The homo/heterodimers of plasma membrane sugar transporters CsSWEET1a and CsSWEET17 mediate growth and cold stress tolerance

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Abstract

Sugars will eventually be exported transporters (SWEET) are involved in plant biological processes. CsSWEET1a and CsSWEET17, were found to be induced by cold acclimation in Camellia sinensis. Particularly, CsSWEET17 was differentially alternatively spliced and its inclusion/exclusion ratio was higher in the cold-resistant cultivar than in the cold-susceptible cultivars. Both CsSWEET1a and CsSWEET17 were located in the plasma membrane, and their interaction was confirmed using yeast two-hybrid and biomolecular fluorescence complementation. The C-terminal of the CsSWEET17, which was different from AtSWEET17, did not affect its plasma membrane localization but promoted its sugar transport activities. Overexpression (OE) of CsSWEET1a and CsSWEET17 resulted in an increased uptake of sugars in Arabidopsis, affecting plant germination and growth. The leaf and seed size of CsSWEET17-OE lines were significantly bigger than wild-type. Moreover, OE of CsSWEET1a and CsSWEET17 significantly reduced the relative electrolyte leakage levels under cold stress. Compared with those in wild-type, the expressions of AtCWINV genes were suppressed in both CsSWEET1a-OE and CsSWEET17-OE lines, indicating the alteration of sugar contents in the cell wall of OE lines. Our results suggest that CsSWEET1a and CsSWEET17 form homo/heterodimers in the plasma membrane to import sugars into the cytoplasm, thereby regulating plant growth and cold tolerance.

KEYWORDS

cold tolerance, CsSWEET1a, CsSWEET17, plant growth, sugar transporter, *Camellia sinensis* (Tea plant)

1 INTRODUCTION

Sugars are important in plant growth and development. They act as the carbon sources for synthesizing cellular compounds and generating energy and as osmoprotectants for improving the cold tolerance of plants (Chen, Cheung, Feng, Tanner & Frommer, 2015c; Wanner & Junttila, 1999). Sugars are products of photosynthesis and are transported from the leaves to all plant tissues, especially the roots and seeds, through sugar transporters (Chen *et al.* , 2015c). Many sugar transporters have been discovered in plants, including sugars will eventually be exported transporters (SWEET) (Chen et al., 2010). The SWEET family of proteins is characterized by seven α -helical transmembrane domains (TMs) and two MtN3/saliva motifs

and have been identified as sugar uniporters that promotes sugar transport across the membrane along the sugar gradient (Chen *et al.* , 2015c). Currently, 17 SWEET members have been identified in *Arabidopsis thaliana* , 21 in*Oryza sativa* , and 13 in *Camellia sinensis* (Chen *et al.* , 2010; Wang *et al* ., 2018; Yuan & Wang, 2013). Xuan *et al.* revealed that *Arabidopsis* SWEETs can form homo- or heterooligomers, which are necessary for transport function (Xuan*et al.* , 2013).

The *AtSWEET1* , a glucose uniporter localized in the plasma membrane of *Arabidopsis* leaves, was the first identified SWEET sugar transporter (Chen *et al.* , 2010). Thereafter, five more members, namely *AtSWEET4/5/7/8/16* that can transport glucose were also identified (Chardon *et al* ., 2013; Chen *et al.* , 2010; Guo *et al* ., 2014; Klemens *et al* ., 2013; Sun, Huang, Yang, Guan & Yang, 2013). Current studies have found that SWEETs play key roles in growth, stress tolerance, and reproductive development of plants. They are involved in phloem loading, nectar secretion, seed filling, pollen nutrition, and embryo nutrition. Although the mechanism of action of *AtSWEET1* remains unknown, the function of*AtSWEET2* belonging to the same subfamily Clade I has been elucidated. AtSWEET2 is sugar transporter protein located to the tonoplast and restricts *Pythium* infection by limiting carbon sequestration at the roots (Chen *et al* ., 2015a). The clade IV member AtSWEET17 is the first sugar transporter to be identified to the tonoplasts that controls the natural changes in the fructose levels in*Arabidopsis* leaves (Chardon *et al.* , 2013). AtSWEET16, which is homologous to AtSWEET17, has been identified as a vacuolar membrane sugar transporter that can transport glucose, sucrose, and fructose. By catalyzing their transport, the sugar metabolism of*AtSWEET16* overexpression (OE) plants were subsequently altered, thereby affecting their germination rate and improving their biomass and cold resistance (Klemens *et al.* , 2013). Guo *et al.* found that AtSWEET16/17 were localized in the vacuolar membrane and mediated the transport of fructose in the roots. Under cold stress, the fructose content in the leaves of *AtSWEET17* -OE lines was reduced by 80% (Guo *et al.* , 2014). CsSWEET16 from *Camellia sinensis* , which is homologous to AtSWEET16, is a sugar transporter localized in the tonoplast membrane and alters the cold tolerance of*Arabidopsis* by promoting sugar compartmentation in the vacuole (Wang *et al.* , 2018). Meanwhile, DsSWEET17, homologous to AtSWEET17, is another sugar transporter localized in the vacuolar membrane of *Dianthus spiculifolius* , affecting sugar metabolism and conferring multiple tolerance to *Arabidopsis* (Zhou, Ma, Feng, Gong & Wang, 2018). In addition to contributing to the loading of the phloem in the source leaves, *atsweet11/12* significantly reduce electrolyte release by down-regulating the expressions of*AtSWEET11 /12* , thereby improving the freezing resistance of*Arabidopsis* (Le Hir *et al* ., 2015).

Recent studies have shown that SWEETs also affect seed size by regulating sugar metabolism. AtSWEET4 mediates the transport of glucose and fructose, thereby accumulating them in *AtSWEET4* -OE, increasing the size of transgenic plants, and improving their freezing resistance (Liu, Zhang, Yang, Tian & Li, 2016). AtSWEET11/12/15 mediate the transfer of sucrose from the seed coat to the embryo, and *atsweet11;12;15* triple mutant showed a severe seed-deficient phenotype because starch accumulated in its seed coat rather than in its embryo (Chen *et al* ., 2015b). Maize*ZmSWEET4c* and its rice ortholog *OsSWEET4* are critical for seed filling, and their mutations are defective in seed filling (Sosso*et al* ., 2015). *OsSWEET11/Os8N3/Xa13* not only affects pollen development, but also plays an important role in the early stage of rice seed filling (Ma *et al* ., 2017).

In our previous study, 13 *CsSWEETs* were identified in*Camellia sinensis,* and their expression patterns in response to different forms of stress and their sugar transport activities in yeast were analyzed. The function of the cold-suppression gene*CsSWEET16* was also studied (Wang *et al.* , 2018). In this study, the functional verification of two cold-induced genes*CsSWEET1a* and *CsSWEET17* was performed. The homo/heterodimers of CsSWEET1a and CsSWEET17 were localized in the plasma membrane. Their constitutive overexpression resulted in the increased uptakes of sucrose, glucose, and fructose in*Arabidopsis* , thereby affecting plant germination and growth and resulting in reduced levels of relative electrolyte leakage (REL) under cold stress. This indicates their significant role in regulating cold tolerance.

2 MATERIAL AND METHODS

2.1 Plant materials

The cold-resistant tea cultivar 'Longjing 43 (LJ43)' and the cold-susceptible tea cultivars 'Damianbai (DMB)'and 'Zhenong 12 (ZN12)' were used. The growth conditions and sampling time were described previously (Wang *et al* ., 2019).

Arabidopsis ecotype Columbia-0 (Col-0) was used as the wild-type (WT) and for the generation of transgenic lines. To generate*CsSWEET1a* -OE lines, the *CsSWEET1a* open reading frame (ORF) was amplified with the primers 5'- CCCAAGCTTATGGGTAATACTGCGCATTTCG -3' and 5'- GCGTCGACCTACTTGCTCGATCGCTTCTCT -3' and was cloned into pMD^{TM} 18-T vector (Takara, Bio Inc., Otsu, Japan). Next, the *CsSWEET1a* ORF was cloned into the super promoter containing vector pCAMBIA S1300 (Lee *et al* ., 2007). To generate *CsSWEET17* -OE lines, the *CsSWEET17* ORF was amplified with the primers 5'-CACCATGGCTAGCTTGAGCTTCATCA-3' and 5'-AGGGTGATCCTTGGTGCTTCCA-3' and was cloned into the pENTR/D-TOPO vector (Invitrogen, Carlsbad, USA). Then, *CsSWEET17* ORF was cloned into the vector pH7FWG2 by LR reaction, as previously described (Wang *et al.* , 2018). The resulting plasmid was introduced into *Agrobacterium* strain GV3101 and transformed into *Arabidopsis* Col-0. The seeds from T3 homozygous lines were used for further analysis.

2.2 Growth conditions

Arabidopsis seeds were surfaced-sterilized in 10% NaClO for 5-10 min, rinsed 5 times with sterile water, and then sown on half-strength Murashige and Skoog (1/2 MS) medium containing 1.5% sucrose. After vernalization treatment at 4 °C in the dark for 2 days, the plates were transferred to the growth chamber under a 12 h light/12 h dark regime (24/22 °C) at a light intensity of approximately 100 μmol $m^{-2}s^{-1}$. For the germination assays, the seeds were grown on 1/2 MS medium with or without sugar for 24 d. The germination rate of the seeds was determined on the third day and every other day after that, for a total of four times. The seeds were grown on 1/2 MS medium with 1.5% sucrose for 18 d, and the whole shoots were immediately collected for measuring their sugar content.

For the cold stress treatment, 7-d-old seedlings were transplanted into a soil mixture consisting of 3:2:1 peat moss: vermiculite: perlite. After 11 days, the plants were subjected to cold acclimation (CA) treatment at 4 °C for 3 d then at 0 °C for 12 h. After the cold treatment, the whole shoots were immediately collected for RNA extraction. For the cold freezing treatment, 7-d-old seedlings were transplanted into a soil mixture consisting of 3:2:1 peat moss: vermiculite: perlite. Then, the 18-d-old plants were subjected to the CA treatment at 4 °C for 3 d, followed by freezing at 0 °C, dropping at a rate of 2 °C h^{-1} until –6 °C for 6 h. The whole shoots were then immediately collected for relative electrolyte leakage (REL) measurements.

2.3 Complementation of yeast EBY.VW4000 and split-ubiquitin yeast two-hybrid (Y2H) assay

The 67 amino acids of the C-terminal of CsSWEET17 protein was removed and the remaining sequence was defined as CsSWEET17_C. To verify the transport activity of CsSWEET17_C, the vector pADH-CsSWEET17_C was constructed. The coding sequence of CsSWEET17_C was amplified with the primers 5'-TGCAGGTCGACTCTAGAGATGGCTAGCTTGAGCTTCATC-3' and 5'-GTACGAAGCTTCAATGGACGTTTTGACA-3' and cloned into ADHpr-Yeplac195. The resulting plasmid was transformed into yeast EBY.VW4000, and yeast complementation assay was performed as previously described (Wang *et al.* , 2018).

For the split-ubiquitin Y2H assay, CsSWEET1a and CsSWEET17 ORFs were cloned into the mating-based split-ubiquitin Nub vector PBT3-STE and Cub vector pPR3-C, respectively (Stagljar, Korostensky, Johnsson & Te, 1998). Then, the plasmids were co-transformed into the yeast strain NMY51. The bait

plasmid toxicity test and the self-activation test were performed. The dot plate experiment was performed on the DDO (SD/Trp-Leu-) and TDO (SD/Trp-Leu-His-) plates.

2.4 Subcellular localization and bimolecular fluorescence complementation (BiFC) assay

To determine the subcellular localization of CsSWEET1a, CsSWEET17, and CsSWEET17_C, their ORFs without stop codons were amplified by PCR and then cloned into the 35s:GFP vector. The isolation and the transformation of rice protoplasts were performed as described previously (Wang *et al* ., 2015). An Olympus FV1000 confocal laser scanning microscope (Olympus, Tokyo, Japan) was used for imaging.

To perform the BiFC assays, CsSWEET1a and CsSWEET17 ORFs were cloned into the N-terminal half (P2YN) of the yellow fluorescent protein (YFP) and into the C-terminal half (P2YC) of YFP (Hou *et al* ., 2018). The plasmids were transformed into the *Agrobacterium* strain GV3101 and transiently expressed in the *Nicotiana benthamiana.*Confocal microscopy images were taken using a Zeiss LSM710 confocal laser scanning microscope (Zeiss, Oberkochen, Germany).

2.5 Measurement of relative electrolyte leakage and sugar content

Whole shoots were collected into a 50-ml-centrifuge tube, in which 15 ml of distilled water was added. After shaking at 150 rpm for 2 h at 25 °C, REL was measured as previously described (Wang *et al.* , 2018). To measure the sugar content, 0.1 g of the fresh leaf sample was ground with liquid nitrogen and extracted with 1.0 ml of distilled water. The methods for measuring the soluble sugar, sucrose, glucose, and fructose were previously described (Wang *et al.* , 2018).

2.6 Total RNA isolation and real-time PCR (qPCR) analysis

Total RNA was extracted from the whole shoot samples using TRIzol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol. Total RNA (1 μg) was used to synthesize the first-strand cDNA for qPCR using the PrimeScript^{TM} RT reagent Kit with gDNA Eraser (Takara, Bio Inc., Otsu, Japan). The cDNA was amplified using a LightCycle 480 machine with SYBR Green I Master Mix (Roche). The*CsPTB* (Hao *et al* ., 2014) and *AtEF* (AT5G19510) were used as the quantitative reference genes and $2^{-\Delta^{\circ}\tau}$ or $2^{-\Delta\Delta^{\circ}\tau}$ method was used to calculate the relative expression of the target gene (Livak & Schmittgen, 2001). The inclusion/exclusion of *CsSWEET17* primer sequences used for qPCR are listed in Supplementary Table S1, whereas the other primer sequences used for qPCR were previously described (Wang *et al.* , 2018).

3 RESULTS

3.1 Expressions of *CsSWEET1a* and*CsSWEET17* in *Camellia sinensis*

Previous studies have shown that the expressions of *CsSWEET1a* and CsSWEET17 were significantly induced by cold stress and natural CA. Notably, *CsSWEET1a* can be specifically induced by low temperature (Wang *et al.* , 2018). Here, we further compared the expression levels of *CsSWEET1a* and*CsSWEET17* transcripts in the three tea plant cultivars with contrasting cold tolerances during natural CA (Wang *et al.* , 2019). As shown in Figure 1a-d, in response to CA, the expression patterns of *CsSWEET1a* and *CsSWEET17* in the three tea plant cultivars were similar. The expression levels of *CsSWEET1a* and*CsSWEET17* were low in the non-acclimation (October, November) and de-acclimation (March) periods and were strongly induced in the CA stage. In the cold-resistant cultivar LJ43, *CsSWEET1a* and*CsSWEET17* reached their highest transcripts levels on Feb 10, 2017 and Jan 11, 2018 (Figure 1a-b) and Jan 17, 2017 and Dec 18, 2017 (Figure 1c-d), respectively. During natural CA, the expression level of *CsSWEET17* in the cold-resistant cultivar LJ43 was always lower than those of two cold-susceptible cultivars DMB and ZN12 (Figure 1c-d).

The previous transcriptome data (Wang *et al.* , 2019), has shown that *CsSWEET17* is an alternatively spliced gene. The third exon was skipped to form a new transcript (Figure 1e). The exclusion expression pattern of *CsSWEET17* was undetectable in October and November. Notably, during CA periods, the inclusion/exclusion ratio of *CsSWEET17* in the cold-resistant cultivar LJ43 was significantly higher than those in the two cold-susceptible cultivars in the two-year repeated experiment (Figure 1f-g). These results indicated that *CsSWEET1a* and*CsSWEET17* might be involved in regulating the cold tolerance of tea plants.

3.2 Subcellular localization of CsSWEET1a and CsSWEET17, and the C-terminal of CsSWEET17 affects sugar transport activity

To determine the subcellular localization of CsSWEET1a and CsSWEET17, the full-length cDNAs of both CsSWEET1a and CsSWEET17 were fused to the 5' end of green fluorescent protein (GFP) and were transiently expressed in rice protoplast. As shown in Figure 2, both CsSWEET1a-GFP and CsSWEET17-GFP localized in the plasma membrane (Figure 2a-h). The 35S:GFP control vector showed fluorescence throughout the whole cell (Figure 2m), and the rice protein OsMCA1 was located on the plasma membrane (Figure 2b, f, j) (Kurusu *et al* ., 2012). However, GFP signals were also detected in the cytoplasm of the cells expressing CsSWEET17-GFP (Figure 2e), indicating that CsSWEET17 expressed not only in the plasma membrane but also in other organelles. CsSWEET17 is homologous to AtSWEET17, which is localized to the vacuolar membrane and mediates the transport of fructose in the roots and leaves of*Arabidopsis* (Chardon *et al.* , 2013; Guo *et al.* , 2014). We found that the subcellular localization of CsSWEET17 was different from that of AtSWEET17. Particularly, the C-terminal of*CsSWEET17* is 192 bp longer than *AtSWEET17* . To determine whether it affects the subcellular localization of CsSWEET17, the extra C-terminal was removed and CsSWEET17_C-GFP was constructed (Figure 2s). Our results showed that even after the removal of the extra C-terminal, the CsSWEET17_C still localized to the plasma membrane (Figure 2i-l), revealing that the subcellular localization features of CsSWEET17 and AtSWEET17 were different and that the function of CsSWEET17 could be different from that of AtSWEET17.

CsSWEET17 has been found to transport glucose, fructose, galactose, mannose, and sucrose in yeast (Wang *et al.* , 2018). To determine the effect of the C-terminal on the transport activity of CsSWEET17, CsSWEET17_C was cloned into the ADHpr-Yeplac195 vector and was transformed into the yeast mutant EBY.VW4000 (Wieczorke *et al* ., 1999). The EBY.VW4000 can grow on maltose-containing medium, and can grow slowly on galactose-containing medium; however, it cannot grow on fructose, sucrose, glucose, and mannose-containing media (Wieczorke*et al.* , 1999). Similar to that of CsSWEET17, the expression of CsSWEET17_C effectively restored the growth of EBY.VW4000 on media supplemented with glucose, mannose, fructose, and sucrose. However, the growth of the yeast cells expressing CsSWEET17_C was slower than that of expressing CsSWEET17 (Figure 2t), indicating that the extra C-terminal of CsSWEET17 can promote its sugar transport activities.

3.3 CsSWEET1a and CsSWEET17 can form homo/heterodimers in the plasma membrane

Xuan *et al* . reported that the AtSWEETs can form homo- or heterooligomeric complexes (Xuan *et al.* , 2013). Because of the similar subcellular localizations of CsSWEET1a and CsSWEET17 (Figure 2a-h), we performed split-ubiquitin Y2H (Figure 3a) and BiFC (Figure 3b-u) assays to confirm the interaction between CsSWEET1a and CsSWEET17. While all combinations grew well on the DDO plate, only the positive control (pTSU2-APP+pNubG-Fe65) and the combination of genes of interest (pBT3-N-CsSWEET1a+pPR3-C-CsSWEET17) survived on the selective TDO plate (Figure 3a). The results of split-ubiquitin Y2H assay showed that CsSWEET1a interacted with CsSWEET17.

To confirm the interaction between CsSWEET1a and CsSWEET17 proteins in vivo, BiFC assay was performed in *Nicotiana benthamiana* . Using the CsSWEET1a fused to the N-terminal YFP fragment (CsSWEET1a-P2YN), and the CsSWEET17 fused to the C-terminal YFP fragment (CsSWEET17-P2YC),

the CsSWEET1a fused to the C-terminal YFP fragment (CsSWEET1a-P2YC), and the CsSWEET17 fused to the N-terminal YFP fragment (CsSWEET17-P2YN), the YFP signal was restored (Figure 3b-i), thereby confirming the in vivo interaction between CsSWEET1a and CsSWEET17. Furthermore, the YFP signal was also restored when the CsSWEET1a was fused to the N- and C- terminals YFP fragment (CsSWEET1a-P2YN+CsSWEET1a-P2YC) and the CsSWEET17 was fused to the N- and C- terminals YFP fragment (CsSWEET17-P2YN+CsSWEET17-P2YC), thereby revealing that CsSWEET1a and CsSWEET17 formed homodimers (Figure 3j-q). In addition to the YFP signal on the cell membrane, a combination of CsSWEET17 (CsSWEET17-P2YN+CsSWEET17-P2YC) also had a YFP signal in the cytoplasm (Figure 3b-i, n-q), which was consistent with the protein localization results of CsSWEET17 (Figure 2e-h). In contrast, the negative controls, consisting of empty P2YC and P2YN vectors, failed to produce any YFP signal (Figure 3r-u). Overall, these results showed that CsSWEET1a interacted with CsSWEET17, and CsSWEET1a and CsSWEET17 formed homo/heterodimers in vivo.

3.4 Import activity of *CsSWEET1a* confers hypersensitivity to sucrose and glucose in *Arabidopsis*

To determine the function of *CsSWEET1a* , transgenic *Arabidopsis* lines constitutively overexpressing *CsSWEET1a* were generated. Three independent T3 homozygous transgenic lines, namely 1a-OE-1, 1a-OE-2, and 1a-OE-3 were confirmed by qPCR. As expected, the transcript level of *CsSWEET1a* were significantly higher in all OE lines than in WT plants, whose *CsSWEET1a* expression was undetectable (Figure 4a). Previous studies have shown that CsSWEET1a can transport sucrose and glucose in yeast (Wang *et al.* , 2018). To investigate its sugar transport activity in plants, we examined the germination efficiency of WT and *CsSWEET1a* -OE lines on media supplemented with sucrose and glucose. There was no difference in the germination efficiency between WT and *CsSWEET1a* -OE plants on 1/2 MS medium without sugar, with 3% sucrose, and with 3% glucose. All exhibited approximately 100% germination rate (Figure 4b). Notably, 24 d after culture, WT and *CsSWEET1a* -OE plants grew normally on the 1/2 MS medium. However, on 3% sucrose and 3% glucose media, all *CsSWEET1a* -OE plants exhibited stress-induced purple leaves and growth retardation (Figure 4c). After lowering the sugar concentration to 1.5% sucrose and 1.5% glucose, the leaves and roots of *CsSWEET1a* -OE plants turned yellow (Figure 4d). No significant difference in the soluble sugars content on the leaves were found between the WT plants and *CsSWEET1a* -OE plants. However, compared to the WT plants, the sucrose, glucose, and fructose contents of three OE lines were significantly increased (Figure 4e), suggesting that the *CsSWEET1a* -OE lines took up more sucrose and glucose, and the import activity of CsSWEET1a conferred hypersensitivity to sucrose and glucose in *Arabidopsis* .

3.5 CsSWEET17 overexpression lines show altered germination efficiency and improved leaf and seed size

To determine the function of *CsSWEET17* , transgenic *Arabidopsis* lines constitutively overexpressing *CsSWEET17* were generated. Three independent T3 homozygous transgenic lines, namely 17-OE-1, 17-OE-2, and 17-OE-3 were confirmed by qPCR. As expected, the transcript level of *CsSWEET17* were significantly higher in all OE lines than in WT plants, whose *CsSWEET17* expression was undetectable (Figure 5a). We monitored the germination efficiency of WT and *CsSWEET17* -OE lines on 1/2 MS medium without sugar, with 3/6% sucrose, 3/4% glucose, and 3% fructose. The OE lines and the WT plants developed similar germination efficiency on the 1/2 MS medium. However, 17-OE-2 and 17-OE-3 lines showed significantly lower efficiencies than those of WT plants after 5 d of germination on 1/2 MS medium supplemented with 6% sucrose, 3/4% glucose, and 3% fructose (Figure 5b). After 3% fructose treatment 18 d, the OE lines did not grow normally after germination, and the leaves turned purple or yellow (Figure 5c). Interestingly, *CsSWEET17* -OE lines grew better than WT plants on media containing 1.5% sucrose, 1.5% glucose, and 1.5% fructose (Figure 5d). No difference in the soluble sugars and sucrose contents between the shoots of WT and *CsSWEET17* -OE plants were found. However, the glucose and fructose levels in 17-OE-2 and 17-OE-3 lines were significantly increased compared to those in WT plants (Figure 5e), indicating that CsSWEET17

had sugar import activity in*Arabidopsis* and could promote plant growth under appropriate sugar treatment concentration.

When grown in the soil, the rosette leaves of the three OE lines were significantly larger than those of the WT plants (Figure 6a-d). The fresh weight of the three OE lines were significantly higher than that of the WT plants (Figure 6e). We further investigated the seed size of the OE lines and found that the seeds of the three OE lines were larger than those of the WT plants (Figure 6f), indicating that*CsSWEET17* promotes vegetative and reproductive growths by transporting and utilizing sugars. *CsSWEET1a* can also transport sucrose and glucose; however, unlike the *CsSWEET17* -OE lines, the rosette leaves and seed sizes of the three *CsSWEET1a* -OE lines showed no significant difference from those of WT (Supplementary Figure S1).

3.6 Overexpression of *CsSWEET1a* and *CsSWEET17* increase freezing tolerance

Because the expressions of *CsSWEET1a* and *CsSWEET17* were significantly induced by cold acclimation and cold stress in tea plants as shown in Figure 1a-d (Wang *et al.* , 2018), we investigated their functions in regulating cold tolerance. Under normal conditions, the REL levels of *CsSWEET1a* -OE and *CsSWEET17* -OE plants showed no significant difference compared to those of WT. After the freezing treatment, the REL levels of the three *CsSWEET1a* -OE lines were significantly lower than those of the WT plants, and the REL levels of the two *CsSWEET17* -OE lines with higher *CsSWEET17*expression levels were also lower than those of the WT plants (Figure 7a-b). At -6°C, the difference in REL values between the three OE lines well correlated with the difference in the transcription levels of*CsSWEET1a* and *CsSWEET17* in transgenic *Arabidopsis* . These results indicated that *CsSWEET1a* -OE and *CsSWEET17* -OE plants were less damaged by freezing, and the overexpression of*CsSWEET1a* and *CsSWEET17* improved the freezing tolerance in*Arabidopsis* .

To further investigate the actions of *CsSWEET1a* and*CsSWEET17* in mediating the sensitivity of *Arabidopsis* to freezing tolerance, we examined the expression levels of the cell wall invertase (CWINV) and vacuolar invertase (VACINV) genes. As shown in Figure 7c-d, after the cold stress treatment, the expression levels of invertase genes in *CsSWEET1a* -OE and *CsSWEET17* -OE plants were significantly lower than those in WT plants, indicating that the overexpression of *CsSWEET1a* and *CsSWEET17* might alter the sugar content in the cell wall, thereby altering the expression level of*AtCWINV1/3/6* .

4 DISCUSSION

The SWEET sugar transporters play important roles in plant growth and development. Many SWEET proteins have been characterized in model plants. However, in *Camellia sinensis* , only the function of*CsSWEET16* had been characterized (Wang *et al.* , 2018). Here, we studied two SWEET sugar transporters in tea plant,*CsSWEET1a* and *CsSWEET17* , which are homologous to*AtSWEET1a* and *AtSWEET17* , respectively. The expression levels of *CsSWEET1a* and *CsSWEET17* have been shown to be induced by cold stress (Wang *et al.* , 2018). In our study, during natural CA, the expression level of *CsSWEET17* in the cold-resistant cultivar was lower than that in the cold-susceptible cultivar (Figure 1a-d). Further, the CsSWEET17 was differentially alternatively spliced between the cold-resistant and cold-susceptible cultivars. This indicates that CsSWEET1a and CsSWEET17 are involved in the cold stress response and are related to the cold tolerance of tea plants, thereby highlighting the important roles of alternative splicing events in the cold response of tea plant.

AtSWEET1 is a low-affinity glucose transporter that is highly expressed in flowers and localized on the plasma membrane, and provides nutrients to gametophyte or nectaries (Chen *et al.* , 2010). The AtSWEET17 is localized to the tonoplast as a fructose-specific transporter and maintains the natural changes in the levels of fructose in the leaves and roots of *Arabidopsis* (Chardon *et al.* , 2013; Guo *et al.* , 2014). The sugar transport activities of CsSWEET1a and 17 in yeast suggest that they should be localized in the plasma membrane (Wang *et al.* , 2018). In this study, we found that CsSWEET17 was localized in the plasma membrane similar

to CsSWEET1a, which was inconsistent with the subcellular localization of AtSWEET17 (Figure 2a-h). In addition to the signal on the cell membrane, numerous small bright GFP signals generated by CsSWEET17-GFP were detected in the cells indicating that CsSWEET17 was not only localized in the cell membrane but could also be localized in other organelles (Figure 2e). We also found out that the extra sequences at its C-terminal did not affect the plasma membrane localization of CsSWEET17 (Figure 2i-l). However, it affected its sugar transport activity (Figure 2t). AtSWEET1 and AtSWEET17 can form heterooligomeric complexes; however, they are only localized on the plasma membrane and vacuole membrane, respectively (Xuan *et al.* , 2013). Therefore, we speculated that AtSWEET17 was localized not only on the vacuolar membrane but may also on the organelles where AtSWEET1 was localized.

Xuan*et al.* found that AtSWEETs could form at least 8 homomers and 47 heteromers through the split ubiquitin Y2H method (Xuan *et al.* , 2013). In our study, we found that CsSWEET1a and CsSWEET17 interacted on the plasma membrane to form homo- and heterodimers (Figure 3). Similar to the results of CsSWEET17 subcellular localization, BiFC assay showed bright signals near the plasma membrane in the cells expressing CsSWEET17, confirming that CsSWEET17 was not localized just in the plasma membrane (Figure 3b, f, n). However, after removing the C-terminal, signals near the plasma membrane disappeared, indicating that the extra C-terminal of CsSWEET17 indeed affected its subcellular localization, although it did not affect the its plasma membrane localization (Figure 2i-l). A single SWEET protein is too small to transport sugar by itself, and thereby must form at least homo- or heterodimers by oligomerization to produce a functional pores to transports sugar (Xuan *et al.* , 2013). Therefore, CsSWEET1a and CsWEET17 form heterodimers to transport sucrose and glucose (Wang*et al.* , 2018). CsSWEET17 may form homodimers to transport sucrose, glucose, fructose, galactose, and mannose, and CsSWEET1a and CsSWEET17 may function synergistically in tea plants.

Sugars, particularly glucose, affect plant growth and development (Dekkers, Schuurmans & Smeekens, 2004; Dekkers, Schuurmans & Smeekens, 2008). Increased AtSWEET16 activity in the *AtSWEET16* -OE lines can transfer excess sugar in the cytoplasm into the vacuole, thereby improve their germination efficiency (Klemens *et al.* , 2013). Here, the overexpression of *CsSWEET1a* and *CsSWEET17* inhibited plant growth and seed germination efficiency in treatments with high sugar concentration, respectively (Figure 4c; Figure 5b-c). During external sugar supplementation, the sugar transporter on the plasma membrane actively pumps external sugar into the cytoplasm, thereby increasing the sugar concentration in the cytoplasm (Buttner & Sauer, 2000). In a medium with 1.5% sucrose, the sucrose, glucose, and fructose contents of the three *CsSWEET1a* -OE lines were significantly higher than those of the WT plants, and the glucose and fructose contents of the two OE lines with higher CsSWEET17 expression levels were significantly higher than those of the WT plants (Figure 4e; Figure 5e). Therefore, plants overexpressing *CsSWEET1a* or *CsSWEET17* can transport more sugars into the cells, leading to sugar accumulation. Moreover, the sugar accumulation in *CsSWEET1a* -OE lines was higher than that in*CsSWEET17* -OE lines, which explained the promoted growth of*CsSWEET17* -OE lines (Figure 5d) and the yellowing of the leaves of*CsSWEET1a* -OE (Figure 4d) in 1.5% sugar concentration treatment. CsSWEET1a can also transport sugars into cytoplasm, whereas CsSWEET17 also functions in regulating the sugar homeostasis in the cytoplasm due to its subcellular localization to other unknown organelles.

Overexpressing *CsSWEET17* in *Arabidopsis* resulted in increased leaf and seed size (Figure 6). During plant growth and under sufficient or high nitrate conditions, *AtSWEET16* -OE lines strictly control the distribution of sugars in the cell, and most photosynthetic sugars can be used in metabolic processes, leading to their rapid growth (Klemens *et al.* , 2013). AtSWEET4 mediates the axial sugar transport during plant development, and the knockdown of*AtSWEET4* reduces the sugar content in axial tissues and the plant biomass (Liu *et al.* , 2016). CsSWEET17 may affect the photosynthetic efficiency of plants by controlling the sugar levels in the cytoplasm, thereby affecting the biomass of plants. SWEET sugar transporter can mediate the sugar transport into the seeds to address its developmental needs. ZmSWEET4c and OsSWEET4 mediate the transfer of hexose from the basal endosperm transfer layer (BETL) into the seeds to maintain their normal growth and development (Sosso *et al.* , 2015). AtSWEET11/12/15 play important roles in the seed filling stage of *Arabidopsis* because they synergistically mediate sucrose efflux during the transfer of sugar from the

seed coat to the embryo (Chen *et al.* , 2015b). Therefore, we speculated that the seed of the *CsSWEET17* -OE plants became larger because it mediates greater sugar transport from the seed coat to the embryo.

When plants were subjected to cold stress, the accumulation of soluble sugars to maintain the cell penetration potential was induced, thereby improving their cold resistance (Nagele, Stutz, Hormiller & Heyer, 2012; Rekarte-Cowie, Ebshish, Mohamed & Pearce, 2008). Over-expressing *CsSWEET1a* and *CsSWEET17* in *Arabidopsis* resulted in decreased RELs under freezing conditions compared to those in WT plants (Figure 7a-b). Because CsSWEET1a and CsSWEET17 are plasma membrane sugar transporters, we speculated that OE lines could transport more sugars from the cell wall into the protoplasts to increase the sugar contents in the cytoplasm, protecting cells from cold damage. Under abiotic stress, photosynthesis is suppressed to reduce the transfer of sucrose, which in turn inhibits the expression of the sucrose invertase (INV) gene (Boyer & McLaughlin, 2006). INVs can be sub-divided into cell-wall INV (CWINV), vacuolar INV (VACINV), and cytoplasmic INV (CINV). Zhang*et al.* found that *TaCWI* expression was down-regulated in low temperature treatment (Zhang *et al* ., 2019). As shown in Figure 7c-d, the gene expression levels of *AtCWINV1/3/6* and*AtVACINV1/2* in OE lines were significantly lower than those of WT plants under cold stress. This indicated that the sugar content in the cell wall of OE lines was lower than that of WT plants because CsSWEET1a and CsSWEET17 can transport more sugars from the cell wall into cytoplasm, and therefore the OE lines maintain the osmotic potential of the cells under cold stress. Furthermore, the induced sugar content in the cytoplasm of the OE lines affects sugar compartmentation and homeostasis between the cytoplasm and vacuole, resulting in the inhibition of *VACINV* expression. Based on these findings, we propose a hypothetical model of CSWEET1a and CsSWEET17 in regulating plant sugar transportation and cold resistance (Figure 8). CsSWEET1a and CsSWEET17 can form homo/heterodimers on the plasma membrane and transport sugars from the cell wall into the cytoplasm to increase its sugar concentration, therefore adjusting osmotic homeostasis to increase cold stress tolerance.

In summary, we have identified the functions of two plasma membrane-localized sugar transporters, namely CsSWEET1a and CsSWEET17, in *Camellia sinensis* . They regulated the growth and cold resistance of transgenic *Arabidopsis* by transporting sugar, which provided a theoretical basis for studying cold resistance of tea plants. Our research also laid the foundation for further research on the biological and physiological processes of the sugar-transporter genes in tea plants.

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AUTHOR CONTRIBUTIONS

LW and XW conceived and designed the research; LY, LW, CD, XH, YY and JZ performed the experiments; LY, LW and XW analyzed and discussed the data; LY and LW wrote the manuscript; all authors read and approved the manuscript.

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FIGURE LEGENDS

FIGURE 1 Expression analyses of *CsSWEET1a* and *CsSWEET17* during natural cold acclimation in the leaves of tea plants. (a-d) The expression levels of *CsSWEET1a* and *CsSWEET17* in the mature leaves of three cultivars in the winter of 2016 to 2017 and of 2017 to 2018. (e) Alternative spliced isoforms of *CsSWEET17* in tea plant. E1-5 represents the five exons. (f-g) The inclusion/exclusion ratio of the CsSWEET17 in the mature leaves of three cultivars in the winter of 2016 to 2017 and of 2017 to 2018. Data are shown as the mean ± SEM (n=3). Statistically significant data from LJ43 are labeled with asterisks (*P <0.05, **P <0.01, ***P <0.001, and ****P <0.0001). All results were calculated using the $2^{-\Delta\Delta\tau}$method and are expressed relative to *CsPTB* expression.

FIGURE 2 Subcellular localization of CsSWEET1a, CsSWEET17, and CsSWEET17_C in rice cell protoplasts and the sugar transport activities of CsSWEET17 and CsSWEET17_C in yeast mutant EBY.VW4000. (a-d) Rice protoplasts were co-transformed using 35S:CsSWEET1a:eGFP and OsMCA1:mKATE. (e-h) Rice protoplasts were co-transformed using 35S:CsSWEET17:eGFP and OsMCA1:mKATE. (i-l), Rice protoplasts were co-transformed using 35S:CsSWEET17_C:eGFP and OsMCA1:mKATE. (m-p) Rice protoplasts were transformed using 35S:GFP. The green signals indicate GFP in a, e, i, m, whereas the red signals indicate plasma membrane marker OsMCA1:mKATE in b, f, j. C, g, k, o, are bright fields whereas d, h, l, p are merged images. (q-s) Transmembrane domains of CsSWEET1a, CsSWEET17, and CsSWEET17_C were predicted by the TOPO2 software (http://www.sacs.ucsf.edu/TOPO2/). (t) Complementation assay in the yeast EBY.VW4000 mutant. Yeast transformants expressing empty vectors, CsSWEET17 and CsSWEET17_C, were cultured on SD (-Ura) media supplemented with 2% maltose, 2% fructose, 2% mannose, 2% glucose, 2% galactose, 2% sucrose, or 1% maltose+0.2% 2-deoxyglucose.

FIGURE 3 Interaction assays between CsSWEET1a and CsSWEET17.(a) Split-ubiquitin Y2H analysis. CsSWEET1a and CsSWEET17 were fused to the PBT3-STE vector (bait) and pPR3-C vector (prey), respectively, and then transformed into the yeast NMY51. The co-expression of CsSWEET1a bait vector with the pPR3-C empty vector and pTSU2-APP with the pPR3-C empty vector were used as negative controls, whereas the co-expression of pTSU2-APP with pNubG-Fe65 was used as positive controls. (b-u) BiFC assay. The co-transformations of (b-e) CsSWEET1a-P2YN and CsSWEET17-P2YC into *N. benthamiana* expressed with a nuclear marker, (f-i) CsSWEET1a-P2YC and CsSWEET17-P2YN, (j-m) CsSWEET1a-P2YN and CsSWEET1a-P2YC, (n-q) CsSWEET17-P2YN and CsSWEET17-P2YC, and (r-u) P2YN and P2YC vectors. The yellow signals indicate YFP in b, f, j, n, whereas the red signals indicate the nucleus in c, g, k, o, s. D, h, l, p, and t are bright fields, whereas e, i, m, q, and u are merged images. Scale bars represent 50 μm.

FIGURE 4 Overexpression of *CsSWEET1a* affects sensitivity of plant growth to sucrose and

glucose. (a) Expressions of CsSWEET1a in the leaves of *Arabidopsis* Col-0 and three *CsSWEET1a* -OE lines. Data are shown as the mean ± SEM (n=3). All values are expressed relative to the *AtEF* expression level using the $2^{-\Delta\Delta\tau}$ method. (b) Germination rate of seeds grown on the 1/2 MS medium without sugar, with 3% sucrose, and with 3% glucose. Data are shown as the mean ± SEM (n=4). (c) WT plants and three OE lines were grown on the 1/2 MS medium without sugar, supplemented with 3% sucrose, or 3% glucose for 24 d. Experiments were performed four times (n=30 each). (d) WT plants and three OE lines were grown on the 1/2 MS medium without sugar, supplemented with 1.5% sucrose, or 1.5% glucose for 18 d. Experiments were performed four times (n=40 each). (e) WT plants and three OE lines were grown on the 1/2 MS medium supplemented with 1.5% sucrose for 18 d, and the whole shoots were immediately collected for the measurements of soluble sugar, sucrose, glucose, and fructose contents. Data are shown as the mean ± SEM (n=3). Student's t-test are indicated by two (P<0.01), three (P<0.001), or four (P<0.0001) asterisks.

FIGURE 5 Germination efficiencies of WT and *CsSWEET17*-OE lines on different sugars. (a), Expressions of CsSWEET17 in the leaves of *Arabidopsis* Col-0 and three *CsSWEET17* -OE lines. Data are shown as the mean ± SEM (n=3). All values are expressed relative to the *AtEF* expression level using the $2^{-\Delta\Delta\tau}$method. (b) Germination rates of seeds grown on 1/2 MS medium without sugar or supplemented with 3/6% sucrose, 3/4% glucose, or 3% fructose. Data are shown as the mean ± SEM (n=4). (c) WT plants and three OE lines were grown on 1/2 MS medium without sugar, or supplemented with 6% sucrose, 4% glucose or 3% fructose for 18 d. Experiments were performed four times (n=30 each). (d) WT plants and three OE lines were grown on 1/2 MS medium without sugar, supplemented with 1.5% sucrose, 1.5% glucose, or 1.5% fructose for 18 d. Experiments were performed four times (n=45 each). (e) WT plants and three OE lines were grown on 1/2 MS medium supplemented with 1.5% sucrose for 18 d, and the whole shoots were immediately collected for the measurements of soluble sugar, sucrose, glucose and fructose contents. Data are shown as the mean ± SEM (n=3). Student's t-test are indicated by one (P<0.05) or two (P<0.01) asterisks.

FIGURE 6 Growth and seed phenotypes of WT plants and *CsSWEET17*-OE lines. (a-b) WT plants and OE lines grown under normal conditions for 18 d. (c-d) WT plants and OE lines grown under normal conditions for 24 d. (e) Fresh weight of the whole shoots of WT plants and OE lines grown under normal conditions for 24 d. Data are shown as the mean ± SEM (n=20). Student's t-test are indicated by one (P<0.05), three (P<0.001), or four (P<0.0001) asterisks. (f) Seed sizes of the WT plants and OE lines grown under normal conditions. Scale bars represent (a-d) 2 cm and (f) 2 mm.

FIGURE 7 Analyses of REL and *AtCWINV* and *AtVACINV* gene expression profiles in Col-0 and OE lines. (a-b) Analyses of REL in the OE lines and WT plants. 18-day-old plants were grown at 4 °C for 3 d, followed by the freezing treatment at 0 °C and dropped at a rate of 2 °C h^{-1} until –6 °C for 6 h. The whole shoots were collected for REL measurement. (c-d) RT-qPCR analysis of the expression levels in the OE lines and WT plants. 18-day-old plants were grown at 4 °C for 3 d and were then treated at 0 °C for 12 h. The whole shoots were collected for RNA extraction. Data are shown as the mean ± SEM (n=4). Student's t-test are indicated by one (P<0.05), two (P<0.01), or three (P<0.001) asterisks. All values were expressed relative to the *AtEF* expression level and were calculated using the $2^{-\Delta\Delta\tau}$ method.

FIGURE 8 The proposed hypothetical model of CSWEET1a and CsSWEET17 in regulating the sugar transportation and cold resistance in *Camellia sinensis*.

ELECTRONIC SUPPLEMENTARY MATERICAL

Table S1 Primer sequences for real-time PCR

Figure S1 Growth and seed phenotypes of WT plants and *CsSWEET1a* -OE lines.