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ORIGINAL PAPER

Fine mapping of the recessive genic male-sterile gene (*Bnms1*) in *Brassica napus* L.

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Abstract A recessive genic male sterility (RGMS) system, S45 AB, has been developed from spontaneous mutation in *Brassica napus* canola variety Oro, and is being used for hybrid cultivar development in China. The male sterility of S45 was controlled by two duplicated recessive genes, named as *Bnms1* and *Bnms2*. In this study, a NIL (near-isogenic line) population from the sib-mating of S45 AB was developed and used for the fine mapping of the *Bnms1* gene, in which the recessive allele was homozygous at the second locus. AFLP technology combined with BSA (bulked segregant analysis) was used. From a survey of 2,560 primer combinations (+3/+3 selective bases), seven AFLP markers linked closely to the target gene were identified, of which four were successfully converted to sequence characterized amplified region (SCAR) markers. For further analysis, a population of 1,974 individuals was used to map the *Bnms1* gene. On the fine map, *Bnms1* gene was flanked by two SCAR markers, SC1 and SC7, with genetic distance of 0.1 cM and 0.3 cM, respectively. SC1 was subsequently mapped on linkage group N7 using doubled-haploid mapping populations derived from the crosses Tapidor × Ningyou7 and DH 821 × DHBao 604, available at IMSORB, UK, and our laboratory, respectively. Linkage of an SSR marker, Na12A02, with the *Bnms1* gene further confirmed its location on linkage group N7. Na12A02, 2.6 cM away from *Bnms1*, was a co-dominant marker. These molecular markers developed from this research will facilitate the marker-assisted selection of male sterile lines and the fine map lays a solid foundation for map-based cloning of the *Bnms1* gene.

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Introduction

Hybrid cultivars have been successfully used to increase double-zero rapeseed production worldwide. Male sterility has been applied to this crop as an effective and economical pollination control system. Plant male sterility has been generally classified into cytoplasmic male sterility (CMS) and genic male sterility (GMS). The advantage of using CMS system is that it can generate a complete male sterile population economically. However, this system involves the development of three lines: male sterile line (A), maintainer (B), and restorer (R), and it usually takes years to develop A and R lines since most CMS systems have stringent restoring-maintaining relationships. In contrast, recessive GMS has many advantages. Firstly, GMS involves only two lines and is transferred feasibly among parental lines, which may result in a shortened breeding cycle. Secondly, for recessive GMS, most breeding lines are restorers, so it is easy to combine any elite lines to produce strong heterosis. Thirdly, GMS does not have the negative cytoplasmic effect on yield as CMS might do. But GMS system has its limitation of being difficult to derive a complete male sterile population. About 50% male fertile plants must be removed from the female lines during hybrid seed production. Development of a complete genic male sterile population with a temporary maintainer B-line of GMS was a breakthrough to

overcome the previous limitation (Chen et al. 1993, 1998). Consequently, several GMS-based hybrids have been released commercially in China.

S45 AB is a recessive genic male sterile line derived from a natural mutant found in the *Brassica napus* canola variety Oro (Pan et al. 1988). Genetic analysis indicated that two duplicate recessive genes, named as *Bnms1* and *Bnms2*, controlled the male sterility in S45 populations since it showed 15:1 segregation ratio in crosses of S45 and fertile cultivars (Pan et al. 1988). S45 AB line is composed of 50% male sterile (ms) plants (i.e. S45 A, Genotype: *Bnms1ms1ms2ms2*) and 50% male fertile (mf) plants (i.e. S45 B, Genotype: *BnMs1ms1ms2ms2*), in which the recessive allele was homozygous at the second locus (Li et al. 1993). S45 AB is maintained by harvesting the progeny from S45 A plants through sib-mating (i.e. S45 A ms plants pollinated by S45 B mf plants). S45 A has a broad spectrum of restoration with almost all normal rapeseed lines restoring the fertility of F_1 hybrids. This wide spectrum of restorers makes it easy to find hybrid combinations with high heterosis (Pan et al. 1988; Li 1995). Moreover, the transfer of RGMS genes to different backgrounds can be facilitated through the use of marker-assisted selection in hybrid breeding programs.

Amplified fragment length polymorphism (AFLP) (Vos et al. 1995) has high effectiveness in the identification of molecular markers that are linked to the gene of interest, particularly when used in combination with bulked segregant analyses (BSA, Michelmore et al. 1991). Normally, 50–100 AFLP loci can be surveyed with every primer pair. By increasing the number of primer pairs, large numbers of AFLP loci can be screened throughout the whole genome, thus enabling AFLP markers linked tightly to the target gene to be found without prior information on plant genome sequences (Xu et al. 2000). In *B. napus*, AFLP technology has been effectively used to map the male sterile locus (Lu et al. 2004; Ke et al. 2004).

In this report we have utilized AFLP technology to identify AFLP markers linked tightly to the *Bnms1* gene and construct a high-resolution map around the *Bnms1* gene.

Materials and methods

Plant materials

RGMS two-type line, S45 AB, was used as materials. S45 AB line has been maintained by full sib-mating (S45 A × S45 B) for 25 generations. The ms plant S45A and the mf plant S45B are therefore near isogenic lines (NIL) that differ nearly only by the ms/mf trait. The fertile plants had yellow, well-developed anthers whereas the sterile plants were characterized by white anthers with no pollen (Fig. 1). A NIL population including 1,974 plants was used for molecular mapping of the *BnMs1* gene. The mf plant was self-pollinated to obtain a segregating population for co-dominant molecular marker analysis. The male fertility was visually scored at flowering time.

DNA extraction and BSA

Genomic DNA was extracted individually from leaves by the CTAB method (Doyle and Doyle 1990). DNA concentration and purity was measured by a Beckman spectrophotometer (Beckman Coulter Inc., Fullerton, CA, USA) at a wavelength of 260 nm versus 280 nm. Final DNA concentration was 25 ng/μl in TE buffer (10 mM Tris, 1 mM EDTA pH 8.0). DNA from 12 ms plants and 12 mf plants was pooled to construct sterile and fertile bulks, respectively. The parental DNA and the two bulks were used for BSA (Michelmore et al. 1991) with the AFLP technique.

AFLP analysis

The AFLP procedure 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 four restriction enzyme combinations (*Eco*RI/*Mse*I, *Eco*RI/*Pst*I, *Pst*I/*Mse*I, *Sca*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 DNA was diluted (1:30), and an aliquot (1 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, PH8.0) 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 2 μl mixture was resolved on 6% denaturing polyacrylamide gels and visualized by the silver nitrate staining.

Cloning and sequencing

AFLP markers were cloned as follows: AFLP fragments were excised from the acrylamide gel and

Fig. 1 Flower morphology of RGMS two-type line S45 AB. **a** A male-sterile bud of S45 A, the size of the bud is about 4.5 mm, the petals and the sepals have been peeled off. **b** A male-fertile bud of S45 B, the size of the bud is about 4.5 mm, the petals and the sepals have been peeled off. **c** Opening flower of a male sterile plant. **d** Opening flower of a male fertile plant

incubated in 20 µl TE (10 mM Tris, 1 mM EDTA pH 8.0) for 10 min at 95°C. After centrifugation, 2 µl of supernatant was used as template for PCR amplification using primers and reaction conditions similar to those used for the AFLP reaction. The amplified products were separated on a 0.8% agarose gel. DNA bands were excised from the gel and purified with UNIQ-10 EZ Spin Column DNA Gel Extraction Kit (Sangon, Shanghai), and the DNA was cloned into the plasmid vector pGEM-T (Promega). The recombinant plasmids were screened using the colony PCR method (Innes et al. 1990). Three correct clones were sequenced using the dideoxy method. We used the Universal GenomeWalker kit (Clontech) to increase the size of the cloned markers by PCR walking (Siebert et al. 1995). The final PCR products were cloned and sequenced as described above.

Conversion of AFLP marker into SCAR marker

To obtain SCAR markers, primers were designed from the sequences of AFLP markers and sequences of extended AFLP markers using the OLIGO program (Rychlik and Rhoads 1989). These primers were used to amplify genomic DNA from the 24 individuals. SCAR amplification was performed in 15 µl reaction volumes, which contained 50 ng template DNA, 1 × amplification buffer, 150 µM dNTPs, 2 mM $MgCl_2$, 5 pmol of each special primer, and 1 U *Taq* DNA polymerase (MBI). The primer sequences and PCR conditions are listed in Table 2.

Mapping

A NIL population with 1,974 individuals was used for mapping the *BnMs1* gene. Two double-haploid (DH) populations, which were from the project of IMSORB (integrated marker system for oilseed rape breeding) and our laboratory, were used to map the SCAR markers to certain linkage group. After the SCAR markers were mapped on the linkage group N7, which was named after the *B. napus* map available at http://www.ukcrop.net/perl/ace/search/BrassicaDB, a number of SSR markers in this region 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 the *BnMs1* gene was constructed. Map order was based on maximum-likelihood estimates. The Kosambi mapping function (Kosambi 1944) was used in calculating genetic distances.

Results

Genetic analysis of the *Bnms1* gene

Of the 1,974 plants developed from the crosses between S45 A and S45 B, the male sterile and the fertile siblings, 997 were male sterile and 977 were male fertile. The population thus displayed a ratio of ms to mf plants that did not differ significantly from 1:1 ($\chi^2 = 0.183$, $P > 0.05$). The segregation of fertile to male sterile plants in the selfing population of the S45 B line (72 individuals, 53 mf plants, 19 ms plants) did not differ significantly from 3:1 ($\chi^2 = 0.019$, $P > 0.05$). The results confirmed that one of the two recessive genes segregated in the S45 AB population, and the gene was designated as *Bnms1*. This result was consistent with observations previously reported (Li et al. 1993).

Screening AFLP markers linked to the *BnMs1* gene

AFLP analysis showed that there were about 50–100 bands with a size range from 50 up to 900 bp in each 3 + 3 primer pair. In the bulked segregant analysis (Michelmore et al. 1991), 1,024 E+3/M+3 and 512 E+3/P+3 and 512 P+3/M+3 and 512 S+3/M+3 primer pairs were used for screening polymorphism between two parents and two bulks. Polymorphic bands that appeared in both S45 B and fertile bulk (BF), but not in S45 A and male sterile bulk (BS) were potentially linked to the gene *BnMs1*. To further examine potential linkage, polymorphic bands were further examined among twenty-four individuals comprising the bulks. Such screening eventually identified seven polymorphic markers linked to the *BnMs1* gene, which were designated as AF1 to AF7 (Table 1), respectively. We then screened 96 individuals in the NIL population for primary linkage analysis. Figure 2 shows a representative amplification profile with two different primer pairs. With the primer pair E-CAA /M-CAC, a 186-bp band (marked as an arrow, Fig. 2a) was present in male fertile individuals and absent in the male sterile individuals; this was designated as AF4 (Table 1). With the primer pair E-ATA/M-GAC, a 241-bp band (shown as an arrow in Fig. 2b) was present in the male fertile individuals and absent in the male sterile individuals; this was designated as AF2. Out of these 96 individual plants analyzed, 2 individuals showed recombination for AF4 marker and no recombination for AF2 marker. These results thus established that the AFLP markers were linked closely to the target gene.

Conversion of AFLP markers to SCAR markers

Several features of the AFLP technique limit its application in large-scale screens for map-based cloning or MAS. To overcome this problem, the AFLP markers linked to the *BnMs1* gene were cloned and sequenced with the objective of converting these into simple PCR-based markers. The sequence data revealed the presence of a selective amplification primer sequence, thereby confirming the correctness of the fragments. Based on the sequence data of the seven AFLP markers, primers were designed for direct amplification of the corresponding loci from genomic DNA. Since the amplified fragments for these loci were small in size, it was not possible to detect polymorphism between ms and mf individuals (data not shown). Thus, PCR-walking was used to extend the sequence into the flanking regions of the original AFLP markers.

Four of these seven markers were successfully extended by PCR walking (AF1, AF3, AF6, and AF7), and four specific primer pairs were designed from the extended sequences (Table 2). Using these primer pairs, PCR reactions were performed among two parents, two bulks, twenty-four individuals comprising the bulks. Eventually, all of these four AFLP markers were successfully converted into SCAR markers. SC1, SC3 and SC6 converted from AF1 (Fig. 3b), AF3 (Fig. 3a) and AF6, respectively, were dominant markers. Primers based on the extended AP7 sequence amplified an

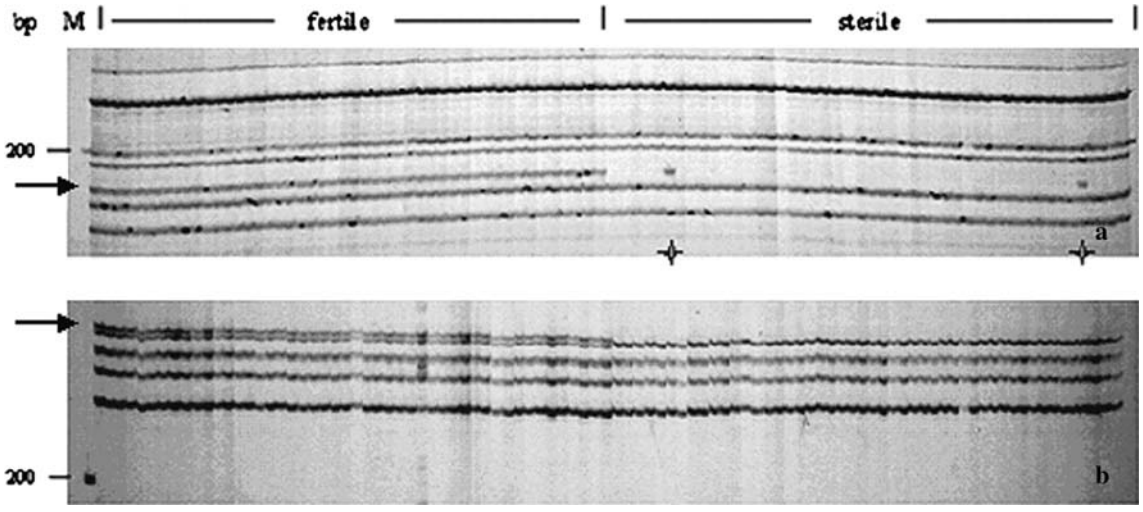

Fig. 2 AFLP profiles from 48 fertile plants and 48 sterile plants. The *arrows* indicate polymorphic band present in fertile individuals but not in individuals. The AFLP markers were AF4 **(a)** and AF2 **(b)**, respectively. + recombinant type *M* 100 bp DNA ladder

Table 1 Description of AFLP markers tightly linked to the *BnMs1* gene

AFLP Marker	Primer combination	Size of marker (bp)	Extended?	Name of the extended marker	Map distance (cM)
AF1	E-ATC/ M-CAG	203	Yes	SC1	0.1
AF2	E-ATA/ M-GAC	241	No	–	0.1
AF3	E-ATC/ P-GCA	211	Yes	SC3	1.6
AF4	E-CAA/ M-CAC	186	No	–	2.3
AF5	P-GTT/ M-CTG	187	No	–	2.3
AF6	S-AAA /M-GCC	284	Yes	SC6	2.3
AF7	E-AGC/ M-GTA	362	Yes	SC7	0.3

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

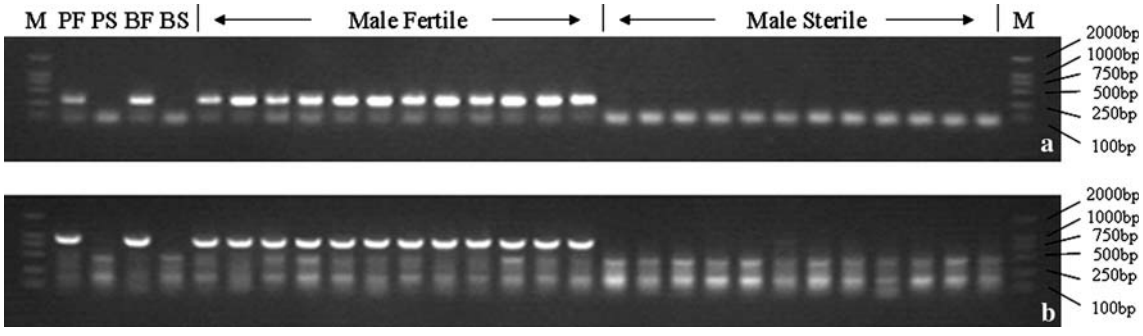

Fig. 3 The amplification results of SC3 (**a**) and SC1 (**b**) on individuals plants. Lanes: *M* DNA molecular weight marker 2000, *PF* S45 B, *PS* S45 A, *BF* male fertile bulk, *BS* male sterile bulk

identical band from both male fertile and male sterile individuals on agarose gels. PCR products were, therefore, resolved on a 6% denaturing polyacrylamide gel. The result showed that products from male fertile plants were two bands while those from male sterile plants were one band. By comparing the sequences of the fragments from the fertile and the sterile plants, a 4-bp deletion was found for the size change, resulting in a co-dominant SCAR marker, SC7.

Mapping

In the mapping experiment, two SCAR markers (SC6 and SC7) were assayed first in the whole NIL population consisting of 1,974 individuals. After all individuals from the mapping populations were tested for their genotypes, 45 individuals displayed recombination between the *Bnms1* gene and SC6, and 6 recombinants of the *Bnms1* gene and SC7 were identified. Furthermore

Table 2 SCAR markers derived from AFLP markers

AFLP markers	AFLP derived SCAR markers					
	Name	Primers (5′ –3′)a	Size (bp)	PCR conditions[b]		Polymorphism
AF1	SC1	ACACGGTGATCCGGTAAGTCGT/ CCAGTAGGAGTCACCGAGATA	1,198	62°C 45 s	72°C 60 s	Dominant
AF3	SC3	TTGATAGGTCTCTCTCCAAA/ TAAAGAGTTGTAACTGATGC	312	55°C 45 s	72°C 30 s	Dominant
AF6	SC6	TGTGGTATGTAGGCAGTGGTTA/ ATGGGAGGAAGAAGAAGCA	1,029	60°C 45 s	72°C 60 s	Dominant
AF7	SC7	GGTCTAGAATAGTTGGCGAG/ CTACCTGAGTACATCTGTGC	580/576[c]	60°C 45 s	72°C 45 s	Co-dominant

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

[b] 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

[c] The size of PCR product linked with *BnMs1* and *Bnms1* respectively

other markers were then analyzed only with these recombinants. The seven AFLP markers and four SCAR markers were all mapped in a 2.6 cM region around the *Bnms1* gene (Fig. 4b). Among these flanking markers of the *Bnms1* gene, SC1 and SC7 were the most closely linked ones. SCAR marker SC1 and AFLP marker AF1 were in the same loci, approximately 0.1 cM from the *Bnms1* gene. On the other side of the *Bnms1* gene, SCAR marker SC7 and AFLP marker AF7 were about 0.3 cM from *Bnms1* gene.

To determine the map location of the *Bnms1* gene in the published *B. napus* genetic map, polymorphism analysis was conducted using the four SCAR markers in the parents of two DH populations derived from Tapidor × Ningyou 7 and DH 821 × DHBao 604 (Yi et al. 2006), respectively. Only SC1 detected polymorphism between the two parents of DH populations. When the DH lines of two mapping populations were surveyed, Mendelian segregation was observed. SC1 was mapped between SNP marker IGF3309b and AFLP marker P10M10–150 on linkage group N7 of the Tapidor × Ningyou 7 reference map (Fig. 4a); In the DH821 × DHBao 604 DH mapping population, SC1 was mapped between two SSR markers Ra2A02 and Na12A02 on linkage group N7 at a distance of 4.9 cM and 2.1 cM, respectively (Fig. 4c).

To further confirm the map location of the *Bnms1* gene and to identify other markers for marker-assisted selection purposes, five SSR markers (Ra2A01, SSR04, SSR22, Ra2A05 and Na12A02) were selected from the region surrounding the *Bnms1* gene on linkage group N7. Only one SSR marker, Na12A02, showed polymorphism between S45 A and S45 B. Na12A02 was used to analyze the mapping population. The result showed that the distance between Na12A02 and *Bnms1* was 2.6 cM on the same side with SCAR marker SC6. We also surveyed the selfing population derived from S45B with Na12A02, and two individuals were found displaying recombination between the *Bnms1* gene and Na12A02 (Fig. 5). The results show that Na12A02 was a co-dominant marker. The evidence described above led to the conclusion that the *Bnms1* gene is located on linkage group N7 of the *B. napus* map.

Discussion

In China, RGMS has become an important hybrid system and several RGMS-based hybrids have already been registered. Because the development of new RGMS lines through conventional backcrossing and selfing is both time and labor consuming, our goal is to establish a set of markers linked to the *BnMs1/Bnms1* locus that will be very useful for marker-assistant selection (MAS) of *Bnms1* gene. We screened 2,560 AFLP primer pairs and identified only seven AFLP markers linked to the *BnMs1* gene. It is not surprising since the two-type line S45 AB have been sib-mated for about 25 generations. S45 A and S45 B are indeed near-isogenic, which may differ only at the *BnMs1/Bnms1* locus and those few tightly linked loci. Meanwhile, other loci that are not linked closely to *BnMs1/Bnms1* locus became homozygous after the long period of sib-mating. Our AFLP analysis of the population suggested that the backgrounds of S45A and S45B were identical.

AFLP markers are generally expensive to generate and dominant in nature, thus limiting their use in large-scale marker-assisted selection in plant breeding. In our study, the AFLP polymorphic markers linked to *BnMs1* were in the size range of 150 to 370 b that was not big enough to reveal any polymorphism inside the sequences of these original AFLP markers; it is expected that we could isolate the flanking sequence of

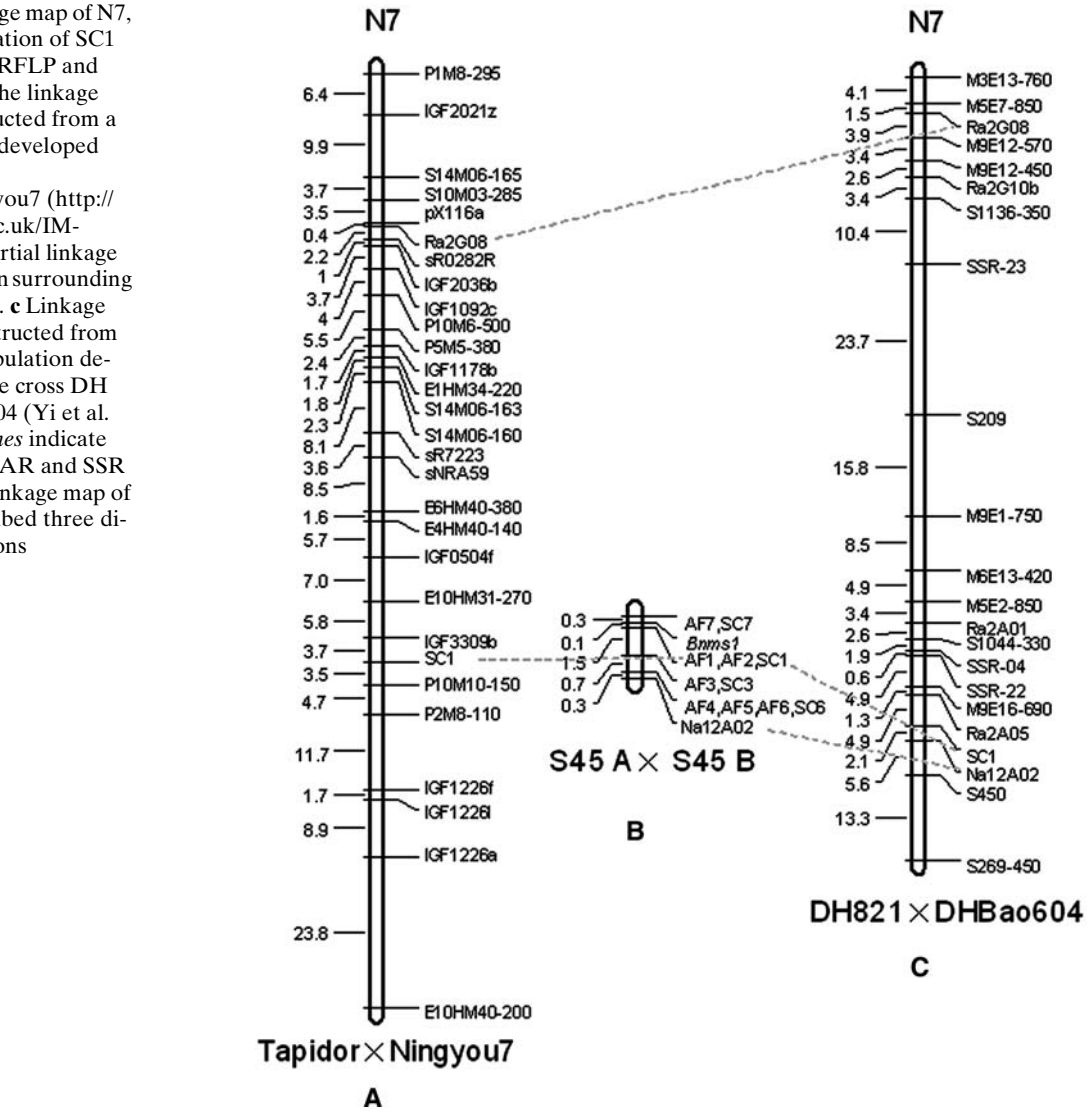

Fig. 4 **a** A linkage map of N7, showing the location of SC1 integrated with RFLP and SNP markers. The linkage map was constructed from a DH population developed from the cross Tapidor × Ningyou7 (http://brassica.bbsrc.ac.uk/IMSORB/). **b** A partial linkage map of the region surrounding the *Bnms1* gene. **c** Linkage map of N7 constructed from another DH population developed from the cross DH 821 × DHBao 604 (Yi et al. 2006). *Dotted lines* indicate the common SCAR and SSR markers in the linkage map of the above described three different populations

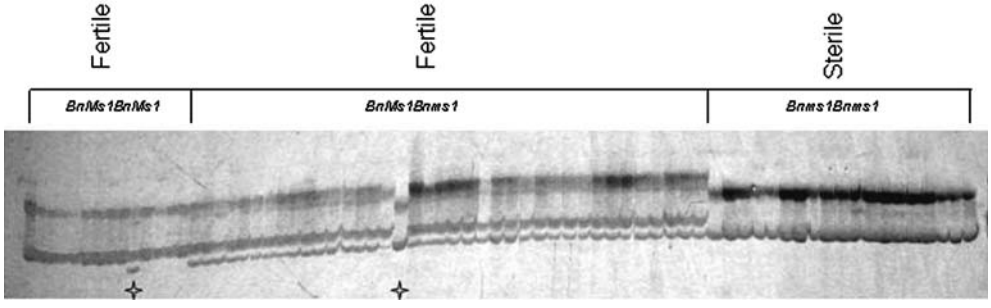

Fig. 5 Analysis of the PCR products obtained using the SSR primer Na12A02 on individual F2 plants. The F2 individual plants are represented as homozygous male fertile (*BnMs1BnMs1*), heterozygous male fertile (*BnMs1Bnms1*) and male sterile (*Bnms1Bnms1*). + recombinant type

these AFLPs and find the sequence positions that result in the polymorphic bands for the AFLP markers. We used the PCR-Walking method to isolate the flanking sequence and four AFLP markers were successfully converted into SCAR markers. The SCAR markers and SSR marker obtained in this study will greatly facilitate the transfer of the recessive GMS allele to desirable genetic backgrounds via marker-assisted selection. PCR-based selection of plants carrying the *Bnms1* allele at the seedling stage should greatly

enhance selection efficiency in backcross breeding. This will also help in the breeding of temporary maintainer B-line with great accuracy in early generations. In the recessive GMS line S45AB, there are two separate ms loci, *Bnms1* and *Bnms2*. It is therefore highly desirable to also develop markers linked to the *Bnms2* gene and this work is in progress presently in our laboratory.

Even though the GMS system is an effective alternative hybrid system in *B. napus*, very little is known regarding the molecular basis of the GMS trait, which limits its wide application to hybrid breeding. Cloning, isolation, and characterization of GMS genes in the future will improve its application in hybrid breeding. Cigan and Albertsen (1997) suggested an interesting strategy to multiply ms line by using an inducible promoter. In this strategy, the native promoter is taken off from the male fertility gene and replaced with an inducible promoter. Such an inducible promoter responds to a chemical. The ms line remains ms (for use in hybrid production) in the absence of the chemical, but is reverted to be mf (for multiplication) with the chemical application. Cloning of the *BnMs1* gene is the first crucial step if we are to apply this strategy in GMS system. Identification of closely linked DNA markers is a prerequisite for map-based cloning of the *BnMs1* gene. In the present study, we have identified AFLP and SCAR markers tightly linked to the *BnMs1* gene at a genetic distance of 0.1 cM. *B. napus* has a nuclear DNA content of approximately 1,200 Mb/1 C (Arumuganathan and Earle 1991). Foisset et al. (1996) estimated the genome length of rapeseed to be 2,486 cM, thus, if the *B. napus* genome has an average of 483 kb/cM, 0.1 cM would correspond to physical distance of less than 50 kb. BAC clones anchoring these SCAR and AFLP markers can be used to construct physical contigs and clone the *Bnms1* gene.

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