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ORIGINAL PAPER

Map-based cloning of a recessive genic male sterility locus in *Brassica napus* L. and development of its functional marker

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Abstract We previously mapped one male-sterile gene (*Bnms3*) from an extensively used recessive genic male sterility line (9012AB) in *Brassica napus* to a 0.14-cM genomic region. In this study, two highly homologous BAC contigs possibly containing the candidate *BnMs3* gene were identified using a map-based cloning strategy. A *BnMs3*-linked SCAR marker (DM1) capable of differentiating the subgenomes between *B. rapa* and the *B. oleracea* aided mapping of *BnMs3* on the contig derived from the *B. napus* chromosome C9. One representative BAC clone was sequenced from each of the two contigs and resulted in a larger number of markers according to the sequence difference between the two clones. To isolate *BnMs3*, these markers were then analyzed in another two BC_1 populations with different genetic backgrounds. This assay allowed for a delimitation of the mutated functional region of *BnMs3* to a 9.3-kb DNA fragment. Gene prediction suggested that one complete open reading frame (ORF, *ORF2*) and partial CDS fragments of *ORF1* and *ORF3* reside in this fragment. Sequence comparison and genetic transformation eventually indicated that *ORF1* (designated as *BnaC9.Tic40*), an analogue of the *Arabidopsis* gene *AT5G16620* which encodes a translocon of the inner envelope of chloroplasts 40 (Tic40), is the only candidate gene of *BnMs3*. Furthermore, two distinct mutation types in *ORF1* both causing the male-sterile phenotype were individually revealed from 9012A and the temporary maintainer line T45. The molecular mechanism of this male sterility as well as the application of *BnMs3*-associated functional and cosegregated markers in true breeding programs was also discussed.

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Introduction

Several major rapeseed (*Brassica napus*) male sterility materials have been harnessed commercially as reliable and efficient pollination-control systems worldwide or in some local regions, including *Ogura* CMS (Ogura 1968), Polima CMS (Yang and Fu 1987), transgenic male sterility (Mariani et al. 1992; Williams et al. 1997) and the Male Sterility Lembke (MSL) (Frauen et al. 2007). Besides these systems, a recessive genic male sterility (RGMS or recessive GMS) line 9012AB (Chen et al. 1993) has also been increasingly adopted in China, due to the remarkable advantages including the capability of generating a complete male-sterile population and almost no restriction of restorer (Ke et al. 2005; He et al. 2008). Initially, the male sterility in 9012AB was regarded to be conditioned by that the interaction of three individual loci (*BnMs3*, *BnMs4* and *BnRf*) (Chen et al. 1998). Thus, recessive homozygosity in both male-sterile loci (*Bnms3ms3ms4ms4*) as well as the

existence of a dominant allele of *BnRf* can result in male sterility, while the fertility will be restored under all the genotypes. According to this inheritance model, a three-line approach (i.e., male-sterile line, temporary maintainer and restorer) was proposed and successfully applied in commercial hybrid production. Recent study, however, suggests that the *BnMs4* locus should be allelic to *BnRf*, turning the trigenic inheritance model to a digenic one (Dong et al. 2010). Based on this modification, two interesting questions can be raised. One is concerning whether the two male-sterile loci (*BnMs3* and *BnRf*) still interact in controlling the male gametophyte development. It was previously regarded that *BnRf* has an epistatic suppression effect on the other two male-sterile genes, according to the trigenic inheritance model (Chen et al. 1998). However, considering that the fertility of the RGMS line can be restored by any of the two genes, we preferred to believe that these two genes act redundantly and independently. The other question is whether the RGMS line 9012AB is caused by mutations of the same gene loci as another three GMS lines, i.e., the RGMS line 7365AB (Huang et al. 2007), the novel GMS system (or nuclear male sterility) patented by Syngenta Participations AG and the MSL system, because, the digenic hereditary model for sterility control in 9012AB is completely consistent with that of the latter three (Frauen et al. 2007; Stiewe et al. 2010; Zu et al. 2010).

To essentially answer these questions, it is first necessary to isolate both *BnMs3* and *BnRf* from 9012AB. Some progress has been made regarding map-based cloning of these two male-sterile loci. For the *BnRf* gene, Xie et al. (2008) first tagged it with several AFLP markers, and then the local genetic map around *BnRf* was improved with some tightly associated and cosegregated markers (Xu et al. 2009). At present, the candidate gene of *BnRf* has been restricted in a 13.8-kb DNA fragment in our group (unpublished). As for the *Bnms3* gene, it was initially mapped in a genetic region of more than 4.3 cM with some genetically loose AFLP and SCAR markers (Ke et al. 2005). Later, it was further delimited to a 0.14 cM fragment by comparative mapping with *Arabidopsis*; however, it still corresponds to a physical distance of 470 Kb in *Arabidopsis* genome (He et al. 2008). In addition, primary cytological observation indicated that the male sterility of 9012A was caused by the persistence of the primary pollen mother cell wall or callose surrounding the tetrads (Yang et al. 1999). Recent electronic microscopy analyses found that the tapetal cells in sterile plants fail to complete the transition from the parietal type to secretory type, because, they apparently lack of electron-dense plastoglobuli (Wan et al. 2010). All these studies have built a strong foundation for eventually understanding the molecular mechanism of male sterility in 9012AB.

Several genes have been successfully isolated by a map-based cloning strategy in several *cruciferae* economically important diploid crops, including the Ogura CMS restorer genes *Rfo* in radish (Brown et al. 2003; Desloire et al. 2003), the dwarf gene *dwf2* gene in *B. rapa* (Muangprom and Osborn 2004), the high-β-carotene gene *Or* gene in *B. oleracea* (Lu et al. 2006). The isolation of another two RGMS genes (the *Bnms1* from S45AB and *BnaC.Tic40* from 7365AB) are presently the only two successful cases in *B. napus* (Yi et al. 2010; Dun et al. 2011). This fact suggested that isolation of target genes via positional cloning approach is still challenging when *B. napus* is involved.

There may be several reasons for this. First, *B. napus* has a highly duplicated amphidiploid genome, resulting from the hybridization of two *Brassica* diploids, i.e. *B. rapa* (containing the *Brassica* A genome) and *B. oleracea* (containing the *Brassica* C genome). Comparative mapping between the *Arabidopsis* and *Brassica* genomes revealed that the *Brassica*-diploid ancestor had undergone an evolutionary process of triplication (Rana et al. 2004; Parkin et al. 2005; Town et al. 2006; Schranz et al. 2006; Lysak et al. 2009; Wang et al. 2011). Chromosomal fragment duplication or loss also occurred in the subsequent polyploidization process of *B. napus* (Parkin et al. 2005; Schranz et al. 2006; Panjabi et al. 2008). As a result, most chromosomal segments in *B. napus* generally have five to seven homologous copies. Second, high sequence similarity is commonly observed between paralogues. Wang et al. (2009) found that the coding sequences of six *FLOWERING LOCUS T* (*BnFT*) paralogues in *B. napus* showed 92–99% sequence identities to each other. A similar phenomenon was also observed between functionally redundant *BnMs1* and *BnMs2* genes of the RGMS line S45AB (Yi et al. 2010). Next, there was a shortage of available DNA library resources as well as a lack of genome sequences of *B. napus*; hence, the development of molecular markers and construction of a physical map from a region of interest were greatly hampered.

Based on the fine genetic mapping of *BnMs3* (He et al. 2008), here we constructed two homologous BAC contigs individually containing *BnMs3* and its paralogue. Using a subgenome-specific SCAR marker, we associated each contig with the subgenome A or C in *B. napus* and mapped *BnMs3* on the latter. Finally, the mutant functional region of *Bnms3* in 9012A was delimited to a 9.3-kb DNA fragment, from which only one predicted ORF can be correlated to *BnMs3* after genetic transformation and sequence analysis.

Materials and methods

Plant materials and mapping populations

Three RGMS two-type lines, 9012AB, DH195AB and GosAB, were the materials for the further mapping of the

target gene. The NIL population 9012AB (hereafter designated as Pop A) was previously described (Ke et al. 2005; He et al. 2008). The fertile plants homozygous in the *BnMs3* locus (designed as 9012F) were selected from the sibmating progeny of a 9012B plant (Wang et al. 2007). The other two RGMS lines, DH195AB and GosAB, were transferred from 9012A but have different genetic backgrounds with 9012A. Here we used the digenic hereditary model to define the individual genotypes of the above materials as reported by Dong et al. (2010). The genotypes of 9012A and 9012B are *Bnms3ms3Rf^{b}Rf^b* and *BnMs3ms3Rf^{b}Rf^b*, respectively. The second mapping population (Pop B for short) was resulted from the three-way cross (9012A//GOSA/9012F). The third population (Pop C for short) was generated by backcrossing 9012A with a fertile F_1 (9012A × DH195B) plant.

Each population contained 16,703 (Pop A, the previous 4,136 individuals were not included), 4,054 (Pop B) and 3,316 plants (Pop C). The segregation between fertile and sterile plants completely fits a 1:1 ration in all the three populations (data not shown). A complete male-sterile population, resulting from the cross between 9012A and the temporary maintainer T45 (*Bnms3ms3Rf^{c}Rf^c*) (Xie et al. 2008), was used for genetic transformation of the candidate *BnMs3* gene. A *B. rapa* cultivar (Aijiaohuang) and a *B. oleracea* cultivar (Zhonggan No. 8) were also used for sequence analysis in this study. Total plant DNA was extracted according to the method described by Doyle and Doyle (1990) with minor modification at seedling stages. The individual male fertility was investigated at the flowering time.

Marker development and assay

Four strategies were employed to saturate the marker density around *BnMs3*. Random AFLP primers were initially screened as described by Ke et al. (2005). Secondly, SSR primers were designed based on the BAC sequences. Thirdly, specific PCR primers were designed on the basis of the BAC sequence. The PCR products individually amplified from 9012A, 9012F, Zhonggan No. 8 and Aijiaohuang were comparatively sequenced. Corresponding SCAR markers were developed according to the sequence difference between the two parents. Finally, SCAR primers were later generated directly in term of sequence differences between two homologous BAC clones. All the markers were firstly assayed between parent lines and then in the populations. Polymorphic markers were finally evaluated with the recombinants from different mapping populations.

Map distances shown in Pop A are given in centi-morgan, representing the percentage of recombinant plants in the total number of plants analyzed. Considering the *BnMs3* locus has been finely mapped in a fairly narrow genetic region in Pop A, the relative genetic distance of a given marker was indicated only by the number of recombinants.

Screening of BAC library, construction of BAC contig and sequencing of BAC clones

The JBnB BAC library constructed from genomic DNA of Tapidor (a winter-type European cultivar) by Dr. Ian Bancroft, John Innes Centre, UK (Rana et al. 2004) was used in this study. This library contains 73,728 clones with the mean insert size of 145 kb, and has probably 7.8-fold redundant representation of 1,200 Mb genome of *B. napus* (Rana et al. 2004). Southern blotting was done as described by Rana et al. (2004), except for that the temperature during hybridization and washing had been modified to 65°C. Two types of DNA probes labeled with α-^{32}P were used in this research. One is the PCR products amplified from the genomic DNA of Tapidor with *BnMs3*-associated molecular markers. The other is the *Hind*III-digested BAC DNA. Plasmid DNA of BAC clones was prepared according to the protocol of QIAGEN Large-Construct Kit (Qiagen, Valencia, USA). Positive BAC clones were further screened by markers and specific primers developed from the *Arabidopsis* syntenic genomic fragment homologous to the *BnMs3* region (He et al. 2008). The overlapping relationship of BAC clones anchored to a specific contig was validated by DNA fingerprinting. The representative BAC clones from the contigs possibly containing *BnMs3* or its paralogue were shotgun sequenced and assembled by Beijing Genomics Institute (BGI).

Gene prediction and plant transformation

The website-based software FGENSH (http://www.softberry.com) was applied to predict the putative ORF from the candidate region. The genomic or coding sequences of predicted genes were submitted to NCBI (http://www.ncbi.nlm.nih.gov) and TAIR (www.arabidopsis.org) for homologue search and basic function analysis. Since the cultivar Tapidor carries the dominant allele of *BnMs3* (data not shown), we directly isolated the genomic sequence of this candidate gene from the BAC clone by long PCR amplification with Phusion® Hot Start High-Fidelity DNA polymerase (NEB, Ipswich, USA). The resulting fragment was inserted to binary vectors pCAMBIA2301 and pFGC5941, respectively. These constructs were confirmed by sequencing and were then introduced into the complete male-sterile F1 plants (from the cross between 9012A and T45) by *Agrobacterium tumefaciens*-mediated genetic transformation according to the method described by Cardoza and Stewart (2003). PCR analysis was used to screen

the positive transformants by examining the presence of the dominant *BnMs3* allele with the primer RCP161, which can amplify a 180-bp fragment in T45, a 172-bp fragment in Tapidor but no product in 9012A, and the antibiotic genes with primers related to kanamycin (pCAMBIA2301) or Basta-resistance gene (pFGC5941) (Table 1).

Sequence isolation from the candidate region

After successfully delimiting *BnMs3* to a 9.3-kb DNA fragment, we attempted to discover the core variations which resulted in the functional alteration in the *BnMs3* locus by comparing target region sequences between the mutant genotypes (*Bnms3*, 9012A and T45) and wild types (*BnMs3*, 9012F and Tapidor). To avoid the possible interference of the homologous sequence, we initially amplified the DNA fragment from the homologous region using PCR primers specifically binding to the homologous BAC. These fragments were individually amplified from 9012A and 9012F and fused to the plasmid PUC18 for sequencing. Comparison of these sequences with the target BAC containing *BnMs3* enabled us to design primers which can exclusively match to the latter. These primers allowed us to obtain the specific fragments conferring *BnMs3* (*Bnms3*) from 9012A, T45 and 9012F. These fragments were also cloned into PUC18 for sequencing. Sequence alignment was performed using the software package of Geneious Pro™ 5.3 (Biomatters, Auckland, New Zealand).

Results

Development of three cosegregated SCAR markers with *BnMs3*

As described in "Introduction", though *Bnms3* has been mapped to a region of only 0.14 cM (He et al. 2008), it was speculated that there remains a large gap between closest flanking markers in *B. napus*. Accordingly, we continued to increase the marker density around *BnMs3* by AFLP technique using the same population described by Ke et al. (2005). Another 23 polymorphic AFLP markers were harvested, 5 of which were further mapped to the genetic region of AR23-AR48 by analyzing the 52 recombination individuals identified previously (He et al. 2008). Among them, three (EC09MC01, SA04TG03 and SA09TG16) were cosegregated with *BnMs3*. One (EA01MC12) was located at the same position as AR48, having three recombination events with *BnMs3*. One (EC03MG10) was cosegregated with AR23, having other three recombination events with *BnMs3*. Other markers distributed outside of the AR23-AR48 region were not further analyzed. Flanking sequences of these five AFLP markers were isolated and submitted to NCBI for BLASTN analysis. Each of them can detect an *Arabidopsis* homologue from the collinear region restricted by AR23 and AR48 (Table 1), supporting our previous conclusion that the genomic region around *BnMs3* in *B. napus* is highly collinear with a fragment on the top end of *Arabidopsis* chromosome 5 (He et al. 2008). However, the *Arabidopsis* orthologues of the three cosegregated SCAR markers still span a large physical interval between AT5G16330 and AT5G16640 (Table 1).

We attempted to separate these *BnMs3*-cosegregated markers by analyzing other 16,703 plants of 9012 NIL (Pop A). To accelerate the genotyping in a large population, we converted all of polymorphic AFLP fragments into stable sequence characterized amplified region (SCAR) markers (i.e., SCR1, SCR2, 9W21 and 9W23, as shown in Table 1) except for EA01MC12. Although other three and two recombination plants were individually screened by 9W23 and AR48 (also for EA01MC12), no recombination event can be revealed by the three cosegregated SCAR markers. Thus, the relative genetic region of *Bnms3* was lowered to 0.058 cM (including the previous 4,136 plants, Fig. 1a), whereas the physical map restricted by 9W23 and EA01MC12 remained unchangeable. This result hints that the recombination in Pop A is severely suppressed between three cosegregated SCAR markers and *BnMs3*. To overcome this difficulty, a possible approach is to map *BnMs3* with new mapping populations.

Mapping of *BnMs3* with new backcross populations

The other two backcross populations individually including 4,054 plants (Pop B) and 3,316 plants (Pop C) were subsequently constructed. For the aim of approaching toward *BnMs3*, only the closest flanking and cosegregated markers from the genetic map (Fig. 1a) were further assessed in the new populations. Considering that EA01MC12 is an AFLP marker, we replaced it with the closest SCAR marker AR48 as a flanking marker. In Pop B, only the marker 9W23 is associated with *BnMs3* and revealed 21 recombination events with it (Fig. 1b). In Pop C, all markers except AR23 showed stable polymorphism with the plant fertility. AR48 and 9W23 were initially assayed in this population, both giving rise to two recombination events with *BnMs3*. With these four plants, SCR2 and 9W21 were finally mapped on the same side of *BnMs3*, each carrying two and one recombination cases, while SCR1 was still cosegregated with it (Fig. 1b).

In summary, integration of the three mapping populations finally enabled the mapping of *BnMs3* between marker 9W21 and EA01MC12 (Fig. 1a, b), corresponding to a 195-kb homologue in *Arabidopsis* chromosome 5 from 5,399 kb to 5,594 kb (Table 1).

Table 1 Major markers (primers) used in this research

Marker designation	Sequences (5′–3′)	Corresponding *Arabidopsis* orthologue[a]
9W23	F: TTTTTTTCGTAAATTGAAGG	*At5g15950*
	R: TTTTTACGTTATGCAAAATACA	
SCR2	F: GAGCTCAAGGCCGGAGATC	*At5g16330*
	R: CGAGACTAAACGAAAATGTGGA	
9W21	F: CATGACGTCAAATAGACGCAAA	*At5g16520–At5g16530*
	R: CATTCATCAATCACGACAACG	
BSR68	F: CCCAAGAAAACCAAAACAAAA	*AT5g16560*
	R: TTGGCACGAGAAATGACTTT	
BSR72	F: TTCGATTCCAGAGTGAATGC	–
	R: ACAGAGGACGACAGCTGGTT	
BSR73	F: CAAAATCAATCGATCATTAGAACA	–
	R: TGCTCAGACTTCAGACCTATCC	
RCP108	F: GGTACGGTGGCTGAGACAAT	*At5g16600–At5g16610*
	R: ATTATCCGAACCGAATCTGC	
DM1	F: AGTCGCTTGCGTTCAATTTC	*At5g16600–At5g16610*
	R: TTTCCAAAATCGGAAACCAG	
BSR111	F: CAAAAGTACAATCCCTTTTGT	*At5g16600–At5g16610*
	R: TCAAAGTTTGCATACAACTCT	
RCP130	F: TTTGAGGCTATCTGGCGAGT	*At5g16610*
	R: GGTTGTGTTGCTGCTTGCTA	
BSR115	F: AATCTCACTCTCCATCTGAG	*At5g16620*
	R: GTAAGCAGGAACATGGTTTG	
RCP137	F: CACCACCGTCATCTTCAT	*At5g16620*
	R: TGGTTTATACACTTCTATGC	
RCP161	F: GAGGGTTACAATGGAGGTAC	*At5g16620–At5g16640*
	R: CTTGCTATCTGAGAAACAACG	
RCP140	F: TTTATCTCAAATCTTTCAGCTG	*At5g16620–At5g16640*
	R: TATGTTTTCTAATACTATATGCTG	
RCP133	F: TAGATGGATGGTGAACAAGT	*At5g16620–At5g16640*
	R: TGTTATAACACCACTGACGT	
RCP144	F: CAAAGCTTTCACCATGAGGA	*At5g16620–At5g16640*
	R: AAGAGAGTTGTATGTGATCAC	
SCR1	F: CGAGGGAGTCGTCTAGGTTC	*At5g16640*
	R: GCTCACACTAGCAGTAGCAACAA	
RCP145	F: TGAGTATATGGTTAGCAGAC	*At5g16640*
	R: CCTCACGCCTTAAGCCTT	
RCP146	F: TAGTCGCTGACTGAACTCA	*At5g16640*
	R: TATGCAGGCTTTGAAGCTG	
MT7	F: GAGTCTGATCAATTCCTCAG	*At5g16670*
	R: TTTGATGAGGTGCGAGGAGA	
MT8	F: GCTTTTCTTATGTGGGTAC	*At5g16690*
	R: TAGCTTGTGGTTAGCTTTG	
9W13 (EA01MC12)	F: TACGGGTCTTGGAGTCAACG	*At5g17020*
	R: CAAGGCCATCAACCATACCA	
RCP150	F: TGGGAATTCAACACTCAATCTAGTGCTACT	*At5g16620–At5g16640*
	R: TAAGGTACCTGAAAGCTTTGAGCTATCTCTCT	
RCP154	F: TAACCCGGGTGAGGCTATCTGGCGAGTAAG	–
	R: TAACCCGGGCAGGTCCTCCAGTTAACCAGA	
RCP170	F: GCGGTCACAAGAGGAGGTAA	*At5g16620*
	R: CAGCCTCAATCCCAGTCTTC	

'–' represents there is no identified paralogue for this marker (primer)

F Forward primer, *R* reverse primer

[a] The product amplified by the marker (primer) from *B. napus* is an orthologue of the marked *Arabidopsis* gene

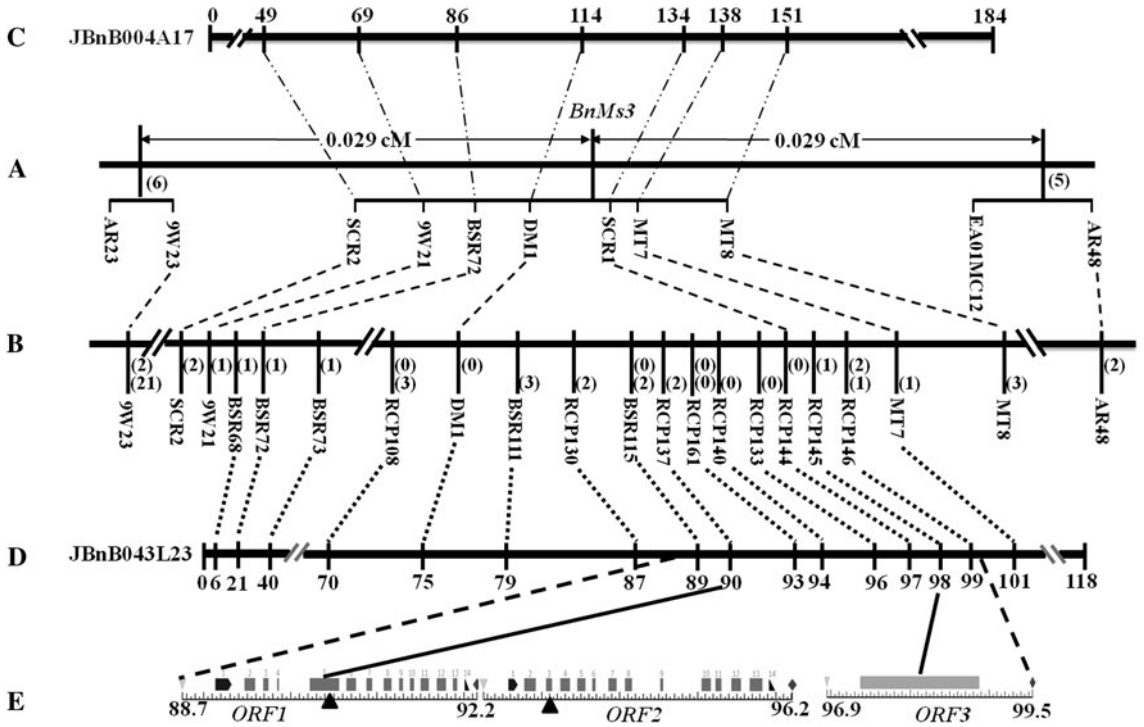

Fig. 1 High-resolution genetic and physical map of the *BnMs3* locus. *Dotted lines* indicate the position of marker on the genetic or physical map. **a** The local fine genetic map of *Bnms3* based on Pop A. The *numbers in parentheses* indicate the number of recombinant between corresponding markers and *BnMs3*. **b** The high-resolution genetic map of *Bnms3* established by integration of Pop B and Pop C. The numbers of recombinant of a marker in Pop B and Pop C are, respectively, shown in the *brackets above* and *below* on the right side, if the marker shows polymorphism in the corresponding population. The position of RCP144 was used to mark the position of SCR1, since they are physically close. Physical maps of BAC clone JBnB004A7 and JBnB043L23 are, respectively, schematically presented in (**c**) and (**d**), from which the *numbers* mark the position of given markers on each clone. **e** Putative *ORFs* from the candidate region predicted by FGENSH. The closest flanking markers were individually located inside *ORF1* and *ORF3* as shown by *solid lines*. Two sequence variations between T45 and Tapidor are marked by *solid triangles*

Primary integration of physical and genetic maps

It seemed feasible to construct the physical map of *BnMs3* by carrying out a chromosome landing strategy, since the candidate region of *BnMs3* has been mapped to a 195-kb fragment in *Arabidopsis* genome, 1.34-fold of the average insertion size (145 kb) of the JBnB BAC library. As an initial step, we used the closest flanking markers as probes, including a 566-bp DNA fragment amplified by 9W21 and a 507-bp fragment amplified by 9W13 which was designed according to the flanking sequences of EA01MC12.

Forty-five positive BAC clones were selected according to their blotting signals on the films. PCR amplification with primer pair 9W13 and 9W21 can screen-out 14 clones and 1 clone, respectively, while no BAC clone can be simultaneously confirmed by these two primers. In this circumstance, the only BAC clone (JBnB004A17) fished by 9W21 was digested by *Hind*III and used as the probe to rescreen the BAC filters. This assay brought about another 12 positive BAC clones, and three of them can be confirmed by marker 9W21 (including JBnB004A17). The cosegregated marker SCR1 can amplify identical fragment from six clones (including the three identified by 9W21). However, there is still no BAC clone which can be confirmed by 9W21 and 9W13 together. This phenomenon can be accepted, since the gap between these two markers is possibly larger than the insertion fragment of these BAC clones. Some *Arabidopsis* genes related PCR primers, designed according to the *Brassica* genomic survey sequences (GSS) or EST sequences located in the candidate *Arabidopsis* homologous region (He et al. 2008), were used together with *BnMs3*-associated SCAR markers to examine the overlapping relationship between all these BAC clones from two hybridization. Finally, a BAC contig consisting of at least four representative clones was established (Fig. S1).

BAC clone sequencing and marker development

From this contig, it is impossible to decide which BAC must contain the candidate gene of *BnMs3*. However, in terms of the distributions of 9W21 and SCR1 in the contig,

the clone JBnB004A17 was first shotgun sequenced. The insert size of JBnB004A17 is about 184 kb, in which the marker 9W21 and SCR1 are located at the position of about 69 kb and 134 kb, respectively (Fig. 1c). The marker SCR2, which was mapped outside of the region of 9W21-*BnMs3*, can also be located in this BAC at 49 kb (Fig. 1c). To map toward *BnMs3*, 23 SSR primers were developed based on the BAC sequence from 69 kb (9W21) to its one end toward SCR1. Population analysis showed that in Pop A, only one SSR primer BSR72 can show reproducible polymorphism, while it was still cosegregated with *BnMs3* (Fig. 1a). In Pop B, all these primers showed monomorphic bands or random segregation with the plant fertility. Luckily, three polymorphic SSR primers (BSR68, BSR72 and BSR73) were mapped between 9W21 and *BnMs3* in Pop C, all having one common recombination event with *BnMs3* (Fig. 1b). Thus, the candidate region of *BnMs3* was reduced for 30 kb thanks to the mapping of BSR73 (at 99 kb).

However, the above SSR markers failed to condense the physical map between *BnMs3* and EA01MC12. Therefore, we tried to develop more markers from the BAC sequence by comparative sequencing. Two blocks individually residing at 100–120 kb and 140–160 kb on the BAC clone were selected for primer design. To differentiate the possible interference from the homologous region, PCR products amplified from the Aijiaohuang (*B. rapa*) and Zhonggan No. 8 (*B. oleracea*) were also sequenced as controls. In this way, three polymorphic markers, including DM1 (at 114 kb), MT7 (at 138 kb) and MT8 (at 151 kb) were developed successfully (Table 1; Fig. 1c). Among them, DM1 showed stable polymorphism in Pop A and Pop C, while it was still cosegregated with *BnMs3* in both populations. Though MT7 and MT8 remained inseparable with *BnMs3* in Pop A (Fig. 1a) and lost their polymorphism in Pop C, they were assigned to the opposite side of AR23 in Pop B, having one and three new recombination events with *BnMs3*, respectively (Fig. 1b). As a result, in combination with BSR73, the development of MT7 promoted the delimitation of *BnMs3* to a 39-kb region from 99 to 138 kb on JBnB004A17.

Identification of the BAC clone containing *BnMs3*

Notably, DM1 generates a bigger fragment from Aijiaohuang than that from Zhonggan No. 8, while it can amplify both bands from the fertile parents and Tapidor. This marker was then evaluated among 50 *B. rapa* and 8 *B. oleracea* cultivars. We found that it can amplify a 198-bp fragment from all the *B. rapa* cultivars and a 160-bp fragment from all the *B. oleracea* cultivars (Fig. S2). Furthermore, the polymorphic bands between the male-fertile and male-sterile parents showed a 100% sequence identity with those from *B. oleracea* cultivars, and the bigger bands from the fertile plants shared a 100% similarity to those from *B. rapa* cultivars. This analysis led to the speculation that DM1 was a subgenome-specific marker and thereby the candidate region of *BnMs3* should origin from the *B. oleracea* subgenome. However, the amplification products of DM1 in two of the four BAC clones in the established contig were completely associated with the band in *B. rapa* (Fig. 2). Consequently, it showed that this BAC contig actually corresponded to a *BnMs3* paralogous region, which originated from the *B. rapa* subgenome, but not the *B. oleracea* subgenome. Localization of several SSR markers developed from JBnB004A17 on the chromosome A10 or C9 of the TNDH population (Long et al. 2007) further supported our speculation (data not shown, SSR primers are listed in Table S1).

As a result, it is necessary to use the smaller DNA fragment amplified by DM1 from Tapidor to screen the BAC library again, which shares a 100% sequence identity with the polymorphic bands from the parents. Twelve positive BAC clones, including JBnB004A7, were selected according to hybridization signals. After PCR analysis with DM1, BSR73 and SCR1 and MT7, a novel BAC contig consisting of six highly overlapping clones from the

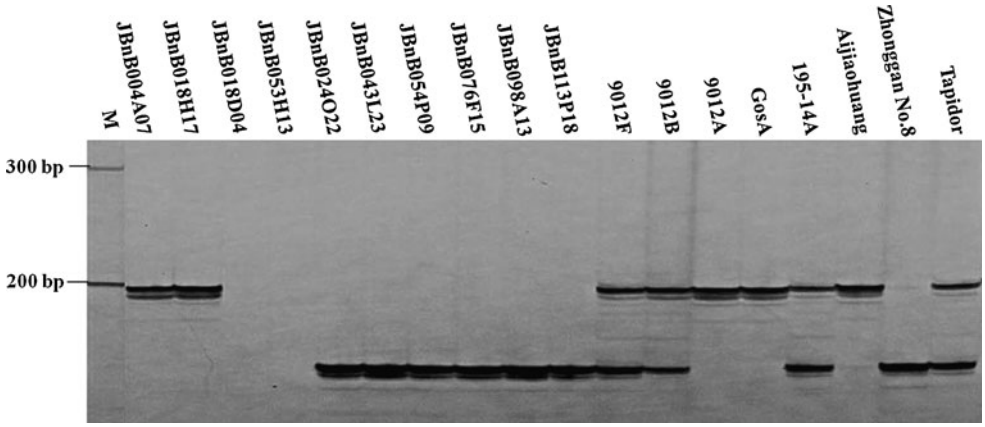

Fig. 2 The amplification pattern of SCAR marker DM1. The first four BAC clones are from the contig spanning the *BnMs3* paralogue, while the following six are from the contig covering *BnMs3*. *M*, GeneRuler™ 100 bp DNA ladder (Fermentas)

Table 2 Sequence alignment of the 9.3-kb fragment restricted by the closest flanking marker (RCP137 and RCP145) from three lines and two homologous BAC clones

Derivation	JBnB043L23 9,246bp[a]	T45 9,252 bp	9012F 8,768 bp	9012A 8,909 bp	JBnB004A17 9,308 bp
JBnB043L23	0, 0	99.9%, 0%[b]	93.3%, 5.9%	79.1%, 10.3%	77.6%, 11.5%
T45		0, 0	93.3%, 6.0%	79.1%, 10.4%	77.5%, 11.6%
9012F			0, 0	82.6%, 5.7%	80.8%, 7.1%
9012A				0, 0	85.5%, 6.1%
JBnB004A17					0, 0

[a] The size of each fragment is listed under the material in the first row

[b] The former number indicates the percentage of sequence similarity between two sequences, while the latter shows the percentage of gap between them

B. oleracea subgenome was rebuilt and confirmed by fingerprinting analysis (Fig. S3). From them, the smaller clone JBnB043L23 containing both closest flanking markers (BSR73 and MT7) and cosegregated marker SCR1, was shotgun sequenced. Its insertion size is about 118 kb, homologous to the *Arabidopsis* region from AT5G16550 to AT5G16700. By comparing the sequences of marker BSR73 and MT7 with JBnB043L23, a 61-kb fragment in the BAC was identified as containing the *BnMs3* gene (Fig. 1d).

The sequence identity between JBnB043L23 and JBnB004A17 was roughly investigated. High sequence similarity was observed on a large scale, while extensive variations including fragment insertion/deletion and massive SNPs were also revealed. According to the sequence difference between two homologous BAC clones, SCAR primers specifically binding to JBnB043L23 as well as SSR primer were designed from the 61-kb region. Interestingly, the majority of them showed stable and reproducible polymorphism in populations directly, and were further examined by the recombination individuals from Pop B and Pop C. This analysis led to our final delimitation of *BnMs3* to a 9.3-kb fragment bracketed by closest flanking markers RCP137 and RCP145, having two and one recombination events with the target gene, respectively (Fig. 1b, d). Sequences of the SCAR and SSR markers in Fig. 1b are listed in Table 1.

Sequence analysis of the candidate region and plant transformation

Gene prediction analysis of the 9.3-kb DNA fragment identified only one complete ORF (*ORF2*) and partial genomic sequences of its two flanking genes (*ORF1* and *ORF3*) (Fig. 1e). Though the recombination events occurred inside of *ORF1* and *ORF3*, they could not be excluded as candidate genes of *BnMs3*, because, their functional mutation regions may exist in the 9.3-kb fragment. *ORF1* is homologous to the *Arabidopsis* gene *AT5G16620*, encoding the translocon of the inner envelope of chloroplast 40 (AtTic40). *ORF3* is the orthologue of the *Arabidopsis* gene *AT5G16640*, encoding a pentatricopeptide repeat (PPR) superfamily protein. Though *ORF2* cannot search homologous gene from *Arabidopsis* genome at the DNA level, tBLASTN analysis showed that it is highly conserved with the serine carboxypeptidase-like (SCPL) family members.

The genomic sequences corresponding to the 9.3-kb BAC fragment were individually isolated from 9012A, 9012F and T45. Alignment of these sequences revealed extensive sequence variations between 9012A and 9012F while high sequence conservation between Tapidor and T45 (Table 2). To facilitate the candidate gene prediction, the sequence differences between Tapidor and T45 were the objective of focus since they are genotyped as *BnMs3* and *Bnms3* at the target locus, respectively. Compared with Tapidor, T45 has a 2-bp deletion in the fifth exon of *ORF1* and the 8-bp insertion in the third exon of *ORF2* (Fig. 1e and Fig. S4). No nucleotide difference was detected in *ORF3* from the 9.3-kb region between Tapidor and T45, excluding its possibility as a candidate gene of *BnMs3*. In Tapidor, the complete proteins of predicted ORF1 and ORF2 include 455 and 461 amino acids, respectively. Both of the deletion and insertion in T45 would create the frameshift mutations, giving rise to the premature proteins only with 222 amino acids in ORF1 and 118 amino acids in ORF2. While in 9012A, it is still difficult to decide the key-sequences associated with the fertility alteration, due to the massive sequence variations between 9012A and 9012F.

It was expected that transformation of male-sterile plants with genomic DNA spanning the dominant *BnMs3* allele should result in the recovery of male fertile transgenic plants. Initially, we only attempted to transform *ORF2*, considering that it is the only complete putative gene in the 9.3-kb fragment. A 5.5-kb DNA fragment, including the last two exon sequences of *ORF1*, complete sequence of *ORF2* and partial exon sequence of *ORF3*, was amplified from the BAC clone JBnB043L23 with primer RCP150 (Table 1). Binary vectors (pCAMBIA2301 and

pFGC5941) carrying this fragment were then individually introduced into the male-sterile plants. One-hundred and eighteen independent positive transformants were obtained from both constructs; however, the male-sterility phenotype of all these plants could not be recovered, indicating that *ORF2* is not the target gene of *BnMs3*. In other words, *ORF1* has been restricted as the only candidate of *BnMs3*. Thus, a 6.4-kb fragment including the complete sequence of *ORF1* was also amplified from JBnB043L23 with primer RCP154 (Table 1) and cloned into pFGC5941. From the 15 transgenic plants obtained now, all the 4 plants having flowers presently are male-fertile. This strongly indicates that *ORF1* can restore the fertility of the male-sterile plants and is the target gene of *BnMs3*. In accordance with the full gene nomenclature for *Brassica* genes (Ostergaard and King 2008) added to the predicted function of *ORF1*, we designated *BnMs3* as *BnaC9.Tic40*, where C9 is used to distinguish it from its paralogue (*BnaA10.Tic40*) on the *B. napus* chromosome A10.

This result was consistent with the observation that the InDel marker (RCP170) correlated to the 2-bp nucleotide variation in *ORF1* can accurately differentiate all the inbred lines (cultivars) carrying *BnMs3* allele from three temporary maintainers (Fig. S5). It also indicated that RCP170 is a functional marker for the *BnMs3* locus, because the 2-bp deletion is the only variation in *ORF1* between T45 and Tapidor. By contrast, the other InDel marker (RCP161) from *ORF2* displayed a random segregation in these lines (data not shown), which hints that the 8-bp deletion in T45 is not responsible for its functional loss in *Bnms3*.

Discussion

In the present study, we described our work on the integration of genetic and physical map of the recessive genic male sterility gene *Bnms3* by a map-based cloning strategy. With the assistance of three mapping populations and the sequences of two homologous BAC clones, we eventually corresponded *BnMs3* to the only candidate gene (*BnaC9.Tic40*).

Differentiation of the target region from highly homologous paralogues

The way to eventual identification of BAC clones containing *BnMs3* twists and turns. In the first two screenings, all the positive clones confirmed by PCR analysis were unfortunately from the paralogous regions. Only in the third screening, a fragment exclusively derived from *B. oleracea* succeeded in identifying the target clones as well as those from the homologous region. The reason remains unknown; however, there is no doubt that the construction of a physical map near *BnMs3* has been much disturbed by the homologous copy from the *B. rapa* subgenome. This dilemma was predominantly resulted from the existence of multiple-copy paralogues in *B. napus*. Actually, it is inevitable that the target BAC clones would be distinguished from the homologues in polyploid when exploiting the positional cloning approach.

Two strategies have been generally used for this differentiation. One is to group the paralogues in terms of their sequence polymorphism. Wang et al. (2009) successfully grouped the BAC clones containing different *BnFT* paralogues in terms of the intron sequence variation and mapped them to six distinct regions. Similarly, two highly conserved BAC clones were grouped to each of the two redundant male sterility genes (*BnMs1* and *BnMs2*) by a subgenome-specific marker (Yi et al. 2010). In this study, the development of a subgenome-specific marker DM1 also set a good example of this strategy. The other possible strategy is to accomplish the physical map from its diploid ancestor, which can also be called haploid-specific chromosome walking or landing. Formanová et al. (2006) introgressed the restorer gene (*Rfp*) of Polima CMS into diploid *B. rapa*, to reduce the negative effect from other paralogues. Another successful application of this strategy came from the physical mapping of a durable major rust resistance gene (*Bru1*) from a highly complicated polyploid ($\sim 12x$) sugarcane (Le Cunff et al. 2008).

Two distinct mutation types in the recessive *Bnms3* allele

The availability of full sequences from two homologous BAC clones and partial sequences from several lines in the vicinity of *BnMs3* and its paralogues provides us an opportunity to investigate the key mutations in the *BnMs3* locus. Initially, we found that the 5,868-bp paralogue (corresponding to JBnB004A17) of *ORF2* from 9012A is completely identical to that from 9012F, sharing 100% sequence similarity with JBnB004A17. This result is consistent with the fact that 9012A is a NIL of 9012F. In addition, it also suggested that this fragment may undergo purification selection, since 9012AB has a distinct genetic background with Tapidor. However, comparison of the 9.3-kb fragment among different lines (Table 2) showed that 9012A has an 85.5% similarity with JBnB004A17, much higher than that with JBnB043L23 (79.1%). In contrast, 9012F has a 93.3% similarity with JBnB043L23, while its similarity with JBnB004A17 is only 80.8%. In most cases, 9012A was found to have an identical sequence with JBnB004A17 in the sites polymorphic between JBnB004A17 and JBnB043L23, whereas 9012B is closely identical to JBnB043L23. Therefore, it can be understood

that why most PCR primers specifically binding to JBnB043L23 can directly show genetic linkage with *BnMs3* in 9012AB. Combining these facts, we hypothesized that the male sterility of 9012A was originally caused by an occasional homeologous reciprocal recombination between non-homologous chromosomes in a *B. napus* line, where the other male-sterility locus *BnRf* has lost its function. This kind of recombination was already found in several Canadian and Australian cultivars of *B. napus* (Osborn et al. 2003; Howell et al. 2008). In this case, the two chromosomal fragments individually distributed on chromosome A10 and C9 were mutually translocated. In the following artificial selections, the translocated locus on chromosome A10 has been recovered while the locus on C9 (from the A10 paralogue) has been maintained, since it has discarded its original function as a dominant *BnMs3* allele, thereby giving rise to male sterility when being homozygous. To effectively apply this male sterility in hybrid breeding, the male-sterile plants were then successively crossed with male-fertile plants heterozygosity in the C9 fragment (including a wild-type C9 fragment and a paralogue translocated from A10). The continuous sib-mating may have caused saturated recombination between these two fragments and fixed massive sequence variations in this region until now, finally leading to the low similarity (82.6%) between 9012A and 9012B and the extremely low recombination frequency around *BnMs3* in Pop A. Distinctively, there was an apparently different story about the origin of the *Bnms3* allele in the maintainer T45. The upstream sequence of *ORF1* was also obtained in T45, which shows no nucleotide difference with Tapidor (data not shown). This fact clearly shows that the only 2-bp nucleotide deletion results in the functional loss of *ORF1* in T45. In contrast to the possible homeologous reciprocal recombination in 9012AB, the *Bnms3* allele from T45 was most likely to be derived from a natural single-locus mutation in *BnaC9.Tic40*. Hence, there are at least two different mutations in the *Bnms3* locus among *B. napus* resources, and both can be used as the donor of the male-sterile lines and their temporary maintainer lines.

Most recently, Dun et al. (2011) has identified that the *BnMs3* gene from another RGMS line (7365AB) also corresponds to *BnaC9.Tic40* (*BnaC.Tic40* in the original paper). Though these two genes were isolated from two different clones (JBnB043L23 and JBnB019O01) from the same BAC library, they seem essentially the same gene. This result is further confirmed by the observation that the *Bnms3* genomic sequence from 9012A has a 100% identity with that from7365A. It suggests that both RGMS lines were likely to be bred from the same original mutation, though the sequence similarity of *BnMs3* between 9012B and 7365B (not open) remains unknown. In addition, both groups hold the opinion that the *Bnms3* mutation in 9012A or 7365A was a product of homeologous recombination between chromosome A10 and C9 in *B. napus*. However, here we reported another completely different mutation type in *Bnms3* as revealed by the maintainer T45. Functional analysis of this mutation will enrich our understanding of how *BnMs3* is exclusively involved in the lipid-related protein transportation in tapetal plastids. Furthermore, compared to that from 9012A (or 7365A), the *Bnms3* allele from T45 has a theoretically better application potential in true breeding programs, because, the abnormal homeologous recombination has occurred in a relatively large region (as indicated by the markers) and possibly caused potential unfavorable mutations on other genes flanking *BnMs3*. By contrast, the 2-bp nucleotide deletion of *Bnms3* in T45 should have no negative effect on other locus.

Possible molecular function of *BnaC9.Tic40*

Tic complex is involved in importing nucleus encoded proteins to the chloroplast stroma and reinserting them to the inner envelope membrane (IEM), where is a major site of lipid and membrane synthesis within the organelle in higher plants (Viana et al. 2010). As a cochaperone, AtTic40 functions in this process by coordinating with the other two members (Tic110 and Hsp93) of the Tic complex, individually depending on its tetratricopeptide repeat (TPR) domain and C-terminal Hsp70-interacting protein/Hsp70/Hsp90-organizing protein (Hip/Hop) domain (Reviewed by Li and Chiu 2010). The premature mutation of *BnaC9.Tic40* in T45 leading to the incomplete TPR domain and the loss of the whole Hip/Hop domain, would likely hamper the interaction between *BnaC9.Tic40* and its one or two colleagues. As a result, the transportation of necessary nucleus encoded proteins to the IEM would be blocked, and subsequently lipid synthesis in IEM would be inevitably suppressed or completely interrupted. This speculation was in consistence with the observation that the tapetal cells of 9012B have an intense accumulation of clear electron-dense plastoglobuli (droplets of lipid in the stroma of chloroplasts) while only small and obscure ones in 9012A during the period from tetrad to the released microspores stage (Wan et al. 2010). Certainly, it is still needed to establish how the chloroplast lipids play a role in regulating the development of microspores. In addition, another motor protein was discovered recently in *Arabidopsis*, which was proved to function in parallel with the motor Hsp93/AtTic40 (Su and Li 2010). Despite it remains to be confirmed whether there is a possible relationship between the *B. napus* Hsp70 orthologue and *BnRf*, these two redundant chaperone systems involved in protein import into chloroplasts are similar to the digenic inheritance model of male sterility in 9012AB. Furthermore, it contributes to our understanding why male gametophytes

can develop normally with only one of the *BnMs3* and *BnRf* genes in the diploid *B. rapa* and *B. oleracea* genomes.

Integration of different GMS systems and application of the markers

Some SSR markers individually associated the *Ms* and *Rf* locus of the GMS system patented by Syngenta (Stiewe et al. 2010) were also found to show a linkage relationship with the *BnRf* and *BnMs3* loci in 9012AB, respectively (data not shown). This result suggested that this GMS line possibly belongs to the same GMS system as 9012AB. Similarly, one SCAR markers (XSC5) associated with *BnRf* in 7365AB (Xiao et al. 2008) can also be mapped closely to the *BnRf* locus in 9012AB (data not shown), consistent with the conclusion that 7365AB may be allelic to 9012AB in the three (presently two) male-sterile-related loci by genetic analysis (Huang et al. 2007). Most recently, one male-sterile locus from 7365A was cloned and characterized as the same gene as the *Bnms3* locus in this research (Dun et al. 2011). Both *Bnms3* loci were independently concluded to be a consequence of homeologous chromosome recombination. Our results proved that the 9012AB and 7365AB are essentially the same and have the identical evolutionarily process in the *Bnms3* locus. As for the Syngenta GMS and MSL systems, it would be easily decided whether it can be classified into the same system as the above two lines by analyzing their genotypes in the *BnMs3* locus.

Integration of different GMS lines into one system greatly broadened the application potentials of the abundant and simple PCR markers associated with *BnMs3* developed here in the breeding process of new RGMS lines and their homologous maintainers by marker assisted-selection (MAS). Although some markers possibly lose their polymorphism in the true breeding materials as shown in this research, it may be easy to harvest markers feasible for a given population due to the high-density marker distribution around *BnMs3*. Especially, the functional marker RCP170 will be valuable for constructing new RGMS lines based on the *Bnms3* allele from the maintainer line T45, and in this process it can also be applied together with the flanking tightly cosegregated ones to control the size of introgression fragment via chromosome recombination.

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