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Functional characterization and expression analysis of cucumber (*Cucumis sativus* L.) hexose transporters, involving carbohydrate partitioning and phloem unloading in sink tissues

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Many hexose transporters (HTs) have been reported to play roles in sucrose-transporting plants. However, little information about roles of HTs in RFOs (raffinose family oligosaccharides)-transporting plants has been reported. Here, three hexose transporters (*CsHT2*, *CsHT3*, and *CsHT4*) were cloned from *Cucumis sativus* L. Heterologous expression in yeast demonstrated that CsHT3 transported glucose, galactose and mannose, with a K_m of 131.9 μM for glucose, and CsHT4 only transported galactose, while CsHT2 was non-functional. Both CsHT3 and CsHT4 were targeted to the plasma membrane of cucumber protoplasts. Spatio-temporal expression indicated that transcript level of *CsHT3* was much higher than that of *CsHT2* and *CsHT4* in most tissues, especially in peduncles and fruit tissues containing vascular bundles. GUS staining of *CsHT3*-promoter-β-glucuronidase (GUS) transgenic Arabidopsis plants revealed *CsHT3* expression in tissues with high metabolic turnover, suggesting that CsHT3 is involved in sugar competition among different sink organs during plant development. The transcript levels of *CsHT3* and cell wall invertase genes increased in peduncles and fruit tissues along with cucumber fruit enlargement, and CsHT3 localized to phloem tissues by immunohistochemical localization; These results suggest that CsHT3 probably plays an important role in apoplastic phloem unloading of cucumber fruit.

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1. Introduction

Phloem unloading in economical sink organs, such as fruits and seeds, is an important process that determines agricultural products yield and quality. Generally, the transfer of sugars from the se/cc complex to adjacent cells can occur via the plasmodesmata through the symplastic route, or apoplasmically across the plasma membrane by transporters through an apoplastic route [1–3]. In most sink tissues, such as roots, potato tubers, sink leaves and vegetative apices [2,4], phloem unloading usually follows the symplastic route [2,5–8]. However, some fruits which accumulate soluble sugars to high concentrations such as tomato, apple and grape, have been shown to employ the apoplastic unloading routes in the whole or part stage of fruit development [9–11].

In most plants, sucrose is the major form of carbohydrate transported in the phloem and distributed to heterotrophic sinks such as fruit. However, in certain species called RFO-transporting plants, raffinose family oligosaccharides (RFOs) are synthesized from sucrose by adding one or more galactose molecules and are the primary translocated carbohydrates [12–14]. Phloem loading strategy of the RFO-transporting plants has been proved to use polymer trapping through symplastic loading pathway in source leaves [15]. However, how the RFOs are unloaded to the sink tissues from the phloem of these plants is still poorly understood.

Cucumber (*Cucumis sativus*) is a typical RFOs-transporting plant with stachyose as the major transporting sugar. Although RFOs are the main transport sugars in cucumber phloem, the major free sugars in the peduncles and fruit tissues are sucrose and hexoses (glucose and fructose), which leads to the conclusion that sucrose rather than stachyose or raffinose is translocated into

Abbreviations: RFOs, raffinose family oligosaccharides; MS, Murashigeand Skoog; DPP, days, post-pollination; GFP, green fluorescent protein; GUS, β-glucuronidase; cwINV, cell wall invertase; SAI, soluble acid invertase.

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the fruit [12,16–18]. The hydrolysis of stachyose or raffinose and phloem unloading in sink organs of RFOs-transporting species is a complicated process. Recently, Hu et al. have shown that phloem unloading in cucumber fruit follows the apoplastic unloading route using different methods [19]. However, the molecular mechanism of this process still need to further study. In particular, few researches about membrane transport proteins involved in this process exist.

To describe the molecular components that contribute to sugar unloading and partitioning in cucumber sink tissues, three genes encoding hexose transporters (*CsTH2*, *CsTH3* and *CsTH4*) were isolated from cucumber fruit. Their functions were analyzed in yeast and their expression patterns were determined by qRT-PCR. A detailed expression pattern of CsHT3 was analyzed by histochemical localization in Arabidopsis plants expressing a *CsHT3* promoter-GUS fusion and its expression pattern was compared with those of cell wall invertase genes during cucumber fruit development. The results are discussed in terms of the physiological roles these proteins may play in carbohydrate partitioning and phloem unloading in cucumber fruit.

2. Materials and methods

2.1. Plant material and bacterial/yeast strains

Cucumber (*C. sativus* L. cv. xintaimici) plants were grown under glasshouse conditions. Tissues were sampled to test gene transcription and translation. For qRT-PCR, different tissues from two-month-old plants were used. Arabidopsis (*Arabidopsis thaliana*) ecotype Landsberg erecta (Ler) were grown in potting soil in the greenhouse or on agar medium in growth chambers at 22 °C and 55% relative humidity under short-day (8 h of light/16 h of dark) or long-day (16 h of light/8 h of dark) conditions, as indicated.

Escherichia coli strain DH5a was used for cloning. Heterologous expression was performed in *Saccharomyces cerevisiae* strain EBY.VW4000.

2.1.1. Cloning and sequencing of cucumber hexose transporter (CsHT) cDNA

To investigate the putative hexose transporters in cucumber fruit, we used the nucleotide sequence of *AtSTP1* (Arabidopsis sugar transporter 1 gene, gene bank accession no. NM_100998) as queries, BLAST searches of the translated cucumber genome (http://cucumber.genomics.org.cn/page/cucumber/blast.jsp). Three putative HT genes, showing high identity at the nucleotide level with *AtSTP1* were designated as *CsHT2*, *CsHT3* and *CsHT4* respectively. The open reading frames (ORFs) of *CsHT2*, *CsHT3* and *CsHT4* were isolated by RT-PCR using ORFs primers as shown in Supplementary Table 2 and total RNA from cucumber fruit. The sequences were cloned into pGEM-T easy vectors (Promega, USA) separately yielded CsHT-Ts and sequenced.

2.2. Heterologous expression of CsHTs in yeast

The open reading frames with appropriate restriction sites of *CsHT2, CsHT3* and *CsHT4* in CsHT-Ts were subcloned into the *Saccharomyces cerevisiae*/*Escherichia coli* shuttle vector pDR196 [20] which contains the URA3 gene for uracil prototrophy and the Met25 promoter for constitutive expression of the introduced transgene. The resulting constructs and the empty vector pDR196 (as a control) were then transferred into the hexose transporter-deficient yeast (*Saccharomyces cerevisiae*) strain EBY.VW4000 [21] according to the method of Morita and Takegawa [22]. Transformed cells were pregrown in liquid SD-ura medium supplemented with 2% (w/v) maltose as sole carbon source to an OD_{623} of 0.6. The drop test for yeast growth was performed according to Loqué's method [23]. Serial dilutions (10×) of yeast cell suspensions were dropped on solid SD-ura medium supplemented with 2% (w/v) maltose or different hexose as sole carbon source. For metabolic inhibitor treatment, 10 μM CCCP was added into the mediums. Cells were incubated 2–5 days at 30 °C prior to photograph.

For the glucose-uptake assays, *Saccharomyces cerevisiae* strain EBY.VW4000, carrying either pDR196/*CsHT3* or empty pDR196, was grown at 30 °C to an OD_{623} of ~0.8 in liquid minimal media containing maltose. Cells were washed twice with 25 mM sodium phosphate buffer (pH4.0) and suspended in the same buffer to an OD_{623} of 20. Uptake assays were initiated by adding ^{14}C-glucose to a final specified concentration. After incubation at 30 °C with shaking for 4 min (except when assaying the time course of the uptake rate), glucose uptake was stopped by adding 4 mL of ice-cold distilled water and filtered on 0.8 μm glass fiber filters (GF/C; Whatman) in a vacuum filtration apparatus. Cells were rapidly washed three times with 4 mL of ice-cold distilled water, and transferred to liquid scintillation vials, and counted.

2.2.1. Quantitative analysis of gene expression by real-time PCR

Expression analysis was performed using real-time PCR analysis with the SYBR green detection protocol (TaKaRa, Japan) and the ABI 7500 system (Bio-Rad, USA). Total RNA was extracted from specified tissues and treated with DNase (Promega), and then reverse-transcribed using an oligo (dT) primer according to the supplier's instructions (Promega, Madison, WI, USA). The cDNA was then used as a template for quantitative RT-PCR analysis. Tubulin mRNA was used as an internal control and relative amounts of mRNA were calculated using the comparative threshold cycle method. Primers used for qRT-PCR analyses are shown in Supplementary Table 2.

2.2.2. Enzyme assay

Cell wall acid invertase (CWI) extracts of peduncles from fruits harvested 0, 3 and 9 days after pollination were prepared as described previously [24]. Acid invertase activities were assayed in the insoluble fractions according to Schaffer et al. [25].

2.2.3. Histochemical localization of CsHT3 by GUS assay

The putative promoter region of *CsHT3* was amplified from cucumber genomic DNA using primers listed in Supplementary Table 2. The PCR product was double-digested with appropriate restriction endonucleases, and ligated into vector pBI121 3′ of the GUS gene to obtain the *pCsHT3-GUS* fusion construct. This construct was transferred into *Agrobacterium tumefaciens* strain LBA4404, which was then used to transform the Arabidopsis plants using the floral dip method according to the protocol of Clough and Bent [26]. For GUS staining, the transgenic Arabidopsis samples were incubated with GUS staining solution overnight at 37 °C as described by Jefferson et al. [27]. After staining, the green tissues were rinsed and dehydrated through an ethanol series before photographed. Some samples were used to prepare paraffin sections for better observation.

2.2.4. Subcellular localization of CsHT:GFP fusion protein

The open reading frames of *CsHT3* and *CsHT4* were amplified with gene-specific primers listed in Supplementary Table 2 and cloned into pEZS-NL vector to generate C-terminal fusions with GFP under control of the Cauliflower Mosaic Virus 35S promoter. Transient expression of the CsHT3-GFP and CsHT4-GFP fusion protein in cucumber protoplasts and onion (*Allium cepa*) epidermal cells were conducted as described by Huang et al. [28] and Hayes et al. [29] respectively. The pEZS-NL empty vector expressing untargeted GFP was used as a control. GFP fluorescence was visualized by Olympus Confocal Laser Scanning Microscope.

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