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Field Evaluation of Transgenic Switchgrass Plants Overexpressing *PvMYB4* for Reduced Biomass Recalcitrance

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Abstract High biomass yields and minimal agronomic input requirements have made switchgrass, *Panicum virgatum* L., a leading candidate lignocellulosic bioenergy crop. Large-scale lignocellulosic biofuel production from such crops is limited by the difficulty to deconstruct cell walls into fermentable sugars: the recalcitrance problem. Our goal in this study was to assess the field performance of switchgrass plants overexpressing the switchgrass MYB4 (*PvMYB4*) transcription factor gene. PvMYB4 transgenic switchgrass can have great lignin reduction, which commensurately increases sugar release and biofuel production. Our results over two growing seasons showed that one transgenic event (out of eight) had important gains in both biofuel (32 % more) and biomass (63 % more) at the end of the second growing season relative to non-transgenic controls. These gains represent a doubling of biofuel production per hectare, which is the highest gain reported from any field-grown modified feedstock. In contrast to this transgenic event, which had relatively low ectopic overexpression of the transgene, five of the eight transgenic events planted did not survive the first field winter. The dead plants were all high-overexpressing events that performed well in the earlier greenhouse studies. Disease susceptibility was not compromised in any transgenic events over the field experiments. These results demonstrate the power of modifying the expression of an endogenous transcription factor to improve biofuel and biomass simultaneously, and also highlight the importance of field studies for "sorting" transgenic events. Further research is needed to develop strategies for fine-tuning temporal-spatial transgene expression in feedstocks to optimize desired phenotypes.

Keywords MYB4 · Field trial · Lignocellulosic biofuel · Switchgrass

Holly L. Baxter and Charleson R. Poovaiah are equal contributors.

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Introduction

Switchgrass (*Panicum virgatum* L.) is a perennial warm season C4 grass that has long been considered a forage crop and is now considered to be a promising dedicated cellulosic bioenergy feedstock. It is widely adapted to eastern North America, produces ample biomass, and has good nutrient and water use characteristics [1]. It has been shown to increase water quality and sequester high amounts of carbon, and its use as a biofuel feedstock would also result in reduced greenhouse gas emissions [2].

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One of the major problems with lignocellulosic biofuel production is the recalcitrance of the cell wall to deconstruction. Given cell walls' natural resistance to degradation, they are difficult to break down into fermentable sugars by enzymatic, chemical, and microbial methods [3]. Lignin, one of the components of the cell wall, provides structural support and integrity for the plant [4] and is one of the main causes of recalcitrance. The processing of lignocellulosic biomass for biofuel production generally requires a pretreatment step to deconstruct the cell wall for a more efficient release of sugars and improved fermentation yields. However, pretreatment is one of the most expensive steps in the production of biofuels and can also create and facilitate the release of inhibitors that impede the fermentation process [5, 6].

Various enzyme-coding genes in the lignin biosynthetic pathway have been modified through genetic engineering to reduce cell wall recalcitrance [7]. Another approach for modifying the lignin pathway is by manipulating transcription factors that might affect the expression of multiple lignin biosynthetic genes. For example, *PvMYB4* codes for an R2-R3-type transcription factor that acts as a transcriptional repressor of many lignin biosynthetic genes. To determine its effect on lignin biosynthesis and cell wall recalcitrance in switchgrass, *PvMYB4* was overexpressed in two "Alamo"-derived switchgrass parental clones (ST1 and ST2) driven by the maize ubiquitin (*ZmUbi*) promoter. Some of the greenhouse-grown transgenic events had lignin reductions of up to 40–50 % relative to the controls, while no changes were observed in the S/G lignin monomer ratio [8]. Increased tillering and reduced plant stature as a result of the *PvMYB4* overexpression was noted in some events. These transgenic plants also had increased saccharification efficiency by up to 300 % without pretreatment and ethanol yield improvements of up to 2.6-fold over the control; however, there was large variation among the independent transformants [8, 9]. Since these initial studies were performed using greenhouse-grown plants, it is essential to follow up with field studies using the biorefinery-relevant senesced material harvested at the end of the growing season. Field experiments will shed light on how plants with altered lignin biosynthesis perform in real-world conditions to evaluate their biotic- and abiotic-stress tolerance, agronomic performance, and biomass production [10, 11].

In this study, we conducted a field experiment using multiple transgenic switchgrass plant events overexpressing *PvMYB4*. These transgenic events were produced and characterized previously under greenhouse conditions [8, 9]. We sought to compare the data from the field and greenhouse experiments to assess the degree of congruency for cell wall traits, sugar release efficiency, and ethanol yield. We also analyzed agronomic performance and rust susceptibility of the transgenic plants in the field.

Materials and Methods

Plants and Field Design

T_0 generation *PvMYB4*-overexpressing plants were produced in two Alamo-derived switchgrass parental clones (ST1 and ST2) as described previously [8, 9]. ST1-derivative plants used in the field study included four independent transgenic events (L1, L2, L6, L8) and three independent non-transgenic ST1 controls derived from tissue culture (ST1 CK-1, CK-2, CK-3). ST2-derivative plants included five independent transgenic events (1A, 1B, 1C, 1D, 1E) and two independent ST2 vector controls (ST2 CK-1, CK-2). All clonal replicate plants were transplanted onto a University of Tennessee-Knoxville farm on July 3, 2012. The field site was 19.1 m×16.8 m containing plants arranged in a randomized block design with blocking on the switchgrass parental clone (ST1 and ST2) (Fig. S1). Three replicate plots were included for each independent control and transgenic event, and four vegetatively propagated T_0 clones of each transgenic event were planted within each replicate plot. Each replicate plot was planted 152 cm apart with 76-cm spacing among the four genetically identical clones within the replicate. The experimental plants were surrounded by border Alamo plants. Transgenic plants in the ST2 background did not survive the first winter; only ST2 control plants survived (Fig. S2). Therefore, all the ST2 control plants were removed from the field in the second growing season (2013) and the experiment was continued as a completely randomized design for the ST1 transgenic and non-transgenic control plants.

Field Maintenance

The field experiment was carried out across two growing seasons in 2012 and 2013 (Fig. S3). Transplants were irrigated as needed during the first growing season for establishment. Soil fertility was not adjusted and weeds were removed by tillage or hand weeding. Panicles at R0-R1 stage [12] below the last node were removed from all plants including non-transgenic controls and border plants according to the conditions required by the USDA Animal and Plant Health Inspection Service Biotechnology Regulatory Services (APHIS BRS) release into the environment permit for the regulated articles.

Agronomic Performance

Aboveground biomass of plants began to senesce in late October to early November for both growing seasons. The first frost in each growing season occurred on November 1 in 2012 and October 25 in 2013. Tiller height, tiller number, and plant width were determined after all aboveground biomass was completely senesced in December of each year. Tiller

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height values for each plant were represented by the tallest tiller (R0 growth stage) from each individual plant, which was measured from the base of the soil to the tip of the top leaf. Plant width was determined by measuring the diameter at the mid-section of each whole plant.

All aboveground (senesced) biomass was harvested from the field in early December of each year. Biomass was oven dried at 43 °C for 96 h and weighed to determine total dry weight biomass yield. Subsamples of dry biomass were milled with a Wiley mill (Thomas Scientific, Model 4, Swedesboro, N.J.) through a 20-mesh (1.0 mm) screen and used for cell wall chemistry and fermentation analyses.

Analysis of Transcript Levels of *PvMYB4* and Lignin Biosynthetic Genes

Tillers at the R0 growth stage were randomly selected from two plants within each transgenic and control replicate. All samples were collected at the same date and time in August 2013. The top portion of each selected tiller (containing two intact top leaves) was sampled by cutting below the top internode and flash freezing in liquid nitrogen. Once transported back to the lab, samples were stored at −80 °C until quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis could be performed. Total RNA isolation and qRT-PCR analysis were performed as described previously [8, 13]. The primer pairs used for the *PvMYB4* transcript analysis were MYB_7X_QRTF2: 5′-AGCCTCCT CGGCAACAAGT-3′ and MYB_7X_QRTR2: 5′-TTGATG TGCGTGTTCCAGTAGTT-3′. The primer pairs used to assess the relative transcript abundance for selected lignin biosynthetic genes (Pv4CL1, PvCAD1, PvCCR1, and PvCOMT1) have been described previously [13].

Cell Wall Chemistry, Sugar Release, and Ethanol Fermentation

Lignin content and composition were estimated using a published method [14] wherein soluble extractives and starch were removed from the biomass samples and the remaining cell wall residues were analyzed. Samples (~4 mg) were pyrolyzed at 500 °C in 80-μl stainless steel cups using an Extrel single quadrupole molecular beam mass spectrometer. Lignin content was estimated from the relative intensities of the lignin precursor peaks. S/G monolignol ratio was determined by dividing the sum of the intensity of syringyl peaks by the sum of the intensity of guaiacyl peaks.

High-throughput pretreatment and enzymatic hydrolysis was performed as previously described [15] with some modifications. Starch and soluble extractives were removed from the biomass samples utilizing amylases followed by ethanol extraction in a Soxhlet extractor [16]. Cell wall residues were loaded into custom-made 96-well metal plates in triplicate. Samples comprised ~1.7 % *w/w* solids in water and were pretreated with condensing steam at 180 °C for 17.5 min. Enzymatic hydrolysis was then performed by sample incubation at 50 °C for 70 h with 70 mg protein/g biomass Ctec2 enzyme cocktail (Novozymes North America, Franklinton, NC) followed by colorimetric assays for glucose and xylose released into the liquid (Megazyme Intl., Bray, Ireland).

Prior to fermentation, samples were analyzed for wet chemistry (carbohydrate composition) using the quantitative saccharification assay described in Fu et al. [17] and Yee et al. [18]. The fermentation methods were described previously in Mielenz et al. [19] and Fu et al. [17]. Briefly, the fermentation experiments were performed in biological triplicate at a dry solid loading of 5.0 % (*w/v*) in a total volume of 20 ml at a pH of 4.8 with a final concentration of 50 mM citrate buffer and 0.063 mg/mL streptomycin. Cellic® Ctec2 was loaded at 20 mg protein per gram dry biomass, Novozyme 188 was loaded at 25 % volume ratio to Ctec2, and Cellic® Htec2 was loaded at 20 % volume ratio to Ctec2. For separate hydrolysis and fermentation (SHF), the biomass and enzymes were incubated at 50 °C and 120 rpm for 5 days. Following the completion of enzymatic hydrolysis, the hydrolysate was fermented with *Saccharomyces cerevisiae* D5A (ATCC 200062) at 35 °C and 150 rpm with a final concentration of 0.5 % *w/v* yeast extract. The simultaneous saccharification and fermentation (SSF) process was similar to that of SHF, but the yeast and enzymes were added simultaneously (fermentation and hydrolysis occurred concurrently) and the process was conducted at 35 °C and 150 rpm. Samples were not removed from the bottles during the fermentation; instead, weight loss was used to monitor the progress [17]. The end point metabolites and simple carbohydrates were measured by HPLC as described in Raman et al. [20], and the yields were calculated as described in Yee et al. [21].

Determination of Wall-Bound Phenolics

Levels of *p*-hydroxybenzaldehyde, vanillin, *p*-coumaric acid, and ferulic acid were quantified using the procedure described in Shen et al. [22]. After removing extractives, 100 mg CWR was subjected to alkaline hydrolysis (2.0 M NaOH, 30 °C, 24 h) to release wall-bound phenolics. Levels of phenolic compounds were quantified using HPLC.

Rust Evaluation

Rust (*Puccinia emaculata*) disease severity was rated from May 31–July 5, 2013, following published methods [11]. Herein, single tillers from three plants within each replicate were chosen at random and tagged for visual assessment of rust infection throughout the growing season. For each selected tiller, the coverage of the leaf tops with rust uredia was visually estimated using the following rating scale: 0=0 %, 1

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≤5 %, 2 ≤10 %, 3 ≤25 %, 4 ≤40 %, 5 ≤55 %, 6 ≤70 %, and 7 ≤100 % of leaf area coverage with uredia. The whole field (transgenic, non-transgenic control, and border plants) was treated for rust on July 1, 2013, and October 11, 2013, with "Quilt" fungicide (Syngenta Canada Inc., Guelph, Ontario) at a rate of 0.21 ml/m^2 because of the high rust incidence throughout the field. The field was also treated for *Bipolaris* with two applications of "Heritage" fungicide (Syngenta Crop Protection, Greensboro, N.C.) on September 6, 2013, and September 24, 2013, at a rate of 20 ml/m^2.

Statistical Analysis

Data for plants in the ST2 background were not included in the analyses since the transgenic plants did not survive past the first growing season. Therefore, the ST1 transgenic events (L1, L6, and L8; L2 was later identified as chimeric for the transgene and excluded from analyses) and controls were analyzed as a completely randomized design for years 1 and 2. Since no significant differences were found among the means of the three independent ST1 non-transgenic controls, they were averaged and used as a single control. Means were analyzed with one-way ANOVA using PROC MIXED in SAS version 9.3 (SAS Institute Inc., Cary, N.C.). Differences with p values less than 0.05 were considered significant, and letter groupings were obtained by Fisher's least significant difference method. Data was evaluated for normality using Shapiro-Wilk test ($p>0.05$) and for equal variance using Levene's test ($p>0.05$).

Results

Field Plants and Design

None of the plants in the ST2 clonal background survived the first field winter except the control plants (Fig. S2). The field experiment was therefore analyzed as a completely randomized design with the ST1 control and transgenic events in 2012 and 2013. Analyses were performed on the ST1 transgenic events (L1, L6, and L8) and the aggregate non-transgenic control (the average of three ST1 non-transgenic control lines).

Transcript Levels of *PvMYB4* and Lignin Biosynthetic Genes

The ST1 transgenic events exhibited a 3–16-fold increase in *PvMYB4* expression level relative to the control during mid-season assessment. The highest level of *PvMYB4* expression was found in transgenic L1 (16-fold) followed by transgenic L8 (7-fold) and L6 (3-fold) events. This is similar to the greenhouse study which found that events L6 and L8 showed intermediate expression levels compared to the relatively high expression observed in L1 [9]. The level of *PvMYB4* transcript abundance in transgenic L2 event was similar to the control (Fig. S4a). Transcript levels of lignin biosynthetic genes *Pv4CL1*, *PvCAD1*, *PvCCR1*, and *PvCOMT1* decreased by ~2.3-, 2.1-, 2.0-, and 2.5-fold, respectively, in transgenic L1 event, while they were either unchanged or reduced up to 1.5-fold in transgenic L2, L6, and L8 events (Fig. S4b).

Agronomic Performance

Transgenic events L6 and L8 did not differ from the control in end-of-season aboveground biomass production in the first year (2012), whereas event L1 exhibited a 70 % yield reduction. In the second year (2013), biomass yield in event L6 was similar to the control, while event L8 had a 63 % increase over the control. Similar to the first year, event L1 had a 79 % yield reduction relative to the control in year 2 (Table 1).

Transgenic events L6 and L8 did not differ from the control in tiller height in year 2, whereas event L1 had a 35 % reduction in height relative to the control. The number of tillers in events L6 and L8 was significantly higher compared to control, with an increase of 21 and 38 %, respectively, whereas event L1 had 17 % fewer tillers than the control. Plant width did not differ among transgenic events L6 and L8 and the control. Event L1 had a 40 % reduction in width compared to the control (Table 1).

Lignin Content and Composition

Pyrolysis molecular beam mass spectrometry (py-MBMS) lignin was decreased after year 1 in transgenic events L1 (8.9 % reduction) and L8 (5.2 % reduction) relative to the non-transgenic control plant (Fig. 1a). The corresponding S/G lignin monomer ratios in events L1 and L8 were decreased by 6.7 and 6.8 %, respectively (Fig. 1b). Event L6 did not differ from the control in lignin content or the S/G ratio (Fig. 1a, b). In the second year, total lignin content was decreased by 5–12 % in the transgenic events relative to the control. The highest reduction was observed in L1 (12 %) followed by L8 (7 %) and L6 (5 %) (Fig. 1c). Event L1 also exhibited the most dramatic reduction in lignin content relative to the control under greenhouse conditions [9]. The S/G lignin monomer ratio for second year-harvested plants decreased by 12 % in event L1, whereas events L6 and L8 were not different from the control (Fig. 1d).

Wall-Bound Phenolic Compounds

The greenhouse study with *PvMYB4*-overexpressing switchgrass plants noted a reduction in several phenolic fermentation inhibitors in the transgenic plants, including *p*-hydroxybenzaldehyde (*P*-HBA), *p*-coumaric acid (*p*-CA),

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Table 1 Morphology and dry biomass yield of field-grown transgenic events during the 2012 and 2013 growing seasons

Year	Event	Dry biomass yield (g/plant)	Tiller height (cm)	Plant width (cm)	Tiller number
2012	L1	11.02±01.88b	ND	ND	ND
	L6	38.26±08.01a	ND	ND	ND
	L8	54.14±08.55a	ND	ND	ND
	Control	36.55±04.81a	ND	ND	ND
2013	L1	82.00±15.10c	94.19±07.21b	69.22±04.68c	79.33±8.01c
	L6	483.67±84.23ab	148.38±09.42a	123.40±11.27ab	116.25±2.50a
	L8	646.67±23.61a	166.16±02.80a	142.66±10.51a	132.83±4.01a
	Control	399.22±63.40b	145.70±11.86a	116.77±09.03ab	95.69±3.78b

Each independent transgenic event (L1, L6, L8) was compared to an average of three ST1 wild-type controls. Values represent the mean of the biological replicates for each transgenic event (n=3) and control (n=9)±standard error. Means were analyzed with a one-way ANOVA, and letter groupings were obtained using Fisher's least significant difference method. Values followed by the same letter do not differ significantly at the 5 % level

ND no difference.

and ferulic acid (FA) [9]. To examine changes in phenolic compounds in field-grown PvMYB4 plants, alkaline hydrolysis was performed on extractives-free cell wall residues to release wall-bound phenolics which were then measured using HPLC. *P*-HBA content was slightly reduced in line L1 (1.8 %) but did not differ among lines L6 and L8 and the control. Vanillin content was similar between each transgenic line and the control. A reduction in *p*-coumaric acid (*p*-CA) was observed in L1 (19.0 %) and L8 (11.8 %) relative to the control but was unchanged in L6. FA content was similar among the transgenic lines and the control. The wall-bound *p*-CA/FA ratio was reduced by 24.3 % in L1 compared to the control (Table 2).

Sugar Release Efficiency

Sugar release efficiency in senesced plant material was determined by enzymatic hydrolysis. Hot water pretreatment was performed on cell wall residues prior to treatment with hydrolytic enzymes. In the first year, event L1 exhibited the highest release in total sugars (24.0 %) compared to the control, with a 28.9 % improvement in glucose release and an 18.6 %

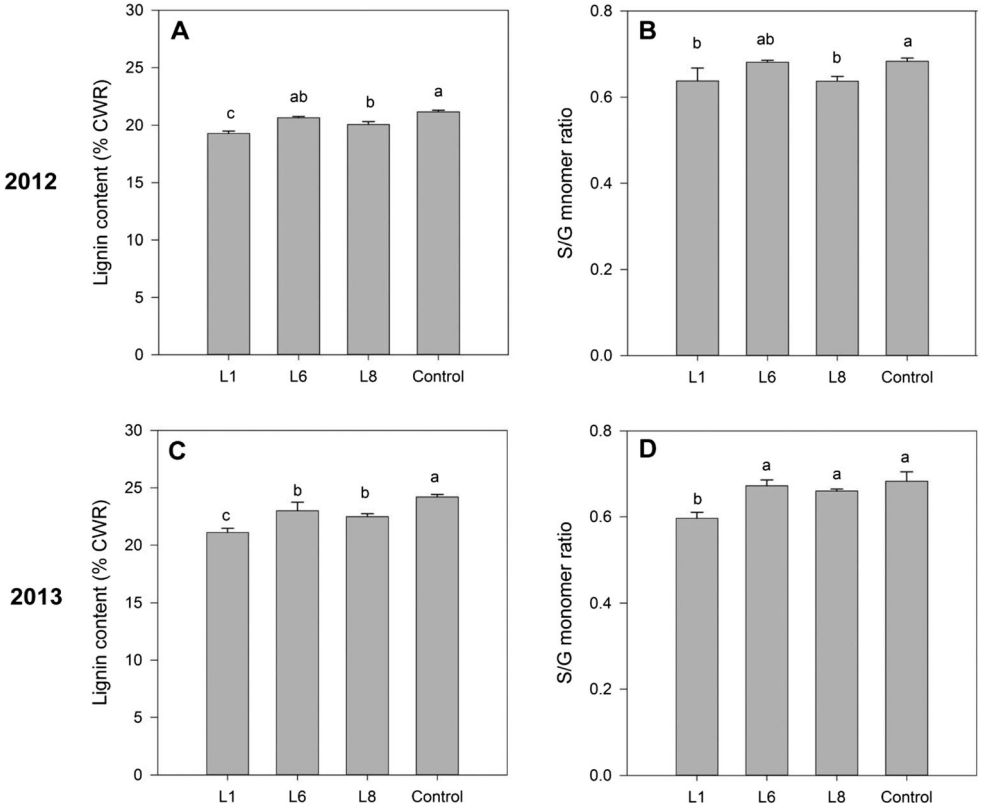

Fig. 1 Lignin content and S/G ratio of senesced biomass samples were estimated by py-MBMS for 2012 (**a**, **b**) and 2013 (**c**, **d**). Each independent transgenic event (L1, L6, L8) was compared to an average of three ST1 wild-type controls. *Bars* represent the mean of the biological replicates for each transgenic event (n=3) and control (n=9)±standard error. Means were analyzed with a 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. *CWR* cell wall residues

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Table 2 Contents of wall-bound phenolics in cell wall residues of senesced transgenic and control samples harvested during the 2013 growing season

Line	*P*-HBA	Vanillin	*p*-CA	FA	*p*-CA/FA ratio
L1	5.56±0.01b	4.44±0.01a	18.74±0.50b	3.40±0.08a	5.52±0.01b
L6	5.63±0.00ab	4.46±0.00a	21.08±0.22ab	3.17±0.14a	6.67±0.00ab
L8	5.62±0.02ab	4.46±0.02a	20.42±0.49b	3.00±0.04a	6.80±0.02ab
Control	5.66±0.02b	4.47±0.02a	23.13±0.76a	3.19±0.06a	7.29±0.02a

Each independent transgenic event (L1, L6, L8) was compared to an average of three ST1 wild-type controls. Values represent the mean of the biological replicates for each transgenic event (n=3) and the control (n=9)±standard error. Means were analyzed with a one-way ANOVA, and letter groupings were obtained using Fisher's least significant difference method. Values followed by the same letter do not differ significantly at the 5 % level. A separate ANOVA was run for each phenolic compound. *P*-HBA, *p*-hydroxybenzaldehyde; *p*-CA, *p*-coumaric acid; FA, ferulic acid

improvement in xylose release. The total sugar release in event L8 was 13.5 % higher than that in the control, with an 18.1 % improvement in glucose release and an 8.4 % increase in xylose release. Event L6 did not differ from the control in glucose, xylose, or total sugar release (Fig. 2a). In the second year, the total sugar release, glucose release, and xylose release in event L1 all showed improvements by 30.7, 54.0, and 12.4 %, respectively, compared with the control. No significant differences in sugar release were observed among transgenic events L6, L8, and the control (Fig. 2b).

Ethanol Yield

Under greenhouse conditions, ethanol yield was increased by up to 2.6-fold in *PvMYB4*-overexpressing plants relative to the controls [9]. The fermentation potential of the senesced field-grown biomass from the first (2012) and second (2013) growing seasons was determined using separate hydrolysis and fermentation (SHF) for unpretreated material and simultaneous saccharification and fermentation (SSF) procedures for hot water-pretreated material. Weight loss from CO_2 release upon venting the fermentation bottles at various time points was used to monitor the progress of the fermentation (data not shown). When the weight loss ceased, the fermentation was deemed complete and end point samples were taken. For the first growing season, the transgenic events (L1, L6, and L8) fermented at a faster rate and had increased end point ethanol yields of 29, 16, and 35 %, respectively, over the ST1 non-transgenic control (Fig. 3a). Of the transgenic events, L8 and L1 had the largest gain in fermentation yield compared to the control, but L1 had reduced biomass yield from the field in comparison to the control. As a result, L8 and a control (ST1 CK-3) were chosen for further screening by SSF that was preceded by hot water pretreatment. Similarly, L8 had a 21 % improvement in ethanol yield over the control (Fig. 4a). The pretreatment conditions had a significant impact on ethanol yield in all plants (transgenic and control) by approximately doubling biofuel yield compared to no pretreatment.

As observed in year 1, senesced year 2 biomass samples had SHF ethanol yields that were higher in L1 (69 %) and L8 (28 %) compared to the control. The apparent gain in L6 was not statistically significant (Fig. 3b). Event L1 had the greatest improvement in ethanol yield, but, as in year 1, the biomass

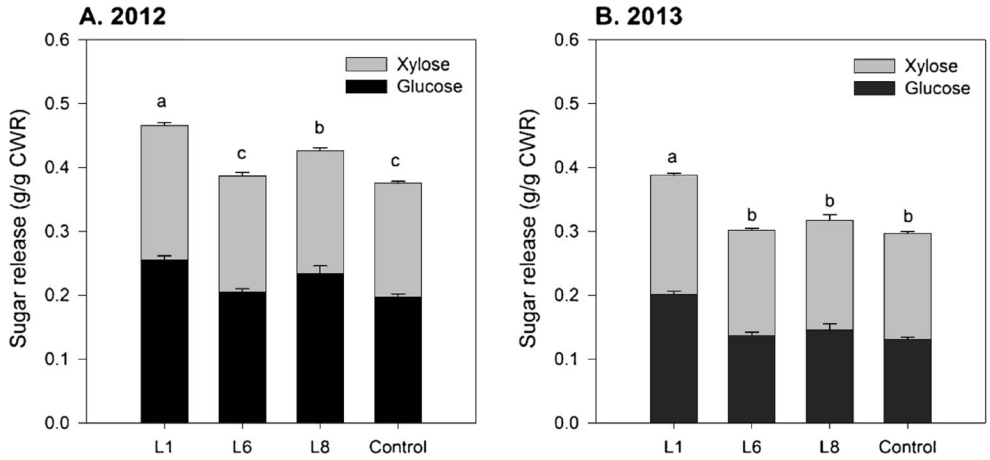

Fig. 2 Sugar release by enzymatic hydrolysis from senesced and pretreated biomass samples for 2012 (**a**) and 2013 (**b**). Each independent transgenic event (L1, L6, L8) was compared to an average of three ST1 wild-type controls. *Bars* represent the mean of the biological replicates for each transgenic event (n=3) and control (n=9)±standard error. Means were analyzed with a 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. *CWR* cell wall residues

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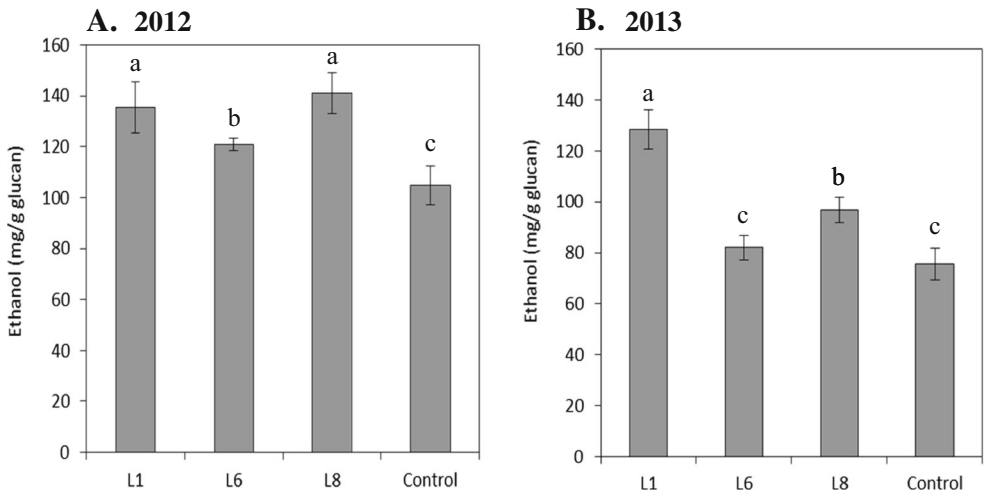

Fig. 3 Ethanol yield by SHF of unpretreated senesced biomass for 2012 (**a**) and 2013 (**b**). Each independent transgenic event (L1, L6, L8) was compared to an average of three ST1 wild-type controls. *Bars* represent the mean of the biological replicates for each transgenic event (n=3) and control (n=9)±standard deviation. Means were analyzed with a 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

yield was significantly reduced in comparison to control. As before, L8 was selected for further fermentation screening using hot water pretreatment and SSF, in which there was a 32 % higher SSF fermentation yield than the control (Fig. 4b). Pretreatment improved the fermentation yield for both the transgenic events and the control by approximately 1.8-fold. Interestingly, regardless of whether or not the biomass was unpretreated or hot water pretreated, the ethanol yields for all samples (transgenic and control) were comparatively higher in the first year than the second, even though the trend of the transgenic having a higher fermentation yield than the control remained regardless of harvest year or pretreatment condition.

Disease Susceptibility

Switchgrass rust caused by *P. emaculata* and leaf spot caused by a *Bipolaris* species were the most prevalent diseases observed throughout the field. Weekly evaluations of rust disease severity began when the first symptoms of rust appeared on plants on May 31, 2013, through the first week of July 2013. Rust symptoms were observed in all transgenic and non-transgenic plants except for L1 (Fig. S5), and the disease severity in the susceptible events ranged from 0–2 % in late May to 11–23 % in early July. Severity of rust damage among L6, L8, and control plants did not significantly differ at any of the six time points assessed (Fig. 5). Because of the severity and rapid progression of the rust throughout the entire field, plants were treated with fungicide to enable downstream analyses. By July 12, 2013, rust incidence had decreased to <5 % coverage of the upper leaves on all plots as plants recovered from the disease. Leaf spot disease caused by *Bipolaris* spp. was observed shortly after plant emergence in May 2013 and continued to progress in severity until treating with Heritage fungicide in

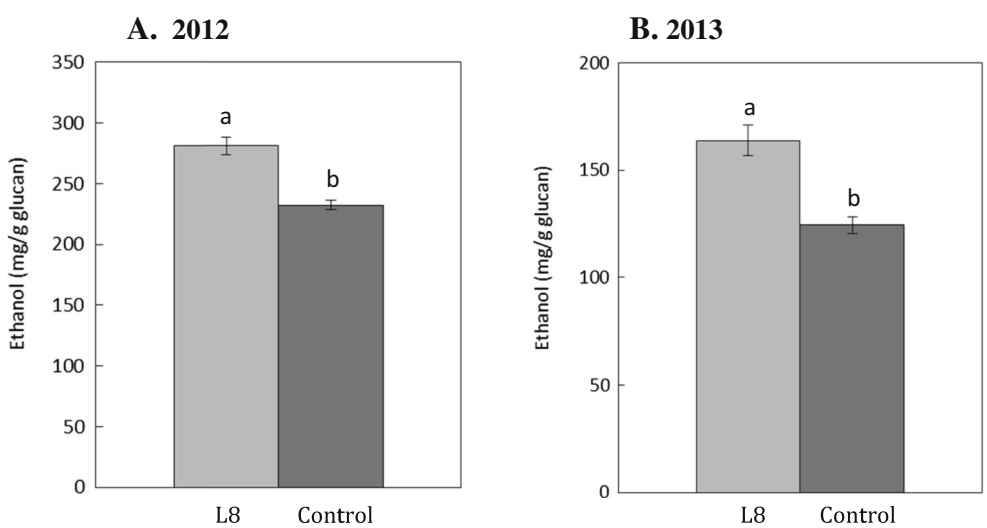

Fig. 4 Ethanol yield by SSF of hot water-pretreated senesced biomass for 2012 (**a**) and 2013 (**b**). The independent transgenic event (L8) was compared to the individual ST1 wild-type control (ST1 CK-3). *Bars* represent the mean of the biological replicates for L8 (n=3) and the control (n=3)±standard deviation. Means were analyzed with a 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

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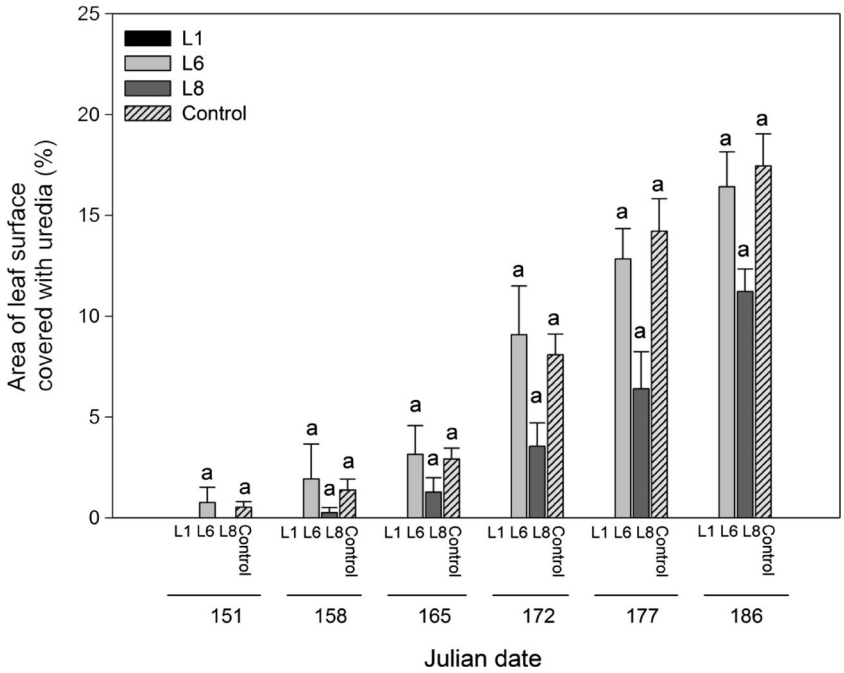

Fig. 5 Rust disease severity during the second growing season (2013). Each independent transgenic event (L1, L6, L8) was compared to an average of three ST1 wild-type controls. *Bars* represent the mean of the biological replicates for each transgenic event (n=3) and control (n=9)±standard error. For each Julian date, means were analyzed with a 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

September 2013 (Fig. S6). There was negligible apparent insect herbivory on any plants during the study.

Discussion

The quest for improved bioenergy feedstocks is leading to the utilization of numerous genetic modifications of lignocellulosic feedstocks. However, the goal of making plant cell walls more easily converted into biofuel and bioproducts without compromising plant robustness in the field is ambitious considering the possible pleiotropic effects of reduced lignin on plant fitness in an agricultural setting [10, 23]. Several studies have shown that genetic manipulation of lignin can be a viable means of reducing recalcitrance in lignocellulosic crops [7–9, 17, 24–28], but many of these experiments have only been conducted in greenhouses where environmental conditions are tightly controlled and the actively growing green tissue was analyzed. Experiments conducted under such conditions cannot predict how transgenic plants might perform in the field. Lignin is essential for normal plant growth and development [29] and also plays a role in several biotic and abiotic stress responses [30]. Negative effects of lignin modification on either of these processes could potentially be exacerbated under field conditions, in which plants are constantly subjected to a variety of biotic and abiotic challenges. Therefore, field experiments are especially important for plants with modified cell walls to ensure that chemotypes with altered lignin or other cell wall constituents are maintained as plants mature across multiple growing seasons.

Transgene Expression: Spatial and Temporal Effects are Crucial for Transcription Factors

At the end of our second field season of assessing the performance of transgenic switchgrass with *PvMYB4* overexpression, there were two surprising results. The biggest surprise was the complete mortality of all five independent ST2 clone-engineered transgenic events over the first winter. The ST2 control plants, however, all survived in the field, indicating that the lack of robustness was genetic background independent; the most likely cause of mortality was the effects of high transgene expression. These ST2 transgenic events all had relatively high transgene expression [8] relative to the ST1 events [13]. Consequently, the ST2 transgenic plants exhibited dramatic reductions in lignin content relative to controls (40 % decrease on average) under greenhouse conditions. We noted that the root systems were diminished in these events relative to controls upon transplantation into the field (data not shown), which indicates that high constitutive overexpression of this transcription factor might be disruptive to the plants, especially the roots. Studies have shown that genetic modification of lignin in perennial herbaceous crops can, in some species, be associated with reduced growth and higher overwinter mortality [10, 23]. When 4CL-downregulated poplar was analyzed across a range of field environments, the transgenic events with the lowest total lignin content generally had reduced rates of survival and growth compared to controls [31]. Taken together, these studies suggest that there may be a threshold in lignin reduction which, after being reached, can result in adverse effects on growth. The role of lignin in roots is largely unknown, as are the effects of its reduction. Above-

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average rainfall was also observed during the 3 months after transplantation (data not shown) which, combined with the weak and underdeveloped root system, may have further contributed to the mortality of the ST2 transgenic plants.

A second surprising result in our study was that low overexpression of the *PvMYB4* transgene could increase both biofuel and biomass in switchgrass. In the field, one ST1 event (L8) maintained a reduction in lignin content (5–7 %), up to a 32 % increase in ethanol, and produced 63 % more biomass relative to the control at the end of the second growing season. The biofuel gain was expected considering the greenhouse results with the ST1 transgenic events [9], but the biomass gain was unexpectedly increased. Furthermore, none of the transgenic events were more susceptible to disease. To our knowledge, this is the first field study reporting the results of a transgenic lignocellulosic grass with modified lignin biosynthesis resulting from the overexpression of a transcription factor.

Both surprises point to the critical importance of tuning the expression of transgenes in biofuel crops, especially if the transgene encodes a regulatory factor such as a transcription factor or microRNA. While gene silencing with constitutive promoters generally results in minimal or no pleiotropic effects on lignocellulosic grass feedstocks grown in the greenhouse [17, 24, 28] as well as under field conditions [11, 32, 33], the overexpression of regulatory factors that influence the expression of multiple genes tends to produce a wider range of phenotypes [25, 34]. Since PvMYB4 is a transcriptional repressor that modulates the expression of several lignin biosynthesis genes, its overexpression has wider-ranging effects on the lignin biosynthetic pathway compared to single-gene silencing [8]. Control of transgene expression, especially when overexpressing transcription factors that impact multiple genes, is important for reducing the pleiotropic effects caused by strong constitutive promoters. Tissue-specific promoters, such as those specific to secondary cell wall biosynthesis, could be used to avoid or minimize such pleiotropic effects [35]. To that end, we have started to "rebuild" the construct, followed by the production of new transgenic switchgrass events for PvMYB4 with the goal of altering lignin in shoots but not roots.

PvMYB4 Overexpression on Phenolic Composition

Greenhouse studies have demonstrated that the phenolic composition of switchgrass, which can be altered by lignin modification, is another important factor affecting recalcitrance [9, 21, 36]. RNAi-mediated gene silencing of COMT resulted in higher levels of certain phenolic acids and aldehydes, some of which were shown to be inhibitory to fermentation microbes [21, 36]. Alternatively, *PvMYB4* overexpression in greenhouse-grown plants resulted in lower levels of phenolic fermentation inhibitors relative to the controls. Specifically, levels of wall-bound *p*-CA and ether-linked FA, as well as the ester-linked *p*-CA/FA ratio, were negatively correlated with SSF ethanol yield of unpretreated material [9]. Among the field-grown ST1 transgenic events, the lines showing significant ethanol gains over the control (L1 and L8) also exhibited a 12–19 % reduction in wall-bound *p*-CA content. Interestingly, event L8 did not exhibit an increased sugar release relative to the control in year 2 but still maintained a significantly higher ethanol yield. We therefore speculate that the increased ethanol production might be associated with changes in phenolic fermentation inhibitors such as coumaric acid or related compounds. L1 also had a lower *p*-CA/FA ratio, which has been reported to be associated with increased sugar release efficiency in switchgrass [22]. Taken together, the field and greenhouse data suggest that the decreased recalcitrance observed in *PvMYB4*-overexpressing switchgrass might be influenced by multiple factors including lower levels of certain phenolic compounds [9].

Lignin Modification and Fitness

Considering the important role of lignin in plant development, yield penalties are of concern whenever lignin biosynthesis is modified [29, 37]. Alterations in growth and morphology were observed in ST1 transgenic events, although these were not as severe as those observed in the ST2 plants. Of the ST1 transgenic events, the most dramatic effects on plant morphology were observed in event L1, which exhibited a reduction in tiller height, plant width, and tiller number, and a corresponding 79 % reduction in biomass relative to the control in year 2. Event L1 had significantly higher expression levels of *PvMYB4* and lower lignin content than events L6 and L8 in the greenhouse [9] as well as in the current field study. Relatively low overexpression of *PvMYB4* was observed in events L6 and L8. These plants were similar to the control in tiller height and width but produced significantly more tillers. This is consistent with the greenhouse studies which reported an increased tillering phenotype in most of the *PvMYB4*-overexpressing events [8, 9]. Growth was not compromised in either of these events; alternatively, the biomass yield was increased in event L8. Increased biomass was also observed in COMT-downregulated switchgrass grown under field conditions [11], demonstrating that biomass yield in transgenic plants with reduced lignin can, in some cases, be enhanced. In the *PvMYB4*-overexpressing plants, it appears that the negative growth effects and yield penalties might be associated with high expression levels and tend to be minor or negligible in the events with low-to-moderate overexpression levels.

Lignin content and composition are strongly influenced by biotic and abiotic stresses [30, 37], and transgenic plants with

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reduced lignin content may or may not retain the low-lignin phenotype when tested under field conditions [31, 38]. In the current study, the total lignin content of the field-grown transgenic ST1 events remained consistently lower than that of the control in fully established plants. The most dramatic changes were observed in the highest *PvMYB4*-overexpressing event (L1) which exhibited a 12 % decrease in both the total lignin content and the S/G lignin monomer ratio compared to the control. Other field studies with lignocellulosic grasses have also confirmed that low-lignin phenotypes associated with lignin-modified transgenic crops can be maintained under field conditions [11, 32, 33].

Lignin acts as a non-degradable physical barrier to pests and pathogens [30], and multiple lignin biosynthetic genes have been shown to be upregulated as a putative defense response to biotic stresses [39]. Consequently, conducting field experiments with lignin-modified plants is important to ensure that altering lignin does not increase disease susceptibility. Recent field studies have shown that lignin can be reduced in grass species without affecting disease susceptibility, specifically to rust [11, 32]. In the current study, *Bipolaris* and rust (*P. emaculata*) were the dominant pathogens observed during the field experiment, both of which have previously been identified as potentially damaging to switchgrass fields [40]. In the second growing season, the first rust symptoms were observed in late May and rapidly progressed in severity until the plot was sprayed with a fungicide in early July. The reduced lignin content did not appear to impact rust susceptibility in any of the transgenic events. Interestingly, L1 was the only event in the field (including transgenic, non-transgenic control, and border plants) that did not show any symptoms of rust for the duration of the field experiment. There have been reports of enhanced resistance to pathogens in plants with modified lignin biosynthesis; the mechanisms are unclear but may be related to downstream effects of lignin suppression on the synthesis of defense-related compounds and/or defense signaling triggered by changes to cell wall integrity [37, 41]. As discussed previously, L1 exhibited a significantly higher expression level of *PvMYB4* than L6 or L8 under greenhouse [9] and field conditions. Decreased lignin might have led to a more dramatic effect on downstream metabolic processes in L1 that could also affect defense. For example, altered phenolic composition resulting from lignin pathway modifications can affect microbial growth in transgenic plants [42, 43]. Such changes could have triggered an enhanced resistance to rust, although further studies would be needed to confirm this. *Bipolaris* was observed in all plants for both years and did not appear to differ in severity among control and transgenic plants based on visual observation. In the second growing season, leaf spot symptoms from *Bipolaris* were mild to moderate in severity in the first few months after plant emergence but worsened in early fall until a fungicide was applied. The severity of fungal pathogens in the field was comparatively worse in the second growing season than in the first, likely from the above-average rainfall and humidity, which are reported to worsen the effects of *Bipolaris* and rust in monocots [40, 44].

Conclusion

In order to achieve sustainability, biofuel feedstocks must be agronomically robust while having cell walls that are relatively easy to convert to biofuels, i.e., low recalcitrance. Obtaining this combination might only be obtained via biotechnology. It appears as if a PvMYB4 transcription factor with optimal expression makes important gains for switchgrass in this regard, a result that should be portable to a number of feedstock species. The results also illustrate the importance of conducting multiyear field studies. The field studies provided crucial information about when, where, and the degree of optimal transgene expression to obtain the desired phenotype: the "Goldilocks Effect." In the case of PvMYB4, if expression is too high, the plants cannot survive in the field. If the expression is too low, no difference from controls are obtained, but when expression is just right, then switchgrass produces high biomass that is more easily converted into biofuel. When extrapolated into an agronomic production scenario, event L8 could yield over twice the biofuel per hectare than its non-transgenic counterpart and without increased disease susceptibility.

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