

Differential expression of two lipid transfer protein genes in reproductive organs of peach (*Prunus persica* L. Batsch)

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Abstract

Two cDNA clones (named *Pp-LTP1* and *Pp-LTP2*) corresponding to different lipid transfer protein (LTP) genes have been isolated from peach (*Prunus persica* L. Batsch) epicarp and ovary, respectively. Sequence analysis revealed that the two fragments share 54% identity at nucleotide level and show common features of plant LTP genes, such as conserved cysteine residues and lipid-binding motifs. Phylogenetic analysis grouped *Pp-LTP1* and *Pp-LTP2* in two distinct clusters, the former with most of LTP genes sequenced in the *Rosaceae* family, the latter only with one almond LTP. Genomic Southern data indicated that a small LTP gene family is present in peach. *Pp-LTP1* and *Pp-LTP2* have been used as gene-specific probes to describe expression in flowers and fruits throughout development. In petals, sepals and stamen only *Pp-LTP1* was expressed whereas transcripts of *Pp-LTP2* strongly accumulated in non-pollinated and pollinated ovary with a decreasing trend in the period of four weeks after pollination. In fruits, a dramatic accumulation of *Pp-LTP1* mRNA was detected in epicarp at all stages of fruit development and, with the exception of the early growth stage, no *Pp-LTP1* transcripts have been detected in mesocarp. When *Pp-LTP2* was used as a probe in the same fruit tissues, a faint hybridisation signal was observed only in epicarp of fruitlets collected at an early growth stage. Infection with *Monilia* induced only a slight increase of *Pp-LTP1* transcript in epicarp of pre-climacteric and climacteric fruits. These results support the hypothesis of multiple roles played by LTPs and, considering that LTPs have been recognised as the major allergen of peach, indicate that *Pp-LTP1* could be related to the allergenicity of peach.

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1. Introduction

Lipid transfer proteins (LTPs) are abundant and widespread in nature and form a broad family of proteins whose role is not clearly defined. In higher plants their function has been associated with the *in vitro* properties of transferring lipids from donor to acceptor membranes [1], but a role in response to abiotic stresses [2–4], in embryogenesis [5], and in defence mechanisms [6,7] has also been postulated. The hypothesis of an involvement of LTPs in plant defence has been proved in transgenic plants [8] and has been recently reinforced by Buhot et al. [9] that demonstrated the

interaction of LTPs with elicitor receptors located on plasma membranes.

The multiple function of LTPs might be related to the presence of small gene families (up to 15–20 genes in *Arabidopsis* [10]) whose members are selectively expressed in plant tissues and encode isoforms with different *pI* (usually close to 9) and molecular weight of 9 kDa (type 1) and 7 kDa (type 2) [11,12]. Most of the studies performed so far in several plant species focused on type 1-LTPs and complete amino acid and gene (or cDNA) sequences have been published and are available in databases. Common features are eight highly conserved cysteine residues forming four disulfide bridges, an intron located at the C-terminus of the protein and a putative signal peptide indicating that the polypeptide (of 90–95 amino acids) is secreted. Transcript accumulation analysis revealed that LTP genes are differentially expressed in plant tissues and that specific mRNA

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accumulation occurs only in aerial part of plants, particularly in leaves and flowers where LTP genes appear developmentally regulated [4,10,13].

Besides their biological functions, LTPs have been recently identified as the major allergen of peach [14,15] and other stone fruits as apricot [16] and plum [17] causing, after ingestion in sensitised population, symptoms usually restricted to the oral cavity (oral allergy syndrome, OAS) although systemic reactions, including anaphylactic shock, have also been reported [18]. Recently, Asero et al. [19] showed that IgE-antibodies to stone (and apple) fruits LTPs react to a broad range of extracts of different plant species such as peanut, walnut, pistachio, broccoli, carrot, tomato, melon and kiwifruit indicating that LTPs represent a genuine pan-allergen with a high degree of cross-reactivity and demonstrating that IgE against LTP is correlated with severe symptoms of food allergy. The 9 kDa peach LTP has been purified and sequence analysis of the protein (named Pru p 1, accession number P81402) shows a high degree of homology (average of 65%) with LTPs from other plant species [14]. The protein extracted from fruit skin, where is predominantly present [20], retains its IgE-binding capacity and demonstrates a high stability following heat treatment [21] and pepsin digestion [19]. Considering that stone fruits are common (and increasing) causes of food allergy diseases and in order to elucidate factors affecting the accumulation of the major allergen, we have approached a molecular study of LTPs in peach. In this paper, we report the isolation of two LTP-related cDNAs and the differential expression of the corresponding genes in reproductive organs of peach.

2. Materials and methods

2.1. Plant material and nucleic acids extraction

From trees of cv. Springcrest, open flowers (separated in petals, sepals, and stamen), non-pollinated and pollinated ovaries, fruits (distinct in epicarp and mesocarp) at S1 (first exponential growth phase), S2 (endocarp lignification), S3 (second exponential growth phase) preclimacteric, climacteric rise, and climacteric peak, as defined by Tonutti et al. [22], have been collected, immediately frozen in liquid nitrogen, and stored at -80°C . Surface-disinfected preclimacteric and climacteric fruits have been wounded and inoculated with 1 mm^2 *Monilia laxa* mycelium plugs. Inoculated and non-inoculated fruits have been maintained at 25°C until the visible lesions on the epicarp reached the diameter of about 1.5 cm.

DNA, from young leaves, and total RNA, from the above mentioned tissues, have been extracted as described by Ruperti et al. [23].

2.2. RT-PCR and gene-specific probe preparation

The first 269 bp cDNA fragment (named *Pp-LTP1*) has been synthesised via PCR (Perkin Elmer GeneAmp[®] System 9700). Briefly, degenerated primers (Sigma Genosys) have been used on epicarp (cv Sentry) poly A+ RNA-derived cDNA (Oligotex mRNA Mini Kit, Quiagen). Primers have been constructed on the basis of Pru p 1 sequence, the allergenic 9 kDa LTP isolated from peach fruit by Pastorello et al. [14]. *Prunus persica* codon usage has been used to limit primers degeneration. LTP-5' sense primer corresponds to N-terminal ITCGQVS sequence: AT[T/C]AC[A/T]TG[C/T]GG[T/A/C]CA[A/G]GT[G/T]TC[T/A]. LTP-3' anti-sense primer corresponds to C-terminal STNCATVQ sequence: [C/A]AC[T/A]GT[A/T/G]GC[G/A]CA[G/A]TT[T/A]GT[T/A]GA. Reaction contained approx. 300 ng of cDNA, 1 U *Taq* DNA polymerase (Pharmacia Biotech), 50 pmol each primer, 200 μM each dNTP, in a final volume of 25 μl of 1X PCR reaction buffer. 30 PCR cycles of denaturation at 94°C for 30 s, primer annealing at 52°C for 60 s and extension at 72°C for 60 s have been performed.

The second 162 bp fragment (named *Pp-LTP2*) has been isolated by RT-PCR on DNA-free RNA (extracted from sepals, petals, stamen, pollinated ovary, and epicarp cv Springcrest) using the Access RT-PCR System (Promega) kit. Retrotranscription has been performed, after denaturation at 94°C for 2 min and oligo (dT)_{12–18} annealing at room temperature for 15 min, on 1 μg of total RNA at 37°C for 50 min. After RNA/cDNA hybrid denaturation, selective amplification of LTP-related cDNAs has been carried out using oligo (dT)_{12–18} and a Family Specific Domain primer [24] designed on TTPDRQ sequence, conserved in all isolated LTPs (5'-ACCACCCCTGACCGCCAG-3'). After activation of *T7* DNA Polymerase at 94°C for 2 min, 40 cycles at 94°C for 30 s, 37°C for 90 s, 68°C for 60 s, and one final cycle at 68°C for 7 min have been performed. PCR products have been separated by gel electrophoresis, purified from gel (QIAquick[®] Gel Extraction Kit, QUIAGEN) and subcloned in P-Gem-T[®] Easy Vector (Promega).

2.3. DNA sequencing and phylogeny

Amplification products have been sequenced according to Sanger et al. [25] at CRIBI, Biology Department, University of Padova using ABI PRISM Big Dye Terminator (Perkin Elmer) Kit. Sequence analysis has been performed by BLASTX algorithm [26]. DNA sequences were analysed with DNASTAR software (DNASTAR, Madison, WI). Predicted amino acid sequences were aligned by MEGALIGN (DNASTAR) using the Clustal method [27] with PAM250 residue weight table and default parameters (Method: Clustal; Multiple

Alignment Parameters–Gap Penalty:10, Gap Length Penalty:10; Pairwise Alignment Parameters–Ktuple:1, Gap Penalty:3, Window:5, Diagonals Saved:5).

2.4. Northern and Southern blot hybridisation analyses

DNA blot analysis (10 µg of restricted genomic DNA) was performed following standard procedures [28]. Membranes were washed with 2 × SSC, 0.1% (w/v) SDS and 1 × SSC, 0.1% (w/v) SDS for 10 min at 42 °C. Northern analysis was carried out on total RNA (15 µg) as described by Tonutti et al. [22].

3. Results

3.1. Cloning of LTP-related cDNAs

Amplification of epicarp RNA-derived cDNA with the selected degenerated primers resulted in two fragments of about 300 and 450 nt. Considering that, on the basis of the primers position in the LTP Pru p 1 sequence, the predicted length had to be 270 nt, only the 300 nt fragment has been isolated, subcloned and sequenced. Using the Family Specific Domain primer and after digestion with *AluI* and *HincII* endonucleases, two fragments of the expected length (about 250 bp) have been detected only when amplification was conducted on pollinated ovary and epicarp cDNAs, respectively. The four fragments have been isolated, subcloned and sequenced.

3.2. Sequence and Phylogenetic analyses

The first isolated clone (269 bp), named *Pp-LTP1* (accession number AY093700), has been sequenced and the deduced aminoacid sequence appeared to be identical to the corresponding Pru p 1 protein with the exception of two aminoacids in position 9 (asparagine instead of alanine) and 19 (valine instead of glycine) (Fig. 1). The sequence shows the characteristics of plant LTPs: the conserved in vitro lipid-binding motifs DRQ and CGV, and the eight cysteine residues responsible for disulfide bridges. Of the four clones obtained by using

the Family Specific Domain primer, the first from epicarp cDNA appeared to be identical to the corresponding sequence of *Pp-LTP1*, and the second shared high homology with a cytochrome oxidase. The two clones (162 bp) derived from pollinated ovary cDNA resulted to be identical each other, showing 54% identity at nucleotide level and 66.5% similarity at deduced aminoacid level with *Pp-LTP1*. This fragment (named *Pp-LTP2*, accession number AY093699) contains the lipid-binding motifs DRQ and CGV and, considering the position of the family specific domain (TTPDRQ) used as a primer, four of the eight cysteines at conserved positions (Fig. 1).

According to the parameters used, the phylogenetic analysis showed that *Pp-LTP1* and *Pp-LTP2* belong to different clusters (Fig. 2). *Pp-LTP1* is grouped with most of LTP genes isolated in other *Rosaceae* species with the exception of almond LTP2 and LTP3: with the latter, *Pp-LTP2* appears to be phylogenetically related. This suggests that, as observed in almond, the sequence difference between *Pp-LTP1* and *Pp-LTP2* is the result of an independent evolution of the two members of the peach LTP family.

3.3. Southern blot analysis

Southern analysis revealed that, in peach, a LTP multigene family is present: in fact, using both *Pp-LTP1* and *Pp-LTP2* as probes, a number of bands are present in DNA restricted with several endonucleases (Fig. 3). Considering the low stringency conditions used, the presence of strong and weak hybridisation bands suggests that *Pp-LTP1* and *Pp-LTP2* belong to two small subfamilies.

3.4. *Pp-LTP1* and *Pp-LTP2* mRNA accumulation in reproductive organs

The two isolated cDNA fragments, sharing 54% nucleotide identity, did not cross react when high stringency conditions were used in preliminary hybridisation experiments (data not shown), allowing to use them as gene-specific probes. Northern blot hybridisations pointed out that *Pp-LTP1* and *Pp-LTP2* are differentially expressed in peach reproductive tissues. In petals, sepals and stamen, collected from open flowers, *Pp-LTP1* transcripts have been strongly detected, whereas *Pp-LTP2* mRNA was totally absent (Fig. 4). In non-pollinated ovary, the most abundant LTP transcripts appeared to belong to *Pp-LTP2* and decreasing amounts *Pp-LTP2* mRNA have been detected in the period of 4 weeks after pollination. In ovaries, the level of *Pp-LTP1* transcript accumulation was lower than that of *Pp-LTP2* and appeared quite similar before and after pollination (Fig. 4). A marked difference in terms of expression pattern has been

Pp-LTP1	ITCGQVSSNLAPCIPYVRVGAVPPACCGNG	30
Pp-LTP2	-----	00
	IRNVNNLARTTPDRQAACNCLKQLSASVP--GVNPNNA	66
	-----TTPDRQSI ^C NCLKQAVNGIYPNANAGLA	29

	AALPGKCGVHIPYKISASTNCATV	90
	AGLPGKCGVINIPYKISPSTDC ^C SIK	54
	* *****	

Fig. 1. Alignment of the deduced aminoacid sequences of *Pp-LTP1* and *Pp-LTP2*. The identical aminoacids are marked with an asterisk, the cysteines residues are shown in bold and underlined, and the lipid-binding motifs are boxed.

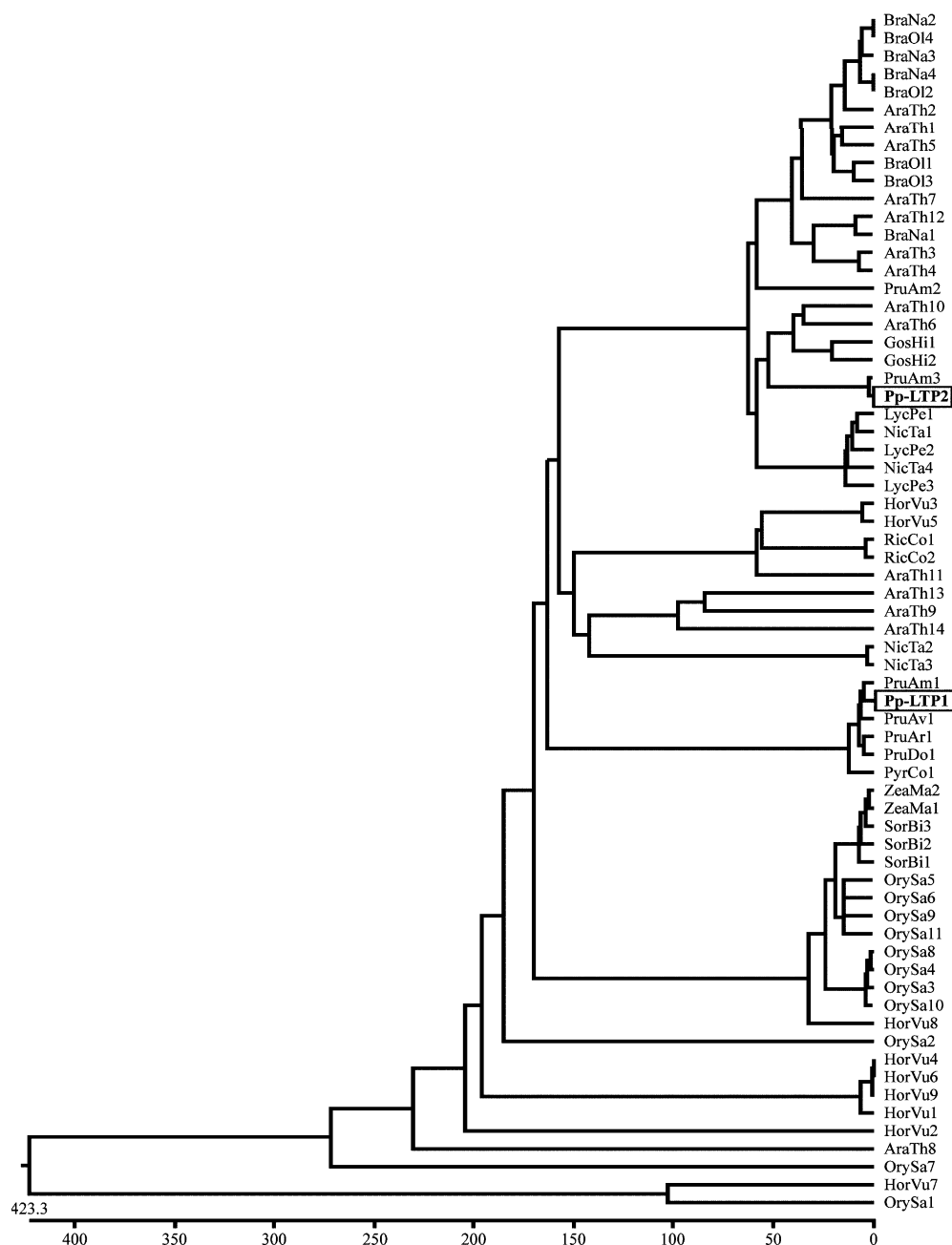


Fig. 2. Phylogenetic tree for 67 plant LTPs, belonging to multigene families, based on alignment of the mature protein sequences available in GenBank database. Plant species are indicated by initial letters of the Latin name followed by a number.

detected between epicarp and mesocarp throughout fruit growth (Fig. 5): in fact, *Pp-LTP1* is constantly expressed in epicarp, whereas in mesocarp no specific transcripts have been detected at S2, S3, and ripening stages. The hybridisation signal detected in mesocarp at S1 is most likely an artefact considering the difficulty to separate mesocarp and epicarp in such small fruits. The strongest hybridisation signal of *Pp-LTP1* in epicarp appeared in correspondence to the S3 stage. Concerning *Pp-LTP2*, no transcripts were detected in mesocarp and

a faint hybridisation band has been observed in epicarp only at S1 stage (Fig. 5). At ripening, no marked difference in terms of *Pp-LTP1* mRNA accumulation has been detected in epicarp of fruits collected at pre-climacteric, climacteric rise and climacteric peak stages (Fig. 5). Treatments with exogenous ethylene carried out on pre-climacteric fruits did not affect transcript levels of *Pp-LTP1* and *Pp-LTP2* in both mesocarp and epicarp (data not shown). When pre-climacteric and climacteric fruits were infected with *Monilia*, only a

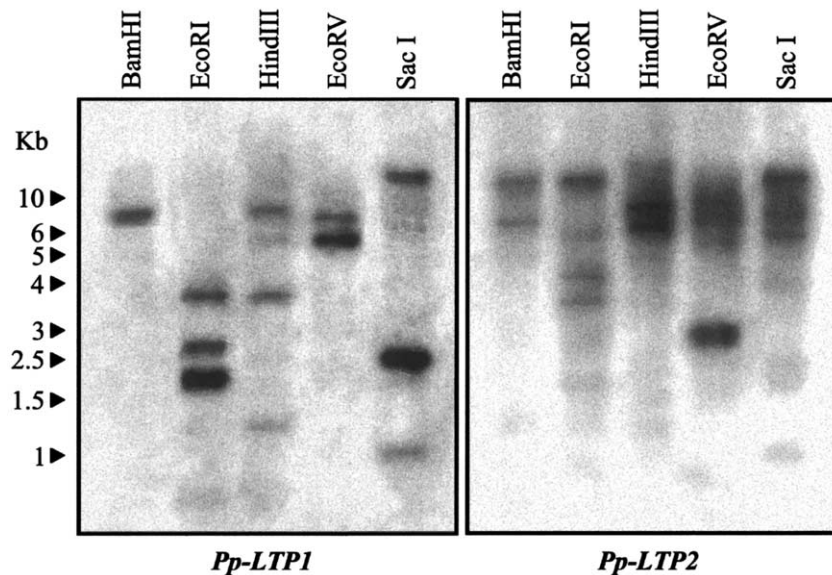


Fig. 3. Southern blot hybridisation analysis of peach (cv Springcrest) DNA using *Pp-LTP1* (left) and *Pp-LTP2* (right) cDNAs as probes. Each lane contains 10 µg of genomic DNA restricted with *Bam*HI, *Eco*RI, *Hind*III, *Eco*RV and *Sac*I endonucleases.

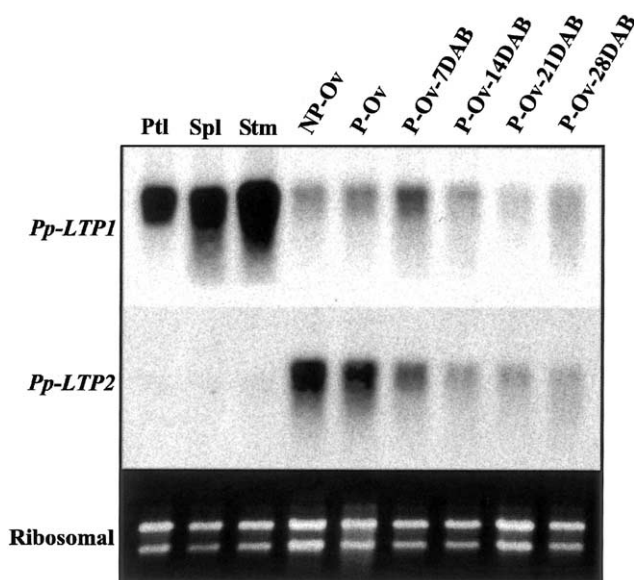


Fig. 4. Northern blot hybridisation analysis carried out with *Pp-LTP1* and *Pp-LTP2* cDNA probes on total RNA (10 µg) extracted from petals (Ptl), sepals (Spl), stamen (Stm), non-pollinated ovary (NP-Ov), pollinated ovary (P-Ov), and pollinated ovary 7 (P-Ov-7DAB), 14 (P-Ov-14DAB), 21 (P-Ov-21DAB), and 28 (P-Ov-28DAB) days after full bloom.

slight increase of *Pp-LTP1* mRNA was detected in climacteric epicarp whereas *Pp-LTP2* transcripts remained undetectable (data not shown).

4. Discussion

As observed in several plant species [11], our results show that also in peach a multigene family encoding

lipid transfer proteins is present and the expression pattern of two members is tissue specific and developmentally regulated. Southern analysis clearly demonstrates the presence of at least 4–5 genes; in almond, a closely related species, LTP gene family is formed by five to six members, with a similarity, among the three isolated cDNAs, lower than 70% [13]. The two isolated peach LTP clones, *Pp-LTP1* and *Pp-LTP2*, share the common features of plant LTPs (positions of cysteine residues, lipid-binding motifs) but show 66.5% of similarity at deduced amino acid level between them. The low similarity value and the results of phylogenetic analysis, that grouped *Pp-LTP1* and *Pp-LTP2* in two distinct clusters, would indicate that these are not duplicated genes, as observed for some members of the LTP family in *Sorghum vulgare* [29], *Brassica oleracea* [30], and *Arabidopsis* [10], but they have independently evolved from an ancestor with some copies of LTP genes. Expression and phylogenetic analyses suggest that the ancestor of Brassicaceae family possessed no less than five LTP gene copies [10,31]. Besides *Pp-LTP1*, constructed on the basis of the 9 kDa Pru p 1 sequence [14], also *Pp-LTP2* appear to encode for the type-1 (9 kDa) LTP: in fact, it lacks the conserved proline and tyrosine residues that characterise the type 2 (7 kDa) LTPs [12] and significantly differs from the 7 kDa LTP sequenced by Conti et al. [32] in apricot, a close-related species to peach.

The differential expression patterns of the two isolated clones reinforce the hypothesis of multiple roles played by LTPs. Differently from *Pp-LTP1*, *Pp-LTP2* transcripts have been strongly detected in non pollinated ovaries and their level declined in the period of four weeks after pollination. Similar results have been

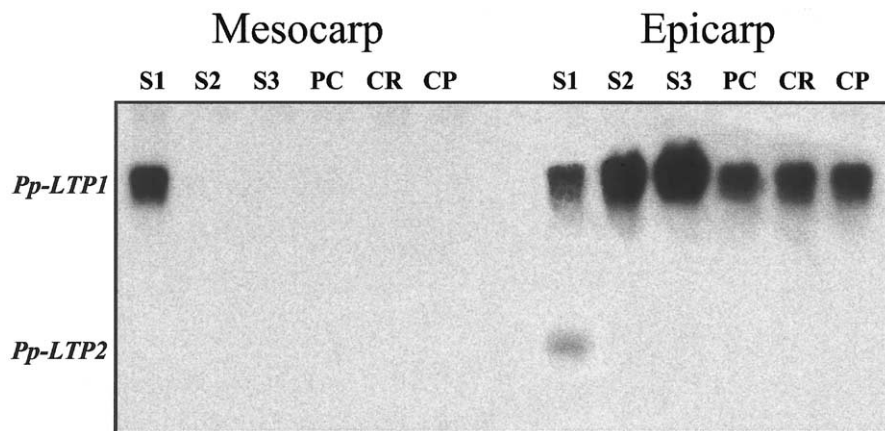


Fig. 5. Northern blot hybridisation analysis carried out with *Pp-LTP1* and *Pp-LTP2* cDNA probes on total RNA (10 µg) extracted from mesocarp (left) and epicarp (right) of fruits collected at S1 (first exponential growth phase), S2 (endocarp lignification), S3 (second exponential growth phase), pre-climacteric (PC), climacteric rise (CR) and climacteric peak (CP) stages.

obtained by Suelves and Puigdomenech [13] in almond where, of the three isolated genes, only LTP 3 (belonging to the same phylogenetic cluster of *Pp-LTP2*) is particularly abundant in mature pistils. The absence of *Pp-LTP2* transcripts in petals, sepals, stamen and in fruit tissues would support the hypothesis that the role of this gene is, within flower structures, highly specific and confined to pistils in which it may have different functions. Besides the involvement of LTPs in embryogenesis [5], it has been recently demonstrated [33] that one of the two proteins necessary for pollen tube adhesion in stylar matrix of lily is a lipid transfer-like molecule which may directly or indirectly function as adhesive agent during pollen tube growth.

In fruits only *Pp-LTP1* is expressed and its transcripts abundantly accumulate in epicarp but not in mesocarp, confirming the findings of Leonart et al. [20] that demonstrated the presence of an allergenic protein, named *Pru p 1* and later identified as a Lipid Transfer Protein, only in the skin of peach fruits. In previous works, LTPs and/or specific transcripts have been detected in cotton elongating fibre [34] and epidermal layers of cutinised organs [35,36] suggesting a role for LTP in cutin transport and deposition, and epicuticular wax secretion [37]. Our findings support this hypothesis: the presence of *Pp-LTP1* transcripts has been observed in epicarp at all stages of developing fruits, but the strongest hybridisation signal appears in correspondence of the S3 stage, when fruits dramatically increase in volume reaching maturation. In apple, cuticular thickness increases during fruit growth with the highest values occurring, in different varieties, before or at harvest [38]. The slight increase of *Pp-LTP1* mRNA in epicarp following infection with *Monilia* in pre-climacteric and climacteric fruits would indicate that this gene (along with *Pp-LTP2*) is not significantly involved in defence mechanisms against this pathogen. Although the active role in vitro of LTPs against bacterial and

fungal pathogens has been proved [7,8], not all members of the LTP family are able to inhibit the growth of fungal pathogens and promoters of different LTP genes differently behave with respect to bacterial pathogens [39].

Considering the abundance and the tissue specificity of transcripts accumulating in epicarp and the results obtained by Leonart et al. [20] indicating that the allergenic activity is largely concentrated in the skin, it is likely that *Pp-LTP1* encodes for the major allergen of peach. Since the LTP family is formed by at least 4–5 genes, we can not rule out that other members are expressed in the epicarp and account for the allergenicity. Our results confirm that, in order to produce hypoallergenic peach-derived food, peeling is strictly required. The similar transcript levels detected throughout ripening suggests that the allergenic potential does not change during the last developmental stage. Moreover, preliminary results would indicate that no major difference exists in terms of *Pp-LTP1* transcript accumulation in peaches maintained, after harvest, at temperature of 4 or 25 °C (Botton et al., unpublished).

Considering the huge number of peach varieties available for breeding and cultivation, future research should attempt to identify genotypes characterised by reduced (or null) level of LTPs in epicarp: this would represent an important step toward the production and commercialisation of hypoallergenic peaches with great benefits for human health.

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