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Different lipid transfer protein mRNA accumulate in distinct parts of *Prunus amygdalus* flower

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Abstract

cDNA clones for three lipid transfer proteins (LTPI, LTPII and LTPIII) have been isolated from *Prunus amygdalus*, Batsch. The degree of similarity between these three LTPs from almond is around 70% and it allows a study of the mRNA accumulation for each one using specific probes. Although the three genes are highly expressed during floral development, significant differences in mRNA levels can be observed in distinct floral organs and in the developing fruit. Southern analysis indicates that in the genus *Prunus*, genes coding for LTP appear as belonging to a small multigenic family where at least three members differ significantly each one forming a distinct subfamily. © 1997 Elsevier Science Ireland Ltd.

Keywords: Prunus; Lipid transfer proteins; Flower expression

1. Introduction

Lipid transfer proteins (LTPs) are small (9 kDa) basic proteins present in high amounts in many plant species. The proteins were identified as a factor that increases the rate of exchange of lipids between organelles in vitro [1] although subsequent experiments have indicated divergent roles for these proteins. Since the first cloning of an LTP cDNA in maize it appeared that the sequence of the protein deduced differed from the purified protein in the N-terminal region indicating the existence of a signal peptide [2] and thus suggesting that the protein may have a different role. In fact, functions proposed for LTPs include defense against bacteria and fungi [3] or cutin biosynthesis [4] and they have been cloned as genes induced by salt stress [5], as tapetum specific [6] or complementing an embryo mutation in carrot [4]. These divergent results have suggested the possibility that similar proteins may be responsible for the different functions and therefore it has become important to know how many genes code

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for LTP. Data about this question are scarce but in a species like barley at least seven genes are present and in at least one case in sorghum two genes are located in tandem [7].

The genus *Prunus* includes interesting plant species for different reasons. Almond, peach, apricot, cherry, etc. are among the species of the genus having a high economic interest and they may be an interesting model for studies in tree species as they have a very low DNA content only twice that of *Arabidopsis thaliana* [8]. In some cases proteins normally encoded by large multigene families such as storage proteins, in *Prunus* they are encoded by a low number of genes [9]. A complete genetic map of almond has recently been published including some characterized cDNAs [10]. Interesting agronomic characters in these species include some characters that depend on flower development. These reasons prompted us to analyze the complexity of LTPs present in the flower of almond. It appears that at least five or six genes coding for LTP are present in the almond genome and three of these genes present specific features of expression in the flower tissues.

2. Materials and methods

2.1. Plant material

Almond (*Prunus amygdalus*, Batsch) fruit and flower samples from the 'Texas' cultivar were collected at different stages of development from crop fields in Departament d'Arboricultura Mediterrània, IRTA (Reus, Spain) and were immediately frozen in liquid nitrogen and stored at -80°C until they were used. During almond flower development four stages have been considered called C, D, E and F corresponding respectively to the appearance of the calyx, corolla and overture of petals with the possibility of seeing the stamen and mature flower with petals completely opened [11].

2.2. Library screening

Polyadenylated RNA, extracted from closed flowers was used to construct a cDNA library in the Uni-ZAP XR λ vector (ZAP-cDNA Synthesis Kit; Stratagene). After in vivo excision, about 500 clones were hybridized with radiolabelled first-strand cDNA from the same tissue. A number of the clones with a high level of expression were sequenced and three different clones with similarity with published LTPs were among the sequences.

2.3. RNA extraction and RNA blot analysis

RNA was extracted from different almond tissues using the methods described by Martin et al. [12] and Haffner et al. [13] depending on the tissue. Then 10 μg of total RNA were separated in a 1.5% agarose-formaldehyde gel and transferred to a nylon membrane (Nytran, Schleicher and Schuell) according to the method originally described by Lehrach et al. [14]. The membranes were hybridized with [^{32}P]dCTP-labelled 3′ non-coding cDNA region amplified for each LTP by PCR and washed according to a protocol described by Church and Gilbert [15].

2.4. DNA preparation and Southern blot analysis

Genomic DNA was extracted from young leaves using the method described by Bernatzky and Tanksley [16]. The DNA was purified by equilibrium centrifugation in a CsCl-ethidium bromide gradient as described by Maniatis et al. [17]. The DNA was digested under standard conditions, electrophoresed on a 0.8% agarose gel and transferred to a nylon membrane (Nytran, Schleicher and Schuell) according to the method originally described by Southern [18]. The membranes were hybridized with [^{32}P]dCTP-labelled 3′ non-coding cDNA region amplified for each LTP by PCR. The washing conditions in LTPI and LTPII were 20 mM Na_2HPO_4, 1% SDS, 1 mM EDTA at 65°C while in LTPIII they were stronger: 5mM Na_2HPO_4, 1% SDS, 1 mM EDTA at the same temperature in order to ensure the specificity of hybridization.

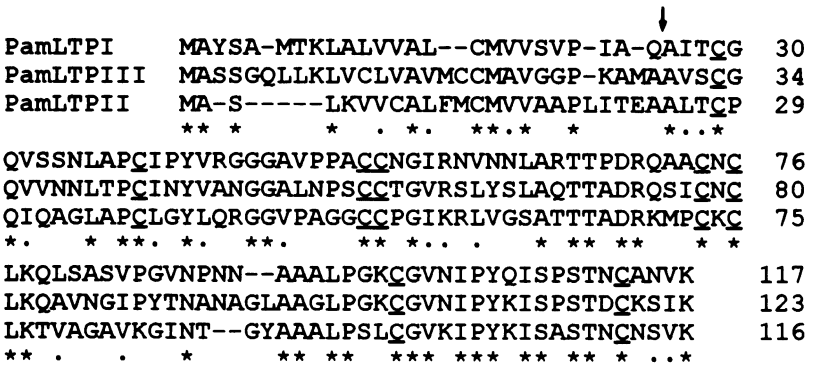

Fig. 1. Alignment of the three *Prunus amygdalus* LTP proteins. The putative signal peptide cleavage site is marked with an arrow. The identical amino acids in all three sequences are marked with an asterisk, and the conservative amino acid replacements are marked with dots. The eight cysteines residues are show in bold and underlined. Gaps (–) are introduced for maximum alignment.

2.5. Sequence alignment and phylogeny

The sequences (excluding the putative signal peptide) of the three almond LTPs were aligned with 49 amino acid sequences of lipid-transfer proteins deduced from cDNAs or genes encoding these proteins in plants. The alignment was analyzed by using parsimony and distance-based methods included the Phylogenetic Inference Package (PHYLIP) program version 3.41 [19]. Accession number of the sequences were compared: *Amaranthus caudatus* (Aca, P80450), *Arabidopsis thaliana* (AthI, M80567; AthII, S29459), *Beta vulgaris* (Bvu, X92748), *Brassica napus* (Bna2, U22174; Bna3, U22175), *Brassica oleracea* (BolI, L33904; BolII, L33905; BolIII, L29767), *Daucus carota* (Dcaep2, P27631), *Eleusine coracana* (Eco, P23802), *Gerbera hybrida* (Ghy, S44100), *Gossypium hirsutum* (Ghi, S78173), *Hordeum annuus* (Han, X92648), *Hordeum vulgare* (HvuI, S49198; HvuII, X68655; HvuIII, S28872; HvuIV, Z66528; HvuVI, U18127; HvuVII, X96716; Hvu1, P07597; Hvu2, P20145), *Lycopersicon esculentum* (Les, S20862), *Nicotiana tabacum* (NtaI, Q03461; NtaII, S22168; NtaIII, U14167), *Oryza sativa* (OsaI, P23096; OsaII, Z23271, Osa2), *Pachyphytum sp* (Psp, L14770), *Pinus taeda* (Pta, U10432), *Prunus amygdalus* (PamI, X96714; PamII, X96715; PamIII, X96716), *Ricinus communis* (RcoI, P10973; RcoII, P10974; RcoIII, P10975; RcoIV, M86353), *Spinacia oleracea* (Sol, M58635), *Senecio odurus* (Sod, L33791), *Sorghum vulgare* (Svu1, X71667; Svu2, X71668), *Triticum aestivum* (Tae, S46250), *Triticum durum* (Tdu, X63669), *Vigna unguiculata* (Vun, X79604), *Zea mays* (ZmaI, P19656; ZmaII, M57249; ZmaIII, S45635), *Zinnia elegans* (Zel, U19266).

3. Results

3.1. Almond lipid transfer protein sequences and genomic structure

In the course of a screening of an almond (*Prunus amygdalus*, Batsch) flower cDNA library searching for genes highly expressed in the floral organs of this plant, different clones containing sequences homologous to lipid transfer proteins (LTP) were found. The clones corresponded to three independent sequences and they were called LTPI, LTPII and LTPIII. An alignment of the three protein sequences deduced from the cDNA is shown in Fig. 1. The three sequences have a relative similarity of 66 (LTPI/II), 67 (LTPI/III) and 68% (LTPII/III) and when aligned they clearly belong to the same protein family. The proteins begin with a sequence of lower similarity that in other species it has been shown to correspond to a signal peptide. The eight conserved cysteine residues present in most LTP sequences reported until now are also present in the three

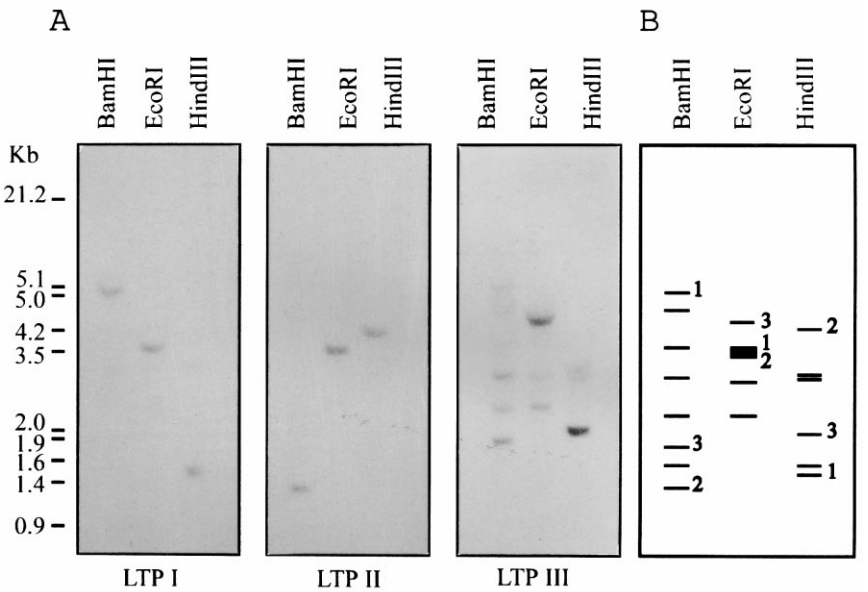

Fig. 2. Southern analysis for *Prunus amygdalus* DNA. Each line contains 5 μg of genomic DNA restricted with *Bam*HI, *Eco*RI and *Hin*dIII. (A) The filters were hybridized with a probe corresponding to the 3′ non-coding cDNA region of each LTP cDNA. (B) Schematic summary of the result of Southern blot analysis of LTP *P. amygdalus* using as a probe the three full-length LTPs cDNA.

almond LTPs and they are surrounded by other similar regions of the proteins. Sequences that have been proposed as responsible for interaction with lipids like the DRQ/K motif at position 69 (LTPI protein) and around the CGV motif at position 99 (LTPI protein) are also present in the three *Prunus* LTPs.

The degree of similarity between the different LTPs from almond flower is low enough in either their coding or their non-coding sequences to allow a study of the mRNA accumulation specific for each one of the LTPs. This is shown on Fig. 2 where the Southern blot using the 3′ cDNA probes for the three LTPs is presented. It is clear that LTPI and LTPII using these probes and appropriate hybridization conditions detect only one gene in the almond genome while LTPIII using similar conditions detects a main set of bands but also other minor bands. In fact, using lower stringency conditions a number of genomic bands are observed that hybridize to the cDNA probes. This is shown in a schematic way in Fig. 2B. In the figure the position of all the bands using any of the three probes is summarized. Our results suggest that five or six genes may encode LTP sequences in the almond genome. Further screenings using the three probes in a different cDNA library (an almond root cDNA library) failed to give LTP sequences different from the three here described that appear to be the most abundantly represented in the mRNA population in both flower and root. All these results also indicate that the three LTPs here identified are encoded by different independent genes in the almond genome. This is confirmed by results from mapping studies that show that LTPI and II map at different chromosome locations (C. de Vicente, personal communication). Lack of polymorphism precluded the mapping of LTPIII.

3.2. Ltp mRNA accumulation in almond floral organs

Using probes specific for each of the three cloned LTPs, RNA blot studies were conducted in order to study the steady state levels of each one of the genes in different parts of the plant. The results are presented in Fig. 3. The mRNA levels

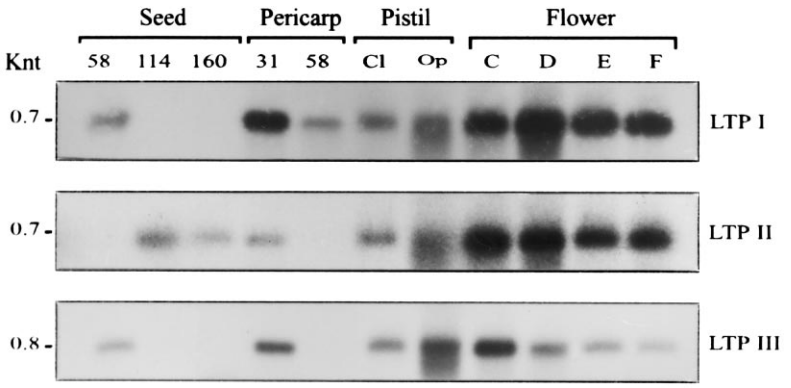

Fig. 3. RNA blot analysis of *Prunus amygdalus* LTPs using a 3′ non-coding cDNA probe. Each lane contains 10 μg of total RNA from different tissues: seed and pericarp in different stages of development, pistil from closed and opened flower and flower from early development (stage C) to completely mature flower (stage F). Numbers indicate days after flowering.

in the flower at different stages of development (stages C to F) show high levels of LTP mRNA accumulation for the three probes and in all cases decreasing with maturation of the whole flower. When the films are developed after long exposure times it is possible to observe that the three LTP mRNAs can be detected in the immature pistil of the developing flower. However at shorter times of exposure it appears that in the mature pistil (Fig. 4) LTPIII is the most abundant component.

Significant differences in mRNA levels can be observed in the developing fruit. While LTPI and LTPIII mRNA are abundant at early stages of seed development, LTPII is present at later stages. The three LTPs here studied are expressed at early pericarp stages while only LTPI is observed at intermediate developmental stages. The results are highly reproducible and in those presented here the autoradiographs result from successive hybridization of the same filters with the corresponding specific probes. The levels of these LTP mRNAs in other organs such as roots or leaves were very low or undetectable (result not shown) although as it was mentioned early, a screening of a root cDNA library with the LTP probes resulted in the cloning of the same sequences here reported.

The analysis of RNA extracted from different organs of the mature flower presented on Fig. 4 indicated that stamen and sepals appear to contain equivalent levels of the three LTP mRNAs, no mRNA can be detected for LTPII in mature pistils and petals and a low mRNA level for LTPI in pistils while LTPIII mRNA seems to be accumulated in the four floral organs but at a higher level in the mature pistil.

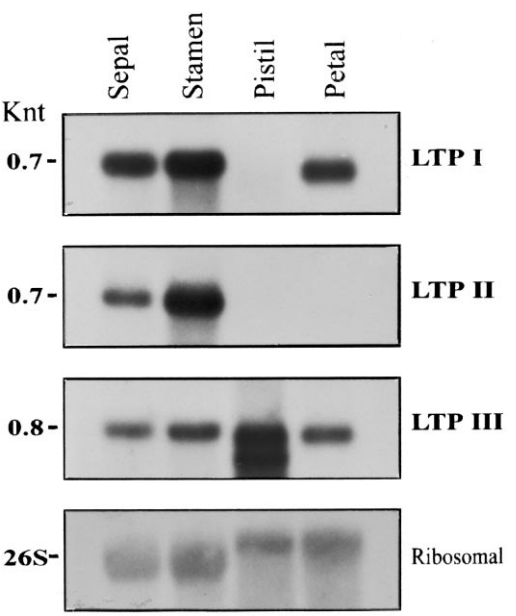

Fig. 4. RNA blot analysis of total RNA preparation from sepal, pistil, stamen and petal from mature flower. The probes used were the 3′ non-coding cDNA region for each LTP cDNA. The cDNA probe for 26S ribosomal from *Zea mays* was used as a control. These films were exposed with an intensifying screen for 20 h.

3.3. Comparison of almond LTP sequences with homologous sequences from other species

The three LTPs here described apparently belong to the same family of proteins but they depart significantly in sequence. They are encoded by three independent genes in the almond genome and they appear to have evolved independently. This result is confirmed when these sequences are aligned and compared with other reported LTP sequences using different algorithms. Fifty-one sequences from 28 plant species could be collected for proteins having either sequence similarity of a possible homologous function. Among these sequences 34 could be grouped systematically using different algorithms based either in parsimony or in distance-based methods. This set of sequences is shown as groups in a phylogenetic tree on Fig. 5 that is the product of the distance-based method Neighbor-joining [19].

Using different algorithms the relative distance of the sequences can vary but a feature was present in all cases and it is shown in the figure. This is the presence of five groups of sequences. In one group (group B in Fig. 5) two *Prunus* LTP sequences (LTPI and LTPIII) appear in the same group as *Daucus carota* belonging also to the class of *Rosidae* but also other sequences from species such as *Gerbera*, *Helyanthus* or *Gossipium* belonging to other classes. Group A is more homogeneous as only *Cruciferae* sequences appear, group C includes *Solanaceae* species but also spinach, group D cereal LTPs and group E are *Hordeum vulgare* sequences. *Prunus* LTPII is grouped with different other LTPs depending on the set of sequences or the algorithm used. A number of other LTPs seem to be more divergent and they cannot be grouped consistently. This result confirms that the three LTP sequences have important sequence differences that appear to have independently evolved.

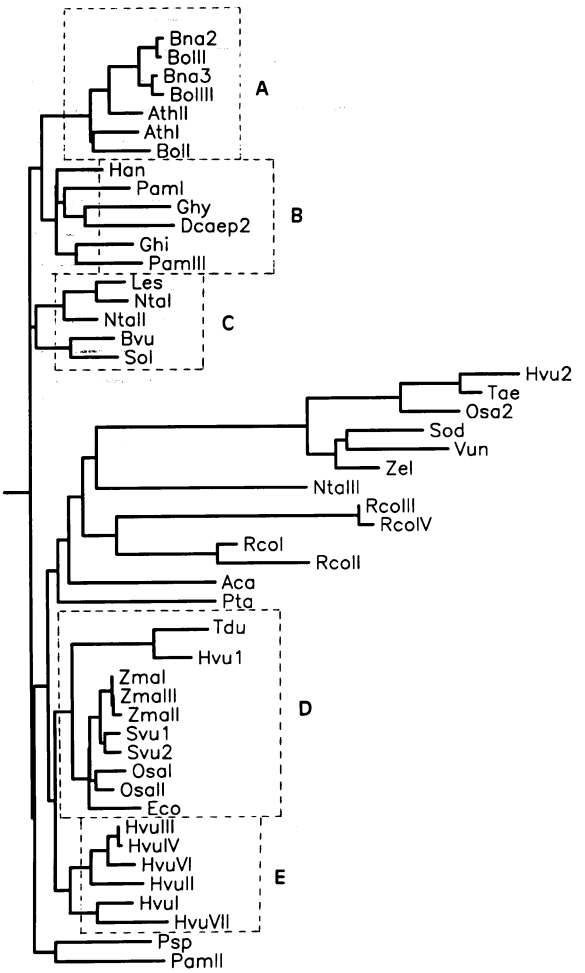

Fig. 5. Neighbor-joining tree for 49 different plant LTPs based on alignment of these mature amino acid sequences. The mature sequence of the protein (excluding the putative signal peptides) were used for the analysis.

4. Discussion

At least three different genes coding for lipid transfer proteins (LTPs) are actively expressed in the floral tissues of *Prunus amygdalus*. The three proteins depart significantly among themselves in their protein sequence although they keep the basic features of all the LTPs described in plants so far. The divergence between the sequences allows to define conditions where specific probes hybridize only with each one. In Southern blots, these probes hybridize with only one band for both LTPI and LTPII and probably with two sequences for LTPIII. When using probes corresponding to common regions and less stringent conditions other bands appear. In total five to six genes appear to form the family of LTP genes in

Prunus and at least three of them are expressed in the flower.

The sequences of the three LTPs here studied are significantly divergent among themselves. The average similarity between LTPs in almond is lower than 70% when in the case of sequences from different *Brassica* species is at least 80% [20] or in a species like tobacco is 82% ([21], AC:Q03461, S22168). This may be an indication that, with the probable exception of LTPIII, each protein is encoded by a single gene in *Prunus* while in other species subfamilies coding for each LTP may exist. This situation may be reflected in the comparative analysis carried out between the different available proteins. When different algorithms of sequence comparison are applied the three *Prunus* LTPs do not cluster in a subgroup as it is the case in some other species. Although in general terms large phylogenetic groups such as monocots and dicots and a number of species keep their expected interspecies relations, the three *Prunus* sequences are among the proteins that depart from their expected classification. In this sense if the function of the protein is the same, it is possible to conclude that the essential features are very few, including mainly the eight cysteine residues and neighboring amino acids. It may also indicate that LTPs were already encoded by multigene families in primordial angosperms and each gene has evoluted independently during the definition of the different geni.

The three genes here studied are expressed in the almond flower and seed. Expression in vegetative tissues is much lower but not inexistent. The presence of LTP transcripts in flower tissues has already been reported in tobacco [21], *Arabidopsis thaliana* [22] and *Gerbera hybrida* [23] although only one component of the LTP family were examined in these cases. Specific expression of these LTPs were also observed in different organs of the flower. The availability of three specific probes in almond flower allows the depiction of the levels of mRNA accumulation for each LTP distinct in specific organs of the flower and seed. This is specially clear in the mature pistil and sepal and in the mature seed. With the data now available it is difficult to draw any conclusion regarding specific functions related to one of the LTPs. In fact, it is possible that the existence of different genes may be important to ensure the presence of LTP protein in all stages of the plant and specifically during flower development. In this sense it is interesting that a species with a small genome such as *Prunus* contains a multigene family coding for LTP with divergent sequences and overlapping patterns of expression. This fact may indicate that it may be important for the plant to ensure a certain level of LTP mRNA in its developing organs although the determinants in the protein sequence are not very high. This argument may lead towards a general transport or defense function for the protein instead than a more specific one.

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