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1 **Anther development in *Arabidopsis thaliana* involves symplastic isolation and apoplastic gating of**
2 **the tapetum-middle layer interface.**

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17

18

19 **Abstract**

20

21 During flowering plant reproduction, anthers produce pollen grains, the development of
22 which is supported by the tapetum, a nourishing maternal tissue that also contributes non-
23 cell autonomously to the pollen wall, the resistant external layer on the pollen surface. How
24 the anther restricts movement of the tapetum derived pollen wall components, whilst
25 allowing metabolites such as sugars and amino acids to reach the developing pollen, remains
26 enigmatic. Here we experimentally show that in *Arabidopsis thaliana*, the tapetum and
27 developing pollen are symplastically isolated from each other, and from other sporophytic
28 tissues, from meiosis onwards. We show that the peritapetal strip (PTS), an apoplastic
29 structure, separates the tapetum and the pollen grains from other anther cell layers and can
30 prevent the apoplastic diffusion of fluorescent proteins, again from meiosis onwards. The
31 formation and selective barrier functions of the PTS require two NADPH oxidases, RBOHE
32 and RBOHC, which play a key role in pollen formation. Together our results suggest that,
33 together with symplastic isolation, gating of the apoplast around the tapetum may help
34 generate metabolically distinct anther compartments.

35 **Introduction**

36 In angiosperms, male gametophytes, called pollen grains, are produced inside specialized
37 floral organs, the anthers. In the model plant *Arabidopsis*, as in most Angiosperms, during
38 pollen formation diploid precursor cells undergo meiosis to produce small haploid cells
39 (microspores) that are initially held together as tetrads by callose, which is subsequently
40 degraded releasing the microspores into the gel-like locular matrix, the composition of which
41 is unclear. Microspores subsequently undergo extensive growth and maturation and
42 gradually acquire the tough external pollen wall which will enable the mature pollen grains
43 to survive the effects of desiccation, solar radiation, and other environmental stresses. The
44 locular matrix is surrounded by a layer of highly metabolically active maternal cells, the
45 tapetum, which after producing key enzymes required for microspore release (Bucciaglia and
46 Smith, 1994; Hird et al., 1993; Stieglitz and Stern, 1973), supplies the pollen with most of the
47 materials necessary for its development including the components of the protective pollen
48 wall (sporopollenin precursors) (Quilichini et al., 2015). Just before pollen maturation is

49 completed, the tapetum undergoes programmed cell death releasing a myriad of substances
50 into the locular matrix, many of which associate with the sporopollenin scaffold at the pollen
51 grain surface to complete the pollen wall (Gómez et al., 2015). Thus, the tapetum and future
52 pollen grains can be functionally described as the zone of active pollen development (ZPD).

53 In *Arabidopsis*, three further maternal sporophytic cell layers surround the tapetum: The
54 middle layer, the endothecium and the epidermis (Fig. 1A). The middle layer lies just outside
55 of the tapetum and its function in pollen development remains unclear. The middle layer is
56 surrounded by the endothecium layer, which ensures release of the mature pollen grains
57 into the environment by enabling anther rupture after pollen maturation (Bonner and
58 Dickinson, 1989). Finally, the external epidermal layer, covered with a functional cuticle,
59 protects the anther itself from environmental stresses (Cheng and Walden, 2005).

60 Throughout the pollen development, the tapetum secretes a plethora of highly specific
61 enzymes and metabolites involved in pollen grain formation, including callase, which
62 mediates microspore release from tetrads (Bucciaglia and Smith, 1994), pollen coat proteins
63 and other pollen coat components (Rejón et al., 2016). It is unknown how these molecules
64 remain corralled within the zone of active pollen development, and are prevented from
65 diffusing into surrounding cell layers.

66 The ability of metabolites and proteins to move symplastically between cells depends on the
67 presence of plasmodesmata which allow continuity between the adjacent cytoplasm. In
68 *Lilium* anthers the tapetum and its neighbouring middle layer 1, have been reported to be
69 symplastically isolated from each other and from other sporophytic cell layers (Clément and
70 Audran, 1995). Thus, molecular movement between the tapetum and the middle layer
71 involves traversing the apoplastic space.

72 Intriguingly, transmission electron microscopy studies at the end of the 20th century have
73 established that an enigmatic electron-dense apoplastic structure exists between the
74 tapetum cells and the middle layer cells. This has been observed in several non-model plant
75 species including the gymnosperm *Pinus banksiana* (Dickinson, 1970), the monocot *Lilium*
76 (Reznickova and Willemse, 1980) and several dicots (Galati et al., 2007; Heslop-Harrison,
77 1969; Platt et al., 1998; Staiger et al., 1994). This structure was named the peritapetal
78 membrane (Dickinson, 1970) or the peritapetal wall (Reznickova and Willemse, 1980). Here

79 we have renamed this structure the peritapetal strip (PTS), since we feel that both the terms
80 “membrane” and “wall” are misleading. The PTS was first observed during meiosis,
81 persisting throughout subsequent pollen development (Dickinson, 1970; Reznickova and
82 Willemse, 1980), and was proposed to contain pollen wall material (sporopollenin) due to
83 similarities in electron density and to resistance to acetolysis (Dickinson, 1970; Dickinson and
84 Bell, 1972; Heslop-Harrison, 1969; Reznickova and Willemse, 1980). Based purely on the
85 microscopical observations, these studies were not able to determine the function or
86 properties of this structure.

87 Here, we provide evidence that pollen maturation in *Arabidopsis* is accompanied not only by
88 symplastic isolation of the ZPD from external maternal tissues, but also by the gating of the
89 apoplast between the ZPD and the middle layer. Using apoplastic fluorescent proteins we
90 observe the presence of an apparent apoplastic barrier between the two zones. We show
91 that the formation of functional PTS requires the NADPH oxidases RBOHE and RBOHC.
92 Abnormal PTS development in the *rbohe rbohC* double mutant correlates with defective
93 selective barrier function at the tapetum/middle layer boundary. An investigation of the
94 composition of this structure leads us to conclude that it may contain phenylpropanoid-
95 containing polymers, including, as previously proposed, sporopollenin.

96

97 **Results**

98 **In *Arabidopsis*, the inner and outer anther cell layers are separated by a peritapetal strip**

99 Live anthers can be examined using confocal microscopy and fluorescent signals from the
100 internal cell layers can be observed successfully (for example, in Fig. 2). Nevertheless,
101 weaker signals are often masked by the autofluorescence of the surrounding tissues. We
102 overcame this limitation by fixing the anthers and making them transparent with
103 ClearSeeAlpha optical clearing method (Kurihara et al., 2021; Ursache et al., 2018).

104 In cleared anthers at various stages of development, we observed a thin fluorescent line
105 between tapetum and middle layer cells when the samples were excited with a UV laser (405
106 nm excitation) (Fig. 1B). By performing a Z-stack acquisition and 3D rendering, we could
107 observe that this line is part of a three-dimensional structure that is reinforced at the
108 external junctions between tapetum cells (Fig. 1C and D). This structure was also fluorescent

109 when excited at 488 nm, 512 nm and 552 nm (Fig. 1 E-G). It was first visible at the stage of
110 meiosis and remained visible up to the late free microspore stage (Suppl. Fig. S1) which
111 coincides with the degradation of the tapetum and middle layer cells (Quilichini et al., 2014).

112 To understand where this structure is located, we used the SCRI Renaissance Stain 2200,
113 which stains the cell walls of all anther cell layers except the tapetum (Matsuo et al., 2013).
114 This allowed us to confirm localisation to the interface between the tapetum and the middle
115 layer (Fig. 1H-J).

116 Transmission electron microscopy (TEM) confirmed the appearance of a thin continuous
117 electron-dense structure within the cell wall between the tapetum and the middle layer cells
118 from meiosis onwards (Fig. 1K-O). Consistent with fluorescent staining, this structure
119 appeared was thicker, darker, and invaginated at the tapetum cell junctions (Figure 1L-N).

120 Overall, this structure appeared similar to the peritapetal membrane observed in other plant
121 species (Dickinson, 1970; Reznickova and Willemse, 1980). Here we have renamed this
122 structure the peritapetal strip (PTS). Although fluorescence staining did not always give the
123 impression that the structure is fully continuous at all developmental stages, no
124 discontinuities were observed using TEM (Fig. 1K-O). Discontinuities in fluorescence signal
125 could therefore suggest a heterogeneity in the chemical composition of this structure (Fig.
126 1D). Thus, in *Arabidopsis thaliana*, the ZPD is separated from outer anther cell layers by a
127 continuous apoplastic structure from the onset of meiosis.

128 **Symplastic movement between the tapetum and middle layer is reduced or lost from** 129 **meiosis onwards**

130 The presence of a PTS prompted us first to investigate connectivity in the maternal anther
131 tissues. Reports have suggested that cytoplasmic connections are lost not only between
132 tapetal cells and pollen precursors, but also between tapetum cells and the middle layer
133 following meiosis (Clément and Audran, 1995; Owen and Makaroff, 1995). Free cytoplasmic
134 green fluorescent protein (GFP) is a small molecule (27 kD) that can move between
135 symplastically connected cells allowing a non-invasive approach to assess the status of
136 symplastic cellular connections (Stadler et al., 2005). We used a GFP variant, mTurquoise2
137 (mTQ2), which retains its fluorescent properties in various pH conditions and which is thus
138 suitable for imaging in both the sym- and apoplast (Stoddard and Rolland, 2019). Free mTQ2

139 expression was driven by the *AMS* promoter, which is active in tapetum cells from meiosis
140 onwards (Truskina et al., 2022). mTQ2 signal was detected exclusively in the tapetum cells
141 from meiosis onwards (Fig. 2A) indicating that this protein cannot freely diffuse from the
142 tapetum to the middle layer. Similarly, when we expressed the free mTQ2 in the middle
143 layer under the *GSO2* promoter, which is active in the middle layer prior to the onset of
144 meiosis and up to the point of tapetum degeneration (Truskina et al., 2022), no fluorescent
145 signal was detected in the tapetum (Fig. 2B), although fluorescence was detected in the
146 endothecium and epidermis. Our results suggest that symplastic movement between the
147 middle layer and tapetum is reduced or lost from meiosis onwards.

148 **The peritapetal strip colocalizes with a functional apoplastic barrier**

149 The symplastic isolation of the tapetum from outer cell layers implies that all molecules
150 entering the ZPD from the middle layer must traverse the apoplast and thus the PTS. To
151 evaluate whether the PTS affects molecular movement across this apoplastic interface, we
152 investigated the diffusion of an apoplastically targeted mTQ2, produced by fusion to the
153 apoplast targeting sequence (aTP) of the *Arabidopsis thaliana* 2S2 protein (Sahoo et al.,
154 2014). The resulting aTP-mTQ2 protein was expressed in the tapetum using *pAMS*. The aTP-
155 mTQ2 fusion protein was able to freely diffuse into the locular matrix and in the apoplast
156 around the tapetum cells but was never observed around the middle layer cells (Fig. 2C). To
157 test movement in the opposite direction, we expressed the aTP-mTQ2 protein fusion in the
158 middle layer under the *GSO2* promoter. In these lines aTP-mTQ2 was detected strongly in
159 the cells of the middle layer but also in endothecium and epidermis cells. Some apoplastic
160 signal was also detected around these cells, but no signal was detected in the locular matrix
161 or around the tapetum cells (Fig. 2D). The apparently poor secretion of aTP-mTQ2 by middle
162 layer cells compared to the tapetum, may be a consequence of their different biological
163 functions, and makes interpretation of *pGSO2-aTP-mTQ2* lines complex. Nonetheless, our
164 experiments suggest that the diffusion of apoplastic proteins is restricted at the middle layer
165 - tapetum interface, supporting the hypothesis that the PTS may prevent diffusion of larger
166 molecules (such as fluorescent proteins) between the ZPD and surrounding sporophytic
167 tissues.

168 **The establishment of the peritapetal strip requires NADPH-oxidases RBOHE and RBOHC**

169 The plant specific RBOH family proteins are plasma-membrane localized NADPH oxidases.
170 When activated, they produce O_2^- in the apoplast, which is then converted into H_2O_2 that
171 can be harnessed by apoplastic peroxidases to fuel ROS-dependant reactions including lignin
172 polymerization (Lee et al., 2013; Marino et al., 2012; Suzuki et al., 2011). Among the 10
173 RBOH-encoding genes in *Arabidopsis thaliana*, *RBOHE* and *RBOHC* have previously been
174 proposed to be important for tapetum programmed cell death at the later stages of the
175 pollen development (Xie et al., 2014). However, *RBOHE* and *RBOHC* are expressed early in
176 the tapetum, from the onset of the meiosis (Xie et al., 2014) (Suppl. Fig. S2A). Thus, it is
177 possible that these enzymes could have other functions during earlier stages of anther
178 development.

179 In line with this, an in-depth analysis of the previously described *rbohe rbohc* double mutant
180 revealed several defects during early stages of anther development. Following the release of
181 microspores from tetrads, both the tapetum and the middle layer cells become swollen
182 (hypertrophied) and irregularly shaped (Fig. 3A and B) in this background. When we imaged
183 the *rbohe rbohc* PTS at 512 or 552 nm, the PTS signal was abnormally diffuse and appeared
184 to be excluded from tapetal cell boundaries (Fig. 3C and D). However, when imaged at 405
185 or 488 nm, the PTS signal was not diffuse but was weaker and more continuous than that
186 observed in wild-type anthers, and appeared to be composed of two layers (Fig. 3C and D).
187 The origin this 'double' PTS signal became clear when we studied cross-sections stained
188 with Toluidine blue which stains lignin-like compounds a blue-green colour (O'Brien et al.,
189 1964). In the *rbohe rbohc* double mutant the apoplastic spaces between the tapetum and
190 the middle layer as well as between the middle layer and the endothecium were filled with
191 deposits of ectopic lignin-like material (Fig. 3E and F). Thus, the 'double' PTS signal appears
192 to originate from an ectopic lignification around the middle layer cells. The defects in the PTS
193 of the *rbohe rbohc* double mutant first appeared at meiosis, prior to the visible hypertrophy
194 of the middle layer and tapetum (Suppl. Fig. S3). The discrepancy between the intensity of
195 PTS fluorescence (weaker in *rbohe rbohc* double mutants than in wild-type, at least at early
196 stages) and toluidine blue staining (strong, turquoise lignin-associated staining in *rbohe*
197 *rbohc* double mutants compared to no visible staining in wild-type anthers), could suggest
198 that the chemical composition of the fluorescent cell wall material in these two backgrounds
199 is different.

200 To analyse the functionality of the PTS, we expressed the apoplastically targeted aTP-mTQ2
201 protein in the tapetum under the *pAMS* promoter in the *rbohe rbohC* double mutant. The
202 *AMS* promoter was expressed normally in this background (Figure S4). In contrast to wild-
203 type anthers in which the aTP-mTQ2 signal was restricted to the ZPD (Fig. 2C), in the *rbohe*
204 *rbohC* double mutant, the aTP-mTQ2 signal was additionally detected in the apoplastic
205 spaces surrounding the middle layer, endothecium and epidermis (Fig. 3G). Thus aTP-mTQ2
206 is able to traverse the PTS in *rbohe rbohC* double mutants indicating defects in the blockage
207 of apoplastic diffusion.

208 When aTP-mTQ2 was expressed in the middle layer using pGSO2 in the *rbohe rbohC* double
209 mutant background, as in wild-type plants the aTP-mTQ2 signal was observed in the middle
210 layer, endothecium and epidermis, predominantly within cells. The GSO2 promoter was
211 expressed normally in this background (Figure S4). However, no obvious signal was observed
212 in the tapetum or locular matrix (Suppl. Fig. S2B). This apparently contradictory result may
213 be due to the relatively low activity of the *GSO2* promoter compared to that of *AMS*, or, as
214 discussed above the apparent lack of strong secretion of aTP-mTQ2 from middle layer cells,
215 combined with the technical constraints of observing low quantities of mTQ2 signal within
216 the extensive apoplast of the ZPD.

217

218 **The PTS is likely to contain phenylpropanoid-pathway derived compounds**

219 The RBOH NADPH oxidases are known to produce ROS required for lignin polymerization
220 during Casparian strip formation in the root endodermis (Fujita et al., 2020; Lee et al., 2013).
221 The presence of a defective PTS in the *rbohe rbohC* double mutant as well as the fluorescent
222 properties of this structure therefore prompted us to ask whether the PTS could be
223 composed of a lignin or lignin-like materials.

224 Apoplastic polymer composition can, to a certain extent be inferred using histochemical
225 stains (Ursache et al., 2018). Thus, to assess the composition of the PTS, we tested a variety
226 of ClearSee-compatible stains for their ability to stain the PTS, and compared this with their
227 ability to stain other structures in the anthers such the anther xylem (composed of lignin),
228 pollen wall (composed of sporopollenin), mature endothecium (containing lignin) and anther
229 epidermis (containing cutin) (Suppl. Figure S5). We found that the PTS could be stained with

230 Auramine-O and Basic Fuchsin, relatively non-specific dyes which also stain lignin-containing
231 xylem and mature endothecium, the epidermal cuticle and the sporopollenin-containing
232 pollen wall. In contrast, Berberine Chloride and the Berberine Hemisulfate stained the PTS
233 only weakly. These dyes also stained the lignin-containing xylem and the lignified
234 endothecium, but not the sporopollenin of the pollen wall or the cuticle. Finally, the
235 lipophilic dye Nile Red was only able to stain epidermal cuticle, but did not stain the PTS,
236 pollen wall, xylem or lignified endodermis (Suppl. Figure S5). Taken together with the fact
237 that the PTS appears well before the initiation of sporopollenin biosynthesis, these
238 experiments suggest that the early PTS may primarily consist of phenolic metabolites
239 resembling lignin. This possibility is also supported by the high intensity of emissions and the
240 broad range of excitation/emission spectra (Donaldson, 2020).

241 Phenolic compounds, are synthesized via the phenylpropanoid pathway from phenylalanine
242 by the consecutive action of the Phenylalanine ammonia-lyase (PAL) and the Cinnamate 4-
243 hydroxylase (C4H) enzymes. The biosynthesis of most, but not all, lignin monomers requires
244 further lignin-specific enzymes such as the Cinnamoyl-CoA reductase (CCR) (Vanholme et al.
245 2019). In *Arabidopsis thaliana* there are four PAL-encoding genes (*PAL1* to *PAL4*), a single
246 *C4H* gene and two CCR-encoding gene homologues (*CCR1* and *CCR2*) (Fraser and Chapple,
247 2011; Vogt, 2010). To further support the hypothesis that the PTS is made from phenolics,
248 we analysed the expression of some of these genes using promoter expression lines
249 containing 3xmVenus reporter lines (Fig. 4, Suppl. Fig. S6)(Andersen et al., 2021). We found
250 that *PAL1*, *PAL2* and *PAL4* are expressed in the epidermis throughout anther development,
251 in the middle layer and the endothecium from the pollen mother cell stage up to the release
252 of the microspores from the tetrads, and in the tapetum from the free microspore stages
253 onwards (Fig. 4, Suppl. Fig. S6). *PAL3* expression was not detected in anthers (Suppl. Fig. S6).
254 *C4H* was expressed in the epidermis, endothecium and the middle layer at all stages of the
255 anther development. Although we were not able to detect strong *CH4* expression in the
256 tapetum (Fig. 4, Suppl. Fig. S6), some expression has been observed from stage 8
257 (microspore release) onwards in another study (Xue et al., 2020). However, because the PTS
258 is observed from much earlier in development our data suggest that the phenolic precursors
259 integrated within PTS at the meiosis stage are likely to be synthesized in the middle layer.

260 The importance of the phenylpropanoid pathway for the PTS formation was further assessed
261 using the mutants of the C4H gene *ref3-2* and *ref3-1*. These missense mutants do not
262 completely abolish the activity of the phenylpropanoid pathway but lead to reduced lignin
263 and potentially other phenolics content (Schilmiller et al., 2009). The *ref3-1* mutant, which is
264 considered to be phenotypically less severe than *ref3-2*, is in the Ler background (Schilmiller
265 et al., 2009); no major differences in the PTS in Ler wild-type plants was detected compared
266 to the Col-0 wild-type (Suppl. Fig. S1 and S7). The stronger *ref3-2* mutant produced a
267 uniformly weak and diffusive fluorescent PTS signal visible using 405 nm and 488 nm
268 excitation wavelengths while when excited at the 514 nm and 552 nm, the signal was almost
269 absent (Fig. 5B and G, Suppl. Fig. S8). The *ref3-1* mutant also produced a very weak signal
270 (Suppl. Fig. S9). These observations strongly suggests that that metabolites from the
271 phenylpropanoid pathway are necessary for the formation of a functional PTS.

272 The expression of lignin biosynthesis genes *CCR1* and *CCR2* (at lower levels) was detected in
273 the tapetum-specific transcriptome (Li et al., 2017b). Due to our inability to produce a
274 flowering *ccr1 ccr2* double mutant in our growth conditions, PTS formation was only
275 assessed in the *ccr1* single mutant (Fig. 5 C and H; Suppl. Fig. S10). *ccr1* single mutants shows
276 reduced endothecium lignification, collapsed xylem vessels and reduced lignin content in
277 stems (Thévenin et al. 2011). A defect in the PTS was apparent at the meiosis stage, with PTS
278 only being visible when excited by the 405 nm and 488 nm wavelengths. At the later stages,
279 the PTS appears to be more discontinuous, and to contain diffusive ‘patches’, particularly
280 when excited at 514 nm and 552 (Suppl. Fig. S10). These results might indicate the presence
281 of a partially defective PTS indicating potential involvement of CCR1-catalysed lignin in its
282 formation.

283 The potential importance of the middle layer in the biosynthesis of phenolic compounds
284 prompted us to investigate the presence of the PTS in plants lacking Receptor-like protein
285 kinase 2 (RPK2) which have previously been reported to lack the correctly specified middle
286 layer, produce a hypertrophied tapetum, and show an inadequate lignification of the
287 endothecium (Cui et al., 2018; Mizuno et al., 2007). Our histological sections confirmed that
288 loss of *rpk2* function causes defects in the formation of the middle layer (Suppl. Fig. S11,12).
289 Our data suggest, as previously proposed, that rather than “lacking” the middle layer, in *rpk2*
290 mutants a “hybrid” cell layer with characteristics of both the endothecium and middle layer

291 is formed (Suppl. Fig. S12 G-J). The PTS signal in the *rpk2* mutants *rpk2-1* and *rpk2-2* appears
292 to be reinforced by ectopic lignin-like material deposited around the entire periphery of
293 these “hybrid” cells adjacent to the tapetum (Fig. 5D, E, I, J; Suppl. Fig. S11 and S12). It is
294 possible that, in the absence of the correctly specified middle layer, PTS defects lead to the
295 compensatory deposition of ectopic lignin-like material as seen in the *rbohe rbohC* double
296 mutant. Indeed, the blue-green Toluidine blue stained lignin was detected in the cross-
297 sections of the *rpk2-2* mutant (Suppl. Fig. S12 E-F). Thus, the middle layer appears to be
298 particularly important for the PTS formation and might contribute to its composition by
299 supplying specific phenolic compounds.

300 Besides lignin, as evoked above and proposed previously, sporopollenin could be a PTS
301 component. This anther-specific polymer is produced in the tapetum from the tetrad stage
302 onwards and is then secreted into the locular matrix before assembling on the pollen
303 surface. Multiple enzymes participate in sporopollenin biosynthesis including MALE
304 STERILITY 2 (*MS2*), POLYKETIDE SYNTHASES A and B (*PKSA/PKSB*), TETRAKETIDE ALPHA-
305 PYRONE REDUCTASES 1 and 2 (*TKPR1/TKPR2*), ACYL-COA SYNTHETASE 5 (*ACOS5*),
306 CYTOCHROME P450 enzymes *CYP703A2* and *CYP704B1* (Quilichini et al., 2015). Since
307 sporopollenin biosynthesis initiates only during microspore release, it is likely that
308 sporopollenin is not a component of the early PTS but might reinforce it later in
309 development. To test this, we analysed the PTS in the sporopollenin biosynthesis mutants
310 *ms2*, *acos5*, *dex2* (which lacks *CYP703A2*), *pkSB* and *tkpr2*. The *ms2* mutant is in the Ler
311 background (Aarts et al., 1997); the PTS in the Ler wild-type background strongly resembles
312 that in the Col-0 wild-type background (Suppl. Fig. S1 and S7). In the *ms2* mutant the PTS
313 appeared intact but contained additional patchy signals not observed in the wild-type
314 anthers (Fig. 6K, P; Suppl. Figure S13). In the *acos5* mutant, additional patchy signals at the
315 free microspore stages were also observed but were not as frequent as in the *ms2* mutant
316 (Fig. 6L, Q, Suppl. Fig.14). In the *dex2* mutant the PTS appeared similar to the wild-type (Fig.
317 6M and R, Suppl. Figure S15). In the *pkSA* and *tkpr2* mutants, we could occasionally observe
318 large spots of strong fluoresce at the PTS (Fig. 6N, S, O, T; Suppl. Fig. S16 and S17). The origin
319 of the patches that appear in these mutants is unclear, but could indicate the presence of a
320 compensatory mechanism triggered by failures in the selective barrier function of the PTS. It
321 should be borne in mind that the single *pkSA* and *tkpr2* mutants analysed here do not

322 completely abolish sporopollenin biosynthesis because the affected genes act redundantly
323 with their paralogues *PKSB* and *TKPR1*, respectively (Quilichini et al., 2015).

324 **Discussion**

325 One of the distinguishing features of the embryophytes, is that haploid reproductive cells
326 develop enclosed within sterile multicellular structures (Niklas and Kutschera, 2010). This
327 feature ensured protection of the developing (macro- or micro-) spores from the
328 environmental stresses associated with terrestrialization. These sterile cell layers not only
329 became important in the process of transferring nutrients to developing spores, but
330 acquired specialized metabolic functions supporting spore development. In the male
331 reproductive structures of higher plants, the tapetum cell layer is the predominant source of
332 highly specific metabolites including pollen wall and pollen coat constituents, enzymes and
333 other proteins necessary for the development of the adjacent pollen grains (Bucciaglia and
334 Smith, 1994; Gómez et al., 2015; Pacini et al., 1985; Quilichini et al., 2015; Rejón et al., 2016).
335 The presence of a highly metabolically active tapetum secreting specialised metabolites may
336 therefore have necessitated reinforced control of molecular movement. Consistent with this
337 idea, we here provide strong evidence for the previously suggested symplastic isolation of
338 the tapetum and the developing pollen from the other sporophytic tissues. In addition, we
339 demonstrate the presence of an apoplastic selective barrier, the PTS, which gates apoplastic
340 diffusion between the tapetum and the outer cell layers of the anther. Our data support our
341 proposition that the tapetum and the developing pollen grains (microspores) constitute a
342 metabolically contained unit termed the zone of active pollen development (ZPD).

343 The PTS is one of only a few apoplastic barriers currently described in plants. Others include
344 the Casparian strip which surrounds the root endodermis and isolates the stele apoplast
345 from that of the root cortex, the cuticle on the surface of the aerial organs and the
346 developing embryo, suberized layers found within the seed coat, and the pollen wall on the
347 surface of pollen grains (Nawrath et al., 2013). These selective barriers are composed of
348 diverse polymers: endodermis-residing barriers are initially composed of lignin and are later
349 reinforced with entire surface-spanning suberin deposits, the cuticle consists of cutin (a
350 complex matrix of aliphatic and phenolic components) and waxes, and the pollen wall
351 contains sporopollenin. These differences may reflect the different permeabilities of each
352 selective barrier. Based on its position, the PTS must permit the diffusion of nutrients such as

353 sugars and amino acids from the mother plant to the developing pollen. In addition, our
354 recent work suggests that the PTS is permeable to small peptides that are perceived in the
355 middle layer and coordinate tapetum and pollen grain development (Truskina et al., 2022).
356 Our current data suggest that the PTS serves to impede the movement of larger molecules
357 such as proteins, possibly acting as a size- and/or charge-specific filter. Nonetheless,
358 analysing the precise biophysical and chemical properties of this filter promises to present a
359 significant technical challenge due to the localisation and extreme thinness of the PTS.
360 Permeability assays to test the capacity of molecules (particularly dyes) with varying sizes
361 and properties to diffuse across the PTS have proved challenging due to difficulty in imaging
362 internal tissues when the external epidermis layer contains cuticle that hinders dye diffusion
363 towards inner cell layers and causes variability in dye penetration. However, optimisation of
364 such assays will undoubtedly be a goal in future studies.

365 Our results show the requirement of RBOH proteins for PTS integrity and functionality,
366 supporting the idea that a ROS-mediated polymerization reaction could occur during PTS
367 formation. Consistent with this finding, we also provide data to support the idea that the PTS
368 contains lignin-like phenolic compounds (Tobimatsu and Schuetz, 2019). The defective PTSs
369 observed in mutants with reduced C4H function strongly suggest the involvement of the
370 phenylpropanoid pathway in PTS formation. The exact composition of the PTS nonetheless
371 remains enigmatic. One of indicators classically used to show the presence of lignin is
372 turquoise staining by the polychromatic stain Toluidine blue. Although this is observed for
373 the ectopic lignification observed in the *rbohe rbohC* and *rpk2* mutants, no such staining is
374 visible at the PTS of wild-type plants. However, the wild-type PTS being extremely thin, it
375 may be difficult to visualise using this stain. In conclusion, further investigations are required
376 to understand the proposed contribution of a lignin-like polymer to PTS construction.

377 Although our data suggest that suberin and cutin can be excluded as potential polymers
378 within the PTS due a lack of staining by the lyophilic Nile Red stain (Suppl. Fig. S5) (Ursache
379 et al., 2018), the juxtaposition of the PTS with the tapetum prompted us to investigate
380 sporopollenin as a potential PTS constituent. The expression of most of the genes known to
381 be involved in sporopollenin biosynthesis initiates in the tapetum at the tetrad stage, later
382 than the initiation of PTS formation. We thus considered that sporopollenin might reinforce
383 the PTS at the later stages of pollen development. This appears plausible since the anther

384 continues to grow throughout pollen development, necessitating continuous PTS
385 reinforcement. Fragmentation of an early PTS containing lignin like molecules, and
386 reinforcement with compositionally distinct polymer (such as sporopollenin) would provide
387 an explanation for the rather discontinuous fluorescence signal that we observe in the wild-
388 type PTS, despite the apparent continuity of the PTS using TEM-based techniques.
389 Furthermore, the defects we observed in the PTS of some mutants defective in
390 sporopollenin biosynthesis, including the apparent deposition of ectopic PTS material,
391 support the idea that the PTS of these mutants may be functionally affected, triggering
392 compensatory mechanisms.

393 In both the *rbohe rbohC* double mutant and the *rpk2* mutants the apparent defects in the
394 PTS are associated with an over-lignification around the middle layer cells, expanding the
395 normal domain of the PTS to the middle layer/endothecium interface (Fig. 3 and 5, Fig. S3,
396 S11 and S12). Despite this, our results suggest that the PTS remains functionally
397 compromised, at least in *rbohe rbohC* double mutants. This situation is very similar to that
398 seen in mutants defective in the formation of the Casparian strip which also undergo
399 compensatory ectopic lignification and suberization but remain functionally defective
400 (Hosmani et al., 2013; Kalmbach et al., 2017; Kamiya et al., 2015; Li et al., 2017a).
401 Furthermore, compensatory lignin in the root endodermis has been shown to differ, in terms
402 of composition, to Casparian-strip lignin (Reyt et al., 2021).

403 Intriguingly, in the root endodermis, localized lignin polymerization required for Casparian
404 strip integrity is achieved through the highly localized activation of RBOH proteins situated
405 adjacent to gaps in the barrier via the SHENGEN (SGN) integrity monitoring pathway (Fujita
406 et al., 2020; Lee et al., 2013). Lignin monomers involved in this process are also thought to
407 be produced by the endodermis (Andersen et al., 2021). By contrast, our data suggest that
408 the ROS necessary for early PTS formation may originate in the tapetum, while phenolic PTS
409 components are produced, at least initially, by the middle layer. Limited diffusion of both
410 ROS and phenolic compounds within the cell wall could provide an elegant mechanism for
411 ensuring the inter-layer positioning of the nascent PTS. Furthermore, our finding that
412 mutants lacking intact PTSs undergo apparently compensatory deposition of lignin, strongly
413 suggesting that, as is the case in the Casparian strip, the integrity of the PTS may be actively
414 monitored. It is tempting to suggest that similar peptide-mediated monitoring mechanisms

415 may be involved in both systems, particularly in light of the recent finding that the receptor
416 kinases GASSHO1(GSO1)/SGN3 and GSO2 coordinate tapetum activity with pollen grain
417 development through their activity in the middle layer (Truskina et al., 2022). However, this
418 possibility requires further investigation.

419

420 **Materials and Methods**

421 **Plant material and growth conditions**

422 Seeds were sown on soil, stratified for 2 days at 4°C and grown in long-day conditions (16h
423 light / 8h dark). Some transgenic plants were examined in the T1 generation; these were first
424 selected on half-strength MS medium with 1% sucrose and 1% agar supplemented with
425 either 50 µg/mL kanamycin or 10 µg/ml glufosinate ammonium (Basta).

426 Mutant alleles used were *rbohe-2 rbohC* (*rbohe-2 rhd2-1*) (Xie et al., 2014), *rpk2-1*
427 (SALK_062412) (Mizuno et al., 2007) , *rpk2-2* (SALK_039514) (Mizuno et al., 2007), *ref3-1*,
428 *ref3-2* (Schillmiller et al., 2009), *ms2* (Aarts et al., 1997), *ccr1-3* (SALK_123689, *ccr1s*) (Mir
429 Derikvand et al., 2008; Panda et al., 2020), *acos5* (SK19167), *dex2-2* (SALK_119582) (Morant
430 et al., 2007), *pksb* (GABI_454C04) (Kim et al., 2010), *tkpr2-1* (SALK_129453) (Grienenberger
431 et al., 2010). The genotyping primers are listed in Suppl. Table 1. The *pPAL1-NLS-3xmVenus*,
432 *pPAL2-NLS-3xmVenus*, *pPAL4-NLS-3xmVenus*, *pPAL4-NLS-3xmVenus*, *pC4H-NLS-3xmVenus*
433 are described in (Andersen et al., 2021). The *pAMS-NLS-3xmVenus* and *pGSO2-NLS-*
434 *3xmVenus* reporter lines are described in (Truskina et al., 2022)

435 **Generation of transgenic plant lines**

436 Gateway MultiSite cloning was used to generate transgenic lines.

437 For the *pRBOHE-NLS-3xmVenus* transcriptional reporter line, the 4105 bp promoter of
438 *RBOHE* from -4105 bp to -1 bp was amplified by PCR from Arabidopsis (Col-0) genomic DNA,
439 inserted into pDONR P4-P1R and recombined with 3xmVenus-N7 pDONR211, OCS
440 terminator pDONR P2R-P3 (containing STOP codon followed by the octopine synthase
441 terminator) and pK7m34GW destination vector (with kanamycin *in planta* resistance), and
442 transformed into Col-0 plants.

443 To create *pAMS-aTP-mTQ2* and *pGSO2-aTP-mTQ2* lines, the ORF encoding the apoplast
444 targeting sequence (aTP) of the *Arabidopsis thaliana* 2S2 protein (Sahoo et al. 2014) and the
445 *mTQ2* ORF sequence lacking the start codon but including the stop codon were separately
446 amplified by PCR and then combined using overlap extension PCR. The resulting *aTP-mTQ2*
447 was inserted into pDONR211 and then recombined with the *pAMS* pDONR P4-P1R or *pGSO2*
448 pDONR P4-P1R, OCS terminator pDONR P2R-P3 and pB7m24GW,3 destination vector (with
449 Basta *in planta* resistance). The resulting constructs were transformed into Col-0 or *rbohe*
450 *rboh*c double mutant plants.

451 To create *pAMS-mTQ2* and *pGSO2-mTQ2* reporter lines, the *mTQ2* ORF containing the start
452 codon and the stop codon was amplified by PCR, inserted into pDONR211 and then
453 recombined with the *pAMS* pDONR P4-P1R or *pGSO2* pDONR P4-P1R, OCS terminator
454 pDONR P2R-P3 and pB7m24GW,3 destination vector (with Basta *in planta* resistance). The
455 resulting constructs were transformed into Col-0 plants.

456 To verify expression of *pAMS* and *pGSO2* in the *rbohe rboh*c mutant background, reporter
457 constructs *pAM3-NLS-3xmVenus* and *pGSO2-NLS-3xmVenus* (as previously described
458 (Truskina et al., 2022)) were transformed directly into Col-0 and *rbohe rboh*c double
459 mutants.

460 The cloning primers are listed in Suppl. Table 1.

461 **Histology**

462 Inflorescences were fixed with FAA (50% (v/v) ethanol, 5% (v/v) acetic acid, 3.7% (v/v)
463 formaldehyde) overnight, dehydrated in a graded series of 50%, 60%, 70%, 85%, 95% and
464 100% of ethanol for 1 h each, then further incubated overnight in 100% ethanol. The
465 samples were then incubated in 50% ethanol/50% Technovit 7100 base liquid (v/v) for 4h
466 and then in 25% ethanol/75% Technovit 7100 base liquid (v/v) overnight. The samples were
467 infiltrated in Technovit 7100 infiltration solution (1g hardener I in 100 ml Technovit 7100
468 base liquid) with vacuum for 2h and then incubated for 6 days. All steps above were
469 conducted at room temperature (RT) with gentle agitation. The samples were polymerized
470 with Technovit 7100 polymerization solution (100 µl Technovit 7100 hardener II in 1,5 ml

471 infiltration solution) at RT for 6 hours. Transverse sections of 3µm were cut using Leica
472 Microtome HM355S.

473 For histological analysis, the sections were stained with 0.01% (w/v) acriflavine in H₂O for 5
474 min, mounted in Vectashield (Vector Laboratories) and observed using TCS SP5 confocal
475 microscopes (Leica) with excitation at 488 nm and emission at 492-551 nm.

476 Alternatively, the sections were stained with the 0.05 % (w/v) Toluidine blue in H₂O for 1
477 minute, mounted in Entellan mounting medium (Sigma) and observed under Zeiss Axio
478 Imager M2 microscope.

479 **Clearsee tissue clearing**

480 Inflorescences were fixed in 4% paraformaldehyde in PBS at 4°C under vacuum for 2h and
481 kept subsequently overnight at 4°C. The samples were washed twice with PBS and cleared
482 with Clearsee Alpha solution (10% (w/v) xylitol powder, 15% (w/v) sodium deoxycholate,
483 25% (w/v) urea and 0.63% (w/v) sodium sulfite) for 1 week changing to a fresh solution
484 every 2 days at RT.

485 Anthers were dissected from the inflorescences, mounted in Clearsee Alpha solution and
486 observed under confocal TCS SP8 confocal microscope (Leica) using 40x oil objective.
487 Autofluorescence was observed using 405 nm excitation with 413-467 nm emission, 488 nm
488 excitation with 492-546 nm emission, 514 nm excitation with 516-570 nm emission, 552 nm
489 excitation with 555-609 nm emission.

490 Alternatively, the samples were stained overnight with either 0.1% (w/v) Auramine O in
491 Clearsee Alpha, 0.2% (w/v) Basic Fuchsin in Clearsee Alpha, 0.05% (w/v) Nile Red in Clearsee
492 Alpha, 0.1% (w/v) Berberine Chloride in Clearsee Alpha, 0.1% (w/v) Berberine Hemisulfate in
493 Clearsee Alpha, 0.5% (v/v) SCRI Renaissance Stain 2200 in Clearsee Alpha. The samples were
494 washed 3 times for 20 min each with Clearsee Alpha solution, dissected, mounted in
495 Clearsee Alpha solution and observed under a confocal TCS SP8 confocal microscope (Leica)
496 using 40x oil objective. The excitation and emission wavelengths were as following:
497 Auramine-O ex. 488 nm, em. 500-570 nm; Basic Fuchsin and Nile Red ex. 552 nm, em. 556-
498 631 nm; Berberine Chloride and Berberine Hemisulfate ex. 488 nm, em. 491-545 nm. For co-
499 localization microscopy, sequential scanning was used with the PTS visualized using 514 nm

500 excitation with 516-570 nm emission, and the SCRI Renaissance Stain 2200 stain visualized
501 using 405 nm excitation with 410-473 nm emission.

502 **Transmission electron microscopy**

503 Flower buds at appropriate developmental stages were fixed with 4 % (w/v) formaldehyde
504 and 2 % (w/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) (PB) under vacuum (0.6
505 bar) at 4°C for 1h during which the vacuum was slowly broken 3 times, then incubated in
506 fresh fixative solution at 4°C overnight. The samples were washed three times in PB,
507 postfixed for 2h in 1 % (w/v) osmium tetroxide in PB at room temperature (RT), rinsed 5
508 times for 5 min in PB and dehydrated under vacuum in a graded ethanol series for 20 min
509 each time, increasing in 5 steps from 30 to 100 % at RT. The samples were then incubated in
510 graded low viscosity SPURR resin in ethanol (33%, 66% and twice 100%) at 4°C for 24h each
511 (including 20min under vacuum). The samples were polymerized in fresh SPURR resin at
512 60°C for 18h. Ultrathin sections (70 nm) were prepared using UC7 Leica Ultramicrotome,
513 placed on formvar-coated grids, then poststained in 2% uranyl acetate and lead citrate.
514 Sections were examined under JEOL 1400 transmission electron microscope at 120kV and
515 imaged with the Gatan Rio 16 camera.

516 **Confocal microscopy**

517 For the 3xmVenus and mTQ2 reporter lines, the anthers were stained with 20 µg/ml
518 propidium iodide solution and examined using TCS SP5 (Leica) TCS SP8 (Leica) confocal
519 microscopes using the 40x oil objectives. The mVenus reporter lines were visualized using
520 excitation at 514 nm and emission at 526-560 nm for mVenus, 605-745 nm for propidium
521 iodide. The mTQ2 reporter lines were visualized by sequential excitation with 448 nm
522 excitation and 452-505 nm emission for the mTQ2, and 514 nm excitation and 632-726 nm
523 for propidium iodide.

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540 **Competing interests:** The authors declare no competing interests.

541 **Figure legends:**

542 **Figure 1. Visualization of the peritapetal strip in *Arabidopsis thaliana* anthers.** (A) In
543 *Arabidopsis thaliana* the developing pollen is surrounded by the four diploid sporophytic cell
544 layers: tapetum, middle layer, endothecium and epidermis. (B-G) The peritapetal strip in
545 anthers at the tetrad stage (post-meiosis) excited at 405 nm (B-D), 488 nm (E), 514 nm (F)
546 and 552 nm (G). (H-J) Localization of the PTS in the anthers (J); the PTS was visualized using
547 514 nm laser (H) and the anther cell walls were stained with the SCRI Renaissance Stain 2200
548 (I). (K-O) Transmission electron microscopy (TEM) of the anther showing the middle layer -
549 tapetum interface at the indicated stages of anther development (staging according to
550 (Sanders et al., 1999). The arrows indicate the peritapetal strip. T = tapetum, ML = middle
551 layer, EN = endothecium, EP = epidermis, Te = tetrads, CW = cell wall, Cyt = cytoplasm, V =
552 vacuole, E = elaioplast. PMC = pollen mother cell stage of pollen development. Scale bars: B-J
553 10 μ m, K-O 500 nm.

554 **Figure 2. Movement of free cytoplasmic mTQ2 and an apoplastically-localized mTQ2 in**
555 ***Arabidopsis thaliana* anthers.** (A) Free cytoplasmic mTQ2 expressed under the tapetum-
556 specific *pAMS* in anthers. (B) Free cytoplasmic mTQ2 expressed under the middle layer-
557 specific *pGSO2* in anthers. (C) The mTQ2 with the apoplast localization signal aTP expressed

558 under the tapetum-specific *pAMS* in anthers. (D) The mTQ2 with the apoplast localization
559 signal aTP expressed under the middle layer-specific *pGSO2* in anthers. PMC = pollen mother
560 cell stage of pollen development. Scale bars: 10 μ m.

561 **Figure 3. The peritapetal strip is structurally and functionally defective in the *rbohe rbohC***
562 **double mutant.** (A-B) Pollen and anther development in the wild-type (A) and the *rbohe*
563 *rbohC* double mutant. Arrows indicate hypertrophied middle layer and hypertrophied
564 tapetum in the mutant. (C-D) The peritapetal strip in the wild-type (C) and the *rbohe rbohC*
565 double mutant (D) at the tetrad stage of anther development visualized at different
566 excitation wavelengths. Arrow indicates 'detached' signal in the mutant. (E-F) Ectopic lignin-
567 like deposition around the middle layer in the *rbohe rbohC* double mutant (F, arrows)
568 compared to the wild-type (E) visualized using the Toluidine blue staining. (G) The mTQ2
569 with the apoplast localization signal aTP expressed under the tapetum-specific *pAMS* in
570 anthers. Arrows indicate mTQ2 signal around endothecium and epidermis cells in the *rbohe*
571 *rbohC* double mutant. Scale bars: (A-B, E-G) 10 μ m, (C-D) 5 μ m.

572 **Figure 4. Expression of genes encoding components of the phenylpropanoid biosynthesis**
573 **pathway in anthers.** (A, E) *PAL1* expression, (B, F) *PAL2* expression, (C, G) *PAL4* expression,
574 (D, H) *C4H* expression at the meiosis and early free microspore stages. (I) Schematic
575 illustrating expression of genes involved in the phenylpropanoid biosynthesis pathway in
576 anthers at different stages of pollen development (see. Figure S6). Scale bars: 10 μ m.

577 **Figure 5. Characterisation of the peritapetal strip in mutants defective in the**
578 **phenylpropanoid biosynthesis pathway, in the *rpk2* mutants and in sporopollenin**
579 **biosynthesis pathway mutants.** The peritapetal strip in the wild-type Col-0 (A, F), *ref3-2* (B,
580 G), *ccr1* (C, H), *rpk2-1* (D, I), *rpk2-2* (E, J), *ms2* (K, P), *acos5* (L, Q), *dex2* (M, R), *pksb* (N, S) and
581 *tkpr2* (O, T) mutants at the tetrad stage using either 405 nm or 512 nm excitation
582 wavelengths. Scale bars: 10 μ m.

583

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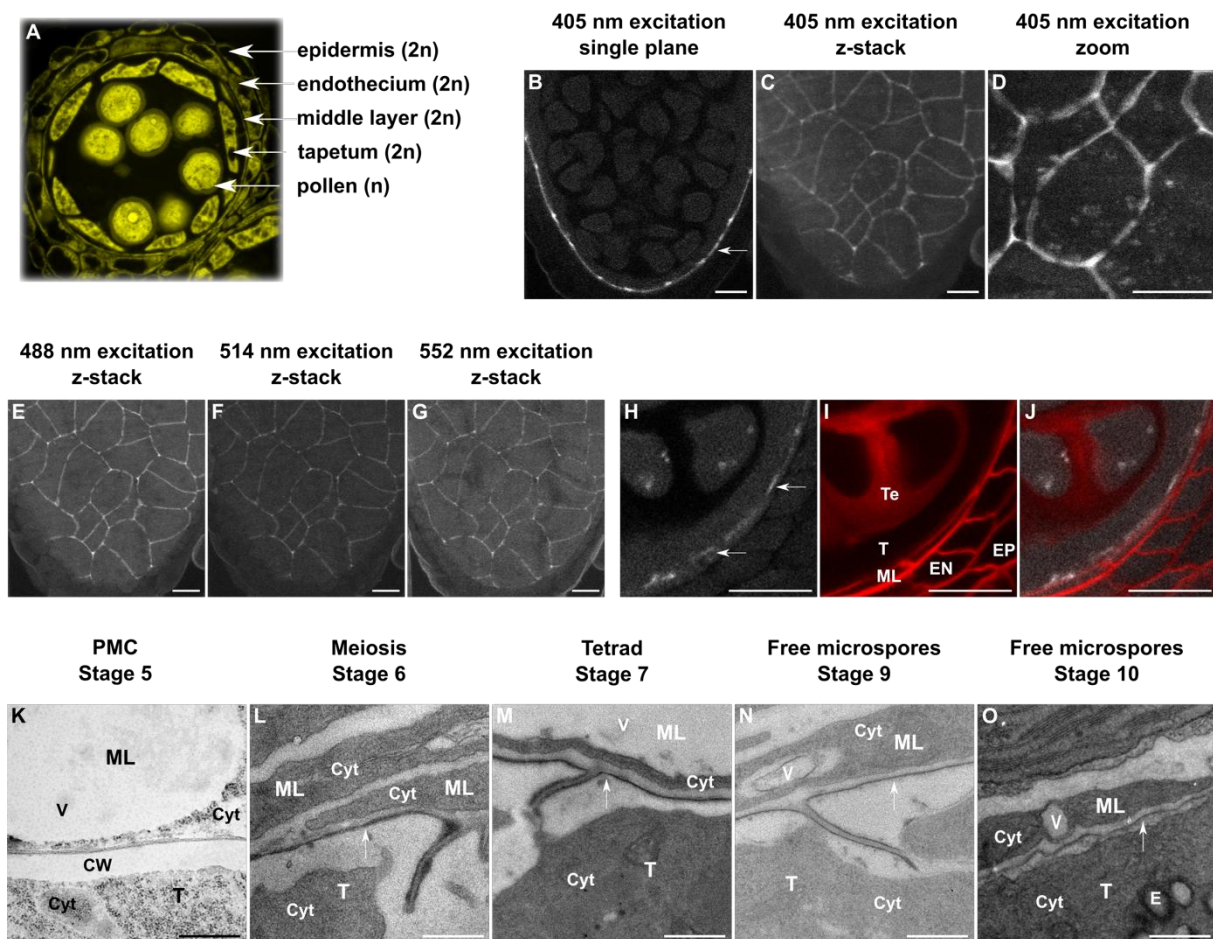
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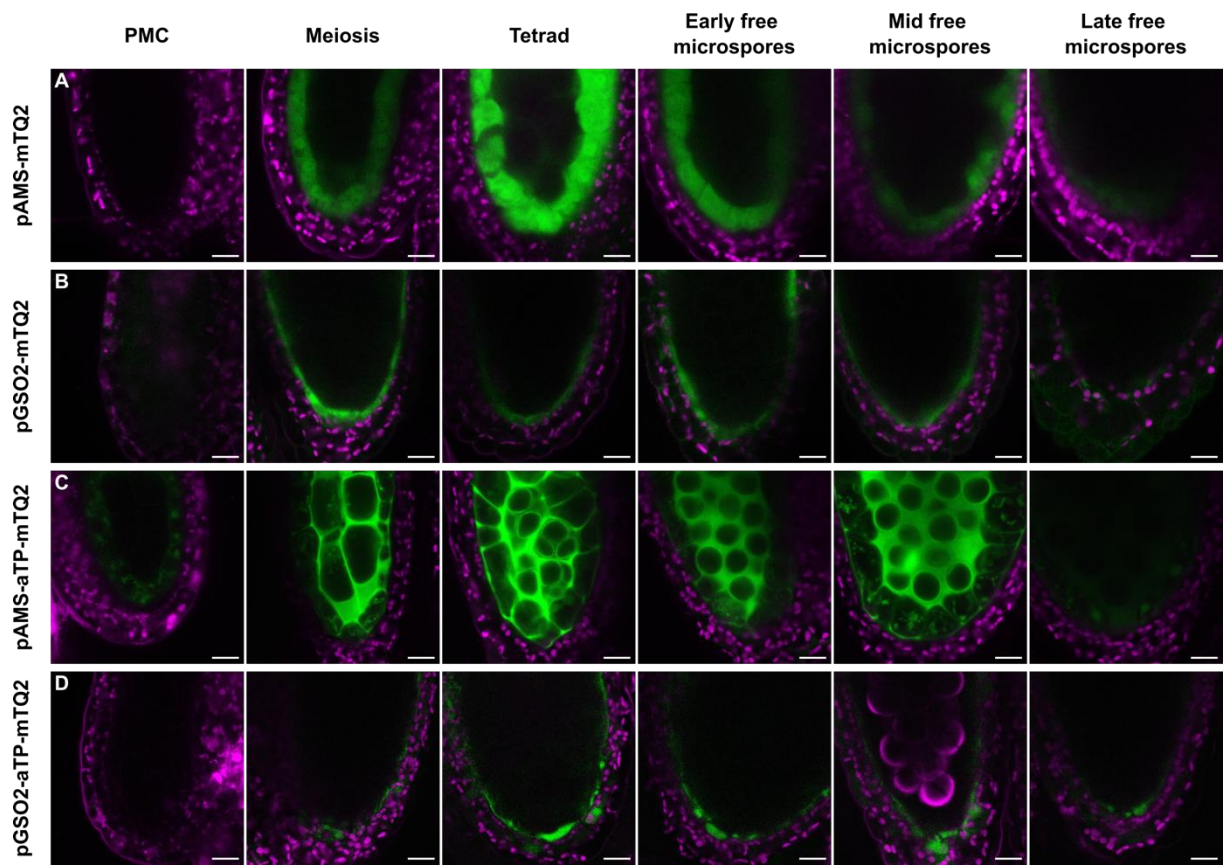
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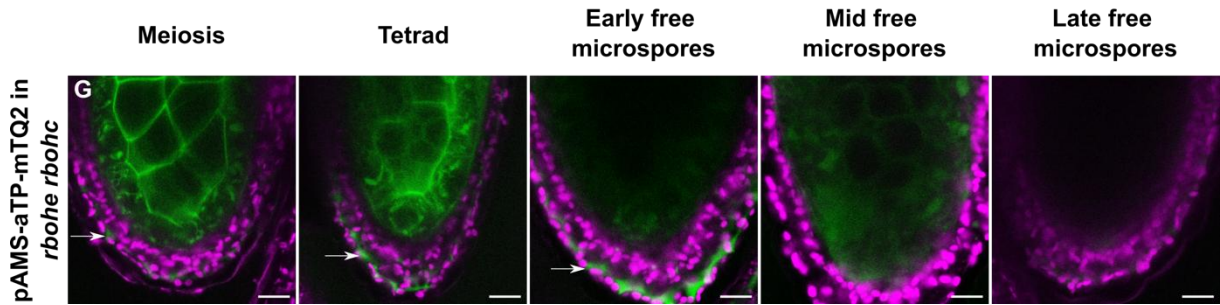
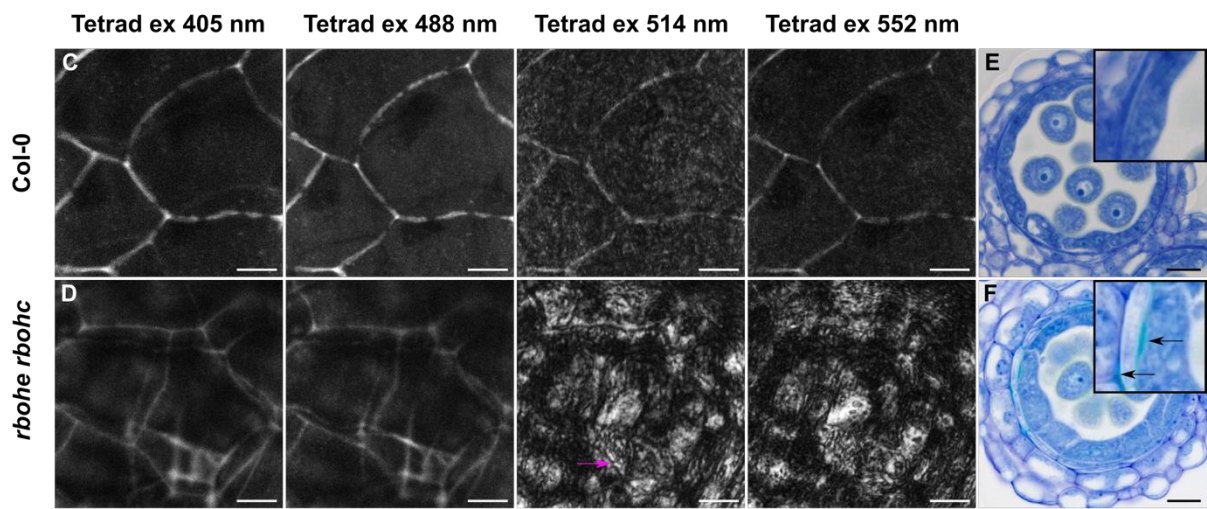
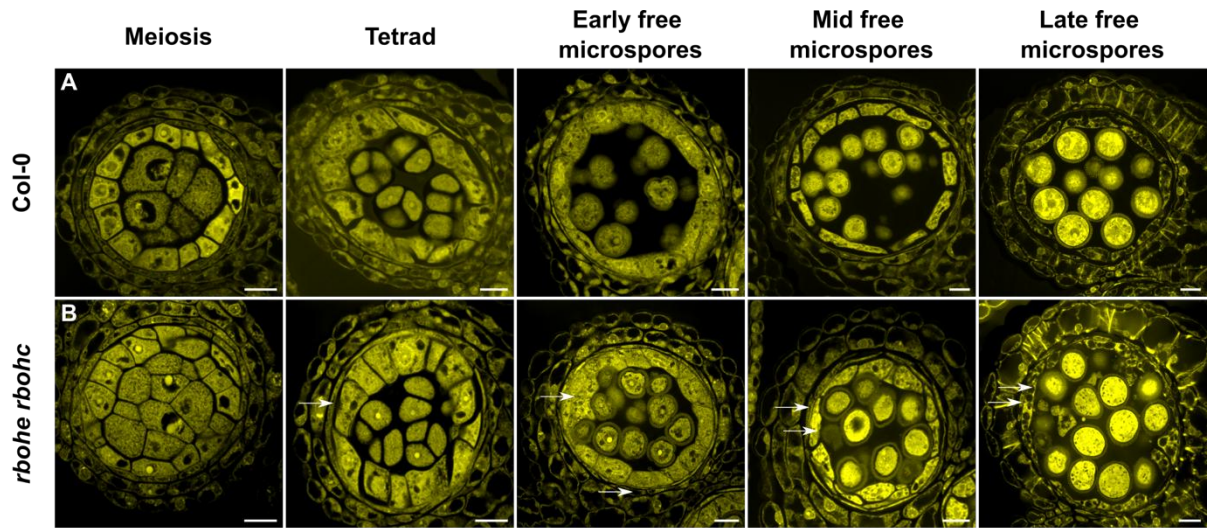
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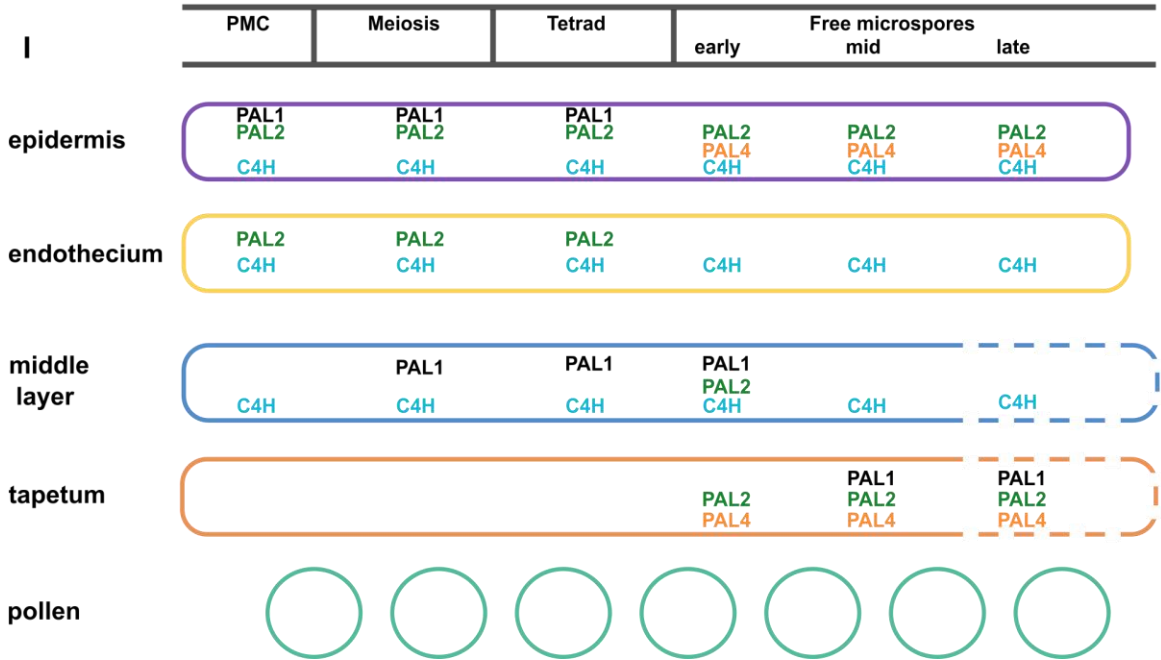
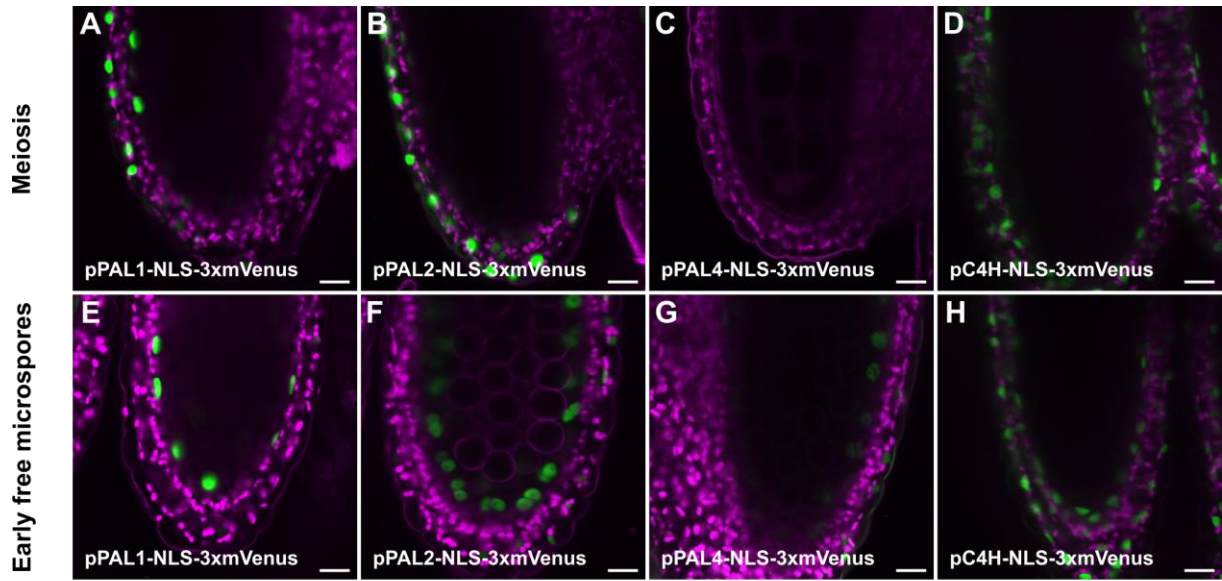


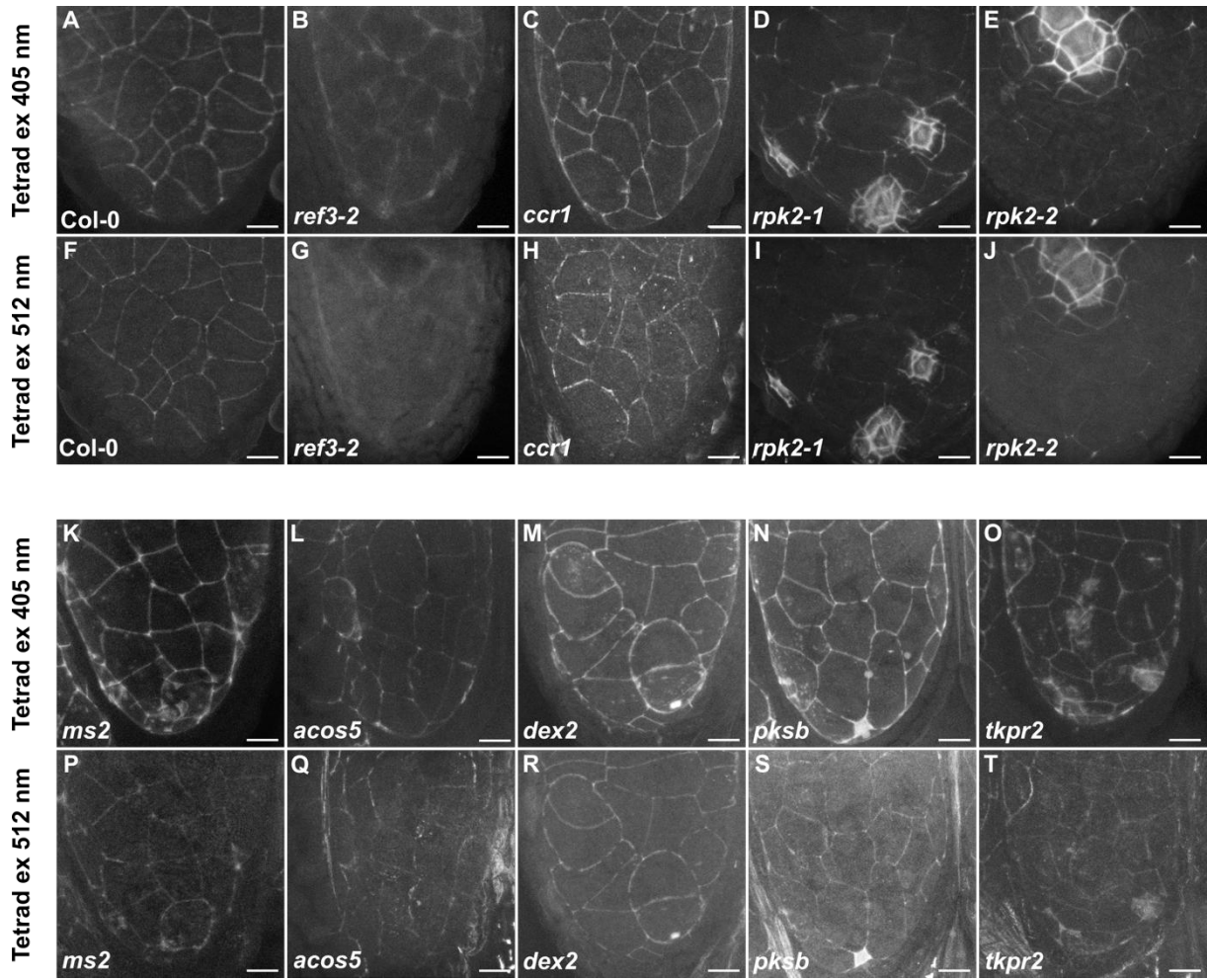
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