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Cloning and sequence analysis of sucrose phosphate synthase gene from varieties of *Pennisetum* species

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ABSTRACT. Sucrose phosphate synthase (SPS) is an enzyme used by higher plants for sucrose synthesis. In this study, three primer sets were designed on the basis of known *SPS* sequences from maize (GenBank: NM_001112224.1) and sugarcane (GenBank: JN584485.1), and five novel *SPS* genes were identified by RT-PCR from the genomes of *Pennisetum* spp (the hybrid *P. americanum* x *P. purpureum*, *P. purpureum* Schum., *P. purpureum* Schum. cv. Red, *P. purpureum* Schum. cv. Taiwan, and *P. purpureum* Schum. cv. Mott). The cloned sequences showed 99.9% identity and 80-88% similarity to the *SPS* sequences of other plants. The *SPS* gene of hybrid *Pennisetum* had one nucleotide and four amino acid polymorphisms compared to the other four germplasms, and cluster analysis was performed to assess genetic diversity in this species.

Additional characterization of the *SPS* gene product can potentially allow *Pennisetum* to be exploited as a biofuel source.

Key words: *Pennisetum* Rich.; Sucrose phosphate synthase; Gene clone; Sequence analysis

INTRODUCTION

Pennisetum Rich. is a fast-growing annual or perennial herb distributed in tropical and subtropical regions, which has multiple tillers and a high biomass and can be grown in barren, sandy, or saline-alkaline soil (Liu, 2009; Zhang, 2012). Elephant grass and hybrid *Pennisetum* are typical examples of this genus. Hybrid *Pennisetum* serves as raw material for energy conversion, with a per hectare yield of nearly 11 tons. It cannot only be directly burned to generate power, but can also be converted into ethanol or gas (Morais et al., 2009; Fan et al., 2012).

Converting plant starches into sugar and then ethanol is a highly efficient method of obtaining energy from biomass (Xu and Liu, 2009). Although this process is often applied to sweet sorghum, it is limited by crop yields. Thus, using perennial grasses that have widespread distribution and high sugar content is a potential solution to the shortage of available plant sources.

Sucrose is the main sugar produced by photosynthesis, and sucrose phosphate synthase (SPS) is a key enzyme in this process. SPS was initially purified as a relatively low abundance and unstable enzyme from wheat germ, and SPS gene expression was thereafter detected in mesophyll cells of maize and rice (Liu et al., 2005; Wei, 2011). Some studies have indicated that the activity of SPS can be improved by transferring foreign genes into crops, resulting in a corresponding increase in sugar production (Worrell et al., 1991); this technology has been applied to sugar beet (Liu et al., 2010), maize (Liu, 2003), and sweet sorghum (Liu et al., 2011). However, there are no reports of the cloning and expression of the *SPS* gene in an energy-rich plant such as *Pennisetum*. Elephant grass (including red and dwarf types), hybrid *Pennisetum* and Taiwan sweet grass are representative varieties of *Pennisetum* Rich. that are widely cultivated in southern China and have high yield, and their SPS activity can potentially be exploited as a source of energy.

In this study, five gene segments were cloned from the genomes of five varieties of *Pennisetum* Rich. using the homologous gene sequence method. Sequence identity and evolutionary conservation were analyzed, including nucleotide and amino acid polymorphisms. The results revealed both conservation and polymorphism in the *SPS* gene of different varieties of *Pennisetum* Rich.

MATERIAL AND METHODS

Plant materials

Five varieties of *Pennisetum* were cultivated in a greenhouse: a *Pennisetum* hybrid (*P. americanus* x *P. purpureum*) was from Beijing, while elephant grass (*P. purpureum* Schum.), red elephant grass (*P. purpureum* Schum. cv. Red), Taiwan sweet grass (*P. purpureum* Schum. cv. Taiwan), and dwarf elephant grass (*P. purpureum* Schum. cv. Mott) were from Hainan.

Total RNA isolation

Fresh young leaves (0.5 g) were flash frozen in liquid nitrogen and ground into a powder. Total RNA was immediately isolated using an RNA extraction kit (Tiangen Biotech, Beijing, China) according to manufacturer instructions, and stored in Tris-HCl/EDTA buffer, pH 8.0, at -80°C until use. The quality of the RNA samples was evaluated by 1.2% agarose gel electrophoresis and the 260/280-nm absorbance ratio, which was determined using a Nano-Drop 2000 spectrophotometer (Thermo Scientific, USA).

First-strand cDNA synthesis and PCR amplification

RNA was reverse-transcribed to cDNA using the First-Strand cDNA Synthesis kit (Tiangen Biotech) according to the manufacturer protocol; the cDNA was stored at -20°C until use.

Three sets of primers were designed on the basis of the SPS coding sequence for maize using the Primer 5.0 software, and synthesized by Sangon Biotech (Shanghai, China). Forward primer (PF) and reverse primer (PR) sequences are shown in Table 1.

Table 1. Primer sequences for cloning sucrose phosphate synthase from *Pennisetum* Rich.

Primer set	Name	Sequence (5'→3')
1	PF1	AACCCTCACAAGCC
	PR1	CGCACGGAAAGATA
2	PF2	AGCAGGTTGGAAATGG
	PR2	GACAGACGGAGTGACAGG
3	PF3	GGCGTTCCCTAAGCATCACA
	PR3	CAGCCGCTCCCTCATCTCAT

PF = forward primer; PR = reverse primer.

The amplification reaction mixture (50 µL) contained the following: 100 ng cDNA, 25 µL 2X Taq Platinum PCR MasterMix (Tiangen Biotech), 2 µL of each primer (10 µM), and 17 µL ddH_2O. The cycling conditions were as follows: 94°C for 2 min; 35 cycles of 94°C for 30 s, 40°-45°C (depending on the primer) for 30 s, 72°C for 1 min; and 72°C for 10 min.

PCR products were resolved by 1.2% agarose gel electrophoresis and purified using a gel extraction kit (Tiangen Biotech). Purified products were ligated into the PMD18-T vector (Takara, Dalian, China), which was used to transform *Escherichia coli* DH5α competent cells. Positive clones were selected on Luria broth/agar plates containing ampicillin (100 µg/mL), IPTG (100 µg/mL), and X-gal (40 µg/mL). After confirming the successful insertion of the amplification product by colony PCR using primer set 1 (PF1, PR1), clones were sequenced.

Data analysis

Sequences obtained for the *Pennisetum SPS* gene were compared to known sequences in the NCBI database using BLASTX. Multiple-sequence alignments and analysis of genetic diversity were performed by using Vector NTI, DNAman, and DNAStar software programs, while ClustalW was used for cluster analysis.

RESULTS

Analysis of the *SPS* gene sequence

PCR products amplified from the genome of five varieties of *Pennisetum* are shown in Figure 1. The band between 1 and 2 kb corresponded to the predicted size of the *SPS* coding sequence.

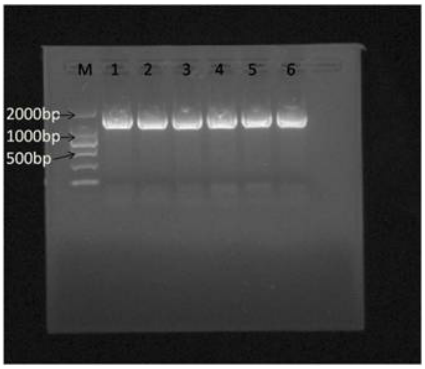

Figure 1. PCR products amplified from *Pennisetum* cDNA (*lanes 1-6*) using primers PF1 and PR1 and an annealing temperature of 40.3°C. Details of the procedure are described in Material and Methods. *Lane M* = DNA marker; *lane 1* = elephant grass; *lane 2* = Taiwan sweet grass; *lane 3* = red elephant grass; *lane 4* = dwarf elephant grass; *lanes 5* and *6* = hybrid *Pennisetum*.

Sequence fragments were spliced using the DNAman software, and the total length was found to be 1408 bp. The *SPS* gene of dwarf elephant grass and *Pennisetum* hybrid encodes 445- and 444-amino acid proteins, respectively, which was also the case for the other three clones. The sequences showed 99.9% identity; using the NCBI BLASTn search, the sequences were found to share identity with *Setaria italica* (foxtail millet; 96%), hybrid sugarcane (88%), maize (87%), sugarcane (86%), sorghum (88%), *Brachypodium distachyon* (purple false brome; 81%), and wheat (80%), indicating that the *SPS* gene in *Pennisetum* was highly homologous to that of other plants (Figure 2).

Description	Max score	Total score	Query cover	E value	Ident	Accession
PREDICTED: Setaria italica sucrose-phosphate synthase-like (LOC10176043	2263	2263	100%	0.0	96%	XM_004971009.1
Saccharum hybrid cultivar ROC22 sucrose phosphate synthase B (SPSB) mR	1644	1644	100%	0.0	88%	JN584485.1
Sorghum bicolor hypothetical protein, mRNA	1628	1628	100%	0.0	88%	XM_002458946.1
Zea mays sucrose phosphate synthase1 (sps1), mRNA >gb|M97550.1|MZES	1550	1550	100%	0.0	87%	NM_001112224.1
Saccharum officinarum mRNA for Sucrose-Phosphate Synthase, partial cds	1511	1511	100%	0.0	86%	AB001337.1
Zea mays full-length cDNA clone ZM_BFb0215J14 mRNA, complete cds	1182	1182	80%	0.0	86%	BT055092.1
PREDICTED: Brachypodium distachyon sucrose-phosphate synthase-like (LC	1061	1061	99%	0.0	81%	XM_003564887.1
Zea mays CL2428_1 mRNA sequence	1016	1424	95%	0.0	84%	AY109574.1
Triticum aestivum sucrose-phosphate synthase 5 mRNA, partial cds	996	996	99%	0.0	80%	AF347067.1
Triticum aestivum cDNA, clone: WT013_F14, cultivar: Chinese Spring	990	990	99%	0.0	80%	AK335612.1
Hordeum vulgare subsp. vulgare cDNA clone: FLbaf138m10, mRNA sequenc	760	760	78%	0.0	79%	AK251897.1

Figure 2. Homology of the *Pennisetum SPS* gene sequence to that of other plant species.

The amino acid sequence identity for the five clones was 99.55%, indicating a highly conserved protein. There was low absolute complexity between individual amino acids that had low identity (Figure 3). The absolute complexity of residues 314-318 in hybrid *Pennisetum* was significantly different from that of four other plant species (Figure 4), suggesting that these residues may confer a property or function unique to *Pennisetum* SPS. Overall, the amino acid sequence shared identity with *S. italica* (96%), maize (90%), sugarcane hybrid (89%), sorghum (89%), sugarcane (87%), *B. distachyon* (81%), and wheat (80%) (Figure 5).

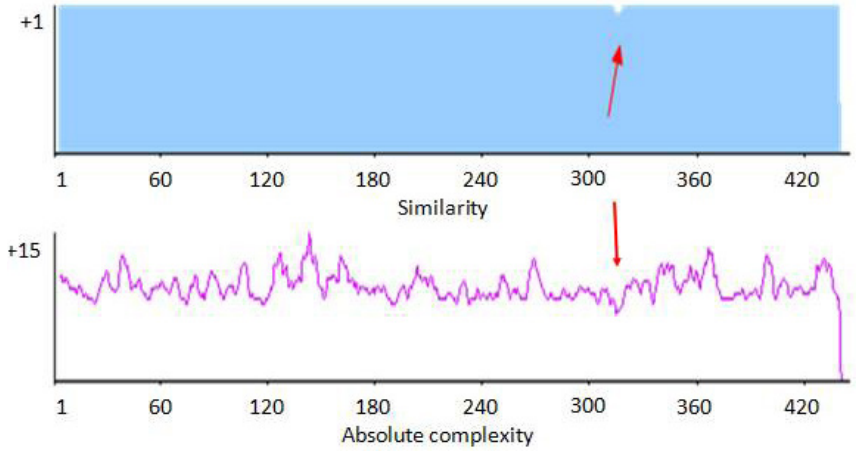

Figure 3. Similarity and absolute complexity of the *SPS* amino acid sequence.

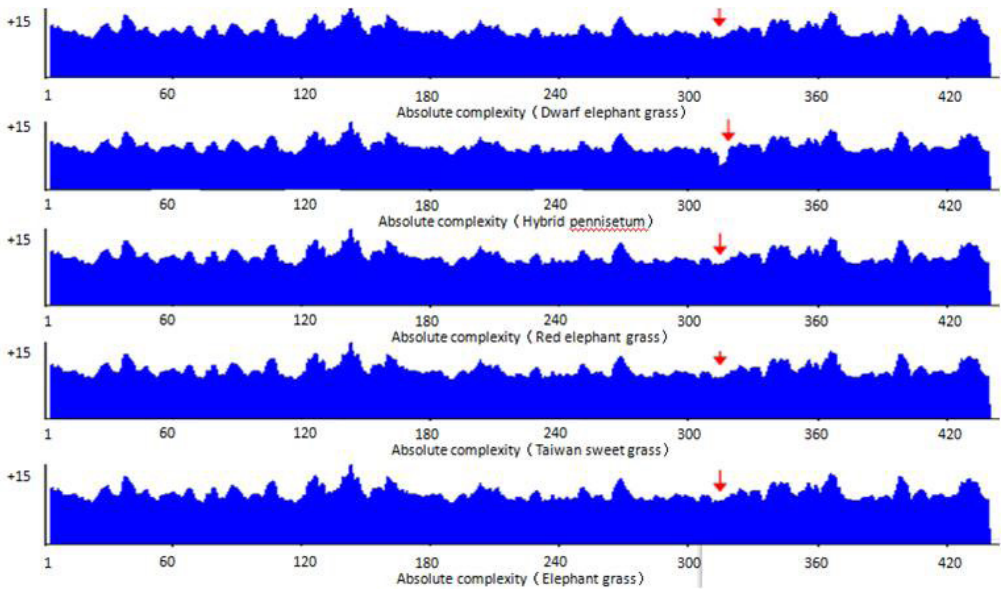

Figure 4. Similarity and absolute complexity of the *Pennisetum* SPS amino acid sequence to that of five plant species.

	Description	Max score	Total score	Query cover	E value	Ident	Accession
☐	PREDICTED: sucrose-phosphate synthase-like [Setaria italica]	873	873	100%	0.0	96%	XP_004971066.1
☐	hypothetical protein SORBIDRAFT_03g043900 [Sorghum bicolor] >gb|EES04	816	816	100%	0.0	89%	XP_002458991.1
☐	TPA: putative sucrose-phosphate synthase family protein [Zea mays]	817	817	100%	0.0	90%	DAA56138.1
☐	sucrose-phosphate synthase [Zea mays] >sp|P31927.1|SPS_MAIZE RecNam	813	813	100%	0.0	89%	NP_001105694.1
☐	sucrose phosphate synthase B [Saccharum hybrid cultivar ROC22]	813	813	100%	0.0	89%	AEO46461.1
☐	Sucrose-Phosphate Synthase [Saccharum officinarum]	786	786	100%	0.0	87%	BAA19241.1
☐	PREDICTED: sucrose-phosphate synthase-like [Brachypodium distachyon]	742	742	100%	0.0	81%	XP_003564935.1
☐	sucrose-phosphate synthase 5, partial [Triticum aestivum]	723	723	100%	0.0	80%	AAQ15109.1
☐	Sucrose-phosphate synthase [Aegilops tauschii]	724	724	100%	0.0	80%	EMT16825.1
☐	RecName: Full=Probable sucrose-phosphate synthase 1; AltName: Full=UDP	701	701	100%	0.0	81%	A2WYE9.2

Figure 5. Homology of the *Pennisetum* SPS amino acid sequence to that of other plant species.

Nucleotide and amino acid polymorphisms among *Pennisetum* varieties

A variation in the nucleotide sequence of the five SPS clones was observed upon comparison using DNAman and DNAstar software programs (Figure 6). A T→C transition at 1017 bp (Figure 6A) resulted in the substitution of Leu by Pro (Figure 7) in hybrid *Pennisetum.* Analysis of the amino acid sequences revealed polymorphisms at four sites. At residue 315, hybrid *Pennisetum* had a Leu residue, while other varieties had Pro; and at residues 441-443, Val-Pro-Leu was found in hybrid *Pennisetum* and dwarf elephant grass, while Ser-Leu-Cys was present in the other three varieties (Figure 7).

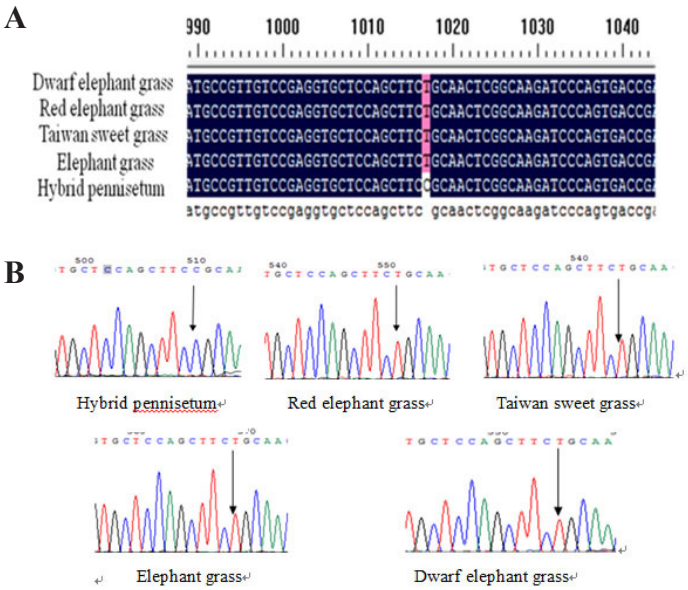

Figure 6. Single nucleotide polymorphism in the *SPS* gene of five *Pennisetum* varieties, as seen in (**A**) sequence alignment and (**B**) chromatograms.

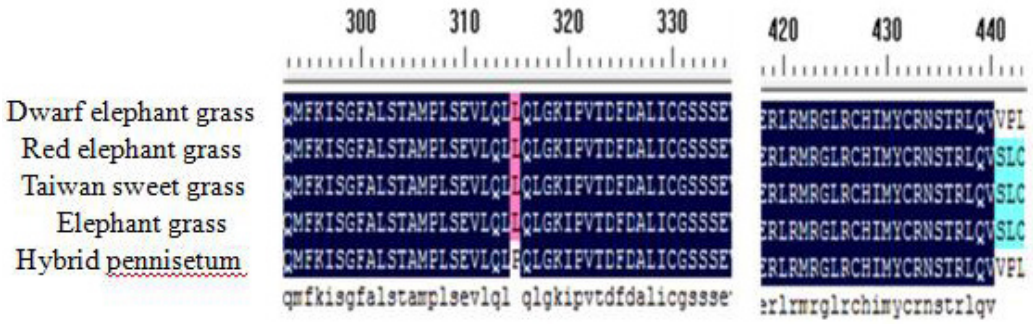

Figure 7. Amino acid polymorphisms in the SPS sequence of five *Pennisetum* varieties.

Genetic diversity of *SPS* gene fragments

A cluster analysis of the five SPS clones was performed using ClustalW. The first cluster included hybrid *Pennisetum*, and another included elephant grass, dwarf and red elephant grasses, and Taiwan sweet grass, which were more closely related (Figure 8A).

Cluster analysis was performed for the SPS amino acid sequences using Vector NTI and Megalign. Hybrid *Pennisetum* and dwarf elephant grass clustered in one group, while the other varieties formed a second group, demonstrating a close relationship between the sequences (Figure 8B).

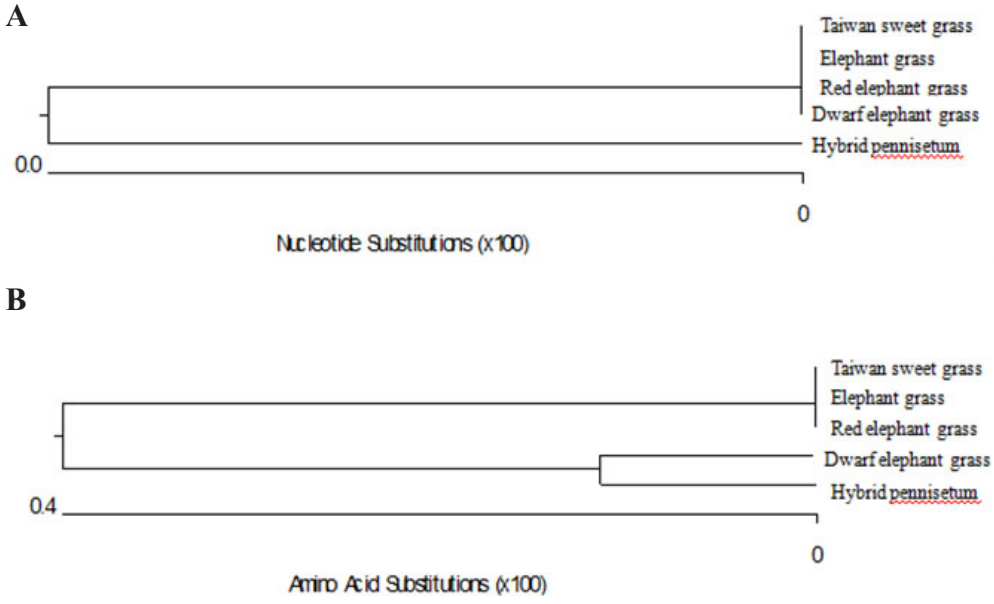

Figure 8. Results of cluster analyses for the *SPS* gene in five varieties of *Pennisetum*. **A.** Nucleotide sequences were compared using Clustal W. **B.** Amino acid sequences were compared using Megalign, revealing two distinct clusters.

DISCUSSION

Homology of the *Pennisetum SPS* gene

SPS is a key enzyme for sucrose synthesis in higher plants. As such, sucrose accumulation is positively correlated with SPS activity (Zhao et al., 2001; Park et al., 2009), and plants with relatively high sugar content such as sugar beet and sugarcane have been shown to have high SPS activity (Li et al., 2010; Ye et al., 2011). The SPS gene family has subfamilies A-C (Langenkämper et al., 2002; Zhou et al., 2006; Huang et al., 2012). In sugarcane, 10 different alleles have been identified (McIntyre et al., 2006); the gene in *Dendrocalamus sinicus* has 90-99% homology with other plants (He et al., 2007; Zhang et al., 2008), indicating a high degree of evolutionary conservation in this gene family. However, considerable diversity at the amino acid level was observed between *Arabidopsis* (Martin et al., 1993) and citrus plants because of alternative splicing (Wu and Liu, 2010b). One study used the target region amplification polymorphism approach to mark the *SPS* gene of sugarcane; the amplified gene exhibited high variability and rich polymorphism, indicating an inherent diversity (Xu et al., 2011). The five clones identified in the present analysis all belonged to *SPS* subfamily B and shared 99.9% homology (Figure 2), indicating a high degree of conservation between different varieties of the same species, but divergence from other non-congeneric types of plant. The *Pennisetum SPS* gene had the highest homology with foxtail millet and hybrid sugarcane (96 and 88%, respectively), but relatively low homology with wheat (80%). Similarly, nucleotide homology of the glycerol-3-phosphate acyltransferase (GPAT) gene from eight varieties of citrus plant was 97.8%, while homology to other species ranged from 72.5 to 98.4% (Wu and Liu, 2010a).

Variation and conservation in amino acid sequences

A high degree of conservation at the amino acid level was also observed between the five SPS clones (99.55%). A T to C transition at 1017 bp resulted in the substitution of Leu by Pro in hybrid *Pennisetum* (Figure 7), while a 3-amino acid polymorphism was also detected: Val-Pro-Leu was found in hybrid *Pennisetum* and dwarf elephant grass, but Ser-Cys-Leu was present in the other three varieties, apparently due to the deletion of a G residue that caused a shift in the reading frame.

Cluster analysis showed divergence between hybrid *Pennisetum* and dwarf elephant grass, possibly due to the single-amino acid polymorphism (Leu *vs* Pro, respectively) at residue 315. The analyses performed using nucleotide and amino acid sequences (Figure 8) showed different clustering patterns. The reason for this is not known; however, it is likely because certain nucleotide substitutions are conservative, and have no effect on the translated sequence.

Certain residues such as Cys, when forming disulfide bonds in the active site of enzymes, tend to be highly conserved (Guo et al., 2007). Studies of the superoxide dismutase and GPAT genes in citrus plants (Wu and Liu, 2010a) as well as in *Pyrus* (pear tree) (Cong et al., 2011) have shown that single nucleotide substitutions can lead to significant variation in the structure and function of the protein, causing divergence between related proteins in the final cluster analysis (Cong et al., 2011). Additional studies in hybrid *Pennisetum*, including mutation analyses, can shed light on the functional significance of the Leu substitution at residue 315.

In this study, the *SPS* gene in five varieties of *Pennisetum* Rich. was cloned and analyzed with respect to intra- and interspecies homology and evolutionary conservation. The *SPS* gene product plays an important role in the sucrose synthesis pathway in plants, and its activity is directly related to sucrose content (Liu et al., 2012). Given that the conversion of sucrose into a biofuel such as ethanol represents an important potential source of energy, future studies should focus on characterizing the functional and biochemical properties of *Pennisetum* SPS, so that *Pennisetum* can be exploited as a renewable energy source.

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