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QTL Mapping of Leaf-Related Traits Using a High-Density Bin Map in *Brassica rapa*

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Abstract: The species *Brassica rapa* includes enormous leafy vegetables with extreme leaf morphological diversity. Leaf traits such as size, shape, weight, and ratio of the leaf blade to the petiole contribute to yield, appearance, and desirability to consumers. These leaf-related traits are controlled by quantitative trait loci (QTLs). The construction of high-density bin maps using low-coverage sequencing is a powerful method for QTL fine-mapping and gene identification. In this study, we performed whole-genome re-sequencing of Wutacai 'Zhongbaye' and Chinese cabbage 'HN53' and 150 F_2 individuals to construct a high-density bin map for QTL mapping of 11 leaf-related traits. The parental lines and F_2 population were re-sequenced at 10x and 1x coverage, respectively. A map containing 565 bin markers was constructed based on parental single-nucleotide polymorphisms and a modified sliding window approach. The total map length was 944.6 cM and the average distance of the bins was 1.65 cM. In total, 60 significant QTLs controlling 11 leaf-related traits were detected. We further identified candidate genes responsible for these complex leaf-related traits. These findings suggest that this cost-effective bin-mapping approach is capable of rapid identification of QTLs and candidate genes, and will thus facilitate the dissection of the underlying molecular basis of leaf morphological variations and accelerate the improvement of *B. rapa* vegetable breeding.

Keywords: *Brassica rapa*; bin map; quantitative trait loci; leaf traits

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1. Introduction

Brassica rapa (*B. rapa*, 2n = 20, AA) is an important leafy vegetable crop in Asia, particularly China, Japan, and Korea. *B. rapa* contains different morphotypes with extreme leaf morphological diversity, including the large curling leaves with knotted leaf surfaces and wide midribs (petioles) of Chinese cabbage, the small round flat leaves with narrow petioles of Wutacai, the smooth leaves with enlarged petioles of Pak choi, and the slender and highly serrated leaves of Mizuna [1,2]. As the leaf is the main edible organ of *B. rapa*, variations in leaf shape, size, weight, and ratio of the leaf blade to the petiole affect the yield, appearance, and desirability of these vegetables to consumers. Understanding the genetic regulation of these complex leaf-related traits is key to breeding *B. rapa* vegetable to satisfy consumer preferences.

The genetic regulation of leaf-related traits has been unraveled through quantitative trait locus (QTL) analysis in *B. rapa*. Genetic linkage maps have been constructed using different molecular markers. For example, simple sequence repeats (SSR) were used to construct genetic linkage maps for the F_2, Doubled Haploid (DH), and Recombinant Inbred Lines (RIL) populations to analyze QTLs for agronomic traits such as leaf-head traits, yield, disease resistance, and leaf color in Chinese cabbage [3–8]; linkage genetic maps were constructed using restriction fragment length polymorphism (RFLP) markers in different genetic populations of Chinese cabbage such as F_2 and RIL for the QTL analysis

of traits such as disease resistance and flowering time [5,9,10]; and, genetic linkage maps were constructed using amplified fragment length polymorphism (AFLP) markers for QTL analysis in the F_2 population for traits related to root growth, leaf-head formation, pubescence, and disease resistance [11–13]. In addition, insertion and deletion (InDel) markers have been used to construct genetic linkage maps [6,14].

A number of QTLs for leaf-related traits have been identified, including leaf size, leaf number, and leaf color. Some well-known candidate genes have been identified based on the analysis of gene functions in trait loci. For example, the genes *BrGRF5*, *BrGA20OX3*, *BrLNG1*, *BrKPP2*, and *BRASSICA RAPA FERREDOXIN-NADP(1)-OXIDOREDUCTASE1*, which control leaf shape; the genes *BrLNG1* and *BrKPP2*, which regulate leaf length (LL); *BRASSICA RAPA FERREDOXIN-NADP(1)-OXIDOREDUCTASE1*, which regulates leaf width (LW); and *BrKAN1*, *BrKAN2*, *BrREV*, *BrPNH*, and other genes related to the adaxial/abaxial polarity of leaves have been identified [1,15]. In addition, genes including *BrSAW1* and *BrTCP*, which are considered candidate genes involved in leaf-head formation in Chinese cabbage, have been identified [3,16]. Yue et al. also identified the heading candidate genes *BrPIN5* and *BrSAURs* [17]. Although the above traditional molecular markers have been widely used to develop genetic linkage maps for *B. rapa* [3,10,18], the resulting map density is still too limited for fine-mapping of complex traits of interest.

The wide availability and low cost of next-generation sequencing (NGS) technologies have allowed researchers to sequence the entire genomes of crops within a short period of time. The high-density single-nucleotide polymorphism (SNP) marker map generated from the low-coverage sequencing-based genotyping method has been applied to QTL mapping of agriculturally important traits. Li et al. constructed a linkage map containing 4253 loci using SNP markers and performed QTL analysis and found that a QTL on chromosome A07 was the main cause of stalk color variation in Zicaitai; in addition, several candidate genes associated with stalk color were identified, with *BrbHLH49* being the best candidate [19]. Similarly, Liu et al. constructed a genetic linkage map using 5392 SNPs and performed QTL analysis to identify two main QTLs associated with the main flower stalk length in Chinese cabbage [20]. However, low-coverage sequencing results in a relatively large proportion of missing data, a small percentage of SNP genotyping sequencing errors, and false-positive SNPs. Bin markers are more informative for a given population compared with conventional molecular markers, RFLP, SSR, InDel, or single SNP markers, and the sliding window approach can help to remove most of the false-positive SNPs caused by sequencing and mapping errors and can increase the accuracy of SNPs. The bin-mapping strategy based on a sliding-window approach was proven to be powerful for high-density genetic map construction and QTL mapping in crops and vegetables such as maize [21], rice [22], sorghum [23], pepper [24], blueberries [25], carrots [26], and watermelons [27]. However, there is limited published data on bin maps for QTLs in *B. rapa*. Sun et al. constructed a bin map using the *Brassica* SNP array for a small DH population (66 lines), which limited the mapping of significant QTLs [28]. Therefore, a large population of 485 F_2 plants was used to construct a map with 36 SNPs and 99 InDel markers for the analysis of QTLs linked to rosette leaf and heading traits in Chinese cabbage.

To further understand the genetic regulation of the complex leaf-related traits in *B. rapa*, 150 F_2 individuals were developed from Wutacai 'Zhongbaye' and Chinese cabbage 'HN53' in this study. SNPs from individuals were generated using whole-genome re-sequencing and used to construct a high-density linkage map using a modified sliding window approach. This study confirmed the effectiveness of the bin-mapping approach for genetically mapping 11 leaf-related traits in *B. rapa*, and further identified candidate genes regulating leaf morphology and development. This research will facilitate understanding of the genetic mechanisms of these complex leaf-related traits and molecular breeding of *B. rapa* vegetable crops.

2. Materials and Methods

2.1. Plant Materials and Phenotype Evaluation

An F_2 population consisting of 150 individuals was obtained from the selfed cross of Wutacai (ssp. *narinosa* cv 'Zhongbaye') as the male parent and Chinese cabbage (ssp. *pekinensis* cv 'HN53') as the female parent. The maternal parent 'HN53' is a heading-type Chinese cabbage that has large leaves with knotted leaf surfaces and enlarged short petioles, whereas the paternal parent 'Zhongbaye' has relatively small, smooth round leaves that are dark green in color, with narrow petioles. All plants were grown in a field trial at the experimental farm of the Chinese Academy of Agricultural Sciences in Beijing, China. In this study, a total of 150 F_2 individuals were randomly selected for the investigation of 11 leaf morphological traits (Table 1). To investigate leaf characteristics, three fully expanded outer leaves were taken from the two parents and 150 F_2 individuals and measured. Of these 11 leaf-related traits, leaf weight traits included LWT and PWT, while leaf size traits included LL, LW, PL, LA, and PA. Leaf shape can be represented by the leaf index, including the LI and the total LL divided by the PL (PI). In addition, the PAI (LA/PA) and PLTI (LWT/PWT) are important for evaluating the appearance or attractiveness of vegetables to consumers. To precisely measure LL, LW, LA, PL, and PA, images of the leaves were scanned and analyzed using ImageJ 1.46r software. The average value of three leaf traits was taken as the trait data. Pearson correlation coefficients were calculated to analyze the relationships between traits.

Table 1. Description of *Brassica rapa* leaf trait measurements.

	Trait Name	Trait Description	Units
LWT	Leaf weight	Total leaf weight	g
PWT	Petiole weight	Weight from the base of the petiole to the bottom of the lamina	g
PLTI	Ratio of total leaf weight to petiole weight	Measured by dividing LWT by PWT (LWT/PWT)	
LL	Leaf length	Length from the base of the petiole to the tip of the lamina	cm
LW	Leaf width	Width of leaves at the widest point	cm
LA	Leaf area	Total leaf surface area	cm^2
PA	Petiole area	Total petiole surface area	cm^2
PAI	Ratio of total leaf surface area to petiole surface area	Measured by dividing LA by PA (LA/PA)	
PL	Petiole length	Length from the base of the petiole to the bottom of the lamina	cm
PI	Ratio of total leaf surface area to petiole surface area	Measured by dividing LL by PL (LL/PL)	
LI	Index of the leaf	Measured by dividing LL by LW (LL/LW)	

2.2. Isolation of Genomic DNA and Re-Sequencing

Sequencing data for the parental lines 'HN53' and 'Zhongbaye' were obtained from a previous study conducted by this research group, referred to as 'sample 19' and 'sample 130' [29], which were sequenced to ~10× coverage. Total genomic DNA of 150 F_2 individuals was isolated from leaf tissues using a CTAB procedure as previously described [30], but with modifications. The extracted DNA was submitted for re-sequencing on an Illumina HiSeq 2500 using 150-bp paired-end reads under a 350-bp insert size library, generating ~1× coverage data.

2.3. Genotyping and Bin-Map Construction

The raw reads were filtered as described previously [29]. Filtered reads were aligned to the *B. rapa* reference genome (Chiifu-401-42) version 3.0. A pooled mapping approach was used to call variations in 150 F_2 individuals, and high-quality SNPs between parents were used as confident polymorphic loci [31]. Finally, ungenotyped loci were imputed based on linkage disequilibrium (LD) using the k-nearest neighbor (k-NN) algorithm [32].

A slightly modified sliding-window approach was used to identify recombination breakpoints and to construct a bin map of F_2 populations [24]. The genotype in each window was called with a window size of 1 Mb and a step size of 400 kb. The ratio of SNPs with 'HN53' and 'Zhongbaye' genotypes was calculated. When >70% of SNPs had one parental genotype, the window was called homozygous; otherwise, the window was called heterozygous. Adjacent windows with the same genotypes were combined into blocks and recombinant breakpoints were determined based on the physical locations where the genotype of the window changed. Based on the recombination breakpoint position, a physical bin map was constructed as described by Han et al. [24]. Bins were used as markers to construct a high-density genetic map using JoinMap 4.0 software [33].

2.4. QTL Analysis of Morphological Traits

All phenotyping data were used for QTL analysis in MAPQTL 4.0 software [34]. First, the interval mapping procedure was performed to detect major QTLs. A 1000× permutation test was performed for each trait to calculate the LOD threshold corresponding to a genome-wide false discovery rate of 5% ($p < 0.05$). Markers with LOD scores equal to or exceeding the threshold were used as cofactors in multiple-QTL-model (MQM) mapping. If new QTLs were detected, the linked markers were added to the cofactor list and the MQM analysis was repeated. If the LOD value of a marker dropped below the threshold in the new model, it was removed from the cofactor list and the MQM analysis was rerun. This procedure was repeated until the cofactor list stabilized.

3. Results

3.1. Variation of Leaf Morphological Traits in the F_2 Population

The maternal parent 'HN53' has large leaves with short, large petioles, while the paternal parent 'Zhongbaye' has relatively small leaves with narrow petioles. The two parental lines showed significant differences in leaf traits (Table 2). A survey of 11 leaf morphological traits in 150 randomly selected F_2 individuals revealed that 'HN53' had the highest phenotypic values for all traits except the LL/LW ratio (LI). In the F_2 population, all 11 leaf-related traits showed a continuous distribution and wide genetic variation (Table 2, Figure 1), indicating that all evaluated traits were quantitatively controlled.

Table 2. Data for 11 leaf morphological traits in the parents and the F_2 population.

	P1	P2	F_2 Means	F_2 Range
LWT (g)	110.89 ± 2.32	14.4 ± 0.44	45.62 ± 16.82	15.10–93.63
PWT (g)	51.12 ± 0.23	9.75 ± 0.58	28.85 ± 10.24	6.33–60.40
PLTI	2.17 ± 0.05	1.48 ± 0.05	1.58 ± 0.14	1.29–2.38
LL (cm)	44.09 ± 0.95	19.22 ± 0.53	33.79 ± 4.70	21.77–43.48
LW (cm)	25.04 ± 0.71	8.32 ± 0.08	17.01 ± 3.08	10.33–26.18
LI	1.76 ± 0.05	2.31 ± 0.06	2.02 ± 0.26	1.45–2.71
PL (cm)	25.15 ± 0.93	13.8 ± 0.20	20.92 ± 3.24	13.92–29.98
PI	1.75 ± 0.04	1.39 ± 0.02	1.63 ± 0.15	1.22–2.05
LA (cm^2)	782.22 ± 8.92	75.01 ± 0.89	335.68 ± 107.95	125.56–627.33
PA (cm^2)	120.36 ± 0.67	18.31 ± 0.47	57.30 ± 16.21	24.84–100.20
PAI	6.50 ± 0.16	4.1 ± 0.13	5.91 ± 1.26	3.40–9.07

Note: P1 represents Chinese cabbage (HN53) and P2 represents Wutacai. See Table 1 for trait abbreviations.

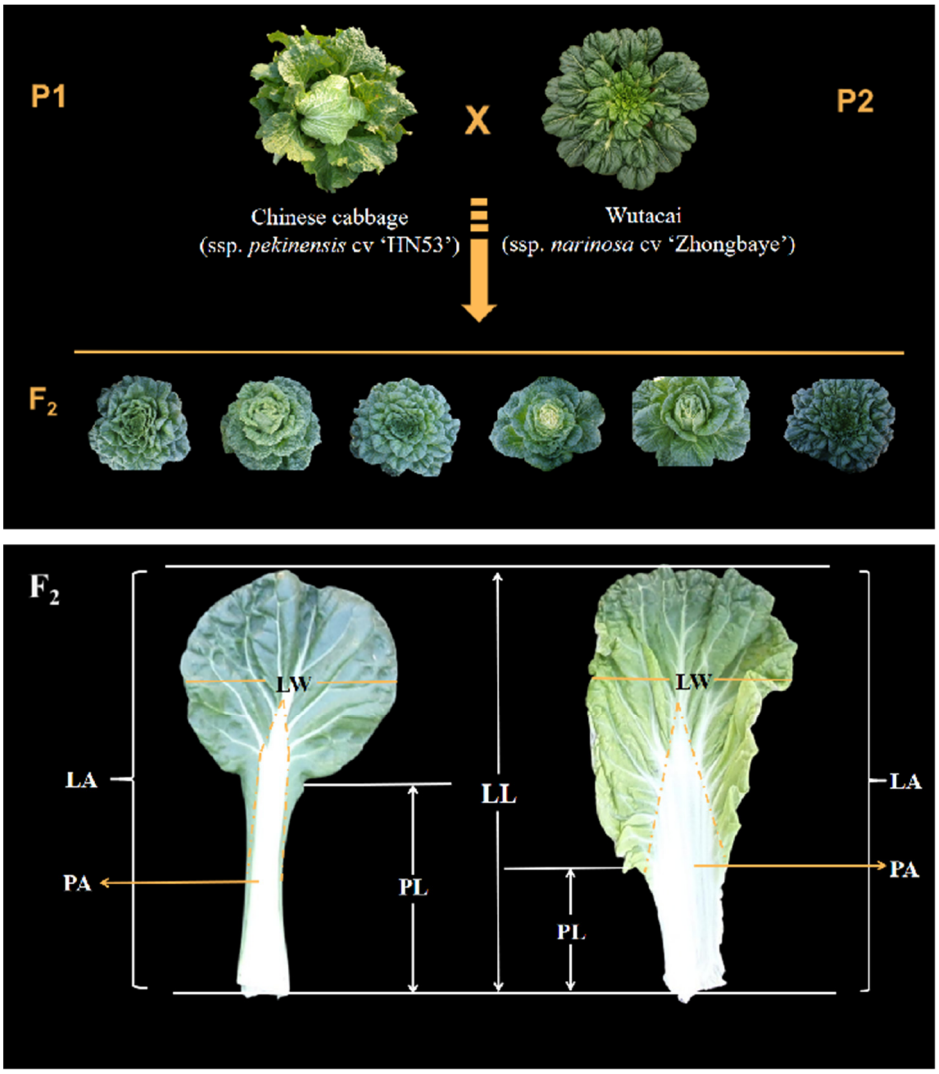

Figure 1. Morphological traits of heading and non-heading cabbage and their F_2 populations are shown on the left. The figure on the right shows two typical plant leaves as examples to describe the different traits measured in this study in relation to leaf morphology. All shapes and their descriptions are listed in Table 1. See Table 1 for trait abbreviations.

Of the 11 leaf-related traits, leaf weight traits included LWT and PWT, and leaf size traits included LL, LW, PL, LA, and PA. Leaf shape can be expressed using the leaf indices LI, PI, PAI, and PLTI. These traits and their phenotypic frequency distribution in the F_2 population and their parents are shown in Figure 2.

Most of the traits showed significant positive correlations with other traits (Figure 3). LWT, PWT, LL, LW, LA, and PA were significantly and positively correlated with each other. The highest correlation coefficient was observed between LWT and PWT (0.97). LI was positively correlated with LL and PL, but negatively correlated with the other traits. PLTI was negatively correlated with PL. PI and PAI were negatively correlated with PL. PA had a low correlation with PLTI and LI.

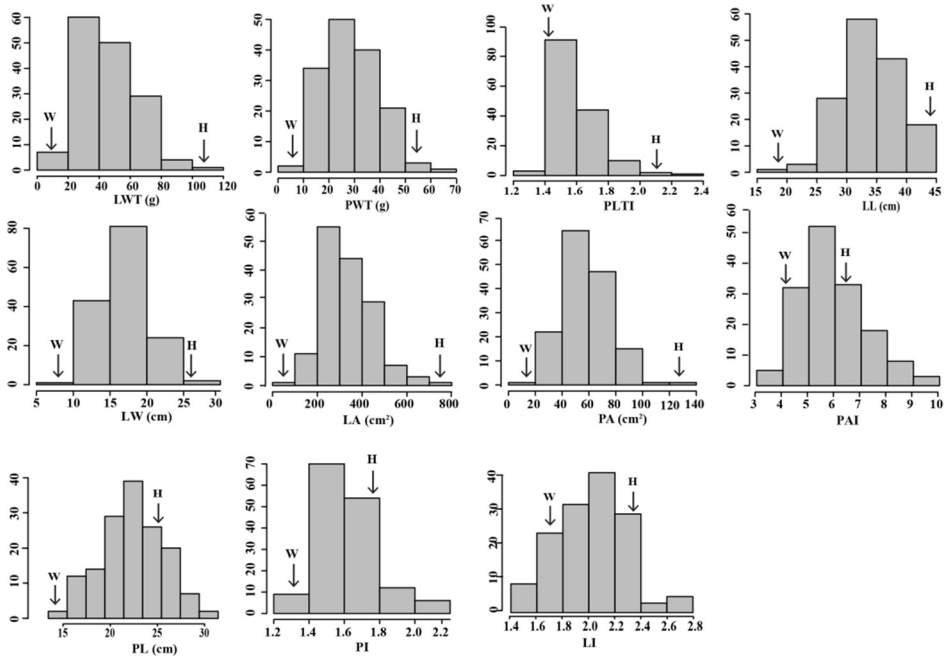

Figure 2. Frequency distributions of 11 traits in the F_2 lines and their parental lines. The vertical axis in each figure represents the number of F_2 lines. W and H represent the parental lines 'Zhongbaye' and 'HN53', respectively. See Table 1 for trait abbreviations.

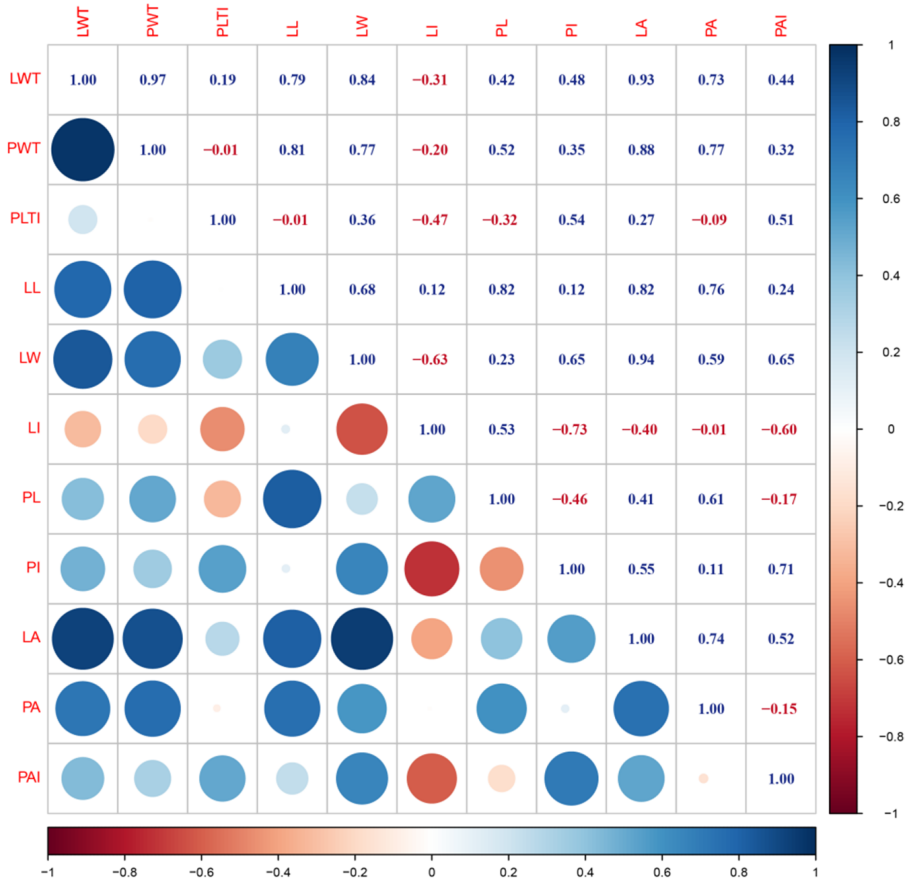

Figure 3. Correlations between morphological traits evaluated in the F_2 population. Blue represents positive correlations and red represents negative correlations. See Table 1 for trait abbreviations.

3.2. Construction of Bin Map Using Low-Coverage Sequencing

One hundred and fifty F_2 individuals were sequenced on an Illumina HiSeq 2500, generating a total of 0.3 billion 150-bp paired-end reads. After filtering out low-quality reads, 80 billion re-sequencing data, with an average of 536 Mb per F_2 individual, were obtained. This was greater than the one-fold coverage of the *B. rapa* genome of each F_2 individual (Table 3). All filtered reads were used to call SNPs using the pooled mapping method, and SNPs between parental lines were defined as confident polymorphic loci [31]. In addition, the *B. rapa* reference genome was used for SNP calling and imputation. A total of 637,647 high-quality SNPs were identified from the 150 F_2 individuals. After imputation, 636,866 high-quality SNPs with an average density of 2.15 SNPs/kb were obtained and used to construct a bin map (Table 4).

Table 3. Overview of sequence data for 150 individuals in F_2 population.

		Total	Average/Plant
Raw datas	Reads	295,559,333	1,970,396
	Bases (bp)	88,667,799,900	591,118,666
After filtering datas	Reads	268,392,401	1,789,283
	Bases (bp)	80,374,268,540	535,828,456

Table 4. Summary of SNPs detected in the F_2 population.

Chromosome	Genotyped SNPs in 150 F_2 Population	Genotyped SNP from Imputation	Chromosome Size (kb)	SNP Density (SNPs/kb)	Genetic Bins
A01	66,254	66,175	29,596	2.24	60
A02	65,193	65,128	31,443	2.07	51
A03	79,985	79,882	38,154	2.09	70
A04	44,924	44,875	21,928	2.05	40
A05	62,950	62,883	28,493	2.21	63
A06	69,416	69,307	29,168	2.38	54
A07	64,161	64,059	28,929	2.21	58
A08	52,645	52,584	22,982	2.29	45
A09	93,194	93,082	45,157	2.06	87
A10	38,925	38,891	20,726	1.88	37
Total	637,647	636,866	296,576	2.15	565

Note: A01–A10 in the table represent the ten Chinese cabbage chromosomes.

To construct the bin map using the sliding window approach, window lengths from 0.5 to 1 Mb and step sizes from 0.1 to 0.5 Mb were first tested in all chromosomes in five random individuals. A window length of 1 Mb and a step size of 0.4 Mb showed a reasonable number of recombination breakpoints per chromosome. This parameter was therefore used to construct a bin map, and a total of 4291 recombination breakpoints were obtained from the 150 F_2 individuals, with an average of 28.6 per F_2 individual. The mean number of crossovers per chromosome for the F_2 population was 2.8. All SNPs were grouped into 565 bins. The physical lengths of the bin markers ranged from 200 kb to 3.1 Mb, with an average of 449.0 kb and a median of 400 kb. In total, 88.13% of bin markers were less than 400 kb in length (Figure 4). A total of 565 bins were used to construct a genetic linkage map. Two bins (bin_246 and bin_249) were unanchored to the linkage map. The total genetic distance of the bin map was 944.6 cM for all 10 chromosomes, and the mean distance of the bins was 1.65 cM. The bin marker order was in agreement with the Chinese cabbage reference genome sequence V3.0, with a few exceptions.

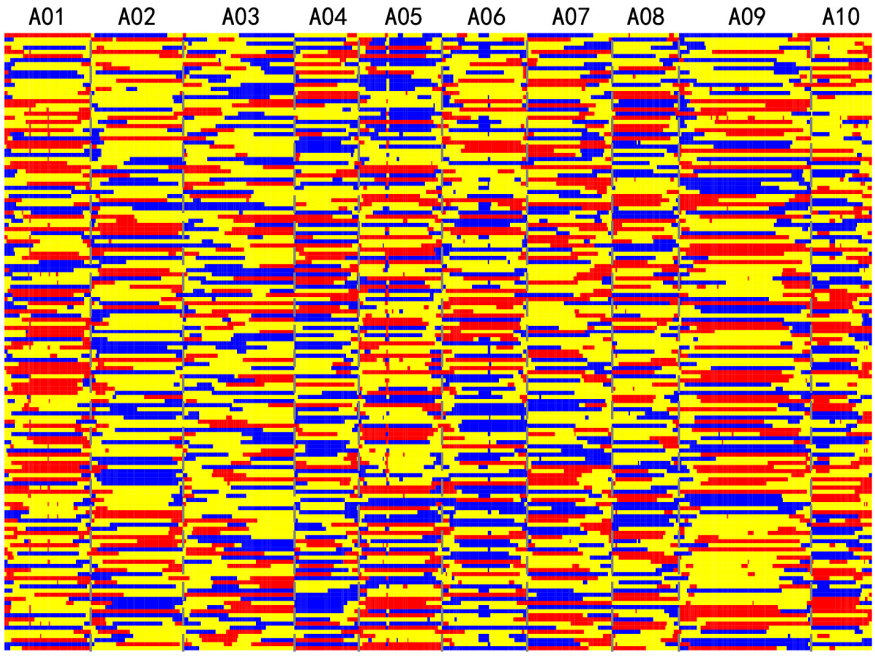

Figure 4. Recombination bin map of the F_2 population. The bin map consists of 565 bin markers. The physical position is based on the 'Chiifu-401-42' sequence. Red color indicates the 'HN53' genotype, blue color indicates the 'Zhongbaye' genotype, and yellow color indicates heterozygotes.

3.3. QTL Analysis

QTL analysis was performed using MAPQTL 4.0 software for MQM. In total, 60 QTLs for 11 traits were identified that satisfied the LOD threshold of more than 3.5 in the F_2 mapping population (Table 5, Figure 5). Eighty percent of these QTLs had positive additive effects. seven QTLs for LA traits showed positive additive effects, especially LA 4, which explained 18.9% of the genetic variation. LA 7 showed high dominance effects compared with other QTLs, except for seven QTLs for LA traits and LWT6, where dominance effects were insignificant (Table 5). For each trait, three to nine QTLs were detected, and these QTLs were unevenly distributed across the 10 chromosomes. Of these QTLs, 41 were linked with leaf-related traits, including LL, LW, LWT, LA, PL, PWT, and PA. A total of nine QTLs for LL were detected on eight chromosomes. The percentage of phenotypic variation explained by individual QTLs ranged from 3% to 11.6%. Six QTLs were detected for LW, of which LW4 showed the highest LOD value. Six QTLs and seven QTLs were detected for LWT and LA, respectively, accounting for 5.6%–18.4% and 4.8%–18.9%, respectively. Three QTLs for PL, six QTLs for PWT, and four QTLs for PA were detected. Of the QTLs for petiole-related traits, PL3, PWT4, and PA3 showed comparatively higher LOD values and were located on chromosome A06. The leaf index traits—namely LI, PI, PAI, and PLTI—can represent leaf shape and attractiveness to consumers and were also used for QTL analysis.

Table 5. QTLs for 11 traits identified using high-density SNP bin map.

Trait	QTL Name	Chr	Peak Position	LOD	2-LOD	Bin	Phenotypic Variation R^2 (%)	Additive Effect	Dominance Effect
LWT	LWT1	A02	13.7	5.79	13.0–16.7	bin_65	7.5	6.17092	0.842102
	LWT2	A03	21.8	4.42	20.5–22.5	bin_121	5.6	−5.44931	6.31162
	LWT3	A04	40.3	7.89	38.3–42.3	bin_215	10.5	7.9973	0.337838
	LWT4	A06	98.6	12.73	98.3–100.3	bin_330	18.4	10.5262	0.69486
	LWT5	A08	27.9	4.65	24.9–27.9	bin_421	5.7	5.95979	−0.752932
	LWT6	A10	19.6	6.89	18.9–20.9	bin_542	9	4.59607	8.22428

Table 5. *Cont.*

Trait	QTL Name	Chr	Peak Position	LOD	2-LOD	Bin	Phenotypic Variation R^2 (%)	Additive Effect	Dominance Effect
PWT	PWT1	A02	13.7	4.09	13.0–17.1	bin_65	5.7	3.02863	1.6585
	PWT2	A03	21.8	5.24	20.5–22.5	bin_121	7.4	−4.17852	3.39901
	PWT3	A04	39.6	5.87	36.3–40.3	bin_214	8	4.17934	0.718021
	PWT4	A06	79.3	11.09	78.3–79.7	bin_319	17.3	5.55048	2.59883
	PWT5	A08	26.9	5.72	24.9–27.9	bin_421	8.2	4.26514	−0.671059
	PWT6	A10	19.6	7.17	18.9–20.9	bin_541	10.5	2.84782	5.51818
PLTI	PLTI1	A01	27.4	6.71	24.4–27.8	bin_10	12.6	0.0685958	−0.0363513
	PLTI2	A02	24.5	5.17	21.8–26.1	bin_70	9.4	0.0403985	−0.0705622
	PLTI3	A03	21.8	6.16	20.1–22.5	bin_121	11.4	0.0807009	−0.022673
	PLTI4	A05	71.8	5.42	70.7–73.4	bin_270	9.9	−0.0292825	0.0991348
	PLTI5	A05	92.7	3.56	88.3–93.0	bin_278	6.3	0.06867	−0.0152701
LL	LL1	A01	125.4	5.12	121.7–126.1	bin_55	4.2	−1.42783	0.132046
	LL2	A04	15.5	4.06	15.1–15.8	bin_195	3.2	0.993059	0.743498
	LL3	A05	93	3.76	91.3–97.0	bin_279	3	0.990827	0.880449
	LL4	A06	45.6	8.47	45.2–46.6	bin_305	7.2	2.53086	0.725966
	LL5	A06	99.6	5.51	98.3–100.3	bin_330	4.1	1.9582	0.237959
	LL6	A07	19.8	6.77	18.4–20.4	bin_363	5.7	1.12922	1.60098
	LL7	A08	20.5	6.96	19.9–21.2	bin_415	5.8	1.61557	0.314969
	LL8	A09	13.1	6.76	8.7–14.1	bin_445	5.5	1.65979	0.187114
	LL9	A10	18.9	12.94	16.5–19.6	bin_540	11.6	0.881175	3.23715
LW	LW1	A01	37.9	6.68	36.2–39.9	bin_19	9.4	0.908156	−1.35427
	LW2	A02	13	7.04	10.2–13.7	bin_64	10	1.2967	0.44839
	LW3	A04	40.3	5.24	38.3–42.3	bin_215	7.2	1.05336	0.712885
	LW4	A06	79.3	9.48	77.7–79.7	bin_319	14	1.51683	0.740417
	LW5	A07	18.4	4.48	17.1–19.8	bin_362	6.1	0.932614	0.686273
	LW6	A08	20.2	3.77	19.9–20.5	bin_414	5.1	0.862757	0.600051
LI	LI1	A01	8.1	4.83	6.1–8.8	bin_4	7.7	−0.0992905	0.0209628
	LI2	A01	110.3	6.99	109.6–112.0	bin_46	11.5	−0.120275	0.0549563
	LI3	A02	13	7.06	11.0–17.1	bin_64	11.7	−0.125431	−0.0224847
	LI4	A04	40.3	4.53	36.3–42.3	bin_215	7.2	−0.0993892	−0.0127891
PL	PL1	A01	8.1	5.89	2.0–8.9	bin_4	10.5	−1.23337	1.07212
	PL2	A01	106.3	8.13	100.4–107.9	bin_40	15.1	−1.61924	0.734733
	PL3	A06	97.3	8.48	93.2–98.3	bin_328	15.8	1.84381	−0.0259659
PI	PI1	A01	30.8	10.75	29.8–31.1	bin_15	18.8	0.0961014	−0.010089
	PI2	A02	13	7.48	11.0–13.7	bin_64	12.4	0.0729022	0.0110429
	PI3	A05	68.1	4.71	67.1–68.4	bin_266	7.5	−0.371918	−0.457907
	PI4	A07	15.1	3.73	14.4–16.1	bin_359	5.8	0.0496321	0.00700846
	PI5	A09	52	4.25	52.0–53.3	bin_468	6.7	0.0541131	0.00126054
LA	LA1	A02	13	6.84	13.0–16.7	bin_64	7.7	39.6736	11.7891
	LA2	A04	40.3	4.37	38.3–42.3	bin_215	4.8	30.1337	9.47975
	LA3	A05	34.9	6.37	33.2–35.9	bin_238	7.2	38.977	14.6048
	LA4	A06	46.2	14.68	45.2–46.6	bin_305	18.9	69.4458	13.5546
	LA5	A07	19.8	5.86	18.4–20.4	bin_363	6.6	32.2879	30.4413
	LA6	A08	26.2	5.68	24.9–27.9	bin_421	6.3	39.7259	−6.26181
	LA7	A10	19.6	10.19	18.9–20.9	bin_541	12	36.7118	57.9985
PA	PA1	A02	34.9	4.22	33.9–36.6	bin_77	4.4	4.22363	2.86465
	PA2	A04	39.6	5.12	36.3–40.3	bin_214	5.3	4.89341	2.52144
	PA3	A06	45.6	18.59	44.5–46.2	bin_305	24.6	11.2716	2.72566
	PA4	A10	8.8	18.29	6.7–9.4	bin_535	24.1	11.1044	0.828217
PAI	PAI1	A01	23.4	5.43	20.6–27.4	bin_9	9.1	0.497833	−0.225617
	PAI2	A05	87.9	5.39	81.5–88.3	bin_276	8.8	0.516711	0.148835
	PAI3	A07	0	3.55	0.0–3.0	bin_339	5.6	0.42485	−0.02513
	PAI4	A08	0	3.61	0.0–3.7	bin_397	5.7	0.408383	−0.0758493
	PAI5	A10	7.7	5.75	5.4–8.8	bin_534	9.8	−0.5165599	0.313028

Note: See Table 1 for trait abbreviations.

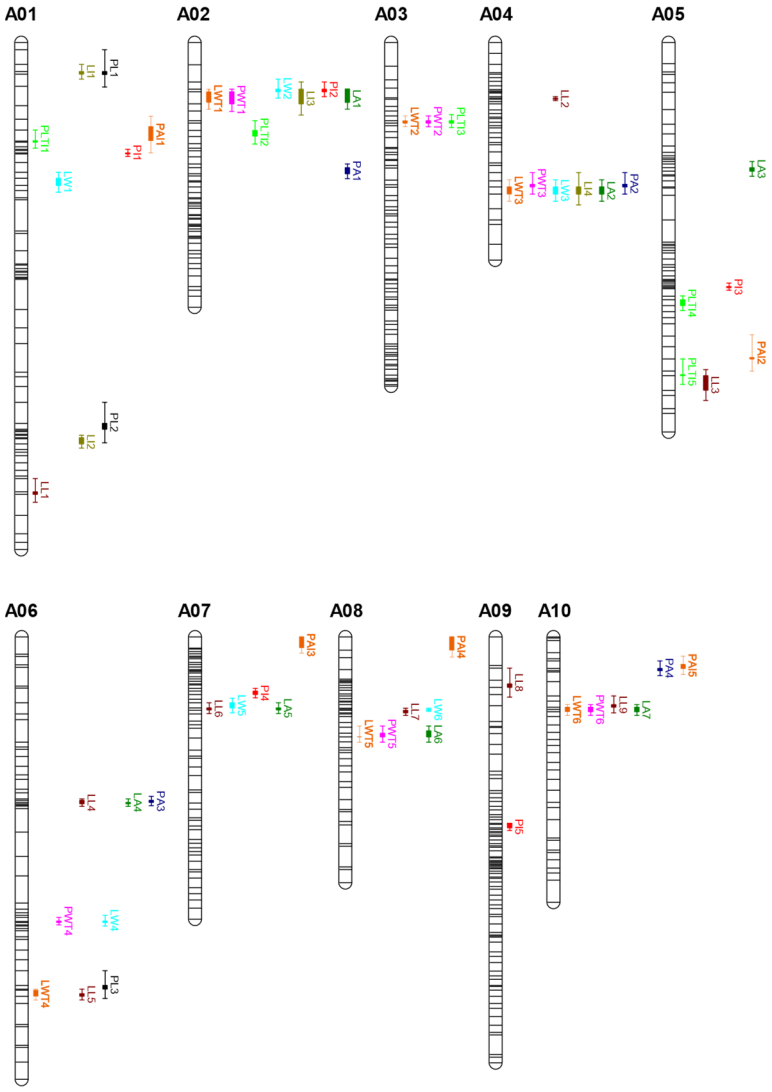

Figure 5. Distribution of the 11 leaf-related traits on the linkage map. The gray bars represent the 10 chromosomes on the linkage map and the colored bars represent potential quantitative trait loci (QTLs) for the 11 leaf-related traits. Bar length indicates the two-LOD confidence interval of potential QTLs. See Table 1 for trait abbreviations.

A total of four QTLs were detected for LI and explained 7.2%–11.7% of the phenotypic variation. Five QTLs were identified for PI, PAI, and PLTI and explained 5.8%–18.8%, 5.6%–9.8%, and 6.3%–12.6% of the phenotypic variation, respectively.

3.4. Co-Localization of QTLs and Candidate Gene Prediction

Most chromosomes contained QTLs for several leaf-related traits in apparent co-localization, except for chromosome A09 (Figure 5). Three QTLs for LWT overlapped with QTLs for PWT, LWT1 and PWT1 were co-located on bin_65 of A02, LWT2 and PWT2 were co-located on bin_121 of A03, and LWT3 and PWT3 were co-located on bin_214−215 of A04, suggesting that the entire LWT was significantly correlated with PWT. Six QTLs for six traits—LWT, PWT, LI, LW, LA, and PI—were co-localized on bin_64−65 of A02. Seven QTLs were detected on A04, and six of the seven QTLs for LWT, LA, PWT, LW, LI, and PA traits were co-located in the 36.3−42.3 cM range on bin_214−215. Previous correlation analyses found that LWT, LA, PWT, LW, PA, and PI traits were positively correlated with each other, but negatively correlated with LI. These results indicate that the co-location of QTLs for leaf-related traits may account for the strong correlations among these traits, and

these traits are possibly controlled by a common region. A previous QTL study identified leaf-related traits (rosette leaf length and PL) and head-related traits on LG A06 [28,35]. In the present study, LL, LA, LWT, and PA were highly correlated with each other, and their QTLs were co-located on A06.

Candidate genes associated with the investigated traits were identified using physical locations on the *B. rapa* reference genome corresponding to the bin markers. As a result, a total of 3170 candidate genes were identified. The functional annotations included transcription factors, kinases, hormonal pathways, and photosystem components. Some of the candidate genes play important roles in controlling leaf shape, leaf size, and leaf morphology through cell proliferation, cell expansion, hormone signaling pathways, or adaxial–abaxial patterning (Table 6). These included some cell-proliferation-related and cell-expansion-related genes, for example, *GRFs*, *WOXs*, *TCPs*, and *BAMs* genes; auxin signaling pathway genes, for example, *ARFs*, *IAAs*, and *SAURs* genes; leaf abaxial-adaxial polarity genes, for example, *ARF*, *PGY*; and ribosome-encoding genes (*RPSs* and *RPLs*) (Table 6). Among these genes, *BrGRF3* (*BraA04g025910.3C*), a homolog gene of *Arabidopsis GRF3*, is located near the peak signal on the co-localized QTL (LWT, LA, PWT, LW, LI, and PA traits) region of chromosome A04. Previous studies have shown that growth-regulating factors (GRFs) are plant-specific proteins that play important roles in regulating leaf size. *GRFs* regulate organ size development through the promotion and/or maintenance of cell proliferation activity [36–38]. Furthermore, previous QTL analysis identified the *BrGRF5* for LL and LI traits in *B. rapa* [1]. In the present study, it was found that the *BrGRF*3 (*BraA04g025910.3C*) allele of 'Zhongbaye' contained three nonsynonymous substitutions and one premature termination compared with that of 'HN53'. In addition, *BrARF5* gene (*BraA06g015500.3C*), the gene homologous to *Arabidopsis ARF5*, was located on co-localized QTL (LL, LA, and PA) regions of A06. This may be a candidate gene because it not only controls leaf adaxial–abaxial asymmetry but also influences organ development [39]. Sequence analysis showed that the *BrARF5* gene of 'Zhongbaye' contained four nonsynonymous substitutions and one alternative splicing site compared with that of 'HN53'.

Table 6. 60 QTLs localized to candidate genes that may be associated with leaf traits.

QTL	Bin	Candidate Gene_ID	Other Name
PLTI1	bin_10	*BraA01g007610.3C*	*ARF16*
LL6, LA5	bin_363	*BraA07g018740.3C*	*ARF10*
LL4, LA4, PA3	bin_305	*BraA06g015500.3C*	*ARF5;IAA2*
PWT4, LW4	bin_319	*BraA06g029140.3C*	*IAA9*
PLTI4	bin_270	*BraA05g030630.3C*	*IAA26*
LW5	bin_362	*BraA07g018500.3C*	*SAUR42*
LL4, LA4, PA3, PI4	bin_305	*BraA06g015440.3C*	*SAUR53*
	bin_359	*BraA07g016220.3C*	
PWT3, PA2	bin_214	*BraA04g025760.3C*	*SAUR45*
LWT3, LW3, LA2, LI4	bin_215	*BraA04g026250.3C*	*SAUR46*
LWT3, LW3, LA2, LI4	bin_215	*BraA04g026370.3C*	*RPS5B*
PL3	bin_328	*BraA06g037340.3C*	*RPL12*
LWT3, LW3, LA2, LI4	bin_215	*BraA04g025910.3C*	*GRF3*
PAI4	bin_397	*BraA08g001700.3C*	*TCP3*
PAI2	bin_276	*BraA05g035610.3C*	*WOX5*
PWT4, LW4	bin_319	*BraA06g029220.3C*	*BAM1*
LL7	bin_415	*BraA08g014070.3C*	*BAM3*
LW5	bin_362	*BraA07g018220.3C*	*PGY1*

Note: See Table 1 for trait abbreviations.

4. Discussion

The bin-map strategy was demonstrated to be efficient for the fast identification of QTLs, with high resolution in cereal crops [21–23]. However, limited studies on QTL bin maps have been conducted in the *B. rapa* vegetable.

In this study, we aimed to investigate the genetic regulation mechanisms underlying complex leaf-related traits in cabbage using a bin map of *B. rapa* constructed using a sliding window approach. The window length was set at 1 Mb instead of fixing the SNP number per window. Our analysis of the F_2 population revealed a total of 4291 recombination breakpoints, with an average of 28 recombination breakpoints per individual. This suggested that about two to three recombination events occurred per chromosome. The constructed bin map contained 565 bin markers spanning a total genetic distance of 944.6 cM, and the average distance between the bins was 1.65 cM, suggesting that the bin map could detect differences in recombination frequency between F_2 plants and that a QTL could be narrowed down to a small interval harboring dozens of genes. We identified 60 QTLs for 11 leaf-related traits distributed across all 10 chromosomes of *B. rapa*, indicating that leaf-related traits are complex quantitative traits controlled by many genes. These results are consistent with previous findings [1,28].

The co-localization of QTLs for different leaf traits is of great interest in understanding the genetic regulation mechanisms underlying complex leaf-related traits in cabbage vegetables. In this study, we observed clear co-localization of QTLs for several leaf traits in some chromosomal intervals (Figure 5). For example, three QTLs for LWT overlapped with QTLs for PWT on A02, A03, and A04. Six QTLs were detected and co-located in the bin interval 214–215 on chromosome 4, including QTLs for the LWT, LA, PWT, LW, LI, and PA traits. Obviously, the co-localization of QTLs indicated that leaf size was correlated with leaf weight and shape. This was in accordance with the fact that most of these traits were significantly correlated with each other. Several studies have reported similar results for leaf-related traits in *B. rapa*. Sun et al. found that QTLs for leaf size traits (LL, LW, and PL), leaf trichome trait, and leafy head traits (head height, head weight, and heading degree) are co-localized, in accordance with the correlation between these traits [28]. QTLs for rosette leaf length and rosette petiole length were co-localized on LG A06 in both investigated populations, suggesting that LL was correlated with PL. Interestingly, the rosette leaf width QTLs were all co-located with the heading degree QTL, revealing a relationship between the rosette leaf and leafy heads, in which the rosette leaf provides nutrients for leafy head formation. These results also indicated that the co-localized QTL regions are more likely to carry important genes that regulate more than one leaf trait.

The leaf and leaf head are the main edible parts of Chinese cabbage, and their quality significantly impacts the commercial value of the vegetable. Therefore, identifying genes associated with leaf shape, leaf head, and leaf abaxial-adaxial polarity could potentially improve the quality of Chinese cabbage. In this study, we analyzed the 60 QTLs linked with leaf-related traits and identified a total of 3170 candidate genes that may be involved in the regulation of these traits. Among these were some genes involved in the regulation of leaf size, leaf shape, and leaf morphology through the regulation of cell proliferation and cell expansion [36,39–41]. *BrGRF5* and *BrTCP4* are reported to be associated with leaf size and leaf heading in *B. rapa* [42]. In this study, *BrGRF3*, *BrTCP3, BrWOX5*, and *BrBAMs* were found in the QTL regions. Genes involved in the auxin signaling pathway play important roles in the curling of Chinese cabbage leaves, and IAAs are involved in leaf curling of Chinese cabbage [43]. Here we found that nine auxin signaling pathway genes (*BrARF16*, *BrARF5*, *BrARF10*, *BrIAA9*, *BrIAA26*, *BrSAU42*, *BrSAU45*, *BrSAU46*, and *BrSAU53*) were located in the QTL regions. In addition, leaf adaxial–abaxial polarity genes are reported to have important roles in regulating leaf curvature and leaf heading in *B. rapa* [29,44–46]. Increasing evidence shows that ribosome-encoding genes such as *PGYs*, *RPSs*, and *RPLs* are involved in the adaxial-abaxial patterning and development of leaves [47–49]. Here, some ribosome-encoding genes such as *PGY1*, *RPL12*, and *RPS5B* were located in the QTL regions. These genes may be important candidate genes for regulating leaf-related traits in *B. rapa*.

Further fine mapping of these QTLs should be conducted in order to narrow down the candidate genes, as the efficiency of QTL mapping depends largely on maker density

and population size. Therefore, larger populations of F_2, BC_1S, or other early-generation crosses combined with a high throughput genotyping method is a cost-effective option.

5. Conclusions

In conclusion, a high-density genetic bin map containing 565 bin markers was constructed. The total map length was 944.6 cM, and the average distance of the bins was 1.65 cM. A total of 60 significant QTLs controlling 11 leaf-related traits were identified. This study demonstrated the use of whole genome re-sequencing and high-density genetic bin mapping in the identification of QTLs and candidate genes in an F_2 population of *B. rapa*. For rapid identification of reliable QTLs and candidate genes associated with complex traits in *B. rapa*, reducing the QTL intervals using larger population sizes and constructing high-density marker maps in early-generation populations will be a cost-effective option in future. Our findings will help to accelerate the improvement of *B. rapa* vegetable crops.

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