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Sugar transporters PpSWEET9a and PpSWEET14 synergistically mediate peach sucrose allocation from source leaves to fruit

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Sugar content is a critical indicator of fruit quality and is mainly controlled by sugar transporters. Sugars will eventually be exported transporters (SWEET) proteins play an indispensable role in sugar allocation between and within plant organs. Sucrose is the major sugar in many fruits and the predominant form of sugar translocated in peach (*Prunus persica*). However, the role of the multiple peach *SWEET* genes in sucrose allocation to fruit remains elusive. In this study, a total of 19 *SWEET* candidates have been identified in the peach genome, and two Clade III SWEET genes, *PpSWEET9a* and *PpSWEET14*, are found to be highly expressed in mature source leaves and branches. Complementation assays, transgene manipulations, and protein interaction studies reveal that PpSWEET9a and PpSWEET14 serve as sucrose efflux proteins and form a heterooligomer that synergistically directs sucrose allocation from source leaves to fruits. Our findings provide insights into the effect of *SWEETs* on sugar accumulation in peach fruit and identify genetic candidates for improving fruit quality.

Sugars not only provide energy and metabolic substrates for growth and development but also serve as osmotic and signaling molecules in response to (a)biotic stresses in plants[1]. In fresh fruit, the soluble sugars, including sucrose, fructose, and glucose, are also central to fruit quality because of their nutritional value and sweetness. The sugar profile (composition and concentration) and balance with organic acids largely determine fruit taste and flavor, which is a major quality indicator. The profile of sugars at fruit maturity mainly depends on their allocation during fruit development and understanding factors that affect this process will assist in the improvement of fruit quality[2].

Sugar is the primary product of photosynthesis and is mainly present as either sucrose in the cytosol or starch in the chloroplast. Sucrose is the predominant transported form of sugar in many plants, although sorbitol plays this role in some members of the Rosaceae family such as apple and pear. Sugars are usually translocated from a source (such as mature leaves) to sink (fruits, seeds, roots, stems, and young leaves) organs by long-distance transport in the phloem[3]. Source loading and sink unloading are two of the pivotal processes for long-distance sucrose translocation and are mediated mainly by SUTs (Sucrose Transporters) and SWEETs (Sugar Will Eventually be Exported Transporters)[4,5]. In source leaves, sucrose is derived from mesophyll cells and imported to sieve element/companion cell (SE/CC) complexes via a symplastic pathway through plasmodesmata, along the gradient of sucrose concentration and the transporters-mediated apoplasmic pathway[6]. SUTs are phloem-specific proteins that act as the symporters of sucrose and H^+ and are mainly responsible for the sucrose uptake from the cell wall space into SE/CC complexes[7,8]. However, sucrose in phloem parenchyma cells (PCs) needs first to be exported to the apoplasmic space via SWEETs prior to loading[9,10]. Symplasmic and apoplasmic pathways for sucrose unloading from SE-CC complexes to PCs occur in sink tissues. In shoot tips and roots, sucrose moves via plasmodesmata-mediated gradient diffusion in the symplasmic path. However, in the apoplasmic pathway, sucrose is released across the plasma membrane of phloem sieves or SE/CC complexes into the apoplast via simple diffusion facilitated by SWEETs[11], and sucrose is transported into PCs by SUT. Sucrose may then be cleaved into glucose and fructose by cell-wall invertase (CWIN) and transported for vacuolar storage mediated by hexose transporters[3]. Thus, SWEETs may act as the central players in mediating sucrose allocation in plants.

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SWEETs are evolutionarily conserved and exist in a wide variety of organisms. SWEET proteins possess a characteristic MtN3/saliva domain, also named the PQ-loop repeat, which constitutes three α-helical transmembrane domains (3-TMs). Eukaryotic SWEETs comprise a tandem repeat of the basic 3-TM unit spaced by a single transmembrane domain, constituting a 3-1-3 TM structure[12]. In plants, SWEETs function as bidirectional uniporters and mediate the translocation of sugars across cell membranes along the substrate concentration gradient. This feature provides an energy-efficient mechanism for loading and unloading sugar[13]. Based on amino acid sequences, SWEETs are divided into four clades. Clade I members (such as AtSWEET1) and II (AtSWEET5, OsSWEET5) mainly transport glucose or galactose, Clade IV (AtSWEET17) could specifically transport fructose, while Clade III appears to transport sucrose and mediate its loading and unloading[14–16]. In Arabidopsis, for example, AtSWEET11 and AtSWEET12 from clade III are specifically expressed in phloem PCs proximal to the SE-CC complexes in leaves and mediate the efflux of sucrose from phloem PCs into the apoplasmic space for its loading and long-distance translocation[9]. Similar functions were observed for ZmSWEET13a, ZmSWEET13b, and ZmSWEET13c in maize[17]. Furthermore, SWEETs from clade III involved in sucrose unloading, such as tomato SlSWEET15, pear PuSWEET15, and Arabidopsis AtSWEET15 increase fruit sucrose content and support the development of fruits and seeds[5,18,19].

Peach (*Prunus persica*) belongs to the Rosaceae family and is an important fruit worldwide. Sucrose dominates the sugar transport and accumulation in peach fruit and directly contributes to the sweetness. Moreover, sucrose metabolism yields hexoses (glucose and fructose) necessary to generate energy and synthesize cellulose, fructan, proteins, and antioxidant compounds. These processes would further affect the flavor (such as sugar profile), texture (cellulose and fructan), and shelf life (antioxidants) of fruit[2,20]. Thus, understanding the mechanism of sucrose allocation in peach fruit is of significance for the regulation and preservation of peach fruit. However, there is little information about SWEETs-mediated sucrose translocation and accumulation in peach fruit. In this study, we performed a genome-wide identification of SWEETs and screened two Clade III SWEET genes, *PpSWEET9a* and *PpSWEET14*, which are preferentially expressed in mature source leaves and branches and at lower levels in fruits and juvenile leaves, and therefore potentially mediating sucrose loading from source leaves. Moreover, complementation assays, transgenes, and protein interaction analysis indicated that both PpSWEET9a and PpSWEET14 served as sucrose efflux proteins and synergistically mediate sucrose translocation from source leaves to fruits as heterooligomers. Our findings not only aid our better understanding of the effect of SWEETs on the sugar accumulation of fruit but also offer genetic candidates for peach fruit improvement.

Results

The SWEET genes in the peach genome

Based on the complete sequencing of the peach genome[21], we screened 19 SWEET candidates that have conserved 3-TMs using MtN3_slv domain sequence (Fig. 1a). Then, 17 SWEET protein sequences from Arabidopsis were used as the reference for phylogenetic analysis of peach SWEETs. These 19 candidates were divided into four clades and named based on their sequence similarity and the number of TM[22]. Proteins with fewer than seven standard TMs were named as SemiSWEETs but considered as SWEET candidates here[22]. Because SemiSWEETs would not form a functional pore alone but function by forming dimeric complexes during sugar transport[13]. Clade I consisted six genes (Prupe.2G307800 [PpSemiSWEET3a], Prupe.2G307700 [PpSemiSWEET3b], Prupe.8G017400 [PpSWEET1], Prupe.8G017500 [PpSemiSWEET1], Prupe.4G072300 [PpSWEET2a], and Prupe.4G155700 [PpSWEET2b]). There were five genes in Clade II (Prupe.5G125100 [PpSWEET7], Prupe.3G034900 [PpSWEET5b], Prupe.8G076100 [PpSWEET5c], Prupe.5G175500 [PpSWEET5a], and Prupe.3G283400 [PpSWEET4]), six genes in Clade III (Prupe.5G146400 [PpSWEET14], Prupe.5G146500 [PpSWEET10], Prupe.1G220700 [PpSWEET15], Prupe.8G253500 [PpSWEET9a], Prupe.2G245600 [PpSemiSWEET9], and Prupe.6G355900 [PpSWEET9b]), and two genes in Clade IV (Prupe.2G118600 [PpSWEET16a] and Prupe.1G133300 [PpSWEET16b]) (Fig. 1a). Genomic structural analysis showed six exons in the coding sequence of 15 candidates, and there were four exons in *PpSemiSWEET1* and *PpSemiSWEET3a*, and five exons in *PpSWEET5c* and *PpSWEET7* (Fig. 1b). The deduced amino acids of these 19 proteins ranged from 137 to 299 in length. The prediction of transmembrane domain revealed four proteins with less than seven TMs, i.e., PpSemiSWEET1 (three TM), PpSemiSWEET3a (four TM), PpSemiSWEET3b (four TM), and PpSemiSWEET9 (six TM) (Fig. 1b).

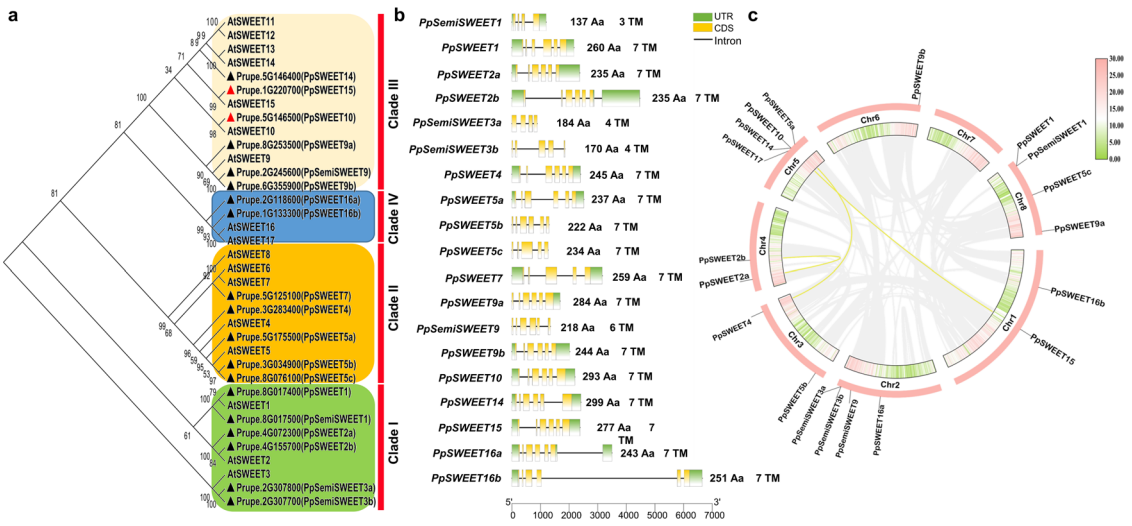

Fig. 1 | Genome-wide identification of SWEETs in peach. a Phylogenetic tree of the amino-acid sequences of 19 *SWEET* candidates in peach and 17 *AtSWEET* genes in Arabidopsis. All the SWEET genes are divided into four clades (I-IV), and numbers near branches represent bootstrap values. **b** Schematic representation of the exon-intron composition in 19 peach *SWEET* candidates. Gene names and predicted numbers of amino acid (Aa) and transmembrane domain (TM) are indicated on the left and right, respectively. **c** Chromosomal position and collinearity relationship of the 19 *SWEET* candidates in the peach genome. The duplication pairs are connected by the pink line. The chromosome number and size scale (million base pairs, Mb) are shown inside the circle, and gene density is indicated outside. The scale bar in the upright indicates gene density.

Two peach *SWEET* candidates were located on chromosomes (Chr) 1 (*PpSWEET16b* and *PpSWEET15*), four on Chr 2 (*PpSWEET16a*, *PpSemiSWEET9*, *PpSemiSWEET3a*, and *PpSemiSWEET3b*), two on Chr3 (*PpSWEET5b* and *PpSWEET4*), two on Chr4 (*PpSWEET2a* and *PpSWEET2b*), four on Chr5 (*PpSWEET7*, *PpSWEET14*, *PpSWEET10*, and *PpSWEET5a*), one on Chr6 (*PpSWEET9b*), and four on Chr8 (*PpSWEET1*, *PpSemiSWEET1*, *PpSWEET5c*, and *PpSWEET9a*). Moreover, gene collinearity analysis revealed three rigor duplication gene pairs, i.e., *PpSWEET2a-PpSWEET2b*, *PpSWEET4-PpSWEET5a*, and *PpSWEET14-PpSWEET15*. Besides, three gene pairs are arranged in a cascade style, i.e., *PpSemiSWEET1-PpSWEET1*, *PpSemiSWEET3a-PpSemiSWEET3b*, and *PpSWEET10-PpSWEET14* (Fig. 1c).

Preliminary activity screening of Clade III *SWEET* genes in sucrose efflux from leaves to fruit

Considering the predominant role of sucrose in contributing to the flavor, texture, and shelf life of fruit[2,20] and the vital role of Clade III SWEETs in sucrose transport, we evaluated the potential of six Clade III genes (i.e., *PpSWEET9a*, *PpSemiSWEET9*, *PpSWEET9b*, *PpSWEET10*, *PpSWEET14*, and *PpSWEET15*) in peach sucrose allocation by analyzing spatial expression profile in juvenile leaves, mature leaves, red mature fruits and branches at the same time-point (Fig. 2a). Interestingly, the lowest expression level for all six genes was observed in fruits relative to other tissues, suggesting they preferentially allocate sucrose from other tissues to fruit. With the exceptions of *PpSWEET9b* and *PpSWEET15*, the remaining four genes were preferentially expressed at the highest levels in the mature leaves and branches and relatively lower in the sink tissues, i.e., juvenile leaves and fruits. Moreover, the expression difference of *PpSWEET9a* and *PpSWEET14* between source and sink tissues was more than 2-fold. By contrast, there was less than a 1.5-fold difference observed for *PpSWEET9a* between source and sink tissues, as well as *PpSWEET10* between juvenile and mature leaves (Fig. 2a). Then, we analyzed the cis-elements in the promoters of *PpSWEET9a* and *PpSWEET14* and this analysis identified enrichment of putative light responsiveness elements, with five out of 15 cis-elements in *ProPpSWEET9a*, and 11 out of 22 in *ProPpSWEET14* (Fig. 2b). RT-qPCR analysis revealed a similar pattern of light responsiveness for both genes. The expression was highly induced by 0.5 h of light exposure and, however, no significant expression increment was observed in the leaves treated with 1 h of light exposure relative to the treatment of 0.5 h (Fig. 2c). Light is essential for leaf photosynthesis and sugar production, which may, in turn, induce the expression of Clade III *SWEETs* for sucrose loading and sugar allocation. Thus, *PpSWEET9a* and *PpSWEET14* genes might have a specific role in sucrose efflux from source leaves into fruit in peach.

PpSWEET9a and PpSWEET14 are located on the plasma membrane and transport sucrose

Sequence alignment revealed standard seven TM structures in pSWEET9a and PpSWEET14, indicating they are plasma membrane proteins with capacity of transporting sucrose alone (Fig. 2d). Subcellular localization analysis showed that both pSWEET9a-YFP and PpSWEET14-YFP signals were located at the periphery of epidermal cells and overlapped clearly with the plasma membrane marker PM-RK-mCherry[23], suggesting their localization at the plasma membrane. However, the YFP signal of the empty vector was dispersed throughout the epidermal cell, including the cell membrane and nucleus (Fig. 3). To examine whether PpSWEET9a and PpSWEET14 could transport sucrose, they were expressed in the sucrose transport-deficient yeast strain SUSY7/*ura3*, which is unable to grow efficiently on medium with sucrose as the sole carbon source[24]. Drop tests showed that all transformants grew well on medium with 2% glucose. However, PpSWEET9a, PpSWEET14, and GhSWEET12, but not empty vector (pDR196), transformants grew well on the medium with 2% sucrose as the sole carbon source (Fig. 4a). GhSWEET12 has been characterized as a sucrose transporter from Clade III in cotton[25]. Thus, both PpSWEET9a and PpSWEET14 are likely to function as a sucrose membrane transporter.

The Arabidopsis *AtSWEET11* and *AtSWEET12* are specifically expressed in phloem PCs proximal to the SE-CC in leaves and contribute to

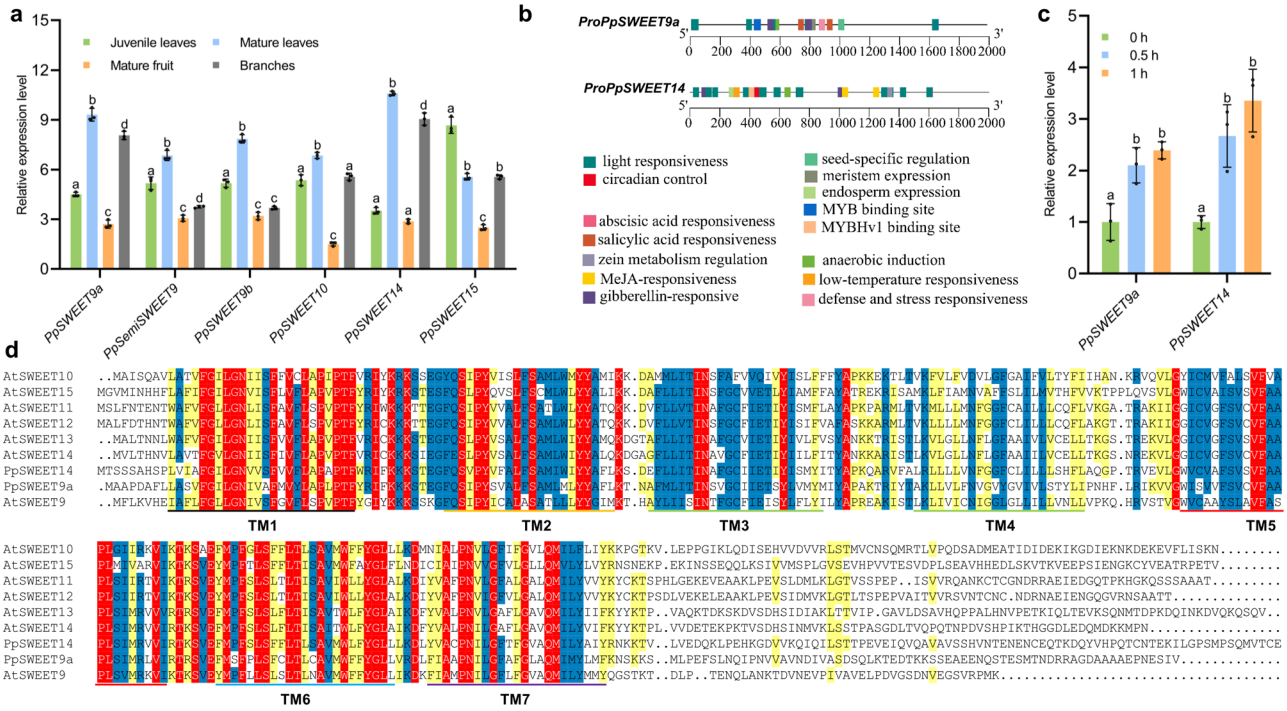

Fig. 2 | Preliminary screen of peach Clade III *SWEET*s for a role in sucrose efflux from leaves to fruit. a Spatial expression pattern of peach Clade III *SWEET*s in juvenile leaves, mature leaves, transporting branches, and mature fruit. **b** Schematic representation of *cis*-element composition in the promoter of *PpSWEET9a* and *PpSWEET14*. **c** Expression pattern of *PpSWEET9a* and *PpSWEET14* in response to 0, 0.5, and 1 h of light exposure after a 24-h-dark treatment in mature leaves. **d** Sequence alignment of PpSWEET9a and PpSWEET14 and Arabidopsis Clade III *SWEET* proteins. Data are means ± standard deviation ($n = 3$). Values followed by different letters are significantly different ($P < 0.05$).

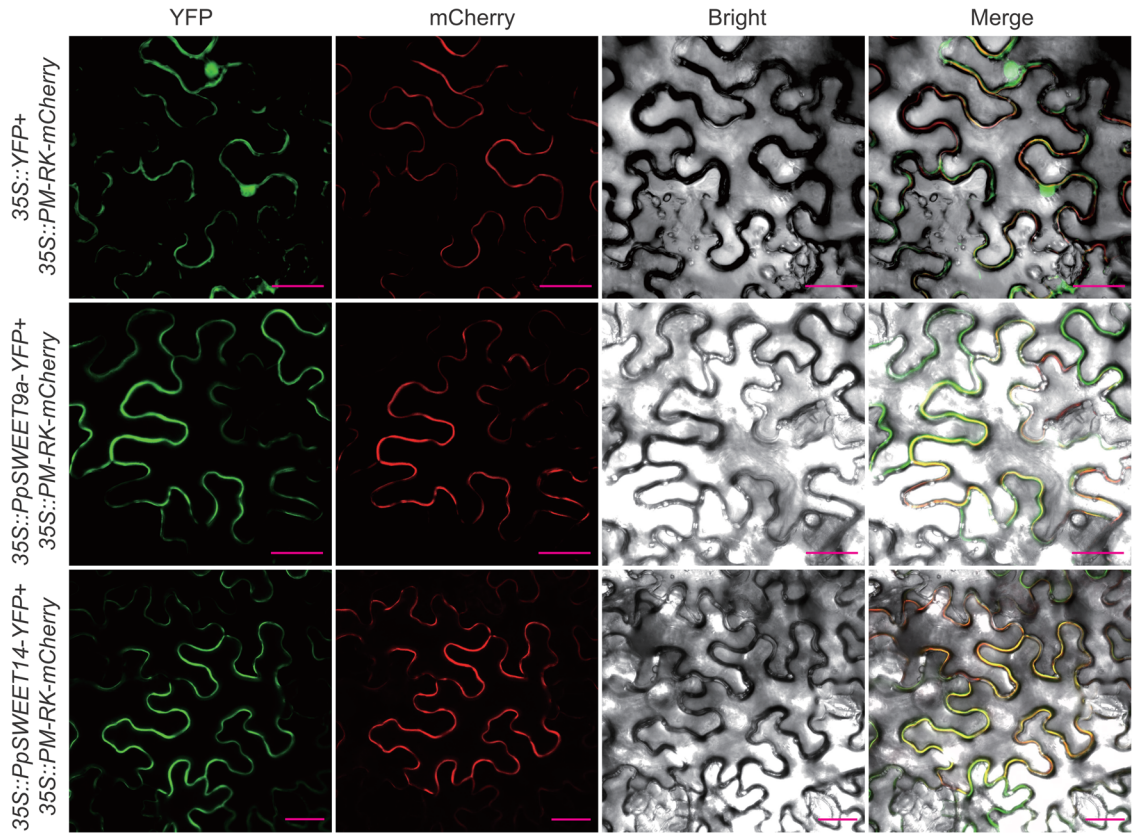

Fig. 3 | Subcellular localization of PpSWEET9a and PpSWEET14 in *Nicotiana benthamiana* leaves. Co-localization of YFP or PpSWEET9a/PpSWEET14-YFP and plasma membrane-localized marker labeled with mCherry. Bars = 40 μm.

sucrose efflux and loading. *atsweet11 atsweet12* loss-of-function double mutant shows compromised sucrose translocation into the phloem as well as more sugar and starch retention in leaves[9]. *PpSWEET9a* and *PpSWEET14* are also preferentially expressed in source leaves and to verify their involvement in the allocation of sucrose from source leaves to sink tissues, we overexpressed *PpSWEET9a* or *PpSWEET14* individually in the *atsweet11 atsweet12* double mutant (Fig. 4b). Under dark conditions, starch synthesized from photosynthesis would be degraded into glucose or maltose in chloroplasts and exported to the cytoplasm. This glucose would then be converted to sucrose through successive enzymatic reactions and, in turn, loaded into the phloem by the SWEETs-mediated apoplasmic pathway[2]. Individual expression of *PpSWEET9a* or *PpSWEET14* significantly reduced starch retention in the leaves of the *atsweet11 atsweet12* double mutant (Fig. 4c, d), indicating that PpSWEET9a and PpSWEET14 likely facilitate starch catabolism in leaves by promoting sucrose efflux.

PpSWEET9a and PpSWEET14 may form a heterooligomer and promote the allocation of sucrose from source leaves to fruits in peach

To provide more genetic evidence of *PpSWEET9a* and *PpSWEET14* function in sucrose allocation, both genes were transiently overexpressed or silenced in the source leaves adjacent to mature fruit (Figs. 5, 6). Consistent with the result of transgenic complementation in Arabidopsis, individual expression of *PpSWEET9a* or *PpSWEET14* significantly reduced starch accumulation in source leaves but not in branches or fruit (Fig. 5a–c). Soluble sugars analysis showed that overexpression of *PpSWEET9a* or *PpSWEET14* did not significantly change the levels of glucose, fructose, or sorbitol in any of the examined tissues (i.e., source leaves, branches, and fruit). However, an obvious change was observed in sucrose accumulation. Sucrose level significantly decreased in source leaves but increased in the fruit of transgenic samples overexpressing *PpSWEET9a* or *PpSWEET14*. By contrast, when *PpSWEET9a* or *PpSWEET14* was silenced, there was an increase in starch and sucrose accumulation of mature leaves but a decrease in fruit sucrose level. Meanwhile, no significance was observed for glucose, fructose, or sorbitol in all tissues (Fig. 6).

It was noted that individual overexpression of *PpSWEET9a* or *PpSWEET14* stimulated the expression of the other (Fig. 5b). Considering their similar roles in the expression pattern of source and sink tissues (Fig. 2a), we speculated that *PpSWEET9a* and *PpSWEET14* may coordinate sucrose efflux in source leaves. When both *PpSWEET9a* and *PpSWEET14* were transiently co-expressed in peach source leaves adjacent to mature fruit, an additive effect on starch and sucrose reduction in source leaves as well as sucrose increase in fruit was observed with co-expression treatment relative to the individual expression treatment (Fig. 5c, d). Moreover, the total gene expression for *PpSWEET9a* and *PpSWEET14* was comparable between PpSWEET14-OE with PpSWEET9a + PpSWEET14-OE groups but different with PpSWEET9a-OE group (Fig. 5a, b). Thus, these results indicated that there was a limited effect for individual PpSWEET9a or PpSWEET14, and high expression of *PpSWEET9a* and *PpSWEET14* together may synergistically mediate sucrose allocation from source leaves to fruit in peach.

Evidence has been provided that SWEET transporter activity could be modulated by forming dimers with interactions between cytosolic C termini with other SWEETs[13]. Due to the synergistic action of PpSWEET9a and PpSWEET14 in sucrose efflux from source leaves, we utilized the split-ubiquitin yeast two-hybrid (SU-Y2H) system to examine whether they could interact and form a dimer. Results showed that PpSWEET9a and PpSWEET14 not only could form homodimers but were also capable of interacting with each other, forming heterodimers (Fig. 7a). Subsequently, a split-luciferase assay was conducted to verify the protein interaction for PpSWEET9a and PpSWEET14. PpSWEET9a or PpSWEET14 were fused with the amino-terminal and carboxyl-terminal halves of luciferase,

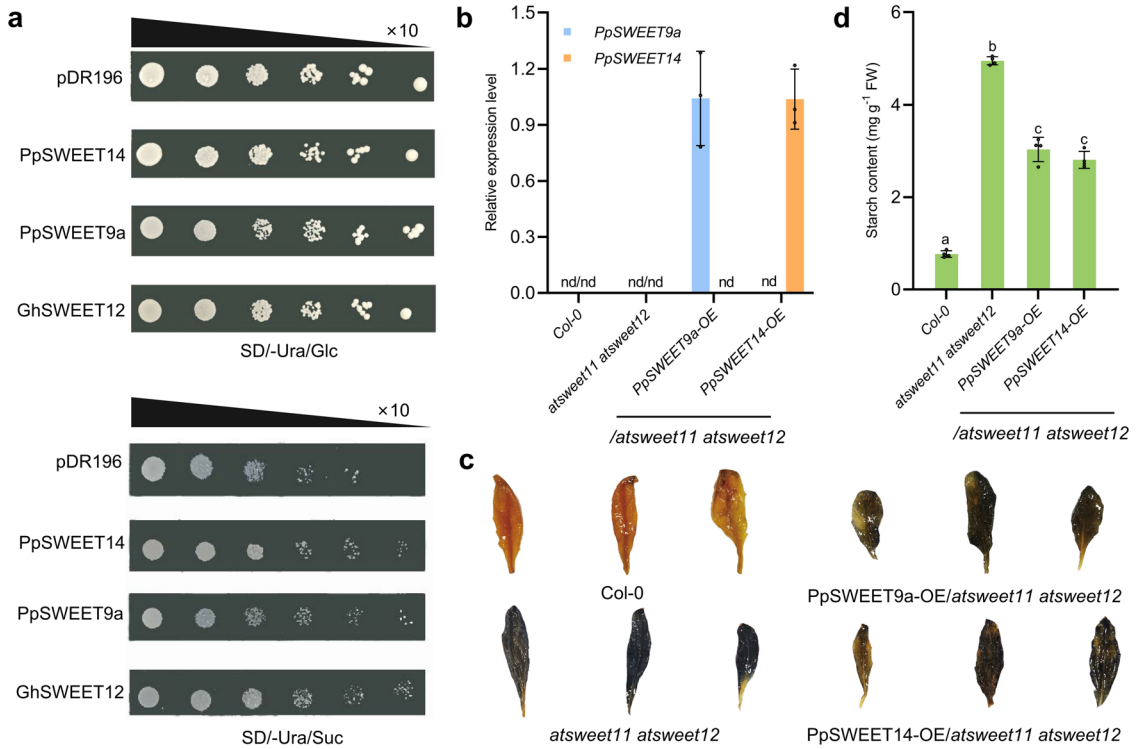

Fig. 4 | Functional characterization of *PpSWEET9a* and *PpSWEET14*. **a** PpSWEET9a and PpSWEET14 function as a sucrose transporter and can rescue the growth of the sucrose uptake-deficient yeast strain SUSY7/*ura3* on 2% (w/v) sucrose medium (SD/-Ura/Suc). The empty vector pDR196 and GhSWEET12 were used as a negative and positive control, respectively, and 2% (w/v) glucose served as the medium control (SD/-Ura/Glc). Serial 10× dilutions were plated on a solid medium. **b** Expression levels of *PpSWEET9a* and *PpSWEET14*, **c** starch staining, and **d** starch content in the leaves of wild-type (Col-0), *atsweet11 atsweet12* double mutant, and transgenic lines overexpressing *PpSWEET9a* or *PpSWEET14* in *atsweet11 atsweet12* mutant. Data are means ± standard deviation ($n = 3$ or 4). Values followed by different letters are significantly different ($P < 0.05$). 'nd' indicates not determined.

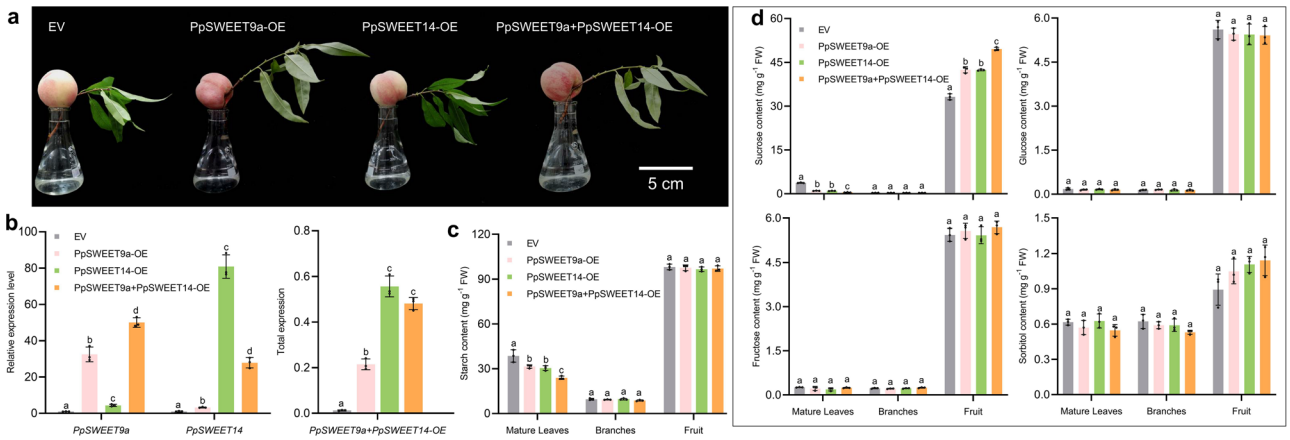

Fig. 5 | Transient overexpression analysis of *PpSWEET9a* and *PpSWEET14* in peach. **a** Images of the empty vector (EV) control, *PpSWEET9a* overexpression (PpSWEET9a-OE), *PpSWEET14* overexpression (PpSWEET14-OE), and co-overexpression of *PpSWEET9a* and *PpSWEET14* (PpSWEET9a + PpSWEET14-OE). **b** Expression levels of *PpSWEET9a* and *PpSWEET14* in different groups of inoculated leaves. The total gene expression was quantified with the value sum of *PpSWEET9a* and *PpSWEET14* absolute expression. **c** Starch content and **d** soluble sugars in the mature leaves (i.e., inoculated leaves), branches, and fruit of different groups. Data are means ± standard deviation (n = 3). Values followed by different letters are significantly different ($P < 0.05$). FW, fresh weight.

resulting in *PpSWEET9a-nLUC*, *PpSWEET14-nLUC*, *PpSWEET9a-cLUC*, and *PpSWEET14-cLUC* constructs. Leaf cells of *N. benthamiana* co-expressing *PpSWEET9a/14-nLUC* and *PpSWEET9a/14-cLUC* exhibited strong fluorescence signals, whereas those co-expressing *nLUC* and *PpSWEET9a/14-cLUC* or *PpSWEET9a/14-nLUC* and *cLUC* showed no signals, indicating that both pSWEET9a and PpSWEET14 could form homodimers and heterodimers in vivo (Fig. 7b). Thus, PpSWEET9a and PpSWEET14 likely form a heterooligomer in the plasma membrane to transport sucrose, and this interaction may enhance their transport activity.

Discussion

Peach is an important fruit crop worldwide, and the yield and quality largely depend on the sugar supply. Sugars are produced via photosynthesis, and their allocation from leaves to fruit is critical for increasing the sugar content

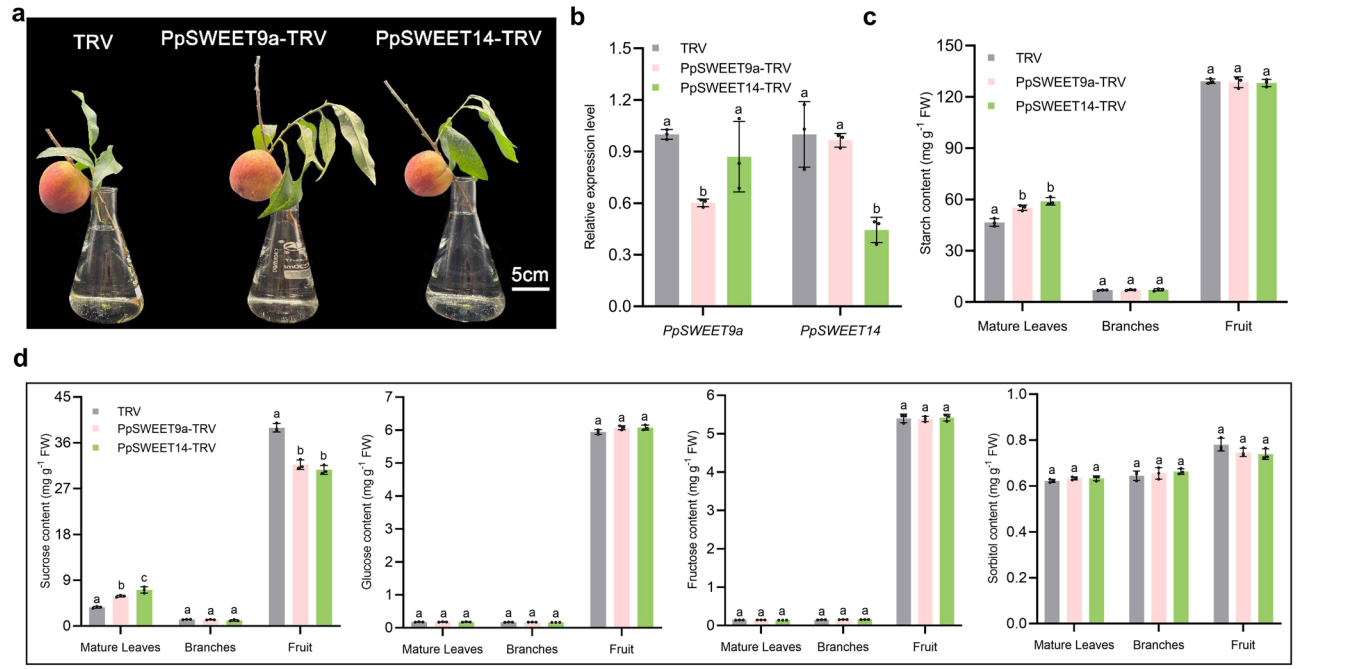

Fig. 6 | Transient silencing analysis of *PpSWEET9a* and *PpSWEET14* in peach. **a** Images of the empty vector (TRV) control and individual silencing of *PpSWEET9a* (PpSWEET9a-TRV) and *PpSWEET14* (PpSWEET14-TRV). **b** Expression levels of *PpSWEET9a* and *PpSWEET14* in the inoculated leaves. **c** Starch content and **d** soluble sugars in the mature leaves (i.e., inoculated leaves), branches, and fruit of different groups. Data are means ± standard deviation ($n = 3$). Values followed by different letters are significantly different ($P < 0.05$). FW, fresh weight.

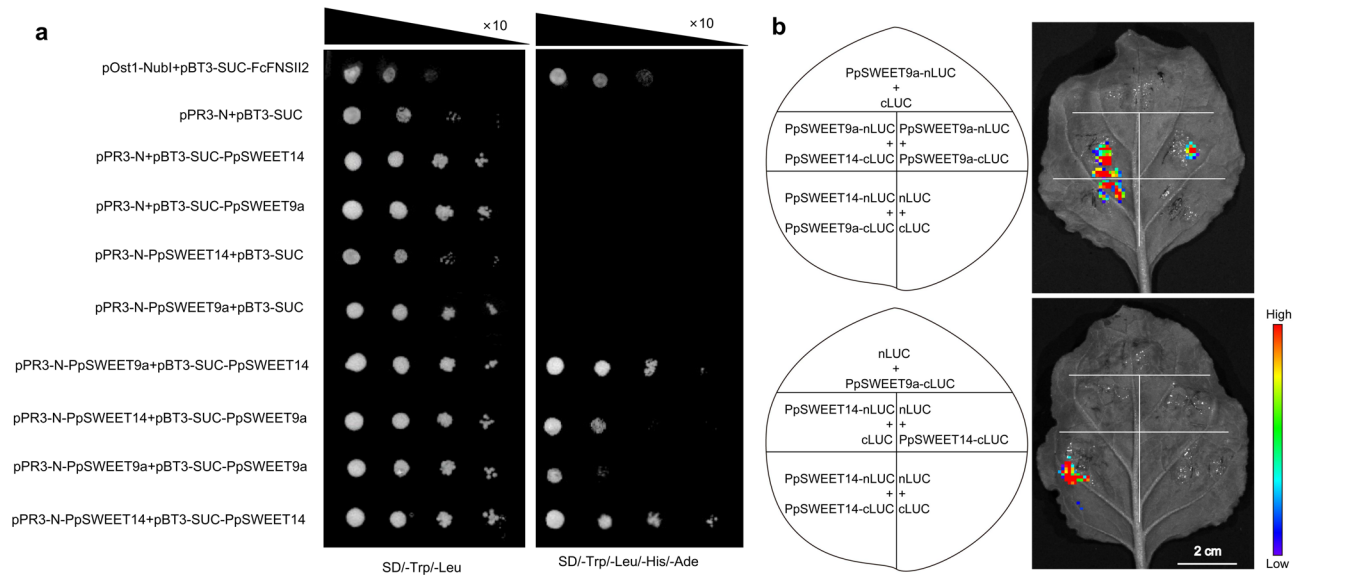

Fig. 7 | Interaction analysis of PpSWEET9a and PpSWEET14. **a** For split-ubiquitin Y2H analysis, yeast cells expressing pBT3-SUC-FcFNSII2 and pOst1-NubI were used as a positive control. pOst1-NubI expressing a fusion protein comprising the wild-type N-terminal half of yeast ubiquitin (NubI) and yeast resident ER protein Ost1. Serial 10 × dilutions were plated on a solid medium and photographed after three days. **b** For split-luciferase complementation analysis, luminescence imaging of tobacco leaves was shown 72 h after co-infiltration with the different constructs.

in mature fruit[2]. SWEETs are evolutionarily conserved sugar transporters and critical for sugar allocation and fruit development[9,13,26]. Sucrose is not only the dominant sugar in peach fruit but also the predominant form of translocated sugar[20]. However, little is known about the role of *SWEETs* in sucrose allocation to fruit in peach. In this study, we report for the first time that PpSWEET9a and PpSWEET14 synergistically mediate sucrose allocation from source leaves to fruits in peach.

Gene duplication is the main force driving *SWEET* family evolution in plants[26,27]. Here we identified 19 *SWEET* candidates, i.e., 15 *SWEETs* and four *SemiSWEETs*, in the peach genome, which is a significantly lower number than in apple (25 SWEETs). Both peach and apple belong to the Rosaceae family, however, the recent whole-genome duplication (WGD) in apple, did not occur in peach and contributes to their different genome size and chromosome number, where the reference genome includes 8 and 17 chromosomes and genome sizes of 227.4 Mb and 643.2 Mb in peach cultivar Lovell and apple cultivar Golden delicious, respectively[21,28]. By contrast, the number of *SWEETs* is comparable in peach and Arabidopsis, but the difference in the clades suggests that segmental duplications may have driven

SWEETs' functional divergence. This speculation was supported by the nonstandard number of TMs in the proteins of PpSemiSWEET1, PpSemiSWEET3a, PpSemiSWEET3b, and PpSemiSWEET9 (Fig. 1b). Phylogenetic analysis showed that the number of Clade I, II, III, IV is 6, 5, 6, and 2 in peach, respectively. However, the corresponding numbers are 3, 5, 7, and 2 in Arabidopsis, respectively. The difference in Clade I *SWEET* members may be attributed to three duplications for peach *SWEET1,2,3* or one duplication for peach *SWEET3* and two dropouts for Arabidopsis *SWEET1,2* (Fig. 1). Also, a larger expansion of Clade I *SWEETs* (10 genes) was shown in the apple genome relative to Arabidopsis[26]. Thus, in response to environmental changes or during development, the duplication and contraction of Clade I SWEETs may have conferred a more flexible or efficient regulation for glucose translocation[14–16]. In addition to being an essential metabolic nutrient and structural component, glucose is also considered a major regulatory molecule affecting developmental and metabolic processes as well as stress tolerance in higher plants[15,29]. By contrast, in peach Clade III *SWEETs*, there was a gene contraction for SWEET11,12, 13, and 14, but a gene expansion for *SWEET9* relative to Arabidopsis (Fig. 1). This difference indicated a functional divergence in sucrose translocation regulation for *PpSWEET9s* and *PpSWEET14*.

Duplicated genes are also prone to diverge in their expression pattern[30,31]. Gene expression and promoter analysis indicated that *PpSWEET9a* and *PpSWEET14* likely respond to light signals and mediate the allocation of sucrose from source leaves to fruit (Fig. 2a–c). Moreover, yeast *SUSY/ura3* complementation assay (Fig. 4a) and subcellular location (Fig. 3) revealed that both PpSWEET9a and PpSWEET14 functioned on the plasma membrane, the major site mediating apoplasmic sugar transport, and transported sucrose. However, the sucrose loading in leaves of Arabidopsis is mainly governed by *AtSWEET11* and *AtSWEET12*[9]. Other members in Clade III, such as AtSWEET13, AtSWEET14, and AtSWEET15, are also involved in the sucrose loading in leaves of Arabidopsis[13,32]. Besides, AtSWEET13 and AtSWEET14 have been demonstrated to transport gibberellin and mediate its signaling during anther dehiscence and germination in Arabidopsis[33]. PpSWEET14 showed a high sequence similarity to AtSWEET11/12/13/14 (Figs. 1a, 2d), which supports the potential involvement of PpSWEET14 in leaves sucrose loading. By contrast, in Arabidopsis, *Brassica rapa*, and *Nicotiana attenuate*, *SWEET9* ortholog is specifically expressed in the nectary tissue and is required for sucrose release during nectar secretion[34]. Although we did not examine the expression of *PpSWEET9a* in flower tissues, we suggest that at least one of the three SWEET9 orthologs is likely to be preferentially or specifically expressed in the nectary tissue of peach. The expansion of *SWEET9* may make up the contraction of *SWEET11/12/13/14* genes. Interestingly, it has been found that *AtSWEET11*, *AtSWEET12*, and *AtSWEET15* are sequentially expressed in the seed coat and endosperm and mediate a cascade of sugar unloading supporting embryo development[19] and our results in peach indicate *PpSWEET9a* is preferentially expressed in source leaves and cooperating synergistically with *PpSWEET14* for sucrose loading. This role for PpSWEET9a and PpSWEET14 in sucrose loading in leaves and allocation to fruit was supported by transgenic complementation for *atsweet11 atsweet12* double mutant as well as transient overexpression and silencing in peach.

It has been suggested that more SWEETs may be recruited to phloem sucrose loading through yet-unknown feedback mechanisms[13]. Consistent with this speculation, individual expression of *PpSWEET9a* and *PpSWEET14* could stimulate the expression of the other one in our peach transient expression assay. Considering the sequence similarity to AtSWEET11/12, *PpSWEET14* is likely to retain the function of *AtSWEET11/12* during peach evolution. By contrast, *PpSWEET9a* diverged in expression regulation and acquired a synergistic expression pattern with *PpSWEET14*. Our observation that PpSWEET9a and PpSWEET14 could form a heterooligomer, which may promote the allocation of sucrose from source leaves to fruits in peach supports this idea of a synergistic mechanism. Arguably, when they were co-expressed in peach source leaves, an enhanced efficiency in sucrose translocation from leaves to fruit was observed in relative to the individual expression of *PpSWEET9a* or *PpSWEET14* (Fig. 5).

In conclusion, genome-wide analysis of *SWEETs* enabled us to identify two Clade III *SWEET* genes, *PpSWEET9a* and *PpSWEET14* preferentially expressed in source leaves and transporting branches. Further analysis revealed that PpSWEET9a and PpSWEET14 served as sucrose efflux proteins and synergistically mediated sucrose translocation from source leaves to fruit via forming a heterooligomer. Our findings will be beneficial to peach breeding for fruit quality improvement.

Methods

Plant materials

P. persica cv. Baifeng grown at Chongqing Yunduo Agricultural Development Co., Ltd. was used in this study. Juvenile leaves, mature leaves, branches, and mature fruits from the same branch were collected for gene expression analysis at the same time. For light-responsiveness analysis, after a 24-dark treatment with blacking bagging, the mature leaves were collected on 0, 0.5, and 1 h of light exposure on a sunny day.

Arabidopsis *atsweet11 atsweet12* double mutant seeds were from the Arabidopsis Biological Resource Center (ABRC). Arabidopsis seeds from wild-type (WT) 'Columbia-0' (Col-0), *atsweet11 atsweet12* double mutant, and transgenic T3 homozygous lines were sterilized and cultured on 1/2 strength MS (Murashige and Skoog) agar medium with 1% sucrose. After a-4d-treatment at 4 °C, these seeds were incubated under illumination conditions (60 μmol m^{-2} s^{-1}). Then, seedlings were transferred to pots (8 × 8 × 7.5 cm) that contained a mixture of soil and perlite (1:1, v: v) in a phytotron (23 °C, 16 h light). For each tissue type, three biological replicates were collected, and each replication included at least five samples. All samples were immediately frozen in liquid nitrogen and stored at −80 °C for analysis.

Identification, phylogenetic analysis, and chromosomal location of *SWEET* genes in peach

To identify peach *SWEET* genes, we used the MtN3/saliva (MtN3_slv) domain (PF03083) as the query sequence and searched against the *Prunus persica* Whole Genome Assembly v2.0 & Annotation v2.1 (https://phytozome.jgi.doe.gov/pz/portal.html) with an E-value cutoff of 1×10^{-20} using the BLASTP program. Multiple alignments were performed with amino acid sequences using the DNAMAN software, and a phylogenetic analysis was conducted with the MEGA 5.0 program. The Arabidopsis SWEETs were retrieved from the TAIR (http://www.arabidopsis.org/) database. Neighbor-joining (NJ) and p-distance methods with the pairwise deletion option were used to deal with gaps in the amino acid sequences, and the bootstrap value was set to 1000. The chromosomal location of *SWEET* genes was obtained from the peach genome annotation GFF file using TBtools[35].

Analysis of gene structure and cis-elements

The gene structure, i.e., exon and intron composition, and transmembrane domain was analyzed using GSDS 2.0 (http://gsds.gao-lab.org/) and HMMTOP software (http://www.enzim.hu/hmmtop/html/submit.php), respectively. The 2000 bp sequences upstream of the initiation codon were used to predict the cis-elements using PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). Gene collinearity was determined using Quick MCScanX Wrapper software[36]. The returned results were collated and visualized using TBtools[35].

Total RNA extraction and reverse transcription-quantitative PCR (RT–qPCR) analyses

The earlier protocol described by Zhou et al.[37] was used for total RNA extraction, first-strand cDNA synthesis, and RT-qPCR operation. Total RNA was extracted using an RNAprep Pure polysaccharide polyphenol plant total RNA extraction kit (TianGen, China) according to the manufacturer's instructions. NanoDrop 2000 spectrophotometer (Wilmington,

USA) was used to assess the RNA purity and concentration. RNA with qualified purity and concentration were reverse-transcribed using the PrimeScript™ RT reagent kit with gDNA Eraser (Takara, Japan) according to the manufacturer's instructions. The expression levels of investigated genes were obtained by RT-qPCR with NovoStart®SYBR qPCR SuperMixn kit (Novoprotein, China). The amplification program was performed as follows: 95 °C for 1 min, followed by 40 cycles at 95 °C for 20 s, 58 °C for 20 s and 72 °C for 30 s. The relative expression level was calculated using the $2^{-\Delta\Delta CT}$ method, and the *Actin* gene of peach and Arabidopsis were used as the reference. Three biological replicates were analyzed for each sample. Primers used are listed in Supplementary Table 1.

Gene isolation and subcellular localization

The sequences of *PpSWEET9a* and *PpSWEET14* were retrieved from the peach genome and their CDS (coding sequences) were cloned using the cDNA synthesized from peach leaves.

The CDS of *PpSWEET9a* or *PpSWEET14* without stop codon were cloned in-frame into PHB-X-YFP vector with C-terminal YFP (i.e., *35S:PpSWEET9a-YFP* or *35S:PpSWEET14-YFP*). The plasm membrane-localized marker PM-RK-mCherry vector was used to specify the plasm membrane localization[23]. The resultant plasmids were transferred into *Agrobacterium tumefaciens* GV3101 (pSoup-p19). The relevant Agrobacterium transformants from overnight growth were inoculated for re-culture until they reached OD_{600} = 0.6 in Luria-Bertani (LB) medium at 28 °C. Then, the cells were pelleted by centrifugation at 3214 g for 5 min and resuspended in filtration buffer (10 mM $MgCl_2$, 10 mM MES, 200 mM acetosyringone, pH=5.6) to OD_{600} = 0.6. The *35S:PpSWEET9a/14-YFP* or PHB-X-YFP empty vector suspension was mixed with PM-RK-mCherry suspension in a 1:1 ratio (v: v). Afterwards, the Agrobacterium suspension was infiltrated into 4-w-old leaves of *Nicotiana benthamiana*. The infiltrated tobacco plants were grown for 3 d in a phytotron (23 °C, 16 h light/8 h dark), and the fluorescence was detected with a confocal laser scanning microscope (Nikon, Tokyo, Japan). Primers used are listed in Supplementary Table 1.

Complementation assays of *PpSWEET9a* and *PpSWEET14* in yeast SUSY/*ura3* and Arabidopsis *atsweet11 atsweet12* double mutant

The CDS of *PpSWEET9a* or *PpSWEET14* were amplified and inserted into the pDR196 vector, which harbors an ampicillin-resistance gene. Primers used are listed in Supplementary Table 1. Then, the sucrose transport-deficient yeast strain SUSY7/*ura3* was transformed with these resultant plasmids for complementation assays using the lithium acetate method[24]. Negative control and positive control were transformed with pDR196 and pDR196-GhSWEET12 vectors, respectively[25]. The transformed yeast strains were screened by SD (synthetic dropout)-Ura solid medium at 30 °C for 3 days. For growth assay, transformants were serially diluted (10-fold) from OD_{600} = 0.6 and cultured on SD-Ura solid media with 2% (w/v) glucose or sucrose as the sole carbon source at 30 °C for 3 days. The growth of various yeast strains was photographed.

The PHB-X-YFP mediated constructs overexpressing *PpSWEET9a* or *PpSWEET14* were individually transformed into Arabidopsis *atsweet11 atsweet12* double mutant by the floral dip method[38]. The rosette leaves from T3 homozygous *35S:PpSWEET9a-YFP* or *35S:PpSWEET14-YFP*/*atsweet11 atsweet12*, *atsweet11 atsweet12*, and Col-0 plants were collected for starch measurement after being subjected to 7h-treatment of darkness.

Transient silencing and overexpression of *PpSWEET9a* and *PpSWEET14* in peach leaves

A virus-induced gene silencing (VIGS) assay was performed as described by Bai et al. [39]. A gene fragment of *PpSWEET9a* or *PpSWEET14* was cloned into the pTRV2 vector (*gene-TRV2*). Then, the resultant plasmids were introduced into *A. tumefaciens* strain GV3101 (pSoup-p19). Primers used are listed in Supplementary Table 1.

For the preparation of the *A. tumefaciens* suspension, Agrobacterium transformants from overnight growth were inoculated for re-culture until OD_{600} = 0.6 in LB medium containing 10 mM MES (pH 6.3) and 20 mM acetosyringone. After centrifugation at 3214 g for 5 min, the collected pellet was resuspended in infiltration buffer (10 mM $MgCl_2$, 10 mM MES, 200 m M acetosyringone, pH 5.6) to OD_{600} = 0.6 and placed in the dark for 3 h at room temperature. For gene silencing analysis, the *PpSWEET9a-TRV2* or *PpSWEET14-TRV2* suspension was mixed with a suspension containing pTRV1 in a 1:1 ratio (v: v).

Peach fruit with one branch and several leaves were collected at the red mature stage, and the branch was immediately placed in ddH_2O (double distilled H_2O) for culture. Then, the Agrobacterium suspension was injected into two mature leaves next to the fruit using a 1 mL syringe without needles. The fruits were cultured at room temperature for 3 days (14 h light [30 μmol m^{-2} s^{-1}]/10 h dark cycles), and the expression of target genes (*PpSWEET9a* and *PpSWEET14*) in the infiltrated leaves was measured by RT-qPCR. The fruit showing overexpressed or silenced target genes in their adjacent leaves were collected for starch and soluble sugar analysis. Fifteen fruits were included in each transgenic analysis. For co-expression of *PpSWEET9a* and *PpSWEET14*, the *35S:PpSWEET9a-YFP* and *35S:PpSWEET14-YFP* suspensions were mixed in a 1:1 ratio (v: v). To ensure an equal copy number of transgene, the total volume of injected solution was controlled at 1 mL per mature leaf for all transient analyses.

Measurement of starch and soluble sugars

Starch staining and quantification were performed as described by Zhou et al. [40]. In brief, leaves were discolored with boiling 95% ethanol and then stained in 5% Lugol's iodine solution (5% [w: v] I_2 and 10% [w: v] KI). For quantification, 100 mg ground fine powder of leaves was rinsed with diethyl ether and 80% ethanol to remove pigments, soluble sugars, and other non-starch substances. Then, the residue was washed and boiled in ddH_2O until the solution became clear. The starch solution was then mixed with I_2 -KI solution (5% [w: v] I_2 and 2% [w: v] KI) and subjected to colorimetric determination at 660 nm. The standard curve was generated using soluble starch (Sigma).

Soluble sugars were extracted and derivatized as described by Hu et al. [41]. Briefly, 100 mg of fruit finely powdered in liquid nitrogen was extracted in 75% methanol, and ribitol was added as the internal standard. After the non-polar metabolites were fractionated into chloroform, 2 μL aliquots of the polar phase were vacuum-dried and derivatized with methoxamine hydrochloride and N-methyl-N-trimethylsilyl-trifluoroacetamide. Finally, fructose, glucose, and sucrose were quantified with GS-MS (gas chromatography-mass spectrometry) 2010SE system (Shimadzu Corporation, Tokyo, Japan).

Protein interaction assays

The SU-Y2H system was used to analyze the formation of SWEET homo- and heter-dimer according to the protocol of Tian et al. [42]. Briefly, the CDS of *PpSWEET9a* or *PpSWEET14* without stop codon was in frame inserted into the *SfiI* sites of the pBT3-SUC vector expressing a recombinant protein with an N-terminal SUC and a C-terminal Cub-LexA-VP16 peptide. Complete CDS of *PpSWEET9a* or *PpSWEET14* were cloned in frame into the *SfiI* sites of the pPR3-N vector to express a recombinant protein with an N-terminal NubG. Then, different pairs of constructs were transformed into NMY51 yeast cells using the lithium acetate method. The transformed yeast cells grown on SD agar medium lacking tryptophan and leucine (SD-Trp/Leu) were serially diluted (10-fold) from OD_{600} = 0.6 and transferred to SD agar medium lacking tryptophan, leucine, histidine, and adenine (SD-Trp/Leu/His/Ade) for a 3-d-culture at 30 °C. Then, the growth of different yeast strains was photographed.

A split-luciferase complementation system was performed as described by Chen et al. [43]. The N-terminal part (nLUC) and C-terminal part (cLUC) of the firefly LUC protein were divided. The complete CDS of *PpSWEET9a* or *PpSWEET14* was in frame cloned into the pCAMBIA-nLUC vector and pCAMBIA-cLUC vector. Primers are listed in Supplementary Table 1. Agrobacterium strains transformed with constructs were mixed in a 1:1 ratio (v: v) and used to infiltrate *N. benthamiana* leaves. After 3 d of growth in a phytotron (23 °C, 16 h light/8 h dark), the infiltrated leaves were sprayed

with luciferin (1 mM) and placed in the dark for 10 min. Then, luminescence detection was conducted using a low-light cooled CCD imaging apparatus (Andor iXon, Grens, Switzerland).

Statistics and reproducibility

Statistical analyses and generation of graphs were performed using GraphPad Prism Software (9.1.0). Data were subjected to one-way ANOVA for statistical analysis using SPSS software (17.0). Experiments were performed with three or four independent repeats.The *n* numbers are indicated in figure legends, where appropriate.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

All relevant data supporting the findings of this study are provided in the main figures and Supplementary Information file. Source data used for all figures in this study can be found in Supplementary Data 1. Any remaining information can be obtained from the authors upon request.

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Author contributions

M.L., M.X.J., and L.P. performed the experiments and collected data. K.Z. and M.L. analyzed the data and prepared the draft. K.Z., W.F.C., and W.P.X. designed the experiments, provided financial funds, and critically revised the article.

Competing interests

The authors declare no competing interests.

Additional information

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