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ORIGINAL PAPER

Towards map-based cloning: fine mapping of a recessive genic male-sterile gene (*BnMs2*) in *Brassica napus* L. and syntenic region identification based on the *Arabidopsis thaliana* genome sequences

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Abstract S45AB, a recessive genic male sterile (RGMS) line, originated as a spontaneous mutant in *Brassica napus* cv. Oro. The genotypes of sterile (S45A) and fertile plants (S45B) are *Bnms1ms1ms2ms2* and *BnMs1ms1ms2ms2*, respectively. In our previous studies, Yi et al. (Theor Appl Genet 113:643–650, 2006) mapped the *BnMs1* locus to a region of 0.4 cM, candidates of which have been identified and genetic transformation is in progress. We describe the fine mapping of *BnMs2* exploiting amplified fragment length polymorphism (AFLP) and amplified consensus genetic marker (ACGM) methodologies, and the identification of a collinear region probably containing *BnMs2* orthologue in *Arabidopsis thaliana*. A near isogenic line (NIL) population S4516AB which segregated for *BnMs2* locus was generated by crossing, allelism testing and repeated full-sib mating. From the survey of 1,024 AFLP primer combinations, 12 tightly linked AFLP markers were obtained and five of them were successfully converted into co-dominant or dominant sequence characterized amplified region (SCAR) markers. A population of 2,650 sterile plants was screened using these markers and a high-resolution map surrounding *BnMs2* was constructed. The closest AFLP markers flanking *BnMs2* were 0.038 and 0.075 cM away, respectively. Subsequently, an ACGM marker was developed to delimit the *BnMs2* locus at an interval of 0.075 cM. We extended marker sequences to perform BlastN searches against the *Arabidopsis* genome and identified a collinear region containing 68 *Arabidopsis* genes, in which the orthologue of *BnMs2* might be included. We further integrated *BnMs2* linked AFLP or SCAR markers to two doubled-haploid (DH) populations derived from the crosses Tapidor × Ningyou7 (Qiu et al., Theor Appl Genet 114:67–80, 2006) and Quantum × No.2127-17 (available in our laboratory), and *BnMs2* was mapped on N16. Molecular markers developed from these investigations will facilitate the marker-assisted selection (MAS) of RGMS lines, and the fine map and syntenic region identified will greatly hasten the process of positional cloning of *BnMs2* gene.

Introduction

Many investigations indicate the significant heterosis for seed yield in rapeseed (*Brassica napus*) (Sernyk and Stefansson 1983; Grant and Beversdorf 1985; Brandle and Mcvetty 1990; Shen et al. 2005). Cytoplasmic male sterility (CMS), an efficient pollination control system, has been widely used and remains the most important component in hybrid rapeseed production in China. However, some of its undesirable characteristics such as unstable nature of sterility, the limitation of restorers, and potential threat of using only one cytoplasmic source necessiate to explore new systems. Recessive genic male sterility (RGMS), one of the promising alternatives to the CMS system, has the following features: complete and stable male sterility, easy transfer of the sterility trait to diverse genetic backgrounds and affluent restorer resources. To date, many double low RGMS lines are widely exploited in China to develop heterotic hybrids.

S45AB, developed from spontaneous mutation in *B. napus* canola variety Oro (Pan et al. 1988), is extensively utilized for hybrid cultivar development in China. Hybrid cultivars

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have been successfully employed to increase double low rapeseed production worldwide. S45A has a broad spectrum of restoration with almost all normal rapeseed lines restoring fertility in F1 hybrids which makes it easy to find suitable hybrid combinations with high heterosis (Pan et al. 1988; Li et al. 1993, 1995).

Genetic analysis indicated that two duplicate recessive genes, designated as *Bnms1* and *Bnms2*, controlled the male sterility in S45 populations as it showed 15:1 segregation ratio in crosses of S45 and fertile cultivars (Pan et al. 1988; Yi et al. 2006). S45AB is maintained by harvesting the progeny from S45A plants through sib-mating (i.e., S45 A ms plants pollinated by S45 B mf plants). In RGMS breeding program, it is both time and labor consuming to select the desired genotypes exclusively on phenotypic characters (Wang et al. 1993; Zhang et al. 1996). In a previous study, Yi et al. (2006) had finely mapped one of the two male sterility genes, *BnMs1* and four SCAR markers were found, which greatly facilitated the breeding work and laid a solid foundation for map-based cloning of *BnMs1*.

AFLP is one of the most efficient molecular marker systems for mapping genes of interest (Lu et al. 2004; Ke et al. 2004; Liu et al. 2005; Hong et al. 2006). However, AFLP markers are relatively expensive and technologically demanding, which limit their application for large-scale screening in both breeding programs and fine mapping. It is, therefore, highly desirable to convert AFLPs to convenient and inexpensive PCR-based markers such as SCAR markers for MAS and recombinant screening.

Map-based cloning has been successfully employed to isolate fertility restorer genes of *Rfk1* and *Rfo* from radish (Imai et al. 2003; Brown et al. 2003). This strategy involves fine scale mapping of the gene in a large segregating population and identification of tightly linked flanking markers that allow quick chromosome walking towards the gene or, preferably, chromosome landing on a large insert clone carrying the target gene (Tanksley et al. 1995). For this approach to be successful, a large number of polymorphic markers are required to delimit the gene within a sufficiently small genetic interval of less than 1 cM (Mayerhofer et al. 2005).

Arabidopsis thaliana (hereafter referred to as *Arabidopsis*) is a close relative of *Brassica* species, and comparative genome analysis between *Arabidopsis* and *Brassica* species can be used to transfer information and resources from the widely studied model organism to this important group of crop plants (Muangprom and Osborn 2004). Giancola et al. (2003) used ACGM approach (Brunel et al. 1999) to develop non-anonymous markers from *Arabidopsis* genome sequences and successfully constructed a precise genetic map around the *Rfo* gene in a segregating radish population. Muangprom and Osborn (2004) found almost perfect collinearity of 13 RFLP markers between *Arabidopsis* and *B. rapa* around a dwarf gene, enabling them to identify a candidate gene in *Arabidopsis* and subsequently clone and characterize the corresponding gene in *B. rapa* (Muangprom et al. 2005; Mayerhofer et al. 2005).

The objectives of this study are: (1) to develop a set of markers (AFLP, SCAR, and ACGM) tightly linked to *BnMs2* for marker assisted selection (MAS) in breeding of RGMS lines; (2) to construct a high-resolution molecular map around *BnMs2* locus; and (3) to identify an *Arabidopsis* collinear region which may carry the homologue of *BnMs2* gene.

Materials and methods

Plant materials and population construction

RGMS two-type line S4516AB, developed and maintained for five generations by full sib-mating, was used as materials. The fertile plants S4516B (genotype: *Bnms1ms1Ms2ms2*) had yellow, well-developed anthers whereas the sterile plants S4516A (genotype: *Bnms1ms1ms2ms2*) were characterized by white anthers without any pollen. Two S4516AB derived NIL populations comprising 262 and 2,650 plants, respectively, were used for molecular mapping of *BnMs2* gene. The male fertile plant was self-pollinated to obtain a segregating population for co-dominant molecular marker analysis. The male fertility was visually scored at flowering time.

S4516AB was derived from the cross S45A × IL362-2-4. IL362-2-4 was a normal *B. napus* line, the genotype of which was *BnMs1Ms1Ms2Ms2*. Two generations of self-pollination generated 35 F3 populations. Some of the F3 populations segregating in an expected 3:1 ratio were maintained by full sib-mating (sterile plants pollinated by fertile plants). The resulting populations were assayed for fertility and those showing 1:1 segregation ratio were the candidate populations and subjected to allelism analysis with S45AB. The segregating locus of one population designated S4516AB was identified as being not allelic to that of S45AB and became a NIL population by continuous full sib-mating.

Allelism analysis

Allelism between S45AB and S4516AB was determined as follows: fertile plants (expected genotype: *Bnms1ms1 Ms2Ms2*) screened from selfed progenies of S4516B for two generations of selfing were crossed with S45B individuals (genotype: *BnMs1ms1ms2ms2*). All the plants in the resulting progenies (expected phenotype: fertile; expected genotypes: *BnMs1ms1Ms2ms2* or *Bnms1ms1Ms2ms2*) were selfed and simultaneously test crossed with S45A (*Bnms1ms1 ms2ms2*). Through assaying the fertility segregation ratio of

the resulting populations, the allelic relationship between S4516AB and S45AB was identified. If S4516AB was not allelic to S45AB, for the subsequent selfed populations the segregation ratio would be 15:1 or 3:1 and for the testcross populations the ratio would be 3:1 or 1:1. However, if S4516AB was allelic to S45AB, which means S4516B genotype was the same as S45B, there would be two types of segregating populations on selfing: one with the ratio of 3:1 and the other composed of completely fertile plants. In the testcross populations, all fertile and 1:1 segregating populations would be observed. The detailed methodology of allelism analysis is shown in Fig. 1a, b.

DNA extraction, BSA, and AFLP

Total DNA was extracted individually from young leaves by the CTAB method (Doyle and Doyle 1990). Final DNA concentration was 25 ng/μl in TE buffer (10 mM Tris, 1 mM EDTA pH 8.0). Equal amounts of DNA from 8 ms plants and 8 mf plants was pooled to construct sterile and fertile bulks, respectively. The two bulks were used for bulk segregation analysis (BSA) (Michelmore et al. 1991)

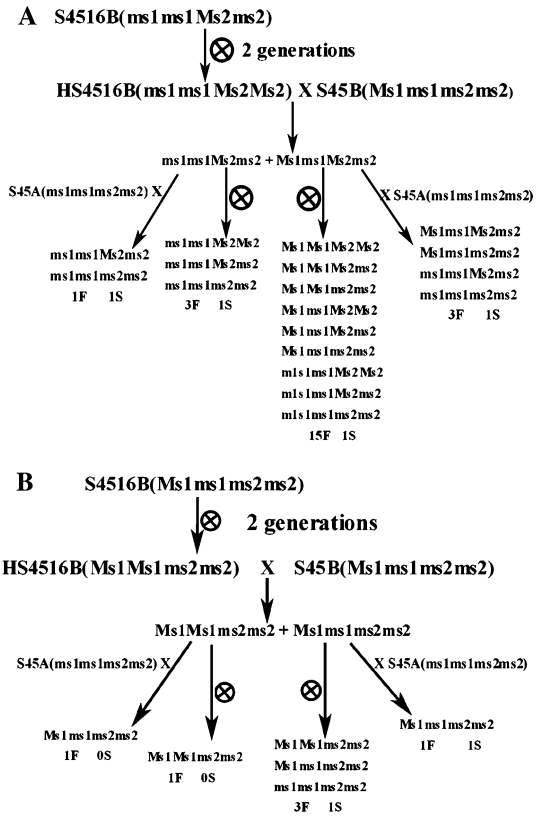

Fig. 1 **a** Supposed segregation performance in the population derived from the cross HS4516B × S45B, in which the genotype of HS4516B is *Bnms1ms1Ms2Ms2*. **b** Supposed segregation performance in the same population, in which the genotype of HS4516B is *BnMs1Ms1 ms2ms2*. *F* fertile, *S* sterile, *HS4516B* homozygous S4516B

with the AFLP technique, which was performed following the protocol developed by Vos et al. (1995) with minor modifications (Lu et al. 2004). Briefly, genomic DNA (250 ng) was digested with two restriction enzyme combinations (*Eco*RI/*Mse*I and *Pst*I/*Mse*I) in a volume of 25 μl. Specific double-stranded adaptors were subsequently ligated to the restriction fragment ends. Pre-amplification reaction was then carried out in a volume of 25 μl including 75 ng each of primers, 5 ng of adaptor-ligated DNA. After amplification, 5 μl of PCR products was analyzed in 1.0% agarose gel and the presence of low-molecular-weight smear indicated successful amplification. The pre-amplified product was diluted (1:30), and an aliquot (3 μl) was used for selective amplification in a volume of 15 μl. Following the selective amplification, 10 μl loading buffer (98% formamide, 0.025% xylene cyanol, 0.025% bromophenol blue, 10 mM EDTA, pH 8.0) was added and thoroughly mixed. The mixture was heated for 5 min at 95°C, and then promptly cooled by placing it on ice. A 1.5–2.0 μl mixture was resolved on 6% denaturing polyacrylamide gels and visualized by the silver nitrate staining.

Sequences and SCAR markers

AFLP fragments were cloned and sequenced as described elsewhere (Ke et al. 2004; Yi et al. 2006). We used the Universal GenomeWalker kit (Clontech, Heidelberg, Germany) to increase the size of the cloned markers by PCR walking (Siebert et al. 1995). Primers of SCAR markers were designed from the sequences of AFLP markers and/or sequences of extended AFLP markers using the Primer 3 program in Biology WorkBench (http://www.biowb.sdsc.edu/CGI/BW.cgi). These primers were used to amplify genomic DNA from 12 fertile plants and 12 sterile plants. The primer sequences and PCR conditions are listed in Table 2.

Genetic mapping

For all the AFLP markers we obtained, a NIL population of 262 individuals was initially used for mapping *BnMs2* gene and a rough map was constructed. Co-segregating AFLP or SCAR markers identified were subsequently analyzed in a larger population of 2,650 recessive individuals which were pooled into 530 sterile bulks. SCAR marker was initially assayed in the whole bulks and the bulks possibly containing recombinants were further examined to detect recombination individuals. Two DH populations, which were from the project of IMSORB (integrated marker system for oilseed rape breeding) and our laboratory, were used to map the AFLP or SCAR markers to a certain linkage group. After *BnMs2* linked markers were mapped on the linkage group N16, a number of simple sequence repeat (SSR)

markers in the linkage group were selected for a polymorphism survey. SSR amplification was performed as described by Lowe et al. (2002). The amplified products were resolved on a 6% denaturing polyacrylamide gel. Data from phenotype survey and AFLP, SCAR, and SSR analyses were combined for linkage analysis using 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 surrounding *BnMs2* gene was constructed. Map order was based on maximum-likelihood estimates.

ACGM markers and comparative mapping with *Arabidopsis*

After genetic mapping in a population of 2,650 plants, sequences of markers less than 1 cM to *BnMs2* were extended to identify putatively homologous sequences within the *Arabidopsis* genome using BlastN searches against the *Arabidopsis* genome database [The *Arabidopsis* Information Resource (TAIR), http:// www.*arabidopsis*.org]. A syntenic interval of *Arabidopsis* genome delimited by the nearest flanking markers of *BnMs2* was identified. *Arabidopsis* genes in this collinear region were selected from the *Arabidopsis* physical map obtained from TAIR using SeqViewer and MATDB database (http://mips.gsf.de/proj/thal/db/index.html). Specific primers designed according to the *Brassica* EST or GSS (available from the *Arabidopsis Thaliana* Integrated Data Base (ATIDB), http://atidb.org/) homologous to genes in the syntenic region were tested for polymorphism among the bulked DNA. Subsequently primers that showed polymorphism among the samples were used for fine mapping *BnMs2* locus by screening 2,650 individuals for sterility and marker segregation.

Results

Allelism analysis between S4516AB and S45AB

Three homozygous fertile plants (expected genotype: *Bnms1ms1Ms2Ms2*) derived from selfed progenies of S4516B were crossed with S45B individuals. Fifteen fertile plants in the resulting progenies were selfed and testcrossed with S45A. After assaying the fertility segregation ratio of the resulting populations, eight selfed populations displayed a ratio of ms to mf plants that did not differ significantly from 15:1 ($\chi^2 = 0.265$, $P > 0.05$; pooled data). Population with only fertile plants was not found. Furthermore, eight corresponding testcross populations showed a ratio of 3:1 ($\chi^2 = 0.034$, $P > 0.05$; pooled data) and no population fully consisting of fertile plants was identified. These data clearly indicated that the segregating locus of S45AB was not allelic to that of S4516AB, and accordingly the genotypes of S45B and S4516B were *BnMs1ms1ms2ms2* and *Bnms1ms1Ms2ms2*, respectively (Fig. 1a).

Screening AFLP markers linked to *BnMs2* gene

In the AFLP assay combined with BSA, two fertile DNA bulks (BF) and two male sterile DNA bulks (BS) were used to identify putative markers linked to the *BnMs2*. The assays involved three commonly used enzymes (*Eco*RI, *Pst*I, and *Mse*I) and a total of 1,024 pairs of primer combinations were used: $E + 3/M + 3$ (512) and $P + 3/M + 3$ (512). Fourteen primer combinations revealed polymorphism between the two fertile and the two sterile DNA bulks, six of which were from $E + 3/M + 3$ and eight from $P + 3/M + 3$. AFLP analysis showed that there were about 50–110 bands with a size range from 40 to 900 bp in each 3 + 3 primer pair. Polymorphic bands that appeared in BF, but not in BS were potentially linked to the gene *BnMs2*. To further examine potential linkage, polymorphic bands were subsequently examined among 32 individuals comprising the bulks. Fourteen markers were screened and 12 polymorphic markers were identified putatively linked to *BnMs2* gene, which were designated as LM01 to LM14 except Lm02 and Lm13 (Table 1), respectively. We failed to confirm Lm02 and Lm13, however, because the background signals were too high and we only obtained smeared bands. Thereafter, 96 individuals in the NIL population were screened for primary linkage analysis (data not shown). Results demonstrated that the 12 AFLP markers were closely linked to *BnMs2* gene.

Conversion of AFLP markers to SCAR markers

For the convenient application in large-scale screening for map-based cloning or MAS, the AFLP markers were cloned and sequenced with the objective of converting them into simple PCR-based markers. With the removal of the AFLP adapter sequences and fill in the cohesive end, the exact sizes of the cloned fragments were obtained (Table 1). Based on the sequence data of the 12 AFLP markers, primers were designed for direct amplification of the corresponding loci from genomic DNA. We successfully converted three AFLPs (Lm06, Lm07, and Lm04) into SCAR markers, of which the former two were confirmed as co-dominant markers and the latter as dominant one (Table 2; Fig. 2). Since some of the amplified fragments for these loci were small in size (for example, the exact sizes of Lm09, Lm10, Lm11, and Lm14 were between 74 and 118 bp), it was not possible to detect polymorphism between ms and mf individuals (data not shown). Thus, several specific primer pairs were designed

Table 1 Description of AFLP markers tightly linked to the *BnMs2* gene

AFLP marker	Primer combination	Size of marker (bp)	Extended for SCAR?	SCAR name derived from AFLP	Map distance (cM)
Lm01[a]	P-CCA/M-CAG	176			0.23
Lm02[a]	P-CTG/M-CTC	123	Yes	Lm02S	0.15
Lm04[a]	E-AAG/M-CCC	224	No	Lm04S	0.26
Lm05	E-AAT/M-GAT	451			1.2
Lm06	P-ACT/M-CCG	280	No	Lm06S	2.3
Lm07[a]	P-GTT/M-CTC	287	No	Lm07S	0.3
Lm08	P-GCA/M-CTA	290			2.3
Lm09[a]	P-TGA/M-CAC	100			0.57
Lm10[a]	P-GCA/M-GGG	98			0.038
Lm11[a]	P-GGA/M-GCG	74			0.075
Lm12	P-ACT/M-GAC	233			11.4
Lm14[a]	E-ACG/M-CCC	118	Yes	Lm14S	0.75

E = *Eco*RI primer, 5′-GACTGCGTACCAATTC-3′, M = *Mse*I primer, 5′-GATGAGTCCTGAGTAA-3′, P = *Pst*I primer, 5′-GACTGCGTACATGCAG-3′

[a] AFLP markers cosegregated with *BnMs2* in the NIL population of 262 plants

Table 2 ACGM and SCAR markers derived from AFLP markers

AFLP or ACGM markers	ACGM or AFLP derived SCAR markers					
	Name	Primers (5′→3′)[a]	Size[b]	PCR conditions[c]		Polymorphism
Lm02	Lm02S	TCACCACTCTCTTTTCTACTGGCTTTG/	382	60°C	72°C	Dominant
		GGCTAACAATGGACGATGAG		30 s	45 s	
Lm04	Lm04S	TTCAAGGAACTCAGTGACAG/	197	59°C	72°C	Dominant
		AACCCAAGGTCGAACCATAG		30 s	30 s	
Lm06	Lm06S	AACCGTTAGAAACACAAGAG/	256	59°C	72°C	Co-dominant
		CTGCAGACTGGCTACTCCTC		30 s	45 s	
Lm07	Lm07S	AATTCCAGAAGTGCCTCTTCCAGACTC/	220	60°C	72°C	Co-dominant
		CAATCTAGACATAGGCTGGAGAAGTCG		30 s	45 s	
Lm14	Lm14S	ATGAGATCATTCGAGGCACG/	572	59°C	72°C	Dominant
		TTAACCCAAAAAATGGGTCC		45 s	60 s	
Yao	Yao	GTCTGCACCTTCCACCTT/	815	54°C	72°C	Co-dominant
		CATCACCACGATAACGAG		45 s	60 s	

[a] Forward and reverse primers are listed in that order

[b] The size of PCR product linked with *BnMs2*

[c] For all PCR programs, denaturation started with 3 min at 94°C , then there were 35 cycles with 30 s at 94°C, and the annealing and extension conditions are as reported on the table for each primer pair

from the extended sequences. Using these primer pairs, PCR reactions were performed among two bulks and 24 individuals. Eventually, another two AFLP markers (Lm14 and Lm02) were successfully converted to dominant SCAR markers. Five SCAR markers viz. Lm02S, Lm04S, Lm06S, Lm07S, and Lm14S (Table 2) were derived from the five AFLP markers Lm02, Lm04, Lm06, Lm07, and Lm14, respectively. We failed to convert the rest of the AFLPs to SCARs because specific primers only amplified monomorphic bands between the two bulks even if we resolved the PCR products on a 6% denaturing polyacrylamide gel.

Genetic mapping of *BnMs2*

In the rough mapping experiment, we genotyped all the 262 plants with the 12 AFLP markers. Results indicated that eight of the 12 AFLP markers co-segregated with *BnMs2* (Table 1). The closest flanking markers were Lm05 and Lm06, which mapped 1.2 and 2.3 cM away from the target

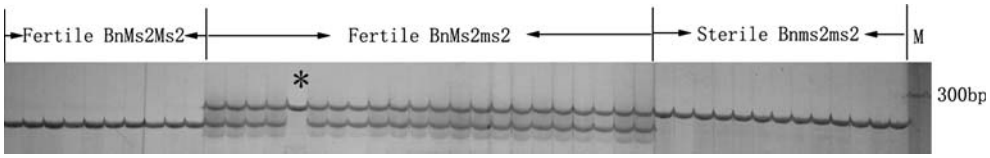

Fig. 2 Analysis of the PCR products obtained using the Lm06S SCAR primer on individual F2 plants. The F2 individual plants are represented as homozygous male fertile (*BnMs2BnMs2*), heterozygous male fertile (*BnMs2Bnms2*) and male sterile (*Bnms2Bnms2*). *Recombinant type **M** 100 bp DNA ladder

gene, respectively. Lm06 was successfully converted to SCAR marker, however, we failed to obtain a SCAR of Lm05 which was on the other side of *BnMs2*.

Fine mapping was then carried out with the eight co-segregated markers. After all individuals from the mapping populations were tested for their genotypes, 60 individuals displayed recombination between *BnMs2* gene and Lm06S. Other markers were then analyzed only with these recombinants. Results showed seven recombination events occurred between *BnMs2* and Lm04S and only one between *BnMs2* and Lm10. No recombinants were found for the remaining six markers, which might be on the opposite side of *BnMs2* gene or cosegregated with *BnMs2* on the same side as Lm06S. We then used dominant SCAR marker Lm14S to test the 530 bulks for recombinants. Fortunately 20 recombinants different from that of Lm06S were detected, which demonstrated Lm14S was not located on the same side as Lm06S. Recombinants for the last five AFLP or SCAR markers were screened out by examining the 20 recombinants. These recombination events were also different from that of Lm06S, which ruled out the possibility that they were on the same side as Lm06S. Eventually the eight AFLP markers and five SCAR markers were all mapped in a 1.0 cM region around *BnMs2* gene (Fig. 3c). Among these flanking markers of *BnMs2* gene, AFLP markers Lm10 and Lm11 were the most closely linked ones, which were 0.038 and 0.075 cM from *BnMs2* gene, respectively.

To determine the map location of *BnMs2* gene in the published *B. napus* reference map, polymorphism analysis was carried out using the 12 AFLP markers or SCAR markers between the parents of two DH populations derived from Tapidor × Ningyou 7 and Quantum × No.2127-17, respectively. AFLP markers Lm02, Lm06, and Lm12 detected polymorphism in the former population and SCAR marker Lm02S in the latter population. These markers were used to survey the corresponding DH populations and Mendelian segregations were observed. All the markers were mapped on linkage group N16 (Fig. 3a, b). To further confirm the map location of *BnMs2* gene and to identify other markers for MAS, five SSR markers (sR32187, Na12A02, Na12A05, sR32187, and BRMS105) were selected from the region surrounding *BnMs2* gene on linkage group N16. Only one SSR marker, Na12A05, showed polymorphism between S4516 A and S4516 B. Na12A05 was used to analyze the mapping population of 262 plants. The result showed that the distance between Na12A05 and *BnMs2* was 9 cM on the same side with SCAR marker Lm06S. Based on these facts, we conclude that *BnMs2* gene was located on linkage group N16 of the *B. napus* map. Yi et al. (2006) had mapped *BnMs1* on N7, substantiating the present allelism analysis between S45AB and S4516AB.

Comparative mapping with *Arabidopsis* for ACGM markers and collinear region

ACGM methodology was used to find closer markers linked to the target gene. By PCR walking we successfully extended all the sequences of the eight AFLP markers used in the fine mapping, the size range of which was approximately from 0.8 to 2.6 Kbp (Table 3). BlastN searches were carried out against the *Arabidopsis* database using a score value of 82 or higher as a cutoff for putative orthology (Lukens et al. 2003). Five of the eight maker sequences had homologues at the bottom of *Arabidopsis* chromosome 1 while marker Lm10 was mapped on chromosome 5 (Table 3). No homologue was found for the other two markers (Lm09 and Lm14). The five markers were perfectly collinear between *B. napus* and *Arabidopsis* and the closest flanking homologues (At1g68710.1 and At1g69920.1) delimited a region containing 121 *Arabidopsis* genes. *Arabidopsis* EST sequences from this region and *Brassica* EST or GSS sequences homologous to this region of *Arabidopsis* chromosome 1 were assayed as potential markers in an attempt to saturate the interval on N16 containing *BnMs2* locus. Altogether 16 primer pairs were designed and tested, however, only one showed polymorphism among the bulked-DNA samples. All the 2,650 plants with this ACGM marker (designated as Yao) were screened for recombinants. Eventually one recombination event was detected between Yao and *BnMs2* locus and marker Yao was mapped on the same side with Lm11. Sequence of Yao from the *B. napus* was further subjected to BlastN search and one homologue (At1g69240) was found in the same syntenic interval. Thus marker Yao and Lm04 (At1g69920.1) delimited a region only containing 68 *Arabidopsis* genes in which the candidate of *BnMs2* might exist (Fig. 3d).

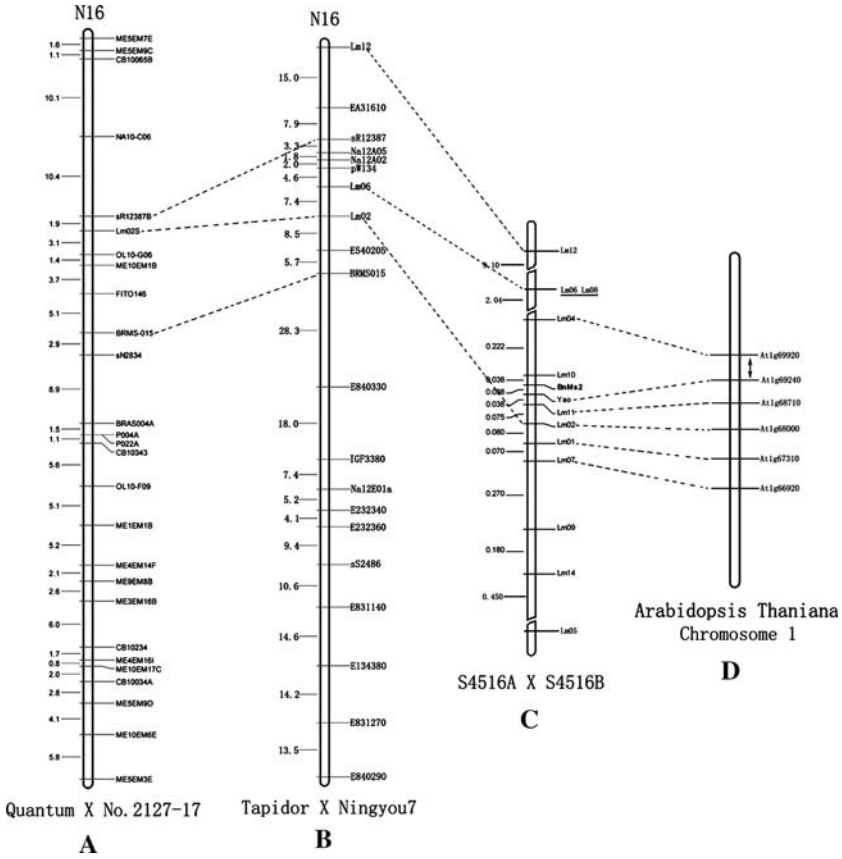

Fig. 3 **a** A linkage map of N16 derived from the cross Quantum × No.2127-17 indicates the position of Lm02S. **b** A linkage map of N16 developed from the cross Tapidor × Ningyou7 (Qiu et al. 2006) shows the location of Lm12, Lm06, Lm02, and Na12A05. **c** Genetic map of the region containing *BnMs2* gene was produced by combining data from the two mapping populations consisting of 262 plants and 2,650 plants, respectively. **d** A partial physical map of Chromosome 1 of *Arabidopsis thaliana* shows the homologues of mapped maker sequences. *Dotted lines* indicate the common AFLPs, SCAR, and SSR in three linkage maps of Tapidor × Ningyou7, Quantum × No.2127-17, and S4516A × S4516B populations, and show the *Arabidopsis thaliana* genes corresponding to specific markers based on sequence homology. *Double arrow-headed line* indicates the collinear interval possibly carrying the orthologue of *BnMs2*

Discussion

In the present study, we describe the construction of a fine scale map of *BnMs2* locus using a combination of BSA, AFLP, and ACGM methodologies. Through comparative mapping with *Arabidopsis*, a syntenic region covering 68 genes was identified in which the homologue of *BnMs2* might be included.

It has been reported that the workload could be effectively reduced by using plants that are recessive at the target locus for linkage analysis (Thomas et al. 1995; Liu et al. 2001; Imai et al. 2003). Thus we chose 2,650 sterile plants (doubly recessive individuals in this study) for detecting recombination events from over 5,000 individuals in a 1:1 segregating NIL population. We prepared 530 bulks and each bulk was composed of five sterile individuals. Recombination bulks were firstly screened out and recombination individuals were subsequently identified after the plants forming the corresponding bulks were assayed. Only one recombinant was detected for most of the five-plant-bulks. For markers less than one centimorgan to the target gene, the bulks consisting of two or more recombinants were not found in our screening. Therefore for fine mapping, one bulk of five recessive plants could be actually seen as one individual for screening recombinants.

In the experiment of mapping the target gene, a population segregating for *BnMs2* locus was generated. Because the SCAR markers were tightly linked to *BnMs1* developed by Yi et al. (2006), it efficiently helped in screening of candidate populations. In fact, genes in two candidate populations were initially identified as being nonallelic to that of S45AB by MAS, however, only S4516AB was confirmed by classical genetic analysis, which suggested molecular markers developed in one population might not show polymorphism in another population. Therefore, it was crucial to conduct allelism analysis to determine the allelic relationship between genes in S45AB and S4516AB. *BnMs2* was eventually mapped on N16 other than N7 on which

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

ACGM and AFLP marker	Extended fragment size in bp	*Arabidopsis* gene name	Identities
Lm01	1,484	At1g67310.1	264, 2e-69, 223/253 (88%), 5 regions,
Lm02	1,368	At1g68010.1	214, 2e-54, 135/144 (93%), 4 regions
Lm04	958	At1g69920.1	101, 2e-20, 105/123 (85%), 1 region
Lm07	1,392	At1g66920	636, 0.0, 575/658 (87%), 3 regions
Lm09	821	Not significant	
Lm10	940	At5g15380	92, 1e-17, 115/138 (83%), 2 regions
Lm11	2,661	At1g68710.1	720, 0.0, 861/1,027 (83%), 4 regions
Lm14	815	Not significant	
Yao	815	At1g69240	196, 2e-49, 237/281(84%), 1 region

In the "Identities" 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 identical in our sequence and in the corresponding *Arabidopsis* sequence; the number of regions is the number of different regions recognized in the *Arabidopsis* sequence

BnMs1 was located. This result was consistent with that of the initial allelism analysis.

Sterility in S45A was controlled by two separate loci: *Bnms1* and *Bnms2*, and both *BnMs1* and *BnMs2* can restore the fertility of S45A independently (Pan et al. 1988; Li et al. 1995). It is generally recognized that these two genes have the same function, thus, it is reasonable to assume that the coding sequences of both genes should be conserved. In the present work, *BnMs2* was mapped on upper part of N16 whereas *BnMs1* was integrated to the lower half of N7 (Yi et al. 2006). These data are in agreement with Parkin et al. (2005) that the lower half of N7 was homologous with N16 and *Arabidopsis* chromosome 1. Moreover, *BnMs1* was defined to the same interval as *BnMs2* on *Arabidopsis* chromosome 1 via comparative mapping (unpublished data from Yi), which led us to speculate that *BnMs1* and *BnMs2* might originate from one gene that diversified during evolution of the amphidiploid bastard *B. napus*.

It has been recognized that the sequence information and genomic resources of *Arabidopsis* could be used for marker development, map-based gene cloning, and candidate gene identification in *Brassica* crops (reviewed in Pflieger et al. 2001; Schmidt 2002; Snowdon and Friedt 2004). In the present study, we have identified AFLP and SCAR markers tightly linked to *BnMs2* gene at a genetic distance of 0.038 cM. Based on *Arabidopsis* sequences, an ACGM marker was developed, which delimited *BnMs2* gene to a smaller genetic interval of 0.075 cM. We previously intended to exploit the candidate gene strategy to clone the target gene. Nevertheless, the homologue of the nearest marker sequence (Lm10) was positioned not in the expected region of *Arabidopsis* chromosome 1 but on chromosome 5, leading to identify a relatively wide interval containing 68 candidates. It has been reported *B. napus* genome has an average of 483 kb/cM (Arumuganathan et al. 1991; Foisset et al. 1996; Yi et al. 2006), 0.075 cM would correspond to physical distance of less than 40 kb. Because *BnMs2* was mapped on N16 belonging to C genome (donated by *B. oleracea*), BAC clones anchoring the closest flanking markers were identified from both *B. napus* and *B. oleracea*. These clones are in the process of sequencing. The partial sequence data (data not shown) indicate that homologues of flanking genes of Lm10 are still located in the candidate region, which might suggest a translocation event had occurred in the *Arabidopsis* genome since the divergence of *Brassica* species and *Arabidopsis*, whereas the *Brassica* species maintained the conserved block.

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