1 ***PbSRT1* and *PbSRT2* regulate pear growth and ripening yet displaying a species-**

2 **specific regulation in comparison to other Rosaceae spp.**

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22 **Abstract**

23 Epigenetic regulation is crucial to ensure a coordinated control of the different events that

24 occur during fruit development and ripening. Sirtuins are NAD^+-dependent histone

25 deacetylases involved in the regulation of gene expression of many biological processes.

26 However, their implications in the Rosaceae family remains unexplored. Accordingly, in

27 this work, we demonstrated the phylogenetic divergence of both sirtuins among Rosaceae

28 species. We then characterized the expression pattern of both *SRT1* and *SRT2* in selected

29 pome and stone fruit species. Both *SRT1* and *SRT2* significantly changed during the fruit

30 development and ripening of apple, nectarine and pear fruit, displaying a different

31 expression profile. Such differences could explain in part their different ripening

32 behaviour. To further unravel the role of sirtuins on the fruit development and ripening

33 processes, a deeper analysis was performed using pear as a fruit model. In pear, *PbSRT1*

34 gene expression levels were negatively correlated with specific hormones (i.e. abscisic acid,

35 indole-3-acetic acid, gibberellin A1 and zeatin) during the first phases of fruit development.

36 *PbSRT2* seemed to directly mediate pear ripening in an ethylene-independent manner. This

37 hypothesis was further reinforced by treating the fruit with the ethylene inhibitor 1-

38 methylcyclopropene (1-MCP). Instead, enhanced *PbSRT2* along pear growth/ripening

39 positively correlated with the accumulation of major sugars ($R^2 > 0.94$), reinforcing the idea

40 that sugar metabolism may be a target of epigenetic modifications during fruit ripening.

41 Overall, the results from this study point out, for the first time, the importance that sirtuins

42 have in the regulation of fruit growth and ripening of pear fruit by likely regulating hormonal

43 and sugar metabolism.

44 **Keywords:** Apple, ethylene, epigenetic regulation, hormonal cross-talk, nectarine,

45 sugars.

1. Introduction

Histone deacetylation is one of the multiple reversible modifications that are involved in the coordinated regulation of gene expression in organisms [1]. This process is mediated by the action of histone deacetylases (HDACs), which include, among others, the sirtuins. Sirtuins (SRTs) are a family of proteins widely conserved from prokaryotes to mammals. They are homologous of the budding yeast *silencing information regulator* 2 (*SIR2*) and catalyse the β-nicotinamide adenine dinucleotide (β-NAD$^+$)-dependent N$^\varepsilon$-acyl-lysine deacetylation on both histone and non-histone protein substrates [2]. Ultimately, such activity is mainly associated to a repression action over specific promoter regions of some key genes leading to an epigenetic regulation of gene expression [3].

Among the eukaryotic sirtuins, a plethora of research in yeasts and mammals (including humans) [4,5] suggest their involvement in a broad range of cellular processes including mitochondrial biogenesis, gluconeogenesis, circadian rhythms, oxidative stress and aging, among others [6]. However, scarce information is available regarding plant sirtuins. Recent evidence confirms the presence and function of SRTs in various plant species (e.g. *Arabidopsis thaliana*, *Oryza sativa*, *Vitis vinifera*, *Glycine max, Musa acuminata* and *Solanum lycopersicum*) suggesting a putative role of these proteins in regulating the growth and development of plants, as well as the plant response to biotic and abiotic stresses [7]. In *A. thaliana*, AtSRT1 is involved in the deacetylation of K9-acetylated histone protein H3 (H3K9ac), sometimes in synergy with the cMyc-binding protein 1 [8], regulating the glycolysis, mitochondrial respiration and also abiotic stress-responsive genes. AtSRT2, in turn, has been associated to the biotic stress response in a salicylic acid biosynthetic-dependent mechanism [9].

The potential involvement of SRTs in regulating fruit development arises from available data on other histone deacetylases. For instance, *AtHD1* mutations led to embryonic

defects and seed set reduction [10] while both silencing and overexpression of *AtHD2A* drastically affected seed development [11,12]. Likewise, the possible role of SRTs in fruit ripening could be extrapolated from the relationship observed between key ripening hormones such as ethylene [13] or ABA [8,14,15] and sirtuin gene expression in Arabidopsis, soybean and *Marchantia polymorpha*. Fu et al. [16] also demonstrated the regulation of ethylene response factors (ERFs) by other histone deacetylases (MaHDA6) in banana fruit.

For grape, the only available study [17] shows that *VvSRT2* is differentially regulated in different plant organs but also at different developmental stages. Similarly, *SlSRT1* and *SlSRT2* in tomato fruit exhibited different expression pattern during growth and ripening but also for the different phenological stages [18], suggesting a possible role of these proteins in modulating fruit ripening. In the later study, changes in the expression of *SlSRT1* during fruit growth paralleled to some extent the typical ethylene production pattern observed in tomato [19].

Despite being one of the most important plant families in terms of number of cultivated species, no information is currently available about the existence of SRTs in Rosaceae species. Among Rosaceae, nectarines, apples and pears are becoming a model for fleshy fruit development and climacteric ripening studies [20]. Accordingly, this study aimed to: i) characterise the presence of SRTs in a range of Rosaceae species; ii) decipher the role of sirtuins during fruit growth and ripening in nectarines, apples and pears; iii) establish the relationship between SRTs gene expression and key ripening related events (sugar accumulation and the hormonal cross-talk) in pear fruit.

2. Material and methods

2.1. Experimental design

2.1.1. Experiment 1: analysis of SRTs gene expression of different species during fruit development and ripening

This experiment was conducted with three different Rosaceae species obtained from organic orchards located near Lleida (Catalonia, Spain). Apples, pears and nectarines, free of physical injuries and rots, were picked at different developmental stages based on days after full bloom (DAFB), considering full bloom the stage when at least 50% of flowers were open. 'Diamond Ray' nectarines (*Prunus persica* (L.) Batch) were picked from an orchard in Gimenells at four different development stages: S1 = 39, S2 = 70, S3 = 94 and S4 = 121 DAFB. 'Golden Reinders' apples (*Malus × domestica* Borkh) were harvested on an orchard located in Vilanova de Segrià at: S1 = 80, S2 = 105, S3 = 125 and S4 = 150 DAFB, whereas 'Williams' pears (*Pyrus communis* L.) were picked from Alcoletge at S1 = 30, S2 = 50, S3 = 70, S4 = 90 and S5 = 125 DAFB. Samples were obtained during the 2018 harvest season and the selection of the different fruit developmental stages was based on that from previous studies [21,22].

2.1.2. Experiment 2: analysis of SRTs gene expression of different pear cultivars at harvest

To further assess sirtuin expression in mature fruit and determine possible gene expression variability among pear cultivars, four different cultivars were selected. 'Williams', 'Conference', 'Flor d'Hivern' and 'Blanquilla' pears (*Pyrus communis* L.) were harvested at commercial harvest date (CHD; about 138, 135, 173, 125 DAFB, respectively) according to grower recommendations and standard maturity indices [21] in an orchard located in Alcoletge (Lleida, Catalonia, Spain). Fruit were selected for uniform size and free of physical injuries and rots. Samples were obtained during the 2019 harvest season.

2.1.3. Experiment 3: analysis of SRTs gene expression of 'Blanquilla' pear cultivar during postharvest storage and in response to 1-MCP treatments

This experiment was conducted using the 'Blanquilla' pear (*Pyrus communis* L.) harvested at commercial harvest date (CHD; 125 d after full bloom, DAFB) in an orchard from Alcoletge (Lleida, Catalonia, Spain) during the 2020 harvest season. Fruit were selected for uniform size and free of physical injuries and rots. Immediately after harvest, fruit were submitted to 1-MCP treatments as previously described by Lindo-García et al. [23]. Briefly, fruit were divided in two batches (control and 1-MCP-treated fruit). 1-MCP treatment was carried out by placing the fruit in a sealed plastic container and applying 300 nL L^{-1} 1-MCP using the Smartfresh^{TM} product (Agrofresh Inc.) for a minimum of 18 h at 0 ºC. The other batch, used as the control, was incubated in the same conditions but without applying 1-MCP. After the treatment, fruit were stored at -0.5 ºC and 90% RH until further sirtuin expression analysis after 0, 15, 30, 60 and 120 d of cold storage.

2.2. Identification of SRT genes, sequence analysis and phylogenetic construction

Sirtuin sequences from the different species presented herein were retrieved from NCBI database. In the case of multiple transcript variants, sequences were named as 'a', 'b', 'c', etc. The duplicates were removed and all protein sequences were analysed for recognizable domains using BLAST-based NCBI conserved domain searches (https://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi) and further verified using the Pfam (http://pfam.xfam.org/) and SMART (http://smart.embl-heidelberg.de/) databases. Domain architecture was illustrate using DOG2.0 software [24] and coloured as indicated. Sirtuin proteins from nectarine, apple and pear were further characterized in terms of their molecular weight (MW), isoelectric point (pI) and subcellular localization. MW and pI values were predicted according to the ProtParam tool from Expasy

146 (https://web.expasy.org/protparam/). Protein sequences were aligned in Geneious Prime

147 (https://www.geneious.com/) using the MUSCLE alignment plugin. Protein localization

148 was predicted by using the WoLF PSORT software (https://wolfpsort.hgc.jp/).

149 The sirtuin proteins from pear, nectarine and apple, together with the sirtuin sequences from

150 other Rosaceae species and families, were submitted to a MUSCLE alignment. Aligned

151 sequences were further used for phylogenetic analysis using MEGA X [25]. The phylogenetic

152 tree was built for best-scoring Maximum Likelihood method and the WAG model [26], using

153 1000 bootstrap replications. All positions containing alignment gaps and missing data were

154 also used. The generated tree was further visualized by using the iTOL (https://itol.embl.de/)

155 tool [27].

156

157 **2.3. RNA extraction, primer design and qPCR analysis**

158 For expression analysis, RNA was extracted from all the samples described above (section

159 2.1). Samples were obtained from 3 or 4 biological replicates (depending on the

160 experiment) of 5 fruit each, immediately ground in liquid nitrogen and stored at -80 ºC

161 until further analysis. RNA was extracted using the Spectrum^{TM} Plant Total RNA Kit

162 (Sigma-Aldrich, St Louis, MO, USA) following the manufacturer's recommendation. Both

163 absence of contaminant DNA and RNA integrity were determined by electrophoresis on an

164 agarose gel by staining with GelRed™ Nucleic Acid Gel Stain (Biotium, Hayward, CA,

165 USA). One µg of RNA was used for cDNA synthesis using the SuperScript IV First-Strand

166 Synthesis System (Invitrogen, Carlsbad, CA, USA). Gene expression analysis was performed

167 as previously described by Baró-Montel et al. [28]. Briefly, the reaction mix consisted of the

168 KAPA SYBR® Fast qPCR Master Mix (Kapa Biosystems, Inc., Wilmington, USA), 100 nM

169 of each primer and the corresponding diluted cDNA. The reaction was performed on a 7500

170 Real Time PCR System (Applied Biosystems) with the following conditions: 10 s at 95 ºC

171 followed by 40 cycles of 95 ºC during 15 s and 60 ºC during 1 min. A melt curve analysis

172 was performed to check primer specific by including a final amplification cycle at 95 °C for

173 15 s, 60 °C for 1 min, 95 °C for 30 s and 60 °C for 15 s. A non-template control (NTC) was

174 included by using DNA-free water instead of cDNA.

175 Primers (Supplementary Table S1) were designed *de novo* using the Primer-BLAST tool

176 form NCBI [29], allowing, when possible, the primers to span and exon-exon junction. When

177 multiple splicing variants occurred for a selected gene, primers were designed to target the

178 multiple variants. Reference genes were selected according previous results on the literature;

179 *Md8283* was selected as an independent reference gene for both pear and apple [30], while

180 *translation elongation factor* 2 (*TEF2*) was used for nectarine samples due to its high

181 statistical reliability [31]. Primer efficiency was calculated by using 3-fold dilutions of the

182 cDNA pool mix. Relative gene expression was expressed as Mean Normalised Expression

183 (MNE) according to Muller et al. [32].

184

185 **2.4. Physiological and biochemical measurements**

186 Fruit diameter from the different cultivars at the different sampling points was determined at

187 the equatorial section of the fruit with an electronic digital calliper (Powerfix, Ilford, UK) and

188 expressed in millimetres (mm). Fruit weight from each individual fruit was also recorded

189 using a digital scale. A total of 6 replicates of 10 fruit each were used for all the sampling

190 points.

191 Ethylene production levels (expressed as nmol Kg^{-1} s^{-1}) in 'Williams' pears (Experiment 1)

192 were recorded along the different phenological stages of the fruit. Four replicates of 5 fruit

193 each, per sampling, were placed in an acclimatised chamber at 20°C into sealed flasks of

194 different volumes (depending on the fruit size) equipped with a silicon septum. Analysis

195 was carried out withdrawing 1 ml gas sample after 2 h incubation and following the

methodology described elsewhere [33]. Ethylene production levels in 'Blanquilla' pears (Experiment 3) was determined as described above in both control and 1-MCP-treated samples to check the efficiency of the 1-MCP treatment. A total of 3 biological replicates were used for each treatment condition.

Sugar content, including glucose, fructose and sucrose of 'Williams' pears (Experiment 1) was determined following the methodology described by Giné-Bordonaba et al. [34]. For the same samples, the hormone profile was analysed by UHPLC-ESI-MS/MS following the same methodology described by Lindo-García et al. [21]. Phytohormone content was expressed on fresh weight (FW) basis (ng g^{-1}). Sugar and hormonal profile were determined on 4 or 6 biological replicates of 5 fruit each per each, respectively, per each development stage.

1-aminocyclopropane-1-carbboxylic acid (ACC) content was determined as previously described by Lindo-García et al. [21]. Extraction was performed on 4 biological replicates of 5 fruit each. Results were expressed as nmol C_2H_4 g^{-1}. 1-aminocyclopropane-1-carboxylic acid synthase (ACS) and 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) activities were analysed following the protocol described by Lindo-García et al. [35]. Enzymes were extracted on 4 biological replicates of 5 fruit each and activities were expressed as C_2H_4 g^{-1} h^{-1}.

2.5. Data analysis

All data were collated and subjected to analysis of variance (ANOVA) using JMP® (v. 13.1, SAS Institute Inc., Cary, NC). Means were compared by analysis of variance (ANOVA). When the analysis was statistically significant, the Tukey's HSD test at the level $p \leq 0.05$ was performed for comparison of means for gene expression for the interaction time/cultivars and for hormones, sugars and ethylene metabolism for the interaction fruit growth and ripening.

221 The comparison of both SRTs expression (*SRT1* and *SRT2*) at each time point and the

222 comparison for control and 1-MCP treated samples was performed using the Student's T-test

223 ($p \leq 0.05$). Correlations between experimental variables were made using the Pearson's

224 product moment correlation ($p \leq 0.05$).

225

226 **3. Results**

227 **3.1   Phylogenetic analysis of plant SRTs**

228 To investigate the molecular evolution and phylogenetic relationship among sirtuins, a

229 phylogenetic analysis of proteins from representative species of the Rosaceae family, and

230 other plant families for which the presence of sirtuins was already described in the literature,

231 was generated. A total of fifty-four protein sequences from six different families (Rosaceae,

232 Poaceae, Solanaceae, Brassicaceae, Vitaceae and Fabaceae) were retrieved from NCBI

233 database, aligned and further used for generating a Maximum-Likelihood phylogenetic tree

234 (Fig. 1A).

235 All the species included had, at least, genes coding for sirtuin 1 (SRT1) and sirtuin 2 (SRT2),

236 with two or more theorical transcript variants (indicated as 'a', 'b', etc). In strawberry, a third

237 sirtuin protein (SRT3) was also identified, while in the case of soybean, SRT3 and SRT4

238 were also included.

239 The phylogenetic results demonstrated that both SRT1 and SRT2 were clearly divided in two

240 main clusters. As an exception, GmSRT1 and GmSRT2 from soybean grouped together,

241 while GmSRT3 and GmSRT4 seemed to diverge from the others and belonged to the SRT1

242 cluster. On the other hand, FvSRT3 from strawberry, clustered with FvSRT2.

243 It is interesting to note that members of either SRT1 or SRT2 tend to cluster based on their

244 family. Many sub-clusters were formed among family members. Hence, sequences from

245 pome fruit, including pear and apple, formed a sister group for both SRT1 and SRT2, while

246 they appeared to form outgroups relative to stone fruit, including nectarine, apricot, almond

247 or cherry, which, in turn, tended to group together. The Poaceae family, which includes rice

248 as a representative species, was the more distantly divergent family with respect to the

249 Rosaceae spp.

250 A detailed examination of sirtuin sequence domains (Fig. 1B) from the Rosaceae species

251 targeted in this study (nectarine, apple and pear) revealed, as expected, the presence of the

252 SIR2 domain (coloured in yellow). The comparison of the SIR2 domain within the sequences

253 of the three different species, demonstrated its conserved distribution among the sequences

254 for either SRT1 or SRT2.

255 Low complexity domains (coloured in blue), typical of eukaryotic proteins and characterized

256 by repeated single amino acids or short amino acids motifs, were only detected on the

257 sequences of SRT1 of both apple and pear. The pI values were quite similar among the

258 analysed protein sequences (Table 1). In fact, the sequences alignment of both SRT1

259 (Supplementary Fig. S1A) and SRT2 (Supplementary Fig. S1B) denoted a high sequence

260 similarity among sirtuin proteins within the targeted species, especially, for those belonging

261 to SRT1 (from 88.2% to 100% of identity). In the case of SRT2 sequences, sequence

262 identities ranged from 56% to 97.7%. An in-depth residue analysis of both SRT1

263 (Supplementary Fig. S1A) and SRT2 (Supplementary Fig. S1B) confirmed that key residues

264 involved in sirtuin activity (including the residues located in the NAD^+ binding site, those

265 involved in the substrate binding and the four cysteines essential for coordinating the zinc

266 atom) were conserved within each sirtuin. However, clear differences were observed when

267 comparing the residues involved in the substrate binding of both SRT1 (Supplementary Fig.

268 S1A) and SRT2 (Supplementary Fig. S1B) from all the three species.

269

270 **3.2   Differential inter and intra species gene expression of SRTs**

3.2.1 SRTs gene expression in different Rosaceae species during fruit development and ripening

Aiming not to compare but rather to elucidate the sirtuin expression profile along fruit development, three important Rosaceae species were selected. Results revealed significant differences in SRTs expression pattern during fruit development/ripening (Fig. 2G-I) for each species (nectarine, pear and apple). In nectarine (Fig. 2G), four different development stages encompassing cell division, pit hardening and cell expansion until full maturity were selected. In this species, the expression levels of *PpSRT1* did not significantly changed during the first phases of fruit growth and a significant down-regulation was observed only at S4 (121 DAFB) if compared to S2 (70 DAFB). No significant changes on *PpSRT2* gene expression occurred during nectarine development/ripening. Significant differences between *PpSRT1* and *PpSRT2* expression levels were especially evident at S2 (70 DAFB).

Completely different expression patterns were observed in pome fruit (Fig. 2H and 2I). In pears (Fig. 2H), five different samplings along fruit development and ripening were also considered. Large differences between expression levels of both sirtuins were obtained along all the analysed sampling points, being the expression of *PbSRT1*, in average, 6.6-fold higher than *PbSRT2*. In fact, expression differences between both sirtuins ranged from 3.5-fold at S5 (125 DAFB) to 14.8-fold at S1 stage (30 DAFB). A general tendency towards an up-regulation of both *PbSRT1* and *PbSRT2* was observed during fruit development/ripening. *PbSRT1* gene expression was significantly up-regulated (1.5-fold) at S5 (125 DAFB) if compared to S1 (30 DAFB), while expression levels of *PbSRT2* progressively increased from S1 (0.01 MNE) to S5 (0.07 MNE).

In the case of apple fruit, only advanced fruit developmental stages (from 80 DAFB to fully ripe fruit) accompanying fruit ripening on-tree were considered. Changes in expression levels in apple (Fig. 2I) were the opposite to those observed in pear. In this case,

296 *MdSRT2* and not *MdSRT1*, was the main sirtuin expressed along on-tree fruit growth and

297 ripening. The difference between both sirtuin expression levels was already evident at initial

298 fruit development stages (S1; 6.4-fold) and further magnified as the fruit developed and

299 ripened (S4; 13.3-fold). Thus, while *MdSRT1* expression levels remained constant during all

300 the fruit growth, *MdSRT2* was clearly up-regulated as fruit ripened on-tree. For instance, the

301 *MdSRT2* expression levels at S4 doubled those from S1.

302

303 3.2.2 SRTs gene expression in different pear cultivars at harvest

304 To further determine the intra species variability in SRT gene expression, four different

305 pear cultivars were harvested at commercial maturity. While *PbSRT1* expression levels

306 remained stable with no significant differences between pear cultivars, *PbSRT2*

307 expression differed significantly (Fig. 3). 'Flor d'Hivern' and 'Williams' were the two

308 cultivars with the lowest *PbSRT2* expression. In these cultivars, *PbSRT1* levels were

309 significantly higher than those of *PbSRT2* (1.8-fold and 1.6-fold for 'Flor d'Hivern' and

310 'Williams', respectively). Regarding 'Blanquilla' and 'Conference', no significant

311 differences were observed when comparing gene expression levels of both sirtuins, but

312 expression levels of *PbSRT2* were significantly higher than those from 'Flor d'Hivern'

313 and 'Williams' cultivars. In fact, 'Conference' pear was the cultivar with the highest

314 levels of *PbSRT2* (2.8-fold, 2.1-fold and 1.4-fold higher expression than that observed in

315 'Flor d'Hivern', 'Williams' and 'Blanquilla', respectively).

316

317 3.3 Relationship between SRTs and hormones participating in fruit development
318 and ripening

319 To decipher the putative involvement of SRTs in fruit development and ripening, we

320 investigated if changes in *PbSRT1* and *PbSRT2* gene expression levels were related to the

321   changes observed in ethylene, its biosynthetic precursors and other plant hormones

322   (Supplementary Table S2) in 'Williams' pears.

323   Significant differences in ethylene production levels were found for the different

324   sampling points (Fig. 4A). At 30 DAFB, ethylene levels were 0.0007 nmol Kg^{-1} s^{-1}, but

325   significantly increased to 0.002 nmol Kg^{-1} s^{-1} reaching a peak at 50 DAFB. Ethylene

326   production decreased thereafter to almost undetectable levels (12.3-fold lower at 70

327   DAFB), which were further sustained during the remaining sampling points.

328   Regarding to the ABA profile (Fig. 4A), the higher levels of this hormone were found at

329   the beginning of the fruit development. At 30 DAFB, its concentration was around 8913

330   ng g^{-1} FW, and significantly decreased thereafter especially between 30 and 50 DAFB (in

331   a opposite way than ethylene production), to progressively decreased later reaching a

332   value of 530.5 ng g^{-1} FW at 125 DAFB.

333   IAA levels remained almost unchanged during all the time course of the experiment (Fig.

334   4A) and no significant differences were found between sampling points.

335   In contrast to ethylene production, ACC levels remained quite stable throughout the entire

336   growing period (Fig. 4B). Levels were around 0.02-0.04 nmol g^{-1} FW, and only

337   significantly increased by 1.96-fold at the last sampling point (125 DAFB). A similar

338   pattern was observed for ACC synthase activity which remained stable along the time

339   course of the experiment. Contrary to ACC and ACC synthase, the ACO activity showed

340   a similar profile to that of ethylene, with the highest activity (0.14 and 0.15 nmol g^{-1} h^{-1})

341   at the initial developmental stages (S1 and S2, respectively) and a progressive decrease

342   thereafter to finally reach 0.024 nmol g^{-1} h^{-1} at S5.

343   Correlation analysis (Fig. 4C) among a wide range of hormones and SRT gene expression

344   levels showed that IAA positively correlated with ethylene ($R^2 = 0.88$, $p = 0.049$) and

345   GA1 ($R^2 = 0.976$, $p = 0.004$). A positive correlation was also observed between ethylene

346   and both GA1 (R^2 = 0.911, p = 0.031) and zeatin (R^2 = 0.883, p = 0.047). On the other

347   hand, *PbSRT1* negatively correlated with ABA (R^2 = -0.930, p = 0.022), IAA (R^2 = -

348   0.883, p = 0.047), GA1 (R^2 = -0.905, p = 0.035) and zeatin (R^2 = -0.886, p = 0.045). No

349   significant correlations were observed between *PbSRT2* expression levels and any of the

350   studied hormones, nor between the two SRTs gene expression and enzymes or precursors

351   involved in ethylene biosynthesis (Fig. 4D).

352

353   **3.4   Relationship between SRTs and sugar changes along fruit development and**

354   **ripening**

355   Monosaccharides (glucose and fructose) levels significantly increased along fruit

356   development (Fig. 5A), being in average 9.25-fold higher at CHD (125 DAFB) than at

357   the first sampling point (30 DAFB). On the other hand, sucrose levels remained stable

358   until 90 DAFB and slightly but significantly increased between S4 to S5 (1.91-fold). The

359   Pearson correlation analysis (Fig. 5B) showed a positive correlation between the content

360   of monosaccharides and sucrose levels (R^2 = 0.892, p = 0.042) and also a positive

361   correlation between the sugars and *PbSRT2* expression levels (R^2 = 0.950, p = 0.013 and

362   R^2 = 0.941, p = 0.017, respectively). Non-significant correlations were obtained in any

363   case with *PbSRT1*.

364

365   **3.5   Ethylene dependent SRTs gene expression during postharvest storage of pears**

366   The effect of ethylene inhibition on the sirtuin expression levels during postharvest

367   storage was assessed in 'Blanquilla' pears untreated and treated with the ethylene

368   inhibitor 1-MCP. The pattern of ethylene production was determined in both control and

369   1-MCP treated samples to verify the efficiency of the treatment (data not shown). *PbSRT1*

370   expression levels significantly decreased both in control and 1-MCP treated fruit during

the first 15 days of cold storage (Fig. 6A). Later, the expression levels significantly

increased regardless of the treatment to reach its maximum expression at 60 d of cold

storage (1.92-fold and 1.84-fold higher compared to 15 d of cold storage). A different

pattern was observed for *PbSRT2* (Fig. 6B). In this case, expression levels also decreased

in both samples during the first days of storage, remained low in 1-MCP-treated fruit, but

slightly increased in control fruit until 120 d, although with no significant differences

between control and 1-MCP treated fruit. Neither *PbSRT1* nor *PbSRT2* were affected by

the ethylene inhibition triggered by the 1-MCP treatment, since no significant differences

were detected at any time point when comparing the control samples with the 1-MCP

treated fruit.

4. Discussion

A tight regulation of gene expression is critical to ensure a precise and coordinated

function of cellular processes that would ultimately enable organisms to growth and adapt

to their environment. The development and ripening process of fruits is a clear example

of the need to maintain a logic and temporal regulation of a particularly set of genes

[36,37]. This epigenetic regulation can be achieved by the modulation of the chromatin

structure though the post-translational modifications of histones [38], which would

influence the degree of packing of the DNA and hence, the regulation of gene expression

[39].

Histone acetylation and deacetylation mediated by histone acetylases (HATs) and histone

deacetylases (HDACs) respectively, are some of the multiple reversible modifications in

which histone and also non-histone substrates can be involved [40]. Many recent studies

have linked the role of these enzymes to many different processes in plants [41].

However, the knowledge on the Rosaceae family is still scarce and there is an increasing

396 need to understand the epigenetic events that may govern the development, ripening and

397 postharvest patterns of this large and economically important family [20].

4.1 Sirtuin sequences diverge between pome and stone fruit within the Rosaceae

399 **family**

400 Sirtuins, are a specific class of HDACs and are widely conserved from bacteria to mammals

401 with the exception of two red algae and some archaea [42]. Plants are characterized by

402 owning, in general, two *SIR2* genes, although in some plant species such as soybean, there

403 have been characterized up to four sirtuin genes [15]. Sirtuins are divided in five different

404 groups [43]; plant SRT1 (SIRT6/7-like protein) belongs to class IV while SRT2 (SRT4-like

405 protein) belongs to class II [44].

406 Our phylogenetic analysis revealed a clear differentiation among SRT1 and SRT2 (Fig. 1A)

407 which points out to an evolutionary divergent function of the two genes. Accordingly,

408 Hollender et al. [45] already suggested different tissue, stage and processes in which

409 Arabidopsis *AtSRT1* and *AtSRT2* were likely involved attending to its expression profiles.

410 The amino-terminal region of proteins contains the signal peptides that ultimately contribute

411 to its unique sub-cellular localization. Thus, the differences on these extensions observed

412 among SRT1 and SRT2 in the three Rosaceae species detailed herein (Fig. 1B and

413 Supplementary Fig. S1A and S1B) reinforce they putative distinct substrate specificity and

414 cellular function.

415 The phylogenetic analysis also evidenced that within the Rosaceae family, stone fruit species

416 grouped together in a species-specific manner and differently to those belonging to pome

417 fruit. To further decipher this question, expression levels of *SRT1* and *SRT2* genes were

418 determined at different stages of fruit growth and ripening (Fig. 2G-I) of three economically

419 relevant Rosaceae spp.; nectarine, apple and pear. In nectarine and pear, the analysis was

420 performed at different key time points during fruit development and ripening based on

421   available literature [21, 22] whereas samplings in apple fruit were exclusively focused on

422   the latest stages of fruit growth generally encompassing the fruit ripening period

423   (Fernandez-Cancelo et al. unpublished). Interestingly, results revealed completely different

424   expression patterns of both genes among species. When comparing both SRTs within each

425   species, expression levels of *SRT1* were, in general, very low in both nectarine and apple,

426   while were highly expressed in pear. Contrary to *SRT1*, *SRT2* was highly expressed in apple

427   but not in pear. These large differences among both sirtuin genes were also reported in other

428   fruit species during its development, including banana [16], tomato [18] and grape [17]. In

429   the latter study, the authors demonstrated a different expression profile in line with a different

430   localization of both proteins and differences on the substrate binding site sequences [17],

431   suggesting then a different role of both sirtuins. Similarly, in this study we demonstrated that

432   both SRT1 and SRT2 of the analysed Rosaceae species were likely located in different

433   organelles within the cell (Table 1), and that the substrate binding site of SRT1 was different

434   from that observed on SRT2 (Supplementary Fig. S1A and S1B).

435

436   **4.2   SRT2 is likely associated to the ripening process in pome fruit**

437   Evidence already exists suggesting the epigenetic regulation of fruit development and

438   ripening mainly through DNA methylation [46]. As an example, the onset of ripening in

439   tomato fruit is associated with changes in the methylation marks of different genes associated

440   to carotenoid accumulation, ethylene perception and fruit softening [47]. Similar evidence

441   also exists for the hypermethylation of key ripening-related genes in pome fruit [48]. Whether

442   the regulation by acetylation/deacetylation is key during fruit development and ripening

443   remains elusive. Our data shows the differential development stage-dependent expression of

444   SRTs (direct comparison of expression levels of SRT1 vs SRT2) among different Rosaceae

445   spp. In pears, *PbSRT1* was already highly expressed during the initial fruit growing stages

446   yet maximum expression levels were found at the fully ripe stage, while expression levels of

447   *PbSRT2* increased progressively as the fruit developed (Fig. 2H). Accordingly, Aiese

448   Cigliano et al. [49] described that in tomato fruit, *SlSRT1* could mediate fruit development by

449   acting during the early development stages while *SlSRT2* seemed to be more involved in the

450   ripening process. Similar findings were also reported in banana [16]. The large increase in

451   *MdSRT2* expression levels observed in our study further reinforce this hypothesis. Indeed,

452   'Golden Reinders' apples in contrast to 'Williams' pears are able to progressively ripen on-

453   tree and to produce ethylene when harvested. We may then hypothesize that the increase in

454   *MdSRT2* gene expression observed in apple is likely associated to a progressive induction of

455   ethylene metabolism on-tree. Accordingly, the low *MdSRT2* expression levels observed in

456   'Williams' pear, may explain at least in part the specific behaviour of some pear cultivars that

457   are unable to produce ethylene even after harvest and need diverse chilling requirement to

458   induce this production.

459   In the case of nectarine, SRT gene expression levels remained low and did not drastically

460   change during fruit growth and ripening yet lower *PpSRT1* expression levels were found at

461   the fully ripe stage. Since SRTs are known to be involved in expanding the life span of

462   multiple organisms [50], the decrease of SRT gene expression levels in nectarine at the time

463   of harvest may be related to the short shelf-life of this fruit, especially in comparison to apples.

464   Under this scenario, a question arises on whether the gene expression levels of SRTs in fully

465   ripe fruit may predict to some extent the shelf-life potential of a given fruit. Future studies

466   analysing SRTs gene expression levels in a wide range of fruit with diverse shelf-life potential

467   are encouraged.

468

469   **4.3   Sirtuin expression is not regulated by an ethylene-dependent mechanism in**

470   **pear fruit**

471 Pears, apart from being an economically important crop, are considered an excellent model

472 to study the physiological events that occur during fruit ripening. Indeed, in comparison to

473 most climacteric fruit, not all pear cultivars are able to ripen once detached from the tree and

474 may need some chilling to initiate ripening [21,51,52]. 'Blanquilla' cultivar for instance, is

475 characterized by its ability to produce ethylene even on-tree and to fully ripen off-tree without

476 chilling [35]. 'Conference' and 'Williams' pears exhibited an intermediate behaviour and

477 need some chilling period to induce ethylene production [21]. Finally, 'Flor d'Hivern'

478 behaves like a non-climacteric fruit, showing an inability to produce ethylene even after long

479 periods of cold-storage [53]. Herein, we investigated if sirtuin gene expression levels at

480 commercial maturity could explain these differential postharvest behaviours. We analysed

481 the differences in SRTs gene expression levels at harvest and show that *PbSRT2* levels did

482 not only differ between species but also within cultivars. Given the known differences in the

483 ripening pattern among the studied cultivars, our results further reinforce the putative

484 involvement of SRT2 in fruit ripening [16,49]. In this sense, *PbSRT2* gene expression levels

485 were not linked to the specific cultivar capability to produce ethylene. This lack of correlation

486 was further confirmed when investigating *PbSRT1* and/or *PbSRT2* gene expression along

487 fruit growth in parallel to the changes observed at the ethylene biosynthetic level for

488 'Williams' pears (Fig. 4B and 4D). In fact, and although some HDACs have been described

489 to be induced by ethylene [54], neither AtSRT1 nor AtSRT2 from Arabidopsis are regulated

490 by ethylene itself at either the gene or protein level [13]. Furthermore, we need to keep in

491 mind the complexity of ethylene metabolism that involves complex perception and signalling

492 processes. In Arabidopsis for instance, both sirtuins interact with the EIN2 Nuclear

493 Associated Protein1 (ENAP1) to mediate the repression of some ethylene-responsive gens

494 [13], while in banana fruit, MaHDA1 binds to MaERF11 to repress the expression of

495 *MaACO1* and ripening [55]. Further studies are needed to better understand the determining

496 regulatory processes especially in relation to pear chilling requirement.

497 The role of ethylene on sirtuins gene expression levels in 'Blanquilla' pears was also

498 investigated by comparing fruit treated with the ethylene inhibitor 1-MCP and untreated fruit.

499 1-MCP is widely used for lengthening the shelf life of climacteric fruit due to its capability

500 to inhibit ethylene perception. In fact, previous studies have already demonstrated the

501 effectivity of this compound on 'Blanquilla' pears [30,56,57]. Our results demonstrated that

502 the gene expression pattern of both sirtuins was mainly altered by cold storage (Fig. 6) but

503 not by 1-MCP treatment, reinforcing then the idea that sirtuin gene expression is not regulated

504 by ethylene in pear fruit. Opposite results were observed for *MaSRT1* in banana fruit [16],

505 likely due to the known differences in ethylene biosynthesis and signalling existing between

506 both species.

507

508 **4.4   Fruit development and ripening-associated hormones negatively correlate**

509 **with *PbSRT1* but not with *PbSRT2***

510 Not only ethylene, but also other hormones play a key role on triggering the different events

511 that take place from seed germination to the fully ripened fruit. Increasing evidence suggest

512 a link between hormonal signalling and epigenetic regulation [58]. To further explore this

513 possibility, we determined the content of the most relevant hormones of 'Williams' pears

514 during fruit development and ripening. Hormonal changes along fruit growth and ripening

515 (Fig. 4A) generally agree with the findings from a previous study [21] yet showing some

516 specificities for the cultivar investigated herein. When exploring the possible link between

517 hormonal changes and SRTs gene expression levels (Fig. 4C), a clear negative correlation

518 between *PbSRT1* and several hormones including ABA, IAA, GA1 and zeatin was found.

519 Some studies have associated auxins in the regulation of the epigenome while also pointed

520   out that some auxin-response gens may be subjected to epigenetic modulation [58].

521   Regarding ABA, it is interesting to note the inverse behaviour existing between the levels of

522   ABA and ethylene production in the first stage of fruit growth (Fig.4A). Between the S1 and

523   S3 stage, a clear decrease in ABA levels was observed in 'Williams' pears concomitantly to

524   a peak of ethylene production and an increase in *PbSRT1* expression levels. These results are

525   in agreement with the work of Setha et al., [59] in cherries and allow us to assume that at this

526   growth stage 'Williams' pears likely behave as a non-climacteric fruit. They also let us to

527   hypothesize, when considering the straight negative relationship exiting between *PbSRT1*

528   expression levels and IAA or ABA content, a putative role of *PbSRT1* in the modulation of

529   the complex ABA-IAA-ethylene cross-talk described during ripening of multiple species

530   [21,60]. This idea would further reinforce the work described in *M. polymorpha* plant [14],

531   suggesting that *PbSRT1* plays a determining role in the preliminary phase of fruit ripening

532   and *PbSRT2* in the final phase of fruit growth/ripening.

533   In our study, no correlations were found between melatonin levels and the expression of

534   SRTs. Even though several studies highlight the relationship between aging, sirtuins and the

535   circadian modulator melatonin [61]; these relationships remain unclear in pears.

536

537   **4.5   Sugar metabolism and *PbSRT2* cooperate in the onset of ripening of pear fruit**

538   Sugar composition is essential to ensure optimal organoleptic properties, provides energy

539   supply for the fruit during development and in turn, becomes essential for the regulation of

540   plant growth processes [62]. Sugars such as sucrose together with ABA and other hormones

541   cooperate to mediate the ripening process by enhancing the expression of ABA-stress-

542   ripening (ASR) transcription factor that in turn, activates the expression of ripening-related

543   genes [63]. In this study, we confirmed that the main sugars in 'Williams' cultivar were both

544   glucose and fructose and that sucrose levels remained low and only significantly started to

545   peaked at harvest (Fig. 5A) reinforcing the idea that an increase in sucrose levels is needed

546   for the fruit to initiate its auto-catalytic ethylene production [35].

547   Sirtuins are involved in energy and sugar metabolism. In Arabidopsis, AtSRT1 represses the

548   glycolytic activity while promotes the mitochondrial respiration [8]. In rice, OsSRT1 is

549   located in the nucleus and is able to mediate the deacetylation of glyceraldehyde-3-phosphate

550   dehydrogenase (GAPDH) as a mechanism to repress the glycolytic pathway [64]. On the

551   other hand, Arabidopsis AtSRT2 has been described in the mitochondria being associated to

552   the regulation of energy metabolism [65]. Our results shown a positive correlation between

553   sugars and *PbSRT2* (Fig. 5B), which is in line with the fact that *PbSRT2* is more expressed

554   as fruit ripens (Fig. 2H) and that glycolytic enzymes were down-regulated during ripening of

555   other pome fruit species (i.e. apple; [66]). Despite correlation does not involved causation,

556   our data together with available literature, suggest a putative involvement of SRTs in sugar

557   metabolism in pear. Thus said, an in-depth examination of how SRTs can regulate sugar

558   metabolism and thereby the ripening process and postharvest behaviour of the different

559   Rosaceae species is envisaged.

560

561   **5. Conclusions**

562   The results presented in this study describe for the first time the presence and expression

563   pattern of both *SRT1* and *SRT2* during development and ripening in three important Rosaceae

564   species. In this line, the phylogenetic analysis of sirtuin sequences revealed an evident

565   divergence between stone and pome fruit but also a high similarity within pome fruit species

566   (apples and pears) that was not sustained when comparing SRT gene expression profile levels

567   during fruit development and ripening. Our data also points out the involvement of sirtuins

568   in orchestrating pear fruit development and ripening. Despite *PbSRT1* may negatively interact

569   with relevant developmental hormones (i.e. ABA), *PbSRT2* seemed to rather mediate the pear

570 ripening process. Such involvement of *PbSRT2* in pear ripening was not mediated by ethylene

571 or ethylene-dependent metabolism nor by other hormones, but rather positively correlated to

572 the sugar accumulation observed during pear development/ripening. Given the plethora of

573 functions in which sirtuins participate, including sugar/energy metabolism, future studies

574 trying to shed light on the actual implications of SRTs during fruit ripening are encouraged.

575

583

584 **Conflict of interests**

585 All authors declare no conflict of interest.

586

587 **Author contributions**

588 JGB, NV and RT and conceived and designed the experiments. NV and VLG carried out the

589 experiments. PM and SMB were responsible for the quantification of hormones. SMB, CL

590 and NT assisted with the statistical analysis and data interpretation. NV and JGB drafted the

591 manuscript and all other authors contributed in improving the final version of the manuscript.

592

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TABLES

Table 1. Characterisation of sirtuin proteins (including protein ID, length (aa), localization, MW and pI) from nectarine, pear and apple species

Gene	Species	Gene ID	Nº of exons	Protein	Protein ID	Protein length (aa)	Localization (score)	MW (Da)	pI
PpSRT1	Nectarine	LOC18780136	14	PpSRT1a	XP_007211402.1	471	Cytoplasm (5) Chloroplast (3)	52768.54	9.28
				PpSRT1b	XP_020418614.1	381	Cytoplasm (7) Nucleus (3)	42878.07	9.39
PpSRT2	Nectarine	LOC18779032	12	PpSRT2a	XP_007211470.2	391	Chloroplast (8) Nucleus (2)	43275.44	8.77
				PpSRT2b	XP_020418106.1	313	Nucleus (8) Chloroplast (3)	35165.99	9.01
PbSRT1	Pear	LOC103963915	14	PbSRT1a	XP_009375070.1	484	Cytoplasm (5) Nucleus 4)	54133.17	9.27
				PbSRT1b	XP_009375071.1	394	Nucleus (4) Cytoplasm (4)	44177.60	9.38
PbSRT2	Pear	LOC103964570	11	PbSRT2a	XP_009375792.1	394	Chloroplast (13) Vacuole (1)	43458.67	8.80
				PbSRT2b	XP_009375793.1	339	Chloroplast (12) Vacuole (2)	37176.66	9.63
MdSRT1	Apple	LOC103426829	14	MdSRT1a	XP_028944666.1	471	Peroxisome (11) Cytoplasm (2)	52687.52	9.30
				MdSRT1b	XP_028944667.1	381	Peroxisome (10) Nucleus (2)	42739.04	9.45
MdSRT2	Apple	LOC103406059	11	MdSRT2a	XP_017179847.1	339	Chloroplast (12) Vacuole (2)	37225.76	9.71
				MdSRT2b	XP_008343302.1	394	Chloroplast (11) Vacuole (2)	43492.70	8.85
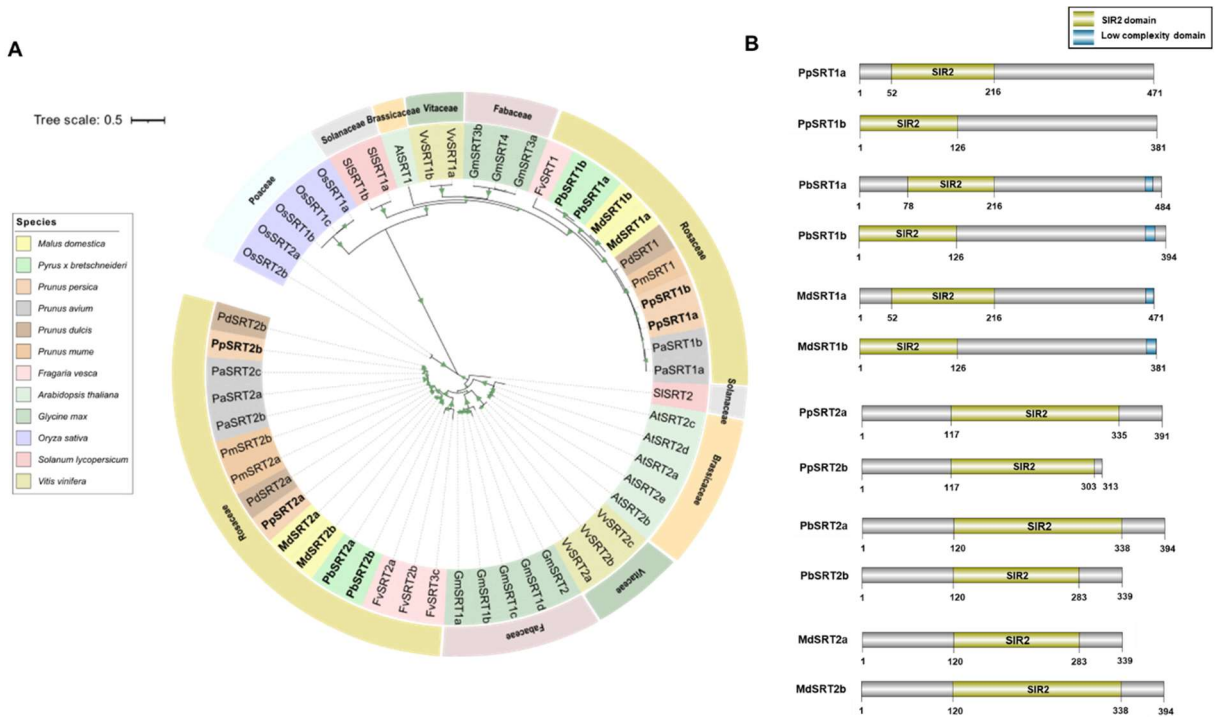
FIGURES

Figure 1

Figure 1. Phylogenetic analysis and domain architecture of sirtuins. (A) Unrooted maximum likelihood phylogenetic tree for sirtuin sequences of different Rosaceae species and other families including Brassicaceae, Solanaceae, Vitaceae, Fabaceae and Poaceae. Protein sequences were aligned using the MUSCLE alignment for subsequent tree building using MEGA X. Different species and families are shown in different colours. Multiple splicing variants are indicated as 'a', 'b', 'c' and 'd'. The size of the green triangles represents the bootstrap values of each node which were, in general, higher that 70%. (B) Domain architecture of sirtuins from apple, pear and nectarine. Location and size of domains are displayed in different colours as indicated. Letters 'a' and 'b' refer to different splicing variants.
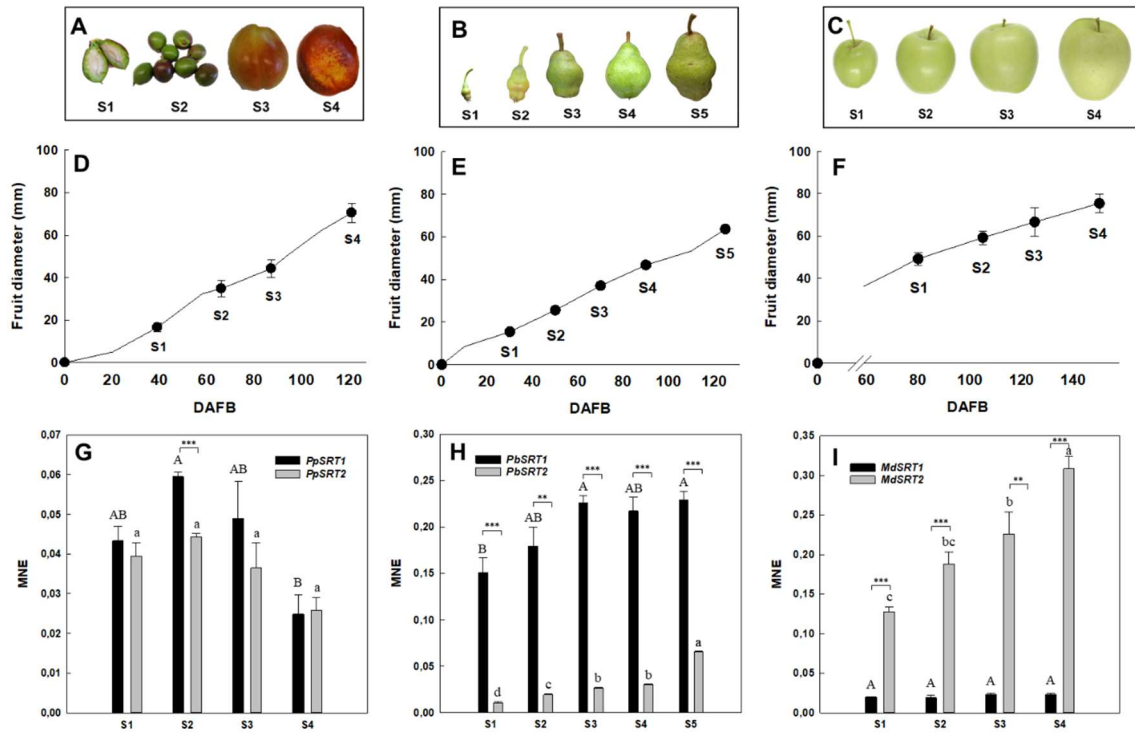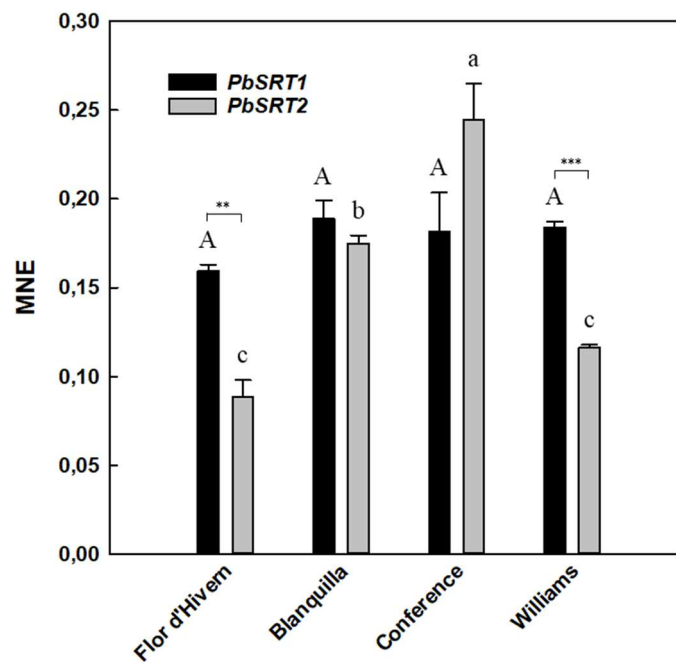
Figure 2

Figure 2. SRT gene expression pattern along the fruit development/ripening. Image of the different phenological stages of (A) nectarine, (B) pear and (C) apple, corresponding to each sampling point (days after full bloom, DAFB). Fruit diameter (mm) was monitored along the fruit growth for (D) nectarine, (E) pear and (F) apple cultivars. Error bars represent the standard errors of the means (n= 6). Relative expression levels of *SRT1* (■) and *SRT2* (▨) at each sampling point for (G) nectarine, (H) pear and (I) apple fruit. Error bars represent the standard errors of the means (n= 4). Lowercase and uppercase letters indicate significant differences ($p \leq 0.05$) for *SRT1* and *SRT2*, respectively, along the fruit growth. Asterisks denote significant differences among *SRT1* and *SRT2* at each sampling point (*$p \leq 0.05$; **$p \leq 0.01$; ****$p \leq 0.001$).

859 **Figure 3**

860

861 **Figure 3.** Relative gene expression levels of *PbSRT1* (■) and *PbSRT2* (▣) of different

862 pear cultivars ('Flor d'Hivern', 'Blanquilla', 'Conference' and 'Williams') at commercial

863 harvest day (*ca.* 173, 125, 135 and 138 DAFB, respectively). Error bars represent the

864 standard errors of the means (n= 3). Lowercase and uppercase letters indicate significant

865 differences ($p \leq 0.05$) for *PbSRT1* and *PbSRT2*, respectively, between cultivars. Asterisks

866 denote significant differences among *SRT1* and *SRT2* in each cultivar (*$p \leq 0.05$; **$p \leq$

867 0.01; ****$p \leq 0.001$).

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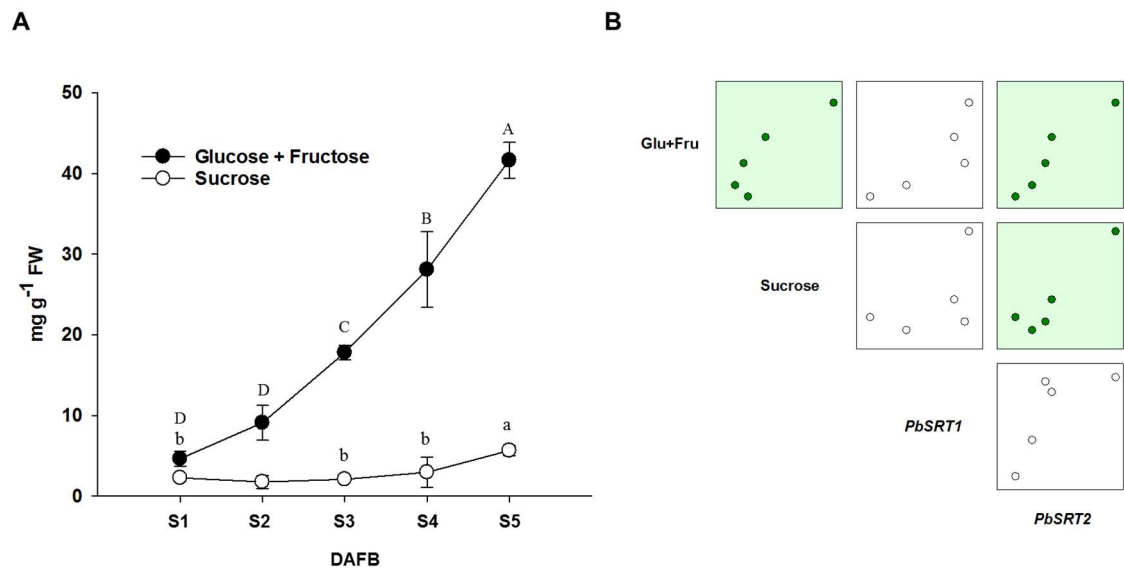
Figure 4

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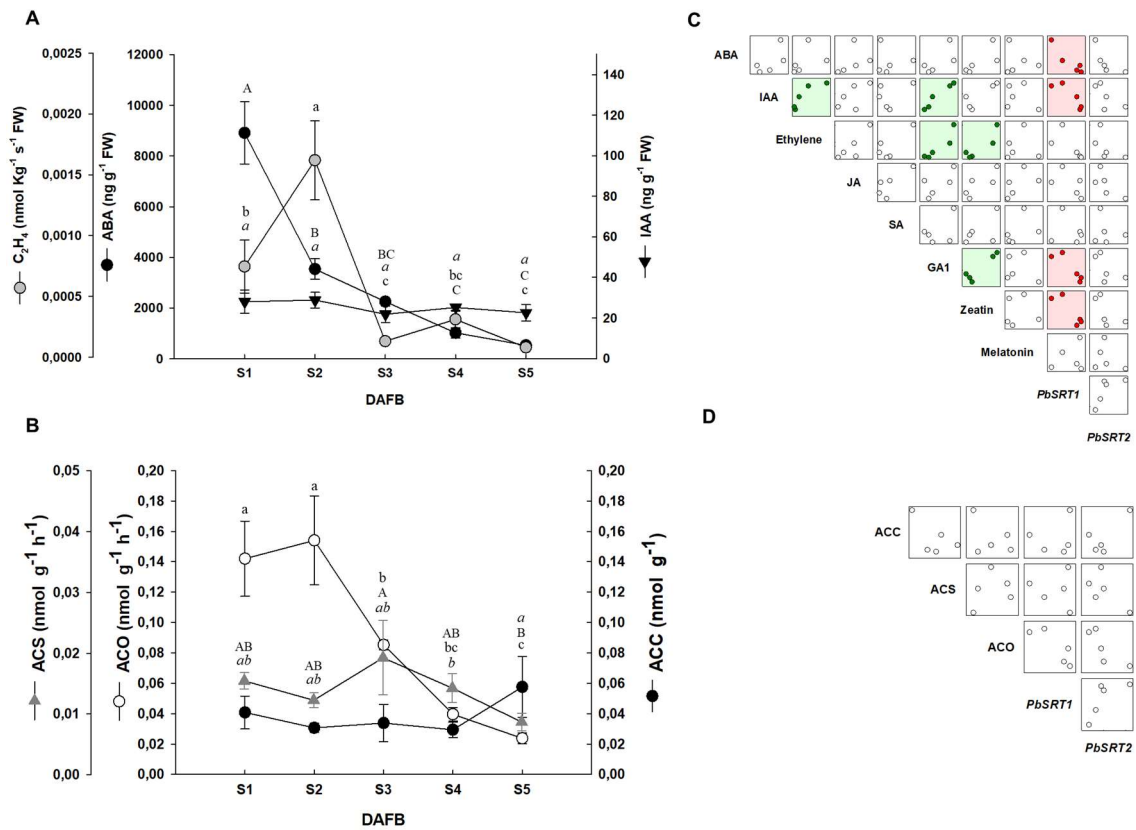
Figure 4. Relationship between temporal changes in hormones (Ethylene (C_2H_4; ⬤ grey),

abscisic acid (ABA; ⬤) and indole 3-acetic acid (IAA; ▼)) and SRTs gene expression

along 'Williams' pear development and ripening (A). Lowercase, uppercase and italic

letters indicate significant differences ($p \leq 0.05$) for ethylene, ABA and IAA, respectively,

along fruit development. (B) ACC synthase (ACS; ▲) and ACC oxidase (ACO; ○)

activity and 1-aminocyclopropane-1-carboxylic acid content (ACC; ⬤). Lowercase,

uppercase and italic letters indicate significant differences ($p \leq 0.05$) for ACC, ACS and

ACO, respectively, along fruit development. Error bars in (A) and (B) represent the

standard error of the means (n=6). Pearson's product moment correlation matrix between

PbSRT1 and *PbSRT2* gene expression levels with (C) hormones or (D) enzymes or

metabolites involved in ethylene biosynthesis. Green and red colours denote significant

($p \leq 0.05$) positive and negative correlations, respectively. Abscisic acid (ABA); indole

883   3-acetic acid (IAA), ethylene (C_2H_4); jasmonic acid (JA); salicylic acid (SA); gibberellin

884   A1 (GA1); 1-aminocyclopropane-1-carboxylic acid (ACC); ACC synthase (ACC); ACC

885   oxidase (ACO); sirtuin 1 (*PbSRT1*) and sirtuin 2 (*PbSRT2*).

886

887 **Figure 5**

888

889 **Figure 5.** Relationship between sugar content and SRTs gene expression along

890 'Williams' pear development and ripening. (A) Content of monosaccharides (glucose and

891 fructose) and sucrose along fruit development. Error bars represent the standard error of

892 the means (n=4). Lowercase and uppercase letters indicate significant differences ($p \leq$

893 0.05) for monosaccharides (glucose and fructose) and sucrose, respectively, along the

894 fruit growth and development. (B) Pearson's product moment correlation between sugars

895 content and *PbSRT1* and *PbSRT2* expression along fruit development. Green colours

896 denote positive and negative significant ($p \leq 0.05$) correlations, respectively.

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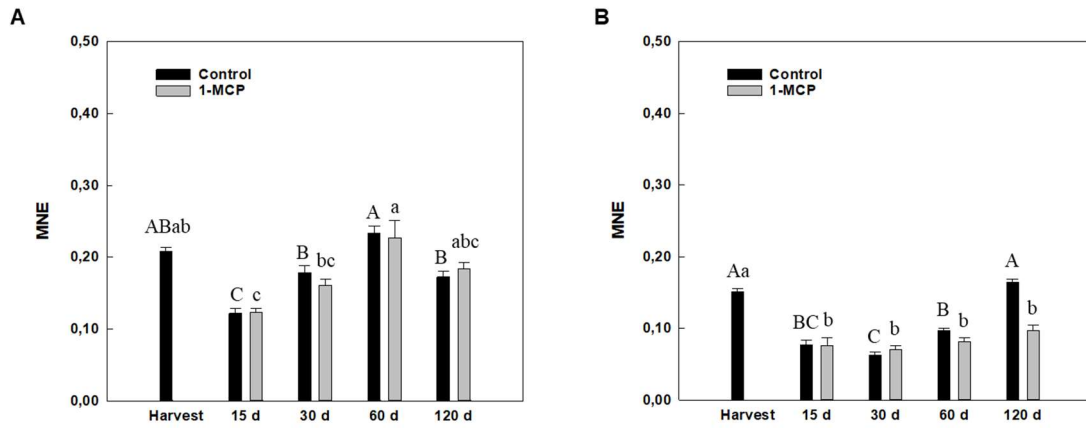
Figure 6

Figure 6. Effect of ethylene inhibition on sirtuin expression during the cold storage of the 'Blanquilla' pear cultivar. (A) *PbSRT1* and (B) *PbSRT2* expression levels of control (■) and 1-MCP (▢) treated samples during the cold storage for 15, 30, 60 and 120 d at -0.5 ºC and 90% RH. Error bars represent the standard error of the means (n=3). Lowercase and uppercase letters indicate significant differences ($p \leq 0.05$) for control and 1-MCP treated samples, respectively, along the fruit cold storage. Lack of asterisks denote no significant differences between treatments for each sirtuin.

SUPPLEMENTARY TABLES

908   **Supplementary Table S1.** Real-time PCR primer set to assess the expression pattern of sirtuins from nectarine, pear and apple cultivars

Gene	Gen ID	Cultivar	Primer name	Primer sequence (5' – 3')	Efficiency	Source
PpSRT1	LOC18780136	Nectarine	PpSRT1-Fw PpSRT1-Rv	CCTTTCATCGTGCAATGCCA AGGCCATCAACATTCTGGCT	2.04	This study
PpSRT2	LOC18779032	Nectarine	PpSRT2-Fw PpSRT2-Rv	GCCCTTAATCCAAAGTGGGC CAGGTCTCTGCTTCATGCCA	1.96	This study
PbSRT1	LOC103963915	Pear	PbSRT1-Fw PbSRT1-Rv	TCTTCAGCTTGATAAGGATGCCA GGGGCCAGGATGACATTTCT	2.08	This study
PbSRT2	LOC103964570	Pear	PbSRT2-Fw PbSRT2-Rv	CAGAGCCTGGTTCAGCTCAT GCACGGTGATGCAACCTATC	2.07	This study
MdSRT1	LOC103426829	Apple	MdSRT1-Fw MdSRT1-Rv	AGCGATCAAAAATGCACAGGC AAGTTCTTGCCGAACCACCA	2.28	This study
MdSRT2	LOC103406059	Apple	MdSRT2-Fw MdSRT2-Rv	CAGAGCCTGGTTCAGCTCAT GCACGGTGATGCAACCTATC	1.99	This study
Md8283	PCP030439	Pear/Apple	Md8282-Fw Md8283-Rv	CTCGTCGTCTTGTTCCCTGA GCCTAAGGACAGGTGGTCTATG	2.05	[30]
TEF2	LOC18778380	Nectarine	PpTEF2-Fw PpTEF2-Rv	GGTGTGACGATGAAGAGTGATG TGAAGGAGAGGGAAGGTGAAAG	2.1	[31]

909

910 **Supplementary Table S2.** Endogenous concentration expressed on fresh weight basis (ng g^{-1} FW) of different hormones in 'Williams' pear cultivar

911 along fruit development and ripening. Means ± standard error followed by the same letter are not significantly different at $p \leq 0.05$ (n=6) along

912 fruit development. Abscisic acid (ABA), indole-3-acetic acid (IAA), jasmonic acid (JA), salicylic acid (SA), gibberellin A1 (GA1).

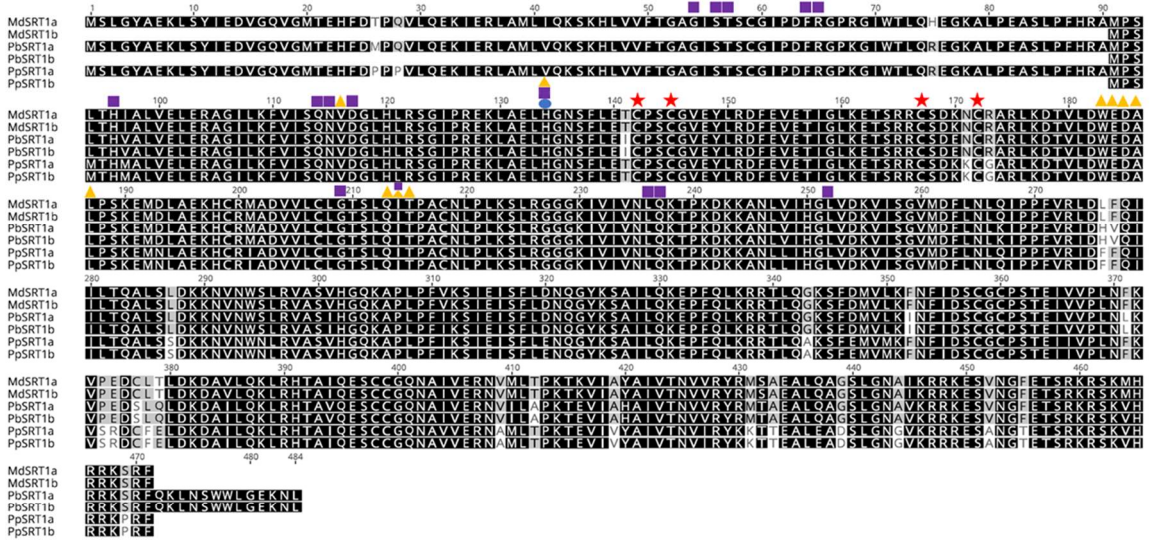|	Hormone profile (ng g^{-1} FW)				
Hormone	30 DAFB	50 DAFB	70 DAFB	90 DAFB	125 DAFB
ABA	8913.96 ± 1226.99 a	3536.43 ± 407.86 b	2254.17 ± 98.89 bc	1019.53 ± 211.61 c	530.51 ± 88.25 c
IAA	28.02 ± 5.69 a	28.77 ± 3.87 a	21.83 ± 4.03 a	25.17 ± 1.81 a	22.55 ± 4.01 a
JA	85.14 ± 8.81 ab	105. 95 ± 13.38 a	87.42 ± 2.90 ab	71.34 ± 6.52 ab	63.97 ± 6.82 b
SA	13.35 ± 2.96 a	27.03 ± 4.72 a	17,19 ± 3.68 a	12.79 ± 0.22 a	15.83 ± 3.70 a
GA1	15.01 ± 5.75 a	16.46 ± 7.76 a	5.68 ± 1.79 a	8.63 ± 4.52 a	7.24 ± 1.73 a
Zeatin	1.52 ± 0.76 a	1.67 ± 1.04 a	0.76 ± 0.40 a	0.54 ± 0.22 a	0.67 ± 0.21 a
Melatonin	3.14 ± 1.74 a	7.04 ± 4.54 a	10.90 ± 4.10 a	4.05 ± 1.68 a	2.73 ± 0.90 a

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914

SUPPLEMENTARY FIGURES

Supplementary Figure S1. (A) SRT1 and (B) SRT2 protein alignment of apple, pear and

nectarine. Protein sequences were aligned in Geneious Prime using the MUSCLE

alignment plugin. Identical residues are shaded in black, and similar amino acids are

shaded in grey. Symbols represent the NAD⁺ binding site ($\blacksquare$), the catalytic histidine ($\bullet$),

the zinc binding site ($\star$) and the substrate binding site ($\triangle$).