Theor Appl Genet (2013) 126:2367–2380
DOI 10.1007/s00122-013-2141-6

ORIGINAL PAPER

Identification of bioconversion quantitative trait loci in the interspecific cross *Sorghum bicolor* × *Sorghum propinquum*

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Received: 14 January 2013 / Accepted: 1 June 2013 / Published online: 9 July 2013

Abstract For lignocellulosic bioenergy to be economically viable, genetic improvements must be made in feedstock quality including both biomass total yield and conversion efficiency. Toward this goal, multiple studies have considered candidate genes and discovered quantitative trait loci (QTL) associated with total biomass accumulation and/or grain production in bioenergy grass species including maize and sorghum. However, very little research has been focused on genes associated with increased biomass conversion efficiency. In this study, *Trichoderma viride* fungal cellulase hydrolysis activity was measured for lignocellulosic biomass (leaf and stem tissue) obtained from individuals in a F_5 recombinant inbred *Sorghum bicolor* × *Sorghum propinquum* mapping population. A total of 49 QTLs (20 leaf, 29 stem) were associated with enzymatic conversion efficiency. Interestingly, six high-density QTL regions were identified in which four or more QTLs overlapped. In addition to enzymatic conversion efficiency QTLs, two QTLs were identified for biomass crystallinity index, a trait which has been shown to be inversely correlated with conversion efficiency in bioenergy grasses. The identification of these QTLs provides an important step toward identifying specific genes relevant to increasing conversion efficiency of bioenergy feedstocks. DNA markers linked to these QTLs could be useful in marker-assisted breeding programs aimed at increasing overall bioenergy yields concomitant with selection of high total biomass genotypes.

Communicated by I. Godwin.

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Introduction

Lignocellulosic biofuels have the potential to increase domestic energy production and reduce the release of greenhouse gases into the atmosphere (Sarath et al. 2008; Schmer et al. 2008). However, for renewable bioenergy sources to become economically viable, substantial increases in overall biomass production as well as net conversion efficiencies must first be realized. Tropical C4-photosynthesis grasses (bioenergy grasses) such as *Sorghum bicolor* (sorghum) have the ability to accumulate large amounts of lignocellulosic biomass coupled to substantial grain and stem sugar yields, of which all can be utilized in bioenergy production (Vermerris 2008). Sorghum also has been shown to have increased nitrogen use efficiency as well as drought tolerance, which are traits that add to sorghum's appeal as a bioenergy feedstock (Heaton et al. 2008). In addition, sorghum has a wide range of genetic and genomic resources (Feltus and Vandenbrink 2012; Paterson 2012) including a fully sequenced genome (Paterson et al. 2009a), Targeting Induced Local Lesions in Genomes (TILLING) populations (Xin et al. 2008), association mapping populations (diversity panels) (Casa et al. 2008; Vandenbrink et al. 2010) and QTL mapping populations (Murray et al. 2008b; Murray et al. 2008a; Feltus et al. 2006; Kong et al. 2013) which can all be deployed in the identification of genes useful in increasing bioenergy production.

By crossing domesticated sorghum (*S. bicolor*) with a wild relative (*Sorghum propinquum*), the opportunity arises

for the introgression and identification of traits or genes that have been lost through the process of plant domestication. For example, seed shattering and tall plant height are two instances of the many traits lost during *S. bicolor* domestication (Paterson et al. 1995). Both of these traits favor increased seed biomass, but may be detrimental to lignocellulose quality and yield. Rhizomes were lost prior to domestication since wild *S. bicolor* is not rhizomatous, but this intriguing trait could theoretically be reintroduced into *S. bicolor* via *S. propinquum*. Often, the domestication process selects for recessive mutant alleles associated with a trait of interest (Lin et al. 1999). Since *S. propinquum* is a non-domesticated relative of *S. bicolor*, it provides opportunity to uncover QTLs and genes for bioenergy conversion efficiency that may have been lost through the process of plant domestication. Clearly, the coupling of specific feedstock with real-world conversion processes is an exciting and open area for research.

Many bioenergy-related QTLs have been identified in grass species. In sorghum, QTLs related to biomass-related traits such as leaf length and width (Xiao-ping et al. 2011), leaf yield and structural composition (Murray et al. 2008a) as well as stem sugar and grain yield (Murray et al. 2008b; Ritter et al. 2008) have all been identified. In addition, stem QTLs for traits such as diameter (Xiao-ping et al. 2011), height (Lin et al. 1995; Murray et al. 2008a; Brown et al. 2006; Feltus et al. 2006; Hart et al. 2001; Kebede et al. 2001; Parh et al. 2008; Pereira and Lee 1995; Rami et al. 1998; Ritter et al. 2008; Shiringani et al. 2010; Chantereau et al. 2001) and tillering (Paterson et al. 1995; Murray et al. 2008a; Feltus et al. 2006; Shiringani et al. 2010) have been identified in sorghum. QTLs associated with flowering time, for which increased duration of vegetative growth has been shown to increase overall biomass accumulation, have also been identified in sorghum (Lin et al. 1995; Brown et al. 2006; Feltus et al. 2006; Hart et al. 2001; Kebede et al. 2001; Parh et al. 2008; Ritter et al. 2008; Shiringani et al. 2010; Srinivas et al. 2009; Chantereau et al. 2001). QTLs associated with the amount of regrowth post-harvest (ratooning) have also been identified (Paterson et al. 1995; Murray et al. 2008a). Genes from these regions have the potential to increase the amount of total biomass harvested from a single planting of a bioenergy crop. QTLs associated with first generation biofuel traits such as grain composition (Rami et al. 1998; Murray et al. 2008b; Fernandez et al. 2008; Winn et al. 2009) as well as grain kernel weight (Paterson et al. 1995; Brown et al. 2006; Feltus et al. 2006; Pereira and Lee 1995; Rami et al. 1998; Murray et al. 2008b; Tuinstra et al. 1997) have also been identified.

Apart from sorghum, QTLs with bioenergy relevance have been identified in closely related grass species such as maize and *Miscanthus*. In maize, QTLs associated with biomass composition as well as forage quality have been identified (Méchin et al. 2001; Roussel et al. 2002; Cardinal et al. 2003; Krakowsky et al. 2005, 2006). QTLs associated with biomass accumulation such as height (Jihua et al. 2007; Austin and Lee 1996b; Khairallah et al. 1998; Koester et al. 1993; Veldboom and Lee 1996a, b; Berke and Rocheford 1995) and flowering time (Austin and Lee 1996b; Khairallah et al. 1998; Koester et al. 1993; Veldboom and Lee 1996a, b; Wisser et al. 2006; Jines et al. 2007) have also been identified and may lead to increased biomass accumulation. Leaf trait QTLs (Koester et al. 1993; Reymond et al. 2003, 2004; Sadok et al. 2007) have been identified and may lead to increased biomass as well as increases in attainable crop density which would lead to more biomass yield per acre. In addition, first generation biofuel crops such as maize are also used in food production. This gives potential for the cross-application of QTLs that have been associated with grain yield (Ribaut et al. 1997, 2007; Ajmone Marsan et al. 1995; Frova et al. 1999; Frascaroli et al. 2007; Stuber et al. 1992; Beavis et al. 1994; Xiao et al. 2005; Li et al. 2007; Austin and Lee 1996a; Austin et al. 2000; Veldboom and Lee 1996a) in the cultivation of superior first generation biofuel feedstocks, which rely on superior grain quality for enhanced production.

While maize and sorghum have proven useful in identifying QTLs associated with bioenergy production, other high-potential bioenergy grass feedstock such as *Miscanthus* have not experienced the same attention given their place outside of the food chain. While QTLs for thermal conversion efficiency (Atienza et al. 2003a; Atienza et al. 2003b), flowering time (Jensen et al. 2008; Atienza et al. 2003c), and plant height (Atienza et al. 2003d, e) have been identified to date, no QTLs for composition have been identified. Recently, three high-resolution linkage maps have been created for *Miscanthus sinensis* (Kim et al. 2012; Ma et al. 2012; Swaminathan et al. 2012) which may lead to further identification of QTLs associated with bioenergy production. Another close relative to sorghum, sugarcane, has also proven difficult to detect QTLs due to a large polyploid genome. Still, QTLs associated with stem sugar production (Méchin et al. 2001; Murray et al. 2008a, b; Lorenzana et al. 2010) have been identified, but few studies have been successful in identifying QTLs associated with composition or forage quality in sugarcane. It is envisioned that bioenergy genes identified in sorghum will provide the rationale and possibly the means to map orthologs in other bioenergy grasses, especially those with complex genomes.

The objective of this study was to identify bioconversion-related QTLs in an interspecific biparental cross between domesticated (*S. bicolor*) and wild (*S. propinquum*) sorghum species. We scanned for QTLs associated with enzymatic hydrolysis rates of cellulose using a fungal

cellulase (*Trichoderma viride*). These hydrolysis rates are a measurement of overall plant digestibility of cellulosic biomass. In addition, we searched for QTLs associated with stem biomass crystallinity index (CI) which we have previously shown to be negatively correlated with conversion efficiency (Vandenbrink et al. 2011). The QTLs we identified highlight candidate markers for use in bioenergy grass breeding applications.

Materials and methods

Plant material

The *S. bicolor* (cv. BTx623) × *S. propinquum* (unnamed accession) population consisted of 161 F_5 RILs mapped with 141 SSR/RFLP markers (Kong et al. 2013). Genetic maps were constructed using MAPMAKER (Lander et al. 1987). Recombination frequencies were converted to centiMorgans using the Kosambi function (Kosambi 1944). The 2010 RILs were planted on May 28, 2010 at the University of Georgia Plant Science Farm near Watkinsville, GA, USA. Seeds were planted using a mechanical planter with each row consisting of a single RIL and the entire population surrounded by a distinct border genotype. Row spacing consisted of 3 m long rows, 8–10 cm between plants and ~76 cm between rows. Plants were harvested between September and November by cutting at ground level at physiological maturity (based on senescence of the primary inflorescence). Tissue was then separated into leaf, stem and inflorescence and dried at ~50 °C for 48 h. Dried plant tissues (leaf and stem) were separately milled to 4 mm using a lab mill (Thomas-Wiley Laboratory Mill Model 4, Thomas Scientific). The 2011 RIL samples were planted on May 16, 2011 at the University of Georgia Plant Science Farm near Watkinsville, GA, USA. Plants were harvested when the primary inflorescence reached physiological maturity, separated into leaf, stem and inflorescence tissue and processed similarly to 2010 samples.

Enzymatic hydrolysis

Enzymatic hydrolysis of sorghum leaf and stem tissue was modified from Vandenbrink et al. (2010). Individual sorghum leaf and stem tissue samples (50 mg) were loaded into 2.0 mL 96-well plates and autoclaved sterilized at 121 °C for 20 min (gravity cycle). Cellulase solution (*T. viride*) contained per liter 74.6 mg *T. viride* cellulase (10 U/mg; crude extract; Sigma-Aldrich SKU #C9422-10KU), 100 mL citric acid buffer (0.5 M, pH 4.8 VWR CAT #JT4093-6) and 7.1 mL ampicillin (1 mg/mL, VWR CAT #EM-2200). Cellulase (0.75 mL, 0.44 μm filter sterilized) was added to each well at an enzyme loading concentration of 20 U/g dried biomass. The 96-well plates were sealed with a silicon cover (Fisher Scientific, CAT #14-222-013) and covered with Scotch 4-in. aluminum foil tape (3 M, St. Paul, MN, USA). Plates were then incubated at 37 °C on a lab-line titer shaker (setting 4) for 48 h. Samples of cellulase hydrolysate (30 μL) were taken at 0, 4, 12, 24 and 48 h and stored at −20 °C.

Dinitrosalicylic colorimetric reducing sugar assay

Quantification of reducing sugars released from enzymatic hydrolysis was done using the dinitrosalicylic acid (DNS) assay. DNS solution contained (per liter) 10 g of dinitrosalicylic acid (Sigma-Aldrich SKU #42260), 10 g of sodium hydroxide (VWR CAT #BDH0292), and 0.5 g of sodium sulfite (Sigma-Aldrich SKU #S0505). The DNS assay was conducted using 50 mL of dilute (1:10) hydrolyzed samples and 50 μl of DNS solution placed in 300 μl PCR plates. PCR plates containing samples were heated to 90 °C for 10 min to promote color change, followed by cooling to 10 °C. Cooled samples were than treated with 17 μL of 40 % potassium sodium tartrate (Rochelle salt; Sigma-Aldrich SKU #217255) to stop the color change. Next 100 μL of sample was transferred to cell culture plates (NUNC CAT #163320) and the absorbance was measured at 585 nm using a plate reader (TECAN GENios Absorbance Microplate Reader). Reducing sugar release was quantified relative to a dextrose standard curve ranging from 0 to 200 μg per reaction. Release rates were obtained as the slope of sugar release at 0, 4, 12 h (HYP12) or 0, 4, 12, 24 h (HYP24).

X-ray diffraction analysis

Biomass crystallinity index (CI) was determine by X-ray diffraction (Rigaku Ultima 4) using a modified Segal method (Segal et al. 1959; Vandenbrink et al. 2011). Stem samples were analyzed in triplicate for 2010 RIL samples. Two scans were performed; from 18.0° to 19° and from 21.8° to 22.8° to reduce the overall scan time and allow for scanning of the large number of samples needed to map QTLs. No significant difference was seen between the abbreviated scans and the full length scans (Student's *T* test, $p = 0.52$). CI was defined according to Vandenbrink et al. (2011):

$$\mathrm{CI} = \frac{I_{002} - I_{\mathrm{am}}}{I_{002}} \times 100,$$

where I_{002} is the diffraction intensity at 22.5° and I_{am} is the diffraction intensity at 18.7°.

QTL mapping

A detailed analysis of genetic map construction for this population and application to QTL analysis has been previously reported (Kong et al. 2013). In this study, QTL scanning for 4, 12, 24 h sugar release as well as 12 and 24 h HYP in both 2010 and 2011 populations was conducted for each phenotype using composite interval mapping (CIM) as implemented by Windows QTL Cartographer V2.5 (Wang et al. 2012). In addition, biomass CI was tested for 2010 stem tissue which we have previously shown to be negatively correlated with hydrolysis rate (Vandenbrink et al. 2011). CIM analysis was conducted using the standard model (Model 6), with a walk speed of 1.0 cM and a window size of 10.0 cM. The regression method used was the Backward Regression Model with the number of background control markers set to 10. Significance for QTL detection was determined by permutation test (significance level = 0.05, 1,000 permutations) with a significant LOD score for leaf tissue being 2.80, and a significant LOD score for stem tissue being 2.78. The QTL region was identified as a 1-LOD interval from the peak of a significant QTL.

Results

Genetic map construction

The linkage map of recombinant inbred lines (RIL) derived from *S. bicolor* (SB) and *S. propinquum* (SP) spans 773.1 cM on 10 linkage groups with an average of 14 markers per linkage group. The average interval between consecutive loci is 5.48 cM, ranging from 0.0 cM between cosegregating markers to 25.7 cM in the largest gap on chromosome 5. Each marker on the genetic map was aligned with the corresponding physical location by virtue of the *S. bicolor* genome sequence (Paterson et al. 2009b). Markers that are not well aligned are possibly due to less recombination information at the end of the chromosome, sequence assembly errors, multiple amplifications of paralogous loci or cryptic structure between *S. bicolor* and *S. propinquum*, as detailed elsewhere (Kong et al. 2013). Detailed analysis of the population including segregation distortion, residual heterozygosity, genetic map construction and a comparison of the genetic map to physical positions has been previously reported (Kong et al. 2013).

Phenotyping

The SB × SP population exhibited large quantitative variation for hydrolysis yield potential (HYP defined as the rate of sugar release over 12 h (HYP12) or 24 h (HYP24) in both leaf (Table 1) and stem tissue (Table 2). In both the 2010 and 2011 experiments, stem tissue had a greater propensity than leaf tissue for reducing sugar release as well as a greater range of sugar releases at 4, 12 and 24 h as well as HYP12 and HYP24. This same trend was seen in both the SB and SP leaf (Table 1) and stem (Table 2) tissue genotypes. In addition, stem biomass CI was measured in 2010 stem tissue in an effort to identify QTLs. CI values of stem tissue range from 42.9 to 58.0 ($\mu = 50.5$). We have previously demonstrated that CI is not associated with hydrolysis rates in leaf tissue and, therefore, it was not included in this study.

Identification of novel bioconversion QTLs

A total of 49 QTLs associated with bioenergy conversion traits were identified in this study. All QTLs identified and their genetic and physical locations can be visualized at the Comparative Saccharinae Genome Resource (CSGR-QTL; http://helos.pgml.uga.edu/cgi-bin/cmap/viewer) and in Figs. 1 and 2, respectively. As described in detail below, QTL locations, associated markers, and proportion of variance explained can be found in Tables 3 and 4 for stem tissue and Tables 5 and 6 for leaf tissue.

Table 1 Leaf enzymatic hydrolysis trait values for S.B. and S.P. parents and recombinant inbred lines (RILs) over two environments

Trait	UGA 2010				UGA 2011			
	S.B.	S.P.	RILs mean	RILs range	S.B.	S.P.	RILs mean	RILs range
4 h (mg)	182.2	132.6	160.9 (3.3)	92.9–291.0	146.0	136.2	157.8 (5.0)	80.5–364.3
12 h (mg)	278.4	186.7	230.0 (5.0)	125.5–383.6	243.6	197.6	237.5 (7.4)	102.8–529.4
24 h (mg)	417.4	249.6	336.2 (6.3)	166.6–517.4	332.0	265.1	327.4 (9.7)	136.0–618.6
HYP (12) (μg/h)	15.2	5.7	9.8 (0.4)	2.3–23.3	10.2	7.3	12.4 (0.4)	2.1–34.7
HYP (24) (μg/h)	13.1	6.1	11.2 (0.2)	2.7–17.2	13.2	9.3	9.7 (0.6)	2.3–19.9

Values in bracket indicate standard error

S.B., *Sorghum bicolor*; S.P., *Sorghum propinquum*; HYP, hydrolysis yield potential (μg/h/1 U *T. viride* cellulase)

Table 2 Stem enzymatic hydrolysis trait values for S.B. and S.P. parents and recombinant inbred lines (RILs) over two environments

Trait	UGA 2010				UGA 2011			
	S.B.	S.P.	RILs mean	RILs range	S.B.	S.P.	RILs mean	RILs range
4 h (mg)	378.6	375.8	438.3 (14.7)	90.8–874.2	281.8	294.8	304.5 (14.4)	63.1–778.8
12 h (mg)	493.6	485.9	560.5 (15.8)	128.7–938.5	444.2	328.9	400.1 (17.5)	75.7–1050.1
24 h (mg)	604.6	566.6	650.7 (17.5)	202.9–1,149.5	575	493.7	534.0 (22.8)	98.5–1243.7
HYP (12) (μg/h)	19.5	17.2	34.8 (1.0)	5.2–57.4	18.8	14.3	23.2 (0.8)	1.2–68.4
HYP (24) (μg/h)	32.9	30.4	19.5 (0.5)	5.1–32.0	27.9	16.9	16.2 (1.2)	1.5–39.4

Values in bracket indicate standard error

S.B, *Sorghum bicolor*; S.P., *Sorghum propinquum*; HYP, hydrolysis yield potential (μg/h/1 U *T. viride* cellulase)

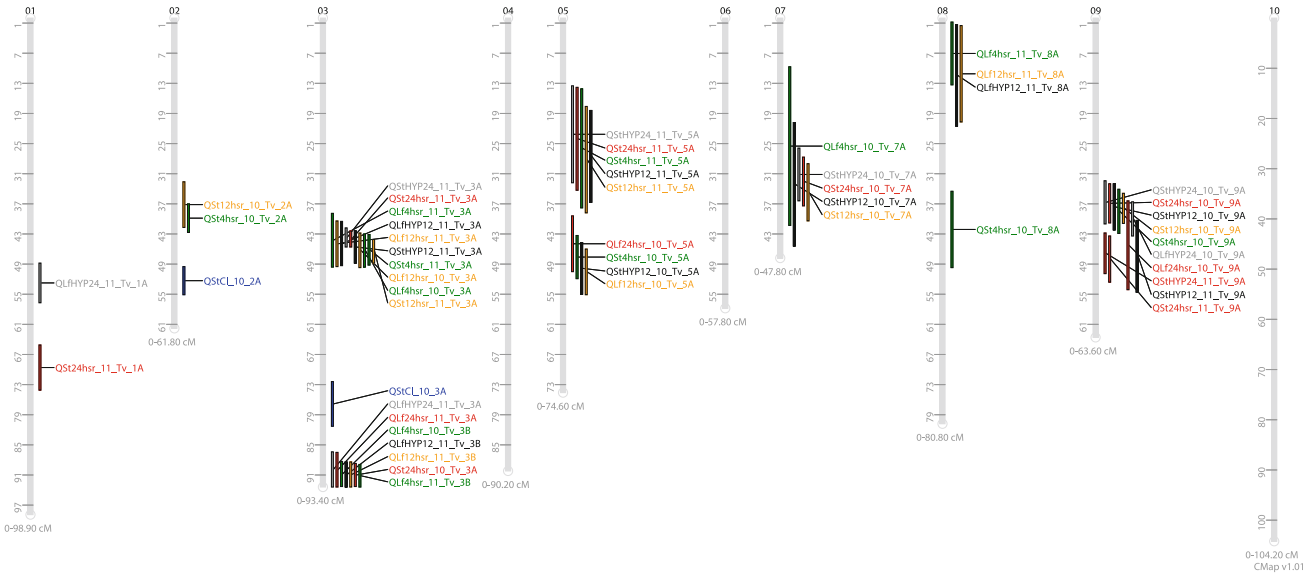

Fig. 1 Genetic map positions of sorghum conversion QTLs. *Green* 4-h release, *yellow* 12-h release, *red* 24-h release, *black* 12-h HYP, *gray* 24-h HYP, *blue* CI, QSt stem tissue, QLf leaf tissue

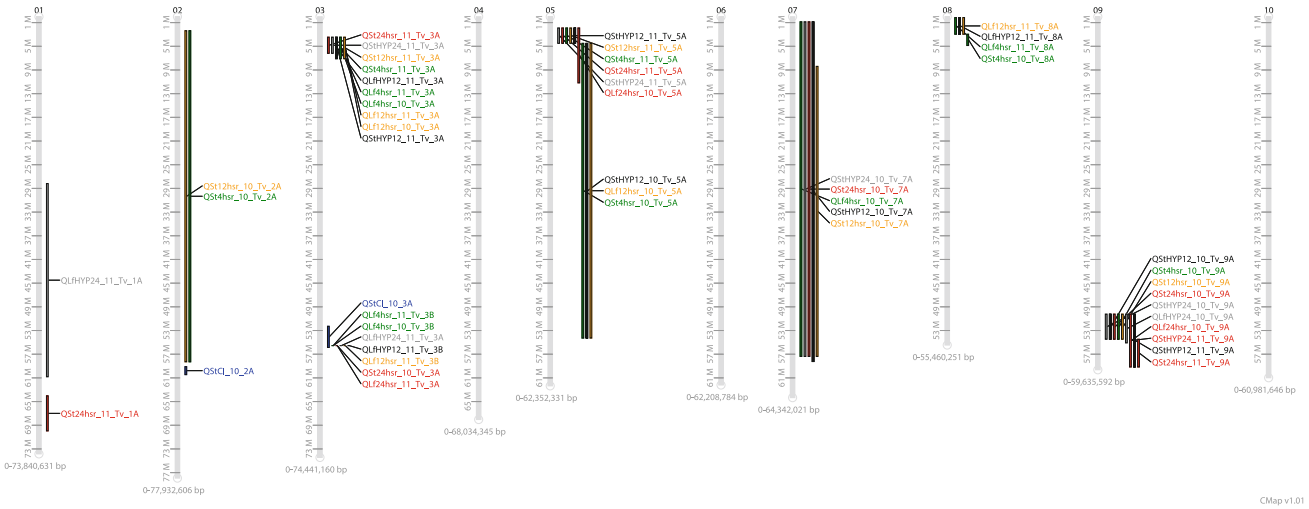

Fig. 2 Physical map positions of sorghum conversion QTLs. *Green* 4-h release, *yellow* 12-h release, *red* 24-h release, *black* 12-h HYP, *gray* 24-h HYP, *blue* CI, QSt stem tissue, QLf leaf tissue

Table 3 Stem 2010 QTLs—location and markers

QTL	Trait category	Chromosome	Peak (cM)	Peak LOD	Left boundary (cM)	Right boundary (cM)	Markers in QTL	Left flanking marker	Right flanking marker	R^2 (%)
QSt4hsr_10_Tv_2A	4-h release	2	37.6	2.9	37.0	42.7	mSbCIR223	TC51638b	CA154181A	8.30
QSt4hsr_10_Tv_5A	4-h release	5	50.7	3.93	43.3	51.9	Xtxp225, S14_460612_aag#3	CA190667c	CA100232a	10.40
QSt4hsr_10_Tv_8A	4-h release	8	43.7	3.4	34.5	49.7	mSbCIR240	Xtxp047	CA105209a	10.41
QSt4hsr_10_Tv_9A	4-h release	9	38.9	2.81	34.1	42.9	TC59518b (2)	CA170467a	TC65153a	8.02
QSt12hsr_10_Tv_2A	12-h release	2	37.5	3.22	32.6	41.7	mSbCIR223	TC51638b	CA154181A	9.04
QSt12hsr_10_Tv_7A	12-h release	7	34.9	3.43	29.0	40.4	S15_224156_ag20	CA193820b	Xcup70	10.05
QSt12hsr_10_Tv_9A	12-h release	9	37.3	4.22	34.9	40.9	TC59518b (2)	CA170467a	TC65153a	10.56
QSt24hsr_10_Tv_3A	24-h release	3	93.3	4.07	88.7	93.3	TC69429a	mSbCIR276	N/A	10.79
QSt24hsr_10_Tv_7A	24-h release	7	30.9	5.04	27.7	37.4	CA193820b, S15_224156_ag20	Xcup33 (2)	Xcup70	14.42
QSt24hsr_10_Tv_9A	24-h release	9	36.1	3.81	33.0	40.8	TC59518b (2)	CA170467a	TC65153a	10.81
QStHYP12_10_Tv_5A	12-h hydrolysis rate	5	51	2.89	44.7	55.0	Xtxp225, S14_460612_aag#3	CA190667c	CA100232a	7.35
QStHYP12_10_Tv_7A	12-h hydrolysis rate	7	36.5	2.82	20.8	45.4	xcup33 (2), CA193820b, S15_224156_ag20, Xcup70	CA148166b	CA107045a	8.94
QStHYP12_10_Tv_9A	12-h hydrolysis rate	9	37.7	3.82	33.0	42.2	TC59518b (2)	CA170467a	TC65153a	9.79
QStHYP24_10_Tv_7A	24-h hydrolysis rate	7	31	5.63	25.9	36.4	CA193820b, S15_224156_ag20	Xcup33 (2)	Xcup70	15.64
QStHYP24_10_Tv_9A	24-h hydrolysis rate	9	36.2	3.24	32.4	41.0	TC59518b (2)	CA170467a	TC65153a	8.85
QStCI_10_2A	Crystallinity index	2	51.9	2.81	49.5	55.1	Xgap84	xcup63	xcup29	7.46
QStCI_10_3A	Crystallinity index	3	77.4	5.12	72.4	81.3	msbCIR278	ca148183a	TC69429a	14.89

Hour release = μg reducing sugar at that time point; release rate = μg/h

LOD logarithm of the odds, R^2 percentage of phenotypic variance explained by the QTL

Table 4 Stem 2011 QTLs—location and markers

QTL	Trait category	Chromosome	Peak (cM)	Peak LOD	Left boundary (cM)	Right boundary (cM)	Markers in QTL	Left flanking marker	Right flanking marker	R^2 (%)
QSt4hsr_11_Tv_3A	4-h release	3	46.2	3.31	43.1	49.2	TC48402a	CA187839a (2)	xisep0101	8.78
QSt4hsr_11_Tv_5A	4-h release	5	26.1	4.43	14.1	37.8	N/A	xtxp065	CA190667c	21.06
QSt12hsr_11_Tv_3A	12-h release	3	46.2	4.2	44.1	48.8	TC48402a	CA187839a (2)	xisep0101	11.62
QSt12hsr_11_Tv_5A	12-h release	5	28.8	4.81	17.6	38.8	N/A	xtxp065	CA190667c	22.61
QSt24hsr_11_Tv_1A	24-h release	1	70.0	2.94	65.1	74.1	Xcup44	CA094128a	CA094069a	8.63
QSt24hsr_11_Tv_3A	24-h release	3	45.0	3.52	42.4	45.6	N/A	CA187839a (2)	TC48402a	11.00
QSt24hsr_11_Tv_5A	24-h release	5	24.9	5.21	13.8	34.3	N/A	xtxp065	CA190667c	25.53
QSt24hsr_11_Tv_9A	24-h release	9	44.2	3.97	43.4	52.6	Xgap32	TC65153a	Xgap206	11.28
QStHYP12_11_Tv_3A	12-h hydrolysis rate	3	46.1	3.5	42.4	48.8	TC48402a	CA187839a (2)	xisep0101	9.89
QStHYP12_11_Tv_5A	12-h hydrolysis rate	5	26.3	5.02	18.4	36.7	N/A	xtxp065	CA190667c	27.98
QStHYP12_11_Tv_9A	12-h hydrolysis rate	9	44.2	2.82	40.3	54.6	Xgap32, TC65153a	TC59518b (2)	Xgap206	8.16
QStHYP24_11_Tv_3A	24-h hydrolysis rate	3	45.0	3.5	41.8	45.6	N/A	CA187839a (2)	TC48402a	11.21
QStHYP24_11_Tv_5A	24-h hydrolysis rate	5	24.0	5.13	13.5	32.8	N/A	xtxp065	CA190667c	26.33
QStHYP24_11_Tv_9A	24-h hydrolysis rate	9	44.2	3.72	42.8	50.9	Xgap32, TC65153a	TC59518b (2)	Xgap206	10.77

Hour release = μg reducing sugar at that time point; release rate = μg/h

LOD logarithm of the odds, R^2 percentage of phenotypic variance explained by the QTL

Table 5 Leaf 2010 QTLs—location and markers

QTL	Trait category	Chromosome	Peak (cM)	Peak LOD	Left boundary (cM)	Right boundary (cM)	Markers in QTL	Left flanking marker	Right flanking marker	R^2 (%)
QLf4hsr_10_Tv_3A	4-h release	3	46.6	2.82	43.0	49.6	TC48402a	CA187839a (2)	Xisep0101	8.22
QLf4hsr_10_Tv_3B	4-h release	3	93.3	4.43	88.3	93.3	TC69429a	mSbCIR276	N/A	12.51
QLf4hsr_10_Tv_7A	4-h release	7	27.8	2.9	9.7	41.3	xcup33 (2), CA193820b, S15_224156_ag20	CA148166b	Xcup70	8.30
QLf12hsr_10_Tv_3A	12-h release	3	46.5	3.02	42.8	49.7	TC48402a	CA187839a (2)	Xisep0101	8.42
QLf12hsr_10_Tv_5A	12-h release	5	52.3	3.01	46.0	55.1	Xtxp225, S14_460612_aag#3	CA190667c	CA100232a	7.75
QLf24hsr_10_Tv_5A	24-h release	5	43.4	2.81	39.4	50.5	CA190667c	Xtxp065	S15_224156_ag20	9.47
QLf24hsr_10_Tv_9A	24-h release	9	40.3	3.46	36.4	54.1	TC59518b (2), TC65153a, xgap32	CA170467a	Xgap206	11.38
QLfHYP24_10_Tv_9A	24-h hydrolysis rate	9	40.7	3.88	36.6	43.4	TC59518b (2), TC65153a	CA170467a	Xgap32	13.17

Hour release = μg reducing sugar at that time point; release rate = μg/h

LOD logarithm of the odds, R^2 percentage of phenotypic variance explained by the QTL

Table 6 Leaf 2011 QTLs—location and markers

QTL	Trait category	Chromosome	Peak	Peak LOD	Left boundary (cM)	Right boundary (cM)	Markers in QTL	Left flanking marker	Right flanking marker	R^2 (%)
QLf4hsr_11_Tv_3A	4-h release	3	43.5	2.8	38.9	49.6	CA187839a (2), TC48402a	CA074959a	Xisep0101	7.01
QLf4hsr_11_Tv_3B	4-h release	3	93.4	6.1	88.9	93.4	TC69429a	mSbCIR276	N/A	15.64
QLf4hsr_11_Tv_8A	4-h release	8	5.4	5	0.8	13.3	xcup27 (2)	Xtxp273	Xtxp047	12.87
QLf12hsr_11_Tv_3A	12-h release	3	44.3	3.53	40.4	49.5	TC48402a	CA187839a (2)	Xisep0101	9.95
QLf12hsr_11_Tv_3B	12-h release	3	93.4	5.21	88.4	93.4	TC69429a	mSbCIR276	N/A	14.45
QLf12hsr_11_Tv_8A	12-h release	8	6.4	3.89	1.5	20.7	xcup27 (2)	Xtxp273	Xtxp047	10.90
QLf24hsr_11_Tv_3A	24-h release	3	92	4.76	86.5	93.4	TC69429a	mSbCIR276	N/A	13.28
QLfHYP12_11_Tv_3A	12-h hydrolysis rate	3	44.1	4.03	40.5	49.3	TC48402a	CA187839a (2)	Xisep0101	7.28
QLfHYP12_11_Tv_3B	12-h hydrolysis rate	3	93.4	5.56	88.4	93.4	TC69429a	mSbCIR276	N/A	15.68
QLfHYP12_11_Tv_8A	12 h hydrolysis rate	8	11.6	3.72	1.3	21.5	xcup27 (2)	Xtxp273	Xtxp047	11.27
QLfHYP24_11_Tv_1A	24 h hydrolysis rate	1	53	2.87	48.8	56.7	mSBCIR288	TC59518b (1)	CA078376a	15.49
QLfHYP24_11_Tv_3A	24 h hydrolysis rate	3	91.6	5.02	86.4	93.4	TC69429a	mSbCIR276	N/A	12.61

Hour release μg reducing sugar at that time point, release rate = μg/h

LOD logarithm of the odds, R^2 percentage of phenotypic variance explained by the QTL

Stem 4-h sugar release

A total of 6 QTLs for 4-h sugar release were detected in the BT × SP population. For the 2010 experiment, 4 QTLs (*QSt4hs_10_Tv_2A, QSt4hs_10_Tv_5A, QSt4hs_10_Tv_8A*, and *QSt4hs_10_Tv_9A*) explained 37.13 % of the phenotypic variance. In the 2011 experiment, 2 QTLs (*QSt4hs_11_Tv_3A* and *QSt4hs_11_Tv_5A*) explained 29.84 % of the phenotypic variance. None of the QTLs corresponded across years. *S. propinquum* contributed the favorable (increased sugar release) allele for three of the 4 loci (*QSt4hs_10_Tv_2A, QSt4hs_10_Tv_8A, and QSt4hs_10_Tv_9A)* in the 2010 experiment and one of the 2 loci (*QSt4hs_11_Tv_3A*) in the 2011 experiment. *S. bicolor* contributed the favorable allele for the remaining loci.

Stem 12-h sugar release

A total of 4 QTLs for 12-h sugar release were detected in the BT × SP population. For the 2010 experiment, 3 QTLs (*QSt12hs_10_Tv_2A, QSt12hs_10_Tv_7A* and *QSt12hs_10_Tv_9A*) explained 29.65 % of the phenotypic variance while in the 2011 experiment two QTLs (*QSt12hs_11_Tv_3A* and *QSt12hs_11_Tv_5A*) explained 34.23 % of the variance. None of the QTLs corresponded across the 2 years. *S. propinquum* contributed the favorable allele for all three loci in the 2010 experiment and *QSt12hs_11_Tv_3A* in the 2011 experiment. *S. bicolor* contributed the favorable allele to *QSt12hs_11_Tv_5A*.

Stem 24-h sugar release

For stem 24-h sugar release, a total of 7 QTLs were identified. For BT × SP, the 2010 experiment contained 3 QTLs, while the 2011 experiment contained 5 QTLs. In the 2010 experiment, 3 QTLs (*QSt24hs_10_Tv_3A, QSt24hs_10_Tv_7A* and *QSt24hs_10_Tv_9A*) explained 36.02 % of the phenotypic variation. In the 2011 experiment, 4 QTLs (*QSt24hs_11_Tv_1A, QSt24hs_11_Tv_3A, QSt24hs_11_Tv_5A* and *QSt24hs_11_Tv_9A*) explained 56.43 % of the phenotypic variation. While two QTLs in each experiment were found on common chromosomes, none of the stem 24-h release QTLs overlapped. *S. propinquum* contributed the favorable allele for *QSt24hs_10_Tv_7A* and *QSt24hs_10_Tv_9A* in 2010 and *QSt24hs_11_Tv_3A,* and *QSt24hs_11_Tv_9A* in 2011. *S. bicolor* contributed the favorable allele to *QSt24hs_10_Tv_3A* in 2010 and *QSt24hs_11_Tv_1A* and *QSt24hs_11_Tv_5A* in 2011.

Stem 12-h hydrolysis yield potential

Six QTLs for 12-h hydrolysis yield potential were identified, 3 in 2010 stem tissue, and 3 in 2011 stem tissue. In the 2010 experiment, 3 QTLs (*QStHYP12_10_Tv_5A, QStHYP12_10_Tv_7A* and *QStHYP12_10_Tv_9A*) explained 26.07 % of the phenotypic variance, while in the 2011 experiment, 3 QTLs (*QStHYP12_11_Tv_3A, QStHYP12_11_Tv_5A* and *QStHYP12_11_Tv_9A*) explained 46.03 % of the phenotypic variation. QTLs identified on chromosome 9 overlapped in both 2010 and 2011 experiments. *S. propinquum* contributed the favorable allele for *QStHYP12_10_Tv_7A* and *QStHYP12_10_Tv_9A* in the 2010 experiment and *QStHYP12_11_Tv_3A* and *QStHYP12_11_Tv_9A* in the 2011 experiment. *S. bicolor* contributed the favorable allele for *QStHYP12_10_Tv_5A* in 2010 and *QStHYP12_11_Tv_5A* in 2011.

Stem 24-h hydrolysis yield potential

A total of 5 QTLs were identified for stem 24-h hydrolysis yield potential. Two QTLs (*QStHYP24_10_Tv_7A* and *QStHYP24_10_Tv_9A*) were identified in the 2010 experiment and three QTLs (*QStHYP24_11_Tv_3A, QStHYP24_11_Tv_5A* and *QStHYP24_11_Tv_9A*) were identified in the 2011 experiment. The two QTLs associated with 24-h HYP in 2010 accounted for 24.49 % of the variation. The three QTLs associated with 24-h HYP in 2011 accounted for 48.31 % of the variation. None of the QTLs identified for 24-h hydrolysis rate overlapped across years. *S. propinquum* contributed the favorable allele for both QTLs in 2010 and *QStHYP24_11_Tv_3A* and *QStHYP24_11_Tv_9A* in 2011. *S. bicolor* contributed the favorable allele for only *QStHYP24_11_Tv_5A* in 2011.

Stem crystallinity index

Stem CI was tested in 2010 stem tissue. Two QTLs (*QStCI_10_2A* and *QStCI_10_3A*) were found on chromosomes 2 and 3, accounting for 22.35 % of the phenotypic variation in CI. *S. propinquum* contributed both favorable alleles for both QTLs.

Leaf 4-h sugar release

Six total QTLs for leaf 4-h sugar release were identified. In 2010 leaf samples, three QTLs (*QLf4hs_10_Tv_3A, QLf4hs_10_Tv_3B* and *QLf4hs_10_Tv_7A*) were identified which accounted for 29.03 % of the phenotypic variance. In the 2011 experiment, 3 QTLs (*QLf4hs_11_Tv_3A, QLf4hs_11_Tv_3B* and *QLf4hs_11_Tv_8A*) accounted for 35.52 % of the phenotypic variation. Two QTLs overlapped across years, both on chromosome 3. *S. propinquum* contributed the favorable allele for *QLf4hs_10_Tv_3A,* and *QLf4hs_10_Tv_7A* in the 2010 experiment and *QLf4hs_11_Tv_3A* in the 2011 experiment. *S. bicolor* contributed favorable alleles for *QLf4hs_10_Tv_3B* in 2010 and

QLf4hs_11_Tv_3B and *QLf4hs_11_Tv_8A* in the 2011 experiment.

Leaf 12-h sugar release

Five QTLs for leaf 12-h sugar release were identified. Two QTLs (*QLf12hs_10_Tv_3A* and *QLf12hs_10_Tv_5A*) were identified in the 2010 leaf samples accounting for a total of 16.17 % of phenotypic variation. Three QTLs (*QLf12hs_11_Tv_3A, QLf12hs_11_Tv_3B* and *QLf12hs_11_Tv_8A*) identified in leaf 2011 samples accounted for a total of 35.30 % of phenotypic variation. One QTL for leaf 12-h sugar release from each population overlapped across years on chromosome 3. *S. propinquum* contributed favorable alleles for *QLf12hs_10_Tv_3A* in the 2010 experiment and *QLf12hs_11_Tv_3A* in the 2011 experiment. *S. bicolor* contributed the favorable allele for *QLf12hs_10_Tv_5A* in the 2010 experiment and *QLf12hs_11_Tv_3B* and *QLf12hs_11_Tv_8A* in the 2011 experiment.

Leaf 24-h sugar release

A total of 3 QTLs for leaf 24-h sugar release were identified. Two QTLs (*QLf24hs_10_Tv_5A* and *QLf24hs_10_Tv_9A*) were identified in leaf 2010 tissue accounting for 20.84 % of phenotypic variation, while a single QTL (*QLf24hs_11_Tv_3A*) was identified in leaf 2011 samples accounting for 13.28 % of phenotypic variation. No QTLs for leaf 24-h sugar release overlapped across the 2 years. *S. propinquum* contributed the favorable allele for *QLf24hs_10_Tv_9A* in the 2010 experiment. *S. bicolor* contributed the favorable allele for *QLf24hs_10_Tv_5A* in 2010 and *QLf24hs_11_Tv_3A* for 2011.

Leaf 12-h hydrolysis yield potential

Three QTLs were identified for 12-h hydrolysis yield potential (*QLfHYP12_11_Tv_3A, QLfHYP12_11_Tv_3B* and *QLfHYP12_11_Tv_8A*), all in 2011 leaf samples and accounting for a total of 34.22 % of phenotypic variation. No QTLs for this trait were found in the 2010 experiment. *S. propinquum* contributed the favorable allele for *QLfHYP12_11_Tv_3A* while *S. bicolor* contributed the favorable allele for *QLfHYP12_11_Tv_3B* and *QLfHYP12_11_Tv_8A*.

Leaf 24-h hydrolysis yield potential

A total of three QTLs were identified for 24-h hydrolysis yield potential. A single QTL (*QLfHYP24_10_Tv_9A*) was identified in the leaf 2010 samples accounting for 13.17 % of phenotypic variation. Two QTLs (*QLfHYP24_11_Tv_1A* and *QLfHYP24_11_Tv_3A*) were identified in the leaf 2011 samples accounting for 28.10 % of phenotypic variation. QTLs for leaf 24-h hydrolysis yield potential did not correspond between years. *S. propinquum* contributed the favorable allele for *QLfHYP24_10_Tv_9A* in the 2010 experiment and *QLfHYP24_11_Tv_1A* in the 2011 experiment. *S. bicolor* contributed the favorable allele to only *QLfHYP24_11_Tv_3A* in the 2011 experiment.

Discussion

Toward the goal of maximizing bioenergy yield from lignocellulosic feedstocks, the identification of traits, pathways and gene variants associated with increased bioenergy production is highly valuable. While much research has been conducted on increased overall biomass accumulation (plant height, tillering, delayed flowering time, and other traits), far fewer studies have been conducted on the genetics of enzymatic conversion efficiency of bioenergy grass feedstocks. For example, a study of *Miscanthus* identified QTLs associated with thermal conversion efficiency (Atienza et al. 2003a, b), yet no QTLs were identified for enzymatic conversion. We believe that these bioprocess-relevant QTL studies are necessary to identify genes and develop genotypes that are favorable to realistic industrial processes.

We have previously shown that *S. bicolor* exhibits a wide range of conversion efficiencies across a broad sampling of diverse genotypes in two environments (Vandenbrink et al. 2010). A second study revealed that conversion efficiency is not enzyme-specific, in that different genotypes react similarly to alternate enzyme treatments (*T. viride* and *A. niger*) (Vandenbrink et al. 2011). This suggests that there is a large amount of genetic diversity relevant to bioprocessing that exists in sorghum, and that these bioconversion genes can be mapped and possibly manipulated (e.g., marker-assisted selection) in an effort to increase conversion efficiencies under varied conversion processes. To our knowledge, no tissue-specific QTLs have been identified for conversion efficiency in sorghum, which was a prime rationale for this study.

Using *T. viride* cellulase as a metric for conversion efficiency, we have identified multiple QTLs linked to increased conversion efficiency and crystallinity of lignocellulosic biomass. A total of 49 QTLs associated with enzymatic conversion efficiency were identified in this study (29 leaf QTLs: Tables 5 and 6; 20 stem QTLs: Tables 3 and 4). Two QTLs for CI were identified in 2010 stem tissue. Due to the labor intensiveness of CI analysis and the lack of correlation between CI and conversion efficiency in leaf (unpublished data), only 2010 stem tissue was analyzed. Stem tissue QTLs were found located on 6 of the 10 chromosomes in the 2010 experiment and only 4

in 2011. In leaf tissue, QTLs were identified on 4 of 10 chromosomes in the 2010 experiment and 3 in 2011. The two CI QTLs were located on two separate chromosomes (2 and 3). As we have previously shown that leaf and stem tissues differ in conversion efficiency and some compositional components (Vandenbrink et al. 2011), it would be interesting to see which QTLs for compositional components overlap with QTLs for conversion efficiency and CI in the BT × SP population.

While conversion efficiency and its genetic control varied substantially between the 2 years studied, our QTL map (Fig. 1) revealed six QTL "hotspots" where 4 or more conversion QTLs overlap. One of the six hotspots only contained QTLs for conversion efficiency in 2011 stem tissue and was located on chromosome 5 (13.4–38.8 cM). The five remaining high-density hotspots contained QTLs for conversion efficiency in both leaf and stem tissues. These hotspots were found on chromosomes 3 (38.9–49.6 cM; 86.4–93.4 cM), 5 (39.4–55.1 cM), 7 (9.7–45.4 cM), and 9 (32.4–54.6 cM). Of the 6 QTL hotspots, 5 contained QTLs for both leaf and stem conversion efficiency while one hotspot contained only stem conversion QTLs. Interestingly, analysis of the QTLs after mapping to the sorghum genome (Fig. 2) revealed that the QTL hotspots found on chromosomes 5 and 7 cover a large portion of the chromosome (~53 and ~57 MB, respectively), which is likely due to low recombination in these chromosomal regions.

In addition to both leaf and stem QTL overlap in the QTL hotspots, there was QTL overlap from both grow-out years which may indicate robust candidate alleles for breeding. Three of the six QTL hotspots contained QTLs from both 2010 and 2011 field studies. The first region on chromosome 3 (38.9–49.6 cM) contained QTLs for both 2010 and 2011 leaf conversion traits, while the second chromosome 3 region (86.4–93.4 cM) contained an overlap of leaf QTLs from the 2 years. The hotspot on linkage group 9 (32.4–54.6 cM) contained QTLs for stem conversion traits identified in both 2010 and 2011 studies. While the existence of QTL hotspots is evident, there were a large number of QTLs that did not overlap across environments, indicating a significant environmental effect that must be considered in any breeding program.

CI has been shown to decrease the ability for cellulase to bind the cellulose substrate Avicel via reduced surface area, thereby decreasing the hydrolysis of lignocellulosics to reducing sugars (Hall et al. 2010). Similarly, a negative correlation between biomass CI and hydrolysis has been previously reported in the grass species *Miscanthus* (Yoshida et al. 2008) and sorghum (Vandenbrink et al. 2011). This same trend has been found in non-grass species such as pine (Chang and Holtzapple 2000). In addition to tissue specificity in leaf and stem enzymatic conversion, CI has been shown to be higher in stem than leaf tissue in both maize and sorghum (Reddy and Yang 2007). CI has previously been shown to be negatively correlated with hydrolysis efficiency stemming from lignocellulosic composition. One could expect that the QTLs for hydrolysis yield would overlap with QTLs for conversion efficiency. While each of the two QTLs identified for CI were located on chromosomes where stem 2010 conversion efficiency QTLs were also located, there was no overlap of the QTLs on chromosomes two or three. This suggests that while CI and enzymatic conversion efficiency are correlated, the QTLs that have the largest effect in the CI and enzymatic conversion efficiency phenotypes are different. In addition, it is likely that these two traits are under separate genetic control in the population that was tested. If CI and enzymatic conversion efficiency are under separate genetic control, it could provide dual routes for genetic manipulation or selection of sorghum for the purpose of increasing lignocellulosic bioenergy production.

Few studies have genetically mapped sugar release loci in bioenergy grasses. Lorenzana et al. (2010) conducted a study of maize and identified QTLs associated with enzymatic glucose release. Dilute acid pretreatment followed by enzymatic hydrolysis of maize stover identified 10 QTLs on 5 chromosomes associated with total glucose release. Interestingly, the QTLs identified in that study tended to be of small effect, with a maximum of 10 % phenotypic variance explained. In this study, we have identified QTLs that tended to explain a larger amount of phenotypic variance, ranging from 7 to 27 %. A Genome Wide Association Study (GWAS) was conducted on glucose release in sorghum (Wang et al. 2011). Wang et al. identified two SSR markers significantly associated with enzymatic glucose release on chromosomes 2 and 4, and these positions did not overlap with the QTLs identified in our study. It should be noted that the study of Wang et al. was not replicated across multiple environments, so it is possible that the markers associated with increased hydrolysis yield were environment specific.

Previous studies have shown that traits relevant to bioenergy conversion may demonstrate tissue specificity. For example, a study of lignocellulosic biomass composition components (cellulose, hemicellulose, lignin) pointed to QTLs specific for leaf and stem tissues (Murray et al. 2008a). In addition, expression profiling of cellulase synthase genes (CESA) in maize has shown tissue-specific expression, which would support tissue-specific QTLs (Appenzeller et al. 2004). While most (5/6) of the high-density QTL hotspots we identified contained QTLs for both leaf and stem conversion traits, the region on chromosome five only contained QTLs for stem conversion efficiency. In addition, a small group of three leaf conversion QTLs overlapped on chromosome 8. It is possible

that genes exist within these regions that are specific to leaf- and stem-specific conversion efficiency. These results suggest that bioenergy grass conversion efficiency might be better understood and improved through analyzing leaf and stem tissues independently for conversion efficiency traits when breeding for superior bioenergy cultivars.

Conclusions

In this study, a total of 20 leaf and 29 stem QTLs were identified for enzymatic conversion efficiency over two grow-out years. In addition, two biomass crystallinity QTLs were identified from stem tissue which has previously been shown to inversely correlate with enzymatic conversion efficiency. Interestingly, the stem crystallinity QTLs did not overlap with conversion efficiency QTLs, which suggests alternate genetic control. We believe that these and undiscovered bioconversion QTLs and genes will help lead to superior feedstocks capable of making lignocellulosic bioenergy a competitive alternative to fossil fuels.

Acknowledgments This research was supported in part by the Clemson Experiment Station project #SC-1700381 to FAF and assigned technical contribution no. 6096.

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