Role of "Well Known" Proteins on Cell Wall Degradation and Softening

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Abstract

Fruit softening has been largely associated with cell wall degradation by a number of loosening proteins. Firmness is a major quality attribute of fresh cherries and also an important factor affecting the susceptibility to postharvest rots. By analyzing the solubilization, depolymerization and composition of pectins and hemicelluloses in cultivars with contrasting firmness we found that the pattern and extent of their wall disassembly was quite similar. No marked pectin or hemicellulose depolymerization was observed and a similar reduction in tightly-bound pectins and hemicellulose was detected in both cultivars during ripening. However, firm cherries presented pectic polymers with lower proportion of neutral sugars compared to uronic acids, suggesting that the variation in total wall polysaccharide and the branching of pectins assembled early in development or the proportion of homogalacturonan (HG) to type rhamnogalacturonan-I (RG-I) may contribute to the differences in firmness between cultivars. Dismantling of the cell wall by the action of relatively "well known" loosening agents is involved in the progressive softening occurring during ripening. Two of these proteins include polygalacturonases (*PG*) which are known to hydrolyze homogalacturonans and expansins (*Exp*) believed to participate in the relaxation of the cell wall by reducing hydrogen bonding between cellulose microfibrils and xyloglucan. We investigated the in vivo roles of these wall-disassembling proteins, by overexpressing *PG* and *Exp1* both alone and in combination in a non-ripening *rin* tomato background. The simultaneous overexpression of *PG* and *Exp1* in *rin* fruit restored softening in these non-ripening fruit. Unexpectedly, *PG* overexpression resulted in higher hemicellulose depolymerization while increased levels of *Exp1* accelerated pectin turnover. This shows that besides their "well known" in vitro functions these proteins act in muro by facilitating the degradation of non-directly targeted wall components, likely by increasing the accessibility of pre-existing wall-degrading proteins to their polysaccharide substrates.

INTRODUCTION

Firmness is a major quality attribute in fruits (Brummell, 2006). It has major implications in consumer acceptability and in fruit storage capacity. Softening also increases the susceptibility to bruising and postharvest rots (Cantu et al., 2008). Textural modifications have been related to changes in turgor pressure and tissue structure. However, in most cases the losses in firmness occurring during ripening have been correlated with the dismantling of the cell walls (Carpita and McCann, 2000). A number of studies have described the sequence of cell wall disassembly during fruit development. In most cases 1) pectin and hemicelluloses are depolymerized, 2) the solubility of CW polysaccharides increases, 3) pectin degree of esterification and pectin neutral sugar content drop, as a number of different cell wall degrading proteins (CWDP) are induced

(Brummell and Harpster, 2001). The extent to which polysaccharide dismantling occurs as well as the specific time at which it takes place depends on the species and even on the cultivar considered (Vicente et al., 2007a,b).

Studies conducted in cherry to date to identify the differences in cell wall structure that could explain the wide range in firmness found in different genotypes have not been conclusive. Soft cherries have been related with low pectin degree of polymerization (Batisse et al., 1994, 1996a,b). However, softening has been observed to occur without marked changes in pectin size or endo-PG action (Barrett and Gonzalez, 1994; Choi et al., 2002a,b). In the first part of this work we evaluated the cell wall composition of cherry cultivars with different firmness with the aim of identifying biochemical factors that may be associated with their textural differences.

Cell wall degradation and softening of fruits has been associated with the action of different cell wall degrading proteins (Brummell, 2006). The in vitro action of several CWDP is well established (Brummell and Harpster, 2001). Although our understanding of the role of these proteins has markedly increased in the last years their in vivo role is far from being elucidated and little is known about the ways they interact to disassemble the cell wall pectic and cellulose-hemicellulose (Cel-Hem) matrices (Vicente et al., 2007c). To get further insights regarding the in vivo role of CWDP in a second part of this work we evaluated the cell wall degradation and softening in *rin* tomato with increased expression of expansin and *PG* either alone or in combination (*PG+Exp1*).

MATERIALS AND METHODS

Cell Wall Differences in Cherry Cultivars with Different Firmness

Cherry fruit cultivars 'Sweetheart' and 'Newstar' were harvested at four different developmental stages, from immature to commercial maturity. Fruit firmness was evaluated by compression tests and cell walls were isolated by extracting 100 g of in ice-cold 80% ethanol (4 ml/g fruit). The fruit was blended in an Omni Mixer homogenizer (Omni International, Kennesaw, GA, USA) and the homogenate was boiled for 30 min, then cooled, and filtered through glass filter paper (Whatman GF/C). The retentate was washed with 95% ethanol. The solids were then resuspended in a mixture of chloroform:methanol (1:1, 3 ml/g fruit), stirred for 15 min and filtered. The insoluble material was washed with acetone, yielding the crude cell wall extract (alcohol insoluble residue, AIR). The AIR was air-dried in a hood and in a vacuum desiccator overnight and then weighed. The AIR were used to perform a serial cell wall fractionation yielding five fractions enriched in different groups of wall components namely water (WSF), CDTA (CSF), Na_2CO_3 (NSF), 1 M KOH (1 KSF) and 4 M KOH-soluble fractions (4KSF) as described in section C. The NSF representing to the most abundant cell wall fraction were then used to determine 1) total uronic acids (UA) and 2) neutral sugars (NS) as indicated in section C. Given that the soft cherries presented higher NS/UA ratios we tested this in two other cherry cultivars having contrasting firmness ('Sunburst', soft; and 'Regina', firm).

Effect of Overexpression of Polygalacturonase (*PG*) and/or Expansin (*Exp1*) on *rin* Tomato Cell Wall Degradation and Softening

Control 'Ailsa Craig', *rin* and transgenic *rin* tomato fruit overexpressing *PG*, *Exp1* or both genes simultaneously (*PG+Exp1*) under the control of an ethylene inducible *E8* promoter were harvested at the mature green stage and transported to the laboratory. The fruit was stored in the presence of ethylene (100 µl L^{-1}, 20°C) for 6 d. During storage we evaluated firmness; we isolated the cell walls (AIR) as indicated above and subsequently performed a fractionation of the AIRs as described in the paragraph "Cell Wall Fractionation". The carbohydrate contents in the pectin rich water soluble fraction and the hemicellulose 1 KSF-soluble fractions were analyzed. We also determined the size exclusion profiles of these fractions as indicated under the title "Cell Wall Analysis".

Cell Wall Analysis

1. Cell Wall Fractionation. AIR fractionation was performed as previously described (Raffo et al., 2011) with minor modifications. Briefly, 1 g of AIR was stirred for 4 h at room temperature with 100 ml of 0.02% (w/v) thimerosal aqueous solution and filtered. The suspension was filtered and the filtrate was saved and designated as water-soluble fraction (WSF). Sequential extraction of the pellet with 0.05 mol/L CDTA in 0.05 M NaOAc/HOAc buffer, pH 6, containing 0.02% (w/v) thimerosal (24 h), 0.1 mol/L Na_2CO_3 containing 0.02 mol/L $NaBH_4$ (24 h), 1 mol/L KOH containing 0.02 mol/L $NaBH_4$ (24 h) and 4 mol/L KOH containing 0.02 mol/L $NaBH_4$ (24 h), yielded the CDTA-soluble fraction (CSF), Na_2CO_3-soluble fraction (NSF), 1 mol/L and 4 mol/L KOH-soluble fractions (1KSF and 4KSF), respectively. The supernatants were recovered after centrifugation at 9,000 rpm at 6°C for 40 min. In the case of the KOH-soluble fractions, pH was adjusted to 5 with glacial acetic acid. All fractions were dialyzed (*MW* cut-off 6,000-8,000 Da) against tap water for 2 days and against distilled water for another day at 4°C. The fractions were recovered by freeze drying.

2. Uronic Acid, Total Carbohydrate and Neutral Sugar Measurements. Uronic acids (UA) were quantitated according to the *m*-hydroxybiphenyl method using galacturonic acid as standard, and expressed as anhydro units (Filisetti-Cozzi and Carpita, 1991). Total carbohydrates were determined by the phenol-H_2SO_4 method using glucose as standard (Dubois et al., 1956). The proportion of neutral sugars was determined after subtracting the uronic acid content from that of total carbohydrates. For this purpose, the phenol-H_2SO_4 reaction was also carried out with a galacturonic acid standard, which showed an absorbance ratio of 0.28 against the same glucose weight.

3. Size-Exclusion Chromatography (SEC). To examine the size distributions of polymers in the NSF, ca. 3 mg of lyophilized samples from each fraction were dissolved in 0.8 ml of 0.4 mg/ml imidazole to which 0.2 ml of 1 mol/L ammonium acetate (pH 5) were added. Solutions were centrifuged, and chromatographed on a low-pressure SEC by employing a 300×9 mm i.d. Sepharose CL-2B column (Sigma Chemical Co., St. Louis, MO, USA) eluted at room temperature with 0.2 mol/L ammonium acetate, pH 5. Fractions were collected and aliquots were assayed for total carbohydrates. Samples from the 1KSF, were dissolved in 0.1 mol/L NaOH, cleaned up by centrifugation, and chromatographed on a 300×9 mm i.d. Sepharose CL-6B column (Sigma Chemical Co., St. Louis, MO, USA) eluted at room temperature with 0.1 mol/L NaOH. Fractions were collected and aliquots were assayed for total carbohydrates (Dubois et al., 1956).

4. Neutral Sugar Composition. Each fraction (ca. 3 mg) was hydrolyzed with 1 ml of 2 mol/L TFA, for 90 min at 120°C in closed-cap vials. The TFA was eliminated by evaporation, and the resulting monosaccharides were reduced to alditols using $NaBH_4$, converted to alditol acetates, and subsequently analyzed using a Hewlett Packard 5890 gas chromatograph (Agilent Technologies Inc., CA, USA) fitted with a capillary column 30 m × 0.25 mm i.d. 0.20 µm, SP-2330 (Supelco Inc., Bellefonte, PA, USA) and equipped with a FID operated at 240°C. The injector temperature was 240°C and the oven temperature was kept isothermally at 220°C. Nitrogen was used as the carrier gas at a head pressure of 15 psi. Samples were injected with a split ratio of 80:1. *Myo*-inositol was used as the internal standard, and the different alditol acetates were identified by comparison with authentical standards. The percentage of the different monosaccharides was calculated by considering that the FID responses are proportional to the molecular weight of the alditol acetates.

Statistical Analysis

Data was analyzed by ANOVA and means were compared by an LSD test at a level of significance of $P<0.05$.

RESULTS AND DISCUSSION

Cell Wall Differences in Cherry Cultivars with Different Firmness

The days from fruit set to commercial maturity differed between the two cultivars tested (73 d for 'Sweetheart' and 58 d for 'Newstar'). 'Sweetheart' fruit were firmer than 'Newstar' cherries at commercial maturity. Interestingly, firmness was already different at early development, suggesting that variations in cell wall structure may contribute to explain the differences in texture (Table 1). Firm 'Sweetheart' cherries presented higher content of total cell wall material than soft 'Newstar' fruit. Both cultivars showed low depolymerization of hemicellulose and tightly bound pectins were detected and a similar pattern of pectin solubilization (data not shown). The clearest differences between the two genotypes, was the relative levels of NS and uronic acids in the NSF. Soft 'Newstar' fruit showed pectic polymers with higher neutral sugar to uronic acid ratio from early development (Fig. 1). This could indicate a lower degree of pectin branching or a higher proportion of homogalacturonan to rhamnogalacturonan I (Carpita and McCann, 2000) in firm 'Sweetheart' fruit. To further test the potential association between cherry texture and pectin NS/UA ratio we analyzed other two cultivars ('Sunburst' and 'Regina') with different firmness (Table 2). In accordance with the previous results the firmer cultivar ('Regina') showed upon maturity a lower ratio of NS to UA in the NSF than soft fruit (Fig. 2).

Rhamnose is present in the backbone of RG-I while *Ara* is the most abundant neutral sugar in cherry cell wall and is located mostly as lateral chains of this polymer (Brummell and Harpster, 2001). The analysis of the proportion of specific neutral sugars in the NSF showed that the molar ratio of *Ara/Rha* was similar in both cultivars (Fig. 3). This indirectly suggests that the side chains of RG-I were similar in all the genotypes and that the differences in the NS/UA ratio between firm and soft fruit may be more likely related to the presence of distinct proportions of branched RG-I and unbranched homogalacturonan. In this scenario firm cherry cultivars may be higher in HG.

Effect of Overexpression of Polygalacturonase (*Pg*) and/or Expansin (*Exp1*) on *Rin* Tomato Cell Wall Degradation and Softening

1. Firmness. Firmness decreased during storage in all cultivars tested (Fig. 4). The control AC showed a 4-fold reduction in firmness after 6 d of storage. Also mutant rin untransformed tomatoes softened significantly, though as expected in a much more restricted manner than wild type fruit. The overexpression of *PG* accelerated softening. A more marked effect was observed by overexpressing *Exp1*. The reduction of firmness was greater in the lines in which *PG* and *Exp1* were simultaneously overexpressed. However, the softening of *rin+PG+Exp1* tomatoes was still lower than that of the wild type fruit. Previous work has shown that the simultaneous suppression of *PG* and *Exp* reduces softening in tomato (Cantu et al., 2008). Herein, we show that the combined overexpression of these two genes can partially restore softening. This indicates *PG* and *Exp1* can cause softening but that other wall degrading proteins are also necessary for tomato softening.

2. Cell Wall Solubilization and Depolymerization. The solubility of pectin in water increased during storage. The fruit overexpressing *PG* and *Exp1* simultaneously showed the largest increase in pectin solubility followed by the single transgenic lines (Fig. 5). The increase in pectin solubility by *PG* overexpression would be expected given the known mode of action of this enzyme. Interestingly, increasing *Exp* expression also resulted in higher pectin solubility suggesting that indirect disruption of the Hem-Cel matrix affects polyuronide extractability. Contrariwise, the *PG* overexpressing lines showed lower content of hemicelluloses soluble in 1 M KOH compared to the untransformed *rin* controls. The indirect effect of *PG* and *Exp1* on non-target polyssacharides was also observed by analyzing the size exclusion profiles of the WSF and 1KSF (Fig. 6). Indeed, overexpression of *PG* not only reduced the mean molecular weight of pectins, but also caused a downshift in hemicelluloses. Unexpectedly, lower

pectin sizes compared to the controls were also found in *rin+Exp1* fruit. Although the potential indirect effect of wall degrading proteins by alteration of wall porosity or accessibility of other enzymes has been repeatedly suggested (Vicente et al., 2007c) not many works have shown direct evidence of these effects.

CONCLUSIONS

In this study we evaluated two aspects of cell wall metabolism that are still poorly understood: 1) the biochemical association between cell wall composition and firmness in the case of cherry fruit and 2) the in muro action of the cell wall degrading proteins polygalacturonase (*PG*) and expansin (*Exp1*) using *rin* tomato lines. Firm cherries presented pectic polymers with lower neutral sugar to uronic acid ratio, suggesting that the branching of polyuronide assembled early in development, or the proportion of branched rhamnogalacturonan to homogalacturnan may contribute to the differences in firmness between cultivars.

In the second part of this work by overexpressing *PG* and *Exp1* in tomato we showed that besides their "well known" in vitro functions these proteins act in vivo by facilitating the degradation of non-directly targeted wall components, likely by increasing the accessibility of pre-existing wall-degrading proteins to their polysaccharide substrates.

Literature Cited

Barrett, D.M. and Gonzalez, C. 1994. Activity of softening enzymes during cherry maturation. J. Food Sci. 59:574-577.

Batisse, C., Fils-Lycaon, B. and Buret, M. 1994. Pectin changes in ripening cherry fruit. J. Food Sci. 59:389-393.

Batisse, C., Buret, M. and Coulomb, P.J. 1996a. Biochemical differences in cell wall of cherry fruit between soft and crisp fruit. J. Agric. Food Chem. 44:453-457.

Batisse, C., Buret, M., Coulomb, P.J. and Coulomb, C. 1996b. Ultrastructure des parois de cerises Bigarreau Burlat de textures differentes au cours de la maturation. Can. J. Bot. 74:1974-1981.

Brummell, D.A. 2006. Cell wall disassembly in ripening fruit. Funct. Plant Biol. 33:103-119.

Brummell, D.A. and Harpster, M.H. 2001. Cell wall metabolism in fruit softening and quality and its manipulation in transgenic plants. Plant Mol. Biol. 47:311-340.

Cantu, D., Vicente, A.R., Dewey Bennett, A.B., Labavitch, J.M. and Powell, A.L.T. 2008. The simultaneous suppression of tomato polygalacturonase and expansin reduces the susceptibility of ripe fruits to *Botrytis cinerea*. Proc. Nat. Acad. Sci. USA 105:859-864.

Carpita, N.C. and McCann, M.C. 2000. The plant cell wall. p.52-108. In: B. Buchanan, W. Gruissem and R. Jones (eds.), Biochemistry & Molecular Biology of Plants. American Society of Plant Physiologists, Rockville, MD.

Choi, C., Toivonen, P., Wiersma, P.A. and Kappel, F. 2002a. Differences in levels of pectic substances and firmness in fruit from six sweet cherry genotypes. J. Am. Pomol. Soc. 56:197-201.

Choi, C., Wiersma, P.A., Toivonen, P. and Kappel, F. 2002b. Fruit growth, firmness and cell wall hydrolytic enzyme activity during development of sweet cherry fruit treated with gibberellic acid (GA_3). J. Hortic. Sci. Biotechnol. 77:615-621.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28:350-356.

Filisetti-Cozzi, T.M.C.C. and Carpita, N.C. 1991. Measurement of uronic acids without interference from neutral sugars. Anal. Biochem. 197:157-162.

Raffo, M.D., Ponce, N.M.A., Sozzi, G.O., Vicente, A.R. and Stortz, C.A. 2011. Compositional changes in 'Bartlett' pear (*Pyrus communis* L.) cell wall polysaccharides as affected by sunlight conditions. J. Agric. Food Chem. 59:12155-12162.

Vicente, A.R., Ortugno, C., Rosli, H., Powell, A.L.T., Greve, C.L. and Labavitch, J.M.

2007a. The temporal sequence of cell wall disassembly events in developing fruits: 2. Analysis of blueberry (*Vaccinium* sp.). J. Agric. Food Chem. 55:4119-4124.
Vicente, A.R., Ortugno, C., Powell, A.L.T., Greve, C.L. and Labavitch, J.M. 2007b. The temporal sequence of cell wall disassembly events in developing fruits: 1. Analysis of raspberry (*Rubus idaeus*). J. Agric. Food Chem. 55:4125-4130.
Vicente, A.R., Saladie, M., Rose, J. and Labavitch, J. 2007c. The linkage between cell wall metabolism and fruit softening: looking to the future. J. Sci. Food Agric. 87:1435-1448.

Tables

Table 1. Firmness (N) in 'Sweetheart' and 'Newstar' cherry fruit during development (DAFS: days after fruit set).

Cultivar	DAFS									
	40	45	47	49	54	57	58	59	67	73
Sweetheart	-	8.0 a	-	3.2 b	-	3.0 c	-	2.7 d	2.4 e	1.8 g
Newstar	1.9 f		1.5 g		0.9 h		0.7 h			

Different letters indicate significant differences based on an LSD test at a level of significance of $P<0.05$.

Table 2. Firmness (g mm^{-1}) in 'Sunburst' and 'Regina' cherry fruit at commercial maturity.

Cultivar	Firmness
Sunburst	415 b
Regina	561 a

Different letters indicate significant differences based on an LSD test at a level of significance of $P<0.05$.

Figures

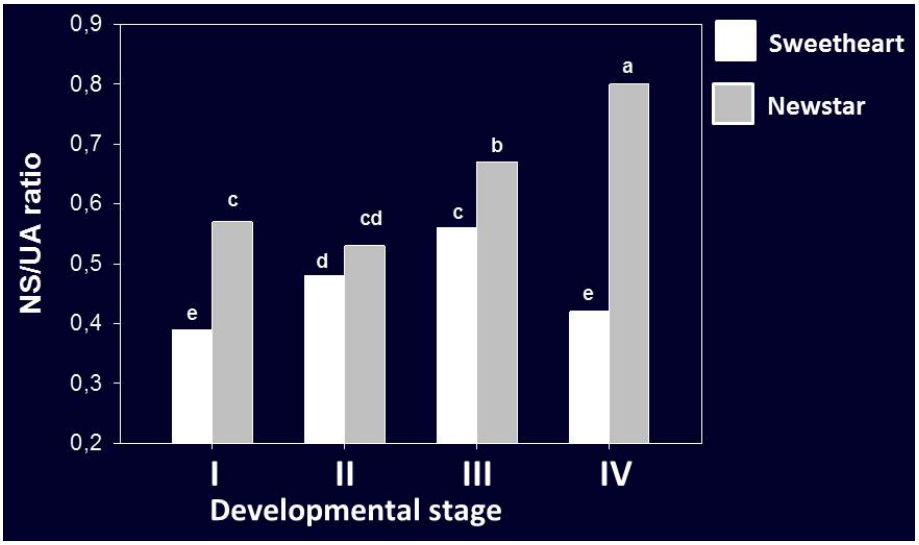

Fig. 1. Neutral sugar (NS) to uronic acid (UA) ratio of the NSF of 'Sweetheart' and 'Newstar' cherry fruit during development (stages: I (cell division/first growth phase), II (endocarp lignification), III (cell expansion/second growth phase), and IV (commercial maturity)). Different letters indicate differences based on an LSD test at a level of significance of $P<0.05$.

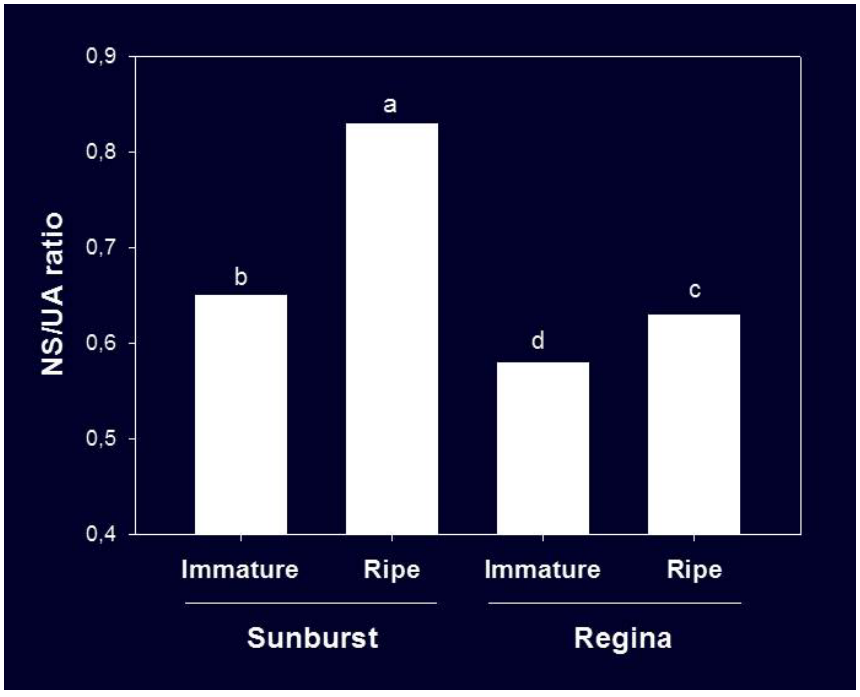

Fig. 2. Neutral sugar (NS) to uronic acid (UA) ratio of the NSF in soft ('Sunburst') and firm ('Regina') cherries. Different letters indicate differences based on an LSD test at a level of significance of $P<0.05$.

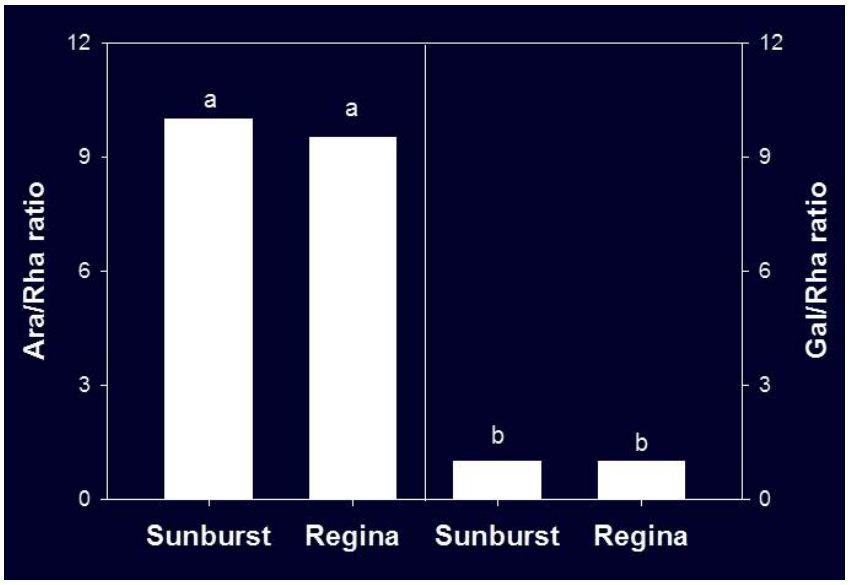

Fig. 3. Arabinose/rhamnose (left) and galactose/rhamnose (right) ratio in soft ('Sunburst') and firm ('Regina') cherries. Different letters indicate differences based on an LSD test at a level of significance of $P<0.05$.

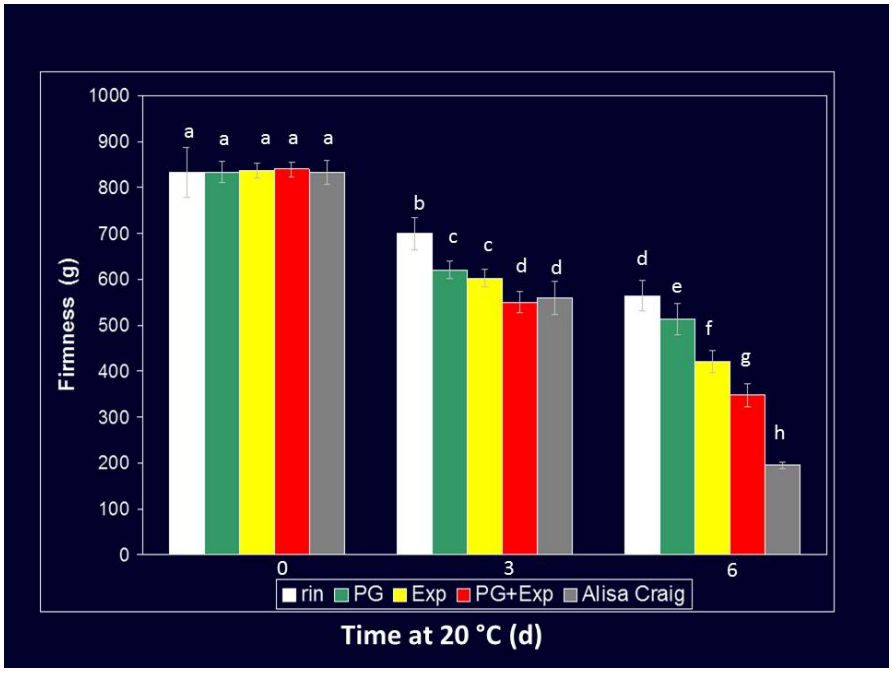

Fig. 4. Firmness or 'Ailsa Craig', or *rin* tomato lines control or overexpressing polygalacturonase (*PG*), expansin (*Exp*) or both genes (*PG+Exp*) during storage at 20°C for 6 d in the presence of ethylene (100 µl L^{-1}). Different letters indicate differences based on an LSD test at a level of significance of $P<0.05$.

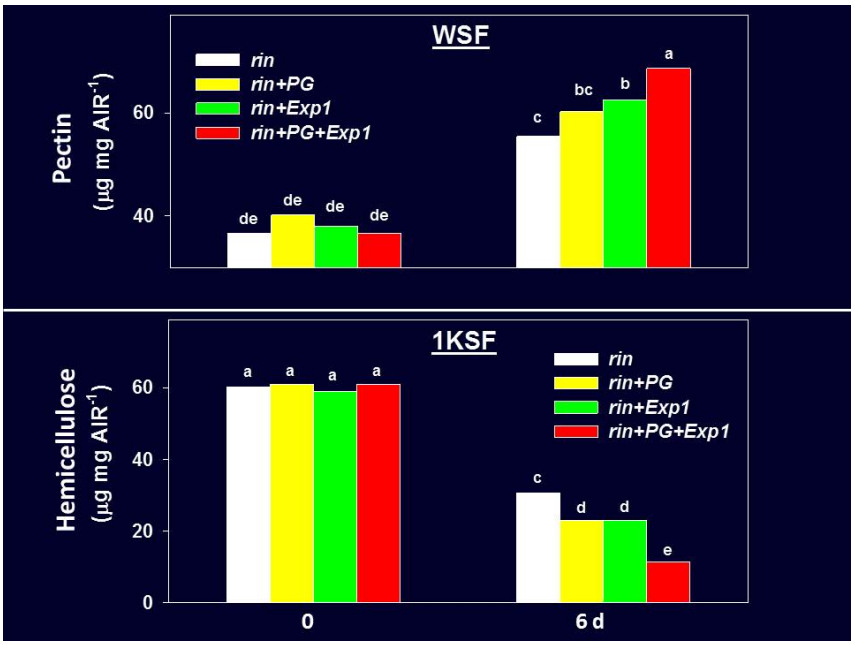

Fig. 5. Pectin (upper panel) and hemicellulose (lower panel) content in the water soluble fraction (WSF) and 1 M KOH soluble fraction (1KSF) of *rin* tomato lines control (*rin*) or overexpressing polygalacturonase (*PG*), expansin (*Exp1*) or both genes (*PG+Exp1*) during storage at 20°C for 6 d in the presence of ethylene (100 µl L^{-1}). Different letters indicate differences based on an LSD test at a level of significance of $P<0.05$.

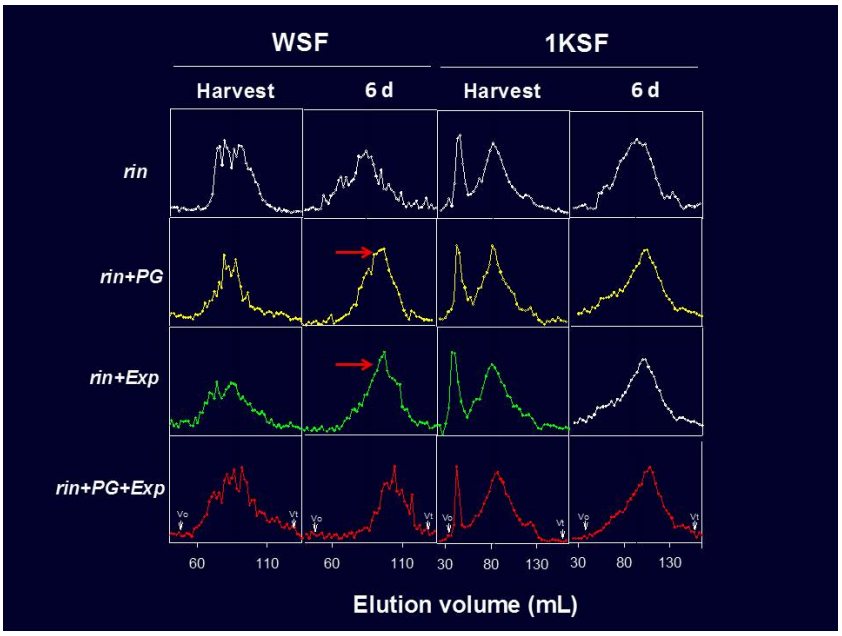

Fig. 6. Size exclusion chromatography profiles of the water soluble fraction (WSF) and 1 M KOH soluble fraction (1KSF) of *rin* tomato lines control (*rin*) or overexpressing polygalacturonase (*PG*), expansin (*Exp1*) or both genes (*PG+Exp1*) during storage at 20°C for 6 d in the presence of ethylene (100 µl L^{-1}). Different letters indicate differences based on an LSD test at a level of significance of $P<0.05$.