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ORIGINAL PAPER

Genetic characterization and fine mapping of a yellow-seeded gene in Dahuang (a *Brassica rapa* landrace)

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Abstract The development of yellow-seeded cultivars in *Brassica rapa* (*B. rapa*) would improve the quality and quantity of available oil. The identification and mapping of the seed coat color gene may aid in the development of yellow-seeded cultivars and facilitate introgression of the yellow-seeded gene into desirable *Brassica napus* (*B. napus*) lines through marker-assisted selection. In the current study, we investigated the inheritance of a yellow-seeded landrace in *B. rapa*, "Dahuang", originating from the Qinghai-Tibetan plateau. Genetic analysis revealed that the phenotype of the yellow-seeded trait in Dahuang is controlled by one recessive gene, termed *Brsc1*. Mapping of the *Brsc1* gene was subsequently conducted in a BC_1 population comprised 456 individuals, derived from (Dahuang × 09A-126) × Dahuang. From a survey of 256 amplified fragment length polymorphism (AFLP) primer combinations, 10 tightly linked AFLP markers were obtained. The closest AFLP markers flanking *Brsc1*, Y10 and Y06, were 0.2 and 0.4 cM away, respectively. Subsequently, using simple sequence repeat (SSR) markers in the reference map, the *Brsc1* gene was mapped on A09 in *B. rapa*. Blast analysis revealed that seven AFLP markers showed sequence homology to A09 of *B. rapa*, wherein six AFLP markers in our map were in the same order as those in A09 of *B. rapa*. The two closest markers, Y10 and Y06, delimited the *Brsc1* gene within a 2.8 Mb interval. Furthermore, Y05 and Y06, the two closest AFLP markers on one side linked to *Brsc1*, were located in scaffold 000059 on A09 of *B. rapa*, whereas the closet AFLP marker on the opposite side of *Brsc1*, Y10, was located in scaffold000081 on A09 of *B. rapa*. Molecular markers developed from these studies may facilitate marker-assisted selection (MAS) of yellow-seeded lines in *B. rapa* and *B. napus* and expedite the process of map-based cloning of *Brsc1*.

Keywords *B. rapa* · Seed coat color · AFLP · SSR · Fine mapping

Introduction

Brassica rapa is one of the most important oilseed crops distributed worldwide with a large ecological amplitude because of its early maturity and freezing resistance. In spite of having a number of desirable agronomic characteristics, most *B. rapa* in China contains high amount of erucic acid in oil and high level of glucosinolates in seed meal, restricting its competitiveness in market. The development of canola-quality varieties is of high priority in *B. rapa* breeding program. Further, the quality and quantity of oil can be improved through developing yellow-seeded cultivars. As comparing to black or brown seeds, yellow-seeded varieties have a significantly thinner seed coat, leading to a low hull proportion and high oil content in seeds and high protein content in the meal (Stringman et al. 1974; Shirzadegan and Röbellen 1985). Therefore, it is important to identify the yellow-seeded germplasm in *B. rapa*. Dahuang is a yellow-seeded *B. rapa*

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landrace originating from Huangyuan county, Qinghai province (Liu 1985). Unlike most cultivated *B. rapa*, Dahuang shows self-compatibility. Another self-compatible plant with a yellow seed coat is yellow sarson, which is an India oleiferous cultivar. When compared to yellow sarson seeds, Dahuang seeds are greater in weight (5 g vs. ~6–7 g for 1,000 seeds) (Liu 1985; Liu 2000). In addition, significant differences have been found in the amino acid sequences, the protein structure and the conserved domain of the *SRK* (S-locus receptor kinase)-exon1 gene between Dahuang and yellow sarson (He et al. 2003). In addition to its yellow seed coat and self-compatibility, Dahuang has many interesting agronomic characteristics, including high oil content (41%) and lodging resistance. Therefore, Dahuang would be a valuable resource for studying seed coat color and self-compatibility in *B. rapa*. To our knowledge, little is known about the seed coat color trait of Dahuang.

Numerous reports have been published concerning the inheritance of seed coat color in *Brassica* species. In *B. rapa*, one or two genes have been found to be responsible for seed coat color (Ahmed and Zuberi 1971; Zhang et al. 2009; Stringam 1980). In *B. juncea*, two genes with duplicate effects have been shown to control the seed coat color, and yellow seeds are produced when both genes are in a homozygous recessive condition (Vera et al. 1979; Lakshmi Padmaja et al. 2005). Inheritance of seed coat color in *B. napus* is quite complicated, and one to four genes have been found to be involved (Liu et al. 1991, 2005; Xiao et al. 2007; Zhang et al. 2010).

Molecular markers have been widely used for the detection of seed coat color genes in *Brassica* species. Recently, one gene was found to simultaneously control seed coat color and hairiness traits in *B. rapa* and was successfully isolated using a positional cloning strategy (Zhang et al. 2009). In *B. juncea*, molecular markers that are tightly linked to the seed coat color genes have been isolated (Negi et al. 2000; Lakshmi Padmaja et al. 2005). In *B. napus*, a single major gene (*pigment* 1), determining over 72% of the phenotypic variation in seed coat color has been identified, and tightly linked RAPD markers have been developed. Using two mapping populations, Zhang et al. (2010) detected two major QTLs (*Bnsc-18a* and *Bnsc-9a*) for seed coat color, wherein *Bnsc-18a* was believed to occur more consistently among different populations (Zhang et al. 2010; Badani et al. 2006).

For better use of the yellow-seeded genetic resource Dahuang, we carried out studies to: (1) investigate the inheritance model of the seed coat color trait in Dahuang, (2) develop AFLP and SSR markers tightly linked with the seed coat color gene in Dahuang, (3) delimit the seed coat color gene to a corresponding region on a certain linkage group of *B. rapa*.

Materials and methods

Plant material and population construction

The *B. rapa* lines 09A-126, 09A-132 and Dahuang were used as materials in the present study. The seed coat color of 09A-126 and 09A-132 is brown, whereas that of Dahuang, a *B. rapa* landrace originating from the Qinghai-Tibetan plateau, is bright yellow. These three *B. rapa* lines have been maintained over six generations by selfing for self-compatible types and by sib-mating for self-incompatible types.

Reciprocal crosses between yellow-seeded (ys) and brown-seeded (bs) parents were carried out to investigate the inheritance of the seed coat color trait. In each cross combination, a single F_1 (ys × bs) and RF_1 plant (bs × ys) was backcrossed with Dahuang to produce the BC_1 and RBC_1 populations, respectively; and a single F_1 and RF_1 plant was simultaneously self-pollinated to obtain the F_2 and RF_2 generations, respectively. Self-pollinated seeds of the individual plant were harvested at the maturity period and visually scored for seed coat color.

The BC_1 population comprising 456 individuals, developed from the cross between Dahuang and 09A-126, was used for mapping the seed coat color gene *Brsc1*. To develop the co-dominant markers linked with *Brsc1*, an F_2 population of 255 plants was constructed by self-pollinating the F_1 plant originating from the cross between Dahuang and 09A-126. Finally, an $F_{2:3}$ generation of 255 families derived from each of the F_2 individuals by self-pollination was used for genotype identification of the F_2 individuals.

DNA extraction and AFLP analysis

Total DNA was extracted individually from fresh leaves by CTAB method (Doyle and Doyle 1990). The final DNA concentration was 50 ng/μl in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Equivalent amounts of DNA from 12 ys and 12 bs individuals were randomly selected to construct two ys and two bs bulks, respectively. The two pairs of bulks were used for bulked segregant analysis (BSA) (Michelmore et al. 1991) in combination with the AFLP technique, which was performed as described by Vos et al. (1995) with minor modifications (Lu et al. 2004). Genomic DNA was restricted with *Pst*I and *Mse*I. Specific double-stranded adaptors were subsequently ligated to the digested DNA fragments. The adaptor-ligated DNA was pre-amplified using the AFLP primers each having one selective nucleotide (P0 and MC). The pre-amplified product was diluted (1:30) and used for selective amplification. The product of the selective amplification was separated and silver stained as described for AFLP markers.

Mapping

A BC_1 population with 456 individuals was used for mapping the *Brsc1* gene. SSR markers in the A genome from the reference maps (Piquemal et al. 2005; Chen et al. 2007; Cheng et al. 2009a, b) were utilized to assign the *Brsc1* gene to a specific chromosome. After an SSR marker was mapped on the linkage group N9 (corresponding to A09 in the A genome of *B. rapa*), a series of SSR markers in this region were selected for a polymorphism survey in our population. SSR amplification was performed as described by Lowe et al. (2002). The amplified products were separated on a 6% denaturing polyacrylamide gel. Data from AFLP as well as SSR markers and individual phenotypes were analyzed with the MAPMAKER/EXP 3.0 program (Lander et al. 1987; Lincoln et al. 1992) and a partial linkage map of the region on the chromosome spanning the *Brsc1* gene was constructed. The genetic distance (cM) was calculated using the Kosambi function (Kosambi 1944).

AFLP marker sequencing and identification of *B. rapa* synteny

The expected AFLP bands were excised from dried polyacrylamide gels, and the DNA was purified following the methods described by Yi et al. (2006). Purified products were ligated into the bacterial plasmid pGEM-T Easy vector (Promega). The transformed clones were screened by M13-specific primers. For each fragment, three positive clones were randomly selected and sequenced by Shanghai Sangon Biotechnology Corporation (Shanghai, China).

After genetic mapping in the BC_1 population, sequences of markers were used to identify putatively homologous sequences within the *B. rapa* genome. Blast analysis was performed using the BRAD database (http://www.brassicadb.org/brad/).

Results

Genetic analysis

The self-pollinated seeds produced on the F_1 (or RF_1) plants of reciprocal crosses between the ys parent Dahuang and the bs parents (09A-126 or 09A-132) were all brown, indicating that the bs trait was dominant over the ys trait. The BC_1 (or RBC_1) progenies developed from the crosses between the F_1 (or RF_1) plant and Dahuang displayed a ratio of bs to ys plants that did not deviate significantly from 1:1. In addition, the bs-to-ys plant ratio in the F_2 progenies was approximately 3:1 (Table 1), indicating that one Mendelian locus controlled the seed coat color trait in our populations, and the ys gene was tentatively designated as the *Brsc1* gene.

Screening of AFLP markers for seed coat color

A cross of (Dahuang × 09A-126) × Dahuang was carried out to establish a BC_1 population for detecting molecular markers linked to *Brsc1*. In the BSA analysis, 256 P + 3/M + 3 AFLP primer combinations were used to screen two ys and two bs bulks. Polymorphism bands that appeared in the bs but not in the ys bulks were subsequently used to amplify 12 ys and 12 bs plants consisting of the bulks. As a result, 10 polymorphism markers linked to the *Brsc1* gene were identified and named Y01–Y10 (Table 2), respectively. Figure 1 shows a representative amplification profile with the primer Y07. In the primary linkage analysis, all of the AFLP markers were used to screen 96 randomly selected individuals in the BC_1 populations. The results indicated that five AFLP markers (Y02, Y04, Y05, Y06 and Y10) showed no recombinant with the *Brsc1* gene.

Genetic mapping of *Brsc1*

In the primary mapping experiment, 96 individuals were screened with 10 AFLP markers. Results indicated that 5 of the 10 AFLP markers (Y02, Y04, Y05, Y06 and Y10) were co-segregated with *Brsc1* (Table 2).

To determine the map location of the *Brsc1* gene in the published *B. rapa* genetic map, polymorphism analysis was conducted in the two bulks of the BC_1 population derived from Dahuang × 09A-126 using the SSR markers from the reference maps (Piquemal et al. 2005; Chen et al. 2007; Cheng et al. 2009a, b). A total of 32 SSR markers were selected. Only CB10022 showed a polymorphism between the two pairs of bulks and the corresponding individuals. Subsequently, CB10022 was used to survey the BC_1 population comprised 456 individuals and Mendelian segregation was observed. The distance between CB10022 and *Brsc1* was 0.8 cM on the same side as the AFLP marker Y07 (Fig. 2c). Because CB10022 was mapped on linkage group N9 (corresponding to A09 of *B. rapa*) of the reference map (Fig. 2a, b), an additional 22 SSR markers on N9 from the reference maps were selected for further analysis, confirming the map location of the *Brsc1* gene. As a result, CB10255 and CB10428 showed polymorphisms between the two pairs of bulks and the corresponding plants. CB10255 and CB10428 were then used to analyze the mapping population. The results showed that CB10255, on the same side with AFLP marker Y01, was 3.5 cM away from *Brsc1*, whereas CB10428 was on the opposite side at a distance of 1.7 cM (Fig. 2c). The evidence described above led to the conclusion that the *Brsc1* gene was located on the linkage group A09 in the *B. rapa* map.

Table 1 Segregation of seed coat color in the BC_1, RBC_1 and F_2 progenies of two crosses

Combination	Population	No. of yellow seed	No. of brown seeds	Expected ratio	$\chi 2$ value
Dahuang × 09A-126	BC_1	212	244	1:1	2.11
	RBC_1	75	95	1:1	0.72
	F_2	57	198	1:3	0.82
Dahuang × 09A-132	BC_1	45	38	1:1	0.43
	RBC_1	45	49	1:1	0.10
	F_2	18	69	1:3	0.65

Table 2 Description of AFLP markers tightly linked to the *Brsc1* gene

AFLP marker	Primer combination	Size of marker (bp)	Map distance (cM)
Y01	P-CAG/M-CCA	179	15.3
Y02	P-TGC/M-CCG	590	1.7
Y03	P-CTG/M-CCG	308	5.9
Y04	P-GCA/M-CCG	320	0.8
Y05	P-CTG/M-CTC	89	0.8
Y06	P-TGA/M-CCT	165	0.4
Y07	P-AGT/M-CCG	288	2.4
Y08	P-GGA/M-CCT	117	5.3
Y09	P-CAG/M-CAA	171	9.1
Y10	P-GGA/M-CCC	87	0.2

M MseI primer, 5′-GATGAGTCCTGAGTAA-3′; *P PstI* primer, 5′-GACTGCGTACATGCAG-3′

In the fine mapping experiment, SSR markers (CB10255 and CB10428) were first used to detect the recombinants in the BC_1 population consisting of 456 plants. After all individuals in the mapping population were tested for their genotypes, 16 individuals displayed recombination between *Brsc1* and CB10255 and another 8 recombinants of *Brsc1* and CB10428 were identified. The 24 recombinants were subjected to genotyping for the co-segregated markers in order to evaluate the genetic distance from the *Brsc1* locus. Results showed that Y10 was on the same side as CB10255, whereas Y06, Y04, Y05 and Y02 on the opposite side of the *Brsc1* gene. Among these flanking markers of the *Brsc1* gene, Y10 and Y06 were the most closely linked ones, which were 0.2 and 0.4 cM away from the *Brsc1* gene, respectively.

We also surveyed the plants in the F_2 population derived from Dahuang as well as 09A-126 with three SSR markers (CB10022, CB10255 and CB10428). For CB10022 and CB10255, different fragments from ys individuals and homozygous bs individuals were obtained, whereas both fragments from heterozygous bs individuals were amplified, indicating that CB10022 and CB10255 are co-dominant markers. Figure 3 shows the amplification profile of CB10022 for individual F_2 plant.

Identification of synteny in the *B. rapa* genome

The closely linked AFLP markers were used for Blast analysis against the BRAD database (http://www.brassicadb.org/brad/). Seven markers showed sequence homology to the A09 of *B. rapa* (Table 3). Except the position of Y08, all the markers that corresponded to the AFLP markers in our map were in the same order as those in A09 of *B. rapa* (Fig. 2). Based on this order, the genomic region containing the *Brsc1* gene was delimited to an interval of approximately 2.8 Mb between 19.3 and 22.1 Mb of A09. We also found that Y05 and Y06 showed sequence homology to scaffold000059 on A09 and Y10 to scaffold000081 on A09 of *B. rapa* (Fig. 4). The amplification sequences of five markers that covered the 19.3–22.1 Mb stretch of A09, available at http://www.brassicadb.org/brad, were selected and used for Blast analysis. The sequence of BrID101127 had highly conserved homologous region on scaffold000081 of A09, BrID10607, BrID10609 and BrID10613 on scaffold000040 of A09, whereas BrID10685 on scaffold000059 of A09 (Fig. 4). Sequence information and the development of PCR-based markers of these three scaffolds may facilitate map-based cloning of the *Brsc1* gene.

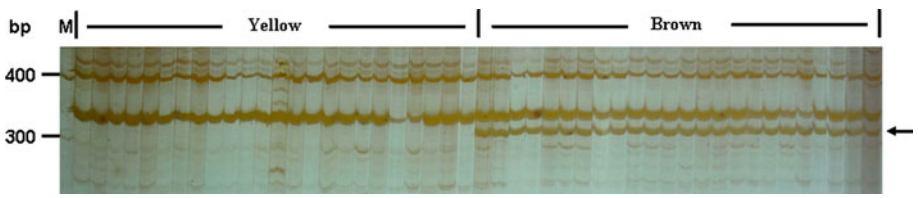

Fig. 1 The AFLP amplification results from 24 ys and 24 bs plants. The *arrows* indicate the polymorphic band present in bs individuals but not in ys individuals. The AFLP marker used was Y07. *M* 100-bp DNA ladder

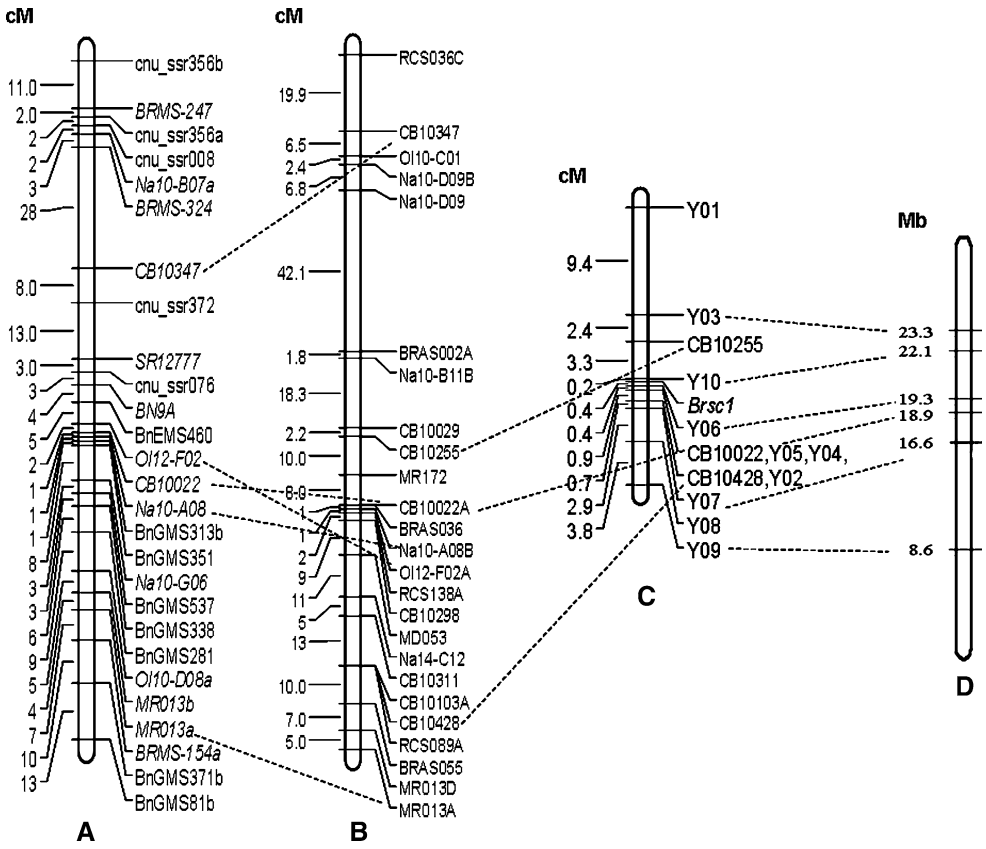

Fig. 2 **a** A linkage map of N9 developed from the cross no. 2,127 × ZY821 (Cheng et al. 2009a, b), indicating the position of CB10022. **b** A linkage map of N9 showing the position of CB10022, CB10255 and CB10428. The linkage map was constructed from six F_2 populations derived from three spring-type rapeseed lines and three winter-type rapeseed lines (Piquemal et al. 2005). **c** A partial linkage map of the region surrounding the *Brsc1* gene. **d** A partial physical map of A09 showing the homologues of the mapped marker sequences. The *dotted lines* indicate the common AFLP and SSR markers in these linkage maps

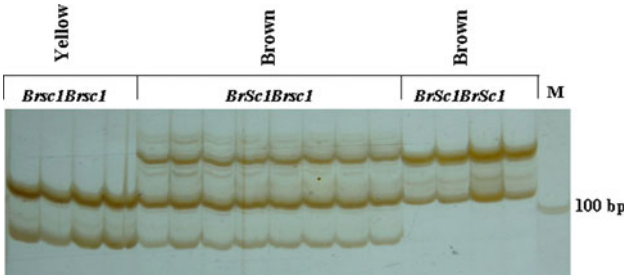

Fig. 3 Analysis of the PCR products obtained using the SSR primer CB10022 on individual F_2 plants. The F_2 plants are represented as ys (*Brsc1Brsc1*), heterozygous bs (*BrSc1Brsc1*) and homozygous bs (*BrSc1BrSc1*) individuals. *M* 100 bp DNA ladder

Discussion

Brassica rapa is one of the most important oilseed crops and has numerous desirable agronomic characters. A *B. rapa* landrace called Dahuang, originating from the Qinghai-Tibetan plateau, exhibits a ys trait. The genetic analysis revealed that the seed coat color in Dahuang is controlled by one recessive gene. Because most presently grown cultivars of *B. napus* are bs, the development of ys rapeseed has been proposed as an effective means to improve canola quality and to increase protein content (Shirzadegan and Röbellen 1985). To date, ys strains in *B. napus* have been generated from interspecific-crosses with related ys *Brassica* species such as *B. rapa*, *B. carinata* and *B. juncea* (Rahman 2001; Rakow et al. 1999). Therefore, Dahuang has become an elite new gene resource for yellow seeds. The development of molecular markers for Dahuang may facilitate the transfer of the yellow seed coat color trait from *B. rapa* to *B. napus*. Compared to normal *B. rapa*, Dahuang has a higher seed weight (about 6–7 g for 1,000 seeds). In addition, Dahuang possesses a higher oil content (41%), better lodging resistance, better shattering resistance and self-compatibility. Therefore, from a breeding perspective, Dahuang is not only an elite new gene resource for yellow seed, but also has many desirable agronomic characteristics.

In the previous studies, a gene controlling the seed coat color in *B. rapa* has been isolated by map-based cloning approach (Zhang et al. 2009). In order to determine the allelism between the seed coat color gene and the *Brsc1* gene, the SCAR markers identified by Zhang et al. (2009) were used to test polymorphism in our mapping population. As a result, no polymorphisms were found in the bulks and the corresponding individuals. Furthermore, Zhang et al. (2009) located the gene controlling the seed coat color trait on R6 of the *B. rapa* genetic map, whereas the *Brsc1* gene was located on linkage group A09 in the present study. Based on these results described above, we deduced that the *Brsc1* gene is not allelic to the seed coat color gene

Table 3 Results of BlastN searches using sequences from the AFLP fragments

AFLP marker	Linkage group (position)	Identities	Scaffold (position)
Y03	A09 (23307858-23308113)	507, e−142, 256/256 (100%)	A09 |26| BGIScaffold000045 (1401564-1401309)
Y10	A09 (22118876-22118939)	103, 1e−21, 62/64 (96%)	A09 |25|BGIScaffold000081 (708851-708788)
Y06	A09 (19352387-19352296)	103, 3e−21, 82/92 (89%)	A09 |23|BGIScaffold000059 (324467-324558)
Y05	A09 (18922812-18922900)	176, 1e−43, 89/89 (100%)	A09 |23|BGIScaffold000059 (754042-753954)
Y07	A09 (16643427-16643193)	466, e−130, 235/235 (100%)	A09 |21|BGIScaffold000022 (2312075-2311841)
Y08	A09 (23300027-23299911)	216, 2e−55, 115/117 (98%)	A09 |26|BGIScaffold000045 (1409395-1409511)
Y09	A09 (8598239-8598398)	285, 4e−76, 156/160 (97%)	A09 |14|BGIScaffold000054 (1187381-1187540)

In the "Linkage group" column, the numbers in the brackets indicate the position of the *B. rapa* homologous sequence corresponding to our sequence. In the "Identity" column, the first and second numbers are the score and the expectation value (*E* value) given by BlastN, respectively; the fraction gives the number of residues that are identical in our sequence and in the corresponding *B. rapa* sequence. In the "Scaffold" column, the numbers in the brackets indicate the position of homologous scaffold sequence corresponding to our sequence

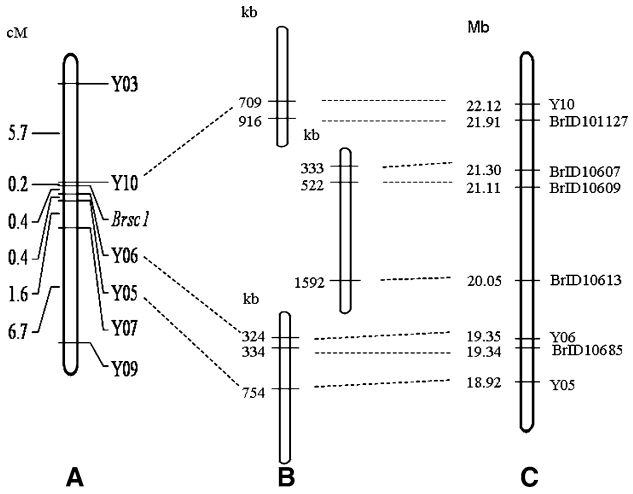

Fig. 4 **a** A partial linkage map of the region surrounding the *Brsc1* gene. **b** The distribution of homologous DNA fragments on the scaffolds of A09. The physical positions of these fragments are indicated on the *left*. The scaffolds from top to bottom are scaffold000081, scaffoldb000040 and scaffold000059, respectively. **c** Some markers between 19.3 and 22.1 Mb, available at http://www.brassicadb.org/brad, are shown. The physical positions of these markers are indicated on the *left*. The *dotted lines* indicate the common markers in these linkage maps

identified by Zhang et al. (2009). Moreover, the present results compared with the published results obtained by other researchers, we found that the seed coat color gene *Y* identified by Xiao et al. (2007) might be the same locus as *Brsc1*, as they were both located in the same linkage group A09 (or N09) of the A genome and two common markers (P-CAG/M-CCA and P-CAG/M-CAA) were detected. Allelic relationships of these seed coat color genes, however, have yet to be verified by fine mapping.

Much of the work was conducted at the same time as the *Brassica rapa* Genome Sequencing Project (BrGSP). Our work greatly benefited from publicly available genome sequences of *B. rapa*. In the present study, a fine scale map of *Brsc1* locus was constructed using a combination of BSA, AFLP and SSR methodologies. Through comparative mapping with *B. rapa*, a syntenic region spanning 2.8 Mb interval was identified in which the homologue of *Brsc1* might be included. Although sequence data are now available for *B. rapa*, there are many 'N' nucleotides in the sequences. Therefore, it might miss some important genomic information if we predict the putative candidate genes in the wide region of 2.8 Mb. We attempted to develop closer markers using the sequence information of the candidate region and constructing a larger population. When the interval covering the *Brsc1* gene is short enough, we will predict the candidate genes within the region.

Seed coat color is usually controlled by maternal genotype, which delays the expression of the phenotype for one generation. In the current study, the seed coat color gene is controlled by one recessive gene, resulting in the selection of the ys individual in the segregating population will be delayed for at least two generations. The development of the co-dominant markers allowed us to easily identify the desirable plant. In this study, the co-dominant SSR markers (CB10022 and CB10255) were identified and can be effectively used in marker-assisted selection for determining the presence of and transferring the seed coat color gene *Brsc1*.

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