



HAL
open science

Sterol 3-beta-Glucosyltransferase TRANSPARENT TESTA15 Controls Seed Development and Flavanol Accumulation through its Role in Vacuole Biogenesis and Maintenance in Arabidopsis

Elodie Akary, Adeline Berger, François Perreau, Anne Frey, Alexandra To, Sylvie Citerne, Hubert Schaller, Samantha Vernhettes, Olivier Grandjean, Nathalie Nesi, et al.

► To cite this version:

Elodie Akary, Adeline Berger, François Perreau, Anne Frey, Alexandra To, et al.. Sterol 3-beta-Glucosyltransferase TRANSPARENT TESTA15 Controls Seed Development and Flavanol Accumulation through its Role in Vacuole Biogenesis and Maintenance in Arabidopsis. 2024. hal-04491046

HAL Id: hal-04491046

<https://hal.inrae.fr/hal-04491046>

Preprint submitted on 5 Mar 2024

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1 RESEARCH ARTICLE

2

3 **Sterol 3-beta-Glucosyltransferase TRANSPARENT TESTA15 Controls Seed**
4 **Development and Flavanol Accumulation through its Role in Vacuole**
5 **Biogenesis and Maintenance in Arabidopsis**

6

7 Elodie Akary,¹ Adeline Berger,¹ François Perreau,¹ Anne Frey,¹ Alexandra To,¹
8 Sylvie Citerne,¹ Hubert Schaller,² Samantha Vernhettes,¹ Olivier Grandjean,^{1,†}
9 Nathalie Nesi^{1,#}, Annie Marion-Poll¹, Loïc Lepiniec¹ and Isabelle Debeaujon^{1,*}

10

11 ¹ Institut Jean-Pierre Bourgin (IJPB), INRAE, AgroParisTech, Université Paris-
12 Saclay, 78000, Versailles, France

13 ² Institut de biologie moléculaire des plantes, CNRS, Université de Strasbourg,
14 67083, Strasbourg, France

15

16 [†] Deceased

17 [#] Present address: Institut de Génétique, Environnement et Protection des Plantes
18 (IGEPP), INRAE, Institut Agro Rennes-Angers, Université de Rennes, Le Rheu,
19 France

20 ^{*} Author for correspondence: isabelle.debeaujon@inrae.fr

21

22 **Short title :**

23 Arabidopsis UGT80B1/TT15 and vacuole function

24

25 **One-sentence Summary :**

26 Arabidopsis UGT80B1/TT15 regulates seed development and flavanol
27 accumulation by modulating tonoplast homeostasis, in collaboration with the
28 TT9/GFS9 protein involved in homotypic vacuole fusion.

29

30 **Material distribution footnote :**

31 The author responsible for distribution of materials integral to the findings presented
32 in this article in accordance with the policy described in the Instructions for Authors
33 (www.plantcell.org) is : Isabelle Debeaujon (isabelle.debeaujon@inrae.fr).

34

35 **ABSTRACT**

36 The Arabidopsis sterol 3-beta-glucosyltransferase UGT80B1/TRANSPARENT
37 TESTA15 (TT15) catalyzes sterol glucoside biosynthesis. Its loss of function causes
38 reduced seed size, defective flavanol, polysaccharide and lipid polyester deposition
39 at the seed coat and reduced seed dormancy. How TT15 controls seed
40 development and physiology is unknown. Here we show that *tt15* mutants exhibit
41 seed lethality with incomplete penetrance and maternal determinism that is
42 correlated with endosperm cellularization defects, together with an increased
43 sensitivity of seed germination to exogenous abscisic acid and paclobutrazol. We
44 also reveal that flavanol deposition in the vacuole during *tt15* seed development
45 triggers premature endothelium cell death. An autoimmune-like syndrome
46 characterized by callose and H₂O₂ accumulation was detected in endothelium at the
47 seed abaxial pole. Similar phenotypes were observed with *tt9/gfs9*, a mutant
48 defective in endomembrane trafficking and homotypic vacuole fusion. Double
49 mutant analysis showed that *tt9* partially rescued *tt15* endothelium phenotypes.
50 Consistent with seed mutant phenotypes, *TT15* promoter activity was detected in
51 endothelium and endosperm and TT15 protein was located mainly at the vacuolar
52 membrane (tonoplast). Using fluorescence recovery after photobleaching, we
53 demonstrated that tonoplast fluidity was increased in *tt15* roots. Altogether our data
54 suggest that TT15 regulates seed development and flavanol accumulation by
55 modulating vacuole biogenesis and maintenance.

56

57 INTRODUCTION

58 Seed development in Angiosperms is initiated by double fertilization, leading to the
59 formation of a diploid embryo and a triploid endosperm. The two siblings develop
60 concomitantly within the surrounding maternal tissue or seed coat (also called
61 testa), which is derived from the post-fertilization differentiation of the two ovule
62 integuments (inner integument or ii, and outer integument or oi) in Arabidopsis.
63 When morphogenesis is completed, the embryo grows at the expense of the
64 nurturing endosperm. The three seed components need to exchange signals to
65 ensure coordinated development, maturation and differentiation that determine final
66 seed traits, among which auxin and sugars play prominent roles (Ingram, 2010;
67 Figueiredo and Köhler, 2016; Robert, 2019). Seed germination begins with the
68 uptake of water during imbibition of quiescent dry seeds and ends up when
69 hypocotyl expansion triggers protrusion of the embryo radicle through the seed
70 envelopes. These ones consist in a dead brown seed coat and a single layer of live
71 endosperm in Arabidopsis. A dormant seed is unable to germinate, even in
72 favourable environmental conditions. The control of germination results from the
73 competitive interaction between embryonic growth potential and mechanical
74 restraint imposed by surrounding tissues. Abscisic acid (ABA) and gibberellins
75 (GAs) are diterpenoid hormones (Supplemental Figure S1) acting antagonistically
76 in seed dormancy and germination control. The ABA/GA balance is an integrator of
77 environmental and metabolic clues favourable to seed germination such as water,
78 oxygen, temperature, light and nitrate (North et al., 2010).

79 Sterols, a class of lipids of terpenic origin (Supplemental Figure S1), play crucial
80 roles in plant development and growth as components of membranes and as
81 precursors for steroidal hormones brassinosteroids (BR) and steroidal specialized
82 metabolites. They are also essential for proper seed development and physiology
83 (Schaller, 2004; Mamode Cassim et al., 2019; Shimada et al., 2021). In most plants
84 and fungi, some animals and a few bacteria, sterols are present not only as free
85 sterols (FS) but also conjugated as steryl glycosides (SG) and acyl steryl glycosides
86 (ASG). The sugar moiety (generally a D-glucose) is attached to the 3 β -hydroxy
87 group at the C3-atom of a sterol. It increases the size of the hydrophilic head-group
88 of the lipid and thus changes its biophysical properties. The conversion of
89 membrane-bound FS to SG is catalyzed by nucleoside diphosphate (NDP)-sugar-
90 dependent sterol glycosyltransferases (Grille et al., 2010). Sterol

91 glycosyltransferases (EC 2.4.1.173) play important roles in plant metabolic plasticity
92 during adaptive responses (Grille et al., 2010; Ferrer et al., 2017). In Arabidopsis,
93 two uridine diphosphate (UDP)-glucose:sterol glucosyltransferases have been
94 identified, namely UGT80A2 and UGT80B1, and their biological functions explored
95 by a reverse genetics approach (Warnecke et al., 1997; DeBolt et al., 2009).
96 Phenotypic analysis of the corresponding single mutants revealed important
97 perturbations in seed development specifically for *ugt80b1*, namely a *transparent*
98 *testa* (*tt*) phenotype (pale seeds compared to brown wild-type seeds), a reduced
99 seed size, a loss of cutin and suberin at the seed coat, but no impact on cellulose
100 biosynthesis in vegetative parts (DeBolt et al., 2009). The *ugt80b1* mutant appeared
101 to be allelic to the *transparent testa15* (*tt15*) mutant previously identified by Focks
102 et al. (1999). Recently, specific roles for both enzymes in polysaccharide
103 accumulation at the level of seed coat epidermal cells (SCE or oi2 cells) were
104 inferred from a thorough cytological, chemical and physico-chemical
105 characterization of *ugt80A2* and *ugt80B1/tt15* mutants. This study revealed that *tt15*
106 oi2 cells do not release properly their mucilage due to a localized increase in the
107 deposition of secondary cell wall polymers at the level of radial oi2 cell walls (Berger
108 et al., 2021). Albeit they are classified in the family 1 of plant UDP
109 glycosyltransferases (UGT), Arabidopsis UGT80B1/TT15 and its paralog UGT80A2
110 are very divergent from other plant UGTs because they do not contain an obvious
111 Plant Secondary Product Glycosyltransferase motif (PSPG) and are more closely
112 related to non-plant UGT families (Caputi et al., 2012). The sequence homology
113 between UGT80B1/TT15 and its orthologs is restricted to the catalytic region
114 including the Putative Steroid-Binding Domain (PSBD) (Warnecke et al., 1999) and
115 the C-terminal PROSITE consensus sequence for family 1 glycosyltransferases
116 (UGT). *In vitro* enzyme assays and the analysis of SG in seeds of single mutants
117 revealed discrepancies between both paralogs. Indeed, if UGT80A2 is responsible
118 for the bulk production of SGs, UGT80B1/TT15 is involved in the production of minor
119 but probably critical SGs, such as campesteryl and brassicasteryl glucosides
120 (Stucky et al., 2015). Another meaningful difference between both paralogs is their
121 subcellular localization as determined by proteomics analyses. UGT80B1/TT15 was
122 detected at the vacuolar membrane or tonoplast (Carter et al., 2004; Jaquinod et
123 al., 2007) and UGT80A2 at the plasma membrane (Marmagne et al., 2007; Zhang
124 and Peck, 2011). On the other hand, both paralogs were shown to be peripheral

125 membrane proteins, consistent with the absence of transmembrane domains
126 (Ramirez-Estrada et al., 2017).

127 Seed coat colour in *Arabidopsis* is conferred by flavanols, a subclass of flavonoids
128 involving proanthocyanidins (also called condensed tannins) and their flavan-3-ol
129 monomers. *Arabidopsis* wild-type seeds synthesize exclusively procyanidins (PC)
130 resulting from the condensation of epicatechin (EC) monomers (Routaboul et al.,
131 2006) (Supplemental Figure S2). PC and EC accumulate as colourless compounds
132 in vacuoles of tannin-producing cells, namely the endothelium (ii1 cells), the
133 micropylar region (a few ii1' cells) and the chalazal pigment strand of the seed coat
134 and are further oxidized as brown pigments by the TT10 laccase upon seed
135 desiccation (Debeaujon et al., 2003; Pourcel et al., 2005). Mutants with altered seed
136 coat colour, thus defective in PC metabolism (*tt* ; *tt glabra* or *ttg* ; *tannin-deficient*
137 *seeds* or *tds* ; *banyuls* or *ban*) were instrumental in establishing many steps of the
138 flavonoid biosynthetic pathway (Koornneef, 1990; Lepiniec et al., 2006). PC
139 production is triggered by ovule fertilization and ends around the heart stage of
140 embryo development (Debeaujon et al., 2003; Figueiredo and Köhler, 2014). It is
141 tightly regulated spatio-temporally by a complex of transcriptional regulators
142 involving a Myeloblastosis (MYB), a basic Helix-Loop-Helix (bHLH) and a WD-
143 repeat (WDR) protein (MBW complex) encoded by the *TT2*, *TT8* and *TTG1* genes,
144 respectively (Lepiniec et al., 2006). *TTG2*, a WRKY-type transcription factor, was
145 proposed to regulate vacuolar transport steps (Johnson et al., 2002; Gonzalez et
146 al., 2016). Upstream the MBW complex, at least two other transcription factors,
147 namely a WIP-type zinc finger and a MADS encoded by the *TT1* and *TT16* genes
148 respectively, control endothelium identity and thus its competency to accumulate
149 flavanols (Nesi et al., 2002; Sagasser et al., 2002). Proanthocyanidins have
150 substantial antioxidant activity, and the ability to chelate metals and to cross-link
151 with proteins and cell wall polysaccharides. These specialized metabolites reinforce
152 coat-imposed dormancy and seed longevity (Debeaujon et al., 2000). Moreover,
153 their role in the regulation of seed size and the control of post-zygotic reproductive
154 barriers is questioned (Garcia et al., 2005; Dilkes et al., 2008; Doughty et al., 2014;
155 Batista et al., 2019; Köhler et al., 2021). The mechanisms mediating flavanol
156 transport to the vacuole in wild-type seeds also are still a matter of debate (Dixon
157 and Sarnala, 2020). The current working model for flavanol trafficking based on
158 *Arabidopsis* and *Medicago* biochemistry and genetics postulates that after

159 biosynthesis by a metabolon anchored at the external side of the ER, EC is
160 glycosylated and transported to the vacuole where it would hypothetically be
161 hydrolyzed by a glycosidase and polymerized to PCs before migrating to the cell
162 wall according to an unknown mechanism (Winkel, 2019; Dixon and Sarnala, 2020)
163 (Supplemental Figure S2). The vacuolar transport of glycosylated EC involves the
164 tonoplastic Multidrug And Toxin Extrusion (MATE) transporter TT12 / Detoxification
165 41 (DTX41) / TDS3 (Debeaujon et al., 2001; Marinova et al., 2007; Zhao and Dixon,
166 2009; Appelhagen et al., 2014) that would be energized by the tonoplastic P_{3A}-
167 ATPase TT13/Autoinhibited H⁺-ATPase isoform 10 (AHA10) / TDS5 (Baxter et al.,
168 2005; Appelhagen et al., 2014; Appelhagen et al., 2015). Another actor is the
169 glutathione S-transferase (GST) TT19/GST26/GSTF12 that may work as a ligandin
170 to protect the flavonoid molecule from oxidative degradation in the cytosol until it
171 reaches its dedicated transporter at the tonoplast (Kitamura et al., 2010). Vesicle
172 trafficking also participates in vacuolar transport of flavanols in Arabidopsis seed
173 coats. The peripheral membrane protein TT9/GREEN FLUORESCENT SEED9
174 (GFS9) localized at the Golgi is involved in vacuole biogenesis and genetically
175 interacts with the trans-Golgi network (TGN)-located ECHIDNA (ECH) protein
176 (Ichino et al., 2014; Ichino et al., 2020). The Arabidopsis AAA ATPase VACUOLAR
177 PROTEIN SORTING4/SUPPRESSOR OF K⁺ TRANSPORT GROWTH DEFECT1
178 (VPS4/SKD1) is a subunit of the endosomal sorting complexes required for transport
179 (ESCRT) machinery involved in the formation of multivesicular bodies (MVB). Seeds
180 expressing a dominant-negative version of AtSKD1 have a *tt* phenotype and
181 mucilage defects (Shahriari et al., 2010a). Flavanol-accumulating cells in the
182 endothelium of various *tt/tds* mutants including *tt15* exhibit defects in biogenesis of
183 the central vacuole, suggesting a link between flavanol accumulation and vacuole
184 morphology (Debeaujon et al., 2001; Abrahams et al., 2003; Baxter et al., 2005;
185 Kitamura et al., 2010; Appelhagen et al., 2014). Anthocyanin flavonoid pigment
186 sequestered in ER-derived vesicle-like structures was shown to be targeted directly
187 to the protein storage vacuole in a Golgi-independent manner in Arabidopsis
188 seedlings (Poustka et al., 2007). On the same line, several works demonstrated that
189 autophagy mechanisms have a role in anthocyanin transport to the vacuole (Külich
190 and Zarsky, 2014; Chanoca et al., 2015). Whether these anthocyanin trafficking
191 routes are also used by flavanols remains to be investigated (Bassham, 2015;
192 Chanoca et al., 2015).

193 The molecular mechanisms by which TT15 and SG regulate seed development and
194 flavanol accumulation in the seed coat are still unclear.
195 Here, we show that *TT15* disruption causes seed lethality with variable penetrance
196 and maternal determinism that is correlated with impaired endosperm
197 cellularization. We also reveal that vacuole biogenesis in endothelial cells is
198 affected, leading to their premature degeneration and death and consequently
199 to a *tt* phenotype which is associated with a polarized accumulation of radical
200 oxygen species (ROS) and callose at the curving zone. Premature endothelium cell
201 death (PECD) is suppressed in the absence of flavanols. The TT15 protein was
202 located mainly at the vacuolar membrane or tonoplast and demonstrated to
203 decrease its fluidity. Consistent with these findings, TT15 was shown to genetically
204 interact with TT9, a protein involved in vacuole biogenesis. We propose that TT15
205 and SG are required for the modulation of vacuole functions required for proper
206 endothelium-endosperm crosstalk and flavanol deposition. As a consequence, they
207 reinforce seed dormancy by increasing seed coat impermeability properties. We
208 also discuss the potential roles of TT15 and SG as links between vacuole, flavonoids
209 and seed development in the establishment of post-zygotic reproductive barriers.
210

211 RESULTS

212 The *tt15* Mutations Cause Seed Lethality and Seedling Developmental Defects 213 with Incomplete Penetrance

214 Previous studies on the characterization of *tt15* mutants have identified the
215 perturbation of several important seed traits and established the pleiotropic
216 complexity of the mutations using only one allele either in Columbia (Col) (Focks et
217 al., 1999; Stucky et al., 2015) or Wassilewskija (Ws) background (DeBolt et al.,
218 2009; Routaboul et al., 2012). To progress further in our understanding of TT15
219 functions, we started our work with an allelic series of three alleles in Col-0 and three
220 alleles in Ws-4 from the Versailles T-DNA collection (Supplemental Table S1). The
221 nature and position of the mutations are shown in Supplemental Figure S3A. The
222 alleles harbour a similar pale grayish brown seed coat colour (Supplemental Figure
223 S3B). Sterol profiling of the *tt15-2* allele in Ws-4 background showed a reduction in
224 SG and ASG (Supplemental Figure S3C), as expected for a mutant affected in a
225 sterol glucosyl transferase and similarly to the previously characterized *tt15* alleles
226 by DeBolt et al. (2009) and Stucky et al. (2015).

227 Reciprocal crosses between *tt15-2* and wild type Ws-4 (Table 1) showed that the
228 seed colour phenotype is maternally inherited, which is consistent with the fact that
229 the seed coat originates from the ovule integuments. They also revealed that the
230 *tt15-2* mutation, which behaves as a recessive trait, exhibits a slightly reduced
231 transmission through the female parent. Indeed the number of F2 plants producing
232 a *tt15* phenotype was lower than the number expected for Mendelian inheritance of
233 a recessive mutation (3:1 ratio *TT15:tt15* seeds).

234

235 **Table 1.** Genetic Determinism and Transmission of the *tt15-2* Seed Coat Colour.
236

Cross (♀ x ♂)	F1 seeds	F2 seeds	F3 seeds	
Ws-4 x <i>tt15-2</i>	[TT15]	[TT15]	57* [TT15]	18 [tt15] a ns
<i>tt15-2</i> x Ws-4	[tt15]	[TT15]	62 [TT15]	11 [tt15] b *

237

238 * Number of F2 plants exhibiting the phenotype

239 a : $\text{Khi}^2 (3 : 1) = 0.04125$

240 b : $\text{Khi}^2 (3 : 1) = 3.84018$

241 ns: not significant

242

243 Systematic observations of *tt15-2* seed batches below binoculars directly after
244 harvest without cleaning (Figure 1, A and B) revealed four main phenotypic classes

245 based on embryo development (Figure 1C). Observations done on a bulk of 3801
246 seeds harvested from three independent *tt15-2* plants established that around
247 88.5% seeds resembled wild-type seeds (class I), 8% were smaller seeds at the
248 cotyledonary stage (class II), 0.8% seeds were mostly at the walking stick stage
249 (class III), and 1.8% were aborted seeds with precocious arrest of embryo
250 development at globular to torpedo stages (class IV). All seeds were able to
251 germinate, except the ones from classes III and IV. Additionally a few seeds from
252 class I (around 0.5% from total seeds) revealed extreme testa weakness at their
253 abaxial pole, letting the embryo partially exit in the course of their development and
254 growth in the silique (Figure 1D). These observations led us to conclude that embryo
255 abortion in *tt15-2* exhibits an incomplete penetrance. Because variable frequencies
256 of seed abortion were frequently observed in the greenhouse upon challenging
257 growth conditions, seed lethality (aborted seed number) was quantified with seed
258 progenies from plants grown in optimal environmental conditions in a growth
259 chamber (Figure 1, E and F). Twenty five siliques (five siliques from five plants)
260 developed from tagged flowers were harvested at 15 daf for *tt15-2*, *tt15-9* and their
261 corresponding wild types Ws-4 and Col-0, respectively. Silique clearing enabled to
262 distinguish aborted seeds as brown and flattened envelopes (Figure 1E,
263 arrowheads). Aborted ovules appearing as white dried structures were not observed
264 in most siliques. Differences in levels of chlorophyll breakdown between WT and
265 mutants and between both WTs were also observed, probably revealing
266 discrepancies in the timing of seed maturation. The mean percentage of seed
267 lethality was significantly higher in mutants compared with corresponding WTs.
268 Abortion was also higher in mutant in Col-0 background than in mutant in Ws-4
269 background (Figure 1F). Abortion scores per silique oscillated between 0% and
270 36.7% for *tt15-2* and between 10% and 60% for *tt15-9* compared with 0% to 5.4%
271 in Ws-4 and 0% to 16.1% in Col-0 (Supplemental Table S3). To determine which seed
272 compartment controls embryo abortion in a *tt15* mutant background, we performed
273 reciprocal crosses between *tt15* and corresponding wild types. Our data showed that F1
274 seed lethality caused by *tt15* is maternally determined (Figure 1G ; Supplemental Table
275 S3).

276 The pattern of seed coat pigmentation in *tt15-2*, with its characteristic dark brown
277 chalaza-micropyle area and pale seed body (Figure 1B, inset ; Supplemental Figure
278 S4A) is similar to the ones of *tt1-4*, *tt9-1* and *tt16-1* mutants (Supplemental Figure

279 S4, B-D, insets). Intriguingly, these three mutants also exhibit some seed abortion.
280 DeBolt et al. (2009) have shown that *ugt80B1*, a *tt15* allele from the University of
281 Wisconsin T-DNA collection (Sussman et al., 2000) exhibited a reduced seed
282 weight. We confirmed these results with our alleles and showed that weight
283 reduction was consistent with a smaller seed size as expected, but without
284 modification in seed shape (Supplemental Figure S5).
285 Other phenotypes expressed with incomplete penetrance concern seedling
286 development. If most mutant seedlings in a plant progeny harbor a wild-type
287 phenotype (Supplemental Figure S6, A-C), a small fraction (between 1 to 2%)
288 exhibits various developmental defects such as an aberrant cotyledon number
289 (tricotily, monocotyly, no cotyledons ; Supplemental Figure S6, D, E and I,
290 respectively), white cotyledon tips (Supplemental Figure S6F), root-like structures
291 at cotyledon tips (Supplemental Figure S6G), transformation of a cauline apex into
292 a root-like apex (Supplemental Figure S6H). Atypical adhesion of the aleurone layer
293 (peripheral endosperm) and seed coat to the root tip and an irregular epidermal
294 surface were also observed (Supplemental Figure S6, H and I). Such phenotypes
295 could be observed with all alleles, especially in seed classes II and III.

296

297 **Sensitivity of Seed Germination to Exogenous Abscisic Acid and** 298 **Paclobutrazol is Increased in *tt15* Mutant Backgrounds**

299 A collection of *tt* mutants affected in flavanol metabolism in the seed coat was
300 previously shown to exhibit a reduced primary dormancy, positively correlated with
301 an increased testa permeability to tetrazolium salts (Koornneef, 1981; Debeaujon et
302 al., 2000). Here we could extend these observations to *tt15* mutant seeds and
303 explored further the germination phenotype using a *tt15* allelic series in *Ws-4* and
304 *Col-0* backgrounds. To avoid a bias due to the seed lethality phenotype (see above),
305 seed lots were cleaned before use to remove the dead seed fractions corresponding
306 to classes III and IV. Freshly harvested seeds from the *tt15* alleles in *Ws-4*
307 background exhibited a reduced primary dormancy. For *tt15* alleles in *Col-0*
308 background, differences in dormancy were less significant than in *Ws-4*
309 (Supplemental Figure S7A). Because a *tt7* mutant affected in the production of
310 dihydroquercetin derivatives (Supplemental Figure S2) was previously
311 demonstrated to be resistant to thermoinhibition (Tamura et al., 2006), we were
312 tempted to investigate *tt15* seed germination tolerance to high temperature. We

313 used a temperature of 34°C previously shown to completely inhibit *Arabidopsis* wild-
314 type seed germination (Tamura et al., 2006), compared to a control temperature of
315 22°C. However the observed differences were not statistically significant
316 (Supplemental Figure S7B). On the other hand, seed coat permeability to
317 tetrazolium salts was increased similarly in all alleles compared with their wild types
318 (Supplemental Figure S7C).

319 To determine whether *tt15* reduced seed dormancy may also have an hormonal
320 component residing in endosperm and/or embryo beside the seed coat
321 physicochemical defects, we assessed *tt15* seed germination response to
322 increasing doses of the dormancy inducer and germination inhibitor ABA after
323 stratification. Germination *sensu stricto* of *tt15* seeds estimated by the percentage
324 of seeds exhibiting radicle protrusion through the seed envelopes was clearly more
325 sensitive to ABA than the corresponding wild types in both accession backgrounds
326 (Figure 2A). Cotyledon greening during photoautotrophic seedling establishment
327 was affected only with the alleles in Ws-4 background and not with the ones in Col-
328 0 (Figure 2B). However hypersensitivity of cotyledon greening to the GA
329 biosynthesis inhibitor paclobutrazol (PAC) was more contrasted between mutants
330 and their wild types, and shared by both accession backgrounds (Figure 2C),
331 possibly pointing to a chloroplast biogenesis defect involving GA in *tt15*,
332 independently from the ABA-related component influencing germination *sensu*
333 *stricto*. Dry Ws-4 seeds exhibited more ABA than Col-0 seeds, which may explain
334 the differential seed dormancy and germination behaviours of Ws-4 and Col-0. On
335 the other hand, *tt15* mutant seeds did not exhibit any reduction in ABA compared
336 with their corresponding wild types (Supplemental Figure S7D). Hypersensitivity to
337 exogenous ABA would explain the increased need for GA to germinate suggested
338 by hypersensitivity to PAC. Thus in *tt15* mutant seeds either GA biosynthesis and/or
339 ABA catabolism upon imbibition may be affected, increasing the ABA/GA ratio, or
340 ABA and/or GA signalling are impacted. Measuring the levels of ABA and GA during
341 imbibition may help answer this question. Another non exclusive option would be
342 that testa permeability to both exogenous germination inhibitors is increased in *tt15*
343 seeds due to their flavanol defect. Indeed *tt4-8* mutant seeds deprived of flavonoids
344 because of a mutation in the chalcone synthase gene (Supplemental Figure S2)
345 exhibited the same dose-response curves to ABA and PAC than *tt15* alleles in Ws-
346 4 (Figure 2D). Altogether these data suggest that *tt15* mutant seeds exhibit a

347 germination syndrome with pleiotropic effects, which physiological analysis is
348 complexified by altered seed coat permeability.

349

350 **Loss of TT15 Affects Endothelium and Endosperm Development**

351 A histological analysis of developing seed structures was undertaken to understand
352 further the mechanisms leading to the seed coat and seed lethality phenotypes in
353 *tt15* mutants. Toluidine blue O (TBO) staining of class-I seed sections revealed that
354 the *tt15-2* endothelium (ii1) layer had dramatically crushed and degenerated in the
355 course of PC accumulation (Figure 3C compared to Figure 3A). PC stained dark
356 blue with TBO fill the vacuole in wild-type endothelial cells (Figure 3B). However in
357 *tt15-2* endothelial cells they appear as a flattened amorphous aggregate (Figure 3,
358 D and F, arrowheads), suggesting that vacuole disruption may have occurred.
359 Intermediate levels of ii1 cell degeneration, apparently taking place in a stochastic
360 way, were observed (Figure 3, E and F, arrows). Aberrant endosperm cellularization
361 and enlarged nodules especially at the chalazal pole were also observed in a few
362 malformed (class III) *tt15-2* seeds around 6 days after flowering (late heart to
363 torpedo stage of embryo development) when the endosperm is supposed to be
364 cellularized (Figure 3, G and H). The same observation was made with *tt15-9* in Col-
365 0 background (Supplemental Figure S8, A-D). A reduction of PC accumulation is
366 not the cause of endothelium crushing because the endothelium of the chalcone
367 synthase null mutant *tt4-8* (Ws-4 background) deprived of any flavonoid including
368 PC exhibits well formed cells (Figure 3, I and J). Interestingly, the endothelium layer
369 of the double mutants *tt15-2 tt4-8* (Figure 3, K and L) and *tt15-2 ban-1* accumulating
370 anthocyanins in place of PC in endothelium (Supplemental Figure S9, C, D, G and
371 H) is not crushed. Altogether these observations strongly suggest that flavanols (EC
372 and/or PC) may specifically induce *tt15-2* endothelium cell death leading to
373 precocious cell crushing. Whole-mount cleared seeds observed with differential
374 interference contrast (DIC) microscopy revealed endothelial cell wall thickening and
375 browning at the abaxial pole of the seed body (curving zone ; Supplemental Figure
376 S4A) in *tt15-2* seeds (Figure 3, M and N). Histochemical staining with the flavanol-
377 specific reagent vanillin enabled to confirm the endothelial nature of the affected
378 layer and confirmed cell wall thickening and browning both in *tt15-2* and *tt15-9*
379 (Figure 3, O-Q ; Supplemental Figure S8, E-G). As previously shown by Pourcel et
380 al. (2005), flavanol oxidation in Arabidopsis seed coat forms brown products. It is

381 therefore very likely that the brown reaction observed here is due to flavanol
382 oxidation, especially as it is absent in *tt4-8* and *tt15-2 tt4-8* backgrounds
383 (Supplemental Figure S9).

384 Some seeds exhibit endothelium rupture probably due to exacerbated mechanical
385 tension after cell wall overthickening (Figure 3R ; Supplemental Figure S8H). This
386 polarized thickening at the abaxial side of the seed body defines a weakness zone
387 which may enable the expanding embryo to be expelled from the testa (Figure 1D).
388 To analyze further the cellular mechanisms at play during endothelium polarized
389 modification, we performed a histochemical detection of reactive oxygen species
390 (ROS) in developing seeds using the fluorochrome DCFH-DA. Interestingly ROS
391 were present and shown to accumulate essentially at the abaxial pole of the
392 endothelium (Figure 4, A and B). During immune response, ROS such as H₂O₂ are
393 known to trigger the accumulation of callose, a β -1,3-linked glucose polymer (Luna
394 et al., 2011). Here, using aniline blue staining, we could also detect ectopic callose
395 deposition in endothelium (Figure 4, C and D).

396

397 **Spatio-Temporal Analysis of Promoter Activity and mRNA Shows that TT15 is** 398 **Expressed in Reproductive and Vegetative Tissues**

399 A 2.0-kb DNA sequence upstream of the ATG translation codon was used to
400 establish the spatio-temporal pattern of *TT15* promoter activity in Arabidopsis Ws-4
401 stable transformants expressing the *pTT15:uidA* construct (Figure 5). As expected
402 from *tt15* mutant seed phenotypes, promoter activity was detected in endothelium
403 (Figure 5, A, J and K), embryo (Figure 5, B, C and L), endosperm (Figure 5, I and
404 L), germinating seeds and young seedlings (Figure 5, F, G and H). Activity was also
405 detected in mucilage layer (oi2 outer integumentary layer ; Figure 5, B, J and K),
406 chalaza-funiculus continuum (Figure 5, A and J), seed abscission zone (Figure 5,
407 B-D) and unfertilized necrotic ovules (Figure 5E). As shown in Supplemental Figure
408 S10, the *TT15* promoter was activated in other reproductive organs (ovule primordia
409 and ovules, gynoecium, style, transmitting tract, nectaries and pollen grains) and
410 also in various vegetative tissues (cauline and root meristems, lateral root primordia,
411 root cap, vascular bundles from cotyledons, leaves, roots and stems, stomata, and
412 hydathodes). The stronger *tt15* mutant phenotypes observed in Ws-4 background
413 compared with Col-0 prompted us to perform a quantitative polymerase chain
414 reaction (qPCR) analysis of *TT15* expression in both accessions (Supplemental

415 Figure S11). This analysis did not reveal any significant differences except in the
416 amount of stored mRNA in dry seeds, which was higher in Ws-4 than in Col-0. A
417 peak of expression was also observed in senescing siliques of both accessions.
418 Interestingly *TT15* mRNA stored in mature seed disappears during imbibition until
419 resuming when early seedling growth occurs. Altogether, our experimental data
420 corroborated by public transcriptomes (Supplemental Figure S12) strongly argue
421 towards a role for *TT15* not only in seed development and germination but also in
422 seedling growth.

423

424 **TT15 Co-localizes with Tonoplast and Amyloplast Markers and is also Present** 425 **in Cytoplasm**

426 To determine the subcellular localization of the *TT15* protein, transgenic *Arabidopsis*
427 plants expressing the green fluorescent protein (GFP)-*TT15* and *TT15*-GFP
428 constructs placed under the control of a dual 35S promoter were generated
429 (Supplemental Figure S13A). Both constructs complemented *tt15-2* seed coat
430 phenotypes, *i.e.* rescued wild-type seed coat colour (Supplemental Figure S13B)
431 and vacuolar deposition of flavanols was restored in the transformants
432 (Supplemental Figure S13C). Seed flavonoid profiling of the *tt15-2* allele showed a
433 strong reduction of flavanols, including epicatechin hexoside (EC-H ; Supplemental
434 Figure S13D), which is consistent with the results from the vanillin histological
435 assay. A significant reduction in the flavonol quercetin 3-O-rhamnoside or QR
436 (Supplemental Figure S13D) was also observed. We detected minor variations in
437 other flavonols as well. All modifications were rescued in transformants.
438 Supplemental Table S2 displays the complete list of flavonoid compounds identified
439 by our UPLC-MS analysis in wild-type mature seeds. After having checked that the
440 same localization pattern was observed with *TT15*-GFP (Supplemental Figure S14),
441 the following part of our study focused on GFP-*TT15*.

442 Confocal imaging microscopy of GFP-*TT15* in developing seeds was realized on
443 the mucilage layer (oi2) of developing seed coat. Imaging at the level of the
444 endothelium (ii1) was not possible due to a weak activity of the 35S promoter in this
445 cell layer. The presence of GFP fluorescence was revealed at the tonoplast, at
446 localized regions of amyloplasts and in the cytosol (Figure 6, A-C ; Supplemental
447 Figure S14A). In cotyledon epidermis, tonoplast fluorescence was particularly
448 conspicuous at the level of stomata, probably because of the presence of numerous

449 vacuolar convolutions in guard cells (Figure 6D ; Supplemental Movie S1). However
450 no obvious signal was observed at the level of chloroplasts. GFP-TT15 at the
451 tonoplast was also observed in seedling roots (Supplemental Figure S14, E-G). As
452 imaged in Supplemental Movies S2 and S3, GFP-TT15 was also located in mobile
453 aggregates or vesicles of various sizes present in the cytosol and also in vacuolar
454 lumen where their movement seemed to be constrained by the tonoplast. The
455 identity of these structures remains to be elucidated.

456 To progress further in the identification of GFP-TT15 intracellular locations,
457 fluorescent markers for various subcellular compartments were introduced in GFP-
458 TT15 transformant background by crosses (Figure 6E ; Supplemental Table S4).
459 GFP-TT15 fluorescence co-localized with the tonoplastic marker mCherry-
460 VAMP711 in epidermis, columella and lateral root cap of seedling roots (Figure 6F).
461 It also partially co-localized with markers mCherry-RabG3c and mCherry-RabG3f at
462 the level of tonoplast but not at the level of multivesicular body (MVB)/prevacuolar
463 compartments (PVC ; Figure 6, G and H). On the other hand, no co-localization of
464 GFP-TT15 was observed with Golgi apparatus, trans Golgi network (TGN)/early
465 endosome (EE), late endosome (LE)/multivesicular body (MVB)/prevacuolar
466 compartment (PVC) and plasma membrane (PM) (Supplemental Figure S15).
467 Intriguingly, the intravacuolar mobile aggregates identified by GFP-TT15 in roots,
468 as mentioned above, happened to co-localize with all our subcellular compartment
469 markers (Figure 6, F-H, white arrowheads ; Supplemental Figure S14, A-F, white
470 arrowheads).

471

472 **Alteration of Vacuole Development in Endothelium Causes a *transparent testa*** 473 **Phenotype**

474 We have shown that *tt15-2* seeds at the heart stage of embryo development (Figure
475 3P) exhibit endothelial cell breakdown causing a strongly reduced flavanol
476 accumulation, compared with wild-type endothelial cells having central vacuoles
477 filled with flavanols (Figure 3O). We failed to detect any significant defect in
478 expression of flavonoid biosynthetic and regulatory genes in developing seeds
479 (Supplemental Figure S16), which suggests that the lower amount of accumulated
480 flavanols in *tt15-2* endothelial cells is rather due to abnormal cell development. To
481 progress further in the identification of the disrupted cellular functions in *tt15-2*, we
482 observed the dynamics of vacuole morphogenesis in endothelial cells during seed

483 development using vanillin-stained flavanols as vacuolar markers (Figure 7). The
484 early spatio-temporal pattern of flavanol deposition is similar between *tt15-2* and
485 wild type (Figure 7, A and E). A difference becomes visible at the globular stage,
486 with endothelial cells stopping vacuole filling and starting a progressive breakdown
487 (Figure 7, B-D and F-H). We named this degeneration process PECD for Premature
488 Endothelium Cell Death, to distinguish it from Programmed Cell Death (PCD)
489 occurring in wild-type endothelium at later stages of seed development, as
490 described by Andème Ondzighi et al. (2008). The PECD syndrome is visible first,
491 and is the most intense at the abaxial pole of the seed (curving zone). From the
492 heart stage onwards, most *tt15-2* endothelial cells (region 2 ; Supplemental Figure
493 S4A) look empty (Figure 7H) by comparison with wild-type cells filled with flavanols
494 (Figure 7D). Only the micropyle (region 1) and chalaza (region 3) exhibit flavanols
495 in *tt15-2* seeds. We observed frequently a few endothelial cells that, apparently with
496 a stochastic pattern, exhibited transient flavanol accumulation. Subcellular
497 organization at the octant stage is similar between both genotypes (Figure 7, I and
498 M). Vacuolar morphology is predominantly roundish with intensely stained tonoplast
499 and intravacuolar aggregates. Differences between *tt15-2* and wild type are obvious
500 from the globular stage onwards (Figure 7, J-L and N-P). Wild-type vacuole
501 morphology evolves from roundish to elongated. Vacuole lumen accumulates
502 substructures resembling small vesicles with stained membranes that start to
503 accumulate flavanols (Figure 7, J and K). At the heart stage, these substructures fill
504 completely the endothelial vacuole, which volume is constrained by the surrounding
505 cell wall (Figure 7L). In *tt15-2* mutant, vacuole morphogenesis is blocked at the
506 globular stage (Figure 7N). Indeed the roundish vacuoles do not elongate and very
507 limited substructural organization of the lumen is taking place. Different stages of
508 evolution can be observed in the same endothelium. The degeneration process
509 ends with cell death as suggested by vacuole disappearance in most endothelial
510 cells at the heart stage.

511

512 **Tonoplast Fluidity is Increased in *tt15* Roots**

513 Knowing that SG and ASG are components of plant tonoplasts (Yoshida and
514 Uemura, 1986; Tavernier et al., 1993; Yamaguchi and Kasamo, 2001) and that they
515 have the ability to efficiently order membranes and thus modulate their fluidity or
516 viscosity (Laloi et al., 2007; Halling et al., 2008; Grosjean et al., 2015) prompted us

517 to investigate whether *tt15-2* tonoplast fluidity was modified. Fluorescence recovery
518 after photobleaching (FRAP) was carried out to quantitatively monitor the lateral
519 diffusion characteristics of the tonoplast in epidermis of Arabidopsis seedling roots
520 (Figure 8). Membrane labeling was done with the fluorescent probe GFP-NRT2.7
521 (Supplemental Table S4). NRT2.7 is an Arabidopsis nitrate transporter located
522 specifically at the tonoplast (Chopin et al., 2007). The probe was introduced in *tt15-2*
523 background by crossing. After having checked that no alteration of GFP-NRT2.7
524 location was observed in the mutant compared with the wild type (Figure 8A),
525 photobleaching was performed with both genotypes at the tonoplast of cells from
526 the root elongation zone (Figure 8B). Two parameters $t_{1/2}$ (recovery half-life) and M_f
527 (mobile fraction) were used to describe membrane lateral mobility in quantitative
528 analysis (Figure 8, C and D). The recovery half-life $t_{1/2}$ provides a measure of the
529 half-life recovery time and M_f indicates the fraction of fluorescent molecules
530 recovered into the bleached region. As shown in Figures 8C and 8D, the level of
531 GFP-NRT2.7 was restored to 83% at 40 s in the bleached ROI in *tt15-2* mutant
532 ($M_f=83\%$; $L_{inf}=67\%$ and $L_{sup}=96\%$), while there was a 79% recovery in that of wild
533 type Ws-4 ($M_f=79\%$; $L_{inf}=63\%$ and $L_{sup}=95\%$). These results revealed that a
534 significantly lower proportion (17%) of GFP-NRT2.7 was present as the immobile
535 fraction (I_f) in *tt15-2* mutant as compared with the 21% in wild type. A significant
536 difference was also observed for the recovery rate, with $t_{1/2}$ of mutant and wild type
537 being 4.505 s and 6.875 s, respectively. Altogether these data demonstrate that
538 decreasing the level of SG increases tonoplast fluidity, thus alters its dynamics.

539

540 **The *tt15* Mutation Partially Rescues the Vacuolar Phenotype of *tt9* in** 541 **Endothelium**

542 Looking for functional relationships between TT15 and other TT proteins which
543 mutants have a similar pattern of flavanol deposition in the seed coat, namely TT9,
544 TT1 and TT16, may put some light on the mechanisms involved in *tt15* endothelium.
545 Here, we focused on TT9 because, as a peripheral membrane protein involved in
546 vacuolar development and trafficking (Ichino et al., 2014), it appeared the most likely
547 candidate to fulfill our objectives.

548 As shown above, *tt15-2* (Figure 1B) and *tt9-1/gfs9-4* (Supplemental Figure S4C)
549 exhibit a similar patterning of seed coat pigmentation, with only the endothelium
550 being defective in PC accumulation. Interestingly whole-mount seed clearing

551 (Figure 9B), TBO-stained sections of developing seeds (Figure 9D) and vanillin
552 assay for flavanol detection (Figure 9E) also revealed strong similarities between
553 *tt9-1* and the *tt15-2* phenotypes presented in Figure 3, notably endothelium
554 degeneration in the course of flavanol accumulation ending with endothelium cell
555 death together with cell wall thickening and browning at the curving zone. (Ichino et
556 al., 2014) previously revealed that *tt9* mutants exhibit vacuole fragmentation using
557 light microscopy and transmission electron microscopy analysis of endothelium.
558 Here, this vacuolar phenotype could be confirmed through the histological detection
559 of flavanols using TBO (Figure 9D arrow) and vanillin (Figure 9, E and G). We did
560 not observe vacuolar fragmentation with *tt15* mutants (Figure 3H and Supplemental
561 Figure S17). These observations prompted us to determine the epistasis
562 relationships between both mutations by constructing the double mutant *tt15-2 tt9-*
563 *1*. Double mutant seeds exhibited a novel seed coat pigmentation (Figure 9L
564 compared with Figure 9, I-K) with an absence of differential patterning between the
565 micropyle-chalaza region and the seed body and a different overall colour.
566 Importantly the *tt9-1* mutation suppressed the *tt15-2* PECD phenotype (Figure 9F).
567 Moreover a novel vacuolar morphology and luminal organization could be observed
568 (Figure 9H), where flavanols are detected only in vesicle-like structures aggregating
569 outside the tonoplast of medium-sized vacuoles. Altogether these data demonstrate
570 that TT15 and TT9 genetically interact in the sense that they both contribute to
571 vacuole development, however in partially overlapping biochemical pathways.
572

573 **DISCUSSION**

574 Here we have identified a function for the sterol-3- β -glucosyltransferase TT15 in
575 regulating vacuolar membrane characteristics and vacuole functions in Arabidopsis.
576 In addition to revealing a seed lethality phenotype with variable penetrance and an
577 increased sensitivity of seed germination to exogenous inhibitors, our work brought
578 novel informations on the cellular mechanism causing the pale seed coat colour
579 phenotype and revealed a genetic interaction between TT15 and TT9, a protein
580 involved in vacuolar biogenesis and vesicular trafficking. The analysis of an allelic
581 series of six *tt15* mutants in two different accessions was important to confirm the
582 robustness of the observed phenotypes and to determine which part of the
583 variations was imparted to the genetic background. Taken together, our data
584 suggest an involvement of the vacuole, SG and flavonoids in the observed traits, as
585 discussed below.

586

587 **TT15 is Essential for Seed Development and Germination**

588 A seed lethality phenotype exhibiting incomplete penetrance and maternal inheritance
589 was observed for both *tt15-2* and *tt15-9* mutant alleles in *Ws-4* and *Col-0*
590 backgrounds, respectively. Moreover an endosperm cellularization defect was
591 detected in the most affected seeds. The percentage of aborted seeds was
592 significantly higher in *Col-0* than in *Ws-4* background. Moreover the proportion of
593 dead seeds was non-mendelian, modulated by environmental conditions and higher
594 when the *tt15-2* mutation was transmitted by the female parent. This developmental
595 syndrome reminds lethality of Arabidopsis F1 hybrids caused by interploidy and
596 interspecific crosses between diverged parents, namely the triploid block. The
597 endosperm plays a central role in this gene dosage-sensitive incompatibility,
598 together with the maternally expressed WRKY transcription factor TTG2 which
599 disruption suppresses the triploid block, decreases endothelial cell elongation
600 leading to smaller seeds and causes precocious endosperm cellularization (Garcia
601 et al., 2005; Dilkes et al., 2008; Burkart-Waco et al., 2013; Köhler et al., 2021). The
602 *tt4* and *tt8* mutations affecting chalcone synthase and a bHLH transcription factor,
603 respectively, also act as maternal suppressors of triploid block (Buer and Muday,
604 2004; Doughty et al., 2014; Zumajo-Cardona et al., 2023), which points to
605 endothelial flavanols as potential inducers of seed lethality in interspecies and
606 interploidy crosses. As *tt15* mutants have small seeds (DeBolt et al., 2009)(this

607 study), a situation mimicking to some extent a maternal-excess scenario where
608 maternally-expressed genes are less imprinted and thus are more expressed in the
609 endosperm is likely and would be consistent with the fact that the mutation is less
610 transmitted through the ovule. However endosperm with defective cellularization
611 reminds a paternal-excess situation, which suggests that genes overexpressed on
612 the paternal side may also interfere with seed development in absence of *TT15*. The
613 fact that *TT15* is expressed both in the endosperm and the seed coat (this study)
614 complexifies the understanding of the *tt15* seed development phenotypes.

615 The moment at which the endosperm cellularizes after a phase of free nuclear
616 divisions without cytokinesis (endosperm proliferation) is a crucial determinant of
617 seed size (Sorensen et al., 2002; Hehenberger et al., 2012; Doughty et al., 2014).
618 Upon fertilization, the auxin phytohormone produced in the central cell of the embryo
619 sac and afterwards in endosperm triggers seed coat differentiation (Figueiredo et
620 al., 2016). Increased biosynthesis and signalling of auxin in the endosperm prevents
621 its cellularization and leads to seed developmental arrest, thus phenocopying the
622 phenotype of paternal-excess triploid seeds (Batista et al., 2019). Moreover
623 flavonoids are negative regulators of auxin transport (Buer and Muday, 2004). In
624 this context, we hypothesize that small seed size, seed lethality and endosperm
625 cellularization defects observed in *tt15-2* may be correlated with a perturbation of
626 auxin homeostasis. Relevant with this hypothesis is the presence of seedlings with
627 abnormal cotyledon number in the progeny of some *tt15-2* plants revealing stem cell
628 niche perturbation (this study). Such a phenotype is regularly observed in situations
629 when auxin intracellular transport is affected, as for instance in mutants affected in
630 sterol homeostasis (Souter et al., 2002), in vacuolar sorting of auxin carriers (Jaillais
631 et al., 2007) or in auxin-mediated ribosomal biogenesis regulating vacuolar
632 trafficking (Rosado et al., 2010). It would be interesting to investigate by a
633 microscopic approach whether auxin sensors such as *DR5-uidA* are deregulated in
634 *tt15* developing seeds.

635 Seeds of *tt15* exhibit a pleiotropic germination syndrome. Consistent with the
636 situation prevailing with mutants which seed coat is affected in flavanol metabolism
637 (Debeaujon et al., 2000) and/or lipid polyester (cutin, suberin) deposition (Molina et
638 al., 2008), previous studies have shown that *tt15* mutant seeds exhibit a reduced
639 seed dormancy and an increased testa permeability to tetrazolium salts (Focks et
640 al., 1999; DeBolt et al., 2009; MacGregor et al., 2015; Loubéry et al., 2018). In our

641 laboratory conditions and with all our alleles in two accession backgrounds (Col-0
642 and Ws-4) we could confirm a reduced primary dormancy and an increased testa
643 permeability to tetrazolium salts. Moreover we observed an hypersensitivity to the
644 germination inhibitors ABA and PAC brought exogeneously, as previously reported
645 for several other *tts* (Debeaujon and Koornneef, 2000; Tamura et al., 2006).
646 Increased testa permeability to ABA and PAC may be responsible for this
647 phenotype, especially knowing that *tt15* cumulates defects in both flavanol and lipid
648 polyester metabolisms (DeBolt et al., 2009). Already we showed here that ABA
649 levels in dry seeds are unmodified. Our analysis also showed the existence of a
650 natural variation for ABA content in dry seeds, with Ws-4 having more ABA than
651 Col-0.

652

653 **TT15 disruption Causes Premature Endothelium Cell Death**

654 Wild-type Arabidopsis endothelium undertakes PCD, starting at the torpedo embryo
655 stage and being effective around the bent-cotyledon stage. It progresses from the
656 abaxial zone (curving zone) towards chalaza and micropyle, as the cellular
657 endosperm expands (Andème Ondzighi et al., 2008). Here, we revealed that the
658 *tt15* endothelium exhibits PECD, a precocious degeneration being visible from the
659 globular stage onwards and ending in cell death. Contrarily to the situation observed
660 in WT PCD, the *tt15* endothelium layer completely collapses, with the exception of
661 a few cells due to a weak penetrance of the phenotype. The cellular mechanisms
662 associated with *ii1* premature cell death progression from the globular stage of
663 embryo development onwards involve the interruption of vacuole development
664 followed by vacuolar collapse probably due to tonoplast lysis. The vacuole is a
665 central player in the execution of cell death (Shimada et al., 2018). Under this
666 scenario, flavanols that have started to accumulate in *ii1* vacuoles may leach into
667 the cytosol and migrate towards the cell walls where they would be oxidized into
668 brown products possibly by resident laccases and peroxidases and cross-link with
669 polysaccharides (Pourcel et al., 2005).

670 We observed premature degeneration leading to cell death at the abaxial pole of
671 the *tt15* endothelium, which was correlated with H₂O₂ production and callose
672 deposition. This cellular response reminds the signature of autoimmunity or lesion
673 mimic syndrome triggered by a spontaneous deregulation of nucleotide-binding
674 domain leucine-rich repeat (NLR) receptors that are normally engaged in effector-

675 triggered immunity upon pathogen attack (Ben Khaled et al., 2015; Freh et al.,
676 2022). The trigger of this polarization may be mechanical stress at the level of the
677 curving zone to which the *tt15* mutant endothelium would respond by exacerbated
678 cell death and wall thickening. Creff et al. (2015) showed that the adaxial epidermis
679 of the outer integument (oi1 cell layer) is a mechanosensitive cell layer responding
680 to the mechanical stress exerted by the expanding embryo and endosperm by cell
681 wall thickening. In this context, we speculate that the endothelium or adaxial
682 epidermis of the inner integument (ii1 cell layer) may also be a mechanosensitive
683 cell layer responding to filial tissue expansion, which pressure would be higher at
684 the curving zone. The reason why this response is exacerbated and leads to a HR-
685 type of cellular mechanism in a *tt15* background deserves to be explored further.
686 On the same line, Burkart-Waco et al. (2013) reported that a perturbation of the
687 communication between endosperm and maternal tissues in Arabidopsis
688 interspecific crosses caused the activation of defense-like responses. Perturbation
689 of *tt15* tonoplast homeostasis through its modified SG composition may possibly
690 affect its resistance and/or the function of resident proteins such as the tonoplast-
691 located mechanosensor PIEZO (Radin et al., 2021). As a corollary, 1-2% *tt15*
692 mature seeds exhibits some vivipary, with an atypical emergence of the embryo
693 from the seed coat at the abaxial pole of the seed suggesting perturbed cell wall
694 integrity at the curving zone. We hypothesize that due to increased thickening
695 caused by callose deposition, the *tt15* cell wall loses its extensibility and thus its
696 resistance to embryo growth, creating a weakness zone.

697 Previous works demonstrated that recombinant TT15 could catalyze the
698 glucosylation of free sterols *in vitro*, which was relevant with a reduction of
699 glucosylated sterols in *tt15* mature seeds (DeBolt et al., 2009; Stucky et al., 2015)
700 (this study). Perturbation of sterol homeostasis is therefore likely to explain the
701 pleiotropic phenotypes of the *tt15* mutants. Sterols serve multiple biological
702 functions, from structural components of membranes to signaling molecules as
703 precursors of BR (Clouse, 2002; Zhang et al., 2015). Shimada et al. (2021) observed
704 that excess sterol led to the development of a darker seed coat due to
705 proanthocyanidin overaccumulation. Cell death-mediated shrinkage of the inner
706 integument was also impaired, resulting in a thicker seed coat and a delayed seed
707 germination. Intriguingly, these phenotypes seem opposite to the ones observed
708 with the *tt15* mutant, which may be explained by the fact that excess free sterols

709 that are toxic to the cell machinery may possibly be neutralized through
710 glucosylation by TT15 (Supplemental Figure S1). Altogether, these observations
711 suggest the existence of a complex interplay between the developing endothelium
712 and endosperm involving TT15, SG and flavanols, which perturbation causes
713 endothelium degeneration and endosperm cellularization defects in a gene- or
714 presumably auxin signal- dosage-dependent manner. In this context, it will be worse
715 investigating whether TT15 works as a hub to integrate auxin-regulated
716 developmental program and vacuole trafficking through the regulation of sterol
717 metabolism, similarly to the situation observed for the ribosomal protein RPL4 by Li
718 et al. (2015).

719

720 **TT15 is Mainly Located at the Tonoplast**

721 Knowing the precise subcellular localization of SGTs is crucial for a thorough
722 understanding of their biological functions. Previous studies in various plant species
723 and with diverse experimental approaches reported multiple subcellular
724 localizations for SGTs, including cytoplasm, PM, ER, Golgi and tonoplast (Grille et
725 al., 2010; Ramirez-Estrada et al., 2017). Our observations of GFP-TT15 signal in
726 stable *Arabidopsis* transformants revealed that the TT15 protein is located primarily
727 at the tonoplast or vacuolar membrane. This localization was ascertained by co-
728 localization with the tonoplast markers endosomal-localized Soluble N-
729 ethylmaleimide-sensitive factor Attachment protein Receptor (SNARE) protein
730 VAMP711 and Rab GTPases RabG3c (Rab7D) and RabG3f (Rab7D) previously
731 characterized by Geldner et al. (2009). Rab GTPases and SNAREs ensure
732 membrane-specific tethering and fusion between transport vesicles and target
733 organelles (Uemura and Ueda, 2014). Previously published vacuole proteomes
734 from *Arabidopsis* cell suspensions (Jaquinod et al., 2007) and rosette leaves (Carter
735 et al., 2004) also mentioned TT15 as being at the tonoplast. Carter et al. (2004)
736 classified it in the “Membrane fusion and remodeling” category. Ramirez-Estrada et
737 al. (2017) demonstrated that TT15 was a peripheral membrane protein, consistent
738 with an absence of transmembrane domain (TMD) in the predicted protein. However
739 they did not detect any consensus amino acid sequences for lipid-mediated
740 reversible post-translational modifications that may be responsible for TT15
741 transient membrane attachment. Therefore recruitment of TT15 to the tonoplast

742 may rather involve protein-protein interaction or another as yet unidentified
743 mechanism (Ramirez-Estrada et al., 2017).

744 The tonoplast-located GFP-TT15 fusion protein was also shown to partially
745 colocalize with amyloplasts from the oi2 integument layer. Interestingly, pioneer
746 work with potato tubers reported SG and ASG formation in the amyloplast
747 membrane (Catz et al., 1985). TT15 may be involved in regulating the association
748 of vacuoles and amyloplasts during oi2 cell layer differentiation, similarly to the
749 situation previously described for graviperception in stem and hypocotyl by Saito et
750 al. (2005) and Alvarez et al. (2016), respectively. To our knowledge, such an
751 association has not been reported to date for the mucilage cell layer, but the fact
752 that TT15 is affected in the differentiation of this layer (Berger et al., 2021) suggests
753 that this may be a plausible hypothesis. This mechanism may promote the
754 remobilization of carbohydrates from starch to fuel the biosynthesis of mucilage.
755 Alvarez et al. (2016) propose the formation of a physical tether between vacuole
756 and amyloplast through tonoplast remodeling. It is also possible that the membrane
757 contact site (MCS) may be a zone of exchange for lipids or other molecules between
758 the amyloplast and vacuole membranes, as illustrated with peroxisome by Shai et
759 al. (2016).

760 Transient expression of a ProCaMV35S-TT15/UGT80B1-YFP fusion infiltrated in
761 *Nicotiana benthamiana* was located both at the PM and in the cytosol by Ramirez-
762 Estrada et al. (2017). On the same line, Pook et al. (2017) located a
763 Pro2xCaMV35S-TT15/UGT80B1-GFP fusion at the PM in stable Arabidopsis
764 transformants. The reason for the discrepancy between our results and these two
765 previous works is unclear. However the fact that in our experimental conditions both
766 GFP-TT15 and TT15-GFP constructs complement the *tt15* mutants and exhibit the
767 same tonoplastic location in accordance with proteomics data from Carter et al.
768 (2004) and Jaquinod et al. (2007) strongly argue towards TT15 being at the
769 tonoplast rather than at the PM. Moreover we did not find any co-localization
770 between TT15-GFP and the PM marker mCherry-NIP1;1. Last but not least, the
771 tonoplastic subcellular localization is relevant with the vacuolar defects observed in
772 *tt15* mutants. We can not rule out the hypothesis that a defect in vacuolar trafficking
773 may affect PM homeostasis (endocytosis, exocytosis) and indirectly explain the cell
774 wall phenotypes observed in *tt15* mutants. An example is provided by the TGN-
775 localized ECHIDNA (ECH) protein which has been shown to be required for the

776 apoplasmic secretion of pectin and hemicellulose in oi2 mucilage cells (Gendre et al.,
777 2011; McFarlane et al., 2013) and in the vacuolar sorting pathway for PC
778 accumulation in the endothelium (Ichino et al., 2020). Interestingly, Gendre et al.
779 (2011) reported that mislocalization of the VHA-a1 vacuolar H⁺-ATPase was
780 contributing to *ech* defects at the cell wall. The TGN is highly dynamic and behaves
781 as a central hub for secretion, endocytosis and recycling (Ebine and Ueda, 2015).
782 In absence of TGN-located ECH, cell wall polysaccharides are mistargeted to the
783 vacuole in place of being secreted to the apoplast (McFarlane et al., 2013), which
784 reveals a trafficking connection between the cell wall and the vacuole, at least for
785 polysaccharides. The *tt15/ugt80b1* mutants have been shown to be affected in
786 polysaccharide accumulation in mucilage layer of Arabidopsis seed coat, with *tt15*
787 specifically strengthening primary and secondary cell wall. Moreover, the amount of
788 the major pectin component of the mucilage, rhamnogalacturonan-I (RG-I), was
789 lower in *tt15* as the amount of hemicellulose (galactoglucomannan or GGM) was
790 higher (Zauber et al., 2014; Berger et al., 2021). Collectively these results are
791 compatible with the fact that TT15 is at the tonoplast as most non-cellulosic
792 polysaccharides including hemicelluloses are synthesized in Golgi before being
793 secreted to the apoplast with the contribution of ECH.

794

795 **TT15 Contributes to Vacuole Biogenesis and Maintenance**

796 The plant tonoplast has been shown to be organized into microdomains (Ozolina et
797 al., 2013; Yoshida et al., 2013) and to contain SG and ASG (Yoshida and Uemura,
798 1986; Tavernier et al., 1993; Yamaguchi and Kasamo, 2001). Albeit membrane lipid
799 homeostasis is recognized as being critical in plant vacuolar trafficking,
800 development and response to biotic and abiotic stresses ((Zhang et al., 2015;
801 Sandor et al., 2016; Boutté and Jaillais, 2020), the biological roles of SG and ASG
802 in these processes remain unclear. Here, by monitoring vacuolar deposition of
803 flavanols in seed coat endothelium with vanillin staining, we revealed that a defect
804 in sterol glucosylation due to *TT15* disruption perturbs vacuole biogenesis. Small
805 vacuoles stop increasing in size from the heart stage onwards, suggesting that
806 tonoplast elongation and/or vesicle fusion has been interrupted in *tt15*. The following
807 step is vacuolar degenerescence and necrotic cell death due to release of vacuolar
808 proteases and flavanols in the cytosol. A relationship between vacuole development
809 and SG has previously been observed in fungi, that may put some light on the

810 mechanisms at play in Arabidopsis. Ergosteryl- β -glucoside (EG) is a major class of
811 glycolipids in fungi. Disruption of the steryl- β -glucosidase Egh1 was shown to cause
812 an abnormally fragmented vacuole morphology in *Saccharomyces cerevisiae*
813 (Watanabe et al., 2015). This phenotype was suggested to be due to the
814 accumulation of EG in the vacuole, pointing to a negative role of EG in vacuole
815 fusion, but the precise mechanism involved is unclear (Hurst and Fratti, 2020).
816 Flavonoid metabolism was also shown to affect vacuolar morphology (Abrahams et
817 al., 2003; Baxter et al., 2005; Kitamura et al., 2010; Rosado et al., 2011; Appelhagen
818 et al., 2014), however the molecular triggers still remain to be identified. We can not
819 preclude that the small amount of flavanols that are accumulated in *tt15* endothelial
820 cells interfere with the vacuolar phenotype caused by SG shortage. Investigating
821 further vacuolar morphology in *tt15 tt4* endothelial cells may help answer this
822 question.

823 We demonstrated in Arabidopsis roots that disruption of the *TT15* gene caused an
824 increase in tonoplast fluidity. Membrane biophysics is characterized by two main
825 parameters : fluidity, as a measure of molecule rotation and diffusion within the
826 membrane ; and order, comprising structure, microviscosity and membrane phases
827 (Sandor et al., 2016). SG exhibit the ability to decrease membrane fluidity and to
828 order membranes into microdomains called lipid rafts, defined as detergent-
829 resistant (DRM) or detergent-insoluble (DIM) membranes (Laloi et al., 2007;
830 Grosjean et al., 2015). Previous studies with plant PM have shown that SG and ASG
831 efficiently order membranes and as a consequence reduce their fluidity (Laloi et al.,
832 2007; Halling et al., 2008; Grosjean et al., 2015). Here, we could extend this
833 observation to the tonoplast using a genetic approach based on the characterization
834 of the *tt15-2* mutant affected in SG formation by FRAP. Our study suggests that SG
835 shortage increases membrane fluidity. The biological significance of this
836 observation is important as membrane fluidity was shown to regulate cellular
837 processes such as membrane fusion in yeast (Hurst and Fratti, 2020) or defense
838 signalling in tobacco (Sandor et al., 2016). The modification of tonoplast fluidity is
839 therefore very likely to also affect the function of tonoplastic membrane proteins and
840 membrane remodeling.

841

842 **Role of TT15 in Flavanol Trafficking and Vacuolar Sequestration**

843 Flavanol deposition is affected in endothelium, not in micropylar and chalazal cells
844 of *tt15* developing seeds, which suggests that endothelium-specific molecular
845 factors that remain to be determined contribute to this phenotype. Potential
846 candidates which disruption gives a similar seed coat pigmentation pattern than *tt15*
847 are TT1, TT9 and TT16 (Nesi et al., 2002; Sagasser et al., 2002; Ichino et al., 2014).
848 Notably a microscopic investigation of developing seeds revealed that the *tt9* mutant
849 exhibited a similar phenotype as *tt15*. This finding prompted us to analyze the
850 functional relationships between TT9 and TT15. The *tt9* mutant previously isolated
851 by Koornneef (1990) and mapped by Shirley et al. (1995) was shown to be allelic to
852 *gfs9* (*green fluorescent seed*) identified as a sorting mutant for vacuolar storage
853 proteins by Fuji et al. (2007). The *tt9/gfs9* mutant exhibits pale seeds, mis-sorting of
854 vacuolar storage proteins, vacuole fragmentation, aggregation of enlarged
855 vesicles, abnormal Golgi morphology and many autophagosome-like structures.
856 AtTT9/GFS9 is localized at the Golgi apparatus, and exhibits strong sequence
857 homology with the *Drosophila melanogaster* Endosomal maturation defective (Ema)
858 protein (Ichino et al., 2014). Ema and its human orthologue C-type LECTin 16A
859 (CLEC16A) cooperatively function with the HOMotypic fusion and Protein Sorting
860 (HOPS) complex and endosomal-localized Soluble N-ethylmaleimide-sensitive
861 factor Attachment protein REceptors (SNARE) proteins to promote lysosomal
862 protein sorting and autophagosome development (Kim et al., 2010; Kim et al., 2012;
863 van Luijn et al., 2015; Pandey et al., 2019). The mechanisms linking endomembrane
864 trafficking to flavanol accumulation in the vacuole (Figure 10A) involve TT9 (Ichino
865 et al., 2014). We discovered that the *tt9-1/gfs9-4* mutant exhibits an endothelium
866 degeneration syndrome that is similar to the one observed in *tt15*. Intriguingly, cell
867 death was suppressed in the double mutant *tt15-2 tt9-1*. Furthermore, vacuole
868 development in *ii1* cells exhibited a novel phenotype that did not recapitulate
869 flavanol accumulation observed in wild type. These observations suggest that : 1) if
870 both TT15 and TT9 function in preserving vacuole homeostasis and cell viability,
871 they act in different genetic pathways, since both proteins have a different
872 subcellular localization ; 2) the normal development of flavanol-accumulating
873 vacuoles is not essential for cell survival, as shown in the double mutant (Figure 10,
874 B and C). TT9 is required for vacuolar development through vesicle and possibly
875 autophagosome fusion at vacuoles (Ichino et al., 2014). Our results point to TT15
876 as being important for tonoplast homeostasis, but vesicle fusion at vacuole does not

877 seem to be affected in *tt15*. Therefore both functions are likely to trigger cell death
878 when disrupted, but for different reasons that would complement each other in the
879 double mutant. Apart from a defective accumulation of flavanols in the seed coat,
880 the *tt9* mutants were reported to exhibit vacuolar development and trafficking
881 defects including vacuole fragmentation, mis-sorting of 12S storage proteins and
882 cytoplasmic accumulation of autophagosome-like structures (Shirley et al., 1995;
883 Ichino et al., 2014). In contrast, *tt15* does not exhibit mis-sorting of storage proteins
884 (Ichino et al., 2014) neither vacuole fragmentation (this study) and vacuole
885 development is only partially restored in a double mutant background. Both *TT15*
886 and *TT9* are peripheral membrane proteins (Ichino et al., 2014; Ramirez-Estrada et
887 al., 2017), meaning that they are likely to move from one subcellular compartment
888 to another.

889 The flavonoid phenotypes of *tt15* and *tt9* seeds are similar. Mutant seeds exhibit a
890 strong reduction in total flavanols (soluble and insoluble flavanols) specifically in
891 endothelial cells, as analyzed with LC-MS and vanillin-based histochemistry
892 (Routaboul et al., 2012; Ichino et al., 2014)(this study). On the other hand, the
893 flavonol fraction remains unchanged apart from quercitrin (quercetin 3-O rhamnose,
894 QR) which is slightly reduced. QR is accumulated essentially in the seed envelopes
895 (Routaboul et al., 2006). The physiological significance of this flavonol phenotype is
896 still unclear. Notably natural variation at the level of the *TT15* locus was observed
897 for both PC and QR seed contents (Routaboul et al., 2012), suggesting an adaptive
898 value for both traits. We can not rule out the hypothesis that flavonoid release from
899 the abnormally developed endothelial vacuoles in *tt15* and *tt9* mutant seed coats
900 triggers cell death. Here, we observed that flavanols (EC and PC) but not
901 anthocyanins or flavonols disrupt cell homeostasis, which may possibly be due to
902 the specific physicochemical properties of decompartmented PC to interact with
903 proteins, carbohydrates and metal ions from the cellular machinery (Hagerman and
904 Butler, 1981; Porter, 1992) and to their cytotoxicity (Dixon and Sarnala, 2020).

905 The expression of a dominant-negative form of *AtSKD1* (*SKD1*^{E232Q}) under the
906 control of the 35S promoter in tobacco induces alterations in the endosomal system
907 leading to cell and plant death (Haas et al., 2007). Interestingly, Arabidopsis plants
908 overexpressing dominant-negative *AtSKD1* constructs under the control of the
909 *GLABRA2* (*GL2*) promoter that restricts expression to trichomes and non-hair cells
910 in the root epidermis, were viable and shown to exhibit a *tt* phenotype and seed

911 mucilage defect (Shahriari et al., 2010a; Shahriari et al., 2010b). Both phenotypes
912 were not characterized further but were supposed to be connected by cell death due
913 to a perturbation in the trafficking of soluble cargo to the vacuole, causing its
914 fragmentation and ultimately cell death (Shahriari et al., 2010b). The same
915 hypothesis may be proposed to explain the *tt15* phenotypes. Thus our results may
916 point to a role played by endosomal and MVB sorting in vacuolar accumulation of
917 PC. On the same line, Gonzalez et al. (2016) showed that TTG2, which disruption
918 causes a *tt* phenotype and an absence of mucilage, controls vacuolar transport of
919 PC by regulating the expression of genes encoding the tonoplast MATE
920 transporter TT12/DTX41 and P_{3A}-ATPase TT13/AHA10. *TT15* regulatory role in the
921 maintenance of endothelial vacuoles (this study) may act at the post-translational
922 level by modulating the activity of either one or both proteins. Relevant with this
923 information, SG have been shown to stimulate the activity of a tonoplast H⁺-
924 ATPase in rice cell cultures (Yamaguchi and Kasamo, 2002). However *tt15*, *tt12*
925 and *tt13* do not exhibit exactly the same endothelial phenotype, which suggest that
926 other actors may also be involved. As a corollary, we did not observe GFP-TT12
927 mislocalization in a *tt15* background. Interestingly the pattern of flavanol
928 accumulation and vacuole morphology in *tt15 tt9* endothelium resembles the one
929 observed previously in *tt12* (Debeaujon et al., 2001; Kitamura et al., 2010;
930 Appelhagen et al., 2015) and *tt13* (Appelhagen et al., 2015). In this context we can
931 not rule out the hypothesis that EC transport activity may be impaired in *tt15*
932 indirectly through perturbation of TT9 function as a mediator of vesicle-vacuole
933 fusion.

934 Altogether, our findings shed light on novel functions of TT15 sterol
935 glucosyltransferase in vacuolar biogenesis and trafficking and their involvement in
936 seed development, germination and flavanol transport to the vacuole. Figure 10
937 proposes a working model visualizing the respective positions of TT15 and TT9
938 proteins at the level of the vacuolar trafficking pathways, together with other major
939 actors of the flavanol trafficking pathway. As a basis for future investigations, we
940 speculate that the modulation of tonoplast fluidity through remodeling of its SG
941 composition by TT15 regulates endomembrane-related mechanisms including the
942 fusion of vesicles and autophagosomes to the tonoplast, microautophagy and the
943 activity of tonoplast-localized membrane proteins.

944

945 **METHODS**

946 **Plant Materials and Growth Conditions**

947 The *Arabidopsis* (*Arabidopsis thaliana*) lines used in this study (Supplemental Table
948 S1) were *tt15-1* (Focks et al., 1999; Appelhagen et al., 2014), *tt15-2* (COB16)
949 (Routaboul et al., 2012), *tt15-6* (DNF6) (Nesi, 2001), *tt15-7* (EAL136) (Berger et al.,
950 2021), *tt15-8* (Salk_021175) (Pook et al., 2017), *tt15-9* (Salk_103581) (Stucky et al.,
951 2015), *tt4-8* (DFW34) (Debeaujon et al., 2003) and *tt9-1/gfs9-4* (Koornneef, 1990;
952 Shirley et al., 1995; Ichino et al., 2014). The *tt15-2*, *tt15-6*, *tt15-7* and *tt4-8* alleles
953 are in wild-type Wassilewskija (Ws-4) accession and were obtained from the INRAE
954 Versailles T-DNA collection (Brunaud et al., 2002). The *tt15-8* and *tt15-9* alleles in
955 wild-type Columbia (Col-0) accession are from the Salk Institute T-DNA collection
956 (Alonso et al., 2003) and were obtained from the NASC stock center. The *tt15-1*
957 allele in Col-2 background and the *tt9-1* allele in *Ler* background were provided by
958 Christophe Benning and Maarten Koornneef, respectively. The double mutant
959 between *tt15-2* and *tt9-1* was obtained by crossing, using *tt15-2* as female. F2
960 plantlets were genotyped for *tt15-2* as described in Berger et al. (2021) and for *tt9-1/gfs9-4*
961 by sequencing after PCR amplification with TT9-F3 and TT9-GST3 primers
962 (Supplemental Table S5). The double mutant with *tt4-8* was selected on the basis
963 of its pale yellow seed colour and absence of anthocyanins in vegetative parts, after
964 checking for the presence of *tt15-2* as described above. For reciprocal crosses, F1
965 hybrid seeds produced by WT mother plants with a *tt15* pollen donor are referred to as WT
966 x *tt15* F1 seeds and those from *tt15* mother plants with a WT pollen donor as *tt15* x WT
967 seeds. For FRAP experiments, the transfer of GFP-NRT2-7 in a *tt15-2* background
968 was done by crossing *tt15-2* with a transformant obtained from Chopin et al. (2007)
969 expressing the cassette in Ws-4 background. For co-localization experiments,
970 fluorescent subcellular compartment markers (Supplemental Table S4) were
971 crossed with a representative transformant expressing GFP-TT15.
972 For *in vitro* cultures, seeds were surface-sterilized, sown in Petri dishes containing
973 Gamborg B5 medium (Duchefa, The Netherlands) supplemented with 3% sucrose
974 and 0.8% agar, stratified for 3 days at 4°C in the dark and grown for 3 to 12 days at
975 20°C with a 16-h light/8-h dark cycle and 70% relative humidity. Plant growth in
976 glasshouse was realized on compost (Tref BV, The Netherlands) fertilized with
977 Plant-Prod nutritive solution (Fertil, France) at around 23°C / 15°C (day/night).
978 Observation of seed lethality was performed on plants grown in controlled conditions

979 in a growth chamber settled at 21°C/18°C (day/night), 16h lighting and 65% relative
980 humidity.

981

982 **Seed Germination Assays**

983 Seed lots from WT (Ws-4, Col-0) and *tt15* mutants to be compared were obtained
984 from plants grown at the same time and in the same environmental conditions.
985 Seeds from a bulk of four plants from each genotype was sown in triplicate in 6-cm
986 Petri dishes (50-60 seeds per dish) on 0.5% (w/v) agarose supplemented with ABA
987 (Junda Pharm Chem Plant Co., China) or the gibberellin biosynthesis inhibitor
988 paclobutrazol (Syngenta, Switzerland). Seeds were stratified at 4°C for three days
989 in the dark and transferred in a growth cabinet (continuous light, 25°C, 70% relative
990 humidity). Germination (emerged radicle) and/or cotyledon greening were scored
991 four days after transfer to light.

992

993 **Constructs**

994 All primers used for plasmid constructs are listed in Supplemental Table S5. For
995 construction of the *ProTT15:uidA* transgene, a region located -2003 to -1 bp relative
996 to the *TT15* translational start codon was amplified with the ProTT15-Sall and
997 ProTT15-Smal primers using a proof-reading Taq polymerase (Phusion ;
998 Thermofisher, USA) and cloned in TOPO vector (Thermofisher, USA). After
999 validation by sequencing, *ProTT15* was digested by Sall-Smal and cloned at the
1000 Sall-Smal sites of the pBI101 binary vector (Clontech, USA) for plant transformation.
1001 To construct the GFP-TT15 translational fusion protein, the *TT15* coding sequence
1002 (CDS) was amplified from clone U22595 (SSP pUni clone BT005834 from the Salk
1003 Institute, USA) with TT15-ATG-attB1 and TT15-END-attB2 primers using Phusion
1004 Taq polymerase. The PCR product was then cloned into pDONR207 by BP
1005 recombination (Gateway BP Clonase enzyme mix, Invitrogen, USA). After validation
1006 by sequencing, TT15 CDS was transferred into the binary vectors pMDC43 and
1007 pMDC83 (Curtis and Grossniklaus, 2003) by LR recombination. Promoter activity
1008 and subcellular localization of translational fusions with GFP were investigated after
1009 stable transformation of Arabidopsis plants according to the floral dip method
1010 (Clough and Bent, 1998). Around fifteen independent transformants per construct
1011 were obtained, among which two were selected for further characterization on the
1012 basis of their representative behaviour.

1013

1014 **Light Microscopy**

1015 Observations were performed with an epifluorescence microscope (Zeiss Axioplan
1016 2, Germany) equipped with Nomarski differential interference contrast optics.
1017 Silique clearing for quantification of seed lethality was performed as described by
1018 Surpin et al. (2003), on 25 siliques per genotype (five siliques from five plants)
1019 harvested 15 days after flowering. Fixation, resin embedding, sectioning and
1020 toluidine Blue O (TBO) staining of seed material were realized as described in
1021 Debeaujon et al. (2003). TBO stains polyphenolic compounds (procyanidins and
1022 lignins) in greenish-blue, pectin in pink and nucleic acids and proteins in purple.
1023 Histochemical detection of GUS activity was performed as described in Debeaujon
1024 et al. (2003), using 2.5 mM potassium ferricyanide and 2.5 mM potassium
1025 ferrocyanide. Developing seeds were cleared by overnight incubation in a chloral
1026 hydrate:glycerol:water (8:1:2, w:v:v) solution. The vanillin assay was used for
1027 specific staining of colorless flavanols in bright red in developing seed coat, as
1028 described by Debeaujon et al. (2000). ROS (H₂O₂) detection was realized according
1029 to Bailly and Kranner (2011) using 5-(and-6)-chloromethyl-2', 7'-dichlorofluorescein
1030 diacetate (DCFH-DA ; Sigma, USA). Developing seeds were incubated for 15 min
1031 in 100 μM DCFH-DA in 20-mM potassium phosphate buffer pH 6.0 at 20°C, and
1032 rinsed three times with buffer for 5-min each before observation under UV light. For
1033 callose detection in developing seeds, young siliques (2-3 dap) were fixed,
1034 hydrated, softened and stained in fresh 0.1% Aniline Blue (Acros Organics, Belgium)
1035 aqueous solution according to Huck et al. (2003). Seed whole mounts were
1036 observed under UV light.

1037

1038 **Confocal Laser Scanning Microscopy**

1039 Observations were performed using a Leica SP5 or a Leica SP8 spectral confocal
1040 laser scanning microscope (Leica Microsystems, Germany) fitted with 20x or 63x
1041 water-immersion objectives. A 488-nm line from an argon laser was used to excite
1042 GFP, and fluorescence was detected in the 501- to 598-nm range. A 561-nm line
1043 from a He-Ne laser was used to excite mCherry, and fluorescence was detected in
1044 the 571- to 668-nm range. Chlorophyll autofluorescence was detected in the 615-
1045 to 715-nm range. Images were false-colored in green (GFP) or magenta (mCherry
1046 or chlorophyll autofluorescence) with the ImageJ software (Schneider et al., 2012)

1047 and processed using the FigureJ plugin (Mutterer and Zinck, 2013). The fluorescent
1048 pH indicator BCECF-AM (Life Technologies, USA) was used to stain the acidic
1049 lumen of the vacuoles according to Scheuring et al. (2015). Seedling roots were
1050 incubated in a 10- μ M BCECF solution during 1 h in the dark and rinsed before
1051 confocal imaging. The wavelengths for excitation and emission were 488 and 520
1052 nm, respectively.

1053 Vacuolar membrane fluidity was determined by FRAP using the NRT2.7 nitrate
1054 transporter fused to GFP (Chopin et al., 2007) as a tonoplastic intrinsic marker in
1055 *Ws-4* and *tt15-2* backgrounds. Images were acquired on epidermal cells of the
1056 elongation zone of 4-day-old seedling roots. Measurements were performed using
1057 the FRAP wizard of Leica SP5 microscope with a 63x objective. Rectangular
1058 Regions of Interest (ROI) of 8.9 μ m² were designed. Fluorescence intensity data
1059 were collected from bleached area (ROI1), total fluorescence area (ROI2) and
1060 background area (ROI3). Pre-bleaching and post-bleaching imaging was performed
1061 with the 488-nm line of an Argon laser at 100% output and 10% transmission. For
1062 bleaching, one scan of ROI1 was done at 100% transmission of 405-nm diode and
1063 458-nm, 476-nm and 488-nm lines of Argon laser with “zoom in” method of bleach.
1064 Scans were done with a minimized time frame of 0.189s. Time course of acquisition
1065 was as follows: pre-bleach: 10 frames, bleach: 1 frame and post-bleach: 200 frames.
1066 Fluorescence intensity data were normalized with the easyFRAP software
1067 (Rapsomaniki et al., 2012) using full-scale normalisation method. T_{half} (half maximal
1068 recovery time) and mobile fraction were computed after curve fitting using single
1069 term equation. Wilcoxon test was used to calculate the statistical significance of T_{half}
1070 and mobile fraction results between *Ws-4* wild type and *tt15-2* mutant (significance
1071 at $P < 0,001$).

1072

1073 **Accession numbers**

1074 Sequence data from this article can be found in the Arabidopsis Genome Initiative
1075 (TAIR) or EMBL/GenBank data libraries under the following accession numbers:
1076 TT15/UGT80B1 (AT1G43620, NP_175027), UGT80A2 (AT3G07020, NP_566297),
1077 TT4 (AT5G13930, NP_196897), TT9 (AT3G28430, BAH57204).

1078

1079 **SUPPLEMENTAL DATA**

1080 **Supplemental Figure S1.** Biosynthesis of steryl glucosides and related end-
1081 products.

1082 **Supplemental Figure S2.** Biosynthesis of flavonoids and related end-products.

1083 **Supplemental Figure S3.** *TT15* mutant alleles used in this study.

1084 **Supplemental Figure S4.** *transparent testa* mutants with a similar pattern of
1085 seed coat pigmentation than *tt15* also exhibit some seed lethality (Supports Figure
1086 1).

1087 **Supplemental Figure S5.** The *tt15* mutant alleles produce smaller seeds than
1088 wild types (Supports Figure 1).

1089 **Supplemental Figure S6.** Phenotypic diversity of *tt15* seedlings (Supports
1090 Figure 1).

1091 **Supplemental Figure S7.** Impact of the *tt15* mutations on seed physiology
1092 (Supports Figure 2).

1093 **Supplemental Figure S8.** The *tt15* mutant seed developmental phenotypes in
1094 Col-0 background are similar to the ones in Ws-4 (Supports Figure 3).

1095 **Supplemental Figure S9.** Flavanol depletion suppresses endothelium browning
1096 and breakdown (Supports Figure 3).

1097 **Supplemental Figure S10.** The *TT15* promoter is active in reproductive and
1098 vegetative organs (Supports Figure 5).

1099 **Supplemental Figure S11.** *TT15* is expressed in vegetative and reproductive
1100 organs similarly in Ws-4 and Col-0 accessions (Supports Figure 5).

1101 **Supplemental Figure S12.** *TT15 in silico* expression data (Supports Figure 5).

1102 **Supplemental Figure S13.** The translational fusions of TT15 with the fluorescent
1103 Protein GFP used for subcellular localization studies complement the *tt15* mutant
1104 (Supports Figure 6).

1105 **Supplemental Figure S14.** Subcellular localization of GFP Fusions with TT15 in
1106 seed coat and root (Supports Figure 6).

1107 **Supplemental Figure S15.** TT15 does not co-localize with Golgi, TGN, late
1108 endosome and plasma membrane markers (Supports Figure 6).

1109 **Supplemental Figure S16.** TT15 absence does not significantly impact flavonoid
1110 gene expression in seeds.

1111 **Supplemental Figure S17.** Vacuole morphology is not affected in absence of
1112 TT15 in roots (Supports Figure 8).

1113 **Supplemental File S1.** Supplemental tables 1 to 5. Mutants (**S1**); Flavonoids
1114 (**S2**); Seed lethality (**S3**); Fluorescent markers (**S4**); Primers (**S5**).

1115 **Supplemental Movie 1** (Supports Figure 5). Z-Stack of GFP-TT15 in a cotyledon
1116 from a 7-day-old seedling, focusing on a stomata (spinning-disk ; 67 frames).

1117 **Supplemental Movie 2** (Supports Figure 5). Dynamics of GFP-TT15 in a root tip
1118 of a 3-day-old Arabidopsis seedling, from the meristematic zone to the elongation
1119 zone (time series 20 planes in 3 s).

1120 **Supplemental Movie 3** (Supports Figure 5). Dynamics of GFP-TT15 in a root tip
1121 of a 7-day-old Arabidopsis seedling, at the elongation zone (spinning-disk ; time
1122 series 167 planes in 24 s).

1123 **Supplemental Movie 4** (Supports Figure 5). Dynamics of GFP-TT15 and
1124 mCherry-GOT1p in a root tip of a 3-day-old Arabidopsis seedling, at the elongation
1125 zone (time series 25 planes in 4 s).

1126

1127 **ACKNOWLEDGMENTS**

1128 We are very grateful to Maarten Koornneef for providing *tt9-1* seeds, Christoph
1129 Benning for *tt15-1* seeds, and Sylvie Ferrario-Méry for seeds of the GFP-NRT2.7
1130 line. We thank Lucille Pourcel, Guillaume De Lagarde and Nathan Leborgne for
1131 contribution to the experimental work. This work was funded by the European
1132 Commission (FOOD-CT-2004-513960 “FLAVO” and FP7 Environment Grant Award
1133 Number 311840 “EcoSeed”) and has benefited from the support of IJPB’s Plant
1134 Observatory technological platforms. The IJPB benefits from the support of Saclay
1135 Plant Sciences-SPS (ANR-17-EUR-0007).

1136

1137 **AUTHOR CONTRIBUTIONS**

1138 E.A., A.B., F.P., A.F., A.T., S.C., H.S., S.V., O.G., N.N. and I.D. performed research
1139 and analyzed data ; E.A., L.L., A.M.P. and I.D. designed research ; E.A. and I.D.
1140 wrote the paper.

1141

1142 **REFERENCES**

1143 Abrahams, S., Lee, E., Walker, A.R., Tanner, G.J., Larkin, P.J., and Ashton, A.R.
1144 (2003). The Arabidopsis TDS4 gene encodes leucoanthocyanidin dioxygenase
1145 (LDOX) and is essential for proanthocyanidin synthesis and vacuole
1146 development. *Plant J* 35, 624-636.

- 1147 Alonso, J.M., Stepanova, A.N., Leisse, T.J., Kim, C.J., Chen, H., Shinn, P.,
1148 Stevenson, D.K., Zimmerman, J., Barajas, P., Cheuk, R., Gadrinab, C., Heller,
1149 C., Jeske, A., Koesema, E., Meyers, C.C., Parker, H., Prednis, L., Ansari, Y.,
1150 Choy, N., Deen, H., Geralt, M., Hazari, N., Hom, E., Karnes, M., Mulholland, C.,
1151 Ndubaku, R., Schmidt, I., Guzman, P., Aguilar-Henonin, L., Schmid, M., Weigel,
1152 D., Carter, D.E., Marchand, T., Risseeuw, E., Brogden, D., Zeko, A., Crosby,
1153 W.L., Berry, C.C., and Ecker, J.R. (2003). Genome-wide insertional mutagenesis
1154 of *Arabidopsis thaliana*. *Science* 301, 653-657.
- 1155 Alvarez, A.A., Han, S.W., Toyota, M., Brillada, C., Zheng, J., Gilroy, S., and Rojas-
1156 Pierce, M. (2016). Wortmannin-induced vacuole fusion enhances amyloplast
1157 dynamics in *Arabidopsis zigzag1* hypocotyls. *J Exp Bot* 67, 6459-6472.
- 1158 Andème Ondzighi, C., Christopher, D.A., Cho, E.J., Chang, S.C., and Staehelin,
1159 L.A. (2008). *Arabidopsis* protein disulfide isomerase-5 inhibits cysteine proteases
1160 during trafficking to vacuoles before programmed cell death of the endothelium
1161 in developing seeds. *Plant Cell* 20, 2205-2220.
- 1162 Appelhagen, I., Thiedig, K., Nordholt, N., Schmidt, N., Huep, G., Sagasser, M., and
1163 Weisshaar, B. (2014). Update on transparent testa mutants from *Arabidopsis*
1164 *thaliana*: characterisation of new alleles from an isogenic collection. *Planta* 240,
1165 955-970.
- 1166 Appelhagen, I., Nordholt, N., Seidel, T., Spelt, K., Koes, R., Quattrochio, F.,
1167 Sagasser, M., and Weisshaar, B. (2015). TRANSPARENT TESTA 13 is a
1168 tonoplast P3A -ATPase required for vacuolar deposition of proanthocyanidins in
1169 *Arabidopsis thaliana* seeds. *Plant J* 82, 840-849.
- 1170 Bailly, C., and Kranner, I. (2011). Analyses of reactive oxygen species and
1171 antioxidants in relation to seed longevity and germination. *Methods Mol Biol* 773,
1172 343-367.
- 1173 Bassham, D.C. (2015). Plant biology: Pigments on the move. *Nature* 526, 644-645.
- 1174 Batista, R.A., Figueiredo, D.D., Santos-Gonzalez, J., and Kohler, C. (2019). Auxin
1175 regulates endosperm cellularization in *Arabidopsis*. *Genes Dev* 33, 466-476.
- 1176 Baxter, I.R., Young, J.C., Armstrong, G., Foster, N., Bogenschutz, N., Cordova, T.,
1177 Peer, W.A., Hazen, S.P., Murphy, A.S., and Harper, J.F. (2005). A plasma
1178 membrane H⁺-ATPase is required for the formation of proanthocyanidins in the
1179 seed coat endothelium of *Arabidopsis thaliana*. *Proc Natl Acad Sci U S A* 102,
1180 2649-2654.

- 1181 Ben Khaled, S., Postma, J., and Robatzek, S. (2015). A moving view: subcellular
1182 trafficking processes in pattern recognition receptor-triggered plant immunity.
1183 *Annu Rev Phytopathol* 53, 379-402.
- 1184 Berger, A., Ralet, M.C., Akary, E., Salle, C., Grandjean, O., Debeaujon, I., and
1185 North, H.M. (2021). Sterol Glucosyltransferases Tailor Polysaccharide
1186 Accumulation in Arabidopsis Seed Coat Epidermal Cells. *Cells* 10.
- 1187 Boutté, Y., and Jaillais, Y. (2020). Metabolic Cellular Communications: Feedback
1188 Mechanisms between Membrane Lipid Homeostasis and Plant Development.
1189 *Dev Cell* 54, 171-182.
- 1190 Brunaud, V., Balzergue, S., Dubreucq, B., Aubourg, S., Samson, F., Chauvin, S.,
1191 Bechtold, N., Cruaud, C., DeRose, R., Pelletier, G., Lepiniec, L., Caboche, M.,
1192 and Lechamy, A. (2002). T-DNA integration into the Arabidopsis genome
1193 depends on sequences of pre-insertion sites. *EMBO Rep* 3, 1152-1157.
- 1194 Buer, C.S., and Muday, G.K. (2004). The transparent testa4 mutation prevents
1195 flavonoid synthesis and alters auxin transport and the response of Arabidopsis
1196 roots to gravity and light. *Plant Cell* 16, 1191-1205.
- 1197 Burkart-Waco, D., Ngo, K., Dilkes, B., Josefsson, C., and Comai, L. (2013). Early
1198 disruption of maternal-zygotic interaction and activation of defense-like
1199 responses in Arabidopsis interspecific crosses. *Plant Cell* 25, 2037-2055.
- 1200 Caputi, L., Malnoy, M., Goremykin, V., Nikiforova, S., and Martens, S. (2012). A
1201 genome-wide phylogenetic reconstruction of family 1 UDP-glycosyltransferases
1202 revealed the expansion of the family during the adaptation of plants to life on land.
1203 *Plant J* 69, 1030-1042.
- 1204 Carter, C., Pan, S., Zouhar, J., Avila, E.L., Girke, T., and Raikhel, N.V. (2004). The
1205 vegetative vacuole proteome of Arabidopsis thaliana reveals predicted and
1206 unexpected proteins. *Plant Cell* 16, 3285-3303.
- 1207 Catz, D.S., J.S., T., and Cardini, C.E. (1985). Steryl glucoside and acyl steryl
1208 glucoside formation in the amyloplast membrane during the development of
1209 potato tuber. *Plant Science* 38, 179-184.
- 1210 Chanoca, A., Kovinich, N., Burkel, B., Stecha, S., Bohorquez-Restrepo, A., Ueda,
1211 T., Eliceiri, K.W., Grotewold, E., and Otegui, M.S. (2015). Anthocyanin Vacuolar
1212 Inclusions Form by a Microautophagy Mechanism. *Plant Cell* 27, 2545-2559.

- 1213 Chopin, F., Orsel, M., Dorbe, M.F., Chardon, F., Truong, H.N., Miller, A.J., Krapp,
1214 A., and Daniel-Vedele, F. (2007). The Arabidopsis ATNRT2.7 nitrate transporter
1215 controls nitrate content in seeds. *Plant Cell* 19, 1590-1602.
- 1216 Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method for
1217 *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J* 16, 735-
1218 743.
- 1219 Clouse, S.D. (2002). *Arabidopsis* mutants reveal multiple roles for sterols in plant
1220 development. *Plant Cell* 14, 1995-2000.
- 1221 Creff, A., Brocard, L., and Ingram, G. (2015). A mechanically sensitive cell layer
1222 regulates the physical properties of the *Arabidopsis* seed coat. *Nat Commun* 6,
1223 6382.
- 1224 Curtis, M.D., and Grossniklaus, U. (2003). A gateway cloning vector set for high-
1225 throughput functional analysis of genes in planta. *Plant Physiol* 133, 462-469.
- 1226 Debeaujon, I., and Koornneef, M. (2000). Gibberellin requirement for *Arabidopsis*
1227 seed germination is determined both by testa characteristics and embryonic
1228 abscisic acid. *Plant Physiol* 122, 415-424.
- 1229 Debeaujon, I., Leon-Kloosterziel, K.M., and Koornneef, M. (2000). Influence of the
1230 testa on seed dormancy, germination, and longevity in *Arabidopsis*. *Plant Physiol*
1231 122, 403-414.
- 1232 Debeaujon, I., Peeters, A.J., Leon-Kloosterziel, K.M., and Koornneef, M. (2001).
1233 The TRANSPARENT TESTA12 gene of *Arabidopsis* encodes a multidrug
1234 secondary transporter-like protein required for flavonoid sequestration in
1235 vacuoles of the seed coat endothelium. *Plant Cell* 13, 853-871.
- 1236 Debeaujon, I., Nesi, N., Perez, P., Devic, M., Grandjean, O., Caboche, M., and
1237 Lepiniec, L. (2003). Proanthocyanidin-accumulating cells in *Arabidopsis* testa:
1238 regulation of differentiation and role in seed development. *Plant Cell* 15, 2514-
1239 2531.
- 1240 DeBolt, S., Scheible, W.R., Schrick, K., Auer, M., Beisson, F., Bischoff, V., Bouvier-
1241 Nave, P., Carroll, A., Hematy, K., Li, Y., Milne, J., Nair, M., Schaller, H., Zemla,
1242 M., and Somerville, C. (2009). Mutations in UDP-Glucose:sterol
1243 glucosyltransferase in *Arabidopsis* cause transparent testa phenotype and
1244 suberization defect in seeds. *Plant Physiol* 151, 78-87.
- 1245 Dilkes, B.P., Spielman, M., Weizbauer, R., Watson, B., Burkart-Waco, D., Scott,
1246 R.J., and Comai, L. (2008). The maternally expressed WRKY transcription factor

- 1247 TTG2 controls lethality in interploidy crosses of Arabidopsis. *PLoS Biol* 6, 2707-
1248 2720.
- 1249 Dixon, R.A., and Sarnala, S. (2020). Proanthocyanidin Biosynthesis-a Matter of
1250 Protection. *Plant Physiol* 184, 579-591.
- 1251 Doughty, J., Aljabri, M., and Scott, R.J. (2014). Flavonoids and the regulation of
1252 seed size in Arabidopsis. *Biochem Soc Trans* 42, 364-369.
- 1253 Ebine, K., and Ueda, T. (2015). Roles of membrane trafficking in plant cell wall
1254 dynamics. *Frontiers in plant science* 6, 878.
- 1255 Ferrer, A., Altabella, T., Arro, M., and Boronat, A. (2017). Emerging roles for
1256 conjugated sterols in plants. *Prog Lipid Res* 67, 27-37.
- 1257 Figueiredo, D.D., and Köhler, C. (2014). Signalling events regulating seed coat
1258 development. *Biochem Soc Trans* 42, 358-363.
- 1259 Figueiredo, D.D., and Köhler, C. (2016). Bridging the generation gap:
1260 communication between maternal sporophyte, female gametophyte and
1261 fertilization products. *Curr Opin Plant Biol* 29, 16-20.
- 1262 Figueiredo, D.D., Batista, R.A., Roszak, P.J., Hennig, L., and Köhler, C. (2016).
1263 Auxin production in the endosperm drives seed coat development in Arabidopsis.
1264 *Elife* 5.
- 1265 Focks, N., Sagasser, M., Weisshaar, B., and Benning, C. (1999). Characterization
1266 of tt15, a novel transparent testa mutant of Arabidopsis thaliana (L.) Heynh.
1267 *Planta* 208, 352-357.
- 1268 Freh, M., Gao, J., Petersen, M., and Panstruga, R. (2022). Plant autoimmunity-fresh
1269 insights into an old phenomenon. *Plant Physiol* 188, 1419-1434.
- 1270 Fuji, K., Shimada, T., Takahashi, H., Tamura, K., Koumoto, Y., Utsumi, S.,
1271 Nishizawa, K., Maruyama, N., and Hara-Nishimura, I. (2007). Arabidopsis
1272 vacuolar sorting mutants (green fluorescent seed) can be identified efficiently by
1273 secretion of vacuole-targeted green fluorescent protein in their seeds. *Plant Cell*
1274 19, 597-609.
- 1275 Garcia, D., Fitz Gerald, J.N., and Berger, F. (2005). Maternal control of integument
1276 cell elongation and zygotic control of endosperm growth are coordinated to
1277 determine seed size in Arabidopsis. *Plant Cell* 17, 52-60.
- 1278 Geldner, N., Denervaud-Tendon, V., Hyman, D.L., Mayer, U., Stierhof, Y.D., and
1279 Chory, J. (2009). Rapid, combinatorial analysis of membrane compartments in
1280 intact plants with a multicolor marker set. *Plant J* 59, 169-178.

- 1281 Gendre, D., Oh, J., Boutte, Y., Best, J.G., Samuels, L., Nilsson, R., Uemura, T.,
1282 Marchant, A., Bennett, M.J., Grebe, M., and Bhalerao, R.P. (2011). Conserved
1283 Arabidopsis ECHIDNA protein mediates trans-Golgi-network trafficking and cell
1284 elongation. *Proc Natl Acad Sci U S A* 108, 8048-8053.
- 1285 Gonzalez, A., Brown, M., Hatlestad, G., Akhavan, N., Smith, T., Hembd, A., Moore,
1286 J., Montes, D., Mosley, T., Resendez, J., Nguyen, H., Wilson, L., Campbell, A.,
1287 Sudarshan, D., and Lloyd, A. (2016). TTG2 controls the developmental regulation
1288 of seed coat tannins in Arabidopsis by regulating vacuolar transport steps in the
1289 proanthocyanidin pathway. *Dev Biol* 419, 54-63.
- 1290 Grille, S., Zaslowski, A., Thiele, S., Plat, J., and Warnecke, D. (2010). The functions
1291 of steryl glycosides come to those who wait: Recent advances in plants, fungi,
1292 bacteria and animals. *Prog Lipid Res* 49, 262-288.
- 1293 Grosjean, K., Mongrand, S., Beney, L., Simon-Plas, F., and Gerbeau-Pissot, P.
1294 (2015). Differential effect of plant lipids on membrane organization: specificities
1295 of phytosphingolipids and phytosterols. *J Biol Chem* 290, 5810-5825.
- 1296 Haas, T.J., Sliwinski, M.K., Martinez, D.E., Preuss, M., Ebine, K., Ueda, T., Nielsen,
1297 E., Odorizzi, G., and Otegui, M.S. (2007). The Arabidopsis AAA ATPase SKD1
1298 is involved in multivesicular endosome function and interacts with its positive
1299 regulator LYST-INTERACTING PROTEIN5. *Plant Cell* 19, 1295-1312.
- 1300 Hagerman, A.E., and Butler, L.G. (1981). The specificity of proanthocyanidin-protein
1301 interactions. *J Biol Chem* 256, 4494-4497.
- 1302 Halling, K.K., Ramstedt, B., and Slotte, J.P. (2008). Glycosylation induces shifts in
1303 the lateral distribution of cholesterol from ordered towards less ordered domains.
1304 *Biochim Biophys Acta* 1778, 1100-1111.
- 1305 Hehenberger, E., Kradolfer, D., and Kohler, C. (2012). Endosperm cellularization
1306 defines an important developmental transition for embryo development.
1307 *Development* 139, 2031-2039.
- 1308 Huck, N., Moore, J.M., Federer, M., and Grossniklaus, U. (2003). The Arabidopsis
1309 mutant *feronia* disrupts the female gametophytic control of pollen tube reception.
1310 *Development* 130, 2149-2159.
- 1311 Hurst, L.R., and Fratti, R.A. (2020). Lipid Rafts, Sphingolipids, and Ergosterol in
1312 Yeast Vacuole Fusion and Maturation. *Front Cell Dev Biol* 8, 539.

- 1313 Ichino, T., Maeda, K., Hara-Nishimura, I., and Shimada, T. (2020). Arabidopsis
1314 ECHIDNA protein is involved in seed coloration, protein trafficking to vacuoles,
1315 and vacuolar biogenesis. *J Exp Bot* 71, 3999-4009.
- 1316 Ichino, T., Fuji, K., Ueda, H., Takahashi, H., Koumoto, Y., Takagi, J., Tamura, K.,
1317 Sasaki, R., Aoki, K., Shimada, T., and Hara-Nishimura, I. (2014). GFS9/TT9
1318 contributes to intracellular membrane trafficking and flavonoid accumulation in
1319 *Arabidopsis thaliana*. *Plant J* 80, 410-423.
- 1320 Ingram, G.C. (2010). Family life at close quarters: communication and constraint in
1321 angiosperm seed development. *Protoplasma* 247, 195-214.
- 1322 Jaillais, Y., Santambrogio, M., Rozier, F., Fobis-Loisy, I., Miege, C., and Gaude, T.
1323 (2007). The retromer protein VPS29 links cell polarity and organ initiation in
1324 plants. *Cell* 130, 1057-1070.
- 1325 Jaquinod, M., Villiers, F., Kieffer-Jaquinod, S., Hugouvieux, V., Bruley, C., Garin, J.,
1326 and Bourguignon, J. (2007). A proteomics dissection of *Arabidopsis thaliana*
1327 vacuoles isolated from cell culture. *Mol Cell Proteomics* 6, 394-412.
- 1328 Johnson, C.S., Kolevski, B., and Smyth, D.R. (2002). TRANSPARENT TESTA
1329 GLABRA2, a trichome and seed coat development gene of *Arabidopsis*, encodes
1330 a WRKY transcription factor. *Plant Cell* 14, 1359-1375.
- 1331 Kim, S., Naylor, S.A., and DiAntonio, A. (2012). Drosophila Golgi membrane protein
1332 Ema promotes autophagosomal growth and function. *Proc Natl Acad Sci U S A*
1333 109, E1072-1081.
- 1334 Kim, S., Wairkar, Y.P., Daniels, R.W., and DiAntonio, A. (2010). The novel
1335 endosomal membrane protein Ema interacts with the class C Vps-HOPS
1336 complex to promote endosomal maturation. *J Cell Biol* 188, 717-734.
- 1337 Kitamura, S., Matsuda, F., Tohge, T., Yonekura-Sakakibara, K., Yamazaki, M.,
1338 Saito, K., and Narumi, I. (2010). Metabolic profiling and cytological analysis of
1339 proanthocyanidins in immature seeds of *Arabidopsis thaliana* flavonoid
1340 accumulation mutants. *Plant J* 62, 549-559.
- 1341 Köhler, C., Dziasek, K., and Del Toro-De Leon, G. (2021). Postzygotic reproductive
1342 isolation established in the endosperm: mechanisms, drivers and relevance.
1343 *Philos Trans R Soc Lond B Biol Sci* 376, 20200118.
- 1344 Koornneef, M. (1981). The complex syndrome of ttg mutants. *Arabidopsis Inf Serv*
1345 18, 1-4.

- 1346 Koornneef, M. (1990). Mutations affecting the testa colour in Arabidopsis.
1347 Arabidopsis Inf Serv 27, 1-4.
- 1348 Küllich, I., and Zarsky, V. (2014). Autophagy-related direct membrane import from
1349 ER/cytoplasm into the vacuole or apoplast: a hidden gateway also for secondary
1350 metabolites and phytohormones? Int J Mol Sci 15, 7462-7474.
- 1351 Laloi, M., Perret, A.M., Chatre, L., Melser, S., Cantrel, C., Vaultier, M.N., Zachowski,
1352 A., Bathany, K., Schmitter, J.M., Vallet, M., Lessire, R., Hartmann, M.A., and
1353 Moreau, P. (2007). Insights into the role of specific lipids in the formation and
1354 delivery of lipid microdomains to the plasma membrane of plant cells. Plant
1355 Physiol 143, 461-472.
- 1356 Lepiniec, L., Debeaujon, I., Routaboul, J.M., Baudry, A., Pourcel, L., Nesi, N., and
1357 Caboche, M. (2006). Genetics and biochemistry of seed flavonoids. Annu Rev
1358 Plant Biol 57, 405-430.
- 1359 Li, R., Sun, R., Hicks, G.R., and Raikhel, N.V. (2015). Arabidopsis ribosomal
1360 proteins control vacuole trafficking and developmental programs through the
1361 regulation of lipid metabolism. Proc Natl Acad Sci U S A 112, E89-98.
- 1362 Loubéry, S., De Giorgi, J., Utz-Pugin, A., Demonsais, L., and Lopez-Molina, L.
1363 (2018). A Maternally Deposited Endosperm Cuticle Contributes to the
1364 Physiological Defects of transparent testa Seeds. Plant Physiol 177, 1218-1233.
- 1365 Luna, E., Pastor, V., Robert, J., Flors, V., Mauch-Mani, B., and Ton, J. (2011).
1366 Callose deposition: a multifaceted plant defense response. Molecular plant-
1367 microbe interactions : MPMI 24, 183-193.
- 1368 MacGregor, D.R., Kendall, S.L., Florance, H., Fedi, F., Moore, K., Paszkiewicz, K.,
1369 Smirnoff, N., and Penfield, S. (2015). Seed production temperature regulation of
1370 primary dormancy occurs through control of seed coat phenylpropanoid
1371 metabolism. New Phytol 205, 642-652.
- 1372 Mamode Cassim, A., Gouguet, P., Gronnier, J., Laurent, N., Germain, V., Grison,
1373 M., Boutte, Y., Gerbeau-Pissot, P., Simon-Plas, F., and Mongrand, S. (2019).
1374 Plant lipids: Key players of plasma membrane organization and function. Prog
1375 Lipid Res 73, 1-27.
- 1376 Marinova, K., Pourcel, L., Weder, B., Schwarz, M., Barron, D., Routaboul, J.M.,
1377 Debeaujon, I., and Klein, M. (2007). The Arabidopsis MATE transporter TT12
1378 acts as a vacuolar flavonoid/H⁺ -antiporter active in proanthocyanidin-
1379 accumulating cells of the seed coat. Plant Cell 19, 2023-2038.

- 1380 Marmagne, A., Ferro, M., Meinnel, T., Bruley, C., Kuhn, L., Garin, J., Barbier-
1381 Brygoo, H., and Ephritikhine, G. (2007). A high content in lipid-modified peripheral
1382 proteins and integral receptor kinases features in the arabidopsis plasma
1383 membrane proteome. *Mol Cell Proteomics* 6, 1980-1996.
- 1384 McFarlane, H.E., Watanabe, Y., Gendre, D., Carruthers, K., Levesque-Tremblay,
1385 G., Haughn, G.W., Bhalerao, R.P., and Samuels, L. (2013). Cell wall
1386 polysaccharides are mislocalized to the Vacuole in echidna mutants. *Plant Cell*
1387 *Physiol* 54, 1867-1880.
- 1388 Molina, I., Ohlrogge, J.B., and Pollard, M. (2008). Deposition and localization of lipid
1389 polyester in developing seeds of *Brassica napus* and *Arabidopsis thaliana*. *Plant*
1390 *J* 53, 437-449.
- 1391 Mutterer, J., and Zinck, E. (2013). Quick-and-clean article figures with FigureJ. *J*
1392 *Microsc* 252, 89-91.
- 1393 Nesi, N. (2001). Analyse génétique et moléculaire du métabolisme des flavonoïdes
1394 dans les graines d'*Arabidopsis thaliana*. Caractérisation du mutants et des gènes
1395 correspondants. In *Sciences (Institut National Agronomique Paris-Grignon)*, pp.
1396 167.
- 1397 Nesi, N., Debeaujon, I., Jond, C., Stewart, A.J., Jenkins, G.I., Caboche, M., and
1398 Lepiniec, L. (2002). The TRANSPARENT TESTA16 locus encodes the
1399 ARABIDOPSIS BSISTER MADS domain protein and is required for proper
1400 development and pigmentation of the seed coat. *Plant Cell* 14, 2463-2479.
- 1401 North, H., Baud, S., Debeaujon, I., Dubos, C., Dubreucq, B., Grappin, P., Jullien,
1402 M., Lepiniec, L., Marion-Poll, A., Miquel, M., Rajjou, L., Routaboul, J.M., and
1403 Caboche, M. (2010). *Arabidopsis* seed secrets unravelled after a decade of
1404 genetic and omics-driven research. *Plant J* 61, 971-981.
- 1405 Ozolina, N.V., Nesterkina, I.S., Kolesnikova, E.V., Salyaev, R.K., Nurminsky, V.N.,
1406 Rakevich, A.L., Martynovich, E.F., and Chernyshov, M.Y. (2013). Tonoplast of
1407 *Beta vulgaris* L. contains detergent-resistant membrane microdomains. *Planta*
1408 237, 859-871.
- 1409 Pandey, R., Bakay, M., Hain, H.S., Strenkowski, B., Yermakova, A., Kushner, J.A.,
1410 Orange, J.S., and Hakonarson, H. (2019). The Autoimmune Disorder
1411 Susceptibility Gene CLEC16A Restrains NK Cell Function in YTS NK Cell Line
1412 and Clec16a Knockout Mice. *Front Immunol* 10, 68.

- 1413 Pook, V.G., Nair, M., Ryu, K., Arpin, J.C., Schiefelbein, J., Schrick, K., and DeBolt,
1414 S. (2017). Positioning of the SCRAMBLED receptor requires UDP-Glc:sterol
1415 glucosyltransferase 80B1 in Arabidopsis roots. *Sci Rep* 7, 5714.
- 1416 Porter, L.J. (1992). Structure and chemical properties of the condensed tannins. In
1417 *Plant Polyphenols*, R.W. Hemingway and P.E. Laks, eds (New York: Plenum
1418 Press), pp. 245-258.
- 1419 Pourcel, L., Routaboul, J.M., Kerhoas, L., Caboche, M., Lepiniec, L., and
1420 Debeaujon, I. (2005). TRANSPARENT TESTA10 encodes a laccase-like enzyme
1421 involved in oxidative polymerization of flavonoids in Arabidopsis seed coat. *Plant*
1422 *Cell* 17, 2966-2980.
- 1423 Poustka, F., Irani, N.G., Feller, A., Lu, Y., Pourcel, L., Frame, K., and Grotewold, E.
1424 (2007). A trafficking pathway for anthocyanins overlaps with the endoplasmic
1425 reticulum-to-vacuole protein-sorting route in Arabidopsis and contributes to the
1426 formation of vacuolar inclusions. *Plant Physiol* 145, 1323-1335.
- 1427 Radin, I., Richardson, R.A., Coomey, J.H., Weiner, E.R., Bascom, C.S., Li, T.,
1428 Bezanilla, M., and Haswell, E.S. (2021). Plant PIEZO homologs modulate
1429 vacuole morphology during tip growth. *Science* 373, 586-590.
- 1430 Ramirez-Estrada, K., Castillo, N., Lara, J.A., Arro, M., Boronat, A., Ferrer, A., and
1431 Altabella, T. (2017). Tomato UDP-Glucose Sterol Glycosyltransferases: A Family
1432 of Developmental and Stress Regulated Genes that Encode Cytosolic and
1433 Membrane-Associated Forms of the Enzyme. *Frontiers in plant science* 8, 984.
- 1434 Rapsomaniki, M.A., Kotsantis, P., Symeonidou, I.E., Giakoumakis, N.N., Taraviras,
1435 S., and Lygerou, Z. (2012). easyFRAP: an interactive, easy-to-use tool for
1436 qualitative and quantitative analysis of FRAP data. *Bioinformatics* 28, 1800-1801.
- 1437 Robert, H.S. (2019). Molecular Communication for Coordinated Seed and Fruit
1438 Development: What Can We Learn from Auxin and Sugars? *Int J Mol Sci* 20.
- 1439 Rosado, A., Sohn, E.J., Drakakaki, G., Pan, S., Swidergal, A., Xiong, Y., Kang, B.H.,
1440 Bressan, R.A., and Raikhel, N.V. (2010). Auxin-mediated ribosomal biogenesis
1441 regulates vacuolar trafficking in Arabidopsis. *Plant Cell* 22, 143-158.
- 1442 Rosado, A., Hicks, G.R., Norambuena, L., Rogachev, I., Meir, S., Pourcel, L.,
1443 Zouhar, J., Brown, M.Q., Boirsdore, M.P., Puckrin, R.S., Cutler, S.R., Rojo, E.,
1444 Aharoni, A., and Raikhel, N.V. (2011). Sortin1-hypersensitive mutants link
1445 vacuolar-trafficking defects and flavonoid metabolism in Arabidopsis vegetative
1446 tissues. *Chem Biol* 18, 187-197.

- 1447 Routaboul, J.M., Kerhoas, L., Debeaujon, I., Pourcel, L., Caboche, M., Einhorn, J.,
1448 and Lepiniec, L. (2006). Flavonoid diversity and biosynthesis in seed of
1449 *Arabidopsis thaliana*. *Planta* 224, 96-107.
- 1450 Routaboul, J.M., Dubos, C., Beck, G., Marquis, C., Bidzinski, P., Loudet, O., and
1451 Lepiniec, L. (2012). Metabolite profiling and quantitative genetics of natural
1452 variation for flavonoids in *Arabidopsis*. *J Exp Bot* 63, 3749-3764.
- 1453 Sagasser, M., Lu, G.H., Hahlbrock, K., and Weisshaar, B. (2002). *A. thaliana*
1454 TRANSPARENT TESTA 1 is involved in seed coat development and defines the
1455 WIP subfamily of plant zinc finger proteins. *Genes Dev* 16, 138-149.
- 1456 Saito, C., Morita, M.T., Kato, T., and Tasaka, M. (2005). Amyloplasts and vacuolar
1457 membrane dynamics in the living graviperceptive cell of the *Arabidopsis*
1458 inflorescence stem. *Plant Cell* 17, 548-558.
- 1459 Sandor, R., Der, C., Grosjean, K., Anca, I., Noirot, E., Leborgne-Castel, N.,
1460 Lochman, J., Simon-Plas, F., and Gerbeau-Pissot, P. (2016). Plasma membrane
1461 order and fluidity are diversely triggered by elicitors of plant defence. *J Exp Bot*
1462 67, 5173-5185.
- 1463 Schaller, H. (2004). New aspects of sterol biosynthesis in growth and development
1464 of higher plants. *Plant Physiol Biochem* 42, 465-476.
- 1465 Scheuring, D., Scholler, M., Kleine-Vehn, J., and Lofke, C. (2015). Vacuolar staining
1466 methods in plant cells. *Methods Mol Biol* 1242, 83-92.
- 1467 Schneider, C.A., Rasband, W.S., and Eliceiri, K.W. (2012). NIH Image to ImageJ:
1468 25 years of image analysis. *Nat Methods* 9, 671-675.
- 1469 Shahriari, M., Hulskamp, M., and Schellmann, S. (2010a). Seeds of *Arabidopsis*
1470 plants expressing dominant-negative AtSKD1 under control of the GL2 promoter
1471 show a transparent testa phenotype and a mucilage defect. *Plant Signal Behav*
1472 5, 1308-1310.
- 1473 Shahriari, M., Keshavaiah, C., Scheuring, D., Sabovljevic, A., Pimpl, P., Hausler,
1474 R.E., Hulskamp, M., and Schellmann, S. (2010b). The AAA-type ATPase AtSKD1
1475 contributes to vacuolar maintenance of *Arabidopsis thaliana*. *Plant J* 64, 71-85.
- 1476 Shai, N., Schuldiner, M., and Zalckvar, E. (2016). No peroxisome is an island -
1477 Peroxisome contact sites. *Biochim Biophys Acta* 1863, 1061-1069.
- 1478 Shimada, T., Takagi, J., Ichino, T., Shirakawa, M., and Hara-Nishimura, I. (2018).
1479 Plant Vacuoles. *Annu Rev Plant Biol* 69, 123-145.

- 1480 Shimada, T.L., Ueda, T., and Hara-Nishimura, I. (2021). Excess sterol accumulation
1481 affects seed morphology and physiology in *Arabidopsis thaliana*. *Plant Signal*
1482 *Behav* 16, 1872217.
- 1483 Shirley, B.W., Kubasek, W.L., Storz, G., Bruggemann, E., Koornneef, M., Ausubel,
1484 F.M., and Goodman, H.M. (1995). Analysis of *Arabidopsis* mutants deficient in
1485 flavonoid biosynthesis. *Plant J* 8, 659-671.
- 1486 Sorensen, M.B., Mayer, U., Lukowitz, W., Robert, H., Chambrier, P., Jurgens, G.,
1487 Somerville, C., Lepiniec, L., and Berger, F. (2002). Cellularisation in the
1488 endosperm of *Arabidopsis thaliana* is coupled to mitosis and shares multiple
1489 components with cytokinesis. *Development* 129, 5567-5576.
- 1490 Souter, M., Topping, J., Pullen, M., Friml, J., Palme, K., Hackett, R., Grierson, D.,
1491 and Lindsey, K. (2002). hydra Mutants of *Arabidopsis* are defective in sterol
1492 profiles and auxin and ethylene signaling. *Plant Cell* 14, 1017-1031.
- 1493 Stucky, D.F., Arpin, J.C., and Schrick, K. (2015). Functional diversification of two
1494 UGT80 enzymes required for steryl glucoside synthesis in *Arabidopsis*. *J Exp Bot*
1495 66, 189-201.
- 1496 Surpin, M., Zheng, H., Morita, M.T., Saito, C., Avila, E., Blakeslee, J.J.,
1497 Bandyopadhyay, A., Kovaleva, V., Carter, D., Murphy, A., Tasaka, M., and
1498 Raikhel, N. (2003). The VTI family of SNARE proteins is necessary for plant
1499 viability and mediates different protein transport pathways. *Plant Cell* 15, 2885-
1500 2899.
- 1501 Sussman, M.R., Amasino, R.M., Young, J.C., Krysan, P.J., and Austin-Phillips, S.
1502 (2000). The *Arabidopsis* knockout facility at the University of Wisconsin-Madison.
1503 *Plant Physiol* 124, 1465-1467.
- 1504 Tamura, N., Yoshida, T., Tanaka, A., Sasaki, R., Bando, A., Toh, S., Lepiniec, L.,
1505 and Kawakami, N. (2006). Isolation and characterization of high temperature-
1506 resistant germination mutants of *Arabidopsis thaliana*. *Plant Cell Physiol* 47,
1507 1081-1094.
- 1508 Tavernier, E., Le Quoc, D., and Le Quoc, K. (1993). Lipid composition of the
1509 vacuolar membrane of *Acer pseudoplatanus* cultured cells. *Biochim Biophys Acta*
1510 1167, 242-247.
- 1511 Uemura, T., and Ueda, T. (2014). Plant vacuolar trafficking driven by RAB and
1512 SNARE proteins. *Curr Opin Plant Biol* 22, 116-121.

- 1513 van Luijn, M.M., Kreft, K.L., Jongsma, M.L., Mes, S.W., Wierenga-Wolf, A.F., van
1514 Meurs, M., Melief, M.J., der Kant, R., Janssen, L., Janssen, H., Tan, R., Priatel,
1515 J.J., Neefjes, J., Laman, J.D., and Hintzen, R.Q. (2015). Multiple sclerosis-
1516 associated CLEC16A controls HLA class II expression via late endosome
1517 biogenesis. *Brain* 138, 1531-1547.
- 1518 Warnecke, D., Erdmann, R., Fahl, A., Hube, B., Muller, F., Zank, T., Zahringer, U.,
1519 and Heinz, E. (1999). Cloning and functional expression of UGT genes encoding
1520 sterol glucosyltransferases from *Saccharomyces cerevisiae*, *Candida albicans*,
1521 *Pichia pastoris*, and *Dictyostelium discoideum*. *J Biol Chem* 274, 13048-13059.
- 1522 Warnecke, D.C., Baltrusch, M., Buck, F., Wolter, F.P., and Heinz, E. (1997). UDP-
1523 glucose:sterol glucosyltransferase: cloning and functional expression in
1524 *Escherichia coli*. *Plant Mol Biol* 35, 597-603.
- 1525 Watanabe, T., Tani, M., Ishibashi, Y., Endo, I., Okino, N., and Ito, M. (2015).
1526 Ergosteryl-beta-glucosidase (Egh1) involved in sterylglucoside catabolism and
1527 vacuole formation in *Saccharomyces cerevisiae*. *Glycobiology* 25, 1079-1089.
- 1528 Winkel, B.S.J. (2019). The subtleties of subcellular distribution. Pointing the way to
1529 underexplored functions for flavonoid enzymes and endproducts. In *Recent*
1530 *Advances in Polyphenol Research* H. Halbwirth, K. Stich, V. Cheynier, and S.
1531 Guideau, eds (John Wiley & Sons Ltd.), pp. 89-107.
- 1532 Yamaguchi, M., and Kasamo, K. (2001). Modulation in the activity of purified
1533 tonoplast H⁺-ATPase by tonoplast glycolipids prepared from cultured rice (*Oryza*
1534 *sativa* L. var. Boro) cells. *Plant Cell Physiol* 42, 516-523.
- 1535 Yamaguchi, M., and Kasamo, K. (2002). Modulation of proton pumping across
1536 proteoliposome membranes reconstituted with tonoplast H⁽⁺⁾-ATPase from
1537 cultured rice (*Oryza sativa* L. var. Boro) cells by acyl steryl glucoside and steryl
1538 glucoside. *Plant Cell Physiol* 43, 816-822.
- 1539 Yoshida, K., Ohnishi, M., Fukao, Y., Okazaki, Y., Fujiwara, M., Song, C., Nakanishi,
1540 Y., Saito, K., Shimmen, T., Suzaki, T., Hayashi, F., Fukaki, H., Maeshima, M.,
1541 and Mimura, T. (2013). Studies on vacuolar membrane microdomains isolated
1542 from *Arabidopsis* suspension-cultured cells: local distribution of vacuolar
1543 membrane proteins. *Plant Cell Physiol* 54, 1571-1584.
- 1544 Yoshida, S., and Uemura, M. (1986). Lipid Composition of Plasma Membranes and
1545 Tonoplasts Isolated from Etiolated Seedlings of Mung Bean (*Vigna radiata* L.).
1546 *Plant Physiol* 82, 807-812.

- 1547 Zauber, H., Burgos, A., Garapati, P., and Schulze, W.X. (2014). Plasma membrane
1548 lipid-protein interactions affect signaling processes in sterol-biosynthesis mutants
1549 in *Arabidopsis thaliana*. *Frontiers in plant science* 5, 78.
- 1550 Zhang, C., Hicks, G.R., and Raikhel, N.V. (2015). Molecular Composition of Plant
1551 Vacuoles: Important but Less Understood Regulations and Roles of Tonoplast
1552 Lipids. *Plants (Basel)* 4, 320-333.
- 1553 Zhang, Z.J., and Peck, S.C. (2011). Simplified enrichment of plasma membrane
1554 proteins for proteomic analyses in *Arabidopsis thaliana*. *Proteomics* 11, 1780-
1555 1788.
- 1556 Zhao, J., and Dixon, R.A. (2009). MATE transporters facilitate vacuolar uptake of
1557 epicatechin 3'-O-glucoside for proanthocyanidin biosynthesis in *Medicago*
1558 *truncatula* and *Arabidopsis*. *Plant Cell* 21, 2323-2340.
- 1559 Zumajo-Cardona, C., Aguirre, M., Castillo-Bravo, R., Mizzotti, C., Di Marzo, M.,
1560 Banfi, C., Mendes, M.A., Spillane, C., Colombo, L., and Ezquer, I. (2023).
1561 Maternal control of triploid seed development by the TRANSPARENT TESTA 8
1562 (TT8) transcription factor in *Arabidopsis thaliana*. *Sci Rep* 13, 1316.
- 1563

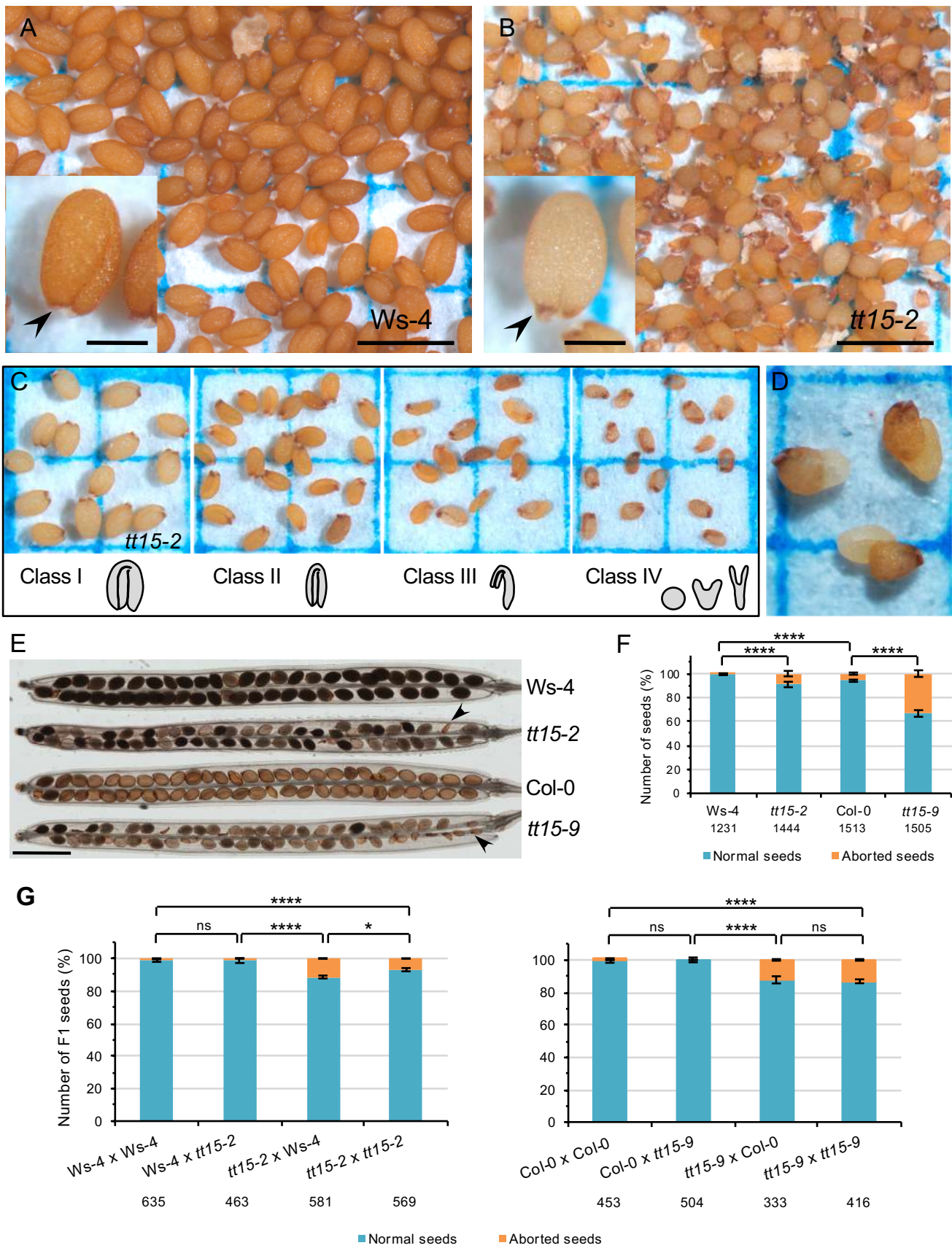


Figure 1. *TT15* Disruption Causes Seed Lethality and Seed Coat Developmental Defects with Incomplete Penetrance and Maternal Inheritance.

(A-B) Mature seed phenotypes showing that *tt15* mutant seeds (**B**) are paler, being pigmented only at the micropyle-chalaza pole (arrowhead in inset) and are smaller than wild-type seeds (**A**). Abnormal seed shapes (from shriveled to aborted seeds) are also observed in (**B**). A normally shaped seed is shown in inset.

(C) Different seed phenotypic classes encountered in a *tt15-2* plant progeny. Schemes refer to prevalent embryo stages observed in mature seeds.

(D) Dry mature seeds of *tt15-2* with embryos emerging from the testa at the level of the curving zone.

(E) and (F) Observation of seed lethality in maturing siliques. **(E)** Cleared siliques. Whole-mounts at 15 days after flowering are shown. Arrowheads point to aborted seeds.

(F) Quantification of aborted seeds realized on 25 siliques per genotype (5 siliques from 5 plants). Sample size is indicated below each bar. Error bars represent standard errors (n=25).

(G) Classification of F1 seeds derived from reciprocal crosses between wild types and corresponding *tt15* mutants based on the seed lethality phenotype. Sample size is indicated below each bar. ns, not significant. Error bars represent standard errors (n=13 for *Ws-4* serie and n=8 for *Col-0* serie, with n being the number of analyzed siliques). Asterisks in **(F)** and **(G)** indicate significant differences for aborted seeds using the non-parametric Mann-Whitney U test (**** $\alpha=0.1\%$ *** $\alpha=1\%$; ** $\alpha=2.5\%$; * $\alpha=5\%$; ns, not significant at $\alpha=5\%$). Bar = 1 mm (250 μ m in insets) in **(A)** and **(B)** and 1.5 mm in **(E)**.

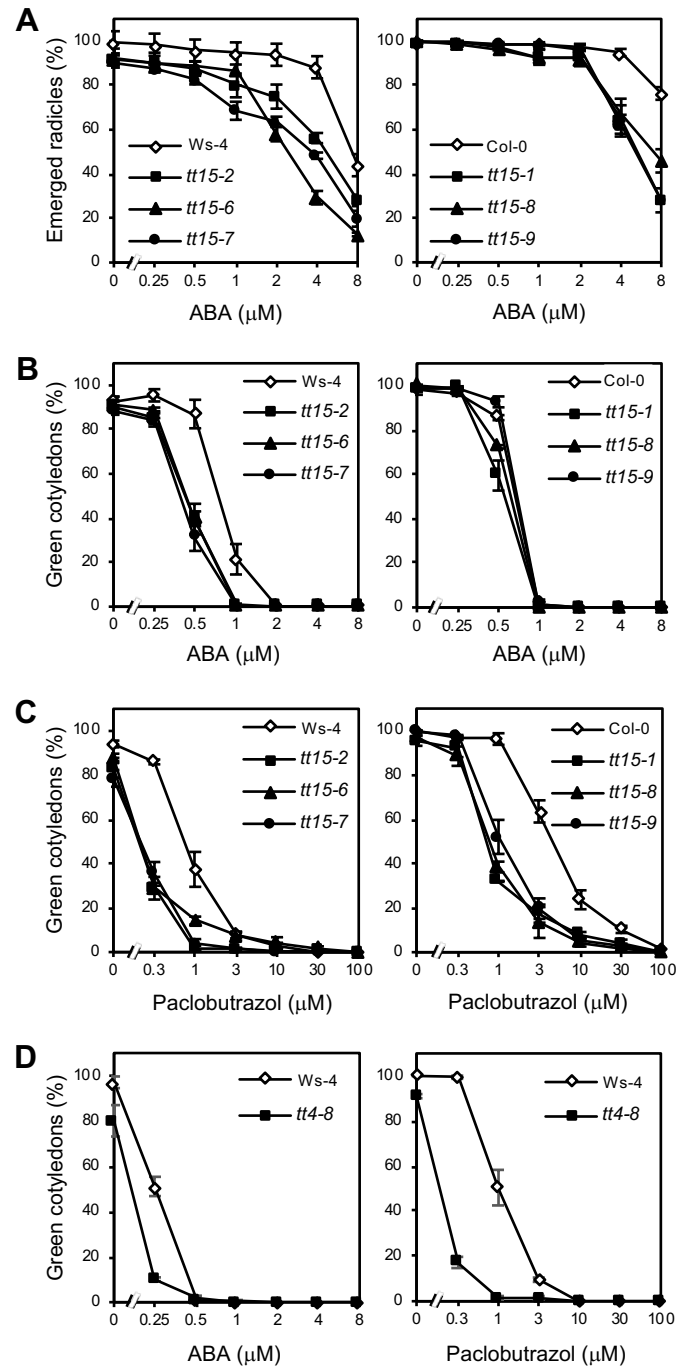


Figure 2. TT15 Depletion Increases Sensitivity of Seed Germination to Abscisic Acid and Paclobutrazol.

(A) and **(B)** Sensitivity to exogenous abscisic acid (ABA), expressed as percentages of emerged radicles and green cotyledons, respectively.

(C) Sensitivity to the gibberellin biosynthesis inhibitor paclobutrazol (PAC).

(D) Sensitivity of *tt4-8* seeds to ABA and PAC.

Error bars represent standard errors ($n=3$).

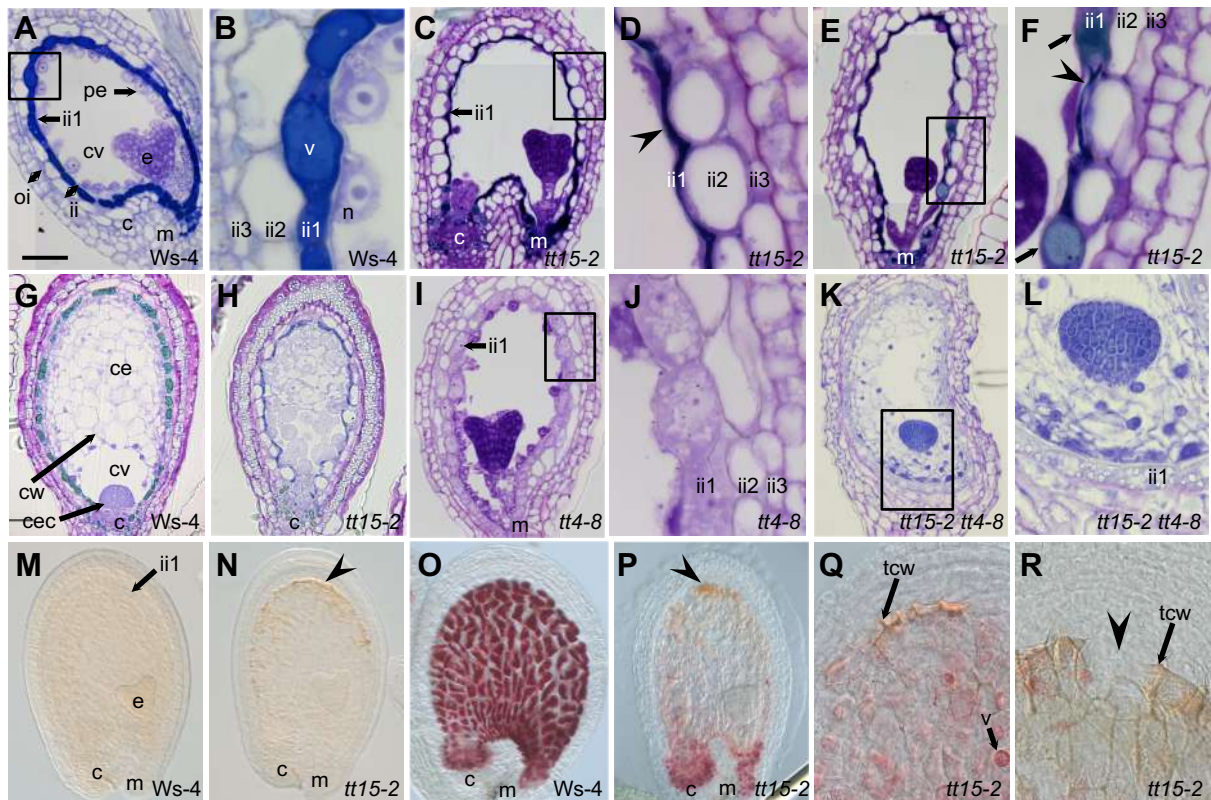


Figure 3. Loss of TT15 Affects Endothelium and Endosperm Development.

(A) to (L) Longitudinal sections of developing seeds stained with toluidine blue O.

(A) and (B) Dark blue-stained flavanols fill the wild-type vacuoles of endothelial cells (ii1 cell layer) at around four days after flowering (daf).

(C) to (F) Most *tt15* endothelial cells flatten and degenerate in the course of flavanol accumulation (arrowheads). (E) and (F) A few ii1 cells exhibit unflattened cells with typical vacuolar structures (arrows) in some seeds.

(G) Wild-type seed with a completely cellularized endosperm at around 6 daf.

(H) Some *tt15* seeds (class III) display strong endosperm defects, with no obvious cellularization and an aberrant chalazal cyst.

(I) and (J) Developing seeds of *tt4-8* deprived of flavonoids do not exhibit flatten endothelium.

(K) and (L) In the double mutant *tt15-2 tt4-8* deprived of flavonoids, endothelium flattening and degeneration is suppressed compared with the situation in *tt5-2* background.

(B), (D), (F), (J) and (L) are magnifications of insets from (A), (C), (E), (I) and (K), respectively.

(M) and (N) whole mount cleared seeds observed with differential interference contrast (DIC) microscopy. (N) Endothelial cells of *tt15* located at the abaxial pole of the seed (curving zone) exhibit oxidized (brown) PAs and cell wall thickening (arrowhead).

(O) to (R) Detection of flavanols with vanillin staining (whole mounts at the heart stage; flavanols stain cherry red). (O) and (P) In *tt15* seeds, flavanols are present mainly at the micropyle and chalaza. Oxidized (brown) flavanols are observed at the abaxial pole (curving zone; arrowhead). (Q) and (R) Curving zone of developing *tt15* seeds at the heart stage stained with vanillin (whole mounts). Arrowheads show thickened brown endothelium cell walls (Q) and a breaking point in the endothelium layer (R).

C, chalaza; ce, cellularized endosperm; cec, chalazal endosperm cyst; cv, central vacuole; cw, cell wall; e, embryo; ii, inner integument; m, micropyle; oi, outer integument; n, nodule; tchw, tannic cell wall; v, vacuole. Bar in (A) = 50 μ m in (A), (C), (E), (G), (H), (I), (K) and (M) to (P), 10 μ m in (B), (D), (F), (J) and (L), and 25 μ m in (Q) and (R).

Akary et al.

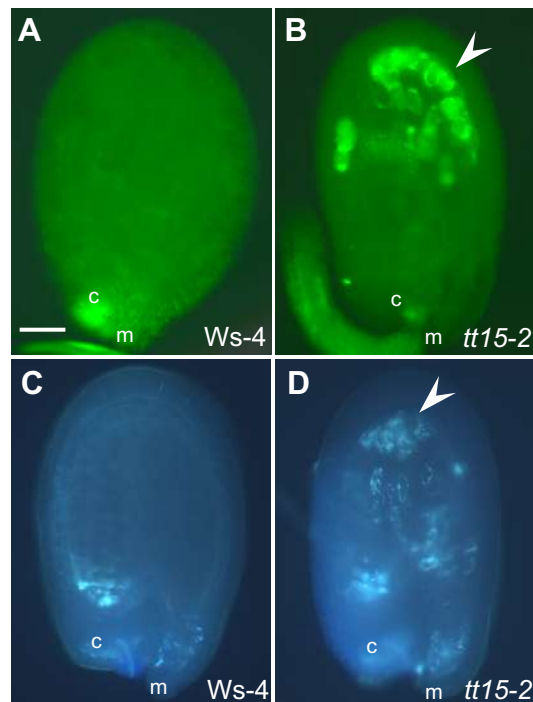


Figure 4. *TT15* Disruption Triggers an Autoimmune-like Response in Seed Coat Endothelium.

Developing seeds observed under UV light are shown (whole mounts).

(A) and **(B)** Detection of ROS with DCFH-DA staining. Arrowhead shows ROS accumulation polarized at the seed abaxial side.

(C) and **(D)** Detection of callose with aniline blue staining. Arrowhead shows ectopic callose deposits.

c, chalaza; m, micropyle. Bar in **(A)** = 50 μm in **(A)** to **(D)**.

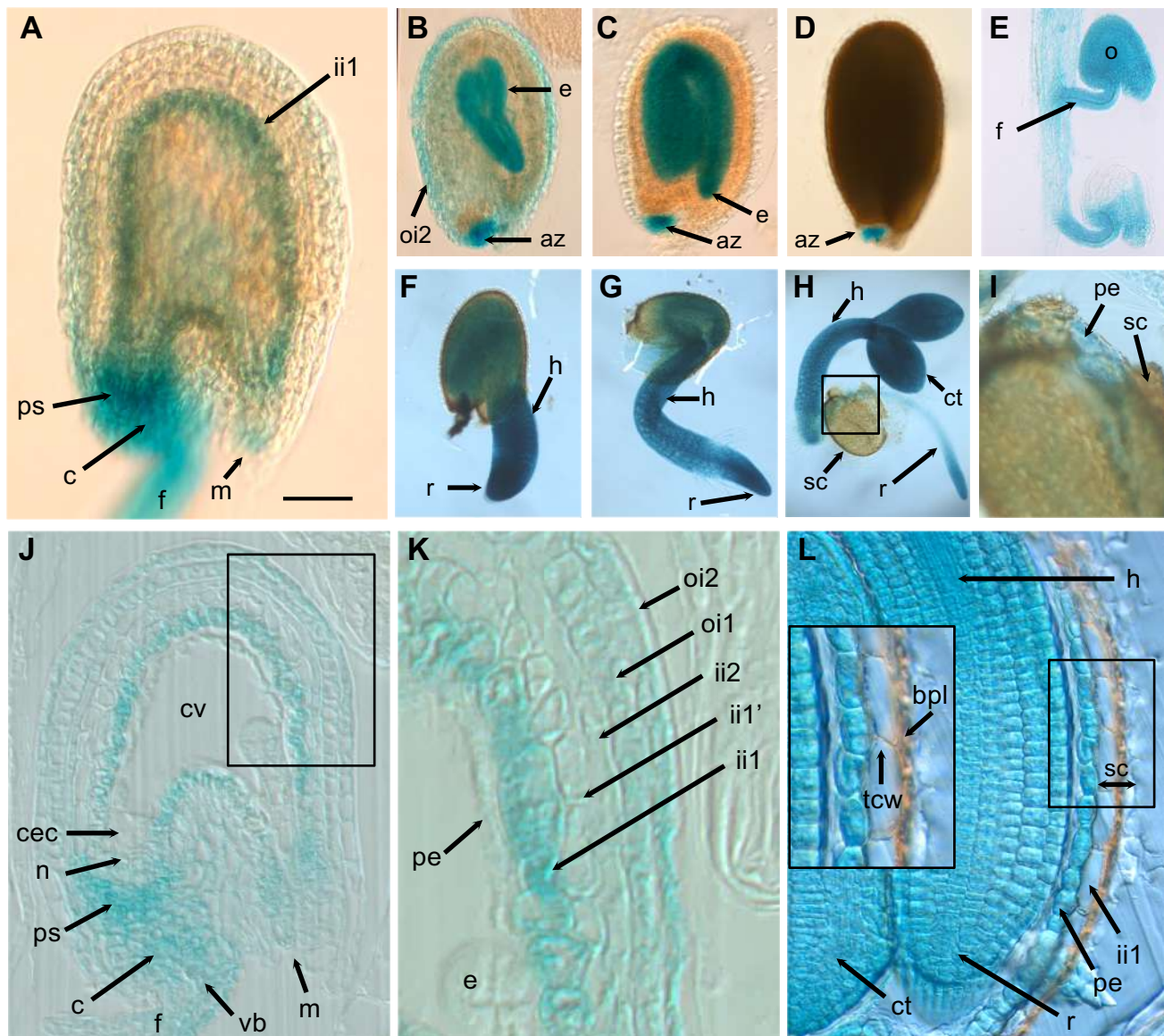


Figure 5. The *TT15* Promoter is Active during Seed Development and Germination.

Arabidopsis transformants expressing the *ProTT15:uidA* construct were analyzed for GUS reporter activity. A 2.0-kb sequence upstream of ATG was used as promoter.

(A) to (D) Developing seeds at the globular (A), torpedo (B), cotyledonary (C) and late maturation (D) stages (whole mounts).

(E) Unfertilized ovules (aborted seeds) from a developed silique (whole mounts).

(F) to (I) Germinating seeds at 32 h (F), 48 h (G) and 72 h (H) after imbibition ; (I) Magnification of the box in (H).

(J) Section of developing seed at the quadrant stage.

(K) Magnification of the box in (J).

(L) Longitudinal section of a mature seed. The inset is a magnification of the boxed area.

az, abscission zone ; bpl, brown pigment layer ; c, chalaza ; cec, chalazal endosperm cyste ; ct, cotyledon ; cv, central vacuole; e, embryo ; f, funiculus ; h, hypocotyle ; ii, inner integument ; m, micropyle ; n, nucellus ; o, ovule ; oi, outer integument ; pe, peripheral endosperm ; ps, pigment strand ; r, radicle ; sc, seed coat ; tcw, tannic cell wall ; vb, vascular bundle. Bar in (A) = 50 μ m in (A) and (J), 140 μ m in (B) to (D), 80 μ m in (E), 250 μ m in (F), 350 μ m in (G), 550 μ m in (H), 200 μ m in (I), 25 μ m in (K), and 50 μ m in (L) (20 μ m in inset).

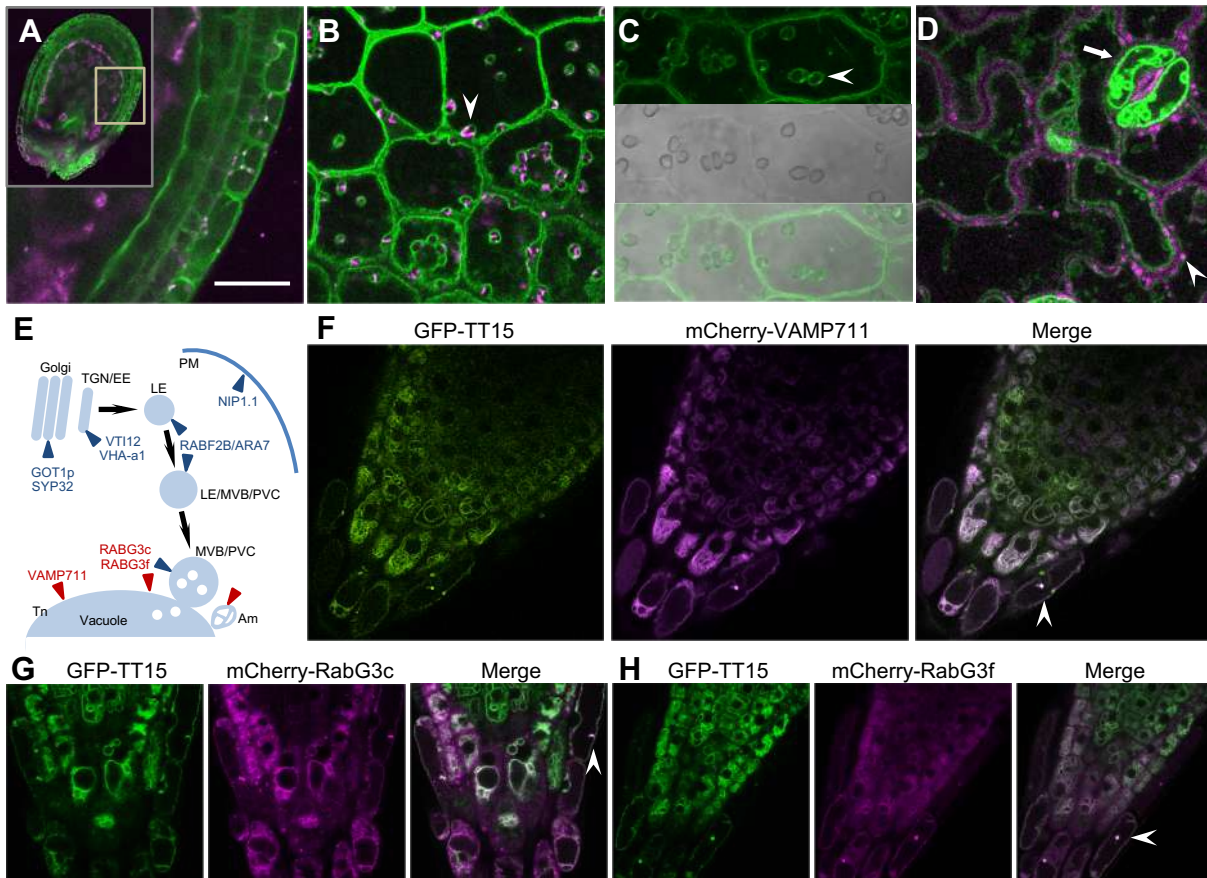


Figure 6. GFP-TT15 Localizes at Tonoplast, Amyloplasts, Cytoplasm and Unknown Intravacuolar Aggregates.

Confocal fluorescence micrographs of Arabidopsis stable transformants are shown.

(A) Transverse optical section of a developing seed at the globular stage expressing GFP-TT15, with magnification at the level of integuments (inset).

(B) and **(C)** Top view of a seed coat epidermis (oi2 cell layer) at the early heart stage of embryo development (around 4 days after flowering) expressing GFP-TT15. Arrowheads show amyloplasts partially co-localizing with GFP-TT15. **(C)** is part of Supplemental Figure 16A (inset).

(D) Cotyledon epidermis from a 3-day-old seedling expressing GFP-TT15, with a stomata (arrow) and chloroplasts (arrowhead). Magenta in **(A)**, **(B)** and **(D)** reveals autofluorescence.

(E) Scheme of subcellular trafficking pathways showing the location of markers used in this study. In red and blue are markers that co-localize or do not co-localize with GFP-TT15, respectively.

(F) to **(H)** Co-localization of GFP-TT15 with various subcellular compartment markers on 5-day-old roots are shown. Arrowheads point to cells with co-localizing intravacuolar aggregates. **(F)** Tonoplast (Tn); **(G)** and **(H)** Multivesicular bodies (MVB) / prevacuolar compartments (PVC).

Bars = 10 μ m; Am, amyloplast; Tn, tonoplast.

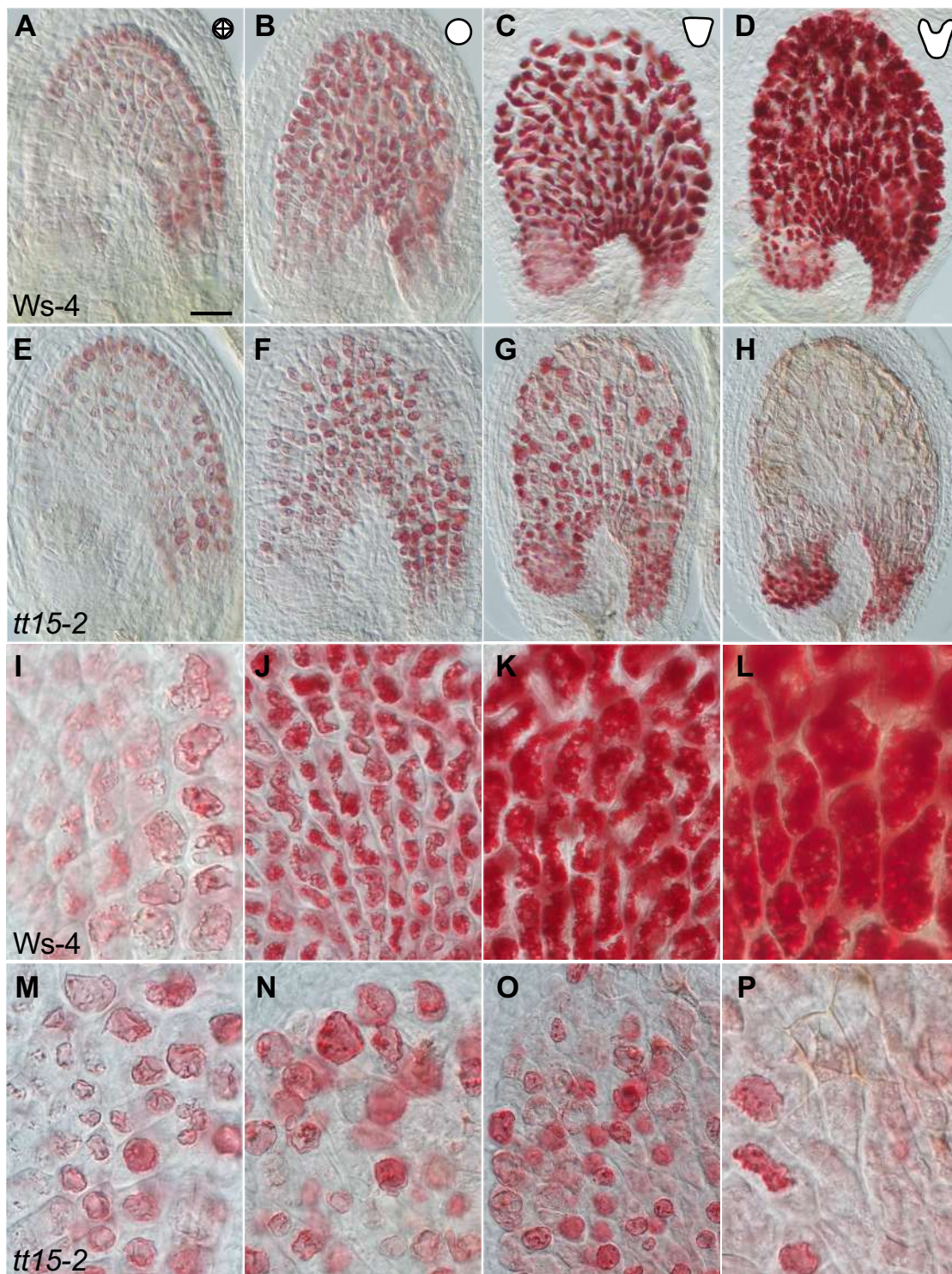


Figure 7. Alteration of Vacuole Development in Endothelium Causes a *transparent testa* Phenotype.

The spatio-temporal pattern of vacuole dynamics in endothelial cells is monitored by following flavanol deposition in vacuoles. Colorless flavanols stain cherry red with vanillin (whole mounts).

(A) to (H) Premature endothelium cell death (PECD) is observed in *tt15-2* mutant seeds from around the globular stage (F) onwards. In comparison, wild-type endothelial vacuoles are filled with flavanols at the heart stage (D).

(I) to (P) Vacuole shape and lumen organization are modified in developing endothelial cells of *tt15-2* mutant seeds.

Bar in (A) = 40 μm in (A), (B), (E) and (F), 50 μm in (C), (D), (G) and (H), 6 μm in (I), (L), (M), (N) and (P), 7 μm in (J), (K), and (O).

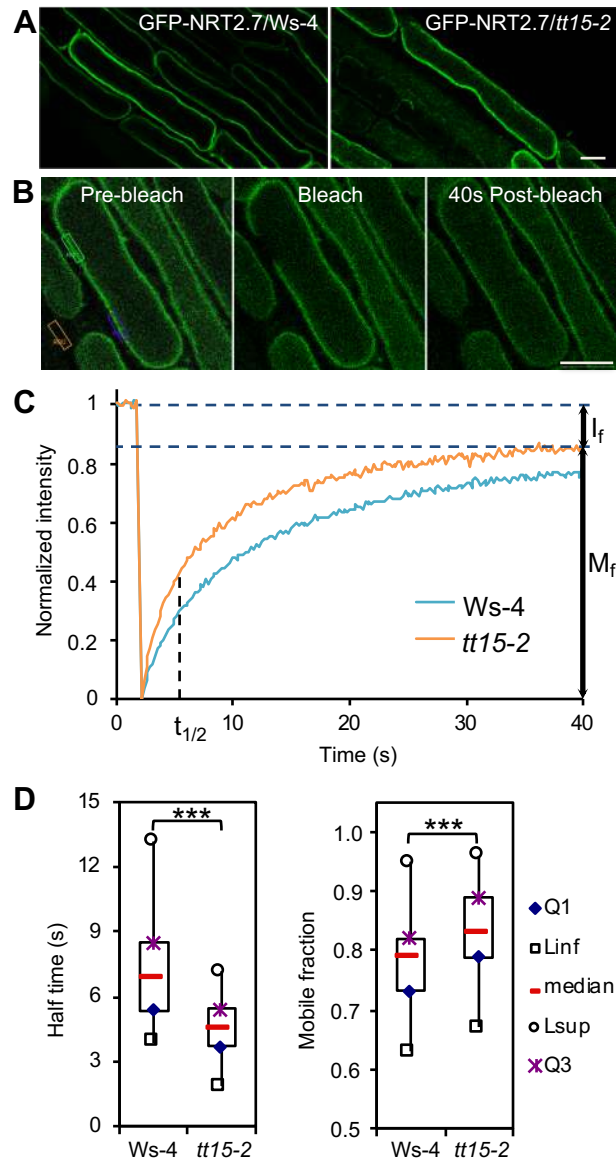


Figure 8. Tonoplast Membrane Fluidity is Increased in *tt15* Mutant Background.

Fluorescence recovery after photobleaching (FRAP) of GFP-NRT2.7 was performed at elongating root epidermal cells of 4 day-old seedlings from Ws-4 wild type and *tt15-2* mutant.

(A) GFP-NRT2.7 subcellular localization is not modified in *tt15-2* background.

(B) Fluorescence-intensity imaging prior to, immediately following, and 40 s after photobleaching. Region of interest 1 (ROI1): bleached area (green); ROI2: unbleached area (purple); ROI3: background area (orange).

(C) Quantitative FRAP analysis. Median values of normalized fluorescence intensities of 66 measurements for each genotype are shown. M_f , mobile fraction ; I_f , immobile fraction ; $t_{1/2}$, recovery half-time.

(D) Box plots of median values of normalized and fitted data for recovery half time and mobile fraction are shown. Asterisks indicate significant differences ($P < 0.001$) between samples by Wilcoxon test.

Bars = 10 μ m in **(A)** and **(B)**.

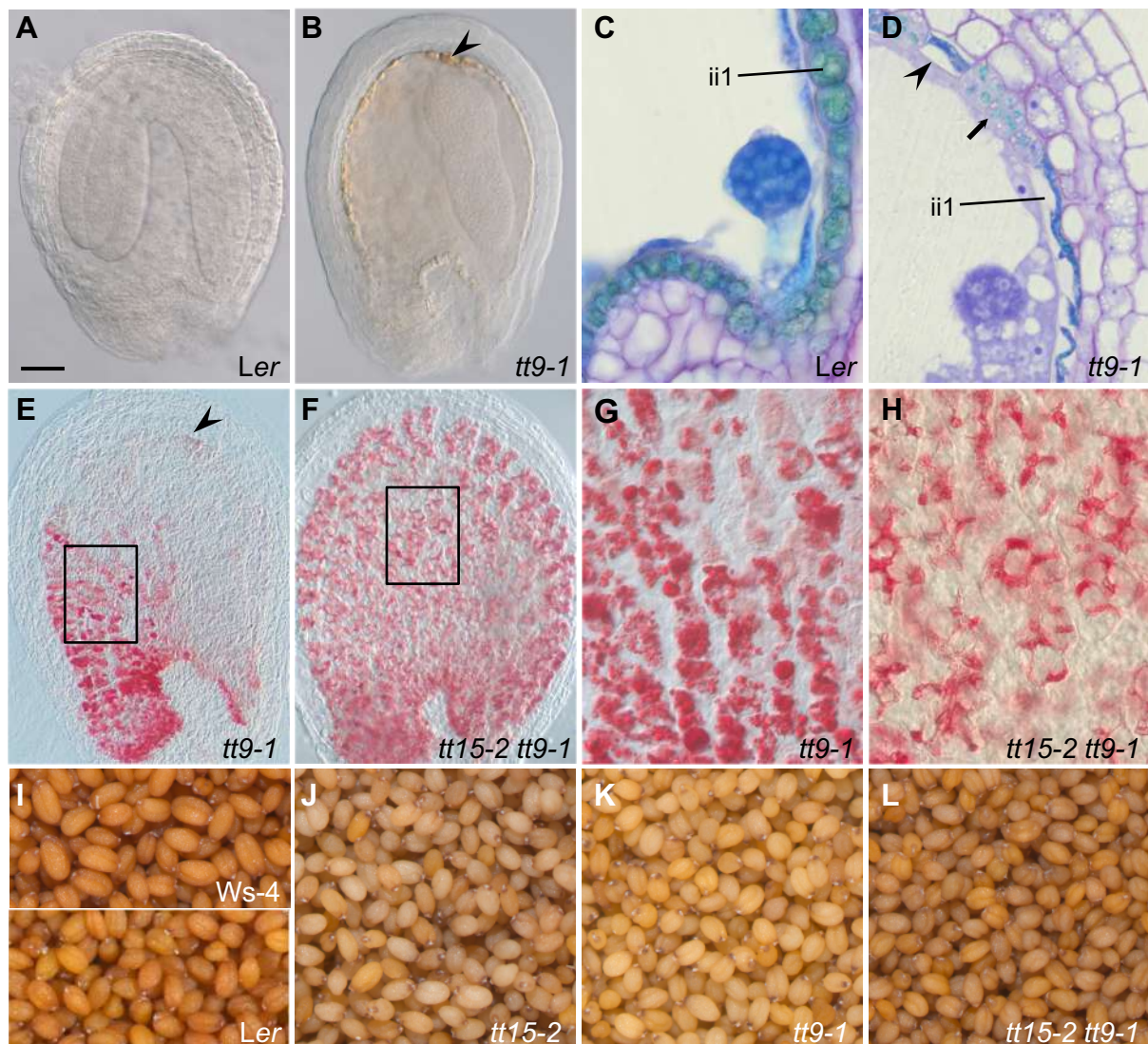


Figure 9. TT15 and TT9/GFS9 Genetically Interact.

(A) to (D) The *tt9-1/gfs9-4* mutant endothelium phenotype resembles *tt15-2* phenotype. (A) Developing wild-type *Ler* seed cleared in chloralhydrate solution (whole mount) (B) Developing seed of *tt9-1* mutant cleared in chloralhydrate solution (whole mount). Endothelial cells exhibit oxidized (brown) PCs and cell wall thickening (arrowhead) mainly at the curving zone (arrows). (C) and (D) Longitudinal sections of developing seeds stained with toluidine blue revealing flavanols in greenish blue. (D) As for *tt15-2*, most *tt9-1* endothelial vacuoles flatten and degenerate in the course of flavanol accumulation (arrowhead). Some cells show fragmented vacuoles typical for *tt9-1* (arrow). ii1, inner integument 1 (endothelium).

(E) to (H) The *tt9-1* mutation partially rescues the *tt15-2* endothelium defects. Detection of flavanols was done with vanillin staining in developing seeds at the heart stage (whole mounts). (E) In *tt9-1* seeds as for *tt15-2*, PCs are present mainly at the micropyle and chalaza and oxidized (brown) PCs are observed at the abaxial pole (curving zone; arrowhead), due to precocious endothelial cell death (PECD). (F) PECD is not observed in the double mutant. (G) and (H) are magnifications of endothelial cells showing vacuole and vesicle morphology (insets in (E) and (F) point to the respective locations).

(I) to (L) Mature seed colours. The double mutant *tt15-2 tt9-1* exhibits a novel pigmentation phenotype.

Bar in (A) = 50 μm in (A), (E) and (F), 25 μm in (B) to (D), 6 μm in (G), 7 μm in (H), and 500 μm in (I) to (L).

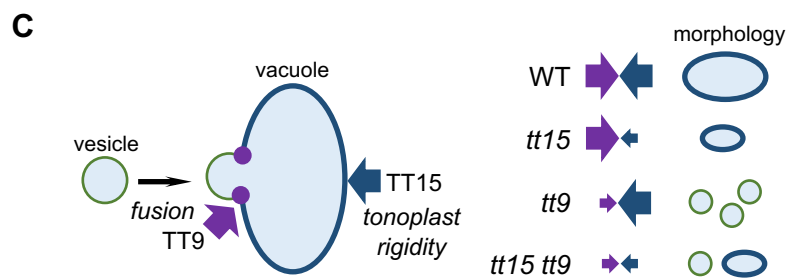
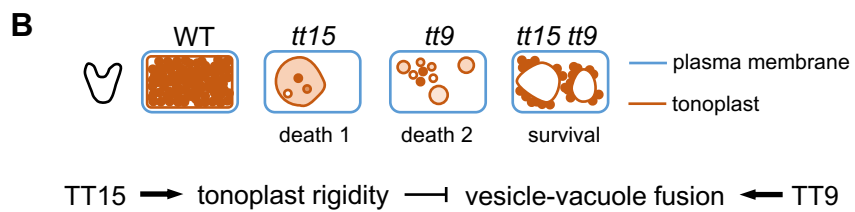
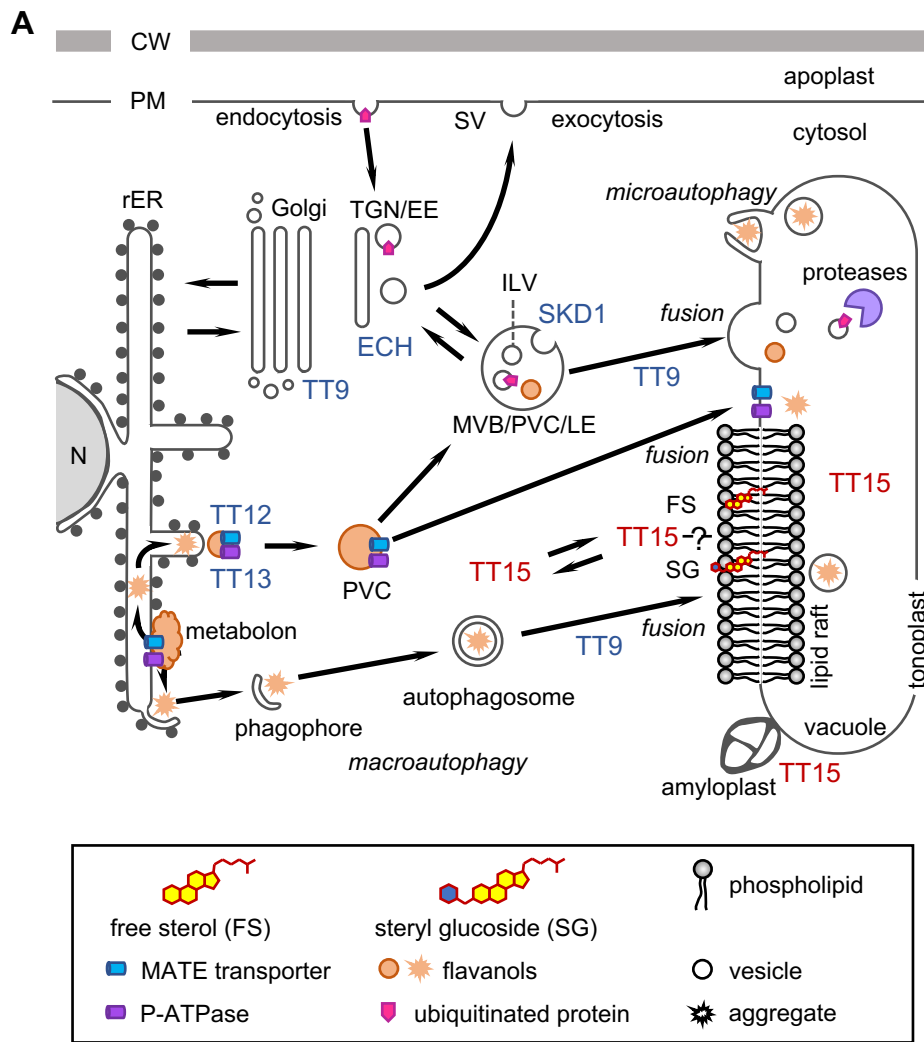


Figure 10. Working Model for the Function of UGT80B1/TT15 in Vacuole Biogenesis and Maintenance at the Seed Coat Endothelium.

(A) Schematics of the endomembrane trafficking system (adapted from Shimada et al., 2018). TT15 subcellular localizations are shown (in red), together with the ones of other endomembrane-related actors involved in the vacuolar sorting pathway for flavanols (in blue). Vacuolar homeostasis, flavanol deposition, degradation of ubiquitinated proteins and autophagy require TT15 for modulation of tonoplast fluidity by glucosylated sterols concentrated at the level of rafts (microdomains). The presence of TT15 at amyloplast-vacuole contact sites suggests functional relationships between both organelles. In absence of TT15, vacuolar homeostasis (biogenesis and maintenance) is disrupted and tonoplast collapses, enabling vacuolar processing enzymes to degrade the cytosolic machinery which leads to cell death. CW, cell wall ; FS, free sterols ; ILV, intraluminal vesicle ; MVB/PVC/LE, multivesicular body/prevacuolar compartment/late endosome ; N, nucleus ; PM, plasma membrane ; rER, rough endoplasmic reticulum ; SG, steryl glucosides ; SV, secretory vesicle ; TGN/EE, trans-Golgi network/early endosome.

(B) Genetic relationship between TT15 and TT9. A schematic interpretation of the situation observed in endothelial cells stained with vanillin at the heart stage of embryo development, is proposed. The accumulation of flavanol-containing vesicles in the vacuole lumen is perturbed differently in *tt15* and *tt9*. The defect is partially suppressed in the double mutant, suggesting that other factors involved in this process, beside TT9 and TT15, are impacted by the mutations. Both *tt15* and *tt9* perturb vacuole biogenesis and maintenance, resulting in precocious vacuole collapse and cell death, however through different genetic routes that compensate each other in the double mutant.

(C) Mechanistic model for the role of TT15 in vacuole dynamics. The increase of tonoplast fluidity caused by *tt15* partially compensates for defective vesicle fusion with the tonoplast due to *tt9*.