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Research Paper

High-density ddRAD linkage and yield-related QTL mapping delimits a chromosomal region responsible for oil content in rapeseed (*Brassica napus* L.)

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Rapeseed (*Brassica napus* L.) is one of the most important oil crops almost all over the world. Seed-related traits, including oil content (OC), silique length (SL), seeds per silique (SS), and seed weight (SW), are primary targets for oil yield improvement. To dissect the genetic basis of these traits, 192 recombinant inbred lines (RILs) were derived from two parents with distinct oil content and silique length. High-density linkage map with a total length of 1610.4 cM were constructed using 1,329 double-digestion restriction site associated DNA (ddRAD) markers, 107 insertion/deletions (INDELs), and 90 well-distributed simple sequence repeats (SSRs) markers. A total of 37 consensus quantitative trait loci (QTLs) were detected for the four traits, with individual QTL explained 3.1–12.8% of the phenotypic variations. Interestingly, one OC consensus QTL (*cqOCA10b*) on chromosome A10 was consistently detected in all three environments, and explained 9.8% to 12.8% of the OC variation. The locus was further delimited into an approximately 614 kb genomic region, in which the flanking markers could be further evaluated for marker-assisted selection in rapeseed OC improvement and the candidate genes targeted for map-based cloning and genetic manipulation.

Key Words: *Brassica napus*, restriction site associated DNA, quantitative trait locus, yield-related trait, oil content.

Introduction

Rapeseed (*Brassica napus* L.) is one of the most important oil crops almost all over the world. It is not only a leading source for vegetable oil (Snowdon 2007), but also a major source for industrial materials such as biofuel and lubricants. In China, rapeseed provides about 40% of the vegetable oil supply (Sun *et al.* 2012). However, due to the great demands for human food and industrial materials in recent years, the production of rapeseed hardly meets the global consumption. Development of cultivars with high yield is one of the most important ways to solve the shortage of rapeseed oil supply. Yield-related traits such as thousand-seed weight (SW), seeds per silique (SS) and silique length (SL) are important determinants of yield and thus target traits in rapeseed breeding and quantitative trait locus (QTL) mapping. Many yield-related QTLs have been identified (Chen *et al.* 2007, 2011, Shi *et al.* 2009, Yang *et al.* 2012, Zhang *et al.* 2012). Of these, only one major QTL for SS (Li *et al.* 2015b, Zhang *et al.* 2011) and one major QTL for SL and SW were mapped and their underlying genes were identified recently (Li *et al.* 2014, Liu *et al.* 2015). Further understanding of the genetic basis of these traits in rapeseed remains essentially needed.

Restriction fragment length polymorphisms (RFLPs) and simple sequence repeats (SSRs) are the major types of markers for linkage map construction in previous QTL analysis. Due to limited RFLP and SSR markers, the density of molecular markers and the mapping resolutions for those oil content (OC) and yield-related QTLs in *B. napus* were relatively low (Chen *et al.* 2007, 2011, Shi *et al.* 2009, Yang *et al.* 2012, Zhang *et al.* 2012), which restrained further utilization in map-based cloning and marker-assisted selection. Those markers with short sequences are difficult to precisely align to the reference genome sequence of *B. napus* (Bus *et al.* 2012). Single nucleotide polymorphisms (SNPs) are currently taking the place of RFLP and SSR markers and becoming predominant in linkage mapping in many crop species (Rowe *et al.* 2011), which are more conducive to substantial improvement of marker density, statistical power, and QTL mapping precision (Jiang and Zeng 1995). In *B. napus*, next generation sequencing (NGS) technologies have been used to discover mass SNPs, in the form of

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genotyping by sequencing (GBS) and/or double-digest restriction-site associated DNA (ddRAD) (Bus *et al.* 2012, Liu *et al.* 2013, 2014, Trick *et al.* 2009). ddRAD-based high-density genetic linkage map SNPs can be used for dissecting the genetic basis of agronomically important traits in oilseed rape.

It is estimated that an increase of 1.0% seed OC is equivalent to an increase of 2.3–2.5% of seed yield in rapeseed (Wang 2004), suggesting seed OC improvement may be more effective to increase oil yield. In the past two decades, 3 to 63 QTLs controlling OC in *B. napus* have been identified in different studies (Chen *et al.* 2010, Delourme *et al.* 2006, Ecke *et al.* 1995, Javed *et al.* 2016, Wang *et al.* 2013). Javed *et al.* (2016) detected a common QTL on chromosome A10 in all four environments. Largely due to relatively long genetic distance, low marker density, or other genomic factors, none of these QTLs for OC has been delimited in a genomic region small enough to facilitate positional cloning.

The objectives of this study were (1) to develop a high-density genetic linkage map using ddRAD and other sequence-based markers; (2) to identify QTLs controlling OC, SL, SS and SW; (3) to delimit the genomic regions for stably expressed QTLs; and (4) to compare QTLs identified in this study with previous ones.

Materials and Methods

Plant materials

Two rapeseed inbred lines, ‘M201’ with high OC and long siliques, and ‘352’ with low OC and short siliques, were selected to develop a population of recombinant inbred lines (RILs) for detection of QTL controlling seed OC and yield-related traits including SL, SS, and SW. An F_1 hybrid from the cross of the two inbred lines was self-pollinated to obtain F_2 plants that were self-pollinated and advanced to the F_6 generation using the single seed descent (SSD) method. A total of 192 recombinant inbred lines (RILs) were obtained as of 2010. Genomic DNA was extracted from 250 mg of young leaves collected from a single plant of each RIL using the cetyltrimethylammonium bromide (CTAB) method.

Experiment design and trait measurement

Using a randomized complete block design, the ‘352’ × ‘M201’ RIL population and their parents were planted in 2011 (Y11) at Hezhen, Gansu Province, China and in 2012 and 2013 (Y12 and Y13) at the experimental farm in Wuhan, Hubei Province, China, respectively. Each RIL or parent was grown in two row plots, with 10–12 plants in each row. Y11 had one replication and seeds were sown in mid-May and harvested at the end of September of the year. Y12 and Y13 each had three replications and seeds were sown in early October and harvested in early May of 2012 and 2013, respectively. Field management followed standard agricultural practices. The distance between the two rows was 30 cm, and the distance between any two neighboring plants was approximately 20 cm. Five mature plants from the center of each plot were selected for trait survey. SL of each plant was based on the average length of 10 well-developed siliques (not including the beak) from the middle of the main inflorescence. SS of each plant was defined as the average number of seeds from 10 well-developed siliques from the middle of the main inflorescence. SW of each plant was defined as the average weight of 1000 well-filled open-pollinated seeds. Threshed seeds were desiccated to minimize the moisture content. OC was measured by nuclear magnetic resonance (NMR) using a previously described method (Burns *et al.* 2003). The OC in each plot was based on an average of five plants.

Preparation and sequencing of ddRAD libraries

ddRAD libraries of the 192 RILs were prepared for paired-end sequencing as previously described (Chen *et al.* 2013), with the exceptions that only 50 ng of genomic DNA was used and inserts with size range of 270–320 bp were recovered. Briefly, 50 ng of genomic DNA was double-digested by *MseI* and *SacI* and indexed adaptors were ligated. After digestion and adapter ligation, ligates of 50 RILs were pooled together and separated on a 2% agarose gel. Fragments in the size range of 350–400 bp were recovered from the gel, which corresponded to 270–320 bp of unligated fragments. Approximately 50 ng of recovered DNA fragments was used as the template for PCR amplification in a 50 µl reaction with the two sequencing primers (Chen *et al.* 2013). The PCR products were separated on a 2% agarose gel and DNA fragments in the size range of 400–450 bp were recovered and sequenced on the HiSeq2000 platform (Illumina, San Diego, CA, USA) with paired-end (PE) reads of 90 bp.

SNP genotyping of the RIL population

The 90 bp PE reads were subjected for quality filtering. Sequence reads from the Illumina runs did not match one of the expected barcodes and sequence reads of poor overall quality (5% base quality score under Q30) were removed from the analysis. The clean data was then parsed into different individuals with the outermost barcodes and remnant restriction sites at both ends exactly matching the adaptors used. The 5 bp barcodes at the 5′ end and the 5 bp error-rich nucleotides at the 3′ end were removed from the PE reads. After trimming, the remaining 80 nucleotides of each PE read were kept for further analysis. A total of 192 RILs were initially used for sequencing and marker discovery. The parents, ‘M201’ and ‘352’, were sequenced together with a much higher coverage (approximately 6-fold) than that of the RILs in this study. The average depth of sequence of the parents were 10× and RILs were 4.5×.

SNP discovery and genotyping were performed exactly following the RFAPtools pipeline (Chen *et al.* 2013). First, a pseudo-reference sequence (PRS) was assembled using the reads from both ‘M201’ and ‘352’ and all RILs. Then,

the 80 bp PE reads of each parent and RIL were aligned to the PRS with the SOAP software (Li *et al.* 2009). Three mismatches to the pseudo-reference sequence were allowed on each end of the PE reads. SNPs were identified for the two parents and all RILs. The threshold of maximum genotype missing rate was set to 25%. SNPs with missing genotypes in either parent were also excluded from further analysis. The sequence data of the RIL population were deposited in NCBI with the Sequence Read Archive accession number PRJNA345453.

SSR and INDEL marker screening and genotyping

Polymorphism between the parents was also screened using previously published 212 SSRs (Cheng *et al.* 2009, Xu *et al.* 2010, Yang *et al.* 2012), and 595 in-house INDELs developed from resequencing data of 22 rapeseed cultivars (Mahmood *et al.* 2016). The 595 INDELs were evenly distributed throughout the A and C subgenomes and uniquely mapped on the reference genomes of *B. napus*. PCR for SSR and INDEL detection was performed as previously described (Cheng *et al.* 2009), and the products were separated on 6% denatured polyacrylamide gels and stained with $AgNO_3$ solution. Polymorphic SSRs evenly distributed on all the 19 linkage groups were selected as anchored markers to compare LGs and QTLs.

Construction of the genetic linkage map

JoinMap 4.0 (Van Ooijen 2006) was used for genetic linkage map construction. SSRs and INDELs were used as anchor markers to assign LGs to specific chromosomes. The maximum recombinant frequency was set to 0.4, and minimum logarithm of odds (LOD) scores of 3.0, and the threshold for goodness of fit was set to ≤5.0, which is a normalized difference of goodness-of-fit chi-squared used to decide whether or not a locus should remain in the linkage group during the process of building the linkage group. CentiMorgan distances were calculated by the Kosambi function for map distance (Kosambi 1943). Pearson's chi-squared test was performed to examine the goodness of fit to the expected 1:1 segregation ratio ($P < 0.05$) for each marker.

Statistical analysis, QTL mapping, meta-analysis and interaction detection

Statistical analyses for all traits were carried out using SAS 9.2 (SAS Institute, Cary, NC, USA). Pearson's phenotypic correlation coefficients were calculated among all traits across the three environments using the CORR procedure. The genetic correlation coefficient was calculated using the general linear model (GLM, $Y_{ijkl} = \mu + r_i + b_{ij} + g_k + e_{ijkl}$, where Y_{ijkl} is an observation of genotype k in replication l of block j in environment i, μ is the general mean, r_i is the effect of the environment i, b_{ij} is the effect of block j in environment i, g_k is the effect of genotype k, and e_{ijkl} is the residual effect of observation, the residual variance is a combination of genotype × location interaction variance and the within location error variance.). The genetic correlation was calculated as: $r_G = \frac{COV_{G_{xy}}}{\sqrt{\sigma^2_{G_x} \times \sigma^2_{G_y}}}$, where $cov_{G_{xy}}$, $\sigma^2_{G_x}$, and $\sigma^2_{G_y}$ were the genetic covariance and variances of a pairwise traits, respectively. The significance of each genetic correlation was determined using a *t*-test of correlation coefficients (Kong 2005). The broad-sense heritability was calculated as: $h^2 = \sigma^2_g / (\sigma^2_g + \sigma^2_{ge} / n + \sigma^2_e / nr)$, where σ^2_g is genotypic variance, σ^2_{ge} is interaction variance of the genotype with the environment, σ^2_e is error variance, n is the number of environments, and r is the number of blocks in each environment. The lines with missing phenotype data were ignored in each trait.

QTLs were detected by composite interval mapping using WinQTL cartographer 2.5 (Zeng 1994). The number of control markers, window size and walk speed were set to 5, 10 and 1 cM, respectively. The LOD threshold for each trait was determined by permutation test with 1000 repetitions t. A QTL was declared when the LOD score was greater than the threshold value, LOD scores corresponding to $P < 0.05$ were used to identify significant QTLs. To avoid missing QTLs with small genetic effects, loci with LOD scores larger than 2.0 but smaller than the threshold in multiple environments were treated as micro-real QTLs (Long *et al.* 2007). These micro-real QTLs might become major QTLs in different environments or in different segregating populations (Long *et al.* 2007). The nomenclature of QTLs followed previous descriptions (Udall *et al.* 2006) with minor modifications. A QTL was named starting with the lowercase letter *q*; followed by an uppercase two-letter designation for the trait name (OC, SL, SS, or SW); an uppercase chromosome set letter (A or C); a chromosome number; a dot; a 1, 2, or 3 (representing 2011, 2012, or 2013, respectively, when the QTL was detected; and a lowercase letter (a, b, c, ...) for one of the multiple QTLs detected in the same linkage group and environment (Yang *et al.* 2012).

To merge and compare QTLs detected in different environments and located in the same chromosome region, either for the same or different trait, meta-analysis was conducted using BioMercator 3.0 (Sosnowski *et al.* 2012). If QTLs for the same trait were detected in multiple environments with overlapping confidence intervals, these QTLs were firstly merged as a consensus QTL and designated with initial letters "*cq*" followed by trait name and linkage group (Yang *et al.* 2012). QTLs for different traits having overlapping confidence intervals were further integrated into unique QTL and designated with initial letters "*uq*" followed by the linkage group. The algorithm of BioMercator software can help to determine the position of the overlapping QTLs based on the variance of these QTLs position and the confidence interval values (Arcade *et al.* 2004).

Epistatic interactions were analyzed based on mixed linear model approaches using QTLNetwork 2.0 (Yang *et al.* 2008). The testing windows, filtration windows and walk

speed were set to 10, 10 and 1 cM, respectively. Both 1D and 2D searches were analyzed with 1000 permutations. $P < 0.05$ was set as the significance threshold.

Comparison of chromosomes between the RIL linkage maps and the B. napus genome sequences

The linkage map constructed in this study was aligned to the *B. napus* genome (version 4.1) (www.genoscope.cns.fr/brassicanapus) through BLAST of the mapped ddRAD sequences and INDEL and SSR amplicon sequences. The E-value was set to ≤1E-5 for all the markers. A Perl script was used to extract the position of best hit in the *B. napus* genome sequence. The orthologous gene(s) in *B. rapa* (http://brassicadb.org/brad/, version 1.5) and *A. thaliana* within the genomic regions delimited by the markers flanking a QTL interval were considered putatively to be associated with the QTL.

Results

Phenotypic variations and genetic correlations among traits

The two inbred lines, 'M201' and '352', and their RILs were grown in three environments to compare OC, SL, SS, and SW. The OC of 'M201' was 42.7 ± 2.92%, 49.6 ± 2.77% and 44.9 ± 2.51% (Mean ± SD) in Y11, Y12, and Y13, respectively. The OC of 'M201' was significantly higher than that of '352' in the three environments ($P < 0.01$), which were 29.8 ± 3.31%, 35.3 ± 2.60% and 30.7 ± 2.09% (Mean ± SD), respectively (**Table 1**). The SL of 'M201' was 5.8 ± 0.47 cm, 6.1 ± 0.41 cm, and 5.9 ± 0.39 cm in Y11, Y12, and Y13 (Mean ± SD), respectively. The SL of '352' was 4.3 ± 0.49 cm, 4.7 ± 0.33 cm, and 4.5 ± 0.31 cm (Mean ± SD) in Y11, Y12, and Y13, respectively (**Table 1**), which were significantly shorter than those of 'M201' ($P < 0.01$). SS and SW did not show significant differences between the two parents in the three environments (**Table 1**).

The distributions of the four traits in the RILs displayed a continuous distribution with transgressive segregation (**Fig. 1**). Normality test indicated that the segregation of the four traits fit a normal distribution model. Two-way ANOVA of individual traits across environments indicated that the genotypes of RILs (G), growing environment (E) and genotype-environment interactions (G × E) had significant effects on the four traits (**Supplemental Table 1**), suggesting that the variations of individual traits were caused by genotype differences among the RILs. In addition, the environments also had a significant effect on the performance of the four traits. The broad-sense heritability of OC, SL, SS and SW was 78.8%, 89.4%, 78.5% and 81.9%, respectively, suggesting that these traits are stable in different environments (**Supplemental Table 1**) and SL was the least affected by environmental variations.

Phenotypic correlation analyses were performed among the four traits. SS showed a negative correlation with SW in Y12, and Y13, but no correlation in Y11. SL showed a significant positive correlation with OC, SS and SW in all three environments (**Supplemental Table 2**). SW showed a significant positive correlation with OC in Y11 and Y12. The genetic correlations among the four traits were also evaluated (**Supplemental Table 3**). OC was positively correlated with SL, SS and SW, respectively. SS showed a weak negative correlation with SW, while SL showed a significant positive correlation with SS, suggesting that longer siliques could produce larger seeds.

ddRAD tag sequencing and SNP discovery in the RIL populations

A total of 68,450,311 high-quality PE90 reads were obtained for the 192 RILs after quality filtering. The number of reads varied from 0.04 and 1.07 million, with an average of 0.36 million reads per RIL (**Supplemental Table 4**). Four RILs with less than 0.10 million reads were excluded from further analysis, and the remaining 188 RILs were

Table 1. Statistical analysis of oil content (OC), sileque length (SL), seeds per silique (SS), and seed weight (SS) for the parental lines and the recombinant inbreed lines (RILs)

Years	Locations	Traits	Parents			RIL population		
			M201(Mean ± SD)	352 (Mean ± SD)	(P value)	Range	Mean ± SD	CV(%)
2011 (Y11)	Gansu	OC (%)	42.7 ± 2.92	29.8 ± 3.31	<0.01	24.6–47.6	35.3 ± 4.22	12.3
		SL (cm)	5.8 ± 0.47	4.3 ± 0.49	<0.01	3.0–9.0	4.8 ± 0.68	15.9
		SS	19 ± 3.13	15 ± 3.22	>0.05	8–17	17 ± 3.27	19.3
		SW (g)	2.98 ± 0.33	2.61 ± 0.29	>0.05	1.36–4.22	2.70 ± 0.47	17.5
2012 (Y12)	Wuhan	OC (%)	49.6 ± 2.77	35.3 ± 2.60	<0.01	29.5–49.3	42.3 ± 3.02	7.0
		SL (cm)	6.1 ± 0.41	4.7 ± 0.33	<0.01	3.6–6.7	5.1 ± 0.57	11.4
		SS	20 ± 2.18	17 ± 2.06	>0.05	10–25	18 ± 2.90	16.2
		SW (g)	3.47 ± 0.49	3.29 ± 0.28	>0.05	2.38–4.58	3.36 ± 0.39	11.6
2013 (Y13)	Wuhan	OC (%)	44.9 ± 2.51	30.7 ± 2.09	<0.001	23.7–46.7	37.6 ± 3.95	10.4
		SL (cm)	5.9 ± 0.39	4.5 ± 0.31	<0.001	3.7–6.3	4.9 ± 0.54	11.2
		SS	16 ± 2.95	14 ± 2.63	>0.05	7–25	15 ± 3.15	21.2
		SW (g)	3.33 ± 0.24	3.02 ± 0.48	>0.05	2.26–4.61	3.30 ± 0.41	12.5

Y11–rapeseed growth environment in 2011 at Hezhen, Gansu Province, China; Y12 and Y13–rapeseed growth environments in 2012 and 2013 at the experimental farm in Wuhan, Hubei Province, China; SD–standard deviation; CV–coefficient of variation.
The *t* test significant level was 0.05.

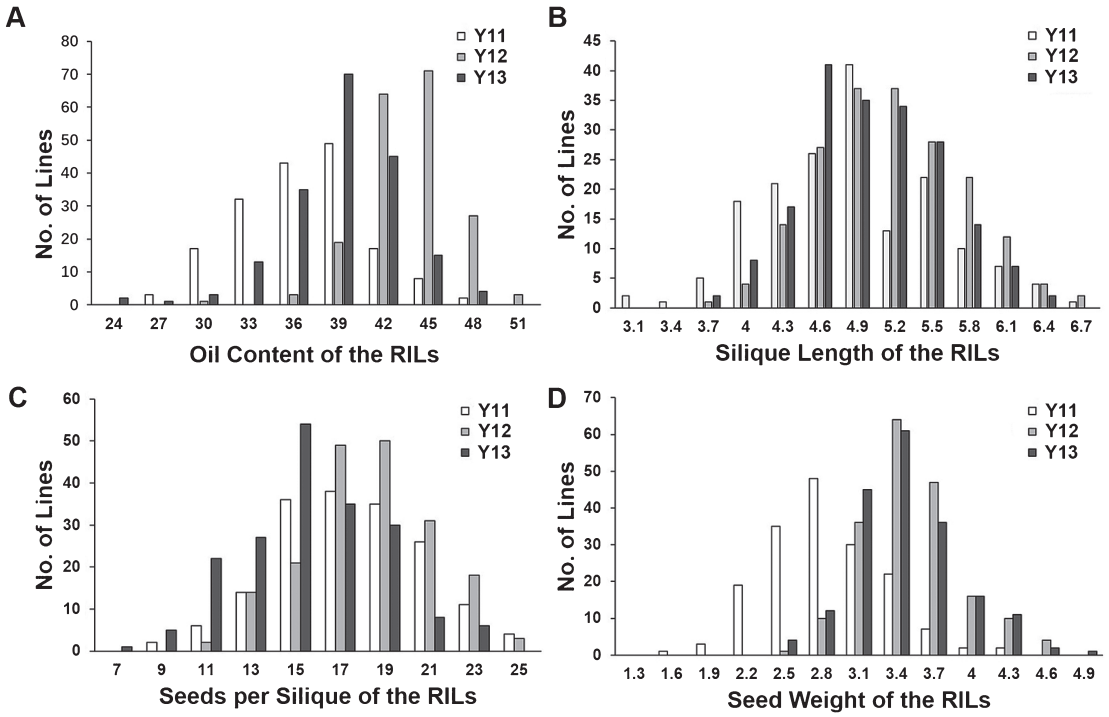

Fig. 1. Phenotypic variation of the four traits in the recombinant inbreed line (RIL) populations in a rapeseed growth environment in 2011 (Y11) at Hezhen, Gansu Province, China and two rapeseed growth environments in 2012 and 2013 (Y12 and Y13) at the experimental farm in Wuhan, Hubei Province, China. (A), (B), (C) and (D) show the distribution of oil content (%), silique length (cm), seeds per silique, and seed weight (gram), respectively.

used for SNP discovery and genotyping. The reads from all RILs were collapsed and used to generate a total of 15 million unique ddRAD tags. A pseudo-reference sequence was assembled using RFAPtools with short reads obtained from sequencing reads of the parents and the whole RIL population as described (Chen *et al.* 2013). All sequence reads from the two parents (352,533 tags from 'M201' and 318,938 tags from '352') and individual RILs were aligned to the pseudo-reference using SOAPsnp with a maximum of three mismatches (Li *et al.* 2008). A total of 1,812 polymorphic ddRAD tags were identified. A subset of 1,700 polymorphic ddRAD markers containing 3,765 SNPs was identified having a maximum missing genotype rate lower than 25%. Of these SNPs, 29.2% and 29.6% were C/T and A/G transitions respectively, and 11.6%, 11.7%, 9.0%, and 8.9% were A/C, G/T, A/T and C/G transversions respectively.

Construction of genetic linkage map

Of the 595 INDELs and 211 SSRs, 126 and 104 showed polymorphisms between 'M201' and '352', respectively, and were genotyped in the RILs. A genetic linkage map containing 1,329 ddRAD markers, 107 INDELs and 90 SSRs was constructed. The linkage map covered a total length of 1610.4 cM and consisted of 19 linkage groups (LGs), with an average distance of 1.06 cM between adjacent loci. The length of LGs ranged from 30.8 (C01) to 181.4 cM (C03), and the average interval between markers ranged from 0.5 (A05) to 6.7 cM (A02). C03 had the maximum number of markers (201). A02 and C02 did not have any SNPs; A02 had 8 INDELs and SSRs and C02 had 11 INDELs and SSRs. We found that five out of the eight markers on A02 had a heterozygous rate ranging from 26% to 66%, which is much higher than the expected heterozygous rate (3.1%) at the F_6 generation. Alignment of '352' short reads to the *B. napus* reference genome indicated that the coverage depth on chromosome A02 was significantly higher (more than 3 fold, $P < 0.05$) than that on chromosome C02 and other chromosomes (**Fig. 2**), suggesting that homeologous nonreciprocal transposition (HNRT) (Zhao *et al.* 2006) or homeologous exchange (HE) (Chaloub *et al.* 2015) from A02 to C02 occurred in the genome of '352'. HNRTs or HEs were also identified between A01 and C01, A04 and C04, A05 and C05, A07 and C06, A09 and C08, and A10 and C09 (**Fig. 2**). In all of these transpositions, only one chromosomal segment was transposed from the C subgenome (C06) to the A subgenome (A07), the others were transposed from the A subgenome to the C subgenome (**Fig. 2**). HNRTs resulted in the presence of a duplication of a chromosomal region and loss of the corresponding homeologous region, which is a common phenomenon in *B. napus* (Chaloub *et al.* 2015, Zhao *et al.* 2006). These HNRTs increased the level of heterozygosity in the RILs (Zhao *et al.* 2006), and thus affected our discovery and genotyping of SNPs.

The sequences of all loci on the genetic linkage map were aligned to the *B. napus* reference genome sequence (**Fig. 3**). If a locus was mapped to multiple positions in the

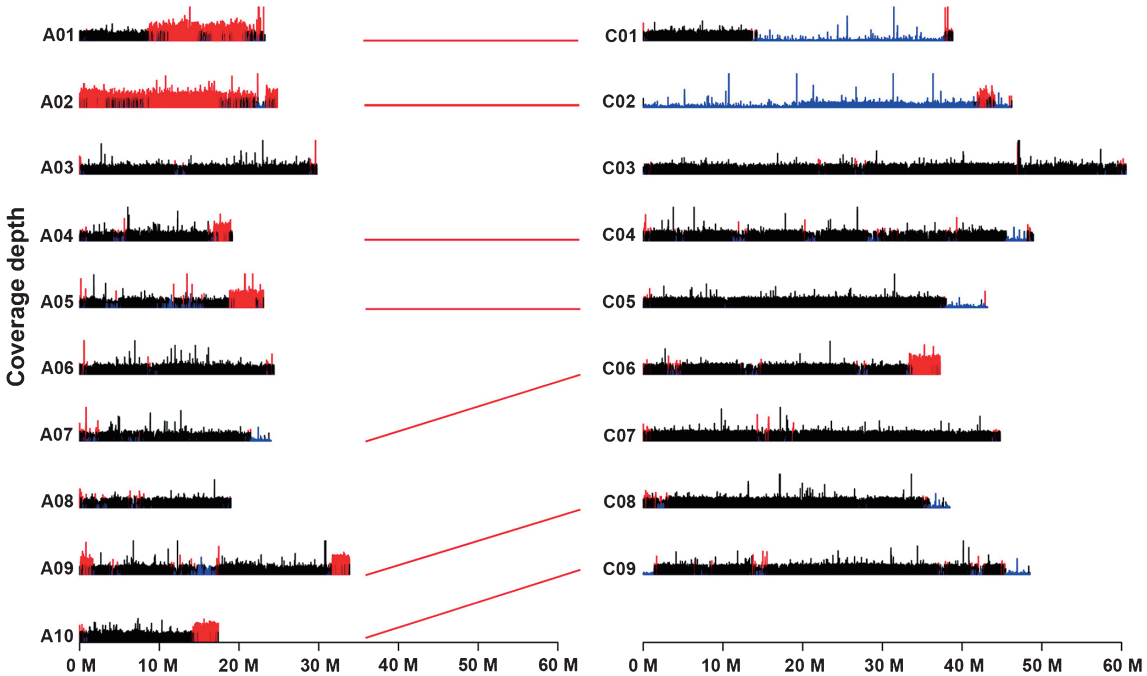

Fig. 2. Homeologous nonreciprocal transpositions (HNRTs) or homeologous exchanges (HEs) between *B. napus* chromosomes A2 and C2. Coverage depth was obtained after mapping Illumina sequence reads to the reference genome of *B. napus*. Segmental HEs are revealed based on sequence read coverage analysis, where a duplication (red) is revealed by geater coverage for a given segment than the rest of the genome (black) and a deletion (blue) by little or no coverage for the corresponding homeologous segment. The X axis indicated the chromosome length, sizes of chromosomes are indicated in Mb. The Y axis showed the coverage depth of sequence reads mapped to the reference. The red lines indicated HNRT or HE occurred between the two chromosomes.

B. napus genome, only the location with the best hit was selected for colinearity analysis. Alignments indicated that all LGs had a good colinearity with the *B. napus* reference genome 'Darmour-*bzh*' with several minor inconsistencies (**Fig. 3**). The inconsistencies such as regions on chromosome A05, C01, C04 and C09 (**Fig. 3**) might be caused by chromosomal rearrangement in '352', or caused by misassembly in the reference genome sequence.

Analysis of the goodness of fit indicated that 411 (26.9%) markers showed a distorted segregation ($P < 0.05$). The distorted segregation markers were unevenly distributed across the 19 chromosomes. The majority of the distorted markers (63.75%) biased towards the parent '352'. Markers on A03, A04, A10, C01, C04 and C09 skewed to 'M201', while markers on A02, A05, A06, A08, C02, C03, C05, C06 and C07 skewed to '352'.

QTLs detected for the four traits

QTL analysis was performed using the phenotypic data collected in two winter environments and one spring environment. A total of 18, 11, 13 and 12 QTLs were detected for oil content, silique length, seeds per silique and seed weight, respectively (**Supplemental Fig. 1**, **Supplemental Table 5**). The 33 QTLs with overlapping confidence intervals were integrated into 15 consensus QTLs (**Table 2**), all of these identified 37 QTLs with 24 located in the A subgenome and 13 in the C subgenome. Most of the QTLs for these four traits individually explained a small fraction of the corresponding phenotypic variation. In addition, most of the 37 QTLs were only in the spring environment (Gansu, Y11) or in one or two winter environments (Wuhan, Y12 and Y13) and explained less 10% of the phenotypic variation (**Table 2**), suggesting that these four traits are controlled by multiple QTLs with minor effects and most of the QTLs are specific to environment. A total of nine SL QTLs were detected: two of them detected both in the spring and semi-winter environments, three only in the spring environment, and four only in the semi-winter environments. The number of the SL QTLs detected in the spring environment was close to that detected in the semi-winter environment. In contrast, much fewer OC, SS, and SW QTLs were detected in the spring environment than in the semi-winter environments, respectively. The differences in the numbers of QTLs detected between the two environments among the four traits might partly reflect the different trait heritability and allele contribution from the parents. For example, 11 of the 12 OC QTLs were identified only in one or two semi-winter environments, and the QTL alleles came from the semi-winter parent "M201", suggesting that some of the 11 OC QTLs might play a role in regulating the adaptability of "M201" to the semi-winter growing conditions. It is worth noting that one consensus QTL for oil content (*cqOCA10b*) was repeatedly detected in the spring and the two winter environments and explaining 9.8–12.8% of the phenotypic variation.

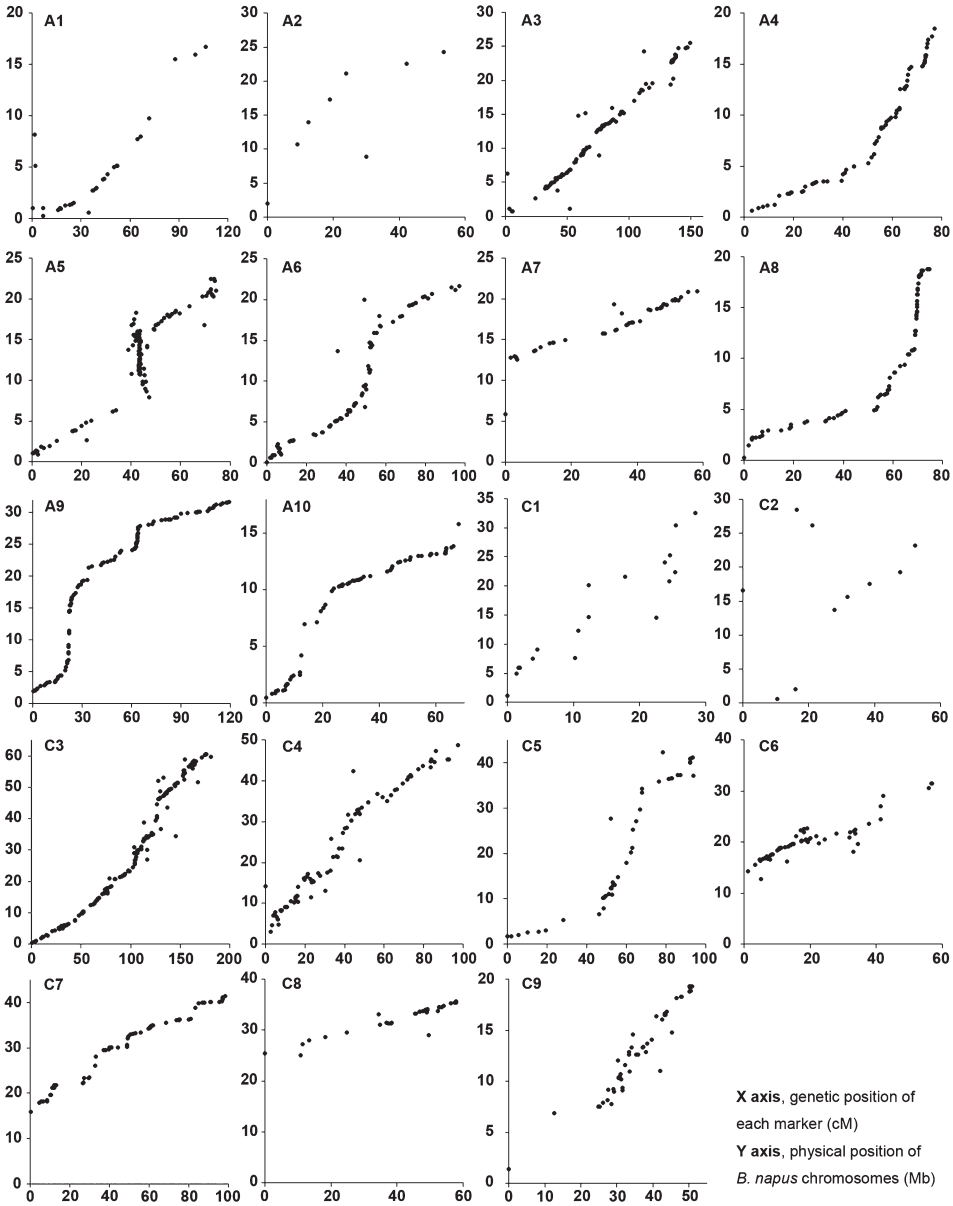

Fig. 3. Alignments between the recombinant inbreed line (RIL) linkage maps and the *B. napus* reference genome sequences. The X axis indicated the genetic position of each marker (cM), and the Y axis indicated the physical position of reference sequence of each corresponding *B. napus* chromosomes (Mb).

QTL of phenotypically correlated yield-related traits may co-localize (Tuberosa *et al.* 2002). We examined the confidence intervals of consensus QTL for different traits by meta–analysis. overlapped consensus QTLs were integrated into seven unique QTL (**Table 3**). These results indicated that these QTLs have pleiotropic effects or tightly linked.

Epistatic interactions between loci

Epistatic interactions were detected for OC, SL, SS and SW. Ten pairs of interactions involving 19 loci were detected for all traits (**Table 4**). These loci distributed on 12 linkage groups were identified to have effects on the four traits. Two loci on A06 (M505-M58 and M1572-M1423) were found to colocalize for *cqSWA6a* and *cqSLA6b*, respectively. These interactions f in total explained less than 1.5% of the phenotypic variation of each trait, suggesting that the effects of digenic interactions are very small and these four traits are primarily controlled by additive effect.

Discussion

OC, SL, SS, and SW are among the mostly studied traits in rapeseed (Chen *et al.* 2007, Javed *et al.* 2016, Qi *et al.* 2014, Wang *et al.* 2013, Yang *et al.* 2012). We assigned QTL regions onto the *B. napus* genome (Version 4.1) (Chalhoub *et al.* 2014) through BLAST analysis of markers linked to the QTLs, and compared our QTLs with those previously reported ones. Of the 12 OC QTLs in this study, *cqOCA1b*,

Table 2. Consensus quantitative trait loci (QTLs) for oil content (OC), sileque length (SL), seeds per silique (SS), and seed weight (SS) obtained by meta-analysis in all three environments in the recombinant inbreed lines (RILs)

Trait	Consensus QTL	LG	Position	CI	LOD	R^2 (%)	Add	Env	QTL in Ref
OC	*cqOCA1b*	A01	21.4	19.4–23.3	2.5–3.8	4.2–6.6	0.78–0.82	Y12/Y13	*oilA1-1* Jiang *et al.* (2014)
	cqOCA10a	A10	47.3	46.4–48.2	4.1–6.8	7.6–11.9	0.84–1.37	Y12/Y13	
	cqOCA10b	A10	57.7	56.9–58.4	5.3–7.6	9.8–12.8	1.08–1.41	Y11/Y12/Y13	
	cqOCC4	C04	71.5	69.8–73.2	2.4–2.6	3.7–4.1	0.58–0.81	Y12/Y13	
	cqOCC8b	C08	37.8	35.9–39.7	2.2–4.5	3.5–7.4	0.74–0.83	Y12/Y13	
SL	*cqSLA1*	A01	25.7	22.5–29.0	2.2–4.1	4.2–7.4	0.14–0.16	Y11/Y12	*sl1* Chen *et al.* (2007)
	cqSLA6b	A06	41.5	40.9–42.1	4.2–4.8	7.0–9.2	0.15–0.21	Y11/Y12	
SS	*cqSSA1b*	A01	44.1	43.6–44.7	2.9–3.6	5.5–6.3	0.68–0.80	Y12/Y13	
	cqSSA6a	A06	5.8	5.4–6.5	3.6–3.9	6.7–7.6	–0.86––0.81	Y11/Y12	
	cqSSC1	C01	25.8	25.4–26.2	2.8–3.1	5.4–6.4	0.71–0.87	Y11/Y12/Y13	
	cqSSC2	C02	38.4	36.8–40.0	3.6–3.8	6.6–6.7	0.81–0.87	Y12/Y13	*cqSLC2* Yang *et al.* (2012)
SW	*cqSWA3c*	A03	113.5	112.3–114.9	2.2–4.4	4.6–7.8	–0.11––0.10	Y11/Y12	
	cqSWA6a	A06	24.6	22.6–26.6	2.4–2.8	3.1–4.1	0.09	Y12/Y13	
	cqSWA6b	A06	31.6	29.6–33.6	2.3–2.4	3.8–4.2	0.08–0.09	Y12/Y13	
	cqSWA7b	A07	56.2	55.8–56.7	4.0–4.1	6.7–7.0	0.11	Y12/Y13	*qSWA7.1* Yang *et al.* (2012)

All QTLs were prefixed with "*cq*", followed by the abbreviated trait name, mapped linkage group, and a small alphabet (a, b, ...) representing multiple QTLs if they are detected for the trait in the same linkage group and same environment. For example, *cqOCA1a* is one of the two QTLs for oil content (OC) detected on linkage A1, and *cqOCA1b* is another one.

CI: the flanking marker closest to the 95% confidence interval; R^2: percentage of the phenotypic variation explained by the QTL.

Additive effects indicate the effects of "M201" allele.

Y11–rapeseed growth environment in 2011 at Hezhen, Gansu Province, China; Y12 and Y13–rapeseed growth environments in 2012 and 2013 at the experimental farm in Wuhan, Hubei Province, China.

QTL in Ref were the QTL detected in previous studies. The references for the QTL were also shown in the same column.

Table 3. Unique quantitative trait loci (QTLs) for oil content (OC), sileque length (SL), seeds per silique (SS), and seed weight (SS) obtained by meta-analysis in all three environments in the recombinant inbreed lines (RILs)

Unique QTL	Consensus QTL	LG	Position	CI
uqA1	*cqOCA1b* *cqSLA1*	A1	22.5	20.8–24.1
uqA3	*cqSSA3* *cqSWA3a*	A3	24.1	19.9–28.3
uqA6a	*cqSLA6a* *cqSSA6b*	A6	35.4	34.6–36.2
uqA6b	*cqSLA6b* *cqSSA6c*	A6	41.6	41.0–42.1
uqA7	*cqOCA7* *cqSWA7a*	A7	50.2	49.4–51.0
cqC1	*cqOCC1b* *cqSSC1*	C1	25.8	25.4–26.2
uqC2	*cqOCC2* *cqSLC2* *cqSSC2*	C2	38.5	37.1–39.8

LG–linkage groups; *CI*–confidence interval for the unique QTLs.

cqOCA7 and *cqOCC2* were co-localized with o*ilA1-1*, *oilA7-1* and *oilC2-3* reported by Jiang *et al.* (2014), respectively; and *cqOCC8a* was co-localized with *qOC-C8-2* in the KN population (Wang *et al.* 2013). *cqOCA1a*, *cqOCA10a* and *cqOCA10b* in A subgenome and *cqOCC1a*, *cqOCC1b*, *cqOCC3*, *cqOCC4* and *cqOCC8b* in C subgenome apparently were new. Of our 9 SL QTLs, *cqSLA1* was co-localized with *sl1* (Chen *et al.* 2007). *cqSLC2* and *cqSLC3b* were co-localized with *cqSLC2* and *cqSLC3b* reported by Yang *et al.* (2012), respectively. The remaining six, *cqSLA4a*, *cqSLA4b*, *cqSLA4c*, *cqSLA6a cqSLA6b*, and *cqSLC3a*, appeared to be new. Only one pleiotropic SS QTL, *cqSSC2*, was co-localized with *cqSLC2* in the study of Yang *et al.* (2012), and the other 7 QTLs were likely new. For SW, *cqSWA3b*, *cqSWA7a* and *cqSWA7b* in this study were co-localized with *qSL.N3-3* (Zhang *et al.* 2011), *TSWA7a-06* (Fan *et al.* 2010) and *qSWA7.1* (Yang *et al.* 2012), respectively. The other 5 SW QTLs, including *cqSWA3a*, *cqSWA3c*, *cqSWA6a* and *cqSWA6b*, and *cqSWC6* apparently were new. In this study three SS QTLs (*cqSSA6a*, *cqSSA6b* and *cqSSA6c*) were detected on A06 and one QTL (*cqSSC1*) on C01 whereas there was no SS QTL detected on A06 and C01 in the previous studies (Chen *et al.* 2011, Qi *et al.* 2014, Shi *et al.* 2009, Zhang *et al.* 2011). In this study, we identified 26 potential new QTLs and 11 QTLs co-localized with previous studies. And the two stably expressed QTLs are great value for breeding cultivars with wide flexibility in different environments. These co-localized and potentially new QTLs acquired in this study facilitate further understanding of these traits and utilization in genetic breeding in the furture.

Large and stable effects of QTL are considered as the key factors for fine mapping and map-based cloning success. OC is most important agronomic trait for rapeseed, it is controlled by complex mechanisms and highly influenced by the environment. So far, only a gene increased oil and oleic-acid contents was cloned in maize (Zheng *et al.* 2008). In oilseed rape, many QTLs for OC have been detected. However, there has no genes controlling seed OC isolated, this is because, most of the detected OC QTLs explain less

Table 4. Epistatic interactions for the four traits

Trait[a]	Chromosome	Marker interval-i	Chromosome	Marker interval-j	Additive by additive effect	R^2 (%)
OC	A03	M301–M551	A04	M836–M394	–4.54	0.77
	A08	M1325–BRGMS2025	C04	M1153–M169	–1.42	0.26
SL	A02	ID9–ID7	A06	M1572–M1423	0.16	0.03
	A03	M301–M551	C07	M463–M130	–0.48	0.09
	A05	M528–M150	C04	M1179–M939	–0.14	0.03
SS	A02	ID108–BNGMS635	C03	M6–M618	–1.73	0.25
	C03	M660–M1619	C03	M1190–M118	–1.84	0.31
SW	A07	M316–M106	C01	M285–M1006	–0.04	0.02
	A06	M505–M58	C04	M1484–M682	–0.11	0.02
	A09	M545–M1590	C03	M318–ID71	0.12	0.02

[a] OC–oil content; SL–silique length; SS–seeds per silique; and SW–seed weight.

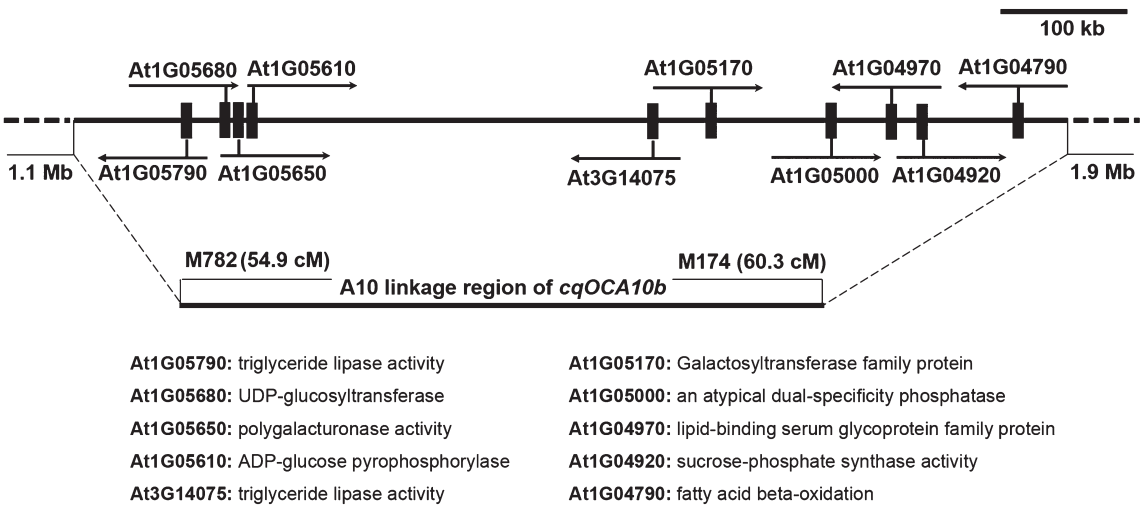

Fig. 4. Linkage and genomic region of *cqOCA10b* on the *B. rapa* genome. The vertical black bars represent some candidate genes.

than 10% of the phenotypic variation (Jiang *et al.* 2014, Sun *et al.* 2012, Wang *et al.* 2013). In this study, a major QTL (*cqOCA10b*) was repeatedly detected in one spring and two winter environments, which may enable us to conduct the fine mapping of candidate genes for OC. The six markers (M174, ID52, M1601, M53, M730 and M782) linked to *cqOCA10b* will be useful for MAS (marker assisted selection) breeding. The interval of *cqOCA10b* between markers M782 and M174 (representing an approximately 6 cM region of chromosome A10) represented approximately 614 kb of the *B. napus* genome, a total of 124 genes (**Supplemental Table 6**) were identified in the region. Our primary candidate gene screening of *B. rapa* and *A. thaliana* orthologues found four genes were directly involved in lipid metabolism activities, i.e., fatty acid beta-oxidation (Wang *et al.* 2008), lipid-binding (Hanada 2011), mono-/di-acylglycerol lipase (Li-Beisson *et al.* 2010), triglyceride lipase activity (Saleh 2008). Six other genes were homologous to sucrose-phosphate synthase (Lutfiyya *et al.* 2007), dual-specificity phosphatase (Roma-Mateo *et al.* 2011), galactosyltransferase (Qu *et al.* 2008), ADP-glucose pyrophosphorylase (Schwarte *et al.* 2015), UDP-glucosyltransferase (Li *et al.* 2015a), and polygalacturonase (Lou *et al.* 2007). The two orthologues genes (*AT3G14075* and *AT1G05790*) likely have directly relationship with the lipid synthesis, these two orthologues genes may be the best choice for the candidate gene (**Fig. 4**, **Supplemental Table 6**). Although more candidate genes may be found, these function-known candidate genes in the A10 OC QTL region are very encouraging. Fine mapping and map-based cloning have been demonstrated to be one of the most efficient ways to dissect these trait-related quantitative trait loci (Miura *et al.* 2011, Takeda and Matsuoka 2008). The QTL with strongly expressed and stable effects was highly suitable for map-based cloning. Thus, validating potential candidate genes is a reliable and feasible strategy for QTL cloning. The information obtained from this study demonstrates potentially novel roles for candidate genes in rapeseed oil accumulation. Future works will provide an opportunity to identify the genes that control seed OC in *B. napus*.

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