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Characterization of a rice gene coding for a lipid transfer protein

(Recombinant DNA; plant; *Oryza sativa*; promoter sequence; gene expression)

Florence Vignols[b], Gertrud Lund[a], Sujata Pammi[a], Dominique Trémousaygue[a], Françoise Grellet[a], Jean-Claude Kader[b], Pere Puigdomènech[c] and Michel Delseny [a]

[a]*Laboratoire de Physiologie et Biologie Moléculaire Végétales, URA CNRS 565, Université de Perpignan, 66860 Perpignan Cedex, France;* [b]*Laboratoire de Physiologie Cellulaire et Moléculaire, URA CNRS 1180, Université Pierre et Marie Curie Paris VI, 75252 Paris Cedex 05, France; and* [c]*Departamento de Genetica Molecular, CID-CSIC, Jordi Girona Salgado, 08034 Barcelona, Spain*

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SUMMARY

The cloning and sequence analysis of a gene that encodes a lipid transfer protein (LTP) from rice is reported. A genomic DNA library from *Oryza sativa* was screened using a cDNA encoding a maize LTP. One genomic clone containing the gene (*Ltp*) was partially sequenced and analyzed. The open reading frame is interrupted by an 89-bp intron. From the results of Southern hybridizations, *Ltp* appears to be a member of a small multigenic family. Transcripts of the corresponding gene were detected in several tissues including coleoptile, leaf, endosperm, scutellum and root. The transcription start point was determined by primer extension. The deduced amino-acid sequence of the *Ltp* product is shown to be homologous to LTPs from other crops.

INTRODUCTION

Plant lipid transfer proteins (LTP) have been identified by their ability to facilitate in vitro movements of lipids between membranes and, thus, have been assumed to participate in membrane biogenesis (Kader, 1993; Yamada, 1992). They have been purified from various plants and the first cDNA corresponding to a maize LTP has been characterized by our group (Tchang et al., 1988). Additional cDNA clones have been isolated from spinach (Bernhard et al., 1991), castor bean (Tsuboi et al., 1991), maize (Arondel et al., 1991) and wheat (Dieryck et al., 1992). In addition, *Ltp*-like cDNAs have been isolated in barley (Mundy and Rogers, 1986), tobacco (Koltunow et al., 1990), carrot (Sterk et al., 1991), tomato (Plant et al., 1991; Torres-Schumann et al., 1992) and rapeseed (Foster et al., 1992). Expression analysis of all these genes revealed complex patterns, characterized by a high cell- and organ specificity. In maize, the expression is restricted to the outer cell layers in the coleoptile and young embryos (Sossountzov et al., 1991). In carrot, a *Ltp*-like gene is expressed in the epidermal cells of somatic embryos (Sterk et al., 1991). Some *Ltp*-like genes have been found specifically expressed in the tapetal cells and the microspores of tobacco (Koltunow et al., 1990) or rapeseed (Foster et al., 1992). Several *Ltp* genes are induced by various stresses, including low temperatures in barley (Hughes et al., 1992), drought and salt treatments in tomato (Plant et al., 1991; Torres-Schumann et al., 1992). Some of them are responsive to abscisic acid (Hughes et al., 1992; Torres-Schumann et al., 1992).

It seems likely that these various expression patterns result from the differential activity of the members of a small multigene family. Evidence for the occurence of

Correspondence to: Dr M. Delseny, Laboratoire de Physiologie et Biologie Moléculaire Végétales, URA CNRS 565, Université de Perpignan, Avenue de Villeneuve, 66860 Perpignan Cedex, France. Tel. (33-68) 668-848; Fax (33-68) 668-499; e-mail: Cooke@univ-Perp.Fr

Abbreviations: aa, amino acid(s); bp, base pair(s); cDNA, DNA complementary to RNA; kb, kilobase(s) or 1000 bp; LTP, lipid transfer protein(s); *Ltp*, gene(s) encoding LTP; nt, nucleotide(s); PAPI, probable amylase/protease inhibitor; oligo, oligodeoxyribonucleotide; ORF, open reading frame; PCR, polymerase chain reaction; SDS, sodium dodecyl sulphate; *tsp*, transcription start point(s); var, variety.

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several proteins have been first obtained in castor bean (Takashima et al., 1988). Preliminary hybridization data also suggested the occurence of several genes in maize (Tchang et al., 1988). Finally, different cDNA clones have been isolated for several species including castor bean (Weig and Komor, 1992) and barley (Jakobsen et al., 1989).

In rice seeds, two proteins homologous to LTPs, previously identified as probable amylase/protease inhibitors (PAPI A and PAPI B) have been isolated (Yu et al., 1988; Park et al., 1991), suggesting that several genes might also occur in this species. In this paper, we report data supporting the existence of several genes as well as the characterization of the first genomic clone encoding a rice LTP. This is the first step towards the analysis of this multigene family; when other members of the family have been characterized, it should become possible to investigate the regulation of each individual gene.

EXPERIMENTAL AND DISCUSSION

(a) Isolation of a rice *Ltp* gene

An *Oryza sativa* (var IR36) genomic library constructed in λ-Dash from *Sau*3A partially digested 15–25-kb fragments was obtained from Dr. Steven Kay and was screened with the ^{32}P-labelled 9C2 cDNA encoding a maize LTP (Tchang et al., 1988). Among several positive clones, clone *16-1* was analysed by *Bam*HI digestion. From this clone, a single hybridizing 3.4-kb fragment was revealed, which was subcloned into pGEM7Z(+) vector and designated pBBO4A. A restriction map of this subclone was generated and the *Ltp* gene was located in the central part of this fragment.

(b) Sequence analysis

From pBB04A, a set of sequentially deleted overlapping clones was produced by exonuclease III limited digestion. Because of extensive overlapping of the clones, sequence could be unambiguously determined essentially from one strand using the dideoxy chain-termination method. Portions of the complementary strand were confirmed using selected oligos. Although the full-length 3.4-kb fragment was completely sequenced, only 1486 bp corresponding to coding and immediately flanking regions are shown in Fig. 1. Comparison with both 9C2 (Tchang et al., 1988) and 6B6 cDNA (Arondel et al., 1991) allowed the delineation of two exons encoding a protein of 117 aa and one 89-bp intron. Analysis of exons/intron splice junctions shows that the typical GT/AG donor-acceptor sites are conserved. The presence of an intron in rice *Ltp* gene was confirmed independently by the characterization of several cDNAs from another rice variety (Nipponbare) and by comparison with rice LTP-related PAPI (F.V., unpublished; Park et al., 1991). The second exon encodes the last two aa of the proteins and contains

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GGCTAAAATATAATGAAACTAAAGATAAGATTTCACATGTAAGTGTAAAACTATTGGAAGTATGTGTCCAATTTTTTCTAGAACTTTTGTGACATTTGCTAATTTGATATATCAGGGTGT   -241

GTATATAAGAAAAGTTTTTGTGTATAGAATATGTTCTCTAATTATAGGTGGACTATTCCGCGTAGTAGCTACATTTGACTTGGTCGATGCATCGGCACAGATGAAATAAATAGCTATTTA   -121
                                                                                                                    Δ
AAGAAACCTACATTCCCGGGAATTCTCATCGATCGATCTCACCTGCAGCAGCAAACGAGCACCACACACCAGCAGCAGCAGCAAGTCGATCGATCGTCAGCACACACGACCAAGATCGAG     -1

ATGGCCCGTGCACAGCTGGTGTTGGTCGCCCTCGTGGCAGCGGCTCTGCTCCTGGCGGGCCCACACACCACCATGGCCGCCATCAGCTGCGGCCAGGTCAACTCCGCCGTGTCGCCCTGC    120
 M  A  R  A  Q  L  V  L  V  A  L  V  A  A  A  L  L  L  A  G  P  H  T  T  M  A  A  I  S  C  G  Q  V  N  S  A  V  S  P  C     40
                                                                                 ⇑
CTCAGCTACGCCCGCGGGCTCCGCCCGTCGGCGGCCTGCTGCAGCGGCGTCAGGAGCCTCAACTCCGCCGCCAGCACCACCGCCGACCGCCGCACCGCCTGCAACTGCCTCAAGAACGTT    240
 L  S  Y  A  R  G  L  R  P  S  A  A  C  C  S  G  V  R  S  L  N  S  A  A  S  T  T  A  D  R  R  T  A  C  N  C  L  K  N  V     80

GCCGGCAGCATCAGCGGCCTCAACGCCGGCAATGCCGCCAGCATCCCCTCCAAGTGCGGCGTCAGCATCCCCTACACCATCAGCCCCTCCATCGACTGCTCCAGgtAATCAAATTTTATA    360
 A  G  S  I  S  G  L  N  A  G  N  A  A  S  I  P  S  K  C  G  V  S  I  P  Y  T  I  S  P  S  I  D  C  S  S                    115
                                                                                 .....................
TCGTTTTGCATGATCGATCATCTAATTAACTAAGTACATATAAATTAATTTCCGCGTGTACGTTTAATTGCagCGTGAACTAATCTGATCGATCGATCGCTACCGGCCGGAGGTGTGACA    480
                                                                                    V  N  *                                 117

TCGCAGGCCGTACATGCACCTGTCGTCGTCTCACGCTTTGTATCTTGTGTTATCTGTGTTTATGCTGAATAAAATGAGAGCTAGCTAGCTAGGTCGATCCATCGATCGCCATGCATGACG    600

AGACTAGCTAGCATTGCTGCTAGATCAGATGCATGCATATGGTTGATCGGCCGGTCACTACGCTATCTGTTTCCTTAATTTATTGTGATCGACTACGTACGTACCTGAGAGCTAGAGATC    720
                  .................
AGTTTCTGTACCATGCATATGCATATGAACTTCTGCATGTACATGCAGTACTACTTGTACTACGTGCGTGTACGTTGTGGATTATACATTATATAGACAGCTCCTTTTGGTATACATATA    840

CATGTTCTACACCTGATTCTCCATCTTCTGGCATTACACATATATACAACTTGGGTAAAAAAACAGTGCTGTGTTAATTTGTCTTGAAATATGTATACTCTAATAATCAATTAATAAATT    960

ACGCCCTTCAGCCCAAAATATATAAGCATTTTTAAAATAGCGTCAAGTCAAATATTTTTAACTTTGATTATTAATAGCAAAAAATCAATAATGTAAATTGATATTACTAAATTTATCATT   1080

AACAAATATCGTAATATGCACTTTTCATTAATATGCTGAGGTTAG...                                                                           1109
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Fig. 1. The nt sequence of rice *Ltp* gene. The deduced aa sequence is shown below the corresponding nt sequence in single-letter code. The aa involved in a putative signal peptide are indicated in italics and position of N-terminal aa of the mature LTP is marked by a arrow. The gap in aa sequence between aa 115 and 116 corresponds to intron and lower-case letters for nt are used to indicate the 5′ and 3′ splice sites. The stop codon is marked by an asterisk. Putative TATA and CAAT boxes in the promoter region and the putative polyadenylation signal in the 3′ region are doubly-underlined. The 5′ end of the *Ltp* transcript (*tsp*) determined by primer extension is marked with an open arrowhead. A+T-rich sequences found upstream all the genes encoding LTPs are underlined. The oligos 5′-GTGAACTAATCTGATCGA (corresponding to nt from +435 to +452) and 5′-CATGTACATGCAGAAGTT (corresponding to nt from +765 to +748) used as primers to amplify the 3′ non-coding region by PCR are overlined by dots. The *Ltp* sequence has been deposited in EMBL database under accession No. Z23271.

the 3′ untranslated sequence. Analysis of the 3′ non-coding region revealed the presence of a polyadenylation signal sequence AATAAA 104 nt downstream from the stop codon.

(c) The promoter region of *Ltp*

The *tsp* of *Ltp* was determined by primer extension. A 18-mer oligo corresponding to the antisense strand from nt +18 to nt +1 (Fig. 1) was used as a primer. Size of the reverse-transcribed product was estimated by gel electrophoresis in parallel with the 5′ region antisense sequence obtained with the same primer (Fig. 2). This experiment located the *tsp* at nt −124. Two putative TATA box-like sequences, $^{-204}$TAATTATA^{-197} and $^{-239}$TATATAA^{-233}, as well as a putative CAAT box-like sequence 89 bp upstream from this second TATA box, were found upstream from the *tsp* position. It should be noted that the distance between the *tsp* and the putative TATA boxes is unusually long.

The 5′ non-coding region of *Ltp* gene contains also two A+T-rich sequences that are present at the same place upstream from the other genes encoding LTPs.

(d) Structure of the rice *Ltp*

The coding region exhibits a high G+C content (70%) which contrasts with the high A+T content of the intron (72%) and of the flanking 5′ and 3′ regions (63% and 60% A+T, respectively). It shows a biased codon usage, which is a character of monocotyledone genes (Murray et al., 1989). Among the 61 sense codons, only 32 are used with a strong preference for those ending in G or C (94%). A similar bias is found in the cDNAs encoding rice LTPs (F.V., unpublished). The aa sequence is Ala+Ser-rich (18% and 15%, respectively), and contains the typical eight Cys residues whose position is highly conserved in plant LTPs (Kader, 1993). The protein contains a putative 26-aa secretion signal sequence and a 91-aa polypeptide that exhibits a high degree of sequence identity when compared to other plant LTPs. The highest homology is obtained with rice PAPI (Yu et al., 1988) and maize LTP (Tchang et al., 1988) (85% and 80% identity, respectively), whereas wheat (Dieryck et al., 1992) and barley LTPs (Mundy and Rogers, 1986) present only 57% and 58% identity, respectively.

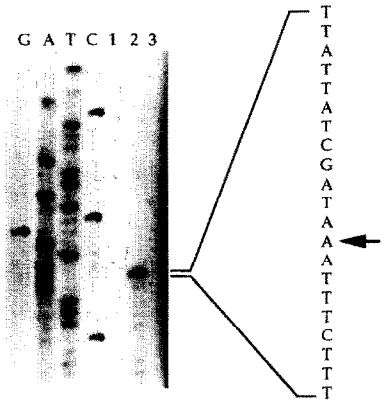

Fig. 2. Mapping of the 5′ end (*tsp*) of *Ltp* transcript by primer extension. The arrow indicates the position of the extended signal. **Methods:** Total RNAs (30 µg) isolated from 7-day-old rice seedlings by phenol/LiCl method (Verwoerd et al., 1989) were reverse-transcribed using the 18-mer 5′-CAGCTGTGCACGGGCCAT, in a primer-extension reaction containing [α-^{32}P]dCTP. Control experiments in which RNAs (lane 1) and primer (lane 3) were omitted from the reaction mixture were also carried out. The product was subjected to electrophoresis in a 6% polyacrylamide gel and compared to the pBBO4A antisense-strand sequence obtained by using the same primer. The arrow indicates the nt determined as *tsp* on the antisense strand. Exposure time of lanes 1, 2 and 3 is 15 days.

(e) *Ltp* as a member of a small multigene family

Southern hybridization using as a probe the fragment described in Fig. 1 (coding region and flanking sequences) strongly suggested the presence of at least three genes encoding LTPs in rice (Fig. 3A, lanes B and E5). This is consistent with the recent isolation of several cDNAs encoding these proteins in another rice variety (F.V., unpublished). Among the bands detected with this probe, a 3.4-kb *Bam*HI fragment corresponds to the insert in pBBO4A.

When a probe corresponding to the promoter-distal region (−360 to −73) was used on the same digestion patterns, a different result was obtained. In each lane except for *Bam*HI digestion (Fig. 3B), only one band was detected, indicating that the members of *Ltp* genes family in rice have divergent promoters.

The hybridization with a probe that recognizes 73 nt in the 5′ leader sequence and the first 162-coding nt indicates that at least two genes of the family are very homologous in their 5′ coding region (Fig. 3C).

(f) Expression of *Ltp* in rice

We examined the expression of *Ltp* in various organs of rice seedlings by Northern hybridization. Since probes containing the coding region recognize other genes encoding LTPs, a gene-specific probe corresponding to *Ltp* 3′ non-coding region was PCR-amplified using primers indicated in Fig. 1. A strong signal corresponding to a 0.7-kb transcript was detected in coleoptile and in leaves (Fig. 4). A weaker signal was also detected in endosperm and in scutellum. An interesting point was the presence of a faint signal in roots, because this observation is in contrast with those made in many other studies, including maize (Sossountzov et al., 1991), which failed to detect any *Ltp* mRNA in roots. No transcript was found in 2-day-old flowers. The same amount of total RNA has been loaded in lanes for roots, endosperm, scutellum, coleoptile and leaf, as confirmed by probing the same blot

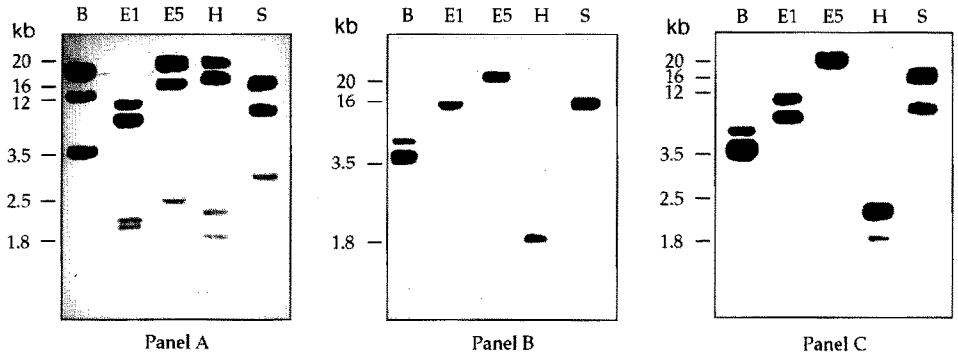

Fig. 3. Southern blot hybridizations of rice genomic DNA with different probes obtained from pBBO4A. (**A**) Hybridization with a probe corresponding to *Ltp* coding region. (**B**) Hybridization with a probe corresponding to promoter region (−360 to −73). (**C**) Hybridization with a probe that recognizes the 5′ coding region of *Ltp* (−73 to +162). **Methods:** Genomic DNA (10 μg) isolated by standard method (Dellaporta et al., 1983) from *Oryza sativa* 7-day-old seedlings was digested with different restriction enzymes, separated on 0.8% agarose gel and transferred onto Hybond N+. The membrane was hybridized following Amersham standard protocol with ^{32}P-labeled probes (5 × SSC/5 × Denhardt solution/0.5% SDS/100 μg per ml sonicated herring sperm DNA, 65°C, 16 h, and washings in 2 × SSC/0.1% SDS and then in 0.2 × SSC) and exposed to X-ray film for 10 days. B, *Bam*HI; E1, *Eco*RI; E5, *Eco*RV; H, *Hin*dIII; S, *Sac*I. The size of hybridizing bands was determined by reference to *Hin*dIII + *Eco*RI-digested fragments of phage λ DNA. SSC is 0.15 M NaCl/0.015 M Na_3-citrate pH 7.6.

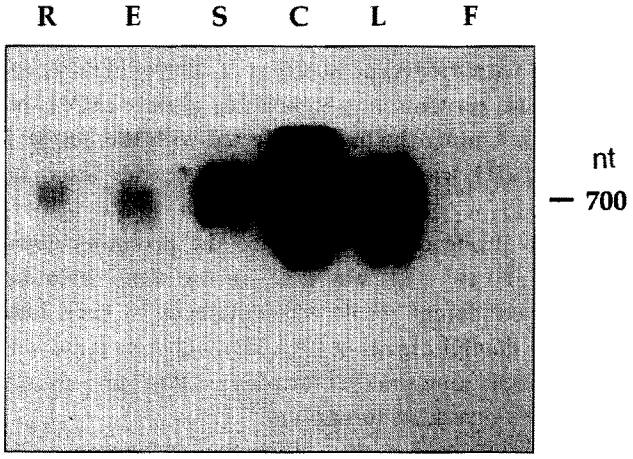

Fig. 4. Northern blot analysis of the expression of rice *Ltp* in different organs. **Methods:** Total RNAs (10 μg) were isolated according to Verwoerd et al. (1989) from 7 day-old seedlings [root (R), endosperm (E), scutellum (S), coleoptile (C), leaf (L)], and from 2-day-old flowers (F) (2 μg for this tissue) and were subjected to electrophoresis in a 2.2 M formaldehyde-1.5% agarose gel by standard method (Sambrook et al., 1989) and transferred onto Hybond N. Northern hybridization was carried out according to Sossountzov et al. (1991) using a gene-specific probe derived from the 3′ untranslated region. Specificity of the probe was determined by hybridization to blots similar to those in Fig. 3 (result not shown) (see Fig. 1). The membrane was exposed to X-ray film for 7 days.

with a rice rDNA probe (Cordesse et al., 1993). Therefore, the different levels of expression represent the relative amount of *Ltp* mRNA in each sample.

(g) Conclusions

This is the first report of a gene coding for a LTP in rice. It has been selected using a maize cDNA and not surprisingly, it encodes a protein very similar in size and aa sequence of LTP from maize and other plants.

A putative 26-aa signal peptide has been deduced from the nt sequence. Signal peptides have been also deduced from all cDNAs and genes encoding LTP characterized until now. The length of this sequence varies from 21 to 27 aa (Kader, 1993). This signal peptide indicates that the protein is targetted to a specific compartment of the cells or secreted. Secretion has been demonstrated in the case of spinach (Bernhard et al., 1991), barley (Mundy and Rogers, 1986, Madrid, 1991) and carrot (Sterk et al., 1991) and recently *Arabidopsis* LTP was located by immuno-electron microscopy within the cell wall (Thoma et al., 1993). A role for LTPs in transporting hydrophobic molecules outside of the cell has been suggested: it might be involved in the biosynthesis of cutin, an important compound for protecting epidermal plant cells (Sterk et al., 1991; Kader, 1993). The recent observation that radish and barley LTP-like proteins have anti-bacterial and anti-fungal activities in vitro (Terras et al., 1992; Molina et al., 1993) also suggests that LTPs might play a role in defence reactions.

The existence of a 89-bp intron in rice *Ltp* gene is in agreement with the presence of a 74-bp intron in maize deduced from cloning of an unspliced cDNA (Arondel et al., 1991). A 133-bp intron interrupts the barley *Ltp1* gene (Linnestad et al., 1991; Skriver et al., 1992) and a 964-bp intron is present in a tobacco gene (Fleming et al., 1992). Interestingly, all these introns are located at the same position and they disrupt the codon determining the third aa before the C-end.

In the present work, the rice *Ltp* gene was found to be preferentially expressed in coleoptile and leaves. Maize, carrot and tobacco *Ltp* genes have also been shown to have a tissue-specific expression pattern (Kader, 1993). The basis for this specificity is not yet known and the characterization of the promoter region should be crucial

in understanding it. Short nucleotide sequences homologous to *Myb* and *Myc* oncogene-binding sites have been detected upstream from the barley *Ltp1* gene and have been suggested to be involved in the regulation of its expression (Linnestad et al., 1991). Although two related sequences were observed in the rice *Ltp* promoter, they present differences (one deletion in *Myb* site, three mutations plus one deletion in *Myc* site) with the consensus of these sites. Such modifications are usually sufficient to disrupt the efficiency of these sites (Roth et al., 1991) and it remains to demonstrate that such sites in *Ltp* promoter are functional by using homologous transgenic plants. In addition rice *Ltp* and barley *Ltp1* genes could be different subclass of the *Ltp* multigene family and be differently regulated. Such a possibility is supported by the Southern blot results which clearly indicate that the promoter of the gene studied here is not closely related to any of the other two or three genes which have a closely related coding sequence. Therefore, the next steps to clarify the regulation of *Ltp* genes are to isolate all the other members of the family, compare their expression pattern using gene specific probes and analyze their respective promoters.

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