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Hybridization of downregulated-COMT transgenic switchgrass lines with field-selected switchgrass for improved biomass traits

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Abstract Transgenic switchgrass (*Panicum virgatum* L.) has been produced for improved cell walls for biofuels. For instance, downregulated caffeic acid 3-*O*-methyltransferase (COMT) switchgrass produced significantly more biomass and biofuel than the non-transgenic progenitor line. In the present study we sought to further improve biomass characteristics by crossing the downregulated COMT T_1 lines with high-yielding switchgrass accessions in two genetic backgrounds ('Alamo' and 'Kanlow'). Crosses and T_2 progeny analyses were made under greenhouse conditions to assess maternal effects, plant morphology and yield, and cell wall traits. Female parent type influenced morphology, but had no effect on cell wall traits. T_2 hybrids produced with T_1 COMT-downregulated switchgrass as the female parent were taller, produced more tillers, and produced 63 % more biomass compared with those produced using the field selected accession as the female parent. Transgene status (presence or absence of transgene) influenced both growth and cell wall traits. T_2 transgenic hybrids were 7 % shorter 80 days after sowing and produced 43 % less biomass than non-transgenic null-segregant hybrids. Cell wall-related differences included lower lignin content, reduced syringyl-to-guaiacyl (S/G) lignin monomer ratio, and a 12 % increase in total sugar release in the T_2 transgenic hybrids compared to non-transgenic null segregants. This is the first study to evaluate the feasibility of transferring the low-recalcitrance traits associated with a transgenic switchgrass line into high-yielding field varieties in an attempt to improve growth-related traits. Our

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results provide insights into the possible improvement of switchgrass productivity via biotechnology paired with plant breeding.

Keywords COMT · Switchgrass · Lignocellulosic biofuel · Transgenic · Hybridization

Introduction

Switchgrass (*Panicum virgatum*, L.), a warm-season C_4 perennial grass species native to North America, has significant potential as a renewable source of fuel because of its adaptation to a wide range of climates, high biomass potential on marginal land, and high nutrient- and water-use efficiency (Parrish and Fike 2005). In addition, switchgrass reduces soil erosion, improves water quality, and its deep root system enhances soil carbon sequestration and improves soil quality (McLaughlin and Walsh 1998; Schmer et al. 2008). As a highly self-incompatible species, switchgrass is considered an obligate outcrosser and relies on wind-mediated pollination for fertilization (Ge et al. 2011). Despite favorable economic and environmental traits, large-scale deployment of switchgrass and other lignocellulosic feedstocks as sustainable and cost-competitive sources of fuel will rely on improvements to feedstock quality, biomass productivity, and other desirable agronomic traits. In particular, recent breeding and biotechnology efforts with switchgrass have focused on improving biomass composition for bioconversion (Fu et al. 2011, 2012; Sarath et al. 2008; Shen et al. 2012) as well as enhancing plant architecture and/or biomass yield (Poovaiah et al. 2015; Vogel et al. 2013; Wuddineh et al. 2015a, b).

The efficiency of converting lignocellulosic biomass into biofuel depends largely on the chemical composition of plant cell walls, particularly the lignin content and composition (Chen and Dixon 2007). Lignin, a heterogeneous phenolic polymer comprised of *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) monomer units, interacts with cell wall polysaccharides to form a complex structure that is resistant to enzymatic breakdown (Boerjan et al. 2003). Several greenhouse and field experiments have shown that cell wall saccharification and biofuel yields of switchgrass and other biofuel feedstocks can be significantly improved by manipulating the lignin biosynthetic pathway (Baxter et al. 2015; Poovaiah et al. 2014). One approach is to reduce the expression of a single gene in the lignin biosynthetic pathway, as demonstrated by switchgrass plants with downregulated caffeic acid *O*-methyltransferase (COMT), an enzyme primarily involved in S-lignin synthesis. Greenhouse and field experiments show that COMT-downregulated switchgrass lines have reduced lignin content and lower S/G lignin monomer ratios, higher sugar release by enzymatic hydrolysis, and up to 38 % higher ethanol yields compared with controls (Fu et al. 2011; Baxter et al. 2014).

We hypothesized that hybrid plants from crosses of altered-cell wall transgenic switchgrass lines with locally-adapted, high biomass accessions would result in high-biomass lines with improved bioconversion efficiency; yet, there are no published studies in this area. With this in mind, T_1-generation COMT-downregulated switchgrass in an 'Alamo' genetic background was hybridized with 'Alamo' and 'Kanlow' accessions from local Tennessee fields based on superior growth performance, and the resulting T_2 hybrid progeny from these crosses were evaluated. The objectives of this study were to (1) evaluate relationships among seed weight, germination rate, and early morphology in T_2 hybrid families, (2) assess the influence of female parent type (T_1 COMT transgenic or field accession) on growth and cell wall traits among T_2 hybrid families, (3) compare plant growth and cell wall traits between transgenic and null segregant (non-transgenic) T_2 hybrids, and (4) examine the relationships among early morphology, reproductive stage morphology, dry weight, and cell wall traits (lignin content, S/G ratios, and total sugar release) in T_2 hybrids.

Materials and methods

Generation and maintenance of hybrid families

Twenty-six accessions from 'Alamo' and 'Kanlow' (lowland switchgrass cultivars) were selected for high biomass, plant height, and vigor from research or production fields in eastern Tennessee in 2010 and propagated in the greenhouse. Transgenic low-recalcitrance lines were vegetatively propagated from two T_1-generation transgenic events (COMT2 and COMT3) that were produced in a previous study by

RNAi-mediated gene silencing of the caffeic acid *O*-methyltransferase (COMT) gene in 'Alamo' seed-derived switchgrass (Fu et al. 2011). In that previous study, two independent T_0 events were crossed to the same 'Alamo' genotype to produce the T_1-generation COMT2 and COMT3 events. Thus, T_1 COMT2 and T_1 COMT3 are considered 'Alamo' half-siblings. T_1 COMT transgenic parents and field-selected clones used for the crosses were maintained in 12 L pots, watered as needed and fertilized every 2 weeks with 100 mg L^{-1} of Peter's 20-20-20 N-P-K fertilizer. Full-sibling and half-sibling T_2-generation hybrid switchgrass families were produced from crosses between transgenic low-recalcitrant lines and field accessions of high-yielding switchgrass.

Flowers were pollinated as they became receptive. Panicles were considered receptive when the distal-most floret opened. Receptive panicles were emasculated and bagged using DelNet pollination bags (508 mm × 457.2 mm; DelStar Technologies, Inc., Middletown, Delaware). Transgenic parents were crossed to wild type field selection parents using either open pollination to generate the half-sibling hybrid families, or mutual pollination to generate full-sibling hybrid families. Mutual pollinations were performed in a greenhouse (16 h photoperiod; 27 °C day temperature and 21 °C night temperature). For mutual pollinations, one or two receptive panicles from each parent plant were placed in a single bag, secured with a twist-tie, labeled, and staked to prevent damage. Crosses made using the mutual pollination method produced full-sibling families under greenhouse or walk-in growth chamber conditions.

Bags were removed for seed harvest after 90 days and dehisced seed in pollination bags was discarded. Panicles were separated and the seeds were threshed by hand on a rubber board. Seeds were counted, weighed, and treated with powdered ethyl mercaptan (CAPTAN®), and stored in air-tight plastic bags at 4 °C for a minimum of 30 days. In total, 9641 seeds were collected from 62 controlled crosses and 94,688 open-pollinated seeds were collected from 35 individual plants (26 wild type field selections and 9 T_1 COMT transgenics) for a total of 62 full-sibling and 35 half-sibling T_2 hybrid families. Of these T_2 hybrid families, 30 (20 full-sibling and 10 half sibling) were used for the current experiments.

Eighteen seeds per family were sown in Fafard 3B potting mix in 7.6 × 7.6 × 5.7 cm containers and maintained in the greenhouse. Germination frequency was determined as the total number of seedlings above the soil line 30 days after sowing (DAS) divided by the total number of seed sown per family. An average of 14 seedlings per family germinated. Seedlings were watered as needed. After the appearance of two true leaves, the seedlings were fertilized every 2 weeks with 100 mg L^{-1} of Peter's 20-20-20 N-P-K fertilizer.

Molecular characterization of hybrid plants

PCR was performed on seedlings from T_2 hybrid families to identify transgenic and non-transgenic null segregant progeny from each cross. A leaf tip from each seedling was collected and total DNA was isolated using a CTAB buffer and two rounds of phenol/chloroform extraction followed by two ethanol washes. DNA was quantified using a BioTek plate analyzer and diluted to 20 ng μL^{-1}. PCR was performed using GoGreen Mastermix according to the manufacturers' instructions using 80 ng of DNA. Two pairs of primers were used to verify transgene status (presence or absence of transgene). *Gus* linker primers (sequences in Fu et al. 2011) were used to confirm presence of the transgene, and *hyg* primers (sequences in Shen et al. 2012) were used to identify the hygromycin selection marker gene. PCR for each T_2 hybrid family was performed with a positive control (the T_1 COMT transgenic parent) and a negative control (the wild type field selection parent). Seedlings showing both amplification products were considered positive (T_2 COMT transgenic), while those with no amplification products were considered negative (non-transgenic null segregant).

Morphology characterization

Morphological measurements were taken on seedlings at 40 and 80 DAS, and on plants of the first reproductive (R1) stage (developmental stages determined using the criteria described by Hardin et al. (2013). Early morphological traits (height at 40 and 80 DAS, number of tillers at 80 DAS) were measured on seedlings maintained in 7.6 × 7.6 × 5.7 cm containers. Height was measured from soil level to the tip of

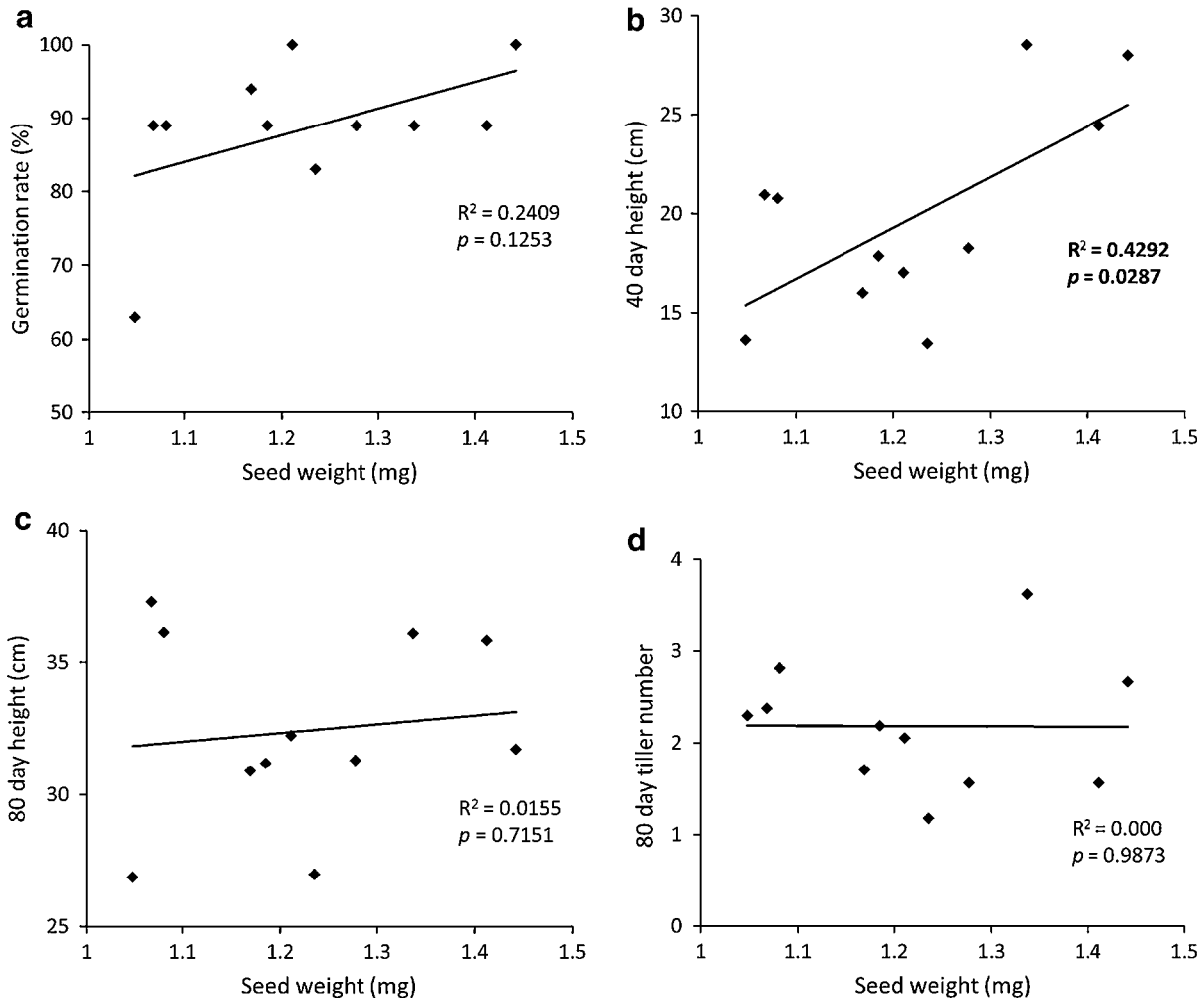

Fig. 1 Regressions between T_2 hybrid family seed weight and **a** germination rate, **b** height at 40 days after sowing, **c** height at 80 days after sowing, and **d** tiller number at 80 days after sowing. Mean seed weight was determined for 11 T_2 hybrid families; each data point represents an average of 15–18 biological replicates per family. *Values in bold* indicate significance at the 5 % level

the tallest leaf. At 80 DAS, plants were transferred to 12-L pots. Based on 80 day morphology measurements, T_2 hybrids at the top 10 % or bottom 10 % of trait (plant height and tiller number 80 DAS) distribution tails were selected for further experiments. The middle 80 % of plants fell into the non-selected category.

Once tillers reached the R1 growth stage, morphological traits (tiller height, leaf width, and number of tillers) were measured on selected plants. After taking morphology measurements, dry biomass was determined for selected and non-selected plants. For non-selected plants, all biomass above the soil level was harvested, dried in an oven at 43 °C for 96 h, and weighed. For selected plants, half of each plant was harvested for dry weight determination and the resulting weight was multiplied by two to estimate the total dry weight yield. The remaining half was maintained for possible future field experiments.

Cell wall chemistry and sugar release

Above-ground tillers at the R1 growth stage were harvested from selected and non-selected plants, dried in an oven at 43 °C for 96 h, and milled with

Table 1 Correlations among morphological and cell wall variables measured on transgenic (shaded) and non-transgenic null segregant (non shaded) T_2 hybrid offspring

	40 day height	80 day tiller no.	80 day height	R1 height	R1 tiller no.	R1 leaf width	Dry weight	Lignin content	S/G ratio	Sugar release
40 day height		**0.59**	0.52	**0.64**	**0.59**	0.20	0.47	0.11	0.45	-0.094
		<.0001	<.0001	**<.0001**	**<.0001**	0.17	<.0001	0.22	<.0001	0.59
80 day tiller no.	0.52		0.40	**0.60**	**0.67**	0.25	0.47	0.17	0.38	-0.033
	<.0001		<.0001	**<.0001**	**<.0001**	0.093	<.0001	0.072	<.0001	0.85
80 day height	**0.61**	0.36		0.26	-0.047	-0.24	0.09	0.12	0.14	-0.21
	<.0001	<.0001		0.051	0.74	0.094	0.28	0.19	0.12	0.22
R1 height	0.36	0.54	0.30		**0.75**	0.37	**0.74**	0.52	**0.57**	-0.005
	0.037	0.001	0.083		**<.0001**	0.010	**<.0001**	<.0001	**<.0001**	0.98
R1 tiller no.	0.51	**0.68**	0.22	0.58		0.35	**0.91**	0.49	**0.57**	0.18
	0.002	**<.0001**	0.21	<.0001		0.015	**<.0001**	0.001	**<.0001**	0.46
R1 leaf width	0.32	0.15	0.14	0.36	0.53		0.34	0.04	0.12	0.39
	0.081	0.43	0.45	0.044	0.002		0.023	0.80	0.50	0.12
Dry weight	0.25	0.31	0.23	0.47	**0.72**	0.38		0.48	**0.58**	0.062
	0.006	0.001	0.013	0.009	**<.0001**	0.049		<.0001	**<.0001**	0.73
Lignin content	0.063	0.15	0.21	-0.048	0.14	-0.043	0.29		0.42	-0.43
	0.54	0.15	0.035	0.82	0.50	0.85	0.004		<.0001	0.008
S/G ratio	0.19	0.017	0.074	0.14	0.33	0.14	0.26	0.39		-0.090
	0.071	0.87	0.47	0.50	0.11	0.52	0.011	<.0001		0.60
Sugar release	-0.028	-0.243	-0.038	-0.26	-0.23	0.039	-0.076	-0.26	-0.39	
	0.88	0.18	0.84	0.40	0.46	0.90	0.69	0.15	0.022	

Values are Pearson correlations with corresponding p values below. n = 163 for transgenic offspring, n = 198 for non-transgenic null segregant offspring. Values in bold represent the strongest (≥0.56) correlations. Morphology measurements were taken at 40 and 80 days after sowing (40 day height, 80 day height and tiller number) and at the first reproductive stage (R1 height, tiller number, and leaf width). Lignin content, S/G (syringyl/guaiacyl) lignin monomer ratio, and sugar release were measured on whole tillers harvested at the R1 stage

a Wiley mill (Thomas Scientific, Model 4, Swedesboro, NJ, USA) through a 0.5 mm screen. Milled samples were used for lignin and sugar release analyses.

Pyrolysis mass beam mass spectrometry (py-MBMS) was used to measure total lignin content and the S/G lignin monomer ratio as previously described (Sykes et al. 2009). After the removal of soluble extractives and starch, the remaining cell wall residues (CWR) were pyrolyzed at 500 °C and resulting vapors were analyzed via MBMS. Lignin content was estimated as the sum of the relative intensities of all lignin precursor peaks. The S/G lignin monomer ratio was estimated as the sum of the intensity of syringyl peaks divided by the sum of the intensity of guaiacyl peaks.

The total sugars released by enzymatic hydrolysis were measured by high-throughput sugar release assays as previously described (Selig et al. 2010). Briefly, soluble extractives and starches were removed from samples (Decker et al. 2012) and resulting CWR were pretreated with condensing steam at 180 °C for 17.5 min, followed by an incubation with 70 mg protein/g biomass Ctec2 enzyme cocktail (Novozymes North America, Franklinton, NC, USA) at 50 °C for 70 h. Colorimetric assays were used to determine the amounts of glucose and xylose released by the samples (Megazyme Intl., Bray, Ireland). The total sugar release reported is the sum of the glucose and xylose release.

Data analysis

All data analysis was performed using SAS Statistical Analysis Software v.9.3 (SAS Institute, Cary, NC, USA). Analysis of variance (ANOVA) was used to partition variance in morphological and cell wall variables within and among families, parent types (COMT transgenic or field accession), family types (full-sibling or half-sibling, and progeny transgene status. Means were separated using Tukey's studentized range test. The REG and CORR procedures were used to perform single linear regressions and correlation analyses, respectively. Chi square goodness of fit

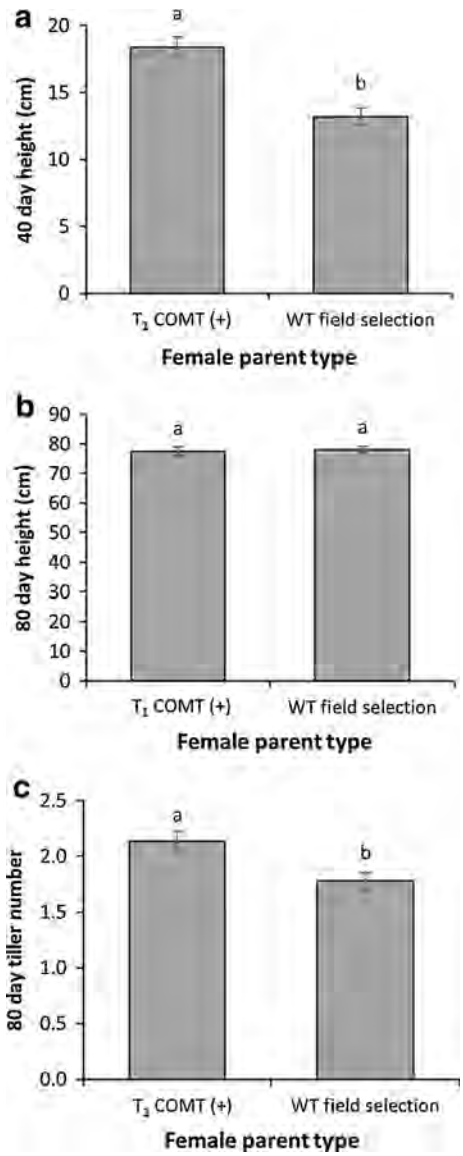

Fig. 2 Effect of female parent type, T_1 COMT transgenic (+) or wild type (WT) field selection, on early morphological traits in T_2 hybrid families. **a** Height at 40 days after sowing, **b** height at 80 days after sowing, and **c** number of tillers at 80 days after sowing. *Bars* represent the mean of biological replicates for T_2 hybrid families generated with either T_1 COMT transgenic as the female parent ($n = 14$) or wild type field selection as the female parent ($n = 16$) ± standard error of the man. Means were analyzed with a one-way ANOVA and letter groupings were obtained using Tukey's studentized range test. *Bars with different letters* are significantly different at the 5 % level ($p < 0.05$)

was performed using PROC FREQ with a hypothesized distribution of equal proportions of COMT (+) and COMT (−) offspring in each family.

Results

Relationships among seed weight, germination rate, and early-growth morphological variables

Final germination was tallied at 30 DAS. Height at 40 and 80 DAS and number of tillers at 80 DAS was determined for T_2 hybrid families. Seed weight did not significantly influence germination rate, but positively influenced seedling height at 40 DAS ($R^2 = 0.43$; Fig. 1b). All early morphological variables (height at 40 and 80 DAS and number of tillers at 80 DAS) were significantly positively correlated with one another, with Pearson correlation coefficients ranging from 0.36 to 0.61 (Table 1).

Effect of female parent type on morphology and cell wall traits

T_1 COMT × field accession hybrids exhibited morphological differences in early (40 and 80 DAS) and late (R1 stage) growth phases, which varied by female parent. T_2 hybrid families produced using the T_1 COMT transgenic line as the female parent were 39 % taller at 40 DAS and had 20 % more tillers at 80 DAS than those families produced using the non-transgenic field switchgrass as the female parent (Fig. 2). The T_1 COMT transgenic line female parent produced hybrids that were 10 % taller at the R1 stage, which produced 113 % more tillers and 63 % more biomass than hybrids coming from seed produced by the field clone (Fig. 3). Female parent type had no effect on lignin content, S/G ratios, or total sugar release in the T_2 hybrid families (Fig. 4).

Transgene segregation

T_2 hybrid families were genotyped for presence (+) or absence (−) of the COMT transgene. Overall, families had a mean of 47 % COMT (+) offspring. Distribution of COMT (−) and COMT (+) T_2 hybrid offspring was not statistically different from 50 % for most families (Table 2). Three families had more COMT (−) offspring than the expected 50 %, while two families had more COMT (+) offspring than expected. Analysis of variance showed that female parent type (T_1 COMT transgenic or wild type field selection) and the interaction of female parent type and family type

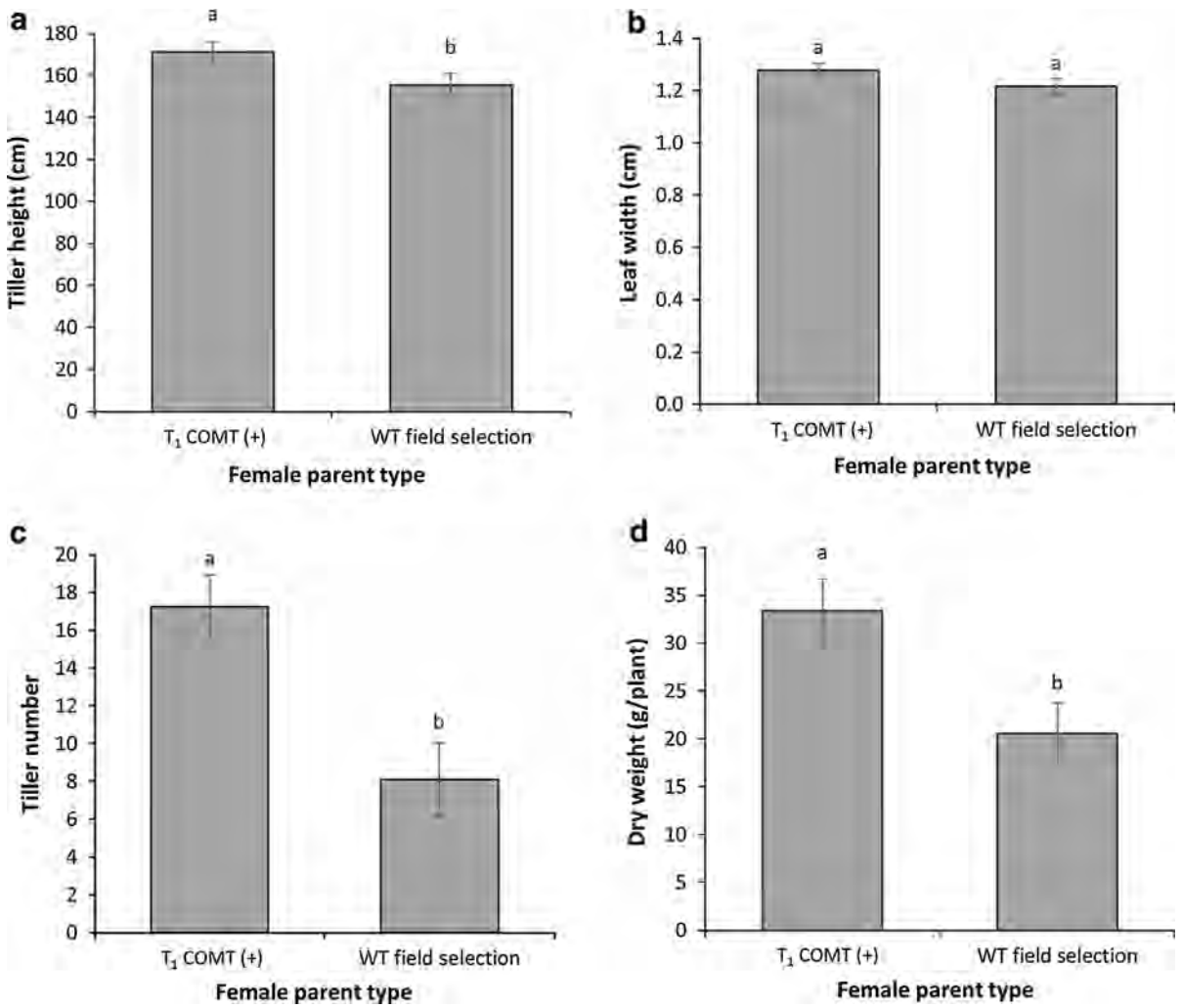

Fig. 3 Effect of female parent type, T_1 COMT transgenic (+) or wild type (WT) field selection, on reproductive-stage morphology and dry weight yield in T_2 hybrid families. Measurements were taken once tillers reached the first reproductive stage (R1). **a** Tiller height, **b** leaf width, **c** number of tillers, and **d** dry weight yield. *Bars* represent the mean of biological replicates for T_2 hybrid families generated with either T_1 COMT transgenic as the female parent (n = 14) or wild type field selection as the female parent (n = 16) ± standard error. Means were analyzed with a one-way ANOVA and letter groupings were obtained using Tukey's studentized range test. *Bars with different letters* are significantly different at the 5 % level ($p < 0.05$)

(full-sibling or half-sibling) were significant sources of variation in the percent of COMT (+) offspring in a family (Table 3). Specifically, the offspring of half-sibling families with a wild type female parent had only 20 % COMT (+) offspring, on average. All other 95 % confidence limits of percent COMT (+) offspring for female parent/family type combinations included 50 % (Table 4).

Effect of transgene status on morphology and cell wall traits

Transgenic seedlings were similar to the null segregants in height at 40 DAS, but were 7 % shorter by 80 DAS (Fig. 5). Once reaching the R1 developmental stage, there were no significant differences between the transgenic hybrids and null segregants in height,

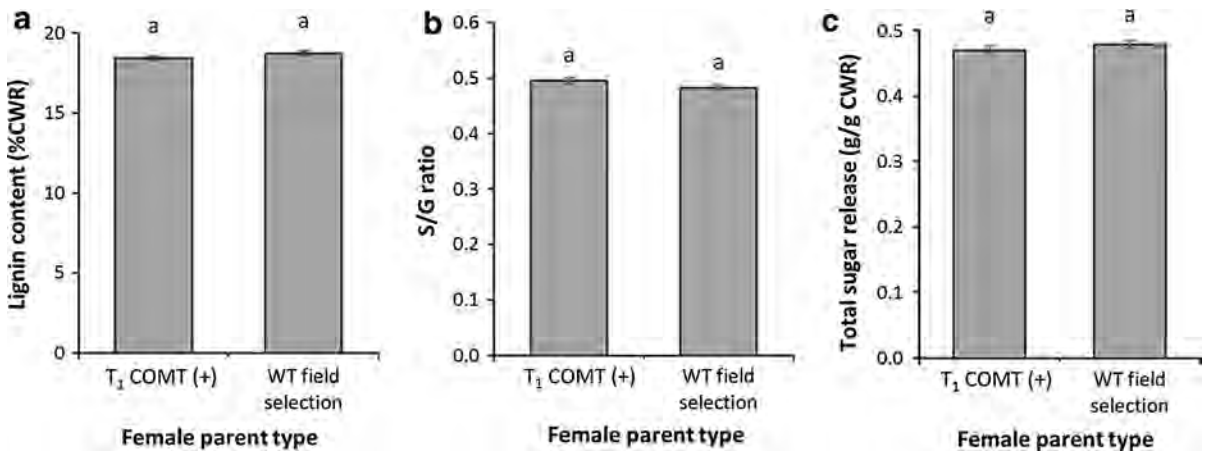

Fig. 4 Effect of female parent type, T_1 COMT transgenic (+) or wild type (WT) field selection, on cell wall traits in T_2 hybrid families. Whole tillers of the first reproductive stage (R1) were used for cell wall analyses. **a** MBMS lignin content, **b** S/G lignin monomer ratio, and **c** total sugars (glucose and xylose combined) released by enzymatic hydrolysis. *Bars* represent the mean of biological replicates for T_2 hybrid families generated with either T_1 COMT transgenic as the female parent ($n = 14$) or wild type field selection as the female parent ($n = 16$) ± standard error. Means were analyzed with a one-way ANOVA and letter groupings were obtained using Tukey's studentized range test. *Bars with different letters* are significantly different at the 5 % level ($p < 0.05$). CWR, cell wall residues; (+), transgenic; WT, wild type

leaf width, or tiller number. However, transgenic hybrids had 43 % less dry weight compared with null segregants (Fig. 6).

As expected, there were clear differences in cell wall chemistry and recalcitrance between the T_2 transgenic hybrids and the non-transgenic null segregants. R1-stage tillers harvested from COMT transgenic hybrids had 7 % less lignin than null segregants, as well as a 12 % reduction in the S/G ratio. Changes in lignin content and composition were accompanied by a 12 % increase in total sugar release (Fig. 7).

Genetic control of morphology and cell wall components

Genetics had a clear influence on T_2 hybrid switchgrass morphology. There were significant differences among families for early morphological variables (height at 40 and 80 DAS and tiller number at 80 DAS), R1 stage morphological variables (height, tiller number, and leaf width) and dry weight yield (Table 5). There was a 6-fold difference in the shortest and tallest families at 40 DAS. At 80 DAS, the difference between the shortest and tallest families was only 1.5-fold (Fig. 8). Families were ranked based on height at 40 and 80 days; spearman rank correlation indicated that 68 % of the variation in 80 day height could be explained by 40 day height ($r_s = 0.68$, $p < 0.001$). There were also some differences among families for cell wall variables (lignin content, S/G ratio, and total sugar release), although, transgene status was much more influential than family for these traits. Conversely, family was more influential than transgene status for growth-related variables (early and R1 stage morphology, dry weight yield; Table 5).

Distribution of transgenic hybrid offspring among the T_2 switchgrass families genotyped ranged from 0 to 100 % (Table 2). In order to differentiate between family (genetic) effects and effects resulting from uneven transgene segregation, we regressed family means for each morphological and cell-wall variable on percent transgenic offspring for each family. There was no significant influence of percent transgenic offspring on any of the morphological variables, cell-wall variables, or family rankings (Table 6). We conclude that family effects on morphological and cell-wall traits can be interpreted as the result of genetic differences between families rather than differences related to uneven transgene segregation between families.

Correlations among morphology, dry weight yield, and cell wall variables

Many morphological and cell wall variables were significantly correlated (Table 1). Most of the early

Table 2 Transgene segregation for T_2 hybrid switchgrass families

Family Number	No. seedlings genotyped	Female parent type	Family type	Percent COMT(+) offspring	χ^2 value
4	14	C	FS	50	0
6	18	C	FS	39	0.89
12	15	C	FS	80	5.4**
13	17	C	FS	53	0.05
16	15	C	FS	33	1.6
18	14	C	FS	36	1.1
20	8	C	FS	50	0
23	14	C	FS	36	1.1
24	14	C	FS	64	1.1
1	9	C	HS	11	5.4**
8	12	C	HS	67	1.3
27	13	C	HS	62	0.69
29	15	C	HS	53	0.067
30	14	C	HS	100	N/A
2	9	W	FS	78	2.7
3	16	W	FS	25	4.0**
7	12	W	FS	42	0.33
9	15	W	FS	27	3.2
10	16	W	FS	63	1
11	17	W	FS	42	0.53
14	12	W	FS	42	0.33
15	12	W	FS	50	0
17	14	W	FS	50	0
22	15	W	HS	0	N/A
26	9	W	HS	33	1
28	12	W	HS	25	3.0

Families are ordered by female parent type and family type

Female parent type: *C* T_1 COMT transgenic, *W* wild type field selection; Family type: *FS* full-sibling, *HS* half-sibling; *Chi square value* Chi square goodness of fit test statistic based on a hypothetical distribution of equal percentages of COMT transgenic (+) and non-transgenic T_2 hybrid offspring; *N/A* Chi square testing was not possible due to an observation of zero (0) in a category

** Chi square goodness of fit test statistics significant at the $\alpha = 0.05$ level

Table 3 Analysis of variance for influence of female parent type and family type on percent transgenic offspring in T_2 hybrid switchgrass families

Source	df	Type III SS	Mean square	F Value	Pr > F	Model R^2
Female parent type[a]	1	2302.7	2302.7	5.8	0.03	0.26
Family type[b]	1	411.0	411.0	1.0	0.32	
Female parent × family type	1	1794.5	1794.5	4.5	0.05	
Error	22	8776.1	389.9			

[a] Female parent type (T_1 COMT transgenic or wild type field selection)

[b] Family type (full-sibling or half-sibling)

Table 4 Mean percent of transgenic T_2 hybrid offspring with 95 % confidence limits for female parent type (T_1 COMT transgenic or wild type field selection) by family type (full-sibling or half-sibling) combinations

Female parent	Family type	n	Percent transgenic offspring	95 % confidence limits
T_1 COMT transgenic	FS	9	49.0 ± 6.7	35.2–62.8
T_1 COMT transgenic	HS	5	58.6 ± 8.9	40.1–77.1
Wild type	FS	9	46.5 ± 6.7	32.7–60.4
Wild type	HS	3	19.3 ± 11.5**	0–43

Family type FS full-sibling, *HS* half-sibling

(40 and 80 DAS) and R1 stage morphological variables were positively correlated with one another. In general, COMT (−) offspring showed more and larger significant trait correlations than COMT (+) offspring. Correlations between height at 40 DAS and height at R1 stage, and height at 40 DAS and dry weight, were twice as strong for COMT (−) offspring than for COMT (+) offspring. Similarly, correlations of dry weight with lignin content and S/G ratio were 0.48 and 0.58, respectively, for COMT (−) offspring, while the same correlations were 0.29 and 0.26 for COMT (+) offspring. There were no significant correlations between R1 morphological traits (height and tiller number) and cell wall traits (lignin and S/G ratio) for COMT (+) offspring, while the same correlations in COMT (−) offspring were significant. Lignin content and sugar release showed a predictable significant negative correlation in COMT (−) offspring, while there was no significant relationship between lignin content and sugar release for COMT (+) offspring.

Discussion

Combining genetic engineering and breeding to incorporate reduced recalcitrance and other key biofuel traits into selected locally-adapted varieties could be a promising strategy for developing biofuel feedstocks that are high-yielding, well-adapted to their environment, and more amenable to bioconversion. To test the feasibility of this approach in a transgenic switchgrass line, we crossed low-lignin COMT-down-regulated switchgrass lines with high-yielding varieties selected from local Tennessee fields and characterized growth and recalcitrance among the resulting T_2 hybrid progeny. These results should provide baseline information to facilitate future biofuel-related breeding efforts with transgenic switchgrass.

Relationships among seed weight, germination rate, and early morphology

Seed weight is an agronomically important trait as it has been shown to be positively associated with higher germination rate, improved emergence, early seedling vigor, and faster growth rates (Das et al. 2004). We examined relationships among seed weight, germination rate, and early morphological variables in T_2 hybrid families. Although there was no significant effect of seed weight on germination rate in our study, seed weight positively influenced seedling height at 40 DAS (Fig. 1b), suggesting that seed weight does influence the initial growth rate (from day 0 to day 40) of young switchgrass seedlings. However, it should be noted that these experiments were performed under optimized greenhouse conditions; therefore, the results might not fully reflect the influence that seed weight would have on early growth traits if grown in a natural field setting where stresses are present.

Effects of female parent type

The use of one particular genetic background over another as the female parent can dramatically influence growth and yield-related traits in hybrid offspring (Bhandari et al. 2014). In our study, there were no morphological or cell wall differences between T_2 hybrid switchgrass families with an 'Alamo' female parent and those with a 'Kanlow' female parent. However, female parent type (T_1 COMT transgenic or field accession) strongly influenced growth but had no effect on cell wall traits among the T_2 hybrids. In general, the T_1 COMT transgenic line used as the female parent had positive effects on growth

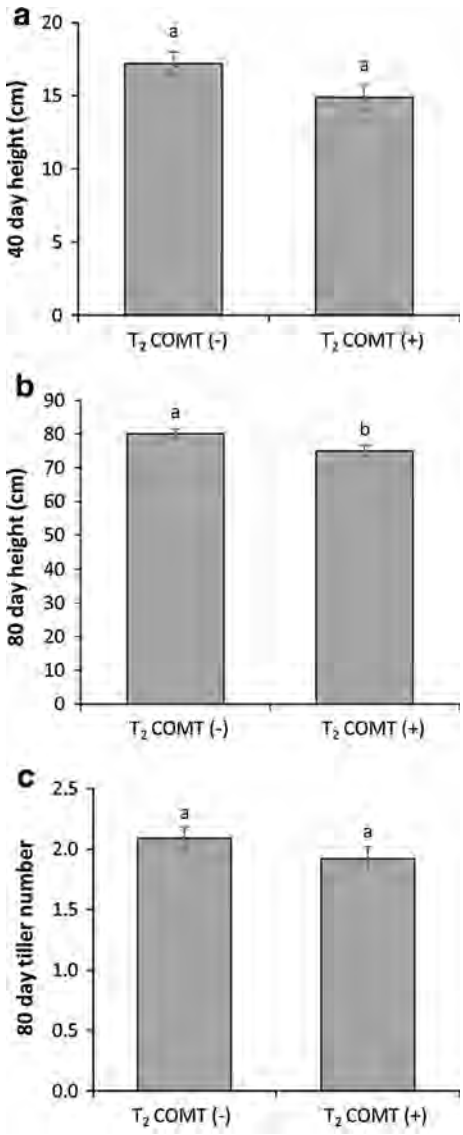

Fig. 5 Effect of transgene status, absence (−) or presence (+) of transgene, on early morphological traits in T_2 hybrid families. **a** Height at 40 days after sowing, **b** height at 80 days after sowing, and **c** number of tillers at 80 days after sowing. *Bars* represent the mean of biological replicates for non-transgenic null segregant ($n = 172$) and T_2 COMT transgenic hybrid families ($n = 140$) ± standard error. Means were analyzed with a one-way ANOVA and letter groupings were obtained using Tukey's studentized range test. *Bars with different letters* are significantly different at the 5 % level ($p < 0.05$)

morphology in the T_2 hybrid families, including improved height at early and late stages of growth, significantly more tillering, and a 63 % increase in dry weight yield over those hybrids produced using the wild type field selection as the female parent (Figs. 2, 3). It might not necessarily be the use of a transgenic plant as a female that leads to improved growth, but that the transgenics used in this study were in a more favorable genetic background compared to that of the field selections. However, it is important to emphasize that these studies were performed under controlled greenhouse conditions. It is possible that the field-selected wild types could become the favored female parent if the T_2 hybrid families were grown under natural field conditions.

Transgene effects

Greenhouse-grown T_1-generation COMT-downregulated switchgrass had reduced lignin, lower S/G lignin monomer ratios, and improved ethanol yield compared with non-transgenic null segregant controls (Fu et al. 2011). In our study, the altered lignin phenotype of the T_1 COMT parent was successfully transmitted to the T_2 transgenic hybrid families as demonstrated by the reduction in lignin content, lower S/G ratio, and higher sugar release relative to the null segregant progeny (Fig. 7). Despite morphological differences among the T_2 hybrid families, the strong effect of transgene status relative to family for lignin content, S/G ratio, and sugar release (Table 5) reiterates the strong genetic control of cell wall chemistry provided by the down-regulated *COMT* gene. Conversely, the strong effect of family relative to that of transgene status for morphological variables indicates that high-performing families can be distinguished before genotyping for presence or absence of the transgene. Genotyping only the top-performing families and individuals saves time and cost in plant breeding programs where narrowing down the number of individuals to maintain and evaluate at early stages is paramount to efficient genetic gain.

Relationships among morphology, dry weight yield, and cell wall traits

Relationships among early- and late- stage morphological traits, dry weight yield, and cell wall traits were analyzed for transgenic and non-transgenic T_2 hybrid families. In general, these data indicated that the non-transgenic null segregant offspring show stronger and more predictable patterns of plant growth, and more predictable relationships between morphology and cell-wall chemistry, than do transgenic offspring. For

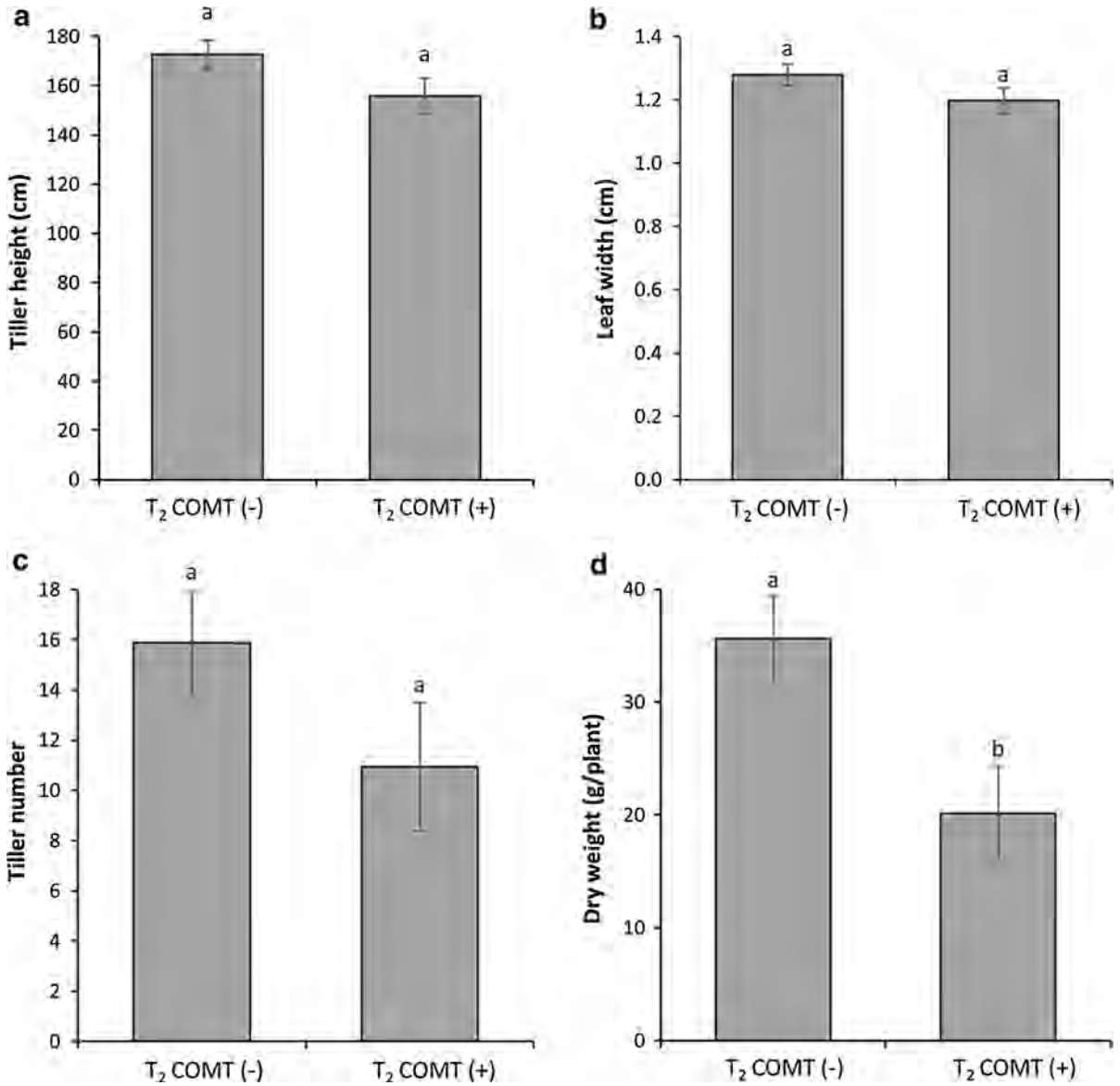

Fig. 6 Effect of transgene status, absence (−) or presence (+) of transgene, on reproductive-stage morphology and dry weight yield in T_2 hybrid families. Measurements were taken once tillers reached the first reproductive stage (R1). **a** Tiller height, **b** leaf width, **c** number of tillers, and **d** dry weight yield. *Bars* represent the mean of biological replicates for non-transgenic null segregant ($n = 46$) and T_2 COMT transgenic hybrid families ($n = 42$) ± standard error. Means were analyzed with a one-way ANOVA and letter groupings were obtained using Tukey's studentized range test. *Bars with different letters* are significantly different at the 5 % level ($p < 0.05$)

both transgenic and non-transgenic offspring, there were significant positive correlations among most early (40 and 80 DAS) and mature (R1 stage) morphological traits, which would suggest that initial selections of hybrid switchgrass families with favorable growth performance could be performed at early stages of development. Dry weight was significantly positively correlated with most morphological variables (Pearson correlation coefficients ranging from 0.23 to 0.91), but was most strongly correlated with R1 tiller number ($r = 0.72$ for transgenic offspring, $r = 0.91$ for non-transgenic offspring) followed by

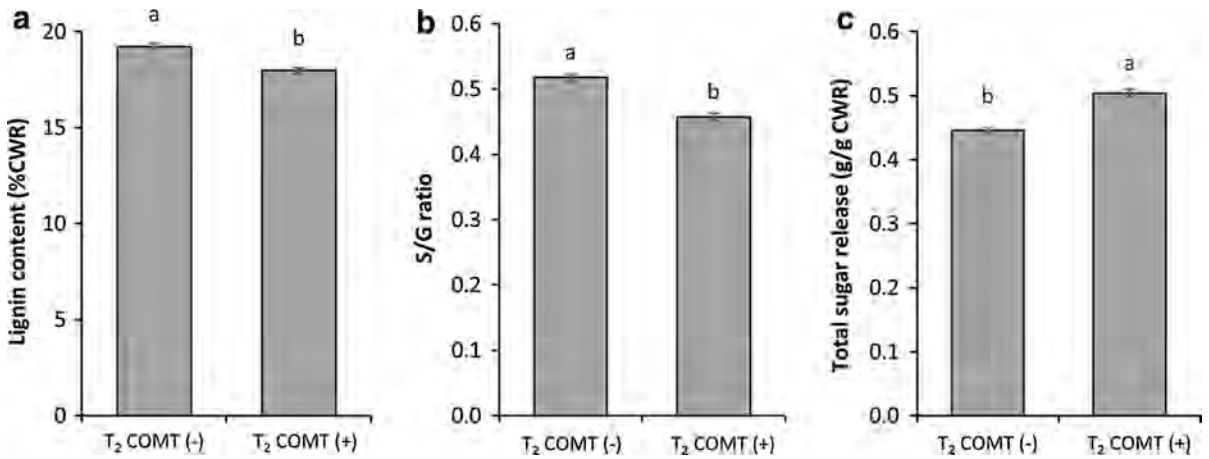

Fig. 7 Effect of transgene status, absence (−) or presence (+) of transgene, on cell wall traits in T_2 hybrid families. Whole tillers of the first reproductive stage (R1) were used for cell wall analyses. **a** MBMS lignin content, **b** S/G lignin monomer ratio, and **c** total sugar (glucose and xylose) released by enzymatic hydrolysis. *Bars* represent the mean of biological replicates for non-transgenic null segregant (n = 104) and T_2-COMT transgenic hybrid families (n = 116) ± standard error. Means were analyzed with a one-way ANOVA and letter groupings were obtained using Tukey's studentized range test. *Bars with different letters* are significantly different at the 5 % level ($p < 0.05$). *CWR* cell wall residues

Table 5 Influence of family and transgene status (presence or absence of transgene) on morphological and cell wall variables in T_2 hybrid switchgrass families

Source	df	40 Day height	80 Day height	80 Day tiller no.	R1 height	R1 leaf width	R1 tiller no.	Dry weight	Lignin content	S/G ratio	Sugar release
Family	27	10.26**	3.31**	7.34**	6.1**	2.37**	5.09**	8.53**	4.35**	5.95**	2.54**
Transgene status	1	0.15	0.93	0	1.68	0.54	0.4	7.98**	38.12**	74.31**	31.21**
Model R^2		0.60	0.33	0.51	0.62	0.39	0.59	0.49	0.46	0.56	0.71

Means were separated using Tukey's studentized range test. Asterisks represent F values significant at the 5 % level. Measurements were taken at 40 and 80 days after sowing, and at the first reproductive stage (R1)

R1 height (r = 0.47 for transgenic offspring, r = 0.74 for non-transgenic offspring). This is in agreement with prior studies that have found switchgrass biomass yield to be most strongly correlated with tillering and/or height compared to other morphological variables (Bhandari et al. 2010; Das et al. 2004). Taken together, these results suggest that tiller height and tiller number could be used as a selection index for identifying potentially high-yielding hybrid switchgrass families for further analyses.

Interestingly, there were no significant correlation between R1 morphological traits (height, tiller number) and cell wall traits (lignin, S/G ratio) for transgenic offspring. However, the same correlations in the non-transgenic null segregant offspring were highly significant (Table 1). This suggests that in non-transgenic switchgrass, favorable plant morphology and high lignin content may be inherited together to some degree. In a breeding program, this would be an important consideration when the goal is to select for families that exhibit reduced recalcitrance (low lignin content) but still display desirable growth characteristics.

In the original greenhouse study, the desired traits were passed on to the T_1-generation transgenic offspring with no apparent growth effects when compared to the corresponding non-transgenic null segregants (Fu et al. 2011), and these results were reproducible under field conditions in fully-established plants (Baxter et al. 2014). In the current study,

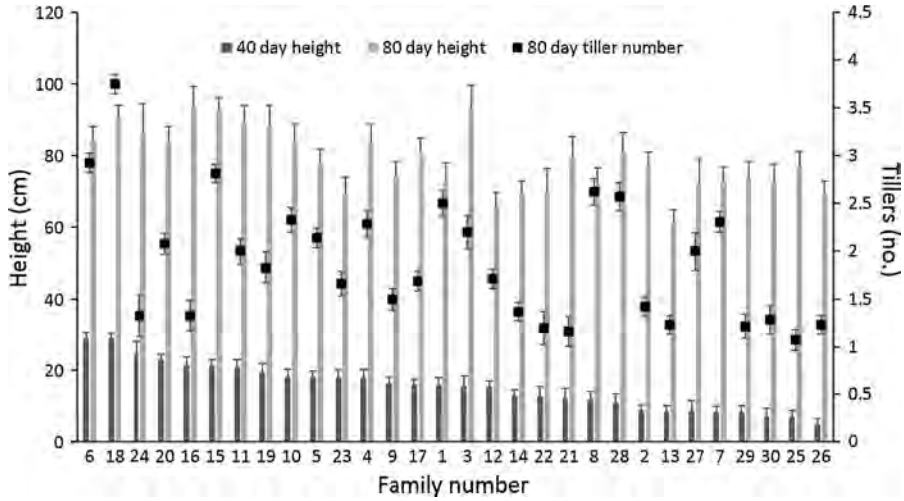

Fig. 8 Least squares means for 40 day height, 80 day height, and 80 day tiller number for T_2 hybrid switchgrass families. Families are ordered by 40 day height. *Error bars* represent standard deviation (n = 8–18 seedlings/family). Measurements were taken at 40 and 80 days after sowing

Table 6 Summary of single linear regressions of family ranks and mean morphological and cell wall variables on percent of T_2 COMT transgenic offspring in each family

Independent variable	*df*	*MS*	*F*	*Pr* > *F*	Model R^2
40 DAS rank	1	31.1	0.42	0.52	0.02
40 DAS height	1	47.8	1.10	0.30	0.04
80 DAS rank	1	39.1	0.56	0.46	0.02
80 DAS height	1	51.4	2.57	0.12	0.09
80 DAS tiller number	1	1.2	3.61	0.07	0.12
R1 height	1	716.7	1.17	0.29	0.06
R1 tiller number	1	36.4	0.54	0.47	0.00
R1 leaf width	1	0.0	0.02	0.90	0.00
Dry weight	1	26.8	0.04	0.84	0.01
S/G ratio	1	0.00	1.94	0.18	0.07
Lignin content	1	0.11	0.11	0.74	0.00

Morphology measurements were taken at 40 and 80 days after sowing (DAS), and at the first reproductive stage (R1). Lignin content and S/G ratio were determined for R1-stage whole tillers

COMT-downregulation in the T_2 generation does appear to affect growth in the transgenic hybrids when compared with the non-transgenic null segregants, as reflected by the decreased dry weight yield (43 %). In our case, the goal is to increase biomass yield relative to the transgenic parent while maintaining the desired cell wall traits. Thus, the ideal T_2 hybrid family would exhibit low recalcitrance while producing biomass amounts at least equal to their T_1 transgenic parent, not necessarily the corresponding T_2 null segregant. Multi-year field experiments will provide an opportunity to compare morphology, biomass yield, and recalcitrance between T_2 hybrid offspring and their T_1 transgenic parent in a realistic environment, and to examine potential tradeoffs between agronomic properties and cell wall recalcitrance.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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