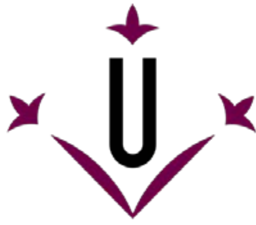

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POSTHARVEST HEAT AND CO_2 SHOCKS INDUCE CHANGES IN CUTICLE COMPOSITION AND CUTICLE-RELATED GENE EXPRESSION IN 'OCTOBER SUN' PEACH FRUIT

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Abstract

Fruit cuticles influence greatly postharvest quality of produce. Yet, few published studies have addressed changes in cuticle composition and related gene expression from a postharvest perspective, particularly for fruit species within the *Rosaceae* family. Because physical treatments have proved effective in improving postharvest potential in some commodities, we were interested in assessing whether such benefits could arise, at some extent, from treatment-related alterations in cuticular components. In this work, commercially ripe 'October Sun' peaches were submitted to heat or CO_2 shocks, and then stored at 0 ºC for two weeks followed by five days at 20 ºC. Wax and cutin composition was analyzed from cuticle samples isolated enzymatically from fruit skin, and expression of selected genes *PpCER1*, *PpLACS1* and *PpLipase*, putatively involved in cuticle deposition, was quantified by quantitative real-time PCR. The amount (g m^{-2}) of the main cuticular wax families identified was higher in treated fruit in comparison with the controls, particularly after the shelf life period. Treatment influence on cutin composition was less clear. Gene expression was generally impaired under cold storage, to recover to a variable extent upon transfer to 20 ºC. The differential gene expression shown in response to treatments may relate to the observed compositional changes in the cuticle.

Keywords: CO_2 shock; cuticle; gene expression; heat shock; peach; postharvest; *Prunus persica* L.

22 ## 1. Introduction

23 Consumer's acceptability of fruit commodities is directly dependent on produce quality.
24 The influence of skin on fruit quality has been often underestimated, fruit surface being
25 regarded uniquely as a major contributor to external appearance and thus to purchase
26 decision. Yet, the properties of surface tissues also have a profound influence on the
27 postharvest potential of fruit. The cuticle is the outermost layer of fruit skin, and it interacts
28 intimately with the external environmental conditions which surround the fruit as well as with
29 the underlying epidermal tissue (Guzmán et al., 2014; Fernández et al., 2016). Current
30 experimental evidence suggest that cuticle characteristics may impact several major aspects of
31 fruit quality, including transpirational water loss, proneness to mechanical injuries and to
32 microbial infections, susceptibility to physiological disorders, and firmness loss (Lara et al.,
33 2014).

34 In spite of the profound influence of cuticle properties on postharvest quality of fruit, and
35 of the world-wide commercial relevance of these crops, limited research efforts have been
36 invested on this topic. Detailed information on the chemical composition of fruit cuticles has
37 been reported for a few species uniquely (Lara et al., 2015), and cuticle biosynthetic pathways
38 as well as the influence thereupon of internal and external factors are poorly understood.
39 Additionally, fruit cuticles keep evolving once the produce is separated from the plant, even if
40 not submitted to any postharvest treatment. Yet, very few reports exist on postharvest
41 modifications in cuticles of fruit species, particularly for those within the *Rosaceae* family.
42 For apple (*Malus* × *domestica* Borkh.), considerable cultivar-associated variability has been
43 demonstrated as to the progress of total wax amounts, properties or chemical components
44 during long-term cold storage (Morice and Shortland, 1973; Dong et al., 2012) or in response
45 to controlled atmosphere storage and subsequent shelf life (Veraverbeke et al., 2001). The
46 composition of cuticular waxes of 'Autumn Gold' and 'Royal Gala' apples was modified

47 throughout postharvest cold storage (Curry, 2008). Interestingly, these postharvest changes
48 were attenuated in apple fruit treated with 1-methylcyclopropene (1-MCP), suggesting that
49 the biosynthesis of cuticular waxes in this species might be under ethylene control.

50 In previous studies, we have shown changes in cuticle composition after harvest of stone
51 fruit species, both in peach (*Prunus persica* L. Batsch.) (Belge et al., 2014a) and in sweet
52 cherry (*Prunus avium* L.) (Belge et al., 2014b), as well as cultivar-related differences within
53 each of both species. We have also reported significant modifications in total yields of cuticle
54 per surface unit of sweet cherry fruit after refrigerated storage for two weeks, as well as in the
55 amounts of the main families of cuticular components (Belge et al., 2014b). Peach fruit
56 deteriorate quickly after harvest when kept at ambient temperature, which results in limited
57 shelf life and postharvest handling possibilities, but they are nonetheless very prone to
58 develop chilling injury when submitted to refrigerated storage (Lurie and Crisosto, 2005).
59 Some physical treatments such as heat and CO_2 shocks, intermittent warming, or storage
60 under high CO_2 levels, have been observed to improve the postharvest potential in peach fruit
61 (Lurie and Crisosto, 2005; Paull and Chen, 2000; Roig et al., 2003; Steiner et al., 2006;
62 Koukounaras et al., 2008; Lurie and Pedreschi, 2014). The question arises whether treatment
63 benefits could be partly ascribed to their impact on fruit cuticle properties. In order to evaluate
64 this possibility, herein we studied the effects of heat and CO_2 shocks on the composition of
65 cuticle composition of peach fruit.

66

67 ## 2. Materials and Methods

68

69 *2.1. Plant material, postharvest handling, and assessment of quality and maturity*
70 *parameters*

71 Samples of melting-type peach (*Prunus persica* L. Batsch., cv. 'October Sun') fruit were
72 harvested in 2011 (September 7th) from a commercial orchard located in Alguaire, near Lleida
73 (NE Spain), at marketable maturity according to the usual indices at the producing area.
74 Uniform and defect-free fruit were divided randomly into three batches. The first of these lots
75 remained untreated and was placed immediately at 0 ºC. The second lot was placed at 0 ºC
76 and treated with a CO_2 shock (30 kPa, 48 h). The third lot was submitted to a hot air (HA)
77 shock (50 ºC, 45 min) in an oven where a water container was also placed in order to prevent
78 sample desiccation, and kept thereafter at 0 ºC. Both untreated and treated samples remained
79 stored at 0 ºC and 92 % relative humidity for fourteen days, and then kept at 20 ºC for five
80 additional days to simulate a commercial retail period.
81 Standard quality parameters were determined on 15 fruit per each analysis date and
82 treatment. Fruit firmness (N) was measured on two opposite sides of the equatorial area of
83 each individual fruit using a hand-held Effegi penetrometer (model FT 327; Milan, Italy)
84 equipped with an 8 mm diameter tip. The weight of the same 15 fruit was assessed at harvest
85 and at each analysis date to determine weight loss (%). Soluble solids content (SSC) and
86 titratable acidity (TA) were assessed in juice pressed from the whole fruit. SSC was
87 determined with a digital hand-held refractometer (Atago, Tokyo, Japan), and results were
88 expressed as % (w/w). For TA determination, 10 mL of juice was diluted in 10 mL of distilled
89 water and titrated with 0.1 M NaOH to pH 8.1; results are given as g L^{-1} malic acid. For the
90 assessment of ethylene production and respiration rates, three fruits per each cultivar were
91 kept individually in 3-L glass jars, continuously aerated with humidified air at a rate of 25 mL
92 min^{-1}. Gas samples (1 mL) of the effluent air were taken with a syringe and injected into a gas
93 chromatograph (model 6890N; Agilent Technologies, Madrid, Spain) equipped with a flame
94 ionization detector and an alumina column (1.5 m × 3 mm). Analyses were conducted
95 isothermally at 100 °C, with N_2 as the carrier gas, in the presence of air and H_2 (45, 400, and

96 45 mL min^{-1}, respectively). The injector and detector were held at 120 and 180 °C,
97 correspondingly, and results are expressed as µg kg^{-1} s^{-1}. Respiration was determined as CO_2
98 production by directly connecting the effluent air from each jar to an infrared gas analyzer
99 (Sensotran S.L., El Prat de Llobregat, Spain). Results are given as µg kg^{-1} s^{-1}.

100

101 *2.2. Cuticle analyses*

102 Cuticular membranes were isolated enzymatically from skin disks excised from the cheek
103 region of five fruit (twelve disks/fruit) using a cork borer (13 mm, i.d.). Sixty skin disks were
104 so obtained per sample (treatment × storage period × shelf life period), corresponding to a
105 surface area of 79.64 cm^2, and then incubated at 37 ºC in 0.2 % (w/v) cellulose, 100 U mL^{-1}
106 pectinase and 1 mmol L^{-1} NaN_3 in 50 mmol L^{-1} citrate buffer (pH 4.0), until no more material
107 was released. The isolated cuticular membranes (CM) were washed in citrate buffer (50 mmol
108 L^{-1}, pH 4.0) at 37 °C, once or twice every day until no material was left in suspension, dried at
109 40 °C, and kept in hermetically capped vials. CM mass was determined gravimetrically, and
110 expressed as g per m^2 of surface area.

111 Waxes were recovered from the sixty dry CM disks (79.64 cm^2/sample) obtained in each
112 case. CM samples were dewaxed by shaking in $CHCl_3$ (1 g L^{-1}) for 24 h at 25 °C, followed by
113 incubation (15 min) in an ultrasonic bath. After filtration, the chloroform extract was
114 concentrated using a rotary evaporator at 50 °C, dried under N_2, and weighed for calculation
115 of total wax yield (g m^{-2}). Free hydroxyl and carboxyl groups were derivatized with N,O-
116 Bis(trimethylsilyl)trifluoroacetamide (BSTFA) in pyridine (2:1, v/v) for 15 min at 100 °C to
117 convert them respectively into their trimethylsilyl (TMSi) ethers and esters. Wax samples
118 were then added eicosane (C_{20}) and dotriacontane (C_{32}) as internal standards, and injected (1
119 µL) in on-column mode into a gas chromatography-mass spectrometry (GC-MS) system for

120 compound identification and quantitation. All procedures and chromatographic conditions
121 were as described previously (Belge et al., 2014a).

122 For the extraction and analysis of cutin monomers, dewaxed cuticular membranes (DCM)
123 were hydrolyzed in 3 mL 1 mol L^{-1} HCl in 100 % MeOH, and esterified in the same solvent
124 for 2 h at 80 °C. The methanolysate was allowed to cool down, and then added 2 mL saturated
125 NaCl. The cutin monomers were extracted three times in 2 mL hexane for 10 min, thoroughly
126 mixed, centrifuged at 20 °C, and the resulting extracts pooled and evaporated to dryness in a
127 N_2 steam. Cutin yields were determined gravimetrically as g m^{-2}. Dry cutin samples were
128 derivatized with BSTFA for 15 min at 100 °C, then added heptadecanoate (C_{17}) and
129 tricosanoate (C_{23}) as internal standards, and injected (1 µL) to GC-FID for quantitative
130 determination. Cutin compounds were identified from their EI-MS spectra after GC-MS
131 analysis using the same chromatographic system as for wax analysis. The chromatographic
132 conditions were as described in previous work (Belge et al., 2014a).
133

134 *2.3. RT-qPCR expression analysis of candidate cuticle-related genes*

135 Total RNA was isolated manually from samples (250 mg) of lyophilized peach skin as
136 described in Chang et al. (1993), and treated with RNase-free DNase I (Qiagen, Hilden,
137 Germany). RNA concentration and purity were verified in a Synergy microplate reader
138 (BioTek Instruments, Inc., Winooski, VT, USA) equipped with the Gen5™ Data Analysis
139 software. cDNA was synthesized from DNA-free RNA (220 ng) by retrotranscription with
140 oligo-$(dT)_{18}$ (Invitrogen, Waltham, MA, USA).

141 The cuticle-related peach genes *PpCER1, PpLACS1* and *PpLipase*, homologues to
142 *PaCER1, PaLACS1* and *PaLipase* identified in sweet cherry, were targeted for expression
143 quantification, on the basis of a previous work (Alkio et al., 2012) showing that their
144 expression in sweet cherry is restricted to the fruit skin and that their up-regulation is parallel

145 to the highest cuticle deposition rates. Three reference genes (*PpSRP19*, *PpPP2A-2* and
146 *PpCAC*) were also selected based on that same previous work. For each cDNA sequence to be
147 submitted to quantitative real-time PCR (RT-qPCR), a set of specific primers was designed
148 (**Table 1**) based on available NCBI databases using the Primer3 on-line primer design
149 software (Rozen and Skaletsky, 2000) and the Oligo Calculator™ on-line tool (Kibbe, 2007).
150 RT-qPCR reactions were prepared in 96-well plates, and run in a qTOWER 2.2
151 quantitative thermal cycler (Analytikjena, Germany). Total reaction volume was 20 µL,
152 containing 150 ng cDNA and 3 µM each primer in 1× SsoAdvanced™ Universal SYBR®
153 Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA). Reaction running conditions
154 were as follows: 95 °C during 30 s; then 40 cycles at 95 °C for 5 s, and 60 °C for 30 s.
155 Dissociation curves were generated for each amplicon at the end of the PCR run by
156 continuous fluorescence measurement (55 °C to 95 °C, with sequential steps of 0.5 °C for 15
157 s), in order to verify amplification specificity and the absence of primer dimers. Each reaction
158 was run at least in triplicate, and the mean threshold cycle (Ct) was used for data analyses.
159 PCR amplification efficiencies were calculated for each primer pair in five-fold serial
160 dilutions spanning five orders of magnitude (1:1-1:625) of cDNAs pooled from all technical
161 replications to generate standard curves. Expression data were normalized by the geometric
162 mean of the expression levels of the reference genes selected (**Table 1**). All procedures for the
163 calculation of amplification efficiencies and for the normalization of relative expression
164 values were as in earlier work (Goulao et al., 2012; Vandesompele et al., 2002).
165
166 *2.4. Statistical analysis*
167 All data were tested by multifactorial analysis of variance (ANOVA), with treatment,
168 cold storage and shelf-life period as the factors, using the Minitab 16 program package, and
169 means were separated by the least significant difference (LSD) test at $P \leq 0.05$.

170

171 **3. Results and Discussion**

172 The common standard indicators used to evaluate the maturity and commercial quality of
173 peach fruit were determined at harvest as well as after storage (**Table 2**). Ethylene production
174 increased after cold exposure in comparison with levels at harvest, but no significant
175 differences were found between treatments upon removal from storage (14+0 samples).
176 However, production rates increased noticeably during the keeping period at 20 ºC, heat-
177 treated fruit displaying lower rates in comparison with both the controls and CO_2-treated
178 samples. Respiration rates after cold exposure were lower than those at harvest for untreated
179 samples, while treated fruit showed higher CO_2 production rates in comparison with the
180 controls, particularly those submitted to the heat shock (**Table 2**). However, respiration rates
181 in heat-treated samples did not show any variation after five days at 20 ºC following cold
182 storage, while a substantial increase was observed for both untreated and CO_2-treated
183 peaches. Upon removal from cold storage, CO_2-treated fruit had higher firmness levels and
184 lower weight loss in comparison with the rest of the samples. After remaining five days at 20
185 ºC subsequent to cold storage, firmness levels were similar regardless of treatment, but heat-
186 treated fruit showed significantly higher weight loss values. Both treatments preserved similar
187 acidity and soluble solid contents upon removal from cold storage in comparison with those at
188 harvest, and led to higher levels of these parameters as compared with untreated fruit after
189 five days of shelf life at 20 ºC (**Table 2**).

190 Clear differences were noted for cuticle yields obtained from fruit skin in each case
191 (**Table 2**). The amount of cuticle per surface area (g m^{-2}) increased significantly after two
192 weeks of refrigerated storage in comparison with values at harvest, regardless of treatment
193 and shelf life period, heat-treated fruit displaying the highest amounts after both 0 and 5 d at
194 20 ºC following cold storage. Simultaneously with the highest cuticle yields, heated fruit also

195 showed however the highest weight loss values (**Table 2**), thus indicating that cuticle amount
196 per surface area is not a key feature by itself for the restriction of transpirational water loss.
197 Water loss must result rather from a combination of a range of factors, including cuticle load,
198 but also cuticle structure, or ratios between different compounds or compound types.
199

200 *3.1. Changes in cuticular waxes and cutin in untreated fruit after refrigerated storage*

201 Cutin, a polymeric polyester comprised mainly of hydroxy and epoxy derivatives of C_{16}
202 and C_{18} fatty acids, constitutes the cuticular matrix in which amorphous waxes and a minor
203 fraction of phenolics are embedded, while the outer side of the cuticle is covered by
204 crystalline as well as amorphous epicuticular waxes (López-Casado et al., 2007). A closer
205 examination of cuticle composition revealed that waxes (**Table 3**) and cutin (**Table 4**)
206 represented respectively around 34 and 11 % over total cuticle at harvest. However, the
207 dynamics of the evolution of each of both cuticle components after the postharvest storage
208 period were rather different.

209 Wax amounts per unit surface area were similar in samples kept at 0 ºC during 14 d in
210 comparison with those at harvest (5.53 and 5.18 g m^{-2}, respectively), but increased noticeably
211 after 5 d at 20 ºC to values around 8.04 g m^{-2}. This increase was also noticed in relative terms,
212 the percentage of waxes rising from 32 to 42 % over total cuticle after the simulated shelf life
213 period (**Table 3**). These data suggest that the increase in cuticle amounts in 14+5 fruit arose
214 mainly from increased wax coverage, since cutin amounts did not show significant
215 differences as compared with 14+0 samples, either in absolute terms (g m^{-2}) or in percentage
216 over total cuticle (**Table 4**). In contrast with the observation for wax content, though, cutin
217 amount was substantially higher after cold storage in relation to harvest.

218 The role of the plant cuticle as a water-proofing barrier has been generally attributed to
219 cuticular waxes. However, the wax content in the surface of untreated fruit was highest for

220 14+5 samples both in absolute and in percentage terms (**Table 3**), although these fruit
221 displayed significantly higher weight loss (**Table 2**). This is clearly indicating that the
222 chemical composition of this cuticle fraction must be more relevant than its total amount for
223 water-loss barrier functions, and therefore the wax compounds present in CM samples were
224 analyzed in detail.

225 Triterpene acids, namely ursolic and oleanolic acids, were the main type of chemical
226 constituents found in the cuticular wax fraction of 'October Sun' peaches, and accounted for
227 over half of total waxes. Alkanes represented roughly 20 % of the wax fraction, *n*-
228 pentacosane (C_{25}), *n*-nonacosane (C_{29}), *n*-heptacosane (C_{27}) and *n*-tricosane (C_{23}) being the
229 main compounds in quantitative terms. The content of fatty acids, alcohols and phytosterols
230 was around 8 %, 3 % and 2 % of total waxes at harvest, respectively (**Table 3**). No significant
231 changes in triterpene or *n*-alkane content (both in absolute and relative terms) were found
232 upon removal from cold storage. However, the amount per surface area (g m^{-2}) increased after
233 5 d at 20 ºC (**Fig. 1**) even though the percentage over total waxes was lower (**Table 3**), owing
234 to higher total cuticle yields in these fruit in comparison with 14+0 samples. The amount of
235 fatty acids per surface area (g m^{-2}) was also higher when the fruit were kept at 20 ºC for 5 d
236 after refrigeration in comparison with values at harvest or upon removal from cold storage
237 (**Fig. 1**), while no significant differences were observed for alcohols or phytosterols.

238 As regards cutin composition, α-monocarboxylic acids and hydroxyacids accounted each
239 for around one third of total cutin monomers identified in the cuticular membranes of
240 'October Sun' peaches at harvest, while α,ω-dicarboxylic acids represented about 13 % of
241 total cutin yields (**Table 4**, **Fig. 2**). The main cutin monomers identified in samples at harvest
242 were C_{18} fatty acid derivatives (54.7 % over total cutin), with smaller contributions of C_{16} and
243 C_{22} fatty acids (10.9 % and 5 %, respectively). The amount of α-monocarboxylic acids,
244 hydroxyacids and α,ω-dicarboxylic acids increased in absolute terms (g m^{-2}) after cold storage

245 in comparison with levels at harvest, and then remained unchanged after the shelf life period
246 (**Fig. 2**). The same pattern of changes was observed for hydroxyacids and α,ω-dicarboxylic
247 acids. However, because the total amount of cutin isolated from cuticular membranes was
248 higher after storage than at harvest, these postharvest increases were not always reflected in
249 terms of relative percentage (**Table 4**).

250 The primary precursors for both waxes and cutin monomers are derivatives of 16 and 18
251 carbon-long fatty acids. These C_{16} and C_{18} fatty acid precursors need to be activated to acyl-
252 CoA derivatives by long-chain acyl-CoA synthetases (LACS) (Lü et al., 2009). For the
253 synthesis of long-chain wax compounds, these precursors are extended to generate very long-
254 chain fatty acids (VLCFA), which then can be released as free VLCFA by acyl-CoA
255 thioesterases (Hunt and Alexson, 2002), or further metabolized through different pathways,
256 one of them including the conversion of aldehydes to alkanes by eceriferum 1 (CER1), an
257 aldehyde decarbonylase constituting the core component of a very long-chain alkane
258 synthesis complex, and involved in epicuticular wax biosynthesis.

259 In this work, the expression of three genes, *Prunus persica* homologues of *P. avium*
260 genes *PaLipase*, *PaCER1* and *PaLACS1*, involved in different steps of wax and cutin
261 deposition in sweet cherry fruit (Alkio et al., 2012) (**Table 1**), was quantified by RT-qPCR to
262 test whether the applied treatments had an impact on gene transcription. The product of the
263 *PaLipase* gene belongs to the GDSL/SGNH superfamily of lipases, which includes acyl-CoA
264 thioesterases involved in cutin and wax production. The functions of *PaCER1* and *PaLACS1*
265 products are related to the biosynthesis of VLCFA-derived waxes (LACS1, CER1) and of
266 cutin monomers (LACS1). This representative set of gene markers was informative,
267 disclosing that gene expression is differentially affected in complementary pathways. The
268 expression level of all quantified transcripts was significantly inhibited at 0 ºC, but while
269 *PpLipase* and *PpLACS1* recovered to initial expression levels during the simulated shelf life

270 period at 20 ºC, *PpCER1* remained inhibited (**Fig. 3**). Results show that the expression of all
271 three genes studied was intensely modified in response to the temperature at which peach fruit
272 are usually stored for commercial purposes.
273
274 *3.2. Changes in cuticular waxes and cutin in response to postharvest CO_2 and heat shocks*
275 The chemical composition of the isolated CM was also analyzed in CO_2- and HA-treated
276 fruit in order to assess possible differences in comparison with untreated controls. Wax
277 amounts per unit surface area (g m^{-2}) were similar in 14+0 samples regardless of treatment. In
278 contrast, although significantly increased cuticular wax contents were detected in 14+5 in
279 comparison with 14+0 samples in all cases, substantial differences were detected among
280 treatments (**Table 3**). The highest wax contents in the fruit surface were found for samples
281 submitted to the heat treatment, both in absolute and in percentage terms. Wax amounts in
282 CO_2-treated samples were lower than those in untreated fruit, even though no differences
283 between both treatments were detected when wax coverage was expressed as a percentage
284 over total cuticle (**Table 3**).
285 Compositional analysis of the cuticular wax fraction of treated and untreated fruit
286 revealed significant modifications in response to the applied treatments. For 14+0 samples,
287 both heat and CO_2 shocks resulted in higher percentage of triterpene acids and phytosterols
288 over total cuticular waxes in comparison with the controls (**Table 3**). In contrast, the
289 percentage of free fatty acids was lower in treated than in untreated fruit, while no significant
290 differences respecting the controls were observed for the content of *n*-alkanes or alcohols.
291 The acyclic, very long-chain compounds present in cuticular waxes, such as *n*-alkanes, fatty
292 acids or fatty alcohols, which can form highly ordered crystalline structures (Reynhardt and
293 Riederer, 1991, 1994), are considered to establish the main barrier against water loss through
294 plant cuticles. Even so, in many reported cases this relationship could not be confirmed (Jetter

295 and Riederer, 2015; Zeisler and Schreiber, 2015; Schuster, 2016). In this study, the acyclic to
296 cyclic compound ratios were lower in treated than in untreated fruit (0.63 in the controls *vs*.
297 0.55 and 0.54 in CO_2- and heat-treated samples respectively) (**Table 3**), with no apparent
298 relationship to weight loss percentages (**Table 2**). In fact, acyclic to cyclic compound ratios
299 were even higher in 14+5 fruit (over 0.70 regardless of treatment), even though water loss
300 were more than two-fold those in 14+0 samples. However, it should be noted that no analyses
301 of cuticular permeability were undertaken in this work. Since stem scar is a major way for
302 water loss (Moggia et al., 2017) and it was not sealed in the fruit samples used herein, caution
303 should be exerted when interpreting these data. This point will require careful attention in
304 future studies.

305 Some differences in cutin loads were also observed in response to the treatments
306 considered. Immediately after removal from cold storage, cutin amounts were significantly
307 lower in heat-treated fruit in comparison with the controls, while those in CO_2-treated samples
308 remained at similar levels as in untreated fruit (**Table 4**). No differences in cutin amount per
309 surface unit were found among treatments after 5 d at 20 ºC, but peaches submitted to a CO_2
310 shock displayed significantly higher percentages of cutin over total cuticle than those
311 untreated or heat-treated (**Table 4**).

312 The composition of the cutin fraction was also modified in comparison with untreated
313 fruit. While no significant differences in the relative amount of α-monocarboxylic acids were
314 observed among treatments upon removal from storage, both heat and CO_2 shocks led to
315 higher percentages relative to the controls after 5 d of simulated retail period (**Table 4**), these
316 increases being noticed also in terms of absolute amounts per unit surface area (**Fig. 2**).

317 Expression levels of *PpLipase*, *PpCER1* and *PpLACS1* responded differentially to the
318 applied treatments, particularly after the simulated shelf life period (**Fig. 3**), suggesting an
319 association with the compositional changes triggered in fruit cuticle. Both CO_2 and heat

320 shocks restored expression of *PpLACS1* gene (also referred to as *CER8*), involved in *n*-alkane
321 biosynthesis from fatty acyl-CoAs (von Wettstein-Knowles, 2016), to levels similar to those
322 in control fruit upon removal from refrigerated storage, while CO_2 shocks decreased the
323 expression of *PpLACS1* five days thereafter. Conversely, both *PpCER1* and *PpLipase*
324 expression generally mimicked the results observed for refrigerated (non-treated) samples.
325 The observed modifications in gene expression levels were not, or not consistently,
326 accompanied by the expected changes in the corresponding cuticular compounds, due to
327 probable rerouting of biological pathways or activation of other members from the same
328 family. Many genes contribute to the synthesis of cuticular components, and the products of
329 some of the involved genes are known to interact through the formation of complexes since,
330 in heterologous systems, the synthesis of particular compounds was shown to require gene
331 coexpression (Bernard et al., 2012). Substantial research efforts will be required in the future
332 for the full understanding of cuticle formation and relationships with postharvest quality of
333 fruit.
334
335

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Table 1. List of primer sequences used for RT-qPCR analysis of candidate cuticle-related gene expression in 'October Sun' peach fruit at harvest and after cold storage following heat and CO_2 shocks.

Gene [a]	Accession no.	Primers		Amplicon (bp)	Efficiency (%)	Reference
PpLipase	JU090714	F: R:	5'-CTTCCTGGGATCAAACTGGTG-3' 5'-AGCAAGCCACTGATGTCACCT-3'	103	93.4	Alkio et al., 2012
PpCER1	JU090728	F: R:	5'-GCTGAAAATGAAGGTGGTGGA-3' 5'-GGCATAAGCAACCTTGGTGAG-3'	118	100	Alkio et al., 2012
PpLACS1	JU090724	F: R:	5'-GGTTGACCGAAACTTGTGGAC-3' 5'-CCATTTCTGGAACCTCATCCA-3'	120	98.4	Alkio et al., 2012
Reference gene [b]	**Accession no.**	**Primers**		**Amplicon (bp)**		**Reference**
PpCAC	JU090733	F: R:	5'-CTCCGCACTTCCTTTTGGTAC-3' 5'-ATCTCGCAGTTCTTCGTGCTC-3'	87	94.9	Alkio et al., 2012
PpPP2A-2	JU090732	F: R:	5'-TCATCAGCTTGTCATGGAAGG-3' 5'-ATTGCAGCCATATTCCCACAC-3'	105	100	Alkio et al., 2012
PpSRP19	JU090731	F: R:	5'-GGGGAGGTCCTTATCCATGTC-3' 5'-GCCACCTAGCGCATTGTATTC-3'	111	91	Alkio et al., 2012

[a] *PpLipase,* a GDSL/SGNH motif lipase of unknown function; *PpCER1*, unknown gene product, putatively involved in *n*-alkane biosynthesis; *PpLACS1*, a long chain fatty acid-CoA synthetase, putatively involved in both wax and cutin deposition.

[b] *PpCAC*, Clathrin adaptor complexes protein; *PapPP2A-2*, Protein phosphatase 2A-2; *PpSRP19*, Signal recognition particle SRP19.

Table 2. Maturity and quality indicators, and amount of cuticle isolated from 'October Sun' peach fruit at commercial harvest and after cold storage following CO_2 and heat shocks.

		Days at 0 ºC + days at 20 ºC					
		14+0			14+5		
	Harvest	Untreated	30 kPa CO_2	50 ºC	Untreated	30 kPa CO_2	50 ºC
Ethylene production ($\mu g\ kg^{-1}\ s^{-1}$)	0.13 d	0.36 c	0.29 c	0.29 c	2.98 a	2.81 a	1.70 b
CO_2 production ($\mu g\ kg^{-1}\ s^{-1}$)	64.2 c	45.9 d	68.6 c	75.5 b	129.9 a	118.9 a	71.1 b
Weight loss (%)	-	1.4 c	1.1 d	1.4 c	3.0 b	3.0 b	3.6 a
Firmness (N)	49.0 b	50.9 b	54.5 a	48.8 b	5.8 c	6.8 c	6.4 c
SSC (%, w/w)	12.8 c	13.2 bc	13.4 bc	12.9 c	13.4 bc	14.0 a	13.6 ab
TA ($g\ L^{-1}$) [a]	8.1 a	6.9 b	7.7 a	7.4 ab	4.8 d	5.3 c	5.6 c
SSC/TA ratio	1.6 c	1.9 c	1.7 c	1.8 c	2.8 a	2.6 ab	2.4 b
Cuticle amount ($g\ m^{-2}$)	15.1 d	17.4 c	17.5 c	18.7 bc	19.2 b	16.7 c	20.5 a
Wax:cutin ratio	3.1	2.4	2.5	3.0	3.7	2.8	4.6

Values represent means of 3 (ethylene and CO_2 production) or 15 replicates. Different letters within a row denote significant differences among treatments and periods at $P \leq 0.05$ (Fisher's LSD test). [a] Titratable acidity is expressed as equivalents of malic acid.

Table 3. Wax yields, and constituents (relative %) identified in cuticles of 'October Sun' peaches at harvest and after cold storage following CO_2 and heat shocks.

	Harvest	Days at 0 ºC + days at 20 ºC: 14+0			14+5		
		Untreated	30 kPa CO_2	50 ºC	Untreated	30 kPa CO_2	50 ºC
Wax (g m^{-2})	**5.18 d**	**5.53 d**	**5.21 d**	**5.78 d**	**8.04 b**	**6.71 c**	**10.64 a**
Wax (% total cuticle)	34.3 c	31.8 c	29.8 c	30.9 c	41.9 b	40.3 b	51.9 a
Triterpenes (% waxes)	**51.92 b**	**51.09 b**	**54.28 a**	**54.57 a**	**48.75 c**	**47.74 cd**	**46.68 d**
Oleanolic acid	22.73	21.50	23.63	23.46	20.91	20.33	19.62
Ursolic acid	29.19	29.59	30.65	31.11	27.84	27.41	27.06
Alkanes (% waxes)	**19.57 ab**	**20.02 a**	**19.22 ab**	**19.53 ab**	**18.96 b**	**18.57 b**	**20.67 a**
Dodecane (C12)	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Tridecane (C13)	0.01	0.01	0.01	0.01	0.01	tr	tr
Tetradecane (C14)	0.01	tr	tr	tr	0.01	0.01	0.01
Pentadecane (C15)	tr	nd	tr	tr	0.01	0.02	0.02
Hexadecane (C16)	0.66	0.71	0.69	0.73	0.38	0.41	0.39
Heptadecane (C17)	tr	tr	0.01	0.01	0.01	0.02	0.01
Octadecane (C18)	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Nonadecane (C19)	0.29	0.28	0.26	0.26	0.32	0.30	0.26
Henicosane (C21)	0.05	0.12	0.08	0.06	0.25	0.26	0.18
Docosane (C22)	0.44	0.41	0.46	0.44	0.57	0.70	0.64
Tricosane (C23)	3.03	3.40	2.86	3.00	3.26	3.36	3.44
Tetracosane (C24)	0.84	1.38	1.03	0.97	1.21	0.79	1.18
Pentacosane (C25)	4.61	4.58	4.53	4.50	4.49	4.34	4.54
Hexacosane (C26)	0.53	0.74	0.61	0.63	0.41	0.53	0.81
Heptacosane (C27)	3.61	3.36	3.53	3.53	3.22	2.99	3.50
Octacosane (C28)	0.67	0.81	0.69	1.05	0.67	0.70	1.75
Nonacosane (C29)	3.95	3.50	3.78	3.65	3.36	3.52	3.26
Triacontane (C30)	0.15	0.14	0.10	0.14	0.13	0.13	0.17
Hentriacontane (C31)	0.70	0.56	0.56	0.53	0.63	0.47	0.49
Fatty Acids (% waxes)	**8.27 c**	**10.26 b**	**8.74 c**	**7.76 c**	**14.25 a**	**14.80 a**	**13.39 a**
C12:0	0.01	0.01	0.02	0.03	0.03	0.02	0.02
C14:0	0.04	0.05	0.05	0.05	0.06	0.06	0.05
C15:0	0.01	0.01	0.01	0.01	0.01	0.01	0.01
C16:0	2.17	2.72	2.23	2.10	3.68	3.82	3.47
C17:0	0.01	0.02	0.02	0.01	0.01	0.02	0.01
C18:0	1.10	1.24	1.07	0.78	1.66	1.91	1.70
C18:1 (9)	2.63	2.34	2.43	2.37	3.12	3.37	2.97
C18:2 (9, 12)	2.30	3.86	2.89	2.39	5.66	5.58	5.15
trans-C18:2 (9, 12)	0.02	0.01	0.02	0.02	0.02	0.01	0.06
Phytosterols (% waxes)	**2.05 a**	**1.56 c**	**1.85 b**	**2.03 a**	**1.84 b**	**1.62 c**	**2.05 a**
Campesterol	1.62	1.15	1.21	1.35	1.36	1.14	1.58
β-Sitosterol	0.43	0.41	0.64	0.68	0.48	0.48	0.47
Fatty Alcohols (% waxes)	**3.25 b**	**3.30 b**	**3.18 b**	**3.50 b**	**3.41 b**	**3.06 b**	**4.21 a**
Tetradecanol (C14)	nd	nd	nd	nd	nd	nd	0.09
Octadecanol (C18)	nd	nd	0.21	0.12	0.07	0.20	0.21
Tricosanol (C23)	1.08	1.17	0.78	0.89	1.11	0.87	0.77
Tetracosanol (C24)	1.18	1.05	1.17	1.24	1.14	1.06	2.05
Hexacosanol (C26)	0.12	0.11	0.09	0.11	0.11	0.10	0.09
Octacosanol (C28)	0.49	0.57	0.54	0.67	0.57	0.47	0.59
Triacontanol (C30)	0.38	0.40	0.39	0.47	0.41	0.36	0.41
Identified wax (%)	**85.06**	**86.22**	**87.27**	**87.38**	**87.21**	**85.79**	**87.00**
Unidentified (%)	**14.94**	**13.78**	**12.73**	**12.62**	**12.79**	**14.21**	**13.00**
Acyclic to cyclic ratio	**0.58**	**0.63**	**0.55**	**0.54**	**0.72**	**0.74**	**0.76**
ACL acyclic compounds [a]	**23.5**	**23.0**	**23.2**	**23.5**	**22.3**	**22.2**	**22.7**

Values represent means of three replicates (nd, non-detectable). Mean values followed by a different lower-case letter within the same row are significantly different at $P \leq 0.05$ (LSD test). [a] ACL, weighted average chain length of acyclic compounds.

Table 4. Cutin yields, and constituents (relative %) identified in cuticles of 'October Sun' peaches at harvest and after cold storage following CO_2 and heat shocks.

	Harvest	Days at 0 ºC + days at 20 ºC: 14+0			14+5		
		Untreated	30 kPa CO_2	50 ºC	Untreated	30 kPa CO_2	50 ºC
Cutin (g m^{-2})	**1.64 c**	**2.32 a**	**2.09 ab**	**1.92 b**	**2.17 ab**	**2.43 a**	**2.31 a**
Cutin (% total cuticle)	10.9 c	13.3 ab	12.0 b	10.3 c	11.3 bc	14.6 a	11.3 bc
α-Monocarboxylic acids (%)	**29.28 b**	**27.44 bc**	**26.34 c**	**30.01 ab**	**28.73 b**	**32.71 a**	**31.53 a**
C14:0	0.05	0.15	0.17	0.15	0.15	0.16	0.14
C15:0	0.05	0.21	0.15	0.16	0.16	0.15	0.13
C16:1 (9)	6.08	5.25	5.36	5.94	5.63	6.92	6.56
C18:1 (9)	10.56	9.37	8.90	9.70	9.12	9.47	9.44
C18:2 (9, 12)	5.71	6.15	5.11	7.18	6.67	9.49	9.02
C20:0	1.62	1.21	1.06	1.42	1.26	1.32	1.37
C22:0	4.78	4.33	4.70	4.60	4.59	4.38	3.95
C26:0	0.42	0.48	0.56	0.56	0.54	0.53	0.38
C28:0	nd	0.29	0.34	0.32	0.33	0.30	0.26
C30:0	nd	nd	nd	nd	0.29	nd	0.28
Hydroxyacids (%)	**30.17 b**	**33.33 a**	**33.91 a**	**31.29 ab**	**33.04 a**	**30.49 b**	**31.17 ab**
2-OH $C_{16:1 (9)}$	2.55	1.98	1.71	2.10	2.02	2.38	2.55
16-OH $C_{16:0}$	0.28	1.11	0.92	1.13	1.34	0.97	0.16
18-OH $C_{18:1 (9)}$	20.15	21.70	22.34	20.42	21.75	19.47	19.98
18-OH $C_{18:2 (9, 12)}$	7.19	8.55	8.94	7.65	7.92	7.67	8.48
α,ω-Dicarboxylic acids (%)	**13.34 b**	**13.54 b**	**14.73 a**	**14.13 ab**	**14.49 a**	**13.72 b**	**13.75 b**
1,16-dioic $C_{16:0}$	1.99	1.76	2.06	1.98	1.87	1.86	1.82
1,18-dioic $C_{18:1 (9)}$	9.39	10.19	10.75	10.37	10.62	9.62	9.44
1,18-dioic $C_{18:2 (9, 12)}$	1.72	1.58	1.92	1.78	1.77	2.24	2.50
1,22-dioic $C_{22:0}$	0.24	nd	nd	nd	0.24	nd	nd
Identified cutin (%)	**72.79**	**74.31**	**74.98**	**75.43**	**76.26**	**76.92**	**76.45**
Unidentified (%)	**27.21**	**25.69**	**25.02**	**24.57**	**23.74**	**23.08**	**23.55**

Values represent means of three replicates (nd, non-detectable). Mean values followed by a different lower-case letter within the same row are significantly different at $P \leq 0.05$ (LSD test).

FIGURE CAPTIONS

Figure 1.

Amount (g m^{-2}) of the main chemical types of wax constituents identified in cuticles of 'October Sun' peaches at harvest and after cold storage following CO_2 and heat shocks (H, at harvest; UT, untreated). Storage time is represented as X+Y (X, days at 0 ºC; Y, days at 20 ºC following cold exposure). Values are the means of three replicates. Bars bearing different letters represent significantly different total wax amounts at $P \leq 0.05$ (LSD test).

Figure 2.

Amount (g m^{-2}) of the main chemical types of cutin monomers identified in cuticles of 'October Sun' peaches at harvest and after cold storage following CO_2 and heat shocks (H, at harvest; UT, untreated). Storage time is represented as X+Y (X, days at 0 ºC; Y, days at 20 ºC following cold exposure). Values are the mean of the replicates. Bars bearing different letters represent significantly different total cutin amounts at $P \leq 0.05$ (LSD test).

Figure 3.

Relative expression of selected candidate genes involved in cuticle biosynthesis in the skin tissue of 'October Sun' peaches after cold storage following CO_2 and heat shocks. For each condition, relative levels of gene expression represent means of three replicates after normalization with the geometric average of the three reference genes. For a given gene, different letters stand for significant differences at $P \leq 0.05$ (LSD test).

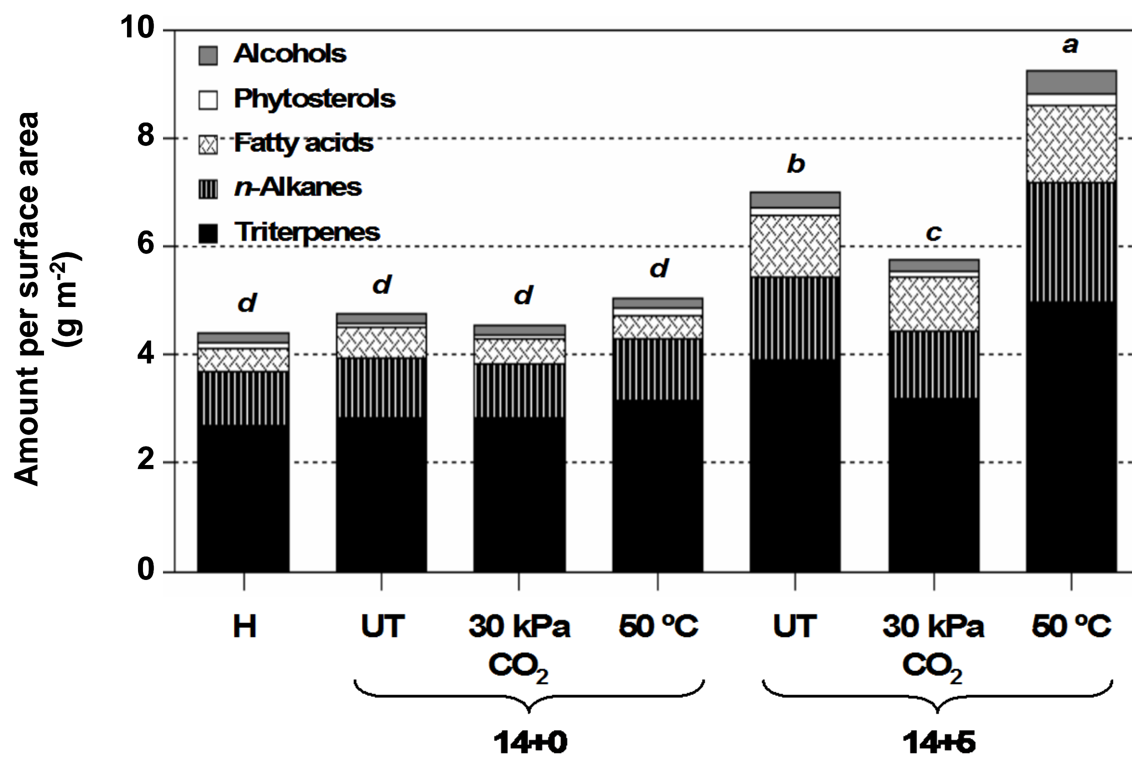

Figure 1

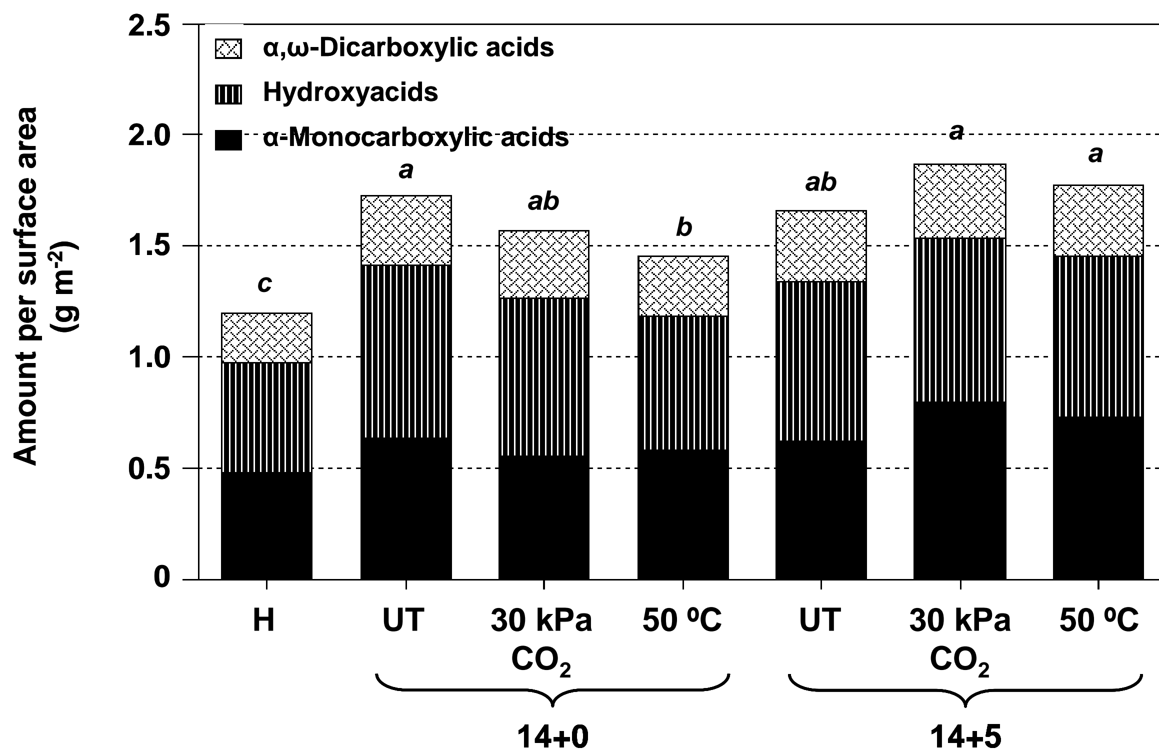

Figure 2

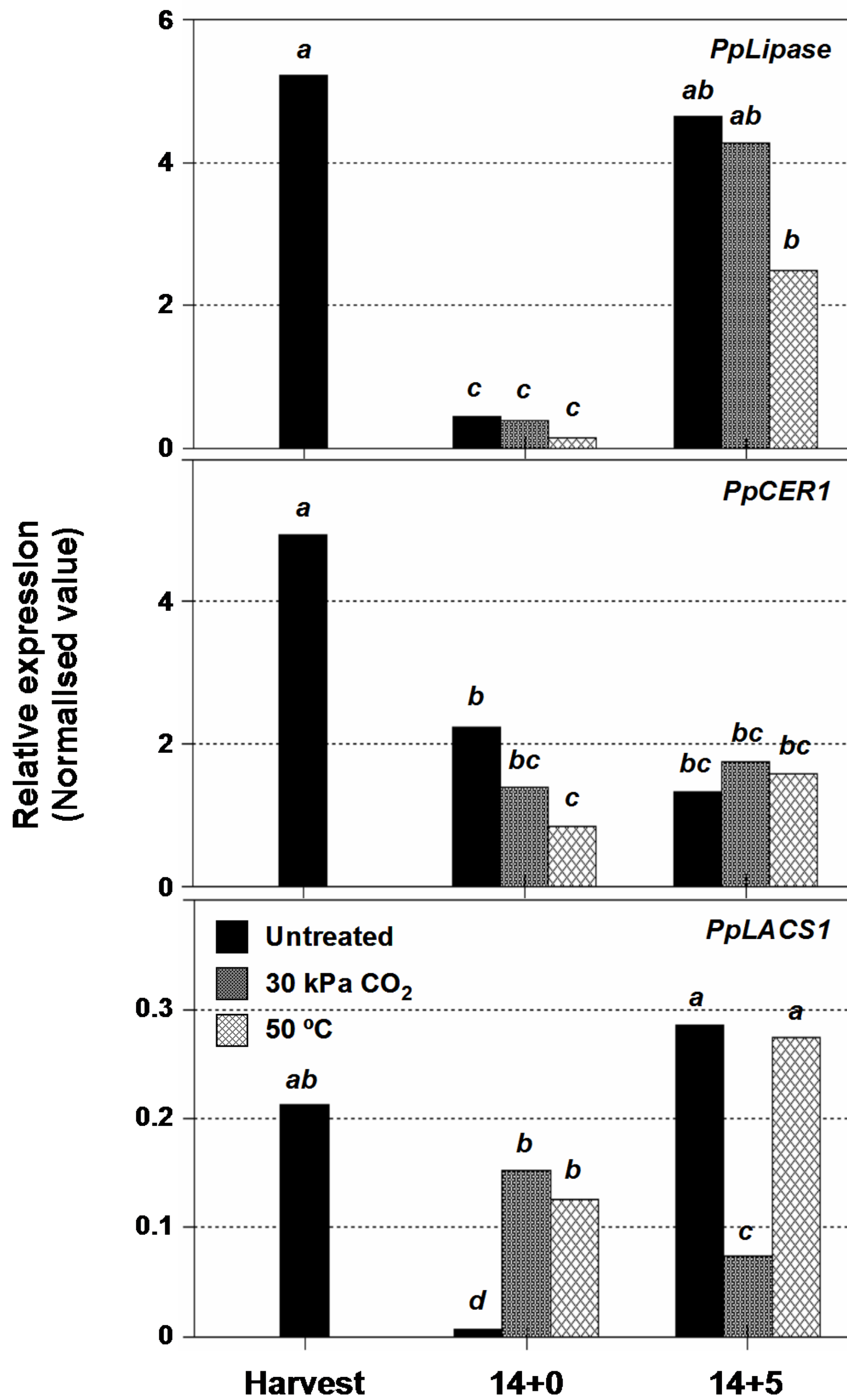

Figure 3