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1 **Ethylene and abscisic acid play a key role in modulating apple ripening after**

2 **harvest and after cold-storage**

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14

## Abstract

An autocatalytic burst in ethylene production generally accompanies ripening of detached apple fruit. However, if apples are left to ripen attached to the tree, some cultivars, will not experience this autocatalytic ethylene production and the fruit will never acquire the organoleptic properties of detached ripened fruit. Accordingly, the present study aimed to understand how the hormonal crosstalk regulates ripening in 'Golden Reinders' apples, ripened on-tree, detached from the tree or after a period of cold storage, following the commercial harvest date. Our results show that during on-tree ripening, ethylene production remained low, and no significant changes were observed in fruit colour or firmness. In fruit ripened detached from the tree, ethylene production was preceded by an increase of indole 3-acetic acid (IAA) and gibberellic acid ($GA_3$) levels, whereas a cold-induced accumulation of jasmonic acid (JA) seem to induce an earlier initiation of the climacteric burst in fruit ripened after cold storage. In both postharvest conditions, ethylene itself was no able to trigger fruit softening and degreening until abscisic acid (ABA) accumulated, pointing out the importance of ethylene and ABA in mediating apple ripening. In contrast, changes in sugars and ROS/antioxidants did not vary among the different ripening scenarios suggesting that none of the measured compounds may act as signalling molecules during apple ripening. Collectively, our results highlight that ethylene together with ABA played a crucial role in triggering ripening-related changes during postharvest ripening of 'Golden Reinders' apples.

**Keywords**: ABA, ACO, degreening, hormonal cross-talk, *Malus* x *domestica*, softening

## 1. Introduction

Fruit ripening is a hormonally regulated process which involves physicochemical changes including softening, flavour acquisition and peel degreening (Forlani et al., 2019). Based on the role of ethylene during ripening, fruit can be classified as climacteric or non-climacteric (Paul et al., 2012). Apples are considered climacteric fruit since the onset of ripening follows an increase in the respiration rate and ethylene production (Giovannoni, 2004). This said, on-tree ripening for most climacteric species is either delayed or inhibited if compared to fruit ripened off-tree (Paul et al., 2012) resulting in fruit unable to reach an optimal organoleptic quality (Lindo-García et al., 2019). Such inhibition has been related to the presence of an inhibiting substance earlier referred as the 'tree factor' (Abeles, 1973), which is thought to be translocated from the leaves to the fruit via the phloem. The 'tree factor' causes an inhibition of the fruit ethylene production impairing the transition from system 1 (autoinhibitory) to system 2 (autocatalytic) ethylene production (Sfakiotakis and Dilley, 1973). Nonetheless, the existence of the 'tree factor' is yet an unproven hypothesis, and other factors such as environmental cues may affect the fruit capacity to produce ethylene (Lin and Walsh, 2008; Fernández-Cancelo et al., 2021).

Recent studies have shown that ABA, in combination with other hormones such as auxins, gibberellins and cytokinins, not only regulate the ripening of non-climacteric fruit (Fuentes et al., 2019), but also participate in a complex hormonal cross-talk with ethylene, triggering climacteric fruit ripening (Liu et al., 2020). An increase of endogenous ABA levels was found to precede the autocatalytic ethylene production and ripening, both during on-tree (Lindo-García et al., 2020b) and post-harvest ripening (García-Pastor et al., 2021) of climacteric fruit, suggesting that the shift from system 1 to system 2 may be, to some extent, triggered by ABA (Mou et al., 2016). Moreover, the regulation of ethylene

64 production by other hormones seems to be dependent on both the fruit variety and the
65 developmental stage (Ferrante et al., 2017). During the first developmental stages the high
66 levels of auxins, GAs and jasmonates appear to act as negative regulators of ethylene
67 metabolism in multiple fruit species, whereas during the last stages of ripening the levels
68 of these hormones drop allowing the onset of ethylene production (Fenn and Giovannoni,
69 2021). Besides phytohormones, other compounds such as sugars and reactive oxygen
70 species (ROS) also participate in fruit ripening signalling networks (Steelheart et al.,
71 2019). Indeed, fruit ripening is *per se* an oxidative process associated with the
72 accumulation of ROS such as $H_2O_2$ (Huan et al., 2016). In tomato, the accumulation of
73 $H_2O_2$ stimulates the induction of ripening-related genes including those encoding ACC
74 oxidase (ACO), ACC synthase (ACS) and polygalacturonases (PG) (Kumar et al., 2016).
75 In pears, an oxidative burst seems to initiate off-tree ripening, likely by triggering the
76 transition from system 1 to system 2 (Lindo-García et al., 2019; 2020a), whereas on-tree
77 pear ripening seems to be regulated by a complex signalling cascade in which sucrose
78 was thought to play a predominant role (Lindo-García et al., 2019). Indeed, the role of
79 sucrose as a ripening signalling molecule is also evident in non-climacteric fruit, such as
80 strawberry, since exogenous sucrose treatment stimulates ABA production and fruit
81 ripening (Jia et al., 2013). However, scarce information is available regarding the
82 influence of ROS and sugar signalling on apple ripening and especially if looking at its
83 possible crosstalk with hormones.
84 Apple (*Malus x domestica Borkh*) is an excellent model to study differences among on-
85 and off-tree ripening since attached fruit show limited or no-ripening whereas fruit that
86 has been cold-stored for relatively long periods, and then rewarmed, generally suffers
87 accelerated ripening (Larrigaudière et al., 1997; Gago et al., 2015). Considering all this
88 information, the aim of this study was to elucidate how the cross-talk between ethylene

and other hormones, ROS and sugars may differentially regulate on-tree and postharvest ripening (immediately after the commercial harvest or after a period of cold storage) in ‘Golden Reinders’ apples.

## 2. Material and methods

### *2.1. Fruit material and experimental design*

Three ripening scenarios, including on-tree ripening and two post-harvest conditions (off-tree and post-cold storage ripening, both at 20ºC) were studied using ‘Golden Reinders’ apples. In all cases, fruit were harvested from a commercial orchard located in Alpicat (Lleida, Catalonia, Spain). For the on-tree study, apples were harvested every 5 days starting from the Optimal Harvest Date (OHD) for 25 days. In the off-tree assay, fruit was picked at the OHD and samples were evaluated every 5 days during storage at 20ºC and 85% of relative humidity (RH) for up to 25 days. In parallel, another batch of fruit was harvested at the OHD and kept at 0 °C and 90 % RH for 2 months. After this period, apples were removed from cold storage, kept in an acclimatized room at 20 °C and analysed each 5 days for 25 days. The OHD corresponded to 160 days after full bloom (DAFB) and was based on commercial standard practices which consider the fruit diameter (75-85mm), background colour (equivalent $H^o$=98-103), firmness (F≥68N) and starch index (4-7) of the fruit (Fernández-Cancelo et al., 2021).

At each evaluation point, 20 apples were used for quality determinations and 20 fruit (4 replicates of 5 apples) for measuring ethylene production. At each time peel and pulp from the same fruit used for ethylene measurements were ground and frozen in liquid nitrogen and kept at -80ºC until further biochemical analysis.

### *2.2. Fruit quality evaluation*

114 Colour, firmness and starch index were measured on 20 individual fruit per ripening
115 scenario and sampling point. In the case of colour and firmness determinations,
116 measurements were made on two opposite sides of each examined fruit using a portable
117 colorimeter (CM-2600d; Konica Minolta Sensing, Japan) and a GÜSS FTA penetrometer
118 (FR Turoni, Foly, Italy), respectively. Starch index was evaluated by dipping equatorial
119 fruit slices in an iodine solution ($I_2$-KI) for ten minutes. The starch index was assigned to
120 each fruit using the starch scale from 1 to 10 developed by the Centre Technique
121 Interprofessionnel des Fruits et Légumes (CTIFL; France).
122

123 *2.3. Ethylene production and ethylene-related metabolites and enzymes*

124 At each sampling point, four replicates of 5 fruit each were placed in an acclimatized
125 chamber at 20 °C in 3.8 L flasks sealed with a silicon septum. After 2 h incubation,
126 ethylene production (nmol $kg^{-1}$ $s^{-1}$) was measured by taking 1 mL of gas from the
127 headspace of the flask with a syringe and injected into a gas chromatograph (GC; Agilent
128 Technologies 6890, Wilmington, Germany) fitted with a FID detector and an alumina
129 column F1 80/100 (2 m × 1/8 × 2.1, Tecknokroma, Barcelona, Spain) as previously
130 described by Lindo-García et al., (2020a).
131 Determination of enzymatic activities (ACO and ACS) and metabolites levels (ACC and
132 MACC) involved in ethylene metabolism were carried out on apple flesh samples
133 according to Bulens et al., (2011) protocols.
134

135 *2.4. Hormonal profiling*

136 Phytohormones were extracted by mixing 100 mg of the apple pulp samples with 200 mL
137 methanol:isopropanol:acetic acid, 50:49:1 (v/v/v) and using ultrasonication and vortexing
138 (Branson 2510 ultrasonic cleaner, Bransonic, USA) for 30 min. Deuterium-labelled

139 internal standards were added. After centrifugation, the pellet was re-extracted using the
140 same procedure and the collected supernatants were merged and filtered through a
141 0.22 mm PTFE filter (Waters, USA) before analyses. Phytohormones were analysed by
142 UHPLC-ESI–MS/MS. The system consisted of an Aquity UPLC™ System (Waters)
143 quaternary pump equipped with an autosampler. An HALO™ C18 (Advanced Materials
144 Technology Inc., USA) column (2.1 × 75 mm, 2.7 µm) was used. Solvent A was water
145 with 0.05 % glacial acetic acid (Sigma-Aldrich, Steinheim, Germany) and solvent B was
146 acetonitrile (Sigma-Aldrich) with 0.05 % glacial acetic acid. Flow rate was set at 0.6 mL
147 min$^{-1}$. Quantification of each sample was adjusted for recovery rate using the deuterium-
148 labelled internal standards (Müller and Munné-Bosch, 2011) and the results expressed on
149 fresh weight basis (µg kg$^{-1}$).
150

151 *2.5. Antioxidants and oxidative stress markers*

152 Based on the previous protocol of Rassam and Laing, 2005, the extraction of ascorbic acid
153 was carried out mixing 2.5 g of frozen pulp tissue with 5 mL of metaphosphoric acid
154 suspension (3% MPA, 8% acetic acid) and centrifuging at 24,000 *g* for 22 min at
155 4ºC. Quantification of ascorbic acid (AsA) and total ascorbic acid was performed as
156 described by Fernández-Cancelo et al., (2021). Dehydroascorbic acid (DHA) was
157 calculated by subtracting the ascorbic acid content from that of total ascorbic acid.
158 Antioxidant capacity (AC) of the apple pulp was determined using frozen tissue as
159 previously described (Giné Bordonaba and Terry, 2008) by mixing 3 g of apple frozen
160 pulp tissue with 10 mL of 79.5% (v/v) methanol and 0.5% (v/v) HCl in Mili-Q water.
161 Sample extraction was held at 20 ºC with constant shaking for 2 h and mixing the samples
162 every 30 min. The extract was centrifuged at 24,000 *g* for 30 min at 20 ºC. Antioxidant

163 capacity was measured by the Ferric Reducing Antioxidant Power (FRAP) assay as
164 described in previous works (Giné-Bordonaba et al., 2016).
165 Hydrogen peroxide concentrations, expressed in mmol $kg^{-1}$, were determined using the
166 PeroxiDetect Kit (Sigma Aldrich, USA) after the extraction of 2.5 g of frozen pulp in
167 10 mL of 5% trichloroacetic acid (TCA) based on the protocol described by Giné-
168 Bordonaba et al. (2019).
169 Malondialdehyde (MDA) was analysed as an index of lipid peroxidation using the
170 thiobarbituric acid reactive substances (TBARS) assay based on the protocol previously
171 described (Martínez-Solano et al., 2005), using 0.5 g of frozen pulp mixed with 4 mL of
172 0.1% trichloroacetic acid (TCA) solution. Absorbance measurements were made at 532
173 nm and 600 nm to avoid the contribution of interfering species to MDA quantification.
174

175 *2.6. Sugars determination*

176 The protocols described by Lindo-García et al., (2019) were used for extracting sugars
177 (sucrose, glucose and fructose) from 2 g of frozen pulp tissue. The supernatants of each
178 extraction were recovered and used for enzyme coupled spectrophotometric
179 determination of glucose and fructose (hexokinase/phosphoglucose isomerase) and
180 sucrose (β-fructosidase) using commercial kits (BioSystems S.A., Barcelona, Spain).
181

182 *2.7. Carotenoids and chlorophylls quantification*

183 Carotenoids and chlorophylls were extracted based on the method described by Alagoz
184 et al., (2020) by mixing 100 mg of freeze-dried flesh tissue with 800 µL of acetone-ethyl
185 acetate (6:4, v/v) solution containing 0.1% butylated hydroxytoluene (BHT), and 0.1%
186 canthaxanthin (0.5 mg $mL^{-1}$) as internal standard. An equal volume of water was added,
187 samples were mixed by inversion, and centrifuged 5 min at 12,000 *g* at 4 ºC. The upper

188 phase was collected and centrifuged again 5 min at 12,000 *g* at 4 ºC. The organic extract
189 was filtered through a 0.22 µm filter and injected (20 µL) on an Agilent 1260 Infinity II
190 liquid chromatograph HPLC fitted with a YMC C30 Carotenoid column (250 mm × 4.
191 mm i.d., 3 µm; Teknokroma, Barcelona, Spain) and a guard column of the same material
192 (10 mm × 4.0 mm, 3 µm). Separation was carried out at a flow rate of 1 mL/min using a
193 binary-gradient elution initially composed by 95% methanol and 5% methyl tert-butyl
194 ether (MTBE), which was increased linearly to 25% MTBE in 15 min, then raised to 40%
195 in 2 min, elevated to 50% in 3 min and finally raised to 100% in 3 min and maintained
196 for 10 min. The temperature of the column was kept at 25 °C and the sample compartment
197 was refrigerated at 10 °C. Detection was performed at 454 nm yet the online spectra was
198 acquired in the 330-700 nm wavelength range with a resolution of 1 nm. Carotenoids and
199 chlorophylls were identified according to their retention time, spectral features, and ratios
200 of maximum absorption peaks (λ). Identified compounds were quantified using an
201 external calibration curve prepared with a canthaxanthin standard stock solution and their
202 canthaxanthin equivalent concentration were expressed as mg $kg^{-1}$.
203
204 *2.8. Statistical data analysis*
205 Data was subjected to analysis of variance (ANOVA) tests using JMP 13.1.0 SAS Institute
206 (Cary, North Carolina, USA). Least significant difference values (LSD; p = 0.05) for the
207 interaction ripening scenario*time were calculated for mean separation using critical
208 values of t for two-tailed tests. Spearman's rank correlation matrix ($p \leq 0.05$) was done
209 using the R corrplot package (Wei et al., 2017).
210
211
212
213

214 **3. Results**

215 *3.1. Fruit quality parameters*

216 Both firmness, colour and starch parameters showed different evolution patterns
217 depending on the ripening scenario. During on-tree ripening, firmness remained around
218 70 N, while during off-tree ripening, firmness dropped from 72 ± 3.9 N to 45 ± 6.0 N
219 after 20 days at 20ºC. Cold storage induced a firmness loss of ca. 14 %, since fruit
220 firmness immediately upon removal from cold storage was 62 ± 6.0 N. After subsequent
221 ripening at 20ºC, fruit firmness remained at 62 ± 6.1 N until day 5 and declined thereafter
222 to reach 51 ± 6.1 N at day 15 or 20 (Figure 1A). A similar behaviour was observed in the
223 colour parameters, which remained unchanged in fruit ripened on-tree. In contrast, both
224 off-tree or post-cold stored fruit showed significant colour changes after 15 days,
225 corresponding with a drop in the *Hue angle* (*Hº* below 95º; Figure S1D) and concomitant
226 to an increase in the carotenoids/chlorophylls ratio (Figure S1C). Fruit ripened on-tree
227 never attained the yellow colour typical from ripe Golden apples. Interestingly, the
228 observed colour changes in detached ripened fruit were not consequence of an
229 accumulation of carotenoids, which levels remained relatively constant under all ripening
230 scenarios (0.06 ± 0.013 mg $kg^{-1}$), but rather caused by a drop in the chlorophyll content
231 of the apple peel to levels below 0.02 mg $kg^{-1}$ (Figure S1B). Although on-tree ripening
232 did not lead to significant changes in fruit firmness or colour, starch index significantly
233 increased from to 4 ± 1.2 to 7 ± 1.0 while the index of absorbance difference ($I_{AD}$; data
234 not shown) showed a downward trend during the 25 days of on-tree ripening. A faster
235 starch degradation was however observed during off-tree ripening, reaching the
236 maximum index value (10) twenty days after harvest. After cold storage, starch index
237 values were already 9 ± 2.0 at removal and rapidly increased to 10 (Figure 1B).

238

239 *3.2. Ethylene metabolism*

240 Accompanying the quality changes described above, ethylene production and
241 biosynthesis significantly differed ($p<0.05$) among the different ripening scenarios. While
242 ethylene production remained below 0.003 nmol $kg^{-1}$ $s^{-1}$ during on-tree ripening, an
243 enhanced ethylene biosynthesis was observed for fruit ripened detached or after cold-
244 storage. The maximum ethylene levels were *ca*. 2 nmol $kg^{-1}$ $s^{-1}$ for both off-tree and post-
245 cold storage ripening yet being reached in post-cold stored fruit 10 days earlier than in
246 off-tree ripened fruit (Figure 2D).

247 The enzymes (ACS and ACO) and intermediates (ACC and MACC), involved in the two
248 last steps of ethylene biosynthesis, remained at basal levels during on-tree ripening
249 (Figure 2), while both off-tree and post cold storage ripening enhanced the activity of
250 ACS and ACO (Figures 2B and 2C). During off-tree ripening, ACS activity increased at
251 a rate of 0.04 ± 0.011 nmol $kg^{-1}$ $s^{-1}$ per day from day 5 until reaching a maximum of 1.20
252 ± 0.181 nmol $kg^{-1}$ $s^{-1}$ at day 25. Likewise, post-cold storage ripening also induced ACS
253 activity, observing an increase from 0.33 ± 0.020 nmol $kg^{-1}$ $s^{-1}$ at day 0 to 1.60 ± 0.175
254 nmol $kg^{-1}$ $s^{-1}$ at day 20, declining thereafter until 1.08 ± 0.248 nmol $kg^{-1}$ $s^{-1}$ at day 25.

255 ACO activity of on-tree ripened fruit remained unchanged. During off-tree ripening, ACO
256 activity remained below 0.50 nmol $kg^{-1}$ $s^{-1}$ during the first 15 days, hence in line with the
257 activity observed during on-tree ripening. However, at day 20, ACO activity in off-tree
258 ripened fruit increased by 6-fold and reached 3.00 ± 0.281 nmol $kg^{-1}$ $s^{-1}$. After cold
259 storage, apples showed higher ACO activity compared with on-tree and off-tree ripened
260 apples, reaching a constant value of 5.70 ± 0.571 nmol $kg^{-1}$ $s^{-1}$ already at day 5.

261

262 *3.3. Hormonal profile*

263 Among the different hormones analysed, ABA was the one showing the greatest
264 differences among the different ripening scenarios and especially when comparing on-
265 tree ripened fruit with those ripened at 20ºC (Figure 3A). During the first 5 days, ABA
266 levels remained below 50 µg $kg^{-1}$ regardless of the ripening scenarios. From day 10
267 onwards, ABA levels followed the same trend in both postharvest scenarios, steadily
268 increasing up to 141 ± 24.0 µg $kg^{-1}$. No significant differences were found in IAA and
269 $GA_3$ levels (Figures 3B and 3C) among the ripening scenarios, except in off-tree ripened
270 apples at day 5 (after harvest or after removal from cold storage) when a peak (151 ± 41.6
271 µg $kg^{-1}$ and 54 ± 20.4 µg $kg^{-1}$, respectively) was observed for both hormones. In the case
272 of jasmonic acid (Figure 3D), the maximum levels were observed immediately after cold
273 storage (10.6 ± 2.26 µg $kg^{-1}$ at day 0), with no clear trends for this hormone observed
274 thereafter.
275
276 *3.4. Oxidative stress markers*
277 The different ripening scenarios tested did not change the fruit antioxidant capacity (AC)
278 or the levels of MDA, which remained constant during the 25 days of ripening (Figures
279 4A and 4E). However, significant differences in AsA, DHA and $H_2O_2$ levels were
280 observed, especially when comparing fruit ripened on-tree and off-tree. Whereas levels
281 of AsA remained below 10 mg $kg^{-1}$ in both postharvest ripening scenarios, on-tree
282 ripening induced the accumulation of AsA levels from 10 ± 1.7 mg $kg^{-1}$ at day 0 to 21 ±
283 3.6 mg $kg^{-1}$ at day 15 and remained stable thereafter (Figure 4B). In the case of oxidised
284 ascorbic acid (DHA), on-tree and off-tree samples showed a similar declining trend from
285 *ca.* 10 mg $kg^{-1}$ to 2 mg $kg^{-1}$, whereas after cold-storage low DHA levels were found
286 immediately upon removal and remaining constrant thereafter at around 2 mg $kg^{-1}$(Figure
287 4D).

288 The three different ripening scenarios studied led to three notably different patterns in
289 $H_2O_2$ (Figure 4C). For example, $H_2O_2$ levels were higher and relatively constant (19 ± 1.9
290 mmol $kg^{-1}$) during the 25 days of apples ripened on-tree. Similar levels were observed in
291 off-tree ripened fruit yet a sharp drop (2-fold) was seen at day 15. Cold storage reduced
292 by *ca*. 40% the content of $H_2O_2$ if compared to the values at harvest. Under this scenario,
293 $H_2O_2$ levels remained unchanged during the first five days (10 ± 0.8 mmol $kg^{-1}$),
294 decreased up to day 10 and remained then stable (4 ± 1.5 mmol $kg^{-1}$) until the end of the
295 shelf-life period.
296
297 *3.5. Sugars content*
298 Fructose was the main sugar found in the apple pulp (51 ± 6.5 g $Kg^{-1}$), followed by sucrose
299 (21 ± 4.3 g $Kg^{-1}$) and glucose (19 ± 1.6 g $Kg^{-1}$). Generally, no significant differences were
300 observed regarding the sugar content among the different ripening scenarios (Figure S2).
301 Indeed, sugar content remained relatively unchanged in all the ripening scenarios.
302
303 **4. Discussion**
304 *4.1. Ethylene: a key element differentiating on- and off-tree ripened apples*
305 Previous studies on 'Golden Reinders' apple have shown that ethylene production during
306 on-tree ripening was to some extent inhibited (Fernández-Cancelo et al., 2021), resulting
307 in some ripening traits, known to be regulated by ethylene, such as colour, firmness or
308 taste not being able to correctly evolve and hence limiting to some extent fruit quality
309 (Harker et al., 2008). In addition, the lack of changes in some ripening-related traits may
310 lead to harvest fruit at an inadequate ripening stage, affecting negatively their storability
311 (Guerra and Casquero, 2010) and organoleptic properties (Echeverría et al., 2004). The
312 absence of colour or firmness changes during on-tree ripening may indicate that apples

313 harvested at advanced harvest dates were younger compared with those harvested near
314 the OHD. However, the observed changes in some ripening parameters such as $I_{AD}$ and
315 starch index, as well as the tendency towards bigger fruit at later harvests (data not
316 shown), confirmed that apples were harvested at different ripening stages (in terms of
317 age).
318 In the present study, the fruit capacity to produce ethylene was strongly influenced by the
319 different ripening scenarios. As observed in 'Conference' pears (Lindo-García et al.,
320 2020b) and 'Gala' apples (Lin and Walsh, 2008), ethylene production during on-tree
321 ripening of 'Golden Reinders' apples remained at basal levels -known as system 1-, and
322 likely preventing fruit ripening. Once apples ripened detached from the tree, such
323 inhibition of the ethylene production capacity disappeared and the transition to system 2
324 occurred, leading to the onset of ethylene production within the two postharvest scenarios
325 investigated (fruit ripened detached immediately after harvest or after cold storage).
326 Although traditionally it has been considered that ethylene production is mainly ruled by
327 ACS (Yoon, 2015), our study suggests that ACO may actually be the main modulator of
328 ethylene production once ethylene metabolism was activated in detached fruit (Figure
329 2C). These results are in agreement with previous studies in apples (Fernández-Cancelo
330 et al., 2021) and pears (Lindo-García et al., 2020a).
331 In this work, some ripening traits such as softening and degreening were triggered after
332 the onset of the fruit ethylene production and mainly during postharvest ripening (Figure
333 1) regardless on whether the fruit was cold-stored or not. Other traits, such as starch
334 content, and the $I_{AD}$, seemed to change even when ethylene remained at basal levels
335 (Figures 1B and 2). Earlier studies suggested that starch degradation in 'Golden' apples
336 is an ethylene-independent process (Blankenship and Unrath, 1988), but the high
337 correlation between starch index and ethylene found herein rather suggest that to some

338 extent ethylene may be involved in starch degradation (Figure S3). Since ethylene
339 production remained low during on-tree ripening, the observed increase in the starch
340 index on-tree was likely associated with a higher sensitivity to ethylene (Johnston et al.,
341 2009) but not ethylene itself. Interestingly, the different starch degradation kinetics
342 among ripening scenarios did not affect sugar levels (Figures S2), hence in agreement
343 with previous studies in 'Golden Reinders' apples (Fernández-Cancelo et al., 2021)
344 showing that these taste-related compounds remained relatively unchanged during the last
345 stages of apple ripening. In this regard, further studies are encouraged to understand starch
346 metabolism in apple fruit and its relationship with ethylene (at the biosynthetic or
347 perception level), especially if considering that starch has been recently pointed out as a
348 key compound differentiating the climacteric *vs.* non-climacteric fruit nature (Chervin,
349 2020).
350
351 *4.2. Hormonal crosstalk and regulation of ethylene biosynthesis*
352 The onset of ethylene production in some climacteric fruit is usually induced by ABA
353 through the activation of genes encoding for ACS and ACO activities (Zhang et al., 2009),
354 both during on-tree (Lindo-García et al., 2020b) and postharvest ripening (García-Pastor
355 et al., 2021). However, scarce information is available on how ABA can regulate apple
356 ripening despite being one the main fruit species consumed worldwide. Our data reveal
357 that other compounds rather than ABA are likely involved in the onset of ethylene
358 production in 'Golden Reinders' apples yet the ABA seemed to play a key role in
359 triggering changes of certain apple ripening-related attributes including firmness and
360 degreening (Figures 1 and S1). Indeed, the high correlation among ethylene, ABA,
361 firmness and colour (Figure S3), points out that both hormones may be required for
362 triggering apple ripening, in a similar way to what has been described in other fruit (Qiao

363 et al., 2021). However, the exact participation of ABA in triggering such changes is still
364 unknown and warrants further studies. For instance, a cold-induced ethylene production
365 seemed to account for the softening occurring during cold storage (ca. 10N after 2 months;
366 Fig. 1) regardless of ABA, since the levels of this hormone remained low and unchanged.
367 This said, once apples were removed from cold storage and left to ripen at 20ºC, softening
368 was initially limited, regardless of the high ethylene production rates, and seemed to
369 accelerate once ABA levels raised. During off-tree fruit ripening, a significant firmness
370 loss was also observed only when ethylene and ABA levels rose simultaneously. These
371 results may suggest that an increase in endogenous ABA may be either needed or
372 facilitating the action of ethylene on trigger some ripening-related changes or as already
373 shown in other fruit (Zhang et al., 2009; García-Pastor et al., 2021; Qiao et al., 2021).
374 Nonetheless, other hormones may be also involved in the regulation of ethylene
375 metabolism/biosynthesis or the activation of ethylene biosynthesis itself (Onik et al.,
376 2018). In this sense, our results showed that the tentative transition from system 1 to
377 system 2 during off-tree apple ripening was preceded by a peak of IAA and $GA_3$ (Figure
378 3B and 3C). Although the specific involvement of those hormones in the regulation of
379 ethylene metabolism remains elusive, it is generally accepted that GAs act as ripening
380 inhibitor reducing both ethylene production and sensitivity (Li et al., 2019), whereas the
381 interplay between auxins and ethylene is highly dependent on the fruit species. Increased
382 auxins levels were found to precede ethylene production in pear (Lindo-García et al.,
383 2020b) and peach (Tatsuki et al., 2013), while in tomato higher auxins levels reduce
384 ethylene production and delay fruit ripening (Li et al., 2016). In 'Golden Delicious'
385 apples, it has been described that IAA is involved in the activation of ethylene
386 biosynthetic genes (MdACS1, MdACS3a and MdACO1) via the expression of the
387 transcription factor MdARF5 (Yue et al., 2020). However, it is also thought that auxins

388 may only control ethylene metabolism during postharvest shelf-life (Busatto et al., 2021).
389 Hence, based on the above-mentioned studies, as well as our findings, it may be feasible
390 to speculate that IAA may be required for the onset of ethylene production, whereas $GA_3$
391 may participate in the modulation of ethylene perception at the first stages of detached
392 apple ripening (Figure 5).
393 Our results also show that cold storage induced not only ethylene production but also
394 enhanced the accumulation of jasmonic acid (Figure 5). Jasmonic acid is considered a
395 key hormone in the regulation of ethylene production (Li et al., 2017) and both hormones
396 act synergistically in stress-response mechanisms induced, for instance, by cold stress
397 (Kazan, 2015). Previous studies suggested that jasmonic acid promotes tolerance to low
398 temperatures by moderating ethylene production and regulating ROS homeostasis
399 (Devireddy et al., 2021). However, in our study, the peak of jasmonic acid observed after
400 removing the fruit from cold storage could partially explain the enhanced ACS and ACO
401 activities, as well as ethylene production, in fruit ripened after cold storage (Figures 2B,
402 2C and 3D). Jasmonic acid not only acts synergistically with ethylene during fruit
403 ripening, but also influences fruit colour stimulating anthocyanins and carotenoids
404 biosynthesis (Liu et al., 2012). This said, the accumulation of jasmonic acid observed in
405 cold-stored fruit did not affect apple colour or the carotenoid content if compared with
406 on-tree or off-tree ripened fruit (Figures 1 and S1). The lack of correlations between JA
407 and carotenoids upon removal from cold storage may be due to the fact that external
408 colour of 'Golden Reinders' apples depends mainly on the chlorophyll content, whose
409 levels seemed to be strongly correlated with ethylene and ABA (Figures 2D, 4A and
410 S1B).
411
412 *4.3. ROS and sugars do not play a key role during apple ripening*

413 Other molecules such as sugars and $H_2O_2$ can participate together with hormones in
414 ripening signalling networks (Decros et al., 2019; Durán-Soria et al., 2020; Morales and
415 Munné-Bosch, 2016). In 'Blanquilla' pears, for instance, oxidative stress markers and
416 sucrose were found to likely act as signals affecting ethylene production and ripening
417 (Lindo-García et al., 2019). However, our results showed that sugars and $H_2O_2$ may not
418 have a clear function or key role during 'Golden Reinders' apples ripening. Indeed and,
419 although ripening is considered an oxidative process (Steelheart et al., 2019), no
420 association was found herein among ripening-related quality changes and the fruit
421 oxidative status (AsA/DHA, antioxidant capacity and $H_2O_2$ levels) of the fruit (Figure 4).
422 Furthermore, the negative correlation between ethylene and $H_2O_2$ (Figure S3) suggest that
423 ethylene may induce the activation of $H_2O_2$-scavenging enzymes (Duque and Arrabaça,
424 1999) accounting, in part, for the observed decline of $H_2O_2$ during postharvest ripening
425 (Figure 4C). The ethylene-mediated activation of such enzymatic antioxidants may be
426 necessary to prevent premature ripening caused by the toxic effect of ROS (Apel and Hirt,
427 2004). For example, previous studies suggested that lipid peroxidation caused enhanced
428 accumulation of specific ROS and initiated to some extent fruit softening in pears (Lindo-
429 García et al., 2020a). However, our data shows that higher levels of $H_2O_2$ did not translate
430 in lower firmness values in 'Golden Reinders' apples. Despite some of the observed
431 correlations between $H_2O_2$ and some ethylene-dependent quality traits (i.e. firmness) or
432 starch, the redox state of the fruit or the accumulation of ROS do not seem to play a clear
433 role in 'Golden Reinders' apple ripening hence in contrast to that suggested in previous
434 studies with other Rosaceae species (Giné-Bordonaba et al., 2017; Vall-llaura et al.,
435 2022).
436

437 ## 5. Conclusion

438 Ethylene is essential for the activation of apple ripening. However, other hormones may
439 also be involved in the modulation of ethylene biosynthesis determining the way by which
440 the fruit ripens attached or detached from the tree. While apples are attached to the tree,
441 ethylene production remained low and consequently no changes in firmness and colour
442 are observed. Once fruit is detached from the tree, the coordinated action of IAA and $GA_3$
443 could be promoting ethylene biosynthesis thereby facilitating fruit ripening. However,
444 our data suggest that under this scenario, ethylene may not able to induce softening and
445 degreening unless ABA levels rose, suggesting that both hormones are required to
446 mediate apple ripening-related changes. Differences in the activation of the ethylene
447 biosynthetic pathway among fruit ripened immediately after harvest or after cold storage
448 were, in turn likely associated with the action of cold storage on endogenous JA levels.
449 JA accumulated as a cold-stress response may induce the anticipation of the onset of
450 ethylene production commonly observed in fruit ripened after cold storage. Studies
451 involving exogenous applications of the studied hormones are encouraged to further
452 unravel the complex hormonal interplay regulating apple ripening.
453

454 **Author's contribution**

455 JGB and PFC conceived and designed the experiments. PFC carried out the experiments. PM
456 and SMB were responsible for the quantification of hormones. GE, CL and NT assisted with
457 the statistical analysis and data interpretation. PFC, CL and JGB drafted the manuscript and
458 all other authors contributed in improving the final version of the manuscript.

459

460 **Declaration of competing interest**

461 The authors declare that they have no known competing financial interests or personal
462 relationships that could have appeared to influence the work reported in this paper.

463

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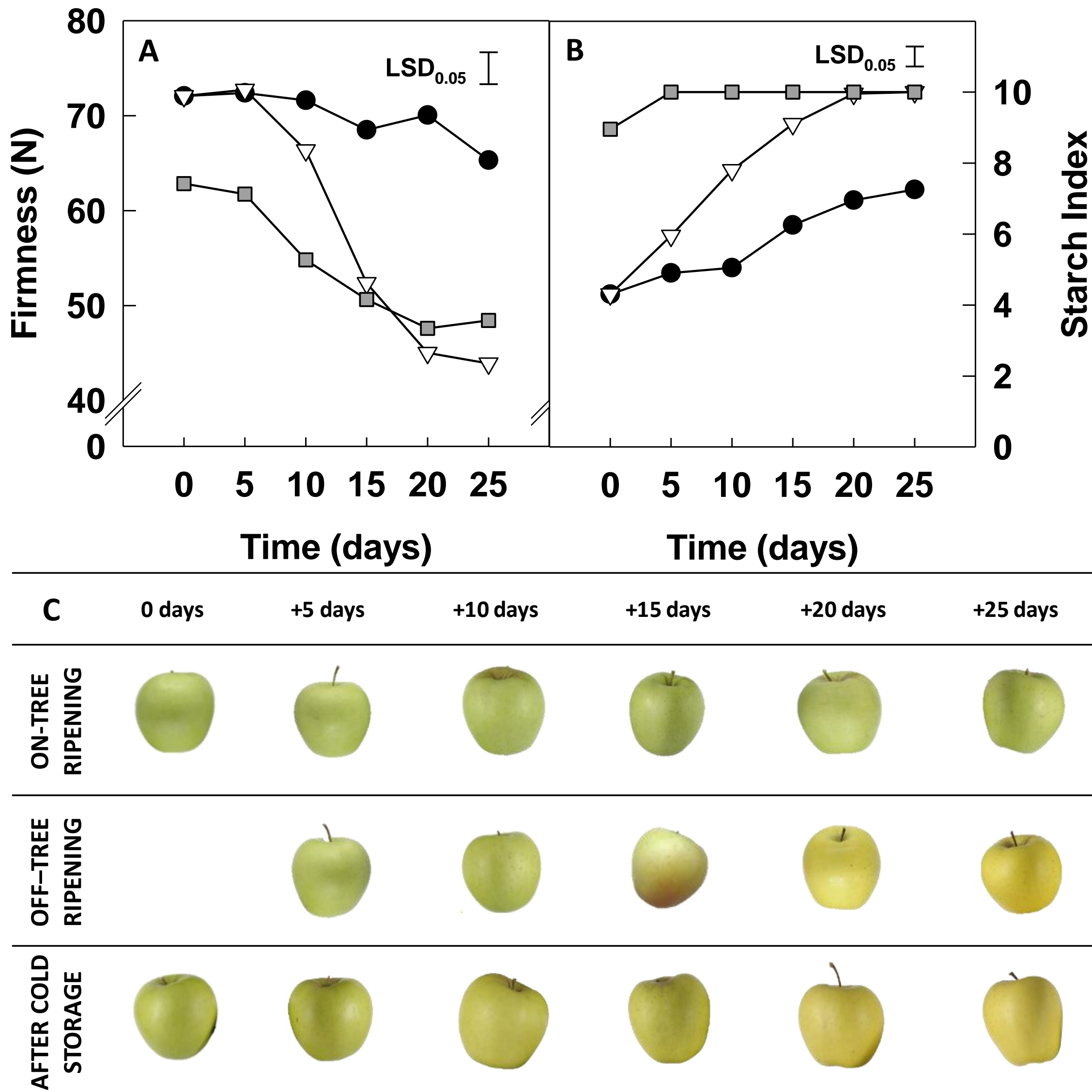
675

676 **Figures**



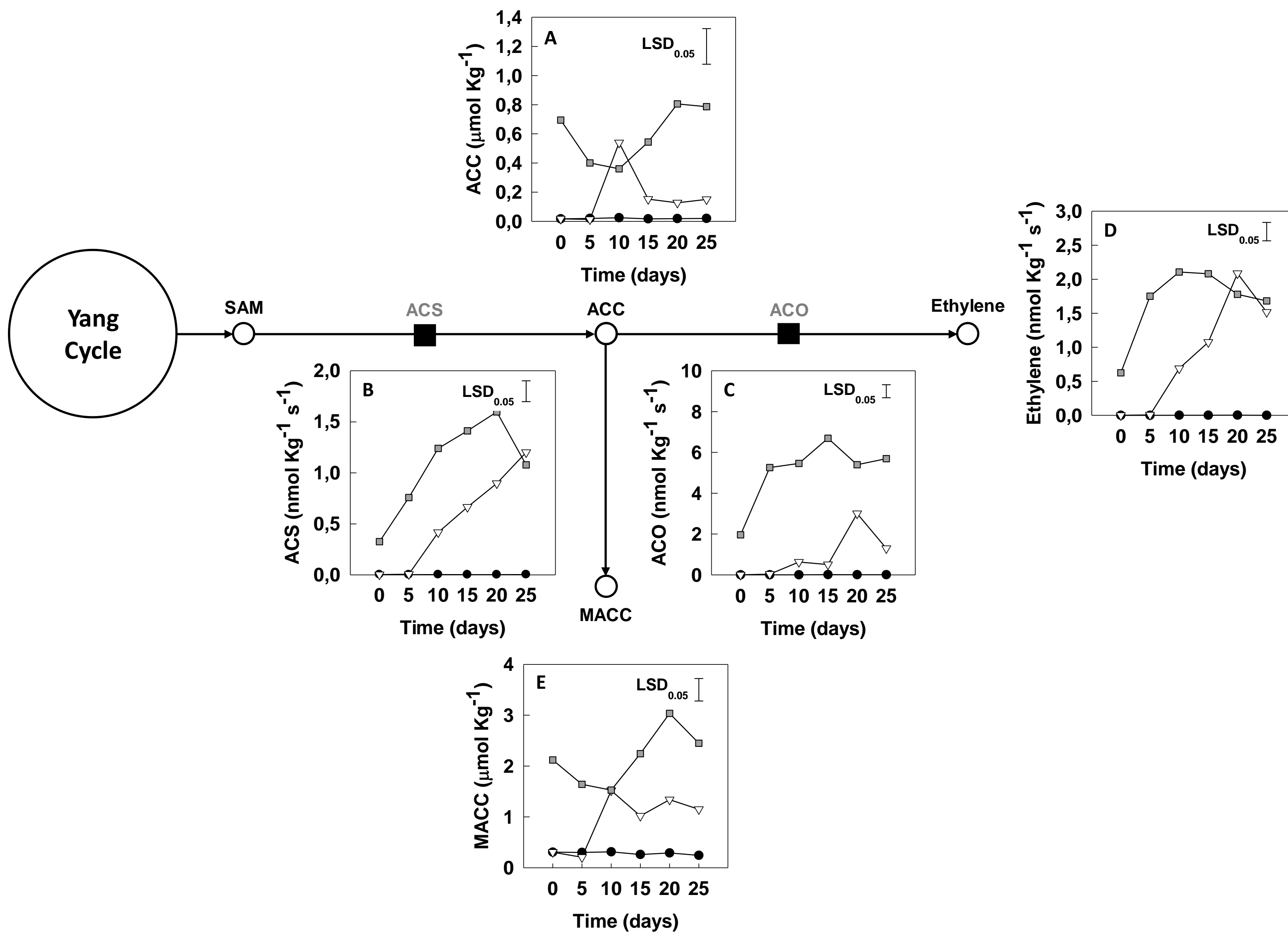

677

678

679 **Figure 1.** Changes in fruit firmness (A) starch index (B) and peel colour (C) during on-

680 tree (●), off-tree (▽) and after cold storage (■) in 'Golden Reinders' apple ripening.

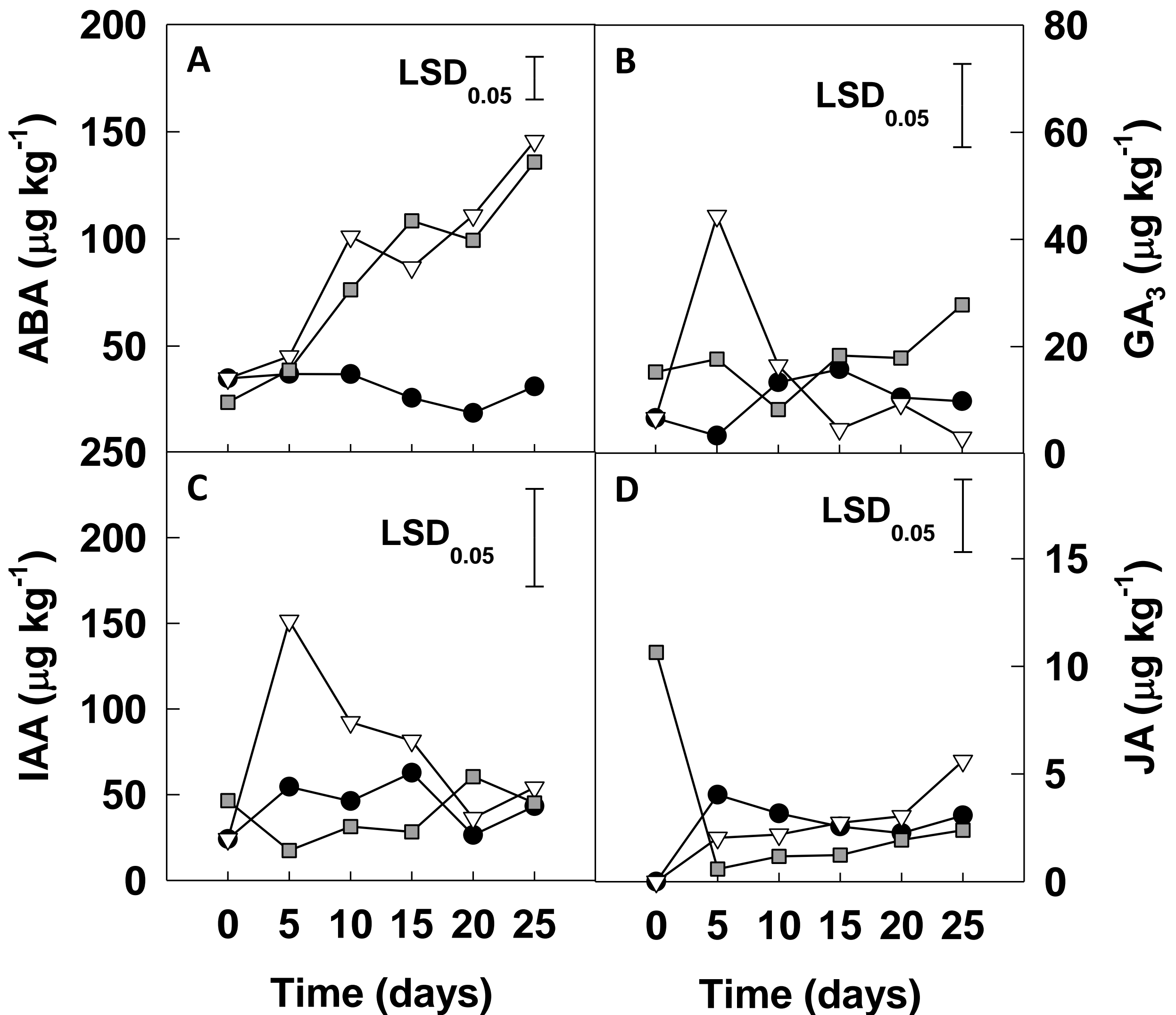
681 Error bars represent the LSD values (p = 0.05) for the interaction ripening scenario*time.



A
LSD0.05
ACC (µmol Kg-1)
Time (days)
Yang Cycle
SAM
ACS
ACC
ACO
Ethylene
MACC
B
ACS (nmol Kg-1 s-1)
C
ACO (nmol Kg-1 s-1)
D
Ethylene (nmol Kg-1 s-1)
E
MACC (µmol Kg-1)

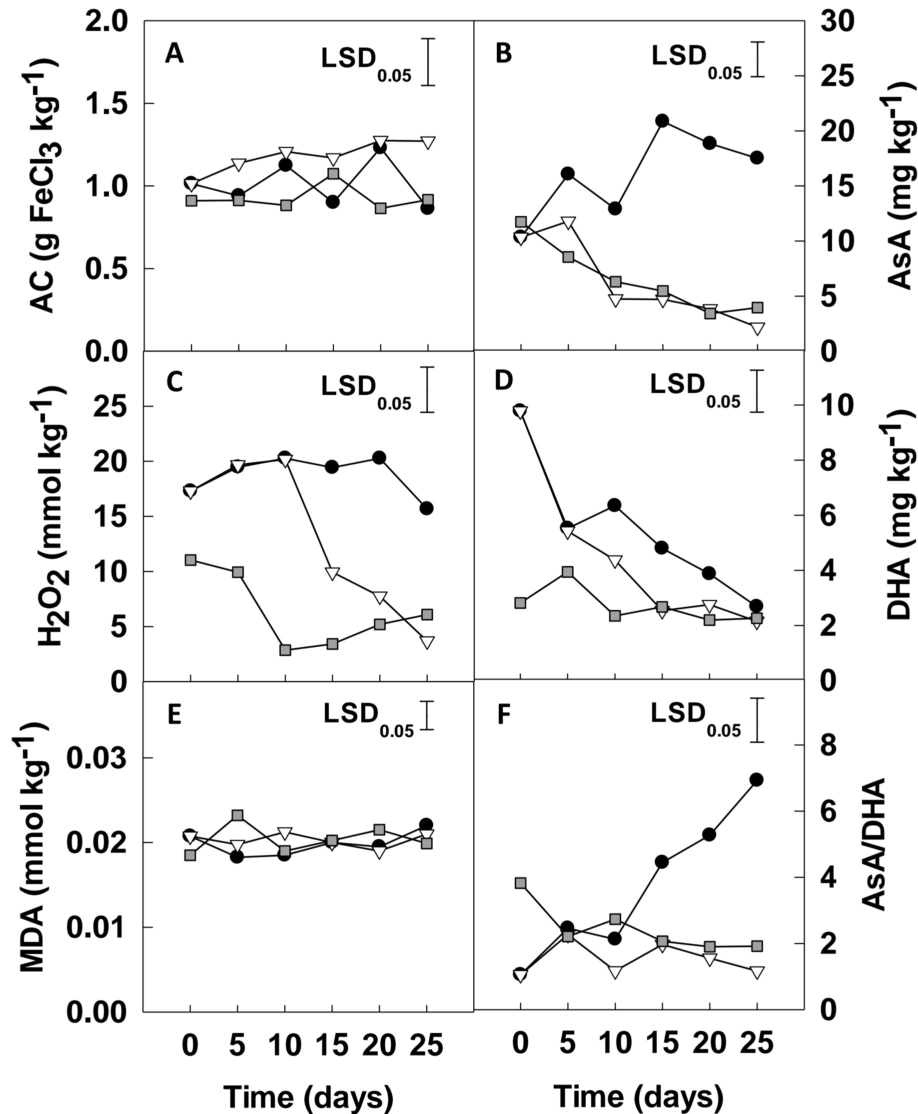
**Figure 2.** Ethylene metabolism scheme showing the ACC content (A), ACC synthase activity (ACS; B), and ACC oxidase activity (ACO; C), ethylene production (D), and MACC (E) in 'Golden Reinders' apple during ripening on-tree (●), off-tree (▽) and after cold storage (■). Error bars represent the LSD values ($p = 0.05$) for the interaction ripening scenario*time. P-values for the interaction ripening scenario*time for figures A, B, C, D and E were: 0.002, <0.0001, <0.0001, <0.0001 and <0.0001 respectively.

687




688

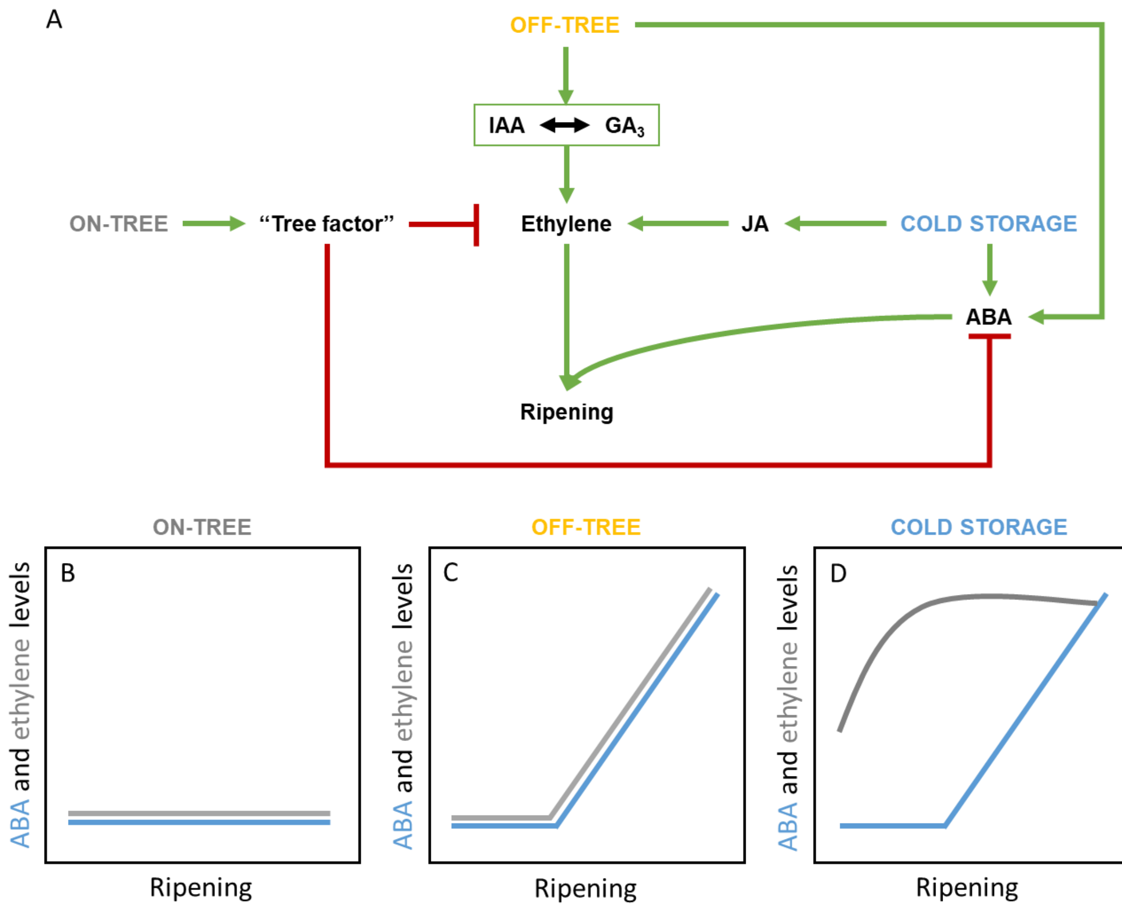
689 **Figure 3.** Changes in endogenous concentration of abscisic acid (ABA; A), gibberellin 3
690 ($GA_3$; B), indole 3-acetic acid (IAA; C) and jasmonic acid (JA; D) in 'Golden Reinders'
691 apple during ripening on-tree (●), off-tree (▽) and after cold storage (-■-). Error bars
692 represent the LSD values (p = 0.05) for the interaction ripening scenario*time. P-values
693 for the interaction ripening scenario*time for figures A, B, C and D were: <0.0001,
694 0.0035, 0.0360 and <0.0001, respectively.




695

696 **Figure 4.** Changes in the levels of antioxidant capacity (AC; A), Ascorbic Acid (AsA;
697 B), hydrogen peroxide ($H_2O_2$; C), Dehydroascorbic acid (DHA; D), malondialdehyde
698 (MDA; E), ratio (AsA/DHA; F) in 'Golden Reinders' apple ripening during on-tree (●),
699 off-tree (▽) and after cold storage (■). Error bars represent the LSD values (p = 0.05) for
700 the interaction ripening scenario*time. P-values for the interaction ripening scenario*time
701 for figures A, B, C, D, E and F were: 0.2231, <0.0001, <0.0001, <0.0001, 0.0679 and
702 <0.0001, respectively.

703




704

705 **Figure 5.** Scheme for the proposed hormonal cross-talk in the three ripening scenarios

706 studied (A): on-tree, off-tree and cold storage. Ethylene (grey) and ABA (blue) levels

707 during were schematized for on-tree (B), off-tree (C) and post-cold storage (D) ripening.

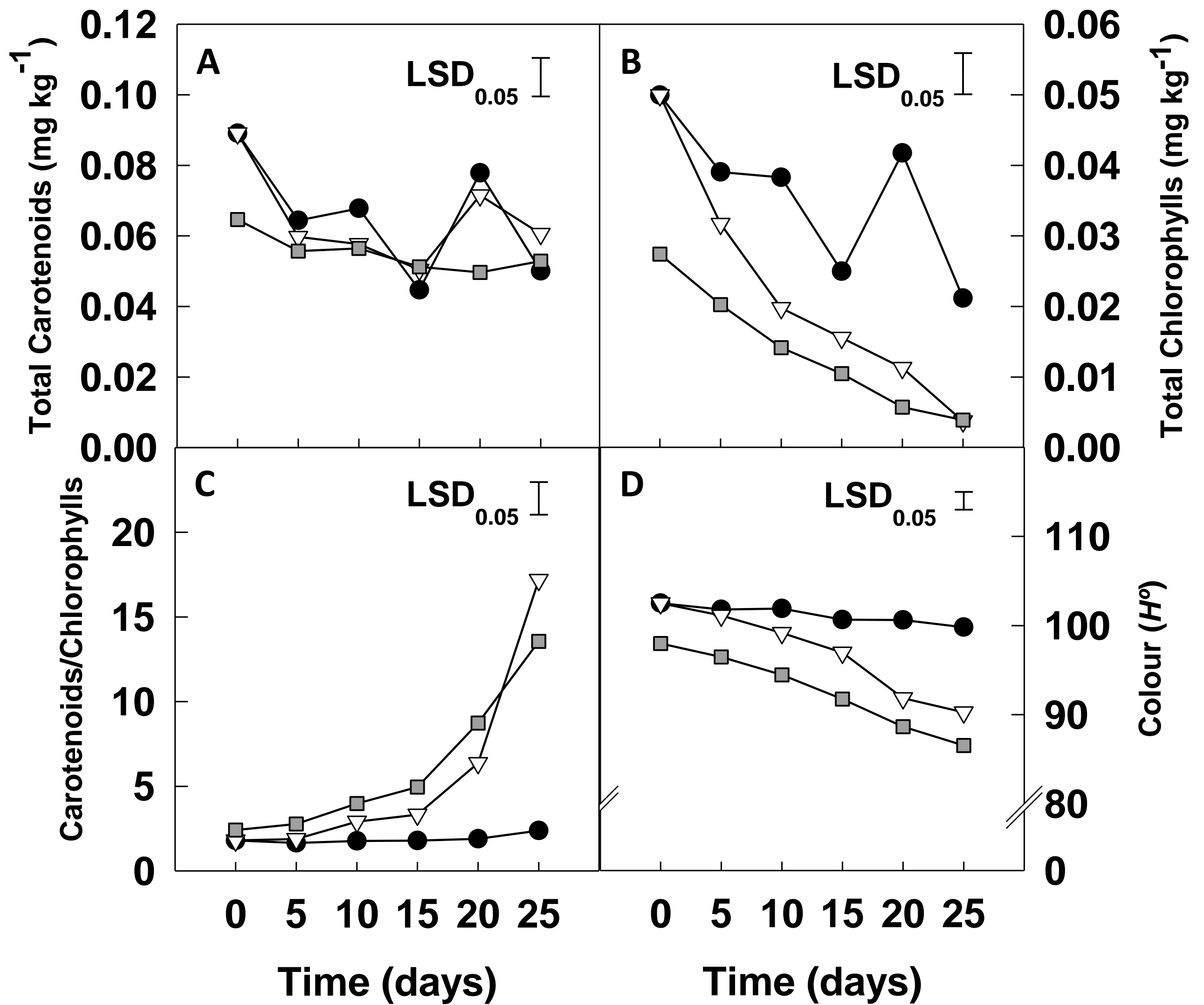
708 Green arrows indicate induction or activation processes whereas red arrows mean

709 inhibition mechanisms. Double-headed arrow symbolize the hypothetical interaction

710 between IAA and $GA_3$.

711

712 **Supplementary Figures**




713

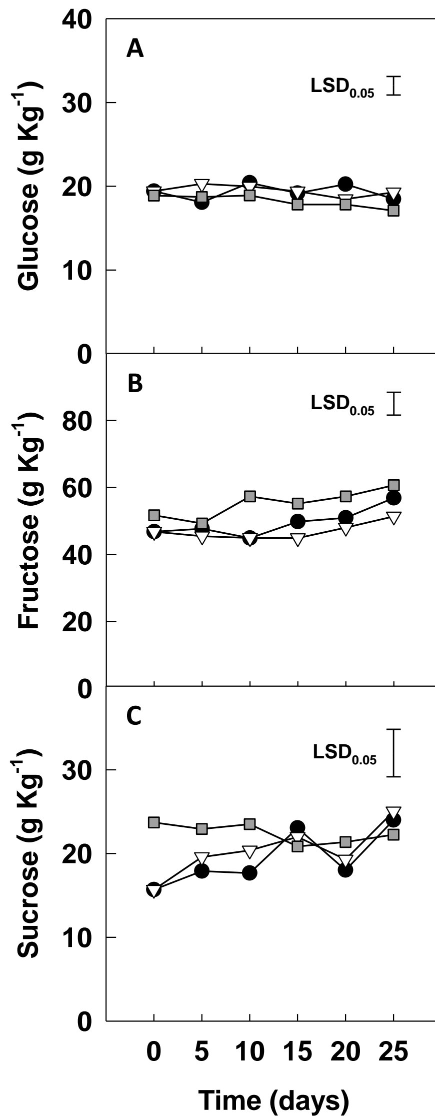
714 **Figure S1:** Changes in carotenoids (A), chlorophylls (B), ratio carotenoids/chlorophylls

715 (C), and objective colour measured as *Hue angle* (*Hº*; D) in 'Golden Reinders' peel during

716 ripening on-tree (●), off-tree (▽) and after cold storage (■). P-values for the interaction

717 ripening scenario*time were <0.0001 in all cases.

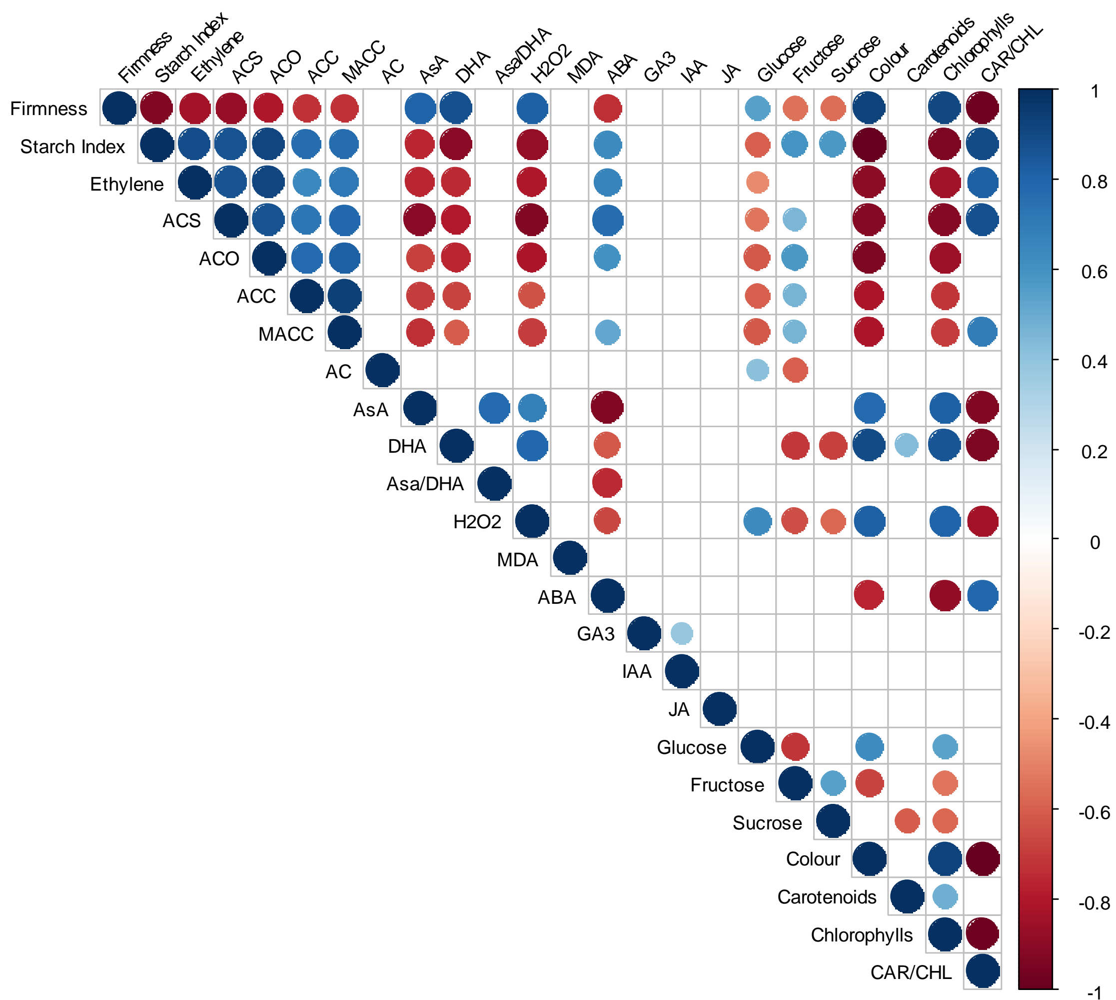
718




719

720 **Figure S2:** Changes in glucose (A), fructose (B) and sucrose (C) in 'Golden Reinders'
721 pulp during on-tree (●), off-tree (▽) and after cold storage (■) ripening. Error bars
722 represent the LSD values (p = 0.05) for the interaction ripening scenario*time. P-values
723 for the interaction ripening scenario*time for figures A, B and C were: 0.5496, 0.5514
724 and 0.1833, respectively.

725




726 **Figure S3:** Bivariate correlations among the different parameters studied during 'Golden
727 Reinders' apples ripening. The size of the circle for each correlation and the colour depict
728 the significance and the correlation coefficient, respectively. Positive correlations
729 coefficients are displayed in blue and negative correlations coefficients in red.