Proteomic, metabalomic, and biochemical analysis of heat treated Valencia oranges during storage

Valeria E. Perotti [a], Hernán A. Del Vecchio [a,1], Alina Sansevich [a], Guillermo Meier [b], Fernando Bello [b], Mariángeles Cocco [b], Sergio M. Garrán [b], Catalina Anderson [b], Daniel Vázquez [b], Florencio E. Podestá [a,*]

[a] Centro de Estudios Fotosintéticos y Bioquímicos (CEFOBI), Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531, 2000 Rosario, Argentina
[b] Estación Experimental Concordia, Instituto Nacional de Tecnología Agropecuaria (INTA), Colonia Yeruá, Argentina

ARTICLE INFO

ABSTRACT

Heat treatment induces defense mechanisms and triggers physiological responses that allow citrus fruit to withstand stressful conditions during storage. The aim of this paper was to identify changes in the proteome and the biochemistry of Valencia oranges (*Citrus sinensis* cv Valencia late) subjected to heat treatment before 0, 30 and 60 d of storage at 5 °C. Heat treatment for 48 h at 37 °C and 90% relative humidity was followed by 24 h at 20 °C. Differentially expressed proteins were detected by two-dimensional differential gel electrophoresis and identified by matrix-assisted laser desorption/ionization MS. Twenty eight different proteins in 40 spots among the 61 analyzed were identified. Functional classification showed that the main affected categories were "Cell rescue, defense, and virulence" and "Metabolism"; while 20% were found to be storage proteins with the rest related to minor categories. Activity of antioxidant enzymes was also evaluated. The most conspicuous change was an increase in superoxide dismutase in both the flavedo and juice sacs. Peroxidases increased in flavedo but decreased in sacs while alcohol dehydrogenase increased in treated flavedo, but not in sacs. Other fermentative enzymes were not affected. Metabolite content showed a higher concentration of sugars in heat treated fruit, while the main acids were slightly or not affected. These results are consistent with the development of a lower degree of fruit susceptibility to fungal pathogens, thus explaining the maintenance of postharvest quality.

1. Introduction

Postharvest storage of citrus fruit is often necessary before commercialization. It may be required to extend shelf life if transportation to distant markets is required and/or it may be applied to control infections. Citrus fruit are often exposed to different chemical treatments to control insect pests or prevent fungal rots as those caused by *Penicillium digitatum,* one of the most common pathogens affecting citrus postharvest. Both the increased emergence of resistant strains (Holmes and Eckert, 1999; Zhu et al., 2006) and the growing demand to reduce the postharvest use of pesticides have brought about the development of alternative approaches to control disease. One of the most widely used methods in postharvest conditioning is heat treatment. The more widespread alternatives include hot water, vapor heat and hot air (Lurie, 1998; Porat et al.,

2004; Sala and Lafuente, 1999). However, regardless of the postharvest treatment applied, ideally it must be effective in controlling disease while maintaining fruit quality.

It is known that heat treatment induces defense mechanisms such as activation of lignin synthesis and other substances such as phytoalexins that inhibit the growth of fungi (Schirra et al., 2000). In addition, exposure to high temperature triggers physiological responses that allow the tissue to withstand subsequent conditions of stress imposed by storage conditions, such as cold. Heat treatments have been shown to improve cold stress hardiness and reduce chilling injury in sensitive fruit (Lara et al., 2009; Lurie and Crisosto, 2005; Sapitnitskaya et al., 2006).

Understanding the biochemical and genetic basis of the response to heat treatment is of great importance for the identification of the factors that may be used in the selection of better storage conditions and/or choosing varieties more resistant to fungal infection and cold stress. In this regard, the proteomic approach represents a powerful tool for identifying and studying differential expression patterns of proteins involved in such responses, since it reveals general changes in the abundance of proteins in response to different developmental and

* Corresponding author. Tel.: +54 341 4371955; fax: +54 341 4371955.
  E-mail address: podesta@cefobi-conicet.gov.ar (F.E. Podestá).
[1] Present address: Department of Biology, Queen's University, Kingston, Ontario, Canada K7L 3N6.

environmental cues (Rampitsch and Srinivasan, 2006; Shi et al., 2008). Throughout the literature proteomic studies based on two-dimensional PAGE (2D-PAGE), coupled with protein identification by MS have been employed to investigate plant (including citrus) responses to various stresses (Amme et al., 2006; Shi et al., 2008).

In this work an integrated study of the metabolic changes taking place in orange fruit (*Citrus sinensis* [L.] Osbeck var. Valencia late) induced by heat treatment followed by cold storage is presented. Comparative analysis of fruit endocarp proteome variations using two-dimensional differential gel electrophoresis (2D-DIGE) was complemented through the evaluation of organoleptic characteristics, levels of enzymatic activities involved in fermentative and antioxidant pathways, and metabolite contents such as sugars, organic acids and alcohols. Combining these different sources of data, it was possible to define some important points of the global response to heat treatment, providing the first analysis at the molecular level of the changes occurring in citrus after heat treatment and cold storage.

2. Materials and methods

2.1. Plant material and treatments

Assays were conducted with orange fruit [*C. sinensis* (L.)Osbeck] cv 'Valencia Late' grown in the Estación Experimental Agropecuaria INTA, Concordia, Entre Ríos, Argentina (EEAC), harvested at full maturity in October/November 2007; and repeated with fruit grown during 2008. Immediately after harvest, fruit were manually selected for uniformity of colour and size and divided into two groups of 50 each. The first group was used as a control and therefore did not receive any treatment. The second group received a heat treatment, which consisted of 48 h at 37 °C and 90% relative humidity followed by 24 h at 20 °C. This treatment is usually applied to oranges from the EEAC because its effectiveness in controlling *Penicillum digitatum* has been demonstrated previously (Cocco et al., 2008). Groups were labelled control (C) and heat treated (HT), respectively. In turn, each group was exposed to variable length storage periods, simulating commercial conditions, and was accordingly further divided into subgroups. The first subgroup corresponded to control and heat treated fruit assayed immediately after treatments (C and HT, respectively), the second to fruit kept at 5 °C for 30 d (C + 30 and HT + 30) and the third to fruit kept at 5 °C for 60 d after applying the treatment (C + 60 and HT + 60). Each subgroup is identified as the first, second and third pair, respectively. Immediately after each treatment, the flavedo (epicarp) and juice vesicle tissue (endocarp) were frozen in liquid nitrogen and stored at −80 °C for further experiments.

2.2. Total protein extraction

Fruit tissue (approximately 3–5 g) was powdered with liquid nitrogen in a mortar and then homogenized with one-fifth volume of extraction buffer I (1 M Tris, 40% (v/v) glycerol, 20 mM MgCl$_2$, 5 mM EDTA, 10 mM β-mercaptoethanol and 1 mM PMSF), in the case of endocarp and with ten volumes of extraction buffer II (100 mM KPi pH 7.0, 1 mM PMSF), in the case of epicarp. The final pH of the crude extracts thus obtained was near 7.0. The homogenates were centrifuged for 15 min at 4 °C in an Eppendorf microcentrifuge at maximum speed. The resulting supernatant was desalted in a Sephadex G-25 column previously equilibrated with an 1/10 dilution of extraction buffer I or with extraction buffer II, correspondingly (Penefsky, 1977). The eluate was used as the source for enzyme activity measurements, nondenaturing electrophoresis and immunoblotting.

2.3. Protein quantitation

Protein concentration was determined in crude extracts by the dye-binding method using the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA) and bovine serum albumin as standard.

2.4. Activity assays

All enzymes were assayed at 30 °C in a Hitachi 150-20 (Hitachi Corp., Tokyo, Japan) spectrophotometer following the oxidation of NAD(P)H at 340 nm in a final volume of 1 mL. The assays were optimized for pH and the concentration of cofactors/substrates, corrected for NADH oxidase activity, and were linear with respect to time and concentration of enzyme assayed. One basic experiment in which enzymatic activities were measured represents the mean of at least 3 determinations per fruit made in 2 fruit from each group. Each experiment was repeated 3 times. The reaction mixtures for each assay were as follows.

Alcohol dehydrogenase (ADH, EC 1.1.1.1) activity was measured in a medium containing 85 mM MES, pH 6.5, 5 mM acetaldehyde and 0.15 mM NADH (Kato-Noguchi, 2000). Pyruvate decarboxylase (PDC, EC 4.1.1.1) activity was determined following NADH consumption in a coupled reaction with ADH. To avoid masking of this enzyme's activity by lactate dehydrogenase (LDH) present in crude extracts, oxamate, an LDH inhibitor, was added to the reaction medium. The reaction medium contained 85 mM MES, pH 6.5, 25 mM NaCl, 2 mM MgCl$_2$, 2 mM DTT, 2 mM thiamine pyrophosphate, 0.15 mM NADH, 50 mM oxamate; 3 U ADH and 10 mM pyruvate (Rivoal et al., 1990). The extracts were incubated for 30 min in the reaction medium in the absence of pyruvate and ADH to allow PDC activation before starting the reaction. Lactate dehydrogenase (EC 1.1.1.27) activity was measured in 50 mM NaPi, pH 7.5, 10 mM pyruvate, 0.2 mM NADH and 1 mM methylpyrazole (Kato-Noguchi, 2000). Methylpyrazole, an inhibitor of PDC, was added to prevent interference by the joint action of this enzyme with ADH. Glutathione reductase (GR, EC 1.8.1.7) determination took place in a medium containing 100 mM NaPi, pH 7.8, 2 mM EDTA, 1 mM oxidized glutathione (GSSG) and 0.1 mM NADPH (Rao et al., 1996). Catalase (CAT, EC 1.11.1.6) activity was measured in a medium containing 100 mM KPi, pH 7.0 and 10 mM H$_2$O$_2$ (Kar and Mishra, 1976). In this case, the reaction was followed by the decrease in absorbance at 240 nm due to H$_2$O$_2$ consumption.

In all cases, except for PDC, reactions were started by the addition of the extract.

2.5. Non-denaturing gel electrophoresis

Non-denaturing gel electrophoresis was performed as described by Bollag and Edelstein (1991), using an 8% (w/v) polyacrylamide solution for the separating gel and 4% (w/v) for the stacking gel. Superoxide dismutase (SOD, EC 1.15.1.1), ascorbate peroxidase (APX, EC 1.11.1.11) and peroxidases (POD, EC 1.11) activities were assayed. Gels, supplemented with 20% (v/v) glycerol and 10% (v/v) ethylene glycol, were run at 12 mA and 4 °C until the tracking dye left the gel. Enzymatic activities were detected by incubating the gels in reaction media until development of coloured bands at 30 °C. For SOD isoforms, gels were equilibrated with 50 mM KPi, 1 mM EDTA, pH 7.8 for 30 min. Gels were then incubated in the same solution supplemented with 120 mM riboflavin, 28 mM tetramethyl ethylene diamine (TEMED) and 2.5 mM nitroblue tetrazolium (NBT) for 20 min in the dark. Finally, gels were placed in distilled water and exposed in a light box for 15 min at room temperature. Observation of cyanide-sensitive or -insensitive isozymes was achieved by incubating gels in 50 mM KPi containing 3 mM KCN (inhibitor of Cu/Zn-SOD) or 5 mM H$_2$O$_2$ (inhibitor of Cu/Zn-SOD and Fe-SOD) for 30 min before staining for SOD activity (Beauchamp and Fridovich,

1971). Non-denaturing PAGE for the detection of APX activity was achieved as described above, except that the carrier buffer contained 2 mM ascorbate. The gels were pre-run for 30 min to allow ascorbate to penetrate the gel before loading the samples. After electrophoretic separation, gels were equilibrated with 50 mM KPi, pH 7.0, containing 2 mM ascorbate for 30 min, followed by incubation in 50 mM KPi, pH 7.0, containing 4 mM ascorbate and 2 mM H_2O_2 for 20 min. The gels were washed with buffer for 1 min and submerged in a solution of 50 mM KPi, pH 7.8, containing 28 mM TEMED and 2.5 mM NBT with gentle agitation. The reaction was continued for 10–15 min and stopped by a brief wash with water (Mittler and Zilinskas, 1993). POD activities were assayed incubating the gels with 50 mM sodium acetate pH 4, 5, 2 mM bencidine (dissolved in dimethylsulfoxide). Reaction was started by adding 3 mM H_2O_2. After the development of purple-stained bands, reaction was stopped by the addition of water (Rao et al., 1996).

Each isoform of the enzymes under study was quantified by densitometric analysis. The gels were scanned, and the area of the bands was obtained by integration using the LabWorks program (Media Cybernetics Inc., Silver Spring, MD). Biological triplicates of the densitometric analysis were used to minimize experimental error. The following standard proteins were used: thyroglobulin (669 kDa); apoferritin (440 kDa); amylase (232 kDa); alcohol dehydrogenase (140 kDa) and BSA (66 kDa) from a calibration kit (GE Healthcare).

2.6. Electrophoresis and immunoblotting

SDS-PAGE was carried out according to the method of Laemmli (1970) using a Bio-Rad mini-gel apparatus (Bio-Rad, Hercules, CA). The final acrylamide concentration was 10% (w/v) for the resolving gel and 5% (w/v) for the stacking gel. The following standard proteins were used: 170 kDa; 130 kDa; 95 kDa; 72 kDa; 55 kDa; 43 kDa; 34 kDa; 26 kDa; 17 kDa and 10 kDa from a commercial kit (Fermentas). Immunoblotting was performed according to the method of Bollag and Edelstein (1991). Samples were run on a denaturing PAGE and transferred to nitrocellulose membrane. The antibodies used for detection were raised in rabbit against *Solanum lycopersicum* purified HSP21 (Polenta et al., 2007). This experiment used biological duplicates.

2.7. Measurement of physiological, chemical and internal quality parameters

The respiratory activity of whole Valencia oranges was measured by infrared gas analysis. Individual fruit was placed in a 160 mL chamber through which air was pumped (at a flow rate of 1 mL/s). Increases in CO_2 in the chamber were monitored by passing the air stream through an infrared gas analyzer (IRGA, Qubit Systems Inc., Kingston, Canada), and from these data, the rate of CO_2 evolution per second and gram fresh weight was calculated. The average temperature was $25 \pm 2\,^{\circ}C$, and the relative humidity was $80 \pm 4\%$. Data shown are the results of measurements on 10 different fruit $\pm$ S.D.

Control and treated fruit were squeezed and the resulting juices were used for the following determinations. Five fruit were used per replication (3 replication samples). The total soluble solid content in the juice was determined with a digital refractometer (expressed as %, w/w), and the titratable acidity was measured by titrating with 0.1 N NaOH to pH 8.2. The results are expressed as percentage of anhydrous citric acid because this is the predominant organic acid in citrus. Maturity index was evaluated as the ratio of total soluble solids:titratable acidity. Juice was extracted from individual fruit (at least 20 fruit per replication, 3 replication samples), and the juice content was expressed as percentage of fruit weight.

Ethanol and acetaldehyde content in juice was determined by gas chromatographic analysis of juice headspace using a gas chromatograph (Shimadzu Mod. GC17A; Shimadzu Corp., Kyoto, Japan) with a Supelco Omegawax 250 column (30 m × 0.25 mm internal diameter) (Supelco, Bellefonte, PA). This experiment was carried out on 5 fruit per replication (3 replication samples).

For electrolyte leakage, 2 fruit per replicate (3 replication samples) were used. Peel was carefully removed, and 1 cm disks were cut with a cork borer. Samples of 10 disks per replicate were placed in a 200 mL glass bottle, washed twice with deionized water and then incubated in 100 mL of deionized water at 20 °C. Conductivity of the incubation medium was measured with a conductivity meter after 3 h of incubation under constant shaking. After readings were taken, the flasks were autoclaved at 120 °C for 20 min and cooled to 20 °C, and the conductivity was measured again for total electrolytes. Data were expressed as (initial conductivity/total conductivity) × 100.

H_2O_2 determination was carried out according to the method of Velikova et al. (2000) in juice sacs extracts. A freshly prepared 1/1000 dilution of a 0.89 M standard solution was added as an internal control and was used as standard.

Weight determination was made using 12 fruit per replication (2 replication samples).

2.8. Protein extraction for 2D electrophoresis

Approximately 2 g of epicarp material was ground in liquid nitrogen using a ceramic mortar and pestle, sand and PVPP, transferred to a SS34 tube containing 5 mL of extraction buffer (100 mM Tris–HCl, pH 8.8, 2% (w/v) SDS, 0.4% (v/v) β-mercaptoethanol, 10 mM EDTA, 1 mM PMSF, 0.9 M sucrose), and 10 mL of ice-cold Tris–HCl-saturated phenol solution, pH 8.8, and then agitated for 30 min at 4 °C. The aqueous phases were back-extracted with extraction media and phenol by vortexing. Tubes were centrifuged at $5000 \times g$ for 15 min at 4 °C and the phenolic phases were transferred to a new tube leaving the interface intact. Proteins were precipitated with 5 volumes of cold 0.1 M ammonium acetate in methanol at −20 °C overnight. Samples were collected by centrifuging at $20,000 \times g$ for 20 min at 4 °C. Afterwards, the pellet was washed with 1.5 mL of cold ammonium acetate/methanol and twice with cold 80% (v/v) acetone. A final wash used 1.5 mL of cold 70% (v/v) ethanol. The pellet was re-suspended in 2D-DIGE buffer (30 mM Tris–HCl, pH 8.5, 7 M urea, 2 M thiourea, 4% CHAPS) and sonicated in an MSE sonicator by applying 3 × 5 s pulses at 20% power, with 1 min intervals, keeping the sample in a water-ice bath.

The differential proteome of citrus under heat treatment was assessed by comparing heat-treated oranges with their respective control: HT versus C; HT + 30 versus C + 30 and HT + 60 versus C + 60. Additionally, the changes produced along the storage periods, within each condition, were analyzed.

2.9. Protein labelling with dyes

In all experiments, proteins were labelled with Alexa 610 (excitation wavelength, 610 nm; emission peak, 628 nm) or Alexa 532 (excitation wavelength, 532 nm; emission peak, 554 nm) after adjusting the pH to 8.5 according to the manufacturer's instructions (Molecular Probes Inc. and Invitrogen Ltd.). Proteins were labelled at a ratio of 100 µg of protein:20 nmol of Alexa protein minimal labelling dye, in dimethylformamide. After vortexing, samples were incubated for at least 2 h on ice. The reaction was quenched by the addition of 1 µL of 1 mM lysine and 20 mM dithiothreitol (DTT). Immediately after, isoelectric focusing (IEF) buffers, pH 3–10 or 5–8, were added to a final concentration of 4% (v/v) (Amersham Biosciences).

2.10. 2D-electrophoresis separation and protein visualization

A 100 μg aliquot of Alexa 532-labelled sample was mixed with 100 μg of Alexa 610-labelled protein prior to 2D gel electrophoresis. A Protean IEF Cell instrument (Bio-Rad, Hercules, CA, USA) was used for IEF with pre-cast immobilized pH gradient (IPG) strips (pH 4–7, linear gradient, 21 cm, Bio-Rad, Hercules, CA, USA). Samples of 450 μL containing the labelled proteins were loaded by in-gel rehydration. The strips were subjected to IEF using the following program: 12 h at 50 V; 1 h at 500 V; 1 h at 1000 V and 8000 V until a final voltage of 68,000 V was reached. The strips were equilibrated for the second dimension separation under continuous shaking in SDS equilibration buffer (375 mM Tris–HCl, pH 8.0, 20% glycerol, 2% SDS, and 6 M urea) for 30 min, in the presence of 130 mM DTT for the first 15 min and afterwards with buffer containing 135 mM iodoacetamide for the last 15 min. The strips were washed briefly with running buffer, then loaded on top of a pre-made Laemmli 15% (w/v) SDS-PAGE gel (21 × 16 cm), and covered with 0.5% (w/v) agarose. Proteins were separated at 1 W per gel for 12–15 h at 15 °C using a Ettan Daltsix Electrophoresis System (GE Healthcare). Gels were scanned using a BioChemi System UVP BioImaging System, applying differential filters for each dye. Data were saved as Tagged Image Files (*.tif). In order to obtain biological replicates, each sample was run in at least three gels using different protein extractions from at least 3 different fruit. In order to obtain samples for mass spectrometry analysis, a preparative gel loaded with 1 mg of protein was run and stained with CBB R-350 (Amersham Pharmacia Biotech AB, Uppsala, Sweden) in methanol:H_2O:acetic acid (3:6:1).

2.11. Gel image analysis

Images were analyzed using Image Master 2D-Platinum (GE Healthcare) using the protocol described by Casati et al. (2005). When necessary, spots were manually edited. A normalization procedure was used to allow for variation in total protein loading onto the gel. Total spot volume was calculated, and each spot was assigned a normalized volume as a proportion of this total value. Normalized spot volumes were compared between Alexa 532- and Alexa 610-labelled samples on each gel. Different thresholds were then applied to identify the proteins with a statistically significant 1.5-fold difference in normalized spot volume ($P < 0.05$) (Casati et al., 2005).

2.12. In-gel digestion and mass spectrometric analysis

Selected protein spots from the 2D-electrophoresis gel were manually excised, transferred to 1.5 mL microcentrifuge tubes and sent to CEBIQUIEM facilities (Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina) for further analyses. Spots were subjected to in-gel digestion (http://donatello.ucsf.edu/ingel.html) with trypsin according to Casati et al. (2005). Mass spectrometry data were obtained using an Ultraflex II (Bruker) matrix-assisted laser desorption/ionization–time-of-flight/time-of-flight spectrometer. Peptide ions were analyzed by the data-dependent method as follows: (1) full MS scan (mass-to charge ratio 800–3000) and (2) MS–MS of the major ions.

2.13. Database search and protein identification

MS–MS data were used to perform protein identifications by searching firstly in a non-redundant protein sequence database (NCBInr) using MASCOT (http://www.matrixscience.com/) (Perkins et al., 1999). The following parameters were used for database searches: taxonomy, *Viridiplantae* (green plants; released December 2008); cleavage specificity, trypsin with 0

or 1 missed cleavage allowed; mass tolerance of 1.2 Da for the precursor ions and a tolerance of 0.7 Da for the fragment ions; allowed modifications, Cys carbamidomethyl (fixed), oxidation of Met (variable).

Only candidates that appeared at the top of the list were considered positive identifications. Peptides were considered as matches if they were classified as 'significant' (i.e. $P < 0.05$, which with search parameters used here equals a MOWSE score of >40).

Alternatively, MS–MS data were used to perform spot identification by MASCOT software against EST-Viridiplantae database. The parameters for these searches and identification criteria were the same as those for the previous search. Identified ESTs translated in one of the six open reading frames (ORF) were compared for similarity via BLASTx (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Only the BLASTx matches with E-values $\leq 10^{-30}$ were selected.

Protein functional classification was done according to the classification of the Munich Information Center for Protein Sequences (MIPS, http://mips.gsf.de).

2.14. Crude extract preparation for GC–MS metabolite analysis

Flavedo or juice sac (600 mg) tissue taken from at least 6 independent fruit (2 replication samples) was powdered with mortar and pestle in the presence of liquid nitrogen and transferred to glass tubes where 8.4 mL of cold methanol (−20 °C) were added. After shaking with vortex, 90 μg of ribitol was added. This latter constituted the internal standard, which was subsequently used for relative quantification of metabolites. The resulting extract was distributed in 6 new glass tubes and incubated at 70 °C for 15 min, with periodic agitation. Subsequently, 750 μL of chloroform were added to each tube, followed by incubation at 37 °C for 5 min. Finally, 1.5 mL of water was added and extracts were centrifuged in a refrigerated microcentrifuge for 15 min at 2200 × g. A volume of 450 μL of the polar phase was transferred to microcentrifuge tubes and dried in a vacuum centrifuge (CentriVap, Labconco) until complete evaporation, leaving a coloured pellet. Samples were stored at −80 °C until derivatization.

2.15. Sample derivatization and chromatography

Samples were thawed, dried in a vacuum centrifuge for another 30 min to ensure no liquid was present. Afterwards, 40 μL of 20 g L^{-1} methoxyamine in pyridine were added. Tubes were vigorously shaken and incubated at 37 °C for 90 min. Finally, 70 μL of N-methyl-N-trimethylsylil-trifluoroacetamide (MSTFA) were added to each tube and incubated at 37 °C for 30 min.

Chromatographic runs were performed by injecting 2 μL of derivatized sample in a 30 m long, 0.25 mm thick VSF MS capillary column using an automatic system (Varian Inc.) coupled to a ThermoQuest mass spectrometer. Data were collected and analyzed using Mass Lab software (ThermoQuest). Spectra obtained from chromatographic runs were used to analyze the individual peaks that showed a significant value. Areas underneath each peak were calculated and expressed relative to the area of the internal standard (ribitol). Following this, data obtained by the technical service were revised using the online software MSRI: Mass Spectra Search (The Comprehensive System Biology Project – CSB) from the Golm Metabolomic Institute (Germany) to confirm the assignments of the compounds found.

2.16. Statistical analysis

Data from the experiments were tested using two-way analysis of variance (ANOVA). Minimum significant differences were calculated by the Holm-Sidak Test ($\alpha = 0.05$) using the Sigma Stat Package. In some cases, it was necessary to transform the variable

to pass Normality and Equal Variance tests (it is indicated in the respective cases). Moreover, in other cases, it was not possible to pass the tests mentioned above, so data were tested using the non-parametric Kruskal–Wallis one-way analysis of variance by ranks method (data were previously expressed in a one factor design at 4 or 6 levels, and the comparisons were done between these means). Minimum significant differences were calculated by the Tukey Test (or Dunn's Method, if the treatment group sizes were unequal) ($\alpha = 0.05$). In the particular cases of phenylalanine in epicarp and boric and octadecanoic acids in endocarp the analysis was carried out comparing two groups by Student's *t*-test or using the non-parametric Mann–Whitney's Rank Sum Test.

3. Results and discussion

3.1. Evaluation of internal quality of HT fruit

One of the goals of this study was to evaluate the effects of the HT on fruit quality. Table 1 summarizes the physiological and chemical parameters for C and HT oranges in three points along the postharvest period. In agreement with the absence of visible external damage, there were no significant differences among internal parameters between treated fruit and their respective controls, with the exception of the volatile compounds ethanol and acetaldehyde, whose increase is evidence of an enhanced fermentative metabolism (see Section 3.10). The increased ethanol level in no case exceeded the levels associated with bad taste (>1000 μL L^{-1}) (Hagenmaier, 2002). It should be noted that although there was a slight induction of fermentative metabolism, the respiration rate did not change as a result of heat treatment. This could be an indication of a higher ATP demand that cannot be satisfied by respiration. No significant differences in weight loss between treated and control fruit were detected.

The rest of the changes observed along the postharvest period were independent of the treatment applied and represented the normal progress towards final maturity (i.e. decrease of titratable acidity; increase of maturity index; increase of electrolyte leakage).

3.2. Total protein and enzymatic activities

SDS-PAGE of crude extracts from control and HT oranges did not show any remarkable qualitative change in the protein pattern (not shown). Moreover, total protein content of fruit on a fresh weight basis was not significantly different among the six points analyzed and it was around 0.5 g kg^{-1}.

3.3. Enzymes involved in fermentative metabolism

The activity of the enzymes responsible for the production of ethanol and acetaldehyde, metabolites that are critical for quality maintenance, were examined. ADH, PDC and LDH were evaluated in both juice sacs and flavedo. In sacs the activity of fermentative enzymes was not modified by heat treatment in comparison with fruit of the same postharvest time, and did not present significant changes along the storage period (Fig. 1, left panel). In contrast, in flavedo, there was an important increment of ADH after heat treatment in all pairs, while LDH and PDC did not appear to be significantly affected by this treatment (Fig. 1, right panel). ADH levels showed a decreasing trend with storage time in both C and HT samples. Progress towards maturation in citrus fruit is characterized by an increasing fermentative metabolism supported by carbohydrate degradation (Goren et al., 2000). The present results show that heat treatment, while leaving this metabolic pathway unmodified in the endocarp, increased the fermentative capacity of the flavedo. Possible reasons for this could be (i) an alteration of mitochondrial function that hampers respiration or (ii) an increased demand for

ATP in an already weakened respiratory pathway. The higher levels of ADH versus LDH in both tissues could be an indication that the alcoholic fermentation pathway seems to be preferred over the lactic one. Although PDC levels were not affected, the activity may be sufficient to provide acetaldehyde for ethanol production. The high values of LDH activity indicate that lactic acid is being actively formed as well (see also Section 3.10).

3.4. Enzymes involved in antioxidant defense metabolism

Reactive oxygen species (ROS) are involved in intrinsic defense mechanisms that constitute the first barrier against pathogen attack (Lamb and Dixon, 1997). ROS can prevent the spread of infection by increasing the resistance of the cell wall or killing the pathogen directly. Additionally, these compounds are able to act as signalling molecules of the host defense pathways against pathogen infection (Dat et al., 2000). It is clear that the response of plants to ROS must rest on a balance between the beneficial effects of this metabolism and its harmful counterpart, the oxidative damage. Given this scenario, three major antioxidant enzymes, APX, POD and SOD were studied by non-denaturing PAGE followed by activity staining, while CAT and GR activity were measured spectrophotometrically.

CAT and GR activities were not affected by treatment in either tissue studied (Fig. 2). GR and CAT activities in juice sacs and GR in flavedo showed no variation. On the other hand, CAT activity levels in flavedo dropped with the storage period, as it had been previously reported to occur in "Fortune" mandarins (Sala and Lafuente, 1999).

The other antioxidant enzymes showed statistically significant alterations between both types of samples. The results show that there was an increase in SOD activity of HT fruit with respect to control in both tissues evaluated, being of 1.24, 1.74, 1.77-fold in flavedo and 2.47, 1.53, 3.74-fold in juice sacs in the three points along the postharvest period (Fig. 3). Other differences among tissues include the isozyme pattern; three reactive bands were observed in the flavedo, while only one was detected in sacs. This form had previously been characterized as a Mn-SOD (Falcone Ferreyra et al., 2006) through inhibition assays using H_2O_2 and cyanide (see Section 2). When these studies were performed in flavedo, only the middle band was inhibited in the presence of H_2O_2 (not shown), an attribute compatible with a Fe-type SOD, while the two other should be classified as Mn-SOD isoforms. The most abundant SOD isoform from flavedo could be the unique form detected in sacs, as suggested by the same electrophoretic mobility and inhibition pattern.

Regarding POD activities, this group of enzymes showed an opposite behavior in both tissues analyzed. Thus, a increase was observed in HT flavedo (2.27, 1.55 and 1.20-fold in each pair, respectively) while a decrease was evident in HT sacs (0.33, 0.65 and 0.45-fold).

Finally, changes in APX activity were more complex to analyze due to the difficulty in selecting a single band from a group of bands that were poorly contrasted against the background. Nevertheless, it was possible to conclude that APX activity practically did not change in sacs while it had an erratic pattern in flavedo (0.70, 1.83 and 0.29-fold in each pair).

The higher SOD activity in HT fruit suggests that superoxide might be effectively eliminated, but also implies that there is a surge in superoxide production brought about by heat treatment. However, more SOD means a consistent production of H_2O_2. A higher capacity of the cured tissue to eliminate H_2O_2 in sacs is not obvious because neither APX activity nor CAT activity showed significant changes in this tissue.

According to Sala (1998), there is an intricate relationship between APX and GR activities. In fact, both enzymes act coordi-

Table 1
Physiological and chemical parameters along the different postharvest conditions. Different letters within each parameter indicate statistically significant differences.

Parameter	C	HT	C + 30	HT + 30	C + 60	HT + 60
Percentage of juice	49.6 ± 0.9^a	49.9 ± 1.7^a	46.6 ± 0.7^b	45.6 ± 1.7^b	49.3 ± 2.4^a	48.6 ± 1.6^a
Soluble solids (%)	13.3 ± 0.1^a	13.1 ± 0.3^a	13.0 ± 0.4^a	13.4 ± 0.2^a	12.4 ± 0.2^b	12.9 ± 0.3^b
Titratable acidity (%)	1.76 ± 0.04^a	$1.60 \pm 0.06^{a,c}$	$1.77 \pm 0.08^{a,e}$	$1.92 \pm 0.03^{d,e}$	1.26 ± 0.11^b	1.36 ± 0.09^b
Maturity index	7.56 ± 0.21^a	8.16 ± 0.49^a	7.31 ± 0.43^a	6.99 ± 0.07^a	9.93 ± 0.92^b	9.50 ± 0.88^b
Weight loss (%)	4.2 ± 0.6^a	3.3 ± 0.6^a	$4.8 \pm 0.9^{a,c}$	$5.0 \pm 0.8^{b,c}$	$4.2 \pm 0.6^{a,d}$	$4.8 \pm 0.6^{b,d}$
Electrolyte leakage (%)	63.9 ± 5.4^a	63.5 ± 1.4^a	64.6 ± 3.0^a	66.1 ± 2.5^a	79.1 ± 9.4^b	73.7 ± 3.3^b
Respiration rate (nmol s^{-1} kg^{-1})	218 ± 30^a	218 ± 42^a	185 ± 52^a	195 ± 50^a	187 ± 38^a	205 ± 75^a
Volatile compounds						
Acetaldehyde (µM)	163 ± 14^a	238 ± 23^b	227 ± 29^c	343 ± 64^d	188 ± 18^a	225 ± 43^b
Ethanol (mM)	5.5 ± 0.5^a	9.1 ± 1.9^b	6.1 ± 0.9^a	10.3 ± 2.3^b	7.1 ± 2.3^a	9.8 ± 2.2^b

nately in the system called APX/GR; APX proceeds by eliminating H_2O_2 excess at the expense of ascorbate oxidation while GR contributes to this system by reducing glutathione, which would maintain high ascorbate levels to support the function of APX. However, this system would not seem to be up-regulated in fruit affected by the heat treatment. It is possible that the potential increase in H_2O_2 is not high enough to induce it or that the basal activities of these two enzymes are high enough to cope with a higher H_2O_2 production. Thus, the accumulation of H_2O_2 in the fruit after the heat treatment cannot be discounted, even though high levels of this compound could be harmful to the plant tissue.

POD activity increased in flavedo whereas the opposite was observed in juice sacs. This result indicates that the accumulation of H_2O_2 could be more pronounced within the fruit, where H_2O_2 would affect the induction of defensive responses. By contrast, POD increase in flavedo accomplishes a protective role against oxidative damages that lead to pitting or spotting. Interestingly, a recent study showed an induction of PAL, peroxidases and other enzymes related to metabolism of phenolic compounds both in albedo and flavedo of oranges subjected to a curing treatment very similar to that evaluated in this work (Ballester et al., 2010).

Overall, the results found for the antioxidant enzymes would agree with the absence of visual damage on the outside of the fruit that might be caused by ROS. Attempts to detect H_2O_2 in sacs were unsuccessful, even when H_2O_2 was added as an internal control to extracts. This may be an indication that the high content of ascorbic acid present in citrus fruits also fulfills a role of protection, since it can react non-enzymatically with superoxide, H_2O_2 and singlet oxygen, minimizing the damage by acting synergistically with other antioxidants (Shao et al., 2008).

3.5. Changes in the fruit endocarp proteome after exposure to heat treatment and along the postharvest period

Preliminary electrophoretic runs before 2D-DIGE analysis revealed that most of the proteins displayed isoelectric points (pIs) between 4.0 and 7.0. About 500 spots were detected on the two-dimensional electrophoresis map carried out with soluble protein extracted from orange fruit of the six different experimental points described in Section 2. The extraction method used was chosen because the phenol extraction procedure is the protocol that habitually produces the highest quality extracts with a reasonable effectiveness according to our experience in plant tissue extraction. Moreover, a similar conclusion (based on better protein extraction and fewer interfering compounds) was reported recently in an analysis of the proteome of orange fruit (Muccilli et al., 2009).

Statistical analysis allowed the identification of significant differences in the proteomic profiles. Proteins with a minimum of 1.5-fold differential expression were subjected to mass spectrometry analyses. Following this procedure, 61 differential protein spots were detected in juice sacs among all comparisons made. Fig. 4 depicts a representative two-dimensional electrophoresis

map showing the spot distribution of proteins from fruit exposed to heat treatment versus control, after a storage period of 60 d. Proteins with differential expression are indicated by the number that appears in Table 2.

3.6. Identification of differentially expressed proteins

The differentially expressed proteins were identified by MS analysis and are listed in Tables 2 and 3. Two approaches were employed to identify the selected spots: searching MS–MS derived peptide sequence against NCBInr protein databases on all available higher plant proteins (*Viridiplantae*), and searching against EST-viridiplantae database. The complete list of peptide sequences from identified proteins is shown in Supplementary Tables 1 and 2.

Tables 2 and 3 show 28 different proteins in 40 spots among the 61 analyzed. Some of these spots matched the same protein present in the database, suggesting that different isoforms of these proteins are present in citrus fruit. Nevertheless, a post-translational modification of the same protein producing changes in molecular mass and/or pI can also account for the different mobility. The same situation has been described by other groups in proteomic studies of citrus fruit (Muccilli et al., 2009; Pan et al., 2009; Yun et al., 2010).

Among a total of 28 identified proteins, only 2 matched proteins belonging to the Citrus genus. All other proteins were identified by matching sequences from other plants, probably due to the relatively low number of Citrus protein sequences present in NCBI database. Thus, 4 belonged to *Arabidopsis thaliana*, 3 to *Vitis vinifera*, 3 to *Ricinus communis* and the remaining belonged to more phylogenetically distant plant species. Nevertheless, all spots identified by searching against EST-databases matched clones of Citrus genus, with the exception of spots 1, 14 (*Populus trichocarpa*) and 10 (*Cryptomeria japonica*). Seven spots corresponded to sequences of unknown function (spot 14), unnamed protein product (spot 44) or hypothetical protein (spots 15, 33, 34, 35 and 37, all of them identified as the same protein).

For several of the identified proteins, experimental Mr and pI were not exactly equal to the predicted values of matching proteins. A variety of factors could explain these differences, such as expression in a different organism or posttranslational modifications (Muccilli et al., 2009; Pan et al., 2009).

To identify the cellular processes involved in the heat response in citrus, the identified proteins were classified into functional groups, following the classification of the Munich Information Center for Protein Sequences (MIPS, http://mips.gsf.de). These results are summarized in Supplementary Table 3. Accordingly, the major categories found to be affected by both heat treatment and throughout the storage period were "Cell rescue, defense, and virulence" and "Metabolism"; while about 20% were found to be storage proteins and the rest related to minor categories (Fig. 5).

There is a notably high relative abundance of proteins involved in pathogen–plant interactions in heat treated samples as spots 5

Table 2

List of differentially expressed proteins in control and HT fruit. The data are grouped in three portions according to the length of storage period (0, 30 and 60 d). The second column shows changes of spot abundance ratios: negative values, if protein levels decrease in HT fruit with respect to control, or positive values, in the opposite case. For each spot, the MASCOT score, the accession number, the protein annotation, the sequence coverage, the number of matched and fragmented peptides and finally the theoretical and experimental relative molecular mass (Mr) and isoelectric point (pI) are indicated. When the search was carried out using the *Viridiplantae* ESTs database, the EST accession number and the E-value associated to the best matching protein obtained via BLASTx are also indicated.

Spot no.	Ratio	MASCOT Score	EST Acc. no. translated ORF	Acc. no. of homologous found in NCBInr	Protein annotation of homologous found in NCBInr [*organism*]	E-value	Coverage %	Matched peptides	Fragmented peptides	Theoretical Mr	Theoretical pI	Observed Mr	Observed pI
C vs HT													
1	−8.81	112	XP_002325470	EEF47403	Regulator of ribonuclease activity A, putative [*Ricinus communis*]	7.E−88	35	5	2	17.97	5.39	20.00	5.30
2	−1.71	73		AAP33012	HSP Class II [*Citrus x paradisi*]		15	1	1	11.19	8.01	21.00	4.90
3	−3.93	173	CB290265	BAA29064.1	Heat shock protein 26 (Type I) [*Nicotiana tabacum*]	4.E−77	36	6	3	29.50	9.14	29.00	5.40
4	−1.55	199	EY668743	EEF47568.1	Heat shock protein, putative [*Ricinus communis*]	6.E−55	32	6	3	31.52	9.55	34.00	5.80
5	−1.78	373	CX297938	ABR68690.1	Oxalate oxidase [*Theobroma cacao*]	5.E−38	54	3	3	11.73	4.77	33.00	5.60
6	+3.27											20.00	4.95
7	+3.74	107	EY696830	NP_180174	Bet v I allergen family protein [*Arabidopsis thaliana*]	1.E−50	47	7		27.68	6.59	37.00	4.85
8	+2.99	272	FC868659	Q9ZWQ8.1	Plastid-lipid-associated protein, chloroplastic [*Citrus unshiu*]	3.E−127	52	7	2	25.60	4.75	40.00	4.85
C + 30 vs HT + 30													
9	−1.5	68	CX297938	ABR68690.1	Oxalate oxidase [*Theobroma cacao*]	3.E−39	54	3	2	11.73	4.77	38.00	5.70
10	−2.09	78	ABW90888	AAK27264.1	Isoflavone reductase-like protein CJP-6 [*Cryptomeria japonica*]	9.E−178	28	8		53.34	8.83	43.00	6.25
11	+1.83	212	CV885795	ABD98820.1	UDP-glucose pyrophosphorylase [*Cucumis melo*]	4.E−134	42	7	4	28.56	6.63	62.00	6.30
C + 60 vs HT + 60													
12	−226.6											17.00	5.15
13	−4.23	116	AAP33012	AAP33012.1	HSP19 class II [*Citrus x paradisi*]	3.E−49	42	2	1	11.19	8.01	27.00	5.50
14	−1.6	122	ABK94100	ABK94100.1	Unknown [*Populus trichocarpa*]	7.E−85	35	3	1	17.03	6.62	29.00	6.00
15	−1.57	146	DR403908	XP_002284334.1	Hypothetical protein [*Vitis vinifera*]	6.E−56	36	4	2	21.75	7.04	38.00	5.20
16	−1.97											44.00	6.15
17	−1.72	205	CX668191	XP_002533363.1	Fructokinase, putative [*Ricinus communis*]	9.E−160	56	11	3	32.84	5.02	46.00	5.20
18	−2.08											50.00	5.00
19	−2.95											52.00	5.50
20	−2.13											55.00	6.40
21	+493	104	EY659853	NP_200632.2	bis(5′-adenosyl)-triphosphatase, putative [*Arabidopsis thaliana*]	2.E−68	32	5	1	33.70	8.80	24.00	5.80

Table 3

List of proteins that differentially change expression levels along the storage period. The data are grouped in three portions according to the condition in which the changes occurred (control samples, HT samples or both). The parameters are the same that were described in Table 2 with exception of the third column, where the comparison made is indicated.

Comparisons made within each condition throughout storage period

Spot no.	Ratio	Comparison	MASCOT Score	EST Acc. no. translated ORF	Acc. no. of homologous found in NCBInr	Protein annotation of homologous found in NCBInr [organism]	E-value	Coverage %	Matched peptides	Fragmented peptides	Computed Mr	Computed pI	Observed Mr	Observed pI
Changes within control samples (C)														
22	+6.45	C vs C+30											15.00	5.35
23	+1.78	C vs C+30											16.00	5.45
24	+1.83	C vs C+30											17.00	5.40
26	+1.95	C vs C+30											21.00	5.15
27	−1.63	C vs C+30											42.00	6.25
28	+1.98	C vs C+30	187		AAQ18140	Enolase [*Gossypium barbadense*]		30	8	1	47.87	6.16	52.00	5.80
	+1.54	C vs C+60												
29	+2.61	C+30 vs C+60	135		AAQ18140	Enolase [*Gossypium barbadense*]		15	4	1	47.87	6.16	52.00	5.75
30	+1.69	C vs C+30	148	EY710132	ABR68690.1	Oxalate oxidase [*Theobroma cacao*]	4.E−38	31	7	2	41.65	9.55	62.00	5.50
31	−1.77	C vs C+60	147	CV718696	ABD66504.1	Actin depolymerizing factor 8 [*Gossypium hirsutum*]	1.E−70	29	3	1	23.49	6.20	21.00	5.40
32	−2.28	C vs C+60											29.00	6.10
34	−2.99	C vs C+60	191	CX297938	XP_002284334.1	Hypothetical protein [*Vitis vinifera*]	2.E−43	54	3	2	11.73	4.77	33.00	5.45
35	+1.93	C vs C+60	191	CX297938	XP_002284334.1	Hypothetical protein [*Vitis vinifera*]	2.E−43	54	3	2	12.00	5.00	34.00	5.35
36	+12.42	C vs C+60	119		BAF46352	Alpha chain of nascent polypeptide associated complex [*Nicotiana benthamiana*]		26	4	2	21.91	4.32	38.00	4.40
	+1.84	C+30 vs C+60												
37	+2.41	C vs C+60	99	EY705259	XP_002284334.1	Hypothetical protein [*Vitis vinifera*]	1.E−51	30	6	3	31.94	9.49	37.00	5.30
38	+2.13	C vs C+60											48.00	5.15
39	+2.6	C vs C+60	164		ABB29926	Fructose-bisphosphate aldolase-like [*Solanum tuberosum*]		8	2	1	38.90	8.32	44.00	6.80
40	−1.85	C vs C+60											47.00	5.70
41	−1.83	C+30 vs C+60	167		AAP33012	HSP19 class II [*Citrus x paradisi*]		42	2	1	11.19	8.01	27.00	5.50
42	−1.71	C+30 vs C+60											21.00	5.00
44	−1.86	C+30 vs C+60	69		CAO64961	Unnamed protein product [*Vitis vinifera*]		18	4		37.93	9.35	24.00	5.15
45	−4.54	C+30 vs C+60	89	CX292452	NP_175665.1	26.5 kDa class I small heat shock protein-like [*Arabidopsis thaliana*]	4.E−68	20	3	1	22.81	6.43	29.00	5.75
46	+1.6	C+30 vs C+60	169		AAV50005	14-3-3 family protein [*Malus x domestica*]		32	6	1	29.79	4.75	41.00	5.65
47	+1.75	C+30 vs C+60											64.00	5.90

Changes within heat treated samples (HT)

48	+1.58	HT vs HT + 30											25.00	6.00
49	+1.68	HT vs HT + 30	120	DR908342	AAS46231.1	Methionine sulfoxide reductase A [*Populus trichocarpa x Populus deltoides*]	8.E−90	29	5	2	24.52	5.64	33.00	6.00
50	+4.36	HT vs HT + 30	86		ABR13881	Arginase [*Malus hupehensis*]		14	5	1	37.15	6.14	46.00	6.00
51	−2.05	HT vs HT + 60	113	CF509972	XP_002513910.1	Annexin. putative [*Ricinus communis*]	5.E−118	36	7	1	28.46	5.67	45.00	5.80
52	−2.26	HT vs HT + 60	90		ABJ80924	Actin [*Pterosperma cristatum*]		11	3	1	38.43	5.37	46.00	5.80
53	+4.04	HT vs HT + 60	274	CX664795	NP_172934.1	Semialdehyde dehydrogenase family protein [*Arabidopsis thaliana*]	2.E−95	28	4	2	28.98	8.35	47.00	5.80
54	−1.88	HT vs HT + 60	188		ABG49457	Actin 1 [*Boehmeria nivea*]		39	8	1	38.54	5.47	49.00	5.45
55	+1.99	HT vs HT + 60	188		ABG49457	Actin 1 [*Boehmeria nivea*]		39	8	1	38.54	5.47	49.00	5.15
56	+1.90	HT vs HT + 60											51.00	5.15
57	+1.53	HT vs HT + 60	317	CX052958	NP_001151807.1	ATP synthase beta chain [*Zea mays*]	3.E−155	57	11	5	30.61	5.92	53.00	5.50
58	+1.57	HT vs HT + 60											58.00	4.85
59	+1.74	HT vs HT + 60											62.00	4.85
60	−3.1	HT + 30 vs HT + 60	112	FC909871	AAC36312.1	CytosoliHTclass II small heat shock protein [*Solanum lycopersicum*]	2.E−49	21	3	1	25.09	9.05	20.00	5.40
61	+15.12	HT vs HT + 60											45.00	6.80

Changes within both types of sample

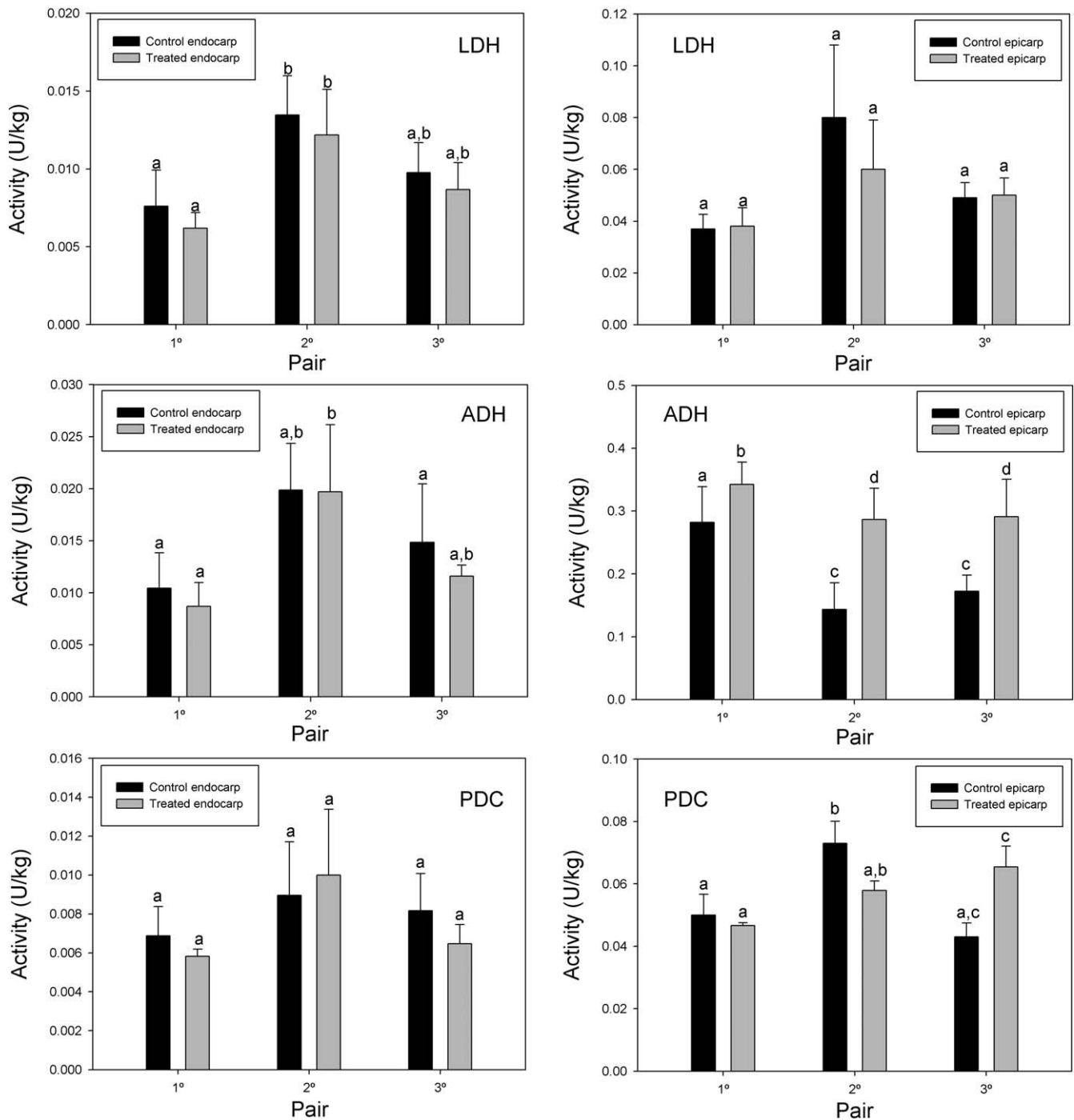|														
25	+3.16	C vs C + 30	81	CV718696	ABD66504	Actin depolymerizing factor 8 [*Gossypium hirsutum*]	1.E−71	29	3	1	23.49	6.20	21.00	5.25
	+3.33	HT vs HT + 30												
33	+1.53	C vs C + 60	188	DR403908	XP_002284334.1	Hypothetical protein [*Vitis vinifera*]	6.E−56	38	5	2	21.75	7.04	32.00	5.75
	+1.56	HT vs HT + 60												
43	−6.63	C + 30 vs C + 60	118		AAP33012	HSP19 class II [*Citrus x paradisi*]		42	2	1	11.19	8.01	27.00	5.50
	−4.65	HT + 30 vs HT + 60												

Fig. 1. Enzymes involved in fermentative metabolism in sacs (left plots) and flavedo (right plots). The activity of enzymes responsible for lactic (LDH) and alcoholic (ADH and PDC) fermentation was analyzed in each experimental pair, in both sacs and flavedo. Activity is expressed in units (U) kg^{-1} of total soluble protein in all cases. For each enzyme, bars with the same letters are not significantly different ($P < 0.05$). Values represent the mean of at least five independent determinations.

and 9 (oxalate oxidase) (Yagami et al., 2000), 7 (Bet v 1 allergen family protein) (Markovic-Housley et al., 2003; Schenk et al., 2009), 8 (plastid-lipid-associated protein) (Vishnevetsky et al., 1999) and 10 (isoflavone reductase-like protein) (Lers et al., 1998). These results might provide a clue to understand the effectiveness of the heat treatment to prevent fungi infection.

On the other hand, 4 of the 15 identified spots differentially expressed between treatments correspond to the large family of small HSPs and apparently their expression levels would be lower after heat treatment. Given these conflicting observations, Western blot assays were carried out, as described below. Furthermore,

spots 41, 43, 45 and 60 showed a similar behavior by decreasing at the end of storage period and all of them were identified as members of the small HSPs family.

3.7. Differences in the proteome of control versus treated fruit

As is apparent in Table 2, the number of proteins with decreased expression after HT was higher than the quantity of those induced by it. Among the latter are the Bet v 1 allergen family protein and a plastid-lipid-associated protein, both immediate response proteins. Two late response proteins are also induced:
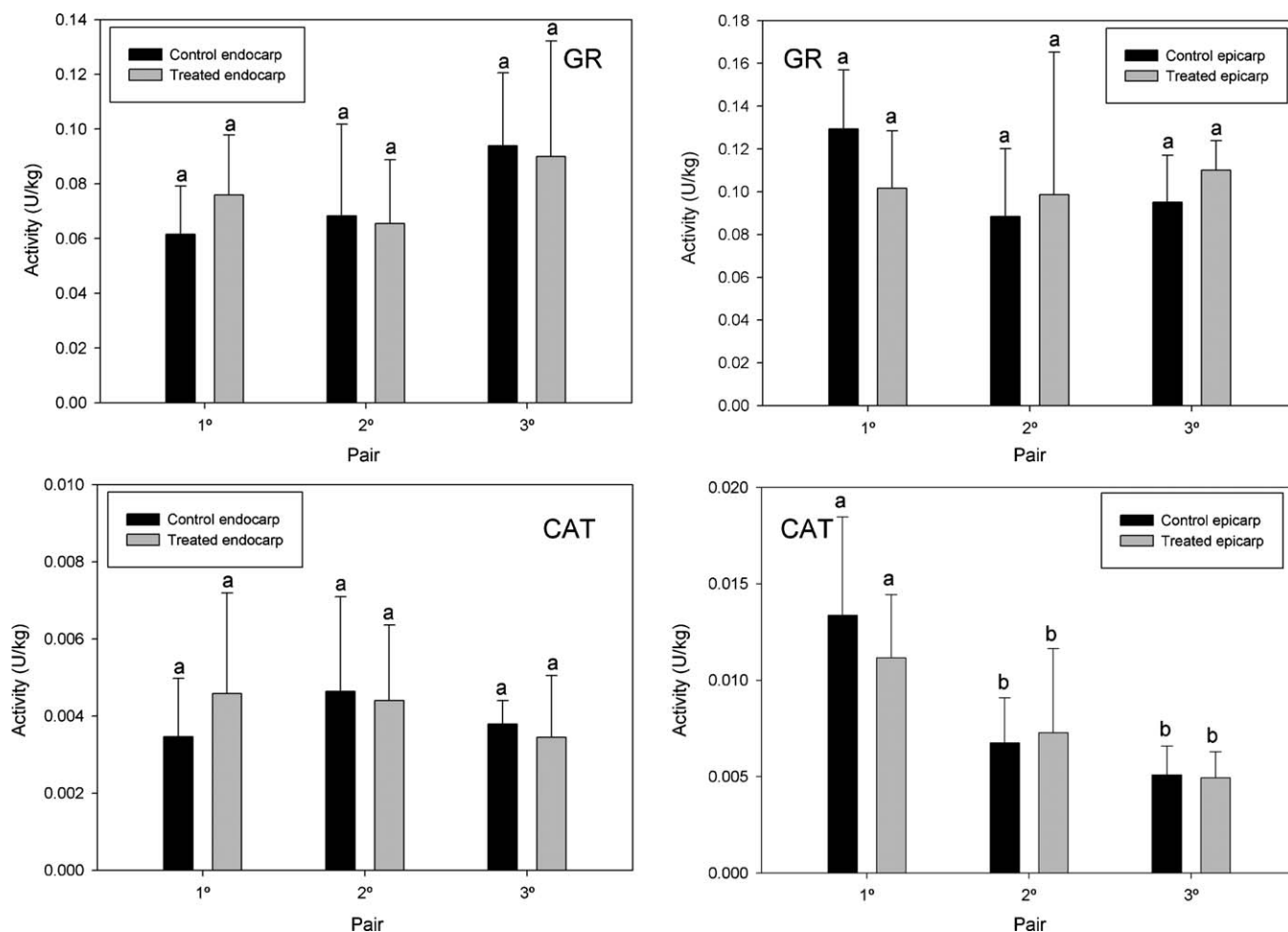
Fig. 2. GR and CAT activities in sacs (left plots) and flavedo (right plots). The activities of GR and CAT were analyzed in each experimental pair, in both sacs and flavedo. Activity is expressed in units (U) kg^{-1} of total soluble protein in all cases. For each enzyme, bars with the same letters are not significantly different ($P < 0.05$). Values represent the mean of at least five independent determinations.

UDP-glucose pyrophosphorylase (induced after 30 d of storage) and a putative bis (5′-adenosyl)-triphosphatase (induced after 60 d).

The Bet v 1 protein belongs to the ubiquitous family of pathogenesis-related plant proteins (PR-10), which are produced in response to various pathogens. This protein is a member of the major birch allergens and has been shown to possess ribonuclease activity (Ines et al., 1996). While the function of this protein in different plant tissues has yet to be elucidated, it has been suggested that it could act as a brassinosteroid carrier in pollen (Markovic-Housley et al., 2003). The biological function of brassinosteroids was defined as regulating the processes of growth, development and gene expression. It is conceivable that such a steroid carrier system could allow rapid trafficking of signalling molecules, which could be readily delivered to their receptor(s) for quick response to external stimuli. It is worth to note that Bet v 1 increase is only evident immediately after the conclusion of HT and is not sustained at later stages (see Table 2).The accumulation of lipid-associated proteins in plastids as well as the biogenesis of structures that sequester hydrophobic compounds has been shown to be stimulated by various stresses (Langenkamper et al., 2001; Leitner-Dagan et al., 2006). This could be an indication of a transient burst in the ripening process induced by heat treatment. Because the genes encoding these proteins are nuclear, their expression must be mediated by some unknown signalling mechanism, which could be linked to an increase in ROS levels in the plastid. H_2O_2 appears to be the most appropriate candidate for this task because of its ability to cross membranes relatively easily (Langenkamper et al., 2001). However, as discussed above, there are no indications of an increase in H_2O_2 in sacs.

Ciereszko et al. (2001) found that the gene for UDP-glucose pyrophosphorylase was induced in Arabidopsis after illumination of leaves previously held in darkness and also under conditions of cold stress. All these conditions resulted in a marked increase in the concentrations of sucrose in the cells, and to some extent, fructose and glucose, pointing to these metabolites as participants in a common transduction mechanism for the regulation of this gene. As discussed below, HT fruit had a higher content of sucrose in sacs, which would be potentially linked to the gene regulation mentioned above.

In the case of bis(5′-adenosyl)-triphosphatase, the strong increase in this enzyme levels may be the result of an overproduction of diadenosine-5′,5‴-P1,P3-triphosphate (Ap3A). One feasible source of Ap3A is the synthesis of phenylpropanoids that proceeds through coumarate:CoA ligase. It has been demonstrated that this enzyme has the capacity to synthesize and use various diadenosine polyphosphates (Pietrowska-Borek et al., 2003). Therefore, induction of this enzyme linked to a higher lignin production could give rise to elevated Ap3A levels, whose degradation would be accounted for bis(5′-adenosyl)-triphosphatase. Accordingly, lower p-coumaric acid levels were detected in treated fruit in the first and third pairs with respect to controls (see Section 3.10), which could be an indication of a higher production of lignin (Wróbel-Kwiatkowska et al., 2007).
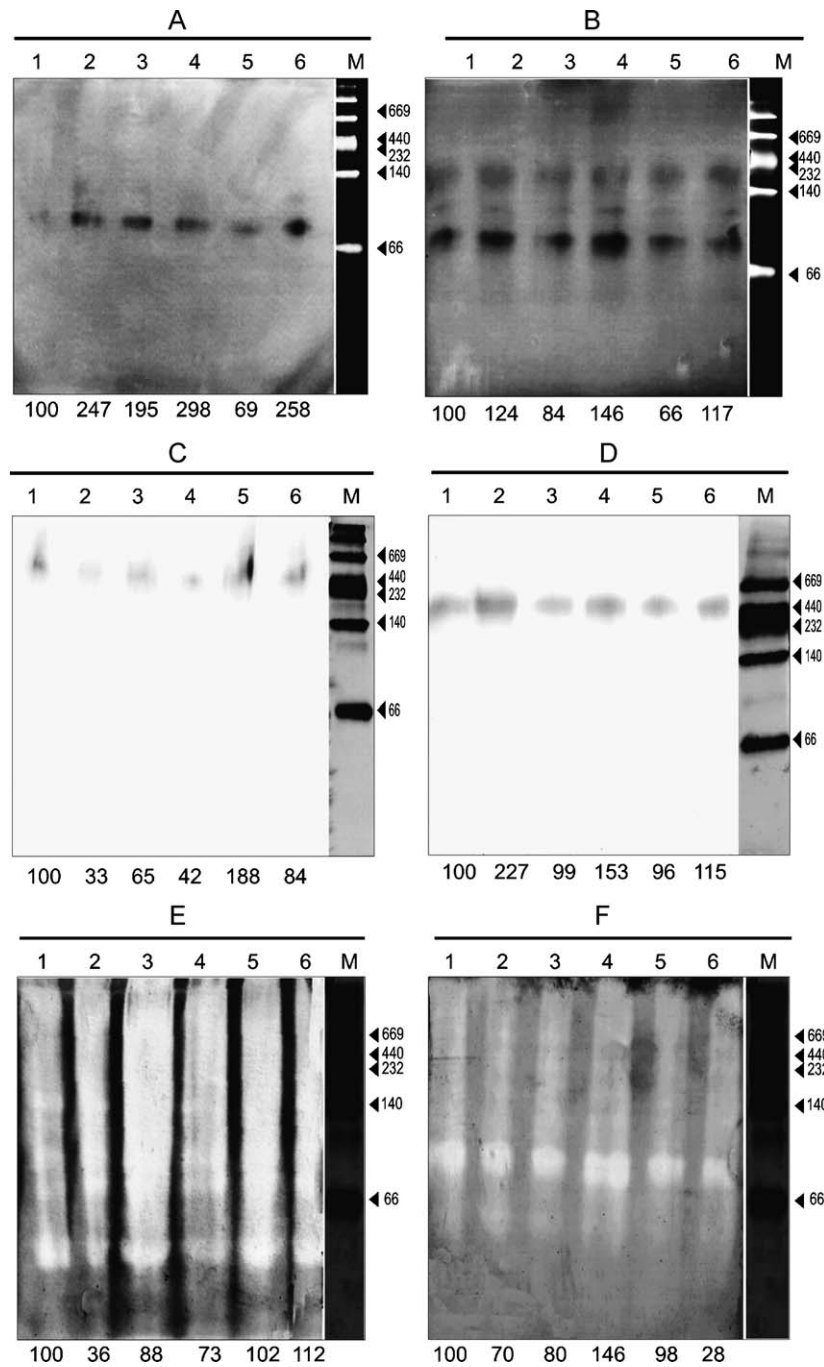
Fig. 3. Analysis of SOD (A and B), POD (C and D) and APX (E and F) enzymes by non-denaturing PAGE stained by enzymatic activity. Gels on the right correspond to flavedo extracts while those on the left correspond to juice sacs extracts. In all cases loading of lanes was as follows: lane 1: crude extracts from control fruit (C); lane 2: crude extracts from HT fruit (HT); lane 3: crude extracts from C samples after 30 d of storage; lane 4: crude extracts from HT samples after 30 d of storage; lane 5: crude extracts from C samples after 60 d of storage; lane 6: crude extracts from HT samples after 60 d of storage. Twenty µg of total protein were loaded in each lane. The numbers below the figures indicate the relative intensities of reactive bands determined by densitometric analysis respect to control samples (100%). The numbers on the right of each gel indicate the mass of the standard molecular weight markers.

Most of the differential spots identified corresponded to a decrease in protein levels in HT fruit compared to controls. Among these, the enzymes oxalate oxidase and isoflavone reductase, both apparently associated with the response to pathogens, decreased in the second pair, while oxalate oxidase decreased in the first pair too. Although this enzyme is not fully characterized, especially with regard to its location and substrates, many observations seem to indicate that it is able to generate H_2O_2 and its activation occurs as a result of pathogenic infection. However, the oxalate oxidase appears to be limited to plant–pathogen interactions only in cer-

tain species (Dumas et al., 1995; Lane, 2002). Decreased levels of this enzyme would be beneficial inasmuch as it would lower the production of ROS. The effect lasts at least for 30 d after the heat treatment, but is not present after 60 d.

On the other hand, the gene encoding for isoflavone reductase showed an induction in response to UV light treatment in grapefruit flavedo (Lers et al., 1998). Moreover, the expression of isoflavone reductase was induced by injury and infection by pathogens. The function of these proteins is not clear yet, although it has been suggested that they could be involved in the response
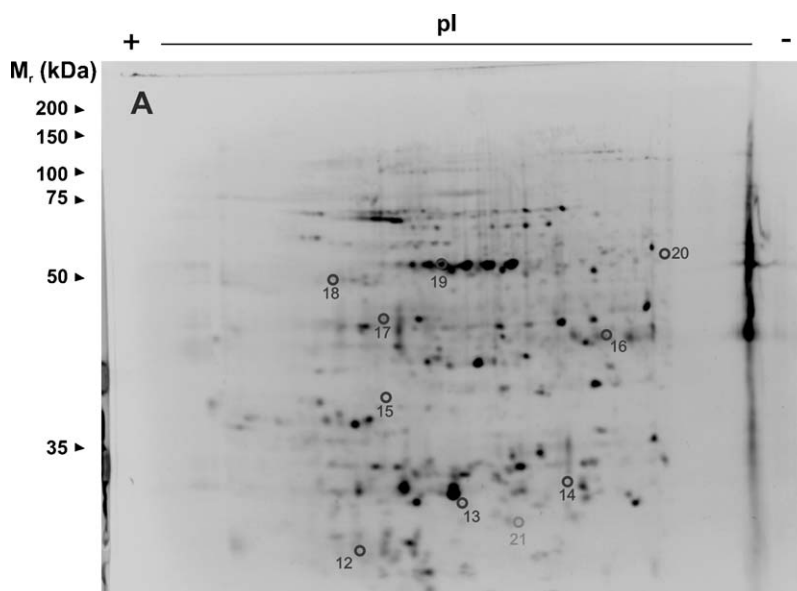
Fig. 4. Two-dimensional electrophoresis maps of total proteins from control fruit after 60 d of storage at 5 °C. This image was acquired by using a 610 nm filter, according to the emission wavelength of the dye Alexa 610 used to label this sample. Proteins were separated over the p*I* range 4–7 in the first dimension and on 15% (w/v) SDS-polyacrylamide gels in the second dimension. The graph represents one example from at least three different gels used for the differential analysis. Protein spots differentially expressed are numbered.
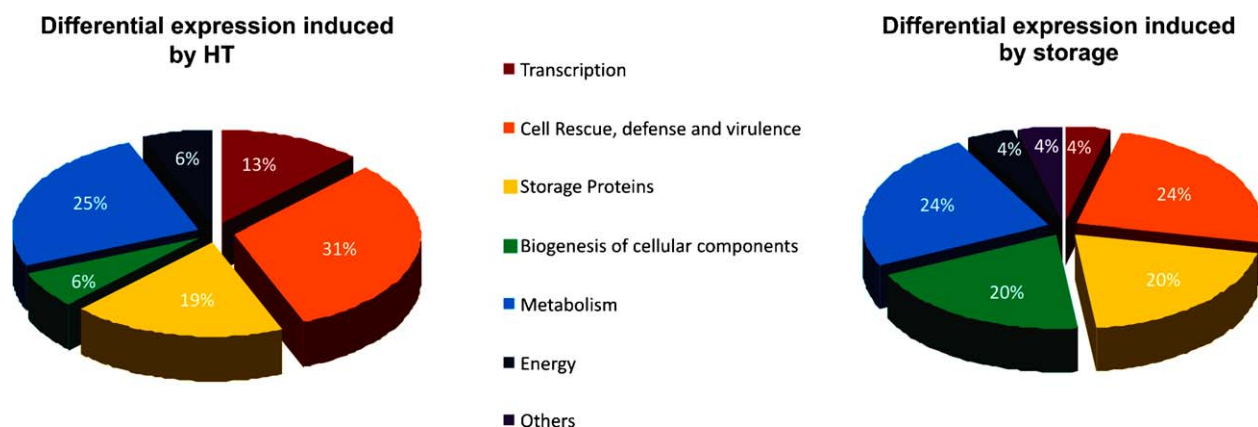
Fig. 5. Functional classification of citrus differentially expressed proteins in the two types of comparisons made. Functional categorization was performed according to the MIPS database (http://mips.gsf.de). The numbers on each fraction indicate the percentage of the detected proteins belonging to each category.

to oxidative stress in Arabidopsis and maize (Petrucco et al., 1996).

In this way, it could be hypothesized that the activity of these two enzymes is more important at a time subsequent to pathogen attack than during the prevention response induced by the heat treatment.

RNA metabolism seems to be affected as well, as shown by an important decrease in the abundance of the regulator of ribonuclease activity A, an inhibitor of RNase E (Zhao et al., 2006). The consequence would be a change in the abundance of plastidic mRNAs, as RNase E has been found in the chloroplasts in Arabidopsis (Schein et al., 2008).

3.8. Changes occurring during storage

With regard to changes that occurred during the storage period (Table 3) only three spots (25, 33 and 43) showed the same pattern in both groups of fruit (controls and treated), which implies that such proteins are clearly affected by storage time. Other proteins varied with time in a treatment-dependent way. In this context, it is of interest to compare our results with those obtained in a recent study in ponkan (*Citrus reticulata*) which succeeded in identifying
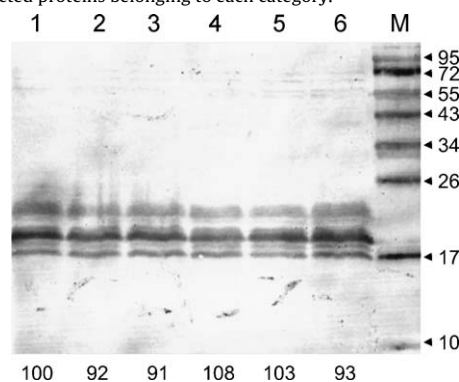

Fig. 6. Immunodetection of small HSPs enzymes from juice sacs extracts using immune serum raised against *Solanum lycopersicum* purified HSP21. Lane 1: crude extracts from control Valencia orange fruit (C); lane 2: crude extracts from HT Valencia orange fruit (HT); lane 3: crude extracts from C samples after 30 d of storage at 5 °C; lane 4: crude extracts from HT samples after 30 d of storage at 5 °C; lane 5: crude extracts from C samples after 60 d of storage at 5 °C; lane 6: crude extracts from HT samples after 60 d of storage at 5 °C. Twenty μg of total protein was loaded in each lane. The numbers below the figures indicate the relative intensities of reactive bands determined by densitometric analysis respect to control samples (100%). The numbers on the right of each gel indicate the mass of the standard molecular weight markers.
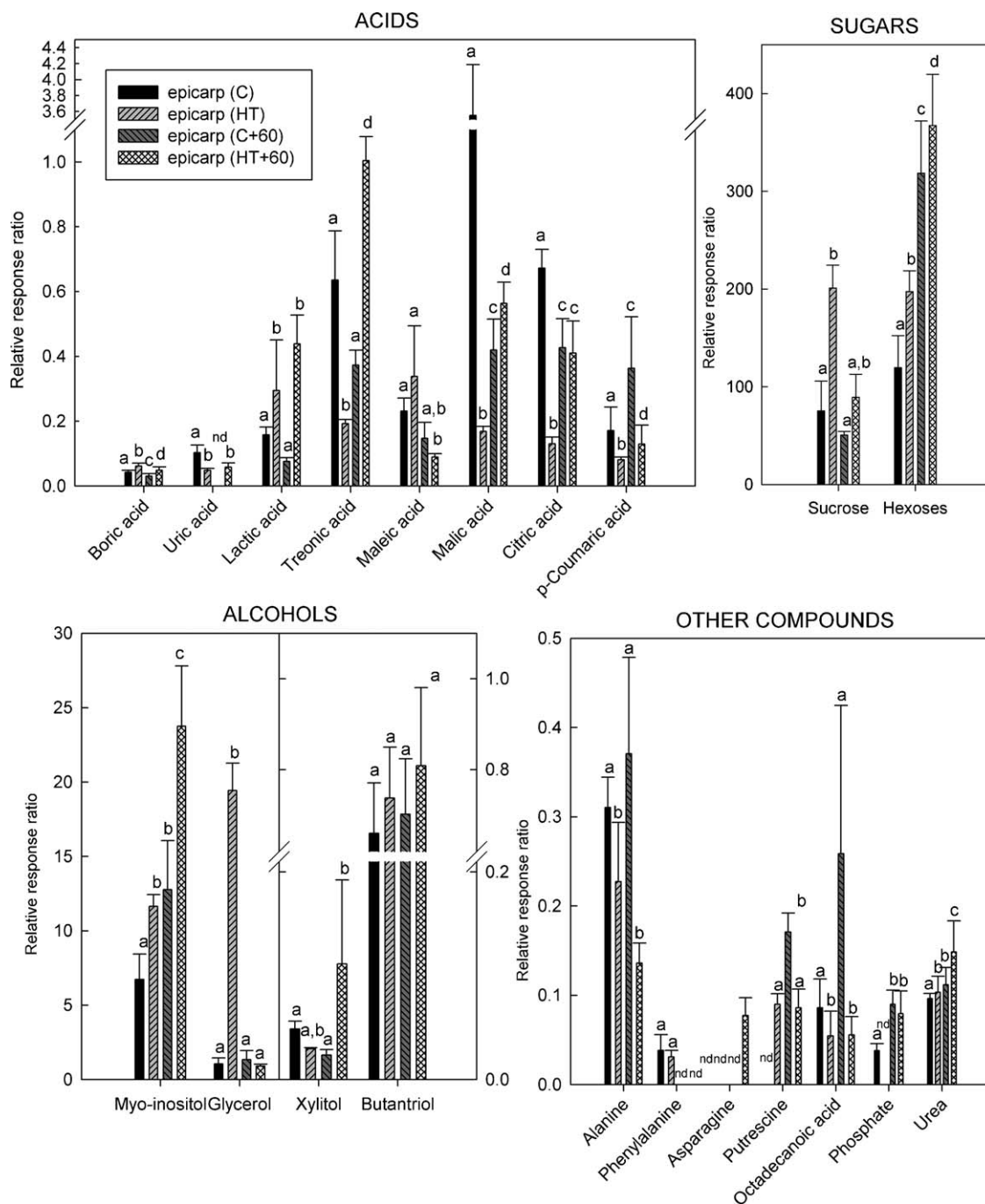
Fig. 7. Quantitation of acids, sugars, alcohols and other compounds in epicarp. Determinations of metabolite concentrations into the first and third pair of flavedo samples were carried out using pools of six independent samples. Variation of levels of metabolites are expressed as the relative response ratio with respect to the ribitol internal standard and normalized to the fresh weight (in grams) of the sample. Statistical analysis was accomplished as described in Section 2. In the following cases it was required to transform the variable: citric, malic, lactic and p-coumaric acids, myo-inositol and alanine, with logarithmic function; urea and octadecanoic acid, with a reciprocal function. Treonic and maleic acids, sucrose, glycerol, xylitol, phenylalanine and phosphate were analyzed by non-parametric methods. For each metabolite, bars with the same letters are not significantly different ($P < 0.05$).

56 differentially expressed proteins during cold storage (Yun et al., 2010). This might be an indication of the different cold susceptibility of both species.

Interestingly, spot 25, which showed an increment of ∼3-fold in both types of samples after 30 d of storage, belongs to the actin-depolymerizing factor (ADF) family. ADF function has been related to ATP hydrolysis in actin assembly to enhance filament dynamics (Carlier et al., 1997). Actin reorganization in response to external and internal cues is likely to be modulated by the spatial distribution and activity of actin-associated proteins. It should be noted that

after contact with fungal hyphae, plant cells typically respond with a proliferation of microfilaments that radiate from the site of contact. This cytoskeletal response is tightly coupled to the resistance mechanism, because it does not occur, or is significantly delayed, in compatible interactions (Staiger et al., 1997). In this case, concerted cytoskeletal rearrangements seem to be occurring during the earlier stages of storage, independently of treatment applied. However, there is evidence that it persists along the storage of cured fruits, as shown the alterations in actin abundance (spots 52, 54 and 55) and other cytoskeletal proteins as annexin (spot 51).
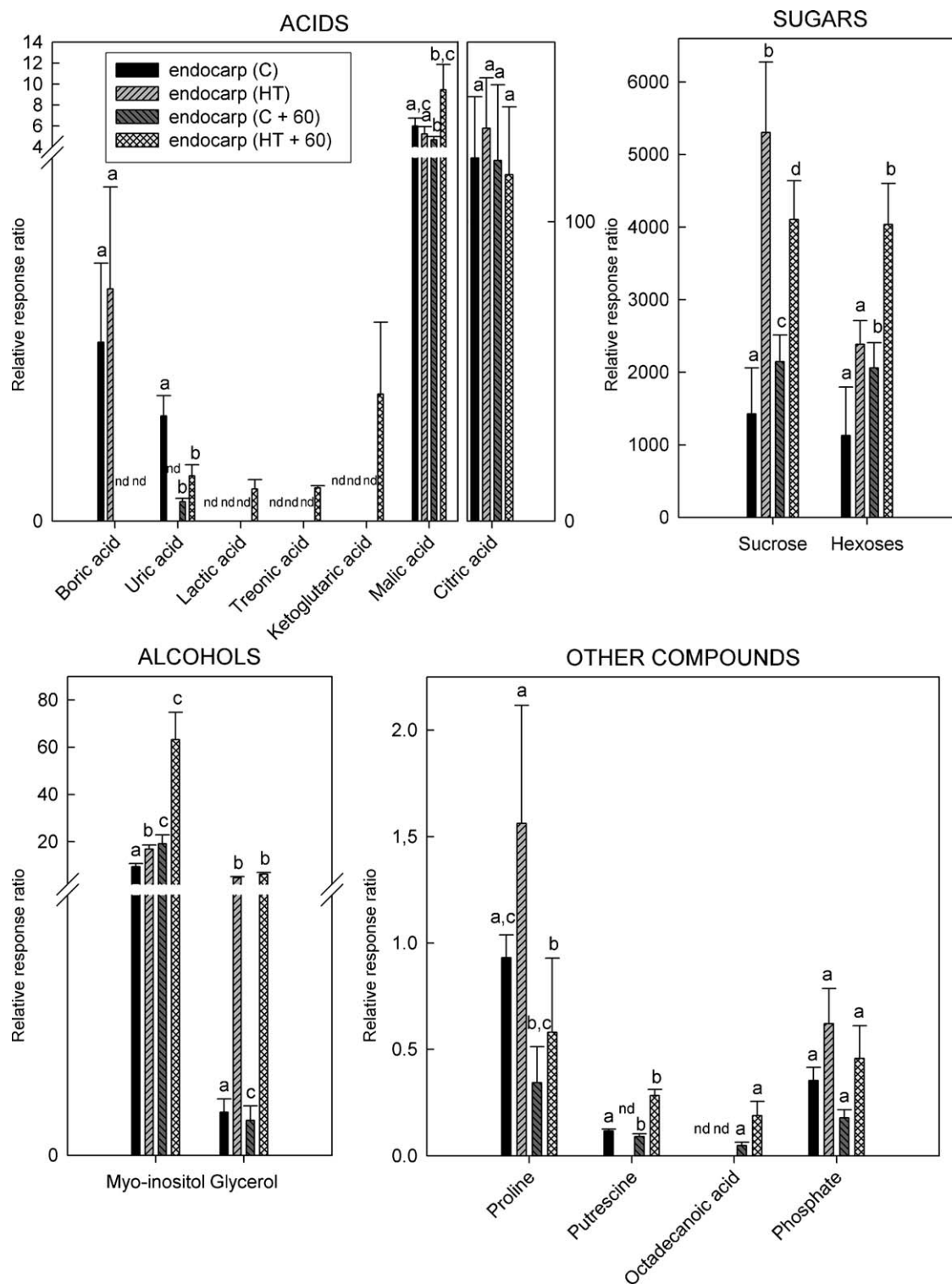
Fig. 8. Quantitation of acids, sugars, alcohols and other compounds in endocarp. Determinations of metabolite concentrations into the first and third pair of juice sacs samples were carried out using pools of six independent samples. Variation of levels of metabolites are expressed as the relative response ratio with respect to the ribitol internal standard and normalized to the fresh weight (in grams) of the sample. Statistical analysis was accomplished as described in Section 2. In the following cases it was required to transform the variable: citric acid, myo-inositol and glycerol, with a logarithmic function. Malic, uric and octadecanoic acids were analyzed by non-parametric methods. For each metabolite, bars with the same letters are not significantly different ($P < 0.05$).

3.9. Immunoblot analysis of small HSPs

The heat stress response is characterized by the induction of a set of mRNAs and proteins that constitute a complex mixture of polypeptides with high and low molecular weight, known as HSPs (Heat Shock Proteins). This response seems to be temporary in some cases or sustained along the time in others.

In order to determine whether the changes observed in the small HSPs abundance by 2D-DIGE analysis in sacs were accurate, immunoblot analyses of control and HT fruit along the storage

period at 5 °C were conducted. Fig. 6 shows that there was not any significant variation in the immunoreactive proteins among the samples analyzed.

The contrasting results of proteomic data versus immunodetection can be analyzed in the following manner. In some samples a single or a small number of proteins may be present in such abundance that they mask many other proteins of interest. These proteins become interfering factors in 2-DE proteomic analysis of regulatory proteins in two ways: (1) they co-migrate with the low-abundance proteins during 2-DE causing the latter to be undetectable (Corthals et al., 2000), and (2) since they account for a substantial proportion of total protein sample and protein loading capacity for a given immobilized pH gradient (IPG) strip is limited, low-abundance proteins may only be taken up by the IPG strip in a small quantity and hence hardly visualized by 2-DE analysis. This problem could be more important yet in preparative gels, where the total quantity of sample load is 5-fold higher than in gels where the analysis is carried out. This fact would explain the unfeasibility of detecting the true protein identity of spots 2, 3, 4, 13, 41, 43 and 45, due probably to the high density of small HSPs in that zone of gel. Previous studies that show a considerable high number of chaperones/heat shock proteins (9.3% of total identified proteins) in the citrus fruit proteome (Katz et al., 2007) support this idea. Moreover, this high relative abundance might well be the cause of the difficulty to detect an appreciable induction of this group of proteins. In addition, the present heat treatment may not be perceived by the orange fruit as a stressful treatment but a long exposition to medium temperatures that do not necessarily elicit a full response to thermal stress.

3.10. Fruit metabolite content

Organic acids, sugars, alcohols and other compounds were measured at 0 and 60 d of storage at 5 °C in control and HT fruit, both in flavedo and juice sacs (Figs. 7 and 8, respectively). GC–MS analysis revealed that a large fraction of the metabolites analyzed was significantly changed in at least one of the conditions studied.

In flavedo, major differences were found in acid levels. Heat treatment produced an increase in boric and lactic acids in both pairs, while the most abundant organic acids, malic and citric, showed an important decrease in the first pair, and then their levels became more similar in the third pair. Boric acid may arise as a consequence of cellular reorganization as it has been reported that one possible function of this acid is in the crosslinking of rhamnogalacturonan II in the cell wall (Matoh, 1997). The lower p-coumaric acid levels detected in HT fruit in the first and third pairs are also an indication of this reorganization, which could involve a higher rate of lignin production. With respect to the behavior along time in flavedo, malic and citric acids from control samples decreased significantly at 60 d of storage while these acids showed the reverse tendency in HT samples. In contrast, the main organic acids did not show important changes in sacs, while lactic, treonic and ketoglutaric acids could only be detected in HT samples of the third pair for this tissue. Nevertheless, titratable acidity decreased in the third pair with respect to the first one in both types of samples (Table 1). In sacs, this parameter is practically a measurement of citric acid content, because of the predominance of this organic acid over the others. The impossibility to detect significant variations in citric acid content by GC–MS measurements in this tissue could be understood by taking into account the higher experimental error associated with this method in comparison with the analytical titration's error (12–20% vs 3–8%, respectively).

Another important difference relative to the heat treatment was the higher sugar content found in HT samples, which was most pronounced for sucrose content in sacs and for hexose levels in flavedo.

The behavior of glycerol was tissue-dependent. It markedly increased in HT sacs from both pairs, and only rose in the first pair of flavedo samples. On the other hand, while myo-inositol increased in HT flavedo from both pairs, it only rose significantly in the first pair of sacs samples. An increase in this metabolite could be the result of elevated synthesis or the result of degradation of inosytol phosphates, such as 1-L-myo-inositol-1-phosphate (MIP). This metabolite is a precursor to compounds connected to essential cellular functions, such as phosphorus storage, signal transduction, actin remodelling, membrane trafficking, stress protection, hormonal homoeostasis and cell wall biosynthesis (Downes et al., 2005; Loewus and Murthy, 2000; Stevenson et al., 2000). Interestingly, Pi levels in sacs showed a profile similar to myo-inositol, although the differences were not statistically significant. It could be hypothesized that both compounds proceed from MIP degradation. If inosytol phosphates are being degraded, the Pi released could be used for increased sugar phosphorylation. If fermentation is boosted by maturation, more sugar metabolism will be needed to support ATP synthesis and that could act as a Pi sink.

Other compounds followed a very different pattern depending on the condition analyzed. Perhaps the most evident change is an increase in putrescine levels in HT flavedo in the first pair, although this pattern was reversed in the third pair. Curiously, levels of this metabolite showed an exactly opposite behavior in sacs. González Aguilar et al. (2000) found a similar trend in the flavedo of Fortune mandarins, where putrescine increased with conditioning, specially with temperatures over 20 °C. Putrescine has been shown to be essential to counteract cold stress in Arabidopsis (Cuevas et al., 2008) and to allow the ABA increase in cold stressed plants (Cuevas et al., 2009). These variations may reflect changes in ABA content in both types of cells, although at this time it is not possible to ascertain whether this is taking place in orange.

4. Concluding remarks

The heat treatment discussed in this paper produces numerous changes in general metabolism of orange fruit in both exocarp and in endocarp, such as alterations in the activities of antioxidant enzymes, induction of key proteins in response to pathogen attack, changes in compounds involved in major metabolic pathways and possibly a cellular reorganization process. All these results lead to a lower degree of susceptibility of the fruit against fungal pathogens, while explaining the maintenance of postharvest quality. Many areas of research remain open after all these findings, for instance the elucidation of compartmental changes and the levels of the enzymes involved in carbohydrate metabolism during the postharvest period and how these are affected by heat treatment.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.postharvbio.2011.05.015.

References

Amme, S., Matros, A., Schlesier, B., Mock, H.-P., 2006. Proteome analysis of cold stress response in *Arabidopsis thaliana* using DIGE-technology. J. Exp. Bot. 57, 1537–1546.

Ballester, A.R., Izquierdo, A., Lafuente, M.T., González-Candelas, L., 2010. Biochemical and molecular characterization of induced resistance against *Penicillium digitatum* in citrus fruit. Postharv. Biol. Technol. 56, 31–38.

Beauchamp, C.O., Fridovich, I., 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal. Biochem. 44, 276–287.

Bollag, D.M., Edelstein, S.J., 1991. Protein Methods, 1st ed. Wiley-Liss Inc., New York.

Carlier, M.F., Laurent, V., Santolini, J., Melki, R., Didry, D., Xia, G.-X., Hong, Y., Chua, N.-H., Pantaloni, D., 1997. Actin depolymerizing factor (ADF/Cofilin) enhances the rate of filament turnover: implication in actin-based motility. J. Cell Biol. 136, 1307–1322.

Casati, P., Zhang, X., Burlingame, A.L., Walbot, V., 2005. Analysis of leaf proteome after UV-B irradiation in maize lines differing in sensitivity. Molec. Cell Proteom. 4, 1673–1685.

Ciereszko, I., Johansson, H., Kleczkowski, L.A., 2001. Sucrose and light regulation of a cold-inducible UDP-glucose pyrophosphorylase gene via a hexokinase-independent and abscisic acid-insensitive pathway in *Arabidopsis*. Biochem. J. 354, 67–72.

Cocco, M., Vázquez, D.E., Albors, A., Cháfer, M., Meier, G.E., Bello, F., 2008. Combinación de tratamientos térmicos y bicarbonato de sodio para el control de Penicillium digitatum en frutos cítricos. Rev. Iberoam. Tecnol. Postcos. 9, 55–62, http://redalyc.uaemex.mx/src/inicio/ArtPdfRed.jsp?iCve=81311226008.

Corthals, G.L., Wasinger, V.C., Hochstrasser, D.F., Sanchez, J.C., 2000. The dynamic range of protein expression: a challenge for proteomic research. Electrophoresis 21, 1104–1115.

Cuevas, J.C., Lopez-Cobollo, R., Alcazar, R., Zarza, X., Koncz, C., Altabella, T., Salinas, J., Tiburcio, A.F., Ferrando, A., 2008. Putrescine is involved in Arabidopsis freezing tolerance and cold acclimation by regulating abscisic acid levels in response to low temperature. Plant Physiol. 148, 1094–1105.

Cuevas, J.C., Lopez-Cobollo, R., Alcazar, R., Zarza, X., Koncz, C., Altabella, T., Salinas, J., Tiburcio, A.F., Ferrando, A., 2009. Putrescine as a signal to modulate the indispensable ABA increase under cold stress. Plant Sign. Behav. 4, 219–220.

Dat, J., Vandenabeele, S., Vranová, E., Van Montagu, M., Inzé, D., Van Breusegem, F., 2000. Dual action of the active oxygen species during plant stress responses. Cell. Molec. Life Sci. 57, 779–795.

Downes, C.P., Gray, A., Lucocq, J.M., 2005. Probing phosphoinositide functions in signaling and membrane trafficking. Trends Cell Biol. 15, 259–268.

Dumas, B., Freyssinet, G., Pallett, K.E., 1995. Tissue-specific expression of germin-like oxalate oxidase during development and fungal infection of barley seedlings. Plant Physiol. 107, 1091–1096.

Falcone Ferreyra, M.L., Perotti, V., Figueroa, C.M., Garrán, S., Anderson, P.C., Vázquez, D.E., Iglesias, A.A., Podesta, F.E., 2006. Carbohydrate metabolism and fruit quality are affected in frost-exposed Valencia orange fruit. Physiol. Plant. 128, 224–236.

Gonzalez-Aguilar, G.A., Zacarias, L., Perez-Amador, M.A., Carbonell, J., Lafuente, M.T., 2000. Polyamine content and chilling susceptibility are affected by seasonal changes in temperature and by conditioning temperature in cold-stored 'Fortune' mandarin fruit. Physiol. Plant. 108, 140–146.

Goren, R., Huberman, M., Zehavi, U., Chen-Zion, M., Echeverria, E., 2000. Sugar utilization by citrus juice cells as determined by [14C]-sucrose and [14C]-fructose feeding analyses. Plant Physiol. Biochem. 38, 507–515.

Hagenmaier, R.D., 2002. The flavor of mandarin hybrids with different coatings. Postharv. Biol. Technol. 24, 79–87.

Holmes, G.J., Eckert, J.W., 1999. Sensitivity of *Penicillium digitatum* and *P. italicum* to postharvest citrus fungicides in California. Phytopathology 89, 716–721.

Ines, S., Karin, H.-S., Gabriel, O.R., Otto, S., Erwin, H.-B., Oscar, V., 1996. Bet v 1 proteins, the major birch pollen allergens and members of a family of conserved pathogenesis-related proteins, show ribonuclease activity in vitro. Physiol. Plant. 96, 433–438.

Kar, M., Mishra, D., 1976. Catalase, peroxidase, and polyphenoloxidase activities during rice leaf senescence. Plant Physiol. 57, 315–319.

Kato-Noguchi, H., 2000. Evaluation of the importance of lactate for the activation of ethanolic fermentation in lettuce roots in anoxia. Physiol. Plant. 109, 28–33.

Katz, E., Fon, M., Lee, Y., Phinney, B., Sadka, A., Blumwald, E., 2007. The citrus fruit proteome: insights into citrus fruit metabolism. Planta 226, 989–1005.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Lamb, C., Dixon, R.A., 1997. The oxidative burst in plant disease resistance. Annu. Rev. Plant Physiol. Plant Mol. Biol. 48, 251–275.

Lane, B.G., 2002. Oxalate, germins, and higher-plant pathogens. IUBMB Life 53, 67–75.

Langenkamper, G., Manac'h, N., Broin, M., Cuine, S., Becuwe, N., Kuntz, M., Rey, P., 2001. Accumulation of plastid lipid-associated proteins (fibrillin/CDSP34) upon oxidative stress, ageing and biotic stress in Solanaceae and in response to drought in other species. J. Exp. Bot. 52, 1545–1554.

Lara, M.V., Borsani, J., Budde, C.O., Lauxmann, M.A., Lombardo, V.A., Murray, R., Andreo, C.S., Drincovich, M.F., 2009. Biochemical and proteomic analysis of 'Dixiland' peach frui (*Prunus persica*) upon heat treatment. J. Exp. Bot. 60, 4315–4333.

Leitner-Dagan, Y., Ovadis, M., Shklarman, E., Elad, Y., Rav David, D., Vainstein, A., 2006. Expression and functional analyses of the plastid lipid-associated protein CHRC suggest its role in chromoplastogenesis and stress. Plant Physiol. 142, 233–244.

Lers, A., Burd, S., Lomaniec, E., Droby, S., Chalutz, E., 1998. The expression of a grapefruit gene encoding an isoflavone reductase-like protein is induced in response to UV irradiation. Plant Mol. Biol. 36, 847–856.

Loewus, F.A., Murthy, P.P.N., 2000. Myo-inositol metabolism in plants. Plant Sci. 150, 1–19.

Lurie, S., 1998. Postharvest heat treatments. Postharv. Biol. Technol. 14, 257–269.

Lurie, S., Crisosto, C.H., 2005. Chilling injury in peach and nectarine: a review. Postharv. Biol. Technol. 37, 195–208.

Markovic-Housley, Z., Degano, M., Lamba, D., von Roepenack-Lahaye, E., Clemens, S., Susani, M., Ferreira, F., Scheiner, O., Breiteneder, H., 2003. Crystal structure of a hypoallergenic isoform of the major birch pollen allergen Bet v 1 and its likely biological function as a plant steroid carrier. J. Mol. Biol. 325, 123–133.

Matoh, T., 1997. Boron in plant cell walls. Plant Soil 193, 59–70.

Mittler, R., Zilinskas, B.A., 1993. Detection of ascorbate peroxidase activity in native gels by inhibition of the ascorbate-dependent reduction of nitroblue tetrazolium. Anal. Biochem. 212, 540–546.

Muccilli, V., Licciardello, C., Fontanini, D., Russo, M.P., Cunsolo, V., Saletti, R., Reforgiato Recupero, G., Foti, S., 2009. Proteome analysis of *Citrus sinensis* L. (Osbeck) flesh at ripening time. J. Proteom. 73, 134–152.

Pan, Z., Guan, R., Zhu, S., Deng, X., 2009. Proteomic analysis of somatic embryogenesis in Valencia sweet orange (*Citrus sinensis* Osbeck). Plant Cell Reports 28, 281–289.

Penefsky, H.S., 1977. Reversible binding of Pi by beef heart mitochondrial adenosine triphosphatase. J. Biol. Chem. 252, 2891–2899.

Perkins, D.N., Pappin, D.J.C., Creasy, D.M., Cottrell, J.S., 1999. Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis 20, 3551–3567.

Petrucco, S., Bolchi, A., Foroni, C., Percudani, R., Rossi, G.L., Ottonello, S., 1996. A maize gene encoding an NADPH binding enzyme highly homologous to isoflavone reductases is activated in response to sulfur starvation. Plant Cell 8, 69–80.

Pietrowska-Borek, M., Stuible, H.-P., Kombrink, E., Guranowski, A., 2003. 4-coumarate: Coenzyme A ligase has the catalytic capacity to synthesize and reuse various (di)adenosine polyphosphates. Plant Physiol. 131, 1401–1410.

Polenta, G.A., Calvete, J.J., González, C., 2007. Isolation and characterization of the main small heat shock proteins induced in tomato pericarp by thermal treatment. FEBS J. 274, 6447–6455.

Porat, R., Pasentsis, K., Rozentzvieg, D., Gerasopoulos, D., Falara, V., Samach, A., Lurie, S., Kanellis, A.K., 2004. Isolation of a dehydrin cDNA from orange and grapefruit citrus fruit that is specifically induced by the combination of heat followed by chilling temperatures. Physiol. Plant. 120, 256–264.

Rampitsch, F., Srinivasan, M., 2006. The application of proteomics to plant biology: a review. Can. J. Bot. 84, 883–892.

Rao, M.V., Paliyath, G., Ormrod, D.P., 1996. Ultraviolet-B- and ozone-induced biochemical changes in antioxidant enzymes of *Arabidopsis thaliana*. Plant Physiol. 110, 125–136.

Rivoal, J., Ricard, B., Pradet, A., 1990. Purification and partial characterization of pyruvate decarboxylase from *Oryza sativa* L. Eur. J. Biochem. 194, 791–797.

Sala, J.M., 1998. Involvement of oxidative stress in chilling injury in cold stored mandarin fruits. Postharv. Biol. Technol. 3, 255–261.

Sala, J.M., Lafuente, M.T., 1999. Catalase in the heat-induced chilling tolerance of cold-stored hybrid Fortune mandarin fruits. J. Agric. Food Chem. 47, 2410–2414.

Sapitnitskaya, M., Maul, P., McCollum, G.T., Guy, C.L., Weiss, B., Samach, A., Porat, R., 2006. Postharvest heat and conditioning treatments activate different molecular responses and reduce chilling injuries in grapefruit. J. Exp. Bot. 57, 2943–2953.

Schein, A., Sheffy-Levin, S., Glaser, F., Schuster, G., 2008. The RNase E/G-type endoribonuclease of higher plants is located in the chloroplast and cleaves RNA similarly to the *E. coli* enzyme. RNA 14, 1057–1068.

Schenk, M., Cordewener, J., America, A., Van't Westende, W., Smulders, M., Gilissen, L., 2009. Characterization of PR-10 genes from eight *Betula* species and detection of Bet v I isoforms in birch pollen. BMC Plant Biol. 9, 24.

Schirra, M., D'Hallewin, G., Ben-Yehoshua, S., Fallik, E., 2000. Host–pathogen interactions modulated by heat treatment. Postharv. Biol. Technol. 21, 71–85.

Shao, H.-B., Chu, L.-Y., Lu, Z.-H., Kang, C.-M., 2008. Primary antioxidant free radical scavenging and redox signaling pathways in higher plant cells. Int. J. Biol. Sci. 4, 8–14.

Shi, J.X., Chen, S., Gollop, N., Goren, R., Goldschmidt, E.E., Porat, R., 2008. Effects of anaerobic stress on the proteome of citrus fruit. Plant Sci. 175, 478–486.

Staiger, C.J., Gibbon, B.C., Kovar, D.R., Zonia, L.E., 1997. Profilin and actin-depolymerizing factor: modulators of actin organization in plants. Trends Plant Sci. 2, 275–281.

Stevenson, J.M., Perera, I.Y., Heilmann, I., Persson, S., Boss, W.F., 2000. Inositol signaling and plant growth. Trends Plant Sci. 5, 252–258.

Velikova, V., Yordanov, I., Edreva, A., 2000. Oxidative stress and some antioxidant systems in acid rain-treated bean plants. Protective role of exogenous polyamines. Plant Sci. 151, 59–66.

Vishnevetsky, M., Ovadis, M., Vainstein, A., 1999. Carotenoid sequestration in plants: the role of carotenoid-associated proteins. Trends Plant Sci. 4, 232–235.

Wróbel-Kwiatkowska, M., Starzycki, M., Zebrowski, J., Oszmianski, J., Szopa, J., 2007. Lignin deficiency in transgenic flax resulted in plants with improved mechanical properties. J. Biotechnol. 128, 919–934.

Yagami, T., Haishima, Y., Nakamura, A., Osuna, H., Ikezawa, Z., 2000. Digestibility of allergens extracted from natural rubber latex and vegetable foods. J. Aller. Clin. Immunol. 106, 752–762.

Yun, Z., Li, W., Pan, Z., Xu, J., Cheng, Y., Deng, X., 2010. Comparative proteomics analysis of differentially accumulated proteins in juice sacs of ponkan (*Citrus reticulata*) fruit during postharvest cold storage. Postharv. Biol. Technol. 56, 189–201.

Zhao, M., Zhou, L., Kawarasaki, Y., Georgiou, G., 2006. Regulation of RraA, a protein inhibitor of RNase E-mediated RNA decay. J. Bacteriol. 188, 3257–3263.

Zhu, J.-w., Xie, Q.-y., Li, H.-y., 2006. Occurrence of imazalil-resistant biotype of *Penicillium digitatum* in China and the resistant molecular mechanism. J. Zhejiang Univ. Sci. A 7, 362–365.