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Additional Information

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2 *TAGL1* regulates cuticle development of tomato
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Transcriptional activity of the MADS-box *ALQ/TAGL1* gene is required for cuticle development of tomato fruit

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Summary:
A ripening transcription factor regulates the cuticle development of tomato fruit as part of the reproductive developmental program.

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Abstract

Fruit development and ripening entail key biological and agronomic events, which ensure the appropriate formation and dispersal of seeds and determine productivity and yield quality traits. The MADS-box *ARLEQUIN/TOMATO AGAMOUS LIKE 1* (*ALQ/TAGL1,* hereafter referred to as *TAGL1*) gene was reported as a key regulator of tomato (*Solanum lycopersicum* L.) reproductive development, mainly involved in flower development, early fruit development and ripening. It is shown here that silencing of *TAGL1/ALQ* gene (RNAi lines) promotes significant changes affecting cuticle development, mainly a reduction of thickness and stiffness, as well as a significant decrease in the content of cuticle components (cutin, waxes, polysaccharides and phenolic compounds). Accordingly, overexpression of *TAGL1/ALQ* significantly increased the amount of cuticle and most of its components, while rendering a mechanically weak cuticle. Expression of genes involved in cuticle biosynthesis agreed with the biochemical and biomechanical features of cuticles isolated from transgenic fruits; it also indicated that *TAGL1/ALQ* participates in the transcriptional control of cuticle development mediating the biosynthesis of cuticle components. Furthermore, cell morphology and arrangement of epidermal cell layers, on whose activity cuticle formation depends, were altered when *TAGL1/ALQ* was either silenced or constitutively expressed, indicating that this transcription factor regulates cuticle development probably through the biosynthetic activity of epidermal cells. Results also support cuticle development as an integrated event in the fruit expansion and ripening processes which characterize fleshy-fruited species as tomato.

86 **Introduction**

87

88 Among the angiosperm species yielding fleshy fruits, tomato (*Solanum lycopersicum*
89 L.) has provided the most relevant discoveries of the last decades about the genetic and
90 physiological mechanisms underlying fruit ripening (Giovannoni, 2004; Seymour et al.,
91 2008; Ariizumi et al., 2013; Seymour et al., 2013). Hence, tomato is currently
92 considered the model species for the study of this complex developmental process,
93 which ensures the formation, maturation and dispersal of seeds, and makes the fruit
94 healthy for human consumption. As with other climateric fruits, ripening of tomato
95 involves biochemical and physiological changes affecting texture, pigmentation,
96 nutritional and organoleptic traits, most of them coordinated and synchronized by
97 ethylene (for a review, see Klee and Giovannoni, 2011). However, ethylene is known to
98 be insufficient to regulate tomato ripening; thus, different regulatory factors must be
99 properly coordinated with ethylene synthesis as part of the genetic network which
100 controls fruit ripening (see review of Seymour et al., 2013). Characterization of several
101 tomato mutants affected in fruit ripening has allowed for the cloning and functional
102 analysis of some of the components in the ripening pathway. It has also highlighted the
103 crucial role of the MADS-box transcription factor encoded by the *RIPENING*
104 *INHIBITOR* (*RIN*) as a main regulator of this process (Vrebalov et al., 2002). Recent
105 evidence has indicated that *RIN* directly controls the expression of target genes involved
106 in a wide range of ripening-related events (Fujisawa et al., 2011; Qin et al., 2012). This
107 transcriptional activity also depends on *COLOURLESS NON RIPENING* (*CNR*), an
108 SBP-box gene (Manning et al., 2006; Martel et al., 2011). Other ripening factors as
109 NON RIPENING (NOR, GenBank accession no. AY573802; Tigchelaar et al., 1973),
110 LeHB1 (Lin et al., 2008), SlAP2a (Karlova et al., 2011) and the MADS-box TOMATO
111 AGAMOUS-LIKE1 (TAGL1; Vrebalov et al., 2009; Giménez et al., 2010) exert their
112 regulatory functions by interacting with RIN (Fujisawa et al., 2011; Qin et al., 2012;
113 Seymour et al., 2013).

114 Cuticle formation of tomato fruit has been considered as an integral part of the
115 fruit ripening program (Saladié et al., 2007). Therefore, composition and mechanical
116 performance of cuticle have been suggested to play an important role during fruit
117 ripening process (Dominguez et al., 2012). The cuticle is synthesised by the epidermal
118 cell layer of fruit pericarp. It is composed by a cutin polymer matrix and cuticular waxes
119 (Pollard et al., 2008). Phenolic compounds, mostly cinnamic acid derivatives and

120 flavonoids, are also present (Hunt and Baker, 1980). In addition, a significant amount of
121 polysaccharides can be found, which represent the portion of epidermal cell wall to
122 which the cuticle is attached (Jeffree, 2006). During fruit development, the cuticle
123 undergoes several biochemical changes and enlarges considerably, surrounding the
124 epidermal cells. The degree of invagination and the relative contribution of each cuticle
125 component affect its biomechanical behaviour as well as the physiological properties of
126 the whole fruit pericarp (Matas et al., 2004; Lopez-Casado et al., 2007; Dominguez et
127 al., 2011; España et al., 2014a). Thus, waxes and flavonoids have been shown to act as
128 fillers stiffening the cuticle (Petracek and Bukovac, 1995; Dominguez et al., 2009). On
129 the other hand, cutin is responsible for the viscoelastic behavior and polysaccharides
130 contribute to the elastic phase (Lopez-Casado et al., 2007, Dominguez et al., 2011).
131 Molecular models for cuticle biosynthetic pathways have been proposed mostly in
132 *Arabidopsis thaliana*; however, there is little information about the genetic and
133 molecular bases which regulate fruit cuticle development in tomato. Some genes have
134 been identified by the characterization of tomato mutants altered in fruit cuticle
135 generation. Among them, *CUTIN DEFICIENT 2* (*CD2*) gene encodes a member of the
136 class IV homeodomain-Leu zipper (HD-ZIP IV) family which regulates cutin
137 biosynthesis (Isaacson et al., 2009, Nadakuduti et al., 2012). *LeCER6* encodes a ß-
138 ketoacyl-coenzyme A synthase involved in cuticular wax composition during fruit
139 development (Vogg et al., 2004; Leide et al., 2007). Like the Arabidopsis AP2
140 transcription factor SHINE1 (SHN1; Aharoni et al., 2004; Kannangara et al., 2007), the
141 tomato SlSHN1, 2 and 3 orthologues have been described as transcriptional regulators
142 of cutin and wax biosynthetis pathways (Mintz-Oron et al., 2008; Shi et al., 2014).
143 Similarly, some key components of the flavonoid pathway have been involved in
144 tomato cuticle development. That is the case of the transcription factor encoded by
145 *SLMYB12* (Adato et al., 2009; Ballester et al., 2009); this regulates flavonoid
146 biosynthesis through controlling CHALCONE SYNTHASE1 (CHS1) and CHS2
147 enzymes, the first step in the flavonoid pathway (O´Neill et al., 1990). In addition, a
148 recent screening of a tomato ethyl methanesulfonate (EMS) collection for mutants
149 displaying glossy and dull fruits has facilitated the isolation of novel genes related to
150 cuticle formation (Petit et al., 2014).

151 There is increasing knowledge about the genetic control of cuticle biosynthesis;
152 yet, the upstream key genes and regulatory mechanisms which integrate cuticle
153 development in the fruit ripening program are still to be characterized. Previous studies

154 have reported significant differences in the fruit cuticle of non-ripening mutants
155 suggesting new roles of *RIN* and *NOR* in the genetic control of fruit ripening with a
156 direct effect on cuticle development (Kosma et al., 2010). Similarly, transcription
157 factors which participate in fruit development and ripening regulation as belonging to
158 AP2/EREBP, MADS-box, MYB and homeodomain-leucine zipper families have been
159 recently involved in different processes of cuticle formation of tomato fruit (see review
160 of Hen-Avivi et al., 2014). It is interesting to note that tomato fruits yielded by
161 *TAGL1/ALQ* silencing plants (RNAi lines) were unable to complete ripening and
162 showed greater firmness than WT fruit. In addition, cuticle of RNAi fruits s displayed a
163 reduced thickness and invagination degree (Gimenez et al., 2010). These results
164 suggested a connection between cuticle formation and the function of *TAGL1/ALQ* as
165 transcriptional regulator of fruit ripening. Furthermore, Matas et al., (2011) have shown
166 that the *TAGL1/ALQ* gene is expressed at high levels in fruit outer epidermis suggesting
167 its role in the development of fruit cuticle. To gain insight into the functional role of
168 *TAGL1/ALQ* in the cuticle development, structural, biomechanical and gene expression
169 analyses have been performed in peels isolated from *TAGL1/ALQ* silencing and over-
170 expression lines. Results show that *TAGL1/ALQ* plays a key role in tomato fruit cuticle
171 development through the genetic control of cuticular component biosynthesis. These
172 results will provide a better understanding of the genetic bases of cuticle formation and
173 of *TAGL1/ALQ* contribution to the tomato's reproductive program, where it is a crucial
174 regulator.
175
176 **Results**
177
178 Previous works have demonstrated the crucial role of *TAGL1/ALQ* gene as
179 transcriptional regulator of flower development and fruit ripening (Itkin et al., 2009;
180 Vrebalov et al., 2009; Giménez et al., 2010). Cuticle formation is currently considered
181 part of the developmental program leading to fruit ripening. Therefore, this paper is
182 aimed at studying the contribution of *TAGL1/ALQ* to the development of fruit cuticle in
183 the tomato model species. With this purpose, the composition and biomechanical
184 properties of cuticles have been analyzed in tomato fruits in which *TAGL1/ALQ* was
185 either silenced by an interference RNA (RNAi) approach or over-expressed (OE) under
186 a 35S-CAMV constitutive promoter. Compared to wild-type fruits of cv. Moneymaker
187 (WT), *TAGL1/ALQ*-RNAi fruits displayed a yellow-orange color and a glossy

188 phenotype; they were also unable to complete normal ripening (Fig. 1A). Yet, fruits
189 yielded by OE lines showed an accelerated ripening and were accompanied by succulent
190 sepals which in turn, developed and ripened like a normal tomato fruit (Fig. 1A).

191 Expression of *TAGL1*/*ALQ* was analyzed in tomato peels, a fruit tissue that
192 includes the epidermis, which is in turn responsible for cuticle biosynthesis.
193 *TAGL1*/*ALQ* transcripts were detected in WT fruit peels at immature green (IG) stage;
194 later, they were accumulated during the ripening process and reached a maximum
195 expression level at red ripe (RR) stage (Fig. 1B). The same expression pattern was
196 found in *TAGL1/ALQ*-OE peels, although the *TAGL1/ALQ* transcript level was higher
197 than in WT ones. On the contrary, no *TAGL1*/*ALQ* expression was observed in peels
198 isolated from *TAGL1/ALQ*-RNAi fruits (Fig. 1B).

199

200 ***TAGL1*/*ALQ* transcript levels affect cuticle development**

201

202 A glossy phenotype as shown by tomato fruits silencing *TAGL1/ALQ* (Fig. 1A)
203 has been recently associated to alterations in the cuticle development and epidermal
204 patterning (Petit et al., 2014). Therefore, developmental changes in cuticle formation
205 promoted by either silencing or overexpression of *TAGL1*/*ALQ* gene were analyzed.
206 Results indicated that, once isolated, cuticles from *TAGL1/ALQ*-RNAi fruits showed a
207 visible pale yellow-orange color together with non-colored patches randomly distributed
208 over their surface; this differed from the homogeneous and intense orange color of
209 cuticles from WT fruits (Fig. 1A). Furthermore, pericarp cross-sections of WT fruits
210 stained with Sudan IV displayed the characteristic cutinization of epidermal cell walls,
211 almost completely surrounded by cuticle material, which is also deposited on the radial
212 walls of the first collenchyma cell layer (Fig. 1A). In contrast, RNAi fruits developed a
213 three-fold thinner cuticle than the WT ones (Table I), and a complete absence of
214 epidermal invaginations (Fig. 1A). It is noteworthy that the thinnest regions of RNAi
215 cuticles coincided with the non-colored patches mentioned above. The amount of cuticle
216 and its four components, i.e. cutin, waxes, polysaccharides and phenolics decreased
217 significantly in tomato cuticles isolated from *TAGL1/ALQ*-RNAi fruits (Table I).
218 Changes affecting cuticular content and thickness also suggested differences in
219 mechanical resistance of *TAGL1/ALQ*-RNAi cuticles. Therefore, several parameters
220 characterizing biomechanical properties of fruit cuticle were measured (Table I).
221 Normalized data showed that only Young's modulus decreased when *TAGL1*/*ALQ* was

222 silenced, while mean values of breaking stress and maximum strain did not differ with
223 respect to WT (Table I), However, non-normalized analytical results showed that
224 cuticles from *TAGL1/ALQ* silencing fruits only withstood 40-50 g before breaking,
225 three-fold less than control cuticles (130 g), indicating that both thickness and
226 composition influence mechanical properties of cuticles. Contrarily, cuticles isolated
227 from tomato fruits constitutively overexpressing *TAGL1/ALQ* showed a more severe
228 cutinization level than those of WT fruits. This is because the cuticle material
229 substantially encased not only the epidermis but also several collenchyma layers, three
230 in the most extreme cases (Fig. 1A). This high level of cutinization was accompanied by
231 the development of epidermal ridges on the cell walls, a feature not observed in WT
232 cuticles (Fig. 1A). Furthermore, cuticle thickness was two-fold increased, as an average,
233 in *TAGL1/ALQ*-OE fruits (Table II). As expected, the total amount of cuticle was also
234 significantly higher than in WT fruits, most likely due to the increased content of cutin
235 and waxes. However, the amounts of polysaccharides and phenolics were similar to WT
236 ones (Table II). In addition to differences related to biochemical composition, thickness
237 and invagination degree of OE fruit cuticles, changes in their biomechanical properties
238 were found. Thus, a significant decrease in Young's modulus and breaking stress was
239 observed (Table II), indicating that constitutive expression of *TAGL1/ALQ* modified the
240 mechanical behavior of fruit cuticle. Normalized data showed that OE cuticles are
241 mechanically different from WT ones, however, non-normalized data indicated that the
242 higher thickness of the former allowed them to withstand a similar breaking stress
243 (about 120 g) to the latter.
244
245 **Compositional and biomechanical properties of cuticles from fruit-like sepals**
246 **developed by *TAGL1/ALQ*-OE plants**
247
248 It was previously reported that ectopic expression of *TAGL1/ALQ* was responsible for
249 the conversion of sepals into succulent organs, which developed and ripened in a fruit
250 fashion (Fig. 1A; Giménez et al., 2010). In this work, the morphological and
251 biochemical features of the cuticle isolated from *TAGL1/ALQ*-OE sepals have been
252 analyzed with the aim to check the contribution of *TAGL1/ALQ* to the homeotic
253 conversion of sepals into fruit-like structures and hence to gain insight into the
254 functional role of *TAGL1/ALQ* as a key regulator of fruit development. Results showed
255 that cuticles isolated from fruit-like sepals overexpressing *TAGL1/ALQ* were extremely

256 delicate, thinner than WT fruit cuticle (Fig. 2A), and therefore hard to isolate. Their
257 morphology and thickness resembled those of cuticles covering WT sepals (Fig. 2A). It
258 is interesting to note that succulent sepals did not develop characteristic epidermal cells
259 (Fig. 2B). Their cuticles had four times less amount of material than those of WT and
260 *TAGL1/ALQ*-OE tomato fruits, despite their fruit-like appearance (Table II).
261 Nevertheless, cell anatomy of succulent sepals was more similar to that of a WT fruit
262 pericarp than to a WT sepal since large oval parenchyma cells, which are characteristic
263 of fruit pericarp, were observed instead of round parenchyma cells typical of WT sepals
264 (Fig. 2B). These results indicated that constitutive expression of *TAGL1/ALQ* in sepals
265 promotes similar developmental changes to those occurring in WT tomato fruits during
266 the ripening process. Said changes mainly affected cell identity and biochemical
267 composition (i.e. content of sugars, carotenoid, lycopene and ethylene). They were
268 almost identical to those observed in the fruits and sepals of tomato *Alq* mutant, which
269 also overexpressed *TAGL1/ALQ* gene (Giménez et al., 2010; Pineda et al., 2010).
270
271 **Pericarp development and cuticle formation of *TAGL1/ALQ* RNAi and OE fruits**
272
273 It is known that fruit cuticle develops from the activity of epidermal cells (Javelle et al.,
274 2011) and that *TAGL1/ALQ* is expressed in this cell layer of fruit pericarp (Fig. 1B;
275 Matas et al., 2011). Therefore, it was analyzed whether cuticular changes promoted by
276 silencing of *TAGL1/ALQ* might be correlated with alterations in the epidermal cells of
277 tomato fruit. Pericarp of red ripe WT fruits consisted in a single epidermal layer
278 composed of elongated cells orderly distributed, followed by two-three hypodermal
279 layers of elongated collenchyma cells and several layers of large parenchyma cells (Fig.
280 3A). This tissue patterning distribution is clearly distinguishable from mature green
281 (MG) stage. However, pericarp tissue development is initiated earlier from 10-12 cell
282 layers forming the carpels, right after the ovary fertilization of flowers at anthesis day
283 (Fig. 3A). As it has been reported (Gillaspy et al., 1993), carpel cells undergo an active
284 period of cell division, which ceases so as to give way to an expansion cell period, both
285 leading to the full development of fruit pericarp (Fig. 3A). Results here obtained
286 showed that repression of *TAGL1/ALQ* in fruit pericarp resulted in a significant
287 decrease of cell number per surface unit in both epidermis and collenchyma tissues;
288 hence cell size in *TAGL1/ALQ* silencing tissues was greater than in WT ones (Fig. 3D;
289 Table III). In addition, the number of collenchyma layers diminished from 3.2±0.4 in

290 WT to 1.3±0.5 in *TAGL1/ALQ*-RNAi pericarps, while the epidermis was not affected
291 (Fig. 3D). All these changes would explain the decreased pericarp thickness previously
292 observed in *TAGL1/ALQ* silencing lines (Vrebalov et al., 2009; Gimenez et al., 2010).
293 On the contrary, constitutive expression of *TAGL1/ALQ* doubled the cell number per
294 surface unit in the parenchyma tissue with respect to WT pericarp, indicating that cell
295 size was clearly smaller in overexpressing lines (Fig. 3A; Table III). Thus, the number
296 of parenchyma layers increased from 19.44 ±0.8 in WT to 26.2±1.84 in *TAGL1/ALQ*-
297 OE fruits while the cell layer number of epidermis and collenchyma tissues did not
298 change with respect to WT (Fig. 3D). Additionally, differentiation of pericarp tissues
299 was anticipated in OE fruits with respect to WT fruits. Thus, the degree of epidermis
300 and collenchyma cell differentiation of OE pericarp at IG stage resembled that observed
301 in WT pericarp at a later developmental stage such as MG (Fig. 3, B and C). Similarly,
302 OE pericarp at MG stage displayed the characteristic tissue patterning of WT pericarp at
303 RR stage (Fig. 3, C and D). It is interesting to point out that epidermis and collenchyma
304 tissues were not differentiated correctly in *TAGL1/ALQ*-RNAi fruits (Fig. 3, C and D).
305 Results described above supported the fact that transcriptional activity of
306 *TAGL1/ALQ* influences tissue patterning of fruit pericarp. Therefore, it was reasonable
307 to think that cuticle formation could also be altered in tomato fruits where *TAGL1/ALQ*
308 expression was modified. As occurred in WT fruits, cuticle formation is visible at IG
309 stage of fruit development in both RNAi and OE plants. Later, cuticle deposition
310 increased at MG stage; cuticle acquired its maximum thickness, and invagination degree
311 at RR stage in WT and OE fruits (Fig. 4). However, cuticle formation was affected at
312 initial stages in RNAi fruits, and it was unable to evolve normally during fruit ripening
313 process. Consequently, *TAGL1/ALQ* pericarp of RR fruits displayed a thin cuticle
314 without invaginations, quite similar to that observed in WT fruits at such an early stage
315 of development as IG (Fig. 4).
316
317 ***TAGL1/ALQ* influences expression pattern of tomato genes involved in cuticle**
318 **development**
319
320 Given the structural and compositional alterations found in cuticles of *TAGL1/ALQ*-
321 RNAi and OE fruits, transcription levels of genes known to be involved in cuticle
322 development were analyzed by quantitative RT-PCR in peels isolated from tomato fruits
323 at IG, MG, BR and RR stages (Figs. 5 and 6). Compared to WT peels, *CD2* was down-

324 regulated in RNAi peels, particularly at BR and RR stages, while it was expressed at a
325 similar level in OE peels except at the end of the ripening process (RR stage) when it
326 was suddenly repressed (Fig. 5). Among the genes involved in wax biosynthesis,
327 *LeCER6* did not show expression changes in RNAi or in OE peels with respect to WT
328 (Fig. 5). However, *SlSHN1* was significantly up-regulated in RNAi from MG stage up
329 to full ripening of tomato fruits (Fig. 5), while expression of *SlSHN3* was clearly
330 induced in OE peels at the early stage of fruit development (IG stage), and later it
331 drastically decreased during fruit ripening stages (Fig. 5).

332 Regarding the genes regulating phenilpropanoid/flavonoid pathway, the
333 transcript levels of *SLMYB12*, *CHS1*, *CAD* and *4CL* were assessed (Fig. 6). In WT
334 fruits, all these genes were up-regulated in tomato peels during the first stages of fruit
335 ripening; they reached a maximum level of transcripts at BR stages and were
336 subsequently down-regulated. Expression of *SLMYB12* and *CHS1* flavonoid
337 biosynthesis genes were significantly inhibited in RNAi lines and did not display the
338 ripening-dependent increase observed in the normal peels. However, no expression
339 alterations were observed for *SlMYB12* gene in OE lines while *CHS1* was down-
340 regulated (Fig. 6). Regarding *CAD* and *4CL*, these were up-regulated in peels of RNAi
341 fruits at IG stage, when these genes are not expressed in WT peels. Contrarily,
342 transcripts of *CAD* and *4CL* were down-regulated at early stages of fruit development of
343 OE lines, although their expression increased during ripening stages, particularly that of
344 *CAD* gene (Fig. 6).

345 In addition to cuticle, epidermal cell walls, which are in direct contact with the
346 cuticular lipids, contribute to the mechanical properties of the tomato fruit exocarp and
347 the resistance of the turgor-driven fruit growth in tomato (Andrews et al., 2002; Bargel
348 and Neinhuis, 2005). Therefore, expressions of *POLYGALACTURONASE* (*PG)* and
349 *PECTIN METHYLESTERASE* (*PME)* genes, which encode major enzymes involved in
350 cell wall degradation (Dellapenna et al., 1986; Smith et al., 1988; Harriman et al.,
351 1991), were analyzed. Both *PG* and *PME* were down-regulated when *TAGL1/ALQ* was
352 silenced in fruit peels. However, *PME* expression was up-regulated in *TAGL1/ALQ*
353 overexpressing peels while *PG* transcript level was similar to that detected in WT peels
354 (Fig. 6). Furthermore, the expression of *PHYTOENE SYNTHASE* (*PSY)*, the major
355 responsible gene for carotenoid biosynthesis (Fray and Grierson, 1993), was
356 significantly repressed in RNAi peels (Fig. 6), which is in accordance with the colorless

357 phenotype of the *TAGL1/ALQ* silencing fruits. Nevertheless, the *PSY* transcript levels
358 were not affected in OE peels (Fig. 6).
359
360 **Discussion**
361
362 ***TAGL1/ALQ* regulates composition and biomechanical properties of fruit cuticle**
363
364 *TAGL1/ALQ* MADS-box gene is particularly relevant for fruit ripening and has been
365 reported as one of the major regulators of fruit development (Itkin et al., 2009; Vrebalov
366 et al., 2009; Giménez et al., 2010). This work delves into the study of fruit development
367 by analyzing the functional role of *TAGL1/ALQ* in cuticle development. With this aim,
368 tomato fruits either silencing or overexpressing *TAGL1/ALQ* have been analyzed at
369 biomechanical, cellular and molecular levels. Results have shown that transcriptional
370 activity of *TAGL1/ALQ* affects the biochemical composition and biomechanical
371 properties of fruit cuticle. Thus, repression of *TAGL1/ALQ* causes a significant decrease
372 in the amount of cuticle and all its components. Reduction of phenolic compounds
373 during ripening is responsible for the pale cuticle colour displayed in RNAi lines. Other
374 transcription factors such as *SlMYB12* and *NOR* have been shown to alter cuticle colour
375 during ripening due to a decrease in phenolics (Adato et al., 2009; Bargel and Neinhuis,
376 2004), /but cuticle deposition was only affected by SLMYB12 as well (Adato et al.,
377 2009). More recently, a significant decrease in cuticle and most of its components,
378 except for waxes, was reported in tomato fruits silencing *CHS* during ripening (España
379 et al., 2014b). The significant reduction in cuticle components known to confer
380 stiffness, i.e. polysaccharides, phenolics and waxes, would explain the lower Young's
381 modulus measured in *TAGL1/ALQ*repressed cuticles. This observation supports the
382 results obtained in the cuticles of *nor* mutants and *CHS* silenced tomatoes (Bargel and
383 Neinhuis, 2004; España et al., 2014b).
384 Overexpression of *TAGL1/ALQ* results in an expected opposite behaviour; that
385 is, an increase in cuticle components, except for phenolics and polysaccharides, which
386 remained unaltered in OE lines. A severe decrease in cuticle biomechanical properties,
387 mainly Young's modulus and breaking stress, is observed after *TAGL1/ALQ*
388 overexpression. The reduced values of Young´s modulus in both RNAi and OE lines
389 would be explained by the decrease in the relative proportion of polysaccharides and
390 phenolics with respect to waxes and cutin detected in these transgenic lines. However,

391 the lower mechanical resistance of the OE cuticle is compensated by the increase in the
392 cuticle thickness. These results, together with the fact that *TAGL1/ALQ* gene is
393 expressed in the fruit's outer epidermis (Matas et al., 2011), suggested that this MADS-
394 box gene participates in cuticle development as part of the genetic program leading to
395 fruit formation (Vrebalov et al., 2009; Giménez et al., 2010).
396

397 ***TAGL1/ALQ* controls cuticle development by regulating biosynthesis of cuticle**
398 **components**
399

400 Expression analyses of genes involved in cuticle biosynthesis were performed in
401 *TAGL1/ALQ* silencing and overexpressing peels, which have allowed for a better
402 understanding of the complexity of the genetic network involved in cuticle formation
403 and the role of *TAGL1/ALQ* as part of this developmental program. Flavonoid genes
404 such as *CHS1* and *SLMYB12* were down-regulated in *TAGL1/ALQ*-RNAi peels, a result
405 which agreed with the low levels of phenolic compounds and suggested a role of
406 *TAGL1/ALQ* in flavonoid biosynthesis. Such a regulatory function may occur through
407 the SLMYB12 transcription factor, which has indeed been proposed as a positive
408 regulator of flavonoid pathway genes such as *CHS1* (Adato et al., 2009; Ballester et al.,
409 2009). Accordingly, increased *CHS1* expression and high levels of the naringenin
410 chalcone flavonoid were detected in fruit-like sepals developed by tomato plants
411 overexpressing *TAGL1* (Itkin et al., 2009).
412 Previous studies have shown that phenylpropanoid/lignin pathway genes, such
413 as *PAL5, 4CL, CAD* and *LCCR1* (Anterola and Lewis, 2002; Vanholme et al., 2010;
414 Wang et al., 2013), were up-regulated in silencing *TAGL1/ALQ* fruit pericarp, which
415 agrees with the increased lignin content observed (Giménez et al., 2010). Similarly,
416 transcript levels of *4CL* and *CAD* genes were induced in RNAi and repressed in OE
417 peels supporting the role of *TAGL1/ALQ* as repressor of lignin biosynthesis in tomato
418 fruits. Such negative regulation could occur either by a direct inhibition of the
419 phenylpropanoid/lignin pathway genes or through an indirect control of flavonoid
420 pathway. Flavonoid genes are down-regulated when *TAGL1/ALQ* is silenced in tomato
421 fruits; thus, the substrate used from both flavonoid and phenylpropanoid/lignin
422 pathways, i.e. *p*- coumaric acid, could be preferentially used for lignin biosynthesis in
423 detriment of flavonoid biosynthesis. If so, TAGL1/ALQ transcriptional factor would act
424 by maintaining a balance between flavonoid and lignin pathways in tomato fruits.

425 Redirection of the metabolic flux between both pathways had previously been reported
426 in silencing Arabidopsis mutants affected in hydroxycinnamoyl-CoA shikimate/quinate
427 hydroxycinnamoyl transferase (*HCT*), a lignin biosynthetic gene (Besseau et al., 2007;
428 Li et al., 2010), and also in *CHS* antisense plants of strawberry (Lunkenbein et al.,
429 2006), supporting a role of *TAGL1/ALQ* mediating the flavonoid and lignin pathways.

430 Decreased *CD2* transcript level assessed in *TAGL1/ALQ* knockout peels was in
431 accordance with the lower amounts of cutin, indicating that cuticle biosynthesis is
432 mediated by *TAGL1/ALQ* transcriptional activity. However, other regulatory genes may
433 be involved in cutin biosynthesis since constitutive expression of *TAGL1/ALQ* did not
434 affect *CD2* expression, although it promoted increased cutin content. Among them,
435 ripening transcription factors as RIN and NOR have been previously involved in wax
436 and cutin composition (Kosma et al., 2010), and therefore could participate in regulating
437 cuticle development.

438 Regarding wax biosynthesis, fruits silencing *TAGL1/ALQ* showed diminished
439 wax content in their cuticles. Expression of genes involved in wax biosynthesis *SlSHN1*
440 and *SlSHN3* displayed an opposite expression pattern during fruit development. While
441 *SlSHN3* was partially inhibited before MG stage, *SlSHN1* was significantly up-regulated
442 after MG stage of RNAi fruits. These results suggest that *TAGL1/ALQ* might regulate
443 wax biosynthesis during fruit development through a temporal expression balance of
444 *SHN* genes. According to this hypothesis, it is known that *SHN1* influences wax
445 accumulation in Arabidopsis by modulating cutin production and changing the physical
446 properties of cuticle (Kannangara et al., 2007). Similarly, *LeCER6* mediates chemical
447 modifications affecting cuticular wax composition of tomato fruits (Vogg et al., 2004;
448 Leide et al., 2007). Taking these observations and the gene expression levels measured
449 in RNAi peels into account, *TAGL1/ALQ is likely to* modulate wax content of fruit
450 cuticle through the control of cutin production rather than regulate expression of wax
451 genes. Together, results reported in this work support that TAGL1/ALQ transcription
452 factor regulates cuticle development of tomato fruit mediating biosynthesis of cuticle
453 compounds such as cutin, wax and flavonoids.

454 On the other hand, constitutive expression of *TAGL1/ALQ* promoted significant
455 developmental changes affecting cell epidermal patterning and biochemical composition
456 of sepals, which turn them into fleshy fruit-like organs (Giménez et al., 2010). Similar
457 results were also reported in the tomato *Alq* mutant, which also overexpressed
458 *TAGL1/ALQ* gene (Pineda et al., 2010). However, developmental features of cuticles

459 from succulent *TAGL1/ALQ*-OE sepals were more similar to cuticles covering leaf
460 surface than to fruit's . It is reasonable to think that cell signals which promote cuticle
461 formation in *TAGL1/ALQ*-OE sepals are sent before sepal cells are converted into carpel
462 cells (Jeffree, 2006). Alternatively, transcriptional activity of other regulatory genes
463 specifically required for the development of tomato fruit cuticle might be absent from
464 sepal organs, which would prevent sepals from developing a fruit-like cuticle.
465
466 **Genetic regulation of fruit cell patterning mediated by *TAGL1/ALQ* influences**
467 **cuticle development**
468
469 Previous results showed that *TAGL1/ALQ* expression begins at early stages of
470 flower development, and that the highest accumulation of *TAGL1/ALQ* transcripts
471 coincides with flower anthesis and RR stage of fruit ripening (Giménez et al., 2010).
472 Furthermore, expression of *TAGL1/ALQ* has been detected in collenchyma and
473 parenchyma of fruit pericarp (Matas et al., 2011). Its repression promoted changes in the
474 size and distribution of cells forming these tissues, indicating that *TAGL1/ALQ* is a
475 crucial step for the development and ripening of a fleshy fruit like tomato (Vrebalov et
476 al., 2009; Giménez et al., 2010). In this work, detailed analyses of cuticles isolated from
477 tomato fruits silencing or overexpressing *TAGL1/ALQ* provide evidence that
478 transcriptional activity of *TAGL1/ALQ* is also a key factor for epidermis and cuticle
479 development of tomato fruits. The cuticle is synthesized by the epidermis (Javelle et al.,
480 2011). Thus, the reduced cuticle thickness, and the alterations in the cuticle
481 development shown by tomato fruits lacking *TAGL1/ALQ* expression might be due to
482 the abnormal biosynthetic activity of epidermal cells, which in turn affects the amount
483 and invagination degree of cuticle. It is also noteworthy that such compositional
484 alterations of *TAGL1/ALQ* repressed cuticles are associated to significant changes in the
485 size, morphology and arrangement of epidermal cells. this indicates that patterning and
486 biosynthetic activity of epidermal cells influence cuticle properties of tomato fruit.
487 Indeed, a close relationship between epidermal cell differentiation and cuticle
488 development has been reported as coordinated in Arabidopsis by SHN1 and two
489 MIXTA-like regulators of epidermal cell morphology (Oshima et al., 2013). Similarly,
490 SlSHN3 transcription factor has been put forward as a link factor between epidermal
491 patterning and fruit cuticle formation in tomato (Shi et al., 2013). In this work, it was
492 proved that *TAGL1/ALQ* controls the expression of the tomato *SlSHN1* and *SlSHN3*

493 genes. Therefore, the function of *TAGL1/ALQ* in the epidermis development could be
494 exercised through regulating the expression pattern of *SlSHN1* and *SlSHN3*, which in
495 turn might act in a redundant manner as Shi et al. (2013) proposed. Taken together, the
496 results hereby gathered support a role of *TAGL1/ALQ* as a transcriptional regulator of
497 fruit cuticle development which links epidermal cell patterning and cuticle formation,
498 most likely through the expression of target genes such as *SlSHN* homologues. Cuticle
499 alterations promoted by *TAGL1/ALQ* silencing also agree with the changes detected in
500 fruit pericarp, particularly with altered structural properties and the reduction of the
501 number of cell layers, which in turn correlated to decreased expression of cell cycle
502 genes as *CDKA1* (Vrebalov et al., 2009; Giménez et al., 2010). Recent studies have
503 shown that *CDKA1*-regulated cell division occurring in fruit pericarp is required for
504 cuticle development in tomato (Czerednik et al., 2012). Therefore, cell division and
505 expansion characterizing fruit pericarp should be accompanied by an appropriate
506 development of fruit cuticle. Hence, *TAGL1/ALQ* could coordinate both processes in a
507 fleshy-fruited species such as tomato.

508 Cuticle development has been suggested as part of the tomato development and
509 ripening programs (Saladié et al., 2007). Furthermore, biochemical composition and
510 mechanical performance of fruit cuticle seem to have a specific impact on the ripening
511 process of this fleshy fruit species (Domínguez et al., 2012). It was hereby reported that
512 cuticles developed by *TAGL1/ALQ* silenced fruits showed alterations in their
513 composition and biomechanical properties, and that these changes are comprehensively
514 associated with those affecting fruit development and ripening. In addition, RIN and
515 NOR do not only represent crucial factors in the genetic network controlling fruit
516 ripening (see review of Klee and Giovannoni, 2011), but they are also likely to act as
517 transcriptional regulators of early fruit development, with a direct effect on cuticle
518 composition (Kosma et al., 2010). MADS-box transcription factors RIN and
519 TAGL1/ALQ have been found to form heterodimers and to act together on ripening
520 process (Leseberg et al., 2008; Itkin et al., 2009; Vrebalov et al., 2009). Presumably,
521 such transcriptional interaction might be necessary to control cuticle development
522 during tomato ripening. Moreover, RIN interacts with the related-ripening transcription
523 factors FRUITFULL (FUL) 1 and FUL 2 to regulate fruit ripening through a subset of
524 RIN target genes different from that controlled by TAGL1/ALQ-RIN complex (Bemer
525 et al., 2012). Given that FUL 1/2 have also been involved in the production of cuticle
526 components (Bemer et al., 2012), TAGL1/ALQ-RIN and FUL1/2-RIN complexes

527 would/might participate in different genetic pathways regulating development of fruit
528 cuticle in tomato.

529 Besides fruit pericarp, *TAGL1/ALQ* induces expression changes of *PSY*, *PME*
530 and *PG* ripening-related genes in fruit peels, indicating that *TAGL1/ALQ* also influences
531 carotenoid biosynthesis and cell wall degradation in this fruit compartment. These
532 results provide new evidence about the relationships between cuticle formation and fruit
533 ripening, two developmental events integrated into a single developmental program
534 mediated by the transcriptional activity of *TAGL1/ALQ* gene.

535

536 **Materials and methods**

537

538 **Generation of *TAGL1/ALQ* transgenic tomato plants**

539

540 The binary plasmids generation mediating the interference RNA (*TAGL1/ALQ*-
541 RNAi) approach and the cloning of the *TAGL1/ALQ* complete open reading frame
542 under the control of the constitutive promoter 35S (*TAGL1/ALQ*-OE) to obtain tomato
543 *TAGL1/ALQ* silencing and over-expression lines respectively, have been previously
544 described by Giménez et al., (2010). The generated binary plasmids were electroporated
545 into *Agrobacterium tumefaciens* LBA 4404 strain and the transformation of tomato (*S.*
546 *lycopersicum* cv. Moneymaker) cotyledon explants was performed as previously
547 described (Ellul et al., 2003).

548 The presence of the transgene was verified in the T_0 generation by PCR using
549 *kanamicine* resistance gene specific primers; homozygous lines were obtained from
550 *TAGL1/ALQ*-RNAi and *TAGL1/ALQ*-OE transgenic plants to use for structural,
551 biochemical and gene expression analyses. WT tomato plants and *TAGL1/ALQ*-RNAi,
552 *TAGL1/ALQ*-OE transgenic lines were grown under greenhouse conditions using
553 standard culture practices with regular addition of fertilizers. The cultivar Moneymaker,
554 used as genetic background, was provided by C.M. Rick Tomato Genetics Resource
555 Center (http://tgrc.ucdavis.edu/).

556

557 **RNA preparation and gene expression analyses**

558

559 Wild-type tomato peels were removed using a peeler; they normally include the
560 cuticle, a single layer of epidermal cells, several layers of collenchyma cells and even

561 parenchyma cell layers. Peels from transgenic fruits were removed as in WT which
562 resulted in tissue sections of similar thickness. Total RNA from three biological
563 replicates of tomato peels was isolated using the Trizol reagent (Invitrogen). 1.2 µg of
564 DNA genomic free-RNA was used for cDNA synthesis using First Strand cDNA
565 Synthesis Kit (Fermentas) according to the manufacturer's instructions. Expression
566 analyses were performed by quantitative real-time PCR from three biological and two
567 technical replicates for each sample. With this aim, SYBR Green PCR Master Mix kit
568 (Applied Biosystems, Foster City, CA, USA) and the 7300 Real-Time PCR System
569 (Applied Biosystems) were used. System Sequence Detection Software v1.2 (Applied
570 Biosystem) was used to calculate gene-specific threshold cycles (Cts) including the
571 endogenous reference (*Ubiquitine3*) for every sample. The ΔΔCt calculation method
572 was used to obtain the results, expressed in arbitrary units, in comparison to a data point
573 from the wild type samples. Relative transcript levels were calculated using *Ubiquitine3*
574 controls. The sequences of the primers used in the expression analyses are listed in
575 Supplemental Table S1.

576

577 **Tissue sectioning and cuticle staining**

578

579 Pericarp pieces of three different fruits at red ripe stage as well as sepals from
580 WT control, *TAGL1/ALQ*-RNAi and OE were collected in two different seasons -
581 autumn and spring -, fixed in a formaldehyde, acetic acid and ethanol solution (1:1:18),
582 dehydrated in an ethanol series (70-95%) and embedded in commercial resin (Leica
583 Historesin Embedding Kit, Heidelberg, Germany) or Paraplast Plus (Sigma). Four to
584 eight µm thick cross-sections were stained with Sudan III, IV or Auramine O in order to
585 visualize the cuticle (Domínguez et al., 2008) and with Toluidine blue O to distinguish
586 the general structure. A minimum of 10 sections per sample was inspected under a light
587 microscope (Nikon, Eclipse E800).

588 Cuticle thickness was estimated from the previous cross-sections using an image
589 capture analysis program (Visilog-Noesis 6.3) also used to calculate cuticle area from
590 small flat pieces of cuticle following the protocol already described in Domínguez et al.,
591 (2008).

592 **Cuticle isolation**

593 Cuticles were enzymatically isolated with an aqueous solution of a mixture of
594 fungal cellulase (0.2% w/v, Sigma, St. Louis, Missouri, USA) and pectinase (2.0% w/v,
595 Sigma), and 1 mM NaN_3 in sodium citrate buffer (50 mM, pH 3.7) (Domínguez et al.,
596 2008). Vacuum was used to facilitate enzyme penetration. Samples were incubated with
597 continuous agitation at 35 °C for at least 14 d, later separated from the epidermis, rinsed
598 in distilled water and stored under dry conditions. Isolated cuticles, which include cutin,
599 waxes, phenolics and small amount of polysaccharides, were used to analyze their
600 components and biomechanical properties.

601 **Cuticle components analysis**

602 Cuticle components, i.e. waxes, cutin, polysaccharides and phenolics, were
603 gravimetrically estimated from 10 samples per genotype after selective removal. Total
604 waxes were extracted by heating at 50 ºC the isolated cuticles for 2 hours in
605 chloroform:methanol (2:1 v/v). Polysaccharides were removed after refluxing dewaxed
606 cuticles in a 6 M HCl solution for 12 h, hence leaving the cutin isolates intact
607 (Domínguez et al., 2009). Phenolic compounds were extracted after cutin
608 depolymerization in 1% methanol/KOH at 65ºC for 16 hours by measuring the
609 absorbance of the solution at 324 nm in a UV-VIS spectrophotometer. The amount of
610 phenolics was estimated from a calibration curve of a naringenin solution dissolved in
611 1% methanol/KOH (Domínguez et al., 2009).

612

613 **Biomechanical tests of isolated cuticles**

614

615 Mechanical properties of fruit cuticles were measured following the protocols
616 already described by Matas et al., (2005). Isolated cuticles were inspected with a
617 microscope to confirm the absence of cracks; afterwards, rectangular segments (3 mm x
618 9 mm) were cut with the aid of a metal block. These segments were fixed between the
619 ends of two hollow stainless-steel needles so that the cuticle formed a flat surface. Each
620 sample was held inside the environmentally controlled extensometer chamber for at
621 least 30 min to equilibrate the temperature and humidity before the extension test.

622 The cross-sectional area of the samples and the length of the exposed surface of
623 the sample between the two supports were measured prior to the mechanical extension
624 tests. Mechanical tests were performed as a transient creep test to determine changes in
625 length of a cuticle segment by maintaining samples in uniaxial tension, under a constant

626 load, for 1200 s, during which the longitudinal extension of each sample was recorded
627 every 3 s by a computer system. Each sample was tested repeatedly using an ascending
628 sequence of sustained tensile forces (from 0.098 N until the breaking-point by 0.098 N
629 load increments) without recovery time (Matas et al., 2005). The tensile force exerted
630 along the sample was divided by the cross-sectional area of the sample in order to
631 determine the stresses. The stress–strain curve was obtained after plotting the applied
632 stress was against the total change in length after 20 min. Young modulus, a measure of
633 stiffness, breaking stress, force needed to break the cuticle, and maximum strain,
634 deformation attained by the cuticle before breaking, were determined for each sample.
635 Strain-time and the corresponding stress–strain curves were calculated for a set of 5–7
636 samples of cuticles at 25°C and 40% RH.
637
638 **Statistics**
639
640 Mean comparison was used to determine whether the measured characteristics of
641 the cuticle varied significantly among genotypes. Analyses were performed using the
642 SPSS software package (IBM SPSS 2010) and data presented as means ± SE.
643
644 **Supplemental Material**
645
646 Table S1: Primers used for real-time quantitative PCR assays.
647
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652
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654
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860 **Figure legends**

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862 **Figure 1.** Phenotypic studies and expression analyses in *TAGL1/ALQ* silencing and
863 overexpresing fruits. A, Fruits, isolated cuticles and cuticle sections stained with
864 Sudan IV to visualize the cuticle of wild-type, *TAGL1/ALQ*-RNAi and
865 *TAGL1/ALQ*-OE tomato plants. Scale bars: 20µm. B, Relative expression of
866 *TAGL1/ALQ* gene in peel from WT, *TAGL1/ALQ*-RNAi and *TAGL1/ALQ*-OE
867 tomato fruits at immature green (IG), mature green (MG), breaker (BR) and red
868 ripe (RR) stages. Data are means ± standard error. Values followed by the same
869 letter (a, b, c) are not statistically significant ($P<0.05$).

870 **Figure 2.** Morphological analysis from *TAGL1/ALQ*-OE sepals. A, Sections of sepal
871 and fruit pericarp from WT and *TAGL1/ALQ*-OE plants harvested at red ripe stage
872 stained with Auramine O fluorescence stain to visualize the cuticle with light
873 microscopy equipped with a FTIC filter. Scale bars: 25 µm. B, Sections of sepal
874 and fruit pericarp from WT and *TAGL1/ALQ*-OE plants harvested at red ripe stage
875 stained with Toluidine for light microscopy observation. e: epidermis, c:
876 collenchyma; p: parenchyma. Scale bars: 50 µm.

877 **Figure 3.** Cell morphology and cell layer patterning of *TAGL1/ALQ*-RNAi and OE fruit
878 pericarp. A, Light microscopy photographs of pericarp sections from WT,
879 *TAGL1/ALQ*-RNAi and *TAGL1/ALQ*-OE fruit at AD, AD+10, IG, MG and RR
880 stages, stained with Toluidine blue. Scale bars: 50 µm. B, 2.5-fold amplification of
881 epidermis and collenchyma cells within rectangles shown at IG stage in A. C, 2.5-
882 fold amplification of epidermis and collenchyma cells within rectangles shown at
883 MG stage in A. D, 2.5-fold amplification of epidermis and collenchyma cells within
884 rectangles shown at RR stage in A. AD: anthesis day, IG: immature green, MG:
885 mature green, RR: red ripe, e: epidermis, c: collenchyma, p: parenchyma.

886 **Figure 4.** Morphological analyses of *TAGL1/ALQ*-RNAi and *TAGL1/ALQ*-OE cuticles
887 during carpel development. Fruit epidermis sections from WT, *TAGL1/ALQ*-RNAi
888 and *TAGL1/ALQ*-OE lines at AD, AD+10, IG, MG and RR stages of development
889 were stained with Sudan III to visualize cuticle. Scale bars: 50 µm. AD: anthesis
890 day, IG: immature green, MG: mature green, RR: red ripe.

891 **Figure 5.** Relative expression of genes related to biosynthetic pathways of cutin (*CD2*)
892 and waxes (*SlCER6, SlSHN1* and *SlSHN3*). The analyses were performed in peel
893 from WT control, *TAGL1/ALQ*-RNAi and *TAGL1/ALQ*-OE tomatoes at immature

894 green (IG), mature green (MG), breaker (BR) and red ripe (RR) stages. Data are
895 means ± standard error. Values followed by the same letter (a, b, c) are not
896 statistically significant (P<0.05).

897 **Figure 6.** Expression studies of genes related with biosynthesis of phenylpropanoids
898 (*4CL, CAD*), flavonoids (*CHS1, SLMYB12*), carotenoids (*PSY*), and with cell wall
899 degradation (*PG, PME*) in peel from WT, *TAGL1/ALQ*-RNAi and OE tomatoes at
900 immature green (IG), mature green (MG), breaker (BR) and red ripe (RR) stages.
901 Data are means ± standard error. Values followed by the same letter (a, b, c) are not
902 statistically significant (P<0.05).

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905 **Tables**

Table I. *Cuticle components and biomechanical parameters of wild-type (cv. Moneymaker) and TAGL1/ALQ-RNAi fruits at red ripe stage*

Values correspond to normalized data, except for thickness. Values are means ± SE. The statistical significance of mean differences was analyzed using a t-Student test: * P < 0.05, ** P < 0.01.

Parameters	Wild type fruits	*TAGL1/ALQ*-RNAi fruits
Thickness (µm)	11.46 ± 0.24	4.02 ± 0.15**
Cuticle	1962.0 ± 72.3	1024.3 ± 46.0**
Cutin	1383.1 ± 51.0	742.1 ± 33.3**
Waxes	69.7 ± 2.6	41.2 ± 1.8**
Polysaccharides	509.2 ± 18.8	236.7 ± 10.6**
Phenolics	109.7 ± 8.0	25.4 ± 5.7**
Young modulus (MPa)	535.0 ± 25.3	457.8 ± 1.3*
Breaking stress (MPa)	34.2 ± 1.4	36.6 ± 4.1
Maximum strain (%)	27.1 ± 4.1	23.4 ± 1.60

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Table II. *Components and biomechanical parameters of cuticles isolated from wild-type (cv. Moneymaker) fruits and their comparison with cuticles from fruits and fruit-like sepals overexpressing TAGL1/ALQ gene*

Values are means ± SE. Statistical significance of mean differences were indicated either by letters (Tukey-b test) with P < 0.05 or by asterisks (T-Student test) with * P < 0.05, ** P < 0.01.

Parameters #	Wild type fruits	*TAGL1/ALQ*-OE fruits	*TAGL1/ALQ*-OE sepals
Thickness (µm)	6.7 ± 0.1	11.9 ± 0.2**	---
Cuticle	1460.2 ± 31.9 b	1742.3 ± 53.8 a	417.0 ± 72.0 c
Cutin	970.4 ± 21.2 b	1209.7 ± 37.4 a	115.9 ± 20.0 c
Waxes	49.6 ± 1.1 b	59.8 ± 1.8 a	31.7 ± 5.5 c
Polysaccharides	440.2 ± 9.6 a	472.7 ± 14.6 a	269.3 ± 46.5 b
Phenolics	64.7 ± 5.1 a	56.1 ± 4.7 a	6.4 ± 1.7 b
Young modulus (MPa)	488.1 ± 51.7	306.6 ± 42.1*	---
Breaking stress (MPa)	36.6 ± 2.4	17.8 ± 1.4**	---
Maximum strain (%)	12.1 ± 1.6	8.2 ± 1.6	---

#Amounts of cuticle and cuticle components (cutin, waxes, polysaccharides and phenolics) are referred as micrograms per square centimeter.

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Table III. *Number of cells from pericarps of wild-type (cv. Moneymaker) and silencing and overexpressing lines at red ripe stage.*

Values are means ± SE. Statistical significance of mean differences were indicated by asterisks (LSD) with * P < 0.05, ** P < 0.01.

	Cell number		
	Epidermis[a]	Collenchyma[b]	Parenchyma[c]
Wild type	9.3±0.9	27±2.6	24±4.3
TAGL1/ALQ-RNAi	7.6±1.5**	18.2±1.5**	24±3.1
TAGL1/ALQ-OE	8.5±1.1	27±2.5	59.2±6.5**

[a] Cell number from epidermis was measured on a line of 300µm.

[b] Cell number from collenchyma was measured on a square area of 50µmx500µm.

[c] Cell number from parenchyma was measured on a square area of 1.25mmx1.25mm.

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