

Quantitative Trait Locus (QTL) Mapping of Sugar Yield-Related Traits in Sugar Beet (*Beta vulgaris* L.)

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Received: 18 February 2018 / Accepted: 4 June 2018 / Published online: 13 June 2018
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Abstract Quantitative trait locus (QTL) mapping of sugar yield-related traits can promote the discovery of new sugar yield-related genes. Subsequently, marker-assisted selection (MAS) can be used to breed new high-yield sugar beet varieties. In this study, we observed the F_1 population (219 individuals) from a cross of *3a* (high-yield, low-sugar, diploid, monogerm, sterile line) and *3b* (low-yield, high-sugar, diploid, polyembryonic, pollinated line) parents located in Gaomi City, Shandong Province, China. A total of four traits (root length, root perimeter, root weight, and sugar content) exhibited a normal distribution. Based on a high-density genetic map, including 3287 specific-length amplified fragment markers and nine linkage groups (LGs) with an overall genetic distance of 1554.64 cM, a total of 32 QTLs were identified for the four aforementioned traits.

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s12355-018-0632-0>) contains supplementary material, which is available to authorized users.

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The QTLs were distributed on LG2, LG3, LG5, LG7, and LG9. The root length was mapped to six regions of LG2. The phenotypic variance explained (PVE) ranged from 6.30% to 8.03%. The root perimeter was mapped to five regions of LG5 and 12 regions of LG7. The largest PVE was on LG5 (7.23%). The root weight was mapped to two regions of LG3 and three regions of LG7. The four sugar content-related QTLs located on LG5 and LG9 had a threshold logarithm of odds (LOD) value of 4.35 and a max PVE of 10.13%, indicating a potentially important QTL for future gene cloning. Using trait-based QTL mapping and chromosomal marker distribution data, we identified 3690 candidate genes including 191 root length, 918 root perimeter, 409 root weight, and 2172 sugar content genes. Our results provide valuable information for additional research in fine mapping, gene functional analysis, pyramid breeding, and MAS.

Keywords High-throughput technology · Specific-length amplified fragment · QTL · Sugar beet

Introduction

Sugar beet (*Beta vulgaris* L., $2n = 18$) is one of the most important sugar crops worldwide and accounts for approximately 25% of global sugar production. Root yield and sugar content are two major economic traits of the sugar beet. Sugar beet breeders have strived to improve the root yield and sugar content with an ultimate goal of increasing sugar yield. However, the root yield and sugar content of the sugar beet are negatively correlated with one another. These traits are controlled by multiple genes and influenced by environmental factors. Therefore, it is necessary to construct a high-density genetic map to determine

the quantitative trait loci (QTLs) of root yield and sugar content. These data lay a foundation for future beet breeding involving the introduction or pyramiding of high-yield and high-sugar genes.

QTL mapping depends on the method of genetic map construction. The earliest genetic maps of sugar beets were established using morphological and isozyme markers, as well as random-amplified polymorphic DNAs (RAPDs), restriction fragment length polymorphisms (RFLPs), amplified fragment length polymorphisms (AFLPs), and expressed sequence tags (ESTs) (Barzen et al. 1995; Halldén et al. 1996; Schondelmaier et al. 1996; Grimmer et al. 2007; Laurent et al. 2007). Molecular marker-based QTL mapping and analysis were also conducted on the sugar beet. For instance, based on 110 AFLP and 25 RFLP markers, Nilsson et al. (1999) identified five QTLs on linkage groups (LGs) 2, 3, and 9 for *Cercospora* leaf spot resistance. Grimmer et al. (2007) mapped a rhizomania resistance gene (RZ4), also on chromosome 3, using AFLPs, single nucleotide polymorphisms (SNPs), and RAPDs. Schneider et al. (2002) used ESTs, AFLPs, and RFLPs to map the sugar content, root yield, and quality-related QTLs in the sugar beet. However, the genetic maps of these QTL mapping studies have low resolution and marker saturation; thus, the accuracy and quantity of the QTLs are limited. Reduced representation genome sequencing (RRGS) is a rapid and cost-effective strategy for high-throughput SNP discovery and genotyping. Specific-length amplified fragment sequencing (SLAF-seq) is a recently developed approach based on RRGS and high-throughput pair-end sequencing (Van Geyt et al. 1990; Pillen et al. 1992; Wagner et al. 1992). This method has been used to construct genetic maps for many species. So far, few studies have mapped the QTLs of traits in the sugar beet using specific-locus amplified fragment sequencing (SALF-seq).

This study was the first to generate SNP primers for the sugar beet using a high-throughput technology platform. In addition, we obtained a large number of chromosome fragments using SLAF-seq technology. A high-density genetic map of the sugar beet was constructed, and QTL mapping analysis was performed on the main traits (i.e., root yield and sugar content).

Materials and Methods

Materials

In April 2015, we obtained an F_1 population of 219 individuals from a cross of 3a (high-yield, low-sugar, diploid, monogerm, sterile line) and 3b (low-yield, high-sugar, diploid, polyembryonic, pollinated line) parents from the

Sugar Beet Institute Trial Site of Hulan District (126°58'E, 45°9'N), Harbin City, Heilongjiang Province, China. In July 2015, the mature F_1 seeds were harvested and directly sown every 0.25 m in 10 m long by 0.67 m wide rows at the Gaomi Test Site (119°51'E, 36°30'N), Shandong Province, China. We provided consistent weed and pest control measures. We chose 219 F_1 individuals, in addition to the two parents, for leaf sampling, genomic DNA extraction, and genotyping.

Methods

Field Trait Survey and Data Analysis

In mid-December 2015, a total of 219 F_1 plants and both parents were surveyed for the following traits separately: root length, root perimeter, root weight, and sugar content per plant. After washing, root length and root perimeter were measured using a tape measure. An electronic crane was used for root weight measurements. A brix meter (ATAGO, Japan) was used for sugar content measurement. We sampled the heart portion of the sugar beet (top of root to the tail) and extracted fluid via a press. The sugar content is equal to the brix percentage multiplied by 0.85. The normal distribution and correlation analysis were performed using SPSS 19.

DNA Extraction and High-Throughput Sequencing

On September 20, 2015, fresh leaf samples were collected from a total of 219 plants. The samples were dipped in liquid nitrogen, and DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method. DNA concentrations were measured and adjusted to equal quantities.

Sequencing was performed using the improved SLAF-seq method (Sun et al. 2013). The number of restriction sites was determined by predicting the different enzyme sites within the sugar beet reference genome (Dohm et al. 2014) using the digestion prediction Perl script (Biomarker Technology Company; Beijing, China). The F_1 samples were subjected to a double digestion (HaeIII and Hpy166II; New England Biolabs; Beverly, MA, USA). SLAF products that ranged from 450 to 500 bp (including the adapter sequence indexes and adaptors) were excised. After dilution, the samples were sequenced using HiSeq 2500 PE100 (Illumina, Inc.; San Diego, CA, USA).

SLAF marker identification and genotyping were performed using procedures described by Sun et al. (2013). Briefly, low-quality reads (quality score < 20e) were excluded. Next, the raw reads were sorted individually according to their duplex barcode sequences. After the

barcodes and terminal 5 bp positions were trimmed from each high-quality read, the clean reads from the same sample were mapped onto the *Beta vulgaris* genome sequence using BWA software (Li and Durbin 2009). Only SLAFs with 2–4 alleles were identified as polymorphic and considered potential markers. All polymorphic SLAFs were regarded as having eight segregation patterns, and all patterns except “aa × bb” were used for genetic map construction of the F_1 population.

Linkage Map Construction

Marker loci were first partitioned into LGs based on their locations in the *Beta vulgaris* genome. Next, the modified logarithm of odds (MLOD) scores between markers was calculated to further confirm the robustness of markers for each LG. HighMap software was utilized to order the SLAF markers and correct genotyping errors (Liu et al. 2014). The initial map order was obtained, and error correction was performed using the SMOOTH algorithm (van Os et al. 2005). The k-nearest neighbor algorithm was used to account for null genotypes, and the maximum likelihood method was used to integrate the skewed markers. The genetic distance was estimated by the Kosambi mapping function (Kosambi 1943).

QTL Analysis

In combination with the graphic and fractal data of the nine LGs and four quantitative traits, QTL mapping was conducted using the interval mapping (IM) model in MapQTL V5.0 (Van Ooijen 2004). We performed the permutation test (PT) with a threshold value of 2.5 or 3. The significance thresholds were determined using 1000 permutations. The percentage of phenotypic variance explained (PVE) by each QTL (expl.%) was calculated based on the population variance found within the segregation population. According to the QTL results of the four economic traits and chromosomal markers, we had identified candidate mRNAs in the region. Gene function annotation and enrichment analysis were performed in the region. The QTL name is notated as, “Q + triad abbreviation + LG

number”; different QTLs within the same LG are expressed numerically.

Results

Phenotypic Analysis

The statistical analysis of the four economic traits in the parents and F_1 population revealed that the root yield and sugar content of the parents were considerably different. The mean values of root length, root perimeter, root weight, and sugar content were 17.75, 21.90 cm, 866.09 g, and 14.55%, respectively. The coefficient of variation (CV) of all traits ranged from 7.90 to 56.79%. The root weight displayed the highest value, whereas sugar content had the lowest value. The absolute values of both skewness and kurtosis for all traits, except root weight, were less than 1. Additionally, the P values from the normality test were larger than the threshold (0.05) for all traits, except for root weight, indicating satisfactory results for QTL detection (Table 1). In general, the F_1 population showed a normal distribution for the four main traits, which met the QTL mapping requirements (Supplementary Fig. 1 to Supplementary Fig. 4).

Data Analysis and QTL Mapping

Based on the high-density genetic map, which spanned a total of 1554.64 cM and had an average inter-marker distance of 0.47 cM (Wang et al. 2018), we identified the QTLs underlying the four sugar yield-related traits. The threshold of the logarithm of odds (LOD) scores for evaluating the statistical significance of QTL effects was determined using 1000 permutations in the MapQTL V5.0 program (Van Ooijen 2004). According to the intervals with LOD scores above 2.5, 32 QTLs were detected for the four traits of interest (Table 2). The root length was mapped to six regions of LG2, most of which were located between 78 and 90 cM. A total of 15 markers were mapped with a PVE of 8.03%. The root perimeter was mapped to LG5 and LG7. The regions with the greatest effect were

Table 1 Statistics analysis of sugar yield-related traits of parents and F_1 population

Traits	Parents				F_1 population				
	3a	3b	Range	Mean	SD	Skewness	Kurtosis	CV (%)	P value
Root length (cm)	22	16	9.5–30	17.15	3.53	0.32	0.29	20.58	0.459
Root perimeter (cm)	26.5	23	13.5–17.3	21.90	4.24	0.39	– 0.13	19.36	0.171
Root weight (g)	1350	900	125–3000	866.09	491.87	1.19	1.80	56.79	0.025
Sugar content (%)	12.4	18.8	11.4–17.3	14.55	1.15	– 0.27	– 0.20	7.90	0.071

“ $P > 0.05$ ” indicates a normal distribution

Table 2 The characteristics of QTLs associated with sugar yield traits

QTL names	Traits	LOD value	Linkage group	Start (cM)	End (cM)	Marker number	PVE (%)
QRL2-1	Root length	3.06	2	37.09	37.36	2	6.30
QRL2-2	Root length	3.13	2	47.54	47.54	2	6.40
QRL2-3	Root length	3.08	2	49.32	50.41	4	6.30
QRL2-4	Root length	3.14	2	50.41	50.71	3	6.40
QRL2-5	Root length	3.35	2	53.77	53.77	2	6.80
QRL2-6	Root length	3.85	2	78.96	90.29	15	8.03
QRP5-1	Root perimeter	3.24	5	40.66	47.31	18	6.82
QRP5-2	Root perimeter	3.11	5	87.70	87.70	2	6.30
QRP5-3	Root perimeter	3.11	5	88.62	88.62	8	6.30
QRP5-4	Root perimeter	3.23	5	92.19	96.06	9	6.58
QRP5-5	Root perimeter	3.57	5	97.91	100.23	6	7.23
QRP7-1	Root perimeter	3.13	7	30.27	30.53	3	6.47
QRP7-2	Root perimeter	3.28	7	41.51	42.70	6	6.70
QRP7-3	Root perimeter	3.09	7	60.40	63.19	5	6.30
QRP7-4	Root perimeter	3.33	7	82.58	82.90	2	7.20
QRP7-5	Root perimeter	3.17	7	94.83	96.21	6	6.50
QRP7-6	Root perimeter	3.30	7	104.69	104.69	6	6.70
QRP7-7	Root perimeter	3.00	7	106.47	106.50	2	6.10
QRP7-8	Root perimeter	3.33	7	107.01	111.94	39	6.77
QRP7-9	Root perimeter	3.32	7	113.73	115.16	7	6.90
QRP7-10	Root perimeter	3.31	7	116.44	124.63	21	6.74
QRP7-11	Root perimeter	3.31	7	124.63	126.01	6	6.78
QRP7-12	Root perimeter	3.43	7	127.63	129.42	5	6.98
QRW3-1	Root weight	2.63	3	132.10	138.17	7	5.72
QRW3-2	Root weight	2.74	3	139.60	141.97	10	5.62
QRW7-1	Root weight	2.68	7	163.58	164.37	3	5.46
QRW7-2	Root weight	2.78	7	167.15	172.32	8	5.78
QRW7-3	Root weight	2.84	7	173.90	177.73	13	5.77
QSC5-1	Sugar content	4.39	5	39.64	40.61	3	8.87
QSC5-2	Sugar content	4.83	5	121.70	122.16	2	9.36
QSC9-1	Sugar content	4.87	9	0	5.21	31	9.72
QSC9-2	Sugar content	5.01	9	8.64	28.97	39	10.13

40–47 cM in LG5 and 107–111 cM in LG7, where a total of 39 markers were located. The PVE ranged from 6.1 to 7.23%. The root weight was mapped to LG3 and LG7. On LG3, 17 markers were located between 132.1 and 141.97 cM. On LG7, 24 markers were located between 163.58 and 177.73 cM. The PVE ranged from 5.46 to 5.78%. The sugar content was mapped according to the intervals with LOD scores greater than 4.35, which were detected as effective QTLs; these QTLs were mapped to LG5 and LG9. The most effective region was 8–28 cM on LG9 (maximum PVE of 10.13% among all the QTLs), where a total of 39 markers were located (Figs. 1, 2, 3, 4, 5, 6, 7).

Candidate Gene Annotation

According to the QTL mapping results based on four traits and the distribution of chromosomal markers, we identified 3690 genes in the candidate region, including 191 root length, 918 root perimeter, 409 root weight, and 2172 sugar content genes (Table 3). We found 143 of 191 root length candidate genes with annotation information, among which 49, 89, 16, 96, and 143 were annotated to the COG, GO, KEGG, SwissProt, and Nr databases, respectively. We found 721 of 918 root perimeter candidate genes with annotation information, among which 196, 320, 53, 416, and 721 had annotation information in COG, GO, KEGG, SwissProt, and Nr, respectively. We found 320 of 409 root

Fig. 1 QTL analysis of the root length trait on LG2. The abscissa axis represents the genetic group; the ordinate axis represents the LOD score

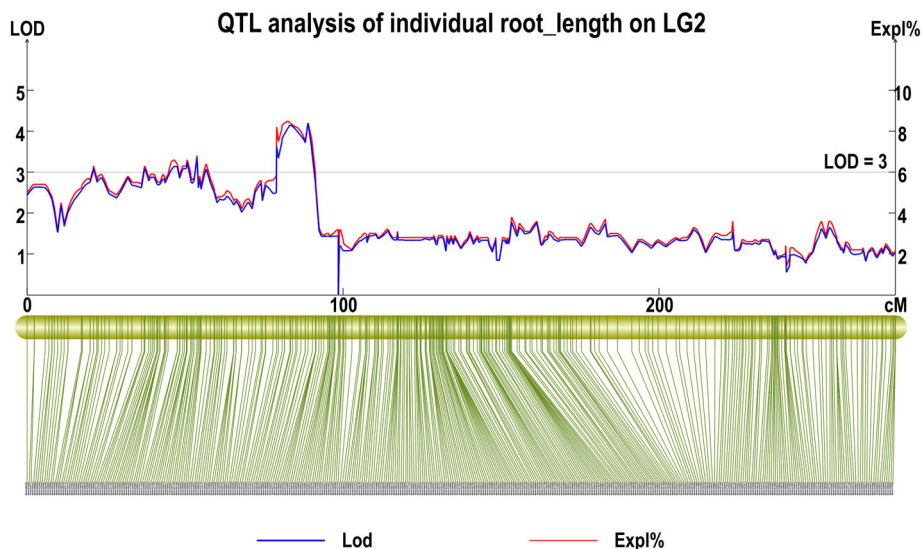


Fig. 2 QTL analysis of the root perimeter trait on LG5. The abscissa axis represents the genetic group; the ordinate axis represents the LOD score

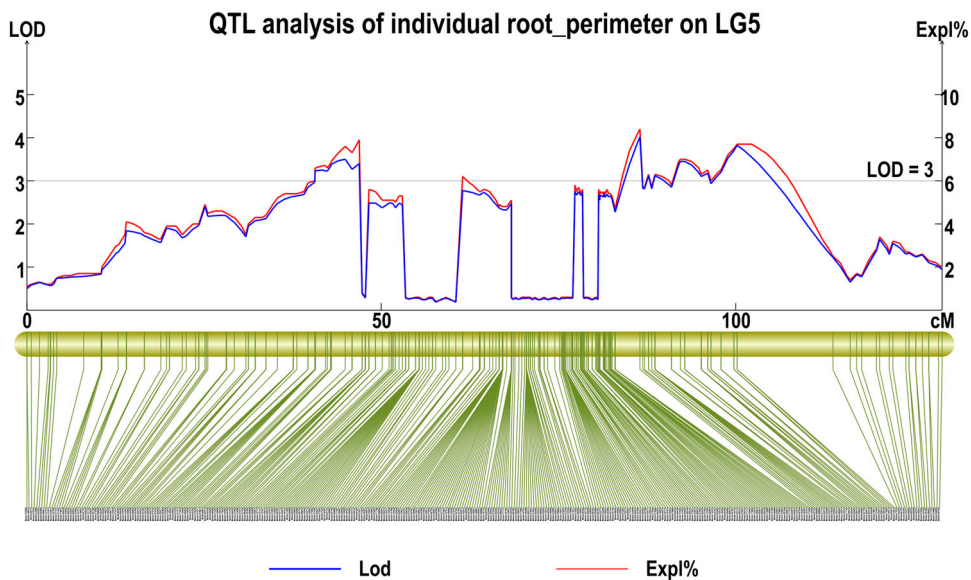


Fig. 3 QTL analysis of the root perimeter trait on LG7. The abscissa axis represents the genetic group; the ordinate axis represents the LOD score

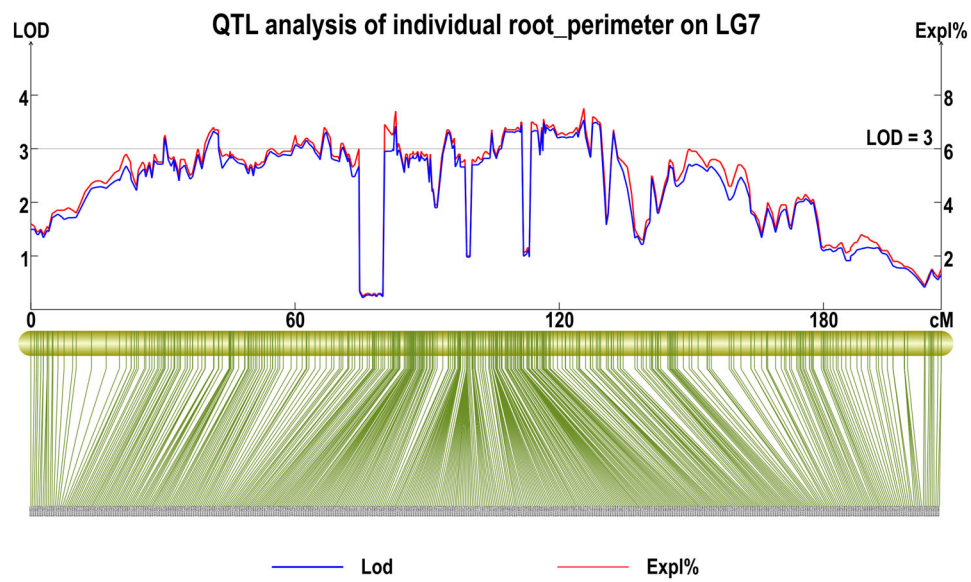


Fig. 4 QTL analysis of the root weight trait on LG3. The abscissa axis represents the genetic group; the ordinate axis represents the LOD score

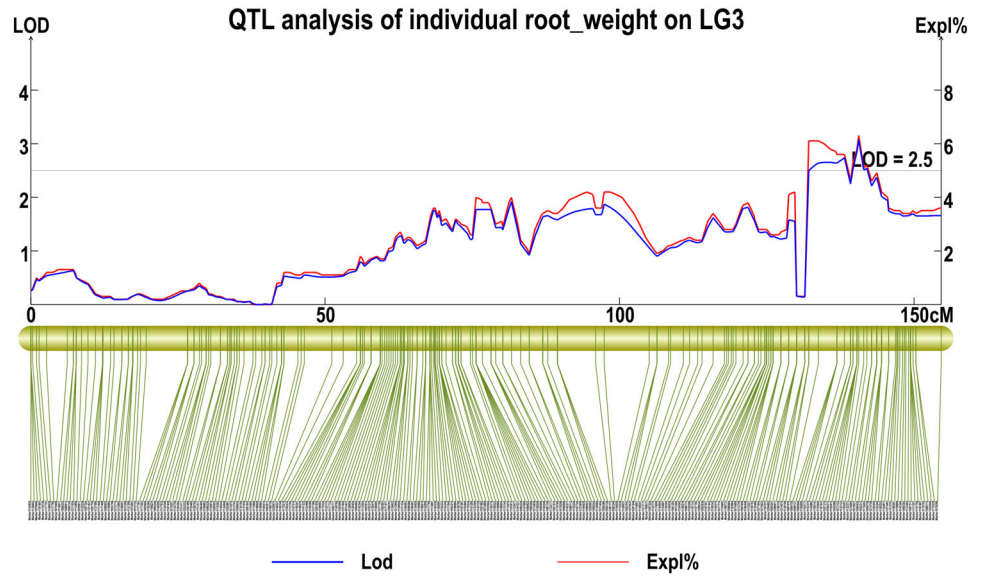


Fig. 5 QTL analysis of the root weight trait on LG7. The abscissa axis represents the genetic group; the ordinate axis represents the LOD score

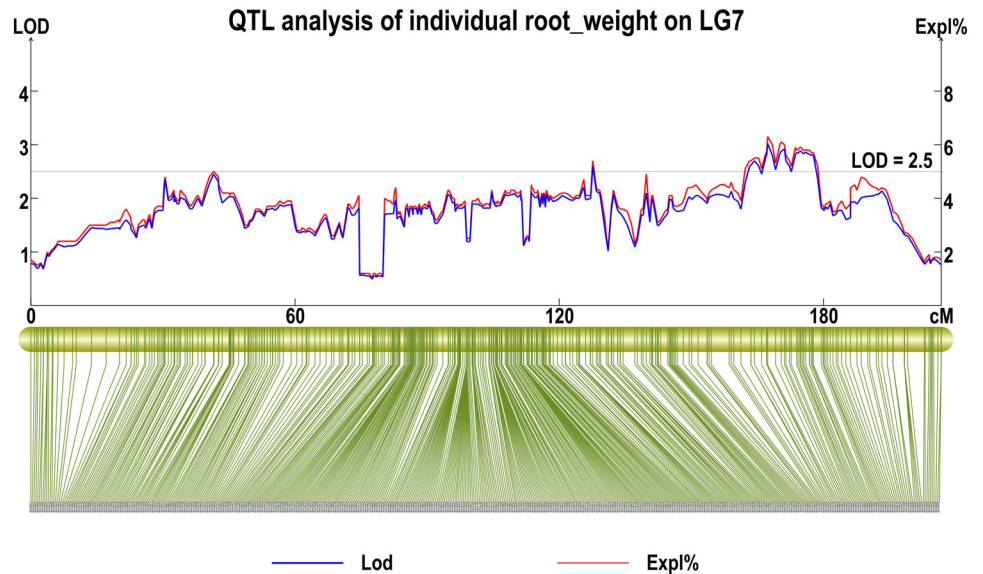


Fig. 6 QTL analysis of the sugar content trait on LG5. The abscissa axis represents the genetic group; the ordinate axis represents the LOD score

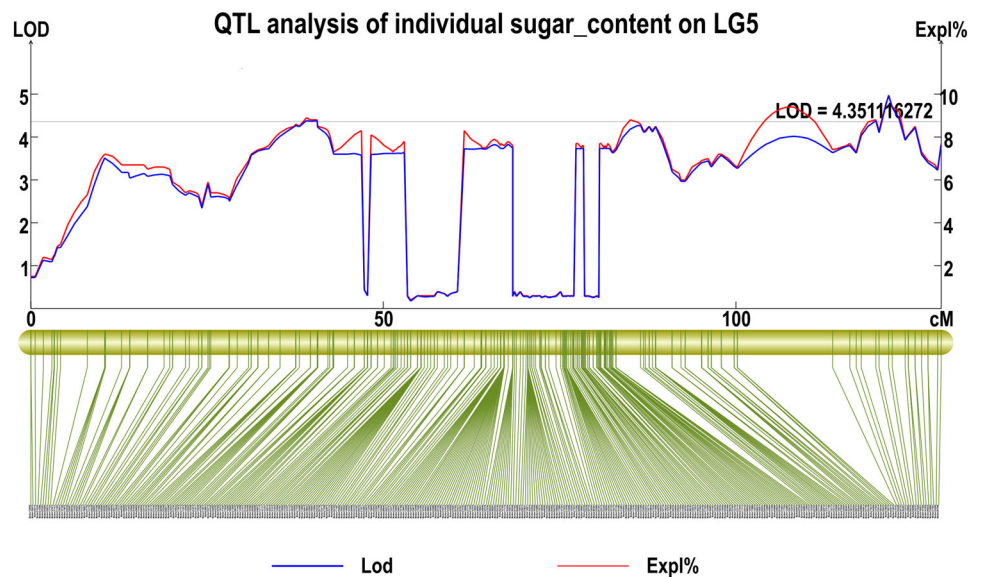
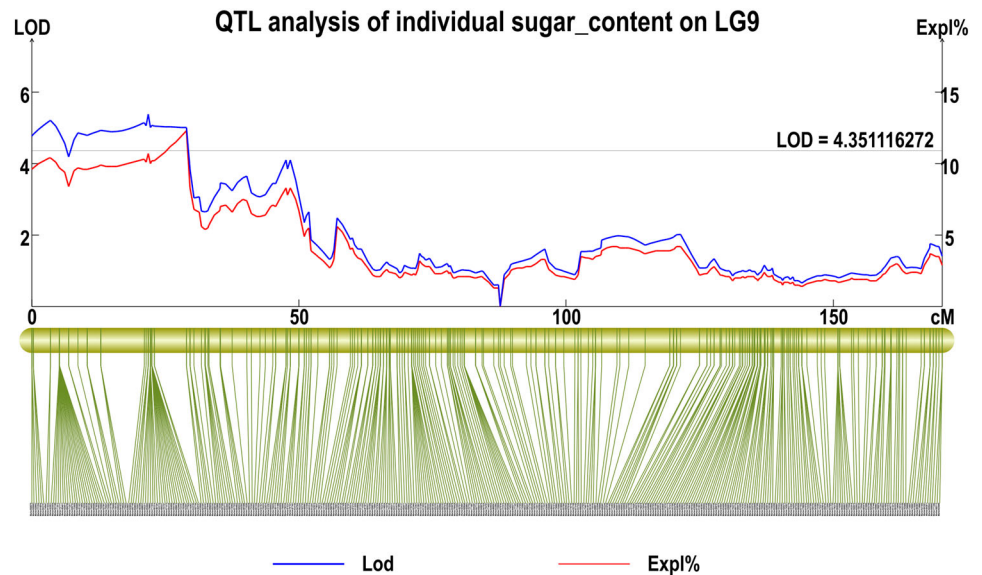


Fig. 7 QTL analysis of the sugar content trait on LG9. The abscissa axis represents the genetic group; the ordinate axis represents the LOD score



weight candidate genes with annotation information, among which 126, 164, 19, 243, and 320 were annotated to the COG, GO, KEGG, SwissProt, and Nr databases, respectively. Lastly, we found 1631 of 2172 sugar content candidate genes with annotation information, among which 639, 874, 142, 1115, and 1631 were annotated in the COG, GO, KEGG, SwissProt, and Nr databases, respectively (Table 4).

Discussion

QTL Mapping for the F_1 Beet Population Using SNP Markers

In this study, we constructed a genetic map using an F_1 sugar beet population because of the relatively high genomic heterozygosity of the parents and the normally distributed major economic traits. These conditions can be proven by our SLAF-seq data. Notably, only approximately 42.18% (20,449/48,478) of the polymorphic SLAF markers were genotyped as “aa × bb” based on the parental SLAF-seq results, which were only suitable for genetic map construction for the homozygous parents. Thus, the F_1 population was more suitable than the F_2 population for the sequencing and mapping of the sugar beet, which is a highly out-crossed crop. In fact, many crops use F_1 populations for genetic and QTL mapping. Zhu et al. (2015) constructed a high-density genetic map using SLAF-seq and identified a QTL for anthracnose resistance in walnut F_1 populations. Wang et al. (2015) constructed cherry linkage maps and identified a QTL for trunk diameter using F_1 populations.

Genetic Map Construction and QTL Mapping

The density and quality of genetic maps are important because they allow the detection of reliable and concise QTL confidence intervals, as well as the identification of functional genes within those intervals.

Previous genetic map construction and QTL mapping studies were not conclusive due to an insufficient number of markers, an inadequate density, large map distances, and few mapped QTLs. For example, Barzen et al. (1992) used RFLP markers to construct a genetic linkage map of the sugar beet to segregate populations of this crop; the map covered 540 cM and contained 9 LGs with 92 genomic markers. In 1996, Halldén et al. published a high-density sugar beet RFLP map covering 621 cM including 413 markers with an average distance of 1.5 cM between markers. In our study, QTL mapping was based on high-throughput genetic mapping (Wang et al. 2017), which spanned a total of 1554.64 cM, with an average inter-marker distance of 0.47 cM. The size of this map is larger than previous maps, which ranged from 446 cM (Schneider et al. 2002) to 1399.88 cM (Wang et al. 2014). The sugar beet genetic map has the highest molecular marker density reported to date.

Previous QTL mapping analysis in sugar beets focused on disease resistance genes. For instance, Setiawan et al. (2000) performed QTL mapping and detected loci on chromosomes 3, 4, 7, and 9 for resistance to *Cercospora* leaf spot. Janssen et al. (2003) mapped QTLs for powdery mildew resistance genes in the sugar beet and identified loci for monogenic resistance. Gidner et al. (2005) conducted QTL mapping using WB41 (a rhizomania-resistant sugar beet strain) and found a new rhizomania resistance gene, RZ3, on chromosome 3. Grimmer et al. (2008)

Table 3 Basic information of mRNA in the trait candidate region

QTL names	Traits	Linkage group	Start	End	mRNA number
QRL2-1	Root length	2	3,531,382	3,771,913	26
QRL2-2	Root length	2	4,901,573	5,060,619	9
QRL2-3	Root length	2	5,238,568	5,445,950	23
QRL2-4	Root length	2	5,314,336	5,470,055	18
QRL2-5	Root length	2	6,202,727	6,420,414	17
QRL2-6	Root length	2	10,593,476	11,813,512	98
QRP5-1	Root perimeter	5	10,641,340	15,651,557	236
QRP5-2	Root perimeter	5	41,636,619	41,643,187	1
QRP5-3	Root perimeter	5	41,687,499	42,020,176	29
QRP5-4	Root perimeter	5	44,324,345	45,905,222	108
QRP5-5	Root perimeter	5	46,415,677	46,956,434	45
QRP7-1	Root perimeter	7	4,503,892	4,990,481	24
QRP7-2	Root perimeter	7	7,193,662	7,717,448	23
QRP7-3	Root perimeter	7	11,587,592	11,958,208	12
QRP7-4	Root perimeter	7	14,208,757	14,245,119	1
QRP7-5	Root perimeter	7	17,516,050	17,600,154	6
QRP7-6	Root perimeter	7	23,333,510	23,811,610	11
QRP7-7	Root perimeter	7	23,965,553	25,608,637	56
QRP7-8	Root perimeter	7	23,994,316	29,437,232	190
QRP7-9	Root perimeter	7	28,900,030	29,934,255	53
QRP7-10	Root perimeter	7	30,177,566	31,885,326	69
QRP7-11	Root perimeter	7	30,634,113	31,885,326	50
QRP7-12	Root perimeter	7	32,081,854	32,176,184	4
QRW3-1	Root weight	3	23,370,062	24,814,505	137
QRW3-2	Root weight	3	24,151,273	25,170,010	111
QRW7-1	Root weight	7	38,875,366	39,059,044	15
QRW7-2	Root weight	7	39,206,122	39,691,409	39
QRW7-3	Root weight	7	39,788,362	40,529,438	107
QSC5-1	Sugar content	5	10,599,611	11,833,219	71
QSC5-2	Sugar content	5	51,550,548	51,635,921	14
QSC9-1	Sugar content	9	35,900,850	45,199,933	1,052
QSC9-2	Sugar content	9	36,113,030	45,213,983	1,035

Table 4 mRNA annotation statistics

Trait name	COG	GO	KEGG	SwissProt	Nr
Root length	49	89	16	96	143
Root perimeter	196	320	53	416	721
Root weight	126	164	19	243	320
Sugar content	639	874	142	1115	1631

identified the rhizomania resistance gene, RZ5, from WB258 (a rhizomania-resistant sugar beet strain). Several studies have mapped QTLs associated with sugar beet root yield and sugar content. Weber et al. (1999, 2000) performed a QTL analysis with yield data from two segregating sugar beet populations. Wang et al. (2017) mapped

17 QTLs of root yield and sugar content on to six linkage groups in the sugar beet. In this study, 32 QTLs were detected for four important traits (root length, root perimeter, root weight, and sugar content) and 3690 genes were identified in the candidate region. Wang et al.'s map

had a lower number of QTLs than our map. Thus, our improved map is more informative and accurate.

Our paper focused on mining the QTLs responsible for underlying traits, and we believe that certain unknown factors may exert adverse effects on QTL mapping. Therefore, it is our opinion that with uniform field management and phenotyping, satisfactory QTLs can be identified using our methods.

SLAF-seq Strategy for QTL Development

The SLAF-seq strategy combines locus-specific amplification and high-throughput sequencing for large-scale SNP discovery and genotyping (Sun et al. 2013). SLAF-seq has been successfully applied in various species and used for genetic and QTL mapping in other crops. For example, Zhang et al. (2016) constructed a high-density genetic map by SLAF-seq and analyzed QTLs for boll weight in upland cotton. The high-density genetic map harbored 5521 SNP markers, which covered a total distance of 3259.37 cM; boll weight had 18 stable QTLs identified. Zhu et al. (2016) constructed a high-density genetic map of the cucumber (3057 SLAFs), which included 4475 SNP markers and detected 15 QTLs for two fruit traits. Using SLAF-seq, Jiang et al. (2015) constructed a high-density genetic map of the wax gourd from a population of 140 F_2 individuals and identified a single locus on chromosome 5 that controlled pericarp color. Mao et al. (2015), through a combination of interval mapping and single locus analysis in two genetic populations, identified 13 QTLs for seedling cold tolerance in Dongxiang (DX) wild rice. Thus, the SLAF-seq strategy proves to be an effective new tool for QTL mapping.

Conclusion

Using high-throughput sequencing technology, the present study selected candidate markers in the F_1 population (219 individuals) of the sugar beet. Based on the high-density genetic map, which spanned a total of 1554.64 cM with an average marker distance of 0.47 cM, QTLs underlying the four economic traits were identified. According to the intervals with LOD scores above 2.5, 32 QTLs were detected for these four traits. The root length was mapped to six regions of LG2, most of which were located between 78 and 90 cM; a total of 15 markers were mapped with a PVE of 8.03%. The root perimeter was mapped to LG5 and LG7, and the PVE ranged from 6.1 to 7.23%. The root weight was mapped to LG3 and LG7, and the PVE ranged from 5.46 to 5.78%. The sugar content was mapped to LG5 and LG9. The most effective region was 8–28 cM on LG9 (maximum PVE of 10.13% among all the QTLs), where a

total of 39 markers were located. According to the QTL mapping results based on four traits and the distribution of chromosomal markers, 3690 genes were identified in the candidate region, including 191 root length, 918 root perimeter, 409 root weight, and 2172 sugar content genes. Thus, QTL mapping of sugar yield-related traits may advance cloning of sugar beet genes and improve future sugar beet yields.

Acknowledgements This work was supported by Fundamental Research Fund for the Provincial Universities Basal Research Project in Heilongjiang Province (KJCXZD201714); Fundamental Research Fund for the Provincial Universities Basal Research Project in Heilongjiang Province (KJCXZD201716); The National Sugar Industrial Technology System Project (CARS-17011306); The National Sugar Industrial Technology System Project (CARS-17011004).

Author contributions HZ and HW designed the study and performed the experiments; WX and ZW performed the experiments; MW, WX, and YX analyzed the data and wrote the manuscript.

Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

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