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SWEET transporters and their potential roles in response to abiotic and biotic stresses in mulberry

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Abstract

Mulberry (*Morus spp*., Moraceae) is a traditional economic crop plant and is also being gradually utilized as a beverage plant. SWEETs (Sugars Will Eventually be Exported Transporter) are important sugar transporters involved in various biological processes and responses to various stresses. However, SWEETs in mulberry are still poorly studied without a comprehensive functional analysis of *SWEETs*. In the present study, a total of 24 *SWEETs* were identified using the *Morus alba (Ma)* genome. Phylogenetic analysis showed that these 24 MaSWEETs were clustered with SWEETs from *Arabidopsis*, *Populus* and *Oryza* and fell into four clades. *MaSWEETs* in the same clade are likely to pose similar intron/exon patterns. These *MaSWEETs* distributed on 12 chromosomes and tandem duplication and block duplication were responsible for the expansion of SWEETs in mulberry. Transmembrane domains and conserved active sites of Tyr and Asp were observed in MaSWEETs. Cis-elements in promoter regions of *MaSWEETs* indicated the possible function of *MaSWEETs* in response to hormones and environment stimulus. *MaSWEETs* showed quite different expression preference in tissues and organs indicating the possible function divergence. In addition, most *MaSWEETs* showed a disturbed expression levels in response to various abiotic stresses and *Ciboria shiraiana* infection. *MaSWEET1a* was functionally characterized as a negative regulator of resistance to *C. shiraiana* infection based on *in vivo* transient overexpression of *MaSWEET1a* in tobacco and down-regulation of *MaSWEET1a/b* in mulberry. Our results provided foundation for further functional dissection of SWEETs in mulberry and a potential regulator for genetic modification.

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INTRODUCTION

Sugars are predominant carbon and energy sources and support plant vegetative and reproductive growth[1]. Transport of sugars across the plant bio-membrane needs the assistance of specific transporters[1,2]. These transporters act as bridges that mediate the distribution of sugars between source–sink organs, which is critical for sugar homeostasis and the cellular exchange of sugar efflux in multicellular organisms[1,3,4].

SWEETs (Sugars Will Eventually be Exported Transporter) and SUTs (sucrose transporters), MSTs (monosaccharide transporters) are the main known sugar transporters in eukaryotes[5] . Unlike SUTs and MSTs, the relatively newly reported sugar transporter SWEETs are pH-independent transporters. SWEETs play important roles in phloem transport and act as bidirectional transmembrane transporters of sugars along the concentration gradient[3,4]. *AtSWEET1* was first identified as a glucose transporter with clear functional characterization[2]. In addition, the SWEET multi-gene family was identified and classified into four clades and the functional divergence of these paralogs were also revealed in *Arabidopsis* at the same time[2]. For example, Clade II *AtSWEET8* contributes to pollen viability and Clade III *AtSWEET15* is involved in leaf senescence. Thereafter, another SWEET in *Arabidopsis* Clade III *AtSWEET9* was characterized as an important transporter involved in nectar secretion[6]. Besides the SWEET family in *Arabidopsis*, the SWEET gene family has been identified in many plants including Tea (*Camellia sinensis, Cs*), tomato (*Solanum lycopersicum*, Sl), wheat (*Triticum aestivum*, *Ta*) barrel medic (*Medicago truncatula*, *Mt*), cabbage (*Brassica rapa, Br*), daylily (*Hemerocallis fulva, Hf*), grapevine (*Vitis vinifera, Vv*), rice (*Oryza sativa*, *Os*) and poplar (*Populus trichocarpa, Pt* and *P. alba* × *P. glandulosa, Pag*)[7–13]. These SWEET homologs belong to the MtN3/saliva family and consist of seven α-helical transmembrane domains (TMs): a tandem repeat of three transmembrane domains (TMs) connected with a linker-inversion TM[2,14].

SWEETs participate in various biological processes including development, flowering, stress responses and plant-pathogen interaction in plants[4,15]. In addition, different SWEET gene family members show functional divergence or redundancy. *In Arabidopsis*, *AtSWEET8* and *13* support pollen development; *AtSWEET11* and *12* provide sucrose to the SUTs for phloem loading and play distinct roles in seed filling; and *AtSWEET9* is essential for nectar secretion[2–4,6,16]. *BrSWEET9* in *Brassica rapa* was also reported to be involved in nectar secretion[17].

Overexpression of *PagSWEET7* promotes secondary growth and xylem sugar content[12]. *OsSWEET11* and *15* have functions affecting pollen development and are key players in seed filling in rice[18]. SWEET homologs also play important roles in abiotic stress responses. Overexpression of *AtSWEET16* promotes freezing tolerance in *Arabidopsis*[19] while *AtSWEET11* and *12* mutants exhibit greater freezing tolerance[20]. *AtSWEET15* could be induced by various abiotic stresses including osmotic, drought, salinity, and cold stresses and overexpression of *AtSWEET15* results in transgenic plants with hypersensitiveness to cold and salinity stresses[21]. *CsSWEET1a* and *CsSWEET16* were also reported to mediate freezing tolerance[22,23]. Recently, more studies have shown that SWEETs are involved in plant-pathogen interaction and are known as susceptibility (S) genes, acting as targets of effector proteins during host–microbe interactions in many plant species[15]. *GhSWEET10,* is induced by *Avrb6*, a transcription activator-like (TAL) effectors from *Xanthomonas citri* subsp. *Malvacearum (Xcm)* and is responsible for maintaining virulence of *Xcm avrb6* and the cotton susceptibility to infections[24]. *OsSWEET11–15* belonging to clade III in rice have been shown to be induced by TAL effector from *Xanthomonas oryzae* and support pathogen growth[25]. In contrast, some *SWEET*s could also function as resistance genes. Overexpression of *IbSWEET10* can promote resistance to *F. oxysporum* in sweet potato[26]. Mutation of *AtSWEET2* resulted in increased susceptibility to the root necrotrophic pathogen *Pythium irregulare*[27].

Mulberry (*Morus spp., Moraceae*) is a traditional economic crop plant and a new beverage plant. In addition, its fruits are rich in nutrient and bioactive components and the ripening process of mulberry fruits along with sugar accumulation and distribution. Mulberry suffers various abiotic stresses and the disasterous fungal disease sclerotiniose which bursts at the early stage of mulberry fruit development[28–30]. Mulberry fruits with sclerotiniose lose their color and flavor and turn pale instead of ripening. *C. shiraiana* is the dominant causal agent of mulberry sclerotiniose in China, and it results in hypertrophy sorosis sclerotiniose. SWEETs as the important transporters involved in sugar homeostasis are expected to be involved in mulberry fruit development and interaction with sclerotiniose pathogens. However, to date, few studies on SWEETs have been reported in mulberry, although the SWEET gene family may play important roles in mulberry fruit development and responses to abiotic and biotic stresses. Mulberry genome information has been released successively since the *Morus notabilis* genome was reported in 2013[31]. The chromosome-level genome of *M.alba* (*Ma*) was released by Jiao et al. and the genome of *M. yunnanensis* was recently released by Xia et al.[32,33]. Released genome information makes it possible to perform genome-wide characterization of the *SWEET* gene family in mulberry. In the present study, a total of 24 *SWEET* genes were identified in the *Morus alba* genome and their phylogenetic classification, conserved motifs, gene structures, distribution on chromosomes, cis-elements in promoter regions and tissue expression profile were revealed. In addition, the responses of *MaSWEET*s to various abiotic stresses and sclerotiniose pathogen infection were also detected. *MaSWEET1a* was functionally characterized as a negative regulator which increased the mulberry susceptibility to *C. carunculoides* infection.

MATERIALS AND METHODS

Plant materials and treatments

The xylem, phloem, fruits at four different developmental stages (S0, inflorescence; S1, green fruits; S2, reddish fruits; S3, purple fruits)) and diseased fruit infected with *C. shiraiana* of *Morus atropurpurea variety Zhongshen 1* (*Mazs*) were collected from the National Mulberry Genebank (NMGB) in Zhenjiang, China for expression profiling. Seedlings of the *M. alba* var. *Fengchi* and tobacco (*Nicotiana Benthamiana*) were grown in a chamber at 22 °C with a 16/8 day/night cycle and 40%–60% humidity. *C. shiraiana* was provided by Professor Zhao and was cultured in potato dextrose agar (PDA) medium.

Tobacco at the four-leaf stage was used for transient overexpression. *M. alba* var. *Fengchi* seedlings at the four-euphylla stage were used for virus-induced gene silencing (VIGS). Four-week-old seedlings with similar growth conditions (~12–15 cm high) were used for treatments under different abiotic stresses. Detailed information for abiotic stress treatments were reported in our previous study[34]. All the above samples were immediately frozen in liquid nitrogen after collection and then stored at −80 °C until use. Three biological replicates were performed for each experiment.

Identification of the SWEET gene family in *Morus alba*

The *M. alba* genome sequences (.fasta) and annotation file (.gff) were generously provided by Professor Jiao, who released this genome information. The Hidden Markov Model (HMM) profiles of the SWEET domain (PF03083) were downloaded from the Pfam database (http://pfam.xfam.org/) and used to search the candidate SWEET proteins in the *M. alba* proteome with HMMER software. In addition, the protein sequences of AtSWEETs, OsSWEETs and PtSWEETs were downloaded from TAIR (www.arabidopsis.org/), TIGR (http://rice.plantbiology.msu.edu/) and phyto-zome (https://phytozome-next.jgi.doe.gov/) respectively, and used as queries to search against the *M. alba* proteome. The Toolbox for Biologists v1.098774[35] was used to analyze the sequence length, molecular weight and theoretical isoelectric point (pI) values of each MaSWEET protein. The distributions of TM helices were predicted by the TMHMM Server v. 2.0 (www.cbs.dtu.dk/services/TMHMM). Prediction of subcellular localization of MaSWEET proteins using the online Tool WoLF PSORT (www.genscript.com/wolf-psort.html)[36].

Chromosomal location and synteny analysis of *MaSWEET*s

Chromosome location information of *MaSWEET*s was extracted based on the *Morus alba* genome annotation file. Tbtools v1.098774 were used to identify syntenic blocks and tandem duplication events using default parameters[37,38]. The results were visualized using Tbtools v1.098774 and both the tandem duplication and block duplication gene pairs were marked.

Sequence alignment and motif analysis

MaSWEETs were aligned using clustal W assembled in MEGA11.0. The alignment result was exported and manually speculated for scanning the MtN3 repeats. The online MEME Suite version 5.5.0 was used to identify 7 conserved motifs from 24 amino acid sequences of SWEET genes in *Morus alba*. The Hidden Markov Model (HMM) profiles of the SWEET domain (PF03083) were downloaded from the Pfam database.

Gene structure and promoter analysis of *MaSWEETs*

The gene structure of each *MaSWEET* was displayed based on the genome sequence and its annotation file using Gene Structure View assembled in Tbtools v1.098774. The upstream 2000 bp sequences were extracted for *in silico* promoter region analysis. Cis-acting elements were predicted using PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Phylogenetic analysis of MaSWEETs

A neighbor-joining (NJ) phylogenetic tree was constructed using full-length SWEETs protein sequences from *A. thaliana*, *P. trichocarpa*, *O. sativa* and *M. alba* using MEGA11.0[26] with JTT + G model and bootstrap test with 1000 replicates.

RNA extraction and RT-qPCR analysis

RNA extraction and cDNA synthesis were performed as in our previous report using Plant RN52 Kit (Aidlab, Beijing, China) and PC54-TRUEscript RT kit (Aidlab, Beijing, China) according to the manual[39]. RT-qPCR (quantitative real-time PCR) was performed to validate the expression patterns of *MaSWEETs* in different tissues, fruit development stages and stresses using ABI StepOnePlus™ Real-Time PCR System (USA). The primers are available in Supplemental Table S1. Actin was used as a reference gene[40]. Graphpad Prism8.0 was used to visualize the RT-qPCR results and to perform T-test and ANOVA. P value < 0.05 was marked as significant. At least three individuals were used and three technical replicates respectively were performed for RT-qPCR.

Transient overexpression of *MaSWEET1a* in *Nicotiana Benthamiana*

The recombinant plasmids *pNC-1304-35S:MaSWEET1a* were constructed using nimble cloning[41]. Both recombinant plasmids *pNC-1304-35S: SWEET1a* and empty vector *pNC-Cam1304-35SMCS*, as the negative control, were transformed into Agrobacterium tumefaciens GV3101 and then transferred into *N. benthamiana* leaves *via* Agrobacterium-mediated transient transformation, as previously reported[41]. Overexpression of *MaSWEET1a* was determined using RT-qPCR by comparing the expression levels of target genes in transgenic plants with those in the negative controls.

Obtaining *MaSWEET1a/b* VIGS Transgenic Mulberry

Virus-induced gene silencing (VIGS) was used to obtain *MaSWEET1a/b* down-regulated mulberry, in accordance with our previous report[42]. *Agrobacterium tumefaciens* containing recombinant plasmids *pTRV2-MaSWEET1a/b*, *pTRV1* and *pTRV2* (negative control) were cultured in transient transformation buffer and then transferred into mulberry leaves by means of pressure injection. Ten independent mulberry plants were injected. The knock-down efficiency for the target genes was determined by RT-qPCR 15 d after injection by comparing the transgenic plants with the negative controls and wild types.

Estimation of plant resistance to *C. shiraiana* infection

Cell death symptoms and the growth condition of *C. shiraiana* were recorded to estimate the resistance of transgenic plants to *C. shiraiana* infection[43,44]. *C. shiraiana* was inoculated at 2 d after infiltration in tobacco and at 10 d after infiltration in mulberry. The cell death symptoms were photographed after inoculation until the sclerotia appeared. The results are representative of at least three biological replicates.

RESULTS

Genome-wide identification and phylogenetic analysis of *SWEET*s in *M.alba*

A total of 24 *SWEET* homologs were identified based on the genome information of *M. alba* and named according to their orthologs in *A. thaliana, P. trichocarpa* or *V. vinifera*[45]. These *MaSWEETs* encode proteins ranging from 197 aa to 304 aa with molecular weight from 21.45 to 34.12 kDa and theoretical isoelectric points from 7.16 to 9.61 (Table 1). Subcellular localization prediction of these MaSWEETs showed that most of them (18/24) distributed on membrane structures such as plasma membrane (PM), tonoplast membrane (TM) and chloroplast thylakoid membrane (CTM). Phylogenetic analysis of MaSWEETs and SWEETs from model plants such as *A. thaliana*, *P. trichocarpa* and *O. sativa* showed that four clades were formed by these SWEETs (Fig. 1). According to previous studies, SWEETs in plants were generally classed as four phylogenetic clades which is in agreement with our results[8]. Major MaSWEETs (10/24) together with AtSWEET1, 2 and 3 belong to clade I. Clade II and IV contain five MaSWEETs each and Clade III contains four MaSWEETs (Fig. 1 and Table 1).

Chromosomal location and gene duplication

*MaSWEET*s distributed on 12 chromosomes except chromosome 1 and 3. Chromosome 5 occupied five *MaSEET*s which formed a gene cluster. Chromosome 5 is the chromosome that had the most *SWEET*s and the following is chromosome 6 and 8 which had four *MaWSEET*s each. There was only one *MaSWEET* locating on chromosome 4 (Fig. 2). In addition, there were three *MaSWEET*s on chromosome 2 and 7 respectively and two *MaSWEET*s on chromosome 1 and 5 respectively. Gene duplication including block duplication and tandem duplication is the main cause for gene family expansion. Tandem duplications were found on chromosome 2, 6 and 12 (linked by red lines in Fig. 2). It is also interesting to find several possible gene clusters such as *MaSWEET1a-b* on chromosome 5, *MaSWEET2a-g* on chromosome 9, *MaSWEET7 a-b* on chromosome 13, *MaSWEET17a-b* on chromosome 12 and *MaSWEET17c-d* on chromosome 6. Two gene pairs (*MaSWEET4b*/*MaSWEET5* and *MaSWEET11b*/ *MaSWEET15*) resulting from block duplications were also marked (linked by black lines in Fig. 2).

Sequence analysis of MaSWEETs

MaSWEETs always located on membrane structures with transmembrane domains. The prediction results of MaSWEETs using DeepTMHMM showed that most MaSWEETs posed seven types of transmembrane helices (TMH) named TMH1-7 (Table 1, Supplemental Fig. S1). Alignment and conserved motif analysis showed that almost all MaSWEETs kept the conserved TMH and active sites Tyr and Asp indicating by red full triangles (Fig. 3). The active residues Tyr and Asp were reported to be involved in forming hydrogen bonds to ensure sugar transport activity[14]. In addition, all MaSWEETs except MaSWEET4a had a conserved Ser in each triple helix bundle (THB) which can be phosphorylated and is important for SWEET activity (Fig. 3). MaSWEET4a replaced Ser with Thr at the first Ser phosphorylation site between TMH1 and TMH2 which may also retain similar activity as Thr is also a common phosphorylation site. All MaSWEETs retained the conserved second Ser phosphorylation site between TMH5 and TMH6 (Fig. 3).

Table 1. SWEET gene family in *Morus alba*.

Clade	Gene name	Accession no.	Gene ID	CDS Size (bp)	Protein physicochemical characteristics				TMHs	Subcellular	MtN3/Saliva (PQ-Loop Repeat)
					Length (aa)	MW (kDa)	pI	Aliphatic index		Localization*	Domain Position
I	*MaSWEET1a*	XM_024170697.1-0	M.alba_G0012049	729	242	26.55	9.61	113.51	7	CTM	6-94, 131-209
I	*MaSWEET1b*	XM_024170698.1-0	M.alba_G0012049	678	225	21.45	9.51	115.74	6	CTM	1-49, 86-164
I	*MaSWEET2a*	XM_024163709.1-0	M.alba_G0019244	708	235	25.94	8.39	129.79	7	CTM	18-104, 137-221
I	*MaSWEET2b*	XM_024164777.1-0	M.alba_G0010863	705	234	25.92	8.98	122.86	7	TM	17-101, 137-218
I	*MaSWEET2c*	XM_024163707.1-0	M.alba_G0019244	774	257	28.58	8.58	132.33	7	PM	54-126, 159-243
I	*MaSWEET2d*	XM_024163712.1-0	M.alba_G0019244	681	226	25.27	8.22	128.89	6	EX	23-95, 128-212
I	*MaSWEET2e*	XM_024163708.1-0	M.alba_G0019244	729	242	26.72	8.49	128.06	7	CTM	39-111, 144-228
I	*MaSWEET2f*	XM_024163703.1-0	M.alba_G0019244	777	258	28.8	8.49	132.56	8	PM	41-127, 160-244
I	*MaSWEET2g*	XM_024163711.1-0	M.alba_G0019244	684	227	25.49	7.62	129.16	7	PM	10-96, 129-213
I	*MaSWEET3*	XM_010099554.2-0	M.alba_G0003063	783	260	29.01	8.89	115.73	7	PM	9-98, 132-216
II	*MaSWEET4a*	XM_010091939.1-0	M.alba_G0009276	735	244	27.45	9.28	109.39	7	CTM	10-98, 134-218
II	*MaSWEET4b*	XM_010113461.2-0	M.alba_G0001536	738	245	27.4	8.98	122.08	7	EX	11-97, 134-216
II	*MaSWEET5*	XM_024168739.1-0	M.alba_G0018295	711	236	26.64	7.63	120.93	7	CY	10-93, 131-127
II	*MaSWEET7a*	XM_010108966.2-0	M.alba_G0005110	774	257	28.32	9.57	128.56	7	CTM	11-95, 134-218
II	*MaSWEET7b*	XM_010108964.1-0	M.alba_G0005109	789	262	28.99	9	124.96	7	CTM	10-97, 134-218
III	*MaSWEET10*	XM_010095631.2-0	M.alba_G0018016	888	295	33.12	8.86	120.31	7	CTM	11-96, 132-216
III	*MaSWEET11a*	XM_010114440.2-0	M.alba_G0016901	804	267	29.63	9.47	124.08	7	CTM	11-99, 135-220
III	*MaSWEET11b*	XM_010095633.2-0	M.alba_G0018015	915	304	34.12	7.57	112.89	7	CTM	12-99, 134-219
III	*MaSWEET15*	XM_010092381.2-0	M.alba_G0006767	885	294	33.42	7.16	109.01	7	CTM	12-99, 133-219
IV	*MaSWEET16*	XM_024167733.1-0	M.alba_G0014617	909	302	33.2	9.08	114.27	7	CTM	20-92, 129-212
IV	*MaSWEET17a*	XM_024171451.1-0	M.alba_G0014614	708	235	26.46	8.71	119.87	5	CY	5-78, 116-198
IV	*MaSWEET17b*	XM_024167902.1-0	M.alba_G0014613	753	250	28.05	8.71	120.88	6	PM	8-93, 131-213
IV	*MaSWEET17c*	XM_024171286.1-0	M.alba_G0008195	720	239	26.72	8.94	111.72	7	CY	6-92, 129-212
IV	*MaSWEET17d*	XM_024171287.1-1	M.alba_G0008196	723	240	27	9.43	122.67	7	CTM	6-92, 127-213

* The subcellular localizations were predicted by WoLFPSORT. PM, plasma membrane; EX, extracellular; CY, cytoplasmic; TM, tonoplast membrane; CTM, chloroplast thylakoid membrane.

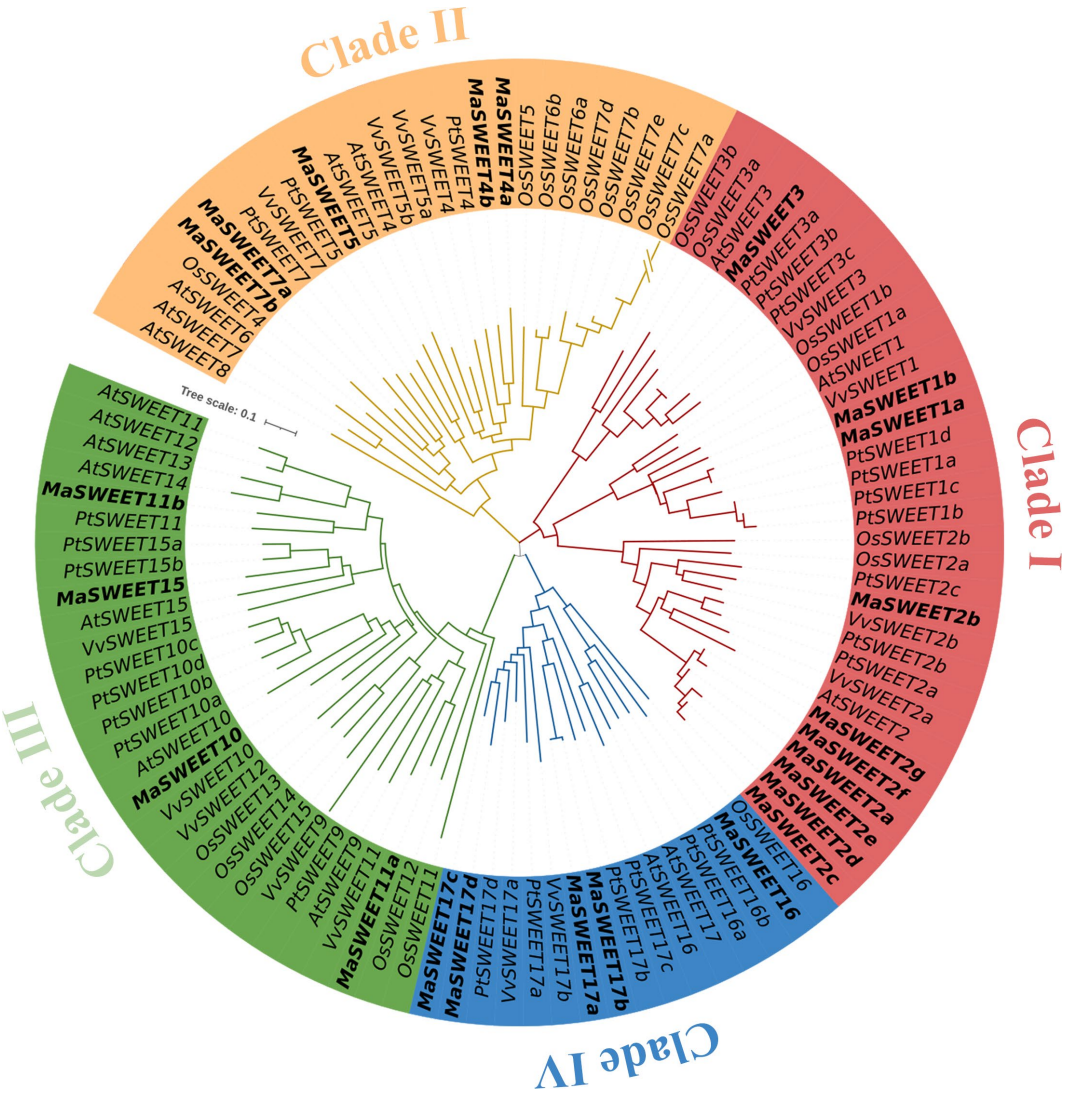

Fig. 1 Phylogenetic relationships of the SWEET family genes in *Arabidopsis*, *Oryza sativa*, *Populus*, *Vitis vinifera*, and *Morus alba*. The sequences of the 104 SWEET proteins from the above four plant species were aligned by Clustal Omega, and the phylogenetic tree was constructed by the MEGA 11.0 using the NJ method with 1000 bootstrap replicates. The proteins from *Arabidopsis*, *Oryza sativa*, *Populus*, *Vitis vinifera*, and *Morus alba* are indicated with the prefixes of At, Os, Pt, Vv, and Ma, respectively.

Gene organization and promoter analysis of *MaSWEETs*

*MaSWEET*s gene structures were identified based on the annotation information of the *M. alba* genome. In summary, there were six *MaSWEET*s with six introns, 12 *MaSWEET*s with five introns and five *MaSWEET*s with four introns (Fig. 4). In addition, genes clustered together based on phylogenetic analysis are likely to show similar gene structures and length. For example, *MaSWEET2a*, *c*, *d*, *e*, *f* and *g* with six introns, *MaSWEET7a* and *MaSWEET7b* with four introns, and *MaSWEET17a* and *MaSWEET17b* with four introns.

Promoter region analysis of *MaSWEET*s indicated the possible function of *MaSWEET*s in response to hormones and environment stimulus. Among all the 22 types of cis-elements identified, most of them are light response elements accounting for 44% of the total elements (Supplemental Table S2). In addition, hormone response elements were also widely identified in the promoters of *MaSWEET*s (Fig. 4c). Most *MaSWEET*s had abscisic acid (ABA), salicylic acid (SA) or methyl jasmonate (MeJA) related response elements in their promoter regions. Especially, *MaSWEET1a-b*, *MaSWEET16*, *MaSWEET17a-d* had cis-elements involved in response to five types of hormones (ABA, SA, MeJA, auxin and gibberellins). Several Myb binding cis-elements were also identified in promoter regions of *MaSWEET2a* and *MaSWEET10* (Fig. 4c, Supplemental Table S2).

Expression profile of *MaSWEET*s in different tissues of mulberry

The tissue or organ expression profiles of *MaSWEET*s were revealed. The *MaSWEET*s with high sequence identity (> 91%) are hard to distinguished by RT-qPCR and were determined by common primers to reveal their total transcription levels. *MaSWEET*s in phylogenetic clade I showed quite similar expression patterns with highest expression levels in leaf and relatively higher expression levels in early stages (S0 and S1) of fruit development except *MaSWEET1a/b* (Fig. 5a–d). *MaSWEET1a/b* showed higher expression levels in fruits during whole fruit development with highest expression level in fruit at S0 stage (Fig. 5a). However, *MaSWEET*s (*MaSWEET10*, *11a-b*, and *15*) in phylogenetic clade III showed preferential expression

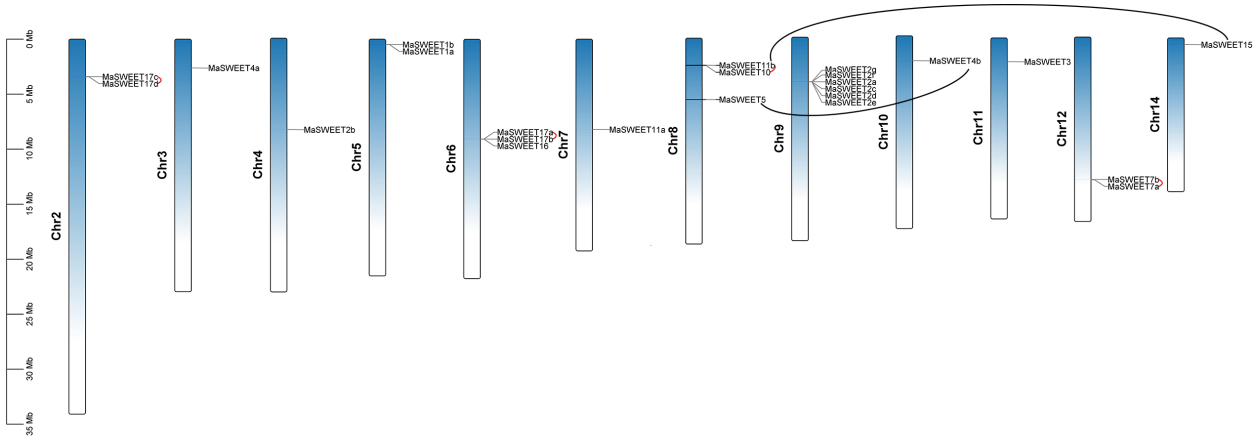

Fig. 2 Distribution of MaSWEET genes in *Morus alba* chromosomes. The tandem gene pairs are linked by red lines. The block duplications gene pairs are marked by black lines. The scale is provided in megabase (Mb).

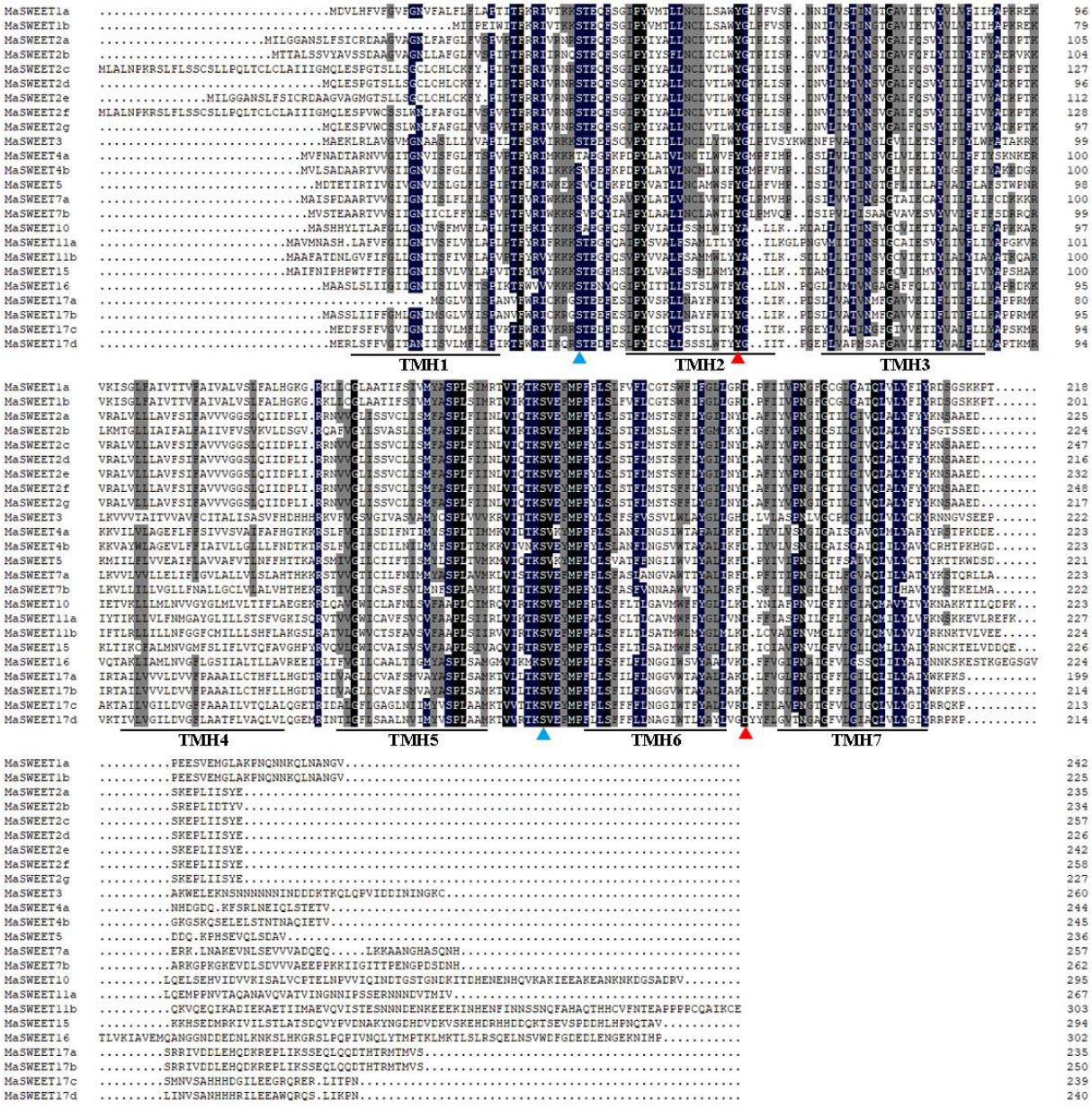

Fig. 3 Multiple sequence alignment of MaSWEET proteins. The positions of the TMHs are underlined. The positions of the active sites of tyrosine (Y) and aspartic acid (D) are indicated by red triangles. The conserved serine (S) phosphorylation sites are indicated by blue triangles.

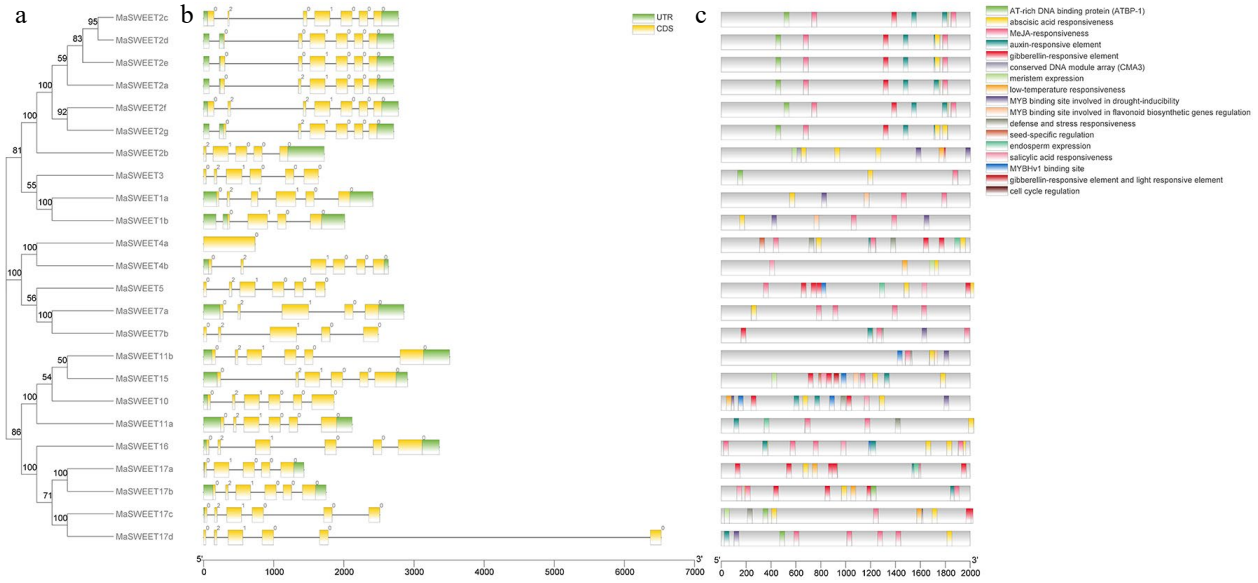

Fig. 4 Gene organization of MaSWEETs and cis-elements in promoter regions of MaSWEETs. (a) Phylogenetic tree using 24 MaSWEETs. (b) Exon/intron structures of *Morus alba* L. SWEETs. (c) Cis-element distribution in the promoter regions of MaSWEETs.

in fruits especially at the late stages (S2 and S3) (Fig. 5j–m). *MaSWEET*s in phylogenetic clade II had different expression patterns in different tissues or organs. *MaSWEET4a* and *b* showed highest expression level in fruit at the S1 stage while *MaSWEET5* showed obviously preferential expression in xylem (Fig. 5e–g). *MaSWEET7a* and *b* showed similar expression pattern with *MaSWEET2* cluster and *MaSWEET3* from phylogenetic clade I. *MaSWEET*s in phylogenetic clade IV showed similar expression pattern with highest expression levels in leaf (Fig. 5b, d, h, i). *MaSWEET16* also had higher expression in fruit with similar expression level at four different development stages (Fig. 5n).

Transcription-level responses of *MaSWEET*s to various stresses

Mulberry sclerotiniose is a fungal disease resulting from fungal pathogen infection. Most (20/24) *MaSWEET*s showed positive or negative responses to the fungal infection. *MaSWEET1a/b*, *MaSWEET2* cluster, *MaSWEET4b*, and *MaSWEET17 a-d* showed a significant decrease of expression levels in diseased fruits with sclerotiniose compared with the expression levels in healthy fruits (Fig. 6). In contrast, *MaSWEET2b*, *MaSWEET3*, *MaSWEET7b*, *MaSWEET10* and *MaSWEET11a-b* showed significant increases of expression levels in diseased fruits. *MaSWEET*s also played roles in response to various abiotic stresses including drought, water logging, cold and high temperature. *MaSWEET1a/b* showed a positive response to drought with significant increasing expression levels while other clade I *MaSWEET*s, *MaSWEET2b* and *3* significantly decreased their expression level under detected abiotic stresses (Fig. 7a–d). In contrast, *MaSWEET16* significantly increased its expression level under detected abiotic stresses. *MaSWEET4a-b*, *MaSWEET5* in phylogenetic clade II and *MaSWEET11a-b* in clade III showed similar response patterns with a significant increase of expression levels in response to low temperature (4 °C), high temperature (40 °C) or drought (Fig. 7e–g). *MaSWEET15* showed high sensitivity for drought and significant increase of expression levels under drought stress. *MaSWEET17a-d* showed a negative response to temperature change with a significant decrease of expression levels under low temperature and high temperature treatments (Fig. 7o–q).

Functional characterization of *MaSWEET1a* in response to sclerotiniose infection

MaSWEET1a/b is quite different from other *MaSWEET*s in clade I based on expression profile analysis, which showed preferential expression in fruits and a negative response to sclerotiniose pathogen (*Ciboria shiraiana*) infection. Our unpublished data indicated *MaSWEET1a* as key genes involved in the pathogen infection process based on comparative transcriptome analysis. Transient overexpression of *MaSWEET1a* in tobacco and VIGS knock-down of *MaSWEET1a/b* in mulberry were performed. RT-qPCR results validated the successful overexpression of *MaSWEET1a* in tobacco and knock-down of *MaSWEET1a/b* in mulberry (Fig. 8b, d). The expression level of *MaSWEET1a* affected the resistance to *C. shiraiana* infection in both tobacco and mulberry (Fig. 8a, d). Overexpression of *MaSWEET1a* decreased the resistance to *C. shiraiana* infection with more severe cell death symptoms observed in OE-line tobacco (Fig. 8a). Knock-down of *MaSWEET1a/b* in mulberry could increase the resistance to *C. shiraiana* infection in mulberry (Fig. 8d). These results proved that *MaSWEET1a* is an important negative regulator of resistance to *C. shiraiana* infection in mulberry.

DISCUSSION AND CONCLUSIONS

Functional studies on SWEETs have revealed that SWEET homologs not only act as loading and unloading transporters of sugars but also play critical roles in various biological processes. Typically, angiosperm genomes contain about 15–25 *SWEET* genes[4]. In the present study, a total of 24 *SWEET* genes were identified and clustered into four clades corresponding with the knowledge of SWEETs in angiosperm. Several SWEETs including *MaSWEET1a/b*, *MaSWEET4a-b*, *MaSWEET10*, *MaSWEET11a-b* and *MaSWEET15* showed preferential expression in fruits indicating their possible roles in fruit

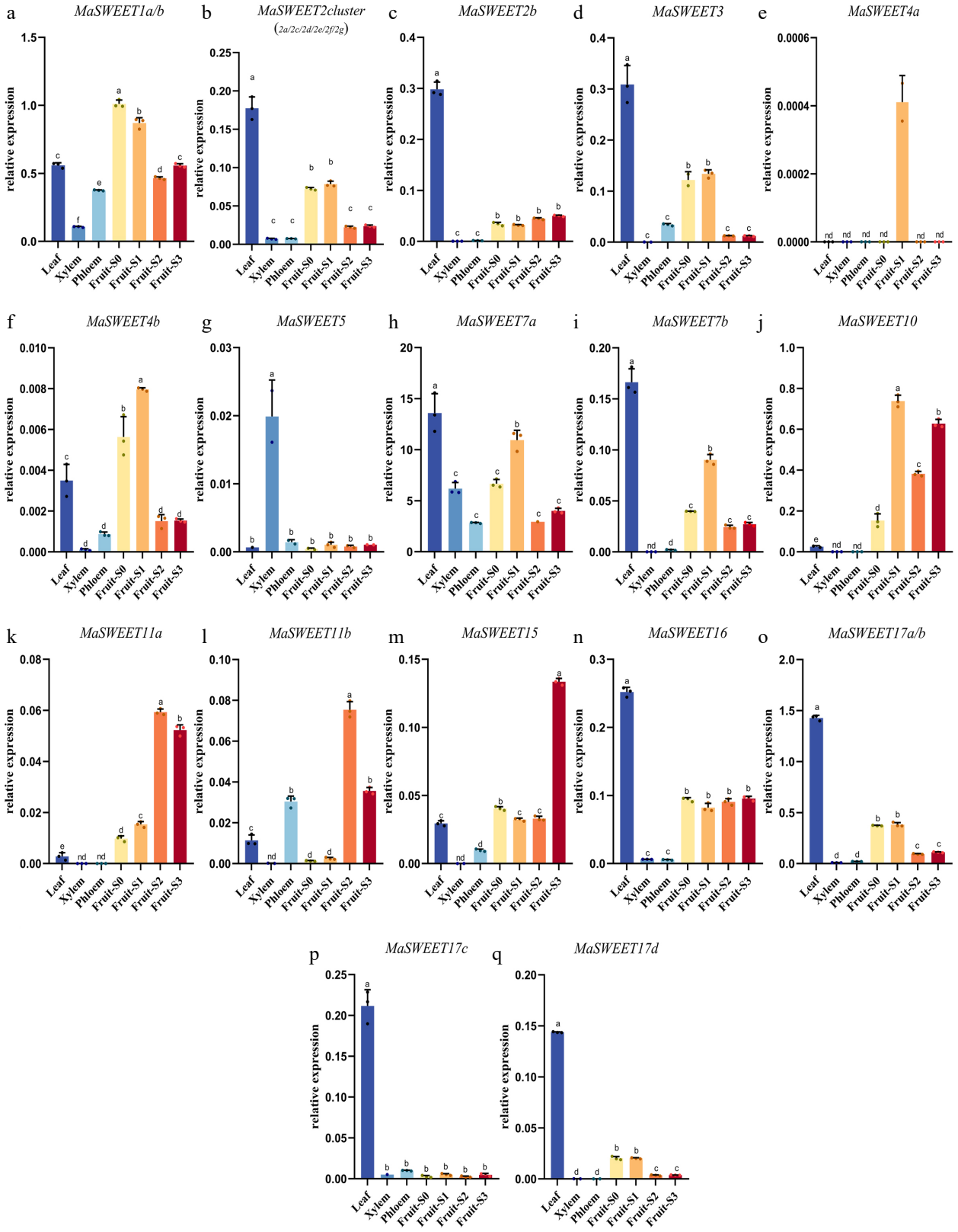

Fig. 5 Transcript levels of MaSWEETs in leaves, xylem, phloem, and different development stages of fruit. Three technical replicates were analyzed. Error bars represent SE. Different letters indicate statistically significant differences (Duncan's test, $p < 0.05$).

development. Similar expression preference of *AtSWEET*s was also reported in *Arabidopsis*[2]. It is noted that fruit-preferential expressed *MaSWEET*s still showed temporal expression difference during fruit development indicating time-course regulation of *MaSWEET*s for fruit ripening in mulberry. Early-stage expressed *MaSWEET1a/b* and *MaSWEET4a/b* further

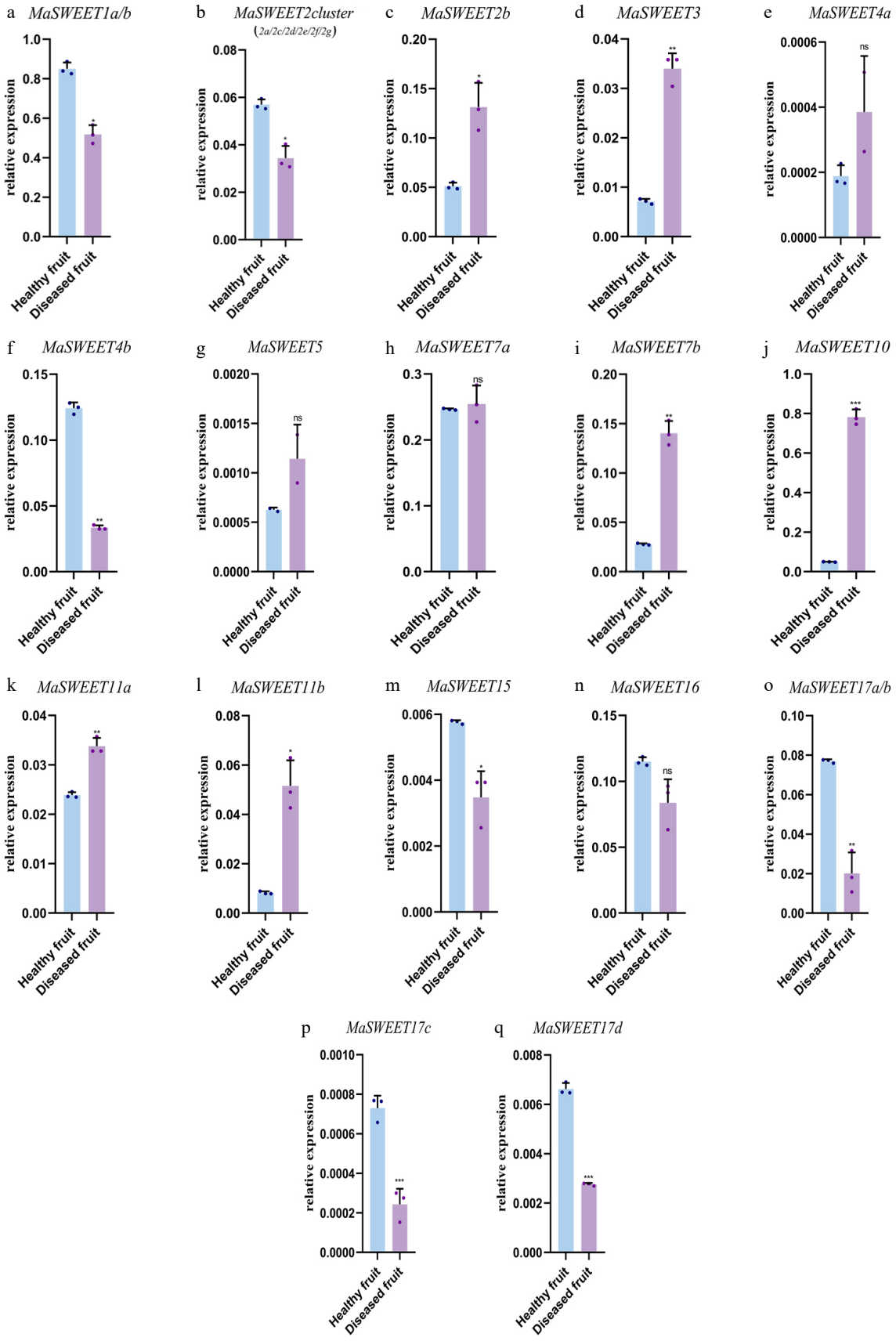

Fig. 6 Expression levels of 24 selected *MaSWEET* genes in response to fungi stress conditions. Three technical replicates were analyzed. Error bars represent SE. Asterisks indicate significant difference as determined by Student's t-test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

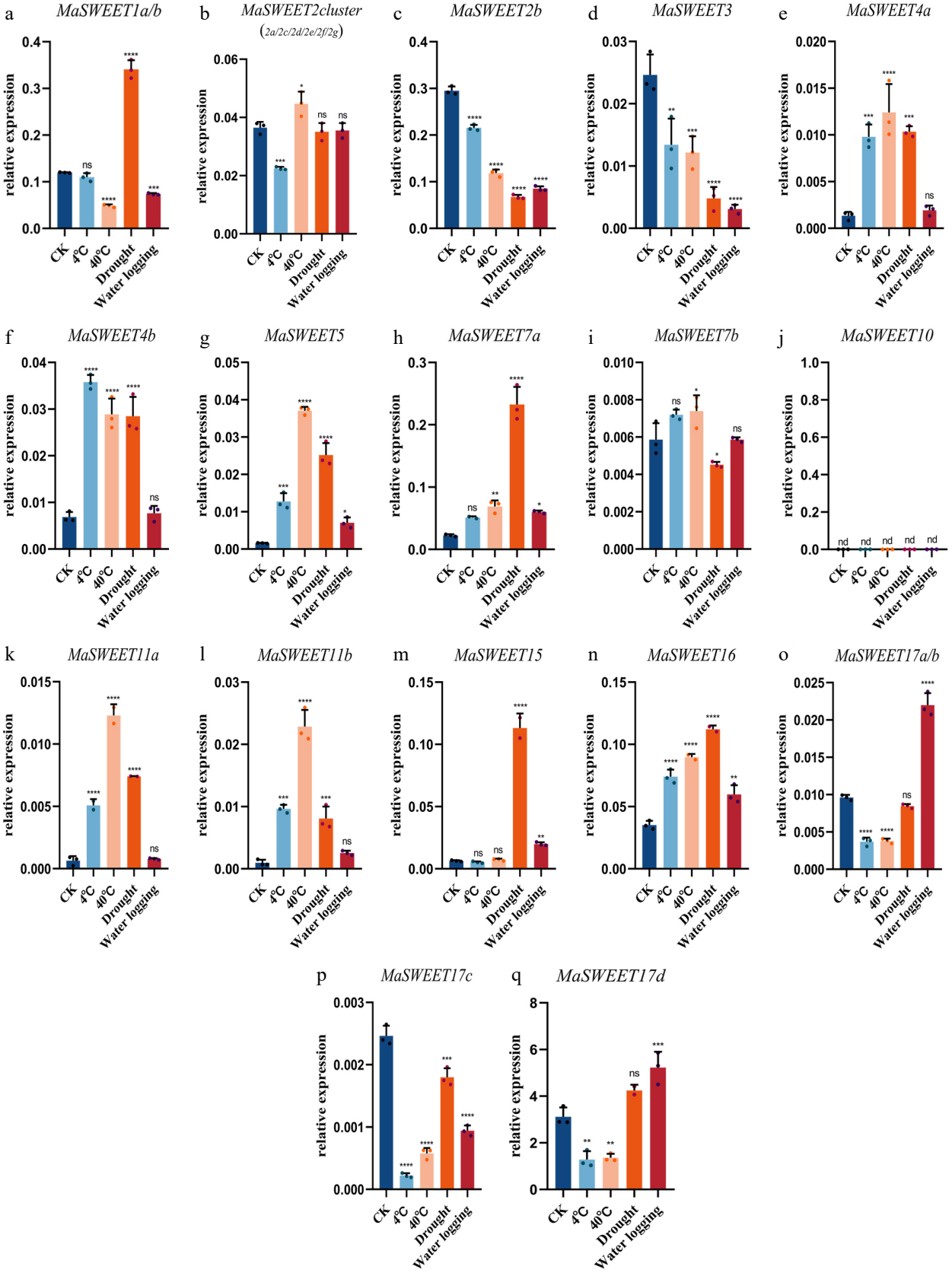

Fig. 7 Expression levels of 24 selected *MaSWEET* genes in response to 4 °C, 40 °C , drought, and flood stress conditions. Three technical replicates were analyzed. Error bars represent SE. Asterisks indicate significant difference as determined by Student's t-test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

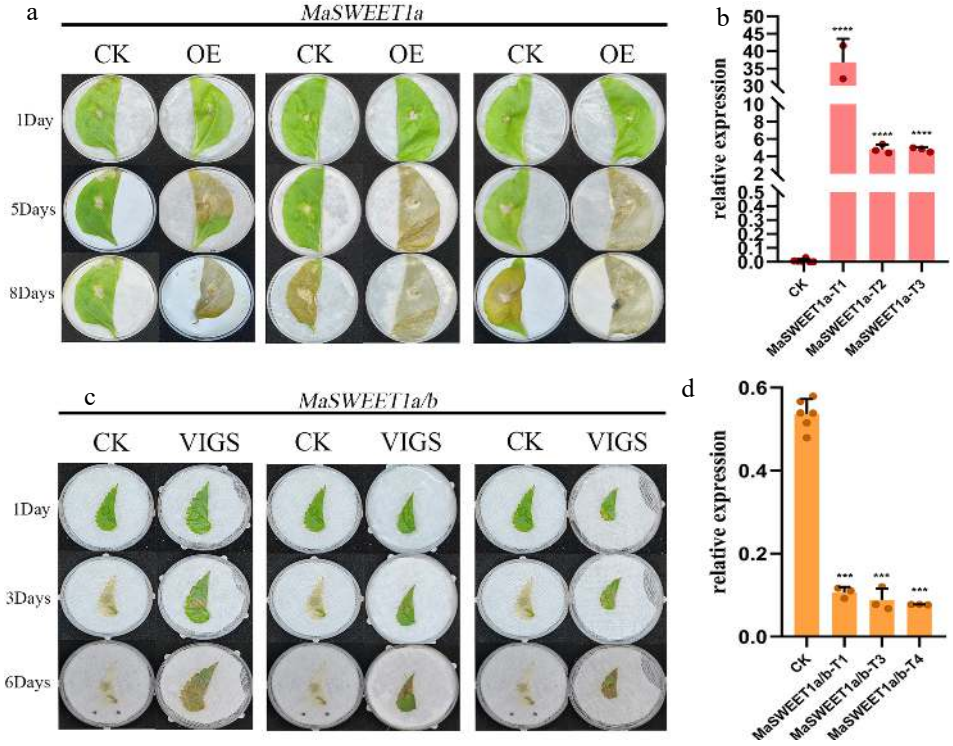

Fig. 8 Functional characterization of *MaSWEET1a/b* in tobacco and mulberry. (a) Damage of tobacco overexpressing *MaSWEET1a* after infection by *C. shiraiana*. (b) Expression levels of *MaSWEET1a* in tobacco. *MaSWEET1a-T1*, *T2*, *T3* are three independent treatments with transient overexpressed MaSWEET1a. (c) Damage of mulberry with knock-down *MaSWEET1a/b* after infection by *C. shiraiana*. (d) The expression level of *MaSWEET1a/b* in mulberry. *MaSWEET1a/b-T1*, *T3*, *T4* are three independent treatments with down-regulation of *MaSWEET1a/b* using VIGS.

differed from late-stage expressed *MaSWEET10*, *MaSWEET11a/b* and *MaSWEET15* in terms of detailed expression patterns during fruit ripening. *MaSWEET2b* and *2* cluster genes (*MaSWEET2a*, *c-g*) showed preferential expression in leaves which is similar with the ortholog *ZjSWEET2.2* in *Ziziphus jujuba*. *ZjSWEET2.2* was reported to be involved in mediating sugar loading in leaves[46]. *MaSWEET3, 7a-b,16* and *17a-d* also showed highest expression levels in leaves indicating their possible roles in sugar source loading or unloading. *MaSWEET11b* showed higher expression levels in phloem and its ortholog *AtSWEET11* in *Arabidopsis* was reported to be involved in sugar phloem loading[3].

Sugar signal is critical for plants in response to various stresses. Previous studies have shown that *SWEET*s participated in abiotic and biotic responses in many plant species including arabidopsis and rice[21,25]. *AtSWEET11*, *12*, *15* and *16* were reported to be involved in affecting cold tolerance in *Arabidopsis*[19–21]. *HfSWEET17* was also reported as a positive regulator of resistance to cold stress in daylily[11]. Cold environment (4 °C) induced expression of *MaSWEET4a*, *4b*, *5*, *11a*, *11b* and *16* in mulberry. Interestingly, these cold-induced *MaSWEET*s can also be induced by high temperature. *MaSWEET15* which is the ortholog of *AtSWEET15* can be induced by drought as well as low or high temperature. *MaSWEET15*, *MaSWEET1a/b*. *4a*, *4b*, *5*, *7a*, *11a*, *11b*, and *16* also showed positive responses to drought. It is obvious that some *SWEET* genes can be induced by different stresses. *AtSWEET15* was also reported to be induced by osmotic, drought and salinity[21]. *MaSWEET4a*, *4b*, *11a*, *11b* and *16* can be induced by low or high temperature and drought indicating their important roles in response to various abiotic stresses in mulberry.

SWEETs were generally thought to 'support the enemy' during infection. SWEETs especially those that function as exporters generally facilitate the export of sugars out of host cells, which support pathogen growth in the apoplasm[15,47,48]. Clade III SWEETs including *AtSWEET11*, *12*, *OsSWEET11* were characterized as negative regulators of resistance to fungal infection and Clade III SWEETs including *OsSWEET11*, *13*, *14* and *GhSWEET10* were characterized as negative regulators of resistance to bacterial pathogen infection[2,24,49,50]. In contrast, clade I *AtSWEET2*, a glucose importer and clade III *IbSWEET10* were reported as positive regulators of resistance to fungal infection[48,51]. Therefore, roles of SWEETs in response to pathogen infection may be quite different. Most *MaSWEET*s were disturbed in diseased fruits that resulted from sclerotiniose pathogen infection. *MaSWEET2b*, *MaSWEET3* in clade I, *MaSWEET7b* in clade II, *MaSWEET10* and *MaSWEET11a-b* in clade III showed significant increase in expression levels in diseased fruits while *MaSWEET1a/b*, *MaSWEET2* cluster (*MaSWEET2a*, *c-g*) in clade I showed significant decrease of expression levels in diseased fruits. *MaSWEET1a* was further validated as a negative regulator of resistance to *C. shiraiana* infection. Given the fact that *MaSWEET1a/b* was repressed in diseased fruits, it is likely that a possible pathway through repression of *MaSWEET1a* exists in mulberry to defense pathogen infection.

In conclusion, we have performed a genome-wide investigation of SWEET genes in *Morus* and a comprehensive analysis including phylogenetic analysis, promoter analysis and expression profile analysis was also carried out. Their possible roles in development and response to abiotic and biotic

stresses were addressed. In particular, the functional role of *MaSWEET1a* in regulation of tolerance to *C. shiraiana* infection was validated using both VIGS knock-down and transient overexpression in tobacco combined with inoculation of *C. shiraiana*. The results in this study provides a foundation for studying the function of the SWEET family in mulberry plants and provides a negative regulator of resistance to *C. shiraiana* infection for further genetic modification.

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Conflict of interest

The authors declare that they have no conflict of interest.

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