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Identification and genetic characterization of an ethylene-dependent polygalacturonase from apricot fruit

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ABSTRACT

During fruit ripening a loss of firmness occurs, which is a key factor limiting postharvest life. In apricot, *Prunus armeniaca* L., a wide range of fruit firmness at commercial maturity has been observed in different cultivars. Endopolygalacturonase (endoPG) activity has been reported to be associated with differences in firmness in many fruit species, but never in apricot. In this paper, we reported the identification of an apricot cDNA (*PaPG*) coding for an endoPG-like protein with 393 amino acids. Protein sequence comparison with known polygalacturonases (PGs) revealed that multiple features as conserved domains and functional residues and a predicted signal peptide were present in PaPG. Moreover, a phylogenetic analysis of this and other plant PGs placed PaPG into a clade containing endoPGs expressed in fruit, abscission and dehiscence zones without a propeptide sequence, very close to PRF5 from peach (*Prunus persica* L. Batsch). *PaPG* gene expression increased during postharvest storage of the fruit, correlating with fruit softening and ethylene release, and it responded to exogenous ethylene treatments. We localized the *PaPG* gene in apricot linkage group 4 after genetic mapping based on SNP analysis, in a position apparently syntenic to the *PRF5* locus from peach. Results obtained offer genetic evidence supporting the hypothesis that *PaPG* and *PRF5* are orthologous genes, and consequently position *PaPG* as a gene of interest for studies on fruit softening in apricot, and contribute to the development of molecular tools for breeding apricots with longer shelf life.

1. Introduction

Apricot (*Prunus armeniaca* L.) is a stonefruit species producing a climacteric fruit with a short shelf life. Fruit ripening involves several processes including metabolic changes, colour shift and softening, which in the main apricot cultivars causes postharvest deterioration that limits the shelf life of the fruit. The loss of firmness occurring during ripening is considered the key factor limiting this deterioration. In order to extend the postharvest period, the fruit is sometimes harvested before ripening and in spite of its climacteric nature it never reaches the optimal quality attributes for consumers. In fruit trees, the peach (*Prunus persica* L. Batsch) has been utilized as a model for climacteric fruit softening and other studies in the Rosaceae due to the considerable physiological, biochemical, genetic and genomic knowledge currently available (Abbott et al., 2002). Recently the genome sequence has become available, released by the International Peach Genome Initiative (IPGI, http://www.rosaceae.org/peach/genome).

Peach cultivars have been classified as melting and non-melting types according to the final phase of fruit ripening named the melting stage, characterized by a loss of firmness occurring more rapidly in melting types, and a higher fruit sensitivity to external damage, which consequently limits storage. The melting trait along with the adherence of the pit to the flesh (clingstone versus freestone) allows the classification of peach varieties into Freestone Melting Flesh (FMF), Clingstone Melting Flesh (CMF) and Clingstone Non Melting Flesh (CNMF). In apricot, although there are differences in firmness at maturity, fruit cannot be classified as melting versus non-melting as in peach, and no information about the genetics of adherence of the pit to the flesh is available.

Tomato has emerged as the main molecular and genetic model for ripening in fleshy climacteric fruit (Giovannoni, 2004). Studies on the biochemical cell wall changes and on the expression of cell wall-degrading enzymes in the ripening-impaired mutants *rin*, *nor* and *Nr* have indicated the involvement of polygalacturonases (PG) in ripening-associated modification of cell walls (Gross and Wallner, 1979; Bird et al., 1988; DellaPenna et al., 1989). Other secreted proteins such as pectin methylesterases,

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β-galactosidases and expansins have also been proposed to affect cell wall modification and softening (Brummell and Harpster, 2001). The phytohormone ethylene plays an essential role in triggering fruit ripening events. Thus ethylene-dependent induction of PG genes has been observed in ripe tomato and other species more closely related to apricot. In peach and nectarine, the maximum expression of endoPG coincides with the climacteric peak and the rapid fruit softening occurring during the melting stage characteristic of most fresh-market cultivars (Lester et al., 1994). Other studies in pear have demonstrated that mRNA accumulation of PG genes coincides with fruit softening events (Hiwasa et al., 2003). More recently, Sekine et al. (2006) have cloned several cDNAs coding for cell-wall hydrolases in pear fruit and showed that the PG-like gene *PcPG1* was overexpressed in both short- and long-term stored fruit. Although enzymatic activity of PG and other cell-wall hydrolases has been reported during apricot fruit storage (Botondi et al., 2003; Dinnella et al., 2006), so far, no PG genes have been identified in this species.

The inheritance of fruit firmness and softening characters has been widely studied in peach. The melting flesh trait (M) segregates in a Mendelian fashion along with the adherence of the flesh to the pit (F), both being traits controlled by a single locus (Bailey and French, 1949). Several linkage analysis approaches for the mapping of agronomic traits in peach have found one or both of *F* and *M* loci in a common region in linkage group 4 (Abbott et al., 1998; Quarta et al., 1998; Dettori et al., 2001; Dirlewanger et al., 2006; Ogundiwin et al., 2009). It has been suggested that multiple alleles of a gene coding for the cell wall-metabolizing enzyme endoPG could control these two traits (Peace et al., 2005). Lester et al. (1994) identified an endoPG-like gene from peach fruit designated *PRF5*, the expression of which was associated with texture differences. A genomic deletion in this gene was found in a non-melting flesh variety, which in addition was associated with the *M* locus in a segregating population (Lester et al., 1996). Callahan et al. (2004) described several genomic deletions affecting an endoPG gene cluster in eight different non-melting cultivars, suggesting that one of the endoPGs coded in this locus determined the differences in loss of fruit firmness among peach cultivars. Several additional studies and reviews have supported the hypothesis of the involvement of endoPG enzymes in the loss of fruit firmness in peach and other species (Fischer and Bennett, 1991; Orr and Brady, 1993; Hadfield et al., 1998; Morgutti et al., 2006). In apricot, a wide range of fruit firmness at commercial maturity has been recorded in several apricot accessions (Bassi and Audergon, 2006; Ruiz and Egea, 2008; Tricon et al., 2009), arguing for the existence of yet unknown genetic factors conditioning this trait. The isolation of genes homologous to the endoPGs described as responsible for the cell wall changes leading to the loss of fruit firmness could help to elucidate this question in apricot.

In this paper, we have identified the *PaPG* gene, an apricot orthologue of the *PRF5* gene of peach coding for an endoPG protein. *PaPG* showed differential expression during ripening, correlating with the loss of fruit firmness and production of ethylene in different cultivars. The information provided in this paper contributes to the unravelling of genetic factors involved in loss of fruit firmness in apricot and to developing molecular tools for breeding apricots with longer shelf life.

2. Materials and methods

2.1. Plant material

The 'Goldrich', 'Currot' and 'Canino' apricot cultivars, and the 'Goldrich' × 'Currot' F_1 family of 81 genotypes, cultivated in an orchard located at the Instituto Valenciano de Investigaciones Agrarias (IVIA), in Moncada (Spain), were used in this study. Fruit were harvested in spring 2009, when all fruit on the tree had completely lost their green colour. For the postharvest storage experiment, fruit from the cultivars 'Goldrich', 'Currot' and 'Canino' were maintained at 20 °C under controlled conditions.

2.2. Isolation of a full-length cDNA coding for a PG protein from fruit mesocarp

In order to identify expressed sequence tags (EST) coding for apricot PG-like proteins, a database similarity search with the BLASTN program (Altschul et al., 1990) was performed, using peach *PRF5* (X76735) as query. The primers endoPG-1 and endoPG-2 (Table 1.1) were designed to amplify the full-length cDNA of a putative PG gene from apricot, based on the ESTs found. Total RNA was isolated from 500 mg of apricot fruit mesocarp using RNeasy® Plant Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. RNA concentration was measured with the Quant-iT™ RiboGreen® RNA Assay Kit (Invitrogen, Carlsbad, CA, USA). About 500 ng of total RNA was reverse transcribed with SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) in a total volume of 20 μL. For cDNA amplification, eight independent 50 μL-PCR reactions containing 0.5 μL of cDNA each were performed, by using the primers endoPG-1 and endoPG-2. A first denaturation step at 95 °C for 5 min was followed by 35 cycles at 95 °C for 30 s, 65 °C for 30 s and 72 °C for 1 min 30 s, and a final extension at 72 °C for 7 min. The pooled PCR products were purified with the High Pure PCR Product Purification Kit (Roche, Basel, Switzerland) and resuspended in 10 mM Tris–HCl pH 8. The purified fragment was sequenced from primers endoPG-1, endoPG-2, endoPG-3, endoPG-4, endoPG-5 and endoPG-6 (Table 1.1), constituting the full-length cDNA sequence of *PaPG*. *PaPG* nucleotide sequence was introduced into the GenBank database with the accession number HQ540310.

2.3. Identification of PaPG protein domains and phylogenetic analysis

The putative signal peptide of the deduced protein sequence of *PaPG* was identified using the SMART program (Schultz et al., 1998). Conserved domains and functional amino acids were obtained from the literature. PaPG protein and 20 known PGs were aligned with ClustalW2 (Larkin et al., 2007). PG proteins employed were: PdPG1 from *Prunus domestica* (ABD33834; Iglesias-Fernández et al., 2007), PRF5 from *P. persica* (CAA54150; Lester et al., 1994), PG from *P. persica* (CAA54448; Lee et al., 1990), PC-PG1 and PC-PG2 from *Pyrus communis* (BAC22688 and BAC22689; Hiwasa et al., 2003), pGDPG-1 from *Malus domestica* (AAA74452; Atkinson, 1994), PG from *Actinidia deliciosa* (AAC14453; Atkinson and Gardner, 1993), MPG1, MPG2 and MPG3 from *Cucumis melo* (AAC26510, AAC26511 and AAC26512; Hadfield et al., 1998), TAPG1, TAPG2 and TAPG4 from *Solanum lycopersicum* (AAA80489, AAB09575 and AAB09576; Kalaitzis et al., 1997), pTOM6 from *S. lycopersicum* (CAA01720; Grierson et al., 1986), SAC66 from *Brassica napus* (CAA90272; Jenkins et al., 1996), Sta44-4 from *B. napus* (P35337; Robert et al., 1993), PGA2 from *Arabidopsis thaliana* (CAA51692; Torki et al., 1999), Npg1 from *Nicotiana tabacum* (CAA50338; Tebbutt et al., 1994), P73 from *Medicago sativa* (AAA62286; Qiu and Erickson, 1996), and P2c from *Aspergillus flavus* (AAA85279; Whitehead et al., 1995). Phylogenetic analysis was performed using programs from the PHYLIP group, PHYLogeny Inference Package (Felsenstein, 1985) in the Mobyle platform (http://mobyle.pasteur.fr/cgi-bin/portal.py). A distance matrix was computed according to the Dayhoff PAM model with Protdist and then used as input for Neighbor, selecting the Neighbor-joining method of clustering. The reliability of different phylogenetic groupings was evaluated by using the bootstrap test (1000 boot-

Table 1
Sequences of primers used.

1. Isolation of a full-length cDNA coding for a PG protein from fruit mesocarp		
endoPG-1	5′-ACGAGGCAAATGGAAAACCGTAGAAG-3′	
endoPG-2	5′-GTCCTTCGTACTGGAGGGCAAACTT-3′	
endoPG-3	5′-TGGCATCCCTAAACAGCCAAATGTTC-3′	
endoPG-4	5′-ATGCCATCGGTGTTAGGGCTGTTAC-3′	
endoPG-5	5′-CTCAGAATGGTCTAAGAATCAAGTC-3′	
endoPG-6	5′-ATTTGATCTCGCTACAAGGGTG-3′	
2. Analysis of *PaPG* gene expression in apricot fruit by real-time PCR		
ACT1	5′-CAGATCATGTTTGAGACCTTCAATGT-3′	
ACT2	5′-CATCACCAGAGTCCAGCACAAT-3′	
3. Tetra-primer ARMS-PCR for SNP detection		
SNP1		
FISNP1	5′-TGCTAGAAATATTCTTTTCCAACATACG-3′	
RISNP1	5′-AATAGGATTTTCGACATTGACCATTATA-3′	
FOSNP1	5′-CAGTTAAAACGGTTACCTTTACTGGTACT-3′	
ROSNP1	5′-AGTTGTAATTAAACTTTGGGAATTGACC-3′	
SNP2		
FISNP2	5′-TAATTACAACTAAATTTTCTCTTACACAT-3′	
RISNP2	5′-GTTTTTATCAAAAACGTTACACTGTC-3′	
FOSNP2	5′-GTACTCAGAATGGTCTAAGAATCAAG-3′	
ROSNP2	5′-CGTGAAGGAAAGAGTTATTGGATTTG-3′	

SSR marker		Reference
4. Markers added to linkage group 4 for mapping PaPG locus in an apricot genetic map		
PaCITA11	F 5′-ACGACGTTGTAAATTGGTAACATTTGTTCATCTTTTTG-3′ R 5′-GGTCAACCAGAGCATACAGGAAATG-3′	Lopes et al. (2002)
pchgms5	F 5′-ACGACGTTGTAAACCAGTAGATTTCAACGTCATCTACA-3′ R 5′-GGTTCACTCTCACATACACTCGGAG-3′	Sosinski et al. (2000)
pchcms5	F 5′-ACGACGTTGTAAACGCCCATGACAAACTTA-3′ R 5′-GTCAAGAGGTACACCAG-3′	Sosinski et al. (2000)
UDAp-404	F 5′-ACGACGTTGTAAACATGAACAGGGTCAAAAGCA-3′ R 5′-TATATCCTTACGCGGCCTCA-3′	Messina et al. (2004)
UDAp-409	F 5′-ACGACGTTGTAAATGGCCACACAAAGATGAAGA-3′ R 5′-GGTTTTGGACTGGTTGAGCA-3′	Messina et al. (2004)
UDAp-416	F 5′-ACGACGTTGTAAATTCAGACTCGAAAACACACATACA-3′ R 5′-TGGAGGAGGTTTATGAGCAA-3′	Messina et al. (2004)
UDAp-418	F 5′-ACGACGTTGTAAACAGAAATAGCCCCAGCACAT-3′ R 5′-TTCTTGCGCCAAAAACAACT-3′	Messina et al. (2004)
UDP96-003	F 5′-ACGACGTTGTAAATTGCTCAAAAGTGTCGTTGC-3′ R 5′-ACACGTAGTGCAACACTGGC-3′	Cipriani et al. (1999)
UDP97-402	F 5′-ACGACGTTGTAAATCCCATAACCAAAAAAAACACC-3′ R 5′-TGGAGAAGGGTGGGTACTTG-3′	Cipriani et al. (1999)

strap replicates). The protein encoded by *pecA* gene from *A. flavus* (P2c) was defined as the outgroup sequence.

2.4. Determination of fruit parameters

Firmness was measured using a Compact Table-Top Universal Tester EZ Test Series (EZ-L) (Shimadzu, Kyoto, Japan). Compression was measured using an 8 mm cylindrical plunger. Twenty fruit per sample were harvested and firmness measured on both sides of each fruit after removing the skin around the assayed area.

Ethylene production was measured using two fruit enclosed in a 350 mL jar, sealed with a rubber cap and kept at 20 °C and 95% RH. After storage for 20 h, the released ethylene was measured by injecting 1 mL gas headspace in a gas chromatograph TRACE GC (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a Poropak QS 80/100 (1.2 m × 0.32 cm i.d.) column and a flame ionization detector. Temperatures in the column, injector, and flame ionization detector were 75 °C, 175 °C, and 175 °C, respectively. Helium was used as carrier gas at a flow rate of 25 mL/min. Ethylene production was determined in 4 replicates, and expressed as $\mu L\, C_2H_4\, kg^{-1}\, h^{-1}$.

Effect on the firmness of the fruit after exposure to different ethylene concentrations was measured as follows: four sets of 20 fruit of 'Goldrich' apricots were stored for 48 h in temperature controlled chambers set at 20 °C, 85% RH with different ethylene concentrations (0, 2, 10 and 100 μL/L). Ethylene exposure chambers consisted of hermetic Perspex cabinets (82 cm × 62 cm × 87 cm) fitted with inlet and outlet ports through which ethylene (Linde Abelló, Valencia, Spain) passed at a rate adjusted to the desired concentration inside the chamber. Gas was allowed to escape from the outlet port through a bubble tube to maintain the proper gas mixture in the chamber. Levels of ethylene, temperature and relative humidity were continuously monitored by means of the ControlTec® system (Tecnidex, Valencia, Spain). Chambers were located inside a 40 m^3 storage room that was also set at 20 °C.

2.5. Analysis of PaPG gene expression in apricot fruit by real-time PCR

PaPG expression was measured in fruit under postharvest storage and in ethylene treated fruit. Extraction of total RNA and synthesis of cDNA was performed as described above (Section 2.2). One microliter of a 10× diluted first-strand cDNA was used for each amplification reaction in a final volume of 20 μL. Quantitative real-time PCR was performed on a StepOnePlus™ Real-Time PCR System using the Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and primers endoPG-5 and endoPG-6. Reaction composition and conditions followed the manufacturer's instructions. Cycling protocol consisted of 20 s at 95 °C, followed by 40 cycles of 3 s at 95 °C for denaturation, and 30 s at 60 °C for annealing and extension. Specificity of the PCR reaction was assessed by the presence of a single peak in the dissociation curve after the amplification and through size estimation of the amplified product by agarose electrophoresis. We used as reference

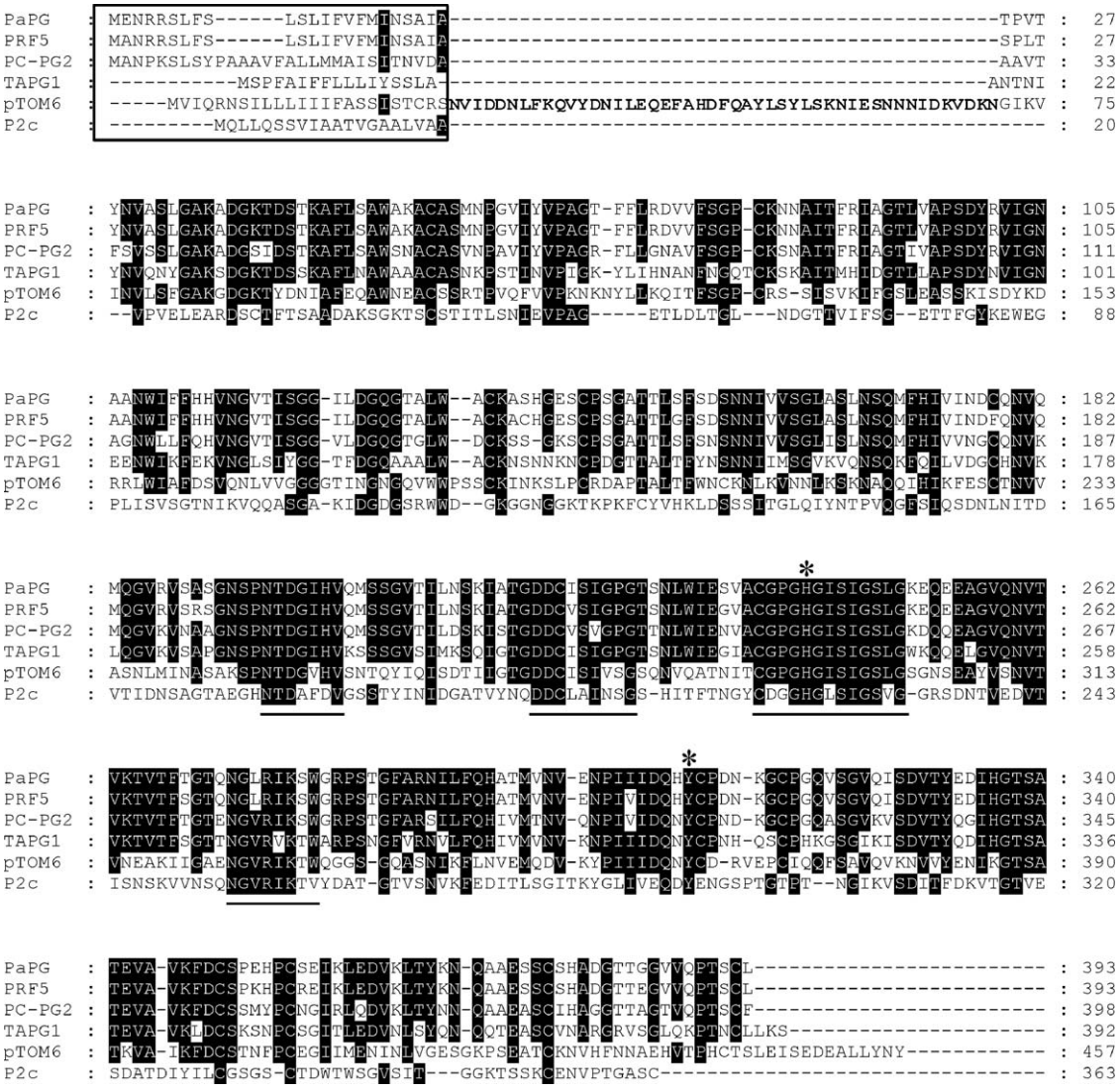

Fig. 1. Alignment of amino acid sequences from PaPG and other PG. Protein accession numbers and bibliographic references are shown in Section 2.3. Identical residues are highlighted in black boxes. The N-terminal signal peptides are framed. The acidic propeptide of tomato pTOM6 is shown in bold. Conserved domains are underlined and essential histidine and tyrosine residues are labelled with an asterisk.

an apricot actin gene (CV044868), amplified with primers ACT1 and ACT2 (Table 1.2). The comparative C_T ($\Delta\Delta C_T$) method was used to quantify *PaPG* RNA, with amplification efficiency similar to the reference actin gene. Results were the average of two independent biological replicates repeated twice.

2.6. Identification of single nucleotide polymorphisms (SNPs) in PaPG for mapping purposes

Genomic DNA was extracted from 50 mg of young leaves from 'Goldrich' and 'Currot' apricots following a CTAB-based method (Doyle and Doyle, 1987). The primers endoPG-5 and endoPG-6 (Table 1.1) were designed from the *PaPG* sequence to amplify a fragment that in the peach homologue *PRF5* includes an intron. PCRs were performed in a GeneAmp® PCR System 9700 thermal cycler (Perkin-Elmer, Waltham, MA, USA) in a final volume of 20 μL, containing 0.8 μM of each primer and 20 ng of genomic DNA. First denaturation was performed at 95 °C for 2 min, followed by 35 cycles at 95 °C for 30 s, 55 °C for 1 min and 72 °C for 1 min 30 s, and a final extension at 72 °C for 7 min. The PCR products of 5–10 separate reactions were pooled and analyzed by agarose gel electrophoresis. A unique band of about 850 bp was obtained from both parents. The PCR product was purified with the High Pure PCR Product Purification Kit (Roche, Basel, Switzerland). After sequencing the PCR fragments from endoPG-5 and endoPG-6 primers, we detected several heterozygous SNPs from both genotypes. Two of them (SNP1 and SNP2) were chosen for mapping purposes.

2.7. Tetra-primer ARMS-PCR for SNP detection

The tetra-primer ARMS-PCR method, which employs modified primers with a mismatch at position −2 from the 3′-terminus for the amplification of SNP-specific PCR fragments (Ye et al., 2001), was used to detect SNP1 and SNP2 polymorphisms in the 'Goldrich' × 'Currot' population of 81 individuals. Primer design was performed using the online tool made available by the authors (URL http://cedar.genetics.soton.ac.uk/public_html/primer1.html). Primers obtained for SNP1 were FISNP1, RISNP1, FOSNP1 and ROSNP1 (Table 1.3), which amplified a common fragment of 231 bp and two specific fragments of 156 bp (G allele) and/or 131 bp (T allele). For SNP2 detection we used primers FISNP2, RISNP2, FOSNP2 and ROSNP2 (Table 1.3). In this case, a common product of 449 bp was obtained and shorter fragments of 198 bp and 250 bp were due to the T and the G alleles, respectively. Amplification

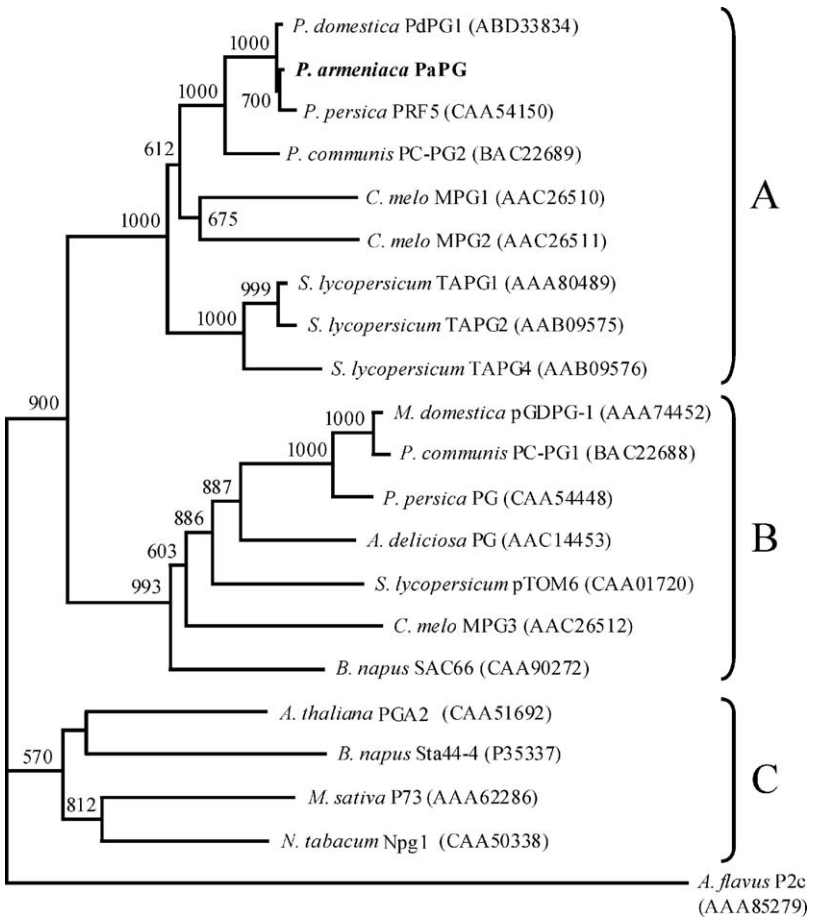

Fig. 2. Phylogram showing the distance between PaPG and 20 PG known sequences. Accession numbers and the species of origin are indicated. The proteins were aligned using ClustalW2 and analyzed as described in Section 2.3. Apricot PaPG protein is highlighted in bold. Bootstrap values higher than 500 (of 1000 samples) are shown for each node.

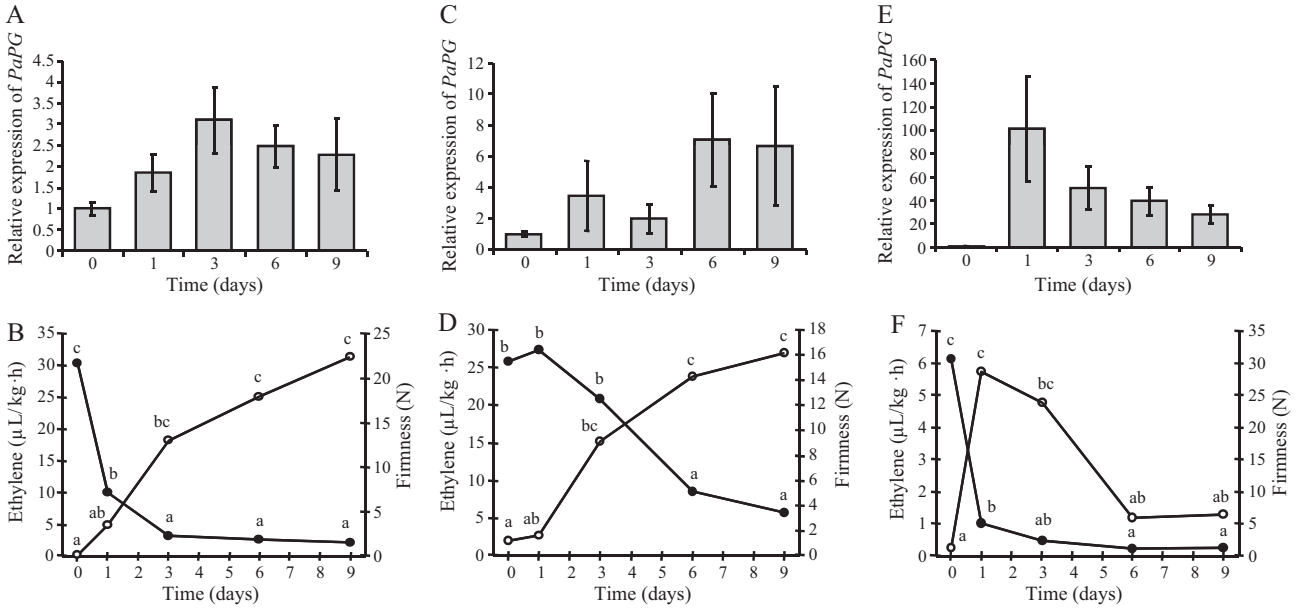

Fig. 3. Time course of *PaPG* transcript levels, ethylene production and firmness during postharvest storage. The relative expression of *PaPG* was measured at different times after harvest in fruit mesocarp from 'Goldrich' (A), 'Currot' (C) and 'Canino' (E) cultivars. The corresponding fruit ethylene production (empty circles) and firmness (filled circles) are shown for 'Goldrich' (B), 'Currot' (D) and 'Canino' (F) cultivars. Real-time PCR expression values are relative to actin. An expression value of 1 is assigned to the starting sample. Data are means from two biological replicates repeated twice, with error bars representing standard deviations. Ethylene or firmness measurements with the same letter are not significantly different ($P < 0.05$) according to Duncan's multiple range test.

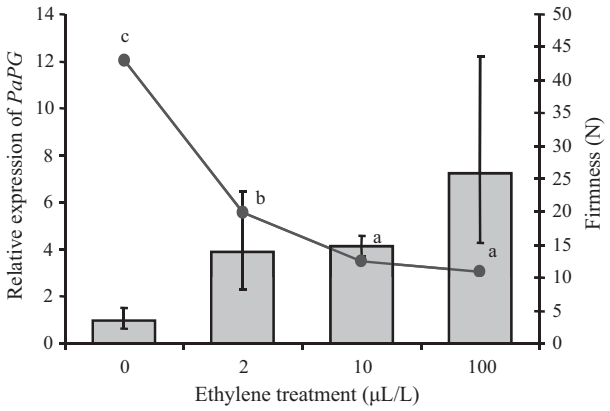

Fig. 4. Effect of exogenous ethylene on *PaPG* expression and fruit firmness. The expression level of *PaPG* (bars) and the fruit firmness (filled circles) were measured after ethylene treatments at different concentrations (0, 2, 10 and 100 μL/L) for 48 h. Real-time PCR expression values and error bars were determined as in Fig. 3. Measurements with the same letter are not significantly different ($P < 0.05$) according to Duncan's multiple range test.

was performed for each of the 81 samples, in a total volume of 10 μL containing 10 μM of each inner primer (FISNP1/RISNP1 or FISNP2/RISNP2), 1 μM of each outer primer (FOSNP1/ROSNP1 or FOSNP2/ROSNP2) and 30 ng of genomic DNA. An initial denaturation at 95 °C for 2 min was followed by 35 cycles at 95 °C for 1 min, an annealing temperature ('Ta') for 1 min, 72 °C for 1 min and a final extension at 72 °C for 2 min. The 'Ta' was 72 °C in the first cycle and decreased 1 °C per cycle until 65 °C and 60 °C for SNP1 and SNP2, respectively. 'Ta' was fixed at this final value until the end of the run. PCR products were separated by 4% (w/v) agarose gel electrophoresis.

2.8. Localization of PaPG SNP alleles and additional simple sequence repeat (SSR) markers on a genetic map

In addition to SNP1 and SNP2 markers combined to localize *PaPG* gene, several SSR markers were added to the published genetic map of 'Goldrich' × 'Currot' population (Soriano et al., 2008). These additional SSRs were PaCITA11 (Lopes et al., 2002), pchgms5 and pchcms5 (Sosinski et al., 2000), UDAp-409 (Messina et al., 2004), UDP96-003 and UDP97-402 (Cipriani et al., 1999). Other three SSR present in the published map (UDAp-404, UDAp-416 and UDAp-418) were screened in this study. The primers employed are described in Table 1.4. PCR amplification of SSR followed Schuelke (2000), using three primers: the specific forward primer of each SSR with M13 (−21) tail at its 5′ end (0.4 μM final concentration), the sequence-specific reverse primer (0.8 μM), and the different fluorescent-labelled M13 (−21) primer (0.4 μM). Fluorescent dye labels used were 6-FAM, VIC, PET and NED (Applied Biosystems, Foster City, CA, USA). Reactions were performed in a final volume of 20 μL, containing 20 ng of genomic DNA, under the following conditions: 94 °C for 2 min, then 35 cycles at 94 °C for 30 s, the optimised

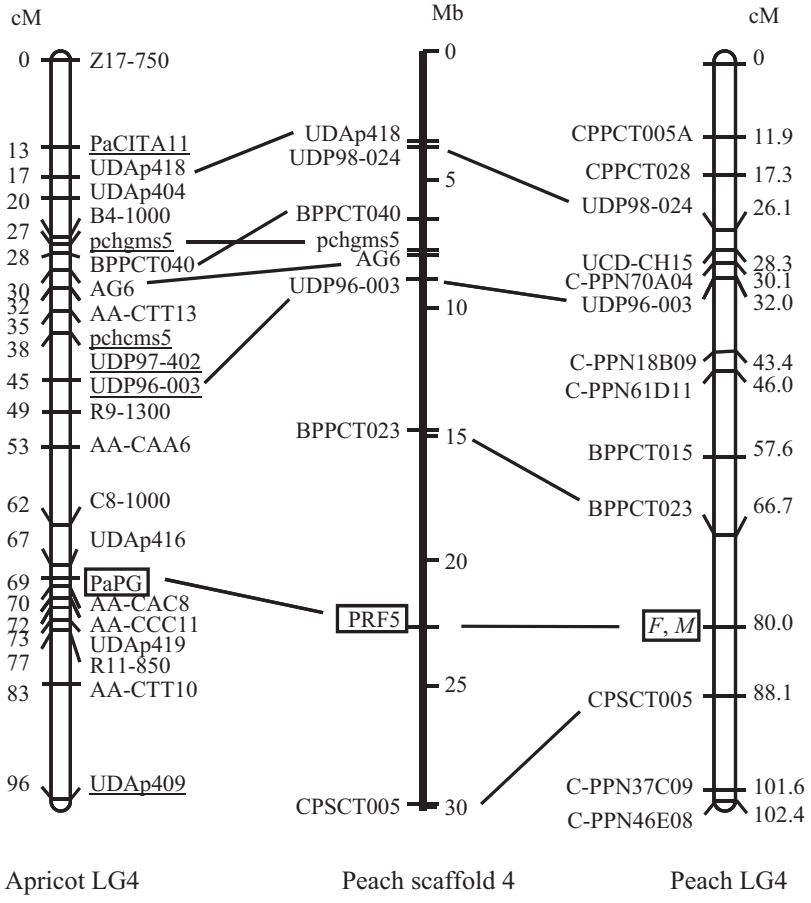

Fig. 5. Genetic mapping of *PaPG* gene. On the left, SNP1 and SNP2 markers were utilized to localize *PaPG* gene on linkage group 4 of 'Goldrich' map from the 'Goldrich' × 'Currot' population (Soriano et al., 2008). The underlined markers have been introduced by this work. On the center, the peach scaffold 4 is represented, showing the position of several molecular markers (obtained from http://www.phytozome.net/cgi-bin/gbrowse/peach/) and *PRF5* gene identified by BLASTN analysis (http://www.phytozome.net/search.php?show=blast&method=Org_Ppersica). On the right, linkage group 4 from peach genetic map showing *F* and *M* loci, elaborated from published data (Ogundiwin et al., 2009). PaPG marker, *PRF5* gene, and *F* and *M* loci are surrounded. Genetic distance is expressed in cM units and physical distance in Mb.

annealing temperature of primer for 1 min and 72 °C for 1.5 min, with a final step at 72 °C for 7 min. Allele specific fragments were identified using an ABI Prism 3130 Genetic Analyzer with the aid of GeneMapper® software, version 4.0 (Applied Biosystems, Foster City, CA, USA).

The endoPG from apricot was mapped using SNP1 and SNP2 combined in a single marker named PaPG. The SSR markers from Table 1.4 were added to the 'Goldrich' × 'Currot' map obtained by Soriano et al. (2008). Mapping of markers was made using the JoinMap® 3.0 software (Van Ooijen and Voorrips, 2001) and the Kosambi mapping function (Kosambi, 1944). Linkage groups were established using as threshold a minimum logarithm of odds (LOD) of 4.0 and recombination frequency lower than 0.4.

3. Results and discussion

3.1. Identification of an orthologue of endoPG gene in apricot

Two overlapping expressed sequence tags (EST) from apricot (accession numbers CV049381 and CB823599) were identified by an *in silico* search of DNA fragments similar to *PRF5* (accession number X76735; Lester et al., 1994). In order to check if both sequences were part of the same transcript in apricot, RNA isolated from ripe fruit was transcribed to cDNA and PCR-amplified with specific primers located on or close to the deduced start and stop codons. A PCR product of 1258 bp was sequenced. The sequence (accession number HQ540310) had only six nucleotide changes with respect to the fused sequence of CV049381 plus CB823599, confirming that both ESTs are partial fragments of the same cDNA. The deduced protein, named PaPG for *Prunus armeniaca* PG, was highly similar to the complete endoPG protein from peach codified by *PRF5* gene, suggesting that the amplified fragment included a complete PG from apricot. An alignment of PaPG with described endoPGs showed that four known conserved domains, and two histidine and tyrosine residues previously described as essential for the enzymatic activity of fungal endoPGs (Caprari et al., 1996; Stratilova et al., 1996) were present in the PaPG protein (Fig. 1). In addition, a 23 residue long signal peptide nearly identical to that of PRF5 was identified in the N-terminal end of PaPG (Fig. 1). Thus, different protein sequence features found in PaPG, including conserved domains and single functional amino acids, supported a putative PG activity for PaPG, which could be consequently involved in the modification of cell wall architecture by hydrolyzing pectin polymers. However PG activity has been found associated to multiple physiological processes requiring cell wall modification as rapid vegetative growth, organ abscission, anther dehiscence, pollen grain maturation and fruit ripening, among others (Hadfield and Bennett, 1998). In order to postulate the involvement of PaPG in one or some of these processes, a more detailed comparison with PGs described at the functional level is required.

A phylogenetic tree was generated from the alignment of PaPG and 20 known PG proteins (Fig. 2). The compared proteins were clustered into three major clades, which accurately reproduced previous studies (Hadfield et al., 1998; Hadfield and Bennett, 1998). Clades A and B contained mostly endoPGs expressed in fruit, abscission and dehiscence zones, whereas clade C grouped exopolygalacturonases (exoPG) that are predominantly expressed in pollen and anthers. Proteins from cluster B are characterized by an acidic propeptide adjacent to the N-terminal signal peptide which is absent in proteins from cluster A, as observed in the tomato pTOM6 endoPG outlined in Fig. 1 (DellaPenna and Bennett, 1988). PaPG appeared in clade A, close to the PGs from the genus *Prunus* PdPG1 (*P. domestica*) and PRF5 (*P. persica*).

PaPG grouped with fruit-ripening related endoPGs, and more specifically PRF5 that has been extensively characterized at the genetic level. The peach *PRF5* gene has been associated with the *melting flesh* (*M*) and *freestone* (*F*) loci controlling the firmness of the ripe fruit and stone adhesion, respectively (Lester et al., 1994, 1996; Callahan et al., 2004; Peace et al., 2005; Morgutti et al., 2006). *PRF5* was not only related to cultivar-specific differences in fruit firmness at harvest time, but interestingly also increased during postharvest storage of the fruit, coinciding with a peak of ethylene production (Lester et al., 1994). *PdPG1* was not investigated during postharvest storage, but had a higher expression during the fruit ripening stage, similar to other plant PGs (Iglesias-Fernández et al., 2007). Thus, based on sequence analysis, PaPG was related to endoPGs involved in fruit softening during maturation and postharvest processes.

3.2. Analysis of PaPG gene expression in apricot fruit

PpPG gene expression was measured in fruit mesocarp from the apricot cultivars 'Goldrich', 'Currot' and 'Canino' during postharvest storage. As shown in Fig. 3A, *PaPG* transcript was present in the mesocarp of recently harvested (zero time of experiment) 'Goldrich' fruit and accumulated during the following days, with a maximum 3-fold increase after three days. Concomitantly with *PaPG* accumulation, fruit firmness decreased and ethylene production increased. After three days, coinciding with the expression peak of *PaPG*, firmness was close to its final lowest value and ethylene production drastically increased (Fig. 3B). *PaPG* followed a similar expression pattern in other cultivars, but with different timing. 'Currot' fruit reached the highest accumulation of *PaPG* after 6 days (Fig. 3C), whereas 'Canino' fruit required only one day for maximum expression and experienced a marked decrease the following days (Fig. 3E). Interestingly, the ethylene burst and firmness loss were delayed in 'Currot' and accelerated in 'Canino' fruit (Fig. 3D and F), mostly coinciding with *PaPG* transcript estimation by quantitative PCR. These results suggest a temporal correlation between the transcriptional activation of *PaPG* gene and the softening of over-ripe fruit triggered by ethylene, as previously found for several other PGs (Lester et al., 1994; Hiwasa et al., 2003). This correlation was observed in the 3 varieties studied although they differed in their postharvest behaviour.

Ethylene has a relevant role in the ripening processes of climacteric fruit, as deduced from its early synthesis and the ripening effect of exogenous applications of this hormone. Moreover ethylene has been shown to regulate PG expression in different species (Sitrit and Bennett, 1998; de Fátima Rosas-Cárdenas et al., 2007; Iglesias-Fernández et al., 2007). In order to test the ethylene-dependent response of *PaPG*, harvested apricot fruit were treated with different ethylene concentrations for 48 h. Exogenous concentrations of ethylene as low as 2 μL/L induced *PaPG* expression significantly, but higher concentrations caused higher transcript accumulation, up to 7-fold induction under 100 μL/L ethylene (Fig. 4). Fruit firmness was inversely correlated with ethylene exposure and *PaPG* expression, as previously observed in the time-course experiment. These results confirm that *PaPG* expression is induced by ethylene and thus PaPG activity could at least partially mediate ethylene-dependent softening of apricot fruit under postharvest storage.

3.3. Genetic mapping of PaPG gene

Two specific primers were designed according to the cDNA sequence of *PaPG*, which should flank a hypothetical intron in case of homologous conservation of intron positions of the peach counterpart *PRF5*. A PCR fragment of 840 bp containing a 573 bp long intron was obtained. Sequence analysis of this fragment in 'Goldrich' and 'Currot' apricots allowed the identification of heterozygous SNPs, which were employed for positioning the *PaPG* gene in a previous genetic map of apricot based on a

'Goldrich' × 'Currot' mapping population (Hurtado et al., 2002; Soriano et al., 2008). A graphic representation of two selected SNPs is shown in Supplementary Fig. 1.

Both, SNP1 and SNP2, were fused in one single marker to properly characterize the parental origin of the two *PaPG* alleles of each individual from the population. The resulting PaPG and additional SSR markers, underlined in Fig. 5 (Cipriani et al., 1999; Sosinski et al., 2000; Lopes et al., 2002; Messina et al., 2004), were subsequently located on the 'Goldrich' × 'Currot' map. Thus, *PaPG* gene was localized on linkage group 4 (Fig. 5), coinciding with the position of *PRF5* in peach chromosome 4, as determined by BLASTN analysis of the peach genome using X76735 sequence as query (http://www.phytozome.net/search.php?show=blast&method=Org_Ppersica), and colocalizing with the *F* and *M* loci as reported in the peach linkage map (Ogundiwin et al., 2009). This result offers genetic evidence supporting the hypothesis that *PaPG* and *PRF5* are orthologous genes, and consequently confers to *PaPG* gene a relevant interest for studies on fruit softening of apricot during postharvest storage.

4. Conclusions

PaPG gene coding for an endoPG-like protein from apricot was considered an orthologue of *PRF5* from peach according to several facts: the phylogenetic analysis of the protein sequence, the specific pattern of *PaPG* gene expression, and finally the syntenic localization of both apricot and peach genes on genetic linkage maps from both species. Relevantly, *PaPG* expression accurately correlated with firmness loss and ethylene production in fruit from three apricot cultivars. To our knowledge, these are the first experimental results associating the expression of a known gene with the natural variability of the fruit firmness trait in apricot. The well documented genetic association of *PaPG* orthologous gene *PRF5* with fruit softening in peach, and the postharvest expression experiments shown here have led to the conclusion that *PaPG* is the most obvious candidate gene described up to now for fruit softening in apricot. The identification and initial characterization of *PaPG* in apricot is a first step in developing genomic tools for breeding apricots with better postharvest response.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.postharvbio.2011.04.003.

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