



Hexose translocation mediated by *SISWEET5b* is required for pollen maturation in *Solanum lycopersicum*

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Abstract

Pollen fertility is critical for successful fertilization and, accordingly, for crop yield. While sugar unloading affects the growth and development of all types of sink organs, the molecular nature of sugar import to tomato (*Solanum lycopersicum*) pollen is poorly understood. However, sugar will eventually be exported transporters (SWEETs) have been proposed to be involved in pollen development. Here, reverse transcription-quantitative polymerase chain reaction (PCR) revealed that *SISWEET5b* was markedly expressed in flowers when compared to the remaining tomato *SISWEETs*, particularly in the stamens of maturing flower buds undergoing mitosis. Distinct accumulation of *SISWEET5b*- β -glucuronidase activities was present in mature flower buds, especially in anther vascular and inner cells, symplasmic isolated microspores (pollen grains), and styles. The demonstration that *SISWEET5b*-GFP fusion proteins are located in the plasma membrane supports the idea that the *SISWEET5b* carrier functions in apoplasmic sugar translocation during pollen maturation. This is consistent with data from yeast complementation experiments and radiotracer uptake, showing that *SISWEET5b* operates as a low-affinity hexose-specific passive facilitator, with a K_m of ~ 36 mM. Most importantly, RNAi-mediated suppression of *SISWEET5b* expression resulted in shrunken nucleus-less pollen cells, impaired germination, and low seed yield. Moreover, stamens from *SISWEET5b*-silenced tomato mutants showed significantly lower amounts of sucrose (Suc) and increased invertase activity, indicating reduced carbon supply and perturbed Suc homeostasis in these tissues. Taken together, our findings reveal the essential role of *SISWEET5b* in mediating apoplasmic hexose import into phloem unloading cells and into developing pollen cells to support pollen mitosis and maturation in tomato flowers.

Introduction

Pollen development is essential for generating male gametophytes that are critical for plant reproduction and crop yield (Mascarenhas, 1989; Ma, 2005). In flowering plants, pollen cells develop within anthers, which are attached to the flower receptacle via filaments in the stamen. Due to active growth and remarkable metabolic activity (Borghi and Fernie, 2017), anther development thus exhibits high sink strength for nutrients during flower formation (Clément et al., 1996; Imlau et al., 1999). Because green sepals and young petals only support limited photoassimilates (Clément et al., 1997; Aschan et al., 2005), pollen growth largely depends on the import of carbon resources, predominantly sucrose (Suc; Ho and Nichols, 1975; Borghi and Fernie, 2017), from source leaves (Goetz et al., 2001; Müller et al., 2010). However, during pollen development, programmed degradation of surrounding maternal anther cells results in complete symplasmic isolation of the male gametophyte (Scott et al., 1991; Clément and Audran, 1995). Accordingly, plasma membrane-localized carriers play an essential role in mediating apoplastic sugar transport for acquiring carbon for pollen development (Truernit et al., 1999; Borghi and Fernie, 2017).

Generally, Suc is first unloaded from the flower terminal phloem and allocated symplasmically to anther cells through vascular tissues and plasmodesmata (PD) in the filament (Mascarenhas, 1989; Borghi and Fernie, 2017). Symplasmic transfer into anther cells has been demonstrated by post-phloem transport of green fluorescent proteins in *Arabidopsis* (*Arabidopsis thaliana*) anthers (Imlau et al., 1999). Subsequently, the symplasmically isolated tapetum and the anther locule mandate result in two apoplastic barriers between the anther inner cells and developing pollen. The tapetum, a nutritious cell layer that encloses the whole pollen sac, has been proposed as a sugar buffer for pollen growth (Goldberg et al., 1993; Castro and Clément, 2007). As the pollen mother cells undergo meiosis, tapetal cells become symplastically isolated due to the degeneration of PDs on their external face toward both the inner cell and the locule (Clément and Audran, 1995; Mamun et al., 2005; Sager and Lee, 2014). The locule is derived from programmed cell death and becomes a closed fluid-filled cavity in which pollen develops (Scott et al., 1991). Consequently, Suc must be apoplastically delivered to fuel the early development of pollen microspores, including being exported from anther inner cells, imported into tapetum cells, released into the locule, and finally imported into pollen cells. During pollen maturation, tapetum cells are programmed to undergo degradation. In this case, the anther inner cells become the major nutritive reservoir for supplying sugars apoplastically (Goldberg et al., 1993; Clément and Audran, 1995; Castro and Clément, 2007).

It has been proposed that sucrose transporters (SUT/SUCs) participate in the apoplastic transport of Suc (Borghi and Fernie, 2017). However, in some species, such as tomato (*Solanum lycopersicum*), a large portion of the

unloaded Suc can be hydrolyzed in filament and anther inner cells by cell wall invertases (CWINs), leading to the production of glucose (Glc) and fructose (Fru; Pressman et al., 2012). Accordingly, the intercellular transport of these hexoses into pollen must be mediated by hexose importers (Slewinski, 2011). While many pollen-specific plasma membrane-localized SUT/SUC- or sugar transporter (STP)-type symporters have been characterized in various species (Ylstra et al., 1998; Truernit et al., 1999; Cheng et al., 2015; Borghi and Fernie, 2017), the major carriers responsible for carrying out apoplastic sugar transfer for pollen development have not been fully identified. Decreased activity of Suc symporters, such as OsSUT1 in rice (*Oryza sativa*; Hirose et al., 2010), LeSUT2 in tomato (Hackel et al., 2006), and AtSUC1 in *Arabidopsis* (Sivitz et al., 2008) only affects pollen germination and pollen tube growth, but not pollen development. While downregulation of the pollen-expressed SUT CsSUT1 in male cucumber (*Cucumis sativus*) flowers led to reduced carbohydrate content and shriveled pollen grains, suggesting that CsSUT1 mediates Suc import into cucumber pollen for their development (Sun et al., 2019).

In contrast, the molecular nature of major hexose transporters likely required for pollen formation, as discussed above, is yet to be determined. Several genes encoding hexose transporters are greatly expressed in pollen cells (Truernit et al., 1999; Schneidereit et al., 2003; Büttner, 2010; Borghi and Fernie, 2017). However, no differences in pollen morphology were observed when the expression of hexose transporters, such as cucumber CsHT1 (Cheng et al., 2015), *Arabidopsis* AtSTP6 (Scholz-Starke et al., 2003), and *Petunia hybrida* PhPMT1 (Garrido et al., 2006) was suppressed. However, high hexose concentrations in the anther loculus would favor the involvement of energy-independent facilitators. For example, members of the large sugar will eventually be exported transporters (SWEETs) facilitator family have been proposed as candidates to mediate hexose import into pollen cells.

Since the first description of the SWEET family was published (Chen et al., 2010), a growing body of evidence indicates that SWEET members are key players in sugar allocation between source and sink organs (Sauer, 2007; Chen, 2013; Eom et al., 2015; Julius et al., 2017). In general, based on amino acid similarity, four clades of SWEET genes have been identified in various plant species (Chen, 2013; Eom et al., 2015). Clades I and II exhibit high transport activity for hexoses (Chen et al., 2010, 2015b), while Clade III members mainly transport Suc (Chen et al., 2012). Some Clade IV SWEET members are located intracellularly to mediate Fru transport across the vacuolar membrane (Chardon et al., 2013; Klemens et al., 2013; Guo et al., 2014). In the source leaves of *Arabidopsis* and maize (*Zea mays*), Clade III SWEET catalyzes passive Suc export from parenchyma cells into the phloem apoplast, prior to active Suc loading by proton-coupled SUC2 (Chen et al., 2012; Bezruczyk et al., 2018). Different sets of SWEET facilitators cooperate with energy-coupled sugar carriers to accomplish apoplastic export or import of Suc or

hexoses to fulfill the development of sink organs, such as Clade III SWEETs for seeds (Chen et al., 2015c; Yang et al., 2018; Wang et al., 2019), SWEET9 for nectarines (Lin et al., 2014), SWEET1 for new leaves (Ho et al., 2019), and SWEET15 for fruits (Ko et al., 2021).

In Arabidopsis, several SWEET-encoding genes are expressed in pollen cells (Chen et al., 2015b). In particular, transcripts of AtSWEET8 (RPG1) and AtSWEET13 (RPG2) preferentially accumulate in male microspores and tapetum cells, since the development of microspore mother cells (Guan et al., 2008; Sun et al., 2013). Disruption of both AtSWEET8 and AtSWEET13 function via T-DNA insertion results in defective pollen wall formation and impaired pollen fertility (Guan et al., 2008), probably due to the lack of sugars for pollen wall formation. Recent studies have highlighted the possibility that SWEET transporters may also participate in sugar unloading into the pollen of crop species which are crucial for food production. Considering the diverse sugar content in pollen cells across plant species, ranging from 20% to 60% in pollen's dry mass (Conti et al., 2016), and various types of sugars used for intercellular translocation (Borghi and Fernie, 2017), further investigation is required to determine the role of SWEET members in pollen development on a crop-specific basis.

In this study, we discovered that tomato *SISWEET5b*, belonging to Clade II SWEETs, is specifically expressed in the stamen of mature flowers. Examination of β -glucuronidase (GUS)- and GFP fusion proteins indicated that *SISWEET5b* functions in the plasma membrane of both pollen and stigma cells. In conjunction with transport activity assays in yeast and analysis of transgenic knockdown tomato lines, we propose an essential role of *SISWEET5b* in hexose translocation into pollen grains, which is required for their maturation and fertility.

Results

SISWEET5b mRNA accumulates specifically in tomato stamens

To determine whether a specific member of the SWEET family is involved in sugar unloading during pollen development in tomato flowers, we performed quantitative RT-PCR for 30 tomato *SISWEET* genes using cDNA isolated from whole flowers at 1 d post-anthesis. *SISWEET5b* transcripts were markedly more abundant in tomato flowers than all other *SISWEET* genes (Figure 1A), while this mRNA was low in abundance in all vegetative organs tested (Figure 1B). When compared to closely related clade II SWEET homologs (48%–80% amino acid identity), only *SISWEET5b* transcripts predominantly accumulated in stamens, but not in petals, sepals, and ovary of flowers (Figure 1C). Within clade II *SISWEETs*, only *SISWEET5b* expression was specifically induced during the late stage (S15, 7 mm) of flower development (Figure 1D; Brukhin et al., 2003). The close association between *SISWEET5b* RNA expression and pollen maturation was not observed for other Clade II SWEETs (Figure 1, C and D), suggesting that *SISWEET5b* is likely involved in pollen

maturation during flower development. Therefore, we focused on the functional characterization of *SISWEET5b*.

SISWEET5b proteins accumulate in unloading cells and pollen grains

Several studies have shown that *SISWEET* protein abundance can be uncoupled to the corresponding mRNA transcripts (Guo et al., 2014; Chen et al., 2015a). To investigate the tissue-specific function of *SISWEET5b* proteins, we created transgenic tomato plants expressing a *SISWEET5b*–GUS fusion protein. The transgene included the complete genomic *SISWEET5b* DNA sequence, containing all introns driven by the native *SISWEET5b* promoter with the GUS coding sequence added as a C-terminal fusion. In T0 transgenic tomato plants, prominent blue staining, for GUS activity, was only observed in mature flowers, but not in roots, leaves, or young flower buds (Figure 2, A–D). In flowers, strong expression of *SISWEET5b*–GUS fusion was also detected in the top region of the style (Figure 2E), pollen grains (Figure 2F), and inner anther cells surrounding the pollen sac (open arrowhead, Figure 2F). During fruit development, distinct GUS activities were also observed in the vascular tissues of the pericarp, columella, and seeds (Figure 2, G and H). Thin sections of GUS-stained flowers were prepared to look at tissue specificity more closely. In both longitudinal and transverse sections of the pistil and stamen, the expression of *SISWEET5b*–GUS was clearly detected in the upper region of the style (arrowhead, Figure 2I) and mature round pollen grains (Figure 2, I–K). In the tomato anther, marked blue staining was also observed in unloading cells around xylem tissues, including in the phloem and surrounding parenchyma cells (Figure 2L). Slight blue staining was also observed in the anther inner cell layer surrounding the pollen sac (open arrowhead, Figure 2K).

Plasma membrane localization of *SISWEET5b*

To examine the subcellular localization of *SISWEET5b*, we transiently expressed *SISWEET5b*–YFP fusion proteins driven by the 35S promoter in Arabidopsis protoplasts. The yellow fluorescence derived from *SISWEET5b*–YFP fusions mainly enclosed cytosolic chloroplasts, as indicated by their red autofluorescence (arrowheads, Supplemental Figure S1A), suggesting its localization to the plasma membrane. When protoplasts were co-expressed with *SISWEET5b*–YFP and the plasma membrane marker AtPIP2A–cyan fluorescent protein (CFP) (Nelson et al., 2007), bright fluorescence resulting from overlapping yellow and blue signals was observed in the merged image and was consistently located outside of the chloroplasts (Figure 3A, arrowheads). These observations were further confirmed by signal distribution and quantification (Figure 3B). To further examine localization in vivo, we transiently expressed *SISWEET5b*–YFP in *Nicotiana benthamiana* leaves. In both normal cells (Figure 3C) and cells subjected to plasmolysis (Supplemental Figure S1B), yellow fluorescence was clearly observed in a lining outside of chloroplasts, indicated by red autofluorescence (arrowheads), demonstrating

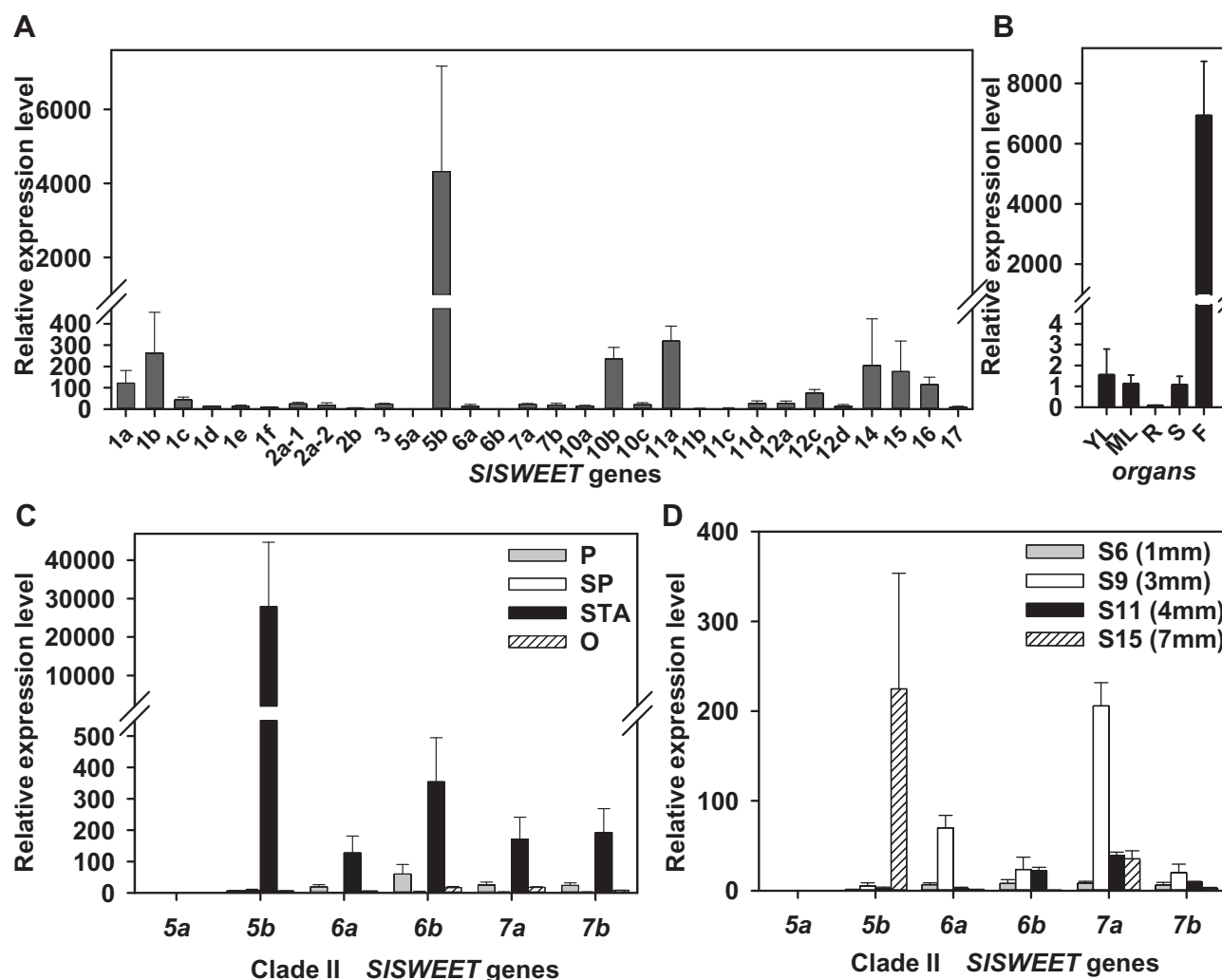


Figure 1 Dominant expression of *SISWEET5b* in stamens of tomato flowers. A, Stable mRNA transcripts of 30 *SISWEET*s in flowers. B, Developmental expression of *SISWEET5b* in tomato. YL, young leaves. ML, mature leaves. R, roots. S, stems. F, flowers. C, Expression of clade II *SISWEET*s in various flower organs. P, petal. SP, sepal. STA, stamen. O, ovary. D, Expression of clade II *SISWEET*s during flower bud development. Stages (S) and sizes (mm) of flower buds are indicated. Total RNA was isolated from the indicated organs of 6-week-old soil-grown tomato plants, and the derived cDNA was used for RT-qPCR with gene-specific primers. Relative expression by normalization to an internal control, *SlActin7*, is shown. Results are presented as the mean $\pm$ standard error (se) from three independent biological replicates. Similar experiments have been repeated for at least three times.

that *SISWEET5b*-YFP was primarily located on the plasma membrane in planta.

Hexose-specific transport activity of *SISWEET5b* in yeast

SISWEET5 belongs to clade II SWEET proteins that generally catalyze the transport of hexoses, such as *Arabidopsis AtSWEET5* (Chen et al., 2010). To verify the sugar transport properties of *SISWEET5b*, we expressed the gene in the baker's yeast mutant EBY4000, which lacks import activities for hexoses and does not grow on hexose-containing media (Wieczorke et al., 1999). As expected, the expression of the control yeast hexose transporter SchXT5 enabled growth on medium containing 2% Glc or Fru (Figure 4A). Neither the empty vector nor the Suc carrier *AtSUC2* restored the growth of the yeast mutant. Similar to SchXT5, the

expression of *SISWEET5b* complemented the growth of EBY4000 mutant cells on both hexose-containing media (Figure 4A). Analysis of [14 C]-glucose ([14 C]Glc) uptake confirmed that yeast cells expressing *SISWEET5b* imported Glc in a time-dependent manner, whereas cells containing the empty vector accumulated much less Glc (Figure 4B). The kinetic analysis of the Glc and Fru transport activity of *SISWEET5b* revealed a K_M value of 36 mM for Glc and 35 mM for Fru (Figure 4C). To address the substrate specificity of *SISWEET5b*, a 10-fold excess of unlabeled sugars was added to the medium to compete with [14 C]Glc. Relative uptake rates showed that only Glc, Fru, and mannose, but not galactose or Suc, could compete for binding and significantly reduce the import of [14 C]Glc (Figure 4D). The decreased Glc uptake in the presence of maltose is probably due to background activity in the EBY4000 yeast strain, as

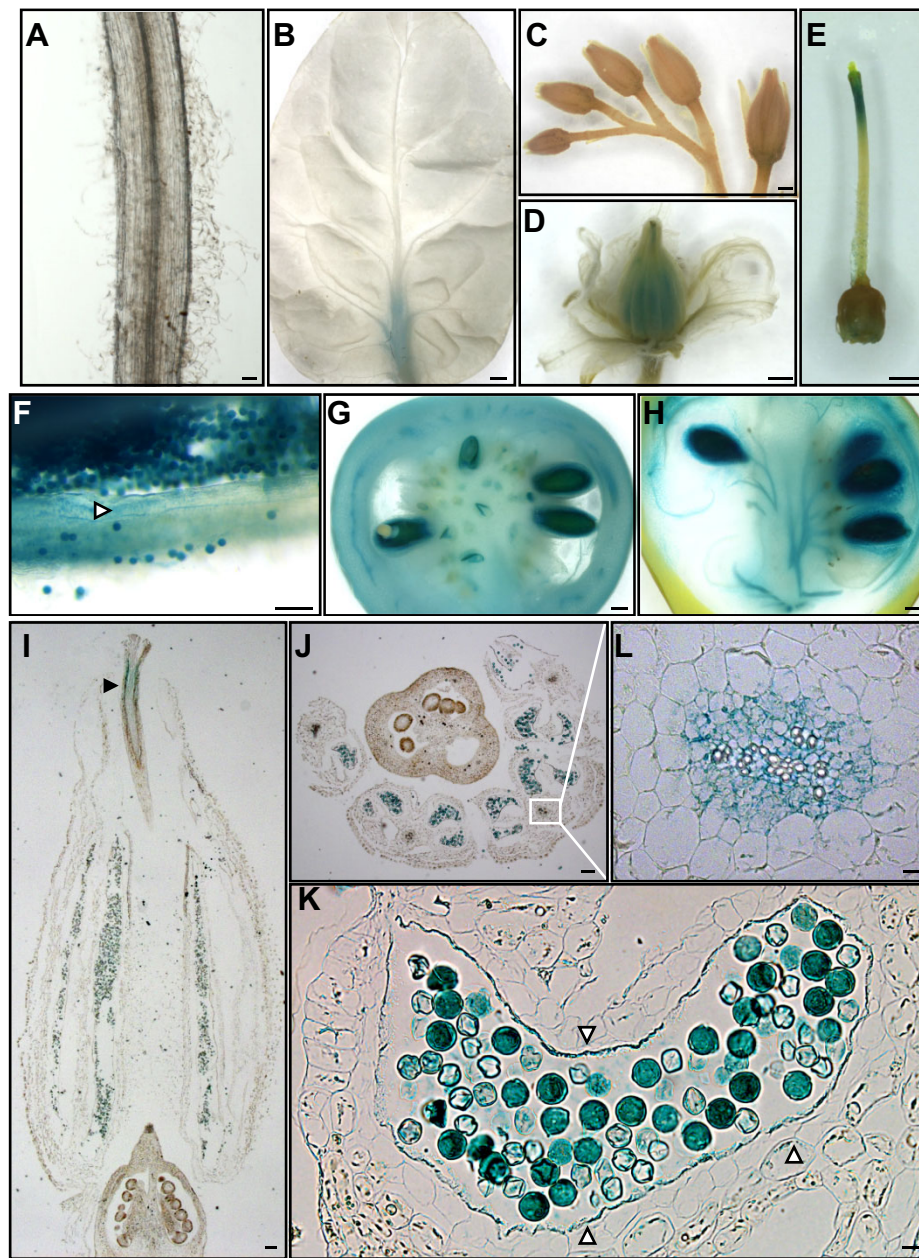


Figure 2 Specific accumulation of *SISWEET5b* in reproductive organs in tomato. Histochemical staining of GUS activity in transgenic tomato plants expressing the *SISWEET5b*–GUS fusion protein driven by the native *SISWEET5b* promoter. A, Mature roots. B, Mature leaflet. C, Flower buds. D, Open flower. E, Pistil. F, Inside of stamen. G, Young green tomato fruit. H, Mature red tomato fruits. I, Longitudinal section of a stamen and pistil. Arrowhead indicates the stained style. J, Transverse section of a stamen. K, Zoom-in picture of J. L, Zoom-in picture of the white box in J. Arrow heads in F and K indicated the anther inner cell layer surrounding the pollen sac. Bars = 100 μ m in A, I, J; 1 mm in B–H; 10 μ m in K, L. Similar patterns have been confirmed for three independent transgenic lines and same experiments have been repeated at least three times.

shown previously (Chen et al., 2010; Ho et al., 2019). Moreover, the Glc transport catalyzed by *SISWEET5b* was very similar under different pH values, ranging from acidic (pH 5) to alkaline (pH 8). Consistently, its uptake activity decreased only slightly upon the addition of the protonophore NH_4Cl (10 mM; Figure 4E). This reduction is probably due to the cell toxicity of this compound (Ho et al., 2019). In summary, these results indicate that *SISWEET5b* is a

proton-independent passive hexose facilitator with low substrate affinity.

Loss of *SISWEET5b* expression impeded seed production

To examine the function of *SISWEET5b* during pollen development, we generated transgenic knockdown tomato mutants expressing an RNA interference (RNAi) construct

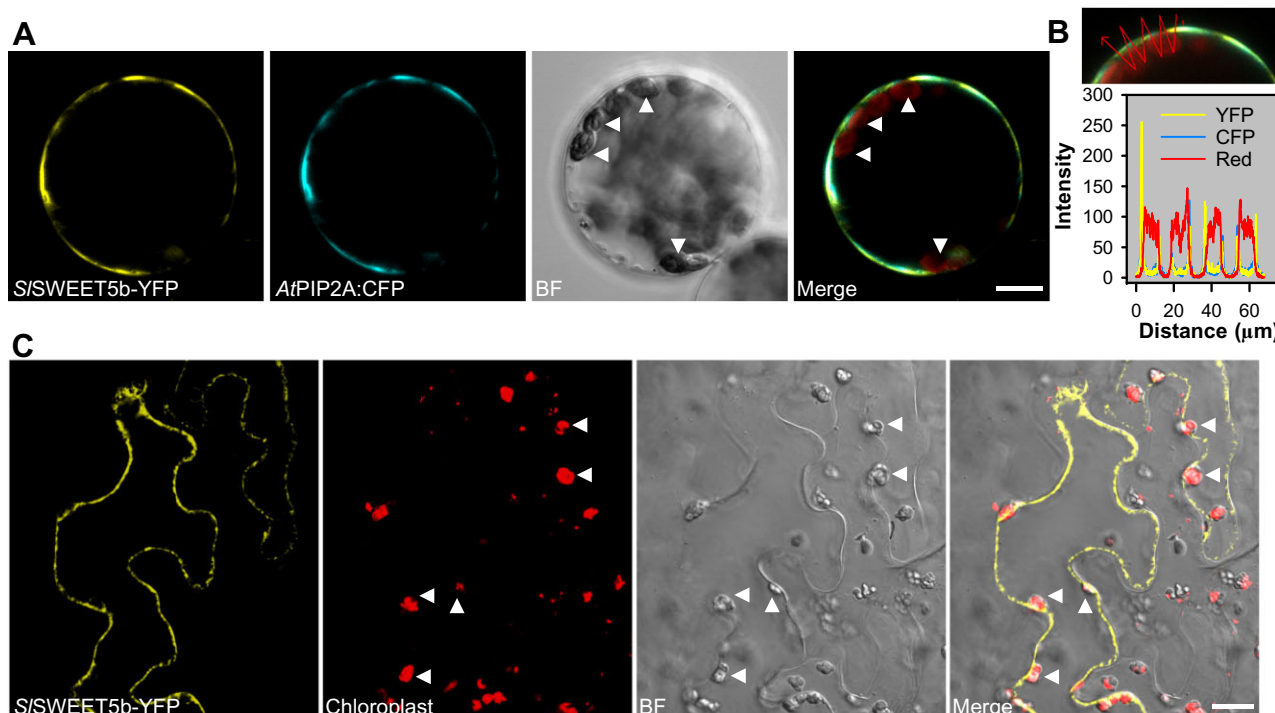


Figure 3 Subcellular localization of *SISWEET5b* on the plasma membrane. A, Localization of *SISWEET5b*-YFP fusions in an Arabidopsis protoplast expressing the PM marker, AtPIP2A-CFP. Signals of yellow fluorescence, cyan fluorescence and BF were detected after 20 h of transfection. The merged image demonstrated that *SISWEET5b*-YFP-derived fluorescence was co-localized with AtPIP2A-CFP in a lining outside of chloroplasts (red autofluorescence, arrowheads), indicating localization to the PM. B, Intensity profile of the indicated line path in the merged image in (A). C, Transient expression of *SISWEET5b*-YFP in *N. benthamiana* leaves. YFP emission and chloroplast red autofluorescence were observed after 36 h of agroinfiltration. The merged image showed that *SISWEET5b*-YFP-derived fluorescence was lined between chloroplasts and the cell periphery, indicating localization to PM. All the images were obtained from the same focal plane. PM, plasma membrane. BF, bright field. Bar = 10 μ m. Similar patterns have been confirmed for at least five cells from four independent transfection experiments.

targeting to *SISWEET5b* (*SISWEET5b*-RNAi). In 20 independent T0 transgenic tomato plants (RNAi-1 to 20), flowers of the RNAi-6, 10, and 16 lines showed significantly decreased levels of *SISWEET5b* mRNA, ranging from 10% to 40% of the expression level in the empty vector controls (Supplemental Figure S2A). When we further examined the expression of other clade II SWEETs, which share high amino acid similarity with that of *SISWEET5b*, no significant changes in silenced flowers were detected (Supplemental Figure S2B), indicating that a high specificity of the silencing intervention to *SISWEET5b* with little effect on other *SISWEET* paralogs. When compared to nonsilenced T0 transgenic plants (T0-NS, lines RNAi-7, -11, 18), no obvious differences in plant size and height were observed in highly silenced lines (T0-HS, line RNAi-6, -10, -16, Supplemental Figure S2C). However, RNAi-6, which had the highest silencing, exhibited a strong decrease in fruit number and fruit fresh weight (Supplemental Figure S3, A and B). These changes were not observed in RNAi-10 and RNAi-16 lines with moderate RNA expression (Supplemental Figure S3, A and B). Despite no consistent difference in average fruit yield and fresh weight between T0-NS and T0-HS lines (combined data from three independent lines; Figure 5, A and B), total seed numbers in T0-HS lines were markedly reduced (Supplemental Figure S3, C; Figure 5, C). Only one seed each was obtained from

RNAi-6 and 16 lines (Supplemental Figure S3C). These results clearly demonstrated that reduced expression of *SISWEET5b* substantially inhibited seed production.

Silencing *SISWEET5b* expression resulted in male sterility

Based on the dominant localization of *SISWEET5b* proteins in pollen and unloading cells (Figure 2, J and L), we assumed that the decreased seed production in T0-HS plants may result from male sterility. The lack of fertile T1 seeds from both RNAi-6 and RNAi-16 lines prevented further characterization of these lines (Supplemental Figure S3C). Therefore, we examined pollen development in T1 plants derived from the moderately silenced RNAi-10 line. Fifteen T1 plants were assayed by reverse transcribed (RT-qPCR) and four lines, RNAi-10-2, -8, -14, and -16, exhibited distinct silencing of *SISWEET5b* gene expression in flowers to approximately 10%–20% that of the wild-type (WT) flowers (Supplemental Figure S4A). Thus, these highly silenced T1 lines (T1-HS) were characterized in more detail. Like the T0 plants, there were no significant differences in plant size (Supplemental Figure S4B) and average fruit fresh weight (Supplemental Figure S4C) observed between WT, T1-NS (T1 plants derived from a T0-NS line, RNAi-7), and T1-HS plants. However, the germination rate of pollen grains from T1-HS plants was

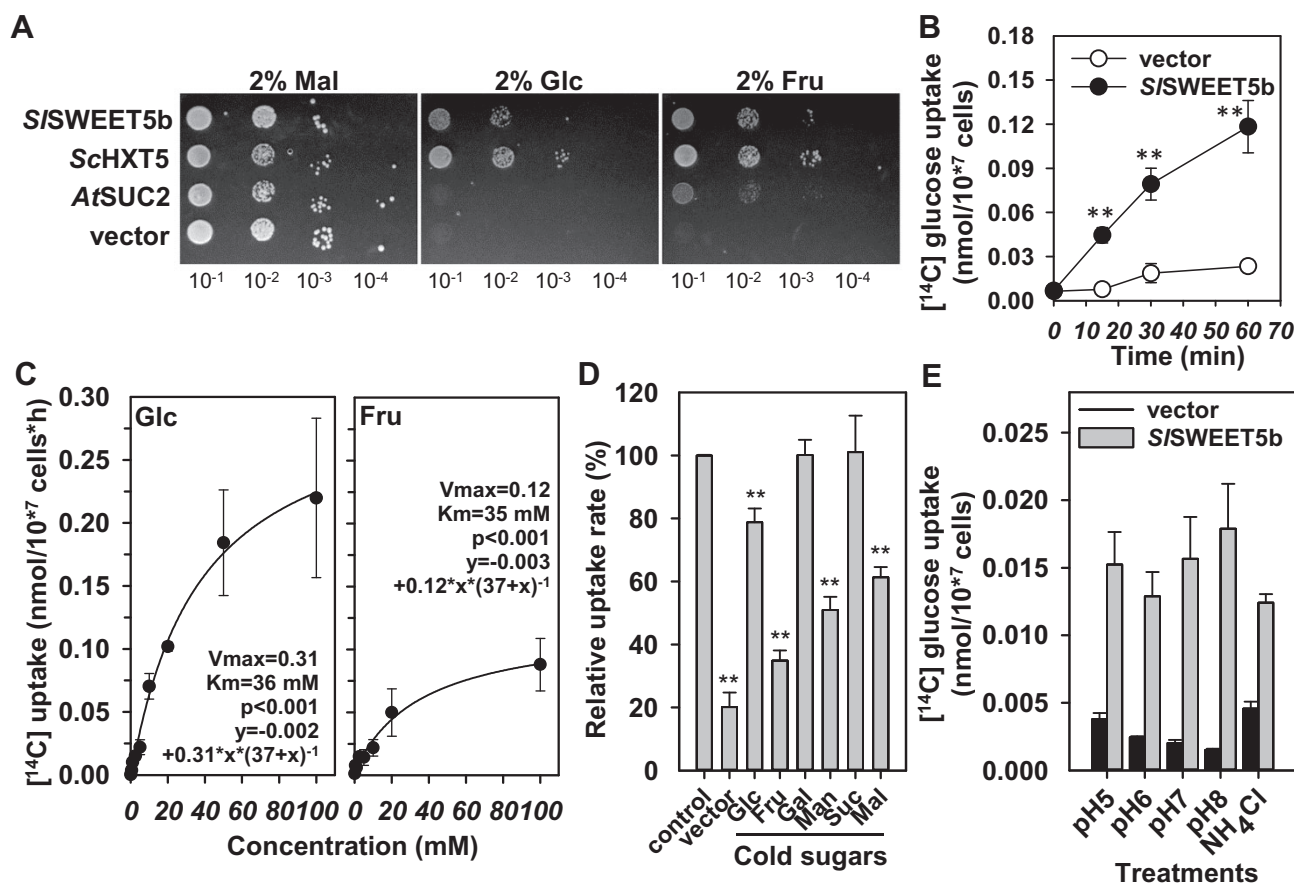


Figure 4 Specific transport activities of *S/SWEET5b* in yeast. A, Complementation of hexose import by *S/SWEET5b* in the yeast mutant. Yeast transformants expressing the yeast hexose transporter (*ScHXT5*), Arabidopsis sucrose symporter (*AtSUC2*) or the empty vector (vector) were serially diluted (10-fold) and cultured on solid media supplemented with 2% Mal, Glc, or Fru. Yeast growth was imaged after incubation at 30°C for 4–6 d. B, Time-dependent uptake activity of *S/SWEET5b* with 1 mM [¹⁴C]Glc. C, Kinetic of Glc or Fru uptake catalyzed by *S/SWEET5b*. D, Substrate specificity of *S/SWEET5b*. Relative uptake rates were measured by incubating *S/SWEET5b*-expressing cells with only 1 mM [¹⁴C]Glc (control) or hot glucose with 10 times concentrated indicated cold sugars. The uptake rate of cells expressing the empty vector (vector) is also shown. E, Insensitivity of *S/SWEET5b* transport to alkaline pH. Yeast cells expressing the empty vector or *S/SWEET5b* were incubated with 1 mM [¹⁴C]Glc and subjected to various pH conditions and treated with NH₄Cl. C–E, Total uptake in 15 min was shown. B–E, Results are mean ± SE (*n* = 3–4 independent colonies) and repeated for two independent transfections. Significant differences from the empty vector (B), control (D), or at pH 5 (E) were determined by Student's *t* test. ***P* < 0.01.

only ~5%, compared to 40% for those from WT plants (Figure 6A). Staining with the DNA-binding dye 4',6-diamidino-2-phenylindole (DAPI) solution (Vector Laboratories Inc.) demonstrated the presence of vegetative and sperm nuclei in WT pollen grains (Figure 6B). In contrast, nuclei were absent in most T1-HS pollen cells, and they were also misshapen (Figure 6B). Up to 80% of T1-HS pollen grains were abnormally shaped and lacked nuclei, compared to <25% of such grains in WT flowers (Figure 6C). Cryo-electron microscopic analysis revealed that pollen grains from T1-HS plants tended to clump and remained adhered to the anther inner layer, with a shrunken, invaginated shape. In contrast, WT pollen grains appeared smooth and round in shape and were freely released into the anther locule (Figure 6D).

Consistent with defective pollen, seed number per fruit was strongly decreased by 90%, in T1-HS plants compared

to that in WT or T1-NS plants (Figure 6E). The small number of T2 seeds that developed in T1-HS plants were subsequently shown to be mostly nontransgenic segregants derived from heterozygous T1 plants. The dry weight of T2 seeds from T1-HS plants was similar to that of WT and T1-NS plants (Figure 6E), indicating that *S/SWEET5b* functions in seed production, but does not participate in seed filling upon fertilization.

Sugar homeostasis was affected in *S/SWEET5b*-silenced stamens

Based on the hexose transport activity of *S/SWEET5b* in yeast (Figure 4), we hypothesized that the defective pollen observed in silenced plants may be due to sugar starvation. To examine this hypothesis, we compared the sugar concentrations in the stamens of open flowers of T1-HS lines. In both WT and silenced flowers, hexoses accumulated in

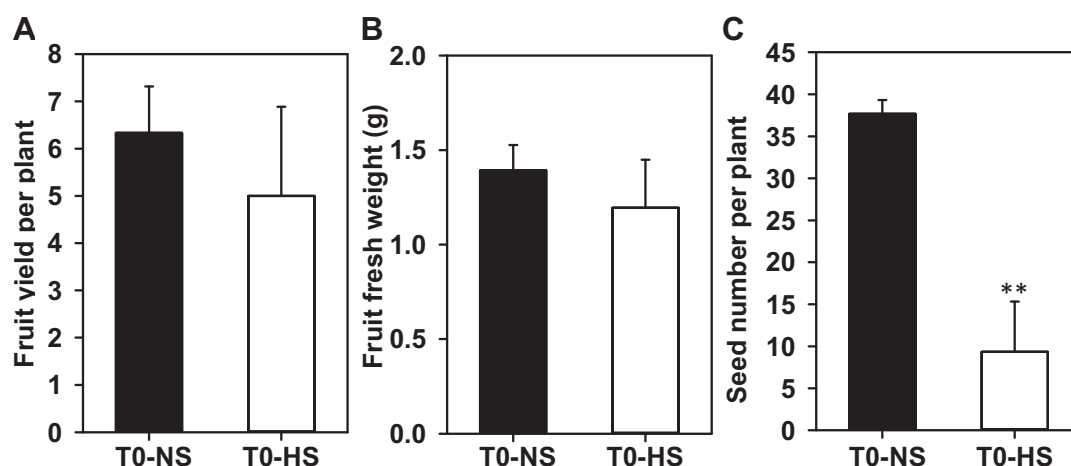


Figure 5 Fruit and seed yields of *SISWEET5b* knockdown tomato plants. Mature red fruits were harvested from T0 transgenic tomato plants expressing the *SISWEET5b*-RNAi construct. A, Fruit yield per plant. B, Average fresh weight per fruit. C, Total seed number per plant. Results are mean $\pm$ SE from three independent T0 transgenic lines where *SISWEET5b* expression was nonsilenced (T0-NS) or highly silenced (T0-HS). Four fruits from each line were averaged. Significant differences from the T0-NS lines were analyzed by Student's *t* test. ***P* < 0.01.

stamens to a level eight-fold that in petals, indicating strong sink activity of tomato stamen during pollen maturation (Figure 6F; Supplemental Figure S4D). However, significant reductions in Suc were observed in the stamens of silenced plants (Figure 6F). Lower Fru and total sugar contents were also observed in the T1-HS stamens. However, sugar concentrations in petals of T1-HS flowers were similar to those of WT flowers (Supplemental Figure S4D), suggesting a specific effect of *SISWEET5b* silencing in stamens. Studies have shown that hexose levels in pollen are regulated by extracellular invertase activity (Goetz et al., 2001; Shen et al., 2019). To further address whether reduced sugar content resulting from *SISWEET5b* silencing affects sugar metabolism, invertase activity in T1-HS flowers was analyzed. Significantly higher activities of CWIN and cytosolic invertase (CIN) were measured in T1-HS flowers than those in WT flowers (Figure 6G). However, there were no differences in vacuolar invertase (VIN) activity among them. In summary, these results confirm that silencing *SISWEET5b* transport activity results in reduced sugar accumulation in the stamen and perturbs sugar metabolism by altering invertase activity.

Discussion

Optimization of molecular properties to support higher fruit yields is of great importance for global food security (Ruan et al., 2012). Tomato (*S. lycopersicum*) is a major fruit crop with a global production value of up to 90 million US\$ (FOASTAT, 2018). In particular, fruit yield in tomato is closely related to pollen fertility (Xu et al., 2017), which critically depends on the continuous supply of assimilates, especially sugars (Clément et al., 1996; Firon et al., 2006; Castro and Clément, 2007). During pollen maturation, high amounts of sugars are translocated to the tomato stamen to support cell wall formation and pollen germination after pollination (Pressman et al., 2012). However, symplastic isolation of developing pollen cells from surrounding nutritive

anther cells, such as tapetum cells (at the early growth stage) or the anther inner cells (during pollen maturation stage; Polowick and Sawhney, 1993; Brukhin et al., 2003), results in the need for a set of plasma membrane STPs to mediate the two steps of sugar flux. During the first transport event, sugars are exported from anther cells into the locular apoplasm, which is followed by sugar import into developing pollen grains for maturation (Clément and Audran, 1995; Borghi and Fernie, 2017). The results presented here indicate that *SISWEET5b* is the primary sugar carrier responsible for hexose translocation for pollen maturation in tomato flowers.

Among the 30 *SISWEET* genes in the tomato genome, *SISWEET5b* was the sole member that showed dominant expression in flowers, while its expression in vegetative tissues was relatively low (Figure 1, A and B). The predominant expression of *SISWEET5b* in tomato stamen (Figure 1C) was similar to that of previously characterized sugar carriers that function in sugar transport during pollen development, such as Arabidopsis AtSWEET8 and AtSWEET13 (Guan et al., 2008; Sun et al., 2013), and cucumber CsSUT1 (Sun et al., 2019). This positive association suggests a specific role for *SISWEET5b* in pollen development. In line with this, we observed that the expression of *SISWEET5b* was greatly induced during the maturation stage of flower buds (S15, 7 mm in Figure 1D), when the formation of microspores and degradation of tapetum cells occur (Brukhin et al., 2003). In the development stage 15 of a tomato flower, carbohydrates stored in tapetum cells are mobilized as soluble sugars to the loculus (Goldberg et al., 1993; Castro and Clément, 2007). This coordinated process leads to the gradual accumulation of sugars during microspore maturation in both tomato locules and pollen grains, reaching maximal levels prior to anthesis (Pressman et al., 2012).

The clear concurrence of tissue-specific *SISWEET5b* expression in the stamen with sugar accumulation in tomato pollen cells (Pressman et al., 2012) indicates that *SISWEET5b* is

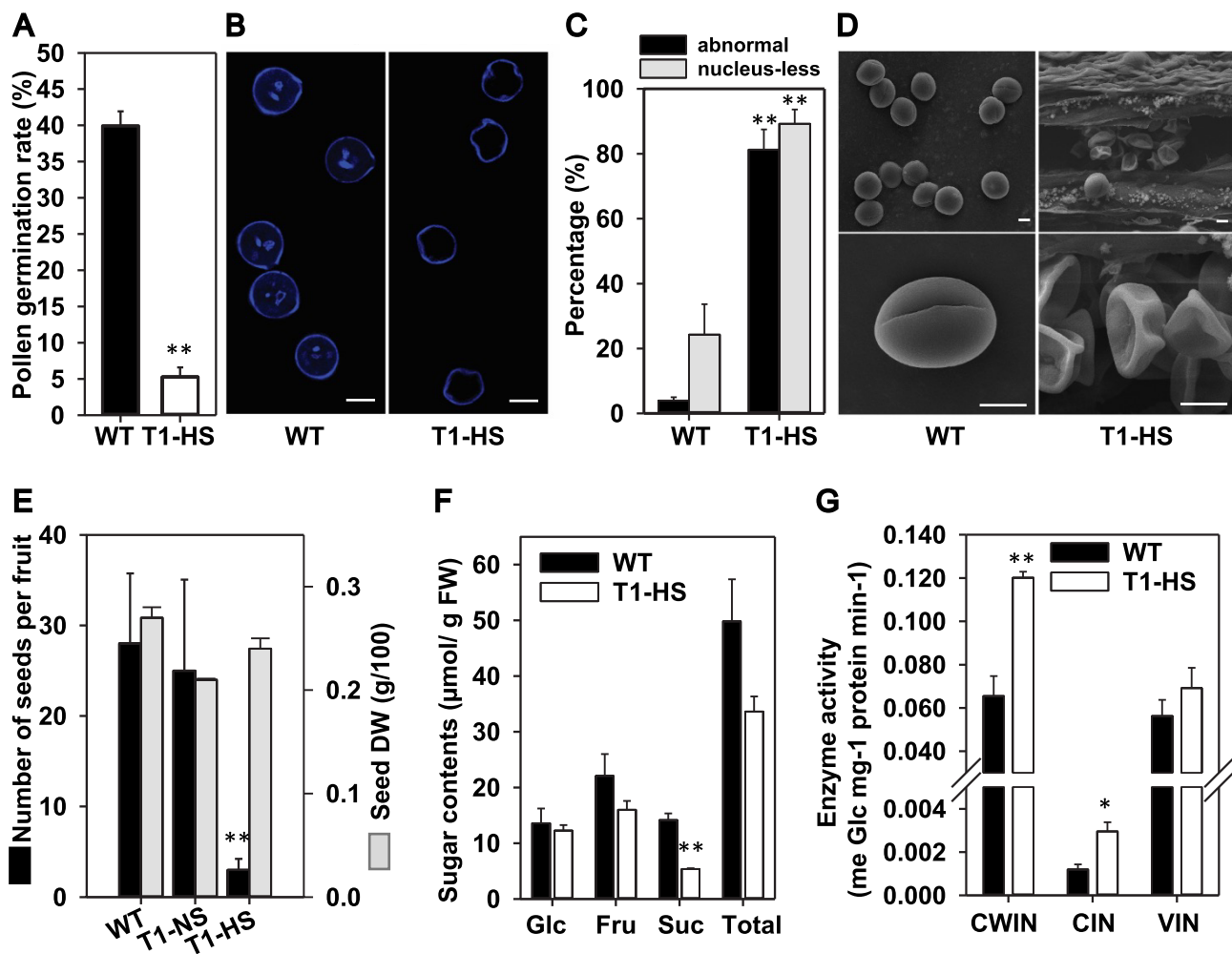


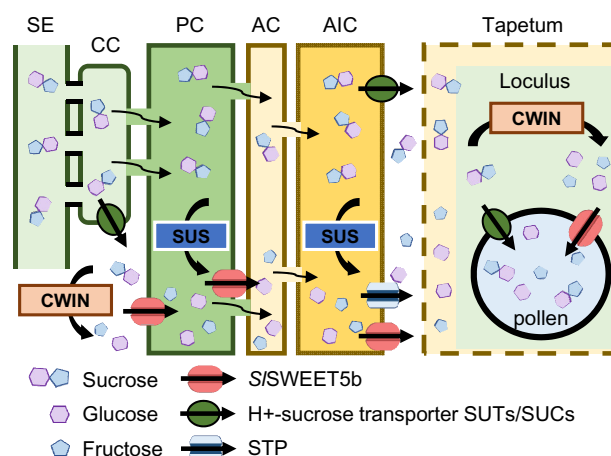
Figure 6 Pollen and seed development in *SISWEET5b*-silenced tomato plants. A, Pollen germination rate. Germinated pollen grains of WT and highly silenced T1 plants (T1-HS) were counted after incubation in the germinating buffer for 4 h. B, Pollen morphology in (A). Freshly harvested pollen grains were stained with DAPI for 30 min and imaged under a confocal microscope. C, Ratio of defective pollen grains in (B). D, Ultrastructure of the tomato *SISWEET5b*-silenced pollen. Stamens were detached from 1-d-anthesis flowers of WT and T1-HS plants and directly frozen on a slide for observation under a cryo-scanning electron microscope. E, Seed yield of T1 plants. Results are mean $\pm$ SE from five independent fruits that were harvested from WT, T1-HS, or nonsilenced T1 plants (T1-NS). F, Sugar concentration of the stamen. G, Invertase activity of flowers. In (F) and (G), stamens or flowers were harvested from the 1-d-anthesis flowers of WT and T1-HS lines. Sugar concentration (Glc, Fru, and Suc; Total, sum of three sugars) and invertase activities (CWIN, CIN, and VIN) were analyzed enzymatically. Results of (A), (C), (E), (F), and (G) are mean $\pm$ SE from three to four biological samples collected from individual independent plants. Similar trends have been repeated in three independent experiments. Bar = 10 μ m in (B and D). Differences from WT were analyzed using Student's *t* test. **P* < 0.05, ***P* < 0.01.

involved in sugar unloading into pollen that is required for maturation. This assumption is supported by the accumulation of *SISWEET5b* proteins in maturing pollen grains, but not in early developing microspores (Figure 2C), as indicated by the analysis of GUS labeling using a whole-gene translational GUS fusion construct (Figure 2). Moreover, the presence of *SISWEET5b* was also detected in the corresponding sugar-unloading cells, including in the vascular tissues (Figure 2L) and the anther inner cell layer (Figure 2, F and K). The anther phloem and the surrounding phloem parenchyma tissue represent the major sugar unloading sites for Suc delivered from maternal filaments (Mascarenhas, 1989). Anther inner cells, symplasmically isolated from pollen cells, would serve as the main sugar reservoir for pollen

maturation, when tapetum cells become degraded (Goldberg et al., 1993; Clément and Audran, 1995; Castro and Clément, 2007). Consistent with this, anther cells contain the highest sugar concentrations within the stamen when compared to those in the locule space and pollen cells (Pressman et al., 2012). Thus, the localization of *SISWEET5b* in cells involved in apoplastic sugar unloading in the stamen highlights the possibility that *SISWEET5b* mediates the entire apoplastic translocation pathway in the stamen, initial unloading into phloem parenchyma cells, sugar efflux from nutritive anther cells, and influx into pollens to support their maturation.

The latter assumption is further supported by independent observations: *SISWEET5b* is located in the plasma

Interestingly, *SISWEET5b* proteins were also detected in different styles (Figure 2I). Our observations are consistent with the stimulated expression of the *SISWEET5b* gene in the style in response to pollination (Shen et al., 2019). Upon pollination, the pollen tube performs rapid tip growth to deliver sperm nuclei to ovules for fertilization (Lord and Russell, 2002). The high energy requirement for pollen tube growth depends on a continuous supply of nutrients in the form of amino acids and sugars (Konar and Linskens, 1966; Borghi and Fernie, 2017), which are secreted by the surrounding tissues (Mascarenhas, 1993; Cheung, 1996; Goetz



et al., 2017). However, an apoplastic barrier with surrounding style tissues (Scott et al., 1991) predicts that plasma membrane-localized STPs are required to allow transport of sugars from style tissue to apoplast and subsequently into elongating pollen tubes (Reinders, 2016; Goetz et al., 2017). We assume that *SISWEET5b* may contribute to sugar unloading from style cells into the transmitting tract, where sugar uptake into growing pollen tubes is mediated by proton-driven SUC/SUT and hexose transporter (HT) type transporters (Cheng et al., 2015; Shen et al., 2019; Li et al., 2020).

It is surprising but explainable that *SISWEET5b*-silenced plants contained decreased levels of Suc in stamen tissue (Figure 6F), although this carrier prefers monosaccharides

and does not transport Suc (Figure 4, C and D). Suc is known to be the major type of sugar present in mature pollen, although Glc and Fru are preferred carbon skeletons that are imported into tomato pollen (Pressman et al., 2012). However, there is evidence to suggest that Suc is constantly resynthesized within tomato pollen during maturation, most likely via fructokinase2 and Suc-phosphate synthase activity (Pressman et al., 2012; Borghi and Fernie, 2017). Such dynamic Suc fluxes might act as a carbon buffer to support pollen wall formation or accumulation of starch (Sun et al., 2013), which functions as an energy reserve for germination and represents a checkpoint for pollen maturation (Datta et al., 2002). In summary, impaired *SISWEET5b* activity likely leads to impaired hexose import and carbohydrate deficiency, which limits both cellular energy provision for mitosis and carbon precursor availability that is required for Suc resynthesis and pollen maturation. Such processes ultimately result in male sterility. Consistently, few viable seeds can be generated from pollen grains carrying severe *SISWEET5b* silencing (Figures 5, C and 6, E).

The decreased *SISWEET5b* activity also affected sugar metabolism in tomato stamen, which was further demonstrated by the induced invertase activities, particularly the CWIN, in *SISWEET5b*-silenced flowers (Figure 6G). Considering no changes in sugar content in the silenced petals (Supplemental Figure S4D) and dominant accumulation of *SISWEET5b* proteins and sugars in tomato stamens (Figures 2 and 6, F; Supplemental Figure S4D), it is likely that the induced cell wall invertase activity occurs in the stamen. This note is supported by the fact that the extracellular invertase, such as *Lin7* in tomato (Proels et al., 2003) and *Nin88* in *N. benthamiana* (Goetz et al., 2001), shows a tapetum/pollen-specific expression pattern in flowers, along with high CWIN activity that can be detected in the maturing anther and pollen (Goetz et al., 2001; Pressman et al., 2012). Indeed, CWIN and STP genes are commonly co-expressed in reproductive organs as recently reviewed by Ruan (2022). The reason why silencing *SISWEET5b* leads to increased CWIN activity remains unknown. We can only speculate that silencing *SISWEET5b* could slow down hexose transport into pollen grains, resulting in low energy state, which may potentially upregulate extracellular invertase activity through an unknown feedback mechanism. In this case, higher CWIN activity in the anther locus may generate a signal that promotes hexose uptake by other types of STPs (Liao et al., 2020). Alternatively, more hexose molecules, due to higher CWIN activity, may increase the sugar gradient across the pollen membrane, which would favor *SISWEET5b*-mediated hexose import into pollen cytosol in the silenced pollen grains. The validity of this speculation is yet to be experimentally examined.

Considering all the above, we propose a functional model of *SISWEET5b* during pollen maturation (Figure 7). During the pollen maturation stage, a large amount of sugar is required for the formation of microspores and a structurally complex pollen cell wall. To fulfill the corresponding high

carbon demand, in addition to symplasmic Suc unloading, the plasma membrane-located *SISWEET5b* protein facilitates Suc unloading from phloem cells by importing apoplasmic hexoses, previously hydrolyzed by CWIN from extracellular Suc. Once sugars are translocated to the anther inner cells, *SISWEET5b* can also promote passive hexose export to the locule, where most of the Suc is converted to Glc and Fru by CWIN. The high monosaccharide concentration in the locular apoplasm allows *SISWEET5b* to catalyze the uptake of hexoses into maturing pollen grains. In tomatoes, pollen develops inside a closed anther cone, where the individual stamen is fused to cover the whole pistil (Brukhin et al., 2003). Because of this morphology, self-fertilization is common in tomato flowers, thereby driving the costs of hybrid seed production. Thus, induced male sterility by inactivation of *SISWEET5b* may provide a new strategy for the development of male sterile lines for commercial F1 hybrid seed production (Du et al., 2020).

Materials and methods

Plant materials and growth conditions

A tomato (*S. lycopersicum*) micro-tom was used in this study. Seeds were sterilized, germinated, and grown in a controlled environment as described previously (Ho et al., 2019). For developmental expression profiling, roots, stems, young leaves (<2 cm long) mature leaves (>3 cm, terminal leaflet), and flowers (1 d post-anthesis) were collected from 5-week-old hydroponically grown tomato plants. Flower organs (sepal, petal, stamen, and ovary) and different stages of flower buds (1, 3, 4, and 7 mm length corresponding to stage 6, 9, 11, and 15 developmental stages, respectively; Brukhin et al., 2003) were harvested from 6- to 7-week-old soil-grown tomato plants. All samples were stored at -80°C before analysis.

RNA expression analysis

Frozen tomato organs were grounded to a powder in liquid nitrogen and total RNA was isolated using TRIsure reagent (Bioline, <http://www.bioline.com/>) along with an RNA purification column (GeneMark, <http://www.genemarkbio.com/>). Quantitative analysis of RNA transcripts was performed as previously described (Ho et al., 2019). Briefly, 5 μg of mRNA was reverse-transcribed (RT) with MMLV (Qiagen) and the derived cDNA products were used for real-time quantitative PCR (qPCR) with gene-specific primers for all 30 *SISWEET* genes (primer sequences are listed in Ho et al., 2019). The expression of the reference gene *SlActin7* (Solyc11g005330) was used to normalize the results from different samples. Relative expression levels were determined using the following equation: $1000 \times 2^{-(C_{tSISWEET} - C_{tSlActin7})}$ (C_t = threshold cycle). For comparison, in some cases, the fold change was calculated by comparing the relative expression to that of the corresponding control.

Observations of GUS fusion proteins

The *S/SWEET5b* (Solyc06g071400) promoter (2,000-bp upstream of ATG) was amplified from genomic DNA using specific primers (SWT5b-promoter-F and SWT5b-promoter-R, Supplemental Table S1) and cloned via *SacI* and *SacII* sites into the binary vector pUTKan (pUTKan-P_{SWEET5b}), which include the gene sequences to express GUS. The *S/SWEET5b* genomic opening reading frame, including all introns (1,110 bp after ATG) but lacking the stop codon, was amplified with primers (SWT5b-gDNA-F and SWT5b-gDNA-R, Supplemental Table S1) and cloned into the pUTKan-P_{SWEET5b} vector via *SacII* and *BamHI* sites. The resulting plasmid pUTKan-P_{SWEET5b}::gS/SWEET5b was transferred into tomato plants via *Agrobacterium* transformation performed by the Transgenic Plant Core Lab in Academia Sinica (<http://abrc.sinica.edu.tw/transplant/index.php>). T0 transgenic plants were regenerated from kanamycin-resistant calli and grown in soil mix after shooting. T1 seeds were collected from the mature red fruits.

Various organs harvested from T0 plants were stained histochemically for GUS activity and imaged after 16 h of incubation as described previously (Ko et al., 2021). For tissue sections, flowers from 8-week-old T0 transgenic plants were collected and vacuum-infiltrated with 2% v/v paraformaldehyde (PFA) buffer (44-mM sodium hydroxide, 0.125% w/v glutaraldehyde, 0.05% v/v Triton X-100, 0.05% v/v Tween-20, 5-g paraformaldehyde, pH 7) for 10 min at 4°C in the dark. Samples were then washed three times with phosphate buffered saline (PBS) (137-mM sodium chloride, 2.7-mM potassium chloride, 4.3-mM disodium hydrogen phosphate, 1.47-mM potassium dihydrogen phosphate, pH 7.4) and stained for 16 h to observe GUS activity. Samples were washed with PBS and vacuum-infiltrated with 4% PFA buffer (87.5-mM sodium hydroxide, 0.25% w/v glutaraldehyde, 0.1% v/v Triton X-100, 0.1% v/v Tween-20, 10 g paraformaldehyde, pH 7.0) for 4 h at 4°C in the dark. Fixed samples were dehydrated in 30% ethanol for 50 min, transferred to 50% v/v ethanol for 50 min, and finally stored in 70% v/v ethanol. Tissue sections were then prepared and imaged by the in situ hybridization core facility in Academia Sinica (<http://abrc.sinica.edu.tw/2010/view/?mid=320&fid=39>).

Localizations of YFP fusion protein

To express the *S/SWEET5b*-YFP fusion protein in *Arabidopsis* protoplasts, the *mYFP* gene was amplified with primers YFP-F and YFP-R (Supplemental Table S1) and cloned into the vector pRT101 via *BamHI* and *XbaI* sites. The *S/SWEET5b* cDNA (714 bp) without the stop codon was amplified using specific primers (*SacI*-SWT5b-F and *BamHI*-SWT5b-dTGA-R, Supplemental Table S1), and cloned into the pRT101-YFP via *SacI* and *BamHI* sites (pRT101-*S/SWEET5b*-YFP). *Arabidopsis* protoplasts were extracted and transformed with the expression construct as described previously (Ko et al., 2021). To mark the position of the inner membranes, the plasma membrane marker AtPIP2A:CFP (1:1) was co-expressed with *S/SWEET5b*-YFP in *Arabidopsis* protoplasts. To transiently express *S/SWEET5b*-YFP in *N. benthamiana*

leaves, the fragment (2,078 bp), including the CaMV35s promoter-*S/SWEET5b*-YFP-CaMV terminator, was digested from the pRT101-*S/SWEET5b*-YFP plasmid and then inserted into the pJH212 binary vector via the *HindIII* site. *Agrobacterium tumefaciens* (EHA105 strain) was transformed with the final binary vector pJH212-*S/SWEET5b*-YFP. Agroinfiltration was performed as previously described (Sosso et al., 2015). After 36–48 h of transfection, epidermal cells were peeled and observed using a Carl Zeiss LSM780 confocal microscope (Instrument Development Center, NCKU). For plasmolysis treatment, epidermal cells were subjected to 1-M sodium chloride for 10 min before observation. YFP fluorescence was visualized by excitation with an argon laser at 514 nm (15% intensity for protoplasts; 10% intensity for tobacco leaf) and emission between 516 and 560 nm (master gain = 800, digital gain = 1). CFP fluorescence was visualized by excitation with an Argon Laser at 458 nm (80% intensity for protoplasts) and emission between 456 and 515 nm (master gain = 800, digital gain = 1). The autofluorescence of chloroplasts was visualized by excitation with a 561 nm laser (1.8% for protoplasts; 30% for tobacco leaf) and emission between 562 and 758 nm (master gain = 750, digital gain = 1).

Yeast growth assay

To express *S/SWEET5b* in yeast, the cDNA was amplified with specific primers (SWT5b-F and SWT5b-R), cloned into the pDONR221-f1 vector, and then transferred to the pDRf1-GW vector using Gateway technology to produce pDRf1-*S/SWEET5b*. A plasmid expressing AtSUC2 was constructed previously (Ho et al., 2020). The yeast strain EBY4000 was then transformed with this plasmid for the growth assay, as described previously (Ho et al., 2019). Briefly, single colonies of yeast transformants were grown to mid-log phase at 30°C in liquid synthetic uracil-deficient medium (SD, including 1.7-g yeast nitrogen base without amino acids, 5-g ammonium sulfate, and 0.01% histidine, leucine, and tryptophan in 1 L) supplemented with 2% maltose (SDM). Cells were collected by centrifugation and diluted to an optical density (OD)₆₀₀ of 0.2 with fresh SDM. Serial dilutions (10⁻¹, 10⁻², 10⁻³, 10⁻⁴) of cell cultures were prepared, and 5 µL of each dilution were spotted on solid SD medium supplemented with 2% maltose (Man), glucose (Glc), or fructose (Fru), respectively. Yeast growth was imaged after 3–5 d of incubation at 30°C.

Radiotracer uptake assays in yeast

The radiotracer uptake assay was slightly modified from that of the previous study (Ho et al., 2019). An overnight cell culture containing the pDRf1-*S/SWEET5b* plasmid was prepared using fresh SDM. Cells were diluted with fresh medium to an OD₆₀₀ of 0.2 and grown at 30°C to an OD₆₀₀ of 0.5. Yeast cells were washed and resuspended to an OD₆₀₀ of 5 in the SP buffer (50-mM sodium phosphate buffer, containing 112 mg of Na₂HPO₄ and 6.8 g of NaH₂PO₄ in 1 L, pH 5) for radiotracer uptake assays as previously described (Ho et al., 2019). For the time-dependent uptake assay, cells were incubated for the indicated time points in the SP hot

buffer. For the kinetic assay, cells were incubated for 15 min in SP buffer containing various concentrations of Glc or Fru (0.2, 1, 2, 5, 10, 20, 40, and 100 mM) with the same molar ratio of μCi of [^{14}C]Glc or [^{14}C]Fru, respectively. For the substrate specificity assay, cells were incubated for 15 min in hot SP buffer (control) or hot buffers supplemented with 10-fold concentrations of other cold sugars (10-mM Glc, galactose, Fru, Man, Suc, or Mal). To examine the effects of various pH values on uptake activities, hot SP buffers with various pH values were freshly prepared using 50-mM sodium phosphate. The cells were then washed with cold pH buffers before incubating for 10 min in the corresponding hot uptake buffers. For treatment with the protonophore NH_4Cl , cells were pre-treated with 10-mM NH_4Cl for 10 min before incubating with the hot SP buffer at pH5. To start all uptake assays (except the kinetic assay), 110 μL of the corresponding SP hot buffers containing 2-mM Glc and 2 μCi of [^{14}C]Glc mL^{-1} were added into an equal volume of cells that were incubated at 30°C. Cells were collected on filter paper (MCE Membrane Filter, 0.45 μm) and washed three times with ice-cold SP buffer by vacuum filtration. Cells on filter papers were then lysed with 4 mL of Rotiszint eco plus (Carl Roth, Germany) in scintillation vials. After 16–18 h of incubation at room temperature, the radioactivity of each vial was quantified using a Tri-Carb 4810TR scintillation counter (Perkin Elmer, USA). Kinetic curves were determined using a single rectangular regression (Sigmaplot version 13).

Establishment of RNAi transgenic plants

To construct a self-complementary hairpin RNA for RNAi post-transcriptional silencing, forward and reverse partial coding sequences of *SISWEET5b* (202- to 501-bp downstream of ATG) were amplified with specific primers (SWT5b-RNAi-SacI-F and SWT5b-RNAi-SpeI-R for the forward; SWT5b-RNAi-NcoI-F and SWT5b-RNAi-SacII-R for the reverse, Supplemental Table S1). The middle intron loop sequence was amplified from the pHellsgate8 plasmid with primers Intron-F and Intron-R (Supplemental Table S1) and cloned into pRT101 (pRT101-intron). The forward and reverse *SISWEET5b* partial sequences were then cloned into the pRT101-intron via SacI/SpeI and NcoI/SacII sites, respectively. The whole RNAi fragment was then cloned into the binary vector pJH212 via the SacI and SacII sites. The resulting plasmid, pJH212-*SISWEET5b*-RNAi, was then transferred into tomato plants by *Agrobacterium* transformation performed by the Transgenic Plant Core Lab in Academia Sinica (<http://abrc.sinica.edu.tw/transplant/index.php>). Eighteen T0 transgenic tomato plants were regenerated and transferred to soil. Flowers of 5- to 6-week-old transgenic plants were collected to examine the expression of *SISWEET5b*. Red fruits from the silenced lines were collected to harvest F1 seeds.

Observation of pollen development

To determine the pollen germination rate, stamens were collected from opened flowers (1 d post-anthesis), placed into 1.5-mL Eppendorf tubes, pressed and mixed well with

100- μL pollen germination buffer (20-mM MES, 3-mM $\text{Ca}(\text{NO}_3)_2$, 1-mM KCl, 0.8-mM MgSO_4 , 1.6-mM boric acid, 24% w/v PEG4000, 2.5% w/v Suc, pH6.0; Lu et al., 2015). The pollen mixture was incubated at 25°C for 2 h on a shaker at 90 rpm. Pollen germination was deemed successful when the pollen tube was longer than the diameter of the pollen (Cheng et al., 2015). To observe pollen nuclei, 20 μL of the above pollen mixtures were centrifuged, and the supernatant was discarded. Pollen grains were first fixed for 30 min by adding 50 μL of Carnoy's fluid (absolute ethyl alcohol: glacial acetic acid = 3: 1 v/v; Lu et al., 2015). Subsequently, treated pollen grains were collected by centrifuging at 100g and stained for 30 min with 20 μL of DAPI solution and observed using a Carl Zeiss LSM780 confocal microscope (Instrument Development Center, NCKU). DAPI fluorescence was visualized by excitation with an argon laser at 405 nm (24% intensity) and emission between 410 and 492 nm (master gain = 700, digital gain = 1). To observe pollen morphology *in vivo*, stamens were freshly collected and observed under a cryo-scanning electron microscopy, which was performed by Cell Biology Core Lab in Academia Sinica (<https://ipmb.sinica.edu.tw/en/core/Plant-Cell-Biology-Core-Lab>).

Sugar content analysis

Stamens and petals were collected from flower buds 7 mm in length and ground into a powder with liquid nitrogen. Samples were extracted in 1-mL 80% v/v ethanol at 80°C for 30 min and centrifuged at 13,000g for 10 min. The supernatant was collected and dried using a Vacufuge Plus concentrator (Eppendorf, NY, USA), and then re-suspended in 50- μL water. To determine contents of Glc, Fru and Suc, 20 μL of the extract were mixed with 190 μL of the reaction buffer (100-mM, pH 7.5, HEPES, 10-mM magnesium chloride, 2-mM adenosine 5'-triphosphate, 0.8-mM NAD^+ , 0.05% v/v glucose-6-phosphate-dehydrogenase [G6P-DH]), where NADH was produced from NAD^+ in the presence of hexose-6-phosphate. In a series of coupled enzymatic reactions, the NADH absorption changes ($\lambda = 340 \text{ nm}$) were determined using a microtiter plate reader, and sugar concentrations were calculated as previously described (Rodrigues et al., 2020). Background absorption (A_0) was measured. Hexokinase, which catalyzes the conversion of hexoses (Glc or Fru) into hexose-6-phosphate, was first added to the mixture, resulting in an increase in NADH absorption (A_1). The absorption change ($A_1 - A_0$) was used to determine Glc concentration. Phosphoglucose isomerase was then added, which converts fructose-6-phosphate to glucose-6-phosphate, and this reacts with G6P-DH and increases NADH absorbance (A_2). The absorption change ($A_2 - A_1$) was used to determine the Fru concentration. Finally, invertase, which hydrolyzes Suc to Glc and Fru, was added, and the resulting monosaccharides reacted with G6P-DH and led to increased NADH absorbance (A_3). The change in absorption ($A_3 - A_2$) was used to determine the Suc concentration.

Invertase activity assay

Fresh tomato flower tissues were harvested, immediately freeze-dried and ground to a powder in liquid nitrogen. Samples were extracted three times with a total of 1.4-mL extraction buffer and centrifuged at 14,000g at 4°C for 10 min. The supernatants were used for activity assays of CIN and VIN, and the pellet was used for the cell wall invertase activity assay as reported previously (Tomlinson et al., 2004) with slight adjustments. In short, 10 µL of supernatant or pellet fractions were added to 180 µL of invertase assay mix. The mixtures were incubated at 30°C for 1 h and then at 85°C for 3 min. Fructosidase assays were performed by adding 100 µL of the mixture to 400 µL of the Fru assay mix. G6P production from Glc was determined by the increase in absorbance at $\lambda = 340$ nm. The extraction buffer, invertase assay mix, and Fru assay mix were prepared as previously described (Tomlinson et al. 2004).

Accession numbers

Sequence data from this article can be found in the Solanaceae Genomics Network data library under locus name_. *SISWEET6a* (Solyc02g086920), *SISWEET6b* (Solyc11g028270), *SISWEET7a* (Solyc08g082770), *SISWEET7b* (Solyc12g055870), *SISWEET5a* (Solyc03g114200), and *SISWEET5b* (Solyc06g071400).

Supplemental data

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

Supplemental Figure S1. *SISWEET5b* functions on the plasma membrane.

Supplemental Figure S2. Phenotypes of T0 transgenic RNAi plants.

Supplemental Figure S3. Fruit and seed production in T0 transgenic plants.

Supplemental Figure S4. Growth of T1 transgenic plants carrying *SISWEET5b*-RNAi construct.

Supplemental Table S1. The list of primers used in this study.

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Conflict of interest statement. None declared.

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