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ORIGINAL ARTICLE

Natural variation and genetic analysis of the tiller angle gene *MsTAC1* in *Miscanthus sinensis*

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Abstract Biomass yield is an important target trait in *Miscanthus* breeding for desirable energy crops. Tiller angle is a key trait of plant architecture because it determines planting density and further influences biomass yield through affecting photosynthesis efficiency. *TAC1*, a major gene involved in tiller and leaf angle control in rice and maize, respectively, has been extensively studied. Nucleotide variation at this gene, *MsTAC1*, was investigated in 33 *Miscanthus sinensis* accessions collected from different areas in China, and one genotype of *Miscanthus* × *giganteus*. A total of 136 loci, including 129 single base substitutions and seven InDels, occurred within the *MsTAC1* gene of 1,874 bp. The genetic diversity at *MsTAC1* is characterized by high nucleotide diversity (π value) and high heterozygosity. Clustering analysis indicated that the phylogenetic tree of the 33 *M. sinensis* accessions was correlated with their geographical sites of origin. The neutrality test revealed no strong selection pressure on coding and non-coding region variations of the *MsTAC1* gene in the accessions. Phenotype evaluations were conducted for tiller angle and five other traits in the *Miscanthus* panels in the first two growth years of 2009 and 2010. Analysis of variance showed significant phenotypic variations in the examined traits. Association analysis using 246 markers detected 88 loci associated with all the test traits in either 1 or 2 years, and 11 of the 88 were year reproducible and thus reliable. These associations indicate that the variation of *MsTAC1* affects the phenotypic value of the tiller angle, tiller number and biomass yield, suggesting that allelic variation in *MsTAC1* affects multiple traits and demonstrates its significance in *Miscanthus* breeding programs.

Keywords Association analysis · Evolution selection · Natural variation · Nucleotide diversity · *Miscanthus* · Tiller angle control

Abbreviations

TAC	Tiller angle gene
Indel	Insert and deletion
CDS	Coding sequence
UTR	Untranslated region

Introduction

Improved cultivars of perennial bioenergy crops are needed to reduce the dependence on traditional fossil energy and decrease greenhouse gas emissions. *Miscanthus* × *giganteus*,

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a sterile triploid hybrid from a spontaneous cross between *M. sacchariflorus* and *M. sinensis*, is an ideal bioenergy crop because of its high biomass yield, low energy inputs, flexible growing season and tolerance to biotic and abiotic stresses (Price et al. 2004; Hastings et al. 2009a, b; Heaton et al. 2009). However, *M.* × *giganteus* is a sterile triploid hybrid that may result in a narrow genetic basis that could reduce its climatic adaptation and overwinter survival in some extreme conditions (Clifton-Brown and Lewandowski 2000). Furthermore, the lack of genetic diversity is a serious risk in the context of potential disease or insects, which may cause extensive damage to planted crops (Jørgensen and Schwarz 2000). Thus, it is necessary to broaden the genetic base of *Miscanthus*. *M. sinensis* occupies a wide range of habitats, including beaches, rocky slopes, riverbanks, roadsides, mountains and other marginal areas (Zhao et al. 2011, 2013a, b). Consequently, the genetic diversity and environmental adaptation of *M. sinensis* would facilitate the development of *Miscanthus* as a bioenergy crop.

Natural variation, including phenotypic and genotypic variation caused by spontaneously arising mutations, has been maintained in nature by all evolutionary processes, including artificial and natural selection within species (Alonso-Blanco et al. 2009). The extent and pattern of DNA sequence variation in natural accessions provide valuable clues about the evolutionary forces acting on a species and evolutionary characteristics (Corre et al. 2002; Akey et al. 2004). The domestication of wild species transformed into modern cultivated crops indicates distinct phenotypic changes or improvement in morphological, physiological and biochemical traits to meet human needs, such as maize, wheat and rice (Peng et al. 1999, 2003; Doebley et al. 2006; Kovach et al. 2007).

Intraspecific variation in biomass yield can be resulted from many components of plant architecture. One such component is the angle between the tillers and erect main stems. Transition from prostrate to erect growth habits or from a spreading tiller angle to a compact tiller angle was also a critical domestication event for grasses (Jin et al. 2008). A plant with narrower tiller angles or more erect growth habits is considered to have a compact plant architecture, which may increase plant density, enhance photosynthesis efficiency and eventually improve grain yield. Therefore, the tiller angle plays an important role in plant architecture, and high yields can be obtained through more dense plantings of plants with a compact tiller angle, which determines the plant's ability to grow and capture light efficiently. Consequently, tiller angle has long attracted attention for achieving ideal plant architecture to improve grain yield (Peng et al. 1999) and is genetically controlled by quantitative trait loci (QTLs) (Yu et al. 2007).

Because of the agronomic and theoretical importance of tiller angle, extensive studies have been conducted, and three genes involved in tiller angle have been cloned, and the molecular basis has been clearly elucidated in rice (Li et al. 2007; Yu et al. 2007; Jin et al. 2008; Tan et al. 2008). *LAZY1* (*LA1*) regulates shoot gravitropism by which the rice tiller angle is controlled and plays a negative role in polar auxin transport (Li et al. 2007). *PROG1* (PROSTRATE GROWTH1), a transcription factor localized in nucleus, encodes a Cys(2)-His(2) zinc-finger protein and controls aspects of wild rice plant architecture, including tiller angle, number of tillers, greater grain number and higher grain yield. An amino acid substitution in the PROG1 protein during domestication led to the transition from the prostrated plant architecture of wild rice to the erect architecture of cultivated rice (Jin et al. 2008; Tan et al. 2008). *TAC* is a major quantitative trait locus controlling the tiller angle in rice. The gene, *tac1*, is a mutation in the 3′-splicing site of a 1.5-kb intron from 'AGGA' to 'GGGA' that decreases the level of tac1, serves as a key control for compact plant architecture with a tiller angle close to zero in rice (Yu et al. 2007). Strong selection has been detected only in *japonica* rice, especially in the 3′-flanking region of the *TAC1* coding region containing the functional nucleotide polymorphism (Jiang et al. 2012). *ZmTAC1*, a putative *TAC1* ortholog, controls the size of the leaf angle in maize, a nucleotide difference in the 5′-untranslated region (UTR) between the compact inbred line ('CTCC') and the expanded inbred line ('CCCC') influences the expression level. The ortholog of *TAC1*, named *MsTAC*, remains to be identified in collections of *M. sinensis*.

Unlike major food crops such as rice, maize and wheat, *M. sinensis* did not undergo drastic morphological modifications for any trait by artificial selection, which makes it useful for dissection of the molecular evolution and function of *MsTAC1*. *M. sinensis* accessions were collected throughout its native range in China (18°39.872′–41°19.730′N for latitude, 100°10.027′–123°41.416′E for longitude, and 3–2,109 m for altitude). Its wide distribution and abundant genetic variation without any artificial selection make *M. sinensis* an attractive resource and model organism for dissection of the natural genetic variation of relevant traits in bioenergy crops (Zhao et al. 2013b). In this study, our specific objectives were (1) to reveal the nucleotide diversity of *MsTAC1* in the *M. sinensis* collection; (2) to elucidate the genetic relationship among these accessions, along with rice, maize and sorghum; (3) to understand whether neutral and selective forces acted on *MsTAC1* gene variation; and (4) to determine association between DNA polymorphism in the *MsTAC1* region and the phenotypic variation of tiller angle. The findings will not only provide important insights into the demographic history and the geographical differentiation pattern of *M. sinensis* within its native geographical range, but also helpful for *Miscanthus* breeding.

Table 1 Origin and geographical parameters in the sites of 33 *M. sinensis* accessions

Acc. code	Original site	North latitude	East longitude	Elevation (m)
MS9	Jianshi County, Hubei Province	30°35.033′	109°42.297′	583
MS13	Xianfeng County, Hubei Province	29°59.417′	109°03.432′	684
MS44	Jiang'an County, Sichuan Province	28°41.072′	105°08.090′	395
MS47	Changning County, Sichuan Province	28°39.202′	104°56.959′	263
MS54	Chengdu City, Sichuan Province	30°04.947′	103°05.044′	672
MS121	Hanzhong City, Shaanxi Province	33°02.995′	107°25.899′	583
MS138	Baoji City, Shaanxi Province	33°55.256′	106°25.370′	968
MS222	Taijiang County, Guizhou Province	26°39.131′	108°13.791′	691
MS240	Guiyang City, Guizhou Province	26°23.971′	106°39.905′	1,096
MS265	Eshan County, Yunnan Province	24°11.510′	102°22.938′	1,556
MS275	Yugan County, Jiangxi Province	28°42.778′	116°49.945′	21
MS281	Jingdezhen City, Jiangxi Province	29°15.672′	117°06.379′	51
MS316	Luoyuan City, Fujian Province	26°23.580′	119°28.717′	107
MS341	Huichang County, Jiangxi Province	25°29.620′	115°45.947′	182
MS344	Huichang County, Jiangxi Province	25°14.616′	115°44.613′	228
MS345	Meixian County, Guangdong Province	24°15.738′	115°57.890′	165
MS382	Wuzhishan City, Hainan Province	18°54.335′	109°30.827′	309
MS383	Wuzhishan City, Hainan Province	18°50.076′	109°30.565′	781
MS384	Wuzhishan City, Hainan Province	18°39.872′	109°34.973′	441
MS393	Nanning City, Guangxi Autonomous Region	22°29.801′	108°24.013′	128
MS400	Guilin City, Guangxi Autonomous Region	24°25.343′	110°12.797′	255
MS401	Guilin City, Guangxi Autonomous Region	24°32.181′	110°23.740′	155
MS403	Guilin City, Guangxi Autonomous Region	24°38.967′	110°25.799′	167
MS416	Fenghuang City, Hunan Province	28°03.960′	109°35.328′	365
MS422	Luxi County, Hunan Province	28°14.846′	109°56.328′	151
MS434	Wuhan City, Hubei Province	30°13.844′	114°19.745′	67
MS470	Zhongjiang County, Sichuan Province	30°37.746′	105°03.713′	370
MS481	Nanchang City, Jiangxi Province	28°41′	115°54′	56
MS500	Qingyuan County, Liaoning Province	41°51.147′	124°56.308′	556
MS503	Qingyuan County, Liaoning Province	41°59.768′	124°29.859′	157
MS505	Benxi County, Liaoning Province	41°14.344′	123°41.169′	219
MS510	Benxi County, Liaoning Province	41°11.163′	123°50.752′	246
MS511	Benxi County, Liaoning Province	41°13.918′	123°45.412′	282
MS616	/	/	/	/

MS616 is *M.* × *giganteus*

Materials and methods

Plant materials and phenotypic evaluation

A total of 33 accessions of *Miscanthus sinensis* Andersson, as well as one *Miscanthus* × *giganteus*, were used in the present study (Table 1). These accessions were collected individually, covered the major distribution areas (Fig. 1), and were expected to represent the wide genetic diversity of *M. sinensis* in China. Over 10 *Miscanthus* rhizomes from an individual clone were sampled. For each *Miscanthus* accession, the rhizome was split evenly and planted in a row with four clonal replicates with a planting density of 1 m × 1 m for a plant in the field on January 18, 2009.

The tiller angle was measured between the main tiller and the vertical in November (Fig. 2). The tiller angle was designated as '0' for the compact plant architecture with an erect stem. The assessments of tiller angle were performed across the replicates. Tiller number was counted in December when growth had stopped. The early harvest in the following year ensured natural senescence, and the biomass dried over winter in the field for a majority of the *Miscanthus* accessions. The genotypes from Hainan were exceptions and stayed green. After drying to constant weight, the weight of leaf and stem was recorded separately and summed to get the total biomass yield for each plant. The averaged biomass yield was used to calculate biomass yield per hectare based on a planting density of 1 m × 1 m

Fig. 1 Geographical distribution of the 33 *M. sinensis* accessions on a map of China. Three *color stars* indicate original sites for *M. sinensis* accessions for the three groups, divided based on the protein sequence

Fig. 2 Phenotype of plant architecture showing the tiller angle variation, relative spread-out (*left*) and compact (*right*) plant architecture at senescence stage

for each accession. The ratio of leaf to stem weight was the average of the duplications for each accession. The phenotypes were measured in 2009 and 2010, the first 2 years after establishment (Table 2).

Analysis of variance (ANOVA) was performed for statistical evaluations of the effects of year (crop age), genotype and their interactions using SAS 9.1 (SAS Institute Inc., Cary, NC, USA) (Table 3).

Table 2 Descriptive statistics of the six phenotypic traits grouped by spread and compact architectures in the 34 *Miscanthus* accessions in 2009 and 2010

	Tiller angle (°)		Tiller number		Leaf weight (Mg ha^{-1})		Stem weight (Mg ha^{-1})		Ratio of leaf to stem		Total biomass yield (Mg ha^{-1})	
	2009	2010	2009	2010	2009	2010	2009	2010	2009	2010	2009	2010
Spread												
Min	50	45	5	11	0.55	1.19	0.25	0.88	0.84	0.78	0.80	2.07
Max	72	65	20	39	8.74	12.54	4.28	9.20	2.21	1.48	13.02	21.74
Mean ± SD	62 ± 7.44	56.57 ± 8.38	13.57 ± 6.50	24.43 ± 8.28	2.49 ± 2.94	4.80 ± 3.77	1.41 ± 1.42	4.02 ± 3.08	1.7 ± 0.48	1.24 ± 0.25	3.90 ± 4.36	8.82 ± 6.72
CV (%)	12.0	14.8	47.9	33.9	118.3	78.7	101.2	76.7	28.5	20.6	111.8	76.2
Compact												
Min	0	0	4	5	0.62	0.22	0.31	0.11	0.93	0.74	1.00	0.34
Max	0	0	25	45	8.27	29.24	3.68	15.13	6.64	5.77	11.72	44.37
Mean ± SD	0	0	12.10 ± 4.93	17.04 ± 9.95	3.31 ± 2.37	6.87 ± 7.04	1.33 ± 0.95	3.44 ± 3.46	2.78 ± 1.39	2.10 ± 1.00	4.65 ± 3.19	10.30 ± 10.28
CV (%)	–	–	40.7	58.4	71.55	102.6	71.5	100.7	50.0	47.5	68.7	99.8

CV, coefficient of variation; –, indicates missing data

DNA sequencing for *MsTAC1*

Total DNA was extracted from young leaves using a CTAB method (Doyle and Doyle, 1990). The published *OsTAC1* sequence (LOC_Os09g0529300) was used to BLAST against the *Sorghum bicolor* genome database (http://www.plantgdb.org/SbGDB/cgi-bin/blastGDB.pl) to obtain the *SbTAC* sequence. The *MsTAC* gene was amplified as two overlapping segments based on the regions conserved between *SbTAC* and *OsTAC*. Two pairs of primers used here were as follows: MsTAC-F, TCATTGGCTGAATTG GAGGA and MsTAC-R, AAATGTTGTCGCATAGGGC to amplify the 3′ end of the gene and MsTAC-up-F, AAGC CAGTGCAACCAAA and MsTAC-up-R, CATCACG GAGCAGAAGG, to amplify the 5′ end. The lengths of fragments amplified with the two primer pairs were 1,455 and 562 bp, with 100 bp overlapping according to the TAC homolog of *Sorghum bicolor*.

Purified PCR products were cloned into the plasmid vector pMD18-T (TaKaRa) and sequenced by the Tsingke BioTech Co., Ltd. To eliminate the *Taq* errors caused by PCR amplification and sequencing and to further confirm the heterozygous loci, at least five independent clones with the target fragment were selected randomly and sequenced individually for each accession. By means of multi-clone sequencing, inter-allelic loci representing true sequence variation were verified, and the loci representing *Taq* polymerase artifacts were removed to confirm the singletons.

Sequence analysis

The obtained sequences were aligned by BioEdit program (Hall 1999) with further manual refinements and special attention to sequence variation and the potential heterozygous loci. Natural variations, including single nucleotide polymorphisms (SNPs) and insertions/deletions (InDels), were included in the analysis. The *MsTAC* CDS corresponded to the conserved coding region of *OsTAC*. The published ortholog protein sequences of *TAC* in rice (Os09g0529300), sorghum (Sb02g030610) and maize (Ku et al. 2011) were obtained from public databases and used as outgroups for the phylogenetic analyses because these species are closely related to *Miscanthus* (Wang et al. 2005). The cluster relationship among the 34 *Miscanthus* accessions was inferred using the neighbor-joining method, and a phylogenetic tree was constructed with MEGA version 5.0 (http://www.megasoftware.net) based on the amino acid sequences (Tamura et al. 2004, 2011).

Genetic variation parameters implemented using DnaSP program version 5.0 (http://www.ub.es/dnasp) (Librado and Rozas 2009) included Singleton sites number (S), indels number (I), the proportion of segregating sites (θ), nucleotide diversity (π), haplotype diversity (hd) and average

Table 3 Analysis of variance (ANOVA) on the examined traits in a set of 34 *Miscanthus* accessions

Source of variation	Tiller angle					Tiller number					Leaf weight				
	df	SS	MS	*F* value	*R* (%)	*df*	SS	MS	*F* value	*R* (%)	*df*	SS	MS	*F* value	*R* (%)
Crop age	1	0.052	0.052	158.45**	12.68	1	1,710.88	1,710.88	133.51**	80.86	1	518.81	518.81	473.92**	77.75
Genotype	33	11.69	0.354	1,083.32**	86.79	33	10,075.77	305.33	23.83**	13.93	33	3,372.29	102.19	93.35**	15.18
Crop age × Genotype	28	0.033	1.19×10^{-3}	3.63**	0.21	27	1,914.58	70.91	5.53**	2.77	26	1,138.15	43.78	39.99**	6.41
Error	126	0.041	3.27×10^{-4}			122	1,563.33	12.81			121	132.46	1.09		
Source of variation	Stem weight					Ratio of leaf to stem weigh					Total biomass yield				
	df	SS	MS	*F* value	*R* (%)	*df*	SS	MS	*F* value	*R* (%)	*df*	SS	MS	*F* value	*R* (%)
Crop age	1	227.60	227.60	748.68**	85.64	1	4.62	4.62	4.10*	32.40	1	1,433.68	1,433.68	831.44**	81.94
Genotype	33	844.61	25.59	84.19**	9.53	33	96.59	2.93	2.60**	16.71	33	7,181.22	217.61	126.20**	12.35
Crop age × Genotype	26	309.85	11.92	39.20**	4.38	30	62.77	2.09	1.86*	8.91	26	2,458.54	94.56	54.84**	5.31
Error	121	36.79	0.30			121	136.44	1.13			121	208.64	1.72		

SS, the sum of squares for the effect of the factors; MS, mean of squares; df, degrees of freedom; R, relative importance (%) = $(MS_{component} - MS_{error}) \times 100/MS_{sum}$

*, ** Significant at $P < 0.05$ and < 0.0001, respectively

number of nucleotide differences (*K*) (Watterson 1975; Nei 1987). Selection in *MsTAC1* and departure from neutrality were also tested at variation loci using Tajima's *D*. The analyzed regions were the full genomic sequences and the CDS region of *MsTAC1*, respectively (Table 4).

Population structure and association analysis

Two hundred and forty-six molecular markers, including 131 polymorphisms from *MsTAC1* and 115 SSRs from the genome (Zhao et al. 2013b), were used to calculate the kinship matrix (*K*) using the SPAGeDi software package (Hardy and Vekemans 2002). STRUCTURE version 2.3 (Pritchard et al. 2009) was used to infer population structure using a run length of burn in period of 10,000, followed by 100,000 MCMC iterations. The number of subpopulations (*K*) was evaluated from one to ten with five replicated runs. The most probable structured population number (*K*) was determined by the method of an ad hoc statistic ΔK based on the rate of change of Ln*P*(*D*) between successive *K* values (Evanno et al. 2005). A good statistical model developed by Yu et al. (2006), the mixed linear model (MLM) approach, which accounts for both population structure (*Q*) and relative kinship (*K*), was adopted to identify marker-trait associations with the TASSEL V2.1 software package (Bradbury et al. 2007).

Results

Nucleotide variations at the *MsTAC1* locus

MsTAC1 was sequenced in a total of 34 *Miscanthus* genotypes. The length of the aligned sequence for each genotype varied between 1,845 and 1,874 bp, with a range from 792 to 813 bp for the CDS. The *MsTAC1* genomic sequence comprises 147 nt in the 5′-flanking region (5′UTR), four exons, three introns, and 186 nt in the 3′-flanking region (3′UTR). SNPs were substantially more common than InDels, and 129 SNP and 7 InDel sites were present across all regions of the gene (Suppl. Table S1).

Of the 129 SNPs, 67 and 62 were located in the coding and non-coding regions, respectively. An average of one SNP per 14 nucleotides was observed in *MsTAC1* gene among the 34 *Miscanthus* accessions. Among the 129 SNP loci, a total of 23 SNP diversity patterns spanning the examined *MsTAC1* sequences were detected. Out of the 23 diversity patterns, 18 (78.3 %) were heterozygotes (Suppl. Table 1). The 67 loci were not evenly distributed across the entire coding region, as lower nucleotide diversity occurred in exon 1 and exon 2, and higher nucleotide diversity was observed in exon 3 and exon 4 (Fig. 3).

Table 4 Summary of DNA sequence variation of the *MsTAC1* CDS and full-length region in *M. sinensis* and rice

Gene region	Groups	Length	*S*	θ	π	hd	*K*	Tajima's *D*	Fu and Li's *D** test	Fu and Li's *F** test
Full length	Group 1	1,866	35	0.00520	0.00478	0.990	8.767	-0.30459^{NS}	-0.18597^{NS}	-0.26184^{NS}
	Group 2	1,845	10	0.00200	0.00156	0.857	2.857	-1.18108^{NS}	-1.32889^{NS}	-1.41888^{NS}
	Group 3	1,856	19	0.00313	0.00377	0.883	6.900	0.82127^{NS}	0.02739^{NS}	0.28739^{NS}
	O. sativa				0.00198	0.791	6.202	1.8358^{NS}	Jiang et al. (2012)	
	indica				0.00086	0.77	2.689	0.1891^{NS}	Jiang et al. (2012)	
	japonica				0.00032	0.529	0.99	−1.805*	Jiang et al. (2012)	
	Wild rice				0.00178	0.927	5.569	-0.6805^{NS}	Jiang et al. (2012)	
CDS	Group 1	813	14	0.00475	0.00432	0.897	3.367	-0.31922^{NS}	-0.05395^{NS}	-0.15700^{NS}
	Group 2	792	10	0.00158	0.00111	0.714	0.857	-1.35841^{NS}	-1.42725^{NS}	-1.52246^{NS}
	Group 3	792	4	0.00155	0.00107	0.533	0.833	-0.96578^{NS}	-1.52158^{NS}	-1.57185^{NS}
	O. sativa				0.00116	0.528	1.478	1.8619^{NS}	Jiang et al. (2012)	
	indica				0.00004	0.045	0.045	-1.1153^{NS}	Jiang et al. (2012)	
	japonica				0.00009	0.113	0.115	-1.5719^{NS}	Jiang et al. (2012)	
	Wild rice				0.00112	0.719	1.427	0.143^{NS}	Jiang et al. (2012)	

L, length of the alignments in which all sequences contain bases, excluding gaps; S, singleton sites number; I, indels number; θ, the proportion of segregating sites; π, nucleotide diversity; hd, haplotype diversity; K, average number of nucleotide difference; Tajima's *D*, Fu and Li's *D**, Fu and Li's *F** value were conducted to investigate the departure from neutrality; NS, not significant

* $P < 0.05$

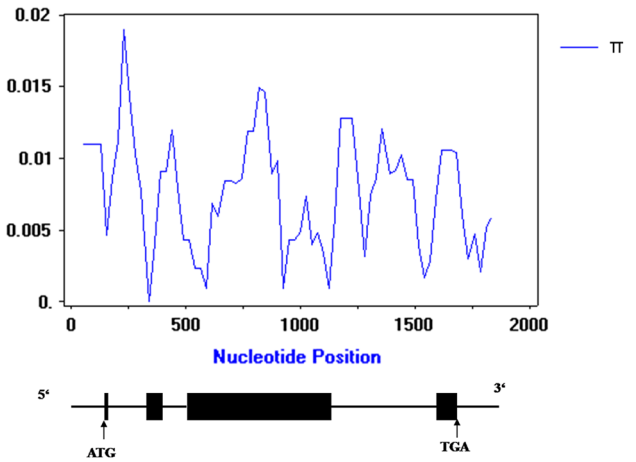

Fig. 3 Sliding-window plot of nucleotide diversity for the 1,874 bp *MsTAC1* region (π value). The window size is 100 bp, step size is 25 bp, and the unit is silent site. The gene structure is shown at the *bottom* of the figure. The *black boxes* and the *thin box* indicate exons and introns, respectively

Functional changes in genes often arise from a variable site in the CDS. Among the 34 *Miscanthus* accessions analyzed, the *MsTAC1* CDS ranged from 792 to 813 bp and encoded proteins between 263 and 270 amino acids. A total of 60 amino acids were involved within the 67 nucleotides in the coding regions. With the *OsTAC1* CDS as a reference, forty non-synonymous substitutions, eleven synonymous substitutions, two non-sense mutations and seven InDels were observed in the 34 *Miscanthus* accessions (Suppl. Table S3). The two InDels in the CDS region, in the 3rd (3 bp) and 4th exon (18 bp), did not cause a frameshift mutation. The deletion at position 82 (L) was detected in the accessions of MS9, MS13, MS44, MS54, MS403 and MS434. The insertions from 247 to 252 positions were only identified in the two accessions MS240 and MS265, which were collected from the YunGui Plateau. Interestingly, heterozygous loci at position 27 (A and T) were found in the accessions MS121, MS503, MS505, MS511, MS382, MS383 and MS384, which were collected from North China and Hainan Island. In addition, the homozygous at position 27 of the amino acid 'T' was only detected in the accessions MS500 and MS510, which were collected from Liaoning province. These highly variable amino acids indicated that the *MsTAC1* might express different phenotypes in diverse genotypes.

Sliding-window plot of nucleotide diversity for the entire length of *MsTAC1*

A sliding-window analysis was conducted to better examine the distribution of variations along *MsTAC1* gene (Fig. 3). Diversity peaks were present in the regions of intron 1, intron 2, intron 3, exon 3 and exon 4 within *MsTAC1* gene. Troughs were observed in exon 1 and 2 within a narrow region, indicating fewer polymorphisms in these regions. Two peaks were observed in the 3rd exon and 3rd intron, respectively. Interestingly, a trough was observed in each neighboring locus between the exons and introns, suggesting that the local connections were conservative.

Genealogical relationships among the *Miscanthus* accessions

The CDSs of *MsTAC1* were used to estimate the phylogenetic relationship of the 34 *Miscanthus* accessions, employing the CDSs of *ZmTAC1*, *OsTAC1* and *SbTAC1* as outgroups. The 34 *Miscanthus* accessions were clearly divided into three groups. Group 1 consisted of 13 accessions, MS47, MS470, MS383, MS344, MS345, MS401, MS393, MS265, MS384, MS281, MS382, MS400 and MS416, which spanned the south and southwest China with latitudes from 18°39.872 to 30°37.746 N and longitude from 102°22.938 to 117°06.379 E. Group 2 contained all of the accessions from Liaoning province, PM500, PM503, PM505, PM510 and PM511; two accessions from the northwestern China, MS121 and MS138; two accessions from Guizhou province, MS222 and MS240; one from Jiangxi province, MS341; and one from Fujian province, MS316. *M.* × *giganteus* was clustered in group 2, and it is presumed to have originated from the north with high latitudes. Accessions from central China were clustered into group 3, which included MS403, MS434, MS13, MS9, MS54, MS44, MS481, MS275 and MS422. The clustering result indicated that phylogenetic tree differentiation of the 33 *M. sinensis* accessions was correlated with their geographical origins. Based on the *TAC1* CDS of *Miscanthus*, sorghum, rice and maize, the phylogenetic analysis revealed that *Miscanthus* has a very close genetic relationship with sorghum and some correlation with maize and rice (Fig. 4), though not as close as with sorghum.

DNA variation patterns in the *MsTAC1* gene of *Miscanthus*

The nucleotide diversity (π) in the *MsTAC1* gene is shown in Table 4, with the entire regions and CDS, respectively (Table 4). Considering the entire length of the *MsTAC1*, the nucleotide diversity (π) was 0.00478 for group 1, and it was much higher than that of group 2 and group 3. The lowest π value was detected in group 2 of 0.00156. Consistent with the full length of *MsTAC1*, the nucleotide diversity of CDS was higher in group 1 (0.00432) than in groups 2 and 3 (0.00111 and 0.00107, respectively). The nucleotide diversity in the entire genomic site was relatively higher than that of CDS in the corresponding groups. Tajima's *D* statistic was calculated to determine whether the gene was subjected to selective constraints. As shown in Table 4, Tajima's *D* statistics were negative in the three groups, either for entire gene or CDS region, except for the full-length sequences from group 3. No significant departure from the neutral expectation was observed in the three groups, indicating that *MsTAC1* was not subjected to natural selection either in the genomic sequences or CDS. This result might indicate no strong selection pressure on the coding and non-coding regions of *MsTAC1* in the *Miscanthus*.

Phenotypic variations

Different climates have already led to altered distributions of species, phenotypic variation, and allele frequencies, and the impact of climate change is expected to intensify. Plant architecture and growth parameters, such as tiller number, tiller angle and biomass yield, are considered important agronomic traits for Miscanthus breeding programs. Therefore, tiller angle and tiller number, leaf weight (including leaf sheath), stem weight, ratio of leaf to stem weight and total biomass yield were scored in 2 years of 2009 and 2010 (Table 2). As shown in Table 2, a wide range of variation was observed in the *Miscanthus* panel for all traits determined.

Among the *Miscanthus* accessions with spread architecture, significant variation was observed for tiller angle ranging from 0° to 72° in 2009 and from 0° to 65° in 2010 (Table 2), exhibiting a more compact and smaller tiller angle in 2010. As indicated by CVs (coefficient of variation), the tiller number displayed relative smaller variation and ranged from 5 to 20 in 2009 and from 11 to 39 in 2010 (Table 2). In 2010, the tiller numbers were generally larger than those in 2009 and the increment varied. The phenotype values of leaf weight, stem weight, ratio of leaf to stem weight and total biomass yield ranged from 0.55 to 8.74 Mg ha^{-1}, 0.25–4.28, 0.84–2.21 and 0.80–13.02 Mg ha^{-1} in 2009, varied from 1.19 to 12.54 Mg ha^{-1}, from 0.88 to 9.20 Mg ha^{-1}, from 0.78 to 1.48, and 2.07 to 21.74 Mg ha^{-1}, respectively. The phenotypic values in 2010 were generally higher than those in 2009.

Among the compact *Miscanthus* accessions, the plants were erect in 2009 and 2010. Compared with the spread architecture plants, the CVs of the examined traits were much higher in 2010 than in 2009 except for the tiller angle. The biomass in 2010 exhibited striking phenotypic variation with more than 100-fold change and variations from 0.34 to 44.37, 0.22 to 29.24, 0.11 to 15.13 Mg ha^{-1} for total biomass, leaf and stem biomass, respectively. The compact *Miscanthus* accessions presented a much higher total biomass with the average of 4.65 ± 3.19 and 10.30 ± 10.28 Mg ha^{-1}, whereas the average of spread accessions was obviously lower, 3.90 ± 4.36 and 8.82 ± 6.72 Mg ha^{-1} in 2009 and 2010, respectively.

Analysis of variance (ANOVA) indicated significant or highly significant effects of year (crop age), genotype and interaction of these two factors on phenotypic variations of the examined traits (Table 3). For tiller angle, we observed that the significant variations were mainly explained by genotype (86.79 %), followed by crop age (12.68 %). The

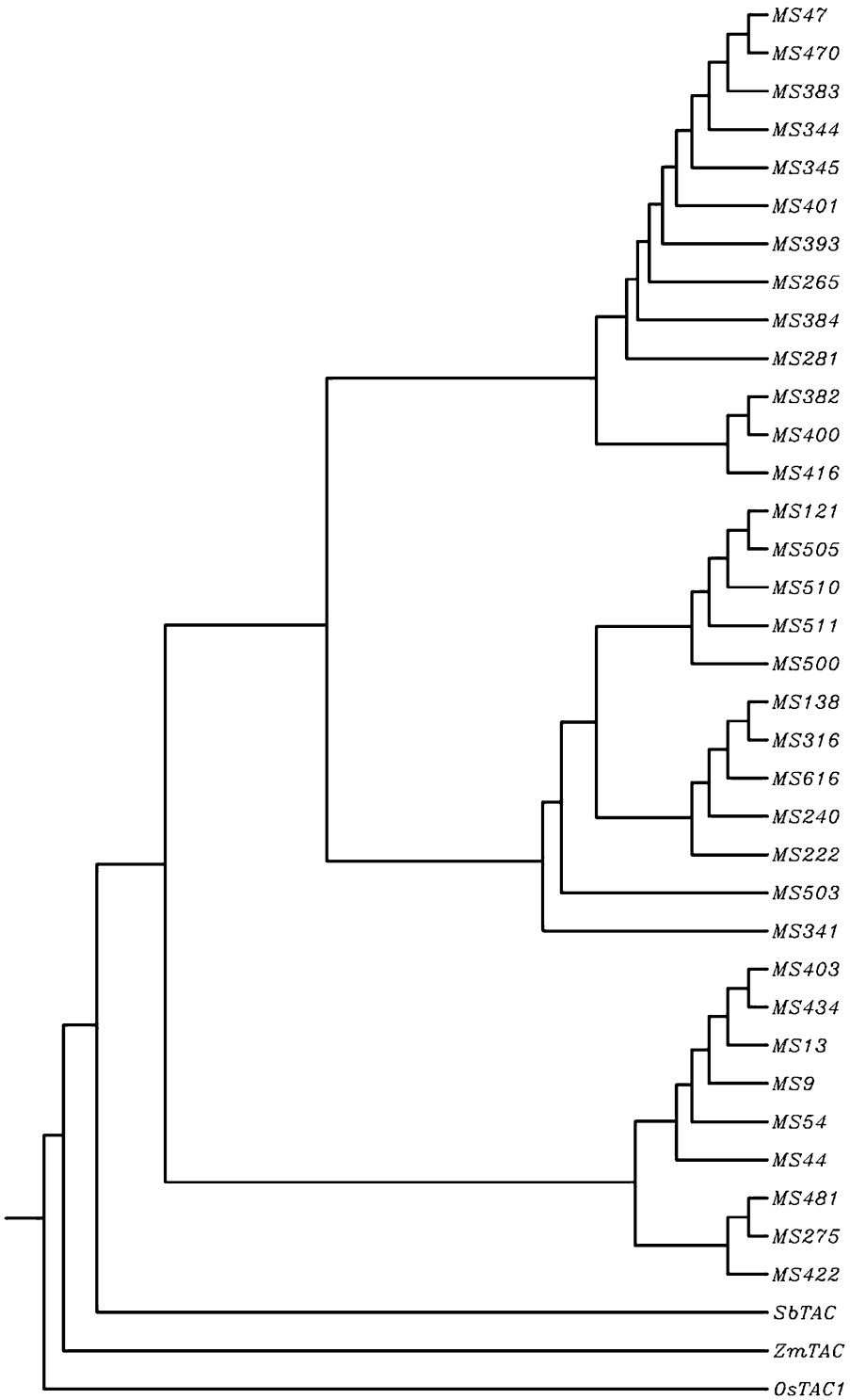

Fig. 4 Phylogenetic tree of *Miscanthus* accessions based on the amino acid sequence of *MsTAC1* with the amino acid sequence of sorghum, maize and rice as outgroups

interaction between crop age and genotype was relatively small (0.21 %). Being in contrast to tiller angle, the four traits, including tiller number, leaf and stem weight and total biomass, the variation explained by crop age, were all significantly higher than by genotype and their interactions (Table 3). The trait, the ratio of leaf to stem weight, presented an entirely distinct pattern and the significant variations from crop age, genotype and their interaction were 32.40, 16.71 and 8.91 %, respectively.

Population structure and relative kinship

The population structure of the 34 *Miscanthus* accessions was ascertained using 131 polymorphisms at *MsTAC1*

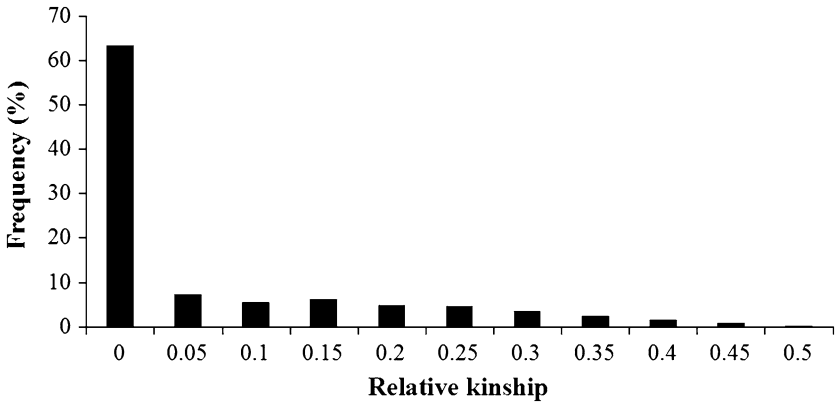

Fig. 5 Distribution of pairwise relative kinship estimates between the *Miscanthus* accessions. Only kinship values in the range from 0 to 0.5 are shown

and 115 SSRs scored as biallelic markers. The structure results of $K = 4$ were the best possible partition as the *Miscanthus* accessions displayed a high consistency with the geographic origin and significant delta K value (Suppl. Fig. S1). The accessions from the south China were grouped into a subgroup, including two from Sichuan province, MS47 and MS470, and three from Hainan province, MS382, MS383 and MS384, in addition to MS400 and MS416 from Guangxi autonomous region and Hunan province. The accessions from the north China and the YunGui Plateau were all assigned to the subgroup. MS393 and MS401, from Guangxi autonomous region, were grouped as a separate subgroup. The fourth group included 12 accessions, most of which were collected from the Wuling Mountain area and the south China.

The relative kinship reflects the approximate genetic identity between pairs of individuals randomly selected over the average probability. A total of 246 markers, 131 polymorphisms in *MsTAC1* and 115 SSRs, were used to estimate the relative kinship in the set of 34 *Miscanthus* accessions. As shown in Fig. 5, 63.2 % of the pairwise kinship estimates were equal to zero. The remaining estimates ranged from 0.05 to 0.5, with a continuously decreasing number of pairs falling in higher estimate categories.

Significant marker-trait association in *Miscanthus*

To identify the polymorphic loci at *MsTAC1* region that affect the traits listed in Suppl. Table S1, association analysis was performed using 246 molecular markers, including 131 SNP or InDel and 115 SSR markers. Taking the population structure data as covariates (Suppl table S2), MLM was used to identify marker-trait associations separately in 2009 and 2010. Markers with high significance of $P < 0.05$ were regarded as associated markers, and the contribution of associated markers to the phenotypic variation (R^2) was presented for all the traits of 2009 and 2010 in Table 5. A total of 88 marker-trait associations were detected in either one or 2 years (Table 5). Among these 88 associations, two, five, three and one were detected in the consecutive 2 years, 2009 and 2010, for tiller angle, tiller number, stem weight and total biomass yield, respectively. In comparison with the 1st year 2009, more associations were observed in the 2nd year 2010 for all the traits except for tiller angle.

The total biomass yield was the sum of leaf weight and stem weight. Forty-four significant associations were identified for these three traits either in one or 2 years, mostly detected in 2010. Forty were the DNA variants located in the non-coding region in *MsTAC1*. The polymorphic sites associated with biomass, leaf weight and stem weight explained 5.2–15.28, 1.37–16.21 and 5.2-14.61 % of the phenotypic variation (Table 5).

Two marker loci, S05 and S08 in the 1st intron, were detected to be associated with tiller angle in the consecutive 2 years, 2009 and 2010. For tiller number, nine and 14 associated loci were observed in 2009 and 2010, respectively. Five of these loci were detected across the 2 years. Each polymorphic locus explained the phenotypic variation from 4.36 to 12.15 % in 2010, whereas the explained phenotypic variations were much higher in 2009 (Table 5).

For leaf weight, only one associated locus ($R^2 = 15.29$ %) was found in 2009, and 11 loci ($R^2 = 1.31$–12.58 %) were detected in 2010. None of these associations were repeatedly detected in the 2 years (Table 5).

Seventeen significant associations were detected for the ratio of leaf to stem weight. Among which, three and 14 were observed in 2009 and 2010, respectively. Again, none of these associations were repeatedly detected in the consecutive 2 years. The phenotypic variation of this trait explained by a single DNA variant varied between 3.22 and 25.93 % (Table 5).

Discussion

Tiller angle (including leaf angle) is one of the most important morphological characters that has a significant effect on the formation of grain yield. In conventional breeding

Table 5 Associations between the polymorphic sites in *MsTAC1* gene and all the examined traits, including tiller angle, tiller number, leaf weight, stem weight, ratio of leaf to stem weight and biomass yield ($P < 0.05$)

Location in MsTAC1 gene	Code	Tiller angle				Tiller number				Leaf weight			
		2009		2010		2009		2010		2009		2010	
		P	Var (%)	*P*	Var (%)	*P*	Var (%)	*P*	Var (%)	*P*	Var (%)	*P*	Var (%)
5′UTR	S01											0.016	12.58
5′UTR	S02							0.018	9.61			0.026	7.7
1st intron	S04									0.038	15.29		
1st intron	S05	0.039	5.60	0.040	5.31								
1st intron	S06												
1st intron	S08	0.05	5.43	0.049	5.16								
3rd exon	S30					0.016	14.8					$1.94E^{-04}$	10.43
3rd exon	S36					0.026	13.15	$7.52e^{-4}$	12.15				
3rd exon	S43							0.002	9.6				
3rd exon	S44							0.003	11.64				
3rd exon	S45												
3rd intron	S48											$7.71E^{-05}$	8.48
3rd intron	S49							0.003	11.0				
3rd intron	S50					0.003	28.61	0.040	6.58			0.001	6.2
3rd intron	S52												
3rd intron	S56												
3rd intron	S58												
3rd intron	S59					0.045	17.06					0.001	4.98
3rd intron	S68							0.003	9.66				
3rd intron	S70					0.045	17.41					0.001	3.99
3rd intron	S71							0.003	9.43				
3rd intron	S74												
3′ UTR	S94					0.003	30.5	0.042	4.90			0.002	2.40
3′ UTR	S95							0.003	8.35				
3′ UTR	S96												
3′ UTR	S100											0.008	1.31
3′ UTR	S101					0.03	14.23	$5.80E^{-04}$	8.12				
3′ UTR	S102					0.044	18.11					0.002	2.15
3′ UTR	S103							0.003	8.10				
3′ UTR	S106												
3′ UTR	S128					0.003	31.41	0.043	4.36			0.002	1.48
3′ UTR	S129							0.003	7.53				
3′ UTR	S131												

Table 5 continued

Location in MsTAC1 gene	Code	Stem weight				Ratio of leaf to stem				Total biomass yield			
		2009		2010		2009		2010		2009		2010	
		P	Var (%)	*P*	Var (%)	*P*	Var (%)	*P*	Var (%)	*P*	Var (%)	*P*	Var (%)
5′UTR	S01	0.050	13.63	0.011	14.61							0.010	14.02
5′UTR	S02	0.024	11.64	0.043	7.01	0.040	10.55			0.047	9.39	0.025	7.88
1st intron	S04	0.033	15.42							0.029	16.21		
1st intron	S05												
1st intron	S06	0.018	17.87							0.033	15.81		
1st intron	S08												
3rd exon	S30	0.039	10.94	$3.53E^{-04}$	14.37							$1.21E^{-04}$	11.48
3rd exon	S36							0.012	9.92				
3rd exon	S43							$1.21E^{-06}$	25.93				
3rd exon	S44							0.042	9.4				
3rd exon	S45					0.001	20.79						
3rd intron	S48			$3.33E^{04}$	15.28							$5.13E^{-05}$	9.8
3rd intron	S49							0.041	8.96				
3rd intron	S50			0.002	12.73							$7.78E^{-05}$	7.43
3rd intron	S52					0.014	13.05						
3rd intron	S56							$5.73E^{-04}$	16.71				
3rd intron	S58							$5.59E^{-04}$	16.23				
3rd intron	S59			0.002	11.92							$7.99E^{-04}$	6.14
3rd intron	S68							0.036	7.03				
3rd intron	S70			0.002	10.92							$8.30E^{-04}$	5.01
3rd intron	S71							3.48	6.71				
3rd intron	S74							$4.68E^{-04}$	12.66				
3′ UTR	S94			0.002	7.67							$9.34E^{-04}$	2.99
3′ UTR	S95							0.031	4.97				
3′ UTR	S96	0.045	11.57										
3′ UTR	S100			0.0247	13.13							0.014	1.37
3′ UTR	S101							0.008	4.54				
3′ UTR	S102			0.002	7.46							$8.98E^{-44}$	2.72
3′ UTR	S103							0.030	4.41				
3′ UTR	S106							$3.77E^{-04}$	8.4				
3′ UTR	S128			0.003	5.2							$9.80E^{-04}$	1.85
3′ UTR	S129							0.028	3.22				
3′ UTR	S131	0.045	11.82										

programs, a plant with smaller tiller angles or erect growth habits is considered to be a compact plant architecture, which may allow planting at a high density, enhance photosynthesis efficiency and improve biomass yield. Os*TAC1* is the main gene controlling tiller angle in cultivated rice and is thus the most important determinant of whether rice plants adopt a spreading or a compact growth habit (Yu et al. 2007). Natural phenotypic variation is the result of genetic variation during evolution in response to environmental selections (Corre et al. 2002). Significant natural phenotypic variation for tiller angle was observed in our *Miscanthus* germplasm. However, the genetic variation of *MsTAC1* was still unknown in Miscanthus. Compared with the ortholog genes previously studied in rice (Jiang et al. 2012), the level of nucleotide diversity determined in the present study for the *MsTAC1* gene was high, which may be caused by the wide geographical range from which the *Miscanthus* accessions were collected. The original habitat presented diverse climatic and environmental factors influencing plant growth and development. Previous studies on the genetic analysis (Zhao et al. 2013b), nutrient dynamics (Yu et al. 2013), cell wall composition and their degradation efficiency (Zhao et al. 2013a) have demonstrated that the *Miscanthus* possesses abundant genetic diversity. Significant correlations were observed between geographical parameters and genetic similarity, indicating that the genetic variations were shaped by geographical habitats. Pairwise kinship estimates were close to zero, indicating that most accessions had no or a weak relationship with other accessions in the *Miscanthus* panel collected from diverse geographic regions.

As for *OsTAC1*, neutrality tests indicated that the Tajima's *D* value of the japonica group for the 3′-flanking region was significantly different from those of the indica and wild rice groups, which were not significantly different from each other. No nucleotide diversity in the *OsTAC1* CDSs of the japonica varieties suggested strong selection of the functional site during domestication of japonica varieties of rice (Jiang et al. 2012). Compared with that of rice, the nucleotide diversity revealed a much higher π value in either the genomic sequence or CDS in the *Miscanthus* accessions based on DNA polymorphism analysis. Neutrality tests indicated that the Tajima's *D* values in all three *Miscanthus* groups were not significant, and the accessions had undergone strong natural selection during the formation and evolution of the *Miscanthus* species.

To clarify the relationship of the *MsTAC1* gene in *Miscanthus*, rice, maize and sorghum, we performed phylogenetic analysis with the amino acid sequence for the homologs of *TAC1*, which revealed that *MsTAC1* is more closely related to its ortholog in sorghum than to maize and rice (Fig. 4). *Miscanthus* has very close genetic relationship with sorghum, and these results demonstrate the feasibility of sorghum as a reference genome sequence for *Miscanthus* (Swaminathan et al. 2010; Dai et al. 2013). The protein sequence presented great variation among the species of rice, maize, sorghum and *Miscanthus*, and also in the genotypes of *Miscanthus*.

Previous studies of *OsTAC1* and *ZmTAC1* demonstrated that different polymorphisms underlie the phenotype diversity in rice and maize (Yu et al. 2007; Ku et al. 2011). In rice, a mutation in the 3′-splicing site of a 1.5-kb intron from 'AGGA' to 'GGGA' decreases the level of tac1 and resulted in compact plant architecture with a tiller angle close to zero. Further sequence verification in the 3′-splicing site of the 1.5-kb intron revealed that the tac1 mutation 'GGGA' was present in 88 compact japonica rice accessions, and *TAC1* with 'AGGA' was present in 21 wild rice accessions and 43 indica rice accessions, all with the spread-out form, indicating that tac1 had been extensively utilized in densely planted rice (Yu et al. 2007). In maize, a nucleotide difference in the 5′UTR between the compact inbred line Yu82 'CTCC' and the expanded inbred line Shen137 'CCCC' influences the expression level of *ZmTAC1*, further controlling the size of the leaf angle. Sequence verification of the change in the 5′UTR revealed *ZmTAC1* with 'CTCC' was present in 13 compact inbred lines and *ZmTAC1* with 'CCCC' was present in 18 expanded inbred lines, indicating that *ZmTAC1* has been extensively utilized in breeding with regard to the improvement of the maize plant architecture (Ku et al. 2011). We did not find the same variation pattern as rice or maize in contrasting phenotypes. In addition, more sequence variation was detected in the *Miscanthus* than in rice and maize. Although the full genomic regions of *MsTAC1* were highly divergent, and no locus was detected that correlated completely for the tiller angle. Most likely, m uch greater variation would have been found if more *Miscanthus* accessions were sequenced, or more than the *MsTAC1* gene is involved in the expression for the tiller angle. Therefore, association analysis was used to identify the *MsTAC1* sequence variation for phenotypic variation of tiller angle. It has been reported that a larger tiller angle is related with tiller number (Xu et al. 2005; Yu et al. 2007; Chen et al. 2012). Significant variations for all the observed traits indicated that the assembled panels are suitable for association analysis. Thus, association analysis was employed for tiller angle, tiller number, leaf weight, stem weight, ratio of leaf to stem weight and total biomass yield. The relative importance of the ANOVA components (Table 3) indicated that the genotype is the key factor determining the phenotypic variation of tiller angle. Two marker-trait associations were detected for tiller angle across the two consecutive years, 2009 and 2010 (Table 5), indicating that the associations are quite reliable. More experiments are still underway in our lab to further confirm the marker-trait associations for tiller angle and other traits.

Because of the perennial nature of the plant, the performances of the 2nd growth year were expected to increase substantially relative to those of the 1st growth year in *Miscanthus*. A discrepancy of the associated polymorphisms between the 2 years was observed in *Miscanthus*. As for the tiller number and the ratio of leaf to stem weight, many more associations were detected in 2010 than in 2009. This is not unexpected. Biomass yield is one of the most important traits and has been intensively studied in *Miscanthus* (Jezowski 2008; Yan et al. 2012; Robson et al. 2013). It is a complex trait associated with many simple traits (Yan et al. 2012). For the three related traits of leaf weight, stem weight and total biomass yield, consistent associations were observed in the three traits with relatively high phenotypic variation explained in 2010. For polymorphism-trait association analysis, the consistent detection of a significant association across various environments using a well-performed statistical model implies that the associations are real positives (Li et al. 2010). Although the small size of the association mapping panel did not guarantee the optimal power of association tests (Yang et al. 2010), *MsTAC1* was found to contain polymorphisms within 34 accessions associated with at least six related traits with a relatively high phenotypic variation. The marker-trait association implied that *MsTAC1* influences the tiller angle and might further be involved in the tiller formation and production of biomass yield. Thereafter, *Miscanthus*, especially the *M. sinensis* germplasm, is an excellent model for association analyses because of the extensive climatic variation across its native range and its nature as a wild species without any artificial selection the geographically diverse germplasm collections.

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