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1 # Self-incompatibility requires GPI anchor remodeling by the poppy
2 # PGAP1 orthologue HLD1

3

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22

23

24 **SUMMARY**

25 Glycosylphosphatidylinositol anchored proteins (GPI-APs) are tethered to the outer leaflet of
26 the plasma membrane where they function as key regulators of a plethora of biological
27 processes in eukaryotes. Self-incompatibility (SI) plays a pivotal role regulating fertilization in
28 higher plants through recognition and rejection of 'self' pollen. Here we used *Arabidopsis*
29 *thaliana* lines engineered to be self-incompatible by expression of *Papaver rhoeas* SI
30 determinants for an SI suppressor screen. We identify *HLD1*/*AtPGAP1*, an ortholog of the
31 human GPI-inositol deacylase *PGAP1*, as a critical component required for the SI response.
32 Besides a delay in flowering time, no developmental defects were observed in *hld1*/*atpgap1*
33 knockout plants, but SI was completely abolished. We demonstrate that HLD1/AtPGAP1
34 functions as a GPI-inositol deacylase and that this GPI-remodeling activity is essential for SI.
35 Using GFP–SKU5 as a representative GPI-AP, we show that the *HLD1*/*AtPGAP1* mutation
36 does not affect GPI-AP production and targeting but affects their cleavage and release from
37 membranes *in vivo*. Our data not only implicate GPI-APs in SI, providing new directions to
38 investigate SI mechanisms, but also identify a key functional role for GPI-AP remodeling by
39 inositol deacylation *in planta*.

40

INTRODUCTION

Addition of a glycosylphosphatidylinositol (GPI) residue to proteins is a highly conserved post-translational modification that plays crucial roles in the biosynthesis and targeting of GPI-anchored proteins (GPI-APs) to the outer leaflet plasma membrane in eukaryotes [1,2]. Over 250 GPI-APs exist in *Arabidopsis thaliana* [3,4]. Several GPI-APs have been shown to be key regulators of cell signaling, growth, morphogenesis, reproduction and pathogenesis in yeast, mammals, and plants [5-7]. GPI-AP biosynthesis, remodeling, and export are well-characterized in yeast and animal cells [8,9]. The GPI anchor is synthesized and transferred to target proteins in the endoplasmic reticulum (ER) [1]. Knockouts of *Phosphatidylinositol glycan anchor biosynthesis* (*PIG*) genes in mouse and Arabidopsis demonstrate that GPI-anchor synthesis is essential for animal and plant viability, male fertility, early embryo and tissue development [6,10,11]. Once the GPI anchor is attached to a target protein, remodeling takes place. In animals, the first step involves the removal of an acyl chain on the GPI inositol, by post-GPI attachment to proteins 1 (PGAP1), a GPI-inositol deacylase [12]. After further processing, GPI-APs are transported to the outer leaflet of the plasma membrane [5,8], where they are retained or released by cleavage [13]. Although homologs of *PGAP1* have been identified in plant genomes [6,14,15], there are no functional data about the biological *in planta* impact of this GPI remodeling and maturation step in plants.

Self-incompatibility (SI) regulates the rejection of 'self' pollen, preventing inbreeding and promoting genetic diversity. Generally, SI is genetically controlled by a polymorphic multi-allelic *S*-locus, with each *S*-haplotype encoding a pair of *S*-determinants [16]. Pistils discriminate between "self" and "non-self" pollen using allele-specific interactions between these two *S*-determinants. In field poppy (*Papaver rhoeas*), the female *S*-determinant PrsS is specifically expressed in the stigma [17], whereas the male *S*-determinant PrpS is specifically expressed in pollen [18]. Briefly, PrpS is a small, novel transmembrane protein [18] and PrsS is a small secreted protein of the cysteine-rich peptide (CRP) family of proteins that include LUREs and rapid alkalinization factors (RALFs) [19]. Cognate PrpS-PrsS interaction triggers a signaling network resulting in pollen tube growth arrest and programmed cell death (PCD) of 'self'-pollen [20]. Recently, we showed that cognate *PrpS* and *PrsS* co-expressed in the self-compatible model plant Arabidopsis rendered Arabidopsis effectively self-incompatible (designated as *At*-SI lines), with no self-seed set, demonstrating that *Papaver S*-determinants are sufficient to confer functional SI in Arabidopsis [21,22].

75 Here we used the Arabidopsis *At*-SI lines as the basis for a forward genetic mutant screen to
76 identify new genes involved in controlling *Papaver* SI. We identified 12 mutant alleles of
77 *highlander1* (*hld1*) exhibiting suppressed SI. We mapped all *hld1* alleles to *AtPGAP1*, a
78 functional ortholog of the mammalian *PGAP1* gene encoding a protein involved in the
79 deacylation of GPI-APs [15]. Our study of *hld1*/*atpgap1* plants and the AtPGAP1 mutation
80 reveals a crucial role of GPI-APs and GPI-remodeling of GPI-APs by AtPGAP1 in the *Papaver*
81 SI response.
82
83 **RESULTS**
84 **Identification of SI-defective *highlander1* mutants**
85 To discover novel players of the *Papaver* SI process, we performed an ethyl methanesulfonate-
86 (EMS-)based forward genetic screen on ~50,000 *At*-SI M1 individuals for suppressors of SI
87 (**Figure S1A, STAR Methods**). Initially, we identified 40 mutants showing wild-type siliques.
88 After excluding mutants with mutations in the *PrpS*$_1$/*PrsS*$_1$ transgenes, or changes in PrpS$_1$–
89 GFP expression, we confirmed in total twelve independent self-compatible SI-repressor
90 mutants with restored normal self-seed set (**Figure 1A; Figure S1B-C**) and named them
91 *highlander1* (*hld1-3/5/6/7/17/18/19/24/25/30/37/39*), after the immortal warrior in the 1980s
92 film of the same name. Pollinating *hld1* pollen onto *At*-SI stigmas resulted in significantly
93 longer siliques (**Figure 1B,** $p<0.001$) and higher seed set (**Figure S1D,** $p<0.001$) than those
94 resulted from the *At*-SI parent line self-pollination. This loss of SI was not observed in the
95 reciprocal cross (**Figure 1B**, p=0.1458, N.S.; **Figure S1D,** p=0.0909, N.S.). Furthermore, after
96 the first backcross (BC) to the *At*-SI parent line, all the heterozygous *hld1* BC1 generation plants
97 were self-compatible (**Table S1**, n= 167). This demonstrates that mutation in the *hld1* mutants
98 affect the male gametophyte (pollen) and that the 50% of the pollen grains carrying the *hld1*
99 mutant alleles are sufficient for full seed set.
100
101 **Whole Genome Sequencing (WGS) identified *AtPGAP1* as the causal gene for all the *hld1***
102 **mutant alleles**
103 To reveal the molecular identity of the *hld1* mutants, we re-sequenced the genome of the self-
104 compatible population of six independent mutants after two backcrosses. SHOREmap analysis
105 [23] revealed six different nonsynonymous mutations in a single gene, *At3g27325* (recently
106 described as *AtPGAP1* by [15]; **Figure S2A; Table S2**). Analysis of the segregating populations
107 revealed 100% linkage between the *AtPGAP1* mutation and suppression of SI (**Table S3**,
108 n=298). Genotyping of the remaining *hld1* mutants identified six additional independent mutant

109 alleles of the same gene (**Table S2**), suggesting that the screen had a high level of saturation
110 and that *AtPGAP1* was the single causal locus for SI suppression in all the mutants identified.
111 The identification of a single causal locus in our suppressor screen was surprising and suggests
112 that other genes involved in SI might either act redundantly, or play important roles for plant
113 viability or fertility.
114
115 **CRISPR-Cas9 confirms that *AtPGAP1* is the causal gene for the *hld1* phenotype**
116 To confirm *AtPGAP1* as the causal gene for SI suppression, we generated two independent
117 CRISPR-Cas9 knockout mutants (*atpgap1-c1* and *atpgap1-c2,* **Figure S2B**) using Arabidopsis
118 $NTP303_{pro}:PrpS_1$–*GFP* (At-$PrpS_1$) as the background. Pollinating *At*-SI stigmas with At-$PrpS_1$
119 pollen resulted in a SI phenotype with no seed set. However, *At*-SI stigmas pollinated with *At*-
120 $PrpS_1$/*atpgap1-c1* or At-$PrpS_1$/*atpgap1-c2* pollen displayed normal siliques and seed set
121 (**Figure 1C, Figure S2C**). This demonstrates that targeted mutation of *AtPGAP1* is sufficient
122 to prevent SI. Consistent with this, the F1 population of an ♀*At*-SI x ♂At-$PrpS_1$/$atpgap1^{+/-}$ cross
123 comprised 99.5% $atpgap1^{+/-}$ mutant plants (n=358; **Figure 1D**), showing that in heterozygous
124 mutants it is almost exclusively *atpgap1* mutant pollen grains, but not their wild-type siblings,
125 that overcome SI. When introgressed into the *At*-SI background line, *atpgap1-c1* and *atpgap1-*
126 *c2* were both sufficient to cause breakdown of SI, comparable to the EMS mutagenesis-derived
127 *hld1* alleles (**Figure S2D, E**). CRISPR-Cas9-generated mutations of *AtPGAP1* phenocopy the
128 EMS-generated *hld1* mutant phenotype, confirming that *AtPGAP1* is the causal gene for *hld1*-
129 mediated SI suppression.
130
131 **PGAP1 is also required for pollen SI in *Papaver***
132 To test whether PGAP1 also functions in the original *Papaver*-SI context, we investigated if
133 the *Papaver rhoeas PGAP1* ortholog *PrPGAP1* is needed for an authentic *Papaver* pollen SI
134 response. As a transgenic approach with this species is not possible, we used an antisense
135 oligonucleotide approach [18,24]. Treatment of *P. rhoeas* pollen with *PrPGAP1* antisense
136 oligonucleotides significantly alleviated SI-induced pollen tube growth inhibition (**Figure 1E**;
137 p<0.0001) and pollen death from >85% to ~45% (**Figure 1F**; p<0.0001), whereas no significant
138 effect was observed when treated with the sense oligonucleotide (**Figure 1E**, p=0.9216; **Figure**
139 **1F**, p=0.3277). This demonstrates that PGAP1 does not just work in the transgenic Arabidopsis
140 *At*-SI context, but that it is required for SI-induced pollen tube growth inhibition and cell death
141 in *Papaver*.
142

143 ***AtPGAP1* regulates SI irrespective of the *S*-alleles involved**

144 Prevention of self-pollination by SI relies on a polymorphic *S*-locus involving *S*-specific self-
145 recognition. Although *AtPGAP1* regulated the $PrpS_1$-$PrsS_1$-based SI response, it was not clear
146 if *AtPGAP1* could also regulate the SI-response involving other cognate *S*-alleles. We therefore
147 examined if the *AtPGAP1* mutation disrupted SI in plants expressing an alternative pair of
148 cognate *S*-alleles, $PrpS_3$ and $PrsS_3$. Pollinating stigmas expressing the $PrsS_3$ (*At*-$PrsS_3$) with *At*-
149 $PrpS_3$ pollen resulted in SI, with a significant reduction in silique length and almost no seed,
150 compared with the control pollination ♀*At*-$PrsS_3$ x ♂Col-0 (**Figure 2A, B**). In contrast,
151 pollinating *At*-$PrsS_3$ with *At*-$PrpS_3$/*atpgap1* pollen resulted in normal silique lengths and seed
152 set (**Figure 2A, B**), showing that mutation of the *AtPGAP1* gene abolishes the $PrpS_3$-$PrsS_3$-
153 based SI. These results demonstrate that *AtPGAP1* regulates SI irrespective of the *S*-alleles
154 involved.

155

156 **AtPGAP1 regulates ectopic "SI-like PCD" in Arabidopsis roots**

157 We recently established that expression of $PrpS_1$-$PrsS_1$ in Arabidopsis roots triggers an ectopic
158 "SI-like PCD" response in vegetative tissues, resulting in root growth inhibition and widespread
159 cell death [25]. We examined if *AtPGAP1* is necessary for this ectopic "SI-like PCD" response
160 outside the reproductive context. While addition of recombinant $PrsS_1$ proteins to
161 $UBQ10_{pro}$:$PrpS_1$ seedlings resulted in root growth arrest and cell death [25], treatment of
162 $UBQ10_{pro}$:$PrpS_1$/*atpgap1* seedling roots with $PrsS_1$ showed no such effect (**Figure 2C-E**). This
163 suppression of root growth inhibition and root cell death reveals that *AtPGAP1* is not only
164 required for SI, but also for the ectopic "SI-like PCD" response independent of the reproductive
165 context.

166

167 ***AtPGAP1* is a functional *HsPGAP1* ortholog**

168 As *At3g27325* had not been annotated as a PGAP1 ortholog at the time when we mapped the
169 *hld1* mutations to this locus, we investigated its possible orthology to genes in animals and
170 fungi. Protein sequence analysis revealed that At3g27325 contains a PGAP1-like domain
171 (PFAM domain ID: PF07819), and is a putative PGAP1 homolog [6,14]. *PGAP1* has been
172 identified in mammals and its ortholog, *Bypass of Sec Thirteen 1* (*Bst1*) in yeast; it encodes a
173 GPI inositol-deacylase [12]. We constructed a phylogenetic tree of 631 predicted PGAP1 protein
174 homologs from >300 eukaryotic species (**Figure 3A, B**). Two major phylogenetic clades were
175 identified for plants; in Arabidopsis, AtPGAP1 (At3g27325) and another PGAP1-like domain
176 containing protein, At5g17670 were classified into different clades (**Figure 3A**). The

177 divergence of these two clades can be traced back to an ancient whole genome duplication event
178 occurring before the radiation of extant Viridiplantae (**Figure 3A, B**) [26]. Comparison of the two
179 homologs, AtPGAP1 and At5g17670 from *A. thaliana* with the *Homo sapiens* PGAP1, revealed
180 that unlike AtPGAP1, which has a similar predicted secondary structure and a conserved ER
181 localization [15] as HsPGAP1, At5g17670 is much smaller in size (**Figure S3A**), and predicted
182 to be a chloroplast-located protein (https://www.uniprot.org/). This suggests that AtPGAP1 is
183 a homolog of HsPGAP1 [14,15], while At5g17670 PGAP1 is not.
184

185 **AtPGAP1 functions as a GPI-inositol deacylase**

186 Mammalian PGAP1 is a GPI-inositol deacylase that removes the inositol acyl chain after the
187 attachment of the GPI anchor to its target protein [12]. HsPGAP1 is ubiquitously expressed in
188 humans [27]; we found AtPGAP1 expression in all the Arabidopsis tissues examined (**Figure**
189 **S3B-D**) [28]. A key feature of PGAP1 is a catalytic serine-containing motif, V***GHSMGG, in
190 the PGAP1-like domain that is highly conserved across the eukaryotic kingdoms, and also
191 present around serine 218 in AtPGAP1 (**Figure 3C**). Mutation of *PGAP1* in mammals results
192 in a "three-footed" structure of GPI-APs that retain an extra acyl chain in addition to the two
193 membrane-anchored non-polar fatty acid tails [12]. This configuration makes GPI-APs in the
194 plasma membrane insensitive to cleavage by phosphoinositide-specific phospholipase C (PI-
195 PLC) and has been used to establish the GPI-inositol deacylase function of PGAP1 [12]. To
196 establish the biochemical function of Arabidopsis AtPGAP1, we examined if a representative
197 GPI-AP in the *atpgap1* mutant background was resistant to PI-PLC treatment. To this end, we
198 introduced the *atpgap1* mutant into a line expressing GFP-tagged SKU5, an established
199 Arabidopsis GPI-AP localizing to the plasma membrane and cell wall [29]. A PI-PLC assay
200 revealed that GFP–SKU5 was highly resistant to PI-PLC-mediated GPI cleavage in the *atpgap1*
201 mutant (**Figure 3D**). This result recapitulates analogous findings obtained in mammalian *pgap1*
202 knockouts [9,12,30], supporting the hypothesis that *GFP–SKU5/atpgap1* is "three-footed" and
203 insensitive to PI-PLC treatment [12]. In summary, AtPGAP1 is an inositol deacylase and a
204 functional ortholog of HsPGAP1, which independently confirms a recently published study [15].
205

206 **AtPGAP1 GPI-inositol deacylase function is required for SI**

207 To examine if AtPGAP1 inositol deacylase activity is required for the SI response, we mutated
208 the conserved serine 218 in the catalytic core of the PGAP1-like domain [12] (**Figure 3C**) to
209 alanine (S218A), and cloned it into the pFAST-Green plasmid vector [31]. As a control we cloned
210 the wild-type *AtPGAP1* cDNA (*cAtPGAP1*) into the same vector and transformed both

211 constructs into the *At*-SI/*hld1* background. In segregating T2 seeds from T1 heterozygous *At*-
212 SI/*hld1/cAtPGAP1* control plants, in contrast to normal Mendelian segregation, only ~50% of
213 the T2 seeds were GFP- (pFAST-)positive. This suggests that the *cAtPGAP1* wild-type
214 transgene was only transmitted *via* the female side, while its transmission *via* the male side was
215 blocked by the restored SI-phenotype (**Figure 3E; Table S4**). This was further verified by the
216 observation that the *At-SI/hld1/cAtPGAP1* transgene was barely transmitted to the F1 seeds
217 after pollination to the stigma of an *At-PrsS*$_1$ plant (**Figure 3E; Table S4**). This shows that the
218 wild-type cDNA of *AtPGAP1* complements the *hld1* mutant phenotype and restores SI. In
219 contrast, selfed heterozygous T1 plants *At*-SI/*hld1/cAtPGAP1(S218A)* expressing the S218A
220 mutant construct displayed normal Mendelian segregation, with ~75% GFP-positive T2 seeds
221 (**Figure 3E; Table S4**), indicating that pollen carrying the S218A mutant allele failed to restore
222 SI. Consistent with this observation, pollinating the *At-PrsS*$_1$ background line or Col-0 wild-
223 type plants with pollen from heterozygous *At*-SI/*hld1/cAtPGAP1(S218A)* plants resulted in a
224 full transmission rate, with ~50% of GFP-positive F1 seeds (**Figure 3E; Table S4**). Thus,
225 unlike the wild-type *AtPGAP1* that rescued the *hld1* phenotype and restored SI, the inositol
226 deacylase-defective S218A *AtPGAP1* allele did not. This demonstrates that the AtPGAP1
227 inositol deacylase activity is essential for SI, implicating a requirement for correctly remodeled
228 GPI-APs in this process.
229

230 **Plasma-membrane localized GPI-APs are required for SI**

231 To assess if a lack of GPI-APs at the plasma membrane had the same SI-suppressing effect as
232 faulty GPI-AP remodeling, we investigated established GPI-AP biosynthesis mutants in the SI
233 context. *SETH1* and *SETH2* (orthologues of *PIG-C* and *PIG-A*) are subunits of the GPI-N-
234 acetylglucosaminyltransferase complex that transfers N-acetylglucosamine to
235 phosphatidylinositol as the first step of GPI anchor biosynthesis [5]. The respective knockout
236 mutants are devoid of GPI-APs at the plasma membrane [5,30], and *seth1-2* and *seth2* mutant
237 alleles are homozygous lethal [10]. Pollen from heterozygous *At-PrpS*$_1$*/seth1-2*$^{+/-}$ and *At-*
238 *PrpS*$_1$*/seth2*$^{+/-}$ plants pollinated onto *At-PrsS*$_1$ stigmas produced short siliques without seeds, so
239 showing no SI-repression phenotype (**Figure 4A, B**). However, *seth1-2* and *seth2* are known
240 to almost completely abolish pollen germination and tube growth (**Figure 4C**) [10], so pollination
241 assays were not suitable to establish their involvement in SI. We therefore used an *in vitro* SI
242 bioassay[32] to examine the effect of these mutants on pollen viability. Addition of PrsS$_1$ to pollen
243 of *At-PrpS*$_1$*/seth1-2*$^{+/-}$ or *At-PrpS*$_1$*/seth2*$^{+/-}$ plants resulted in ~50% pollen grain death in contrast
244 to > 98% death in *At-PrpS*$_1$ pollen (**Figure 4D-F**), demonstrating that SI-induced cell death

does not occur in *SETH1* or *SETH2* knockouts. This demonstrates that the SI response depends on at least two processes in the GPI-anchoring pathway: the first step of GPI anchor synthesis facilitated by SETH1/2, and the later GPI remodeling by the inositol deacylase, AtPGAP1, suggesting the presence of GPI-APs at the plasma membrane being indispensable for SI-PCD.

GFP–SKU5 targeting to the plasma membrane is not affected by lack of *PGAP1*

We next asked the question whether non-remodeled GPI-APs in *atpgap1* mutants were actually still correctly targeted to the plasma membrane. In mammals and yeast, GPI-inositol deacylation by PGAP1 [12] is important for efficient sorting of GPI-APs to exit the ER [5,15,33]. However, knockout of *PGAP1* only delays, but does not prevent, transport of GPI-APs to the plasma membrane, so the functional significance of GPI deacylation remains unclear [9,34]. Similar to the effect of *PGAP1* knockout in animals [12,30], the *AtPGAP1* mutation had no obvious effect on overall GFP–SKU5 protein expression levels (**Figure S4A, B; Figure 5A, B**). Confocal imaging of GFP–SKU5 signals in 4-day-old GFP–SKU5/WT and GFP–SKU5/*atpgap1* seedling roots and quantification showed that GFP–SKU5 was targeted to the plasma membrane at similar levels in the *atpgap1* mutant (**Figure 5A, B**), suggesting that normal levels of this GPI-AP are targeted to the plasma membrane in the *atpgap1* mutant (**Figure 5B**). Nevertheless, there was a significantly increased proportion of intracellular GFP–SKU5 signal in the *atpgap1* mutant (****, $p<0.0001$, **Figure 5C**), consistent with delayed transport of GPI-APs observed in *pgap1* mutant mammalian and plant cells [9,15]. Importantly, our data show that GFP–SKU5 is still able to reach the plasma membrane, as has been reported for other GPI-APs in mammalian and plant *pgap1* mutants [9,15].

It is well established that lack of GPI-APs at the plasma membrane strongly affect plant development. Both *seth1* and *seth2* mutants are homozygous lethal, and the hypomorphic *gpi8-1* allele of AtGPI8, a PIG-K ortholog, exhibits reduced accumulation of GPI-APs and disturbed growth and stomata formation (**Figure 5D**) [35]. In contrast, mutation of AtPGAP1 had no major effect on vegetative plant development (**Figure 5D, E**). However, we observed that flowering time was delayed in *atpgap1* plants (**Figure S4D**), though less strongly than in *gpi8-1* [35]. While the absence of major developmental phenotypes in *atpgap1* plants is surprising, given observations in animals, they are in line with a recently published analysis of AtPGAP1 in Arabidopsis [15].

PrpS localization at the plasma membrane is not affected in *atpgap1* mutants

278 One reason why SI might be defective in *atpgap1* mutants could be that PrpS cannot localize
279 to the plasma membrane, as some GPI-APs have been shown to play a crucial role in the proper
280 localization of other plasma membrane proteins [36,37]. However, confocal imaging of PrpS–GFP
281 in wild-type and *atpgap1* mutant roots and pollen tubes showed that PrpS–GFP did reach the
282 plasma membrane (**Figure 5F, G**). Similar to PrpS immunolocalization reported in *Papaver*
283 pollen tubes [18], PrpS–GFP was localized to the endomembrane system as well as the plasma
284 membrane; its localization was similar in wild-type and *atpgap1* mutant pollen tubes (**Figure**
285 **5G**). This provides evidence that lack of PGAP1 does not perceptibly affect the localization of
286 PrpS, so is unlikely to be a reason for the failure of SI.
287
288 **Inositol deacylation by AtPGAP1 is required for release of GFP–SKU5**
289 Besides their localization in the outer leaflet of the plasma membrane, many GPI-APs in
290 mammals are cleaved and released into the extracellular space; this can be mediated by PLC
291 activity and is important for many cellular processes in mammals, including adhesion,
292 proliferation, survival and oncogenesis [9,13]. We therefore examined a role for AtPGAP1
293 mediated GPI-AP remodeling *in planta*. In wild-type Arabidopsis, we detected GFP–SKU5 in
294 both membrane and soluble fractions (**Figure 6A**); this is consistent with a report that SKU5
295 can be cleaved and released into the apoplast [29]. In contrast, in the *atpgap1* mutant, GFP–SKU5
296 was exclusively detected in the membrane fraction (**Figure 6A**). Moreover, using Triton X-
297 114, GFP–SKU5 was detected primarily in the aqueous phase in wild type, whereas most GFP–
298 SKU5 was present in the detergent phase in the *atpgap1* mutant (**Figure 6B**). Thus, in the
299 absence of AtPGAP1, GFP–SKU5 was more hydrophobic and was retained in the membrane.
300 This provides evidence that cleavage and release of GFP–SKU5 (and likely other GPI-APs)
301 from the membrane requires GPI inositol deacylation by AtPGAP1 *in vivo*.
302
303 **AtPGAP1 regulates SI possibly by affecting PLC-mediated release of GPI-APs**
304 As cleavage of membrane-bound GPI-APs is prevented by the "three-footed" configuration in
305 mammalian and yeast *PGAP1* mutants [9,12,30], it is conceivable that the prevention of SI by the
306 *AtPGAP1* mutation is caused by failure to cleave GPI-APs at the plasma membrane.
307 Mammalian *pgap1* cell lines lacking a GPI inositol deacylase have GPI-APs that are resistant
308 to PLC-mediated cleavage *in vitro* [12]. Although extracellular PI-PLCs were proposed by
309 mammalian and plant researchers to allow cleavage of GPI-APs at the plasma membrane [38-40],
310 the actual PLCs have not yet been identified. However, in Trypanosomes, a GPI-PLC at the
311 external face of the plasma membrane has been identified as responsible for cleavage of the

GPI-anchor of a variable surface glycoprotein [41]. We therefore investigated whether PLC inhibitors might prevent SI, using the *in vitro* SI-bioassay. We could not test their effect on SI-induced pollen tube growth inhibition as pollen tube growth requires PLC activity [42,43], so we examined if they prevented SI-induced death of pollen. *At-PrpS$_1$* pollen pretreated with U73122 had significantly alleviated SI-induced cell death (a reduction of ~40% at 10 μM, $p<0.001$; and ~63% at 25 μM, $p<0.0001$; **Figure 6C**). Pretreatment with ET-18-OCH3 and C48/80 also showed similar alleviation of SI-induced death of incompatible pollen (**Figure S5**). The reduction in death by PLC inhibitors shows that a PLC/PLC-like activity is required for SI-induced pollen death, suggesting a possible role for cleavage of GPI-APs by PLC/PLC-like enzyme during SI. As SI is prevented by the "three-footed" GPI-AP configuration resulting from the *AtPGAP1* mutation, our evidence is not only consistent with the idea that GPI-APs play a key role in SI, but that the prevention of their release from the plasma membrane is likely to be responsible for the breakdown of SI in the *atpgap1* mutant plants.

DISCUSSION

Over 250 GPI-APs have been predicted in Arabidopsis [4,44]; despite recent advances in our knowledge of GPI-APs in plants, information on their functional roles remains limited. Here we identified a plant *PGAP1* orthologue as essential for the *Papaver* SI-response. Although GPI-APs are involved in many important developmental processes in eukaryotes [31,35,45,46], the functional role of GPI remodeling at a whole-organism phenotypic level remains unclear. Our findings reveal that prevention of GPI-AP deacylation of restores self-fertility in an SI line. This points to a pivotal role for correctly remodeled GPI-anchored proteins in incompatible pollen recognition and/or rejection in *Papaver* SI. These data provide an important demonstration of the functional importance of GPI-remodeling and inositol deacylation *in planta*. Our findings open up new avenues to explore the role of GPI-AP modifications in plant cells. While the specific GPI-APs involved and the mechanistic consequences of their remodeling remain to be elucidated, our characterization of the impact of loss of this GPI-remodeling factor on SI provides valuable insights into the roles of the plant GPI pathway and raises several interesting questions.

Key differences and similarities between PGAP1 function in plants and animals

In animals, little is known about the physiological functions of PGAP1 apart from its biochemical role in deacylation in the GPI-anchoring pathway [12] and the pathological consequences of PGAP1 mutations [30,34,45,46]. *PGAP1* knock-out mice display serious

346 developmental defects [34] and humans with a null mutation in *PGAP1* suffer from intellectual
347 disabilities and encephalopathy [30,34,45,46]. Thus, retention of an extra acyl chain in GPI anchors
348 causes severe developmental defects in mammals. A recent report identified a *PGAP1*
349 orthologue (*AtPGAP1/At3g27325)* in *A. thaliana* [15] and provided evidence that it functions as
350 a GPI inositol-deacylase in plant cells, though it did not provide any data relating to biological
351 function in a whole-plant context. Both this study and ours show that, in contrast to mammalian
352 *PGAP1* mutants, Arabidopsis *hld1/atpgap1* mutant plants display largely normal development
353 [15]. Why there is this major difference between plants and mammals is an interesting question
354 to be addressed in the future. The various *hld1/atpgap1* mutant alleles isolated from our SI
355 suppressor screen provide a valuable resource to investigate this question.
356
357 We found that in our *atgap1* knockout plants, the GPI-AP reporter GFP–SKU5 was present at
358 the plasma membrane at levels comparable to the wild type. This is similar to the situation in
359 animal *pgap1* knockout cells where, although transport of GPI-APs was delayed, plasma
360 membrane levels of GPI-APs were not significantly decreased [9,12]. Although it was recently
361 reported that GPI-APs accumulated in the ER in an *atpgap1* knockout [15], this ER retention was
362 less extreme in our study. This difference is likely to be due to the use of the strong 35S
363 promoter, as over-expression will exacerbate ER retention, which in turn may explain the strong
364 ER-localized signal observed in the earlier study [15]. Our approach, using stable transformants
365 with a native promoter is likely to be nearer to the endogenous expression levels. Nevertheless,
366 both studies show that GPI-APs can successfully reach the plasma membrane in the absence of
367 *AtPGAP1* function.
368

369 Loss of AtPGAP1 does not cause a deficiency of GPI-APs or PrpS at the plasma 370 membrane

371 Our identification of *AtPGAP1* implicates involvement of GPI-APs in the SI response. Many
372 GPI-APs, for example, the widely studied GPI-AP LORELEI (LRE) and LORELEI-LIKE GPI-
373 APs (LLGs) LLG1/2/3, play central roles in plant growth and development [47,6] and their
374 presence at the plasma membrane is critical for their function in regulating normal development.
375 The absence of a severe developmental phenotype in the *atpgap1* mutants suggests that
376 critically important GPI-APs are largely correctly expressed, localized, and functional in the
377 absence of AtPGAP1. Thus, loss of PGAP1 function does not affect the targeting of crucial
378 GPI-APs required for normal development. This provides strong evidence that GPI-AP
379 targeting to the plasma membrane is not likely to be the defect underlying SI breakdown in

380 *atpgap1* mutants (**Figure 6D**). Another theoretical possibility is that the lack of GPI-APs affect
381 PrpS localization, as it is well established in both animal and plants that some GPI-APs act as
382 chaperones and can play a crucial role in the correct localization of plasma membrane proteins
383 [36,37]. For example, in Arabidopsis, the absence of the GPI-AP LLG1 affects plasma membrane
384 localization of the Receptor-like Kinases (RLKs) FERONIA (FER) and FLAGELLIN-
385 SENSING 2 (FLS2) [36,37]. Although PrpS is a pollen plasma membrane-localized protein, it is
386 clearly not a GPI-AP, so cannot be a substrate of AtPGAP1 [48]. However, a possible explanation
387 of why SI is defective is that PrpS is not localized to the plasma membrane in the *atpgap1*
388 mutants because a crucial GPI-AP that acts as a chaperone is missing. As our data show that
389 PrpS–GFP reaches the plasma membrane and has a very similar localization in wild-type and
390 *atpgap1* mutant pollen tubes and root cells, the loss of the SI phenotype is unlikely to be due to
391 the incorrect localization of PrpS. Thus, it seems that neither GPI-AP nor PrpS localization is
392 defective in the *atpgap1* mutant plants (**Figure 6D**).
393
394 **Implication of a potential role for GPI-APs in cell-cell signaling in *Papaver* SI**
395 GPI-APs also play a key role in cell-cell signaling as co-receptors, including enhancing
396 receptor-ligand interactions through association with partner receptor-like kinases (RLKs) and
397 their ligands [36,37]. Malectin-like RLKs (*Cr*RLK1Ls) play critical roles in many interactions [49]
398 and their interaction with GPI-APs in regulating plant reproduction, including pollen tube
399 reception by the female gametophyte [47,50-52] has been intensely studied. For example LRE
400 functions as a co-receptor for the *Cr*RLK1L, FER [36], interacting with the ligand, RALF1.
401 Pollen-specific-expressed LLG2/3 interacts with FER homologs ANXUR1/2 (ANX1/2) and
402 Buddha's Paper Seal 1/2 (BUPS1/2), and responds to RALF4/19 peptide signals to regulate
403 pollen tube growth [53]. Thus, these RLKs and GPI-APs form a receptor-coreceptor complex to
404 perceive RALF peptide signals. While PrpS is not a GPI-AP or a RLK, it interacts with its *S*-
405 specific ligand, PrsS to mediate the SI signaling pathway [20]. Thus, its activity and interaction
406 with PrsS or other proteins at the extracellular face of the plasma membrane might be regulated
407 by GPI-APs (**Figure 6D**). Through comparison with what is known about well characterized
408 GPI-APs, our data here raise the possibility that GPI-APs may be involved in PrpS interaction
409 with its ligand PrsS.
410
411 As the ectopic "SI-like PCD" response in Arabidopsis roots [25] also requires AtPGAP1, it
412 appears that broadly expressed GPI-APs, rather than those specifically involved in
413 reproduction, are involved in SI. Interestingly, *SKU5*, which regulates directional root growth,

414 is a member of a gene family containing pollen-expressed *SKS11/12*, which control pollen tube
415 growth [54]. As well as playing a key role in cell-cell signaling during reproduction and
416 development, GPI-APs also participate in pathogen responses [37,47,55]. An important component
417 of innate immunity in plants is the interaction of RLKs with ligands to activate signaling to
418 defense mechanisms that restrict pathogen invasion [56]. For example, FLS2 is a leucine-rich
419 repeat RLK that recognizes Pathogen-Associated Molecular Patterns (PAMPs), e.g. flagellin
420 [57]. The GPI-AP LLG1 plays a key role in these interactions, by constitutively associating with
421 PAMP RLKs in the presence of flagellin. In this way, LLG1 plays an important role in plant
422 innate immunity, functioning as a key component of PAMP-recognition immune complexes,
423 modulating their ability to regulate disease responses [37]. As the LLG1 clearly participates in
424 several signaling pathways, this raises the possibility that some GPI-APs, receptors and
425 signaling ligands have some common evolutionary origin.
426
427 Although the genetic basis of the *Papaver* and *Brassica* SI systems are quite different, they both
428 utilize highly polymorphic plasma-membrane localized *S*-determinants (PrpS and SRK) that
429 interact with highly polymorphic ligands (PrsS and SCR/SP11), which results in the recognition
430 and rejection of "self" pollen. PrsS and SCR/SP11are cysteine-rich peptides, which are thought
431 to be distantly related to the defensins utilized in plant innate immunity. Like the plant innate
432 immune system, *Papaver* SI triggers a signaling pathway leading to PCD. While the
433 components involved differ, there are distinct broad similarities between SI and the plant
434 immune response [58,59], which raises the intriguing scenario of possible evolutionary parallels
435 between SI systems and plant innate immunity systems. This has been proposed several times
436 [60-63]. Our findings here, showing that GPI-APs play a critical role in regulating *Papaver* SI, hint
437 of further evidence for this functional diversification of key components involved in cell-cell
438 recognition and signaling pathways regulating growth/development and that innate immunity
439 in plants may also be utilized by these SI systems. Furthermore, as the Brassica SRK is a
440 receptor kinase, it raises the possibility that GPI-APs may potentially be also involved in
441 regulating this SI system. Thus, our study raises unanswered questions leading to several new
442 avenues of research to be explored in the future.
443

444 **How might PGAP1 work in *Papaver* SI?**

445 It has been proposed that the lack of GPI deacylation disturbs GPI anchor-mediated signal
446 transduction in animals [34]; here we show that prevention of deacylation of GPI-APs in the
447 *atpgap1* mutants affects SI, which utilizes signaling to reject incompatible pollen. Cleavage and

448 release of GPI-APs from the plasma membrane is prevented by the "three-footed" configuration
449 in mammalian *pgap1* mutants [9,12,30]; our data suggest that cleavage of GPI-APs may play an
450 important functional role in the SI context. Our observations on GFP–SKU5 suggest that GPI-
451 APs still reach the plasma membrane in *atpgap1* mutants but cannot be released into the
452 extracellular space owing to their "three-tailed" configuration. Furthermore, our data on
453 alleviation of SI by PLC inhibitors are consistent with a model in which prevention of SI by the
454 *AtPGAP1* mutation is caused by a failure to cleave GPI-APs at the plasma membrane (**Figure**
455 **6D**). How this cleavage affects GPI-APs and their function in plants will be an intriguing aspect
456 for future research. It is tempting to speculate that cleavage of GPI-APs by PLC-like enzymes
457 is required for PrpS-PrsS function in the SI process (**Figure 6D**). As such, our findings pave
458 the way for both the identification and characterization of particular GPI-APs as well as putative
459 PLC-like enzymes in plants involved in the SI response.
460
461 In conclusion, our study opens new avenues for research into the involvement and regulation
462 of GPI anchoring in SI, as well as the investigation of the functional implications of GPI-
463 remodeling in a plethora of GPI-APs in plant signaling processes in general.
464
465
466
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476
477 **Author Contributions**
478 Z.L., M.K.N., V.E.F-T., and M.B. designed the study. Z.L., F.X., and M.T. performed the
479 research and analyzed the data. Z.T. contributed to the PGAP1 phylogeny analysis. F.C. and
480 L.S. contributed to the analysis of WGS data. Z.L., V.E.F-T., M.K.N. and M.B. wrote the
481 manuscript with input from all the other authors.
482
483
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485
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487 of this paper self-identifies as an underrepresented ethnic minority in science.
488

489 **Main Figure Titles and Legends**
490
491 **Figure 1. Identification of *hld1* as a male gametophytic mutant that overcomes SI.**
492 (A) Mutants were screened for a defective SI phenotype, with normal siliques and seed set.
493 Upper panel: Inflorescences from *hld1-7* mutants had normal length siliques like Col-0 plants,
494 in contrast to *At*-SI. Lower panel: Fluorescence imaging shows that $PrpS_1$–GFP expression in
495 *hld1* mutant pollen is similar to expression in *At*-SI pollen.
496 (B) *Hld1* stigmas were pollinated with self-pollen (selfing) or *At*-SI pollen (♂*At*-SI x ♀), and
497 *At*-SI stigmas were pollinated with *hld1* pollen (♀*At*-SI x ♂). *At*-SI stigmas pollinated with *At*-
498 SI pollen were SI controls for these pollinations. Silique length measurements showed that the
499 *hld1* mutants used as the male parent had significantly longer silique lengths than the *At*-SI
500 parent line. *Hld1* mutants used as the female parent pollinated with *At*-SI pollen displayed a
501 normal SI phenotype of short silique lengths. N=9-11. One-way ANOVA. ***: $p<0.001$. NS:
502 not-significant, $p>0.05$.
503 (C) *At*-SI or Col-0 stigmas were pollinated with *At-PrpS₁* pollen containing the CRISPR-Cas9-
504 derived *AtPGAP1* mutant allele (*atpgap1-c1* or *atpgap1-c2*). Significant increases in silique
505 lengths were observed when *AtPGAP1* was mutated, in both heterozygous and homozygous
506 mutants. N=12-16. One-way ANOVA. ***: $p<0.001$. NS: not-significant, $p>0.05$.
507 (D) Segregation analysis of the F1 population of ♀*At*-SIx♂*At-PrpS₁/atpgap1⁺ᐟ⁻* showed that
508 ~100% of the progenies are *atpgap1* heterozygous mutants, demonstrating that when pollinated
509 onto *At*-SI stigmas, only *atpgap1⁻* mutant pollen can bypass the SI response, resulting in
510 successful fertilization and seed set.
511 (E, F) *Papaver rhoeas* pollen was treated with an antisense oligonucleotide designed against
512 *PrPGAP1* (*PrPGAP1-AS*), which alleviated SI-induced pollen tube growth inhibition (E;
513 Results = median with interquartile range; n = the number of pollen tubes measured; one-way
514 ANOVA) and death (F; Results = mean ± SD; n = 5 biological replicates, with ~100 pollen
515 tubes counted for each sample in each replicate; two-way ANOVA.); the sense oligonucleotide
516 (*PrPGAP1-S*) had no significant effect. ****: $p<0.0001$. NS: not-significant, $p>0.05$. PrGM
517 acted as a negative control, and ~no pollen tube death was observed.
518 See also **Figure S1** and **S2**; **Table S1, S2** and **S3**.
519
520 **Figure 2. *AtPGAP1* regulates SI in an *S*-specific manner and regulates ectopic "SI" in**
521 **roots.**
522 (A-B) *At-PrpS₃* pollen containing *atpgap1-c1, atpgap1-c2, or hld1-7* mutant allele was
523 pollinated onto *At-PrsS₃* or Col-0 stigmas. Silique lengths (A) and seed set (B) were measured
524 and showed that *S₃*-specific inhibition was prevented in the mutant lines. N=10-20. One-way
525 ANOVA. ***: $p<0.001$.
526 (C) Treatment of *UBQ10pro:PrpS₁* seedlings with $PrsS_1$ resulted in root growth inhibition; in
527 contrast, when *UBQ10pro:PrpS₁/atpgap1* seedlings were given the same treatment, no inhibition
528 was observed (upper panel). Mock treatment did not affect any of the seedling roots (lower
529 panel). White lines indicate the position of root tips when treated. Bar = 0.5 cm.
530 (D) Quantification of the increases in root length 24h after treatment; 12-20 seedlings from
531 three independent experiments were measured for each treatment. Results = mean ± SD. One-
532 way ANOVA with multiple comparison test. Different letters indicate $p<0.05$.
533 (E) Treatment of *UBQ10pro:PrpS₁* seedlings with $PrsS_1$ resulted in root cell death indicated by
534 propidium iodide (PI) signals (white); when *UBQ10pro:PrpS₁/atpgap1* seedlings were treated
535 with $PrsS_1$ protein, minimal cell death was observed, comparable with what was observed in
536 Col-0 seedlings. Bar = 100 μm.
537
538 **Figure 3. AtPGAP1 is a GPI inositol deacylase and this function is required for SI.**

539 (A, B) A phylogenetic tree of 631 predicted PGAP1 protein homologues from >300 eukaryotic
540 species identified two major phylogenetic clades in plants; Two putative Arabidopsis homologs
541 AtPGAP1 and At5g17670 classified into different clades (red triangles). Star indicates a
542 genome duplication event. Detailed information of the 631 PGAP1-like domain-containing
543 proteins used for this phylogeny tree is contained in **Table S6**.
544 (C) Upper panel: cartoon of AtPGAP1 protein secondary structure. The PGAP1-like domain is
545 indicated by the grey box. Black boxes indicate transmembrane domains. Middle panel: amino
546 acid sequence alignment around the conserved lipase motif in the PGAP1-like domain in
547 several higher plants, human and yeast. The grey box indicates the lipase motif with the
548 catalytic site Serine 218 indicated (star). Bottom panel: amino acid motif logo shows that the
549 lipase motif of the PGAP1-like domain is conserved across eukaryotic kingdoms.
550 (D) PI-PLC assays used pelleted membrane proteins (P) separated from soluble (S) proteins
551 after centrifugation. GFP–SKU5 (~120 kDa) was enriched in the pellet (P) fractions in both
552 wild type (WT) and *atpgap1* mutant in the mock (buffer control) treatment. After PI-PLC
553 treatment, GFP–SKU5 was present in the soluble (S) fraction in WT samples, demonstrating
554 cleavage by PI-PLC, while GFP–SKU5 from the *atpgap1* mutants was found in the pellet,
555 demonstrating that in the *atpgap1* mutant this GPI-AP was resistant to PI-PLC treatment. This
556 supports the idea that in the *atpgap1* mutant a persistent inositol-linked acyl chain makes GPI-
557 APs insensitive to cleavage by PI-PLC. Aquaporin (~26 kDa) is shown as a control for
558 membrane extraction; loading control is stain-free signals of PROTEAN TGX Stain-Free
559 Precast gels (Bio-Rad).
560 (E) *Hld1* mutants were transformed with *AtPGAP1$_{pro}$:mCherry–cAtPGAP1* or
561 *AtPGAP1$_{pro}$:mCherry–cAtPGAP1(S218A)* cloned in the pFAST-Green plasmid vector
562 backbone, which expresses GFP in the seeds. So GFP signals report transgene transmission in
563 the seeds in the F1 generation. Representative images of segregating seeds resulted from
564 pollinations by pollen from T1 heterozygous plants onto self-stigmas (upper panel), *At-PrsS$_1$*
565 stigmas (middle panel) or Col-0 stigmas (lower panel). Numbers indicate the ratio of GFP-
566 positive seeds (number of GFP seeds/total seeds). Bar = 5 mm. Unlike the wild-type HLD1 that
567 rescued the *hld1* phenotype and restored SI, the inositol deacylase-defective S218A HLD1
568 allele did not, therefore resulting in normal Mendelian segregation of GFP signals in the T2
569 seeds.
570 See also **Figure S3**, **Table S4** and **S6**.
571
572 **Figure 4. Evidence for involvement of the GPI-anchoring pathway in the regulation of SI-**
573 **induced death of pollen.**
574 (A-C) GPI-anchoring pathway mutants, *seth1-2* and *seth2*, were introduced into *At-PrpS$_1$*
575 background and pollen from these plants was pollinated onto *At-PrsS$_1$* or Col-0 stigmas.
576 Pollinating *At-PrsS$_1$* stigmas with *At-PrpS$_1$/seth1$^{+/-}$* or *At-PrpS$_1$/seth2$^{+/-}$* pollen resulted in short
577 siliques (A) and no seed set (B). N=13-17. (C) Genotyping the F1 seedlings of ♀Col-0 x ♂*At-*
578 *PrpS$_1$/seth1$^{+/-}$* and ♀Col-0 x ♂*At-PrpS$_1$/seth2$^{+/-}$* revealed that mutation of *seth1* or *seth2*
579 abolished pollen fertilization.
580 (D-F) The *seth1-2* and *seth2* mutations were introduced into *At-PrpS$_1$* background, and pollen
581 from these plants was treated with *At*-GM buffer (D) or PrsS$_1$ proteins (E). Samples were co-
582 stained with FDA and PI 6h after treatment. Quantification of PI positive ratios (F) showed that
583 all pollen samples were ~fully viable after treatment with *At*-GM, but after treatment with PrsS$_1$
584 proteins, in contrast to the ~95% death induced in *At-PrpS$_1$* pollen, only minimal (~5%) pollen
585 death was observed for Col-0 or *At-PrpS$_1$/atpgap1-c1* pollen. Bar = 100 μm. Three biological
586 replicates were carried out, with 100-200 pollen grains counted for each sample in each
587 replicate.
588

589 **Figure 5. Mutation of AtPGAP1 does not affect the targeting of GFP–SKU5 and PrpS$_1$ to**
590 **the plasma membrane, and has no major effect on plant development.**
591 (A) Representative confocal images of GFP–SKU5 signals of 4-day-old *GFP–SKU5/WT* or
592 *GFP–SKU5/atpgap1-c1* seedling root meristem epidermis. GFP–SKU5 signals were observed
593 at the plasma membrane (PM) and endoplasmic reticulum (ER).
594 (B) Quantification of GFP fluorescence at the PM showed no significant difference between
595 WT and the *atpgap1* mutant.
596 (C) A significant increase (****, p<0.0001, student's t-test) in the ER/PM GFP signal ratio was
597 observed in the *atpgap1* mutant.
598 (D, E) The *gpi8-1* mutant was established to have reduced accumulation of GPI-APs; this
599 affects both stomata formation, and plant growth [35]. This *gpi8-1* was employed as a negative
600 control here. (D) Cotyledon epidermis of 5-day-old seedlings was labelled with FM4-64. The
601 *gpi8-1* mutation resulted in has clusters of stomata, which were not observed in Col-0, nor the
602 *atpgap1/hld1* mutants. (E) The *gpi8-1* mutation resulted in a significant delay of plant
603 development which was not observed in Col-0, nor the *atpgap1/hld1* mutants.
604 (F, G) Representative confocal images of PrpS$_1$–GFP signals of seedling epidermis (F) and
605 pollen tubes (G) from WT or *atpgap1-c1* plants. The right hand box outlined in yellow shows
606 a zoomed image of a part of the region of the pollen tube including the plasma membrane
607 indicated in the left hand image. No observable difference of the PrpS$_1$–GFP signals could be
608 seen in the WT and *atpgap1*. White bar = 10 μm; yellow bar = 1 μm.
609 See also **Figure S4**.
610
611 **Figure 6. Evidence and a model for a requirement of GPI-APs at the plasma membrane**
612 **for SI.**
613 (A) Membrane (M) and soluble (S) fractions from WT and *atpgap1* (*atpgap1-c1)* plants were
614 extracted and localization of GFP–SKU5 (~120 kDa) proteins were analyzed on western blots.
615 In the WT, GFP–SKU5 was detected in both fractions, but in the *atpgap1* mutant, GFP–SKU5
616 was only detected in the membrane fraction, consistent with defective cleavage. Aquaporin
617 (~26 kDa) is shown as a control for membrane extraction; loading control shows signals from
618 a PROTEAN TGX Stain-Free Precast gel (Bio-Rad).
619 (B) Whole proteins were extracted using 2% Triton X-114 extraction buffer, partitioned into
620 aqueous (A) and detergent (D) phases. Western blot analysis showed that GFP–SKU5 (~120
621 kDa) was primarily enriched in the aqueous phase in the WT, but in the *atpgap1* mutant it was
622 primarily detected in the detergent phase, revealing that mutation of AtPGAP1 resulted in an
623 increase of GFP–SKU5 hydrophobicity. Aquaporin (~26 kDa) is shown as a control for
624 membrane extraction; loading control shows signals from a PROTEAN TGX Stain-Free Precast
625 gel (Bio-Rad).
626 (C) Col-0 and *At-PrpS$_1$* pollen grains were pretreated with the PLC inhibitor, U73122, or
627 solvent (Mock) before subject to SI induction (PrsS$_1$) or control treatment (AtGM) *in vitro* and
628 samples were co-stained with FDA and PI 6h after treatment. Four independent experiments
629 were carried out; 100-200 pollen grains were counted for each treatment. *At-PrpS$_1$* pollen
630 treated with recombinant PrsS$_1$ and mock solvent had high levels of death; treatment of At-
631 PrpS1 pollen with recombinant PrsS1 and U73122 resulted in significant lower levels of death.
632 This suggests that cleavage of GPI-APs by PLC is required for mediating SI-induced death of
633 pollen.
634 (D) GPI-anchoring is a post-translational modification involving several phases which have
635 been established in mammals and yeast. First, biosynthesis of the GPI involves a series of events
636 [steps 1-10]. Once the core GPI is assembled [step 10], the target precursor protein (blue sphere)
637 is transferred to the GPI by a GPI transamidase complex [step 11]. The nascent immature GPI-
638 AP then undergoes remodeling; the first of these is deacylation [step 12], involving PGAP1,

639 which we identified in this study (indicated in pink). After this, several other PGAP genes (not
640 shown) mediate further modification during the transport from ER to the Golgi and then to the
641 plasma membrane. The genes involved in GPI biosynthesis identified in humans are indicated
642 in black; genes identified in plants are indicated in green. Knockouts of genes early in the
643 biosynthetic pathway in mammals result in lack of expression of GPI-APs at the plasma
644 membrane; in plants homozygous knockouts of the orthologs of *PIG-C* and *PIG-A, SETH1* and
645 *SETH2*, are lethal. We show in this study that mutation of *SETH1* or *SETH2* in *At-PrpS$_1$* pollen
646 alleviates SI-induced pollen death. By analogy to animal systems, it is likely that the *seth1/2*
647 mutants lack GPI-APs at the plasma membrane (grey outline). Our data implicate GPI-APs at
648 the plasma membrane are required for the SI response, as the normal SI-PCD response is
649 prevented in their absence. After generation of the nascent GPI-APs, the acyl chain linked to
650 inositol is removed by the GPI-deacylase, PGAP1 [step 12]. In the *atpgap1/hld1* mutant, this
651 step does not occur. As a consequence, the GPI-APs retain their inositol-linked acyl chain
652 (indicated in pink), giving a 3-footed configuration responsible for retaining the GPI-AP in the
653 plasma membrane in mammalian cells. Here we provide evidence that this is also the case in
654 plants. We show that knockout of PGAP1 completely prevents SI, resulting in full fertility. This
655 demonstrates that inositol deacylation, required for maturation of GPI-APs, is critical for the SI
656 response. We propose that during an incompatible SI induction when the ligand PrsS interacts
657 with plasma-membrane localized PrpS, this *S*-specific interaction may require interaction
658 (direct or indirect) with (unknown) GPI-APs. *Cartoon adapted from* [9].
659 See also **Figure S5**.
660
661
662 **STAR METHODS**
663
664 **RESOURCE AVAILABILITY**
665 **Lead contact**
666 Further information and requests for resources and reagents should be directed to and will be
667 fulfilled by the lead contact, Moritz Nowack (Moritz.Nowack@psb.vib-ugent.be).
668
669 **Materials availability**
670 All unique/stable reagents generated in this study are available from the Lead Contact with a
671 completed Materials Transfer Agreement.
672
673 **Data and code availability**
674 • cDNA sequence for *PrPGAP1a* and *PrPGAP1b* data have been deposited at the
675 GenBank/EMBL database and are publicly available as of the date of publication.
676 Accession numbers are listed in the key resources table. All data generated or analyzed
677 during this study are included in this published article (and its supplementary
678 information files). All data reported in this paper will be shared by the lead contact upon
679 request.
680 • This paper does not report original code.
681 • Any additional information required to reanalyze the data reported in this paper is
682 available from the lead contact upon request.
683
684 **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
685 *Arabidopsis thaliana* plants were grown as described in[25]. Briefly, chlorine gas sterilized seeds
686 were sown out on LRC2 plates (2.15 g.L^{-1} MS basal salts (Duchefa Biochemie), 0.1g.L^{-1} MES,
687 pH adjusted to 5.7 with KOH, 1.0 % Plant Tissue Culture Agar NEOGEN), and kept at 4 °C for
688 three days before transfer to a growth chamber for vertical growth with continuous light emitted

689 (white fluorescent lamps, intensity 120 μmol.m^2.s^{-1}), at 22 ºC. Seedlings were transferred to
690 Jiffy pots in soil and grown under glasshouse conditions under a 16h light/8h dark regime at 22
691 ºC.

692 *Papaver rhoeas* plants raised from seed of known SI genotypes derived from the Suttons
693 ' Shirley Single Mixed' cultivar were used as experimental material for obtaining mRNA for the
694 cloning of *P. rhoeas PrPGAP1* gene and for pollen used for the antisense/sense oligonucleotide
695 experiments. *P. rhoeas* plants were field grown. Fresh, dry pollen was collected from newly
696 opened flowers and stored over silica gel at -20 ºC, until needed.

697

698 **METHOD DETAILS**

699 **EMS mutagenesis and mutant screening of plant material**

700 The self-incompatible *Arabidopsis thaliana* (*At*-SI) lines provide an easy macroscopic readout
701 of SI-PCD, as self-pollinated *At*-SI lines are largely sterile and do not form elongated siliques.
702 T2 seeds of *At*-SI lines were collected and sown on LRC2 plates containing BASTA (10 μg.ml^{-}
703 ^{1}). Single insertion transgenic *At*-SI lines were obtained by selecting lines showing ~75% of
704 BASTA resistance. Homozygous lines were obtained by selecting lines whose T3 seeds showed
705 100% BASTA resistance. As only ~50 seeds could be harvested from an *At*-SI plant, selected
706 *At*-SI lines were propagated for two further generations to collect enough seeds for EMS
707 mutagenesis. EMS mutagenesis was carried out as described in[64]. M1 *At*-SI seeds after EMS
708 mutagenesis were sown in Jiffy pots, germinated and grown in greenhouse. The first screen was
709 carried out ~10 days after flowering. Mutants showing longer siliques were selected. For M1
710 plants that did not show this phenotype, the primary inflorescences were cut back and a second
711 screen was carried out ~2 weeks later. In total, ~50,000 M1 plants were screened and 40 mutants
712 were identified. After eliminating those with transgene mutations, altered PrpS$_1$–GFP
713 expression levels, or pseudo-fertilization, 12 mutants remained; they were named *highlander1s*
714 (*hld1s*).

715 To eliminate the effect of the mosaic nature of the M1 genetic background, M1 *hld1*
716 mutants (male) were backcrossed with the parent *At*-SI line (female) to obtain the backcross1
717 (BC1) generation of *hld1* mutants. To reduce the number of background single-nucleotide
718 polymorphisms (SNPs) caused by EMS mutagenesis, BC1 *hld1* mutants were backcrossed
719 again with the parent *At*-SI line to obtain the BC2 generation (see Figure S1).

720

721 **WGS, backcrosses and SNP analysis to identify the causal gene for the *hld1* mutants**

722 As the *hlds* were gametophytic mutations, after backcrossing with the non-mutagenised parent,
723 any unlinked SNP generated by the EMS mutagenesis segregated 1:3 in the pool of mutant
724 individuals of the F1 population of the second backcross (BC2), whereas the causative SNP
725 segregated 1:1[66]. Calculating the SNP ratio in the BC2 population allowed us to identify the
726 causal mutation region. For each of the *hld1* mutant lines (*hld1-3/5/7/19/24/25*), leaf disc
727 samples from 50 self-compatible BC2 plants were pooled, followed by DNA extraction using
728 a CTAB-based protocol[65], and whole genome re-sequencing (WGS) using Illumina next
729 generation sequencing (NGS) platform. The *At*-SI line was also sequenced as the background
730 control. SNPs were identified using SHOREmap[23]. Analysis of all the SNPs of the six *hld1*
731 mutants revealed six different nonsynonymous mutations in a single gene, *At3g27325*.
732 *At3g27325* is the only gene whose mutation was found in all the six mutants analyzed, therefore
733 was analyzed further as a promising candidate gene.

734

735 **Cloning, transgenic and T-DNA lines**

736 All the expression vectors were generated using Greengate cloning[67], Gibson assembly (New
737 England BioLabs), or Gateway cloning (Invitrogen). High-fidelity Phusion DNA polymerase

738 (New England BioLabs) was used for all the DNA fragment amplification. All the clones were
739 verified through Sanger sequencing.

740 The expression clone pFASTGreen-AtPGAP$_{pro}$:mCherry–cAtPGAP1 and pFASTGreen-
741 AtPGAP1$_{pro}$:mCherry–cAtPGAP1(S218A) was generated using Greengate cloning, during
742 which entry clones pGG-A-AtPGAP1$_{pro}$-B, pGG-B-mCherry-C, pGG-C-cAtPGAP1-D or
743 pGG-cAtPGAP1(S218A)-D, pGG-D-linker-E, pGG-E-G7T-F, and pGG-F-linker-G were
744 cloned into Greengate destination vectors pFAST-GK-AG[25]. The Greengate entry clone pGG-
745 B-mCherry-C, pGG-D-linker-E, pGG-E-G7T-F, and pGG-F-linker-G were obtained from PSB
746 Plasmid Vector Collection (https://gatewayvectors.vib.be/collection). The Greengate entry
747 clone pGG-A-AtPGAP1$_{pro}$-B was obtained through Gibson assembly. The 2755 bps upstream
748 of At3g27325.2 translational start site was amplified as the AtPGAP1 promoter (AtPGAP1$_{pro}$)
749 sequence. As there is a BsaI restriction site within the AtPGAP1$_{pro}$ sequence, two AtPGAP1$_{pro}$
750 fragments were separately amplified using primer sets 665/666 and 667/668 (detailed primer
751 information can be found in Supplemental Table S5), respectively, with Col-0 Arabidopsis
752 gDNA as the template to mutate the BsaI site, and assembled into BsaI linearized pGGA000
753 entry vector backbone using Gibson assembly. To make the Greengate entry clone pGG-C-
754 cAtPGAP1-D, AtPGAP1 cDNA (cAtPGAP1) was amplified and cloned into pJET1.2 using
755 CloneJET PCR Cloning Kit (ThermoFisher).

756 There are three different AtPGAP1 splice variants according to TAIR
757 (https://www.arabidopsis.org) annotation. The coding region of AtPGAP1.1 was amplified in
758 this experiment. As there are 3 BsaI restriction sites within the coding region of cAtPGAP1.1,
759 cAtPGAP1.1 was amplified through a two-step protocol. Four cAtPGAP1.1 fragments were
760 amplified using primer sets 732/675, 676/677, 678/679, and 680/683 with Arabidopsis seedling
761 cDNA as the template. These four DNA fragments were assembled into cAtPGAP1.1 using
762 overlap PCR. The resulting PCR products were cloned into pJET plasmid vector backbone to
763 obtain the Greengate entry clone pGG-C-cAtPGAP1-D. To make the Greengate entry clone
764 pGG-C-cAtPGAP1(S218A)-D, two cAtPGAP1(S218A) fragments were amplified using
765 primer sets 732/834, 833/683 with pGG-C-cAtPGAP1-D as the template. Mutations in the
766 primers were designed to change Serine 218 to Alanine. These two PCR fragments were
767 assembled into cAtPGAP1(S218A) using overlap PCR, after which the resulting PCR products
768 were cloned into pJET plasmid vector backbone to obtain the Greengate entry clone pGG-C-
769 cAtPGAP1(S218A)-D. The expression vectors were transformed into GV3101 *Agrobacterium*
770 *tumefaciens* competent cells. The floral-dipping method was adopted to stably transform
771 homozygous *At-SI/hld1-7* Arabidopsis plants to obtain At-SI/hld1/cAtPGAP1 and At-
772 SI/hld1/cAtPGAP1(S218A) lines.

773 To generate new *AtPGAP1* mutant alleles, 4 gRNAs (Table S5) were designed using
774 CRISPOR[68] and cloned into BbsI linearized Greengate entry vectors pGG-A-AtU6-26-B, pGG-
775 B-AtU6-26-C, pGG-C-AtU6-26-D, and pGG-D-AtU6-26-E respectively via Gibson assembly.
776 The resulting Greengate entry modules were assembled into pFASTGK-pUbi-Cas9-AG
777 together with pGG-E-linker-G to generate expression vector pFASTGK-CRISPR-AtPGAP1.
778 This was transformed into *At-PrpS$_1$* background line via Agrobacterium-mediated floral-
779 dipping, as the non-mutagenized parent line *At*-SI is self-incompatible and unfeasible for
780 transformation. *Atpgap1-c1* and *atpgap1-c2* alleles were screened from T2 Cas9-free seedlings
781 and used for further experiments. *At-PrpS$_3$/atpgap1* and *UBQ10$_{pro}$:PrpS$_1$/atpgap1* were
782 generated by crossing *At-PrpS$_3$*[22] or *UBQ10$_{pro}$:PrpS$_1$*[25] with *atpgap1* (*atpgap1-c1*, *atpgap1-c2*,
783 and *hld1-7* in the WT background).

784 The expression clones *UBQ10$_{pro}$:PrpS$_1$–GFP* were generated using Gateway cloning, by
785 recombining entry vectors pEN-L4-UBQ10$_{pro}$-R1, pEN-L1-PrpS$_1$-L2 [25] and pEN-R2-GFP-R3
786 into Gateway destination vector pB7m34GW using LR Clonase II plus enzyme (Invitrogen).
787 The expression vectors were transformed into GV3101 *Agrobacterium tumefaciens* competent

788 cells and transformed to wild-type Col-0 Arabidopsis plants. AtPGAP1 mutant allele was
789 introduced into *UBQ10$_{pro}$:PrpS$_1$–GFP* line by conventional crossing.

790 Seeds of T-DNA lines, *seth1-2* (SAIL_674_B03) and *seth2* (SALK_039599) were
791 obtained from The Nottingham Arabidopsis Stock Center (NASC). We introduced *seth1-2* and
792 *seth2* mutant alleles[10] into the *At-PrpS$_1$* background by crossing and generated heterozygous
793 *At-PrpS$_1$/seth1-2$^{+/-}$* and *At-PrpS$_1$/seth2$^{+/-}$* lines. Primers used for genotyping these two T-DNA
794 mutants can be found in Table S5. Genotyping of plants was carried out using Phire Plant Direct
795 PCR Kit (Thermo Scientific) according to the manufacturer's instructions.
796

797 **Cloning of *PrPGAP1***

798 To clone the *P. rhoeas PrPGAP1* gene, a degenerate primer set 961/965 was designed based
799 on the highly conserved region revealed by sequence alignment of *A. thaliana*, *Papaver*
800 *somniferum*, and *Oryza sativa PGAP1* genes. PCR using *P. rhoeas* DNA as template resulted
801 in a DNA product of ~3300 base pairs, which was subsequently cloned into the pJET1.2 cloning
802 vector (ThermoFisher). Sanger sequencing and subsequent sequence alignment analysis
803 showed >90% identity between the *PGAP1* coding regions of *P. somniferum* and *P. rhoeas*.
804 Therefore, instead of 5' and 3' Rapid Amplification of cDNA Ends (RACE) experiments, we
805 cloned the *PrPGAP1* directly, using primer sets (1098/1088, 1086/1093, 1103/1111,
806 1112/1106, see Table S5), which were designed according to mRNA sequence of *P. somniferum*
807 *PGAP1*. The cDNA sequences of two *PrPGAP1* alleles (*PrPGAP1a* and *PrPGAP1b*) were
808 obtained and have been deposited in the GenBank database under the accession number
809 MZ781963 and MZ781964.
810

811 **Triton X-114 assay, PI-PLC assays, and Western blots**

812 Three-day-old *A. thaliana* seedlings were collected into a 2 mL tube with 3 steel beads (3 mm)
813 and frozen in liquid N_2. Frozen samples were homogenized using a grinding mill (Retsch MM
814 400) at 20 Hz for 3 x 40 s. For Triton X-114 assays, 2% Triton X-114 extraction buffer [100mM
815 Tris-HCl pH=7.5, 150mM NaCl, 2mM EDTA, 10% glycerol, 2% Triton X-114 (Sigma-
816 Aldrich), 1x cOmplete protease inhibitor (Roche)] was added to the homogenized samples.
817 Triton X-114 was pre-processed as described in [69] before being added to extraction buffer.
818 Samples were centrifuged at 21,000g at 4 °C, and the supernatant retained. Protein
819 concentrations were determined using Bradford (Bio-Rad) assay. To separate the detergent and
820 aqueous phases, samples were incubated at 37 °C for 10 mins followed by centrifugation at
821 21,000g for 10 mins[70]. The aqueous upper phase was transferred to a new tube. Proteins from
822 the detergent and aqueous phases were precipitated using MeOH/$CHCl_3$[71] and dissolved in 1x
823 loading buffer.

824 PI-PLC assays were modified from protocols published by[71,72]. In brief, membrane protein
825 extraction buffer [100 mM Tris-HCl pH 7.5, 25% (w/w) sucrose, 5% (v/v) glycerol, 5 mM
826 EDTA, 5 mM KCl, 2x cOmplete protease inhibitor (Roche)] was added to the homogenized
827 samples. Samples were centrifuged (600 g, 3 mins) and supernatants were kept as whole protein
828 extracts. For the membrane fraction, supernatants were diluted with equal volume of water and
829 centrifuged at 21,000g (2 h., 4 °C) and pellets resuspended in PI-PLC treatment solution [10
830 mM Tris-HCl pH 7.5, 5% (v/v) glycerol]. PI-PLC (ThermoFisher) was added to a final
831 concentration of 2 units.mL^{-1}. For the mock control, 50% glycerol was added. Samples were
832 incubated at 37 °C for 1.5 h, followed by centrifugation (21,000g for 2 h., 4 °C). The upper
833 phase was transferred to a new tube and precipitated using MeOH/$CHCl_3$[71]; the pellet was
834 dissolved in 1x loading buffer.

835 SDS-PAGE and Western blot were carried out as described in[21] with minor modifications.
836 Proteins were separated using 4-20% Mini-PROTEAN TGX Stain-Free Precast gels (Bio-Rad).
837 Stain-free signals were used as "loading controls". Primary antibodies were incubated with

838 membranes overnight at 4 ºC in the PBS buffer containing 0.1% Tween-20 and 5% skimmed
839 milk powder. Anti–GFP (Takara), and anti-Aquaporins (Agrisera) were used at 1:1000 dilution,
840 and anti-α-Tubulin (Sigma-Aldrich) were used at 1:4000 dilution. Secondary HRP-conjugated
841 anti-Mouse (GEHEALTH) and anti-Rabbit (GEHEALTH) antibodies were incubated with
842 membrane for 1 h at RT with a 1:5000 dilution in the PBS buffer containing 0.1% Tween-20
843 and 5% skimmed milk powder. WesternBright ECL HRP substrate (ADVANSTA) was used
844 for detection.
845
846 ***A. thaliana* seedling treatment with recombinant PrsS proteins**
847 Recombinant PrsS protein treatment was performed as described in[25]. Briefly, PrsS proteins
848 were dialysed in 1/5 LRC2 liquid medium at 4 ºC and 10 µl PrsS proteins (10 ng.µl^{-1}) added
849 onto root tips of 4-day-old seedling on LRC2 plates. The plates were kept horizontal for 30 min
850 before being placed back vertically in the growth chamber for 24 h before root length
851 measurement. $PrsS_1$ protein provided the "SI" treatment for the transgenic *A. thaliana lines*
852 (*UBQ10*$_{pro}$:*PrpS*$_1$), with matching/cognate *S*-alleles in the seedling and protein; treatment with
853 LRC2 liquid medium or recombinant $PrsS_3$ proteins provided the controls.
854
855 **Arabidopsis pollen SI *in vitro* bioassay**
856 *A. thaliana* pollen was placed onto an 8-well chambered coverglass (Thermo Scientific Nunc
857 Lab-Tek) and *A. thaliana* pollen germination medium [AtGM; 15% (w/v) sucrose, 0.01% (w/v)
858 H_3BO_3, 5 mM KCl, 1 mM $MgSO_4$, 2.5 mM $CaCl_2$, 2.5 mM $Ca(NO_3)_2$, and 10% PEG3350,
859 pH=7.0 adjusted using KOH] modified from[73] containing 10 ng.µl^{-1} recombinant PrsS proteins
860 (SI treatment) was added. For controls, the PrsS proteins were omitted from the AtGM solution.
861 Pollen grains were incubated at RT (~22 ºC) for 6h and then fluorescein diacetate (FDA, 2
862 µg.ml^{-1}) and propidium iodide (PI, 5 µg.ml^{-1}) were added. Samples were examined using
863 fluorescence microscopy (for setting up details, see the following Imaging section). For each
864 sample, 100-200 pollen grains/tubes were scored for the number of FDA-positive (live) and PI-
865 positive (dead).
866 For treatment with PLC inhibitors (U73122, ET-18-OCH3, C48/80[74,75]), a similar
867 procedure was used. *A. thaliana* pollen was added to AtGM and pretreated with each PLC
868 inhibitor for 1h prior to the induction of SI (addition of 10 ng.µl^{-1} recombinant PrsS protein).
869 For the control, mock treatment, the equivalent volume of solvent was used.
870
871 ***Papaver rhoeas* pollen SI bioassay with antisense oligonucleotide treatment *in-vitro***
872 As a transgenic approach with *Papaver rhoeas* is not possible, we used an alternative approach
873 to knockdown *PrPGAP1* by incubating *P. rhoeas* pollen with phosphorothioated antisense
874 oligonucleotide designed against *PrPGAP1*[18,24]. *P. rhoeas* pollen was hydrated for 0.5 h and
875 incubated with poppy pollen growth medium (PrGM) containing 3 µM phosphorothioated
876 oligonucleotides (Antisense-PrPGAP1 or Sense-PrPGAP1, designed using *S*fold[76], Table S5)
877 and 0.5% (v/v) cytofectin (T201007H, Genlantis) for 2 h at RT before SI induction. Pollen
878 viability were assessed using FDA (2 µg.ml^{-1}) and PI (5 µg.ml^{-1}) 10 h after SI treatment. Pollen
879 tube lengths were measured using Fiji (https://fiji.sc/)[77]. Due to the limit of the imaging field
880 size, pollen tubes which were longer than 600 µm (very rarely observed) were all recorded as
881 600 µm.
882
883 **PGAP1 Phylogeny analysis**
884 PGAP1 domain-containing proteins comprising 631 proteins across three eukaryotic kingdoms
885 (Table S6), were downloaded from Pfam (http://pfam.xfam.org/family/PGAP1). Multiple
886 sequence alignment was performed using MAFFT (version 7.187). IQ-TREE (version 1.7-

beta7) was used for the maximum-likelihood tree inference with 1000 bootstrap replicates (under the model of 'JTT+R', -alrt 1000 -bb 1000).

Imaging, image analysis and figure preparation

For PI staining *A. thaliana* seedling roots were mounted in 1/5 LRC2 medium containing 5 $\mu g.ml^{-1}$ PI imaged (ex 561 nm, em 580-700 nm) with a PlanApochromat 20x objective (n/a 0.8) using a Zeiss LSM710 microscope.

For the *in-vitro* pollen SI bioassays, *A. thaliana* or *P. rhoeas* pollen was co-stained using FDA (2 $\mu g.ml^{-1}$; ex 561 nm, em 580-550 nm) and PI (5 $\mu g.ml^{-1}$; ex 488 nm, em 500-700 nm) and imaged with a Fluostar VISIR 25x/0.95 objective using a Leica SP8 confocal laser scanning system with a HyD detector.

GFP–SKU5 in *A. thaliana* seedling roots was imaged with a HCPL APO CS2 40x/1.10 objective using a Leica SP8 confocal laser scanning system with a HyD detector (ex 488 nm, em 500-550 nm). All images were processed and analyzed using Fiji (https://fiji.sc/)[77].

Quantification and Statistical analysis

The statistical details of experiments can be found in the corresponding Figure legends. The results of statistical tests can be found in the corresponding Results section. Statistical tests were carried out using GraphPad Prism 8.0 for Windows.

Supplemental Excel tables

Table S6: List of PGAP1-like domain-containing proteins used for the phylogeny analysis. Related to STAR Methods.

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