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Volatile compound and gene expression profiles associated with the storage of two peach fruit genotypes differently sensitive to chilling injuries

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Abstract: The patterns of volatile organic compounds (VOCs) emission and the expression of genes associated with the lipoxygenase (LOX) pathway have been studied in harvested peach fruit of two cultivars ('Flaminia', FL, and 'Red Haven', RH) during and after cold storage. Two temperature storage conditions have been applied for two weeks: 0.5 and 5.5°C, the latter recognized to be an inducer factor of chilling injury (CI) of the flesh. Fruit were also monitored during 3 days of shelf-life (SL) at room temperature after removing from the cold storage. A different behaviour between cultivars has been observed in terms of internal browning (more evident in FL after 2 weeks) and extractable juice (already reduced in RH at the end of 1 week of storage at 5.5°C). Although some common responses have been observed (e.g. a general increase of 2-hexenal and 2-hexenal-E at the end of both cold storage conditions), LOX pathway-associated volatiles (aldehydes, alcohols, esters) showed different trends in relation to the genotype and the applied stress, with apparently no specific correlations with the incidence of CI. The expression level of five LOX pathway-associated genes (*PpLOX1, PpLOX4, PpHPL1, PpADH1, PpAAT1*) have been analysed and the results point out that a genotype-dependent behaviour is present, but specific responses (up-regulation of *PpLOX1* and *PpAAT1* during SL) appear to be present in both cultivars. In addition, the expression of two *C-repeat-binding factors* (*PpCBF1* and *PpCBF6*), recognized to be involved in the responses of plant tissues to low temperature stress, showed marked changes in relation to the applied temperature, suggesting that these genes might play a regulatory role in the overall metabolism of cold stored peaches.

1. Introduction

Peach fruit are highly perishable at ambient temperature and they are normally stored under refrigeration, making possible to maintain commercial quality up to 2-4 weeks, depending on the specific cultivars and

storage conditions (Ramina *et al*., 2008). However, low temperatures may lead to the development of chilling injury (CI), which negatively affect fruit quality shattering important organoleptic quality traits such as taste and flavor, as well as inducing the appearance of physiological disorders such as internal browning and mealiness/woolliness (Crisosto and Mitchell, 2002; Lurie and Crisosto, 2005). CI symptoms typically evolve during post-storage/shelf-life conditions and can also compromise the fruit capability to ripen after storage, resulting in quality levels not compatible with marketability. Different cvs possess variable levels of tolerance/susceptibility against CI (Brizzolara *et al*., 2018; Nilo-Poyanco *et al*., 2018). For peach fruit a specific range of temperatures, called "killing zone" (2.2-7.6°C), has been associated to CI symptoms which tend to appear more rapidly and to become more severe than at 0-1°C (Crisosto and Valero, 2008). Several works investigated the causes of CI onset arguing that a key aspect in peach fruit response to "killing zone" temperatures is the lower ethylene synthesis that, in turn, may only in part activate the ripening machinery (Fernández-Trujillo *et al*., 1998; Walsh *et al*., 2001; Zhou *et al*., 2001; Pons *et al*., 2015; Wang *et al*., 2017).

One of the most important ripening-related quality traits for fruit in general and, especially, for peaches is the profile of volatile organic compounds (VOCs). During peach fruit ripening a number of different VOCs (e.g. alcohols, terpenes, esters, lactones) are synthesized and play a role in making fruit attractive and for consumer acceptance (Aubert and Milhet, 2007; Yang *et al*., 2009). The lipoxygenase (LOX) pathway, by using fatty acids as precursors, is responsible for the production of specific VOCs that play important roles in fruit ripening, also acting as precursors for other metabolites. The LOX pathway has been also studied in relation to the physiological responses of peaches to cold storage and the changes in terms of overall organoleptic quality (Ortiz *et al*., 2009; Zhang *et al*., 2011). In addition to the description of VOC profiles and enzyme activity pattern, these works correlate specific LOX pathway-associated gene expression with the onset of CI. Indeed, Zhang *et al*. (2011) suggested that the decreased levels of fruity note volatiles in peaches with CI, were the consequence of modifications in expression of two lipoxygenase (*PpLOX1* and *PpLOX2*) and one alcohol acyltransferase (*PpAAT1*) genes.

The different metabolic profiles of peach fruit stored under CI-inducing or "safe" low temperatures have been also related to molecular mechanisms effective to control the expression of structural and regulatory genes. Considering the latter, the C-repeat (CRT) / dehydration-responsive element (DRE) binding factor 1 (CBF/DREB1) is recognized to be involved in plant responses to abiotic stresses (Thomashow, 2010). Plant CBF proteins are encoded by multigene families and specific members show distinct expression patterns in relation to low-temperature stress. The involvement of CBF in responses to cold storage has been reported in tomato fruit, in which an up-regulation of *LeCBF1* has been associated with a reduction of CI during storage (Zhao *et al*., 2011).

Differential expression of 6 isolated *CBF* genes has been observed in peaches stored under refrigeration (Liang *et al.*, 2013). These authors demonstrated that *PpCBF* 1/5/6 were up-regulated during cold storage at 0°C and this was accompanied by a decrease of CI symptoms.

In this work, we report the effects of 2 weeks of cold storage (0.5 and 5.5°C), followed by shelf-life at room temperature (20°C) on the profiles of LOX pathway-derived VOCs and the expression of LOX-pathway and *CBF* genes in peaches of two different genotypes ('Flaminia' and 'Red Haven', a typical Italian cultivar from Tuscany region and a worldwide commercially important cultivar, respectively).

2. Materials and Methods

Fruit material, sampling procedure and CI symptoms evaluation

Peach (*Prunus persica L. Batsch*) fruit belonging to 'Red Haven' (RH, yellow fleshed) and 'Flaminia' (FL, yellow-fleshed, +35 days from RH harvest) have been harvested at flesh firmness values of about 36 and 44 N for RH and FL, respectively, from a commercial orchard located at Casciana Terme (Pisa, Tuscany, Italy). The fruit selection, the refrigeration treatments and the sampling procedures are those described by Brizzolara *et al*. (2018). Briefly, after harvest fruit were selected for homogeneity in terms of fruit size and peel color and were analysed using NIR technology (NIR-Case, SACMI, Italy), which has been previously calibrated for firmness (manual penetrometer), total soluble solids (TSS) (optical refractometer) and peel color (arbitrary scale) for each cv. Peaches (thirty for each treatment) have been stored in cold chambers at two low temperature conditions (0.5 and 5.5±0.5°C) and, as non-refrigerated control, at 20±0.5°C. Relative humidity under cold storage was kept at 85% and monitored using a TGU-4500

(Tinytag Ultra 2, Gemini, United Kingdom) data logger. Peaches were sampled at harvest (T0) and after 1 and 2 weeks of cold storage. Nine fruit per treatment were additionally kept for 3 days under shelf life (SL, 20°C) conditions to investigate the post-storage phase. Firmness (N) was measured on nine fruit using a manual penetrometer (GY-3, Newtry) equipped with an 8 mm tip.

Chilling injury symptoms have been evaluated following the same protocol described by Brizzolara *et al*. (2018) using an arbitrary scale. Briefly, a total of nine peaches has been evaluated for each sampling time and treatment. For internal browning evaluation, peaches have been cut in two halves and, on each part, brown areas have been assessed with the open source software 'ImageJ', for a total of 18 measurements (2 for each sampled fruit) for every thesis and sampling time. Half fruit has been considered as representing 50% of the whole fruit, and browning incidence (%) has been calculated as the average of the nine measured fruit. Juiciness has been assessed by processing with a juice extractor (Moulinex Juice Extractor JU350B27) 50 g of pooled mesocarp tissue from three peaches (in triplicates, nine fruit in total), and it has been expressed as percentage of extracted juice normalized on control levels. At each sampling time, three fruit per replicate were processed and a random pool of mesocarp tissue was immediately frozen in liquid nitrogen and stored at -80°C. Powdered tissue has been used for molecular analyses, while for VOC analysis, mesocarp samples of the same three fruit were crushed in a 1M NaCl solution by means of a T25 Ultra-Turrax^{R} (IKA, Germany) and the obtained puree was immediately frozen in liquid nitrogen and stored at -80°C.

Volatile organic compounds (VOCs) analysis

For aroma volatile compound analysis, the same method previously adopted by Brizzolara *et al*. (2017, 2018) was used. Analyses have been performed using Agilent Technologies (6890N, United States) equipment: a gas chromatograph coupled with mass spectrometer (5973 Network Mass Selective Detector, Agilent Technologies) and equipped with an autosampler (MPS2, Gerstel Multipurpose sampler, Germany) has been used. Peach samples were incubated at 40°C for 2 h, volatile compounds were sampled for 45 min using solid phase micro extraction (SPME) fiber (Supelco Inc., Bellefonte, PA, United States) technology, with a polydimethylsiloxane / divinylbenzene (PDMS/DVB, 1 cm long, 65 µm thickness, 0.357 µL volume) sorptive coating. The fiber was desorbed into the split/splitless liner of the GC for 5 min in splitless mode, setting at 250°C the injector temperature. The GC was equipped with a 30 m × 0.25 mm i.d. capillary column (HP-5MS, 5% phenyl methyl siloxane) having a film thickness of 0.25 µm. Helium was employed as carrier gas with a flow rate of 1.2 mL min^{-1}. The GC oven heating program started at 40°C and increased to 250°C at a rate of 5°C min^{-1} with a total run time of 32.5 min. Each sample was analysed in triplicate. A mass spectrometer (5973 Network Mass Selective Detector, Agilent Technologies) coupled to the GC was used as detector for compounds quantification/identification.

Compound identification has been performed deconvoluting and analysing each chromatogram using the automated mass spectral deconvolution and identification system (AMDIS, National Institute of Standards and Technology, Gaithersburg, MD, United States). Each chromatographic peak spectra has been compared with those of the National Institute for Standards and Technology (NIST98, Version 2.0, United States) data bank considering only results with 90%, or more, of matching. Peak retention indices (RI) have been used to optimize data bank screening. Raw peak areas have been normalized on the sum of the areas of all the identified peaks for each specific chromatogram.

Total RNA extraction and qRT-PCR analysis

Frozen mesocarp samples were grounded to powders using ceramic mortar and pestle pre-cooled with liquid nitrogen. 100 mg of grounded tissue were weighted. Total RNA extraction was carried out following the protocol of Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, Italy), including DNA digestion with the On-Column DNase I Digestion Set (Sigma-Aldrich, Italy). RNA concentration and purity were determined with Nanodrop 2000 spectrophotometer (Thermo Scientific, Italy), verifying an absorbance ratio 260/280 nm between 1.8-2 and 260/230 nm between 1.3-2. Integrity of the RNA extracted was demonstrated by the existence of intact ribosomal bands on a 1% (weight/volume) agarose gel. Next, reverse transcription (RT) of the RNA templates to cDNA was carried out in a final volume of 20 µL using iScript™ cDNA Synthesis (Bio-rad Inc, Italy) following the manufacturer instructions. The experimental design of the relative expression analysis included three biological replicates. The approach followed was sample maximization according which, in one run of the qPCR reaction, one gene should be tested on all the samples in the experimental set (Helleman

et al., 2007). Additionally, in each qPCR reaction a non-template control (NTC) was amplified as a negative control. Analyses were performed by ABI Prism 7300 sequence detection system (applied biosystem). The reaction system consisted of 5 µl of KiCqStart™ SYBR® Green qPCR ReadyMix™, with ROX™ (Sigma-Aldrich, Italy), 3 ng of cDNA template, 0.3 µl of revers and forward primers in working solution of 10 nM. DDW (Sigma-Aldrich, Italy) was added to reach a final volume of 10 µl. The RT-qPCR cycle was set to as follows: initial denaturation at 95°C for 2 min, followed by 40 cycles of amplification with denaturation at 95°C for 15s and annealing and elongation at specific temperature suited to the primer melting temperature (Tm) for 1 min. Following the 40 cycles a melt cycle was performed at 95°C for 15 s, 60°C for 1 min, 95°C for 15s and 60°C for 15s. Expression levels were normalized employing ubiquitin C (PpUBC) housekeeping gene as a reference. Genes and primers have been selected based on previously published work dealing with LOX pathway gene expression in peach fruit (Zhang *et al*., 2010; 2011).

Statistical analyses

Presented data produced with all the employed analytical techniques have been analysed using one-way analysis of variance (ANOVA) statistical tools ($p \leq 0.05$) and Tukey's Honest Significant Difference (HSD) *post hoc* test in order to identify compounds or genes significantly differing in terms of relative intensity or expression level between tested treatments and sampling times. Before performing the analysis, ANOVA assumptions have been tested for all the investigated genes and compounds.

3. Results and Discussion

Technological parameters and CI incidence after cold storage and during shelf life conditions

Both low temperatures were effective in slowing down the loss of firmness rate in both cultivars. At the end of 1 and 2 weeks of storage higher firmness values have been detected in FL fruit stored at 0.5°C than at 5.5°C (Table 1) and, although not statistically significant at the end of the first storage week, a similar trend has been observed in RH fruit. After 3 days of shelf-life (SL) at room temperature following cold storage, fruit were still firmer than control samples. These data confirm previously published researches on peach fruit firmness changes under and after cold storage (Zerbini *et al*., 2011; Cano-Salazar *et al*., 2012; Wang *et al*., 2013).

Considering the CI incidence, a marked difference has been observed between the two considered cultivars (Table 1). As far as internal browning is concerned, RH fruit did not show any symptoms at the end and after both cold storage conditions. Instead, FL peaches developed a marked incidence of mesocarp browning after 2 weeks storage + SL, with some limited symptoms under control conditions (20°C). The two cultivars behaved differently also in terms of extractable juice. In fact, FL peaches showed no difference of this parameter among samples after 1 week of storage, and a significant reduction of extractable juice was detected in the sample kept at 5.5°C for 2 weeks + SL when compared with both control (20°C) and 0.5°C + SL. On the other hand, RH fruit after 1 week at 0.5°C and 5.5°C + SL displayed significant lower values of extractable juice than control fruit. These samples were also those showing sig-

Table 1 - Flesh firmness, incidence of internal browning and extractable juice values in peaches during two weeks of cold storage and subsequent three days of shelf life

	0 week	1 week					2 weeks				
	T0	0.5	5.5	20	0.5 + SL	5.5 + SL	0.5	5.5	20	0.5 + SL	5.5 + SL
A - Flaminia											
Firmness (N)	44.4±7.9 a	45.1±5.3 a	26.8±1.5 bc	5.6±3.7 e	45.1±5.3 a	26.8±1.5 bc	39.8±3.6 a	22.0±2.2 cd	4.5±2.3 e	35.8±3.6 ab	12.8±2.2 de
Int. Browning (%)	0.0 d	0.0 d	0.0 d	0.0 d	0.0 d	0.0 d	0.0 d	0.0 d	11.0±1.0 c	60.0±4.5 a	51.0±3.6 b
Extractable juice (%)	78.1±4.1 c	77.7±4.2 c	80.5±5.0 bc	85.7±1.4 bc	87.1±4.3 bc	86.7±5.0 bc	84.3±7.2 bc	83.1±4.8 bc	92.4±1.6 ab	100.0±3.8 a	78.6±3.8 c
B - Red Haven											
Firmness (N)	36.3±4.1 ab	41.2±3.4 a	36.1±2.5 ab	2.8±1.0 d	41.1±3.3 a	36.0±2.6 ab	40.7±2.5 ab	29.3±7.3 bc	1.7±1.5 d	38.6±2.5 ab	20.8±7.3 c
Int. Browning (%)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Extractable juice (%)	88.1±0.7 c	91.9±1.4 bc	92.5±2.7 abc	100.0±4.3 a	98.4±2.6 ab	88.9±2.5 c	88.8±1.2 c	90.1±4.9 bc	95.5±0.7 ab	93.0±1.4 abc	78.2±3.8 d

'Flaminia' (A) and 'Red Haven' (B) were analysed at harvest (0), at the end of 1 and 2 weeks of cold storage at 0.5 and 5.5°C, and after three additional days of shelf life (0.5 and 5.5°C + SL). Samples kept at 20°C represent the room temperature control. The average values of nine replicates ± SD for each treatment and measured parameter are reported. Letters indicate the results of Tukey's Honest Significant Difference (HSD) post hoc test, performed independently for each cultivar.

nificantly reduced values after 2 weeks of storage +SL (Table 1).

These data concerning CI incidence under and after cold storage are in agreement with the published literature concerning the effects of different temperatures and the role played by the genetic background (Lurie and Crisosto, 2005; Zhang *et al*., 2011; Pons *et al*., 2015; Bustamante *et al*., 2016).

Cold storage effects on LOX pathway-related VOCs

As general trend, during peach fruit ripening volatile compounds associated with green-notes, such as C6 aldehydes and alcohols, tend to decrease while the levels of molecules associated with fruity-notes, such as esters and, in particular, lactones follow an opposite trend (Defilippi *et al*., 2009; Zhang *et al*., 2011; Brizzolara *et al*., 2018).

Considering aldehydes, synthesized through the activities of lipoxygenase (LOX) and hydroperoxide lyase (HPL), three compounds have been targeted: hexanal, 2-hexenal and 2-hexenal-E (Fig. 1). 2-hexenal and 2-hexenal-E significantly decreased from harvest to the second week in control fruit (kept at 20°C) (Fig. 1), confirming what has been previously demonstrated in other works (Defilippi *et al*., 2009; Ortiz *et al*., 2010). Differently from hexanal, 2-hexenal and 2-hexenal-E showed an increasing trend, compared to controls, in both cultivars after 1 week of storage either at 0.5 and 5.5°C. In addition, these two compounds showed, in general, a decreasing trend during SL at room temperature, reaching the control levels (Fig. 1).

Moving downstream in the LOX pathway, C6 aldehydes produced by LOX and HPL are used by other enzymes as precursor for the production of C6 alco-

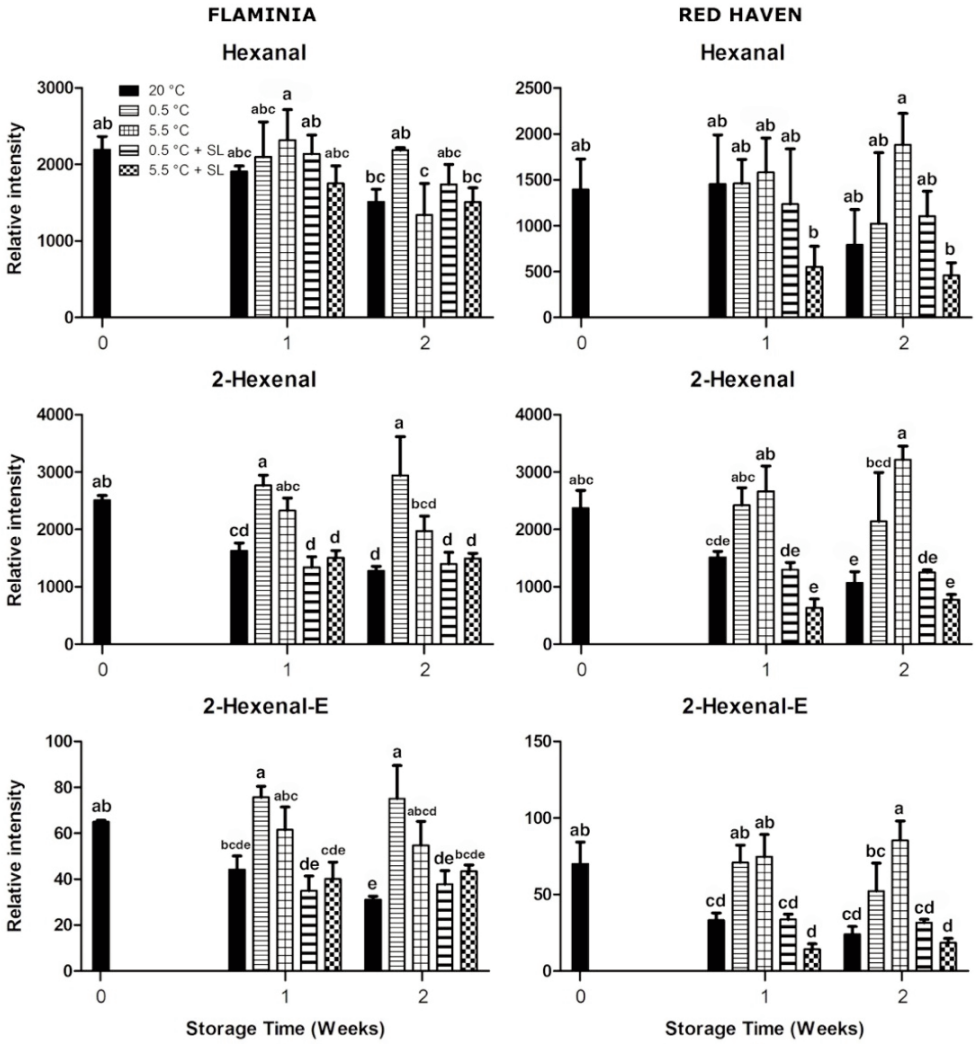

Fig. 1 - Analysis of targeted LOX pathway-derived aldehydes. Mesocarp samples (cv. Flaminia, left panel; cv. Red Haven, right panel) were analysed at harvest (0), at the end of 1 and 2 weeks of cold storage at 0.5 and 5.5°C, and after three additional days of shelf life (0.5 and 5.5°C + SL). Samples kept at 20°C represent the room temperature control. The average value of three biological replicates is reported with bars representing SD. Letters indicate the results of Tukey's Honest Significant Difference (HSD) post hoc test, performed independently for each cultivar.

hols. Specifically, aldehyde dehydrogenase (ADH) is the enzyme responsible for the production of alcohols from aldehydes (Lara *et al*., 2003; Defilippi *et al*., 2005). Considering the results on C6 alcohols level, hexanol, 2-hexen-1-ol and 3-hexen-1-ol accumulation trends are reported in figure 2. Interestingly, a temporary increase of these three compounds was detected after 1 week at 20°C only in FL. After 1 week of cold storage, hexanol and 3-hexen-1-ol resulted significantly reduced only in FL after both 0.5 and 5.5°C treatments. In both cultivars, 2-hexen-1-ol significantly increased in 0.5°C stored samples after 2 weeks when compared with control fruit (Fig. 2). While for hexanol similar levels have been detected in both cultivars, 2-hexen-1-ol and 3-hexen-1-ol were generally higher in RH and FL peaches, respectively.

Our result only in part confirm those reported by Zhang *et al*. (2011), who showed an increase of 2-hexenol and 3-hexenol, in peaches during shelf life following 2 weeks of cold storage at 0°C.

In the LOX pathway, the addition of an acyl moiety to alcohol, reaction catalysed by acyl transferase (AAT), results in the production of esters (Lara *et al*., 2003; Defilippi *et al*., 2005). This enzyme drives the production of these important compounds that impart fruity notes (Schwab *et al*., 2008). In addition to C6, also C5 compounds, including 2-pentenal, can be synthetized from an additional branch of the LOX

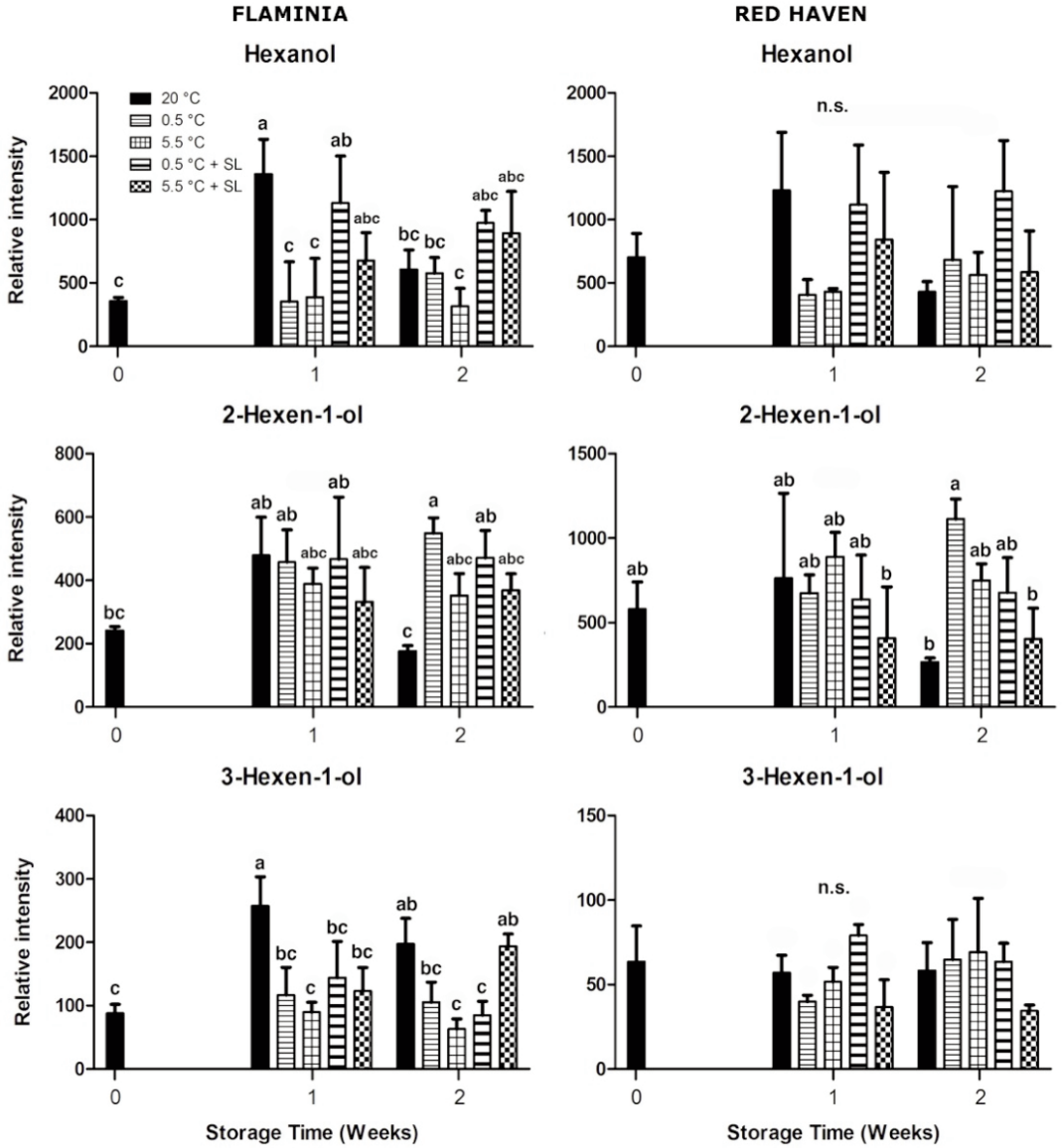

Fig. 2 - Analysis of targeted LOX pathway-derived alcohols. Mesocarp samples (cv. Flaminia, left panel; cv. Red Haven, right panel) were analysed at harvest (0), at the end of 1 and 2 weeks of cold storage at 0.5 and 5.5°C, and after three additional days of shelf life (0.5 and 5.5°C + SL). Samples kept at 20°C represent the room temperature control. The average value of three biological replicates is reported with bars representing SD. Letters indicate the results of Tukey's Honest Significant Difference (HSD) post hoc test, performed independently for each cultivar.

pathway (Gardner *et al.*, 1996; Nielsen *et al*., 2004; Veronico *et al*., 2006; Shen *et al*., 2014 a) and specific pentyl esters, derivatives of C5 compounds, have been considered. The targeted analysis that have been performed includes hexyl, 2-hexenyl, 3-hexenyl, 2-pentenyl and 4-pentenyl acetate compounds (Fig. 3). In general, FL fruit showed no significant difference among samples after 1 week of storage regarding hexyl, 2-hexenyl and 3-hexenyl acetate. The most significant change has been observed in RH cv concerning the levels of 2-hexenyl acetate that showed (as in FL samples) a decreasing trend during postharvest ripening at room temperature and increased at the end of 1 and 2 weeks of cold storage under both low temperature conditions. A similar increasing trend of 2-hexenyl acetate was also observed in cold stored FL samples, even though the differences are not statistically significant for both temperatures and

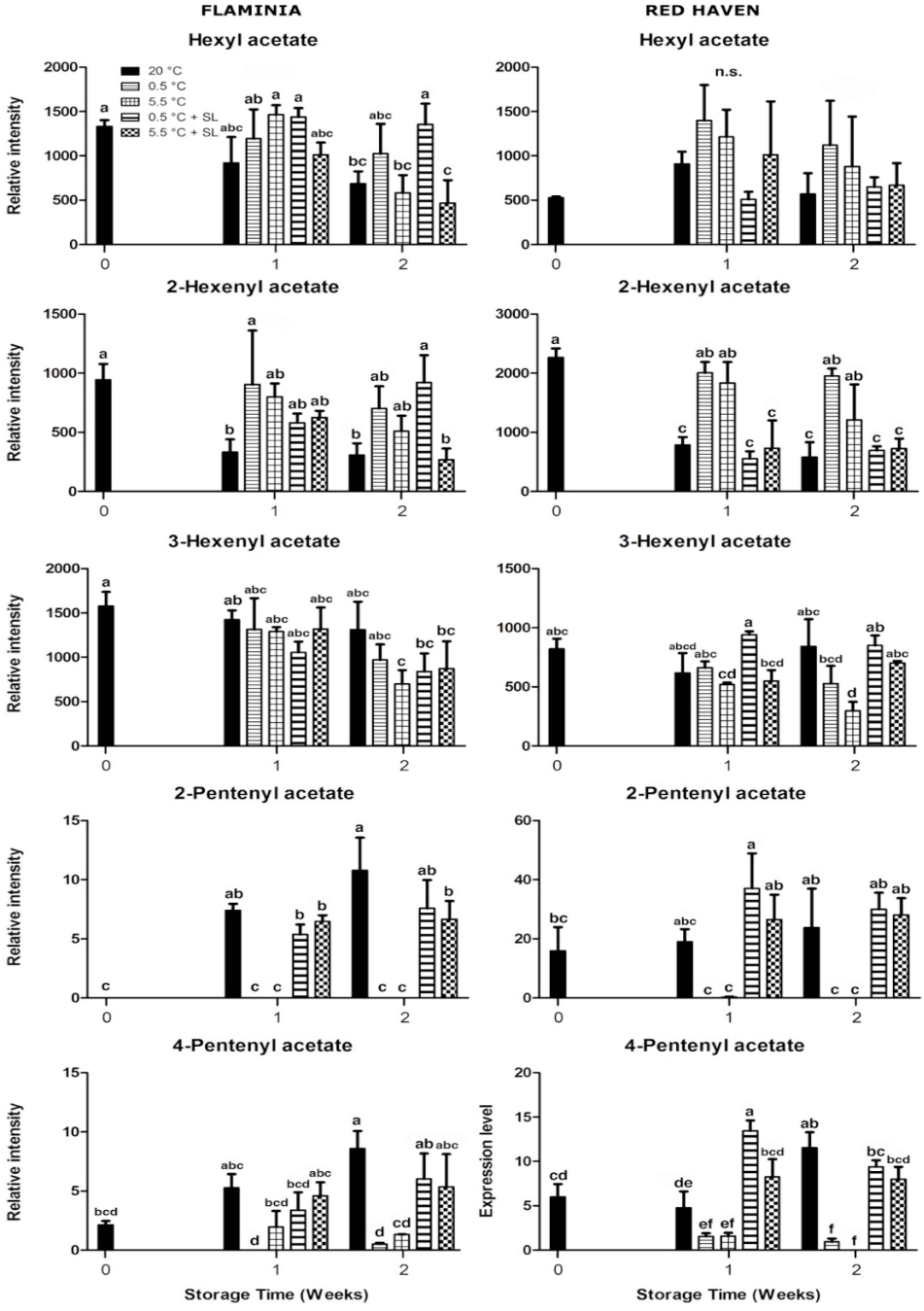

Fig. 3 - Analysis of targeted LOX pathway-derived esters. Mesocarp samples (cv. Flaminia, left panel; cv. Red Haven, right panel) were analysed at harvest (0), at the end of 1 and 2 weeks of cold storage at 0.5 and 5.5°C, and after three additional days of shelf life (0.5 and 5.5°C + SL). Samples kept at 20°C represent the room temperature control. The average value of three biological replicates is reported with bars representing SD. Letters indicate the results of Tukey's Honest Significant Difference (HSD) post hoc test, performed independently for each cultivar.

sampling times.

Interestingly, the two targeted pentyl esters (2-pentenyl and 4-pentenyl acetate), which show an increasing trend during postharvest ripening at 20°C, were markedly reduced by cold storage. 2-pentenyl acetate, in particular, showed almost undetectable levels in all samples at the end of cold storage at both temperatures. A general recover of both pentyl esters levels during SL was observed, with values reaching those of the controls (Fig. 3). Previous works demonstrate that chilling injured peaches show a lack of esters production and it has been argued that this could be the result of an inhibition of AAT1 activity (Ortiz *et al*., 2009). Also in other fruits, CI appearance has been linked to ester biosynthesis: this is the case of papaya in which CI symptoms appearance is accompanied by lower activities of LOX and AAT (Galli *et al*., 2008). Our data do not clearly confirm this behaviour although a general trend of lower hexyl and 2-hexenyl acetate in 5.5°C FL samples stored for 2 weeks was observed.

Expression analysis of LOX pathway genes

The expression of genes involved in the LOX pathway (*PpLOX1*, *PpLOX4*, *PpHPL1*, *PpADH1* and *PpAAT1*) is reported in figure 4.

Concerning *PpLOX1* and *PpLOX4* gene expression, specific pattern of accumulation of these transcripts has been identified in the two cultivars and different conditions. In FL control peaches (20°C) *PpLOX1* and *PpLOX4* revealed an identical trend showing a significant increase from harvest to the first week followed by a decrease during the second week. On the other hand, in RH control fruit the expression levels of these two genes showed a stable (*PpLOX4)* or decreasing (*PpLOX1)* trend in 2 weeks after harvest. *PpLOX1* appeared to be inhibited at the end of both low temperature storage conditions in FL, and a significant increase of its expression level has been recorded during SL in all samples. This increase during SL has been observed also in RH fruit. *PpLOX4* expression was significantly reduced and increased in FL and RH peaches, respectively, after 1 week of cold storage at both temperatures. An up-regulation of this gene was detected in both cultivars in 5.5°C + SL samples after 1 week of storage.

Considering *PpHPL1*, a decreasing trend of expression was detected in FL control fruit only. Genotype-related difference has been also observed when comparing the expression trends of cold stored peaches with significant increases in both SL samples of RH fruit after 1 week of storage.

PpADH1 and *PpAAT1* genes revealed a similar expression trend, almost mirroring the pattern detected for *PpLOX1* (Fig. 4). A peak of expression after 1 week has been detected in FL control samples for both genes, while in RH control peaches *PpADH1* expression showed a decreasing trend and *PpAAT1* expression remained unchanged. Besides the reduced expression of *PpADH1* detected in cold treated FL samples after 1 week, limited or non-significant changes of *PpADH1* expression levels were detected in cold stored and SL samples of RH (1 and 2 weeks) and FL (2 weeks). Interestingly, in both cultivars *PpAAT* gene expression showed a significant up-regulation in all SL fruit of either temperatures, if compared to samples collected at the end of cold storage. This behaviour is similar to that observed for *PpLOX1*. Thus, the expression of these two genes seems highly sensitive to temperature conditioning with a general decrease observed during cold storage and an expression recovery during SL at room temperature.

In general, our expression data confirm what reported in literature on peach fruit ripening in particular concerning *PpHPL1*, that shows a decreasing trend, paralleled by reduced amount of C6 aldehydes (Zhang *et al*., 2010; Shen *et al*., 2014 b). A decreasing *HPL* expression level during ripening has been observed also in other fruit species, such as tomato (Howe *et al*., 2000). The expression of *LOX1* and *4*, *ADH1* and *AAT1* during ripening appears to be different in relation to the genotype. In fact, the transient up-regulation of these genes observed in FL control fruit after 1 week of storage (followed by a down-regulation) was not observed in RH fruit.

Considering the effect of cold stress on *LOX* genes, an induction on *PpLOX1* and *PpLOX3* expression in peach fruit during shelf life after storage at 0.5 and 5.5°C, flanked by higher ethylene synthesis, has been reported by Zhang *et al*. (2011). In our trials, the effect of "rewarming" (shelf life at room temperature after cold storage) is clearly evident when considering the expression trend of *PpLOX1* (thus confirming the data by Zhang *et al*., 2011) but not of *PpLOX4*, suggesting that members of this multigene family have different regulatory mechanisms and may be selectively involved in the responses of peaches to postharvest temperature conditioning.

As observed for *PpLOX1, PpAAT* seems highly sensitive to temperature conditioning showing a marked expression recovery during post-cold storage SL. Similarly, Ortiz *et al*. (2009) reported an enhancement of *PpAAT* gene expression after cold storage,

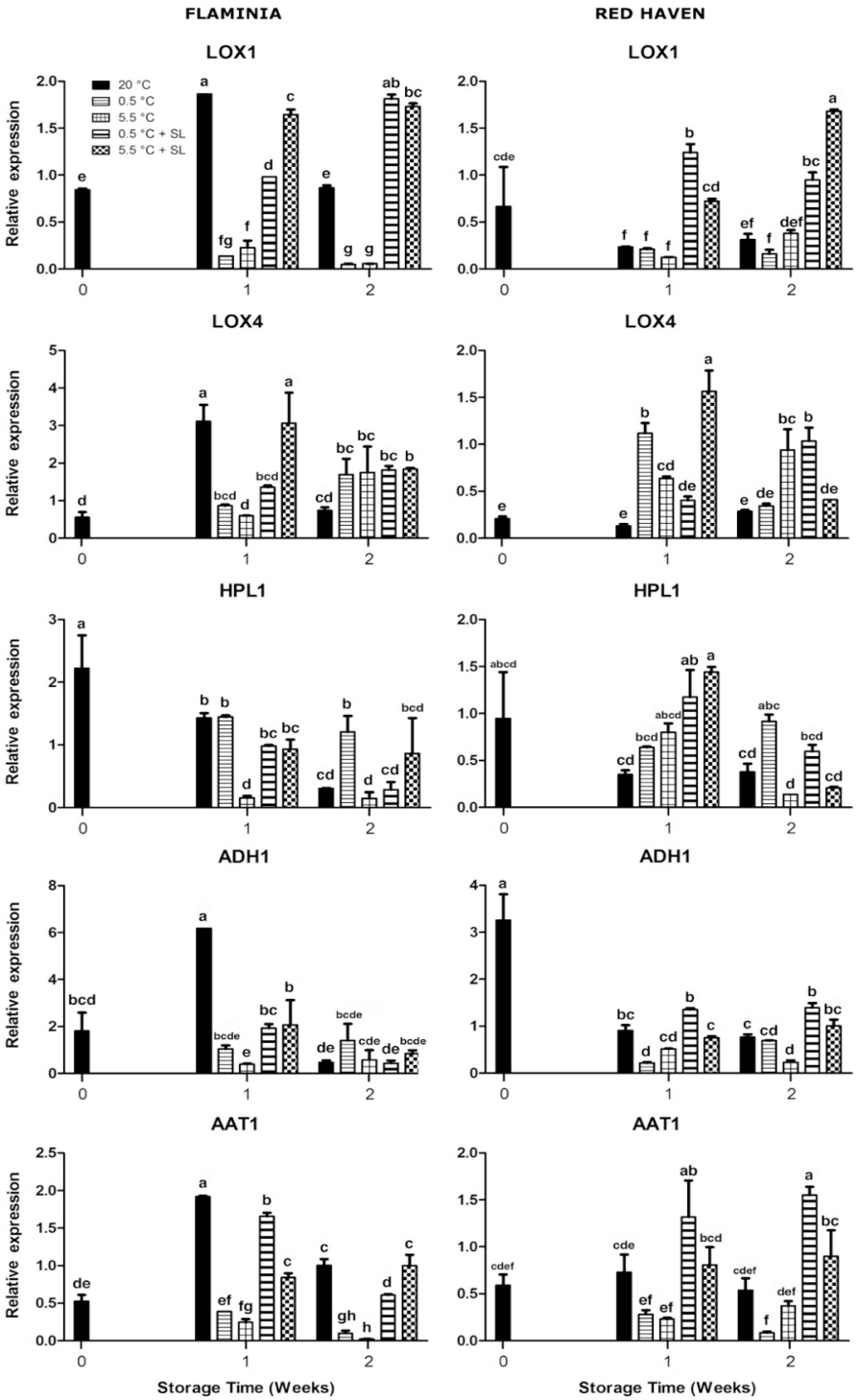

Fig. 4 - RT-qPCR analysis of selected LOX pathway-related genes. Mesocarp samples (cv. Flaminia, left panel; cv. Red Haven, right panel) were analysed at harvest (0), at the end of 1 and 2 weeks of cold storage at 0.5 and 5.5°C, and after three additional days of shelf life (0.5 and 5.5°C + SL). Samples kept at 20°C represent the room temperature control. The average value of three biological replicates is reported with bars representing SD. Letters indicate the results of Tukey's Honest Significant Difference (HSD) post hoc test, performed independently for each cultivar.

indicating a renewed ability of synthetizing esters after cold stress. Our results (Figs. 3, 4) confirm this observation.

Considering the possible relationships between CI and LOX pathway, Zhang *et al*. (2011) reported that in peach fruit LOX pathway genes showed lower transcript levels in fruit stored at a CI-inducing temperatures (e.g. 5°C) compared to fruit kept at 0°C, with transcript abundance that tends to decrease with extended storage periods and the onset of CI symp-

toms. Our gene expression data do not show any clear relationship with CI incidence, while, considering VOCs, only for hexyl acetate and 2-hexenyl acetate significantly lower concentrations have been detected in samples with CI symptoms.

Expression analysis of CBF genes

The expression pattern of two *C-repeat-binding factors* genes (*PpCBF1* and *PpCBF6*) has been analysed in FL and RH samples in relation to cold storage treatments (Fig. 5).

As a general trend the two genes appeared to be significantly up-regulated at the end of 1 and 2 week storage either at 0.5 and 5.5°C in both cultivars. In FL samples after 1 week and in RH fruit after 2 weeks, it appears that the lower the storage temperature the higher is the expression of these two genes. During 20°C SL after cold storage, either at 0.5 and 5.5°C, the expression of these genes markedly decreases reaching the level of the control, confirming that *CBF* gene expression is highly responsive to temperature conditions. It is interesting to note that RH peaches showed higher levels of expression, in particular considering *PpCBF1*.

Similar results in terms of expression pattern during refrigeration of *PpCBF* genes have been previously reported in peach fruit, confirming that these regulatory genes are generally sensitive to low temperatures (Liang *et al*., 2013). Specifically, *PpCBF1/6* appeared to be induced exclusively under cold storage, revealing very low or non-detectable levels during post-storage shelf life, and their level of expression was significantly higher under CI-delaying temperature (0°C), when compared to chilling inducing conditions (5°C). Indeed, this paper reports that the up-regulation of *CBF* genes correlated with a decreasing level of CI symptoms, such as flesh browning, as well as with a reduction of firmness loss. Our data clearly show that *CBF* expression is higher at 0.5°C than at 5.5°C in FL after 1 week and in RH after 2 weeks of storage, highlighting the different behaviour and regulatory mechanisms characterizing peach genotypes. It has been reported that *CBF* genes in peach fruit are activated over different time periods, with *PpCBF5* being activated at high levels in several hours, *PpCBF6* later within three days and *PpCBF1* requiring 1 week to be expressed at high levels (Liang *et al*., 2013). This temporal distribution of *CBF* genes activation during cold storage suggests that each gene plays a specific role, and it has been argued that while *PpCBF5* seemed to be associated with an early response to low temperatures, *PpCBF1/6* appeared to be more involved in protection to prolonged cold storage. However, the possible relationship between the expression trends and levels of these *CBF* genes and the sensitivity to postharvest cold stress of peach fruit remains to be elucidated. This is an important topic for future research in elucidating regulatory mechanisms involved in low temperature responses considering that the over-expression of peach *PpCBF1* allowed Wisniewski *et al.* (2011) to get to an increased freezing tolerance of apple trees of about 4-6°C. Moreover, the overexpression of several *CBF* genes in different plant species, including *Arabidopsis*, grape, potato and apple, appears to increase plant tolerance to cold stress (Novillo *et al*., 2007; Pino *et al*., 2008; Siddiqua and Nassuth, 2011;

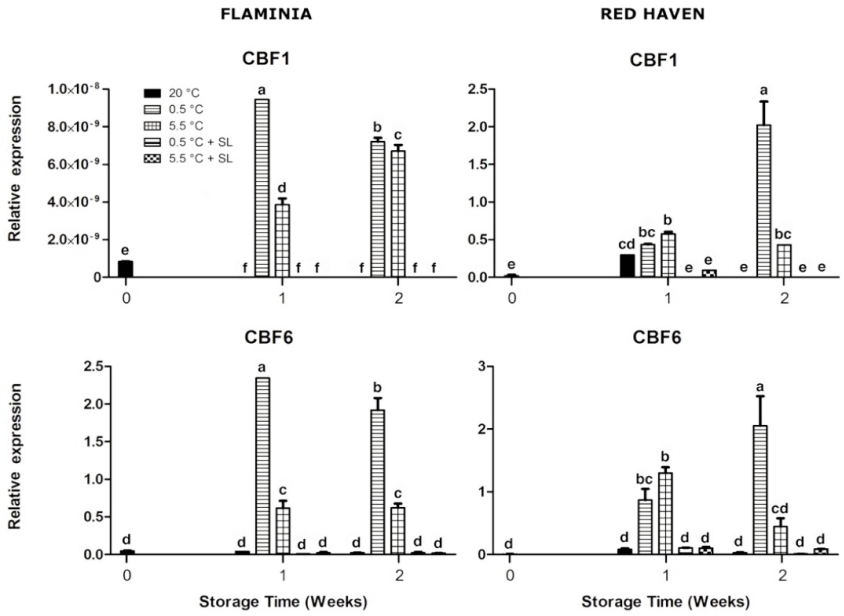

Fig. 5 - RT-qPCR analysis of two C-repeat-binding factors (CBF) genes. Mesocarp samples (cv. Flaminia, left panel; cv. Red Haven, right panel) were analysed at harvest (0), at the end of 1 and 2 weeks of cold storage at 0.5 and 5.5°C, and after three additional days of shelf life (0.5 and 5.5°C + SL). Samples kept at 20°C represent the room temperature control. The average value of three biological replicates is reported with bars representing SD. Letters indicate the results of Tukey's Honest Significant Difference (HSD) post hoc test, performed independently for each cultivar.

Takuhara *et al*., 2011).

4. Conclusions

Peach genotypes show marked difference in terms of responses to cold storage, with some cvs less susceptible to CI and others developing an array of symptoms during shelf life after a few days/weeks of refrigeration. This different behavior observed here in RH and FL peaches, as already reported in a previously published paper (Brizzolara *et al*., 2018), appears to be associated with different expression pattern of two *CBF* genes (*PpCBF1* and *PpCBF6*), recognized to be involved in the responses of peaches to cold storage.

If the general picture of the regulatory role played by *CBF* genes has been described in plants, the possible mechanisms involving CBF transcription factors in modulating molecular and physiological responses of fruit tissues to postharvest cold stress remain to be elucidated. Peaches represent an interesting model for such studies, based on the fact that the expression of the two *CBF* genes analyzed in the present paper appears to be differently affected by temperatures more (5.5°C) or less (0.5°C) inducing the appearance of CI symptoms.

In addition to the expected effect of genotype, specific genes of the LOX pathway also show different expression pattern depending on the storage temperature and the shelf-life, with some of them (e.g. *LOX1, ADH and AAT1*) representing good candidates for additional studies aimed at identifying metabolic and molecular markers of CI onset and incidence in cold stored peaches.

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