# CLONING, EXPRESSION AND GENETIC TRANSFORMATION OF SUCROSE PHOSPHATE SYNTHASE (SPS) GENE IN SACCHARUM SPONTANEUM L.

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Sucrose phosphate synthase (SPS) is a key enzyme catalyzing sucrose metabolism in plants. In this study, we isolated the *SPS* cDNA from *Saccharum spontaneum* and designated as *SsSPS* (GenBank accession no. MF398541). The full-length of *SsSPS* cDNA was 4153-bp with an opening reading frame (ORF) of 3132 nucleotides, which encoded a 1043-amino acid protein. The nucleotide sequences alignment showed that it had 98%, 97% and 87% homology with *S. officinarum*, *Setaria italica* and *Lolium perenne*, respectively. Moreover, the *SsSPS* was detected to express in leaf and stem tissues of *S. spontaneum* and exhibited a predominant expression in the stem tissue. However, there was no significant difference in the expression level of *SsSPS* between young leaves and mature ones. Additionally, we generated transgenic *S. spontaneum* using *Agrobacterium*-mediated transformation. Our data will provide a valuable foundation for further study of the potential role of SPS in plants.

**Keywords:** *Saccharum spontaneum* L., *SPS* gene, cloning, expression, tissue culture, genetic transformation

## INTRODUCTION

It has long been recognized that fossil energy is drying up around the world, which not only aggravates global warming but also it even causes wars because of its over-utilization. Likewise, there is high consumption of fossil fuels in our country. China has become the world's second largest oil consumer and the top third importing country (Xie et al., 2007). At present, the lack of energy has been a constraint factor on economic development in China, which may even be a threat to its national security (Li et al., 2007). Ethyl alcohol from sugarcane has been commercialized successfully as a biomass energy resource, but there is still a huge development prospect to develop substitution for sugarcane as a kind of biomass energy resource in China (Li et al., 2014).

*Saccharum spontaneum* L., which belongs to *Saccharum*, Gramineae, is a perennial herbaceous plant with developed rhizome and 1-2 meters height of stem as a promising biomass energy resource. Meanwhile, *S. spontaneum* has excellent resistance to various abiotic stresses, strong endurance under barren environment with high biomasses. Furthermore, *S. spontaneum* has a moderate level content of sucrose, indicating a tremendous potential value as a biomass energy resource by promoting its sucrose content using molecular breeding (Chang et al., 2010). However, the genetic engineering on the sucrose metabolic related pathway of the *S. spontaneum*, which is essential for acceleration of the breeding processing of energy grasses, has not yet been conducted.

There are three enzymes involved in the metabolism of sucrose in plants, among which sucrose phosphate synthase (SPS) is a key enzyme catalyzing the synthesis of sucrose (Huang et al., 2012). SPS was discovered in wheat for the first time, and then detected in rice and maize

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(Hawker, 1971; Liu et al., 2005). Previous studies indicated that the activity of SPS had a positive correlation with accumulation of sucrose, and most (85%) of total sugar was presented as a form of sucrose in stem of *Sorghum bicolor* (L.) Moench (Gnansounou et al., 2005; Yang et al., 2009). In general, the *SPS-A* gene was increasingly expressed along with the growth of *S. bicolor*, which was considered to be the most essential reason for sucrose accumulation. The high activities of SPS enzyme were also detected in other plants with high sucrose contents, such as sugar beet and tomato, etc. (Hesse et al., 1995; Sun and Wang, 2005). Recently, the research on cloning and expression of *SPS* gene has been fast developing. Klein et al. (1993) cloned the *SPS* gene and analyzed its expression level in root, petiole and leaf tissue of spinach finding that *SPS* was expressed in an organ-specific manner and predominantly localized in the leaf. Wang et al. (2013) found that *SPS* gene had the highest expression in flower and the lowest expression in leaf of *Lycium barbarum* L. In our study, we got *SPS* cDNA sequence from *S. spontaneum* through homology-based cloning and analyzed the expression patterns of *SPS* gene in *S. spontaneum*. Furthermore, we optimized the tissue culture system of *S. spontaneum* and gained transgenic plants overexpressing *SsSPS* gene. Our study on *SPS* gene in *S. spontaneum* will help us to better understand the synthesis, regulation and metabolism of sucrose and lay the foundation for *Agrobacterium*-mediated transformation and further breeding of new varieties of *S. spontaneum* with high sucrose content.

## MATERIALS AND METHODS

### PLANT MATERIALS AND GROWTH CONDITIONS

Seeds of three wild germplasms (A1, A16 and A19) of *S. spontaneum* derived from Sichuan, all of which showed good comprehensive traits like relatively high biomass and upper secondary content of sucrose according to the data of Tang (2016), were grown in a greenhouse in Beijing (39°28'–41°05'N, 115°25'–117°35'E). Total RNA was extracted from the leaves of *S. spontaneum* (A1) using the TRIzol reagent method (Invitrogen, Carlsbad, CA, USA), and used for gene cloning. For gene expression analysis, total RNA from young leaves, mature leaves and middle part of stems of *S. spontaneum* (A16, A19), which had grown for 5 months, were isolated. Meanwhile, the meristem of stem tip of A19 was surface-disinfected with 75% alcohol and cut into small pieces 5 mm in length for callus induction. *E. coli* DH5α, *Agrobacterium tumefaciens* EHA105

and expression vector pCAMBIA-Ubi1301 were preserved in our laboratory for vector construction and transformation.

### MOLECULAR CLONING AND BIOINFORMATICS ANALYSIS

Total RNA was extracted from leaves of *S. spontaneum* using the TRIzol reagent method and used for cDNA synthesis using a Transcript RT-PCR Kit (Tiangen Biotech Co., Ltd., China) with an oligo-dT primer. The cDNA was amplified using the primers (Forward: 5'-GGCTGGAGAACATGT CGTGGAGGAT-3'; Reverse: 5'-AGCGCAGTG CTT GAATTCTCATTGTC-3') designed from the highly conserved regions of *SPS* gene from other plant species. Based on the fragment, the gene-specific primers (5'-GCTGCACCTCCTGTGAAGGAGATTA-3'; 5'-TCCTAGCGAGGTTCCAGATCCTCCA-3') were designed to amplify the 3'-end and 5'-end of the *SPS* cDNA, respectively. The conserved sequences, 3'-end and 5'-end sequences, were compared to find an overlap region, and the full-length cDNA was finally acquired after sequence assembly. Afterwards, the sequence similarities of both nucleotide and amino acid sequences were compared using basic local alignment search tool (BLAST) (http://blast.ncbi. nlm.nih.gov/Blast.cgi). Multiple sequences alignment analysis with previously identified SPS proteins from other six plant species was performed using DNAMAN software. Protein structures of SPS were predicted using ProtParam software (http://web. expasy.org/protparam/). Conserved Domain Search Service at NCBI was used for conserved domain predication (http://www.ncbi.nlm.nih.gov/Structure/ cdd/wrpsb.cgi).

### GENE EXPRESSION ANALYSIS

The total RNA from stem and leaf of *S. spontaneum* were respectively isolated, and then used for reverse transcription using Takara PrimeScript® RT reagent kit with gDNA Eraser (TaKaRa, China). Primers of real-time PCR (qRT-PCR) were designed according to Ye et al. (2011). The 25S rRNA gene was used as an internal control (Yang et al., 2012). The sequence of gene-specific primers and internal reference gene were as follows: (SPS-F: 5'-TCTTCTCAAACCCCCGTAAGC-3', SPS-R: 5'-CAGTTCACGATGTTCACCAAATG-3'; 25S rRNA-F: 5'-GCAGCCAAGCGTTCATAGC-3', 25S rRNA-R: 5'-CCTATTGGTGGGTGAACAATCC3'). ABI 7500 FAST Real-Time PCR System (Applied Biosystems, USA) was used for gene expression analysis.

## CALLUS INDUCTION AND TISSUE CULTURE

The meristem from stem tip of *S. spontaneum* was used as explant for callus induction. The meristems were sterilized by immersion in 75% (v/v) alcohol for 1 min followed by 5% (v/v) sodium hypochlorite for 1h with gentle shaking. To optimize the process of callus induction, the sterilized explants were plated on diverse callus induction mediums which were different from each other in terms of 2,4-D concentration (1, 2, 3, 4 mg/L). The callus initiation was cultivated at 25°C in the dark for 4 weeks. Each subculture was conducted on a subculture medium at 25°C in the dark. Finally, calluses were grown in MS medium + 1 mg/mL KT + 30 g/L sucrose + 8 g/L agar + 3 mg/L 2,4-D at 25°C with 16 h light and 8 h dark for differentiation.

## VECTOR CONSTRUCTION AND TRANSFORMATION

The ORF of *SsSPS* cDNA was amplified and inserted into the expression vector, pCAMBIA-UBI1301. The resulting vector harboring a *Hyg* (hygromycin phosphotransferase), which was used as a selectable marker and an intron interrupted *gus* (*β-glucuronidase* gene), was then transferred into *Agrobacterium* strain EHA105. Calluses derived from the meristem of stem tip (A19) were used for genetic transformation and transgenic plantlets were placed into rooting medium composed of MS medium + 2 mg/L NAA + 30 g/L sucrose + 8 g/L agar + 3 mg/L 2,4-D at 25°C with 16 h light and 8 h dark. The detailed procedures of transformation, selection and regeneration were performed according to Zhu et al. (2006).

## RESULTS

### CLONING AND BIOINFORMATICS ANALYSIS OF *SSSPS*

In the present study, we first isolated the *SPS* cDNA from *S. spontaneum*. A 2500-bp cDNA fragment from *S. spontaneum* was amplified by homologous cloning (Fig. 1a). Based on the cDNA fragment, 5' RACE and 3' RACE were used to generate the cDNA ends sequence (Figs. 1b, 1c). A 4153-bp full-length cDNA was acquired after sequence assembly and confirmed by sequencing (Fig. 1d). The *S. spontaneum SPS* cDNA, which we named *SsSPS*, contained an open reading frame (ORF) of 3132-bp encoding a 1043-amino acid protein. The deduced protein has a predicted molecular mass of 116.95 kDa.

The amino acid sequence of *SsSPS* was 95% identical to *Saccharum officinarum* (ABX59534.1), 90% to *Setaria italica* (XP_004973187.1), 86% to *Bambusa oldhamii* (AAR16190.1) and *Lolium perenne* (BAE80113.1), 85% to *Oryza brachyantha* (XP_006659287.1) and 84% to *Brachypodium distachyon* (AAQ15106.1) (Table 1). Like other SPS amino acids, SsSPS harbored similar conserved motifs, including GT1-sucrose synthase and glycosyl transferase-GTB-type-superfamily which corresponded to the process of sucrose synthesis and glycosyl transferring, respectively (Fig. 2a). Multiple alignment analysis of the SsSPS amino acids with 6 other SPSs (SsSPS, SiSPS, SoSPS, BdSPS, ObSPS, BoSPS and LpSPS) demonstrated a high sequence conservation (Fig. 2b).
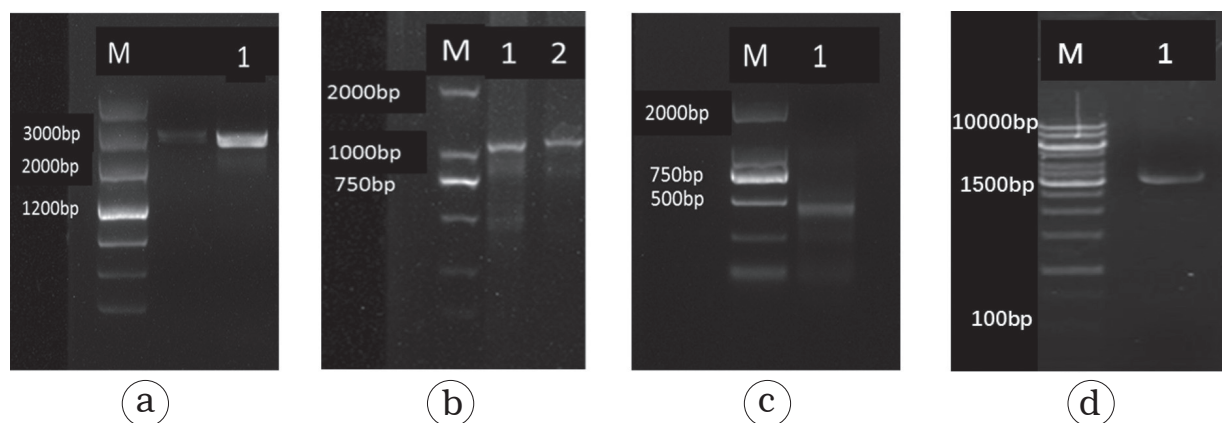


**Fig. 1.** Molecular cloning of *SPS* cDNA from *S. spontaneum*. (**a**) Amplification of core fragments. (**b**) Amplification of 3'RACE fragment. (**c**) Amplification of 5'RACE fragment. (**d**) Amplification of full-length cDNA. M – marker.

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TABLE 1. The blast result of nucleotide sequence (NS) and amino acid sequence (AS) of the *SPS* from *S. spontaneum*.

| Similar species (Blast) | Query coverage (%) (NS, AS) | E-value (NS, AS) | Identity (%) (NS, AS) | Accession NO. (NS, AS) |
|---|---|---|---|---|
| *Saccharum officinarum* | 97%, 99% | 0,0 | 98%, 95% | EU269038.1,ABX59534.1 |
| *Setaria italica* | 97%, 99% | 0,0 | 97%, 90% | XM_004973130.1,XP_004973187.1 |
| *Bambusa oldhamii* | 97%, 99% | 0,0 | 87%, 86% | AY445835.1,AAR16190.1 |
| *Lolium perenne* | 96%, 99% | 0,0 | 87%, 86% | AB232784.1,BAE80113.1 |
| *Oryza brachyantha* | 97%, 99% | 0,0 | 85%, 85% | XM_006659224.1,XP_006659287.1 |
| *Brachypodium distachyon* | 97%, 94% | 0,0 | 85%, 84% | XM_003573633.2,AAQ15106.1 |
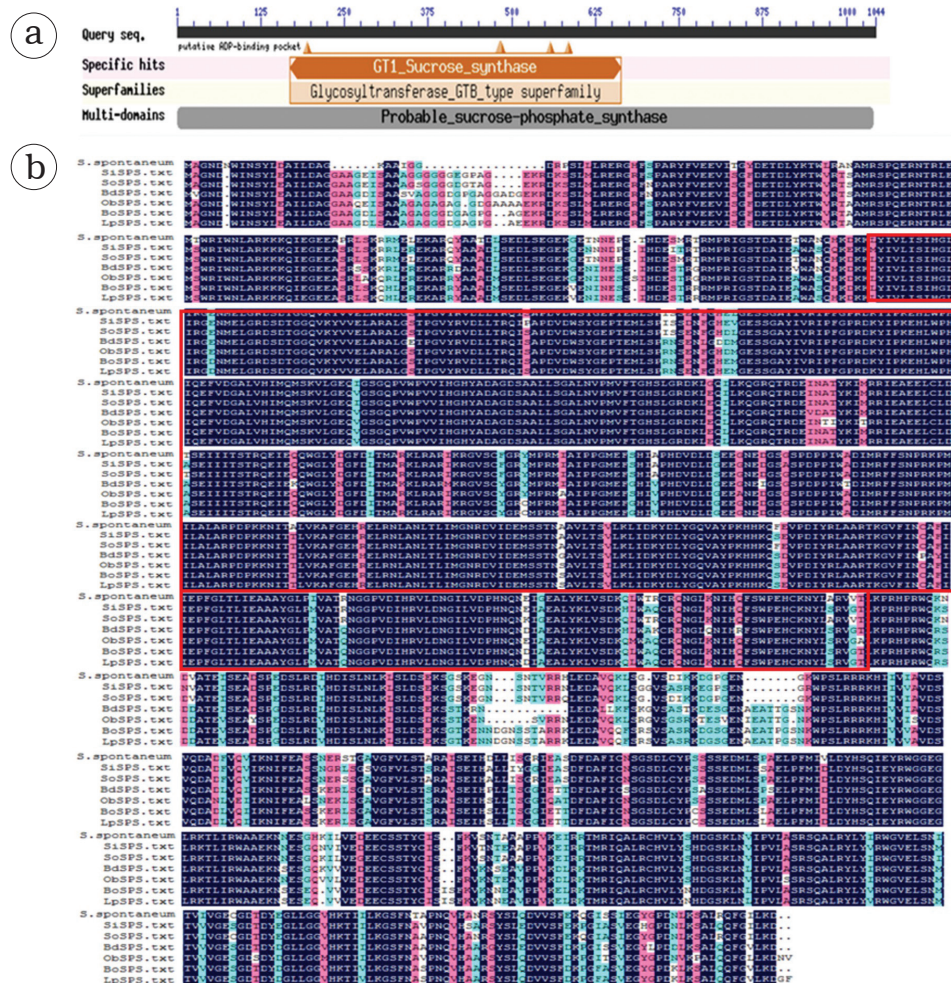


**Fig. 2.** (**a**) Conserved domain prediction of SsSPS. (**b**) Multiple alignment of the deduced amino acid sequences of SPS from different plant species. Conversed motifs for SsSPS are boxed. Protein IDs or accession numbers are as follows: *Setaria italica* SPS (XP_004973187.1), *Saccharum officinarum* SPS (ABX59534.1), *Brachypodium distachyon* SPS (AAQ15106.1), *Oryza brachyantha* SPS (XP_006659287.1), *Bambusa oldhamii* SPS (AAR16190.1) and *Lolium perenne* SPS (BAE80113.1).

## EXPRESSION ANALYSIS OF *SSSPS* GENE

In our study, the expression of *SsSPS* gene was studied in different tissues (stem and leaf) by qRT-PCR. A single peak in the fluorescent melt curves of the amplified products of the target gene and 25S rRNA gene as a reference gene indicated that the primer pairs were specific (Fig. 3a). Based on that, we found that the *SsSPS* was expressed in both stem and leaf of *S. spontaneum*, and had a significantly higher expression level in the stem. Young leaves and mature leaves were not significantly different from each other in the expression level of *SsSPS* gene (Fig. 3b).
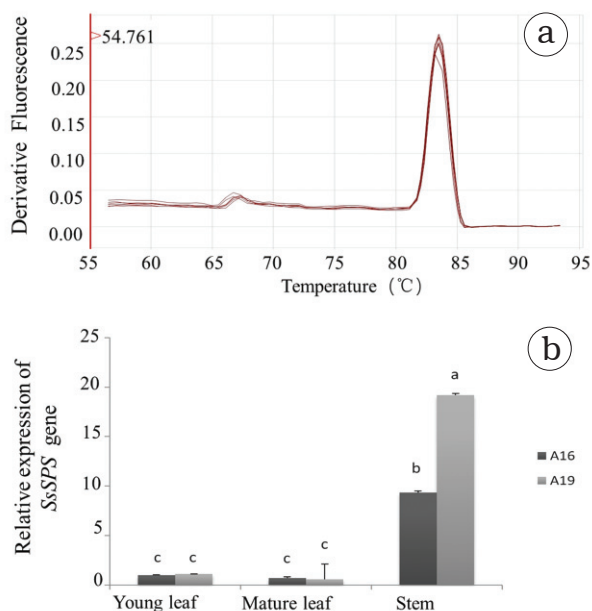


**Fig. 3.** (**a**) Fluorescent melt curves of 25S rRNA as reference gene. (**b**) Expression patterns of *SPS* gene from leaf and stem tissues of *S. spontaneum*. Note: The data represent the mean ± S.E of three replicate samples. Columns with different letters indicate significant differences at P < 0.05.

## OPTIMIZATION OF TISSUE CULTURE SYSTEM

To construct and optimize tissue culture system of *S. spontaneum*, the meristem from stem tip was used as explants and subjected to MS medium with different concentrations of 2,4-D (1 mg/L, 2 mg /L, 3 mg/L, 4 mg/L). The callus of 3 mg/L and 4 mg/L 2,4-D could be induced with higher rate, better and quicker growth (Fig. 4a). Comparing the concentrations of 3 mg/L to 4 mg/L 2,4-D, the calluses were better produced in the subculture medium with 3 mg/L 2,4-D (Fig. 4b). Then the differentiation rate of callus with 3 mg/L 2,4-D was correspondingly higher induced in the differential medium (Fig. 4c).
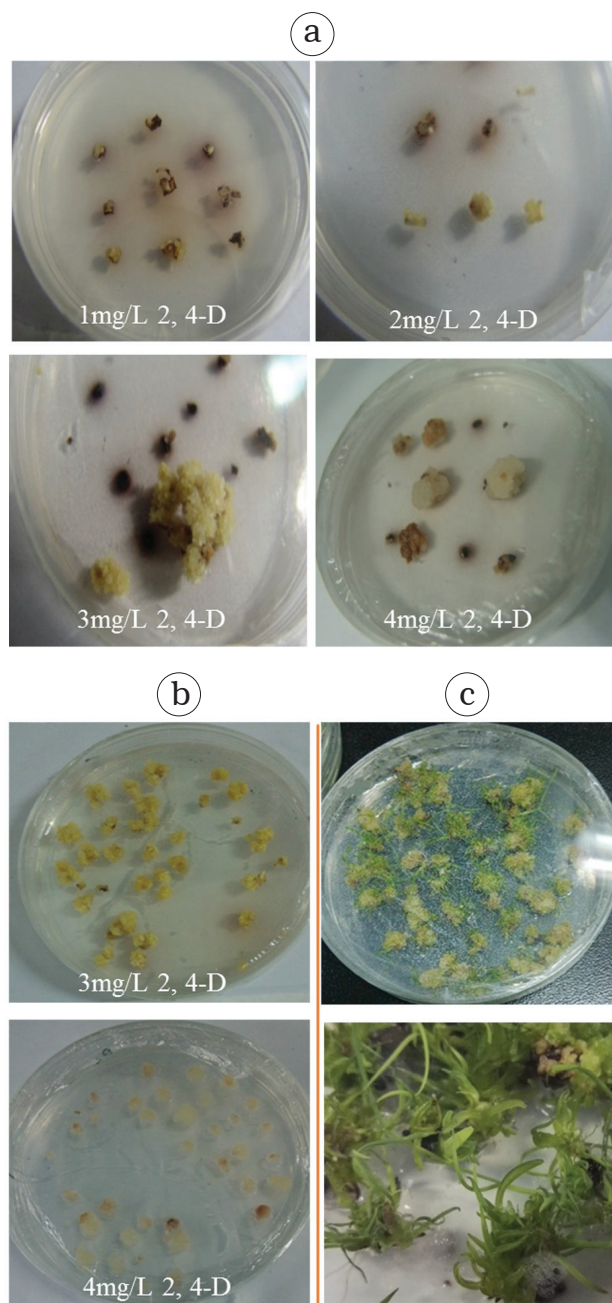


**Fig. 4.** Optimization of tissue culture system of *S. spontaneum* by different concentrations of 2,4-D. (**a**) Selection of optimum concentrations of 2,4-D for high efficiency of tissue culture system. (**b**) Callus from subculture medium. (**c**) Differentiation status from callus by 3 mg/L 2,4-D.

## VECTOR CONSTRUCTION AND TRANSFORMATION

Transgenic *S. spontaneum* plants expressing the *SPS* gene were generated by transforming *S. spontaneum* with the binary vector Ubi1301-SsSPS. The vector carried the *Hyg* (hygromycin phosphotransferase gene) as a selectable marker

and the transgene was constitutively expressed under the control of maize ubiquitin promoter and 35S promoter (Fig. 5a). The procedure of the genetic transformation was based on the *Agrobacterium*-mediated method. The callus from MS induction medium with 3 mg/L 2,4-D, which had a better color and structure, was used for transformation (Fig. 5b). The callus generated green spots within 1 week. After 2 weeks of cultivation, the green spots on callus developed into kanamycin-resistant transgenic adventitious shoots with a high regeneration frequency (Fig. 5c). One month later, vigorous plantlets selected by 50 mg/L *hyg* B (hygromycin B) were generated from callus on the differentiation medium (Fig. 5d). The regenerated green shoots were rooted on MS medium supplemented with 50 mg/L *hyg* B for further selection (Fig. 5e).

## DISCUSSION

### BIOINFORMATICS ANALYSIS OF *SSSPS*

As sucrose-phosphate synthase (SPS) is a key enzyme catalyzing the synthesis of sucrose in plants, a study on it is definitely of importance for *S. spontaneum* as a quite potential and promising biomass energy resource, and so is the study on genes encoding it. Currently, the *SPS* gene has been cloned in many species, such as *Spinacia oleracea*, *Citrus reticulata* and *Dendrocalamus sinicus* (Liu and Li, 2003; Zhang et al., 2008). With the further in-depth research, molecular characteristics of SPS are believed to be crucial for playing a physiological role of regulating photosynthetic carbon flux into sucrose (Sawitri et al., 2016). A predicted molecular mass of 116.95 kDa for SsSPS is similar to the molecular mass of SPS from *Arabidopsis thaliana* (117.32 kDa), *Medicago sativa* (117.89 kDa), *Dendrocalamus oldhami* (119.33 kDa) and *Oncidium hybridum* (118.67 kDa) (Bausher, 2002), which corresponds to Macrae et al. (2004) that plant SPSs have a molecular mass around 120 kDa. Previous studies suggested that *SPS* had an abundant genetic diversity, variation and was classified into four families in gramineae, including A, B, C and D (Huang et al., 2011; Xu et al., 2011). In addition, *SPS* genes from various families were significantly different on the nucleotide sequences other than the functional role (Liu et al., 2005). In the present study, nucleotide sequence alignment showed that *SPS* from *S. spontaneum* shared a high homology (98%) with SPS-A family but presented a low homology (65%) with SPS-B family of sugarcane. Therefore, it is suggested that *SsSPS* belonged to SPS-A family. Meanwhile, we found that there were two function domains, GTl-sucrose-synthase and glycosyl transferase-GTB-type superfamily corresponding to the process of sucrose synthesis and glycosyl transfer which was the central region containing glycosyl transferase domain responsible for the catalytic function of SPS (Macrae et al., 2004). Similarly, studies have shown that SPS of maize also has two function domains
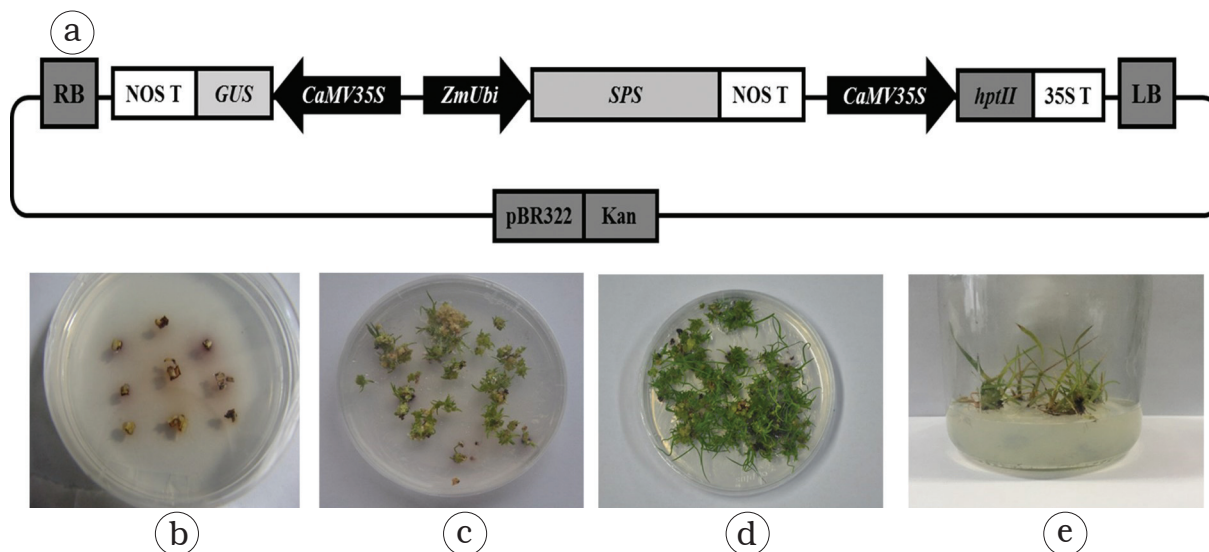


**Fig. 5.** Genetic transformation of sucrose phosphate synthase (*SPS*) gene from *S. spontaneum*. (**a**) Schematic diagram of vector conduction. (**b**) Callus from meristem on MS medium by 3 mg/L 2,4-D. (**c**) Phenotype of callus for 2 weeks on differentiation medium. (**d**) Phenotype of callus for 1 month on differentiation medium. (**e**) Rooting culture of regeneration seedlings.

(Lunn et al., 2000). The results of analyzing signal peptide and transmembrane domain of SPS gene in *S. spontaneum* showed that there was no transmembrane domain and signal peptide, suggesting that the whole peptide chain only played a role in the cytoplasm and did not enter subcellular organelle. These studies would provide an insight into molecular mechanism of SsSPS for affecting plant sucrose biosynthesis.

### EXPRESSION ANALYSIS OF *SSSPS* GENE

Real-time PCR method, a common method to study gene expression at RNA level, was chosen to study the expression of *SPS* gene in different tissues based on some advantages like better specificity, higher sensitivity and so on (Mohamad et al., 2013). In this study, an organ-specific expression pattern of *SsSPS* gene was emphasized (Fig. 3b), which had also been reported in sugarcane (Ye et al., 2011) and sweet sorghum (Yang et al., 2009). Interestingly, *SPS* gene was predominantly expressed in tap root of sugar beet (Hesse et al., 1995), or fruits and flowers of *Lycium barbarum* (Wang et al., 2013). Given the above references, we could hypothesize that the tissue with a higher expression level of *SPS* gene presumably had higher sugar contents in plant. Combining the expression level of *SsSPS* gene with the data on sucrose content of *S. spontaneum* by Tang (2016), we found a relatively positive correlation between them, which was in agreement with findings of Komatsu et al. (1999) showing that sucrose gradually accumulated and eventually comprised about 65% of the total sugars in the mature citrus fruits where the expression of *CitSPS1* and *CitSPS2* genes rapidly increased during the mature stage in the edible tissue. Otherwise, the expression pattern of *SPS* at different development stages of plants was also explored. Yu et al. (2007) analyzed the developmental stages-specific expression of *SPS* gene in melon fruit by Northern blot and found that the expression of *SPS* gene began to be present 25 days after pollination, and the expression level increased along with the ripening degree of fruit. Li et al. (2015) found that the expression level of cacao *SPS* gene showed a rising trend with the development of leaves and buds. All of this evidence indicates that the expression of *SPS* gene has spatial and temporal specificity and also depends on plant species.

### OPTIMIZATION OF TISSUE CULTURE SYSTEM AND TRANSFORMATION

In order to establish an efficient *Agrobaterium*-mediated transformation system for *S. spontaneum*, we optimized tissue culture system by selecting most suitable concentration of 2,4-D in the basal induction medium. We then used this culture system in a callus-based *S. spontaneum* transformation system. However, the browning occurring in calluses of our study resulted in a low product rate during the process of callus induction. According to previous studies, the phenomenon of browning was generally attributed to polyphenol oxidase (PPO) acting on natural substrate phenolic compounds, which is one of the three major problems in tissue culture (Li et al., 2005). Additionally, some studies found that medium with glucose as a carbon source could effectively prevent browning, followed by maltose, sucrose worst (Yan et al., 2003). The reason for browning in this experiment may be related to medium with sucrose. Our result exhibited that higher concentration of 2,4-D in this experiment had a higher rate of product and less browning compared to the result caused by low concentrations of 2,4-D group. Previous studies also demonstrate that the auxin 2,4-D or IAA can delay the synthesis of phenol and reduce browning (Xu et al., 1997). Therefore, the 3 mg/L 2,4-D was an optimal hormone concentration for callus induction; whereas rooting efficiency was not as good as we had expected on the rooting medium in our study, which could be caused by explant types, strains of *Agrobacterium* and also various conditions of tissue culture. Many studies have been conducted to improve the capacity of plantlet regeneration by optimizing the factors of the regeneration medium, especially PGRs (Zhao et al., 2011). Also, there are still a lot of factors like plant genotype and selection marker genes, influencing the accomplishment in *S. spontaneum* transformation. In our view, the main reason for the low success rate of transformation in *S. spontaneum* may be largely due to the technical immaturity of tissue culture and transformation in *S. spontaneum* for which there are few cases reported.

### CONCLUSION

We successfully cloned the full-length cDNA of a SPS ortholog with 5'UTR and 3'UTR, which encoded sucrose phosphate synthase in *S. spontaneum*. Bioinformatics analysis of *SsSPS* indicated that it was a member of the SPS-A gene family. Expression analysis of *SsSPS* gene by qRT-PCR exhibited a predominant expression in the stem tissue. 3 mg/L 2,4-D was more suitable to construct and optimize the tissue culture system of *S. spontaneum* when the meristem from stem tip was used as explants. Our work serves as a foundation for further breeding process of *S. spontaneum* with a high sucrose content.

## AUTHORS' CONTRIBUTIONS

## ACKNOWLEDGEMENTS

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