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Polygalacturonase activity and expression of related genes during ripening of strawberry cultivars with contrasting fruit firmness

Natalia M. Villarreal, Hernán G. Rosli, Gustavo A. Martínez, P. Marcos Civello *

IIB-INTECH (Instituto de Investigaciones Biotecnológicas-Instituto Tecnológico de Chascomús) (CONICET-UNSAM), Camino de Circunvalación Laguna, Km 6. (B7130IWA) Chascomús, Argentina

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Abstract

Fleshy fruits soften during ripening mainly as a consequence of the catabolism of cell wall components. In strawberry (*Fragaria* × *ananassa* Duch), the depolymerization and solubilization of pectins increase during ripening and contribute to fruit softening. In the present paper, we report the cloning and expression analysis of two polygalacturonase (PG) putative cDNAs: *FaPG1* and *T-PG*. The former seems to be the same sequence of previously reported PG in strawberry, while *T-PG* cDNA has a deletion of 85 bp that cause a frame shift and would produce an inactive protein. Measurement of total PG activity and expression of *FaPG1* and *T-PG* were performed in strawberry cultivars with contrasting softening rates. The softest cultivar (Toyonaka) showed the higher total PG activity in all ripening stages analyzed. The analysis by RT-PCR revealed that both genes express in the three cultivars, though the expression pattern was different. In the firmer cultivars (Selva and Camarosa) the expression of *T-PG* was considerably higher than the expression of *FaPG1*, while the opposite occurred in the softest cultivar (Toyonaka). Therefore, the higher PG activity detected in Toyonaka correlates with the enhanced expression of *FaPG1* gene, while the low PG activity found in the firm cultivars correlates with a higher expression of *T-PG*, a gene that could encode a truncated protein without PG activity. The influence of auxins on both the expression of *PG* genes and the total PG activity during strawberry fruit ripening was also analyzed.

Keywords: Cell wall; *Fragaria*; Pectin; PG; Softening

1. Introduction

The plant cell wall is a dynamic structure that shows many changes throughout differentiation and development of different organs. The softening that takes place during ripening of fleshy fruits has been largely associated to degradation of different cell wall components (Brummell and Harpster, 2001). The disassembly of cell wall facilitates the pathogen attacks and increases the susceptibility to decay. Studies made mainly in climacteric fruits have shown that modification of cell wall polymers is a consequence of the coordinated action of cell wall-modifying enzymes and proteins such as polygalacturonase (PG), pectate lyase, pectin methylesterase, β-galactosidase, α-L-arabinofuranosidase, endo-(1,4)-β-D-glucanase, β-xyloxidase, expansin, xyloglucan endotrasglucosylase and endo-mannanase (Brummell and Harpster, 2001). The role of an individual cell wall modifying enzyme has been analyzed in different fruits by using genetic engineering. In tomato, suppression of PG activity rendered fruit with altered pectin metabolism but similar softening in relation to controls (Smith et al., 1988) and overexpression of PG gene in *rin* tomato fruit caused the solubilization of cell wall polyuronides but did not affect fruit softening (Giovannoni et al., 1989). On the contrary, reduction or increment of expansin abundance early in ripening decreased or increased, respectively, fruit softening (Brummell et al., 1999). In strawberry, fruit with antisense suppression of a putative pectate lyase gene showed a significant reduction of pectin solubilization and softening (Jiménez-Bermúdez et al., 2002). Differently, strawberry lines with down-regulated expression of *cel1*, which encodes for an endo-β-1,4-glucanase, showed no appreciable reduction of ripening-related fruit softening (Wolley et al., 2001). As the modification pattern of cell wall polymers can vary among species, the set of genes determinant of fruit softening could be different in different plant species.

The high softening rate of strawberry, a non-climacteric fruit, contributes to its fast postharvest decay. The biochemical basis of cell wall degradation in strawberry has not been clearly established yet and contradictory results have been reported

* Corresponding author. Tel.: +54 2241 424049; fax: +54 2241 424048.
E-mail address: civello@intech.gov.ar (P.M. Civello).

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in different cultivars. In the case of hemicellulose metabolism, an increased cellulase activity (Abeles and Takeda, 1990) and expression of endo-β-1,4-glucanase genes (Llop-Tous et al., 1999) were detected during ripening. However, depolymerization of hemicelluloses was found in Dover cultivar (Huber, 1984) but it was absent or very low in Camarosa and Pajaro (Rosli et al., 2004). On the other hand, the percentage of water-soluble pectins increases during strawberry ripening (Huber, 1984; Rosli et al., 2004), but the magnitude of pectin depolymerization depends on the cultivar analyzed (Rosli et al., 2004). In the case of PG, data reported have been quite contradictory. The existence of the enzyme in strawberry has been called in doubt for long time. The first studies performed in Dover cultivar did not detect PG activity (Huber, 1984), but three isoenzymes were found later in Toyonaka (Nogata et al., 1993). Three polygalacturonase-related genes have been cloned: *spG* (Redondo-Nevado et al., 2001), *D15* and *B4* (Salentijn et al., 2003). Two of these clones, *spG* and *D15*, correspond apparently to the same gene (Salentijn et al., 2003), but contradictory data about its expression have been reported.

The objective of this work was to isolate PG cDNAs from strawberry and to characterize their expression and the PG enzyme activity in cultivars with contrasting firmness, as an approach to find out the possible role of PG in strawberry softening. The influence of auxins on gene expression and PG activity was also discussed.

2. Materials and methods

2.1. Plant material

Strawberries (*Fragaria* × *ananassa* Duch.) were obtained from local producers (La Plata, Buenos Aires Province, Argentina). Three cultivars were selected by their different fruit firmness: Selva, Camarosa and Toyonaka. Fruit were harvested at different ripening stages according to the external coloration degree: large green (LG), white (W), 25% red (25% R), 50% red (50% R), 75% red (75% R) and 100% red (100% R). Samples were washed, drained and, after removing the calyx and peduncle, frozen in liquid nitrogen and stored at −80 °C until used.

2.2. Fruit firmness

The firmness was measured using a Texture Analyzer (TA.XT2, Stable Micro Systems Texture Technologies, Scarsdale NY) fitted with a 3 mm flat probe. Each fruit was penetrated 7 mm at a rate of 0.5 mm s^{-1} and the maximum force developed during the test was recorded. Thirty fruit of each cultivar at each ripening stage were assayed, and each fruit was measured twice in opposite sides of the equatorial zone.

2.3. cDNA library screening

A cDNA library constructed from ripe strawberry fruit cv. Chandler (Civello et al., 1999) was screened by using a tomato PG probe prepared from pTOM6 clone. For screening, 4.2×10^5 pfu were plated and plaque lifts were performed with Hybond-N+ nylon membranes (Amersham-Pharmacia). The membranes were fixed at 80 °C for 2 h and cross-linked with an UV-Stratalinker Model 1800 (Stratagene), and then were prehybridized at 42 °C for 4 h in a hybridization solution (50% (v/v) formamide, 6× SSPE, 5× Denhart's solution, 0.15 μg L^{-1} denatured salmon sperm DNA and 0.5% (w/v) SDS). The solution was replaced by another fresh aliquot, the labelled probe 1 was added and the membranes were hybridized at 42 °C overnight. The hybridized membranes were washed once at 42 °C for 30 min and twice at 50 °C for 30 min in 25 mL of 1× SSC with 0.1% (w/v) SDS. The blot was exposed to X-ray film (X-OMAT AR, Kodak) with an intensifying screen at −80 °C, and the film was developed according to manufacturers' recommendation. Positive plaques were carried through two additional rounds of screening for purification. After this, phagemid DNA was excised and the clones were sequenced.

2.4. Cloning of T-PG

The longest PG putative clone obtained in the library screening was not complete, but truncated in the 5′ end. Amplification of the 5′ end was performed using the BD Smart™ RACE cDNA Amplification Kit (Clontech), with a reverse gene-specific primer 3*T-PG* (5′CATGACCTGGTCCACAACTTAC3′) and the Universal Primer A Mix (Clontech). Products of PCR were analyzed by electrophoresis in agarose gels and bands of interest were purified from the gel with GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences), cloned into pGEM-T Easy Vector (Promega) and sequenced.

2.5. Nucleotide sequencing and analysis

DNA was completely sequenced using T3, T7, SP6 and internal primers. A sequencer Perkin-Elmer Applied Biosystems 377 with a 3700 and 3100 capillary column was used (HHMI Biopolymer and W.M. Keck Biotechnology Resource Laboratory, Yale University). The sequencing reactions utilized fluorescently labelled dideoxynucleotides (Big Dye Terminators) and Taq FS DNA polymerase in a thermal cycling protocol. Sequence analyses were carried out using the EditSeq and Megalign programs included in the DNASTAR 4.05 software package. Nucleotide and amino acid deduced sequences were compared with the GenBank database using the BLAST program (Altschul et al., 1997).

2.6. RNA isolation and reverse transcription

Total RNA was isolated from frozen fruit using the hot borate method (Wan and Wilkins, 1994). First strand of cDNA was obtained by using the following mixture: total RNA 1 μg, dNTPs 0.03 mmol L^{-1}, 1 μL Moloney murine leukemia virus RT (200 U/μL; Promega), 5 μL 5× reaction buffer (250 mmol L^{-1} Tris–HCl, 375 mmol L^{-1} KCl, 15 mmol L^{-1} $MgCl_2$, 50 mmol L^{-1} DTT, pH 8.3), 330 pmol of random primers (Biodynamics S.R.L., Buenos Aires, Argentina) and dis-

tilled water up to a total volume of 25 μL. The reaction mixture was incubated at 38 °C for 1.5 h.

2.7. Semiquantitative RT-PCR

PCR amplification was done with 2.4 μL of reverse transcription reaction as template, 4 μL of 10× buffer Taq polymerase (500 mmol L^{-1} KCl; 200 mmol L^{-1} Tris–HCl; pH 8.4), 0.05 mmol L^{-1} dNTPs, 1.5 mmol L^{-1} $MgCl_2$, 1 U Taq polymerase (Invitrogen), 12.5 pmol of primers 5*T-PG* (5′ATACTGCAGGCGCCACCAATTG3′) and 3*T-PG* (5′CATGA-CCTGGTCCACAACTTAC3′) corresponding to PGs, or Rib5 (5′ACCGTAGTAATTCTAGAGCT3′) and Rib3 (5′CCACTATCCTACCATCGA-AA3′) corresponding to 18S rRNA from strawberry, in a total volume of 40 μL. The amplification conditions for ribosomal primers were 15, 20, 25 and 30 cycles (94 °C, 1 min; 66 °C, 1 min and 72 °C, 1 min) and a final elongation at 72 °C for 7 min. In the case of PG, the amplification was performed under the same conditions, except for the numbers of cycles that were 18, 22, 26 and 30.

2.8. Southern blotting

Products of semiquantitative RT-PCR were analyzed by electrophoresis in agarose gels 1.2% (w/v) for 18S rRNA and 2.0% (w/v) for PGs. After running, gels were incubated twice in a denaturing solution of 0.5 mol L^{-1} NaOH, 1.5 mol L^{-1} NaCl during 15 min, rinsed with distilled water and incubated twice in a neutralization solution of 0.5 mol L^{-1} Tris–HCl, 3 mol L^{-1} NaCl during 15 min. DNA was then transferred to a Hybond-N+ nylon membrane (Amersham-Pharmacia Biotech UK, Buckinghamshire, UK), and cross-linked with an UV-Stratalinker Model 1800 (Stratagene). Membranes were prehybridized with 25 mL of hybridization solution at 42 °C for 4 h and then hybridized overnight at 42 °C with the denatured radiolabelled probe 3 or probe 4. The membranes were washed twice at 42 °C and once at 50 °C for 30 min in 25 mL of 1× SSC with 0.1% (w/v) SDS. The blot was exposed to X-ray film (X-OMAT AR, Kodak) with an intensifying screen at −80 °C. Once determined the zone of lineal amplification, the bands corresponding to amplification products of 18S rRNA (25 cycles) from each ripening stage of a same cultivar were analyzed by densitometry (GEL PRO ANALIZER VERSION 3.0) and the ratio among them was calculated. This made possible to calculate the PCR mix volumes necessary to equalize rRNA quantities among samples. The appropriate volumes of PCR reaction mix corresponding to PG amplification (26 cycles) were loaded in an agarose gel (2%, w/v) and electrophoresed. The gels were transferred as described above, and the blots were hybridized with PG probe 3.

2.9. Northern blotting

Total RNA (10 μg) was resolved by electrophoresis on 2% (w/v) formaldehyde denaturing agarose gel. To ensure that equal amounts of RNA per lane were loaded, gels were stained with ethidium bromide and individual lanes were evaluated for comparable fluorescence levels upon exposure to a UV light source. After running, the RNA was transferred to Hybond-N+ nylon membranes, hybridized with probe 2 and washed as described above in Southern blotting.

2.10. Probe preparation

Probe 1 was prepared from a PG cDNA clone from tomato fruit (pTOM6; Accession number A24194). This clone was cut with HindIII and the fragment of approximately 740 bp was purified and used as a template in a random primer labelling reaction using [^{32}P]dATP. This probe was used for the screening of the strawberry cDNA library. The search allowed cloning a cDNA fragment of 1008 bp, which showed homology with PG. This fragment (probe 2, Accession number DQ458990) was purified and labelled and then it was used in Northern experiments.

Probe 3 (Accession number DQ458991) was prepared from a PCR reaction using 1.5 μL of reverse transcription mixture as template. Reaction was done with 12.5 pmol of primers 5*T-PG* and 3*T-PG*, 2.5 μL 10× Taq polymerase buffer (500 mmol L^{-1} KCl; 200 mmol L^{-1} Tris–HCl; pH 8.4), 0.05 mmol L^{-1} dNTPs, 1.5 mmol L^{-1} $MgCl_2$, 1 U Taq polymerase (Invitrogen) and distilled water up to 25 μL. The conditions of amplification were: 94 °C, 3 min; 35 cycles (94 °C, 1 min; 66 °C, 1 min; 72 °C, 1 min) and a final elongation at 72 °C for 7 min. A fragment of 375 bp was gel purified using the GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences) and the product was used as template in a random primer labelling reaction using [^{32}P]dATP. This probe was used for Southern blotting experiments.

Probe 4 (Accession number X15590) was prepared in a similar way to probe 3 except that primers were Rib5 and Rib3 (corresponding to 18S rRNA from strawberry). This probe was used for Southern blotting experiments.

2.11. PG activity

The methodology to measure PG activity was adapted from Nogata et al. (1993). Frozen strawberries (10 g) were homogenized in an Omnimixer with 30 mL of the following buffer: 0.05 mol L^{-1} sodium acetate/acetic acid, 1% (w/v) PVPP, pH 6.0. The mixture was centrifuged at 12,000 × *g* for 30 min and the supernatant was discarded. The pellet was washed twice with 30 mL of buffer A (0.05 M sodium acetate/acetic acid pH 6.0). Then, the sample was centrifuged at 12,000 × *g* for 30 min, the supernatant was discarded and the pellet was extracted with 30 mL of buffer A containing 1 mol L^{-1} NaCl. The mixture was stirred for 2 h and then centrifuged at 12,000 × *g* for 30 min. The supernatant was dialyzed overnight with buffer A. All the steps were done at 4 °C. The dialyzed extract was used to determine PG activity, using polygalacturonic acid as substrate. A volume of 700 μL of enzymatic extract was incubated at 37 °C with 700 μL of 0.3% (w/v) polygalacturonic acid in buffer A. Three hundred microliters were taken at 0, 6, 12 and 24 h from each reaction mixture. In the control reactions, 700 μL of buffer A was added instead of enzymatic extract. The amount of galacturonic acid released was determined with 2-cyanoacetamide

(Gross, 1982), and the PG activity was expressed as nanomol of galacturonic acid released per second and per kilogram of fruit. Three independent extracts were prepared from each condition analyzed (ripening stage or auxin treatment), and the PG activity of each extract was measured twice.

2.12. Auxin treatment

The auxin treatment was performed on fruit halves of Selva cultivar, maintaining the other halves as control. Sixteen fruit at the white stage were used in each experiment. In a set of experiments, the achenes were removed from one-half of each fruit using sharp tweezers, maintaining the other half as control. In other experiments, a lanolin paste containing 1 mmol L^{-1} naphthalene acetic acid (NAA) and 1% (v/v) dimethyl sulfoxide was applied over one-half of the fruit surface, while a similar paste without NAA was applied over the other half as control. The peduncle of each fruit was immersed in a micro-centrifuge tube containing distilled water to avoid dehydration, and then the fruit were held at 20 °C for 3 d. After the treatment, the calyx and peduncle were removed and the treated and control halves were cut apart, frozen in liquid nitrogen and stored at −80 °C until use. The anthocyanin content, PG activity and PG gene expression were analyzed. The entire experiment was repeated twice.

2.13. Anthocyanins

Frozen fruit (10 g) were ground with mortar and pestle in presence of liquid nitrogen. Approximately 0.3 g of the resultant powder was poured into 3 mL of HCl–methanol 1% (v/v) and held at 0 °C for 10 min. The slurry was centrifuged at 1500 × *g* at 4 °C for 10 min, the supernatant was saved and its absorbance at 515 nm was measured. The amount of anthocyanins was expressed as micromol of pelargonidine-3-glucoside per kilogram of fruit, by using $E_{molar} = 3.6 \times 10^6$ mol L^{-1} m^{-1} (Woodward, 1972). Three replicates were done per each sample (auxin treatment) analyzed.

2.14. Sequence analysis and phylogeny

The deduced protein sequences were aligned using the CLUSTAL W version 1.8 software (Thompsom et al., 1994). For the construction of consensus trees, the alignments were analyzed using the Maximun Parsimomy (MP) method from the TNT program (Tree Analysis Using New Technology. Version 1.0. P. Goloboff, J.S. Farris and K. Nixon) with random sequence addition. Three independent runs were performed with the MP method. The support for phylogenetic clades was assessed by bootstrap analysis using a data set of 1000 replicates.

2.15. Accession numbers

The GenBank accession numbers of the sequences used in this study are as follows: Alfalfa (*Medicago sativa*, Q40312), Cotton (*Gossypium hirsutum*, Q39786), Oenothera (*Oenothera organensis*, P24548), Tobacco (*Nicotiana tabacum*, Q05967), Maize (*Zea mays*, P26216), Pga2,5 (*Arabidopsis thaliana*, O48729), FaPG1 (*Fragaria* × *ananassa*, DQ458990), Pga1,5 (*A. thaliana*, 3212847), Pga1,4 (*A. thaliana*, O22816), Pga1,2 (*A. thaliana*, O22818), MPG2 (*Cucumis melo*, AF062466), PRF5 (*C. melo*, P48979), Pga1,1 (*A. thaliana*, O22817), ***TAPG1 (*Solanum lycopersicum*, Q40135), TAPG2 (*S. lycopersicum*, Q96487), TAPG3 (*S. lycopersicum*, O22310), TAPG4 (*S. lycopersicum*, Q96488), TAPG5 (*S. lycopersicum*, O22313), Peach-gen (*Prunus persica*, Q43063), pGDPG (*Malus* × *domestica*, P48978), Kiwi (*Actinidia chinensis*, P35336), Avocado2 (*Persea americana*, Q02096), pAVOpg (*P. americana*, L06094), TPG2 (*S. lycopersicum*, P05117), B. napus (*Brassica napus*, Q42399), Pga1,6 (*A. thaliana*, O23147), Pga1,7 (*A. thaliana*, O22935), cedar (*Cryptomeria japonica*, P43212), D15 (*Fragaria* × *ananassa*, AY282613), B4 (*Fragaria* × *ananassa*, AY280662), spG (*Fragaria* × *ananassa*, AF380299) and T-PG (*Fragaria* × *ananassa*, DQ458991).

Table 1
Change of fruit firmness during ripening of three cultivars of strawberry fruit

Stage	Selva	Camarosa	Toyonaka
Large green	20.15 a	20.34 a	13.39 b
White	12.42 a	11.31 a	2.93 b
25% red	5.52 a	3.62 b	1.16 c
50% red	4.30 a	2.77 b	0.95 c
75% red	3.23 a	2.00 b	0.89 c
100% red	1.83 a	1.39 b	0.74 c

Values with different letters indicate significant differences among fruits at the same ripening stage from different cultivars ($P = 0.05$).

2.16. Experimental design and statistical analysis

Data of PG activity, firmness, and anthocyanin content were analyzed by ANOVA, and the means were compared by the LSD test at a significance level of 0.05.

3. Results

3.1. Fruit firmness

Strawberry softened fast and the main firmness reduction was observed between the large green and 25% R stages. Among the selected cultivars, Selva was the firmest and Toyonaka the softest (Table 1). Firmness values in Selva and Camarosa were similar in immature stages (LG and W), while they were different in more advanced ripening stages. The firmness of Toyonaka fruit was lower in all stages analyzed, even at 100% R. The difference of firmness among cultivars is such that Toyonaka fruit at 25% R are as soft as Camarosa and Selva fruit at 100% R stage.

3.2. Polygalacturonase activity

The PG activity was detected in all the ripening stages analysed (Fig. 1). In Selva, PG activity was low and remained constant from LG to W stage, slightly increased in 50% R and decreased in 100% R stage. In Camarosa, PG activity showed

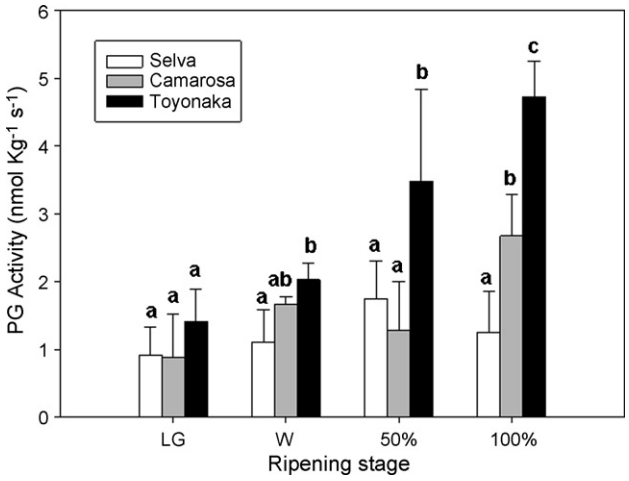

Fig. 1. Change of total polygalacturonase activity during ripening of strawberry fruit cultivars with different softening rate. Protein extracts were prepared from large green (LG), white (W), 50% red (50% R), and 100% red (100% R) fruit. Columns with different letters indicate significant differences ($P = 0.05$) among cultivars.

almost no changes from LG to 50% R, but increased at 100% R stage. Instead, in Toyonaka the PG activity increased continuously from LG to 100% R. The activity of the enzyme was considerably higher in the softest cultivar from 50% R stage on. At this stage, Toyonaka PG activity was ca. 2.5-fold higher than in the other cultivars, while at 100% R stage its activity was ca. 1.8- and 3.8-fold higher than in Camarosa and Selva, respectively.

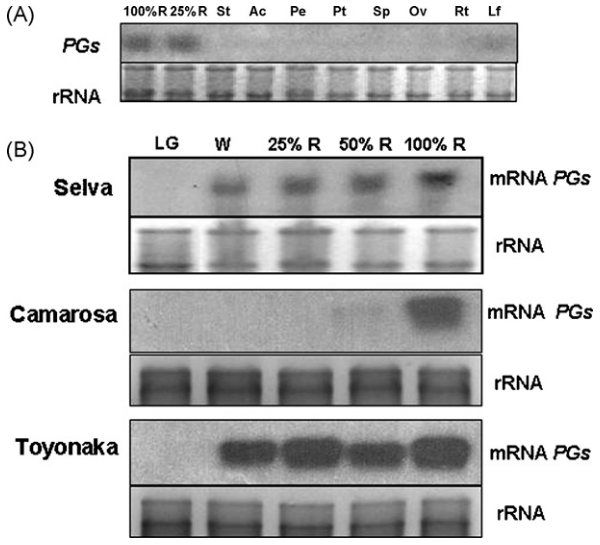

Fig. 2. Northern-blot analysis of *PG* expression: (A) in different tissues of Selva cultivar, (B) during ripening of strawberry cultivars with contrasting fruit firmness. Total RNA (10 μg) was extracted from stamen (St), achenes (Ac), petals (Pe), petioles (Pt), sepals (Se), ovaries (Ov), roots (Rt), leaves (Lv) and from fruit at different ripening stages: large green (LG), white (W), 25% red (25% R), 50% red (50% R), and 100% red (100% R). The RNA was electrophoresed and then hybridized with radiolabelled probe 2 (Section 2). The gel was stained with ethidium bromide to check that there was approximately the same amount of RNA per lane.

3.3. Cloning of FaPG1 and analysis of PG expression in different tissues and ripening stages by Northern blot

A screening of a cDNA library from ripe strawberry fruit was performed in order to obtain new clones encoding putative polygalacturonases. To do that, a heterologous probe from tomato (fragment of pTOM6, probe 1) was used. The search allowed cloning a cDNA fragment of 1008 bp (named *FaPG1*), which was homologous to *spG*, a genomic clone reported by Redondo-Nevado et al. (2001) and *D15*, a cDNA clone isolated by Salentijn et al. (2003). The cloned fragment was then utilized to analyze the expression of *PGs* by Northern blot in different tissues of Selva cultivar (Fig. 2A). A strong hybridization signal was detected in 25% R and 100% R fruit, while a faint signal was found in leaf. Instead, no expression of *PGs* was detected in other vegetative (root and stem) and reproductive (sepal, petal, stamen,

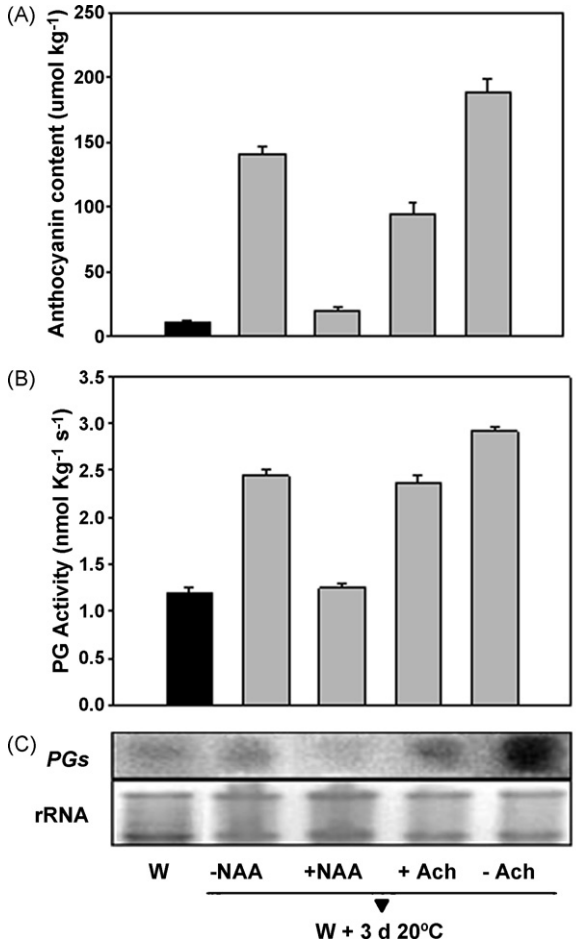

Fig. 3. Effect of auxin treatment and elimination of achenes on anthocyanin content (A), polygalacturonase activity (B) and *pg* expression (C). Halves of intact white fruit (W) from Selva cultivar were treated with NAA and incubated at 20 °C for 3 d (+NAA), maintaining the non-treated halves as a control (−NAA). Similarly, halves of white fruit were deachened and incubated at 20 °C for 3 d (−Ach), maintaining the achened halves as controls (+Ach). After 3 d of incubation, the anthocyanin content and PG activity were determined. Bars in graphs A and B indicate standard deviations. Also, total RNA (10 μg) from fruit samples was extracted and analysed by northern hybridization with probe 2. The gel was stained with ethidium bromide to check that equal RNA amounts had been loaded in each lane (bottom panel).

achene and ovary) tissues. The analysis of *FaPG1* expression in three cultivars during fruit ripening revealed different patterns (Fig. 2B). In Selva, PG mRNA accumulated from W stage, maintained its level until 50% R, and then increased slightly in 100% R fruit. In Camarosa, PG mRNA was not detected in LG, W and 25% R stages, while the expression was very low in 50% R and increased in 100% R stage. In the softest cultivar, Toyonaka, no PG mRNA was detected in LG stage but the expression increased in W stage and remained high until the end of ripening.

3.4. Effect of auxin on PG expression and total polygalacturonase activity

The auxins produced in the achenes of strawberry fruit delay the receptacle ripening (Given et al., 1988) and regulate negatively the expression of most of ripening-related genes (Manning, 1994; Aharoni et al., 2002).

The influence of these hormones on the expression and activity of PG was analyzed by removing the achenes or by application of naphthalene acetic acid (NAA) to halves of white fruit from Selva cultivar. The effect of hormone treatments on fruit ripening was followed by measuring the anthocyanin amount (Fig. 3A). The NAA-treated halves (+NAA) accumulated less anthocyanins than the corresponding controls (−NAA). The level of anthocyanins increased in the deachened halves (−Ach) in comparison with intact fruit halves (+Ach). The PG activity increased in untreated halves (−NAA; +Ach) after 3 d at 20 °C, but the activity remained at similar levels than in initial W fruit in NAA treated halves (Fig. 3B). In turn, the deprivation of endogenous auxins caused by achene elimination increased slightly PG activity in comparison with that observed in intact fruit halves. The effect of auxins on PG expression was analyzed by Northern blot (Fig. 3C). An increase of PG expression was found in untreated halves after 3 d at 20 °C. In contrast, the expression of PG was reduced in NAA-treated halves, and the elimination of achenes (−Ach) caused an increase of PG mRNA regarding the control (+Ach).

3.5. Cloning of T-PG

A RACE reaction was performed in order to obtain the 5′ end of *FaPG1* gene. The experiment allowed cloning two cDNA fragments of different length. Both fragments showed homology with the *spG* gene reported in Chandler cultivar (Redondo-Nevado et al., 2001) and with *D15* clone (Salentijn et al., 2003). The other strawberry gene reported by Salentijn et al. (2003), *B4*, is quite divergent of the four strawberry sequences shown in Fig. 4, and was not included in this alignment. The alignment of the cDNAs cloned showed differences with the predicted cDNA from *spG* gene (Fig. 4). Once aligned with *spG* cDNA, both *FaPG1* and *T-PG* include the sequence 5′GGTCTAGCCATT3′ at the position 454 which corresponds to the amino acid sequence GLAI (Fig. 5). This nucleotide sequence is present in the *spG* genomic clone, but it was absent in the predicted cDNA because was considered as part of an intron (Redondo-Nevado et al., 2001). However, the cDNAs isolated in our laboratory and *D15* cDNA clone do present this sequence, indicating that it corresponds to a gene coding region. In addition, *T-PG* was shorter than *spG*, *FaPG1* and *D15* cDNAs because of a deletion of 85 bp between the positions 454 and 538 of *spG* (Fig. 4). Interestingly, the deletion introduced a stop codon at 574 position in *T-PG*, which caused in turn that this shorter predicted protein lost all the domains characteristic of PGs (Torki et al., 2000). Therefore, while the product of *FaPG1* would be a PG very similar to that predicted for *spG* and D15, the protein corresponding to *T-PG* would not encode for an active PG. As *FaPG1* and *T-PG* were very similar, except for the mentioned deletion, was not possible to design specific probes to study their expression by Northern blot, but the problem was solved by using a semiquantitative RT-PCR approach. Specific primers (5*T-PG* and 3*T-PG*) were designed to amplify a DNA sequence that includes the 85 bp deletion zone found in *T-PG*. RT-PCR experiments allowed amplifying two fragments of 460 and 375 bp (Fig. 6A) from both LG and 100% R fruit of Selva. The fragments were purified, cloned and sequenced, showing complete

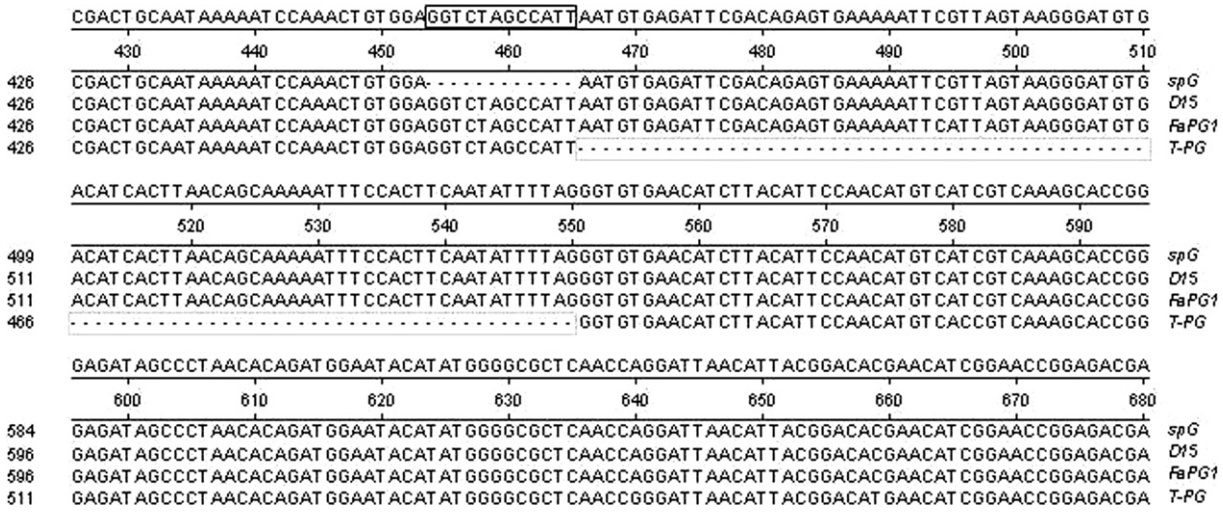

Fig. 4. Alignment of nucleotide sequences corresponding to fragments of *T-PG*, *FaPG1*, *D15* and *spG*. The sequence 5′GGTCTAGCCATT3′ is in black box, and was predicted originally as part of an intron (Redondo-Nevado et al., 2001). The gap of 85 bp of *T-PG* is in gray box.

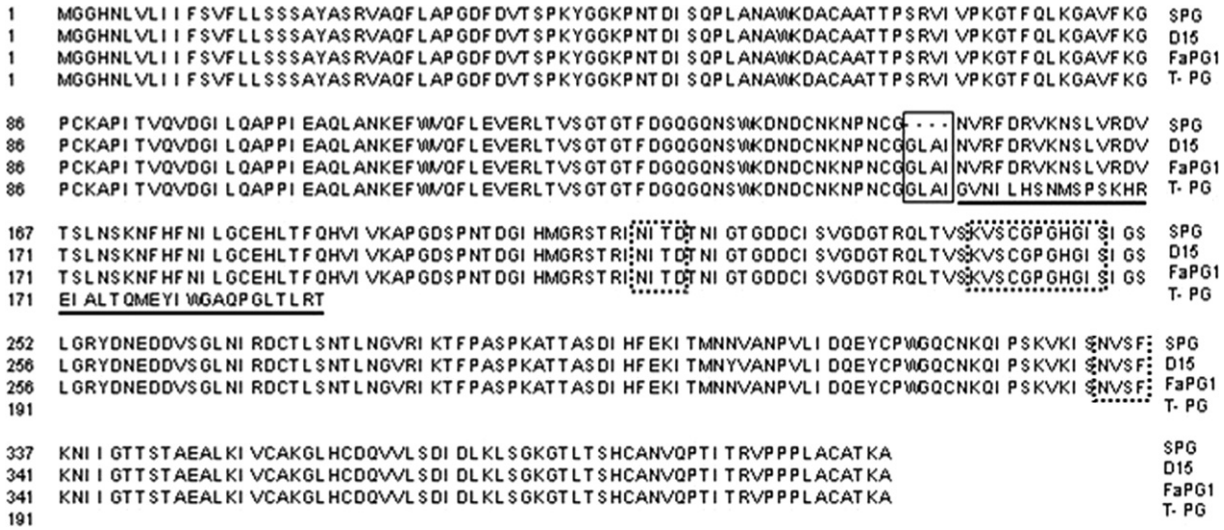

Fig. 5. Comparison among predicted proteins from clones *T-PG*, *FaPG1*, *D15* and *spG*. The box indicates the sequence GLAI, which is lacking in sPG; the sequence underlined in T-PG would not correspond to PG proteins. The PG active site and the *N*-glycosylation sites are indicated in dash boxes.

homology with *FaPG1* and *T-PG*, respectively. The shorter fragment, which hybridized with both fragments, was used as a probe in semiquantitative RT-PCR experiments (probe 3).

3.6. Expression of FaPG1 and T-PG by semiquantitative RT-PCR

The expression of both mRNAs was analyzed in Selva, Camarosa and Toyonaka cultivars in ripening stages ranging from LG to 100% R. The three cultivars showed different expression patterns of *FaPG1* and *T-PG* (Fig. 6B). In Selva, the expression of *T-PG* started in LG stage and remained high until the end of ripening, while the expression of *FaPG1* was undetectable in LG and W stages, slight in 25% R and 50% R and higher in 100% R. In Camarosa, the expression of *T-PG* increased from W to 50% R and decreased slightly in 100% R stage. A similar pattern was detected for *FaPG1* expression but it started from 25% R stage. It is noteworthy that the expression of *T-PG* was higher than that of *FaPG1* in all ripening stages for Selva and Camarosa. Instead, the amount of *FaPG1* mRNA was considerably higher than *T-PG* mRNA during ripening of Toyonaka cultivar. *FaPG1* expression was low in LG, increased in W stage and maintained at high levels until the end of ripening. Regarding *T-PG*, its expression was very low, in all stages analyzed though slightly increased at the end of ripening in this soft cultivar.

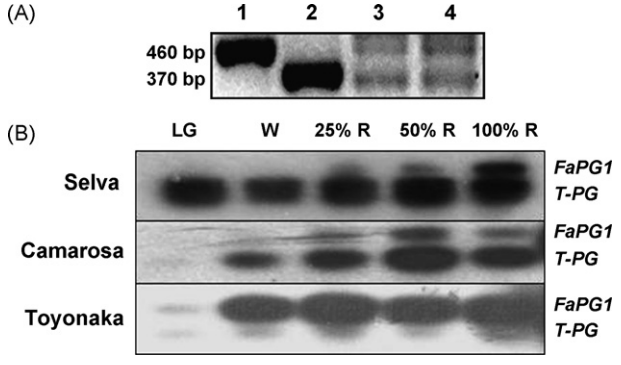

Fig. 6. (A) Amplification of *FaPG1* and *T-PG* by RT-PCR. The primers used, 5*T-PG* and 3*T-PG*, flanked the zone of the deletion present in *T-PG*. Lanes 1 and 2: positive controls using clones *FaPG1* and *T-PG*, respectively, as templates. Lanes 3 and 4: amplification products obtained using RT-PCR mixture from large green (LG) and 100% red (100% R) fruit, respectively, from Selva cultivar. (B) Semiquantitative RT-PCR and Southern blot analysis of the expression of *FaPG1* and *T-PG* in three strawberry cultivars with contrasting fruit firmness. The following ripening stages were used: large green (LG), white (W), 25% red (25% R), 50% red (50% R) and 100% red (100% R).

3.7. Phylogenetic tree

Until now, there were some controversial results about the classification of PGs in strawberry. Redondo-Nevado et al. (2001) classified the deduced spG protein as a Clade A polygalacturonase, whereas Salentijn et al. (2003) classified the same gene, which they called *D15*, as a Clade C PG. In this work, we report the cloning of *FaPG1*, which sequence is very similar to *spG* and *D15*.

As an attempt to get insight about the classification of *FaPG1* (*spG*) the amino acid sequences of FaPG1, 26 other angiosperm PGs and 1 gymnosperm PG (from cedar) were aligned using the CLUSTAL W version 1.8 software (Thompsom et al., 1994). An alignment edited to represent the informative portion of the sequences was used to construct a phylogenetic tree using the Maximun Parsimomy (MP) method with cedar as out-group. The statistical reliability of the tree obtained (Fig. 7) was supported by performing a bootstrap analysis with 1000 replicates.

The sequences of 27 PGs used in the analysis are distributed on the three characteristic clades of PG (Hadfield and Bennett, 1998). The phylogenetic tree placed FaPG1 (spG) on Clade C, which comprise genes expressed in pollen that are thought to encode exo-PGs (Hadfield and Bennett, 1998). This result is in agreement with the classification of spG proposed by Salentijn et al. (2003).

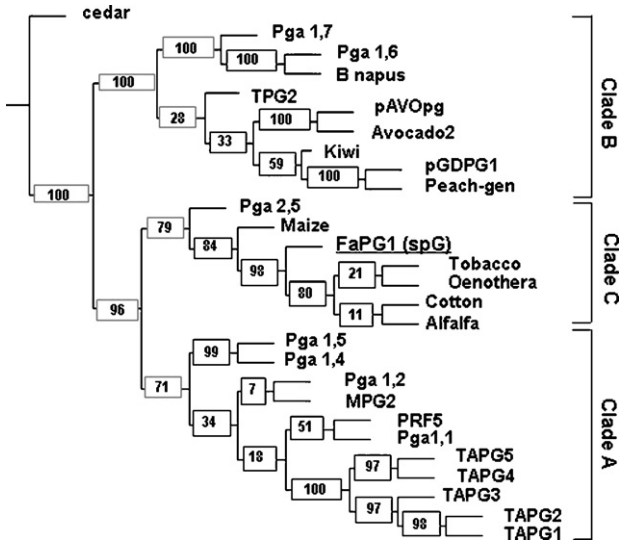

Fig. 7. Phylogenetic tree inferred from the alignments of the plant PGs. The consensus tree was obtained by the MP method. A bootstrap analysis of 1000 replicates was performed, and percentage bootstrap values are indicated in the tree.

4. Discussion

Strawberry showed a continuous softening during ripening in all the cultivars analyzed. Fruit softening in Toyonaka cultivar is intense and the fruit is very soft at ripe stage. On the contrary, fruit from Camarosa and Selva cultivars are firm even at 100% R stage, which make these varieties particularly apt for commercialization. Firmness loss of fleshy fruits has been largely associated to solubilization and depolymerization of cell wall polysaccharides (Rose et al., 1998; Brummell and Harpster, 2001; Brummell et al., 2004; Manrique and Lajolo, 2004). In the particular case of strawberry, total amount of cell wall decreased during ripening, but the correlation between the content of the cell walls and the softening rate of cultivars with contrasting firmness is not clear (Rosli et al., 2004), indicating that other factors in addition to the cell wall amount should be considered. That work suggested that strawberry softening could be association with a decrease of hemicelluloses during the last stage of development and the beginning of ripening, and to solubilization and depolymerization of pectins at the end of ripening. Moreover, significant pectin depolymerization was found in the softest cultivar (Toyonaka) while no depolymerization of these fractions was detected in Camarosa.

The increment of PG activity and the corresponding gene expression during ripening has been reported in many fruits (DellaPenna et al., 1986; Hadfield et al., 1998; Hadfield and Bennett, 1998; Asif and Nath, 2005). However, the presence of polygalacturonase activity in strawberry has been controversial. Huber (1984) found an increment of polyuronide solubilization and a limited pectin depolymerization during ripening of Dover cultivar, though no PG activity could be detected. However, Nogata et al. (1993) detected a low polygalacturonase activity in fruit from Toyonaka cultivar and described the presence of three isoenzymes. These contrasting results could be ascribed to the different sensitivity of PG enzyme assay and the relative PG amount in the cultivars used in both studies. In the present study we have used a very sensitive method to detect the galacturonic acid released by the enzyme (Gross, 1982), and the PG activity was detected in the three cultivars analyzed. The highest PG activity was found in Toyonaka cultivar, at all the ripening stages. The enzyme activity increased continuously in this soft cultivar, while it remained at lower levels in Selva and Camarosa. According to these data, a higher softening rate correlates with a higher PG activity during ripening. Moreover, the higher PG activity correlates with the higher degree of pectin depolymerization observed during fruit ripening of Toyonaka cultivar (Rosli et al., 2004). A similar situation was described in pears and peaches. In the first case, pear cultivars with soft texture presented higher pectin depolymerization and PG activity (Hiwasa et al., 2004). In peaches, melting cultivars showed an increased PG activity during ripening while the activity of the enzyme was not detected in non-melting cultivars (Pressey and Avants, 1978).

In order to analyze the expression of putative polygalacturonase genes, a screening of a cDNA library from ripe strawberry was performed. The isolated cDNA fragment (1008 bp, named *FaPG1*) was truncated in its 5′end and it was homologous to *spG* gene, previously described by Redondo-Nevado et al. (2001) and to *D15* (Salentijn et al., 2003). Northern-blot analysis indicated that PG expression was strongly related to fruit tissues. Moreover, our results indicate that auxins repress PG gene expression and the increase of PG enzyme activity. In strawberry, the fruit growth and ripening are regulated mainly by auxins produced in the achenes. Auxins stimulate receptacle expansion during fruit development, and later inhibit fruit ripening (Given et al., 1988). As the strawberry matures, a reduced tissue auxin concentration activates or de-represses the expression of ripening-related genes (Given et al., 1988). The inhibitory effect of auxins on PG expression found in this work is similar to that reported for other cell wall-related genes in strawberry, such as β-galactosidase (Trainotti et al., 2001), pectate lyase (Benítez-Burraco et al., 2003), endo-β-1,4-glucanase (Harpster et al., 1998) and β-xylosidase (Martínez et al., 2004). The expression pattern of s*pG* gene has been analyzed in Chandler cultivar during ripening by Redondo-Nevado et al. (2001). The authors detected that *spG* was expressed only in white stage and suggested that the gene might be involved in the production of oligosaccharins, molecules involved in fruit ripening activation, rather than in the degradation of pectins. More recently, Salentijn et al. (2003) cloned two polygalacturonase genes (*D15* and *B4*), being the former identical to *spG*. However, their results differ substantially from those of Redondo-Nevado et al. (2001), since they found an increase of *D15* expression during ripening of three cultivars with different texture, and even detected a higher expression of *D15* in ripe fruit of the softest cultivar. As regard to *B4* gene, the authors found that it was upregulated in the soft cultivar. The deduced amino acid sequence of *B4* cDNA is homologous to *A. thaliana* (e.g. NP 190464) and to microbial PGs (e.g. NP 346995), but the PG active site of the deduced protein differs from the one present in known PG proteins (Salentijn et al., 2003).

In the three cultivars analyzed in the present work the expression of PG started at different ripening stages and increased during ripening, suggesting a possible role in softening. Northern-blot analysis revealed that the expression of PGs began early at W stage and at a high level in Toyonaka, while it was delayed or at low level in Camarosa and Selva, respectively. Nevertheless, the correlation between polygalacturonase expression and softening was only partial, since Selva cultivar showed earlier accumulation of PG mRNA than Camarosa but its fruit were firmer.

Our screening of polygalacturonase genes allowed cloning *FaPG1*, homologous to *spG* clone reported by Redondo-Nevado et al. (2001), and *T-PG*, which were identical except for a deletion of 85 bp present in *T-PG* (Fig. 4). This deletion would cause a frame shift and the appearance of a codon stop 105 bp after the deletion, which would generate a shorter protein. The predicted T-PG protein lacks the PG active site and the *N*-glycosylation sites (pattern PS00502 and PS00001 of the prosite data base at http://www.expasy.org/prosite, respectively) as well as the plant PG consensus sequences (Torki et al., 2000). As *FaPG1* and *T-PG* are almost identical except for the 85 bp deletion, the probes used in Northern-blot analysis did not allow differentiating the expression of both genes. Instead, an analysis by RT-PCR with primers that flanked the deletion zone allowed determining that both genes are expressed in fruit tissue (Fig. 6A). When semiquantitative RT-PCR analysis was performed, a different expression pattern for both genes (*FaPG1* and *T-PG*) was detected in the three cultivars (Fig. 6B). Interestingly, Selva and Camarosa, the firmest cultivars, showed a higher expression of *T-PG* in relation to *FaPG1* in all ripening stages. Otherwise, in Toyonaka, the softest cultivar, the expression of *FaPG1* was higher than that of *T-PG*. If it is assumed that the product of *FaPG1* is an active PG while that of *T-PG* is a protein lacking PG activity, then the higher accumulation of *FaPG1* mRNA in the softest cultivar correlates with its higher PG activity. On the contrary, Selva and Camarosa showed higher expression of *T-PG* that could produce a truncated protein without PG-activity, which would explain the lower PG activity found in both cultivars. Moreover, the accumulation of this inactive protein during Selva fruit ripening could explain the apparent discrepancy between the expression relatively high of PGs (in comparison with Camarosa) detected by Northern blot (Fig. 2) and the low PG activity found in this cultivar. According to our knowledge, this is the first report on the accumulation of a PG-like transcript in fruits that would encode for a putative inactive PG. However, a similar situation was found in the case of endo-β-mannanases from tomato. In most of tomato cultivars, there is an increment of this enzyme activity during fruit ripening. However, the cultivar Walter exhibits no endo-β-mannanase activity, though the corresponding gene (*LeMAN4*) is transcribed in the fruit and the resultant truncated protein is localized in the cell wall (Banik et al., 2001). In this cultivar the nucleotide sequence of *LeMAN4* has a two-nucleotide deletion starting at 1209 bp, which causes a frame-shift resulting and then the translation of a protein truncated at the C-terminal amino acid (Bourgault and Bewley, 2002).

In a previous work, it had been suggested that strawberry fruit could be closely related to pectin solubilization and depolymerization rather than to hemicellulose or cellulose catabolism (Rosli et al., 2004). In this sense, the higher polygalacturonase activity could be associated to the remarkable degradation of pectic polymers detected in Toyonaka cultivar at the end of ripening (Rosli et al., 2004). Another enzyme that could participate of pectin disassembly is pectate lyase This protein may contribute to pectin depolymerization by cleaving de-esterified homogalacturonans by a mechanism of β-elimination that differs from that of PGs. Jiménez-Bermúdez et al. (2002) have shown that strawberry lines with antisense expression of a pectate lyase gene generated firmer fruit with lower cell wall swelling and pectin solubility. Taken together, these data support the hypothesis that the metabolism of pectins could contribute significantly to strawberry fruit softening, particularly at the end of ripening. In the present work, we have found that three strawberry varieties with contrasting fruit firmness differ in the expression pattern of two PG-related genes: *FaPG1* and *T-PG*. Interestingly, the higher PG activity found in the softest cultivar correlates with the enhanced expression of *FaPG1* and a very low expression of *T-PG*. On the contrary, the other two firm cultivars express *T-PG* preferentially, encoding for an inactive PG truncated protein. The differential expression of these genes could be one of the features that determine the different softening rate observed in strawberry cultivars.

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