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ORIGINAL PAPER

Exploiting comparative mapping among *Brassica* species to accelerate the physical delimitation of a genic male-sterile locus (*BnRf*) in *Brassica napus*

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Abstract The recessive genic male sterility (RGMS) line 9012AB has been used as an important pollination control system for rapeseed hybrid production in China. Here, we report our study on physical mapping of one male-sterile locus (*BnRf*) in 9012AB by exploiting the comparative genomics among *Brassica* species. The genetic maps around *BnRf* from previous reports were integrated and enriched with markers from the *Brassica* A7 chromosome. Subsequent collinearity analysis of these markers contributed to the identification of a novel ancestral karyotype block F that possibly encompasses *BnRf*. Fourteen insertion/deletion markers were further developed from this conserved block and genotyped in three large backcross populations, leading to the construction of high-resolution local genetic maps where the *BnRf* locus was restricted to a less than 0.1-cM region. Moreover, it was observed that the target region in *Brassica napus* shares a high collinearity relationship with a region from the *Brassica rapa* A7 chromosome. A *BnRf*-cosegregated marker (AT3G23870) was then used to screen a *B. napus* bacterial artificial chromosome (BAC) library. From the resulting 16 positive BAC clones, one (JBnB089D05) was identified to most possibly contain the $BnRf^{e}$ allele. With the assistance of the genome sequence from the *Brassica rapa* homolog, the 13.8-kb DNA fragment covering both closest flanking markers from the BAC clone was isolated. Gene annotation based on the comparison of microcollinear regions among *Brassica napus*, *B. rapa* and *Arabidopsis* showed that five potential open reading frames reside in this fragment. These results provide a foundation for the characterization of the *BnRf* locus and allow a better understanding of the chromosome evolution around *BnRf*.

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Introduction

The recessive genic male sterile (RGMS) line 9012AB was discovered in 1991 as a spontaneous mutant in a *Brassica napus* accession (Chen et al. 1993). A tri-genic hereditary model subsequently proposed to interpret sterility inheritance in 9012AB indicates that its male sterility is controlled by the interaction of two recessive male-sterile genes (*Bnms3* and *Bnms4*) with one epistatic suppressor gene (*BnRf* or *BnEsp*) (Chen et al. 1998). In male-sterile plants, a homozygous recessive sterile gene can be found at both loci (*Bnms3ms3ms4ms4*). Fertility can be restored by the presence of dominant alleles at either of the sterile genes or by homozygous recessive alleles at the *BnRf* locus. According to this model, crosses between the homozygous male-sterile lines (*Bnms3ms3ms4ms4RfRf*) and the temporary maintainers (*Bnms3ms3ms4msrfrf*) would generate complete male-sterile populations. In turn, these populations can be used as female parents to be pollinated by restorer lines, giving rise to fertile three-way

hybrids. This approach for hybrid production greatly overcomes the inevitable restriction of previously reported RGMS lines, such as S45AB and 117AB, in which 50% of the fertile plants would be artificially removed from the female lines before flowering (Chen et al. 1998; Ke et al. 2005). Thus, the application of 9012AB in hybrid production was widely promoted in China, and a series of commercial cultivars were released (Chen et al. 2007).

However, recent studies on both systematic genetic analyses and molecular marker integration suggested that the previously designated *BnMs4* locus should be allelic to the *BnRf* locus (Dong et al. 2010; Zu et al. 2010). Accordingly, a major modification of the tri-genic hereditary model was raised in which the previously appointed dominant *BnMs4* allele (hereafter designated as $BnRf^{a}$) is indeed the third allele of the *BnRf* locus, in addition to $BnRf^{b}$ (corresponding to the previously dominant *BnRf* allele from 9012A) and $BnRf^{c}$ (corresponding to the previously recessive *Bnrf* allele from the temporary maintainer), with a dominance relationship of $BnRf^{a} > BnRf^{b} > BnRf^{c}$ (Dong et al. 2010). In this new hereditary explanation, plants carrying the most dominant allele $BnRf^{a}$ whether in homozygosity or heterozygosity would always show the fertile phenotype. $BnRf^{c}$ is also a recessive allele as restorer for normal pollen fertility; however, its ability can be suppressed by the existence of the $BnRf^{b}$ allele. The multiple-allele *BnRf* locus shows an identical genetic pattern with the other two male-sterile loci reported in *B. napus* (Song et al. 2006) and *B. rapa* (Feng et al. 2009). Though the male-sterile locus in *B. napus* is obviously differentiated with the *BnRf* locus in this research, the possibility remains that the one from *B. rapa* has the same evolutionary origin as the *BnRf* locus, considering that some simple sequence repeats (SSR) markers linked to it (Feng et al. 2009) show a genetic linkage with *BnRf* as well (data not shown). Advancement of the digenic sterility control model would effectively simplify the breeding strategy involved with this RGMS line.

In recent years, efforts toward understanding the molecular mechanism of sterility control in 9012AB have been taken. First, cytological observations suggested that the arrest of microsporogenesis occurred at the tetrad stage, with unscheduled apoptosis of the tapetal cells resulting in inadequate secretion of callase to degrade callose surrounding the tetrads, thereby leading to the collapse of male gametophyte development (Wan et al. 2010). Second, attempts toward final isolation of the genes responsible for male sterility in 9012AB have been carried out. For example, the genetic map of *BnMs3* was gradually narrowed by continual efforts (Ke et al. 2005; Huang et al. 2007; He et al. 2008). Now, the *BnMs3* locus has been cloned and characterized as a member of the protein import complex responsible for the transport of nuclear-encoded proteins to the chloroplast stroma (Dun et al. 2011). As for the *BnRf* locus, it was first tagged by several amplified fragment length polymorphism (AFLP) markers (Xie et al. 2008) and was then anchored to the *B. napus* A7 chromosome (Xiao et al. 2008). The local map around *BnRf* was later improved with some tightly associated or cosegregated markers (Xu et al. 2009). However, because some of the *BnRf*-associated markers were population specific, it was difficult to compare their relative genetic distances from the target gene, and their physical positions on the chromosome are yet to be determined. Therefore, integrating these markers into one population would be very informative and help to construct a physical map around *BnRf*. Finally, expression analysis of the downstream genes in *BnMs3*- and *BnRf*-directed pathways using the *Arabidopsis* orthologs as a reference led investigators to hypothesize that *BnMs3* and *BnRf* may have opposite reactions in pollen formation (Zhu et al. 2010). Thus, isolation of the *BnRf* locus would be a premise to test this speculation. Moreover, it would contribute to understanding the molecular mechanism of male sterility in 9012AB and enrich our knowledge of microgametogenesis in amphidiploid *B. napus*.

Brassica species represent the closest crop plant relatives to the model plant *Arabidopsis*. Comparative mapping studies using common molecular markers have revealed the existence of conserved blocks between *Brassica* and *Arabidopsis* (Parkin et al. 2005; Schranz et al. 2006; Panjabi et al. 2008; Wang et al. 2011a). Identification of conserved regions in *Arabidopsis* enables the identification of molecular markers and candidate genes for accelerating positional cloning in *Brassica* species (Brown et al. 2003; Desloire et al. 2003; Snowdon et al. 2007). Examples of the use of this strategy are provided by the map-based cloning of the dwarf gene *DWF2* in *B. rapa* (Muangprom et al. 2005) and the *Bnms1* and *Bnms3* genes in *B. napus* (Yi et al. 2010; Dun et al. 2011). However, such applications of collinearity may be complicated by the highly duplicated nature of *Brassica* genomes and the occurrence of rearrangements within collinear blocks (Mayerhofer et al. 2005; Snowdon et al. 2007; Formanova et al. 2010). Therefore, it is necessary to examine and validate the microcolinearity between a specific target region in the *Brassica* genome and the corresponding region in *Arabidopsis* before utilizing the latter genome as a stepping stone for the positional cloning of a *Brassica* gene (Zhang et al. 2009). Most recently, the release of the diploid *B. rapa* genome sequence opened a new age for crop research in *Brassica* species (Wang et al. 2011b), which would absolutely accelerate and improve the gene cloning strategy in *B. napus* by directly providing the reference sequence of the A genome of this family. As a result, much more sequence and structure information can be achieved around a target locus by comparative genomics between

B. napus and *B. rapa* than between *B. napus* and *Arabidopsis*. However, use of the *B. rapa* genome sequence must be carefully considered in the regions where evolutionary divergences have occurred, because massive genome rearrangements have been detected between these two species (Jiang et al. 2011).

In the present study, we report our study on physical mapping of the *BnRf* locus via a classic map-based cloning strategy in combination with comparative mapping between *B. napus* and *Arabidopsis* as well as *B. rapa*. The *BnRf*^{c} allele was finally delimited to a 13.8-kb DNA fragment in the *B. napus* genome, which contains five predicted open reading frames (ORF). These results will greatly facilitate the final isolation of this male-sterile locus in *B. napus*.

Materials and methods

Plant materials and recombinant screening

The RGMS two-type line 9012AB and the temporary maintainer T45 used in our study have been described by Ke et al. (2005) and Xie et al. (2008), respectively. According to the recently modified hereditary pattern of this RGMS line, the genotypes of 9012A, 9012B and T45 are *Bnms3ms3Rf*^{b}*BnRf*^{b}, *BnMs3ms3Rf*^{b}*Rf*^{b} and *Bnms3ms3Rf*^{c}*Rf*^{c}, respectively (Dong et al. 2010). GosAB, another RGMS two-type line bred in our group, has a distinct genetic background with 9012AB, but shares the same genotype as 9012AB (i.e., *Bnms3ms3Rf*^{b}*BnRf*^{b} for GosA and *BnMs3ms3Rf*^{b}*BnRf*^{b} for GosB). DH206, a double haploid *B. napus* breeding line developed in our group, has a genotype of *BnMs3Ms3Rf*^{a}*Rf*^{a} in the two RGMS loci (Dong et al. 2010). We previously obtained a backcross population including 180 plants from the cross (9012A × T45) × T45 for a rough map of the *BnRf* locus (Xie et al. 2008). To conduct a high-resolution local genetic map, we expanded this population (hereafter referred to as Population I) and constructed another similar BC_1 population (hereafter referred to as Population II) from the cross (GosA × T45) × T45. After validation of the allelism between *BnMs4* and *BnRf*, a backcross population used for mapping the previously designated *BnMs4* locus was also harnessed for integration of the fine genetic map around *BnRf* (Dong et al., unpublished data). It was generated as follows: first, some F_2 fertile plants from the cross between 9012A and DH206 were backcrossed with 9012A. The BC1 families displaying a 1:1 fertility segregation were further genotyped with two *BnMs3* tightly flanking markers (AR23 and AR28; He et al. 2008). Only those families where both markers showed the recessive homozygosity at the *BnMs3* locus were kept because the *BnRf*^{a} allele is responsible for the fertility segregation in them. Then one fertile plant (genotyped as *Bnms3ms3Rf*^{a}*Rf*^{b}) from these BC1 families was randomly selected to pollinate 9012A again. The resulting BC_2 progenies are hereafter referred to as Population III. Total plant DNA was extracted according to the method described by Doyle and Doyle (1990) with minor modification at seedling stages. Individual male fertility was investigated at flowering time.

Marker development and genotyping

We selected molecular markers that were mapped on *Brassica* chromosome A7 for polymorphism analysis, because they evolutionarily originated from the same chromosome of a common diploid ancestor (http://www.brassica.info/resource/maps/lg-assignments.php). These markers included SSR from *B. napus* (Piquemal et al. 2005; Long et al. 2007; Cheng et al. 2009) and *B. rapa* (Choi et al. 2007) and insertion/deletion (InDel) markers (originally designated as IP marker) from *B. juncea* (Panjabi et al. 2008). Furthermore, we designed more SSR primers according to the sequences of some BAC clones physically located in the *B. rapa* chromosome A7 (http://www.brassica-rapa.org). These BAC-derived SSR primers were named according to their BAC code. All the SSR and InDel markers were first evaluated by the two parents of Population II and subsequently in individuals of this population.

To identify a possible syntenic region around *BnRf* in *Arabidopsis*, we aligned the sequences of all previously reported SCAR markers (Xie et al. 2008; Xiao et al. 2008; Xu et al. 2009) with *Arabidopsis* genome sequences using the BLASTN program of The Arabidopsis Information Resource (TAIR, http://www.arabidopsis.org), with the *E* value of ≦1E−07 as a cutoff to decide whether the homolog exists in *Arabidopsis* (Parkin et al. 2005). If no homolog could be searched directly, the marker sequence was then submitted for NCBI BLASTN analysis (http://www.ncbi.nlm.nih.gov/) to fish *B. rapa* BAC or BAC ends, which may show high sequence similarity with this marker. To determine whether these identified BAC clones were anchored in the established *B. rapa* BAC contigs (http://www.brassica-rapa.org), we aligned the end sequences from an individual BAC or every BAC of a contig with the *Arabidopsis* genome. With this method, we could indirectly determine the *Arabidopsis* sequence matching to the marker.

After identification of a syntenic *Arabidopsis* genomic interval around *BnRf*, more InDel markers were developed from this collinear interval to examine possible intronic polymorphism in our mapping populations. InDel markers were designed according to the *Brassica* expressing sequence tags (EST) or genomic survey sequences, which showed strong nucleotide conservation with the exon sequences of the corresponding *Arabidopsis* genes (http://gbrowse.arabidopsis.org/cgi-bin/gbrowse/arabidopsis). A

relatively even primer distribution was primarily considered, and an initial comparison with a homologous *Arabidopsis* genomic sequence was also done to avoid having one primer anchor two exons of a putative *Arabidopsis* gene (He et al. 2008). These primers were named according to their homologous *Arabidopsis* genes for the convenience of subsequent comparative mapping between *B. napus* and *Arabidopsis*. These primers were initially surveyed among four parent lines (9012A, GosA, T45 and DH206), and polymorphic markers were subsequently scored in all three large populations. Amplification with SCAR, SSR and InDel primers was individually performed as described before (Piquemal et al. 2005; Panjabi et al. 2008; Xie et al. 2008). The amplified products were resolved on a 6% denaturing polyacrylamide gel.

Genetic mapping

All the polymorphic SCAR, SSR and previously reported InDel primers (Panjabi et al. 2008) were assayed only in 180 plants from Population II for integration of different genetic maps of *BnRf*. Data from marker genotypes and an individual phenotype survey were combined for linkage analysis using the MAPMAKER/EXP 3.0 program (Lander et al. 1987; Lincoln et al. 1992), and a partial linkage map surrounding the *BnRf* gene was constructed. Because the newly developed InDel markers were all cosegregated in this rough genetic map, they were not shown in the map and were assayed only in the three large populations to construct high-resolution maps of *BnRf*. Map distances are given in centimorgans (cM) and represent the percentage of recombinant plants in the total number of plants analyzed (Desloire et al. 2003). The genetic linkage map was drawn using MAPDRAW_V2-2 (Liu and Meng 2003).

Identification and sequence analysis of the candidate clone

The John Innes Centre JBnB BAC library (Rana et al. 2004) constructed from genomic DNA of a winter-type European cultivar Tapidor (carrying the *BnRf* ^{c} allele in the *BnRf* locus) was used to construct the physical map. A genomic DNA fragment was isolated from 9012A using a *BnRf*-cosegregated dominant InDel marker (AT3G23870). The fragment was labeled with α-^{32}P and used as a probe to screen the four nylon filters, including all 73,728 clones of the library, according to the method described by Rana et al. (2004), except that hybridization and washing were done at a high stringency condition (0.1 × SSC and 0.1% SDS at 65°C). Plasmid DNA of positive BAC clones was prepared according to the protocol of the QIAGEN Large-Construct Kit (Qiagen, Valencia, CA, USA). Positive clones were genotyped by polymerase chain reaction (PCR) amplification with the co-dominant markers around *BnRf*, which can distinguish the alleles of *BnRf* ^{b} and *BnRf* ^{c}. If the markers can amplify the same fragment from a given BAC clone as from T45, which is specifically linked to the *BnRf* ^{c} allele from T45, this clone would be considered a candidate around *BnRf*. The mutual overlapping relationship of these candidate clones were established by comparing their genotypes for every marker with the genetic position of the respective marker on the map. BAC clones on this contig containing both flanking markers would be regarded as the candidates containing *BnRf*.

Long-fragment PCR was employed to amplify the BAC DNA in a PTC-200 Thermocycler (MJ Research, Waltham, MA, USA) using Phusion® High-Fidelity DNA Polymerase (Finnzymes Oy, Finland) and the primers designed from the closest flanking marker sequences. Then, the specific DNA fragment was recovered from the gel with the DNA Gel Extraction Kit (Tiangen, Beijing, China) and sequenced with an ABI Prism3730 DNA sequencer (Applied Biosystems, Foster City, CA, USA). Software packages FGENSH (http://www.softberry.com) and GlimmerHMM (Majoros et al. 2004) were both applied to predict the putative ORF from the candidate region. The genomic or coding sequences of the predicted genes were used as queries for homolog search and basic function analysis in NCBI (http://www.ncbi.nlm.nih.gov) and TAIR (http://www.arabidopsis.org) by BLASTN analysis.

Comparison of physical maps around *BnRf* with the *B. rapa* genome

The sequences of all the markers (between AT3G24315 and XSC5) were submitted to the Web site database (http://brassicadb.org/brad/index.php, chromosome v1.1) for BLASTN analysis with the *B. rapa* genome. Based on the highly matched orthologs, we can compare the physical distance variation and the order among these orthologs between the two species. The gene annotation in *B. rapa* can also be used to estimate the accuracy of gene prediction in *B. napus*, especially when the candidate region of the *BnRf* locus has been located at a small region in *B. napus*. The information on gene prediction in *B. rapa* can be downloaded from the Web site (http://brassicadb.org/cgi-bin/gbrowse/cbgdb11/).

Results

Population fertility segregation and primary integration of public markers around *BnRf*

Population I consisted of 2,055 male-fertile and 2,003 male-sterile plants (including the previous 180 plants

reported by Xie et al. (2008), $\chi^2_{1:1} = 0.64$, $P = 0.42$). Population II consisted of 1,857 male-fertile and 1,815 male-sterile plants ($\chi^2_{1:1} = 0.46$, $P = 0.50$). Population III included 954 male-fertile and 898 male-sterile plants ($\chi^2_{1:1} = 1.64$, $P = 0.20$). Obviously, the plant fertility of all the three population statistically segregated as a Mendelian 1:1 ratio, as a result of segregation of the *BnRf* locus. According to the genotype definition of parents based on the modified digenic hereditary model, the fertile and sterile plants in Population I and II are, respectively, genotyped as *Bnms3ms3Rf*^{c}*Rf*^{c} and *Bnms3ms3Rf*^{b}*Rf*^{c}, and individually *Bnms3ms3Rf*^{a}*R*^{b} and *Bnms3ms3Rf*^{b}*Rf*^{b} in Population III.

The polymorphism between GosA and T45 was much higher than that between 9012A and T45, as revealed by the published *BnRf*-associated markers. Hence, 188 plants from Population II were randomly selected for the integration of molecular markers from different maps. Initially, four SCAR markers associated with *BnRf* were directly united (Fig. 1), including SC1 (Xie et al. 2008), WSC5, WSC6 (Xu et al. 2009) and XSC5 (Xiao et al. 2008). The other seven SSR and three InDel markers assigned to the *Brassica* A7 chromosome (Table 1) were also mapped in Population II, although all of them had a much larger genetic distance with *BnRf* (Fig. 1). From the 22 pairs of SSR primers designed according to the sequences of BAC clones positioned on the *B. rapa* A7 chromosome, only three of them (KBrH001J06, KBrB022C05 and KBrB080N15) showed loose genetic linkage with *BnRf* in Population II (Fig. 1; Supplementary Table 1). In summary, we totally integrated four SCAR, ten SSR and three InDel markers on a rough local map of *BnRf* on the basis of Population II (Fig. 1).

InDel marker development from a novel F block encompassing *BnRf*

Sequences of the above PCR markers or their related BAC sequences were submitted to TAIR for identification of putative orthologs. Two SCAR markers (XSC5 and WSC5), three SSR-corresponded BAC sequences and 3 InDel markers can search homologous sequences directly from the *Arabidopsis* genome (Table 1). Other markers showing no homology with *Arabidopsis* were then used as queries to identify related *B. rapa* BAC (or BAC end) sequences in NCBI, harvesting one end of the *B. rapa* BAC clone KBrH059E14 homologous to WSC6. Because KBrH059E14 has been anchored to the *B. rapa* contig1232 (http://www.brassica-rapa.org), from which many BAC ends corresponded to the *Arabidopsis* genome sequence between *AT3G23920* and *AT3G24350*, WSC6 could be located indirectly at the same homologous region. Overall, it can be clearly seen that these homologs are individually

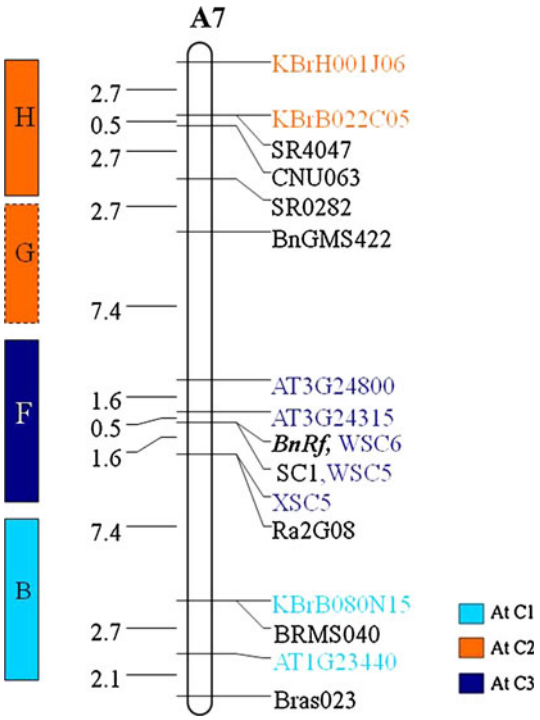

Fig. 1 Comparative mapping between *B. napus* and *Arabidopsis* around *BnRf* The *bars in different colors* on the *left* corresponded to different *Arabidopsis* chromosome fragments as shown in the *lower right corner*. The *letters* in the *bars* indicate different AK blocks proposed by Schranz et al. (2006). The *dashed line bar* shows the block H, which was not detected in this study but identified previously. Markers indicated in different colors can be individually aligned to the blocks in the same color. Genetic distances are shown in cM (color figure online)

distributed on three *Arabidopsis* chromosomes (Table 1), and there seems to be no large *Arabidopsis* region syntenic to the *BnRf* locus. Nevertheless, the orthologs of the four markers (XSC5, WSC6, AT3G24315 and AT3G24800) that link most closely to *BnRf* all fall into a relatively narrow region of *Arabidopsis* chromosome 3 and have a consistent arrangement order with that of the related markers on the map (Fig. 1; Table 1). Although WSC5 also hit the *Arabidopsis* gene *AT3G42556*, it is not considered here because *AT3G42556* is annotated as a transposable element.

According to the definition of the conserved blocks from the ancestral karyotype (AK) by Schranz et al. (2006), the chromosomal fragment covering *BnRf* can be schematically represented by three neighboring blocks H, F and B (Fig. 1). Among these, block F possibly contains the ancestor of the *BnRf* locus. To test this speculation, we designed a total of 122 pairs of InDel primers from the 355-kb fragment between *Arabidopsis* gene *AT3G23590* and *AT3G24315* as restricted by marker XSC5 and AT3G24315. Of these primers, five were identified as markers in all the three populations (AT3G23910-1 and

Table 1 The *BnRf*-linked markers reported previously or developed from public *Brassica* chromosome A7 and their homologous region in *Arabidopsis*

PCR marker	Marker types	Linkage group	Homologous region in *Arabidopsis* genome	References
CNU063	SSR	N7	Not significant	Long et al. (2007)
SR0282	SSR	N7	Not significant	Long et al. (2007)
Ra2G08	SSR	N7	Not significant	Long et al. (2007)
Bras023	SSR	N7	Not significant	Long et al. (2007)
BnGMS422	SSR	N7	Not significant	Cheng et al. (2009)
SC1	SCAR	N7	Not significant	Xie et al. (2008)
WSC5	SCAR	N7	*AT3G42556*	Xu et al. (2009)
WSC6	SCAR	N7	Not significant	Xu et al. (2009)
XSC5	SCAR	N7	*AT3G23590*	Xiao et al. (2008)
SR4047	SSR	R7	Not significant	Choi et al. (2007)
BRMS040	SSR	R7	Not significant	Choi et al. (2007)
KBrH001J06	SSR	R7	*AT2G20220-AT2G20320*	Developed here
KBrB022C05	SSR	R7	*AT2G19530-AT2G19710*	Developed here
KBrB080N15	SSR	R7	*AT1G25240-AT1G25580*	Developed here
AT3G23800	InDel	J7	*AT3G23800*	Panjabi et al. (2008)
AT3G24315	InDel	J7	*AT3G24315*	Panjabi et al. (2008)
AT1G23440	InDel	J7	*AT1G23440*	Panjabi et al. (2008)

AT3G23910-2 were developed from the same sequence homologous to the *Arabidopsis* gene *AT3G23910*), eight were polymorphic in Population I and II, and two could only reveal polymorphism in Population II (Fig. 2).

High-resolution mapping of the *BnRf* locus

These polymorphic InDel markers were subsequently analyzed in the respective full populations. A flanking marker strategy (Tanksley et al. 1995) was applied to improve the efficiency of genotyping in a large population. Finally, a total of 138 recombinants were detected in Population I by SC23 and Ra2G08 (Xu et al. 2009). Sixty recombinants were revealed in Population II by XSC5 and AT3G24315, and 23 were identified in Population III by AT3G24030 and AT3G23700. All the recombinants from the respective populations were then genotyped by those inside polymorphic markers as well as the three SCAR markers (SC1, XSC5 and WSC6). In combination with the marker genotypes and recombinant fertility, three high-resolution genetic maps around the *BnRf* locus were eventually constructed. In Population I, *BnRf* was restricted to the narrowest genetic region of 0.232 cM, with 0.206 cM for WSC6 and 0.026 cM for AT3G23900 (Fig. 2a). In Population II, *BnRf* was mapped between AT3G24240 and AT3G23760, with the smallest genetic distances of 0.057 cM and 0.057 cM, respectively (Fig. 2c). In Population III, *BnRf* was bracketed by the closest flanking markers AT3G23900 and AT3G23910-2, with a map distance of 0.054 cM and 0.108 cM, respectively (Fig. 2b). Some markers, such as AT3G23900 and AT3G24240, were cosegregated with *BnRf* in one population, but could be separated in the other two populations. Taking all the three genetic maps into account, we found that the *BnRf* locus was bracketed by the closest flanking markers AT3G23900 and AT3G2910-1 (or AT3G2910-2), with the other three markers (SC1, WSC5 and AT3G23870) cosegregating with *BnRf*.

Identification of BAC clones covering the $BnRf^c$ allele

As shown in Fig. 2, the arrangement order of the InDel markers between XSC5 (homologous to *AT3G23590*) and AT3G23910 was basically consistent with that of their homologs in *Arabidopsis*, except for a small fragment inversion or alternatively a translocation occurring between AT3G23800 and AT3G23910. A similar result was observed in the genomic region between AT3G23920 and AT3G24240, while there was an obvious inversion event between *B. napus* and *Arabidopsis* (Fig. 2). These facts not only strongly support that the genomic region around *BnRf* originated from the conserved block F, but there is also good microcolinearity between *B. napus* and *Arabidopsis* in this block. Consequently, we can conclude that the homolog of *BnRf* in *Arabidopsis* is possibly located from *AT3G23800* to *AT3G23910*, including no more than ten predicted genes (*AT3G23800* itself can be excluded because the InDel markers homologous to it was distributed outside of marker AT3G23900). In *Arabidopsis*, this region covers about a 60-kb DNA fragment, much less than

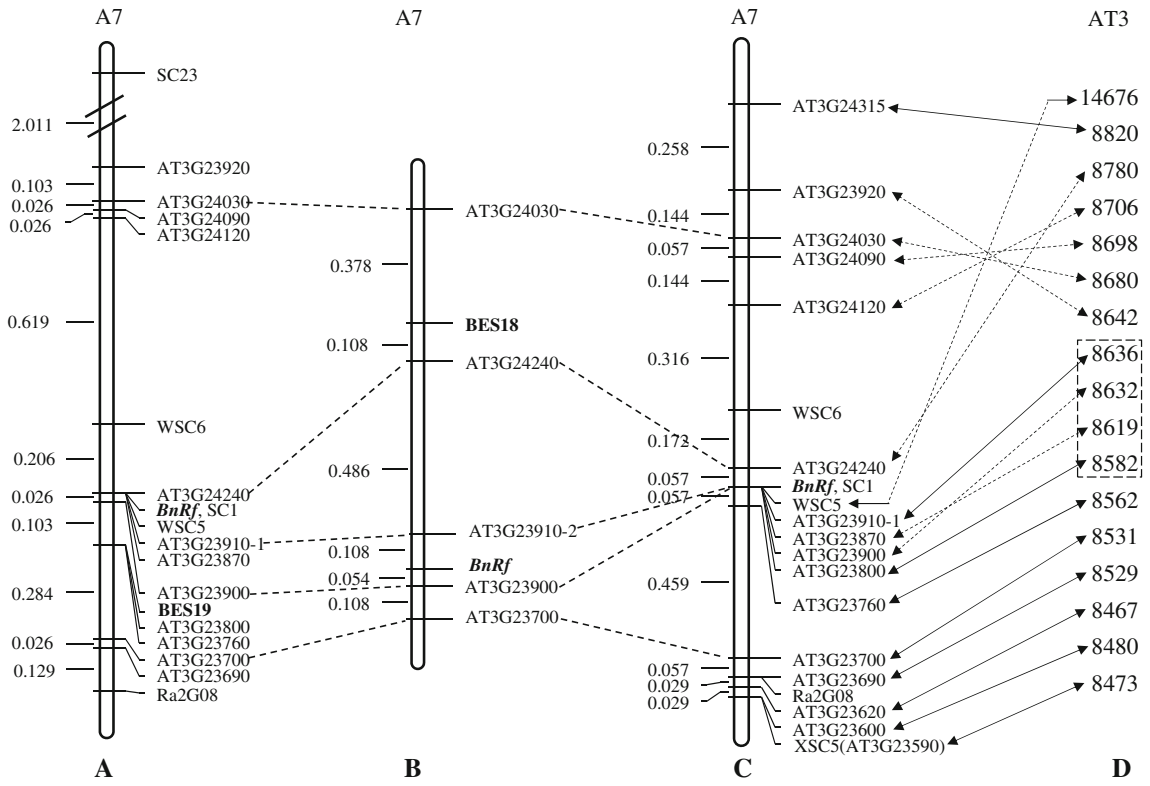

Fig. 2 High-resolution genetic maps around *BnRf* and their comparison with *Arabidopsis*. **A** The genetic map from Population I; **B** the genetic map from Population III (Dong et al., unpublished data); **C** the genetic map from Population II; **D** the physical position of *Arabidopsis* fragments homologous to the *BnRf*-linked markers. *Dotted lines* between **A** and **C** indicate the polymorphic markers shared by all three populations. *Dotted lines with double arrowhead* between **C** and **D** depict the inversion or translocation of orthologs between *B. napus* and *Arabidopsis*, while the *solid line with double arrowhead* indicates the orthologs with good microcollinearity between them. The *dashed line box* emphasizes the *Arabidopsis* orthologs related to the closest flanking and cosegregated markers. Genetic distances are shown in cM for *B. napus* and physical distances are shown in kb for *Arabidopsis* genes according to the TAIR database

the mean insert size (145 kb) of the JBnB BAC library (Rana et al. 2004).

This analysis convinced us to use the cosegregated marker AT3G23870 to screen the JBnB BAC library as a chromosome landing strategy. Sixteen positive clones were identified by Southern blotting with a high stringency. From them, seven overlapping clones (JBnB003N05, JBnB026K23, JBnB089D05, JBnB100O04, JBnB134D11, JBnB148D20 and JBnB174I06) were further verified by the five co-dominant *BnRf*-linked InDel markers listed in Table 2 (except for AT3G24030 and AT3G24120, which are regarded to be too far from AT3G23870), forming a contig covering the *BnRf* ^{c} allele. In this contig, JBnB089D05 covering both flanking markers (AT3G23900 and AT3G24240) was regarded as one of the candidate BAC clones. This conclusion was further proved by the fact that two markers (BES18 and BES19, Dong et al., unpublished data), which individually originated from end sequences of JBnB089D05, can be mapped outside of *BnRf* either in Population I or in Population III (Fig. 2a, c).

A physical map around the *BnRf* ^{c} region

From the *Arabidopsis* orthologs, it seemed that the physical gap between the closest flanking markers AT3G23900 and AT3G23910-2 may be relatively small in *B. napus*. Hence, we adopted a long-fragment PCR method to isolate the candidate sequence from the BAC clone JBnB089D05 instead of shotgun sequencing. In addition, because the marker AT3G23870 was cosegregated with *BnRf*, it could be used as a bridge between AT3G23900 and AT3G23910-2. PCR amplification with two primer combinations individually designed from the previously described three marker sequences generated two fragments. One was from AT3G23900 to AT3G23870 with a size of about 9.5 kb, and the other was from AT3G23870 to AT3G23910-2 with a size of 4.3 kb. Sequencing and assembling of these two fragments gave rise to the complete 13.8-kb sequence between AT3G23900 and AT3G23910-2 (Fig. 3a).

Results of gene prediction for the 13.8-kb fragment varied between FGENSH and GlimmerHMM. In this

Table 2 Details of the markers from high-resolution genetic maps

Markers	Primer sequences(5′–3′)[a]	Corresponding EST or GSS	Size[b] (bp)	Polymorphism	E value[c]	Position[d]
AT3G24315	CCCTCAGCTCCCTTCTGATGA CTGTTCCACCACCAAGGAGAA	Panjabi et al. (2008)	424	Dominant	2e−33	4,102,087–4,102,536
AT3G23920	AGGTAGAACACCCGTGCAAT GTCTTGCTCGGGGTATGAAG	AF319168	274	Dominant	1e−149	4,202,805–4,203,079
AT3G24030	TTGAGCTTAAACCTAGCGTGA GACTTGGCAGCTTCTACGG	EX101924	235/209	Co-dominant	1e−115	4,284,361–4,284,569
AT3G24090	CTAGATTCCTCGGCTGGTCA AGCAATAGAGGTTCCGCAAG	EX062180	230	Dominant	1e−127	4,288,160–4,288,389
AT3G24120	TAGCAGCTTGCTCGTCAAAG GCTCAGATGGAAGTCACAGAAG	DX904761	209/220	Co-dominant	6e−57	4,309,652–4,309,861
WSC6	TCTTTGCATCTAGATTATCCCCATA CATATGAGCTTTGGTGATTTGTG	Xu et al. (2009)	937	Dominant	0	4,459,842–4,460,687
BES18	CGGCTCTGGATCAAGGAGAT AGTGAAAAATAACGTGTGGTTGG	FP013234	200	Dominant	1e−109	**4,463,861–4,464,060**
AT3G24240	CGTCGCTGGATCTTATGGTT ACAGAAACGCACACACATCA	BZ481819	254/236	Co-dominant	2e−38	4,484,731–4,484,939
SC1	TTAACTGCGATATAAAGGAGG CTGCAG CAGGTAGAGGGA	Xie et al. (2008)	173	Dominant	–	–
WSC5	GTCGTACGTTGCTCGTCAAG CATATGAGCTAAGACTTCTCCACTG	Xu et al. (2009)	559	Dominant	2e−92	3,288,875–3,289,109
AT3G23910-1	AGACATCATTGACGCTGCTG TTGTGTCTAGCAATGCAGAGTG	EV043067	506	Dominant	1e−121	**4,514819–4,515,434**
AT3G23910-2	GAGACATCATTGACGCTGCT CTCAAAGGGTACATAAAACTC	EV043067	256	Dominant	1e−139	**4,515069–4,515,434**
AT3G23870	TGAAGGAGGGCATGGATACT GCTCCCAAAGGTGTGACTAGA	EE484347	217	Dominant	3e−99	**4,519,123–4,519,341**
AT3G23900	TTGGCATTGTCTGATTGAT GCGTTGCTATCATGGGAAGT	CX277970	194/205	Co-dominant	2e−47	**4,532,414–4,532,628**
BES19	CCGGACGTGTTAGTTTTGTG GGAGTATGGACGGTGGAAAA	FP013235	224	Dominant	1e−54	**4,591,449–4,590,894**
AT3G23800	AGGAGGCAGTGGGTTTCTCT GTCTCATGCCGAGGTTGGTA	EX104994	232	Dominant	4e−61	4,606,629–4,606,859
AT3G23760	CCAAGAGACCTGTTCAATGG TGAAAACGCAGTCTCAAATTCT	EV214979	312/307	Co-dominant	1e−105	4,636,679–4,636,991
AT3G23700	TGGTCGGTTCAACACTTCCT CGGTAAATACATCCCCAACG	EX123653	212/214	Co-dominant	7e−93	4,675,906–4,676,077
AT3G23690	CCAGATCCTGAAGCAAGTCA TCAGGCAACTGATAGCCACA	EV124342	153/166	Co-dominant	4e−54	4,679,169–4,679,337
AT3g23620	GAACTCCCAAGACCCACAAA GTAGCGCTTGTCTTCGTTCC	EX042534	203	Dominant	1e−91	4,802,977–4,803,183
AT3G23600	GGAGATCCTTTTGATGCCTCT TGGCTTCAATTACTGGCTTTG	EX125964	241	Dominant	5e−39	4,811,397–4,811,642
XSC5	CTCATGATGGCTCGTCTGAT GAATTCACCAAGGACCTGCA	Xiao et al. (2008)	215	Dominant	1e−106	4,823,602–4,823,818

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

[b] The sequences of the four dominant markers XSC5, AT3G23600, AT3G23620 and AT3G24315 were isolated from GosA, while that of AT3G23910-2 was isolated from DH206. Sequences of all the other markers were obtained from 9012A (dominant) or 9012A and T45, respectively (co-dominant)

[c] BLASTN analysis between the marker sequences (from 9012A or GosA) and the *B. rapa* genome sequences

[d] Position of the *B. rapa* homologs on chromosome A7, and '–' indicates that no homolog was identified on *B. rapa* A7. The closest flanking markers and BAC end-derived markers as well as their orthologs are shown in bold type

circumstance, sequence comparison between this candidate fragment and the *Arabidopsis* genome can be used as a reference. As shown in Supplementary Fig. 1, high sequence conservation was observed in the coding regions of gene *AT3G23870* (87% sequence similarity) and *AT3G23880* (87% sequence similarity). Consistent results were also displayed for the gene *AT3G23900* and *AT3G23910*, although the homologous *B. napus* sequences were not complete due to their respective distribution on each end of the fragment. Comparatively, GlimmerHMM provided a much closer result with similarity analysis between *B. napus* and *Arabidopsis* than did FGENSH; therefore, the former's prediction was adopted in this research (Fig. 3b). Of the five ORFs predicted by GlimmerHMM, *G1* and *G5* are not full-length coding sequences (CDS) individually homologous to gene *AT3G23900* and *AT3G23910*. However, these two ORFs cannot be excluded as the candidate gene of *BnRf*, because their functional regions are probably located in the 13.8-kb region even though the recombinant events had been detected inside both of these ORFs. *G3* and *G4* are the orthologs to gene *AT3G23870* and *AT3G23880*, respectively. *G2* cannot identify a homolog whether by CDS or translated protein sequence, but it is supported by a *B. napus* EST (CN735355) just like the other four ORFs (data not shown). In *Arabidopsis*, *AT3G23870* encodes a hypothetical protein without function annotation, but contains a conserved domain with undefined function (DUF803), which is possibly involved in the transportation of magnesium. *AT3G23880* is a member of the F-box gene family, while its function remains unknown. AT3G23900 is an RNA recognition motif (RRM)-containing protein, while AT3G23910 is highly associated with an RNA-directed DNA polymerase (reverse transcriptase). Presently, it is still difficult to predict which of the five genes should play a key role in development of the male gametophyte; however, we would focus on *G3* and *G4* first, because the other three genes seem to have a much more universal function in *Arabidopsis*.

Comparison of physical maps around *BnRf* between *B. napus* and *B. rapa*

Because *BnRf* had been mapped to the chromosome A7 of *B. napus* (Xiao et al. 2008), we compared the physical map around *BnRf* with the *B. rapa* genome. As shown in Table 2, except for one SCAR markers (SC1), all other markers from AT3G24315 to XSC5 can search highly conserved orthologs in *B. rapa*. Moreover, all these homologs, with the exception of the homolog of WSC5, are physically distributed in the same order as the markers in a 723-kb region from 4,102,087 to 4,823,818 bp in the A7 chromosome. According to the positions of orthologs corresponding to the closest flanking markers (AT3G23900 and AT3G910-2), the *BnRf* locus can be physically delimited to about a 17.8-kb fragment from 4,484,731 to

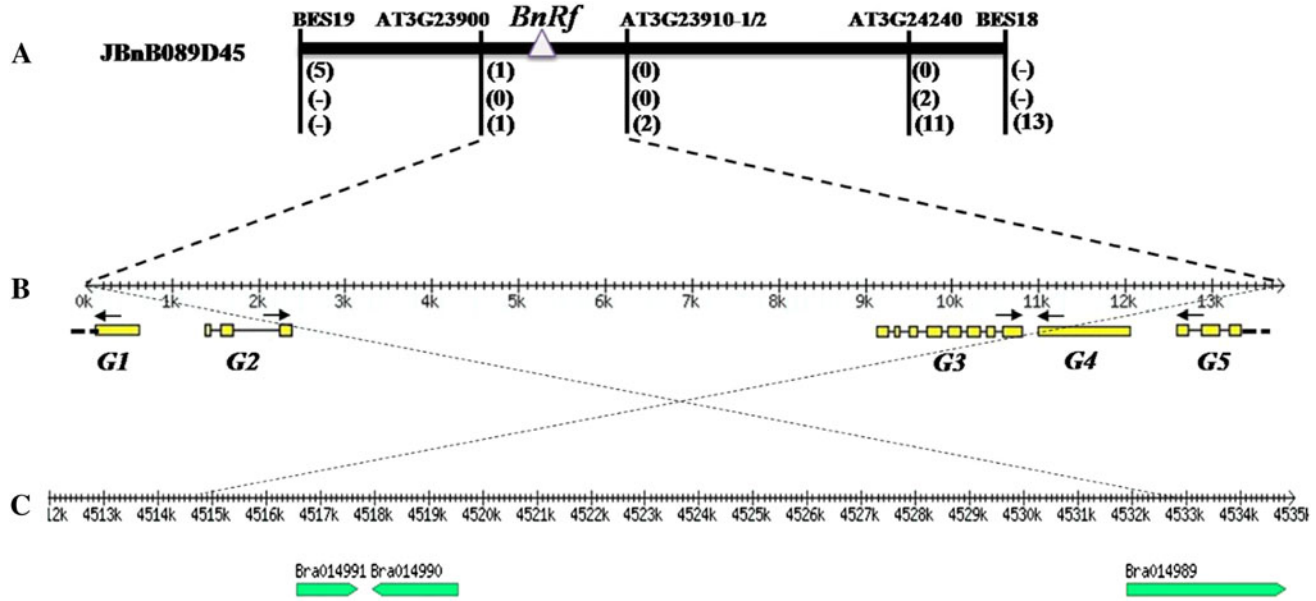

Fig. 3 Physical map around the *BnRf* locus and comparison with *B. rapa* homolog. **a** A schematic representation of the BAC clone JBnB089D05 and the distribution of the five *BnRf*-associated markers on it. Recombination events respective to each marker in Populations I, II and III are shown individually by *numbers in the brackets* from up to down. '–' means the marker does not show polymorphism in a given population. **b** ORFs from the candidate region were predicted by GlimmerHMM with the direction marked by *a line with a single arrowhead*. **c** Gene annotation from the *B. rapa* orthologous region (http://brassicadb.org/cgi-bin/gbrowse/cbgdb11). The *dotted lines* between **b** and **c** delimit the borders of *B. rapa* homolog, which is shown reversely to the BAC

4,534,214 bp. Although this fragment is almost 4 kb larger than the candidate region in *B. napus*, the two regions share high sequence conservation. In *B. rapa*, however, this fragment is annotated with only three putative genes individually homologous to *G1*, *G3* and *G4* (http://brassicadb.org/cgi-bin/gbrowse/cbgdb11), while the homologs to *G2* and *G5* are not predicted (Fig. 3c). The arrangement of the four genes in *B. rapa* is completely consistent with that in *B. napus*. These results emphasize that the physical map and gene prediction around the *BnRf* ^{c} allele are plausible.

Discussion

In the present study, we reported our work on high-resolution genetic mapping of one male-sterile locus related to the RGMS line 9012AB, and subsequent physical mapping of its one allele. First, we integrated the previously reported *BnRf*-linked markers, public InDel markers and SSR markers (also including three developed here) into one genetic map. Comparative mapping with *Arabidopsis* led to the identification of a novel conserved AK block where the *BnRf* locus evolved. Second, the development of massive InDel markers from the F block enabled us to construct three high-through local genetic maps around the target locus based on three large backcross populations. Finally, we successfully anchored the *BnRf* ^{c} alele to a BAC contig and eventually physically delimited it to a 13.8-kb fragment of a clone, including five putative genes. Additionally, comparison of the physical map around *BnRf* with the *B. rapa* genome further supported our mapping results and candidate analysis. We believe our study has made a solid foundation for future isolation of the *BnRf* locus.

Comparative genomic analysis between *Arabidopsis* and *B. napus* identified 21 syntenic blocks shared by the genomes of these two species that could be duplicated and reshuffled to represent 90% of the *Brassica napus* genome (Parkin et al. 2005). Comparative linkage mapping and chromosome painting in the close relatives of *Arabidopsis* indicated that the introduction of AK would facilitate comparative genomics in the *Brassicaceae* (Boivin et al. 2004; Lysak et al. 2006). Subsequently, Schranz et al. (2006) unified the concept of AK with the 21 syntenic blocks in *Brassica* and updated the AK with 24 conserved genomic blocks (A–X). In the present study, we identified a novel block F encompassing the *BnRf* locus on *B. napus* chromosome A7, which was not previously detected on this chromosome (Schranz et al. 2006; Wang et al. 2011b). A previous report suggested that the top end of the *B. napus* A7 chromosome was composed of three continuous conserved blocks (H–G–B) from the AK (Schranz et al. 2006). From our discovery of the novel F block, we concluded that this chromosomal region should evolve from four conserved ancestral blocks (H–G–F–B) (Fig. 1). Although block G was not identified in our study, probably due to the absence of markers located in this region, it has been commonly identified in the A7 chromosome in *Brassica* (Parkin et al. 2005; Schranz et al. 2006; Panjabi et al. 2008). In addition, our results also indicate that this novel block F is possibly the product of an extra segment duplication after the triplication process in the *Brassica* diploid species, because it has been found in chromosome A1, A3, A5, C1, C3 and C5 in *B. napus* (Parkin et al. 2005). Furthermore, the existence of this F block in the *B. rapa* genome A7 suggests that it was evolutionarily generated before formation of the amphidiploid *B. napus*.

Map-based cloning of a mutant gene may become problematic and tedious without the presence of high-density molecular markers around the target gene. Although the *BnRf* locus was finely mapped with a lot of SCAR markers (Xu et al. 2009), information about their related physical positions remains unknown. Here, the identification of a conserved region promised a possibility to increase the marker density around *BnRf*. Most of the InDel primers developed from block F were primarily anchored to different potential exons for discovering the possible intron polymorphism, due to the fact that introns are more variable than exons (Yang et al. 2007; Park et al. 2010). As expected, this strategy was considered to be relatively effective in the present study, because 15 newly developed InDel markers allowed us to successfully construct three high-resolution maps near the *BnRf* locus. More importantly, comparison between the InDel markers and their homologs in *Arabidopsis* allowed estimation of the physical positions of these markers in *B. napus*. Identification of the F block encompassing the *BnRf* locus and utilization of the microcollinearity between *B. napus* and *Arabidopsis* played a central role in the eventual physical mapping of the target gene. Although release of the *B. rapa* genomic sequence would reduce the importance of this step by directly providing the reference sequence of the subgenome A of *B. napus*, it is still an effective approach for gene mapping in the subgenome C in *Brassica*, as well as for other species without a reference genome sequence.

The establishment of high-resolution genetic and physical maps around *BnRf* provided an insight for investigation of regional chromosomal evolution. Comparative mapping between *B. napus* and *Arabidopsis* revealed perfect microcolinearity around *BnRf* in block F, although it was interrupted by several chromosomal rearrangement events after their divergence from a common ancestor (Fig. 2). These events included one segment inversion from *AT3G23910* to *AT3G24315*, with one border located between *AT3G23910* and *AT3G23920* and the other between *AT3G24240* and *AT3G24315*. We speculated that this inversion possibly resulted from the fusion and

subsequent breakage of a chromosomal loop between *AT3G23910* and *AT3G24255*, because these two genes were highly conserved paralogs. The other three events belonged to small fragment translocations such as the movement of *AT3G23900* to the outside of *AT3G23870*, the translocation of the transposable element *AT3G42556* (WSC5), and the translocation of marker SC1. Comparative sequence analysis between *B. napus* and *B. rapa* confirmed these major chromosomal evolution events around the *BnRf* locus, including the inversion and translocations of *AT3G23900* and *AT3G42556*. Thus, these chromosomal evolutions should have occurred in the diploid ancestor of *B. rapa*. In addition, alterations in small fragments also take place after the divergence between *B. rapa* and *B. napus*, such as insertion of marker SC1 and size variation in the *BnRf* candidate region between *B. rapa* and *B. napus*. The transposable elements were hypothesized to be responsible for these divergences, because they have been revealed to be the main reason for the difference in physical size between *Arabidopsis* and *B. rapa* (Wang et al. 2011a, b).

The other male-sterile locus *BnMs3* in 9012AB has been cloned by Dun et al. (2011). Molecular characterization suggested that it was involved in protein transportation of the inner chloroplast stroma membrane (Dun et al. 2011). As speculated from the modified digenic hereditary model of sterility control in 9012AB, *BnRf* most likely functions in a redundant way as does *BnMs3* in developmental control of microgametogenesis in *B. napus*. However, according to the five predicted genes located in the candidate region of the BAC clone, it remains difficult to establish their possible relationships with each other and their speculated gene functions. So in the next step, we would attempt to isolate the target sequences from different inbred lines that differ in the *BnRf* allele. Also, we will confirm the candidate gene by transforming the sterile allele ($BnRf^{b}$) to the temporary maintainers or the restorer allele ($BnRf^{a}$) to the male-sterile plants, together with haplotype analysis among these inbred lines.

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