

1 **Melon ethylene-mediated transcriptome and methylome dynamics provide insights to** 2 **volatile production**

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16 **Abstract**

17 During climacteric ripening large-scale transcriptional modifications are governed by ethylene. While
18 ripening-related chromatin modifications are also known to occur, a direct connection between these
19 factors has not been demonstrated. We characterized ethylene-mediated transcriptome modification,
20 genome methylation dynamics, and their relation to organoleptic modifications during fruit ripening in the
21 climacteric melon and an ethylene repressed line where the fruit-specific *ACC oxidase 1 (ACO1)* gene
22 was targeted by antisense. The *ACO1* antisense line exhibited mainly reduced transcriptional repression of
23 ripening-related genes associated with DNA CHH hypomethylation at the onset of ripening. Additionally,
24 transcription of a small set of ethylene-induced genes, including known ripening-associated genes, was
25 inhibited by *ACO1* repression and this inhibition was associated with CG hypermethylation. In the *ACO1*
26 antisense line, the accumulation of aromatic compounds, which are mainly derived from the catabolism of
27 amino acids, is known to be inhibited. One of the ethylene-mediated transcriptionally up-regulated genes,
28 *CmTHA1*, encoding a threonine aldolase, exhibited differential cytosine methylation. Threonine aldolase
29 catalyzes the conversion of L-threonine/L-allo threonine to glycine and acetaldehyde and thus is likely
30 involved in threonine-dependent ethyl ester biosynthesis. Yeast mutant complementation and incubation
31 of melon discs with labeled threonine verified *CmTHA1* threonine aldolase activity, revealing an
32 additional ethylene-dependent amino acid catabolism branch involved in climacteric melon ripening.

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35 **Introduction**

36 Ethylene, widely known as the ripening hormone, underlines climacteric fruit ripening. Widely studied
37 climacteric fruits include tomato and melon where following seed maturation ripening competence is
38 achieved and a burst of autocatalytic ethylene orchestrates coordinated changes in fruit pigmentation,
39 aroma, cell wall modifications and tissue softening (Giovannoni, 2004; Yano and Ezura, 2016). This
40 transition is accompanied by reprogramming of expression of thousands of genes (Alba et al., 2005; Saladié
41 et al., 2015; Shin et al., 2017; Yano et al., 2018). Fruit ethylene biosynthesis is dependent on the activity
42 of multiple genes including transcription factors (TFs) and changes in histone-mediated repression of
43 MADS-box and/or NAC domain TFs (Giovannoni et al., 2017; Rios et al., 2017; Lu et al., 2018). From a
44 biochemical flux perspective autocatalytic ethylene involves transcriptional up-regulation of
45 *cystathionine-γ-synthase* (*CGS*), the first committed step in synthesis of methionine, the amino acid
46 precursor of ethylene (Alba et al., 2005). Much of our knowledge of climacteric fleshy fruit ripening
47 derives from studies in tomato. Melon is also widely studied as it exhibits extreme genotypic and
48 phenotypic variation including in climacterism (Burger et al., 2010; Gur et al., 2017; Galpaz et al., 2018).
49 In contrast to tomato, fruit carotenoid accumulation is ethylene independent in melon and controlled by a
50 ‘golden’ SNP in the *CmOr* gene (Tzuri et al., 2015). Introduction of a ‘golden’ SNP harboring allele into
51 tomato significantly elevates fruit nutritional value through increased carotenoid accumulation (Yuan et
52 al., 2015; Yazdani et al., 2019).

53 The *ACO1* antisense ethylene deficient mutant has proven an important tool in the study of climacteric
54 melon fruit ripening (Ayub et al., 1996; Pech et al., 2008). In particular, *ACO1* antisense fruits fail to
55 degrade chlorophyll and retain green rind color. The stay-green (SGR) phenotype is mediated by reduced
56 activity of the ethylene-regulated SGR protein resulting in reduced chlorophyll degradation (Alba et al.,
57 2005; Barry et al., 2008; Shimoda et al., 2016). *ACO1* antisense also results in greatly reduced fruit
58 aromatic volatile compounds including acetate esters and ethyl esters (Homatidou et al., 1992; Bauchot et
59 al., 1998; Flores et al., 2002). During melon ripening, aroma is largely attributed to ester biosynthesis
60 involving the conversion of aldehydes into alcohols by alcohol dehydrogenases (ADHs) (Manríquez et
61 al., 2006; Jin et al., 2016) followed by activity of alcohol acyltransferases (AATs) (Shalit et al., 2001; El-
62 Yahyaoui et al., 2002; El-Sharkawy et al., 2005). Several amino acid metabolism pathways and associated
63 ethylene-regulated genes underlying ester biosynthesis have been identified in melon. These include
64 metabolism of branched-chain amino acids, methionine and phenylalanine (Wyllie et al., 1995; El-
65 Yahyaoui et al., 2002; El-Sharkawy et al., 2005; Gonda et al., 2010; Gonda et al., 2018). Acetaldehyde is
66 synthesized from pyruvate, the end product of glycolysis via pyruvate decarboxylase (PDC) (Tietel et al.,

67 2011). Recently CmPDC1, a ripening induced melon pyruvate decarboxylase has been shown to be
68 involved in acetaldehyde biosynthesis and downstream ester accumulation (Wang et al., 2019).
69 Acetaldehyde has been shown to be a limiting factor in ethanol and derived ester levels in feijoa and
70 strawberry (Pesis and Avissar, 1990; Pesis et al., 1991). Similar observations were made in apple for
71 different aldehydes (De Pooter et al., 1983). Goulao and Oliveira (2007) hypothesized that threonine
72 aldolase activity might also serve as a limiting factor in acetaldehyde-derived volatile biosynthesis during
73 apple ripening based on transcriptional up-regulation of a putative L-allo-threonine aldolase gene. Due to
74 their contributions to fleshy fruit quality, there is growing interest in biosynthesis of aromatic compounds,
75 though limited variance is observed in modern cultivars of some important species such as tomato
76 (Tieman et al., 2017).

77 Recently fleshy fruit development has been shown to be dependent upon chromatin remodeling. For
78 example, during early tomato fruit and floral development, *SIFIE* and *SIEZI*, members of the polycomb
79 repressive 2 (PRC2) complex, play critical roles in fruit development as demonstrated by their repression
80 (How Kit et al., 2010; Liu et al., 2012; Bucher et al., 2018). The PRC2 complex, which belongs to the
81 polycomb group (PcG), catalyzes trimethylation of histone H3 lysine 27 (H3K27me3), negatively
82 regulating gene expression (Holec and Berger, 2012). Additional regulators found in plants include
83 histone variants. Histone H3.1 is enriched in silent genomic regions enriched with both H3K27me3 and
84 DNA methylation, negatively correlating with gene expression; in contrast, H3.3 associates with actively
85 transcribed genes in *Arabidopsis* (Stroud et al., 2012). DNA methyltransferase inhibition promotes
86 premature ripening in tomato, suggesting an active remodeling mechanism prevents premature ripening
87 through DNA methylation (Zhong et al., 2013). During tomato fruit ripening, *SIDML2*, a DEMETER-like
88 DNA demethylase is transcriptionally up-regulated, and its repression or gene editing resulted in genome-
89 wide hypermethylation, including in the promoters of ripening transcription factors resulting in inhibition
90 of ethylene biosynthesis and ripening repression (Liu et al., 2015; Lang et al., 2017). Further genetic
91 evidence of an active gene suppression mechanism during early fruit developmental stages was
92 demonstrated through manipulation of the PRC2 member *SIMSII* (Liu et al., 2016).

93 DNA CHH methylation typical of heterochromatic transposable elements (TEs) is significantly increased
94 in fruits. In addition CHH methylation exhibits dynamics during fruit development and is affected by
95 ripening mutants and has been associated with gene expression changes (Zhong et al., 2013; Corem et al.,
96 2018; Lu et al., 2018). An example of interaction among multiple chromatin remodeling components was
97 demonstrated by a mutation in the rice CHH methyltransferase gene *OsDRM2*, resulting in the loss of
98 H3K27me3 and de-repression of genes (Zhou et al., 2016). In contrast to chromatin regulation of the
99 ripening transition and ethylene biosynthesis (Giovannoni et al., 2017; Lu et al., 2018), the downstream

100 involvement of chromatin dynamics in the ethylene response of climacteric fruits remains poorly
101 understood.

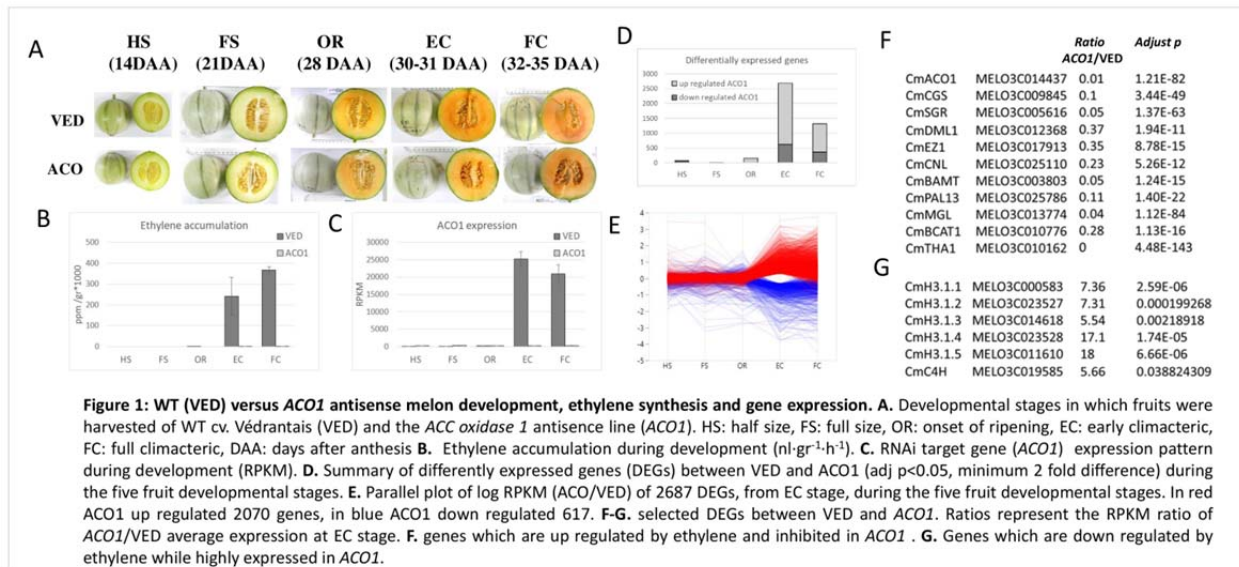
102 To test whether there is a direct link between ethylene-regulated genes and chromatin dynamics we
103 performed transcriptome and methylome comparisons of wild-type Védraçais (VED) and *ACO1*
104 repressed melon fruit during early ripening and discovered ethylene-dependent methylome dynamics
105 associated with ripening gene expression. A subset of this data suggested a role of threonine aldolase
106 (CmTHA1) in melon ripening and fruit quality. Functional analysis confirmed that CmTHA1 plays a role
107 in plant secondary metabolism, specifically production of volatile compounds integral to melon fruit
108 quality.

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110 **Results**

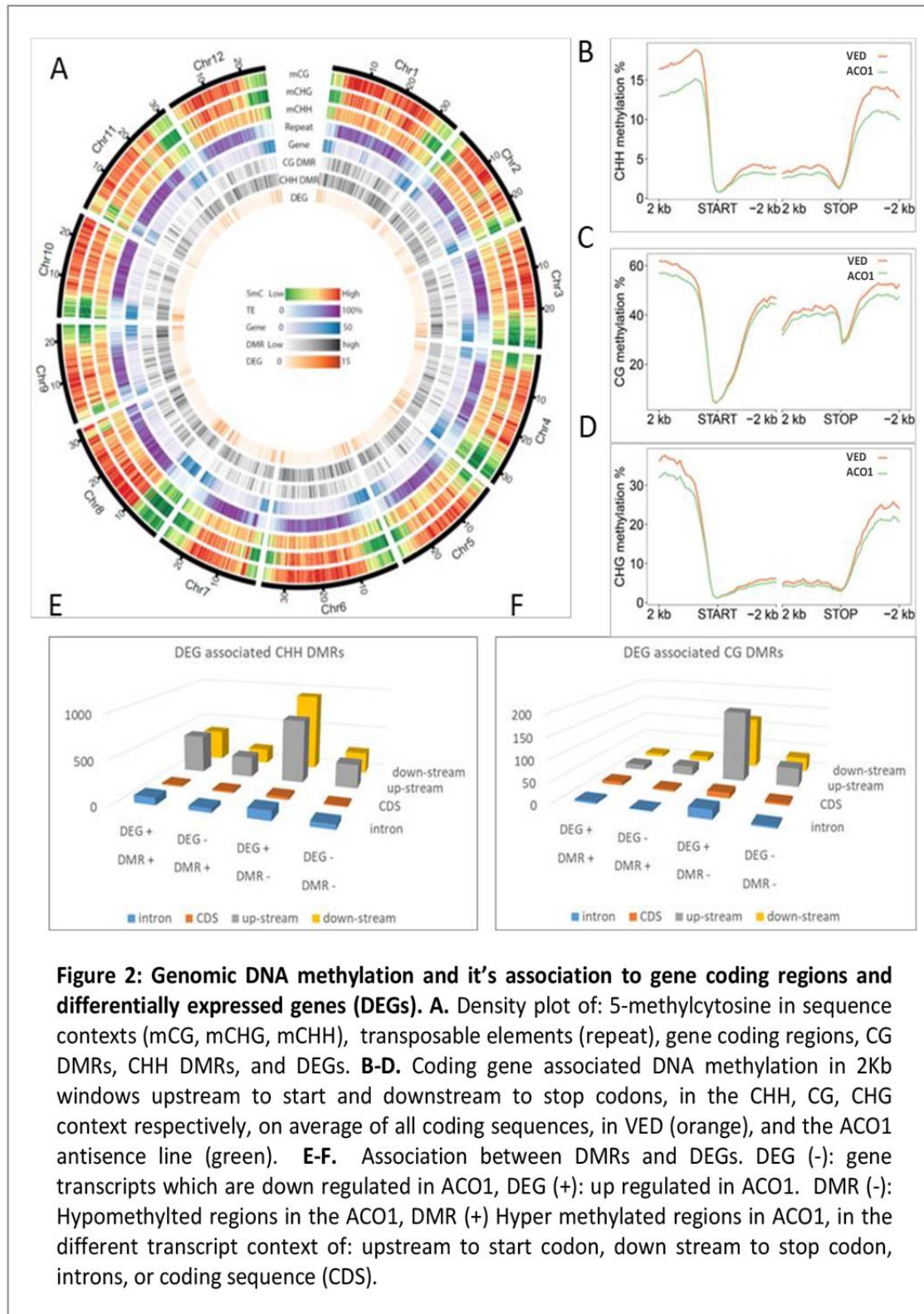
111 To determine the effect of ethylene on transcriptional regulation during melon fruit development, RNA-
112 Seq transcriptome profiling was performed on mesocarp tissue at five stages: Half Size (HS), Full Size
113 (FS), Onset of Ripening (OR), Early Climacteric (EC), and Full Climacteric (FC; Fig. 1A, Supplemental
114 Tables S1-S3). Significant ethylene accumulation in VED was detected at the EC and FC stages, while
115 the *ACO1* antisense line exhibited >99% reduction in ethylene evolution (Fig. 1B). The analysis
116 confirmed abundant mRNA accumulation of *ACO1* in VED (*MELO3C014437*;
117 <http://cucurbitgenomics.org/>), correlating with ethylene accumulation, while in the *ACO1* antisense line
118 *ACO1* mRNA was dramatically reduced (Fig. 1C). Ethylene accumulation in VED at EC was
119 accompanied by differential expression of 2,687 genes when compared to *ACO1* antisense (adjusted
120 $p < 0.05$, minimum 2-fold difference), of which 2,070 were up-regulated and 617 were down-regulated in
121 *ACO1* compared to VED (Fig. 1D-E), indicating that the predominant ethylene effect during VED fruit
122 ripening manifests as down-regulation of fruit gene expression.

123 **Dynamics of chromatin remodeling factors**



124 The role of DNA methylation in ethylene-mediated gene expression differences was of interest due to
 125 recent reports regarding the role of DNA methylation and chromatin remodeling during fruit ripening
 126 (Zhong et al., 2013; Liu et al., 2015; Lang et al., 2017; Lu et al., 2018). DNA bisulfite sequencing was
 127 performed to determine cytosine (C) methylation in EC fruit from both genotypes (Supplemental Tables
 128 S4-S6). Similar to previous reports in Arabidopsis (Zhang et al., 2006), hypermethylated sites in melon
 129 were also found mainly in transposable element (TE)-rich heterochromatic regions (Fig. 2A). Total
 130 cytosine methylation in the EC tissue of VED was 23%, while the *ACO1* mutant exhibited 4% less
 131 genome-wide methylation (19%) in all cytosine context (Supplemental Table S6). This hypomethylation
 132 included regions associated with coding sequences and again in all cytosine contexts, and generally
 133 within a window spanning 2 kb up- and downstream of coding sequence (Fig. 2B-D). Methylome analysis
 134 between VED and *ACO1* antisense EC fruit revealed 52,426 differentially methylated regions (DMRs) in
 135 the CHH context and 9,866 in the CG context (Fig. 2A, Supplemental Table S7). 46.5% of the CHH and
 136 38% of the CG DMRs were found to associate with gene regions defined as ± 2 kb from the ends of
 137 coding sequences. Total methylation of gene-associated DMRs was decreased by 27% in the *ACO1*
 138 antisense line compared to VED (calculated by the sum of average methylation in each site multiplied by
 139 total DMR length), Suggesting ethylene regulates active CHH methylation leading to transcriptional
 140 repression. Of the 2,687 DEGs at the EC stage between VED and *ACO1* antisense, 1,988 (74%) were
 141 associated with DMRs (Supplemental Table S7). We noted a general tendency toward hypomethylation of
 142 genes not repressed by ethylene in *ACO1* antisense fruit (Fig. 2E-F), most prevalently in the CHH
 143 context.

144 Although most of the genes in this study consist of up-regulated DEGs (as compared to VED)
 145 associated with hypomethylated DMRs in the EC tissue of *ACO1* antisense in the CG or CHH context, 30



146 DEGs did show CG hypermethylation associated with transcriptional down-regulation in the *ACO1*

147 antisense. These genes include: *CmSGR* (*MELO3C005616*), *CmCGS* (*MELO3C009845*), and *CmEZI*
148 (*MELO3C017913*), the ortholog of tomato *SIEZI* involved in H3K27me3-mediated gene silencing (Table
149 S8; How kit et al 2010) and could be targets of DNA demethylase activity. *SIDML2*-dependent
150 hypomethylation is the main mechanism underlying DNA methylation dynamics during fruit ripening
151 characterized to date in tomato (Liu et al., 2015; Lang et al., 2017). Melon climacteric ripening is also
152 associated with increased expression of a DNA glycosylase/demethylase, *CmDML1* whose expression is
153 reduced to 37% of WT with *ACO1* repression (*MELO3C012368.2*, Fig. 1F, Supplemental Fig. S1).
154 Unlike the tomato ripening-upregulated *SIDML2* gene which is an ortholog of Arabidopsis *REPRESSOR*
155 *OF SILENCING1* (*ROS1*), *CmDML1* is the ortholog of Arabidopsis *DEMETER* (*DME*) (Zemach et al.,
156 2010; Zhong et al., 2013). It is especially noteworthy that loss of *AtDME* function can occur with some
157 loci still becoming hypomethylated, likely due to RNAi-mediated DNA methylation dynamics (Hsieh et
158 al., 2009; Zemach et al., 2010). As such, while decreased *ACO1* genome methylation is not consistent
159 with the observed reduction of *CmDML1* expression in the antisense line, alternative
160 methylation/demethylation mechanisms likely contribute to maintaining relative hypomethylation in the
161 absence of ethylene. Likely candidate genes contributing to this phenomena (e.g methyltransferases)
162 were not revealed by our transcriptome analysis but the reduction in *CmCGS* necessary for downstream
163 S-adenosyl-methionine (SAM) production (the methyl donor for DNA methylation) may provide a
164 general mechanism for reduced methylation in *ACO1* antisense fruit.

165 Ethylene also influences histone variant transcriptional dynamics which could further influence gene
166 expression. All five H3.1 histone variants found in melon (*MELO3C000583*, *MELO3C023527*,
167 *MELO3C014618*, *MELO3C023528*, and *MELO3C011610*, Fig. S2) displayed ethylene-dependent down-
168 regulation in VED, which is arrested in *ACO1* antisense fruit, resulting in a 5-18 fold expression increase
169 in their relative mRNA abundances in the *ACO1* antisense line as compared to VED (Fig. 1G). The
170 increased abundance of H3.1 histones which are generally associated with gene silencing could also be
171 responsible for reduced expression of some genes repressed in *ACO1* ethylene reduced fruit.

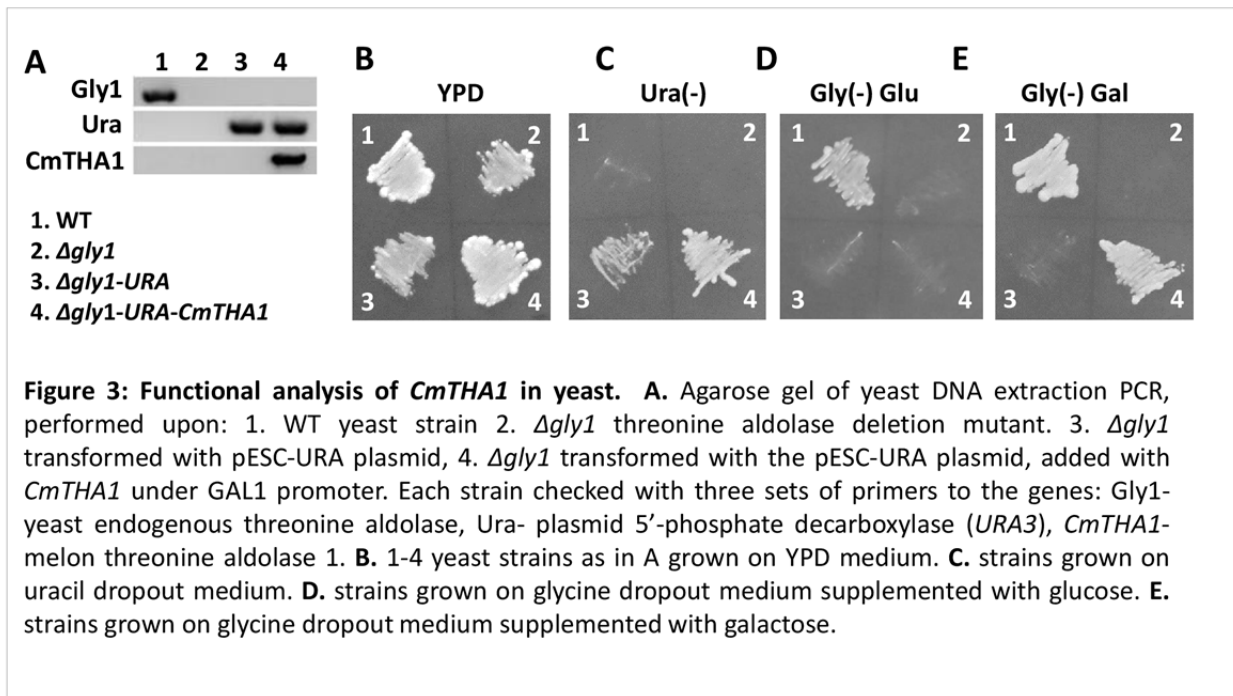
172 **Genetic regulation of aromatic compound accumulation**

173 Volatiles are a significant component of consumer appreciation of ripe fruit (Gonda et al., 2016). Amino
174 acid catabolism results in the production of a wide array of volatiles in melon. L-phenylalanine derived
175 volatiles represent an important group of melon volatiles, and key pathway genes are both regulated by
176 ethylene and have associated DMRs revealed in this study. For example, *CmCNL* (*MELO3C025110*)
177 encoding an (E)-cinnamic acid:coenzyme A ligase involved in (E)-cinnamaldehyde biosynthesis, and
178 *CmBAMT* (*MELO3C003803*) encoding a benzoic acid:S-adenosyl-L-methionine carboxyl
179 methyltransferase involved in methyl benzoate biosynthesis (Gonda et al., 2018), exhibited strong

180 ethylene-dependent up-regulation (Fig. 1F) in addition to DMRs in both the CG and CHH contexts
181 associated with *CmBAMT* (Supplemental Table S7). Two additional genes, *phenylalanine ammonia-lyase*
182 *13* (*CmPAL13*, *MELO3C025786*) and *cinnamate 4-hydroxylase 1* (*CmC4H1*, *MELO3C019585*),
183 previously suggested to be involved in melon rind phenylpropanoid synthesis (Feder et al., 2015), were
184 also regulated by ethylene. *CmPAL13* was up-regulated by ethylene, while *CmC4H1* mRNA was down-
185 regulated (Figs. 1F,G), suggesting the involvement of these genes in ethylene-mediated metabolic
186 alternations resulting in channeling flux toward cinnamic acid and downstream volatiles in the maturing
187 fruit. *CmC4H1* associated with a CHH DMR, while *CmPAL13* associated with DMRs in both the CHH
188 and CG contexts (Supplemental Table S7). L-methionine-derived volatiles are dependent upon the *L-*
189 *methionine- γ -lyase* (*CmMGL*, *MELO3C013774*), which is involved in ethyl ester biosynthesis through
190 L-isoleucine (Gonda et al., 2013). In addition, branched-chain amino catabolism in melon derive from α -
191 keto acids of L-isoleucine, L-leucine and L-valine, through the activity of the enzyme encoded by
192 *CmBCAT1* (*MELO3C010776*), a branched-chain amino acid transaminase which is highly expressed in
193 climacteric ripening melon fruits (Gonda et al., 2010). These two genes, *CmMGL* and *CmBCAT1*, also
194 exhibited strong ethylene-dependent transcriptional up-regulation during VED fruit ripening (Fig. 1F) and
195 a CHH DMR is associated with *CmMGL* expression changes (Supplemental Table S7).

196 **Functional validation of *CmTHAI*'s role in melon fruit volatile synthesis**

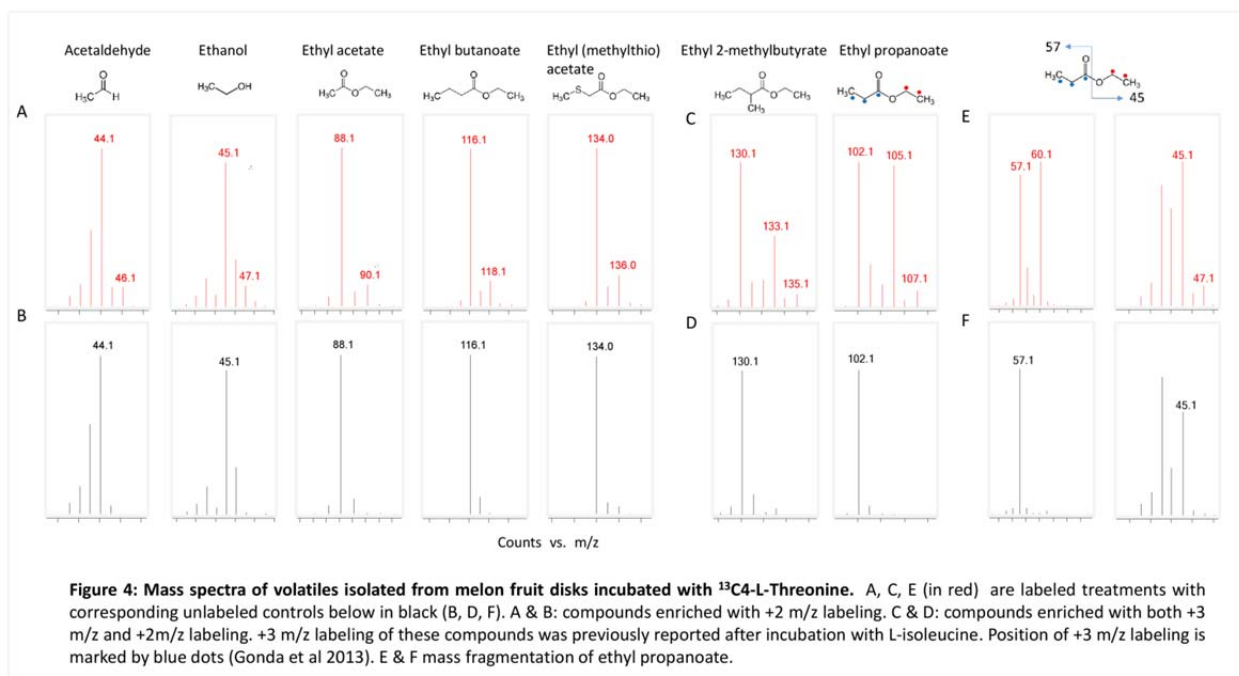
197 L-threonine contributes to volatile biosynthesis via L-isoleucine, which is catabolized into esters (Gonda
198 et al., 2013). Here we show that ethylene regulates *CmTHAI* (*MELO3C010162*, Fig. 1F), which encodes
199 a putative L-allo threonine aldolase. *CmTHAI* associated with four DMRs in both the CHH and CG
200 contexts (Supplemental Table S7). The melon genome harbors two homologs to this gene, *CmTHA2*
201 (*MELO3C017520*) and *CmTHA3* (*MELO3C004421*). *CmTHAI* displayed the greatest mRNA
202 accumulation in EC fruit of VED and was the only one significantly affected by ethylene, suggesting its
203 involvement in ripening (Supplemental Fig. S3). Functional assessment of *CmTHAI* was performed via
204 yeast complementation in a similar manner as previously described (Jander et al., 2004), using $\Delta gly1$, a
205 yeast deletion mutant in strain BY4741 (Giaever et al., 2002). Gly1 is a low specificity threonine aldolase
206 catalyzing the cleavage of both L-threonine and L-allo-threonine to glycine (Liu et al., 1997). $\Delta gly1$ was
207 transformed independently with vector pESC-URA ($\Delta gly1$ -URA) and with the same vector harboring
208 *CmTHAI* coding sequence under the galactose inducible Gal1 promoter ($\Delta gly1$ -URA-*CmTHA*).
209 Transformed strains were verified by PCR (Fig. 3A). Confirmed strains were grown on yeast extract-
210 peptone-dextrose (YPD) control medium (Fig. 3B) along with different dropout media. Growth upon
211 uracil dropout medium verified the ability of the pESC-URA vector to facilitate recovery from uracil
212 auxotrophy (Fig. 3C). Glycine dropout medium supplemented with glucose verified that $\Delta gly1$ is indeed a



213 glycine auxotroph as well as the two $\Delta gly1$ -URA and $\Delta gly1$ -URA-CmTHA transformants (Fig. 3D).
214 Changing glucose to galactose, allowing Gal1 promoter activation, confirmed that CmTHA1 could relieve
215 $\Delta gly1$ glycine auxotrophy (Fig. 3E), indicating that CmTHA1 harbors THA activity.

216 To test the threonine aldolase catalytic activity in melon, fruit disks were incubated with $^{13}\text{C}_4,^{15}\text{N}$ -L-
217 threonine, followed by GC-MS analysis. Two types of labeling were detected. The +2m/z labeling was
218 detected in acetaldehyde, confirming aldolase activity. Similar +2 m/z labeling was also detected in
219 ethanol, and the ethyl esters (ethyl acetate, ethyl butanoate, ethyl (methylthio) acetate, ethyl 2-
220 methylbutyrate, and ethyl propanoate) (Fig. 4). In addition, stronger +3 m/z labeling was observed for
221 ethyl 2-methylbutyrate and ethyl propanoate, both of which were previously observed to be labeled
222 similarly after incubation with $^{13}\text{C}_6$ -L-isoleucine and positioned in the aldehyde derived moiety (Fig. 4C-
223 D) (Gonda et al., 2013). Relatively low labeling of the +2 m/z fraction was anticipated to occur due to
224 higher affinity of CmTHA1 to L-allo threonine and/or availability of acetaldehyde biosynthesized from
225 pyruvate. The ester +2 m/z labeling position by the mass fragments of ethyl propanoate is a clear
226 indication of its derivation from ethanol (Fig. 4E-F).

227



228 Discussion

229 Climacteric melon ripening transcriptome activity shifts toward down-regulation of gene 230 expression via increased gene methylation.

231 Changes in DNA methylation have been implicated in ripening control in tomato (Zhong et al., 2013), a
232 climacteric fruit whose ripening is dependent upon ethylene. In general tomato ripening occurs in the
233 context of demethylation of ripening gene promoters at regions at or adjacent to transcription factor
234 binding sites. The role of ethylene in genome methylation changes has not been specifically examined in
235 prior studies. Here we show the effect of ethylene upon DNA methylation through examination of
236 climacteric (VED) and non-climacteric melon where the latter is achieved via antisense repression of the
237 ethylene synthesis gene *ACO1* (Ayub et al., 1996). Chromatin remodeling factor genes are among those
238 responding to ethylene in this system, suggesting a role for ethylene-mediated histone changes during
239 melon ripening (Fig. 1G). Chromatin methylation during ripening in VED was also recently reported at
240 rates of 75%, 63% and 91% hypomethylation in the CG, CHG and CHH contexts, respectively, in DMRs
241 during the VED transition from unripe to ripe (Lu et al., 2018). The same report analyzed two non-
242 climacteric varieties that trended toward hypermethylation of DMRs in the CHH context during the
243 immature and unripe to fully ripe transition. It is important to note that Lu et al. (2018) specifically
244 compared unripe (20 DAA) and ripe (40 DAA) VED fruit, spanning a developmental transition
245 encompassing both ethylene-dependent and independent physiological and biochemical changes. The
246 long duration between compared stages precluded any direct correlation with the climacteric or ripening
247 ethylene induction in VED. Changes in sugars, carotenoids, organic acids and part of cell wall

248 metabolism all occur in this period and are known to be ethylene independent in melon (Ayub et al.,
249 1996; Pech et al., 2008), and their relationship to chromatin dynamics remain unclear. Neither has any
250 direct relationship between the plant hormone ethylene and methylome changes been established even
251 though ethylene is a likely candidate for mediating such ripening-related changes in climacteric fruit.
252 Here we characterized methylome changes in identically aged fruit at the early ripening stage, EC (30-31
253 DAA) in WT and ethylene repressed fruit to address whether ethylene is involved in the methylation
254 dynamics of ripening genes. We observed that ethylene-mediated methylome changes are substantial and
255 the main effect on DNA methylation associated with ethylene in WT climacteric melon fruit ripening is
256 hypermethylation.

257 Ethylene mainly caused down-regulation of genes which are also associated with CHH hypermethylation
258 in WT (Fig. 1D). In addition, 30 WT ethylene induced genes are associated with CG hypomethylation,
259 suggesting possible DNA demethylase involvement. This possibility was supported by the observation of
260 ethylene-dependent up-regulation of *CmDML1* (Fig.1F), the orthologue of Arabidopsis *DME*. Tomato
261 ripening occurs with transcriptional up-regulation of a DNA demethylase, *SIDML2*, the orthologue of
262 Arabidopsis *ROS1*. This observation is not necessarily surprising in the context of recently reported
263 evidence of convergent evolution of different regulatory targets in the evolution of fruit ripening (Lu et
264 al., 2018). AtDME functions in the CG hypomethylation-dependent activation of gene expression (Hsieh
265 et al., 2009). We show ethylene-dependent transcriptional up-regulation of *CmDME1* associated with a
266 corresponding decrease in CG methylation and transcriptional up-regulation of *CmSGR*, *CmCGS*, and
267 *CmEZ1*, suggesting that these genes are possible CmDME1 targets.

268 Further investigation into possible DNA methylation regulators based on melon fruit gene expression
269 changes suggests possible CmCGS involvement. In tomato, ethylene-regulated transcriptional up-
270 regulation of *cystathionine-γ-synthase* (*CGS*), the first committed step in methionine biosynthesis,
271 controls the biochemical flux toward ethylene and is part of the ethylene autocatalytic mechanism (Alba
272 et al., 2005). From methionine, ethylene is synthesized through S-adenosyl-L-methionine (SAM) which
273 also serves as the main methyl donor for cytosine methylation catalyzed by DNA methyltransferases.
274 Disruption of this pathway, including at SAM biosynthesis in Arabidopsis, results in genome
275 hypomethylation leading to de-repression of TEs and activation of gene expression (Rocha et al., 2005;
276 Groth et al., 2016; Meng et al., 2018; Yan et al., 2019). We demonstrate CmCGS also shows ethylene-
277 dependent transcriptional up-regulation (Fig. 1F), and as such that *CGS*-dependent autocatalytic ethylene
278 is conserved between tomato and melon. As an ethylene synthesis biochemical flux regulator, CmCGS is
279 a limiting factor in SAM biosynthesis, consistent with the decreased capability of *ACO1* antisense to

280 down-regulate gene expression (Fig. 1D-E) through cytosine methylation (Fig. 2) due to limited
281 availability of the necessary methyl donor.

282 In Arabidopsis, H3.1 histone variants were shown to associate with silent areas of the genome (Stroud et
283 al., 2012). The ethylene-dependent transcriptional down-regulation of all melon H3.1 variants (Fig. 1G,
284 Supplemental Fig. S3) suggests the ethylene-dependent transcription activation (Fig. 1D) is additionally
285 mediated at least in part by down-regulation of H3.1 histone variants.

286 **CmTHA1 contributes to melon fruit volatile production**

287 Ethylene is known to regulate different amino acid catabolism pathways involved in fruit aroma
288 including: L-methionine (via CmMGL), branched-chain amino acids (via CmBCAT1) and L-
289 phenylalanine (via CmBAMT, CmCNL). In addition, responsiveness of *CmPAL13* and *CmC4H1* to
290 ethylene suggested additional upstream flux channeling that may influence levels of these amino acids
291 and their volatile metabolic products (Fig.1 F-G).

292 While the final steps in volatile ester biosynthesis involving AHD and AAT are largely understood,
293 earlier steps in aldehyde formation remain uncertain. Recently CmPDC1 was found to mediate
294 acetaldehyde biosynthesis in ripening melon fruit though additional factors are certainly involved (Wang
295 et al., 2019). We demonstrate here that one such factor is CmTHA1 as demonstrated by +2 m/z labeling
296 of acetaldehyde originating from threonine (Fig. 4). The resulting +2 m/z labeled compounds follow the
297 general paradigm of ester biosynthesis in which acetaldehyde is converted to ethanol by ADH and
298 subsequent AAT-dependent incorporation into ethyl esters (ethyl acetate, ethyl butanoate, ethyl
299 (methylthio) acetate, ethyl 2-methylbutyrate, and ethyl propanoate).

300 **Summary**

301 We investigated basic chromatin remodeling at the level of DNA cytosine methylation specifically as
302 influenced by ethylene during fruit ripening through comparison of early ripening WT climacteric (VED)
303 and ethylene repressed transgenic (*ACO1* antisense) melon fruit. It is well known that modern cantaloupe
304 melon varieties produce reduced aromatic compounds as a consequence of breeding efforts focused on
305 increased shelf life through limiting ethylene biosynthesis and/or perception (Aubert and Bourger, 2004;
306 Obando-Ulloa et al., 2008). We demonstrate that melon fruit severely limited in ripening ethylene
307 production have substantially altered gene expression at the initial ripening stage and that many genes
308 altered in expression are associated with DMRs, the majority of which are associated with
309 hypomethylation in ethylene-repressed fruit and elevated gene expression as compared to WT. A smaller
310 subset of genes show elevated expression with lower methylated DMRs in WT, a phenomena also

311 reported in tomato (Lang et al., 2017). As ethylene is known to be important in melon volatile synthesis
312 we focused efforts on characterization of ethylene and DMR-associated genes contributing to volatile
313 synthesis. We identified and demonstrated the function of CmTHA1, a previously uncharacterized L-allo
314 threonine aldolase contributing to volatile ester synthesis. The more comprehensive picture of ethylene
315 effects on gene expression resulting from this study should prove helpful in designing breeding strategies
316 focused on ethylene-regulated ripening components such as volatile synthesis to target and reverse the
317 negative effect of ethylene reduction on aroma.

318

319

320 **Materials and methods**

321 **Plant material and ethylene measurements**

322 Seeds of the *ACO1* antisense line (Ayub et al., 1996), along with Védraçais (VED) were kindly provided
323 by Maria-Carmen Gomez-Jimenez, Plant Physiology department, University of Extremadura, Spain.
324 Plants were grown in a randomized design in pots inside a greenhouse under standard conditions during
325 spring 2013 at Newe Ya'ar Research Center. Flowers were tagged at anthesis. Fruits were collected
326 during development according to Table S1. Ethylene emission was measured as previously described
327 (Galpaz et al., 2018).

328 **RNA-Seq library preparation, sequencing and data analysis**

329 RNA extraction, library preparation and sequencing were performed according to the methods described
330 previously (Zhong et al., 2011; Feder et al., 2015). Two to four biological replicates were performed for
331 each sample. Raw RNA-Seq reads were processed using Trimmomatic (Bolger et al., 2014) v0.36 to
332 remove adaptor and low-quality sequences. The cleaned reads were then aligned to the ribosomal RNA
333 database (<https://www.arb-silva.de/>) with bowtie (Langmead, 2010) v1.0.0 (parameter '-v 3') to filter out
334 rRNA reads. The final cleaned reads were aligned to the melon genome v3.5.1 (Diaz et al., 2015) using
335 HISAT (Kim et al., 2015) allowing up to two mismatches. Raw counts for each gene were then derived
336 from the alignments and normalized to reads per kilobase of transcript, per million mapped reads
337 (RPKM). Differentially expressed genes between WT climacteric (VED) and ethylene repressed
338 transgenic (*ACO1* antisense) melon fruit at each of the five developmental stages, half size (HS), full size
339 (FS), onset of ripening (OR), early climacteric (EC), and full climacteric (FC), were identified with the
340 DESeq2 package (Love et al., 2014). Genes with adjusted *P* value <0.05 and fold-change ≥ 2 were defined
341 as differentially expressed genes.

342 **Whole-genome bisulfite sequencing and data analysis**

343 DNA for bisulfite sequencing was extracted from isolated nuclei following Zhong et al. (2013). Bisulfite
344 library construction and sequencing were performed at the Roy J. Carver Biotechnology Center,
345 University of Illinois, Urbana-Champaign. Raw bisulfite sequencing reads were first processed to collapse
346 duplicated read pairs into unique read pairs. The resulting reads were then processed to remove adaptor
347 and low-quality sequences using Trimmomatic v0.36. Two biological replicates were combined for the
348 downstream analysis to obtain a better coverage of the genome. The method for the whole genome
349 methylation analysis was same as described previously (Zhong et al., 2013). Briefly, before alignment,
350 each base cytosine in the reads and the double-strand genome (G in reverse strand) was replaced with
351 thymine (or A if reverse strand in the genome). The converted reads were aligned respectively to the two
352 converted strands of the genome using bowtie allowing up to two mismatches, and reads aligned to
353 multiple locations were excluded from the analysis. Alignments from the two strands were combined and
354 the original read sequences in the alignments were recovered. Finally, methylation status of each cytosine
355 in the melon genome was calculated on the basis of the alignments. A sliding-window approach with a
356 100-bp window sliding at 50-bp intervals was used to identify context-specific DMRs. Windows with
357 fewer than 5, 4 and 20 sequenced cytosine sites ($\geq 4\times$ coverage) in the CG, CHG and CHH contexts,
358 respectively, were discarded. For each window, a Kruskal–Wallis test was performed, and the *P* values
359 were corrected using Benjamini-Hochberg method (Benjamini and Hochberg, 1995). Windows with
360 corrected *P* value < 0.05 were identified as DMRs and overlapping DMRs were concatenated.

361 **Yeast transformation**

362 *Δgly1* yeast obtained from the knockout library in the BY4741 background (Giaever et al., 2002), along
363 with pESC-URA, were kindly provided by Maya Schuldiner's lab at Weizmann Institute of Science.
364 Coding sequence of *CmTHA1* was obtained from cDNA of ripe fruit of Charentais melon, following PCR
365 with the *THA1*-F/R primers. pESC-URA was linearized using *Sma*I/*Hind*III restriction enzymes,
366 following insertion of the PCR product with NEBuilder (New England BioLabs). Growing media,
367 including custom made Kaiser synthetic complete Gly(-) dropout, were obtained by Formedium. Primer
368 sequences used in this study are listed in Supplemental Table S9.

369 **Labeled L-threonine feeding and CG-MS analysis**

370 Plants were grown in winter 2019 in greenhouse at Newe Ya'ar. Ripe fruits were collected, left at room
371 temperature for 48h. Fruit mesocarp discs (approximately 1g each) were incubated with 100mM L-
372 threonine-¹³C₄, ¹⁵N (Sigma) for 12 hours, after which tissue was frozen in liquid nitrogen and ground.
373 Sample preparation for GC-MS was performed according to Gonda et al 2013. Data analysis was
374 performed with the MassHunter software (Agilent).

375

376 **Accession numbers**

377

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383

384 **Conflict of interest**

385 The authors declare no conflict of interest.

386

387 **Supporting information**

388 Additional Supporting Information may be found in the online version of this article.

389

390 **Supplemental Figure S1.** Phylogenetic tree of the DML glycosylase gene family.

391 **Supplemental Figure S2.** Phylogenetic tree of H3.1 and H3.3 histone variants.

392 **Supplemental Figure S3.** Melon threonine aldolase fruit gene expression.

393 **Supplemental Table S1.** Fruit sampling, measurements, RNA-Seq preparation and statistics.

394 **Supplemental Table S2.** RNA-Seq sample correlation.

395 **Supplemental Table S3.** RNA-Seq gene expression (RPKM).

396 **Supplemental Table S4.** DNA bisulfite sequencing statistics.

397 **Supplemental Table S5.** DNA bisulfite coverage percentage.

398 **Supplemental Table S6.** DNA methylation percentage.

399 **Supplemental Table S7.** Differentially methylated regions associated with protein-coding genes.

400 **Supplemental Table S8.** Putative CmDML1 targets.

401 **Supplemental Table S9.** Primers used in this study.

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403

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