Journal of Experimental Botany, Vol. 60, No. 15, pp. 4315–4333, 2009
doi:10.1093/jxb/erp267 Advance Access publication 4 September, 2009

Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Biochemical and proteomic analysis of 'Dixiland' peach fruit (*Prunus persica*) upon heat treatment

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Received 22 July 2009; Revised 10 August 2009; Accepted 12 August 2009

Abstract

Shipping of peaches to distant markets and storage require low temperature; however, cold storage affects fruit quality causing physiological disorders collectively termed 'chilling injury' (CI). In order to ameliorate CI, different strategies have been applied before cold storage; among them heat treatment (HT) has been widely used. In this work, the effect of HT on peach fruit quality as well as on carbon metabolism was evaluated. When fruit were exposed to 39 °C for 3 d, ripening was delayed, with softening inhibition and slowing down of ethylene production. Several differences were observed between fruit ripening at ambient temperature versus fruit that had been heat treated. However, the major effects of HT on carbon metabolism and organoleptic characteristics were reversible, since normal fruit ripening was restored after transferring heated peaches to ambient temperature. Positive quality features such as an increment in the fructose content, largely responsible for the sweetness, and reddish coloration were observed. Nevertheless, high amounts of acetaldehyde and low organic acid content were also detected. The differential proteome of heated fruit was characterized, revealing that heat-induced CI tolerance may be acquired by the activation of different molecular mechanisms. Induction of related stress proteins in the heat-exposed fruits such as heat shock proteins, cysteine proteases, and dehydrin, and repression of a polyphenol oxidase provide molecular evidence of candidate proteins that may prevent some of the CI symptoms. This study contributes to a deeper understanding of the cellular events in peach under HT in view of a possible technological use aimed to improve organoleptic and shelf-life features.

Key words: Chilling injury, heat treatment, peach, post-harvest, *Prunus persica*.

Introduction

Ripening of fleshy fruits is a dynamic transitional period that encompasses a myriad of biochemical and physiological changes that transform the mature fruit into a ready-to-eat fruit. Significant progress has been made in characterizing the molecular components of the fruit ripening process, including ethylene biosynthesis and perception, cell wall depolymerization, signal transduction, and pigment accumulation (Giovannoni, 2001, 2004). The peach (*Prunus persica* L. Batsch; Rosaceae family) is a climacteric fruit whose ripening process is controlled by the production of

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Abbreviations: 2D-DIGE, two-dimensional fluorescence difference gel electrophoresis; ACO1, AAC (aminocyclopropane-1-carboxylic acid) oxidase 1; ADH, alcohol dehydrogenase; AI, acid invertase; ALD, aldolase; CAC, citric acid cycle; CI, chilling injury; DTT, dithiothreitol; FK, fructokinase; G3PDH, glycerol-3-phosphate dehydrogenase; GK, glucokinase; G6PDH, glucose-6-phosphate dehydrogenase; HSP, heat shock protein; HT, heat treatment; LDH, lactate dehydrogenas, NI, neutral invertase; NAD-MDH, NAD-malate dehydrogenase; NAD-ME, NAD-malic enzyme; NADP-ME, NADP-malic enzyme; PDC, pyruvate decarboxylase; PEPC, phosphoenolpyruvate carboxylase; PCK, phosphoenolpyruvate carboxykinase; PFK, ATP-dependent phosphofructokinase; PFP, PP_i-dependent phosphofructokinase; PK, pyruvate kinase; PPDK, pyruvate orthophosphate dikinase; PPO, polyphenol oxidase; QRT-PCR, quantitative real-time reverse transcription-PCR; SAMS, *S*-adenosylmethionine synthetase; SDH, sorbitol dehydrogenase; TP, triose phosphate; TPI, triose phosphate isomerase; UGPase, UDP-glucose pyrophosphorylase.

ethylene, although other hormones are also involved in this process (Trainotti *et al.*, 2007). Changes in the chemical composition and the physical characteristics of the fruit take place during ripening, which lead to easily perceivable alterations in fruit texture, firmness, pigmentation, aroma, and sweetness. Transcriptome analyses of the peach ripening process have contributed to understanding the role of transcriptional gene regulation during the transition from the pre-climacteric to the climacteric phase in this fruit (Trainotti *et al.*, 2006). Studies on changes in the sugar and organic acid metabolism occurring during peach fruit ripening after harvest contributed to the identification of important components of the carbon metabolism operating during this process (Borsani *et al.*, 2009).

While ripening prepares fleshy fruit for human conumption, marketing and shipping of fleshy fruits require a lengthy storage period. Refrigeration is used to slow ripening and to extend fruit market life; however, several fruits can develop chilling injury (CI) during storage at low temperature. In peach, CI includes internal and external browning, flesh breakdown, woolliness, reddish discoloration, loss of ability to ripen, and increased incidence of decay when stored for >2–3 weeks at temperatures <8 °C (Brummell *et al.*, 2004; Lurie and Crisosto, 2005, and references therein). In order to alleviate CI symptoms, different pre-harvest and post-harvest treatments have been applied to peach (Lurie and Crisosto, 2005, and references therein; Wang *et al.*, 2006). Among them, exposure to subletal high temperature after harvest has been widely used, as this treatment increases the tolerance to subsequent chilling and also delays ripening. In addition, this treatment also reduces pathogen levels and disease development in several fruits (Saltveit, 1991; Lurie, 1998, 2006; Ferguson *et al.*, 2000; Paull and Chen, 2000; Budde *et al.*, 2006; Wang *et al.*, 2006; Jin *et al.*, 2009). The rationale for the use of heat treatment (HT) is that exposure to a high temperature triggers physiological responses that allow the tissue to cope in a better way with subsequent stress conditions. However, peach molecular responses to HT are not completely understood and the effect of HT on carbon metabolism during ripening has not yet been analysed. Some of the beneficial effects of HT in reducing CI have been mainly reported to be contingent on heat shock proteins (HSPs) Sabehat *et al.*, 1996, 1998; Rozenzvieg *et al.*, 2004; Polenta *et al.*, 2007). Studies on citrus, grapefruit, pomegranate, and banana suggested that compounds or enzymes that are induced by HT may also be involved in protection from CI (Porat *et al.*, 2002*a*, *b*; Mirdehghan *et al.*, 2007; Chen *et al.*, 2008). Moreover, several putative heat-induced chilling tolerance genes have been isolated in grapefruit and citrus fruit (Sanchez-Ballesta *et al.*, 2003; Sapitnitskaya *et al.*, 2006).

The understanding of the biochemical and genetic basis of CI is of great importance and so is the identification of the factors that may prevent it in each type of fruit. In this regard, a recent macroarray study identified woolliness response genes in peach fruit after cold storage (González-Agüero *et al.*, 2008). Another approach to understand better and prevent CI would be the study of the fruit changes that take place after a successful treatment that prevents some of the CI symptoms. This kind of study would be very useful in order to identify possible candidates involved in protection from or alleviation of CI. In the present work, an integrated study of metabolic changes induced by post-harvest HT in peach cv 'Dixiland' was carried out. The impact of HT on peach was assessed through the evaluation of organoleptic characteristics, level of enzymes involved in central metabolic pathways, and the contents of metabolites such as sugars, sorbitol, fermentation products, and organic acids. Moreover, in order to identify candidate proteins that may prevent some of the CI symptoms, a comparative analysis of the fruit mesocarp proteome variations after HT was also evaluated using two-dimensional differential gel electrophoresis (2D-DIGE). By these approaches, several candidate proteins that may be directly correlated with CI symptoms were identified, which would be useful in future identification of varieties more resistant to CI.

Materials and methods

Plant material and treatments

Assays were conducted with peach fruits [*P. persica* (L.) Batsch] cv 'Dixiland' grown in the Estación Experimental Agropecuaria INTA, San Pedro, Argentina (Budde *et al.*, 2006), during 2005 and harvested in 2006, and repeated with fruits grown during 2006 and harvested in 2007. The flesh firmness of the fruits at harvest was typically at 52.1±6.5 N (Table 1), which corresponded to ~93 d after bloom. Immediately after harvest, fruits were manually selected for uniformity of colour, size, and firmness, and divided into two groups: one was kept in a chamber at 20 °C and 90% relative humidity for 7 d and the other was held in a chamber at 39±1 °C and 90% relative humidity for 3 d followed by 3 d at 20 °C. Samples were taken immediately after harvest (R0) and after 3 d and 7 d in the chamber at 20 °C (R3 and R7, respectively); and also after 3 days at 39±1 °C (HT) and after 3 d at 20 °C (HT+3).

About 20–30 fruits from each group were used for colour, firmness, solid soluble content (SSC), and acidity measurements. Representative mesocarp tissue was also collected

Table 1. Quality attributes of 'Dixiland' peach after heat treatment

Different letters within each parameter indicate statistically significant differences.

Parameter	R0	HT	HT+3
Firmness (N)	52.1±6.5 a	53.0±7.7 a	4.3±1.2 b
Ground colour			
H value	103.1±2.3 a	92.4±1.9 b	73.6±6.0 c
Pulp colour			
H value	97.0±2.0 a	93.3 ±1.9 b	85.2± 2.5 c
Soluble solids (°Brix)	10.6±0.9 a	10.3±0.8 a	11.4±1.2 a
Acidity [H^+] (M)	0.14±0.01 a	0.08±0.01 b	0.10±0.01 c

from the different sample fruits, immediately frozen in $N_{2(l)}$ and stored at – 80 °C for further experiments.

Determination of fruit quality traits and metabolite measurements

Flesh firmness, SSC, ground colour, and titratable acidity (TA) were determined as previously described (Borsani *et al.*, 2009).

D-Glucose, D-fructose, sucrose, sorbitol, malic acid, citric acid, ethanol, and acetaldehyde were determined as previously described (Borsani *et al.*, 2009). The level of each metabolite is expressed as g per 100 g of fresh peach mesocarp.

CO_2 respiration rate

CO_2 production was measured in fruit enclosed in 1.5 l sealed jars. Air samples of 3 ml were withdrawn from the jars and injected through a sampling valve with a 1 ml loop into a gas chromatograph (Hewlett Packard 5980 Series II), equipped with a thermic conductivity detector and with a 3.048 m Porapack Q column (80/100). The nitrogen carrier gas flow rate was 30 ml min^{-1}, the hydrogen carrier flow was 30 ml min^{-1}, and air flow was 400 ml s^{-1}. Injector and oven temperatures were 100 °C and 120 °C, respectively. Calibration was carried out with a 1520 ppm CO_2 standard (Alfa Gas, Argentina). The CO_2 production rate was expressed in μg per kg of fresh weight per second (μg kg^{-1} s^{-1}).

Protein extraction for enzyme assays

For enzyme activity measurements, total protein from peach mesocarp tissue was extracted using a buffer containing 400 mM TRIS-HCl, pH 8.5, 5 mM EDTA, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 20% (v/v) glycerol, 10 mM ascorbic acid, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1% (v/v) Triton X-100, 10 μg ml^{-1} leupeptin, and 10 μg ml^{-1} chymostatin in a ratio of buffer:fresh tissue of 0.3 ml:1 g. A 10 μl aliquot of protease inhibitor cocktail (Sigma, St Louis, MO, USA) was used per g of fresh tissue. In the case of the crude extract prepared for invertase measurements, 400 mM HEPES-NaOH, pH 8.8, was used instead of TRIS-HCl, pH 8.5. Samples were completely ground in a cold mortar in the presence of insoluble polyvinyl polypyrrolidone (PVPP, Sigma) and centrifuged at 10 000 *g* for 15 min at 4 °C. The supernatant of crude extracts was desalted, according to Penefsky (1977), in a cold Sephadex G-25 column pre-equilibrated with a buffer of the same composition as the extraction buffer but containing 100 mM TRIS-HCl, pH 7.5 or 100 mM HEPES-NaOH, pH 8.5.

Protein extraction under denaturing conditions

Approximately 2 g of mesocarp material was ground in liquid nitrogen using a ceramic mortar and pestle, sand and PVPP, transferred to a SS34 tube containing 10 ml of extraction buffer (100 mM TRIS-HCl, pH 8.8, 2% (w/v) SDS, 0.4% (v/v) 2-mercaptoethanol, 10 mM EDTA, 1 mM PMSF, 0.9 M sucrose), and 10 ml of ice-cold TRIS-HCl, pH 8.8-saturated phenol, and then agitated at 4 °C for 30 min. The aqueous phases were back-extracted with extraction media and phenol by vortexing. Tubes were centrifuged at 5000 *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. The samples were collected by centrifugation at 20 000 *g* at 4 °C for 20 min. Next, 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 resuspended in 2D-DIGE buffer (30 mM TRIS-HCl, pH 8.5, 7 M urea, 2 M thiourea, 4% CHAPS) for 2D-DIGE or diluted in 0.25 M TRIS-HCl, pH 7.5, 2% (w/v) SDS, 0.5% (v/v) β-mercaptoethanol, and 0.1% (v/v) bromophenol blue, and boiled for 2 min for SDS–PAGE.

Protein quantitation

Protein concentration was determined in crude extracts by the method of Bradford (1976) using the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA) and bovine serum albumin as standard.

Enzyme assay

The activity of enzymes was measured spectrophotometrically in a final volume of 1 ml at 30 °C and 340 nm using a UNICAM Helios b spectrophotometer (UNICAM Instruments, Cambridge, UK). The reaction mixtures used for each enzyme were as follows:

(i) Alcohol dehydrogenase (ADH): 85 mM MES, pH 6.5, 5 mM acetaldehyde, and 0.15 mM NADH (Kato-Noguchi, 2000).

(ii) ATP-dependent phosphofructokinase (PFK): 50 mM TRIS-HCl, pH 7.5, with 5% (w/v) polyethylene glycol (PEG), 5 mM $MgCl_2$, 0.5 mM ADP, 1 mM dithiothreitol (DTT), 4 mM fructose-6-phosphate, 0.15 mM NADH, 0.2 U of aldolase, 1 U of triose phosphate isomerase (TPI), and 0.1 U of glycerol-3-phosphate dehydrogenase (G3PDH), starting the reaction with ATP (Lara *et al.*, 2004).

(iii) Fructokinase (FK): 0.1 M TRIS-HCl, pH 8.5, 5 mM $MgCl_2$, 0,5 mM NAD, 1.5 U of phosphoglucose isomerase, 10 mM fructose, 2 mM ATP, and 2 U of glucose-6-phosphate dehydrogenase (G6PDH). The reaction was started with ATP (Mustroph and Albrecht, 2003).

(iv) Glucokinase (GK): 0.1 M TRIS-HCl, pH 8.5, 5 mM $MgCl_2$, 0.5 mM NAD, 10 mM glucose, 2 mM ATP, and 2 U of G6PDH. The reaction was started with ATP (Mustroph and Albrecht, 2003).

(v) Invertases: neutral invertase (NI) activity was assayed in an incubation mixture containing 200 mM HEPES-NaOH, pH 7.5, 200 mM sucrose, and an aliquot of the protein extract to be tested. The mixture was incubated at 30 °C for different times and the progress of the reaction was followed detecting the amount of glucose produced by using the glucose oxidase/horseradish peroxidase assay. Acid invertase (AI) was assayed under the conditions described above, although the reaction mixture contained 100 mM acetic acid/sodium acetate buffer, pH 5.0. In the case of AI, prior to glucose determination, the aliquot was neutralized (Vargas *et al.*, 2007).

(vi) Lactate dehydrogenase (LDH): 50 mM NaPi, pH 7.5, 10 mM pyruvate, 0.2 mM NADH, and 1 mM methylpyrazole, which inhibits pyruvate decarboxylase (PDC) activity (Kato-Noguchi, 2000).

(vii) NAD-malate dehydrogenase (NAD-MDH) was assayed in the oxaloacetate (OAA) reduction direction using a 50 mM imidazole, pH 6.8, medium containing 1 mM OAA and 0.15 mM NADH (Lara *et al.*, 2004).

(viii) NAD-malic enzyme (NAD-ME): 50 mM HEPES, pH 7.3, 2 mM NAD, 2 mM L-malate, 5 mM DTT, 75 mM CoA, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 10 U of malate dehydrogenase (MDH). After the rapid increase in the absorbance at 340 nm, the subsequent steady increase is attributable to the decarboxylation of L-malate by the NAD-ME (Lara *et al.*, 2004).

(ix) NADP-malic enzyme (NADP-ME): 50 mM TRIS-HCl, pH 7.5, 0.5 mM NADP, 10 mM L-malate, and 10 mM $MgCl_2$. The reaction was started with malate (Detarsio *et al.*, 2003).

(x) Pyruvate decarboxylase (PDC): 85 mM MES, pH 6.5, 25 mM NaCl, 1 mM $MgCl_2$, 2 mM DTT, 2 mM thiamine pyrophosphate, 0.15 mM NADH, 50 mM oxamate, 3 U of ADH, and 10 mM pyruvate, where oxamate acts as an inhibitor of lactate dehydrogenase (LDH) activity. Crude extracts were pre-incubated for 30 min in the reaction medium in the absence of pyruvate, NADH, and ADH (Rivoal *et al.*, 1990).

(xi) Phosphoenolpyruvate carboxylase (PEPC): 100 mM TRIS-HCl, pH 8.0, 20% (v/v) glycerol, 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 4 mM phosphoenolpyruvate (PEP), 0.15 mM NADH, and 10 U of MDH (Lara *et al.*, 2004).

(xii) Phosphoenolpyruvate carboxykinase (PCK): 50 mM HEPES, pH 7.3, 4 mM PEP, 10 mM $NaHCO_3$, 2.5 mM $MgCl_2$, 2.5 mM $MnCl_2$, 0.15 mM NADH, 10 U of MDH, and 3 mM ADP (Lara *et al.*, 2003).

(xiii) Pyruvate kinase (PK): 25 mM HEPES, pH 7.2, 20 mM KCl, 10 mM $MgCl_2$, 2 mM PEP, 0.15 mM NADH, 0.5% (w/v) PEG, 2 mM DTT, 1 mM ADP, and 0.4 U of LDH. The reaction medium was previously bubbled with helium in order to remove the bicarbonate and avoid interference by endogenous PEPC and MDH. Enzymatic activity was corrected for interference by PEP phosphatase activity by omitting ADP from the reaction mixture (Falcone *et al.*, 2006).

(xiv) PPi-dependent phosphofructokinase (PFP): 50 mM TRIS-HCl, pH 7.5, with 5% (w/v) PEG, 5 mM $MgCl_2$, 0.5 mM $NaPP_i$, 1 mM DTT, 4 mM fructose-6-phosphate, 2 mM fructose-2,6-bisphosphate, 0.15 mM NADH, 0.2 U of aldolase, 1 U of TPI, and 0.1 U of G3PDH, starting the reaction with NaPPi (Lara *et al.*, 2004).

(xv) UDP-glucose pyrophosphorylase (UGPase): 80 mM HEPES, pH 7.8, 5 mM $MgCl_2$, 0.6 mM NAD, 1 mM UDP-glucose, 0.5 mM PPi, 1 U of G6PDH, and 1 U of phosphoglucomutase. The reaction was started by PPi addition (Sowokinos *et al.*, 1997).

Gel electrophoresis

SDS–PAGE was performed in 10% (w/v) polyacrylamide gels according to Laemmli (1970). Proteins were visualized with Coomassie blue or electroblotted onto a nitrocellulose membrane for immunoblotting. Bound antibodies were located by linking to alkaline phosphatase-conjugated goat anti-rabbit IgG according to the manufacturer's instructions (Bio-Rad, Hercules, CA, USA). The antibodies used for detection were the following: 1:200 anti-*Amaranthus viridis* PEPC (Colombo *et al.*, 1998); serum against the α-subunit of NAD-ME (diluted 1:1000) from *A. hypochondriacus* (Long *et al.*, 1994); 1:1000 anti-*Zea mays* L. pyruvate orthophosphate dikinase (PPDK; Chastain *et al.*, 2000); 1:200 anti-cucumber PCK (Walker *et al.*, 1995); 1:10 anti-*Z. mays* recombinant NADP-ME (Saigo *et al.*, 2004), and 1:200 anti-*Nicotiana tabacum* HSP70 (Lara *et al.*, 2005). The molecular masses of the polypeptides were estimated from a plot of the log of molecular mass of marker standards versus the migration distance. Quantification of the intensity of the bands was conducted by image analysis software in at least three independent blots. The mean value of the immunoreactive bands in recently harvested peaches (R0) was arbitrarily set at 100%.

RNA isolation and RT-PCR

Total RNA from different samples of peaches was isolated from 4 g of tissue using the method described by Meisel *et al.* (2005). The integrity of the RNA was verified by agarose gel electrophoresis. The quantity and purity of RNA were determined spectrophotometrically according to the method described by Sambrook *et al.* (1989). First-strand cDNA was synthesized with MoMLV reverse transcriptase following the manufacturer's instructions (Promega, Madison, WI, USA) and using 3 μg of RNA and oligo(dT).

Quantitative real-time reverse transcription-PCR (QRT-PCR)

Relative expression was determined by performing QRT-PCR in an iCycler iQ detection system and the Optical System Software version 3.0a (Bio-Rad, Hercules, CA,

USA), using the intercalation dye SYBRGreen I (Invitrogen) as a fluorescent reporter, with 2.5 mM $MgCl_2$, 0.5 μM of each primer, and 0.04 U μl^{-1} of GoTaq Polymerase (Promega). PCR primers were designed based on peach fruit cDNA sequences published in GenBank and *P. persica* expressed sequence tag (EST) databases (TIGR Plant Transcript Assemblies; http://plantta.tigr.org, Childs *et al.*, 2007), with the aid of the web-based program 'primer3' (http://www.frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) in such a way as to produce amplicons of 131–226 bp in size (Supplementary Table S1 available at *JXB* online). The primer and amplicon sequences were further analysed using peach EST databases (ESTree Database, http://www.itb.cnr.it/estree/, Lazzari *et al.*, 2008; and GDR Genome Database for Rosaceae, http://www.bioinfo.wsu.edu/gdr/, Jung *et al.*, 2008). A 10-fold dilution of cDNA obtained as described above was used as template. PCR controls were performed in the absence of added reverse transcriptase to ensure RNA samples were free of DNA contamination. Cycling parameters were as follows: initial denaturation at 94 °C for 2 min; 40 cycles of 96 °C for 10 s; and 58 °C for 15 s; 72 °C for 1 min, and 72 °C for 10 min. Melting curves for each PCR were determined by measuring the decrease in fluorescence with increasing temperature (from 65 °C to 98 °C). The specificity of the PCRs was confirmed by melting curve analysis using the software as well as by agarose gel electrophoresis of the products. Each RNA sample was run in triplicate and repeated in at least two independent sets of treatments, generating a total of six replicates per gene per sample. Relative gene expression was calculated using the 'Comparative $2^{-\Delta\Delta CT}$' method (Livak and Schmittgen, 2001) and elongation factor 1 (*ef1*) as reference gene. Results were expressed in relation to the values obtained for recently harvested peaches (R0).

ef1 was chosen among different control genes widely used following the indications of Brunner *et al.* (2004). To test whether *ef1* behaves as a housekeeping gene in the analysed samples, the gene expression index was plotted against the sample, and linearity and low slope were verified (Brunner *et al.*, 2004). *Actin* was also tested but its expression varied upon HT (data not shown). In comparison, in potato among seven common housekeeping genes tested during biotic and abiotic stresses, *ef1* was also detected as the most stable gene (Nicot *et al.*, 2005).

Statistical analysis

Data from the quantitative real-time experiments were tested using one-way analysis of variance (ANOVA). Minimum significant differences were calculated by the Bonferroni, Holm–Sidak, Dunett, and Duncan tests (α=0.05) using the Sigma Stat Package.

2D-DIGE

The differential proteome of peach fruit under HT was assessed by comparing heat-treated peaches with those ripening at ambient temperature of the same age: R3 versus HT and R7 versus HT+3. Additionally, the heat-treated proteome was compared with that of samples transferred to 20 °C after HT (HT versus HT+3).

Protein labelling with dyes

In all experiments, proteins were labelled with Alexa 610 (excitation, 612 nm; emission peak, 628 nm) or Alexa 532 (excitation, 532 nm; emission peak, 554 nm) after adjusting the pH to 8.5 using the supplier's instructions (Molecular Probes Inc. and Invitrogen Ltd). Proteins were labelled at the 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 addition of 1 μl of 1 mM lysine and 20 mM DTT, and 4% (v/v) of isoelectric focusing (IEF) buffers pH 3–10 or 5–8 was added (Amersham Biosciences).

Two-dimensional gel electrophoresis

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, 17 cm, Bio-Rad, Hercules, CA, USA). Samples of 300 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. Focused gel strips were equilibrated in SDS equilibration buffer (375 mM TRIS-HCl, pH 8.0, 20% glycerol, 2% SDS, and 6 M urea), first with buffer containing 130 mM DTT for 15 min and afterwards with buffer containing 135 mM iodoacetamide for 15 min. The strips were washed briefly with running buffer, then loaded on top of a prepared SDS–PAGE Laemmli gel cast with 15% (w/v) acrylamide, and covered with 0.5% (w/v) agarose. Proteins were separated at 15 mA per gel for 12–15 h at 15 °C using a Hoefer TMSE 600, 18×16 cm (Amershan, Uppsala, Sweden), and the gels were scanned using a BioChemi System UVP BioImaging System. Data were saved in tiff format. In order to obtain biological replicates, each sample comparison was run in at least three different gels using different protein preparations from different fruit. To excise samples for mass spectrometric analysis, a preparative gel loaded with 1 mg of protein was run.

Gel image analysis

Images were analysed using Image Master 2D-Platinum (GE Healthcare) using the protocol described in Casati *et al.* (2006). When necessary, spots were manually edited. A normalization procedure was used to allow for variation in total protein loading onto the gel(s). Total spot volume was calculated, and each spot was assigned a normalized spot volume as a proportion of this total value. Normalized spot volumes were compared between Alexa 532- and Alexa 610-labelled samples on each gel. Difference 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.*, 2006).

In-gel digestion, mass spectrometry, and database searching

Before spot picking the gel was stained using Coomassie Blue stain. Gel spots of interest were manually excised from gels 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 (donatello.ucsf.edu/ingel.html) with trypsin according to Casati *et al.* (2006). The mass spectrometric data were obtained using a MALDI-TOF-TOF spectrometer, Ultraflex II (Bruker).

The spectra obtained were submitted for National Center for Biotechnology Information (NCBI) database searching using MASCOT (www.matrixscience.com, Perkins *et al.*, 1999) and analysed as previously described (Casati *et al.*, 2006). Only candidates that appeared at the top of the list were considered positive identifications. Peptides were considered as matches either if they were classified as 'significant' (*i.e.* $P < 0.05$, which with our search parameters equals a MOWSE score of ⩾40) or if they showed 'homology' (a MOWSE score between 15 and 39, depending on the sequence of the peptide) and at the same time represented a protein with a theoretical molecular weight corresponding to the apparent molecular weight after SDS–PAGE (Casati *et al.*, 2006). Protein functional classification was done according to literature data.

Results

In this study, peach fruit exposed immediately after harvest (R0) to 39 °C for 3 d (HT) were analysed in comparison with fruit allowed to ripen at 20 °C for 3 d (R3) or 7 d (R7). In order to analyse if the HT affects subsequent ripening at 20 °C, treated peaches (HT) were moved to 20 °C for 3 d, simulating shelf life (named HT+3). Quality parameters, carbon metabolism, and the differential proteome were analysed in all the samples to assess how HT affects central metabolism and to identify candidate proteins involved in heat-induced CI tolerance in peach.

Fruit quality traits determinations after HT and subsequent ripening at 20 °C

'Dixiland' peaches were harvested at an average firmness of 52.1±6.5 N (Table 1, Fig. 1A). For the group of fruit kept at ambient temperature, the ripening process took place normally, which was evidenced by a decrease in the firmness, reaching values <15 N at 7 d after harvest (Fig. 1A). In contrast, after 3 d of HT, fruit maintained their firmness, which rapidly decreased after transfer to 20 °C, reaching values even lower than those of fruit of practically the same post-harvest age not exposed to high temperature (HT+3 versus R7, Fig. 1A). In addition, the epidermis colour was modified after high temperature exposure, reaching an orange-reddish colour when transferred to ambient temperature (Budde *et al.*, 2006; Table 1). A similar trend was observed in the pulp coloration upon exposure to 39 °C (Budde *et al.*, 2006; Table 1).

The level of the transcript encoding 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase 1, *ACO1*, involved in ethylene synthesis, was also analysed by QRT-PCR. It was previously shown that *ACO1* levels are well correlated to

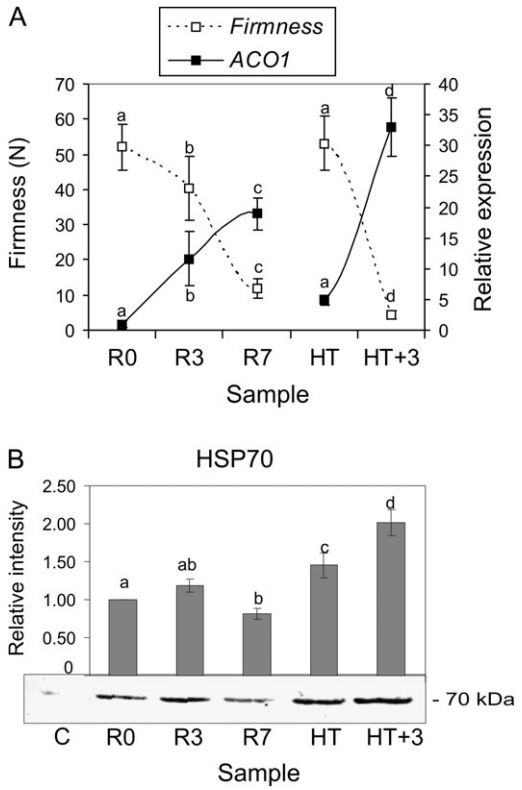

Fig. 1. (A) Relationship between fruit firmness and *ACO1* level of fruit kept at 20 °C or after HT. Fruit collected from the tree (R0) were allowed to ripen at 20 °C during 3 d and 7 d (R3 and R7) or maintained at 39 °C for 3 (HT) and then transferred to 20 °C for another 3 d (HT+3). Fifteen fruit were used for firmness determination (values represent the mean ±SD). Expression analysis of *ACO1* involved in ethylene biosynthesis in peach fruit was carried out by QRT-PCR. The means of the results obtained, using three independent mRNAs as template, are shown. Each reaction was normalized using the C_t values corresponding to *P. persica* elongation factor 1 mRNA. The *y*-axis shows the fold difference in a particular transcript level relative to its amount found in peaches after harvest (R0). Standard deviations are shown. (B) Western blot of the different fruit samples using antibodies against maize HSP70. A 25 μg aliquot of total peach soluble protein was added per lane. As control (C), 25 μg of *Zea mays* crude extract was loaded. The molecular mass of the immunoreactive bands is shown on the right and expressed in kDa. The quantification of the immunoreactive bands is expressed as a percentage of the amount found after harvest (R0, n=2). Standard deviations are shown. Bars with the same letters are not significantly different (P <0.05).

ethylene production (Borsani *et al.*, 2009). Figure 1A shows that the higher the firmness of the fruit, the lower the level of *ACO1*. HT for 3 d prevents the *ACO1* increase that usually occurs during fruit ripening at ambient temperature, as fruit exposed to HT displayed a much lower level than fruit of the same post-harvest age maintained at ambient temperature (HT versus R3, Fig. 1A). However, once heat-treated fruits are transferred to 20 °C a rapid increase in *ACO1* level occurs, reaching values even higher than those found in R7 (Fig. 1A). The levels of HSP70 estimated by western blot were also analysed in the different fruit samples, detecting higher levels of expression in HT and HT+3 samples with respect to fruit of the same post-harvest age (R3 and R7, respectively, Fig. 1B). The high level of this HSP in heat-treated fruits is indicative that the HT applied was sensed by the fruit, provoking induction of specific proteins (Fig. 1B).

As regards the total SSC, no prominent differences were observed among the different samples (Table 1). Total acidity levels decreased after heat exposure, although this parameter was partially restored when fruit were removed from high temperature and maintained at 20 °C for 3 d (Table 1). Total acidity matched with the levels of malic acid which were half in fruits exposed to HT with respect to peaches of the same post-harvest age kept at ambient temperature (Fig. 2). Citric acid content also correlated with total amounts of acidity after HT, presenting 20% lower values in fruit exposed to HT compared with peaches of the same post-harvest age (Fig. 2).

Regarding the sugar content, sucrose and both hydrolytic products, glucose and fructose, were quantified (Fig. 2). The sucrose level was not affected by the HT and thus fruit exposed to heat displayed the same sucrose level as fruit of the same post-harvest age kept at room temperature (Fig. 2). Interestingly, higher levels of both glucose and fructose were observed in HT and HT+3 fruits in comparison with peaches that ripen at ambient temperature (Fig. 2). After HT, the sorbitol content did not change significantly and practically the same level of this metabolite was found in the HT+3 sample (Fig. 2). Thus, when compared with fruit of the same post-harvest age not subjected to HT, significantly higher levels of this metabolite were found, reaching 10 times higher levels in the HT+3 sample (Fig. 2).

Finally, metabolites produced by alcoholic fermentation were also quantified. The intermediate product, acetaldehyde, was affected by the heat exposure (Fig. 2), with HT+3 fruit having nearly twice the amount as fruit of practically the same age and nearly five times the value of fruit recently harvested (Fig. 2). In contrast, the quantity of the final product ethanol remained undetectable in all samples analysed (data not shown). On the other hand, an increase in the fruit respiration rate after HT was observed. In this way, HT fruit (24.8 ± 0.9 μg CO_2 kg^{-1} s^{-1}) displayed a nearly 40% higher respiration rate than that found at harvest (R0; 16.9 ± 0.4 μg CO_2 kg^{-1} s^{-1}) or after 3 d at 20 °C (R3; 13.0 ± 0.3 μg CO_2 kg^{-1} s^{-1}). In addition, HT+3 fruit also displayed a higher respiration rate (21.7 ± 0.4 μg CO_2 kg^{-1} s^{-1}) than peaches of almost the same post-harvest age (R7; 18.0 ± 0.4 μg CO_2 kg^{-1} s^{-1}).

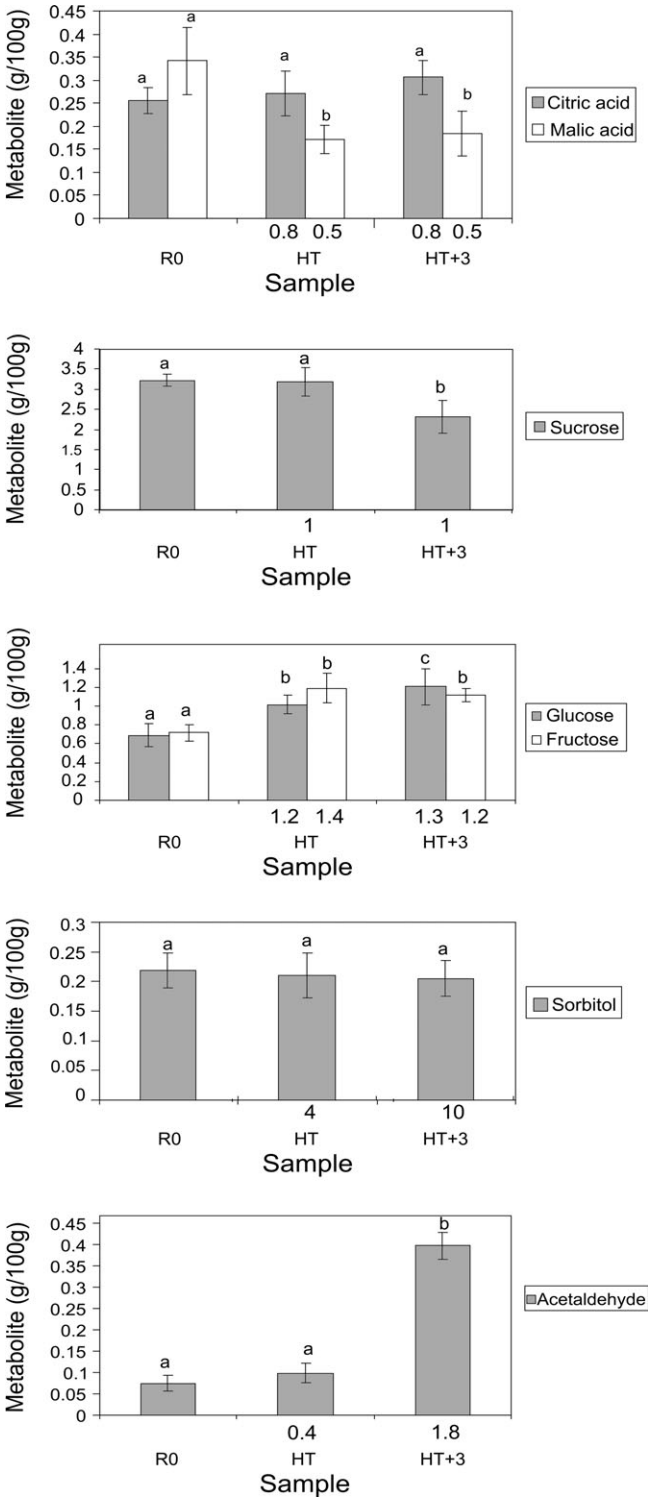

Fig. 2. Quantitation of sugars, organic acids, and fermentation products in peach fruit exposed to HT. Amounts of metabolites are expressed in g per 100 g of fruit fresh weight. Fruit were detached from the plant and transferred to 39 °C for 3 d (HT) and then transferred to 20 °C for 3 d (HT+3). Values represent the mean of at least 14 independent determinations ±SD. For each metabolite, bars with the same letters are not significantly different (P <0.05). Below each bar, the ratio of the metabolite level relative to the unheated sample of the same post-harvest age is indicated (HT versus R3 and HT+3 versus R7; Borsani *et al.*, 2009).

Enzymes involved in sugar metabolism after HT and subsequent ripening at 20 °C

In order to elucidate how sugar metabolism is affected by high temperature exposure, several enzymatic activities were studied after HT (Fig. 3). In addition, the levels of transcripts encoding many of these proteins, when the sequences were available, were also quantified by QRT-PCR (Fig. 4). Transcript expression levels obtained by QRT-PCR were normalized using *ef1* as the reference gene, and results for the different samples were expressed in relation to the values obtained for recently harvested peaches (R0).

Sucrose may be catabolized through either invertase or sucrose synthase (SS) pathways. Both NI and AI were modified upon fruit exposure to high temperature. AI activity could not be detected in peach fruit after HT or after the heated fruits were transferred to 20 °C (Fig. 3). On the other hand, NI activity was induced when peach fruit under HT were transferred to 20 °C with respect to both HT fruit and peaches of practically the same post-harvest age (Fig. 3). Four different transcripts encoding NIs were detected in peach EST databases and their expression levels were evaluated by QRT-PCR (Fig. 4). The data obtained show that the increase in NI activity observed in HT+3 is preceded by a large increase in the levels of *NI2*, *NI3* and *NI4*, with a 12- and 17-fold rise occurring in *NI3* and *NI4*, respectively, in HT with respect to fruit of the same post-harvest age (Fig. 4). In the case of *NI3* and *NI4*, this induction declined after transfer to ambient temperature. Nevertheless, the amounts of *NI2* and *NI3* remained at higher levels of expression in comparison with untreated fruit (Fig. 4). The level of the transcript encoding SS was also dramatically affected by the heat treatment. A 10-fold decrease was measured for *SS* in HT with respect to fruit of practically the same post-harvest age (Fig. 4). The amount of *SS* was partially restored after transfer to 20 °C, with values almost three times lower than in fruit of practically the same post-harvest age (Fig. 4). A similar trend was observed for the enzyme UGPase. The activity of UGPase decreased nearly 4-fold in HT fruit with respect to fruit of the same post-harvest age (Fig. 3) and remained low after transfer to 20 °C (HT+3) (Fig. 3).

Regarding sorbitol metabolism, the transcript encoding sorbitol dehydrogenase (*SDH*) was significantly decreased in HT and HT+3 samples in relation to fruit of the same post-harvest age (Fig. 4), in agreement with the high levels of sorbitol in peaches exposed to HT with respect to untreated fruit (Fig. 2).

In addition, the effect of HT on several enzymes catalysing key steps of the glycolytic pathway was analysed. The level of fructokinase transcript (*FK*) was significantly reduced in HT+3 peaches (Fig. 4). On the other hand, glucokinase (GK) and both PFP and PFK activities were very similar to the activities measured in untreated fruit of the same post-harvest age (Fig. 3). In contrast, PK activity decreased in HT samples with respect to peaches of the same post-harvest age, followed by a nearly 2-fold increase after transfer to 20 °C (Fig. 3).

Finally, the activity of fermentative enzymes was practically not modified by HT in comparison with fruit of the same post-harvest age (Fig. 3). In contrast, upon transfer of

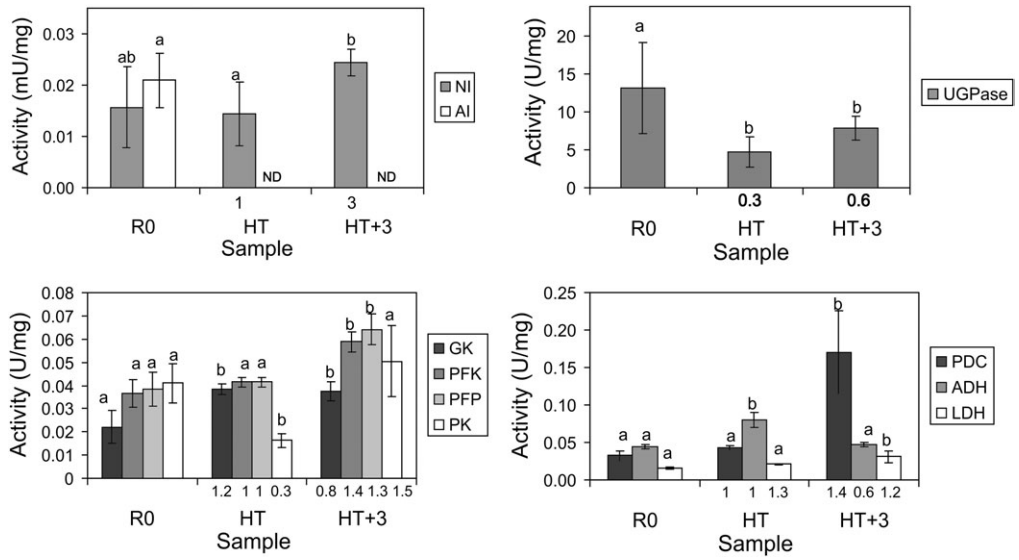

Fig. 3. Sucrose degradation, and glycolytic- and fermentation-related enzymes activity after HT and subsequent ripening. The activity of NI and AI UGPase and enzymes involved in key steps of glycolysis (GK, PFP, PFK and PK); and alcoholic (PDC and ADH) and lactic (LDH) fermentation were analysed. Peach fruit were collected immediately after harvest (R0) and exposed to 39 °C for 3 d (HT) followed by 3 d at 20 °C (HT+3). Activity is expressed in international units (U) per mg of total soluble protein in all cases except for invertases, where activity is expressed in mU per mg of total protein. For each enzyme, bars with the same letters are not significantly different (P <0.05). Values represent the mean of at least six independent determinations using different fruits collected at different times after harvest ±SD. ND, activity not detected. Below each bar, the ratio of the activity of the sample relative to the activity in the untreated sample of the same post-harvest age is indicated (HT versus R3 and HT+3 versus R7; Borsani *et al.*, 2009).

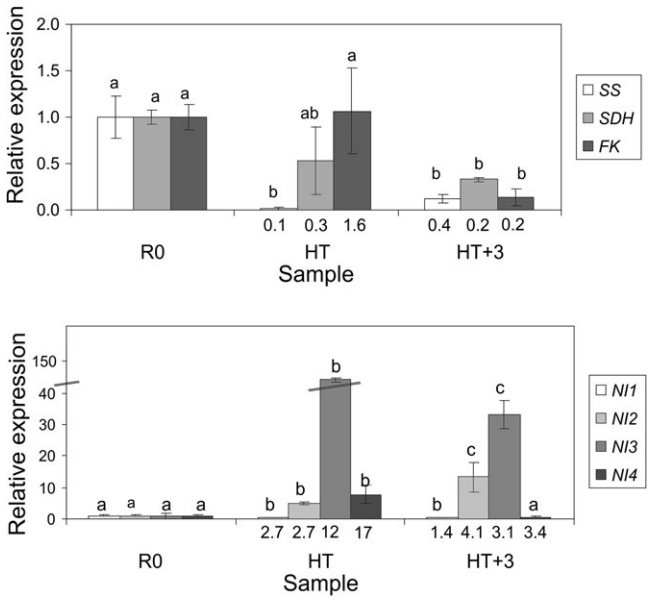

Fig. 4. Expression analysis, assessed by QRT-PCR, of transcripts encoding enzymes involved in peach fruit carbon metabolism after HT and subsequent ripening at ambient temperature. Peach fruits were collected immediately after harvest (R0) and exposed to 39 °C for 3 d (HT) followed by 3 d at 20°C (HT+3). For each sample, means of the results obtained, using three independent RNAs as a template, are shown. Each reaction was normalized using the C_t values corresponding to *P. persica* elongation factor 1 mRNA (Table 1). The *y*-axis shows the fold difference in a particular transcript level relative to its amount found in peaches analysed after harvest (R0). Standard deviations are shown. For each transcript analysed, bars with the same letters are not significantly different (P <0.05). Below each bar, the ratio of the level of the particular transcript relative to the level in untreated peaches of the same post-harvest age is indicated (HT versus R3 and HT+3 versus R7; Borsani *et al.*, 2009).

the heated peaches to 20 °C for 3 d (HT+3), a decrease in ADH activity as well as an increase in PDC activity was observed (Fig. 3).

Enzymes involved in organic acid metabolism after heat treatment and subsequent ripening

The levels of a number of proteins associated with malate metabolism were evaluated by activity measurement as well as by semi-quantification by western blot analysis in peaches subjected to HT (Fig. 5).

PEPC and PCK are involved in the OAA production from PEP. OAA is later used for malate generation by MDH. PEPC activity increased in HT samples with respect to fruit of practically the same post-harvest age, a value that was maintained in HT+3 samples (Fig. 5A). Levels of PEPC immunoreactive protein correlated with changes in activity (Fig. 5B). In the case of PCK, a decrease in activity was observed in HT and HT+3 samples (Fig. 5A). However, the modification in PCK immunoreactive protein during the HT did not correlate with PCK activity (Fig. 5B). In this case, two PCK immunoreactive bands were detected, and the lower PCK band was particularly increased in HT+3 samples (Fig. 5B). NAD-ME was practically not affected by the HT at both the protein and activity levels, while NADP-ME activity in HT and HT+3 peaches was nearly half of that in fruits of the same age (Fig. 5A), which correlated with the levels of immunoreactive protein (Fig. 5B).

Finally, PPDK, an enzyme involved in PEP/pyruvate interconvertion, was affected by the HT. Significant increases in the levels of immunoreactive protein were observed in fruit subjected to 39 °C (HT) and also after transfer to 20 °C for 3 d (HT+3, Fig. 5B).

2D-DIGE analysis of proteins modified upon heat treatment followed by MS/MS identification

To analyse the response of the peach proteome to the high temperature treatment, 2D-DIGE analysis was conducted using pH 4.0–7.0 strips and 15% SDS–PAGE. Preliminary electrophoretic runs were conducted with pH 3.0–10.0 strips, which revealed that the major proportion of the proteins displayed isoelectric points (pIs) between 4.0 and 7.0. About 600 spots were detected on the two-dimensional electrophoresis map carried out with soluble protein extracted from peach fruit after harvest, kept at either 20 °C or 39 °C during 3 d. The left panel of Fig. 6C shows the typical protein pattern obtained for the peach fruit proteome, corresponding to fruit exposed to 39 °C for 3 d (HT).

From the 2D-DIGE analysis the following results could be observed: 57 polypeptides were affected by temperature by a factor of ±1.5 (*t*-test with significance P <0.05, Table 2) of which 52 proteins were differentially expressed between fruits exposed to HT or after transfer to 20 °C (HT+3), versus fruits kept at 20 °C and taken at different time points (R3 and R7, Fig. 6A). Five proteins were differentially expressed when HT samples were compared against HT+3 (Fig. 6A). Forty-four differential proteins were identified by fingerprinting mass analysis (Table 2). Missing identifications could be explained by the low abundance of the spots or the fact that the protein was not present in the available databases. From the analysed spots, 20 proteins had already been described in the *Prunus* spp. genus, with half of the proteins already identified in peach (*P. persica*). Among identified spots, a large number (93%) have been proposed to play a role in plant metabolism such as the defence and stress response, cytoskeleton organization, primary metabolism, transcription and translation regulation, and protein storage and catabolism, and only 7% of the identified proteins do not have a function assigned yet (Fig. 6B).

The category of proteins participating in biotic or abiotic stress responses was the one with the most proteins differentially expressed (Fig. 6B). Twenty-seven percent of the identified proteins corresponded to the large family of HSPs and exhibited molecular masses <20 kDa. Among the HSPs detected, six different isoforms were recognized based on MS-TOF-TOF spectra. With the exception of spot 85, all of the HSPs identified increased in peaches subjected to

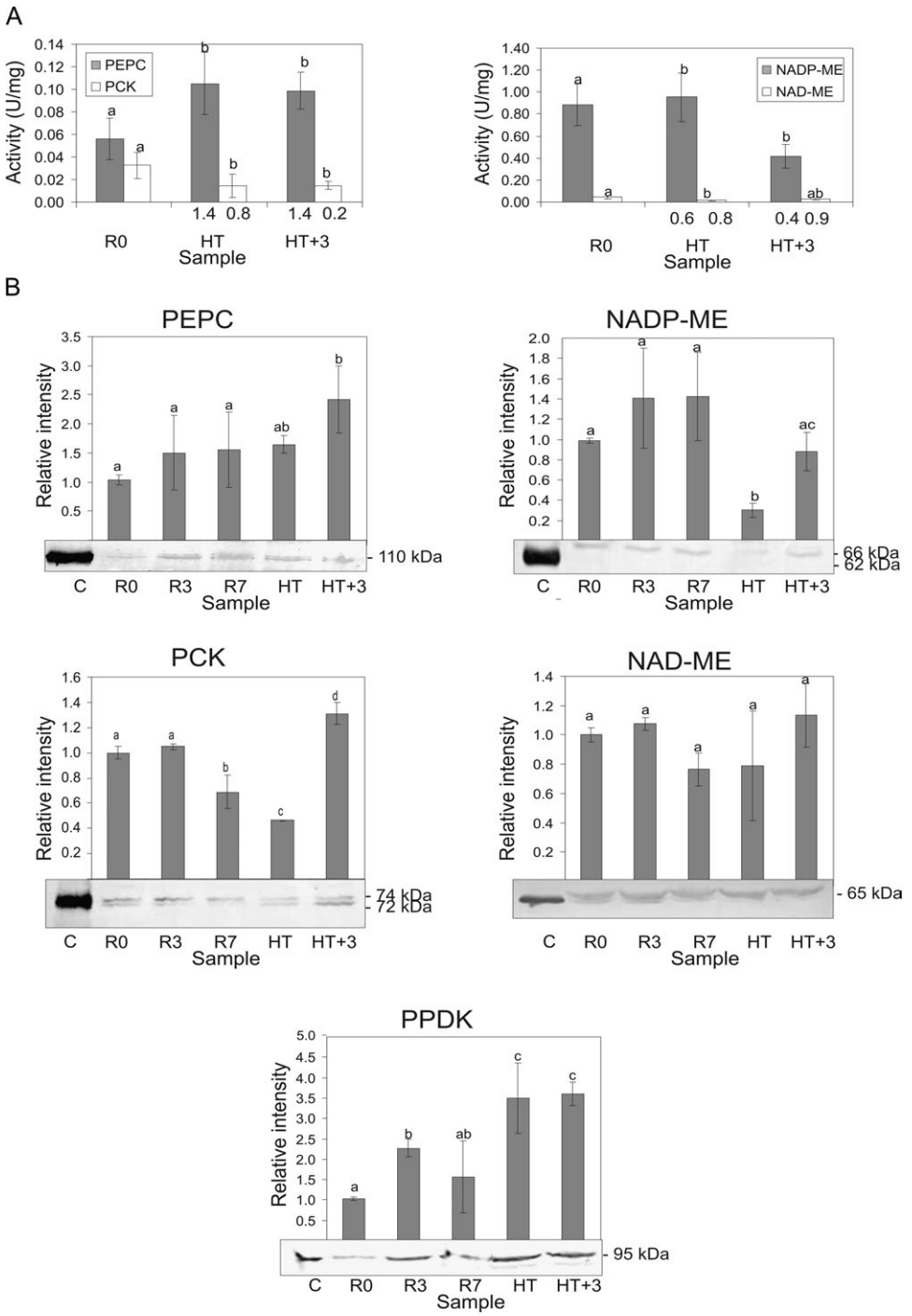

Fig. 5. Enzymes involved in organic acid metabolism in peach fruit exposed to HT. (A) Activity assays of enzymes involved in the consumption of malate (NADP-ME and NAD-ME) and PEP metabolism (PCK and PEPC). Activity is expressed in international units (U) per mg of total soluble protein. For each enzyme, bars with the same letters are not significantly different (P <0.05). Values represent the mean of at least six independent determinations in different fruits ±SD. Fruit were collected (R0) and kept for 3 d at 39 °C (HT) and followed by 3 days at 20 °C, simulating shelf life (HT+3). Below each bar, the ratio of the activity of the sample relative to the activity in the untreated sample of the same post-harvest age is indicated (HT versus R3 and HT+3 versus R7; Borsani *et al.*, 2009). (B) Western blot analysis of proteins involved in malate metabolism, using antibodies against PEPC, PCK, NAD-ME, NADP-ME, or PPDK. A 25 μg aliquot of total soluble protein was added per lane. As assay control (C), 25 μg of *Zea mays* (for PCK, NADP-ME, or PPDK western blots) or *Portulaca oleracea* (for PEPC or NAD-ME western blots) crude extracts were loaded in the first lane. Molecular masses of the immunoreactive bands are shown on the right (kDa). The quantification of the immunoreactive bands is expressed in relation to the amount in fruits sampled after harvest (R0) and is shown below each western blot (n=2 or 3). Standard deviations are shown. For each enzyme, bars with the same letters are not significantly different (P <0.05).

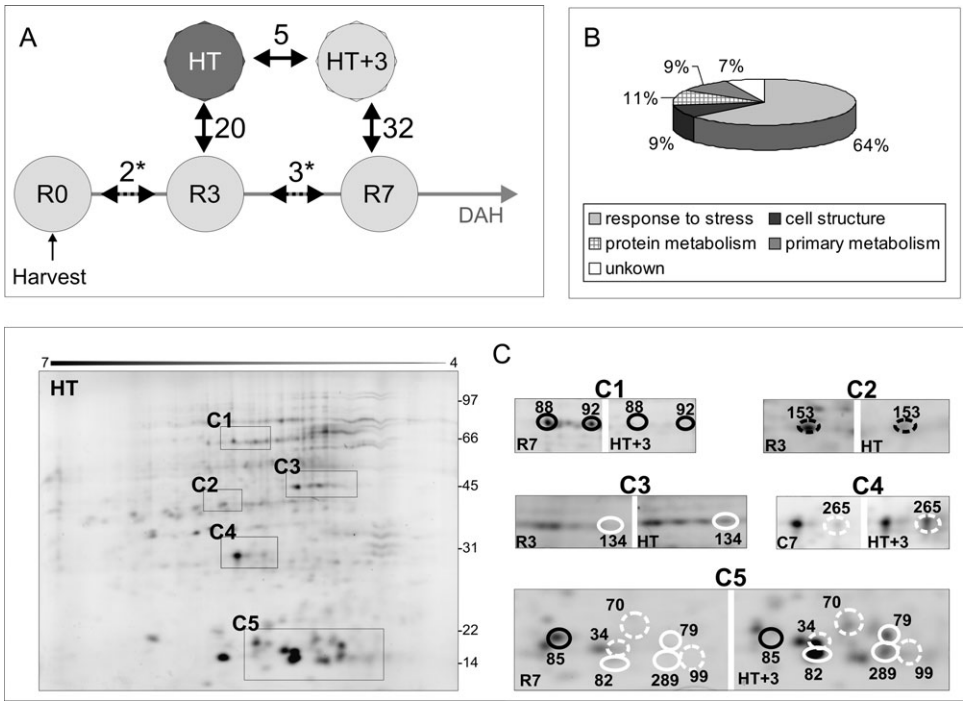

Fig. 6. Differential proteome of peach fruit ripening at 20 °C or subjected to heat treatment. 2D-DIGE was conducted using 75 μg of proteins extracted with the phenol method from peach fruit. (A) Scheme of sample collection and treatment. Samples were collected from peach at harvest (R0) and allowed to ripen at 20 °C for 3 d or 7 d (R3 and R7, respectively) and compared with peach subjected to 39 °C for 3 d (HT) and then transferred to 20 °C (HT+3). Arrows point towards the samples involved in each comparison and the numbers nearby indicate the amount of spots differentially expressed between the samples. Comparisons indicated with discontinuous arrows and numbers with an asterisk were analysed in Borsani *et al.* (2009). (B) Functional classification of differentially expressed proteins in samples subjected to HT. (C) A representative 2D-DIGE gel showing the spot distribution of proteins from fruit exposed to 39 °C during 3 d (HT). As examples of proteins with differential expression, the relative abundances of some and not all spots annotated by the number that appears in Table 2 are shown with magnification in squares C1–C5. The relative size of the different spots amplified is conserved. The graph represents one example from at least three different gels used for the differential analysis. The first dimension was carried out using 18 cm immobilized pH gradient strips (pH 4–7), acidic side to the left; and the second dimension was on 15% (w/v) SDS–PAGE, low molecular mass at the bottom. The relative abundance of proteins was determined. The protein spots with significant changes in intensities (P <0.05) were considered to be different (Table 2).

HT, being differentially expressed in HT fruits, HT+3 fruits, or both, with respect to peach fruit kept at 20 °C (Fig. 6C, Table 2). Within the induced HSPs, half of the proteins were up-regulated (with ratios of, for example, 87 in the case of spot 67), while the other half exclusively appeared after the HT (Table 2). The relative abundance of pathogen-related (PR) proteins is shown in Fig. 6C5. The major cherry allergen, Pru av 1.0202 (spot 79), was induced after the HT. The MS analysis from two different spots matched the protein present in the Uni-prot database with accession number Q6QHU3, suggesting that at least two different isoforms of a protein identified as a major cherry allergen are present in peach fruit after HT. Analysis of the available ESTs reveals the existence of more than one transcript encoding the major cherry allergen in peach. Nevertheless, a post-translational modification of the same protein producing changes in molecular mass and/or pI cannot be ruled out. Another PR protein, identified as major-allergen Pru p1 (spot 69), was also induced upon HT (Table 2). Proteins induced by or related to abscisic acid (ABA) were also increased in peaches subjected to temperature stress treatment (spots 134 and 265, Fig. 6C3 and C4, Table 2) while another one was repressed (spot 247, Table 2). Other proteins involved in the stress response such as temperature-induced lipocalin (spot 95), isoflavone reductase (spots 15 and 16), and enzymes involved in antioxidant metabolism (spots 291 and 88) were repressed in HT+3 samples in relation to samples of practically the same post-harvest age (R7) (Fig. 6C1, Table 2).

The four polypeptides related to actin metabolism identified in the present work were repressed in HT+3 samples in comparison with peaches kept for the same period of time at 20 °C (R7) that were never exposed to 39 °C (spots 91, 96, 11, and 12, Table 2). On the other hand, different proteins involved in several steps of protein metabolism were also affected by the temperature treatment applied, including a polypeptide involved in protein catabolism through the ubiquitin system, which was induced by HT in HT samples as well as in HT+3 samples (spot 289, Fig. 6C5, Table 2). In addition, a translation initiation factor was also induced in HT samples (spot 266, Table 2). On the other hand, putative glycine-rich RNA-binding

Table 2. Identification of differentially expressed proteins from 'Dixiland' peach subjected to HT as well as the trend observed in the protein abundances

Recently harvested fruits (R0) were divided into two groups, one was kept at 20 °C for 3 d (R3) or 7 d (R7) and the other was subjected to a 3 d 39 °C treatment (HT) and then transferred for 3 days at 20 °C (HT+3, Fig. 6).

Spot no.	Ratio[a]	Accession no.		Homologous protein assignment	Score[b]	SC (%)	QM[c]	Computed		Observed	
		NCBI	Uniprot					pI	MW	pI	MW
				R3 versus HT							
				Decrease in HT							
153	1.8	gil37051117	Q76KV5	*S*-Adenosylmethionine synthetase-2	102	37%	10	6.3	37.8	6.0	40
				Increase in HT							
480	1.8	gil2677828	O50002	Cysteine protease	88	14%	3	6.4	39.9	4.8	31
490	1.5	gil2677828	O50002	Cysteine protease	22	12%	3	6.4	39.9	4.4	30
70	49.5	gil6969974	Q9M6R2	LMW heat shock protein	41	5%	1	5.4	18.2	5.2	22
69	2	gil82492265	Q2I6V8	Major allergen Pru p 1	74	54%	7	5.8	17.6	5.6	21.5
67	1.5	gil1235898	Q39930	17.7 kDa heat shock protein	157	31%	6	6.2	17.7	6.0	22
82	10.2	gil41059801	Q6RFM0	Small heat shock protein	102	29%	2	6.0	17.4	5.3	17
241	1.5	–	–	–	–	–	–	–	–	4.6	30
79	HT	gil44409451	Q6QHU3	Major cherry allergen Pru av 1.0202	92	10%	1	5.0	17.4	4.9	21.5
83	HT	gil5257560	Q9XGS6	Cytosolic class II LMW heat shock protein	139	29%	4	5.6	17.6	5.1	21
289	HT	gil136640	P25866	Ubiquitin-conjugating enzyme E2	30	11%	1	5.7	17.4	5.0	17
34	HT	AY500559.1		Small heat shock protein	56	54%	7	6.0	17.3	5.4	21.5
134	HT	gil73762178	Q30E95	Type II SK2 dehydrin	41	28%	6	5.4	28.5[d]	5.1	43
243	HT	gil84619272	Q2P9V0	Soluble inorganic pyrophosphatase	197	27%	6	5.8	26.7	4.8	29
266	HT	gil829282	P69039	Eukaryotic initiation factor 5A-1	69	44%	4	6.3	15.9	5.5	23
221	HT	–	–	–	–	–	–	–	–	5.4	32
263	HT	–	–	–	–	–	–	–	–	5.2	24
259	HT	–	–	–	–	–	–	–	–	5.0	25
63	HT	–	–	–	–	–	–	–	–	4.6	66
190	HT	–	–	–	–	–	–	–	–	5.6	40
				R7 versus HT+3							
				Decrease in HT+3							
247	1.5	gil110288693	Q7XGB3	TB2/DP1, HVA22 family protein	67	30%	8	7.6	39.3	6.3	3
291	2.5	gil1420938	Q41712	Cytosolic ascorbate peroxidase	109	11%	2	5.6	27.1	5.8	28
85	R7	gil41059801	Q6RFM0	Small heat shock protein	126	42%	7	6.0	17.4	5.5	22
86	R7	gil34851124	Q6YNS1	Putative glycine-rich RNA-binding protein	158	64%	9	7.8	17.4	5.6	20
90	R7	gil145340839	A4RR80	Predicted protein	74	39%	7	5.1	22.6	5.9	22
95	R7	gil77744891	Q38JC5	Temperature-induced lipocalin	204	41%	7	5.6	21.4	5.5	23
11	R7	gil126215670	Q0DLA3	Actin-depolymerizing factor 7	116	25%	2	6.3	16.1	6.3	21
12	R7	gil125550577	A2XZM6	Hypothetical protein	128	26%	2	6.3	15.5	6.4	21
13	R7	gil147866185	A5C9Q0	Hypothetical protein	156	32%	5	6.8	18	6.6	21
87	R7	gil170743	Q41553	HMW glutenin subunit Ax2	81	18%	8	6.2	88.6	6.1	27
15	R7	gil3243234	O81355	Isoflavone reductase-related protein	152	25%	7	6.0	34	6.4	33
16	R7	gil3243234	O81355	Isoflavone reductase-related protein	136	23%	6	6.0	34	6.4	35
88	R7	gil3282505	O81103	Polyphenol oxidase precursor	250	15%	8	6.4	67.4	5.9	65
92	R7	gil1708924	P51615	NADP-dependent malic enzyme	71	11%	7	6.1	65.6	5.6	65
91	R7	gil56181504	Q5PU47	Putative actin 1	208	29%	10	5.6	40.3	5.3	43
96	R7	gil23955912	Q8H6A3	Actin	344	45%	14	5.3	41.9	5.3	43
111	R7	gil118482898	A9PAG0	Unknown	52	17%	4	5.5	25.6	5.6	28
112	R7	gil2970051	O64438	ARG10	75	17%	3	5.6	25.8	5.6	27
89	R7	–	–	–	–	–	–	–	–	5.7	22
98	R7	–	–	–	–	–	–	–	–	5.5	30
				Increase in HT+3							
70	5.1	gil6969974	Q9M6R2	LMW heat shock protein	65	26%	1	5.4	18.2	5.2	23
67	87	gil1235898	Q39930	17.7 kDa heat shock protein	157	31%	6	6.2	17.7	6.0	22
34	5.1	AY500559.1		Small heat shock protein	191	54%	7	6.0	17.4	5.4	21.5
99	4.1	gil44409451	Q6QHU3	Major cherry allergen	165	26%	3	5.0	17.4	4.8	17
265	2.1	gil16588758	Q93WZ6	Abscisic stress ripening-like protein	55	49%	5	5.7	20.7	5.5	31
327	1.5	gil2246378	Q7DLI5	Plastid protein	91	20%	4	7.6	22.8	5.5	21
82	HT+3	gil41059801	Q6RFM0	Small heat shock protein	102	29%	2	6.0	17.4	5.3	17

Table 2. *Continued*

Spot no.	Ratio[a]	Accession no.		Homologous protein assignment	Score[b]	SC (%)	QM[c]	Computed		Observed	
		NCBI	Uniprot					pI	MW	pI	MW
79	HT+3	gil44409451	Q6QHU3	Major cherry allergen	81	10%	1	5.0	17.4	4.9	21.5
289	HT+3	gil136640	P25866	Ubiquitin-conjugating enzyme E2	30	11%	1	5.7	17.4	5.0	17
330	1.9	–	–	–	–	–	–	–	–	5.8	21.5
207	2.1	–	–	–	–	–	–	–	–	5.3	42
208	HT+3	–	–	–	–	–	–	–	–	5.3	41
				HT versus HT+3							
				Increase in HT+3							
82	1.5	gil41059801	Q6RFM0	Small heat shock protein	102	29%	2	6.0	17.4	5.3	17
83	1.5	gil5257560	Q9XGS6	Cytosolic class II LMW HSP	139	29%	4	5.6	17.6	5.1	21
99	1.5	gil44409451	Q6QHU3	Major cherry allergen	165	26%	3	5.0	17.4	4.8	17
216	1.6	–	–	–	–	–	–	–	–	6.1	33
243	1.5	–	–	–	–	–	–	–	–	6.2	29

[a] Ratio of increase or decrease of target protein as indicated in the table. When the spot is found in only one condition of the comparison, the name of the sample where it is present is shown instead of a number.
[b] Score: Mascot-MOWSE score. SC, sequence coverage; QM, queries matched
[c] Number of peptides matched in databases.
[d] Molecular weight deduced from a partial sequence.
– No sequences were found in the databases.

protein and α-type proteasome subunit were repressed upon the treatment (spots 86 and 111, Table 2).

Regarding primary metabolism, decreases in NADP-ME (spot 92, also detected by western blot analysis, Fig. 5B) and *S*-adenosylmethionine synthetase (SAMS; spot 153) in peaches exposed to HT with respect to fruits of the same age were observed (Table 2 and Fig. 6C1, C2).

Discussion

Quality attributes of peach fruit exposed to HT: reversible softening inhibition and changes in coloration

Some quality-determining factors were modified in peach fruit exposed to HT in relation to fruits allowed to ripen at 20 °C (Figs 1, 2). Pulp and epidermis of peach exposed to HT turned reddish (Table 1), as previously reported (Budde *et al.*, 2006). Similarly, colour changes due to carotenoid accumulation were observed in heated tomato and in transgenic tomato overexpressing a HSP (Neta Sharir *et al.*, 2005). On the other hand, as in tomato exposed to high temperature (Lurie and Klein, 1991), HT fruit showed the same firmness as fruit after harvest (R0) and in contrast to peach of the same post-harvest age at 20 °C (R3), which were less firm (Fig. 1). However, this softening inhibition by HT is reversible since HT fruit transferred to ambient temperature (HT+3) soften even more quickly than unheated fruit (Fig. 1A). This effect of HT on peach firmness may be due to the inhibition of ethylene production, as previously shown (Budde *et al.*, 2006). This ethylene decline may take place through the regulation of different steps of the biosynthesis since a decrease in *ACO1* (Fig. 1A) and SAMS protein (Table 2) was observed in the HT sample with respect to peaches kept at 20 °C. The decrease in ethylene biosynthesis is reversible, which is in relation to the restoration of both *ACO1* and SAMS levels when the heat stress is removed (Fig. 1A, Table 2). Moreover, the higher level of *ACO1* detected in HT+3 peaches versus untreated peaches of the same post-harvest age may be directly correlated to higher ethylene production (Budde *et al.*, 2006) and to the rapid softening once the heated peaches are allowed to ripen at ambient temperature (Fig. 1A). The reversible softening inhibition induced by HT indicates that this treatment may be used for preserving peach quality during post-harvest storage, probably before cold storage. To evaluate this possibility, the metabolic changes induced by HT in important carbon compounds that contribute to the overall organoleptic quality of fresh peach were evaluated.

Sugar and organic acid metabolism in peach fruit under HT

When peach fruit are exposed to 39 °C, sucrose utilization via SS or invertase is modified (Fig. 7), with *SS* transcripts (Fig. 4) and UGPase activity (Fig. 3) drastically down-regulated with respect to unheated fruits. While AI activity is decreased upon HT to undetectable levels, NI activity is not modified (Fig. 3). However, among the four *NI* genes deduced from EST databases, three transcripts (*NI2*, *NI3*, and *NI4*) are clearly induced (Fig. 4). Invertases are classified according to their subcelullar distribution in vacuolar, cell wall, cytosolic, and, recently, mitochondrial and plastidic NI (Szarka *et al.*, 2008; Vargas *et al.*, 2008). In grape berry, five *NI* genes (*VvNI* genes) are expressed in the fruit mesocarp (Nonis *et al.*, 2008). Motifs responsive to heat stress (HSEs) have been found in the *VvNI1* and *VvNI4* promoters, and motifs responsive to low temperatures (LTRs) in the *VvNI3* promoter (Nonis *et al.*, 2008).

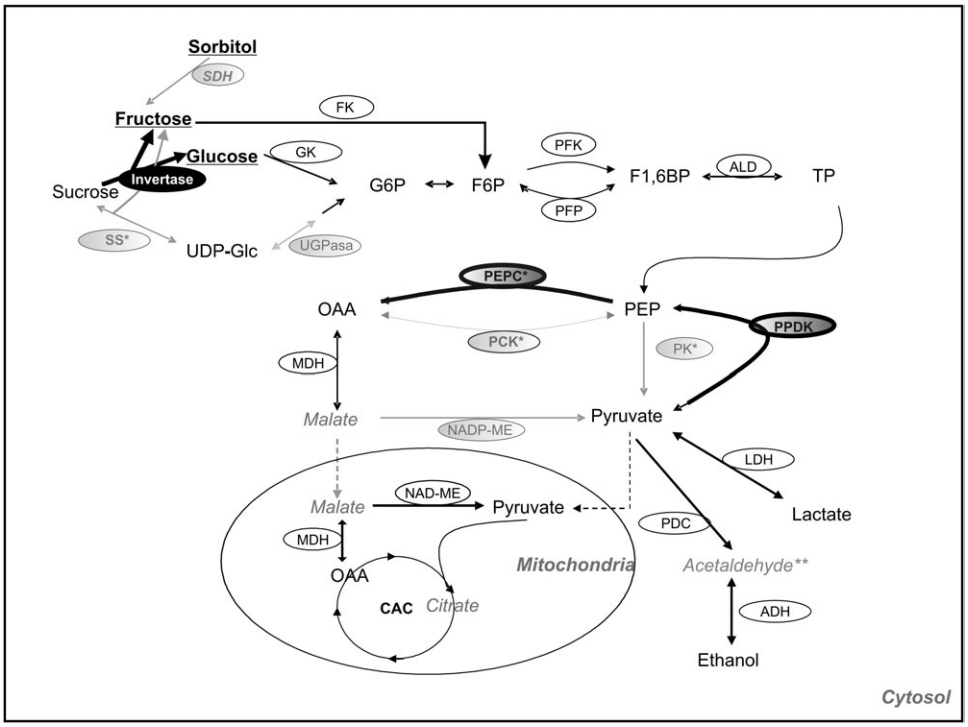

Fig. 7. Simplified metabolic scheme and changes observed after HT in relation to typical peach metabolism during post-harvest ripening. The scheme shows the reactions analysed in the present study involving the metabolism of sugars and major organic acid. Only metabolites that are used as substrates for the next enzyme reaction are shown. The principal metabolites and enzymes that are increased after HT are indicated in bold (metabolites are underlined and enzymes appear on a dark background), while the foremost metabolites and enzymes that decrease due to this treatment are shown in grey (metabolites are in italics and enzymes on a white background). Cases in which enzyme or metabolite levels affected by HT are restored in HT+3 samples to values found in untreated fruit of the same post-harvest age are indicated with an asterisk. Acetaldehyde is marked with ** since its level in HT+3 fruit is much higher than that in R7 fruit. The enzyme invertase is particularly distinguished since specific transcripts encoding this enzyme are induced and others are repressed. The change in enzyme levels was measured by transcript, protein, or activity quantitation depending on the enzyme. ALD, aldolase; CAC, citric acid cycle; TP, triose-phosphate; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; F1,6BP, fructose-1,6-bisphosphate; OAA, oxaloacetate.

In peaches under HT, the great increase in *NI3* and *NI4*, which are highly homologous to *VvNI3* and *VvNI1*, respectively (Fig. 4), suggests that HSEs may be conserved in at least some NI peach promoters. Future studies regarding the activity and localization of invertase isoforms may elucidate not only the role of each isoform in sucrose utilization but also its function under HT and/or CI protection in peach fruit. In addition, future research in this respect may help to understand their role in the network of sugar accumulation, sensing, and signalling.

Besides changes in sucrose cleavage enzymes (Figs 3, 4), sucrose levels are not significantly modified under HT with respect to untreated peaches (Fig. 2). Conversely, the levels of both fructose and glucose are higher in fruit exposed to 39 °C than in peaches kept at 20 °C (Fig. 2). If this increase in hexoses is not explained by a decrease in the amount of sucrose, one reasonable explanation is a decrease in the rate of hexose consumption. In this regard, several enzymes involved in the first part of the glycolytic pathway are not modified upon HT in terms of activity level (GK, PFK, and PFP; Fig. 3). However, in fruit kept at 39 °C (HT), the activity of PK diminished (Fig. 3), which may limit the glycolytic flux and, thus, may be responsible for the increase in glucose and fructose levels (Fig. 7). On the other hand, PPDK immunoreactive protein is increased after HT (Fig. 5). A partial substitution of PK activity by PPDK may restore the glycolytic pathway under HT (Fig. 7), as in the case of tissues under stress conditions such as phosphate starvation (Theodorou and Plaxton, 1993) or hypoxia (Huang *et al.*, 2005). Further use of pyruvate for fermentation is not affected in HT peaches, as accounted for by maintenance of the activity of the fermentative enzymes (Fig. 3). In this respect, the decrease in acetaldehyde content in HT with respect to R3 samples (Fig. 2) may be due to a higher volatilization rate under high temperature conditions.

Regarding organic acid contents in peach under HT, malate and citrate levels were lower than in fruit allowed to ripen at 20 °C (Fig. 2). While NAD-MDH (data not shown) and NAD-ME (Fig. 5A) showed the same activity in HT and in recently harvested fruit, NADP-ME decreased (Fig. 5A). In addition, PEPC, catalysing the synthesis of OAA, was increased due to HT, at both the activity and protein level (Fig. 5A, B). These observations, together with a nearly

40% increase in the respiration rate in heated with respect to unheated peach, suggest that under HT organic acid respiration is preferred over sugar metabolism. Thus, PEP may be converted to either pyruvate through PPDK or to OAA and malate via PEPC and MDH, which is later respired, probably in the mitochondria (Fig. 7). It is worth mentioning that, under HT, a higher sugar/acid ratio was also observed in several fruits (Paull and Chen, 2000, and references therein). Alternatively, utilization of intermediates of the citric acid cycle (CAC) in different biosynthetic reactions, such as nitrogenous compounds, during the HT supports the necessity for higher levels of PEPC (Fig. 5) and also explains a putative drain of malate for the synthesis of C_4-carbon skeletons.

Finally, sorbitol levels found in peach after harvest are maintained in heated samples, in contrast to the low levels detected during ripening at 20 °C (Fig. 2). In peach trees, sorbitol and sucrose are the major photosynthetic products transported along the phloem pathway to various sink tissues (Loescher *et al.*, 1990); thus in peach fruit sorbitol is imported and not synthesized. As the *SDH* transcript is reduced in HT peach fruit (Fig. 4), the decrease in sorbitol metabolization may conserve the levels of this metabolite (Fig. 7) and may contribute to the protection against heat damage. Although there are many studies showing increases in sorbitol contents in response to low temperature (Loescher, 1987; Deguchi *et al.*, 2002), its increase after HT has not been reported yet. However, *in vitro* studies have shown the effect of this compound on increasing the heat stability of particular enzymes (Smirnoff and Stewart, 1985).

Sugar and organic acid metabolism in heated peach fruit transferred to ambient temperature: reversible or irreversible metabolic changes?

An important desirable feature of the HT, as a technological tool used to prevent CI and to delay fruit ripening, is that changes in fruit metabolism induced by the treatment should be reversed once the HT exposure is suppressed and, more importantly, normal ripening should continue. In this regard, heated peaches were also analysed after a period of 3 d at ambient temperature. NI activity (Fig. 3) and *SS* levels (Fig. 4) are increased after transferring heated peaches to ambient temperature, in accordance with the decrease in sucrose level in HT+3 with respect to HT fruit (Fig. 2).

When fruits are transferred to 20 °C after the HT, the glycolytic flux is restored with an increase in PK activity (Fig. 3), showing the capacity of the fruit to readapt to variable environments (Fig. 7). PDC is also greatly induced in HT+3 with respect to HT fruit, while ADH is decreased (Fig. 3). This differential response of both enzymes involved in alcoholic fermentation explains the high levels of acetaldehyde in HT+3 peach fruit (Fig. 2). Regarding organic acid metabolism, the content of malate and citrate remains as low in HT+3 as in HT fruit (Fig. 2) and no prominent changes in organic acid metabolism were found comparing HT versus HT+3 samples (Fig. 5).

Overall, a 3 d treatment of 39 °C applied to peach is effective in keeping the levels of fruit firmness found at harvest. HT leads to changes in carbon metabolism (Fig. 7). However, several of them are restored after transfer to 20 °C during 3 d, simulating shelf life, indicating that the majority of the changes are reversible. Among the differences detected in HT+3 fruit, high levels of glucose and fructose, and reddish coloration are positive modifications contributing to fruit sweetness and attractiveness for the consumers, with lower acidity affecting fruit taste. With respect to acetaldehyde, higher levels of this metabolite do not precisely constitute a detrimental attribute. Acetaldehyde has been associated with enhancement of aroma volatile production and for many years it has been exogenously applied to improve fruit aroma (Pesis, 2005). Acetaldehyde has also been proposed to prevent CI symptoms in various fruit through altering membrane function and levels of free SH groups which inhibit the browning process (Pesis, 2005).

Differential proteome of 'Dixiland' peach fruit subjected to air HT

In the present work, 57 differentially expressed proteins among the heated and unheated peach mesocarp proteome samples were detected by 2D-DIGE analysis, whose molecular masses were encompassed between 60 kDa and 15 kDa (Fig. 6). Amongst them, 95% of the proteins exhibited a molecular mass between 17 kDa and 43 kDa. As expected from a HT, the functional category of defence and stress response appears greatly represented in the present study (64%, Fig. 6B).

In the present study, a polyphenol oxidase (PPO) precursor (spot 88) and ascorbate peroxidase (APX, spot 291) were repressed in HT+3 samples compared with unheated fruit of the same post-harvest age (Table 2). PPO is involved in the browning process, one of the symptoms of CI (Lurie and Crisosto, 2005) affecting the appearance, the taste, and the nutritional value (Valero *et al.*, 2003). Therefore, the repression of PPO due to HT observed in HT+3 fruits would be an additional advantage of this treatment apart from delaying fruit softening. Other treatments applied to peach fruit, such as the use of salicylic acid and yeast, induced the expression of PPO, superoxide dismutase (SOD), and catalase (CAT) (Chan *et al.*, 2007). As fruit ripening is an oxidative process in which PPO, CAT, SOD, and APX are systematically induced (Jimenez *et al.*, 2002; Sarry *et al.*, 2004), it is not surprising that 2D-DIGE analysis did not reveal the induction of the antioxidant machinery in fruit exposed to HT since the levels of antioxidant enzymes may already be high in fruit ripening at this stage.

In our study, two cysteine proteases (spots 480 and 490) were increased in HT samples with respect to peaches kept at 20 °C (R3). During citrus fruit ripening a cysteine protease was induced (Alonso and Granell, 1995). Plant cysteine proteases function in many aspects of cellular regulation, including plant defence and response to different stresses (Estelle, 2001; Harrak, 2001). Interestingly, in

tomato a dual function for a cysteine protease was described, as an enzyme in the cytosol and as a transcriptional factor regulating ACC synthetase 2 (*ACS2*) gene expression (Matarasso *et al.*, 2005).

In heated peach, the induction of samll HSPs (smHSPs) of 17–18 kDa (Fig. 6C5, Table 2) and HSP70 (Fig. 1B) may participate in the acquisition of tolerance against some CI symptoms. SmHSPs from tomato are involved in the control of pectin depolymerization and juice viscosity during fruit ripening (Ramakrishna *et al.*, 2003). Heating tomato prevented CI after prolonged cold storage, in part due to induction of smHSP synthesis caused by the HT. This effect was not induced by other stress conditions such as drought and anaerobiosis (Sabehat *et al.*, 1996, 1998). Similarly, in grapefruit, a short HT, but not UV, wounding, and anaerobiosis, improved chilling tolerance by increasing the expression of various HSP cDNAs (Rozensvieg *et al.*, 2004). In addition, the effect of salicylic acid in alleviating CI of peach has been attributed, at least in part, to induction of HSPs (Wang *et al.*, 2006). Moreover, decreased levels of two HSP70s and one HSP100 were found in woolly fruit kept at 4°C (González-Agüero *et al.*, 2008).

Finally, other stress-related proteins have been induced in response to HT, such as major allergen, major cherry allergens, and a dehydrin protein (spots 69, 79, 99, and 134, respectively; Table 2). Studies on peach and on several other woody plant species identified these proteins as responsive to low temperature (Wisniewski *et al.*, 1996; Renaut *et al.*, 2008). Dehydrins would participate in dehydration events occurring upon freezing, drought, and salinity stress (Close, 1997).

Concluding remarks

The present work provides a great insight into acid and sugar accumulation in 'Dixiland' peach subjected to HT, showing the complexity of the mechanisms regulating sugar metabolism, and constitutes the first proteomic analysis of peach subjected to HT. In peach fruit kept at 39 °C, ripening is impaired, with maintenance of fruit firmness and a decrease in ethylene production. Upon return of heat-stressed fruit to moderate temperature, ripening recovers. Collectively, 2D-DIGE analysis reveals that the induction of HSPs, allergen proteins, dehydrin, and other proteins involved in the stress response and repression of PPO caused by HT may constitute the molecular basis for the protection against chilling stress in peach fruit. In order to evaluate the final impact on peach quality and the effects of HT in preventing CI, the relevance of the differential proteins and metabolites identified must be analysed during cold storage of peach fruit, as well as in different peach varieties with differential sensitivity to CI.

Supplementary data

Table S1. Sequences of the oligonucleotide primers used for real-time RT-PCR.

Acknowledgements

This work was funded by grants from Agencia Nacional de Promoción Científica y Tecnológica (PICT No. 15-20278) and INTA (PNFRU 3192). CSA, MFD, and MVL, are members of the Researcher Career of CONICET. COB and RM are members of INTA. The authors thank Dr Paula Casati (CEFOBI-UNR) for her kind help with the 2D-DIGE experiments and analysis.

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