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Genetic control of yield related stalk traits in sugarcane

K. S. Aitken · S. Hermann · K. Karno · G. D. Bonnett · L. C. McIntyre · P. A. Jackson

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Abstract A major focus of sugarcane variety improvement programs is to increase sugar yield, which can be accomplished by either increasing the sugar content of the cane or by increasing cane yield, as the correlation between these traits is low. We used a cross between an Australian sugarcane variety Q165, and a *Saccharum officinarum* accession, IJ76-514, to dissect the inheritance of yield-related traits in the complex polyploid sugarcane. A population of 227 individuals was grown in a replicated field trial and evaluated over 3 years for stalk weight, stalk diameter, stalk number, stalk length and total biomass. Over 1,000 AFLP and SSR markers were scored across the population and used to identify quantitative trait loci (QTL). In total, 27 regions were found that were significant at the 5% threshold using permutation tests with at least one trait; individually, they explained from 4 to 10% of the phenotypic variation and up to 46% were consistent across years. With the inclusion of digeneic interactions, from 28 to 60% of the variation was explained for these traits. The 27 genomic regions were located on 22 linkage groups (LGs) in six of the eight homology groups (HGs) indicating that a number of alleles or quantitative trait alleles (QTA) at each QTL contribute to the trait; from one to three alleles had an effect on the traits for each QTL identified. Alleles of a candidate gene, *TEOSINTE BRANCHED 1* (*TB1*), the major gene controlling branching in maize, were mapped in this population using either an SSR or SNP markers. Two alleles showed some association with stalk number, but unlike maize, *TB1* is not a major gene controlling branching in sugarcane but only has a minor and variable effect.

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K. S. Aitken · G. D. Bonnett · L. C. McIntyre
CSIRO Plant Industry, Queensland Bioscience Precinct,
306 Carmody Rd, St Lucia, QLD 4067, Australia

S. Hermann
BSES Limited, Meiers Rd, Indooroopilly, QLD 4068, Australia

K. Karno
School of Integrated Biology, University of Queensland,
Brisbane, Australia

P. A. Jackson
Davies Laboratory, CSIRO Plant Industry, Private Mail Bag,
Aitkenvale, QLD 4814, Australia

K. S. Aitken (✉) · S. Hermann · K. Karno ·
G. D. Bonnett · P. A. Jackson
Co-operative Research Centre for Sugar Industry Innovation
through Biotechnology, University of Queensland, Level 5,
John Hines Building, St Lucia, QLD 4072, Australia
e-mail: karen.aitken@csiro.au

Introduction

Modern sugarcane hybrids are highly complex polyploids derived from interspecific hybridisation between *Saccharum officinarum* and its wild relative *S. spontaneum* (Roach 1984). *S. officinarum* ($x = 10$) is an octoploid ($2n = 80$) (D'Hont et al. 1996), and *S. spontaneum* ($x = 8$) has a range of ploidy levels, which vary between 6 and 12 (Sreenivasan et al. 1987). *S. officinarum* and *S. spontaneum* differ for many morphological traits with *S. officinarum* having few thick stalks and high sucrose content, whereas *S. spontaneum* has many thin stalks yielding little or no sucrose. A major trait incorporated into modern sugarcane from *S. spontaneum* was the increased ratooning ability that the wild species had over the cultivated *S. officinarum*. Typically, sugarcane is clonally propagated and harvested

through a number of years called ratoon crops. The modern cultivars due to their hybrid origin have increased yield of ratoon crops from the initial planting (Daniels and Roach 1987).

A major focus of sugarcane variety improvement programs is obtaining higher sugar yields. Work by Milligan et al (1990) determined that cane yield was the most important determinant of sucrose yield and become increasingly important in ratoon crops. Further, they identified stalk number as being the primary determinant of cane yield and again increasing in importance in ratoon crops. Stalk diameter was more important than stalk length in affecting stalk weight regardless of crop age. They concluded that selection for increased sucrose content should emphasise cane yield with concentration on stalk number. These results indicate that an understanding of the relationship among sugar yield components could increase the efficiency of selection for sugar yield. In recent decades, the increase in sugar yield has been achieved primarily by increasing cane yield rather than sugar content (Jackson 2005). Identification of markers linked to both yield and sucrose traits could help increase sugar yield through both an increase in cane yield and sucrose content.

There have been a number of studies on marker–trait associations in sugarcane, although due to high ploidy, these have been limited by the number of markers generated and subsequently low genome map coverage. Another difficultly with sugarcane is that due to the heterozygous nature of this outcrossing species and the high ploidy level, it is expected that many alleles would be segregating at key loci for the traits of interest. These quantitative trait allele (QTA) effects may be small compared to a diploid species, especially for traits that have been selected for in modern cultivars for several generations.

Molecular markers have been used to determine the location of QTL for a number of yield traits in sugarcane. Two interspecific populations derived from basic germplasm were used with a candidate gene approach to identify markers associated with sucrose content (Ming et al. 2001). A number of QTL were detected for stalk weight and stalk number, although the maps generated all had low genome coverage (Ming et al. 2002). A further QTL study carried out on a selfed population of the cultivar R570 identified 16, 15 and 11 QTL for stalk diameter, stalk length and stalk number, respectively, although only one QTL was consistently detected in two years data. They also identified a number of interactions, which, when included in the model, explained from 30 to 51% of the variation for these traits (Hoarau et al. 2002). These QTL were located onto a partial AFLP map with 11 of the QTL placed onto homology groups.

In this study, we report the identification of molecular markers linked to yield traits in an Australian cultivar using an extensive genetic map (Aitken et al 2005) constructed using AFLP and SSR markers. This represents the most extensive study on yield traits using the most organised and highest genome coverage map of a sugarcane cultivar to date. The combination of three years of data from two separate replicated field trials with single row 5 m plots allows an accurate estimation of phenotype data. In an attempt to start to identify the genes that underlie the QTL, we extensively tested the candidate gene *TEOSINTE BRANCHED 1* (*TB1*) for association with any of the yield traits measured. *TB1* has been shown in maize × teosinte crosses to map to a region, which contains a QTL controlling vegetative branching (Doebley et al 1995). The expression of mutant phenotypes of *TB1* correlated with changes in axillary branching in maize (Hubbard et al. 2002).

Materials and methods

Mapping population and phenotypic data

The *S. officinarum* clone IJ76-514 ($2n = 80$) was used as the female in a cross, made in 1999, with Q165 ($2n \sim 115$), an Australian cultivar and elite parent. Two hundred and twenty-seven progeny clones were evaluated in two field trials. Both trials were of a randomised complete block design with four replicates of single row 5 m plots at the Kalamia estate, Ayr (147.4°E, −19.5°S), Queensland (Aitken et al. 2005). The trials were planted in two separate locations in September 2000 and August 2001 and ratooned twice. Stalk weight was measured in the first location in August 2001 and stalk weight, height, diameter and stalk number measured at the second location a few days prior to harvest in the plant crop in July 2002 and again in the second ratoon crop in July 2004. The number of millable stalks, stalk number (SN), was counted for the whole row plot. Four stalks were randomly sampled to measure stalk diameter (SD) and stalk length (SL). SD was recorded at 1 m from the stalk base. SL was measured from the first visible dewlap leaf to the stalk base. Tonnes of cane per hectare (TCH) was estimated from the product of stalk weight and stalk number.

Genotyping

Generation of the marker data and construction of the genetic map of Q165 were reported by Aitken et al. (2005). AFLPs and SSRs were used to generate 2,238 polymorphic markers that were scored across this population. Of these, 1,365 were present in Q165 and were used to generate the map reported by Aitken et al. (2005). AFLP markers were named by the primer combination consisting of the three selective

nucleotides in the *Eco*RI primer followed by the three selective nucleotides in the *Mse*I primer followed by numbers of polymorphic bands in descending molecular weight order. SSR markers were labeled "m" and then the name and identity number from the Sugarcane Microsatellite Consortium collection and a letter denoting the allele by descending molecular weight. A total of 951 simplex (present only once in the genome and segregating 1:1) and 123 duplex markers (present twice in one parental genome) formed 136 linkage groups (LG), of which 127 LG could be assigned into eight homo(eo)logous groups (HG). This number of HGs is equivalent to the basic chromosome number of *S. spontaneum* (D'Hont et al. 1996). For two of the HGs, two sets of small LGs (assumed to be inherited from *S. officinarum*) aligned to single larger LG (assumed to be inherited from *S. spontaneum*) forming in total the 10 basic chromosomes of *S. officinarum* (Aitken et al. 2005).

Analysis of trait data

Phenotypic data from each experiment were analysed using the SAS statistical package (SAS Institute Inc., NC, USA) as in the study by Aitken et al. (2006). In brief, for analyses of variance, the following model was assumed:

$$y_{ij} = \mu + b_j + g_i + e_{ij}$$

where μ, b_j, g_i and e_{ij} are the grand mean, block effect, genotype effect and error effect, respectively. Genotypes were considered to be random effects, generating variance component $\sigma_g{}^2$.

Analyses of covariance were done for selected pairs of traits. This was done in the same manner as the analyses of variance except that sums of cross products and mean cross products were determined, with appropriate covariance components and mean cross products substituted for variance components and mean squares.

Broad sense heritabilities (h^2) for each trait were determined from (Falconer and Mackay 1996)

$$h^2 = \sigma_g^2 \Big/ \sigma_p^2,$$

where σ_g^2 = genetic variance and σ_p^2 = phenotypic variance. Phenotypic variance was determined from

$$\sigma_p^2 = \sigma_g^2 + \sigma_e^2 / r,$$

where σ_e^2 = error variance and r = number of replicates. Genetic correlations between characters X and Y, $r_{g(x,y)}$, were determined from

$$r_{g(x,y)} = \mathrm{COV}_{g(x,y)} \Big/ \left(\sigma_{g(x)} \cdot \sigma_{g(y)}\right),$$

where

$\mathrm{COV}_{g(x,y)}$ Genetic component of covariance between characters X and Y,

$\sigma_{g(x)}$ and $\sigma_{g(y)}$ Genetic standard deviation for characters X and Y, respectively.

QTL detection

Detection of putative QTL was carried out for each marker using a one-way analysis of variance to identify significant associations between the presence or absence of a marker and the trait. Composite interval mapping was also carried out using QTL Cartographer v1.15, but as only simplex markers can be included in this analysis and no additional QTA were identified, only single marker analysis is presented. Both simplex (S) and multiplex (present as two or more copies per genome) (M) markers were used for this analysis. As sugarcane is a polyploid, up to 12 alleles were considered to be segregating at any given locus, suggesting that only the most significantly different alleles were likely to be detected. Other alleles at that locus may also contribute to the trait resulting in many quantitative trait alleles (QTAs) of small effect. The threshold used for detection of a QTA was calculated using 1,000 permutations, where the trait values are randomly permuted among the progeny, destroying any relationship between the trait values and the genotypes of the marker loci and the analysis carried out as previously described to generate a distribution of statistic values, which we would expect if there were no QTL linked to any of the marker loci (Churchill and Doerge 1994). The statistic values generated are used to determine thresholds for identification of a QTA. The QTAs detected are identified as suggestive or significant and correspond to the genome-wide probabilities proposed by Lander and Kruglyak (1995), where suggestive linkage corresponds to the detection of one false QTL at random in a genome scan and significant linkage one false QTL expected to occur 0.05 times in a genome scan. This allowed the detection of putative QTA, which can be verified in further generations. The QTA effect was estimated as the average difference in phenotype of the individuals carrying the marker compared to those without. The proportion of the total phenotypic variance explained was calculated using the sums of squares provided by the analysis of variance for significant associations.

To identify epistatic interactions, every simplex marker was tested for digenic linear × linear interactions; only interactions between unlinked markers were considered. Because of the relatively small sample size, estimations of the four class means are poor; so, a stringent threshold of $P < 0.00001$ was used to identify an interaction. The phenotypic variance explained by all the QTAs was determined by multiple regression.

Testing *TEOSINTE BRANCHED 1* as a candidate gene

To test *TB1* as a candidate gene for any of the QTAs identified, targeted marker discovery was performed on the sugarcane homologues (*SoTB1*) of the maize *TB1* gene, which is present in a single copy in rice. The sugarcane homologues were identified by comparison of sequences from rice, maize, sorghum and sugarcane using BLAST searches in genbank database (AY286002; AF415048; AJ293562). This gene has been shown to be associated with QTL controlling vegetative branching in maize × teosinte crosses (Hubbard et al. 2002). We used several different techniques to find simplex markers in *SoTB1* alleles including gene-specific SSRs, ecotilling and the Sequenom Mass Array SNP detection system. Primers for all products leading to the identification of simplex markers are listed in Table 1. Directed SSR analysis was carried out essentially as described by Aitken et al. (2005). Primers were used to a previously identified *Saccharum* microsatellite (mSSCIR76; AJ293562), which was found to have homology to *SoTB1* using BLAST searches in the genbank database. Ecotilling was carried out essentially as described by McIntyre et al. (2006). Two overlapping fragments *SoTB1a* (amplified with 5′-GGACTTACCGCTTTACCAACA-3′ and 5′-GACT ACTTGCCTTGGCCGGCTTCTTCCC-3′) and *SoTB1b* (amplified with 5′-GCAGCCTCTCCGTCGACGGCAA GC-3′ and 5′-GTTCTGCTGAAAGACGACTCCACC GAG-3′) were used to screen ~1,100 bp gene sequence. Detection using the Sequenom Mass Array platform® was carried out at the Australian Genome Research Facility (Brisbane, Australia) according to standard quantitative SNP genotyping protocols.

Results

Quantitative trait analysis

For all traits, the genotype effect was highly significant ($P < 0.0001$) in both plant crop and second ratoon. Furthermore, broadsense heritabilities were high, indicating good control of within-trial environmental variation and experimental error (Table 2). For all traits apart from stalk diameter, the heritability decreased by a significant amount from the plant crop to the ratoon crop. Estimated genetic correlations were highest between stalk diameter and stalk weight (1.02) for both plant and ratoon crops. They were also generally high between stalk numbers, stalk weight, stalk diameter and TCH for both years with stalk number being negatively correlated with stalk weight and stalk diameter (Table 2). Stalk length was only consistently correlated with TCH. The decrease in TCH from the 2002 plant crop to the 2004 second ratoon crop appears to be due to a decrease in stalk weight driven by a decrease in stalk length.

Marker trait associations identified with simplex markers

At a significance level of $P < 0.003$, which corresponded to the suggestive level from the permutation analysis, a total of 140 associations were found for all traits. Of these, 83 (59%) were also significant at $P \leq 0.001$, which corresponded to the significant level from the permutation analysis (Tables 3, 4). The 140 associations corresponded to 68 different markers, of which 64 were distributed onto 20 LGs, with four markers remaining unlinked (Table 3). At this level of significance, a single marker–trait association was detected on 2 of the 20 LGs, whereas for the other 18 LGs, from 2 to 56 marker–trait associations were detected. At a lower significance, threshold of $P \leq 0.005$, all of the 20 LGs were represented by more than one marker–trait association.

As many of the marker–trait associations involved closely linked markers and a number of the traits studied were highly correlated, they probably reflect the effects of the same QTA, although the presence of several marker–trait associations over a large distance on a LG means that more than one QTA at that location cannot be ruled out. A group of significant markers on a LG was considered as one QTA with two exceptions; two QTAs were identified on LG12a in HG2 and LG3 in HG4 as they were either end of a large LG in the case of LG12a and, on LG3, were of opposite effect.

Table 1 Primers used to identify simplex alleles in *SoTB1*

Allele	Mapping technique	Forward primer	Reverse primer	Extension primer
TB1-115	Ecotilling	GCAGCCTCTCCGTCGACGGCAAGC	GTTCTGCTGAAAGACGACTCCACCGAG	–
TB1-ssr1	Directed ssr	TCCACCGAGTTCCCATTG	GCGAACCAAGGAGAAGCA	–
TB1-82	Mass array	TTGCCAAAGCCTAGGACCAC	ATCGTCGTCCATGAACATGC	TTCTTGCATCCT TCCTC
TB1-84	Mass array	GTTTCTTGCATCCTTCCTC	CCGAGCTCCAACCATTTGA	GAATTGGAGGA GAGGGA

Table 2 Genetic and phenotypic correlations of the four related traits for the plant crop and second ratoon, as well as broad sense heritability (H^2), genetic variance and means for each trait crop year

Traits	Traits[a]					H^2		$\sigma^2 g$		Units	Mean	
	SN	SWT	SD	SL	TCH	2002	2004	2002	2004		2002	2004
SN	0.69	−0.68	−0.69	0.17	0.96	0.83	0.74	4.13	5.71	m	11.93	13.22
SWT	−0.61	0.72	1.02	0.35	0.71	0.88	0.73	1.81	0.46	kg	7.31	4.34
SD	−0.65	1.02	0.82	−0.13	0.30	0.88	0.86	7.05	7.53	mm	28.52	27.27
SL	−0.11	0.56	0.20	0.61	0.61	0.85	0.61	591.3	231.3	cm	306.47	178.66
TCH	0.44	0.76	0.56	0.58	0.52	0.71	0.45	472.6	147.6	T/ha	142.58	93.85

[a] Genetic correlations between traits in the plant crop (2002) and in the second ratoon crop (2004) are given below and above the diagonal, respectively. On the diagonal is indicated the phenotypic correlation of a trait between years. Traits: *SN* stalk number, *SWT* stalk weight, *SD* stalk diameter, *SL* stalk length, *TCH* tonnes of cane/hectare

Each individual marker–trait association explained from 4 to 10% of the total phenotypic variation. The distribution of the magnitude of the effect was typical with an asymmetrical shape skewed towards QTAs of small effect. Out of the total of 140 marker–trait associations for all traits, fifty explained 4%, forty-four 5%, seventeen 6%, fourteen 7%, seven 8%, six 9% and two 10% of the phenotypic variation. All the markers explaining 6% or more of the phenotypic variance were significant at $P \leq 0.001$. This was also the case for the majority of markers with an r^2 of 5% (30/44), but only four markers that explained 4% of the phenotypic variation were significant at this level.

Using the SSRs to align the LGs within the HGs, these 22 QTAs on 20 LGs could be condensed into 16 locations called QTL (Table 3). Each of these QTL had from one to three LGs, which probably represent different alleles of the same locus. Only the most significant marker within a group of markers is reported in Table 3, although from 1 to 55 marker–trait associations were detected for each of the QTA (data not shown). All of the QTAs that were assigned a map position had more than one marker significant at that QTA. Out of the 26 locations for QTAs (22 located to linkage groups and 4 unlinked markers), 13 (50%) were associated with more than one trait at a $P \leq 0.003$ in at least one of the two years. The majority of the individual marker–trait associations formed two groups of trait effects, which were in accordance with the sign of the genetic correlations. Group one (LG12a and LG59 in HG2 and LG63 in HG3) all increased stalk weight, stalk diameter, height and TCH with a reduction in stalk number. Group two (LG38, LG8 in HG2, LG4 in HG3, LG3, LG80 in HG4, LG55 in HG5 and unlinked Aaccag31 and Aagcat17) all decreased stalk diameter and stalk weight and increased stalk number as expected. The marker–trait associations on LG8, HG2 and LG3, HG4 both increased height and resulted in increased TCH. LG4, HG3 and unlinked Aaccag31 had no effect on TCH and the QTAs on LG38, LG35, HG2, LG80, HG4, LG55, HG5 and unlinked Aagcat17 all decreased both height and TCH.

Marker–trait associations with multiplex markers

All of the 239 multiplex markers were tested for association with a trait as for simplex markers. A total of 27 multi-dose markers were associated with from one to five traits. Seven of these multiplex markers were SSRs and could be assigned to an existing QTL by aligning with the map (Table 3). Nine of the AFLP markers fitted the expected segregation ratio for duplex markers and could be putatively assigned to a linkage group (Table 3) or formed a new LG. These resulted in 14 QTAs, of which six had already been identified using the simplex markers–trait associations (Table 3). These results plus the SSR markers indicate that, in these cases, multiplex and simplex markers may reveal the same QTA. So, in total, at least seven new genetic factors were added using the multiplex markers.

Interactions between unlinked markers

For all the related traits over all years of data, 195 interactions between two unlinked markers were significant at $P \leq 10^5$, which could be condensed to 73 specific interactions, as in many cases linked markers exhibited the same interaction. The 73 specific interactions mapped to 63 locations, of which 54 could be placed on the Q165 genetic map (Aitken et al. 2005) and were represented by from 1 to 32 interactions between two markers. Twenty (32%) of the 63 locations were at QTAs and 14 were detected by more than one trait. One location on LG38 HG2 was involved in 75 (39%) of the interactions. Only 2% of the interactions were between two markers that were not located at QTAs. The majority of the interactions (58%) was between two QTAs, and in total, 98% of the interactions involved at

Table 3 Significant associations between either the most significant simplex marker located within a group of markers on a linkage group or an unlinked simplex or mutliplex marker and agronomic traits

Marker[a,b]	lg/hg[d]	QTL	Stalk weight 01		Stalk weight 02		Stalk weight 04		Stalk diameter 02		Stalk diameter 04	
			r^2	Effect	r^2	Effect	r^2	Effect	r^2	Effect	r^2	Effect
mSSCIR36g	19/1	Q1	2*	0.36	2*	0.46	2*	0.21	3*	0.93	6******	1.42
mSSCIR43 h	27/1	Q2										
Aaccac36	11/2	Q3	4****	0.52	4**	0.55	4***	0.30	2*	0.79		
Acccac24	12a/2	Q4	2*	−0.35	2*	−0.45	2*	−0.24	3**	−1.02	4****	−1.13
Aggctc24	35/2	Q4	4***	−0.50	3*	−0.47			4****	−1.15	2*	−0.79
Aggctc31	6/2	Q4			2*	−0.45			2*	−0.85		
AB16-11	12a/2	Q5	6******	0.61	6****	0.72	3**	0.26	4****	1.15	2*	0.83
Acgctt27	38/2	Q6	10*********	−0.81	9*********	−0.88	5*****	−0.36	7*******	−1.53	3**	−1.03
Aggctg10	8/2	Q6					3**	−0.29	2*	−0.83	4****	−1.18
Actctg12	59/2	Q7					5******	0.37			5*****	1.24
Agccac4	11/3	Q8										
Aggcac21	45/3	Q9										
Acactg8	69/3	Q9					3***	−0.30	3*	−0.90	3**	−1.00
Acgcag22	4/3	Q10	4***	−0.49	4****	−0.62	4****	−0.32	7******	−1.47	6******	−1.45
Aaccat27	63/3	Q11	7******	0.65	7*******	0.79	3**	0.27	7*******	1.47	2*	0.78
mSMC1120c	3/4	Q12										
Aaccac22	3/4	Q13	4***	−0.49	4**	−0.55	2*	−0.23	4***	−1.09	3***	−1.08
Acccat13	80/4	Q13	3*	−0.40	3**	−0.52	3**	−0.27	5****	−1.21	5******	−1.25
mSSCIR27a	47/4	Q14										
mSSCIR39i	40/5	Q15	3**	−0.45	3**	−0.50	2*	−0.24	2*	−0.86	3**	−0.97
Agcctg1	55/5	Q15	5****	−0.53	5****	−0.63	4*****	−0.34	3*	−0.94		
mSCCIR15d	71/6	Q16					2*	−0.25				
Aaccag31	u[e]	Q17	4**	−0.48	3**	−0.50	3**	−0.27	4****	−1.14	2*	−0.78
Aagcat17	u	Q18	2*	−0.39	2*	−0.45	4****	−0.32				
Aagcta16	u	Q19										
Aggctc23	u	Q20	4****	−0.51	5*****	−0.65			4**	−1.08	2*	−0.8
MD[c]												
mSSCIR54 m	u/2	Q5	8********	−0.73	7*******	−0.80	4***	−0.31	7******	−1.49	3**	−1.04
mSSCIR1q	u/2	Q5										
mSMC2055b	u/3	Q9	2*	−0.41	2*	−0.49			5*****	−1.37	6******	−1.48
Aagcta38	u	Q21	5***	−0.44	5*****	−0.53	3***	−0.24	2*	−0.70		
Acgctt23	38/2	Q6	10*********	−0.81	9*********	−0.88	5*****	−0.35	6******	−1.45	3*	−0.95
Agccat37	u	Q22	3*	−0.37	2*	−0.38	3**	−0.26	3**	−0.95	4****	−1.03
Aggcac13	48/4	Q10										
Aaccag5	u	Q23										
Acgctt7	19/1	Q1	5****	0.57	4****	0.65	2*	−0.23	4****	1.23	2*	0.92
Acactg12	u	Q24										
Acgcac13	u	Q25										
Agccac19	u	Q26										
Aggcac31	u	Q27										

Marker[a,b]	lg/hg[d]	QTL	Stalk no. 02		Stalk no. 04		Stalk height 02		Stalk height 04		TCH02		TCH 04	
			r^2	Effect	r^2	Effect	r^2	Effect	r^2	Effect	r^2	Effect	r^2	Effect
mSSCIR36g	19/1	Q1			3**	−1.02								
mSSCIR43 h	27/1	Q2	9********	−1.28	2*	−0.93					7*******	−13.72		
Aaccac36	11/2	Q3					2*	7.41						

Table 3 continued

Marker[a,b]	lg/hg[d]	QTL	Stalk no. 02		Stalk no. 04		Stalk height 02		Stalk height 04		TCH02		TCH 04	
			r^2	Effect	r^2	Effect	r^2	Effect	r^2	Effect	r^2	Effect	r^2	Effect
Acccac24	12a/2	Q4	2*	0.66										
Aggctc24	35/2	Q4			2*	0.91					4****	−10.74		
Aggctc31	6/2	Q4									5****	−10.97		
AB16-11	12a/2	Q5					4***	9.96	2*	5.19	6******	12.41		
Acgctt27	38/2	Q6	2*	0.64	4*****	1.27	5****	−11.28			4***	−10.32		
Aggctg10	8/2	Q6	4****	0.9	2*	0.86					4****	10.7		
Actctg12	59/2	Q7												
Agccac4	11/3	Q8									4****	−10.85		
Aggcac21	45/3	Q9					5*****	11.92	5*****	8.74				
Acactg8	69/3	Q9	5*****	0.95	4*****	1.26	2*	7.83			3**	9.56	3**	6.32
Acgcag22	4/3	Q10	4***	0.81	4*****	1.26	2*	7.92	3*	6.48				
Aaccat27	63/3	Q11			2*	−0.8	3**	8.78	3**	7.21	4***	9.95		
mSMC1120c	3/4	Q12	5****	−0.94										
Aaccac22	3/4	Q13	6******	1.04	4****	1.16			4****	8.23			2*	5.27
Acccat13	80/4	Q13							4****	−8.18	3**	−9.46		
mSSCIR27a	47/4	Q14			2*	0.93	4***	9.92			2*	7.09	4****	7.6
mSSCIR39i	40/5	Q15	2*	0.63	2*	0.91	3**	8.71	7*******	10.11	2*	7.74	2*	5.52
Agcctg1	55/5	Q15					6******	−12.28	2*	−5.62	3*	−8.58	2*	−5.13
mSCCIR15d	71/6	Q16											4****	−7.71
Aaccag31	u[e]	Q17			4****	1.22								
Aagcat17	u	Q18					3*	−8.57	4*****	−8.48			3*	−5.96
Aagcta16	u	Q19									5*****	−11.7		
Aggctc23	u	Q20							4**	−9.13	3**	−9.13		
MD[c]														
mSSCIR54 m	u/2	Q5			4****	1.17	2*	−8.36			2*	−7.22		
mSSCIR1q	u/2	Q5			2*	0.82	3**	7.88	3**	5.59	5*****	12.78	4****	8.19
mSMC2055b	u/3	Q9	2*	0.68										
Aagcta38	u	Q21					3**	−7.94			4****	−8.65		
Acgctt23	38/2	Q6	2*	0.63	4****	1.14	5****	−11.39			3*	−8.66		
Agccat37	u	Q22												
Aggcac13	48/4	Q10							4****	−8.5				
Aaccag5	u	Q23							5******	−9.41				
Acgctt7	19/1	Q1									5*****	12.5		
Acactg12	u	Q24									6******	9.65	6******	7.13
Acgcac13	u	Q25											5****	7.39
Agccac19	u	Q26											4****	−6.96
Aggcac31	u	Q27					5*****	10.2			2*	6.65		

When an association was significant for a given year, the association for the other year was reported up to $P = 0.05$. The corresponding percentage of variance (r^2) is also given as well as the phenotypic effect attributed to an individual marker

Level of significance for marker–trait association: *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.005$, ****$P \leq 0.001$, *****$P \leq 0.0005$, ******$P \leq 0.0001$, *******$P \leq 0.00005$, ********$P \leq 0.00001$, *********$P \leq 0.000005$

[a] The most significant marker of the group of simplex markers associated with the trait

[b] All SSR markers start with an m all other markers are AFLPs

[c] Multiplex markers

[d] The linkage group and homology group from Aitken et al. (2005)

[e] Unknown location

Table 4 Number of simplex, multiplex and interaction-QTAs detected for yield components and the estimated proportion of phenotypic variance explained

	Stalk weight						Stalk diameter			
	2001		2002		2004		2002		2004	
Associations detected	<0.001[e]	<0.003[g]	<0.001	<0.003	<0.001	<0.003	<0.001	<0.003	<0.001	<0.003
Simplex markers	17	24	20	25	3	10	15	23	5	9
Simplex markers common to 2 years	16	23	17	23	2	8	2	4	2	4
Deduced S-QTAs[a]	4	8	6	7	3	5	3	7	3	6
Deduced S-QTAs common to 2 years	4	7	4	7	2	3	2	2	2	2
M-QTAs[b]	4	6	5	6	2	2	4	6	3	5
M-QTAs common to 2 years	4	6	4	6	1	1	1	2	1	2
Int-QTA pairs[c]	59		11		5		10		17	
deduced int-QTA pairs	14		8		5		4		10	
Int-QTA common to two years	5		5		2		3		2	
r^2 of a multiple regression										
S-QTAs	17% (203)[f]	23% (199)	22% (203)	25% (201)	14% (219)	17% (217)	19% (206)	26% (204)	14% (219)	24% (215)
S-QTAs and M-QTA[d]	20% (193)	27% (190)	24% (194)	29% (192)	18% (219)	20% (217)	21% (204)	32% (200)	27% (217)	36% (213)
Int-QTA	51% (196)		52% (193)		39% (180)		41% (199)		56% (194)	
S-QTAs, M-QTAs, int-QTAs	53% (186)	55% (185)	57% (190)	57% (190)	43% (179)	44% (178)	43% (197)	53% (193)	59% (191)	60% (191)

	Stalk number				Stalk length				TCH			
	2002		2004		2002		2004		2002		2004	
Associations detected	<0.001[e]	<0.003[g]	<0.001	<0.003	<0.001	<0.003	<0.001	<0.003	<0.001	<0.003	<0.001	<0.003
Simplex markers[a]	4	10	7	13	2	8	4	5	5	11	1	2
Simplex markers common to 2 years	0	1	0	1	1	1	4	4	0	0	0	0
deduced S-QTAs	3	4	3	5	2	3	3	4	3	7	1	2
Deduced S-QTA common to 2 years	0	1	0	1	1	1	1	1	0	0	0	0
M-QTAs[b]	0	0	0	2	2	2	1	2	3	5	1	1
M-QTL common to 2 years[b]	0	0	0	0	0	0	1	2	1	2	0	0
Int-QTA pairs[c]	29		19		1		10		22		10	
Deduced int-QTA pairs	16		9		1		6		13		3	
Int-QTA common to 2 years	0		0		0		0		0		0	
r^2 of a multiple regression												
S-QTAs	20% (194)[f]	23% (194)	9% (227)	15% (222)	11% (210)	16% (210)	14% (221)	14% (167)	18% (200)	26% (200)	7% (219)	10% (218)

Table 4 continued

Associations detected	Stalk number				Stalk length				TCH			
	2002		2004		2002		2004		2002		2004	
	<0.001[e]	<0.003[g]	<0.001	<0.003	<0.001	<0.003	<0.001	<0.003	<0.001	<0.003	<0.001	<0.003
S-QTAs and M-QTA[d]	20% (194)	23% (194)	9% (227)	15% (222)	19% (209)	19% (208)	19% (221)	20% (166)	25% (197)	32% (197)	15% (218)	19% (217)
Int-QTA	50% (189)		40% (186)		12% (212)		15% (213)		54% (191)		23% (218)	
S-QTAs, M-QTAs, int-QTAs	54% (189)	54% (189)	43% (186)	45% (186)	28% (209)	29% (208)	27% (213)	29% (162)	56% (189)	59% (189)	29% (217)	32% (216)

[a] One simplex marker per cluster

[b] QTA detected with multiplex markers

[c] Interactions detected at $P \leq 0.00001$

[d] QTA identified with simplex and multiplex markers

[e] Corresponds to a QTA detected at the significant level

[f] In parenthesis, the total degrees of freedom of the regression model representing the number of progeny with no missing molecular data across all factors

[g] Corresponds to a QTA detected at the suggestive level

least one QTA. Seven of the interactions were detected in more than one year's data (Table 4).

Consistency of QTA detected between years

Of the 38 QTAs detected using both simplex and multiplex markers at the suggestive level, 18 (45%) were detected in more than 1 year with at least one of the traits (Table 3). These included 12/26 simplex QTAs and 6/14 multiplex associations. To further determine the stability of the effects of the marker–trait associations, the stringency was reduced; at $P \leq 0.005$, 21 (53%) marker–trait associations were significant across years, and at $P \leq 0.01$, 26 (65%) marker–trait associations were significant across years (data not shown). The directions of the effects were the same for all QTAs regardless of whether they were significant in both years or not.

For the digenic interactions, out of a total of 73, seven were detected in more than one year's data and were represented by 67 interactions. As for the QTAs, the direction of the effect was the same in the two years data for all the interactions detected regardless of whether they were significant or not.

QTA contribution to genetic variance

The r^2 was calculated using multiple regressions for different models, which included only simplex QTAs, simplex and multiplex QTAs, interactions alone and then all three types together (Table 4). Depending on which trait/year was considered, the simplex QTAs at $P \leq 0.003$ explained from 10% (two QTAs) to 26% (seven QTAs) of the phenotypic variance. With the addition of the multiplex QTAs, up to 12% more variation was explained.

Apart from stalk length, the digenic interactions explained more of the phenotypic variance than the combined simplex and multiplex markers. The variation explained ranged from 12% (one interaction pair, $df = 212$) to 56% (10 interaction pairs, $df = 194$). When all effects were included into a single model, the proportion of the total phenotypic variance explained ranged from 29 to 60%, with stalk length having the lowest variation explained (approximately one-third) and the remaining traits having approximately one half of the variation explained. Higher levels of variation were explained by all traits apart for stem diameter in the plant crop compared to the second ratoon crop. The reduced variation explained in the second ratoon crop was probably due to poorer plant performance and higher environmental variation.

Each phenotypic trait of an individual plant was the product of a number of QTAs with both positive and negative effects. To determine how the QTAs contribute to the phenotype, the phenotypes of individuals within the

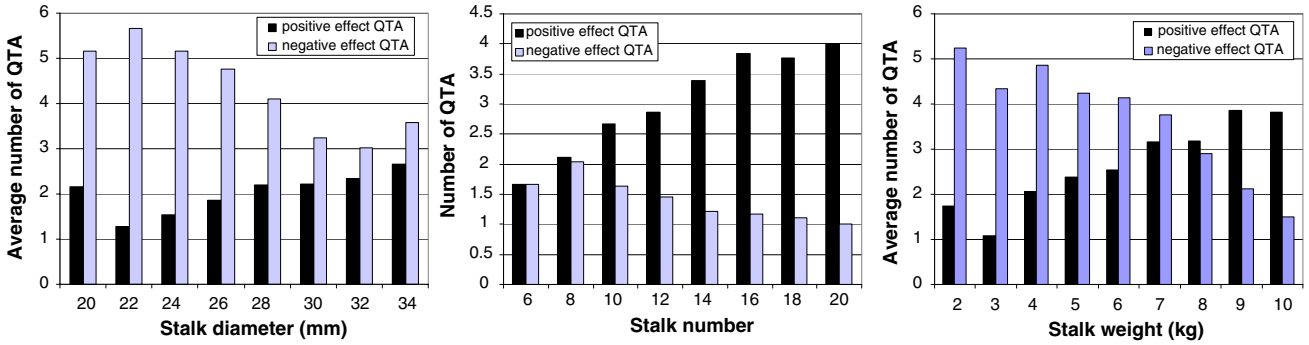

Fig. 1 Frequency distribution of both negative and positive QTAs identified in Q165 for different stalk traits averaged across two years

population were grouped for each trait, and the average number of positive and negative QTAs detected in both years was plotted (Fig. 1). In all cases, the number of negative QTAs diminished and positive QTAs increased as the measured value of the trait increased.

Candidate gene contribution

To test the approach of identification of candidate genes that underlie QTL for these traits, we investigated sugarcane homologues (*SoTB1*) of the maize *TB1* gene, as this is known to have a major effect on stalk architecture in maize and rice. Initially, we used directed SSRs, with primers specific to *SoTB1*. These primers produced five products in each parent, with only three products in common (i.e. seven different products). We screened 227 progeny and found that only one of these products (TB1-ssr1), inherited from Q165, segregated as a simplex marker. TB1-ssr1 was positioned on LG25 in HG4 and was not associated with a QTA. However, other linkage groups within this homology group did contain QTA in a similar position. In an attempt to identify more simplex markers that could be mapped to the other LGs in this HG, we performed sugarcane eco-tilling, which should allow identification of all single nucleotide polymorphisms. Sugarcane *SoTB1* alleles were amplified in two parts (A and B), and products from each progeny were subjected to CELI digestion. We identified 20 putative SNPs in the first 654 bp fragment (A) and 15 putative SNPs in the second 568 bp fragment (B). However, once again, only one SNP (TB1-115) segregated as a simplex marker, and this SNP was positioned in the same map location as TB1-ssr1, indicating that both techniques had identified the same allele. This also suggested that there were no other simplex SNP markers present in *SoTB1* alleles. Finally, we used Sequenom Mass Array (SMA) technology to look for associations between the traits and SNP dosage and perhaps identify map positions for duplex markers. When we screened 93 progeny for four SNP markers using SMA, we found a further two simplex markers (TB1-82 and TB1-84). Both of these SNPs were present in Q165 but absent in IJ76-514. As with the previous alleles, they mapped to HG4 in a location homologous to TB1-115 marker on LG3 (TB1-82) and LG73 (TB1-84). The remaining two SNPs were multidose and could not be mapped. Interestingly, LG3 contained a QTA for stalk number and stalk height (see Table 3); detailed analysis of this region showed that other markers besides TB1-82 mapped more closely to the QTA.

Single marker regression was used to test the role of *SoTB1* in controlling the traits scored. The analysis determined that only TB1-82 was associated with stalk traits including TCH in a single year and stalk number in both years. However, these associations were only at a low significance level of $P \leq 0.01$ and explained from 6 to 8% of the variation (data not shown).

Discussion

In total, 167 simplex and multiplex markers were significantly associated with at least one of the traits at $P \leq 0.003$. These markers clustered into 38 locations (QTAs), of which the majority was detected by more than one marker and could be placed on the genetic linkage map generated by Aitken et al. (2005). These explained individually from 4% to 10% of the phenotypic variation, which is similar to the amounts detected in other studies in sugarcane for these traits (Ming et al. 2002; Hoarau et al 2002). Twenty-nine of these QTAs could be condensed using the SSRs on the linkage map to 16 mapped locations (QTL). Another 11 markers could not be placed on the map resulting in at least 27 loci involved in these traits.

The majority of these QTAs had an effect on more than one trait; in all cases, effects on stalk weight were associated with a corresponding effect on stalk diameter. The majority of the QTAs detected caused a decrease in stalk weight and diameter and a corresponding increase in stalk number. This is not surprising because of the high correlation between the traits. The QTA reducing stalk diameter could be inherited from the wild type *S. spontaneum* cane,

which has many thin stalks. *S. spontaneum* germplasm is also more polymorphic, and therefore, larger numbers of simplex markers were probably detected corresponding to these parts of the genome. Four QTAs were detected that increased stalk weight and stalk diameter and decreased stalk number corresponding to the *S. officinarum* characteristics. Fifteen of these QTAs had an effect on TCH, seven of them increased TCH and eight decreased TCH. Four of the QTAs that increased TCH also increased stalk weight through an increase in stalk diameter or stalk height. Two decreased stalk weight but increased stalk number and the third and most consistent QTA for TCH at the unlinked multiplex marker Acactg12 had little or no effect on any other trait measured (Table 3).

The variance explained by all simplex and multiplex QTAs ranged from 15 to 36%. This increased substantially when the digenic interactions were added to the model to as much as 60% (stalk diameter 2004) (Table 4). A high percentage of the interactions involved at least one QTA and seven were consistent between years indicating strong evidence that epistasis plays a role in the genetic control of these traits. Interactions have also been detected in other sugarcane studies with similar levels of variation explained (Hoarau et al. 2002; Ming et al. 2002), although this study, with greater genome structure and coverage in the linkage map, allowed more of the interactions to be located to the map and detected a greater number of interactions between existing QTAs.

The majority of the QTAs affected more than one trait; since all the traits apart from stalk length are highly correlated, this was expected. These pleiotropic effects of the QTAs could be due to the same loci having an affect on the different traits or could be due a number of different QTAs affecting different traits located in the same genomic region. This association of a QTA with more than one trait was also observed by Ming et al. (2002). A number of the QTAs while highly significant for one trait only had a minor or no significant effect on other traits.

Sugarcane breeders concentrate on both increasing the sugar content and increasing the biomass, as both increase the final sugar yield. There is evidence that breeders have been more successful in increasing biomass than sugar content (Jackson 2005). There was no correlation between the stalk traits and the sugar measurement traits in this population; the highest correlation was between stalk number and brix (0.21, 0.002). Twelve loci were mapped in this population for sugar-related traits (brix and pol) (Aitken et al. 2006). Nine out of the twenty-two QTAs mapped for the stalk-related traits map to similar locations to those for brix or pol measures of sugar accumulation. This co-location of QTAs for both yield-related traits and sugar accumulation was also noted in a study by Ming et al. (2002), where one QTA for sugar yield, stalk weight, ash content and pol mapped to the same location on a genetic map generated from a cross between *S. spontaneum* and a hybrid (*S. officinarum* × *S. spontaneum*). In another study on a selfed population generated from a sugarcane cultivar R570 again QTA for stalk number and brix were located to the same LG (Hoarau et al. 2002). There are not enough SSRs in common between this map and the Q165 map to determine if any of these QTAs locate to the same region. The nine QTAs identified in this study formed three groups, group 1 contained five QTAs, which all increased sucrose with a resulting decrease in yield due to an increase in stalk number with a corresponding decrease in stalk diameter and stalk weight. Group 2 contained two QTAs, which increased sucrose content with a decrease in stalk number and an increase in stalk diameter and weight. Group 3 contained two QTAs, which decreased sucrose content and yield with an increase in stalk number and a corresponding decrease in stalk diameter and stalk weight. These QTAs had the expected effect corresponding to the negative correlation between stalk number and stalk weight and diameter, but sucrose content both increased and decreased with an increase in stalk number and a decrease in stalk diameter and weight. This reflects the lack of correlation between sucrose content and yield components in this population and indicates that it is possible to increase sucrose content without a corresponding decrease in yield.

The high ploidy combined with sugarcane's heterozygosity results in a number of alleles being detected at each locus that affects a trait as well as a number of loci being detected for each trait studied. Individual alleles have a small effect on the trait, and it is a combination of these alleles that result in the phenotype. In all traits studied, both positive and negative effects were detected. These results are similar and in the same range as other studies that detected QTAs in sugarcane (Ming et al. 2002; Hoarau et al 2002), although in this study, due to greater genome organization, more QTAs could be aligned into QTL. When the number of positive and negative QTAs in individual plants was analysed for each trait, the trend for all traits was for the accumulation of positive effect QTAs and a decline in the number of negative effect QTAs (Fig. 1). The data indicate that it should be possible to select for, for example increase stalk weight, by selecting for the positive QTAs and away from the negative QTAs and that the positive effect QTAs are additive. This would indicate that cultivars with high stalk weight have accumulated positive effect QTAs. Within this population, the individuals with the highest stalk weight had on average 1.6 QTAs with negative effects and 4 QTAs with positive effects.

The consistency of QTA effects across years was to a large extent dependant on the significance level used to

assess the data. At the genome wide level of 5%, calculated from permutation testing, from seven QTAs for stalk weight to zero QTAs for TCH were consistent between years. This increased to a consistence of detection across years of 95% for stalk weight to 20% for TCH at an individual detection level of 5%. Obviously, this reflects the high genotype × crop-cycle interactions detected for traits like TCH. It also indicates that for a polyploidy heterozygous individual like sugarcane the numerous small effect QTAs identified are at the limit of statistical detection for a population size of 227 individuals and thus appear to fluctuate between years. All the QTAs that were consistent between years were detected at $P \leq 0.001$. A larger population size would increase the statistical power for detection of these QTAs, but field trials, which are needed to test these types of traits, are costly. In addition, it may be possible to identify more consistent yield component traits.

Correlation of QTL with *SoTB1*

To better understand the molecular basis of sugarcane stalk traits, we mapped several alleles of *SoTB1* and looked for association with QTAs. All three simplex alleles were mapped to HG4 in three homo(eo)logous LGs. Only one allele *TB1*-82 was significantly associated with yield traits including stalk number and TCH, although it only explained a small amount of the variation, from 6 to 9%. Because of the small number of progeny tested for these markers, this is probably an over estimation of the variation explained. QTAs of similar size were also identified on other LGs in this region. This indicates that while alleles of *SoTB1* are associated with stalk number, there are a number of other loci on other HGs that have an equal or greater effect on this trait. QTL analysis of a cross between maize and the highly tillered wild relative teosinte determined that five gene regions controlled the major morphological differences between these two species (Doebley et al. 1995). *TB1* mutants of maize resemble teosinte, and their phenotype is due to the presence of secondary and tertiary axillary branching as well as to an increase in the length of each node, rather than to an increase in the number of nodes (Hubbard et al. 2002). These results indicate that, in sugarcane, like maize, *TB1* is one of a number of genes that contribute to stalk morphology, but unlike maize, it is not the major candidate for the control of tillering in the population studied. Our results are similar to those obtained by Doust et al. (2004) who studied the effect of *TB1* on tillering in foxtail millet. They found that *TB1* had only a minor and variable effect on tillering or branching and identified a number of possible alternative candidate genes including hormone biosynthesis pathway genes.

Our results show that, in total, 27 regions of the sugarcane genome have effects on yield traits. For individual traits, from two to eight QTAs were identified, the majority of these had an effect on more than one trait. The candidate gene *SoTB1* mapped to one of these QTAs but mapped to a different homo(eo)gous group than the largest effect QTA. Future work will use comparative mapping of our sugarcane maps with sorghum to identify new potential candidate genes.

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