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RESEARCH PAPER

Genome-wide association mapping identifies an *SNF4* ortholog that impacts biomass and sugar yield in sorghum and sugarcane

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Abstract

Sorghum is a feed/industrial crop in developed countries and a staple food elsewhere in the world. This study evaluated the sorghum mini core collection for days to 50% flowering (DF), biomass, plant height (PH), soluble solid content (SSC), and juice weight (JW), and the sorghum reference set for DF and PH, in 7–12 testing environments. We also performed genome-wide association mapping with 6 094 317 and 265 500 single nucleotide polymorphism markers in the mini core collection and the reference set, respectively. In the mini core panel we identified three quantitative trait loci for DF, two for JW, one for PH, and one for biomass. In the reference set panel we identified another quantitative trait locus for PH on chromosome 6 that was also associated with biomass, DF, JW, and SSC in the mini core panel. Transgenic studies of three genes selected from the locus revealed that Sobic.006G061100 (*SbSNF4-2*) increased biomass, SSC, JW, and PH when overexpressed in both sorghum and sugarcane, and delayed flowering in transgenic sorghum. *SbSNF4-2* encodes a γ subunit of the evolutionarily conserved AMPK/SNF1/SnRK1 heterotrimeric complexes. *SbSNF4-2* and its orthologs will be valuable in genetic enhancement of biomass and sugar yield in plants.

Keywords: Association mapping, juice yield, resequencing, sorghum, sugar yield, sugarcane, sugar content, *SNF4*.

Introduction

Sorghum [*Sorghum bicolor* (L.) Moench] is a unique crop in that it is primarily used as feed/industrial crop in developed countries and as an important staple food across much of Africa, Asia, and Latin America (Williams and Capps, 2020). For example, in the USA, sorghum is equally split between industrial and feed uses (Williams and Capps, 2020). Sorghum is also a

Abbreviations: AMPK, AMP-activated protein kinase;DF, days to 50% flowering;GWAS, genome-wide association study;JV, juice volume;JW, juice weight;LD, linkage disequilibrium;MC, mini core;PH, plant height;QTL, quantitative trait locus;RS, reference set;SNF, sucrose non-fermenting;SNP, single nucleotide polymorphism;SnRK1, SNF1-related protein kinase 1,SSC, soluble solid content;SY, sugar yield.

genetic model and genome reference species for C_4 grasses of the Panicoideae PACCAD clade (Mullet *et al.*, 2014) because of its diverse germplasm, genetics and genomics platform, broad adaptation, being a diploid self-pollinator that enables inbred/hybrid breeding, and worldwide utilization as a grain, forage, sugar, and bioenergy crop (Paterson *et al.*, 2009; Mullet *et al.*, 2014; McCormick *et al.*, 2018). Both sugarcane (*Saccharum officinarum*) and sorghum can accumulate large amounts of sugar in the mature internodal stems (Welbaum and Meinzer, 1990; Hoffmann-Thoma *et al.*, 1996). Total sugar content (or total soluble solid content; SSC) in sorghum and sugarcane is most commonly measured using °Brix, which is proportionally correlated with the concentrations of sucrose and total sugar (Calviño *et al.*, 2008; Lingle *et al.*, 2012; Kawahigashi *et al.*, 2013; McKinley *et al.*, 2016).

Quantitative trait loci (QTLs) related to sugar yield have been extensively mapped in sorghum (Murray *et al.*, 2008a, 2008b, 2009; Ritter *et al.*, 2008; Shiringani *et al.*, 2010; Guan *et al.*, 2011; Felderhoff *et al.*, 2012; Mace *et al.*, 2019) and sugarcane (Ming et al., 2001, 2002; Aitken *et al.*, 2006). One such locus is the *Dry Stalk* (*D*) locus on sorghum chromosome 6, controlling the pithy/juicy stem trait (Burks *et al.*, 2015; Casto *et al.*, 2018; Fujimoto *et al.*, 2018; Xia *et al.*, 2018; Zhang *et al.*, 2018). The locus contains a NAC transcription factor (Sobic.006G147400) located within 50896168–50898604 bp; the functional *D* allele has been associated with dry stems and the mutant allele with juicy stems (Casto *et al.*, 2018; Fujimoto *et al.*, 2018; Xia *et al.*, 2018; Zhang *et al.*, 2018). However, genes regulating sucrose accumulation and sugar and biomass yield have not been identified in either sorghum or sugarcane (Brenton *et al.*, 2020).

The main objective of this study was to identify and genetically confirm the gene responsible for increased sugar and biomass yield as measured by SSC, juice yield, and biomass in both sorghum and sugarcane. Using the sorghum mini core collection (MC) (Upadhyaya *et al.*, 2009) as the mapping panel with 6 094 317 single nucleotide polymorphisms (SNPs) (Wang *et al.*, 2021), we have mapped a pleiotropic locus responsible for biomass, plant height, SSC, juice weight, and sugar yield, and genetically confirmed that the underlying gene is *SbSNF4-2*, encoding the γ subunit of the AMP-activated protein kinase (AMPK)/sucrose non-fermenting 1 (SNF1)/SNF1-related protein kinase 1 (SnRK1) heterotrimeric complexes, which typically consist of a catalytic α, a scaffolding β, and an activating γ subunit and play central regulatory functions in metabolism, stress signaling, and development (Broeckx *et al.*, 2016; Li and Shee, 2016).

Materials and methods

Plant materials and phenotyping

The 242 accessions of the sorghum MC (Upadhyaya *et al.*, 2009) and 304 accessions of the sorghum reference set (RS) (Ramu *et al.*, 2013) were phenotyped in rainy and post-rainy seasons; in post-rainy seasons, the plants were either irrigated or not irrigated (Table 1) at ICRISAT in Hyderabad, India. The plants were grown in an alpha design with three replications. Each single-row plot was 4 m long, with a row spacing of 75 cm and plant spacing within rows of 10 cm. Ammonium phosphate was applied at 150 kg ha^{-1} before planting, and 100 kg ha^{-1} of urea was applied as a top dressing 3 weeks after planting. For the post-rainy season with irrigation, field plots were irrigated five times at equal intervals, each with 7 cm of water. For MC, two weeks after anthesis, five representative plants were weighed to measure fresh biomass. After weighing, juice was extracted from each batch of five plants and the SSC, juice volume (JV), and juice weight (JW) were measured; SSC was measured with a handheld refractometer (Ritter *et al.*, 2008). Sugar yield (SY) was calculated by multiplying JW by SSC (Felderhoff *et al.*, 2012). For both MC and RS, plant height (PH) and flowering time (days to 50% flowering; DF) were also recorded.

Initial genotyping and association mapping

The 265 500 SNP markers developed by Morris *et al.* (2013) were used in an association analysis with MC and RS using the mixed linear model (J.Yu *et al.*, 2006) as implemented in TASSEL (Bradbury *et al.*, 2007) v5.0. We used a mixed linear model with kinship index, which was generated with SNP markers developed in a previous study (Wang *et al.*, 2013). An association between a marker and a trait was declared if multiple SNPs were linked with the trait in the same locus in more than one environment. Candidate genes containing linked SNP markers were identified based on information in the *S. bicolor* genome Sbi1.4 (http://www.plantgdb.org/SbGDB/.

Genotyping by resequencing and association mapping

Genome resequencing of 244 MC accessions (mini core plus controls) and genome-wide association study (GWAS) were performed as described

Table 1. Testing environments for days to 50% flowering (DF), plant height (PH), biomass, soluble solid content (SSC), juice volume (JV), and juice weight (JW) used in this study

1	2	3	4	5	6	7	8	9	10	11	12
2006 PRi	2009 PRi	2010 PRi	2011 PRi	2010 PR	2011 PR	2007 R	2008 R	2009 R	2010 R	2011 R	2012 R
DF	DF	DF	DF	DF	DF	DF	DF	DF	DF	DF	DF
PH	PH	PH	PH	PH	PH	PH	PH	PH	PH	PH	
		BM	BM	BM	BM				BM	BM	BM
		SSC	SSC	SSC	SSC				SSC	SSC	SSC
		JV	JV	JV	JV				JV	JV	JV
		JW	JW	JW	JW				JW	JW	JW

PRi, post-rainy season with irrigation; PR, post-rainy season without irrigation; R, rainy season.

in Wang *et al.* (2021). A total of 6 094 317 SNPs on chromosome 1–chromosome 10 were identified after filtering based on the criteria of minor allele frequency of 0.05 or greater and missing data rate of 10% or less in the population. GWAS was performed using 6 094 317 SNPs and 957 449 indels. Kinship matrix (*K*) was generated using EMMAX (Kang *et al.*, 2010) and was used to perform GWAS analyses with a Q matrix calculated with STRUCTURE 2.3.4 (Pritchard *et al.*, 2000) as the covariate variable. The modified Bonferroni correction was used to determine the genome-wide significance thresholds of the GWAS, based on a nominal level of α=0.05, which corresponds to a *P*-value of 8.2E-09, or $-\log_{10}(P)$ values of 8.08. Candidate genes were identified using the reference genome *Sorghum bicolor* v3.1.1 (Paterson *et al.*, 2009; McCormick *et al.*, 2018) curated at Phytozome (Goodstein *et al.*, 2012) v13 (https://phytozome-next.jgi.doe.gov/).

Sorghum and sugarcane genetic transformation

Vector and construct preparation

To make the pUBI1301 vector, pCAMBIA1301 was digested with *Pst*I/*Eco*RI to remove the *Pst*I/*Eco*RI fragment. This left the *Hin*dIII site intact. The linearized pCAMBIA1301 without the *Pst*I/*Eco*RI fragment was blunt-ended and self-ligated. The new vector was digested with *Hin*dIII and ligated with the *Hin*dIII fragment from pAHC25 (Christensen and Quail, 1996) containing the maize ubiquitin 1 (UBI) promoter and the *GUS* gene, which can be replaced by a transgene through *Xma*I/*Sac*I digestion. The coding sequences of candidate genes were synthesized by Bio Basic Inc. (Amherst, NY, USA) or Synbio Technologies (Monmouth Junction, NJ, USA) and were delivered in pUC57 flanked by *Xma*I and *Sac*I. Both synthesized gene and pUBI1301 were digested with *Xma*I and *Sac*I to produce the transgene constructs used in sorghum and sugarcane transformation.

Agrobacterium preparation

Competent *Agrobacterium tumefaciens* strain LBA4404 cells were transformed with the above transgene constructs using electroporation. A single colony from transformed *Agrobacterium* cells was inoculated into 10 ml Luria broth with 50 mg l^{-1} kanamycin and grown for 48 h at room temperature. An aliquot of the cultured cells was subsequently inoculated into 200 ml Luria broth with 50 mg l^{-1} kanamycin and grown for another 48 h. This culture was harvested for sugarcane transformation, described below.

Sorghum genetic transformation

The procedure described by Liu and Godwin (2012) was followed for genetic transformation of sorghum. Immature panicles from Tx430 were collected 12–15 d after pollination. Seeds were removed from the panicles and soaked in 70% ethanol (v/v) for 5 min while shaking at 200 rpm. The soaked seeds were then drained, transferred to 50% commercial bleach, and shaken for 10 min before being washed five times with sterile water. Immature embryos 1.0–2.0 mm in length were isolated on to petri dishes containing callus induction medium [CIM: Murashige and Skoog (MS) medium supplemented with 1 g l^{-1} L-proline, 1 g l^{-1} L-asparagine, 1 g l^{-1} KH_2PO_4, 0.16 mg l^{-1} $CuSO_4$, and 1 mg l^{-1} 2,4-dichlorophenoxyacetic acid] with scutella facing upward. The embryos were incubated in the dark in a tissue culture room before microprojectile transformation. Six embryos were placed at the center of a shallow petri dish containing osmotic medium (MS medium supplemented with 0.2 M D-sorbitol and 0.2 M D-mannitol) and stored for 2–3 h in the dark prior to bombardment, which was performed using a biolistic system (PDS 1000/He, Bio-Rad, Hercules, CA, USA). Plasmid DNA delivery was performed using 0.6 μm gold particles (0.42 mg per shot). The distance from the filter holder to the target cells was adjusted to 12 cm and 1100 psi rupture disks were used. Up to 5 μg of each pNPTII (UBI::NPTII) plasmid and each of the above transgene construct plasmids were mixed and then equally loaded into the receptacle for six shots. After bombardment, immature embryos were kept on osmotic medium for 3–4 h before being transferred on to CIM. After immature embryos had recovered on CIM for 3–4 d, they were transferred to selective regeneration medium (MS medium supplemented with 1 mg l^{-1} 6-benzylaminopurine, 1 mg l^{-1} indole-3-acetic acid, 0.16 mg l^{-1} $CuSO_4$, and 30 mg l^{-1} geneticin G418) and placed under lights in a tissue culture room. Immature embryos were subcultured every 2 weeks until putative transgenic shoots grew to 4–6 cm. These shoots were then moved to selective rooting medium (MS medium supplemented with 1 mg l^{-1} 1-naphthaleneacetic acid, 1 mg l^{-1} indole-3-acetic acid, 1 mg l^{-1} indole-3-butyric acid, 0.16 mg l^{-1} $CuSO_4$, and 30 mg l^{-1} geneticin G418) for 4 weeks without subculture. Rooted plantlets were transferred into plastic pots in the greenhouse and were transferred into the soil in the glasshouse after 7 d in plastic pots.

Sugarcane genetic transformation

The procedure described by Mayavan *et al.* (2015) was used for genetic transformation of sugarcane. Sugarcane cultivars Ho 02-113 and Co. 290 were provided by Jeffrey W. Hoy and Kenneth Gravois of Louisiana State University, and L 01-299 was provided by Garrie Landry. Sugarcane setts approximately 7 cm long were incubated in 1% carbendazim solution for 1 h and then rinsed several times with sterile water. *Agrobacterium* cultures as described above were pelleted and resuspended in infiltration medium (MS with 5% sucrose, 0.1% Silwet L-77, and 100 μM acetosyringone) with an OD_{600} of 0.6. The axillary bud was gently pricked five times to a depth of 1 mm using a sterile 22 gauge hypodermic needle. The pricked setts were vacuum-infiltrated for 5 min at 500 mm Hg in the suspension and then incubated in the suspension for 5 h at room temperature. The setts were then removed, air-dried briefly on sterile paper towels, and incubated (co-cultivated) at room temperature for 18 h in a desiccator under complete darkness. After co-cultivation, the setts were washed with sterile double-distilled water containing 500 mg l^{-1} cefotaxime to kill residual *A. tumefaciens* before being transferred to tissue culture boxes and partially immersed in 100 ml of sterile distilled water with 20 mg l^{-1} hygromycin and 500 mg l^{-1} cefotaxime. The antibiotic-laced water was replaced weekly to avoid bacterial growth. After ~30 d, putative transgenic shoots that had grown were planted in pots in the greenhouse.

Phenotypic evaluation of transgenic plants

Transgenic sorghum plants were grown in Sanya, Hainan and Fengyang, Anhui, China. Transgenic sugarcane and control setts were grown in Lafayette, Louisiana, USA. For the transgenic sorghum, SSC was measured with a handheld refractometer either 6 weeks after anthesis or at harvest. To measure SSC, plants were harvested and their fresh biomass was weighed before stripping off the leaves and pressing for juice. JW and JV were recorded and SSC was then measured. In addition, PH, DF, and the number of basal tillers were recorded. For the transgenic sugarcane, all tillers from each sett were tested by PCR for the presence of the hygromycin resistance gene using the primers 5′-GATGTTGGCGACCTCGTATT-3′ and 5′-GATGTAGGAGGGCGTGGATA-3′. Canes were harvested after growing for 10 months (the first 6 months in the greenhouse and the last 4 months outside) for the first season and 14 months for the second season. PH and fresh weight were recorded. To measure JW, an internode of ~5 cm in length was weighed and pressed with a handheld cane juice presser. The resultant juice was weighed and SSC was measured with again a handheld refractometer. The number of tillers was also recorded. For statistical analysis, a *t*-test between transgenic and control plants was performed with Microsoft® Excel 365 using 'Two Sample Assuming Unequal Variances' from its Analysis ToolPak. Two-tailed *P*-values were used in comparisons.

For reverse transcription–PCR analysis of the transgenic sorghum, RNA Prep Pure Plant kit (Tiangen Co, Beijing, China) was used for RNA extraction, according to the manufacturer's instructions. The analyses were conducted as described previously (Li *et al.*, 2016). Transgene transcript levels were quantified by normalization with both *ACTIN* and *eIF4a1* transcript levels according to the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). Forward and reverse primers for *ACTIN* were 5′-ACGGCCTGGATGGCGACGTACATG-3′ and 5′-GCAGAAGGACGCCTACGTTGGTGAC-3′ and for *eIF4a1* they were 5′-TGGCGAAGGATGGTTTCTAAGT-3′ and 5′-GGAGCGGCACATAGCCTACTC-3′, respectively. Each biological replicate was run in three technical replicates and used to calculate the standard error.

Results

Phenotypic analyses

Environments 3 and 5 were applied in the same year (2010), with or without irrigation, respectively, in the same location; environments 4 and 6 were applied similarly in 2011 (Table 1). We found that irrigation did not affect DF (P=0.533 and 0.436, respectively) and increased PH by 17% (P=8.4808E-14) between environments 4 and 6, but did not affect PH between environments 3 and 5 (P=0.208). Irrigation increased biomass by 31% between environments 3 and 5 (P=4.081E-11) and by 16% between environments 4 and 6 (P=0.000992), and increased JW by 51% between environments 3 and 5 (P=1.177E-09) and by 27% between environments 4 and 6 (P=0.00104), but decreased SSC by 6.5% between environments 3 and 5 (P=0.000121) and by 27% between environments 4 and 6 (P=2.801E-47).

Pearson's correlation coefficients (R) among SY, SSC, JV, JW, PH, DF, and biomass were calculated with phenotypic data averaged across the replicates in each environment. Correlation coefficients were calculated for each environment and were then averaged across the testing environments shown in Table 1 to generate the final numbers in Table 2. All traits were positively and significantly (P=0.001) correlated (Table 2). Although there were slight variations among the environments, the overall trend was similar in all. For example, SSC was less strongly correlated with the other traits, although it was more strongly correlated with SY and biomass than with JV or JW, and SY was highly correlated with JV/JW and biomass, with correlation coefficients of 0.97 and 0.90, respectively (Table 2).

Table 2. Pearson's correlation among days to 50% flowering (DF), plant height (PH), biomass, soluble solid content (SSC), juice volume (JV), juice weight (JW), and sugar yield (SY)

	DF	BM	SSC	JV	JW	SY
PH	0.65	0.58	0.58	0.44	0.37	0.42
DF		0.73	0.71	0.58	0.49	0.54
BM			0.73	0.92	0.89	0.90
SSC				0.60	0.57	0.68
JV					0.98	0.97
JW						0.97

All numbers are significant at P=0.001.

GWAS

A frequency distribution of all data used in association mapping is presented in Supplementary Fig. S1. As the data were not normally distributed, we used a procedure described by Templeton (2011) to transform all the phenotypic data. Although normality was improved in the transformed data (Supplementary Fig. S2), the *Dw3* (Multani *et al.*, 2003) gene used as the association mapping control (Morris *et al.* (2013) was not detected in the transformed data (Supplementary Fig. S2). Consequently, only untransformed phenotypic data were used in this study.

Two association mapping panels were used, namely MC and RS, although only MC accessions were resequenced. PH and DF were phenotyped in both panels. Our main interest was to identify QTLs that were stable across environments. Therefore, only association peaks with multiple markers detected in two or more environments were analyzed. As in Morris *et al.* (2013), we used *Dw3*/*SbPGP1*/Sobic.007G163800 (Multani *et al.*, 2003) on sorghum chromosome 7 as a positive control for GWAS using MC with SNPs from the resequencing. *Dw3* encodes an auxin transporter that modulates polar auxin transport in the stem (Multani *et al.* , 2003). PH in MC was measured in 11 environments (six post-rainy and five rainy; Table 1) and the *Dw3* peak was detected in four of the six post-rainy environments but none of the five rainy environments (Supplementary Fig. S3). Association analysis with height in two of the post-rainy environments, PH2 and PH6, showed that there were three genes covered by the peak, Sobic.007G163800 (*Dw3*/*SbPGP1*/ABC transporter B family member 1), Sobic.007G163901 (hypothetical protein), and Sobic.007G164000 (ABC transporter F family member 1) (Supplementary Fig. S4). Based on RNA-seq results (McCormick *et al.*, 2018), only Sobic.007G163800 is highly expressed in the internodes (Supplementary Table S1), whose growth is impacted by *Dw3*, which consequently determines PH (Multani *et al.*, 2003). Therefore, Sobic.007G163800 is the *Dw3* gene, which is also supported by the fact that Sobic.007G163800 sequence is identical to *Dw3* (AY372819) (Multani *et al.*, 2003).

Using the criteria of association with multiple SNP markers in two or more environments with P-values below the Bonferroni P-values of 8.2E-09 at α=0.05, or $-\log_{10}(P)$ values greater than 8.08, we identified three QTLs for DF (one was detected in two environments with below threshold P-values and two with above threshold P-values), one for PH detected in two environments, one for biomass, and two for JW (Table 3). QTLs detected in more than two environments with slightly higher P-values are also presented in Table 3. For all traits except SSC, marker association was specific to either rainy or post-rainy environments (Table 3). The PH marker 10:9572427

Table 3. Top SNP markers in each locus associated with days to 50% flowering (DF), plant height (PH), biomass, soluble solid content (SSC), and juice weight (JW)in more than two environments

	1	2	3	4	5	6	7	8	9	10	11	12
	2006 PRi	2009 PRi	2010 PRi	2011 PRi	2010 PR	2011 PR	2007 R	2008 R	2009 R	2010 R	2011 R	2012 R
DF	**1:15456196**[a]		1:15456196	**10:9017856**	1:15456196	10:9017856		7:60157503	7:60157503		7:60157503	**7:60157503**
			10:		**10:**							
			9017856		**9017856**							
PH		**10:9572427**	3:72315482	3:72315482	10:9572427	3:72315482						
			10:9572427	10:9572427								
BM			2:77584294	7:3640254	**2:77584294**	2:77584294					6:55619784	6:55619784
			7:3640254		7:3640254							
SSC						2:58272758					2:58272758	
JW				10:3206383	10:3206383						**2:2697058**	**2:2697058**
											6:55626409	6:55626409

[a] 1 (before the colon) is the chromosome number and 15456196 is the SNP marker position in bp; all other data in the table are presented in the same fashion. Bold text indicates that the *P*-value is below the threshold.

falls in a seedling height QTL (10:9381364–9806030) mapped by Maulana *et al.* (2017) curated in the Sorghum QTL Atlas (Mace *et al.*, 2019), but no other loci in Table 3 colocalized with previously mapped QTLs curated in the Sorghum QTL Atlas.

GWAS with the RS panel identified another height QTL on chromosome 6 (Fig. 1A). Although not linked to height in the MC panel, this QTL was subsequently found to be associated with DF (Fig. 1B), biomass (Fig. 1C), JW (Fig. 1D), and SSC (Fig. 1E) in the MC panel. Examination of the genomic region covered by this pleiotropic QTL in all the five traits (Fig. 1F–J) revealed seven genes in both the v1.4 (Fig. 1F) and v3.1.1 (Fig. 1J) genomes. Among the five traits in Fig. 1F–J, SNPs in the coding region of one of the two genes in the middle, Sb06g014920 or Sobic.006G061100, consistently showed lower association *P*-values, although for JW and SSC, SNPs from the proximal promoter region of the other gene (i.e. Sobic.006G061000 or Sb06g014910) also displayed tight association. From Fig. 1G, it was also observed that SNPs in the promoter region of Sobic.006G060800 exhibited tight association with DF. Therefore, Sobic.006G060800, Sobic.006G061000, and Sobic.006G061100 were chosen for transgenic analysis.

Transgenic analysis

To perform genetic transformation in sorghum, the binary vector pCAMBIA1301 (Hajdukiewicz *et al.*, 1994) was re-engineered to contain the maize ubiquitin promoter from pAHC25 (Christensen and Quail, 1996). The cDNAs of the three genes were synthesized and inserted after the introduced promoter to drive the expression of the transgene. Four transgenic sorghum plants overexpressing each of Sobic.006G060800 or Sobic.006G061000 were generated, and one Sobic.006G061100-overexpressing sorghum plant (named 42-1) was produced. Evaluation of the T_1 plants demonstrated that no plants from among the Sobic.006G060800 or Sobic.006G061000 overexpressers displayed any significant phenotypic difference from control plants, whereas progeny plants from the lone Sobic.006G061100 overexpresser were taller and produced more biomass and more sugar in their stems (Fig. 2). Sobic.006G061100 was thus determined to be the underlying gene responsible for PH, biomass, JW, and SSC in this pleiotropic locus. Based on sequence homology, Sobic.006G061100 is most similar to *AtSNF4* (AT1G09020, *AtSnRK1βγ*, *AKINβγ*, *KINβγ*) (Broeckx *et al.*, 2016) in Arabidopsis. Since *AtSNF4* is most similar to Sobic.001G005600 in sorghum and second most similar to Sobic.006G061100, hereafter Sobic.001G005600 will be named *SbSNF4-1* and Sobic.006G061100 will be named *SbSNF4-2*.

The *SbSNF4-2* overexpression construct was used to transform sorghum again and also to transform sugarcane. We were able to produce one other transgenic *SbSNF4-2* overexpressing sorghum plant (named 42-2). *SbSNF4-2*

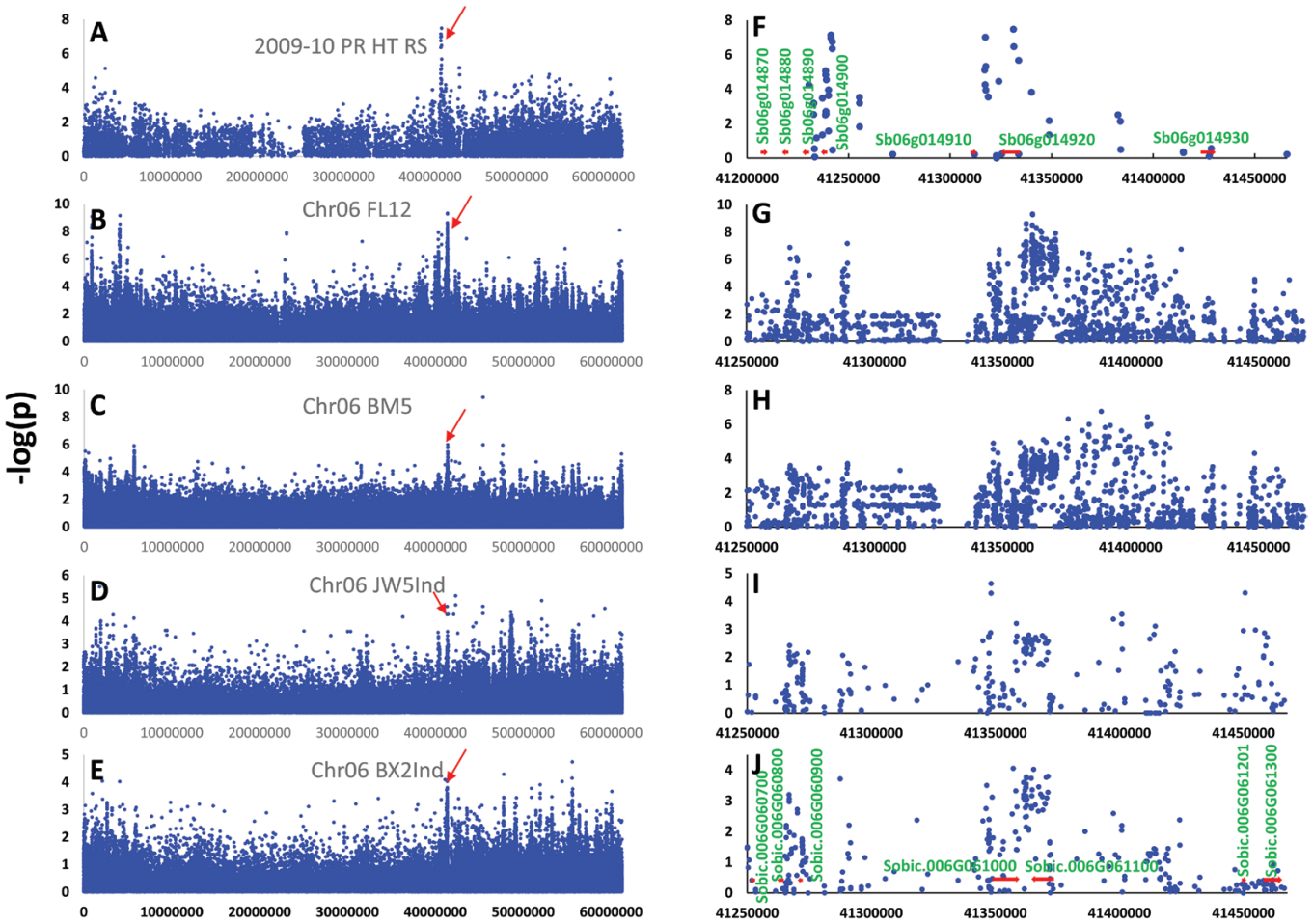


Fig. 1. A quantitative trait locus on chromosome 6 associated with plant height, flowering time, fresh biomass, juice yield, and SSC in sorghum. (A–E) Manhattan plots for plant height in the reference set panel (A), and for days to 50% flowering (B), fresh biomass (C), juice weight (D), and SSC (E) in the mini core panel. (F–J) Genomic region associated with plant height (F), flowering time (G), fresh biomass (H), juice weight (I), and SSC (J). The location of the genes was based on Sbi1.4 (http://www.plantgdb.org/SbGDB/) (F) and *Sorghum bicolor* v3.1.1 (https://phytozome-next.jgi.doe.gov/info/Sbicolor_v3_1_1) (J).

expression was confirmed in the two transgenic sorghum lines (42-1 and 42-2) by quantitative PCR (Supplementary Fig. S5). Inoculation of 300 setts each from Ho 02-113, Co. 290, and L 01-299 sugarcane clones produced one putative transgenic sett (Ts-1) from Ho 02-113. The two transgenic sorghum lines (42-1 T_2–T_3 and 42-2 T_1–T_2) and sugarcane (Ts-1) were evaluated for two growing seasons. The overall trends were similar: the transgenic plants outperformed controls, but only results from the most recent seasons (T_2 for 42-2 and T_3 for 42-1) are presented here. Flowering of the two transgenic sorghum lines was delayed by 1 week compared with the controls (20 July versus 13 July). PH, biomass, JW, SSC, and calculated SY based on SSC (Felderhoff *et al.*, 2012) were all higher in the two transgenic plants compared with the controls (Fig. 3A), similar to the transgenic sugarcane line Ts-1 (Fig. 3B), which showed increases of 36%, 46%, 47%, 17%, and 75% in PH, cane weight, JW, SSC, and SY, respectively (Fig. 3C). In transgenic sorghum, SY increased by 77% in 42-1 and 85% in 42-2 plants on average, while there were average increases of 80% and 88% in PH, 265% and 306% in biomass, 29% and 46% in JW, and 36% and 25% in SSC in 42-1 and 42-2, respectively (Fig. 3C). The increase in PH in both 42-1 and 42-2 was brachytic, as the number of internodes was not affected (Supplementary Fig. S6). We measured juice sugar composition in 42-1 and found that although the proportion of sucrose was 85% in 42-1 compared with 94% in the control, stem juice from 42-1 plants contained 72% more sucrose per unit of juice on average relative to the control (Supplementary Table S2) due to the increased SSC/sugar content. Two factors contributing to the increased biomass were increased height and tiller number (Fig. 3D) in transgenic sorghum, although tiller number was not significant in transgenic sugarcane, in which the tiller numbers were 25 for Ts-1 and 22 for the control. Overexpression of *SbSNF4-2* did not adversely affect grain yield per plant in either 42-1 or 42-2 (Fig. 3E), although 1000-seed weight in 42-1 plants was significantly higher (Fig. 3E). These results demonstrated

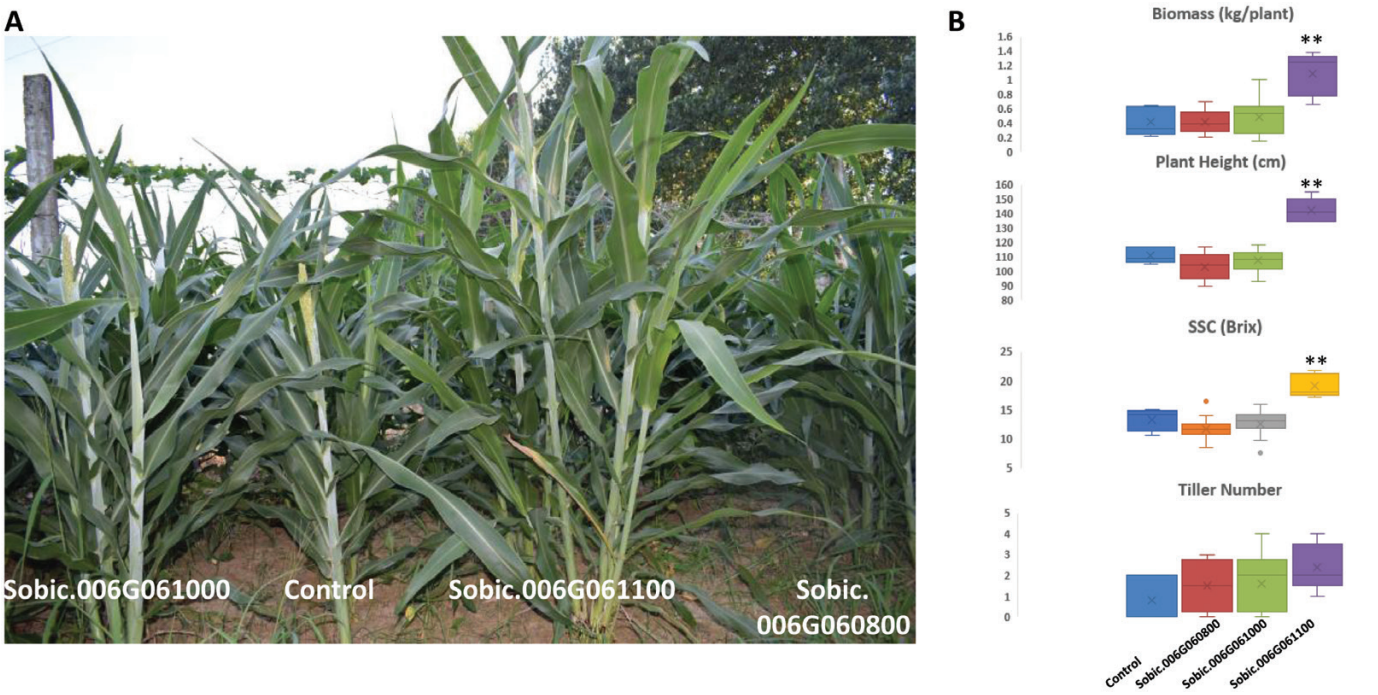


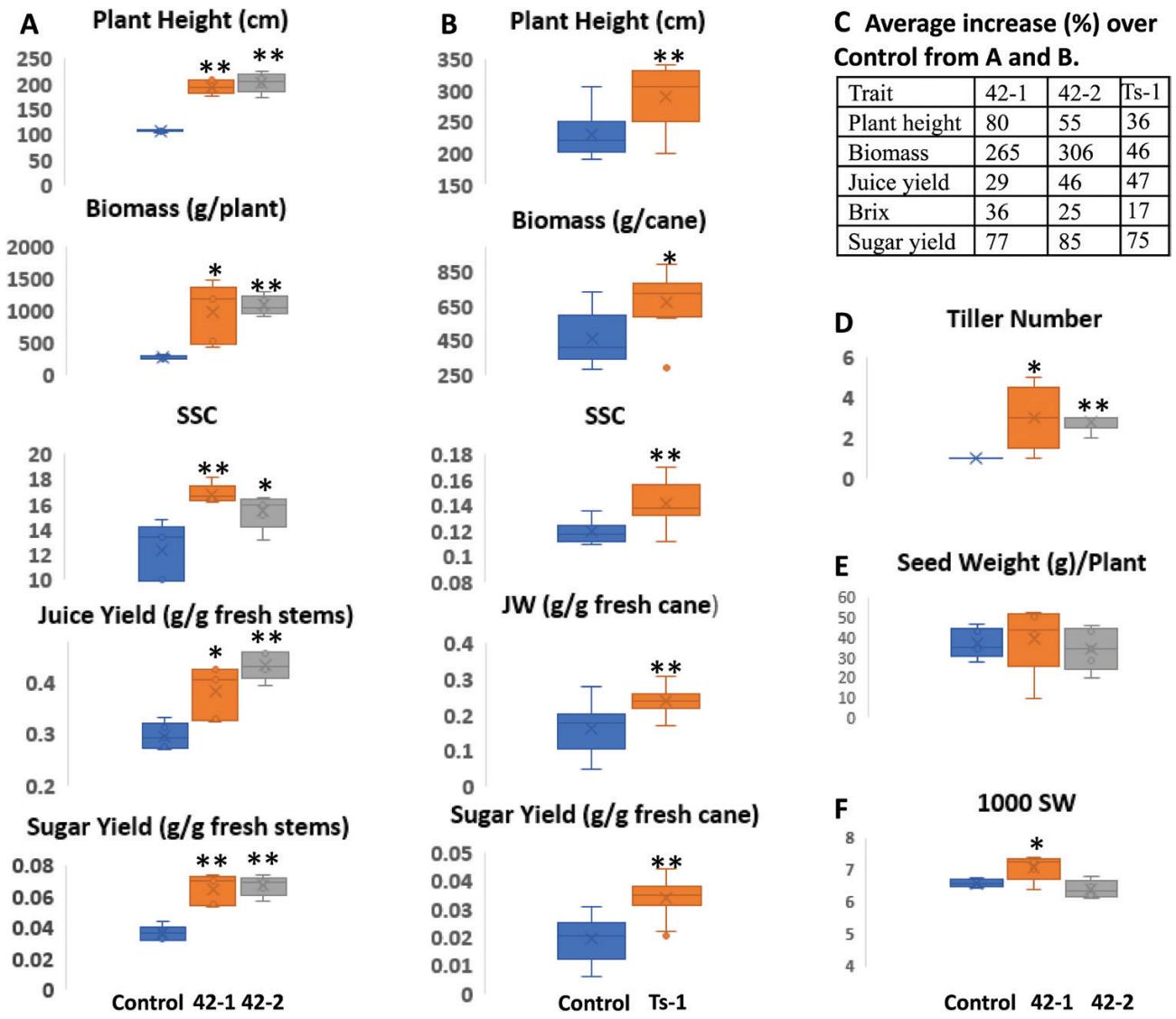
Fig. 2. Phenotypic differences between control and transgenic sorghum plants overexpressing Sobic.006G060800, Sobic.006G061000, and Sobic.006G061100. (A) Sobic.006G061100 plants showed greater height and later flowering. (B) Boxplots displaying phenotypic difference in fresh biomass, plant height, and SSC (Brix). The increased tiller number was not statistically significant. The × inside each box in the boxplot represents the mean and the horizontal line represents the median value of each data group. Significant differences from the control are indicated with asterisks: $^{**}P<0.01$.

that overexpression of *SbSNF4-2* can increase SY by 75–85% in both sorghum and sugarcane due to consistent increases in JW and SSC in both species (Fig. 3C). This is significant because juice yield is a primary limiting factor for higher SY and ethanol yield in bioenergy production (Lueschen *et al.*, 1991; Carvalho and Rooney, 2017). The increased biomass, JW, and consequently SY were achieved without negatively affecting reproductive traits such as number of seeds per plant (Fig. 3E, F; Supplementary Fig. S7) and the increased height was not associated with decreased stem thickness in transgenic plants (Supplementary Fig. S7).

Genetic analysis of the SbSNF4-2 *locus*

To understand the evolutionary history of the region, we analyzed nucleotide diversity (π) with a window size of 50 kb. We found that the mean π for chromosome 6 was 0.003095, but 0.002272 for the arbitrarily chosen region of 217 kb of 41 250 032–41 467 613 bp shown in Fig. 1G–J, and 0.005363 in the *SbSNF4-2* coding region of 41 363 743–41 371 945 bp. Diversity in the *SbSNF4-2* coding region was 73% higher than the average for the whole chromosome 6 and 136% higher than the average in the 217 kb region. We further calculated π in the 217 kb region with a step size of 10 and window size of 50 bp using TASSEL v5.0 (Bradbury *et al.*, 2007). Again, the average π in the *SbSNF4-2* coding region (0.2459) was 88% higher than in the 217 kb region (0.1308). The *SbSNF4-2* coding region also fell into a linkage disequilibrium (LD) block covering the *SbSNF4-2* coding and downstream regions (Fig. 4A, 4B). As expected, LD was more extensive in the region among wild sorghums (Fig. 4C). This increased diversity maintained in *SbSNF4-2* in an LD block may reflect the result of evolution (Slatkin, 2008) or a population bottleneck (Hamblin *et al.*, 2006; Mace *et al.*, 2013), depending on whether domestication of sorghum caused the bottleneck (Smith *et al.*, 2019). The LD in *SbSNF4-2* can be another piece of evidence that it was the candidate gene, because LD can be used to identify candidate genes mapped by GWAS (Sulem *et al.*, 2008).

Next, we selected the SSC, juice yield (JW), and *SbSNF4-2* coding region indels (all intronic; no exonic indels were found) to test whether any haplotypes were associated with the traits. Seven indel-based haplotypes were identified, which were divided into two clades: haplotypes 1 and 2 in one clade and haplotypes 3–7 in the second clade (Fig. 5A). The results indicated that the average juice yield and SSC of accessions with haplotypes 3–7 were significantly higher than those with haplotypes 1 or 2 in rainy seasons (Fig. 5B, C). For individual haplotypes, average juice yield and SSC of accessions with haplotypes 5 or 6 were significantly higher than those with haplotypes 1 or 2 in rainy seasons (Fig. 5D, E). However, there was no significant difference between haplotypes in post-rainy environments (Supplementary Fig. S8). We repeated the examination with *SbSNF4-2* exonic SNPs and found nine SNP-based haplotypes. We found that accessions with haplotype



Trait	42-1	42-2	Ts-1
Plant height	80	55	36
Biomass	265	306	46
Juice yield	29	46	47
Brix	36	25	17
Sugar yield	77	85	75

Fig. 3. Phenotypic differences of transgenic sorghum (42-1 and 42-2) and sugarcane (Ts-1) relative to controls. (A, B) Plant height, biomass, juice yield (JW), SSC, and sugar yield in sorghum (A) and sugarcane (B). (C) Percentage increases in the values presented in A and B relative to controls. (D–F) Tiller number (D), seed weight per plant (E), and 1000-seed weight (F) in sorghum. The × inside each box in the boxplots represents the mean and the horizontal line represents the median value of each data group. Significant differences from the controls are indicated with asterisks: $^{**}P<0.01$, $^{*}P<0.05$.

2 were more likely to produce more juice and higher SSC compared with the most common haplotype, haplotype 1, in rainy environments (Supplementary Fig. S9) but not in post-rainy environments (Supplementary Fig. S10). Three SNPs at positions 41 364 171, 41 365 808, and 41 369 438 are non-synonymous (Supplementary Fig. S11A). In haplotype 9, the non-synonymous substitution of C by T (G by A in the negative strand) in position 41 365 808 results in a missense mutation replacing arginine (R) with histidine (H), which is the last amino acid residue of cystathionine-β synthase (CBS) domain 3 (Supplementary Fig. S11A, B). Accessions with haplotype 9 produced more juice in rainy environments (Supplementary Fig. S9B) and higher SSC in post-rainy environments (Supplementary Fig. S10C) compared with haplotype 1 accessions. The other two non-synonymous SNPs each occurred in only one accession and were not included in this analysis. The simultaneous effect of haplotypes on both JW and SSC could be due to the significant correlation between the two traits (R=0.57; Table 2).

Discussion

We found that SSC was highly correlated with SY, biomass, and JV or JW in this study. In an evaluation of a sorghum recombinant inbred line population from Rio×BTx623 in Texas, USA, Murray *et al.* (2008b) found that the correlation coefficients of SSC/Brix with SY, biomass, and SW were 0.58, 0.34 and 0.27, respectively. Similarly, Kanbar *et al.* (2021) found that

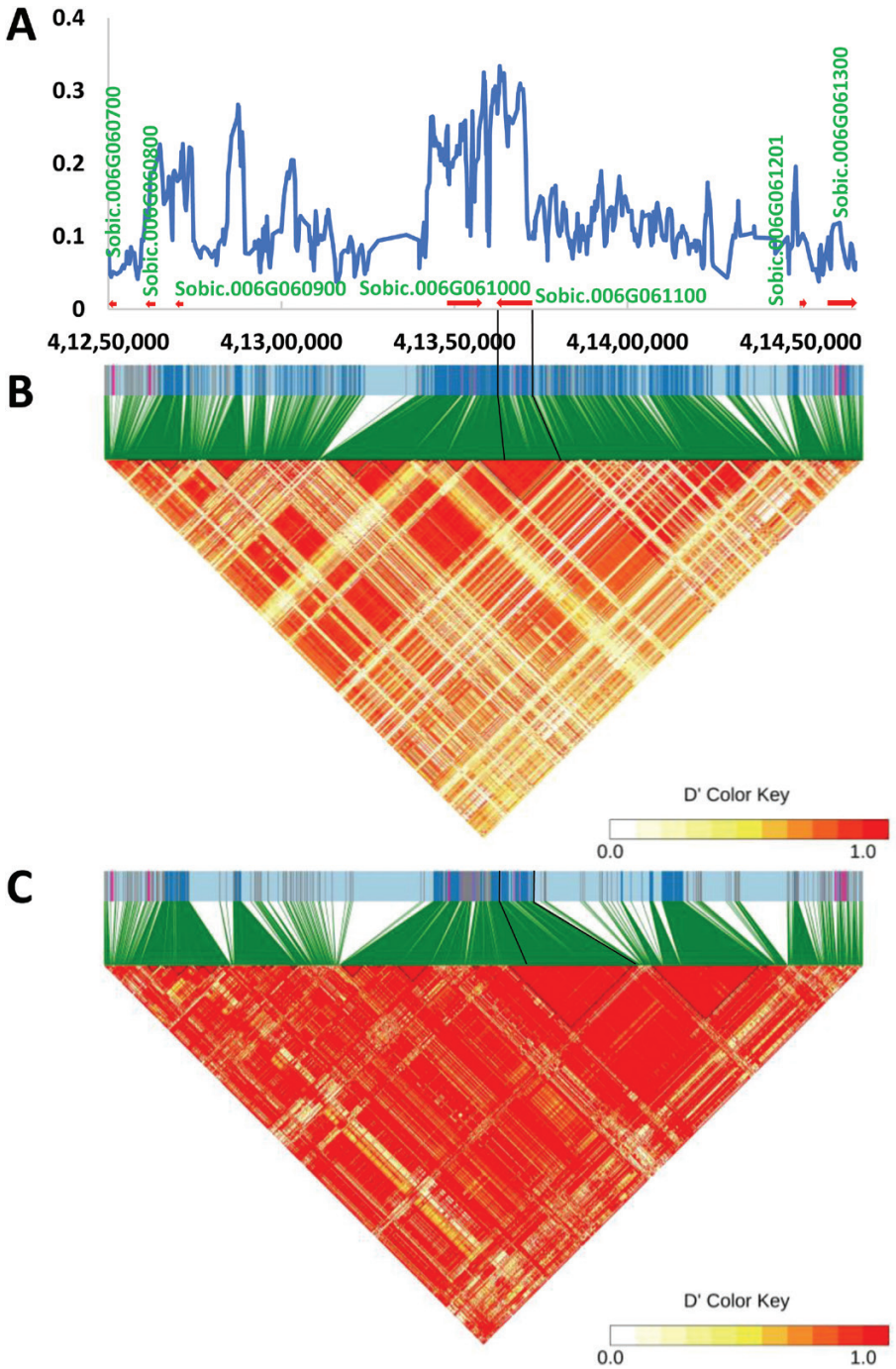


Fig. 4. Diversity in the *SbSNF4-2* region of the *S. bicolor* genome. Nucleotide diversity (A) and linkage disequilibrium in landrace (B) and wild (C) accessions in the arbitrarily chosen region of *SbSNF4-2* of 41 250 032–41 467 613 bp on sorghum chromosome 6. The location of the genes in A was based on *Sorghum bicolor* v3.1.1, available at https://phytozome-next.jgi.doe.gov/info/Sbicolor_v3_1_1.

the correlation coefficients of SSC/Brix with SY, biomass, and JW were 0.75, 0.54, and 0.60, respectively, in an evaluation of 14 sorghum genotypes under temperate conditions. The high correlation between SY and JW may be the reason that SY can be increased rapidly by selecting for higher juice yield (Slewinski, 2012). This correlation has important practical implications for plant breeding.

In this study, using GWAS, we have identified a pleiotropic *SbSNF4-2* that delays DF and increases biomass, SSC, JW, and PH when overexpressed in sorghum. *SbSNF4-2* has similarity with *AtSNF4* and *PRKAG1/2/3* (γ1/2/3), the γ subunits of the conserved AMPK/SNF1/SnRK1 heterotrimeric complexes from Arabidopsis and humans, respectively. AMPK/SNF1/SnRK1 complexes typically consist of a catalytic α, a

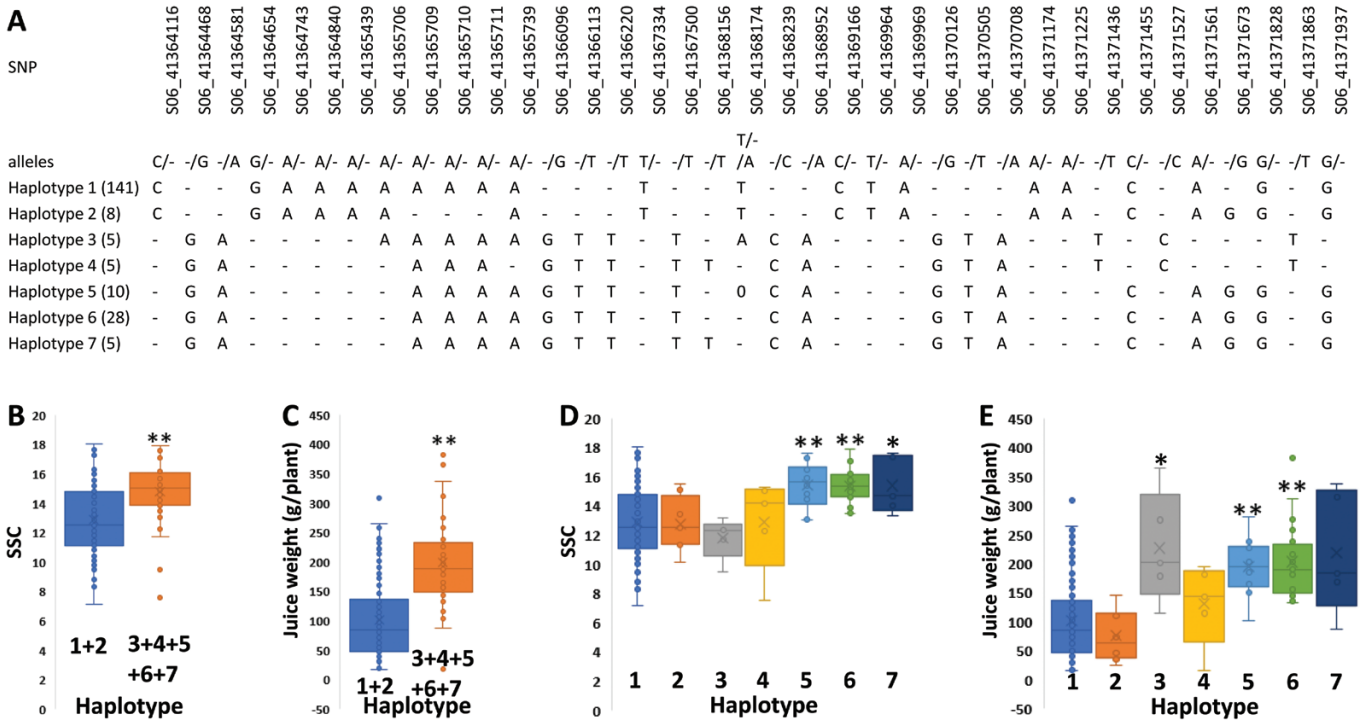


Fig. 5. Haplotype analysis. Association of indel haplotypes 1 + 2 and 3–7 (A) with SSC (B) and juice weight (C), and haplotypes 1–7 with juice weight (D) and SSC (E) in rainy environments. The × inside each box in the boxplots represents the mean and the horizontal line represents the median value of each data group. For each haplotype in A, the number in parentheses is the number of accessions with that haplotype. Asterisks indicate significant differences between haplotypes 1 + 2 and 3–7 (B, C) or from haplotype 1 (D, E): ***P*<0.01, **P*<0.05.

scaffolding β, and an activating γ subunit and play central regulatory functions in metabolism, stress signaling, and development (Broeckx *et al.*, 2016; Li and Sheen, 2016). In yeast and mammals, the γ subunit acts as the complex's energy-sensing module by controlling the activity of the α subunit (Hedbacker and Carlson, 2008; Crozet *et al.*, 2014). The γ (or βγ in plants; Ramon *et al.*, 2013) subunit typically contains four highly conserved CBS motifs (Supplementary Fig. S11B) that can bind adenine nucleotides and hence function as the cellular energy-sensing module of the complex (Crepin and Rolland, 2019). In Arabidopsis, *AtSNF4* is essential for SnRK1 heterotrimeric formation (Kleinow *et al.*, 2000), and loss-of-function mutation in *AtSNF4* is lethal (Ramon *et al.*, 2013; Gao *et al.*, 2016). No *AtSNF4* overexpression studies have been found in the literature.

The γ subunits have been most intensively studied in humans. SNF4-2 has the same sequence identity/similarity of 29%/50% with the human γ1 (NP_002724), γ2 (AJ249976), and γ3 (NP_059127) subunits. Overexpression of $\gamma1^{H151R}$ (Schönke *et al.*, 2015), γ2 (Arad *et al.*, 2003), or γ3 (H.Yu *et al.*, 2006) in mice increases glycogen content. Interestingly, cardiac overexpression of a mutant γ2 (T400N) in mice also significantly increases cardiac mass, beginning at age 2 weeks (Banerjee *et al.*, 2010). Similarly, in mice with cardiac overexpression of the wild-type and mutant (R302Q) γ2 gene, the weight of the hearts of transgenic mice averages 296.67 mg, twice that of wild-type mice (140 mg) and nearly three times that of non-transgenic mice (117 mg) (Sidhu *et al.*, 2005). Furthermore, knock-in expression of the mouse mutant γ2 (R299Q; the same as R302Q in human γ2) subunit causes chronic AMPK activation as well as ghrelin signaling-dependent hyperphagia, obesity, and impaired pancreatic islet insulin secretion. In both homozygous male and female transgenic knocked-in mice, bodyweight is doubled after 30 weeks compared with wild-type mice (Yavari *et al.*, 2016). The increased bodyweight in homozygous transgenic mice is the result of greater glucose requirement caused by greater whole-body glucose utilization and increased glucose uptake in gastrocnemius muscle (Yavari *et al.*, 2016). This is similar to the effect of overexpressing *SNF4-2* in sorghum and sugarcane, which increased fresh biomass through increased PH and tillering (Fig. 3).

In conclusion, we have presented evidence that *SbSNF4-2* increases juice SSC, extractable JW, plant biomass, and PH when overexpressed. First, we mapped SY, JW, SSC, biomass, and PH to a single genetic locus on sorghum chromosome 6 covering *SbSNF4-2* (Sobic.006G061100). Second, overexpression of *SbSNF4-2* almost perfectly recreated the mapped phenotypic traits. In human γ subunit studies, overexpression of γ2 increases bodyweight, similar to the increased biomass in *SNF4-2* overexpressing plants observed in this study. The *SNF4-2* gene will be important in enhancing sugar production as well as other economically important traits in plants. Further studies

are warranted to better understand the mechanism behind the phenotypic changes and to determine whether SNF4-2 acts alone or through the SnRK1 complex to effect such changes.

Supplementary data

The following supplementary data are available at *JXB* online.

Table S1. RNA-seq results of three genes in the *Dw3* peak.

Table S2. Sugar content in stem juice in sorghum transgenic line 42-1.

Fig. S1. Phenotypic distribution of days to 50% flowering, plant height, biomass, SSC, juice volume, and juice weight in the 12 testing environments described in Table 1.

Fig. S2. Frequency distribution of plant height in PH2 and PH6 testing environments, Manhattan plot of SBI-07 for plant height in the mini core panel showing *Dw3* peaks in PH2 and PH6, and details of the *Dw3* region in PH2, PH6 and the three genes.

Fig. S3. Manhattan plot for plant height in the mini core panel showing *Dw3* peaks in post-rainy environments HT2, HT3, HT5, and HT6 on sorghum chromosome 7.

Fig. S4. Manhattan plots of SBI-07 for plant height in the mini core panel showing *Dw3* peaks in HT2 and HT6, and details of the *Dw3* region in HT2, HT6, and the three genes.

Fig. S5. Fold change expression of *SbSNF4-2* in transgenic and control sorghum plants, calculated by the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001).

Fig. S6. Pairs of internodes and panicles from control and transgenic 42-1 sorghum.

Fig. S7. Number of seeds per plant and stem diameter of transgenic 42-1 and 42-2 and control sorghum.

Fig. S8. Association of haplotypes 1–7 with SSC and juice weight in post-rainy environments.

Fig. S9. Association of haplotypes 1–9 with juice weight and SSC in rainy environments.

Fig. S10. Association of haplotypes 1–9 with juice weight and SSC in post-rainy environments.

Fig. S11. *SbSNF4-2* mRNA and protein sequences.

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Author contributions

Conceptualization: HDU, JL, and YHW; mini core and reference set phenotyping: HDU, KS, CLLG, KG, RK, and SKS; sugarcane transformation: JL; transgenic sugarcane evaluation: CSP; sequencing preparation: LW; sorghum transformation: LW and LG; transgenic sorghum evaluation: YL and RM; initial GWAS: YHW; bioinformatics and GWAS from resequenced data: JL; coordination: YHW; writing—original draft: YHW; writing—review and editing: YHW, HDU, and JL.

Conflict of interest

The authors declare no conflicts of interest.

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Data availability

All data are available in the main text or the supplementary data published online.

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