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ORIGINAL PAPER

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Molecular validation of multiple allele inheritance for dominant genic male sterility gene in *Brassica napus* L

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Abstract Dominant genic male sterility (DGMS) has been playing an increasingly important role, not only as a tool for assisting in recurrent selection but also as an alternative approach for efficient production of hybrids. Previous studies indicate that fertility restoration of DGMS is the action of another unlinked dominant gene. Recently, through classical genetic analysis with various test populations we have verified that in a DGMS line 609AB the trait is inherited in a multiple allelic pattern. In this study, we applied molecular marker technology to provide further validation of the results. Eight amplified fragment length polymorphism (AFLP) markers tightly linked to the male sterility allele (Ms) were identified in a BC_1 population from a cross between 609A (a sterile plant in 609AB) and a temporary maintainer GS2467 as recurrent parent. Four out of the eight markers reproduced the same polymorphism in a larger BC_1 population generated with microspore-derived doubled haploid (DH) parents (S148 and S467). The two nearest AFLP markers SA12MG14 and P05MG15, flanking the Ms locus at respective distances of 0.3 centiMorgan (cM) and 1.6 cM, were converted into sequence characterized amplified region (SCAR) markers designated SC6 and SC9. Based on the sequence difference of the marker P05MG15 between S148 and a DH restorer line S103, we further developed a SCAR marker SC9f that is specific to the restorer allele (Mf). The map distance between SC9f and Mf was consistent with that between SC9 and Ms allele. Therefore, successful conversion of the marker tightly linked to Ms into a marker tightly linked to Mf suggested that the restoration for DGMS in 609AB is controlled by an allele at the Ms locus or a tightly linked gene (regarded as an allele in practical application). The Ms and Mf-specific markers developed here will facilitate the breeding for new elite homozygous sterile lines and allow further research on map-based cloning of the Ms gene.

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Introduction

Dominant genic male sterility (DGMS) is often seen in higher plants including some important crops, such as rice, wheat and *Brassica* species (Liu 1992). DGMS inherits in a dominant fashion, but its fertility can be restored in many cases. For the control of fertility restoration, three genetic models have been proposed. The fertility is regulated: (1) by multiple alleles at one locus, (2) by two linked genes, or (3) by two unlinked genes. In *B. napus*, several cases of DGMS have been reported (Li et al. 1985; Mathias 1985; Wang et al. 2001; Hu et al. 2002; Song et al. 2005). The double dominant gene inheritance characterized by Li et al. (1985, 1988, 1990) has gained broad acceptance. This model suggests that an unlinked inhibitory gene Mf (or Rf in previous reports) suppresses the expression of a male sterility gene Ms. Evidence from ingenious genetic test studies in *B. napus* (Liu 1992; Zhou and Bai 1994; Hu et al. 2004) and studies conducted in *Brassica rapa* (Dong et al. 1998, 1999) and other crops further supports this model. Besides this unlinked digenic model, however, a multiple allele inheritance model has been identified in Chinese cabbage (*B. rapa* ssp.*pekinesis*) (Zhang et al. 1990; Xu et al. 2003), one of the ancestral species of *B. napus*, implying that these two genetic models might also exist in *B. napus*. In our previous report, we presented strong evidence to suggest that the multiple allele model likely exists in *B. napus* (Song et al. 2005). Through genetic tests, we found that the inhibitory (or restoration) genes in the fertile plants

from 609AB and in the restorers tested are allelic to the Ms allele. Nevertheless, it needs further molecular evidence to verify our previous finding.

Molecular marker technology is extensively used in genetic mapping, map-based gene cloning, genetic diversity analysis, variety protection and breeding selection. Various types of markers have been developed to map important traits involving resistance (Barret et al. 1998; Pilet et al. 1998; Plieske et al. 1998; Manzanares-Dauleux et al. 2000), quality (Somers et al. 1998; Tanhuanpää et al. 1998; Schierholt et al. 2000; Gupta et al. 2004) and seed coat color (Negi et al. 2000; Sabharwel et al. 2004; Liu et al. 2005). The respective restorer genes for Ogu cytoplasmic male sterility (CMS) (Hansen et al. (1997), Pol CMS (Jean et al. 1997, 1998) and Tour CMS (Janeja et al. 2003) in *Brassica* have been characterized with various molecular markers and are even localized to a particular linkage region. The markers developed have been employed to assist the practical selection in breeding program of gene introgression (Delourme et al. 1998) and resistance improvement (Varshney et al. 2004). The genes responsible for recessive genic male sterility in *B. napus* (Ke et al. 2004) and *Brassica rapa* (Ying et al. 2003) have been flanked with amplified fragment length polymorphism (AFLP) markers. In addition, the gene for DGMS in *B. napus* has been mapped in a linkage group with a nearest marker at a distance of 3.7 centiMorgan (cM) (Lu et al. 2004a, b), which is converted into a sequence characterized amplified region (SCAR) marker. The multiple allele inheritance results in six genotypes on the whole, of which four share an identical fertile phenotype. The determination of a desired genotype traditionally takes more resources and two growing seasons or more. Therefore, the development of markers tightly linked to Ms gene for DGMS will make the associated breeding more efficient.

Because of its stable and complete sterility performance, extensive distribution of restorers, diverse cytoplasmic sources and the availability of 100% sterile plant population, genic male sterility controlled by recessive or dominant genes is gaining importance in rapeseed breeding, not only being an assistant part but an alternative approach for hybrid seed production. DGMS has been extensively used to construct recurrent selection populations for improvement of important quantitative agronomic traits. The three-line approach proposed by Li et al. (1985) has been demonstrated to be practical for hybrid production, and two commercial hybrids have been registered and released in China. In such a three-line system, a temporary maintainer, which cannot successively maintain an entire sterile population as a maintainer in the CMS system, is utilized to cross to the homozygous sterile line to produce a population with 100% sterile individuals, with which the hybrid seed production is subsequently carried out. Thus, manual removal of fertile plants in sterile line, a step that is required in a two-line approach, can be obviated. Whether the maintainer is homologous to the sterile line, however, is critical to the unification and heterosis performance of commercial F_1 hybrid. Therefore, it is necessary to determine the inheritance pattern of DGMS before designing a breeding program to make both homologous.

In this study, we attempted to investigate the genetic model of DGMS in a newly bred sterile line 609AB. We described the development and evaluation of AFLP markers tightly linked to Ms, and the direct conversion of one Ms marker into an Mf marker, which led us to conclude that markers linked to Ms are also linked to Mf.

Materials and methods

Plant materials

The 609AB (provided by Jiangxi Academy of Agricultural Sciences, Jiangxi, China) is a newly bred DGMS line from a spontaneous mutant. It is completely male sterile, with withered anthers and normal pistil, but to some extent it shows ecotypic sensitivity (mainly to temperature) in some other genic backgrounds. It is a homozygous sterile type, including sterile plants (609A, MsMs) and fertile plants (609B, MsMf)). A doubled haploid (DH) sterile line S148 was developed from the 609B with isolated microspore culture and maintained with tissue culture method. A DH temporary maintainer line S467 was purified from GS2467, a breeding line previously identified as a temporary maintainer, and a DH restorer S103 was generated from a F_1 plant between 609A and a restorer line RS4.

Population construction for marker development and validation

BC_1 populations were constructed to develop and validate AFLP markers tightly linked to Ms, and to further verify the inheritance pattern. A plant of 609A was crossed as female parent to the maintainer GS2467, resulting in a sterile F_1 population. A segregating population designated as Popu1 was obtained by backcrossing a sterile F_1 plant with GS2467. To validate markers developed and to characterize allelism between Ms and Mf, another two BC_1 populations were generated with three microspore-derived DH lines. The S467 was crossed to the S148 and the resulting F_1 was backcrossed to S467, producing a population designated as Popu2. We produced another population Popu3 by crossing the restorer S103 to the sterile line S148 and subsequently backcrossing to S148. This population specifically segregated at the restorer locus. Plants were first grown in plastic culture plates with safe soil medium and were transplanted to a field when plants had reached to 4–5 leaf age. The fertility was determined during flowing and the young leaves of each individual were collected for DNA extraction.

DNA preparation

Total DNA from each individual plant was extracted using a modified CTAB method (Doyle and Doyle 1990). The method was modified for the mini-preparation of large numbers of individuals. DNA concentration and purity were measured by a Beckman spectrophotometer at a wavelength of 260 versus 280 nm and was adjusted to 50 ng/μl with sterilized double-distilled water (ddH_2O). To identify molecular markers for the Ms allele, we prepared two sterile bulks and two fertile bulks, each by pooling equal quantities of DNA from six individuals of the BC_1 population Popu1.

AFLP marker analysis

Bulked segregation analysis (Michelmore et al. 1991) was applied according to a double-screening strategy (Jean et al. 1998) so as to reduce the influence of background and increase the identification efficiency. The two sterile bulks and two fertile bulks constructed from Popu1 were in parallel subjected to AFLP analysis to identify putative markers linked to the Ms allele. AFLP analysis was carried out as described in Vos et al. (1995) with minor modifications. In brief, digestion reaction was performed in a volume of 12.5 μl with 100 ng genomic DNA, 2.5 U of the rare-cutting enzyme *Eco*RI (or *PstI, Sac*I) and 1.5 U of the frequent-cutting enzyme *Mse*I (MBI Fermemtas). The specific double-stranded *Eco*RI (or *PstI*, *SacI*) and *Mse*I adaptors were subsequently ligated to the restriction fragment ends. The ligation product was diluted (1:10) with sterilized ddH_2O, 5 μl of which was employed for the pre-amplification reaction in a volume of 25 μl, including 50 ng each of primers EA (or P0, SA), and MC or MG, each having one selective nucleotide except P0. The pre-amplified DNA was diluted (1:40), 2 μl of which was used for selective amplification with M + 3 (*Mse*I-adaptor primer with three additional selective nucleotides at the 3′ ends; the other ends are similar) and E + 3 (P + 3, S + 3) primers. The PCR products were then separated on 6% denaturing polyacrylamide gels and visualized by the silver staining system (Promega, Madison, WI.).

Cloning of AFLP markers and conversion to SCAR

Candidate AFLP fragments were separately recovered from gel (Ke et al. 2004). Recovered DNA was used for another round PCR in conditions similar to the second selective amplification of AFLP. And amplified PCR products were purified with UNIQ10 Column DNA Collection Kit (Sangon) and were cloned into pGEM-T vector (Promega, Madison) or pMD18-T vector (TaKaRa Bio.) as described (Molecular cloning: a laboratory manual, 3rd edn.) and sequenced (Beijing Sunbiotech Co., Ltd). Based on the sequence information, PCR primers for SCAR were designed using a primer design program Oligo 5 (National Biosciences, Inc., Plymouth, MN., USA).

PCR walking for the isolation of the flanking regions of AFLP markers

PCR walking (Siebert et al. 1995) was used to extend the regions adjacent to a specific marker locus. The methodology was simplified as a procedure similar to that of AFLP. The restriction reaction was performed at 37°C for 18 h in a volume of 10 μl with 200 ng DNA, 10U endonuclease, 1 μl of the Y + TANGO buffer (Fermentas MBI). It was then added with 10 μl of the ligation mixture with 10 U T4 DNA ligase, 2 μl of the ligation buffer, 2 μl of 50% PEG 4,000, and 2 μl of the 25 μM adaptor (duplex strands processed with single strand 5′-GTAATACGACTCACTATAGGGCACGCGTGGT CGACGGCCCGGGCTGGT-3′ and 5′-ACCAGCCC-NH_2-3′). The ligation reaction proceeded first for 1–2 h at room temperature and then at 4°C overnight. The walking step included two rounds of PCR amplification of the adaptor-ligated DNA. In the first PCR amplification step, 2 μl of the diluted ligation liquid (10×) was used in a reaction volume of 20 μl containing 50 ng each of the adaptor-specific primer AP1 (5′-GTAATACG ACTCACTATAGGGC-3′) and the gene-specific primer (Table 1). The PCR was performed in a PTC-225 Thermocycler (MJ Research, Waltham, Mass.) and it consisted of seven cycles of 2 s at 94°C, 3 min at 72°C, 32 cycles of 2 s at 94°C, 3 min at 67°C followed by a final extension step of 4 min at 72°C

In the second PCR amplification step, 4 μl of 30-fold PCR product from the first PCR was used in a volume of 50 μl with 100 ng each of the adaptor-specific primer AP2 (5′-ACTATAGGGCACGCGTGGT-3′) and the nested gene-specific primer (Table 1). The PCR parameters were: six cycles of 2 s at 94°C, 3 min at 72°C, 22 cycles of 2 s at 94°C, 3 min at 67°C followed by an additional extension step of 10 min at 72°C.

Table 1 Gene-specific primers and enzymes for PCR walking

PCR	Markers	Primers (5′–3′)	Enzymes
First round	SA12MG14	W6R1: AGTCAGAGTCAAGGTATCATATTCAAC′	*Sca*I
	P05MG15	W9F1: TGCAGCCACAAATCCGACAAGTACGAG	*Dra*I
		W9R1: CAGCAAAACAGGGACAAAAGTAATCCT	*Ssp*I
Second round	SA12MG14	W6R2: ACATTCGATGATCTTGAGCCGGTGTTT	
	P05MG15	W9F2: AGGATTACTTTTGTCCCTGTTTTGCTG	
		W9R2: CTCGTACTTGTCGGATTTGTGGCTGCA	

PCR products from the second PCR were retrieved, cloned and sequenced as mentioned earlier. The SCAR marker primers were designed based on the sequences obtained from the marker sequence and the flanking sequence extended by PCR walking.

Conversion of a marker tightly linked to Ms into a marker tightly linked to Mf

With the primer pairs used as SCAR markers tightly linked to Ms, or redesigned based on the flanking sequence, a PCR amplification was performed on restorer S103 along with S148 at higher annealing temperature. The products were retrieved, cloned and sequenced as above. Based on the sequence difference between the line S148 and S103, the primers were designed and analyzed on the population Popu3.

Data analysis

The putative markers that revealed consistent reproducible polymorphism between the two bulk pairs were further verified among the individuals from which the bulks were constructed. Each putative marker was confirmed in Popu1 first and further in Popu2. Linkage analysis was conducted on the segregation data of all markers and fertility trait using the software package MAPMAKER/EXP 3.0 (Lander et al. 1987; Lincoln et al. 1992). The recombination frequencies between the Ms allele and the molecular markers were calculated using two-point analysis. The most likely map order was determined by three-point or multi-point analysis with a minimum LOD threshold of 4.0 and a maximum recombination fraction of 0.3. The Kosambi map function (Kosambi. 1944)) was employed to convert the recombination frequencies into centiMorgans.

Results

Inheritance of DGMS in 609AB

For the DGMS, the sterile lines can be classed into two types: the homozygous and the heterozygous. 609AB was confirmed as a homozygous line, since crossing its sterile plants (MsMs) with temporary maintainers (msms) produced populations of 100% sterile individuals (Msms) and selfing its fertile plant (MsMf) resulted in a segregation ratio of three fertile: one sterile.

We recently identified the restorer allele (Mf) for the DGMS in 609AB (*B. napus*) (Song et al. 2005). To examine the allelism of Ms and Mf in 609AB, 609B was crossed to temporary maintainers, resulting in F_1s segregating in an expected ratio of 1:1. The fertile plants (msMf) in the F_1s population were selected to backcross to maintainers (msms). As a result, all the BC_1s (msMf + msms) were fertile, which suggested that the Mf is allelic to the Ms (Fig. 1a); otherwise they would generate one-fourth sterile plants, as would the digenic pattern expected (Fig. 1b). Furthermore, the Mf alleles in restorers (MfMf) were also proven to be allelic to Ms, since all testcrosses made between the restored F_1s (MsMf) and maintainers shared a 1:1 segregation ratio in agreement with the expectation of the multiple allele inheritance (Fig. 1c) rather than a 3:1 segregation ratio as the digenic pattern expected (Fig. 1d). Therefore, the DGMS in 609A is conditioned by a locus with three alleles, Ms, Mf, and ms.

AFLP marker development

Popu1 was initially constructed to identify AFLP markers linked to Ms. Ninety four plants were randomly selected, including 48 sterile plants and 46 fertile plants.

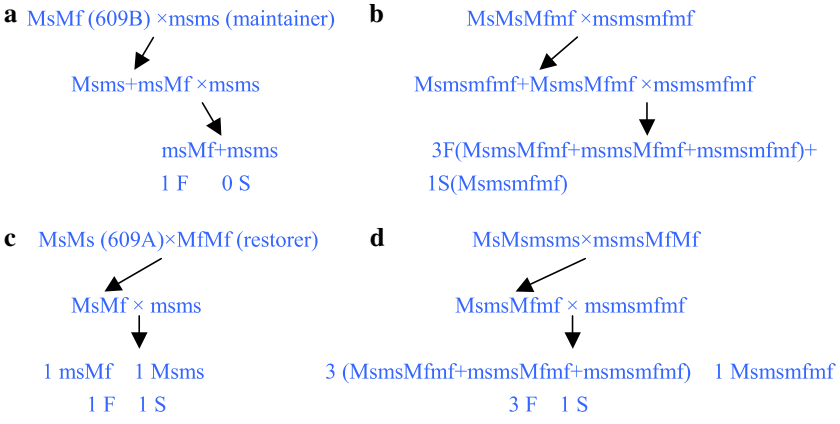

Fig. 1 Comparison of fertility segregation in genetic test populations between the multiple allelic inheritance (MAI) and unlinked digenic inheritance (UDI) of DGMS in 609AB. **a, b** Supposed segregation performance in populations from crosses between 609B and maintainers and from backcrosses between selected fertile plants in F_1 and maintainers based on MAI (**a**) and UDI (**b**). **c, d** Supposed segregation performance in populations from crosses between 609A and restorers and from test crosses between F_1 plants and maintainers based on MAI (**c**) and UDI (**d**). F: fertile. S: sterile

Two pairs of the bulks were in parallel subjected to perform AFLP analysis. We analyzed a total of 1,536 pairs of primer combinations including *Mse*I + C + 2 or *Mse*I + G + 2 with either of *Pst*I + 3, *Sac*I + A + 2 or *Eco*RI + A + 2. The selected combinations were analyzed again beginning from the digestion of DNA and to confirmation of consistently reproducible polymorphism between the two pairs of bulks. The candidate combinations were first examined in a small sample of 24 plants selected from Popu1 and then in the other plants. Consequently eight AFLP markers were observed as being closely linked to the Ms allele. The map distance for each marker was evaluated (Table 2). Based on the linkage analysis in Popu1, all the markers were located on one side of the Ms locus, with SA13MC06, SA12MG14 being closest to it at a distance of 1.1 cM (Fig. 2a)

To confirm the exact order and linkage relationship among the markers and the Ms allele, similar AFLP analyses of eight markers identified in Popu1 were performed in a larger population Popu2, which consisted 190 sterile plants and 196 fertile plants. Four markers lost their polymorphism, and the SA12MG14, formerly observed in Popu1 as being residing on the same side with P05MG15, was identified on the other side of the Ms locus. Thus the Ms allele was flanked with the two closest AFLP markers SA12MG14 and P05MG15 on either side at distances of 0.3 and 1.6 cM, respectively (Fig. 2b).

Conversion of AFLP markers to SCARs

To transfer the two closest AFLP markers into SCARs, the fragments of SA12MG14 and P05MG15 were cloned and sequenced. After removal of adaptor the sequence their actual sizes were 182 bp and 78 bp, respectively. Based on their sequences, we designed primers for PCR walking to amplify sequences adjacent to these two markers. As a result, the sequence of P05MG15 locus was stretched on both directions to a total 1873 bp; whereas the sequence of SA12MG14 locus was extended

Table 2 Primers, fragment sizes and map distances of AFLP markers for Ms allele in Popu1

Marker names	Primers	Approximate marker size (bp)	Map distances (cM)
EA07MG12	E + ATC/M + GCG	100	5.3
EA09MC11	E + ACA/M + CCC	260	3.2
SA13MC06	S + AGA/M + CTT	90	1.1
SA02MG14	S + AAT/M + GGT	80	4.3
SA12MG14	S + ACG/M + GGT	210	1.1
P03MC15	P + CAC/M + CGC	420	2.2
P04MC11	P + CAG/M + CCC	200	4.3
P05MG15	P + CCA/M + GGC	100	3.2

Map distances were calculated from recombination frequencies. *E EcoR*I primer. 5′-GACTGCGTACCAATTC-3′; *S Sac*I primer. 5′-GACTGCGTACAAGCTC-3′; *P Pst*I primer. 5′-GACTGCGTACATGCAG-3′; *M Mse*I primer: 5′-GATGAGTCCTGAGTAA-3′

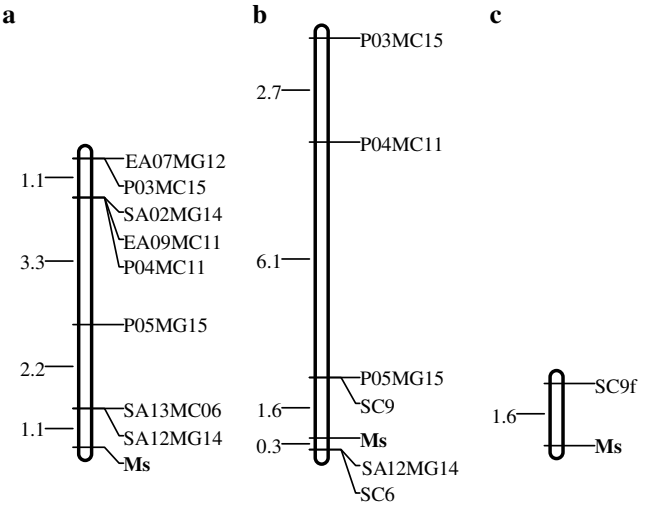

Fig. 2 Comparison of genetic linkage relationship of AFLP and SCAR markers analyzed on the three populations: **a** Popu1; **b** Popu2; **c** Popu3

on one direction to a size of 544 bp. Based on the extended sequences, primer pairs (SC9 for P05MG15 and SC6 for SA12MG14, Table 3) were designed and both allowed generating dominant polymorphism specific to the Ms allele from the sterile parent and the sterile individuals in the Popu2 (Fig. 3). The two SCARs exhibited consistent with each of the corresponding AFLP markers.

Development of SCAR markers tightly linked to the Mf allele

A primer pair was designed on outsides of both primer sites from which the SCAR marker SC9 for Ms allele was developed. With the primer pair SC9-3 (F: 5′-TCCGAGCTTGAGCCCTTGTCT-3′, R: 5′-GCGGC GCGTTGTATTCTTCTC-3′) for locus P05MG15, PCR amplification was performed at gradient annealing temperatures of 55–67°C and generated one identical band between the two parents: S148 and S103, but showed a larger product at lower annealing temperature and absent at higher annealing temperature for S467 (Fig. 4). The fragments amplified from the two parents S148 and S103, from which the Popu3 was developed, were retrieved, purified, cloned and sequenced. Fragments from both parents shared the length of 1,215 bp, suggesting that they were amplified from the same locus. Based on information from the sequence alignment, a primer pair (F: 5′-TATCTGGGAGGTGACAATGTTCT-3′, R: 5′-GTACTGGTTGGATTAGTGGCTGT-3′) was designed to amplify the marker locus specific to the Mf allele instead of the Ms allele. The PCR amplification conducted with 40 cycles and an annealing temperature of 66°C produced a band of 456 bp, which was present in fertile plants and absent in sterile plants in Popu3 (Fig. 5). Linkage analysis showed this SCAR marker SC9f was tightly linked to Mf, and the map distance was

Table 3 Primer sequence, annealing temperature and PCR product length of SCARs developed

SCAR names	Markers	Sequences of primer pairs 5′–3′	Annealing temperature (°C)	Product length
SC9	P05MG15	F:AAGATGTTAACCCGAGAGAT R: CTAAGAAACTTCGCCACAC	62	899
SC6	SA12MG14	F:AGCAAATCTCATCAATAACA R: ATACAAAACCCACCACA	57.5	493

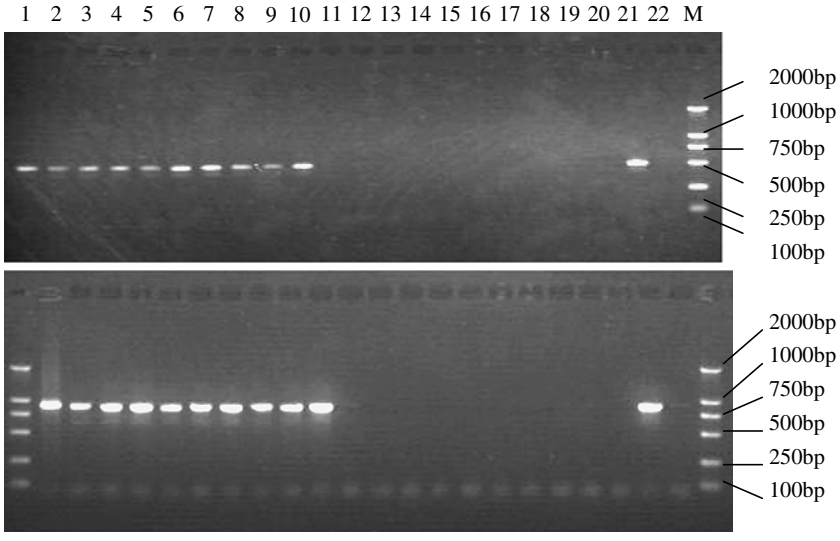

Fig. 3 Amplification patterns of the SCAR markers SC6 (*top*) and SC9 (*bottom*) in a subset of population Popu2. *1–10 lanes*: sterile individuals, *11–20 lanes*: fertile individuals, *lane 21*: S148, *lane 22*: S467, and *lane M*: DNA ladder

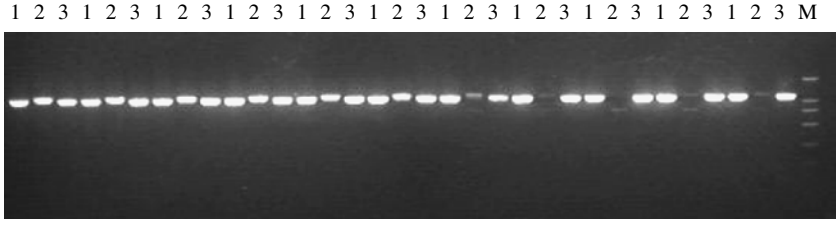

Fig. 4 Amplification pattern of SC9-3 at gradient annealing temperatures between 55–67°C (from *left* to *right*, with products at the temperature of 55°C omitted because of insufficient lanes at a row) on S148 (*lane 1*), S467 (*lane 2*) and S103 (*lane 3*), showing an identical band between S148 and S103 along the range of annealing temperature. *Lane M* is DNA ladder (2,000, 1,000, 750, 500 bp)

consistent with that between the corresponding SC9 and Ms (Fig. 2b, c).

Discussion

Inheritance of DGMS in *B. napus*

Previous studies indicate that two unlinked dominant genes regulate the DGMS in *B.napus* . However, classical genetic analysis with diverse segregating populations has been applied and verified that the gene responsible for the fertility restoration of DGMS is an allele at the Ms locus (Song et al. 2005). This is confirmed not only in the 609AB but also in the earlier reported DGMS line (data not shown). We presumed here that two possibilities may account for the different conclusions: either there may exist two kinds of restorer genes, the unlinked which has been identified before (Li et al. 1985; Zhou and Bai 1994; Hu et al. 2004) and the allelic which was identified here, that is, the sterility of DGMS may be controlled by two loci with multiple alleles, or the previous results are likely affected by ecotypic fertility restoration, for the sterility of DGMS is sometimes broken in some genetic backgrounds. In addition, the inference based on the expected segregation ratios is easily affected by the experimental conditions and the sizes of populations. In the present report, using the molecular marker technology, the inheritance of the fertility restoration for DGMS in

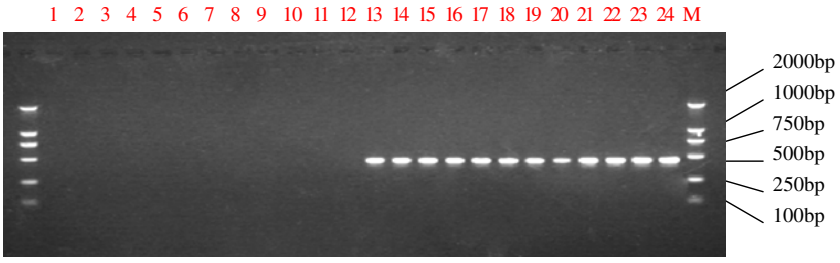

Fig. 5 The amplification pattern of the SC9f in subset of Popu3, *lanes 1 to 12*: sterile individuals; *lanes 13 to 24*: fertile individuals; *lane M*: DNA ladder

609AB was further characterized. The marker tightly linked to Ms allele proved to be linked to Mf allele, indicating that the restorer gene for DGMS is an allele at the Ms locus. Even so, a tightly linked dominant restorer gene or a pseudo-allele one could not be excluded before the associated genes are finally cloned.

AFLP marker development and conversion to SCAR

Out of 1536 primer combinations, only eight AFLP markers were polymorphic in the bulks selected from Popu1. The success rates were very low in contrast to some previous reports of AFLP analyses (Negi et al. 2000; Janeja et al. 2003; Liu et al. 2005). The two parents used in this study were not intentionally selected for AFLP analysis while the breeding for an elite sterile line was mainly taken into consideration. The populations of BC_1 generation possess potential difference from only one allele unlike near isogenic line (NIL), DH and F_2 population with genotypes determined by F_3 generation assessment, which can exhibit either difference of the two alleles at a locus. As to the present investigation, only BC_1 can be used as the segregating population, because the F_1 generation was sterile and cannot be employed to develop F_2 or DH population.

Although four AFLP markers developed based on Popu1 lost their polymorphism in examining Popu2, the remaining four markers included the nearest two, localized on both sides of the Ms locus, with map distances of 0.3 and 1.6 cM, respectively. Compared to previous research (Lu et al. 2004a, b), the distance of the marker to Ms is much nearer. The reasons that why the marker orders in the two populations were a little different might be the presence (in an individual of pupu1) of double exchanges on both sides flanking the Ms. The nearest two AFLP markers were successfully converted into SCARs SC6 and SC9, which are much more easily manipulated and more valuable in breeding for new DGMS lines with marker-assisted selection and in the next step of map-based cloning of the Ms.

To develop markers tightly linked to the Mf allele, we believe that AFLP analysis is a practical and effective technology as we did in the development of markers for Ms. In this case for validation of the allelism between Ms and Mf, however, it was not the best choice. Basing on the assumption that the Mf is allelic to the Ms, with the identical primer pair of an Ms marker the amplified region from the restorer parent was homologous to that from the sterile parent. PCR amplification of SC9-3 only produced a band in the sterile line S148 and the restorer S103, two parents of Popu3. Further, we recovered and sequenced the DNA fragment of SC9f, and the sequence alignment result indicated that the 456 bp sequence of the Mf marker SC9f was completely consistent with that of the expected PCR product (data not shown). These results suggested that the Mf marker was converted from the very Ms marker. Accordingly, we not only developed a SCAR marker for restorer breeding but also validated that the Mf is likely an allele at the Ms locus.

Application of DGMS

Whether DGMS was inherited by multiple allele or inherited by double unlinked genes, it can be used in a three-line approach for large-scale commercial production of rapeseed hybrid. This approach would generate a population of 100% sterile individuals with a temporary maintainer, with which the hybrid seed production is subsequently carried out, thus avoiding manual removal of fertile plants in the sterile line when using a two-line approach. Though there needs artificially eliminating the fertile plants in the propagation of the complete sterile line, that does not add much to overall cost of hybrid seed production because of the high reproducibility of rapeseed (Fu 1995). Nevertheless, the discrimination of the inheritance models is critical to the breeding of new homozygous sterile lines homologous to the maintainers, or of new maintainers homologous to the homozygous sterile lines. Compared to the digenic inheritance, the multiple allele inheritance has fewer genotypes in the segregation populations involved in the breeding program, of which there is only one fertile genotype capable of segregating sterile and fertile plants in selfed progenies. This is a very useful characteristic that could simplify the breeding process.

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References

Barret P, Guerif J, Reynoird JP, Delourme R, Eber F, Renard M, Chevre AM (1998) Selection of stable *Brassica napus–Brassica juncea* re-

combinant lines resistant to blackleg (*Leptosphaeria maculans*) 2 A 'to and fro' strategy to localise and characterise interspecific introgressions on the B. napus genome. Theor Appl Genet 96:1097–1103
Delourme R, Foisset N, Horvais R, Barret P, Champagne G, Cheung WY, Landry BS, Renard M (1998) Characterisation of the radish introgression carrying the Rfo restorer gene for the Ogu-INRA cytoplasmic male sterility in rapeseed (*Brassica napus* L.). Theor Appl Genet 97:129–134
Dong ZS, Liu CS, Jing JS, Zhuang SQ, Ran LG (1998) Selection and breeding of double dominant nuclear sterility of 896AB in *Brassica campestris* L. Acta Agron Sin 24(2):187–192
Dong ZS, Liu CS, Jing JS, Ran LG, Zhang XS, Dong J-G, Liu XX (1999) Genotype identification of restorer of double dominant genic sterilie line 896 AB in *Brassica campestris* L. Acta Agron Sin 25(2):193–198
Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. Focus 12:13–15
Fu TD (1995) Breeding and utilization of rapeseed hybrid. Hubei Science and Technology Press, Wuhan, pp 42–135
Gupta V, Mukhopadhyay A, Arumugam N, Sodhi YS, Pental D, Pradhan AK (2004) Molecular tagging of erucic acid trait in oilseed mustard (*Brassica juncea*) by QTL mapping and single nucleotide polymorphisms in FAE1gene. Theor Appl Genet 108:743–749
Hansen M; Hallden C, Nilsson N, Sall T (1997) Marker-assisted selection of restored male-fertile *Brassica napus* plants using a set of dominant RAPD markers. Mol Breed 3:449–456
Hu SW, Yu CY, Zhao HX, Lu M (2002) Development of GMS homozygous two-type line 803AB from dominant GMS Shaan-GMS in *Brassica napus* L. Acta Agric Boreali-occidentalis Sin 11(4):25–27
Hu SW, Yu CY, Zhao HX, Lu M, Zhang CH, Yu YJ (2004) Identification and genetic analysis of the fertility restoring gene for dominant genic male sterility accession "Shaan-GMS" in *Brassica napus* L. J Northwest Sci-Tech Univ Agric For (Nat Sci Ed) 32(4):9–12,18
Janeja HS, Banga SS, Lakshmikumaran M (2003) Identification of AFLP markers linked to fertility restorer genes for tournefortii cytoplasmic male-sterility system in *Brassica napus*. Theor Appl Genet 107:148–154
Jean M, Brown GG, Landry BS (1997) Genetic mapping of nuclear fertility restorer genes for the 'Polima' cytoplasmic male sterility in canola (*Brassica napus* L.) using DNA markers. Theor Appl Genet 95:321–328
Jean M, Brown GG, Landry BS (1998) Targeted mapping approaches to identify DNA markers for the 'Polima' CMS of canola (*Brassica napus* L). Theor Appl Genet 97:431–438
Ke LP, Sun YQ, Liu PW, Yang GS (2004) Identification of AFLP fragments linked to one recessive genic male sterility (RGMS) in rapeseed (*Brassica napus* L.) and conversion to SCAR markers for marker-aided selection. Euphytica 138:163–168
Kosambi DD (1944) The estimation of map distance from recombination values. Ann Eugen 12:172–175
Lander E, Green P, Abrahamson J, Barlow A, Daley M, Lincoln S, Newburg L (1987) Mapmaker: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174–181
Li SL,Qian YX, Wu ZH (1985) Inheritance and utilization of genetic male sterility in rapeseed(*Brassica napus* L.). Acta Agric Shanghai 1(2):1–12
Li SL, Qian YX, Wu ZH, Stefansson.BR (1988) Genetic male sterility in rape (*Brassica napus* L.) conditioned by interaction of genes at two loci. Can J Plant Sci 68:1115–1118
Li SL, Zhou XR, Zhou ZJ, Qian YX (1990) Inheritance of Genetic male sterility (GMS) and its utilization in rapeseed (*Brassica napus* L.). Crop Res 4(3):5–8
Lincoln S, Daly M, Lander E (1992) Constructing genetic maps with MAPMAKER/EXP 3.0. Whitehead Institute Technical Report, 3rd edn. Whitehead Technical Institute, Cambridge
Liu DF (1992) The theoretical studies on the restoration for dominant genic male sterility in plants. Heriditas 14(6):31–36
Liu ZW, Fu TD, Tu JX, Chen BY (2005) Inheritance of seed colour and identification of RAPD and AFLP markers linked to the seed colour gene in rapeseed (*Brassica napus* L.). Theor Appl Genet 110:303–310
Lu GY, Yang GS, Fu TD (2004a) Molecular mapping of a dominant genic male sterility gene Ms in rapeseed (*Brassica napus* L.). Plant breed 123:262–265
Lu GY, Yang GS, Fu TD (2004b) Linkage map construction and mapping of a dominant genic male sterility gene (Ms) in *Brassica napus*. Acta Genet Sin 31(11): 1309–1315
Manzanares-Dauleux MJ, Delourme R, Baron F, Thomas G (2000) Mapping of one major gene and of QTLs involved in resistance to clubroot in *Brassica napus*. Theor Appl Genet 101:885–891
Mathias R (1985) A new dominant gene for male sterility in rapeseed *Brassica napus*. Z Pflanzencichitg 94(2):170–173
Michelmore RW, Paran I, Kesseli RV (1991) Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. Proc Natl Acad Sci USA 88:9828–9832
Negi MS, Devic M, Delseny M, Lakshmikumaran M (2000) Identification of AFLP fragments linked to seed coat colour in *Brassica juncea* and conversion to a SCAR marker for rapid selection. Theor Appl Genet 101:146–152
Pilet ML, Delourme R, Foisset N, M Renard (1998) Identification of QTL involved in field resistance to light leaf spot (*Pyrenopeziza brassicae*) and blackleg resistance (*Leptosphaeria maculans*) in winter rapeseed (*Brassica napus* L.). Theor Appl Genet 97:398–406
Plieske J, Struss D, Röbbelen G (1998) Inheritance of resistance derived from the B-genome of *Brassica* against Phoma lingam in rapeseed and the development of molecular markers. Theor Appl Genet 97:929–936
Sabharwal V, Negi MS, Banga SS, Lakshmikumaran M (2004) Mapping of AFLP markers linked to seed coat colour loci in *Brassica juncea* (L.). Czern Theor Appl Genet 109:160–166
Siebert PD, Chenchick A, Kellogg DE, Lukyanov KA, Lukyanov SA (1995) An improved PCR method for walking in uncloned genomic DNA. Nucleic Acids Res 23:1087–1088
Schierholt A, Becker HC, Ecke W (2000) Mapping a high oleic acid mutation in winter oilseed rape (*Brassica napus* L.). Theor Appl Genet 101:897–901
Somers DJ, Friesen KRD, Rakow G (1998) Identification of molecular markers associated with linoleic acid desaturation in *Brassica napus*. Theor Appl Genet 96:897–903
Somers DJ, Rakow G, Rimmer SR (2002) *Brassica napus* DNA markers linked to white rust resistance in *Brassica juncea*. Theor Appl Genet 104:1121–1124
Song LQ, Fu TD, Yang GS, Tu JX, Ma CZ (2005) Genetic Verification of Multiple Allelic Gene for Dominant Genic Male Sterility in 609AB (*Brassica napus L.*). Acta Agron Sin 31(7):869–875
Tanhuanpää P, Vilkki J, Vihinen M (1998) Mapping and cloning of FAD2 gene to develop allele-specific PCR for oleic acid in spring turnip rape (*Brassica rapa ssp oleifera*). Mol Breed 4:543–550
Varshney A, Mohapatra T, Sharma RP (2004) Development and validation of CAPS and AFLP markers for white rust resistance gene in *Brassica juncea*. Theor Appl Genet 109:153–159
Vos P, Hogers R, Bleeker M, Reijans M, Van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 23:4407–4414
Wang TQ, Huang ZS, Tian ZP, Dai WD (2001) The sterility identification and genetics analysis of newly bred GMS line Qianyou 2AB in *B napus* L. Southwest China J Agric Sci 14(1):46–49
Xu M, Bai MY, Wei YT (2003) Transfer male sterile gene of Chinese cabbage to inbred line 97A407. China Vegetable (2):8–10
Ying M, Dreyer F, Cai DG, Jung C (2003) Molecular markers for genic male sterility in Chinese cabbage. Euphytica 132:227–234
Zhang SF, Song ZH, Zhao XY (1990) Breeding of interactive genic male sterile line in chinese cabbage (*Brassica pekinensis* Rupr) and utilization model. Acta Hortic Sin 17(2):117–125
Zhou YM, Bai H-H (1994) Identification and genetic studies of the inhibition of dominant male sterility in *Brassica napus*. Plant Breed 113:222–226