Short title: Pollen galactose sensitivity via SWEET5 and GALK

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The hexose transporter SWEET5 confers galactose sensitivity to Arabidopsis pollen germination via the galactokinase GALK

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Author Contributions

J.W. and L.-Q.C. conceived and designed the experiments. W.J., Y.-C.Y., and Y.L. conducted experiments. J.W., and L.-Q.C wrote the manuscript.

One-sentence summary

SWEET5 mediates pollen galactose sensitivity via GALK that is required for efficient galactose uptake in pollen during pollen germination.

Abstract

Galactose is an abundant and essential sugar used for the biosynthesis of many macromolecules in different organisms, including plants. Galactose metabolism is tightly and finely controlled since excess galactose and derivatives are inhibitory. In Arabidopsis, root growth and pollen germination were strongly inhibited by excess galactose. However, the mechanism of galactose induced inhibition during pollen germination remains obscure. In this study, we characterized a plasma-membrane localized transporter, AtSWEET5, that transports glucose and galactose. SWEET5 protein level started to accumulate at the tricellular stage of pollen development and peaked in mature pollen before rapidly declining after pollen was germinated. SWEET5 levels are responsible for the dosage-dependent sensitivity of galactose and galactokinase (GALK) is essential for the inhibitory effects of galactose during pollen germination. Overall, SWEET5 and GALK contribute to the maintenance of galactose metabolic homeostasis during pollen germination. SWEET5 serves as a major low-affinity hexose transporter at the early stage of pollen germination.

Key words

SWEET, GALK, galactose sensitivity, galactose inhibition, pollen germination, Gal-1-P

Introduction

Carbon flux, which is dependent on carbon supply and demand, needs to be well-controlled for plant growth and development (Hofmeyr and Cornish-Bowden, 2000; Bezrutczyk et al., 2018). Plants have developed sophisticated mechanisms to sense the sugar levels and adjust these in response to developmental or environmental cues (Ruan, 2014). Plant sensitivity to various concentrations of sugar varies depending on the specific sugar involved. For example, in *Arabidopsis thaliana,* a low 10 mM concentration of galactose can trigger an inhibitory effect on Col-0 root growth (Yamada et al., 2011; Egert et al., 2012), while the much higher 167 mM (presented as 3%) concentration of glucose or 100 mM sucrose still promotes root growth (Kircher and Schopfer, 2012; Singh et al., 2014). Additionally, during *in vitro* pollen germination, 60 mM galactose inhibits pollen germination (Hirsche et al., 2017), while 100 mM glucose does not affect the pollen germination rate (Rottmann et al., 2018) and high levels of sucrose (250-578 mM) are required for successful pollen germination (Wang et al., 2008; Rottmann et al., 2018). However, the mechanism by which galactose inhibits plant growth has not been well-characterized. Sugar transporters and enzymes are key components in the supply-demand system to maintain carbon flux control (Ruan, 2014; Julius et al., 2017). The mutants of the two hexose transporter STPs (Sugar Transport Protein), AtSTP1 and AtSTP13, have shown a galactose-tolerant phenotype for root growth (Sherson et al., 2000, Yamada et al., 2011). The transporter that is involved in the process of galactose-suppressed pollen germination, however, remains unexplored.

STPs are important during early stage gametophyte development (Truernit et al. 1999) and pollen tube growth but not in the initial phase of pollen germination (Scholz-Starke et al. 2003, Buttner, 2007). Facilitator SWEETs (Sugar Will Eventually be Exported Transporter) have been shown to transport different sugars including galactose. OsSWEET5 (Zhou et al., 2014) and CsSWEET7a (Li et al., 2021) have been demonstrated to transport galactose. So far, all plant SWEETs with a measured Km, e.g. AtSWEET1 (Chen *et al*., 2010), AtSWEET12 (Chen *et al*.,2012), AtSWEET17 (Guo *et al*., 2014), SlSWEET1a (Ho *et al*., 2019) and CsSWEET7a (Li et al., 2021), have been found to function as low-affinity glucose or sucrose transporters, ranging from ~10 to ~120 mM. It is not unusual to observe that a SWEET transports different sugars but with different efficiency and Kms (Chen *et al*., 2010, 2012, Kuanyshev et al., 2021). For example, SWEET7 has a Km of ~74 mM to glucose but ~308 mM to xylose (Kuanyshev et al., 2021). AtSWEET5/VEX, belonging

78 to SWEET family Clade II that primarily transports hexose (Chen et al., 2010; Eom et al., 2015),
79 is strongly expressed in the pollen vegetative cell (Engel et al., 2005), but no other details have
80 been reported. We hypothesized that SWEET5 might be responsible for the galactose-suppressed
81 pollen germination.
82
83 Once galactose enters a cell, it is activated by galactokinase (GALK), a cytosolic enzyme, which
84 phosphorylates α-D-Gal into α-D-Gal-1-P at the C-1 position (Cardini and Leloir, 1953). Gal-1-P
85 can be further converted into UDP-Gal through a reversible reaction catalyzed either by Gal-1-P
86 uridylyltransferase (GALT) in the presence of UDP-Glc (mainly used in non-plant species) (Leloir,
87 1951) or by UDP-sugar pyrophosphorylase (USP) using UTP as a substrate (mainly found in plants)
88 (Feusi et al., 1999; Kotake et al., 2004). The activated UDP-Gal can be converted reversibly into
89 UDP-Glc by UDP-Glc epimerase (GALE/UGE) (Maxwell et al., 1960; Seifert et al., 2002). Only
90 a single copy of the GALK gene was reported to exist in Arabidopsis (Sherson et al., 1999; Yang
91 et al., 2009; Egert et al., 2012), and a galactose-insensitive phenotype in root growth was observed
92 in *galk* (Egert et al., 2012). As GALK is the first enzyme to catalyze galactose, we analyzed its
93 role together with SWEET5 transporter during pollen germination.
94
95 We show the plasma membrane localized SWEET5 transports both glucose and galactose. Data
96 from the current study indicate SWEET5 and GALK play critical roles in controlling galactose
97 response during pollen germination. SWEET5 transports galactose that can be phosphorylated by
98 GALK, resulting in pollen germination inhibition through yet unknown mechanisms.
99

100 **Results**

101 **SWEET5 transports galactose**

102 To determine which SWEET in the mature pollen stage is able to transport galactose, we surveyed
103 the expression and protein accumulation of all SWEETs in Arabidopsis (Mergner et al., 2020).
104 Among all 17 members of the SWEET family, several were found to be expressed in mature pollen,
105 but only SWEET5, SWEET11 and SWEET12 proteins were detectable there (Supplemental Figure
106 S1). SWEET11 and SWEET12 have been reported to be transporters of sucrose (Chen et al., 2012),
107 while SWEET5 is the most likely transporter of galactose, since its homolog OsSWEET5 has been

108 found to transport galactose (Zhou et al., 2014). To test whether SWEET5 transports galactose,
109 we conducted growth assays with a yeast strain EBY.VW4000 lacking 17 hexose transporters
110 (Wieczorke et al., 1999). Yeast cells expressing SWEET5 grew on the medium containing glucose
111 and galactose, but not fructose (Figure 1A), with slower growth on galactose than on glucose. To
112 test whether SWEET5 transports galactose less efficiently than glucose, we conducted radio-tracer
113 uptake, which showed that the uptake rate of galactose is lower than that of glucose (Fig. 1B). To
114 further confirm glucose is the favored substrate than galactose to SWEET5, we conducted sugar
115 competition experiment. Even though ten times higher concentration of galactose was used as a
116 competitor, it was insufficient to compete with glucose uptake. Similarly, sorbitol also failed to
117 compete with glucose uptake significantly. By contrast, the same concentration of glucose
118 significantly reduced glucose uptake (Fig. 1C). These results support that galactose is not
119 efficiently transported by SWEET5 relative to glucose.
120
121 Additionally, we did the kinetic analysis of SWEET5. The Km for galactose uptake of SWEET5
122 was calculated as 671 ± 58.4 mM (Figure 1D), however, we do not know whether osmolarity will
123 affect the physiological performance of yeast cells when cultured with a high concentration (>500
124 mM). Therefore, to test if the osmolarity will affect yeast uptake and thus calculated Km, we
125 compared galactose uptake with and without osmolarity adjusted by mixing different designed
126 concentrations of galactose with matched concentrations of sorbitol to reach a total concentration
127 of 500 mM and observed no significant osmotic effects on galactose uptake. (Supplemental Figure
128 S2).
129
130 **SWEET5 contributes to galactose inhibition of Arabidopsis pollen germination**
131 To test how galactose affects pollen germination under our setting, we performed pollen
132 germination experiments using pollen germination medium (PGM) supplemented with 60 mM
133 galactose compared with that supplemented with or without other sugars. The pollen germination
134 rate of Col-0 was dramatically reduced from 57% to 11% when PGM with 60 mM Galactose
135 (Figure 1E and 1F), while remained unchanged when PGM was supplemented with either 60 mM
136 glucose or fructose (Supplemental Figure S3). The galactose suppression was barely detectable in
137 *SWEET5* loss-of-function mutants created from T-DNA insertion (*sweet5*) or CRISPR/CAS9
138 editing (Figure 1E and 1F; Supplemental Figure S4). The phenotypes of the mutants were fully

139 complemented by transformation with a construct carrying the *SWEET5* promoter driving either
140 endogenous *SWEET5* or a synonymous m*SWEET5* that carries mutations to avoid gRNA
141 recognition in CRISPR mutant lines (p*SWEET5*:*mSWEET5/sweet5_crispr*; Figure 1E and 1F;
142 Supplemental Figure S4D). Accordingly, the SWEET5 overexpression lines
143 (p*SWEET5*:*SWEET5*/Col-0) were more sensitive to galactose than Col-0 (Figure 1E and 1F). To
144 test whether in vivo pollen germination is affected in *sweet5* mutant, we compared pollen
145 germination and pollen tube growth of *sweet5* with that of Col-0 on Col-0 pistil after hand-
146 pollination. No obvious differences in the pollen germination/pollen tube growth (aided by aniline
147 blue staining) in vivo were observed at 2 hours or 6 hours after pollination (Supplemental Figure
148 S4G), suggesting the galactose content at the apoplasm of stigma cells is not high enough to trigger
149 pollen inhibition under normal condition. It is worth noting that glucose has a marginal effect on
150 pollen germination (Supplemental Figure S3) and thus is negligible, even if present. However, it
151 is not known if galactose content will rise to affect pollen germination under stress conditions. For
152 example, soluble galactose was significantly increased in coffee plant leaves under heat stress,
153 likely due to cell-wall modifications (Lima et al., 2013). Moreover, there were no observable
154 morphological phenotypes in *sweet5* mutant under normal conditions, which may be due to
155 functional redundancy with other plasma membrane-localized hexose transporters (STPs, PMTs)
156 detected at either the RNA or protein level in pollen (Supplemental Figure S5). Among those
157 hexose transporters that were detected at the protein level, many can transport glucose and
158 galactose (Supplemental Table S1).
159
160 **SWEET5 is mainly expressed at the late stages of pollen development**
161 To determine the timing of the initiation of SWEET5-associated galactose inhibition, we examined
162 the spatial and temporal accumulation patterns of SWEET5 using transgenic lines harboring a
163 SWEET5 translational fusion with β-glucuronidase (GUS) or yellow fluorescent protein (YFP)
164 driven by its native SWEET5 promoter (p*SWEET5*:*gSWEET5-GUS* or p*SWEET5*:*gSWEET5-YFP*).
165 SWEET5 was almost exclusively found in the anthers and mature pollen at late stages of flower
166 development (Figure 2A), although GUS staining was detected at the early seedling stage with a
167 preference in the vein. The SWEET5 protein accumulation pattern was carefully assessed in the
168 flowers. Flowers at different developmental stages (Bowman, 1994) were imaged using
169 fluorescence microscopy to detect SWEET5 protein levels using the reporter YFP. As shown in

170 Figure 2C, SWEET5 started to accumulate in immature pollen grains from flower stage 11, in
171 which pollen mitosis I and II occur (Cecchetti et al., 2008) and peaked at flower stage 13 (anthesis).
172 The YFP signal was also observed over the course of male gametophyte development by isolating
173 developing pollen grains. The SWEET5-YFP protein was first detected at the tricellular pollen
174 (TCP) stage and peaked at the mature pollen grain (MPG) stage, while it was undetectable in both
175 uninucleate microspores (UNM) and bicellular pollen (BCP), as delineated by PI staining of the
176 cell wall and nucleus (Figure 2B). The observed pattern of SWEET5-YFP protein agrees with the
177 reported gene expression profiling of *SWEET5* in Arabidopsis pollen over different developmental
178 stages (Honys and Twell, 2004), namely that *SWEET5* was highly expressed at the tricellular stage
179 and mature pollen stage. Additionally, SWEET5 protein level was substantially reduced in
180 germinated pollen/pollen tube (marked by white arrow) compared with non-germinated pollen
181 grains after *in vitro* pollen germination (Figure 2D). The subcellular localization of SWEET5 was
182 determined using pollen from transgenic plants harboring a SWEET5 (CDS) translational fusion
183 with a green fluorescent protein (GFP) driven by its native SWEET5 promoter
184 (p*SWEET5*:*cSWEET5-GFP*). SWEET5 is localized to the plasma membrane, and also
185 endomembrane compartments as delineated by Nile red staining for the lipid droplets (Figure 2E).
186 It is not unusual to observe that strong fluorescence throughout the cell, including endomembrane,
187 when a tagged protein is abundant in pollen (Tunc-Ozdemir et al., 2013). This has been similarly
188 observed for other plasma membrane localized proteins in pollen (Frietsch et al., 2007; Tunc-
189 Ozdemir et al., 2013; Hamilton et al., 2015). SWEET5 localization was further transiently
190 examined using the reporter mVenus in *N. benthamiana* leaves (Gookin and Assmann, 2014).
191 SWEET5 protein was localized to the pavement cell periphery enclosing both chloroplast and
192 Golgi apparatus (Figure 2F), which suggests SWEET5 localized to the plasma membrane,
193 consistent with published Arabidopsis SWEETs except for SWEET2, SWEET16, and SWEET17
194 that are localized to the tonoplast (Klemens et al., 2013; Guo et al., 2014; Chen et al., 2015a).
195

196 **Galactose sensitivity is GALK-dependent during pollen germination**

197 To further elucidate whether GALK is involved in the galactose sensitivity during pollen
198 germination, we first surveyed whether GALK is accumulated in the pollen. The RNA transcripts
199 of *GALK* are abundant in all pollen developmental stages, preferentially in microspore and
200 bicellular pollen stages (Honys and Twell, 2004). The protein level of GALK is relatively

201 consistent across all tissues, including mature pollen grains (Supplemental Figure S6A). Therefore,
202 we tested pollen germination of the loss-of-function *galk* mutant (Egert et al., 2012) on PGM
203 containing galactose. The *galk* mutant showed a galactose-tolerant phenotype similar to that of
204 *sweet5* (Figure 3A; Supplemental Figure S7A), although it is a knock-down mutant with *an* 80%
205 reduction in mature pollen RNA transcripts (Supplemental Figure S6C). The *galk* galactose-
206 tolerant phenotype was fully complemented by *GALK* expressed under control of the pollen-
207 specific *LAT52* promoter (Figure 3A; Supplemental Figure S7A). Notably, the *galk*
208 complementation line showed a much stronger galactose sensitivity than Col-0, which is likely
209 due to the over-expression of GALK in the complementation line (Supplemental Figure S6C). As
210 expected, the GALK and SWEET5 double mutant *sweet5 × galk* showed a similar galactose-
211 tolerant phenotype to their individual single mutants, which indicates that GALK and SWEET5
212 function in the same pathway way. As Gal-1-P is suggested to exert cellular toxicity from many
213 studies in animals (Lai et al., 2009), it is reasonable to speculate that pollen sensitivity to galactose
214 may be due to the accumulation of potential cytotoxic Gal-1-P, which depends on GALK activity
215 (de Jongh et al., 2008). To test this possibility, we compared the effects Gal-1-P on pollen
216 germination, 6 mM Gal-1-P was sufficient to mimic the suppression effect produced by 60 mM
217 galactose in Col-0 (Figure 3B; Supplemental Figure S7B). Gal-1-P can be converted into UDP-
218 Gal by USP in plants (Feusi et al., 1999). To test whether the USP-catalyzed reaction could
219 alleviate galactose suppression, we generated over-expression lines of AtUSP driven by the pollen-
220 specific *pLAT52* promoter in Col-0 background. However, USP overexpression lines failed to
221 improve the pollen germination rate under 60 mM galactose condition (Supplemental Figure S8).
222
223 **Galactose inhibition is dosage-dependent**
224 To determine the range of concentration at which galactose inhibits pollen germination, pollen
225 from different genotypes were germinated on PGM with galactose concentrations ranging from 0
226 to 600 mM. In Col-0, the pollen germination rate was not affected under 0.6 mM galactose, started
227 to decline significantly at 6 mM, followed by a substantial decrease at 60 mM and further reduced
228 to almost zero at 600 mM (Figure 4A, Supplemental Figure S7C). A time-course pollen
229 germination experiment of Col-0 further confirmed that the pollen germination rate was
230 significantly affected in the presence of 6 mM galactose starting from four hours after germination
231 (Supplemental Figure S7D). However, no significant difference was observed between Col-0 and

232 *sweet5* mutants at 6 mM (Figure 4A, Supplemental Figure S7C). *sweet5* mutants were generally
233 tolerant to galactose up to 60 mM. Consistent with the observation, the SWEET5 overexpression
234 lines were more sensitive to ≥ 6 mM galactose than Col-0, with pollen germination rates reduced
235 to near zero by 60 mM galactose. These results suggest that SWEET5 is responsible for galactose
236 flux at the higher concentration range. To assess the differences in galactose uptake between Col-
237 0 and *sweet5*, we conducted ^{14}C-galactose tracer uptake assays in pollen with various galactose
238 concentrations. Pollen grains germinated in a liquid PGM for 45 mins (the hydration period, Wang
239 *et al*., 2008), minimizing the interference from transporters expressed on pollen tubes. Less ^{14}C-
240 galactose was taken up by *sweet5* pollen than by Col-0 pollen at 60 mM galactose concentration
241 (Figure 4B). By contrast, differences were not prominent at lower amounts of galactose (Figure
242 4B).
243 Galactose at 10 mM was reported to strongly inhibit root growth of Col-0 (medium containing no
244 sucrose, Yamada et al., 2011; Egert et al., 2012), while higher galactose (60 mM) is needed to
245 strongly inhibit Col-0 pollen germination (medium containing 15% (~ 439 mM) or 19.8 % (~578
246 mM) sucrose, Hirsche et al., 2017 or current study). To test whether the sucrose in the medium
247 may differentially affect the observed galactose response, which may lead to a biased comparison,
248 we conducted a galactose dosage-dependent assay for root growth using Col-0. Root growth was
249 not affected at 1 mM galactose but was strongly inhibited at 6 mM or higher without sucrose in
250 the medium (Supplemental Figure S9). By contrast, a significant root reduction was only observed
251 at 60 mM galactose when 0.5% or 1% sucrose was supplemented in the medium. Therefore, the
252 level of inhibitory galactose concentration should be considered in the context of sucrose presence
253 or absence.
254
255 **Discussion**
256
257 **Pollen is a simple system to study the control of galactose flux**
258 While pollen has a fundamental role in the sexual reproduction of flowering plants, pollen
259 germination together with pollen tube growth has served as a model system to study single cell
260 growth and morphogenesis (Feijó et al., 2004). In Arabidopsis, pollen grains are highly
261 homogeneous and have strikingly low transcriptional complexity (on average, 6044 genes
262 expressed in mature pollen; Rutley and Twell, 2015) compared to sporophytic tissues or purified

263 sporophytic cells (e.g., 13,222 genes expressed in stomatal guard cells, and 11,696 genes expressed
264 in root hair cells; Bates et al., 2012, Becker et al., 2014). Our work to understand relationships
265 among galactose transport, GALK, and galactose sensitivity sheds light on developing alternative
266 strategies to attenuate galactose suppression by fine-tuning their relationships.
267
268 Over the past several decades, extensive efforts have been made to study galactose toxicity in
269 humans (galactosemia) and yeast; however, the mechanism underlying the toxicity is far from
270 understood (Lai et al., 2009). Considerably less attention has been paid to galactose inhibition by
271 plant scientists. In the current study, we found that the plasma membrane-localized hexose
272 transporter AtSWEET5 is responsible for galactose sensitivity during pollen germination. A high
273 concentration of sucrose at 19.8% is needed in the medium for *in vitro* pollen germination, which
274 may be partially cleaved into glucose and fructose by the Arabidopsis cell-wall invertase 2 before
275 import into pollen (Hirsche et al., 2009). As SWEET5 prefers to transport glucose (Figure 1B),
276 glucose may competitively suppress galactose uptake, resulting in much less galactose transported
277 in pollen. Thus, a high concentration of galactose, such as 60 mM, is needed to result in a severe
278 inhibitory effect on pollen germination. This concept was supported by the reduced galactose
279 response during root growth of wild-type plant in presence of 0.5 % sucrose (Supplemental Figure
280 S9). Even when 60 mM external galactose was used, the estimated concentration was only about
281 135 μM in Col-0 pollen after incubation with 60 mM galactose for 45 min. This was calculated
282 based on the uptake of 200 pmol galactose (Figure 4B) determined from 20 flowers that yielded a
283 combined 40,000 pollen (2000 pollen per flower), and the mean pollen volume deduced from the
284 Arabidopsis pollen diameter of 20.72 μm (De Storme et al., 2013).
285
286 **Model of galactose transport and metabolism during *in vitro* germination of pollen**
287 From the data presented in this study and literature reports, a schematic model of galactose
288 sensitivity in pollen was illustrated in Figure 5: SWEET5, as a uniporter, transports galactose
289 across pollen membrane across a concentration gradient. If galactose is not sufficiently transported
290 into pollen grains due to a lack of enough SWEET5 protein, a galactose-tolerant phenotype can be
291 observed. Similarly, the lack of AtSTP1 and AtSTP13 have shown a galactose-tolerant phenotype
292 of root growth in conditions of up to 100 mM galactose (Sherson et al., 2000, Yamada et al., 2011).
293 Once galactose is taken up by cells, it will first be phosphorylated by GALK to produce the

294 potentially toxic Gal-1-P. A galactose detoxification pathway that stores excess galactose in the
295 vacuoles was proposed in the *galk* mutant (Egert et al., 2012). This pathway of detoxification
296 cannot explain the galactose-tolerant phenotype of *galk* pollen, because vacuoles are nearly absent
297 from mature pollen grains (Pacini et al., 2011). The disrupted Gal-1-P production may therefore
298 be responsible for the galactose tolerance of the *galk* mutant during pollen germination. Though
299 possible free galactose accumulation by *galk* pollen, similar to *galk* leaves (Egert et al., 2012), may
300 negatively impact galactose influx during pollen germination and obscure our understanding.
301
302 Our study suggested that Gal-1-P is responsible for the galactose sensitivity observed during pollen
303 germination, which is supported by the results of Gal-1-P effect on *in vitro* pollen germination.
304 However, caution should be taken when interpreting these results, because it is still unclear how
305 Gal-1-P can be taken up by pollen as no such transporters have been identified. Gal-1-P showed a
306 significant increase as the external galactose concentration rises beyond the inhibitory levels in
307 corn and barley coleoptiles (Roberts et al., 1971). In animals or yeast, Gal-1-P has been reported
308 to potentially inhibit enzymes such as phosphoglucomutase (de Jongh et al., 2008), UDP-glucose
309 pyrophosphorylase (UGPase, catalyzing the reversible conversion from Glc-1-P to UDP-Glc) (Lai
310 et al., 2003), and inositol monophosphatase (Bhat, 2003) *in vitro,* however, no *in vivo* targets of
311 Gal-1-P have been identified. In limited studies in plants, Gal-1-P has only been reported to inhibit
312 UGPase activity in the crude extract from oat coleoptiles, but not from the azuki bean epicotyl,
313 suggesting that galactose sensitivity appears to vary depending on the species (Yamamoto et al.,
314 1988). We attempted to measure the galactose-associated metabolites upon galactose treatment in
315 *sweet5* and *galk*, however, no clear conclusions can be drawn due to the difficulties of separating
316 glucose and galactose conjugated phosphates and nucleotide sugars using an LC-MS/MS system.
317
318 **SWEET5 orthologs may play essential roles in hexose-utilizing pollen**
319 Compared to the proton sugar symporters, SWEET5 likely functions as a uniporter, facilitating
320 substrate movement along a substrate concentration gradient (Chen et al., 2015b). The
321 physiological importance of SWEET family members was underscored mainly by their
322 involvement in multiple processes requiring sugar transport capability, for instances, SWEET11
323 and SWEET12 in apoplasmic phloem loading (Chen *et al*., 2012); SWEET9 in nectar secretion
324 (Lin *et al*., 2014); and SWEET11, SWEET12, and SWEET15 in seed filling (Chen *et al*., 2015c).

325 In the current work, we showed that SWEET5 is highly expressed in the mature pollen but
326 dramatically declines during the transition from germinated pollen to pollen tubes, suggesting that
327 SWEET5 functions during the early stages of pollen germination. By contrast, several STPs are
328 primarily expressed in pollen tubes but are almost absent in mature pollen grains (Rottmann et al.,
329 2018). For example, STP10 is a pollen tube-specific hexose transporter (Rottmann et al., 2016);
330 the glucose transporter STP9 is weakly expressed in mature pollen grains and is primarily
331 expressed in growing pollen tubes (Schneidereit et al., 2003). Therefore, SWEET5 and STPs may
332 have complementary roles during pollen germination. As reported, high concentrations of sugars
333 (ranging from 2% to 19.8%) are needed for successful germination *in vitro* in various species
334 (Ylstra et al., 1998; Stadler et al., 1999; Wang et al., 2008) since pollen germination is an energy-
335 demanding process (Reinders, 2016). However, the effect of different sugars on *in vitro* pollen
336 germination depends on the species. For example, sucrose and hexose differentially affect *in vitro*
337 pollen germination, ranging from growing equally well on glucose, fructose, or sucrose for petunia
338 (Ylstra et al., 1998), cucumber (Cheng et al., 2015), and pearl millet (Reger et al., 1992), to a
339 nearly complete inhibition by glucose or fructose for Arabidopsis (Hirsche et al., 2017). But for
340 some species, such as tobacco, either sucrose or glucose can support pollen germination, while
341 only sucrose can promote pollen tube growth (Goetz et al., 2017). Galactose alone can also
342 promote pollen germination, for example in *Picea wilsonii* (Zhou et al., 2020) and cucumber
343 (Cheng et al., 2015) to a similar extent as sucrose. Therefore, the lack of a phenotype in *sweet5*
344 under normal conditions may be due to the preference of Arabidopsis pollen during the initial
345 phase of germination for sucrose. Considering SWEET5 also transports glucose, it is rational to
346 speculate that SWEET5 orthologues may be essential for pollen development, and therefore seed
347 production in the species that prefer to use hexose as a carbon source during pollen germination or
348 pollen tube growth.
349
350 **Conclusions**
351
352 We have presented SWEET5 as a plasma membrane-localized hexose transporter that is highly
353 expressed in mature pollen grains. GALK catalyzes the synthesis of Gal-1-P from galactose that
354 can be transported by SWEET5. The pollen grains of *sweet5* and *galk* mutants are tolerant to
355 galactose inhibition during pollen germination in vitro, suggesting that SWEET5 and GALK play

356 a critical role in the sensitivity of pollen germination to galactose in Arabidopsis. Pollen
357 germination studies served as a tool to elucidate SWEET5 biological functions and provide a
358 model system that facilitates research to uncover mechanisms underlying galactose sensitivity.
359 The future work associated with either SWEET5 or its homologs from species that favor different
360 carbon sources for pollen germination, will offer new insights into the carbon skeleton and/or
361 energy supply mechanisms responsible for successful reproduction.
362

363 **Materials and Methods**

364 **Constructs for yeast expression**

365 The CDSs of *AtSWEET5* were amplified by RT–PCR using gene specific primers named by gene
366 (Supplemental Table S2**,** P1 and P2) and were subsequently cloned into the donor vector
367 pDONR221-f1 by BP clonase (Invitrogen) before transfer to pDRf1-GW (Loque et al., 2007) by
368 LR clonase (Invitrogen).
369

370 **GUS and YFP fusion constructs under native promoters**

371 To construct *SWEET5* fused with GUS (p*SWEET5*:*gSWEET5-GUS*), the fragment including the
372 *SWEET5* native promoter sequence (1801 bp upstream of ATG) and genomic sequence of
373 *SWEET5* was amplified (P3 and P4) and cloned into pDONR221-f1 by BP reactions followed by
374 recombination into the vector pMDC163 carrying the GUS gene (Curtis and Grossniklaus, 2003)
375 by LR reaction. For the SWEET5 overexpression construct (p*SWEET5*:*gSWEET5-YFP*), the entry
376 clone from above was transferred into pEarleyGateTW1 (Wang et al., 2016) by LR reactions.
377

378 **eGFP fusion constructs under different promoters for strength comparisons and mutant**
379 **complementation**

380 The *SWEET5* (1216 bp upstream of ATG) native promoter, and *LAT52* promoter (602 bp;
381 Muschietti et al., 1994) were amplified with specific forward primers (P5-P8) containing a *BamH*I
382 restriction site and the reverse primers containing an *Xba*I restriction site and sub-cloned into the
383 eGFP-containing vector pGTKan3 (Kasaras and Kunze, 2010) via *BamH*I and *Xba*I restriction
384 sites. The CDS of *SWEET5, GALK*, and *USP* by their own specific primers (P9-P14) were
385 seamlessly subcloned into corresponding pGTKan3_promoter constructs by In-Fusion® (Takara)

386 following the linearization at *Xba*I and *Pst*I restriction sites. For the genome editing construct of
387 *SWEET5*, gRNA that targets the first exon of SWEET5 (Supplemental Figure S4D) was cloned
388 into p*YAO*:*hSpCas9* (Yan et al., 2015). For the synonymous complementation construct of Cas9
389 containing *sweet5_crispr,* synonymous *SWEET5* driven by pSWEET5 was amplified by primer
390 P15 and P16 based on pDONR221_pSWEET5-*SWEET5* with gRNA targeting the region modified
391 through site-directed mutagenesis before transferring into pEarleyGate301 (Earley et al., 2006) by
392 LR reactions.
393
394 **Construct for subcellular localization**
395 For subcellular localization of SWEET5, after the vector pDOE-13 (Gookin and Assmann, 2014)
396 was linearized by *SanD*I + *AatI*I restriction enzymes at MCS3, the CDS of SWEET5 was amplified
397 by primers P17 and P18 and subcloned into it by In-Fusion® (Takara). The agroinfiltration-based
398 assays were performed as previously described (Gookin and Assmann, 2014). The *N. benthamiana*
399 leaf disks were collected 72 h post infiltration for confocal imaging. Three independent trials were
400 conducted.
401
402 **Plant materials and growth conditions**
403 The Arabidopsis Col-0 plants were grown under controlled temperature (22°C) with a 16-h light
404 (100-150 $\mu Em^{-2}s^{-1}$)/ 8-h dark photoperiod. The flowers at stage 13 (fully opened) were collected
405 at around 5 hours into the light period for different experiments. T-DNA mutants of *galk* (GABI-
406 Kat 489D10), and *sweet5* (CS853155) were obtained from NASC or ABRC. Homozygous lines
407 were genotyped using primers of P19-P24 and used in related experiments. The floral dip method
408 (Clough and Bent, 1998) was used to generate all the transgenic lines used in this study. At least
409 15 T_1 lines were generated for each construct and at least 3 randomly selected lines were
410 propagated to generate T_3 homozygous seeds.
411
412 **Yeast complementation assay and ^{14}C uptake assay**
413 Yeast complementation and yeast ^{14}C uptake assays were conducted following the previously
414 described method (Chen et al., 2010). ^{14}C-glucose (0.1 μCi D-[U-^{14}C] glucose; 275 mCi/mmol) ,
415 ^{14}C-galactose (0.1 μCi D-[1-^{14}C] galactose; 56.2 mCi/mmol) was added per sample and four
416 independent transformants were used for each uptake experiment. For concentration- and time-

417 dependent uptake of [14C] galactose for SWEET5, 5 galactose concentrations (25, 50, 100, 250,
418 500 mM), and 4 time points (10 sec, 2.5, 5, and 10 min) for each concentration were used to collect
419 yeast cell samples and measure galactose uptake. Sugar uptake at 10 sec was normalized as 0.
420

421 ***In vitro* pollen germination**

422 The *in vitro* pollen germination assay was conducted according to a previously described method
423 (Wang et al., 2008) with minor modifications. The sucrose only basic pollen germination medium
424 (PGM) was composed of 19.8% (w/v) Suc (15% (w/v) Suc in liquid PGM), 1.5 mM boric acid,
425 0.8 mM $MgSO_4$, 1 mM KCl, 5 mM MES, 0.05% (w/v) lactalbumin hydrolysate, 10 μM myo-
426 inositol, 5 mM $CaCl_2$ and 1.5% (w/v) agarose. The pH was adjusted to 5.8 using 1 mM Tris (pH
427 = 8). The medium solution was heated to boiling on a heat plate, and then 500 μl was spread evenly
428 onto 75 × 25 mm glass slides. Slides were cooled down at room temperature before placing in an
429 opaque, sealed slide box. The medium was made freshly for each germination experiment. Six
430 flowers at stage 13 were collected from each genotype to spread pollen gently onto a ~1 cm^2 area
431 of the germination medium. The slide box was kept at room temperature for 8 hours in the dark
432 before samples on slides were imaged with a compound microscope. Pollen with pollen tubes
433 longer than the diameter of the pollen grain (about 20 μm) was considered as germinated pollen.
434

435 **Pollen galactose uptake**

436 The liquid pollen germination method was adopted from a previously described method (Wang et
437 al., 2008) with minor modifications for ^{14}C-galactose uptake. For each trial, 20 flowers (at least
438 2000 pollen grains collected per flower) at stage 13 for each genotype were collected into a 2 ml
439 tube, and then 1 ml of liquid PGM was added in and vortexed vigorously for 60 s to release mature
440 pollen grains into the solution. Subsequently, the pollen grains were counted using a
441 hemocytometer. Equal amounts of pollen for all tested genotypes were collected by centrifuging
442 at 15,000 g for 1 min. The pollen pellet was resuspended in 30 μl of liquid PGM containing ^{14}C-
443 galactose (0.3 μCi hot galactose) as well as different concentrations of cold galactose (ranging
444 from 0 to 60 mM), and subsequently cultured in a petri dish (35 mm in diameter). The petri dish
445 was covered and placed in the dark for 45 min before pollen was washed using 1 ml of ice-cold
446 liquid PGM and collected into a 1.5 ml tube. The pollen was precipitated at 15,000 g for 1 min and
447 the pellet was washed by 1 ml ice-cold pollen isolation buffer (PIB, composed of 100 mM $NaPO_4$,

448 pH 7.5, 1 mM EDTA, and 0.1% (v/v) Triton X-100) 5 more times. The pellet was resuspended in
449 100 µl PIB and transferred to a scintillation vial containing 5 ml Ultima Gold XR Scintillation
450 liquid (PerkinElmer). Radioactivity for each vial was measured by liquid scintillation spectrometry.

451 **GUS histochemical analysis**

452 GUS staining was performed as previously described (Chen et al., 2012). Twelve-day-old
453 seedlings were used, and inflorescences and siliques were collected for histochemical GUS
454 staining.

455

456 **Microscopy imaging**

457 A Zeiss Apotome.2 (Carl Zeiss, Thornwood, NY, USA) was used for fluorescence acquisition.
458 Different FL filter sets (YFP, CFP, RFP and GFP) were used to image samples as needed. A Zeiss
459 LSM 710 confocal microscope (Carl Zeiss, Thornwood, NY, USA) was used to image samples.
460 Argon laser excitation wavelength and emission bandwidth were 405 nm and 425-550 nm for
461 aniline blue, 458 nm and 480–520 nm for mTq2 (cyan), 488 nm and 500–550 nm for mVenus
462 (yellow), and 633 nm and 633-740 nm for chlorophyll autofluorescence (red) respectively. Image
463 acquisition parameters were held consistent. Raw data from each channel were not altered beyond
464 equal signal increases.

465

466 **RNA isolation and RT-qPCR**

467 RNA isolation from pollen was performed as previously described with modifications (Lu, 2011).
468 For each independent sample, pollen grains from 50 flowers at stage 13 were collected. For
469 samples under galactose treatment, pollen grains from 50 flowers were germinated in liquid PGM
470 for 45 mins as described above in pollen galactose uptake. RNA was isolated using Trizol
471 (Invitrogen) as instructed by the manufacturer. First-strand cDNA was synthesized using oligo(dT)
472 and M-MuLV reverse transcriptase (NEB). Real-time qPCR was performed using PowerUp™
473 SYBR™ master mix (Applied Biosystems) according to the manufacturer's instructions on a
474 CFX96 Real-Time PCR Detection System (Bio-Rad) using gene specific primers (P25-P30). The
475 expression values were normalized to *ACTIN8* expression values in each repeat, and subsequently
476 normalized to Col-0 using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

477

478 **Statistical analysis**

479 The differences between the two subjects were determined using the two-tailed Student's t-test
480 with equal variance. The differences among multiple subjects were assessed using one-way
481 ANOVA followed by multiple comparison tests (Fisher's LSD method). All statistical analysis
482 was performed using SPSS 26 statistical software (SPSS Inc, Chicago, IL, USA).
483
484 **Accession numbers**
485 Sequence information from this article can be found in the Arabidopsis Genome Initiative or
486 GenBank/EMBL databases under the following accession numbers: SWEET5 (AT5G62850),
487 GALK (AT3G06580), USP (AT5G52560).
488
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494
495
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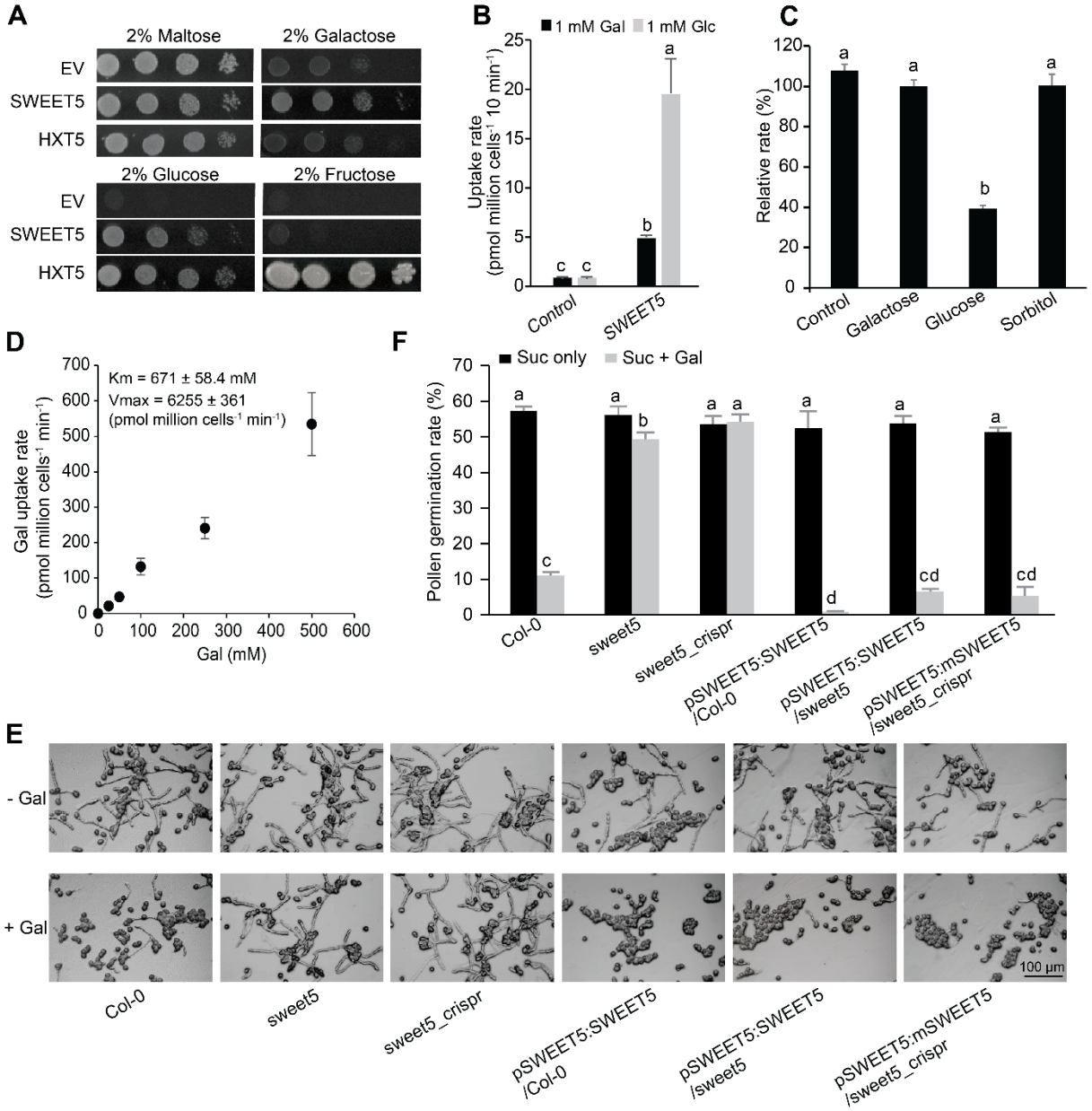

A
2% Maltose
2% Galactose
EV
SWEET5
HXT5
2% Glucose
2% Fructose
EV
SWEET5
HXT5
B
1 mM Gal
1 mM Glc
Uptake rate (pmol million cells-1 10 min-1)
Control
SWEET5
C
Relative rate (%)
Control
Galactose
Glucose
Sorbitol
D
Km = 671 ± 58.4 mM
Vmax = 6255 ± 361
(pmol million cells-1 min-1)
Gal uptake rate (pmol million cells-1 min-1)
Gal (mM)
F
Suc only
Suc + Gal
Pollen germination rate (%)
Col-0
sweet5
sweet5_crispr
pSWEET5:SWEET5 /Col-0
pSWEET5:SWEET5 /sweet5
pSWEET5:mSWEET5 /sweet5_crispr
E
- Gal
+ Gal
100 µm
Col-0
sweet5
sweet5_crispr
pSWEET5:SWEET5 /Col-0
pSWEET5:SWEET5 /sweet5
pSWEET5:mSWEET5 /sweet5_crispr

703

704

705 **Figure 1.** Characterization of Arabidopsis SWEET5.

706 (A) Functional analysis of SWEET5 by EBY.VW4000 yeast complementation assay. The empty
707 vector (EV) as a negative control, or HXT5 as a positive control.

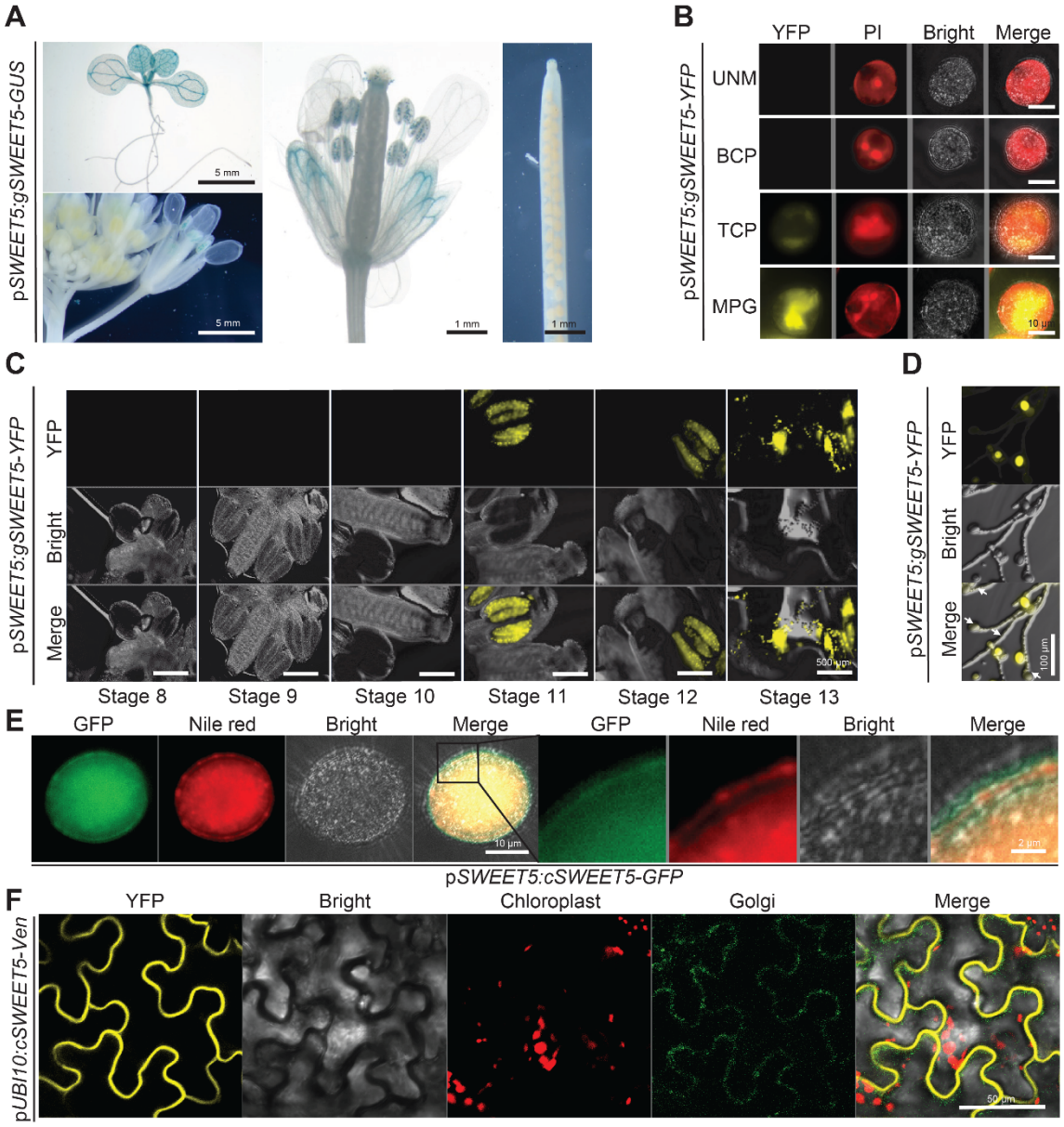
708 (B) SWEET5-mediated uptake of 1 mM ^{14}C-galactose and ^{14}C-glucose over 10 minutes in yeast
709 cells. Cells transformed with an empty vector were used as a negative control (mean ± SE, n = 4).

710 (C) Substrate competition uptake analysis of SWEET5 in yeast over 10 min. Different 50 mM cold
711 sugars competed with 5 mM cold glucose (including ^{14}C-glucose) that was normalized to 100 %
712 (mean ± SE, n = 4).

713 (D) Kinetics of ^{14}C-galactose accumulation by SWEET5 in yeast (mean ± SE, n = 4). An empty
714 vector served as a negative control and was subtracted.

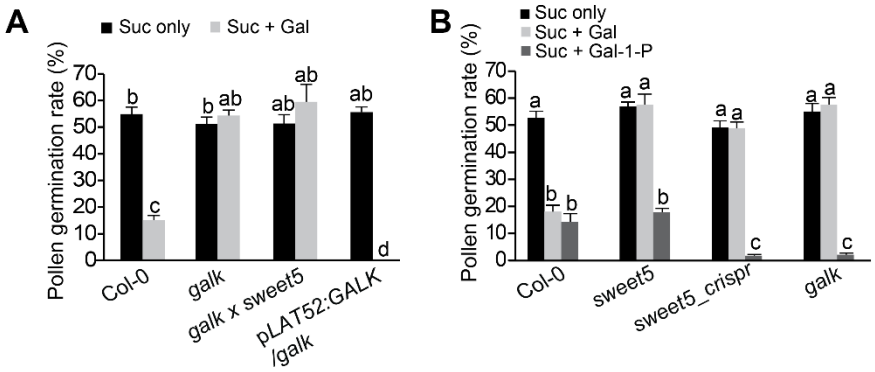
715 (E) *In vitro* pollen germination assay for various genotypes on sucrose-based medium with or
716 without 60 mM galactose. The pictures were taken 8 hours post-germination.

717 (F) Statistical analysis of pollen germination rates for various genotypes. The means were
718 calculated from multiple repeats (± SE, n ≥ 9), with over 450 pollen grains/tubes counted in total.
719 The statistically significant differences among different samples were determined using one-way
720 ANOVA followed by multiple comparison tests and were represented by different letters (P <0.05).


A
pSWEET5:gSWEET5-GUS
5 mm
5 mm
1 mm
1 mm
B
YFP
PI
Bright
Merge
pSWEET5:gSWEET5-YFP
UNM
BCP
TCP
MPG
10 µm
C
pSWEET5:gSWEET5-YFP
YFP
Bright
Merge
500 µm
Stage 8
Stage 9
Stage 10
Stage 11
Stage 12
Stage 13
D
pSWEET5:gSWEET5-YFP
YFP
Bright
Merge
100 µm
E
GFP
Nile red
Bright
Merge
GFP
Nile red
Bright
Merge
10 µm
2 µm
pSWEET5:cSWEET5-GFP
F
YFP
Bright
Chloroplast
Golgi
Merge
pUBI10:cSWEET5-Ven
50 µm

721

722 **Figure 2.** Tissue-specific accumulation and cellular localization of SWEET5.
723 (A) SWEET5 tissue-specific expression was evaluated. Twelve-day-old seedlings, inflorescences
724 and siliques from about one-month old Arabidopsis carrying p*SWEET5*:*gSWEET5-GUS* were
725 histochemically stained 4 h for GUS activity.
726 (B) SWEET5 localization in pollen grain was examined using fluorescence microscopy. Images
727 were captured using the YFP filter for YFP, the RFP filter for propidium iodine (PI) staining, and
728 under bright field. SWEET5 was detected in the tricellular pollen grain (TCP) and mature pollen
729 grain (MPG) but absent in early stages of uninucleate microspores (UNM) and bicellular pollen
730 (BCP).
731 (C) SWEET5 accumulation in different floral stages of Arabidopsis was examined using
732 fluorescence microscopy. The signals started to be detected from stage 11.
733 (D) SWEET5 accumulation was detected after pollen germination in p*SWEET5*:*gSWEET5-YFP*
734 *lines*. Pictures were taken 8 hours post-germination using a fluorescence microscope. White arrows
735 pointed to germinated pollen with much weaker fluorescence than un-germinated pollen.
736 (E) SWEET5 cellular localization was examined in mature pollen carrying p*SWEET5*:*cSWEET5-*
737 *GFP*.
738 (F) SWEET5 cellular localization was examined 72 hours after infiltration of *Nicotiana*
739 *benthamiana* leaves with *pUBI10*:*cSWEET5-mVenus*. Pictures were taken using confocal
740 microscopy.
741



742

743 **Figure 3.** Involvement of GALK in galactose-inhibited pollen germination

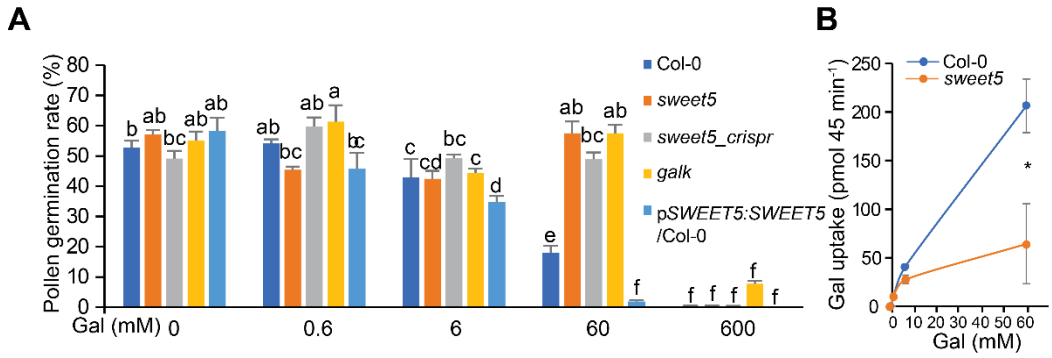
744 (A) Examination of *in vitro* pollen germination on sucrose-based medium with 0 mM or 60 mM

745 galactose for various genotypes. (means ± SE, n ≥ 6 (over 300 pollen grains/tubes counted) and *P*

746 <0.05).

747 (B) Effects of galactose-1-phosphate (6 mM) on *in vitro* pollen germination (means ± SE, n ≥ 6

748 (over 300 pollen grain/tubes counted) and *P* <0.05).



749

750 **Figure 4.** Concentration-dependent galactose response.

751 (A) *In vitro* pollen germination of various genotypes on medium supplemented with different
752 concentrations of galactose (ranging from 0 to 600 mM). The means (± SE) of at least 6 repeats
753 were plotted (over 300 pollen grains/tubes tested) ($P < 0.05$).
754 (B) Concentration-dependent galactose uptake in pollen of Col-0 and *sweet5* (means ± SE, n=3
755 and $P < 0.05$). Pollen germinated on liquid medium containing 0 mM, 0.6 mM, 6 mM, or 60 mM
756 galactose in addition to 0.3 µCi ^{14}C-galactose per sample for 45 min.
757

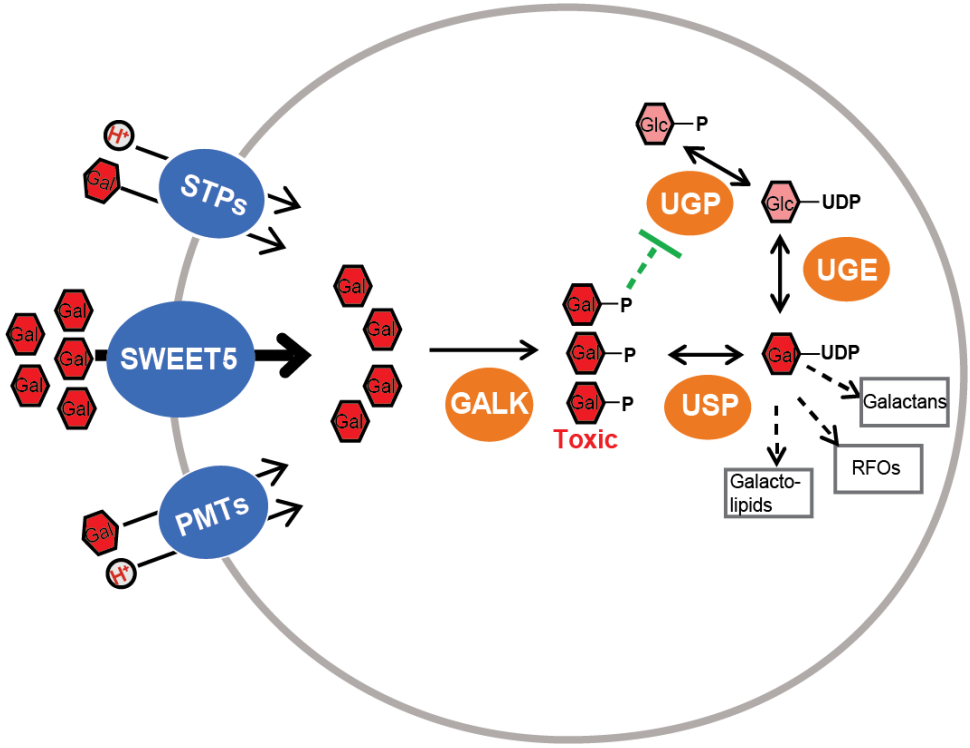


Figure 5. Schematic representation of galactose transport and metabolism in Arabidopsis pollen. A simplified pollen structure was drawn for clarity. Arrows represent positive regulation and bar-headed lines indicate negative regulations. The black lines represent galactose metabolic flux. The thick black line means higher flux. The green dotted line is for hypothetical regulation. Galacto-lipids are predominantly found in plastid membrane. RFOs (Raffinose Oligosaccharides) mainly include raffinose and stachyose containing one and two Galactose moieties, respectively. Galactans represents the cell wall-bound galactose.