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Improving biomass production and saccharification in *Brachypodium distachyon* through overexpression of a sucrose-phosphate synthase from sugarcane

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Running title
Sucrose-phosphate overexpression in Brachypodium

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Abstract

The substitution of fossil by renewable energy sources is a major strategy in reducing CO_2 emission and mitigating climate change. In the transport sector, which is still mainly dependent on liquid fuels, the production of second generation ethanol from lignocellulosic feedstock is a promising strategy to substitute fossil fuels. The main prerequisites on designated crops for increased biomass production are high biomass yield and optimized saccharification for subsequent use in fermentation processes.

We tried to address these traits by the overexpression of a sucrose-phosphate synthase gene (*SoSPS*) from sugarcane (*Saccharum officinarum*) in the model grass *Brachypodium distachyon*. The resulting transgenic *B. distachyon* lines not only revealed increased plant height at early growth stages but also higher biomass yield from fully senesced plants, which was increased up to 52 % compared to wild-type. Additionally, we determined higher sucrose content in senesced leaf biomass from the transgenic lines, which correlated with improved biomass saccharification after conventional thermo-chemical pretreatment and enzymatic hydrolysis. Combining increased biomass production and saccharification efficiency in the generated *B. distachyon SoSPS* overexpression lines, we obtained a maximum of 74 % increase in glucose release per plant compared to wild-type. Therefore, we consider *SoSPS* overexpression as a promising approach in molecular breeding of energy crops for optimizing yields of biomass and its utilization in second generation biofuel production.

Abbreviations

SoSPS

Sucrose-phosphate synthase from sugarcane (*Saccharum officinarum*)

T-DNA

Transfer DNA

Ubi

Ubiquitin promoter

HEPES

4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid

Introduction

The combination of an increasing, global energy demand and decreasing fossil energy resources that can be conventionally explored not only results in higher atmospheric CO_2 levels with their observed and expected impact on climate change (Ash et al., 2013), but also fosters explorations for fossil energy sources in sensitive ecosystems (Finer et al., 2008; Jernelov, 2010; Sojinu et al., 2010). To face these challenges and to mitigate climate change, a key strategy is the substitution of fossil by renewable energy sources. Because liquid fossil fuels have a predominant role in the transport sector, second generation biofuels from lignocellulosic feedstock reveal a high potential in substituting fossil fuels (Sims et al., 2010). The production of the biofuel ethanol, which is a key player in the biofuel market (Vimmerstedt et al., 2012), from lignocellulosic biomass is mainly restricted by the high recalcitrance of the plant cell wall towards degradation, which is determined by cellulose crystallinity but also lignin and hemicellulose content (Chen and Dixon, 2007; Hall et al., 2010; Yoshida et al., 2008). Different approaches have been launched to improve efficiency of lignocellulosic ethanol production ranging from cell wall modifications in feedstock via molecular breeding (Fu et al., 2011; Pauly and Keegstra, 2010; Wilkerson et al., 2014), physical and chemical pretreatment to break and degrade the tight polymer network of the cell wall (Blanch et al., 2011; Socha et al., 2014; Xu and Huang, 2014) and identification of advanced enzymes for cell wall hydrolysis (Arfi et al., 2014; Inoue et al., 2014; Zhang et al., 2012), to engineering of improved microorganisms for fermentation of cell wall-derived saccharides (Chung et al., 2014; Hasunuma et al., 2013).

Besides saccharification and efficient fermentation, the availability of lignocellulosic feedstock is a decisive factor to meet the projected increased demand for renewable energy in general and lignocellulosic ethanol in special. Because the currently available biomass waste form agriculture or forestry industries might not be sufficient, an extended cultivation of bioenergy crops has been considered as a favourable solution (Somerville et al., 2010). To decrease the competition of arable land of crops, which are cultivated for food and feed production versus energetic utilization, a major prerequisite on energy crops is a high biomass yield.

In our study, we tried to address these two demands on lignocellulosic feedstock for biofuel production, improved saccharification and biomass yield, by following a strategy of overexpressing a sucrose-phosphate synthase (SPS) gene in the model grass *Brachypodium distachyon* for metabolic engineering. SPS (EC 2.4.1.14) catalyses UDP-glucose and fructose-6-phospahte conversion into sucrose-6-phosphate, which can then be hydrolysed to sucrose by

a sucrose-phosphate phosphatase (Huber and Huber, 1996). Especially in grasses, SPS has a major function in regulating sucrose biosynthesis (Castleden et al., 2004). Because sucrose is the main photosynthetic product and, therefore, a decisive factor for plant growth, productivity has been correlated with SPS activity. For maize (*Zea mays*) and rice (*Oryza sativa*), genetic studies have shown a linkage between yield and plant growth QTLs (quantitative trait loci) and SPS activity (Causse et al., 1995; Ishimaru et al., 2004; Prioul et al., 1999); and in sugarcane (*Saccharum officinarum*), sucrose accumulation in stems correlated with SPS activity (Grof et al., 2007; Zhu et al., 1997). Therefore, *SPS* overexpression has been used to modify plant development. Heterologous overexpression of an *SPS* gene from maize in tomato (*Lycopersocum esculentum*) resulted in increased fruit yield (Laporte et al., 1997; Micallef et al., 1995) where best results were obtained with an SPS activity in transgenic plants which was approximately twice as high as in wild-type plants (Laporte et al., 2001). Similar to tomato, overexpression of a maize *SPS* gene increased tuber weight and total yield in transgenic potato (*Solanum tuberosum*) plants (Ishimaru et al., 2008) as well as increased plant height and biomass production but also delayed flowering in transgenic tobacco (*Nicotiana tabacum*) lines (Coleman et al., 2010; Park et al., 2008).

Unlike dicotyledonous plants, results from *SPS* overexpression in grasses are relatively limited, but overexpression of a maize *SPS* gene in rice, which resulted in taller plants at early growth stages (Ishimaru et al., 2004), indicated the applicability of this molecular breeding strategy for improving biomass production in grasses. Because results from *SPS* overexpression on yield and saccharification of senesced, dry biomass from grasses have not been reported, but would be very important to increase efficiency of second generation ethanol production, we focussed on these traits in the transgenic *B. distachyon* lines generated in this study. We chose the model grass *B. distachyon* to test the effect of *SPS* overexpression on biomass yield and saccharification due to efficient transformation protocols (Christiansen et al., 2005), easy cultivation and fast generation cycles (Draper et al., 2001), but also similarities in cell wall composition and biotechnological biomass application with other monocotyledonous crops (Bevan et al., 2010; Gomez et al., 2008; Meineke et al., 2014). Because of the high, SPS-dependent sucrose production in sugarcane (Grof et al., 2007; Zhu et al., 1997), we selected the sucrose-phosphate synthase B gene (*SoSPS*) from sugarcane, which showed highest homology to successfully applied *SPS* genes from maize and rice (Huang et al., 2013), for heterologous overexpression in *B. distachyon*.

Our results revealed a positive effect of *SoSPS* overexpression at early stages of *B. distachyon* growth and increased yield of senesced biomass at the final developmental stage of the plant.

In addition, the sucrose content in senesced leaf biomass was higher than in control samples from wild-type plants, which correlated to improved biomass saccharification. Therefore, we propose *SoSPS* expression as a promising strategy for combining improved biomass production and saccharification in designated grasses and monocotyledonous energy crops for lignocellulosic biofuel production.

Materials and Methods

Plant material

B. distachyon (inbred line Bd21 (Vogel et al., 2006)) was cultivated as described in Meineke et al. (2014). For determination of biomass production, plants that reached their final developmental stage due to complete, natural senescence with subsequent drying for 2 weeks without irrigation were harvested after 4 month. For quantification of sucrose content and saccharification efficiency, plant material was separated into stem biomass, including all nodes, and leaf biomass. Roots and spikelets were excluded from this analysis. Biomass was homogenized with a mill fitted with a 0.2 mm mesh screen for the determination sucrose content and saccharification efficiency.

Cloning and *B. distachyon* transformation

To generate a vector construct for the overexpression of the sugarcane (*S. officinarum*) sucrose-phosphate synthase B gene *SoSPS* (GenBank accession No. JN584485.1) under control of the ubiquitin promoter from maize (*Zea mays*), *SoSPS* was amplified from sugarcane complementary DNA (cDNA) using primers in PCR reactions that provide DNA recombination sequences (*attB* sites) at their 5' and 3' ends (SoSPS-5'attB: 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGCC GGGAACGAGTGGA; SoSPS-3'attB: 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCCATGCCGCTAGAAGTCTTGG) for subsequent utilization with the Gateway cloning technology (Life Technologies, USA). For cDNA synthesis, RNA was isolated from sugarcane leaves using peqGOLD TriFast (Peqlab, Germany) and used with the Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, USA) according to the manufacturer's instructions. After introduction into the donor vector pDONR221 (Life Technologies) via BP Clonase-mediated recombination, *SoSPS* was introduced into the monocotyledonous plant expression vector p7i586 (DNA Cloning Service, Germany), via LR Clonase-mediated recombination. This vector also provided C-terminal fusion with the fluorescence tag mCherry (Shaner et al., 2004) after successful expression and resistance to the herbicide Basta, which was conferred by the *bar* gene (Block et al., 1987) under control of the double 35S promoter from the cauliflower mosaic virus. The *SoSPS* expression vector was transformed into *Agrobacterium tumefaciens* strain GV3101. *Agrobacterium*-mediated *B. distachyon* transformation followed the protocol from Christiansen et al. (2005) and produced four independent *Ubi:SoSPS* lines. For detailed studies, only homozygous lines in the T3 generation were used.

Gene expression analysis

To confirm expression of the *SoSPS-mCherry* fusion construct in transformed *B. distachyon* lines, RNA was isolated from wild-type and four generated *Ubi:SoSPS* lines using peqGOLD TriFast (Peqlab) according to the manufacturer's instructions. For cDNA synthesis, the Maxima First Strand cDNA Synthesis Kit (Thermo Scientific) was used. The expression of *Actin* (Primer, 5'Act: 5'-GCTGGGCGTGACCTAACTGAC and 3'Act: 5'-ATGAAAGATGGCTGGAAAA GGACT; primer sequences derived from *B. distachyon Actin* sequence, GenBank accession No. XM_003578821) was used as reference for the expression of the *SoSPS-mCherry* fusion construct (5'SoSPS: 5'-CTGTGGACTGCTACCAAGAC and 3'mCherry: 5'-GCTTGACGTAGGCCTTCGAG) in PCR reactions using the Q5 High-Fidelity DNA Polymerase (New England Biolabs, USA). Identity of amplified PCR products was confirmed by DNA sequencing.

Southern blot analysis

Genomic DNA from *B. distachyon* wild-type and four generated *Ubi:SoSPS* lines was digested with the restriction enzymes *Hind*III (Thermo Scientific, USA), separated on a 1.0 % Agarose/TBE gel and blotted onto a Hybond NX membrane (GE Healthcare, UK). A DIG (dioxygenin)-labelled (Roche Diagnostics, Germany) DNA probe specific for the *bar* resistance gene of the plant expression vector (Primer for PCR amplification: 5'bar: 5'-GCACCATCGTCAACCACTACATC; 3'bar: 5'-AAACCCACGTCATGCCAGTTC) was used for overnight hybridization at 68°C. Hybridization and washing of blots were performed according to the manufacturer's instructions.

Confocal laser-scanning microscopy

Leaf samples were mounted between a microscope slide and coverslip in water. Micrographs were captured using the LSM 780 confocal laser-scanning microscope (Carl Zeiss Microimaging, Germany). For localization of SoSPS-mCherry, the fluorochrome of the fusion protein was excited at 561 nm by using a diode-pumped solid-state laser. Emission filtering was achieved using a 586- to 638-nm bandpass filter. Image processing was performed using integral functions of the ZEN 2010 (Carl Zeiss Microimaging) operating software.

Sucrose-phosphate synthase activity assay

Protein extraction and sucrose-phosphate synthase activity assay were performed according to Baxter et al. (2003) with some modifications. 0.5 g leaf tissue from four-week-old

B. distachyon wild-type and four generated *Ubi:SoSPS* lines were ground in liquid nitrogen with 1 mg of insoluble polyvinyl polypyrrolidone (all chemicals purchased at Sigma-Aldrich, Germany if not indicated otherwise). Four volumes of extraction buffer (50 mM HEPES/KOH pH 7.5, 10 mM $MgCl_2$, 1 mM EDTA, 2 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride, 1 mM benzamidine, 5 mM ε-amino-n-caproic acid, 0.1% (v/v) Triton X-100, and 10% (v/v) glycerol) were added and samples were centrifuged at 4°C and 12,000 x g for 2 min. Supernatants were transferred to spin columns (Vivaspin 6, 50.000 MWCO, GE Healthcare, USA), and samples were filtered at 4°C and 4,000 x g for 3 h. After re-dissolving the proteins in 800 µl assay buffer (50 mM HEPES-KOH pH 7.5, 20 mM KCl, and 4 mM $MgCl_2$), 200 µl aliquots, additionally containing either 12 mM UDP-glucose, 10 mM fructose-6-phosphate, and 80 mM glucose-6-phosphate for sucrose synthesis or no hexose phosphates to provide blank values, were incubated at 25°C for 20 min. To stop the reaction, samples were incuabted at 95°C for 5 min, followed by a centrifugationt 4°C and 12,000 x g for 5 min. 5 M KOH was added to supernatants (ratio 1:1 (v/v)) to destroy unreacted hexose phosphates and incubated at 95°C for 5 min. The fructosyl moiety of sucrose was quantified using the anthrone test (Lunn and Furbank, 1997). Four Volumes of 0.14 % anthrone reagent in 14.6 M H_2SO_4 were added; and absorbance was measured at 620 nm. Amounts of sucrose-6-phosphate were calculated using a standard curve with 0 – 300 nm sucrose.

Determination of sucrose content

The extraction and determination of sucrose in *B. distachyon* leaf and stem biomass was based on the description by Stitt et al. (1989). Sucrose was extracted from 7.5 mg of lyophilized plant material by a double treatment with 80 % ethanol (250 µl and 150 µl) for 30 min at 95°C. For the third extraction step, 50% ethanol was used. After centrifugation at 1500 rpm for 10 min, the supernatants of each step were collected and combined. The absorbance of a 200 µl reaction mixture containing 2.3 mM ATP, 2.3 mM NADP, 0.23 U glucose-6-phosphate dehydrogenase (Roche, Germany) and 5 µl sample extract in HEPES/KOH buffer (100 mM HEPES/KOH, 3 mM $MgCL_2$, pH 7.0) was measured in a 96 well plate until the OD was stable. 4 µl hexokinase (9000 U ml^{-1}), phosphoglucose isomerase (600 U ml^{-1}; both from Roche Diagnostics) and invertase (dissolved in HEPES/KOH buffer; Sigma, Germany) were added in a sequential manner. The absorbance was measured after addition of each enzyme until it was stable. The generated NADPH is considered equimolar to glucose; and glucose amounts were calculated by using the following equation: µmol NADPH = ΔOD/(2.85*6.22). The sucrose content was calculated based on the determined glucose amounts.

Determination of saccharification efficiency

The saccharification efficiency was determined by the release of glucose from *B. distachyon* biomass after thermo-chemical pretreatment with diluted sulphuric acid followed by enzymatic hydrolysis with the enzyme mixture Accellerase 1500 (Genencor, Netherlands). Glucose concentration in the supernatant of processed biomass was determined with a refractive index detector connected to a HPLC system. Biomass pretreatment, hydrolysis as well as glucose quantification was performed according to the description in Meineke et al. (2014).

Statistical analysis

Descriptive statistics including the mean and the standard error of the mean (SE) along with the Tukey range test for multiple comparison procedures in conjunction with an ANOVA were used to determine significant differences. $P < 0.05$ was considered significant.

Results

Genetic and biochemical characterization of transgenic *B. distachyon* lines

From *Agrobacterium*-mediated transformation of *B. distachyon* calli, we obtained four *Ubi:SoSPS* lines, which we analysed in homozygous T3 generations. Southern blot analysis confirmed integration of the T-DNA into the genome and genetic independence of the four lines (Supplemental Fig. 1A). The sugarcane sucrose-phosphate gene *SoSPS* fused to the fluorescence gene *mCherry* was expressed in all four lines (Supplemental Fig. 1B), which correlated with detection of SoSPS-mCherry in cytosolic strands of the respective *B. distachyon* lines but not the wild-type (Supplemental Fig. 1C). A cytosolic localization of SoSPS was expected from previous results and the general formation of sucrose in the cytosol of leaf cells (Huber and Huber, 1992). All transgenic *B. distachyon* lines with confirmed *SoSPS-mCherry* expression revealed a significantly higher sucrose-phosphate synthase activity in leaf tissue than the wild-type, except for line #1 where a tendency of higher sucrose-phosphate synthase activity was determined (Fig. 1).

Early plant growth

Because the overexpression of *SPS* genes could increase plant growth (Baxter et al., 2003; Ishimaru et al., 2008; Park et al., 2008), we tested for similar phenotypes in the transgenic *B. distachyon Ubi:SoSPS* lines. Two-week-old *Ubi:SoSPS* plants were about 15 % (line #1, 3 and 4) to 25 % (line #2) higher than wild-type (Supplemental Fig. 2). We also observed an increase in plant height ranging from 18 % (line #4) to 25 % (line #2) for the *Ubi:SoSPS* lines after three weeks of growth (Fig. 2), but not at later stages of plant growth. Not only plant height but also the number of leaves of the three-week-old plants reflected the accelerated development of *Ubi:SoSPS* lines at relatively early growth stages compared to wild-type. Whereas wild-type plants showed 3 and only occasionally 4 leaves at this growth stage, leaf number was increased to 4 to 5 in *Ubi:SoSPS* lines (Supplemental Fig. 3).

Senesced biomass

For characterization for senesced biomass, which would be preferably used for biotechnological applications like ethanol fermentation, we defined parameters for the selection of two *Ubi:SoSPS* lines for subsequent experiments with senesced biomass: i) significantly higher sucrose-phosphate synthase activity than wild-type (Fig. 1), ii) significant increase in plant height and number of leaves during early plant growth compared to wild-

type (Fig. 2 and Supplemental Fig. 3), and iii) number of T-DNA integrations into the genome (Supplemental Fig. 1) to evaluate a putative effect on phenotype determination. Based on these parameters, we selected *Ubi:SoSPS* lines #2 and #4. Both lines revealed i) significantly higher sucrose-phosphate synthase activity (Fig. 1) and ii) a significant increase in plant height and number of leaves with line #2 constantly showing strongest increase (Fig. 2 and Supplemental Figs. 2 and 3). Concerning the number of T-DNA integrations, line #2 revealed multiple T-DNA integration whereas line #4 was the only line with a single T-DNA integration (Supplemental Fig. 1).

The total dry weight of senesced biomass excluding roots was increased in *Ubi:SoSPS* lines #2 and #4 by 22 % and 52 %, respectively, compared to wild-type (Fig. 3A). Separation of the total biomass into the three organs stem, leaf and spikelet revealed a relative shift form spikelet to leaf biomass whereas the relative proportion of stem biomass remained stable (Fig. 3B). As a result, the relative proportion of leaf biomass increased from 14 % in wild-type to about 20 % in the two *Ubi:SoSPS* lines. To further examine the decrease in the relative proportion of spikelet biomass in the *Ubi:SoSPS* lines, we determined the number of kernels per plant and the kernel weight. Whereas the total number of kernels per plant was not significantly different between wild-type and *Ubi:SoSPS* lines (about 50 kernels per plant), we observed a drop of the kernel weight from about 2 mg in wild-type to about 1.7 mg in the two *Ubi:SoSPS* lines (Supplemental Fig. 4).

As expected from *SoSPS* overexpression, we determined higher sucrose levels in senesced leaf biomass of *Ubi:SoSPS* lines than wild-type, where the sucrose content increased by 52 % for line #2 and 22% for line #4 (Fig. 4A). The sucrose level in stem biomass was generally lower than in leaf biomass and was not different between wild-type and *Ubi:SoSPS* lines (Fig. 4A). The increase in sucrose content of leaf biomass from *Ubi:SoSPS* lines correlated with improved saccharification efficiency, which was measured by the release of glucose from biomass after thermo-chemical pretreatment with diluted sulphuric acid and subsequent hydrolysis using the commercially available enzyme mix Accellerase 1500. We determined an increase in relative saccharification efficiency of leaf biomass by 10 % for *Ubi:SoSPS* line #2 and by 5 % for line #4 whereas relative saccharification efficiency of stem biomass was not changed comparing wild-type and *Ubi:SoSPS* lines. Considering only senesced, dry leaf and stem biomass for saccharification in processes leading to lignocellulosic ethanol production, the combination of higher leaf and stem biomass production (Fig. 3) and improved saccharification of leaf biomass of the *Ubi:SoSPS* lines (Fig. 4B) resulted in an increase in saccharification per plant by 37 % for line #2 and by 74 % for line #4.

Discussion

In our study, we showed that overexpression of the sugarcane sucrose-phosphate synthase gene *SoSPS* in the model grass *B. distachyon* was able to improve biomass yield and saccharification, which are two important traits for lignocellulosic feedstock and its utilization in biomass fermentation for ethanol production. Whereas previous studies mainly concentrated on growth phenotypes and altered sucrose content as well as SPS activity in developing plants due to *SPS* overexpression (Coleman et al., 2010; Galtier et al., 1993; Ishimaru et al., 2008; Ishimaru et al., 2004; Park et al., 2008), we were especially interested in effects on senesced, dry biomass because it would represent the favoured state of the biomass for harvesting and subsequent utilization in biorefineries for ethanol fermentation. Even though a main source of lignocellulosic feedstock could be stover and straw from major field crops like maize, wheat (*Triticum aestivum*) and rice or designated energy crops like *Miscanthus* x *giganteus* (Dohleman and Long, 2009; Heaton et al., 2008) and switchgrass (*Panicum virgatum*) (Keshwani and Cheng, 2009), which all belong to the economically important family of Poaceae, results from *SPS* overexpression in grasses were rather limited. Ishimaru et al. (2004) showed an increase in plant height of about 25 % in 30-days-old rice lines overexpressing maize *SPS* but no results of possibly increased biomass yield. We observed a similar increase in plant height in 21-days-old *B. distachyon* line with *SoSPS* overexpression as in rice and could additionally show higher biomass yield from senesced plants, confirming previous calculations on the linkage between plant height and biomass production (Niklas and Enquist, 2001). Because increased plant growth due to *SPS* overexpression was also shown for tobacco and potato (Baxter et al., 2003; Ishimaru et al., 2008), the developmental effect of *SPS* overexpression seems to be a general effect directly linked to higher SPS activity, which would be related to a higher capacity in carbon assimilation and carbon partitioning (Galtier et al., 1993; Huber and Huber, 1992; Worrell et al., 1991). Consequently, *SPS* overexpression and increased SPS activity has been associated with modifying sink capacity in leaves and is reflected by the increase in the sucrose/starch ratio in respective transgenic lines (Baxter et al., 2003; Galtier et al., 1993; Ono et al., 1999). These observations combined with the fact that sucrose production can be an important factor in limiting carbon export from leaves (Foyer and Galtier, 1996) would also explain the higher sucrose content in leaves and the shift of the biomass ratio from spikelets to leaves in the *SoSPS* overexpression *B. distachyon* lines. Unlike in tobacco with an increased number of flowers in *SPS* overexpression lines (Baxter et al., 2003), we did not find alterations in flower numbers as indicated by the unchanged total number of kernels per plant. However, the kernel

weight was reduced in the *SoSPS* overexpression *B. distachyon* lines, which was in contrast to previous findings in tomato with an increased fruit yield with maize *SPS* overexpression (Laporte et al., 2001; Laporte et al., 1997) and might be the consequence of altered carbon partitioning reflected by the increased sucrose content of leaves. This negative effect on kernel yield should be considered and tested in those approaches where *SoSPS* overexpression would be used to generate possible dual-use plants providing grain for food and feed production and improved biomass conversion, which we demonstrated in the generated *SoSPS* overexpression *B. distachyon* lines with a significant increase in saccharification efficiency of senesced, dry biomass. Because higher saccharification directly correlated with the increased sucrose content in leaf biomass of the *SoSPS* overexpression lines, an acid-catalysed hydrolysis of sucrose during biomass pretreatment with diluted sulphuric acid (Bower et al., 2008) would explain elevated glucose release from processed, transgenic biomass. However, even without complete chemical hydrolysis, increased amounts of sucrose in optimized biomass could directly contribute to higher ethanol production because this disaccharide can be hydrolysed to glucose and fructose by invertases, which are secreted by *Saccharomyces cerevisiae* during biomass fermentation (Gascon et al., 1968) and subject to metabolic engineering for improved ethanol yield (Basso et al., 2011). Ethanol yield also depends on the performance of hexose transporters, which enable the uptake of the two monosaccharides into *S. cerevisiae* cells where they can be utilized for ethanol fermentation (De La Fuente and Sols, 1962; Zuchowska et al., 2015).

Compliance with Ethical Standards

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Conflict of Interest: The authors declare that they have no conflict of interest.

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Figure legends

Figure 1 Sucrose-phosphate synthase activity of *Ubi:SoSPS B. distachyon* lines.
For determination of sucrose-phosphate synthase activity, four-week-old leaves from *B. distachyon* wild-type (wt) and *SoSPS* overexpression lines (*Ubi:SoSPS*, C-terminal fusion of SoSPS with fluorochrome mCherry) #1, #2, #3 and #4 were used. Letters a, b indicate groups with significant difference, $P < 0.05$ based on Tukey's test with $n = 3$ biological independent experiments. Error bars represent ± SE.

Figure 2 Early stage growth phenotype of *Ubi:SoSPS B. distachyon* lines.
Plant height of three-week-old plants from *B. distachyon* wild-type (wt) and *SoSPS* overexpression lines (*Ubi:SoSPS*). Values represent the mean of three independent biological experiments with $n = 6$ individual plants for each line in each experiment. Letters a, b, c indicate groups with significant difference, $P < 0.05$ based on Tukey's test. Error bars represent ± SE.

Figure 3 Biomass production of *Ubi:SoSPS B. distachyon* lines.
All experiments were done with biomass of fully senesced and dried biomass of 4-month-old of *B. distachyon* wild-type (wt) and *SoSPS* overexpression lines *Ubi:SoSPS* #2 and #4. **(A)** Total biomass production (excluding roots). **(B)** Ratio between spikelet, leaf and stem biomass. Values represent the mean of three independent biological experiments with $n = 6$ individual plants for each line in each experiment. Letters a, b, c indicate groups with significant difference, $P < 0.05$ based on Tukey's test. Error bars represent ± SE.

Figure 4 Sucrose content and saccharification efficiency of *Ubi:SoSPS B. distachyon* lines.
All experiments were done with biomass of fully senesced and dried biomass of four-month-old of *B. distachyon* wild-type (wt) and *SoSPS* overexpression lines *Ubi:SoSPS* #2 and #4. **(A)** Relative sucrose amounts in leaf and stem biomass determined in enzymatic assays. **(B)** Saccharification efficiency determined by release of glucose from pretreated and hydrolysed leaf biomass. (A, B) Letters a, b, c, d indicate groups with significant difference, $P < 0.05$ based on Tukey's test with $n = 3$ biological independent experiments. Error bars represent ± SE.

Figure 1 New

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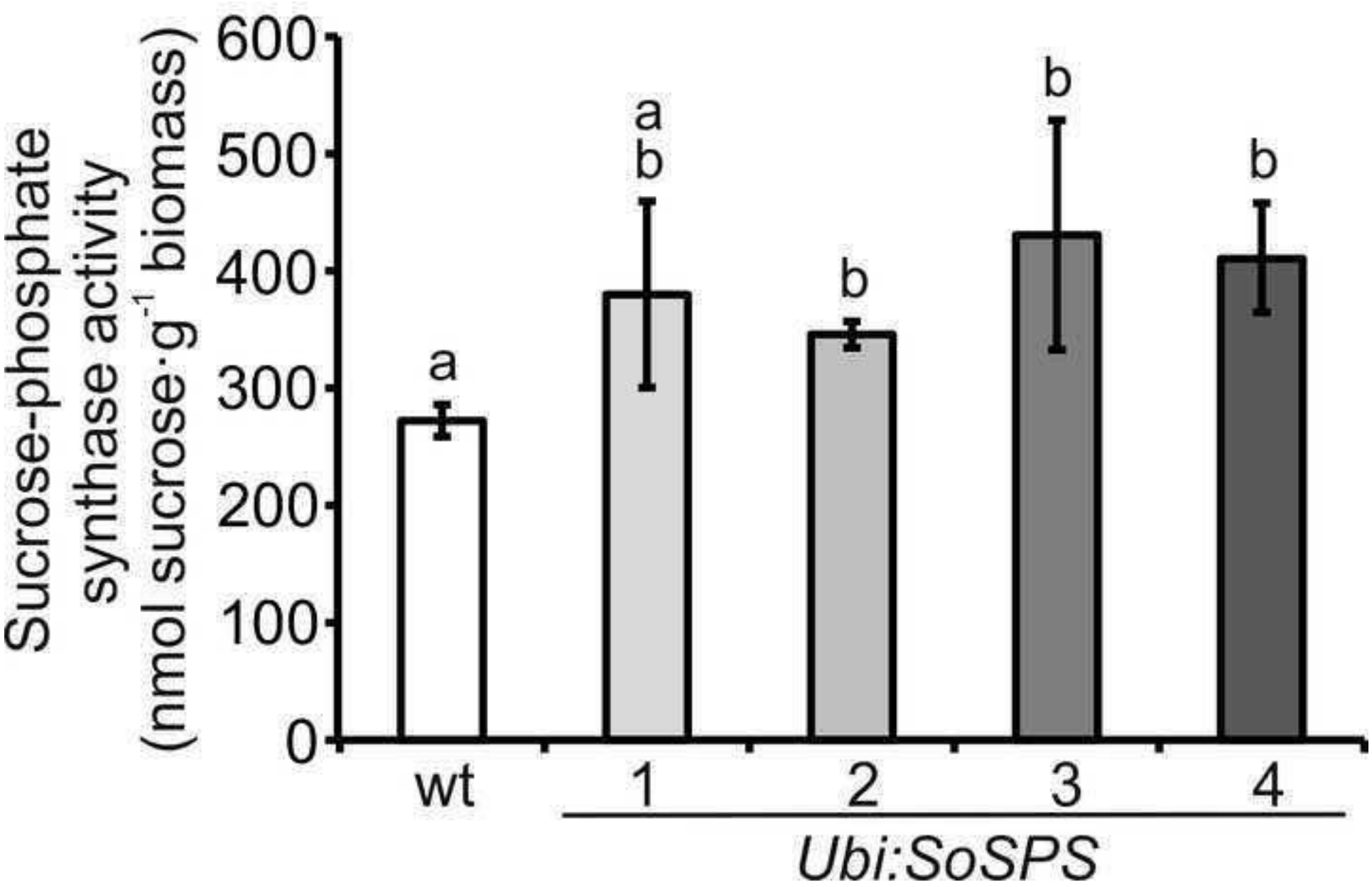

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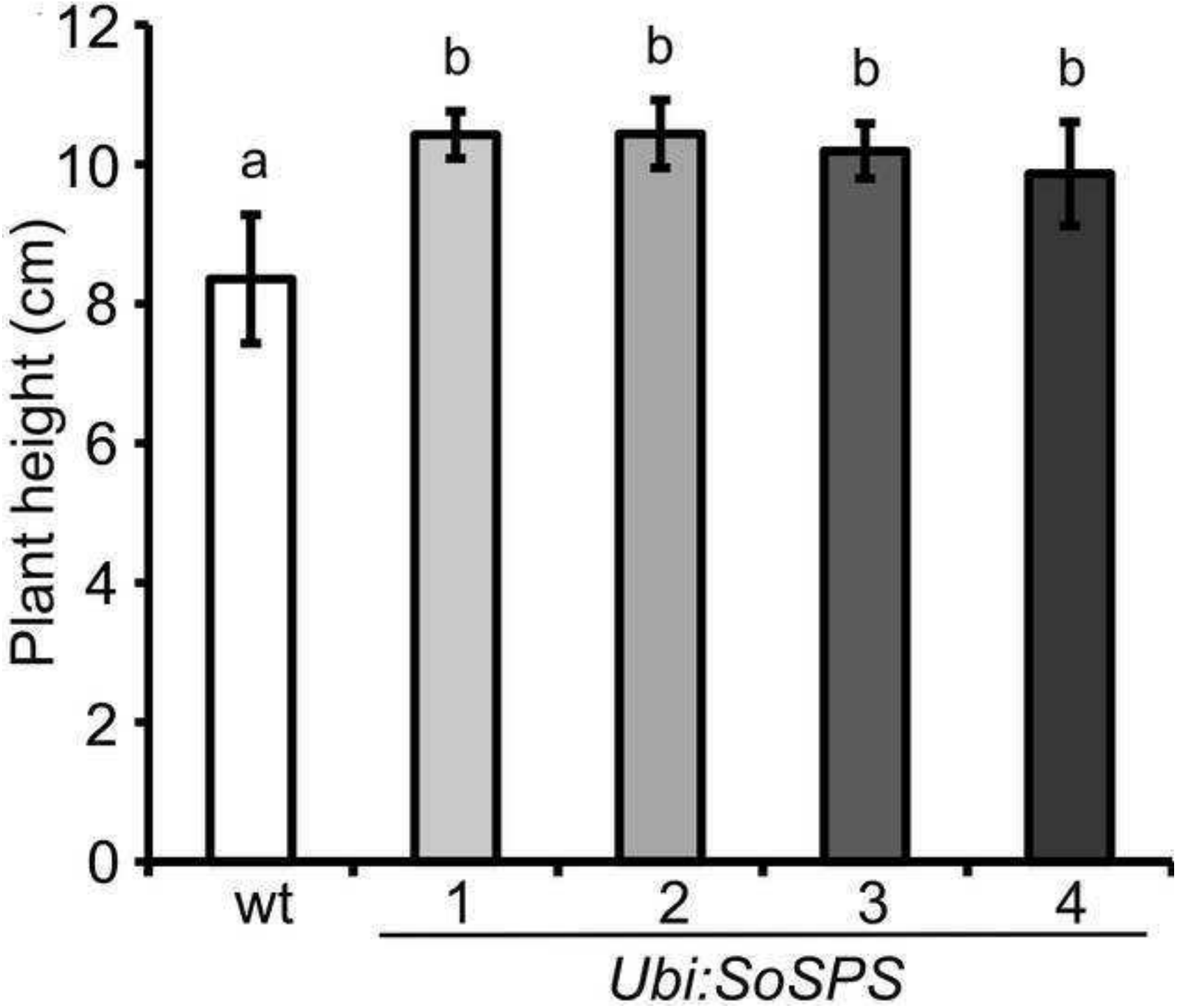

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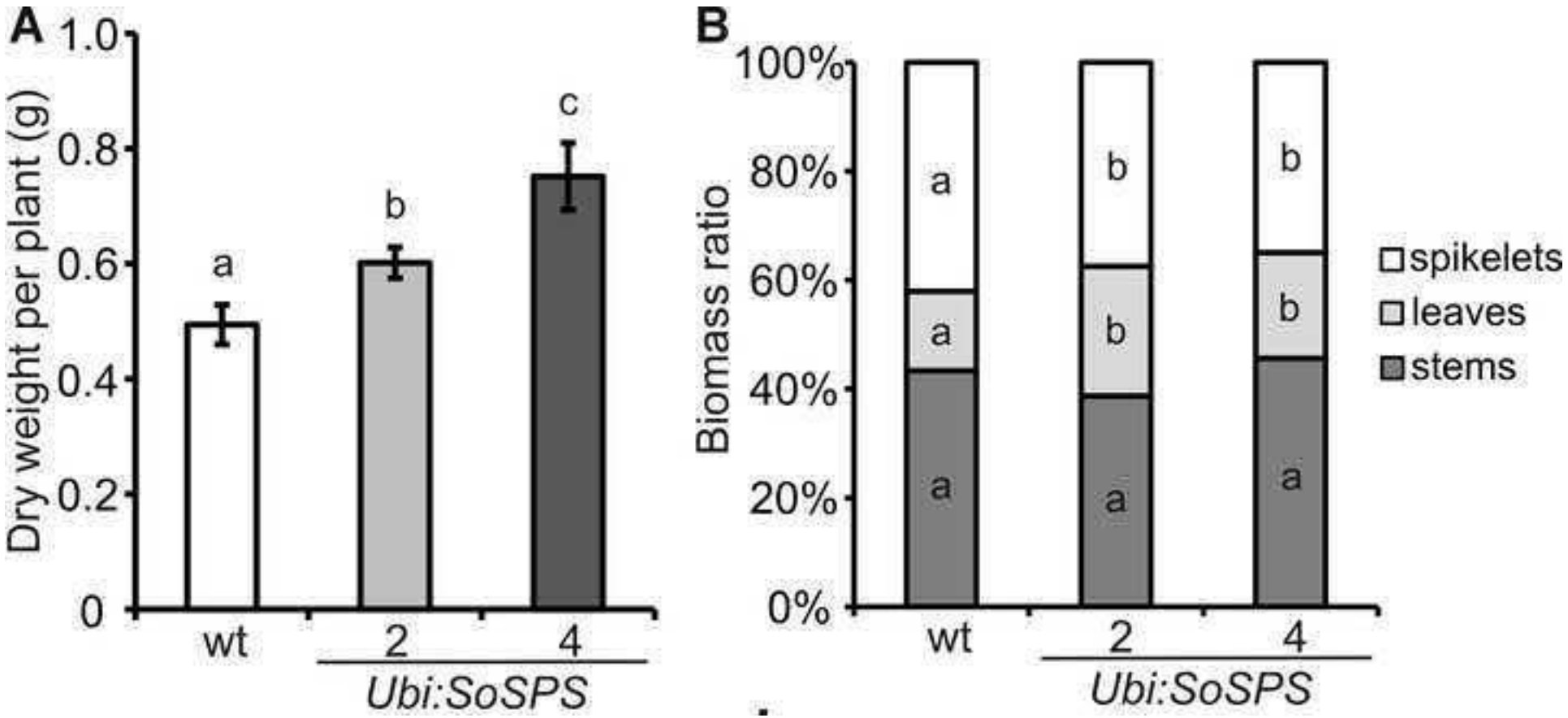

Figure 4

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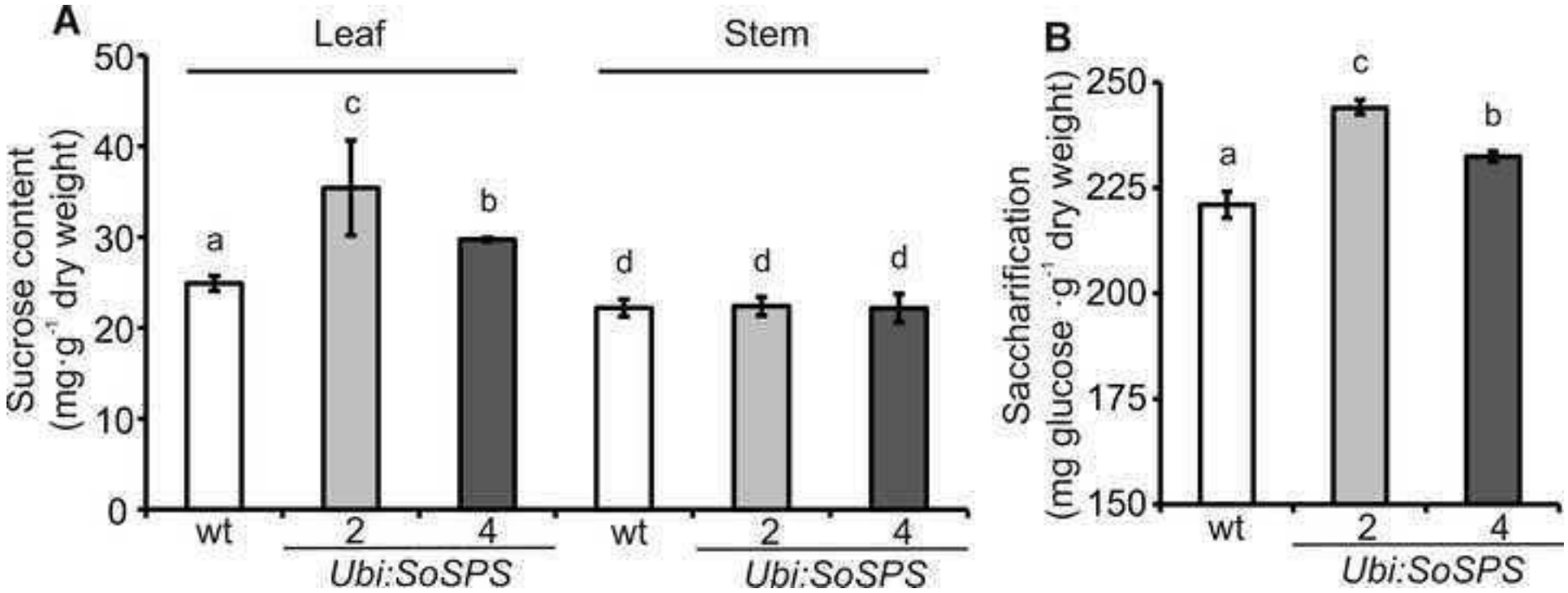
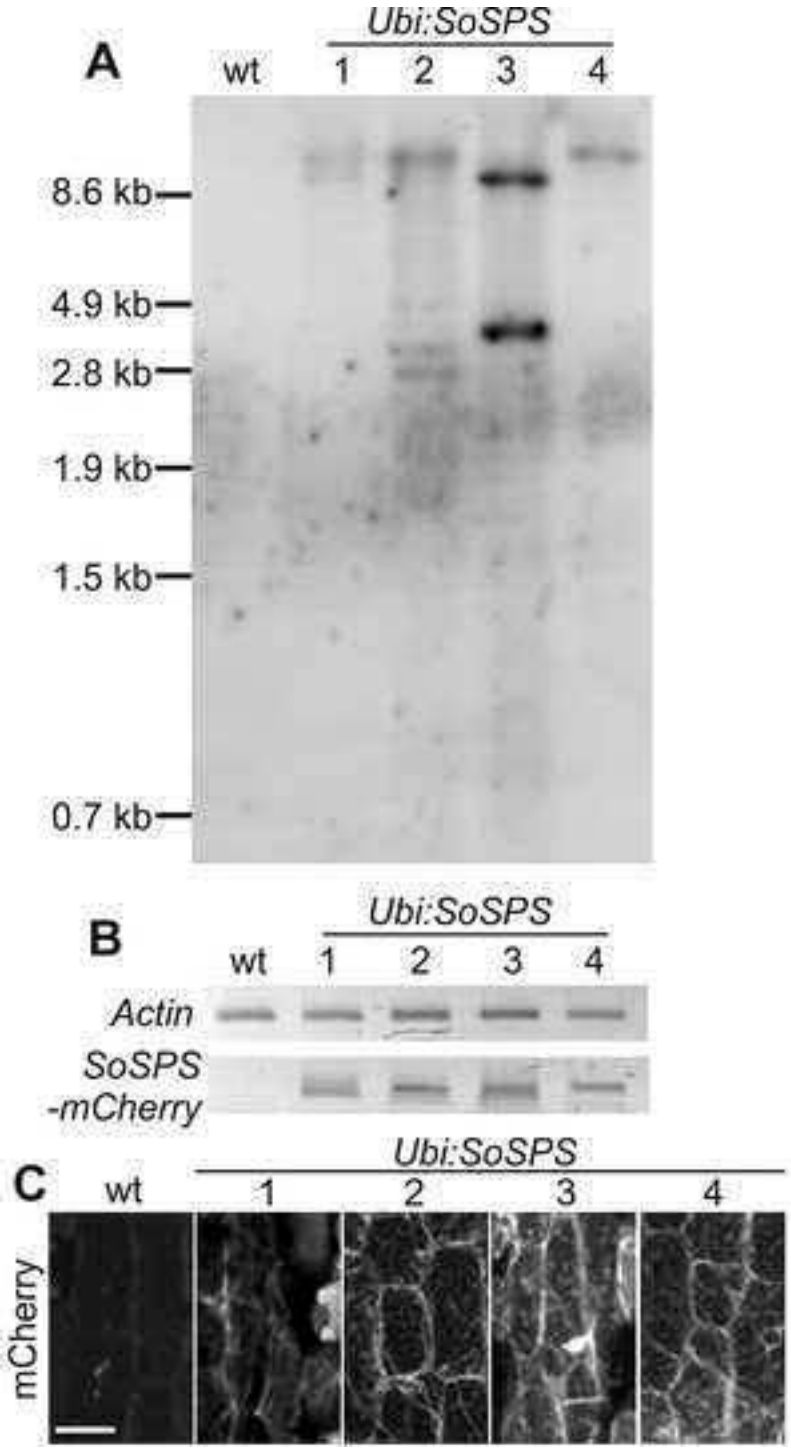
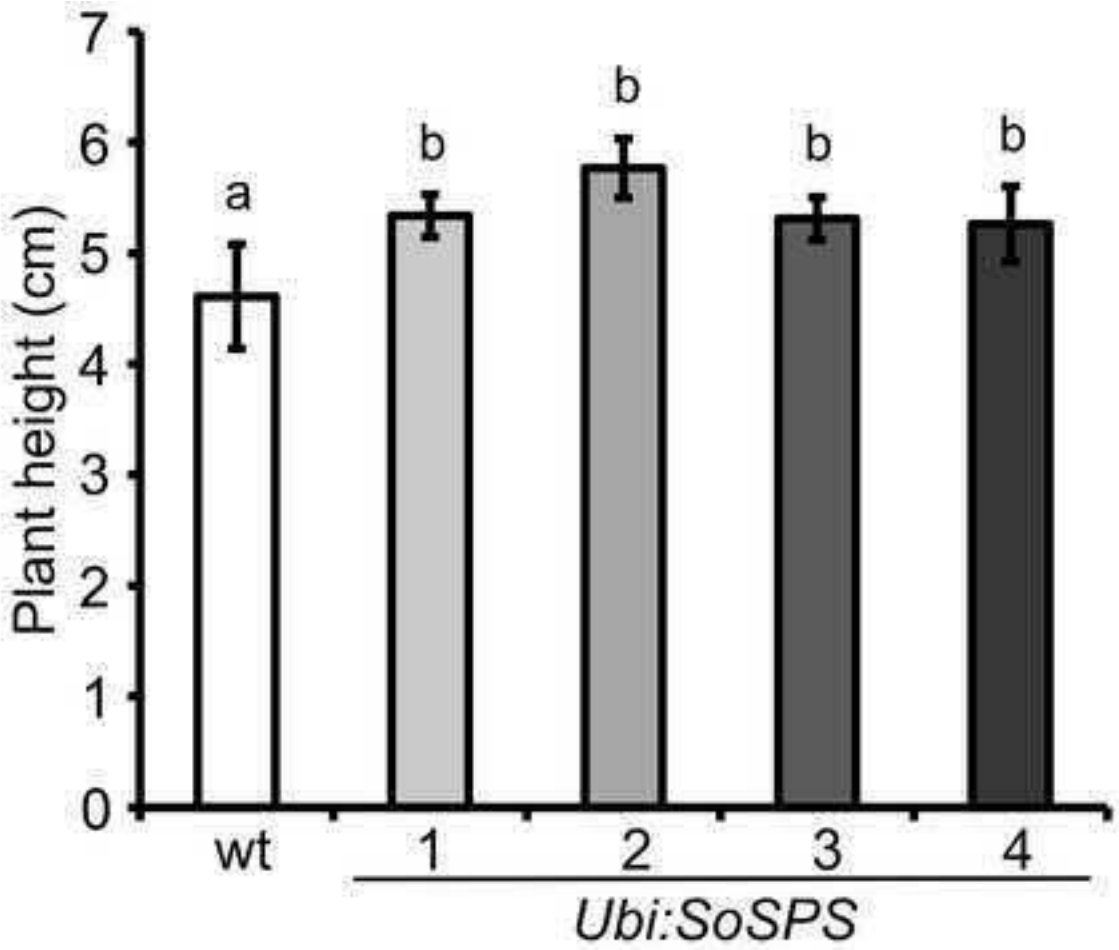


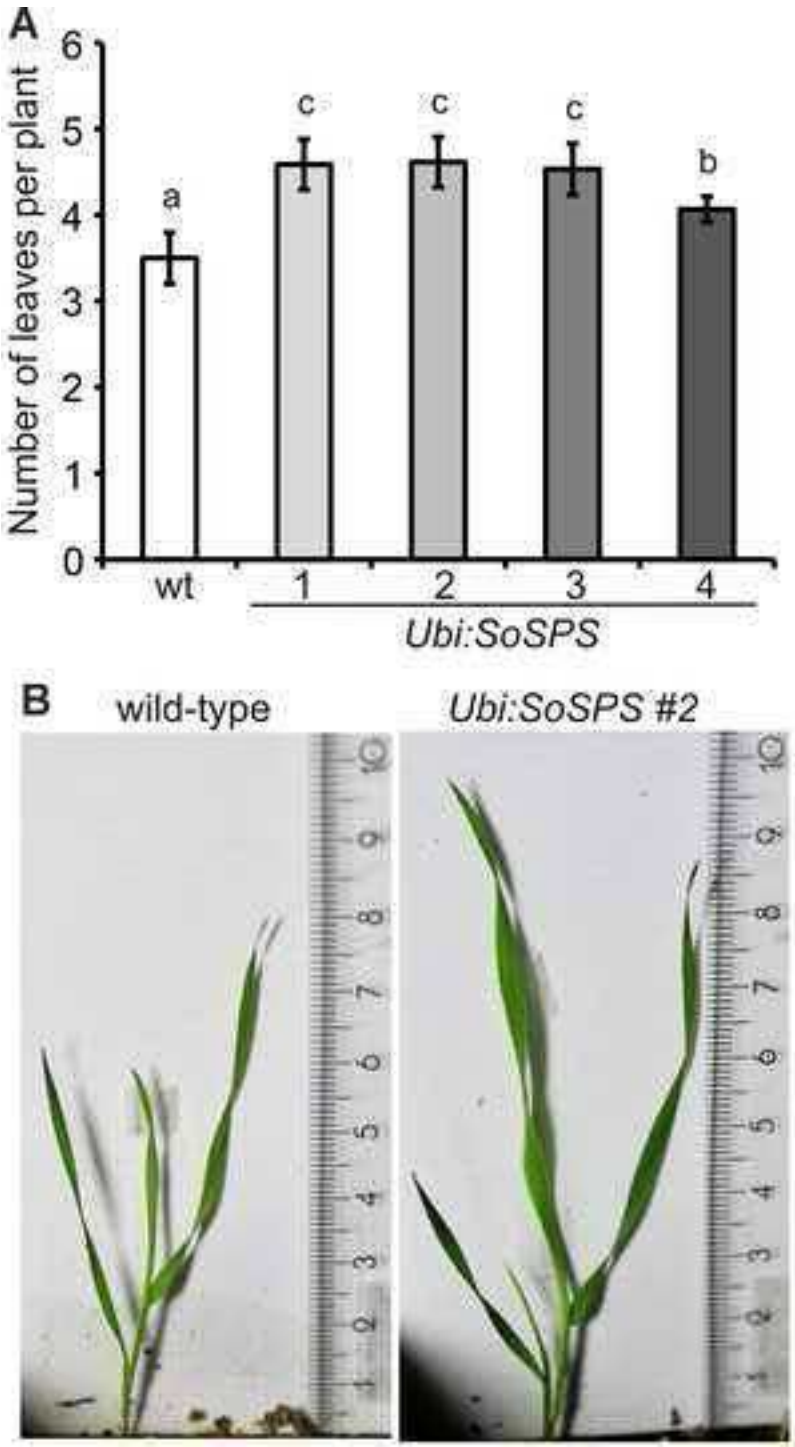


Supplemental Figure 1. Genetic and microscopic characterization of *Ubi:SoSPS B. distachyon* lines.

For all experiments, four-week-old leaves from *B. distachyon* wild-type (wt) and *SoSPS* overexpression lines (*Ubi:SoSPS*, C-terminal fusion of SoSPS with fluorochrome mCherry) #1, #2, #3 and #4 were used. **(A)** Southern Blot analysis of *Ubi:SoSPS* lines and wt using *Hind*III-restricted genomic DNA from leaves. For determination of T-DNA integration into the genome after Agrobacterium-mediated transformation, a DIG-labelled internal probe of the *bar* resistance gene was used. Fragment sizes according to the DIG-labelled DNA Molecular Weight Marker VII (Roche Diagnostics). **(B)** Expression of the *SoSPS-mCherry* fusion construct in leaf tissue of *Ubi:SoSPS* lines. Gene expression in non-transgenic wt was used as control. RNA was isolated from leaves and used as template in cDNA generation. *Actin* gene expression served as reference. **(C)** Confocal laser-scanning microscopy to localize SoSPS-mCherry in epidermal leaf cells of *Ubi:SoSPS* lines. Scale bar = 20 µm.

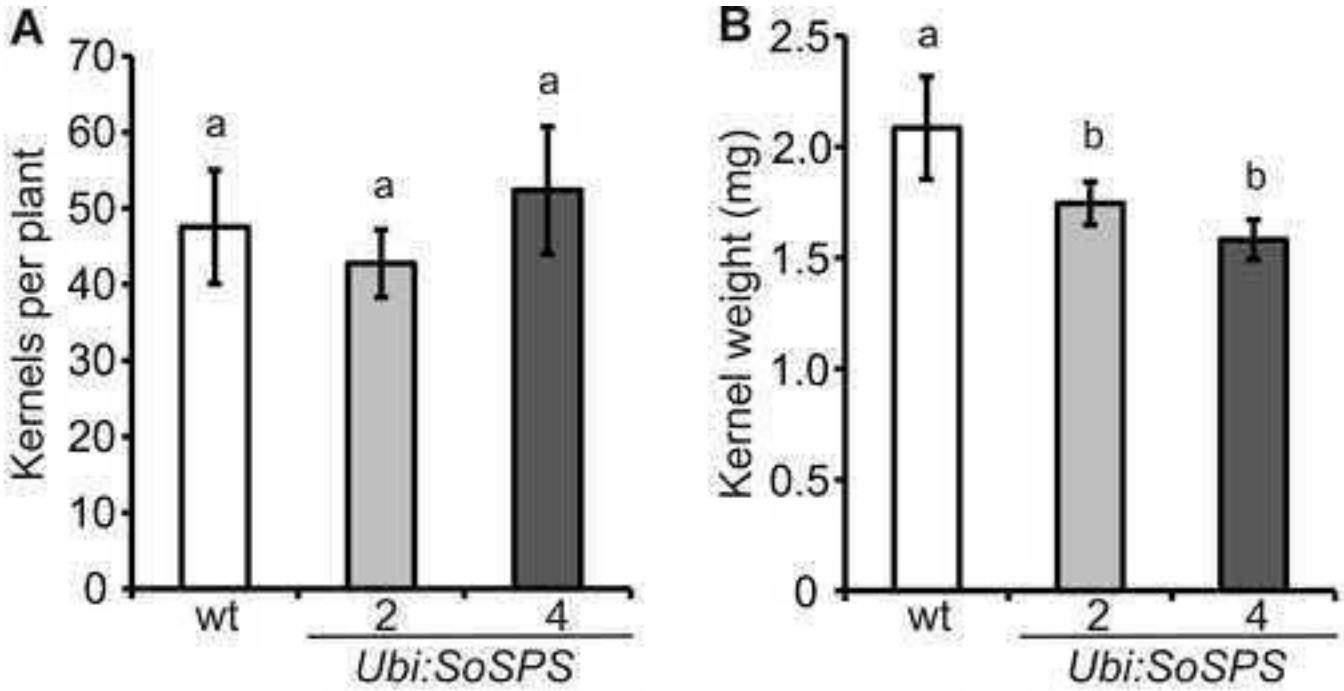


Supplemental Figure 2. Early stage growth phenotype of *Ubi:SoSPS B. distachyon* lines. Plant height of *B. distachyon* wild-type (wt) and *SoSPS* overexpression lines (*Ubi:SoSPS*) after 2 weeks of cultivation. Values represent the mean of three independent biological experiments with $n = 6$ individual plants for each line in each experiment. Letters a, b indicate groups with significant difference, $P < 0.05$ based on Tukey's test. Error bars represent ± SE.



Supplemental Figure 3. Number of leaves in early growth stage *Ubi:SoSPS B. distachyon* lines.

Three-week-old plants from *B. distachyon* wild-type (wt) and *SoSPS* overexpression lines (*Ubi:SoSPS*) were used. **(A)** Number of leaves of 3-week-old plants. Values represent the mean of three independent biological experiments with $n = 6$ individual plants for each line in each experiment. Letters a, b, c indicate groups with significant difference, $P < 0.05$ based on Tukey's test. Error bars represent ± SE. **(B)** Representative photographs of differences in plant height and number of leaves of 3-week-old wt and *Ubi:SoSPS* plants. Scale indicates height in cm.



Supplemental Figure 3. Kernel development of *Ubi:SoSPS B. distachyon* lines.

All experiments were done with biomass of fully senesced and dried biomass of 4-month-old of *B. distachyon* wild-type (wt) and *SoSPS* overexpression lines *Ubi:SoSPS* #2 and #4. **(A)** Kernel development indicated by the total number of kernels per plant and **(B)** the weight of a single kernel. Values represent the mean of three independent biological experiments with $n = 6$ individual plants (A) and $n = 6$ kernel pools from 6 plants (B) for each line in each experiment. Letters a, b indicate groups with significant difference, $P < 0.05$ based on Tukey's test. Error bars represent ± SE.