

Cloning and Expression Analysis of Tomato *SISPS1* Gene

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Abstract Sucrose is the main product of plant photosynthesis and participates in many physiological and biochemical reactions of plants. Sucrose phosphate synthase is one of the key rate-limiting enzymes necessary for sucrose to enter various metabolic pathways, and its activity directly reflects the ability of sucrose synthesis in plants. In order to study the mechanism of sucrose synthesis in tomato, this study cloned a sucrose phosphate synthase gene from tomato pulp and named it *SISPS1*. Through bioinformatics analysis, it was found that its cDNA is 3488bp in length, encoding 1054 amino acids, and there are 3 functional domain. There are 4 *SPS* genes in tomato, and their homology is relatively high. In Salicaceae crops, *SISPS1* protein is closely related to potatoes, peppers and eggplants. The results of tissue-specific analysis found that *SISPS1* was expressed in all tomato tissues, among which the expression was relatively high in the seedling stage and different development stages of the fruit. In order to further study the mechanism of *SISPS1* in tomato growth and sucrose accumulation, an overexpression and knockout vector of *SISPS1* was constructed and transformed into Micro-Tom to obtain transgenic positive lines. Analysis of the expression level of *SISPS1* on the obtained transgenic lines showed that the expression levels of the overexpression lines at the seedling stage, fruit green ripe stage and ripening stage were significantly up-regulated compared with the wild type, and the enzyme activity and sucrose content increased. Compared with the wild type, the expression of *SISPS1* at the seedling stage, green ripe stage and ripening stage of the knockout line was significantly down-regulated, and the enzyme activity and sucrose content were reduced. As for the mechanism of *SISPS1* in tomato growth and fruit sucrose accumulation, functional verification is needed in the future.

Keywords Tomato; Clone; *SISPS1*; Expression; Analysis

Solanum lycopersicum belongs to *Lycopersicon* of Solanaceae, commonly known as tomato, and is a fruit and vegetable that is very popular among consumers. Carbohydrates affect the growth and development of fruit, and as signal molecules participate in regulating the expression of genes related to fruit ripening and the process of stress tolerance (Liao et al., 2020). The sugars in tomato fruit basically were glucose, fructose and sucrose. Sucrose is not only the main product of plant photosynthesis, but also participates in the physiological and biochemical processes of plants. There are three kinds of enzymes associated with sucrose metabolism in higher plants, sucrose phosphate synthase (SPS), sucrose synthase (SuSy), invertase (INV), among which, SPS is a rate-limiting enzyme and plays a certain role in sucrose metabolism. Its activity directly reflects the ability of sucrose synthesis in plants (Huber and Huber, 1996; Harbron, 1981).

At present, *SPS* genes have been successively cloned and studied in higher plants, such as *Beta vulgaris*, *Cucumis melo*, *Manihot esculenta*, *Dendrobium officinale*, *Cerasus humilis*, *Mangifera indica*L, *Dimocarpus longan* and so on (Hao, 2012; Hou et al., 2008; Huang et al., 2018; Meng et al., 2013; Wang et al., 2017; Bai et al., 2019; Shuai et al., 2020). The current research results have found that *SPS* participates in plant growth, fruit ripening and other processes, and has the effect of responding to abiotic stress. However, at present, the transcriptional regulation process of *SPS* in plants, the molecular mechanisms and pathways involved in various biological functions are still unclear. Therefore, it is of great significance to analyze the function and regulation mechanism of *SPS* in sucrose metabolism and plant growth, which can provide a theoretical basis for increasing crop yield

and improving crop quality.

In this study, *SISPS1* gene was cloned from tomato fruit and analyzed by bioinformatics. Real-time quantitative polymerase chain reaction (qPCR) was used for tissue specificity analysis. In addition, overexpression and knockout vectors were constructed, and Micro-Tom was transformed by agrobacterium-mediated method. *SISPS1* expression analysis, SPS enzyme activity determination and sucrose determination were performed on the obtained transgenic lines.

1 Results and Analysis

1.1 Cloning and bioinformatics analysis of *SISPS1* gene

Using tomato fruit genomic DNA and the whole gene cDNA obtained by reverse transcription as templates, primers were designed to clone the full length of *SISPS1* gene and cDNA. The target fragment was detected by agarose gel electrophoresis. The size of cDNA was about 3 488 bp, which was consistent with the expected results (Figure 1). Using ORF finder on NCBI, it was found that the CDS region of *SISPS1* was 3 165 bp, encoding 1 054 amino acids.

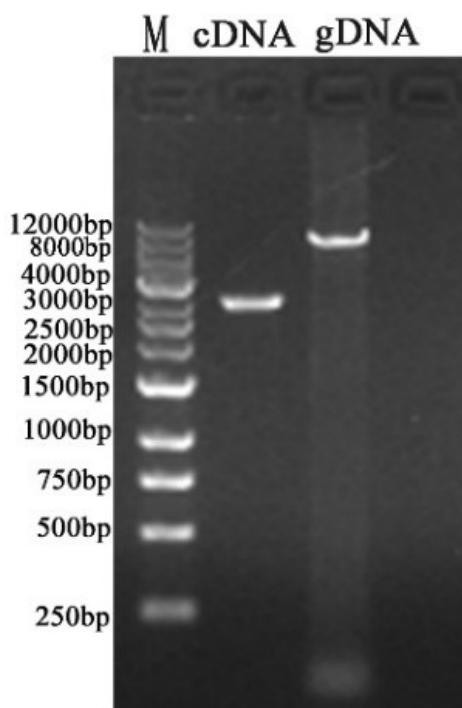


Figure 1 Full-length electrophoresis analysis of tomato *SISPS1* gene

Note: M: 1 kb DNA Ladder; g-DNA: Genomic DNA

Through the GENE BANK search of NCBI, it was found that there were four SPS with high protein homology in tomato, namely *SISPS1*, *SISPSB*, *SISPS4* and *SISPSA2*. Through the protein homologous sequence alignment analysis of these four genes (Figure 2), it was found that *SISPS1* had high homology with the other three proteins, indicating that there may be some functional similarities among them. By predicting the domain of *SISPS1* protein (Figure 3), it can be found that it had 3 domains, namely *Sucrose_synth*, *Glycos_transf_1_4* and *S6PP*. The secondary structure of *SISPS1* protein was predicted through the online website. The prediction results of the secondary structure of the protein were as follows: α -helix accounted for 40.7%, extended chain accounted for 13.76%, irregular curl accounted for 38.99%, and the tertiary structure of the protein was further constructed (Figure 4). The protein sequences of Solanaceae crops were obtained from NCBI and a phylogenetic tree was constructed (Figure 5). The results showed that *SISPS1* was closely related to *Solanum tuberosum*, *Capsicum annuum* and *Solanum melongena*.



Figure 2 Sequence alignment of SISPS1 and its homologous gene protein
 Note: Solyc07g007790: SISPS1 Solyc08g042000: SPSA2 Solyc09g092130: SPSB Solyc11g045110: SISPS4; Red box: Conservative sequences; Yellow box: More conservative sequences than white

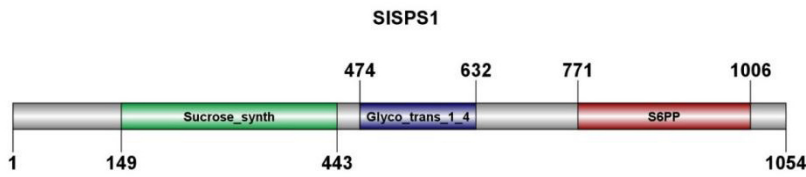


Figure 3 SISPS1 protein domain prediction

Note: Sucrose_synth: sucrose synthase; Glycos_transf_1_4: glycosyltransferase group 1; S6PP: sucrose-6-phosphohydrolase

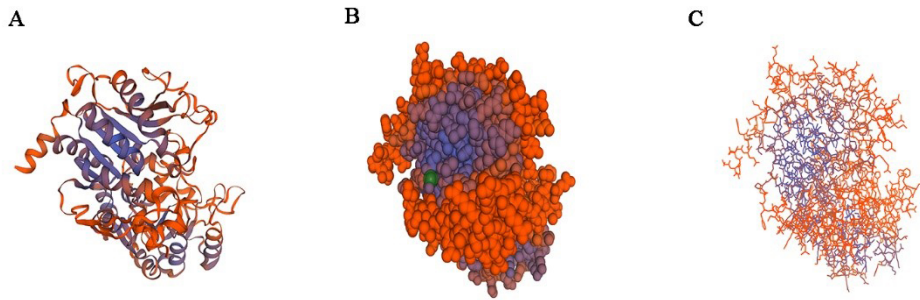


Figure 4 F Tertiary structure of SISPS1 protein

Note: A: Surface-shaped three-level structure diagram; B: Spherical three-level structure diagram; C: Linear three-level structure diagram

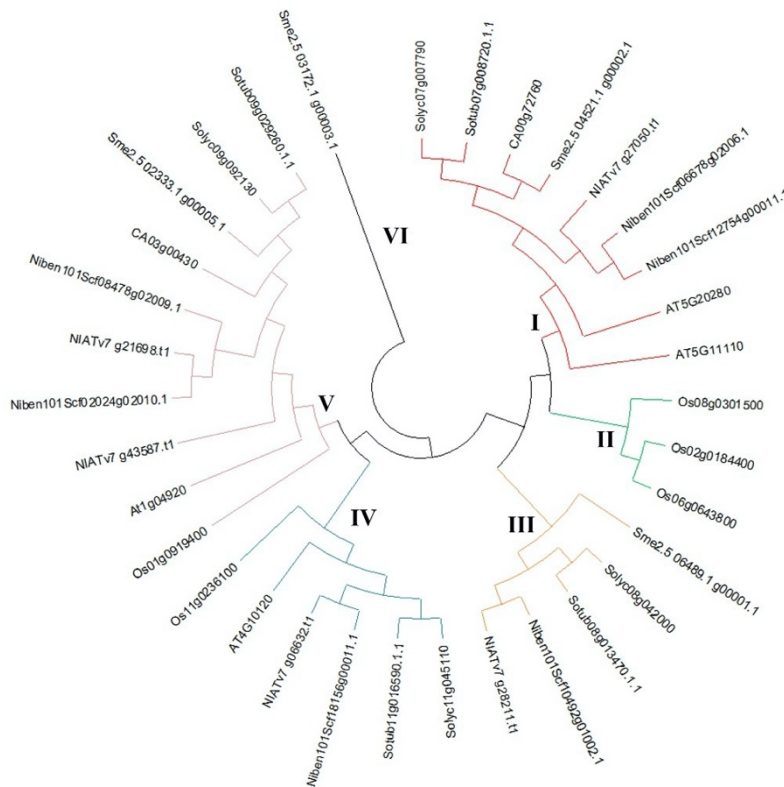


Figure 5 Protein evolutionary tree of SISPS1

1.2 SISPS1 tissue specificity analysis

After detecting the expression of *SISPS1* in root, stem, leaf, seedling, flower and fruit (green ripe stage, slight maturity stage, half-ripe stage and ripening stage) of Micro-Tom, qPCR results showed that *SISPS1* was expressed in all tomato tissues, and there were differences in expression patterns among different tissues, among which the expression was relatively high in the seedling stage and different development stages of the fruit (Figure 6). At

the same time, the SPS enzyme activity in Micro-Tom tissues at different stages was measured. The results showed that the SPS enzyme activity was also relatively high in fruit and seedling, and the SPS enzyme activity was the highest in the half-ripe stage of fruit (Figure 7). It was speculated that SPS may play an important role in tomato fruit development and carbohydrate accumulation and metabolism.

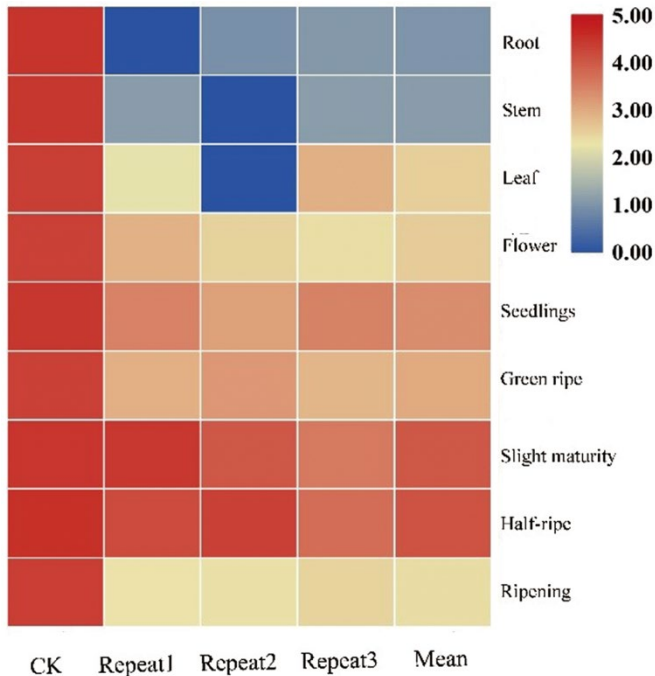


Figure 6 Expression analysis of *SISPS1* in different organs of tomato model plant Micro-Tom

Note: Set the expression level of *SISPS1* in the root to 1, use *elf* as the internal reference gene, and set 3 repeats

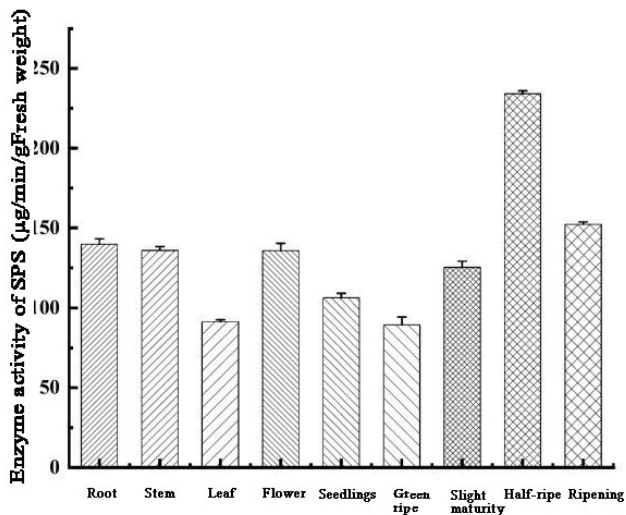


Figure 7 SPS enzyme activity analysis

1.3 Construction and analysis of *SISPS1* overexpression and knockout vector

The *SISPS1* gene was cloned and constructed by using pCAMBIA1300-pYAO-cas9 and pC1300-35S-4X Myc vectors, and the knockout vector and overexpression vector were constructed respectively. After sequencing correctly, it was transferred to *Agrobacterium* GV3101. The tomato model plant Micro-Tom was transformed by *Agrobacterium* mediated method, and the overexpression and knockout positive lines were obtained, which can be used for subsequent phenotypic analysis and the research of the mechanism of tomato growth and response to high temperature stress.

After identifying the DNA level of the obtained *SISPS1* overexpression transgenic lines, the expression of *SISPS1* in the transgenic lines was detected by qPCR. The results showed that the expression of *SISPS1* at the seedling stage of the overexpression line was significantly up-regulated compared with the wild type. At the same time, the expression of *SISPS1* at fruit green mature stage and mature stage of the overexpression line was also significantly up-regulated compared with the wild type (Figure 8). The SPS enzyme activities of the overexpression lines at seedling stage, green mature stage and red mature stage were measured. It was found that compared with the wild type, the enzyme activities of the overexpression lines increased significantly at seedling stage, green mature stage and red mature stage (Figure 9). The content of sucrose in the overexpression lines was further determined. The results showed that the content of sucrose at the seedling stage, green mature stage and red mature stage of the overexpression lines was significantly higher than that of the wild type (Figure 10).

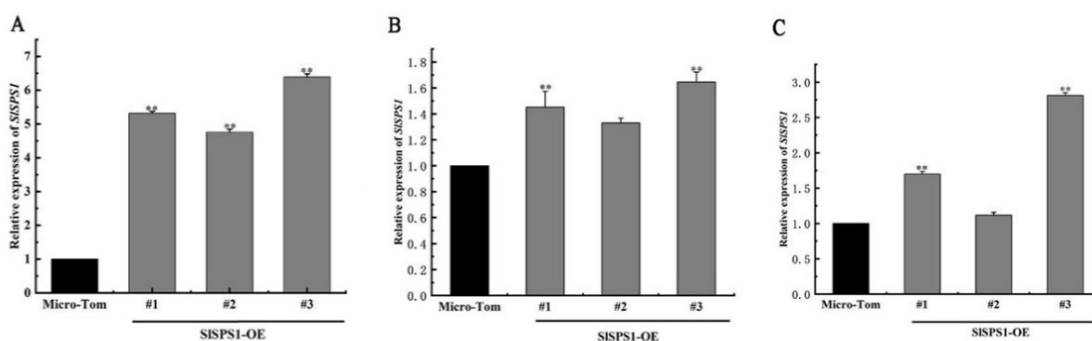


Figure 8 Analysis of expression level of overexpression line SISPS1
 Note: A: Seedling period; B: Green mature period; C: Red mature period

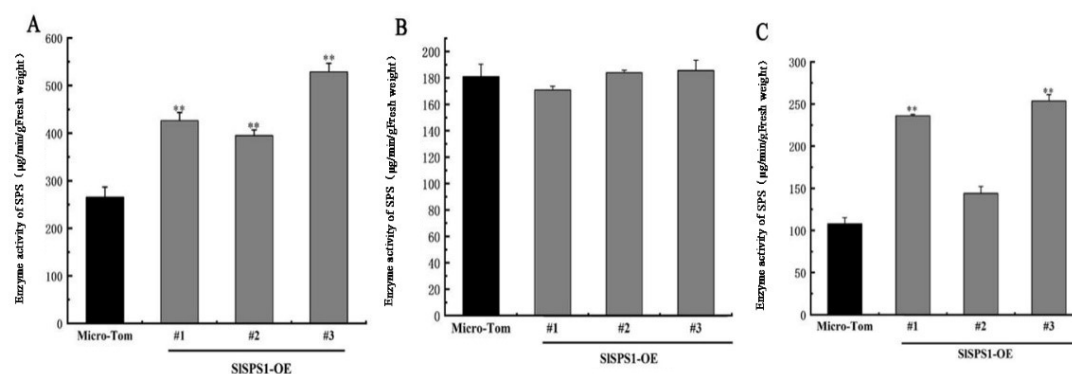


Figure 9 SPS enzyme activity of overexpression line
 Note: A: Seedling period; B: Green mature period; C: Red mature period

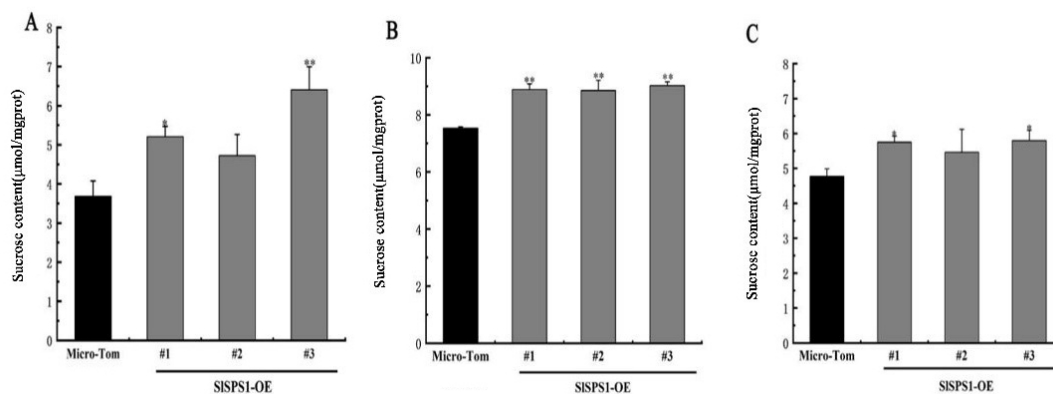


Figure 10 Over-expression plant sucrose content
 Note: A: Seedling period; B: Green mature period; C: Red mature period

Similarly, the DNA level of the knockout lines was identified and sequenced successfully. After obtaining the homozygous lines, the expression of *SISPS1* in the transgenic lines was detected by qPCR. The results showed that the expression of *SISPS1* at the seedling stage, green mature stage and red mature stage of the knockout line was significantly down-regulated compared with the wild type (Figure 11). The SPS enzyme activities of the knockout lines at seedling stage, green mature stage and red mature stage were measured. It was found that the enzyme activities of the knockout lines tended to decrease compared with the wild type (Figure 12). The content of sucrose in the knockout lines was measured. It was found that the content of sucrose at the seedling stage, green mature stage and red mature stage of the knockout lines was lower than that of the wild type (Figure 13).

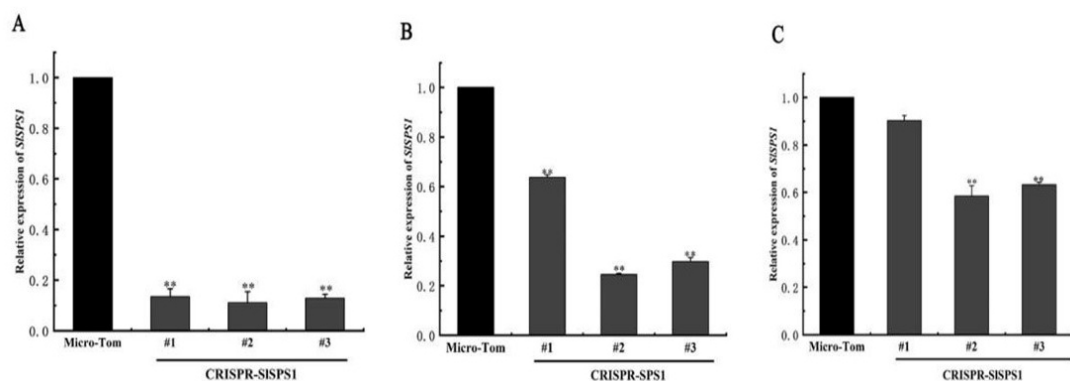


Figure 11 Sucrose content of overexpression line
 Note: A: Seedling period; B: Green mature period; C: Red mature period

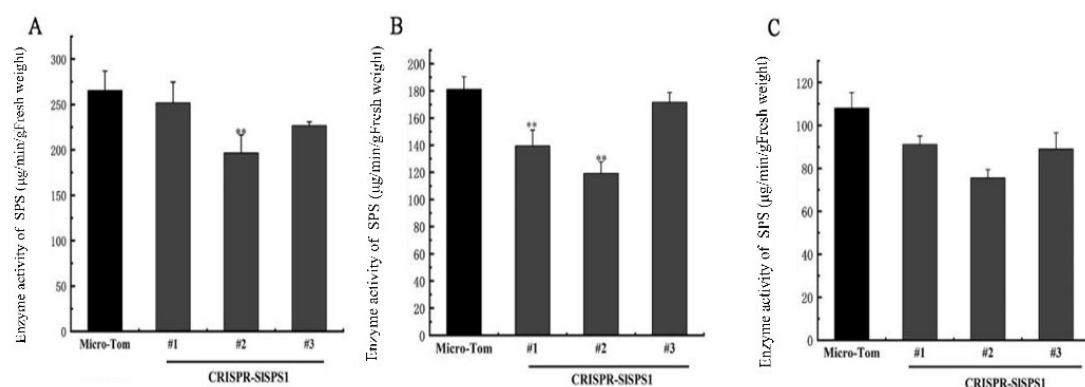


Figure 12 SPS enzyme activity of knockout line
 Note: A: Seedling period; B: Green mature period; C: Red mature period

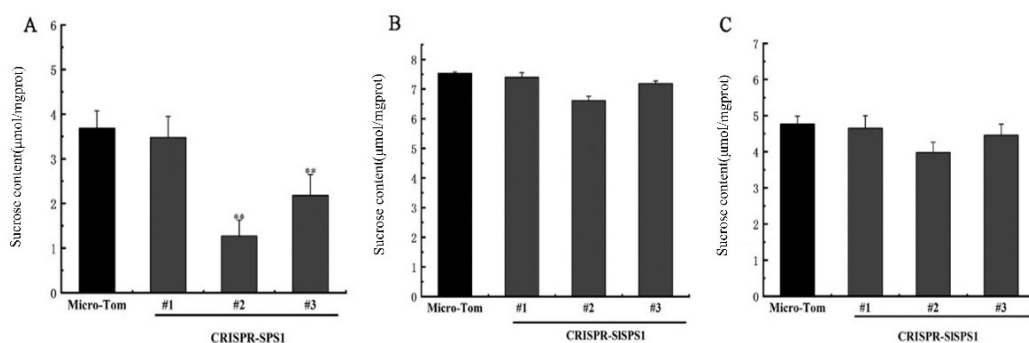


Figure 13 Knockout line SPS enzyme activity
 Note: A: Seedling period; B: Green mature period; C: Red mature period

2 Discussion

Sucrose is the main product of plant photosynthesis and plays an important role in plant growth, signal transduction and adaptation to environmental stress (Jiang et al., 2015). The activity of sucrose phosphate synthase directly reflects the ability of sucrose synthesis in plants. At present, *SPS* genes in higher plants have been cloned and studied. *SPS* gene was first found in *Zea mays* and *Vicia faba* (Hawker, 1971). Subsequently, it was found that *SPS* was involved in the regulation of carbon distribution in tomato leaves. When *SPS* activity increased in tomato leaves, sucrose increased and starch decreased (Worrell et al., 1991). During the development of tomato fruit, sucrose accumulation was related to the increase of *SPS*, and *SPS* would affect the sucrose accumulation process of tomato fruit (Dali et al., 1992). During the ripening process of *Litchi chinensis*, sucrose would accumulate in the aril. There are four *SPS* genes in *Litchi chinensis*, belonging to different *SPS* gene families, and their expression patterns were different. *LcSPS1* and *LcSPS4* were highly expressed in flowers, while *LcSPS3* was highly expressed in mature leaves (Wang et al., 2018). In this study, it was also found that *SISPS1* was expressed in tomato, and it was relatively high at seedling stage and different development stages of fruit. It was speculated that *SISPS1* played a certain role in tomato fruit development and carbohydrate accumulation and metabolism. The expression of *SISPS1* in the transgenic lines was analyzed. It was found that the expression levels of *SISPS1* in the overexpression lines at the seedling stage, fruit green ripe stage and ripening stage were significantly up-regulated compared with the wild type, and the enzyme activity and sucrose content increased. Previous studies have found that overexpression of *Arabidopsis thaliana SPS* gene would affect its growth and fiber development process, and the stem height of transformed plants increased significantly, which was related to internode elongation, stem diameter increase, fiber lengthening and biomass increase, and the *SPS* enzyme activity of transformed plants also increased (Park et al., 2008). Compared with the wild type, the expression of *SISPS1* at the seedling stage, green ripe stage and ripening stage of the knockout line was significantly down-regulated, and the enzyme activity and sucrose content were reduced. Therefore, it could be speculated that *SISPS1* gene played a certain role in tomato growth and sucrose accumulation.

Then the transgenic line was further applied to the study of sucrose accumulation, tomato growth and response to high temperature, so as to analyze the role of *SISPS1* in tomato growth and fruit sucrose accumulation, so as to analyze the function and regulation mechanism of *SPS* in sucrose metabolism and plant growth.

3 Materials and Methods

3.1 Experimental materials

Tomato fruit was used as experimental material in this study. In addition, the root, stem, leaf, flower, seedling, green ripe stage, slight maturity stage, half-ripe stage and ripening stage of Micro-Tom in the same growth period were selected for tissue expression specificity analysis. After quick freezing with liquid nitrogen, the above samples were stored in an ultra-low temperature refrigerator at -80°C.

3.2 Main reagents

RNA extraction was used Biospin Plant Total RNA Extraction Kit (DNA-free) of Bioer Technology Co., Ltd. The reverse transcription kit was Hifair®II 1st Strand cDNA Synthesis SuperMix for qPCR (gDNA digester plus) of Yeasen Biotech Co., Ltd. The fluorescent quantitative qPCR reagent was TB Green series of TaKaRa. *Escherichia coli* competent cells were purchased from Weidi Biotech Co., Ltd. Sucrose assay kit and *SPS* enzyme activity assay kit were purchased from Nanjing Jiancheng Bioengineering Institute.

3.3 Main instruments and equipment

Applied Biosystems Quantstudio5 real-time fluorescence quantitative PCR instrument (ABI company of the United States), micro nucleic acid protein analyzer (IMPLEN company), high-speed centrifuge (Eppendorf company of Germany), PCR instrument, thermostat water bath, enzyme-labeled instrument, ultra-clean bench, shaker, constant temperature culture chamber and autoclave.

3.4 RNA extraction and reverse transcription

Preparation before RNA extraction: RNAase-free products were used during RNA extraction, which were dried after high-pressure steam sterilization. The RNA extraction process of tomato fruit referred to Biospin Plant Total RNA Extraction Kit (DNA-free) of Bioer Technology Co., Ltd., and after extraction, gel electrophoresis was used for examination., with total RNA as template, according to Hifair® II 1st Strand cDNA Synthesis SuperMix for qPCR (gDNA digester plus) kit of Yeasen Biotech Co., Ltd. for reverse transcription.

3.5 Bioinformatics analysis

The open reading frame of *SISPS1* gene was found by using the tomato database Sol Genomics Network, and the homologous sequence of SISPS1 protein was searched by using the Blastp of NCBI. (<https://prosite.expasy.org/>) was used to predict the domain of SISPS1 protein, (<https://www.predictprotein.org/>) was used to predict the protein structure. Using the protein sequence of Solanaceae, the phylogenetic tree was drawn by MEGA-X software.

3.6 Gene cloning

Using tomato fruit cDNA as a template, primers were designed on both sides of the 5'UTR and 3'UTR of tomato *SPS* gene mRNA (Table 1) to clone the full length of the *SISPS1* gene. The PCR reaction system was 25 μ L, the ratio of each reactant should refer to the instructions of I-5TM2X High-Fidelity Master Mix 25 μ L. PCR reaction procedure was as follows: pre denaturation at 98°C for 5 min; Denaturation at 98°C for 10 s; Annealing at 58°C for 10 s; Extension at 72°C for 30 s, and for 10 cycles; Denaturation at 98°C for 10 s; Annealing at 58°C for 10 s; Extension at 72°C for 60 s, and for 10 cycles; Denaturation at 98°C for 10 s; Annealing at 58°C for 10 s; Extension at 72°C for 90 s, and for 10 cycles. After amplification, agarose gel electrophoresis was used to detect the amplified products. After the PCR products were cut and recovered, the target fragments were connected with the vectors, and then the connected products were transformed into *E. coli* competent cells. The colonies were selected for identification and sequencing, and the plasmids were extracted.

3.7 The genetic transformation process of tomato

Soak the seeds with sterile water overnight or 5 h; sterilize with 20% sodium hypochlorite (12 \pm 2) min; wash with sterile water for 4~6 times, each time about 5 min; sown on 1/2MS medium and cultivate in the dark for 2~3 d. After exposure, transfer to 26~28°C light culture for about 5~7 d, and the cotyledons began to unfold. When the true leaves did not appear and the cotyledons just emerged from the seed coat, the cotyledons were cut into leaf discs. Each cotyledon was cut into two pieces and connected to the nursing culture medium, about 60~80 pieces each time. After 3 d of low light pre culture, the leaf discs would expand significantly. During the pre-culture period, the engineering bacteria should be prepared. It was best to draw a line 2 d in advance, and then pick a single bacteria and shake the bacteria under the dark condition of 26°C for about 16 hours to OD600=0.6~0.8. Collect the bacterial solution at 5 000 r/min for 8 min, adjust OD600=0.4 with culture medium, 20 mL each time, and add 300 μ mol/L (375 μ mol/L) AS. The prepared engineering bacterial solution should be used within 1 h. Immerse the pre cultured leaf disc explants for 10 min, suck them dry with sterile filter paper, place the leaf back upward on the nursing medium containing filter paper (do not pinch), and incubate in the dark at 26°C for 2 d. Transfer to selective regeneration medium, culture under light with leaf back upward; Transfer once every 10 d, wait for the regenerated buds to grow to about 1~2 cm (including the leaf part), cut off the albino explants (each bud should not be separated), and transfer the resistant buds into the fresh selective regeneration medium. Part of the non budding leaf discs would turn white, the cut ends would turn brown and there was no sign of healing. Such leaf discs should be removed in time. For leaf discs with the ability to grow regenerated buds, small callus would appear in the incision, and then buds would be differentiated. The emergence of buds would be in order. Pay attention to distinguish and mark them. When the resistant bud stem grew to 0.5~1 cm, it was cut and placed in the rooting medium. When growing in the field, slowly open the bottle cap 3 d before transplanting, put it in the sterilized culture soil and wrap it in a transparent bag, uncover the culture after it grew, and finally transplant it to the field.

3.8 Real-time fluorescence quantitative PCR detection

Using *eIF* gene as an internal reference gene, real-time fluorescent quantitative PCR was used to detect the expression of *SISPSI* gene. The primers (Table 1), and the calculation method of relative expression amount were $2^{-\Delta\Delta CT}$ method.

3.9 SPS enzyme activity and sucrose determination

The extraction and determination were carried out according to the method of sucrose phosphate synthase (SPS) determination kit and sucrose determination kit of Nanjing Jiancheng Bioengineering Institute.

Table 1 Primers and sequences used in the experiment

Primer name	Sequence	Usage
SPS-ORF-F	GGAAACGATTGGATTAACAGTT	ORF amplification
SPS-ORF-R	TTATCCTTTGAGTACCGCTAGT	
SISPS-QF2	CTGGTGGTCAGGTGAAGTATGTT	Fluorescence quantitative primer
SISPS-QR2	TCTAGTAAGCAAGTCAACCCGATA	
eIF-F	ATCCTTCAGAGCGGTGTTCA	Internal reference primer
eIF-R	ATCTCAAGAGCCTCTGGTGG	
HYG-F	TCGTTATGTTTATCGGCACCTT	Transgenic resistance identification
HYG-R	GGGCGTCGGTTTCCACTAT	
CR-SPS-F	AGAATCTCTGTATTTTTAGC	Knockout material identification
CR-SPS-R	TACACAACGCATCAAACAA	
SPS-OE-F	AACGCCGTCAAGAACGTGAG	Identification of overexpression materials
SPS-OE-R	CCTCGCAAGTTCAACAACATAC	

3.10 Data processing

Excel was used for data sorting, SPSS was used for data statistics and analysis, and Origin was used for drawing.

Authors' contributions

ZDW was the designer of the experiments and the executor of the research. ZDW and LYH completed the data analysis and wrote the first draft of the manuscript. ZH, YXD and ZLY participated in the experimental design and the analysis of experimental results. ZWM was the conceiver and the person in charge of the project. ZYY guided the experimental design, data analysis and paper revision. All authors read and approved the final manuscript.

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