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Research article

Effects of heat treatment on enzyme activity and expression of key genes controlling cell wall remodeling in strawberry fruit

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ABSTRACT

Modification of cell wall polymers composition and structure is one of the main factors contributing to textural changes during strawberry (*Fragaria x* ananassa, Duch.) fruit ripening and storage. The present study aimed to provide new data to understand the molecular basis underlying the postharvest preservation of strawberry cell wall structure by heat treatment. Ripe fruit (cv. Aroma) were heat-treated in air oven (3 h at 45 °C) and then stored 8 days at 4 °C + 2 days at 20 °C, while maintaining a set of non-treated fruit as controls. The effect of heat stress on the expression pattern of key genes controlling strawberry cell wall metabolism, as well as some enzymatic activities was investigated. The expression of genes proved to be relevant for pectin disassembly and fruit softening process (*FaPG1*, *FaPLB, FaPLC*, *FaAra1, FaβGal4*) were down-regulated by heat treatment, while the expression of genes being involved in the reinforcement of cell wall as pectin-methylesterase (*FaPME1*) and xyloglucan endo-transglycosilase (*FaXTH1*) was up-regulated. Total cell wall amount as well as cellulose, hemicellulose, neutral sugars and ionically and covalently bounded pectins were higher in heat-stressed fruit compared to controls, which might be related to higher firmness values. Interestingly, heat stress was able to arrest the *in vitro* cell wall swelling process during postharvest fruit ripening, suggesting a preservation of cell wall structure, which was in agreement with a lower growth rate of *Botrytis cinerea* on plates containing cell walls from heat-stressed fruit when compared to controls.

1. Introduction

The plant cell wall not only determines the shape of cells and acts as an effective physical barrier against pathogens, but also it is a dynamic structure able to change its architecture and composition as a response to biotic and abiotic stimuli (Cantu et al., 2008a; Le Gall et al., 2015).

Commercial strawberry (*Fragaria x ananassa*, Duch.) is a soft fruit highly appreciated by consumers because of their organoleptic properties as well as nutritional quality (Reganold et al., 2010). Nevertheless, its high softening rate leads to a fast postharvest deterioration, both challenging fruit transportation and reducing fruit shelf life. Hence, the understanding of changes in cell wall metabolism in response to physical or chemical stimuli is a matter of interest.

Cell wall disassembly is the main process leading to softening of strawberry fruit and, in a great extension, loss of fruit firmness is due to coordinated action of enzymes involved in pectins disassembly such as polygalacturonases (PGs), pectate lyases (PLs), β-galactosidases (β-Gal) and arabinofuranosidases (Aras) (Posé et al., 2011; Paniagua et al., 2016). However, the contribution to strawberry fruit firmness of proteins acting over the hemicellulosic fraction such as β-xylosidases (β-Xyl) and xyloglucan endo-transglycosylases/hydrolases (XHTs) should not be discarded (Bustamante et al., 2006; Nardi et al., 2014).

Pectins represent around 60% of strawberry cell wall, with homogalacturonans (HGs) being the main polymers (Villarreal et al., 2016; Paniagua et al., 2017). Homogalacturonans are exported to the apoplast with a great percentage of methyl esterification at C-6 of galacturonic acid residues and the de-esterification process is catalyzed by pectin methylesterases (PMEs) (Pelloux et al., 2007). Demethylated HGs from

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pectins can bind Ca^{2+} and connect each other in order to reinforce cell wall pectins, but also represent the substrate of pectin degrading enzymes such as PGs and PLs (Posé et al., 2011). In that sense, pectin methylesterase activity represents a key control point for both the assembly and disassembly of pectin networks (Pelloux et al., 2007). Moreover, it has been shown that the ectopic over-expression of *FaPME1* in the woodland strawberry (*Fragaria vesca*) rendered fruit with pectin oligogalacturonides showing lower degree of methyl esterification and being more resistant to *Botrytis cinerea* infection than wild type (Osorio et al., 2008).

In this way, the application of abiotic stimuli that might up-regulate endogenous PMEs in strawberry fruit, while down-regulated pectin degrading genes proved to be relevant in the strawberry softening process by transgenic and biochemical approaches (Rosli et al., 2009; Posé et al., 2011, 2015; Paniagua et al., 2016), will be of great interest both from a fundamental and an applied point of views. Several reports have shown that heat treatment is an effective technique to preserve strawberry fruit quality during post-harvest storage (Vicente et al., 2002, 2005; Lara et al., 2006). As regards heat treatment effect on strawberry cell wall metabolism, although it has been reported that it is able to increase total PME activity (Vicente et al., 2005), as well as decreased other enzymes activities (as PG, β-Gal, β-Xyl, Lara et al., 2006; Martínez and Civello, 2008), the information about the action of this physical stimulus on the expression pattern of relevant genes controlling cell wall metabolism during postharvest ripening is still scarce. Particularly the effect of heat treatment on the expression of genes strongly related to strawberry fruit firmness as *FaPME1*, *FaPLC*, *FaβGal4*, among others, has not been studied.

In this framework, our hypothesis is that heat stress will be able to change the fruit cell wall composition and possibly, its architecture through an up-regulation of endogenous PMEs, while down-regulates the expression of genes involved in strawberry cell wall disassembly. We proposed to make a contribution to the actual knowledge of strawberry cell wall response to heat treatment during postharvest fruit ripening, both from biochemical and molecular approaches. The already known positive effect of heat treatment in the preservation of strawberry fruit quality is discussed in relation to results obtained in terms of its effects on gene expression, enzyme activities, cell wall swelling, *in vitro* pathogen growth and cell wall content.

2. Materials and methods

2.1. Fruit material and heat treatment

Three hundred strawberry (*Fragaria* × *ananassa*, Duch, cv Aroma) fruits were harvested at commercial ripening stage (75–90% red) from local producers (La Plata, Buenos Aires Province, Argentina) and immediately transported to the laboratory. Fruits of similar size and without visible damage were selected, the peduncle of each fruit was cut at 30 mm from the receptacle base, and fruit were classified according to size and shape. Two hundred and forty fruit were put in fourteen plastic trays (a mean of seventeen strawberries per tray) and covered with PVC film. A set of 120 fruits (seven trays) was used for each condition analyzed according to: Control (C): 3 h, air at room temperature and; Heat treatment (H): 3 h, air oven at 45 °C (according to Vicente et al., 2002). Fifty heat-treated fruits along with fifty control fruits (three trays per each condition) were taken immediately after each treatment (0 days or i) and used to measure firmness as described below, then were cut into small pieces, frozen in liquid nitrogen and stored at −80 °C until use. Seventy heat-treated fruits along with seventy control fruit (four trays per each condition) were taken after 8 days of cold storage (4 °C) + 2 days at 20 °C (shelf condition) (final or f), and were used to measure firmness, weight loss and fruit decay. Then, fruits were cut into small pieces, frozen in liquid nitrogen and stored at −80 °C until use.

2.2. Firmness

Fruit firmness was measured for each condition at initial (i) and final (f) time using a texture analyzer (Texture Analyzer, TA. XTPlus, Stable Micro Systems Texture Technologies). Fruit were compressed 0.8 mm with a 25 mm diameter cylinder Perspex probe and the maximum force (in Newton, N) developed during the test was recorded. Sixty fruits were used for each condition and time of analysis (i and f), and two measures per fruit were made (each fruit was rotated in a 180° angle between measures).

2.3. Weight loss and fruit decay evaluation

Seventy heat-treated fruit (divided in four trays) along with seventy control fruit (divided in four trays) were used to evaluate weight loss as well as fruit decay.

For weight loss assay, the weight of each fruit was recorded immediately after treatment and during storage. For fruit decay assay, fruit were classified in agreement with the absence (no rots) or presence (rots) of macroscopic fungal growth after visually evaluation for each condition at the end of storage (8 days at 4 °C and 2 days at 20 °C).

2.4. Anthocyanins, total sugar content, phenolic compounds, pH and titratable acidity

All these fruit quality parameters were measured according to Villarreal et al. (2010) with slight modifications.

For anthocyanins, 5 g of frozen fruit were ground in presence of liquid nitrogen with a mortar and pestle. The resultant powder (approximately 0.3 g) was poured into 3 ml of 1% (v/v) HCl in methanol and kept at 0 °C during 10 min. The slurry was centrifuged for 10 min at 1500 × g at 4 °C, the supernatant was recovered and its optical density (OD) was measured at 515 nm. The quantity of anthocyanins was shown as micromoles of pelargonidin-3-glucoside per kilogram of fruit ($E_{molar} = 3.6 \times 10^6\ M^{-1}\ m^{-1}$). For total sugar content, 5 g of frozen strawberry fruit were pulverized with a mortar and pestle in liquid nitrogen, and 0.4 g of the powder was suspended with 6 ml of ethanol. The mixture was centrifuged at 9000 × g during 10 min at 4 °C, and an aliquot of 1 ml from the supernatant was diluted to a final volume of 50 ml with distilled water. Then, 0.1 ml of extract was mixed with 1 ml of 0.2% (w/v) anthrone in 72% (v/v) H_2SO_4. The mixture was incubated for 12 min at 100 °C and cooled in a bath of ice-water. Total sugar content was measured spectrophotometrically (λ = 625 nm) and results were expressed as grams of glucose per kilogram of fresh fruit.

For total phenolic compounds, frozen fruit (5 g) were ground in presence of liquid nitrogen with a mortar and pestle. Then, 1 g of the powder was suspended with 6 ml of absolute ethanol and the mixture was centrifuged at 9000 × g during 10 min at 4 °C. One milliliter of the resultant supernatant was brought to 5 ml with distilled water. The solution obtained was used to determine total phenolic compounds with the Folin-Ciocalteu reagent. A standard curve was built using 10 mg ml^{-1} gallic acid in 100% (v/v) ethanol and phenolic compounds content was expressed as grams of phenol per kilogram of fruit.

For anthocyanins, total sugars and total phenolic compounds measurements, three independent samples per condition (control and heat-treated fruit, at initial and final time) were analyzed.

Titratable acidity was expressed as milliequivalents of H^+ per kilogram of fresh fruit. Two independent samples per condition were analyzed and each sample was titrated in duplicate.

2.5. Cell wall enzymes activity

For each enzymatic measurement, three independent extracts from treated- and control fruit both immediately after treatment and after cold + shelf storage, were prepared, and activity of each extract was measured twice.

2.5.1. Pectin methylesterase

Total PME activity was measured according to Vicente et al. (2005) with modifications. Five grams of frozen fruit were homogenized with 15 ml of 1 M NaCl and 1% (w/v) PVPP. The suspension was stirred during 4 h at 4 °C and then centrifuged at 12,000 × g for 30 min at 4 °C. The supernatant was collected, adjusted to pH = 7.5 with 0.1 M NaOH and used for assaying the enzyme activity in a mixture containing 30 µl of enzymatic extract, 185 µl of 1% (w/v) of partially methylated (≥85% esterified) citrus pectins pH = 7.5 (as enzyme substrate), 45 µl of 0.01% bromothymol blue in phosphate buffer pH = 7.5 and 40 µl of distilled water pH = 7.5. The reduction of OD measured at λ = 620 nm was followed spectrophotometrically during 30 min keeping the reaction-mixture at 37 °C. A calibration curve was built using different volumes of 0.01 M galacturonic acid pH = 7.5 as standard. PME activity was expressed as µmol of demethylated galacturonic acid (GalA) generated per second and per kilogram of fruit.

2.5.2. Polygalacturonase

Total PG activity was assessed according to Villarreal et al. (2008). Five grams of frozen strawberries were homogenized with 15 ml of 0.05 M sodium acetate/acetic acid, 1% (w/v) PVPP, pH = 6.0. The mixture was centrifuged at 12,000 × g for 30 min at 4 °C and the supernatant was eliminated while pellet was saved. After washed two times with 15 ml of buffer 0.05 M sodium acetate/acetic acid pH = 6.0, pellet was centrifuged at 12,000 × g during 30 min at 4 °C, the supernatant was discarded and the sample was suspended with 15 ml of 0.05 M sodium acetate/acetic acid pH = 6.0 containing 1 M NaCl. The mixture was stirred during 2.5 h at 4 °C and then centrifuged at 12,000 × g for 30 min at 4 °C. The supernatant was dialyzed overnight with 0.05 M sodium acetate/acetic acid pH = 6.0 at 4 °C. The dialyzed extract was used to determine PG activity in a mixture containing 700 µl of enzymatic extract and 700 µl of 0.3% (w/v) polygalacturonic acid in 0.05 M sodium acetate/acetic acid pH = 6.0. The colorimetric reaction was assayed using 100 µl of enzyme mixture, 0.1 M sodium tetraborate pH = 9.0 and 1% (w/v) 2-cianoacetamide in a total volume of 1.1 ml. A calibration curve was built using different volumes of 0.001 M galacturonic acid as standard. Results were expressed as nmol of galacturonic acid released per second per kilogram of fruit.

2.5.3. β-Xylosidase and β-galactosidase

Total β-Xyl activity was measured according to Villarreal et al. (2010), while β-Gal activity was assayed according to Trainotti et al. (2001). Five grams of frozen strawberries were homogenized with 15 ml of 0.05 M sodium acetate/acetic acid, 1M NaCl, 1% (w/v) PVPP, pH = 6.0. The mixture was stirred during 2 h at 4 °C and then centrifuged at 10,000 × g for 10 min. The supernatant was saved to determine both enzyme activities. For β-Xyl activity, a mixture of p-nitrophenyl-β-D-xylopyranoside, 0.05 M sodium acetate/acetic acid pH = 4.5 and extract enzyme was used in 1.5 ml as final volume. Meanwhile, for β-Gal activity, 0.03 M p-nitrophenyl-β-D-galactopyranoside, 0.05 M sodium acetate/acetic acid pH = 4,5 and extract enzyme were put in contact in a volume of 2 ml. Reactions were stopped at different times using 500 µl of 1% (w/v) Trizma base and 500 µl of 0.4 M Na_2CO_3 for β-Xyl and β-Gal, respectively. For both enzymes, activity was expressed as nmol of p-nitrophenol released per second per kilogram of fruit.

2.5.4. α-L-Arabinofuranosidase

Total α -Ara activity was measured as described by Rosli et al. (2009). Five grams of frozen strawberries were homogenized with 30 ml of 0.05 M sodium acetate/acetic acid (pH = 6.0), 1 M NaCl, 0.05% (v/v) Triton X-100, 10 mM EDTA, 2 mM PMSF, 1% (w/v) PVPP. Samples were stirred at 4 °C during 4 h, and then centrifuged 30 min at 10,000 × g at 4 °C. The supernatant was saved and used to determine α-L-arabinofuranosidase activity, using 4-nitrophenyl-α-L-arabinofuranoside as substrate. The reaction mixture was prepared as follows: 250 ml of 3 mM 4-nitrophenyl-α-L-arabinofuranoside in 150 mM citrate buffer (pH = 4.5) and 300 ml of enzymatic extract. The mixture was incubated at 37 °C, aliquots were taken at different times, and the reaction was stopped by freezing samples in liquid nitrogen. The quantity of 4-nitrophenol released was determined by measuring the OD at 410 nm using p-nitrophenol to perform a standard curve and, results were expressed as nmol of 4-nitrophenol released per second per kilogram of fruit.

2.6. Degree of esterification

The degree of pectin esterification (DE) was determined according to Galetto et al. (2010) with slight modifications. The DE was expressed as the mole ratio of methanol released after alkaline hydrolysis per 100 mg of AIR to mole of total GalA per 100 mg of AIR. Approximately 60 mg of AIR and 25 ml of phosphate buffer (pH = 7.5) were shaken and 25 ml of 1.0 M KOH were added. Then, solution was incubated at room temperature for 30 min, the pectin hydrolysates were neutralized with H_3PO_4 to pH = 7.5 and then diluted to 100 ml. Methanol content in aliquots of the hydrolyzed pectin samples were measured through its oxidation to formaldehyde using the enzyme alcohol oxidase and, followed by condensation with 2,4-pentanedione to obtained a colored product. Absorbance was measured at 412 nm, and different amounts of methanol were used to build a standard curve.

2.7. RNA isolation, reverse transcription and real-time PCR assays

The 2-butoxyethanol method (Manning, 1991) was used for total RNA isolation from 5 g of frozen strawberry fruit, then treated with DNAase I (Promega) and purified with chloroform:octanol (24:1). Five independent RNA extractions were performed for treated and control fruit and first strand of cDNA was obtained by using 1 µg of total RNA, 1 µl of Moloney murine leukemia virus RT (200 U μl^{-1}; Promega), 0.03 mM dNTPs, 330 pmoles of random primers (Biodynamics S.R.L., Buenos Aires, Argentina), 5 µl of: 250 mM Tris-HCl, 375 mM KCl, 15 mM $MgCl_2$ and 50 mM DTT (pH = 8.3) and distilled water up to a total volume of 25 µl. Sequences of primers used, sizes of amplified fragments, accession numbers, and EC numbers (when correspond) are listed in Table STI. The amplification reactions were performed using Fast Start Universal SYBR Green Master Rox 2× (Roche) according to the manufacturer's instructions, in a Step One Plus Real-Time PCR System (Applied Biosystems). Real Time-PCR conditions were: 95 °C during 10 min, 40 cycles of 95 °C for 15 s, 60 °C for 1 min, and a melting curve from 60 °C to 95 °C at 0.3 °C increments was introduce. A dilution series was built to estimate the amplification efficiency of each pair of primers (85–100%), using cDNA from 50% red strawberry fruit samples as template. For each gene analyzed, a negative water control was included in each run. The relative expression level corresponding to mean of five biological replicates was normalized against the expression level of *FaGAPDH1* gene (which showed constant expression level throughout all condition analyzed). Relative expression levels were calculated conforming to the method described by Pfaffl (2001) and expressed in arbitrary units ± SEM.

2.8. Isolation of cell wall polysaccharides

All measurements were performed according to Villarreal et al. (2010). For strawberry cell wall polysaccharides isolation, 5 g of frozen fruit were homogenized with 20 ml of absolute ethanol and boiled with reflux during 30 min. The resultant homogenate was vacuum filtered and the residue was washed three times with 15 ml of absolute ethanol. Afterwards, the residue was dried for 12 h at 37 °C and weighed. Three independent strawberry cell wall extracts (expressed as Alcohol Insoluble Residues or AIRs) were obtained for heat-treated and control fruit at final time of assay (f). For extraction and quantification of pectins, 50 mg of AIRs were homogenized in 50 ml of distilled water

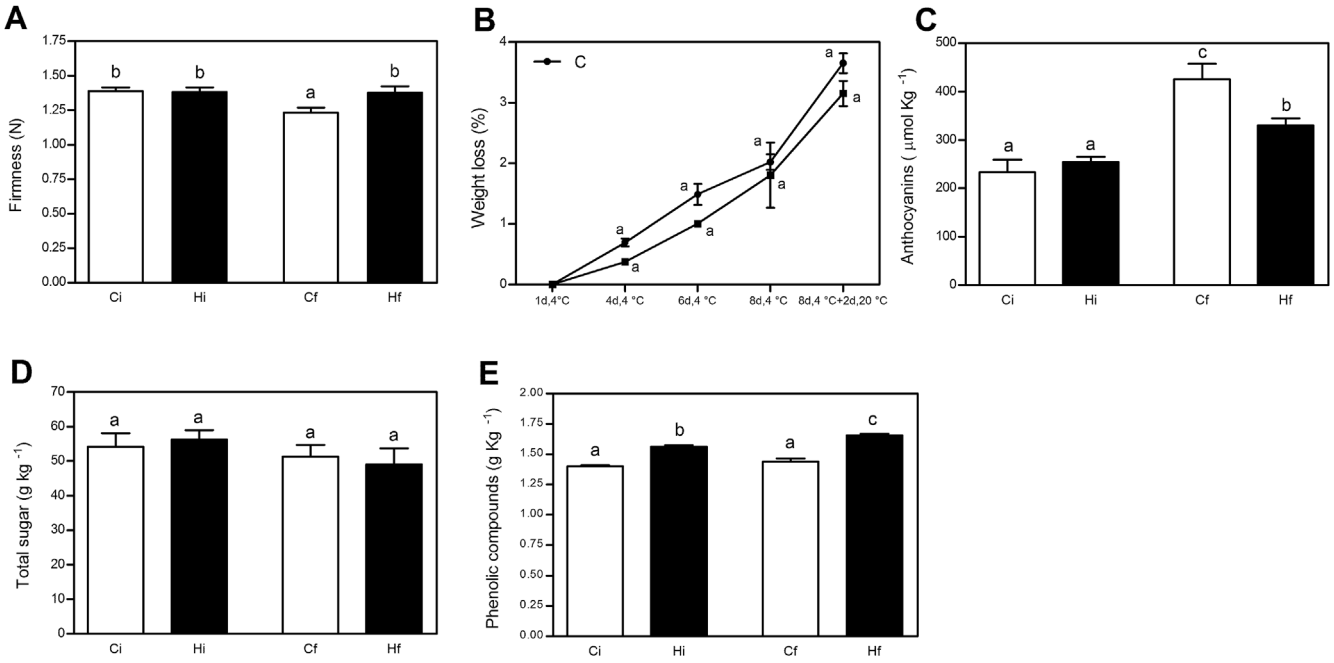

Fig. 1. Effect of heat stress on fruit firmness level (A), weight loss (B), anthocyanins (C), total sugars (D) and phenolic compounds content (E) compared with controls both immediately after assay (i) and after 8 days at 4 °C + 2 days at 20 °C of storage (f). Ci, Cf: control fruit at i and f; Hi, Hf: heat-treated fruit at i and f. Bars represent the mean ± SEM of three replicates. For A and C-E different letters indicate statistically significant differences at $P < 0.05$ (One-way ANOVA and Tukey), for B different letters indicates statistically significant differences at $P < 0.05$ between heat-treated and controls (One-way ANOVA and Dunnett).

and shaken overnight at 20 °C. The homogenate was vacuum filtered and the solid was washed three times with 5 ml of distilled water. Then, filtrates obtained were pooled and named as water-soluble pectins (WSP). The residue was subsequently suspended in 25 ml of 0.05 M sodium acetate which contained 0.04 M EDTA, pH = 4.5 and shaken at 20 °C for 4 h. The vacuum filtered homogenate was reserved and the solid was washed three times with 2.5 ml of the same buffer. The filtrates were pooled and named as EDTA-soluble pectins (ESP). Lastly, the residue was suspended in 25 ml of 0.05 M HCl and heated at 100 °C with agitation during 1 h. The homogenate was cooled and vacuum filtered, and the residue was washed three times with 2.5 ml of 0.05 M HCl. The filtrates were pooled and named as HCl-soluble pectins (HSP). Uronic acid concentration of each fraction was evaluated by the m-hydroxydiphenyl method using galacturonic acid (GalA) as standard.

Neutral sugars (NS) concentration was measured over total pectins (extracted from AIR in a similar way of HSP extraction) by the anthrone method with glucose as standard.

For extraction and quantification of hemicelluloses and cellulose, the washed residue from pectin extraction was mixed with 50 ml of 4 M NaOH and shaken for 8 h 20 °C. The vacuum filtered homogenate was washed three times with 5 ml of 4 M NaOH. Then, filtrates were joined and named as hemicellulose fraction. The remained solid was considered as cellulose. For quantification of hemicelluloses and cellulose samples were completely hydrolyzed with 66% v/v H_2SO_4 at 37 °C for 1 h and estimated as glucose with the anthrone method.

2.9. In vitro cell wall swelling

Five milligrams of AIRs (from strawberry ripening stages as well as control and heat-treated fruit) were suspended in 2 ml of sterile distilled water and shaken horizontally for 24 h at 22 °C. Tubes were then placed vertically, and cell wall swelling was assessed based on the height of the sedimented AIR layer according to Villarreal et al. (2016). Three independent AIR extracts were used for each condition analyzed.

2.10. In vitro pathogens growth rate assay

Botrytis cinerea strain (B05.10 of the IIB-INTECH Fungal Culture Collection IFCC 458/02) was routinely maintained in potato-dextrose agar slants at 4 °C and used for each assay. Previously to inoculation, mycelium was grown at 24 °C in solid Czapek-Dox medium (50 g glucose, 2 g $NaNO_3$, 1 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.5 g KCl, 0.05 g $FeSO_4.7H_2O$, 20 g agar, pH = 5.5–6.0). In a previous work, a growth curve was performed, transferring a 4 mm^2 agar plug from the edge of a 5-day-old actively growing culture of *B. cinerea* to the solidified plates containing agar 0.8% (w/v) and 0.02, 0.1, 0.2 or 2 mg ml^{-1} of cell walls (AIRs) from non-treated fruit (Villarreal et al., 2016). Subsequently, plates were incubated at 24 °C, and the fungus growth area was measured after 24, 48 and 72 h. According to growth curve results, next assays were developed using 0.2 mg ml^{-1} of AIRs from heat-treated fruit and controls. The size of the *B. cinerea* growth areas (mm^2) were measured using the Image-Pro®Plus software (Media Cybernetics Inc., San Diego, CA), after 24, 72 and 120 h. Three independent AIRs extractions were used for each heat-treated and control fruit, and five technical replicates of each of them were made.

2.11. Statistical analysis

Data for fruit decay assay were analyzed by Chi-squared (χ^2) analysis to test the dependence between treatment and rots development, while results for cell wall and polymers content were analyzed by Student's t-test. Data corresponding to the rest of experiments was analyzed by One-way ANOVA, and means compared with Tukey as post-test (except for Weight loss and *B. cinerea* growth where Dunnett was used as post-test) at a significance level of 0.05.

3. Results and discussion

3.1. Fruit firmness, weight loss, anthocyanins, total sugars and phenolic compounds

In order to determine if chosen heat treatment conditions were

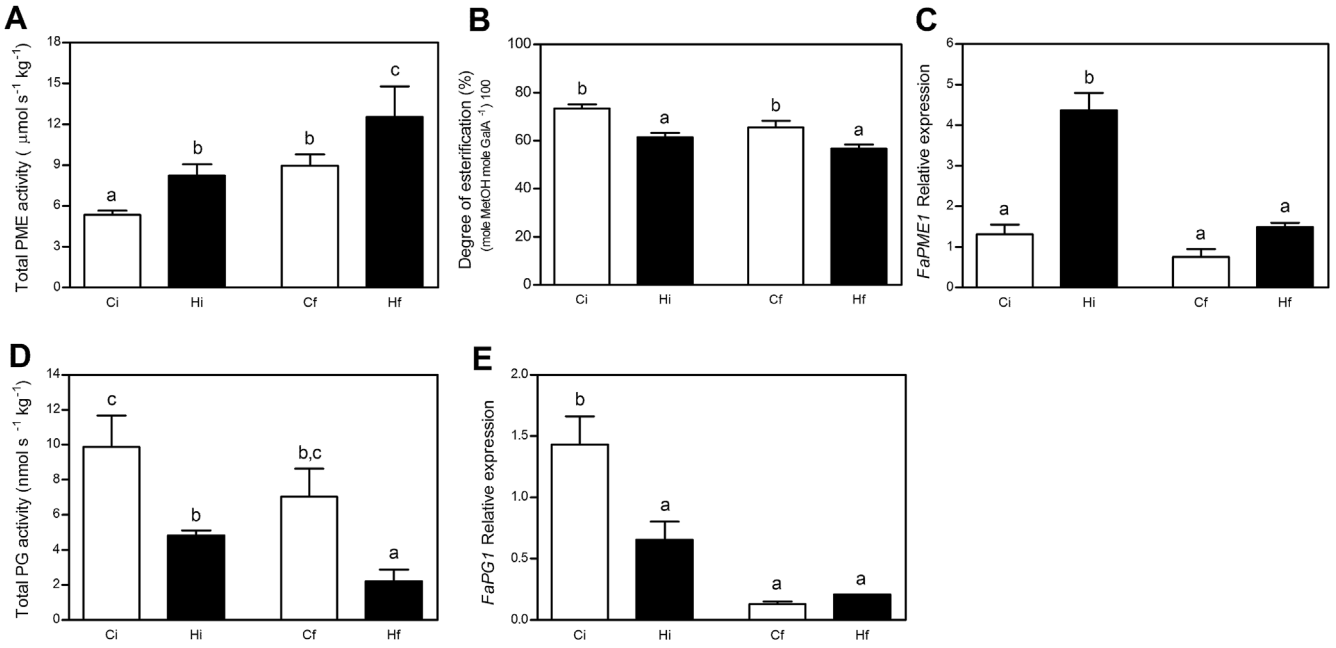

Fig. 2. Effect of heat treatment on PME activity (A), Degree of esterification (B), *FaPME1* gene expression (C), PG activity (D) and *FaPG1* gene expression (E). Relative expression values represent the expression level of each target gene normalized against *FaGAPDH1* abundance and compared with controls. Bars represent the mean ± SEM of three replicates for total enzyme activity and, five replicates for Real Time PCR assay. Different letters indicate statistically significant differences at $P < 0.05$ (One-way ANOVA, Tukey).

effective to delay postharvest fruit ripening in Aroma cultivar, a set of common parameters was evaluated.

Immediately after treatment (i) both heat-stressed and control fruit showed the same firmness level. After 8 days of incubation at 4 °C + 2 days at 20 °C (f), a decrease on firmness level in control fruit was detected, while firmness of heat-treated fruit remained constant and were significantly higher than controls (Fig. 1A). As well, anthocyanins accumulation was studied as a typical parameter used to follow strawberry ripening during postharvest (Villarreal et al., 2010). No differences in that group of pigments were found at initial time between treated- and control fruits. On the other hand, after cold + shelf storage, anthocyanins levels increased both in control and treated fruit, but a significant lower accumulation of these pigments was observed in heat-treated fruit than in controls (Fig. 1C). In this manner, the effectiveness of heat stress to delay postharvest strawberry fruit ripening in Aroma cultivar was proved. Moreover, our results are in agreement with reported on Selva cultivar by Vicente et al. (2002).

After 1 day at 4 °C, neither heat-treated nor control fruits showed weight loss respect to the weight recorded immediately after treatment (Fig. 1B). Then, after 4, 6 and 8 days at 4 °C fruits from control and heat treatment showed an increase in weight loss (1–2%), although the accumulated losses did not differ statistically between them. Once fruits were brought to 20 °C (8 days at 4 °C + 2 days at 20 °C), all fruits reached an average of 4% of weight loss relative to initial values, not showing significant differences between control and heat-treated.

Considering total sugar content, no differences between treated- and control fruit were observed both immediately after treatment (i) and after storage (f) (Fig. 1D). Previous studies have shown that strawberry fruit did not accumulate appreciable amount of sugars after harvest, and starch degradation account only for 3% of total sugars in ripe fruit (Cordenunsi et al., 2003; Souleyre et al., 2004). In that sense, no differences (as it was detected) or a slightly increase of fruit sugar content during storage might be expected for control fruits. Concerning heat-treatment effect on total sugar accumulation during storage, it seems to be cultivar dependent, as it was reported that heat treatment increased sugar content in Pajaro cultivar (Lara et al., 2006), while no differences between controls and treated fruit were detected in Selva cultivar (Vicente et al., 2002).

As regards phenolic compounds, no changes were detected during storage of control fruits but notably, heat treatment increased the content of these molecules before and after storage (Fig. 1E). In these sense, a reinforcement of cell wall in roots of tomato through induction of phenolic compounds by elicitors (Mandal and Mitra, 2007) and, an increase in total phenolic content and an induced resistance against *B. cinerea* by heat treatment in strawberries has been reported (Jin et al., 2016). In the present work, an increase in cell wall phenolic compounds as result of heat stress could be related to higher fruit firmness (Fig. 1A) and, lower growth of *B. cinerea* observed in heat-treated fruits (Fig. 5).

On the other hand, it was observed that pH of strawberry juice was not significantly modified under any condition analyzed, and no difference on titratable acidity was found either (Table STII). Moreover, hot air treatment did not affect external appearance, rendering fruits with healthier appearance than controls (data not shown).

3.2. Heat stress modifies enzyme activity and expression of relevant genes controlling strawberry cell wall metabolism

The central role of pectin matrix in the softening process of strawberry fruit has been thoroughly proved both by functional characterization of genes using transgenic plants and from biochemical approaches (Jiménez-Bermúdez et al., 2002; Osorio et al., 2008; Quesada et al., 2009; Paniagua et al., 2016, 2017).

Accordingly, we decide to study the response to heat stress of two central pectin-modifying activities in strawberry fruit (pectin methylesterase and polygalacturonase) as well as evaluating the possible correspondence with the expression pattern of genes involved in the resistance to *B. cinerea* (*FaPME1*; Osorio et al., 2008) and fruit firmness (*FaPG1*; Villarreal et al., 2008; Quesada et al., 2009). We observed that total PME activity increased after storage both for control and heat-treated fruits but, heat treatment provoked a significant increase in endogenous pectin methylesterase activity compared to controls both immediately after treatment (i) and after 8 day at 4 °C + 2 day at 20 °C (f) (Fig. 2A). Moreover, we study the effect of heat treatment on the degree of esterification of pectins and we found that, in agreement with

PME activity, it was detected lower DE on heat-treated fruit than in controls both before and after storage (Fig. 2B).

Furthermore, a compelling up-regulation of *FaPME1* was detected in heat-treated fruit immediately after treatment when compared with controls (Fig. 2C). *FaPME1* expression is specific for strawberry fruit ripening (Castillejo et al., 2004), and transgenic strawberries over-expressing this gene showed oligogalacturonides with a reduced degree of esterification in comparison with those from wild-type fruit (Osorio et al., 2008). Moreover, authors reported that reduced esterification was necessary to elicit defense responses in strawberry. To our knowledge, the present work constitutes the first one reporting the up-regulation of *FaPME1* by heat treatment.

With respect to polygalacturonase, heat treatment significantly decreased total PG activity both immediately after stress application (i) and after cold + shelf storage (f) when compared with controls (Fig. 2D). In agreement, *FaPG1* gene expression was remarkably down-regulated in heat-treated fruit compared to controls previously to storage (Fig. 2E). In strawberry, *FaPG1* gene expression has been associated to fruit ripening as well as textural differences between cultivars (Quesada et al., 2009; Villarreal et al., 2008). Recently, it has been reported that silenced *FaPG1* strawberry fruit shows remarkably modifications in the nanostructure of pectins, which correlates with a significant reduction in fruit softening (Posé et al., 2015). It is worth to mention that the not correspondence between enzymes activities and *FaPME1* and *FaPG1* expression detected at the end of storage (f), might be because of the contribution of other PME and PG genes. Nevertheless, we proposed that the effect of heat stress up- and down-regulating *FaPME1* and *FaPG1* respectively just after treatment, would be contributing to maintain pectin structure during storage, as homogalacturonans with less degree of esterification will be able to bind Ca^{2+} and connect each other in order to reinforce cell wall pectins, which will be benefit by a decrease on PG activity and *FaPG1* expression.

Heat treatment effect on the expression pattern of three ripening-related pectate lyase genes (*FaPLA*, *FaPLB* and *FaPLC*, Benitez-Burraco et al., 2003) was also investigated. Heat stress effect on *FaPLC* expression was particularly interesting as this gene was functional characterized trough antisense transgenic strawberry plants and a delay of fruit softening was showed (Jiménez-Bermúdez et al., 2002). In the present work, no changes in *FaPLA* expression was detected immediately after assay (i) and after storage (f) both for control and treated fruits and, heat stress did not change *FaPLA* expression compared to controls (Fig. 3A). Nevertheless, heat-treatment provoked a significant down-regulation of *FaPLB* and *FaPLC* expression with regard to not-treated fruits prior storage (Fig. 3B and C).

The effect of heat treatment in side chain metabolism of pectins and hemicelluloses was also studied. As regards, being β-galactosidase and α-arabinofuranosidase activities involved in degradation of galactans and arabinans from plant cell wall (Redgwell et al., 1997a), the effect of heat treatment on enzyme activities, as well as related gene expression, was evaluated.

A significant decrease on β-gal activity was detected on heat-treated fruits compared to controls immediately after assay (i), then activity decreased for controls remaining at the same low level for heat-treated fruit (f). In strawberry, it has been isolated four cDNAs encoding β-galactosidase genes (Trainotti et al., 2001; Paniagua et al., 2016). While *FaβGal1* showed an expression pattern related to fruit ripening, *FaβGal2* and *FaβGal3* expressed mainly in immature green fruits (Trainotti et al., 2001). As regards *FaβGal4*, it has been shown that the antisense down-regulation of this gene increases cell wall galactose levels and reduces fruit softening in transgenic strawberry fruits (Paniagua et al., 2016). In the present work, the effect of heat treatment on the expression pattern of these genes was evaluated. No differences in the expression level of *FaβGal1* was detected between heat-stressed and controls both immediately after treatment and after storage (Fig. 4B). Nevertheless, *FaβGal1* expression decreased significantly after storage both for control and treated fruits explaining, at least in part, the reduction on β-Gal activity observed after storage (Fig. 4A). Heat stress seems not to provoke a change in the expression level of *FaβGal2 and FaβGal3* respecting control fruits (Fig. 4C–D). Remarkably, the expression of *FaβGal4* was significantly down-regulated by heat treatment as regards controls prior to storage (Fig. 4E).

Respecting α-Ara activity, we detected a significant reduction on heat-stressed fruit with regard to controls at initial time (i) (Fig. 5A). Interestingly, the expression of a gene associated with strawberry fruit ripening (*FaAra1*, Rosli et al., 2009) was significantly down-regulated immediately after assay in heat-treated fruit compared to controls (Fig. 5B).

Additionally, the effect of heat stress on β-xylosidase activity as well as on the expression of some relevant genes for strawberry hemicelluloses metabolism was evaluated. While no effect of heat treatment was observed for *FaXyl1* gene expression (Fig. 6B), it was detected a significantly reduction on total β-xylosidase activity on heat-treated fruit when as regards controls both immediately after treatment (i) and after incubation (f) (Fig. 6A). Notably, *FaXTH1* expression was significantly up-regulated by heat stress (Fig. 6C), while *FaXHT2* gene expression was down-regulated (Fig. 6D). Regarding heat treatment effect on expansin expression, no statistical differences between controls and heat-treated fruit were found for *FaExp2* mRNA accumulation, while an up-regulation of *FaExp5* expression was detected in heat-stressed fruit immediately after treatment, compared to controls (Fig. 6E–F).

Xyloglucan endotransglycosylase/hydrolase (XTH) enzymes can likely have two different catalytic activities in agreement with their effects on xyloglucans. While xyloglucan endo-hydrolase (XEH) activity produces irreversible chain curtailment, xyloglucan endo-transglycosylase (XET) activity involves the non-hydrolytic cleavage and ligation of xyloglucan chains (Eklöf and Brumer, 2010). It has been proposed that hemicellulose-cellulose interaction is mainly modulated by two groups of proteins, xyloglucan endo-transglycosylases/hydrolases

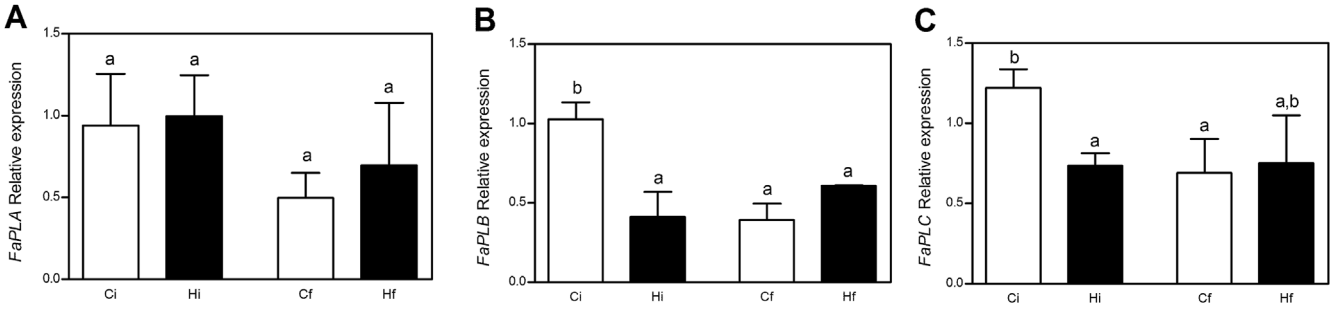

Fig. 3. Effect of heat treatment on the expression pattern of genes encoding putative pectate lyases, *FaPLA* (A), *FaPLB* (B) and *FaPLC* (C). Relative expression values represent the expression level of each target gene normalized against *FaGAPDH1* abundance and compared with controls. Bars represent the mean ± SEM of five replicates. Different letters indicate statistically significant differences at $P < 0.05$ (One way ANOVA, Tukey).

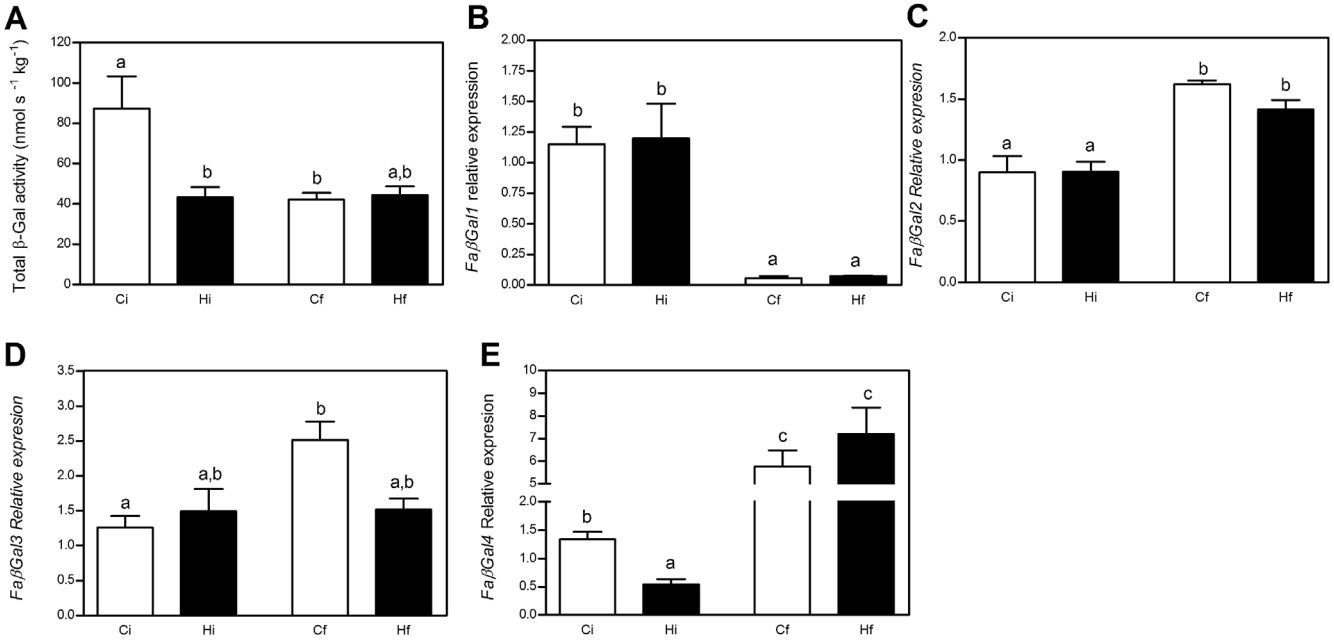

Fig. 4. Effect of heat treatment on total β-Gal activity (A) and *FaβGal1* (B), *FaβGal2* (C), *FaβGal3* (D), *FaβGal4* (E) gene expression. Relative expression values represent the expression level of each target gene normalized against *FaGAPDH1* abundance and compared with controls. Bars represent the mean ± SEM of three replicates for total enzyme activity and, five replicates for Real Time PCR assay. Different letters indicate statistically significant differences at $P < 0.05$ (One-way ANOVA, Tukey).

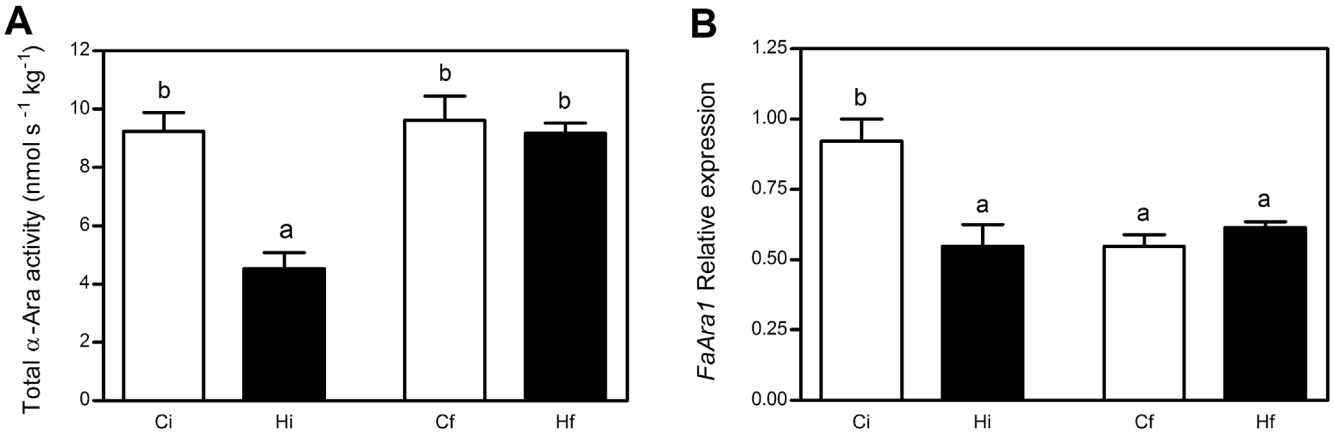

Fig. 5. Effect of heat treatment on α-Ara activity (A) and *FaAra1* gene expression (B). Relative expression values represent the expression level of each target gene normalized against *FaGAPDH1* abundance and compared with controls. Bars represent the mean ± SEM of three replicates for total enzyme activity and, five replicates for Real Time PCR assay. Different letters indicate statistically significant differences at $P < 0.05$ (One-way ANOVA, Tukey).

(XTH) and expansins (Eklöf and Brumer, 2010; Sampedro and Cosgrove, 2005).

Hemicellulose and cellulose account for 10% and 30% of strawberry cell wall respectively (Villarreal et al., 2016), and the alteration of gene expression related to this cell wall fraction in response to heat stress and its role in the acquisition of thermotolerance is interesting to be discussed. It has been shown in Chinese cabbage (*Brassica rapa* L.) that several genes encoding proteins from the XTH family, expansins and pectin esterases were up-regulated by heat treatment (Yang et al., 2006). Authors proposed that this up-regulation by permissive high temperature as 37 °C corresponds to acquired thermotolerance, which might involve cell wall strengthening. A study developed in grapevine fruit, revealed an increase of XETs mRNA accumulation by heat treatment (Rienth et al., 2014), and in *Agrostis* grass species it has been shown that expansin is strongly up-regulated in response to temperature of 40 °C (Xu et al., 2008). In a previous work, we cloned *FaXTH1* and *FaXHT2* cDNAs from strawberry fruit, and our studies suggested that the putative enzyme FaXTH1 would have XET as exclusive activity, being involved in strawberry hemicellulose reinforcement; while FaXTH2 might have both XET/XEH activities (Nardi et al., 2014). Moreover, in that work we cloned both promoter regions, and found potential regulatory elements associated with stress-related responses. Taken together, these studies suggest that in order to tolerate high temperatures, plant cell wall needs to become more elastic to maintain cellular functions during heat stress, and our results of an up-regulation of *FaXTH1* and *FaExp5* (a fruit-specific gene, Harrison et al., 2001), in heat-treated fruit compared to controls, might be related to these suggestions.

3.3. Cell wall amount and composition

To evaluate the influence of heat stress on strawberry cell wall amount and composition, we decided to perform a sequential isolation of main strawberry cell wall polysaccharides. Because differences in

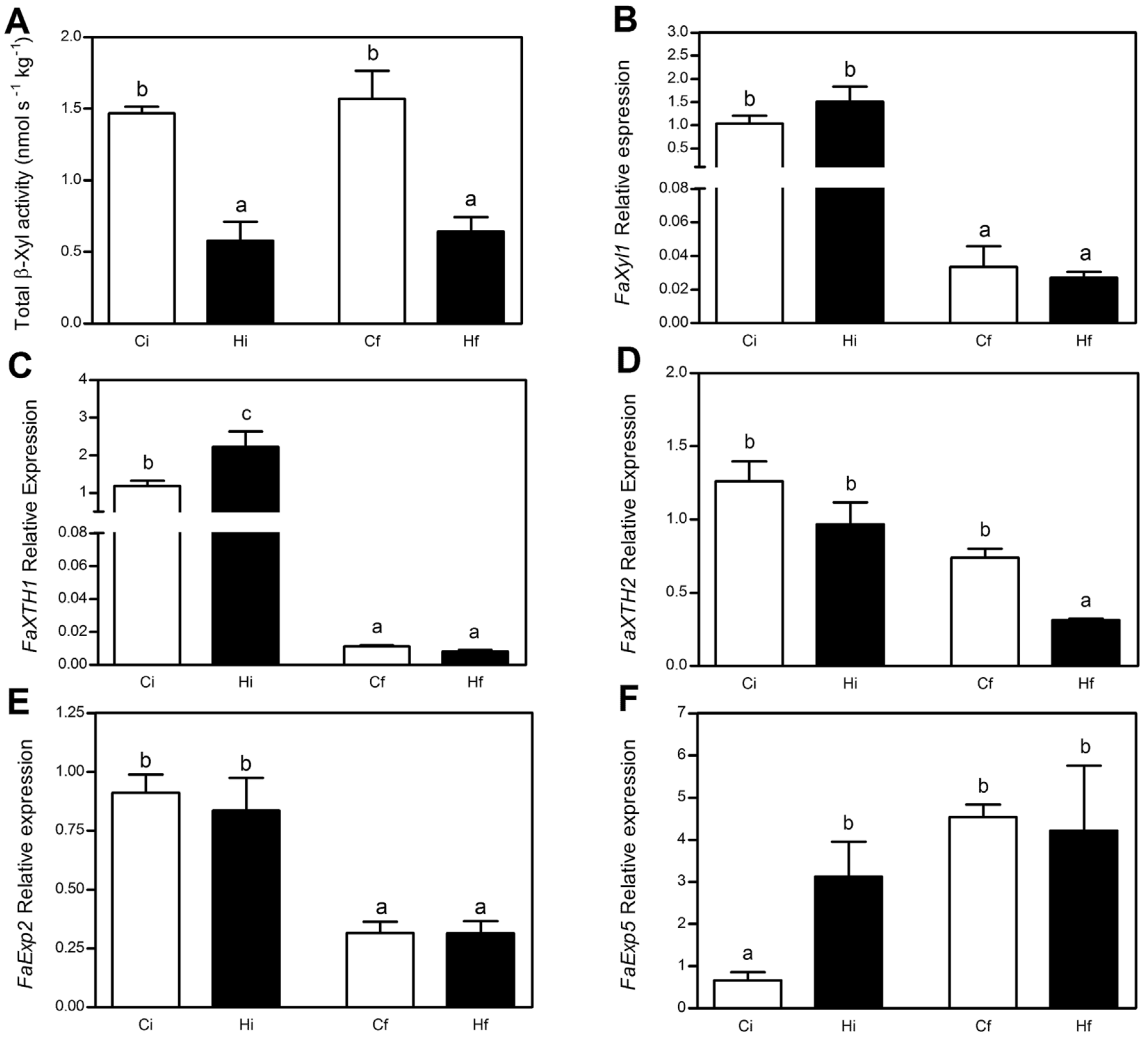

Fig. 6. Effect of heat stress on β-Xyl activity (A), *FaXyl1* (B), *FaXTH1* (C) and, *FaXTH2* gene expression (D), *FaExp2* (E) and *FaExp5* (F). Relative expression values represent the expression level of each target gene normalized against *FaGAPDH1* abundance and compared with controls. Bars represent the mean ± SEM of three replicates for total enzyme activity and, five replicates for Real Time PCR assay. Different letters indicate statistically significant differences at $P < 0.05$ (One-way ANOVA, Tukey).

firmness levels between heat-treated- and control were detected after cold + shelf storage (Fig. 1A), studies were performed using these samples. Nevertheless, in order to discard collateral effects of the heat treatment on the fruit water content (which might be translate as an increase in cell wall content per fresh fruit weight), we analyzed cell wall contents just after the heat treatment (i) and no significant differences on AIR amount (as mg of AIR per 100 mg of fruit^{-1}) were found on heat-treated fruits when compared to controls (Supp. Fig. IA). Moreover, total pectin content immediately after treatment was also measured and no differences were detected between controls and heat-treated fruits (Supp. Fig. IB). Then, cell walls (as Alcohol Insoluble Residues, AIRs) were prepared from each treatment and controls after cold + shelf storage. As a result, higher amount of cell wall was detected in heat treated fruit than in controls (Fig. 7A).

AIRs from control and treated fruits after storage were sequentially fractionated to yield water soluble- (WSP), ionically- (ESP) and covalently- (HSP) bound pectins, as well as base-soluble hemicelluloses and an insoluble cellulose residue.

Concerning pectins, a slightly higher content of galacturonic acid (GalA) from WSP was detected (Fig. 7B). Both for ionically- and covalently bounded pectins (ESP and HSP) it was detected a higher amount of GalA in treated fruit than in controls (Fig. 7C and D). Neutral sugars content was analyzed on total pectins and a higher content was detected on heat-treated fruits in comparison with controls (Supp. Fig. IC).

Finally, the analyses of hemicellulose and cellulose amount revealed a significant higher content of these polysaccharides in heat-stressed fruit when compared to controls (Fig. 7E and F).

Our results show that heat treatment modifies both cell wall amount and fruit firmness after storage (Figs. 1A and 7A) suggesting that both parameters might be related. When cell wall fractioning was done, we found that heat stress modified the relative amount of each polysaccharide fraction. As relevant results, we detected that heat-treated samples showed a significantly higher content of galacturonic acid in EDTA-soluble (ESP) and HCl-soluble fractions (HSP) (Fig. 7C and D), a higher content of hemicelluloses and cellulose (Fig. 7E and F), as well as a higher amount of neutral sugars in total pectins (Supp. Fig. IC), suggesting a decreased solubilization of cell wall polymers in stressed fruit, which also might be related with fruit firmness.

Furthermore, our results of higher PME activity (along with higher *FaPME1* expression and lower DE, Fig. 2A–C) together with lower PG activity and down-regulation of *FaPG1* expression (Fig. 2D–E) in heat-treated fruits agree with a higher ESP amount observed.

As regards neutral sugars metabolism, it has been proposed that *FaPG1* not only degrades HGs backbone during strawberry fruit ripening, but also reduces the number of side-chains of pectins from

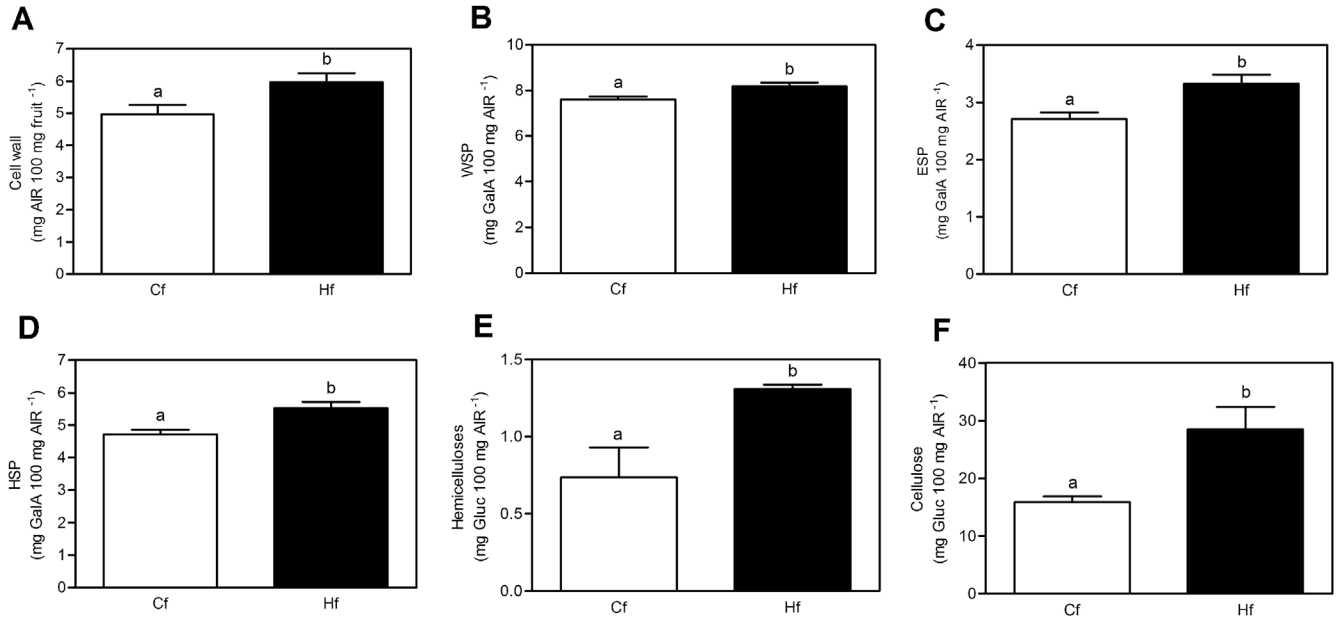

Fig. 7. Effect of heat stimulus on strawberry cell wall content per 100 mg of fruit (A); GalA-WSP (B), GalA-ESP (C), GalA-HSP (D), hemicelluloses (E) and cellulose (F) content per 100 mg of AIR compared with controls after 8 days at 4 °C + 2 days at 20 °C of storage (f). Cf: control fruit at f; Hf: heat-treated fruit at f. Bars represent the mean ± SEM of three replicates. Asterisks indicate significant differences between control and heat-stressed fruit (*: $P < 0.05$, Student's t-test).

EDTA soluble fraction as well as the length of side chain from HCl-soluble fraction (Posé et al., 2015). In the present work, heat stress reduced PG, β-Gal and α-Ara activities (Figs. 2A, 4A and 5A), as well as down-regulated the expression of *FaPG1* as well as two genes related with strawberry side chain metabolism and fruit softening as *FaβGal4* and *FaAra1* (Paniagua et al., 2016; Rosli et al., 2009). Conjointly, these results might explain, at least partially, the higher neutral sugar amount detected in heat treated fruits with respect to controls (Supp. Fig. IC).

Finally, results of up- and down-regulation of *FaXTH1* and *FaXTH2* by heat stress respectively (Fig. 6C and D), as well as less β-xyl activity, could be related to a higher hemicellulose amount in treated fruits as regards controls.

3.4. In vitro cell wall swelling

The effect of heat stress on *in vitro* strawberry cell wall swelling was analyzed, as this process has been associated to a number of chemical features and particular properties of cell wall components and middle lamella.

Same swelling of AIR was found in controls and heat-treated fruit at initial condition, while a higher cell wall swelling was observed in heat-stressed fruit when compared to controls after cold + shelf storage (Fig. 8A).

As well, we analyzed the *in vitro* swelling process during strawberry fruit ripening, to be used as a reference. AIR from small green (SG), large green (LG), white (W), 50% red (50% R), 80% red (80% R), 100% red (100% R) and overripe (OR) fruit were obtained and the cell wall swelling assay was performed (Fig. 8B). In this case, an increase of swelling was detected from SG to 100% R stages and then, a significant decrease was observed in OR stage (Fig. 8B–C). The grade of *in vitro* swelling of AIRs from controls and heat-treated fruit at initial time (Ci, Hi) was similar to that detected for AIRs from 80% red fruit. However, after storage (8 days at 4 °C + 2 days at 20 °C) the swelling of AIRs from controls decrease to the level of over-ripe fruit, while that of heat-treated fruit (Hf) remained at similar level than initial (Hi, Ci) (Fig. 8C).

In vitro cell wall swelling has been related to higher pectin solubility, higher hydrolysis of cell wall side branches, as well as loss of cellulose-hemicellulose network (Brummell, 2006; Redgwell et al., 1997a, 1997b). In several fruit species like kiwi, tomato and also strawberry, *in vitro* cell wall swelling increased between unripe and ripe stages (Redgwell et al., 1997b). In the present work, higher wall swelling was observed in AIR from heat-stressed fruit when compare to non-treated fruit after cold + shelf storage (Fig. 8A). In that sense, it has been reported that AIR's swelling in kiwi fruit peak during ripening but decrease in overripe stages (Redgwell et al., 1997b). Accordingly and as first report in strawberry, we decided to study *in vitro* AIR swelling all along fruit ripening, and it was revealed a gradual increase of swelling process peaking at 100% red and decreasing in overripe stage (Fig. 8B). Remarkably, when comparing the height of the settled aqueous suspensions of AIR from control and heat-stressed fruit immediately after assay (Ci and Hi) and heat-treated fruit after storage (Hf), no significant differences between them was found, and heights where comparable to that observed for AIR from 80% red fruit (Fig. 8C). In contrast, AIR's height from control fruit after storage (Cf) was similar to that detected for overripe strawberry fruit (Fig. 8C). These results suggest that heat stress is able to arrest strawberry *in vitro* swelling process, explaining the greater swelling observed in heat-stressed fruit after cold + shelf storage (similar to an 80% red fruit) when compared with controls (Fig. 8A).

While swelling process associated to fruit ripening would be determined by the three factors mentioned before, it has been proposed that late in ripening (overripe stages), cellulose and hemicellulose are modified by specific enzyme action as xyloglucanases or xyloglucan endo-transglycosylases causing some collapse of microfibrillar structure and reducing wall swelling (Redgwell et al., 1997b). Our results agree with these suggestions, since a down- and up-regulation of *FaXTH1* and *FaXTH2* respectively, together with fewer amounts of hemicellulose and cellulose in controls after storage (Cf) when compared to heat-stressed fruit (Hf) were observed.

3.5. Pathogen's growth on isolated cell walls and rots development

Considering that heat stress modified strawberry cell wall composition and feasibly its structure, we decided to study heat stress effect on the capacity of the necrotrophic fungus *B. cinerea* to grow on agar plates containing cell wall material (AIRs from control and heat-treated fruit) as the only carbon source. The radial *in vitro* growth of *Botrytis* was significantly lower when using cell wall from heat-stressed fruit when

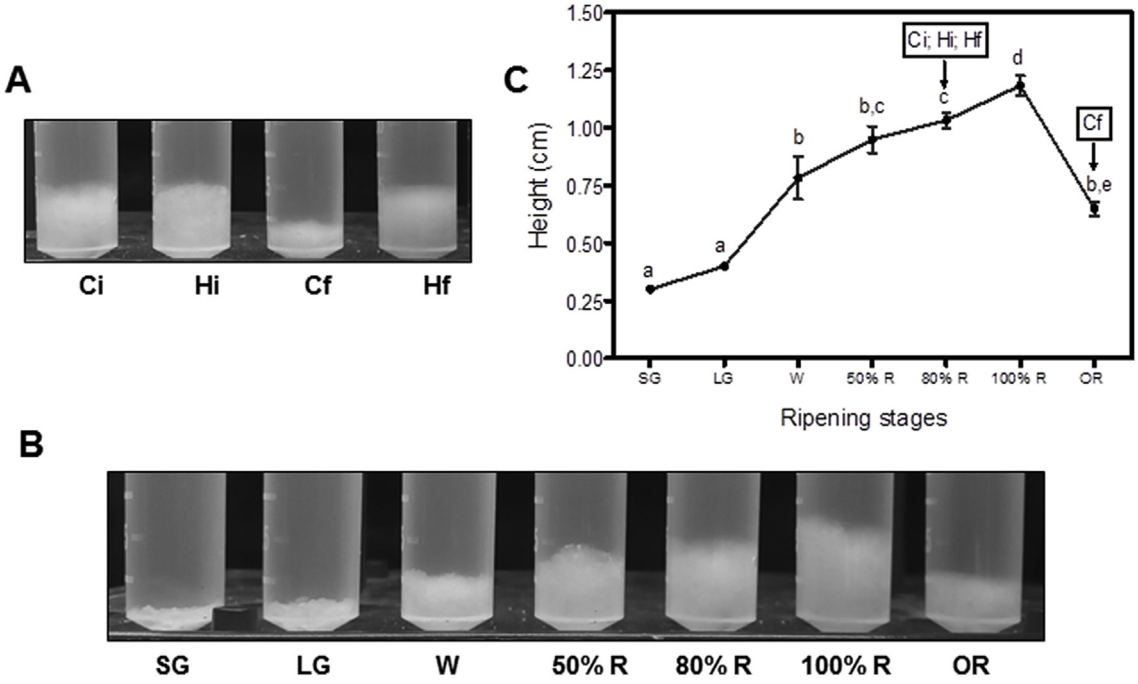

Fig. 8. *In vitro* swelling of AIR isolated from control and heat-stressed fruit both immediately after heat treatment (i) and after 8 days at 4 °C + 2 days at 20 °C of storage (f) (A) and, from different strawberry fruit ripening stages (B). Graphical representation of the height of settled aqueous suspensions of AIRs during strawberry fruit ripening and, comparison with control and heat-treated fruit at i and f (C). Images are representative of three replicates. Different letters indicate significant differences between ripening stages ($P < 0.05$, One-way ANOVA, Tukey).

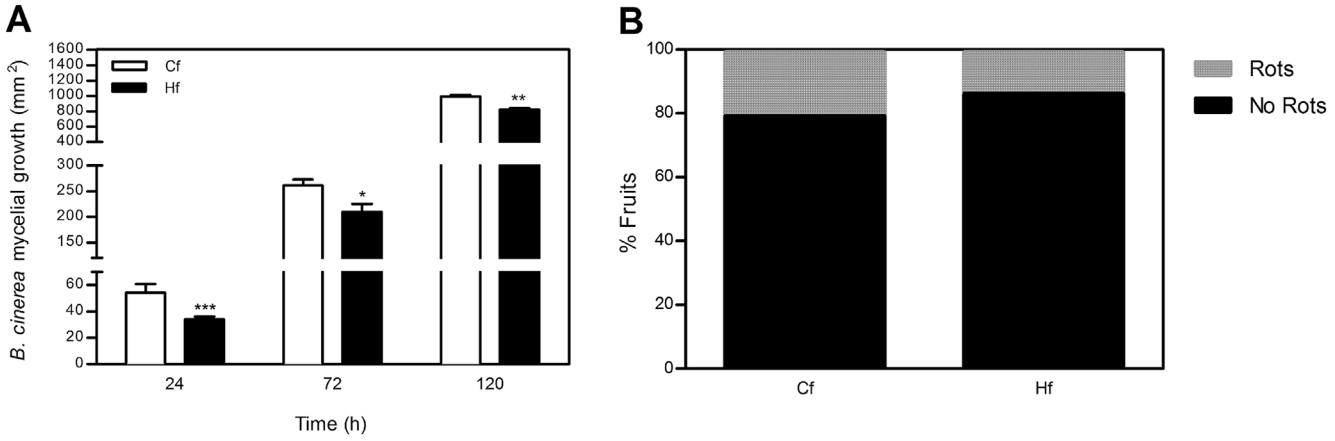

Fig. 9. *Botrytis cinerea* growth on plates containing 0.8% (w/v) agar and 0.2 mg ml^{-1} of AIRs from control and heat-treated fruit (A), and rots development (No Rots %, black columns; Rots %, grey columns) in control and heat-treated fruit (B) after 8 days at 4 °C + 2 days at 20 °C of storage (f). Bars represent the mean ± SEM of three biological replicates (A). Each datum corresponds to the percentage of fruit decay from a seventy heat-treated and seventy controls fruits (divided in four trays) (B). Asterisks indicate significant differences between treatments and controls (*: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$, One-way ANOVA, Dunnett for A; Chi-square analysis with $\alpha = 0.05$ and 1 degree of freedom, for B).

compared with controls, at each time point analyzed (24, 72 and 120 h of pathogen growing; Fig. 9A). In this sense, it has been proved that transgenic tomato fruits with simultaneous suppression of polygalacturonase and expansin expression show and alteration on the cell wall disassembly process which in turns, decrease the infection by *B. cinerea* (Cantu et al., 2008b). Moreover, authors assessed pathogen's biomass in liquid cultures containing AIRs from transgenic and wild type fruits using a monoclonal antibody and detected that *Botrytis* growth was significantly reduced when cell walls from transgenic plants were used. Accordingly, although the *in vitro* reduction of *B. cinerea* growth in a medium containing AIRs from heat-treated fruits compared to controls might be explained by different mechanisms, from an altered cell wall structure and composition that will reduce the digestibility of pathogen cell wall degrading enzymes to the presence of growth inhibitors in the fruit cell wall, results obtained suggested that heat stress would be limiting the accessibility of *Botrytis* to its substrates.

Finally, decay was evaluated on heat-treated and control fruits and results revealed that after 8 days of storage at 4 °C + 2 days at 20 °C, (Fig. 9B). Despite statistical analysis showed no dependence between fruit decay and treatments ($\chi^2 = 0.8840$, 1 degree of freedom, *P*-value = 0.3471), results revealed that 20% of control fruit showed rots, while 13% of heat-treated fruit were affected. Hence, although no statistically differences were detected, the observed trend in the reduction of rots development in heat-treated fruits could be related with a more preserve cell wall structure and also with a higher accumulation of phenolic compounds as its was shown (Fig. 1E).

4. Conclusions

Taking into account the results obtained through the various approaches performed in the current work, we suggested that the positive effect of heat treatment to preserve strawberry fruit quality during storage, is closely related to the up-regulation of genes involved in cell wall reinforcement (as *FaPME1* and *FaXTH1*), and down-regulation of genes previously proved to be key in fruit cell wall disassembly as *FaPG1*, *FaPLB, FaPLC*, *FaAra1, FaβGal4*. Likewise, results of *in vitro* cell wall swelling and *Botrytis cinerea* growth, as well as modifications on cell wall content and polysaccharides composition suggest a changed on strawberry cell wall structure by heat treatment.

In this manner, controlled heat-stress reveals itself not only as a postharvest treatment which preserves the main organoleptic properties of strawberry, but also as a tool which might contribute to the understanding of the mechanism underlying the response of this soft fruit's cell wall to an abiotic stimulus.

Author contributions.

NMV designed the study with inputs from GAM and PMC. SEL, NCO, MM, JLB and NMV performed the experimental studies. All results and data were analyzed and interpreted by NMV, SEL, MM, GAM and PMC. NMV wrote the manuscript with the contribution of GAM and PMC.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.plaphy.2018.07.015.

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