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Heterologous expression of the apple hexose transporter MdHT2.2 altered sugar concentration with increasing cell wall invertase activity in tomato fruit

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Summary

Sugar transporters are necessary to transfer hexose from cell wall spaces into parenchyma cells to boost hexose accumulation to high concentrations in fruit. Here, we have identified an apple hexose transporter (HTs), MdHT2.2, located in the plasma membrane, which is highly expressed in mature fruit. In a yeast system, the MdHT2.2 protein exhibited high ^{14}C-fructose and ^{14}C-glucose transport activity. In transgenic tomato heterologously expressing *MdHT2.2*, the levels of both fructose and glucose increased significantly in mature fruit, with sugar being unloaded via the apoplastic pathway, but the level of sucrose decreased significantly. Analysis of enzyme activity and the expression of genes related to sugar metabolism and transport revealed greatly up-regulated expression of *SlLIN5,* a key gene encoding cell wall invertase (CWINV), as well as increased CWINV activity in tomatoes transformed with *MdHT2.2*. Moreover, the levels of fructose, glucose and sucrose recovered nearly to those of the wild type in the *sllin5*-edited mutant of the *MdHT2.2*-expressing lines. However, the overexpression of *MdHT2.2* decreased hexose levels and increased sucrose levels in mature leaves and young fruit, suggesting that the response pathway for the apoplastic hexose signal differs among tomato tissues. The present study identifies a new HTs in apple that is able to take up fructose and glucose into cells and confirms that the apoplastic hexose levels regulated by HT controls CWINV activity to alter carbohydrate partitioning and sugar content.

Introduction

Sugars in plant cells are essential molecules that not only provide energy and building blocks for growth and development but also constitute osmotic and signalling molecules (Ruan, 2014). In fruit crops, the concentration of soluble sugar is also a determinant of fruit quality, especially sweetness. Sugar content and composition depend on the regulation of sugar translocation, synthesis and metabolism (Li *et al*., 2018; Ruan, 2014).

In the majority of plants, sucrose (Suc) is produced in photosynthetically active leaves (source, loading) and translocated to support nonphotosynthetic tissues (sink, unloading), such as the developing seed, fruit and tuber. In source leaves, Suc moves from photosynthetic cells into sieve element–companion cell (SE-CC) complexes through a symplastic loading pathway via plasmodesmata along the Suc concentration gradient or through an apoplastic loading pathway via transporters (Comtet *et al*., 2017; Patrick, 1997). After long-distance phloem transport, the Suc in phloem moving into sink cells needs to be transferred from SE-CC complexes to utilization sites/storage tissues (Ruan, 2014). There are two pathways for Suc unloading: the symplasmic pathway and the apoplasmic pathway. In the symplasmic pathway, which typically occurs in shoot tips and roots, Suc moves along the concentration gradient from SE-CC complexes directly to surrounding parenchyma cells (PCs) via plasmodesmata (Patrick, 1997). In the apoplasmic pathway, Suc is first released across the plasma membrane of phloem sieves or SE-CC complexes into the apoplasmic space between cells, possibly via simple diffusion facilitated by a newly discovered family of Suc uniporters, SWEETs (Abelenda *et al*., 2019). Suc can then be transported into storage PCs by Suc transporters (SUTs or SUCs). Suc can also be converted into fructose (Fru) and glucose (Glc) by cell wall invertases (CWINVs) and then transported into storage cells by hexose transporters (HTs) (Slewinski, 2011). It is now well accepted that the phloem unloading capability plays an important role in the partitioning of photoassimilate, thereby to a large

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extent determining crop output and quality as well as fruit sugar content (Chen *et al*., 2017; Patrick, 1997). Although phloem unloading mechanisms have been extensively studied (Lalonde *et al*., 2003), how unloading processes regulate sugar metabolism and content remains poorly understood.

In most fruits, Suc unloading occurs via the apoplasmic pathway during the maturation stage, for example, in apple (Zhang *et al*., 2004), pear (Li *et al*., 2017) and kiwifruit (Chen *et al*., 2017), whereas in grape and tomato, the Suc unloading pathway shifts from a symplasmic pathway to an apoplasmic pathway during fruit development (Ruan and Patrick, 1995; Zhang *et al*., 2006). In apoplasmic unloading, the SUT protein (e.g. *Arabidopsis*, *AtSUC2*; rice, *OsSUT3*; sorghum, *SbSUT5*) performs an indispensable function in taking up Suc that leaks from the apoplastic space into PCs (Milne *et al*., 2018; Scofield *et al*., 2007). Nonetheless, how Suc moves into sink cells is also important, and in cooperation with HTs, the CWINV-mediated hydrolysis of Suc has been suggested to play an essential role in determining sink strength and regulating the balance between source and sink in ripening tomato fruit (Jin *et al*., 2009; Nguyen-Quoc and Foyer, 2001; Ruan, 2014; Wan *et al*., 2018; Wang and Ruan, 2012). Indeed, an increase in CWINV activity enhances hexose concentration, delays leaf senescence and increases the weight of seeds in tomato (Jin *et al*., 2009; Liu *et al*., 2016). In maize (Bi *et al*., 2017) and rice (Hirose *et al*., 2002), CWINVs play a pivotal role in carbohydrate unloading into developmental seeds, which is related to increased grain filling and yield. Moreover, three *SlHT* genes in tomato fruit have been found to colocalize with QTLs for sugar accumulation (Prudent *et al*., 2011), a relationship that was further verified by RNAi mutants of these three *SlHT*s (McCurdy *et al*., 2010), which decreased hexose concentrations to 55% of those of the wild type. HTs have also been reported to increase sink strength as a mechanism for stress resistance (Lemonnier *et al*., 2014; Sade *et al*., 2013). Although it is well known that the CWINV-HT system acts as a gateway where sugar is taken up into fruit, it remains unknown whether HT regulates CWINV activity in fruit.

Apple (*Malus domestica* Borkh.) and other Rosaceae tree fruits synthesize sorbitol (Sor) and Suc in leaves; they both are then translocated and unloaded into the cell wall space of developing fruit via the apoplasmic pathway (Zhang *et al*., 2004). Sor is transferred into the cytosol of PCs by the Sor transporter, and Suc is mostly cleaved into hexoses via CWINV before being moved into fruit PCs (Zhang *et al*., 2004; ;Li *et al*., 2012). HT is needed for the efficient movement of Glc and Fru from cell wall spaces into PCs because Sor transport is inhibited by high concentrations of hexose (Gao *et al*., 2003; Li *et al*., 2018), while fructose content is more than 50% of the soluble sugar in apple fruit cells (Li *et al*., 2018). Particularly in the late stage of fruit development, strong HT activity is expected to result in hexose uptake against a high chemical gradient due to the high concentrations of hexose in fruit PCs. Among 30 candidate *MdHTs*, *MdHT2.2* was found to be highly expressed in fruit, and both its mRNA and protein levels were significantly and positively correlated with sugar levels in apple fruit (Li *et al*., 2016; Wei *et al*., 2014). Thus, *MdHT2.2* likely encodes a key transporter that loads hexose into PCs in apple fruit.

To determine the function of MdHT2.2 in hexose unloading and its link to sugar metabolism in fruit, we performed an analysis of *MdHT2.2* expression characteristics and sugar transport activity, and then heterologously expressed *MdHT2.2* in Micro-Tom tomato (*Solanum lycopersicum* cv. Micro-Tom) to investigate its effect on sugar metabolism in fruit. The results confirmed that MdHT2.2, as a sugar/H^+ symporter, mainly functions in the uptake of Fru and Glc from the cell wall space and the movement of these sugars into cells. Heterologous expression of *MdHT2.2* in tomato increased the contents of Fru and Glc but decreased the Suc content in ripening fruit due to increased expression of *SlLIN5*, a key gene encoding a CWINV in tomato fruit (Jin *et al*., 2009; Liu *et al*., 2016), and increased CWINV activity. In addition to having a high soluble sugar content and large fruit size, the transgenic tomatoes also showed other advantageous traits, such as thinned pericarp, dwarfness and early flowering. The present study identifies a new low-affinity HT in apple that is able to take up Fru and Glc into cells and implies that the apoplastic hexose level controls CWINV activity, affecting carbohydrate partitioning and sugar content. The results offer new insight into the regulation of the mechanism of carbohydrate partitioning and unloading in fruit, which will be helpful for improving fruit quality and yield.

Results

Isolation, homology analysis and expression assay of *MdHT2.2*

The MdHT2.2 (MD15G1193400/MDP0000154362) ORF was cloned from the cDNA of ripening apple fruit. The gene was localized to chromosome 15 of the *Malus* genome, and the mRNA sequence is 2943 bp. The complete open reading frame, as cloned from 'Gala' apple, is 1569 bp and encodes 522 amino acids. Phylogenetic analysis of the predicted MdHTs and HTs/STPs from other species (Figure S1A) indicated that different members of the same subfamily share high homology. The putative amino acid sequence of MdHT2.2 shows approximately 77% homology with the predicted peptide of AtSTP14, a galactose transporter involved in drought/senescence-mediated cell wall recycling (Poschet *et al*., 2010). The predicted transmembrane structure indicated that MdHT2.2 has 12 transmembrane domains with a cytoplasm-exposed loop (Figure S1B).

To examine the spatiotemporal expression levels of *MdHT2.2*, qRT-PCR was performed with RNA extracted from different tissues of apple plants. *MdHT2.2* was found to be highly expressed in flowers and mature fruits (especially the flesh) (Figure 1a); however, in tissues with low hexose accumulation (e.g. leaves and roots), its transcript levels were correspondingly very low (Figure 1a). The trend of the change in *MdHT2.2* expression was highly similar to that of sugar accumulation (especially fructose and sucrose) during fruit development (Figures 1b and S2, Table S1). In mature fruit, the enriched parenchymal cell region had a much higher expression level of MdHT2.2 than the region of the petal and sepal vascular bundles (Figure S3). To determine the relationship between *MdHT2.2* expression and sugar content, fruit flesh was exposed to different sugars, and mRNA transcript levels were assayed. Compared to the control, which was exposed to sorbitol, *MdHT2.2* expression was significantly induced by Fru and Glc (Figure 1c). These results indicate that *MdHT2.2* might play an important role in the control of sugar accumulation and the uptake of hexose in apple fruit.

Subcellular localization of the MdHT2.2 protein

To examine the location of the MdHT2.2 protein, its ORF was cloned and linked to the GFP reporter gene to produce fusion proteins MdHT2.2-GFP and GFP-MdHT2.2 driven by the

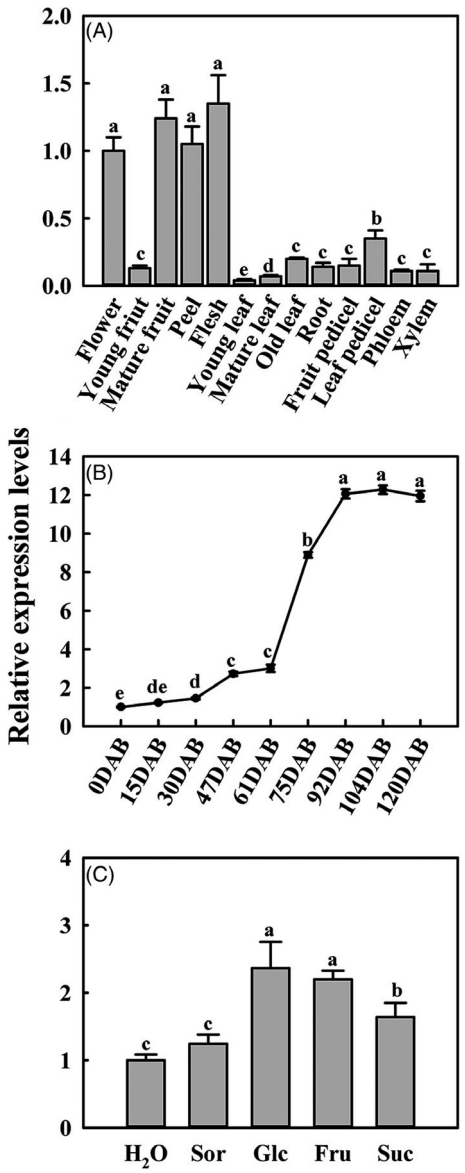

Figure 1 Spatiotemporal expression level analysis of *MdHT2.2*. (a) Relative *MdHT2.2* expression levels in different tissues from flower, young fruit, mature fruit, mature fruit peel and flesh, young leaf, mature leaves, old leaf, root, fruit pedicel, leaf pedicel, phloem and xylem of apple trees. The expression level in flower was set to 1. (b) Trends in relative expression of *MdHT2.2* at different developmental stages of apple fruit. Samples from 0 days after bloom (DAB) were set to 1. (c) Change in the relative transcript level of *MdHT2.2* after treating flesh samples for 6 h with 2% exogenous sugar (Sor: sorbitol; Glc: glucose; Fru: fructose; and Suc: sucrose, with ddH_2O as a negative control, set to 1). Expression levels were calculated relative to that of *MdActin*. Bars represent the mean value ± SE ($n \geq 3$). Different letters indicate significant differences.

CaMV35S promoter. Confocal laser scanning microscopy (CLSM) showed the green fluorescence signal to be outside the vacuole (Figure 2), clearly demonstrating plasma membrane localization for MdHT2.2.

Functional characterization of MdHT2.2 in yeast

To examine the transport property of MdHT2.2, we cloned the *MdHT2.2*-ORF into the vector pYST2.0 in either the sense or antisense orientation and expressed it in the HTs-deficient yeast mutant EBY.VW4000, which is able to grow normally only on maltose (Wieczorke *et al*., 1999). EBY.VW4000 was transformed with pYST2.0-*MdHT2.2* in the sense orientation to analyse growth-based complementation, and EBY.VW4000 transformed with *MdHT2.2* in the antisense orientation was used as a control. Expression of the *MdHT2.2* gene in the antisense orientation in this null mutant background did not restore growth on test sugar-containing media (Figure 3a).

The expression of *MdHT2.2* in the sense orientation allowed the cells to grow on 2% Fru, Glc, galactose (Gal) and xylose (Xyl) (Figure 3a), and the fastest growth ratio was observed on Fru; on 0.2% Fru- or Glc-containing medium, however, the transformed yeast grew very slowly. These results showed that the expression of recombinant *MdHT2.2* could complement multiple deletions of endogenous yeast HT genes and that MdHT2.2 is a HTs with Fru and Glc transport activity. This finding was supported by transport assays that determined the transport capacity for ^{14}C-labelled hexose; the transport capacity for ^{14}C-Glc was slightly lower than that for ^{14}C-Fru, and transformed yeast cells were barely able to take up galactose and xylose (Figure 3b). The Km of MdHT2.2 for Fru was calculated to be approximately 220.91 μm (Figure 3c, inset). Additionally, a low concentration of the proton uncoupler carbonyl cyanide-m-chlorophenylhydrazone (CCCP) significantly reduced Fru uptake (Figure 3b), suggesting that sugar uptake via MdHT2.2 is driven by a proton gradient across the plasma membrane. Taken together, the results of heterologous expression in yeast indicate that MdHT2.2 is an energy-dependent, low-affinity monosaccharide/H^+ symporter specific for Fru and Glc.

Heterologous expression of *MdHT2.2* altered the growth status and sugar content of tomato plants

To further understand the function of *MdHT2.2* in fruit sugar regulation, we heterologously expressed *MdHT2.2* in tomato (*S. lycopersicum* cv. Micro-Tom), which exhibits a short life cycle and a symplastic-to-apoplastic unloading phase change in fruit (Ruan and Patrick, 1995). Three homozygous lines (L5, L11 and L12) were obtained after evaluation at the RNA and protein levels (Figure S4C,D). The transgenic tomato plants displayed a significant dwarf phenotype with decreased height, stem diameter and internode length (Figure S4A, Table 1). However, the ripening fruit of the transgenic lines was larger than the wild-type fruit (Figure S4B), and the fruit weight, transverse diameter and seed number were correspondingly notably increased (Table 1). Although the longitudinal diameter, carpel thickness and thousand-seed weight did not change, the pericarp thickness decreased significantly, by approximately 35% (Table 1). The sugar concentration in the transgenic fruit was assayed at three stages of fruit development: young fruit [15 days after bloom (DAB)], breaker-stage fruit (30 DAB) and ripening fruit (45 DAB) (Figure 4). The Suc concentrations in the fruit of transgenic lines were markedly higher than those in young (15 DAB) and breaker-stage (30 DAB) wild-type fruit. The Glc, Fru and starch concentrations were unchanged in those two stages. In ripening fruit (45 DAB), in which Suc is unloaded by the apoplastic pathway (Ruan and Patrick, 1995), the Suc concentrations were clearly decreased in the three transgenic lines; as expected, the Glc and Fru concentrations increased by approximately 29% and 15%, respectively, compared to those in the wild type. The soluble solid content

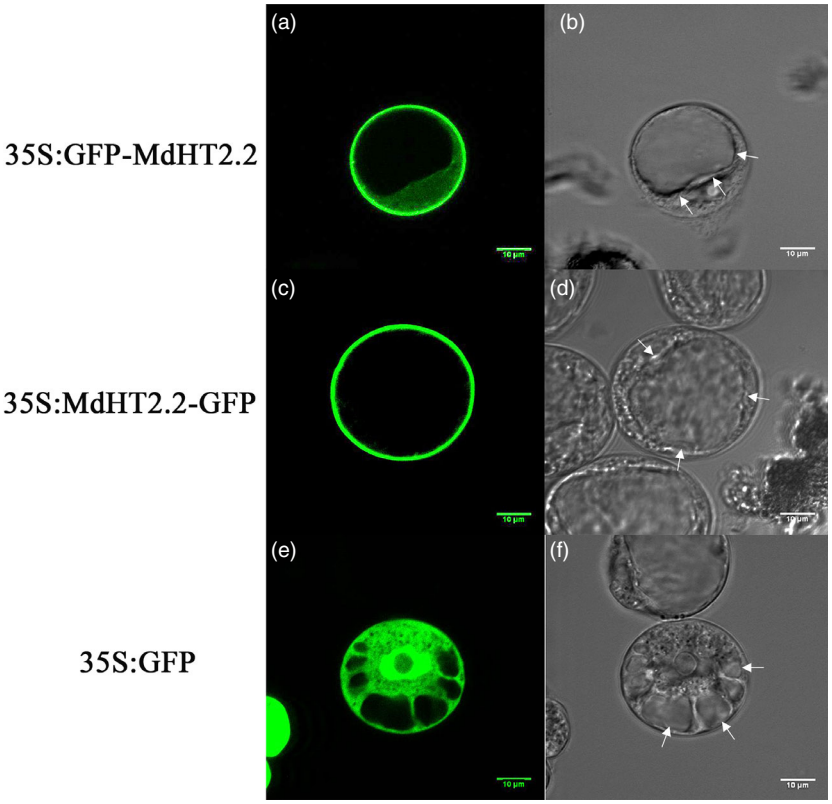

Figure 2 Subcellular localization of C/N-terminal GFP fusion proteins in *Arabidopsis* protoplasts. (a, c) GFP expressed in the protoplast membrane, which is indicated by the green ring outside the tonoplast. (b,d) Bright-field images of the left side images. (e,f) CaMV35S:GFP was widely expressed in the protoplast as the control. The tonoplast is indicated by white arrows.

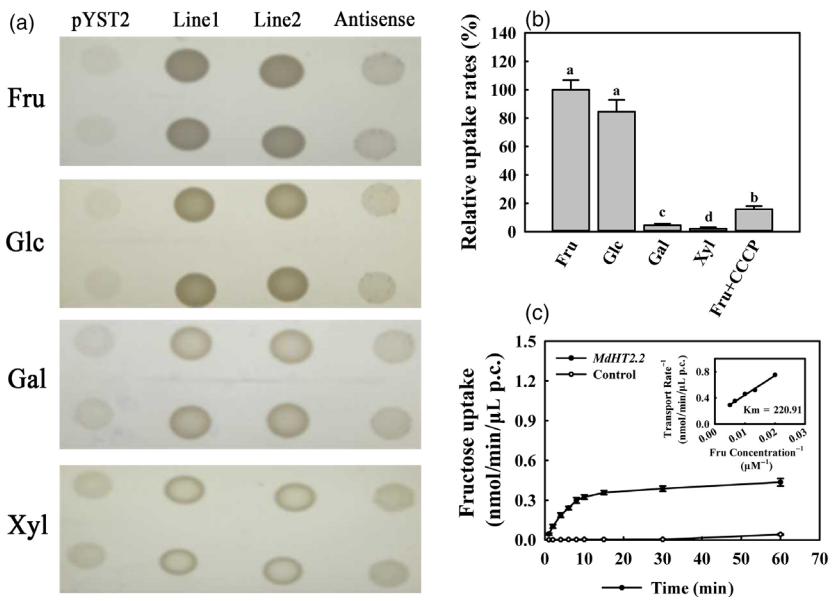

Figure 3 Heterologous expression of *MdHT2.2* in yeast. (a) Growth complementation of a yeast mutant strain. The HT-deficient yeast mutant carrying the empty pYST2.0 vector or the *MdHT2.2*-antisense recombinant plasmid could not grow normally on 4 monosaccharides, whereas the *MdHT2.2*-sense mutant yeast grew normally on fructose and glucose but slowly on galactose and xylose. (b) Relative uptake rates of feeding with 100 μM [^{14}C]-labelled exogenous sugars. For the inhibition assay, 50 μM CCCP (proton pump uncoupler) was applied for 30 s before the addition of [^{14}C] fructose. The fructose uptake rate was set to 100%. Different letters indicate significant differences. (c) Time course of the sugar uptake assay by the strain carrying pYST2.0-*MdHT2.2* (black spot) and pYST2.0 empty vector (black rings, as negative control) at 100 μM exogenous [^{14}C] fructose at pH = 5.5. Uptake rates were determined after the addition of fructose. The inset image shows a typical Km = 220.91 μM. Bars represent the mean value ± SE ($n \geq 4$).

(SSC) and starch content were also higher than those in the wild type (Figure 4). Additionally, the concentrations of citric acid and malic acid at 15 and 30 DAB were similar between transgenic lines and wild type, but at 45 DAB, malic acid level was less in the transgenic lines, opposite to citric acid (Figure S5).

Table 1 Phenotypic characteristics of transgenic tomato fruit and plants

	Phenotype	WT	L5	L11	L12
Fruit	Fruit number (/plant)	11 ± 1.22	12.8 ± 1.64	11.4 ± 1.67	12.8 ± 2.35
	Single fruit weight (g/fruit)	2.48 ± 0.19	3.45 ± 0.48*	3.19 ± 0.21*	3.35 ± 0.31*
	Transverse diameter (mm)	16.45 ± 1.25	18.40 ± 1.19*	17.86 ± 1.75*	18.28 ± 1.40*
	Vertical diameter (mm)	16.38 ± 0.66	17.35 ± 1.20	16.99 ± 1.62	16.42 ± 0.59
	Pericarp thickness (mm)	2.08 ± 0.25	1.16 ± 0.15*	1.48 ± 0.23*	1.28 ± 0.11*
	Carpel thickness (mm)	1.10 ± 0.14	1.13 ± 0.15	1.11 ± 0.12	1.11 ± 0.17
	Average seed number	16.09 ± 1.97	25.75 ± 2.54*	24.66 ± 3.93*	25.87 ± 2.94*
	Thousand-seed weight (g)	2.76 ± 0.20	2.53 ± 0.34	2.58 ± 0.25	2.52 ± 0.18
Plant	Height (cm)	18.76 ± 0.92	13.76 ± 0.54*	12.54 ± 0.87*	13.46 ± 0.71*
	Stem diameter (mm)	6.39 ± 0.61	5.13 ± 0.54*	5.44 ± 0.62*	5.52 ± 0.69*
	Internode length (cm)	3.96 ± 0.14	2.99 ± 0.26*	3.10 ± 0.12*	3.23 ± 0.23*

The seeds of 10–50 independent fruits from each line were counted for the 1000-seed weight. Values are the mean ± SE ($n \geq 3$). The asterisk indicates $P \leq 0.05$.

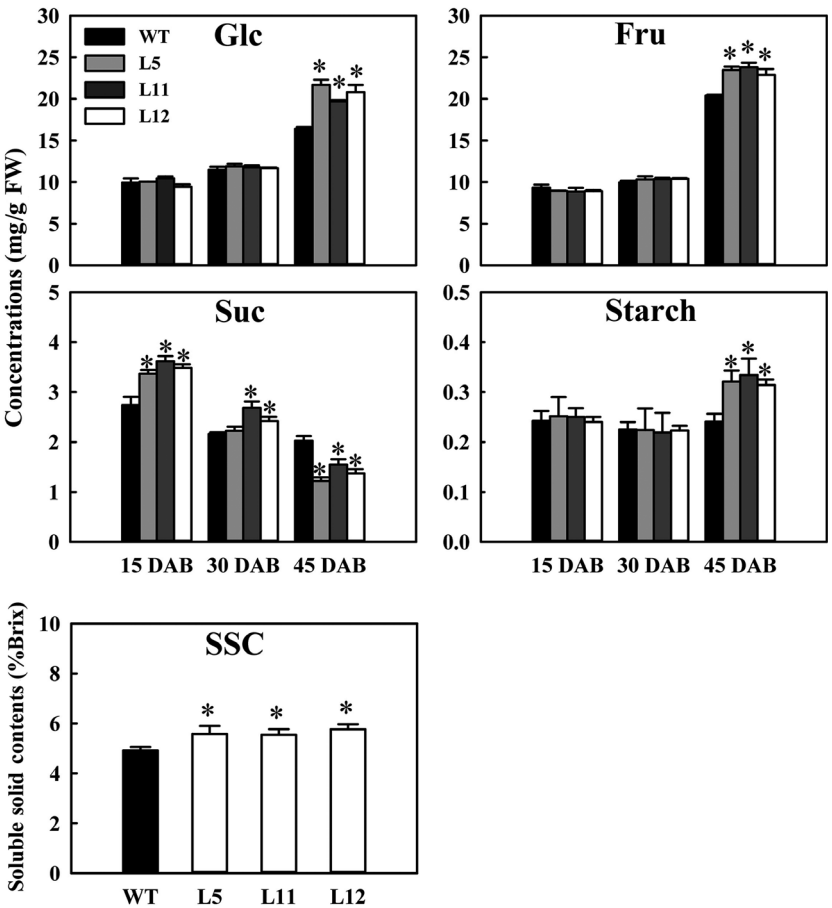

Figure 4 Sugar concentrations in tomato fruit. Glucose (Glc), fructose (Fru), sucrose (Suc) and starch concentrations at 15 DAB (young fruit), 30 DAB (breaker-stage fruit) and 45 DAB (ripening fruit), and SSC (soluble solid content) in 45-DAB fruit. Bars of different colours represent different lines. Bars represent the mean value ± SE ($n \geq 4$). The asterisk indicates $P \leq 0.05$.

Enzyme assays and expression levels of members of the sugar metabolism pathway in fruits of the transgenic lines

To determine why the sugar concentration changed in the transgenic tomato fruit expressing *MdHT2.2* (especially ripening fruits with decreased Suc), enzyme activity and gene expression related to sugar metabolism were detected in developing fruit. CWINV activities in both WT and transgenic lines were increased during fruit development, although activity in the transgenic lines was increased by less than twofold compared to that of the wild type at 45 DAB (Figure 5a). Staining of acid invertase in 45-DAB fruit also indicated greater activity in transgenic lines than in the wild type (Figure 5b). In contrast, the activities of neutral

invertase (NINV), vacuolar invertase (VINV) and sucrose synthase (SUSY), all of which are related to Suc dissociation, were significantly reduced in 45-DAB fruit of the transgenic lines compared with that of the wild type, and sucrose phosphate synthase (SPS), which is involved in Suc synthesis, showed increased activity (Figure 5a). These results indicate that the decreased Suc concentration in the *MdHT2.2*-transgenic fruit was due to increased cleavage of Suc into hexose via CWINV in the cell wall space. The lower activities of CWINV and NINV compared to the wild type in the leaves of the transgenic lines (Figure S7) were consistent with the increased Suc concentration, as shown in Figure S6.

To further identify key genes that contribute to these activities in the transgenic lines, the transcript abundance of key genes encoding these enzymes and transporters was detected. Among the four genes encoding tomato CWINV proteins, *SlLIN5* was highly expressed in fruit, especially in the transgenic lines at 30 and 45 DAB, and CWINV activity was high, even though *SlLIN8* expression was significantly increased only at 45 DAB. Indeed, the expression level of *SlLIN5* was more than 10 times greater than that in the wild type (Figure 6); the changes in *SlLIN6/7* expression levels were inconsistent with the changes in CWINV activity in developing fruit. Correspondingly, the expression levels of *SlNINV*, *SlVINV* and *SlSUSY* were decreased in the 45-DAB fruit of *MdHT2.2*-transgenic tomato compared with those in the wild type, but the expression of *SlSPS* increased in the transgenic lines. In addition, three key HTs, *SlHT1/2/3,* exhibited significantly decreased expression in the transgenic fruits, while expression of the tonoplast sugar transporter *SlTST2* was significantly increased in 15- and 30-DAB fruit of the transgenic lines. The Suc transporters *SlSUT1* and *SlSUT2*, which move Suc into the cytosol, exhibited decreased expression in the transgenic tomato fruit, especially at 45 DAB. These findings further suggest that Suc cleavage in the cell wall space was increased in *MdHT2.2*-transgenic tomato fruit but that the capacity for Suc absorption via SUT decreased.

In mature leaves of the transgenic lines, the expression patterns of *SlLIN5/8* and *SlNINV* were consistent with the decreased activities compared with the wild type (Figure S8), and the *SlHT1/2/3* and *SlSUSY1/4* expression levels were obviously down-regulated. The expression of *SlTST1* in the transgenic leaves decreased to less than 40% of the expression in the wild type, whereas the opposite was observed for *SlTST2*.

Sugar concentration in the *sllin5* mutant of *MdHT2.2*-transgenic tomato fruit

To confirm whether up-regulated *SlLIN5* expression was mainly responsible for altering the sugar content in ripening tomato fruit with expressed *MdHT2.2*, we produced *sllin5* mutant lines from the wild type and the *MdHT2.2*-transgenic line L5 using the CRISPR-Cas9 method. Special target sequences for CRISPR-Cas9 were designed at the 5′-end of the *SlLIN5* ORF. We thereby obtained lines with mutations of *sllin5*, and sequencing revealed that editing the target genes had produced frameshift mutants (Figure 7a). Two mutant lines for the wild type (WT-*sllin5*-1 and WT-*sllin5*-2) and L5 (L5-*sllin5*-1 and L5-*sllin5*-2) were employed to investigate CWINV activity and sugar concentration in fruit. In ripening fruit of WT-*sllin5*-1 and WT-*sllin5*-2, the activities of CWINV were less than 50% of that in the wild type, and the concentrations of Fru, Glc and SSC were significantly decreased;

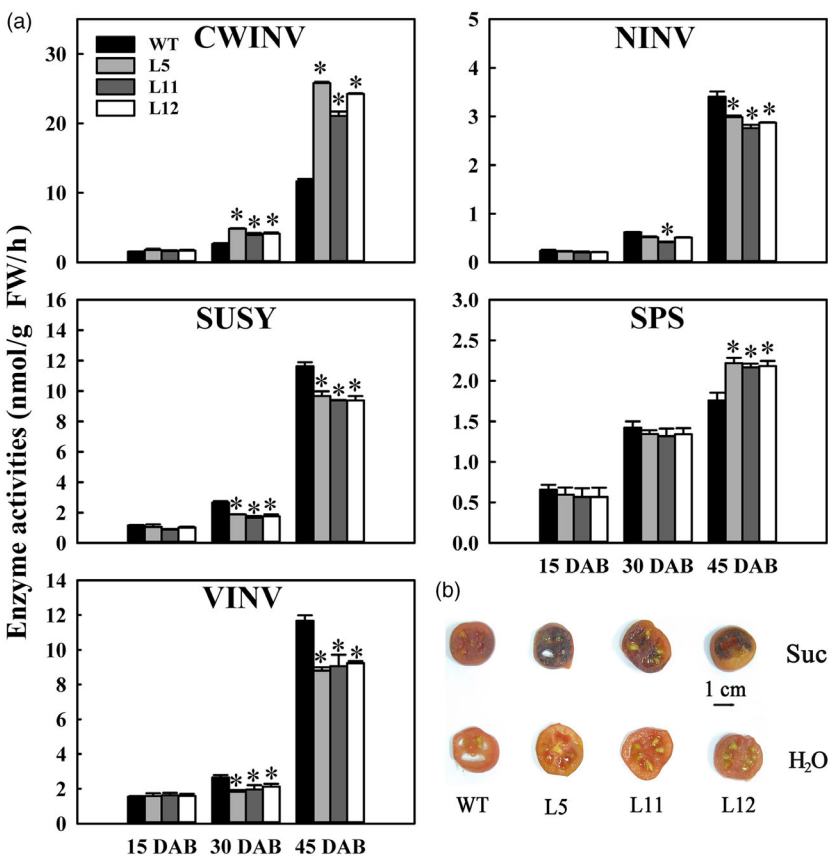

Figure 5 Assay of enzymes involved in sugar metabolism in tomato fruit. (a) Enzyme activity of CWINV (cell wall invertase), NINV (neutral invertase), SUSY (sucrose synthase), SPS (sucrose phosphate synthase) and VINV (vacuolar invertase) in tomato fruit at 15 DAB (young fruit), 30 DAB (breaker-stage fruit) and 45 DAB (ripening fruit). Bars of different colours represent different lines. Bars represent the mean value ± SE ($n \geq 4$). The asterisk indicates $P \leq 0.05$. (b) Acid invertase activity staining of ripening tomato fruit in 45 DAB (ripening fruit). Black bar = 1 cm.

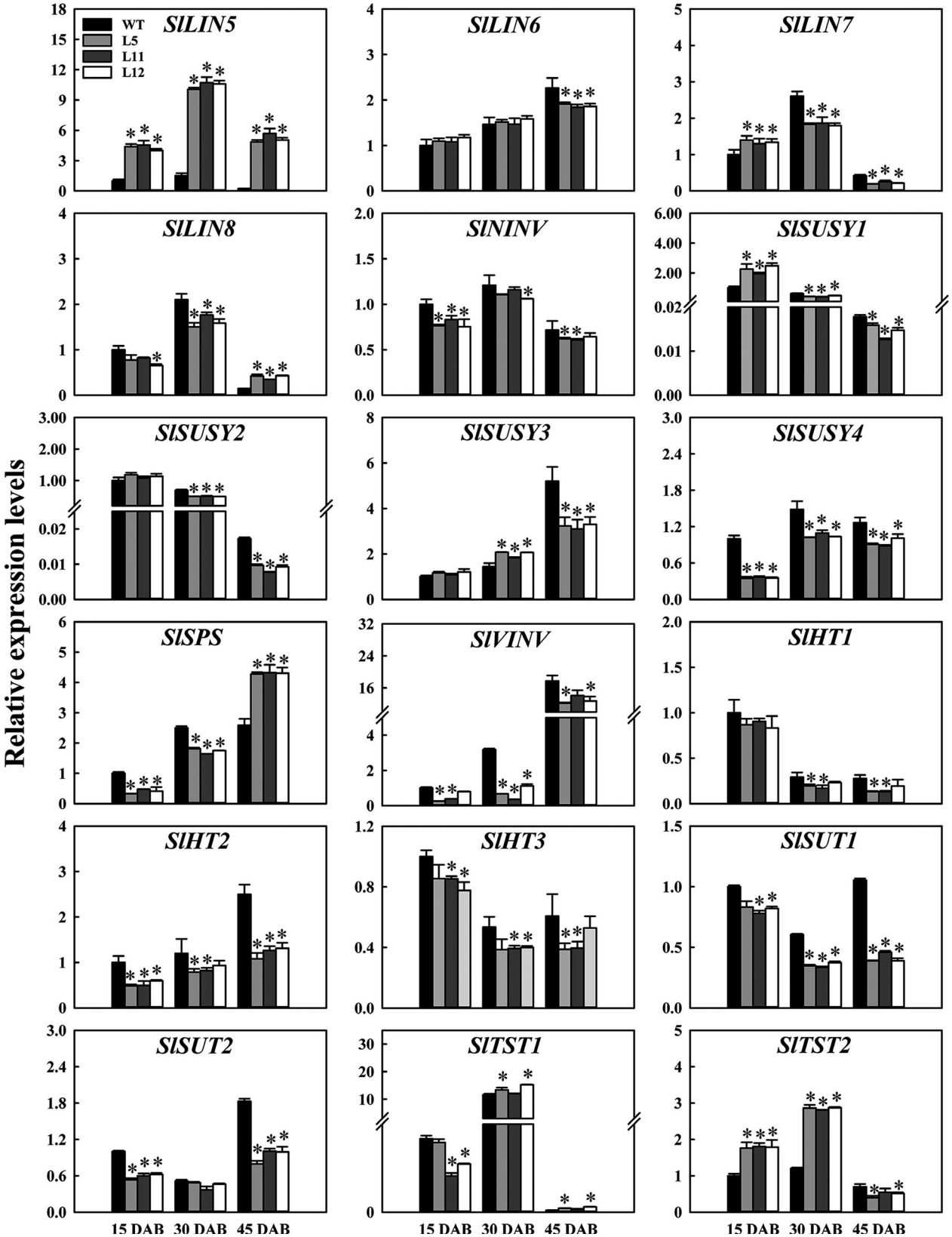

Figure 6 Relative expression levels of several enzymes and sugar transporter genes in the unloading pathway at different developmental stages of tomato fruit. Total RNA was isolated from fruit samples collected at 15 DAB (young fruit), 30 DAB (breaker-stage fruit) and 45 DAB (ripening fruit), and expression levels were calculated relative to that of *SlActin*. The expression of WT at 15 DAB was set to 1. Bars of different colours represent different lines. Bars represent the mean value ± SE ($n \geq 3$). The asterisk indicates $P \leq 0.05$.

however, the Suc concentration was nearly 1.5-fold that in the wild type (Figure 7b–d). In the fruit of L5-*sllin5*-1 and L5-*sllin5*-2, CWINV activities decreased to approximately 50% of that in L5 (as the parent), but the sugar concentration returned to that of the wild type, and the Suc concentration was slightly higher than that of the wild type (Figure 7b–d). To examine the reason CWINV activity in L5-*sllin5*-knockout plants had not decreased to that in WT-*sllin5*-knockout plants, the expression levels of other *CWINV* genes were detected. *SlLIN8* showed much more up-regulated expression than did transgenic line L5 (Figure S9). These results suggest that *SlLIN8* performs complementary functions for *SlLIN5* in tomato fruit overexpressing *MdHT2.2*.

Discussion

MdHT2.2, as a new hexose transporter, was involved in the unloading and uptake of fructose and glucose in apple fruit

Functional characterization of HTs has mostly been performed in model plants, for example, *Arabidopsis* and rice (Slewinski, 2011). Most of these transporters take up hexose from the apoplasm in source or sink tissues to meet the carbon requirements of specialized cell types such as guard cells, pollen grains and pollen tubes (Rottmann *et al*., 2016; Slewinski, 2011), though the hexose concentrations in these tissues/cells in physiological contexts are very low and rarely exceed 10 mM. However, little is known about HTs expressed in organs that accumulate hexose to high concentrations (more than 100 mM), such as sugar beet taproots (Jung *et al*., 2015) and fruit flesh (e.g. apple, Li *et al*., 2018; grape, Afoufa-Bastien *et al*., 2010; watermelon, Ren *et al*., 2018; tomato, McCurdy *et al*., 2010). The present study builds on our knowledge of HTs expressed in apple fruit (Wei *et al*., 2014), in which hexose accumulates to concentrations of up to 300 mM. In apple fruit, Suc and Sor enter the PCs via the apoplastic pathway after being released from the SE-CC complex (Zhang *et al*., 2004) and contribute to Suc import via hydrolysis into hexose by CWINV (Zhang *et al*., 2004; ;Li *et al*., 2012). As Sor transport is inhibited by high hexose concentrations (Gao *et al*., 2003), high HT activity is needed to move Glc and Fru from the cell wall spaces into PCs in apple fruit (Wei *et al*., 2014).

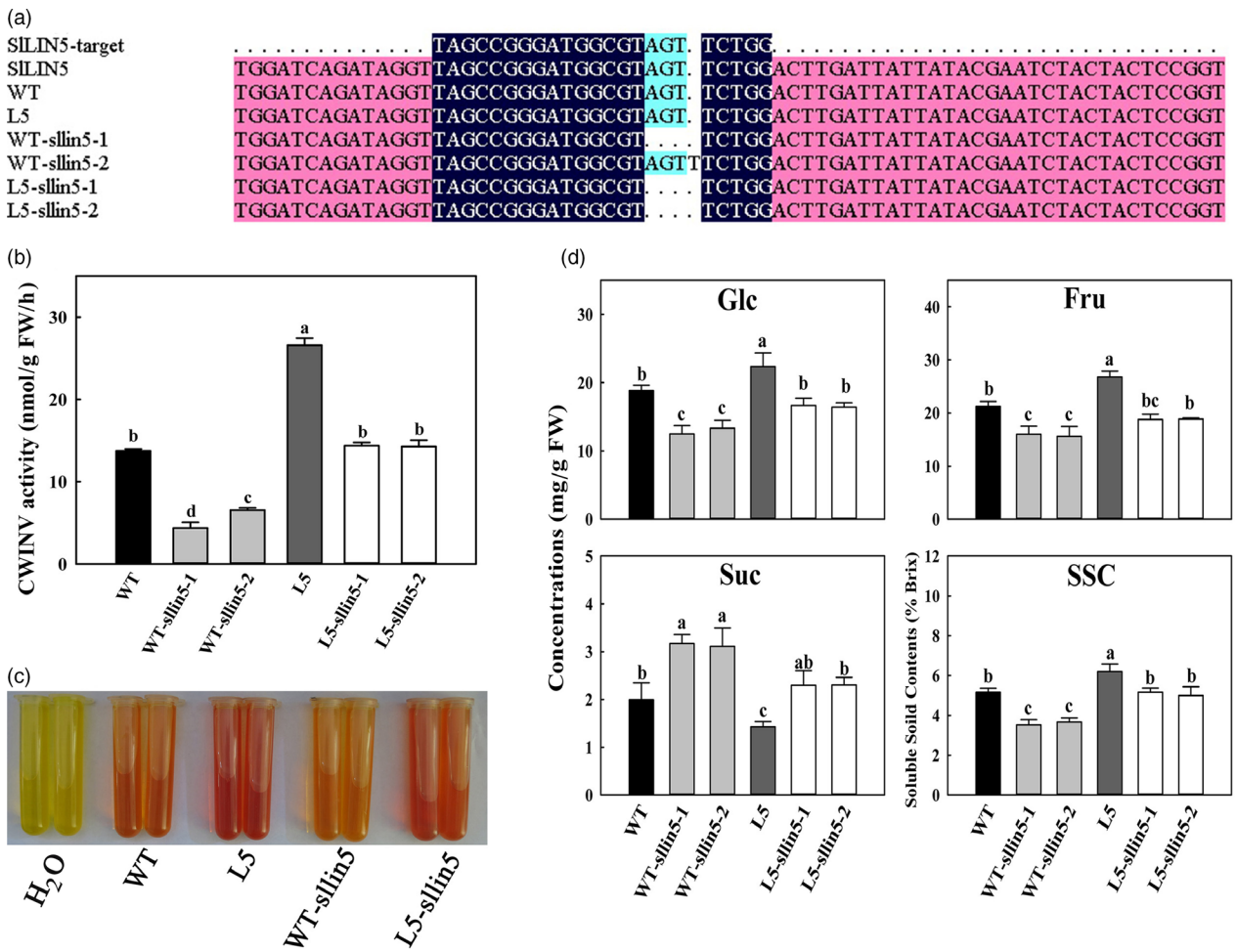

Figure 7 The sugar concentration and CWINV enzyme assay of *lin5*-knockout mutant lines. (a) The DNA sequence of the target region; *SlLIN5* was the target sequence chosen from CRISPR direct (http://crispr.dbcls.jp/) and used with the CRISPR-Cas9 system in this study. The *SlLIN5* sequence is the DNA sequence in the reference genome database of NCBI. Sequences were aligned using DNAMAN. The dark region is the target sequence, the blue region was the difference in sequence between those lines, the target sequences of WT and L5 were the same as the control, the edited lines WT-*sllin5*-1 and L5-*sllin5*-1/2 had 4 bases missing, and WT-*sllin5*-2 had 1 base insert. (b,c) CWINV activities of ripening fruit in different lines (WT, *MdHT2.2*-heterologous expression line L5, WT-*sllin5*-knockout lines, L5-*sllin5*-knockout lines). (d) glucose (Glc), fructose (Fru) and sucrose (Suc) concentrations and SSC (soluble solid content) in ripening fruit. Bars represent the mean value ± SE ($n \geq 4$). Different letters indicate significant differences at $P \leq 0.05$.

Phylogenetic analysis (Figure S1) demonstrated that MdHT2.2 shares high amino acid sequence identity with AtSTP14 in *Arabidopsis*, which is a galactose substrate-specific transporter located at the plasma membrane (Poschet *et al*., 2010). In our study, MdHT2.2-GFP and GFP-MdHT2.2 fusion proteins were localized to the plasma membrane in *Arabidopsis* cells, but the MdHT2.2 protein exhibited different substrate specificity for hexose in yeast. Similar results have been reported for TST homologous proteins from different plants with different substrate specificities (Jung *et al*., 2015; Ren *et al*., 2018). As shown for most apple fruit (Li *et al*., 2018), the soluble sugar content of the mature fruit of 'Gala' apple contains more than 50% Fru, followed by Suc, though much Fru leaks into the apoplastic space because of Suc apoplastic unloading, the high Fru concentration gradient or cell damage. The high transport activity of the MdHT2.2 protein for Fru supports the requirement that leaked Fru and Glc be efficiently transported into cells. The patterns for Fru and Glc were similar in transgenic tomatoes heterologously expressing *MdHT2.2*. These results indicate that MdHT2.2 is a new sugar/H^+ symporter with high transport activity for Fru in addition to Glc.

In apple fruit, the apoplastic unloading of Suc and Sor can be affected by the unloading activity of the fruit. In many species (e.g. mature tomato fruit) that employ apoplastic unloading for Suc in sink cells, Suc is mainly converted to Glc and Fru by CWINV in the cell wall space and then transported into PCs by HT. However, in apple fruit, the expression of *MdCWINV*s was lower in developing fruit than in shoot tips (Li *et al*., 2012), and we postulate that to avoid the inhibition of Sor uptake by Suc-derived Glc and Fru, most Suc is directly transported into PCs by plasma membrane-bound SUCs in apple fruit (Li *et al*., 2016, 2018) that partly derived Glc and Fru are transported into cells by high-activity MdHT2.2. During the late stage of fruit development, in addition to hexose derived from unloaded Suc, more Fru would passively leak from cells into the extracellular space and need to be taken up by the sugar transporter (Fillion *et al*., 1999), which is consistent with the higher level of *MdHT2.2* expression. MdHT2.2 in apple fruit mainly functions in both the unloading and uptake of Fru and Glc from the cell wall space.

Heterologous expression of *MdHT2.2* altered sugar concentration and metabolism in tomato fruit

In tomato fruit, three HT genes have been colocalized with QTLs for sugar accumulation (Prudent *et al*., 2011). This relationship was further verified by RNAi knockdowns of the three *SlHT*s (McCurdy *et al*., 2010), which are highly homologous to *MdHT2.2*. Although overexpressing or silencing in apple plants is the best way to identify the function of *MdHT2.2* in controlling the sugar content in fruit, apple transformation is very difficult, and there is a 4- to 6-year juvenile phase for fruit set. Therefore, we heterologously expressed *MdHT2.2* in tomato to determine its role in controlling sugar concentration in fruit. As expected, the concentrations of Fru and Glc in mature fruit increased significantly due to *MdHT2.2* overexpression, but the Suc concentration markedly decreased. This result was expected, as it was previously reported that the post-translational elevation of CWINV activity by silencing its inhibitor increased tomato fruit hexose levels and reduced Suc concentrations (Jin *et al*., 2009). Suc is involved in apoplasmic unloading in mature tomato fruit (Ruan and Patrick, 1995), and the Suc that is transferred from SE-CC complexes into the apoplastic space is mainly converted into hexose and then transported into PCs by the CWINV-HT system (Jin *et al*., 2009; Wang and Ruan, 2012). The overexpression of *MdHT2.2* increased the transport capacity for Fru and Glc from the apoplastic space into PCs, which led to a decrease in instantaneous sugar concentration in the apoplastic space. In transgenic tomato, CWINV, which is typically considered a sink-specific enzyme (Wan *et al*., 2018), was greatly increased with up-regulated expression of *SlLIN5*, a key gene encoding a CWINV protein in tomato fruit (Jin *et al*., 2009), and *SlLIN8* expression was also obviously increased in ripening fruit (Figure 6). As a result, more Suc was converted to Fru and Glc by the increased CWINV activity in the cell wall space, and more hexose molecules were transported by MdHT2.2 into cells and accumulated in the transgenic tomato fruit; in contrast, less Suc was moved into PCs by SUT. The decreased expression of *SlSUT1* and *SlSUT2*, two key Suc transporters involved in Suc unloading into PCs (Milne *et al*., 2018) that can be induced by increasing the Suc concentration (Milne *et al*., 2018), is consistent with the observed decreased concentration of Suc in the apoplastic space of the transgenic tomato fruit. This was further verified by the decreased gene expression and enzyme activities of SUSY and NINV (Figures 5 and 6), both of which regulate sugar metabolic homeostasis and are induced by Suc (Li *et al*., 2018; Nguyen-Quoc and Foyer, 2001). Overall, these results suggest that the alteration in sugar concentration (increased hexose and decreased Suc) in the mature fruit of tomato heterologously expressing *MdHT2.2* can be mainly attributed to up-regulated *SlLIN5* expression and increased CWINV activity. This interpretation was confirmed by the fact that in the *sllin5* mutants in the *MdHT2.2*-transgenic background produced by the CRISPR-Cas9 method, the concentrations of Fru and Glc were restored almost to the wild-type levels, and the Suc concentration was slightly higher than that in the wild type; however, the CWINV activities in the L5-*sllin5* mutant lines were reduced to the level in the wild type. For these *sllin5* mutant lines in the *MdHT2.2* background, greater up-regulation of *SlLIN8* expression would be the main reason CWINV activity decreased to that of the WT-*sllin5* knockout.

Nonetheless, Fru and Glc concentrations did not increase in the leaves and young fruit of *MdHT2.2*-transgenic tomato when compared to wild-type tomato but rather decreased slightly, even though Suc levels were significantly increased. Photosynthesis was unchanged in the mature leaves of *MdHT2.2*-transgenic tomato, but the enzyme activities of CWINV and NINV, both of which cleave Suc to hexose (Nguyen-Quoc and Foyer, 2001), were significantly decreased, and the expression levels of *SlLIN5/8*, *SlNINV* and *SlSUSY1/4* were reduced. Therefore, the increased Suc concentration in the leaves of transformed plants might be partly due to decreased Suc decomposition. *MdHT2.2* should be expressed in all living cells of these transgenic plants, including phloem SE-CC cells and leaves, because expression was driven by the constitutive CaMV35S promoter (Gittins *et al*., 2001). Decreased CWINV activities would decrease the hexose content in the apoplastic space around the phloem SE-CC complexes of mature leaves, which would decrease the amount of hexose in phloem as much as possible because Fru and Glc cannot be transported in the phloem (Liu *et al*., 2016). Interestingly, as an adaptive response to extracellular hexose signalling, the pathway regulating *SlLIN5* expression would differ in leaves and mature fruit in tomato.

In the early stages of tomato fruit development, Suc moves from the SE-CC complex directly to surrounding PCs via plasmodesmata; in mature leaves, Suc is loaded via the symplastic pathway (Ruan and Patrick, 1995). The increased Suc

concentration in mature leaves should be the main reason the Suc concentration was higher in the young fruit of *MdHT2.2*-expressing plants than in wild-type plants because long-range Suc transport in the symplastic system is determined by its chemical gradient from source to sink (Comtet *et al*., 2017; Patrick, 1997). Additionally, up-regulated *SlTST2* expression (as in leaves) also enhanced Suc accumulation in the vacuole of young transgenic tomato fruit, but its expression decreased in mature fruit. These results indicate that the different changes in sugars between young and mature fruit of *MdHT2.2*-expressing tomato might be related to the unloading pathway of Suc and the system sensing the extracellular hexose signal.

In summary, *MdHT2.2* mainly functions in efficiently taking up Glc or Fru from the cell wall space and moving these sugars into cells in apple fruit. In mature apple fruit, hexose (especially Fru) accumulates to concentrations of up to 300 mM, and Suc is unloaded via the apoplastic pathway (Zhang and Turgeon, 2009). A high level of *MdHT2.2* expression in mature fruit meets the requirement for transferring Fru and Glc from cell wall spaces into PCs. Although we did not directly confirm the function of *MdHT2.2* at the genetic level in apple plants, the fact that heterologous expression increased fruit size and Fru and Glc contents in mature tomato fruit demonstrates its role in hexose unloading in fruit. Interestingly, the overexpression of *MdHT2.2* decreased relative hexose levels in the apoplastic space of mature tomato fruit, up-regulating the expression of *SlLIN5/8* and the activity of CWINV but decreasing the Suc concentration. This result offers new insight into the regulatory mechanism by which apoplastic hexose signalling regulated by HT controls Suc partitioning and transport and the sugar content in fruit through the apoplastic unloading pathway of Suc. Conversely, in mature leaves where Suc is mainly loaded via the symplastic pathway, CWINV activity and *SlLIN5/8* expression decreased with *MdHT2.2* overexpression, suggesting that the response pathway of the apoplastic hexose signal differs between tomato leaves and mature fruit. This mechanism will be better understood when the factors regulating *SlLIN5* expression and how apoplastic hexose signals sense and transmit in different plant tissues are verified. In addition to having a high sugar content and large fruit size, transgenic tomatoes heterologously expressing *MdHT2.2* also showed other advantageous traits, such as early flowering, dwarfness, thinned pericarp, and effective pollination and fertilization. These results suggest that *MdHT2.2* is a potential candidate gene for use in improving fruit quality.

Materials and methods

Plant material

The samples of 'Gala' apple (*M. domestica*) were the same as those used in our previous report (Wei *et al*., 2014). At 0, 15, 30, 47, 61, 75, 92, 104 and 120 DAB, fruits were sampled between 3:00 PM and 4:00 PM. On each collection date, six apples per replicate were harvested from three trees, with a total of five replicates. The fruits were immediately weighed, cut into small pieces after removing the core and frozen on-site in liquid nitrogen; the pedicel, peel and flesh were sampled at 120 DAB. Additionally, flowers, young leaves, mature leaves, old leaves and roots were collected. All frozen samples were stored at −80 °C.

Fresh tissues from the fruit of 'Gala' apple (104 DAB) were collected using a 1.0-cm-diameter cork borer and cut into discs of 0.3 cm thick. As described by Hancock *et al*. (2003), fresh samples were preincubated in 20 mL of buffer containing 2% sugar, 20 mM MES (pH 5.5), 5 mM $MgCl_2$, 2 mM KCl, 1 mM $CaCl_2$ and 1 mM $CaSO_4$. The sugar sources were Sor, Glc, Fru and Suc, with ddH_2O as the negative control. Following incubation for 6 h on a rotary shaker (100 r.p.m.) at 25 °C, samples were washed with sterile water and surface-dried on filter paper, followed by immersion in liquid nitrogen for later investigation of *MdHT2.2* expression.

Tomato (*S. lycopersicum* cv. Micro-Tom) was grown in a culture room at 70% relative humidity with 16 h of light at 25 °C and 8 h of darkness at 22 °C. The flowers were tagged at full bloom to determine fruit age. Fruits were sampled in this study at 15 (young fruit), 35 (breaker-stage fruit) and 45 (mature fruit) DAB.

Cloning of *MdHT2.2*

The *MdHT2.2* sequence (MD15G1193400/MDP0000154362) was retrieved from the *Malus* Genome Database (http://www.rosaceae.org). Total RNA was extracted from mature fruits of 'Gala' apple, and cDNA was synthesized using PrimeScript™ II Reverse Transcriptase (Takara, Dalian,China). *MdHT2.2* was cloned using specific primers, and the cDNA was obtained as the template (Table S2).

Protoplast isolation and subcellular location

Protoplasts were obtained from *Arabidopsis* cell suspension cultures as described by Schirawski *et al*. (2000). The protoplasts of *Arabidopsis thaliana* suspension cells were centrifuged at 80 ***g*** for 5 min, resuspended and digested by pectolyase and cellulase, filtered through a sterilized 40-mm stainless steel sieve, washed several times with medium B and diluted. *MdHT2.2* was cloned into pGWB405 and pGWB406 vectors with C-terminal GFP and N-terminal GFP, respectively. Both of these vectors carry a kanamycin resistance gene and the CaMV35S promoter. The recombinant plasmids were purified using a Qiagen Midi Kit (Qiagen, Hilden, Germany). For transfection, 7.5 μg of plasmid was used per 2.5×10^5 protoplasts. The plasmid was immediately mixed twice with the protoplasts and PEG-containing solution. The mixture was left for 10 min at room temperature, after which the protoplasts were incubated in 4.5 mL of culture medium in 6-well plates in the dark at 24 °C and 70% humidity. GFP fluorescence was visualized by CLSM.

Heterologous expression of *MdHT2.2* in yeast

MdHT2.2-ORF was inserted into the pYST2.0 vector, which carries the ampicillin-resistance gene, and transferred into the sugar transporter-deficient yeast strain EBY.VW4000 (Wieczorke *et al*., 1999). The growth of EBY.VW4000 expressing *MdHT2.2* was assessed on culture media containing 2% concentrations of different sugars; controls were transformed with the vector carrying *MdHT2.2* in the antisense orientation. For uptake experiments, ^{14}C-labelled sugars were used. A single clone of EBY.VW4000 containing the *MdHT2.2* gene or empty vector was precultured on maltose–amino acid medium [0.67% (w/v) yeast nitrogen base, 1% (w/v) casamino acids, 0.002% (w/v) tryptophan and 2% (w/v) maltose] to an A_{600} of 0.8, and transport tests were performed as described by McCurdy *et al*. (2010). For inhibitor assays, a final concentration of 50 μM CCCP (as a proton uncoupler) was added to yeast cells 30 s before the addition of [^{14}C] Fru.

Tomato plant transformation

MdHT2.2-ORF was inserted into the gateway vector pGWB402 carrying the CaMV35S promoter and kanamycin resistance, and

the recombinant plasmid was transformed into EHA105 competent cells for plant transformation. The transformation methods for Micro-Tom tomato are according to Sun *et al*. (2006). Tomato plants were grown at 25 °C, and the 16-h photoperiod was supplemented with lamps at 120 μmol/m^2/s. The transgenic lines were screened by kanamycin resistance and PCR analysis, and the homozygous lines were confirmed without character segregation until the T_3 generation.

For the CRISPR-Cas9 experiment, the target sequence (TAGCCGGGATGGCGTAGTTCTGG) for *SlLIN5* was designed using CRISPR direct (http://crispr.dbcls.jp/), and the sequence specificity was confirmed by BLAST in NCBI. After checking the specificity in Micro-Tom tomato, the target sequence was synthesized and cloned into the pHSE401 vector as described by Xing *et al*. (2014). After transforming the plasmid into EHA105, the transgenic lines were screened by hygromycin resistance and sequencing, and the T_2 generation was used in this study.

Enzyme assay and expression analysis

The exact methods described by Li *et al*. (2012) were used to extract and determine the activities of CWINV, NINV, VINV, SUSY and SPS in tomato fruit samples in this study. Soluble proteins were measured using Coomassie blue, and enzyme activities were expressed on a protein basis. Staining for tomato fruit acid invertase was performed according to Wang and Ruan (2012).

Total RNA was isolated from frozen samples, and cDNA was synthesized using PrimeScript™ II Reverse Transcriptase (Takara, Dalian,China). Gene-specific primers were checked in NCBI and examined by RT-PCR and melting curve analysis. PCR products were quantified using a LightCycler® 96 real-time PCR detection system (Roche, Basel, Switzerland) with LightCycler Ultra SYBR Mixture (CWBIO, Beijing, China). *MdActin* for apple and *LeActin* for tomato were used for the normalization of target gene transcripts using the $2^{-\Delta\Delta C_t}$ method. The primers are listed in Table S2.

Protein detection and protein blot analysis

Protein measurements were conducted according to previous protocols (Yang *et al*., 2018). Total protein concentrations were determined with protein assay kits (Bio-Rad, California, USA) using bovine serum albumin as a standard. Specific monoclonal antibodies were raised against a peptide (NVARAIKNPFRNLLC) of MdHT2.2 protein, and this antibody was raised in rabbits (GenScript, Nanjing, China). *Actin* was monitored using a monoclonal antibody (CWBIO). The procedures for Western blotting followed Sun *et al*. (2018). The antigen–antibody complexes were detected using Clarity™ Western ECL Substrate (Bio-Rad) according to the manufacturer's instructions.

Sugar and starch concentration measurement

As previously described (Li *et al*., 2018; Yang *et al*., 2018), soluble sugars and hexose phosphates were extracted in 75% methanol with ribitol added as an internal standard and then derivatized sequentially with methoxyamine hydrochloride and N-methyl-N-trimethylsilyl-trifluoroacetamide. After derivatization, the metabolites were analysed using a Shimadzu GCMS-2010SE (Shimadzu Corporation, Tokyo, Japan) with a DB-5MS capillary column (20 m × 0.18 mm × 0.18 μm) and a 5-m Duraguard column (Agilent Technology, California, USA). The residue after 75% methanol extraction for GC-MS analysis was re-extracted three times with 80% (v/v) ethanol at 80 °C, and the pellet was retained for the enzymatic determination of starch as Glc equivalents.

Statistical analysis

All data were analysed using IBM SPSS Statistics 21 (IBM Corp., Chicago, USA) and graphed with Sigma Plot 12.0 software (Systat software, California, USA). Data were analysed using independent *t*-tests at a significance level of $P \leq 0.05$. Values are presented as the means ± standard error (SE) in at least biological triplicate for each measurement.

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Conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

M.L. and F.M. conceived and supervised this study; M.L., Z.W. and L.C. designed the experiments; Z.W., X.W., J.Y., H.L., K.Z., Y.Z. and B.M. performed the experiments; Z.W. and M.L. wrote the original draft; and all authors reviewed and edited the manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Phylogenetic tree analysis and transmembrane structure prediction of MdHT2.2.

Figure S2 Changes in glucose (Glc), fructose (Fru) and sucrose (Suc) concentrations at different developmental stages of apple fruit; samples were compared to 0 days after bloom (DAB).

Figure S3 Relative expression levels of *MdHT2.2* in enriched parenchymal cells, the petal and sepal bundle vascular region of ripening fruit.

Figure S4 Phenotype and identification of transgenic tomato plants.

Figure S5 Malic acid and citric acid concentrations in tomato fruit at 15 DAB (young fruit), 30 DAB (breaker-stage fruit), and 45 DAB (ripening fruit).

Figure S6 Changes in glucose (Glc), fructose (Fru), sucrose (Suc), starch, malic acid and citric acid concentrations in mature leaves of *MdHT2.2*-expressing tomato.

Figure S7 Enzyme assay of CWINV (cell wall invertase), NINV (neutral invertase), SUSY (sucrose synthase), SPS (sucrose phosphate synthase) and VINV (vacuolar invertase) in mature tomato leaves.

Figure S8 Relative expression levels of several enzymes and sugar transporter genes in mature tomato leaves.

Figure S9 Relative expression levels of *SlLIN6*, *SlLIN7* and *SlLIN8* in ripening tomato fruit of *lin5*-knockout mutant lines.

Table S1 Correlation analysis between *MdHT2.2* expression level and sugar concentrations.

Table S2 Primers used in this study.