Short title: Pollen galactose sensitivity via SWEET5 and GALK

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The hexose transporter SWEET5 confers galactose sensitivity to Arabidopsis pollen 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 upon excess galactose. However, the mechanism of galactose induced inhibition during pollen germination remains obscure. In this study, we characterized AtSWEET5 as a glucose and galactose plasma-membrane transporter localized in the pollen. SWEET5 protein level start to accumulate since 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 GALK is essential for the inhibitory effects of galactose during pollen germination. The unexpected observation that GALK is required for efficient galactose uptake in pollen may reveal an unknown regulatory mechanism for galactose transporters. Overall, SWEET5 and GALK contribute to the maintenance of galactose metabolic homeostasis during pollen germination, and galactose transport is positively regulated by GALK. The study of SWEET5 upon galactose condition also suggests SWEET5 is 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 itself 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 of sucrose still promote root growth (Kircher and Schopfer, 2012; Singh et al., 2014). Additionally, during *in vitro* pollen germination, 60 mM of galactose inhibits pollen germination (Hirsche et al., 2017), while 100 mM of glucose does not affect the pollen germination rate (Rottmann et al., 2018a) and high levels of sucrose (250-578 mM) are required for successful pollen germination (Wang et al., 2008; Rottmann et al., 2018a). 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 controlled (Ruan, 2014; Julius et al., 2017). The mutants of the two hexose transporter STPs (Sugar Transport Protein), AtSTP1 and AtSTP13, has 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 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 sugar transporters, ranging from ~10 to ~120 mM. AtSWEET5/VEX, belonging to SWEET family Clade II that primarily transports hexose (Chen et al., 2010; Eom et al., 2015), is strongly expressed in the pollen vegetative cell (Engel et al., 2005),

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

100 **Results**

101 **SWEET5 transports galactose and contributes to galactose inhibition of Arabidopsis pollen**
102 **germination**

103 To determine which SWEET in the mature pollen stage is able to transport galactose, we surveyed
104 the expression and protein accumulation of all SWEETs in Arabidopsis (Mergner et al., 2020).
105 Among all 17 members of the SWEET family, several were found to be expressed in mature pollen,
106 but only SWEET5, SWEET11 and SWEET12 proteins were detectable there (Figure 1A).
107 SWEET11 and SWEET12 have been reported to be transporters of sucrose (Chen et al., 2012),
108 while SWEET5 is the most likely transporter of galactose, since its homolog OsSWEET5 has been

109 found to transport galactose (Zhou et al., 2014). To test whether SWEET5 transports galactose,
110 we conducted growth assays with a yeast strain EBY.VW4000 lacking 17 hexose transporters
111 (Wieczorke et al., 1999). Yeast cells expressing SWEET5 grew on medium containing glucose
112 and galactose, but not fructose (Figure 1B). The Km for galactose uptake of SWEET5 was
113 calculated as 671 ± 58.4 mM (Figure 1C), however, caution should be taken in interpreting this
114 Km due to uncertain physiological effects on yeast when cultured with a high concentration (>500
115 mM). The pollen germination rate of Col-0 was dramatically reduced from 57% to 11% when
116 pollen germination medium (PGM) was supplemented with 60 mM Galactose (Figure 1D and 1E),
117 while remained unchanged when PGM was supplemented with either 60 mM glucose or fructose
118 (Supplemental Figure S1). The galactose suppression was barely detectable in *SWEET5* loss-of-
119 function mutants created from T-DNA insertion (*sweet5*) or CRISPR/CAS9 editing (Figure 1D
120 and 1E; Supplemental Figure S2). The phenotypes of the mutants were fully complemented by
121 transformation with a construct carrying the *SWEET5* promoter driving either endogenous
122 *SWEET5* or a synonymous m*SWEET5* that carries mutations to avoid gRNA recognition in
123 CRISPR mutant lines (p*SWEET5*:*mSWEET5/sweet5_crispr*; Figure 1D and 1E; Supplemental
124 Figure S2D). Accordingly, the SWEET5 overexpression lines (p*SWEET5*:*SWEET5*/Col-0) were
125 more sensitive to galactose than Col-0 (Figure 1D and 1E). In pollen of *sweet5* mutant, there were
126 no observable morphological phenotypes under normal conditions, which may be due to functional
127 redundancy with other plasma membrane-localized hexose transporters (STPs, PMTs) detected at
128 either the RNA or protein level in pollen (Supplemental Figure S3). Among those hexose
129 transporters that were detected at the protein level, many can transport glucose and galactose
130 (Klepek et al., 2005; Klepek et al., 2010; Poschet et al., 2010; Rottmann et al., 2018b).
131
132 **SWEET5 is mainly expressed at the late stages of pollen development**
133 To determine the timing of the initiation of SWEET5-associated galactose inhibition. we examined
134 the spatial and temporal accumulation patterns of SWEET5 using transgenic lines harboring a
135 SWEET5 translational fusion with β-glucuronidase (GUS) or yellow fluorescent protein (YFP)
136 driven by its native SWEET5 promoter (p*SWEET5*:*gSWEET5-GUS* or p*SWEET5*:*gSWEET5-YFP*).
137 SWEET5 was almost exclusively found in the anthers and mature pollen at late stages of flower
138 development (Figure 2A), although GUS staining was detected at the early seedling stage with a
139 preference in the vein. The SWEET5 protein accumulation pattern was carefully assessed in the

140 flowers. Flowers at different developmental stages (Bowman, 1994) were imaged using
141 fluorescence microscopy to detect SWEET5 protein levels using the reporter YFP. As shown in
142 Figure 2C, SWEET5 started to accumulate in immature pollen grains from flower stage 11, in
143 which pollen mitosis I and II occur (Cecchetti et al., 2008) and peaked at flower stage 13 (anthesis).
144 The YFP signal was also observed over the course of male gametophyte development by isolating
145 developing pollen grains. The SWEET5-YFP protein was first detected at the tricellular pollen
146 (TCP) stage and peaked at the mature pollen grain (MPG) stage, while it was undetectable in both
147 uninucleate microspores (UNM) and bicellular pollen (BCP), as delineated by PI staining of the
148 cell wall and nucleus (Figure 2B). Additionally, SWEET5 protein level substantially reduced in
149 germinated pollen compared with non-germinated pollen grains after *in vitro* pollen germination
150 (Figure 2D). The subcellular localization of SWEET5 was determined using pollen from
151 transgenic plants harboring a SWEET5 (CDS) translational fusion with green fluorescent protein
152 (GFP) driven by its native SWEET5 promoter (p*SWEET5*:*cSWEET5-GFP*). SWEET5 is localized
153 to the plasma membrane (PM), and also endomembrane compartments as delineated by Nile red
154 staining for the lipid droplets (Figure 2E), which has been similarly observed for other plasma
155 membrane localized proteins in pollen (Hamilton et al., 2015). SWEET5 localization was further
156 transiently examined using the reporter mVenus in *N. benthamiana* leaves (Gookin and Assmann,
157 2014). SWEET5 protein was localized to the pavement cell periphery enclosing both chloroplast
158 and Golgi apparatus (Figure 2F), which suggests SWEET5 localized to the plasma membrane,
159 consistent with published Arabidopsis SWEETs except for SWEET2, SWEET16, and SWEET17
160 that are localized to the tonoplast (Klemens et al., 2013; Guo et al., 2014; Chen et al., 2015a).
161

162 **Galactose sensitivity is GALK-dependent**

163 To further elucidate whether GALK is involved in the galactose sensitivity during pollen
164 germination, we first surveyed whether GALK is accumulated in the pollen. The protein level of
165 GALK is relatively consistent across all tissues, including mature pollen grains (Supplemental
166 Figure S4A). Therefore, we tested pollen germination of the loss-of-function *galk* mutant (Egert
167 et al., 2012) on PGM containing galactose. The *galk* mutant showed a galactose-tolerant phenotype
168 similar to that of *sweet5* (Figure 3A; Supplemental Figure S5A), although in mature pollen it is a
169 knock-down mutant with 80% reduction in RNA transcripts (Supplemental Figure S4B and S4C).
170 The *galk* galactose-tolerant phenotype was fully complemented by *GALK* expressed under control

171 of the pollen-specific *LAT52* promoter (Figure 3A; Supplemental Figure S5A). Notably, the *galk*
172 complementation line showed a much stronger galactose sensitivity than Col-0, which is likely
173 due to the over-expression of GALK in the complementation line (Supplemental Figure S4C). As
174 expected, the GALK and SWEET5 double mutant *sweet5 × galk* showed a similar galactose-
175 tolerant phenotype to their individual single mutants, which indicates that GALK and SWEET5
176 function in the same pathway way. As Gal-1-P is suggested to exerts cellular toxicity from many
177 studies in animals (Lai et al., 2009), it is reasonable to speculate that pollen sensitivity to galactose
178 may be due to the accumulation of potential cytotoxic Gal-1-P, which depends on GALK activity
179 (de Jongh et al., 2008). To test this possibility, we compared the effects Gal-1-P on pollen
180 germination, 6 mM Gal-1-P was sufficient to mimic the suppression effect produced by 60 mM
181 galactose in Col-0 (Figure 3B; Supplemental Figure S5B). Gal-1-P can be converted into UDP-
182 Gal by USP in plants (Feusi et al., 1999). To test whether the USP-catalyzed reaction could
183 alleviate galactose suppression, we generated over-expression lines of AtUSP driven by the pollen-
184 specific *pLAT52* promoter in Col-0 background. However, USP overexpression lines failed to
185 improve the pollen germination rate under 60 mM galactose condition (Supplemental Figure S6).
186
187 **Galactose inhibition is dosage-dependent**
188 To determine the range of concentration at which galactose inhibits pollen germination, pollen
189 from different genotypes were germinated on PGM with galactose concentrations ranging from 0
190 to 600 mM. In Col-0, the pollen germination rate was not affected under 0.6 mM galactose, started
191 to decline significantly at 6 mM, followed by a substantial decrease at 60 mM and further reduced
192 to almost zero at 600 mM (Figure 4A, Supplemental Figure S5C). A time-course pollen
193 germination experiments of Col-0 further confirmed that the pollen germination rate was
194 significantly affected in the presence of 6 mM galactose starting from four hours after germination
195 (Supplemental Figure S5D). However, no significant difference was observed between Col-0 and
196 *sweet5* mutants at 6 mM (Figure 5A, Supplemental Figure S5C). *sweet5* mutants were generally
197 tolerant to galactose up to 60 mM. Consistent with the observation, the SWEET5 overexpression
198 lines were more sensitive to ≥ 6 mM galactose than Col-0, with pollen germination rates reduced
199 to near zero by 60 mM galactose. These results suggest that SWEET5 is responsible for galactose
200 flux at the higher concentration range.

201 Galactose at 10 mM was reported to strongly inhibit root growth of Col-0 (medium containing no
202 sucrose, Yamada et al., 2011; Egert et al., 2012), while higher galactose (60 mM) is needed to
203 strongly inhibit Col-0 pollen germination (medium containing 15% (~ 439 mM) or 19.8 % (~578
204 mM) sucrose, Hirsche et al., 2017 or current study). To test whether the sucrose in the medium
205 may differentially affect observed galactose response, which may lead to biased comparison, we
206 conducted a galactose dosage-dependent assay for root growth using Col-0. Root growth was not
207 affected at 1 mM galactose but was strongly inhibited at 6 mM or higher without sucrose in the
208 medium (Supplemental Figure S7). By contrast, a significant root reduction was only observed at
209 60 mM galactose when 0.5% or 1% sucrose was supplemented in the medium. Therefore, the level
210 of inhibitory galactose concentration should be considered in the context of sucrose presence or
211 absence.
212
213 **GALK positively regulates galactose uptake in pollen independent of SWEET5 expression**
214 **level**
215 To assess the differences in galactose uptake among Col-0, *sweet5*, *galk*, and SWEET5
216 overexpression line, we conducted ^{14}C-galactose tracer uptake assays in pollen under various
217 galactose concentrations. Pollen grains were germinated in a liquid pollen germination system for
218 45 mins (the hydration period, Wang *et al*., 2008) to minimize the interference from transporters
219 expressed on pollen tubes. Less ^{14}C-galactose was taken up by *sweet5* pollen than by Col-0 pollen
220 at 60 mM galactose concentration; relative to the ~200 pmol galactose taken up by Col-0 pollen,
221 *sweet5* utilized only ~50 pmol when 60 mM of galactose was externally supplied (Figure 4B).
222 Differences were not prominent at lower amounts of galactose (Figure 4B); however, the SWEET5
223 overexpression lines had significantly higher ^{14}C-galactose uptake than Col-0 under these
224 conditions (Figure 4C). Further, *sweet5* and *sweet5_crispr* pollen did not have significantly
225 reduced ^{14}C-galactose uptake under lower galactose conditions (Figure 4C), which is consistent
226 with involvement of other galactose transporters, that have high-affinity. Finally, ^{14}C-galactose
227 uptake was dramatically decreased in *galk* as well as *galk*×*sweet5* under all three tested galactose
228 conditions (Figure 4C), suggesting a positive feedback regulation of GALK on galactose
229 transporters, including SWEET5. The fact that the double *galk*×*sweet5* mutant phenocopies the
230 *galk* single mutant in both galactose uptake and *in vitro* pollen germination indicates that GALK
231 is epistatic to SWEET5 in controlling galactose uptake by pollen. However, the level of *SWEET5*

232 expression cannot explain the decrease of galactose uptake in *galk*, since expression of *SWEET5*
233 as well as other hexose transporters (STP 6 and 8) were significantly upregulated in *galk* (Figure
234 4D; Supplemental Figure S8).
235
236 **Discussion**
237
238 **Pollen is a simple system to study the control of galactose flux**
239 Over the past several decades, extensive efforts have been made to study galactose toxicity in
240 humans (galactosemia) and yeast; however, the mechanism underlying the toxicity is far from
241 understood (Lai et al., 2009). Considerably less attention has been paid to galactose inhibition by
242 plant scientists. In the current study, we found that the PM-localized hexose transporter
243 AtSWEET5 is responsible for galactose sensitivity during pollen germination. A high
244 concentration of sucrose at 19.8% is needed in the medium for in vitro pollen germination, which
245 may be partially cleaved into glucose and fructose by the Arabidopsis cell-wall invertase 2 before
246 imported into pollen (Hirsche et al., 2009). As SWEET5 also transports glucose (Figure 1B),
247 glucose may competitively suppress galactose uptake, resulting in much less galactose transported
248 in pollen. Thus, a high concentration of galactose, such as 60 mM, is needed to result in a severe
249 inhibitory effect on pollen germination. This concept was supported by the reduced galactose
250 response during root growth of wild type plant in presence of 0.5 % sucrose (Supplemental Figure
251 S7). Even when 60 mM external galactose was used, the estimated concentration was only about
252 135 μM in Col-0 pollen after incubation with 60 mM galactose for 45 min. This was calculated
253 based on the uptake of 200 pmol galactose (Figure 4B) determined from 20 flowers that yielded a
254 combined 40,000 pollen (2000 pollen per flower), and the mean pollen volume deduced from the
255 Arabidopsis pollen diameter of 20.72 μm (De Storme et al., 2013). While pollen has a fundamental
256 role in the sexual reproduction of flowering plants, pollen germination together with pollen tube
257 growth have served as a model system to study single cell growth and morphogenesis (Feijó et al.,
258 2004). In Arabidopsis, pollen grains are highly homogeneous and have strikingly low
259 transcriptional complexity (on average, 6044 genes expressed in mature pollen; Rutley and Twell,
260 2015) compared to sporophytic tissues or purified sporophytic cells (e.g. 13,222 genes expressed
261 in stomatal guard cells, and 11,696 genes expressed in root hair cells; Bates et al., 2012, Becker et
262 al., 2014). Our work to understand relationships among galactose transport, GALK, and galactose

263 sensitivity sheds light on developing alternative strategies to attenuate galactose suppression by
264 fine-tuning their relationships.
265
266 **Model of galactose transport and metabolism during pollen *in vitro* germination**
267 From the data presented in this study and reported from the publication, a schematic model of
268 galactose sensitivity in pollen was illustrated in Figure 5: SWEET5, as a uniporter, transports
269 galactose across pollen membrane across concentration gradient. If galactose is not sufficiently
270 transported into pollen grains due to a lack of enough SWEET5 protein, a galactose-tolerant
271 phenotype can be observed. Similarly, the lack of AtSTP1 and AtSTP13 have shown a galactose-
272 tolerant phenotype in conditions of up to 100 mM galactose (Sherson et al., 2000, Yamada et al.,
273 2011). Once galactose is taken up by cells, it will first be phosphorylated by GALK to produce the
274 potentially toxic Gal-1-P. A galactose detoxification pathway that stores excess galactose in the
275 vacuoles was proposed in the *galk* mutant (Egert et al., 2012). This pathway of detoxification
276 cannot explain the galactose-tolerant phenotype of *galk* pollen, because galactose uptake is
277 significantly decreased in *galk* pollen (Figure 4C) and vacuoles are nearly absent from mature
278 pollen grains (Pacini et al., 2011). Both decreased galactose uptake and the disrupted Gal-1-P
279 production may therefore be responsible for the galactose tolerance of the *galk* mutant during
280 pollen germination. Though possible free galactose accumulation by *galk* pollen, similar to *galk*
281 leaves (Egert et al., 2012), may negatively impact galactose influx during pollen germination and
282 obscure our understanding; the positive relationship between GALK and galactose uptake
283 activities suggests a yet-unknown mechanism associated with post-transcriptional and/or
284 translational regulation of PM-localized transporters.
285
286 Our study suggested that Gal-1-P is responsible for the galactose sensitivity observed during pollen
287 germination, which is supported by the results of Gal-1-P effect on *in vitro* pollen germination.
288 However, caution should be taken when interpreting these results, because it is still unclear how
289 Gal-1-P can be taken up by pollen as no such transporters have been identified. Gal-1-P showed a
290 significant increase as the external galactose concentration rises beyond the inhibitory levels in
291 corn and barley coleoptiles (Roberts et al., 1971). In animals or yeast, Gal-1-P has been reported
292 to potentially inhibit enzymes such as phosphoglucomutase (de Jongh et al., 2008), UDP-glucose
293 pyrophosphorylase (UGPase, catalyzing the reversible conversion from Glc-1-P to UDP-Glc) (Lai

294 et al., 2003), and inositol monophosphatase (Bhat, 2003) *in vitro,* however, no *in vivo* targets of
295 Gal-1-P have been identified. In limited studies in plants, Gal-1-P has only been reported to inhibit
296 UGPase activity in the crude extract from oat coleoptile, but not from the azuki bean epicotyl,
297 suggesting that galactose sensitivity appears to vary depending on the species (Yamamoto et al.,
298 1988). The double mutant of AtUGP1 and AtUGP2 exhibited dramatic growth defects and male
299 sterility (Park et al., 2010). Similarly, strong male sterility was also observed in the mutants that
300 lack USP to catalyzes the reversible conversion of monosaccharide-1-P and UDP-monosaccharide
301 (Schnurr et al., 2006; Kotake et al., 2007; Geserick and Tenhaken., 2013). However, the limited
302 production of UDP-Glc, UDP-GlcA, and UDP-Ara are not likely to be responsible for the *usp*
303 phenotype (Geserick and Tenhaken, 2013), since overexpressing UGPase for UDP-Glc synthesis
304 and overexpressing UDP-glucose dehydrogenase for UDP-GlcA synthesis have failed to rescue
305 the *usp* mutant (Geserick and Tenhaken, 2013), and the mutant of UDP-xylose-4-epimerse, which
306 contributes mostly to UDP-Ara production, does not differ from the wild type (Burget et al., 2003).
307 Therefore, the conversion between Gal-1-P and UDP-Gal might be important for the *usp*
308 phenotype. We tested this possibility by overexpressing USP; however, it failed to alleviate
309 galactose suppression during pollen germination. The pool of UDP-Gal may result not only from
310 the reaction catalyzed by USP, but also the reversible conversion of UDP-Gal and UDP-Glc
311 catalyzed by the epimerase of UGE. The lack of both UGE2 and UGE3 is responsible for a male
312 sterile phenotype resembling the *usp* phenotype (Rosti et al., 2007). Additionally, low endogenous
313 UGE1 activity has been suggested to be responsible for the more severe galactose inhibition, along
314 with an increase in the UDP-Gal content (Dormann and Benning, 1998). Thus, the dynamically
315 quantitative analysis of metabolites, including Gal-1-P, Glc-1-P, UDP-Gal and UDP-Glc, may help
316 to elucidate the role of USP or UGE in galactose sensitivity. We attempted to measure these
317 galactose-associated metabolites upon galactose treatment in *sweet5*, however, no clear
318 conclusions can be drawn due to the difficulties of separating glucose and galactose conjugated
319 phosphates and nucleotide sugars using a LC-MS/MS system.
320
321 **SWEET5 orthologs may play important roles in hexose-utilizing pollen**
322 Compared to the proton sugar symporters, SWEET5 likely functions as a uniporter, facilitating
323 substrate movement along a substrate concentration gradient (Chen et al., 2015c). The
324 physiological importance of SWEET family members was underscored mainly by their

325 involvement in multiple processes requiring sugar transport capability, for instances, SWEET11
326 and SWEET12 in apoplasmic phloem loading (Chen *et al*., 2012); SWEET9 in nectar secretion
327 (Lin *et al*., 2014); and SWEET11, SWEET12, and SWEET15 in seed filling (Chen *et al*., 2015b).
328 In the current work, we showed that SWEET5 is highly expressed in the mature pollen but dramatic
329 declines during the transition from germinated pollen to pollen tubes, suggesting that SWEET5
330 functions during the early stages of pollen germination. By contrast, several STPs are primarily
331 expressed in pollen tubes but are almost absent in mature pollen grains (Rottmann et al., 2018a).
332 For examples, STP10 is a pollen tube-specific hexose transporter (Rottmann et al., 2016), the
333 glucose transporter STP9 is weakly expressed in mature pollen grains and is primarily expressed
334 in growing pollen tubes (Schneidereit et al., 2003). Therefore, SWEET5 and STPs may have
335 complementary roles during pollen germination. As reported, high concentrations of sugars
336 (ranging from 2% to 19.8%) are needed for successful germination *in vitro* in various species
337 (Ylstra et al., 1998; Stadler et al., 1999; Wang et al., 2008) since pollen germination is an energy-
338 demanding process (Reinders, 2016). However, the effect of different sugars on *in vitro* pollen
339 germination depends on the species. For example, sucrose and hexose differentially affect *in vitro*
340 pollen germination, ranging from growing equally well on glucose, fructose, or sucrose for petunia
341 (Ylstra et al., 1998), cucumber (Cheng et al., 2015), and pearl millet (Reger et al., 1992), to a
342 nearly complete inhibition by glucose or fructose for Arabidopsis (Hirsche et al., 2017). But for
343 some species, such as tobacco, either sucrose or glucose can support pollen germination, while
344 only sucrose can promote pollen tube growth (Goetz et al., 2017). Galactose alone can also
345 promote pollen germination for example in *Picea wilsonii* (Zhou et al., 2020) and cucumber
346 (Cheng et al., 2015) to a similar extent as sucrose. Therefore, the lack of a phenotype in *sweet5*
347 under normal conditions may be due to the preference of Arabidopsis pollen during the initial
348 phase of germination for sucrose. Considering SWEET5 also transports glucose, it is rational to
349 speculate that SWEET5 orthologues, in the species that prefer to use hexose as a carbon source
350 during pollen germination or pollen tube growth, may be essential for pollen development, and
351 therefore seed production.
352
353 **Conclusions**
354

355 We have presented SWEET5 as a PM-localized hexose transporter that is highly expressed in
356 mature pollen grains. SWEET5 in synergy with GALK is responsible for the galactose sensitivity
357 observed during pollen germination in Arabidopsis. Pollen germination studies served as a tool to
358 elucidate SWEET5 biological functions and provide a model system that facilitates research to
359 uncover mechanisms underlying galactose sensitivity. The future comparison of the effect of
360 galactose or glucose on pollen germination, associated with either SWEET5 or its homologs from
361 species that favor different carbon sources for pollen germination, will offer new insights into the
362 carbon skeleton and/or energy supply mechanisms responsible for successful reproduction.
363

364 **Materials and Methods**

365 **Constructs for yeast expression**

366 The CDSs of *AtSWEET5* were amplified by RT–PCR using gene specific primers named by gene
367 (Supplemental Table S2**,** P1 and P2) and were subsequently cloned into the donor vector
368 pDONR221-f1 by BP clonase (Invitrogen) before transfer to pDRf1-GW (Loque et al., 2007) by
369 LR clonase (Invitrogen).
370

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

372 To construct *SWEET5* fused with GUS (p*SWEET5*:*gSWEET5-GUS*), the fragment including the
373 *SWEET5* native promoter sequence (1801 bp upstream of ATG) and genomic sequence of
374 *SWEET5* was amplified (P3 and P4) and cloned into pDONR221-f1 by BP reactions followed by
375 recombination into the vector pMDC163 carrying the GUS gene (Curtis and Grossniklaus, 2003)
376 by LR reaction. For the SWEET5 overexpression construct (p*SWEET5*:*gSWEET5-YFP*), the entry
377 clone from above was transferred into pEarleyGateTW1 (Wang et al., 2016) by LR reactions.
378

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

381 The *SWEET5* (1216 bp upstream of ATG) native promoter, and *LAT52* promoter (602 bp;
382 Muschietti et al., 1994) were amplified with specific forward primers (P5-P8) containing a *BamH*I
383 restriction site and the reverse primers containing an *Xba*I restriction site and sub-cloned into the
384 eGFP-containing vector pGTKan3 (Kasaras and Kunze, 2010) via *BamH*I and *Xba*I restriction

385 sites. The CDS of *SWEET5, GALK*, and *USP* by their own specific primers (P9-P14) were
386 seamlessly subcloned into corresponding pGTKan3_promoter constructs by In-Fusion® (Takara)
387 following the linearization at *Xba*I and *Pst*I restriction sites. For the genome editing construct of
388 *SWEET5*, gRNA that targets the first exon of SWEET5 (Supplemental Figure S1D) was cloned
389 into p*YAO*:*hSpCas9* (Yan et al., 2015). For the synonymous complementation construct of Cas9
390 containing *sweet5_crispr,* synonymous *SWEET5* driven by pSWEET5 was amplified by primer
391 P15 and P16 based on pDONR221_pSWEET5-*SWEET5* with gRNA targeting the region modified
392 through site-directed mutagenesis before transferring into pEarleyGate301 (Earley et al., 2006) by
393 LR reactions.
394
395 **Construct for subcellular localization**
396 For subcellular localization of SWEET5, after the vector pDOE-13 (Gookin and Assmann, 2014)
397 was linearized by *SanD*I + *AatI*I restriction enzymes at MCS3, the CDS of SWEET5 was amplified
398 by primers P17 and P18 and subcloned into it by In-Fusion® (Takara). The agroinfiltration-based
399 assays were performed as previously described (Gookin and Assmann, 2014). The *N. benthamiana*
400 leaf disks were collected 72 h post infiltration for confocal imaging. Three independent trials were
401 conducted.
402
403 **Plant materials and growth conditions**
404 The Arabidopsis Col-0 plants were grown under controlled temperature (22°C) with a 16-h light
405 (100-150 $\mu Em^{-2}s^{-1}$)/ 8-h dark photoperiod. The flowers at stage 13 (fully opened) were collected
406 at around 5 hours into the light period for different experiments. T-DNA mutants of *galk* (GABI-
407 Kat 489D10), and *sweet5* (CS853155) were obtained from NASC or ABRC. Homozygous lines
408 were genotyped using primers of P19-P24 and used in related experiments. The floral dip method
409 (Clough and Bent, 1998) was used to generate all the transgenic lines used in this study. At least
410 15 T_1 lines were generated for each construct and at least 3 randomly selected lines were
411 propagated to generate T_3 homozygous seeds.
412
413 **Yeast complementation assay and ^{14}C uptake assay**
414 Yeast complementation and yeast ^{14}C uptake assays were conducted following the previously
415 described method (Chen et al., 2010). ^{14}C-galactose (0.5 μCi D-[1-^{14}C] galactose; 56.2 mCi/mmol)

416 was added per sample and four independent transformants were used for each uptake experiment.
417 For concentration- and time-dependent uptake of [14C] galactose for SWEET5, 5 galactose
418 concentrations (25, 50, 100, 250, 500 mM), and 4 time points (10 sec, 2.5, 5, and 10 min) for each
419 concentration were used to collect yeast cell samples and measure galactose uptake. Sugar uptake
420 at 10 sec was normalized as 0.
421
422 ***In vitro* pollen germination**
423 The *in vitro* pollen germination assay was conducted according to a previously described method
424 (Wang et al., 2008) with minor modifications. The sucrose only basic pollen germination medium
425 (PGM) was composed of 19.8% (w/v) Suc (15% (w/v) Suc in liquid PGM), 1.5 mM boric acid,
426 0.8 mM $MgSO_4$, 1 mM KCl, 5 mM MES, 0.05% (w/v) lactalbumin hydrolysate, 10 μM myo-
427 inositol, 5 mM $CaCl_2$ and 1.5% (w/v) agarose. The pH was adjusted to 5.8 using 1 mM Tris (pH
428 = 8). The medium solution was heated to boiling on a heat plate, and then 500 μl was spread evenly
429 onto 75 × 25 mm glass slides. Slides were cooled down at room temperature before placing in an
430 opaque, sealed slide box. The medium was made freshly for each germination experiment. Six
431 flowers at stage 13 were collected from each genotype to spread pollen gently onto a ~1 cm^2 area
432 of the germination medium. The slide box was kept at room temperature for 8 hours in the dark
433 before samples on slides were imaged with a compound microscope. Pollen with pollen tubes
434 longer than the diameter of the pollen grain (about 20 μm) was considered as germinated pollen.
435
436 **Pollen galactose uptake**
437 The liquid pollen germination method was adopted from a previously described method (Wang et
438 al., 2008) with minor modifications for ^{14}C-galactose uptake. For each trial, 20 flowers (at least
439 2000 pollen grains collected per flower) at stage 13 for each genotype were collected into a 2 ml
440 tube, and then 1 ml of liquid PGM was added in and vortexed vigorously for 60 s to release mature
441 pollen grains into the solution. Subsequently, the pollen grains were counted using a
442 hemocytometer. Equal amounts of pollen for all tested genotypes were collected by centrifuging
443 at 15,000 g for 1 min. The pollen pellet was resuspended in 30 μl of liquid PGM containing ^{14}C-
444 galactose (0.3 μCi hot galactose) as well as different concentrations of cold galactose (ranging
445 from 0 to 60 mM), and subsequently cultured in a petri dish (35 mm in diameter). The petri dish
446 was covered and placed in the dark for 45 min before pollen was washed using 1 ml of ice-cold

447 liquid PGM and collected into a 1.5 ml tube. The pollen was precipitated at 15,000 g for 1 min and
448 the pellet was washed by 1 ml ice-cold pollen isolation buffer (PIB, composed of 100 mM $NaPO_4$,
449 pH 7.5, 1 mM EDTA, and 0.1% (v/v) Triton X-100) 5 more times. The pellet was resuspended in
450 100 µl PIB and transferred to a scintillation vial containing 5 ml Ultima Gold XR Scintillation
451 liquid (Perkinelmer). Radioactivity for each vial was measured by a liquid scintillation
452 spectrometry.
453
454 **GUS histochemical analysis**
455 GUS staining was performed as previously described (Chen et al., 2012). Twelve-day old seedlings
456 were used, and inflorescences and siliques were collected for histochemical GUS staining.
457
458 **Microscopy imaging**
459 A Zeiss Apotome.2 (Carl Zeiss, Thornwood, NY, USA) was used for fluorescence acquisition.
460 Different FL filter sets (YFP, CFP, RFP and GFP) were used to image samples as needed. A Zeiss
461 LSM 710 confocal microscope (Carl Zeiss, Thornwood, NY, USA) was used to image samples.
462 Argon laser excitation wavelength and emission bandwidth were 458 nm and 480–520 nm for
463 mTq2 (cyan), 488 nm and 500–550 nm for mVenus (yellow), and 633 nm and 633-740 nm for
464 chlorophyll autofluorescence (red) respectively. Image acquisition parameters were held
465 consistent. Raw data from each channel were not altered beyond equal signal increases.
466
467 **RNA isolation and RT-qPCR**
468 RNA isolation from pollen was performed as previously described with modifications (Lu, 2011).
469 For each independent sample, pollen grains from 50 flowers at stage 13 were collected. For
470 samples under galactose treatment, pollen grains from 50 flowers were germinated in liquid PGM
471 for 45 mins as described above in pollen galactose uptake. RNA was isolated using Trizol
472 (Invitrogen) as instructed by the manufacturer. First-strand cDNA was synthesized using oligo(dT)
473 and M-MuLV reverse transcriptase (NEB). Real-time qPCR was performed using PowerUp™
474 SYBR™ master mix (Applied Biosystems) according to the manufacturer's instructions on a
475 CFX96 Real-Time PCR Detection System (Bio-Rad) using gene specific primers (P25-P34). The
476 expression values were normalized to *ACTIN8* expression values in each repeat, and subsequently
477 normalized to Col-0 using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

478

479 **Statistical analysis**

480 The differences between the two subjects were determined using the two-tailed Student's t-test
481 with equal variance. The differences among multiple subjects were assessed using one-way
482 ANOVA followed by multiple comparison tests (Fisher's LSD method). All statistical analysis
483 was performed using SPSS 26 statistical software (SPSS Inc, Chicago, IL, USA).

484

485 **Accession numbers**

486 Sequence information from this article can be found in the Arabidopsis Genome Initiative or
487 GenBank/EMBL databases under the following accession numbers: SWEET5 (AT5G62850),
488 GALK (AT3G06580), USP (AT5G52560).

489

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495

496

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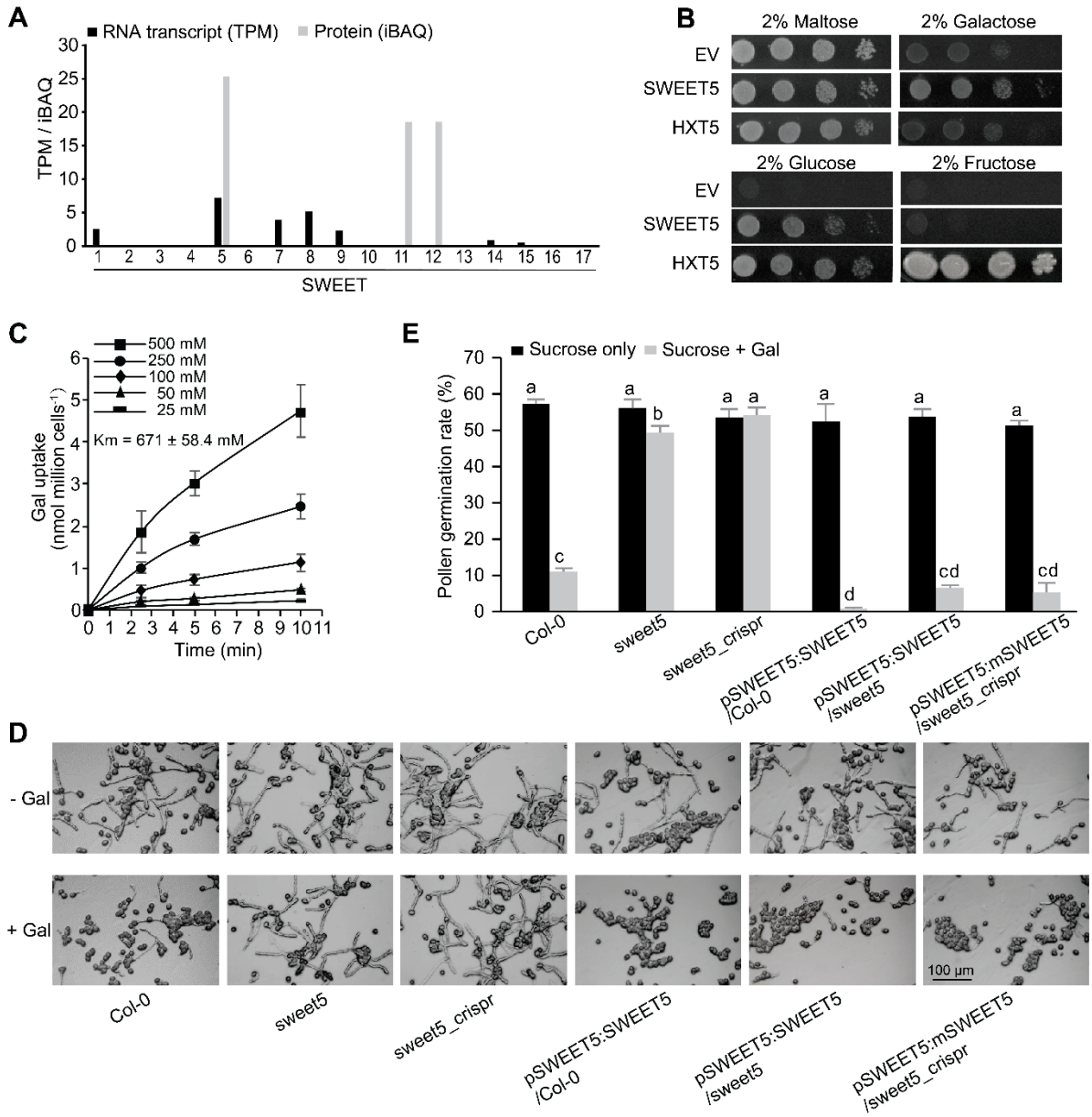

A
RNA transcript (TPM)
Protein (iBAQ)
TPM / iBAQ
SWEET
B
2% Maltose
2% Galactose
2% Glucose
2% Fructose
EV
SWEET5
HXT5
C
500 mM
250 mM
100 mM
50 mM
25 mM
Km = 671 ± 58.4 mM
Gal uptake (nmol million cells-1)
Time (min)
E
Sucrose only
Sucrose + Gal
Pollen germination rate (%)
Col-0
sweet5
sweet5_crispr
pSWEET5:SWEET5 /Col-0
pSWEET5:SWEET5 /sweet5
pSWEET5:mSWEET5 /sweet5_crispr
D
- Gal
+ Gal
100 µm

722

723

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

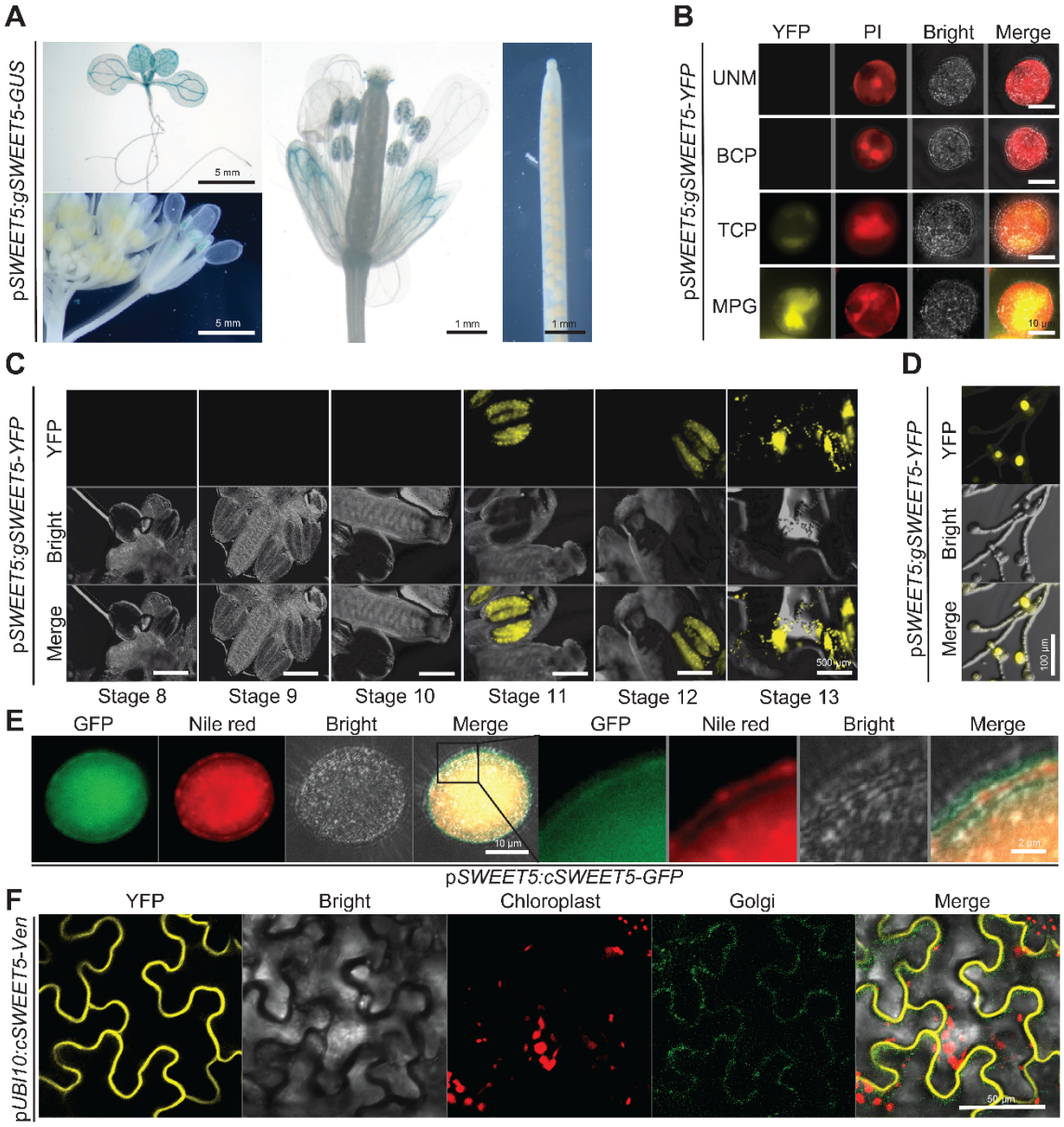
725 (A) 17 SWEET transcripts and protein levels in Arabidopsis mature pollen. Data were retrieved
726 from athena.proteomics.wzw.tum.de.

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

729 (C) Concentration-dependent uptake of ^{14}C-Galactose over time by yeast transformed with
730 SWEET5 or an empty vector as a negative control (mean ± SE, n = 4). The negative control is
731 subtracted.

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

734 (E) Statistical analysis of pollen germination rates for various genotypes. The means were
735 calculated from multiple repeats (± SE, n ≥ 9), with over 450 pollen grains/tubes counted in total.
736 The statistically significant differences among different samples were determined using one-way
737 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
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

738

739 **Figure 2.** Tissue-specific accumulation and cellular localization of SWEET5.

740 (A) SWEET5 tissue-specific expression was evaluated. Twelve-day old seedlings, inflorescences
741 and siliques from about one-month old Arabidopsis carrying p*SWEET5*:*gSWEET5-GUS* were
742 histochemically stained 4 h for GUS activity.

743 (B) SWEET5 localization in pollen grain was examined using fluorescence microscopy. Images
744 were captured using the YFP filter for YFP, the RFP filter for propidium iodine (PI) staining, and
745 under bright field. SWEET5 was detected in the tricellular pollen grain (TCP) and mature pollen
746 grain (MPG) but absent in early stages of uninucleate microspores (UNM) and bicellular pollen
747 (BCP).

748 (C) SWEET5 accumulation in different floral stages of Arabidopsis was examined using
749 fluorescence microscopy. The signals started to be detected from stage 11.

750 (D) SWEET5 accumulation was detected after pollen germination in p*SWEET5*:*gSWEET5-YFP*
751 *lines*. Pictures were taken 8 hours post-germination using a fluorescence microscope.

752 (E) SWEET5 cellular localization was examined in mature pollen carrying p*SWEET5*:*cSWEET5-*
753 *GFP*.

754 (F) SWEET5 cellular localization was examined 72 hours after infiltration of *Nicotiana*
755 *benthamiana* leaves with *pUBI10*:*cSWEET5-mVenus*. Pictures were taken using confocal
756 microscopy.

757

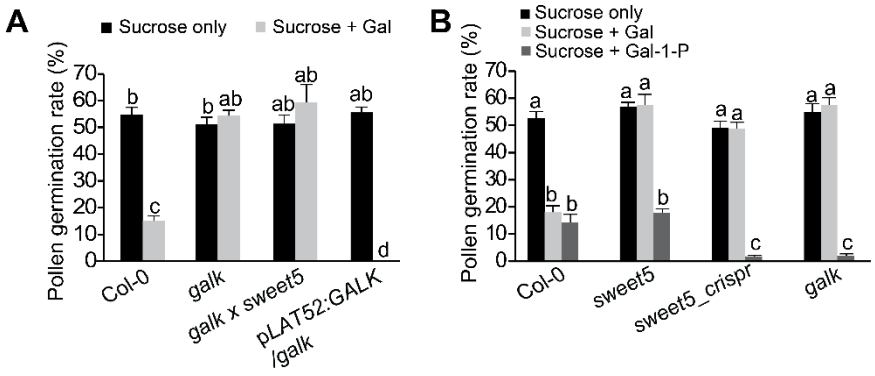
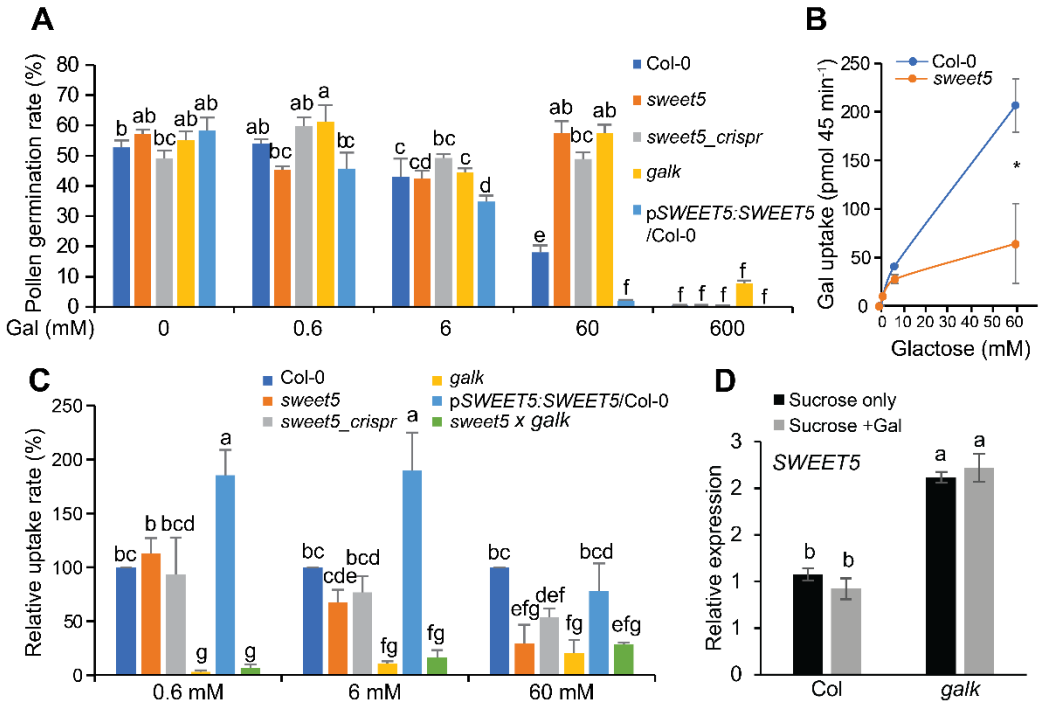


Figure 3. Involvement of GALK in galactose-inhibited pollen germination

(A) Examination of *in vitro* pollen germination on sucrose-based medium with 0 mM or 60 mM galactose for various genotypes. (means ± SE, n ≥ 6 (over 300 pollen grains/tubes counted) and $P < 0.05$).

(B) Effects of galactose-1-phosphate (6 mM) on *in vitro* pollen germination (means ± SE, n ≥ 6 (over 300 pollen grain/tubes counted) and $P < 0.05$).



765

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

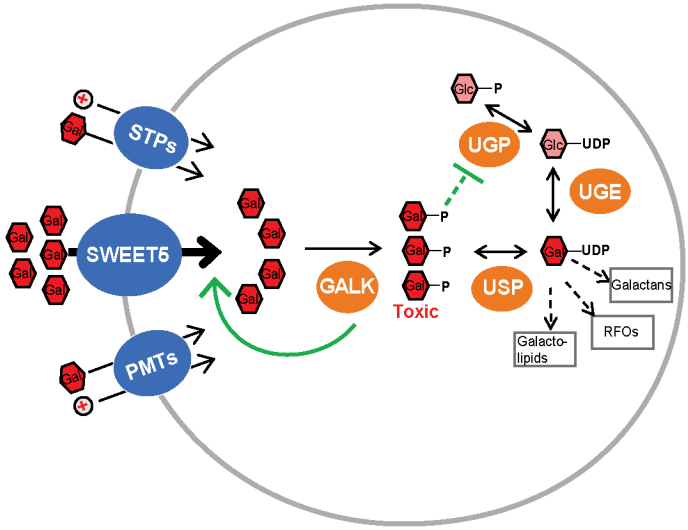
767 (A) *In vitro* pollen germination of various genotypes on medium supplemented with different
768 concentrations of galactose (ranging from 0 to 600 mM). The means (± SE) of at least 6 repeats
769 were plotted (over 300 pollen grains/tubes tested) ($P < 0.05$).

770 (B) Concentration-dependent galactose uptake in pollen of Col-0 and *sweet5* (means ± SE, n=3
771 and $P < 0.05$). Pollen germinated on liquid medium containing 0 mM, 0.6 mM, 6 mM, or 60 mM
772 galactose in addition to 0.3 μCi ^{14}C-galactose per sample for 45 min.

773 (C) Relative ^{14}C-galactose uptake by mature pollen grains of various genotypes under different
774 galactose concentrations (means ± SE, n=3). Pollen germinated on liquid medium containing 0.6
775 mM, 6 mM, or 60 mM galactose in addition to 0.3 μCi ^{14}C-galactose per sample for 45 min. The
776 ^{14}C-galactose uptake rate for individual samples was normalized to Col-0, whose uptake rate was
777 converted to 100%, for each concentration.

778 (D) Expression of *SWEET5* of Col-0 and the *galk* mutant tested by RT-qPCR with or without
779 galactose treatment (means ± SE, n=3 and $P < 0.05$). Pollen germinated on liquid medium
780 containing 0 mM, or 60 mM galactose for 45 min. *ACT8* was used as an internal control gene.

781 The gene expression level of Col-0 under sucrose only condition was normalized to 1. Different
782 letters represent significant difference ($P < 0.05$).



783

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

791

792