Research Article

Transgenic switchgrass (*Panicum virgatum* L.) biomass is increased by overexpression of switchgrass sucrose synthase (*PvSUS1*)

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Sucrose synthase (SUS) converts sucrose and uridine di-phosphate (UDP) into UDP-glucose and fructose. UDP-glucose is used by the cellulose synthase to produce cellulose for cell wall biosynthesis. For lignocellulosic feedstocks such as switchgrass, the manipulation of cell walls to decrease lignin content is needed to reduce recalcitrance of conversion of biomass into biofuels. Of perhaps equal importance for bioenergy feedstocks is increasing biomass. Four SUS genes were identified in switchgrass. Each gene contained 14 or 15 introns. *PvSUS1* was expressed ubiquitously in the tissues tested. *PvSUS2* and *PvSUS6* were highly expressed in internodes and roots, respectively. *PvSUS4* was expressed in low levels in the tissues tested. Transgenic switchgrass plants overexpressing *PvSUS1* had increases in plant height by up to 37%, biomass by up to 13.6%, and tiller number by up to 79% compared to control plants. The lignin content was increased in all lines, while the sugar release efficiency was decreased in *PvSUS1*-overexpressing transgenic switchgrass plants. For switchgrass and other bioenergy feedstocks, the overexpression of *SUS1* genes might be a feasible strategy to increase both plant biomass and cellulose content, and to stack with other genes to increase biofuel production per land area cultivated.

Supporting information available online

1 Introduction

In higher plants, photosynthates are transported from source tissues (photosynthetically active mature leaves) to sink tissues (photosynthetically less active shoot apex, roots, tubers, fruits, seeds, and stems) via phloem loading/unloading, which mostly consists of the disaccharide sucrose. Sucrose synthase (SUS), a glycosyltransferase, catalyzes the reversible conversion of sucrose to uridine di-phosphate (UDP) to UDP-glucose and fructose [1]. The role of SUS in phloem loading and unloading and in carbon partitioning for storage and metabolism in the plant cell has been well characterized in many species of higher plants [2, 3]. The breakdown of sucrose by SUS is correlated with the sink strength of starch storage organs [4, 5], e.g. SUS activity is high in potato tubers [5] and maize kernels [6].

At the cellular level, SUS can be accumulated in the cytosol or associated with the plasma membrane. Cytosolic SUS is mainly associated with metabolic tasks such as respiration and synthesis of starch [2]. Plasma membrane-associated SUS is linked to the synthesis of cell wall polysaccharides callose and cellulose [7]. SUS has been found to accumulate to high levels along the plasma membrane

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and in microtubules beneath the secondary cell wall thickening in tracheary elements [8]. Maize *ZmSUS1* is membrane-associated and is expressed during stem elongation [9]. Functionally, SUS has been identified as an integral part of the cellulose synthase machinery and the catalytic unit that is part of the plasma membrane-associated rosette structure involved in cellulose synthesis [10]. All four identified SUS genes in barley identified to date exist in both soluble and membrane associated forms, although their exact functions have not been elucidated [11].

Overexpression of *SUS* genes has been established to increase cellulose content in diverse species such as *Acetobacter xylinum* [12] and poplar [13]. Biomass and lignin content did not change in poplar plants overexpressing SUS [13]. Biomass was increased in tobacco [14] and cotton [15] when SUS was overexpressed. However, when SUS was downregulated in carrot, there was decreased cellulose content [16]. However, whether or not these particular isoforms were associated with membranes has not been established.

In our study, we identified four *SUS* genes in the lignocellulosic biofuel feedstock switchgrass (*Panicum virgatum* L.) and compared their sequences to previously identified *SUS* genes. Subcellular localization of *PvSUS1* was studied using a translational fusion construct to a fluorescent protein gene. Stable transgenic switchgrass plants were made whereby overexpression of *PvSUS1* yielded variable results including plants with increased biomass and cellulose content. Our findings indicated *PvSUS1* is likely to be membrane-associated and could play a role in cellulose synthesis, which renders it a good candidate for improving biomass and biofuel yield for sustainable energy production, likely in concert with other genetic modifications.

2 Materials and methods

2.1 Bioinformatic and cluster analyses

Rice and maize SUS sequences were used for BLAST searches against the switchgrass sequence database (www.phytozome.net) to identify putative switchgrass members of the SUS gene family. Exon–intron structure was predicted by comparing genomic sequences with mRNA and complementary DNA (cDNA) sequences. To identify conserved domains in SUS proteins, a search was done with the InterPro program using the Pfam database [17].

To enable cluster analysis, derived amino acid sequences of SUS genes were obtained from Genbank. Protein accession numbers of SUS genes from *Eucalyptus*, poplar, sugarcane, potato, beet, cotton, pea, *Arabidopsis*, *Phaseolus*, *Vigna*, *Lolium*, bamboo, maize, rice, barley, tomato, and *Physcomitrella* were used: EgSUS1

(ABB53601), EgSUS3 (ABB53602), PtrSUS1 (AAR03498), PtrSUS2 (ABW76422), SoSUS2 (AAM68126), StSUS (AAO67719), StSUS2 (AAO34668), BvSUS (AAK65960), GhSUS1 (ACV72640), GaSUS3 (AAD28641), Ps-NeSUS (AAC28107), MsSUS (AAC17867), BvSUS2 (AAR19769), AtSUS1-6 (NP_197583, NP_199730, NP_192137, NP_566865, NP_198534, NP_177480), PvSUS (ACP17902), VaSUS (BAH56282), SbSUS2 (ACM69042), CcSUS2 (ABI17891), MeSUS (ABD96570), PtrSUS2 (ABW76422), LpSUS (BAE79815), BoSUS1-4 (AAV64256, AAL50571, AAL50570, AAL50572), ZmSUS1 (NP_001105323), ZmSUS2 (NP_001105194), ZmSUS-SH1 (NP_001266691), OsSUS1-7 (P31924, P30298, Q43009, Q10LP5, H6TFZ4, Q6K973, Q7XNX6), HvSUS1-4 (P31922, P31923, CAZ65725, AEH16642), Le-FSUS (AAA34196), PpSUS (XP_001781447). The amino acid sequences were aligned using the online version of MAFFT v 7.0 [18]. Model testing for producing the best-fit model was done using ProtTest v 2.4 [19]. The maximum likelihood tree was constructed by PhyML [20] using the model identified by ProtTest and bootstrapping with 100 trees. The resulting tree was visualized in FigTree v.1.4.0 (http://tree.bio.ed.ac.uk/software/figtree) and rooted using the *Physcomitrella patens* gene as the outgroup.

2.2 PvSUS1 cloning

RNA was extracted from E2 to E3 stage [21] stem and leaf of ST1 switchgrass, a clonal genotypye of cultivar Alamo, using TRIREAGENT (Molecular Research Center, Inc. Cincinnati, OH, USA) following the manufacturer's guidelines. cDNA was synthesized using random hexamers and oligo dT using iScript™ cDNA synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) after DNase treatment (Promega Corporation, Madison, WI, USA). *PvSUS1* was amplified from the cDNA using the primers CP33 and CP34 (Table 1). *PvSUS1* was cloned into pCR8-GW-TOPO vector (Life Technologies, Carlsbad, CA, USA) for sequence verification. *PvSUS1* was subsequently cloned into pANIC-10A vector [21] using the Gateway recombination reaction to produce the overexpression vector subsequently used for stable switchgrass transformation, where the gene was driven by maize ubiquitin-1 promoter.

2.3 Subcellular localization experiments

To construct plasmids for subcellular localization *PvSUS1* was amplified with restriction sites *Hind*III and *Bam*HI using the primers CP210 and CP211 (Table 1) and then subcloned into the vector pSAT6-RFP-C1 [22] at *Hind*III and *Bam*HI restriction sites to generate the plasmid pSAT6-RFP-C1-*PvSUS1*. The procedure for transient expression in living onion epidermal cells using particle bombardment was done as described previously [23] with slight modifications. Briefly, 10 μg of pSAT6-RFP-C1, sub-

Table 1. Primer sequences used in cloning, PCR confirmation and qPCR switchgrass sucrose synthase gene experiments

Primer no.	Primer name	Primer sequence 5′ → 3′
CP33	PvSUS1-R	TCACTTGCTGGAGGGCTCTCCCTC
CP34	PvSUS1-F	ATGGGGGAAGCTGCCGGCGACCGC
CP43	PCR-F	TCGATGCTCACCCTGTTGTTTGG
CP44	PCR-R	CGATCATAGGCGTCTCGCATATCTC
CP101	PvSUS1-qRT-F1	AATTGTTCGCAAGTGGATCTC
CP102	PvSUS1-qRT-R1	ACGTCATCGGTGTAAGTCTC
CP104	PvSUS2-qRT-F1	TCGTGTTCCATTCAGAAATGAG
CP105	PvSUS2-qRT-R1	GCAACATCCTCAGTGTATGTC
CP181	PvSUS1-F	CCCTCCAGCAAGTGAGG
CP182	PvSUS1-R	CTCCGAATGAGGAAGGAAAC
CP210	PvSUS1-HindIII-F	AAGCTTTAATGGGGGAAGCTGCCGGCG
CP211	PvSUS1-BamHI-R	GGATCCTCACTTGCTGGAGGGCTCTCC
CP277	PvFRK-F1	ATCGTTGTTGCCTGAATGA
CP278	PvFRK-R1	GCACCGCCAAGATATCAAT
CP181	PvSUS1-F	CCCTCCAGCAAGTGAGG
CP182	PvSUS1-R	CTCCGAATGAGGAAGGAAAC
CP295	PvSUS4-qF4	TCCCAACTCTGTTTGCTTAC
CP296	PvSUS4-qR4	AACCTAATCTTGTCGGAACG
CP285	PvSUS6-qF2	AGATTTGTTCCCCATCAAGC
CP286	PvSUS6-qR2	TTTACTGAAGAGAGACCGGG
	PvAct-RT-F1	CAAGATTTGGAGATCCCGTG
	PvAct-RT-R1	AATGCTCCACGGCGAACAG

cellular localization control plasmid DNA and 10 μg pSAT6-RFP-C1-*PvSUS1* were coated on 0.6 μm gold particles, bombarded into onion epidermal cells and incubated for 48 h before observation under the epifluorescence microscope using a Texas Red filter set (Em-D560/40, BS-595DCLP, and EM-D630/60). The cells were plasmolyzed with one or two drops of 40% sucrose solution and visualized after 30 min.

2.4 Switchgrass transformation and plant growth

Switchgrass callus generated from immature inflorescence was transformed with the pANIC-10A binary vector harboring the *PvSUS1* overexpression cassette using the *Agrobacterium*-mediated transformation method described previously [24]. The switchgrass ST1 clone, which was derived from the cultivar "Alamo" genotype [25], was transformed and transgenic tissue was selected on MS medium [26] supplemented with 60 mg/L hygromycin-B (EMD Millipore, Billerica, MA, USA). The transgenic hygromycin resistant callus was regenerated on MS medium supplemented with 0.9 μM kinetin. Regenerated shoots were transferred to a hormone-free MS medium for root development. The regenerated plants were PCR-screened using the primers CP43 and CP44 (Table 1) to assay for the presence of the insert. The plants were transferred to 12 L pots in the greenhouse after acclimatization. For plant biomass experiments, the primary transformants were clonally propagated and the plants were grown from a single tiller in triplicate at the same growth

stage. The plants were grown for 4 months in the greenhouse before all above ground biomass was harvested. Plant height and tiller number were taken before harvesting. Plant biomass was dried in the oven for 3 days at 40°C and weighed. Prior to lignin determination and sugar release assays, the dry biomass was milled using a Wiley mill and passing through a 20-mesh sieve.

2.5 Quantification of transgene expression levels by qPCR

RNA was extracted from leaves from *PvSUS1* overexpressors and control plants at E4 developmental stage [21, 27] using TRIREAGENT (Molecular Research Center Inc.) according to manufacturer's instructions. Following extraction, 5 μg of total RNA was treated with 1 unit of DNase I (Promega Corporation) in a 10 μL reaction volume. The reaction was incubated at 37°C for 30 min and then incubated at 65°C with 1 μL stop solution for 1 min to inactivate the DNase.

For expression analysis of *PvSUS* genes, RNA was extracted from leaf blade, leaf sheath, internode, node, and panicle of R0 stage [21] switchgrass plants. One microgram of RNA was used for synthesizing cDNA after DNase treatment. cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Quantitative PCR was performed on the ABI HT7900 instrument (Applied Biosystems) with the Power SYBR I kit for PCR (Applied Biosystems) according to manufacturer's instructions.

Each reaction was performed with 1 μL of 1:100 dilutions of the first cDNA strands in a total volume of 10 μL. The reactions were incubated at 95°C for 10 min to activate the hot start recombinant polymerase, followed by 40 cycles of 10 s at 95°C, 30 s at 56°C, and 30 s at 72°C. The specificity of the PCR amplification was checked with a heat dissociation protocol (from 60 to 95°C) following the final cycle of the PCR. The results obtained for the different samples analyzed were standardized to the constitutive *PvActC* gene expression level [28]. The primers used for each gene is listed in Table 1. Data were analyzed using a one-way analysis of variance (ANOVA), and letter groupings were obtained using Fisher's least significant difference method.

2.6 Lignin and sugar release assays

Total lignin content was determined by high throughput pyrolysis molecular beam mass spectrometry. Briefly, ~4 μg of dry biomass was loaded in 80 μL stainless steel cups and pyrolyzed at 500°C in a quartz reactor using a Frontier py2020 autosampler. The resulting pyrolysis vapors were analyzed using a custom Extrel single quadrapole molecular beam mass spectrometer [29].

Sugar release efficiency was determined by high throughput screening as described previously [30]. Briefly, dry, milled biomass (–20 mesh) was dispensed in triplicate into custom 96-well Hastelloy reactor plates. Biomass-only controls, sugar standards, enzyme-only, and blank wells were included in each plate. The total capacity of each well in the plate was 417 μL. Subsequently, 300 μL water was added, and the plates were sealed with polytetrafluoroethylene (PTFE) film tape containing a silicone-based adhesive (3M Corporation, St. Paul, MN, USA), stacked and pretreated in a steam chamber to 180°C for 40 min. The plates were rapidly cooled with water, centrifuged, and the tape was removed in order to add 40 μL enzyme in citrate buffer (1.0 M, pH 5.0) to each well. An enzyme cocktail consisting of Novozymes Cellic CTec2 cellulase (Novozymes North America, Franklinton, NC, USA) and Novo188 β-glucosidase (Novozymes) was loaded at 70 mg protein/g initial biomass, and 2.5 mg protein/g initial biomass, respectively. The plates were resealed and incubated for 3 days at 54°C. The plates were analyzed for glucose and xylose release using enzyme-linked sugar assay kits (Megazyme International).

2.7 Histology

In order to qualitatively assay for cellulose, 7 μm sections of stem tissues from the 2nd internode of R1 stage plants were fixed and embedded with JB4® embedding kit (Polysciences, Inc. Warrington, PA, USA) The tissues were stained with one drop of calcofluor-white (comprising 1 g/L calcofluor-white M2R and 0.5 g/L Evans blue; Sig-

ma–Aldrich, St. Louis, MO, USA), and one drop of 10% potassium hydroxide was applied on top of the slides and a cover slip was applied. The slide was then left to stand for 1 min before being examined by fluorescence microscopy using blue light excitation and 4',6-diamidino-2-phenylindole (DAPI) filters (Ex-D360/40, BS-400DCLP, and Em-D460). All samples were treated identically.

3 Results

3.1 Phylogenetic analysis of SUS proteins

Switchgrass SUS homologous deduced amino acid sequences were found using rice and maize SUS genes [9, 31] in the Phytozome switchgrass cDNA database. Four switchgrass SUS sequences *PvSUS1* (Pavirv00026879m), *PvSUS2* (Pavirv00046631m), *PvSUS4* (Pavirv00031455m), and *PvSUS6* (Pavirv00054750m) were identified. The identified model LG + I + G for the tree, with a gamma distribution of 0.959 and proportion of invariable sites of 0.137, was selected using Akaike information criterion. SUS sequences from monocots and dicots fell into distinct groups as described by Barrero-Sicilia et al. and Komatsu et al. [11, 32]. Cluster and sequence analyses indicated the presence of four different SUS genes in switchgrass (Fig. 1 and Supporting information, Fig. S1). Monocot group 1 was comprised of *PvSUS1* and *PvSUS2* along with orthologs from maize *ZmSUS1* and *ZmSUS*-SH1, rice *OsSUS1*, *OsSUS2*, and *OsSUS3* and barley *HvSUS1* and *HvSUS2*. SUS group 2 contained *PvSUS4* along with *ZmSUS4* from maize, *OsSUS4* from rice, and *HvSUS3* from barley. SUS group 3 was comprised of *PvSUS6* and orthologous sequences from rice *OsSUS5*, *OsSUS6*, and *OsSUS7* and from barley *HvSUS4*. Each of the SUS groups contained at least one member from switchgrass. Switchgrass sequences were deposited into Genbank – PvSUS1 (KF729952), PvSUS2 (KF729953), PvSUS4 (KF729954), and PvSUS6 (KF729955).

3.2 Characterization of switchgrass sucrose synthases

PvSUS1 clustered with *ZmSUS1*, *OsSUS1*, and *OsSUS3* (98.4, 95.6, and 90.5% identity, respectively). *PvSUS2* appeared to be closely related to *ZmSUS*-SH1 and *OsSUS2* (87.5 and 84.7%, respectively). A high percentage of amino acid identity was found between *PvSUS4*, *ZmSUS2*, and *OsSUS4* (94.6 and 93.7%, respectively) while identity percentage of *PvSUS6* with *OsSUS7*, *OsSUS5*, and *OsSUS6* was 89.3, 89.1, and 80.8%. The identity of *PvSUS1* with *PvSUS2, PvSUS4*, and *PvSUS6* was 71.9, 70.5, and 53.8%, respectively (Supporting information, Table S1). The molecular mass of *PvSUS1*, *PvSUS2*, and *PvSUS4* were deduced to be approximately 92 kDa whereas *PvSUS6* had a higher molecular mass of
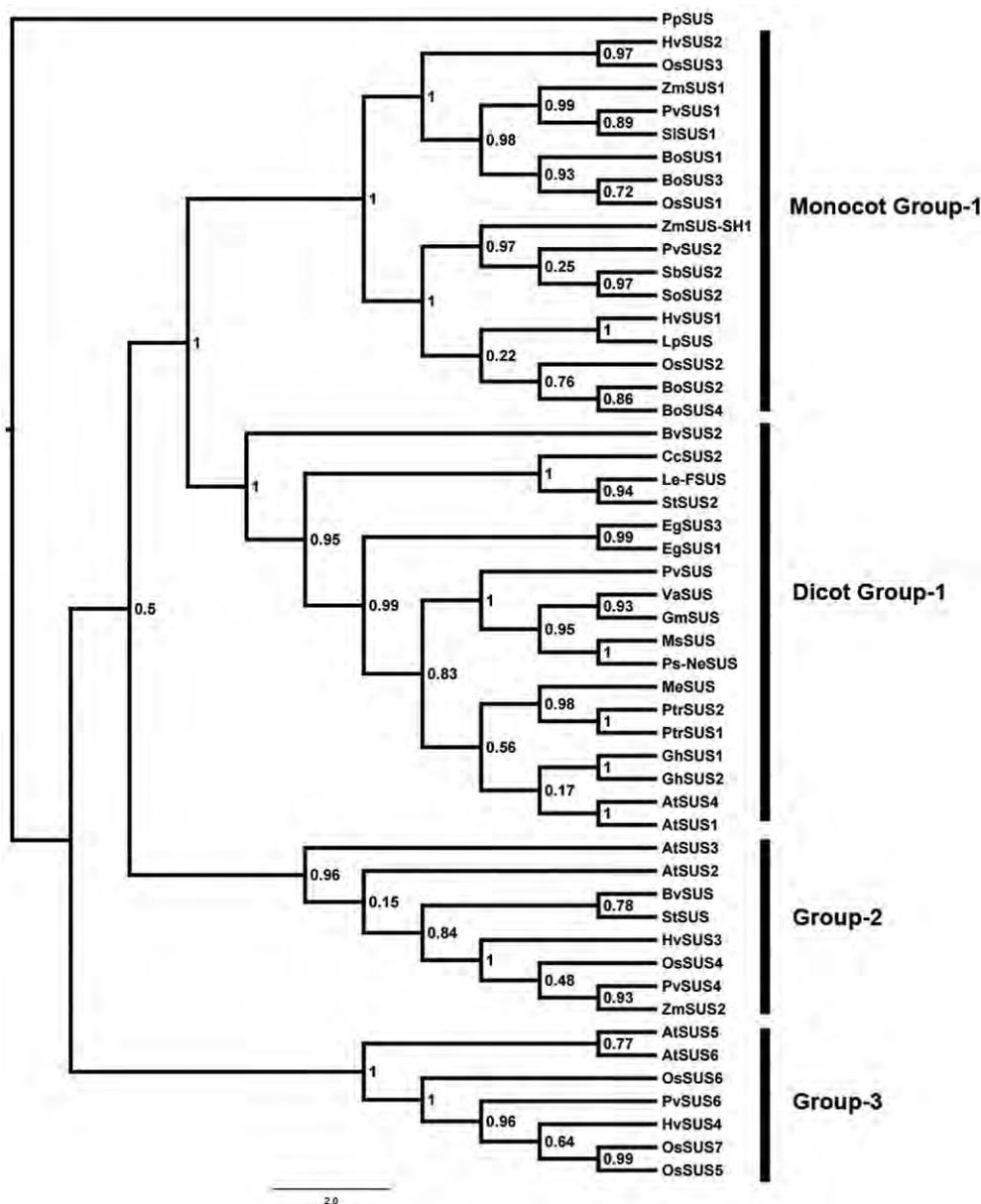
Figure 1. Maximum likelihood tree of switchgrass sucrose synthase deduced amino acid sequences and other known sucrose synthase amino acid sequences. Protein accession numbers of SUS genes from *Eucalyptus*, *Manihot,* poplar, sugarcane, potato, beet, cotton, pea, *Arabidopsis*, *Phaseolus*, *Vigna*, *Lolium*, bamboo, maize, rice, barley, tomato, and *Physcomitrella* are EgSUS1 (ABB53601), EgSUS3 (ABB53602), PtrSUS1 (AAR03498), PtrSUS2 (ABW76422), SoSUS2 (AAM68126), StSUS (AAO67719), StSUS2 (AAO34668), BvSUS (AAK65960), GhSUS1 (ACV72640), GaSUS3 (AAD28641), Ps-NeSUS (AAC28107), MsSUS (AAC17867), BvSUS2 (AAR19769), AtSUS1-6 (NP_197583, NP_199730, NP_192137, NP_566865, NP_198534, NP_177480), PvSUS (ACP17902), VaSUS (BAH56282), SbSUS2 (ACM69042), CcSUS2 (ABI17891), MeSUS (ABD96570), LpSUS (BAE79815), BoSUS1-4 (AAV64256, AAL50571, AAL50570, AAL50572), ZmSUS1 (NP_001105323), ZmSUS2 (NP_001105194), ZmSUS-SH1 (NP_001266691), OsSUS1-7 (P31924, P30298, Q43009, Q10LP5, H6TFZ4, Q6K973, Q7XNX6), HvSUS1-4 (P31922, P31923, CAZ65725, AEH16642), Le-FSUS (AAA34196), PpSUS (XP_001781447). PvSUS1 and PvSUS2 fall in to SUS Group 1; PvSUS4 in Group 2, and PvSUS6 in Group 3. Bootstrapping values are indicated as node values.

97 kDa. Amino acid sequence analysis revealed all four switchgrass contain the GT-B glycosyltransferase domain (Table 2).

The predicted intron/exon structures of the SUS genes were analyzed between the 5′-untranslated region (UTR) and 3′-UTR including the non-coding exon. Comparison between the genomic sequence and the cDNA sequence revealed that all the genes had 14 or 15 coding exons (Fig. 2). Nearly, all the introns had similar spacing, but of various sizes. *PvSUS1* and *PvSUS2* have large introns in the 5′-non-coding region and *PvSUS1* has a 1.34 kb intron between 4th and 5th exons. Non-coding exons were not

Table 2. Nucleotide and protein features of the identified switchgrass sucrose synthase genes and corresponding proteins

Name	Number of exons	Length of genomic sequence (bp)	Length of coding sequence (bp)	Length of protein sequence (aa)	N-Terminal regulating domain	GT-4 domain (aa)	C-Terminal extension	Molecular mass (kDa)
PvSUS1	14	7405	2451	816	1–282	283–767	768–816	92.95
PvSUS2	15	7691	2409	802	1–274	275–748	748–802	91.76
PvSUS4	14	5829	2436	811	1–277	278–763	763–811	92.63
PvSUS6	14	4185	2556	851	1–282	283–768	768–851	97.02

found in *PvSUS4* and *PvSUS6*. In addition, in *PvSUS6* the 3′-non-coding region is split by an intron.

Bioinformatic prediction using the TargetP program [33] predicted a putative mitochondrial targeting peptide (mTP) of 13 and 9 amino acid residues for PvSUS1 and PvSUS2, respectively. The mTP signal was not predicted for PvSUS4 and PvSUS6 by the program. However, the program Predotar [34] predicted a putative mTP for PvSUS2 only. The N-terminal "regulatory" or non-catalytic domain contained the serine phosphorylation sites at S-15 and S-170 [35] (Supporting information, Fig. S1).

3.3 Expression analysis of *PvSUS* genes

In the R0 stage, transcriptional abundance was detected in all tissues assayed for the four *PvSUS* genes relative to that of switchgrass *PvActinC* (ActC). Among these genes,

PvSUS1 was expressed ubiquitously with higher expression levels in internodes, nodes, and roots. Transcript level of *PvSUS2* was high in internodes (Fig. 3). The expression of *PvSUS4* is relatively high in roots compared to other tissues tested; expression is lower level in all tissues relative to the other *PvSUS* genes. *PvSUS6* was especially highly expressed in roots (Fig. 3).

3.4 Sub-cellular localization of PvSUS1

We performed subcellular co-localization assay in onion epidermal cells to determine subcellular distribution of *PvSUS1* and its association with the plasma membrane. The open reading frame of *PvSUS1*, translationally fused to the DsRed fluorescent protein (RFP), was cloned to be under the control of CaMv35S promoter (pSAT6-RFP-C1-*PvSUS1*), and transiently expressed in onion epidermal
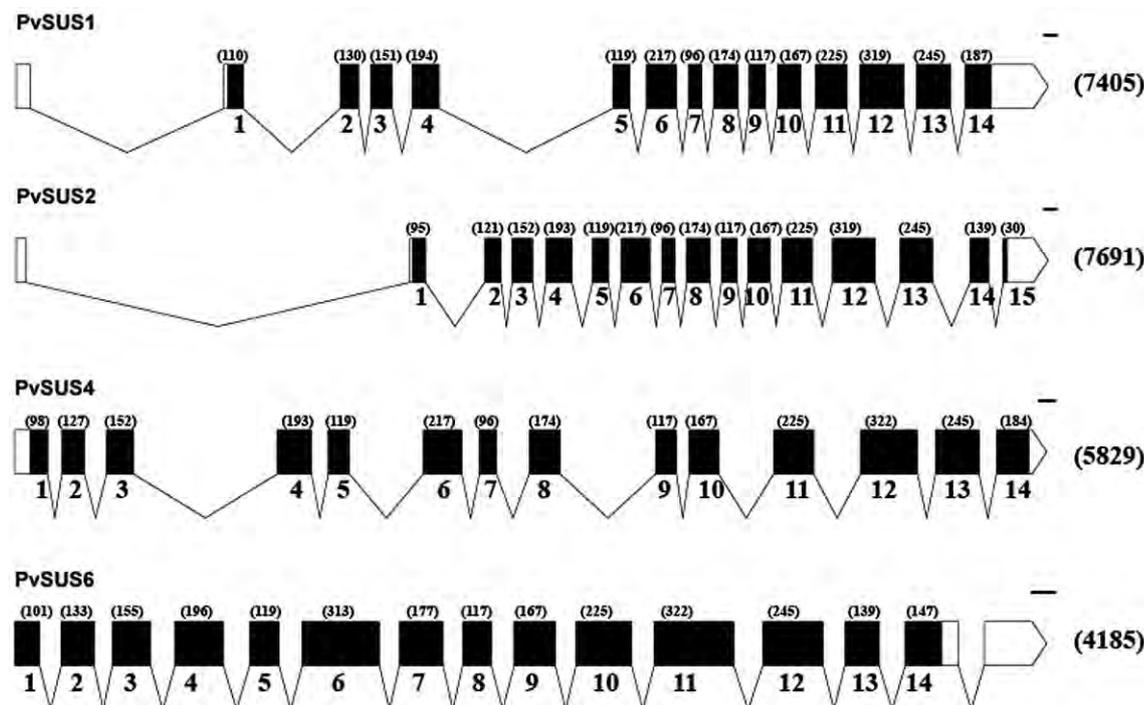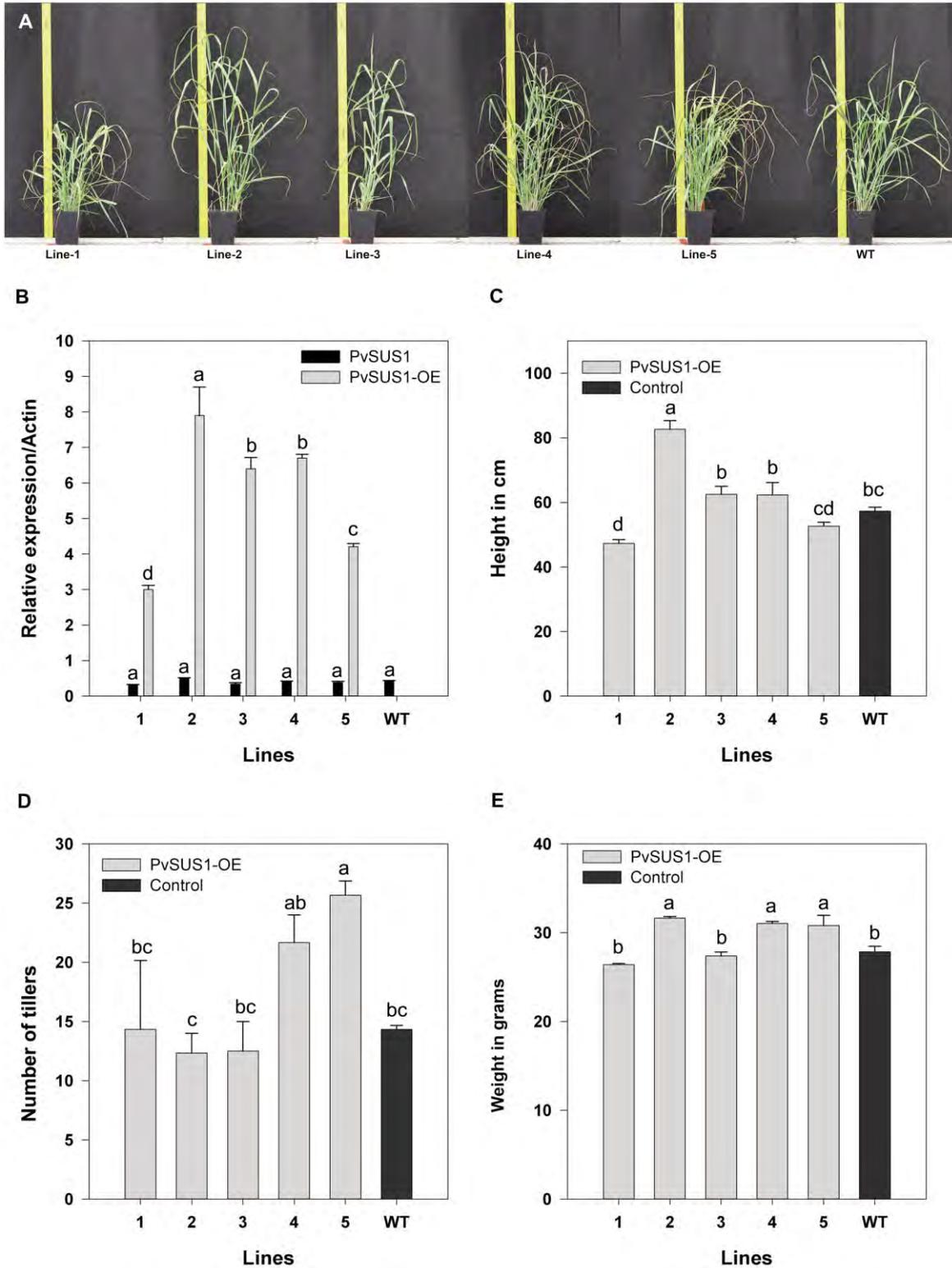

Figure 2. Intron–exon structure of switchgrass sucrose synthase genes. Solid black boxes represent coding exons, whereas hollow boxes represent non-coding exons. Introns are indicated by lines. Coding exons are numbered below the exons and the sizes of the coding exons are numbered in parenthesis above the exons. The rightmost numbers represent the number of bases comprising each genomic sequence. Scale bar represents 100 bp.
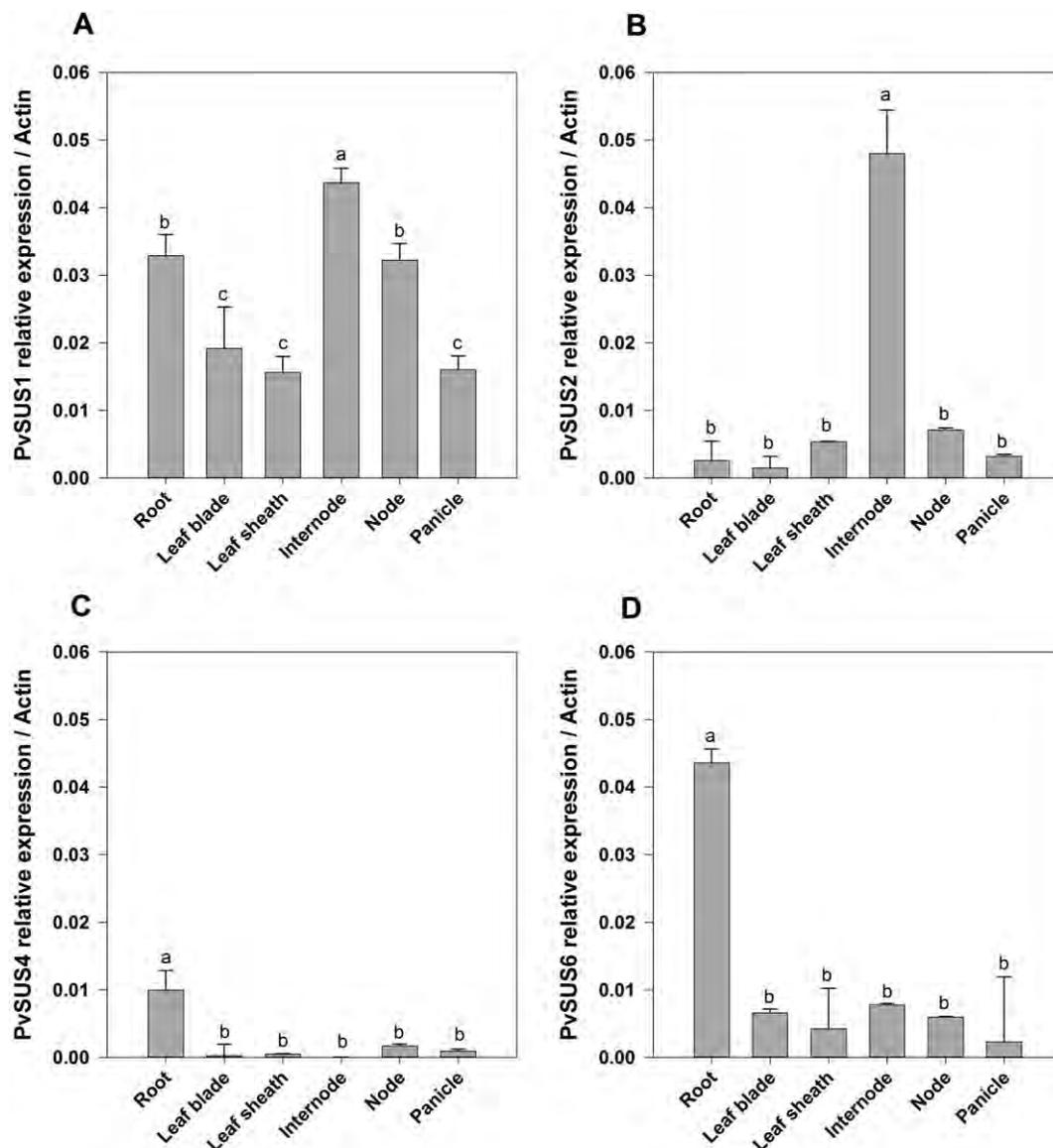
Figure 3. Fold changes in the relative expression profiles of four *PvSUS* genes in various switchgrass tissues. Relative expression level of (**A**) *PvSUS1* ($p<0.0015$), (**B**) *PvSUS2* ($p<0.0001$), (**C**) *PvSUS4* ($p = 0.0023$), and (**D**) *PvSUS6* ($p<0.0001$). Relative transcript levels were measured by qPCR and standardized to the constitutive *PvACTC* gene expression level. The mRNA was extracted from roots, leaf blades, leaf sheaths, internodes, nodes, and panicles at the R0 growth stage. All data shown are means + SE ($n = 3$). Means were analyzed with one-way ANOVA, and letter groupings were obtained using Fisher's least significant difference method. Bars with the same letter do not differ significantly at the 5% level.

cells. The plasmid pSAT6-RFP-C1 containing 35S-*RFP* was used as control for subcellular localization studies. RFP fluorescence was seen throughout the cytoplasm in cells with control plasmid (Supporting information, Fig. S2) while in RFP-tagged *PvSUS1* fluorescence was observed predominantly along the plasma membrane with some expression in the cytoplasm (Supporting information, Fig. S2). When epidermal cells were plasmolyzed with 40% sucrose solution, fluorescence was observed throughout the cytoplasm in cells with control plasmid (Supporting information, Fig. S2) and fluorescence was

seen predominantly along the plasma membrane in cells with RFP tagged *PvSUS1* (Supporting information, Fig. S2) along with low levels of fluorescence in the cytoplasm. While the fusion of the RFP sequence at the N-terminus rather than the C-terminus could have affected subcellular localization, there was no evidence of off-targeting. Nonetheless, the possibility of non-native targeting should be considered when interpreting these results.

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Figure 4. Phenotype, relative expression, height, tiller, and dry biomass weight of *PvSUS1* transgenic plants. (**A**) Regenerated plants in the greenhouse. (**B**) Relative expression levels of *PvSUS1* transcripts in transgenic switchgrass lines. (**C**) Plant height. Line 2 were significantly taller compared to controls (*p*<0.0001). (**D**) Tiller number. Lines 4 and 5 had more tillers compared to controls (*p* = 0.0379). (**E**) Dry biomass weight in *PvSUS1* transgenic plants. Lines 2, 4, and 5 had significantly greater biomass compared to controls (*p* = 0.0007). Data shown are means + SE (*n* = 3). Different letters on the bars of figures indicate significant differences at the *p* ≤ 0.05 level as tested by least significant difference (LSD) grouping using SAS software (SAS Institute, Inc.).

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3.5 *PvSUS1* overexpression in transgenic switchgrass in the ST1 clonal genotype

Five independent transgenic lines were regenerated from hygromycin-resistant calli generated in different plates and at different times, confirming their independence. Genomic PCR confirmed that all plants were stably transgenic and with no T-DNA backbone present in the plants (data not shown). Quantitative polymerase chain reaction (qPCR) demonstrated that the *PvSUS1US* was overexpressed between a two- and eightfold increase in the transgenic plants (Fig. 4). The level of expression of the endogenous *PvSUS1* was not affected and was similar to expression levels in non-transgenic control (Fig. 4).

Greenhouse-grown plant height of vegetatively reproduced T0 plants was significantly increased in line 2 (37%) compared to the control, whereas height was significantly decreased in lines 1 and 5 (21 and 12%, respectively) (Fig. 4). There was no significant difference in the other two lines compared to control plants. Line 5 had a significant increase in number of tillers (79%) compared to controls, whereas other lines were not significantly different from controls (Fig. 4). Differences on dry weight basis were significant in lines 2, 4, and 5 compared to control
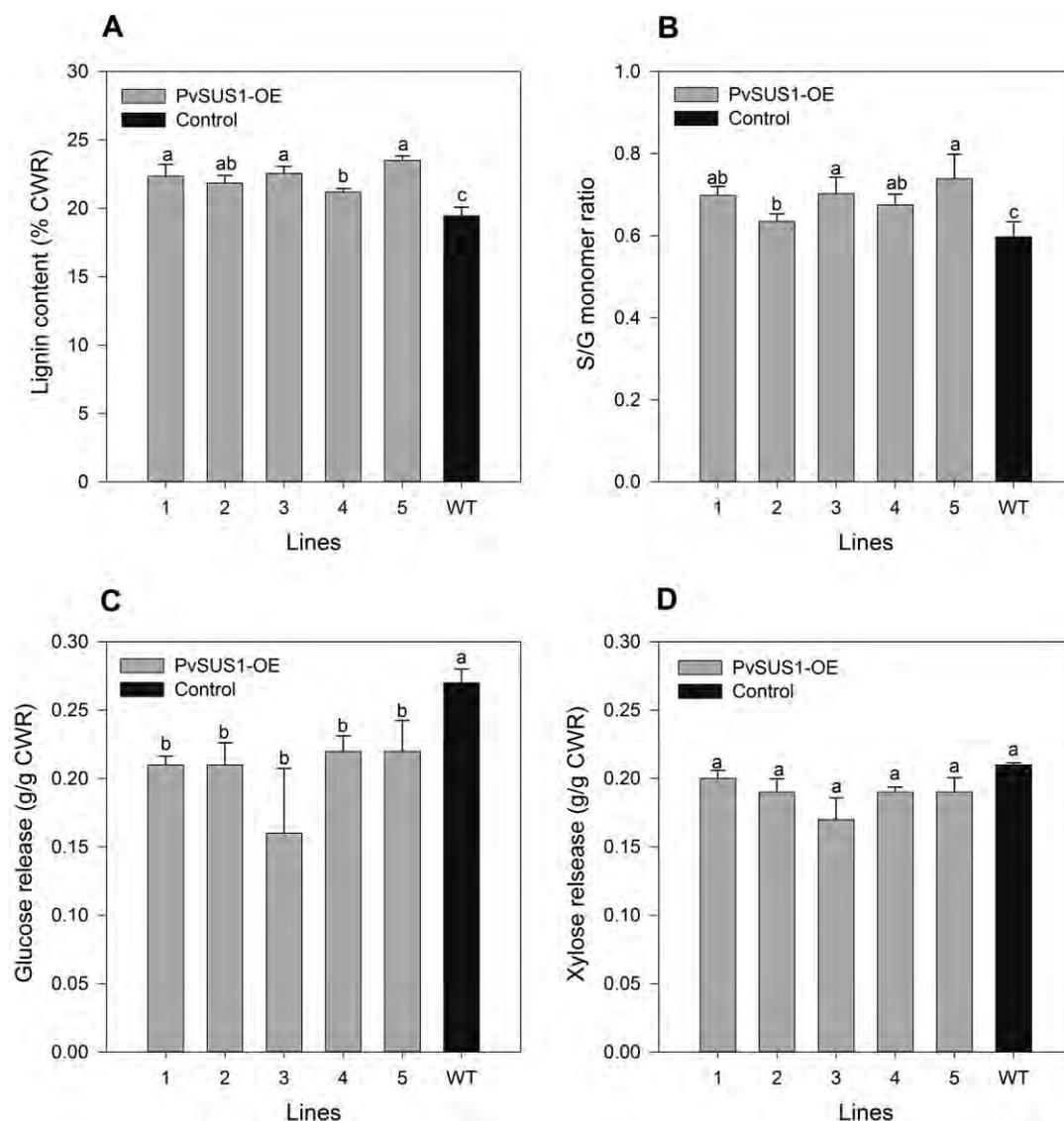
Figure 5. Lignin content, S/G ratio, and sugar release efficiency of *PvSUS1* overexpressing transgenic plants. Lignin content (%) and S/G ratio was determined by MBMS. (**A**) Lignin content, and (**B**) S/G ratio were statistically significantly different at the $p = 0.05$ level in all transgenic events relative to the control. (**C**) Glucose release efficiency was significantly different in all transgenic events relative to the control ($p = 0.031$). (**D**) Xylose release efficiency was not significantly different ($p = 0.689$). All data are means + SE ($n = 3$). Means were analyzed with one-way ANOVA, and letter groupings were obtained using Fisher's least significant difference method. Bars with the same letter do not differ significantly at the 5% level.

(Fig. 4). Percentage of increase in dry weight in lines 2, 4, and 5 was 13.6, 11.5, and 10.6%, respectively.

Various cell wall analyses provided some insight into how overexpression of *PvSUS1* could be used to alter cell walls for potentially improved biofuels. Lignin content increased in switchgrass plants overexpressing *PvSUS1* compared to control plants (Fig. 5). The S (syringyl lignin):G (guaiacyl lignin) monomer lignin ratio was also increased in the transgenic plants compared to controls (Fig. 5). All transgenic plants had lower glucose release (Supporting information, Fig. S2C) efficiency compared to control plants. Xylose release efficiency was not significantly different from control plants (Fig. 5).

We quantified the transcript level of fructokinase in the transgenic plants to determine if fructose produced by the breakdown of sucrose was recycled into the hexose-phosphate pool. All lines except line 4 had decreased levels of fructokinase (Supporting information, Fig. S3). Calcofluor-white staining showed an apparent increased fluorescence in *PvSUS1* plants indicating that there was an increase in deposition of cellulose in cell walls. Additionally, the cell walls appeared to be thicker in transgenic relative to controls (Supporting information, Fig. S4).

4 Discussion

In this study, we identified switchgrass SUS genes *PvSUS1*, *PvSUS2*, *PvSUS4*, and *PvSUS6* that have homologues with other species. For example, barley has four SUS genes with *HvSUS1* and *HvSUS2* in group 1, *HvSUS3* in group 2, and *HvSUS4* in group 3 [11] corresponding to SUS1 (Group 1), SUSA (Group 2), and NG (Group 3) groups [15, 32]. Although rice and *Brachypodium* have at least six SUS isoforms, only four SUS isoforms were identified in switchgrass during our search in the Phytozome switchgrass database. Given that lowland switchgrass is an allotetraploid, there are likely additional SUS genes in the species that were not discovered in our study.

Expression analysis revealed that *PvSUS1* is ubiquitously expressed in all the tissues. *PvSUS1* falls in the same clade as *OsSUS1*, *OsSUS3*, *ZmSUS1*, and *HvSUS2*. *PvSUS1* expression was similar to that of the homologous gene *OsSUS1* in rice [31], in which there was high expression in internodes. *PvSUS2*, which was similar to *OsSUS2*, *HvSUS2*, and *ZmSUS-SH1* was highly expressed in internodes. While *HvSUS2* and *ZmSUS-SH1* are highly expressed in embryos, the expression patterns of *PvSUS2* was more similar to that of *OsSUS2* [31]. *PvSUS4* transcript levels were very low in all tissues except roots and were not similar to other homologous genes in rice (*OsSUS4*) and barley (*HvSUS3*). *HvSUS3* was mainly expressed in dry kernels and was induced by dehydration in leaves [11]. Expression of *PvSUS6* was similar to *HvSUS4*, *OsSUS5*, and *OsSUS6* in leaf blades and internodes, but *PvSUS6* expression was highest in roots com-

pared to other tissues, in contrast to the expression patterns of its homologs.

Serine phosphorylation sites were predicted at S-15 and S-170 for PvSUS1, S-10 and S-162 for PvSUS2, S-13 and S-165 for PvSUS4, and S-12 and S-169 for PvSUS6. Phosphorylation at S-15 increases membrane association and SUS catalytic activity at acidic pH [36]. However, the phosphorylation of Ser-170 in maize SUS1 promoted SUS activity by increasing SUS ubiquitinylation [37, 38]. The N-terminal regulatory region contains the cellular targeting regions, which enable SUS to associate with the membrane [39]. Membrane-associated forms of SUS have been implicated in the synthesis of both callose and cellulose [7] and these functions have been identified in maize and barley [6, 11, 40]. *PvSUS1* is 98% identical to *ZmSUS1* and contains the sequence that has been shown in maize to be involved in membrane association [9]; therefore *PvSUS1* was chosen for subcellular localization and overexpression studies in switchgrass. In our plasmolysis experiments after 40% sucrose treatment, the RFP fluorescent signal appeared to be co-localized with the retracted plasma membrane indicating possible association with the plasma membrane. N-terminal fusion appeared to have not influenced the subcellular localization, which was congruent to similar experiments of SUS subcellular localization *Arabidopsis* (Chen Masters Thesis, http://hdl.handle.net/2429/43856). SUS has been identified to be a component of the cellulose synthase biosynthesis machinery in Asuki bean [10] and is expressed along with cellulose synthase during secondary cell wall formation in poplar [13]. Elevation of cellulose levels in plants overexpressing *PvSUS1* along with its association with the plasma membrane supports its purported role in cellulose production and carbon partitioning.

Several switchgrass plants that overexpressed *PvSUS1* had increased plant biomass. SUS overexpression has been associated with increased plant height and biomass in other species. In cotton when SUS was overexpressed, plants had increased plant biomass, fiber quality, and fiber yield [15]. In tobacco and poplar, overexpression of SUS was associated with increased plant height and soluble sugar content [14, 41]. The role of SUS in the control of sink strength is consistent with the increase of plant height and biomass when overexpressed and is corroborated by the reduced plant size in carrots by the suppression of SUS [16]. While we still do not know the exact mechanism controlling plant height, tillering, and biomass in switchgrass or any other species, the variable results among our switchgrass lines could be due to transgene position effects and/or expression.

Lignin content was increased in the transgenic switchgrass plants in our study. There was no significant increase in lignin content in SUS-overexpressing tobacco and poplar, even though there was an increase in biomass in these species [13, 42]. In other transgenic tobacco,

there has been a positive association between lignin content and biomass [43]. Increased lignin content could be from the cross-talk between the lignin biosynthesis pathway and cellulose biosynthesis pathway. Decrease in glucose- and xylose-release efficiency seem to be associated with increase in lignin content because of the occlusion effect of lignin on sugar release efficiency; lignin is known to shield cellulose from enzymatic hydrolysis of cellulose [44].

In the presence of UDP, SUS breaks down sucrose to UDP-glucose and fructose. UDP-glucose is a substrate for cellulose synthase to produce cellulose and callose for cell wall synthesis [7]. While UDP-glucose is available immediately for cellulose synthesis, fructose is recycled into the hexose-phosphate pool [41]. The first step in this pathway is the phosphorylation of fructose to fructose-6-phosphate, catalyzed by the enzyme fructokinase. Accumulation of fructose causes inhibition of SUS activity by feedback inhibition [45]. Transcript levels of fructokinase in *PvSUS1*-overexpression transgenic switchgrass were decreased in all lines except in line 4 (Supporting information, Fig. S3) and was not correlated with the increase in biomass. Anti-sense suppression of SUS did not change levels of fructokinase and glucokinase in carrots [16]. Therefore, it is possible that there is an additional mechanism present for fructose recycling. Thus, the mechanisms behind the altered SUS-related phenotypes are still largely unknown. While the SUS-transgenic switchgrass might not be immediately useful as improved bioenergy feedstocks, they might be used as a crossing partner with other transgenic switchgrass lines. One trait that has been altered in transgenic switchgrass is lignin. Thus, one especially attractive partner to stack with SUS1 is a transgenic switchgrass line with relatively low overexpression of the switchgrass MYB4 transcription factor, in which total lignin is decreased [25]. Another crossing partner for SUS would be switchgrass in which the COMT gene is silenced via RNAi, which resulted in plants with higher biomass and altered lignin under field conditions [46]. In both cases, the increased saccharification of MYB4 and COMT transgenics might complement the increase in lignin and higher biomass of SUS transgenics in transgene stacks. Therefore, we have begun to make such crosses for subsequent lab and field analysis in search of switchgrass plants that have increased saccharification and biomass compared with their non-transgenic counterparts.

5 Concluding remarks

In conclusion, we have identified four isoforms of SUS in switchgrass. Expression analyses indicate that the four isoforms may have specific roles in different tissues. PvSUS1 is probably associated with the plasma membrane and is the highest expressor of the SUS genes we analyzed. Overexpression of *PvSUS1* increased biomass in transgenic switchgrass, to the degree of increased biomass reported in other species. These results have significant implications in improving biofuel yield in bioenergy crops, especially in transgene stacks for increasing saccharification and biomass.

6 References

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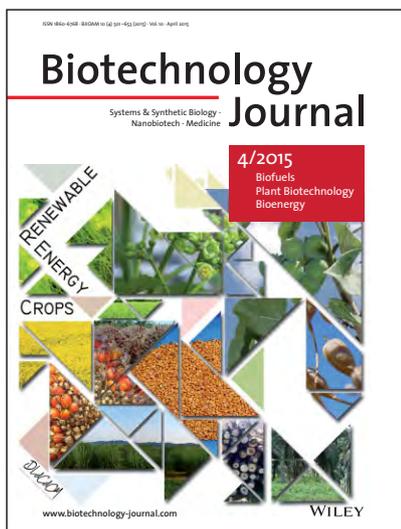
Cover illustration

Special issue: Renewable Energy Crops, edited by Margit Laimer, Fatemeh Maghuly, Johann Vollmann and Nicolas Carels. Given the growing importance of environmental advantages of biofuel plants reducing greenhouse gas emissions as compared to fossil fuel consumption, second and third generation biofuels today are produced from both annual and perennial non-food biofuel crops, e.g. switchgrass, poplar and *Miscanthus*, or from new and non-food oil crops, like *Jatropha*, *Camelina* and oil palm. To achieve an economic production of biofuels for our future need of sustainable energy, genetic improvement of the plant material must be obtained by a range of different biotechnologies.

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