

Genome-wide identification and expression analysis of *SWEET* gene family in daylily (*Hemerocallis fulva*) and functional analysis of *HfSWEET17* in response to cold stress

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Abstract

Background: The *SWEET* (Sugars Will Eventually Be Exported Transporters) was a newly discovered family of sugar transporters whose members exist in a variety of organisms and is highly conserved. The *SWEET* genes have been reported to be involved in growth and development in many plants, but little is known about in daylily (*Heimerocallis fulva*), which is an important perennial flower.

Results: In this study, a total of 19 daylily *SWEET* genes were identified and named based on its homologous genes in Arabidopsis and rice. Phylogenetic analysis classified these *HfSWEET* genes into four clades (Clade I to IV). The conserved motifs and gene structures showed that the *HfSWEET* genes were very conservative during evolution. Chromosomal localization and synteny analysis found that *HfSWEET* genes were unevenly distributed in eleven chromosomes and there were five pairs of segmental duplicated events and only one pairs of tandem duplication event. The expression patterns of the 19 *HfSWEET* genes showed that the expression levels of most *HfSWEET* genes were increased under low temperatures. Furthermore, *HfSWEET17* was overexpressed in tobacco and cold resistance of transgenic plants were much higher than in wild-type tobacco.

Conclusion: This study is the first report of identifying the *SWEET* gene family in daylily at the genome-wide level, and most of the genes were expressed in different temperature. The overexpression further showed that *HfSWEET17* genes was participate in daylily low-temperature response. The results of this study provide a basis for further functional analysis of the *SWEET* gene family in daylily.

Introduction

As the substrate of carbon and energy metabolism, sugar provides energy sources for plant growth and development^[1]. Sugar also participates in various signal transduction pathways in plants^[2]. But sugar can not independently cross the plant biomembrane system, assistance of the transport function of corresponding sugar transporters is needed^[3]. *SWEET* protein is a kind of sugar transporter that is largely pH-independent and bidirectional transmembrane transport of sugar along concentration gradient^[4–5]. It can selectively transport monosaccharides or disaccharides in cells or plasma membrane^[6]. It is widely found in prokaryotes, plants, humans and other animals^[7–8]. The typical structures of eukaryotic *SWEET* proteins consist of seven transmembrane helices, harboring two MtN3/saliva domains, also known as PQ-loop-repeat^[9]. However, the *SWEET* protein in prokaryotes contains only three transmembrane helical proteins and one MtN3/saliva domain^[10]. This difference may indicate that the eukaryotic *SWEET* protein evolved by replicating and fusing the basic 3-TM unit present in the prokaryotic semi-*SWEET* protein^[11].

The first identified plant *SWEET* transporter is At*SWEET1*, which acts as a single glucose transporter and involves in flower development by supplying nutrients to the gametophyte or nectaries^[6]. Arabidopsis (*Arabidopsis thaliana*) At*SWEET8* has also been shown to be critical for plant pollen viability^[12]. In addition to, numerous studies have shown that *SWEET* genes are involved in multiple biological processes, such as reproductive development, modulating gibberellins, disease resistance, abiotic stress, seed and fruit development^[13–16]. Genome-wide identification and analysis of *SWEET* gene family have been reported in a

variety of plant species, such as *Gossypium hirsutum*, *Arabidopsis*, *Sorghum bicolor*, rice (*Oryza sativa*), *Litchi chinensis*, *Glycine max* and so on^[9,11,17–19].

Daylily (*Hemerocallis fulva*) is a herbaceous perennial plant, with edible, medicinal and ornamental value, it is widely cultivated worldwide. In recent years, daylily has attracted biological investigation, there is a growing number of reports on daylily molecular mechanisms and gene function analysis^[20–23].

Our previous study analyzed the characteristics of daylily *HfSWEET2a* gene and its expression level changes at low temperature^[24]. Besides, there is no report on *SWEET* gene in daylily. In the current study, given the molecular mechanism of daylily gene family remains poorly understood, we performed whole genome-wide analysis to identify *SWEET* genes in daylily and analyze the phylogenetic relationships, gene structures, chromosomal localization, conserved motifs and domains of the *SWEET* genes in detail. Then the expression characteristics of daylily *SWEET* gene family members at different low temperatures stages were studied. Beyond that *HfSWEET17* gene was introduced into tobacco by *Agrobacterium*-mediated method to investigate its function. The results of this study provided some clues for understanding the function and cold response of *SWEET* gene.

Results

Identification of the daylily SWEET Gene Family

Through the screening, a total of 19 *SWEET* genes were obtained in daylily (GenBank accession No. OM264165–OM264183, Additional file 1: Table S1), named as *HfSWEET1–HfSWEET17* according to their identity percentage with *Arabidopsis AtSWEETs* and rice *OsSWEETs*. Gene characteristics, including the complete ORFs, number of amino acids (AA), molecular weight (MW), isoelectric point (pI) and so on were analyzed (Table 1). The results showed that the ORFs of the 19 *HfSWEET* genes ranged from 699 bp to 900 bp in length, encoding proteins 232 aa to 299 aa. *HfSWEET7* protein had the smallest MW with 25.764 kDa, and the largest one was *HfSWEET16* with 32.976 kDa. The pI ranged from 4.74 (*HfSWEET17*) to 9.64 (*HfSWEET16*), which indicated that most of the *HfSWEET* protein were basic proteins. The instability index ranged from 27.15 (*HfSWEET1b*) to 48.05 (*HfSWEET12*), both stable and unstable proteins were present in *HfSWEET* protein. All of the *HfSWEET* proteins were hydrophobic protein (Grand average of hydropathicity, GRAVY > 0). The results of the number of transmembrane helix analysis showed that majority of the *HfSWEET* proteins contained 7 transmembrane domains, only *HfSWEET4a* contained 6 transmembrane domains. These results indicated that the basic properties of the proteins encoded by members of the daylily *HfSWEET* gene family were different.

Phylogenetic Analysis of the HfSWEET Gene Family

In order to investigate the evolutionary relationships among *HfSWEET* proteins and *SWEET* proteins from *Arabidopsis* and rice (Additional file 2: Table S2), a neighbor-joining phylogenetic tree was constructed using MEGA 7 software. Results showed that the *HfSWEET* proteins were clearly divided into four clades (Clades I, II, III, and IV) (Fig. 1). The largest clade was Clade II, which consisted of seven *HfSWEET* proteins (*HfSWEET4a/4b/4c/5/6a/6b/7*); The second clade is Clade III, which contained six *HfSWEET* proteins

(HfSWEET12/13a/13b/14a/14b/15); Clade I contained four HfSWEET proteins (HfSWEET1a/1b/2a/3b); and Clade III were the fewest, containing only HfSWEET proteins (HfSWEET16/17). Compared with Arabidopsis, the similarity of SWEET gene between daylily and rice is higher, indicating that SWEET genes in daylily was more closely related to rice than to Arabidopsis.

Table 1
Information about daylily SWEET genes

Gene name	ORF length (bp)	AA (aa)	MW(kDa)	pI	II	AI	GRAVY	THM	MtN3/saliva (PQ-loop repeat) domain position
HfSWEET1a	735	244	27.162	9.26	40.21	111.80	0.656	7	7-95, 132-214
HfSWEET1b	762	253	28.351	9.01	27.15	105.49	0.519	7	7-95, 132-214
HfSWEET2a	699	232	25.908	8.81	42.55	124.78	1.018	7	18-100, 138-218
HfSWEET3b	714	237	26.322	9.44	38.27	117.22	0.700	7	7-98, 132-217
HfSWEET4a	774	257	28.370	9.30	28.83	127.35	0.800	6	10-95, 133-217
HfSWEET4b	735	244	26.993	8.89	37.65	130.82	0.942	7	10-97, 133-217
HfSWEET4c	735	244	26.959	8.95	34.37	132.87	0.941	7	10-98, 133-217
HfSWEET5	714	237	25.932	8.63	31.03	131.52	0.891	7	11-96, 134-213
HfSWEET6a	714	237	26.235	9.21	44.96	133.59	0.957	7	9-98, 133-217
HfSWEET6b	840	279	30.460	9.03	35.24	118.14	0.669	7	11-96, 133-217
HfSWEET7	711	236	25.764	9.22	36.30	134.49	1.106	7	10-95, 133-213
HfSWEET12	786	261	29.196	8.94	48.05	122.53	0.721	7	14-98, 132-218
HfSWEET13a	825	274	30.706	9.20	29.62	118.43	0.667	7	12-99, 133-214
HfSWEET13b	870	289	32.278	5.74	34.67	116.99	0.516	7	12-82, 134-215
HfSWEET14a	861	286	32.412	8.80	33.51	123.36	0.593	7	12-99, 133-215

AA: Number of amino acids; MW: Molecular weight(kDa); pI, isoelectric point

II: Instability index; AI: Aliphatic index; GRAVY: Grand average of hydropathicity

THM: Prediction of the number of transmembrane helix

Gene name	ORF length (bp)	AA (aa)	MW(kDa)	pI	II	AI	GRAVY	THM	MtN3/saliva (PQ-loop repeat) domain position
HfSWEET14b	861	286	32.276	8.80	31.49	122.38	0.596	7	12-99, 133-215
HfSWEET15	870	289	32.116	5.49	38.42	121.45	0.724	7	12-98, 134-215
HfSWEET16	900	299	32.976	9.64	34.03	112.04	0.442	7	7-91,128-211
HfSWEET17	723	240	26.934	4.74	43.23	119.67	0.730	7	7-90,128-212
AA: Number of amino acids; MW: Molecular weight(kDa); pI, isoelectric point									
II: Instability index; AI: Aliphatic index; GRAVY: Grand average of hydropathicity									
THM: Prediction of the number of transmembrane helix									

Conserved motifs and conserved domains analyses of HfSWEETs

The conserved motifs and conserved domains were analyzed to further understand the characteristics of *HfSWEETs*. The results of conserved motif analysis showed that a total of 10 motifs were identified and named motif 1 to 10 (Fig. 2). Motif 1 to 5 were detected in all HfSWEET proteins except HfSWEET12, which lacking Motif 5. Motif 6 was detected in two members in each of Clade I, II, and III, but not in any member of Clade IV. Motif 7 was detected in the members of the Clade II and Clade III. Motif 8 and motif 9 were only detected in some members of the Clade II. Motif 10 was only detected in HfSWEET3b, HfSWEET12 and HfSWEET16 protein.

The results of conserved domain analysis showed that all the HfSWEET proteins harbored two MtN3/saliva domains or PQ-loop superfamily at the similar positions (Fig. 2). These MtN3/saliva domains ranged from 70 aa to 91 aa, and most of them were approximately 85 aa in length. The position of the MtN3/saliva domains in the protein were shown in Table 1. In addition, there are no other conserved domains.

Gene structure analysis of HfSWEETs

In order to elucidate the structural characteristics of daylily *SWEET* genes, the exon-intron organization was analyzed. The result showed that five or six exons were exist in most *HfSWEETs* (Fig. 3). The *HfSWEET* genes in the Clade I, III and IV all contained 6 exons; Majority *HfSWEET* genes in the Clade II contained 5 exons, while *HfSWEET5* and *HfSWEET6a* containing 6 exons, and *HfSWEET7* containing 7 exons. In general, the introns length of *HfSWEET* members in Clade II and IV were longer than that in Clade I and III. These results revealed that *HfSWEET* genes in the same clade share similar gene structure.

Chromosomal localization and syntenic analysis of HfSWEETs

According to the gene loci information, the 18 *HfSWEET* genes were unevenly distributed in eleven chromosomes of daylily and the detailed chromosomal locations were shown in the Figure 4. By contrast, *HfSWEET16* was distributed on a scaffold whose exact locations on chromosome was not determined. Chromosome 2 and 9 had the largest number of *HfSWEETs* (three genes), followed by chromosomes 1, 3, 4, 5 and 10 (two genes on each chromosome), and the minimum number was found on chromosome 8 and 11 (one gene). Except for the *HfSWEET7* gene, other *HfSWEET* genes were located in the middle and lower part of the chromosomes.

According to the results of collinearity analysis, there was five pairs of segmental duplicated events of *HfSWEET* genes in daylily genomes. The most frequently duplicated gene was *HfSWEET13a*, which duplicated three times, which corresponding to *HfSWEET13b*, *HfSWEET14b*, and *HfSWEET15*, respectively. *HfSWEET4a/7* and *HfSWEET4a/4b/4c* may also be generated by fragment duplication. In addition to, *HfSWEET14a/14b* was clustered into tandem duplication events. Based on the above results, some *HfSWEET* genes were probability generated by gene segmental or tandem duplication. The results of collinearity analysis between daylily and Arabidopsis and rice show that seven *HfSWEET* homologous protein genes appear in the last three chromosomes of Arabidopsis (Fig. 5), but there were nine *HfSWEET* genes that can find corresponding paralogous genes on six chromosomes on rice. It can be seen that the relationship between daylily and rice is closer than that of Arabidopsis.

Expression profiles of *HfSWEETs* under low temperature

To obtain insights into the physiological functions of the *HfSWEETs* in response to low temperature stress, the expression patterns of 19 *HfSWEETs* under different temperature (25 °C as CK, low temperature treatments: 10 °C, 5 °C and 0 °C) were measured by qRT-PCR analysis. The results showed that the expression patterns were different among the 19 *HfSWEETs* (Fig. 6). Compared with the CK (25 °C), with the decrease of temperature, the relative expression levels of nine *HfSWEETs* increased first and then decreased, but three *HfSWEETs* showed contrary expression trend. The relative expression levels of five *HfSWEETs* (*HfSWEET3b*, *HfSWEET5*, *HfSWEET14b*, *HfSWEET16*, *HfSWEET17*) were higher than CK at all lower temperatures. Among them, the expression level of *HfSWEET5* and *HfSWEET17* rose steadily as the temperature drops. However, three *HfSWEETs* (*HfSWEET1a*, *HfSWEET12*, *HfSWEET13b*) were lower than CK at all lower temperatures and the expression level of *HfSWEET1a* gradually decreased with the decrease of temperature. In general, the relative expression of majority *HfSWEETs* were up-regulated by low-temperature treatment, and most of them was highest at 10 °C or 0 °C, which were 1.43–57.95 times than CK.

Subcellular localization analysis of *HfSWEET17* protein

The *HfSWEET17* had the highest relative expression level in the daylily *SWEET* family, and the expression level of it gradually increased with the decrease of temperature. In order to explore the function of *HfSWEET17* in daylily, the subcellular localization of *HfSWEET17* protein was studied. *HfSWEET17* protein was transiently expressed as translational GFP (green fluorescent protein) fusion proteins in tobacco leaf epidermal cells. Confocal images of transient expression of GFP fusion protein in protoplasts was showed that 35S:*HfSWEET17*-GFP fusion protein was mainly presented in the cytoderm (Fig. 7). This result suggested that *HfSWEET17* protein was cytoderm-localized.

Ectopic expression of HfSWEET17

To further explore the function of *HfSWEET17* in the response to low temperature stress, it was chosen to be ectopically expressed in tobacco through *Agrobacterium*-mediated transformation. Under normal conditions (25 °C), the leaf size of transgenic plants was significantly larger than those of the WT plants (Fig. 8). When exposed to cold stress condition, all lines received mild cold injury, chlorosis and leaf margins slightly curled before the temperature drops to 5 °C, but no significant difference between transgenic and WT plants was observed. When the temperature reached 0 °C, all lines were wilted, but transgenic plants showed significantly better status than the WT plants under low temperature treatment (Fig. 8).

The level of REL and the activity of POD were measured. In normal condition, the REL and POD were not significantly different between the WT and transgenic plants. With the decrease of temperature, REL and POD of leaves from all transgenic and WT plant leaves showed a trend of first increasing and then decreasing (Fig. 9). The transgenic plants showed significantly lower REL values of 1.13-, 1.26-, 1.23-, and 1.08-fold under 15 °C, 10 °C, 5 °C, 0 °C, respectively, than that of WT plants. The activity of POD was significantly increased in transgenic plants compared with that of WT plants and was 1.32-, 1.51-, 1.15-, 1.42-, and 1.2-fold higher under 20 °C, 15 °C, 10 °C, 5 °C, 0 °C, respectively.

Discussion

Plant SWEETs play significant roles in physiological metabolism, growth, and development by regulating sugar transport and distribution^[5]. For example, they are involved in pollen wall formation, anther dehiscence, seed development and responses of various abiotic stresses^[25–27]. Recently, the *SWEET* gene families from some plant species have been reported. A growing number of evidence suggests SWEETs play important roles in low-temperature response^[16, 28]. In the present study, we identified and characterized *SWEET* gene family in daylily by genome-wide analysis and investigated their expression patterns under low temperature treatment.

SWEET gene family in daylily

In this study, we successfully identified 19 *HfSWEETs* based on daylily genome and named them as *HfSWEET1–HfSWEET17* based on their homologies in *Arabidopsis* and rice (Table 1). The length of *HfSWEET* proteins ranged from 232 aa to 299 aa, which was similar to that has been reported in other plants, such as 229–300 aa in litchi, 233–308 aa in tomato, and 234–301 aa in *Gossypium hirsutum*^[8, 11, 19]. Phylogenetic analysis divided 19 *HfSWEETs* into four clades (Clade I to IV) which is consistent with the results in *Arabidopsis*, *Litchi chinensis*, and *Gossypium hirsutum*^[6, 8, 19]. Each clade contained 4, 7, 6, and 2 *HfSWEET* members in daylily, respectively (Fig. 1), which was similar to other plants^[29–30]. The results of the intron–exon location analysis showed that the number and distribution of the introns and exons of *HfSWEETs* were highly conserved, and most *HfSWEETs* possessed five or six exons (Fig. 3). It has been indicated that the results of conserved motif analysis were similar to those of phylogenetic analysis^[30–31]. Our results were consistent with those reports. The *HfSWEETs* in each clade harbored some special conserved motifs (Fig. 2), which suggested that they might have different functions in daylily.

Further chromosomal localization and synteny analysis showed that 18 *HfSWEET*s were unevenly distributed on eleven chromosomes of daylily, and only one (*HfSWEET16*) was distributed on scaffold. Collinearity analysis showed there were segmental duplicated events and tandem duplication events in the daylily *HfSWEET* gene family (Fig. 4). This suggested that *HfSWEET*s in daylily might have evolved from gene duplication. Gene duplication, including whole-genome duplication, tandem gene duplication and segmental duplication events, can be a crucial factor for plant gene family evolution^[32], and the latter two have been suggested to represent the main causes of gene family expansion in plants^[33]. Following gene duplication, duplicated gene pairs can undergo different functions^[34]. Combined with the above analysis of the characteristics of *HfSWEET*s, we speculated that the expansion of *HfSWEET* genes might play an important role in various gene functions of *HfSWEET*^[34].

Expression patterns and function diversity of *HfSWEET*s in response to low temperature stress

The differential expression analysis of *SWEET*s can help to explore the special functions of *SWEET* proteins. The expression of *SWEET* genes has been shown to change in response to chilling stress in several plant species^[28,31]. Analyzing the expression pattern of 19 *HfSWEET*s under low temperature treatment, we found that compared with the control group (25 °C), the expression levels of all *HfSWEET*s in the low temperature (10 °C, 5 °C, 0 °C) treatment group were changed and the relative expression levels of most *HfSWEET*s were increased (Fig. 6), suggesting that more than one *HfSWEET* gene were responsive to low-temperature stress. The expression patterns of 19 *HfSWEET*s were different, the relative expression of most of them was highest at 10 °C or 0 °C, suggesting that these genes may have functional redundancy.

Retained duplication genes were generally believed to be those involved in neofunctionalization, subfunctionalization, and nonfunctionalization, among which, neofunctionalization and subfunctionalization can lead to the differential spatial and temporal expression of duplication genes^[35]. In the present study, the expression patterns of the pairs of duplicated genes in daylily under low temperature stress were various. For example, some duplicated genes, such as *HfSWEET4a/4b* and *HfSWEET4a/7* were the same, whereas that of some duplicated genes like *HfSWEET13a/13b* and *HfSWEET14a/14b* were significantly different. These results indicated that some duplicated *HfSWEET* genes were functionally similar may due to nonfunctionalization during gene replication, while some duplicated *HfSWEET* genes may have developed neofunctions or subfunctions and were functionally different^[32, 35]. These results were consistent with results reported for litchi and apple^[19, 36].

Ectopic expression of *HfSWEET17* improved cold stress tolerance in transgenic tobacco

In *Arabidopsis*, *AtSWEET17* is a vacuolar fructose transporter that participates in the regulation of fructose levels and controls leaves fructose content^[37–38], and is critical for root development and drought tolerance^[39]. *DsSWEET17* from *Dianthus spiculifolius* affected the sugar metabolism and conferred multiple tolerance in transgenic *Arabidopsis*^[40]. In the present study, the *HfSWEET17* gene was highly expressed under low temperature treatment (Fig. 6). To further evaluate the roles of *HfSWEET17* gene in response to cold stress condition, the *HfSWEET17* from daylily was transformed into tobacco. Morphological observations revealed that, the leaf size of the *HfSWEET17*-overexpressed lines was obviously larger than those of the WT

plants under the non-stress growth condition (Fig. 7), indicating that *HfSWEET17* may promote nutrition and reproductive growths by transporting and utilizing sugars, which was consistent with the experimental results of Yao et al^[41]. Under 0 °C treatment condition, *HfSWEET17*-overexpressed plants showed significantly better status than the WT plants (Fig. 8), indicating that transgenic plants were less damaged by chilling.

Analysis of physiological indices showed that the *HfSWEET17*-overexpressed tobacco exhibited lower REL and higher POD under cold stresses compared to the WT plants, which conferred cold tolerance in transgenic tobacco. These results indicated that *HfSWEET17* from daylily positively regulates cold stress in tobacco. Similar studies have previously reported that the overexpression of *CsSWEET17* gene from *Camellia sinensi* increased sugar transport in Arabidopsis, thus affecting plant germination and growth, and improving freezing resistance^[41]. However, the roles of *HfSWEET17* was limited such as the transgenic plants did not produce enough influence to reverse tobacco performance under cold stress in this study. The effect may be made even more pronounced through the formation of homo- or heterodimers by oligomerization^[10]. However, the biological function of this potential interaction remain to be further investigated.

Conclusions

In summary, this study identified the *SWEET* gene family in daylily at the genome-wide level. Nineteen *HfSWEET* genes were identified and comprehensively characterized, including phylogenetic analysis, conserved motifs prediction, exon-intron structure, chromosomal localization, and synteny analysis. Phylogenetic analysis classified 19 *HfSWEET* genes into four clades (Clade I to IV). We also focused on the expression patterns of all the *HfSWEET*s under low temperature treatments, which indicated that they may involve in low temperature stress signaling pathway regulation. Furthermore, the overexpression of *HfSWEET17* gene improved cold stress tolerance in transgenic tobacco. This study laid the foundation for elucidate the functions of the *HfSWEET* genes in daylily under low-temperature response.

Materials And Methods

Plant material

Daylily cultivar ‘Golden Doll’ was grown in the botanical garden of Shanghai Institute of Technology, Shanghai, China. As the material of this experiment, it has the advantages of long flowering period and strong multiple resistance in Shanghai, China. Plants used for samples collection were under the same integrated botanical garden management practices. Leaves were collected and immediately frozen in liquid nitrogen and kept at -80 °C for experiment.

Identification and molecular cloning of SWEET gene family in daylily

The *SWEET* family member domain Hidden Markov Model (MtN3_slv, PF03083.15) from the Pfam (<http://pfam.xfam.org/>) database^[42], was used to retrieve daylily genome database (unpublished) by HMMER3.0 and SPDE software^[43–44]. The results were sequentially sorted to remove redundancy, and candidate genes of daylily *SWEET* gene family members were preliminarily obtained. Then, the candidate

sequences were identified by SMART (<http://smart.embl-heidelberg.de>) and NCBI-CCD (<https://www.ncbi.nlm.nih.gov/cdd>)^[45–46].

Leaf total RNA was extracted using the Quick RNA isolation Kit and the quality of the RNA was analyzed by 1.5% (w/v) agarose gel electrophoresis and NanoDrop One. The first-strand cDNA was synthesized using M-MuLV First Strand cDNA Synthesis Kit. The coding sequences of daylily SWEET genes were amplified from cDNA using gene-specific primers (Additional file 3: Table S3). PCR amplification was carried out using Taq DNA Polymerase Kit in a PCR Thermal Cycler (Bio-Rad, S1000, USA). All PCR products were purified with the Prep Column PCR Product Purification Kit, then the purified PCR products were sequenced and the consensus sequences were deposited in GenBank (Additional file 1: Table S1). All the above kits and primers were provided by Sangon, Shanghai, China.

Sequence analyses

ProtParam (<https://web.expasy.org/protparam/>) was used to analyze the amino acids, molecular weights, and theoretical isoelectric point of daylily SWEET gene family members. Transmembrane domains were predicted by TMHMM Server v2.0, and the MtN3/saliva (PQ-loop repeat) domain position was searched by NCBI-CCD.

Phylogenetic analysis

Arabidopsis AtSWEET and rice OsSWEET protein sequences were obtained from TAIR database (<https://www.arabidopsis.org/>) and NCBI database (<https://www.ncbi.nlm.nih.gov>), respectively. Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) was used to perform homologous sequence alignment of the protein sequences of daylily, rice and Arabidopsis (Additional file 2: Table S2)^[47]. Based on the results of sequence alignment, a neighbor-joining phylogenetic tree was constructed by MEGA7.0 with 500 bootstrap replicates^[48].

Gene structure analysis and prediction of conserved motifs and domains

The exon-intron structures were analyzed by GSDS (<http://gsds.cbi.pku.edu.cn>). The MEME (<http://meme-suite.org/>) was used for conserved protein motif prediction, and the NCBI conserved domain database was used to predict the conserved domains of *SWEET* gene family members of daylily.

Chromosomal distribution and gene synteny analysis

The positions of daylily *SWEET* genes on chromosomes were obtained from the daylily genome annotation files (unpublished). Arabidopsis and rice genome were both obtained from Ensembl plants (<https://plants.ensembl.org/index.html>). Furthermore, the synteny analysis among members of the daylily *SWEET* family members and the synteny analysis between daylily and Arabidopsis and rice were constructed using the MCScanX and TBtools^[49–50].

Expression profiles of SWEET genes in daylily

The ‘Golden Doll’ daylily was moved to an indoor incubator at a constant temperature and cultured at 25 °C (control group, CK), 10 °C, 5 °C and 0 °C with a 12 h photoperiod for 24 h respectively. Samples were

collected from fully expanded functional leaves. All samples were frozen in liquid nitrogen immediately after collection and stored at -80 °C. Primer based on cDNA sequences of daylily *SWEET* family members were design by Primer5 (<https://sg.idtdna.com/pages/tools/primerquest>) (Additional file 3: Table S3). UBQ was used as the internal reference for real-time quantitative PCR (qRT-PCR)^[51].

Total RNA was extracted from leaves by the Quick RNA isolation Kit (Sangon, Shanghai), and the first-strand cDNA was synthesized using M-MuLV First Strand cDNA Synthesis Kit (Sangon, Shanghai). Real-time quantitative PCR amplification was performed by AceQ qPCR SYBR Green Master Mix (Vazyme Biotech). Amplification was initiated with a denaturation step of 5 min at 95 °C, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. Fluorescence signals were detected at the end of every cycle. All reactions were performed using the Real-Time PCR Detection System (QuantStudio 5, USA), and data were analyzed using QuantStudio™ Design and Analysis Software. All reactions were performed in triplicate. Changes in gene expression were calculated using the $2^{-\Delta\Delta C_t}$ method^[52]. Statistical analysis was performed by SPSS 20 software, and one-way ANOVA was performed for the relative expression of *HfSWEETs* under different temperatures.

Construction of HfSWEET17 transient expression vectors and subcellular localization

The open reading frame (ORF) of HfSWEET17 was amplified using primers containing the *EcoRI*/*SpeI* restriction sites, and the expression vector 35S:HfSWEET17-YFP was constructed using pc131-YFP vector framework. The recombinated plasmids were then transformed into *Agrobacterium Tumefaciens* strain GV3101 through shock transformation^[53]. *Agrobacterium tumefaciens* was cultured and injected into tobacco (*Nicotiana benthamiana*), and the fluorescence distribution in leaf cells was observed under confocal laser microscope (Leica STELLARIS 5, Germany) after 48 h dark culture. The primers used were listed in Additional file 3: Table S3.

Generation of transgenic plants with HfSWEET17

The ORF of *HfSWEET17* was inserted into the *EcoRI*/*SalI* restriction sites of pCAMBIA1301. The obtained plasmid was transformed into *Agrobacterium tumefaciens* strain GV3101. Generation of transgenic tobacco was performed following the of leaf dish transformation method. Transgenic plants were selected using hygromycin B (50 mg/L) and confirmed by PCR analysis. The positive plants were harvested and sown. Each generation of transgenic plants was verified by PCR to ensure that *HfSWEET17* gene was inserted into the tobacco genome. The T3 transgenic tobacco plants and wild-type tobacco (WT) plants were cultivated under the same growing condition.

Nine-leaf stage plants were placed in a light incubator with 12 h photoperiod, and the samples were collected after 48h of incubation at 25 °C as control group (CK). Then, the culture temperature was lowered to 20 °C, 10 °C, 5 °C, 0 °C for 48h, respectively. Fully expanded leaves were collected after each treatment. Each group was set up with six biological replicates. Then, the level of relative electrolyte leakage (REL) and the activity of peroxidase (POD) were measured for each sample. Using WT tobacco plants served as the negative control, the cold resistance of transgenic plants was analyzed.

Abbreviations

SWEETs: Sugar Will Eventually be Exported transporters; qRT-PCR: Quantitative real-time polymerase chain reaction; ORFs: Open reading frames; REL: Relative electrolyte leakage; POD: Peroxidase; AA: Amino acids; MW: Molecular weight; pI: Isoelectric point; II: Instability index; AI: Aliphatic index; GRAVY: Grand average of hydropathicity; THM: Prediction of the number of transmembrane helix; GFP: Green fluorescent protein; WT: Wild-type; CK: Control group.

Declarations

Ethics approval and consent to participate

All methods used in the manuscript were performed in accordance with relevant guidelines and regulations.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and analysed during the current study are available in the GenBank repository, GenBank accession is No. OM264165–OM264183 and all sequences were provide in Additional file 1.

Competing interests

The authors declare that there are no conflict of interest.

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Authors' contributions

QQ was responsible for the guidance of experiments and the revision of manuscript. DH was responsible for the designed and performed of experiments, carried out data analysis and wrote manuscript. YC was responsible for the culture of experimental samples. All authors reviewed the manuscript.

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Figures

Figure 1

Phylogenetic tree of SWEETs from daylily, Arabidopsis, and rice

The protein sequences of the 54 SWEETs from daylily, Arabidopsis, and rice were aligned by Clustal Omega, and the phylogenetic tree was constructed by the MEGA7.0 using the neighbor-joining method with 500 bootstrap replicates. SWEETs of daylily, Arabidopsis, and rice were prefixed with Hf, At, and Os, respectively, and represented by Black circles, hollow circles, and five-pointed star, respectively.

Figure 2

Phylogenetic relationships, conserved motif and conserved domain analyses of HfSWEET proteins

(A) The neighbor-joining phylogenetic tree of putative HfSWEET proteins was constructed by MEGA7 with 500 bootstrap replicates. The daylily *SWEETs* were classified into four clades (Clades I, II, III, and IV). (B) The motif composition of HfSWEET proteins. Ten motifs were displayed in different colored rectangles. (C) The domain composition of HfSWEETs. Green rectangles represent MtN3/saliva domain and yellow rectangles represented PQ-loop domain.

Figure 3

Gene structures of 18 *HfSWEETs* from daylily.

The exon-intron structures were analyzed by GSDS (<http://gsds.cbi.pku.edu.ch>). Exons were represented by green boxes, upstream/downstreams were represented by blue boxes, and introns were represented by black lines.

Figure 4

Locations and duplications of *HfSWEETs* on daylily chromosomes.

The chromosome location of *HfSWEET* genes were shown by short grey lines. The red lines indicate segmentally duplicated genes, and the tandemly duplicated genes are boxed.

Figure 5

The synteny analysis between daylily and Arabidopsis and rice genome.

Syntenic relationship between daylily and Arabidopsis and rice genome shown on the chromosome maps. The gray line represents the collinearity among all members, and the green line represents the collinearity among the members of the SWEET family.

Figure 6

Expression profiles of *HfSWEETs* under different temperatures.

A represented 25 °C for the control group, while B, C and D represented 15 °C, 10 °C and 5 °C, respectively. The colored bar represents the average of the relative expression levels. Statistical analysis was performed by SPSS 20 software, and one-way ANOVA was performed for the relative expression of *HfSWEETs* under different temperatures.

Figure 7

Subcellular localization of HfSWEET17 protein.

HfSWEET17-GFP fusion protein and GFP alone (as control) were constructed using pc131-YFP vector framework and transiently expressed in *Nicotiana benthamiana* leaves using shock transformation. Protein localization was examined 48 h after dark culture and representative images are shown. Bar = 10 µm.

Figure 8

Phenotypic changes of WT and transgenic plants under cold treatment.

Nine-leaf age plants were placed in a light incubator with 12 h photoperiod, and cultured at 25 °C for 48 h as control group (CK). Then, the culture temperature was lowered to 20 °C, 15 °C, 10 °C, 5 °C, 0 °C for 48h, respectively.

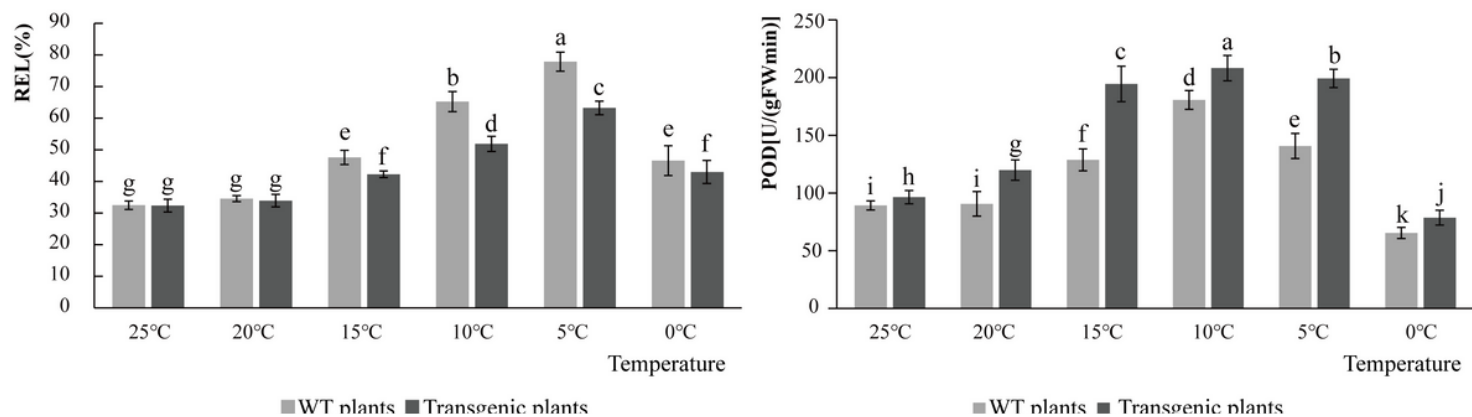


Figure 9

REL and POD changes of WT and transgenic plants under cold treatment. Nine-leaf age plants were cultured at each temperature for 48 hours. Columns denote mean the average under each temperature, and standard error were marke.

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