SRAP Molecular Marker of Sugar Content and Juice Yield in Sweet Sorghum

Li Jinwang, Chen Quiling, Chen Peng, Li Oujing, Lv Jianpeng, Duan Xiaifei, Luo Feng, Gao Jianming, Sun Shoujun, Pei Zhongyou

Key Laboratory of Crop Genetics and Breeding, Tianjin Agricultural University, Tianjin, 300384, China

Corresponding author Email: zhongyoupe@tiau.edu.cn


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Abstract As an important energy crop, sweet sorghum (Sorghum bicolor L. Beauv) has been recognized around the globe. The present work utilized recombined inbred F₂,₃ population derived from the hybridization of 141 sweet sorghum “W455” and grain sorghum “Xinliang 52” as starting material to locate the molecular markers associated with sugar content by using BSA method combined with SRAP molecular markers. The results showed that the sugar content belonged to a single gene with additive-dominant effects or single gene with additive effect and the juice yield was controlled by poly-genes. A SRAP marker (M3E7-S248) linked to the sugar content gene was found to be located on chromosome 7. From the comparison of nucleotide sequences, we found that it had 79% similarity with sorghum adenosine diphosphate glucose pyrophosphorylase subunit SH2. Three SRAP markers M8E2-J727 (M8E2-J712), M8R12-J241 and F13E9-J150 linked to juice yield gene were found located on chromosome 6. However, other two SRAP markers namely M8E2-J712 and M8E2-J727 were found co-dominant. The nucleotide sequencing results showed that the marker M8E2-J712 was highly consistent with the oxygen capture enhancement protein in chloroplasts of many plants. The marker M8E2-J727 was 99% consistent with a sequence of mRNA of sorghum bicolor unknown functional protein. The markers M8R12-J241 and F13E9-J150 were both highly consistent with the unknown gene sequences on chromosome 6.

Keywords Sweet sorghum; Bulk segregant analysis (BSA); SRAP molecular marker

Background Sweet sorghum (Sorghum bicolor L. Moench) has excellent traits like drought resistance, water logging resistance, saline-alkaline tolerance, poor soil tolerance, high temperature resistance and dry-hot wind resistance, etc. It is also referred to as “the macroergic crop” (Li, 2004). Another outstanding feature is that it can produce 6000-7500 kg of sugar-rich stems per hectare (Zhang, 2011) and the synthesized carbohydrates can produce 47.9 L of alcohol every day through fermentation (Ta, 2011). Therefore sweet sorghum is honored as “the best competitor in bio-energy system” (Yang, 2004).

In recent years, bulk segregant analysis (BSA), as a widely-used method to quickly and high-efficiently search for molecular markers linked tightly to target genes, has been applied to plants like corn, rice and soybean. By combining simplified genomic sequencing technology and BSA, Beijing Biomarker Company has independently researched and developed Super BSA which has been extensively applied to plants like corn, rice, sorghum and Arabidopsis. SRAP (Sequence-Related Amplified Polymorphism) is characterized by its simplicity, stability and moderate yield. It is fit for molecular biological studies like gene mapping and cloning of different plants for various kinds of researches. Genetic maps of high saturation can be structured more efficiently and quickly by integrating SRAP molecular markers and BSA method. Liu et al. (2009) successfully screened out molecular markers linked to disease-resistant genes of cotton by SRAP-BSA method. It shows that the use of BSA method and SRAP markers has become mature and has been recognized by all.

The development of molecular biology in recent years, especially the application of molecular marker technology in sweet sorghum related studies, has brought opportunities for sugar content genetic breeding of sweet sorghum. Ritter et al. (2008) used the F₂ population derived from the hybridization of sweet sorghum R9188 and grain sorghum R9403463-2-1 as material. By SSR and AFLP molecular markers, they found the QTL’s for higher
sucrose and total sugar content located on the 1st, 5th and 6th chromosomes. However, the QTL's for higher sucrose content and yield were located on the 10th chromosome, and the QTL's for higher glucose content were on the 7th chromosome. Taking 234 F_{2:3} families obtained from the hybridization of grain sorghum LR625 with low stem sugar content and sweet sorghum Rio with high stem sugar content as material, Lu et al. (2015) constructed a genetic linkage map containing 92 SSR markers and 28 INDEL markers. Among them, 7 QTLs related to sugar content were distributed in the LG-3, LG-5, LG-7 and LG-9 linkage groups of sorghum and the total phenotypic variation explained was 62.45%; 8 QTLs related to juice yield were distributed in the LG-1, LG-2, LG-2, LG-3, LG-6, LG-7 and LG-9 linkage groups of sorghum and the total phenotypic variation explained was 99.75%.

The purpose of this study was to use the classical bulked segregant analysis (BSA) combined with SRAP molecular marker technique to study the molecular markers linked to sugar content and juice yield of sweet sorghum in order to screen out the marker sites associated with them and to provide foundation for the molecular marker assisted (MAS) breeding of sweet sorghum and the mapping and cloning of the genes related to sugar content and juice yield.

1 Results and Analysis
1.1 Phenotypic analysis of sugar content and juice yield in F_{2:3}
In this study, the frequency distribution of sugar content and juice yield characters of 141 plants in F_{2:3} population was studied (Figure 1). The sugar content histogram showed three-peak distribution which can be inferred that the sugar content belonged to a single gene with additive-dominant effects or single gene with additive effect. However, the juice yield histogram showed three-peak distribution with no obvious peak effect which can be inferred that the juice yield was controlled by poly-genes.

![Figure 1 Frequency distribution histogram of sugar and juice yield characters in F_{2:3}](image)

1.2 SRAP markers of sugar content and juice yield genes
1.2.1 SRAP primer screening
The study used the high sugar DNA pool (GT), low sugar DNA pool (DT), high juice DNA pool (GZ), low juice DNA pool (DZ) and two parents W455 (P1) and Xinliang 52 (P2) as templates to screen 224 pairs of SRAP primers. A total of 219 pairs of primers were able to amplify the bands which accounted for 97.8% (Figure 2 as partial primer amplification). Among them, 5 pairs of primers with specific bands were screened out from sugary population P1, P2, GT and DT, and 9 pairs of primers with specific bands were screened out from juice yield population P1, P2, GZ and DZ.

1.2.2 Analysis of SRAP marker acquisition and sequence of sugar content
This study used 5 pairs of specific primers which were related to sugar content to identify the primers of 7 individual plants from GT and DT, respectively. It was found that only primer combination Me3/Em7 had specificity between GT and DT plants. Furthermore, the primer was used to verify 141 plants in F_2 population and one-way ANOVA and chi-square test were carried out. It was found that this primer could amplify a marker band about 250 bp. The average sugar content of a single plant with this band was 2.6% higher than that without this band, and the difference between these two was extremely significant. It can be inferred that this marker was linked to the gene or QTL that controlled sugar content, and it explained 11.6% of the total variation (Table 1).
Figure 2 SRAP primer screening agarose gel electrophoresis
Note: M: 100 bp ladder marker; Other lanes, every four lanes are of a same primer; The DNA sequence of every four lanes are high sugar DNA pool, low sugar DNA pool, high juice DNA pool and low juice DNA pool, respectively; Arrow for difference banding

Table 1 statistics analysis results of five markers

<table>
<thead>
<tr>
<th>Primer Combinations</th>
<th>High No.</th>
<th>Low No.</th>
<th>High mean value (%)</th>
<th>Low mean value (%)</th>
<th>Difference value (%)</th>
<th>Chi-square test (P)</th>
<th>Explaining the degree of variation (R²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Me3/Em7</td>
<td>103</td>
<td>38</td>
<td>8.7</td>
<td>6.1</td>
<td>2.6</td>
<td>0.559</td>
<td>117/1007.2=11.6%</td>
</tr>
<tr>
<td>Me8/Em2-1</td>
<td>114</td>
<td>27</td>
<td>14.2</td>
<td>9.3</td>
<td>4.9</td>
<td>0.109</td>
<td>0.052/0.324=16.0%</td>
</tr>
<tr>
<td>Me8/Em2-2</td>
<td>45</td>
<td>96</td>
<td>16.3</td>
<td>11.8</td>
<td>4.5</td>
<td>0.058</td>
<td>0.062/0.324=19.1%</td>
</tr>
<tr>
<td>Me8/R12</td>
<td>40</td>
<td>101</td>
<td>15.9</td>
<td>12.2</td>
<td>3.7</td>
<td>0.330</td>
<td>0.054/0.324=16.7%</td>
</tr>
<tr>
<td>F13/Em9</td>
<td>37</td>
<td>104</td>
<td>16.0</td>
<td>12.3</td>
<td>3.7</td>
<td>0.697</td>
<td>0.037/0.324=11.4%</td>
</tr>
</tbody>
</table>

Me3/Em7 was found to be 248 bp in size by sequencing. It was named M3E7-S248 and compared in GenBank database with BLAST alignment. There was a 97% consistency between No.1~No.248 base sequences of M3E7-S248 and No.86737~No.86497 base sequences of sorghum SB_BBc0005H14 clone, and a 79% consistency with No.35941~No.35695 base sequences of sorghum adenosine diphosphate glucose pyrophosphorylase subunit SH2. It was identical with No.31197048~No.31197295 interbase sequences on chromosome 7 of sorghum bicolor by comparing it with the genome sequence of sorghum. Therefore, the marker might be located near No.31197171 base on chromosome 7.

1.2.3 Analysis of SRAP marker acquisition and sequence of juice yield
Nine pairs of specific primers related to juice yield were used to identify the primers of 7 individual plants from GZ and DZ respectively. It was found that three primer combinations Me8/Em2, Me8/R12 and F13/Em9 had specificity between GZ and DZ plants. Furthermore, the primer combinations were used to verify 141 plants in F2 population and one-way ANOVA and chi-square test were carried out. It was found that all the primers could amplify specific bands.

(1) Amplification of primer Me8/Em2 in 141 individual plants of F2 population revealed that there were 2 specific bands Me8/Em2-1 and Me8/Em2-2 in the region of 700~800 bp (the arrow in Figure 3 indicates the difference band).

Figure 3 Amplification of primer Me8/Em2 in F2 population (The arrow is different band)
Note: M: 100 bp ladder marker; A: W455; B: Xinliang 52; C: High sugar pool; D: Low sugar pool; E: High juice pool; F: Low juice pool; The other is F2 individuals
The average juice yield of a single plant with Me8/Em2-1 was 4.9% higher than that without this band, which explained 16.0% of the total variation. The average juice yield of a single plant with Me8/Em2-2 was 4.7% lower than that without this band, which explained 19.1% of the total variation. It can be inferred that this marker was linked to the gene or QTL that controlled juice yield (Figure 3; Table 1)

Me8/Em2-1 was found to be 727 bp in size by sequencing. It was named M8E2-J727 and by BLAST, it was found that there was a 99% consistency between a sequence of 257 bp in size of M8E2-J727 and a sequence of the mRNA of sorghum bicolor unknown functional protein. There was a high degree of consistency with No.29909723~No.29910438 interbase sequences on chromosome 6 of sorghum bicolor by comparing it with the genome sequence of sorghum. Therefore, the marker might be located near No. 29910081 base on chromosome 6.

Me8/Em2-2 was found to be 712 bp in size by sequencing. It was named M8E2-J712 and there was a 99% consistency between No. 365~No. 621 bases of it and No.1~No.257 bases of mRNA of sorghum unknown functional protein. By comparing this mRNA sequence by BLAST, the results showed that it had certain homologous regions with the mRNA sequence of oxygen capture enhancement protein in chloroplasts of many plants. It was identical with No. 29909752~No. 29910438 interbase sequences on chromosome 6 of sorghum bicolor by comparing it with the genome sequence of sorghum. Therefore, the marker might be located near No. 29910095 base on chromosome 6. It can be inferred that the marker Me8/Em2-2 might regulate the water content in sorghum stems by regulating photosynthesis and eventually influence the juice yield of the stems.

By comparing the sequences of M8E2-J712 and M8E2-J727, the results showed that they were highly consistent with each other except for the differences of some small sections of sequences between different alleles of the same sequence. Furthermore, chi-square test was carried out at F2 population segregation ratio (25:75:45) and the results showed that $X^2 (1:2:1) = 5.69$, $p = 0.058 > 0.05$ which was in line with the segregation ratio 1:2:1. Thus, it can be inferred that these two markers were different alleles in one gene locus and were codominant markers.

(2) It was found that Me8/R12 could amplify a marker about 250 bp. The average juice yield of a single plant with this band was 3.7% lower than that without this band, which explained 16.7% of the total variation (Table 1). It was found to be 241 bp in size by sequencing and named M8R12-J241. By BLAST, it was found that there was a 85% consistency between the sequences of this section and a section of sequence of sorghum bicolor SB_Bbc0169M22 and SB_Bbc0188M08 clones and there was a 90% consistency between the sequences and a section of sequence of sorghum bicolor SB_Bbc000-5H14 clone. It was identical with No.14289504~No.14289744 interbase sequences on chromosome 6 of sorghum bicolor by comparing it with the genome sequence of sorghum. Therefore, the marker might be located near No. 142896245 base on chromosome 6.

(3) It was found that F13/Em9 could amplify a band about 150 bp. The average juice yield of a single plant with this band was 3.7% lower than that without this band, which explained 11.4% of the total variation (Table 1). It was found to be 150 bp by sequencing and named F13E9-J150. By comparing it with the genome sequence of sorghum by BLAST, it was found that there was a high degree of consistency with No. 38927145 ~ No. 38926996 interbase sequences on chromosome 6 of Sorghum bicolor. The marker might be located near No. 38927071 base on chromosome 6.

2 Discussion
To be more reliable, this study selected F2:3 population to analyze the characters of sugar content and juice yield. In the distribution histogram of F2:3, there were nearly three peaks in the sugar content histogram. If the effect of a certain quantitative character on the main gene is large enough, the character in the population separated by the main gene will be obviously a mixture of multi-peak distribution. When the allele of the main gene is incomplete dominance, there will be three peaks in F2:3. Thus, it can be inferred that the sugar content belonged to a single gene with additive-dominant effects or single gene with additive effect. This was consistent with the results of Liang and Li (2002) who concluded in their study that the additive effect was the absolutely dominant factor in
sugar content of sweet sorghum stems. And this study also found a marker M3E7-S248 linked to sugar content on chromosome 7.

The marker M3E7-S248 was highly consistent with sorghum adenosine diphosphate glucose pyrophosphorylase subunit. Adenosine diphosphate glucose pyrophosphorylase is mainly located in storage organs of plants and plays a key role in the synthesis of plant starch. The activity of the enzyme is positively correlated with the rate of grain filling and starch accumulation (Jiang et al., 2002). It can be inferred that AGPase not only determined the rate of starch synthesis, but also affected the size of the sink strength (Cheng et al., 2001). This study indicated that AGPase might be related to the sugar content in the juice of sorghum stems. Thus, it would be necessary to further clone the sequences of the gene, analyze its expression pattern and genetic law, and to study its correlation with the sugar content in the juice of sorghum stems.

Juice yield is an important indicator of the qualitative traits of sweet sorghum which is always ignored by people. Only when both the sugar content and juice yield are high, can it indicate that the absolute sugar content is high. It can be seen that there are three peaks in juice yield histogram and the distribution pattern is not the standard form 1:2:1 with no obvious peak effect. It can be inferred that the juice yield was a qualitative trait controlled by poly-genes. This indicated that the genetic basis of juice content was relatively complex, which was consistent with the previous research results that juice content was easily affected by environmental conditions, and the heritability was low (Cao and Pang, 2009) and also the results by Ma and Xu (1989) and Li and Ma (1993) that juice yield was a qualitative trait controlled by poly-genes.

According to the results of the experiment, the sequences of markers linked to sugar content and juice yield can be further cloned and the expression pattern and genetic law can be analyzed. It might provide references for the studies on molecular markers of sugar content and juice yield of sweet sorghum in the future.

3 Materials and Methods

3.1 Materials

The parental materials used in this experiment were provided by Key Laboratory of Genetics and Breeding in Department of Agriculture of Tianjin Agricultural University. The experiment were conducted using grain sorghum “Xinliang 52” with lower sugar content and juice yield as the female parent and sweet sorghum “W455” with higher sugar content and juice yield as the male parent to generate F1 seeds after hybridization. F2 population was generated after the inbreeding of F1. The F2 generation was sown by ear-to-row method and the F2:3 population was generated (141 plants).

3.2 Measure of sugar content and juice yield

According to the standard of 10 F2 plants per row, the total fresh weight of the 10 plants was weighed. The 10 plants were mixed and squeezed and the total juice weight of the 10 plants was weighed. The sugar content of the mixed juice was measured with reference to 3,5-dinitrosalicylic acid colorimetric method by Zhang et al. (2005). The system was scaled down to 1/1000 of the original. The formula of juice yield was: juice yield = total juice weight / total fresh weight.

3.3 Extraction of genomic DNA

Taking “W455” (P1), “Xinliang 52” (P2) and their hybridized single plants of F2 generation as materials, the experiment took young leaves before heading for extraction of genomic DNA from these two parents and 141 single plants of F2 generation by improved CTAB method (Zhang et al., 2012).

3.4 Establishment of high and low sugar, high and low juice DNA pools

According to the sugar content and juice yield of F2:3 segregated population, 7 single plants with high sugar content were selected from F2 population, and their DNA was mixed with the same amount to establish the high sugar DNA pool (GT). 7 single plants with low sugar content were selected to establish the low sugar DNA pool (DT). 7 single plants with high juice yield were selected to establish the high juice DNA pool (GZ). And 7 single
plants with low juice yield were selected to establish the low juice DNA pool (DZ).

3.5 Synthesis of SRAP primers
The primer was designed with reference to the primer sequences published by Ferriol et al. (2003) and Li et al. (2003). The primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd. (Table 2).

Table 2 List of SRAP primers used in this experiment

<table>
<thead>
<tr>
<th>Forward primer</th>
<th>Sequence (5'-3')</th>
<th>Reverse primer</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Me1</td>
<td>TGAGTCCAAACCGGATA</td>
<td>Em1</td>
<td>GACTGCGTACGAATTAAT</td>
</tr>
<tr>
<td>Me2</td>
<td>TGAGTCCAAACCGGAGC</td>
<td>Em2</td>
<td>GACTGCGTACGAATTTGC</td>
</tr>
<tr>
<td>Me3</td>
<td>TGAGTCCAAACCGGAAT</td>
<td>Em3</td>
<td>GACTGCGTACGAATTTGAC</td>
</tr>
<tr>
<td>Me4</td>
<td>TGAGTCCAAACCGGACC</td>
<td>Em4</td>
<td>GACTGCGTACGAAATTAAC</td>
</tr>
<tr>
<td>Me5</td>
<td>TGAGTCCAAACCGGAAG</td>
<td>Em5</td>
<td>GACTGCGTACGAATTTGCA</td>
</tr>
<tr>
<td>Me6</td>
<td>TGAGTCCAAACCGGAAC</td>
<td>Em6</td>
<td>GACTGCGTACGAATTTCCA</td>
</tr>
<tr>
<td>Me7</td>
<td>TGAGTCCAAACCGGAGC</td>
<td>Em7</td>
<td>GACTGCGTACGAATTTACC</td>
</tr>
<tr>
<td>Me8</td>
<td>TGAGTCCAAACCGGACT</td>
<td>Em8</td>
<td>GACTGCGTACGAATTTCA</td>
</tr>
<tr>
<td>Me9</td>
<td>TGAGTCCAAACCGGAGG</td>
<td>Em9</td>
<td>GACTGCGTACGAATTTGTA</td>
</tr>
<tr>
<td>Me10</td>
<td>TGAGTCCAAACCGGAAA</td>
<td>Em10</td>
<td>GACTGCGTACGAATTTGTC</td>
</tr>
<tr>
<td>F11</td>
<td>GTAGCACAAGCCGGAGC</td>
<td>R11</td>
<td>CGCACGTCGATTTAATC</td>
</tr>
<tr>
<td>F12</td>
<td>GTAGCACAAGCCGGACC</td>
<td>R12</td>
<td>CGCACGTCGATTTCCA</td>
</tr>
<tr>
<td>F13</td>
<td>CGAATTCGTACGGGATA</td>
<td>R13</td>
<td>CGTAGCGCGTCAATTATG</td>
</tr>
<tr>
<td>F14</td>
<td>CGAATTCGTACGGGAGC</td>
<td>R14</td>
<td>CGTAGCGCGTCAATTACC</td>
</tr>
<tr>
<td>F15</td>
<td>CGAATTCGTACGGGCAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F16</td>
<td>CGAATTCGTACGGGAAT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.6 SRAP reaction system
The reaction system of 20μL SRAP-PCR: 1 ng/μL genome DNA, 0.25 μmol/L forward primer and reverse primer, 0.04 U/μL Taq polymerase, 0.2 mmol/L dNTPs and 1.5 mmol/L MgCl2.

3.7 SRAP reaction procedure
The PCR amplification procedure: 94°C 5 min, 94°C 1 min, 35°C 1 min, 72°C 2 min 15 s, 5 cycles; 94°C 1 min, 50°C 1 min, 72°C 1 min 15 s, 35 cycles; 72°C 10 min. After PCR reaction, 2% agarose gel was used to detect.

3.8 Data statistics and analysis
Distribution histograms of sugar content and juice yield were portrayed respectively by SPSS software and one-way ANOVA was carried out.

3.9 Recovery, purification, cloning, sequencing and comparison of target bands
The agarose gel purification kit and cloning kit of Sangon Biotech (Shanghai) Co., Ltd. were used to purify by gel slices and clone, and sequenced by Sangon Biotech. The tested sequences were compared with those in nucleotide sequence database in BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) on NCBI website (http://www.ncbi.nlm.nih.gov/).

Authors’ contributions
LJW, CQL and LOJ were the executors of design and research of this study; LJW, CP and CQL were responsible for data analysis and first draft writing; LOJ, LJP and DXF participated in experiment design and result analysis; SSJ, PZY, GJM and LF were the persons in charge, conceiving the experiment and directing experiment design, data analysis, paper writing and revision. All the authors have read and agreed with the final manuscript.

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