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ORIGINAL PAPER

Fine mapping of a recessive genic male sterility gene (*Bnms3*) in rapeseed (*Brassica napus*) with AFLP- and *Arabidopsis*-derived PCR markers

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Abstract 9012AB, a recessive genic male sterility (RGMS) line developed from spontaneous mutation in *Brassica napus* (Chen et al. in Acta Agron Sin 24:431–438, 1998), has been playing an increasing role in hybrid cultivar development in China. The male sterility of 9012AB is controlled by two recessive genes (designated *Bnms3* and *Bnms4*) interacting with one recessive epistatic suppressor gene (*esp*). Previous study has identified seven AFLP markers, six of which were co-segregated with the *Bnms3* gene in a small population (Ke et al. in Plant Breed 124:367–370, 2005). By cloning these AFLP markers and their flanking sequences, five of the six co-segregated markers were successfully converted into sequence characterized amplified region (SCAR) markers. For fine mapping of the *Bnms3* gene, these SCAR markers were analyzed in a NIL population of 4,136 individuals. The *Bnms3* gene was then genetically mapped to a region of 0.56 cM, with 0.15 cM from marker SEP8 and 0.41 from marker SEP4, respectively. BLAST analysis with these SCAR marker sequences identified a collinear genomic region in *Arabidopsis* chromosome 5, from which two specific PCR markers further narrowed the *Bnms3* locus from an interval of 0.56 to 0.14 cM. These results provide additional information for map-based cloning of the *Bnms3* gene and will be helpful for marker-assisted selection (MAS) of elite RGMS lines and maintainers.

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Introduction

Male sterility, as an effective and economical pollination control system, is worldwide used for the utilization of rapeseed heterosis. Cytoplasmic male sterility (CMS) systems are still convenient for the production of hybrid seeds because of their mode of maintenance and restoration of fertility (Budar and Pelletier 2001). But genic male sterility (GMS) systems are regarded as a promising alternative to CMS, due to several advantages, such as the stable and complete male sterility, no negative cytoplasmic effect on yield as CMS might do (Yi et al. 2006). Especially, recessive GMS (RGMS) systems have another remarkable advantage that hybrids with strong heterosis are easily bred since most lines can be used as restorers. However, for some RGMS lines, such as S45AB (Pan et al. 1988) and 117AB (Hou et al. 1990), their wide application in the production of commercial hybrids is limited by the inability to generate a completely male-sterile population. Fortunately, this difficulty can be completely eliminated when a novel RGMS line, 9012AB (Chen et al. 1993) is involved. Genetic analysis indicated that the male sterility of 9012AB is controlled by two recessive genes (*Bnms3* and *Bnms4*) interacting with one recessive epistatic suppression gene (*Bnesp*) (Chen et al. 1998). Homozygous recessive sterile gene at both loci (*Bnms3ms3ms4ms4*) can result in male sterility, but the fertility can be restored when the *Bnesp* gene is in recessive homozygosity or either of the sterile genes is not recessively homozygous (Chen et al. 1998). A three-line hybrid

production model based on this RGMS system has been well-documented (Ke et al. 2005; Huang et al. 2007). The great breakthrough of this model is that a 100% sterile population (*Bnms3ms3ms4ms4Espesp*) can be obtained, with which a restorer line is then used to cross to generate commercial F1 hybrid seeds. This kind of male sterility system has excited a great deal of interest and has been increasingly used for rapeseed hybrid production. In recent years, several high oil content and double-low quality hybrid cultivars with great yield potential have been released in China (Chen et al. 2003; Sun et al. 2004, 2005). However, due to the complex genotypes involved in segregation generations when transferring three recessive genes simultaneously, traditional breeding programmes for novel RGMS lines and their maintainers are very laborious and time-consuming. It is expected that this procedure can be greatly accelerated through the use of MAS in hybrid breeding programs.

The abnormal pollen development of sterile plants in 9012AB was reported by Yang et al. (1999a). The male sterility phenotype is characterized by the persistent existence of the primary pollen mother cell (PMC) wall or callose surrounding the tetrads and thus arrest of microspore release (Yang et al. 1999a). Nevertheless, nothing is available about the molecular mechanism of the *Bnms3* gene in degradation of the PMC wall or callose. Isolation of the *Bnms3* gene is therefore regarded as an important work, not only for developing specific PCR markers for MAS but for understanding the mode of action of these genes. Previous studies in our group have tagged one of the sterile genes (*Bnms3*) with seven AFLP markers, six of which were identified to be co-segregated with the target gene in a population involving 115 individuals (Ke et al. 2005). However, it is not suitable to use AFLP markers in large scale genotype identification, since AFLP technique is relatively expensive and complicated. Hence, for practical use in MAS or fine mapping the need remains for simple PCR-based markers, such as sequence characterized amplified region (SCAR) or sequence-tagged site (STS) markers.

Comparative mapping between model plants and major crops provides a new strategy for exploiting the public information resource of model plants. *B. napus* and *Arabidopsis thaliana* (hereafter referred to as *Arabidopsis*), which share recent common ancestry, are both members of *Brassicaceae* family (Yang et al. 1999b). Therefore, the completed genome sequence of *Arabidopsis* can be efficiently used for identifying useful marker and targeting genic region (Desloire et al. 2003), and especially it has shown that the sequence identity between *Arabidopsis* and *B. napus* reaches 86% (Cavell et al. 1998). Recently, more and more sequence data from *B. rapa*, *B. oleracea* and *B. napus* are available from public websites, which are then comparatively mapped in *Arabidopsis* genome and hereby can be also used for gene location in *B. napus*.

In this study we reported our work on fine mapping of the *BnMs3* gene by using AFLP-derived SCAR markers and making use of the collinearity between *B. napus* and *Arabidopsis*.

Materials and methods

Plant materials

The recessive epistatic GMS two-type line 9012AB developed by Chen et al. (1993), was used as materials. By full sib-mating (9012A × 9012B) for more than 20 generations, 9012A plants (male-sterile, *Bnms3ms3ms4ms4EspEsp*) and 9012B plants (male-fertile, *BnMs3ms3ms4ms4EspEsp*) can be regarded as near isogenic lines (NIL) that differ nearly only by the male fertility/sterility trait. This NIL population was then used for molecular mapping of the *Bnms3* gene. All the seeds were orderly programmed in the seed plot, and the seedlings with five to six leaves were transplanted to the cropland in Huazhong Agricultural University. The male fertility of each plant was visually assessed during flowering.

Isolation of genomic DNA

Genomic DNA was extracted individually from leaves using a modified CTAB method (Doyle and Doyle 1987). DNA concentration and purity were measured by a Beckman spectrophotometer (Beckman Coulter Inc., Fullerton, USA). Final DNA concentration was adjusted to 25 ng/μl in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The fertile and sterile DNA bulks were constructed by pooling equivalent amounts of DNA from ten male-fertile and ten male-sterile plants, respectively.

Cloning and sequencing of AFLP fragment

Bnms3 linked AFLP fragments were excised from the polyacrylamide gel. The gel slices containing DNA fragments were placed in a 0.5-ml eppendorf tube with 30 μl of double-distilled water, then crushed with a pipette tip and boiled at 95°C for 10 min. The tubes were centrifuged at 14,000 rpm for 5 min and the supernatant was used as template for selective amplification with the same primer combination as before. The re-amplified products were separated on a 1.2% agarose gel. A UNIQ-10 EZ Spin Column DNA Gel Extraction Kit (Sangon, Shanghai, China) and a pGEM-T Easy Vector (Promega, Madison, USA) were used to purify and clone the polymorphism fragments, respectively. White colonies growing on Amp^+/X-gal/IPTG LB solid media plates were selected and cultured overnight in LB liquid media. To each DNA fragment, three random

positive colonies were sequenced with an ABI Prism3730 DNA sequencer (Applied Biosystems, Foster City, USA).

Conversion of AFLP markers to SCAR markers

The software Primer3 (Steve and Skaletsky 1996) was used for sequence analysis and SCAR primer design. PCR amplification from the genomic DNA of the two DNA bulks and 30 individuals was carried out in a 15 µl mixture, containing 50 ng template DNA, 1 × PCR Buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 1.0 U *Taq* (MBI Fermentas) and 5 pmol each of forward and reverse SCAR primers. Annealing temperature of SCAR primers was optimized first in a PTC-200 Thermocycler (MJ Research, Waltham, USA), using the following cycling parameters: one cycle of 3 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at an annealing temperature, and 1 min at 72°C. An amount of 10 µl PCR product of each sample was examined in a 1.5% agarose gel to confirm whether the SCAR primers were converted successfully or not.

For the AFLP marker that could not be converted directly, the standard protocol of GenomeWalker™ Kits (Clonetech, Madison, USA) developed from suppression PCR (Siebert et al. 1995) was adopted to isolate the adjacent genomic regions. Target fragments obtained by PCR Walking were then purified, cloned and sequenced just as the re-amplified AFLP polymorphic fragments. Based on the flanking sequences, polymorphic SCAR primers were expected to be obtained.

Linkage analysis

The SCAR markers were identified in the mapping population. Considering that six AFLP markers are all cosegregated with *Bnms3* in a previous small population, we firstly analyzed 1,500 individuals to decide the relative distance between the SCAR markers and the target gene, and then used the outside one to genotype other individuals of the NIL mapping population. The individuals showing recombinant events were selected and subsequently used to analyze all the left SCAR and AFLP markers. The individual fertility phenotype and the data of linked molecular markers were combined for linkage analysis using the software package MAPMAKER/EXP 3.0 (Lander et al. 1987; Lincoln et al. 1992). The Kosambi mapping function (Kosambi 1944) was used in calculating genetic distances. A local linkage map of the region on the chromosome surrounding the *BnMs3* gene was drawn using Mapdraw V2.5 (Liu and Meng 2003).

Sequence similarity analysis with *the Arabidopsis* genome

In order to identify a putative syntenic region around the *BnMs3* gene in *Arabidopsis* genome, sequence similarity between the molecular markers and *Arabidopsis* genome was analyzed using the BLAST programs of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). Then the linear arrangement relationship of these molecular markers in *B. napus* genome was compared with that of their homologues in *Arabidopsis* genome, to decide whether the synteny exists or not around *BnMs3* between these two related species.

Development of specific PCR primers from the *Arabidopsis* syntenic region

Specific PCR primers were developed from the *Arabidopsis* genomic region syntenic to the DNA segment around the *BnMs3* locus. Considering the nucleotide sequence difference between these two species, we developed the specific primers not only based on the *Arabidopsis* genomic sequences directly, but predominantly on the published EST (expressed sequence tag) or GSS (genomic survey sequence) of *B. napus*, then of *B. rapa*, or *B. oleracea*, which are comparatively located in the *Arabidopsis* syntenic region. All the ESTs and GSSs were obtained from the public *Arabidopsis thaliana* Integrated Database (ATIDB, http://atidb.org/cgi-perl/gbrowse/atibrowse/). Specific PCR primers were then designed by hand or software Primer3, according to these published sequences or conjunct sequences generated by adjacent ones. A relatively even primer distribution was primarily considered, and an initial comparison with homologous *Arabidopsis* genomic sequence was also done to avoid one primer anchoring two exons of a putative *Arabidopsis* gene. These *Arabidopsis*-derived specific primers were all analyzed and detected as the SCAR primers described above.

Results

Fertility segregation in 9012AB NIL population

A total of 4,136 individuals were obtained from a full sib-mating cross between 9012A and 9012B, of which 2,085 plants were male-fertile and 2,051 plants male-sterile. This segregation of fertility/sterility was consistent with the expected ration of 1:1 ($\chi^2 = 0.28$, $P = 0.75–0.50$), confirming the deduction that only one male sterile gene segregates in 9012AB NIL population (Chen et al. 1998).

Conversion of AFLP markers to SCAR markers

The seven AFLP markers linked to the *Bnms3* gene developed previously (Ke et al. 2005) were then cloned and sequenced. With a removal of the AFLP adapter sequences and a filling of the cohesive ends, the exact size of each marker was shown in Table 1. Based on these AFLP

Table 1 Characterization of AFLP markers tightly linked to the *BnMs3* gene

AFLP marker	Original size (bp)	Extended fragment size (bp)	SCAR conversion	Homologous region in *Arabidopsis* genome	Location in *Arabidopsis* chromosome 5 (bp)	*E* value
E01P12	149	333	SEP4	At5g11630	3740461–3742140	6e−35
E07P04	339	817	SEP7	At5g08470	2735926–2743057	2e−81
E07P13	267	161	SEP5	At5g19140	6423275–6426127	6e−09
E09P06	227	1713	SEP8	At5g17670	5821062–5822738	3e−52
E09P09	104	1170	–	At5g07475	2364746–2365606	5e−09
E12P03	262	176	SEP10	–	–	–
E13P09	95	1687	–	At5g02770	609379–611677	2e−30

marker sequences, SCAR primers were designed to amplify the corresponding loci from genomic DNA. Among them, four AFLP markers (E07P13, E07P04, E09P06 and E12P03) were converted to dominant SCAR markers directly, designated as SEP5, SEP7, SEP8 and SEP10 (Tables 1, 2), respectively. Unfortunately, specific primers derived from the other three markers (E13P09, E09P09 and E01P12) could just amplify a monomorphic band between the fertile and sterile bulks. This suggested that it was not feasible to convert them directly. So, the flanking sequences of these three AFLP markers were isolated by suppression PCR.

According to the standard PCR walking protocol, these three AFLP markers were all successfully extended (Table 1), and new SCAR primers were designed based on these sequences. PCR analysis showed that only the SCAR primer SEP4 derived from the extended E01P12 sequence could amplify the original polymorphism (Fig. 1a), whereas several pairs of SCAR markers designed from the flanking sequences of E13P09 and E09P09 still revealed monomorphic bands between fertile and sterile plants, indicating that their SCAR conversion failed.

Linkage analysis

According to the primary analysis of 1,500 individuals, two SCAR markers (SEP5 and SEP10) were identified as the outside markers flanking *Bnms3*. Subsequently they were used to assay other individuals of the whole NIL mapping population. A total of 44 recombination events for SEP10, and eight for SEP5 were detected. These recombinants were further characterized by using the other three SCAR markers (SEP4, SEP7 and SEP8) and all the AFLP markers. Linkage analysis showed that all the AFLP markers and SCAR markers span a genetic region of 1.44 cM, four of which were distributed on one side of the *Bnms3* gene, eight on the other side (Fig. 2a). Among these flanking markers of the *Bnms3* gene, SEP4 and SEP8 were the most closely linked SCAR ones, with a genetic distance of 0.41 and 0.15 cM, respectively. Expectedly, all the five AFLP markers were mapped at the same position as their corresponding SCAR markers. The two AFLP markers, not converted to SCAR markers, were both mapped between SCAR marker SEP7 and SEP10.

Table 2 SCAR markers derived from AFLP markers and *Arabidopsis* syntenic region

SCAR marker	Direction	Sequences (5′ → 3′)	Annealing temp. (°C)	Polymorphism	Production size (bp)	Map distance (cM)
SEP4	Forward Reverse	CGTGCGAATTCAAATTTCTGAATA TCTCGATGTCGATCGCTAT	54	Dominant	286	0.41
SEP5	Forward Reverse	CTGCAGTGTCCTAAAAGTCT GACCGAATTCATCGGTTTTTTC	56	Dominant	267	0.19
SEP7	Forward Reverse	5′-TCACGGACCTGAGAGATGGATG 5′-CAGCAGCGTGTTCCCTAC	67	Dominant	339	0.73
SEP8	Forward Reverse	5′-GAATTCACAACTGAAACTAGC 5′-CTGCAGCTGTTGGTTTTG	56	Dominant	227	0.15
SEP10	Forward Reverse	5′-GAATTCACGCCAAGCAATTA 5′-CTGCAGCACACAAAAAGGTT	58	Dominant	262	1.1
AR23	Forward Reverse	TTGTTCGCAAATGGTTTTTC TATGGCCATCTCCAGACTGA	60	Dominant	268	0.07
AR48	Forward Reverse	TTCAACTCTCTCCGGAACCTG CGGTAGCGGTTGCTTCTGTT	67	Dominant	451	0.07

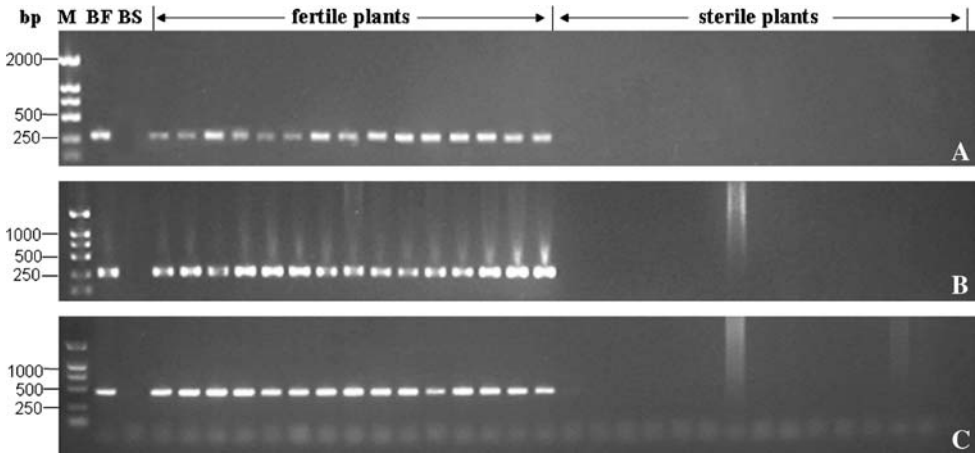

Fig. 1 The amplification results of AFLP-derived SCAR markers (**a**) and *Arabidopsis*-derived PCR markers (**b**, **c**) associated with the *BnMs3* gene. **a** SEP4. **b** AR23. **c** AR48. *Lanes*: *M* DNA Ladder 2000, *BF* fertile DNA bulk, *BS* sterile DNA bulk

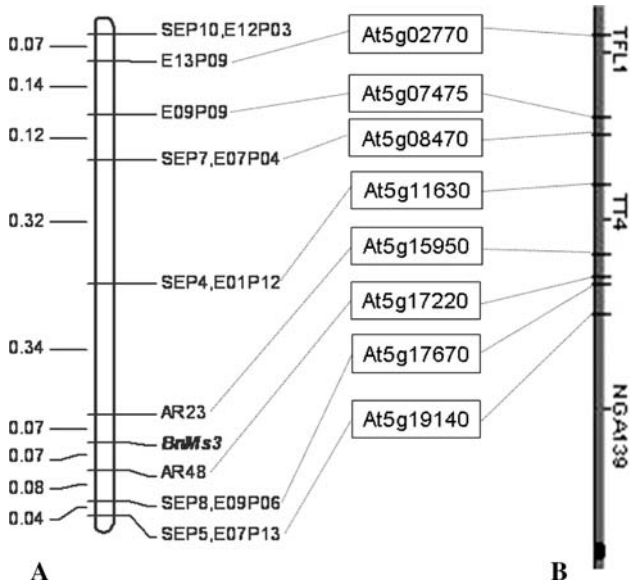

Fig. 2 **a** A genetic linkage map of the *BnMs3* gene and associated molecular markers according to this research. **b** The distribution of homologous DNA segments on *Arabidopsis* chromosome 5 (this figure was drawn with the SeqViewer tool available on http://www.arabidopsis.org/servlets/sv). The detailed *Arabidopsis* regions homologous to the PCR markers flanking the *BnMs3* gene are listed with rectangle boxes. The *dotted lines* indicate the relationship between these linked markers and their homologues

BnMs3-linked markers match *Arabidopsis* chromosome 5

For increasing the accuracy in BLAST analysis, we also extended the sequences of the other four AFLP markers (Table 1), though they had been converted to SCAR markers directly. Sequences of all the seven AFLP markers were submitted to the NCBI website for BLAST search. Six AFLP extended sequences were found to have highly conserved homologues in *Arabidopsis* genome. All of the homologues were putative genes predicted by *Arabidopsis* Genome Initiative (AGI), spanning a large region from At5g02770 to At5G19140 on *Arabidopsis* chromosome 5 (Table 1; Fig. 2b). Moreover, the arrangement order of the molecular markers linked to *Bnms3* was consistent with that of their homologues in *Arabidopsis* (Fig. 2a, b). This indicates that the syntenic region was perfectly collinear between *B. napus* and *Arabidopsis*. Accordingly, we concluded that SCAR/AFLP markers that are tightly linked to *Bnms3* in *B. napus* should match this single genomic region in *Arabidopsis* 5, and some specific PCR primers from this region are expected to map the *Bnms3* gene more closely.

Arabidopsis-derived specific primers map *Bnms3* more closely

To narrow down the target interval, we attempted to develop specific PCR markers from the putative syntenic genomic region in *Arabidopsis*. The nearest SCAR markers flanking *Bnms3* were SEP4 and SEP8, of which the orthologous regions in *Arabidopsis* were positioned in AT5G11630 and AT5G17670, respectively (Fig. 2a, b). Much more attention was thereby paid on the segment delimited by these two *Arabidopsis* genes. Unfortunately, a total of more than 600 AGI-predicted genes reside in this region, suggesting that it is fairly difficult and expensive to obtain markers from such a large physical region directly. However, considering the *Bnms3* gene has been restricted in a genetic region of only 0.56 cM by SEP4 and SEP8, we hypothesized that the ratio of physical to genetic distance is evenly distributed around the *Bnms3* locus. Thus, in this little genomic interval of *B. napus*, every 0.1 cM distance corresponds to an average of 108 Arabidopsis genes. Then we concluded that the *Arabidopsis* gene most likely homologous to *Bnms3* may be located around AT5G16050. Based on this analysis, we paid more attention to the homologous *B. napus* (*B. rapa* or *B. oleracea*) ESTs or GSSs mapped near AT5G16050, varying from AT5G14800 to AT5G17300 (altogether 250 genes).

The corresponding sequences located in this region were downloaded and analyzed, from which a total of 50 specific

primers were designed. After PCR amplification and agarose gel electrophoresis, three primers (AR23, AR35 and AR48) were identified to show stable polymorphism between fertile and sterile DNA bulks. Of them, AR23 was directly developed from the end sequence of a genomic DNA TAC (transformation-competent artificial chromosome) clone JBnY027D02R (EM: DU108405), which shares a high sequence similarity with At5g15950. While AR48 was designed according to the conjunct sequence between a *B. napus* EST (EM: CD817558) and *B. oleracea* GSS (EM: BZ066619), which is homologous with At5g17220. AR35 was also from the conjunct sequence of two GSSs (EM: DX062660, EM: DU126320) homologous to At5g16715. Further analysis indicated that, AR23 and AR48 are cosegregated with the *Bnms3* gene in 30 individuals (Fig. 1b, c), whereas AR35 shows to randomly segregate with it. Primer AR23 and AR48 were subsequently analyzed by all the 52 recombination events. Three individuals showing recombinant event were confirmed between AR23 and *Bnms3*, while another three ones between AR48 and *Bnms3*. Linkage analysis indicated the two specific primers were both closely linked to the *Bnms3* gene with a genetic distance of 0.07 cM. Eventually, aided by the *Arabidopsis*-derived polymorphic markers, the *Bnms3* gene was finely mapped in a genetic region of 0.14 cM (Fig. 2a).

Discussion

With almost all fertile rapeseed lines restoring the fertility of F1 hybrids, the key step of breeding three-line RGMS hybrids is to develop elite male-sterile lines and their homologous maintainers. One of our purposes, therefore, is to obtain a set of markers which can be conveniently and cheaply used for MAS. AFLP is a powerful technique for the identification of associated markers; but the relatively long costly procedure makes it economically unacceptable for routine application in plant breeding programmes (Mienie et al. 2005) or genotyping in large scale. In the present study, based on our previous study, five SCAR markers and two specific PCR markers tightly linked with the *BnMs3* gene were developed. These markers will be valuable in breeding novel sterile lines and maintainers, particularly when used in combination with the molecular markers tightly linked with the *Bnms4* gene and the *Bnesp* gene.

Extensive genome collinearity has been observed between *Arabidopsis* and *B. napus* (Parkin et al. 2005). This relationship can help us to exploit the *Arabidopsis* genome information for developing more closely linked markers. In our work, six of the seven AFLP markers linked to *Bnms3* were located to the *Arabidopsis* chromosome 5 by homologous sequence analysis, and the arrangement of these markers in *B. napus* is consistent with their homologues in *Arabidopsis*. This result strongly suggested that the genomic segment around *Bnms3* between *B. napus* and *Arabidopsis* may also locate in a collinear region. Therefore, it was possible to exploit the *Arabidopsis* genomic sequence to develop specific PCR markers, as shown in map-based isolating the *Rfo* gene in radish (Desloire et al. 2003). Specific PCR markers derived from this putative collinear region mapped the *Bnms3* gene more closely, proving the authenticity of this collinear region and the feasibility of exploiting sequence information from it. Otherwise, in order to raise the specificity and success probability of PCR amplification, we designed the PCR primers preferentially on the sequence resource of *B. napus* or its related *Brassica* species (*B. rapa* or *B. oleracea*). This strategy was considered to be very effective, because about 80% primers from this way could generate a single and clear band, whereas primers originated from *Arabidopsis* genome sequence generally could obtain several non-specific and weaker bands (data not shown). Only 2 of 50 specific PCR primers showing tightly linkage with the *Bnms3* gene were identified, suggesting that it was still difficult to develop polymorphic markers directly. Indeed, we were not surprised at this result, because fertile and sterile plants in 9012AB are NILs, and the DNA sequence polymorphism surrounding the *Bnms3* gene between them is likely to concentrate on few nucleotides or single one. Consequently, only the primers anchoring the nucleotide variation itself or the occasional length polymorphic fragment can be detected showing polymorphism here. Thus, in the next step, we expected to develop more useful markers (including SNP markers) from these fifty specific primers, by comparatively sequencing the monomorphic PCR products between the fertile and sterile plants.

For many organisms, map-based or positional cloning is the primary strategy for identifying and characterizing genes with unknown biochemical products (Formanova et al. 2006). Construction of a high resolution genetic map of the target gene is a prerequisite in this process. Though some nuclear genes associated with male sterility have been genetically mapped in *B. napus* recently (Song et al. 2006; Yi et al. 2006; Formanova et al. 2006), no related gene has been isolated by map-based cloning, partially due to the difficulty of fine mapping in the complex polyploidy genome. Huang et al (2007) reported that the *Bnms3* gene was restricted in a genetic region of 0.4 cM, and the nearest marker in one side was an AFLP marker (EA09P06) identified by Ke et al (2005). In this study, a new PCR marker located between *Bnms3* and EA09P06 (SEP8) was developed, which greatly narrowed down the chromosome fragment containing the target gene. We believed that the positional cloning of the *Bnms3* gene will be greatly expedited by this research result, together with the mapping information published by Huang et al (2007).

An approximate 8 Mb region on the top of *Arabidopsis* chromosome 5 has been identified to display strong conservation of gene content and gene order with *B. napus* genome, and evidence also illustrated that in this region every one cM in *B. napus* genomic region is equivalent to an average of 285 kb *Arabidopsis* genomic sequence (Parkin et al. 2002). In this study, sequence comparison also indicated that the DNA segment syntenic to the *Bnms3* locus just lies in this defined *Arabidopsis* genomic region. From this result, it can be concluded that the two closest markers (AR23 and AR48) should theoretically delimit a genetic distance of about 1.65 cM in *B. napus*, since they span a genomic region of 470 kb in *Arabidopsis* genome. Obviously, it was significantly different from the actual genetic distance of 0.14 cM. This divergence suggested that the recombination may be severely suppressed around the *Bnms3* locus, and the physical distance between these two markers may be much larger than the expected 58 kb, which can be concluded from the average physical to genetic ratio of 494 kb/cM in *B. napus* according to previous reports (Arumuganathan and Earle 1991; Lombard and Delourme 2001). This deduction has been confirmed when using these two markers as probes to screen bacterial artificial chromosome (BAC) clones of *B. napus* (not published). It has been reported that the recombination frequency around a particular locus is generally dependent upon the population used (Casselman et al. 2000; Bentolila and Hanson 2001; Imai et al. 2003), and the fine mapping studies are best conducted with not one, but several crosses, and preferably in early generations (Bentolila and Hanson 2001). Consequently, the next work under consideration is to investigate the recombination frequency of these markers tightly linked to the *Bnms3* gene in different crosses. Alternatively, it may be also an efficient approach by assaying these markers in a much larger population to obtain enough recombinant events, and the target gene will be physically mapped in a narrow region.

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References

Arumuganathan K, Earle ED (1991) Nuclear DNA content of some important plant species. Plant Mol Biol Rep 9:208–218

Bentolila S, Hanson MR (2001) Identification of a BIBAC clone that co-segregates with the petunia restorer of fertility (*Rf*) gene. Mol Genet Genomics 266:223–230

Budar F, Pelletier G (2001) Male sterility in plants: occurrence, determinism, significance and use. C R Acad Sci III 324:543–550

Casselman A, Vrebalov J, Conner JA, Singhal A, Giovannoni J, Nasrallah ME, Nasrallah JB (2000) Determining the physical limits of the *Brassica S* locus by recombinational analysis. Plant Cell 12:23–33

Chen FX, Hu BC, Li QS (1993) Discovery and study of genic male sterility (GMS) material 9012A in *Brassica napus* L (in Chinese). Acta Agric Univ Pekinensis 19(Suppl.):57–61

Chen FX, Hu BC, Li C, Li QS, Chen WS, Zhang ML (1998) Genetic studies on GMS in *Brassica napus* L: I. Inheritance of recessive GMS line 9012A. Acta Agron Sin 24:431–438

Chen FX, Hu BC, Li QS, Hou SM, Wu XJ, Fei WX, Li C, Chen WS (2003) Breeding of Wanyou 14, a recessive epistatically genic male sterile hybrid rapeseed (*Brassica napus*) with double low. Chinese J Oil Crop Sci 25:63–65, 68

Desloire S, Gherbi H, Laloui W, Marhadour S, Clouet V, Cattolico L, Falentin C, Giancola S, Renard M, Budar F, Small I, Caboche M, Delourme R, Bendahmane A (2003) Identification of the fertility restoration locus, *Rfo*, in radish, as a member of the pentatricopeptide repeat protein family. EMBO Rep 4:588–594

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 19:11–15

Formanova N, Li XQ, Ferrie AMR, DePauw M, Keller WA, Landry B, Brown GG (2006) Towards positional cloning in *Brassica napus*: generation and analysis of doubled haploid *B.* rapa possessing the B. napus pol CMS and Rfp nuclear restorer gene. Plant Mol Biol 61:269–281

Hou GZ, Wang H, Zhang RM (1990) Genetic study on genic male sterility (GMS) material No. 117A in *Brassica napus* L. (in Chinese). Oil Crop China 2:7–10

Huang Z, Chen YF, Yi B, Xiao L, Ma CZ, Tu JX, Fu TD (2007) Fine mapping of the recessive genic male sterility gene (*Bnms3*) in *Brassica napus* L. Theor Appl Genet 115:113–118

Imai R, Koizuka N, Fujimoto H, Hayakawa T, Sakai T, Imamura J (2003) Delimitation of the fertility restorer locus *Rfk1* to a 43-kb contig in *Kosena* radish (*Raphanus sativus L.*). Mol Genet Genomics 269:388–394

Ke LP, Sun YQ, Hong DF, Liu PW, Yang GS (2005) Identification of AFLP markers linked to one recessive genic male sterility gene in oilseed rape, *Brassica napus* L. Plant Breed 124:367–370

Kosambi DD (1944) The estimation of map distances from recombination values. Ann Eugen 12:172–175

Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newburg L (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1(2):174–181

Lincoln S, Daly M, Lander E (1992) Constructing genetic maps with Mapmaker/Exp 3.0. Whitehead Institute Technical Report, Cambridge, MA, USA

Liu RH, Meng JL (2003) Mapdraw, a Microsoft Excel macro for draw genetic linkage maps based on given genetic linkage data. Hereditas (Beijing) 25:317–321

Lombard V, Delourme R (2001) A consensus linkage map for rapeseed (*Brassica napus L.*): construction and integration of three individual maps from DH populations. Theor Appl Genet 103:491–507

Mienie CMS, Liebenberg MM, Pretorius ZA, Miklas PN (2005) SCAR markers linked to the common bean rust resistance gene *Ur-13*. Theor Appl Genet 111:972–979

Pan T, Zeng FY, Wu SH, Zhao Y (1988) A study on breeding and application GMS line of low eruci acid in rapeseed (*B. napus*). Oil Crop China 3:5–8

Parkin IAP, Lydiate DJ, Trick M (2002) Assessing the level of collinearity between *Arabidopsis thaliana* and *Brassica napus* for *A. thaliana* chromosome 5. Genome 45:356–366

Parkin IAP, Gulden AG, Lukens L, Trick M, Osborn TC, Lydiate DJ (2005) Segmental structure of the *Brassica napus* genome based on comparative analysis with *Arabidopsis thaliana*. Genetics 171:765–781

Siebert PD, Chenchick A, Kellogg DE, Lukyanov KA, Lukyanov SA (1995) An improved PCR method for walking in uncloned genomic DNA. Nucleic Acids Res 23:1087–1088

Song LQ, Fu TD, Tu JX, Ma CZ, Yang GS (2006) Molecular validation of multiple allele inheritance for dominant genic male sterility gene in *Brassica napus* L. Theor Appl Genet 113:55–62

Steve R, Skaletsky HJ (1996) Primer3: A software component for picking PCR primers. Source code. http://www-genome.wi.mit.edu/genome_software/other/primer3.html

Sun CC, Zhao H, Wang WR, Li YL, Qian XF, Fang GH (2004) Breeding of a recessive genic male sterile (GMS) hybrid variety Huyouza No. 1 with double low in *Brassica napus* L. Chin J Oil Crop Sci 26:63–65

Sun CC, Wang WR, Li YL, Zhou XR, Qian XF (2005) Breeding of a double-low recessive genic male sterile hybrid Huyouza No. 2 in *Brassica napus* L. Acta Agric Shanghai 21:1–3

Yang GS, Qu B, Fu TD (1999a) Cytological study of microsporogenesis in three recessive genic male sterility lines of *Brassica napus* L. J Huazhong Agric Univ 18:520–523

Yang YW, Lai KN, Tai PY, Li WH (1999b) Rates of nucleotide substitution in angiosperm mitochondrial DNA sequences and dates of divergence between *Brassica* and other angiosperm lineages. J Mol Evol 48:597–604

Yi B, Chen YL, Lei SL, Tu JX, Fu TD (2006) Fine mapping of the recessive genic male-sterile gene (*Bnms1*) in *Brassica napus* L. Theor Appl Genet 113:643–650