



Article

Functional Analysis of the Apple SPS Gene Family in Response to Abiotic Stresses

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Abstract: Sucrose phosphate synthase (SPS) is an important link in the process of sugar metabolism. In addition, it is also involved in abiotic stresses in plants. In order to study the SPS gene family and its role in abiotic stress, we identified the *MdSPS* gene family members by bioinformatics methods such as correlation analysis, the HMM method, and the Clustering method, and analyzed the transient expression of *MdSPS* genes by quantitative real-time fluorescence analysis (qRT-PCR). The *MdSPS* gene family consists of a total of 19 members divided into three subfamilies distributed on 14 chromosomes in apples. The *MdSPS* gene family has 12 collinearity gene pairs, indicating significant duplication. Most members of this family contain a large number of plant hormone response elements, light-inducible elements, and abiotic stress response elements 2kb upstream of the promoter. Codon bias analysis shows that there are 28 high-frequency codons and no codons with strong preference in this family. Gene chip results showed that only *MdSPS2*, *MdSPS3*, *MdSPS11*, and *MdSPS17* were up-regulated in roots, and they were all members of subfamily C. The qRT-PCR analysis showed that all members of this family responded significantly to drought stress, salt stress, and low temperature stress. Interestingly, the relative expression of *MdSPS12* was significantly down-regulated under salt stress and low temperature stress. In addition, the expression of *MdSPS3*, *MdSPS8*, *MdSPS11*, and *MdSPS17* was more than 20 fold higher than that of the control under drought stress, salt stress, and low temperature stress. These four genes could be candidates for molecular breeding in the *MdSPS* family.

Keywords: *Malus domestica*; sucrose phosphate synthetase (SPS); abiotic stress; expression analysis



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1. Introduction

The apple (*Malus domestica*) is one of the most widely cultivated fruits in the world. It is a woody perennial, a representative fruit tree of the Rosaceae family, and one of the four major fruits in the world. It is rich in minerals, vitamins, and phenolic compounds, making it a fruit with high nutritional value [1].

The main sugars in apple fruit are starch, sucrose and fructose [2]. Sucrose, as a soluble sugar, originates from the carbohydrate products of apples. It promotes the division of fruit cells, increases the sweetness of the fruit, and enhances the flavor of the fruit during the ripening stage [3]. Sucrose alters the developmental stages of fruit trees through interactions with various phytohormones. It is also an important carbohydrate for the plant [4]. Unlike other plants, 60–70% of the photoassimilates in apple leaves are sorbitol, and only about 20% are sucrose [5–7]. Although sucrose is low in the fruit, which is an important component of fruit sugar metabolism. In apple fruit, sorbitol and sucrose are unloaded from each sieve tube-companion cell complex into the cell interstitial space [8]. Fruit cells utilize the sucrose cycle and sorbitol dehydrogenation as major sources of metabolic energy.

Sucrose synthesis includes sucrose phosphate synthase (SPS), sucrose synthase (SuSy), and sorbitol dehydrogenase (SDH) in higher plants [9]. Among them, SPS is a soluble rate-limiting enzyme for plant sucrose synthesis, and fructose 6-phosphate (F6P) and UDP-glucose (UDPG) are catalyzed by sucrose phosphate synthase to synthesize sucrose 6-phosphate (S6P), the activity of which is related to sucrose accumulation and directly reflects the ability of sucrose synthesis in plants [10]. In addition, sucrose phosphate synthetase and sucrose phosphorylase exist as a complex in plants, which means that the process of sucrose synthesis is an irreversible reaction in plants [11].

Cardini et al. first discovered *SPS* in wheat germ in 1955 [12] and subsequently cloned members of the *SPS* gene family in maize [13], rice [14], Arabidopsis [15], sugarcane [16], and other species. In addition, several studies have shown that the *SPS* family has a certain regulatory effect on abiotic stress: the expression of sucrose phosphate synthase (*OsSPS*) in rice plants subjected to waterlogging stress showed a decreasing trend in grain and leaves [17], and the activity of sucrose phosphate synthase was significantly increased in leaves under drought stress [18] and in the roots of rice seedlings under saline stress [19]. The low temperature of 12–14 °C increased the content of *SPS* in cold-sensitive maize series [20], and the activity of sucrose phosphate synthetase increased in maize treated with the sulfonyleurea herbicide nicosulfuron compared with the control [21]. Low temperature stress significantly increased the activity of wheat *SPS* [18], and this increase increased with decreasing temperature [22]. *SISPS1* was positively regulated by dehydration under water stress [23] and showed higher activity in tomato *SPS* under heat stress [24]. Exogenous high-temperature spray treatment of spermidine promotes up-regulated *SPS* expression in lettuce [25]. Sugar beet sucrose phosphate synthetase activity was inhibited at low night temperatures [26]. Storage inhibits cold damage and maintains high sucrose phosphate synthase activity in zucchini fruits at high relative humidity [27]. Trehalose treatment increased sucrose phosphate synthase activity in apples [28] and peaches [29].

At present, the *SPS* gene family has been preliminary analyzed in apples. The published apple *SPS* gene family focuses on the role of *SPS* genes in sugar metabolism [30]. Recent studies have shown that water stress treatment of apple fruit during the developmental period reduces SPS activity within the fruit. This led to a decrease in fruit sucrose content and affected fruit quality [31]. Therefore, the activity of *SPS* due to non-stress affects the expression of *SPS*, which in turn affects the sucrose level. In other words, the *SPS* gene family is of high research value in abiotic stress. In this context, we used bioinformatics methods such as correlation analysis, the HMM method, and the Clustering method to identify the apple *SPS* gene family and to investigate the expression profiles and functions of apple *SPS* genes under abiotic stress. It provides a theoretical basis for the genetic improvement of apple stress resistance genes and fruit quality research.

2. Materials and Methods

2.1. Materials and Treatments

The experimental material was Gala-3 (GL-3) apple in a test tube plantlet, and the medium was MS + 30 g·L⁻¹ sucrose + 0.3 mg·L⁻¹ 6-BA + 0.2 mg·L⁻¹ IAA. Each triangular flask was inoculated with a single stem segment from a single apple bud. The apple seedlings were grown using the paper bridge method. The incubation conditions of the artificial climate chamber were as follows: light at 28 °C for 16 h, dark at 25 °C for 8 h, light intensity of 200 μmol·m⁻²·s⁻¹. After 32 days of succession, apple seedlings with stable growth and no contamination were selected as experimental materials. Experimental treatments were performed in MS liquid medium containing 10% polyethylene glycol 6000 (PEG 6000), 200 mmol·L⁻¹ sodium chloride (NaCl), and a low temperature (4 °C). Controls were untreated. The incubation conditions were 28 °C/25 °C (light/dark) (4 °C for low-temperature treatment) for 16 h/8 h (light/dark). The apple seedlings treated at 4 °C were incubated in a low-temperature plant incubator (Percival LT36VL, Perry, IA, USA). The length of treatment was 24 h. Each group treated a total of 30 bottles of apple seedlings, and every 10 bottles of apple seedlings was 1 biological replicate. The treated

apple seedling leaves were collected, weighed, and wrapped in aluminum foil. The leaves were rapidly frozen in liquid nitrogen and stored at -80°C .

2.2. Identification of SPS Gene Family Members in Apple

Arabidopsis Thaliana SPS gene IDs were obtained using known literature, and the gene length, CDS sequence, amino acid number, and cDNA length of each gene were obtained from NCBI. Apple SPS gene family members were screened by homologous gene comparison using phytozome13 (<https://phytozome-next.jgi.doe.gov/>, accessed on 16 November 2022) and the corresponding protein, CDS sequence, and full-length gene sequence under known gene landing sequences were retained. The SPS [Sucrose-synth (PF00862) and Glycos-transf-1 (PF00534)] candidate genes that did not contain the characterized structural domains were again filtered by the functional structural domain filtering of the HMMER (<https://www.ebi.ac.uk/Tools/hmmer/>, accessed on 17 November 2022) online software [32], and the screening resulted in 19 apple SPS gene family members. Based on the filtered SPS amino acid sequences, their basic information, such as amino acid number, molecular weight, isoelectric point, hydrophilicity, etc., were analyzed using Expasy ProtParam (<https://web.expasy.org/protparam/>, accessed on 17 November 2022) online software tool [33].

2.3. Chromosome Mapping, Subcellular Mapping and Secondary Structure Analysis of Apple SPS Gene Family

The genomic data were analyzed using TBtools software v2.096 [34] and visualized and mapped using the MG2C (http://mg2c.iask.in/mg2c_v2.1/, accessed on 25 November 2022) website. Subcellular localization prediction of the SPS gene family was performed using WoLFPSORT (<https://www.genscript.com/wolf-psort.html>, accessed on 25 November 2022). Secondary structure analysis of the SPS gene family proteins using PRABI (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html, accessed on 26 November 2022).

2.4. Phylogenetic Tree Construction and Collinearity Analysis of SPS Family

The SPS family members of *Arabidopsis thaliana*, grape (*Vitis vinifera*), rice (*Oryza sativa*), peach (*Prunus persica*), wheat (*Triticum aestivum*), and maize (*Zea mays*) were screened by phytozome13, and the amino acid sequences of the SPS genes of the known species were converted to fasta format using Clustalx.exe v1.83 [35], and the phylogenetic tree was constructed using MEGA 7.0 software [36]. The default value of Neighbor-Joining was 1000. The results were analyzed by MCScanX tool in TBtools. Using TBtools, the annotation information of the apple genome was analyzed with the MCScanX tool, and the results were visualized with Advanced Circos. The genome annotation information of *Arabidopsis thaliana*, maize, rice, and apple was imported into TBtools software for comparative analysis among species, and then the results were visualized using Multipul Synteny plot.

2.5. Motif, Gene Structure, and Cis-Acting Element Analysis of Apple SPS Gene Family

Multiple comparisons were performed using DANMAN software v6.0.40 to avoid duplicate sequences, and conserved motifs were analyzed using MEME online software (<https://meme-suite.org/meme/tools/meme>, accessed on 10 January 2023). The apple genome GFF3 file was analyzed using TBtools software to extract and analyze the gene structure of the SPS family. The 2000 bp promoter sequence of the apple SPS gene family was extracted using TBtools software. The cis-functional elements were screened on the PlantCARE website (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>, accessed on 12 January 2023) and the results were visually analyzed [37]. The phylogenetic tree, motif sequences, gene structures, and cis-functional elements of the apple SPS gene family were visualized and analyzed using TBtools software.

2.6. Codon Bias Analysis of MdSPS Family

The CodonW application (<http://codonw.sourceforge.net>, accessed on 15 January 2023) was used for codon bias analysis of the *MdSPS* gene, and Excel 2010 was used to organize the analyzed data. The results were entered into Origin 2021 software for correlation analysis and into RStudio software (<https://www.rstudio.com/products/rstudio/download/>, accessed on 15 January 2023) for relative synonymous codon usage analysis.

2.7. Analysis and Prediction of Interaction Network of Apple SPS Gene Family Proteins

The *MdSPS* family protein sequences were constructed using the Protein-Protein Interaction String Database on the Internet (https://cn.string-db.org/cgi/input?sessionId=bQ-2lYDwXJ23e&input_page_show_search=on, accessed on 18 January 2023).

2.8. Expression Analysis of Apple SPS Family Gene Chip

The expression of apple was downloaded from GEO data (GSE42873) in NCBI database (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE42873>, accessed on 25 February 2023), and the transcriptome data of apple roots, stems, leaves, flowers, fruits, seeds, and seedlings were selected, and the genes were extracted using GDR (<https://www.rosaceae.org/>, accessed on 26 February 2023) gene, after that, it will be compared between the Excel used to organize the data and TBtools used to draw the gene expression heatmap.

2.9. qRT-PCR Analysis of Apple SPS Family

RNA was extracted from leaves using the plant extraction kit RNAlplant-RTR2303 (Zhongkeritai Biotechnology Co., LTD., Beijing, China) according to the instruction manual. The cDNA was synthesized using the Prime Script RT reagent kit (Perfect Real Time) reverse transcription kit (TaKaRa Biotechnology, Lanzhou, China), and the LightCycler[®] 96 qRT-PCR system (Roche, Basel, Switzerland) was used. The primers were amplified by PCR under different treatments, and the reference gene was *GAPDH* (GenBank accession no. CB973647). The amplification reaction system was 20 μ L: cDNA 1 μ L, upstream and downstream primers 1 μ L, SYBR 10 μ L, ddH₂O 7 μ L. 19 primers for apple *SPS* genes were designed (Table 1) and synthesized online by Sangon Biotech (Shanghai, China). The relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method [38]. Three biological and technical replicates were established for this experiment.

Table 1. Apple *SPS* family of real-time fluorescent quantitative primers.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
<i>GADPH</i>	TTCTCGTTGAGGGCTATTCCA	CCACAGACTTCATCGGTGACA
<i>MdSPS1</i>	GGACACGGACTACGAAGGATTGC	AGTGCCAGGACATCGGATAGAGG
<i>MdSPS2</i>	TTGACCCACTTCCACCCTGAAATTG	TCCAGCCTTGCCATTGTGAAGATG
<i>MdSPS3</i>	TTGACCCACTTCCACCCTGAAATTG	TCCAGCCTTGCCATTGTGAAGATG
<i>MdSPS4</i>	TGACTGGATTGGTTGAGTGCTATGC	AATGTAACCAGCGACTACGACAAGG
<i>MdSPS5</i>	CCTCTGGCATGGACTTCAGTAATG	AACCGCATCACTTCTGACCATATCG
<i>MdSPS6</i>	AACCTTGCTCGTGCTCTGGCTAATAC	TGGGTTCAACGTAGCTGGAGTC
<i>MdSPS7</i>	TGACACCAAGGGAGAGGGATGAAG	CAAGGATGACCGAGCAGCAATTC
<i>MdSPS8</i>	ATGGAGATGGGGATGGTGAAGGAG	CTGGCAAGGGCAAGTATCATAGGC
<i>MdSPS9</i>	CCCTACGCAGAGAAGCAGAAACG	ATGTCCAGCCTTGCCATTGAGAAG
<i>MdSPS10</i>	GTGTGAAGCCAGGAGCCAAGAC	AGTGTAGACAAGGTTGCATCGGAAG
<i>MdSPS11</i>	AACTAATCGAGTGAGCAACGGTGAG	CCGCAAGTCATAGCCTCCACAAC
<i>MdSPS12</i>	GGGAATCACACAGAGGGCTCAAAC	CCAATATGCTCGTCTGCTGCTCAG
<i>MdSPS13</i>	CTTCAGGCTCCAGATCCCTCTACC	CTCCAGTGTCAGGCAAACCCAAG
<i>MdSPS14</i>	ACCAACCTCAGCAGTCCCTCAG	TGACGCAGCAGCAATTCCATAGG
<i>MdSPS15</i>	GCCGTTTCAGTCCGACTCGTTAC	ATCCGCCAGCACATATTCTCCAAC
<i>MdSPS16</i>	GGTGGTTGTCGCTGGAGAATGC	AGTTTCGGTTCGTGTGGAGTTGAC
<i>MdSPS17</i>	GCCGAGGATGCGATCAAGGAAC	ACCCAATTTGAGTGCCATCAGTGAG
<i>MdSPS18</i>	TCTTGCTCCTTTCGTGGCTCTTG	GCACTCTCCGTCAATAAGCTCTTCC
<i>MdSPS19</i>	CAGGCGGGCAGGTAGTTACATTC	CCTTGCTTCGGGTATGAGTCTTGTC

2.10. Statistical Analysis

Excel 2010 was used to sort the experimental data, and Origin 2021 software was used for graphing. SPSS 26 was used for the analysis of variance and multiple comparisons of experimental data. Duncan's method was used to analyze the significance difference, and the significance level was $p < 0.05$.

3. Results

3.1. Chromosome Mapping and Physicochemical Property Analysis of Apple SPS Gene Family

19 apple SPS genes were identified by homologous comparison of the HMMER model, which were named *MdSPS1*~*MdSPS19*. Location of the apple SPS family on the chromosome (Figure 1). They are located on each of the 14 chromosomes of the apple. There are three genes on chromosomes 2 and 15, two genes on chromosome 13, and one gene on each of the remaining 11 chromosomes. *MdSPS2* and *MdSPS3* are located on chromosome 2 and closely spaced, while *MdSPS12* and *MdSPS13* are located on chromosome 13 in the same condition.

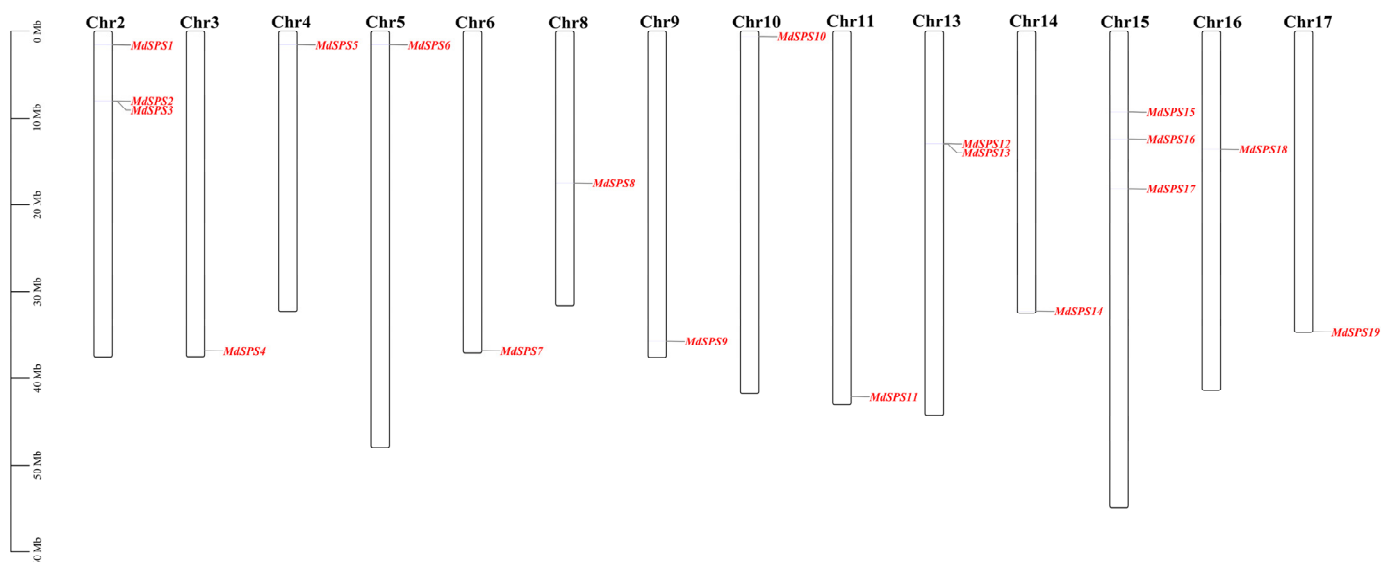


Figure 1. The distribution of SPS gene family members of chromosomes in apple. The left scale indicates the chromosome length (Mb).

Table 2 shows that the amino acid length of the *MdSPS* family ranges from 305 to 1065 aa, among which the protein sequence of *MdSPS12* is the shortest and that of *MdSPS5* is the longest. The relative molecular weight of the protein ranges from 34,488.82 to 119,183.48 Da. The isoelectric point is 5.67~7.21. The instability index of *MdSPS3* is the lowest (31.51) and that of *MdSPS15* is the highest (49.04). The value of the grand average of hydropathicity ranges from -0.092 to -0.492 .

Table 2. Physicochemical properties of SPS gene family in apple.

Gene Name	Gene Accession	GDR	Amino Acid/aa	Molecular Weight/Da	pI	Instability Index	Aliphatic Index	Grand Average of Hydropathicity
<i>MdSPS1</i>	MD02G1022300	MDP0000288876	1055	118,220.19	6.08	47.86	89.10	-0.420
<i>MdSPS2</i>	MD02G1100500	MDP0000160578	806	92,581.30	6.00	32.68	94.08	-0.269
<i>MdSPS3</i>	MD02G1100600	MDP0000872262	807	92,569.29	6.05	31.51	94.46	-0.269
<i>MdSPS4</i>	MD03G1291300	-	306	34,951.07	6.02	32.57	83.92	-0.158
<i>MdSPS5</i>	MD04G1013500	MDP0000783676	1065	119,183.48	6.15	42.60	83.77	-0.481
<i>MdSPS6</i>	MD05G1006400	MDP0000256965	1024	115,199.99	6.76	46.62	88.32	-0.444

Table 2. Cont.

Gene Name	Gene Accession	GDR	Amino Acid/aa	Molecular Weight/Da	pI	Instability Index	Aliphatic Index	Grand Average of Hydropathicity
MdSPS7	MD06G1237200	-	899	101,283.99	6.79	37.77	85.52	−0.270
MdSPS8	MD08G1157000	-	663	74,815.07	6.17	46.50	85.78	−0.492
MdSPS9	MD09G1280200	-	833	94,483.07	6.37	34.36	87.94	−0.300
MdSPS10	MD10G1002300	-	516	57,616.61	6.69	43.70	95.35	−0.296
MdSPS11	MD11G1307000	MDP0000126946	812	92,891.09	5.77	38.93	89.37	−0.290
MdSPS12	MD13G1164000	-	305	34,488.82	5.67	43.93	106.10	−0.092
MdSPS13	MD13G1164200	-	639	72,997.50	6.03	34.68	89.72	−0.254
MdSPS14	MD14G1244000	MDP0000212593	898	101,084.66	6.81	39.45	83.44	−0.276
MdSPS15	MD15G1127900	MDP0000174537	1057	118,011.50	6.04	49.04	84.71	−0.465
MdSPS16	MD15G1164900	-	1055	118,134.12	6.09	47.40	89.00	−0.412
MdSPS17	MD15G1223500	MDP0000250070	807	92,371.03	5.87	32.26	95.18	−0.266
MdSPS18	MD16G1164000	-	850	97,377.40	5.99	35.33	89.46	−0.229
MdSPS19	MD17G1287000	-	843	95,854.58	7.21	39.45	86.65	−0.323

3.2. Subcellular Localization Prediction and Secondary Structure Analysis of the Apple SPS Family

The subcellular localization prediction of apple SPS proteins is shown in Table 3. The expression of MdSPS proteins is mainly concentrated in the cytoplasm, chloroplast, nucleus, and plasma membrane. And a small amount of expression in mitochondria and peroxisome. All members were expressed in the cytoplasm; MdSPS9 and MdSPS12 were the highest. Except for MdSPS2 and MdSPS3, other members of the gene family were also highly expressed in chloroplasts. Only MdSPS10 and MdSPS14 were expressed in peroxisomes.

Table 3. Prediction of apple SPS protein subcellular localization.

Protein	Cytoplasm	Chloroplast	Nucleus	Plasma Membrane	Mitochondria	Peroxisome
MdSPS1	3	3	6	1	-	-
MdSPS2	7	-	2	2	2	-
MdSPS3	11	-	2	-	-	-
MdSPS4	2	4	-	3	-	-
MdSPS5	4	1	7	1	-	-
MdSPS6	4.5	2	3.5	1	-	-
MdSPS7	2.5	3	3.5	4	-	-
MdSPS8	8	3	2	-	-	-
MdSPS9	12	1	-	-	-	-
MdSPS10	6	3	-	1	1	1
MdSPS11	5	3	-	2	3	-
MdSPS12	12	1	-	-	-	-
MdSPS13	8	2	-	2	2	-
MdSPS14	4	2	3	2	-	2
MdSPS15	6	2	4	1	-	-
MdSPS16	3	3	6	1	-	-
MdSPS17	6	-	3	-	4	-
MdSPS18	7	6	-	-	-	-
MdSPS19	11	2	-	-	-	-

“-” indicates that the protein is not predicted to be expressed at this site.

The secondary structure analysis of the apple SPS gene family proteins is shown in Table 4. The results showed that the apple SPS gene family proteins all contain an alpha helix, a beta turn, a random coil, and an extended strand. The alpha helix and random coil are the main components of the secondary structure of the proteins in this gene family. Alpha helix accounted for 34.50–64.71%, random coil accounted for 17.32–39.55%, followed by extended strand, and beta turn accounted for the smallest proportion.

Table 4. The secondary structure of SPS protein sequence in apple.

Protein	Alpha Helix/%	Extended Strand/%	Beta Turn/%	Random Coil/%
MdSPS1	41.90	13.36	6.45	38.29
MdSPS2	54.47	12.41	6.45	26.67
MdSPS3	54.52	12.89	6.82	25.77
MdSPS4	64.71	10.46	7.52	17.32
MdSPS5	40.66	13.24	6.57	39.53
MdSPS6	41.21	14.16	6.74	37.89
MdSPS7	48.50	12.24	6.45	32.81
MdSPS8	45.55	10.86	6.79	36.80
MdSPS9	52.70	12.85	6.60	27.85
MdSPS10	34.50	19.19	7.95	38.37
MdSPS11	52.71	13.42	6.77	27.09
MdSPS12	44.59	15.41	5.57	34.43
MdSPS13	52.58	13.15	7.20	27.07
MdSPS14	49.78	12.03	6.68	31.51
MdSPS15	40.02	13.81	6.62	39.55
MdSPS16	42.18	13.27	6.64	37.91
MdSPS17	54.52	12.64	6.20	26.64
MdSPS18	52.35	13.88	6.94	26.82
MdSPS19	52.79	11.86	6.05	29.30

3.3. Construction of Phylogenetic Tree of Apple SPS Gene Family

According to related studies, the *SPS* gene family was divided into four subgroups based on phylogenetic analysis in higher plants [39]. To further understand the evolutionary relationship between *MdSPS* and other species, we performed phylogenetic analysis of 97 protein sequences from seven species, including *Arabidopsis thaliana*, grape (*Vitis vinifera*), rice (*Oryza sativa*), peach (*Prunus persica*), wheat (*Triticum aestivum*), maize (*Zea mays*), and apple (*Malus domestica*). Sucr_P_syn_N (TIGR02472) was found in subgroups A, B, and D, and sucr_synth (cl30294) was found in subgroup C. All members of subgroup D were monocotyledons. *OsSPS6*, *TaSPS7*, *TaSPS19*, *TaSPS27*, and *ZmSPS6* all have the DUF1752 superfamily domain (cl07240) (Figure 2).

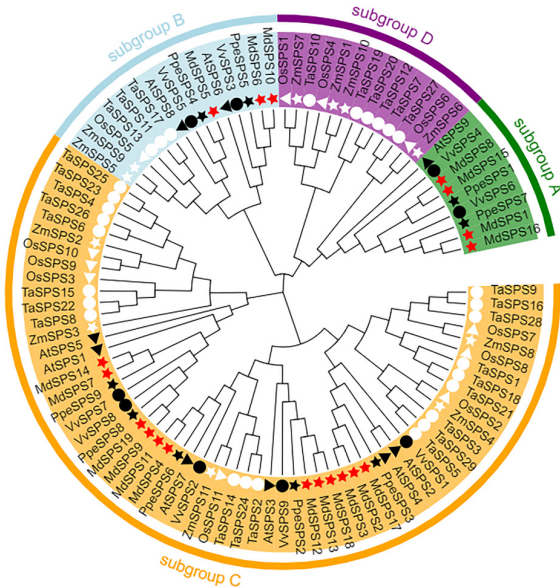


Figure 2. Phylogenetic tree analysis of *SPS* genes in rice (*Os*), wheat (*Ta*), maize (*Zm*), *Arabidopsis thaliana* (*At*), grape (*Vv*), peach (*Ppe*), and apple (*Md*). White triangles represent rice, white circles represent wheat, white stars represent maize, black triangles represent *Arabidopsis*, black circles represent grapes, black stars represent peaches, and red stars represent apples.

The apple *SPS* gene family is divided into three subfamilies. Subfamily A contains *MdSPS1*, *MdSPS8*, *MdSPS15*, and *MdSPS16*; subfamily B contains only *MdSPS5*, *MdSPS6*, and *MdSPS10*; and subfamily C contains most of the genes of the apple *SPS* family, namely *MdSPS2*, *MdSPS3*, *MdSPS4*, *MdSPS7*, *MdSPS9*, *MdSPS11*, *MdSPS12*, *MdSPS13*, *MdSPS14*, *MdSPS17*, *MdSPS18*, and *MdSPS19* were 12 genes. Subgroup D contains no members of the apple *SPS* family.

3.4. Synteny Analysis of *SPS* Genes Family

The analysis of the synteny between the *SPS* gene families of apple, maize, *Arabidopsis thaliana* and grape is shown in Figure 3. There was abundant replication between apple and all species, and gene pairs existed in all genes (excepting *MdSPS6*, *MdSPS8*, *MdSPS12*, *MdSPS13*, and *MdSPS19*). There were more collinear gene pairs with *Arabidopsis* and grape, but fewer collinear gene pairs with maize. This is probably because apples are very distant from maize.

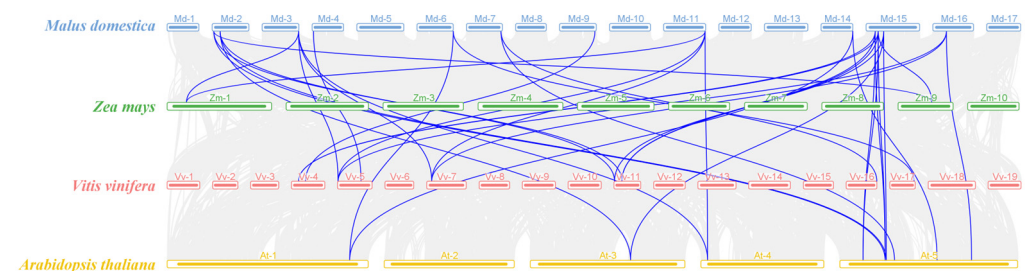


Figure 3. Synteny analysis of *SPS* genes in apple, maize, *Arabidopsis thaliana* and grape. The gray lines in the background represent collinear blocks in the genomes of apple, maize, *Arabidopsis* and grape, while the blue lines highlight genetic pairs of the apple *SPS* genes.

As shown in Figure 4, the replication phenomenon of 19 *MdSPS* genes was obvious, and there were 12 pairs of collinearity genes. *MdSPS3*, *MdSPS5*, and *MdSPS13* have no collinearity information. *MdSPS15* and *MdSPS16* are on the same chromosome and close to each other. This indicates that there may be a genetic linkage between the two genes. All other collinear relationships between genes occur between two chromosomes.

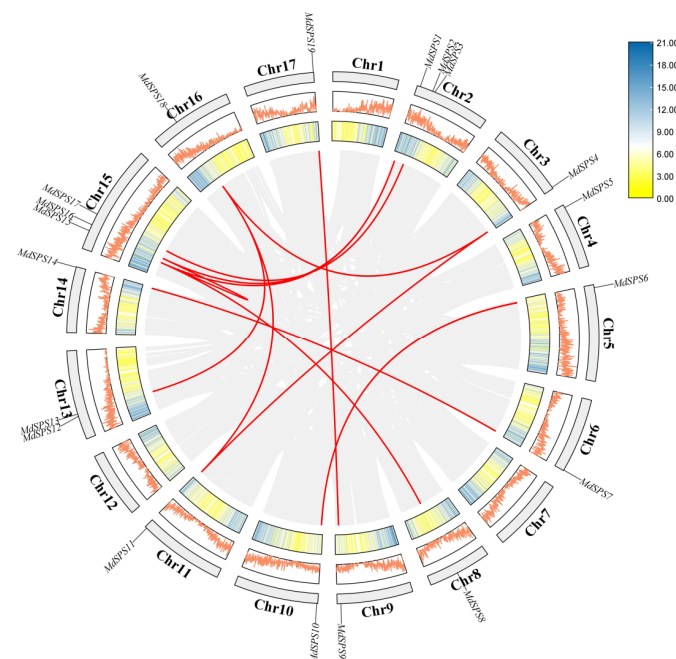


Figure 4. Collinearity analysis of apple *SPS* gene family. The red lines represent duplicate pairs of *MdSPS* genes.

3.5. Motif Analysis of Apple SPS Gene Family and Prediction of Promoter Cis-Acting Elements

A total of 25 motifs were obtained by motif analysis of the apple SPS family proteins (Figure 5a). All proteins contained motif 7, indicating that they have similar functions. Motif 4, motif 11, motif 12, motif 14, motif 18, and motif 21 were found only in subgroup C. Motif 1, motif 7, motif 9, and motif 19 were found in subgroups A and B. Except for MdSPS4 and MdSPS12, all of the other genes contained seven or more motifs.



Figure 5. Motif analysis and promoter *cis*-acting element analysis of apple SPS gene family. (a) Motif analysis of MdSPS proteins. (b) Upstream *cis*-acting element analysis of 2000bp MdSPS genes. (c) Intron-exon structure of MdSPS genes.

To determine the function of *cis*-acting elements in the MdSPS family, *cis*-acting element analysis was performed on the 2000 bp promoter sequence upstream of the family (Figure 5b). *Cis*-acting elements of the apple SPS gene family promoter include hormone response, light response, and stress response.

Structure analysis of 19 genes in the apple SPS family showed the following results (Figure 5c): The number of exons in this gene family ranges from 6 to 15, with MdSPS4 and MdSPS12 having no upstream and downstream gene sequences, MdSPS13 and MdSPS18 having no upstream gene sequences, and MdSPS3 and MdSPS14 having no downstream gene sequences, while all of the other genes contain upstream and downstream. The number of exons in subgroup C is quite different, which may be related to their function. The number of exons in MdSPS11, MdSPS18, and MdSPS19 is up to 15, while the number of exons in MdSPS4 is the lowest, with only 7. Except for MdSPS8, the other three genes in subgroup A all contain 13 exons. However, there were differences in the distribution, length, and number of coding regions in the same subpopulation.

All 19 SPS genes in apples contain hormone-responsive and light-responsive elements (Figures 5c and 6). Anaerobic induction elements were found in 15 genes, and defense

and stress-responsive elements were found in five genes. Ten genes, including *MdSPS3*, *MdSPS4*, *MdSPS5*, *MdSPS9*, *MdSPS10*, *MdSPS13*, *MdSPS14*, *MdSPS15*, *MdSPS18*, and *MdSPS19*, contain low temperature responsive elements. Drought-inducible elements were found in nine *MdSPS* genes: *MdSPS3*, *MdSPS4*, *MdSPS6*, *MdSPS7*, *MdSPS8*, *MdSPS9*, *MdSPS10*, *MdSPS14*, and *MdSPS15*. The wound response element is present in only one gene, *MdSPS13*. *MdSPS2*, *MdSPS11*, *MdSPS16*, and *MdSPS17* all contain hormone-responsive, light-responsive, anaerobic-induction, defense, and stress-responsive elements. Six elements of hormone responsive, light responsive, anaerobic induction, low temperature responsive, drought inducible, defense and stress responsive elements appeared in *MdSPS3* and *MdSPS10*.

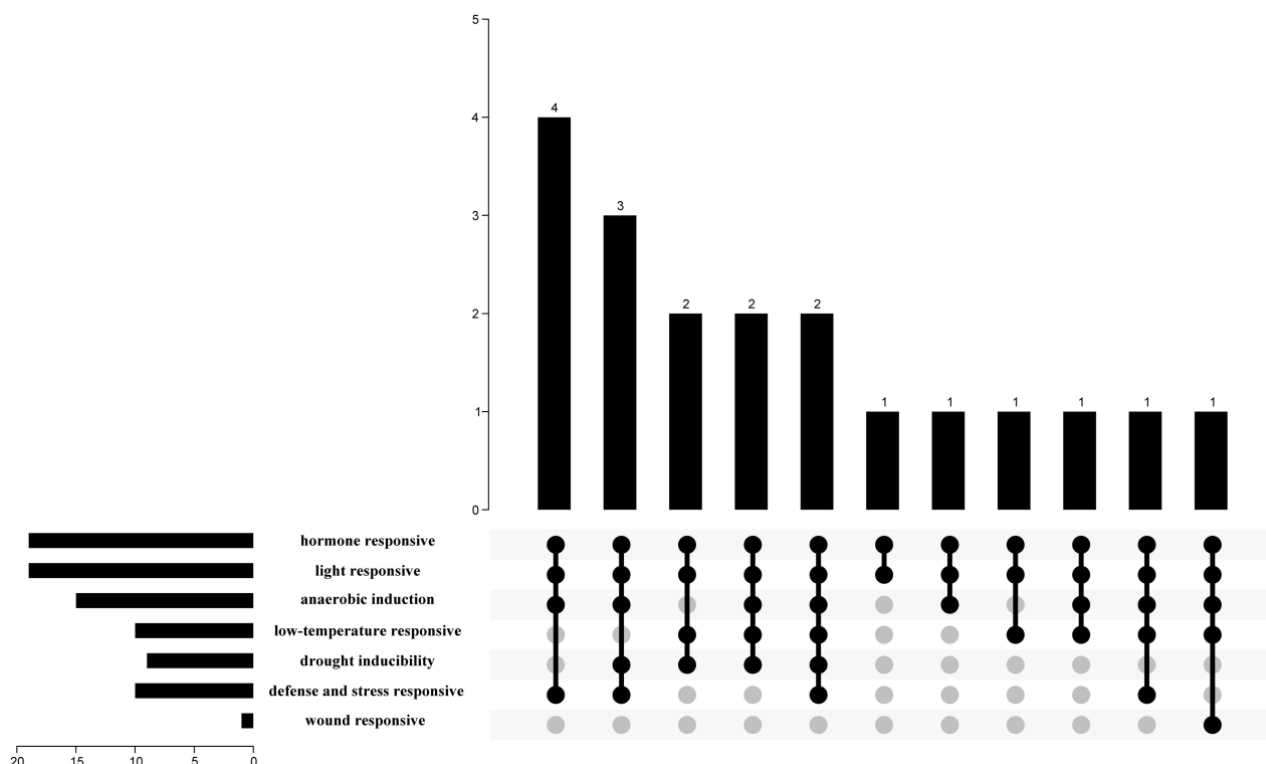


Figure 6. Cis-acting element analysis of apple SPS genes. The left abscissa represents the number of *MdSPS* that the *cis*-acting element responds to. The right ordinate represents the number of *MdSPS* in which homeopathic components are grouped. The dot plot at the bottom right represents a collection of *cis*-acting elements.

3.6. Codon Bias Analysis of Apple SPS Family

In Table 5, the RSCU values of 19 CDS sequences in the apple SPS family are calculated. The codon of apple SPS mainly prefers to end in G and U. There are 28 high-frequency codons (RSCU > 1) in the CDS sequence of apple sucrose phosphate synthetase. Thirteen of them end in U, nine in A, five in G, and one in C. However, the RSCU values of these high-frequency codons are all less than 2.0, indicating that there is no codon with strong preference in apple SPS genes. Interestingly, the AGA codon, which translates arginine, was used most frequently (1.947). The frequency of the ACG codon translating threonine was the lowest, 0.419.

Correlation analysis of the apple SPS family shows that T3s is negatively correlated with C3s, A3s, G3s, and GC3s. C3s was positively correlated with G3s, CAI (codon adaptation index), CBI (codon preference index), Fop (optimal codon usage frequency), and GC3s (Figure 7).

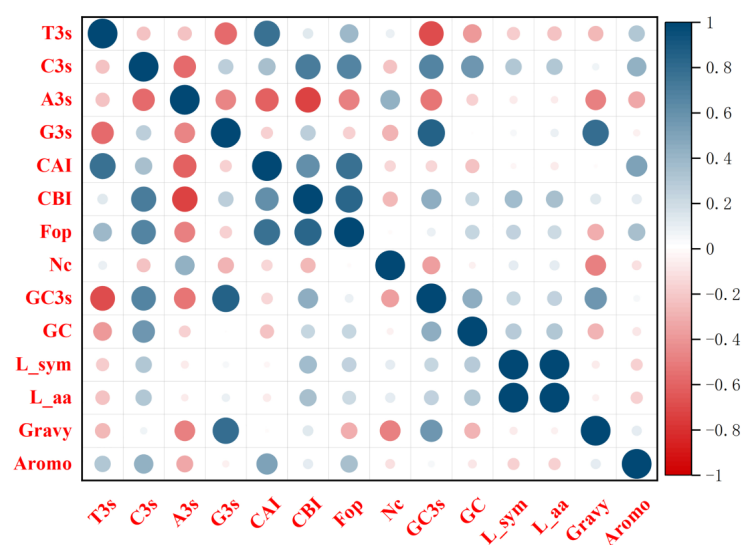


Figure 7. Correlation analysis of *MdSPS* genes. T3s represents the amount of the third T of the codon in the amino acid containing the synonymous codon ending in T. C3s represents the amount of the third C of the codon in the amino acid containing the synonymous codon ending in C. A3s represents the amount of the third A of the codon in the amino acid containing the synonymous codon ending in A. G3s represents the amount of the third G of the codon in the amino acid containing the synonymous codon ending in G. CAI stands for codon adaption index. CBI stands for codon bias index. Fop stands for frequency of optimal codon. Nc stands for effective number of codon. GC3s represents the frequency of occurrence of G or C in the third base of the codon. GC represents the amount of G and C in a gene. L_sym stands for number of synonymous codon. L_aa represents total number of amino acids. Gravy stands for grand average of hydropphilicity. Aromo represents aromaticity of proteins. The darker the circle, the larger the correlation, and vice versa. Blue represents positive correlation, red represents negative correlation, and white represents no correlation.

Table 5. Apple *SPS* genes codon RSCU value.

Amino Acid	Condon	RSCU	Amino Acid	Condon	RSCU
Phe	UUU	1.053	Ala	GCU	1.388
	UUC	0.947		GCC	0.864
Leu	UUA	0.762	Tyr	GCA	1.237
	UUG	1.385		GCG	0.508
	CUU	1.342	His	UAU	1.091
	CUC	0.912		UAC	0.909
Ile	CUA	0.543	Gln	CAU	1.237
	CUG	1.055		CAC	0.763
	AUU	1.324	Asn	CAA	1.108
	AUC	0.867		CAG	0.892
Met	AUA	0.809	Lys	AAU	1.116
	AUG	1		AAC	0.884
Val	GUU	1.469	Asp	AAA	0.902
	GUC	0.821		AAG	1.098
	GUA	0.561	Glu	GAU	1.292
	GUG	1.149		GAC	0.709
Ser	UCU	1.302	Cys	GAA	1.018
	UCC	0.796		GAG	0.982
	UCA	1.306	Trp	UGU	0.993
	UCG	0.554		UGC	1.007
Arg	AGU	1.061	Arg	UGG	1
	AGC	0.982		CGU	0.704

Table 5. Cont.

Amino Acid	Condon	RSCU	Amino Acid	Condon	RSCU
Pro	CCU	1.242	Gly	CGC	0.485
	CCC	0.811		CGA	0.801
	CCA	1.327		CGG	0.545
	CCG	0.618		AGA	1.947
Thr	ACU	1.263		AGG	1.519
	ACC	0.918		GGU	0.944
	ACA	1.401		GGC	0.889
	ACG	0.419		GGA	1.375
				GGG	0.79

3.7. Interaction Analysis of Apple SPS Gene Family Proteins

Interactions between 19 MdSPS proteins were predicted (Figure 8). 12 kinds of MdSPS proteins, including MdSPS2, MdSPS5, MdSPS6, MdSPS7, MdSP10, MdSPS11, MdSPS14, MdSPS15, MdSPS16, MdSPS17, MdSPS18, and MdSPS19, interact with each other to form a protein interaction network. MdSPS5, MdSPS6, MdSPS10, MdSPS15, and MdSPS16 had different interactions with seven proteins.

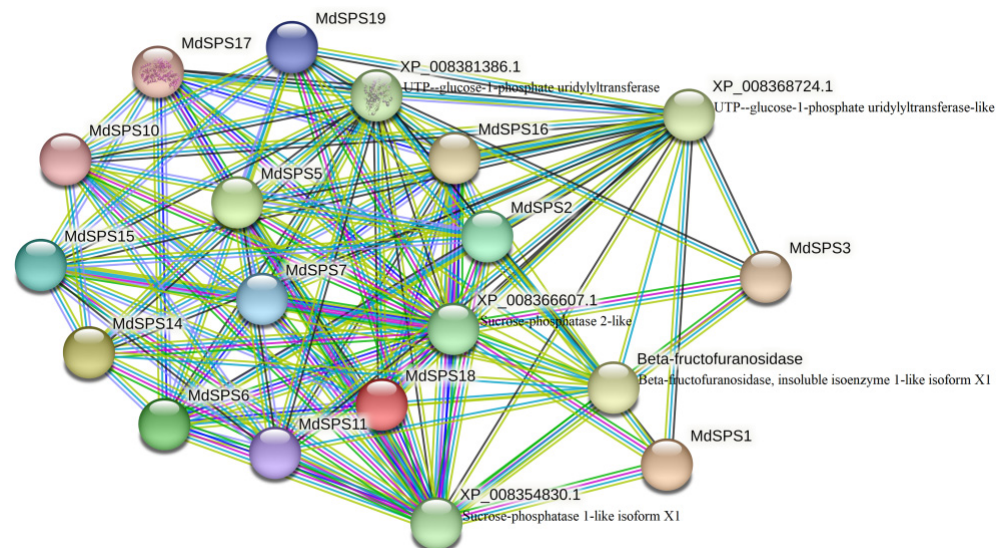


Figure 8. Protein interaction analysis of apple SPS proteins. Nodes represent proteins, and lines between nodes represent interactions between proteins, with different colors corresponding to different types of interactions.

By predictive analysis of interactions with other proteins, MdSPS were associated with sucrose phosphatase, UTP-glucose-1-phosphate uridylyltransferase and beta-fructofuranosidase. They are involved in the glycogen storage mechanism.

3.8. Analysis of Expression Patterns in the Apple SPS Gene Family

A total of nine MdSPS genes were comparatively analyzed in the GEO database. The corresponding gene numbers of GDR loci were MDP0000288876, MDP0000160578, MDP0000872262, MDP0000783676, MDP0000256965, MDP0000126946, MDP0000212593, MDP0000174537, and MDP0000250070. As can be seen from Figure 9, there were significant differences in the phenological quantities of the different organs. Except for MdSPS14, the other four genes had high expression in different organs, while MdSPS14 was almost not expressed in different tissues. In subgroup a, both MdSPS1 and MdSPS15 were highly expressed in seeds, and MdSPS1 was also highly expressed in flowers and fruits. In subgroup b, MdSPS5 had higher expression in fruit and seeds, and the expression in seeds

was the highest among the nine genes. In contrast, *MdSPS6* was only highly expressed in seedlings.

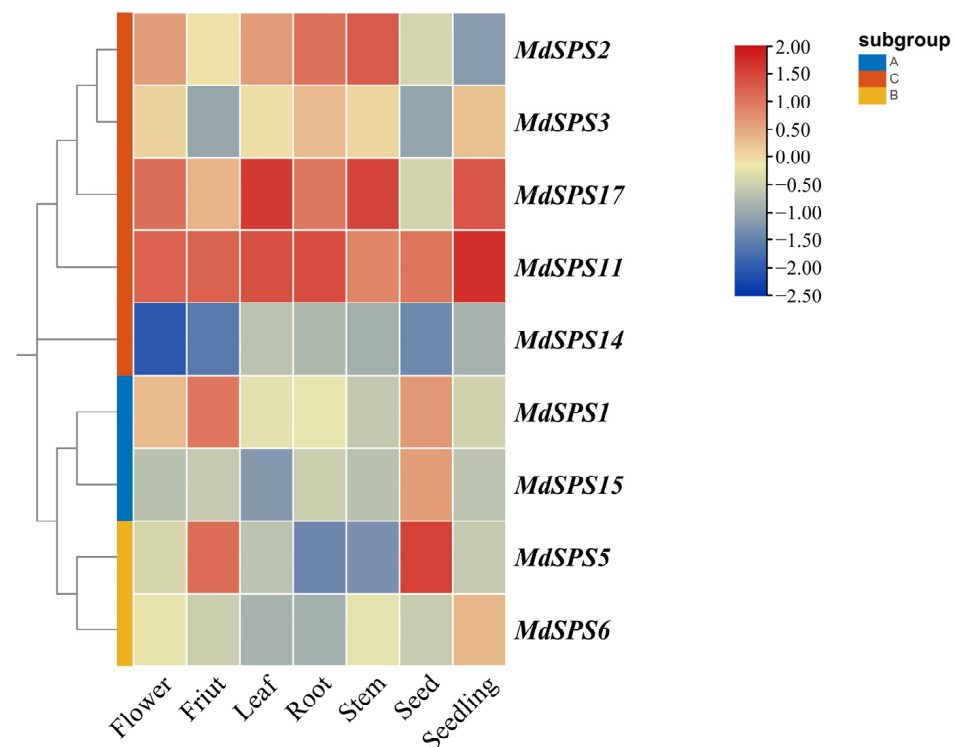


Figure 9. Tissue expression analysis of apple *SPS* genes. Heat map experiments were performed using Gene Chip microarrays from apple tissue expression database (GEO: GSE42873). Red represents high gene expression, and blue represents low gene expression. Flower data were M67, fruit data were M20, leaf data were M14, root data were GD, stem data were X8877, seed data were 4442 × 2596, and seedling data were X4102.

3.9. Quantitative Fluorescence Analysis of Apple *SPS* Gene Family

The *SPS* family members were induced in the leaves of apple seedlings under different abiotic stress conditions, and the expression pattern analysis by qRT-PCR is shown in Figure 10. Significant differences in the relative expression levels of 19 *MdSPS* family members were observed under PEG, NaCl stress, and low-temperature stress. The highest relative expression of *MdSPS4* was observed after 24 h of PEG treatment, which was 288.7-fold higher than that of the control. In addition, the expression of all *MdSPS* members was up-regulated, indicating that the *SPS* gene family played an active role in regulating drought stress in apples. The relative expression of *MdSPS17* was the highest after NaCl treatment for 24 h, which was 158.7-fold higher than that of the control. The relative expression of *MdSPS3* was the highest in the 4 °C -treated apple leaves for 24 h, which was 27.1-fold higher than that of the control. The relative expression of *MdSPS4* was also the highest after PEG treatment, which was 288.7-fold higher than that of the control. Interestingly, the relative expression of *MdSPS12* was down-regulated under NaCl, and its relative expression was also suppressed at 4 °C, whereas the expression of other *SPS* genes was up-regulated. It is hypothesized that *SPS* genes (except *MdSPS12*) play an important positive regulatory role in salt stress and low temperature stress. On the contrary, *MdSPS12* may play a negative regulatory role in plant salt stress and low temperature stress.

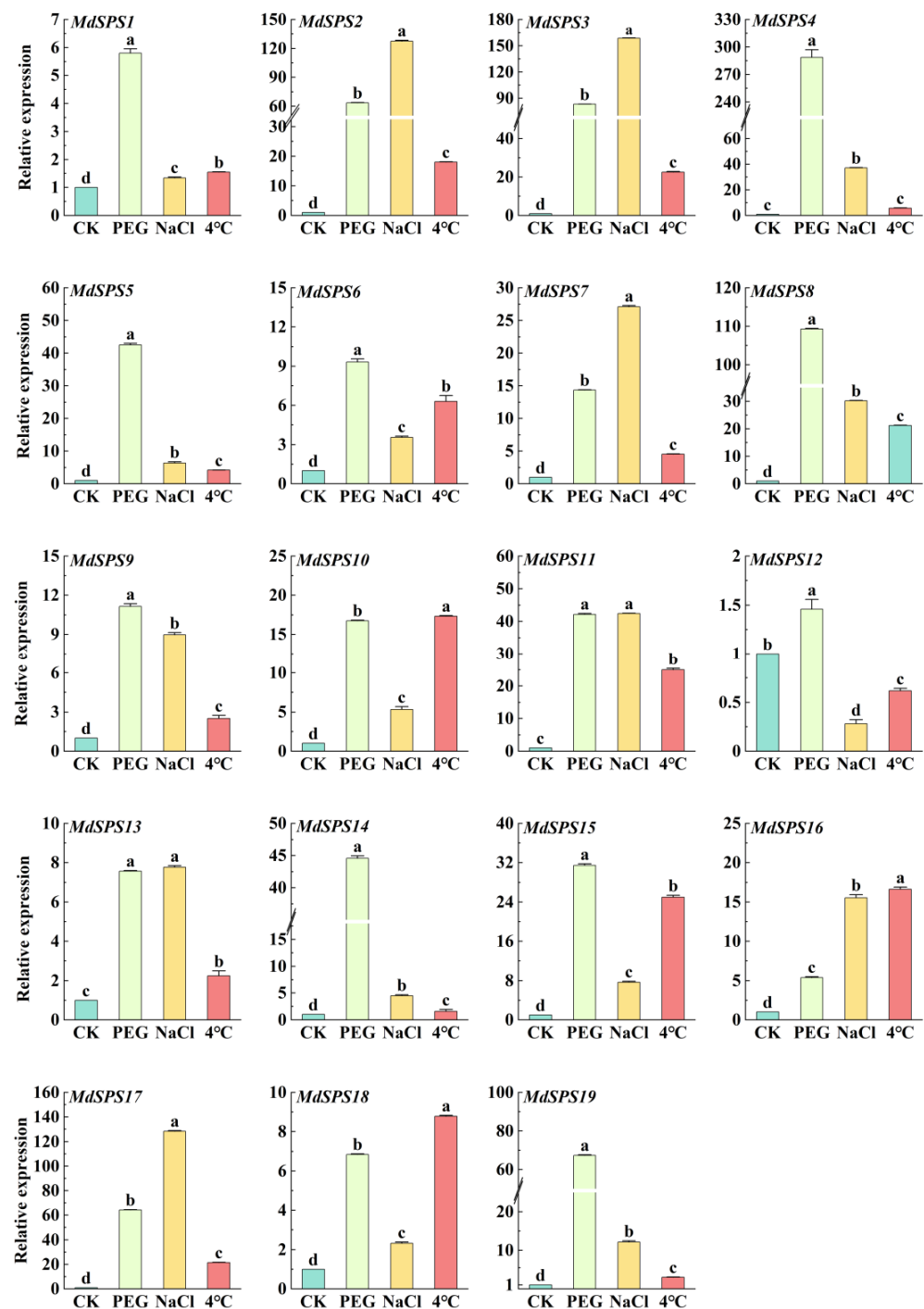


Figure 10. Quantitative expression analysis of *SPS* gene family in apple. Apple seedlings growing for 40 days were treated with 10% PEG, 200 mmol·L⁻¹ NaCl and 4 °C. The test-tube seedlings treated with distilled water in the growth chamber were used as the control. Three replicates were set for each treatment. The internal reference gene was *GAPDH* (GenBank accession no.CB973647). Different lowercase letters indicate a significant difference at the 0.05 level, and the same lowercase letter indicates no statistical difference.

4. Discussion

The identification and cloning of *SPS* gene families have been reported in many plants, including lychee (*Litchi chinensis* Sonn) [40], peach (*Prunus persica* (L.) Batsch) [41], cassava (*Manihot esculenta* Crantz) [42], rice (*Oryza sativa* L. cv. Nipponbare) [43], and sugarcane (*Saccharum officinarum* and *Saccharum spontaneum*) [44]. In this study, bioinformatics meth-

ods were used to identify and screen the apple genome database. Finally, 19 members of apple *SPS* gene family were obtained and named *MdSPS1-MdSPS19*. Previous studies have shown that subfamily D exists only in ligneous monocotyledonous plants. A, B, and C are widespread not only in monocots but also in dicotyledon plants [39]. The same phenomenon occurs in the apple *SPS* gene family, where phylogenetic analyses have classified 19 genes into three subfamilies. Subfamilies A, B, and C contain 4, 3, and 12 genes, respectively.

The distribution of the *SPS* gene was different in different species. In tomatoes, *SlSPS* were distributed in the plasma membrane [45]. In *Actinidia chinensis* and *A. eriantha*, *SPS* genes are distributed in the cytoplasm and nucleus [46]. The nucleus, cytoplasm, mitochondria, and chloroplasts are the main distribution regions of *SPS* proteins in sugarcane [47]. *GmSPS* proteins are predicted to be localized and distributed in the nucleus and cytoplasm [48]. We found that the results of subcellular localization prediction analysis were similar to the results of sugarcane studies. Apple *SPS* proteins were mainly distributed in the cytoplasm, chloroplasts, mitochondria and nucleus. However, *MdSPS10* and *MdSPS14* proteins were predicted to be localized in peroxisomes, which may be related to their functions.

Previous studies have shown that the number of exons in the wheat *SPS* family ranges from 12 to 14 [39]. In cassava, the numbers of exons and introns were 11–14 and 10–13, respectively [42]. The numbers of exons and introns were 12–14 and 11–13, in lychee, respectively [40]. The members of subfamily A had similar results in apple *SPS* gene structure. However, some members of subgroup B and subgroup C have large differences, which may be due to the addition or deletion of exons and introns in the genes during the evolutionary process. The same phenomenon occurred in soybean (*Solanum lycopersicum*) [48] and sugarcane (*Solanum lycopersicum*) [47]. For motif analysis we found that genes with similar evolutionary relationships in the *MdSPS* family have similar gene structures. For example, *MdSPS2*, *MdSPS3*, and *MdSPS17* have the same motif and the same number of exons.

Apparently, the same situation occurred in our study. We analyzed the cis-acting elements of the apple *SPS* promoter and found a large number of light-responsive, hormone-responsive, and stress-related elements in its 2000 bp promoter. Similar results were found in potato, *Actinidia chinensis*, and *A. eriantha*. All *SPS* members were rich in hormone-responsive elements, stress-responsive elements, and light-responsive elements in potato [49]. In *Actinidia chinensis* and *A. eriantha*, 2000 bp upstream of the *SPS* gene promoter had a large number of elements related to light response, hormone response and stress [46].

Plants have different response mechanisms to different stresses, and the *SPS* gene family has been suggested to play an important role in regulating plant stress [50,51]. With the prolongation of the stress time, the soluble sugar content increases slowly until it decreases, which may be due to the fact that the arrangement of the lipid bilayer on the protoplasmic membrane is damaged by the prolongation of the stress time and the permeability of the plasma membrane is changed [52]. Water stress affects not only the distribution of carbohydrates but also the size of the sucrose pool [53,54]. Dehydration of protoplasts and cell walls leads to cell collapse, which is associated with mechanical damage and metabolic disorders [55]. Previous studies have shown that *SPS* activity tends to change significantly with the onset of stress. The relative expression of *GmSPS1* in soybean leaves increased significantly under water deficit conditions [56], and *SPS* activity in *Phaseolus vulgaris* L. cv Linden leaves decreased by about 50% due to water deficit [57]. Keller's study on carbohydrate metabolism in the leaves of pigeonpea (*Cajanus cajan*) under early drought stress indicated that after 27 days of drought stress, the activity of decomposing enzymes such as amylase and *SPS* synthesis of sucrose in the leaves of the plant increased significantly [58]. The activity of *SPS* in cucumber (*Cucumis sativus* L.) increased under short-term drought stress [59]. We found that *MdSPS* has similarities with soybean *SPS* genes under drought condition stress [56]. The relative expression levels of all *SPS* genes

were significantly up-regulated and all were significantly different from CK compared with 10% PEG treatment in apples. However, contrary to some of the previous findings [57], this result may be due to the short-term drought caused by the short treatment time in this experiment. This suggests that *MdSPS* is more sensitive to short-term drought stress, and it is hypothesized that the SPS gene plays an active role in the response to short-term drought stress in apples.

In jujube fruits, the activity of SPS increased significantly with the increase in salinity stress [60]. In tomato (*Solanum lycopersicum*) salt stress, the relative expression of *SISPS4* increased with time, but the expression of *SISPS1* and *SISPS2* was opposite [45]. SPS activity in cotton (*Gossypium hirsutum* L.) leaves increased in a short period of time under medium to high salt stress [61]. In our study, the relative expression levels of the remaining *MdSPS* genes, except for *MdSPS12*, increased to different degrees under salt stress for 24 h and were significantly higher than those of the control group under this treatment. The phenomenon of the decrease in the relative expression of *MdSPS12* under salt stress was similar to that of tomato *SISPS1* and *SISPS2*, which might have similar conserved motifs [45].

When plants were subjected to osmotic water loss under environmental stress, the activity of SPS increased rapidly, and a large amount of sucrose was synthesized or starch was hydrolyzed and resynthesized into sugars [62]. The increase in these soluble sugars can increase the concentration of cell fluid and buffer the excessive dehydration of the cytoplasm. In addition, at low temperatures, the high concentration of cell fluid can also protect the cytoplasmic colloid from freezing and reduce mechanical damage to the membrane [63]. SPS activity in the leaf tissue of spinach (*Spinacia oleracea*) increased significantly under cold stress at 5 °C [64]. Fruits of peach (*P. persica*) showed a significant increase in SPS activity over a two week period when stored at 5 °C or 10 °C [65]. The relative expression level of SPS in chickpea GPF2 (cold-sensitive) increased at 4 °C for 72 h [66]. The relative expression of most of the apple *MdSPS* genes was significantly higher than that of CK after 24 h of treatment at 4 °C, but there was no significant difference in *MdSPS4*, although it was higher than that of CK. It might be caused by the insensitivity of *MdSPS4* to low temperature stress. Moreover, the relative expression of *MdSPS12* was significantly decreased at low temperatures.

5. Conclusions

Nineteen *SPS* genes were identified in apples, which can be categorized into three subfamilies. All of *MdSPS*s contain light-responsive elements and hormone-responsive elements. Second, the expression levels of *MdSPS* proteins varied in different tissues. *MdSPS5* and *MdSPS15* had the highest expression in seeds; *MdSPS1* had the highest expression in fruits; and *MdSPS17* had a lower expression in seeds. The results of qRT-qPCR showed that most of the *SPS* genes were significantly different from CK, except for *MdSPS4*, which was found in apples. Finally, qRT-qPCR results showed that most of the *SPS* genes were significant compared with CK except for *MdSPS4*, but *MdSPS12* was significantly lower than that of CK under salt stress and low temperature stress. In addition, the expression of *MdSPS3*, *MdSPS8*, *MdSPS11*, and *MdSPS17* was more than 20 fold higher than that of the control under drought stress, salt stress, and low temperature stress. These four genes could be candidates for molecular breeding in the *MdSPS* family. Overall, the three stresses analyzed in this experiment are only one aspect of abiotic stresses, and the response mechanisms of apple *SPS* genes to more adversity stresses need to be further investigated.

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