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Effects of postharvest treatments on gene expression in *Prunus persica* fruit: Normal and altered ripening

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ABSTRACT

Peach (*Prunus persica*) fruit have a short shelf-life, and the most common method employed to delay ripening and increase their postharvest life is cold storage. However, after extended storage at low temperature some cultivars have alterated ripening processes, resulting in a lack of juice and a woolly texture. To improve our understanding of the molecular mechanisms involved in the responses of peach fruit to cold storage we determined gene expression changes of fruit (cv. O'Henry) under different postharvest conditions: ripening (5 days at 21 °C), cold storage (21 days at 4 °C) and induction of woolliness (21 days at 4 °C followed by 5 days at 21 °C).

Cluster analyses of genes differentially expressed between treatments revealed unique patterns associated with biological processes that operate during postharvest treatments. Genes up-regulated during postharvest ripening and woolliness include components of ethylene, and aroma biosynthesis as well as oxidative stress response. During cold storage treatment and woolliness, several genes linked to the oxidative stress response increased in abundance, suggesting changes in redox status. Quantitative RT-PCR analysis showed a sequential increase levels of mRNAs encoding key components of cellular stress response. Moreover, after 21 days of cold storage, expression of genes encoding oxidoreductase, catalase, superoxide dismutase and gluthatione reductase was still significantly higher than before cold treatment, suggesting that fruit cells were able to respond to the increased production of ROS that was induced by extended cold storage. In the woolly fruit, up-regulation of stress response genes was accompanied by down-regulation of key components of metabolic pathways that are active during peach ripening. The altered expression pattern of these genes might account for the abnormal ripening of woolly fruit.

1. Introduction

The ripening of *Prunus persica* fruit involves a series of physiological and biochemical changes that induce a dramatic alteration in colour (chlorophyll degradation and carotenoid biosynthesis), texture (cell wall dismantling) and flavour (sugar formation and volatile compound biosynthesis) (Giovannoni, 2001). Once ripeness has been reached, the texture of the mesocarp continues to change and soften, and thus the firmness of the fruit is rapidly lost (Brummell, 2006). Peaches have a short shelf-life, and the most common method employed to delay ripening and increase postharvest life is cold storage, using temperatures ranging from 0 to 8 °C. However, this treatment negatively affects fruit quality due to the development of physiological disorders such as chilling injury (CI) or internal breakdown, which include woolliness or lack of juice, as well as browning, (Lill et al., 1989; Lurie and Crisosto, 2005). Woolliness has been attributed to an imbalance between the activities of cell wall degrading enzymes, which leads to an accumulation of de-methyl esterified pectins that are not subsequently depolymerized. Furthermore, it has been proposed that high molecular weight pectins form a gel structure along with cell wall calcium, binding free water and contributing to the mealy phenotype (Buescher and Furmanski, 1978; Von Mollendorff and de Villiers, 1988; Dawson et al., 1992; Lurie et al., 1994; Zhou et al., 2000).

Because of the high economic value of numerous cultivars of *P. persica*, in the last decade this species has emerged as a model to identify and characterize molecular components associated with fruit ripening (Trainotti et al., 2003, 2006; Falara et al., 2011; Nilo et al., 2012), and preservation of fruit quality during postharvest storage (Crisosto et al., 1999; Campos-Vargas et al., 2005). Recently, Vizoso et al. (2009) described the differential expression of genes associated with plastids, mitochondria, endoplasmic membrane and ribosomes when comparing woolly and juicy fruit.

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Gonzalez-Aguero et al. (2008) identified a set of genes differentially expressed in juicy and woolly peaches, including genes putatively involved in intracellular trafficking and cell wall metabolism that were repressed in woolly fruit. In addition, Ogundiwin et al. (2008) also found genes differentially expressed between cold and non-cold treated samples that might be related to the control of chilling injury. In cold-treated samples, stress-induced genes, ripening related genes, and genes involved in amino acid transport were up-regulated, while genes encoding heat shock proteins (HSPs) were down-regulated (Pegoraro et al., 2010).

Given the current knowledge of the transcriptional response associated with woolliness in peaches, in the present work we attempted to widen our understanding of the different processes that could be linked to this physiological phenomenon, through the identification of transcripts that are differentially accumulated not only in juicy and woolly peaches, but also in the different conditions of storage and ripening of the fruit. To this end, a collection of 1463 peach unigenes obtained from peaches stored at four different postharvest conditions (Vizoso et al., 2009) were spotted onto nylon membranes to generate a cDNA macroarray. The strategy of the hybridization assays aimed to identify gene expression changes among fruit ripened for 5 days at 21 °C, fruit stored for 21 days at 4 °C and fruit stored for 21 days at 4 °C and then ripened for 5 days at 21 °C. Moreover, for a set of selected genes, low temperature-regulated gene expression was studied by storing fruit at 4 °C for different periods of time. The results that we present here support the hypothesis that woolliness in *P. persica* involves gene expression changes that are consistent with abnormal fruit ripening. Transcriptional changes that were induced after cold storage and woolliness can be useful in defining the cellular processes that affect peach quality during postharvest storage.

2. Materials and methods

2.1. Plant material and postharvest conditions

Fruit were harvested from 8-year-old peach trees (*P. persica* L. Batsch cv. O'Henry) grown on Nemaguard rootstocks in a commercial orchard located in the Aconcagua Valley, Chile. Change in fruit ground colour was a harvesting index. In general, large to medium size fruit were selected and transported to a packing facility where peaches were handled following standard packing procedures used for long distant markets, including cooling at 15–20 °C for 2 h, washing, sizing and packing as described in Campos-Vargas et al. (2005). Additional details on fruit quality and physiological parameters are described in Table 1. Peaches were exposed to four different treatments that simulate postharvest conditions: (1) packing; (2) packing plus 5 days at 21 °C (ripening); (3) packing plus 21 days at 4 °C (cold storage); and (4) packing plus 21 days at 4 °C followed by 5 days at 21 °C (woolliness). Ripening and woolliness treatments were applied to fruit in order to obtain juicy and woolly fruit samples, whereas samples obtained after cold storage treatments allowed us to evaluate the effects of low temperature on gene expression. At the end of the different treatments, 10 fruit from each condition were halved, and one quarter was immediately frozen in liquid nitrogen and stored at −80 °C. In the case of fruit exposed to ripening and woolliness treatments, one quarter was used for the quantitative measurement of juice content as described by (Campos-Vargas et al., 2005). Fruit were classified as woolly when they contained less than 10% (w/w) of juice, and as juicy when this value was above 10% (w/w). Three fruit exposed to ripening and woolliness treatments that showed the highest and the lowest percentage of juice, respectively, along with three fruit from packing and cold storage treatments, were selected for RNA extraction, macroarray hybridizations, and quantitative real time RT-PCR (qPCR) assays.

In order to determine the relative transcript levels of the selected genes during cold storage, 'O'Henry' peach fruit were selected at the packing facility and stored for 0, 0.5, 1.0, 1.5, 2.0, 4.0, 7.0, 14.0 and 21 days at 4 °C. Afterwards, three fruit from each time point were selected for RNA extraction and qPCR assays.

2.2. Preparation of DNA macroarrays

EST clones were selected from two peach fruit cDNA libraries: a ripe peach fruit (cv. Loring) and a cDNA library constructed from mesocarp RNAs of peach (cv. O'Henry) that were exposed to four postharvest treatments as described in Vizoso et al. (2009). Clones were picked from −80 °C stocks and grown overnight in 96-well plates in 200 μL of LB plus 50 μg mL^{-1} of ampicillin. Overnight cultures were used directly as templates in PCRs containing 1× *Taq* buffer, 0.25 mM each of dATP, dCTP, dGTP, and dTTP, 2.5 U of *Taq* DNA polymerase, and 0.2 μM of T3 and T7 forward/reverse primers. Aliquots (5 μL) of each reaction were loaded onto a 1% (w/v) agarose gel to determine product quality. A 20 μL aliquot of the PCR products was arrayed in 96-well plates and mixed with an equal volume of 50% dimethylsulphoxide (DMSO). PCR products (70 ng) were single spotted onto Immobilon NY+ nylon membranes (10 cm × 7 cm) (Millipore, Billerica, MA, USA) using an 8-pin print-head (Arraylt model SSP015) and the arraying robot Versarray Chip Writer Compact (Bio-Rad, Hercules, CA, USA). The membranes were treated as described in (Gonzalez-Aguero et al., 2005), and the cDNA was fixed to the membrane by UV cross-linking using an Ultraviolet Crosslinker CL-1000 (UVP). In addition to the selected ESTs, the following controls were spotted onto membranes: (i) a fragment of the vector pBluescript II obtained by amplification with T3 and T7 universal primers; (ii) several aliquots of 50% DMSO; (iii) *Pp-Expansin* (GenBank: AB029083 [GenBank]), a gene that shows a decreased expression in woolly peaches compared with juicy peaches (Obenland et al., 2003); and (iv) cDNA from a *Bacillus subtilis* gene *dap* (obtained from the ATCC; number 87486), which hybridizes to an in vitro synthesized poly(A) RNA that was added to the fruit mRNA (dilution 1/200) prior to the labelling process and that was used as a spike mRNA to normalize expression data between membranes (Kane et al., 2000).

2.3. RNA extraction and mRNA isolation

Total RNA was isolated from peach fruit as described by (Meisel et al., 2005). The amount and quality of the RNA were assessed by measuring the $A_{260/280}$ and $A_{260/240}$ ratios, and by electrophoresis in a 1.2% formaldehyde-agarose gel. The common yield was 20–40 μg of total RNA per g of tissue. The mRNAs were purified using an Oligotex Mini kit (Qiagen, German Town, MD, USA), following the recommendations of the manufacturer.

2.4. Probe preparation and macroarray hybridizations

The ^{32}P-labelled probes from fruit samples exposed to the four postharvest treatments were prepared from poly(A)$^+$ RNAs by incorporation of [α-^{32}P]dCTP during first-strand cDNA synthesis. Unincorporated radioactive nucleotides were removed using the QIAquick Nucleotide Removal kit (Qiagen). The labelled cDNA products were denatured and immediately used for membrane hybridization according to the protocol described by (Gonzalez-Aguero et al., 2008). Membranes were sealed in plastic bags and placed in an Imaging Screen-K (Bio-Rad) for 72–96 h.

Table 1
Physiological parameters of peach fruit at different postharvest conditions.

Postharvest condition	Firmness (N)	TSS[a]	Respiration rate (mL CO_2 kg^{-1} h^{-1})	Ethylene production (μL C_2H_4 kg^{-1} h^{-1})	Juice content (%)
Packing	72.5	10.7	28.8	1.6	–[b]
Ripening	7.9	10.6	110.8	4.7	31.5
Cold storage	43.2	11.1	94.3	1.0	–[b]
Woolliness	6.8	10.4	98.2	18.1	0.0

[a] Total soluble solids.
[b] Content of juice could not be quantified in these samples because they are in a non ripening state. Data was obtained from Campos-Vargas et al. (2005).

2.5. Macroarray experimental design and data analysis

Macroarray experiments were performed in two independent labelling/hybridization events of cDNA probes from each condition and biological replicates. Radioactive images of hybridized membranes were obtained using a Personal Molecular Image FX scanner (Bio-Rad) at a 50 μm/pixel resolution. Intensity values were measured using VersArray Analyzer v.4.5.1.46 (Bio-Rad).

To eliminate inconsistent behaviour between replicate membranes, we calculated the Pearson correlation coefficient in order to compare global intensities among replicates, and selected those with a correlation value over 0.5. In order to discard low quality spots, we calculated the mean intensity value of solvent spots ($N = 66$) and used it as the background intensity value. Then, we calculated the net intensity of each spot by subtracting the background value. Spots that showed a net intensity < (background + (2 × background standard deviation)) were considered as low quality spots and were removed. After filtering the data, net intensity values were normalized against the mean intensity values for the *dap* spike.

The Significance Analysis of Microarrays (SAM) method (Tusher et al., 2001) was applied to detect differentially expressed genes between each of the postharvest treatments and the packing treatment. In all cases, we used a false discovery rate (FDR) of 0.1 as the threshold for differential expression. After SAM analysis, the normalized intensity values of genes that significantly changed their expression levels in at least one of the postharvest conditions were calculated as a ratio by using the expression values of the packing sample as a reference, and then subjected them to a hierarchical cluster analysis, using the MeV v4.0 software, with Pearson distance and average linkage. All data is MIAME compliant and the genes described have been deposited in NCBI's Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO number GPL13211.

Differentially expressed genes between each of the postharvest treatments and the packing treatment were analysed to determine whether any GO categories were significantly enriched compared to the entire list of probes on the macroarray. In doing so, we assigned *Arabidopsis thaliana* EntrezGene IDs to the entire set of *P. persica* spotted sequences in order to use the GO based on the *A. thaliana* annotation. Then, we selected the best tBlastX hits between peach cDNAs and the *A. thaliana* transcriptome. Thus, enrichment for specific gene ontology (GO) categories was performed on all those cDNA features that had associated GO identifiers. Enrichment of categories from the GO Cellular Component and Biological Process ontologies was calculated using the R software environment and the FunNet package with the Functional Analysis option (Prifti et al., 2008). A *p*-value of 0.05 was adjusted for multiple testing errors by applying the Storey FDR correction approach (Storey and Tibshirani, 2003).

2.6. Quantitative real-time RT-PCR (QPCR)

Total RNA (2 μg) was used as a template for reverse transcription reactions to synthesize single stranded cDNA using MMLV-RT reverse transcriptase (Promega) and an oligo(dT) primer (Promega), according to standard procedures. RNA was spiked (1:2000) with an in vitro synthesized *Bacillus subtilis dap* mRNA (ATCC 87486) to monitor the efficiency of reverse transcription and to normalize between replicates. The specific forward and reverse oligonucleotide primers for target genes were designed using Primer Premier 5.0 software (Premier Biosoft International), based on GeneBank database sequences, the sequence of each primer is shown in Table 2. Real-time RT-PCR reactions were performed in a Light-Cycler system (Roche) using SYBR Green to monitor cDNA amplification. Equal amounts of cDNA, corresponding to a 1/10 dilution of the cDNA, were used in each reaction, which contained 5 μL of Platinum SYBR Green I SuperMix-UDG (Invitrogen) 0.5 μL of BSA 20× (Invitrogen) and 5.0 pmol of forward and reverse primers in a total volume of 10 μL. The following standard thermal profile was used: 2 min at 50 °C, 2 min at 95 °C, 40 repeats of 5 s at 95 °C and 15 s at primer annealing 59–64 °C, and a final stage of 15 s at 72 °C. Data were analysed using Light-Cycler Software (v3, Roche). Efficiency was determined for each sample and gene by LinReg PCR v7.5, using data obtained from the exponential phase of each individual amplification plot as described in (Ramakers et al., 2003). Only the reactions in which transcripts were reliably detectable (Cp values below 30) and the reaction efficiency was above 1.5 were used for data evaluation. Three technical replicates were done for each combination of cDNA and primer pair, and the quality of the PCR reactions was checked through analysis of the dissociation and amplification curves. Products were resolved using a 2% agarose gel electrophoresis to confirm the expected size of the DNA fragments. Results were analysed via the Pfaffl equation (Pfaffl, 2001) to calculate the relative expression of each target gene after fruit treatments, normalized to the spike RNA. Real-time PCRs were performed in samples from at least three independent biological experiments and data were subjected to statistical analyses. We applied a one way ANOVA and a posteriori LSD Fisher test and *p*-values < 0.05 were considered as statistically significant.

3. Results and discussion

3.1. Effects of postharvest treatments on gene expression

Peaches (*P. persica* cv. O'Henry) were exposed to four different treatments that simulate postharvest conditions: packing, ripening, cold storage and woolliness. Thirty-six labelled probes corresponding to the four postharvest conditions *x* three experimental replications *x* three biological replicates were hybridized under the same conditions onto nylon macroarrays containing 1463 ESTs from two peach fruit cDNA libraries. Gene expression values were measured and filtered as described in Section 2. The results showed that 92.7% of the clones ($N = 1356$) fulfilled all quality filter criteria and were selected for further analysis. To identify transcripts that exhibited significant changes in their abundance at each postharvest treatment when compared to packing, we performed a SAM analysis and selected transcripts with an FDR < 10%. This led to the identification of 243 ESTs (225 up-regulated and 18 down-regulated) whose expression changed in

Table 2
Primers used for quantitative real-time PCR assays.

Target	Primer sense (5′–3′)	Primer antisense (5′–3′)
Endopolygalacturonase	GTCATCTGGTGTCACAATC	ACCCTCAGTTGTTCCATC
1-Aminocyclopropane-1-carboxylate oxidase	TGAGAACTGGGGCTTCTTTG	CTTGAACCTCTGCTCCAAGC
Expansin 3	AAAGGCGAAGATACCGACAC	GGCCTGCCTATGTTCTTCAC
S-adenosylmethionine synthetase 1	ATGAGGGTCACCCAGACAAG	TCTTGGTGCAGGTTTCACAG
Oxidoreductase	GGACGGATGGAGTCTCGGATG	TGACCCTGAAGCCCAGCAAA
Small heat shock	AACGACAAGTGGCACAGGAT	CACAGTCACGGTGAGAACCC
Gluththione reductase	GCCAGATGCACCTGAGATTG	ATACGCCTCGTCACTGAACG
Vesicle-associated membrane protein	GCAAAGCAGGTGGTCTCAGG	TTAAGGCTATTGGCAGGGGCT
Glycosyltransferase	AATTTACGGCATCTATGTTTGC	GACAGCTGGGTAAAGAACAGC
Secretory carrier membrane protein	TTTCTCTTCCATGGCTGGTC	GTTTTTGGCAGGAGCAACAC
Catalase	CTCGTGGTTTTGCAGTGAAG	TCTCTTGGATGTGCGACTTG
Superoxide dismutase [Mn]	GAGCGCCATCAAGTTCAATG	ACCACCTCCTTGACCAACAG
DAP spike	TTGCATTAGAGCACGGAGTC	GCGTATCTGAAGCGTTTGG

response to the ripening treatment, 60 ESTs (3 up-regulated and 57 down-regulated) whose expression changed after 21 days of cold exposure and 242 ESTs (156 up-regulated and 86 down-regulated) whose expression changed after the woolliness treatment (Fig. 1). The entire list of 481 postharvest-regulated ESTs is provided in Supplementary data.

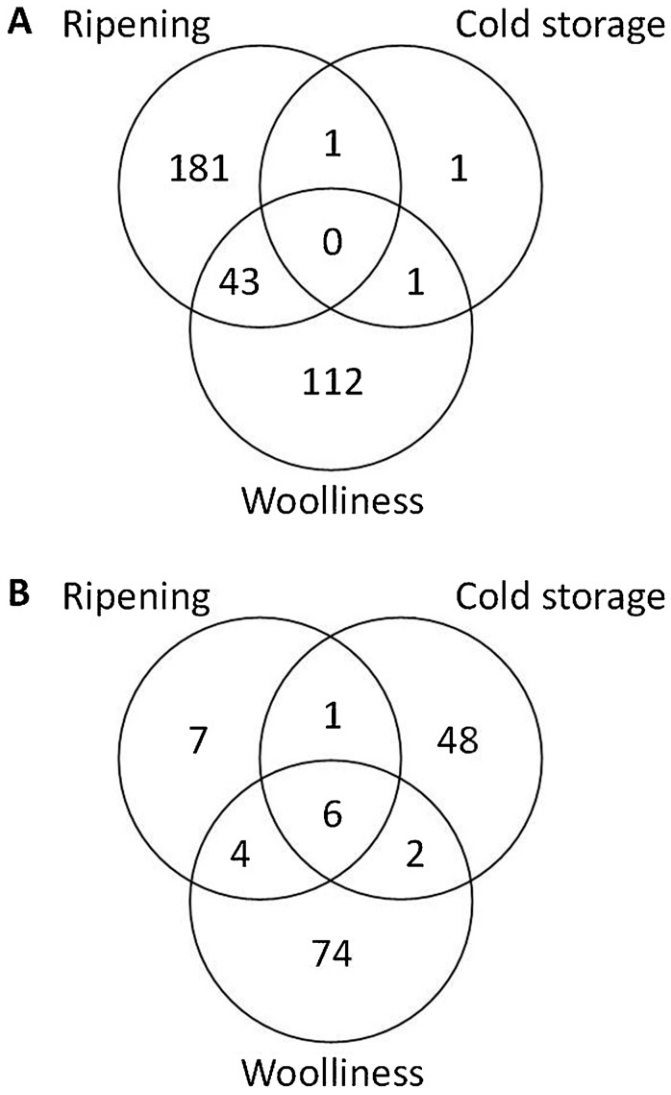

Fig. 1. Venn diagrams showing the number of genes differentially regulated by each treatment using packing as the reference. The number of probes up (A) and down (B) regulated resulting from macroarray analysis is indicated.

For functional categorization, we identified homologs in *A. thaliana* for 95% of the genes that responded to the postharvest treatments and used the bioinformatic tool FunNet (Prifti et al., 2008, 2010) to examine whether there was a significant enrichment for particular biological processes. Our results were consistent with previous work on gene expression changes during peach ripening and woolliness (Gonzalez-Aguero et al., 2008; Vizoso et al., 2009; Nilo et al., 2010) and validate the effectiveness of our fruit treatments. Thus, in the set of 243 ESTs differentially expressed between packing and ripening, three functional processes, ethylene biosynthesis, glucan metabolism and auxin-mediated signalling, showed significant enrichment ($p < 0.05$, Table 3). These results are consistent with the activation of genes involved in the synthesis of sugars and signalling molecules, two processes that are directly linked to the transformation of peaches into a mature, edible and fully flavoured fruit (Ziliotto et al., 2008; Prinsi et al., 2011).

From the set of 60 genes that changed expression between packing and cold storage, FunNet identified four significantly enriched functional themes ($p < 0.05$, Table 3): carbohydrate biosynthesis, transport, oxidation–reduction and virus response. These last two categories include transcripts of genes encoding manganese superoxide dismutase, catalase and glutathione reductase, three enzymes that are directly linked to the control of redox changes. Thus our results are consistent with data from previous reports showing that these proteins are activated by exposure to low

Table 3
Significantly enriched biological process among the genes differentially expressed between packing and the postharvest treatments.

GO identifier[a]	Term[b]	*p*-Value[c]
Ripening (Pkg vs. Pkg plus 5 days at 21 °C)		
GO:0009693	Ethylene biosynthetic process	4.81e−03
GO:0009734	Auxin-mediated signalling pathway	2.88e−02
GO:0044042	Glucan metabolic process	2.88e−02
GO identifier[a]	Term[b]	*p*-Value[c]
Cold storage (Pkg vs. Pkg plus 21 days at 4 °C)		
GO:0055114	Oxidation reduction	4.66e−02
GO:0006810	Transport	4.48e−02
GO:0009615	Response to virus	4.66e−02
GO:0016051	Carbohydrate biosynthetic process	3.14e−02
GO identifier[a]	Term[b]	*p*-Value[c]
Woolliness (Pkg vs. Pkg plus 21 days at 4 °C followed 5 days at 21 °C)		
GO:0009733	Response to auxin stimulus	2.91e−02
GO:0048193	Golgi vesicle transport	4.99e−02
GO:0006950	Response to stress	3.72e−02
GO:0050896	Response to stimulus	1.83e−02

[a] Enriched gene ontology (GO) identifiers among the genes differentially expressed between the different comparisons.
[b] Description of the enriched GO identifier.
[c] *p*-Values for GO-term enrichment.

temperatures (Sevillano et al., 2009), suggesting that the cold storage protocol induces changes in the redox status of the fruit cells.

Finally, stress response, Golgi vesicle transport, response to auxin and abiotic stimulus were significantly enriched in the set of 242 genes differentially expressed between woolliness and packing conditions ($p < 0.05$, Table 3). As previously observed in the fruit that went through cold storage, an important number of the enriched genes in this condition are involved in the response to stress agents. The involvement of stress response related genes suggests that low temperatures could alter the normal metabolism of the fruit in a non-reversible manner, causing cellular stress or damage that prevents the fruit from being able to ripe normally.

3.2. Cluster analysis and qPCR validation of macroarray data

To compare the gene expression patterns and determine whether ripening, cold storage or woolliness conditions might be separated into distinct groups based on their gene expression profiles, we performed a hierarchical clustering of the 481 ESTs that were differentially expressed in at least one postharvest treatment relative to packing condition (Fig. 2). This analysis allowed us to identify three distinct patterns of gene expression. The first group (blue bar) contains genes that increased their expression levels during ripening when compared to packing condition. Even though in woolly fruit these genes were also up-regulated, the magnitude of the changes was lower compared to the ripening fruit. Accordingly, the fold-change in their expression levels between ripening and packing differed by 2.1 or more than the fold-change between cold storage and packing and by 1.6 or more than the fold-change between woolliness and packing treatments. We analysed the gene composition of this cluster and found transcripts encoding proteins known to be involved in ethylene metabolism, including S-adenosyl methionine synthetase (SAM) and 1-aminocyclopropane-1-carboxylate oxidase (ACO-1), which have been shown to increase their expression levels during ripening (Vizoso et al., 2009). Genes involved in cell wall metabolism include transcripts encoding expansins (EXP) and endopolygalacturonase (EndoPG), which are first detected at the onset of the climacteric period, reaching their maximum at later stages of softening (Trainotti et al., 2003, 2006). Woolliness in peaches is believed to be caused by an altered activity of cell wall enzymes during cold storage, which affects the metabolism of cell wall polysaccharides (Lurie and Crisosto, 2005) and leads to an imbalance in pectin degradation. Consistently, EXP and EndoPG have been reported to be less abundant in woolly fruit when compared to juicy fruit, leading to further altered cell wall metabolism (Zhou et al., 2000; Obenland et al., 2003; Brummell et al., 2004).

The activation of genes involved in aroma production, was also detected within this cluster. We identified genes encoding lipoxygenase (LOX) and an alcohol acyl transferase, two enzymes that are involved in the generation of volatile esters during ripening of melon, apple and peach (Yahyaoui et al., 2002; Souleyre et al., 2005; Zhang et al., 2011), and enoyl-CoA hydratase, which has been implicated in the β-oxidation pathway. In apple, the expression of the enoyl-CoA hydratase gene was shown to peak simultaneously with the production of esters, known as secondary metabolites that play a major role in fruit quality (Sugimoto et al., 2008). Our results suggest that woolly fruit might have lower expression levels of genes involved in aroma production, and therefore, lower levels of the volatile compounds that would normally confer the characteristic aromatic properties of a ripe fruit.

In this group of genes (blue bar), the increased expression level of a gene encoding GDP-mannose pyrophosphorylase (GMPase), an enzyme that catalyzes one of the first steps of the Smirnoff–Wheeler pathway, suggests that de novo synthesis of ascorbic acid (AsA) was up-regulated during ripening. Conklin et al. (1997) demonstrated that reduced GMPase activity leads to ascorbate deficiency in *A. thaliana*, whereas expression of an Acerola GMPase in tobacco resulted in up to 100% increased levels of AsA (Badejo et al., 2007). In tomato the induction of GMPase activity was concomitant with the increase of AsA level in leaves, green fruit and red fruit (Cronje et al., 2012), suggesting that GMPase is an important control point in ascorbate biosynthesis. It has been reported that the expression level of tomato GMPase is up-regulated by temperature stresses. Moreover, overexpression of this enzyme elevated AsA levels and enhanced the tolerance to temperature stress in transgenic tobacco plants (Wang et al., 2011), this effect being consistent with AsA playing a central function in protecting plant cells from oxidative stress (Horemans et al., 2000). Given that fruit ripening is an oxidative process (Wang and Jiao, 2001), it is expected to detect an increment in AsA synthesis (Alhagdow et al., 2007; Cruz-Rus et al., 2011). Thus, based on the abnormal ripening observed in woolly fruit it will be of interest to determine whether there is a correlation between the level of AsA and the development of peach woolliness.

In order to confirm the predicted expression pattern of the genes in this cluster, we quantified by means of qPCR assays the abundance levels of the transcripts for four selected genes: endopolygalacturonase, expansin, aminocyclopropane-1-carboxylate oxidase and S-adenosylmethionine synthase. In all four cases, the measurements registered expression changes that were equivalent to those previously observed in our macroarrays experiments (Fig. 2).

The second group (green bar) includes genes whose expression levels were increased during cold storage and remained high after the woolliness treatment. Relative expression values of these genes differed by 1.5-fold or more than the fold change between ripening and packing treatments. As for the previous cluster, the levels of expression of three selected genes were used to confirm the predicted expression pattern of this cluster (Fig. 2). Although expression changes of ESTs encoding oxidoreductase and small heat shock proteins did not pass the significance threshold, changes in their relative transcript abundance closely parallel the pattern of expression obtained via the macroarray. In agreement with previous reports that linked oxidative stress and increased levels of ROS with chilling injury (Prasad et al., 1994; Apel and Hirt, 2004; Cao et al., 2009; Sevillano et al., 2009), a transcript encoding glutathione reductase (GR) was up-regulated in this cluster. GR plays a key role as a detoxification enzyme by maintaining the intracellular glutathione pool in the reduce state to function as an important antioxidant that prevents ROS damage by scavenging free radicals (Gill and Tuteja, 2010). Other genes in this cluster encode proteins with roles in stress tolerance, such as a small heat shock protein (sHSP), and a lipid transfer protein. sHSPs increase in abundance following exposure to low temperature, and unlike the HSPs produced in response to high temperature stress, which function as molecular chaperones, they have a cryoprotective effect, participating in membrane protection and facilitating protein refolding achieved by HSP70 and HSP100 (Sun et al., 2002; Mogk et al., 2003; Timperio et al., 2008). Heat treatment of peach can prevent chilling injury after cold storage (Infante et al., 2008). In heated peaches, the induction of sHSP synthesis participated in the acquisition of tolerance against chilling injury (Lara et al., 2009). Similarly, in tomato, heat treatment increased the synthesis of sHSPs (Sabehat et al., 1998), which appear to prevent chilling injury by modulating pectin depolymerization and juice viscosity during tomato ripening (Ramakrishna et al., 2003). Lipid transfer proteins also seem to have an important role in stabilizing membranes by enhancing the transfer of phospholipids between membranes and possibly by binding acyl chains (Zhang et al., 2010). Both biotic and abiotic stress are able to modify the expression level of lipid transfer proteins (Sapitnitskaya et al., 2006; George and Parida, 2010),

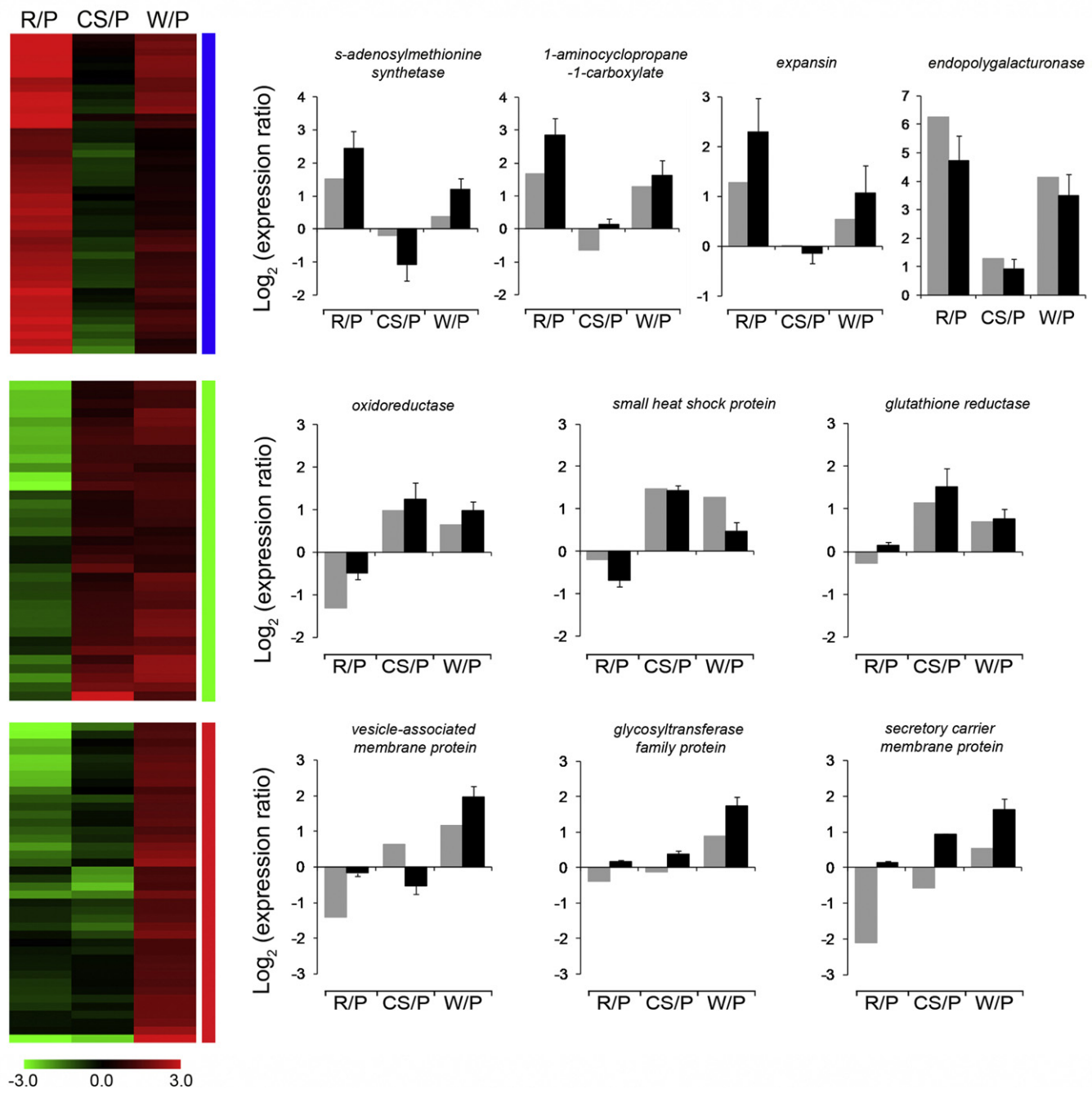

Fig. 2. Cluster analysis of macroarray data and confirmation of gene expression changes by qPCR. Hierarchical clustering was used to group the differentially expressed genes (false discovery rate < 10%) based on similar expression patterns over the three postharvest treatments examined (ripening, cold storage and wolliness) using as reference the packing treatment (indicated above the columns, R/P = ripening/packing; CS/P = cold storage/packing; W/P = wolliness/packing). Gene expression patterns are evident across the rows. Increased and decreased expression compared with the mean expression of the packing sample for each gene is shown in red and green, respectively. Green indicates $\log_2$ ratios < 0, and red indicates values >0. Three main gene groups were resolved and indicated by colour bars shown to the right of the figure. (B) Graphs indicate genes that displayed a similar expression pattern validated by RT-qPCR. *y* axis: $\log_2$ of expression ratio between each postharvest treatment and packing; *x* axis: postharvest treatments.

suggesting that they play a role in activating the defense mechanism of the cell. For instance, in tobacco, the overexpression of this gene enhances resistance to plant pathogens (Sarowar et al., 2009). Moreover, low-temperature-regulated lipid transfer proteins with cryoprotective roles have been identified in barley and cabbage (Molina et al., 1996; Hincha et al., 2001).

Finally, the third group (red bar) contains genes that increased their expression only after woolliness treatment, and the fold change in their transcript abundance differed 1.7-fold from the fold-change of ripening or cold storage treatments relative to packing. With the aim of confirming the observed gene expression pattern of this cluster, the relative abundance of the transcripts of three genes was examined by qPCR, revealing that the pattern of expression obtained via qPCR correlates with the macroarray data (Fig. 2). Several genes with potential functions in stress response were also identified in this cluster. Among them, two ESTs encoding the enzyme ascorbate peroxidase (APX) were up-regulated in woolly peach. APX catalyses the conversion of H_2O_2 to water with ascorbate serving as the electron donor (Asada, 1992). Thus, it plays a key role regulating H_2O_2 levels and H_2O_2 signalling in plant cells

(Asada, 1999; Apel and Hirt, 2004). In *A. thaliana* an APX1-deficient mutant accumulated more hydrogen peroxide and was significantly more sensitive to stress than wild type plants (Koussevitzky et al., 2008). Moreover, APX seems to have a key role protecting fruit against chilling stress (Wang et al., 2005; Galli et al., 2009). Up-regulation of ROS scavenging enzymes, GR and APX in woolly fruit suggests that they were able to respond to the excess of ROS generated during the ripening process and/or the extended cold storage at low temperatures. However, this response appears to be insufficient to prevent the abnormal ripening of the woolly fruit. A different group of genes in this cluster encode proteins with potential functions in stress adaptation. This is the case of DEAD-box RNA helicase, which has a crucial role in the stress response in *A. thaliana*. DEAD-box RNA helicase seems to function as a RNA chaperone, since it can use energy derived from ATP hydrolysis to actively disrupt misfolded RNA structures (Tanner and Linder, 2001; Lorsch, 2002). In *A. thaliana*, the mutants for this gene are more sensitive to chilling and freezing stress and fail to accumulate transcripts of cold binding factor (CBF) genes during cold exposure. Interestingly, two genes known to be regulated by the CBF pathway in other species (Lee et al., 2005) were found in this cluster. One of them encodes a β-1,3-glucanase, a pathogenesis-related protein that has been implicated as a biosensor for abiotic stressors such as wounding and cold (Hincha et al., 1997). The other gene encodes an Early Light-Induced Protein (ELIP), which increases its expression level in response to a variety of stress-related signals (Bhalerao et al., 2003; Provart et al., 2003). In ripening tomato fruit, the ELIP gene is induced during chloroplast-to-chromoplast transition (Bruno and Wetzel, 2004), although the precise role of the ELIP proteins in oxidative stress responses remains to be elucidated.

Within this group, we also identified genes encoding proteins of the endomembranous system, such as a secretory carrier membrane protein (SCAMP), a vesicle-associated membrane protein (VAMP) and a Golgi SNARE protein. It is known that the endomembrane system plays important roles in the biogenesis of the cell wall, plasma membrane and vacuole and the control of development and plant responses to biotic and abiotic stresses (Surpin and Raikhel, 2004). Changes in the expression of these genes might be explained by a role of the endomembrane system in cell wall remodelling since ripening of peach fruit involves a series of cell wall modifications, including changes in pectin metabolism, carried out by the secretion of several of the enzymes (Lurie and Crisosto, 2005), and possibly the deposition of newly synthesized cell proteins (Trainotti et al., 2003, 2006). On the other hand, changes in the expression of genes involved in endomembrane trafficking have been previously reported in woolly peach (Gonzalez-Aguero et al., 2008; Nilo et al., 2010), suggesting that alteration in the abundance of the endomembrane system components might have a role in the development of woolliness in peach.

Abnormal fruit ripening observed after cold storage might be seen as oxidative stress symptoms caused by a major failure of the tissue antioxidant protection systems. Therefore, it has been proposed that prolonged cold storage increases production of ROS above normal or mild levels. Under this condition the antioxidant defense system may only be moderately up-regulated causing an imbalance between oxidative and antioxidative reactions that result in permanent tissue damage (Arora et al., 2002; Wang et al., 2006; Galli et al., 2008, 2009). The analysis of our microarray data indicated that cold stored fruit were able to respond to the increased production of ROS by up-regulating key components of the ROS scavenging system, such as the enzymes CAT, SOD and GR (Apel and Hirt, 2004), in addition to genes encoding proteins with roles in stress tolerance, such as, sHSPs. Even though, AsA synthesis might be affected by a diminished expression of GMPase during cold storage the activities of other antioxidant enzymes such as SOD and CAT might have been enough to protect the tissue from ROS damage. Further analysis of the contents of ascorbate, glutathione and phenolics, as well as quantification of enzymatic activities should be carried out to determine whether up-regulation of the cellular antioxidant system leads to an adequate defense response that is able to prevent the effect of cold storage on the normal ripening process.

In addition to alter the efficiency of the antioxidant system, prolonged cold storage of peach might have affected the expression level of genes involved in fruit ripeness. In this regard, down-regulation of cell wall remodelling and ethylene synthesis enzymes have been previously reported in woolly peach fruit (Lurie and Crisosto, 2005). The analysis of our microarray data revealed that several genes encoding enzymes with potential roles in peach ripeness were significantly down-regulated in the woolly fruit (Supplementary data). Among them, we found key components of the organic acid metabolism, such as NADP dependent malic enzyme (NADP-ME) and phosphoenolpyruvate carboxykinase (PEPCK). High activity levels and increased transcript abundance have been determined by these two enzymes during postharvest ripening of 'Dixiland' peach fruit (Lombardo et al., 2011). Down-regulation of a gene encoding sucrose synthase (SS), an enzyme involved in sucrose degradation during ripening, suggests an alteration in sugar metabolism of the woolly fruit. Lombardo et al. (2011) reported that in 'Dixiland' peach fruit, constant levels of sucrose during ripening were accompanied by an increment in the abundance of transcripts encoding SS and sucrose phosphate synthase (SPS2), an enzyme involved in sucrose synthesis. Therefore, a cycle for sucrose synthesis and degradation processes was suggested, which might control other important physiological functions as has been shown in different plant tissues (Roby et al., 2002). Two genes encoding enzymes involved in the synthesis of carotenoids, β-carotene hydroxylase and hydroxymethylbutenyl diphosphate synthase (GCPE), were also down-regulated in the woolly fruit. β-Carotene hydroxylase has been reported to be up-regulated during peach fruit ripening as part of the pigment biosynthesis pathway responsible for the yellow flesh colour (Trainotti et al., 2006). An additional down-regulated gene encodes bZIP DNA binding protein, a protein with potential, but still undefined roles, in fruit ripening (Costa et al., 2010). Taken together, our gene expression data revealed that different components of metabolic pathways involved in normal peach fruit ripening displayed an altered expression pattern in the woolly fruit.

3.3. Gene expression analysis of stress related genes during low temperature exposure

To study the temporal course of low temperature regulation of gene expression, we exposed peach fruit to 4 °C for different times (from 0 to 21 days) and measured the relative expression of a set of five genes encoding components of the antioxidant defense system (Fig. 3). Three of the selected genes, small heat shock protein, oxidoreductase and glutathione reductase, increased their transcript abundance after cold storage and woolliness treatments as shown by our macroarray analysis (Fig. 2, second cluster, green bars). Fig. 3 shows that the relative abundance levels of small heat shock protein, oxidoreductase and catalase mRNAs were significantly increased ($p < 0.05$) after 2 days of treatment, compared to the unexposed control fruit. Both small heat shock protein and oxidoreductase mRNAs reached maximum levels (fold change 3.5- and 3.6, respectively) after 7 days and then declined by the 14th and 21st day of cold exposure, respectively, suggesting that these two mRNAs accumulated coordinately in response to low temperatures. Meanwhile, catalase mRNA reached its maximum level only after 21 days (fold change = 3.5) of cold exposure. Superoxide dismutase [Mn] and glutathione reductase mRNAs accumulated at later time points during cold exposure. Superoxide dismutase

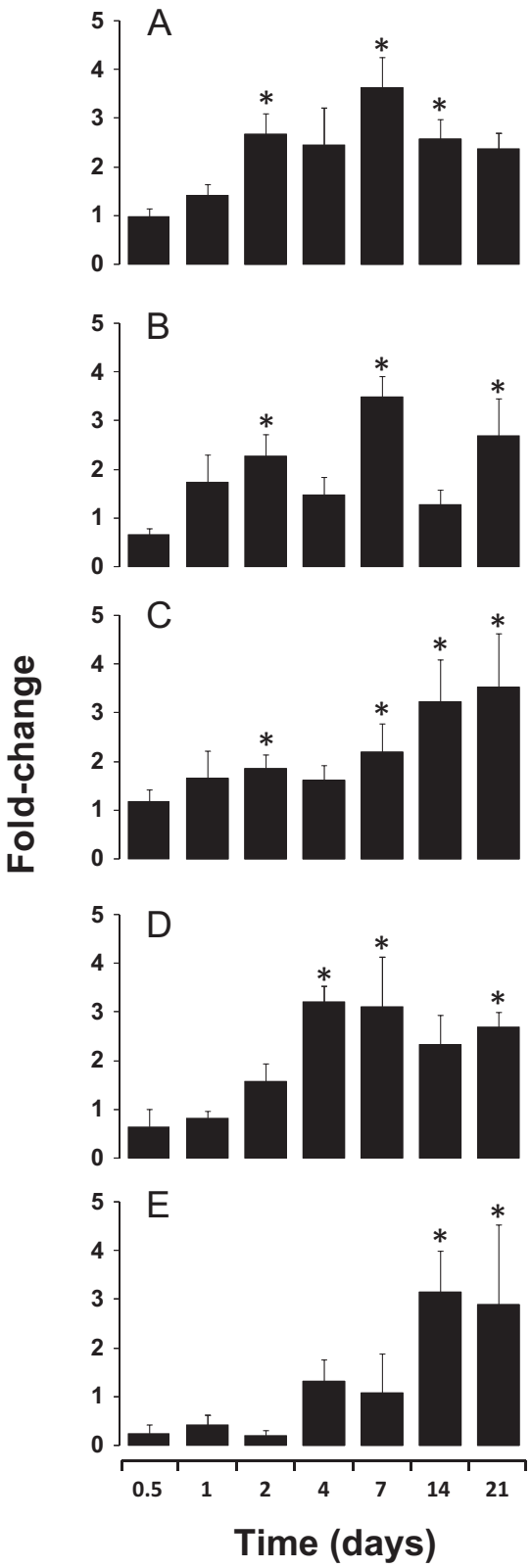

Fig. 3. Quantitative analysis of the expression of stress response genes in fruit exposed to low temperature. The expression levels of five selected genes involved in stress response, small heat shock protein (A), oxidoreductase (B), catalase (C), superoxide dismutase [Mn] (D) and glutathione reductase (E) were quantified in fruit exposed at 4 °C, from 0 to 21 days. For each gene, transcript abundance was normalized to that of dap spike mRNA. Data are presented as fold change relative to 0 h. *$P < 0.05$ (Mann–Whitney U statistic test).

[Mn] mRNA significantly increased its relative abundance level ($p < 0.05$) after 4 days, when it reached its maximum level (3.2-fold change), whereas the relative abundance of glutathione reductase mRNA increased significantly only by the 14th and 21st day of cold exposure, reaching its maximum after 14 days (3.2-fold change). These data indicate that the low temperature treatment of peaches resulted in a sequential increase of the amount of mRNAs encoding key components of the cellular oxidative stress response. Moreover, our data indicate that after 21 days of cold exposure, the expression of genes encoding oxidoreductase, catalase, superoxide dismutase and gluthatione reductase was still significantly higher than before cold treatment, suggesting that fruit cells were able to respond to the increased production of ROS that was induced by the extended cold storage.

4. Conclusions

Our analyses have yielded a list of *P. persica* genes and their expression profiles in different postharvest conditions. During ripening, we observed the up-regulation of transcripts related to ethylene biosynthesis and the auxin mediated signalling pathways. Cold storage modified the abundance of genes linked to oxidative stress response, suggesting the occurrence of changes in the redox status of the fruit cells. The development of woolliness was correlated with the up-regulation of genes involved in stress response, indicating that woolly fruit was able to respond to the oxidative stress of ripening. However, we cannot discard the fact that woolly fruit might still be affected by an excess of oxidative stress even after they were removed from low temperatures and ripened at 21 °C. Woolliness was also accompanied by the down-regulation of key enzymes involved in different metabolic pathways that underlie the process of peach ripeness. Transcriptional changes in these genes might account for the abnormal ripening observed in woolly fruit. The up-regulation of five genes involved in redox response at different times of exposure to cold, supports the idea that storage of peach fruit at low temperatures induces the generation of ROS in unripe peaches and the up-regulation of key components of the cellular oxidative stress response. Further studies are needed to understand chilling injury and the development of practical applications to prevent it, including studies on the mechanisms by which antioxidant defense enzymes respond to cold and prevent woolliness in certain fruit, and not in others, as well as analysis of the transcriptional regulation of enzymes involved in the metabolic pathways of peach ripening.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.postharvbio.2012.08.002.

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