Expression of genes encoding cell wall modifying enzymes is induced by cold storage and reflects changes in pear fruit texture

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Abstract

Preclimacteric ‘Rocha’ pears stored under chilling conditions, had a larger increase of ACO (1-aminocyclopropane-1-carboxylate oxidase) activity and softened faster than those treated with ethylene. Non-treated fruit did not ripen or soften, acquired a rubbery texture, and showed barely detectable levels of ACO activity. The transcript accumulation of seven genes encoding cell wall modifying enzymes was followed during fruit growth, ripening, and senescence, and in fruit that failed to ripen, by quantitative real-time PCR. Transcripts from ‘Rocha’ pear polygalacturonase1 and 2 (PcPG1, PcPG2), β-galactosidase (PcβGAL) and β-xylosidase (PcXYL) genes accumulated up to 1000-fold at the climacteric onset, while low transcript levels were detected in growing fruit. In fruit that did not ripen, this transcript accumulation was lower compared with fruits that ripened normally. Transcripts for expansin1 and 2 (PcEXPA1, PcEXPA2) accumulated in growing fruit, but about 10-fold more in fruit after rewarming. Xyloglucan endotransglucosylase/hydrolase (PcXTH) had the highest basal expression levels in all samples, showing only a small increase during fruit growth and ripening. PcEXPA2 and PcXTH transcripts accumulated in untreated fruit, 21 d after harvest, to levels similar to those of fruit that ripened normally. Since in untreated fruit ACO activity was barely detectable, it is likely that the activation of these genes might occur at very low ethylene levels. Results suggest that PcXTH and PcEXPA2 gene induction might be associated with cell wall maintenance during ‘Rocha’ pear development and ripening, while PcEXPA1, PcPG1, PcPG2, PcβGAL, and

PcXYL expression is likely to be related to cell wall disassembly and loosening.

Key words: Cell wall, cold storage, fruit ripening, gene expression, pear, softening.

Introduction

‘Rocha’ pear (Pyrus communis L.) is a climacteric fruit that produces large amounts of ethylene during ripening. Fruit is harvested commercially at the mature preclimacteric stage and stored for several months at low temperatures. This induces in fruit, after rewarming, uniform ripening and the development of good aroma and texture characteristics through the induction of ethylene biosynthesis.

The pathway of ethylene biosynthesis is well established in plants. The first step, the conversion of S-adenosylmethionine into 1-aminocyclopropane-1-carboxylic acid (ACC) is catalysed by ACC synthase (ACS). ACC oxidase (ACO) catalyses the final step of the pathway oxidizing ACC to ethylene (Kende, 1993). It has been shown that ethylene affects the transcription of several ripening-related genes, including those related to softening (Alexander and Grierson, 2002). Fruit softening occurs during ripening as a consequence of progressive cell wall modification and disassembly by enzyme action (Giovannoni, 2001; Rose and Bennett, 1999). Pectins and hemicelluloses, two of the major cell wall components, undergo solubilization and depolymerization (Fischer and Bennett, 1991). In fruit, polygalacturonases (PG) are one of the best studied
enzymes that act on pectins by cleaving the galacturonic acid backbone (Brummell and Harpster, 2001). Accumulation of PG mRNA in tomato and pear is induced by ethylene (Sitrit and Bennett, 1998; Hiwasa et al., 2003a). Experiments with transgenic tomato plants showed that PG activity is responsible for polyuronide depolymerization and solubilization, but it is neither necessary nor sufficient for fruit softening (Smith et al., 1988; Giovannoni et al., 1989). β-galactosidases (β-GAL) contribute to the net loss of galactosyl residues from cell wall polymers resulting in fruit textural changes (Brummell and Harpster, 2001; Gross and Sams, 1984). In tomato, β-galactosidases are encoded by a family of at least seven members, from which the ethylene inducible TBG4 is responsible for the major part of losses of cell wall galactose during ripening (Smith and Gross, 2000). In Japanese pear (Pyrus pyrifolia) a β-GAL gene, induced at the onset of ripening, has so far been isolated (Tateishi et al., 2001).

Xyloglucan endotransgalactosylase/hydrolases (XTH), α-expansins (EXPA), and β-xylidosidases (XYL) are some of the best-studied enzymes, able to disassemble the cellulose-xyloglucan matrix, that have been associated with the fruit-softening process. XTH catalyses the cleavage of internal xyloglucan matrix, that have been associated with the fruit-softening process. XTH catalyses the cleavage of internal xyloglucan matrix, that have been associated with the fruit-softening process.

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Materials and methods

Plant materials and treatments

Fully-grown pear fruit (Pyrus communis L. cv ‘Rocha’) of similar size were harvested 104 d after flower anthesis (DAF) (sample H), which corresponds to the commercial harvesting time for storage of this pear cultivar. One group of these fruits was kept in air at 23 °C; another group was treated with 100 μl l⁻¹ ethylene for 24 h and then kept in air at 23 °C (Avelar and Rodrigues, 2000); and the third group was stored at 0 °C for 60 d and transferred to 23 °C (rewarming) where they were allowed to ripen. Samples of five fruit were taken every 3 d from each group. The first sample, from cold-stored fruits, was taken 4 h after rewarming and followed during ripening until days 12 and 15, when fruit were, respectively, overripe and completely senescent. Ethylene-treated fruit were followed until day 18, when they reached complete senescence. Non-treated fruit were followed for 21 d. Fruits that failed to ripen, even after 21 d at room temperature, were named FR21. Small growing fruit were also harvested and grouped as ‘G1’ (2–3 cm diameter, 60 DAF) and ‘G2’ (4–6 cm, 90 DAF). The five fruit taken at each time point were used for firmness measurements. Fruit mesocarp was frozen in liquid nitrogen, stored at −80 °C and used for RNA preparation and protein extraction for ACO activity assays.

Fruit firmness was evaluated using a Fruit Pressure Tester (Bellevue) fitted with an 8 mm diameter plunger. Fruits were peeled and punctured on two opposite sides. The values presented correspond to the average of the values obtained from five fruits from each ripening stage.

Extraction and assay of ACC oxidase from pear fruit

The method used for ACC oxidase extraction and activity assay was essentially that of Vioque and Castellano (1994). All the products used were purchased from Sigma Chemical Co. (St Louis, MO). For each extraction, 100 g of mesocarp from a pool of five fruit was used. ACC oxidase was assayed in 4 ml vials that were sealed and CO₂ was injected to a final concentration of 16%. The ethylene produced was determined by gas chromatography. Total protein content in the ACC oxidase enzyme extracts was estimated according to Lowry et al. (1951) using a BSA standard curve. All determinations were made in triplicate and results were expressed as means and standard deviations (SD).

RNA extraction and cDNA library construction

Frozen pear material was ground in liquid nitrogen using a mortar and pestle. Total RNA was purified according to the hot-urate method (Wan and Wilkins, 1994). The quantity and the quality of the RNA were evaluated by gel electrophoresis and A₂₆₀/𝐴₂₈₀ ratios. mRNA was purified using the PolyATtract™ mRNA Isolation System kit (Promega, Madison, WI). From the mRNA obtained from all the growing and ripening stages, a pool of a total of 10 μg was used for library construction with ZAP Express cDNA synthesis and ZAP Express cDNA Gigapack III Gold cloning kits (Stratagene, La Jolla, CA).

cDNA isolation and analysis

The cDNA fragments of PcACO and PcPG1, containing the complete open reading frame, and partial cDNA fragments of PcXTH and PcXYL were obtained by random screening of a cDNA library and a subtracted library (Fonseca et al., 2004). The cDNAs of PcPG2, PcβGAL, PcEXP1, and PcEXP2 were obtained by PCR as described by Fonseca et al. (2002). All the cDNA fragments obtained were sequenced in an ABI 310 sequencer (PE Applied Bionics, Foster City, CA). The nucleotide and deduced amino acid sequences for all the genes were compared with those existing in the NCBI database by using the BLASTN and BLASTX programs (Altschul et al., 1990).
Quantitative real-time PCR

Quantitative real-time PCR was performed using a LightCycler® instrument (Roche Diagnostics, Mannheim, DE) with gene-specific primers. SYBR Green was used as fluorescent dye to measure DNA amplification products derived from the mRNA present in the fruit tissues. Twenty µg of total RNA, obtained from a pool of five fruits from each sample, were reverse transcribed with 20 µl of AMV (Boehringer Mannheim, DE), at 42 °C for 90 min, in the presence of poly(dT) sequences in a total volume of 20 µl. After a 100 times dilution, 2 µl of the diluted cDNA was used as a target in the reaction mixture and added to a mix containing 2 µl of Master SYBR Green 10X concentrated (containing FastStart Taq DNA polymerase, reaction buffer, dNTP mix with dUTP instead of dTTP, SYBR Green I dye, and 10 mM MgCl₂) and 1 µM of each gene-specific forward and reverse primers to a final volume of 20 µl, according to the instructions provided on the LightCycler–FastStart DNA Master SYBR Green 1 version 5.0 (Roche Diagnostics, Mannheim, DE). The amplification was carried out with the following cycling parameters: 10 min hot start at 95 °C, 45 cycles of denaturation at 95 °C, annealing at 56 °C for 5 s and extension at 72 °C for 8 s, followed by extension at 72 °C for 10–14 s, depending on the amplicon size. Fluorescence detection was performed at the elongation phase. For melting curve determination, the annealing temperature was 65 °C for 30 s. The melting curves were checked for single peaks, and the product size was confirmed in an agarose gel. The primers forward and reverse used for amplification of each gene were, respectively: β-actin: ACTQF (5'-CTCGACCTCGAGATGGTGT-3') and ACTQR (5'-CATGATGTCGCTGAAGAGGA-3'); PcACO: ACOQF (5'-AATGACCACTCCTTACACGATA-3'), ACOQR (5'-GGTTCACTGT-AGTCAATAACACA-3'); PcβGAL: GALQF (5'-CATCAATGTGCAGCTTCAGGA-3'); GALQR (5'-CTGAATTCGACCTTTCAATTGTA-3'); PcPG1: PG1QF (5'-ACGTTCAAGCTTCGCTCTCA-3'), PG1QR (5'-CGTTCTTCTCAGTACACCA-3'); PcPG2: PG2QF (5'-GATTGAAAACGTCCATGTGGA-3'), PG2QR (5'-TGCAAGGATACATGGAACCTACA-3'); PcXTH: XTHQF (5'-CCCATCTACAACACTGCACTGA-3'), XTHQR (5'-ACACCTCA- AAGGATAATGAACTCT-3'); PcEXP1: EX1QF (5'-CATCGGCATCCATCAGTGT-3'), EX1QR (5'-GACCTCGCCATGCCCA-TGT-3'); PcEXP2: EX2QF (5'-CATGTTGGTGCCACCGCTTGTC- T-3'), EX2QR (5'-TGATTTGGGTGACCCCTGTTTT-3'); PcXYL: XYLQF (5'-CTCTTGAATAGGCTTGACCA-3'), XYLQR (5'-CATCCTTGGCGAGACAATCATA-3'). For each gene a calibration curve was performed by measuring the fluorescence of six serial dilutions of a plasmid carrying the fragment to amplify that served as the positive control. Triplicate quantitative PCR experiments were performed for each sample and the obtained expression values were normalized against β-actin.

Results

Sequence isolation and analysis

By random screening of a ‘Rocha’ pear cDNA library a 942 bp cDNA containing the complete open reading frame (ORF) of the PcACO1 gene was isolated (AJ504857), encoding a polypeptide of 314 amino acids. At the nucleotide level the PcACO cDNA presents 98% homology with that of ACO cDNA of the ‘Passe-Crassane’ cultivar (X87097). A full-length cDNA containing the complete ORF encoding for PcPG1 (1383 bp, encoding a polypeptide of 460 amino acids) (AJ504855) was also isolated that had homology at the nucleotide level of 98% with PC-PG1 cDNA isolated from ‘La France’ pear fruit (AB084461). A partial cDNA of PcXTH was isolated (342 bp, encoding a polypeptide of 114 amino acids) (AJ504876) presenting homology of 96% with PC-XTH1 (AB095368) isolated from the ‘La France’ pear cultivar. A 712 bp fragment containing a partial fragment of PcXYL (AJ811690) was isolated from ‘Rocha’ pear and had at the nucleotide level, the highest homology (84%) to Prunus persica β-β-xylulokinase cDNA (AJ504974). The PcβGAL (AX392011), PcPG2 (AX392013), PcEXPA1 (AX392017), and PcEXP2 (AX392019) cDNA sequences are protected by world patent WO0216613 (Fonseca et al., 2002).

Fig. 1. Fruit firmness during ripening of cold and ethylene-treated fruit after harvest, compared with untreated fruit. Harvest (H); days after harvest (3–21), except on cold-stored fruit where the numbers represent days after return to room temperature. Vertical bars correspond to SD of three replicates.

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The firmness evaluated after fruit harvest, did not decrease in fruit kept in air (non-treated), even after 21 d at room temperature. The highest firmness corresponded to day 21 when the fruit had a rubbery texture (Fig. 1). The fastest decrease in firmness was found in fruits after cold storage. In these fruit, firmness reached undetectable levels in 15 d, coincident with the time at which fruit were brown inside and completely senescent. Pears treated with 100 µl l⁻¹ ethylene presented a similar softening rate, but fruit reached firmness zero after 18 d, three days later than cold-treated fruit, though for some points the differences were not statistically significant (Fig. 1).

ACC oxidase activation by ethylene and cold storage and ACO gene expression

In growing pear fruit, ACO activity was below the threshold detection levels and at harvest, residual enzyme activity could be detected (Fig. 2). In fruit treated with 100 µl l⁻¹ ethylene ACO activity gradually increased from day 3 to reach a peak on day 12, though much lower than the values obtained in cold-stored fruits (Fig. 2). ACO activity of cold-stored fruit started to increase 4 h on rewarming and increased 10-fold in 6 d, being 10-fold more active than
in ethylene-treated fruit after the same period of time. Instead of declining, ACO activity in chilled fruit continued to increase until fruit were senescent (day 15) (Fig. 2). In non-treated fruit that did not ripen, ACO levels increased on day 12 to double that of fruit at harvest, returning to levels below the limit of detection afterwards (Fig. 2).

Real-time PCR analysis of cDNA accumulation showed that PcACO transcript relative levels were very low in leaves and petioles and in G1 and G2 young fruit (Fig. 3A). During fruit growth, PcACO transcript levels increased almost 100 times (from 8.8×10⁻¹¹ to 7.7×10⁻⁹) until fruit reached commercial harvesting size. After rewarming, the accumulation of PcACO transcripts was even faster, increasing 300-fold in the first 4 h. In these fruit ACO activity increased greatly between 4 h and 3 d after transfer to room temperature, showing that the PcACO transcript accumulation pattern in cold-stored fruit precedes that of ACO activity (Figs 2, 3A). In non-treated fruit, kept for 21 d at room temperature (FR21), PcACO transcript levels were 6-fold higher than those of fruit at harvest, but much lower than in fruit after 4 h rewarming (Fig. 3A).

**Temporal expression pattern of genes encoding cell wall modifying enzymes**

Temporal expression patterns of the seven genes encoding cell wall modifying enzymes were analysed by real-time PCR (Fig. 3). Expression in leaves and petioles was analysed to evaluate how these genes are expressed in organs other than fruit. PcβGAL, PcPG1, PcPG2, and PcXYL transcripts accumulated during fruit growth until the commercial harvest stage (between G1 and H) by 10–100-fold. After cold storage and rewarming, transcript accumulation increased up to 8-fold in the first 4 h, followed by an increase from 10–100-fold in the first 3 d (Fig. 3B, C, D, H). During fruit ripening, transcript levels of PcβGAL, PcPG1, PcPG2, and PcXYL genes remained almost constant (Fig. 3B, C, D, H). PcXTH transcript basal levels were very high in all the organs studied and from all the genes studied PcXTH is the least ripening related. Transcript accumulation increased about 4-fold in the latest growing stages and a similar increase occurred 3 d after rewarming (Fig. 3G).

Compared with the other genes, PcEXPA1 and PcEXPA2 transcript accumulation were very high in leaves and petioles. PcEXPA2 was expressed at higher levels than PcEXPA1 in all stages of fruit growth and ripening (Fig. 3E, F). PcEXPA1 transcript accumulation was higher in the initial growth stage (G1) while PcEXPA2 transcripts accumulated to a greater extent in the final growth stage (G2), declining again in fully grown fruit (Fig. 3E, F). Four hours after rewarming, transcript levels of both PcEXPA genes increased 2-fold and, after 3 d, a 6-fold and 24-fold increase was detected for PcEXPA1 and PcEXPA2, respectively. In late ripening/senescent fruit, transcript levels of PcβGAL, PcPG1, PcEXPA2, PcXTH, and PcXYL decreased about 2-fold between days 12 and 15, while PcPG2 and PcEXPA1 transcripts increased 2-fold (Fig. 3B–H).

Non-treated fruits that failed to ripen and did not soften, acquiring a rubbery texture, were assayed to quantify transcript accumulation 21 d after harvesting (FR21, Fig. 3). Like PcACO, PcβGAL, and PcXYL transcript levels were between those of fruit at harvest and those of fruit 4 h after rewarming (Fig. 3A, B, H). PcPG1 and PcEXPA1 transcript abundance was between that of fruit 4 h and 3 d after rewarming (Fig. 3C, E). In FR21 fruit, accumulation of PcEXPA2 and PcXTH transcript was similar to that of fruit that ripened normally (Fig. 3F, G). By contrast, PcPG2 transcript abundance in FR21 fruit decreased 5-fold relative to that of fruit at harvest (Fig. 3D).

**Discussion**

**Cold storage as an inducer of ethylene synthesis**

Some of the genes encoding for cell wall modifying enzymes are induced by ethylene (Alexander and Grierson, 2002). Therefore ACO1 transcript accumulation that directly reflects ACO1 mRNA levels was measured, and ACO activity was quantified. The comparison of cold storage, exogenous ethylene treatment, and the absence of treatment showed that ACO activity in ‘Rocha’ pear was much higher in cold-stored fruit than in ethylene-treated ones (Fig. 2). Although both groups of fruit were able to ripen, cold-stored fruit achieved a quicker and higher climacteric burst and softened faster than ethylene-treated fruit, showing that the chilling treatment provides optimal conditions for a normal climacteric ripening in ‘Rocha’ pear. In the ‘Passe-Crassane’ pear cultivar it was shown that gene expression can be induced either by exogenous ethylene or by chilling (Lelièvre et al., 1997).

In cold-stored fruit, the PcACO transcript accumulation pattern preceded that of ACO activity. In untreated fruit, ACO activity was not detectable after 21 d at room temperatures, whereas in ethylene-treated fruit, ACO activity was not detectable after 21 d at room temperature (FR21). Therefore mRNA levels was measured, and ACO activity was quantified. The comparison of cold storage, exogenous ethylene treatment, and the absence of treatment showed that ACO activity in ‘Rocha’ pear was much higher in cold-stored fruit than in ethylene-treated ones (Fig. 2). Although both groups of fruit were able to ripen, cold-stored fruit achieved a quicker and higher climacteric burst and softened faster than ethylene-treated fruit, showing that the chilling treatment provides optimal conditions for a normal climacteric ripening in ‘Rocha’ pear. In the ‘Passe-Crassane’ pear cultivar it was shown that gene expression can be induced either by exogenous ethylene or by chilling (Lelièvre et al., 1997).

In cold-stored fruit, the PcACO transcript accumulation pattern preceded that of ACO activity. In untreated fruit, ACO activity was not detectable after 21 d at room
temperature although transcript accumulation was higher than in fruit at harvest (Fig. 3A). According to Alexander and Grierson (2002) an increase in the de novo synthesis of ACO1 might stimulate the ethylene biosynthetic pathway, with the ethylene produced inducing ACS genes, resulting in ACC and subsequent ethylene production. In non-treated pears, however, a moderately high level of PcACO transcript accumulation had no effect on ACO activity, suggesting that, even if undetectable ethylene amounts were produced, they were not sufficient to initiate the chain. Immediately after harvesting, ‘Rocha’ fruit respond to exogenous ethylene stimuli, probably because the threshold to initiate ripening by activating ACS was reached, enabling the transition from system 1 to system 2 of the ethylene biosynthesis pathway, in a similar way to that described for tomato (Barry et al., 2000).

Fig. 3. Changes in the induction of ACO (A) and genes encoding for cell wall modifying enzymes (B–H). Values on the y-axis correspond to gene/actin ratio in an exponential scale. Graphics have different scales. Leaves (LF); petioles (PT); growing stages (G1 and G2); harvest day (H); fruit 4 h after rewarming (4 h); days after rewarming (numbers from 3–15); non-treated fruit that failed to ripe after 21 d at room-temperature (FR21). Bars correspond to the average of three replicates and SD is represented by vertical bars.
**Fruit softening and expression of genes encoding cell wall modifying enzymes**

Although not all ripening-associated events are ethylene dependent in climacteric fruit, ethylene production in fruit serves as a good parameter for comparison among the ripening phases of different fruit. In pear, softening has been related to pectin depolymerization leading to the disassembly of primary cell wall (Dick and Labavitch, 1989; Martin-Cabrejas et al., 1994). It is accepted that in fruits in general, softening occurs as a consequence of enzyme-driven solubilization and depolymerization of pectins and hemicelluloses (Fischer and Bennett, 1991). The high homology levels confirm the likely function of the cDNA clones isolated from 'Rocha' as encoding for cell wall modification enzymes, being PGs, βGAL, EXPs, XYL, and XTH. To the best of our knowledge, this is the first time that the expression of such a set of genes was followed during pear fruit growth, ripening and senescence, and also in fruit unable to ripen.

*PcβGAL, PcPG1, PcPG2,* and *PcXYL* present a similar pattern of transcript accumulation. It increased during fruit growth, but more strongly after climacteric onset (Fig. 3B, C, D, H). In unripe fruit (FR21) that remained firm, the transcript level of *PcβGAL, PcPG1,* and *PcXYL,* remains similar to that of fruit before entering climacteric (Fig. 3B, C, H). The transcript accumulation pattern occurs in parallel with softening, was similar to that of *PcACO* and correlated with ACO activity. It is not clear if β-GAL can be regulated by ethylene (Smith and Gross, 2000). Although there is no information on β-GAL enzyme activity, these results agree with those suggesting that strong suppression of β-GAL activity, early in ripening, is able to prevent softening (Smith et al., 2002). It might be that, as well as in tomato, β-GAL can play a role on 'Rocha' pear fruit softening. Though *PcXYL* was more expressed after climacteric onset, the blockage of ethylene biosynthesis in tomato by using 1-methylcyclopropene suggested that the regulation of the two xylosidase isoforms isolated are independent of ethylene action (Itai et al., 2003). Recently, it has been suggested that pear and tomato β-xylidosases can play an important role in cell wall depolymerization during fruit ripening (Itai et al., 1999, 2003).

In tomato, *PG* mRNA accumulation is induced by ethylene even at low levels (Sitrit and Bennett, 1998) and in ‘La France’ pears, the expression of two *PG* genes can be enhanced by exogenous ethylene (Hiwasa et al., 2003a). The results obtained for ethylene non-treated ‘Rocha’ pears suggest that the barely detectable ACO activity in these fruits was sufficient to produce the ethylene necessary to activate low levels of transcription of *PcPG1* but not of *PcPG2,* nor to trigger the softening process (Figs 2, 3C, D). Recent studies on different pear cultivars showed that differential expression of *PG* is strongly correlated with differences of softening behaviours (Hiwasa et al., 2004). PG-suppressed fruit maintain tissue integrity probably due to the retention of high molecular weight polyuronides (Langley et al., 1994; Brummell and Labavitch, 1997) although in tomato PG was neither necessary nor sufficient for fruit softening (Brummell and Harpster, 2001).

Both *PcEXPAs* transcripts accumulated during fruit growth and were highly induced after rewarming of fruit previously chilled, *PcEXPA2* transcripts being more abundant than those of *PcEXPA1* (Fig. 3E, F). These results are similar to those obtained by Hiwasa et al. (2003b) for the homologues of *PcEXP6* and *PcEXP3* reported for the 'La France' pear cultivar. The high levels of *PcEXPA2* transcripts in FR21 fruit (Fig. 3F), where ACO activity was not measurable (Fig. 2), suggests that *PcEXPA2* induction does not necessarily require ethylene. Even though it can be ethylene inducible as has been shown in 'La France' pears, the accumulation of expansin mRNAs is suppressed by the blockage of ethylene biosynthesis (Hiwasa et al., 2003b). The relatively low *PcEXPA1* transcript accumulation in fruit that acquired a rubbery texture agree with that reported for the tomato homologue LeEXP1, the suppression of which resulted in an enhanced firmness throughout ripening and an increase in fruit shelf life and fruit paste viscosity (Brummell et al., 1999, 2002).

The high accumulation of *PcXTH* transcripts in all samples (Fig. 3G), as well as in rubbery textured fruit, indicate that *PcXTH* is expressed at high basal levels. It was found that in three different pear cultivars with different softening behaviour, *XTH* expression was constitutive (Hiwasa et al., 2004), suggesting that the enzyme plays an important role in cell wall turnover and maintenance rather than softening (Campbell and Braam, 1999). Although XTH activity seems to be induced by ethylene in kiwifruit (Redgwell and Fry, 1993), *PcXTH* as well as *PcEXPA2* transcript accumulation levels seem not to be affected by the absence of ethylene production in untreated fruit.

Taken together, these results suggest that *PcXTH* and *PcEXPA2* gene induction might be associated with cell wall maintenance during 'Rocha' pear development and ripening, while *PcEXPA1, PcPG1, PcPG2, PcβGAL,* and *PcXYL* might be related to cell wall disassembly and loosening. It is important to note that this work focuses on a limited group of genes. Others that encode enzymes playing significant roles on pear fruit cell wall modifications might exist. Further investigation is needed in order to elucidate the mechanisms behind the regulation of these genes and to understand to what extent the activity of each enzyme depends or is correlated with the others. Even if a complete understanding of the softening process in climacteric fruits is still far away, integrative studies on gene expression and enzyme activity might bring important contributions to our knowledge of this process.
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