Identification of AFLP markers linked to *Ms*, a genic multiple allele inherited male-sterile gene in Chinese cabbage

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As a first step toward cloning the genic multiple allele inherited male-sterile gene *Ms* in Chinese cabbage (*Brassica rapa* L. ssp *pekinensis* (Lour.) Olsson), we attempted the molecular tagging of this male-sterility locus using the amplified fragment length polymorphism (AFLP) technique. A BC₁ mapping population (130 plants) segregating for male sterility/fertility was constructed for the tagging using the bulked segregant analysis (BSA) method. Screening 144 primer combinations, four AFLP markers were identified to be tightly linked to the *Ms* gene, the nearest one of which, AFLP01, was converted to a SCAR marker for the marker-assisted selection (MAS). Finally, a genetic map of the *Ms* gene was constructed with four AFLP markers and one SCAR marker, covering a total interval of 13.9 cM. Two markers AFLP01(SCAR01) and AFLP04 flanked the *Ms* locus at distances of 2.3 and 7.8 cM, respectively.

Key Words: Chinese cabbage, multiple allele inheritance, genic male sterility, AFLP, SCAR.

Introduction

Chinese cabbage (*Brassica rapa* L. ssp *pekinensis* (Lour.) Olsson) which originated in China is an important vegetable crop in extratropical, subtropical and tropical regions of the world. It is a diploid species with $2n=20$ chromosomes, and has a haploid genome size of about 550 Mbp (Arumuganathan and Earle 1991). It is a typical cross-pollinated plant with hermaphroditic flowers and obvious heterosis.

Male-sterile lines are ideal for the cross-breeding of Chinese cabbage. Male-sterile materials in Chinese cabbage can be classified into genic male sterility (GMS) and cytoplasmic male sterility (CMS) (Van der Meer 1987, Michael 2004). Most CMS lines in Chinese cabbage were bred from Ogu or Polima CMS source (Zhang *et al.* 2006). The male sterile lines with the Ogu CMS source cannot be used in the hybrid seed production for some defects, such as the leaf etiolation and the nectary degeneration (Ren and Cao 1992). On the other hand, those which were bred from the Polima CMS source have been used in hybrid seed production in China, but the thermal-sensitive sterility limited its performance in the large scale production (Zhang *et al.* 2001).

Compared to CMS, GMS in Chinese cabbage has more obvious advantages, such as stable and complete sterility performance, extensive distribution of restorers, and no negative cytoplasmic effects. Genic male sterility in Chinese cabbage was mostly considered as being controlled by a

monogenic recessive gene or a dominant gene. If a testcross is employed during the selection of maintaining lines of the male sterility, the percentage of male sterile plants in these lines only reaches 50%. A pronounced drawback to using such male-sterile resources is the required manual removal of the fertile plants from the female parent line in seed production stage.

Feng *et al.* (1995) obtained four stable hereditary lines of Chinese cabbage comprising 100% male sterile plants by crossing male sterile plants with male fertile plants between AB lines. Later, a classical genetic analysis was designed and carried out to reveal the mechanism of inheritance of the male-sterility locus, and a genetic hypothesis of a genic multiple allele male-sterile gene was proposed (Feng *et al.* 1996). This hypothesis could satisfactorily explain most of our data. This model proposes the existence of a single locus with three alleles, the “*Ms*” allele for male sterility, the “*ms*” allele for fertility, and the “*Ms^f*” for fertility restoration. The dominant-recessive > relationship among these alleles was proposed to be $Ms^f > Ms > ms$. According to this genetic model, in order to obtain a male sterile line with 100% male sterile plants (*Msms*), a temporary maintainer line (*msms*) is crossed with the homozygous sterile line (*MsMs*) (Fig. 1). Hybrid seed production could be subsequently carried out by using this population as the female parent. Thus, the manual removal of fertile plants in the sterile line, a necessary step in a two-line system, could be obviated.

Following this model, the male-sterile allele could be easily transferred into normal fertile lines. Thus, this allele could be used to breed new male-sterile lines in Chinese cabbage. This model may also be used to guide the research on male sterility in other crops, such as rape (Song *et al.* 2006).

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Fig. 1. Genetic model of genetic multiple allele male sterile line in Chinese cabbage

The model proposed by Feng *et al.* (1996) has been shown to be practical for hybrid seed production. The allele has been transferred to Chinese cabbage of different ecotypes (Wang *et al.* 2005, Feng *et al.* 2007). Some high quality male-sterile lines have been bred successfully. With these male-sterile materials, two commercial hybrids have been registered and released in China.

Molecular marker techniques have been widely used in genetic mapping, map-based cloning, genetic diversity analysis, variety protection, and breeding selection. Some studies of the male sterility in *Brassica* crops have been published. Various molecular markers that are linked to the restorer genes for Ogu, Pol and Tour CMS in *Brassica* have been identified (Hansen *et al.* 1997, Jean *et al.* 1997, 1998, Janeja *et al.* 2003). Miao *et al.* (2003) identified four sequence-tagged site (STS) markers that are tightly linked to a recessive male-sterile gene in Chinese cabbage; A group of homologs of the male-sterility or fertility gene have been isolated, and the relevant biological functions have been revealed (Wang *et al.* 2005, Cao *et al.* 2006, Jiang and Cao 2007, Li *et al.* 2008, Tian *et al.* 2009); Zhang *et al.* (2008) identified a random amplified polymorphic DNA (RAPD) marker that is tightly linked to a dominant male sterile gene and converted it into a sequence characterized amplified region (SCAR) marker. Wang *et al.* (2000), Ke *et al.* (2005), Yi *et al.* (2006), Hong *et al.* (2006), Lei *et al.* (2007) and Huang *et al.* (2007) identified a set of molecular markers that are linked to the male sterile gene in *Brassica napus*. Wang *et al.* (2000a, 2000b) identified 12 amplified fragment length polymorphism (AFLP) markers, a SCAR marker and an extended random primer amplified region (ERPAR) marker that are closely linked to a dominant male-sterile gene in *Brassica oleracea*, and mapped the gene to linkage group 09, corresponding to chromosome 3 of *B. oleracea*.

The "Multiple-allele model" of male sterility created a new way to use the genic male-sterile materials in Chinese cabbage; however, the special inheritance model of this locus results in six total genotypes, four of which present identical fertile phenotypes, while the other two are sterile. Traditionally, it takes more work and time to determine the

desired genotype through a testcross. Marker-assisted selection is an efficient way to solve the problem, though tightly-linked and easily-detected molecular markers are needed for this type of analysis.

In the present study, we focused on constructing an AFLP genetic map of the *Ms* gene. The application of one converted SCAR marker to MAS is also discussed.

Materials and Methods

Plant materials and development of the mapping population

The AB01-1 is a male sterile plant of a previously bred AB line of Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis* (Lour.) Olsson) with sterile plants (AB01-1, *MsMs*) and fertile plants (AB01-2, *MsfMs*) segregated in a ratio of 1 : 1. It is a complete and stable male-sterile plant, with withered anthers and a normal pistil, which was employed as the female parent of the mapping population. The male parent was SD2 (*msms*), a fertile inbred line in Chinese cabbage.

A BC₁ population was constructed to tag the genic multiple allele inherited male-sterile locus. A plant of AB01-1 was crossed to SD2, resulting in a F₁ population with 100% male-sterile plants. A segregating population was obtained by backcrossing a sterile F₁ plant with SD2. The sterility was determined during flowering.

DNA extraction and preparation of bulks

Genomic DNA was isolated from fresh leaves of parents and BC₁ plants following the procedure of Pierre and Marechal-Drouard (1992) with minor modifications. Grind 1.5 g of the sample in liquid nitrogen, then transfer the sample into a centrifuge tube containing 10 ml of extraction medium (100 mM sodium acetate, 50 mM EDTA, 500 mM NaCl, 2% soluble PVP, 20 mM cysteine, and 1.4% SDS) and incubate at 65°C for 30 min; add 5 ml of potassium acetate (5 M, pH=4.8) and incubate on ice for 30 min; centrifuge at 10,000 g at 4°C for 10 min; decant the supernatant into another centrifuge tube; add 0.6 vol. isopropanol and incubate at -20°C for 20 min; centrifuge at 10,000 g at 4°C for 10 min; pour off the supernatant, add 25 ml of 100% ethanol, and wash for one h; centrifuge at 10,000 g at 4°C for five min; pour off the ethanol and pre-dissolve DNA pellet in 500 µl of water; add 50 µg of RNase (10 mg/ml) and incubate at 37°C for 30 min; add 50 µl of NaAc (3 M) and 500 µl of isopropanol; centrifuge at 10,000 g at 4°C for 15 min; wash with 70% (v/v) ethanol and air dry for 5 min; redissolve DNA pellet in 50 µl of ddH₂O.

A spectrophotometer was used to quantify DNA quantification. Fertile and sterile bulks were prepared for BSA (bulk segregant analysis) by pooling equal quantities of DNA from ten fertile (*msms*) and ten sterile (*Msms*) individuals.

AFLP analysis

AFLP analysis was performed using the method described by Vos *et al.* (1995) with minor modifications. One hundred and forty four primer combinations were tested for

polymorphism analysis between the bulks. Genomic DNA (500 ng) was digested for 1 h at 37°C in a total volume of 25 µl (5 U *Pst*I, 5 U *Mse*I, 10 mM Tris-HAc, 10 mM MgAc, 50 mM KAc, 5 mM DTT, 50 ng/µl BSA). After heat-inactivation of restriction enzymes at 75°C for 15 min, 25 µl of a solution containing 5 pmol *Pst*I-adapters, 50 pmol *Mse*I-adapters, 1 U T4 DNA-ligase, 1 mM ATP in 10 mM Tris-HAc, 10 mM MgAc, 50 mM KAc, 5 mM DTT, 50 ng/µl BSA was added and incubated at 37°C for 3 h. A 5-µl aliquot of a tenfold dilution of the restriction/ligation mixture was pre-amplified with primers complementary to the adaptor sequence and including one additional selective nucleotide at the 3' end of an *Pst*I directed primer (P+G) and an *Mse*I directed primer (M+C). Pre-amplification reaction contained 0.2 mM dNTP, 1×PCR buffer (20 mM Tris-HCl (pH 8.4); 20 mM KCl; 10 mM (NH₄)₂SO₄; 1.5 mM MgCl₂), 27 ng each of *Pst*I+G/*Mse*I+C, and 1 U of *Taq* DNA polymerase. The sequences of adaptors and pre-amplification primers are presented in Table 1. The amplicon was diluted 50-fold and used as template DNA in selective amplification reactions, which were carried out using with two primers additional selective nucleotides at the 3' end for all the enzyme digests. To identify markers, 144 primer combinations were made with the 12 *Pst*I + GNN and 12 *Mse*I + CNN primers (Table 2). Selective PCR was performed with the aforementioned 1×PCR buffer, 0.2 mM dNTP, 30 ng *Mse*I+CNN, 15 ng *Pst*I+GNN, and 0.4 U of *Taq* DNA polymerase. All amplifications were performed in a BIO-RAD iCycler with the PCR conditions described by Vos *et al.* (1995). PCR products were mixed with an equal volume of loading dye (98% formamide, 10 mM EDTA, 0.001% each of xylene cyanol and bromphenol blue). Samples were denatured at 94°C for 5 min and separated on a 6% denaturing polyacrylamide gel at 85 W. After electrophoresis, the gel was developed using a silver staining kit (Bioneer, Daejeon, Korea).

Conversion of AFLP markers to SCAR markers

Gel pieces containing the AFLP markers were sliced and boiled for 5 min in 100 µl of sterile water. After centrifugation, a 5-µl aliquot of the supernatant was amplified with the corresponding selective primer combination and the same PCR conditions that were used in the selective amplification. The amplicons were separated on a 1.0% agarose gel, then eluted with a gel extraction kit (Tiagen, Beijing, China). The eluted AFLP fragments were cloned into the pGEM-T Easy Vector system I (Promega, USA). The AFLP clones were sequenced from both ends using an ABI PRISM 377 automated sequencer. Primer pairs for developing SCAR markers were designed based on the two terminal sequences using the Primer3 program (Rozen and Skaletsky 1996). PCR conditions were optimized with the pre-amplification products of the two parents and the two bulks as templates. Subsequently, these conditions were used to amplify genomic DNA from the 20 BC₁ plants that constituted the two bulks. PCR products were separated on a 2% agarose gel and a 6% denaturing polyacrylamide gel. SCAR

Table 1. Sequences of adapters and primers that were used in the AFLP analysis

Enzyme	Adapter sequence (5' > 3')	Pre-amplification primers (5' > 3')
<i>Pst</i> I	Oligo 1 ctcgtagactgcgtacatgca	gactgcgtacatgcag
	Oligo 2 catctgacgcatgt	
<i>Mse</i> I	Oligo 1 gacgatgagtcctgag	gatgagtcctgagtaac
	Oligo 2 tactcaggactcat	

Table 2. Sequences of selective-amplification primers used in the AFLP analysis

	1	2	3	4	5	6	7	8	9	10	11	12
P+	ca	cg	ct	ga	gc	gt	ta	tg	tt	ac	at	ag
M+	ca	cg	ct	ga	gc	gt	ta	tg	tt	ac	at	ag

P: pre-amplification primer for *Pst*I; M: pre-amplification primer for *Mse*I

markers were considered successful when a fragment was amplified with the primer pairs and showed polymorphism between fertile and sterile plants under the optimized condition in the AFLP marker analysis.

Linkage analysis

Linkage analysis of markers and traits was carried out using MAPMAKER version 3.0 (Lander *et al.* 1987). A genetic map was constructed based on a minimum LOD score of 3 and a maximum recombination fraction of 0.4. Map distances in centimorgans (cM) were calculated using the Kosambi mapping function (Kosambi 1944).

Results

Phenotyping of male fertility and sterility

To tag the male sterile gene, a BC₁ population was used. Only two phenotypes, male sterile and fertile, were observed. Male sterile and fertile plants could be distinguished unambiguously via visual examination. The BC₁ population (130 plants) segregated for male sterility (61) and fertility (69) according to the expected 1 : 1 ratio for monogenic inheritance.

AFLP analysis

To identify markers linked to the *Ms* gene, AFLP analysis was carried out with BSA. We tested 144 *Pst*I/*Mse*I primer combinations for DNA amplification polymorphism in the two bulks. The *Pst*I+GTA primers did not yield a product when combined with any of the 12 *Mse*I+CNN primers. The remaining 132 primer combinations yielded approximately 40 bands per primer combination. The frequency of polymorphism between two parents was around 18%, with a range of 2–30%. Of the 1,590 polymorphic loci detected between the two parents, 10 displayed polymorphism with respect to the two bulks, in which four were shown in Fig. 2. The size of the amplified fragments ranged from 50 to 800 bases. Primer

combinations yielding polymorphic amplification patterns between the bulks were further examined in individual BC₁ plants that were used to prepare the bulks. A total of 40 individuals (20 of each type) were examined. Four primer combinations gave polymorphic amplification patterns in individual plants; the sizes of the polymorphic amplicons ranged between 154 and 380 bp.

Conversion of AFLP markers into SCAR markers

The polymorphic fragments were eluted from the gel, re-amplified with the respective primers, ligated with a cloning vector, and transformed into *E. coli*. The polymorphic AFLP markers were cloned and sequenced. The sequences were subjected to BLAST analysis. None of them showed significant homology in the BLAST results.

Based on sequence of the AFLP clones, SCAR primers

were designed for PCR amplification. Only AFLP01 was successfully converted into a SCAR marker, which was subsequently named SCAR01 (Table 3). SCAR01 primers gave one amplicon of 255 bp in PCR. This band was present almost exclusively in sterile individuals and absent in fertile individuals (Fig. 3). When 130 BC₁ individuals were tested with the SCAR primers, the amplicon was obtained in 59 sterile plants; only one out of the 69 fertile plants produced the amplicon. As such, a strong association was found between the SCAR01 marker and the male sterility.

Linkage analysis

In the present study, a total of 130 BC₁ plants were screened with four AFLP markers and one SCAR marker. All of these markers were found to segregate normally within the mapping population (Table 4). The linkage map that

Fig. 2. AFLP profiles showing the polymorphic amplification patterns that were associated with the multiple allele inherited male sterility trait obtained with primer combinations AFLP01 PggT/Mcac, AFLP02 Pggc/Mcct, AFLP03 Pgga/Mcgc, AFLP04 Pgca/Mctt. The position of polymorphic band is indicated with an arrow. Pf fertile parent, Ps male sterile parent, Bf fertile bulk, Bs male sterile bulk.

Table 3. Details of PCR conditions and SCAR primers for amplifying male sterility-specific AFLP markers

Name	Acc. No.	Sequence of primer pairs (5'-3')	Size	Tm
SCAR01	FJ851467	F:CACGGATCATCAGCTCCATGT; R:GGTAGAAAACCAATCGAACCAAGA	255 bp	58°C

Fig. 3. Segregation of SCAR01 marker in BC₁ individuals. M DL2000 DNA ladder, Ps male sterile parent, Pf fertile parent, Bf₁–Bf₁₀ ten fertile BC₁ individuals, Bs₁–Bs₁₀ ten sterile BC₁ individuals. Recombinants are indicated by asterisks.

Table 4. Segregation of AFLP markers in the BC₁ population

S.no.	Name	Primer combination	Segregation pattern		Expected ratio	χ^2 ($\chi^2_{0.05,1}=3.84$)
			Absent	Present		
1	AFLP01	Pggt/Mcac	70	60	1:1	0.623
2	AFLP02	Pggc/Mcct	71	59	1:1	0.931
3	AFLP03	Pgga/Mcgc	69	61	1:1	0.377
4	AFLP04	Pgca/Mctt	69	61	1:1	0.377

was constructed with these markers is presented in Fig. 4. Linkage analysis of the segregation data for these markers was performed with MAPMAKER version 3.0; our results indicate that all markers map to the same linkage group with a minimum LOD score of 3. We could localize these markers and the *Ms* locus to an interval of 13.9 cM. *Ms* was found to be flanked by the AFLP01 (SCAR01) and AFLP04 markers at genetic distances of 2.3 and 7.8 cM, respectively.

Discussion

Since this genic multiple allele inherited male-sterile material has so many advantages, considerable attention has been paid to it. Based on the proposed inheritance model of multiple alleles, the breeding for a male sterile line with 100% male sterility and 100% male sterile plants is easy; it requires more time and space to select plants with desired genotypes, however, because a testcross must be involved at each generation. The identification of molecular markers that are closely linked to the *Ms* gene allows for a more efficient and earlier selection of the desired plants. For practical use, we sought to establish a tightly linked SCAR marker in this research.

Molecular markers tightly linked to genes that are responsible for traits of economic importance are valuable in marker-assisted selection breeding programs. The *Ms*-specific markers identified here should greatly enhance the selection efficiency in backcross breeding by using a PCR-based selection of plants carrying *Ms* in an early seedling stage. Though only one SCAR marker has been developed, only three recombinants were detected in 130 BC₁ plants; this result suggests that this SCAR marker can correctly predict the genotype in all but 2.3% of the samples. Furthermore, it can be easily detected on agarose gels. Therefore, this SCAR marker can be used in backcrosses to breed male-sterile lines carrying an *Ms* gene.

Markers closely linked to specific traits can be easily identified by bulked segregant analysis (BSA) if an appropriate population is available. BSA, devised by Michelmore *et al.* (1991), has been the most powerful and widely employed method for the molecular tagging of traits. For tagging qualitative traits that can be easily classified into two or more discreet classes, BSA is particularly efficient. The advantage of this approach is that the likelihood of identifying false positive markers is very small (Michelmore *et al.* 1991,

Fig. 4. Genetic linkage map of the *Ms* locus generated with the BC₁ population of AB01-1/SD2. The genetic distance in centimorgans (cM) was calculated using the Kosambi mapping function.

Meksem *et al.* 1995, Li *et al.* 1998). The combination of the AFLP technique and BSA has been effectively used to generate molecular markers that are closely linked to the genes of interest in many crops (Chantret *et al.* 2000, Miftahudin *et al.* 2002, Asnaghi *et al.* 2004). In most of these studies, F₂ populations were used to establish pools (Gallego *et al.* 1998), however, the dominant male-sterile gene results in a F₁ population with 100% male-sterile plants. In the present case, we employed BSA and used BC₁ plants to assemble DNA pools. We successfully identified four AFLP markers and one SCAR markers that are closely linked to the *Ms* gene in Chinese cabbage.

Although AFLP markers are very useful for linkage mapping, the generation of these markers is often expensive and labor intensive. Therefore, these markers cannot be conveniently used for large-scale marker-assisted plant breeding. For practical purposes, these markers need to be converted to SCAR markers. SCAR markers are an ideal choice for MAS, because they are detected by single genetically defined loci, are identified as distinct bands in agarose gels, are easier to score, are less sensitive to reaction conditions, and are more reproducible. Many successful cases of such conversions have been reported (Shan *et al.* 1999, Negi *et al.* 2000, Miftahudin *et al.* 2002). Not all AFLP markers can be successfully converted, however, perhaps because undesired AFLP fragments contaminate the isolation of true fragments from gels. As demonstrated by Xu *et al.* (2001), the following factors probably cause this contamination. First, there is a difference in mobility between the visible, single ³³P-labelled-DNA strand and its invisible, complementary DNA strand (Vos *et al.* 1995); thus it is possible that other invisible AFLP bands can contaminate the isolation of a desired AFLP marker. Second, the presence of neighboring AFLP fragments may allow for contamination. In the

present study, we used the silver staining to visualize AFLP fragments. We believe that the most likely reason for contamination in the present study is neighboring AFLP fragments.

As the first step towards the map-based cloning of the *Ms* gene, we identified markers flanking the *Ms* gene: AFLP01 (SCAR01) and AFLP04 at a 10.1 cM interval on the genetic linkage map. These two tightly flanking markers will be useful as starting points for gene isolation and cloning (Tanksley *et al.* 1989).

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