Genetic manipulation of lignin reduces recalcitrance and improves ethanol production from switchgrass

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Switchgrass is a leading dedicated bioenergy feedstock in the United States because it is a native, high-yielding, perennial prairie grass with a broad cultivation range and low agronomic input requirements. Biomass conversion research has developed processes for production of ethanol and other biofuels, but they remain costly primarily because of the intrinsic recalcitrance of biomass. We show here that genetic modification of switchgrass can produce phenotypically normal plants that have reduced thermal-chemical (≤180 °C), enzymatic, and microbial recalcitrance. Down-regulation of the switchgrass caffeic acid *O*-methyltransferase gene decreases lignin content modestly, reduces the syringyl:guaiacyl lignin monomer ratio, improves forage quality, and, most importantly, increases the ethanol yield by up to 38% using conventional biomass fermentation processes. The down-regulated lines require less severe pretreatment and 300–400% lower cellulase dosages for equivalent product yields using simultaneous saccharification and fermentation with yeast. Furthermore, fermentation of diluted acid-pretreated transgenic switchgrass using *Clostridium thermocellum* with no added enzymes showed better product yields than obtained with unmodified switchgrass. Therefore, this apparent reduction in the recalcitrance of transgenic switchgrass has the potential to lower processing costs for biomass fermentation-derived fuels and chemicals significantly. Alternatively, such modified transgenic switchgrass lines should yield significantly more fermentation chemicals per hectare under identical process conditions.

biofuel crop | cellulosic ethanol | lignin modification | *Panicum virgatum*

Lignocellulosic biomass is an abundant, domestic, renewable feedstock source that can be converted to liquid transportation fuels and other chemicals by fermentation. Cellulosic ethanol is a promising near-term technological option to reduce transportation sector greenhouse gas emissions (1). Because lignocellulosic biomass is made up of the complex structures of cellulose, hemicellulose, and lignin, such feedstock is highly recalcitrant to bioconversion of its carbohydrates into ethanol compared with starch (2, 3). Current biomass fermentation processes for fuels and chemicals have a relatively high cost primarily because of this recalcitrance, which in turn has limited commercialization of biomass ethanol (4). To achieve sustainable energy production, it is necessary to overcome the chemical and structural properties of biomass that inhibit its deconstruction in dedicated bioenergy crops (5).

The conversion of lignocellulosic biomass to ethanol is a three-step process that involves pretreatment followed by polysaccharide hydrolysis to simple sugars followed by sugar fermentation to ethanol (6). The presence of lignin in cell walls negatively impacts these conversion steps (7, 8). Examination of natural variation in alfalfa, switchgrass, canarygrass, and sorghum has shown that decreased lignin levels improve in vitro enzyme hydrolysis (9, 10). Lignin pathway modification in alfalfa generated transgenic lines with increased enzymatic sugar release essentially proportional to the extent of lignin down-regulation (11). Although transgenic approaches have been used to characterize the lignin biosynthetic pathway and to improve cell wall traits, most of the research has been conducted with dicot species in the context of forage quality and paper pulping (8, 12); only limited information is available for perennial monocot species (13, 14). To date, there has been no report regarding fermentation of improved plants for ethanol production in any genetically modified perennial biofuel crop.

Switchgrass (*Panicum virgatum* L.) is a dedicated energy crop identified by the US Department of Energy (15). It is native to the United States and is a productive perennial C4 species, with a broad cultivation range, that requires relatively minimal agronomic inputs as a biofuel crop (15, 16). Field studies demonstrated that switchgrass grown and managed as a biomass crop produces 540% more renewable energy than energy consumed in its production and has significant environmental benefits (16). Genetic improvement of switchgrass to reduce intrinsic recalcitrance to fermentative bioprocessing would improve biofuel and chemical production processes and have a profound positive impact on the nascent bioenergy industry.

We show here that down-regulation of the caffeic acid 3-*O*-methyltransferase (*COMT*) gene in the lignin pathway leads to the generation of transgenic switchgrass plants with a normal growth phenotype that have reduced lignin content, altered lignin composition, improved forage quality, increased saccharification efficiency, and increased ethanol production yield on substrate compared with the controls. Moreover, the transgenic plant materials require less severe pretreatment and much lower cellulase dosages to obtain ethanol yields equivalent to yields in controls. These transgenic switchgrass lines and the approach are valuable for developing improved cultivars of biofuel crops.

Results

Down-Regulation of the *COMT* Gene in Switchgrass. Based on earlier results of lignin modification in alfalfa and other species (14, 17, 18), we chose to down-regulate the *COMT* gene in switchgrass using the widely distributed variety "Alamo." We constructed a cDNA library from 2-mo-old switchgrass seedlings and isolated a *COMT* cDNA containing a 1,086-bp ORF. Partial sequences of the ORF were used to construct an RNAi vector, which was transferred into *Agrobacterium tumefaciens* strain EHA105, and transgenic switchgrass plants were obtained by *Agrobacterium*-

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Data deposition: The switchgrass COMT coding sequence in this paper has been deposited in GenBank with the (accession no. HQ645965).

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mediated transformation of embryogenic calli. RT-PCR and quantitative real-time PCR analyses of selected transgenic plants revealed significantly reduced transcript abundance (Fig. 1 *A* and *B*) in most of the lines, with more than 90% reduction observed in the lines T0-2 and T0-3 (Fig. 1*B*). To determine whether reduced *COMT* transcript resulted in the reduction of COMT enzyme activity, crude enzyme extracts prepared from the transgenic and control plants were assayed with two preferred COMT substrates, 5-OH coniferaldehyde and caffeyl aldehyde (Fig. 1*C*). Except for line T0-9, all transgenic plants showed significant reduction in COMT enzyme activity. The most strongly down-regulated lines, T0-2 and T0-3, had 22–25% and 25–28% residual enzyme activity, respectively, depending on the substrates used (Fig. 1*C*). T0 lines T0-2, T0-3, and a moderately down-regulated line T0-12 were chosen for further analysis. Because of the outcrossing requirement of switchgrass, these lines were outcrossed with a wild-type plant to obtain progeny seeds designated as T1 lines. Both COMT RNAi-positive and -negative (null segregant) plants were identified from the progeny of each cross, and the negative plants were used as controls for analyses of the corresponding T1 transgenic plants.

Effects of *COMT* Suppression on Lignin Content, Composition and Plant Growth. We examined lignin content and lignin monomer composition in whole tillers (including stem, leaf, and sheath) and stems of transgenic switchgrass. In the T0 generation, transgenic lines T0-2, T0-3, and T0-12 showed significant reduction in their acetyl bromide (AcBr) lignin content (12.2% for T0-2, 14.7% for T0-3, and 6.4% for T0-12), syringyl (S) and guaiacyl (G) lignin monomer content, and S/G ratios for whole tillers (Table 1). Even after outcrossing with wild-type plants, the T1 generation of the two most down-regulated lines, T1-2 and T1-3, showed reductions in both AcBr lignin, at 11.4–13.4%, and S and G lignin content similar to the reductions seen in the respective T0 lines. The resulting S/G ratio was essentially identical in the T1 and T0 lines, at 0.37–0.40, versus 0.69–0.71 in controls (Table 1). The stem material had similar levels of reduction in lignin content and a higher S/G ratio (Table S1).

The composition of the complex cell wall polysaccharides was evaluated to determine potential broader impacts of *COMT* down-regulation. The T0 and T1 lines exhibited small variations in the levels of cellulose, at −3% to −5% for T0 lines and +3% for T1 lines compared with controls (Table S2). The cellulose crystallinity index (CrI) and degree of polymerization (DP) of the T1-2 and T1-3 lines were essentially identical to those of controls (Fig. S1 *A* and *B*). Similarly, the stem material for T0 and T1 transgenic lines showed a −1% to +3% change in cellulose content compared with controls (Table S3). Apparently, the reduced lignin content has minor or negligible impact on cellulose content or structure.

Both T0 and T1 transgenic plants showed normal growth and development (Fig. 2*A*), and for both whole tillers and stems the fresh weight, dry weight, and height of T1-2, T1-3, and T1-12 transgenics were similar to those of the controls (Fig. 2*B* and Table S4). Interestingly, T0-2 and T0-3 and their progeny T1-2 and T1-3, which had the greatest degree of COMT down-regulation, showed brownish coloration at the basal internode of the stem (Fig. 2*D*), and cross-section analysis (without staining) revealed strong coloration at the basal mature internodes (Fig. 2*F*). This coloration diminished in the upper (younger) internodes and was not observed in other tissues such as leaf blade, leaf sheath, and leaf midrib.

Impact of Lignin Down-Regulation on Enzymatic Hydrolysis and Fermentation. A common approach to evaluate the acceptability of plant material for bioconversion to products is the saccharification assay in which the sample is exposed to cellulases and other enzymes that specifically hydrolyze cell wall carbohydrates. Compared with control plants, both T0 and T1 lines showed significant increases in saccharification efficiency with or without mild acid pretreatment (Fig. S2). In the T0 generation, the transgenic lines (except T0-9) showed variously increased levels in enzymatic saccharification efficiency with and without pretreatment (Fig. S2*A*). Similarly, in the T1 generation, T1-2 and T1-3 plants had 16.5–21.5% increase in saccharification efficiency with mild pretreatment and 29.2–38.3% increase without pretreatment (Fig. S2*B*). We also analyzed in vitro true dry matter digestibility (IVTDMD) and neutral detergent fiber digestibility (NDFD), which measures the amount of forage material that can be digested by the rumen of animals and hence is an important indicator of forage quality. The COMT-suppressed plants were more digestible (Fig. S3). In particular, transgenic lines T0-2 and T0-3 had a more than 9% increase in IVTDMD and an 11% increase in NDFD; such a large increase in forage quality has significant value, because switchgrass can serve as a dual-purpose (bioenergy/forage) crop.

Although the impact of lignin pathway modifications on plant material usually is evaluated only with the saccharification assay, such an approach fails to determine the true bioconversion potential by ignoring the response of the fermentation microorganism. Therefore, we examined the potential of the transgenic and unmodified switchgrass to support ethanol production by a combination of enzymatic hydrolysis and yeast fermentation of hydrolyzed carbohydrates to ethanol, termed "simultaneous saccharification and fermentation" (SSF). Fermentation of the transgenic and control switchgrass by SSF without pretreatment demonstrated that transgenic lines T0-2, T0-3, and T0-12, T1-2, T1-3, and T1-12 produced more ethanol per gram of biomass than did their representative control plants (Fig. S4*A*). However, the yield on substrate was low because of the lack of pre-

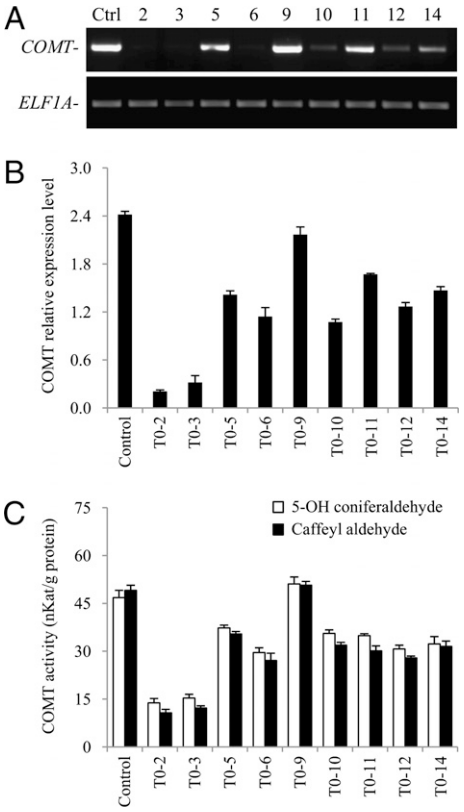

Fig. 1. Analysis of transgenic switchgrass plants. (*A*) RT-PCR gel analysis of *COMT* transcripts. (*B*) Quantitative real-time PCR analysis of *COMT* transcript levels. (*C*) COMT enzyme activity in extracts of transformed plants.

Table 1. Lignin content and composition in T0- and T1-generation transgenic switchgrass plants

Plant line	Acetyl bromide lignin (mg/g CWR)	Thioacidolysis yield (μmol/g CWR)	G lignin (μmol/g CWR)	S lignin (μmol/g CWR)	S/G
Control	192.1 ± 2.5	155.3 ± 2.7	89.5 ± 1.1	63.1 ± 1.3	0.71
T0-2	168.7 ± 1.3**	97.9 ± 3.1**	68.3 ± 1.5**	26.3 ± 1.3**	0.39**
T0-3	163.9 ± 2.2**	102.0 ± 1.3**	70.7 ± 0.6**	28.1 ± 0.5**	0.40**
T0-12	179.8 ± 1.1**	132.5 ± 2.6**	78.5 ± 1.4**	51.2 ± 1.0**	0.65**
Control	185.4 ± 1.2	151.9 ± 1.6	87.7 ± 0.9	60.6 ± 0.7	0.69
T1-2	164.3 ± 1.1**	115.7 ± 1.9**	80.9 ± 0.8**	31.3 ± 0.9**	0.39**
T1-3	160.5 ± 1.2**	104.1 ± 2.2**	73.5 ± 1.0**	27.5 ± 0.8**	0.37**
T1-12	176.3 ± 0.8**	134.3 ± 4.8**	80.0 ± 2.8**	52.8 ± 1.5**	0.66**

Values are means ± SE (n = 3). Tillers (4 cm above the soil surface) at the E4 stage were collected from each T0- or T1-generation plant. CWR, cell wall residue.
**P <0.01.

treatment processing required to open up the plant structure and dramatically improve accessibility of biomass enzymes to the substrate (19, 20).

The transgenic switchgrass lines and their corresponding controls were pretreated under moderate (<190 °C) dilute acid conditions, as discussed later, for the production of ethanol by SSF. Initial pretreatment followed by fermentation was tested with whole tillers of the superior lines T1-2 and T1-3. Under these conditions, the transgenic lines showed similar significant increases in ethanol yield per gram (38% and 30%, respectively) (Fig. 3*A*). Stem material also was tested because it contains more lignin than leaves. Stems from both T0 and T1 lines were analyzed by SSF after the same dilute acid pretreatment. As shown in Fig. 3*A*, both T0 and T1 lines outproduced the relevant controls with 25–35% more ethanol on a weight basis. On a weight basis, stem material produced a larger amount of ethanol than did tiller, with an average increase in ethanol of 59% (Fig. 3*A*). Examination of cellulose content showed stem material (Tables S2 and S3) contained about 18% more cellulose than whole tillers; this additional cellulose can account for only a portion of the additional yield. The remainder of the difference probably results from structural differences in the highly porous stem material and the more compact leaf and sheath material, which would ferment more slowly because of limited enzyme accessibility. The time course of the fermentation of T1 whole tillers was examined by recording the weight loss caused by CO_2 escape with time; the plot (Fig. 3*B*) shows that the transgenic switchgrass had a fermentation pattern similar to the control but was able to produce ethanol more quickly, reaching a higher level by the end of the fermentation. The most notable weight losses occur in the first 2–3 d of fermentation, and more so with the COMT lines, indicating that cellulose was more accessible in the transgenic material.

Examination of Recalcitrance Changes in the Transgenics. To evaluate further the degree of reduced recalcitrance of the COMT down-regulated plants, we analyzed responses of the transgenic biomass to thermal-chemical and biochemical challenges. Thermal-chemical testing, commonly called "pretreatment," involved exposing the switchgrass to various combinations of time and temperature (150–180 °C) conditions in the presence of 0.5% H_2SO_4, yielding different degrees of treatment severity. These conditions are not to be confused with very high temperature thermochemical gasification. Washed, pretreated biomass solids then were fermented in a SSF mode by yeast plus enzymes. As shown in Fig. 3*C*, the severity of pretreatment clearly impacts the level of ethanol in both the control and transgenic switchgrass. However, the transgenic line consistently yielded more fermentation ethanol regardless of the conditions, with differences in yield ranging from 14–28% more ethanol per gram of cellulose except under the most severe condition. Pretreatment conditions are routinely quantified by combined severity (CS) calculations (21) as:

$$\log \mathrm{CS} = \log(\mathrm{t} \bullet \exp[(\mathrm{T} - 100)/14.75]) - \mathrm{pH}$$

The results in Fig. 3*C* are presented with regard to the combined severity value in Fig. 3*D* demonstrating that the transgenic plant material, in this case the T1-2 line, produced more ethanol when pretreated by a variety of increasingly severe conditions. This increase in ethanol continued until the severity of pretreatment was high enough to decrease yield for both the transgenic material and the control switchgrass, probably because of excessive acid-catalyzed carbohydrate degradation (22).

As a second test to characterize the apparent decreased recalcitrance of the transgenic lines, we evaluated the relative impact of enzyme levels in the control and transgenic lines. Although cellulase levels of 15 filter paper enzyme units (FPU) (23)

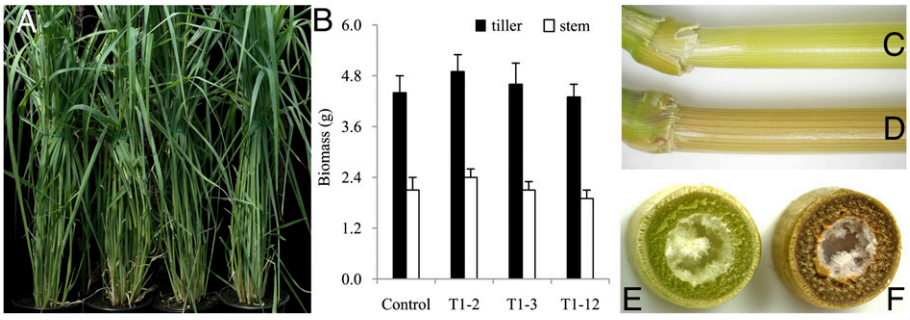

Fig. 2. Phenotype of T1-generation transgenic switchgrass plants. (*A*) Greenhouse-grown switchgrass. Plants shown left to right are control, transgenic T1-2, T1-3, and T1-12. (*B*) Dry weight (DW) of tillers and stems of control and transgenic switchgrass. Values are means ± SE (n = 5). (*C–F*) Basal internode coloration of control (*C* and *E*) and transgenic line (*D* and *F*).

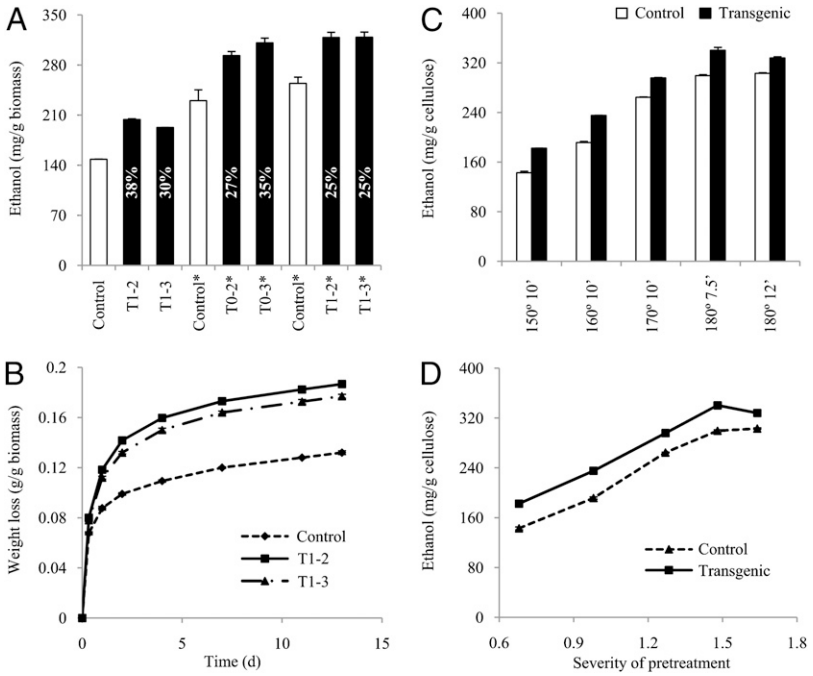

Fig. 3. Bioconversion of transgenic switchgrass to ethanol. (*A*) Fermentation of T1 whole tillers and T0 and T1 stem material after pretreatment at 180 °C, for 7.5 min with 0.5% H_2SO_4. T0 is the original transformant, T1 represents progeny derived from T0. The percent is the relative ethanol yield for the transgenic line compared with the appropriate control. *Stem material. (*B*) Time course of fermentation of whole plant material in *A* measured by weight loss of fermentation bottles. (*C*) Impact of dilute acid pretreatment on ethanol yields of control and the transgenic line T1-2. The *x* axis shows temperature and time of exposure of the plant matter to 0.5% H_2SO_4. (*D*) Analysis of the severity of pretreatment (obtained from the equation in the text) versus ethanol yield for transgenic T1-2 (solid line) and control (dashed line). Fermentations were in triplicate or quadruplicate (stems).

per gram cellulose were used in previous studies, here the enzyme level was modified to 5–60 FPU per gram cellulose using one batch of the pretreated T1-2 line or appropriate control. Fig. 4*A* shows the results of the time course of fermentation by weight loss and endpoint ethanol yield per gram cellulose for both materials. The similar weight loss values were matched with similar ethanol yields, confirming the value of time course data. The COMT down-regulated material consistently responded with more ethanol more quickly for all enzyme loadings. The resulting ethanol yield per gram cellulose is shown in Fig. 4*B*. Ethanol yield for less severely pretreated material is shown in Fig. S4*B*. Although the control switchgrass required 15 or 60 FPU for production of a certain level of ethanol, the transgenic material required only 5 or 15 FPU for equivalent ethanol yield for producing the same levels, respectively (Fig. 4*B* and Fig. S4*B*). The two enzyme–response curves for the control and the transgenic materials are shown in Fig. S4*C*. The data are verified in part by the overlapping fermentation curves (Fig. 4*A*). Therefore, the transgenic line requires 300–400% less enzyme than the nontransgenic material to produce an equivalent ethanol yield per unit of cellulose. The increased susceptibility of the transgenic line to commercial cellulases is not a result of a specific pretreatment condition, because, as shown in Fig. S4*B*, reducing the severity of the pretreatment of both nontransgenic and transgenic switchgrass did not affect the improved susceptibility of the transgenic line. Analysis of multiple conditions of pretreatment and enzyme dosage yielded a data matrix which was used to generate 3D susceptibility curves (Fig. 4*C* and Table S5) for both switchgrass materials. The transgenic switchgrass (orange plane) outproduced the control switchgrass (blue plane) under all process conditions tested, which included four enzyme levels and five pretreatment severity conditions. The results indicate that the susceptibility of the transgenic material is an intrinsic characteristic of the plant line.

We undertook a third approach to characterize the transgenic line's apparent improved susceptibility to bioconversion, the use of a consolidated bioprocessing (cbp)-capable microorganism that is characterized primarily by production of its own biomass hydrolyzing enzymes (24). *Clostridium thermocellum* is a well-studied cbp microorganism with regard to cellulose hydrolysis and fermentation using its multivariate cellulosome (25). Duplicate 1-L fermentations of the transgenic T1-3 line and control switchgrass demonstrated the production of multiple products of acetate, ethanol, and minor levels of lactic acid from 2 g [dry base (db)] biomass, with the transgenic line producing 18% more fermentation products than the control material on a gram product per gram cellulose basis (Fig. 4*D*). In addition, analysis showed that after *C. thermocellum* fermentation, the control switchgrass had 27.2 ± 0.84% of the cellulose remaining, while the transgenic line had only 14.1 ± 2.1% of the cellulose remaining. The results support the observed higher yields of fermentation products for the COMT transgenic line substrate.

Discussion

Development of next-generation commercially useful bioenergy cultivars requires that they exhibit improved fermentation production without compromised biomass yield. Evaluation of the highly suppressed COMT transgenic lines demonstrated they had both normal growth and development and superior susceptibility to bioprocessing. For example, the only phenotypic change we observed between the control and the COMT down-regulated lines was the brownish to reddish color in the basal internode and its cross-sections in the severely down-regulated lines. This color change is a useful trait that can be used easily as a phenotypic maker during the breeding and selection process and also can be used for checking purity of the cultivar and crop stands.

Another critical aspect of using transgenics to develop novel cultivars is the transmission of the desirable trait to the next

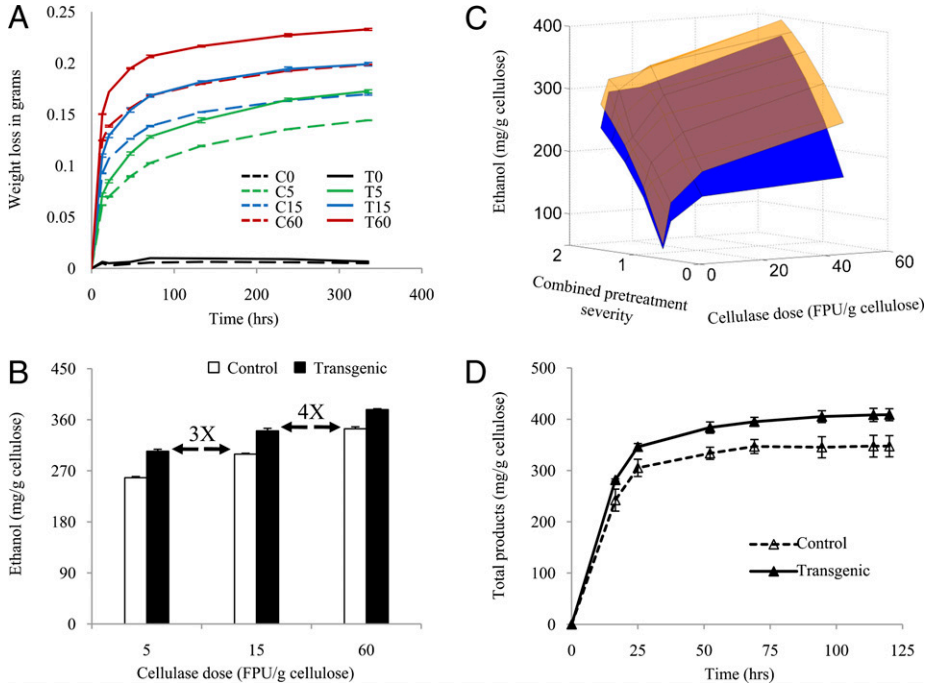

Fig. 4. Impact of cellulase dosages on bioconversion of transgenic and control switchgrass. Samples in *A* and *B* were pretreated at 180 °C for 7.5 min with 0.5% H_2SO_4. (*A*) Time course of fermentation of controls (C, dashed line) and transgenic switchgrass line T1-2 (T, solid line). Cellulase doses were 0, 5, 15, and 60 FPU/g cellulose. (*B*) Final ethanol yield from fermentation in *A* for control and transgenic switchgrass, with comparison of similar ethanol yields with different enzyme dosages. (*C*) Biplanar plot of enzyme dosages of 2.5, 5, 15, and 60 FPU/g cellulose versus the severity of the pretreatment impact on ethanol yield for control (blue) and transgenic (orange) switchgrass. (*D*) Total yield of products (ethanol, acetate, and trace of lactic acid) from control and transgenic switchgrass line T1-3 from *C. thermocellum* fermentation. Fermentation data in *A*–*C* are from triplicate samples and in *D* are from duplicates.

generation, because in some cases the T0 generation may have carryover effects from tissue culture (26). Analyses of T1 generation transgenics revealed that the plants inherited the desirable traits from the parents and showed reduced lignin, increased sugar release, and improved ethanol yield. In addition to increased ethanol production, transgenic switchgrass showed increased forage digestibility, which is beneficial for improving daily weight gain of livestock. Therefore, the material has the potential to be used as a dual-purpose crop and offers more flexibility for farmers.

It is commonly accepted that biomass recalcitrance is the major hurdle for production of biofuel and biochemicals from lignocellulosic materials (5). Here we document the development of an improved dedicated, perennial feedstock that has multifaceted reduced recalcitrance. Regardless of the processes used (i.e., with or without pretreatment, various pretreatment conditions, different enzyme dosages, fermentation by a cbp microorganism), and irrespective of the materials analyzed (i.e., stem vs. whole tiller), the transgenics consistently showed significantly improved fermentation yields. Thus, these switchgrass lines can improve the economic viability of various bio-based fermentation-derived fuels and chemicals by greatly improving the energy, cost, and land-use efficiency of their production. For example, the improved switchgrass lines produced 30–38% more ethanol by SSF for whole plants. Such a large improvement in ethanol yield will have a net effect of reducing land use by about one third or producing significantly more fermentation product per hectare.

COMT-associated brown midrib mutants have been identified in maize (*bm3*) and sorghum (*bmr12*), and a fermentation study for *bmr12* sorghum was reported (10). The COMT mutant of sorghum showed 15% reduction in lignin content and 21% increase in conversion of cellulose to ethanol (10). Possibly because of differences in plant species or growth stage, the increase in ethanol yield in transgenic switchgrass was relatively larger than in the sorghum mutant. Because switchgrass is an obligate outcrossing and polyploid species, the likelihood of finding *bm*- or *bmr*-like mutations in natural switchgrass populations is very low. Thus, transgenic modification remains the most effective method for targeted improvement of this important biofuel species. Because the sorghum double mutant (COMT and cinnamyl alcohol dehydrogenase) exhibited additive effects in lignin reduction and improvement in ethanol yield (10), we anticipate that combinations of different transgenes may further benefit biofuel production.

The transgenic switchgrass requires lower chemical and energy inputs in the form of lower pretreatment severity compared with wild type. Reduction of pretreatment severity also will reduce production of acid-catalyzed sugar degradation products furfural and hydroxymethylfurfural that decrease available fermentable sugars and inhibit fermentation rate and yields (27). Enzymes are the single largest processing cost component for bioconversion of biomass after the biomass itself (28). Significantly, the transgenic lines require only one-quarter to one-third the level of enzymes for equivalent ethanol fermentation compared with the unmodified switchgrass. Therefore, use of the transgenic switchgrass lines as feedstock can reduce the cost of biomass processing by 21–25% for enzymes alone after excluding biomass and capital charges (28, 29). Furthermore, because this three- to fourfold increased susceptibility to cellulases is unrelated to the pretreatment conditions, the down-regulated switchgrass lines simultaneously provide feedstock with both lower energy and processing costs for biomass fermentation. The *C. thermocellum* fermentation is particularly interesting because it demonstrates improved bioconversion of washed, pretreated transgenic switchgrass on a mass basis without any added cellulases, further supporting development of even lower-cost biomass-based fuels and chemicals.

The reduced recalcitrance of these improved switchgrass lines will impact more than biomass ethanol production. Newly emerging bio-based fuels such as butanol, isobutanol, and fermentation "green gasoline" will require a biomass-based feedstock for improved economics and long-term sustainability. Because switchgrass is a perennial crop that easily survives for more than 10 y and allows the harvest of large annual production of biomass, utilization of the transgenic approach probably is one of the most effective and economical ways for feedstock improvement. Together with the development of new processing and conversion methods, this technology will enable the development of an economic and efficient industry converting lignocellulosic biomass into liquid fuels.

Materials and Methods

Plant Materials and Growth Conditions. A lowland-type switchgrass cultivar, Alamo (2n = 4× = 36), was used for genetic transformation and lignin modification. Switchgrass plants were grown in the greenhouse at 26 °C with 16 h light (390 $\mu E \cdot m^{-2} \cdot s^{-1}$). The vegetative development of switchgrass was divided into four elongation stages (E1, E2, E3, and E4) according to the criteria described by Moore et al. (30). T1-generation plants were obtained by crossing T0 transgenics with a wild-type Alamo plant. Isolation of switchgrass COMT cDNA sequences, construction of RNAi vector, and generation of transgenic switchgrass plants are described in *SI Materials and Methods*.

Molecular Characterization of Transgenic Plants. Molecular analysis of the transgenics was performed as described in *SI Materials and Methods*.

Biochemical Characterization of Transgenic Plants. Assay of COMT activity and determination of lignin content and composition were performed as described in *SI Materials and Methods*.

NMR and Gel Permeation Chromatography Analysis of Cellulose. Isolation of cellulose, NMR, and gel permeation chromatography (GPC) analysis of cellulose were performed as described in *SI Materials and Methods*.

Measurement of Forage Quality. Forage quality analysis was performed as described in *SI Materials and Methods*.

Determination of Saccharification Efficiency. Saccharification efficiency was determined as described in *SI Materials and Methods*.

Pretreatment and Fermentation. Pretreatment and fermentation was performed as described in *SI Materials and Methods*.

Statistical Analysis. Triplicate samples were collected for each transgenic line. Data from each trait were subjected to one-way ANOVA. The difference between transgenic and control plants was evaluated by Dunnett's test. SEs are provided in all tables and figures as appropriate.

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