

# Comprehensive Analysis of Evolutionary, Characterization and Expression for Sugar Transporter Family Genes in *Nelumbo nucifera*

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## Research article

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# Abstract

## Background

The sugar transporter, an important class of transporter for sugars function, play regulators of many processes associated with growth, maturation and senescence processes in plant.

## Results

In this study, a total of 35 NNUSTs were identified in the *Nelumbo nucifera* genome. Furthermore, all NNUSTs genes identified were grouped according to conserved domains and phylogenetic analysis. Additionally, we identified 316 ST genes in other 10 representative plants, and performed a comparative analysis with *Nelumbo nucifera*, including evolutionary trajectory, gene duplication, and expression pattern. A large-scale analysis across plants and alga suggested that the ST family could have been originated from STP and Glct, expanding to form STP and SFP by dispersed duplication. Finally, quantitative real-time polymerase chain reaction and cis-elements analysis showed that the NNUSTs were response to abiotic stresses.

## Conclusions

This provides useful resources when exploring the molecular evolution and mechanisms of NNUSTs in plants.

## Background

In plants, sugars (sucrose, monosaccharides, polyols) play crucial roles in plant growth and development. It constitutes not only osmotic and signal molecules but also metabolites and nutrients. Movement of sugars on the whole-plant has two ways[1]. One way: loading and unloading of transport tissues[1]; In long-distance carbon partitioning, sugar alcohols (mannitol, sorbitol and galactinol) can be transported on top of sucrose[2]. Another way: the sugar transporters (STs) have controlled allocation of sugars into sink cells and sources, such as mediate the transport of sucrose[3, 4], monosaccharides[5] or polyols [6, 7]. The different sink cells, organelles of source or more biochemical have existed sugars for the transport of hexoses into the the vacuoles[8], chloroplast[9] and the Golgi apparatus[10]. Thus they have constructed sink and source organs[11]. Sugar transporters (STs) are critical for both phloem loading in source tissue and sucrose transport into sink cells[12]. In fauna and flora studies, some ST genes have been identified, such as hexose transporters in *Juglans regia* [13], *Vitis* [14, 15] and a few polyol transporters in *Malus domestica*[16], *Prunus cerasus*[17] and *Olea europea* [18]. The sugar transporters mainly sucrose transporters (SUTs) and monosaccharide transporters (MSTs) have been identified in many plants including *Arabidopsis thaliana*[19], rice (*Oryza sativa*)[20], wheat (*Triticum aestivum*)[21], populus[22], sorghum (*Sorghum bicolor*)[23], *Medicago truncatula*[24], tomato[25] and *Solanum lycopersicum*[26, 27], and from woody plants such as *Rosa hybrida* [28]. Recently, a novel sugar

transporter SWEET (Sugars Will Eventually Be Exported Transporter) family that can transport sugars was recently identified from *Arabidopsis* and *Oryza sativa*.

The MSTs play a critical role in long-distance sugar partitioning or sub-cellular sugar distribution and catalyze the transport of hexoses, but also polyols and in one case also pentoses and tetroses. MSTs contained seven distinct subfamilies (STP, VGT, PLT, INT, SFP, TMT, Glct). The sugar transport protein (STP) were responded to dehydration 6-like (ERD6L) and tonoplast monosaccharide transporter. STPs were also encoded by a far more diverse multigenic family in plants[5]. In previous study, there are 53 and 74 MSTs members were indentified in *Arabidopsis* and pear, respectively[29]. Most STP members are sink-specific and responsible for sink development. In plants, the STPs play important roles were proved by related studies in sugar accumulation. Such as, the three hexose transporters were RNAi-mediated knockdown in tomato that result in an obvious decrease of hexoses content in fruits, which proved that they controlled of hexoses into tomato fruit[30]. In addition, other MSTs subfamilies are identified as tonoplast-located MSTs. In *Arabidopsis* and rice, AtTMTs(tonoplast monosaccharide transporter), *AtVGT1* (vacuolar glucose transporter) and OsTMTs, which played important roles in vacuole sugar partition that were proved to import hexoses into the vacuole . In previous report it indicated that AtERDL6 as a tonoplast-localized glucose exporter and release glucose from vacuoles into the cytosol [31]. In addition to the above-mentioned functions, the suger transporters are also involved in plant responses to abiotic stress. In *Arabidopsis*, the expression of SUC1 and SUC2 were up-regulated by low temperature[32]. In rice, SUT1 and SUT2 were involved in salt and drought stress responses [33, 34]. A recent study also showed that AgSUT1, which encodes a high affinity sucrose/H<sup>+</sup> transporter, reduced the sensitivity of celery to salt stress[35]. Although the expression patterns and functional analysis of the sugar transporter gene family have been previously studied in a variety of plants, knowledge on the sugar transporter gene family is lacking in lotus root.

Lotus root (*Nelumbo nucifera* Gaertn), which originated from India and China, is an aquatic herb vegetable[36]. Lotus has been cultivated in China for over 2000 years and has gradually developed into two ecotypes due to its different biological properties (rhizome, flowers and seeds); the temperate type produces an enlarged rhizome and the tropical type has a long period of florescence. Lotus root, lotus powder and lotus seed are acknowledged nourishing food, rich in starch, protein, vitamins and mineral substances, with high edible value and medicinal value and deeply loved by consumers [37, 38]. Starch is the main storage material of lotus root, accounting for more than 70% of the dry matter weight. It is the main factor that affects the quality of lotus root product[39].

In plants, sugar transporters play an important role in sugar transport, absorption and utilization, and affect the growth and development of plants. Sucrose synthesized in the source organ enters the phloem under the action of sugar transporter, and after a long distance transportation, it enters the sink organ through the euplast or extracellular pathway. In the storage organ of lotus root, the material of the synthetic starch from the sucrose, which produced by the synthesis or degradation of the starch in the photosynthetic tissue. It through the sugar transporters into the storage organ and synthesizes starch

with the action of a series of enzymes. Therefore, the sugar transporters play an important role in the synthesis of lotus root starch.

However, little information on the sugar transporter gene family expression in different tissues and developmental roots of lotus root is available. Because of the vital functions of the sugar transporter in lotus root, it is of considerable importance to investigate the sugar transporter gene family in lotus root. In this study, we comprehensively analyses of drought, exogenous hormones, extreme temperatures and salinity influences of NNUST. In addition, based on cis-regulatory elements and qRT-PCR , we will further analysis of NNUST roles in *N. nucifera*. Finally, we constructed NNUST genes interaction networks, and analyze NNUST genes expression patterns through comparative genomics. These studies the first report on ST genes in *N. nucifera* and extends our understanding of the roles of the ST genes family in evolution tress responses.

## Result

### Identification and Phylogenetic relationship of the ST gene family in *N. nucifera*

To identify the all putative ST proteins in the *N. nucifera* genome, we used the HMM profile of the MFS domain (PF07690) and Blastp to search against the database. A total of 35 STs were identified and subjected to Pfam and SMART analyses, which resulted in the TableS.1. The ST genes were clustered into seven group, including STP, VGT, PLT, INT, SFP, TMT and Glct. The SUT sub family were not found in *N. nucifera*. To investigate the classification and phylogenetic relationship of the ST gene family in *N. nucifera*, we used the ST proteins to construct a phylogenetic tree. Based on the phylogenetic tree (Fig.1), all the ST proteins were consistented with *Arabidopsis* groups.

To explore the diversity in each group, we were identified motifs by MEME program. As showed in Fig. 1, the ST proteins that share common motif 1-10, which were conversed in ST. The motif 1, 4, 7, 8 and 10 were representative MFS domain. The each subgroup that share similar motifs and motif compositions were clustered into the same group. The result that ST gene family has highly conserved domains and motifs . (Fig.4).

### Evolution and Expansion of ST in different plant species

To investigate the evolution of the ST gene family in the plant kingdom, we selected 9 Angiospermae (6 eudicots, and 1 basal angiosperm), 1 Pteridophyta and 1 Bryophyta species for comparative analysis (Fig.2). Based on the whole-genome level, the number of ST in each species was counted (Fig.2a). The land plants have a relatively large number of ST gene. In addition, species that have a larger genome seem to contain a greater number of ST gene except for *Am. Trichopoda* and *P.patens*. In all species, the densities of ST proteins in *A. thaliana* (0.3926 number/Mb) were the highest, followed by *B.rapa* (0.2403 number/Mb) and *C. papaya* (0.1704 number/Mb), which were higher than those in lower plants. The reason is that *Vitis vinifera*, *P. trichocarpa*, and *C. papaya* did not undergo  $\alpha$  and  $\beta$  duplications and *Am. trichopoda*, a basal angiosperm, did not undergo the  $\gamma$  duplication event. Furthermore, due to specific

WGT events, there were more ST gene family members in some species [40, 41]. Meanwhile, we found that no ST were detected in *V. carteri* and *C. reinhardtii*. Then we constructed phylogenetic tree of the ST genes to analyze the evolutionary relationships of these species (Fig.2b). The phylogenetic tree showed that the ST gene family formed five distinct groups (STP, VGT, PLT, INT, SFP, TMT and Glct), which is consistent with the result for *A. thaliana*. The expansion happened in the evolutionary process from low plants to high plants, and the density of ST proteins increased as the plants evolved. From algae to angiosperm, the ST gene family has highly conserved domains and motifs. According to our findings, the evolutionary history of ST in the plant kingdom was constructed.

### **Chromosomal distribution and synteny analysis of NNUST genes**

The NNUST genes were unevenly mapped on the 11 megascaffolds (Fig.3). Some megascaffolds have more genes, whereas others have few. The megascaffold1 contained the largest number of NNUST genes (11). In other megascaffolds, the numbers of ST genes in megascaffolds6 (4), followed by megascaffolds2 and megascaffolds3(3), which megascaffolds10, megascaffolds14, megascaffolds5 and megascaffolds8 have only one gene. The NNUST duplicate genes were identified with PlantDGD. The duplicate genes were derived from four modes of gene duplication, including 7 of whole-genome duplications (WGD), 2 of tandem duplications (TD), 7 of transposed duplications (TRD), 17 of dispersed duplications (DSD). These results indicated the dispersed duplication may be a major driving force for NNUST genes evolution.

To investigate the evolution of NNUST family, three dicots (*Arabidopsis*, *S.tuberosum* and grape) and one monocots (maize) were constructed four comparative microsynteny maps with *N. nucifera* (Fig. 4). The collinear gene pairs showed syntenic relationship in maize (135), followed by grape(107), *Arabidopsis*(89), *S.tuberosum*and(22) (TableS.2). In these syntenic gene pairs, we found that some genes correspond to at least 4 collinear genes, especially in maize and grape, such as NNUSTP7 and NNUSFP5. Some NNUST collinear gene pairs (correspond to at least 4 collinear genes) identified in *N. nucifera* /*Arabidopsis*, *N. nucifera*/grape and *N. nucifera*/maize, indicated that these genes may already exist before the ancestral divergence and play an important role of NNUST gene family during evolution. In contrast, the subgroups of INT collinear gene pairs were not identified between *N. nucifera* and all of the other four species, indicate that may occurred after the divergence of dicotyledonous and monocotyledonous plants

To further investigated the evolution footprint of the NNUST family, the Ka/Ks ratios of the NNUST gene pairs were calculated between *N. nucifera* and *Arabidopsis* (TableS.3). All collinear gene pairs NNUST gene pairs had Ka/Ks < 1, suggesting that the NNUST gene family might have purifying selective pressure during evolution (Fig.S1).

### **Comparative expression pattern analysis on the ST genes in different tissues from *N. nucifera***

Compared different Sugar transporters in different tissues expression, we investigate the divergence expression patterns (Fig.5, TableS.4). We detected different MST gene expression levels, including the

leaves, petioles, flowers and rhizome. In SFP family, all genes showed relatively low levels in leaves and petioles, of which the expression of *NNUSFP3* gene was slightly higher; The expression of *NNUSFP3* gene was the highest in the rhizome, followed by *NNUSFP4* and *NNUSFP5*, and *NNUSFP2* was hardly expressed. In pGlcT family, all genes showed relatively levels in flowers, of which the expression of *NNUpGlcT2* and *NNUpGlcT4* is the highest. *NNUpGlcT4* was predominant expressional member in this gene family and is expressed in all four tissues, followed by *NNUpGlcT5*. In STP family, *NNUSTP6* gene is a low active member in the family, because it is undetectable in our study. *NNUSTP3* and *NNUSTP9* were the main members for the family, showed relatively levels in all four tissues, of which the expression of *STP9* gene was highest, especially in flowers. *NNUSTP1*, *NNUSTP4*, *NNUSTP5*, *NNUSTP7*, *NNUSTP8* expressions were detected in leaves, but these genes do not express or express less in other tissues. In INT and PLT family, some genes (including *NNUINT2*, *NNUINT3*, *NNUPLT1*, *NNUPLT2*) were detected only in leaves. Both *INT4* and *PLT4* gene have higher expression only in flowers. *INT1* and *PLT3* gene do not express or express less in all four tissues. In tMT family, all genes expressions were detected in all four tissues, and *NNUtMT2* is the most active. In VGT family, both *NNUVGT1* and *NNUVGT2* were highly expressed in leaves and flowers, but less expressed in other tissues.

### **Cis-elements and Interaction Network Analysis among ST Proteins in *N. nucifera***

The ST gene of cis-elements were identified at 1.5 kb promoter regions. Then, we analyzed the cis-elements by the Plant Cis-acting Regulatory DNA Elements (PLACE) website. We identified the 10 most common cis-elements in ST genes in *N. nucifera*(Fig.S2).

A total of 10 common cis-regulatory elements were identified in the promoter regions of the MST and SUT genes, which were highly conserved among all the studied MST and SUT genes in *N. nucifera* (Fig.S2). Three common cis-regulatory elements, ARBE, the TGACG-motif and the GARE-motif, were responsive to plant hormones, including ABA, JA and GA. Some common cis-regulatory elements were responsive to both abiotic and biotic stresses, including one fungal elicitor-responsive elements (W-box), a light-responsive element pathogen (G-Box), low-temperature responsiveness (LTR), defense and stress responsiveness (TC-rich repeats) and a drought-responsive element (MBS), indicating the importance of ST genes in stress tolerance. The ST genes were responsive to various stresses including drought, cold and salinity, which may be due to upstream gene specificity and the binding of corresponding cis-elements that regulate the expression of ST genes.

### **Expression profiles of ST genes under abiotic stresses in *N. nucifera***

In plant, ST gene family plays very important roles in development as well as in stress responses. Abiotic stress such as drought, extreme temperatures, and salinity adversely affects plant growth and crop productivity. So we chosed NaCl, PEG, ABA and cold treatments to identify the stress-responsive ST genes (Fig.6, TableS.5). Under ABA treatment, 23ST genes were upregulated. Meanwhile, 2ST genes were down-regulated. Among the 31 ST genes, the *NNUSFP5*, *NNUSTP2*, *NNUSTP4* and *NNUSTP5*, their expression was over 6 times than that of the control at 8h. Under NaCl treatment, 25 ST genes were upregulated. We found that especially the *NNUSFP2*, *NNUSFP3*, *NNUSTP3*, *NNUSTP4*, *NNUSTP6*, *NNUSTP7* and *NNUVGT2*,

their expression was upregulated at 8h or 16h but were downregulated at 24h. Under PEG treatment, we found that the *NNUSTP5* and *NNUSTP8* were reached the highest at 24h and over 6 times than that of the control. Including the 12 ST genes were upregulated at 8h. Under Cold treatment, 7 ST genes were upregulated at 8h and reached the highest. Among the 31 ST genes, especially the *NNUINT3*, *NNUSTP2* and *NNUSTP5*, their expression was over 5 times than that of the control at 8h; the expression of *NNUINT1*, *NNUINT2*, *NNUSTP3*, *NNUSTP5* and *NNUSTP8* was over 4 times than that of the control at 24h. Specifically, we observed that *NNUVGTs* were not responded or less to the four stress treatments.

To further investigate the connection between these ST genes, correlation and co-regulatory networks were established based on the PCCs of the relative expression of the genes (Fig.6b). NNUST gene pairs with PCC values that were significant at the 0.05 significance level and were greater than 0.5 were collected and visualized to construct hormones and abiotic stresses coregulatory networks (Fig.S3). All the gene pairs with positive significant correlations were shown in the co-regulatory network, a total of 35 nodes. The NNUST genes interaction network shows a very complicated correlation with other genes *N. nucifera*, which may indicate that NNUST genes are involved in many fundamental mechanisms and regulate many downstream factors and/or are regulated by many upstream genes. The expansion of the gene family depicted in the network, could help plants adapt to the changing environment by increasing cooperation and obtaining new functions.

## Discussion

In plants, sugar production through photosynthesis is a vital process, and sugar status modulates and coordinates internal regulators and environmental cues that govern growth and development. ST was likely involved in growth, development and responses to stress environment.

These genes are likely to be retained if genes products are involved in complex regulatory network [42, 43]. The previous reports suggest that gene duplicates are retained more frequently than the classical model permits and that new function or expression arises through the processes of neo- and subfunctionalization. In our study, we identified 35NNUST genes encoding putative sugar transporters in the the *N. nucifera* genome. The NNUSTP(13) genes were preferly retained in *N. nuciferaa*. The duplication genes experienced one of three fates: subfunctionalization, neofunctionalization, or non-functionalization (deletion or pseudogenization). In subfunctionalization, expression or function present in a progenitor gene is partitioned between daughter genes through complementary mutations to regulatory or coding regions[44]. In neofunctionalization, related or novel function may arise in one of the duplicates through initial relaxation of purifying selection with accumulation of mutations conferring new function under either neutral or positive selection[45]. The ST genes were provided opportunities to gain functional diversification.

Based on sequences of cis-elements and motif analyzed, ST gene promoters contained common cis-regulatory elements and several common motifs, domains. The MFS domain was originally believed to function primarily in the uptake of sugars but subsequent studies revealed that drug efflux systems,

Krebs cycle metabolites, were all members of the MFS. These observations led to the probability that the MFS is far more widespread in nature and far more diverse in function than had been thought previously. ST common motifs, such as DOFCOREZM that DNA-binding may play an important role in the regulation of gene expression in terms of activity levels for the ST genes. Such as, the AtSUT2 expression is regulated by the close co-operation of binding sites for a putative HD-Zip transcription factor and a DOFCOREZM protein [46]. In previous studies, concentration of sugar-responsive elements were regulated by some transporter gene. The transcriptional regulation via sugars, Such as the transcriptional regulation of VvHT1 by glucose was demonstrated[47, 48]. In ST gene family members, the MYBCOREATCYCB1 promoter which is required for transcriptional regulation of sucrose-dependent induction of Cyclin D3 gene expression[49]. In addition, the MYBCOREATCYCB1 promoter also found in ST members of pear, which indicated that different species might have a different transcriptional regulation mechanism in the ST gene family.

The great diversity of land plants on Earth today provides an chance to study gene family evolution in major lineages that differently in function complexity and life histories[50, 51]. In plants, gene families of evolution which prevalently combined of tandem, segmental and whole genome duplication (polyploidy) events. *N. nucifera* experienced whole genome triplication (WGT) event [52, 53]. The ancient of ST gene family was found in the moss lineage, which diverged from the vascular plants >410 million years ago [54]. In our study, the ST gene family was detected in *P. patens*, which was consist with ancient of ST from the moss lineage. Meanwhile, the ST of gene family shared similarly evolution with ST. Gene family expansion in the plants often due to multiple gene duplications arising from ancestral genes. In *Arabidopsis*, the large expansion of the ST gene families are due to tandem duplications. However, ST gene duplications in the *N. nucifera* belong to dispersed duplication(FigS.4). So, we can conclude that the ST family experienced large expansions resulting from the WGDs, that most of the ST family began to expand after divergence of bryophyta and chlorophyta.

## Conclusions

In conclusion, we comprehensive analysed the evolutionary pattern, gene synteny, gene duplication or losses, stress treatments and interaction network of ST genes involved in the sugar transport pathway. A total of 316 ST genes were identified among 10 representative species. The ST expansion happened in the evolutionary process from low plants to high plants. The analysis of promoter sequences indicated that different species have different transcriptional regulation in the ST gene family. The expression analysis revealed that most ST genes are expressed during development and responded to different stress treatment. Our study can provide comprehensive functional characterization of ST genes genes by reverse genetic approaches and molecular genetics research.

## Methods

### Retrieval of genome sequences

The *N. nucifera* and 10 representative genome sequences were used for comparative analyses in this study. The genome sequences of *N. nucifera* were downloaded from NCBI genome database ([https://www.ncbi.nlm.nih.gov/genome/14095genome\\_assembly\\_id=58790](https://www.ncbi.nlm.nih.gov/genome/14095genome_assembly_id=58790)). The *Arabidopsis* sequences were downloaded from TAIR (<http://www.arabidopsis.org/>). The sequences of the other 9 species were downloaded from phytozome[55].

### **Identification and characterization of ST gene family**

Pfam database was used to identify ST genes from all protein sequences of the examined species, with a threshold of e-value <1e-5. ST genes have the typical MFS domain (PF07690). The retrieved ST candidates were further verified by using SMART. MEME was used to search for conserved motifs[56].

Sequence alignment and phylogenetic analyses Multiple sequence alignment was performed using MUSCLE with default parameters[57]. Based on alignment, we generated phylogeny using previously reported method [58]. Phylogenetic analyses were conducted using Maximum Likelihood (ML).

### **Syntenic analysis**

The NNUST genes were mapped to *N. nucifera* chromosomes using Circos. Multiple Collinearity Scan toolkit (MCScanX) was exhibited the syntenic relationship of the NNUST genes and other selected species, the syntenic analysis maps were constructed using the Dual System Plotter software(<https://github.com/CJ-Chen/TBtools>). Non-synonymous (ka) and synonymous (ks) were calculated using KaKs\_Calculator 2.0.

### **Conditions and Treatments and qRT-PCR**

Experimental samples were used cultivar *N. nucifera* seedlings which grew in plastic container containing water in a controlled- environment growth chamber. Seedlings at the leaf stage were transferred to growth chambers set at 4 °C under the same condition as cold treatments. Other treatments: (1) control; (2) ABA 5mg/L; (3) polyethylene glycol (PEG)300mM(4) NaCl 6g/L. The control and stress-treated plants were collected, frozen in liquid nitrogen, and stored at -70 °C and used for bioassays.

Total RNA was isolated from treated leaves using the total RNA kit (Tiangen, Beijing, China). Total RNA was reverse transcribed into cDNA for RT-PCR (Takara, Dalian, China). cDNA was then diluted 1:20 with ddH<sub>2</sub>O as template for qRT-PCR. Three independent PCR reactions according to the manufacturer on the 7500 Fast Real-Time PCR System. All the specific primers according to the NNUST gene sequences. The qRT-PCR assays were performed with three biological and technical replicates. The gene expression levels were calculated with the  $2^{-\Delta\Delta CT}$  method[59].

## **Abbreviations**

**WGT:** whole genome triplication

**ST:** Sugar transporter

**SUT:**sucrose transporter

**MST:** monosaccharide transporters

**FPKM:** Fragments per kilobase of exon per million fragments mapped

**PCC:**Pearson Correlation Coefficient

## Declarations

### 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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### Author contributions

P. W. conceived and designed the experimental design. LJ.L(LiangJun Li) and YX.Z. contributed to the experimental work. All the authors read and approved the final manuscript.

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## Figures

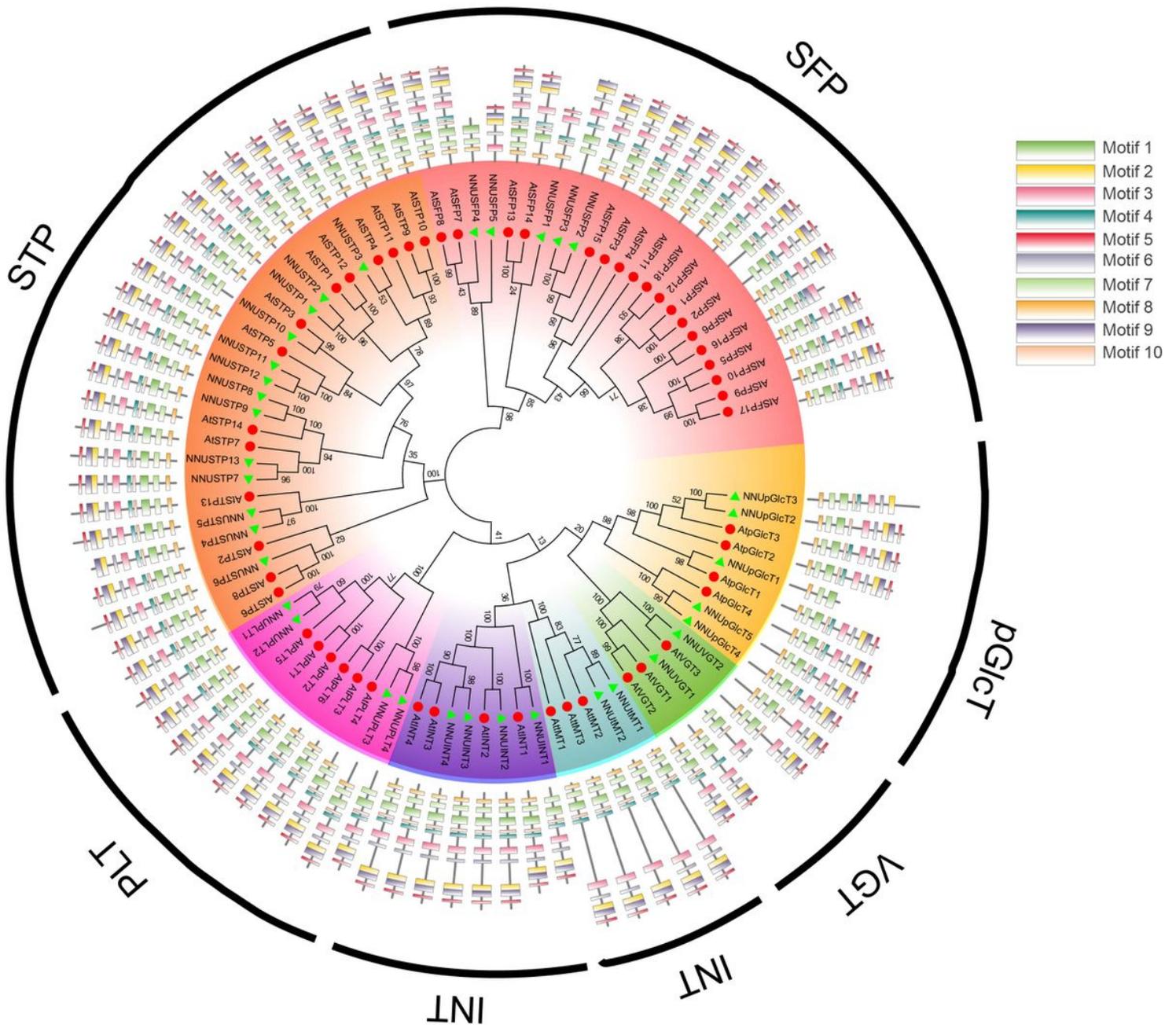
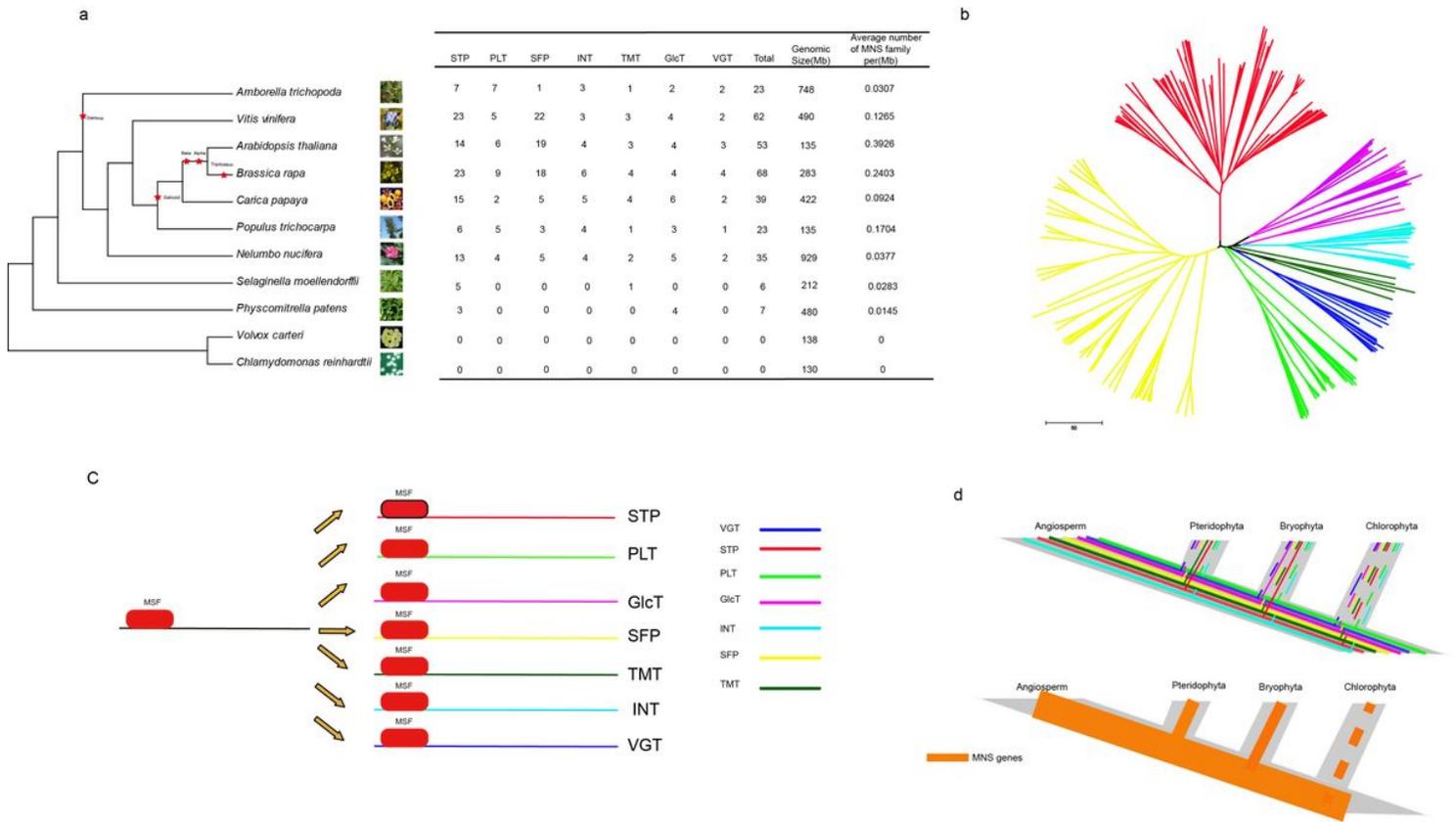


Figure 1

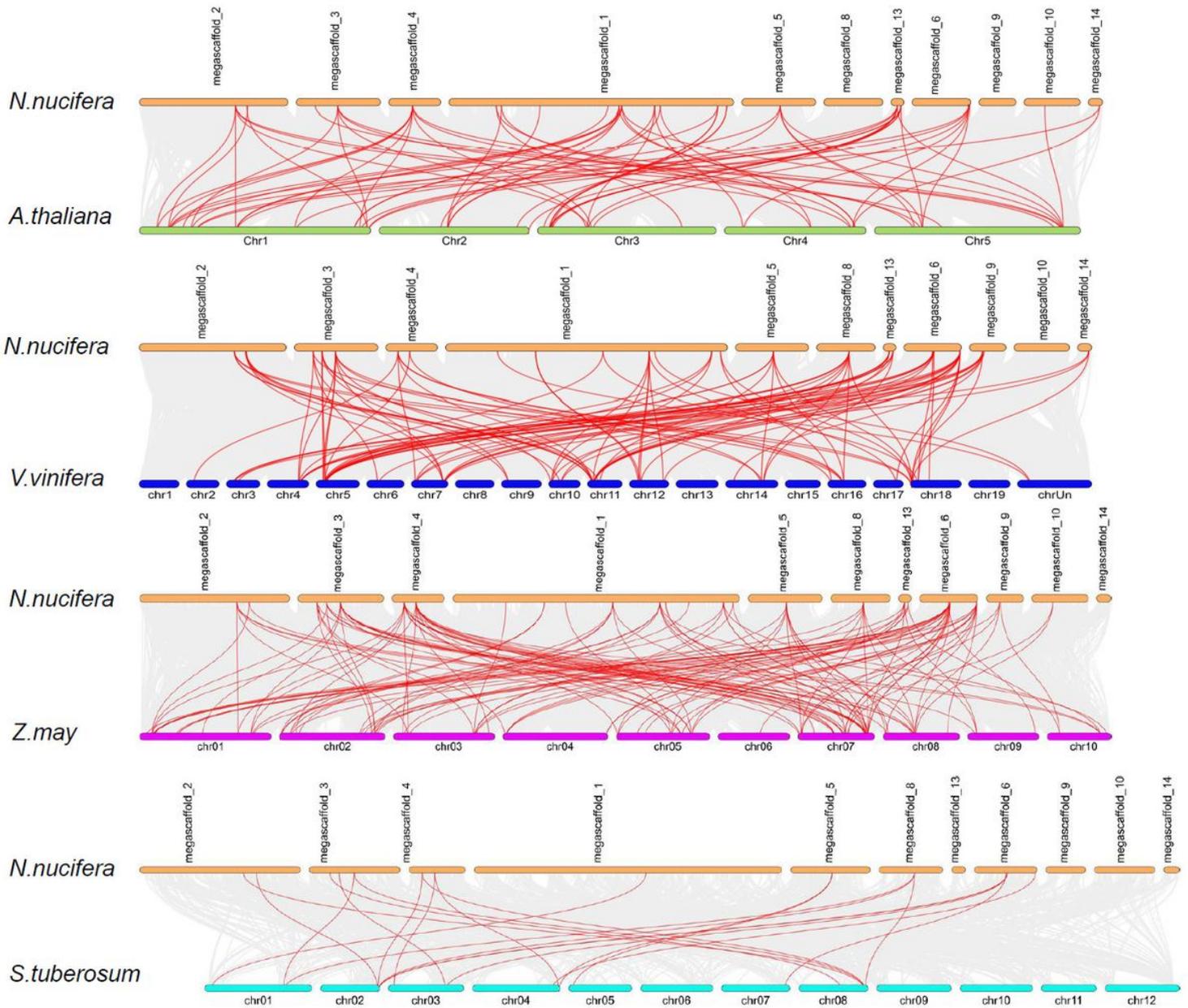
Phylogenetic relationships and conserved motif compositions of NNUST proteins.



**Figure 2**

The analysis of NNUST genes evolution (a) comparison of the percentage of ST gene in representative species. (b) Phylogenetic relationships among MST genes; (c,d) The evolutionary pattern of STs

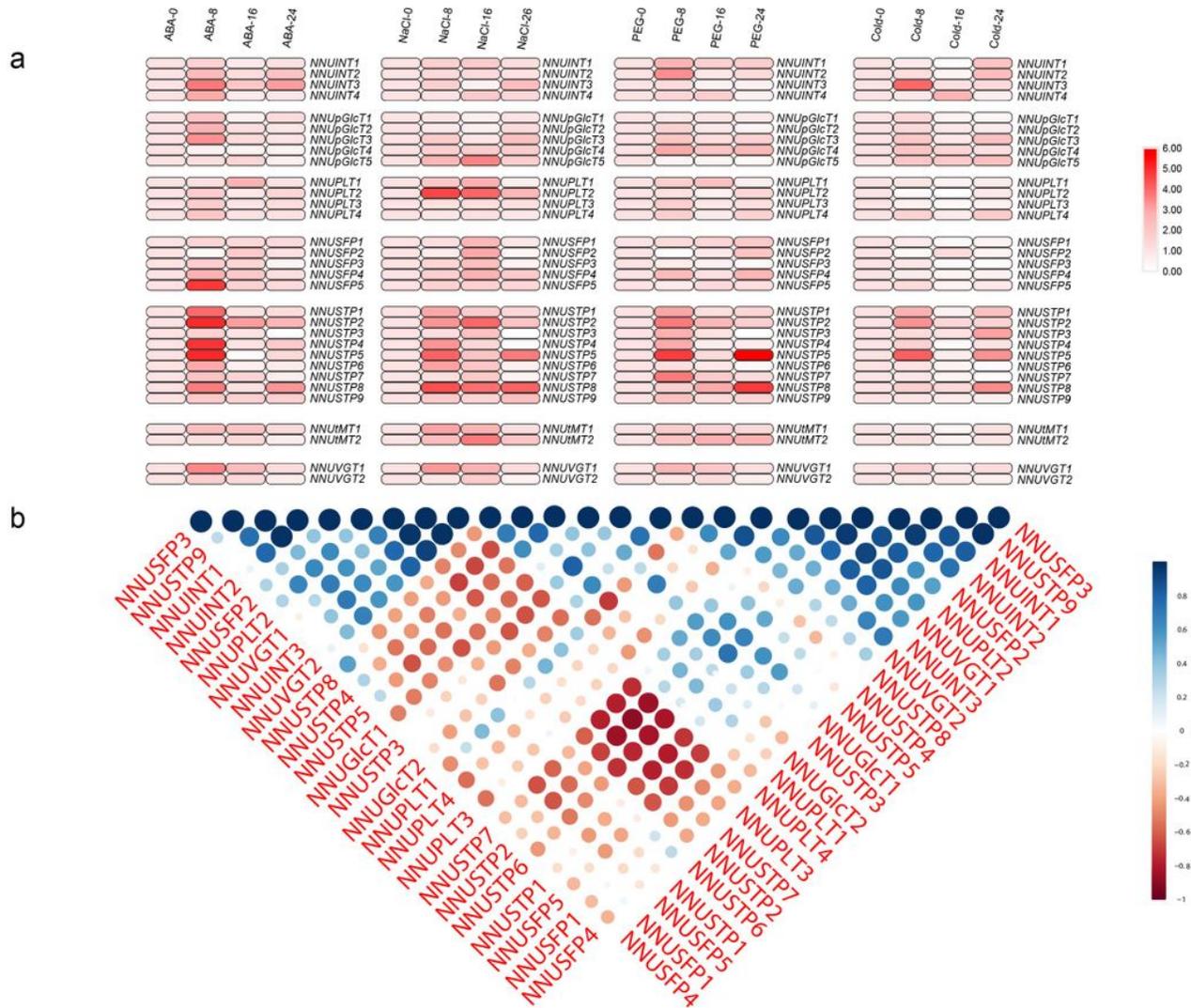




**Figure 4**

Synteny of NNUSTs in maize, grape, *S. tuberosum* and *Arabidopsis*. Gray lines in the background indicate the collinear blocks within *N. nucifera* and other plant genomes, while the red lines highlight the syntenic NNUST gene pairs.





**Figure 6**

Expression analysis of NNUSTs under abiotic stresses. Heat map representation and hierarchical clustering of NNUSTs during a ABA stress, NaCl stress, PEG stress, cold stress. (b) Correlation analysis by using the R package program.

## Supplementary Files

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