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Study on fine mapping of QTL for juice yield traits of sweet sorghum (*Sorghum dochna*)

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Abstract: The stem juice yield is a key factor that influences both the biological and economic production of sweet sorghum [*Sorghum dochna* (Forssk.) Snowden]. To elucidate upon the genetic basis of the stem juice yield, an F_5 population developed from a cross between the low juice yielding Xinliang52 (XL52) and high juice yielding W455 lines, were used in a quantitative trait locus (QTL) analysis. A main effect of the QTL controlling stem juice yield was separated with an SSR marker called Xtxp97, which explained 46.7% of the phenotypic variance. In addition, F_5 and F_6 populations were constructed with XL52 and W452 as the parents to further verify the QTLs, and a significant correlation was found between the juice yield trait and the Xtxp97 marker. Based on the progeny tests of 29 recombinants, *QJy-sbi06* was located in a region of about 21.2 kb on chromosome 6, where a candidate gene encoding an NAC transcription factor (sobic.006G147400) was identified. Combining the different population association analysis and sequencing technology showed that XL52 inserted a 1.8 kb transposon in the NAC to directly interrupt and inactivate the juice yield gene. This study also demonstrated that the colour of the leaf midribs was controlled by a single gene and was significantly positive correlated with juiciness ($r = 0.784$, $P < 0.01$). These results could lay the foundation for map-based cloning of *QJy-sbi06* and provide genes or QTLs for breeding sorghum lines with a high juice yield and quality.

Keywords: juice yield; NAC; QTL; *Sorghum dochna*; transposon

Sweet sorghum is a C_4 crop with a high photosynthetic efficiency. The species has rapid growth and high biomass productivity (Evans et al. 2013; Mullet et al. 2014; McCormick et al. 2018). As a variety of common grain sorghum, it shows a wider range of adaptability and resistance to harsh climate and soil conditions (Almodares & Hadi 2009). Moreover, it has been utilised as a unique energy crop characterised by high amounts of soluble solid in the stem and is also regarded as a forage crop in agroecosystems (Paterson et al. 2009; Alhajturki et al. 2012; Mathur et al. 2017; Kanbar et al. 2020, 2021a, b). The stem juice yield is essential in providing a guarantee for the stalk transportation and storage of water and nutrients (Slewinski 2012).

A correlation analysis between the stem juiciness (or pithy) with the colour of the leaf midribs (green or white) was observed 100 years ago (Hilson 1916). Previous studies have shown that the leaf midribs and stem juiciness were controlled by a single locus, named *D* or *Dry*, as a main genetic factor (Hilson 1916; Swanson & Parker 1931; Rangaswami et al. 1937). Farmers in Ethiopia can estimate the stem juice yield in the early stages of sorghum growth by the colour of the leaf midrib (Teshome et al. 1997; Lekgari 2010). Using a population of 98 recombinant

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inbred lines (RILs, F_7) derived from a cross between the stay-green sorghum cultivar B35 (higher stem juice rate) and the non-stay-green sorghum Tx7000 (low stem juice rate) and using the colour of the leaf midribs (*D* gene) as a model, the QTL site related to the stay-green was located on chromosome 6 between marker UMC34b and TXS1030 (Xu et al. 2014). Hart et al. (2001) found that the gene controlling the juice yield was closely linked with the Xtxp97 marker on chromosome 4 by using an F_{6-8} population from a cross between BTx623 and IS3620C and taking the colour of the leaf midribs of each plant as a research target. Zhai et al. (2014) designed two populations to precisely locate the gene of the stem juice yield of sorghum in the 51.18 M and 51.9 M region on chromosome 6 by observing the colour of the leaf midribs.

Two basic approaches to identify the causal gene of complex quantitative traits can be used: linkage mapping based on a recent recombination in the parental population and genome-wide association mapping (GWAS) based on historical recombination in natural diverse populations (Wang et al. 2018). Some quantitative trait loci for the midrib colour, sugar yield and juice yield have been confirmed by a genome-wide association study (GWAS) at 51.8 Mb on chromosome 6 (Burks et al. 2015). Zhang et al. (2018) cloned the *Dry* gene controlling the stem juice retention from sorghum, which encodes a plant-specific NAC (petunia NAM, *Arabidopsis* ATF1 ATF2 and CUC2) transcription factor and, provided evidence for the origin of sweet sorghum. The sweet sorghum stem juice yield is an important agronomic trait influencing the sugar yield of sweet sorghum. In this experiment, grain sorghum XL52, sweet sorghum W452 and W455 were used as the parents to create two recombinant inbred line populations, to identify and clarify the gene for the sorghum juice yield.

MATERIAL AND METHODS

Population development. A total of 96 F_5 RILs of sorghum derived from the XL52 and W455 cross used were planted in 2013. A total of 94 F_6 RILs of sorghum derived from the XL52 and W455 cross were planted in 2014. XL52 is a white leaf midrib variety with a low stem juice yield, and W455 is a wax leaf midrib variety with a high stem juice yield (Pei et al. 2010).

The verification populations (139 F_5 and 142 F_6) were developed with XL52 and W452 wax as the parents through the reciprocal cross, which were planted in early May in 2013 and 2014, respectively. Each line was derived from a single F_2 plant following a single seed descent method. W452 is a wax leaf midrib variety with a high stem juice yield (Pei et al. 2010).

The plant materials were planted in the field experimental station of Tianjin Agricultural University (39°N, 117°E), China. The planting spacing was 0.5 m between the plants in a row and 0.25 m between the rows with 16 plants per row. The field management followed local agricultural practices.

Measurements of phenotypic values. Stalks of sorghum (not including the leaves, ears and roots) were collected at the maturity stage. The total fresh weight was weighed, and then the stalks were pressed and mixed. The total juice weight was weighed and calculated by:

$$\text{Juice yield} = \text{total juice weight/total fresh weight of the stalks}$$

The colour of the leaf midribs was determined by observing the middle of the leaves at the flowering stage. If the colour showed all white with no trace of green or wax, it was denoted as "white (B)"; otherwise, it was denoted as "wax (L)".

Construction of genetic linkage map QTL analysis. Genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method (Doyle & Doyle 1990). A Join Map (Ver. 3.0) was employed to calculate the genetic distances among the markers and to construct the linkage map (Ooijen & Voorips 2001). QTL mapping was performed by the composite interval mapping (CIM) method using Map QTL (Ver. 4.0) software with logarithm of odds (LOD) values over 3.0 (Ooijen et al. 2002). All bar charts and statistical analysis were produced with the software of Graph Pad Prism (Ver. 9.0) and SPSS (Ver. 19.0). The fine mapping figure was plotted with PowerPoint from Microsoft Office 2007. The sequencing results were compared with the National Center for Biotechnology Information (NCBI) database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using DNAMAN software sequence splicing. The threshold of significant QTLs in the CIM was determined by a 1 000-permutation test at $P < 0.05$. The location of each QTL was described according to the QTL LOD peak and flanking region with 95% confidence. The same DNA fragment amplified by the Simple sequence repeat (SSR) markers in XL52 was scored 0, and W455 was scored 2. A positive QTL additive value indicated that the allele was

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Table 1. Phenotypic performance for the juice yield between two different recombinant inbred lines (RILs)

Population	Year	Max	Min	Mean	SD	Kurtosis	Skewness
		(%)					
XL52/W455	2013	53.8	6.7	0.335	0.119	–0.924	–0.299
	2014	64.5	3.3	0.265	0.125	0.209	0.742
XL52/W452	2013	48.6	6.0	0.309	0.099	–0.251	–0.543
	2014	58.8	4.0	0.290	0.110	–0.545	0.040

Pooled data in 2013 and 2014 during the beginning of May were used in the table; RIL sample size 96, replications $r = 2$, standard deviation (SD) between the mean of 96 lines in 2013 of XL52 × W455; RIL sample size 94, replications $r = 2$, SD between the mean of 94 lines in 2014 of XL52 × W455; RIL sample size 139, replications $r = 2$, SD between the mean of 139 lines in 2013 of XL52 × W452; RIL sample size 137, replications $r = 2$, SD between the mean of 137 lines in 2014 of XL52 × W452

from XL52, while a negative QTL additive value indicated that the allele was from W455.

Marker development. The SSR primers used in this experiment refer to the primer sequences published by Bhattramakki (2000), Kong et al. (2000) and Li et al. (2009). The SSR markers were designed using the NCBI Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and Primer premier 6. The sequence-related amplified polymorphism (SRAP) primers used in this experiment refer to the primer sequences published by Ferriol et al. (2003) and Li et al. (2003). The markers are listed in Table S1 and Table S2 in the Electronic Supplementary Material (ESM).

RESULTS

Phenotypic variation in the populations. The statistical analysis on the juice yield traits of the mapping population was summarised in Table 1. The phenotypic values for the juice yield trait showed continuous variation and were normally distributed in the populations of XL52 × W455 and XL52 × W452. Significant differences were observed between the parents, and the progenies showed transgressive segregation (Figure1).

Mapping and validation of QTL for the juice yield. The SRAP markers Me8/Em2-1 and Me8/

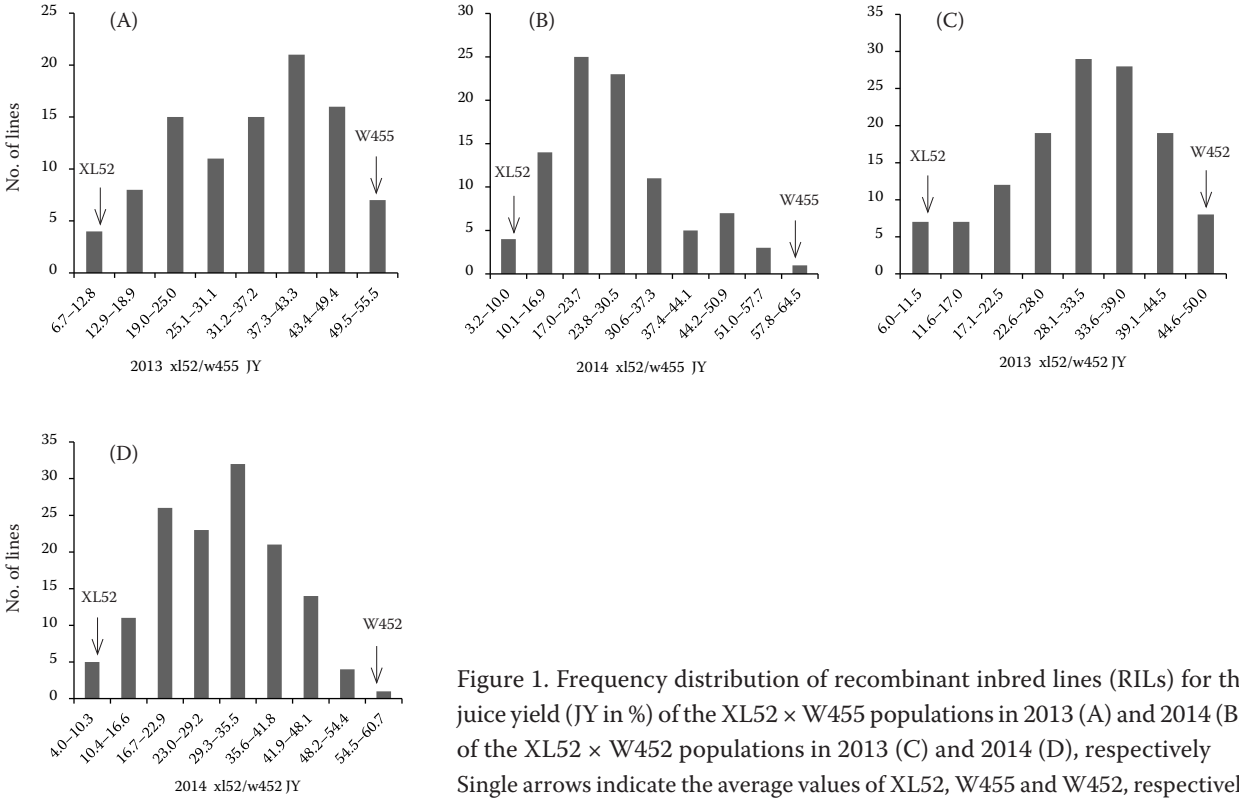

Figure 1. Frequency distribution of recombinant inbred lines (RILs) for the juice yield (JY in %) of the XL52 × W455 populations in 2013 (A) and 2014 (B), of the XL52 × W452 populations in 2013 (C) and 2014 (D), respectively
Single arrows indicate the average values of XL52, W455 and W452, respectively

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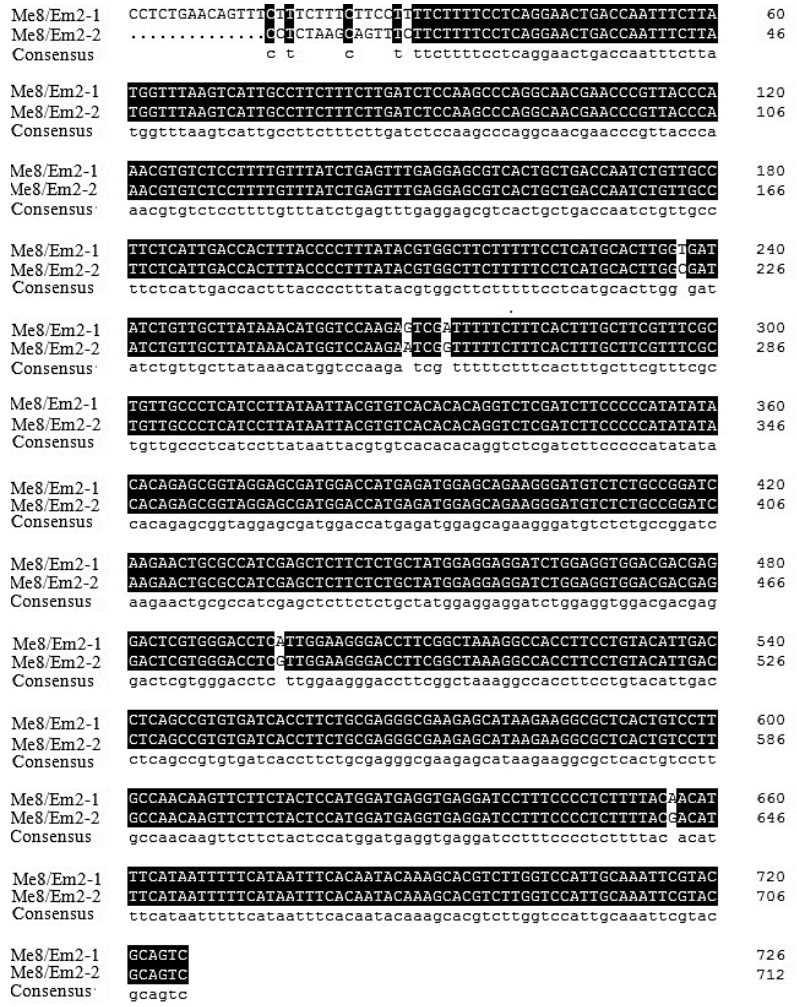

Figure 2. BLAST results of M8E2-J727 with M8E2-J71

Em2-2 were closely linked with the juice yield trait of sorghum after the sequencing and comparison, and the sequences showed extremely high consistency and belonged to the alleles (Supplementary Data 1 in the ESM). The differences in the individual bases in the sequence may be due to substitution mutations. Compared with the sorghum genome sequence (reference genomic sequences BLAST), the amplified sequences of the markers Me8/Em2-1 and Me8/Em2-2 had a high consistency with the sequence between bases 29909723 and 29910438 on chromosome 6 (Figure 2). Therefore, marker M8E2-720 was designed at this position.

Preliminary, a QTL was identified for the juice yield in the F_5 (XL52 × W455) population. A 70 cM genetic linkage diagram (Figure 3) consisting of 15 markers (14 pairs of SSR primers and 1 pair of SRAP primers) was constructed with an average distance of 4.7 cM among the markers. The QTL was located between

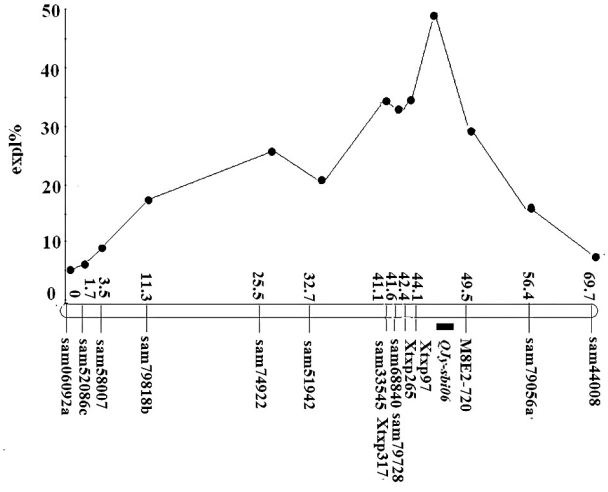

Figure 3. Quantitative trait loci for the juice yield were detected on chromosome 6 in the (XL52 × W452) F_5 population

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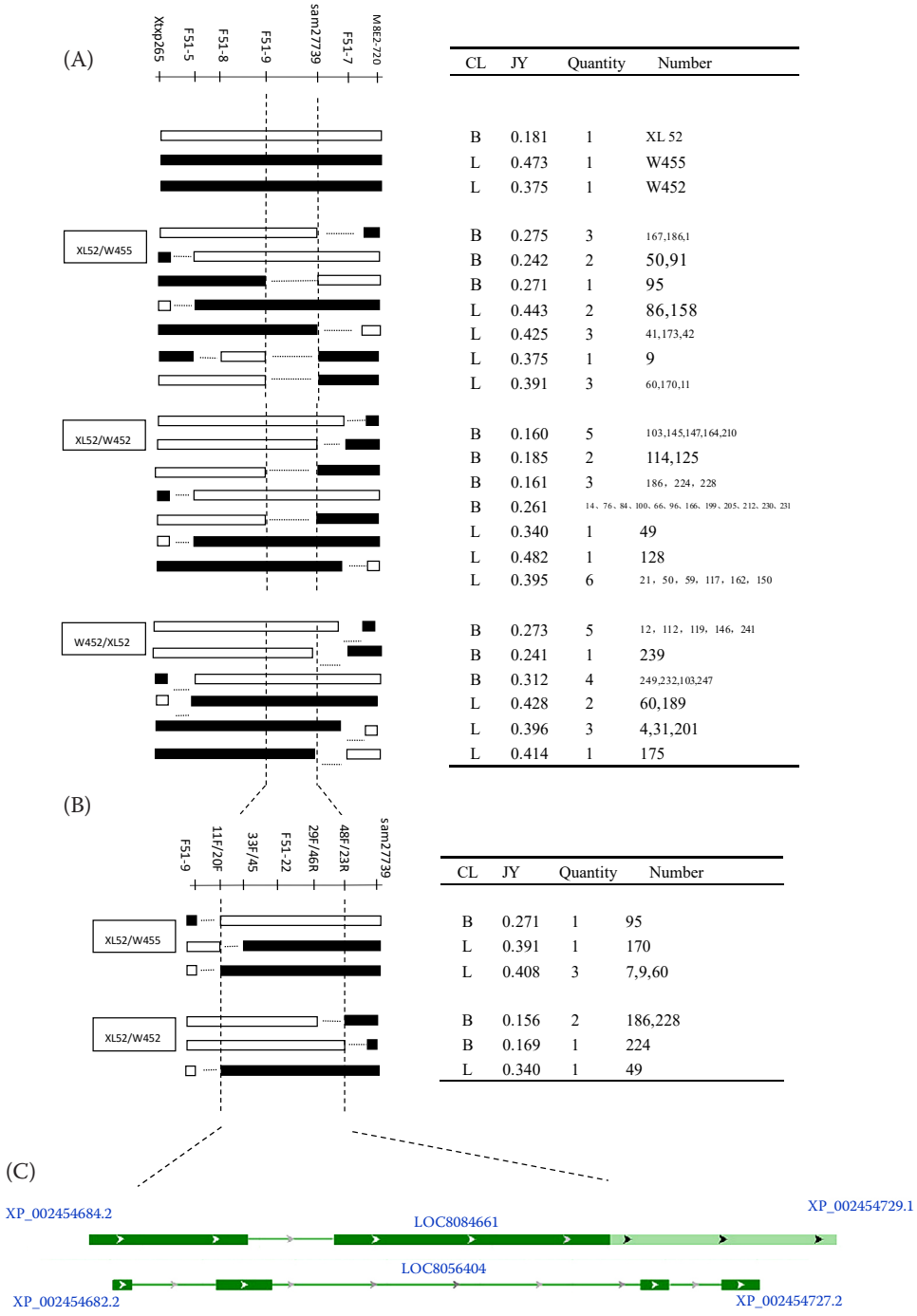
M8e2-720 and Xtxp265 on chromosome 6 within the 1 M and co-separated from markers Xtxp97, which explained 46.7% of the juice yield.

The F_5 (XL52 × W452) and derived F_6 populations were used to confirm the QTL, which accounted for more than 10% of the F_2 phenotypic variation.



CL	JY	Quantity	Number
B	0.181	1	XL 52
L	0.473	1	W455
L	0.375	1	W452
B	0.275	3	167,186,1
B	0.242	2	50,91
B	0.271	1	95
L	0.443	2	86,158
L	0.425	3	41,173,42
L	0.375	1	9
L	0.391	3	60,170,11
B	0.160	5	103,145,147,164,210
B	0.185	2	114,125
B	0.161	3	186，224，228
B	0.261		14、76、84、100、66、96、166、199、205、212、230、231
L	0.340	1	49
L	0.482	1	128
L	0.395	6	21，50，59，117，162，150
B	0.273	5	12，112，119，146，241
B	0.241	1	239
B	0.312	4	249,232,103,247
L	0.428	2	60,189
L	0.396	3	4,31,201
L	0.414	1	175

CL	JY	Quantity	Number
B	0.271	1	95
L	0.391	1	170
L	0.408	3	7,9,60
B	0.156	2	186,228
B	0.169	1	224
L	0.340	1	49

Figure 4. Fine mapping of the positioning of the juice yield: (A) indicates the exchange of primers M8E2-270, F51-7, sam27739, F51-9, F51-8, F51-5 and Xtxp265; (B) indicates the exchange of primers sam27739, 48F/23R, 29F/46R, F51-22, 33F/45, 11F/20F and F51-9; (C) indicates the compression result

CL is short for vein colour, B for white vein and L for waxy vein; juice yield (JY) is short for the JY of the sorghum stalk; quantity indicates the number of single plants of the same type; number indicates the mark of the individual plants with the corresponding traits in the population

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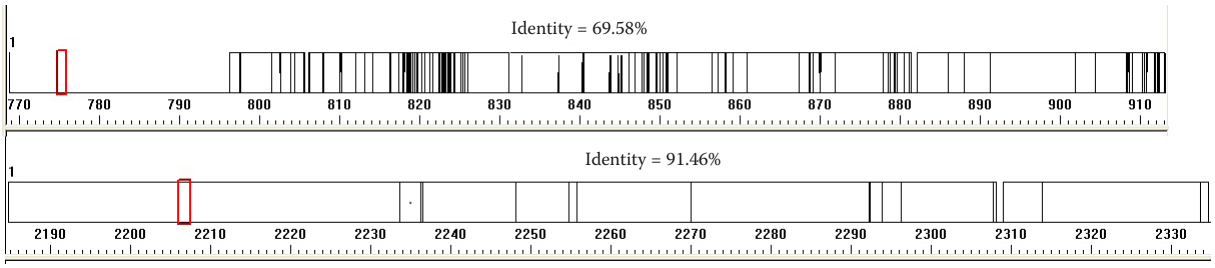

Figure 5. Similarity comparison of the target zones

The first result shows the similarity comparison of XL52 and W452; the second result shows the similarity comparison of XL52 and BTx623; W455 is consistent with the W452 sequence in the target area

The results showed that the juice yield was significantly correlated with marker Xtxp97 in F_5 $r = 0.691$, $P < 0.01$, and in F_6, $r = 0.509$, $P < 0.001$.

Fine mapping of the juice yield. After rescanning the three populations using markers M8E2-720 and Xtxp265, single plants with the same genotype as XL52 were selected (Figure 4A). Eight SSR markers were developed in the region and employed to genotype the recombinants that delimited *QJy-sbi06* to an interval flanked by markers sam27739~F51-10, which ranged the existing single exchange plants within 51.71–51.87 M (Figure 4B). Finally, markers 11F/20F and 48F/23R were used to reduce the region in 21.214 kb (Figure 4C).

Resequencing of target regions and prediction of candidate genes. The target regions of XL52 and W452 were sequenced in combination again. The reference sequence genome of BTx623 was published by the NCBI (Paterson et al. 2009). The sequence of two parents was obtained by splicing the sequencing with the DNAMAN software. It was found that the sequence similarity of the target region of the two parents was 69.58%, and the sequence similarity of W452 and BTx623 (wax vein, high juice yield) was 91.46% (Figure 5). In addition to some locations on the bases of the insertions, deletions and replacement, the biggest difference between XL52 and W452 appeared near the right end in the sequence by inserting a 1.8 kb of transposons, and this transposon in the genome of sorghum randomly exists and has full repeat at least 30 times (Paterson et al. 2009).

Combining the sequence comparison and BLAST, two candidate genes were found in the associated target interval, LOC8056404, and the NAC transcription factor LOC8084661 (Figure 4). No difference was observed in LOC8056404 between XL52 and W452, but both were different from BTx623. The preliminarily result showed that there was little difference in the juice yield regulation between XL52 and W452. Compared with BTx623, W452 had one segment difference: GGATGGCGACCAATGTGGTGGTG-GACCTGCGGGTCCGACACGGTCACCAAGCCC-CTCCGACGCCATGCGCGCCGCCATGGCCGCG in LOC8084661, and XL52 inserted a 1.8kb transposon in the middle of the NAC to directly interrupt and inactivate the gene (Figure 6).

Correlation analysis of candidate genes in different sorghum varieties. In this study, we also discussed the relationship between the pulse colour and the juice yield. A total of 71 sorghum varieties were measured for the leaf midrib colour, and 47 varieties showed a statistical significance (Table S3 in the ESM). The colour of the leaf midribs had a significantly positive correlation with the juiciness ($r = 0.784$, $P < 0.01$) (Figure 7). Among in the

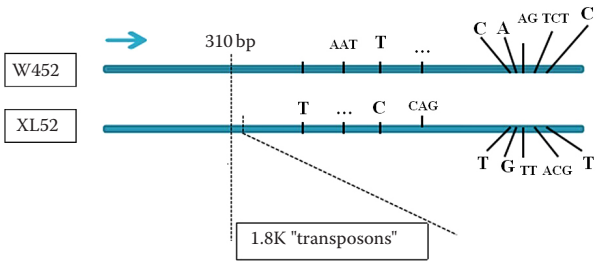

Figure 6. Diagram of the NAC transcription factor structural between the parents

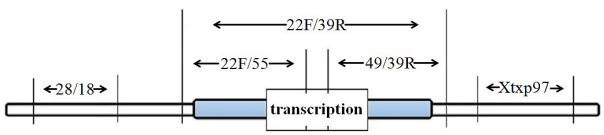

Figure 7. Diagram of the primer location in the fine mapping of the juice yield

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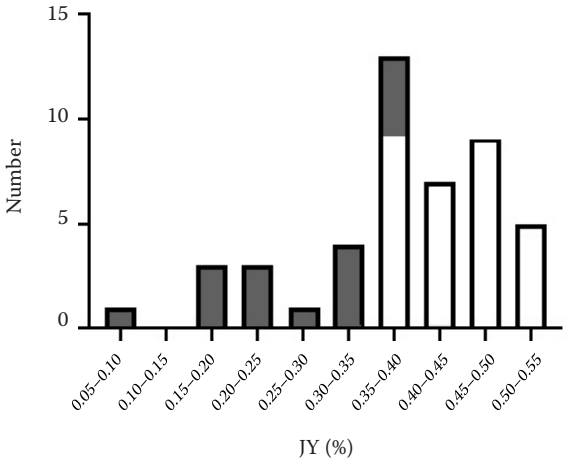

Figure 8. Juice yield (JY) distribution of the different varieties
The black bar chart means white midrib with a low juice yield, and the white bar chart means wax midrib with a high juice yield

populations, including two evaluating populations, the highest correlations were observed in the XL52 × W455 F_5 population. The correlation coefficients of XL52 × W452 F_5 and XL52 × W452 F_6 populations were $r = 0.745$, $P < 0.01$; $\chi^2 = 5.18$, $0.05 > P > 0.01$; $r = 0.646$, $P < 0.01$; $\chi^2 = 1.3$, $P > 0.05$, respectively, implying that fitting the 1 : 1 segregation through the chi-square testing.

The correlation between the juice yield and candidate genes was verified in 71 varieties of sorghum (Figure 8). Different primers were designed in both sides of the NAC transcription factor. 22F/39R was successfully obtained to amplify the complete NAC gene. In addition, since the two ends of the segment W452 and BTx623 were different, the primers were designed to be 28/18.

The results showed that the markers 28/18, Xtxp97, 22F/55, 22F/39R, and 49/39R were significantly and positively correlated with the juice yield, the highest correlations of 0.725 ($P < 0.01$) were found between 49/39R and the juice yield. Furthermore, the results demonstrated that the NAC transcription factor was the candidate gene that most likely controlled the juice production.

DISCUSSION

This experiment proved that the colour of the leaf midribs is a quality trait controlled by a single gene, in which the white of the main veins is dominant compared with the waxy succulent type, while the yield of the sorghum stalk is controlled by one major gene and several micro-genes. As shown in Figure 4, the traits of the main pulse colour and the juice yield of the leaves are heritable, which belongs to the nuclear gene inheritance. The main vein colour of the leaves had different colour changes in the natural population – white (B), waxy (LB) and waxy (L) in the 71 different sorghum varieties. As the leaf colour deepens, the juice yield is higher (Teshome et al. 1997). However, Zhang et al. (2018) observed at least three kinds of leaf midrib colour, namely green, yellow and white, but did not detect the midrib colour and the allele. Any separation between the close correlation is likely to be highly effective in the visual selection that results in the phenotype of the leaf midrib.

Earlier studies have shown that the pulse colour is closely related to the juiciness rate site (Hilson 1916; Swanson & Parker 1931; Rangaswami et al. 1937). The juice yield is a complex quantitative trait locus associated with the plant height, biological yield and sugar content (Xu et al. 2000; Hart et al. 2001; Burks et al. 2015; Han et al. 2015). In this study, the gene controlling sorghum juice yield was located at 21.214 kb of chromosome 6, consistent with the result of Zhai et al. (2014). At the same time, an NAC transcription factor in this region was identified as a candidate gene.

Zhang et al. (2018) showed that the juiceless sorghum Ji2731 contained a functional Dry gene, while the Dry gene was missing in the sweet sorghum E-tian, which resulted in the difference in the sorghum juice yield. However, in this study, the low juice line has a 1.8-kb insertion which disrupted the function of the NAC gene. The reason for the different results is that different populations were used in the study and the reasons for the loss of the NAC function are also different. To verify the experimental results, a single plant with a low yield, but with a NAC gene, could be selected for multiple back cross with W452. After the "transgenic" individual plant is constructed, the transcriptome differences can be compared with W452 to obtain downstream genes regulated by the NAC, so studies related to the regulation of the juice yield expression can be carried out.

It has been reported that sweet sorghum can be found in almost all major common sorghum varieties (Ritter et al. 2007; Ali et al. 2008; Wang et al. 2009). Some sweet sorghum varieties are found in different areas with a high juice yield, high sugar content and high yield (Mullet et al. 2014; Anami et al. 2015;

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Mathur et al. 2017). Therefore, the NAC transcription factor identified in this study is likely to provide a theoretical basis and reference for the cultivation of dual-use sorghum varieties as biofuels and food crops in the future. Meanwhile, sweet sorghum is considered a bioenergy crop with a potential evolutionary relationship (Calviño & Messing 2012). The collinearity between the genomes of major food crops (Paterson et al. 2009) and other biofuel crops with more complex genomes (such as willow twigs and sugarcane) (Wang et al. 2010) needs to be further studied. There was a high correlation between the juice yield and sugar content in the sweet sorghum. In this study, this was not only correlated with many characteristics, but also has a certain promoting effect on the sugar content. The differences in the sugar content between the two parents can be further observed, perhaps due to the result of the multiple effects (Srinivas et al. 2009).

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