

# Deciphering the metabolic pathways influencing heat and cold responses during post-harvest physiology of peach fruit

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## ABSTRACT

Peaches are highly perishable and deteriorate quickly at ambient temperature. Cold storage is commonly used to prevent fruit decay; however, it affects fruit quality causing physiological disorders collectively termed ‘chilling injury’ (CI). To prevent or ameliorate CI, heat treatment is often applied prior to cold storage. In the present work, metabolic profiling was performed to determine the metabolic dynamics associated with the induction of acquired CI tolerance in response to heat shock. ‘Dixiland’ peach fruits exposed to 39 °C, cold stored, or after a combined treatment of heat and cold, were compared with fruits ripening at 20 °C. Dramatic changes in the levels of compatible solutes such as galactinol and raffinose were observed, while amino acid precursors of the phenylpropanoid pathway were also modified due to the stress treatments, as was the polyamine putrescine. The observed responses towards temperature stress in peaches are composed of both common and specific response mechanisms to heat and cold, but also of more general adaptive responses that confer strategic advantages in adverse conditions such as biotic stresses. The identification of such key metabolites, which prime the fruit to cope with different stress situations, will likely greatly accelerate the design and the improvement of plant breeding programs.

*Key-words:* *Prunus persica*; chilling injury; stress.

## INTRODUCTION

Peach (*Prunus persica* L.) is the third most important fruit worldwide among the fruit-producing rosaceous crops [Food and Agriculture Organization of the United Nations: Statistics (FAOSTAT)]. At ambient temperature, harvested peaches

deteriorate quickly and refrigeration is therefore extensively used to delay ripening and to extend fruit shelf life. However, cold storage affects peach quality causing physiological disorders that are collectively termed ‘chilling injury’ (CI; Lurie & Crisosto 2005). CI includes internal and external browning, flesh breakdown, lack of juiciness (mealiness or woolliness), reddish discolouration (flesh bleeding), black pit cavity, loss of flavour, loss of ability to ripen and increased incidence of decay. Our comprehension of the complex molecular events that underlie the response to cold treatment leading to CI in peach fruit has grown dramatically since the adoption of proteomic and transcriptomic strategies (González-Agüero *et al.* 2008; Ogundiwin *et al.* 2008; Vizoso *et al.* 2009; Dagar, Friedman & Lurie 2010; Nilo *et al.* 2010; Zhang *et al.* 2010; Dagar *et al.* 2013; Pavez *et al.* 2013). In parallel, a variety of pre- and post-harvest treatments have been applied to peach to prevent or at least alleviate CI symptoms, including pharmacological treatment with salicylic acid (SA), methyl jasmonate,  $\gamma$ -aminobutyric acid (GABA) or gibberellic acid; the use of modified atmospheres; and the exposure to sub-lethal high temperatures (Lurie & Crisosto 2005; Budde *et al.* 2006; Lurie 2006; Wang *et al.* 2006; Jin *et al.* 2009; Cao *et al.* 2010; Lara *et al.* 2009, 2011; Pegoraro *et al.* 2010, 2011; Yang *et al.* 2011, 2012).

Heat treatment (HT) has been proved to be an efficient post-harvest technology given that it preserves organoleptic quality, decreases pathogen levels and disease progression while at the same time increasing the tolerance to subsequent chilling occurring on cold storage of fruits (Saltveit 1991; Ferguson *et al.* 2000; Paull & Chen 2000; Budde *et al.* 2006; Lurie 2006; Wang *et al.* 2006; Jin *et al.* 2009). For this reason, we have focused our previous studies on the characterization of the biochemical and molecular responses to HT in ‘Dixiland’ peach fruit. Fruit exposure to 39 °C for 3 d delayed ripening via an inhibition of softening and a decelerated ethylene production (Lara *et al.* 2009). Moreover, positive changes in quality markers such as an increase in both fructose content and in reddish colouration were observed in treated fruit (Lara *et al.* 2009). In addition, when preheated peaches were transferred to ambient temperature normal fruit ripening was restored. Proteomics and transcriptomic approaches were

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used to reveal the response of the mesocarp to HT (Lara *et al.* 2009; Bustamante *et al.* 2012; Lauxmann *et al.* 2012). By these means in heated peaches, 57 differentially expressed soluble proteins were identified, of which 70% were stress and defence proteins that could potentially be involved in CI protection (Lara *et al.* 2009). On the other hand, among the 127 heat responsive genes identified in heated peaches, a high percent also respond to cold, thus constituting candidate genes involved in the better preparation of heated-peach fruit for cold storage (Lauxmann *et al.* 2012). In addition, several proteins were differentially detected in the apoplast of peach fruits subjected to HT that could mediate cold tolerance in HT-cold combined treatments (Bustamante *et al.* 2012). Furthermore, HT also reduced levels of proteins involved in CI symptoms such as HSPs70 and HSP100 associated with woolliness or polyphenol oxidase (PPO) responsible for browning (Lurie & Crisosto 2005; González-Agüero *et al.* 2008; Lara *et al.* 2009).

On the basis of the combined evidence summarized above, the current hypothesis is that HT of peach fruit after harvest triggers physiological responses that prime the fruit to better cope with subsequent cold stress. While the above hypothesis on the CI protection induced by HT is now generally accepted, the mechanism by which this is achieved remains unclear with many details still to be elucidated. In this regard, there has been an increasing trend in using metabolomic methods in the field of fruit development, ripening and post-harvest handling, which has been very valuable in deciphering metabolic processes underlying biochemical key steps in fruit biology (Carrari *et al.* 2006; Fait *et al.* 2008; Lombardo *et al.* 2011; Osorio *et al.* 2011, 2012; Rohrmann *et al.* 2011; Lara & Drincovich 2012; Dai *et al.* 2013). In the present work, the metabolite profiles of 'Dixiland' peach fruit during post-harvest at 20 °C (normal ripening conditions), 0 °C (regular cold storage), 39 °C (HT) or combination of post-harvest conditions were compared, allowing the identification of key metabolites, such as galactinol, raffinose, proline and putrescine, as being highly responsive to these treatments. The relative expression levels of transcripts encoding enzymes involved in the metabolism of these key compounds allowed the identification of regulatory mechanisms in response to heat and cold, which may mediate the acclimation to cold observed following the HT. These results are discussed in the context both of post-harvest physiology of fruits and also within the broader framework of organismal priming as a preparation for future environmental conditions.

## MATERIAL AND METHODS

### Plant material

Assays were conducted using peach fruit (*P. persica* L. Batsch) cv. Dixiland grown in the Estación Experimental Agropecuaria INTA, San Pedro, Argentina (Budde *et al.* 2006; Borsani *et al.* 2009). Peach fruits were harvested when the fruits reached a flesh firmness of typically  $67.3 \pm 6.3$  N at approximately 102 d after bloom (Borsani *et al.* 2009). Immediately after harvest, fruits were manually selected for

uniformity of colour, size and firmness, divided into three pools and subjected to different post-harvest conditions. One group was allowed to ripen in a chamber at 20 °C and 90% relative humidity for 7 d when fruit flesh firmness reached values lower than 15 N (Borsani *et al.* 2009). Samples were collected at harvest (H), and after 3, 5 and 7 d after harvest, and named as H3, H5 and H7, respectively. Another group was held in a chamber at  $39 \pm 1$  °C and 90% relative humidity for 3 d (HT), followed by 3 d at 20 °C (HT+3). Alternatively, after the HT, fruits were transferred to 0 °C for another 2 d (HT+R2). A third group was subjected to refrigeration. For this purpose, fruits were kept in a chamber at 0 °C and 90% relative humidity for up to 5 d followed by 2 d at 20 °C. Within this group, samples were taken after 3 and 5 d in the chamber at 0 °C (R3 and R5, respectively), and after 2 d at 20 °C following the 5 d 0 °C incubation (R5+2). A schematic representation of Dixiland peach fruit post-harvest treatments and sample collection is shown in Fig. 1a. Five separate pools, each one composed of three different fruits of each post-harvest treatment, were used for further analysis. Samples were immediately frozen in liquid N<sub>2</sub> and stored at -80 °C for further experiments. The complete metabolic profile during ripening and post-harvest storage shown in the present work corresponds to fruits collected during 2007/2008, and similar results were obtained with fruits collected during 2006/2007 (not shown). The average temperature of the hottest week during fruit development was 25.9 °C (with maximum temperatures of 35.3 °C).

### Fruit quality trait determination

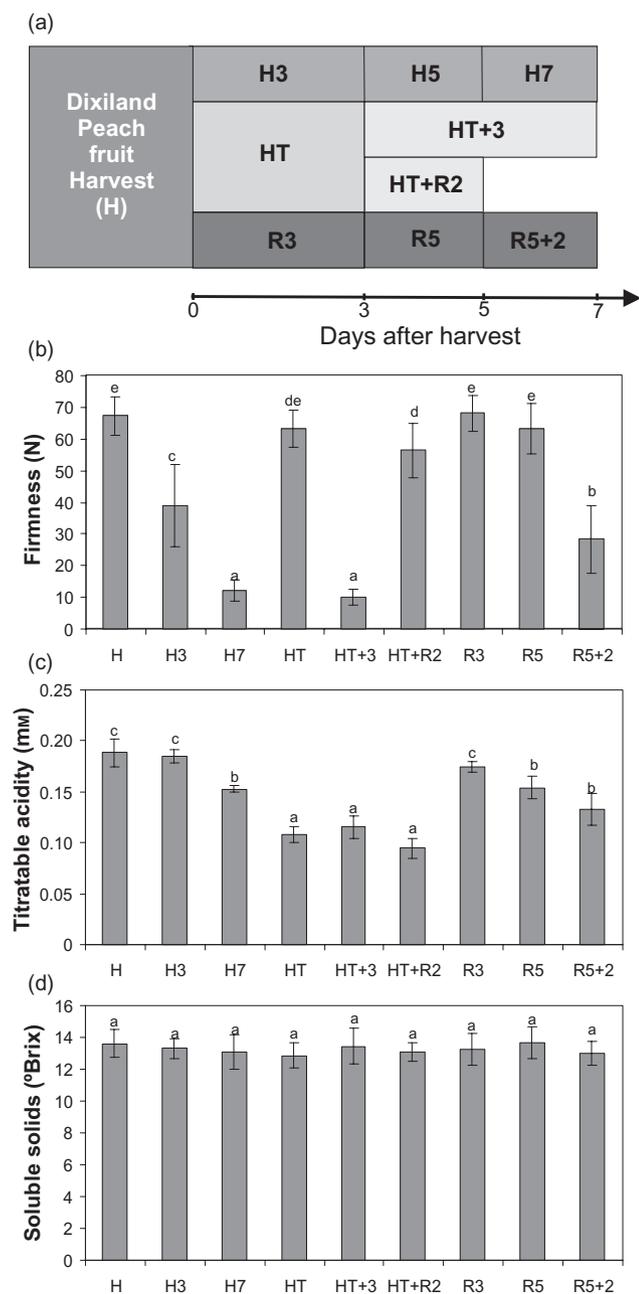
Flesh firmness was evaluated with a penetrometer (Effegi 327; Milan, Italy) with a 7.9 mm tip and expressed in newton (N). Measurements were carried out on two opposite sides of each individual fruit after peel removal. Soluble solid content (SSC) was measured with a refractometer (Atago N1 0–32 °C; Minato-Ku, Tokyo, Japan). Titratable acidity (TA) was determined in three pools of three fruits each by titration with 0.1 M NaOH and expressed as mm.

### RNA isolation and RT-PCR

Total RNA from different peach samples was isolated employing 4 g of tissue using the method described by Meisel *et al.* (2005). RNA integrity was verified by agarose electrophoresis and by isolation and sequence determination of the complete coding sequences for some known transcripts. RNA quantity and purity were determined spectrophotometrically. 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 RT-PCR

Relative expression was determined by performing quantitative real-time RT-PCR (QRT-PCR) in an iCycler iQ detection system and the Optical System Software version



**Figure 1.** Schematic representation of sample collection of Dixiland peach fruit upon post-harvest treatments (a) and quality parameters (b–d). Studies were conducted using fruits harvested (H) and allowed to ripen normally at 20 °C for 3, 5 and 7 d (H3, H5 and H7, respectively); after the application of a heat treatment of 39 °C for 3 d (HT) and after 3 d at 20 °C following the HT (HT+3) or after 2 d at 0 °C following the HT (HT+R2). Alternatively, after harvest, a third group of fruits was kept at 0 °C during 3 or 5 d (R3 and R5, respectively). A subgroup of the R5 fruits was transferred to 20 °C for 2 d (R5+2). Organoleptic attributes: fruit firmness (b), total titratable acidity content (c) and soluble solids (d). Values represent the mean of 15 independent determinations using different fruits (standard deviations are shown). In the case of acidity estimation, determinations were carried out using pools of three fruits. Bars with the same letters are not significantly different ( $P < 0.05$ ).

3.0a (Bio-Rad, Hercules, CA, USA) using the intercalation dye SYBRGreen I (Invitrogen, Carlsbad, CA, USA) as a fluorescent reporter, with 2.5 mM MgCl<sub>2</sub>, 0.5 μM of each primer and 0.04 U μL<sup>-1</sup> GoTaq polymerase (Promega). PCR primers were designed based on peach fruit cDNA sequences published in GenBank and *P. persica* EST databases (TIGR Plant Transcript Assemblies; <http://plantta.tigr.org>), with the aid of the web-based program 'primer3' (<http://bioinfo.ut.ee/primer3/>) to produce amplicons of 150–250 bp in size (Supporting Information Table S1). Primers and amplicon sequences were further analysed using peach EST databases (ESTree Database, <http://www.itb.cnr.it/estree/>, and GDR Genome Database for Rosaceae, <http://www.rosaceae.org/>; 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 DNA-free RNA samples. Cycling parameters were as follows: initial denaturation at 94 °C for 2 min; 35 cycles at 96 °C for 10 s and 58 °C for 15 s, and 72 °C for 1 min and 72 °C for 10 min. Melting curves for each PCR reaction were monitored by measuring fluorescence quenching with increasing temperature (from 65 to 98 °C). PCR specificity was confirmed by software-based melting curve analysis as well as by agarose gel electrophoresis of the products. Relative gene expression was calculated using the 'Comparative 2<sup>-ΔΔCT</sup>' method (Livak & Schmittgen 2001) and elongation factor-1α from *P. persica* (*EF-1α*, Supporting Information Table S1) as reference gene. Each assay was run in three technical replicates and repeated at least three times using different biological replicates.

### Metabolite measurements

Gas chromatography–mass spectrometry (GC-MS) metabolite analysis was carried out essentially as described by Roessner-Tunali *et al.* (2003) and Lombardo *et al.* (2011). Representative mesocarp tissue of peach fruits (250 mg) was ground using ceramic mortar and pestle pre-cooled with liquid nitrogen and extracted in 3 mL of methanol. Internal standard (180 μL, 0.2 mg ribitol mL<sup>-1</sup> water MilliQ) was subsequently added for quantification purposes. The mixture was extracted for 15 min at 70 °C (vortexing every 3 min) and mixed vigorously with pre-cooled water MilliQ (1.5 mL). After centrifugation at 2200 × *g*, an aliquot of the supernatant (50 μL) was transferred to a reaction tube (1.5 mL) and vacuum dried. Tubes were filled with argon gas and stored at -80 °C. Samples were derived and GC-MS performed as described by Roessner-Tunali *et al.* (2003). Mass spectra were cross-referenced with those in the Golm Metabolome Database (Kopka *et al.* 2005). Five independent determinations, composed of three different fruits of the same stage, were performed to each sample analysed. Data presented are normalized to the harvested peach fruit (H) and expressed as log<sub>2</sub> ratios to H using the MultiExperiment Viewer software (MeV v4.4.1, <http://www.tm4.org/>; Saeed *et al.* 2003). Data from these experiments are provided in the supporting information material in accordance to recent recommendations

for reporting metabolite data (Supporting Information Table S2; Fernie *et al.* 2011).

Principal component analysis (PCA) was performed on data sets obtained from metabolite profiling and correlation analysis between metabolites based on Pearson's correlation using the software package XLSTAT (Microsoft Excel).

### Statistical analysis

Data presented were analysed using one-way analysis of variance (ANOVA). Minimum significance differences were calculated by the Bonferroni, Holm-Sidak, Dunnett and Duncan tests ( $\alpha = 0.05$ ) using the Sigma Stat Package (San Jose, CA, USA).

## RESULTS

### Quality traits of peach fruit during post-harvest ripening, heat exposure or cold refrigeration

Peaches cv. 'Dixiland' were harvested (H) at  $67.3 \pm 6.3$  N mean firmness and subjected to different post-harvest treatments (Fig. 1a). Fruits allowed to ripen at 20 °C reach firmness values lower than 12 N, which usually occurred 7 d after harvest (H7; Fig. 1b). When fruits were exposed to 39 °C for 3 d, ripening was delayed by softening inhibition. However, following transfer to 20 °C for 3 d (HT+3), peaches softened as those of untreated fruits of essentially the same post-harvest age (H7; Fig. 1b). In contrast, when heat-exposed fruits were refrigerated, they maintained their firmness (HT+R2; Fig. 1b). A similar trend was observed in the cold-stored fruits for 3 or 5 d (R3 and R5) in which softening was prevented by the cold and restored after transfer to normal ripening temperatures (R5+2) (Fig. 1b).

Fruit TA decreased during ripening at 20 °C in H7 (Fig. 1c). In peaches exposed to 39 °C, acidity decreased to a greater extent than in ripened fruits (H7) and it was maintained after removal from the heat to either 20 or 0 °C (HT+3, HT+R2; Fig. 1c). In contrast, cold-stored fruits exhibited a similar pattern of acidity change as those ripening at 20 °C (Fig. 1c). By contrast, total SSC was not modified during the ripening process at 20 °C or at any other post-harvest temperature (Fig. 1d).

### Metabolite profiles in fruits show dramatic variation upon post-harvest treatments

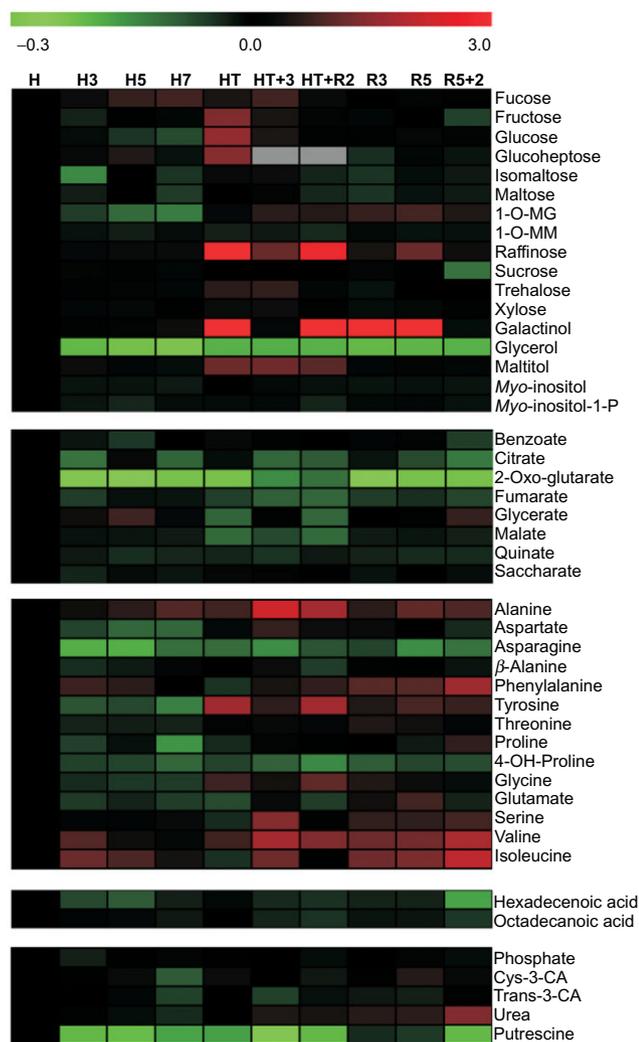
In order to assess the metabolic profiles of 'Dixiland' peach fruits under the different conditions during the post-harvest, metabolites were extracted from representative mesocarp samples and subjected to GC-MS analysis to detect primary metabolite levels. By this technique, 47 metabolites were unambiguously identified and their levels relative to those detected in harvested fruit (H) were measured (Supporting Information Table S3). To test whether changes are significant among samples, data were analysed using one-way ANOVA, with values regarded as significantly different at the threshold of  $P < 0.05$ . To reveal the changes induced by heat

or cold exposure during the post-harvest period, metabolite contents from peaches exposed to 39 °C (HT) for 3 d or to 0 °C (R3) were compared with those from peaches with the same post-harvest life at 20 °C (H3; Fig. 1a). Heat-treated peaches followed by 3 d at 20 °C (HT+3) or by refrigeration (HT+R2) were compared with H7 and H5, respectively (Fig. 1a). Similarly, peaches kept at 0 °C for 5 d (R5) and then removed and maintained at 20 °C for 2 d were compared with those ripening at 20 °C for 5 d (H5) and 7 d (H7), respectively (Fig. 1a). Thus, in order to identify metabolite changes that occur due to temperature treatments, relative metabolite content to H (Supporting Information Table S3 and Fig. 2) and ratios between samples of the same post-harvest age (Fig. 3) are presented.

The metabolic profile during normal ripening of fruits kept at 20 °C has been previously described by our group (Lombardo *et al.* 2011) and can be briefly described as it follows. Essentially, an increase in the sugar fructose and a decrease in glycerol and 1-methyl-glucoside during ripening were accompanied by a decrease in the organic acid citrate and 2-oxo-glutarate and an increase in glycerate. The amino acid composition was also modified during ripening, with increases in Asn, Asp, Tyr, Glu, Pro and 4-OH-Pro, and decreases in Phe, Ile and Ala relative to the amounts at harvest (H). A significant decrease in putrescine was also observed during ripening (Supporting Information Table S3). As illustrated in the heat map presented in Fig. 2, metabolism is globally reprogrammed during cold storage or heat exposure with respect to normal ripening. It is noticeable that 81% of the 47 metabolites quantified were significantly affected by temperature conditions during the post-harvest period.

### Metabolites altered due to HT

HT had a profound effect on the level of sugars and sugar alcohols (Fig. 2). Galactinol exhibited the greatest increase with 18.5-fold higher levels in HT than in H3 (Fig. 3). While removal from the heat and transfer to 20 °C (HT+3) resulted in a re-establishment of the levels found in H7, exposure to 0 °C prevented the decrease in galactinol, with 11.6-fold higher levels in HT+R2 than in H5 (Fig. 3). Raffinose, maltitol, fructose, glucose and 1-O-methyl-glucoside as well as the disaccharides maltose, isomaltose and trehalose followed a similar trend of increase in HT fruits, with raffinose exhibiting the largest increase within this group (8.5-fold with respect to H3) (Fig. 3). After transferring to 20 °C (HT+3) or to 0 °C (HT+R2), these metabolites remained elevated with the exception of fructose and glucose and the three disaccharides, which returned to the levels found in H5 and H7 fruits (Figs 2 & 3). By contrast, glucoheptose, which in HT fruits was twice as high as in ripening peaches, was no longer detected after heat removal in both HT+3 and HT+R2 (Fig. 2), while fucose, which is increased during fruit ripening (Lombardo *et al.* 2011), followed the same pattern as during ripening with the exception of HT+R2 in which the increase was lower than in H5 fruits (Fig. 2). Other sugars and alcohol sugars such as *myo*-inositol, *myo*-inositol-1-P, glycerol, 1-O-methyl-mannoside, xylose and sucrose did not exhibit



**Figure 2.** Distribution of metabolites analysed by gas chromatography–mass spectrometry (GC-MS) in peach fruit harvested and kept at different temperatures. The graph shows the relative level of each metabolite to its amount found in harvested peach fruit (H). Normalized values are shown on a colour scale (shown at the top of the figure), which is proportional to the content of each metabolite. Mean values of five independent determinations for each stage were expressed as the ratio between  $\log_2$  and H using the MultiExperiment Viewer software (MeV v4.4.1; Saeed *et al.* 2003). Grey colour indicates not determined value. Relative values for each metabolite peak area are provided in Supporting Information Table S3. 1-O-MG, 1-O-methyl-glucoside; 1-O-MM, 1-O-methyl-mannoside; Cys-3-CA, cys-3-caffeoylquinic acid; trans-3-CA, trans-3-caffeoylquinic acid.

changes in their relative levels with respect to fruits ripening at ambient temperature when kept at 39 °C for 3 d (Fig. 2).

Heat exposure for 3 d (HT) also affected organic acids, with citrate doubling its relative amount, and 2-oxo-glutarate, malate and glycerate reducing in half their relative amount with respect to H3 (Figs 2 & 3). Following transfer to 20 °C, but not to 0 °C, these metabolites presented similar levels to those of ripened fruits (H7) with the exception of 2-oxo-glutarate, which was increased 4- and 2.5-fold in HT+3 and

HT+R2, respectively (Fig. 3). Cold exposure also reduced the increased levels of citrate induced by heat, but to levels lower than in H5 fruits (Fig. 3). In addition, a slight decrease in fumarate was also observed in HT+3 and HT+R2 peaches (Fig. 2).

Ten out of the 14 amino acids identified were modified upon heat exposure (Fig. 2). Tyr, Asp, Ala and Gly were increased in HT, HT+3 and HT+R2, with respect to fruit of the same post-harvest age at 20 °C H3, H7 and H5, respectively (Figs 2 & 3). In contrast, Val showed an increase when HT fruits were transferred either to 20 °C (HT+3) or to 0 °C (HT+R2), while Pro, Glu and Ser only increased in HT+3 with respect to H7 (Figs 2 & 3). On the other hand, Phe and 4-OH-Pro decreased in HT fruits with respect to H3, with Phe increasing following 3 d at 20 °C (HT+3) being even higher than in H7, and 4-OH-Pro diminishing in HT+2R (Figs 2 & 3).

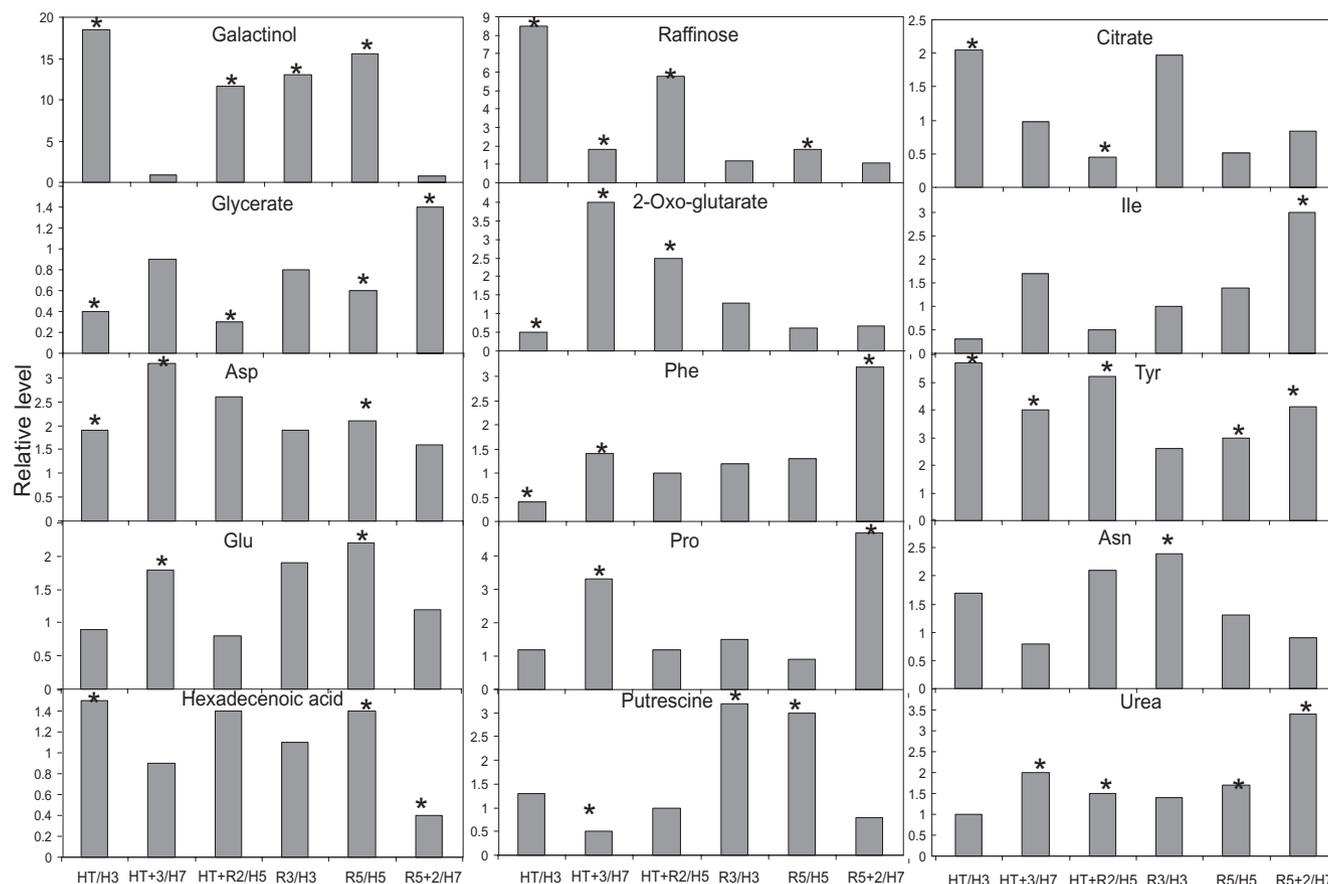
Regarding the two fatty acids detected, an increase in hexadecenoic acid in HT and a decrease in octadecanoic acid in HT+R2 were detected (Figs. 2 & 3). Within the miscellaneous group, urea was augmented after removal from the heat and transfer to both 20 °C (1.9-fold enlargement with respect to H7) and 0 °C (1.5-fold increase with respect to H5) (Fig. 3). Finally, the usual decrease in putrescine that takes place during fruit ripening was exacerbated when heated fruits were transferred to 20 °C for 3 d (HT+3) (Figs 2 & 3).

### Metabolite changes in response to cold exposure

On the other hand, significant changes in the metabolome of fruit mesocarp were also observed upon refrigeration, which affected the levels of several functional groups (Figs 2 & 3; Supporting Information Table S3).

Twelve out of 17 sugars and alcohol sugars detected by GC-MS were not affected in peach mesocarp upon refrigeration in comparison with harvested fruits ripening at 20 °C (Supporting Information Table S3; Fig. 2). Regarding the sugars that did vary and similar to heat exposure, cold storage for 3 and 5 d also resulted in a considerable increase in galactinol of 12.6- and 15.6-fold with respect to fruits of the same post-harvest age at 20 °C (H3 and H5), respectively (Fig. 3). After cold removal and transferring to 20 °C, relative levels of galactinol returned to those observed in ripe fruits (H7) (Fig. 3). Raffinose was increased in cold-stored fruits as well, but to a lesser extent (about twice as high in R5 as in H5) (Fig. 3). On the other hand, sucrose decreased by 50% after cold removal (R5+2) (Fig. 2). The decrease that usually occurs in 1-O-methyl-glucoside during ripening (H to H7) was prevented in cold-stored fruits (R3, R5 and R5+2), which displayed values even higher than those of harvested fruits (H) (Fig. 2). In contrast, the usual increase in fucose of ripening was prevented in cold-stored fruits (R5) and even after removal to 20 °C (R5+2) (Fig. 2).

The main organic acids in fruit mesocarp, malate and citrate, together with saccharate, fumarate, 2-oxo-glutarate and quinate, were not affected by cold storage following similar patterns than those in fruits ripening at 20 °C (Figs 2



**Figure 3.** Main metabolites altered by heat and/or cold treatments during the post-harvest of Dixiland peach fruits. The ratio between the levels in treated fruits relative to the levels in peaches of the same post-harvest age kept at 20 °C is indicated. Asterisks indicate that the samples compared in the ratio are statistically different. Relative values for each metabolite peak area are provided in Supporting Information Table S3.

& 3). In contrast, benzoate was slightly increased in R5, and glycerate was slightly decreased in R5 and increased thereafter in R5+2, with respect to H5 and H7, respectively (Fig. 2).

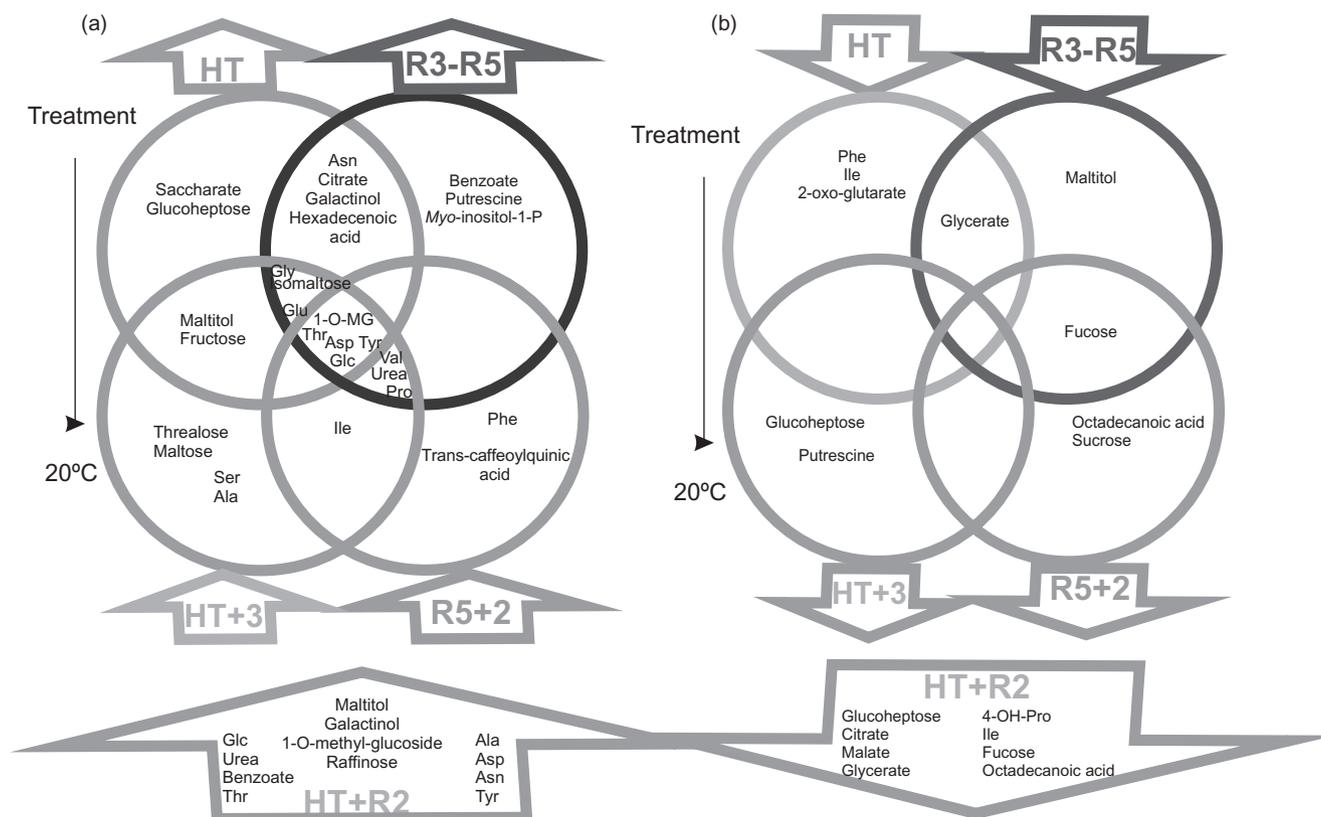
Cold storage additionally resulted in an increase in 11 out of the 14 amino acids identified, with Tyr (2.9-fold in R5), Asn (2.4-fold in R3), Glu (2.2-fold in R5) and Asp (2.1-fold in R5) showing the greatest increases with respect to fruits of the same post-harvest age ripening at 20 °C (Figs 2 & 3). Following cold removal and transfer to 20 °C, while some amino acids such as Pro (4.7-fold), Tyr (4.1), Phe (3.2) and Ile (3-fold) remained increased with respect to ripe fruits (H7), others such as Ser, Gly, Thr and Asp returned to the values of non-refrigerated fruits (Figs 2 & 3). Conversely, Ala,  $\beta$ -Ala, 4-OH-Pro and Ser were unaffected by refrigeration at 0 °C (Fig. 2).

The monounsaturated fatty acid hexadecenoic acid increased by 50% following cold refrigeration for 5 d (R5) with respect to H5 and decreased after transferring to 20 °C (R5+2) to a third of the amount detected in ripe fruits (H7) (Fig. 3). By contrast, octadecanoic acid decreased in R5 with respect to H5 (Fig. 2).

Finally, among the miscellaneous compounds, the decrease of putrescine characteristic of normal ripening was prevented by the cold with R3 and R5 peaches showing the accumulation of this polyamine to levels three times as high as those observed in fruits at 20 °C H3 and H5 (Fig. 3). Nevertheless, following transfer from the cold, this decrease in putrescine took place declining to levels as low as those in ripe fruits (H7). Cold storage increased the relative level of urea in R5 (1.5-fold) and in R5+2 (2.6-fold) fruits with respect to H5 and H7, respectively (Fig. 3).

### Comparison of heat and cold responses

In order to easily visualize the common and distinct metabolites modulated by heat and/or cold treatments during the post-harvest, metabolites are presented in two Venn diagrams, one showing the metabolites that were increased (Fig. 4a) and the other those that were decreased (Fig. 4b) by the treatments. The compounds modulated by the combination of both heat and cold treatments (HT+R2) are presented separately from the Venn diagram at the bottom.



**Figure 4.** Venn diagrams comparing the differentially metabolites that are increased (a) and decreased (b) in Dixiland peach fruits under different post-harvest storage conditions. Cold storage at 0 °C (R3 and R5) and heat treatment (HT) at 39 °C were conducted. Following these treatments, fruits were transferred to 20 °C (HT+3 and R5+2). The metabolites included display statistically significant ( $P < 0.05$ ) changes with respect to fruit of the same post-harvest age at 20 °C. At the bottom of the figures, the metabolites that display significant changes in heat-exposed fruits followed by cold storage (HT+R2) are also shown. 1-O-MG, 1-O-methyl-glucoside; Raff, raffinose.

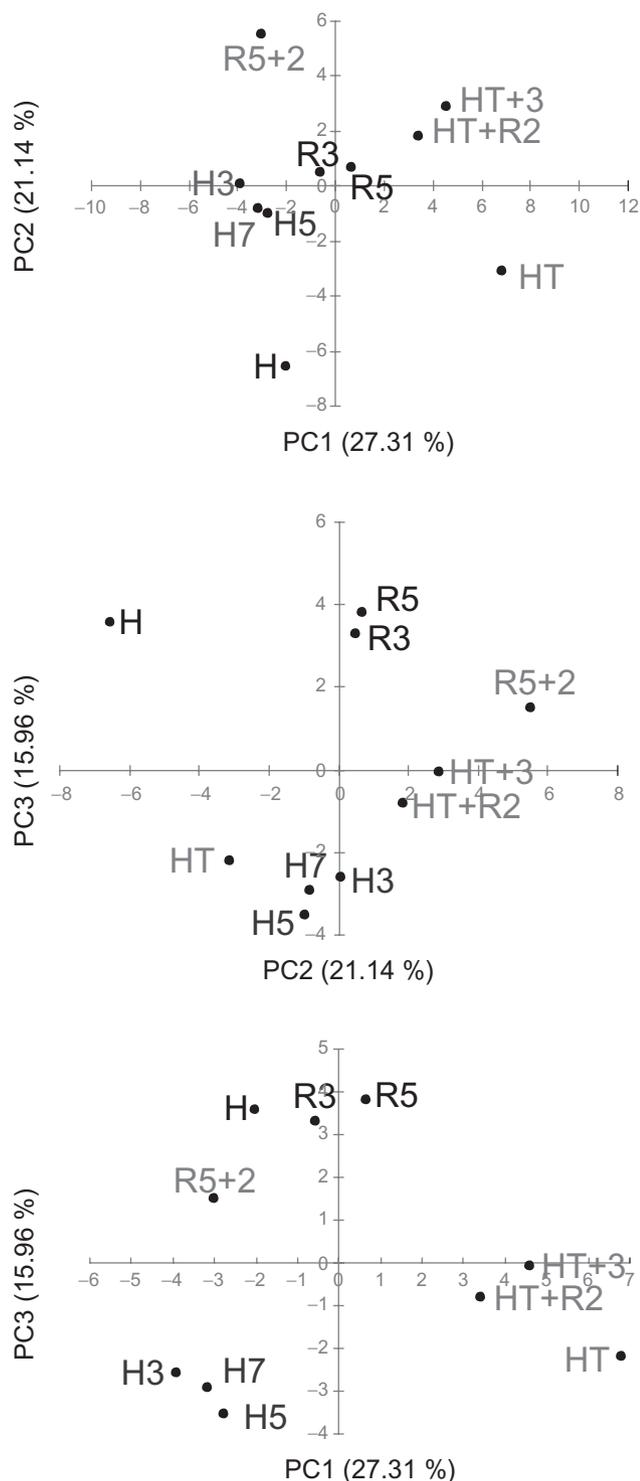
Of the 27 metabolites that were increased, 8 were only increased in heat-exposed fruits (HT and HT+3), 5 were only increased in cold-stored peaches (R3, R5 and R5+2) and 17 were affected by both heat and cold treatments (Fig. 4a). As expected, when both treatments were applied sequentially (HT+R2), 8 (galactinol, raffinose, Glc, 1-O-methylglucoside, Asn, Asp, Tyr and Thr) out of these 13 metabolites were induced (Fig. 4a). In addition, urea and benzoate, which were only induced by refrigeration, and maltitol, which was only increased in heated fruits (HT), remained augmented when both treatments were applied to the fruits (HT+R2) (Fig. 4a). On the other hand, the amino acid Ala that was altered in neither HT nor R3 or R5 fruits was increased in HT+R2 fruits (Fig. 4a).

The number of metabolites that were decreased by heat and/or cold was considerably lower than the number of compounds that were increased. While only glycerate was decreased in both heated (HT) and cold-stored fruits (R3-R5), four metabolites were decreased by heat (HT fruits) and five by cold (R3-R5 peaches) (Fig. 4b). When HT preceded refrigeration, glycerate, Ile and fucose were decreased, as in the case of heated or cold-stored fruits (Fig. 4b). Nevertheless, glucoheptulose and octadecanoic acids, which were only decreased after transferring to 20 °C the heated and

refrigerated fruits, respectively, were decreased in HT+R2 fruits. It is interesting to note that three of the eight metabolites (37.5%) that were decreased in HT+R2 peaches were decreased neither in heated (HT and HT+3) nor cold-exposed peaches (R3, R5 and R5+2) (Fig. 4b).

### Principal component and correlation analyses

The data set obtained by GC-MS during peach ripening and post-harvest treatments (Supporting Information Table S3) was examined by PCA (Fig. 5), with three principal components explaining 64.4% of the overall variance of the metabolite profiles (27.3, 21.1 and 16.0% for PC1, PC2 and PC3, respectively). PCA reveals different groups of samples according to the different post-harvest conditions. Harvested fruits (H) changed their metabolomes when ripening at 20 °C, with H3 to H7 stages grouping together and separately from H. Cold-stored peaches (R3 and R5) group together and separately from HT fruits. Interestingly, heat-treated fruits (HT) removed from the cold to either 20 °C (HT+3) or to 0 °C (HT+R2) group together and separately from other conditions (Fig. 5). In addition, the contribution of metabolites to each principal component is shown in Supporting Information Table S4. Regarding PC1, saccharate



**Figure 5.** Principal component analysis of 47 identified metabolites by gas chromatography–mass spectrometry (GC-MS) data. Peach fruit samples during the post-harvest ripening process and during different post-harvest treatments were analysed: harvest (H); during the ripening progress at 20 °C for 3, 5 and 7 d (H3, H5 and H7, respectively); during a heat post-harvest treatment of 39 °C during 3 d [heat treatment (HT)] followed by 3 d at 20 °C (HT+3) or by 2 d at 0 °C (HT+R2); and during refrigerated storage at 0 °C for 3 or 5 d (R3 and R5, respectively) followed by 2 d at 20 °C (R5+2). The variance explained by each component (%) is given within parentheses.

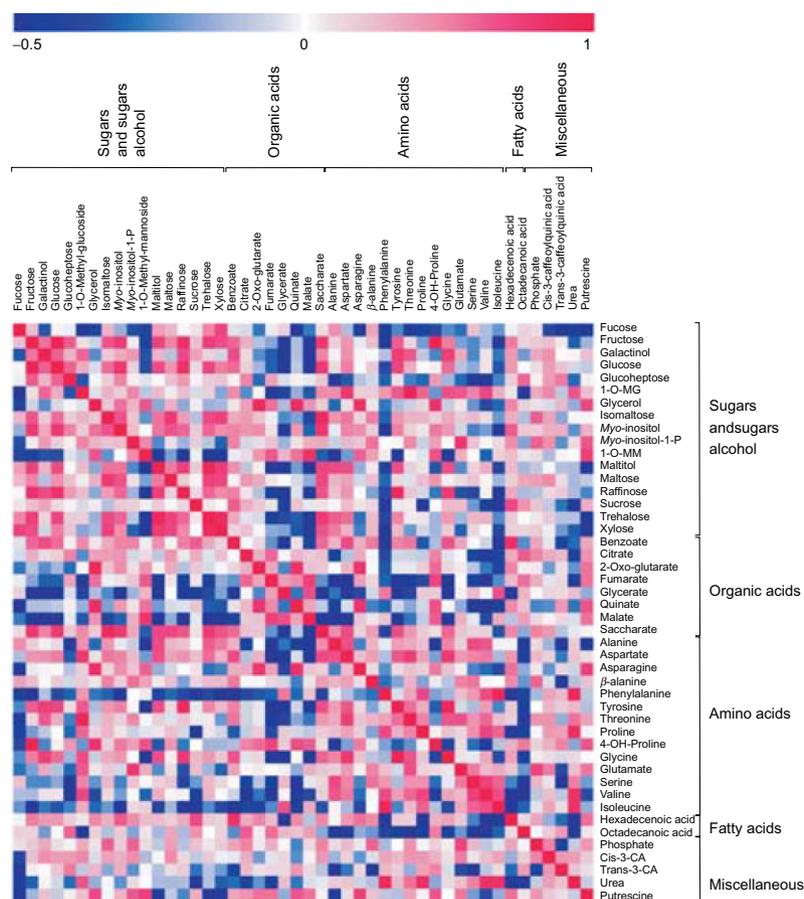
(6.14%), maltitol (6.12%), malate (5.84%), Tyr (5.5%), fructose (5.32%) and raffinose (5.31%) are the variables that contribute the most to this component. On the other hand, the main contributors to PC2 are Val (7.93%), Phe (6.02%), Ile (5.88%), hexadecenoic acid (5.78%), urea (5.75%) and fumarate (5.3%). Finally, putrescine (8.3%), glutamate (7.74%), Thr (7.34%), 1-O-methyl-glucoside (7.01%) and *myo*-inositol-1P (6.93%) are the main variables contributing to PC3.

Correlation analysis was also performed on the entire data set of metabolites during peach ripening and post-harvest treatments by the calculation of the Pearson's correlation coefficients for each metabolite pair. A total of 1081 pairs were analysed, from which 259 resulted in significant correlation coefficients ( $P < 0.05$ ). Out of these, 201 were positive and 58 negative (Fig. 6). This analysis showed that sugars were strongly and positively associated within each other (raffinose with fructose and glucose; glucose with fructose; trehalose with xylose and maltitol), with some amino acids (raffinose positively correlated with Tyr; and 1-O-methyl-glucoside with Thr) and some organic acids (isomaltose positively correlated with saccharate, 1-O-methyl-mannoside with malate). In addition, it was identified that urea positively correlated with amino acids such as Pro, Val, Ile and Phe, and Phe also correlated with Ile. The evaluation of the behaviour of the metabolite network also revealed that glycerol was positively associated with Asn, 4-OH-Pro, quinate and  $\alpha$ -oxo-glutarate. Finally, among highly positively correlations, the pairs quinate-Asn, quinate- and  $\alpha$ -oxo-glutarate, and benzoate-hexadecenoic acid were also found. By contrast, malate correlated negatively with raffinose, Tyr and maltitol; glycerate with raffinose, benzoate and 4-OH-Pro, while hexadecenoic displayed negative associations with Ile and Phe (Fig. 6).

### Analysis of transcripts encoding enzymes involved in raffinose metabolism

In view of the fact that galactinol and raffinose were the metabolites that exhibited the greatest increase in heat-treated or cold-stored fruits, the relative level of transcripts encoding enzymes involved in raffinose metabolism was studied by QRT-PCR (Fig. 7). The trisaccharide raffinose, along with stachyose and verbascose, belongs to the raffinose family of oligosaccharides (RFOs). RFOs are synthesized from Suc by the subsequent addition of Gal moieties donated by galactinol in the reaction catalysed by raffinose synthase (RS) (Peterbauer & Richter 2001). Galactinol synthase (GolS) catalyses the first committed step in the biosynthesis of RFOs (Fig. 8a). On the other hand, the initial enzymes in the RFO catabolism are the  $\alpha$ -galactosydases (Peters *et al.* 2010), which hydrolytically remove the terminal galactose moiety of the RFOs. A formerly unidentified group of proteins in germinating seed embryos called 'Seed Imbibition Proteins (SIPs)' is now also included within this group (Carmi *et al.* 2003).

Peach database search and comparison with already known  $\alpha$ -galactosyltransferases (Carmi *et al.* 2003), along with phylogenetic relationship analysis (Supporting Information



**Figure 6.** Visualization of metabolite-metabolite correlations during peach fruit post-harvest ripening process and during different post-harvest treatments. Correlation coefficients were calculated by applying Pearson's correlation. Out of 1081 pairs of metabolites analysed, 259 resulted in significant correlations ( $P < 0.05$ ). From these, 201 were positive and 58 negative. Each square represents the correlation between each metabolite heading the column and the metabolite heading the row with a colour scale (colour scale key at the top of the figure). Metabolites were grouped by compound class in the same order as shown in Supporting Information Table S3. 1-O-MG, 1-O-methyl-glucoside; 1-O-MM, 1-O-methyl-mannoside; Cys-3-CA, cys-3-caffeoylquinic acid; Trans-3-CA, trans-3-caffeoylquinic acid.

Fig. S1), allowed the identification of two transcripts encoding putative SIPs (called *PpSIP1* and -2), and a third peach sequence encoding a putative RS (called *PpRS*). The phylogenetically classification of these enzymes places *PpSIP1* and *PpSIP2* in different branches of the  $\alpha$ -gals/SIPs which catalyse raffinose hydrolysis, while placing *PpRS* within the cluster of RFO synthases in the branch of RS (Supporting Information Fig. S1). Therefore, the relative expression levels of *PpSIP1*, *PpSIP2* and *PpRS* were investigated in peach fruit during the post-harvest at different temperatures, together with *PpGolS* (Figs 7 & 8a).

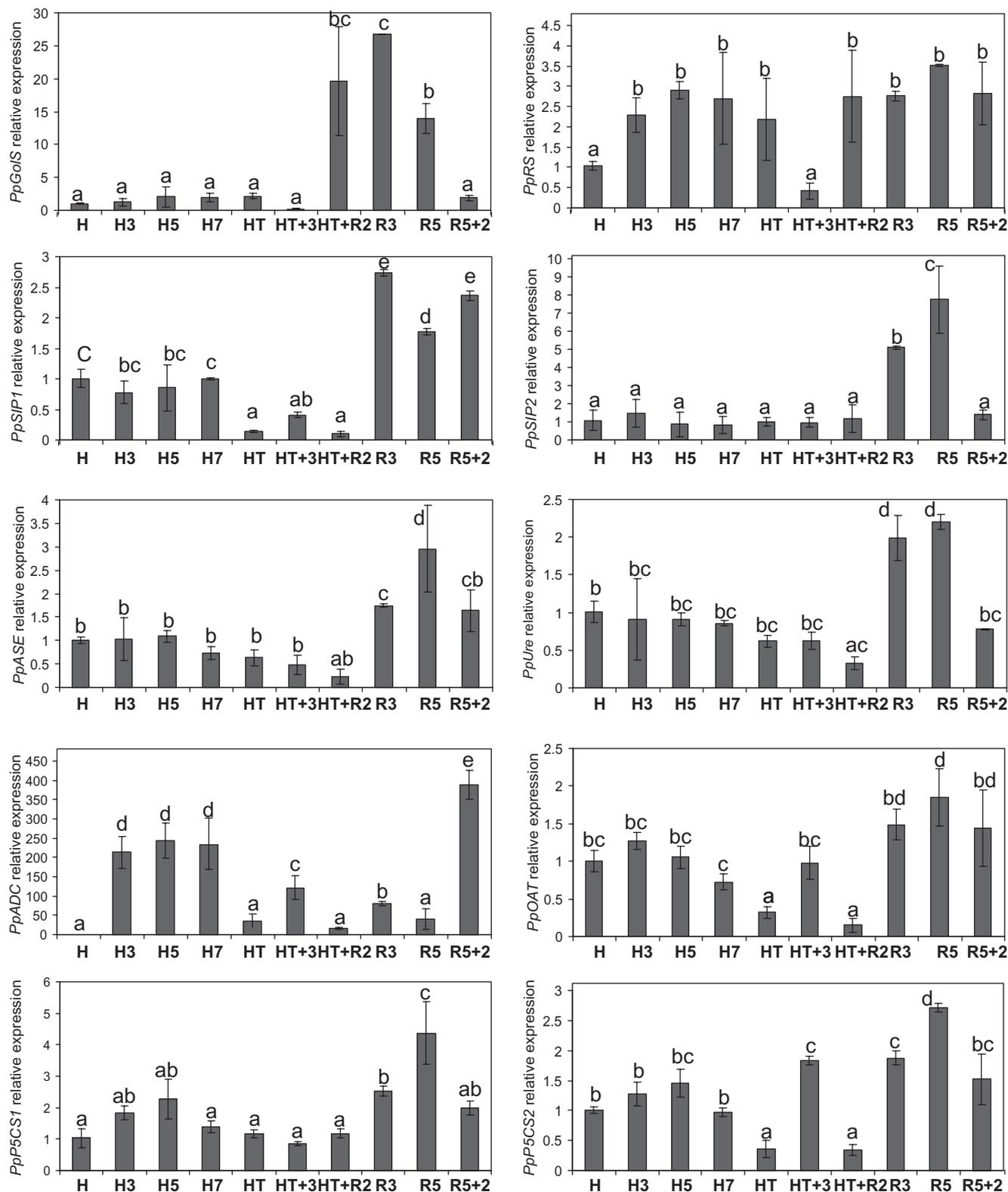
The level of the transcript encoding a *PpGolS* (*PpGolS*) was dramatically increased after refrigeration of harvested fruit (R3 and R5) or after a HT (HT+R2), showing R3, R5 and HT+R2 increases of 22-, 7- and 10-fold in *PpGolS* with respect to fruits of the same post-harvest age kept at 20 °C, respectively (Fig. 7). On the other hand, the relative expression of *PpGolS* was not modified with respect to peach fruit ripening at 20 °C (H, H3-H7; Fig. 8).

Neither *PpSIP1* nor *PpSIP2* was modified during peach fruit ripening at ambient temperature (H versus H3-H7) and

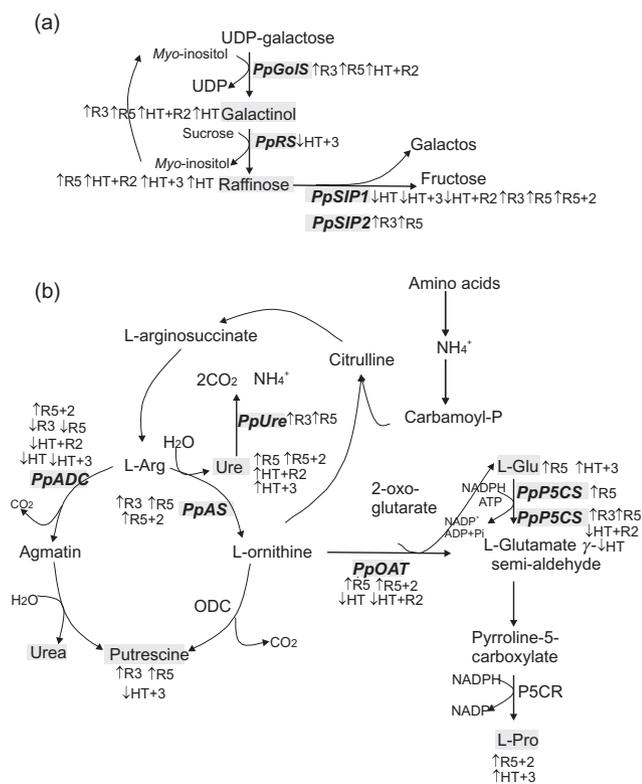
both transcripts increased in refrigerated fruits for 3 and 5 d with respect to fruits of the same post-harvest age at 20 °C (Fig. 8). Nevertheless, while *PpSIP2* was not modified either in R5+2 or in any group of fruits exposed to 39 °C (HT, HT+3, HT+R2) (Fig. 7), *PpSIP1* was induced 2.4-fold in R5+2 and notably decreased by 82 and 88% in HT and HT+R2, with respect to fruits at 20 °C, respectively (Fig. 7). Regarding *PpRS*, the relative expression of this transcript was induced during ripening and decreased after heat-treated peaches were transferred to 20 °C for 2 d (HT+3; Fig. 7).

### Relative quantification of transcripts encoding enzymes involved in the metabolism of some nitrogenous compounds

Because urea was significantly increased in cold-stored fruits (R3, R5 and R5+2) and after removal from the heat (HT+3 and HT+R2), the expression profile of transcripts encoding arginase (*PpASE*) and urease (*PpUre*) involved in urea synthesis and hydrolysis, respectively, was also investigated (Figs 7 & 8b). Both transcripts followed the same expression



**Figure 7.** Expression analysis of transcripts encoding enzymes involved in raffinose family of oligosaccharides (RFOs) and in putrescine, proline and urea metabolisms in peach fruit during the post-harvest at different temperatures. For each sample, means of the results obtained using three independent RNA biological replicates as templates are shown. Each reaction was normalized using the  $C_t$  values corresponding to *Prunus persica* elongation factor 1 $\alpha$  mRNA (Supporting Information Table S1). Y axis refers to the fold difference in a particular transcript level relative to its amount found in peaches analysed after harvest (H). Standard deviations are shown. For each transcript analysed, bars with the same letters are not significantly different ( $P < 0.05$ ).



**Figure 8.** Representative scheme of pathways significantly affected by heat and cold treatments during the post-harvest of peach fruit: (a) galactinol and raffinose family oligosaccharide (RFO) and (b) arginine and derived nitrogenous compounds. Simplified metabolic scheme and changes observed after heat, cold, and combined heat and cold treatments. ↓ and ↑ represent increase and decrease, respectively, in metabolite or transcript levels in the indicated storage condition with respect to fruit of the same post-harvest age kept at 20 °C. Metabolites and transcripts measured in this work are shown with a grey background. The names of some enzymes are shown. (a) Galactinol synthase (GoIS) and raffinose synthase (RS) catalyse the synthesis of galactinol and raffinose, respectively, while seed imbibition proteins (SIPs;  $\alpha$ -galactosydases) remove the terminal galactose moiety of raffinose. (b) Arginine is hydrolysed in the mitochondria by arginase (ASE) to urea and ornithine. Urea that leaves the mitochondria is hydrolysed by cytosolic urease (Ure) to ammonia which can be re-assimilated. Mitochondrial ornithine aminotransferase (OAT) transfers the side chain amino group of ornithine to 2-oxo-glutarate generating glutamate and L-glutamate-semi-aldehyde which spontaneously renders pyrroline-5-carboxylate (P5C). P5C is then transformed to L-Pro by P5C reductase (P5CR). Pro can also be synthesized from glutamate via  $\Delta$ 1-pyrroline-5-carboxylate synthetase (P5CS). Alternatively, L-ornithine can be decarboxylated by ornithine decarboxylase (ODC) to produce putrescine. This polyamine can alternatively be synthesized from L-Arg by the sequentially action of arginine decarboxylase (ADC) and agmatinase, which also produces urea.

profile, remaining constant during normal ripening (H–H7), being not affected by the HT (HT+3, HT+R2) and temporally induced by cold refrigeration for 3 and 5 d (R3 and R5), and reverted following transferring to 20 °C (R5+2).

In addition, to elucidate possible steps controlling the relative levels of putrescine and proline under heat and cold exposure, the relative expression of transcripts encoding enzymes involved in their metabolism was analysed. Putrescine in plants can be formed either directly from Orn in a single reaction catalysed by Orn decarboxylase (ODC) or by decarboxylation of Arg via the Arg decarboxylase (ADC) pathway (for a review, see Tiburcio *et al.* 1997). ADC hydrolyses L-Arg conducting to the synthesis of urea (Fig. 8b). In plants, proline is synthesized from either glutamate or ornithine via  $\Delta$ 1-pyrroline-5-carboxylate synthetase (P5CS) or ornithine  $\delta$ -aminotransferase (OAT), respectively (Fig. 8b; Verbruggen & Hermans 2008). In the present work, the relative expression of the transcripts *PpADC*, *PpOAT* and of two P5CSs (*PpP5CS1* and -2) was assessed by QRT-PCR (Fig. 7).

While the transcript encoding arginine decarboxylase (*PpADC*) was dramatically induced during fruit ripening (Fig. 7), this increase was prevented in fruits exposed to heat for 3 d (HT), and this relative level remained unchanged following transfer to 0 °C for 2 d (HT+R2) but was increased after transfer to 20 °C (HT+3). Cold treatment (R3 and R5) also prevented the increase in *PpADC* during ripening, but an even greater increase than the one observed during ripening was observed following transfer from the cold (R5+2). By contrast, neither *PpOAT* nor *PpP5CS1* and *PpP5CS2* were modified during ripening at 20 °C. In cold-stored fruits (R5), *PpP5CS1* was twice as high as in H5 peaches (Fig. 7), but approximated control levels following 2 d at 20 °C (R5+2). However, both *PpOAT* and *PpP5CS2* were repressed by heat exposure for 3 d (HT), maintained at low levels following refrigeration (HT+R2), but recovered following return to ambient temperature (HT+3) (Fig. 7). By contrast, cold refrigeration after harvest induced *PpOAT* (R5) and *PpP5CS2* (R3 and R5) levels when compared with fruits of the same post-harvest age under 20 °C ripening conditions (H7).

## DISCUSSION

### Common and distinct metabolomic responses of peach mesocarp to heat and cold facilitate the elucidation of the metabolic basis of the heat-induced CI protection

In the present work, metabolic profiling analyses in heat-, cold-, and sequentially heat- and cold-exposed peach fruits after harvest are presented. A short period of cold storage was selected to avoid the presence of CI symptoms and, thus, to address the metabolic changes in the fruit in response to the cold but not due to the injury *per se*. According to their behaviours depending on the cold or heat or combination of both treatments, different metabolic groups can be identified in order to decipher, at the metabolite level, the basis of the CI prevention or alleviation when HT is used prior to refrigeration.

One group of metabolites (A) is composed of 20 compounds which exhibit a common response to both heat and cold. Members of this group are increased due to both HT and short cold exposure, with the exception of glycerate that

decreases (Figs 3 & 4). Many of the metabolites in this group participate in protein and membrane protection during heat, drought, oxidative damage and other types of stress (Rudolph & Crowe 1985; Colaço *et al.* 1992; Bachmann, Matile & Keller 1994; Kaplan & Guy 2004; Schramm *et al.* 2006; Nishizawa, Yabuta & Shigeoka 2008). Because the exposure to a short cold treatment induces a defensive response, which is subsequently overcome by prolonged cold exposure generating CI in the fruit, it is highly probable that metabolites from group A are involved in the cold-induced protection invoked by HT. Thus, the application of a HT, without causing the injury generated by cold, primes the fruit with a battery of metabolic components to better cope with the subsequent cold stress. Group A can be subdivided in (1) metabolites induced in HT and R3/R5 that return to normal levels after transferring the fruits to 20 °C (galactinol, Asn, citrate and hexadecenoic acid); (2) metabolites induced in HT and R3/R5 and kept increased in HT+3 and R5+2 (Thr, Asp, Tyr, 1-O-MG and glucose); (3) metabolites induced in HT and R3/R5 and kept increased in HT+3 but not in R5+2 (raffinose, isomaltose, Gly and Glu); and (4) metabolites induced in HT+3, R3 and R5 and kept increased in R5+2 (Val, Pro and urea; Figs 3 & 4).

Galactinol and raffinose are the metabolites that show the greatest increase in response to either HT or cold storage for a short period of time (Fig. 3; Supporting Information Table S3). Thus, in peach fruit the accumulation of galactinol and raffinose may be crucial for the HT-induced protection against CI. We have previously demonstrated for the first time the presence of raffinose in peach fruits and its importance, together with galactinol, in stone formation or seed maturation (Lombardo *et al.* 2011). The present study constitutes the first attempt to reveal the importance of these compounds in the protection against CI in peach. In other plant species, raffinose and galactinol were identified as key antioxidants or signals mediating stress responses, and they are often increased during cold acclimation (Nishizawa *et al.* 2008; Korn *et al.* 2010; Valluru & Van den Ende 2011; Davik *et al.* 2013). Moreover, galactinol has been proposed as biomarker for cold tolerance in strawberry (Davik *et al.* 2013). Thus, galactinol and raffinose are emerging as essential compounds in plant stress tolerance, and their metabolism and cellular function should be analysed in more details in the future.

Several different amino acids such as Asn, Asp, Thr, Tyr, Gly, Pro and Glu also belong to group A. The increase in the level of free amino acids in heated fruits could be a consequence of protein proteolysis, which has been reported in heat-treated cells (Ferguson *et al.* 1994). Proteomic and transcriptomic studies of HT peach revealed the increase of both proteins and transcripts involved in protein metabolism, like the ubiquitin-26S proteasome system (Lara *et al.* 2009; Lauxmann *et al.* 2012). Regarding Tyr, this amino acid, together with the other aromatic amino acids, is a precursor for the synthesis of defence compounds, such as flavonoids, acting as antimicrobial agents in plant defence, protectants against reactive oxygen species and insect and herbivore-feeding repellants, among others (Celenza 2001; Yu & Jez

2008). On the other hand, Pro accumulation can serve as an adaptive mechanism to chilling stress in higher plants by osmotic regulation and/or prevention of proteins from degradation (Hare, Cress & Staden 1999). In grapefruit and tomato, increases in Pro have been associated with improved resistance to chilling during cold storage (Purvis 1981; Zhao *et al.* 2009). In cold-stored peach, Shang *et al.* (2011) used GABA treatment to alleviate CI and demonstrated that the effect was due to the induction of endogenous GABA and Pro. In our work, the increase of Pro was recorded even following cold removal (Figs 3 & 4), although the protective role of Pro could be of importance at this time, when CI symptoms appear in the fruit.

Another metabolite of group A is the hexadecenoic acid, which may be involved in the stabilization of membranes through modification of the lipid composition (Hazel 1995; Wongsheree, Ketsa & Van Doorn 2009). In keeping with this observation, the induction of a transcript encoding  $\Omega$ -6 fatty acid desaturase was previously detected in heated peaches (Lauxmann *et al.* 2012), being this the possible link to the observed accumulation of such monounsaturated fatty acid. Moreover, enhanced chilling tolerance due to the increase in unsaturated fatty acids has been reported in 'Beijing 33' peaches (Zhang & Tian 2009).

A second group (B) is composed of those metabolites that increased following HT but not by cold such as saccharate and glucopheptose (in HT), maltitol and fructose (in HT and HT+3), and trehalose, maltose, Ser and Ala (in HT+3) (Figs 3 & 4). Particular attention should be paid to the metabolites of this group since when HT is applied prior to cold storage, the heated fruit enters the cold with an 'extra' set of compounds that are not induced by the cold, and thus could be involved in the HT protection against CI. Within this group, of particular interest is maltitol, which is induced by HT, decreased by cold, and increased in the sequentially application of heat and cold (HT+R2; Fig. 4). Maltitol is a sugar conjugate (4-O- $\alpha$ -glucopyranosyl-D-sorbitol), and its increase may be related to the sorbitol increase previously reported in heated peaches (Lara *et al.* 2009). Although to our knowledge the response of maltitol to stress has not been described in higher plants, its accumulation due to drought stress has been reported in the moss *Physcomitrella patens* (Erxleben *et al.* 2012). Considering its chemical structure, maltitol could act as antioxidant and osmoprotectant, and it may constitute another example of the evolutionary conserved trait of compatible solutes in widely diverse organisms.

A third group (C) is composed of compounds modified by cold but not by heat. It encompasses the increases of benzoate, putrescine and *myo*-inositol-1-P (in R3-R5), Phe and *trans*-caffeoylquinic acid (in R5+2), and decreases in maltitol (in R3-R5), fucose (in R3-R5 and R5+2), octadecanoic acid and sucrose (in R5+2). The metabolites in this group may be involved in the protection against cold, but also in the development of the CI symptoms. In this respect, changes in polyamine metabolism have been described not only in response to cold but also to various abiotic stresses (Bouchereau *et al.* 1999; Cook *et al.* 2004; Kaplan *et al.* 2004). Despite the fact that its exact biological function is still

unknown, it appears that putrescine accumulation under cold stress is essential for proper cold acclimation and survival at freezing temperatures (Cuevas *et al.* 2008). Therefore, the greater levels of putrescine (the variable that contributes the most to PC3; Supporting Information Table S4) in cold-stored fruits with respect to ripening fruits could be beneficial under cold-storage conditions. On the other hand, the cold induction of benzoate, which is a precursor of several important benzenoid compounds, including the defence signalling SA and its derivatives, and floral scent constituents, such as phenylethylbenzoate and benzylbenzoate methylbenzoate (D'Auria, Chen & Pichersky 2002; Boatright *et al.* 2004; Lu 2009), may be an extra positive feature of the cold treatment in addition to the delay in ripening.

Finally, when analysing the metabolome of fruits subjected to both heat and cold treatments (HT+R2), a resulting complex behaviour after heat and/or cold treatment can be recognized. In the case of maltitol, which increases in HT and HT+R2 and decreases in R3-R5, it seems that the heat response prevails over the cold treatment. On the other hand, there are some metabolites that are modulated by cold but not by heat, and the modification in their levels is also observed in HT+R2. Examples are benzoate and fucose, which are increased and decreased, respectively, in both R3-R5 and HT+R2 (Figs 3 & 4). Additionally, a group of metabolites can be recognized in which the opposite response to cold and heat occurs, that is, putrescine increased in R3-R5, decreased in HT+3 and was unmodified in HT+R2. However, a major group of metabolites modified in HT+R2 belongs to group A (conserved common response to both heat and cold), such as galactinol, raffinose, 1-O-methylglucoside, Glc, Thr, Asp, Asn, Tyr, glycerate and urea, which may be involved in the heat-induced CI protection, as discussed earlier. Finally, it is interesting to note that there are metabolites with unpredictable responses such as malate and 4-OH-Pro, which are only modified when both treatments are sequentially applied to fruits; or the case of citrate that increases in both heat and cold but does not vary in HT+R2. These results indicate complex responses to heat and cold of the enzymes involved in the metabolism of these compounds. Although each case deserves specific follow-up studies to disentangle the complex responses to post-harvest treatment, the results indicate that the response to heat followed by cold treatment can not be predicted considering the response to the application of heat and cold separately.

### **There may be different routes (enzyme modifications) to the same end (metabolite change)**

Heat and cold induce the accumulation of several common metabolites (group A; Fig. 4); however, the results obtained in the present work indicate that in some cases the effect may be driven by different genes (Figs 7 & 8). In this respect, in a previous study more than 90% of analysed genes responding to heat were also modified by cold, from which nearly 60% followed the same and nearly 40% opposite response to heat and cold (Lauxmann *et al.* 2012).

In fruits exposed to cold (R3 and R5) and in HT+R2, the relative amounts of *PpGolS* paralleled those of galactinol (Figs 3, 7 & 8a). In contrast, in heated peaches (HT), galactinol was significantly accumulated (Fig. 3) but *PpGolS* was not modified (Fig. 7). *GolS* gene expression has been closely associated with *GolS* activity and galactinol accumulation (Sprenger & Keller 2000; Taji *et al.* 2002; Nishizawa *et al.* 2008). However, under HT, the increase in galactinol seems to be mediated by other mechanism/s. One possibility is the presence of other *PpGolS*s in peach fruit, in addition to the *PpGolS* analysed in the present work which responds to cold (Fig. 7), which may mediate the increase in galactinol due to the HT. In this context, it is worth mentioning that we identified another transcript encoding *GolS* in the peach databases, but we were not able to amplify this transcript in peach fruit (data not shown). However, the mentioned hypothesis is in relation to the presence of several *GolS* genes in *Arabidopsis* (*AtGolS1-7*), from which only one transcript (*AtGolS3*) but not *AtGolS1* and *-2* increased in response to cold (Taji *et al.* 2002), while *AtGolS1* and *-2* are regulated by a heat shock transcription factor (HSF) (Panikulangara *et al.* 2004; Busch, Wunderlich & Schöffl 2005; Schramm *et al.* 2006). Moreover, in fruits like grapevine berries, *VvGolS1* is transactivated by HSFA2 (Pillet *et al.* 2012). On the other hand, it may also be possible that the regulation of *PpGolS* activity may be exerted at a level other than transcription following HT. Finally, considering the results obtained in the present work, the increase in galactinol under HT may be linked to a decrease in its conversion to raffinose due to the decrease in *PpRS* detected only in HT+3 peach fruits, as discussed below (Figs 7 & 8a).

The increase of raffinose in HT, HT+3, HT+R2, R5 and R5+2 fruits together with the expression of *PpRS* and *PpSIP1* and *-2* (Fig. 8a) indicates that RS may not be a main point controlling raffinose synthesis, at least at the transcriptional level. Instead, the flux in this pathway seems to be controlled by the level of galactinol via *GolS* and the further hydrolysis of raffinose by *PpSIPs*. Concerning this hydrolysis, *PpSIP2* responds to cold greatly increasing in R3 and R5, while *PpSIP1* responds to both cold and heat in positive and negative manners (Fig. 8a). Overall, our results suggest that the increase in galactinol and raffinose levels may be mediated by different mechanisms following exposure to cold and heat but that both mechanisms operate at the transcriptional level. Following cold treatment, an increase in *PpGolS* may provide more galactinol for raffinose synthesis, which would lead to an increase in these compounds even when *PpSIP1* and *-2* involved in the hydrolysis of raffinose are induced (Fig. 8a). By contrast, under HT, the increase in galactinol and raffinose may be mediated by a decrease in raffinose hydrolysis mediated by a decrease in *PpSIP1* and a decrease in *PpRS* in HT+3 (Fig. 8a). Moreover, this differential regulation supports the use of a HT before cold storage, and may be linked to the greater relative levels of raffinose detected in HT+R2 than in R3 or R5 peaches (Fig. 3; Supporting Information Table S3).

In addition, a differential regulation due to heat and cold has also been identified for nitrogenous-containing compounds such as putrescine, Pro and urea. While putrescine

accumulation under stress has been traditionally correlated with changes in ADC activity in several plants (Chattopadhyay *et al.* 1997; Shen, Nada & Tachibana 2000), in peach, the elevated content encountered in cold-stored fruits can not be associated to its biosynthesis through ADC (Figs 3, 7 & 8b). In contrast, considering that *PpASE* is induced under refrigeration (Fig. 7), it is probably that in peach the ornithine decarboxylase (ODC) pathway is also active, as it is the case of wheat (Ye *et al.* 1997). By contrast, the decreased levels of putrescine in HT+3 agree with the relative lower levels of *PpADC* transcripts (Figs 3, 7 & 8b).

Pro derives from Glu or Orn via the intermediate L-glutamate semi-aldehyde (Fig. 8b). The increases in *PpASE*, *PpOAT*, *PpP5CS1* and -2, and Glu in R3-R5 and/or R5+2 likely account for the greater levels of Pro in R5+2 fruits. By contrast, the increase in Pro after removal from the heat (HT+3) seems to be regulated only through the increased flux through the Glu pathway (Fig. 8b).

The increase in urea in cold-exposed peaches and after transferring to 20 °C (R5 and R5+2; Fig. 3), which significantly contributes to separation of the samples in PC2 (Supporting Information Table S4), is in close accordance with the induction of *PpASE* (Figs 3, 7 & 8b). Under 0 °C storage, *PpUre* was also induced in R3 and R5 (Fig. 7), regulating the accumulation of urea that was elevated at R5 and R5+2 (Fig. 3). By contrast, neither of these transcripts were modified under HT (Figs 7 & 8a). Thus, it again appears that the increase of a given metabolite is mediated by differential regulation of transcripts involved in its metabolism under cold and heat (Fig. 8a).

Finally, it is interesting to note that, apart from the transcriptional or post-transcriptional regulation of the several enzymes analysed, the activity of such enzymes may be directly affected by the temperature treatment *per se*. Such temperature regulation of enzyme activity would probably affect the fluxes of the metabolic routes.

### Perspectives of metabolomic studies with the aim of fruit quality improvement

The primary metabolic profile of peach fruits during different post-harvest temperature treatments reveals the metabolic basis of the benefits of applying a HT prior to refrigeration. The combination of high and low temperatures during the post-harvest period results in an overlap of changes in metabolites responding to heat and cold which may participate not only in the CI alleviation but also in the protection of stored fruits from biotic stress. The study also reveals how metabolic pathways respond similarly to heat and cold, although the control of their fluxes may be exerted at different levels. Finally, the use of metabolomic tools leads to knowledge that will accelerate the design and the improvement of plant-breeding projects, with the aim of obtaining fruits with higher content of a catalogue of metabolites, such as amino acids, sugars (raffinose), polyols (galactinol) and polyamines (putrescine), which could render fruits more resistant to a wide variety of stress situations and with better quality properties for consumers.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Figure S1.** Phylogenetic tree based on amino acid sequences showing relationships between alkaline  $\alpha$ -gals/seed imbibition proteins (SIPs) and raffinose family of oligosaccharide (RFO) synthases.

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

**Table S2.** Overview of the metabolite reporting data.

**Table S3.** Relative levels of 47 metabolites analysed by GC-MS during post-harvest at different temperatures.

**Table S4.** Variable contribution to principal components (%) presented in the PCA graphs shown in Fig. 5.