

Research Paper

Identification and application of markers closely linked to the restorer gene (*Rfm*) in rapeseed (*Brassica napus* L.)

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The Mutsu-Isuzu cytoplasmic male sterility (MI CMS) system is one of the three-line hybrid systems used in China. As we know, the hybrid system is tightly associated with the yield variation in F_1 heterosis, while the restorer gene for the MI CMS (*Rfm*) has not been finely mapped for further application in marker-assisted selection (MAS). In this study, the sets of near-isogenic lines (NILs) of *Rfm* in two different genetic backgrounds were hybridized with the genome-wide 60 K single-nucleotide polymorphism (SNP) chip of *Brassica* for screening the possible associated genomic region of *Rfm*. Through screening genotypes with SNP loci and sequencing the candidate loci, one 2.5 Mb physical region (covering three scaffolds) on chrA09 was identified as the candidate for the *Rfm* region. Then, the SSR markers for the target scaffolds were used to detect the recombination in an F_2 population and narrowed the *Rfm* gene within the genetic distance of 0.52 cM, equivalent to a 350 kb physical segment. Moreover, the markers were tested to improve new elite restoration lines and to assess the percentage of hybrid seeds. Our results could potentially accelerate the map-based cloning of the *Rfm* gene to benefit rapeseed breeding.

Key Words: restorer gene, MI CMS, rapeseed (*Brassica napus* L.), near-isogenic lines (NILs), marker-assisted selection (MAS).

Introduction

The plant cytoplasmic-male-sterility (CMS) system is one of the most important ways to improve crop yield and harness the F_1 heterosis. This system has been reported in more than 320 plant species since its discovery (Chen and Liu 2014). New artificial CMS systems have been continuously created to satisfy the increasing demands for breeding new varieties (Yamagishi and Bhat 2014). Now, plant CMS systems are not only a valuable means to explore the interaction mechanisms between organelle genes and nuclear genes (Chen and Liu 2014) but also a tool for hybrid breeding and seed multiplication. The restorer gene (*Rf*) is able to confer male fertility to the sterile line; thus, mapping and cloning this gene are the primary steps for molecular studies on the CMS system. Until now, more than ten *Rf* genes in plant CMS systems have been cloned using the mapping method (Gaborieau *et al.* 2016).

Rapeseed (*Brassica napus* L.) is one of the most important oil crops globally, and its *Rf* genes have been well stud-

ied in two widely used CMS systems. One of them is *Ogu* CMS, in which the cytoplasm was discovered in radish (*Raphanus sativus* L.) (Ogura 1968). The combination of the sterile cytoplasm and the restorer gene in the rapeseed *Ogu* CMS system are transferred from radish (Yamagishi and Bhat 2014). Due to the large size of the radish segment inserted into the rapeseed genome with the *Rfo* gene, some unexpected traits (such as high glucosinolate content) were inevitably introgressed into the rapeseed genome. Therefore, it is difficult to produce high-quality restorer lines (Primard-Brisset *et al.* 2005). For the purpose of finding closely linked molecular markers to the fertility restorer gene, a sequence characterized amplified region (SCAR) marker derived from a random amplified polymorphic DNA (RAPD) marker was first screened by Mikolajczyk *et al.* (2008), and then its marker-assisted selection (MAS) function for the *BnRfo* gene was confirmed in an F_2 population. The allele-specific marker was designed to breed restorer lines according to the sequence specific to the *Rfo* gene (Hu *et al.* 2008). The *BnRfo* gene was eventually physically located on the linkage group N19 of the C-genome (Feng *et al.* 2009). The closely flanking simple-sequence repeat (SSR) markers for the *BnRfo* gene were identified by Hu *et al.* (2008). The second CMS system in rapeseed is *pol* CMS, which is derived from a spontaneous event (Fu 1981).

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Several restriction fragments length polymorphism (RFLP) markers linked to the *Rfp* gene on linkage group 18 were found (Jean *et al.* 1997). The *BnRfp* gene was transferred to *B. rapa* and anchored to a 4.3 Mb chromosome region by RFLP markers (Formanova *et al.* 2006, 2010). Liu *et al.* (2012), Yun *et al.* (2011) and Zeng *et al.* (2008) narrowed the genetic distances from 10 cM to 0.02 cM, which was equivalent to 29.2-kb physical distance, using the SSR, amplified fragment length polymorphism (AFLP) and SCAR markers. Finally, the *Rfp* gene was cloned and clarified to function by reducing transcript levels of the cytoplasm gene (Liu *et al.* 2016).

Single-nucleotide polymorphism (SNP) markers in rapeseed were recently developed. Researchers (Bancroft *et al.* 2011, Delourme *et al.* 2013, Trick *et al.* 2009) identified that the heritable SNP variation could be used as genetic markers in genetic studies and gene mapping. The release of the sequences of the A and C genomes in *B. rapa* (<http://brassicadb.org/brad/>) (Wang *et al.* 2011) and *B. oleracea* (<http://www.ocri-genomics.org/bolbase/>) (Liu *et al.* 2014) promoted the development of SNP markers. Then, the commercialization of the Brassica Infinium SNP array launched the era of SNP markers in genome-wide association studies (GWASs) on phenotype explanation and high-efficiency germplasm screening (Snowdon and Iniguez Luy 2012). Since a 60 K *Brassica* chip was first used in GWAS on seed weight and seed quality in rapeseed (Li *et al.* 2014), it has greatly contributed to identifying and cloning candidate genes of this oil crop in recent years.

There are CMS systems other than *Ogu* CMS and *pol* CMS (such as MI CMS, 681A CMS, Nsa CMS) utilized in rapeseed hybrid breeding processes (Fu *et al.* 1989, Liu *et al.* 2005, Wei *et al.* 2010). For the MI CMS system, the sterile line (A line) was developed by the continuous backcrossing with Mutsu as the female parent and Isuzu as the male recurrent parent (Fu *et al.* 1989). Mutsu and Isuzu were rapeseed varieties known to have sterile cytoplasm and sterile nuclear genes, respectively. The name of MI was derived from the first letters of Mutsu and Isuzu. The restorer line (R line) was obtained using massive existing germplasm by testcrossing with the A line and inspecting the fertilities of the F₁s. To date, several high-yield rapeseed varieties have been bred through this system and have contributed to Chinese rapeseed production. However, the molecular studies on the MI CMS system were on the initial stage, which limited its application. The objectives of the present research were to: (1) identify the candidate genomic region of the *Rfm* gene by SNP chip; (2) confirm the closely linked molecular markers to the *Rfm* gene; and (3) test the application value of these markers in breeding.

Materials and Methods

Two sets of NILs (T84-RR and T84, ZS9-RR and ZS9) for the *Rfm* gene were used, in which T84 and ZS9 were the recurrent parents in each set of NILs. The construction pro-

cess of the NILs was described in a previous publication (Long *et al.* 2011). T84 and ZS9 had the same genotype *N(rr)* but a different genetic background. T84-RR and ZS9-RR had the same genotype *S(RR)*, which had the genetic background of T84 and ZS9, respectively. Here, *S* and *N* in the genotypes indicate sterile and normal cytoplasm, respectively, and *R* and *r* indicate the dominant and recessive nuclear restorer genes, respectively. Two elite lines (D231, D238) with the *N(rr)* genotype were used as the other genetic backgrounds to breed new restorer lines. NingA7, a sterile line with the genotype *S(rr)* of the MI CMS system, was used as the tester to identify the existence of the *Rfm* gene. NingR7 is a classic restorer line with the genotype *S(RR)*. The commercial seeds of two varieties (Ningza No15 and Ningza No19) were used for the purification test (the percentage of real hybrid seeds). Each variety had three batches (named B15-1, B15-2, B15-3 and B19-1, B19-2, B19-3).

The F₁ produced from T84-RR × T84 was selfed to develop a T84-F₂ population. A 100-individual population (34 sterile and 66 fertile plants, named T84-SG) was randomly chosen from T84-F₂ for verifying the SNP loci. Four F₂ populations (named Pop1, Pop2, Pop3 and Pop4) were developed separately from NingR7 × D231, D231 × NingR7 and NingR7 × D238, D238 × NingR7, respectively. All materials were provided by Jiangsu Academy of Agriculture Sciences and planted at the breeding base. The fertility was recorded at the flowering stage according to Liu *et al.* (2012).

Genomic DNA was extracted from leaves using the cetyltrimethyl-ammonium-bromide (CTAB) method (Porebski *et al.* 1997). For purity assessment, the 2 cm long sprouts of single germinated seeds of each batch were cut as the tissue to extract DNA. DNA concentrations of all samples were determined and diluted to 50 ng/μl. For the NILs, ten plants of each line were used, and equal amounts of DNA from five plants of a line were mixed as a copy. Therefore, each NIL line had two copies of DNAs.

Eight DNA samples of NILs (i.e., two copies of each set) were hybridized with the Brassica 60 k Illumina[®] Infinium SNP array in 2013. The detailed descriptions of the procedure of SNP genotyping and mapping were provided in previous reports (Li *et al.* 2014, Wang *et al.* 2016). The location of each SNP on the chromosome was obtained using Illumina BeadStudio genotyping software. Specifically, in the present experiment, the following SNP loci were kicked out from the large data of genotyping results if: (1) the SNP loci that did not coincide between the two copies of each line or between the lines with the same genotype (T84-RR and ZS9-RR or T84-rr and ZS9-rr); (2) the SNP loci were same between T84-RR and T84-rr or between ZS9-RR and ZS9-rr; and (3) the SNP loci were heterozygous genotypes. The left SNP loci were considered as the candidate loci.

Three SNPs were randomly selected from the candidate SNPs and used to verify their linkage relationships with the fertility of the T84-SG by sequencing. Primers (Table 1) were designed based on their corresponding probe sequences in SNP, and PCR was conducted on the individual DNA

Table 1. Primers for the fragments corresponding to the three SNP loci

No.	SNP loci	Forward Primer (5'–3')	Reverse Primer (5'–3')	Product length
1	Bn-A09-p33406111	GTGTTTCAGGCAACTGGGAGA	CGTTCCTGGGATACGCATTGTG	309
2	Bn-A09-p33427256	GTGTGTGTGTACCCGTTGAATC	TCCTTTGTGAACATCCCTTATC	346
3	Bn-A09-p35476619	GCAGGCGAGTCTCAGCAATG	GGAACTCCACCGGACATCAC	399

samples of T84-SG. For the PCR assay, 1 µl DNA was used as the template in 20 µl reaction mixtures containing 1× buffer mix with 0.5 u *pfu* enzyme and 0.25 µM of each primer. The PCR procedure was as follows: an initial denaturation 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 extension of 10 min at 72°C. The nucleotides of the target SNP loci of each line were recorded after sequencing the PCR products.

As the SSR markers are universal between *B. rapa* and *B. napus* (Shi *et al.* 2014), the probe sequences of the candidate SNPs were finely located on the *B. rapa* physical map by blasting against the *B. rapa* scaffolds data (BRAD, <http://brassicadb.org/brad/blastPage.php>). A total of 118 pairs of SSR primers covering the target *B. rapa* scaffolds were selected from the high-density linkage map to detect polymorphism in parents and to create a map using all individuals of the T84-F₂ population. The SSR amplification was performed as described by Shi *et al.* (2014). The PCR products were separated on 6 % (w/v) denaturing polyacrylamide gels and visualized by silver staining.

The polymorphic SSR fragments were cloned and sequenced as described previously (Yi *et al.* 2006). By subjecting the amplified sequences to a BLAST search against the *B. napus* genome (<http://brassicadb.org/brad/blastPage.php>), each polymorphic SSR marker had its physical location noted on the reference genome. The linkage maps were constructed by converting the recombination events into genetic distances (cM).

The conventional method and the MAS were applied to select new restorer lines from Pop1, Pop2, Pop3 and Pop4. In the conventional way, lines from Pop1 to Pop4 were self-fertilized to produce F₃-lines that did not show fertility segregation. We selected 200 F₃-lines with no fertility segregation from Pop1 and Pop3 (with the sterile cytoplasm gene) and randomly selected 200 F₃-lines from Pop2 and Pop4 (with the fertile cytoplasm gene) as the new restorer lines. In the MAS method, the SSR marker W14 was used for PCR on each plant in the Pop1-Pop4 populations. We selected 200 lines with the T84-RR band-type from Pop1 to Pop4. All the newly selected restoration lines from the two methods were crossed with NingA7, and a line was regarded as the correct restoration line if its corresponding F₁ plants were fully fertile. To assess the purity by the conventional method, 300 seeds of each batch were planted in pots (one seed per pot), and the percent of fertile plants (observed in the next year) was calculated. In addition, in the MAS method, the seeds with heterozygous (*Rr*) bands were considered as pure F₁ hybrids.

Results

Phenotype of sterile/fertile lines and genetic analysis of the *Rfm* gene

The buds of MI CMS sterile and fertile plants showed great differences in their phenotypes (Fig. 1). Buds from sterile plants were slim with shorter and shriveled stamens but normal pistils. However, buds from fertile plants appeared to be strong, with green plump stamens and regular pistils. The T84-F₂ population, including 2,324 individuals with sterile cytoplasm, was developed and had 1,748 fertile and 576 sterile plants. The segregation ratio of fertility and sterility in this population was coincident with the expected 3:1 ratio ($\chi^2 = 0.057$, $P > 0.05$), which supports that there is only one dominant locus for the *Rfm* gene in the MI CMS system.

Identifying the associated genomic region for the *Rfm* by SNP chip

The genotype of each SNP for NIL samples was analyzed by the Illumina BeadStudio genotyping software. Over 97% of SNP loci showed hybridization signals, and 48,715 (93.40%) and 49,889 (96.65%) SNPs had identical genotypes for each of the two sets of NILs, respectively. There were 856 loci (412 loci as AA/BB and 444 loci as BB/AA) showing the homozygous opposite genotypes on T84-RR/T84 and 672 loci (323 loci as AA/BB and 349 loci as BB/

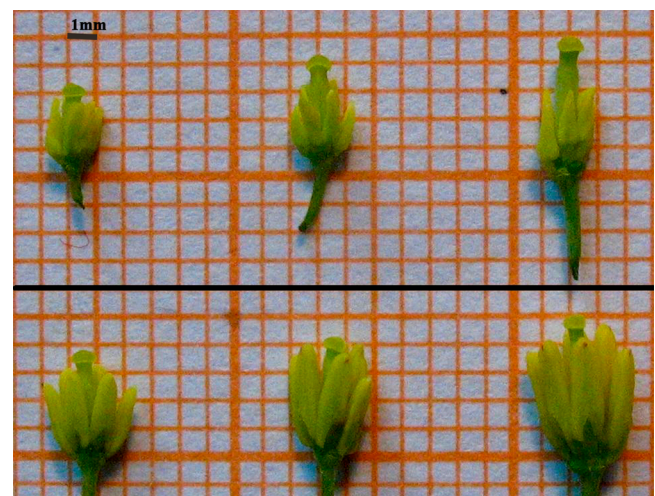


Fig. 1. Phenotypes of the sterile/fertile plants of the MI CMS system. The upper half of the figure shows buds of different lengths without sepals and petals from sterile plants, and the lower half shows buds from fertile plants. The background is the coordinate paper, and the smallest frames are the 1 mm-length squares. Bar = 1 mm.

AA) showing similar genotypes on ZS9-RR/ZS9. By selecting the intersections of the putative SNP loci screened from each background, 33 candidate SNP loci (**Supplemental Table 1**) were preliminarily decided as the possible SNP markers for the *Rfm* gene, among which 32 loci were concentrated into 2.5 Mb physical region on chromosome A09 and the other was on a scaffold.

Verifying results from SNP chip by sequencing

To confirm the results from the SNP chip, three SNPs (Bn-A09-p33406111, Bn-A09-p33427256 and Bn-A09-p35476619) were randomly chosen for sequencing. The specific primers were designed based on their probe sequences and used to clone the corresponding fragments of the T84-SG lines. Nucleotides on the targeted SNP loci are shown in **Table 2**, which demonstrate that the hit genomic region by SNP chip was correct. To map the *Rfm* gene more precisely, the target region on chromosome A09 was aligned onto three scaffolds (Scaffold000053, Scaffold000077 and Scaffold000082), which have high-density SSR markers and were used in the following experiment.

Linkage map construction of the *Rfm* gene

A total of 118 SSR markers (26, 50 and 42 markers covering the three scaffolds of the *B. rapa* genome, respectively, **Supplemental Table 2**) were selected from the pool (Shi *et al.* 2014) and used to detect polymorphism between the parents (T84-RR and T84). There were only 3, 3 and 4 polymorphic markers in the respective scaffolds (**Supplemental Table 2**), which were then sorted on the linkage map based on the recombination events after detecting all plants in T84-F₂ (**Fig. 2**). The *Rfm* gene was located in Scaffold000077 with both-side flanking markers. Through blasting the specific fragments against the *B. napus* genome, the polymorphic SSR markers were physically located (**Fig. 2**). The nearest SSR markers (W14 and W117) narrowed the possible location of the *Rfm* gene within a 352 kb region. The co-dominant markers (W14) closely linked to the *Rfm* gene could clearly distinguish plants having homozygous (*RR*, *rr*) and heterozygous (*Rr*) genotypes (**Fig. 3**).

Utilization of the markers in breeding

By checking the fertilities of the F₁ hybrids produced by the conventional method, it was clarified that 99.75% of the

Table 2. Sequencing of the three candidate SNPs in the T84-SG population

Plants of T84-SG	SNP1	SNP2	SNP3	Plants of T84-SG	SNP1	SNP2	SNP3	Plants of T84-SG	SNP1	SNP2	SNP3
F-1	G/A	G/A	T/G	S-1	A	G	T	S-35	A	G	T
F-2	G/A	G/A	T/G	S-2	A	G	T	S-36	A	G	T
F-3	G	G/A	T/G	S-3	A	G	T	S-37	A	G	T
F-4	G/A	G/A	T/G	S-4	A	G	T	S-38	A	G	T
F-5	G/A	G/A	/	S-5	A	/	T	S-39	A	G	T
F-6	G/A	G/A	T/G	S-6	A	G	T	S-40	A	G	T
F-7	G/A	/	T/G	S-7	A	G	T	S-41	/	G	T
F-8	G/A	G/A	T/G	S-8	A	G	T	S-42	A	G	T
F-9	G	A	G	S-9	A	G	T	S-43	A	G	/
F-10	G/A	G/A	T/G	S-10	A	G	T	S-44	A	G	T
F-11	/	G/A	T/G	S-11	A	G	T	S-45	A	G	T
F-12	G/A	G/A	T/G	S-12	A	G	T	S-46	A	G	T
F-13	G	A	G	S-13	A	G	T	S-47	A	G	T
F-14	G	A	G	S-14	/	/	/	S-48	A	G	T
F-15	G/A	G/A	T/G	S-15	A	G	T	S-49	A	G	T
F-16	G/A	G/A	T/G	S-16	A	G	T	S-50	A	G	T
F-17	G/A	G/A	T/G	S-17	A	G	T	S-51	A	/	T
F-18	G	A	G	S-18	A	G	T	S-52	A	G	T
F-19	G	G/A	T/G	S-19	A	G	T	S-53	A	G	T
F-20	G	A	G	S-20	A	G	T	S-54	A	G	T
F-21	G/A	G/A	T/G	S-21	A	G	T	S-55	A	G	T
F-22	G/A	G/A	T/G	S-22	A	G	T	S-56	A	G	T
F-23	G/A	G/A	T/G	S-23	A	G	T	S-57	/	G	/
F-24	G/A	G/A	T/G	S-24	A	G	/	S-58	A	G	T
F-25	G/A	/	T/G	S-25	A	G	T	S-59	A	G	T
F-26	G	G/A	T/G	S-26	A	G	T	S-60	A	G	T
F-27	G/A	G/A	T/G	S-27	/	G	T	S-61	A	G	T
F-28	G/A	G/A	T/G	S-28	A	/	T	S-62	A	/	T
F-29	G/A	G/A	/	S-29	A	G	T	S-63	A	G	T
F-30	G	G/A	T/G	S-30	A	G	T	S-64	A	G	T
F-31	G/A	G/A	T/G	S-31	A	G	T	S-65	A	G	T
F-32	G	A	G	S-32	A	G	T	S-66	A	G	T
F-33	G/A	G/A	T/G	S-33	A	G	T				
F-34	G/A	G/A	T/G	S-34	A	G	T				

S: Sterile, F: Fertile, /: not detected, SNP1: Bn-A09-p35476619, SNP2: Bn-A09-p33406111, SNP3: Bn-A09-p33427256.

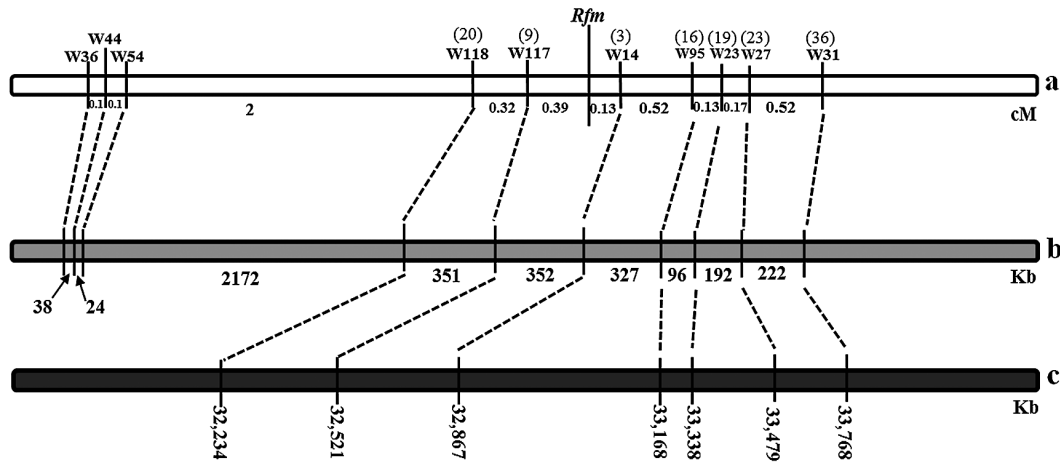


Fig. 2. Genetic and physical map of the *Rfm* gene. (a) Genetic distance (cM) of the *Rfm* gene with the linked SSR markers. The *Rfm* gene was mapped to the region between SSR markers W117 and W14. (b) The physical distances (kb) of the markers on the chromosome of *B. rapa*. (c) The physical locations (kb, the vertical numbers below the lines) of the markers mapped on chromosome A09 of *B. napus*. Note: three markers on Scaffold000053 (W36, W44 and W54) were not anchored to the *B. napus* A09 chromosome.

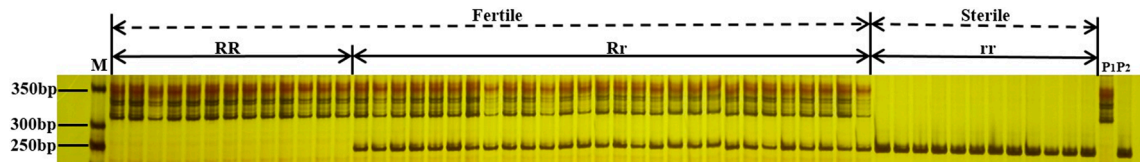


Fig. 3. Genotyping the single plants in the F_2 population by the SSR marker W14. The individual plants were grouped as *RR*, *Rr* and *rr* by their genotypes, and the gel showed the clear distinguishable band types. The *rr* genotype with the sterile cytoplasm showed the sterile phenotype, and the *RR* (homozygous), *Rr* (heterozygous) with the sterile cytoplasm showed the fertile phenotype.

Table 3. Comparison of two ways to breed new restorer lines

Ways	Conventional way				MAS			
	Dependent				Independent			
Cytoplasm dependence	Dependent				Independent			
Hybridization	NingR7×D231	NingR7×D238	D231×NingR7	D238×NingR7	NingR7×D231	NingR7×D238	D231×NingR7	D238×NingR7
Methods	Selecting fertile lines				Selecting <i>RR</i> genotype by markers			
Identification stage	Flowering stage				Seedling stage			
Total lines	200	200	200	200	200	200	200	200
Fertile F_1	200	199	49	52	198	200	199	197
Percentage (%)	100	99.5	24.5	26	99	100	99.5	98.5
Labor work	much				less			

/: none.

new-bred restoration lines from the sterile cytoplasm Pop1 and Pop3 populations and 25% of the lines from the fertile cytoplasm Pop2 and Pop4 populations belonged to the *RR* genotype. In contrast, 98% of the new-bred restoration lines produced by the MAS method had homozygous *Rfm* genes (Table 3). For purity assessment, there was only a 1.0~2.3% difference (Table 4) between the two methods on assessing the purity of the commercial seeds of Ningza No15 and Ningza No19, showing the feasibility of the MAS method.

Discussion

The SNP chip technique facilitates the test of trait-controlling genome segments in rapeseed. As rapeseed is a tetraploid crop with a combination of A and C sub-genomes

Table 4. Purity assessment of the commercial hybrid seeds by the two methods

Variety	Batch	Assessing methods (%)		Difference between two methods (%)
		By the conventional method	By MAS	
Ningza No15	B15-1	92.67	91.33	1.3
	B15-2	95.67	96.67	1.0
	B15-3	96.00	93.67	2.3
Ningza No19	B19-1	89.67	91.33	1.7
	B19-2	91.67	90.33	1.3
	B19-3	93.33	91.00	2.3

with high homology (Chalhoub *et al.* 2014), it is difficult to identify suitable markers for specific chromosomes without adequate sequence data. The previous molecular markers,

such as RFLP and RAPD, had intrinsic shortages and were limited in fine mapping. The AFLP markers with high polymorphism were considered to be a prospective method for mapping (Ke *et al.* 2004, Lu *et al.* 2004), and the release of A and C sub-genome sequences enriched the number of SSR markers (one for every 2~3 kb physical distance) (Shi *et al.* 2014); however, there is still a large amount of laborious works required to screen AFLP and SSR markers on parents and the segregation population. The high-scaled SNP markers were developed through sequencing more contrasting genotypes by next-generation sequencing (NGS) (Trick *et al.* 2009), which accelerated the commercial application of the SNP chip technique. With excellent characteristics, such as high-amount processing, easy detection and lower cost, the newly developed SNP chip was quickly applied to linkage map construction and GWAS (Delourme *et al.* 2013). In the present work, the gene location controlling the fertility of the MI CMS system was efficiently identified using the SNP chip technique.

The NILs are the lines with almost the same genetic background except for the loci controlling the objective trait. In this work, except for the SNPs identified on A09, there were an additional 800 SNPs on the other chromosome locations that had the expected genotypes in the T84-background NILs and 600 SNPs in the ZS9-background, which could theoretically be regarded as potential candidate objects. This result showed that there were some *Rfm*-unlinked segments transferring into the recurrent parent during the NIL construction process, which may disturb the identification of the *Rfm*-controlling region. However, we were able to identify the putative genomic region for the *Rfm* gene by taking the intersection of two different genetic backgrounds. Therefore, it is better to construct NILs with different genetic backgrounds. Moreover, the results of this work also demonstrated that it is an effective method to confirm the associated genomic region by combining the SNP chip and the NILs method. However, there is a limitation in this study. The target gene would be difficult to map finely because the very high homology between the NILs was an obstacle for developing the polymorphic markers (Keurentjes *et al.* 2007). In this work, less than 10% (10/118) of SSR markers were confirmed as effective. An alternative method is to change the mapping population to that with the genetically diversified parents.

ChrA09 may be a hot chromosome for the restorer genes in rapeseed CMS systems. Before our localization of the *Rfm* gene, the *Rfp* gene for the *Pol* CMS was mapped on the site of 36~37 Mb on ChrA09 (Liu *et al.* 2012); meanwhile, the *Rfn* gene for the *Nap* CMS was also finely mapped at the 41.5~42.5 Mb physical region on the same chromosome (Gaborieau *et al.* 2016). Although *Rfn* and *Rfp* were thought to be allelic genes (Li *et al.* 1998), they were shown to be different pentatricopeptide repeat (PPR) genes with just a 125 kb distance (Liu *et al.* 2016, 2017). Moreover, they were shown to function with a post-transcriptional mechanism to restore male sterility. The results of this work will

speed up *Rfm* gene cloning and clarify the relationships of these restoration genes in different rapeseed CMS systems.

The closely linked markers for the *Rfm* gene would facilitate the breeding process. First, these markers could upgrade the conventional method to the MAS method for breeding new restoration lines and confirm the lines with the *Rfm* at the early stage. Moreover, the costs of restoration of *Rfp* and *Rfn* on the sterile cytoplasm were demonstrated (Dufay *et al.* 2007, Montgomery *et al.* 2014). The MAS method could help breed the restoration lines with the fertile cytoplasm efficiently and avoid the negative effect of sterile cytoplasm on yield. Second, assessing the purities of commercial seeds is another valuable application for the markers of *Rfm*. Although some methods, such as using the isozymes or the not-so-close markers (Čurn and Žaludová 2007), were developed before, the effectiveness of testing was not satisfactory. The results from the present marker evaluation showed a good accordance with the fact and demonstrate the great potential to apply these markers in the hybrid seed industry based on its use in the early-stage and its accurate evaluation.

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