Expression of a bacterial 3-dehydroshikimate dehydratase (QsuB) reduces lignin and improves biomass saccharification efficiency in switchgrass (Panicum virgatum L.)

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Abstract

Background: Lignin deposited in plant cell walls negatively affects biomass conversion into advanced bioproducts. There is therefore a strong interest in developing bioenergy crops with reduced lignin content or altered lignin structures. Another desired trait for bioenergy crops is the ability to accumulate novel bioproducts, which would enhance the development of economically sustainable biorefineries. As previously demonstrated in the model plant Arabidopsis, expression of a 3-dehydroshikimate dehydratase in plants offers the potential for decreasing lignin content and overproducing a value-added metabolic coproduct (i.e., protocatechuate) suitable for biological upgrading.

Results: The 3-dehydroshikimate dehydratase QsuB from Corynebacterium glutamicum was expressed in the bioenergy crop switchgrass (Panicum virgatum L.) using the stem-specific promoter of an O-methyltransferase gene (pShOMT) from sugarcane. The activity of pShOMT was validated in switchgrass after observation in-situ of beta-glucuronidase (GUS) activity in stem nodes of plants carrying a pShOMT::GUS fusion construct. Under controlled growth conditions, engineered switchgrass lines containing a pShOMT::QsuB construct showed reductions of lignin content, improvements of biomass saccharification efficiency, and accumulated higher amount of protocatechuate compared to control plants. Attempts to generate transgenic switchgrass lines carrying the QsuB gene under the control of the constitutive promoter pZmUbi-1 were unsuccessful, suggesting possible toxicity issues associated with ectopic QsuB expression during the plant regeneration process.

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**Background**

The development of biorefineries to reduce our dependence on nonrenewable fossil fuel resources requires production of dedicated bioenergy crops that can be grown with few inputs on marginal lands. Other desired traits for bioenergy crops include high biomass yields, stress resilience, reduced recalcitrance to conversion into biofuels and bioproducts, and the accumulation of valuable co-products [1, 2]. Switchgrass has long been recognized as an ideal crop for bioenergy purposes considering its pest and disease resistance, high biomass yields, growth performance on poor soils due to relatively low requirements for added fertilizers, carbon sequestration capacity via its extensive root system, drought tolerance, and efficient water use [3]. As a consequence, significant efforts have been implemented for the improvement of switchgrass via breeding and genetic transformation [4, 5].

Lignin is a major polymer in plant biomass that negatively impacts the conversion of cell wall polysaccharides into advanced bioproducts, and several engineering approaches have been established to modify lignin content and its monomeric composition [6, 7]. For example, the heterologous expression of a bacterial 3-dehydroshikimate dehydratase (QsuB) targeted to plastids resulted in strong lignin reductions (up to 50%) in Arabidopsis [8]. One explanation for this observation is the possible reduction of the cytosolic shikimate pool needed for the synthesis of \( p \)-coumaroyl-shikimate catalyzed by hydroxycinnamoyl CoA: shikimate hydroxycinnamoyl transferase (HCT) during lignin biosynthesis (Fig. 1).

In switchgrass, several HCT gene candidates have been proposed to have a role in lignin biosynthesis based on the HCT activity measured with the corresponding recombinant enzymes and their expression profile in lignifying cell suspension cultures [9, 10]. In fact, more than 90% reduction in transcript levels of either \( \text{PvHCT1} \) or \( \text{PvHCT2} \) had no effect on lignin content, but simultaneous downregulation of both genes resulted in slight decreases of lignin content (5–8%) based on the yield of lignin monomers released after thioacidolysis [11]. These results not only indicate a role for HCT in lignin biosynthesis in switchgrass, with \( \text{PvHCT1} \) and \( \text{PvHCT2} \) being redundant, but also suggest the involvement of additional HCTs with similar functions.

In this work, we report on the expression of QsuB in switchgrass using the promoter of a sugarcane O-methyltransferase gene (\( \text{pShOMT} \)) [12]. Several switchgrass QsuB transformation events show reduction of
lignin content and decreased cell wall recalcitrance. A significant increase in the content of protocatechuate accumulated in biomass was also observed.

Results
Molecular characterization of the pShOMT::QsuB switchgrass lines
A total of eight independent transformation events were regenerated after Agrobacterium-mediated transformation of switchgrass using a DNA construct that contains the plastid-targeted QsuB coding sequence fused downstream of the pShOMT promoter. The QsuB transgene was detected by PCR using gDNA from each transformant (Fig. 2a), and QsuB expression was validated by qPCR performed on cDNA synthesized from RNAs obtained from the first internode of each line at the E2 stage (Fig. 2b). A DNA construct consisting of pShOMT fused upstream of the GUS reporter gene was also transferred to switchgrass. Analysis of internodes and nodes from switchgrass plants harboring the pShOMT::GUS construct at the E4 stage suggested that pShOMT is mainly active in the nodes, whereas little activity was observed in the internodes (Figure S1). Under controlled growth conditions, all transgenic lines did not show any particular phenotype nor growth defect and were visually indistinguishable from each other or compared to non-transformed wild-type plants.

Protocatechuate content in pShOMT::QsuB switchgrass
Protocatechuate (PCA), the product of QsuB activity, was extracted from the total aboveground biomass of switchgrass plants at the E5 stage and quantified. Compared to control plants carrying the pShOMT::GUS construct, PCA was significantly increased by ~2–3-fold in four independent pShOMT::QsuB lines, reaching up to 380 μg/g dry weight (Fig. 3). This data shows that expression of plastid-targeted QsuB in transgenic switchgrass enabled the conversion of endogenous 3-dehydroshikimate into PCA.

Lignin content and biomass saccharification efficiency in pShOMT::QsuB switchgrass
Total lignin content in the biomass from the pShOMT::QsuB switchgrass lines was measured using the Klason method. Compared to control lines containing the pShOMT::GUS construct, several pShOMT::QsuB lines showed significant reductions of lignin content ranging from 12 to 21% (Fig. 4a). Inspection of stem sections treated with phloroglucinol-HCl for the staining of lignin did not reveal any differences between the different pShOMT::QsuB lines and the control pShOMT::GUS lines (data not shown). However, on leaf blade sections, reductions in the intensity of the typical red staining were observed in the case of the pShOMT::QsuB lines compared to controls, especially in thick fibers located in the abaxial zone (Fig. 4b).

The recalcitrance towards enzymatic degradation of the biomass of the engineered switchgrass was evaluated by measuring the amount of sugars released from cell wall residues after pretreatment with hot water followed by a 72-h hydrolysis using a commercial cellulase...
cocktail (CTec2). As shown in Fig. 5, higher amount of reducing sugars was obtained for several pShOMT::QsuB lines compared to the pShOMT::GUS control lines, with significant increases ranging between 21 and 30%.

Discussion

Here, we describe the successful expression of the bacterial 3-dehydroshikimate dehydratase QsuB gene under the control of pShOMT in switchgrass. We show that the resulting plants display 12–21% reduction in lignin, a 2–3-fold increase in the bioaccumulation of PCA and a 5–30% increase in saccharification efficiency.

pShOMT was previously shown to be preferentially active in stem vascular tissues in sugarcane, rice, maize, and sorghum [12], making it a good promoter candidate to express QsuB specifically in lignifying tissues within vascular bundles. Similar to previous observations made in sugarcane, we were able to detect GUS activity in stem nodes from switchgrass lines carrying a pShOMT::GUS construct. Nevertheless, an apparent reduction of lignin content observed in some discrete regions of leaf blades (i.e., fibers on the adaxial zone) from plants carrying the pShOMT::QsuB construct indicate that pShOMT is also active in leaf cells with secondary wall accumulation (Fig. 4b). In addition to pShOMT, attempts to generate transgenic switchgrass lines with constructs containing QsuB under the control of the constitutive promoter of the maize ubiquitin1 gene (pZmUbi-1) was unsuccessful, whereas only a single event was obtained.

![Fig. 3](image1.png)

**Fig. 3** Protocatechuate (PCA) content measured in the biomass of switchgrass pShOMT::QsuB transgenic lines. Values are means ±SE of three biological replicates (n = 3). Asterisks indicate significant differences from a line containing the pShOMT::GUS construct using the unpaired Student’s t-test (*P < 0.05).

![Fig. 4](image2.png)

**Fig. 4** a Klason lignin content measured in cell wall residues (CWR) obtained from biomass of switchgrass lines containing the pShOMT::QsuB construct. A line containing the pShOMT::GUS construct was used as control and analyzed thrice since measurements were carried out in three separate batches. Values are means ±SE of four biological replicates (n = 4). Asterisks indicate significant differences from the line containing the pShOMT::GUS construct using the unpaired Student’s t-test (*P < 0.05). b Representative pictures of leaf blade cross-sections stained with phloroglucinol-HCl from lines containing either the pShOMT::GUS or the pShOMT::QsuB construct. Note the reduction of the staining specifically in thick fibers located in the leaf abaxial zone for the pShOMT::QsuB line (red arrows). Scale: black bar = 200 μm.
with a pZmCesa10::QsuB construct containing the promoter of the maize cellulose synthase gene CESA10 involved in secondary cell wall formation [13] (Figures S2, S3). This is possibly the result of toxicity occurring during the plant regeneration process when using these two pZmUb1::QsuB and pZmCesa10::QsuB constructs. Considering that QsuB diverts lignin biosynthesis, using the promoter of a lignin biosynthetic gene to drive QsuB expression may be more suited spatial-temporally during plant development. Interestingly, the single pZmCesa10::QsuB line showed a reduction of total lignin content as well as reduced phloroglucinol staining in leaf fibers (Figure S2E, F). Obtaining more switchgrass transgenic lines as well as reduced phloroglucinol staining in leaf fibers is essential to validate the effectiveness of pZmCesa10 in driving QsuB expression to reduce lignin content.

The exact mechanism by which QsuB expression reduces lignin in switchgrass is still unresolved; in particular, whether the cytosolic pools of shikimate precursors of lignin in switchgrass is still unresolved; in particular, whether the cytosolic pools of shikimate exist in the cytosol, and if so, whether these pools are regulated by a specific gene or set of genes. Further research is needed to elucidate the role of the cytosolic pool of shikimate in the regulation of lignin biosynthesis in switchgrass.

The overproduction of PCA in switchgrass lines expressing QsuB probably results from a partial conversion of the endogenous pool of 3-dehydroshikimate catalyzed by QsuB activity. Notably, increases in PCA titers (2–3-fold compared to control switchgrass) are smaller than those previously reported in Arabidopsis and tobacco plants containing the QsuB gene under the control of the promoter of the Arabidopsis cinnamyl alcohol dehydrogenase (CAD). This work highlights the potential of QsuB engineering strategy to enhance PCA titers in switchgrass biomass because several techno-economic analyses demonstrated the benefits of producing co-products in planta to render bioenergy crops economically sustainable [1, 24, 25]. In fact, several studies have already reported on the use of PCA as carbon source or pathway intermediate for the production of diverse valuable products such as bioactive compounds, mevalonate, and methyl ketones [22, 26–30].

### Conclusion

The QsuB engineering approach has been established in switchgrass. This work highlights the fact that selecting an adequate promoter to drive QsuB expression should be an important parameter for successful engineering of other crops with this gene via tissue culture-dependent transformation methods. Considering that pShOMT activity is induced in the leaf and root by key regulators of biotic and abiotic stress responses such as salicylic acid, jasmonic acid and methyl jasmonate [12], it will be essential to field test our engineered pShOMT::QsuB switchgrass to assess its agronomic performance and resilience to environmental stress.

### Methods

#### Vector construction and plant transformation

The promoters pShOMT [12], pZmCesa10 (2.6 kb located upstream the start codon of the maize CESA10
gene [GenBank: AY372244.1], and pZmUbi-1 [31] were synthesized with the following flanking restriction sites: 5′-AscI / 3′-AvrII for pShOMT and 5′-HindIII / 3′-AvrII for pZmCesa10 and pZmUbi-1 (Genscript, Piscataway, NJ). Promoter sequences were released by enzyme digest and ligated into the binary vector pA6-GW [32] pre-digested with either AscI/AvrII or HindIII/AvrII to generate respectively the pA6-pShOMT-GW, pZmCesa10-GW, and pA6-pZmUbi-1-GW binary vectors. The entry vector pDONR221-schl-QsuB containing the gene encoding the 3-dehydroshikimate dehydratase QsuB from Corynebacterium glutamicum preceded with the nucleotide sequence of a chloroplast transit peptide [8] was LR recombined with the pA6-pShOMT-GW, pA6-pZmCesa10-GW, and pA6-pZmUbi-1-GW vectors using the Gateway cloning technology (Thermo Fisher Scientific, Waltham, MA) to generate the constructs pA6-pShOMT-schl::QsuB, pA6-pZmCesa10-GW-schl::QsuB, and pA6-pZmUbi-1-GW-schl::QsuB, respectively. A nucleotide sequence encoding the beta-glucuronidase (GUS) gene from E. coli was amplified from pCAMBIA1301 using primers flanked with attB1 (5′) and attB2 (3′) Gateway recombination sites, and inserted into the pA6-pShOMT-GW and pA6-pZmCesa10-GW vectors by Gateway cloning to generate the constructs pA6-pShOMT::GUS and pA6-pZmCesa10::GUS, respectively. Cloning primers are listed in Table S1. The binary vectors were transformed into Agrobacterium tumefaciens strain AGL1 for switchgrass (Panicum virgatum L.) transformation which was performed at the University of Missouri’s Plant Transformation Core Facility as previously described [33], where embryogenic calli used for transformation were induced from mature seeds of switchgrass cultivar Alamo-A4 (Hancock Farm & Seed Company, Dade City, FL). Hygromycin B (Life Technologies, Foster City, CA) was added to the selection medium at 50 mg/L.

Plant growth conditions
Four transgenic switchgrass plants for each event were transferred to 2-gal pots containing Pro-Mix soil and grown in a room at 22 °C and 60% humidity using a light intensity of 250 μmol/m²/s and 16 h of light per day.

PCR genotyping
Genomic DNA was extracted from leaf tissue obtained from one of the clones from each event using the Plant DNeasy plant mini kit (Qiagen, Carlsbad, CA). PCR primers specific to the QsuB gene were used to detect the transgene, and primers specific to the switchgrass PvUBQ6 gene (GenBank: FE609298.1) were used to assess the quality of the gDNA. All the primers used in this study are listed in Table S1.

RT-qPCR
Total RNAs were extracted from the first internode collected from plants at the E2 stage [34] using the TRIzol reagent (Thermo Fisher Scientific, Waltham, MA) and cDNA synthesis was conducted using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) as previously described [35]. RT-qPCR was performed as described previously using 40 cycles consisting of 5 s at 95 °C for denaturation and 15 s at 60 °C for annealing and amplification [35]. The relative quantification of QsuB transcripts was calculated using the 2−ΔΔCT method and normalized to the reference gene PvUBQ6 (GenBank: FE609298.1). The results are the average from two biological replicates which were each analyzed in technical replicates. RT-qPCR primers are listed in Table S1.

Lignin assays
The Wiesner histochemical test using phloroglucinol-HCl, a reagent that reacts with coniferaldehyde groups in lignin, was performed on transverse sections of stems and leaf blades from plants at the E2 stage as previously described [36, 37]. For Klasen lignin measurements, whole switchgrass plants were cut at the E5 stage (no visible flag leaf) 3 cm from the bottom, and biomass was dried in an oven at 50 °C for 7 days. Dried biomass was ground with a Model 4 Wiley Mill equipped with a 1-mm mesh (Thomas Scientific, Swedesboro, NJ). Grinded biomass was extracted as previously described [8] and Klasen lignin was measured using the standard NREL biomass protocol [38].

Saccharification assays
Grinded and extracted biomass obtained from plants at the E5 stage was ball-milled to a fine powder using a Mixer Mill MM 400 (Retsch Inc., Newtown, PA) and stainless-steel balls. For saccharification assays, four biological replicates of 10 mg of fine biomass powder from each line was pretreated with liquid hot water followed by a 72-h enzymatic hydrolysis using 1% w/w Cellic CTec2 enzyme mixture (Novozymes, Denmark) as previously described [35]. Hydrolysates were used for measurement of reducing sugars using the 3,5-dinitrosalicylic acid (DNS) assay [39].

Protocatechuate measurements
Whole switchgrass plants were cut 3 cm from the bottom at the E5 stage (no visible flag leaf), and biomass was dried in an oven at 50 °C for 7 days. Dried biomass was ground with a Model 4 Wiley Mill equipped with a 1-mm mesh (Thomas Scientific, Swedesboro, NJ). An aliquot of the grinded biomass was ball-milled to a fine powder using a Mixer Mill MM 400 (Retsch Inc., Newtown, PA) and stainless-steel
Histochemical GUS assays
Stem and leaf sections were obtained manually from plants at the E4 stage using a razor blade. GUS assays were conducted on plant sections using 2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuroneide (Sigma-Aldrich, St. Louis, MO) as substrate for 48 h at 37 °C as previously described [41]. After incubation, sections were dehydrated in 95% (v/v) ethanol prior to observation of the GUS staining in 70% (v/v) ethanol.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12870-021-02842-9.

Additional file 1: Figure S1. Representative pictures showing GUS activities in various tiller sections of switchgrass lines harboring the pZmCesa10::QsuB construct. (A) Representative pictures showing GUS activities in various tiller sections of switchgrass lines harboring the pZmCesa10::QsuB construct. (B) Characterization of a switchgrass line harboring the pZmCesa10::QsuB-5 line containing the pZmCesa10::QsuB construct was used as control. Values are means ± SE of three biological replicates (n = 3). Asterisks indicate a significant difference from the control using the unpaired Student’s t-test (P < 0.001). (C) Klason lignin content measured in cell wall residues (CWR) obtained from the biomass of the switchgrass line pZmCesa10::QsuB-5. A line containing the pZmCesa10::GUS construct was used as control. Values are means ±SE of four biological replicates (n = 4). Asterisks indicate a significant difference from the control using the unpaired Student’s t-test (P < 0.005). (F) Representative pictures of stem and leaf blade cross-sections stained with chlorogenic acid-HCl from line pZmCesa10::QsuB-5 and a line containing the pZmCesa10::GUS construct. Note in the leaves the reduction of the staining specifically in thick fibers located in both the adaxial and abaxial zones for the line pZmCesa10::QsuB-5 (red arrows).

Additional file 2: Table S1. Oligonucleotides used in the study.

Additional file 3: Figure S3. Full length unprocessed images of PCR gels used for Figs. 2a and S2B. Note that the seven transformants obtained with the pZmUB8-t::QsuB construct were all false positives and did not contain the QsuB gene (see purple rectangle on the PCR gel).

Additional file 4: Figure S5. Representative pictures showing GUS activities in various tiller sections of switchgrass lines harboring the pZmCesa10::QsuB construct. GUS expression is mostly observed in internodes, especially in developing vascular bundles (red arrows). Scale: White bars = 2 mm, black bar = 400 μm, N: node; IN: internode, IS: internode transverse section.
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