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ORIGINAL PAPER

Identification and mapping of a major dominant quantitative trait locus controlling seeds per silique as a single Mendelian factor in *Brassica napus* L.

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Abstract One putative quantitative trait locus (QTL) for seeds per silique (*SS*), *cqSS*.A8, was identified using a double haploid (DH) population in *Brassica napus*, and near-isogenic lines (NILs; BC_3F_1) for *cqSS*.A8 were developed. However, the flanking markers from *cqSS*.A8 showed no significant difference using single-marker analysis, even though the frequency distribution of *SS* in the BC_3F_1 was bimodal, suggesting that one novel locus existed. In this study, we characterized the effects of this locus in the NILs and used a published linkage map to determine its location. A three-step approach was designed for mapping the locus in the NILs (BC_3F_2): (1) determining the individual BC_3F_2 genotype at the locus using a progeny test; (2) identifying amplified fragment length polymorphism (AFLP) markers linked to the locus using a combination of AFLP and bulked segregant analysis; and (3) determining the location and effects of this locus. QTL analysis in the BC_3F_2 revealed that this locus explained 85.8 and 55.7 % of phenotypic variance for *SS* and *SL*, respectively. Its additive and dominant effects on *SS* were 6.1 and 5.7, respectively. The locus was validated using a DH population by composite interval mapping and located to linkage group C9 (designated as *qSS*.C9). Mapping *qSS*.C9 was undertaken using 230 extremely low-*SS* plants of a BC_4F_1 population containing 807 plants. We found that *qSS*.C9 delimited a 1.005-Mb interval including 218 predicted genes in the reference *Brassica rapa* (Chiifu-401). These results will greatly facilitate map-based cloning of *qSS*.C9 and seed yield improvement in rapeseed.

Abbreviations

AFLP	Amplified fragment length polymorphism
CB-NILs	Consecutive backcrossing near-isogenic lines
DH	Double haploid
HIF-NILs	Heterogeneous inbred family near-isogenic lines
LOD	Logarithm of odds score
NIL	Near-isogenic line
QTL	Quantitative trait locus
SCAR	Sequence characterized amplified region
SSR	Simple sequence repeats
SS	Seeds per silique
SL	Silique length
SW	1,000-seed weight
TP-MBS	Trait performance and marker-assisted background selection
TP-NILs	Trait performance near-isogenic lines

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Introduction

Rapeseed (*Brassica napus* L.), derived from the mesopolyploids *Brassica oleracea* ($n = 9$) and *Brassica rapa* ($n = 10$) (Nagaharu 1935), is cultivated worldwide as an oilseed crop and it provides 15 % of the world's edible vegetable oil (Fu 2004). Therefore, exploitation of cultivars with high seed yield is an important focus in rapeseed

breeding programs. Seeds per silique (*SS*) is one of the three direct components of yield (siliques per plant, seeds per silique, and seed weight) in rapeseed and has always received much attention. Many quantitative trait locus (QTL) have been mapped for *SS* in previous studies. Analysis of quantitative data using a double haploid (DH) population revealed three QTL for *SS*, which explained 6.8, 8.2, and 3.6 % of phenotypic variance individually (Radoev et al. 2008). Shi et al. (2009) detected QTL for *SS*, which occurred in 13 linkage groups (A1, A2, A3, A4, A5, A7, A8, A9, C2, C3, C6, C8, C9) from 10 natural environments, and found that the identified QTL each could explain 2.4 to 20.5 % of phenotypic variance. Zhang et al. (2011) used two *B. napus* lines, Y106 exhibiting extremely high *SS* and HZ396 with extremely low *SS*, to detect QTL for silique traits and found two consensus QTL for *SS*, *cqSS*.A8 and *cqSS*.C3, which explained 9.4 and 11.4 % of the phenotypic variance, respectively. Although primary mapping populations have been widely used for QTL mapping in rapeseed, it is still difficult to utilize the existing mapping results in the fine mapping and cloning of these QTL (Zamir 2001). The immediate cause is the genetic background noise, which is comprised of other QTL (Korff et al. 2004; Xing et al. 2008; Zamir 2001). To block the genetic background noise and elucidate the characteristics of a QTL of interest, it is vital to exploit near-isogenic lines (NILs) (Tanksley and Nelson 1996). An advanced backcross strategy has been widely used for developing NILs in tomato, rice, maize, and wheat (Alpert et al. 1995; Ashikari et al. 2005; Liu et al. 2006; Szalma et al. 2007). To date, however, no NIL of a QTL for *SS* in *B. napus* has been successfully developed.

In terms of successful examples of developing NILs, reliable QTL mapping is a prerequisite for the use of this strategy (Alpert et al. 1995; Xing et al. 2008). To obtain a reliable QTL map, besides reliable phenotypic data, a reliable linkage map is necessary to serve as a map of the chromosomes; a linkage map can be produced from a considerable number of molecular markers evenly distributed on every chromosome or linkage group. Thus far, however, it has proven difficult to construct a relatively saturated linkage map in the absence of a complete genome sequence for *B. napus*, although many simple sequence repeats (SSR) markers (Xu et al. 2010; Li et al. 2010) and single nucleotide polymorphisms (Bancroft et al. 2011) have been identified in recent years. Clearly, an unsaturated linkage map affects the results of QTL mapping. In some cases, the confidence interval of a QTL of interest may be a large gap due to a lack of polymorphic markers in the QTL region during the construction of a linkage map. Thus, a QTL based on an unsaturated linkage map causes an undetected error in the location of the QTL, even for a major one. In this case, it can be a useful alternative to develop NILs via trait performance and marker-assisted background selection (TP-MBS).

In our previous study, one putative QTL for *SS*, *cqSS*.A8, was identified in *B. napus* using a DH population derived from HZ396 × Y106 (Zhang et al. 2011). To define *cqSS*.A8, NILs (BC_3F_1) for *cqSS*.A8 were first developed by successive crossing and backcrossing of HZ396 (low SS) with Y106 (high SS), using HZ396 as the recurrent parent. However, flanking markers from *cqSS*.A8 showed no significant difference based on single-marker analysis even though the frequency distribution of *SS* in the BC_3F_1 population was bimodal, which would suggest that one novel locus existed. In the present study, we aimed to characterize the effects of this novel locus in the NILs (BC_3F_2 and BC_4F_1) and used a published linkage group to determine the location.

Materials and methods

Development of NIL for undetected QTL via TP-MBS

In our previous study, one putative QTL for *SS*, *cqSS*.A8, was mapped to the interval between the amplified fragment length polymorphism (AFLP) marker EA02MG05-210 and the SSR marker CB10364 in linkage group A8 using a DH population derived from HZ396 × Y106 in *B. napus* (Zhang et al. 2011). To define *cqSS*.A8, NILs for *cqSS*.A8 were developed by successive backcrossing. A cross was made between HZ396 with low *SS* and Y106 exhibiting high *SS* (Zhang et al. 2011). The resulting F_1 plants were backcrossed with HZ396 as the male parent. During the backcrossing process, the progenies of each backcross were first selected by EA02MG05-210 and CB10364, supplemented by trait performance for high *SS* (i.e., estimating *SS* as a mean of the first 10 well-developed siliques of the main raceme immediately above the first side branch) for each plant, because the positive allele encoding *cqSS*.A8 originated from the parental line Y106. The plants that had greater than 26 seeds per silique were selected (Fig. 1). The selected individuals were then analyzed by 86 anchor microsatellite markers (Table S1) evenly distributed on the 19 linkage groups in *B. napus* (Zhang et al. 2011) for background selection. Progenies obtained by backcross of the high-*SS* individual with the highest recovery rates were selected for further crossing. After the third backcrossing, all flanking markers including AFLP markers (EA02MG05-210, SA10TC11-100, SA03TC09-370, SA03TC09-650, SA08TC07-370) and SSR markers (CB10026, CB10364, BnGMS373) from *cqSS*.A8 showed no significant difference using single-marker analysis, even though frequency distribution of *SS* in the BC_3F_1 population was bimodal, which would suggest that there is one

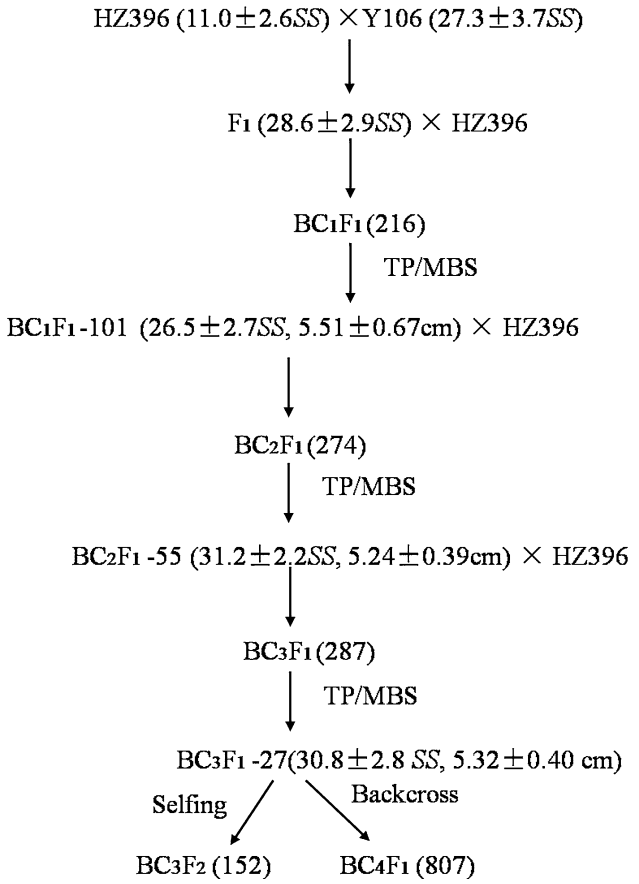

Fig. 1 Procedure for developing near-isogenic lines (NILs) using trait performance and marker-assisted background selection (TP-MBS). *Values in parenthesis* are the numbers of the plants tested or seeds per silique or silique length of selected individuals at each stage in the procedure for developing NILs

novel locus. To obtain NILs of this novel locus, the selected plants with high *SS* in the BC_3F_1 population were analyzed using markers EA02MG05-210 and CB10364 for background selection. Thus, one plant (BC_3F_1-27), which had 30.8 ± 2.8 seeds per silique and 98.8 % similarity with HZ396 besides one SSR marker pMR181 (which also showed no significant difference based on single-marker analysis), was selfed and backcrossed to obtain the populations of BC_3F_2 and BC_4F_1, respectively.

Field trial and trait measurement

A total of 152 BC_3F_2 plants, together with their parents HZ396 and Y106, were grown during the rapeseed growing season of 2010 on the experimental farm of Huazhong Agricultural University, Wuhan, China. Twelve plants per row were grown, with a distance of 0.15 m between plants within a row and 0.3 m between rows. The field was equipped with netting to prevent birds from eating the crop. The main racemes of all the BC_3F_2 plants were open pollinated to keep a full record of fertilization rates to estimate *SS* and silique length (*SL*), and the first side branches were self-pollinated to obtain BC_3F_3 seeds. All 807 BC_4F_1 plants were grown at the same experimental farm with the same planting density as that of BC_3F_2 plants.

The BC_3F_3 families of each of the 152 BC_3F_2 plants, together with parental lines and recombinants from BC_4F_1, were grown in a netted field. There were two rows per plot consisting of 24 plants, and the length and width of the rows were 1.8 and 0.3 m, respectively. The field trial was carried out in May 2011 in Gansu, China. The seeds were sown by hand and the field management followed standard agricultural practice. From each BC_3F_3 family and recombinant, 20 plants were sampled for the progeny test.

We measured *SS* and *SL* using the method described by Radoev et al. (2008). At the mature stage, the first 10 well-developed siliques of the main raceme immediately above the first side branch were harvested from each plant. The siliques were air-dried for about 2 weeks, and the mean *SS* and *SL* were estimated from the 10 siliques. We did not evaluate 1000-seed weight (*SW*) in Gansu, a spring-type rapeseed growing area in northwestern China, because the plant materials were semi-winter-type rapeseed and were extremely sensitive to frost damage during the silique maturity stage.

Data analysis

Analyses of variances were performed using PROC MIXED (SAS Institute Inc., Cary, North Carolina, USA), where genotype was treated as a random effect. The heritability (h^2) of *SS* and *SL* in the BC_3F_2 population was calculated as: $h^2 = [V_{F2} - 1/2(V_{P1} + V_{P2})]/V_{F2}$, where V_{F2}, V_{P1}, and V_{P2} are the phenotypic variance of BC_3F_2, P_1, and P_2, respectively.

Use of bulked extremes to identify AFLP markers linked to the locus

Genomic DNA from the parents and the BC_3F_2 and BC_4F_1 individuals was extracted using a modified cetyltrimethyl ammonium bromide method (Doyle and Doyle 1987) and preserved at −20 °C. Before polymerase chain reaction, the DNA was diluted to 50 ng/μL with double distilled water.

Bulked segregant analysis (Michelmore et al. 1991) combined with the AFLP technique (Vos et al. 1995) were used to identify molecular markers linked to the locus. Based on the genotypes identified by a progeny test, 10 plants that had homozygous genotypes for the Y106 allele and another 10 plants that had homozygous genotypes for the HZ396 allele from the BC_3F_2 population were

randomly selected for constructing low-*SS* bulk and high-*SS* bulk. Each bulk was pooled by equivalent amounts of DNA from the 10 plants. The two bulks were digested with two restriction enzyme combinations, *Eco*RI/*Mse*I and *Sac*I/*Mse*I. A total of 512 primer combinations (256 [*Eco*RI + 3]/[*Mse*I + 3] and 256 [*Sac*I + 3]/[*Mse*I + 3]) were screened. The AFLP procedure, electrophoresis, and silver staining were performed following the protocol described by Lu et al. (2004). AFLP markers were named according to the primer combination.

Development of SCAR and SSR markers linked to the locus

The production of clones and sequences of the AFLP fragments exhibiting polymorphism was performed as described by He et al. (2008). Primer 3 (http://www.biowb.sdsc.edu/CGI/BW.cgi) was used to design primers for sequence characterized amplified region (SCAR) markers based on these AFLP fragments sequences.

The genome sequence of *B. rapa* is available (Cheng et al. 2011; Wang et al. 2011b) (The Brassica database, BRAD; http://brassicadb.org), so all polymorphic fragments around a given QTL were sequenced to identify putatively homologous regions using a BLASTN search against the *Brassica* genome database. Because *B. rapa* is one of the diploid progenitors of *B. napus*, the nucleotide repeats distribution is, to a certain extent, in accordance with the patterns in *B. rapa*. The SSR primers were designed according to the homologous regions, as described by Cheng et al. (2009). When a given QTL was mapped to a linkage group in *B. napus*, several SSR markers in the reference maps (Li et al. 2010; Wang et al. 2011a) were selected for a polymorphism survey.

The SCAR amplification followed Xie et al. (2008), and SSR amplification was performed as described by Piquemal et al. (2005). The amplified products were resolved on a 6 % denaturing polyacrylamide gel.

Determining the location and effects of the locus

We designed a three-step approach for mapping the locus: (1) determining the location and the effects of this locus in the BC_3F_2; (2) using a published linkage group to determine the location; and (3) further mapping the locus using the extremely low-*SS* plants in the BC_4F_1. The local molecular linkage map was constructed based on the BC_3F_2 population using Mapmaker 3.0 (Lincoln et al. 1992) and the Kosambi function (Kosambi 1944) to calculate the genetic distance. *SS* performance of BC_3F_3 was used to determine the individual BC_3F_2 genotype at the QTL. Then the QTL was treated as a marker and directly located within the linkage group using Mapmaker 3.0. Interval mapping was performed on the BC_3F_2 data with the use of Mapmaker/QTL 1.1 (Lander and Botstein 1989). The DH population derived from the cross of HZ396 × Y106 (Zhang et al. 2011) was used again to locate the linked markers to a specific linkage group. A linkage map was constructed using Mapmaker/EXP V3.0 (Lincoln et al. 1992). A minimum logarithm of odds (LOD) score of 3.0 and a maximum distance of 40 cM were used to group loci into the linkage groups. The values of recombination fractions were converted into genetic map distances (cM) by means of the Kosambi mapping function (Kosambi 1944).

QTL analysis was performed by composite interval mapping (Zeng 1994) using WinQTL Cartographer 2.5 software (http://statgen.ncsu.edu/qtlcart/WQTLCart.htm). The experiment-wise LOD threshold was determined by permutation analysis (Churchill and Doerge 1994) with 1,000 repetitions. LOD scores corresponding to $P = 0.05$ (3.1 for DH) were used for identifying significant QTL. The additive effect and the percentage of variance (R^2) explained by individual QTL were estimated. For the designation of QTL, we followed the recommendation of McCouch et al. (1997). By assuming that all extremely low *SS* were homozygous for the recessive allele (HZ396) at the locus, the recombinations in the BC_4F_1 were determined by the method as described by Xing et al. (2008).

Results

Phenotypic variations in the BC_3F_2 population of HZ396 × Y106

The frequency distributions of *SS* and *SL* in the BC_3F_2 population are shown in Figs. 2 and 3. *SS* showed a bimodal distribution, with 23 seeds per silique as the

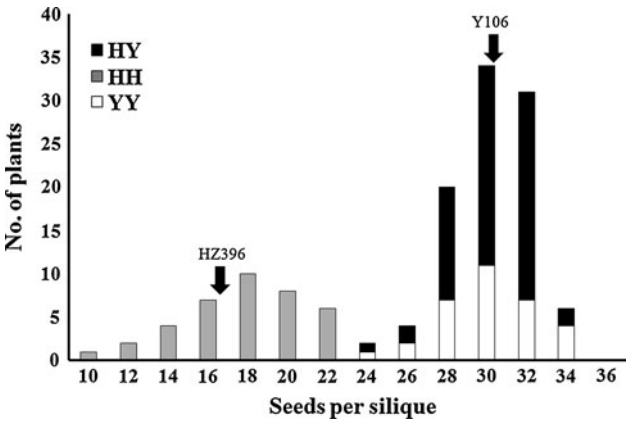

Fig. 2 Frequency distribution of seeds per silique in the BC_3F_2 population. The three genotypes of homozygote Y106 (YY) and HZ396 (HH) and heterozygote (HY) were identified by a progeny test

boundary. The frequency distribution of *SL* deviated from normality and appeared to have a bimodal pattern. A significant positive correlation between *SS* and *SL* ($r = 0.74$) was observed.

In the BC_3F_3 families of each BC_3F_2 individual, *SS* displayed three phenotypic types: 40 families with uniformly low *SS*, 35 families with uniformly high *SS*, and 77 families with variant *SS*. Considering that the recurrent parent HZ396 has low *SS*, the three types were matched to three genotypes: homozygote for HZ396 allele (HH), homozygote for Y106 allele (YY), and heterozygote (HY) (Table 1). A Chi-square test showed that the ratio of the three genotypes fit the Mendelian segregation ratio (1:2:1) of a single gene ($\chi^2 = 0.355$, $P > 0.05$), suggesting that one locus existed in the BC_3F_2 population. All individuals in the BC_3F_2 population fell between the mean of HZ396 *SS* minus twofold its standard deviation and the mean of Y106 *SS* plus twofold its standard deviation, indicating that no transgressive segregation was observed for *SS*.

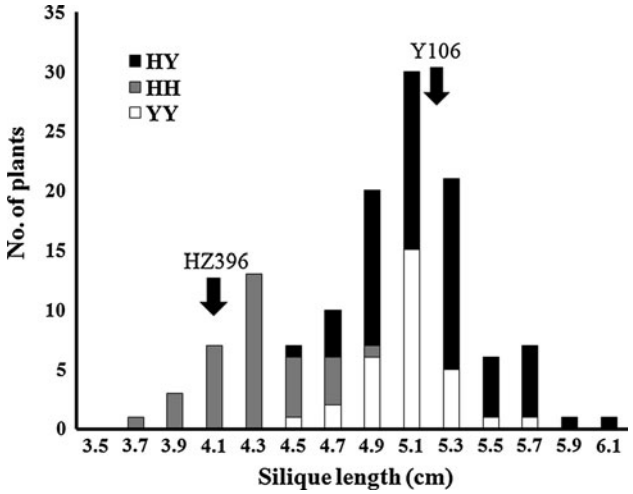

Fig. 3 Frequency distribution of silique length in the BC_3F_2 population. The three genotypes of homozygote Y106 (YY) and HZ396 (HH) and heterozygote (HY) were identified by a progeny test

Significant differences among the three genotypes demonstrated that genetic influence on *SS* was pervasive (Table S2). The high heritabilities of *SS* and *SL* in the BC_3F_2 population (0.89 and 0.77, respectively) confirmed this. Multiple comparisons among the three genotypes showed that the average *SS* of the YY genotype (29.7 ± 2.32) was significantly higher than that of HH (17.5 ± 2.72; Table 1). The average *SS* of heterozygotes (29.3 ± 1.83) was similar to that of YY, suggesting that the locus controlling the high-*SS* characteristic is fully dominant. For *SL*, similar results were found (Fig. 3; Table 1). These findings indicated that one dominant locus controlled the segregations of both *SS* and *SL* in the BC_3F_2 population.

Construction of the local linkage map using AFLP markers linked to the *SS* locus

To develop markers linked to the locus, we utilized an AFLP assay combined with bulked segregant analysis. Three AFLP markers linked to the locus were identified by bulked segregation analysis (Table 2). The three markers were used to genotype the BC_3F_2 population, and a local linkage map, including the *SS* locus determined based on the performance of their progenies, was constructed (Fig. 4a). Given that AFLP has limitations in genotype analysis, we attempted to convert the two AFLP markers EA08MC13 and SA09MC04, which were closely linked to the *SS* locus at a distance of 0.60 and 5.12 cM, respectively, into SCAR markers. The AFLP marker SA09MC04 successfully converted into codominant SCAR marker SCC9-005 (annealing temperature = 60 °C) (see Table 5).

QTL analysis in the BC_3F_2 population of HZ396 × Y106

QTL analysis based on the BC_3F_2 population showed that this locus explained 85.8 % of phenotypic variance with additive and dominant effects of 6.1 and 5.7 seeds per

Table 1 Seeds per silique and silique length of the three genotypes of 152 individuals from the population of BC_3F_2

Traits[a]	Genotype	No. of plants	Range	Mean ± SD[b]
SS	Homozygote for Y106 allele (YY)	35	23.3–33.6	29.7 ± 2.32(A)
	Heterozygous (HY)	77	23.6–33.3	29.3 ± 1.83(A)
	Homozygote for HZ396 allele (HH)	40	10.3–22.8	17.5 ± 2.72(B)
SL	Homozygote for Y106 allele (YY)	35	4.49–5.67	5.09 ± 0.245(A)
	Heterozygous (HY)	77	4.46–5.98	4.99 ± 0.379(A)
	Homozygote for HZ396 allele (HH)	40	3.60–5.03	4.24 ± 0.300(B)

The genotypes were determined by progeny test

[a] *SS*, *SL*, *SW*, seeds per silique, silique length, 1,000-seed weight, respectively. The same is as below

[b] A and B indicate ranking by Duncan test at $P \leq 0.001$

Table 2 Description of AFLP markers that are linked to the *SS* locus

AFLP markers	Primer combinations	Fragment size (bp)	SCAR conversion
EA08MC13	E-ATG/M-CGA	137	–
SA09MC04	S-ACA/M-CAG	204	SCC9-005
SA07MC15	S-ATC/M-CGC	220	–

E = *Eco*RI primer, 5′-GACTGCGTACCAATTC-3′; S = *Sac*I primer, 5′-GACTGCGTACAAGCTC-3′; M = *Mse*I primer, 5′-GATGAGTCCTGAGTAA-3′

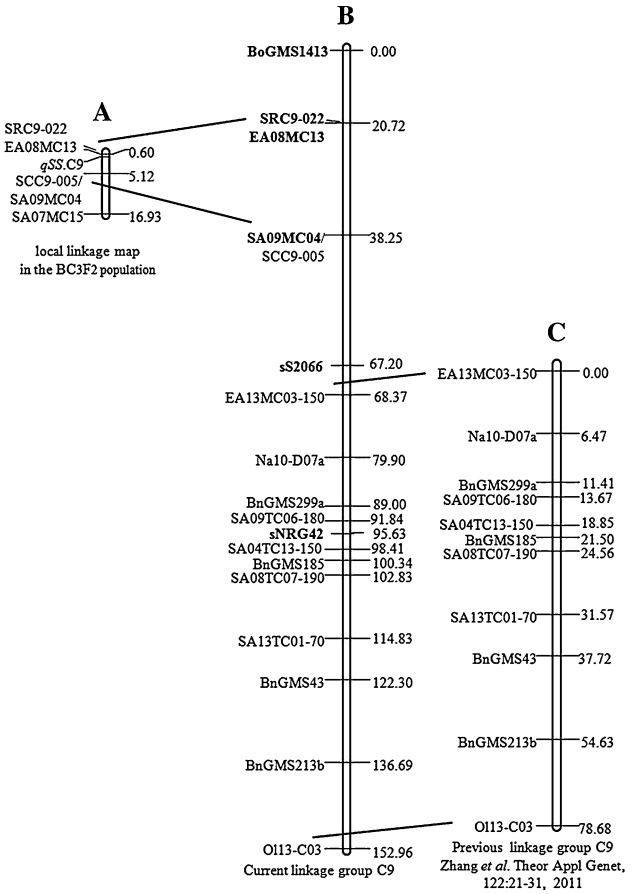

Fig. 4 Local linkage map of the QTL *qSS*.C9 in the BC_3F_2 population. **a** *qSS*.C9 was mapped as a codominant marker based on a progeny test and **b** positioned in the current linkage map of C9 of the present study and **c** previous linkage map of C9 of Zhang et al. (2011)

silique, respectively (Table 3). The locus, explaining 55.7 % of phenotypic variance of *SL*, was detected in the same interval, which suggested that the locus was major for *SS* and relatively minor for *SL*.

Validation of QTL in the DH population of HZ396 × Y106 by composite interval mapping

To determine the location of the *SS* locus in *B. napus*, two closely linked markers, EA08MC13 and SCC9-005, were used for screening the DH population derived from HZ396 × Y106 (Zhang et al. 2011). As a result, the two markers were mapped in linkage group C9 (Fig. 4b).

Table 3 Genetic effects on seeds per silique and silique length in the BC_3F_2 population by Mapmaker/QTL

Traits	LOD	*A*	*D*	*D*/*A*	R^2 (%)
SS	54.2	6.1	5.7	0.94	85.8
SL	22.6	0.40	0.45	1.13	55.7

LOD logarithm of odds score, *A* additive effect, positive additive effect means Y106 allele increasing trait values; *D* dominant effect; R^2 percentage of the phenotypic variance explained by the QTL

To validate the results based on the BC_3F_2 population, QTL analysis was further performed in the DH population using composite interval mapping. Three QTL, *qSS*.C9, *qSL*.C9, and *qSW*.C9, which influenced *SS* and *SL* as well as *SW* in the four different environments, were identified within the same interval in linkage group C9 with overlapping peaks (Table 4). A comparison of the current (Fig. 4b) and previous (Fig. 4c) linkage map of C9 showed that the QTL region not covered by the previous linkage map of C9 might explain the undetected error in the location of the QTL *qSS*.C9.

The QTL *qSS*.C9, which explained 57.77 % of the phenotypic variance of *SS*, showed a major effect, while the QTL *qSL*.C9 and *qSW*.C9, which explained 29.14 and 37.30 % of the phenotypic variance of *SL* and *SW*, respectively, exhibited relatively minor effects, in accordance with the results in the BC_3F_2 population. Interestingly, the additive effects of *qSS*.C9 and *qSL*.C9 (5.68 seeds per silique and 0.43 cm, respectively) were positive, whereas the additive effect of *qSW*.C9 (−0.47 g) showed a negative effect, which helps to explain the positive correlation between *SS* and *SL* ($r = 0.73$) and the negative correlation between *SS* and *SW* ($r = -0.62$) (Zhang et al. 2011). The results in the DH population combined with those in the BC_3F_2 population suggest the existence of a single locus with pleiotropic effects for *SS* and *SL* (for simplicity, we will refer to *qSS*.C9 and *qSL*.C9 as *qSS*.C9) and a probably linked QTL *qSW*.C9 for *SW* in linkage group C9.

Development of SSR markers linked to the locus

To enrich the markers linked to the locus, SSR markers surrounding this region were selected from linkage group C9 in two recent reference maps (Li et al. 2010; Wang et al. 2011a) to analyze the two parents. Only three SSR markers (BoGMS1413, sS2066, and sNRG42) showed polymorphisms between HZ396 and Y106. These markers were then tested on the plants from the DH population.

Table 4 Genetic effects of the *qSS*.C9 detected in four environments using a doubled haploid population derived from a cross between HZ396 and Y106 (Zhang et al. 2011)

Traits[a]	Interval markers	Peak position	Confidence interval	2007			2008						2009		
				Wuhan			Wuhan-I[d]			Wuhan-II[d]			Gansu		
				LOD	A^b	R^2 (%)[c]	LOD	A	R^2 (%)	LOD	A	R^2 (%)	LOD	A	R^2 (%)
SS	EA08MC13/ SCC9-005	21.91	19.7–24.2	22.2	4.3	47.70	27.1	6.2	65.35	19.5	5.4	60.99	22.1	6.8	57.03
SL	EA08MC13/ SCC9-005	21.91	19.3–24.1	10.9	0.34	23.45	15.0	0.46	36.04	12.5	0.48	36.70	8.7	0.42	20.38
SW	EA08MC13/ SCC9-005	21.91	19.8–24.5	12.1	−0.46	37.38	13.78	−0.47	37.21						

[a] For abbreviation, see Table 1

[b] Additive effect: positive additivity indicate that the QTL allele originated from the parental line Y106; negative additivity means that the QTL allele originated from the parental line HZ396

[c] Percentage of the phenotypic variance explained by the QTL

[d] Wuhan-I, the plant materials planted on 21st September in Wuhan; Wuhan-II, the plant materials planted on 6th October in Wuhan

Table 5 Characterization of markers linked to *qSS*.C9 and *Brassica rapa* syntenic region

Markers	Sequences (5′–3′)	Fragment size (bp)	SSR motifs	Homologous region or gene in *Brassica rapa* and E value
BoGMS1413	TGGGCTTCTTCTTACTTACCT TGCTCTGTCTATTATTCGTTTG	291	(TC)13	15590534–15589938, E−134
SRC9-022	TTGTGGTTTTTCTCTCCGTGATGCT TCGAGGCAGAACCATCGGGGT	211	(GGAT)4	14875375–14875897/Bra009015, 4E−73
SCC9-005/SA09MC04	GCAGCAACTTGGTTTCAGGT CAGCCCTTGGCTTAGACACT	204	–	13870257–13870472/Bra008797, 7E−38
sS2066	AATTAAGGGACCACGCAACA CCAGAACCCTCTTGATTCACTT	242	(TG)9	10325617–10325859, 9E−15
EA13MC03-150	–	150	–	10230503–10230634, 4E−48

Results indicated that all the SSR markers were located in linkage group C9 (Fig. 4b), which further confirmed that this locus is located in linkage group C9.

The region around the locus *qSS*.C9 showed a linear relationship with the *B. rapa* genome. Most of the markers were connected to chromosome A10 of *B. rapa* by BLASTN (http://brassicadb.org/brad/) (Table 5; Fig. 5). The closest markers flanking *qSS*.C9, EA08MC13 and SCC9-005, were positioned in the region between Bra008797 and Bra009015. The corresponding sequences located in this region were downloaded and analyzed, from which a total of 138 specific microsatellite primers were designed (data not shown). Only one microsatellite marker linked to *qSS*.C9, SRC9-022, was identified by bulked segregation analysis (Table 5). Linkage analysis of the BC_3F_2 population indicated that the dominant microsatellite marker, SRC9-022, was closely linked to *qSS*.C9, with a genetic distance of 0.60 cM (Fig. 4a).

Mapping of *qSS*.C9 using the extremely low-*SS* plants

The 152 plants in the BC_3F_2 population could be divided into two subgroups, with 23 seeds per silique as the boundary (Fig. 2). HZ396 alleles at *qSS*.C9 exhibited low *SS*, unlike Y106 alleles and heterozygous alleles. To further map *qSS*.C9, 230 plants with extremely low *SS* (<16 seeds per silique), which accounted for about a quarter of the 807 plants in the BC_4F_1 population, were used. All the BC_4F_1 plants were tested by the markers SRC9-022 and SCC9-005. One recombination event was detected between the locus *qSS*.C9 and SRC9-022 or EA08MC13, and 16 recombinants of the locus *qSS*.C9 and SCC9-005 were identified (Fig. 5). However, no polymorphic marker was left for the recombinants. Thus, aided by the sequences of the closest markers SRC9-022 and SCC9-005, the locus *qSS*.C9 was mapped in a region of 1.005 Mb, which includes 218 predicted genes in *B. rapa*.

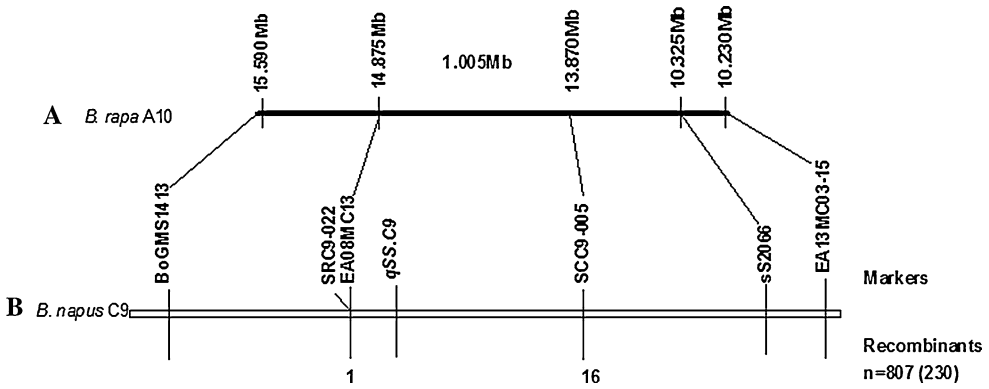

Fig. 5 Genetic and comparative mapping of *qSS*.C9. Genetic mapping of *qSS*.C9 (**b**): the *qSS*.C9 locus was restricted to the region between markers EA08MC13 and SCC9-005 using 230 extremely low-*SS* (<16 seeds per silique) plants of a BC_4F_1 population containing 807 plants. The number of recombinants between the markers and *qSS*.C9 is indicated under the linkage map. Comparative mapping of *qSS*.C9 (**a**): the *qSS*.C9 locus was delimited a 1.005-Mb interval in the reference *Brassica rapa* A10

Discussion

Developing NIL of undetected QTL using TP-MBS

Usually, there are three strategies for QTL-NIL development. As reported by Tanksley and Nelson (1996), consecutive backcrossing (CB-NILs) combined with marker-assisted selection is a conventional method that has been widely used in crops (Alpert et al. 1995; Ashikari et al. 2005; Liu et al. 2006; Szalma et al. 2007). The alternative method is to search NILs according to trait performance (TP-NILs) in recombinant inbred lines (Zhang et al. 2006). The third strategy is the heterogeneous inbred family (HIF-NILs) method (Tuinstra et al. 1997), whose genetic principle is based on molecular marker screening for a small heterozygous genomic region. Comparing the genetic principles of the three methods reveals that the two key elements to NIL development are target QTL tracking and background similarity selection. In a certain sense, to develop a NIL is to satisfy the two elements, whether using trait performance or molecular marker screening. Target QTL tracking in TP-NILs selection is based on phenotypic screening, whereas that in CB-NILs and HIF-NILs selection are based on molecular marker screening.

In this study, we constructed NILs of *qSS*.C9 by tracking the target QTL using trait performance and background similarity selection using marker-assisted background selection (TP-MBS-NILs) (Fig. 1), because the QTL *cqSS*.A8 was a relatively minor QTL as compared with *qSS*.C9 (Table S3, Table 4). This method provides a valuable reference for NIL development, especially in cases where there is an undetected error in the location of a QTL of interest (Fig. 4). When using the method of TP-CB-NILs, researchers must pay attention to the following aspects in the target QTL screening. The first important aspect is the existence of a large phenotypic difference, such as HZ396 with low *SS* and Y106 exhibiting high *SS* in this study. This made the existence of a major QTL possible. Certainly, it would be better to evaluate the existence of a major QTL using classic genetic analysis. The inheritance analysis of *SS* using the joint segregation analysis of multiple populations showed that major genes of *SS* existed in the cross of HZ396 × Y106 (Zhang et al. 2010). Another important aspect is that the target trait has a medium to high heritability. As described by Zhang et al. (2011), the heritabilities of *SS* and *SL* in the DH population were about 0.80. The third but less important aspect is that there is a complete dominant/recessive character in an agronomic trait of interest. The high *SS* was almost completely dominant over the low *SS* in the cross between HZ396 and Y106 (Zhang et al. 2008, 2010) in the present study. Thus, using phenotypic selection in the consecutive backcrossing made selection of target genes possible. For background similarity selection using the method of TP-MBS-NILs, it is important to select molecular markers that are evenly distributed on each chromosome or linkage group. Hence, TP-MBS-NILs provides a valuable method for NIL development in the absence of a complete genome sequence.

Causes of a negative correlation for *SS* and *SW*

The causes of a significant negative correlation for *SS* and *SW* (Liu 1987; Zhang et al. 2011), which are two important components of seed yield in rapeseed, have received much attention over the years. One possible reason is tight linkage of genes that control independent traits, known as linkage drag (Brown 2002). Analysis of quantitative data in rice by Fukuoka et al. (2009) revealed linkage drag between blast resistance (*Pi21*) and poor grain quality (lower stickiness and hardness of cooked rice). Cao et al. (2010) detected a strong linkage drag of

low seed oil content and low seed erucic acid content in linkage group A8 in *B. napus*. Here, the positive allele of *qSS*.C9 was inherited from Y106, whereas the positive allele of *qSW*.C9 was derived from HZ396 in the same region of linkage group C9 (Table 4), a result that reflected linkage drag between *SS* and *SW*, which is an important reason for the negative correlation between *SS* and *SW* ($r = -0.62$) in the DH population derived from HZ396 × Y106 (Zhang et al. 2011). The other possibility is the existence of a negative pleiotropic effect for *SS* and *SW*. Pleiotropy occurs when a single gene affects two or more distinct traits. There are some reports of QTL showing pleiotropic effects in crops. Xue et al. (2008) cloned a QTL, *Ghd7*, with pleiotropic effects for spikelets per panicle, heading date, and plant height in rice. Tian et al. (2006) also mapped a QTL, *gpa7*, that controlled five panicle traits. In this study, the QTL *qSS*.C9 showed pleiotropic effects for *SS* and *SL*, as revealed by correlation analysis and the peak overlap of interval mapping or composite interval mapping. These results suggest that pleiotropic effects occur widely in crops. Because we did not measure *SW* in Gansu in the progeny test, we cannot estimate the pleiotropic effects for *SW* by the QTL *qSS*.C9. The distinction of negative pleiotropic effects by the QTL *qSS*.C9 and genetic effects by *qSW*.C9 is sometimes difficult to detect using a primary mapping population. This issue requires further research to evaluate the likely negative pleiotropic effect for *SS* and *SW* with a progeny test and to estimate the genetic effects of *qSW*.C9 for *SW* using an advanced backcross strategy.

QTL *qSS*.C9 cloning strategy

To date, major QTL located in linkage group C9 for *SS* have not been reported. Therefore, the QTL *qSS*.C9 reported in this study is a novel major QTL for *SS*, although the QTL was undetected due to the QTL region not being covered by a previous linkage map of C9 in a primary QTL mapping population (Zhang et al. 2011). Moreover, the QTL *qSS*.C9 explained 85.8 % of phenotype variance for *SS* in the BC_3F_2 population. This made it possible to isolate *qSS*.C9 using a positional cloning strategy based on the extremely low-*SS* plants.

The mapping of *qSS*.C9 to linkage group C9 will facilitate the identification of additional linked markers. According to the reference maps (Li et al. 2010; Wang et al. 2011a), we identified three SSR markers (BoGMS1413, sS2066, and sNRG42) that were mapped to linkage group C9 (Fig. 4). Recently, a high-resolution genetic map comprising 23,037 single nucleotide polymorphisms was constructed for *B. napus* (Bancroft et al. 2011). However, none of the single nucleotide polymorphisms located in the orthologous region in *B. rapa* (from B10.07C3_1 Bin to X_C9_7 Bin) showed polymorphisms between low-*SS* bulk and high-*SS* bulk (data not shown). Resolution of genetic mapping could not be improved for *qSS*.C9 due to lack of polymorphic markers, even though there are recombinants in this region (Fig. 5). This limitation forced us to apply an effective molecular marker strategy, such as development of sequenced restriction-site associated DNA markers (Baird et al. 2008), to close the distance of *qSS*.C9 in the absence of a complete genome sequence.

Through map-based cloning of qualitative traits, such as male sterility (Dun et al. 2011; Yi et al. 2010), in *B. napus*, it was recognized that an accurate genetic map and comparative mapping with molecular markers linked to target genes will facilitate fine mapping of the target genes. In our study, *qSS*.C9 corresponding to the orthologous region in *B. rapa* was located in the fragment between Bra008797 and Bra009015 (Table 5, Fig. 5). Much more attention was thereby given to the segment delimited by the two *B. rapa* genes. Furthermore, the whole genome sequence of *B. oleracea* will be available in the near future. These genetic resources from the progenitor species of *B. napus* will facilitate the map-based cloning of *qSS*.C9.

Potential application of *qSS*.C9 in marker-assisted selection

Although it is not possible to know whether the QTL *qSS*.C9 is in the same location of linkage group C9 as reported by Radoev et al. (2008) and Shi et al. (2009) due to a lack of public markers, we strongly believe that the mapping of *qSS*.C9 will be helpful for marker-assisted selection breeding because *qSS*.C9 accounted for a large proportion of phenotypic variance for *SS* and was stable across environments (Table 4). Positive *qSS*.C9 homozygotes could result in about 6.0 seeds per silique compared with negative homozygotes (Table 3). Theoretically, the positive allele of *qSS*.C9 could be transferred to current rapeseed cultivars. Moreover, this QTL is a fully dominant locus controlling *SS*, which indicated that the heterozygote has higher *SS* than the HZ396 homozygote. Hence, in theory, *qSS*.C9 could be transferred to female or male parents to produce a better hybrid. The closely linked marker SRC9-022 and SCAR marker SCC9-005 (Fig. 5) have good potential for genotype analysis in marker-assisted selection breeding.

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