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1 **ABCG transporters export cutin precursors for the formation of the plant cuticle**

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24 will be fulfilled by the Lead Contact, Christiane Nawrath (christiane.nawrath@unil.ch).

25

26 **Summary**

27 The plant cuticle is deposited on the surface of primary plant organs, such as leaves,
28 fruits and floral organs forming a diffusion barrier and protecting the plant against
29 various abiotic and biotic stresses. Cutin, the structural polyester of the plant cuticle,
30 is synthesized in the apoplast. Plasma membrane-localized ABC-transporters of the G
31 family have been hypothesized to export cutin precursors. Here we characterize
32 *SIABCG42* of tomato representing an orthologue of *AtABCG32* in Arabidopsis.
33 *SIABCG42* expression in Arabidopsis complements the cuticular deficiencies of the
34 Arabidopsis *pec1/abcg32* mutant. RNAi-dependent downregulation of both tomato
35 genes encoding proteins highly homologous to *AtABCG32* (*SIABCG36* and
36 *SIABCG42*) leads to reduced cutin deposition and formation of a thinner cuticle in
37 tomato fruits. By using a tobacco (*Nicotiana benthamiana*) protoplast system, we show
38 that *AtABCG32* and *SIABCG42* have an export activity for 10,16-dihydroxy
39 hexadecanoyl-2-glycerol, a cutin precursor *in vivo*. Interestingly, also free ω -hydroxy
40 hexadecanoic acid as well as hexadecanedioic acid were exported furthering the
41 research on the identification of cutin precursors *in vivo* and the respective
42 mechanisms of their integration into the cutin polymer.

43

44

45 **Keywords**

46 ABC-transporter, acyl lipid, cutin, cuticle, wax, tomato, Arabidopsis, transport, plasma
47 membrane, diffusion barrier

48 **Introduction**

49 A crucial step in the evolution of land plants has been the development of the
50 cuticle, a particular surface structure of plant organs in primary growth stage that
51 determines the surface properties of the organ [1]. The cuticle represents a diffusion
52 barrier limiting transpirational water loss and other abiotic stresses and is playing an
53 important role in plant defense by its impact on a wide variety of biotic interactions [1].

54 The main structural component of the cuticle is the polyester cutin that is
55 composed of C16 and C18 fatty acids and their oxygenated derivatives, in particular
56 hydroxy fatty acids, epoxy fatty acids and dicarboxylic acids, as well as glycerol and
57 minor amounts of aromatic acids. 10, 16-dihydroxy hexadecanoic acid (10,16-diOH
58 C16:0) is an aliphatic monomer present in the cutin of many species that may be very
59 abundant, e.g. representing typically 70–80% in tomato fruit cutin and 60% of the
60 Arabidopsis petal cutin [2-4]. Waxes composed of very long chain fatty acids and their
61 derivatives as well as flavonoids and terpenoids impregnate the cutin polyester to form
62 a functional cuticle. The structure of the cuticle and the composition of cutin and wax
63 components vary according to species, organ, and developmental state [5].

64 A lot of progress has been made in the elucidation of cutin precursor formation.
65 Briefly, fatty acids are synthesized in the plastid and are oxygenated at the ER. The
66 transfer of acyl-CoA activated fatty acid derivatives to glycerol-3-phosphate by the
67 glycerol-3-phosphate acyltransferases GPAT4, GPAT6, GPAT8 that have a
68 phosphatase activity in addition to the acyltransferase activity result in mono acyl-2-
69 glycerols [6-8]. In addition, numerous other precursors are generated by the action of
70 acyltransferases of the BAHD family, including DEFICIENT IN CUTIN FERULATE
71 (DCF) and DEFECTIVE IN CUTICULAR RIDGES (DCR) [9, 10].

72 The polymerization of cutin takes place in the apoplast within the cuticle and in
73 the outermost layer of the cell wall. Several mechanisms of cutin polymerization and
74 assembly have been investigated recently [5, 11]: Specific members of the GDSL-
75 lipase family, such as CUS1 in tomato, are required for cutin synthesis acting via a
76 trans-esterification mechanism [3, 12-15]. In addition, $\alpha\beta$ hydrolases, such as
77 BODYGUARD, were proposed to structure the cuticle via a yet unknown mechanism,
78 possibly polymerization [16, 17]. In addition, based on *in vitro* studies, a non-enzymatic

79 polymerization mechanism has been postulated that might be facilitated by pectin [18].
80 The finding that CUS1 of tomato can use 10, 16-diOH C16:0-2-glycerol as substrate
81 to perform cutin oligomer/polymer synthesis by transesterification *in vitro* reinforced
82 the model that mono hydroxyacyl-2-glycerols are cutin precursors *in vivo* [14].

83 For the export of cutin precursors, ATP-binding cassette (ABC) transporters of
84 the G family have been proposed [19, 20]. Whereas half-size ABCGs seem to be
85 ubiquitous, full-size or PDR-type ABCGs having a double nucleotide binding domain
86 (NBD)-transmembrane domain (TMD) structure are restricted to plants and fungi [21,
87 22]. ABC transporters are capable of translocating molecules across cellular
88 membranes against the electrochemical gradient using ATP as energy source. Despite
89 being present in organisms of all kingdoms, plants have a highly enlarged number of
90 the ABC-transporters of the C- and G-family in comparison to other eukaryotes,
91 including mammals [23]. These are associated with the high number of metabolites
92 that need to be transported within in the cell and have to be distributed within the plant
93 organism or to its surfaces, serving as signals, such as plant hormones, being
94 constituents of protective barriers or compounds toxic to biotic aggressors [24]. ABC-
95 transporter diversity has been related to the developmental changes as well as
96 adaptations to the aerial environment of terrestrial plants that keeping a sessile lifestyle
97 acquired unique features to protect themselves against various abiotic and biotic
98 stresses [19, 24].

99 Characterization of the *abcg11* mutant of Arabidopsis deficient in the AtABCG11
100 half-size transporter revealed its broad implication in the export of aliphatic molecules
101 of the cuticle, including cutin monomers and aliphatic wax molecules [25-28]. While for
102 the export of aliphatic wax molecules a dimerization with AtABCG12 is necessary, cutin
103 precursors may be transported by the AtABCG11 homodimer, although
104 heterodimerization with other AtABCG half transporters cannot be excluded [26]. The
105 AtABCG13 half transporter is required for the formation of floral cutin in Arabidopsis
106 [29]. Interestingly, while the ABCG11-type transporter is broadly present in all land
107 plants and has often an increased number of homologues, AtABCG13 has been not
108 identified in monocots and early diverging land plant lineages [30].

109 In addition, the full-size transporter ABCG32 of Arabidopsis has non-redundant
110 functions in the formation of cutin in different organisms, particularly for cutins rich in

111 C16:0 monomers. The *pec1/atabcg32* mutant had reduced amounts of oxygenated
112 cutin monomers (approximately 50%) in flowers and structural modifications of the
113 interface of the cuticle with the cell wall in petals [31]. In Arabidopsis leaves that have
114 a particular cutin rich in unsaturated C18 DCAs *AtABCG32* plays a more pronounced
115 role in the early developing stages where the cutin has a higher proportion of C16
116 monomers [32]. In monocots having a mixed C16/C18 cutin composition, the knockout
117 in the ABCG32-orthologue ABCG31 leads to an approximately 50% reduction in cutin
118 and is associated with profound changes in cuticle thickness and ultrastructure [33,
119 34]. Considering the complexity of pathways involved in cutin formation a thorough
120 investigation of the potential export functions of ABCG transporters for cuticular lipids
121 is pertinent.

122 Here we show that *SIABCG42* of tomato is a true orthologue of *AtABCG32* since
123 *SIABCGG42* expression under the control of the native *AtABCG32* promoter can
124 complement the cutin deposition and the permeability of the cuticle in *pec1/atabcg32*
125 flowers. Both *AtABCG32* and *SIABCG42* have very similar substrate specificities in the
126 *Nicotiana benthamiana* protoplast assay exporting 10,16-diOH C16:0-2-glycerol, but
127 also unesterified ω -hydroxylated C16:0 fatty acid as well as C16:0 dicarboxylic acid
128 (DCA). *AtABCG11*, in contrast, does only transport 10,16-diOH C16:0-2-glycerol and
129 the unesterified ω -OH C16:0 acid, but not C16:0 DCA. Downregulation of *SIABCG42*
130 together with the highly homologous gene, *SIABCG36*, in tomato leads to a decrease
131 in fruit cuticle thickness and cutin amount, *i.e.* the amount of aliphatic monomers, in
132 correlation to the expression level. Furthermore, cell size and surface structure were
133 altered in the strongest downregulated line. These results show that ABCG32
134 transporters export various fatty acids and their derivatives, potentially supplying
135 several pathways of cutin formation with precursors.

136

137 Results

138 SIABCG42 is a functional orthologue of AtABCG32

139 While in monocots and many dicot species, such as *Brassicaceae*, only a single
140 gene with high homology to *AtABCG32* exists [30, 33, 34], tomato as well as other
141 *Solanaceae* species own a second gene encoding a protein with high homology to
142 *AtABCG32* [35] (Figure S1A). Following the nomenclature by Ofori et al., *SIABCG36*
143 (*Solyc05g018510*) encodes the closest *AtABCG32* homologue, a protein of 75%
144 identity and 85% similarity to *AtABCG32* [35]. *SIABCG42* (*Solyc06g065670*) encodes
145 for a protein of 69% identity and 79 % similarity to *AtABCG32* and was thus called
146 *AtABCG32*-like. This nomenclature differs from the one used by Lashbrooke et al. who
147 named the *Solyc06g065670* gene product the *ABCG32* homologue in tomato [36].

148 Although both, *SIABCG36* and *SIABCG42*, are expressed in the epidermis
149 during tomato fruit development in accordance with a potential function cutin
150 biosynthesis [37], *SIABCG36* has a much higher expression level than *SIABCG42*
151 (Figure S1B) [37]. *SIABCG42* has nevertheless been reported to be regulated in its
152 expression by the transcription factors MIXTA and ER4.1, similarly as other cutin
153 biosynthesis genes [36, 38]. The question whether *SIABCG42* indeed represents a
154 functional homologue of *AtABCG32* was addressed by expressing *SIABCG42* in the
155 *pec1/atabcg32* mutant under the control of the *AtABCG32* promoter.

156 Characterization of the floral cutin composition of several transgenic lines
157 expressing *pAtABCG32::GFP-SIABCG42* in *pec1/abcg32*, including *pec1/SIABCG42-*
158 *L1* and *pec1/SIABCG42-L2* revealed complementation of the cutin deficiencies of the
159 *pec1* mutant (Figure 1A, Figure S2) [31]. Although not all transgenic lines did
160 complement the amount of floral cutin to 100% (Figure 1A), the expression of the GFP-
161 tagged *SIABCG42* transporter was able to complement the Arabidopsis cutin
162 composition, *i.e.* not only cutin monomers present in tomato, such as 10,16-dihydroxy
163 hexadecanoic acid (10,16-diOH C16:0), but also unusual cutin monomers typical for
164 Arabidopsis cutin, *i.e.* 9,12-octadecadiendioic acid (C18:2 DCA) present in Arabidopsis
165 flowers (Figure 1A, Figure S2).

166 Furthermore, the barrier properties of the petal cuticle were restored in both
167 *pec1/SIABCG42* lines, as evaluated by TB staining (Figure 1B). The ultrastructure of

168 the petal cuticle in the *pec1/abcg32* mutant complemented with *pAtABCG32::GFP-*
169 *SIABCG42* had the same structure as the one complemented with *pAtABCG32::GFP-*
170 *AtABCG32* [31] (Figure 1C). The cuticular ridges were regular and filled by electron-
171 dense cell wall material similar to Arabidopsis WT.

172 The complementation of the Arabidopsis *pec1/abcg32* mutant with
173 *pAtABCG32::GFP-SIABCG42* demonstrates that the SIABCG42 transporter can exert
174 AtABCG32 function in Arabidopsis that is hypothesized to be the export of cutin
175 precursors [31].

176 **AtABCG32 and SIABCG42 export mono-10,16-diOH C16:0-2-glycerol**

177 In order to investigate whether AtABCG32 and SIABCG42 indeed have an
178 export activity for cutin precursors, the *N. benthamiana* protoplast system was used
179 that had been previously developed for the characterization of other ABC-transporters
180 of Arabidopsis and tomato [39-41]. AtABCG32 fused to GFP at its N-terminus fully
181 complemented *pec1/atabcg32* mutant phenotypes and was thus used for the activity
182 study in *N. benthamiana* [31] as well as an analogous construct generated for
183 SIABCG42. In addition, constructs were generated to abolish transport activities by
184 mutating the N-terminal Walker A motif required for ATP binding and hydrolysis,
185 resulting in GFP-AtABCG32-M and GFP-SIABCG42-M, respectively (Figure S3A) [42].
186 Constructs for the expression of GFP-AtABCG32 and GFP-ABCG32-M as well as
187 GFP-AtABCG32 and GFP-ABCG32-M under the control of the CaMV-35S promoter
188 were *Agrobacterium*-transfected into *N. benthamiana* (Figure S3). Expression and
189 localization at the plasma membrane (PM) of all the ABCG proteins epidermal cells of
190 *N. benthamiana* were investigated by confocal laser microscopy. The expression of
191 AtABCG32 and AtABCG32-M was in general stronger than of SIABCG42 and
192 SIABCG42-M. Nevertheless, all ABCG transporters were at least partially localized at
193 the PM based on the co-localization of the FP-tagged ABCGs with the PM-marker
194 PIP2A-mCherry expressed under the CaMV-35S promoter as well as their localization
195 adjacent the propidium iodine-stained cell wall (Figure S4) [43, 44]. FP-labeled
196 proteins, PIP2A-mCherry as well as the FP-labelled ABCGs were often additionally
197 localized to the endoplasmic reticulum, in particular when the expression level was
198 high (Figure S4), probably due to saturation of the secretion system.

199 For studying the transport activity *N. benthamiana* cells transfected either with
200 the functional or mutated GFP-AtABCG32 and GFP-SIABCG42 were loaded with [³H]-
201 10,16-diOH C16:0-2-glycerol, the most likely *in vivo* substrate, together with the
202 unrelated substrate [¹⁴C]-indole acetic acid ([¹⁴C]-IAA). Both *N. benthamiana* cells
203 expressing the functional GFP-AtABCG32 and GFP-SIABCG42 revealed significantly
204 enhanced export of 10,16-diOH C16:0-2-glycerol in comparison with the vector control
205 (Figure 2A and 2C, Figure S5). This was not the case for the auxin, IAA, an indolic
206 substrate of ABCB-type ABC transporters (Figure 2B and 2D) [40], indicating substrate
207 specificity for fatty acid compounds. Importantly, the Walker A mutated proteins
208 revealed no export activity with 10,16-diOH C16:0-2-glycerol clearly indicating the
209 ATP-dependency of this transport process and excluding indirect effects, like activation
210 of secondary transport systems (Figure 2A and 2B). In summary, this dataset indicates
211 that GFP-AtABCG32 and GFP-SIABCG42 catalyze the plasma membrane export of
212 10,16-diOH C16:0-2-glycerol in an ATP-dependent manner.

213 Previously, YFP-tagged AtABCG11 had been shown to complement the
214 *atabcg11* mutant phenotypes (Bird et al., 2007). Therefore, YFP-AtABCG11 was
215 expressed under the control of the CaMV-35S promoter in *N. benthamiana* cells
216 (Figure S3A). Expression and localization at the PM of *N. benthamiana* cells was
217 verified by confocal microscopy, as for the other ABCGs, see above (Figure S4). An
218 export activity for [³H]-10,16-diOH C16:0-2-glycerol, but not for [¹⁴C]-IAA could be seen
219 with YFP-AtABCG11, similar to these of AtABCG32 and SIABCG42 (Figure 2C and
220 2D; Figure S5).

221 Thus, all functional ABCGs transporters investigated here, *i.e.* AtABCG32,
222 SIABCG42 and AtABCG11, transported specifically 10,16-diOH C16:0-2-glycerol in an
223 ATP-dependent manner.

224 **AtABCG32 and SIABCG42 transport also unesterified oxygenated fatty acids**

225 In order to investigate further the specificity of AtABCG32 and SIABCG42 as
226 well as AtABCG11, several commercially available ¹⁴C-radiolabeled molecules that are
227 also typical components of cutin, *i.e.* ω-OH C16:0 ([¹⁴C]-ω-OH C16:0) and C16:0 DCA
228 ([¹⁴C]-C16:0 DCA) were tested in double labeling experiments with [³H]-10,16-diOH
229 C16:0-2-glycerol.

230 Interestingly, under our conditions the amount of [¹⁴C]-ω-OH C16:0 molecules
231 was reduced in the assay supernatant of the vector control in a time-dependent
232 manner (Figure 2E; Figure S5). This apparent negative export (quantified by
233 measuring radioactivity in the supernatant) represents a net import of unspecifically
234 exported substrate, likely by yet uncharacterized import systems present in tobacco
235 cells. However, again expression of GFP-AtABCG32, GFP-SIABCG42 and YFP-
236 AtABCG11 significantly reduced this net import seen in the control, which can only be
237 caused by an export activity for [¹⁴C]-ω-OH-C16:0 (Figure 2E; Figure S5).

238 Similar as for [³H]-10,16-diOH C16:0-2-glycerol, [¹⁴C]-C16:0 DCA was released
239 from the tobacco cells carrying the control construct and this release was enhanced
240 by the presence of GFP-AtABCG32 and GFP-SIABCG42, but not by YFP-AtABCG11.
241 These results indicate an export activity for C16:0 DCA of GFP-AtABCG32 and GFP-
242 SIABCG42, but not of YFP-AtABCG11 (Figure 2F; Figure S5).

243 In summary, reduced import of [¹⁴C]-ω-OH C16:0 and enhanced export of [¹⁴C]-
244 C16:0 DCA indicate an export activity of non-glycerol bound aliphatic C16:0
245 compounds for AtABCG32 and SIABCG42. An export of [¹⁴C]-ω-OH C16:0, but not of
246 [¹⁴C]-C16:0 DCA demonstrates that ABCG11 has only a partially overlapping substrate
247 specificity with AtABCG32 and SIABCG42 under the same assay conditions.

248 ***SIABCG36/42* mutants have reduced cutin deposition in the fruit cuticle**

249 Tomato plants downregulated in the expression of *SIABCG36* and *SIABCG42*
250 were generated by an RNAi approach. In the T2 generation *SIABCG42* expression
251 was downregulated in expanding tomato fruits at 10 days after anthesis (DPA) by 95%
252 in the strongest RNAi line expressing the *SIABCG42*-RNAi-fragment (Figure S6A).
253 Interestingly, the expression of homologue *SIABCG36* was also reduced by 70%.
254 Therefore, this RNAi-line was called *slabcg36/42-a*. In the T3 generation that was used
255 for the complete characterization presented here the RNAi silencing was slightly less
256 strong (90% for *SIABCG42* and 60% for *SIABCG36*). Downregulation of *SIABCG36* by
257 the *SIABCG36*-RNAi fragment led to the reduction of *SIABCG36* expression by 60%
258 in the strongest line of the T3 generation (10 DPA) (Figure 3A). As *SIABCG42*
259 expression was also reduced in this line it was called *slabcg36/42-b*. Since the degree
260 of downregulation of *SIABCG36* was similar between the RNAi-plants *slabcg36/42-a*

261 and *slabcg36/42-b*, differences in the phenotypes between both lines are likely due to
262 the different reduction in *SIABCG42* expression, although changes in the level of RNAi-
263 dependent downregulation of both *SIABCG36* and *AtABCG32* during fruit development
264 may not be excluded [45].

265 Fruit cuticles of *slabcg36/42-a* and *slabcg36/42-b* were characterized at 20 DPA
266 and in Red Ripe stage (~50 DPA). Although macroscopically the fruits did not show a
267 difference to WT, the cuticle of both RNAi lines was strongly altered. While in WT not
268 only the epidermis, but also subepidermal layers are cutinized, the cutinization of
269 subepidermal layers is rather remnant in the RNAi lines at both developmental stages,
270 as visualized by Sudan IV staining of cross-sections of the pericarp (Figure 3C).
271 Furthermore, the cuticle thickness of the epidermis was reduced in the *slabcg36/42-b*
272 and even more in the *slabcg36/42-a* at 20 DPA. Overall the cutinization was reduced
273 by 35% in *slabcg36/42-b* and by 45% *slabcg36/42-a* at 20 DPA, when measuring the
274 cutinized, *i.e.* Sudan VI-stained area. This reduction correlated well to the degree of
275 the downregulation in the RNAi lines. The cutinization was also reduced in the RNAi
276 lines at Red Ripe stage, even when less pronounced (19% and 24%, respectively).

277 Transmission Electron Microscopy (TEM) revealed alterations in the shape of
278 the epidermal cells. In WT epidermal cells have a broad base and a conical top. At 20
279 DPA the epidermal cells were rounder in *slabcg36/42-b*, whereas in *slabcg36/42-a* the
280 epidermal cells were much flatter and the conical top was barely visible (Figure 3C).
281 These changes could also be well seen when the size of the ultrathin sections was
282 quantified. The cells of WT and the intermediate downregulated RNAi-lines showed a
283 high variability in size depending on where the section was taken. The *slabcg36/42-a*
284 RNAi line having a rather flat shape showed less variability in section size that was
285 overall smaller both at 20 DPA and in the Red Ripe stage (Figure 3C; Figure S6B),
286 which corresponded well with the much smoother surface structure as visualized by
287 Scanning Electron Microscopy (SEM) in both developmental stages (Figure 3C).

288 Alterations in cell shape which are likely a secondary effect of a reduced
289 cutinization were much more pronounced in the stronger *slabcg36/42* line indicating
290 that below a certain threshold potentially other regulatory mechanisms were activated.
291 Altered surface morphology based on flatter epidermal cells had also been observed

292 in other strong cutin mutants, such as in *CUS1*-, *SISHN3*- and *SIMIXTA-RNAi* lines [3,
293 36, 46].

294 The cuticular polyester of tomato fruits was characterized by GC-MS analysis
295 at 20 DPA and the Red Ripe fruits stage. The principal cutin monomers were the C16
296 monomers 10,16-dihydroxy C16:0, ω -OH-C16:0, and C16:0 DCA. The only C18
297 monomer that could be detected was C18:0 DCA, which had very low abundance
298 (Figure 4A and 4B). Other monomers of low abundance were not characterized in this
299 study. The changes in the visible cutinization of the epidermal cell wall in *slabcg36/42-*
300 *b* and *slabcg36/42-a* correlated to a reduction in the cutin amount (Figure 4A and 4B)
301 as well as amount of the hydroxylated C16:0 acids, *i.e.* ω -OH C16:0 and 10, 16-diOH
302 C16:0 that were reduced by approximately by 17% in *slabcg36/42-b* and 27% in
303 *slabcg36/42-a* at 20 DPA as well as by 15% and 25% in Red Ripe tomato fruits,
304 respectively (Figure 4A and 4B). 16:0 DCA and C18:0 DCA were not reduced in
305 *slabcg36/42-b*, but reduced by approximately 50% in *slabcg6/42-a* in both
306 developmental stages (Figures 4A and 4B). The lack in reduction of C16:0 DCA and
307 C18:0 DCA was also observed in line *slabcg36/42-c*, another line carrying the
308 construct for silencing *SIABCG36* that has an intermediate expression level for both
309 genes (Figures S6A and S6C).

310 In summary, *slabcg36/42-RNAi* plants showed a reduction in all oxygenated
311 cutin monomers investigated. The reason that C16:0 DCA and C18:0 DCA were only
312 reduced in the strongest downregulated line might be due to differences in the affinity
313 of the *SIABCG36* and *SIABCG42* transporters to different cutin precursors.

314 The reduction in 10, 16-diOH C16:0 was not accompanied by an alteration in
315 the esterification level of its OH groups. The vast majority was esterified on both mid-
316 chain and ω -OH position indicating that the reticulation pattern of the cutin polyester is
317 not altered in *slabcg36/42-RNAi* plants in both stages of fruit development (Figure 4C).
318 In this feature the cutin of *slabcg36/42-RNAi* plants was similar to this of the *gpat6*
319 mutant of tomato that also does not have an alteration in the esterification pattern of
320 10, 16-diOH C16:0 [12].

321 Furthermore, the amount of ester-bound glycerol in the cutin was only reduced
322 by approximately 15–20% in the stronger downregulated *slabcg36/42-a* line in both

323 stages of fruit development (Figure 4D) indicating only minimal changes in the
324 structure of its fruit cutin.

325 **Wax increase restores cuticle permeability in SIABCG36/42 compromised plants**

326 The deposition of aliphatic wax components was assessed in *slabcg36/42*-RNAi
327 plants revealing an increase of 20% and 30% at 20 DPA in *slabcg36/42-b* and
328 *slabcg36/42-a*, respectively (Figure 5A). Particular, the odd-chain length alkanes (C29
329 and C31) were increased. In Red Ripe tomato fruits the variation in wax content was
330 higher, not revealing a significant increase (Figure 5B). Thus, wax deposition was likely
331 only indirectly affected by the downregulation of *SIABCG36* and *SIABCG42*.

332 Isolated, but not dewaxed cuticles of tomato fruits of *slabcg36/42*-RNAi lines did
333 not exhibit an increased permeance to water, similar as seen in some other tomato
334 fruit cuticle mutants [47] (Figure 5C). Only after the removal of wax from cuticles of
335 tomato fruits at 20 DPA an increase in water permeance was identified. No differences
336 were remarked in Red Ripe fruits in which the cuticle was over all less altered. The
337 increased permeances of dewaxed cuticles at 20 DPA support the understanding that
338 wax deposition plays a crucial role as barrier against transpirational water loss [48].
339

340 Discussion

341 ABCG transporters export cutin monomers

342 For the formation of the cuticular polyester cutin, the export of cutin precursors,
343 from the epidermal cell to the apoplast, is essential. Genes encoding ABC-transporters
344 of the G family are essential for cutin formation in many plant species, including
345 monocots and dicots [25, 27, 28, 31, 34, 49]. Here we demonstrate that the ABCG half-
346 size transporter AtABCG11 and the fully-size transporter AtABCG32, which are both
347 required for cutin formation in Arabidopsis [25, 27, 28, 31] show export activity with
348 ester-bound and free oxygenated fatty acids in a *N. benthamiana* protoplast system
349 (Figure 2). In addition, SIABCG42, a homologue of AtABCG32 in tomato that is further
350 characterized in these studies, exhibits similar export activity to AtABCG32.

351 The export activity for 10,16-diOH C16:0-2-glycerol of the investigated ABCG-
352 transporters is well in accordance with our current understanding of cutin
353 polymerization [7, 14, 50]. These results are also supported by the reduced 10,16-
354 diOH C16:0 amounts in the cutin of the respective mutants as well as the restoration
355 of the amounts of 10,16-diOH C16:0 in floral cutin by complementation of the
356 *Arabidopsis pec1/abcg32* mutant with AtABCG32 or SIABCG42 or the *abcg11* mutant
357 with AtABCG11 [25, 27, 28, 31] as also shown in these studies (Figure 1 and Figure
358 4).

359 Interestingly, AtABCG32 and SIABCG42 exported not only 10,16-diOH C16:0
360 ester-bound to glycerol in the *N. benthamiana* protoplast system, but also the
361 unesterified ω -OH C16:0 acid and C16:0 DCA suggesting that they recognize the
362 aliphatic chain of the oxygenated fatty acid and not the configuration at the C1-carbon
363 (Figure 2F). Whether AtABCG32 and SIABCG42 transport other types of acyl derivatives
364 than glycerol-bound derivatives during cutin synthesis needs future investigations.

365 The question whether AtABCG32 and SIABCG42 transport also molecules
366 having a C18 backbone could not be directly addressed due to the lack of radiolabeled
367 oxygenated C18 substrates commercially available. The complementation of C18:1
368 DCA and C18:2 DCA in the flower cutin of Arabidopsis by SIABCG42 and the reduction
369 of the C18:0 DCA when SIABCG36 and SIABCG42 are strongly downregulated argue
370 for a transport activity of molecules with a C18-backbone.

371 The export activity for 10,16-diOH C16:0-2-glycerol and free ω -OH C16:0 in *N.*
372 *benthamiana* protoplasts expressing AtABCG11 (Figure 2E) supports in general the
373 hypothesis that AtABCG11 exports cutin monomers as homodimer [26]. Interestingly,
374 the substrate specificity of AtABCG11 differed from this of AtABCG32 and SIABCG42
375 since it did not export C16:0 DCA in the employed heterologous transport system
376 arguing for an altered *in vivo* substrate specificity. However, at the moment we cannot
377 rule out the theoretical but unlikely possibility that a functional heterodimerization with
378 a tobacco half-size ABCG isoform or the lack of an Arabidopsis heterodimeric ABCG
379 half forcing homodimerization has an influence on substrate specificities. Thus,
380 ultimate proof of an altered substrate specificity for AtABCG11 awaits its *in*
381 *vivo* confirmation.

382 While ABC-transporters are well characterized to transport a wide variety of
383 metabolites, including many plant hormones, little is known about plant ABC-
384 transporters transporting acyl lipids. Transport of acyl lipids from the endoplasmic
385 reticulum to the chloroplast depends on the multicomponent ABC transport system
386 with TGD1/AtABCG114 being predicted to be the membrane permease of the ABC
387 transporter that is localized to the inner plastidial membrane and is necessary to build
388 the plastidial membrane system for photosynthesis [51]. The substrates of this
389 transport system have not yet been identified, but are hypothesized to be phosphatidic
390 acids [51]. The peroxisomal AtABCD1 that is required for β -oxidation exhibits transport
391 activity for CoA-bound fatty acids, but not for free fatty acids [52]. Beyond cutin
392 biosynthesis numerous ABCG half-size transporters are required for the formation of
393 other polymers in the apoplast that are rich in lipids, such as suberin and
394 sporopollenine [24]. Suberin is a polyester with a high amount of very-long chain fatty
395 acids and fatty alcohols as well hydroxycinnamic acids. While the simultaneous
396 knockout of AtABCG2, AtABCG6, AtABCG20 affects suberin synthesis in roots and
397 seeds, the knockout of AtABCG1 and AtABCG16 affects pollen formation, potentially
398 because of a function in sporopollenine formation [53]. The StABCG1 potato loss-of-
399 function mutant that is most similar to AtABCG1 and ABCG16 of Arabidopsis affects,
400 however, suberin formation in potato tubers [54]. Contribution of AtABCG1 to suberin
401 formation in Arabidopsis could recently be shown [55]. The lack of information about
402 the dimerization pattern of ABCG half transporters to functional transporters as well as
403 the lack in knowledge about the structure of suberin precursors *in vivo* rendered the

404 characterization of the activity of suberin transporters difficult. Nevertheless, an
405 enhanced ATP-hydrolyzing activity in the presence of very long chain fatty acids (C24-
406 C30) and alcohols (C26-C30) of the purified AtABCG1 supported the hypothesis that
407 this ABCG transporter exports suberin precursors in an unesterified form [55].
408 Questions about the dimerization partners as well as identity of the substrates *in vivo*
409 remain open, similarly as in our studies of AtABCG11 in cutin synthesis.

410 Plant ABCG-transporters may thus transport esterified and potentially also unesterified
411 acyl-lipids across membranes.

412 **AtABCG32 and SIABCG42 may transport particular derivatives of fatty acids**

413 The cuticle of Arabidopsis petals is characterized by cuticular ridges having an
414 amorphous cuticle proper and a more electron dense cuticular layer within the ridges
415 [56]. In Arabidopsis, *AtABCG32* loss-of-function specifically affects the formation of
416 regular cuticular ridges as well as the cuticle of young expanding leaves in the
417 presence of functional AtABCG11 and AtABCG13 transporters [31]. The phenotypes
418 of the *pec1/abcg32* mutant may be associated with the lack of particular cutin
419 monomers exported by AtABCG32 that contribute to structuring the cuticle, as
420 discussed in Bessire et al. and Fabre et al. [31, 32]. The tomato fruit has a thick
421 reticulated cuticle that is also characterized by its content of cell wall components [57,
422 58]. The restoration of the ultrastructure of cuticular ridges in Arabidopsis by
423 SIABCG42 points towards similar transport activities that are important for structuring
424 the cuticle (Figure 1C).

425 In addition, *atabcg11* and *atabcg13* mutants have a strongly reduced cutin load
426 and altered cuticle structure in leaves/stems and flowers, respectively, despite their
427 functional AtABCG32 [25, 28, 29] which shows that ABCG-transporters do not function
428 fully redundantly in cutin formation. Whether this partial lack in redundancy is related
429 to a particular type of acyl lipid-derived cutin precursors that are transported by a
430 specific transport system will remain open to future studies.

431 **AtABCG32 orthologues of tomato are required for tomato fruit cutin formation**

432 The modifications of the cuticle in *slabcg36/42*-RNAi plants comprise a reduced
433 amount of cutin and a thinner cuticle at 20 DPA in correlation to the expression level

434 as well as a reduced amount of 10, 16-diOH C16:0 as well as ω -OH-C16:0 in the
435 tomato fruit cutin (Figure 4A). Differences in the affinity of AtABCG32 and SIABCG36
436 and/or SIABCG42 might explain the lack in reduction of DCAs in the intermediate
437 downregulated lines. Interestingly, AtABCG11 did not show a transport activity with
438 C16:0 DCA in the protoplast system potentially pointing also to differences in affinity
439 for this compound class among ABCGs required for cutin synthesis.

440 The molecular structure of the cutin of *slabcg36/42*-RNAi lines was unaltered similarly
441 to the tomato *gpat6* mutant having also a reduced cutin amount, but no alterations in
442 the esterification pattern of 10,16-diOH C16:0 (Figure 4C). This stands in contrast to
443 the *cus1* mutant, where the esterification pattern of 10,16-diOH C16:0 was strongly
444 altered [12]. The observed reduction in aliphatic cutin monomers in the *slabcg36/42*-
445 RNAi lines and no other changes in cutin structure and composition support well the
446 results of the SIABCG42 export activity for all oxygenated fatty acid derivatives.

447 The strongly down-regulated *slabcg36/42*-RNAi line exhibited in addition to the
448 strongly reduced amount of cutin a particular strong cell flattening as well as a smaller
449 cell size indicating broader effects on the properties of the cuticle and cell shape
450 (Figure 3). Similar observations have also been made in other strong cutin mutants,
451 including in *slmixta*, *slshine3*, *slcyp86a69* and *slgpat6* mutants of tomato [36, 46, 50].
452 The phenomenon might be related to alterations in regulatory pathways after sensing
453 the lack in cell wall/cuticle integrity. There might, however, be some differences in the
454 activated pathways among different cutin mutants. Indeed, unlike *slshine3* and *slgpat6*
455 mutants of tomato, no surface glossiness of the tomato fruits was observed in the
456 *slabg36/42*-RNAi plants and the amount of glycerol was rather lower than higher in
457 comparison to the *gpat6* mutant having a board range of changes in the gene
458 expression of cutin biosynthesis genes [50, 59].

459 Tomato fruits of *slabcg36/42*-RNAi plants showed an increase in cuticular wax
460 accumulation at 20 DPA likely compensating for the reduced cutin deposition since the
461 permeability of the isolated cuticles was not significantly increased (Figure 5). Only
462 dewaxed cuticles, *i.e.* cutin, of 20 DPA tomato fruits showed a significant increase in
463 permeability highlighting the importance of wax for the impregnation of the cuticle [60].
464 An increase in aliphatic wax components has also been reported for *gpat6* and *cus1*

465 mutants of tomato [12, 59]. The increase in wax highlights that *SIABCG36* and
466 *SIABCG42* are not required for wax export similarly as *AtABCG32* [31].

467 Tomato fruits of *slabcg36/42*-RNAi plants in the Red Ripe stage exhibited still a
468 significant reduction in the amount of cutin. However, the differences were weaker
469 indicating that other transport systems likely contribute to cutin formation in later stages
470 of tomato fruit development during which the gene expression of both *SIABCG36* and
471 *SIABCG42* is low. In agreement with this also the cuticle of Red Ripe tomato fruits
472 exhibited a better functionality, including better sealing properties to water and no
473 increase in wax accumulation, similar as seen in mutants compromised in cutin
474 biosynthesis in tomato fruits [47]. However, the surface of the strongest RNAi line
475 remained flat indicating also persisting impact of the reduction in *SIABCG36* and
476 *SIABCG42* expression on the cuticular morphology in Red Ripe tomato fruits (Figure
477 3C).

478 Since both *SIABCG36* and *SIABCG42* were downregulated in the RNAi
479 downregulated plants little can be said about the exact contribution of *SIABCG36* or
480 *SIABCG42* to the observed phenotypes. Remarkably, despite the more distant relation
481 of *SIABCG42* than *SIABCG36* to *AtABCG32*, *SIABCG42* was consistently mentioned
482 to be regulated together with other cutin biosynthetic genes, i.e. by *MIXTA* as well as
483 by *ER4.1* [36, 38]. A more detailed analysis of the specific roles of *SIABCG36* and
484 *SIABCG42* in cutin biosynthesis will be the subject of future studies.

485 **Transport of acyl lipid precursors by *AtABCG* transporters plays an essential** 486 **role in cuticle formation**

487 The current model of cutin synthesis could be greatly consolidated by our finding
488 that the full-size ABCG-transporters *AtABCG32* and *AtABCG42* export 10,16-diOH
489 C16:0-2-glycerol being cutin precursors *in vivo* [5]. That also the half-size ABCG-
490 transporter *AtABCG11* export acyl lipids represent an additional important piece of
491 information despite the remaining uncertainties about the substrate specificity due to
492 the impact of dimerization of the half transporters on the active site. Numerous
493 questions that concern the elucidation of the export specificities *in planta* remain open,
494 i.e. possible redundancies and specificities of the considerable number of other ABCG
495 half transporters involved in cutin formation.

496 In the view of the broad specificity of the ABCG transporters associated with
497 cutin synthesis for substrates containing an oxygenated acyl chain the questions
498 remains open how the range of structures that represent cutin precursors *in vivo* is
499 defined. Whether also unesterified monomers can be polymerized in the apoplast *in*
500 *vivo* will need further investigations as well as the generation of potentially other more
501 complex precursors containing an acyl chain within the epidermal cell. Up to now only
502 the reaction mechanism of one enzyme polymerizing cutin in the apoplast has been
503 characterized [14] that belongs to a large gene family encoding GDSL-type
504 lipases/esterase proteins [61]. Furthermore, the reaction mechanism of members of
505 the BODYGUARD clade, classified as $\alpha\beta$ -hydrolases, which have been suggested to
506 be required for cutin synthesis await characterization [16, 17].

507 Our findings support the model of a strongly diversified cutin biosynthesis
508 pathway, including a high diversity of precursors, transport systems and polymerization
509 mechanisms (Figure 6). Given the importance of cutin for the formation of a functional
510 cuticle redundancies in the cutin biosynthetic pathway make sense, but also functional
511 specializations can be expected.

512 **Conclusions**

513 The long-standing hypothesis that half-size and full-size ABCG transporters
514 export the precursor 10, 16-diOH C16:0-2-glycerol for cutin formation [25, 31] has been
515 experimentally confirmed by using a *N. benthamiana* protoplast system. Thus, direct
516 transport of acyl lipids has been measured for the first time. The identification of
517 unesterified hydroxy acids and dicarboxylic acids as potential substrate will require
518 further research featuring the identity precursors and mechanisms of cutin
519 polymerization and cuticle formation *in vivo*.

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532 **Author contributions**

533 Conceptualization, C.N., M.G.; Methodology, C.E.P., I.G., M.G.; Investigation, C.E.P.,
534 I.G., L.C., D.D.B., A.M., V.Z., B.B.; J.L.; Validation and Formal Analysis, C.E.P., I.G.,
535 M.G., LS; Resources, I.M.S.S., M.H.C., A.A.; Supervision, M.G., C.N.; Writing, C.N.;
536 Funding acquisition, C.N.

537 **Declaration of interests**

538 The authors declare no competing interests.

539 **Figure legends**

540 **Figure 1. *SIABCG42* complements *pec1/atacbg32* mutant phenotypes**

541 **(A)** Transgenic *pec1* lines expressing *SIABCG42* (*pec1 SIABCG42-L1* and *L2*) under
542 the native *PEC1* promoter complement the monomer composition (left) as well as cutin
543 amount (right) in flowers of the *pec1* mutant. Statistically significant differences are
544 indicated by different letters (Two-way ANOVA, Tukey HSD multiple comparison,
545 $P < 0.05$, $n = 5$ replicates). DW, dry weight. The analysis was performed twice with similar
546 results and a representative data set is shown. Selected monomers are presented
547 here. See Figure S2 for complete data set. **(B)** The cuticle permeability is restored in
548 the *pec1 SIABCG42-L1* and *L2* lines. Toluidine blue (TB) staining is shown in flowers
549 of different genotypes. Scale bar = 1 mm. **(C)** The structure of cuticle in petals in the
550 *pec1 SIABCG42-L2* line is similar to WT, as seen in *pec1* complemented with
551 *AtABCG32* (*pec1 AtABCG32*) [22]. Transmission electron micrographs (TEM) of petals
552 in different genotypes highlighting the cuticular proper (CP), cuticular layer (CL) and
553 cell wall (CW) of the cuticular ridges of the petal cuticle. Black arrows indicate electron
554 dense areas in the cuticular layer; white arrows indicate the lack of this electron dense
555 area. Scale bars represent 1 μm . See also Figure S2.

556 **Figure 2. ABCG transporters required for cutin formation export C16:0**
557 **derivatives**

558 **(A, B)** In transport assays using *N. benthamiana* protoplasts, AtABCG32 and
559 SIABCG42 show transport activity with 10,16-diOH C16:0-2-glycerol in contrast to
560 transport-incompetent versions mutated in the Walker A domain (AtABCG32-M and
561 SIABCG42-M). Percentage of initial export across the plasma membrane of
562 protoplasts expressing the empty vector (Control), GFP-AtABCG32, GFP-AtABCG32-
563 M, GFP-SIABCG42, and GFP-SIABCG42-M with different substrates measured after
564 20 min are shown: (A) [³H]-labeled 10, 16-diOH 16:0-2-glycerol; (B) [¹⁴C]-labeled indole
565 acetic acid (IAA) used as unrelated compound for comparison. **(C-F)** Heterologous
566 expression of AtABCG32 and SIABCG42 as well as AtABCG11 in *N. benthamiana*
567 protoplasts reveals export activity across the plasma membrane for 10,16 diOH C16:0
568 as well as ω -OH C16:0, while C16:0 DCA is only exported by AtABCG32 and
569 SIABCG42. Percentage of initial export after 20 min of different substrates across the
570 plasma membrane of protoplasts expressing the empty vector (Control), YFP-
571 AtABCG11, GFP-AtABCG32 and GFP-SIABCG42. Three radiolabeled C16:0
572 substituted acids were used as substrates: (C) 10, 16-diOH 16:0-2-glycerol; (E) ω -OH
573 C16:0; (F) C16:0 dicarboxylic acid (C16:0 DCA). (D) Radiolabeled indole acetic acid
574 (IAA) was used as unrelated compound for comparison. Data represented as mean \pm
575 standard error of the mean; multiple t-tests followed by Holm-Sidak multiple
576 comparisons correction. Letters indicate significance groups; a = $P < 0.001$ and b = $P <$
577 0.05; ns: not significant. See also Figure S3, Figure S4, and Figure S5.

578 **Figure 3. Reduced expression of AtABCG32-homologous genes and fruit cuticle**
579 **formation in *slabcg36/42*-RNAi plants**

580 **(A)** Expression analysis of *SIABCG36* and *SIABCG42* in selected RNAi lines, i.e.
581 *slabcg36/42-a* (*36/42-a*) and *slabcg36/42-b* (*36/42-b*) normalized to wild-type (WT)
582 show significant differences between the RNAi lines in SIABCG42 expression as well
583 as of both genes to WT. Real time-qPCR was performed with two biological replicates
584 for 2 plants belonging to the same T3 line and three technical replicates by sample.
585 Error bars represent SD. Analyses were performed with 10 DPA fruits. Statistically
586 significant differences are indicated by different letters (Two-way ANOVA, Tukey HSD
587 multiple comparison, $P < 0.05$, $n = 4$ replicates). The experiments were repeated twice

588 with similar results. **(B)** and **(C)** Reduced cutinization and altered epidermal cell shape
589 were revealed in fruits of *slabc36/42* plants. **(B)** Reduction of the cutinization of the
590 tomato fruit cuticle as visible by Sudan IV staining. Representative pictures (shown in
591 **C**) were quantified by measuring the Sudan stained area in 5 pictures of 250 μm x 70
592 μm in size. Statistically significant differences between cuticle areas are indicated by
593 different letters (One-way ANOVA, Tukey HSD multiple comparison, $P < 0.05$, $n = 15$
594 pictures). **(C)** Cross sections of the fruit exocarp are shown in light microscopy and
595 transmission electron microscopy (TEM) as well as of the fruit surface in cryo-
596 scanning-electron microscopy (SEM). Two developmental stages are shown: 20 DPA
597 and Red Ripe. Sudan IV staining revealed cuticle and cutinized cells of the first exocarp
598 cell layer. WT, wild type. Transmission electron microscopy (TEM) images showing
599 cuticular layer and epidermal cells structure. EC: epidermal cell, CW: cell wall, CL:
600 Cuticular layer. White bars = 10 μm . Black bars = 50 μm . See also Figure S1 and
601 Figure S6.

602 **Figure 4. Reduced cutin deposition in *slabcg36/42* fruits**

603 The amount of the most abundant and selected minor cutin monomers (left) and the
604 total cutin amount (right) present in isolated fruit cutins were quantified in 20 DPA-old
605 **(A)** and in Red Ripe **(B)** fruits. Experiments were repeated at least twice and a
606 representative set is shown here. Statistically significant differences between amount
607 of cutin monomers and total cutin amounts are indicated by different letters (Two-way
608 ANOVA, Tukey HSD multiple comparison, $P < 0.05$, $n = 4$ replicates). DW, dry weight.
609 **(C)** Esterification levels of the different OH groups in 10,16-diOH C16:0 of cutin from
610 fruits of wild-type (WT) and *slabcg36/42* lines are not different. Data were statistically
611 analyzed by Two-way ANOVA, Tukey HSD multiple comparison, $P < 0.05$, $n = 5$
612 replicates. **(D)** Glycerol content in fruit cutin from the *slabcg36/42-a* plants is reduced.
613 Statistically significant differences are indicated by different letters (Two-way ANOVA,
614 Tukey HSD multiple comparison, $P < 0.05$, $n = 5$ replicates). See also Figure S6.

615 **Figure 5. Wax deposition and water permeances of *slabcg36/42* fruit cuticles**

616 Increased deposition of wax components (left) and total wax (right) of *slabcg36/42*
617 tomato fruits in different stages **(A)** 20 DPA and **(B)** Red Ripe fruits. Statistically
618 significant differences are indicated by different letters (Two-way ANOVA, Tukey HSD

619 multiple comparison, $P < 0.05$, $n = 5$ replicates). **(C)** Only dewaxed fruit cutins of
620 *slabcg36/42* lines harvested at 20 DPA show increased permeability to water. The
621 permeability of waxy and dewaxed cuticles was measured of 20 DPA and Red Ripe
622 (RR) fruits. Student's *t*-test was used for assessing significant differences of wax
623 components and water permeabilities when compared to WT (* = $p < 0.05$).

624 **Figure 6. Schematic diagram of the function of ABCG-transporters in cutin**
625 **formation**

626 Full-size ABCG transporters, ABCG32 (AtABCG32 and its tomato orthologue
627 SlABCG42), as well as half-size transporter AtABCG11 (G11) can export various types
628 of cutin precursor classes across the plasma membrane. Individual substrate
629 specificities for which evidence was presented in this study by transport assays in *N.*
630 *benthamiana* (bold) and by the analysis of loss/gain of function mutants are indicated
631 below the transporters. Hypothesized precursors are marked by a question mark.
632 Whether only esterified or also unesterified forms of oxygenated fatty acid are exported
633 *in vivo* needs to be elucidated. Cutin precursors are exported to the apoplast where
634 cutin is formed by different cutin synthases. The action of the ABCG transporters in
635 cutin formation is highlighted in red. ABCG transporters that were characterized in this
636 study are indicated in orange, other ABCG transporters that may be present, i.e.
637 ABCG13 in petals, are indicated in grey. Blue ovals, CUTIN SYNTHASES of the
638 GDSL-family; green ovals, BODYGUARD isoforms (having a hypothetical role in cutin
639 polymerization); different shades of blue or green symbolize enzymes with potentially
640 different enzymatic activities; cell wall material (CW) is depicted in brown, cutin in black
641 and wax in grey; gradation indicates the complex composition of the cell wall/cuticle
642 continuum.

643

644 **STAR METHODS**

645 **RESOURCE AVAILABILITY**

646 **Lead contact**

647 Further information and requests for resources and reagents should be directed to and
648 will be fulfilled by the Lead contact Christiane Nawrath (christiane.nawrath@unil.ch).

649 **Materials Availability**

650 Plasmids generated in this study will be made available by the Lead contact. [³H]-
651 10,16-diOH C16:0 2-glycerol cannot be shared because of the regulations for shipment
652 of radioactive material.

653 **Data and Code Availability**

654 Original data for the Figures in the paper will be made available upon request by the
655 Lead contact.

656

657 **EXPERIMENTAL MODEL AND SUBJECT DETAILS**

658 **Plant material**

659 *Arabidopsis thaliana* accession Col-0, *Solanum lycopersicum* L. accession Micro-Tom
660 and *Nicotiana benthamiana* were used in this work. The *Arabidopsis thaliana* mutant
661 *pec1-2* was described previously [31]. All investigated genes are listed in Table S1.
662 The primers for the identification of the T-DNA insertion are described in Table S2.

663 **Growth conditions**

664 *A. thaliana* plants were grown on soil in a Percival growth chamber under
665 continuous light (100 mmol. m⁻². s⁻¹) at 20°C and 65% humidity for propagation,
666 transformation, toluidine blue staining and floral cuticle analysis. *N. benthamiana* was
667 grown on soil in a walk-in growth chamber at 63% humidity in a 16 h light/8 h dark cycle
668 and 28 °C and 25°C, respectively. *S. lycopersicum* cv Micro Tom plants were grown on
669 soil in the greenhouse, in which natural day light was supplemented with a sodium
670 vapor bulb (250 μmoles/m²/s) for 16 h (6 am to 10 pm), having a temperature of
671 minimally 25°C for 12 h (6 am to 18 pm), 23°C for 3 h (6 pm to 9 pm) and 21°C for 9 h
672 (9 pm to 6 am).

673 *S. lycopersicum* flowers were labelled at anthesis and fruits collected after 20-25
674 days for the 20 DPA stage and after 50 to 55 days for Red Ripe (RR) stage.

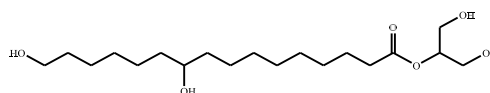
675

676 METHOD DETAILS

677 Chemical synthesis of tritium-labelled 2-mono (10,16- 678 dihydroxyhexadecanoyl)glycerol ($[^3\text{H}]$ -10,16-diOH C16:0 2-glycerol)

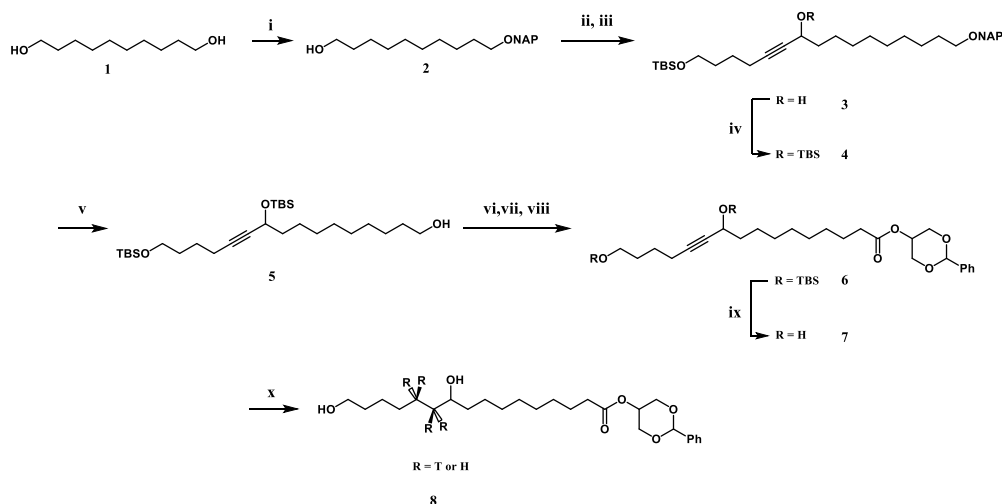
679 $[^3\text{H}]$ -10,16-diOH C16:0 2-glycerol was synthesized from a custom-synthesized
680 precursor that was supplied to Quotion Bioreserch, Cardiff.

681 A) Overview of the chemical synthesis of $[^3\text{H}]$ 682 10,16-diOH C16:0 2-glycerol



683 $[^3\text{H}]$ -10,16-diOH C16:0 2-glycerol was synthesized in 7 steps from 1,10-decanediol **1**
684 as shown in Scheme 1. **2** was prepared by protecting one of the hydroxyl groups of
685 decane-1,10-diol with NAPBr. The other free alcohol was oxidized to the aldehyde by
686 a Dess-Martin oxidation, to which was added the lithium acetylide 6-((tert-
687 butyldimethylsilyl)oxy)hex-1-yne to afford the propargylic alcohol **3**. This alcohol was
688 TBS protected to give triether **4**. The NAP group was removed with DDQ and H₂O to
689 give the primary alcohol **5**. The alcohol was subjected to a step-wise oxidation to the
690 carboxylic acid, via successive Dess-Martin and Pinnick oxidations, which was then
691 reacted with cis-5-hydroxy-2-phenyl-1,3-dioxane by Steglich esterification to form
692 product **6**. Both TBS groups were removed with 20% aq. HF at 0 °C, to afford product
693 **7**. The final product was afforded by reduction of **7** with tritium gas and Pearlman's
694 catalyst. Due to the requirements for dedicated facilities when working with tritium,
695 tritium labelling was performed from precursor **7** by Quotient Bioresearch following a
696 protocol developed in our laboratory for the deuteration of **7**, but substituting deuterium
697 by tritium. HRMS of the tritiated product showed a mixture of isotopically labelled
698 products, in which the number of incorporated tritium atoms ranged from 0 to 4.

699 The radioactivity of **8** prevented its purification by recrystallization, so it was performed
700 by HPLC. Non-isotopically labelled 10,16-diOH C16:0 2-glycerol was used to establish
701 suitable HPLC conditions that prevented the migration of the ester from the secondary
702 to a primary position of glycerol.

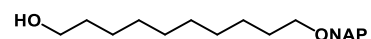


703

704 **Scheme 1.** Synthesis of [³H]-10,16-diOH C_{16:0} 2-glycerol. (i) NaH, 2-
 705 (Bromomethyl)naphthalene, DMF/THF, 22 °C, 62 %; (ii) Dess-Martin periodinane,
 706 CH₂Cl₂, 22 °C; (iii) 6-((*tert*- butyldimethylsilyl)oxy)hex-1-yne, *n*-BuLi, THF, -78 °C, 73
 707 % (over two steps); (iv) TBSCl, imidazole, DMF, 22 °C, 76 %; (v) DDQ, H₂O, CH₂Cl₂,
 708 22 °C, 64 %; (vi) Dess-Martin periodinane, CH₂Cl₂, 22 °C; (vii) NaClO₂, NaHPO₄
 709 (aqueous, pH 3.5), 2-methyl-butene, *t*-BuOH, 22 °C; (viii) *cis*-hydroxy-2-phenyl-1,3-
 710 dioxane, DMAP, EDC-HCl, 22 °C, 87 % (over three steps); (ix) 20 % aq. HF, MeCN, 0
 711 °C, 69 %; (x) T₂, 20 % Pd(OH)₂/C, THF.

712 B) Experimental procedures for the chemical synthesis of precursor 7

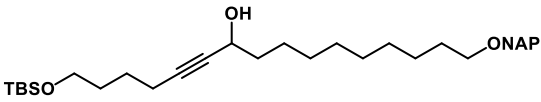
713 10-(Naphthalene-2-ylmethoxy)decan-1-ol (2)



714 To a suspension of NaH (60% in oil, 3.44 g, 0.086 mol) in dry DMF (40 ml) at 0 °C
 715 under an atmosphere of argon was added slowly a solution of 1,10-decanediol (15.00
 716 g, 0.086 mol) in a mixture of THF and DMF (30 and 40 ml respectively). The resulting
 717 mixture was let to warm slowly to 20 °C. After 2 h, a solution of NAPBr (9.52 g, 0.043
 718 mol) in dry DMF (30 ml) was added dropwise. The reaction was stirred for 16 h and
 719 excess reagent was quenched by addition of ice until bubbling ceased. The mixture
 720 was extracted with Et₂O (3 × 200 ml). The combined organic phases were washed with
 721 sat. aq. NaCl (200 ml), dried with MgSO₄, filtered, concentrated and purified by flash
 722 chromatography (EtOAc/heptane 1:4) affording **2** as a white solid (8.44 g, 62%). **R_f**
 723 (EtOAc/heptane 1:4) = 0.16; **mp**: 60–62 °C; **¹H NMR** (400 MHz, CDCl₃) δ 7.86–7.80
 724 (m, 3H), 7.78 (s, 1H), 7.51–7.43 (m, 3H), 4.67 (s, 2H), 3.63 (t, *J* = 6.6 Hz, 2H), 3.51 (t,
 725 *J* = 6.7 Hz, 2H), 1.69–1.60 (m, 2H), 1.60–1.51 (m, 2H), 1.44–1.23 (m, 12H); **¹³C NMR**
 726 (101 MHz, CDCl₃) δ 136.4, 133.4, 133.1, 128.2, 128.0, 127.8, 126.4, 126.2, 125.9,

727 125.9, 73.1, 70.7, 63.2, 32.9, 29.9, 29.7, 29.7, 29.6, 29.5, 26.3, 25.9; **HRMS (MALDI+)**
728 C₂₁H₃₀O₂, m/z [M+Na⁺] 337.2138, found 337.2140.

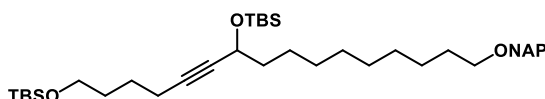
729 1-((*tert*-Butyldimethylsilyl)oxy)-16-(naphthalene-2-ylmethoxy)hexadec-5-yn-7-ol (**3**)

730 To a mixture of DMP (6.63 g, 15.63 mmol) in
731 CH₂Cl₂ (25 ml) was added slowly a solution 
732 of **2** (3.78 g, 12.02 mmol) in CH₂Cl₂ (20 ml). The resulting mixture was stirred for 1.5 h
733 and poured into an aqueous solution of Na₂S₂O₃ (35 g in 200 ml). The mixture was
734 stirred vigorously for 5 min and extracted with Et₂O (200 ml). The organic phase was
735 washed with sat. aq. NaHCO₃ (200 ml), dried with MgSO₄ and concentrated, and used
736 in the subsequent reaction without further purification.

737 To a solution of **2** (3.32 g, 15.64 mmol) in THF (15 ml) at -78 °C under an atmosphere
738 of nitrogen was added dropwise *n*-BuLi (6.7 ml, 2.7 M in pentane, 18.05 mmol). The
739 resulting mixture was allowed to warm slowly to 20 °C and a solution of the crude
740 aldehyde (3.760 g, 12.0 mmol) in dry THF (15 ml) was added. The resulting mixture
741 was stirred for 16 h, poured into sat. aq. NH₄Cl (100 ml) and extracted with CH₂Cl₂ (3
742 × 100 ml). The combined organic phases were dried with MgSO₄, filtered, concentrated
743 and purified by flash chromatography (EtOAc/heptane 1:9) affording **3** as a yellow oil
744 (4.12 g, 65%); *R_f* (EtOAc/heptane 1:9) = 0.17; **¹H NMR** (400 MHz, CDCl₃) δ 7.86–7.80
745 (m, 3H), 7.78 (s, 1H), 7.51–7.45 (m, 3H), 4.67 (s, 2H), 4.34 (tt, *J* = 8.9, 4.1 Hz, 1H),
746 3.62 (t, *J* = 6.0 Hz, 2H), 3.51 (t, *J* = 6.7 Hz, 2H), 2.23 (td, *J* = 6.7, 1.7 Hz, 2H), 1.72–
747 1.53 (m, 8H), 1.48–1.25 (m, 12H), 0.91 (s, 9H), 0.05 (s, 6H); **¹³C NMR** (101 MHz,
748 CDCl₃) δ 136.36, 133.44, 133.08, 128.24, 127.99, 127.82, 126.40, 126.16, 125.91,
749 125.88, 85.41, 81.69, 73.09, 70.67, 62.90, 62.79, 38.35, 32.06, 29.94, 29.66, 29.64,
750 29.61, 29.42, 26.35, 26.10 (3C), 25.36, 25.27, 18.64, 18.48, -5.14 (2C); **HRMS**
751 **(MALDI+)** C₃₃H₅₂O₃Si, m/z [M+H⁺] 525.3578, found 525.3593.

752 1,7-Bis((*tert*-Butyldimethylsilyl)oxy)-16-(naphthalen-2-ylmethoxy)hexadec-5-yne (**4**)

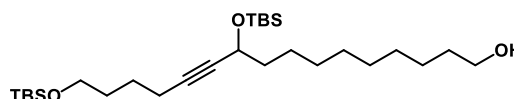
753 To a solution of **4** (4.12 g, 7.86 mmol) in dry
754 DMF (20 ml) under an atmosphere of
755 nitrogen was added imidazole (0.803 g, 11.80
756 mmol) and TBSCl (1.54 g, 10.22 mmol). The reaction mixture was stirred for 18 h,
757 poured into sat. aq. NH₄Cl (100 ml) and was extracted with CH₂Cl₂ (3 × 100 ml). The
758 combined organic phases were dried with MgSO₄, filtered, concentrated and purified



759 by flash chromatography (EtOAc/heptane 1:19) affording **4** as a colourless oil (3.54 g,
760 71%); R_f (EtOAc/heptane 1:19) = 0.33; $^1\text{H NMR}$ (400 MHz, CDCl_3) δ 7.85–7.80 (m,
761 3H), 7.78 (s, 1H), 7.50–7.43 (m, 3H), 4.67 (s, 2H), 4.31 (tt, J = 6.6, 1.9 Hz, 1H), 3.61 (t,
762 J = 6.0 Hz, 2H), 3.51 (t, J = 6.6 Hz, 2H), 2.21 (td, J = 6.9, 1.9 Hz, 2H), 1.68–1.50 (m,
763 8H), 1.37 (m, 4H), 1.28 (d, J = 2.9 Hz, 8H), 0.90 (s, 9H), 0.89 (s, 9H), 0.12 (s, 2H), 0.10
764 (s, 3H), 0.04 (s, 6H); $^{13}\text{C NMR}$ (101 MHz, CDCl_3) δ 136.36, 133.44, 133.08, 128.22,
765 127.98, 127.81, 126.38, 126.14, 125.89, 125.86, 84.27, 82.37, 73.08, 70.67, 63.36,
766 62.78, 39.17, 32.09, 29.95, 29.69, 29.67, 29.62, 29.42, 26.36, 26.10 (3C), 26.01 (3C),
767 25.49, 25.31, 18.65, 18.46, 18.43, -4.28, -4.81, -5.16 (2C); **HRMS (MALDI+)**
768 $\text{C}_{39}\text{H}_{66}\text{O}_3\text{Si}_2$, m/z $[\text{M}+\text{Na}^+]$ 661.4443, found 661.4445.

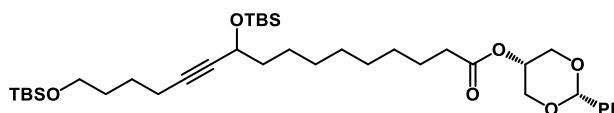
769 10,16-Bis((*tert*-butyldimethylsilyl)oxy)hexadec-11-yn-1-ol (**5**)

770 To a solution of **4** (3.47 g, 5.431 mmol) in
771 CH_2Cl_2 (85 ml) was added DDQ (1.85 g, 8.15
772 mmol) and H_2O (20 ml). After 16 h, the mixture was poured into sat. aq. NaHCO_3 (100
773 ml), and was extracted with CH_2Cl_2 (3 \times 100 ml). The combined organic phases were
774 dried with MgSO_4 , filtered, concentrated and purified by flash chromatography
775 (EtOAc/heptane 1:9) affording **5** as a colourless oil (1.95 g, 72%); R_f (EtOAc/heptane
776 1:4) = 0.29; $^1\text{H NMR}$ (400 MHz, CDCl_3) δ 4.31 (tt, J = 6.6, 2.0 Hz, 1H), 3.66–3.58 (m,
777 4H), 2.21 (td, J = 6.9, 2.0 Hz, 2H), 1.67–1.50 (m, 8H), 1.45–1.24 (m, 12H), 0.90 (s, 8H),
778 0.89 (s, 9H), 0.11 (s, 3H), 0.09 (s, 3H), 0.04 (s, 6H); $^{13}\text{C NMR}$ (101 MHz, CDCl_3) δ
779 84.30, 82.36, 63.36, 63.23, 62.81, 39.17, 32.96, 32.11, 29.68, 29.64, 29.56, 29.41,
780 26.11 (3C), 26.02 (3C), 25.88, 25.48, 25.32, 18.67, 18.48, 18.45, -4.28, -4.81, -5.15
781 (2C); **HRMS (MALDI+)** $\text{C}_{28}\text{H}_{58}\text{O}_3\text{Si}_2$, m/z $[\text{M}+\text{Na}^+]$ 521.3817, found 521.3825.



782 *cis*-2-Phenyl-1,3-dioxan-5-yl 10,16-bis((*tert*-butyldimethylsilyl)oxy)hexadec-11-ynoate
783 (**6**)

784 To a mixture of DMP (1.25 g, 2.95
785 mmol) in CH_2Cl_2 (30 ml) was added
786 slowly a solution of **5** (1.13 g, 2.27
787 mmol) in CH_2Cl_2 (30 ml). The resulting mixture was stirred for 2.5 h and poured into a
788 solution of $\text{Na}_2\text{S}_2\text{O}_3$ (45 g) in sat. aq. NaHCO_3 (250 ml). The mixture was stirred
789 vigorously for 5 min and then extracted with Et_2O (200 ml). The organic phase was
790 washed with sat. aq. NaHCO_3 (200 ml), dried with MgSO_4 , concentrated and used in
791 the subsequent reaction without further purification.

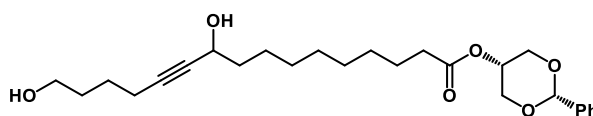


792 To a solution of the crude aldehyde (1.24 g) in *t*-BuOH (100 ml) was added 2-methyl-
793 2-butene (3.180 g, 4.8 ml, 45.34 mmol) and an aqueous solution (27 ml) of NaH₂PO₄
794 (2.18 g, 18.14 mmol) and NaClO₂ (0.267 g, 2.95 mmol). After 15 h, a buffer aqueous
795 solution of NaH₂PO₄ was added (0.66 M, 100 ml) and the mixture was extracted with
796 CH₂Cl₂ (3 × 150 ml). The combined organic phases were washed with sat. aq. NaCl
797 (200 ml), dried with Na₂SO₄, filtered, concentrated, and used in the subsequent
798 reaction without further purification.

799 To a solution of the crude carboxylic acid (1.47 g) in dry CH₂Cl₂ (100 ml) under an
800 atmosphere of nitrogen was added *cis*-5-hydroxy-2-phenyl-1,3-dioxane (0.531 g, 2.946
801 mmol), DMAP (0.472 g, 3.852 mmol) and EDCI (0.660 g, 3.399 mmol). After 15 h, the
802 reaction mixture was concentrated, taken on silica and purified by flash
803 chromatography (EtOAc/hexane 1:9), affording **17** as a transparent oil (0.609 g, 40%);
804 **R_f** (EtOAc/heptane 1:9) = 0.22; **¹H NMR** (400 MHz, CDCl₃) δ 7.55–7.47 (m, 2H), 7.41–
805 7.32 (m, 3H), 5.56 (s, 1H), 4.74–4.69 (m, 1H), 4.34–4.13 (m, 5H), 3.61 (t, *J* = 6.1 Hz,
806 2H), 2.43 (t, *J* = 7.6 Hz, 2H), 2.21 (td, *J* = 6.8, 1.8 Hz, 2H), 1.73–1.50 (m, 8H), 1.47–
807 1.21 (m, 10H), 0.90 (s, 9H), 0.89 (s, 9H), 0.11 (s, 3H), 0.10 (s, 3H), 0.06–0.03 (m, 6H);
808 **¹³C NMR** (101 MHz, CDCl₃) δ 173.99, 137.97, 129.19, 128.41 (2C), 126.16 (2C),
809 101.35, 84.27, 82.33, 69.26 (2C), 65.83, 63.33, 62.78, 39.14, 34.52, 32.08, 29.53,
810 29.37 (2C), 29.24, 26.08 (3C), 26.00 (3C), 25.47, 25.29, 25.06, 18.64, 18.45, 18.42, -
811 4.30, -4.82, -5.17 (2C); **HRMS (MALDI+)** C₃₈H₆₆O₆Si₂, *m/z* [M+Na⁺] 697.4290, found
812 697.4243.

813 *cis*-2-Phenyl-1,3-dioxan-5-yl 10,16-dihydroxyhexadec-11-ynoate (**7**)

814 To a solution of **6** (0.975 g, 1.445 mmol)
815 in MeCN (145 ml) at 0 °C was added 20%
816 aq. HF (6 ml, 69 mmol). The resulting



817 mixture was stirred at 0 °C for 4 h. TMSOEt (33 ml) was added, and the mixture was
818 stirred for 30 minutes, poured into sat. aq. NH₄Cl (200 ml) and extracted with CH₂Cl₂
819 (3 × 200 ml). The combined organic phases were dried with MgSO₄, filtered,
820 concentrated and purified by flash chromatography (EtOAc/hexane 6:4), affording **7** as
821 a white solid (0.354 g, 55%); **R_f** (EtOAc/heptane 6:4) = 0.16; **mp**: 50–52 °C; **¹H NMR**
822 (400 MHz, CDCl₃) δ 7.58–7.46 (m, 2H), 7.45–7.31 (m, 3H), 5.56 (s, 1H), 4.72 (s, 1H),
823 4.38–4.25 (m, 3H), 4.17 (d, *J* = 13.2, 2H), 3.65 (t, *J* = 6.3 Hz, 2H), 2.43 (t, *J* = 7.5 Hz,
824 2H), 2.25 (td, *J* = 6.8, 2.0 Hz, 2H), 1.74–1.54 (m, 10H), 1.50–1.24 (m, 10H); **¹³C NMR**

825 (101 MHz, CDCl₃) δ 174.04, 137.94, 129.23, 128.44 (2C), 126.17 (2C), 101.38, 85.16,
826 81.89, 69.28 (2C), 65.87, 62.84, 62.47, 38.27, 34.52, 31.91, 29.43, 29.29, 29.24, 29.19,
827 25.26, 25.06, 25.05, 18.62; **HRMS (MALDI+)** C₂₆H₃₈O₆, m/z [M+Na⁺] 469.2561, found
828 469.2563.

829 1,3-Dihydroxypropan-2-yl 10,16-dihydroxyhexadecanoate-11,11,12,12-*t*₄ (**8**)

830 The final product **8** (for chemical structure: see Scheme 1) was synthesized by Quotient
831 Bioresearch by adapting a protocol developed in our laboratory for the deuteration of
832 **7**, shown below. This was done by substituting deuterium by tritium, as well as
833 modifying the method of purification. The radioactivity of **8** prevented its purification by
834 recrystallization, so it was instead purified by HPLC (Column: Zorbax Eclipse XDB-C8
835 5 μm 150 x 4.6 mm. Elution was carried out combining eluent A (5 mM NH₄Ac in H₂O)
836 and eluent B (5 mM NH₄Ac in 90% aq. MeCN) in the following fashion: 20% B for 1
837 minute, gradual increment of B to 30% for 4 minutes, gradual increment of B to 40%
838 for 15 minutes, then sudden reduction of B to 20% and hold for 3 minutes. Total run
839 time: 23 min). **TOF MS ES⁻** C₁₉H₃₄T₄O₆, m/z [M+FA-H⁺] 415.2971, found 415.2947.
840 **Radiochemical purity (RCP):** 91.6 % **Specific activity:** 86 Ci/mmol

841 **C) Procedure for the deuteration of 7 supplied to Quotient Bioresearch**

842 1,3-Dihydroxypropan-2-yl 10,16-dihydroxyhexadecanoate-11,11,12,12-*d*₄

843 To a solution of **7** (0.169 g, 0.378 mmol) in dry THF (20 ml) under an atmosphere of
844 nitrogen was added 20% Pd(OH)₂/C (40 mg). A deuterium atmosphere was installed
845 by bubbling D₂ through the solution for 5 minutes. The reaction mixture was stirred
846 under a D₂ atmosphere for 15 h filtered through Celite, concentrated and recrystallized
847 from EtOAc and heptane, affording **9** as a white solid (96 mg, 69%); **mp:** 56–58 °C; **¹H**
848 **NMR** (400 MHz, MeOD) δ 4.94–4.88 (m, 1H), 3.70 (dd, *J* = 12.4, 5.3 Hz, 2H), 3.65 (dd,
849 *J* = 12.0, 6.0 Hz, 2H), 3.55 (t, *J* = 6.6 Hz, 2H), 3.52–3.48 (m, 1H), 2.38 (t, *J* = 7.4 Hz,
850 2H), 1.68–1.59 (m, 2H), 1.58–1.49 (m, 2H), 1.48–1.24 (m, 16H); **¹³C NMR** (101 MHz,
851 MeOD) δ 175.34, 76.53, 62.99, 61.70 (2C), 38.38, 35.15, 33.64, 30.79, 30.59, 30.37,
852 30.17, 26.78, 25.98; **HRMS (MALDI+)** C₁₉H₃₄D₄O₆, m/z [M+Na⁺] 389.2812, found
853 389.2827.

854 **Isolation of *N. benthamiana* protoplasts**

855 Protoplasts were prepared as previously described by Geisler et al., 2005 [62]. Shortly,
856 Agrobacterium-infiltrated leaves of four-weeks-old *N. benthamiana* plants were

857 abraded with abrasive paper P500 and the abaxial part was put in contact with ½ MCP
858 medium (500 mM D-Sorbitol, 10 mM 2-(N-morpholino) ethanesulfonic acid (MES), 1
859 mM CaCl₂, pH 5.6). Incubated leaves in digestion buffer (MCP with 1% cellulose YC,
860 1% pectolyase Y23, 1% bovine serum albumin (BSA)) for 90 min at 30°C. Solution was
861 filtered through a 320-mesh stainless steel filter and protoplasts were purified using
862 MCP medium with 100% Percoll pH 6 cushion at the bottom of the tube. Tubes were
863 centrifugated for 10 min at 4°C at 1500 rpm, no brake. Protoplasts resuspended in up
864 to 5 mL of 100% Percoll pH 6. A gradient was built from bottom to top by overlaying
865 carefully: protoplasts in 100% Percoll pH 6, 1 Vol. Percoll 25% (in CP), and 5 mL MCP
866 and centrifuged at 1200 rpm, 10 min, 4°C, no brake. Protoplasts were recovered from
867 the Percoll 25%/MCP interphase.

868 **Generation of constructs**

869 All primers that are mentioned in the following description of the cloning strategies are
870 listed in Table S2. Constructs for the expression of SIABCG42 in *A. thaliana*: Genomic
871 DNA from *S. lycopersicum* cv Micro Tom was isolated by 500 mg of grinded young
872 leaves, extraction with CTAB buffer (1.4 M NaCl, 100 mM Tris– HCl pH 8, 20 mM EDTA
873 pH 7.8, 2% CTAB and 0.2% 2-mercaptoethanol) and a subsequent purification using
874 phenol/chloroform/isoamyl alcohol (25:24:1). The 8209 bp-long *SIABCG42*
875 (*Solyc06g065670*) gene was amplified from genomic this DNA using the primers
876 SIABCG42 attB1 F and SIABCG42 attB2 R, and recombined into pDONR 207 to
877 generate *pENTRY L1-SIABCG42-L2*. To generate *pAtABCG32::GFP-SIABCG42*, the
878 CaMV35S promoter was removed from pMDC43 [63] by restriction enzyme digestion
879 with HindIII and KpnI. A 2043 bp-long fragment of the native *ABCG32/PEC1* promoter
880 was amplified using the primers pAtABCG32+HindIII F and pAtABCG32+KpnI R and
881 cloned into pMDC43, creating the plasmid pMDC43_ *pABCG32::GFP*. *pENTRY L1-*
882 *SIABCG42-L2* was recombined with the destination vector *pAtABCG32::GFP* giving the
883 final construct *pAtABCG32::GFP-SIABCG42* for the expression of a SIABCG42 fusion
884 protein having eGFP at the N-terminus. *pAtABCG32::GFP* was used as control.
885 Constructs were transformed in *Agrobacterium tumefaciens* (GV3101mp90) and then
886 into *Arabidopsis thaliana* accession *pec1-2* using the floral dip method [64]. Shortly,
887 inflorescences were dipped into a 5% sucrose/0.05% Silwet L-77 solution containing
888 *Agrobacterium* for 30 s and kept under high humidity for 24 h. Transformed seeds were

889 selected based on their resistance to 150 $\mu\text{g mL}^{-1}$ of hygromycin on $\frac{1}{2}$ Murashige and
890 Skoog (MS) medium.

891 Constructs for the heterologous expression of the ABCG transporters in *N.*
892 *benthamiana*: The plasmid for the expression of YFP-AtABCG11 under the control of
893 the CaMV 35S promoter (pEARLYGATE_p35S::YFP-WBC11) was kindly provided by
894 Lacey Samuels [25]. The pMDC43_p35S::GFP-AtABCG32 plasmid for the expression
895 eGFP-AtABCG32 was previously described [31]. To express GFP-SIABCG42 under
896 the control of the CaMV35S promoter, the pENTRY L1-SIABCG42-L2 was recombined
897 with pMDC43 resulting in pMDC43_p35S::GFP-SIABCG42. The mutated versions of
898 pMDC43_p35S::GFP-AtABCG32 and pMDC43_p35S::GFP-SIABCG42 for the
899 expression of GFP-AtABCG32-M and GFP-SIABCG42-M were generated using the Q5
900 (r) Site-Directed Mutagenesis kit (NEB) following the manufacturer instructions and
901 were named pMDC43_p35S::GFP-AtABCG32_M and pMDC43_p35S::GFP-
902 SIABCG42_M, respectively. Primers were designed by using the NEBaseChanger™
903 tool for changing two nucleotides in the AtABCG32 and SIABCG42 sequences inducing
904 K174L and K165L mutations, respectively, in the Walker A1 motif of the transporters.
905 Constructs were transformed in *Agrobacterium tumefaciens* (GV3101 mp90). Leaves
906 of 4-weeks-old *N. benthamiana* plants were transfected following the protocol
907 described by Leuzinger et al., 2013 [65] : An *Agrobacterium* solution was prepared at
908 a final OD₆₀₀ of 0.8 for construct diluted in the AS medium (1M MES-KOH, pH 5.6; 3M
909 MgCl₂, 150mM acetosyringone). Co-infiltration with a vector encoding the silencing
910 suppressor P19 from the tomato bushy stunt virus [66] was performed for high level
911 target gene expression (final OD₆₀₀ of 0.1). For co-localization with PIP2A-mCherry
912 construct pm-rk (ARBC stock number CD3-1007) was transformed in *Agrobacterium*
913 *tumefaciens* (GV3101 mp90) used at a final OD₆₀₀ of 0.4-0.5 [43].

914 Constructs for RNAi-dependent gene silencing of SIABCG36 and SIABCG42 tomato:
915 *S. lycopersicum* L. SIABCG36 (*Solyc05g018510*) and SIABCG42 (*Solyc06g065670*)
916 gene sequences were obtained from the annotation of the tomato genome (ITAG v2.4).
917 Silencing fragments were designed in sequences encoding the transmembrane
918 domains having for weak sequence homology to other ABCG genes by using the SGN
919 VIGS tool (<https://vigs.solgenomics.net/>). Fragments were amplified from *S.*
920 *lycopersicum* cv “Micro-Tom” leaf cDNA that was prepared as described below (gene
921 expression analysis). pENTRY L1-fragSIABCG36-L2 was generated by cloning the 224

922 bp-fragment of *Solyc05g018510* resulting from a PCR reaction using the primers
923 fragSIABCG36 F and fragSIABCG36 R into pDONR207. *pENTRY L1-fragSIABCG42-*
924 *L2* was generated by cloning the 234bp fragment of the *Solyc06g065670* gene, that
925 was generated by PCR reaction using the primers fragSIABCG42 F and fragSIABCG42
926 F in the pDONR207 vector. Both pENTRY plasmids were recombined with the
927 destination vector pK7GWIWG2 [67] resulting in pK7GWIWG2-fragSIABCG36 and
928 pK7GWIWG2-fragSIABCG42. Constructs were transformed in *Agrobacterium*
929 *tumefaciens* (GV3101).

930 Transformation of *S. lycopersicum* cv Micro Tom was performed as described by
931 Cardenas et al., 2019 [68]. Shortly, cotyledon explants were excised from 7-day-old *in*
932 *vitro* grown tomato seedlings, and placed on co-cultivation medium (3% Glucose,
933 0.02% KH₂PO₄, 75 µg/mL DTT, 2µg/mL Zeatin, 1µg/mL IAA, 100µM acetosyringone)
934 plus 3% GelRite and preincubated for 24 h at room temperature in the dark. Co-
935 cultivation of the explants with the transformed *A. tumefaciens* strains (OD₆₀₀ = 0.3) was
936 carried out for 48 h under dark conditions in liquid co-cultivation medium. Explants were
937 transferred to shoot induction medium: MS medium containing dithiothreitol (DTT - 75
938 µg mL⁻¹), Kanamycin (50 µg mL⁻¹), Zeatin (2 µg mL⁻¹), indole-3-acetic acid (IAA - 1 µg
939 mL⁻¹), and Timentin (5000 µg mL⁻¹) for 2 to 3 weeks. Timentin was used to remove
940 *Agrobacterium* from the explants. Once the explants developed callus, they were
941 transferred to the shoot elongation medium containing DTT (75 µg mL⁻¹), Kanamycin
942 (50 µg mL⁻¹), Zeatin (1 µg mL⁻¹), Zeatin riboside (1 µg mL⁻¹), IAA (1 µg mL⁻¹), and
943 Timentin (250 µg mL⁻¹). Subsequently, well-developed shoots were excised and
944 transferred to rooting medium containing Kanamycin (50 µg mL⁻¹), and Timentin (250
945 µg mL⁻¹). After 3-4 weeks, plantlets with roots were transplanted to sterile soil and
946 covered to conserve humidity for a week when they were finally transferred to
947 greenhouse conditions (16 h light/24°C and 8 h dark/20°C). Transgenic lines were
948 selected by qPCR. Plants in the T3 generation were used for further analysis.

949 **Gene expression analysis**

950 Quantitative real time PCR was performed with three biological replicates from several
951 tomato plants ($n \geq 4$) for each genotype. RNA isolation was performed from two 0.5 cm
952 exocarp discs for each sample using the ReliaPrep™ RNA Miniprep Systems
953 (Promega) following the guidelines of the manufacturer. RNA was reverse transcribed
954 using the Moloney Murine Leukemia Virus Reverse Transcriptase RNase H

955 Minus, *Point Mutant* (M-MLV RT(H-)) (Promega). Quantitative real-time PCR was
956 performed using 200 nM of the appropriated primers and the Select Master Mix
957 (Applied Biosystems). Using the QuantStudio™ 3 Real-Time PCR System (Applied
958 Biosystems) with the following parameters: UDG Activation at 50°C for 2 min,
959 denaturation at 95°C for 2 min, 40 amplification cycles with a denaturation step at 95°C
960 for 15 s, an annealing step at 60°C for 30 s and an amplification step at 72°C for 30 s.
961 Data acquisition and analysis were done using the QuantStudio™ Software V1.3
962 (Applied Biosystems). *TIP41* (SGN-U321250) based on Expósito-Rodríguez et al.
963 (2008) [69] was used as reference gene to calculate relative transcript expression using
964 gene expression analysis. Primers for measuring silencing annealed outside the region
965 of the fragment used for silencing to assure that only the endogenous gene is being
966 measured. All primers are listed in Table S1B.

967 **Transport assays**

968 Transport assays were performed as previously described by Geisler et al., 2005 [62].
969 Leaves of 4-weeks-old *N. benthamiana* plants were agroinfiltrated with the following
970 constructs: *35S::YFP-AtABCG11*, *35S::GFP-AtABCG32*, *35S::GFP-SIABCG42*, and
971 the mutated versions *35S::GFP-AtABCG32-M*, *35S::GFP-SIABCG42-M*. The *P19*
972 silencing inhibitor (control) was always co-infiltrated. After four days protoplasts were
973 prepared. Two radiolabelled substrates, i.e. one [³H]-labelled and one [¹⁴C]-labelled
974 were added at this stage: [³H]-10,16-diOH C16:0 2-glycerol and [¹⁴C]- ω -OH C16:0,
975 [¹⁴C]-C16:0 DCA or [¹⁴C]-indole-3-acetic acid (IAA; control) were added to the
976 protoplast to yield a final concentration of 1 μ M each. The mixture was incubated on
977 ice for 15 min for substrate intake. External radioactivity was removed by separating
978 protoplasts using a 50%/25%/5% Percoll gradient built from bottom to top: protoplasts
979 in Percoll pH 6, 1 Volume, 1 Vol. Percoll 25%, 5 ml Percoll 5%. Protoplasts were
980 recovered from the 25%/5% Percoll interphase. For the export experiment, protoplasts
981 were diluted 1:3 with Betain buffer and incubated at 25°C. Aliquots of 70 μ L were taken
982 at 0, 5, 10, 15, and 20 min. Aliquots were centrifuged in a silicon oil gradient (50 μ L
983 33% Percoll/ 200 μ L silicon oil AR200) for 30 s at 9000 rpm, and supernatants were
984 objected to scintillation counting. Indicated relative uptake was calculated as the
985 radioactivity normalized to the first time point (0 min). Experiment were repeated at
986 least four times, and data are presented as means with the standard error of the mean
987 (SEM) of all technical replicates.

988 **Microscopy studies**

989 *Confocal imaging:*

990 Leaf discs of *N. benthamiana* were observed 2 or 3 days after agroinfiltration at the
991 laser scanning confocal microscope, Zeiss LSM880 (Carl Zeiss, Germany) with setting
992 parameters for the observation of YFP (λ_{ex} : 516; λ_{em} : 529), GFP (λ_{ex} : 475; λ_{em} : 503),
993 mCherry (λ_{ex} : 587; λ_{em} : 610) and propidium iodide (PI) (λ_{ex} : 535; λ_{em} : 617) or with the
994 Leica SP5 (Leica, Germany) with the setting parameters for GFP (λ_{ex} : 488; λ_{em} : 505-
995 530) and for PI (λ_{ex} : 488; λ_{em} : 610 – 620). Leaf discs were incubated for 20 min in PI
996 (20 μ g/ml) and washed 10 min in water before observation to visualize the cell wall.

997 *Light microscopy:* Three tomato fruits of each line and developmental stage were
998 harvested and small equatorial pericarp pieces of each fruit were fixed in a
999 formaldehyde, acetic acid and ethanol solution (FAA solution, 1:1:18). Serial
1000 dehydration steps were performed for 1h each in ethanol (50% to 100%). Pieces were
1001 embedded in the commercial resin Technovit 7100 following the manufacturer
1002 instructions. Samples were cross-sectioned into 8 μ m thick slices using a Leica
1003 (Wetzlar, Germany) microtome (RM2125). Sudan IV was used to distinctively stain the
1004 cuticle using the protocol described by Buda et al. (2009) [70]. Sudan IV stock solution
1005 (0.1% w/v in isopropyl alcohol) was diluted 3:2 with distilled water, mixed well, allowed
1006 to sit at room temperature for 30 min, and filtered through a Whatman filter paper to
1007 remove precipitates. Sections were incubated in the Sudan IV solution for 10 min, and
1008 subsequently rinsed first with 50% isopropyl alcohol and distilled water. Slices were
1009 mounted in distilled water with a cover slip, and viewed immediately. A minimum of five
1010 slices per sample were inspected under light microscope (DM5500, Carl Zeiss).
1011 Microphotographs were taken with a Nikon camera (DXM1200) coupled to the light
1012 microscope. Cuticle area was estimated from a minimum of 15 measurements using
1013 an image capture analysis program (Fiji v 2.0.0) using a square area of 250 x 70 μ m
1014 [71].

1015 *Transmission electron microscopy (TEM):* Fully open flowers of *A. thaliana* and 1.2 mm
1016 discs of the tomato fruits were fixed 2.5% glutaraldehyde solution in 0.1M phosphate
1017 buffer pH 7.4 (PB) for 2 h at room temperature (RT). They were rinsed three times for
1018 5 min in PB buffer and post-fixed in a fresh mixture of osmium tetroxide 1% (EMS) with
1019 1.5% potassium ferrocyanide in PB buffer for 2 h at RT. The samples were then washed
1020 two times in distilled water and dehydrated in acetone solution at graded concentrations

1021 (30%–40 min; 50%–40 min; 70%–40 min; 100%–3 x 1 h). The infiltration with Spurr
1022 resin at 33% in acetone for 12 h, Spurr 66% in acetone for 12 h, Spurr 100% for 1h,
1023 Spurr 100% twice for 8 h. The *Arabidopsis* flowers were then dissected in the resin
1024 under a binocular. Petals discs were placed in molds filled with resin and then
1025 polymerized for 48 h at 60°C. Ultrathin sections of 60 nm thick were cut on a Leica
1026 Ultracut (Leica Mikrosysteme GmbH), picked up on a copper slot grid 2x1 mm (EMS)
1027 coated with a polystyrene film (Sigma Aldrich). Sections were post-stained with 4%
1028 uranyl acetate in distilled water for 10 min, rinsed several times with distilled water
1029 followed by Reynolds lead citrate in water for 10 min and rinsed several times with
1030 distilled water. Micrographs were taken with a transmission electron microscope FEI
1031 CM100 (FEI, Eindhoven, The Netherlands) at an acceleration voltage of 80 kV with a
1032 TVIPS TemCamF416 digital camera (TVIPS GmbH) using the software EM-MENU 4.0
1033 (TVIPS GmbH).

1034 *Scanning electron microscopy*: tomato fruit pieces were investigated by cryo-scanning
1035 electron microscopy directly without solvent treatment. For cryo-scanning electron
1036 microscopy, we used a Quorum system PP3000T (Quorum Technologies Ltd) attached
1037 to a Quanta 250 FEI scanning electron microscope (FEI Company). The tomato pieces
1038 were mounted on aluminum stubs using a mixture of Tissue-Tek and colloidal graphite,
1039 frozen in nitrogen slush at –210°C and then transferred to the preparation chamber of
1040 the Quorum system. The sample was freeze-dried at –80°C for 10 min, and then
1041 sputter-coated with platinum at 10 mA for 25 s. After transfer on the cryostage at
1042 –140°C in the scanning electron microscope, imaging was performed, at the tomato
1043 equatorial area, at 10 keV using an Everhart-Thornley electron detector.

1044 Scanning and transmission electron micrographs were taken from at least 3 different
1045 fruits of for minimally two different plants per line.

1046 **Isolation of tomato fruit cuticles**

1047 Cuticles were isolated from the fruits of wild-type and transgenic lines by incubating
1048 tomato halves in 2% pectinase and 0.2% cellulase in 0.02 M citrate buffer pH 3.7.
1049 Incubation was performed at 37°C for at least 2 weeks with regular changes of the
1050 enzymatic mixture [72].

1051 **Chemical analyses**

1052 *Cutin analysis:* The protocol for the determination of ester-bond lipids previously
1053 described in Li-Beisson et al., 2013 [73] was adapted. For the analysis of floral cutin of
1054 Arabidopsis 20 fully open flowers from four to six plants were collected. For the analysis
1055 tomato cutin four discs of isolated cuticles (1 cm diameter) from 8 tomatoes coming
1056 from two different plants per line were used. Four samples per line and per stage were
1057 analyzed. All plant samples were delipidated by an initial incubation in 7 mL isopropanol
1058 /0.01% butylated hydroxytoluene (BHT) for 10 min at 85°C and then subsequent
1059 washes in methanol:chloroform with 0.01% BHT (1:2, 1:1, 2:1, respectively), finishing
1060 by 24 h in 100% methanol/0.01% BHT. Samples were then dry under a gentle stream
1061 of nitrogen at 30°C and kept for 3 days in a desiccator. Depolymerization was
1062 performed by base catalyzed transesterification in dry methanol containing 15% (v/v)
1063 methyl acetate and 6% (v/v) sodium methoxide, and ω -pentadecalactone (2.5 μ g) and
1064 methyl heptadecanoate (5 μ g) were added to the reaction medium as an internal
1065 standard. Reactions was incubated for 2 h at 60°C. 3.5 mL dichloromethane, 0.7 mL
1066 glacial acetic acid and 1 mL 0.9% NaCl (w/v) Tris 100 mM pH 8.0 were added to each
1067 sample and subsequently vortexed for 20 s. After centrifugation (1500 g for 2 min), the
1068 organic phase was collected, washed with 2 mL of 0.9% NaCl, and dried over sodium
1069 sulfate. The organic phase was then recovered and concentrated under a stream of
1070 nitrogen. Monomers were derivatized by acetylation using 20 μ L of anhydrous pyridine
1071 and 20 μ L of acetic anhydride and incubated at 60°C for 2h. Tomato fruit samples were
1072 injected in split mode (30:1), while Arabidopsis samples were injected split-less out of
1073 hexane into a gas chromatograph coupled to a mass spectrometer and a flame
1074 ionization detector (Agilent 6890N GC Network systems) equipped with a HP-5
1075 capillary column (30 m, 0.32 mm ID, 0.25 μ m film thickness, J&W Scientific)
1076 (temperature program: 2 min at 50°C, increment of 20°C/min to 140°C, of 3°C/min
1077 310°C, held for 10 min). Peaks quantified on the basis of their FID ion current.

1078 *Wax analysis:* Wax components were analyzed by submerging the freshly peeled discs
1079 in 7 mL of chloroform for 30 s twice, and then derivatized by sylation, with
1080 BFTSA/pyridine (1:1) at 70°C for 1 h. Samples were injected out of chloroform in a gas
1081 chromatograph coupled to a mass spectrometer and a flame ionization detector
1082 (Agilent 6890N GC Network systems) on a HP-1 column (30 m, 0.32 mm ID, 1 μ m film
1083 thickness, (Agilent)) (temperature program: 50°C for 2 min, raise by 40°C min⁻¹ to

1084 200°C, held for 2 min at 200°C, raised by 3°C min⁻¹ to 320°C, and held for 30 min at
1085 320°C). Peaks were quantified on the basis of their FID ion current.

1086 *Glycerol determination in cutin:* Glycerol was released by mild methanolysis using the
1087 protocol described by Graça et al., 2002 [74] with slight modifications. Isolated cutin
1088 slices (3 mm x 3 mm) were stirred at room temperature in a mixture of 50 mM sodium
1089 methoxide in dry methanol with the internal standard 1,2,4-butanetriol [75]. After 20 h,
1090 the extract was dried with a nitrogen flow, silylated with 1% N, O-
1091 bis(trimethylsilyl)trifluoroacetamide/trimethyl chlorosilane, and analyzed by gas
1092 chromatography-flame ionization detection.

1093 *Ester-linkage pattern of 10,16-dihydroxy hexadecenoic acid in cutin:* Free hydroxy
1094 groups were derivatized by benzyl etherification by a procedure adapted from a
1095 procedure developed for organic alcohols [76]. Isolated dewaxed cutins were mixed
1096 with 15 µg of 2-benzyloxy-1-methylpyridinium triflate and 1.68 µg of magnesium oxide
1097 in 1 µL of trifluorotoluene in a 7 mL screw-capped glass tube at 90°C overnight. Cutin
1098 was then washed extensively with dichloromethane and dried. By comparing different
1099 sizes of cutin samples and cutin powders, verification that the same amount of
1100 derivatized cutin monomer was performed, indicating that cutin diffusion barriers did
1101 not hinder the reaction.

1102 **Cuticle permeability assays**

1103 For studying the permeability of the cuticle of *Arabidopsis* leaves, a drop (5 µL) of an
1104 aqueous solution of 0.05% w/v toluidine blue (TB)/0.001% Tween 20 was applied on
1105 expanding leaves for 45 min. Fully open inflorescences were dipped in the same TB
1106 solution for 45 min. TB was washed away with running water.

1107 For water loss assays, at least 10 isolated cuticles and dewaxed cuticles (cutins) of
1108 tomato fruits were mounted to stainless steel transpiration chambers as described
1109 previously [77] with the exception that the exposed area was 28 mm² and not 1.13 cm².
1110 Permeances for water (m s⁻¹) were calculated from the slopes of regression lines fitted
1111 to the plots water amount lost versus time using the equation $P = \text{water loss} / (\text{time} \times$
1112 $\text{exposed area} \times \text{driving force})$. Since humidity outside the chamber was 0 the driving
1113 force corresponded to pure water in the chamber, which is 1 g cm⁻³.

1114 **QUANTIFICATION AND STATISTICAL ANALYSIS**

1115 For the chemical analyses of the cutin and wax composition, the glycerol amount, the
1116 gene expression, cuticle area and water permeability measurements, presented values
1117 are the mean \pm standard deviation. Two-way ANOVA with Tukey HSD multiple
1118 comparison, $P < 0.05$ were performed to highlight differences among the genotypes. For
1119 the transport assays, data are represented as mean \pm SEM; multiple t-tests followed by
1120 Holm-Sidak multiple comparisons correction were performed to highlight the differences
1121 between Control and other constructs. ns: not significant. Letters were assigned to
1122 differently significant groups; a = $p < 0.001$ and b = $p < 0.05$. Number of repetitions and
1123 replicates are mentioned for each experiment in the method details.

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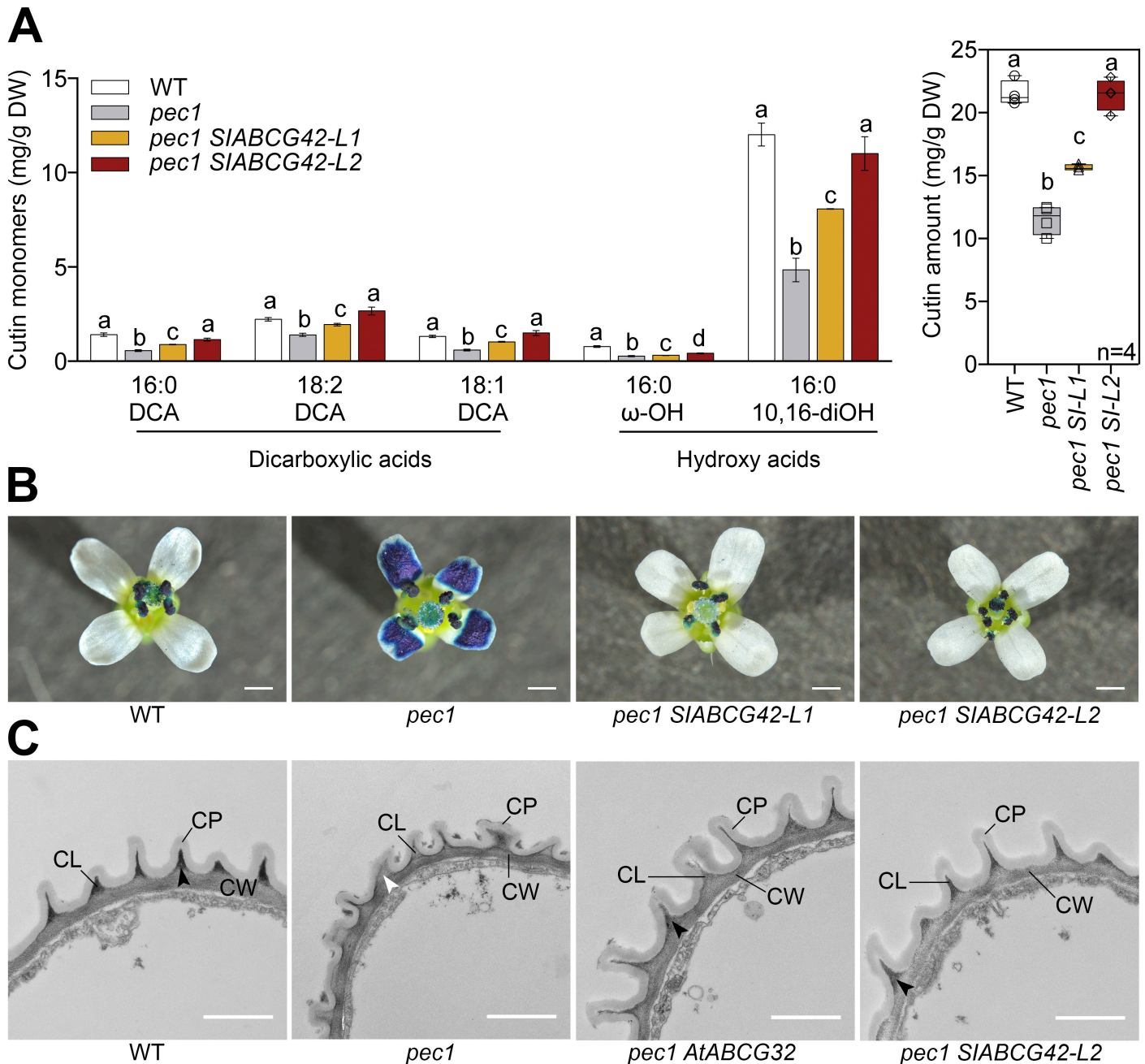


Figure 1. *SIABCG42* complements *pec1/atabcg32* mutant phenotypes

(A) Transgenic *pec1* lines expressing *SIABCG42* (*pec1 SIABCG42-L1* and *L2*) under the native *PEC1* promoter complement the monomer composition (left) as well as cutin amount (right) in flowers of the *pec1* mutant. Statistically significant differences are indicated by different letters (Two-way ANOVA, Tukey HSD multiple comparison, $P < 0.05$, $n = 5$). DW, dry weight. The analysis was performed twice with similar results and a representative data set is shown. Selected monomers are presented here. See Figure S2 for complete data set. **(B)** The cuticle permeability is restored in the *pec1 SIABCG42-L1* and *L2* lines. Toluidine blue (TB) staining is shown in flowers of different genotypes. Scale bar = 1 mm. **(C)** The structure of cuticle in petals in the *pec1 SIABCG42-L2* line is similar to WT, as seen in *pec1* complemented with *AtABCG32* (*pec1 AtABCG32*) [22]. Transmission electron micrographs (TEM) of petals in different genotypes highlighting the cuticular proper (CP), cuticular layer (CL) and cell wall (CW) of the cuticular ridges of the petal cuticle. Black arrows indicate electron dense areas in the cuticular layer; white arrows indicate the lack of this electron dense area. Scale bars represent 1 μ m.

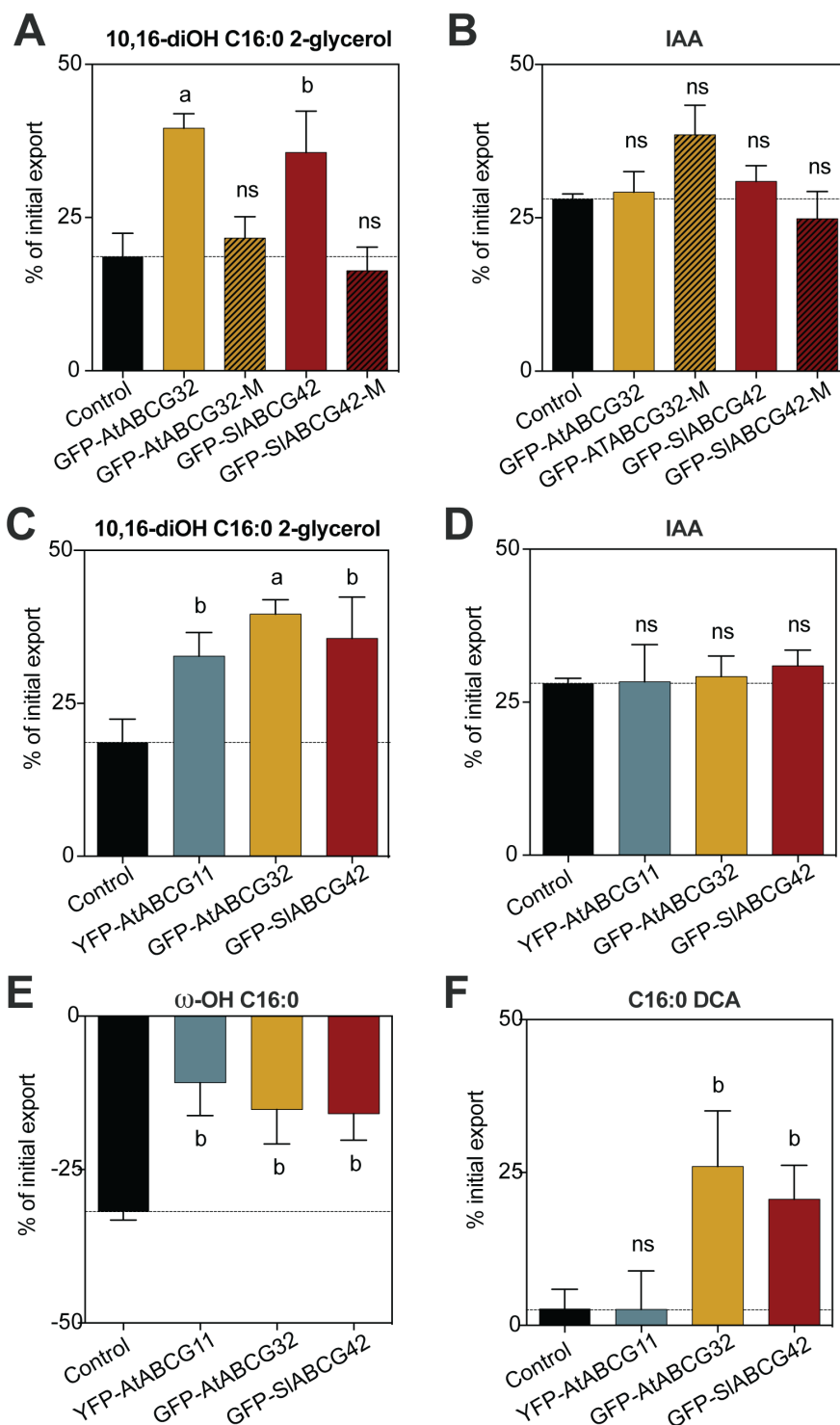


Figure 2. ABCG transporters required for cutin formation export C16:0 derivatives

(A, B) In transport assays using *N. benthamiana* protoplasts, AtABCG32 and SIABCG42 show transport activity with 10,16-diOH C16:0-2-glycerol in contrast to transport-incompetent versions mutated in the Walker A domain (AtABCG32-M and SIABCG42-M). Percentage of initial export across the plasma membrane of protoplasts expressing the empty vector (Control), GFP-AtABCG32, GFP-AtABCG32-M, GFP-SIABCG42, and GFP-SIABCG42-M with different substrates measured after 20 min are shown: (A) [3 H]-labeled 10, 16-diOH 16:0-2-glycerol; (B) [14 C]-labeled indole acetic acid (IAA) used as unrelated compound for comparison. (C-F) Heterologous expression of AtABCG32 and SIABCG42 as well as AtABCG11 in *N. benthamiana* protoplasts reveals export activity across the plasma membrane for 10,16 diOH C16:0 as well as ω -OH C16:0, while C16:0 DCA is only exported by AtABCG32 and SIABCG42. Percentage of initial export after 20 min of different substrates across the plasma membrane of protoplasts expressing the empty vector (Control), YFP-AtABCG11, GFP-AtABCG32 and GFP-SIABCG42. Three radiolabeled C16:0 substituted acids were used as substrates: (C) 10, 16-diOH 16:0-2-glycerol; (E) ω -OH C16:0; (F) C16:0 dicarboxylic acid (C16:0 DCA). (D) Radiolabeled indole acetic acid (IAA) was used as unrelated compound for comparison. Data represented as mean \pm standard error of the mean; multiple t tests followed by Holm-Sidak multiple comparisons correction. Letters indicate significance groups; a = $P < 0.001$ and b = $P < 0.05$; ns: not significant.

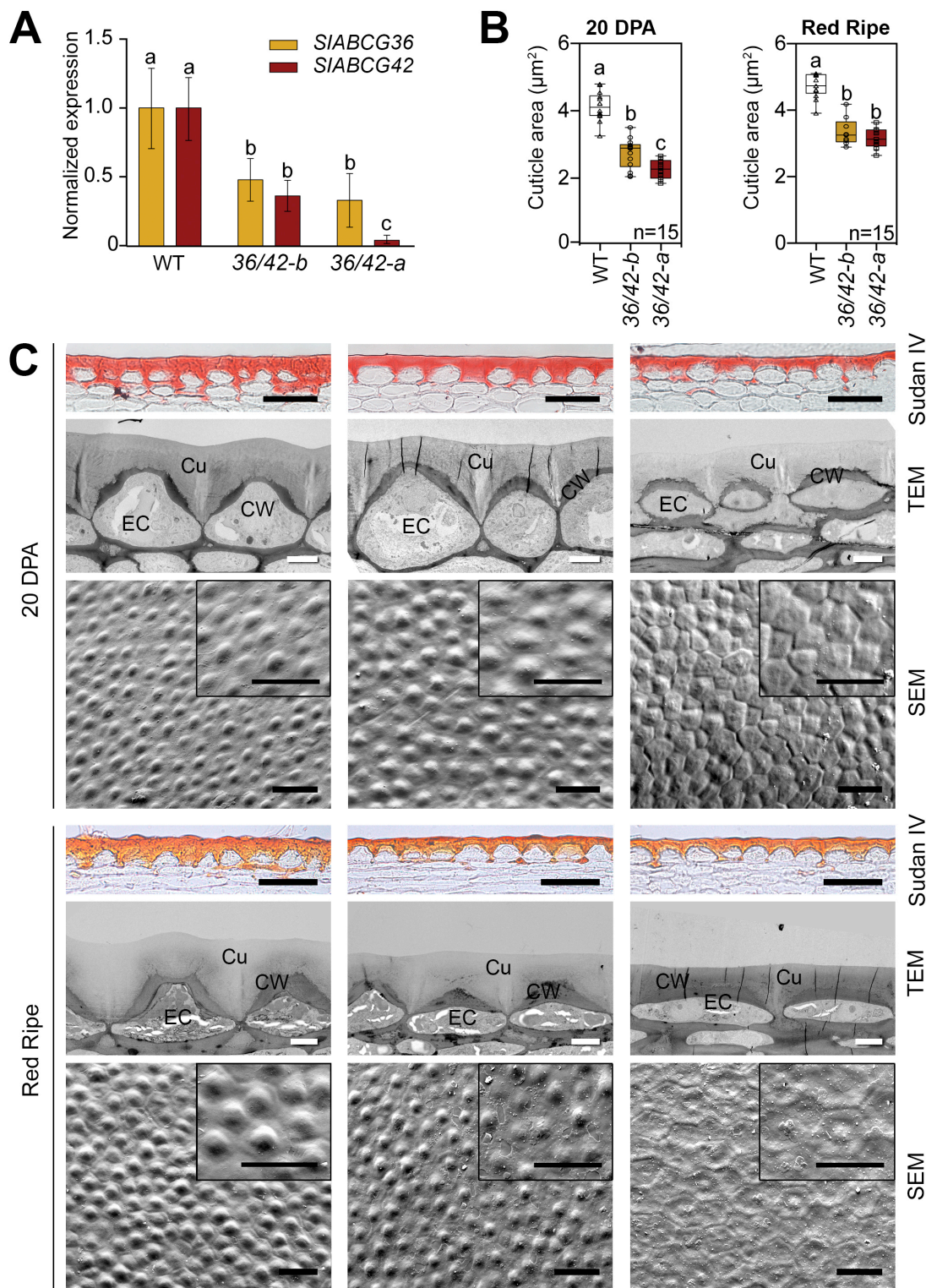
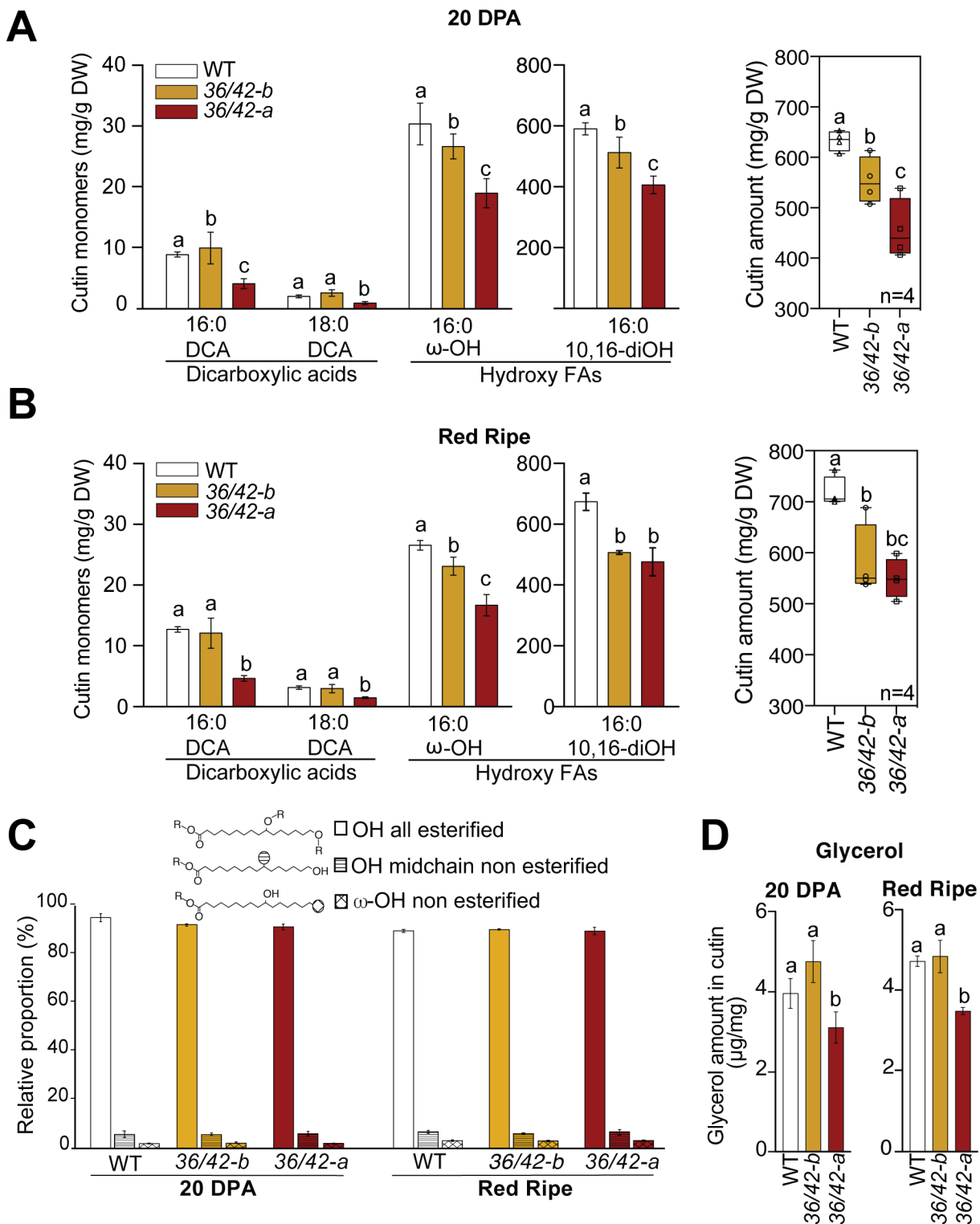


Figure 3. Reduced expression of *AtABCG32*-homologous genes and fruit cuticle formation in *slabcg36/42* plants

(A) Expression analysis of *SIABCG36* and *SIABCG42* in selected RNAi lines, i.e. *slabcg36/42-a* (*36/42-a*) and *slabcg36/42-b* (*36/42-b*) normalized to wild-type (WT) show significant differences between the RNAi lines in *SIABCG42* expression as well as of both genes to WT. Real time-qPCR was performed with two biological replicates for 2 plants belonging to the same T3 line and three technical replicates by sample. Error bars represent SD. Analyses were performed with 10 DPA fruits. Statistically significant differences are indicated by different letters (Two-way ANOVA, Tukey HSD multiple comparison, $P < 0.05$, $n = 4$). The experiments were repeated twice with similar results. **(B)** and **(C)** Reduced cutinization and altered epidermal cell shape were revealed in fruits of *slabcg36/42* plants. **(B)** Reduction of the cutinization of the tomato fruit cuticle as visible by Sudan IV staining (representative picture in **(C)**) was quantified by measuring the Sudan stained area in 5 pictures of $250 \mu\text{m} \times 70 \mu\text{m}$ in size. Statistically significant differences between cuticle areas are indicated by different letters (One-way ANOVA, Tukey HSD multiple comparison, $P < 0.05$, $n = 15$). **(C)** Cross sections of the fruit exocarp are shown in light microscopy and transmission electron microscopy (TEM) as well as of the fruit surface in cryo-scanning-electron microscopy (SEM). Two developmental stages are shown: 20 DPA and Ripe Red. Sudan IV staining revealed cuticle and cutinized cells of the first exocarp cell layer. WT, wild type. Transmission electron microscopy (TEM) images showing cuticular layer and epidermal cells structure. EC: epidermal cell, CW: cell wall, CL: Cuticular layer. White bars = $10 \mu\text{m}$. Black bars = $50 \mu\text{m}$.



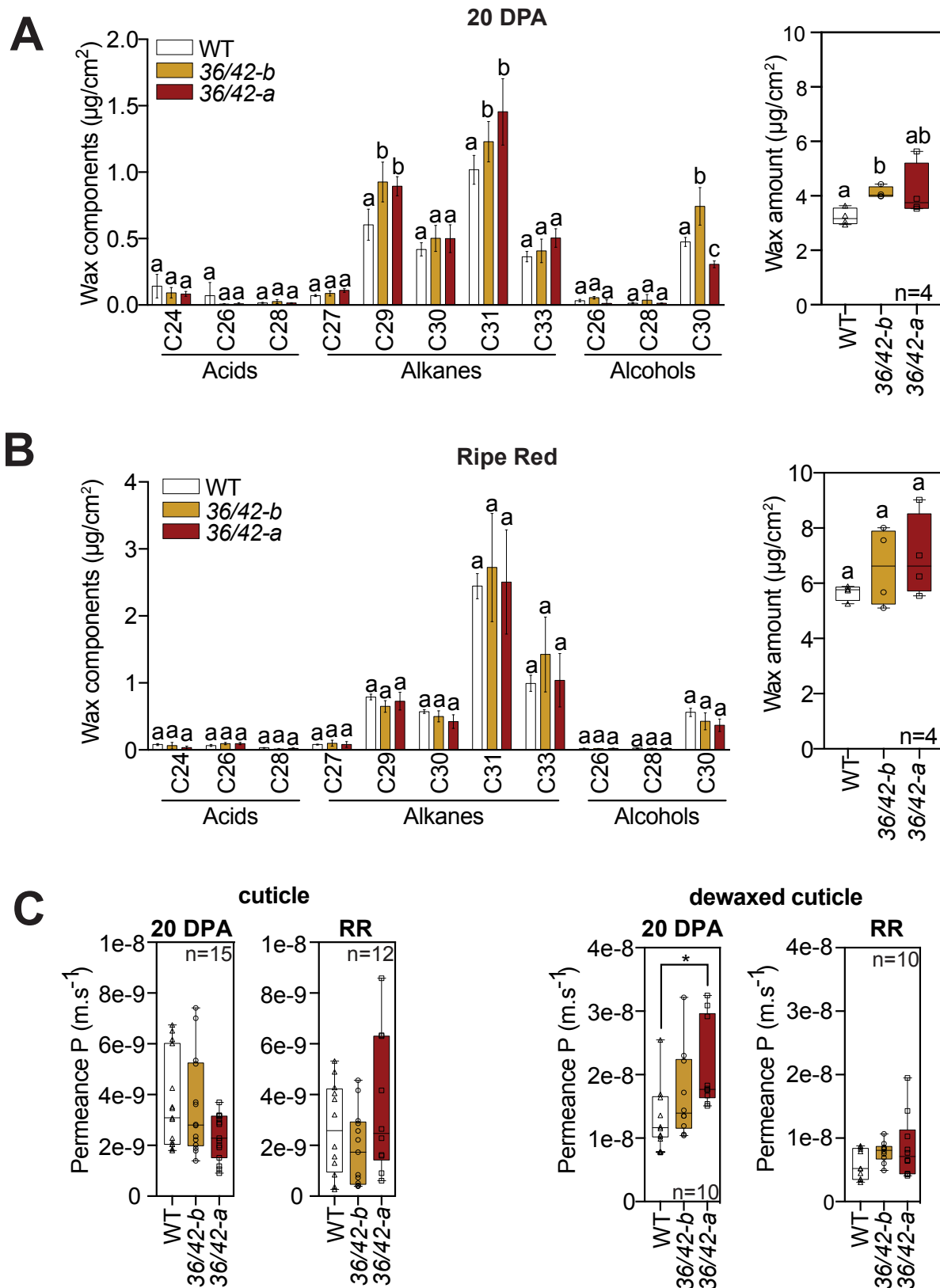
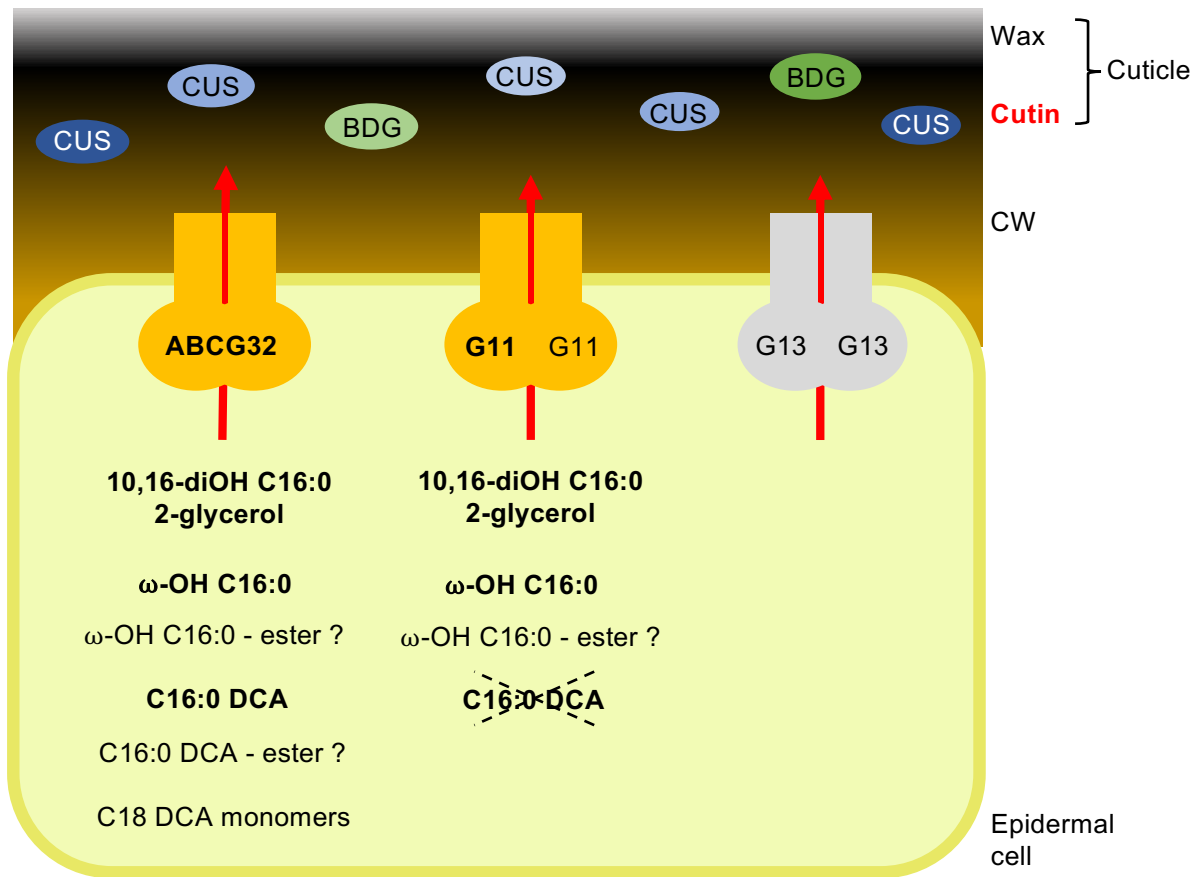


Figure 5. Wax deposition and water permeances of *slabcg36/42-RNAi* fruit cuticles

Increased deposition of wax components (left) and total wax (right) of *slabcg36/42* tomato fruits in different stages (**A**) 20 DPA and (**B**) Red Ripe fruits. Statistically significant differences are indicated by different letters (Two-way ANOVA, Tukey HSD multiple comparison, $P < 0.05$, $n = 5$). (**C**) Only dewaxed fruit cutins of *slabcg36/42* lines harvested at 20 DPA show increased permeability to water. The permeability of waxy and dewaxed cuticles was measured of 20 DPA and Red Ripe (RR) fruits. Student's t -test was used for assessing significant differences of wax components and water permeabilities when compared to WT (* = $p < 0.05$).



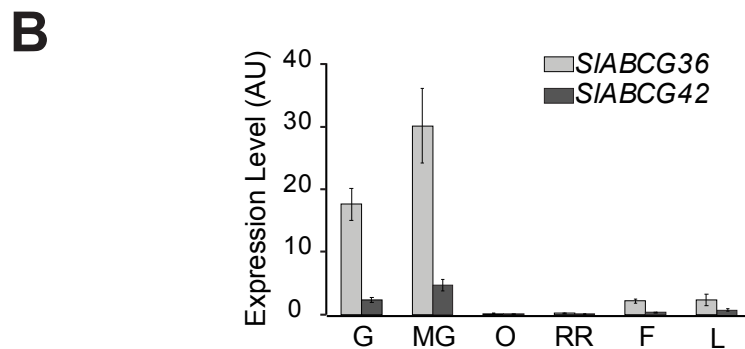
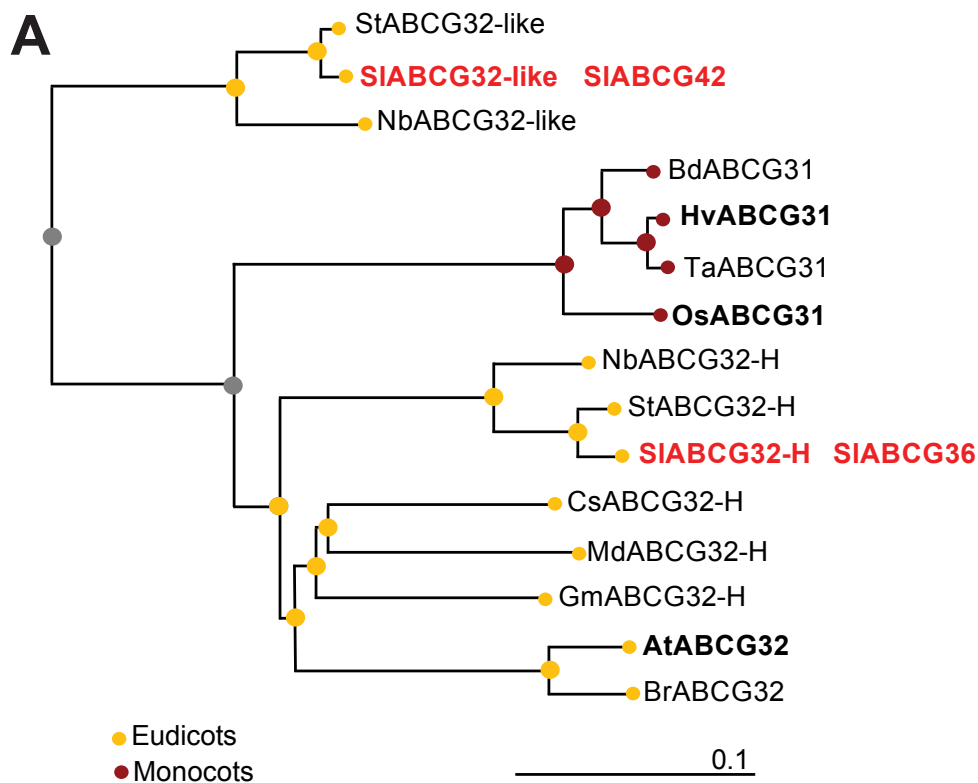


Figure S1. Two *AtABCG32* homologues exist in tomato, Related to Figure 1 and Figure 3

(A) Neighbor Joining-based phylogenetic tree representing proteins of monocots and eudicots having homology to *AtABCG32* based on a BLAST search using blastp (protein-protein BLAST), using selected species. Results were filtered to match records with percentage identity between 65 and 100%. Tree was generated using the Blast tree view tool. Scale bar represents the number of differences between species (0.1 means 10% differences between two sequences). ABCG32-like: Nb, *Nicotiana benthamiana* BAR94054.1; SI, *Solanum lycopersicum* XP_010322739.1 (SIABCG42; gene product of Solyc06g065670); St, *Solanum tuberosum* |XP_006353655.1|. ABCG32-H: At, *Arabidopsis thaliana* |O81016.1| (Bessire et al, 2011), Br, *Brassica rapa* |XP_009140751.1|; Cs, *Cucumis sativus* |XP_004139333.1|; Gm, *Glycine max* |XP_003524521.1|; Md *Malus domestica* |XP_028950701.1|; Nb, |BAR94048.1|, SI, |XP_010321067.1| (SIABCG36; gene product of Solyc05g018510) ; St, |XP_006338166.1|. ABCG31-H: Bd, *Brachypodium dista*, XP_003569645.1; Hv, *Hordeum vulgare* |KAE8773549.1| (Chen et al. 2011); Os, *Oryza sativa*, |Q8GU87.3| (Chen et al, 2011, Garroum et al., 2016), Ta, *Triticum aestivum*, |CDM81797.1|. Red, *S. lycopersicum* proteins (Ofori et al., 2018); bold, experimentally characterized members.

(B) Expression of the ABCG32 homologues, *SIABCG36* and *SIABCG42*, in different organs and fruit developmental stages in WT tomato plants. G, green fruits; MG, mature green fruits; O, orange fruits; RR, ripe red fruits; L, young leaves; F, fully open flowers. Histograms represent the mean expression level (in arbitrary units (AU) for 4 different samples for 3 different WT plants, and three technical replicates by sample. Error bars represent SD.

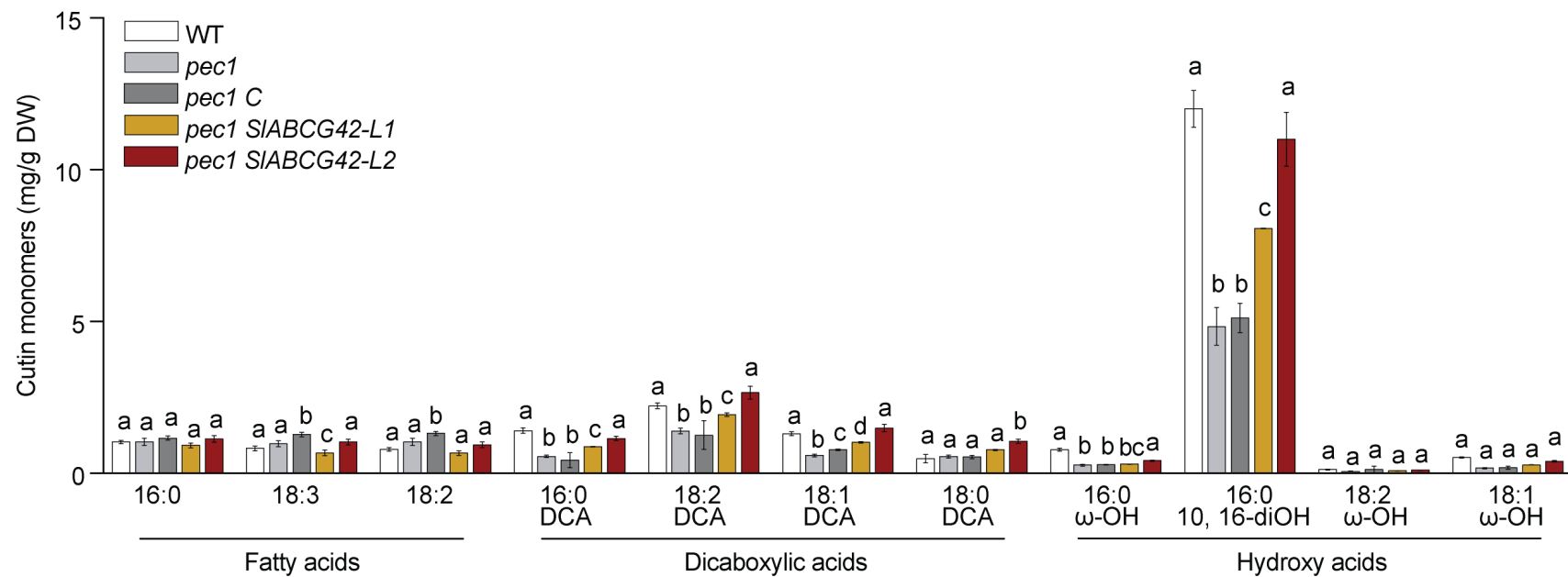


Figure S2. SIABCG42 complements *pec1/acbg32* mutant cutin composition, Related to Figure 1

Transgenic *pec1* lines expressing *SIABCG42* (*pec1 SIABCG42-L1* and *L2*) under the native *PEC1* promoter complement the monomer composition of flowers of the *pec1* mutant. *pec1 C*, *pec1* mutant transformed with the empty vector. Statistically significant differences are indicated by different letters (Two-way ANOVA, Tukey HSD multiple comparison, $P < 0.05$, $n = 5$ replicates). The analysis was performed twice with similar results and a representative data set is shown.

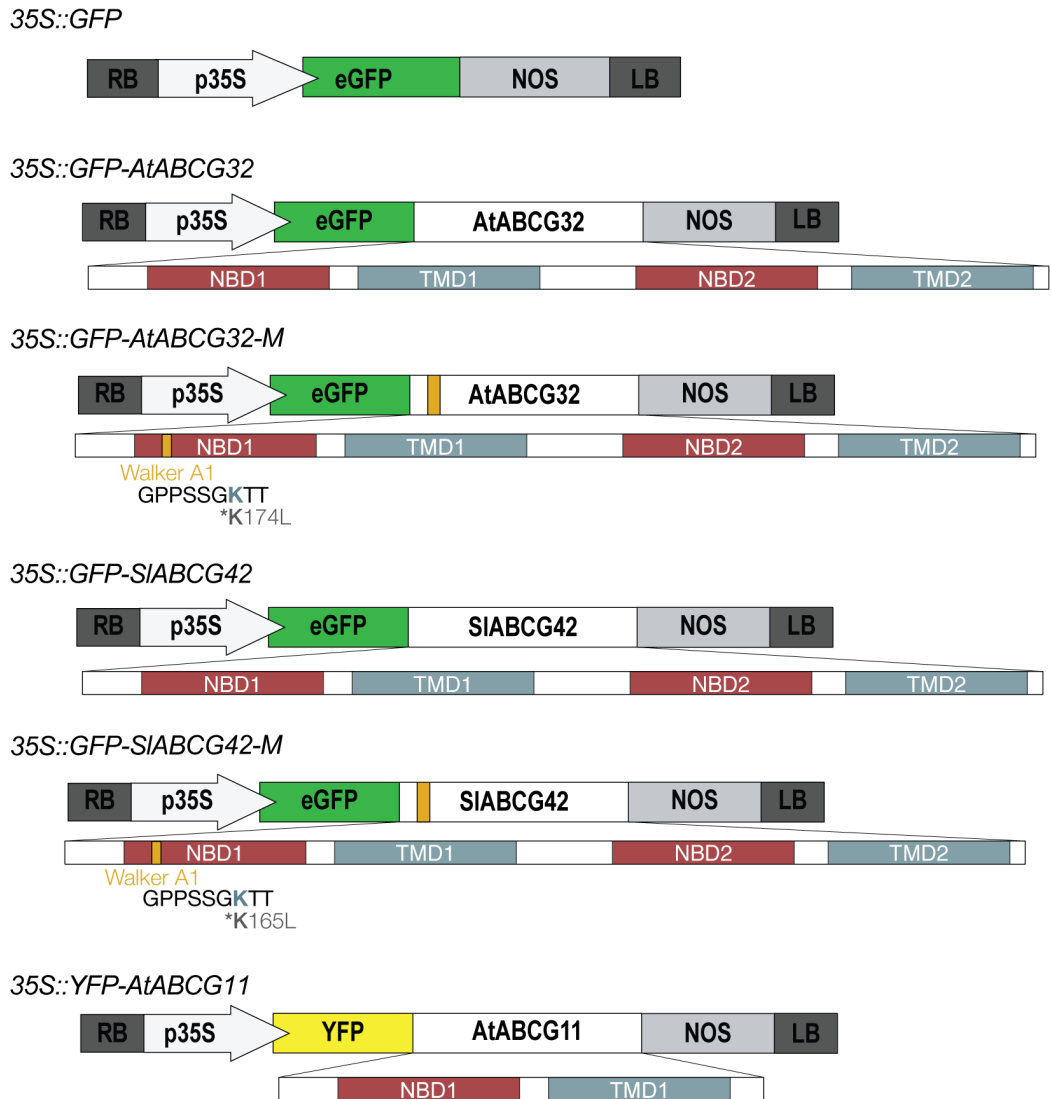


Figure S3. Schematic diagrams of the constructs used for the heterologous expression of ABCG transporters in *N. benthamiana*. Related to Figure 2.

Schematic diagrams of the constructs as well as the main domains of ABCGs and the location of the mutation (K174L) in the Walker A1 motif underneath each construct. NBD: Nucleotide-Binding domain; TMD: transmembrane domain.

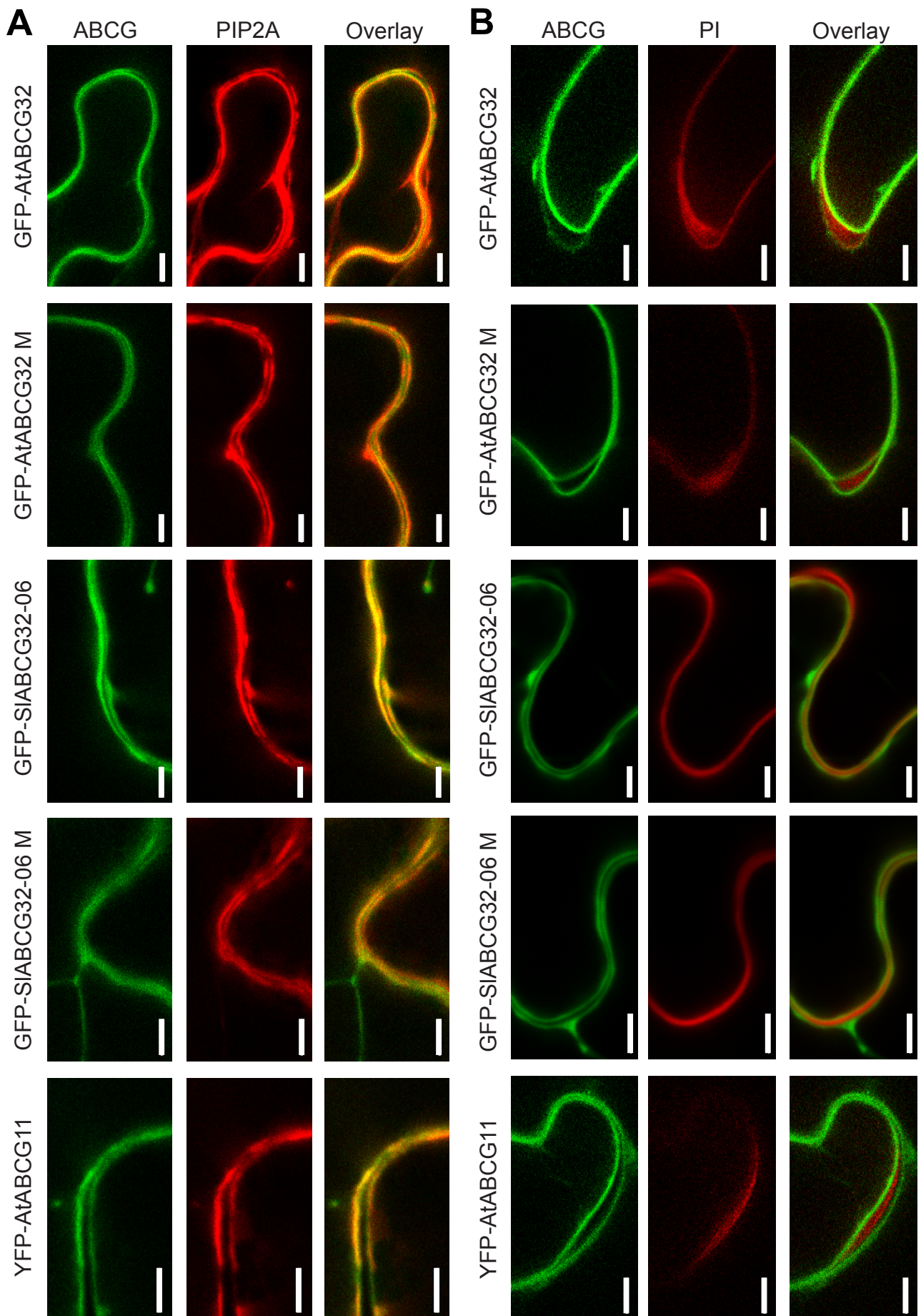


Figure S4. Localization of *Arabidopsis* ABCG transporters in *N. benthamiana*, Related to Figure 2

FP-tagged ABCG transporters were localized in *N. benthamiana* leaf epidermal cells using confocal-laser scanning microscopy. Co-localization of the FP-tagged ABCG-transporters with the mCherry-tagged plasma membrane marker PIP2A is presented in A. Localization of the FP-tagged ABCG-transporters adjacent to the cell wall visualized with propidium iodide is shown in B. Scale bars represent 5 μm .

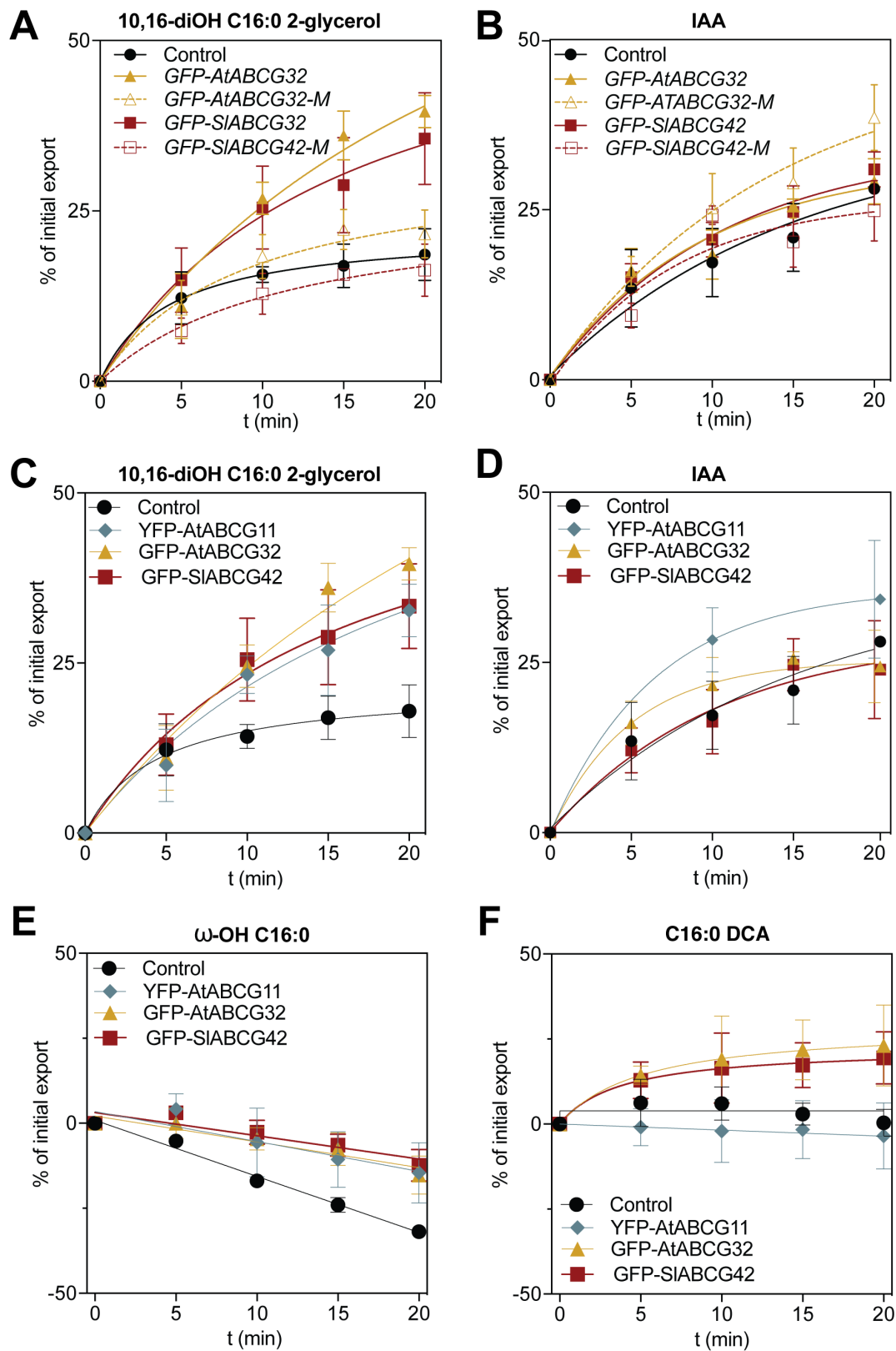


Figure S5. ABCG transporters required for cutin formation export fatty acid derivatives, Related to Figure 2

Heterologous expression of AtABCG32 and SIABCG42 as well as AtABCG11 in *N. benthamiana* protoplasts reveals export activity across the plasma membrane for 10,16 diOH C16:0 as well as ω -OH C16:0, while C16:0 DCA is only exported by AtABCG32 and SIABCG42. Percentage of initial export of different substrates across the plasma membrane of protoplasts expressing the empty vector (Control), GFP-AtABCG32, GFP-AtABCG32-M, GFP-SIABCG42, GFP-SIABCG42-M, and YFP-AtABCG11 at 0, 5, 10, 15 and 20 min. Three radiolabeled C16:0 substituted acids were used as substrates: **(A,C)** [3 H]-10,16-diOH 16:0-2-glycerol; **(E)** [14 C]- ω -OH C16:0; **(F)** [3 H]-C16:0 dicarboxylic acid (C16:0 DCA). **(B,D)** [14 C]-labeled indole acetic acid (IAA) was used as unrelated compound for comparison. Data represented as mean \pm standard error of the mean.

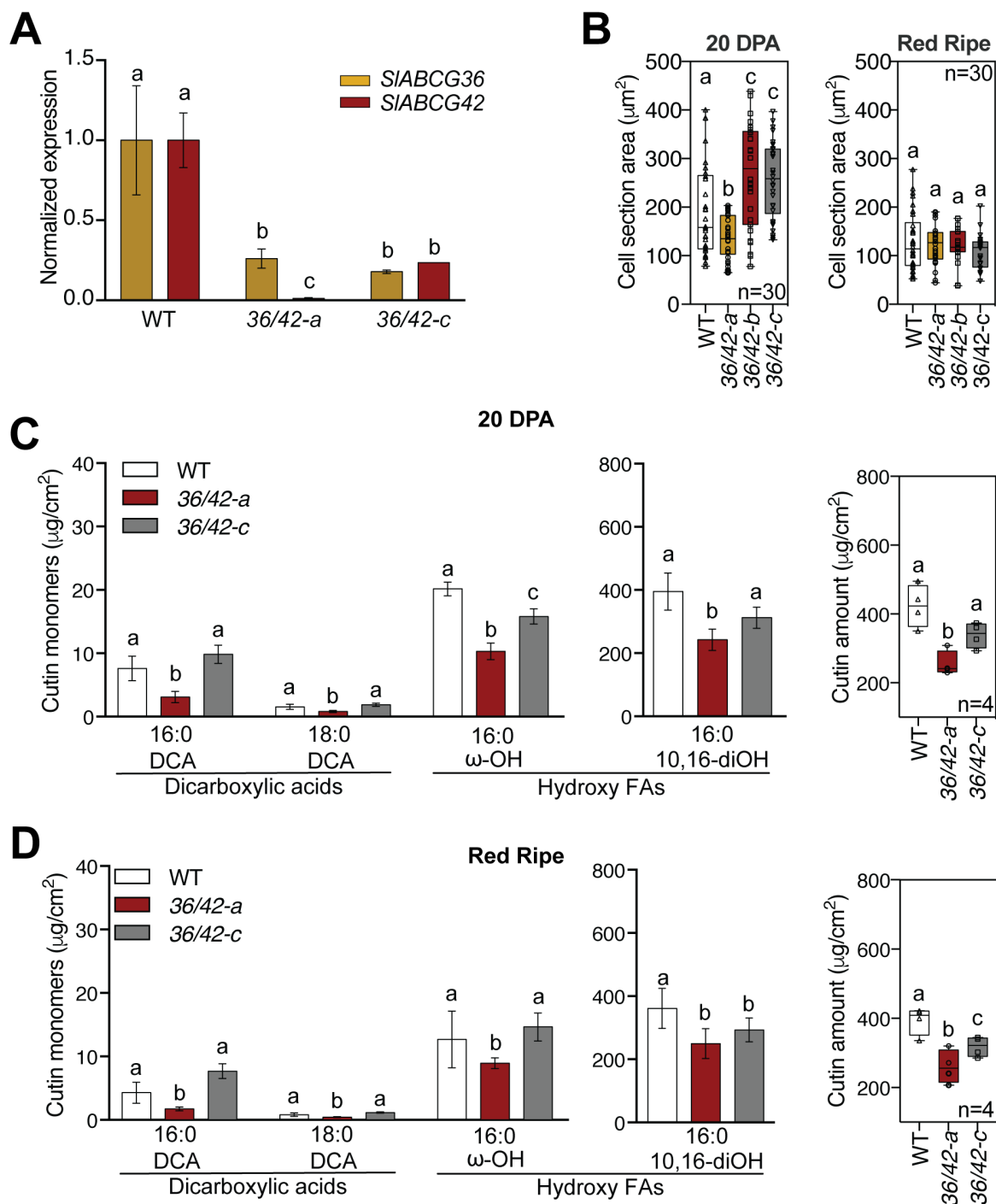


Figure S6. Reduced expression of *AtABCG32*-homologous genes as well as alterations in cell shape and reduction in cutin deposition in *slabcg36/42* plants, Related to Figure 3 and Figure 4

(A) Expression analysis of *SIABCG36* and *SIABCG42* in selected RNAi lines, i.e. *slabcg36/42-a* (*36/42-a*) and *slabcg36/42-c* (*36/42-c*) normalized to wild-type (WT) show significant differences between the RNAi lines in *SIABCG42* expression as well as of both genes to WT. Real time-qPCR was performed with two biological replicates for 2 plants belonging to the same T2 line and three technical replicates by sample. Error bars represent SD. Analyses were performed with 10 DPA fruits. Statistically significant differences are indicated by different letters (Two-way ANOVA, Tukey HSD multiple comparison, $P < 0.05$, $n = 4$ replicates). **(B)** Cell shape was evaluated by measuring the size of ultrathin sections of epidermal cell pictured in transmission electron microscopy. A high variability in cell section size is generated by the conical cells in WT and the intermediate downregulated *slabcg36/42* line, while the homogenous flat cells result in a small cell section size of low variability in the strongest downregulated line at 20 DPA. Statistically significant differences between cell section areas are indicated by different letters (One-way ANOVA, Tukey HSD multiple comparison, $P < 0.05$, $n = 30$ pictures) **(C)** and **(D)** The amount of cutin monomers (left) and the total cutin amount (right) of isolated cutins were quantified in 20 DPA-old **(C)** and in Red Ripe **(D)** fruits of the T2 plants characterized in **(A)**. Statistically significant differences between amount of cutin monomers and total cutin amounts are indicated by different letters (Two-way ANOVA, Tukey HSD multiple comparison, $P < 0.05$, $n = 4$ replicates).

Gene name	Gene number
<i>AtABCG32/PEC1</i>	<i>At2g26910</i>
<i>AtABCG11</i>	<i>At1g17840</i>
<i>SlABCG36</i>	<i>Solyc05g018510</i>
<i>SlABCG42</i>	<i>Solyc06g065670</i>

Table S1. List of genes mentioned in this study, Related to Star Methods

Primer name	Primer sequence	Reference
pec1-2_LP	GTTGTGATGCATTGTTGTTCCG	SALK primer design (SALK line SALK_025696.51.80.x)
pec1-2_RP	AAGCCTCGTCGTGGAGTATG	
LBAi	TGGTTCACGTAGTGGCCATCG	
pAtABCG32+HindIII F	<i>ATAAGCTTAGAAAGTCTCGGGAGGAAAC</i>	Bessire et al., 2011 ^{S1}
pAtABCG32+KpnI R	<i>TTAGGTACCAATCTCCGCGGCGGCGCAACAGAA</i>	
SIABCG42 attB1 F	<u>GGGGACAAGTTTGTACAAAAAGCAGGCTAC</u> ATGTCAACAA GAGGTGAAAATGG	Present work
SIABCG42 attB2 R	<u>GGGGACCACTTTGTACAAGAAAGCTGGGTTTTACACAAAT</u> AGCCAGTCGGAT	
SDM_AtABCG32 F	AAGTTCAGGGttGACAACATTACTCTTAG	Present work
SDM_AtABCG32 R	GGAGGACCTAGTAGTAGTG	
SDM_SIABCG42 F	TAGCTCTGGAAttAACAACTTGCTTTTGGCACTTG	Present work
SDM_SIABCG42 R	GGCGGACCCAAAAGCAGC	
fragSIABCG36 F	<u>AAAAAGCAGGCTAC</u> ACATACCAGTGACCAAGT	Present work
fragSIABCG36 R	<u>AGAAAGCTGGGTTAAGAGCTGAAAAAATTTGAAAAT</u>	
fragSIABCG42 F	<u>AAAAAGCAGGCTACA</u> AACCTGTCTACTGGAG	Present work
fragSIABCG42 R	<u>AGAAAGCTGGGTTGGCTGCTCTTTCACGATA</u>	
TIP41 qPCR F	AGATGAACTGGCAGATAATGG	Expósito-Rodríguez et al., 2008 ^{S2}
TIP41 qPCR R	CATCAACCCTAAGCCAGAAA	
SIABCG36 qPCR F	GACTACTGCAATCACACCCA	Present work
SIABCG36 qPCR R	CCATCGGATAGTTTCACCAG	
SIABCG42 qPCR F	CGCTCTTCGGTATGATGACA	Present work
SIABCG42 qPCR R	AACACCATCAGCGAGCGTAA	

Table S2. Primers used in this study, Related to Star Methods

Special information about the primer sequences are indicated as follows: italic, restriction enzymes sites; underlined, sequence corresponds to AttB sites for recombination cloning; lower case, exchanged nucleotides.

Supplemental References

- S1. Bessire, M., Borel, S., Fabre, G., Carraca, L., Efremova, N., Yephremov, A., Cao, Y., Jetter, R., Jacquat, A.C., Metraux, J.P., et al. (2011). A member of the PLEIOTROPIC DRUG RESISTANCE family of ATP binding cassette transporters is required for the formation of a functional cuticle in Arabidopsis. *Plant Cell* 23, 1958-1970.
- S2. Exposito-Rodriguez, M., Borges, A.A., Borges-Perez, A., and Perez, J.A. (2008). Selection of internal control genes for quantitative real-time RT-PCR studies during tomato development process. *BMC Plant Biology* 8, 131.