Phylogenetic Relationships of Sucrose Transporters (SUTs) in Plants and Genome-wide Characterization of *SUT* Genes in Orchidaceae

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Research Article

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Abstract

Background

Sucrose is the primary form of photosynthetically produced carbohydrates transported long distance in many plant species, which significantly affects plant growth, development and physiology. Sucrose transporters (SUTs or SUCs) are a group of membrane proteins that play vital roles in mediating sucrose allocation within cells and at the whole plant level.

Results

In this study, we investigated the relationship of SUTs in 24 representative plant species and performed a comprehensive analysis of *SUT* genes in three sequenced Orchidaceae species, *Dendrobium officinale*, *Phalaenopsis equestris*, and *Apostasia shenzhenica*. All the SUTs from 24 plants were classified into three groups and five subgroups: subgroups A, B1, B2.1, B2.2, and C, based on the evolutionary relationships. A total of 22 *SUT* genes were identified in Orchidaceae species, among which *D. officinale* had 8 genes (*DenSUT01-08*), *P. equestris* had 8 genes (*PeqSUT01-08*) and *A. shenzhenica* had 6 genes (*PeqSUT01-06*). For the 22 Orchidaceae *SUTs*, each of the subgroups A, B2.2 and C contains three genes, whereas the *SUT* genes were significantly expanded in the monocot-specific subgroup B2.1 which contained 12 genes. To shed light into sucrose partitioning and functions of sucrose transporters in Orchidacea species, we analysed water-soluble sugar content and performed RNA sequencing of different tissues of *D. officinale*, including leaves, stems, flowers and roots. The results showed that although total content of water-soluble polysaccharides was highest in the stems of *D. officinale*, the sucrose content was highest in flowers. Moreover, gene expression analysis showed that most of the *DenSUTs* were expressed in flowers, among which *DenSUT01*, *DenSUT07* and *DenSUT06* had significantly high expression levels.

Conclusions

These results indicated that stems are used as main storage sinks for photosynthetically produced sugar in *D. officinale*, and that the *DenSUTs* mainly take functions in the cellular machinery and development of floral organs. Our findings provide valuable information on sucrose partitioning and the evolution and functions of *SUT* genes in Orchidaceae and other species.

Background

Photoassimilated carbohydrates are produced by autotrophic source tissues such as leaves, and moved to heterotrophic sink tissues such as roots, stems, flowers and seeds. Sucrose is the major transport form of photosynthetically produced sugar in many plant species due to its non-reducing nature and insensitivity to degradation [1]. Long distance sucrose transport along the phloem sap requires the across of a series of membranes. Sucrose transporters (SUTs or SUCs) play vital roles in transmembrane transport during phloem loading and unloading as well as in sucrose allocation within plants and between pathogens and beneficial symbionts [2].

Plant sucrose transporters are members of the major facilitator superfamily (MFS), typically with 12 transmembrane (TM) domains divided into two regions by a hydrophilic cytoplasmic loop [3–4]. Most of the sucrose transporters identified to date are sucrose/H + symporters including vacuolar SUTs, except for several sucrose facilitators in some leguminous plants [5]. The first plant *SUT* gene, *SoSUT1*, was isolated from spinach

using the yeast complementation system [6]. With the increasing availability of plant genomes and molecular information, a growing number of *SUT*s have been identified in many plant species including both monocot and dicot plants, such as *Arabidopsis* [7], rice [8], *Populus* [9–10], wheat [11], maize [12], pear [13], cacao [14], tomato [15], cotton [16] and weeds [17]. However they are absent in the unicellular chlorophyte alga *Chlamydomonas rheinhardtii* and *Volvox carterii* [18].

According to the genomes of grasses, the *SUT* genes were originally classified into five proposed groups including SUT1-SUT5 [2, 19–20]. The SUT1 clade is dicot specific with members expressed in the plasma membrane of sieve elements or companion cells [7, 21–22]. SUT2 and SUT4 encompass both dicot and monocot plants, whereas SUT3 and SUT5 are both monocot specific. The SUT2 transporters are mainly expressed in plasma membrane of SEs and found in vegetative sink organs [23–24]. Members of the SUT4 clade are identified in both plasma membrane and the vacuole [25–26]. Recent studies have divided the *SUT*s into two subfamilies (Ancient Group 1 and Ancient Group 2) and three types (Type I, Type II and Type III) [18, 27]. *SUT* family genes play essential roles in phloem loading and unloading, pollen development, fruit ripening, ethylene biosynthesis and seed development and germination in many plants [10, 12, 26, 28–29]. Besides, the *SUT* genes also involved in various physiological processes and sucrose exchanges between plants and symbiotins, pathogens and fungi [2, 30–31]. For example, in *Arabidopsis*, *AtSUC5* is predominantly expressed in seeds, whereas *AtSUC1* and the mutant *atsuc9* both expressed in floral organs, and facilitates anthocyanin accumulation and floral transition [28, 32]. The rice *OsSUT2* is expressed in seeds which involve in the germination of embryos [33–34]. The activity and expression of sucrose transporters are regulated by genetic, molecular and physiological factors.

The family Orchidaceae is one of the largest families in angiosperms, with over 25,000 species and 880 genera, representing ~ 10% of the flowering plants [36]. Many of them are economically important for their unmatched ornamental and medical value. Moreover, the orchids are model systems for elucidating floral evolution in angiosperms and symbiotic activities between plants and fungi [35, 37]. To date, the genomes of three Orchidaceae species, *Dendrobium officinale*, *Phalaenopsis equestris*, and *Apostasia shenzhenica*, have been sequenced and published, which greatly promoted the genetics and genomics of orchids [37–40]. However, the roles of sucrose transporters in orchids are still unknown. In the present study, we performed genome-wide identification and characterization of the *SUT* gene families in the three sequenced Orchidaceae species. Transcriptome sequencing and water-soluble sugar content analysis was also conducted in *D. officinale*. Our findings have shed light into the evolution, expression, and functions of *SUT* genes in Orchidaceae.

Results And Discussion

Genome-wide identification of *SUT* genes in Orchidaceae species

The SUTs are prevalent in plants and play fundamental roles in plant growth, development and stress tolerance [19,41-42]. To understand the potential roles of SUTs in orchids, the three sequenced Orchidaceae species, *D. officinale*, *P. equestris*, and *A. shenzhenica*, were used for genome-wide identification and characterization of *SUT* genes. The HHM profile of the SUT protein was used as a query to perform an HMMER search against the genome assemblies of the three species. Bioinformatics analysis identified a total of 22 *SUT*s from the three species, which were designated '*DenSUT*' for *D. officinale*, '*PeqSUT*' for *P. equestris*, and '*ApoSUT*' for *A. shenzhenica*, with a serial number (Table 1, Table S1). Among them, *D. officinale* had 8 genes (*DenSUT01-08*), *P. equestris* had 8 genes (*PeqSUT01-08*) and *A. shenzhenica* had 6 genes (*PeqSUT01-06*). The results agree with previous reports that plant sucrose transporters are encoded by relatively small gene families.

According to the phylogenetic tree, the 22 *SUT* genes from three orchids were classified into four subgroups, subgroups A, B2.1, B2.2 and C (Fig. 1, Table 1). Subgroup A included three genes, *DenSUT01*, *PeqSUT01* and *ApoSUT01*. There were also four genes in subgroup C (*DenSUT03*, *DenSUT04*, *PeqSUT08* and *ApoSUT02*) and three genes in subgroup B2.2 (*DenSUT02*, *PeqSUT03* and *ApoSUT03*), respectively. However, the *SUT* genes were significantly expanded in the monocot-specific subgroup B2.1 which comprised 12 genes. Phlogenetically, sucrose transporters of *D. officinale* were more close to that of *P. equestris* than *A. shenzhenica*.

The molecular weights of the *SUT* genes ranged from 51.22 to 106.90 kD with pI values ranged between 4.95 and 10.12. Most of these genes were ~500 aa or ~600 aa in length with 11-13 introns and 12-14 extrons, whereas there were several genes with only 4-5 introns/extrons. Previous studies indicate that plant sucrose transporters are usually consisted of 500-600 aa, with molecular weight of 55-60 kD [15,43], which is consistent with the findings in the present study. Detail information of the *SUT* genes, including name, coding protein, CDS length, molecular weight and PI value, is shown in Table 1.

Table 1

Physical and molecular characteristics of *SUT* genes in *A. shenzhenica*, *D. officinale*, and *P. equestris*

Gene Name	Scaffold location (bp)		Subgroup	Length (bp)	Size (aa)	MW (kDa)	pI	Extron	Intron
ApoSUT01	1215667	1232090	A	16423	458	49879.49	7.5	12	11
ApoSUT02	644564	658003	C	13439	532	56556.26	9.6	5	4
ApoSUT03	1021836	1041725	B2.2	19889	589	64293.55	5.39	13	12
ApoSUT04	328657	335790	B2.1	7133	488	51878.16	8.85	13	12
ApoSUT05	534128	538346	B2.1	4218	477	51224.4	9.05	14	13
ApoSUT06	314008	320943	B2.1	6935	499	52812.97	8.36	14	13
DenSUT01	1939695	1953824	A	14147	716	78785.55	8.55	13	12
DenSUT02	11559848	11579293	B2.2	19445	571	62989.08	4.95	14	13
DenSUT03	83114	90826	C	7712	216	22730.49	10.12	5	4
DenSUT04	74903	90826	C	7712	216	22730.49	10.12	5	4
DenSUT05	10800848	10810268	B2.1	9420	984	106898.82	8.93	14	13
DenSUT06	3505256	3512511	B2.1	7255	177	18812.02	8.79	14	13
DenSUT07	29089	32887	B2.1	3798	492	52983.52	9.06	14	13
DenSUT08	397815	406603	B2.1	8788	470	50102.76	8.38	14	13
PeqSUT01	247532	268499	A	20967	461	50349.07	7.51	13	12
PeqSUT02	14129	22513	B2.1	8384	240	26208.15	9.3	8	7
PeqSUT03	3166955	3194083	B2.2	27128	611	65924.14	6.19	14	13
PeqSUT04	2062452	2066080	B2.1	3628	499	53546.81	8.32	14	13
PeqSUT05	58510	76466	B2.1	17956	500	53040.17	8.24	14	13
PeqSUT06	523303	531370	B2.1	8067	492	52933.39	9.11	14	13
PeqSUT07	659976	662642	B2.1	2666	489	52440.8	9.17	12	11
PeqSUT08	10082423	10107132	C	24709	413	43952.87	9.02	7	6

Phylogenetic relationship of the SUT proteins in major plant species

To provide insight into the evolution of *SUT* gene families, we performed phylogenetic analysis using 24 representative plant species, including green alga, mosses, lycophytes, gymnosperms, monocots and dicots.

Detailed information of the *SUTs* see Methods. The SUT domain sequence and neighbour-joining method was used with 1000 bootstraps to construct the phylogenetic tree. In this study, the *SUT* genes of several eukaryotic chlorophyta were clustered in a special branch, which was defined as outgroups. The *SUTs* from the 24 species were classified into three groups and five subgroups: subgroups A, B1, B2.1, B2.2, and C (Fig. 1). Group A contained at least one member from mosses, lypophytes and angiosperms including both monocots and dicots. Group B was the largest group which is divided into three subgroups; subgroup B1 was made up of *SUT* genes from exclusively dicot species, corresponding to the SUT1 clade by Lalonde & Frommer [20]. Subgroup B2.2 contained both monocot and dicot species that are also present in the SUT2 group reported by Lalonde & Frommer [20]. Whereas subgroup B2.1 was monocot-specific expansion clade containing members from SUT3 and SUT5 reported by Kühn & Grof [2]. Group C contained mosses, lypophytes and angiosperms including both monocots and dicots, corresponding to SUT4 clade [20]. According to previous studies, the SUT1 and SUT2 proteins mainly play roles in phloem loading and unloading, sucrose transport to sink cells, and sucrose exchanges with microbes [2, 30,31,44-45]. While SUT4 proteins are involved in various physiological processes such as circadian rhythms and responses to dehydration and photosynthesis [46-47]. In recent studies, the *SUTs* were classified into two subfamilies (Ancient Group 1 and Ancient Group 2) and three types (Type I, Type II and Type III) [18,27]. Type I clade is dicot specific which corresponds to the SUT1 group [2] and Type III clade contains both monocots and dicots which corresponds to the SUT4 group [20]. Type II (A) is composed of monocot and dicot species that are also reported in the SUT2 group by Lalonde & Frommer [20], whereas the monocot specific Type IIB contains members from SUT3 and SUT5 reported by Kühn & Grof [2].

Sucrose transporters have been identified in primary terrestrial plants including both lypophyte and moss, with 6 *SUTs* in *Selaginella lepidophylla* and 7 *SUTs* in *Physcomitrella patens*; however, none was identified in green alga *Chlamydomonas reinhardtii* [18]. There were 6-10 *SUT* genes in monocot see crops such as rice (6 genes), maize (10 genes) and sorghum (8 genes). In contrast, in another monocot species, *Ananas comosus*, only 3 *SUTs* were identified. For most dicot species, 4-9 *SUTs* were identified. These results revealed that the number of sucrose transporters remains largely stable during the evolution from lower plants to terrestrial plants. This conclusion is consistent with previous studies on *SUT* gene identification and evolution [18,20,27]. However, the *SUTs* were expanded in several species such as *Triticum aestivum* (18 genes) and *Glycine max* (14 genes), which may be the result of whole genome polyploidization. The *SUT*s of some monocot species were expanded in subgroups B2.1; for example, there were 5 *ZmaSUT*s in subgroup B2.1, whereas 3 *ZmaSUT*s were identified in subgroup A, and only one was identified in subgroup B2.2 and C. Likewise, the *SUTs* from dicot species were expanded in subgroup B1 such as *GmaSUT*s, *AtSUT*s and *DcaSUT*s. The characean algae *Chlorokybus atmosphyticus* contains one *SUT* homolog which is basal to all the streptophyte *SUTs* [18]. We also identified one *SUT* (*VcaSUT01*) in chlorophyta *Volvox carteri*. Thereby, the origin of the sucrose transporters predates the divergence between green alga and the ancestors of terrestrial plants.

Conserved motifs analyses of the *SUT* genes

The diversity of motif compositions in sucrose transporters of Orchidaceae species was assessed using the MEME programme; a total of 10 conserved motifs were identified. The distribution of these 10 motifs in the SUT proteins is shown in Figure 2. The motif, motif2 was the most conserved SUT domain, which was identified in all of the SUT proteins except PeqSUT08 and DenSUT06. Besides, motif10 was observed in 17 SUT proteins, whereas absent from PeqSUT08, ApoSUT05, DenSUT06, DenSUT08, and PeqSUT02. All the three members in group A contained the same four motifs, motif10, motif2, motif5 and motif9. Moreover, Group B members shared the same motif,

motif5, except for DenSUT07; likewise, motif4 was also commonly owned by all group B SUTs except for ApoSUT04 (Fig. 2). Among the 12 SUTs in subgroup B2.1, three motifs were commonly owned, i.e. motif2, motif3, and motif5. There were eight sucrose transporters that had all 10 motifs, among which five for *P. equestris* (PeqSUT03, PeqSUT04, PeqSUT05, PeqSUT06 and PeqSUT07), two for *A. shenzhenica* (ApoSUT02 and ApoSUT06), whereas *D. officinale* had only one (DenSUT05). The sucrose transporters in each subgroup shared several unique motifs, indicating that the SUT proteins within the same subgroups may have certain functional similarities. In addition, motif distribution of the SUTs suggested that those genes were largely conserved during evolution.

Water-soluble sugar content in *D. officinale*

Photosynthetically produced sugar is not just a resource of carbon skeletons but also an energy vector and signaling molecule, which has major impacts on plant growth, development and physiology [48-49]. After synthesis in mesophyll cells of leaves, sucrose needs to be loaded to the phloem parenchyma cells or apoplasm of mesophyll cells, then transported in specialized networks [i.e. sieve element/companion cell complexes (SE/CCC)], and finally unloaded to distal sink organs [2,30,49]. Unlike other monocot crops such as maize, rice, and wheat that uses seeds as main storage sinks, the endosperms of most orchid seeds are significantly degenerated. As a result, Orchidaceae plants are highly dependent on symbiotic fungi to complete their life cycle, especially at the stage of seed germination and seedling growth due to nutrient deficiency [50-52]. To shed light into sucrose partitioning and functions of sucrose transporters in Orchidacea species, we analysed water-soluble sugar content in different tissues of *D. officinale*, including leaves, stems, flowers and roots, using the GC-MS/MS method. The results showed that the content of water-soluble polysaccharides varies significantly among different tissues (Fig. 3). The amount of total water-soluble polysaccharides was highest in the stems of *D. officinale*, with approximately 116.17 mg/g, followed by leaves with approximately 113.23 mg/g; flowers had approximately 88.08 mg/g, whereas roots have a significantly lower level of water-soluble polysaccharides, only ~26.66 mg/g (Fig. 3a). These indicated that stems were the major sink organs for sugar storage in *D. officinale*. Because the *D. officinale* is an epiphytic plant in its natural habitation which usually experiences drought stress [53-54], thus the high amount of sugar in stems may help to maintain osmic pressure to improve drought tolerance. The content of sucrose also varies greatly among different tissues. Nonetheless, sucrose content was highest in flowers, approximately 28.1 mg/g, followed by leaves (with ~18.13 mg/g) which are the major source tissues for the photosynthetically assimilated sucrose. The amount of sucrose in stems is ~13.77 mg/g, and that of roots is also the lowest containing only ~7.82mg/g (Fig. 3b). Previous studies show that the developing pollen grains are strong sink tissues, which require sucrose to provide energy for maturing, germination and growth [55-56]. These results showed that although total sugar content was highest in the stems, the photoassimilated sucrose was mainly transported to support the growth and physiology of floral organs in *D. officinale*.

Expression patterns of the *SUT* genes in different tissues of *D. officinale*

Sucrose transport systems play vital roles in carbon partitioning, plant development, inter-/intra cellular communications and environmental adaptations. The *SUT* genes are not only involved in sucrose transport, but also play essential roles in pollen germination, fruit ripening, and ethylene biosynthesis in many species [10,28-29,47]. To further understand the roles of the *SUT* genes in orchids, we investigated expression profiles of *DenSUT*

genes in *D. officinale*. RNA sequencing (RNA-seq) was performed using different tissues of *D. officinale* including leaves, stems, flowers and roots. The FPKM expression of *DenSUT* genes in four different tissues is provided in Table S2. The expression levels of different *DenSUT* genes in the four *D. officinale* tissues are represented in different colours and are shown in Fig. 4.

The *Arabidopsis AtSUC1* is expressed in germinating pollens where it is translationally regulated and facilitates anthocyanin accumulation; while the mutant *atsuc9* promotes floral transition by manipulating sucrose uptake [28,32]. *AtSUC1* is also expressed in the parenchymatic cells of the style and anthers, which guides modulates water availability around the region and finally results in pollen-tube towards the ovule and anther opening [55]. Recent studies have also described the roles of *NtSUT3* and *LeSUT2* in sucrose uptake during pollen development and pollen tube growth [9,56]. In the present study, RNA-seq showed that most of the *DenSUTs* were expressed in flowers, among which three genes, *DenSUT01*, *DenSUT08*, and *DenSUT06* had significantly high expression levels. In agreement with the expression profiles, sucrose accumulation also predominantly occurs in the flowers, with approximately 28.1 mg/g. These result indicated that these genes mainly took function in the cellular machinery and development of floral organs. Phylogenetically, *DenSUT01*, *DenSUT08*, and *DenSUT06* were classified in subgroup A and the monocot-specific expanded subgroup B2.1, respectively.

In leaves, sucrose is mainly synthesized in the mesophyll cytoplasm, maybe also in organelles such as vacuoles and plastids [57]. Once released to the leaf apoplasm, sucrose is actively loaded into the SE-CCCs via a sucrose/H+ mechanism in apoplasmic loading species [58]. The analysis of transgenic and mutant plants indicates that dicot members of the SUT1 clade and monocot members of the SUT3 clade are essential for apoplasmic loading of SE-CCC [59-61]. In maize, *ZmSUT1* plant an important role in efficient phloem loading [61]. The inhibition of sucrose transporters results in starch accumulation in the epidermal cell [62]. The sucrose transporter SUC2 is crucial for sucrose allocation, the null mutant of which in *Arabidopsis* led to compromise health of the plant [63]. After loaded into the SE-CCC, energy-driving reloading is required along the whole process of long terminal sucrose transport from source to sink. In *D. officinale*, sucrose content was ~18.13 mg/g in the leaves, ranked second among four tissues. However, only one gene, *DenSUT02*, was significantly expressed in the leaves, which was also expressed in flowers and roots. We deduce that *DenSUT02* may play a potential role in phloem loading in *D. officinale*. Nonetheless, other sugar transporters such as SWEETs and MSTs are also likely involved in sucrose transport.

In well studied grass stems, immature internodes are considered as utilization sinks, whereas the fully elongated mature internodes are storage sinks where the sucrose accumulates [64-66]. The plasmamembrane-localized sucrose transporters are promising candidates for sucrose uptake in stems. For example, all of the *SbSUTs* in sorghum are active in sucrose uptake, although the expressed sites of different *SUTs* in internodes may vary [19,66-67]. The *SbSUTs* are localized to sieve elements in both developing and mature sorghum stems [68], which is consistent with the localization of wheat *TaSUT1* and rice *OsSUT1* proteins in SE-CCCs in mature stems [69-70]. In the present study, three genes, *DenSUT03*, *DenSUT05* and *DenSUT07*, were expressed in *Dendrobium* stems. *DenSUT05* and *DenSUT07* were both moderately expressed in stems and flowers, whereas *DenSUT03* was slightly expressed in the stems and significantly expressed in the roots. In addition, *DenSUT01* and *DenSUT08* were also slightly expressed in the roots. The expression of *DenSUTs* was also analysed in flower, stem and leaf *D. officinale* using qRT-PCR (Fig. 5). The results were largely consistent with that from RNA-seq analysis. However, the functions of *SUT* genes in *D. officinale* and the other two Orchidaceae species remain to be verified.

Conclusions

In conclusion, we performed a comprehensive study of the phylogenetic relationship of the SUTs in 24 plant species and a genome-wide characterization of the *SUT* genes in three Orchidaceae species. The *SUTs* were classified into three groups and five subgroups. We identified a total of 22 *SUT* genes in the three orchids, 8 *DenSUTs*, 8 *PeqSUTs*, and 6 *ApoSUTs*. The functions of the *SUTs* in *Dendrobium* were analysed. The results showed that most of the *DenSUTs* had high expression levels in flowers. Although total content of water-soluble sugars was highest in the stems, the sucrose content was highest in flowers. We proposed that stem were used as major sinks for sugar storage in *D. officinale*, and the *DenSUTs* mainly take functions in floral organs. Our findings provide important insights into the evolution patterns in plants and advanced our knowledge of sucrose partitioning and potential functions of *SUT* genes in Orchidaceae species.

Methods

Identification and characterization of the SUT proteins in Orchidaceae

The genomes, genes and corresponding protein sequences of three sequenced Orchidaceae species, *D. officinale* [38-39], *P. equestris* [40], and *A. shenzhenica* [37], were downloaded from Chinese Herbal Plant Genome Database (http://herbalplant. ynau.edu.cn/html/Genomes/3.html) and OrchidBase (http://orchidbase.itps.ncku. edu.tw/est/home 2012.aspx). All members in SUT family contains GPH_sucrose (TIGR01301) domain, the seed sequence of which was downloaded from TIGRFAMS database (http://tigrfams.jcvi.org/cgi-bin/ index.cgi). ClustalW [71] was used for sequence alingment and a Hiden Markov Model (HMM) [72] was constructed for SUT proteins. The HMMER program was used to search for SUT proteins in all *D. officinale*, *P. equestris*, and *A. shenzhenica* proteins with a cutoff E-value of $1e^{-4}$ using the HMM as a query. If the location of two *SUT* genes on the genome is less than 10KB, it is considered as the homologous gene generated by fragment replication; if not, it is considered as the homologous gene generated by genome-wide replication. After a comprehensive check, the candidate proteins that only contained fragmental SUT domains were eliminated. Protparam (http://web.expasy.org/protparam/) website was used to simulate the molecular weight of each gene and the theoretical isoelectric point (pI) of each protein was also predicted.

Phylogenetic analysis of the *SUT* genes

The amino acid sequences of SUT proteins identified in three Orchidaceae species (*A. shenzhennica*, *D. officinale*, *P. equestris*) and 21 other species have been used in the phylogenetic analysis, including alage, moss, lypophyte, and angiosperms: *Chlamydomonas reinhardtii* (*Cre*), *Volvox carteri* (*Vca*), *Physcomitrella patens* (*Ppa*), *Selaginella moellendorffii* (*Smo*), *Aquilegia coerulea* (*Aco*), *Picea abies* (*Pab*), *Brachypodium distachyon* (*Bdi*), *Oryza sativa* (*Osa*), *Zea mays* (*Zma*), *Vitis vinifera* (*Vvi*), *Eucalyptus grandis* (*Egr*), *Malus domestica* (*Mdo*), *Carica papaya* (*Cpa*), *Cucumis sativa* (*Csa*), *Daucus carota* (*Dca*), *Solanum lycopersicum* (*Sly*), *Asparagus officinalis* (*Aof*), *Populus trichocarpa* (*Ptr*), *Arabiqopsis thaliana* (*AT*), *Glycine max* (*Gma*), and *Theobroma cacao* (*Tca*). The protein sequences were downloaded from PFAM database (https://phytozome.jgi.doe.gov/) and phytozome (https://phytozome.jgi.doe.gov/). MEGA6 (V6.0, Tokyo Metropolitan University, Tokyo, Japan) was used to systematically analyze the protein sequences of SUTs. First, CLUSX2 in MEGA6 was used for multiple sequence alignment, and then the maximum likelihood (ML) method with the Jones-Taylor-Thornton (JTT) model was used to construct the phylogenetic tree with. Moreover, 1000 bootstrap replicates and a partial deletion with a site coverage cutoff of 70% was used for gap treatment. Phylogenetic trees were visualized using FigTree v1.4.2 (http://tree.bio.ed. ac.uk/software/figtree/).

Gene structure and motif analyses

The Gene Structure Display Server tool (http://gsds.cbi.pku.edu.cn/, v2.0) was used to analyse gene structure of all the SUTs identified in *D. officinale*, *P. equestris*, and *A. shenzhenica*. MEME software (http://meme.nbcr.net/meme/, v4.11.0) was used to search for motifs in SUT proteins with a motif window length from 10 to 100 bp, maximum number of motifs was set at 20, and the motif exist in at least three SUT proteins was identified as the true motif.

Analysis of *SUT* gene expression in different tissues of *D. officinale*

Firstly, we performed RNA-seq on different tissues of *D. officinale*. The 3-year old *D. officinale* plants were grown in glasshouses on Mulberry Field Base of Zhejiang Academy of Agriculture Science (Hangzhou, China). Four different tissues, inluding roots, stems, leaves and flowers, were collected and frozen in liquid nitrogen, and then stored at -80 °C until used. Each tissue was sampled for three independent times. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturers' instruction. The library preparations were sequenced on an Illumina Hiseq 2000 platform and 150 bp paired-end reads were generated for the 12 samples. Then the expression profile of all *Dendrobium* genes was obtained with FPKM (Fragments Per Kilobase of exon per million fragments Mapped) value using software Cufflinks (http://cole-trapnell-lab.github.io/cufflinks, v2.2.1) under the guidance of annotated gene models with a GFF file. The *SUT* gene expression profile from each sample was analysed using the HemI program (http://hemi.biocuckoo.org/) with the average hierarchical clustering method.

Determination of total water-soluble polysaccharide content

The 3-year old *D. officinale* plants were grown in glasshouses on Mulberry Field Base of Zhejiang Academy of Agriculture Science (Hangzhou, China). Four *D. officinale* tissues inluding roots, stems, leaves and flowers (three replicates for each tissue) were collected and dried in an oven at 105℃ until constant weight. The 12 samples were shattered into fine powders in independently way by a mixer mill (MM 400, Retsch). The total polysaccharide was extracted using water extraction and alcohol precipitation method, and the content of total polysaccharide was measured using phenol-sulfuric acid method.

Total polysaccharide extraction: about 0.05g from each sample were weighed, and added in 1mL water, and fully homogenized. The sample was then extracted in water bath at 100℃ for 2h, centrifuged at 10000g for 10min after cooling, and reserved supernatant. 0.2mL supernatant was collected and slowly added with 0.8mL anhydric ethanol. After well mixed, the mixture was stored overnight at 4℃. After centrifuged 10000g for 10min, the supernatant was discarded, 1mL water was added into the precipitation, fully mix and dissolved.

Calculation of total polysaccharide cotent: Preheat the Microplate Reader for more than 30min and adjust the wavelength to 490nm. 200μL supernatant was extracted, and 100μL reagent and 0.5mL concentrated sulfuric acid were added. After well mixed, it was incubate in 90℃ water for 20min. 200μL mixture was extracted and added into the enzyme label plate and the absorbance value A was determined at 490nm. Glucose was used as the reference. The regression equation under standard conditions was y = 7.981x-0.0037, R2 = 0.9973, x represented for glucose content (mg/mL), y represented for the absorbance value. Total polysaccharide (ug/g dry weight) = (A+0.0037)÷7.981×V1÷V2×V3÷W×1000 =626.49×(A + 0.0037)÷W. V1: The redissolved volume after alcohol precipitation, 1mL; V2: The volume of alcohol precipitation, 0.2ml; V3: The volume of water added during extraction, 1mL; W: Sample weight, g; 1000: The conversion coefficient from mg to μg.

Determination of sucrose content

After dried, the 12 samples were shattered into fine powders in independently way by a mixer mill (MM 400, Retsch). 20 mg of powder was diluted to 500 μL with methanol: isopropanol: water (3:3:2 V/V/V). The extracts was centrifuged at 14,000 rpm under 4°C for 3 min. 50μL of the supernatants were mixed with internal standard (ZZBIO, Shanghai ZZBIO CO., TD.) and evaporated under nitrogen gas stream, then transferred to the lyophilizer for freeze-drying. The residue was used for further derivatization. The sample of small molecular carbohydrates was mixed with 100 μL solution of methoxyamine hydrochloride in pyridine (15 mg/mL). The mixture was incubated at 37 °C for 2 h. Then 100 μL of BSTFA was added into the mixture and kept at 37 °C for 30 min after vortex-mixing. The mixture was diluted and analyzed by GC-MS/MS according to the description by Gómez-González et al. [73] and Sun et al. [74], with modifications. Agilent 7890B gas chromatograph coupled to a 7000D mass spectrometer with a DB-5MS column (30 m length × 0.25 mm i.d. × 0.25 μm film thickness, J&W Scientific, USA) was employed for GC-MS/MS analysis of sugars. Helium was used as carrier gas at a flow rate of 1 mL/min. Injections were made in the split mode with a ratio of 3:1 and the injection volume was 3 μL. The oven temperature was set at 170°C for 2min, then raised to 240°C at 10°C/min, raised to 280°C at 5°C/min, raised to 310°C at 25°C/min and held for 4 min. All samples were analyzed in selective ion monitoring mode. The injector inlet and transfer line temperature were 250 °C and 240 °C, respectively.

RNA Extraction and qRT-PCR Analysis

Total RNA was extracted from three *D. officinale* tissues, i.e. flowers, stems and leaves, using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturers' instruction. DNase I was used to purify the potential contaminating genomic DNA. The quality of total RNAs was checked with 1% denaturing agarose gels and the NanoDrop 2000 spectrophotometer (ThermoFisher Scientific, Beijing, China). First-strand cDNA synthesis was performed with PrimeScript reverse transcriptase (TaKaRa Biotechnology, Dalian, China), using the RNA was used as the template. Gene-specific primers wre designed with Primer Premier 5.0 program (Table S3). The *DnActin* (comp205612_c0) gene was used as an internal standard for normalizing the gene expression data [75]. The expression levels of *DenSUTs* were analyzed in a qRT-PCR assay, which was completed with the SYBR Green qPCR kit (TaKaRa Biotechnology, Dalian, China) and the Stratagene Mx3000P thermocycler (Agilent, Santa Clara, CA, USA). The PCR program was as follows: 95℃ for 10 min then 40 cycles of 95℃ for 15 s and 60℃ for 60 s. The relative *SUT* gene expression levels were calculated with the $2^{-\triangle\triangle Ct}$ method [76]. The analysis included three biological replicates, each with three technical replicates. The expression levels in different tissues were visualized in a histogram using the average values.

Statistical Analyses

Statistical analysis was performed to calculated the average values and standard errors for the three replicates. SPSS software (vs. 16.0) was used to determine the significant differences of sugar content among different tissues using a one-way ANOVA procedure and post hoc analysis. P value=0.05 indicates a significant dierence and is represented by an asterisk (*); p value=0.01 indicates a very significant dierence and is represented by two asterisks (**).

Abbreviations

SUTs/SUCs: Sucrose transporters; TM: transmembrane; MEME: Multiple Em for Motif Elicitation; SE: Sieve element; CCC: Companion cell complexes; SWEETs: Sugars Will Eventually be Exported Transporters; MSTs:

Monosaccharide transporters; RNA-seq: RNA sequencing; qRT-PCR: quantitative real-time polymerase chain reaction.

Declarations

Authors' Contributions

YZW and CBS conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft. YC analyzed the data, authored or reviewed drafts of the paper, approved the final draft. QZW and HJW analyzed the data, contributed reagents/materials/analysis tools, authored or reviewed drafts of the paper, approved the final draft. CBS conceived the experiments, authored or reviewed drafts of the paper, approved the final draft.

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Availability of data and materials

The following information was supplied regarding data availability: The raw data of RNA-seq experiment is deposited in Sequence Read Archive (NCBI): SUB8609885. All data and material used in this study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

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Figures

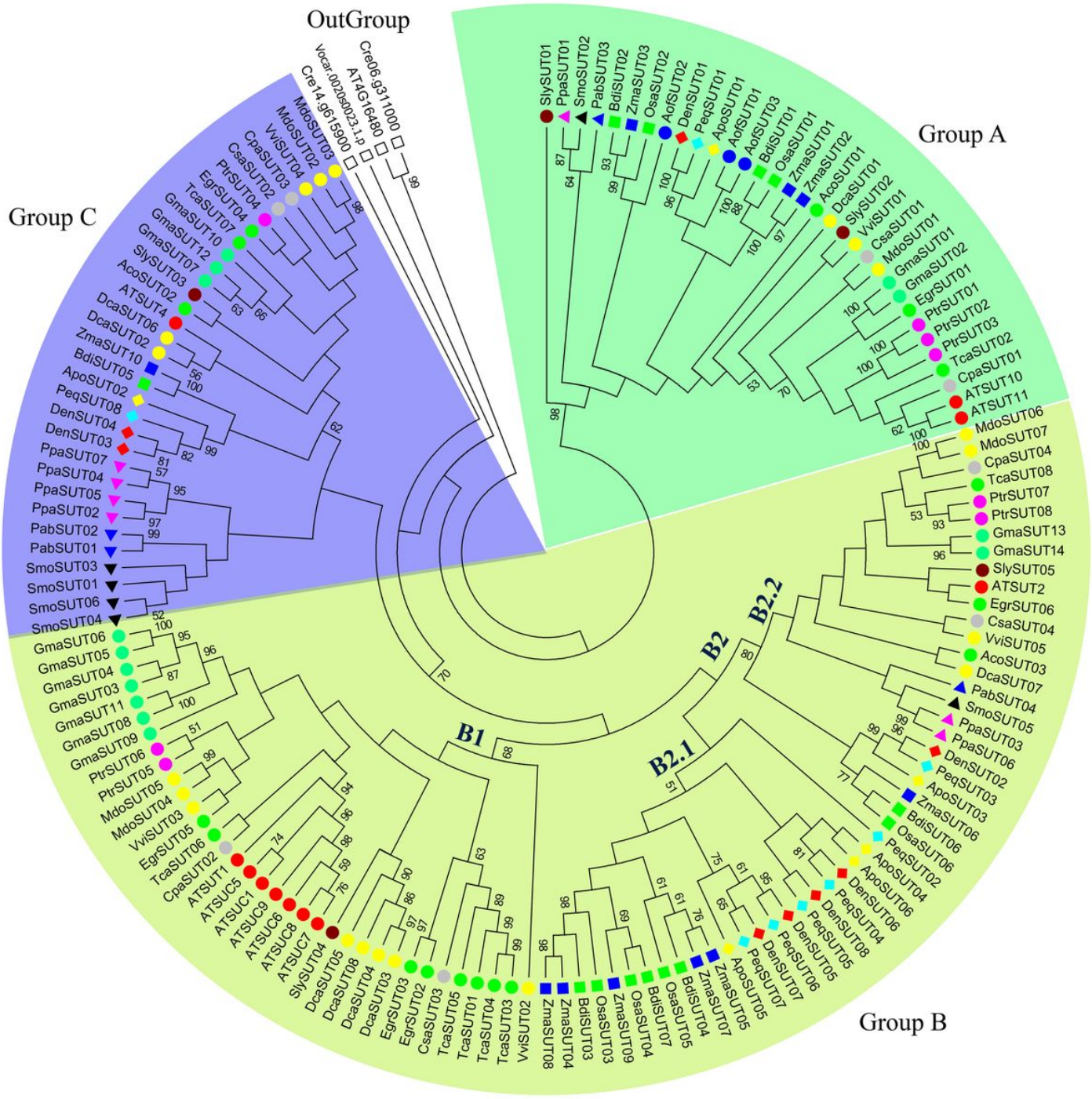

Figure 1

Phylogenetic analysis of SUT gene families from 24 representative plant species. The phylogenetic tree was constructed using MEGA 6.0 with the maximum likelihood (ML) method and 1000 bootstrap replicates. All SUT sequences were grouped into three groups and five subgroups (A, B1, B2.1, B2.2, and C). Green, blue, and yellow green shades indicate groups A, B, and C, respectively. The gene code prefixes are as follows: Chlamydomonas reinhardtii (Cre), Volvox carteri (Vca), Physcomitrella patens (Ppa), Selaginella moellendorffii (Smo), Aquilegia coerulea (Aco), Picea abies (Pab), A. shenzhennica (Apo), D. officinale (Den), P. equestris (Peq), Brachypodium distachyon (Bdi), Oryza sativa (Osa), Zea mays (Zma), Vitis vinifera (Vvi), Eucalyptus grandis (Egr), Malus domestica (Mdo), Carica papaya (Cpa), Cucumis sativa (Csa), Daucus carota (Dca), Solanum lycopersicum (Sly), Asparagus officinalis (Aof), Populus trichocarpa (Ptr), Arabiqopsis thaliana (AT), Glycine max (Gma), and

Theobroma cacao (Tca). Individual species are distinguished by circle, triangle, square, or rhombus in different colors.

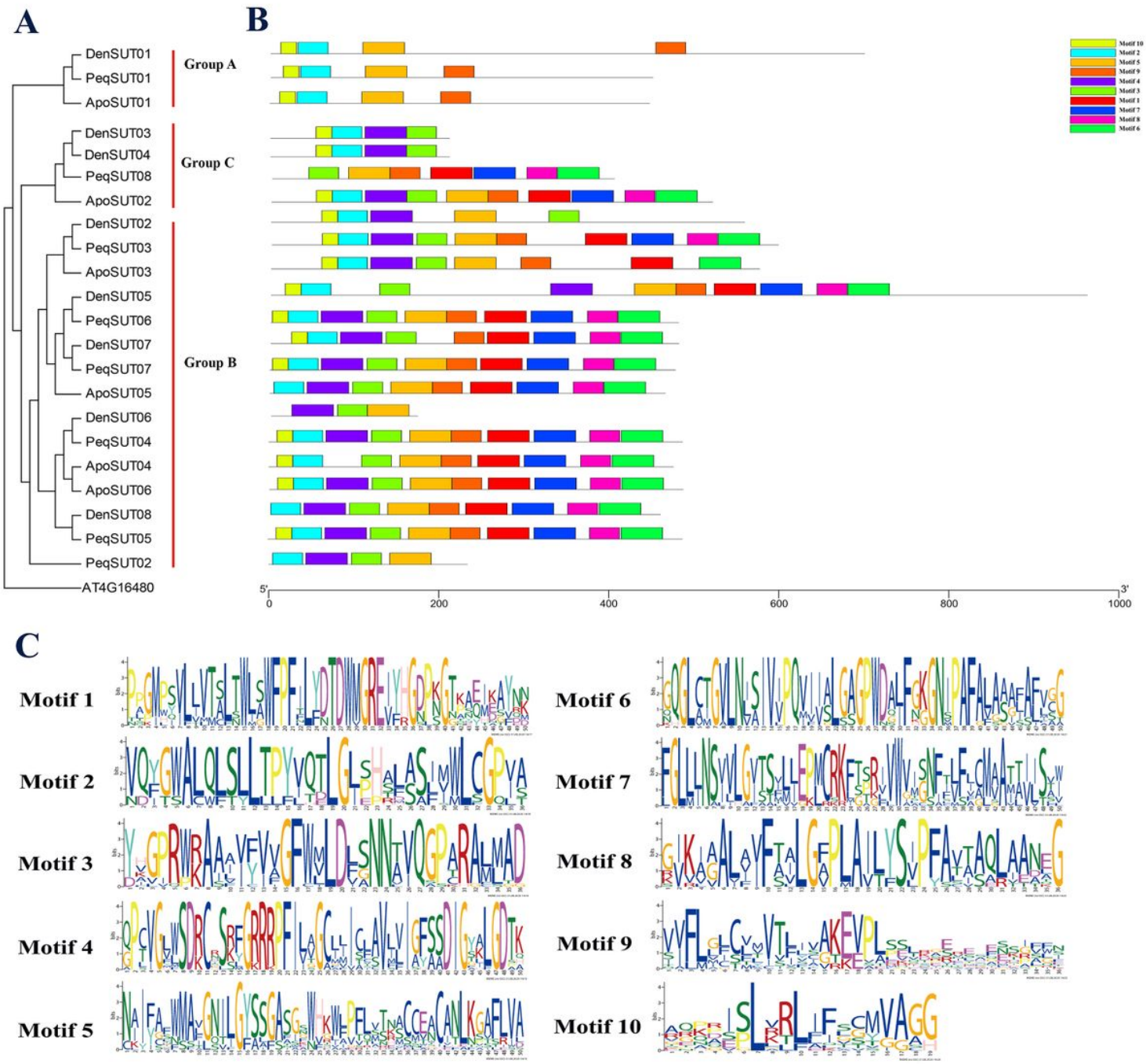

Figure 2

Phylogenetic and conserved motif analyses of the SUT proteins from A. shenzhennica (Apo), D. officinale (Den), and P. equestris (Peq). (A): Phylogenetic tree of the SUT proteins using AT4G16480 as outgroup; (B): Schematic presentation of the conserved motifs in Orchidaceae SUTs; (C) Sequence logos of all the 10 motifs

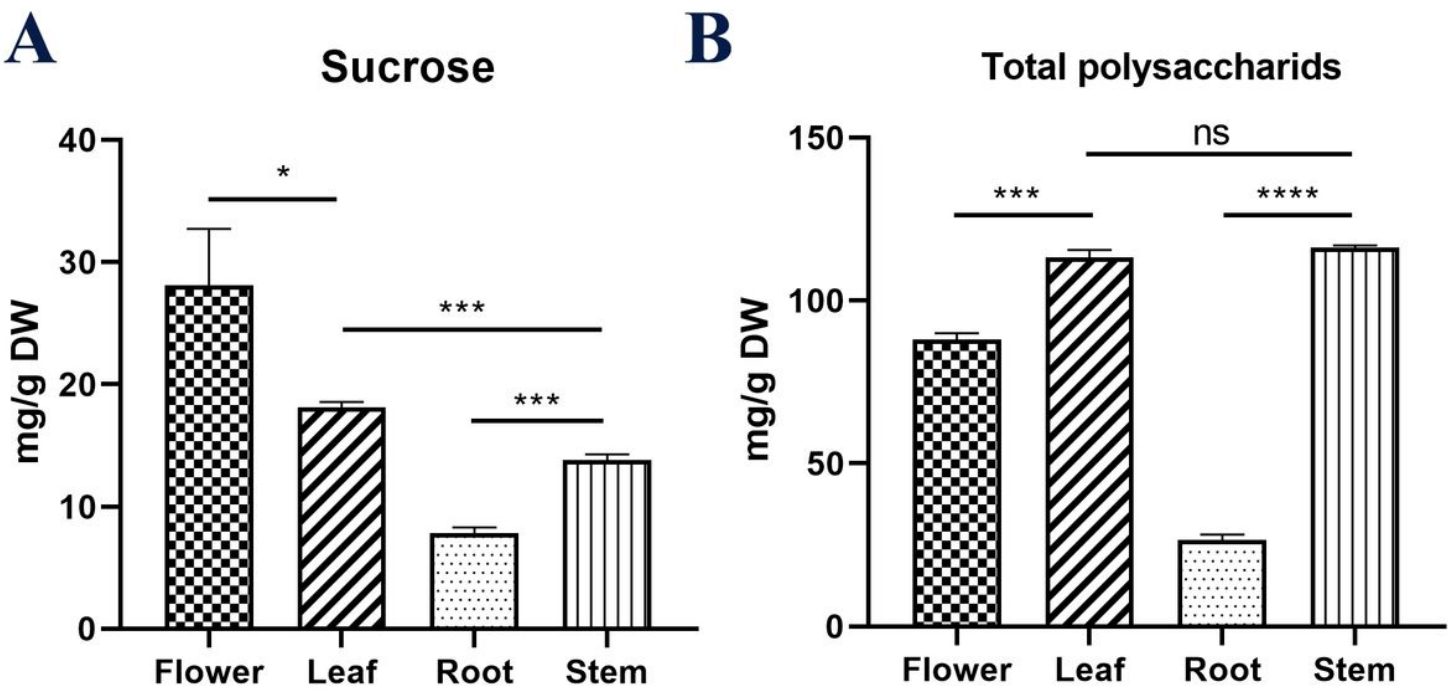

Figure 3

Histogram of water-soluble sugar content (mg/g) in different tissues of D. officinale including flower, stem, leaf and root; (A): Sucrose content (B): Total polysaccharide content.

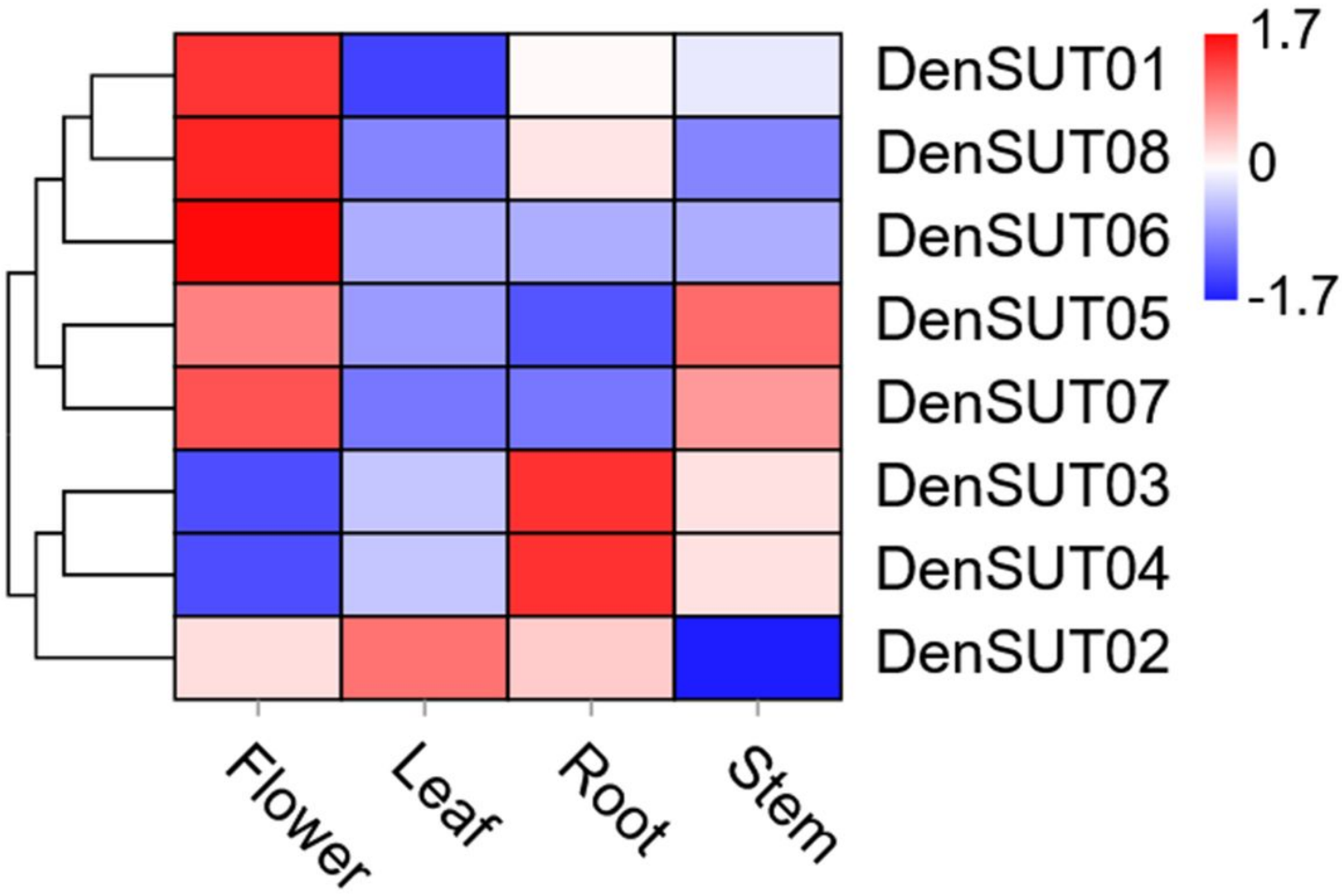

Figure 4

Hierarchical clustering of gene expression profiles of D. officnale SUTs in different tissues including flower, stem, leaf and root. The FPKM values were visualized in the heat map.

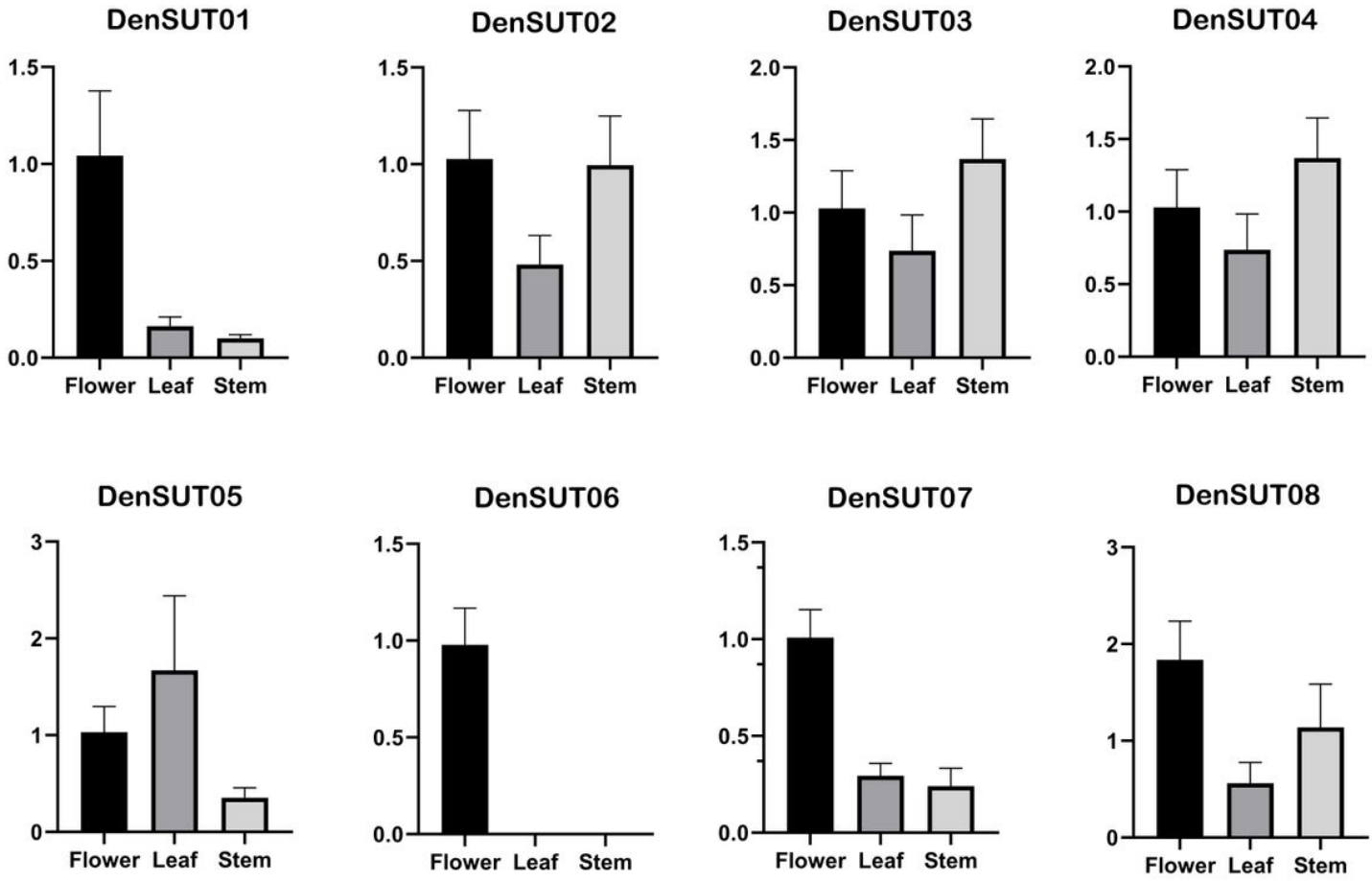

Figure 5

Expression levels of DenSUT genes in different tissues of D. officinale determined by qRT-PCR analysis. The results are shown as means ± SDs of three independent experiments. The presented gene expression levels are relative to the expression of the reference gene.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS1.xlsx
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