Plant Biotechnology Journal

Plant Biotechnology Journal (2013) 11, pp. 709–716 doi: 10.1111/pbi.12061

RNA interference suppression of lignin biosynthesis increases fermentable sugar yields for biofuel production from field-grown sugarcane

Je Hyeong Jung[1], Wilfred Vermerris[1,2,3,4], Maria Gallo[1,2,3,†], Jeffrey R. Fedenko[1], John E. Erickson[1] and Fredy Altpeter[1,2,3,*]

[1]*Agronomy Department, University of Florida, IFAS, Gainesville, FL, USA*
[2]*Plant Molecular and Cellular Biology Program, University of Florida, IFAS, Gainesville, FL, USA*
[3]*Genetics Institute, University of Florida, Gainesville, FL, USA*
[4]*Department of Microbiology and Cell Science, University of Florida, IFAS, Gainesville, FL, USA*

Received 8 December 2012;
revised 28 January 2013;
accepted 30 January 2013.
**Correspondence* (fax +352-392-7248; email altpeter@ufl.edu)
†Current address: College of Tropical Agriculture and Human Resources, University of Hawai'i-Mānoa, 3050 Maile Way, Honolulu, HI, 96822, USA.

Keywords: caffeic acid *O*-methyltransferase (COMT), field performance, lignocellulosic biofuel, saccharification efficiency, *Saccharum*, sugarcane.

Summary

The agronomic performance, cell wall characteristics and enzymatic saccharification efficiency of transgenic sugarcane plants with modified lignin were evaluated under replicated field conditions. Caffeic acid *O*-methyltransferase (*COMT*) was stably suppressed by RNAi in the field, resulting in transcript reduction of 80%–91%. Along with *COMT* suppression, total lignin content was reduced by 6%–12% in different transgenic lines. Suppression of *COMT* also altered lignin composition by reducing syringyl units and *p*-coumarate incorporation into lignin. Reduction in total lignin by 6% improved saccharification efficiency by 19%–23% with no significant difference in biomass yield, plant height, stalk diameter, tiller number, total structural carbohydrates or brix value when compared with nontransgenic tissue culture–derived or transgenic control plants. Lignin reduction of 8%–12% compromised biomass yield, but increased saccharification efficiency by 28%–32% compared with control plants. Biomass from transgenic sugarcane lines that have 6%–12% less lignin requires approximately one-third of the hydrolysis time or 3- to 4-fold less enzyme to release an equal or greater amount of fermentable sugar than nontransgenic plants. Reducing the recalcitrance of lignocellulosic biomass to saccharification by modifying lignin biosynthesis is expected to greatly benefit the economic competitiveness of sugarcane as a biofuel feedstock.

Introduction

Sugarcane (*Saccharum* spp. hybrids) is a highly productive, perennial C_4 grass and a major feedstock for global bioethanol and sugar production (Tew and Cobill, 2008). In 2010, more than 1.7 billion tonnes of sugarcane were produced on 23.9 million hectares worldwide, and its production, on a dry weight basis, is higher than any other crop in the world (FAOSTAT; http://faostat3.fao.org/home/index.html#DOWNLOAD).

Sugarcane accumulates up to 50% of its dry weight as sucrose in the internodes (Waclawovsky *et al.*, 2010). Sucrose-derived ethanol production from sugarcane has been successfully commercialized in Brazil, with high environmental sustainability and average production costs that are 24% lower than the cost of ethanol production from maize in the USA (Crago *et al.*, 2010; Goldemberg, 2007). Sugarcane stalk harvest followed by sucrose extraction generates a large amount of lignocellulosic residue (green tops, leaf litter and bagasse) comprising 55% of total aboveground biomass (Somerville *et al.*, 2010; Tew and Cobill, 2008; Vermerris, 2011). Including both sucrose and lignocellulosic biomass from sugarcane as feedstocks for ethanol production will boost the ethanol yield per land area while increasing sustainability and adding environmental benefits (Goldemberg, 2007; Leite *et al.*, 2009; Somerville *et al.*, 2010).

The presence of lignin in the cell wall is a major problem for cellulosic ethanol production because it limits the accessibility of cellulose and hemicellulose and reduces the activity of cellulolytic enzymes (Jørgensen *et al.*, 2007; Mansfield *et al.*, 1999). Therefore, an energy-intensive pretreatment of the biomass is typically required for disruption of the cell wall matrix and degradation of lignin. Such pretreatments may adversely affect downstream ethanol production by degrading sugars and generating inhibitory molecules (Alvira *et al.*, 2010; Yang and Wyman, 2008). Therefore, reduction in lignin content or modifications of its structure are attractive targets to enhance the production of cellulosic biofuel.

Lignin is formed from the polymerization of three monolignols, *p*-coumaryl, coniferyl and sinapyl alcohol. After incorporation of these monolignols into the lignin polymer, they are referred to as *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units, respectively (Bonawitz and Chapple, 2010). Grass lignin also contains considerable amounts of the hydroxycinnamic acids such as *p*-coumarate and ferulate. Ferulate plays a role in interconnecting hemicellulosic cell wall polysaccharides and lignin (Grabber, 2005; Grabber *et al.*, 1996), whereas *p*-coumarate, esterified primarily to the gamma carbon of sinapyl alcohol (Ralph *et al.*, 1994), enhances the incorporation of this monolignol in the growing lignin polymer (Hatfield *et al.*, 2008). Among

monolignol biosynthetic genes, caffeic acid *O*-methyltransferase (*COMT*) encodes the enzyme that catalyses *O*-methylation at the C5 position of 5-hydroxyconiferaldehyde and 5-hydroxyconiferyl alcohol, yielding sinapaldehyde and sinapyl alcohol, respectively (Bout and Vermerris, 2003; Humphreys *et al.*, 1999; Louie *et al.*, 2010; Osakabe *et al.*, 1999). COMT deficiency or suppression in brown midrib mutants (maize *bm3* and sorghum *bmr12*) or in transgenic plants reduces both S units and total lignin content, consistent with the role of COMT in sinapyl alcohol biosynthesis (Barrière *et al.*, 2004; Fu *et al.*, 2011; Guo *et al.*, 2001; Marita *et al.*, 2003; Palmer *et al.*, 2008).

Down-regulation of lignin biosynthetic gene(s) or the identification of mutants with reduced lignin content is a viable strategy to improve saccharification efficiencies and/or cellulosic ethanol yields as demonstrated with a number of crops (Chen and Dixon, 2007; Dien *et al.*, 2009; Saballos *et al.*, 2008) including transgenic switchgrass (Fu *et al.*, 2011; Saathoff *et al.*, 2011; Xu *et al.*, 2011) and sugarcane (Jung *et al.*, 2012).

Caffeic acid *O*-methyltransferase–suppressed transgenic sugarcane lines were previously generated in our laboratory by RNA interference (RNAi), and they showed significant reductions in total lignin and S subunit content (Jung *et al.*, 2012). Fermentable glucose yields were significantly increased in the transgenic sugarcane lines, following enzymatic hydrolysis of the lignocellulosic biomass. A moderate reduction in lignin (up to 8%) did not compromise biomass production under greenhouse conditions (Jung *et al.*, 2012).

To our knowledge, data on field performance of transgenic C_4 grass species with modified lignin content or composition have not been reported. Such data are critically important to evaluate the growth and feedstock performance following production under realistic growing conditions. In this study, cell wall characteristics, saccharification efficiencies and plant growth performance of *COMT*-suppressed sugarcane lines were evaluated in a field trial under USDA-APHIS permit 11-040-120n.

Results

COMT gene suppression in transgenic sugarcane lines

Caffeic acid *O*-methyltransferase gene suppression through RNAi was evaluated 6 months after initiation of the field trial. Transcript abundance of *COMT* was significantly reduced by 80%, 89%, 92% and 91% in the transgenic lines T41, T23, T31 and T4, respectively, compared with wild-type (WT) sugarcane (Figure 1).

Effects of *COMT* suppression on lignin content and composition

Total lignin content in stalk samples of transgenic and control plants grown in the field was determined using the acetyl bromide (AcBr) method. There was no significant difference in total lignin content of the WT, the nontransgenic tissue culture control (NT) or the *npt*II transgenic control (TC) (Table 1). However, the transgenic lines had 4.5%–11.1% lower total lignin content compared with WT. In comparison with the corresponding TC, the level of total lignin reduction was 5.5%, 7.5%, 11.2% and 12.0% in the transgenic lines T41, T23, T31 and T4, respectively (Table 1).

The S subunit content was significantly reduced by 16% and 49% in T41 and T4, respectively, compared with the TC control (Table 1). There were no significant differences in G subunit content among the transgenic lines, WT or the TC line (Table 1).

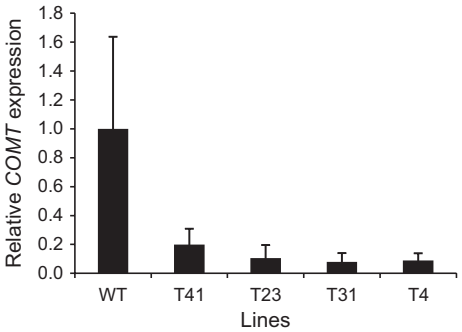

Figure 1 Real-time RT-PCR analysis of Caffeic acid *O*-methyltransferase (*COMT*) expression level in transgenic sugarcane.WT: wild-type sugarcane; T41, T23, T31 and T4: transgenic sugarcane lines. Error bars represent the 95% confidence intervals of the $2^{-\Delta\Delta Ct}$ value ($n = 3$).

Table 1 Lignin content and composition in transgenic sugarcane

Line	AcBr lignin mg/g DW	G units μmol/g AcBr lignin	S units μmol/g AcBr lignin	S/G molar ratio
WT	192.2 ± 1.9[a]	97.4 ± 5.4[a]	143.9 ± 3.5[a]	1.48[a]
NT	193.3 ± 2.1[a]	na	na	na
TC	194.3 ± 1.7[a]	102.4 ± 4.0[a]	150.0 ± 4.8[a]	1.47[a]
T41	183.6 ± 0.7[b]	106.8 ± 1.6[a]	125.4 ± 2.6[b]	1.17[b]
T23	179.8 ± 1.2[b]	na	na	na
T31	172.5 ± 0.8[c]	na	na	na
T4	170.9 ± 1.3[c]	107.2 ± 5.7[a]	77.1 ± 1.0[c]	0.72[c]

Lines include: WT, wild-type sugarcane; NT, nontransgenic control derived from tissue culture; TC, transgenic control harbouring the *npt*II gene; T41, T23, T31 and T4: transgenic sugarcane lines.

G, guaiacyl subunit; S, syringyl subunit; DW, dry weight; na, not analyzed.

Values are means ± standard error of the mean; different letters within the same column indicate significant differences among means ($n = 3$, $P < 0.05$) as determined by Tukey's test.

Total lignin content was analyzed using the acetyl bromide (AcBr) method.

Due to a reduction in S subunits, T41 and T4 transgenic lines had significantly lower S/G molar ratios of 1.17 and 0.72, respectively, compared with 1.47 for the TC and 1.48 for the WT.

Effects of *COMT* suppression on cell wall carbohydrates and cell wall-bound hydroxycinnamic acids

The consequences of *COMT* suppression on other cell wall components, such as cellulose, hemicellulose and cell wall-bound hydroxycinnamic acids, were investigated in the two transgenic lines, T41 and T4, which represented the lines with the least and most reduction in lignin content, respectively, versus the WT and the TC (Table 2). The amounts of glucose, mostly derived from cellulose, did not differ significantly between the transgenic and control plants. The amount of xylose, the major hemicellulose component, was significantly increased in T4 compared with T41 or control plants, while the amount of arabinose was not different among the lines. The total amount of cell wall sugars did not differ significantly between the transgenic and control plants.

The content of cell wall-esterified *p*-coumaric acid (*p*CA) and ferulic acid (FA) was evaluated following mild alkaline hydrolysis.

Table 2 Composition of structural carbohydrates of the cell wall in control and transgenic sugarcane plants

	Glucose	Xylose	Arabinose	Total sugar
Line	mg/g DW	mg/g DW	mg/g DW	mg/g DW
WT	462.0 ± 17.5[a]	234.9 ± 6.2[b]	7.7 ± 0.3[a]	704.6 ± 23.6[a]
TC	446.1 ± 2.6[a]	228.6 ± 1.1[b]	7.5 ± 1.0[a]	682.3 ± 4.5[a]
T41	429.0 ± 2.7[a]	223.6 ± 0.6[b]	9.5 ± 0.1[a]	662.1 ± 3.1[a]
T4	441.8 ± 5.0[a]	250.2 ± 0.8[a]	9.2 ± 0.1[a]	701.2 ± 5.4[a]

Lines include: WT, wild-type sugarcane; TC, transgenic control harbouring the *npt*II gene; T41 and T4, transgenic sugarcane lines.
DW, dry weight.
Values are means ± standard error of the mean; different letters in the same column indicate significant differences among means ($n = 3$, $P < 0.05$) as determined by Tukey's test.

The esterified *p*CA content was significantly decreased by 8% and 32% in T41 and T4, respectively, compared with the WT (Table 3). Esterified FA did not differ significantly between the transgenic and control plants.

Effect of lignin reduction on saccharification efficiency

Enzymatic hydrolysis following dilute-acid pretreatment was performed to evaluate the bioconversion efficiency of lignocellulosic biomass from field-grown transgenic sugarcane into directly fermentable sugars. The time course of saccharification describes elevated saccharification efficiencies of the transgenic biomass compared with WT and TC during the entire 72-h period of enzymatic hydrolysis (Figure 2a). A maximum saccharification rate was reached at 72 h of enzymatic hydrolysis, and saccharification efficiencies for T41 and T4 were 23.2% and 32.4% higher than for the TC control. Cellulose in the transgenic lines was converted to glucose more rapidly compared with WT or the TC control. Transgenic lines T41 and T4 had saccharification efficiencies of 49.2% and 54.8%, respectively, after only 24 h of enzymatic hydrolysis, values which exceeded the 48.4% and 46.7% conversion for WT or TC at 72 h of enzymatic hydrolysis, respectively.

To evaluate the effect of enzyme dosage on the saccharification of transgenic and control sugarcane plants, hydrolysis was performed with different cellulase loadings of 5, 20 and 60 filter paper unit (FPU) per gram of cellulose. All lines showed the highest saccharification efficiencies at the highest cellulase loading (60 FPU) (Figure 2b). However, lignocellulosic biomass from transgenic sugarcane plants was more effectively converted to glucose than that of WT or the TC control, regardless of enzyme dosage. For T41 and T4, saccharification efficiencies at 5 FPU were similar to those at 20 FPU for WT or the TC control. Furthermore, at 20 FPU, saccharification efficiencies of 49.9% and 55.9% in T41 and T4 were significantly higher than those obtained with 60 FPU in the WT or TC control, respectively.

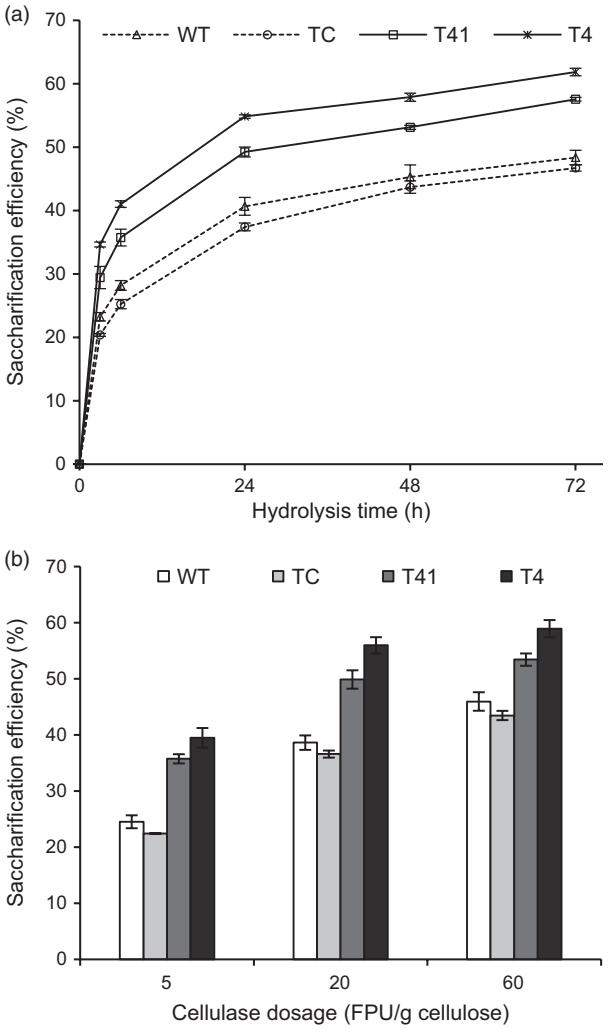

Figure 2 Enzymatic saccharification performance in wild-type (WT), transgenic control harbouring *npt*II gene alone (TC) and transgenic sugarcane lines (T41 and T4). (a) Time course of enzymatic hydrolysis of ground extract-free stalk sample with 60 filter paper unit (FPU)/g cellulose following dilute-acid pretreatment. (b) Saccharification efficiencies at different cellulase dosages (5, 20 and 60 filter paper unit (FPU)/g cellulose). Error bars represent standard error of the mean ($n = 3$).

Table 3 Content of cell wall-bound *p*-coumaric acid (*p*CA) and ferulic acid (FA) after mild alkaline hydrolysis of the control and transgenic sugarcane

	*p*CA	FA
Line	mg/g DW	mg/g DW
WT	14.4 ± 0.3[a]	2.2 ± 0.2[a]
TC	14.5 ± 0.3[a]	2.3 ± 0.1[a]
T41	13.3 ± 0.3[b]	2.4 ± 0.2[a]
T4	9.9 ± 0.1[c]	2.7 ± 0.1[a]

Lines include: WT, wild-type sugarcane; TC, transgenic control harbouring the *npt*II gene; T41 and T4, transgenic sugarcane lines.
DW, dry weight.
Values were means ± standard error of the mean; different letters in the same column indicate significant differences among means ($n = 3$, $P < 0.05$) as determined by Tukey's test.

Growth performance of transgenic sugarcane grown under field conditions

Clonally produced rootstocks derived from the vegetative progeny (V1) of transgenic events and control plants established well

and were grown for 7 months under field conditions in Citra, FL (Figure 3a,b). The transgenic line, T41, with 6% lignin reduction, displayed no significant difference in biomass production compared with the TC and NT lines (Table 4). Stalk length and diameter were also not significantly different between T41, TC and NT lines. However, lines of T23, T31 and T4 with 8%, 11% and 12% reduction in lignin, respectively, displayed a 21%, 64% and 65% reduction in biomass, respectively, compared with the TC line. This reduction in biomass can primarily be attributed to reduced stalk diameter and height in T31 and T4 compared with the controls. In comparison with the original CP88-1762 WT plants, both the NT tissue culture controls and the TC control lines had an 18% reduction in biomass.

For the transgenic lines, the number of internodes per stalk was not statistically different from WT or the control lines, indicating that the developmental stage was similar among the lines at the time of harvest (Table 4). The number of stalks per plant was not different among the lines, except in transgenic line T31, which had fewer stalks per plant (Table 4). The amount of soluble solids in the stalk did not differ significantly between the transgenic lines T41 and T23 and control lines. However, transgenic lines T31 and T4 displayed significantly reduced concentrations of soluble solids in their stalks compared with the control lines (Table 4).

Diseases, pests and lodging were monitored monthly. A minor occurrence of orange rust (*Puccinia kuehnii*) was observed during the main growth period and was rated between 0 and 1 in all of the lines, with no significant differences between the lines. A pink sugarcane mealybug (*Saccharicoccus sacchari)* infestation was observed in August, and the pest was eliminated by pesticide application after scoring. The rate of mealybug infestation did not significantly differ between the transgenic and control plants, ranging from 10% to 37% of the internodes being infested. The transgenic and control plants did not show any lodging throughout the growing season until an isolated thunderstorm hit the field on 10 October 2011 with a maximum wind speed of 68 km/h. After the storm, lodging was most severe in the part of the field facing the prevailing wind direction during the severe thunderstorm, with no significant difference between transgenic lines and WT or TC controls (Figure 3).

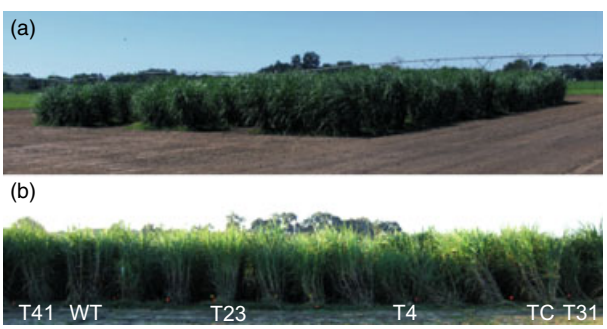

Figure 3 Field trial of the Caffeic acid *O*-methyltransferase (*COMT*)-suppressed transgenic sugarcane lines. (a) The field trial of transgenic sugarcane 6 months after establishment, 1 October 2011. (b) Sugarcane growth in block 1 at the time of harvest, 26 October 2011. WT: wild-type sugarcane; TC: transgenic control harbouring the *nptII* gene; T41, T23, T31 and T4: transgenic sugarcane lines. Border plants were removed before harvest.

Table 4 Growth characteristics of the transgenic sugarcane lines under field conditions

Line	No. of Internodes	Biomass (kg/plant)	Stalk height (cm)	Stalk diameter (mm)	No. of Stalks per plant	Soluble solids (° Brix)
WT	18.5^{a}	18.5^{a}	204^{a}	28.2^{a}	20.5^{a}	20.0^{a}
NT	17.6^{a}	15.2^{b}	180^{bc}	27.5^{a}	18.7^{a}	19.9^{a}
TC	18.3^{a}	15.7^{b}	185^{b}	26.5^{ab}	19.8^{a}	20.4^{a}
T41	18.2^{a}	15.7^{b}	182^{b}	25.1^{bc}	22.8^{a}	20.3^{a}
T23	18.9^{a}	12.4^{c}	191^{ab}	23.7^{cd}	23.0^{a}	20.1^{a}
T31	17.9^{a}	5.5^{d}	161^{dc}	21.6^{de}	14.0^{b}	17.5^{b}
T4	18.7^{a}	5.3^{d}	155^{d}	19.8^{e}	19.0^{a}	17.2^{b}

Lines include: WT, wild-type sugarcane; NT, nontransgenic control derived from tissue culture; TC, transgenic control harbouring the *npt*II gene; T41, T23, T31 and T4, transgenic sugarcane lines.

Values are means; different letters in the same column indicate significant differences among means ($n = 3$, $P < 0.05$) as determined by Fisher's least significant differences.

Discussion

To our knowledge, this is the first report on the field performance of a transgenic C_4 grass with modified cell wall composition. The field-grown *COMT*-suppressed transgenic sugarcane lines showed improvement in bioconversion efficiency of mature lignocellulosic biomass to fermentable glucose. Suppression of *COMT* transcripts ranging from 80% to 91% resulted in a reduction in lignin content by 6% to 12% in different transgenic lines and an improvement of saccharification efficiency by 19% to 32% compared with nontransgenic and transgenic (*npt*II-only) controls. The transgenic lines required one-third of the hydrolysis time and 3- to 4-fold less enzyme to produce an equal or higher amount of glucose than control plants. These findings are consistent with earlier reports on greenhouse-grown *COMT*-suppressed transgenic switchgrass (Fu *et al.*, 2011) or sugarcane (Jung *et al.*, 2012). *COMT* suppression in switchgrass increased ethanol production by 38% or enabled reduced pretreatments and 3- to 4-fold less enzyme loading to produce an equal amount of ethanol compared with the control. Elevated biofuel production from lignocellulosic sugarcane biomass will boost the sucrose-derived biofuel yields for this prime biofuel crop. Accelerated rates of bioconversion, less severe pretreatment conditions and/or lower enzyme loadings to achieve yields of fermentable sugars equal to or higher than with wild-type biomass are expected to significantly reduce biofuel production costs.

The level of RNAi-induced *COMT* suppression in the different transgenic lines that was observed earlier under greenhouse conditions (Jung *et al.*, 2012) was stably maintained under field conditions. Reductions in total lignin and S subunit content in the field-grown transgenic lines were consistent with those previously reported for greenhouse-grown plants. Suppression of *COMT* in transgenic sugarcane specifically resulted in a significant reduction in S subunit content without affecting G subunit content, similar to COMT-deficient maize *brown midrib* (*bm3)* and sorghum *bmr12* mutants (Barrière *et al.*, 2004; Palmer *et al.*, 2008).

The transgenic sugarcane lines displayed reduced *p*-coumaric acid (*p*CA) levels. *p*CA is primarily esterified to S lignin subunits in grasses (Grabber *et al.*, 1996; Ralph *et al.*, 1994). The reduced

*p*CA content most likely resulted from a reduction in S subunits in the *COMT*-suppressed transgenic sugarcane plants, similar to maize *bm3* and sorghum *bmr12* mutants (Marita *et al.*, 2003; Palmer *et al.*, 2008; Piquemal *et al.*, 2002), and *COMT*-suppressed transgenic maize (Piquemal *et al.*, 2002). Ferulic acid (FA) cross-linking of the cell wall in grass species negatively affects enzymatic hydrolysis (Grabber *et al.*, 1998). FA is esterified to arabinoxylans, and xylans are interconnected by radical coupling of esterified FA into FA dimers or trimers. Furthermore, esterified FA is incorporated into lignin, forming an extensive lignin–ferulate–polysaccharide complex (Grabber, 2005; Ralph, 2010). Elevated levels of FA were not observed in the sugarcane lines with *COMT* suppression.

In this study, there was no difference in the amount of glucose in the cell wall of transgenic sugarcane lines analysed, while xylose was significantly increased in the transgenic line T4 that displayed the greatest lignin reduction. Similarly, xylose amounts in the stem of *COMT*-suppressed transgenic switchgrass were increased in both T0 and T1 plants (Fu *et al.*, 2011). However, it is unclear whether there is a compensatory increase in cell wall carbohydrates for lignin reduction as proposed in 4-coumarate/CoA ligase (4CL)-suppressed transgenic poplar (Hu *et al.*, 1999). The maize *bm3* mutant, cinnamyl alcohol dehydrogenase (CAD)- or 4CL-suppressed transgenic switchgrass did not exhibit an increase in cell wall carbohydrates (Marita *et al.*, 2003; Saathoff *et al.*, 2011; Xu *et al.*, 2011). Furthermore, no increase in the amount of cellulose or the transcription level of cellulose biosynthetic genes was observed in a set of *Arabidopsis* lignin mutants (Vanholme *et al.*, 2012).

Plant biomass yield is one of the most important factors for determining the economic viability of a lignocellulosic feedstock. Despite the beneficial effects of reducing lignin for ethanol conversion, blocking the monolignol biosynthetic pathway may be associated with impaired growth and development of the transgenic plants. Particularly, suppression of hydroxycinnamoyl CoA shikimate/quinate hydroxycinnamoyl transferase (HCT), *p*-coumarate 3′-hydroxylase (C3′H) or cinnamoyl CoA reductase (CCR) frequently leads to developmental arrest, dwarfism, collapsed xylem vessels and/or abnormal flowering (Gallego-Giraldo *et al.*, 2011; Goujon *et al.*, 2003; Piquemal *et al.*, 1998; Ralph *et al.*, 2006; Shadle *et al.*, 2007; Srinivasa Reddy *et al.*, 2005). These negative effects may result from disruption of nonlignin metabolite biosynthesis, such as coniferaldehyde derivatives and/or shikimate derivatives, which influence cell growth and defence mechanisms (Bonawitz and Chapple, 2010; Gallego-Giraldo *et al.*, 2011). Interestingly, *COMT*-suppressed plants were reported to display normal plant growth in a variety of species including tobacco, alfalfa, maize and switchgrass under greenhouse conditions, and poplar under field conditions (Chen and Dixon, 2007; Fu *et al.*, 2011; Pilate *et al.*, 2002; Pinçon *et al.*, 2001; Piquemal *et al.*, 2002). However, complete knockout of COMT in maize and sorghum *brown midrib* mutants was generally associated with lower biomass yields (Lee and Brewbaker, 1984; Miller *et al.*, 1983; Oliver *et al.*, 2005a,b; Sattler *et al.*, 2010). In our results, 80% suppression of *COMT* and 6% reduction in lignin did not cause significant reduction in biomass production or related traits, such as stem diameter, height, tillering and accumulated sugars compared with non-transgenic tissue culture–derived control plants. However, *COMT* suppression of 91% and a 12% reduction in lignin compromised biomass production under both greenhouse (Jung *et al.*, 2012) and field conditions. These results indicate that adverse effects on plant growth can be avoided by targeted suppression of specific lignin biosynthetic gene(s), which do not cause pleiotropic effects, and by determining the level of lignin modification that allows high biomass yield along with improved conversion performance. This strategy is facilitated by the range of gene expression/suppression that is typically found among different transgenic events. The tolerance to reduced lignin may also differ among different species, and even, within a species. In *brown midrib* mutants, agronomic traits in the mutant cultivar or hybrid are influenced by the interaction between the specific gene and the genetic background (Pedersen *et al.*, 2005; Sattler *et al.*, 2010). For example, a sorghum COMT-deficient *bmr12* near-isogenic line in the genetic background of Early Hegari-Sart exhibited similar plant height and dry matter yield as the wild-type counterpart, unlike what was observed for the comparison in the background of Atlas, Kansas Collier or Rox Orange (Oliver *et al.*, 2005a).

Differences in tiller number or biomass yield between transgenic line T31 and nontransgenic controls may be associated with somaclonal variation, which can be minimized by reducing the period in tissue culture (Taparia *et al.*, 2012a,b) or eliminated by a single backcross (Bregitzer *et al.*, 2008). Although line T4 produced the least amount of biomass, it displayed the highest saccharification efficiency and the lowest lignin content among the transgenic sugarcane lines. This would suggest its use as a parent in future breeding efforts to explore if further improvements can be achieved by crossing *COMT*-down-regulated sugarcane lines (e.g. T4) to genetically diverse accessions (e.g. high biomass-type energycanes). Among the transgenic sugarcane lines evaluated under both greenhouse and field conditions, T41 has the greatest promise for crop improvement because its field performance was comparable to control lines, but with a 19% to 23% increase in saccharification efficiency. Improving the saccharification efficiency of lignocellulosic sugarcane biomass by modifying lignin biosynthesis will greatly benefit the biofuels industry.

Experimental procedures

Field design

Caffeic acid *O*-methyltransferase–suppressed transgenic sugarcane RNAi lines were generated using the commercially important cultivar CP88-1762, as previously described (Jung *et al.*, 2012). Rootstocks of clonal propagules of the V1 (the first generation of vegetative progeny from transgenic plants), tissue culture control, transgenic control and the original CP88-1762 (wild-type) plants were transplanted on 30 March 2011 at the University of Florida, Plant Science Research and Education Unit, Citra, Florida, USA, under USDA/APHIS permit 11-040-120n. The field design was a randomized block design with three replications. Four plots of transgenic lines representing four independent transformation events, three plots of wild-type, nontransgenic controls derived from tissue culture and the transgenic control harbouring the *npt*II gene were included in each replicate. Each plot consisted of one row with five clonal propagules planted per plot. Spacing between plants, rows and blocks was 90, 150 and 450 cm, respectively. Each block was surrounded by one row of wild-type sugarcane plants. Weeds were removed manually during the establishment phase. Acephate (Orthene 97) (AMVAC, Los Angeles, CA) was applied in August 2011 for insect control. Plots were fertilized with 40 kg/ha N, 15 kg/ha P and 60 kg/ha K at planting and with 70 kg/ha N, 15 kg/ha P and 60 kg/ha K on 9

May 2011. Plots were irrigated once or twice a week with a rate of 10 mm depending on rainfall and harvested on 26 October 2011 for the determination of biomass and compositional analysis.

Evaluation of plant performance

Pests and diseases [e.g. sugarcane brown leaf rust (*Puccinia melanocephala*), orange leaf rust (*P. kuehnii*) and pink sugarcane mealybug (*Saccharicoccus sacchari*)] were monitored at monthly intervals. Leaf rust was rated using a 0–4 scale (Comstock *et al.*, 2010). For evaluating the rate of mealybug infestation, six stalks per plant were randomly selected and the number of infested internodes was recorded. The rate of infestation was calculated by the number of infested internodes over the total number of internodes. The lodging incidence was monitored every month. The lodging susceptibility was scored in every plant using a 0–7 scale with 0 being no lodging (0–20° deviation from erect), and the score increasing with every 10° lodging, until 7 which equated to complete lodging.

The fresh weight of the harvested biomass was determined on site immediately after the plot harvest. The number of stalks was counted for each plant, and five stalks were collected for milling. The leaves and leaf sheaths were removed from the stalks, the number of internodes was counted, and stalk height, from the shoot apical meristem to the soil surface, was measured. Stalk diameter was measured at the centre of the 8th internode from the base of the plant. Internodes 1–4 below the shoot apical meristem were removed and the remaining portion of the stalk was crushed to extract juice using a custom-made juice extractor. Percentage soluble solid in the extracted juice (°Brix) was measured using a PAL-1 portable refractometer (ATAGO U.S.A., Inc., Bellevue, WA). Crushed stalks were dried at 45 °C and stored for further analysis.

Gene expression analysis

The third internode below the shoot apical meristem was collected from one of the millable stalks in each plot. RNA extraction, cDNA synthesis and quantitative real-time RT-PCR were performed as previously described (Jung *et al.*, 2012).

Sample preparation for the evaluation of cell wall composition and saccharification efficiency

Crushed stalks, dried at 45 °C as described above, were ground using a Wiley mill (Thomas Scientific, Swedesboro, NJ) with a 1.0-mm sieve, and the samples were further passed through a 0.42-mm sieve. Soluble extract was removed from the samples by three successive extractions with 50% ethanol (v/v) and sonication at 45 °C for 30 min. Extract-free samples were dried at 45 °C until constant weight was achieved to analyse lignin, hydroxycinnamic acids and cell wall polysaccharides, and the evaluation of saccharification efficiencies.

Lignin content and composition

Total lignin content was measured using the acetyl bromide (AcBr) method as previously described (Jung *et al.*, 2012). Lignin composition was determined by thioacidolysis (Robinson and Mansfield, 2009). Thioacidolysis derivatives for lignin monomers were analysed using a Varian 3800 gas chromatograph (GC) coupled to the Varian 1200 mass spectrometer (MS) (Varian, Walnut Creek, CA). One microlitre of the sample was injected onto a Factor-4 VF-5 ht column (35 m, 0.25 mm i.d.) (Varian) with helium (1.2 mL min^{-1}) as carrier gas. GC conditions were as follows: initial column temperature 130 °C, held for 3 min, ramped at 3 °C min^{-1} to 250 °C, and held for 5 min; injector temperature, 250 °C; and 1 : 2 split ratio. The mass spectrometer was operated in electron impact mode at 70 eV. The detector was operated at 1.0 kV. Mass spectra were recorded every 0.2 s at a scanning range of *m/z* 50–550, and the data were recorded and analysed using Varian MS Workstation software. Thioacidolysis monomers, G and S, were quantified by integration of the respective peak areas, and employing the response factors of G and S against an internal standard, tetracosane (IS) as follows: G versus IS, 0.47; and S versus IS, 0.53 (Yue *et al.*, 2012).

Analysis of hydroxycinnamic acids

Ester-linked cell wall-bound *p*-coumaric acid (*p*CA) and ferulic acid (FA) were determined following Hatfield *et al.* (1999) with modifications. Briefly, 25 mg of extract-free sample was treated with 1.7 mL of 2 M NaOH, and 20 μL of 2-hydroxycinnamic acid (1 mg/mL in 2 M NaOH) (Sigma-Aldrich, Saint Louis, MO) was added as an internal standard. The reaction was carried out at room temperature for 20 h in the dark, then acidified with 0.3 mL 12 M HCl. Samples were extracted three times with an equal volume of diethyl ether, and supernatants were combined and dried under a stream of N_2. Derivatization was performed by adding 40 μL methoxyamine hydrochloride (20 mg/mL in pyridine) (Sigma-Aldrich) and incubating at 37 °C for 90 min. The sample was trimethylsilylated by adding 60 μL of MSTFA [*N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide] (Sigma-Aldrich) and incubating at 37 °C for 30 min. *p*CA and FA were identified and quantified using GC-MS with a Factor-4 VF-5 ht column (35 m, 0.25 mm i.d.) (Varian). One microlitre of the sample was injected, and helium was used as carrier gas (1.2 mL/min). The injector temperature was 220 °C; the split ratio was 1 : 10. The oven temperature was held for 4 min at 70 °C and increased to 250 °C at 10 °C min^{-1} without holding, then ramped at 40 °C min^{-1} and held constant for 2 min. MS operating procedure was the same as that for lignin compositional analysis except for a scanning range of *m/z* 45–650. Peaks from derivatized *p*CA, FA and internal standard were identified by characteristic mass spectrum ions obtained from the reference compounds. Concentrations of *p*CA, FA and internal standard were determined using the standard curve. The concentrations of *p*CA and FA were normalized with the concentration of internal standard among the samples.

Cell wall carbohydrates and starch

Cell wall carbohydrates in the stalk samples were determined according to the National Renewable Energy Laboratory (NREL) protocol by Sluiter *et al.* (2008). Three hundred milligrams of extract-free sample was hydrolysed with 72% H_2SO_4 at 30 °C for 1 h and then treated with 4% H_2SO_4 at 121 °C for 1 h. Liberated monomeric sugars were identified and quantified with an Agilent/HP 1090 HPLC equipped with an RI detector (Agilent Technologies, Santa Clara, CA). The HPLC analysis was carried out using a HPX-87H column (Bio-Rad, Hercules, CA), operating at 50 °C with a 0.004 M H_2SO_4 mobile phase at a flow rate of 0.4 mL min^{-1}. Starch content in the sample was determined by the NREL procedure by Ehrman (1996). Glucose yield was measured using a YSI glucose analyzer (YSI Life Science, Yellow Springs, OH) following enzymatic digestion of the extracted starch, and its value was adjusted by multiplying 0.9 for the weight gained by hydration.

Dilute-acid pretreatment and enzymatic hydrolysis

Extract-free samples possessing 0.1 g of cellulose were pretreated as previously described (Chen and Dixon, 2007). The amount of cellulose was defined as the amount of glucose from cell wall carbohydrate corrected by subtracting the amount of glucose from starch (Selig *et al.*, 2008). Five sets of pretreated samples were prepared per sugarcane line to evaluate saccharification performance at different hydrolysis times and enzyme dosages.

The enzymatic hydrolysis was performed according to the NREL protocol by Selig *et al.* (2008) with modifications. The pretreated sample was suspended in 8 mL distilled water and placed in a preweighed 50-mL polypropylene tube. Ten millilitres of 0.1 M sodium citrate buffer (pH 4.8) and 200 μL of 2% (w/w) sodium azide (Sigma-Aldrich) were added to each tube. The enzymatic hydrolysis was performed by adding the appropriate volume of Kerry Biocellulase W (Kerry Bioscience, Cork, Ireland) and Novozyme 188 β-glucosidase (Sigma-Aldrich). One set of the pretreated samples was used as the no-enzyme blank (no addition of cellulolytic enzymes). The appropriate volume of distilled water was added to each tube to bring the total mass of each sample to 20 g, assuming all solutions and the biomass sample have 1.000 g/mL specific gravity. The hydrolysis was carried out for 168 h in a shaking incubator at 50 °C and 250 rpm. The time course of enzymatic hydrolysis with 60 FPU/g cellulose of Kerry Biocellulase W and 64 pNPGU/g cellulose of Novozyme 188 β-glucosidase was monitored by measuring glucose yields at the time points of 0, 3, 6, 24, 48 and 72 h. The effect of enzyme loading on saccharification was evaluated with Kerry Biocellulase W loadings of 5, 20 or 60 FPU/g cellulose. Novozyme 188 β-glucosidase loading was constant as 64 pNPGU/g cellulose. One millilitre of the hydrolysed sample was collected for each treatment and time point and filtered through a 0.2-μm syringe filter. Glucose yields were analysed using the YSI glucose analyzer (YSI Life Science). Saccharification efficiency was calculated as the ratio of glucose released following the enzymatic hydrolysis to the amount of glucose present in the cell wall before the hydrolysis. The glucose amount derived from starch in the stalk was subtracted to calculate the glucose amount in the cell wall. Glucose yields were adjusted by multiplying 0.9 for the weight gained by hydration.

Statistical analysis

ANOVA was performed using Proc GLM in SAS™ Version 9.3 (SAS Institute Inc., Cary, NC). Statistical significance among the means for plant growth performance was determined using Fisher's protected LSD test at $P < 0.05$. Significant differences ($P < 0.05$) of means for the content of cell wall components among the samples were determined using Tukey's test. For the gene expression analysis, the t-test was used to determine whether the means of ΔCt were significantly different between wild-type and a transgenic line ($P < 0.05$).

Acknowledgements

We would like to thank USDA-NIFA (grant # 2009-10001-05117) and Syngenta Biotechnology Inc. for their financial support of this work, Dr. Robert Gilbert EREC, Belle Glade, FL, for providing donor plants of sugarcane cultivar CP88-1762, Dr. James Preston, Department of Microbiology and Cell Science University of Florida for providing Kerry Biocellulase W and Novozyme 188 β-glucosidase, Dr. Janice Zale for her help with editing this manuscript and Sun Gro Horticulture for donation of the Fafard #2 potting mix. The University of Florida is gratefully acknowledged for providing funding for the GC–MS equipment.

References

Alvira, P., Tomás-Pejó, E., Ballesteros, M. and Negro, M.J. (2010) Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: a review. *Bioresour. Technol.* **101**, 4851–4861.

Barrière, Y., Ralph, J., Méchin, V., Guillaumie, S., Grabber, J.H., Argillier, O., Chabbert, B. and Lapierre, C. (2004) Genetic and molecular basis of grass cell wall biosynthesis and degradability. II. Lessons from *brown-midrib* mutants. *C. R. Biol.* **327**, 847–860.

Bonawitz, N.D. and Chapple, C. (2010) The genetics of lignin biosynthesis: connecting genotype to phenotype. *Annu. Rev. Genet.* **44**, 337–363.

Bout, S. and Vermerris, W. (2003) A candidate-gene approach to clone the sorghum *brown midrib* gene encoding caffeic acid *O*-methyltransferase. *Mol. Genet. Genomics* **269**, 205–214.

Bregitzer, P., Dahleen, L.S., Neate, S., Schwarz, P. and Manoharan, M. (2008) A single backcross effectively eliminates agronomic and quality alterations caused by somaclonal variation in transgenic barley. *Crop Sci.* **48**, 471–479.

Chen, F. and Dixon, R.A. (2007) Lignin modification improves fermentable sugar yields for biofuel production. *Nat. Biotechnol.* **25**, 759–761.

Comstock, J.C., Glynn, N.C. and Davdison, R.W. (2010) Sugarcane rusts in Florida. *Proc. Int. Soc. Sugar Cane Technol.* **27**, 1–9.

Crago, C.L., Khanna, M., Barton, J., Giuliani, E. and Amaral, W. (2010) Competitiveness of Brazilian sugarcane ethanol compared to US corn ethanol. *Energy Policy*, **38**, 7404–7415.

Dien, B., Sarath, G., Pedersen, J., Sattler, S., Chen, H., Funnell-Harris, D., Nichols, N. and Cotta, M. (2009) Improved sugar conversion and ethanol yield for forage sorghum (*Sorghum bicolor* L. Moench) lines with reduced lignin contents. *Bioenerg. Res.* **2**, 153–164.

Ehrman, T. (1996) Determination of starch in biomass samples by chemical solubilization and enzymatic digestion. In *Laboratory Analytical Procedure (LAP)*, Procedure #16 Golden, Colorado: National Renewable Energy Laboratory.

Fu, C., Mielenz, J.R., Xiao, X., Ge, Y., Hamilton, C.Y., Rodriguez, M. Jr, Chen, F., Foston, M., Ragauskas, A., Bouton, J., Dixon, R.A. and Wang, Z.Y. (2011) Genetic manipulation of lignin reduces recalcitrance and improves ethanol production from switchgrass. *Proc. Natl Acad. Sci. USA*, **108**, 3803–3808.

Gallego-Giraldo, L., Escamilla-Trevino, L., Jackson, L.A. and Dixon, R.A. (2011) Salicylic acid mediates the reduced growth of lignin down-regulated plants. *Proc. Natl Acad. Sci. USA*, **108**, 20814–20819.

Goldemberg, J. (2007) Ethanol for a sustainable energy future. *Science*, **315**, 808–810.

Goujon, T., Ferret, V., Mila, I., Pollet, B., Ruel, K., Burlat, V., Joseleau, J.-P., Barrière, Y., Lapierre, C. and Jouanin, L. (2003) Down-regulation of the *AtCCR1* gene in *Arabidopsis thaliana*: effects on phenotype, lignins and cell wall degradability. *Planta*, **217**, 218–228.

Grabber, J.H. (2005) How do lignin composition, structure, and cross-linking affect degradability? A review of cell wall model studies. *Crop Sci.* **45**, 820–831.

Grabber, J.H., Quideau, S. and Ralph, J. (1996) *p*-Coumaroylated syringyl units in maize lignin: Implications for β-ether cleavage by thioacidolysis. *Phytochemistry*, **43**, 1189–1194.

Grabber, J.H., Ralph, J. and Hatfield, R.D. (1998) Ferulate cross-links limit the enzymatic degradation of synthetically lignified primary walls of maize. *J. Agric. Food Chem.* **46**, 2609–2614.

Guo, D., Chen, F., Inoue, K., Blount, J.W. and Dixon, R.A. (2001) Downregulation of caffeic acid 3-*O*-methyltransferase and caffeoyl CoA 3-*O*-methyltransferase in transgenic alfalfa: impacts on lignin structure and implications for the biosynthesis of G and S lignin. *Plant Cell*, **13**, 73–88.

Hatfield, R.D., Wilson, J.R. and Mertens, D.R. (1999) Composition of cell walls isolated from cell types of grain sorghum stems. *J. Sci. Food Agric.* **79**, 891–899.

Hatfield, R., Ralph, J. and Grabber, J. (2008) A potential role for sinapyl *p*-coumarate as a radical transfer mechanism in grass lignin formation. *Planta*, **228**, 919–928.

Hu, W.-J., Harding, S.A., Lung, J., Popko, J.L., Ralph, J., Stokke, D.D., Tsai, C.-J. and Chiang, V.L. (1999) Repression of lignin biosynthesis promotes cellulose accumulation and growth in transgenic trees. *Nat. Biotech.* **17**, 808–812.

Humphreys, J.M., Hemm, M.R. and Chapple, C. (1999) New routes for lignin biosynthesis defined by biochemical characterization of recombinant ferulate 5-hydroxylase, a multifunctional cytochrome P450-dependent monooxygenase. *Proc. Natl Acad. Sci. USA*, **96**, 10045–10050.

Jørgensen, H., Kristensen, J.B. and Felby, C. (2007) Enzymatic conversion of lignocellulose into fermentable sugars: challenges and opportunities. *Biofuels, Bioprod. Biorefin.* **1**, 119–134.

Jung, J.H., Fouad, W.M., Vermerris, W., Gallo, M. and Altpeter, F. (2012) RNAi suppression of lignin biosynthesis in sugarcane reduces recalcitrance for biofuel production from lignocellulosic biomass. *Plant Biotechnol. J.* **10**, 1067–1076.

Lee, M.H. and Brewbaker, J.L. (1984) Effects of brown midrib-3 on yields and yield components of maize. *Crop Sci.* **24**, 105–108.

Leite, R.C.C., Leal, M.R.L.V., Cortez, L.A.B., Griffin, W.M. and Scandiffio, M.I.G. (2009) Can Brazil replace 5% of the 2025 gasoline world demand with ethanol? *Energy*, **34**, 655–661.

Louie, G.V., Bowman, M.E., Tu, Y., Mouradov, A., Spangenberg, G. and Noel, J.P. (2010) Structure-function analyses of a caffeic acid *O*-methyltransferase from perennial ryegrass reveal the molecular basis for substrate preference. *Plant Cell*, **22**, 4114–4127.

Mansfield, S.D., Mooney, C. and Saddler, J.N. (1999) Substrate and enzyme characteristics that limit cellulose hydrolysis. *Biotechnol. Prog.* **15**, 804–816.

Marita, J.M., Vermerris, W., Ralph, J. and Hatfield, R.D. (2003) Variations in the cell wall composition of maize *brown midrib* mutants. *J. Agric. Food Chem.* **51**, 1313–1321.

Miller, J.E., Geadelmann, J.L. and Marten, G.C. (1983) Effect of the brown midrib-allele on maize silage quality and yield. *Crop Sci.* **23**, 493–496.

Oliver, A.L., Pedersen, J.F., Grant, R.J. and Klopfenstein, T.J. (2005a) Comparative effects of the sorghum *bmr-6* and *bmr-12* genes: I. Forage sorghum yield and quality. *Crop Sci.* **45**, 2234–2239.

Oliver, A.L., Pedersen, J.F., Grant, R.J., Klopfenstein, T.J. and Jose, H.D. (2005b) Comparative effects of the sorghum *bmr-6* and *bmr-12* genes: II. Grain yield, stover yield, and stover quality in grain sorghum. *Crop Sci.* **45**, 2240–2245.

Osakabe, K., Tsao, C.C., Li, L., Popko, J.L., Umezawa, T., Carraway, D.T., Smeltzer, R.H., Joshi, C.P. and Chiang, V.L. (1999) Coniferyl aldehyde 5-hydroxylation and methylation direct syringyl lignin biosynthesis in angiosperms. *Proc. Natl Acad. Sci. USA*, **96**, 8955–8960.

Palmer, N., Sattler, S., Saathoff, A., Funnell, D., Pedersen, J. and Sarath, G. (2008) Genetic background impacts soluble and cell wall-bound aromatics in *brown midrib* mutants of sorghum. *Planta*, **229**, 115–127.

Pedersen, J.F., Vogel, K.P. and Funnell, D.L. (2005) Impact of reduced lignin on plant fitness. *Crop Sci.* **45**, 812–819.

Pilate, G., Guiney, E., Holt, K., Petit-Conil, M., Lapierre, C., Leple, J.-C., Pollet, B., Mila, I., Webster, E.A., Marstorp, H.G., Hopkins, D.W., Jouanin, L., Boerjan, W., Schuch, W., Cornu, D. and Halpin, C. (2002) Field and pulping performances of transgenic trees with altered lignification. *Nat. Biotechnol.* **20**, 607–612.

Pinçon, G., Maury, S., Hoffmann, L., Geoffroy, P., Lapierre, C., Pollet, B. and Legrand, M. (2001) Repression of *O*-methyltransferase genes in transgenic tobacco affects lignin synthesis and plant growth. *Phytochemistry*, **57**, 1167–1176.

Piquemal, J., Lapierre, C., Myton, K., O'Connell, A., Schuch, W., Grima-pettenati, J. and Boudet, A.-M. (1998) Down-regulation of cinnamoyl-CoA reductase induces significant changes of lignin profiles in transgenic tobacco plants. *Plant J*, **13**, 71–83.

Piquemal, J., Chamayou, S., Nadaud, I., Beckert, M., Barrière, Y., Mila, I., Lapierre, C., Rigau, J., Puigdomenech, P., Jauneau, A., Digonnet, C., Boudet, A.-M., Goffner, D. and Pichon, M. (2002) Down-regulation of caffeic acid *O*-methyltransferase in maize revisited using a transgenic approach. *Plant Physiol.* **130**, 1675–1685.

Ralph, J. (2010) Hydroxycinnamates in lignification. *Phytochem. Rev.* **9**, 65–83.

Ralph, J., Hatfield, R.D., Quideau, S., Helm, R.F., Grabber, J.H. and Jung, H.-J.G. (1994) Pathway of *p*-coumaric acid incorporation into maize lignin as revealed by NMR. *J. Am. Chem. Soc.* **116**, 9448–9456.

Ralph, J., Akiyama, T., Kim, H., Lu, F., Schatz, P.F., Marita, J.M., Ralph, S.A., Srinivasa Reddy, M.S., Chen, F. and Dixon, R.A. (2006) Effects of coumarate 3-hydroxylase down-regulation on lignin structure. *J. Biol. Chem.* **281**, 8843–8853.

Robinson, A.R. and Mansfield, S.D. (2009) Rapid analysis of poplar lignin monomer composition by a streamlined thioacidolysis procedure and near-infrared reflectance-based prediction modeling. *Plant J*, **58**, 706–714.

Saathoff, A.J., Sarath, G., Chow, E.K., Dien, B.S. and Tobias, C.M. (2011) Downregulation of cinnamyl-alcohol dehydrogenase in switchgrass by RNA silencing results in enhanced glucose release after cellulase treatment. *PLoS ONE*, **6**, e16416.

Saballos, A., Vermerris, W., Rivera, L. and Ejeta, G. (2008) Allelic association, chemical characterization and saccharification properties of *brown midrib* mutants of sorghum (*Sorghum bicolor* L. Moench). *Bioenerg. Res.* **1**, 193–204.

Sattler, S.E., Funnell-Harris, D.L. and Pedersen, J.F. (2010) Brown midrib mutations and their importance to the utilization of maize, sorghum, and pearl millet lignocellulosic tissues. *Plant Sci.* **178**, 229–238.

Selig, M., Weiss, N. and Ji, Y. (2008) Enzymatic saccharification of lignocellulosic biomass. In *Laboratory Analytical Procedure (LAP)*, Technical Report NREL/TP-510-42629. Golden, CO: National Renewable Energy Laboratory.

Shadle, G., Chen, F., Srinivasa Reddy, M.S., Jackson, L., Nakashima, J. and Dixon, R.A. (2007) Down-regulation of hydroxycinnamoyl CoA: shikimate hydroxycinnamoyl transferase in transgenic alfalfa affects lignification, development and forage quality. *Phytochemistry*, **68**, 1521–1529.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D. and Crocker, D. (2008) Determination of structural carbohydrates and lignin in biomass In *Laboratory Analytical Procedure (LAP)*, Technical Report NREL/TP-510-42618). Golden, Colorado: National Renewable Energy Laboratory.

Somerville, C., Youngs, H., Taylor, C., Davis, S.C. and Long, S.P. (2010) Feedstocks for lignocellulosic biofuels. *Science*, **329**, 790–792.

Srinivasa Reddy, M.S., Chen, F., Shadle, G., Jackson, L., Aljoe, H. and Dixon, R.A. (2005) Targeted down-regulation of cytochrome P450 enzymes for forage quality improvement in alfalfa (*Medicago sativa* L.). *Proc. Natl Acad. Sci. USA*, **102**, 16573–16578.

Taparia, Y., Fouad, W., Gallo, M. and Altpeter, F. (2012a) Rapid production of transgenic sugarcane with the introduction of simple loci following biolistic transfer of a minimal expression cassette and direct embryogenesis. *In Vitro Cell Dev. Biol. Plant* **48**, 15–22.

Taparia, Y., Gallo, M. and Altpeter, F. (2012b) Comparison of direct and indirect embryogenesis protocols, biolistic gene transfer and selection parameters for efficient genetic transformation of sugarcane. *Plant Cell Tiss. Org. Cult.* **111**, 131–141.

Tew, T.L. and Cobill, R.M. (2008) Genetic improvement of sugarcane (*Saccharum* spp.) as an energy crop. In *Genetic Improvement of Bioenergy Crops*, (Vermerris, W. ed), pp. 273–294. New York, NY: Springer.

Vanholme, R., Storme, V., Vanholme, B., Sundin, L., Christensen, J.H., Goeminne, G., Halpin, C., Rohde, A., Morreel, K. and Boerjan, W. (2012) A Systems biology view of responses to lignin biosynthesis perturbations in *Arabidopsis*. *Plant Cell*, **24**, 3506–3529.

Vermerris, W. (2011) Survey of genomics approaches to improve bioenergy traits in maize, sorghum and sugarcane. *J. Integr. Plant Biol.* **53**, 105–119.

Waclawovsky, A.J., Sato, P.M., Lembke, C.G., Moore, P.H. and Souza, G.M. (2010) Sugarcane for bioenergy production: an assessment of yield and regulation of sucrose content. *Plant Biotechnol. J.*, **8**, 263–276.

Xu, B., Escamilla-Treviño, L.L., Sathitsuksanoh, N., Shen, Z., Shen, H., Percival Zhang, Y.H., Dixon, R.A. and Zhao, B. (2011) Silencing of 4-coumarate: coenzyme A ligase in switchgrass leads to reduced lignin content and improved fermentable sugar yields for biofuel production. *New Phytol.* **192**, 611–625.

Yang, B. and Wyman, C.E. (2008) Pretreatment: the key to unlocking low-cost cellulosic ethanol. *Biofuels, Bioprod. Biorefin.* **2**, 26–40.

Yue, F., Lu, F., Sun, R.-C. and Ralph, J. (2012) Syntheses of lignin-derived thioacidolysis monomers and their uses as quantitation standards. *J. Agric. Food Chem.* **60**, 922–928.