CRISPR/Cas9 gene editing uncovers the role of CTR1 and ROS1 in melon fruit ripening and epigenetic regulation

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Abstract

Melon (*Cucumis melo* L.) has emerged as an alternative model to study fruit ripening due to the coexistence of climacteric and non-climacteric varieties. The previous characterization of a major QTL *ETHQV8.1* sufficient to trigger climacteric ripening in a non-climacteric background allowed the identification within the QTL interval of a negative regulator of ripening *CmCTR1*-like (MELO3C024518), and a putative DNA demethylase *CmROS1* (MELO3C024516), the orthologue of *DML2*, a DNA demethylase regulating fruit ripening in tomato. To understand the role of these genes in climacteric ripening, we generated homozygous CRISPR knockout mutants of *CmCTR1*-like and *CmROS1* in a climacteric genetic background. The climacteric behavior was altered in both loss-of-function mutants in two summer seasons with an advanced ethylene production profile compared to the climacteric wild type, suggesting a role of both genes in climacteric ripening in melon. Single cytosine methylome analyses of the *CmROS1* knockout mutant revealed DNA methylation changes in the promoter regions of key ripening genes as *ACS1*,

39 *ETR1* and *ACO1*, and ripening associated-transcription factors as *NAC-NOR*,
40 *RIN* and *CNR*, suggesting the importance of *CmROS1*-mediated DNA
41 demethylation for triggering fruit ripening in melon.

42 **Introduction**

43
44 During the ripening process, fleshy fruits undergo physiological and metabolic
45 changes affecting color, flavor, firmness, and aroma. These changes are driven
46 by phytohormones and developmental factors and occur in a highly coordinated
47 manner with a direct impact on fruit quality and shelf-life (Giovannoni, 2001).
48 One of the main promoters of fruit ripening is the volatile hormone ethylene.
49 Depending on the involvement of this hormone during ripening, fruits have been
50 traditionally divided into i) climacteric, characterized by an increase in
51 respiration and ethylene production at the onset of ripening and ii) non
52 climacteric, presenting low levels of both ethylene production and respiration
53 rate across the process (McMurchie *et al.*, 1972). Dissecting the regulatory
54 network underlying the control of fruit ripening has been a major goal due to its
55 biological significance but also for its commercial value (Giovannoni *et al.*, 2017;
56 Wang *et al.*, 2020).

57 Important advances in the understanding of the molecular mechanisms
58 underlying climacteric fruit ripening have been made in the model species
59 tomato (Giovannoni, 2007) Ripening related mutants allowed the identification
60 of several transcription factors that are upstream regulators of ethylene
61 dependent or independent ripening. Among them *RIPENING INHIBITOR (RIN)*,
62 *NON-RIPENING (NOR)*, and *COLORLESS NON-RIPENING (CNR)*(Vrebalov *et al.*,
63 *et al.*, 2002; Manning *et al.*, 2006; Giovannoni, 2007).

64 Recent studies demonstrated that DNA methylation levels play an important
65 role at the onset of fruit ripening in tomato(Zhong *et al.*, 2013). Modulation of
66 DNA methylation levels is governed by DNA methylases and demethylases.
67 The enzymatic removal of methylcytosine in plants is initiated by a family of
68 DNA glycosylases/lyases, including DEMETER (DME), Repressor of silencing 1
69 (ROS1), DEMETER-like2 (DML2) and DEMETER-like3 (DML3), firstly
70 characterized in the model plant *Arabidopsis thaliana* (Zhu, 2009). In tomato,
71 SIDML2 is induced upon the onset of ripening leading to a global DNA
72 hypomethylation during ripening (Zhong *et al.*, 2013; Liu *et al.*, 2015; Lang *et*

73 *al.*, 2017) Knockout using CRISPR/Cas9 system and knockdown RNAi mutants
74 in this species revealed that *SIDML2* is required for normal fruit ripening by the
75 activation of ripening-induced genes and repression of several ripening-
76 repressed genes (Zhong *et al.*, 2013; Lang *et al.*, 2017). Nonetheless, the
77 tomato model is not universal as different transcriptional positive feedback
78 circuits controlling ripening in climacteric species were identified (Lü *et al.*,
79 2018).

80 Melon (*Cucumis melo* L.) has emerged as an alternative model to study fruit
81 ripening since both climacteric (e.g. *cantalupensis* types as ‘Védrantais’ (VED))
82 and non-climacteric (e.g. *inodorus* types as ‘Piel de Sapo’ (PS)) genotypes
83 exist. The recent characterization of a major QTL in chromosome 8 of melon,
84 *ETHQV8.1*, which is sufficient to activate climacteric ripening in a non-
85 climacteric background, allowed the identification of candidate genes related to
86 fruit ripening in a genomic interval of 150 kb that contained 14 annotated genes
87 (Pereira *et al.*, 2020). Some of these genes are highly expressed in fruits and
88 contain multiple non-synonymous polymorphisms distinguishing the climacteric
89 VED from the non-climacteric PS genotype.

90 One of the candidates (*CmROS1*, MELO3C024516) encodes the homolog of
91 the main DNA demethylase *ROS1* in Arabidopsis, which targets mainly
92 transposable element (TE) sequences and regulates some genes involved in
93 pathogen response and epidermal cell organization (Yamamuro *et al.*, 2014; Le
94 *et al.*, 2014). The closest orthologue in tomato, *SIDML2* is crucial for the DNA
95 demethylation of fruit ripening genes including ethylene synthesis and signaling
96 (Lang *et al.*, 2017).

97 The other candidate gene is *CONSTITUTIVE TRIPLE RESPONSE 1* (*CTR1*) a
98 serine/threonine kinase (*CmCTR1-like*, MELO3C024518). This kinase interacts
99 physically with ethylene receptors as a negative regulator of the ethylene signal
100 transduction pathway (Kieber *et al.*, 1993). In the absence of ethylene, *CTR1* is
101 activated, preventing the downstream transduction pathway; when ethylene is
102 present, the ethylene receptor terminates the activation of *CTR1*, leading to the
103 ethylene responses (Binder, 2008). In tomato, the silencing of *CTR1* promoted
104 fruit ripening, validating its role as a negative regulator of the ethylene signal
105 transduction pathway (Fu *et al.*, 2005).

106 In this study, we aimed to better understand the role of the two *ETHQV8.1*-
107 containing candidate genes *CmROS1* and *CmCTR1*-like in fruit ripening by
108 obtaining CRISPR/Cas9-induced loss-of-function mutants in a climacteric melon
109 genotype. Furthermore, we characterized the role of *CmROS1* in DNA
110 methylation homeostasis during fruit ripening.

111 **Materials and methods**

112 **CRISPR/Cas9 vector Construction**

113 To target *CmROS1* (MELO3C024516) two different guide RNAs (gRNA) of 20
114 nucleotides in length separated by 188 bp were designed using Breaking Cas
115 tool (<https://bioinfogp.cnb.csic.es/tools/breakingcas/>) (Table S1). The two
116 oligonucleotides generated for each gRNA were annealed and cloned in the
117 sites *BbsI* and *BsaI* into the plasmid ptandemgRNA. The construct was verified
118 by sequencing and then digested with *SpeI* and *KpnI* to release the cassette
119 that was then inserted into the same sites in the pB7-Cas9-TPC-polylinker
120 binary vector. Cloning vectors were gently provided by Prof. Puchta (KIT,
121 Germany).

122 For *CmCTR1*-like (MELO3C024518) we used the pEn-CHIMERA vector
123 provided by Prof. Puchta (KIT, Germany) to generate the entry construct. A
124 single gRNA of 20 nucleotides was designed using Breaking Cas tool
125 (<https://bioinfogp.cnb.csic.es/tools/breakingcas/>) (Table S1). Cloning steps of
126 the gRNA and transfer to the pDe-Cas9 binary vector were performed as
127 previously described (Schiml and Puchta, 2016).

128 **Agrobacterium mediated plant transformation**

129
130 *Agrobacterium tumefaciens* (strain AGL0) cells were transformed with the
131 binary CRISPR/Cas9 construct. Plant transformation was performed by co-
132 cultivation of the Agrobacterium culture with one-day-old cotyledons of VED as
133 previously described (Castelblanque *et al.*, 2008) including the following
134 modifications: cotyledons were dissected as in (García-Almodóvar *et al.*, 2017),
135 regeneration media was supplemented with 6-benzylaminopurine (BA) and
136 Indole-3-acetic acid (IAA) and *Agrobacterium* was co-cultured with the explants

137 during a period of three days. Transgenic plants containing the *bar* gene were
138 selected by L-Phosphinothricin (PPT) resistance and were grown in a growth
139 room under a 12-h light/12-h dark cycle at 28 °C.

140

141 **Detection of mutations**

142 Genomic DNA from leaves of in vitro plantlets (T0) and from young leaves of T1
143 and T2 plants was extracted using the CTAB method with some modifications
144 as described in (Pereira *et al.*, 2018). The transgene presence was detected by
145 PCR using specific primers targeting Cas9. Genomic regions flanking gRNA1
146 and gRNA2 of *CmROS1* were amplified by PCR using specific primers. For
147 detection of mutations in *CmCTR1*, a region targeting the gRNA was amplified
148 with specific primers. All primers are listed in Table S2. Mutations were detected
149 by sequencing the amplified fragments and identified by double peaks in the
150 sequence chromatograms. Purified PCR products were cloned into p-Blunt II-
151 TOPO vector (Life Technologies) and sequencing of colonies using M13F and
152 M13R primers was performed to confirm the mutations.

153

154 **Generation of T2 plants and phenotyping of climacteric ripening traits**

155 Ploidy level of T0 plants was evaluated by flow-cytometry analysis and selected
156 T0 plants for each gene were grown under greenhouse conditions (25°C for 16
157 hours and 22°C for 8 hours) and self-pollinated. T1 seedlings were screened for
158 the presence of Cas9 by PCR. After segregation, non-transgenic homozygous
159 edited T1 plants were selected and grown under greenhouse conditions to
160 obtain the T2 seeds for the phenotypic assay.

161 Edited T2 *CmROS1* (n=8) and *CmCTR1* plants (n=8) were grown randomized
162 under greenhouse conditions (24°C for 16 hours and 22°C for 8 hours) at
163 Caldes de Montbui (Barcelona) in 2020 and 2021. VED plants were used as a
164 wild type control plant (n=8). Plants were weekly pruned and manually
165 pollinated to obtain one fruit per plant. The harvest date was determined
166 following two criteria: either abscission date, when the fruit abscised from the

167 plant, or 5 days after the formation of the abscission layer when it was not
168 complete.

169 Ripening-related traits were evaluated as described in Pereira 2020 in two
170 consecutive summer seasons (2020 and 2021). Production of aroma (ARO),
171 chlorophyll degradation (CD) and abscission layer formation in the pedicel of
172 the fruit (ABS) were daily evaluated and firmness was measured at harvest
173 time. The visual inspection of melon fruits, attached to the plant, was performed
174 daily, from approximately 20 days after pollination (DAP) until harvest. In
175 addition, individual pictures of the fruits were obtained weekly. ARO, ABS and
176 CD were recorded as 0 = absence and 1 = presence. The aroma production
177 was evaluated every day by smelling the fruits. The firmness of fruit flesh was
178 measured at harvest using a penetrometer (Fruit TestTM, Wagner Instruments),
179 in at least three regions of the fruit (distal, proximal and median), and the mean
180 value was registered.

181 **Ethylene production**

182 Ethylene production *in planta* was measured in the 2020 summer season using
183 non-invasive gas chromatography – mass spectrometry (GC-MS) method, as
184 described in (Pereira *et al.*, 2017). The ethylene peak was monitored before
185 ripening from 20 DAP until harvest. The atmosphere of the chamber containing
186 the fruit was measured every day.

187 The ethylene peak was characterized by four traits, measured as described in
188 Pereira 2020: maximum production of ethylene in the peak (ETH), earliness of
189 ethylene production (DAPE), earliness of the ethylene peak (DAPP), and width
190 of ethylene peak (WEP).

191 **Epigenomics**

192 DNA was extracted from fruit flesh of ROS1-CRISPR-2 and the wild-type VED
193 at different ripening stages (15, 25 and 30 DAP and harvest point) following the
194 CTAB protocol(Doyle and JJ, 1990) adding a purification step using
195 Phenol:Chloroform:Isoamyl alcohol (25:24:1). For each time point, three
196 biological replicates were analysed. Bisulfite conversion, BS-seq libraries and

sequencing (paired-end 100 nt reads) were performed by BGI Tech Solutions (Hong Kong). Mapping was performed on melon genome v3.6.1 (Ruggieri *et al.*, 2018) using Bismark v0.14.2 (Krueger and Andrews, 2011) and the parameters: --bowtie2, -N 1, -p 3 (alignment); --ignore 5 --ignore_r2 5 --ignore_3prime_r2 1 (methylation extractor). Only unique mapping reads were retained. The methylKit package v0.9.4 (Akalin *et al.*, 2012) was used to calculate differential methylation in 100 bp non-overlapping windows (DMRs). Significance of calculated differences was determined using Fisher's exact test and Benjamin-Hochberg (BH) adjustment of p-values (FDR<0.05) and methylation difference cutoffs of 40% for CG, 20% for CHG and 20% for CHH. Differentially methylated windows within 100bp of each other were merged to form larger DMRs. 100bp windows with at least six cytosines covered by a minimum of six (CG and CHG) and ten (CHH) reads per comparison were considered.

211

212 **Statistical analyses**

213 All the statistical analyses and graphical representations were obtained using
214 the software R v3.2.3 (R Development Core Team, 2011) with the RStudio
215 v1.0.143 interface (*RStudio: Integrated development environment for R*, 2012).

216

217 **Results**

218

219 **Generation of CRISPR/Cas9 knockout mutants in candidate genes for** 220 ***ETHQV8.1* and inheritance of the editions**

221

222 To investigate the role of *CmROS1* (MELO3C024516) and *CmCTR1*-like
223 (MELO3C024518) genes in the fruit ripening process in melon, we knocked
224 them out using the CRISPR/Cas9 gene editing system in a climacteric genetic
225 background (VED).

226 A strategy with two target sites in exon 2 was used for *CmROS1* (Fig. 1). We
227 obtained 15% transformation efficiency, recovering in total 59 transgenic rooted
228 plants. From the transgenic plants, almost half of them (46%) were edited.
229 Multiple independent transgenic plants were genotyped by sequencing the
230 genomic DNA spanning both target sites. Most of the editions (75%) occurred in
231 target 1 (gRNA1) whereas only a few editions (25%) were obtained for target 2
232 (gRNA2). Several different insertions and deletions were obtained in T0 plants

with biallelic or heterozygous mutations (Fig. S1), with several plants carrying the same mutation (+1 bp). A diploid biallelic line with an insertion of 1 bp and a deletion of 23 bp that were predicted to generate truncated proteins was selected for further work (Fig. 1A).

The selected biallelic T0 line was self-pollinated to obtain non-transgenic (Cas9 free) plants carrying homozygous editions. After segregation, T1 lines homozygous for the 1 bp insertion (ROS1-CRISPR-1) or the 23 bp deletion (ROS1-CRISPR-2) were selected for further study (Fig. 1A and C).

A different CRISPR Cas9 strategy was used to target the *CmCTR1*-like gene. A single target site was selected in exon 6 of *CmCTR1*-like (Fig. 1B). For this target gene we obtained 12% of transformation efficiency. Transgenic T0 plants were screened for mutations in the target site and 40% were edited showing mainly large or small deletions (Fig. S1). From the edited T0 plants, a biallelic line carrying a 11 bp deletion and a 1 bp insertion was selected and self-pollinated to segregate out the Cas9 transgene. The genetic editions were stably transmitted to T1 plants. After segregation, a homozygous edited line carrying the 11 bp deletion (CTR1-CRISPR-1), which is predicted to generate a premature termination codon, and the homozygous line with 1 bp insertion (CTR1-CRISPR-2), generating a frame shift, were grown under greenhouse conditions for the characterization of fruit ripening related traits (Fig. 1B and C).

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Figure 1: Schematic representation of the target sites for CRISPR/Cas9 and selected CRISPR edited lines. **(A)** Position of the gRNA target sites (red triangle) and selected mutations in the CRISPR lines for *CmROS1* **(B)** Position of the gRNA target sites (red triangle) and selected mutations in the CRISPR lines for *CmCTR1*-like **(C)** Fruit phenotype at harvest time of the wild type VED and CRISPR edited lines.

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***CmROS1* and *CmCTR1* edited plants show altered ripening phenotypes**

ROS1-CRISPR-1/2 and CTR1-CRISPR-1/2 were evaluated and characterized for ripening related traits in two consecutive summer seasons (2020 and 2021). However, the line CTR1-CRISPR-2 was only characterized in 2021 due to a powdery mildew infection of some replicates in 2020 that prevented its evaluation. Overall, the fruit appearance (shape, weight and colour) of the CRISPR edited lines did not show major differences with the wild-type VED at

271 harvest time and no significant changes were detected in the flesh firmness
 272 (Fig. 1C, Table S3). To better characterize the ripening process, we measured
 273 ethylene production *in planta* in 2020 with a non-invasive methodology allowing
 274 observing the phenotype of the downstream effects of this hormone.

275 The phenotypic characterization revealed a significant earliness of the
 276 climacteric symptoms for all the edited lines showing the same ripening
 277 behaviour pattern in both years (Fig. 2, Table S3). In 2020, the earliest
 278 climacteric symptom was sweet aroma production (EARO), which in the
 279 CRISPR edited lines for both genes appeared around two days before VED.

280 The initiation of the rind color change, which is attributed to chlorophyll
 281 degradation (ECD), was appreciated almost simultaneously with the detection
 282 of the abscission layer formation (EALF) and both ripening-related traits arose
 283 in both *CmROS1* edited lines two days before VED. The CTR1-CRISPR-1
 284 edited line exhibited the earliness of the ripening related traits all at the same
 285 time, which differed significantly from VED, arising around three days before
 286 than VED for ECD and EALF and two days for EARO.

287 During the second summer season, we evaluated all the CRISPR edited lines.
 288 In general, the environmental conditions delayed ripening of both VED and
 289 mutant plants (around 4-5 days later in 2021). Despite this environmental effect,
 290 all CRISPR edited lines displayed significant advances of about three days in
 291 the ripening-related traits ECD, EARO and EALF when compared to VED (Fig.
 292 2). Moreover, during this year, the line CTR1-CRISPR-2 was evaluated, and
 293 the dataset showed the same behaviour for both CTR1 edited lines. ROS1
 294 edited lines also showed the same pattern between them.

295 We also monitored fruit ethylene emission daily in 2020 without altering the
 296 ripening process (Fig. 2C). The CRISPR edited lines showed a different
 297 ethylene production pattern compared to wild-type VED, with both *CmROS1*
 298 edited lines showing the same profile. In *CmROS1* mutant lines, ethylene
 299 production started two days before the wild-type VED and with an increment of
 300 2.7 to 3-fold of ethylene production (Fig. 2C and Table S3).

301 For *CmCTR1* edited lines, ethylene measurements for CTR1-CRISPR-2 were
 302 not available due to the infection with powdery mildew of some of the replicates
 303 of this line at around 20 DAP. The CTR1-CRISPR1 line showed a significant
 304 difference in the earliness of ethylene production (DAPE) and earliness of

305 ethylene peak (DAPP). In this line, ethylene was detected around three days in
306 advance of wild-type VED. Similarly, the peak of ethylene production was also
307 advanced three days in CTR1-CRISPR1 compared to wild-type VED, however
308 this advancement was not accompanied by a significant difference in the
309 maximum quantity of ethylene produced (Fig. 2C and Table S3). Overall, these
310 results demonstrate that both candidate genes are involved in melon fruit
311 ripening.

312
313 **Figure 2:** Evaluation of climacteric ripening associated traits in CRISPR edited
314 lines and VED (in two consecutive years) and ethylene emission rates. **(A)**
315 Earliness of chlorophyll degradation (ECD), Earliness of production of aroma
316 (EARO) and Earliness of abscission layer formation (EALF) in 2020 **(B)** ECD,
317 EARO and EALF in 2021 **(C)** Ethylene production in attached fruits from 25
318 days after pollination (DAP) until harvest in 2020.

319 320 **Characterization of the ROS1-CRISPR and VED methylome at different** 321 **fruit ripening stages**

322
323 To better understand at the molecular level the role of *CmROS1* in DNA
324 demethylation and fruit ripening in melon, we generated single-cytosine
325 resolution methylomes by whole genome bisulfite sequencing from fruits of
326 ROS1-CRISPR-2 and the wild-type VED plants at 15, 25 and 30 DAP as well as
327 at harvest (H) point (Fig. 3A).

328 When comparing the global methylation level along ripening in VED, we found
329 that methylation at CG and CHG contexts declines along fruit ripening, showing
330 around 2,000 and 4,000 hypomethylated regions (DMR) respectively in the CG
331 and CHG context at harvest time compared to the first stage of ripening (i.e. H
332 vs 15 DAP) (Fig. 3B). Interestingly, these changes were more often associated
333 with promoter and intergenic regions (Fig. 3D).

334 In order to evaluate the role of ROS1 in the observed DNA methylation
335 dynamics, we compared the methylation level in the three contexts of ROS1-
336 CRISPR-2 and the wild-type VED plants at the same ripening stage (Fig. 3C). In
337 this way, we identified numerous changes in DNA methylation levels for the
338 three sequence contexts. In total (CG, CHG and CHH context together), we
339 found 16,968 hypermethylated DMRs at 15 DAP, 26,497 at 25 DAP, 19,928 at
340 30 DAP and 43,156 at H time relative to VED. Overall, CRISPR-ROS1 line is

341 associated with hypomethylation of CG and hypermethylation of CHG DMRs
342 (Fig. 3C).

343 To further investigate the targets of ROS1 we focused on the hypermethylated
344 DMRs in the CRISPR-ROS1 line (Table S4). Moreover, in CHH context at H
345 time there are changes in the number of DMRs annotation between VED and
346 the edited line. Among the CHH hypermethylated regions in CRISPR-ROS1
347 compared to VED at H time, 14% are associated with TEs, 46% with intergenic,
348 6% in promoter regions (defined as 1 kb upstream transcriptional start sites),
349 and 33% in genic regions (Fig. 3D).

350

351 **Figure 3:** General methylation and DMR regions at different ripening stages of
352 VED and CRISPR-ROS1 line. **(A)** Fruit ripening stages **(B)** number of DMRs
353 along ripening in VED **(C)** number of DMRs in VED vs CRISPR-ROS1 at the
354 same time point of ripening **(D)** DMRs annotation in VED along ripening **(E)**
355 DMRs annotation in VED vs CRISPR-ROS1 at the same time point of ripening

356

357 Notably, Gene ontology (GO) enrichment analysis of genes associated with
358 hypermethylated DMRs at H time in the CRISPR-ROS line compared to VED
359 and hypomethylated along ripening in VED context revealed an
360 overrepresentation of genes related to response to stress in CRISPR-ROS1
361 compared to VED (Table S5).

362

363 ***CmROS1* targets promoter regions of key genes involved in ripening**

364 We have further analysed the methylation level of key genes known to
365 participate in the ripening process in the three contexts. Changes were found at
366 different stages of ripening in the promoter region of genes involved in the
367 ethylene biosynthesis or signaling pathway: *ACS1* (MELO3C016340.2), *ETR1*
368 (MELO3C003906.2) and *ACO1* (MELO3C014437) as well as in ripening
369 associated-transcription factors: *NAC-NOR* (MELO3C016540), *RIN*
370 (MELO3C026300.2) and *CNR* (MELO3C002618.2) (Fig. 4).

371 Notably, the promoter region of *ACS1* appeared hypomethylated on the three
372 sequence contexts in the CRISPR-ROS1 line compared to VED in all the time
373 points studied along ripening. Furthermore, hypomethylation of the *ACO1*
374 promoter (CG and CHH context) was observed at 25 and 30 DAP and the *ETR1*

375 promoter region (CHG and CHH context) at 30 DAP. In contrast, CHG
376 hypermethylation of *NAC-NOR* was found from the earliest stage until 30 DAP
377 in the mutant and hypomethylated at 30 DAP in the CHH context. For the other
378 two transcription factors, we observed CHH hypomethylation of *RIN* and *CNR*
379 promoter regions at H time. In combination, these results provide strong
380 evidence that *CmROS1* modulates DNA methylation levels of ripening genes in
381 melon, which may have major consequences for fruit ripening.

382
383 **Figure 4:** DNA methylation levels of ethylene related genes and ripening
384 associated transcription factors for VED and CRISPR-ROS1 at different fruit
385 ripening development stages in the three contexts **(A)** *ACO1* in CHH context at
386 25 (left) and 30 DAP (right) **(B)** *ACS1* in CHG context at 25 (left) and 30 DAP
387 (right) **(C)** *ETR1* in CHG context at 30 DAP **(D)** ripening associated transcription
388 factors in CHH context: *NAC-NOR* at 30 DAP, *RIN* at H point and *CNR* at H
389 point.

390 Discussion

391 Advances in genome editing have been obtained applying the CRISPR/Cas
392 technology in several plant species. However, among the Cucurbitaceae family
393 studies were only reported in watermelon for herbicide resistance (Tian *et al.*,
394 2016, 2018) and cucumber for virus resistance (Chandrasekaran *et al.*, 2016).
395 More recently, edited plantlets with a disruption of a visual reporter gene
396 (*CmPDS*), which could not be carried to the next generation, were generated in
397 melon using CRISPR/Cas9 (Hooghvorst *et al.*, 2019). To our knowledge,
398 hereby we report for the first time the generation of melon knockout mutants for
399 an agronomic important trait such as fruit ripening and the inheritance of the
400 introduced mutations to the following generations using CRISPR/Cas9.

401 Melon is considered a recalcitrant species for genetic transformation. In this
402 study, we obtained on average 15% transgenic plants and from these, 40% and
403 46% were successfully edited plants for our target genes *CmROS1* and
404 *CmCTR1* using two or one gRNA strategy, respectively. The edited plants
405 carried several types of editions nearby the protospacer adjacent motif (PAM)
406 sequence of the target gRNA. As reported for other species (Feng *et al.*, 2014),
407 biallelic edited plants were obtained (70% of the edited plants), suggesting early
408 editions during developmental stages.

409 In accordance with the mutations induced by Non-homologous end Joining
 410 pathway, the sequence analysis of the edited lines revealed that the most
 411 frequent editions were insertions and deletions with more than one independent
 412 event exhibiting the same edition. All the gRNA used here successfully induced
 413 mutations in the target genes. However, editions in *CmROS1* were mainly
 414 obtained in gRNA1 suggesting a higher edition efficiency for this gRNA. In
 415 addition, in contrast to the observations reported by Hooghvorst et. al., base
 416 pair substitutions were not obtained for any of the genes targeted in this study.

417 Improving fruit quality and shelf life has been one of the main challenges for
 418 agriculture. During the last decades, advances in understanding the ripening
 419 process were approached by conventional breeding and genetic engineering
 420 tools. For instance, CRISPR knockout mutants in tomato have proved the
 421 importance of master ripening regulator genes(Ito *et al.*, 2015).

422 More recent studies showed that epigenetic regulation plays a key role in fruit
 423 ripening (Lü *et al.*, 2018). The balance of global DNA methylation/demethylation
 424 is altered during fruit ripening and these alterations are governed by DNA
 425 demethylases. In tomato, more than 200 promoters of ripening-related genes,
 426 including master regulators, ethylene related genes, fruit softening, and
 427 carotenoids synthesis genes, are regulated by DNA demethylation at the onset
 428 of ripening (Zhong *et al.*, 2013).

429 In Arabidopsis, the protein repressor of silencing 1 (*ROS1*), which belongs to
 430 the subfamily of bifunctional 5-methylcytosine DNA glycosylases/lyases, has
 431 been characterized as the main sporophytic DNA demethylase(Gong *et al.*,
 432 2002). In tomato, there are four genes (*SIDML1*, *SIDML2*, *SIDML3* and *SIDML4*)
 433 encoding putative DNA demethylases, being *SIDML2* the closest ortholog to
 434 Arabidopsis *ROS1* gene. Furthermore, *SIDML2* expression is highly correlated
 435 with fruit ripening (Zhong *et al.*, 2013; Liu *et al.*, 2015). In melon, we have
 436 identified four putative *ROS1* homologues (MELO3C024516, MELO3C021451,
 437 MELO3C002241 and MELO3C009432) (Fig S2). The gene MELO3C024516
 438 locates in the previously identified ripening QTL interval *ETHQV8.1* and
 439 therefore was edited in this study. The CRISPR-*ROS1/2* lines, carrying loss-of-
 440 function homozygous alleles of MELO3C024516, showed an advance in
 441 climacteric ripening compared to the wild type, suggesting a role of this gene in
 442 the complex regulation of climacteric ripening in melon. Interestingly, RNA-seq

expression analysis of several fruit ripening stages in wild type climacteric VED shows that the four putative ROS1 genes have a similar expression profile along ripening (Fig. S3), suggesting that more than one DNA demethylase may be involved in this process. Unlike in tomato, in which the CRISPR SIDML2 mutant showed an inhibitory effect on fruit ripening (Lang *et al.*, 2017), the *CmROS1* knockout melon fruit ripens ahead of the wild type VED. Our methylome analysis of the climacteric variety VED showed an overall demethylation in CG and CHG context along fruit ripening, similar to what has been reported in tomato (Liu *et al.*, 2015; Lang *et al.*, 2017), orange (Huang *et al.*, 2019) and strawberry (Cheng *et al.*, 2018). Furthermore, hypomethylation levels in the promoter regions of key ripening genes (e.g. *ACS1*, *ETR1*, *ACO1*) are in agreement with the phenotype displayed by the *CmROS1* CRISPR lines. Also, genes related to biotic stress response were also found to be hypomethylated in ROS1 vs VED at harvest, suggesting a possible role of this DNA demethylase in stress-response genes, as reported for *ROS1*, *DML2*, *DML3* in response to biotic stress in Arabidopsis (Le *et al.*, 2014; Halter *et al.*, 2021).

Both mutant lines of CTR1-CRISPR promote fruit ripening in melon in agreement to the phenotype described when silencing *LeCTR1* in tomato fruits (Fu *et al.*, 2005) and the previously described role of *CTR1* as a negative regulator of ethylene signaling in other species (Binder, 2008). This second candidate gene of the QTL *ETHQV8.1* plays also an important role in the ripening process as a negative regulator affecting the initiation of the ripening process but without affecting other important traits such as firmness.

To our knowledge, this is the first time that the CRISPR technology has been implemented on genes involved in agronomically important traits in melon. The implementation of this technology in this species and the inheritance of the editions to the following generations is of high interest and a valuable resource not only for researchers but also for breeders. We have functionally validated two genes involved in the complex regulation of fruit ripening and studied in depth the role of the DNA demethylase ROS1 in fruit ripening. However, as mutants for both candidate genes *CmROS1* and *CmCTR1-like* showed an

475 altered ripening phenotype, further studies are needed to identify which of them
476 is the candidate for *ETHQV8.1*.

477

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A

ROS1 (MELO3C024516)

5' 3'

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gRNA1

AACATCTAGTCCAGCTAGTCAGGGAGCTGCCAATTCTCAAGAAAACGTGAGAACTAAAAGGAAATATGTCCGAAAGAATGCAGCCAACAAATCCCTGGAAAACCCTCTTGAACGAG

GAACCTCTTGATCCAGTCACCCAGCTGGTTCTTTGGAAAATTCAAGAGGTCTAAGGACATACACTCGAAAGCGTGGCGTCAACATAATAGAAACAGGGCGATCGACTGATATGG

gRNA2

VED

AACCAAGAACATCTAGTCCAGCTAGTCAGGROS1-CRISPR-1 AACCAAGAACATCTAGTCCAGCTA**T**GTCAGG (+1bp)

ROS1-CRISPR-2 AACC-----AGG (-23bp)

B

CTR1-like (MELO3C024518)

5' 3'

CCATATAGTGTACAGTCGACCCC
gRNA1

VED

TTCTCCATATAGTGTACAGTCGACCCC

CTR1-CRISPR-1 TTCT-----ACAGTCGACCCC (-11bp)

CTR1-CRISPR-2 TTCTCCATATA**A**GTGTACAGTCGACCCC (+1bp)**C**

A**Summer season 2020** **Summer season 2021** **B**

Ethylene related genes

Ripening associated TF