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ORIGINAL PAPER

Fine mapping and candidate gene analysis of the nuclear restorer gene *Rfp* for *pol* CMS in rapeseed (*Brassica napus* L.)

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Abstract The Polima (*pol*) system of cytoplasmic male sterility (CMS) in rapeseed is widely used in China for commercial hybrid seed production. Genetic studies have shown that its fertility restorer gene (*Rfp*) is monogenic dominant. For fine mapping of the *Rfp* gene, a near isogenic line comprising 3,662 individuals of $BC_{14}F_1$ generation segregating for the *Rfp* gene was created. Based on the sequences of two SCAR markers, SCAP0612ST and SCAP0612EM2, developed by Zhao et al. (Genes Genom 30(3):191–196, 2008) and the synteny region of *Brassica napus* and other *Brassica* species, 13 markers strongly linked with the *Rfp* gene were identified. By integrating three of these markers to the published linkage map, the *Rfp* gene was mapped on linkage group N9 of *B. napus*. Using these markers, the *Rfp* locus was narrowed down to a 29.2-kb genomic region of *Brassica rapa*. Seven open reading frames (ORFs) were predicted in the target region, of these, ORF2, encoding a PPR protein, was the most likely candidate gene of *Rfp*. These results lay a solid foundation for map-based cloning of the *Rfp* gene and will be helpful for marker-assisted selection of elite CMS restorer lines.

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Abbreviations

BC	Backcross
CMS	Cytoplasmic male sterility
NIL	Near isogenic line
ORF	Open reading frame
MAS	Marker-assisted selection
DH	Doubled haploid
PPR	Pentatricopeptide repeat

Introduction

Cytoplasmic male sterility (CMS) is a widespread maternally inherited trait in higher plants that results from the expression of novel open reading frames (ORFs) located in the mitochondrial genome (Hanson and Bentolila 2004). In many instances, specific dominant nuclear genes, termed restorers of fertility (*Rf*), have been identified that can suppress the male sterility phenotype and restore fertility in plants carrying CMS mitochondrial genomes. In general, such nuclear restorer genes are specific for a particular form of CMS and function by down-regulating the expression of the corresponding novel, CMS-associated ORF (Chase 2007; Schmitz-Linneweber and Small 2008). CMS/*Rf* systems thus provide an ideal model for studying genetic interactions and cooperative functions between the nuclear and mitochondrial genomes in plants, in addition to being a practical tool for facilitating hybrid seed production.

There are four main sources of CMS in *B. napus*: *pol*, *nap*, *ogu*, and *tour* cytoplasms. Currently, *pol* CMS is most extensively used for the production of hybrid seeds in China. The *pol* cytoplasm from the cultivar 'Polima' (Fu 1981) confers complete male sterility to many *B. napus*

cultivars and partial sterility to the rest (Fan et al. 1986). *Pol* CMS system can be used both in a three-line system (*pol* CMS sterile line, maintainer line and restorer line) and a two-line system (*pol* temperature-sensitive cytoplasmic male sterility sterile line and restorer line) in order to produce commercial hybrid rapeseed (Fu et al. 1990; Yang et al. 1995). The restorer line plays an important role in both of the pollination control systems and for this reason breeders pay particular attention to the restorer line during the application of *pol* CMS.

Genetic analyses indicate that the *Rfp* gene is dominant and monogenic, controlling pollen fertility (Yang et al. 1996). To date, most of the studies have focused primarily on screening or developing molecular markers tightly linked to the *Rfp* locus, and a number of molecular markers have been reported. Jean et al. (1997) identified ten RFLP and one RAPD molecular markers linked to the *Rfp* gene, four of which were mapped to linkage group 18 reported by Landry et al. (1991). Based on one RFLP marker identified by Jean et al. (1997), Formanová et al. (2006) mapped the *Rfp* gene in *B. rapa*; a syntenic interval of 4.3 Mb on chromosome 1 of *Arabidopsis* genome was also identified after sequence alignment, and then the *Rfp* gene was found to be localized to a chromosomal region equivalent to a 115-kb segment of *Arabidopsis* chromosome 1 (Formanová et al. 2010). In our laboratory, a number of AFLP, RAPD and SCAR markers linked to the *Rfp* gene have been identified. Wang et al. (2000) discovered two RAPD markers and converted these two markers into SCAR markers. Liu et al. (2007) also identified an AFLP marker and a RAPD marker. Zeng et al. (2009) found a total of 13 AFLP markers, one of which was mapped on the linkage group N18 in the doubled haploid (DH) population derived from a cross between Quantum and No. 2127-17 (Chen et al. 2007). Moreover, Li et al. (2011) developed an SSR marker strongly linked to the *Rfp* locus from six *B. rapa* BAC clone sequences corresponding to the target region. Nevertheless, much still remains unknown about the mechanism of fertility restoration conferred by the *pol* CMS system. Thus, cloning and characterization of the fertility restorer gene *Rfp* for *pol* CMS will be particularly significant in marker-assisted selection (MAS) of elite CMS restorer lines and will provide more insights into the mechanism for restoring fertility found in CMS plants.

Comparative mapping between model plants and major crops provides a new strategy for exploiting the public information resource of model plants. *Brassica* and *Arabidopsis thaliana*, which share a recent common ancestry, are both members of the *Brassicaceae* family (Yang et al. 1999). As an increasingly greater number of *B. rapa*, *B. oleracea* and *B. napus* sequence information is available on public database, especially with the completion of *B. rapa* genome sequencing (Wang et al. 2011; http://brassicadb.org/brad/), these sequence information and genomic resources can then be comparatively mapped in *A. thaliana* or *B. rapa* genome, and thus provide gene location in *B. napus* and other *Brassica* species. Based on the collinearity between radish and *Arabidopsis*, Brown et al. (2003) and Desloire et al. (2003) cloned the *Rfo* and the *Rfk* genes of radish CMS Ogura (*ogu*) and Kosena (*kos*) using a map-based approach. Yi et al. (2010) cloned the *BnMs1* and *BnMs2*, and Dun et al. (2011) and Li et al. (2012) the *BnMs3* by map-based cloning approach while relying on microsynteny between *B. napus* and *Arabidopsis.*

Most of the *Rf* genes that have been identified for CMS in various plant species have been found to encode pentatricopeptide repeat (PPR) proteins (Bentolila et al. 2002; Brown et al. 2003; Desloire et al. 2003; Akagi et al. 2004; Komori et al. 2004). Such proteins are characterized by a tandem array of repeats of degenerate 35-amino acid motifs and are thought to specify the sites of transcript modification events, such as nuclease processing and editing by binding to specific sequences on RNA molecules (Schmitz-Linneweber and Small 2008). It thus seems likely that *Rfs* will encode the related PPR proteins which also down-regulate the expression of their corresponding CMS-associated ORFs by binding to and mediating the processing events on a different set of mitochondrial transcripts.

The objectives of this study were to develop additional molecular markers linked to the *Rfp* gene, fine map the *Rfp* gene by using the collinearity of those markers between *B. napus* and other *Brassica* species and analyze the candidate genes of *Rfp* for future functional validation. This information will help to achieve map-based cloning of the *Rfp* gene.

Materials and methods

Plant materials and fertility scoring

The *pol* CMS line, 1141A, was chosen as the female parent to cross with Huayehui, a *pol* CMS restorer line, and a maintainer line named Wu108 was used as a recurrent parent to backcross with the F_1 from 1141A × Huayehui in this study. The fertile plants among progeny of the backcross (BC) population were backcrossed 14 times with Wu108 until a near isogenic line (NIL) population was obtained. The genotypes of 1141A, Wu108, Huayehui and NIL population were identified as S(*rfprfp*), N(*rfprfp*), N(*RfpRfp*) and 1/2 S(*Rfprfp*) +1/2 S(*rfprfp*), respectively.

Individual fertility in the NIL was assessed by observing a minimum of three flowers per plant during the flowering period. The fertility of each individual was checked again 10 days later. Fertility was classified to seven levels as described by Yang and Fu (1991). According to this method, the fifth and sixth levels are regarded as fertile, while zero to fourth levels are considered as sterile.

Isolation of genomic DNA and construction of DNA bulks

Young leaves form each individual of the NIL population were collected for genomic DNA extraction. Genomic DNA was extracted each plant according to a modified version of the cetyltrimethyl ammonium bromide (CTAB) procedure described by Doyle and Doyle (1987). Fertile bulks (BF) and sterile bulks (BS) were constructed by pooling equal quantities of DNA from 12 fertile and 12 sterile individuals, selected randomly from fertile and sterile segregants.

Development of locus-specific PCR primers from *Brassica* species syntenic region

Locus-specific markers were developed based on the sequences of two SCAR markers, SCAP0612ST and SCAP0612EM2, developed by Zhao et al. (2008) and Scaffold sequence of *B. rapa* and Scaffold sequence of *B. oleracea* (Wang, personal communication) at the region syntenic to the DNA fragment around the *Rfp* locus. Specific PCR primers were designed by software Primer Premier 5.0 (http://www.premierbiosoft.com/primerdesign/). PCR amplification from the genomic DNA of the two DNA bulks 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 0.2 μmol each of primer. Annealing temperature of SCAR primers was optimized in Applied Biosystems Veriti^{TM} 96 Thermal Cycler (Life technologies, 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, 45 s at 72 °C; and a final cycle of 10 min at 72 °C. The PCR products were analyzed on 6 % polyacrylamide gel or 1 % agarose gel. The polymorphic markers were considered putatively linked to the target gene and subsequently confirmed by the NIL segregation population.

Linkage analysis and map construction

The molecular marker data and corresponding individual's fertility/sterility trait in the NIL were analyzed using MAPMAKER 3.0 software (Lander et al. 1987). An LOD score of 3.0 was used as the lower limit for map construction. The recombination values were converted into genetic map distance (cM) by means of Kosambi mapping function (Kosambi 1944). One DH population derived from the cross HZ396 × Y106 (Zhang et al. 2010), available in our laboratory, was used to determine the map location of the *Rfp* gene in the published *B. napus* reference map.

Candidate gene identification and sequence analysis

The online system FGENESH (http://www.softberry.com) was used to identify predicted ORFs in the delimited gene region. Their putative functions were bioinformatically analyzed by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The speculated candidate gene was amplified from Huayehui and 1141A. Specific primers were designed according to the Scaffold of *B. rapa*. PCR products were purified using a DNA Gel Extraction Kit (Tiangen, Beijing, China) for TA cloning. Purified PCR products were introduced into the pMD18-T Simple Vector (Takara, Dalian, China) and transformed into *E. coli* strain DH5α. The recombinant plasmids were sequenced by BGI (Beijing Genomics Institute, China). Sequence alignment was performed with the BLAST network service (National Center for Biotechnology Information, NCBI) and ClustalW (European Bioinformatics Institute, EBI).

Results

Genetic analysis of the *Rfp* gene

An NIL population, comprised of 3,662 individuals, was successfully constructed after the fertile plants among progeny of the BC population were backcrossed with Wu108 14 times. Phenotypic evaluation revealed the segregation of 1,835 sterile individuals and 1,827 fertile individuals. The χ^2 test showed that the segregation ratio of fertility and sterility was consistent with the expected ratio of 1:1($\chi^2 = 0.01$ and $P > 0.05$), confirming the deduction that the parent line carried only one *Rfp* locus (Zhao et al. 2008; Li et al. 2011).

SSR and SCAR molecular marker developing

The sequences of two SCAR markers, SCAP0612ST and SCAP0612EM2, obtained from Zhao et al. (2008) were compared with the sequence assembly of *B. rapa* and *B. oleracea* (http://brassicadb.org/brad). Both of them were found to have highly conserved homologs on the Scaffold of *B. rapa* and *B. oleracea*. Based on the sequence information of these two Scaffolds, a total of 86 SSR and 115 SCAR primer pairs were designed. Of them, four SSR markers and nine SCAR markers showed polymorphism between BF and BS (Table 1). These markers also showed polymorphism among the 12 fertile and 12 sterile individuals selected randomly from the NIL population.

Linkage analysis and fine mapping

According to the primary analysis of 470 individuals from the NIL population, two markers, Kbr (Li et al. 2011) and

Table 1 Details of the molecular markers linked to the *Rfp* gene

Marker	Primer type	Direction	Sequences (5′–3′)	Polymorphism	Production size (bp)	Annealing temp. (°C)
Os20	SSR	Forward	CGCTAAACAGCGACTCCAAT	Co-dominant	426	56
		Reverse	TGCAAATAAATCTTGTACCACCA			
Os31	SSR	Forward	GCAGTCTAGGGCTGATGAGG	Dominant	338	56
		Reverse	CTGCTCCCCAGTTATCAACC			
Os36	SSR	Forward	AAGCCCTAAGCCTTCATCGT	Co-dominant	179	56
		Reverse	CGGTGCTCAAGAAACGATCT			
Brs06	SSR	Forward	ATATGGAGAGTGCCGCTACG	Dominant	242	56
		Reverse	TGTCATTGGCTTCTGCTGTC			
BrIP14	SCAR	Forward	TGCTCCAACATTGCTTTCTG	Co-dominant	491	63
		Reverse	TTCCTGCCGATCCTACCG			
BrIP30	SCAR	Forward	GGAATGAGATGTGTGTGGCAG	Dominant	348	60
		Reverse	TTACCAAGTTTTGTCATCATCCA			
BrIP33	SCAR	Forward	AATCATTTGGCGATTTCCAC	Co-dominant	408	58
		Reverse	TTAGCTTCGGCTCATTGTCAT			
BrIP35	SCAR	Forward	ATCACTCCCGCCGATAGAAT	Co-dominant	509	56
		Reverse	GTGAACCCGTCTATCATCGTG			
BrIP43	SCAR	Forward	TTCCTCTCCACTCCAACAAGC	Dominant	873	58
		Reverse	TGACCTTGCGAGTTCCATCT			
BrIP53	SCAR	Forward	CGTTTGGACAAGTTTTCACG	Dominant	166	59
		Reverse	CTTTGAACTATTGCCGGTGA			
BrSC08	SCAR	Forward	ACTTGGTTTGGTGCTATCGT	Dominant	373	57
		Reverse	GCTGAATCGTCTGCTCCCT			
BrSC15	SCAR	Forward	TAGCCGATTGAATGACAGCA	Co-dominant	337	60
		Reverse	GTCTAAGCAAAACCTGCCACT			
BrSC47	SCAR	Forward	CTCTCCTCACTTCTCCGATT	Dominant	271	62
		Reverse	AAATCATTCCTTTCCTACGG			

BrIP14, were identified as the outside markers flanking the *Rfp* gene. Subsequently, they were used to assay other individuals of the whole NIL segregation population. A total of 47 recombination events for Kbr and 8 for BrIP14 were detected. These recombinants were further characterized by using other markers. The segregating data of the 14 markers and the fertile trait were used to construct the linkage map. Linkage analysis showed that all the markers spanned a genetic region of 1.39 cM. Eight of the markers were located on one side of the *Rfp* gene, three were on the other side, and three of them (Os31, BrSC08, BrSC15) were co-segregated with the *Rfp* gene. The linkage map and genetic distances for the *Rfp* gene and 14 markers are presented in Fig. 1a. The order of these markers was consistent with the physical distance of their homologs in *B. rapa* (data not shown). This suggested that the syntenic region was well collinear between *B. npaus* and *B. rapa*. Accordingly, we concluded that the *Rfp* gene was located in 29.2-kb genomic region of *B. rapa*. Subsequently, the corresponding region of *B. napus* was sequenced through the overlapping PCR. The result confirmed that this region was strongly collinear between *B. npaus* and *B. rapa*.

To determine the location of the *Rfp* gene in a published *B. napus* genetic map, some of the markers linked to the *Rfp* gene were screened between the parents of one DH populations derived from the cross HZ396 × Y106. Three SSR markers designated Os20, Os31 and Os36 showed polymorphic bands between the two parents HZ396 and Y106, and they were used to screen the population of 140 DH lines. As a result, these three SSR makers were all mapped on linkage group N9 (Fig. 1b). This finding confirmed the assumption that the *Rfp* gene exists in the A genome of *B. napus* (Yang and Fu 1991).

Candidate gene identification and sequence analysis

A total of seven ORFs were predicted in this 29.2-kb target region. Six of them could highly match with *Arabidopsis* proteins by blastp except ORF5 (Altschul et al. 1990) (Table 2; Fig. 2). Of these, ORF2 was the most likely gene

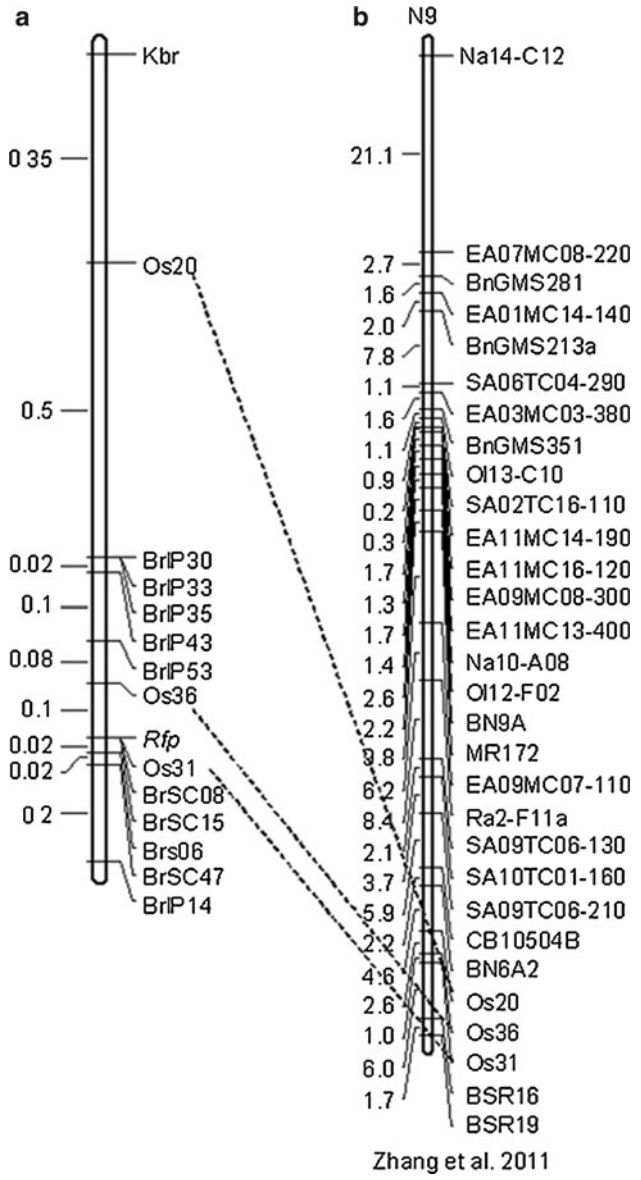

Fig. 1 **a** The genetic linkage map of the *Rfp* gene and associated molecular markers. Recombination distances are indicated on the *left side* of linkage group in centiMorgans (cM), and the marker names are shown on the *right side*, **b** The linkage map of N9 showing the location of SSR markers Os20, Os31 and Os36. The linkage map was constructed from a DH population developed from the cross HZ396 × Y106. *Dotted lines* indicate common markers

related to fertility restoration, which encoded a PPR protein. To identify ORF2 as the *Rfp*, we cloned and sequenced it in both Huayehui and 1141A. DNA sequencing analysis revealed that there were some nucleotide polymorphisms between Huayehui and 1141A in the coding region (data not shown). Subsequently, based on the nucleotide polymorphisms, a pair of primers SCP4 was designed to amplify the *ORF2* allele of the fertile parent, whose sequences were as follows—F: GGCTAATGGATG TCAACCCA; R: CTTCATCTAAGCGCTTGTCG.

Table 2 Gene annotation of the 29.2-kb DNA fragment

Number	Homologous *Arabidopsis* gene and *E* value	Gene annotation
ORF1	AT1G13030, 1e−34	Sphere organelles protein-related
ORF2	AT1G12300, 4e−121	PPR containing protein
ORF3	AT1G 13020, 1e−65	Eukaryotic translation initiation factor
ORF4	AT1G 12980, 3e−45	Enhancer of shoot regeneration 1
ORF5	AT3G 62330, 0.31	Zinc knuckle (CCHC-type) family protein
ORF6	AT1G 12970, 5e−170	Leucine-rich repeat family protein
ORF7	AT1G 12950, 2e−73	MATE efflux family protein

Genotype analysis for SCP4 among the NIL population revealed that the SCP4 genotypes exhibited co-segregation with phenotypes. The segregation in some NIL individuals and the polymorphism between BF and BS are shown in Fig. 3. This candidate gene was further analyzed with the subcellular localization prediction programs TargetP v1.1 (Emanuelsson et al. 2000) (http://www.cbs.dtu.dk/services/TargetP) and MitoProt II (Claros and Vincens 1996) (http://ihg.gsf.de/ihg/mitoprot.html). The results indicated that ORF2 was targeted to mitochondria.

Discussion

The highly duplicated nature of the *Brassica* genomes poses a significant challenge to the isolation and identification of genes through positional cloning approaches. *Brassica napus* is an amphidiploid species comprising homologous A and C genomes, which are thought to have evolved through a common hexaploid ancestor (Osborn et al. 1997). Previous studies indicated that the linkage groups N9 and N18 of *B. napus* are primarily homologous (Parkin et al. 2005). The *Rfp* gene has previously been mapped to *B. napus* linkage group N18 (Landry et al. 1991) using RFLP markers (Jean et al. 1997). Zeng et al. (2009) also concluded that the *Rfp* gene was located on linkage group N18 (Chen et al. 2007), while Li et al. (2011) found the *Rfp* gene located on linkage group N9 of a published DH mapping population (Qiu et al. 2006). In this study, we mapped the *Rfp* gene on linkage group N9 (Zhang et al. 2011) using three newly developed SSR markers. Our results together with that of Li et al. (2011) and Yang and Fu (1991) provided more evidence that the *Rfp* gene might exist in the A genome of *B. napus*.

It has been recognized that the sequence information and genomic resources of *Arabidopsis* and other *Brassica*

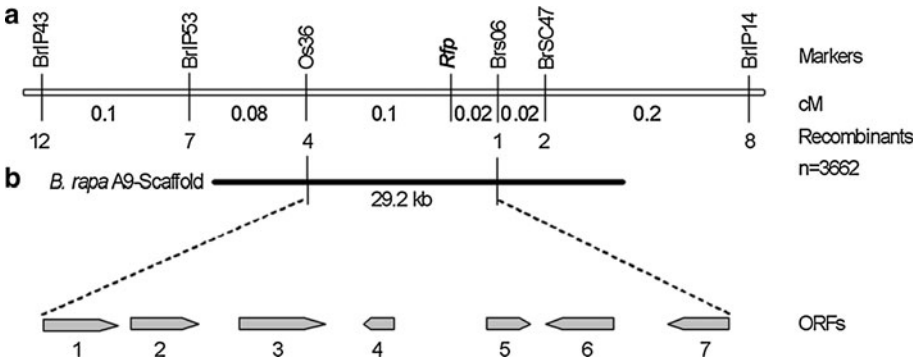

Fig. 2 Genetic and physical maps of the *Rfp* gene and candidate gene identification. **a** Fine mapping of the *Rfp* gene. The *Rfp* gene was restricted to the region between markers Os36 and Brs06 using 3662 individuals. The number of recombinants between the markers and the *Rfp* locus is indicated under the linkage map. **b** Candidate region of the *Rfp* locus and the predicted gene from http://www.softberry.com. The *Rfp* locus was narrowed down to a 29.2-kb region in the *B. rapa* Scaffold. There were seven predicted ORFs in this region

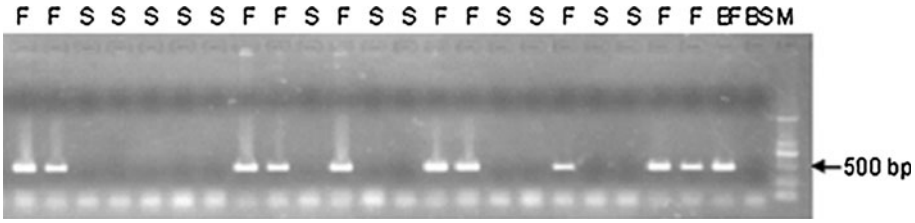

Fig. 3 Co-segregation analysis in the NIL population using SCP4 marker. *F* fertile plant, *S* sterile plant, *BF* fertile bulks, *BS* sterile bulks, *M DL2000* marker

species could be used for marker development, map-based gene cloning and candidate gene identification in *B. napus* (Yi et al. 2010). This strategy has been successfully used to develop markers tightly linked to recessive genic male sterility gene (*BnMs2, BnMs3*) (Lei et al. 2007; He et al. 2008) and map-based cloning *BnMs1, BnMs2* and *BnMs3* (Yi et al. 2010; Dun et al. 2011; Li et al. 2012) in *B. napus* and fertility restorer gene *Rfo* in radish (Brown et al. 2003). In the present study, we constructed a high-resolution map of the *Rfp* locus in *B. napus* using the syntenic region of *B. rapa* or *B. oleracea*. These results will speed up map-based cloning of the *Rfp* gene.

Although the *pol* CMS system is a widely used hybrid system of *B. napus* in China, its mechanism of control at the molecular level remains unclear. Cloning and characterization of the *Rfp* gene will provide more insights into the mechanism for restoring fertility in CMS plants. The most significant result of this work is fine physical mapping of the *Rfp* locus to a DNA fragment of 29.2 kb. Additionally, we identified a PPR gene as the candidate for the *Rfp* gene in the target region. This is an important step toward map-based cloning this gene and will aid in understanding the fertility restoring mechanisms at the molecular level. The functional studies of the *Rfp* gene will be done for the next step, including analysis of gene expression pattern and gene identification with *Agrobacterium*-mediated transformation technology and so on. Additionally, the newly developed markers tightly linked with the *Rfp* will be very useful in the MAS of elite CMS restorer lines.

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