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Multiple mRNA coding for phospholipid-transfer protein from *Zea mays* arise from alternative splicing

(Recombinant DNA; maize; cDNA cloning)

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SUMMARY

We have isolated a novel cDNA coding for maize phospholipid-transfer protein. The cDNA sequence is similar to the first one obtained by Tchang et al. [J. Biol. Chem. 263 (1988) 16849–16855] differing only by a small number of nucleotide substitutions and insertions. One of these insertions is 74 bp long and is flanked by consensus intron splicing sequences. The protein coded by the two cDNA has identical amino acids except in the C terminus. This difference derived from the presence of the 74-bp insert. The possible existence of an alternative splicing mechanism that could introduce heterogeneity in the sequence of these proteins is proposed.

INTRODUCTION

Phospholipid-transfer proteins (PLTPs) are cytosolic proteins able to transfer in vitro phospholipids between different membranes (for review, see Wirtz, 1982). It has been shown that PLTPs participate in membrane biogenesis in vivo (Kader, 1985). They are abundant and have been purified from a number of both prokaryotic and eukaryotic organisms (Kader, 1985). The cloning of a cDNA corresponding to the maize protein has recently been reported (Tchang et al., 1988). Southern analysis indicates that PLTP is coded by a small number of genes in the maize genome (Tchang et al., 1988).

Comparison between the deduced aa sequence of maize PLTP and its aa sequence reveals a signal peptide of 27-aa (Tchang et al., 1988). This opens the question on the subcellular localization of the protein generally considered as cytosolic (Kader, 1985). One possibility is the existence of PLTP isoforms differing in their molecular mass as it has been shown in rat adrenal (Pastuszyn et al., 1987) and liver tissues (Ossendorp et al., 1990).

Here we report the sequence of a new cDNA coding for maize PLTP. This sequence was compared with the previous one obtained by Tchang et al. (1988).

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Abbreviations: aa, amino acid(s); bp, base pair(s); cDNA, DNA complementary to RNA; kb, kilobase(s) or 1000 bp; nt, nucleotide(s); oligo, oligodeoxyribonucleotide; ORF, open reading frame; PLTP, phospholipid transfer protein; *PLTP*, gene encoding PLTP.

EXPERIMENTAL AND DISCUSSION

(a) cDNA and aa sequences

The cloning and sequencing of a cDNA clone (9C2) coding for a phospholipid-transfer protein detected in a cDNA library from the pure maize inbred line W64A have

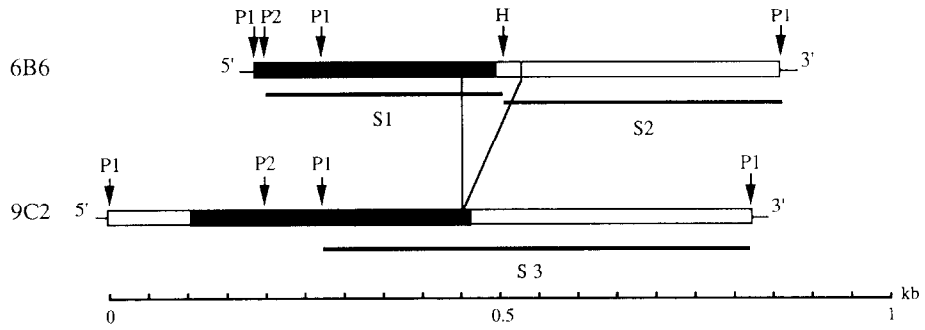

Fig. 1. Partial restriction map of 6B6 and 9C2 cDNA inserts corresponding to the maize PLTP. Only relevant restriction sites are shown. The probes (used in Figs. 4 and 5) are: S1, *Pvu*II-*Hin*fI fragment; S2, *Hin*fI-*Pst*I fragment, both isolated from 6B6. S3 corresponds to the largest *Pst*I-*Pst*I fragment of 9C2. Blackened bar, coding region; open bar, noncoding region; line, pBR322; H, *Hin*fI; P1, *Pst*I; P2, *Pvu*II.

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9C2   AGCTGCCATTGCCATCTACTGACCTGTGACCTCCGCATTCCCAAACAGCC  50
6B6   ---------------------------------------------------

9C2   CGAGCGAGCGAGCTAGCAGAGCGGCGGCAGGCACCTCCCTCCTCAAGGAAC 101
6B6   ---------------------------------------------------

9C2   ATG GCC CGC ACG CAG CAG TTG GCA GTA GTG GCG ACC GCC 141
6B6   --- --- --- --- --- --- --- --- --- --- --- --- ---

9C2   GTG GTG GCC TTG GTG CTG CTG GCG GCG GCG ACC TCG GAG 179
6B6   --- --- --- --- --- --- --- --- --- --- --- --- ---

9C2   GCG GCC ATC AGC TGC GGG CAG GTG GCG TCG GCC ATC GCG 218
6B6   --- --- --C AGC TGC GGG CAG GTG GCG TCG GCC ATC GCG  31

9C2   CCC TGC ATC TCC TAC GCG CGC GGC CAG GGC TCG GGG CCC 257
6B6   CCC TGC ATC TCC TAC GCG CGC GGC CAG GGC TCG GGG CCC  70

9C2   TCC GCC GGC TGC TGC AGC GGC GTC AGG AGC CTC AAC AAC 296
6B6   TCC GCC GGC TGC TGC AGC GGC GTC AGG AGC CTC AAC AAC 109

9C2   GCC GCC CGC ACC ACC GCC GAC CGC CGC GCC GCC TGC AAC 333
6B6   GCC GCC CGC ACC ACC GCC GAC CGC CGC GCC GCC TGC AAC 148

9C2   TGC CTC AAG AAC GCC GCC GCC GGC GTC AGC GGC CTC AAC 374
6B6   TGC CTC AAG AAC GCC GCC GCC GGC GTC AGC GGC CTC AAC 187

9C2   GCC GGT AAC GCC GCC AGC ATC CCC TCC AAG TGC GGC GTC 413
6B6   GCC GGT AAC GCC GCC AGC ATC CCC TCC AAG TGC GGC GTC 226

9C2   AGC ATC CCC TAC ACC ATC AGC ACC TCC ACC GAC TGC TCC 452
6B6   AGC ATC CCC TAC ACC ATC AGC ACC TCC ACC GAC TGC TCC 265

9C2   AGG --- --- --- --- --- --- --- --- --- --- --- --- 455
6B6   AGG TAC TCG CGT CGC ATG CAT GCT AGC GCT GAC TAG TGA 304

9C2   ----------------------------------GTGAACTGAACCC 468
6B6   GCTAGTACTGACTCCATGACCGTGCATATATATGCAGGGTGAACTGAACCC 355
          *****
9C2   TAAACGACGACGACGGCGCCGGCGGCCGCCTGAAGACGATCCATATCTATC 519
6B6   TAAACGACGACGACGGCGCCGGCGGCCGCCTGAAGACGATCCATATCTATC 406

9C2   CCGCCGTCG---TCGG--CGCCCGCGGATCGGACCGTCATGACCATATCTA 565
6B6   CCGCCGTCGACGTCGGCGCGCCCGCGGATCGGACCGTCATGACCATATCTA 457

9C2   CCTACGCATGATCACTACTTGAATGTCTACTACTGCTACGTACTACTACTG 616
6B6   CCTACGCATGATCACGACTTGCATGTCTACTACTGCTACGTACTACTACTG 508

9C2   AATAAAAAACCTCTCCGCGCACATCGTCCTTGTGTTTGTGTGTGTGAGAGA 667
6B6   AATAAAAAACCTCTCCGCGCACGTCGTCCTTGTGTTTGTGTGTGAGAGAGA 559

9C2   GAGAGGAGACGGAGAGCCGGCCATGTCCTTTTGGCTGTTTGTTTGGTTTAT 718
6B6   GAGAGGAGACGGAGAGCCGGCCATGTCCTTTTGGCTGTTT----GGTTTAT 606

9C2   TTTTGAGTTCATGCGGTCTTTATTTATTTCCATGTACGCTGCTTCCTCG-- 767
6B6   TTTTGAGTTCATGCGGTCTTTATTTATTTCCATGTACGCTGCTTCCTCGTA 657

9C2   TACCCACTTTAAATTTGTACCGATGCATGCATGTTCTTGATGATACAAAAA 818
6B6   TACCCACTTTAAATTTGTAC---------------------------------- 677

9C2   AAAA                                                 822
6B6   ----
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Fig. 2. Nucleotide sequence of 6B6 and 9C2 cDNAs. The 6B6 cDNA was isolated from a cDNA library prepared from poly(A)$^+$ RNA extracted from 7-day-old maize (E41 double hybrid) coleoptiles from seedlings grown in the dark. The library was constructed in the *Pst*I site of pBR322 as described (Tchang et al., 1988; Stiefel et al., 1988). The nt sequence was determined according to Sanger et al. (1977). The sequences of the two inserts are aligned with gaps which were introduced to achieve maximum homology. Nucleotide changes between the two sequences are boxed. The start and stop codons, and the polyadenylation site are underlined. The *Hin*fI site is marked with asterisks. The oligo sequence, 5′-CACGGTCATGGAGTCAGTA, used to make antisense oligo probe is doubly underlined. All nt and aa sequence analyses and comparisons were carried out on CITI2 (Paris) databases. GenBank accession number is M57249.

been reported (Tchang et al., 1988). A cDNA (6B6) from a library obtained from another variety, the double hybrid E41, has also been isolated using maize anti-PLTP antibodies. The restriction map of the insert (Fig. 1) is compared with that of 9C2. The main restriction sites are conserved on both cDNAs, however, a *Hin*fI site is only present in 6B6 cDNA. A comparison of 9C2 and 6B6 nt sequences is presented in Fig. 2. Although the 5′-end of 6B6 cDNA is incomplete, the two cDNAs present a high homology particularly in the coding region. Nevertheless, there are several differences in the 3′-noncoding region which are insertions or deletions corresponding to the duplication of small sequences. These variations probably result from the different origins of the two cDNAs. Similar observations have been reported for a number of other sequences obtained from different maize varieties (Prat et al., 1987; Stiefel et al., 1988).

However, the main difference between the two cDNAs is due to a 74-bp insertion at the end of the coding region of the 6B6 cDNA. The point of insertion of this sequence is flanked by the nt sequences 5′-AGGT and AGGGT-3′ at the 5′ and 3′ ends, respectively. Preceding the later one an A + T-rich sequence is present. These patterns have the feature of plant intron splicing consensus sequence (Brown, 1986; Wiebauer et al., 1988). Part of the insertion in 6B6 is in frame with the coding sequence. The ORF is interrupted by two stop codons and is longer than the 9C2 one (Fig. 3). Consequently, the insert alters the C-terminal sequence of the protein. The deduced aa sequence of 6B6 is incomplete in 5′ region, but otherwise the aa sequences are perfectly identical except in the C terminus. Indeed, the last two

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9C2  M A R T E Q L A V V A T A V V A L V L L A A A T S E A A  28
6B6  - - - - - - - - - - - - - - - - - - - - - - - - - - -

9C2  I S C G Q V A S A I A P C I S Y A R G Q G S G P S A G C  56
6B6  - S C G Q V A S A I A P C I S Y A R G Q G S G P S A G C  27

9C2  C S G V R S L N N A A R T T A D R R A A C N C L K N A A  84
6B6  C S G V R S L N N A A R T T A D R R A A C N C L K N A A  55

9C2  A G V S G L N A G N A A S I P S K C G V S I P Y T I S T 112
6B6  A G V S G L N A G N A A S I P S K C G V S I P Y T I S T  83

9C2  S T D C S R V N - - - - - - - - -                      120
6B6  S T D C S R Y S R R M H A S A D                        99
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Fig. 3. Comparison of the aa sequences deduced from 6B6 and 9C2 cDNA. The numbering starts at the first aa of the 9C2 sequence. The extra C-terminal sequence in the 6B6 protein is boxed. The signal peptide is underlined.

residues are different (aa 92 and 93) and the protein deduced from 6B6 cDNA contains 8 aa more than the mature PLTP. The presence of four charged aa in this extra region alters the character of the protein at its C terminus. The prediction of its secondary structure using different methods (Chou and Fasman, 1974; Garnier et al., 1978) indicates the possible formation of an extra coiled or helical structure in this fragment.

(b) Southern analysis

To check whether the additional sequence fragment in the 6B6 clone was present in the genome of the two maize varieties studied, Southern-blot analysis was carried out on the variety lacking the insertion (W64A). Advantage was taken from the fact that the additional sequence found in the 6B6 clone has a unique *Hin*fI site (Fig. 1). DNA from the W64A variety was digested with *Hin*fI, electrophoresed and transferred to nylon membrane. It was hybridized with probes S1 and S2 corresponding to 5′ and 3′ regions from the *Hin*fI site in 6B6 (Fig. 1), respectively. The other probe used is the longest *Pst*I fragment (S3) of 9C2 cDNA which overlaps the place where the insertion is observed (Fig. 1). The result is shown in Fig. 4. On the one hand, the two probes flanking the *Hin*fI site detect only one band each. On the other hand two bands are detected when using the probe overlapping this sequence.

The result described above indicates that in the W64A pure inbred line the *PLTP* gene has a *Hin*fI site which is not present in the cDNA. This site is located at a similar position as the one observed in the 6B6 cDNA. The presence of this site in the genome of the E41 variety is inferred by its appearance in the cDNA sequence. It is therefore possible to conclude that the insert containing the *Hin*fI site exists in both maize varieties. Nevertheless, the insert is selectively spliced in 9C2 cDNA. It can be noticed that probes S1 and S2 reveal only a single band in the Southern blot indicating that PLTP is encoded by only one gene.

(c) Northern analysis

To demonstrate that a mRNA corresponding to 6B6 cDNA is present in the variety W64A, a Northern blot

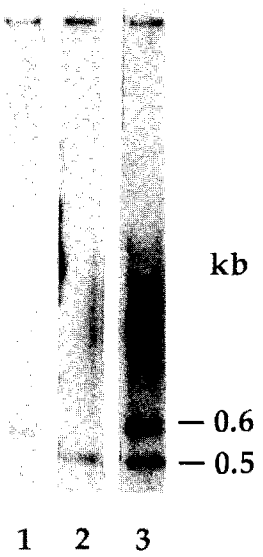

Fig. 4. Southern-blot analysis of W64A DNA. DNA (10 μg) was digested with *Hin*fI, electrophoresed in 0.8% agarose gel and transferred to nylon membranes (Hybond N, Amersham). The probes were radioactively labelled by random priming (Feinberg and Vogelstein, 1983). The membrane was hybridized as previously described (Tchang et al., 1988). Lanes: **1, 2, 3** hybridized with probes S1, S2, S3, respectively (see Fig. 1).

hybridized with a specific oligo probe was realized. An antisense oligo probe (19 mer) was synthesized from part of the 6B6 insert sequence (Fig. 2). The probe was hybridized to a Northern blot of poly(A)$^+$ RNA extracted from W64A. A 0.9-kb RNA was detected (Fig. 5; lane 1). The weak intensity of the signal confirmed that this mRNA is low-abundant. An hybridization of an identical membrane with a probe corresponding to 9C2 (Fig. 1; S3) revealed an abundant mRNA of 0.9 kb corresponding to PLTP as previously shown by Tchang et al. (1988) (Fig. 5; lane 2). These two mRNAs cannot be separated on the same gel because they differ by 74 nt only. These results indicate that, in the variety W64A, two classes of mRNA corresponding to PLTP are present: one is predominant and one is minor, corresponding to 6B6 sequence.

(d) Conclusions

The existence of multiple mRNAs coding for PLTP and transcribed by a single gene in maize is reported. Two different cDNAs have been isolated. Besides smaller differences coming from variations already observed among different maize varieties, the two cDNA differ by an insertion of 74 bp in one of them. The presence of consensus sequences flanking the insert suggests that it corresponds to an intron. The results indicate that the *PLTP* gene has at least one intron corresponding to the 74-bp insert in W64A as well as in E41. Consequently, the insert is not linked to the maize variety used.

Our results allow to propose that the two mRNA species arise from an alternatively spliced pre-mRNA. The differ-

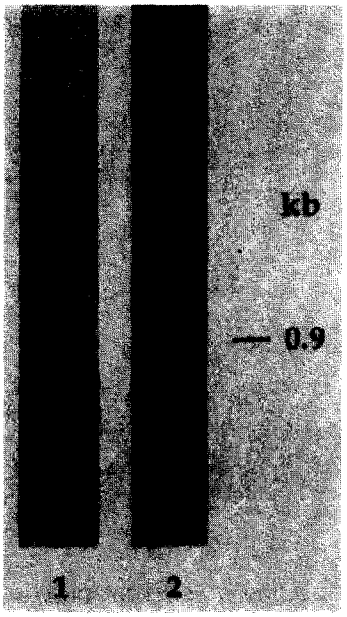

Fig. 5. Northern-blot analysis of W64A RNA. Poly(A)$^+$ RNA (10 μg; lane **1**) and total RNA (10 μg; lane **2**) were analyzed on 1.5% agarose gel and transferred to nylon membrane (Hybond N, Amersham). Lane **1**, the filter was hybridized with an antisense oligo probe made from part of the 6B6 insert sequence (Fig. 2). This probe was labelled with [γ-^{32}P]ATP by T4 polynucleotide kinase (Boehringer-Mannheim) as described by Sambrook et al. (1989). Hybridization was performed as described by Zeff and Geliebter (1987). Lane **2**, the filter was hybridized with probe S3 (Fig. 1) as previously described (Tchang et al., 1988). Exposure time is two weeks and overnight for lanes **1** and **2**, respectively.

ence observed in the C terminus of the proteins suggests the existence of PLTP isoforms which could have different subcellular locations. Recently it has been shown that part of the sequence in the C terminus of a protein can be crucial for its cellular targeting (Munroe and Pehlman, 1987; Nilsson et al., 1989). In other respects the production of heterogeneity in the aa sequences by alternative splicing is well documented in animal systems leading to proteins which are development- and tissue-specific (for review see Breitbart et al., 1987). Unfortunately, information on this mechanism in plants is lacking. The present observation indicates a new mechanism that may be of interest for the regulation of plant gene expression at the splicing level.

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