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ORIGINAL PAPER

Fine mapping of the recessive genic male sterility gene (*Bnms3*) in *Brassica napus* L.

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Abstract The *Brassica napus* oilseed rape line, 7-7365AB, is a recessive epistatic genic male sterile (RGMS) two-type line system. The sterility is controlled by two pairs of recessive duplicate genes (*Bnms3* and *Bnms4*) and one pair of recessive epistatic inhibitor gene (*Bnrf*). Homozygosity at the *Bnrf* locus (*Bnrfrf*) inhibits the expression of the two recessive male sterility genes in homozygous *Bnms3ms3ms4ms4* plants and produces a male fertile phenotype. This line has a good potential for heterosis utilization but it is difficult to breed heterotic hybrids without molecular markers. To develop markers linked to the *BnMs3* gene, amplified fragment length polymorphism (AFLP) technology was applied to screen the bulks of sterile and fertile individuals selected randomly from a population of near-isogenic lines (NIL) consisting of 2,000 plants. From a survey of 1,024 primer combinations, we identified 17 AFLP markers linked to the *BnMs3* gene. By integrating the previous markers linked to the *BnMs3* gene into the genetic map of the NIL population, two markers, EA01MC12 and EA09P06, were located on either side of the *BnMs3* gene at a distance of 0.1 and 0.3 cM, respectively. In order to use the markers for male sterile line breeding, five AFLP markers, P05MG05, P03MG04, P11MG02, P05MC11$_{250}$, and EA09P06, were successfully converted into sequence characterized amplified region (SCAR) markers. Two of these, P06MG04 and sR12384, were subsequently mapped on to linkage group N19 using two doubled-haploid mapping populations available at our laboratory derived from the crosses Tapidor × Ningyou7 and Quantum × No2127-17. The markers found in the present study should improve our knowledge of recessive genic male sterility (RGMS), and accelerate the development of male sterile line breeding and map-based cloning.

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Introduction

A key problem in utilizing heterosis in rapeseed breeding is that of producing hybrid seeds economically. Currently, the main approaches for rapeseed hybrid production are based on male sterility. The trait can be classified into cytoplasmic male sterility (CMS) and genic male sterility (GMS) according to whether the genetic resources are cytoplasmic or nuclear. CMS is preferred, because it is easier to find maintainers of male sterility. Several three-line systems have been identified in *Brassica napus*. However, there are some defects with some of them such as sensitivity to temperature, incomplete sterility, and the limited availability of restorer lines (Fu and Tu 2002). On the other hand, GMS has more advantages, such as stable and complete male sterility, rich sources of cytoplasm, and ease of transfer of male sterility genes. The major drawback to utilizing GMS is that the offspring of GMS plants pollinated by heterozygous pollinators always segregate in a 1:1 ratio and this requires the eradication of male fertile plants from the maternal line. In recent years, studies have been carried out on some GMS systems to generate an absolutely male sterile population.

The GMS systems currently in use are classified into three kinds according to their inheritance; GMS with

double recessive genes (Hou et al. 1990; Tu et al. 1997), GMS controlled by interacting recessive genes (Chen et al. 1998), and GMS which is inherited as a dominant gene (DGMS) (Li et al. 1985, 1990). There are at least two origins of independent recessive duplicate genes (RDSG) in *B. napus* L., 9012A and S45A, which are non-allelic (Tu et al. 1997;Chen et al. 1998). The 9012A and 7-7365A have the same inheritance model, their RDSG are allelic (data not shown), and their sterility is controlled by two pairs of recessive duplicate genes (*Bnms3* and *Bnms4*). However, one pair of recessive epistatic inhibitor gene (*Bnrf*) at the *Bnrf* locus (*Bnrfrf*) can inhibit the expression of the recessive male sterility trait in homozygous *Bnm3m3m4m4* plants and show male fertility in the phenotype (Chen et al. 1998). Accordingly, the plants with the *Bnms3ms3ms4ms 4rfrf* genotype are male fertile, while the plants with the *Bnms3ms3ms4ms4Rf_* genotype are male sterile. According to genetic theory, a new model for heterosis utilization has been designed: two-type lines (*Bnms3ms3ms4ms4RfRf/BnMs 3ms3ms4ms4RfRf* or *Bnms3ms3ms4ms4RfRf/Bnms3ms3Ms 4ms4RfRf*), complete maintainers (*Bnms3ms3ms4ms4rfrf*) and restorers (most open pollinators). This model overcomes the shortcomings of recessive genic male sterility (RGMS) and generates an absolutely sterile population for hybrid seed production.

Based on this model, the breeding program will be complex, and molecular marker technology can profitably be employed to identify the target genotypes. Amplified fragment length polymorphisms (AFLPs, Vos et al. 1995) are an effective technology to identify markers linked to the target gene, particularly when used in combination with bulk segregant analysis (BSA, Michelmore et al. 1991). However, this technology is relatively expensive, complicated in procedure and not easy to use in large-scale selection. Therefore, successful conversion to co-dominant markers is necessary and very desirable in a breeding program.

Many molecular markers associated with important traits in *B. napus*, especially with GMS, have been identified. Six AFLP markers linked to the *Ms* gene in RGMS 117A (Ke et al. 2004), seven AFLP markers associated with the *BnMs1* gene in RGMS S45A (Yi et al. 2006), eight AFLP markers associated with the *Ms* gene in DGMS 609A (Song et al. 2006) have been identified and some have been converted into sequence characterized amplified region (SCAR) markers. So far none of the RGMS genes in *B. napus* has yet been cloned. To isolate and clone the *Bnms3* gene, it is imperative to construct a fine map of the *Bnms3* gene and to find the nearest linked markers. The aims of the present work were to identify some markers linked to the male sterility gene in this model system, to construct a high-resolution map around the *Bnms3* gene and convert them to SCAR markers for marker-assisted selection.

Materials and methods

Plant materials and population construction

The RGMS two-type line, 7-7365AB (*Bnms3ms3ms4ms 4RfRf/BnMs3ms3ms4ms4RfRf*), was used in this study. This line has been sib-mated for 15 generations and the male sterile plants, 7-7365A (*Bnms3ms3ms4ms4RfRf*), and the male fertile plants, 7-7365B (*BnMs3ms3ms4ms4RfRf*), were used to construct a near-isogenic lines (NIL) population of 2,000 individuals, to identify markers linked to the *BnMs3* gene. An F_2 population, which was obtained by selfing 7-7365B (*BnMs3ms3ms4ms4RfRf*) was used for co-dominant molecular marker analysis.

DNA extraction and bulked segregant analysis

Genomic DNA was extracted by CTAB method (Doyle and Doyle 1990). Young leaves were removed at flowering time, and equal quantities of DNA from ten sterile plants and ten fertile plants was pooled to form the sterile bulks (BS) and fertile bulks (BF), respectively. DNA concentration was adjusted to 50 ng/μl.

AFLP analyses

Genomic DNA was isolated from the NIL and the F_2 populations as described previously. It was digested with *Eco*RI/*Mse*I and *Mse*I/*Pst*I, ligated to adapters and, after fivefold dilutions, the adapter-ligated DNA was pre-amplified with AFLP primers (EA: 5′-GAC TGC GTA CCA ATT CA-3′; P0: 5′-GAC TGC GTA CAT GCA G-3′; MC: 5′-GAT GAG TCC TGA GTA AC-3′; MG: 5′- GAT GAG TCC TGA GTA AG-3′) using the following cycling parameters: 20 cycles of 30 s at 94°, 60 s at 56°, and 60 s at 72°. A 5 μl products were analyzed in a 1.0% agarose gel and the presence of a low-molecular weight smear indicated successful amplification. After 10-30-fold dilutions of pre-amplified DNA, the products were prepared for selective amplification. The following cycling parameters were used for selective amplification, 1 cycle of 30 s at 94°, 30 s at 65°, and 60 s at 72°. The annealing temperature was then lowered by 0.7° per cycle during the first 12 cycles, and then 23 cycles were performed at 94° for 30 s, 56° for 30 s, and 72° for 60 s (Negi et al. 2000). Selective amplification products were separated on 6% polyacrylamide denaturing sequencing gel, fixed in 10% acetic acid for 30 min, rinsed with H_2O for 20 min, then visualized by silver staining system, and dried on glass plates (Lu et al. 2001, 2004).

Conversion of AFLP markers to SCAR markers

After silver staining, putative marker fragments were excised from the dried polyacrylamide gel (Cho et al.

1996), dissolved in 40 µl deionized water, boiled for 15 min, and centrifuged at 10,000 rpm for 1 min. The supernatant was used for selective amplification, using the same conditions as the original AFLP reaction (Mienie et al. 2005). The products were separated on 1.0% agarose gel, purified using a UNIQ-10 EZ Spin Column DNA Gel Extraction Kit, and then cloned to the pGEM-T easy vector (Promega, Madison, WI, USA) The two clones were sequenced, and based on these sequences, the specific primers were designed with the software Primer3 and amplified in sterile and fertile individuals. Finally, the PCR products were detected on 1.5% agarose gel to test whether or not there was polymorphism between the phenotypes.

Mapping

A NIL population with 2,000 individuals was used for mapping the *Bnms3* gene. Two doubled-haploid mapping populations derived from the crosses Tapidor × Ningyou7 (Qiu et al. 2006) and Quantum × No2127-17, available at our laboratory, were utilized to locate the AFLP markers to a specific linkage group. Because the AFLP markers were mapped to linkage group N19, all the simple sequence repeat (SSR) markers in this region were selected for the polymorphism survey. SSR amplification was performed as described by Lowe et al. (2002). The specific AFLP and SSR fragments that showed reproducible polymorphism between sterile and fertile individuals were regarded as dominant or co-dominant markers, respectively. These marker data and individual phenotypes were analyzed with the MAPMAKER/EXP 3.0 program (Lander et al. 1987; Lincoln et al. 1992). A minimum LOD score of 3.0 was used for map construction. Map distances were calculated using Kosambi's (1944) mapping function.

Results

Genetic analysis of the *Bnms3* gene

The NIL population derived from the crosses between 7-7365A and 7-7365B consisted of 2,000 individuals in all, which included 1,008 fertile and 992 sterile plants. The segregation ratio of fertile and sterile agreed with a 1:1 ratio ($\chi^2 = 0.13$ and $P > 0.05$). There were 214 individuals in total in the segregating F_2 population derived from selfing 7-7365B, comprising 157 fertile and 57 sterile plants, which did not differ significantly from a three fertile to one sterile ratio ($\chi^2 = 0.31$ and $P > 0.05$). The results showed that one of the two recessive genes segregated in the 7-7365AB population and it was named *BnMs3*.

Identification of AFLP markers linked to the *BnMs3* gene

In order to identify the markers linked to the *BnMs3* gene, 1,024 pairs of selective primer combinations of EA + 2/MC + 2, EA + 2/MG + 2, P0 + 3/MC + 2, and P0 + 3/MG + 2 were screened for two bulks, BF (ten male fertile individuals) and BS (ten male sterile individuals). The combinations that showed reproducible polymorphism were used to test the twenty individuals comprising the bulks. Finally, 15 primer combinations showed polymorphism and they amplified 17 polymorphic bands (Table 1); all were dominant markers. The NIL population of 2,000 individuals was used for map construction (Fig. 1b). Two markers, EA01MC12 and EA09P06, were located on either side of the *BnMs3* gene at a distance of 0.1 and 0.3 cM, respectively.

Conversion of AFLP markers into SCAR markers

For utilization in marker-assisted selection, the AFLP markers were converted into SCAR markers. All the AFLP markers above were cloned and sequenced. Based on the sequences, PCR primers were designed, amplified in the two bulks and in individuals of the NIL population. Subsequently, five SCAR markers were converted successfully (Table 2) and all amplified polymorphic bands in the fertile bulk and the fertile individuals only. These results indicated the dominant nature of the markers. The nearest marker, EA-CA/P0-CTG identified by Ke et al. (2005), was also converted into a SCAR marker successfully.

Table 1 AFLP markers associated with the *BnMs3* gene and their sizes

AFLP marker designation	Approximate size of marker (bp)	Primer combination
P05MG05	150	P0-CCA/MG-TA
P05MG02	150	P0-CCA/MG-AT
P06MG09	100	P0-CTG/MG-CA
P03MG04	250	P0-CAC/MG-AG
P04MG04	190	P0-CAG/MG-AG
EA01MC12	200	EA-AA/MC-CG
P05MC11	350	P0-CCA/MC-CC
P05MC11	250	P0-CCA/MC-CC
P03MC11	220	P0-CAC/MC-CC
EA12MG11	100	EA-CG/MG-CC
EA15MG04	90	EA-GC/MG-AG
P03MG15	220	P0-CAC/MG-GC
P11MG02	210	P0-GTT/MG-AT
P11MG05	150	P0-GTT/MG-TA
P15MG07	250	P0-ACA/MG-TC
P15MG07	150	P0-ACA/MG-TC
P06MG04	350	P0-CTG/MG-AG

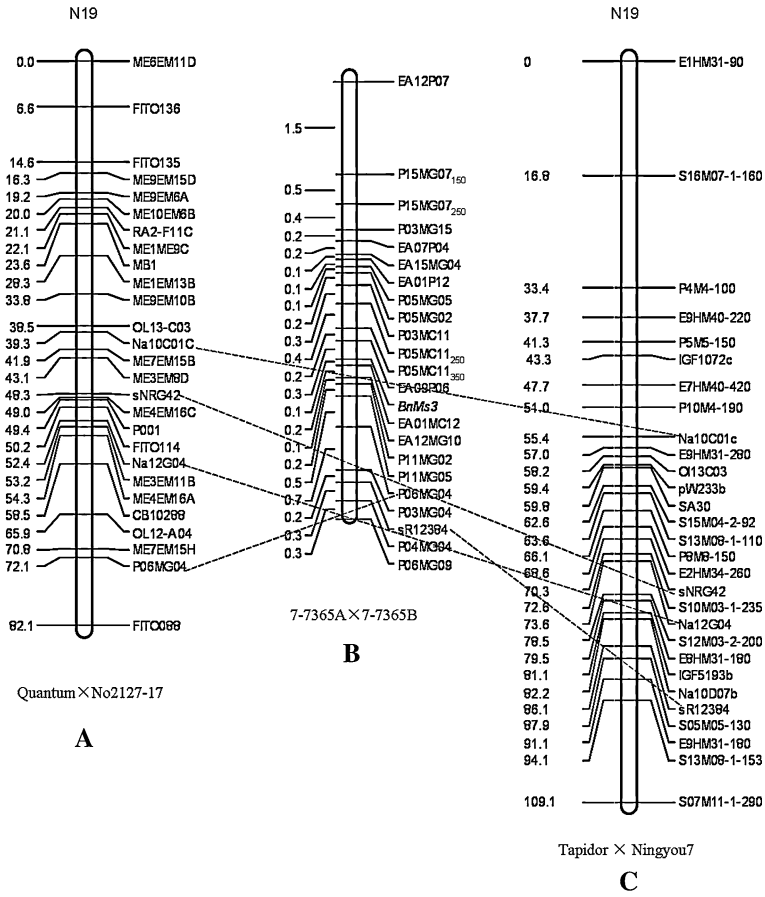

Fig. 1 Partial linkage maps of *B. napus* indicated the relative location of the *BnMs3* gene on linkage group N19 of the two reference maps. **a** Partial linkage group N19 from Quantum × No2127-17, the linkage map was mainly consisted of SSR markers and SRAP markers. **b** Linkage map of the region surrounding the *BnMs3* gene from the NIL population. **c** Partial linkage group N19 from Tapidor × Ningyou7, the map was mainly consisted of RFLP markers, SNP markers, and SSR markers. *Dotted lines* indicated the relationship of three genetic maps

Table 2 SCAR markers developed from AFLP fragments linked to the *BnMs3* gene

AFLP primer combinations	SCAR names	Primers (5′–3′)[a]	Size (bp)	PCR conditions[b]
P0-CCA/MG-TA	SC1	CAGCCAAATTGCCTGTAGTG/TAAGTATCCAAATCCACCCG	168	55°C 72°C 45 s 45 s
P0-CAC/MG-AG	SC2	TGCAGCACACAACAATAGAC/GAACAATAGCGAGTCAAAGG	234	55°C 72°C 45 s 45 s
P0-CCA/MC-CC_{250bp}	SC3	CCACCGTTCCAGGCCTTACT/TTCTCTGGATCAGTAGCCAC	236	59°C 72°C 45 s 45 s
P0-GTT/MG-AT	SC4	TGCAGGTTACCCTCTTTGGC/GGAAAGACAACAACCCCAAG	203	57°C 72°C 45 s 45 s
EA-CA/P0-CTG	SC5	CTGCAGCTGTTGGTTTTGAA/TCACAACTGAAACTAGCAGC	182	55°C 72°C 45 s 45 s

[a] Forward and reverse primers listed in that order

[b] PCR conditions for all primers were 3 min at 94°C, 35 cycles of 30 s at 94°C (Piao et al. 2004)

Mapping

All AFLP markers including those identified by Ke et al. (2005) and an SSR marker were mapped to a region of 7.1 cM around the *BnMs3* gene with an average distance of 0.3 cM. Among these flanking markers associated with the *BnMs3* gene, EA01MC12 and EA09P06 were the closest linked, at a distance of 0.1 and 0.3 cM, respectively (Fig. 1b).

In order to determine the location of the *BnMs3* gene in the published *B. napus* genetic map, all of the markers linked to the *BnMs3* gene were screened in the four parents of two DH populations derived from Tapidor × Ningyou7 and Quantum × No2127-17. Only P06MG04 showed polymorphic bands between the two parents of Quantum × No2127-17, and it was used to screen the population of 258 DH lines. As a result, P06MG04 was mapped to linkage group N19 of Quantum × No2127-17, and was located between an SSR marker FITO088 and a sequence related amplification polymorphism (SRAP) marker ME7EM15H. The ME7EM15H marker was nearest to P06MG04 at a distance of 1.3 cM (Fig. 1a).

To further confirm the location of the *BnMs3* gene and to identify other markers for marker-assisted selection purposes, all the SSR markers from that region on N19 of two reference maps were used to screen the F_2 population

derived from selfing 7-7365B. Only sR12384 from the reference map of Tapidor × Ningyou7 (Fig. 1c) showed polymorphism as a co-dominant marker between the sterile and fertile individuals. sR12384 was genotyped in the mapping population and was located at a distance of 2.0 cM from the *BnMs3* gene. All evidence showed that the *BnMs3* gene was located on linkage group N19 of the *B. napus* genetic map.

Discussion

In the present study, AFLP technology was used in combination with BSA to identify markers linked to the *BnMs3* gene. We screened 1,024 AFLP primer combinations in all and identified 17 AFLP markers linked to the *BnMs3* gene. We constructed a high-resolution map around the *BnMs3* gene, and found there were 22 markers around the *BnMs3* gene region, some of which were closer than any previously reported markers. In the previous study by Ke et al. (2005), seven AFLP markers were identified as being tightly linked to the *BnMs3* gene in a small population. However, with an enlarged population size, we found that some of them did not co-segregate with the gene. We used a large NIL population to allow fine mapping, which not only provided information on some available SCAR and co-dominant SSR markers, but also allowed us to locate the *BnMs3* gene on linkage group N19. Due to different genetic backgrounds in the two populations, only four markers showed polymorphism in the NIL population. EA01MC12 and EA09P06 were on either side of the *BnMs3* gene with distances of 0.1 and 0.3 cM, respectively. According to the estimate of Foisset et al. (1996), this distance of 0.1 cM would be <50 kb, which could be helpful for isolating the *Bnms3* gene by means of a map-based cloning strategy. Fine mapping and cloning of the *Bnms3* gene in *B. napus* will definitely promote the molecular-based study of the RGMS.

Amplified fragment length polymorphism markers are not adapted for large-scale application in plant breeding. We have tried to convert them into usable markers, such as SCAR, cleaved amplified polymorphic sequence (CAPS). Based on the sequences of polymorphic bands, we designed PCR primers. Fortunately, some SCAR markers (SC1, SC2, SC3, SC4, and SC5) were converted successfully, and they were located on either side of the *BnMs3* gene, which would be useful for new RGMS lines in marker-assisted selection. However, others, especially the nearest marker, EA01MC12, could not be directly converted into SCAR markers, so it might be necessary to employ PCR-walking to isolate the flanking regions in order to convert them.

Recessive genic male sterility is a very important tool to facilitate hybrid seed production in *B. napus*. The 7-7365AB is a new type of RGMS, the sterility of which is not affected by the environment to allow traces of pollen contamination. The strategy for using this material consists in crossing line 7-7365A with a temporary maintainer line to generate a whole male sterile population for hybrid seed production. Selection of a homozygous two-type line is the foundation while the crux is a temporary maintainer line. Sometimes there are many genotypes in the offspring from the crosses between 7-7365A and other varieties posing, which causes problems in effectively choosing the homozygous two-type line and the temporary maintainer. Among the F_2 population, there are 64 different genotypes but only three are male sterile and one is the temporary maintainer. There would need to be a very large F_2 population to be 95% certain of combining these and acquiring the target genotypes. Many researchers will make use of the molecular markers linked to the *BnMs-ms* locus and the *BnRf-rf* locus to select the temporary maintainers and homozygous two-type lines. Now that markers very tightly linked to the *BnMs3* gene have been identified, they could be very useful for marker-assisted selection for the improvement of new RGMS lines. Sometimes, the procedure involved in backcross breeding is very complicated, involving successive backcrossing and selfing, in order to distinguish the different genotypes. sR12384, a co-dominant SSR marker, can distinguish the genotypes of *BnMs3Ms3*, *BnMs3ms3*, and *Bnms3ms3* precisely. Combined with the co-dominant markers linked to the second gene *BnMs4* and the third gene *BnRf*, it will be easy to choose the double heterozygous plants (*BnMs3ms3Ms4ms4*) and the temporary maintainer, used to backcross continuously, while the selfing will be used once at the last step. That could greatly help to shorten the breeding time and save more space. Work on identifying the co-dominant markers associated with the *BnMs4* and *BnRf* genes is in progress in our lab.

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