Down-regulation of the Sucrose Transporter *CsSUT1* Causes Male Sterility by Altering Carbohydrate Supply[1][OPEN]

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In plants, male sterility is an important agronomic trait, especially in hybrid crop production. Many factors are known to affect crop male sterility, but it remains unclear whether Suc transporters (SUTs) participate directly in this process. Here, we identified and functionally characterized the cucumber (*Cucumis sativus*) CsSUT1, a typical plasma membrane-localized energy-dependent high-affinity Suc-H^{+} symporter. *CsSUT1* is expressed in male flowers and encodes a protein that is localized primarily in the tapetum, pollen, and companion cells of the phloem of sepals, petals, filaments, and pedicel. The male flowers of *CsSUT1*-RNA interference (RNAi) lines exhibited a decrease in Suc, hexose, and starch content, relative to those of the wild type, during the later stages of male flower development, a finding that was highly associated with male sterility. Transcriptomic analysis revealed that numerous genes associated with sugar metabolism, transport, and signaling, as well as with auxin signaling, were down-regulated, whereas most *myeloblastosis* (*MYB*) transcription factor genes were up-regulated in these *CsSUT1*-RNAi lines relative to wild type. Our findings demonstrate that male sterility can be induced by RNAi-mediated down-regulation of *CsSUT1* expression, through the resultant perturbation in carbohydrate delivery and subsequent alteration in sugar and hormone signaling and up-regulation of specific *MYB* transcription factors. This knowledge provides a new approach for bioengineering male sterility in crop plants.

The utilization of male sterility is of importance for hybrid seed production. Many mutations leading to male gametophyte sterility have been shown to interfere with tapetal programmed cell death, meiosis, and mitosis, etc., supporting pivotal roles for these processes in the production of functional pollen grains (Niu et al., 2013; Ko et al., 2014; Han et al., 2018). Abnormal meiosis in the naked oat (*Avena nuda*) mutant *zbs1* also resulted in male sterility by forming defective callose walls and cell plates (Shi et al., 2016; Yang et al., 2016).

The knockout of the *Magnesium Transporter 5* resulted in Mg^{2+} deficiency, which led to defects in male gametophytes in Arabidopsis (*Arabidopsis thaliana*; Xu et al., 2015).

Carbohydrates also play critical roles in male gametophyte development by providing nutrients for normal growth, and may also influence development as signaling molecules during this process (Clement and Audran, 1995). Numerous male sterile lines have been shown to involve perturbations in carbohydrate metabolism. For example, in wheat, a decline in invertase activity alters carbohydrate metabolism, resulting in reduced starch accumulation within the pollen, leading to pollen abortion (Dorion et al., 1996). The rice (*Oryza sativa*) *carbon starved anther* (*csa*) mutant is also male sterile through a perturbation in sugar partitioning (Zhang et al., 2010). Overexpression of *GhCK1*, which encodes a cotton (*Gossypium hirsutum*) casein kinase, exhibits starch synthase kinase activity, perturbs Glc homeostasis, and triggers abscisic acid accumulation in cotton flower buds, leading to anther abortion (Min et al., 2013).

Suc, hexoses, and trehalose also function as signal molecules that participate in control of nutrient homeostasis, stress response, and organ development (Paul et al., 2008; Lastdrager et al., 2014). In addition, hexokinase 1 (HXK1) and Suc non-fermenting-1-related

protein kinase (SnRK1) were reported to be related to sugar signaling, as two dependent pathways (Moore et al., 2003; Broeckx et al., 2016; Sakr et al., 2018). Although the relationship between trehalose-6-phosphate and Suc signaling in plant remains to be fully resolved, it is known that trehalose-phosphate synthase (TPS) plays an important role in controlling the ratio of trehalose-6-phosphate and Suc (Figueroa and Lunn, 2016; Sakr et al., 2018).

Auxin signaling is also closely related to the development of male floral organs and has crosstalk with sugar signaling (Eveland and Jackson, 2012; Lin et al., 2016). Overexpression of the strawberry (*Fragaria x ananassa*) auxin biosynthesis gene, *FvYUC6*, delays flowering and leads to male sterility (Liu et al., 2014). It was also reported that auxin could reverse male sterility caused by high temperature in Arabidopsis (Sakata et al., 2010).

Generally, Suc transporters (SUTs)/Suc carriers (SUCs) belong to the major facilitator superfamily, and are divided into four distinct clades (Sauer, 2007; Kühn and Grof, 2010). The high-affinity SUT1/SUC2 clade members are targeted to the plasma membrane and function in phloem loading, long-distance transport, unloading, and stress resistance (Williams et al., 2000; Patrick and Offler, 2001; Sivitz et al., 2008; Srivastava et al., 2008; Jia et al., 2015). By contrast, the low-affinity SUT2/SUC3 and SUT4/SUC4 clade members are thought to function in sink organ development, seed germination, stress response, and so forth (Hackel et al., 2006; Chincinska et al., 2008; Frost et al., 2012; Li et al., 2012). Finally, the overall importance of these SUTs in mediating Suc transport has been amply demonstrated.

As pollen grains are symplasmically isolated from the anther (Clement and Audran, 1995; Lemoine et al., 1999; Zhang et al., 2010), Suc is delivered to the microspore, from the phloem, via a SUT/SUC, or by a monosaccharide transporter when Suc is hydrolyzed into hexoses by an extracellular invertase (Goetz et al., 2001; Schneidereit et al., 2005). Some *SUTs* from tomato (*Solanum lycopersicum*; Hackel et al., 2006), Arabidopsis (Sivitz et al., 2008), and rice (Hirose et al., 2010) are expressed in pollen and support a link between Suc transport and pollen development and viability. SlSUT2 in tomato plays a role in pollen tube growth (Hackel et al., 2006), and disruption of *OsSUT1* and *AtSUC1* in rice and Arabidopsis, respectively, impairs pollen germination (Sivitz et al., 2008; Hirose et al., 2010).

Expression of *SUT* genes is regulated by transcription factors. For example, the apple (*Malus domestica*) ABA-responsive transcription factor, MdAREB2, directly binds to the promoters of *MdSUT2* and amylase genes that are involved in soluble sugar accumulation (Ma et al., 2017). In addition, several transcription factors, such as MYB21, MYB24, MYB57, and MYB108, are involved in the regulation of male fertility in Arabidopsis (Cheng et al., 2009; Mandaokar and Browse, 2009; Song et al., 2011).

Presently, the question of whether SUTs directly affect male flower development and/or pollen formation remains to be established. Here, we identified a cucumber (*Cucumis sativus*) Suc transporter gene, *CsSUT1*, which was expressed primarily in the male flower; the gene encoded a plasma membrane-targeted protein that was localized on the tapetum, pollen, and companion cells (CCs) in the phloem of sepals, petals, filaments, and the pedicel of the male flower. Down-regulation of *CsSUT1* expression resulted in a decrease in Suc, hexose, total soluble sugars, and starch levels in the male flower. In addition, this reduction in *CsSUT1* expression gave rise to a decline in sugar metabolism, a reduction in expression of transport-related genes, and altered sugar and auxin signaling, and the up-regulation of the expression of several *MYB* transcription factors. These findings support the hypothesis that CsSUT1 plays a central role in male flower development, and that a block in this transport step can lead to male sterility.

RESULTS

CsSUT1 Encodes a Typical Suc Transporter and Is Expressed Primarily in Male Flowers

The gene encoding CsSUT1 was isolated from cucumber stage 12 male flowers (Accession no. MG324290), and its 1,488-bp open reading frame encoded a protein of 495 amino acid residues, with a predicted molecular mass of 46 kD (Supplemental Fig. S1). The predicted CsSUT1 amino acid sequence exhibited a modular structure typical for major facilitator superfamily members, comprising 12 transmembrane-spanning regions (Supplemental Fig. S2). A phylogenetic analysis indicated that CsSUT1 belongs to the SUT1 subfamily (Aoki et al., 2003; Kühn and Grof, 2010) of high-affinity Suc transporters (Supplemental Fig. S3; Supplemental Table S1). Another two cucumber Suc transporters, CsSUT2 and CsSUT4 (http://cucumber.genomics.org.cn/page/cucumber/index.jsp), belong to the SUT2 subfamily and the SUT4 subfamily, respectively (Supplemental Fig. S3). These findings established CsSUT1 as a classic SUT1-clade Suc transporter.

The molecular function of CsSUT1 was verified by heterologous expression in a Suc uptake-deficient yeast mutant, SUSY7/*ura3* (Riesmeier et al., 1992). The drop test revealed that *CsSUT1*-SUSY7/*ura3* transformants could rescue mutant yeast growth on Suc medium (Supplemental Fig. S4A). Yeast strains harboring the full-length *CsSUT1* displayed linear ^{14}C-Suc uptake characteristics (Supplemental Fig. S4B); the optimum pH for transport was 3.5 (Supplemental Figure S4C) and the $K_{0.5}$ value was 75.6 μM (Supplemental Figure S4D).

Assay involved in testing the specificity of CsSUT1 for its substrate indicated that only Suc caused an inhibition of ^{14}C-Suc uptake (Supplemental Fig. S4E). Furthermore, Suc transport was strongly

inhibited by p-chloromercuribenzene sulfonate (SH group inhibitor), and the protonophore carbonyl cyanide m-chlorophenylhydrazone (an uncoupler of transmembrane proton gradients; Supplemental Fig. S4E). Additional ^{14}C-Suc transport assays also showed that CsSUT1 can mediate Suc uptake when expressed in *Xenopus* oocytes (Supplemental Fig. S4F). Taken together, these findings indicated that CsSUT1 functions as a high-affinity, energy-dependent Suc-H$^+$ symporter.

Expression of *CsSUT1* occurred mainly in cucumber male flowers, but also in roots, stems, and female flowers, to some extent, with low expression in leaves and fruits (Fig. 1A). *CsSUT1* expression increased gradually with male flower development, reaching a peak at stage 12, and then decreased sharply at the time of flower opening (Fig. 1B; Table 1). At stage 12, *CsSUT1* was highly expressed in the anther, especially in pollen, with lower levels in sepals and petals, whereas expression levels of *CsSUT2* and *CsSUT4* were very low in all of these tissues (Fig. 1C).

CsSUT1 Is a Plasma Membrane Protein Detected Primarily in Tapetum, Pollen, and CCs in the Phloem of Male Flowers

Immunolocalization studies indicated that CsSUT1 was primarily detected in the tapetum, pollen in the anther, and CCs of the phloem located in the pedicle, sepals, petals, and filaments of stage 10 male flowers (Fig. 2, A–N). Similar results were obtained in other stages of male flower development (Supplemental Fig. S5). Transient expression of a CsSUT1-green fluorescent protein (GFP) fusion protein construct in cucumber protoplasts (Fig. 2O) and tobacco (*Nicotiana tabacum*) epidermal cells (Supplemental Fig. S6) demonstrated that it was targeted to the plasma membrane. These findings are consistent with previous SUT1 localization studies (Kühn et al., 1997; Knop et al., 2004; Schmitt et al., 2008). Taken together, our

Table 1. *Characteristics used to define the developmental stages of cucumber male flowers*

Stage	Length of Floral Bud (mm)	Morphological Indications
1–7	<1	Anther expands
8	1–1.5	Locules differentiate morphologically
9	2–3	Microsporocytes initiate
10	3–4	Meiosis in anthers
11	5–10	Uninuclear pollen appears
12	>11	Mature pollen is formed
13	Open	Pollen release

results established that CsSUT1 is a plasma membrane protein that is located primarily in the tapetum, pollen, and CCs of the phloem of the male flower, implicating a potential role in cucumber male flower development.

Down-regulating *CsSUT1* Expression Leads to Male Sterility

To further study the function of CsSUT1, we constructed an RNAi expression vector (Fig. 3A) that was used for *Agrobacterium*-mediated transformation of cucumber cotyledons (Cheng et al., 2015; Li et al., 2017a). Five of 30 transgenic lines were variously down-regulated, and pollen germination in these lines was impacted (Supplemental Fig. S7). In T1 plants, *CsSUT1*-RNAi 14-1 and 18-21 lines were selected for further study (Fig. 3B). There were no visible differences in general plant morphology between wild type and these *CsSUT1*-RNAi lines with respect to growth, stem diameter, and leaf size, with the exception of the male flowers (Supplemental Fig. S8).

Immunolocalization studies indicated that the CsSUT1 signal in male flowers of the *CsSUT1*-RNAi lines was very weak compared with wild-type male flowers (Supplemental Fig. S9). In these *CsSUT1*-RNAi lines, male flower development was perturbed; these
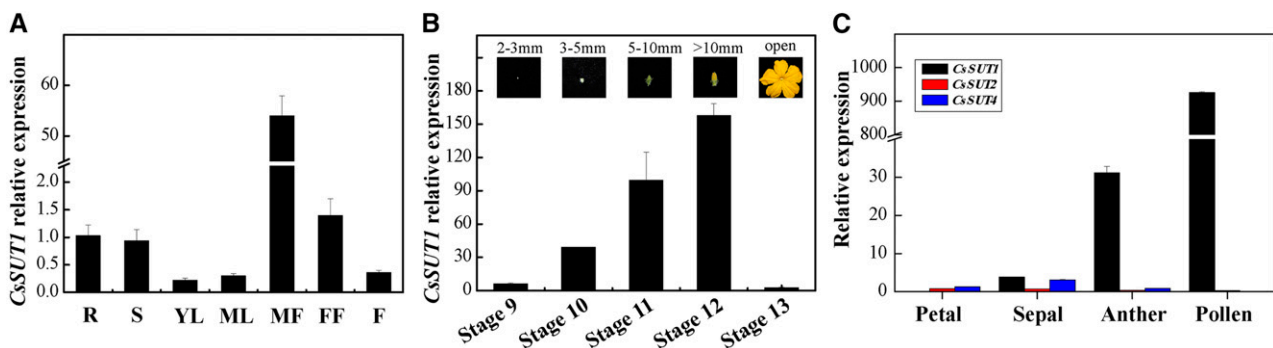

Figure 1. Spatio-temporal expression of *CsSUT1* in cucumber. A, RT-qPCR analysis of *CsSUT1* in different plant organs. B, RT-qPCR analysis of *CsSUT1* at various stages of male flower development. Images in (B) were obtained from the male flower at various developmental stages, illustrated by different floral bud lengths. C, Expression analysis of three cucumber *SUTs* in sepals, petals, anthers, and pollen of stage 12 male flowers. Error bars represent ± SEM ($n = 3$). Abbreviations: R, root; S, stem; YL, young leaf; ML, mature leaf; MF, male flower; FF, female flower; F, fruit.
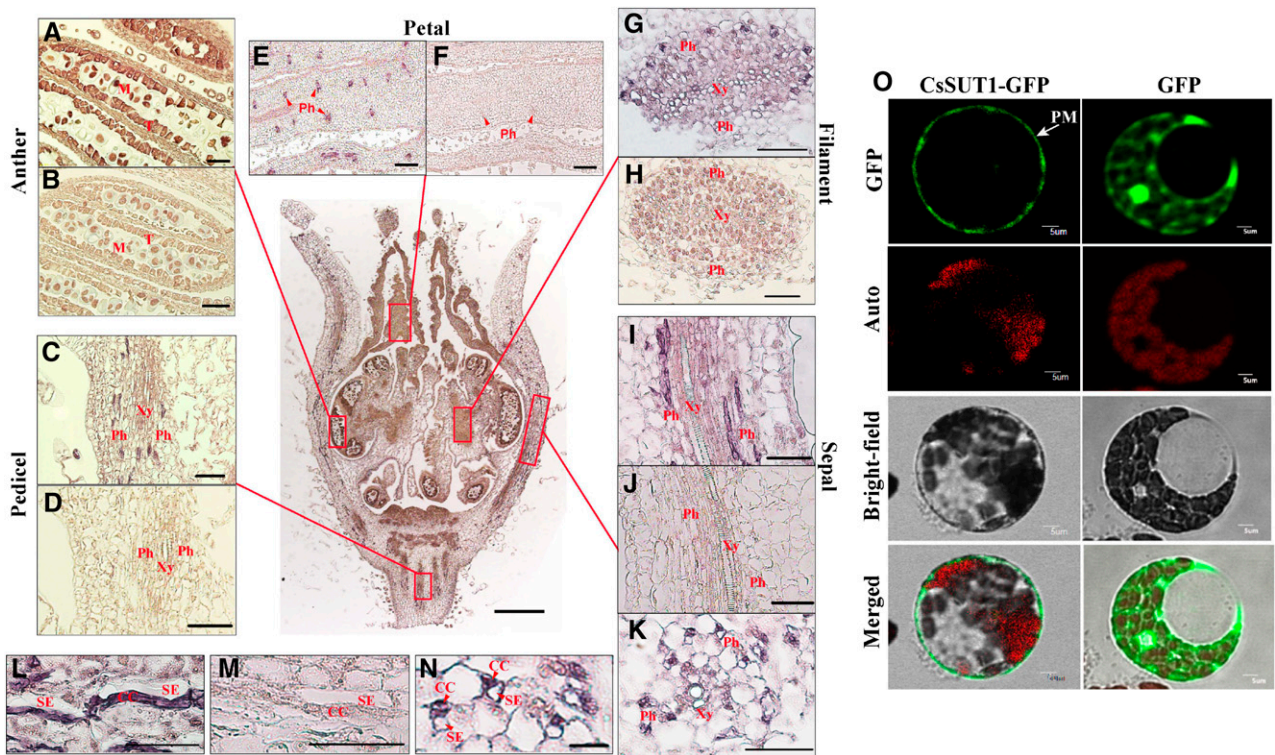
Figure 2. Histological and cellular localization of CsSUT1. A–N, Immunolocal detection of CsSUT1 in stage 10 male flowers using a CsSUT1-antiserum. A, C, E, G, I, K, L, N, Immunolabel detected in the tapetum and microspores in anthers, and phloem cells in sepals, petals, filaments, and the pedicel. B, D, F, H, J, M, Control sections incubated with preimmune serum were devoid of label. (G), (H), (K), and (N) are transverse sections, and the others are longitudinal sections. (L), (M), and (N), are the close-ups of (I), (J), and (K), respectively. O, Plasma membrane localization of CsSUT1-GFP fusion protein in protoplasts derived from cucumber cotyledons. GFP served as a control. Abbreviations: T, tapetum; M, microspore; Ph, phloem; Xy, xylem; SE, sieve element; PM, plasma membrane; Auto, chlorophyll autofluorescence. Scale bars = 50 μm in (A–N) and 5 μm in (O).

flowers grew more slowly and were smaller than wild type, at all stages of development, and did not open at anthesis when *CsSUT1* was strongly down-regulated (Fig. 3, C and D).

Transverse sections taken from stages 10 to 12 male flowers indicted that the cell walls of anther cells developed abnormally, the microspores became enlarged and accumulated less storage material, and hollow and fragmented pollen grains were extruded into a narrow anther space. In addition, the tapetum layer was thinner, more irregular, and disappeared earlier in *CsSUT1*-RNAi line anthers than in the wild type, especially in *CsSUT1*-RNAi 14-1 (Fig. 3E).

Ultrastructural observations revealed that, in the final stage of development, the microsporocyte of *CsSUT1*-RNAi 14-1 line developed abnormally and formed empty and shriveled pollen, whereas in wild-type male flowers the microsporocyte formed normal pollen grains (Fig. 3F). Moreover, in *CsSUT1*-RNAi lines pollen viability was greatly reduced (Fig. 3G). At the scanning electron microscope level, relative to the regular anther wall and mature pollen grains of wild type, in the *CsSUT1*-RNAi lines, the anther walls appeared distorted, and pollen grains were shriveled, displayed abnormal shapes, and adhered together with

the contorted anther wall; the stronger the knockdown the more shrunken the pollen grains, and in the most severe case, there was no normal pollen (Fig. 3H).

Taken together, these findings provided support for the hypothesis that CsSUT1 plays a crucial role in cucumber male flower development, and down-regulating *CsSUT1* causes male sterility in cucumber.

Carbohydrate Metabolism in Male Flowers of CsSUT1-RNAi Lines Is Perturbed

Carbohydrate analyses revealed that Suc, Fru, Glc, and starch decreased gradually in the *CsSUT1*-RNAi lines, relative to wild-type plants, from stages 10 to 12. Interestingly, the levels of stachyose and raffinose were low, with little difference between *CsSUT1*-RNAi and wild-type male flowers (Fig. 4). Based on iodine–potassium iodide (I_2-KI) staining, starch accumulation in petals, anthers, and pollen of the *CsSUT1*-RNAi lines was lower compared with these same wild-type floral tissues (Fig. 4).

To further explore the cause of cucumber male sterility, we sequenced the mutant transcriptome (wild type as the control). RNA sequencing (RNA-seq) data
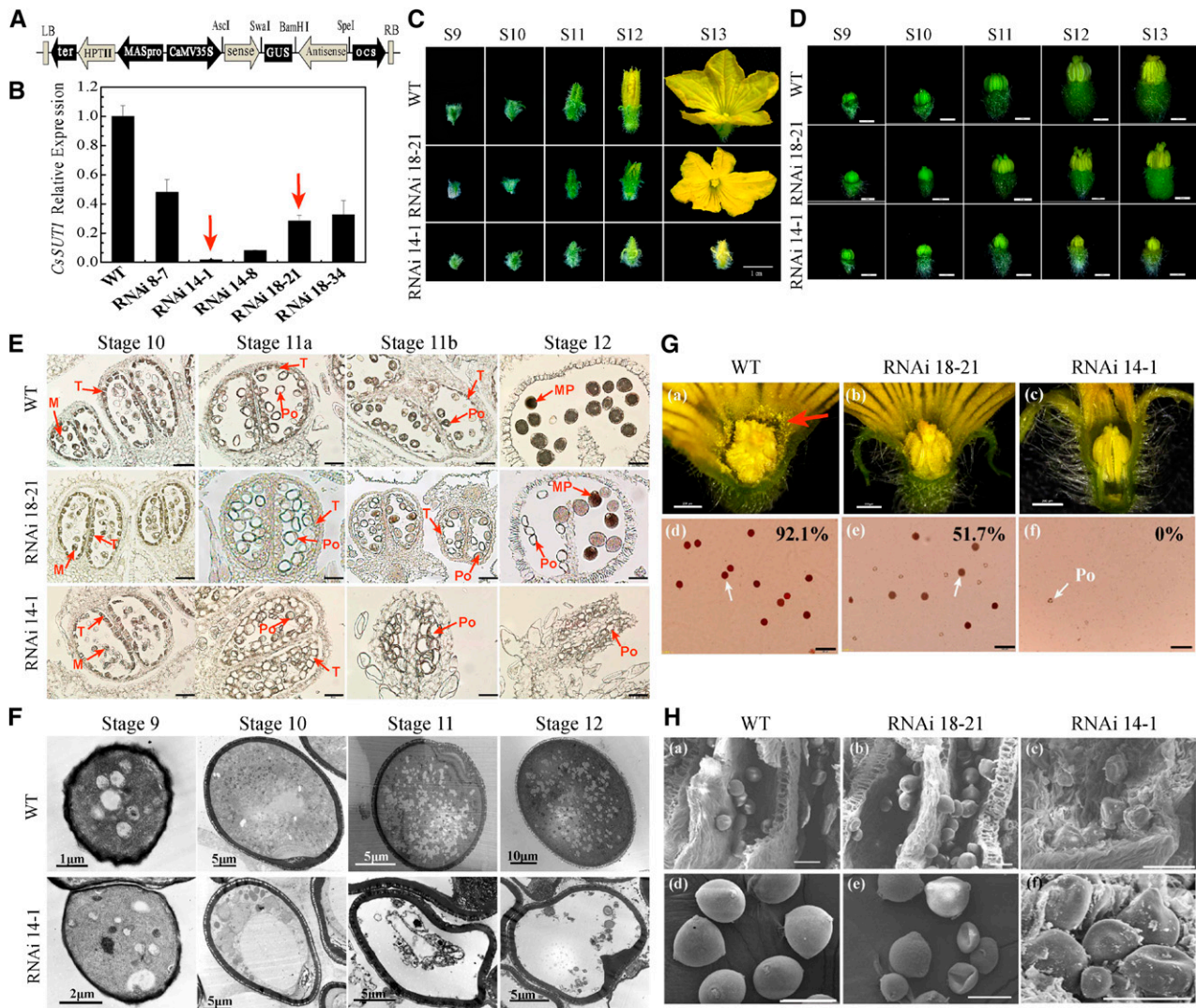
Figure 3. Morphological and structural characterization of *CsSUT1*-RNAi male flowers. A, Schematic illustration of the *CsSUT1*-RNAi expression vector used in this study. B, RT-qPCR analysis of *CsSUT1* transcripts present in stage 12 male flowers of wild-type (WT) and *CsSUT1*-RNAi lines. Error bars = ± SEM (*n* = 3). Two lines were selected for further research (red arrows). C, Morphological features of male flowers at different developmental stages in wild type and *CsSUT1*-RNAi lines. D, Anther development in wild type and *CsSUT1*-RNAi male flowers. E, Transverse sections illustrating anther and pollen development in wild type and *CsSUT1*-RNAi lines. F, Transmission electron micrographs of developing pollen from wild type and *CsSUT1*-RNAi 14-1 plants. G, Phenotype of male flowers at anthesis stage (a–c), and triphenyltetrazolium chloride stained pollen grains (d–f, white arrows), with percentage viability (%) of wild type and *CsSUT1*-RNAi lines. Red arrow in (a) indicates abundant pollen. H, Scanning electron micrographs of anthers (a–c) and pollen grains (d–f) from stage 12 male flowers of wild type and *CsSUT1*-RNAi lines. Abbreviations: M, microspore; MP, mature pollen; Po, pollen; T, tapetum. Scale bars = 1 cm in (C), 200 μm in (D) and (G), and 50 μm in (E) and (H).

revealed that, in 12th stage male flowers of *CsSUT1*-RNAi lines, relative to wild-type plants, of the total of expressed genes, 1604 genes were up-regulated and 1728 were down-regulated (Fig. 5A). In *CsSUT1*-RNAi lines, of the differentially expressed genes (DEGs), most were enriched in biological processes of carbon metabolism, starch and Suc metabolism, and plant hormone signal transduction (Fig. 5B). Further analysis indicated that most of the genes related to sugar and carbon metabolism and sugar transport were down-regulated, such as *cell wall invertase* (*CWINV3*), *Suc synthetase3* (*SUS3*), *sugar transport protein10* (*STP10*), *sugar will eventually be exported transporter5a* (*SWEET5a*), *HXK1*, *polygalacturonase* (*PG*), and *pectate lyase* (*PEL*; Fig. 5C). This result was also confirmed by reverse transcription-quantitative PCR (RT-qPCR; Fig. 5D). In addition, genes related to sugar signaling, such as *HXK1*, *fructokinase2* (*FK2*), and *SnRK1* underwent a reduction in expression, whereas expression of Fru-1,6-bisphosphatase (*FBP*) and *TPS* was increased (Fig. 5C).
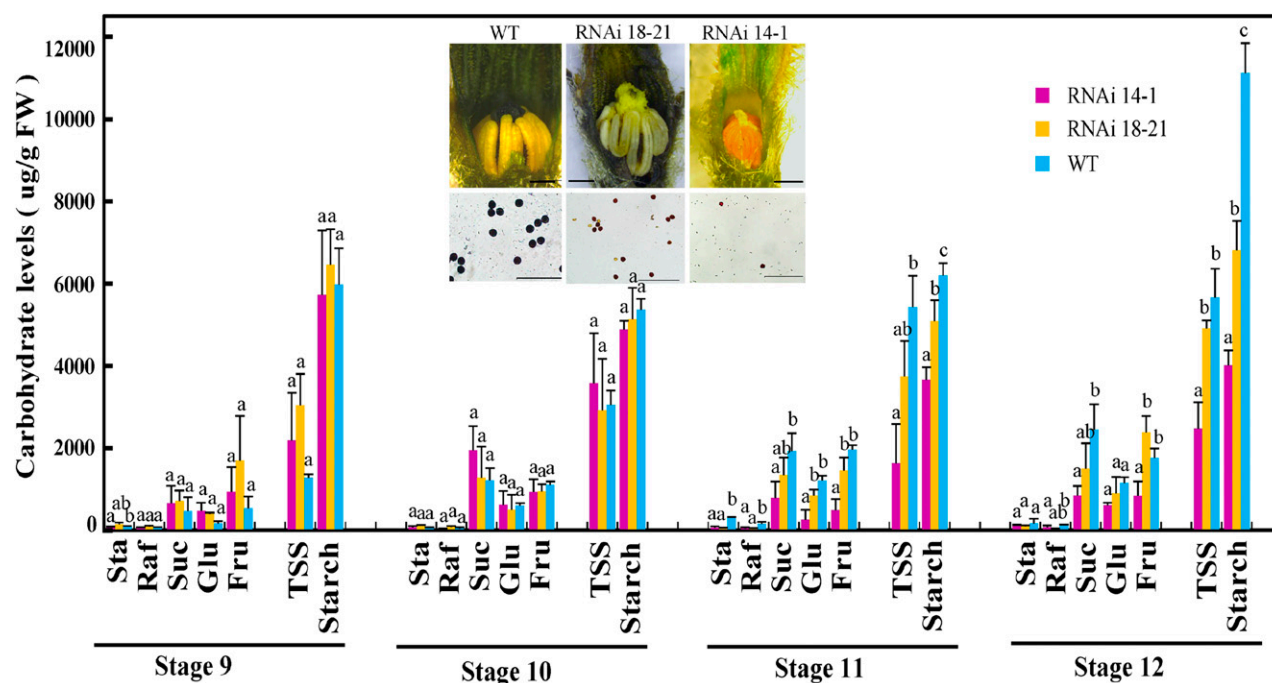
Figure 4. Carbohydrate levels analysis in male flowers from wild-type (WT) and *CsSUT1*-RNAi lines. Data presented are means ± SEM ($n = 3$) with units of μg/g fresh weight (FW). An ANOVA was undertaken using Tukey Honestly Significant Difference test, $n = 3$; the letters above the bars indicate significant differences ($P < 0.05$). Inset: Starch levels in male flowers and pollen of wild-type and *CsSUT1*-RNAi lines, detected by I_2-KI staining. Abbreviations: Sta, stachyose; Raf, raffinose; TSS, total soluble sugar. Scale bars = 1 mm in inset figure.

Auxin Signaling and MYB Transcription Factors May Contribute to Male Sterility

In the *CsSUT1*-RNAi lines, genes encoding proteins involved in auxin biosynthesis, auxin-induced genes, and genes of numerous auxin response proteins (except for *IAA29* and *IAA15*) were down-regulated; expression of an auxin carrier gene and an IAA regulator were up-regulated (Fig. 6A). Interestingly, two MYB-binding sites are located in the *CsSUT1* promoter, and our transcriptomic analysis indicated that numerous *MYB* transcription factors were up-regulated (except for a few) in the *CsSUT1*-RNAi lines, especially *Csa1G024160*, *Csa1G488750*, *Csa1G042350*, *Csa1G482740*, and *Csa3G168940* (Fig. 6B). Proteins encoded by these genes shared high sequence similarity with GhMYB24 (Li et al., 2013b) and AtMYB24 (Yang et al., 2007), respectively (Fig. 6C), for which overexpression was previously reported to cause male sterility in Arabidopsis. Thus, our findings are consistent with the hypothesis that both auxin signaling and MYB transcription factors participate in the development of male sterility in the *CsSUT1*-RNAi lines.

DISCUSSION

CsSUT1 Encodes a Plasma Membrane Protein and Is Expressed Primarily in Male Flowers

Suc transporters in various plants have been widely shown to play important roles in phloem loading in source organs (Hackel et al., 2006; Srivastava et al., 2008), unloading in sink organs, as well as influencing sink organ development (Lemoine et al., 1999; Kühn et al., 2003; Chincinska et al., 2008). In this regard, SUT1 of many crops has been shown to be targeted to the plasma membrane (Riesmeier et al., 1992; Sauer and Stolz, 1994; Kühn et al., 1997; Knop et al., 2004; Schmitt et al., 2008) and most of them are pH dependent with higher affinity at more acidic external pH, except for a ZmSUT1 from maize (*Zea mays*), which was reported as being pH independent (Chandran et al., 2003; Knop et al., 2004; Baker et al., 2016).

In this study, we isolated a *CsSUT1* from the cucumber male flower, which encodes a plasma membrane-localized Suc-H$^+$ symporter (Fig. 2O; Supplemental Fig. S4 and S6). *CsSUT1* was expressed primarily in the later stages of male flower development (Fig. 1), which is similar to that of *Brassica napus BnSUT1c* and rice *OsbHLH142* (Li et al., 2013a; Ko et al., 2014). CsSUT1 shared high sequence similarity with Arabidopsis Suc transporter AtSUC1 (Supplemental Figs. S2 and S3), which is observed mainly in pollen grains of ecotype *Columbia-0* (Sivitz et al., 2008; Feuerstein et al., 2010). Various *SUT* genes are also expressed in pollen grains and have been reported to affect pollen germination and/or tube elongation (Hackel et al., 2006; Sivitz et al., 2008). It is worth mentioning that another cucumber Suc
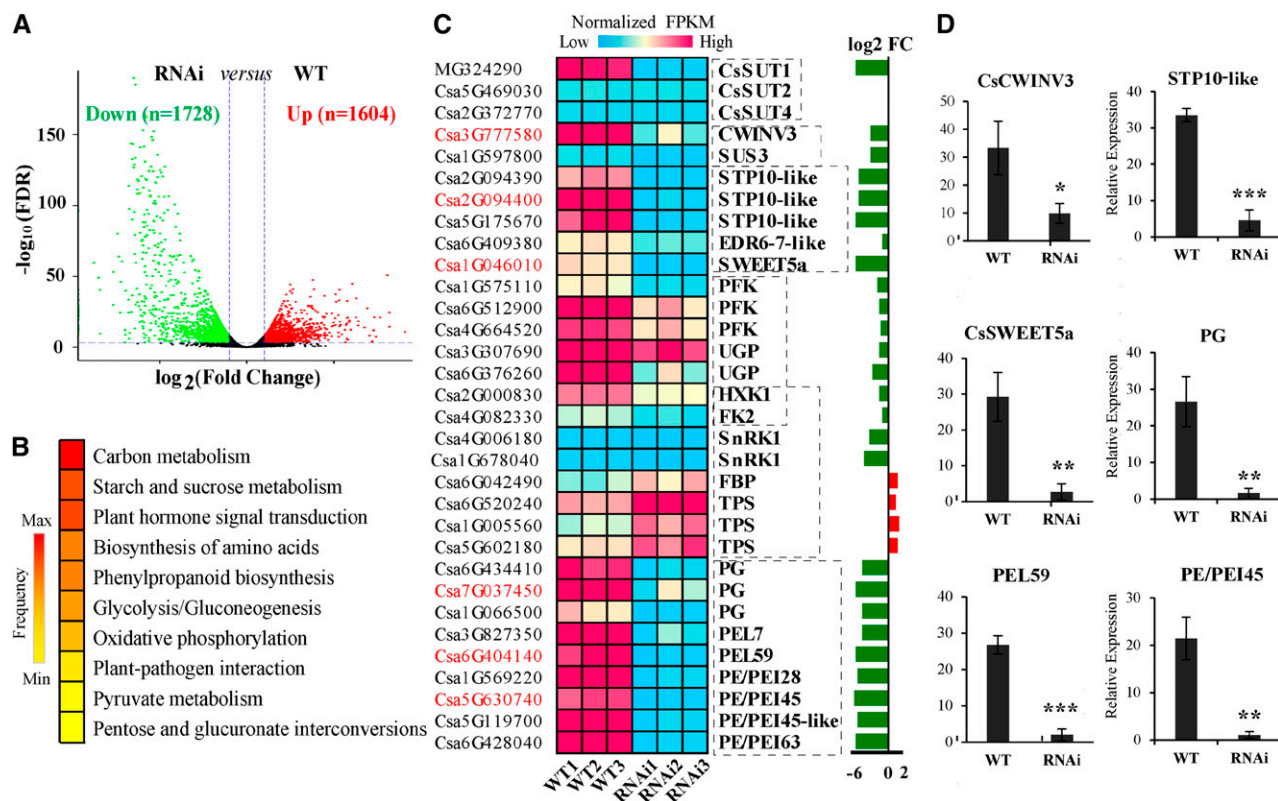
Figure 5. Identification of genes related to carbohydrate metabolism in the cucumber male flower. A, Volcano plot of down-regulated (Down) and up-regulated (Up) DEGs developed based on RNA-Seq data from *CsSUT1*-RNAi lines versus wild-type (WT) lines. B, Functional categories of DEGs in RNA-Seq data identified by Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis; the top ten categories are shown. 'Frequency' stands for the percentage of genes in a certain category to the total DEGs. C, The DEGs involved in carbohydrate metabolism, transport, and signaling. D, Expression of several genes related to carbohydrate metabolism in (C), verified by RT-qPCR. α-Tubulin (AJ715498) was used as the normalization control. Error bars = ± SEM ($n = 3$). Student's t test, *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Abbreviations: FC, fold change; SWEET, sugar will eventually be exported transporter; PFK, phosphofructokinase; UGP, UTP-Glc-1-phosphate uridylyltransferase; HXK1, hexokinase 1; FK2, fructokinase 2; SnRK1, SNF1-related protein kinase; FBP, Fru-1,6-bisphosphatase; TPS, alpha-trehalose-phosphate synthase; PG, polygalacturonase; PEL, pectate lyase; PE/PEI, pectinesterase/pectinesterase inhibitor; FPKM, fragments per kilobase of transcript per million fragments mapped; Min, minimum; Max, maximum.

transporter gene, *CsSUT2*, is expressed in mature leaves and participates in the phloem loading (Ma et al., 2019).

Previous studies have established that many Suc transporters are localized to the phloem sieve elements and/or CCs where they function in the loading of Suc into the sieve tube system (Chandran et al., 2003; Kühn et al., 2003; Knop et al., 2004), whereas some SUTs are localized to xylem parenchyma or pollen grains (Decourteix et al., 2006; Schmitt et al., 2008; Sivitz et al., 2008). In this study, strong CsSUT1 signals were primarily detected in the pollen, tapetum, and CCs of the phloem located in the pedicel, sepals, petals, and filaments (Fig. 2, A–N; Supplemental Fig. S5). These locations indicate that CsSUT1 likely plays a role in regulation Suc delivery from the phloem into the tapetum and pollen. Collectively, our findings establish that CsSUT1 is a plasma membrane-localized high-affinity and energy-dependent Suc-H⁺ symporter that appears to performs a nonredundant function in male flower development.

Down-regulating *CsSUT1* Expression Induced Male Sterility

Developing floral organs act as strong sinks; however, both the tapetum and pollen are symplasmically isolated. Hence, these male organs must obtain nutrients, via the apoplasm, to fuel their development (Clement and Audran, 1995; Lemoine et al., 1999; Xu et al., 2015), and here, Suc uptake into the cytoplasm of these cells requires the presence of functional SUT proteins. Indeed, several studies have shown that *SUTs* are expressed in pollen, and play a role in pollen germination (Lemoine et al., 1999; Hackel et al., 2006; Sivitz et al., 2008). For example, antisense inhibition of the tomato *SlSUT2* reduced pollen tube growth (Hackel et al., 2006) and the Arabidopsis *Atsuc1* mutant had impaired pollen germination (Sivitz et al., 2008). In addition, the growth and development of male floral organs, such as sepals, petals, filaments, and pollen, are entirely dependent on sugar unloading from the
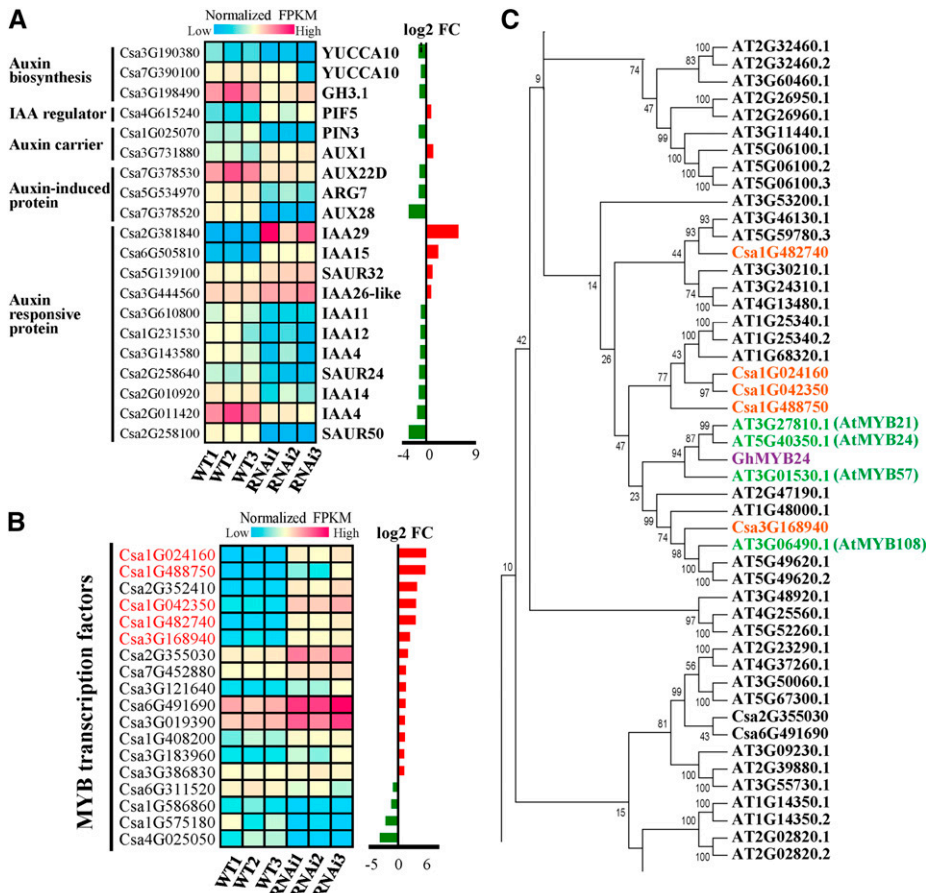
Figure 6. Identification of auxin signaling and *MYB* transcription factor genes whose expression patterns were changed in flowers of *CsSUT1*-RNAi compared to wild-type (WT) plants. A, Heat map of DEGs involved in auxin metabolic pathways, including auxin biosynthesis, transport, and response. B, Heat map showing normalized FRKM values of *MYB* transcription factors; five genes that changed significantly are highlighted in red. C, Phylogenetic analysis of MYB transcription factors. The five cucumber proteins highlighted in red font shared high sequence similarity with Arabidopsis AtMYB21, 24, 57, 108 (green font), and cotton GhMYB24 (purple font). The genes presented in (A) and (B) were selected with an FPKM value $\geq$ 10 before down-regulation or after up-regulation. Abbreviations: FC, fold change; PIN3, PIN-formed 3; PIF5, phytochrome-interacting factor 5; AUX1, auxin transporter-like protein; FPKM, fragments per kilobase of transcript per million fragments mapped.

phloem, and in this context, CsSUT1 would appear to be essential to this process.

It is important to note, however, that in contrast with our findings for *CsSUT1*, suppression of other plant *SUTs* has not been reported to give rise to pollen abortion or induction of male sterility. Thus, male sterility obtained by suppression of *CsSUT1* and the consequential reduction in nutrient delivery to the tapetum and pollen and other male floral tissues may offer an effective strategy for cucumber hybrid seed production. This would greatly facilitate cucumber, as well as other crops, breeding efficiency by obviating the need for artificial emasculation and also remove the challenges associated with preventing self-crossing caused by insect pollination.

Carbon Undersupply Is the Main Cause for Male Sterility

The mechanisms underlying crop male sterility can be very complex. Many genetic factors, as well as hormones, photoperiod, temperature, and various forms of stress (Takeno et al., 1996; Kobayasi and Atsuta, 2010; Han et al., 2018), along with a perturbation to carbohydrate supply, can affect anther development, thereby leading to male sterility. As an example, we previously mapped a male sterility gene, *ms-3*, in a cucumber

mutant that is defective in microsporogenesis (Han et al., 2018).

Many studies have shown that genes associated with either sugar metabolism or transport are related to male sterility. For example, alteration in invertase activity affected pollen development, pollen tube growth, and pollination through alterations in sugar signals (Goetz et al., 2001; Wang and Ruan, 2016). In cucumber and Arabidopsis, both CsHT1 and AtSTP10, two homologous monosaccharide transporters, play key roles in pollen tube growth (Cheng et al., 2015; Rottmann et al., 2016). Silencing *OsUgp2*, a gene encoding a rice UDP-Glc pyrophosphorylase that is expressed preferentially in pollen, can result in failure to accumulate starch, leading to sterile pollen (Mu et al., 2009).

Our study established that down-regulating *CsSUT1* caused a significant reduction in sugar levels within male flowers (Fig. 4). Meanwhile, the expression of many genes related to carbohydrate metabolism and sugar transport were also down-regulated, such as *CWINV3*, *SUS3*, *STP10*, *UTP-Glc-1-phosphate uridylyltransferase*, *PG*, and *PEL*. In addition to the up-regulation of *FBP* and *TPS*, numerous genes related to sugar signaling, such as *HXK1*, *FK2*, and *SnRK1*, also underwent a reduction in expression (Fig. 5C). These genes participate in many physiological and biochemical processes, such as *AtHXK1*, a gene

encoding a well-known Glc sensor, that is involved in regulation of nutrient, light, hormone, and sugar signaling networks in plant development and growth (Moore et al., 2003; Cho et al., 2006). Antisense inhibition of *SnRK1* causes abnormal pollen development and male sterility in transgenic barley (Zhang et al., 2001). Taken together, these studies suggest that both the carbohydrate supply and sugar signaling, likely acting together, may regulate the growth and development of male flowers.

Auxin Signaling and MYB Transcription Factors May Underlie Male Sterility

Auxin is closely related to male sterility (Yang et al., 2012; Liu et al., 2014). It has been shown that, in strawberry, overexpressing *FvYUC6*, an auxin biosynthesis gene, can lead to male sterility (Liu et al., 2014). In Arabidopsis, the AUXIN RESPONSE FACTOR17 is essential for pollen development and the *arf17* mutant exhibited a male-sterile phenotype (Yang et al., 2013). In our study, knockdown of *CsSUT1* caused not only carbon undersupply, but also changes in genes associated with hormone signaling, notably auxin signaling. In this regard, we demonstrated that, for *CsSUT1*-RNAi relative to wild-type plants, at the 12th stage of male flower development expression of genes related to auxin biosynthesis (*YUCCA10* and *GH3.1*), auxin transport (PIN-formed 3 [*PIN3*]), auxin-induced proteins, and auxin response proteins (except for *IAA29* and *IAA15*, etc.), were down-regulated, whereas expression of an IAA regulator gene (*phytochrome-interacting factor 5*, *PIF5*) and an auxin carrier gene (*auxin transporter-like protein1*) were up-regulated (Fig. 6A). Our results are similar to the cotton male sterility induced by high temperature, in that both sugar levels and the expression of genes related to auxin signaling were significantly altered (Min et al., 2014).

MYB transcription factors can influence anther and pollen development (Yang et al., 2007; Zhang et al., 2010; Song et al., 2011), and are involved in sugar-regulated gene expression (Lu et al., 2002) or sugar signaling (Chen et al., 2017). In our study, a MYB-binding site was identified in the *CsSUT1* promoter, and most *MYB* transcription factor genes in the DEGs were up-regulated in the *CsSUT1*-RNAi lines. The five most substantially up-regulated genes encode proteins that shared high sequence similarity with homologous to Arabidopsis MYB21, MYB24, MYB57, MYB108, and cotton GhMYB24 (Fig. 6, B and C). These Arabidopsis and cotton genes have been reported to participate in the development of male floral organs (Cheng et al., 2009; Huang et al., 2017). For example, in Arabidopsis, overexpression of *AtMYB24* causes aberrant anther development (Yang et al., 2007); *AtMYB108* acts together with *AtMYB24* to regulate stamen maturation (Mandaokar and Browse, 2009); overexpression of the cotton gene *GhMYB24* leads to Arabidopsis flower malformation and male sterility, and it is able to

recover, partially, male fertility in the *myb21 myb24* double mutant (Li et al., 2013b). Thus, misexpression of these *MYB* transcription factor genes in the developing flowers of our *CsSUT1* RNAi lines may have contributed to the observed male sterility phenotype.

CONCLUSION

In summary, down-regulating *CsSUT1* expression causes consequential reduction in nutrient delivery, with a subsequent alteration in sugar and auxin signaling, along with misexpression of specific *MYB* transcription factors, thereby leading to male sterility in cucumber.

MATERIALS AND METHODS

Cloning of *CsSUT1*

Wild-type cucumber (*Cucumis sativus* cv 'Xintaimici') plants and *CsSUT1*-RNAi lines were grown under greenhouse conditions. Total RNA was isolated from cucumber stage 12 male flowers, using a Plant RNA extract kit (Huayueyang), and subsequently reverse transcribed to complementary DNA (cDNA) using an oligo(dT) primer (Tiangen), according to the manufacturer's instructions. Stage division of male flowers was as described by Bai et al., 2004, with slight modification (Table 1). Full-length *CsSUT1* coding sequence was amplified from the cDNA, using the primer pairs *CsSUT1F* and *CsSUT1R* (Supplemental Table S2). The resultant PCR product was cloned into pMD19-T (TaKaRa), and confirmed by sequencing.

Heterologous *CsSUT1* Expression in Yeast and *Xenopus* Oocytes

The *CsSUT1* open reading frame was subcloned into the yeast expression vector pDR196 (Cheng et al., 2015) and then transformed into yeast strain SUSY7/*ura3* (Riesmeier et al., 1992). Yeast carrying the pDR196 empty vector served as the control. Drop test and Suc uptake assays were preformed, as previously described (Cheng et al., 2015). The medium for drop tests was supplemented with 2% (w/v) Glc or Suc, as the sole carbon source; for Suc uptake assays the medium contained 0.1 μCi ^{14}C-Suc (26 GBq mmol^{-1}; PerkinElmer). The *CsSUT1* coding region was also subcloned into the *Xenopus* oocyte expression vector, pCS107, linearized with AscI (New England Biolabs) and 1 mg then used as template for complementary RNA (cRNA) synthesis. Suc uptake assays were then performed, as previously described (Chen et al., 2010).

RT-qPCR Analysis

Total RNA was extracted from the specified tissues of wild type or *CsSUT1*-RNAi lines, respectively, and reverse transcribed into cDNA. Gene-specific primers and internal control (tubulin mRNA) primers (Supplemental Table S2) were used to amplify PCR products on an ABI 7500 system (Bio-Rad). Three biological replicates (samples from three individual plants) were performed and relative amounts of mRNA were calculated using the $2^{-\triangle\triangle CT}$ method (Livak and Schmittgen, 2001).

Immunolocalization Assays

CsSUT1-specific antiserum was produced in rabbits (two specific peptide fragments MEHGGVVSKGMASDPSSC and CSENQFDPLEIDEEATPF, derived from the CsSUT1 protein sequence, were chosen to synthesize polypeptides), by the Beijing Biotech Company, and antiserum specificity was confirmed by immunoblot, using yeast proteins extracted from SUSY7/*ura3* yeast cells (Supplemental Fig. S1). Male flowers were fixed (Wang et al., 2014), and paraffin sections treated with alkaline phosphatase immunoglobulin G-horse radish peroxidase (AP IgG-HRP) and examined with an Olympus BX53 microscope.

Subcellular Localization Assays

Full-length *CsSUT1* was subcloned into PUC-SPYNE and pCAMBIA 1300 vectors to produce a CsSUT1-GFP fusion protein and then expressed, respectively, in cucumber mesophyll protoplasts (Huang et al., 2013), or transformed into *Agrobacterium* and then introduced into tobacco epidermal cells (Li et al., 2017b). GFP fluorescence was visualized at 488 nm wavelength, and red chlorophyll auto-fluorescence was visualized at 546 nm wavelength using an Olympus confocal laser scanning microscope.

Construction of a CsSUT1-RNAi Vector and Cucumber Transformation

Two fragments of *CsSUT1* were generated through PCR amplification, using specific primers containing AscI (5′ end) and SwaI (3′ end) sites, and SpeI (5′ end) and *Bam*HI (3′ end) sites. These fragments were inserted, in reverse orientation, into the pFGC1008 vector (Lü et al., 2017) to form the construct *35S-CsSUT1-RNAi*. *Agrobacterium*-mediated transformation of *Cucumis sativus* cv Xintaimici was then performed, as previously described (Cheng et al., 2015; Li et al., 2017a).

Pollen and Soluble Sugar and Starch Assays

Pollen germination assays (Cheng et al., 2015) were observed under an Olympus BX53 microscope. Pollen viability was assessed using a 2% (w/v) triphenyltetrazolium chloride staining solution. Sugar and starch measurements were performed, as previously described (Lü et al., 2017). Male flowers, at stage 12, were harvested and immersed in the I_2-KI staining solution for several minutes, and then washed in distilled water and photographed to assay for starch.

Scanning and Transmission Electron Microscopy Observations

Stage 12 anthers were collected and processed, as previously described (Cheng et al., 2015), and observed under a HITACHI SU8010 scanning electron microscope. Anthers were also fixed, washed, embedded, and stained, as previously described (Sui et al., 2017), and examined under a HITACHI-7500 transmission electron microscope.

RNA-seq

Stage 12 male flowers of wild type and *CsSUT1*-RNAi lines were sampled for total RNA extraction. RNA library construction and sequencing were performed on an Illunima HiSeq X-ten platform (Biomarker Biotechnology Co.). Each sample yielded more than 6 gigabytes of data. Clean data were aligned to the reference cucumber genome Chinese Long v2.0 via HISAT2. Quantification of gene expression levels were estimated by fragments per kilobase of transcript per million fragments mapped (FPKM). A corrected P value < 0.01 and fold change > 2 were set as the threshold for significantly differential expression.

Statistical Analysis

Student's *t* test and Tukey's Honestly Significant Difference test were used to evaluate the significant differences based on the IBM_SPSS_Statistics software. Data presented are means ± SE of three independent experimental replicates.

Accession Numbers

Accession numbers of the major genes/proteins mentioned in this paper can be seen in Fig. 5, Fig. 6, and Supplemental Table S1.

Supplemental Data

The following supplemental information is available:

Supplemental Figure S1. Immunoblot of proteins extracted from SUSY7/ *ura3* yeast strains probed with CsSUT1 antiserum.

Supplemental Figure S2. Alignment of CsSUT1 and selected Suc transport proteins from Arabidopsis.

Supplemental Figure S3. Suc transporter (SUT) phylogenetic tree analysis.

Supplemental Figure S4. Suc transport characteristics of CsSUT1 assayed in yeast and *Xenopus* oocytes.

Supplemental Figure S5. Immunological detection of CsSUT1 in different developmental stages of cucumber male flowers using CsSUT1-antiserum.

Supplemental Figure S6. Plasma membrane localization of CsSUT1-GFP fusion protein in tobacco leaf epidermal cells.

Supplemental Figure S7. Analysis of *CsSUT1*-RNAi lines in T_0 cucumber plants.

Supplemental Figure S8. Phenotype comparison between wild-type (WT) and *CsSUT1*-RNAi cucumber lines.

Supplemental Figure S9. Immunological detection of CsSUT1 protein in male flowers of wild-type (WT) and *CsSUT1*-RNAi lines.

Supplemental Table S1. GenBank accession numbers of the genes/proteins used for phylogenetic analysis.

Supplemental Table S2. Primers used in this study.

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