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A peptide-mediated, multilateral molecular dialogue for the coordination of pollen wall formation

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Abstract

The surface of pollen grains is reinforced by pollen wall components produced non-cell autonomously by tapetum cells that surround developing pollen within the male floral organ, the anther. Here we show that tapetum activity is regulated by the GASSHO (GSO) receptor-like kinase pathway, controlled by two sulfated peptides, CASPARIAN STRIP INTEGRITY FACTOR 3 (CIF3) and CIF4, the precursors of which are expressed in the tapetum itself. Coordination of tapetum activity with pollen grain development depends on the action of subtilases, including AtSBT5.4, which are produced stage-specifically by developing pollen grains. Tapetum-derived CIF precursors are processed by subtilases, triggering GSO-dependent tapetum activation. We show that the GSO receptors act from the middle layer, a tissue surrounding the tapetum and developing pollen. Three concentrically organized cell types therefore cooperate to coordinate pollen wall deposition through a multilateral molecular dialog.

38 **Significance Statement**

39 Pollen viability depends on a tough external barrier called the pollen wall. Pollen wall
40 components are produced by tapetum cells which surround developing pollen grains within
41 the anther. Precise coordination of tapetum activity with pollen grain development is
42 required to ensure effective pollen wall formation. Here we reveal that this is achieved
43 through a multidirectional dialogue involving three distinct cell types. We show that peptide
44 precursors from the tapetum are activated by proteases produced stage-specifically in
45 developing pollen grains. Unexpectedly, we found that activated peptides are perceived not
46 in the tapetum, but in the middle layer, which encloses the developing tapetum and pollen
47 grains, revealing an unsuspected role for this enigmatic cell layer in the control of tapetum
48 development.

49 **Introduction**

50 Pollen grains, housing dispersible male gametophytes, form a critical element in plant sexual
51 reproduction. The pollen from a single plant can be carried by wind or pollinators towards
52 multiple other individuals allowing efficient exchange of genetic material. However, the
53 release of pollen grains into the terrestrial environment requires protection from
54 dehydration, sunlight and other environmental stresses. Protection is provided by a
55 multilayered pollen wall with sporopollenin, one of the most resistant biological polymers
56 known, as the main constituent of the outermost layer (the exine) (1).

57 Pollen is produced in the anthers from diploid precursor cells, the pollen mother cells (PMC).
58 Each PMC undergoes meiosis forming four haploid cells. These are temporarily held together
59 as tetrads before they are released as individual immature microspores (2, 3). The
60 subsequent assembly of the pollen wall is a highly dynamic multistep process involving the
61 sporophytic tissues that surround developing pollen grains. In Arabidopsis, these comprise
62 four concentric layers of cells: the tapetum (most internal cell layer), middle layer,
63 endothecium and epidermis (outer cell layer) (Fig. 1A). The tapetum contributes the most to
64 pollen wall formation, by producing the biochemical precursors of sporopollenin and of
65 other wall components (1, 3, 4). However, the developing pollen grains are not in direct
66 contact with the tapetum but are suspended a matrix of largely unknown composition (often
67 called the locular fluid), which is presumably also produced by the tapetum (5). The tapetum

68 cells export pollen wall components, which must then traverse this matrix for deposition on
69 the pollen surface to form an intact and functional pollen wall (Fig. 1B).

70 The export of pollen wall components from the tapetum is under developmental control. It
71 terminates with programmed cell death of the degenerating tapetum, liberating the final
72 components of the pollen wall. Although the critical contribution of the tapetum to pollen
73 wall development is uncontested, the question of whether, and if so how it is coordinated
74 with pollen grain development, as suggested in the literature (6, 7), remains unanswered.

75 Two extracellular structural barriers have recently been shown to be developmentally
76 monitored to ensure physical integrity prior to their functional deployment. These are the
77 embryonic cuticle (necessary to prevent water loss from the seedling surface at
78 germination), and the Casparian strip (necessary for the regulation of water and ion
79 homeostasis in roots). The integrity of both the nascent Casparian strip and the nascent
80 embryonic cuticle, depends on intercellular signaling pathways involving GASSHO (GSO)
81 receptor-like kinases (RLKs) and CASPARIAN STRIP INTEGRITY FACTOR (CIF)-related sulfated
82 peptide ligands (8–10). In both cases, the diffusion of post-translationally processed ligands
83 across the barrier into tissues containing functional receptor complexes signals defects in
84 barrier integrity and triggers gap-filling responses.

85 Here we show that the proper tapetum function necessary for the formation of a third
86 extracellular structure, the pollen wall, is controlled through a molecular dialogue between
87 the middle layer, the tapetum, and the developing pollen grains. This dialogue involves two
88 previously functionally uncharacterized CIF peptides (CIF3 and CIF4) and their cognate
89 receptors GSO1 and GSO2.

90

91 **Results**

92

93 *The receptors GSO1 and GSO2 and the sulfated peptides CIF3 and CIF4 are necessary for*
94 *normal tapetum development and pollen wall formation.*

95

96 We noticed in previous crossing experiments that pollen from *gso1 gso2* double mutant
97 flowers form large clumps. Scanning Electron Microscopy confirmed that in contrast to wild-
98 type mutant pollen, which is released as individual grains (Figure 1C and D), *gso1-1 gso2-1*
99 double mutant pollen tends to form large fused masses of misshapen grains (Figure 1E and

100 F) with some free non-fused pollen grains remaining. This phenotype was confirmed in the
101 independent *gso1-2 gso2-2* double mutant carrying alternative T-DNA insertions, and was
102 rescued by complementation with the *GSO1* wild-type sequence (Figure S1A-B). The single
103 *gso1* and *gso2* mutants produced normal pollen (Figure S1C-D).

104 Five ligands of GSO1 and/or GSO1 and GSO2 have already been identified; TWISTED
105 SEED1 (TWS1) involved in embryonic cuticle formation (9), CIF1 and CIF2 involved in
106 Casparian strip formation (8, 10) and CIF3 and CIF4 (11). While pollen of *tw1* and *cif1 cif2*
107 double mutants was unaffected, simultaneous loss of *CIF3* and *CIF4* function phenocopied
108 the *gso1 gso2* pollen phenotype (Figure S2A-B). Occasional pollen adhesion was also
109 observed for single *cif4* but not for the *cif3* single mutant (Figure S1E, F, M, N), suggesting
110 that these genes do not act fully redundantly. Because CIF3 and CIF4 belong to a family of
111 sulfo-peptides that rely on post-translational tyrosine sulfation for GSO binding and activity
112 (8, 9, 11), we suspected involvement of the unique Arabidopsis tyrosine protein
113 sulfotransferase (TPST). Similar pollen defects were indeed observed in the *tpst* mutant,
114 although, as reported in the seed, the phenotype tended to be less severe than that caused
115 by loss of ligand or receptor function (Figure S2C-D). Despite their dramatic phenotype, the
116 *gso1-1 gso2-1*, *cif3 cif4*, and *tpst* mutants were not sterile, as viable pollen was detected
117 using Alexander staining (Figure S3A-H) and pollen located at the periphery of the clusters
118 was still able to germinate (Figure S3I-L). Furthermore, plants left to self-fertilize produced
119 viable seeds, although seed number per silique was reduced compared to wild type (Figure
120 S3M).

121 We traced the origin of the pollen defects by histological analyses. Anther
122 development appeared normal in all backgrounds until stage 8 (staging according to (12))
123 the point of microspore release (see Figure 1B). From this stage onwards pollen grains were
124 uniformly distributed within the locular matrix of wild-type anthers. Tapetal cells were well-
125 defined and thinned progressively until they underwent cell death, prior to the release of
126 pollen grains between stages 12 and 13 (Figure 1G; (13)). In contrast, in *gso1 gso2*, *cif3 cif4*
127 and *tpst* mutants the apparent volume of locular matrix surrounding the developing pollen
128 grains was dramatically reduced. The swollen and hypertrophied tapetum occupied a much
129 larger volume resulting in an apparent “crowding” of the developing pollen grains and a lack
130 of locular fluid (Figure 1H, Figure S2). Further aspects of this phenotype were related to the
131 deposition of pollen wall components. In wild-type stage 8 anthers, the pollen wall stains

132 reddish purple with toluidine blue (Figure 1I)(14, 15). The reddish-purple staining is masked
133 in subsequent stages by progressive deposition of the exine, which is visible in toluidine
134 blue-stained sections as a yellow halo around developing pollen grains (Figure 1J). This is
135 also observed for peripheral pollen grains of *gso1 gso2*, *cif3 cif4* and *tpst* mutants. However,
136 the walls of more internal pollen grains continue to stain reddish purple, suggesting
137 abnormal/reduced exine deposition (Figure 1 K-L and Figure S4A-D).

138 Both cryo-scanning (SEM) and transmission electron microscopy (TEM) analyses of
139 pollen walls showed that many pollen grains were physically fused in the mutant
140 backgrounds via a shared exine (Figure 1 O-P, R; Figure S4E-L), whereas the exine layers of
141 neighboring pollen grains were clearly separated by locular matrix in wild-type anthers
142 (Figure 1 M-N, Q). Furthermore, the normal exine structure of bacula (pillars) and tecta
143 (connecting the apices of the bacula on the outer surface of the pollen grain) was difficult to
144 discern in mutant pollen grains, particularly those not in direct contact with the tapetum.
145 The bacula were often shorter in mutants (Figure 1M-R; Figure S4E-L).

146 In wild-type anthers, in addition to appearing yellow in toluidine-blue stained
147 sections (Figure 1J), the sporopollenin-containing exine can be stained with the dye
148 Auramine-O. In anthers, this staining is restricted to pollen grains, the epidermal cuticle and
149 to a thin band, of unknown origin, between the tapetum and the middle layer (Figure 1S). By
150 contrast, in *gso1 gso2*, *cif3 cif4* and *tpst* mutants, yellow coloration and Auramine-O staining
151 were also observed between the tapetum cells, extending to cell junctions with the middle
152 layer (Figure 1L, T, Figure S4B, D, M, N). TEM analyses confirmed ectopic accumulation of
153 electron-dense osmophilic material resembling sporopollenin both between and behind the
154 tapetal cells of mutant anthers (Figure 1V and Figure S4O, P).

155 Tapetum cells become polarized as they differentiate (5). In order to address whether
156 GSO1 signaling might influence tapetum polarization, we analyzed the expression and
157 localization of the ABCG26 transporter involved in the secretion of pollen wall components
158 from the tapetal cells. We found that an ABCG26:mTQ2 fusion protein expressed from the
159 endogenous *ABCG26* promoter was detected both in intracellular compartments (putatively
160 vacuoles possibly due to lytic cleavage of the fluorescent protein) and at the cell surface, at
161 what appears to be the plasma membrane, where it was polarly localized towards the
162 locular matrix in wild-type plants (Figure S5C), as previously reported for the rice ABCG26
163 homologue (16). ABCG26:mTQ2 plasma membrane polarity was established at the early free

164 microspore stage (stage 8; Figure S5A-C). In contrast, in the *gso1 gso2*, *cif3 cif4* and *tpst*
165 mutants ABCG26:mTQ2 plasma membrane signal was detected predominantly between
166 tapetal cells indicating a lack of cell polarization (Figure S5D-M). No change in expression
167 was observed for the sporopollenin biosynthesis genes (Figure S6), suggesting that the
168 accumulation of pollen wall material in the mutants is likely not due to enhanced
169 sporopollenin biosynthesis, but may be due to polarity defects.

170 In summary, GSO1/2, CIF3/4 and TPST all appear to be necessary for normal tapetum
171 function. Tapetum function in turn is necessary for normal pollen grain spacing within the
172 locular fluid and for the targeted and regular deposition of pollen wall components onto the
173 surface of developing pollen grains, suggesting that GSO-mediated signaling is necessary for
174 the coordination of these processes.

175

176 *GSO receptors are produced in the middle layer while CIF3 and CIF4 are expressed and*
177 *sulfated in the tapetum cells*

178 To understand the organization of the GSO signaling pathway in developing anthers,
179 we studied the expression of pathway components. Transcriptional reporters revealed
180 expression of *GSO1* and *GSO2* in the middle layer (Figure 2 A-B and Figure S7). Despite the
181 difficulty of counterstaining the inner cell layers of the anther wall, middle layer nuclear
182 signals can be clearly distinguished due to their flattened form (Figure 2 A-B). Expression was
183 initiated prior to meiosis of the pollen mother cell, at around stage 5 (Figure S7), significantly
184 earlier than pollen wall deposition, and was maintained until the degradation of the middle
185 layer at stage 11 (13). Translational reporter constructs revealed the presence of GSO1 and
186 GSO2 proteins at the periphery of middle layer cells, consistent with plasma membrane
187 localization (Figure 2 D-E and Figure S7). The GSO1 translational reporter fully
188 complemented the pollen and tapetum phenotypes of *gso1 gso2* mutants (Figure S1B),
189 confirming that this expression pattern accurately reflects native *GSO1* expression in the
190 anther. Although both transcriptional reporters and fusion proteins were occasionally seen
191 in the endothecium, neither gene expression nor protein accumulation was ever detected in
192 the tapetum. Protein accumulation appeared uniform within the membrane of middle layer
193 cells, similar to the situation previously reported in the embryo epidermis (9).

194 In contrast, transcriptional reporters *pCIF3-NLS-3xmVenus* and *pCIF4-NLS-3xmVenus*
195 revealed that the expression of *CIF3* and *CIF4* is restricted to the tapetum and initiates at

196 around the onset of PMC meiosis (Figure 2C, Figure S8). Tapetal expression is distinguished
197 from that in other cell layers by the presence of characteristic double nuclei. While *CIF3*
198 expression diminishes shortly after microspore release, *CIF4* expression continues until
199 tapetum degradation at stage 11 (Figure S8). Expression of the *CIF4* ORF either under its own
200 promoter, or under the tapetum-specific *AMS* (17) (Figure S9), and *SHT* (18) (Figure S9)
201 promoters, fully complemented the *cif3 cif4* phenotype (Figure S1G, H, J, O, Q) confirming
202 that these expression patterns likely reflect the native expression of *CIF3* and *CIF4*.

203 To explore the spatial requirement for TPST, which acts cell autonomously in the
204 Golgi apparatus during peptide secretion (19), we expressed the *TPST* ORF under the
205 tapetum-specific *AMS* promoter in the *tpst-1* mutant. All transformed lines showed a
206 complete or nearly complete complementation of the pollen and tapetum phenotypes of
207 *tpst-1*, consistent with TPST activity being required for the production of sulfated peptide
208 ligands in the tapetum (Figure S1L, R).

209 In summary, our results show that normal pollen wall deposition and tapetum
210 function depend on the activity of *CIF3* and *CIF4* peptides sulfated and secreted from the
211 tapetum, and their cognate receptors, *GSO1* and *GSO2* in the anther middle layer.

212

213 *A pollen specific subtilisin serine-protease, SBT5.4, can cleave the extended C-terminus of the*
214 *CIF4 precursor.*

215 Analysis of the embryonic cuticle integrity signaling in seeds revealed a bidirectional
216 signaling pathway in which an inactive, sulfated precursor of the TWS1 peptide requires C-
217 terminal subtilase-mediated processing to release the mature and bioactive TWS1 peptide as
218 a ligand for *GSO1/2* (9). Like TWS1, both *CIF3* and *CIF4* possess C-terminal extensions (Figure
219 3A) suggesting that they may also require C-terminal processing for activation. We found
220 that both the full length *CIF4* ORF, and a truncated version lacking the sequence encoding
221 the part of the precursor C-terminal to the predicted active peptide, could complement the
222 *cif3 cif4* mutant phenotype when expressed under the *CIF4* promoter or under the tapetum-
223 specific *SHT* promoter in developing anthers (Figure S1G, I, J, K, O, P). The C-terminal
224 extension of *CIF4* is thus not required for activity. Our finding that C-terminal extensions in
225 *CIF*-class ligands impair receptor binding (9), and the apparent contribution of the free C-
226 terminus of *CIF* peptides to receptor/co-receptor complex formation (11), suggest that the
227 C-terminal extension needs to be removed for bioactivity. We therefore tested the

228 hypothesis that, as in the seed, subtilases could be involved in activating CIF peptides in the
229 anther. We found that *SBT5.4*, one of numerous subtilase-encoding genes expressed in
230 developing anthers (including *SBTs 1.8, 3.7, 3.12, 3.16, 3.18, 4.13, 4.15, 5.1, 5.4-5.6*, Figure
231 S10), showed strong and apparently specific pollen-localized expression from stage 8, when
232 the pollen wall starts to be laid down, until pollen maturity (Figure 2F, Figures S11, S12).
233 *SBT5.4* is thus a good candidate for CIF activation.

234 *SBT5.4* loss-of-function mutants did not show any defects in pollen development
235 (Figure S13), possibly due to the presence of functionally redundant proteases (Figure S10).
236 Furthermore, a triple mutant lacking function of *SBT5.4, 5.5* and *5.6*, all of which showed
237 similar expression patterns in the developing pollen, also failed to show any pollen
238 phenotype (Figure S12, 13).

239 We therefore tested directly whether *SBT5.4* is able to cleave the CIF4 precursor. N-
240 terminally GST-tagged proCIF4 expressed in *E. coli* was co-incubated with *SBT5.4:(His)6*
241 transiently expressed in tobacco (*N. benthamiana*) leaves and purified from cell wall
242 extracts. Several cleavage products were generated upon co-incubation with *SBT5.4*, but not
243 with extracts from untransformed plants, indicating that proCIF4 is processed by *SBT5.4*
244 (Figure 3B). To confirm cleavage at sites relevant for CIF4 maturation, a synthetic CIF4
245 peptide extended by three amino acids of the precursor at either end was used as substrate
246 for recombinant *SBT5.4*. Mass spectrometry analysis of cleavage products revealed specific
247 cleavage only at the C-terminal processing site between His89 and Gly90 (Figure 3C, D).
248 *SBT5.4*, a subtilase specifically expressed in pollen grains from stage 8 onwards, is thus able
249 to remove the C-terminal extension for CIF4.

250 To further confirm the potential role of *SBT5.4* we expressed it under the *AMS*
251 promoter which drives expression specifically in the tapetum, from the point of PMC meiosis
252 onwards (Figure S9) (and thus earlier than the initiation of endogenous *SBT5.4* expression in
253 pollen grains). This led to strong pollen phenotypes (Figure 3). Pollen in these lines tended to
254 fuse together in a mass (Fig. 3E-H). However, defects in these lines were very distinct from
255 those observed in *gso1 gso2* or *cif3 cif4* mutants. Firstly, tapetum hypertrophy was either
256 weaker or absent (Figure 3Q). Secondly, and again unlike the situation in loss of function
257 *gso1 gso2* or *cif3 cif4* mutants where the reticulate patterning of the pollen wall is still visible
258 in most peripheral pollen grains (Figure 1C-F), the patterning of the pollen wall was severely
259 compromised in lines expressing the *pAMS:SBT5.4* construct (Figure 3F, H). Defects varied

260 from loss/fusion of tecta, giving rise to a “broken” appearance, to an apparent massive de-
261 regulation of pollen wall deposition, giving pollen grains a “lumpy” appearance (Figure 3F,
262 H). Deregulation of pollen wall deposition could be observed as early as the tetrad stage in
263 the *pAMS-SBT5.4* lines. At this stage the organization of the primexine, which that serves as
264 a scaffold for the pollen wall formation, was abnormal (Figure 3I-L). At later stages, the
265 bacula appeared to form very close to each other resulting in abnormally dense exine (Figure
266 3 N, P). Bacula also appeared to be poorly attachment to the pollen surface (Figure 3P).
267 Thus, the ectopic expression of the SBT5.4 leads to strong deregulation of the pollen wall
268 formation.

269 Our results strongly support the hypothesis that the strict spatial and temporal
270 regulation of CIF-cleaving SBT activity in developing pollen grains is a critical factor in
271 ensuring the organized deposition of the pollen wall.

272

273 *GSO1 expression in the tapetum interferes with pathway function.*

274 Our results suggest a model in which tapetum-derived peptides, processed by pollen derived
275 subtilases, must diffuse to the middle layer to activate GSO-mediated signalling (Figure 4G).
276 To test this model further, we expressed full length GSO1 and a truncated version lacking the
277 cytoplasmic kinase domain (GSO1 Δ Kinase) under the tapetum-specific *AMS* promoter.
278 Δ Kinase versions of LRR-RLKs have previously been shown to provoke dominant negative
279 phenotypes, possibly through ligand sequestration (20). Consistent with this, wild-type
280 plants transformed with *pAMS:GSO1- Δ Kinase* showed a strong loss of function phenotype
281 identical to the *gso1 gso2* mutant (tapetum hypertrophy with both ectopic and defective
282 pollen wall deposition) (Figure 4A-C). This could be explained by the sequestration of
283 activated CIF peptides, which according to our model need to diffuse around tapetum cells
284 to reach their receptors in the middle layer. In contrast, wild-type plants transformed with
285 *pAMS:GSO1* presented a different phenotype with disrupted pollen wall patterning, frequent
286 pollen death, pollen grain adhesions, but no tapetum hypertrophy (Figure 4D-F). These
287 phenotypes are reminiscent of those observed upon *SBT5.4* expression under the *AMS*
288 promoter, suggesting that ectopic GSO1 expression in the tapetum may cause a deregulation
289 of pathway activation.

290

291 **Discussion**

292 The coordination of tapetum activity (polar secretion of nutrients, enzymes, pollen wall
293 components, and the locular matrix) with pollen development (growth, and initial patterning
294 of the pollen wall) is likely a key factor in ensuring the generation of viable pollen grains.
295 Taken together our data indicate that this coordination involves a molecular dialogue
296 between the tapetum, the developing pollen grains and the middle layer. The localization of
297 GSO1 and GSO2 receptors on the membrane of middle-layer cells, and expression of CIF3
298 and CIF4 precursor-encoding genes in the tapetum, initiate significantly before microspore
299 release from tetrads and the onset of pollen wall deposition (Figure 2G, S7 and S8).
300 However, our phenotypic data show that this early production of signaling pathway
301 components, which is also observed in the seed system (9), may “poise” the signaling system
302 for action, but is not necessary for early stages of tapetum or pollen grain development,
303 since loss of function phenotypes in both ligand and receptor mutant backgrounds only
304 appear after microspore release.

305 In contrast, our results suggest that the trigger in the pathway is the production of
306 pollen-derived CIF-activating proteases including SBT5.4, the microspore-specific expression
307 of which is synchronized perfectly with the onset of pollen wall deposition (Figure 2G, S12).
308 The importance of accurately timing pathway activation is underlined by the dramatic
309 effects of expressing SBT5.4 early in the tapetum, which would be predicted to trigger a
310 precocious activation of GSO-mediated signalling through constitutive ligand activation. The
311 highly disrupted deposition of pollen-wall components observed in these lines, including the
312 difficulty of wall components to anchor to the pollen surface could be a consequence of a
313 lack of coordination of pollen wall component production by the tapetum with microspore
314 release from tetrads.

315 Our model predicts that subtilases produced in immature pollen grains mediate the
316 timely processing and liberation of activated CIF3/4 peptides in the pollen wall or locular
317 matrix. Activated peptides must then diffuse between tapetum cells to the middle layer
318 where they can bind to GSO1 and GSO2, ultimately leading to the activation of the tapetum,
319 and the organized secretion of pollen wall components into the anther locule. Either pollen
320 wall completion, or specific changes in the tapetum, presumably subsequently attenuate
321 signaling activity by separating subtilases from their substrates, thus adjusting tapetum
322 activity to the needs of the pollen grains (Figure 4G).

323 This model raises the intriguing question of why GSO1 and GSO2 are expressed in the
324 middle layer, rather than in the tapetum. One possible explanation is that separating pro-
325 peptide and receptor production prevents untimely receptor-ligand interaction in the
326 secretory pathway. However our published data suggest that C-terminally extended CIF
327 peptides show impaired receptor binding (9) and, consistent with this, co-expression of the
328 TWS1 precursor and the GSO receptors in the embryo epidermis does not lead to receptor
329 activation. A second possibility is that GSO signaling could interfere with other signaling
330 pathways if it occurs in the tapetum, for example through competition for SERK co-receptors
331 which are known to interact with GSO1 and GSO2 but also with other receptors involved in
332 anther development (11, 21). A final possibility is that the directional output of GSO signaling
333 from the middle layer helps to polarize secretion from the tapetum, as suggested by the
334 ectopic deposition of sporopollenin in pathway mutants.

335 In conclusion, our work has revealed that a complex peptide-mediated dialogue
336 spanning three distinct cell types is necessary to synchronize secretion and deposition of
337 pollen wall components within the anther locule. The middle layer, an understudied and
338 poorly understood anther tissue, plays an important role in this dialogue, apparently acting
339 as an organizing hub for the activation and associated polarization of the neighboring
340 tapetum.

341

342 **Materials and Methods**

343 **Plant material and growth conditions**

344 Seeds were sown on half-strength MS (Murashige Skoog) medium with 1% sucrose and 1%
345 agar, stratified for 2 days at 4°C and grown for 7 days in short-day conditions (8h light / 16h
346 dark). Seedlings were then transferred to soil and grown for 3 more weeks in short-day
347 conditions (8h light / 16h dark) and subsequently transferred to long-day conditions (16h
348 light / 8h dark) to promote flowering. Some of the transgenic lines described were examined
349 in the T1 generation; these were first selected on half-strength MS medium with 1% sucrose
350 and 1% agar supplemented with either 50 µg/mL kanamycin or 10 µg/ml glufosinate
351 ammonium (Basta).

352 For *promoter::GUS(uidA)* reporter lines, seeds were incubated over night at -75 °C, re-
353 suspended in 0.1 % agar, stratified for 48 hours at 4°C in the dark, and sown on either half-

354 strength MS (Murashige Skoog), 1% sucrose, 0.6 % agar plates, or a mix of potting compost
355 with 3.6 % (v/v) sand and 7.2 % (v/v) perlite. Plants were grown under short-day conditions
356 (12 h photoperiod) at 22 °C and 100-120 μE white light.

357 Mutant alleles used were *gso1-1* (SALK_064029) *gso2-1* (SALK_130637) (9, 22), *gso1-2*
358 (SALK_103965) *gso2-2* (SALK_143123) (10), *cif3-2* (GABI_516E10) *cif4-1* (CRISPR mutant line)
359 (11), *tpst-1* (SALK_009847) (19), *sbt5.4* (*At5g59810*, SALK_025087), *sbt5.5* (*At5g45640*,
360 SALK_107233), *sbt5.6* (*At5g45650*, CRISPR mutant generated as described below) (Fig. S13);
361 genotyping primers used are listed in Suppl. Table 1. The *pGSO1:GSO1-mVenus* and
362 *pGSO2:GSO2-mVenus* lines are described in (9).

363 **Generation of transgenic plant lines**

364 Gateway technology (Invitrogen™) was used for the production of genetic constructs. For
365 the NLS-3xmVenus transcriptional reporter lines, the following promoter fragments were
366 amplified by PCR from Arabidopsis (Col-0) genomic DNA: *pSBT5.4* -2639 bp to -1 bp, *pSBT5.5*
367 -1903 bp to -1 bp, *pSBT5.6* -4144 bp to -1 bp, *pAMS* -2618 bp to -1 bp, *pSHT* -859 bp to -1 bp,
368 *pGSO1* -5583 bp to -1 bp, *pGSO2* -3895 bp to -1 bp, *pCIF3* -2092 bp to -1 bp, *pCIF4* -2201 bp
369 to -1 bp. The fragments were inserted into pDONR P4-P1R and recombined with *NLS-*
370 *3xmVenus-N7* pDONR211, *OCS* terminator pDONR P2R-P3 (containing STOP codon followed
371 by the *OCTOPINE SYNTHASE* terminator) and pK7m34GW destination vector (with
372 kanamycin *in planta* resistance); this yielded plasmids containing *pSBT5.4-NLS-3xmVenus*,
373 *pSBT5.5-NLS-3xmVenus*, *pSBT5.6-NLS-3xmVenus*, *pAMS-NLS-3xmVenus*, *pSHT-NLS-*
374 *3xmVenus*. To construct *pGSO1-NLS-3xmVenus*, *pGSO2-NLS-3xmVenus*, *pCIF3-NLS-*
375 *3xmVenus*, and *pCIF4-NLS-3xmVenus*, promoter regions cloned into pDONR P4-P1R vectors
376 and *NLS-3xmVenus* in pDONRZ were integrated into the pB7m24GW,3 destination vector
377 (with Basta *in planta* resistance and with the 35S terminator) by LR reaction. These
378 constructs were transformed into the Col-0 background.

379 The promoter::GUS(*uidA*) reporter lines were generated by amplifying promoter fragments
380 of *SBT4.15* (2634 bp) and *SBT5.4* (1592 bp) from Arabidopsis (Col-0) genomic DNA. Promoter
381 fragments were digested with *NotI/XhoI* and *NotI/Sall*, respectively, and cloned into pGreen
382 0029 (23) containing a promoterless β -glucuronidase (*GUS*):green fluorescent protein (*GFP*)
383 fusion reporter gene from pCambia 1303 (CAMBIA GPO Box 3200 Canberra, ACT 2601
384 Australia) followed by the 282 bp *OCS* terminator.

385

386 To produce *GSO1* mis-expression constructs, the *GSO1* genomic fragment lacking the
387 cytoplasmic kinase-encoding domain (from the ATG (+1 bp) to +2850 bp) was inserted into
388 pDONR211 and recombined with *pAMS* pDONR P4-P1R (see above), *OCS* terminator pDONR
389 P2R-P3 (see above) and pK7m34GW destination vector to yield the *pAMS:GSO1-ΔKinase*
390 construct. Similarly, the entire *GSO1* genomic fragment from ATG (+1 bp) to the STOP codon
391 (+3826 bp) was inserted into pDONR211 and recombined with *pAMS* pDONR P4-P1R (see
392 above), *OCS* terminator pDONR P2R-P3 and pK7m34GW destination vectors to yield the
393 *pAMS:GSO1* vector. These constructs were transformed into Col-0 background.

394 The *CIF4* complementation construct was produced by amplifying the *CIF4* genomic
395 fragment including the promoter region and the entire coding sequence from -4242 bp to +
396 309 bp (including the STOP codon) and introducing it into pDONR211. Alternatively, a
397 truncated version of the *CIF4* genomic fragment from -4242 bp to +267 bp with an artificial
398 STOP codon before the C-terminal extension was introduced into pDONR211. The *CIF4*
399 terminator fragment from +310 bp to + 2541bp was inserted into pDONR P2R-P3. Genomic
400 fragments were recombined into the pK7m34GW destination vector to yield *pCIF4:CIF4* or
401 *pCIF4:CIF4_truncated* constructs which were then transformed into the *cif3-2 cif4-1* double
402 mutant.

403 To produce *CIF4* expression constructs, *CIF4* coding sequence from +1 bp (ATG) to +309 bp
404 (STOP codon) was introduced into pDONR211. The construct was recombined with either
405 *pAMS* pDONR P4-P1R or *pSHT* pDONR P4-P1R and with *OCS* terminator pDONR P2R-P3 and
406 pK7m34GW destination vectors to yield the *pAMS:CIF4* and *pSHT:CIF4* constructs.
407 Alternatively, a truncated version of the *CIF4* coding sequence including N-terminal part
408 from +1 bp (ATG) to +267 bp (and therefore lacking the C-terminus that is removed in the
409 mature peptide) was introduced into pDONR211. The construct was recombined with *pSHT*
410 pDONR P4-P1R and with *OCS* terminator pDONR P2R-P3 and pK7m34GW destination vectors
411 to yield the *pSHT:CIF4_truncated* construct. These constructs were transformed into the
412 *cif3-2 cif4-1* double mutant.

413 To produce the *TPST* complementation construct, a plasmid containing the *TPST* ORF in
414 pDONR211 (9) was recombined with the *pAMS* pDONR P4-P1R, *OCS* terminator pDONR P2R-

415 P3 and the pB7m34GW destination vector to yield the *pAMS:TPST* construct. This construct
416 was introduced into the *tpst-1* background.

417 For ectopic expression of SBT5.4 in the tapetum, the *SBT5.4* coding sequence from +1 bp
418 (ATG) to +2337 bp (STOP codon) was introduced into pDONR211. The construct was
419 recombined with *pAMS* pDONR P4-P1R and with OCS terminator pDONR P2R-P3 and
420 pK7m34GW destination vectors to yield the *pAMS:SBT5.4* construct. This construct was
421 transformed into the Col-0 background.

422 For the generation of the CRISPR allele of *SBT5.6* the method described by Stuttmann and
423 co-workers was used (24). Two guides cutting at the beginning of the gene coding sequence
424 were designed and inserted into the pDGE332 and pDGE334 shuttle vectors. The two shuttle
425 vectors were then recombined into the pDGE347 recipient vector containing FastRed
426 selection marker. The construct was transformed into the *sbt5.4 sbt5.5* double mutant
427 background. The transgenic plants were selected with FastRed and analyzed for the
428 presence of the mutation. The CRISPR allele was shown to contain a 104 bp deletion in the
429 second exon of the *SBT5.6* gene.

430
431 To generate the *pABCG26:ABCG26-mTQ2* translational reporter line, the *ABCG26* genomic
432 fragment including the promoter (-583 bp to -1 bp) and the coding sequence with introns up
433 to but not including the STOP codon was amplified by PCR from Arabidopsis (Col-0) genomic
434 DNA and cloned into the pENTR 5'-TOPO vector. The *mTQ2* ORF with four alanine codons for
435 a small N-terminal linker replacing the start codon was cloned into the pDONR211 vector.
436 The *pABCG26:ABCG26* pENTR 5'-TOPO, the *mTQ2* pDONR211 vector were recombined with
437 the OCS terminator pDONR P2R-P3 vector and pB7m34GW destination vector. The construct
438 was transformed into the Col-0, *gso1-1 gso2-1*, *cif3-2 cif4-1* and *tpst-1* backgrounds.

439
440 For plant transformation, the constructs were first introduced into *Agrobacterium*
441 *tumefaciens* strains C58pMP90 by electroporation, and then transformed into Col-0 plants or
442 the respective mutant genotypes by floral dip as described in (25).

443 Primers used are listed in Suppl. Table 1.

444 **Histology**

445 Inflorescences were fixed with FAA (50% (v/v) ethanol, 5% (v/v) acetic acid, 3.7% (v/v)
446 formaldehyde) overnight, dehydrated in a graded series of 50%, 60%, 70%, 85%, 95% and
447 100% ethanol for 1 h each, then further incubated overnight in fresh 100% ethanol. The
448 samples were then incubated in 50% ethanol/50% Technovit 7100 base liquid (v/v) for 4h
449 and then in 25% ethanol/75% Technovit 7100 base liquid (v/v) overnight. The samples were
450 infiltrated in Technovit 7100 infiltration solution (1g hardener I in 100 ml Technovit 7100
451 base liquid) with vacuum for 2h and further incubated for 6 days. All steps above were
452 conducted at room temperature (RT) with gentle agitation. The samples were polymerized
453 with Technovit 7100 polymerisation solution (100 µl Technovit 7100 hardener II in 1,5 ml
454 infiltration solution) at RT for 6 hours. Transverse section of 3µm were cut using a Leica
455 Microtome HM355S.

456 For histological analysis, the sections were stained with 0.01% (w/v) acriflavine in H₂O for 5
457 min, mounted in Vectashield (Vector Laboratories) and observed using TCS SP5 or TCS SP8
458 confocal microscopes (Leica) with excitation at 488 nm and emission at 492-551 nm.

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

462 For histochemical staining of GUS activity (26) in the *promoter::GUS(uidA)* reporter lines,
463 plants were vacuum-infiltrated with 2 mM each of potassium ferrocyanide, potassium
464 ferricyanide and 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc, Biochemical
465 DIRECT) in 50 mM sodium phosphate buffer pH 7.0 with 0.1% Triton X-100 (v/v) for 2 min at
466 80 mbar. After 24 h at 37°C, the tissue was destained in a graded ethanol series (20, 35, 50,
467 70, 80, 90% (v/v)). Pictures were taken with a Nikon D5000 digital camera and at a dissecting
468 microscope (Stemi SV11; Carl Zeiss Microscopy, Jena, Germany) using a SPOT RT KE Color
469 Mosaic Camera model 7.2 with SPOT software (Visitron Systems; Puchheim, Germany).

470 **Clearsee tissue clearing**

471 Inflorescences were fixed in 4% paraformaldehyde in PBS at 4°C under vacuum for 2h and
472 subsequently incubated overnight at 4°C. The samples were washed twice with PBS and
473 cleared with Clearsee Alpha solution (27) (10% (w/v) xylitol powder, 15% (w/v) sodium

474 deoxycholate, 25% (w/v) urea and 0.63% (w/v) sodium sulfite) for 1 week changing to a fresh
475 solution every 2 days at RT.

476 After clearing, the samples were incubated in 0.1% (w/v) Auramine O dissolved in Clearsee
477 Alpha overnight, washed 3 times for 20 min each with Clearsee Alpha solution. The anthers
478 were dissected from the inflorescences, mounted in Clearsee Alpha solution and observed
479 using a confocal TCS SP5 or TCS SP8 confocal microscope (Leica). Auramine O was excited at
480 488 nm and the emission was collected at 500-570 nm.

481 **Scanning and cryo-scanning electron microscopy**

482 Pollen grains were observed in the scanning electron microscope SEM FEG FEI Quanta 250 at
483 5kV voltage or, alternatively, using SEM HIROX 3000 at 10 kV.

484 For cryo-SEM, anthers were immobilized on a specimen mount coated with modeling clay
485 and rapidly frozen in liquid nitrogen for at least 15 s under vacuum. The specimen was
486 transported under vacuum into the cryo-SEM MEB FEG FEI Quanta 250 precooled to -140°C.
487 The frozen anthers were fractured at -140°C, then sublimed at -90°C to remove ice
488 accumulations, immediately refrozen to -140°C and coated with gold. The anthers were
489 observed at 5 kV.

490 **Transmission electron microscopy**

491 Flower buds at appropriate developmental stages were fixed with 4 % (w/v) formaldehyde
492 and 2 % (w/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) (PB) under vacuum (0.6
493 bar) at 4°C for 1h during which the vacuum was slowly broken 3 times, then incubated in
494 fresh fixative solution at 4°C overnight. The samples were washed three times in PB,
495 postfixed for 2h in 1 % (w/v) osmium tetroxide in PB at room temperature (RT), rinsed 5
496 times for 5 min in PB and dehydrated under vacuum in a graded ethanol series (30 to 100%
497 v/v) with 20 min incubations in each bath at RT. The samples were then infiltrated with low
498 viscosity Spurr resin (EMS Catalog #14300) using a graded series in ethanol (33%, 66% and
499 twice 100% v/v Spurr resin) at 4°C with each incubation lasting 24h (including 20min under
500 vacuum). The samples were polymerized in fresh Spurr resin at 60°C for 18h. Ultrathin
501 sections (70 nm) were prepared using UC7 Leica Ultramicrotome, placed on formvar-coated
502 grids, then poststained with 2% uranyl acetate and lead citrate. Sections were examined

503 under JEOL 1400 transmission electron microscope at 120kV and imaged with the Gatan Rio
504 16 camera.

505 **Confocal microscopy**

506 For the mVenus-expressing transcriptional and translational reporter lines, anthers were
507 stained with 20 µg/ml propidium iodide solution and examined using TCS SP5 (Leica) or ZEISS
508 710 confocal microscopes with excitation at 514 nm and emission at 526-560 nm for
509 mVenus, and 605-745 nm for propidium iodide.

510 For the *pABCG26:ABCG26-mTQ2* reporter lines, the anthers were stained with 20 µg/ml
511 propidium iodide solution and examined using ZEISS 710 confocal microscopes with
512 sequential excitation at 458 nm for mTQ2 and emission peak at 490 nm, as well as with
513 excitation at 514 nm and emission peak at 642 nm for propidium iodide. 40 anthers per
514 genotype from the same developmental stage were imaged.

515 **Peptide cleavage assay and cleavage product analysis**

516
517 The *SBT5.4* ORF was amplified by PCR from a RIKEN full-length cDNA clone (RAFL19-27-C18).
518 Primers included *KpnI* and *XbaI* restriction sites and six terminal His codons in the reverse
519 primer. The PCR product was cloned between the CaMV 35S promoter and terminator in
520 pART7 (28). The expression cassette was then transferred into the *NotI* site of the binary
521 vector pART27, and introduced into *Agrobacterium tumefaciens* (C58C1). C-terminally His-
522 tagged SBT5.4 was transiently expressed in *N. benthamiana* by agro-infiltration as described
523 (29). The leaf apoplast was extracted four to five days after infiltration with 50 mM
524 Na₂HPO₄/NaH₂PO₄ pH 7.0, 200 mM KCl, 1 µM pepstatin, 10 µM E64. Extracts were subjected
525 to affinity chromatography on 500 µl Ni-NTA agarose slurry according to protocols provided
526 by the manufacturer (Qiagen; Hilden, Germany).

527
528 The *CIF4* ORF, without the sequence coding for the signal peptide, was amplified by PCR
529 from cDNA using primers that included a *BamHI* site at the 5'-end, and six His codons and an
530 *EcoRI* site at the 3'-end. The PCR product was cloned downstream and in frame with the
531 glutathione S-transferase (GST) ORF in pGEX-3X (GE Healthcare). The expression construct
532 was transfected into *E. coli* BL21-CodonPlus(DE3)-RIL (Agilent Technologies). Cells were
533 grown in 250 ml LB medium at 37 °C and 200 rpm, until OD₆₀₀ reached 0.6 to 0.8.

534 Subsequently, the culture was cooled down to 30 °C and after 20 min at 200 rpm, 1 mM IPTG
535 was added to induce protein expression. Cells were harvested by centrifugation after 2 hrs,
536 and lysed by sonication in in 50 mM Na₂HPO₄/NaH₂PO₄ pH 7.0, 150 mM NaCl, 1 mM PMSF, 5
537 mM benzamidine hydrochloride, and a spatula tip DNaseI. Four mM imidazole was added to
538 the cleared lysate, and the recombinant protein was purified on Ni-NTA agarose following
539 the manufacturer's instructions (Qiagen). The eluate (3x 500 µl) was concentrated by
540 ultrafiltration (Vivaspin concentrators, 10 kDa molecular weight cut-off; Sartorius; Göttingen,
541 Germany) and dialyzed against 50 mM Na₂HPO₄/NaH₂PO₄ pH 6.0, 300 mM NaCl.

542

543 Two and a half µg GST-CIF4-His in a total volume of 10 µl 50 mM Na₂HPO₄/NaH₂PO₄ pH 6.0,
544 300 mM NaCl were incubated at 25°C with recombinant SBT5.4 at the indicated
545 concentrations. As negative control, apoplastic washes from *N. benthamiana* plants that had
546 been agro-infiltrated with the empty pART27 vector were subjected to a mock-purification,
547 and an equal volume of the final fraction was used in the assay. The reaction was stopped
548 after 2 hrs by addition of 4x SDS-PAGE sample buffer. The digest was analyzed by SDS-PAGE
549 (15%) followed by Coomassie-Brilliant Blue (Instant Blue; Abcam, Cambridge, UK) staining.

550 For mass spectrometric analysis of the cleavage site, 6 µg of a synthetic CIF4 peptide
551 extended at both the N- and the C-terminus by three precursor-derived amino acid residues
552 were digested with 50 ng recombinant SBT5.4 and a mock-purified fraction as a control. The
553 reaction was stopped after 1 hour at 25°C by addition of 1% trifluoroacetic acid. Reaction
554 products were purified on C18 stage tips equilibrated in 0.5% (v/v) acetic acid as described
555 (30). Peptides were eluted in 0.5% (v/v) acetic acid, 50% (v/v) acetonitrile, evaporated to
556 dryness in a SpeedVac concentrator (Savant Instruments; Holbrook, NY), and stored at -20°C
557 until further analysis. Digests were analyzed by LC-MS/MS using an UltiMate 3000 RSLCnano
558 system (Dionex, Thermo Fisher Scientific) coupled to an Orbitrap mass spectrometer
559 (Exploris 480, Thermo Fisher Scientific). The Exploris 480 was operated under XCalibur
560 software (version 4.4, Thermo Fisher Scientific) and calibrated internally (31). Peptides were
561 trapped on a pre-column (C18 PepMap100, 5µm x 5mm, Thermo Fisher Scientific) and then
562 eluted from a 75 µm x 250 mm analytical C₁₈ column (NanoEase, Waters) by a linear gradient
563 from 2 to 55% acetonitrile over 30 min. MS spectra (m/z = 200-2000) were acquired at a
564 resolution of 60,000 at m/z = 200. Data dependent MS/MS mass spectra were generated for
565 the 30 most abundant peptide precursors using high energy collision dissociation (HCD)

566 fragmentation at a resolution of 15000 and a normalized collision energy of 30. Peptides
567 were identified by a Mascot 2.6 search (Matrix Science, UK) and quantified using peak areas
568 calculated from extracted ion chromatograms in XCalibur 4.4 (Thermo Fischer Scientific).

569

570 **Pollen viability and germination assays, seed count.**

571 Pollen viability was tested by staining mature anthers and pollen with Alexander solution
572 (32). For the pollen germination assays mature pollen grains were transferred onto medium
573 containing 0.5% low melting agarose, 18% sucrose, 0.01% boric acid, 1 mM CaCl₂, 1mM
574 Ca(NO₃)₂, 1 mM KCl, 0.03% casein enzymatic hydrolysate, 0.01% ferric ammonium citrate,
575 0.01% myo-inositol and 0.25 mM spermidine, pH 8.0 for 8h at 25°C. The images were taken
576 with Zeiss Axio Imager M2 microscope.

577 For the seed number analysis, seeds were counted in the 4th-8th silique of the respective
578 plants. 50 siliques per genotype were counted. Unpaired two-tailed t-tests were used for
579 statistical analysis.

580

581 **qRT-PCR**

582 Whole inflorescences containing unopened flowers (from the first closed flower upwards)
583 were collected with 4 independent replicates per genotype. RNA was extracted using the
584 Spectrum Plant Total RNA kit (Sigma-Aldrich) and the DNA was removed using the TURBO
585 DNA-free kit (Invitrogen). The cDNA was generated using SuperScript IV VILO Master Mix
586 (Thermo Fischer) with 500 ng RNA per sample. The cDNA was then diluted 1:50. The qRT-
587 PCR was performed using Applied Biosystems Fast SYBR Green Master Mix. The PCR reaction
588 was as follows: 95°C for 10 min, 50 cycles of 95°C for 15 s, and 60°C for 30 s. Expression of
589 *PP2AA3* gene (*At1g13320*) was used as standard (33, 34). Similar results were obtained
590 when the *AP2M* gene (*AT5G46630*) was used as the standard (33, 35). The PCR efficiency
591 was calculated from the standard curve amplification data: *ACOS5* 108%, *PKSA* 111%, *PKSB*
592 93%, *MS2* 100%, *TKPR2* 86%, *CYP703A2* 101%, *CYP704B1* 83%, *PP2AA3* 97%, *AMS* 94%.
593 Expression levels are presented as $E^{-\Delta Ct}$ with $\Delta Ct = Ct_{GOI} - Ct_{PP2AA3}$. Primers used are listed in
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595

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620

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721 **Figure legends**

722

723 **Fig. 1. GSO1 and GSO2 receptor-like kinases are required for pollen wall formation and tapetum**
724 **development. (A)** Inside the anther the haploid (n) pollen are surrounded by four diploid (2n)
725 sporophytic cell layers: tapetum, middle layer, endothecium and epidermis. **(B)** At the end of the
726 tetrad stage (stage7) the tapetum cells start to synthesize and release sporopollenin precursors into
727 the locular matrix where they ultimately attach to the surface of the pollen grains. When pollen wall
728 construction is finished (stage 11), the tapetum cells degrade and the resulting cellular debris also
729 adhere to the pollen surface. Pollen wall structural components are indicated: The exine is composed
730 of nexine, bacula and tectum. PMC: pollen mother cell. **(C-F)** Scanning electron microscopy (SEM)
731 images of the wild-type (C-D) and the *gso1-1 gso2-1* (E-F) mature pollen. **(G-H)** Pollen and anther
732 development in wild-type anthers (G) and *gso1-1 gso2-1* anthers (H) from the tetrad stage (stage 7)
733 to pollen release (stage 13). The anther cross-sections are stained with acriflavine. **(I-L)** Pollen wall
734 formation in wild-type (I-J) and the *gso1-1 gso2-1* mutant (K-L) anthers shown by toluidine blue
735 staining at stage 8 (I, K) and stage 10 (J, L). Black arrow indicates reddish purple staining (toluidine
736 blue) on the surface of the pollen grains in the *gso1-1 gso2-1* mutant; red arrow indicates ectopic
737 yellow color detected between tapetum cells in the *gso1-1 gso2-1* mutant. **(M-P)** Transmission
738 electron microscopy (TEM) of the pollen wall in wild-type (M-N) and *gso1-1 gso2-1* (O-P) pollen.
739 Arrow indicates fused pollen wall in the mutant. **(Q-R)** Cryo-SEM of the pollen wall in wild-type (Q)
740 and *gso1-1 gso2-1* (R) pollen. Arrows indicate the pollen walls. **(S-T)** Pollen wall material stained with
741 Auramine-O in wild-type (S) and *gso1-1 gso2-1* (T) anthers. Arrows indicate ectopic staining in the
742 mutant. **(U-V)** TEM showing ectopic deposition of sporopollenin-like material around the tapetum
743 cells in the *gso1-1 gso2-1* mutant (V) (arrow) compared to the wild-type (U). Scale bars: C, E 50 μ m;
744 G-H 20 μ m; D, F, I-L, S-T 10 μ m; M-R, U-V 1 μ m. P = pollen, T = tapetum, ML = middle layer.

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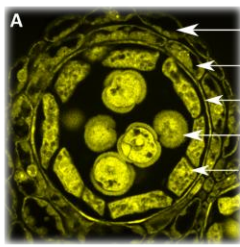
746 **Fig. 2. The GSO signaling pathway involves three different cell types. (A-B)** Expression of the *GSO1*
747 and *GSO2* genes in anthers using *pGSO1-NLS-3xmVenus* and *pGSO2-NLS-3xmVenus* reporter lines. **(C)**
748 Expression of *CIF4* in anthers using *pCIF4-NLS-3xmVenus* reporter line. **(D-E)** Protein localization of
749 the *GSO1* and *GSO2* receptor-like kinases in anthers using *pGSO1:GSO1-mVenus* and *pGSO2:GSO2-*
750 *mVenus* reporter lines. **(F)** Expression of *SBT5.4* in anthers using *pSBT5.4-NLS-3xmVenus* reporter
751 line. **(G)** Schematic illustration of the expression of GSO pathway components in anther cell types
752 during development. **(A-F)** Propidium iodide (PI) staining is shown in purple, mVenus signal is shown
753 in green. Insets: 2x magnification. Scale bars: A-F 20 μ m.

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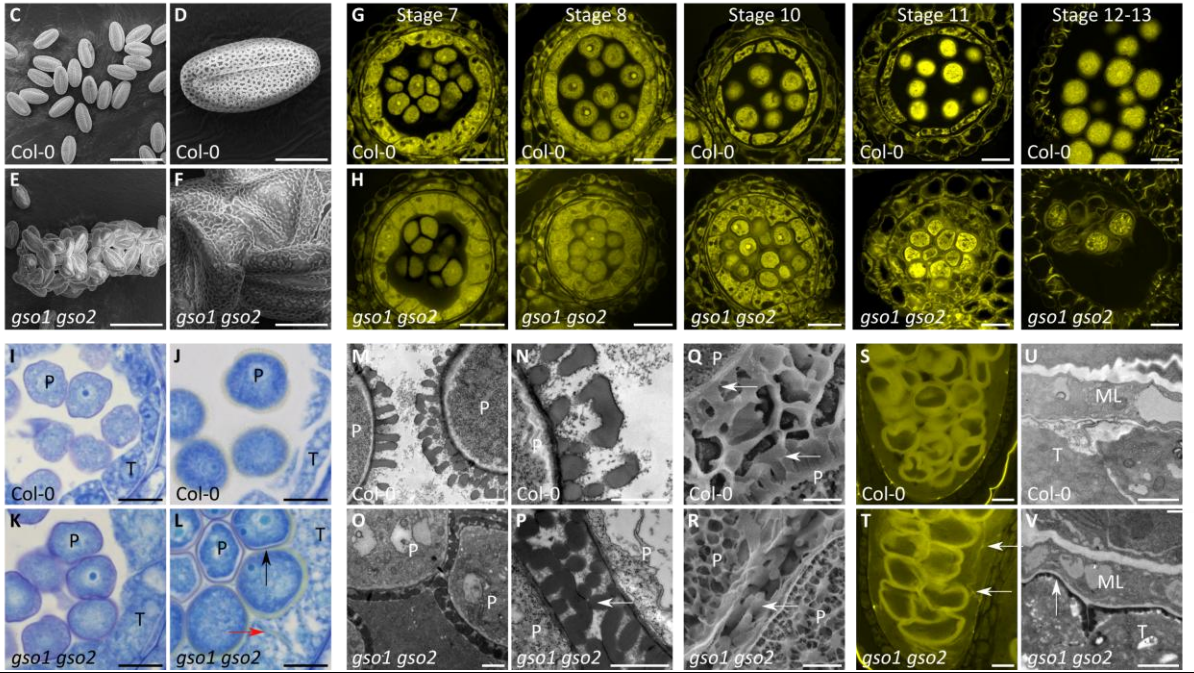
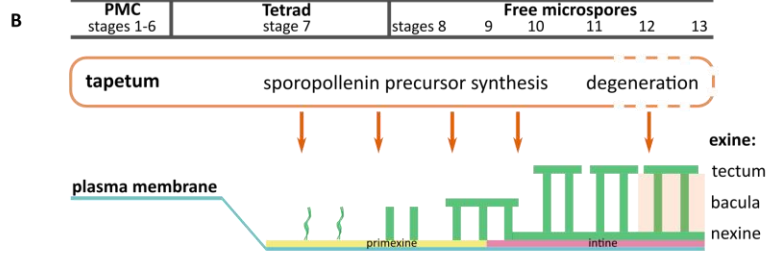
755 **Fig. 3. SBT5.4 can process the CIF4 precursor, and ectopic SBT5.4 expression leads to deregulated**
756 **pollen wall formation. (A)** Sequence alignment of the C-termini of CIF family precursors, mature
757 peptide sequences in bold face. Fully, strongly and weakly conserved residues (Clustal W; Gonnet
758 Pam250 matrix) are highlighted in red, blue and green, respectively. Arrow heads indicate processing
759 sites. **(B)** Coomassie-stained SDS-PAGE showing digests of recombinant GST-CIF4 (the full length and
760 a truncated version of the precursor are marked by blue and black arrow heads, respectively) with
761 SBT5.4 purified from tobacco leaves. The molar subtilase:substrate ratio is indicated for each lane;
762 cleavage products highlighted by asterisks. **(C, D)** Cleavage of a synthetic CIF4 precursor peptide by
763 SBT5.4. Bar graphs show relative abundance of the substrate peptide (top row) and fragments
764 thereof for the control (C) and SBT5.4 (D) digests as the percentage of all peptides detected by
765 MS/MS analysis. Arrow heads mark N- and C-terminal processing sites flanking the mature CIF4
766 peptide. The cleavage product generated by SBT5.4-specific processing of the C-terminal extension is
767 highlighted in dark blue. **(E-H)** Scanning electron microscopy (SEM) images of *pAMS:SBT5.4* pollen
768 from two independent lines in (E, F) and (G, H). Similar phenotypes were observed in 4 independent
769 lines. **(I-P)** Transmission electron microscopy (TEM) of the pollen wall formation in the wild-type (I, K,
770 M, O) and the *pAMS:SBT5.4* (J, L, N, P) anthers at the tetrad stage (stage 7) and the free microspore
771 stage (stage 9). Black arrows indicate putative detached primexine in the *pAMS:SBT5.4* tetrads. **(Q)**
772 Pollen and anther development in the *pAMS:SBT5.4* line at different developmental stages. The
773 anther cross-sections are stained with acriflavine. Scale bars: E, G, Q 20 μm ; F, H, I-J, M-N O 5 μm ; K-
774 L, O-P 1 μm .

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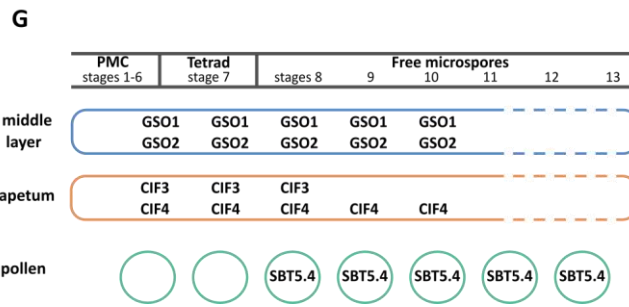
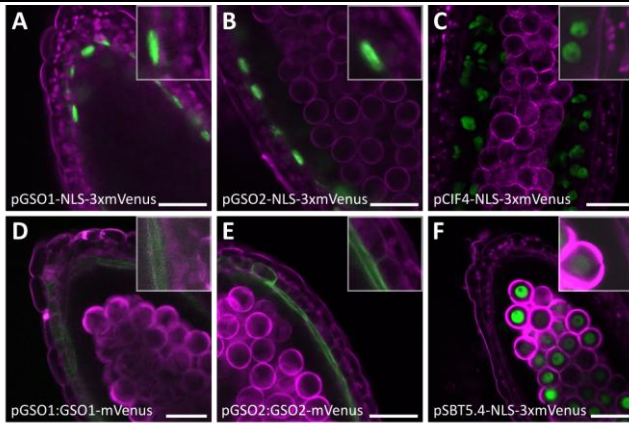
776 **Fig. 4. The complex spatial organization of GSO pathway components is necessary for controlled**
777 **tapetum development. (A-C)** Scanning electron microscopy (SEM) images and histological cross-
778 section of anthers of the *pAMS:GSO1- Δ Kinase* line. Phenotype confirmed in 7 independent lines. **(D-**
779 **F)** Scanning electron microscopy (SEM) images and histological cross-section of anthers of the
780 *pAMS:GSO1* line. Phenotype confirmed in 5 independent lines. **(G)** Taken together our data support
781 the following model for the coordination of pollen wall formation with tapetum development. The
782 CIF precursors produced in the tapetum are processed by the pollen-derived subtilases. The
783 activated peptides diffuse between tapetum cells to bind GSO receptors in the middle layer which
784 induces downstream signaling leading to polarized sporopollenin secretion towards the pollen grains.
785 The completion of the pollen wall on the pollen surface prevents the interaction of subtilases with
786 their substrates, the uncleaved peptides are inactive and this attenuating signaling. The anther cross-
787 sections are stained with acriflavine. Scale bars: 10 μm .



epidermis (2n)
 endothecium (2n)
 middle layer (2n)
 pollen (n)
 tapetum (2n)



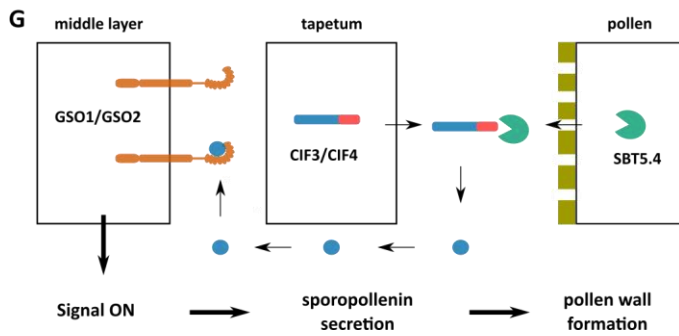
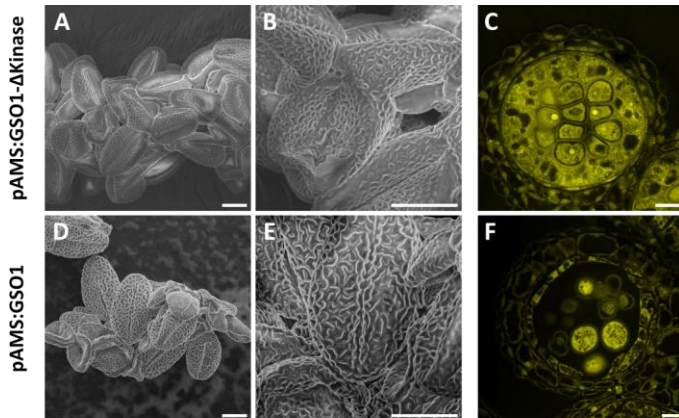
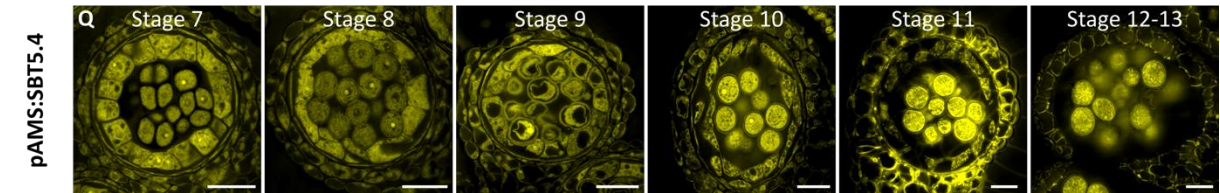
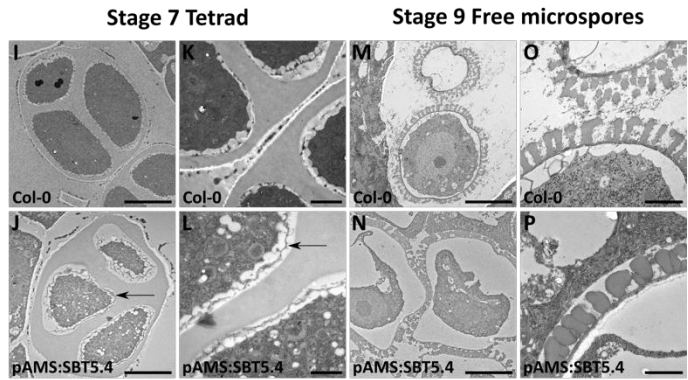
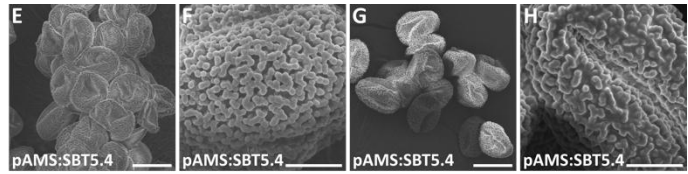
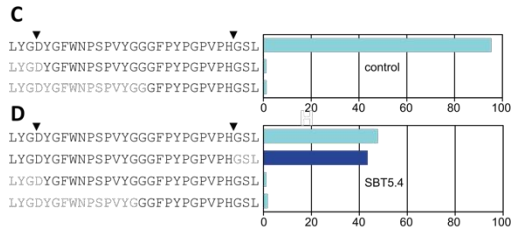
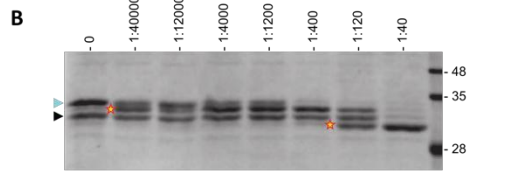
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CIF1 ...RLLRMNTKDYGNNS-**PS**PRLERP--PFKLI**PN**▼
 CIF2 ...RILRMNSRDYGHSS-**PK**PKLVRP--PFKLI**PN**▼
 CIF3 ...IWEVFHGDYGSWS-**PT**PKIPRR--SPAPI**PH**DDFAPPRLKA
 CIF4 ...SFLPKLYGDYGFWN-**PS**PVYGGGFYPGVP**H**GSGLPIQRHKPK▼
 TWS1 ...SEMKVGLEDY**N**FPVD**PT**TKASI-K**PG**PIEH**G**TPLN**PI**PKPPS...



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