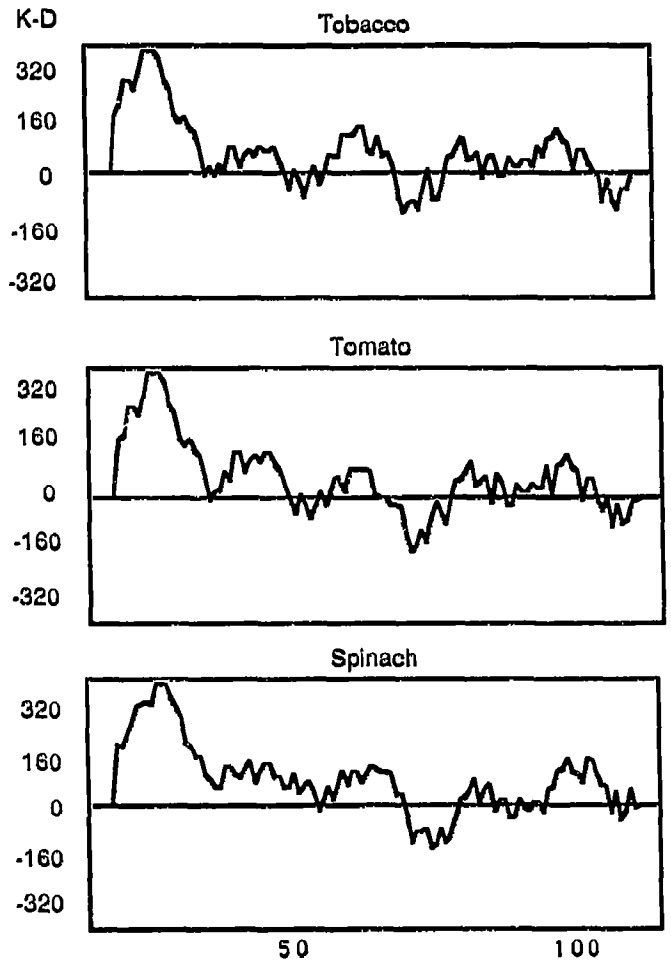

Fig. 2. Hydropathy profiles of pro-LTPs.

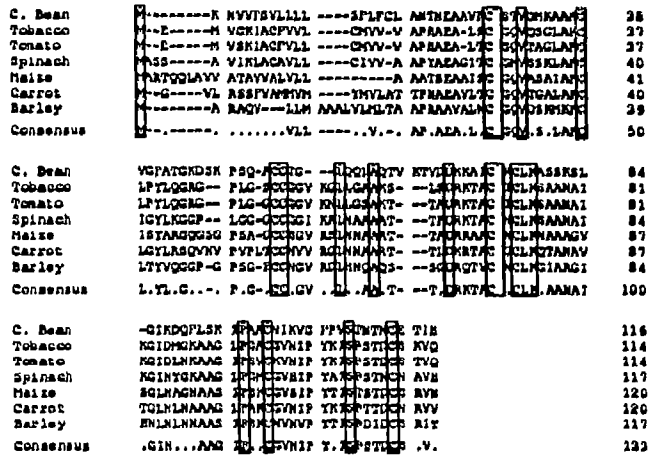

Fig. 3. Amino acid sequence comparison of 7 pro-LTPs from the DNA sequence available in data bases. The alignment was carried out using the protein analysis program of GeneWorks (IntelliGenetics Inc.). The amino acid residues conserved in all the proteins are boxed. The consensus amino acid residues were determined by majority.

features in those plant LTPs [11]. Fig. 3 shows an alignment of plant pro-LTPs deduced from their DNA sequences available in data bases. Like the other LTPs, the TobLTP has 8 cysteines at conserved positions and the highly charged regions of DRK from residues 66–68 and of LKSAA from residues 74–78. A phylogenic tree was drawn for the mature plant nsLTPs currently available in protein data bases (Fig. 4). LTPs from rice and barley seeds were originally described as probable amylase/protease inhibitors [21,22]. An α-amylase inhibitor I-2 from Indian millet seeds was found to show co-identity with plant nsLTPs [23]. The carrot LTP was originally found as a secretory protein from cultured tissues [13]. They all now appear to be LTPs on the basis of amino acid sequence homology. We assume that LTPs and some amylase/protease inhibitors appear to have evolved from a common ancestor and should be re-classified to the same family. As demonstrated in the castor bean LTPs [14], the amino acid sequence identity among LTPs well reflects the evolutionary distance between plant species.

In order to confirm the ability of TobLTP to transfer lipids between membranes in vitro, the cDNA was cloned in pMAL-cRI to produce pMAL-Tob and expressed in *E. coli* JM 83. Although some other expression vectors, such as pKK223-3 (Pharmacia), pGEMEX-1 (Promega) and pTV118N (Takara, Japan) were initially tested, the gene product was not efficiently expressed for some as yet unknown reasons: perhaps this LTP is relatively susceptible to proteolytic digestion in *E. coli*. In the pMAL vector system, the target protein

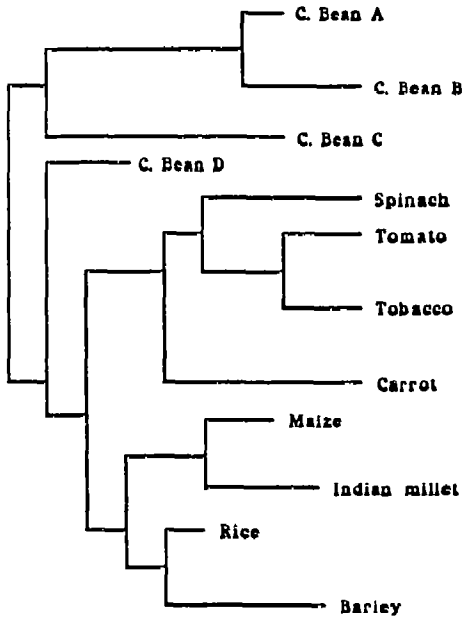

Fig. 4. Phylogenic tree of plant LTPs. The branch lengths, which reflect the evolutional distance, are roughly proportional to vertical lines. The dendrogram was constructed on the program ODEN (DNA Research Center, National Institute of Genetics, Mishima, Japan) using the simultaneous alignment and phylogeny method of Hein [24].

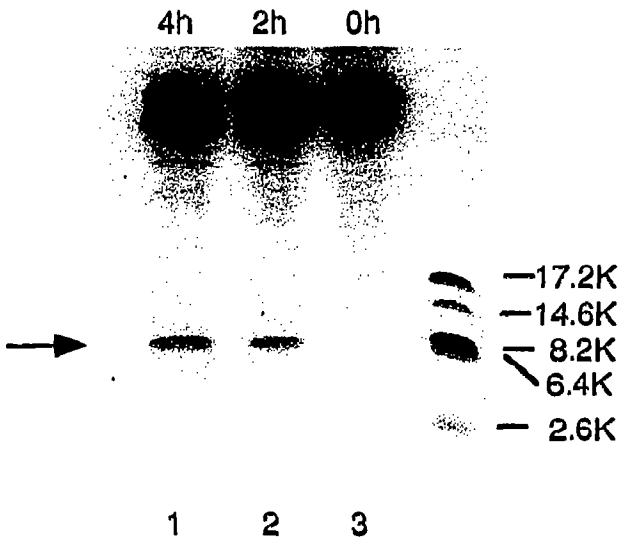

Fig. 5. Digestion of MBP–TobLTP with factor Xa and separation of the TobLTP domain. MBP–TobLTP was concentrated and dialyzed against factor Xa buffer. Factor Xa cleavage was carried out at a w/w ratio of 0.4% of the amount of MBP–TobLTP at room temperature for 6 h. The products were analyzed by SDS-PAGE (17.5%). (Lane 1 and 2) 10 μg of MBP–TobLTP were digested for 4 h and 2 h, respectively; (lane 3) 0 h digestion. The arrow indicates the separated TobLTP domain. Size markers are indicated at the right side.

is fused to the maltose binding protein (MBP), and the fusion protein is easily purified to homogeneity by one step of affinity column chromatography using the amylose resin. The obtained fusion protein is afterwards subjected to factor Xa cleavage at the junction site. The total yield of the fusion protein was estimated to be a maximum value of 20 mg/l. Fig. 5 shows the appearance of TobLTP with an expected size (approximately 9 kDa) after factor Xa digestion. After about 6 h digestion at room temperature, the recovery of the TobLTP was over 90%, as determined by SDS-PAGE. The resulting TobLTP contained 4 extra amino acid residues (I-S-E-F) derived from the vector at the N-terminal. The digestion mixture was directly used to test its lipid transfer ability. As shown in Table I, as increasing amounts of TobLTP were added to the reaction mixture, more and more ^{3}H label (phosphatidylcholine) was transferred to the acceptor mitochondria. Since cholesteryloleate is known not to be transferred by LTP, [^{14}C]cholesteryloleate found in the precipitated mitochondria represents the non-specific value. The activity in the control containing MBP and the fusion without the processed TobLTP is the background value. As a positive control, the $(NH_4)_2SO_4$ precipitate of spinach soluble proteins was used. Since salts may affect lipid transfer activity, TobLTP in TB (factor Xa buffer) was dialyzed against PB to desalt. The result indicated that the desalting step is not necessary.

Since the mRNA for carrot LTP was found to be abundant in the flower organ [13], we used a cDNA

Table I

Lipid transfer activity[a] of TobLTP expressed in *E. coli*

Sample	Amount of protein (μg)	Transfer activity (%)[b]
TobLTP in TB[c]	20	1.0
	40	1.7
TobLTP in PB[c]	20	0.8
	30	1.2
	40	1.5
Spinach $(NH_4)_2SO_4$ precipitate	5,000	1.0
	12,500	2.4
Factor Xa	1	0.1
Control[d]	200	0.1

[a] Liposomes containing [^{3}H]phosphatidylcholine and [^{14}C]cholesteryloleate were incubated for 30 min at 30°C with mitochondria in the presence or absence (background control) of TobLTP. The transfer activity is expressed as a percentage of phosphatidylcholine from liposomes to mitochondria. In this experiment, 1.5–2.0% cholesteryloleate, indicating the cross-contamination, was recovered in the mitochondrial pellet.

[b] 100% corresponds to 150 nmol of lipids.

[c] The fusion of MBP–TobLTP was cleaved with factor Xa and the protein mixture was used for the assay. The amount of TobLTP was estimated from the amount of the fusion and the cleavage efficiency (>90%). Protein was suspended in TB (20 mM Tris-Cl, pH 8.0, 100 mM NaCl, 2 mM $CaCl_2$, 10% glycerol) or PB (10 mM phosphate, pH 7.0, 10% glycerol) and stored at −70°C until use.

[d] Contains MBP (100 μg) + fusion protein (100 μg).

library made for mRNA from tobacco flower organ for screening of the TobLTP gene. LTPs are assumed to play a role in membrane biogenesis and/or cutin synthesis during differentiation, including flower development [13]; we believe TobLTP would have similar functions to those proposed for the other plant LTPs. Using TobLTP synthesized in *E. coli*, we are now raising antibodies to analyze the localization of TobLTP, which will give a clue towards an understanding of the in vivo function of TobLTP. We will also use the antibodies to identify TobLTP in the tobacco suspension medium with which we will investigate our hypothesis that LTPs may play a role in the plant defence response.

The above results provide direct evidence that the tobacco cDNA clone really encodes a plant LTP. In this paper, it was also demonstrated that a functional TobLTP was expressed in *E. coli*. The establishment of the expression system in *E. coli* makes it feasible to elucidate the in vivo role of plant LTPs, and to obtain a substantial amount of LTP for detailed structural studies, such as NMR analysis and X-ray crystallography.

Acknowledgements: We thank Dr. Allan Capllan, University of Idaho, for reviewing our manuscript and S. Tsuboi, Tokyo University, for helping us to conduct the lipid transfer assay.

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