https://doi.org/10.1093/plphys/kiac068 PLANT PHYSIOLOGY 2022: 189: 388–401

Plant Physiology®

Research Article

Hexose transporter SWEET5 confers galactose sensitivity to Arabidopsis pollen germination via a galactokinase

Jiang Wang [1,2], Ya-Chi Yu [1], Ye Li [1,3,†] and Li-Qing Chen [1,2,*,‡]

1 Department of Plant Biology, University of Illinois at Urbana-Champaign, Urbana, Illinois, 61801, USA
2 Carl R. Woese Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801, USA
3 Biotechnology Research Institute, Chinese Academy of Agricultural Sciences, Beijing 100081, China

*Author for correspondence: lqchen77@illinois.edu
†Present address: Key Laboratory of Agricultural Products Chemical and Biological Processing Technology, Zhejiang University of Science and Technology, Hangzhou 310023, China
‡Senior author.

J.W. and L.-Q.C. conceived and designed the experiments. J.W., Y.-C.Y., and Y.L. conducted experiments. J.W. and L.Q.C. wrote the manuscript.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (https://academic.oup.com/plphys/pages/general-instructions) is: Li-Qing Chen (lqchen77@illinois.edu).

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 its derivatives are inhibitory to plant growth. In Arabidopsis (*Arabidopsis thaliana*), root growth and pollen germination are 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, Arabidopsis Sugars Will Eventually be Exported Transporter 5, that transports glucose and galactose. SWEET5 protein levels started to accumulate at the tricellular stage of pollen development and peaked in mature pollen, before rapidly declining after pollen germinated. SWEET5 levels are responsible for the dosage-dependent sensitivity to galactose, and galactokinase is essential for these inhibitory effects during pollen germination. However, sugar measurement results indicate that galactose flux dynamics and sugar metabolism, rather than the steady-state galactose level, may explain phenotypic differences between *sweet5* and Col-0 in galactose inhibition of pollen germination.

Introduction

Carbon flux, 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 sugars varies depending on the specific sugar involved. For example, in Arabidopsis (*Arabidopsis thaliana*), a low concentration of galactose (10 mM) 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). Likewise, 60 mM galactose inhibits *in vitro* pollen germination (Hirsche et al., 2017), while 100 mM glucose does not

Received September 07, 2021. Accepted January 15, 2022. Advance access publication February 21, 2022

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. The transporter that is involved in the galactose-suppression of pollen germination, however, remains unexplored.

Sugar transporters and enzymes are key components in the supply-demand system that maintains carbon flux control (Ruan, 2014; Julius et al., 2017). Mutants of the two hexose transporters, Sugar Transport Protein 1 (AtSTP1) and AtSTP13, show a galactose-tolerant phenotype for root growth (Sherson et al., 2000, Yamada et al., 2011). 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 Sugars Will Eventually be Exported Transporters (SWEETs) transport different sugars, including galactose. So far, all plant SWEETs with a measured Km, for example, Arabidopsis AtSWEET1 (Chen et al., 2010), AtSWEET12 (Chen et al., 2012), AtSWEET17 (Guo et al., 2014), tomato SlSWEET1a (Ho et al., 2019), and cucumber CsSWEET7a (Li et al., 2021), have been found to function as low-affinity glucose or sucrose transporters (with Km values ranging from ~10–120 mM). It is not unusual to observe that a SWEET transports different sugars but with different efficiency and Km values (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/Vegetative cell Expressed 1*, belonging to SWEET family Clade II that primarily transports hexoses (Chen et al., 2010; Eom et al., 2015), is strongly expressed in the pollen vegetative cell (Engel et al., 2005), but no other details have been reported. OsSWEET5 (Zhou et al., 2014) and CsSWEET7a (Li et al., 2021) have been demonstrated to transport galactose. Taken together, we hypothesized that SWEET5 might be responsible for galactose suppression of pollen germination.

Once galactose enters a cell, it is activated by the cytosolic enzyme galactokinase (GALK), which phosphorylates α-D-Gal into α-D-Gal-1-P at the C-1 position (Cardini and Leloir, 1953). Gal-1-P can be further converted into UDP-Gal through a reversible reaction catalyzed either by Gal-1-P uridylyltransferase in the presence of UDP-Glc (mainly used in nonplant species; Leloir, 1951) or by UDP-sugar pyrophosphorylase (USP) using UTP as a substrate (mainly found in plants) (Feusi et al., 1999; Kotake et al., 2004). The activated UDP-Gal can be converted reversibly into UDP-Glc by UDP-Glc epimerase (GALE/UGE) (Maxwell and Derobichonszulmajster, 1960; Seifert et al., 2002). Only a single copy of the *GALK* gene was reported in Arabidopsis (Sherson et al., 1999; Yang et al., 2009; Egert et al., 2012), and a galactose-insensitive phenotype in root growth was observed in *galk* (Egert et al., 2012). As GALK is the first enzyme to catalyze galactose, we analyzed its role together with the SWEET5 transporter during pollen germination.

We show the plasma membrane-localized SWEET5 transports both glucose and galactose. Data from this study indicate that SWEET5 and GALK play critical roles in controlling the response to galactose during pollen germination. SWEET5 transports galactose, which can then be phosphorylated by GALK, resulting in inhibition of pollen germination through yet unknown mechanisms.

Results

SWEET5 transports galactose

To determine which SWEET is able to transport galactose in mature pollen, we surveyed the expression and protein accumulation of all SWEETs in Arabidopsis (Mergner et al., 2020). Among all 17 members of the SWEET family, several were found to be expressed in mature pollen, but only SWEET5, SWEET11, and SWEET12 proteins were detectable (Supplemental Figure S1). SWEET11 and SWEET12 have been reported to preferentially transport sucrose (Chen et al., 2012). SWEET5 is the most likely transporter of galactose, since its homolog OsSWEET5 has been found to transport galactose (Zhou et al., 2014). To test if SWEET5 transports galactose, we conducted growth assays with yeast (*Saccharomyces cerevisiae*) strain EBY.VW4000, which lacks 17 hexose transporters (Wieczorke et al., 1999). Yeast cells expressing *SWEET5* grew on medium containing glucose and galactose, but not fructose (Figure 1A). Radiotracer uptake assays showed that SWEET5 could transport both galactose and glucose (Figure 1B). In a sugar competition experiment, galactose was insufficient to compete with glucose in uptake, even at 10 times higher galactose concentration. In contrast, the same concentration of glucose significantly reduced glucose uptake, while sorbitol failed to significantly compete with glucose uptake. (Figure 1C). These results indicate that galactose is less efficiently transported by SWEET5 relative to glucose.

A kinetic analysis found that SWEET5 exhibited an apparent Km of 671 ± 58 mM for galactose uptake (Figure 1D). The possibility that high osmolarity, introduced by high concentrations of galactose, affects Km estimation was excluded by the finding that there was no significant difference in Km analyzed with or without the osmolarity adjusted (Supplemental Figure S2).

SWEET5 contributes to galactose inhibition of Arabidopsis pollen germination

To test how galactose affects pollen germination under our experimental conditions, we performed pollen germination experiments using pollen germination medium (PGM) supplemented with 60 mM galactose and with or without other sugars. The pollen germination rate of Col-0 was dramatically reduced, from 57% to 11%, on PGM with 60 mM galactose (Figure 1, E and F), while remaining unchanged when PGM was supplemented with either 60 mM glucose or fructose (Supplemental Figure S3). The galactose suppression

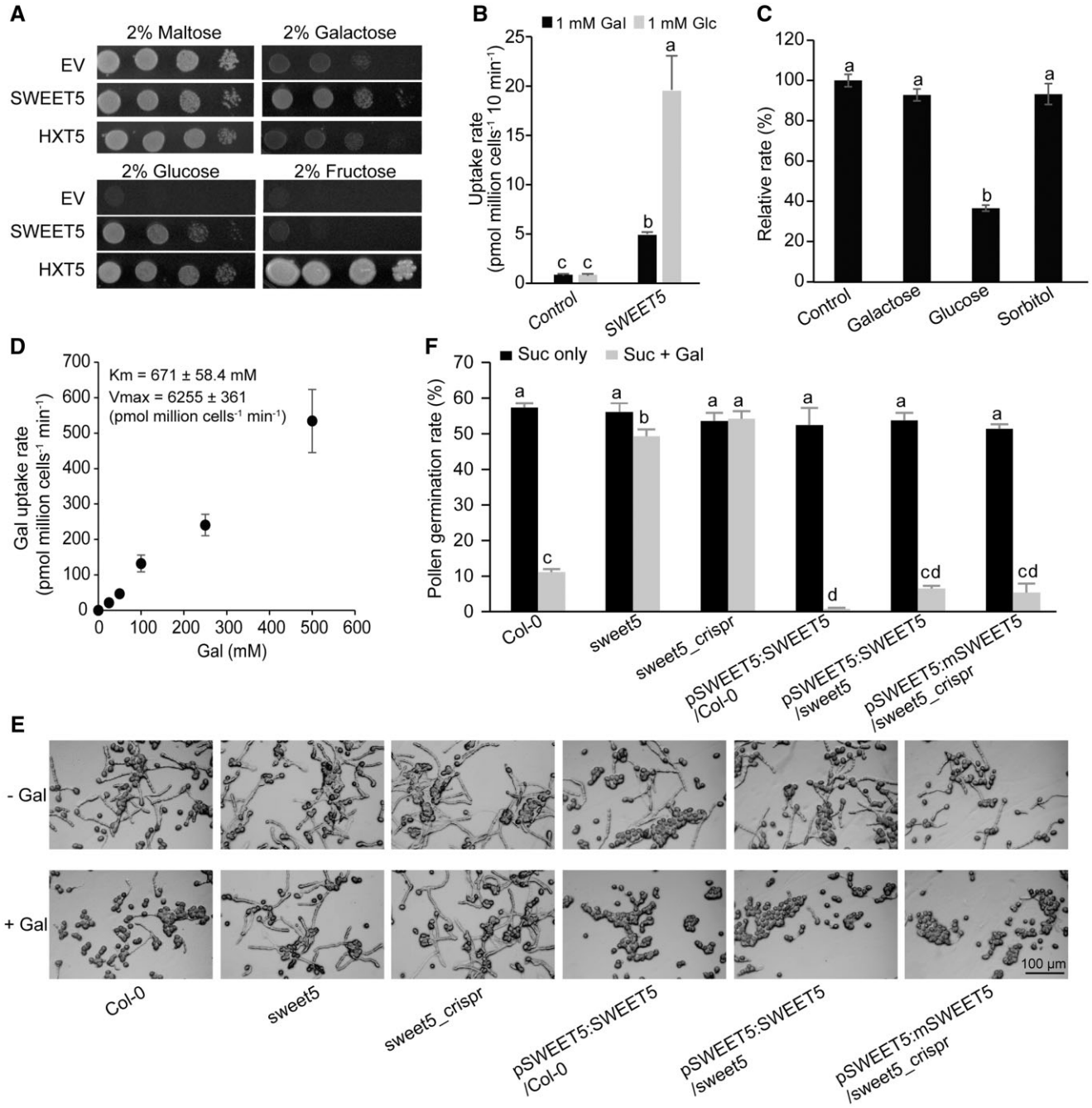

Figure 1 Characterization of Arabidopsis SWEET5. A, Functional analysis of SWEET5 by EBY.VW4000 yeast complementation assay. The empty vector (EV) as a negative control, or HXT5 as a positive control. The pictures were taken on Day 5 after spotting yeast cells on galactose-containing medium, but on Day 3 after spotting yeast cells on other sugar-containing mediums. B, SWEET5-mediated uptake of 1 mM ^{14}C-galactose (Gal) and ^{14}C-glucose (Glc) over 10 min in yeast cells. Cells transformed with an EV were used as a negative control (mean ± SE, n = 4). C, Substrate competition uptake analysis of SWEET5 in yeast over 10 min. Different 50 mM cold sugars competed with 5 mM cold Glc (including ^{14}C-Glc). The control uptake without competing sugar was normalized to 100% (mean ± SE, n = 4). D, Kinetics of ^{14}C-Gal accumulation by SWEET5 in yeast (mean ± SE, n = 4). An EV served as a negative control and its values were subtracted. E, *In vitro* pollen germination assay of various genotypes on sucrose (Suc)-based medium with or without 60 mM Gal. The pictures were taken 8-h postgermination. F, Statistical analysis of pollen germination rates for various genotypes. The means were calculated from multiple repeats (±SE, $n \geqslant 9$), with over 450 pollen grains/tubes counted in total. The statistically significant differences among different samples in (B), (C), and (F) were determined using one-way ANOVA followed by multiple comparison tests (Fisher's LSD test) and were represented by different letters ($P < 0.05$).

was barely detectable in *SWEET5* loss-of-function mutants created by either T-DNA insertion (*sweet5*) or clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (CAS9) editing (Figure 1, E and F; Supplemental Figure S4). The phenotypes of the mutants were fully complemented by transformation with a

construct carrying the *SWEET5* promoter driving either endogenous *SWEET5* or a synonymous *mSWEET5* that carries mutations to avoid gRNA recognition in CRISPR mutant lines (p*SWEET5*:*mSWEET5*/*sweet5_crispr*; Figure 1, E and F; Supplemental Figure S4D). Accordingly, the SWEET5 overexpression lines (p*SWEET5*:*SWEET5*/Col-0) were more sensitive to galactose than Col-0 (Figure 1, E and F). To test whether pollen germination is affected in the *sweet5* mutant, we compared *in vivo* pollen germination/pollen tube growth of *sweet5* with that of Col-0 on Col-0 pistil after hand-pollination. No obvious differences in the *in vivo* pollen germination/pollen tube growth were observed (aided by aniline blue staining) at 2 or 6 h after pollination (Supplemental Figure S4G). This suggests that apoplasmic galactose content of the stigma cells is not high enough to trigger inhibition of pollen germination under normal conditions. However, it is not known if galactose content will increase under stress conditions to affect pollen germination. For example, soluble galactose was significantly increased in coffee (*Coffea arabica*) plant leaves under heat stress, likely due to cell-wall modifications (Lima et al., 2013). Moreover, there were no observable morphological phenotypes in the *sweet5* mutant under normal conditions, which may be due to functional redundancy with other plasma membrane-localized hexose transporters, such as STPs and polyol/monosaccharide transporters (PMTs), that were detectable at either the RNA or the protein level in pollen (Supplemental Figure S5). Among those hexose transporters that were detected at the protein level, many can transport both glucose and galactose (Supplemental Table S1).

SWEET5 is mainly expressed at the late stages of pollen development

To determine the timing of SWEET5-associated galactose inhibition, we examined the spatial and temporal accumulation patterns of SWEET5 using transgenic lines harboring a SWEET5 translational fusion with β-glucuronidase (GUS) or with yellow fluorescent protein (YFP) driven by its native SWEET5 promoter (*pSWEET5*:*gSWEET5-GUS* or p*SWEET5*:*gSWEET5-YFP*). SWEET5 was almost exclusively found in the anthers and mature pollen at late stages of flower development (Figure 2A), although GUS staining was detected at the early seedling stage with a preference in the vein. The SWEET5 protein accumulation pattern was carefully assessed in the flowers. Flowers at different developmental stages (Bowman, 1994) were imaged using fluorescence microscopy to detect SWEET5 protein levels using the reporter YFP. As shown in Figure 2C, SWEET5 started to accumulate in immature pollen grains from flower stage 11, in which pollen mitosis I and II occur (Cecchetti et al., 2008), and peaked at flower stage 13 (anthesis). The YFP signal was also observed over the course of male gametophyte development by isolating developing pollen grains. The SWEET5-YFP protein was first detected at the tricellular pollen stage and peaked at the mature pollen grain (MPG) stage, while it was undetectable in both uninucleate microspores (UNMs) and bicellular pollen (BCP), as delineated by propidium iodide (PI) staining of the cell wall and nucleus (Figure 2B). The observed pattern of SWEET5-YFP protein agrees with the reported gene expression profiling of *SWEET5* in Arabidopsis pollen over different developmental stages (Honys and Twell, 2004), namely that *SWEET5* was highly expressed at the tricellular stage and mature pollen stage. Additionally, the SWEET5 protein level was substantially reduced in germinated pollen/pollen tube (marked by white arrow) compared with nongerminated pollen grains after *in vitro* pollen germination (Figure 2D).

Subcellular localization of SWEET5 was determined using pollen from transgenic plants harboring a SWEET5 (CDS) translational fusion with a green fluorescent protein (GFP) driven by its native SWEET5 promoter (*pSWEET5*:*cSWEET5-GFP*). SWEET5 was localized to the plasma membrane and in endomembrane compartments, as delineated by Nile red staining of the lipid droplets (Figure 2E). It is not unusual to observe strong fluorescence throughout the cell, including endomembrane, when a tagged protein is abundant in pollen (Tunc-Ozdemir et al., 2013). This has been similarly observed for other plasma membrane-localized proteins in pollen (Frietsch et al., 2007; Tunc-Ozdemir et al., 2013; Hamilton et al., 2015). SWEET5 localization was transiently examined using the reporter mVenus in *Nicotiana benthamiana* leaves (Gookin and Assmann, 2014). In pavement cells, SWEET5 protein localized to the periphery enclosing both chloroplast and Golgi apparatus (Figure 2F), which suggests SWEET5 localized to the plasma membrane. This was consistent with Arabidopsis SWEETs, except for SWEET2, SWEET16, and SWEET17 that localize to the tonoplast (Klemens et al., 2013; Guo et al., 2014; Chen et al., 2015a).

Galactose sensitivity is GALK dependent during pollen germination

Since GALK is the first galactose catalytic enzyme and may be involved in galactose sensitivity during pollen germination, we first surveyed whether GALK accumulates in pollen. The RNA transcripts of *GALK* are abundant in pollen at all developmental stages, and preferentially in the microspore and BCP stages (Honys and Twell, 2004). The protein level of GALK is relatively consistent across all tissues, including MPGs (Supplemental Figure S6A). We next tested pollen germination of the loss-of-function homozygous *galk* mutant (Supplemental Figure S6B; Egert et al., 2012) on PGM containing galactose. The *galk* mutant showed a galactose-tolerant phenotype similar to that of *sweet5* (Figure 3A; Supplemental Figure S7A), although *galk* is a knockdown mutant with an 80% reduction in mature pollen RNA transcripts (Supplemental Figure S6C). The *galk* galactose-tolerant phenotype was fully complemented by *GALK* expressed under the control of the pollen-specific *LAT52* promoter (Figure 3A; Supplemental Figure S7A). Notably, the *galk* complementation line showed a much stronger galactose sensitivity than Col-0, which is likely due to the over-expression of *GALK* in the complementation line

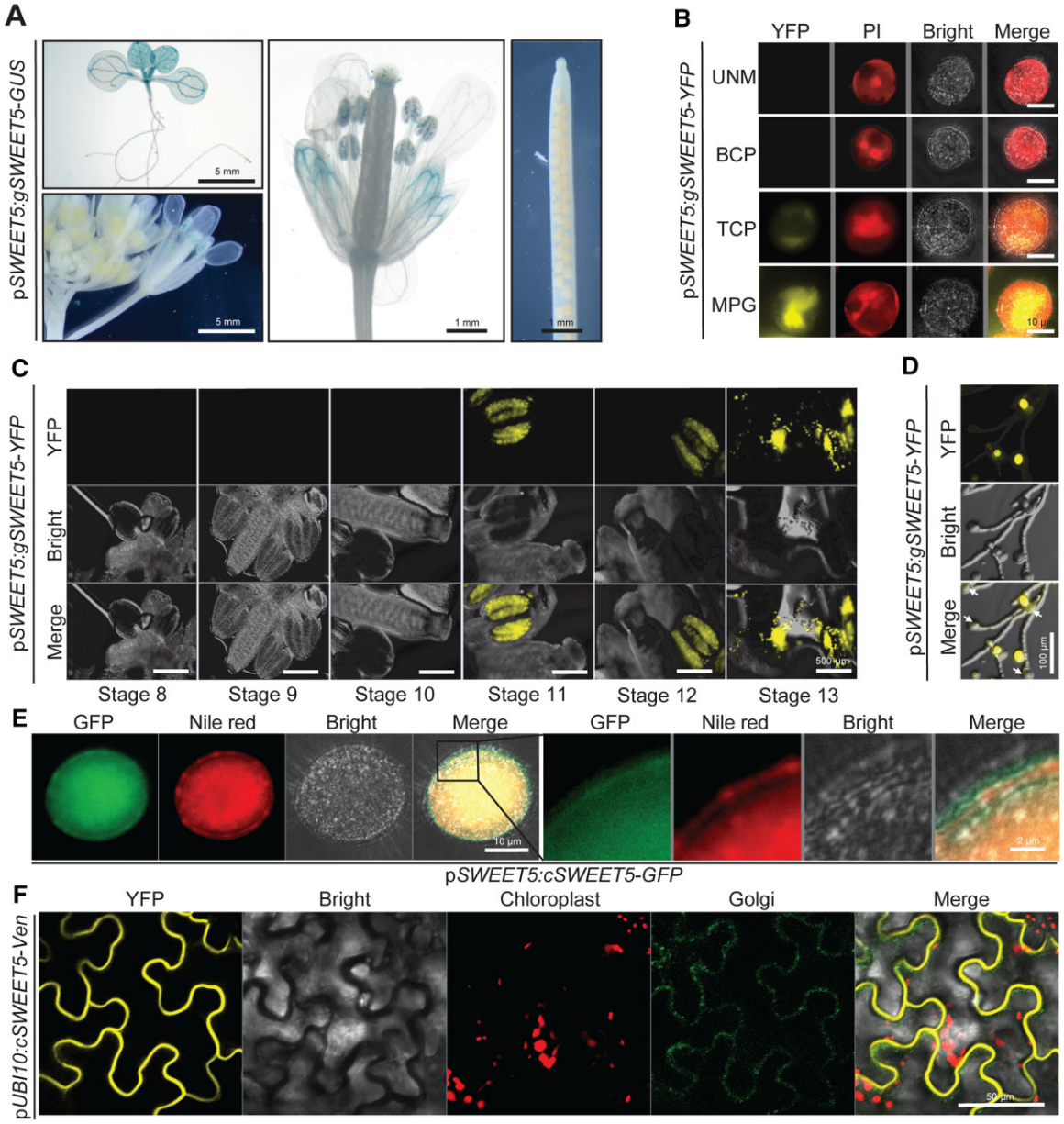

Figure 2 Tissue-specific accumulation and cellular localization of SWEET5. A, SWEET5 tissue-specific expression was evaluated. Twelve-day-old seedlings and inflorescences and siliques from ∼1-month-old Arabidopsis carrying *pSWEET5:gSWEET5-GUS* were histochemically stained for 4 h for GUS activity. B, SWEET5 localization in pollen grain was examined using fluorescence microscopy. Images were captured using the YFP filter for YFP, the RFP filter for PI staining, and under bright field. Exposure time and histogram range were set as 500 ms and 0–65,000 for YFP, 20 ms and 24,000–65,000 for RFP, and 300 ms and 0–25,000 for the bright field. SWEET5 was detected in the tricellular pollen grain and MPG, but absent in early stages of UNMs and BCP. C, SWEET5 accumulation in different floral stages of Arabidopsis was examined using fluorescence microscopy. The signals started to be detected from stage 11. Exposure time and histogram range were set as 150 ms and 0–16,000 for YFP, and 500 ms and 0–16,000 for the bright field. D, SWEET5 accumulation was detected after pollen germination in *pSWEET5:gSWEET5-YFP* lines. Pictures were taken 8 h postgermination using a fluorescence microscope. Exposure time and histogram range were set as 1,000 ms and 4,900–40,000 for YFP, 4 ms and 0–28,000 for the bright field. White arrows point to germinated pollen with much weaker fluorescence than un-germinated pollen. E, SWEET5 cellular localization was examined in mature pollen carrying *pSWEET5:cSWEET5-GFP*. Images were captured using the GFP filter for GFP, the RFP filter for nile red (lipid) staining, and under the bright field. Exposure time and histogram range were set as 1.5 ms and 5,000–9,000 for GFP, 90 ms and 29,000–46,000 for RFP, and 500 ms and 450–3,600 for the bright field. F, SWEET5 cellular localization was examined 72 h after infiltration of *N. benthamiana* leaves with *pUBI10:cSWEET5-mVenus*. Pictures were taken using confocal microscopy. Argon laser excitation wavelength and emission bandwidths were 488 nm and 500–550 nm for mVenus (yellow), 633 nm and 633–740 nm for chlorophyll autofluorescence (red), and 458 nm and 480–520 nm for mTq2 (cyan; Golgi), respectively.

(Supplemental Figure S6C). As expected, the *sweet5* × *galk* double mutant showed a similar galactose-tolerant phenotype to their individual single mutants, which indicates that GALK and SWEET5 function in the same pathway. As many studies in animals suggest that Gal-1-P causes cellular toxicity (Lai et al., 2009), it is reasonable to speculate that pollen sensitivity to galactose may be due to the accumulation of potential cytotoxic Gal-1-P, which depends on GALK activity (de Jongh et al., 2008). To test this possibility, we compared the effects of Gal-1-P on *in vitro* pollen germination. Six millimolar Gal-1-P was sufficient to mimic the suppression effect produced by 60 mM galactose in Col-0 (Figure 3B; Supplemental Figure S7B). Gal-1-P can be converted into UDP-Gal by USP in plants (Feusi et al., 1999). To test whether the USP-catalyzed reaction could alleviate galactose suppression, we generated over-expression lines of *AtUSP* driven by the pollen-specific *pLAT52* promoter in Col-0 background. However, *USP* overexpression lines failed to improve the pollen germination rate <60 mM galactose (Supplemental Figure S8).

Galactose inhibition is dosage dependent

To determine the range of concentration at which galactose inhibits pollen germination, pollen from different genotypes were germinated on PGM with galactose concentrations ranging from 0 to 600 mM. For Col-0, the pollen germination rate was not affected <0.6 mM galactose, started to decline significantly at 6 mM, substantially decreased at 60 mM, and was further reduced to almost zero at 600 mM (Figure 4A; Supplemental Figure S7C). A time-course pollen germination experiment of Col-0 further confirmed that the pollen germination rate was significantly affected by 6 mM galactose starting from 4 h after germination (Supplemental Figure S7D). However, no significant difference was observed between Col-0 and *sweet5* mutants at 6 mM (Figure 4A; Supplemental Figure S7C). The *sweet5* mutants were generally tolerant to galactose up to 60 mM, while Col-0 was not. Consistent with this observation, the SWEET5 overexpression lines were more sensitive to $\geqslant 6$ mM galactose, with pollen germination rates reduced to near zero by 60 mM galactose. These results suggest that SWEET5 is responsible for galactose flux at the higher concentration range. To assess the differences in galactose uptake between Col-0 and *sweet5*, we conducted ^{14}C-galactose tracer uptake assays by exposing pollen to the various concentrations of galactose. It is worth noting that the procedure we adopted was developed by Wang et al. (2008). Importantly, germinating pollen was collected after 45 min in liquid PGM (the hydration period) to minimize interference from transporters expressed during pollen tube growth. Less ^{14}C-galactose was taken up by *sweet5* pollen than by Col-0 pollen at 60 mM galactose concentration (Figure 4B). In contrast, differences were not prominent at lower amounts of galactose (Figure 4B).

Galactose at 10 mM was reported to strongly inhibit root growth of Col-0 (medium containing no sucrose, Yamada et al., 2011; Egert et al., 2012), while higher galactose (60 mM) is needed to strongly inhibit Col-0 pollen germination (medium containing 15% (~439 mM) or 19.8% (~578 mM) sucrose, Hirsche et al., 2017 or this study). To test whether the sucrose in the medium may differentially affect the observed galactose response, which may lead to a biased comparison, we conducted a galactose dosage-dependent assay for root growth using Col-0. Without sucrose in the medium, root growth was not affected at 1 mM galactose but was strongly inhibited at 6 mM or higher galactose (Supplemental Figure S9). In contrast, significant root reduction was only observed at 60 mM galactose when 0.5% or 1% sucrose was supplemented in the medium. Therefore, the level of inhibitory galactose concentration should be considered in the context of the presence or absence of sucrose.

Sugar content in pollen was altered in the presence of galactose

As galactose inhibition of pollen germination is dependent on the concentration of galactose, it is reasonable to

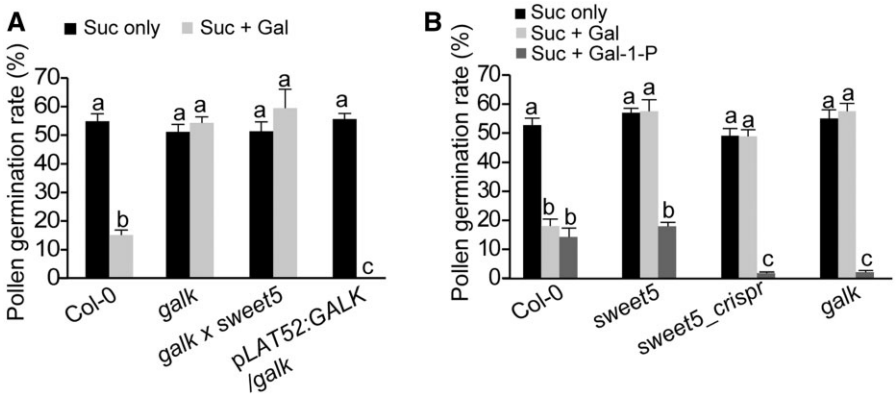

Figure 3 Involvement of GALK in Gal-inhibited pollen germination. A, Examination of *in vitro* germination of pollen from various genotypes on Suc-based medium with 0 or 60 mM Gal. (means ± SE, $n \geqslant 6$ [over 300 pollen grains/tubes counted]). B, Effects of Gal-1-phosphate (Gal-1-P, 6 mM) on *in vitro* pollen germination (means ± SE, $n \geqslant 6$ [>300 pollen grain/tubes counted]). The statistically significant differences among different samples in (A) and (B) were determined using one-way ANOVA followed by multiple comparison tests (Fisher's LSD method) and were represented by different letters ($P < 0.05$).

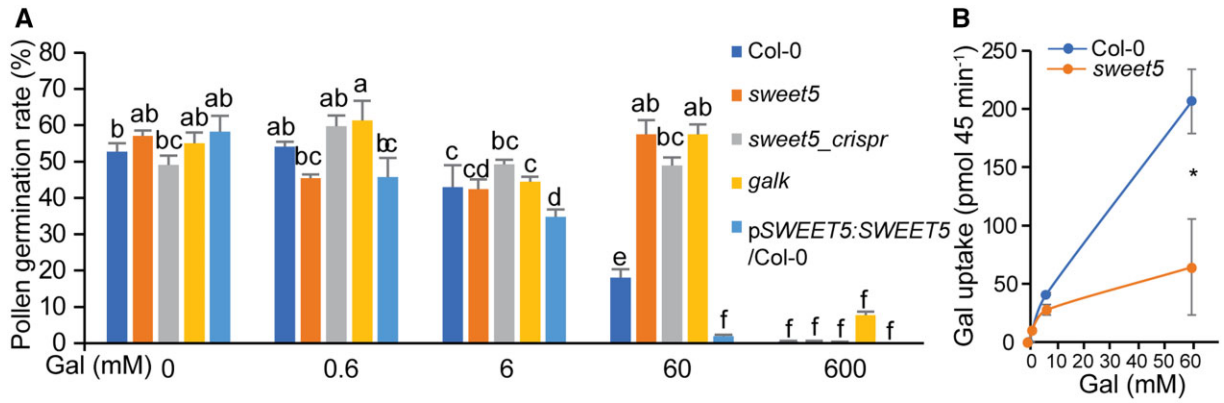

Figure 4 Concentration-dependent Gal response. A, *In vitro* pollen germination of various genotypes on medium supplemented with different concentrations of Gal (ranging from 0 to 600 mM) (The means [±SE] of at least six repeats were plotted [over300 pollen grains/tubes tested]). B, Concentration-dependent Gal uptake in pollen of Col-0 and *sweet5* (means ± SE, $n = 3$). Pollen germinated on liquid medium containing 0, 0.6, 6, or 60 mM Gal in addition to 0.3-μCi ^{14}C-Gal per sample for 45 min. The statistically significant differences among different samples in (A) were determined using one-way ANOVA followed by multiple comparison tests (Fisher's LSD method) and were represented by different letters ($P < 0.05$). The statistically significant differences in (B) were determined using Student's *t* test ($P < 0.05$).

hypothesize that some difference in the galactose content between Col-0 pollen and *sweet5* pollen may explain the differential responses to galactose. To test this hypothesis, we quantified galactose and other sugars, including glucose, fructose, and sucrose, in both MPGs and pollen grains hydrated with or without galactose. Unexpectedly, galactose contents were not significantly different between Col-0 and *sweet5* mutant under either one of the conditions (Figure 5A). However, galactose content was relatively abundant in MPGs (~20-nmol/mg fresh weight (FW)), and it further increased to ~60-nmol/mg FW after hydration. There were no significant differences observed with or without supplemented galactose (Figure 5A). These data suggest that the steady-state galactose level in pollen is well-controlled by developmental and dynamical adjustment of its metabolism in response to changes between developmental stages, in galactose supplies, and/or galactose influx (Figure 4B). Thus, it is unlikely that only the steady-state galactose level can explain phenotypic differences in galactose inhibition.

Both glucose and sucrose content was significantly decreased in Col-0 pollen in the presence of galactose, relative to its absence, while their contents were not significantly different in the *sweet5* mutant (Figure 5, B and C). In addition, the fructose level also exhibited a similar, albeit insignificant, trend (Figure 5D). Therefore, the reduction in glucose, fructose, and sucrose levels in Col-0 pollen in the presence of galactose corresponds with the galactose-inhibited Col-0 pollen germination. In contrast, the unaltered glucose, fructose, and sucrose levels in *sweet5* pollen in the presence of galactose correspond with the uninhibited *sweet5* pollen germination, supporting the hypothesis that abundant sugar is needed for successful pollen germination (Goetz et al., 2017). Notably, gas chromatography–mass spectrometry (GC–MS) showed that the overall glucose and fructose content is much higher than the sucrose content, but caution is needed when interpreting the relative amounts of sucrose, glucose and fructose, which may be observed differently when measured using different methods, such as liquid chromatography-tandem mass spectrometry (LC–MS/MS) (Arbona et al., 2009).

The above results indicated that either pollen hydration or galactose addition alters glucose and fructose accumulation. Sucrose in plants can be catalyzed by either sucrose synthase (SUS) or invertase (INV), but none of the six *SUS* genes is highly expressed in either MPGs or hydrated pollen (Qin et al., 2009). We assessed the transcript levels of the alkaline/neutral INVs (*A/N-InvH and A/N-InvD*) and the *cell-wall INVs* (*cwINV2* and *cwINV4*), which are highly expressed in Arabidopsis pollen (Mergner et al., 2020). The transcript level of *A/N-InvH*, encoding a cytosol-localized alkaline/neutral INV H (Battaglia et al., 2017), was significantly reduced in Col-0 but not in *sweet5* upon galactose treatment (Figure 5E), consistent with the reduced glucose and fructose levels observed in Col-0 pollen under galactose treatment. The transcript levels of other INVs were not significantly affected under our treatments (Supplemental Figure S10). Overall, the galactose influx controlled by SWEET5, rather than the steady-state galactose level, may be responsible for the observed galactose inhibition phenotype, which corresponds with differentially accumulated internal glucose, fructose, and sucrose levels during *in vitro* pollen germination.

Discussion

Pollen is a simple system to study the control of galactose flux

While pollen has a fundamental role in the sexual reproduction of flowering plants, pollen germination together with pollen tube growth has served as a model system to study single-cell growth and morphogenesis (Feijo et al., 2004). In Arabidopsis, pollen grains are highly homogeneous and have strikingly low transcriptional complexity. On average, 6,044

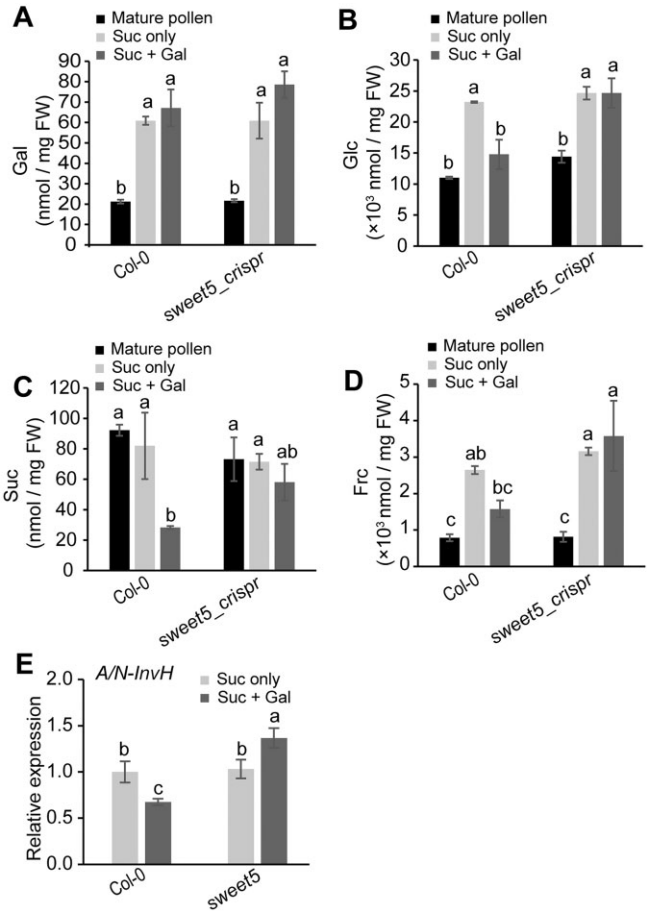

Figure 5 Internal sugar content was altered upon Gal inhibition. A, Gal, (B) Glc, (C) Suc, and (D) Frc content measured from Col-0 and *sweet5* mutant pollen grains collected directly off fresh flowers (mature pollen) or after treatments with or without 60 mM Gal during in vitro germination. Pollen was germinated on a liquid medium containing Suc and 0 or 60 mM Gal for 45 min (means ± SE, $n = 3$). E, Expression of *A/N-InvH* in Col-0 and *sweet5* tested by RT-qPCR with or without Gal treatment (means ± SE, $n = 3$ and $P < 0.05$). Pollen was germinated on liquid medium containing Suc and 0 or 60 mM Gal for 45 min. *ACT8* was used as an internal control gene. The gene expression level of Col-0 under Suc only condition was normalized to 1. The statistically significant differences among different samples in (A–E) were determined using one-way ANOVA followed by multiple comparison tests (Fisher's LSD method) and were represented by different letters ($P < 0.05$).

genes are expressed in mature pollen (Rutley and Twell, 2015), compared to sporophytic tissues or purified sporophytic cells (e.g. 13,222 genes expressed in stomatal guard cells; 11,696 genes expressed in root hair cells; Bates et al., 2012; Becker et al., 2014). Our work to understand relationships among galactose transport, GALK, and galactose sensitivity sheds light on developing alternative strategies to attenuate galactose suppression, possibly by fine tuning these relationships.

Over the past several decades, extensive efforts have been made to study galactose toxicity in humans (galactosemia) and in yeast; however, the mechanism underlying the toxicity is far from understood (Lai et al., 2009). Considerably less attention has been paid to galactose inhibition in plants. In the current study, we found that the plasma membrane-localized hexose transporter AtSWEET5 is responsible for galactose sensitivity during pollen germination. In Arabidopsis, a high concentration of sucrose (19.8%) is needed in the medium for *in vitro* pollen germination, which may be partially cleaved into glucose and fructose by cwINV2 before import into pollen (Hirsche et al., 2009). As SWEET5 prefers to transport glucose (Figure 1C), glucose may competitively suppress galactose uptake, resulting in less galactose transported in pollen. Thus, a high concentration of galactose, such as 60 mM, is needed to result in a severe inhibitory effect on pollen germination. This concept was supported by the reduced galactose response during root growth of wild-type plants in the presence of 0.5% sucrose (Supplemental Figure S9). Even when 60 mM galactose was used, the galactose content in pollen was not significantly changed compared to the sucrose-only control (Figure 5A), but galactose influx is significantly lower in *sweet5* under the same conditions (Figure 4B). These results support that galactose level is tightly controlled within a cell and that the rate of galactose metabolism is rapidly adjusted based on the availability and transport of galactose across the plasma membrane.

Model of galactose transport and metabolism during *in vitro* germination of pollen

From the data presented in this study and from the literature, a schematic model of galactose sensitivity in pollen is illustrated in Figure 6: SWEET5, as a uniporter, transports galactose across the pollen membrane down a concentration gradient. If galactose is not sufficiently transported into pollen grains, due to a lack of enough SWEET5 protein, a galactose-tolerant phenotype can be observed. Similarly, the lack of AtSTP1 and AtSTP13 creates galactose-tolerant root growth in up to 100 mM galactose (Sherson et al., 2000, Yamada et al., 2011). Once galactose is taken up by cells, it will first be phosphorylated by GALK to produce the potentially toxic Gal-1-P. A galactose detoxification pathway that stores excess galactose in the vacuoles was proposed in the *galk* mutant (Egert et al., 2012). However, this pathway of detoxification cannot explain the galactose-tolerant phenotype of *galk* pollen, because vacuoles are nearly absent from MPGs (Pacini et al., 2011). Therefore, the disrupted Gal-1-P production may be responsible for the galactose tolerance of the *galk* mutant during pollen germination. It is possible that free galactose may accumulate in *galk* pollen, similar to in *galk* leaves (Egert et al., 2012), and this galactose may negatively impact galactose influx during pollen germination and obscure our understanding.

Our study suggested that Gal-1-P is likely responsible for the galactose sensitivity observed during pollen germination, which is supported by the results of the effect of Gal-1-P on *in vitro* pollen germination. However, caution should be taken when interpreting these results, because it is still unclear how Gal-1-P can be taken up by pollen, as no such transporters have been identified. Gal-1-P did show a

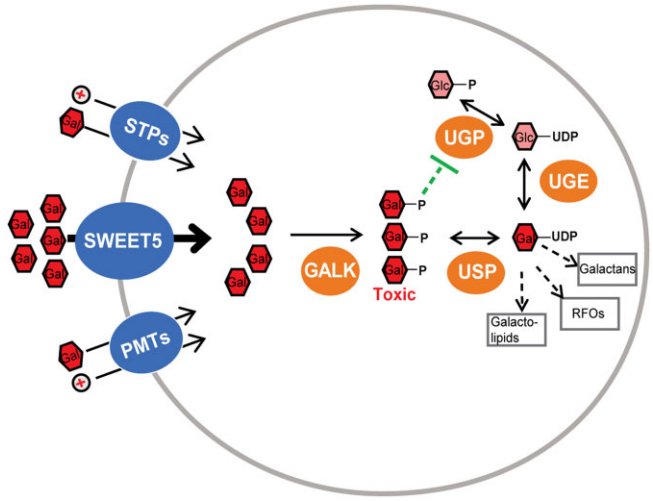

Figure 6 Schematic representation of Gal transport and metabolism in Arabidopsis pollen. A simplified pollen structure was drawn for clarity. Blue ovals represent plasma membrane-localized Gal transporters. Orange ovals represent enzymes during Gal metabolism. Black boxes represent downstream Gal-containing compounds. Arrows represent positive regulation, and bar-headed lines indicate negative regulations. The black lines represent the Gal metabolic flux. The thick black line means the higher flux. The green dashed line is for the hypothetical regulation. The black dashed lines represent the indirect metabolic flow. Galacto-lipids are predominantly found in plastid membranes. Raffinose Oligosaccharides mainly include raffinose and stachyose containing one and two Gal moieties, respectively. Galactans represent the cell wall-bound Gal.

substantial increase when the external galactose concentration exceeded the inhibitory levels in maize (*Zea mays*) and barley (*Hordeum vulgare*) coleoptiles (Roberts et al., 1971). In animals and yeast, Gal-1-P has been reported to potentially inhibit enzymes such as phosphoglucomutase (de Jongh et al., 2008), UDP-glucose pyrophosphorylase (UGPase, catalyzing the reversible conversion from Glc-1-P to UDP-Glc; Lai et al., 2003), and inositol monophosphatase (Bhat, 2003); however, no *in vivo* targets of Gal-1-P have been identified. In limited studies in plants, Gal-1-P has only been reported to inhibit UGPase activity in the crude extract from oat (*Avena sativa*) coleoptiles, but not from azuki bean (*Vigna angularis*) epicotyl, suggesting that galactose sensitivity appears to vary depending on the species (Yamamoto et al., 1988). We attempted to measure the galactose-associated metabolites upon galactose treatment in *sweet5* and *galk*. However, no clear conclusions can be drawn due to the challenges in separating glucose- and galactose-conjugated phosphates and nucleotide sugars using an LC–MS/MS system. Additionally, we found that *A/N-InvH* was downregulated in Col-0 but not in *sweet5* by galactose addition, which is consistent with the changes in glucose, fructose, and sucrose levels (Figure 5) and that the mutant phenotype of *A/N-InvH* shows a reduced number of seeds per silique (Battaglia et al., 2017). However, further investigation into what directly triggers these changes is required.

SWEET5 putative orthologs may play essential roles in hexose utilization in pollen

Compared to the proton sugar symporters, SWEET5 likely functions as a uniporter, facilitating substrate movement along a substrate concentration gradient (Chen et al., 2015b). The physiological importance of SWEET proteins was underscored mainly by their involvement in multiple processes requiring high sugar transport capability. For instance, SWEET11 and SWEET12 in apoplasmic phloem loading (Chen et al., 2012); SWEET9 in nectar secretion (Lin et al., 2014); and SWEET11, SWEET12, and SWEET15 in seed filling (Chen et al., 2015c). In this work, we showed that SWEET5 is highly expressed in mature pollen but dramatically declines during the transition from germinated pollen to pollen tubes, suggesting that SWEET5 functions during the early stages of pollen germination. In contrast, several STPs are primarily expressed in pollen tubes but are almost absent in MPGs (Rottmann et al., 2018). For example, STP10 is a pollen tube-specific hexose transporter (Rottmann et al., 2016); and the glucose transporter STP9 is weakly expressed in MPGs and is primarily expressed in growing pollen tubes (Schneidereit et al., 2003). Therefore, SWEET5 and STPs may have complementary roles during pollen germination. High concentrations of sugars (ranging from 2% to 19.8%) are needed for success *in vitro* germination in various species (Ylstra et al., 1998; Stadler et al., 1999; Wang et al., 2008), since pollen germination is an energy-demanding process (Reinders, 2016). However, the effect of different sugars on *in vitro* pollen germination depends on the species. For example, sucrose and hexose differentially affect *in vitro* pollen germination, ranging from growing equally well on glucose, fructose, or sucrose for petunia (*Petunia hybrida*; Ylstra et al., 1998), cucumber (*Cucumis sativus*; Cheng et al., 2015), and pearl millet (*Pennisetum glaucum*) (Reger et al., 1992), to a nearly complete inhibition by glucose or fructose for Arabidopsis (Hirsche et al., 2017). But for some species, such as tobacco (*Nicotiana tabacum*), either sucrose or glucose can support pollen germination, while only sucrose can promote pollen tube growth (Goetz et al., 2017). Galactose alone can also promote pollen germination, for example, in spruce (*Picea wilsonii*; Zhou et al., 2020) and cucumber (Cheng et al., 2015), to a similar extent as sucrose. Therefore, the lack of a phenotype in *sweet5* under normal conditions may be due to the preference for sucrose of Arabidopsis pollen during the initial phase of germination. Considering SWEET5 also transports glucose, it is rational to speculate that SWEET5 putative orthologs may be essential for pollen development, and therefore seed production, in species that prefer to use hexose as a carbon source during pollen germination or pollen tube growth.

Conclusions

We have presented SWEET5 as a plasma membrane-localized hexose transporter that is highly expressed in MPGs. GALK catalyzes the synthesis of Gal-1-P from the galactose that can be transported by SWEET5. The pollen

grains of *sweet5* and *galk* mutants are tolerant to galactose inhibition during pollen germination *in vitro*, suggesting that SWEET5 and GALK play a critical role in the sensitivity of pollen germination to galactose in Arabidopsis. Pollen germination can serve as a tool to elucidate SWEET5 biological functions and provide a model system that facilitates research to uncover mechanisms underlying galactose sensitivity. Future work with SWEET5 and its homologs from species that favor different carbon sources for pollen germination will offer insights into the carbon skeleton and/or energy supply mechanisms responsible for successful reproduction.

Materials and methods

Constructs for yeast expression

The CDS of *AtSWEET5* were amplified by RT-PCR using gene-specific primers named by the gene (Supplemental Table S2, P1 and P2) and was subsequently cloned into the donor vector pDONR221-f1 by BP clonase (Invitrogen, Waltham, MA, USA) before transfer to pDRf1-GW (Loque et al., 2007) by LR clonase (Invitrogen).

GUS and YFP fusion constructs under native promoters

To construct *SWEET5* fused with GUS (p*SWEET5*:g*SWEET5-GUS*), the fragment including the *SWEET5* native promoter sequence (1,801-bp upstream of ATG) and genomic sequence of *SWEET5* was amplified (P3 and P4) and cloned into pDONR221-f1 by BP reactions followed by recombination into the vector pMDC163 carrying the *GUS* gene (Curtis and Grossniklaus, 2003) by LR reaction. For the SWEET5 overexpression construct (p*SWEET5*:g*SWEET5-YFP*), the entry clone from above was transferred into pEarleyGateTW1 (Wang et al., 2016) by LR reactions.

eGFP fusion constructs under different promoters for mutant complementation

The *SWEET5* (1,216-bp upstream of ATG) native promoter and *LAT52* promoter (602 bp; Muschietti et al., 1994) were amplified with specific forward primers (P5–P8) containing a BamHI restriction site and the reverse primers containing an XbaI restriction site and sub-cloned into the eGFP-containing vector pGTKan3 (Kasaras and Kunze, 2010) *via BamHI and XbaI* restriction sites. The CDS of *SWEET5*, *GALK*, and *USP* by their own specific primers (P9–P14) were seamlessly subcloned into corresponding pGTKan3_promoter constructs by In-Fusion (Takara, Shiga, Japan) following the linearization at XbaI and PstI restriction sites. For the genome editing construct of *SWEET5*, gRNA that targets the first exon of SWEET5 (Supplemental Figure S4D) was cloned into p*YAO:hSpCas9* (Yan et al., 2015). For the synonymous complementation construct of Cas9 containing *sweet5_crispr*, synonymous *SWEET5* driven by pSWEET5 was amplified by primer P15 and P16 based on pDONR221_pSWEET5-*SWEET5* with gRNA targeting the region modified through site-directed mutagenesis before transferring into pEarleyGate301 (Earley et al., 2006) by LR reactions.

Constructs for subcellular localization

For subcellular localization of SWEET5, after the vector pDOE-13 (Gookin and Assmann, 2014) was linearized by SanDI + AatII restriction enzymes at MCS3, the CDS of SWEET5 was amplified by primers P17 and P18 and subcloned into it by In-Fusion (Takara). The agroinfiltration-based assays were performed as previously described (Gookin and Assmann, 2014). The *N. benthamiana* leaf disks were collected 72-h postinfiltration for confocal imaging. Three independent trials were conducted.

Plant materials and growth conditions

The Arabidopsis (*A. thaliana*) Col-0 plants were grown under controlled temperature (22°C) with a 16-h light (100–150 $\mu molm^{-2}$ s^{-1})/8-h dark photoperiod. The flowers at stage 13 (fully opened) were collected at around 5 h into the light period for different experiments. T-DNA mutants of *galk* (GABI-Kat 489D10), and *sweet5* (CS853155) were obtained from NASC or ABRC. Homozygous lines were genotyped using primers of P19–P24 and used in related experiments. The floral dip method (Clough and Bent, 1998) was used to generate all the transgenic lines used in this study. At least 15 T_1 lines were generated for each construct and at least three randomly selected lines were propagated to generate T_3 homozygous seeds.

Yeast complementation and ^{14}C uptake assays

Yeast complementation and yeast ^{14}C uptake assays were conducted following the previously described method (Chen et al., 2010). ^{14}C-glucose (0.1-μCi D-[U-^{14}C] glucose; 275 mCi/mmol), ^{14}C-galactose (0.1-μCi D-[1-^{14}C] galactose; 56.2 mCi/mmol) was added per sample and four independent transformants were used for each uptake experiment. For concentration- and time-dependent uptake of [14C] galactose for SWEET5, 5 galactose concentrations (25, 50, 100, 250, and 500 mM), and 4 time points (10 s, 2.5, 5, and 10 min) for each concentration were used to collect yeast cell samples and measure galactose uptake. Sugar uptake at 10 s was normalized as 0.

In vitro pollen germination

The *in vitro* pollen germination assay was conducted according to a previously described method (Wang et al., 2008) with minor modifications. The sucrose only basic PGM was composed of 19.8% (w/v) Suc (15% (w/v) Suc in liquid PGM), 1.5- mM boric acid, 0.8 mM $MgSO_4$, 1 mM KCl, 5 mM MES, 0.05% (w/v) lactalbumin hydrolysate, 10-μM myo-inositol, 5 mM $CaCl_2$, and 1.5% (w/v) agarose. The pH was adjusted to 5.8 using 1 mM Tris (pH = 8). The medium solution was heated to boiling on a heat plate, and then 500 μL was spread evenly onto 75 × 25 mm glass slides. Slides were cooled down at room temperature before placing in an opaque, sealed slide box. The medium was made freshly for each germination experiment. Six flowers at stage 13 were

collected from each genotype to spread pollen gently onto a ~1 cm^2 area of the germination medium. The slide box was kept at room temperature for 8 h in the dark before samples on slides were imaged with a compound microscope. Pollen with pollen tubes longer than the diameter of the pollen grain (~20 μm) was considered as germinated pollen.

Pollen galactose uptake

The liquid pollen germination method was adopted from a previously described method (Wang et al., 2008) with minor modifications for ^{14}C-galactose uptake. For each trial, 20 flowers (at least 2,000 pollen grains collected per flower) at stage 13 for each genotype were collected into a 2 mL tube, and then 1 mL of liquid PGM was added in and vortexed vigorously for 60 s to release MPGs into the solution. Subsequently, the pollen grains were counted using a hemocytometer. Equal amounts of pollen for all tested genotypes were collected by centrifuging at 15,000 *g* for 1 min. The pollen pellet was resuspended in 30 μL of liquid PGM containing ^{14}C-galactose (0.3-μCi hot galactose) as well as different concentrations of cold galactose (ranging from 0 to 60 mM), and subsequently cultured in a petri dish (35 mm in diameter). The petri dish was covered and placed in the dark for 45 min before pollen was washed using 1 mL of ice-cold liquid PGM and collected into a 1.5-mL tube. The pollen was precipitated at 15,000 *g* for 1 min and the pellet was washed by 1-mL ice-cold pollen isolation buffer (PIB, composed of 100 mM $NaPO_4$, pH 7.5, 1 mM EDTA, and 0.1% (v/v) Triton X-100) 5 more times. The pellet was resuspended in 100 μL PIB and transferred to a scintillation vial containing 5 mL Ultima Gold XR Scintillation liquid (PerkinElmer, Waltham, MA, USA). Radioactivity for each vial was measured by liquid scintillation spectrometry.

Sugar quantification using GC–MS

MPGs were collected from more than 1,000 plants using a vacuum cleaner method (Johnson-Brousseau and McCormick, 2004). For mature pollen samples, pollen grains were resuspended in 2-mL ice-cold PIB right after collection. For sucrose only or sucrose + galactose pollen samples, pollen was collected as described in the galactose uptake experiment. Pollen pellets from each treatment were washed by 1-mL ice-cold ddH_2O 3 times before being stored in a −80°C freezer. Pollen samples (~10 mg) were homogenized in 100-μL ddH_2O with Fisherbrand Bead Mill 4 Homogenizer (Thermo Fisher Scientific, Waltham, MA, USA), and then supernatants were collected after being centrifuged and saved for further processing.

Both standards and samples were dried and derivatized first with 50-μL methoxyamine hydrochloride (Sigma-Aldrich, MO, USA) (40 mg/mL in pyridine) for 90 min at 50°C, then with 50-μL MSTFA + 1% TMCS (v/v) (Thermo Fisher Scientific, Waltham, MA, USA) at 50°C for 120 min. Chromatograms were acquired using a GC–MS system (Agilent Inc., Santa Clara, CA, USA) consisting of an Agilent 7890B gas chromatography and an Agilent 5977A MSD. Gas chromatography was performed on a ZB-5MS (60 m × 0.32 mm I.D. and 0.25-μm film thickness) capillary column (Phenomenex, Torrance, CA, USA). The inlet and MSD interface temperatures were 250°C, and the ion source temperature was adjusted to 230°C. An aliquot of 1 μL was injected with the split ratio of 10:1. The helium carrier gas was kept at a constant flow rate of 2.4 mL/min. The temperature program was: 5-min isothermal heating at 70°C, followed by an oven temperature increase of 5°C min^{-1} to 310°C and a final 10 min at 310°C. The mass spectrometer was operated in positive electron impact (EI) mode at 69.9 eV ionization energy at *m/z* 30–800 scan range. Mass spectra were recorded in the combined scan/selected ion monitoring (SIM) mode. SIM mode was tracking *m/z* 307 for fructose, *m/z* 319 for glucose and galactose, *m/z* 361 for sucrose. Calibration curves were obtained for the 50–0.01 μM concentration range. Target peaks were evaluated using the Mass Hunter Quantitative Analysis B.08.00 (Agilent Inc., CA, USA) software.

GUS histochemical analysis

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

Microscopy imaging

A Zeiss Apotome.2 (Carl Zeiss, Thornwood, NY, USA) was used for fluorescence acquisition. Different FL filter sets (YFP, CFP, RFP, and GFP) were used to image samples as needed. A Zeiss LSM 710 confocal microscope (Carl Zeiss, Thornwood, NY, USA) was used to image samples. Argon laser excitation wavelength and emission bandwidths were 405 nm (70% intensity) and 425–550 nm for aniline blue (gain 600), 458 nm (100% intensity) and 480–520 nm for mTq2 (cyan; gain 850), 488 nm (50% intensity) and 500–550 nm for mVenus (yellow; gain 700), and 633 nm (50% intensity) and 633–740 nm for chlorophyll autofluorescence (red; gain 600) respectively. Image acquisition parameters were held consistent. Raw data from each channel were not altered beyond equal signal increases.

RNA isolation and RT-qPCR

RNA isolation from pollen was performed as previously described with modifications (Lu, 2011). For each independent sample, pollen grains from 50 flowers at stage 13 were collected. For samples under galactose treatment, pollen grains from 50 flowers were germinated in liquid PGM for 45 min as described above in pollen galactose uptake. RNA was isolated using Trizol (Invitrogen) as instructed by the manufacturer. First-strand cDNA was synthesized using oligo(dT) and M-MuLV reverse transcriptase (NEB, Ipswich, MA, USA). RT-qPCR was performed using PowerUp SYBR master mix (Applied Biosystems, Waltham, MA, USA) according to the manufacturer's instructions on a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using gene-specific primers (P25–P38). The expression values were normalized to *ACTIN8* expression values in each repeat, and

subsequently normalized to Col-0 using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Statistical analysis

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

Accession numbers

Sequence information from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: SWEET5 (AT5G62850), GALK (AT3G06580), USP (AT5G52560), A/N-InvH (AT3G05820), A/N-InvD (AT1G22650), cwINV2 (AT3G52600), and cwINV4 (AT2G36190).

Supplemental data

The following materials are available in the online version of this article.

Supplemental Figure S1. The levels of 17 SWEET transcripts and proteins in Arabidopsis mature pollen.

Supplemental Figure S2. Comparison of galactose uptake with or without osmolality adjusted using yeast transformed with SWEET5.

Supplemental Figure S3. Pollen is sensitive to galactose, but not to glucose or fructose.

Supplemental Figure S4. Characterization of *sweet5* and *sweet5_crispr* mutants and complementation lines.

Supplemental Figure S5. STP and PMT transcripts and protein levels in Arabidopsis mature pollen.

Supplemental Figure S6. Characterization of *galk* mutant and complementation lines.

Supplemental Figure S7. Phenotypes of pollen germination under various conditions.

Supplemental Figure S8. Over-expression of USP did not alleviate galactose sensitivity of Arabidopsis pollen.

Supplemental Figure S9. Root growth of Col-0 on 1/2 MS medium containing 0%, 0.5%, or 1% sucrose and supplemented with a range of galactose from 0 to 60 mM.

Supplemental Figure S10. RT-qPCR analysis of INVs upon galactose treatment.

Supplemental Table S1. Substrate specificity and affinity pollen grain-expressed PM-bound hexose transporters.

Supplemental Table S2. Primers used in this study.

Acknowledgments

We thank Dr. Yaxin Li for help with sample collection and thank Dr. Qi Xie for providing us with p*YAO:hSpCas9* vector. We thank Dr. Alexander Vladimirovich Ulanov for the GC–MS analysis of sugar components in pollen. We thank Dr. Doug Allen and Dr. Shrikaar Kambhampati for their great efforts in helping to analyze sugar phosphate and nucleotide sugar using LC–MS/MS, although data were not presented. We also thank Mrs. Anita K. Snyder for editing.

Funding

This work was supported by startup funds from the University of Illinois at Urbana-Champaign to Dr Chen (to J.W., Y.-C.Y., and L.-Q.C.). Y.L. was supported by the China Scholarship Council (No. 20160325007).

Conflict of interest statement. The authors declare that there is no conflict of interest.

References

Arbona V, Iglesias D, Talon M, Gomez-Cadenas A (2009) Plant phenotype demarcation using nontargeted LC-MS and GC-MS metabolite profiling. J Agric Food Chem **57**: 7338–7347

Bates G, Rosenthal D, Sun J, Chattopadhyay M, Peffer E, Yang J, Ort D, Jones A (2012) A comparative study of the *Arabidopsis thaliana* guard-cell transcriptome and its modulation by sucrose. PLoS One **7**: e49641

Battaglia M, Martin M, Lechner L, Martinez-Noel G, Salerno G (2017) The riddle of mitochondrial alkaline/neutral invertases: a novel Arabidopsis isoform mainly present in reproductive tissues and involved in root ROS production. PLoS One **12**: e0185286

Becker J, Takeda S, Borges F, Dolan L, Feijo J (2014) Transcriptional profiling of Arabidopsis root hairs and pollen defines an apical cell growth signature. BMC Plant Biol **14**: 197

Bezrutczyk M, Yang J, Eom J, Prior M, Sosso D, Hartwig T, Szurek B, Oliva R, Vera-Cruz C, White F, et al. (2018) Sugar flux and signaling in plant-microbe interactions. Plant J **93**: 675–685

Bhat P (2003) Galactose-1-phosphate is a regulator of inositol monophosphatase: a fact or a fiction? Med Hypotheses **60**: 123–128

Bowman J (1994) Arabidopsis: An Atlas of Morphology and Development, Springer-Verlag, New York, NY

Buttner M (2007) The monosaccharide transporter(-like) gene family in Arabidopsis. Febs Lett **581**: 2318–2324

Cardini C, Leloir L (1953) Enzymic phosphorylation of galactosamine and galactose. Arch Biochem Biophys **45**: 55–64

Cecchetti V, Altamura M, Falasca G, Costantino P, Cardarelli M (2008) Auxin regulates Arabidopsis anther dehiscence, pollen maturation, and filament elongation. Plant Cell **20**: 1760–1774

Chen H, Huh J, Yu Y, Ho L, Chen L, Tholl D, Frommer W, Guo W (2015a) The Arabidopsis vacuolar sugar transporter SWEET2 limits carbon sequestration from roots and restricts Pythium infection. Plant J **83**: 1046–1058

Chen L, Cheung L, Feng L, Tanner W, Frommer W, Kornberg R (2015b) Transport of sugars. Ann Rev Biochem **84**: 865–894

Chen L, Hou B, Lalonde S, Takanaga H, Hartung M, Qu X, Guo W, Kim J, Underwood W, Chaudhuri B, et al. (2010) Sugar transporters for intercellular exchange and nutrition of pathogens. Nature **468**: 527–U199

Chen L, Lin I, Qu X, Sosso D, McFarlane H, Londono A, Samuels A, Frommer W (2015c) A cascade of sequentially expressed sucrose transporters in the seed coat and endosperm provides nutrition for the Arabidopsis embryo. Plant Cell **27**: 607–619

Chen L, Qu X, Hou B, Sosso D, Osorio S, Fernie A, Frommer W (2012) Sucrose efflux mediated by SWEET proteins as a key step for phloem transport. Science **335**: 207–211

Cheng J, Wang Z, Yao F, Gao L, Ma S, Sui X, Zhang Z (2015) Down-regulating CsHT1, a cucumber pollen-specific hexose transporter, inhibits pollen germination, tube growth, and seed development. Plant Physiol **168**: 635–647

Clough S, Bent A (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J **16**: 735–743

Curtis M, Grossniklaus U (2003) A gateway cloning vector set for high-throughput functional analysis of genes in planta. Plant Physiol **133**: 462–469

de Jongh W, Bro C, Ostergaard S, Regenberg B, Olsson L, Nielsen J (2008) The roles of galactitol, galactose-1-phosphate, and phosphoglucomutase in galactose-induced toxicity in *Saccharomyces cerevisiae*. Biotechnol Bioeng **101**: 317–326

Earley K, Haag J, Pontes O, Opper K, Juehne T, Song K, Pikaard C (2006) Gateway-compatible vectors for plant functional genomics and proteomics. Plant J **45**: 616–629

Egert A, Peters S, Guyot C, Stieger B, Keller F (2012) An Arabidopsis T-DNA insertion mutant for galactokinase (AtGALK, At3g06580) hyperaccumulates free galactose and is insensitive to exogenous galactose. Plant Cell Physiol **53**: 921–929

Engel M, Davis R, McCormick S (2005) Green sperm. Identification of male gamete promoters in Arabidopsis. Plant Physiol **138**: 2124–2133

Eom JS, Chen LQ, Sosso D, Julius BT, Lin IW, Qu XQ, Braun DM, Frommer WB (2015) SWEETs, transporters for intracellular and intercellular sugar translocation. Curr Opin Plant Biol **25**: 53–62

Feijo J, Costa S, Prado A, Becker J, Certal A (2004) Signalling by tips. Curr Opin Plant Biol **7**: 589–598

Feusi MES, Burton JD, Williamson JD, Pharr DM (1999) Galactosyl-sucrose metabolism and UDP-galactose pyrophosphorylase from *Cucumis melo* L-fruit. Physiol Plant **106**: 9–16

Frietsch S, Wang Y, Sladek C, Poulsen L, Romanowsky S, Schroeder J, Harper J (2007) A cyclic nucleotide-gated channel is essential for polarized tip growth of pollen. Proc Natl Acad Sci USA **104**: 14531–14536

Goetz M, Guivarch A, Hirsche J, Bauerfeind M, Gonzalez M, Hyun T, Eom S, Chriqui D, Engelke T, Grosskinsky D, et al. (2017) Metabolic control of tobacco pollination by sugars and invertases. Plant Physiol **173**: 984–997

Gookin T, Assmann S (2014) Significant reduction of BiFC non-specific assembly facilitates in planta assessment of heterotrimeric G-protein interactors. Plant J **80**: 553–567

Guo W, Nagy R, Chen H, Pfrunder S, Yu Y, Santelia D, Frommer W, Martinoia E (2014) SWEET17, a facilitative transporter, mediates fructose transport across the tonoplast of Arabidopsis roots and leaves. Plant Physiol **164**: 777–789

Hamilton E, Jensen G, Maksaev G, Katims A, Sherp A, Haswell E (2015) Mechanosensitive channel MSL8 regulates osmotic forces during pollen hydration and germination. Science **350**: 438–441

Hirsche J, Engelke T, Voller D, Gotz M, Roitsch T (2009) Interspecies compatibility of the anther specific cell wall invertase promoters from Arabidopsis and tobacco for generating male sterile plants. Theor Appl Genet **118**: 235–245

Hirsche J, Fernandez J, Stabentheiner E, Grosskinsky D, Roitsch T (2017) Differential effects of carbohydrates on Arabidopsis pollen germination. Plant Cell Physiol **58**: 691–701

Ho LH, Klemens PAW, Neuhaus HE, Ko HY, Hsieh SY, Guo WJ (2019) SlSWEET1a is involved in glucose import to young leaves in tomato plants. J Exp Bot **70**: 3241–3254

Hofmeyr J, Cornish-Bowden A (2000) Regulating the cellular economy of supply and demand. Febs Lett **476**: 47–51

Honys D, Twell D (2004) Transcriptome analysis of haploid male gametophyte development in Arabidopsis. Genome Biol **5**: e1000621

Johnson-Brousseau SA, McCormick S (2004) A compendium of methods useful for characterizing Arabidopsis pollen mutants and gametophytically- expressed genes. Plant J **39**: 761–775

Julius B, Leach K, Tran T, Mertz R, Braun D (2017) Sugar transporters in plants: new insights and discoveries. Plant Cell Physiol **58**: 1442–1460

Kasaras A, Kunze R (2010) Expression, localisation and phylogeny of a novel family of plant-specific membrane proteins. Plant Biol **12**: 140–152

Kircher S, Schopfer P (2012) Photosynthetic sucrose acts as cotyledon-derived long-distance signal to control root growth during early seedling development in Arabidopsis. Proc Natl Acad Sci USA **109**: 11217–11221

Klemens P, Patzke K, Deitmer J, Spinner L, Le Hir R, Bellini C, Bedu M, Chardon F, Krapp A, Neuhaus H (2013) Overexpression of the vacuolar sugar carrier AtSWEET16 modifies germination, growth, and stress tolerance in Arabidopsis. Plant Physiol **163**: 1338–1352

Kotake T, Yamaguchi D, Ohzono H, Hojo S, Kaneko S, Ishida H, Tsumuraya Y (2004) UDP-sugar pyrophosphorylase with broad substrate specificity toward various monosaccharide 1-phosphates from pea sprouts. J Biol Chem **279**: 45728–45736

Kuanyshev N, Deewan A, Jagtap SS, Liu J, Selvam B, Chen LQ, Shukla D, Rao CV, Jin YS (2021) Identification and analysis of sugar transporters capable of co-transporting glucose and xylose simultaneously. Biotechnol J 2100238

Lai K, Elsas L, Wierenga K (2009) Galactose toxicity in animals. Iubmb Life **61**: 1063–1074

Lai K, Langley S, Khwaja F, Schmitt E, Elsas L (2003) GALT deficiency causes UDP-hexose deficit in human galactosemic cells. Glycobiology **13**: 285–294

Leloir L (1951) The enzymatic transformation of uridine diphosphate glucose into a galactose derivative. Arch Biochem Biophys **33**: 186–190

Li Y, Liu H, Yao X, Wang J, Feng S, Sun L, Ma S, Xu K, Chen LQ, Sui X (2021) Hexose transporter CsSWEET7a in cucumber mediates phloem unloading in companion cells for fruit development. Plant Physiol **186**: 640–654

Lima R, dos Santos T, Vieira L, Ferrarese M, Ferrarese O, Donatti L, Boeger M, Petkowicz C (2013) Heat stress causes alterations in the cell-wall polymers and anatomy of coffee leaves (*Coffea arabica* L.). Carbohydr Polym **93**: 135–143

Lin I, Sosso D, Chen L, Gase K, Kim S, Kessler D, Klinkenberg P, Gorder M, Hou B, Qu X, et al. (2014) Nectar secretion requires sucrose phosphate synthases and the sugar transporter SWEET9. Nature **508**: 546–549

Livak K, Schmittgen T (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. Methods **25**: 402–408

Loque D, Lalonde S, Looger LL, von Wiren N, Frommer WB (2007) A cytosolic trans-activation domain essential for ammonium uptake. Nature **446**: 195–198

Lu Y (2011) RNA isolation from Arabidopsis pollen grains. Bio-protocol **1**: e67

Maxwell E, Derobichonszulmajster H (1960) Purification of uridine diphosphate galactose-4-epimerase from yeast, and the identification of protein-bound diphosphopyridine nucleotide. J Biol Chem **235**: 308–312

Mergner J, Frejno M, List M, Papacek M, Chen X, Chaudhary A, Samaras P, Richter S, Shikata H, Messerer M, et al. (2020) Mass-spectrometry-based draft of the Arabidopsis proteome. Nature **579**: 409–414

Muschietti J, Dircks L, Vancanneyt G, Mccormick S (1994) LAT52 protein is essential for tomato pollen development - pollen expressing antisense LAT52 RNA hydrates and germinates abnormally and cannot achieve fertilization. Plant J **6**: 321–338

Pacini E, Jacquard C, Clement C (2011) Pollen vacuoles and their significance. Planta **234**: 217–227

Qin Y, Leydon A, Manziello A, Pandey R, Mount D, Denic S, Vasic B, Johnson M, Palanivelu R (2009) Penetration of the stigma and style elicits a novel transcriptome in pollen tubes, pointing to genes critical for growth in a pistil. PLoS Genet **5**: e1000621

Reger B, Pressey R, Chaubal R (1992) In vitro chemotropism of pearl-millet pollen tubes to stigma tissue - a response to glucose produced in the medium by tissue-bound invertase. Sex Plant Reprod **5**: 201–205

Reinders A (2016) Fuel for the road - sugar transport and pollen tube growth. J Exp Bot **67**: 2121–2123

Roberts R, Heishman A, Wicklin C (1971) Growth inhibition and metabolite pool levels in plant tissues fed D-glucosamine and D-galactose. Plant Physiol **48**: 36–42

Rottmann T, Fritz C, Sauer N, Stadler R (2018) Glucose uptake via STP transporters inhibits in vitro pollen tube growth in a HEXOKINASE1-dependent manner in *Arabidopsis thaliana*. Plant Cell **30**: 2057–2081

Rottmann T, Zierer W, Subert C, Sauer N, Stadler R (2016) STP10 encodes a high-affinity monosaccharide transporter and is induced under low-glucose conditions in pollen tubes of Arabidopsis. J Exp Bot **67**: 2387–2399

Ruan Y (2014) Sucrose metabolism: gateway to diverse carbon use and sugar signaling. Ann Rev Plant Biol **65**: 33–67

Rutley N, Twell D (2015) A decade of pollen transcriptomics. Plant Reprod **28**: 73–89

Schneidereit A, Scholz-Starke J, Buttner M (2003) Functional characterization and expression analyses of the glucose-specific AtSTP9 monosaccharide transporter in pollen of Arabidopsis. Plant Physiol **133**: 182–190

Scholz-Starke J, Buttner M, Sauer N (2003) AtSTP6, a new pollen-specific H^+-monosaccharide symporter from Arabidopsis. Plant Physiol **131**: 70–77

Seifert G, Barber C, Wells B, Dolan L, Roberts K (2002) Galactose biosynthesis in Arabidopsis: genetic evidence for substrate channeling from UDP-D-galactose into cell wall polymers. Curr Biol **12**: 1840–1845

Sherson S, Gy I, Medd J, Schmidt R, Dean C, Kreis M, Lecharny A, Cobbett C (1999) The arabinose kinase, ARA1, gene of Arabidopsis is a novel member of the galactose kinase gene family. Plant Mol Biol **39**: 1003–1012

Sherson S, Hemmann G, Wallace G, Forbes S, Germain V, Stadler R, Bechtold N, Sauer N, Smith S (2000) Monosaccharide/proton symporter AtSTP1 plays a major role in uptake and response of Arabidopsis seeds and seedlings to sugars. Plant J **24**: 849–857

Singh M, Gupta A, Laxmi A (2014) Glucose control of root growth direction in *Arabidopsis thaliana*. J Exp Bot **65**: 2981–2993

Stadler R, Truernit E, Gahrtz M, Sauer N (1999) The AtSUC1 sucrose carrier may represent the osmotic driving force for anther dehiscence and pollen tube growth in Arabidopsis. Plant J **19**: 269–278

Truernit E, Stadler R, Baier K, Sauer N (1999) A male gametophyte-specific monosaccharide transporter in Arabidopsis. Plant J **17**: 191–201

Tunc-Ozdemir M, Rato C, Brown E, Rogers S, Mooneyham A, Frietsch S, Myers C, Poulsen L, Malho R, Harper J (2013) Cyclic nucleotide gated channels 7 and 8 are essential for male reproductive fertility. PLoS One **8**: e51944

Wang R, Liu M, Yuan M, Oses-Prieto J, Cai X, Sun Y, Burlingame A, Wang Z, Tang W (2016) The brassinosteroid-activated BRI1 receptor kinase is switched off by dephosphorylation mediated by cytoplasm-localized PP2A B ′ subunits. Mol Plant **9**: 148–157

Wang Y, Zhang W, Song L, Zou J, Su Z, Wu W (2008) Transcriptome analyses show changes in gene expression to accompany pollen germination and tube growth in Arabidopsis. Plant Physiol **148**: 1201–1211

Wieczorke R, Krampe S, Weierstall T, Freidel K, Hollenberg C, Boles E (1999) Concurrent knock-out of at least 20 transporter genes is required to block uptake of hexoses in *Saccharomyces cerevisiae*. FEBS Lett **464**: 123–128

Yamada K, Kanai M, Osakabe Y, Ohiraki H, Shinozaki K, Yamaguchi-Shinozaki K (2011) Monosaccharide absorption activity of Arabidopsis roots depends on expression profiles of transporter genes under high salinity conditions. J Biol Chem **286**: 43577–43586

Yamamoto R, Inouhe M, Masuda Y (1988) Galactose inhibition of auxin-induced growth of monocotyledonous and dicotyledonous plants. Plant Physiol **86**: 1223–1227

Yan L, Wei S, Wu Y, Hu R, Li H, Yang W, Xie Q (2015) High-efficiency genome editing in Arabidopsis using YAO promoter-driven CRISPR/Cas9 system. Mol Plant **8**: 1820–1823

Yang T, Bar-Peled L, Gebhart L, Lee S, Bar-Peled M (2009) Identification of galacturonic acid-1-phosphate kinase, a new member of the GHMP kinase duperfamily in plants, and comparison with galactose-1-phosphate kinase. J Biol Chem **284**: 21526–21535

Ylstra B, Garrido D, Busscher J, van Tunen A (1998) Hexose transport in growing petunia pollen tubes and characterization of a pollen-specific, putative monosaccharide transporter. Plant Physiol **118**: 297–304

Zhou Y, Cui X, Hu A, Miao Y, Zhang L (2020) Characterization and functional analysis of pollen-specific PwSWEET1 in *Picea wilsonii*. J For Res **31**: 1913–1922

Zhou Y, Liu L, Huang W, Yuan M, Zhou F, Li X, Lin Y (2014) Overexpression of OsSWEET5 in rice causes growth retardation and precocious senescence. PLoS One **9**: e94210