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Research article

Overexpression of hexose transporter CsHT3 increases cellulose content in cucumber fruit peduncle

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ABSTRACT

Hexose transporters play many important roles in plant development. However, the role of hexose transporter in secondary cell wall growth has not been reported before. Here, we report that the hexose transporter gene *CsHT3* is mainly expressed in cells with secondary cell walls in cucumber. Spatiotemporal expression analysis revealed that the transcript of CsHT3 mainly accumulates in the stem, petiole, tendril, and peduncle, all of which contain high cellulose levels. Immunolocalization results show that CsHT3 is localized at the sclereids in young peduncles, shifts to the phloem fiber cells during peduncle development, and then shifts again to the companion cells when the development of secondary cell walls is almost completed. Carboxyfluoresce unloading experiment indicated that carbohydrate unloading in the phloem follows an apoplastic pathway. Overexpression of *CsHT3* in cucumber plant can improve the cellulose content and cell wall thickness of phloem fiber cells in the peduncle. The expression of cellulose synthase genes were increased in the *CsHT3* overexpression plants. These results indicated that CsHT3 may play an important role in cellulose synthesis through promoting the expression of cellulose synthase genes.

1. Introduction

Sugars play many important roles in plant, such as providing energy source for cellular metabolism and acting as the main component of carbon skeletons and the signal material recognized by cells (Eastmond, 2006). Plant cell wall is mainly composed of cellulose, hemicellulose, and pectin polysaccharides, all of which are sugar polymers (Taylor-Teeples et al., 2015; Zhong and Ye, 2015). In plants, almost all cells have a primary wall surrounding the plasma membrane to determine cell shape. Specialized plant cell types, such as vessels, xylary fibers, phloem fibers, and sclereids, produce secondary cell walls, which provide mechanical support to the plant and the ability to transport solutes (Taylor-Teeples et al., 2015; Didi et al., 2015). Cellulose is the main component of secondary cell walls and fibers (Zhong and Ye, 2015; Abidi et al., 2010). In general, sucrose is the main source of carbon supplied to the fibers (Tarczynski et al., 1992). A recent study has found that the sucrose transporter SWEET12 participates in cotton fiber elongation (Sun et al., 2019). However, the cellulose macromolecule is the outcome of polymerization reactions between glucose units (Abidi et al., 2010). In addition, analysis of the sugar composition of cell wall polysaccharides in Arabidopsis leaves showed that the main monosaccharide components are glucose, xylose, galactose, rhamnose, arabinose, mannose, and fucose (Lee et al., 2007). Therefore, glucose and galactose probably play important roles in cellulose synthesis and cell wall formation. In Arabidopsis, the hexose transporter STP14 plays a role in recycling of cell wall-derived galactose during different developmental processes (Poschet et al., 2010). However, whether any hexose transporter is involved in cellulose synthesis and cell wall formation remains unclear to date.

In our previous research, we found that the hexose transporter CsHT3 (has glucose and galactose transport activity) is highly expressed in the phloem fiber cells of cucumber fruit (*Cucumis sativus*) peduncle, indicating that this protein has a special function (Cheng et al., 2015a). Cucumber fruit can grow fast in a few days. Thus, cucumber needs a tough peduncle to support its increasing weight, and the phloem fiber in the peduncle also needs to grow fast. Large amounts of carbohydrates are required to be transported from the source to the fruit and the fruit peduncle during cucumber fruit enlargement. Different from most plants, cucumber is a typical raffinose family oligosaccharide-transporting plant with stachyose and raffinose as the major transporting sugars (Hu et al., 2009, 2011; Dai et al., 2011). Although stachyose is the main transport sugar in cucumber phloem, the major free sugars in

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the peduncle and fruit tissues are sucrose and hexoses (glucose and fructose) (Hu et al., 2009; Handley et al., 1983). Stachyose and raffinose are apparently metabolized to sucrose and galactose in the fruit peduncles, and the released galactose is converted into UDP-glucose before distribution or rapidly transferred directly (Dai et al., 2006, 2011; Gross and Pharr, 1982; Smart and Pharr, 1981). The released galactose and glucose need to be transferred and the hexose can be absorbed as a raw material for the growth of phloem fiber cells, indicating that hexose transporters are important in cucumber fruit peduncle during fruit development.

In the present study, we combined qRT-PCR, immunohistochemical localization, and transgenic technology to analyze the function of CsHT3 during cucumber fruit peduncle development. Results indicated that the localization of CsHT3 shifts to different cell types in the phloem during peduncle development and that overexpression of *CsHT3* improves the cellulose content and cell wall thickening of phloem fiber in the cucumber fruit peduncle.

2. Materials and methods

2.1. Plant materials

Cucumber (wild type: *Cucumis sativus* L. cv. Xintaimici and transgenic lines) plants were grown under plastic house conditions from March to June in Wuhan (central China). The plastic house condition is approximately 26°C–30 °C/18°C–23 °C day/night average temperatures and 60%–80% of the air relative humidity, with a natural sunlight (midday average photonflux density of 700–800 $\mu mol\, m^{-1}\ s^{-1}$). Tissues were sampled for testing of gene transcription. Cucumber fruit peduncles at different developmental stages (stages 9–12; 0, 3, 6, and 9 DAA) were harvested for temporal expression and immunolocalization. The division of peduncle development stages is the same as the division of ovary/fruit development stages, such as stage 9–11 means the peduncle connected to stage 9–11 of cucumber ovary development (Bai et al., 2004).

2.2. qRT-PCR

RNA extraction and reversion were based on the report of Cheng et al. (2018). The primers for qRT-PCR analysis are shown in Supplementary Table S1. *TUA* was used as the control gene. SYBR green (TaKaRa) and ABI7500 system (Bio-Rad) were used to perform qRT-PCR. The mean expression level of relevant genes was calculated by the $2^{-\Delta\Delta\ Ct}$ method (Livak and Schmittgen, 2001).

2.3. Western blot analysis

Membrane proteins from yeast cells expressing CsHT3 (KP113694), CsHT9 (XP_011657575.1), CsHT12 (XP_004146580.1), CsHT16 (XP_011658192.1), or empty PDR196 vector were prepared, and 100 μg of each preparation was separated on SDS–PAGE, blotted onto nitrocellulose, and then incubated with anti-CsHT3 or anti-CsHT16 antiserum.

2.4. Immunolocalization of CsHT3 during the development of cucumber fruit peduncle

The CsHT3 antibody was prepared as previously described (Cheng et al., 2015a). To detect the antibody's quality and specificity, we used the proteins extracted from cucumber plant lysis and yeast expression *CsHT3*, other *CsHTs*, or empty vector as templates in Western blot.

For immunohistochemical analysis, cucumber fruit peduncles at different developmental stages were sliced into transverse paraffin sections (14 μm) and used as previously described (Cheng et al., 2015a, 2015b). The specimens were viewed with a Nikon eclipse 80i microscope.

2.5. Fluorescent imaging of CF

The loading and fluorescent imaging of CF were conducted as previously described (Ruan et al., 2001). Peduncles were collected after 48 h. Tissue was sectioned by hand (~1 mm), and glycerol was used as a mounting solution to prevent dye loss. Sections were viewed and imaged via confocal laser scanning microscopy (FV1200, Olympus).

2.6. Construction of expression vector and generation of transgenic cucumber plants

To construct the *CsHT3* overexpression vector pBI121, we isolated the coding region of CsHT3 from ‘Xintaimici’ cucumber fruit through RT-PCR using the primers listed in Supplementary Table S1. The DNA was digested with XbaI/SmaI and cloned into the pBI121 vector under the control of the CaMV *35S* promoter. Then, the vector was transformed into *Agrobacterium tumefaciens* LBA4404. *A. tumefaciens* with the *CsHT3* overexpression vector was transformed into the cucumber pure line ‘Xintaimici’ by using fresh expanding cotyledon disk transformation as previously described (Cheng et al., 2015b). The regenerated plants were screened by RT-PCR analysis.

2.7. Determination of cellulose content

The 12 DAA fruit peduncles of CsHT3 overexpression plants and wild-type plants were harvested and incubated in 80% ethanol at 65 °C overnight. Tissues were exchanged with acetone. Dry cell wall materials were ball milled to fine powder. Cellulose was measured by anthrone-H_2SO_4 colorimetry according to Xiao et al. (2016). D-Glc (Sigma) was used as a standard for calculation of cellulose content in samples.

2.8. Bioinformatics analysis

Primers were designed using the Primer Premier 5.0. Sequence homology search in GenBank was performed with the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/ (Madden et al., 1996););.

2.9. Statistical analysis

Student's *t*-tests were performed using the algorithm embedded in Microsoft Excel, and significance was evaluated at the 5% level ($P < 0.05$) for all comparisons. For each treatment, the standard error of the mean (SE) was calculated based on at least three biological replicates.

3. Results

3.1. Spatiotemporal expression analysis of CsHT3 during peduncle development

In our previous research, we found a high expression of *CsHT3* in the cucumber fruit peduncle (Cheng et al., 2015a). To detect the detailed expression motif of *CsHT3* during peduncle development, we performed qRT-PCR and found that *CsHT3* expression was upregulated at stage 11 and remained high at stage 12. However, the expression decreased at the day of anthesis (DAA), increased again during fruit enlargement (3–6 DAA), and then decreased again at 9 DAA (peduncle connected to the commodity maturity fruit) (Fig. 1).

3.2. CsHT3 localization to different cell types of phloem during peduncle development

Immunolocalization was performed to detect the expression/location of CsHT3 in the peduncles during cucumber fruit development. To detect the specificity of anti-CsHT3 antiserum, we performed Western blot using the proteins extracted from plant lysis and yeast expression,

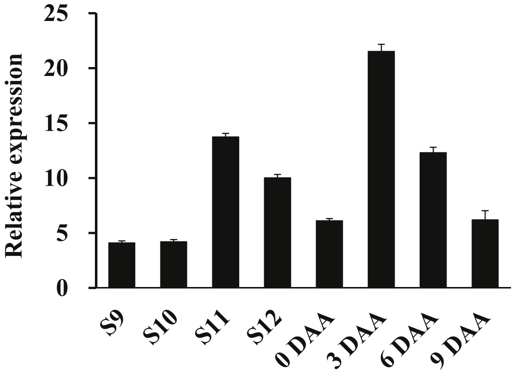

Fig. 1. Developmental expression profile of *CsHT3* in developing cucumber fruit peduncles. S9–S12, four developmental stages of ovary; DAA, day after anthesis; *TUA* (α-tubulin) was used as the control. Error bars represent the SE for four technical replicates of three biological replicates.

different *CsHT* genes, or the empty vector. A specific band was only detected at about 55 KD in plant lysis or cells expressing CsHT3 (Fig. 2A; Supplementary Fig. S1). Immunolocalization results show that the CsHT3 protein was dispersed among the parenchyma cells of the external phloem in the peduncles connected to the early-development ovaries (stages 9–11) (Fig. 2B). A partially enlarged image indicates that the dispersed cells are sclereids (Fig. 2C). The expression of CsHT3 shifted to the PF cells at stage 11 and concentrated on the PF cells at stage 12. However, CsHT3 expression gradually decreased in the PF cells and appeared in the cells around the SE after anthesis (Fig. 2B). Upon close observation, these cells were determined as companion cells (CCs) (Fig. 2C). No signals can be detected in the histological sections incubated with their respective pre-immune sera (Supplementary Fig. S2).

3.3. Phloem unloading in cucumber fruit peduncles following an apoplastic pathway

The phloem unloading in the cucumber fruit follows an apoplastic pathway (Hu et al., 2011). However, the carbohydrate unloading in the peduncle remains unclear to date. We loaded the phloem-mobile

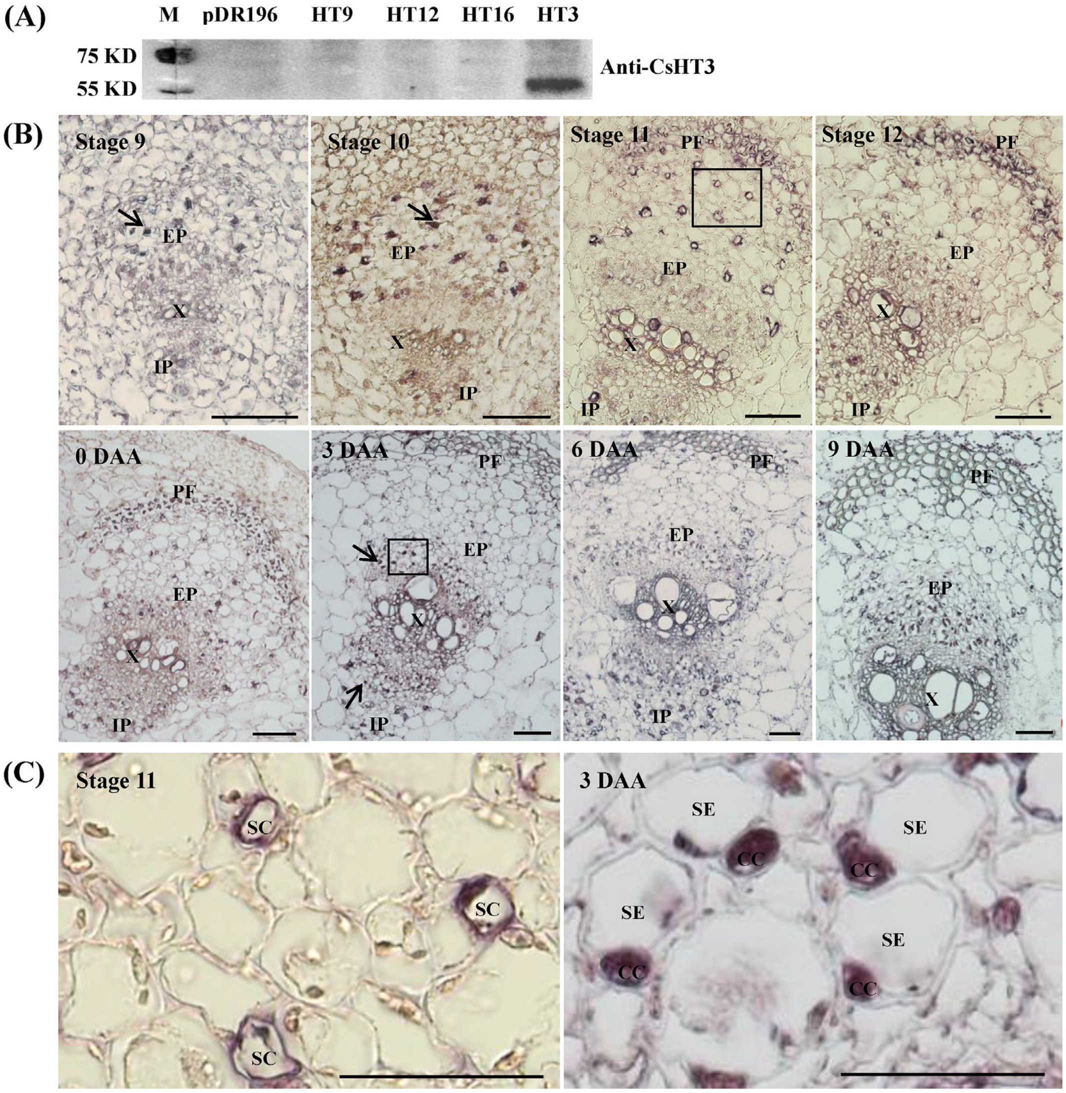

Fig. 2. *Immunolocalization of CsHT3 in cucumber fruit peduncles at different developmental stages. **(A)** Western blot to test the quality of anti-CsHT3 antiserum,* a specific band was detected in cells expressing CsHT3 when anti-CsHT3. *(B) Cross-sections of peduncles.* Bar = 100 μm. *(C) Close-up of the boxes in (a).* Bars = 50 μm. PF, phloem fiber; EP, external phloem; IP internal phloem; X, xylem; SE, sieve element; SC, sclereid; CC, companion cell; PP, phloem parenchyma cell; *Arrows, phloem-specific cells.*

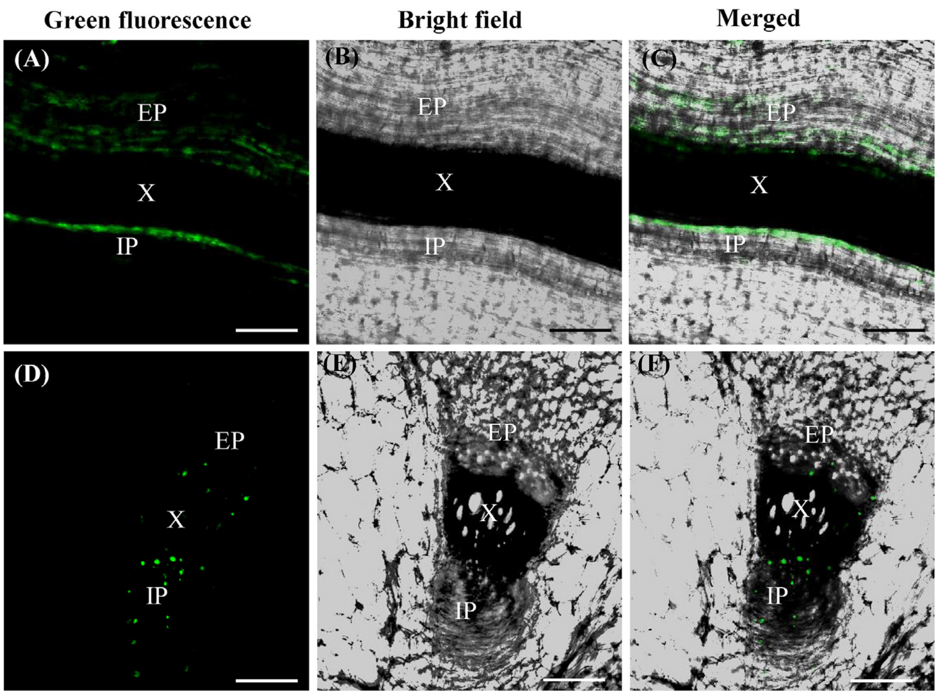

Fig. 3. Phloem unloading follows an apoplasmic pathway in the peduncle, as revealed by monitoring the movement of the symplasmic dye carboxyfluorescein (CF). CF fluorescence was detected in 9 DAA fruit peduncles after 24 h feeding of carboxyfluorescein diacetate. CF fluorescence was only restricted in the EP and IP cells. (A–C) transverse sections; (D–F) longitudinal sections; EP, external phloem; IP, internal phloem; X, xylem. Bars = 200 μm.

symplasmic tracer carboxyfluorescein (CF) into the peduncle connected to the 6 DAA fruit. Results revealed that CF was restricted to the phloem cells without diffusing into the surrounding tissues (Fig. 3), indicating that carbohydrate unloading in the phloem also follows an apoplastic pathway similar to that in fruits and that sugar transporters are involved in this process.

3.4. Overexpression of CsHT3 can improve the cellulose content in cucumber fruit peduncle

To test the role of *CsHT3* during the development of cucumber fruit peduncle, we generated a *CsHT3* overexpression construct (cloning the coding region of *CsHT3* into the restriction site of XbaI and SmaI in the pBI121 vector under the control of the CaMV35S promoter) and obtained more than 10 OE-*CsHT3* lines (T1). Two independent transgenic lines for each transformation construct were selected for the subsequent generation. The expression of *CsHT3* significantly increased in the OE-*CsHT3* lines compared with the wild type (Fig. 4A). The phenotypes of peduncle in the overexpression lines were detected, and results showed that the cell wall of PF in OE-*CsHT3* was thicker than that in the wild type (Fig. 4B–C). The cellulose content in the peduncle of OE-*CsHT3* was also higher than that in the wild type (Fig. 4D). However, the other cell wall, but PF cells, did not become thicker in *HT3-OE* plants (Supplementary Fig. S3).

To analyze further the co-expression of *CsHT3* and cellulose synthase genes, we searched for the relative genes using the Cucurbit Expression Atlas and found four cellulose synthase-like protein genes (*CSLD5-1*, *CSLD5-2*, *CSLE1*, *CSLG2*) that were highly expressed in the cucumber fruit peduncle (Supplementary Fig. S4). Then, the expression patterns of these genes during peduncle development were detected.

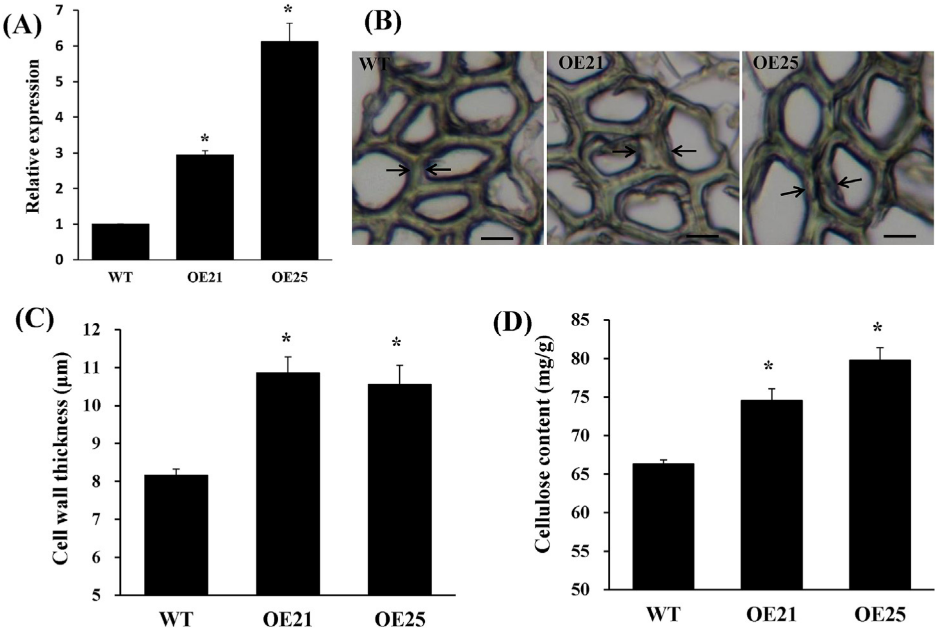

Fig. 4. Biological function analysis of *CsHT3* in cucumber peduncle. (A) qRT-PCR detected the relative expression of *CsHT3* in transgenic cucumber lines. (B) Microscopic observation of peduncle PF cell of *CsHT3* transgenic lines; arrows indicate the cell wall between two cells. (C) Cell wall thickness statistics of peduncle PF cells. (D) Cellulose content in peduncle. OE, *CsHT3* overexpression lines; Bar = 10 μm. The results represent at least three independent biological repetitions. The asterisks in A, C and D indicate statistically significant as compared with WT. $^*P < 0.5$.

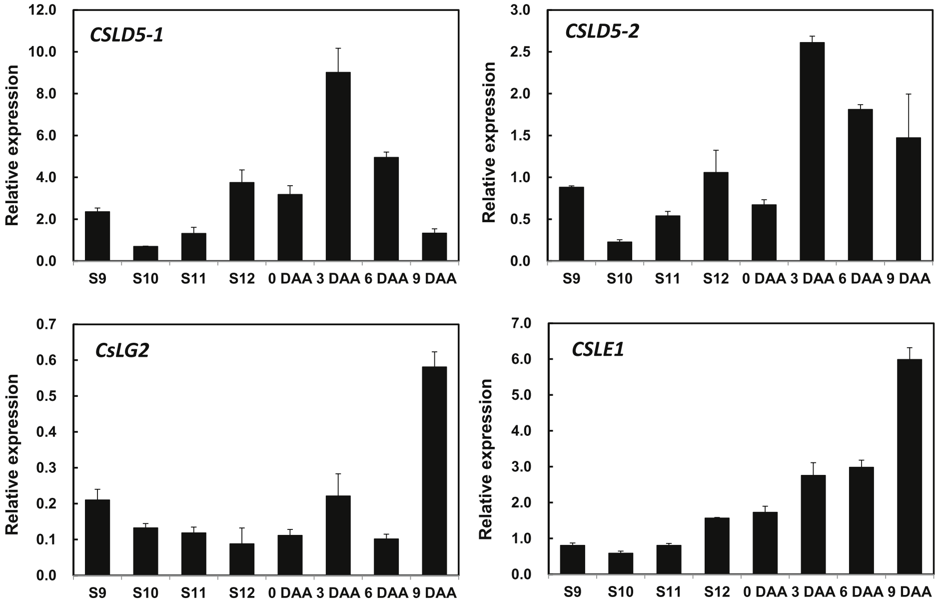

Fig. 5. Developmental expression profile of cellulose synthase-like protein genes in developing cucumber peduncles. S9–S12, four developmental stages of ovary; DAA, day after anthesis; *TUA* (α-tubulin) was used as the control. Error bars represent the SE for three technical replicates of three biological replicates.

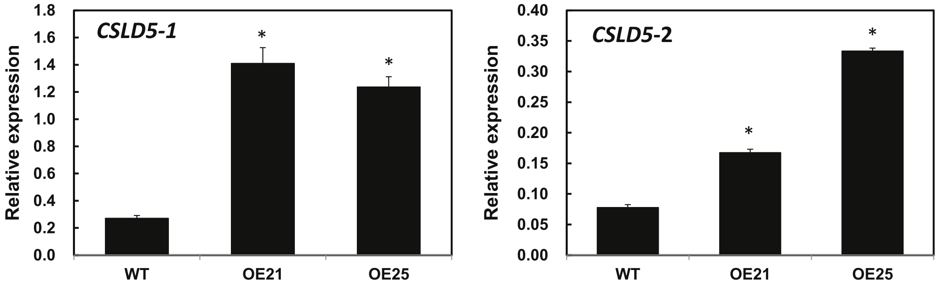

Fig. 6. Relative expression of cellulose synthesis-like protein genes (in the transgenic cucumber lines. Results represent at least three independent biological repetitions. Asterisks indicate statistically significant as compared with WT. $^{*}P < 0.5$.

CSLD5-1 and *CSLD5-2* showed similar expression patterns to *CsHT3* (Fig. 5). Furthermore, the relative expression levels of *CSLD5-1* and *CSLD5-2* in the transgenic cucumber lines were analyzed. The expression of both *CSLD5-1* and *CSLD5-2* significantly increased in the OE-*CsHT3* lines compared with the wild type (Fig. 6).

4. Discussion

In plants, STP/HT is a multigene family belonging to the monosaccharide transporter-like superfamily (Büttner, 2007; Doidy et al., 2012). The family members of STP/HTs are distributed and specifically regulate hexose transport in different tissues and cell types (Poschet et al., 2010; Slewinski, 2011; Büttner and Sauer, 2000; Rottmann et al., 2016). However, a functional analysis of STP/HT genes in the peduncle has yet to be conducted. In this study, we described the expression patterns and functional properties of the STP/HT gene *CsHT3* in the cucumber fruit peduncle. Our results demonstrated that the expression and localization of CsHT3 shifted to different cell types during fruit peduncle development. We also determined that CsHT3 plays an important role in cellulose synthesis in the cucumber fruit peduncle.

4.1. Expression and localization patterns of the CsHT3 gene

Most of the known hexose transporters show sink-specific expression as expected from their postulated role in sink support (Cheng et al., 2015b; Rottmann et al., 2016; Büttner, 2010). In our previous research, RT-PCR results revealed significant levels of *CsHT3* transcripts in all of the cucumber organs tested, including roots, stems, leaves, female flowers, male flowers, fruit, and peduncle (Cheng et al., 2015b). In the present study, we focused on the expression pattern of *CsHT3* in developing cucumber fruit peduncles and found that the expression of *CsHT3* increased in the peduncle during ovary development and fruit enlargement (Fig. 1). Immunohistochemical localization results revealed that CsHT3 was mainly localized at the sclereid and the phloem fiber cells before they became completely fibrotic and then translocated to the CCs (Fig. 2). This result indicates that CsHT3 plays an important role in fiber synthesis and secondary cell wall formation. Furthermore, we searched for the detailed expression data in the Cucumber transcriptome database and found that the transcript of CsHT3 mainly accumulated in the stem, petiole of old leaf, peel of old fruit, fruit peduncle, and tendril, all of which contain relatively high cellulose levels (Supplementary Fig. S5). The expression of CsHT3 was especially high in the tendril (Supplementary Figs. S5A–B). Fiber ribbons can be found in specialized cells of tendril coils (Gerbode et al., 2012).

4.2. Role of hexose transporters in the development of cucumber fruit peduncle

In the Cucurbitaceae family, most photoassimilates translocated in the phloem are stachyose and raffinose; however, these assimilates can hardly be detected in fruit (Hu et al., 2009; Mitchell et al., 1992). Therefore, most of the sugar is possibly metabolized to sucrose and galactose, which are released in fruit peduncles (Dai et al., 2006; Smart and Pharr, 1981). In our previous research, we have found a galactose-specific transporter (CsHT4) and a broad-spectrum hexose (glucose, galactose, and mannose) transporter (CsHT3) in the cucumber peduncle (Cheng et al., 2015a). The expression patterns of cell wall invertase gene *CscwINV*3 and *CsHT3* are similar during cucumber fruit peduncle development (Fig. 1 and Supplementary Fig. S6). Immunolocalization results indicated that CsHT3 was mainly localized to the phloem-specific cells of peduncles (Fig. 2 and Supplementary Fig. S2). These results suggest that galactose, which is released from the hydrolysis of stachyose and raffinose in the peduncle, is released to the apoplastic space first and then transported to specific cells by CsHT3 and CsHT4 for metabolism.

The phloem-mobile symplasmic tracer CF was loaded into the peduncle. CF was restricted to the phloem cells without diffusing into the surrounding tissues, indicating that carbohydrate unloading in the phloem also follows an apoplastic pathway similar to that in fruits (Fig. 3). Galactose can hardly be detected in the peduncle and fruit tissues, indicating that the release, transport, and metabolism of galactose involve continuous and fast processes (Smart and Pharr, 1981). Rapid metabolism and transport are important to maintain sink strength and promote fruit development and enlargement (Bermudez et al., 2014; Bihmidine et al., 2013; Cook and Evans, 1983).

In our previous research, we performed the immunolocalization of CsHT3 in the peduncle at one stage (about 1–2 days before the anthesis) and found that CsHT3 is restricted to the phloem fiber cells (Cheng et al., 2015a). However, in the present research, we found an interesting result when different developmental peduncles were used to perform this experiment. In young peduncles connected to the early developmental stage of ovaries (stages 9–11), CsHT3 was mainly localized at the sclereids, which were dispersed among the parenchyma cells of the external phloem, and was then translocated to the PF cells at stage 12 (Fig. 2). After anthesis, CsHT3 was transferred again to the CCs of the peduncle (Fig. 2). The locations of CsHT3 in the sclereids and PF cells suggest that CsHT3 plays an important role in the development of PF cells and in the maintenance of peduncle toughness. A thick secondary cell wall, the main component of which is cellulose, is formed during the developmental of sclereids and PF cells in the cucumber fruit peduncle. Glucose is an important substrate for cellulose synthesis (Prassinos et al., 2005), whereas galactose is a component of primary cell wall polysaccharides (Poschet et al., 2010). Cucumber fruit develops from an enlarged inferior ovary and rapidly grows to immense proportions. The rapid increase in cucumber fruit weight needs the high tenacity of the peduncle for support. Therefore, the glucose and galactose transporter CsHT3, which is localized to the PF cells of the peduncle, may play an important role in this process. The cellular structure and cellulose content of the peduncle were detected in transgenic cucumber plants. Results indicated that the overexpression of *CsHT3* can increase the cell wall thickness of PF cells and cellulose content in the peduncle (Fig. 4). The expression of cellulose synthase-like protein genes with similar expression patterns to *CsHT3* significantly increased in the *CsHT3*-overexpressing peduncles compared with the wild type (Figs. 5 and 6, Supplementary Fig. S4). These results indicate that *CsHT3* and cellulose synthase genes are synergistically involved in the secondary cell wall synthesis of cucumber fruit peduncle. In addition to the peduncle, we also observed other phenotypes of the *CsHT3* overexpression plants and found that overexpression of *CsHT3* by 35S promoter also can improve the seedling growth (Supplementary Fig. S7). Hexose transporters usually transport a broad spectrum of hexose substrates. They usually play multiple roles in plant development, indicating that the expression of *HTs* can be regulated as needed to perform different biological functions. However, the exact mechanisms by which these genes are regulated are still obscure. Future research should focus on the regulatory mechanisms of hexose transporters.

Author contributions

Jintao Cheng and Zhilong Bie conceived and designed the experiment, analyzed the data. Jintao Cheng and Suying Wen performed the experiment and wrote the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.10.034.

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