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Full Length Research Paper

Molecular mapping of *MS-cd1* gene in Chinese kale

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A dominant male sterility (DGMS) line 79-399-3 was developed from spontaneous mutation in *Brassica oleracea* var. *capitata* and has been widely used in the production of hybrid cultivar in China. In this line, male sterility is controlled by a dominant gene *Ms-cd1*. In the present study, primary mapping of *Ms-cd1* was conducted by screening a segregating population developed by four times backcrossing of *B. oleracea* var. *alboglabra* into a male sterile *B. oleracea* var. *italica* line harboring *Ms-cd1*. Bulked segregation analysis (BSA) was performed for 226 BC_4 individuals using SRAPs regarding of male sterility and fertility. Using 800 SRAP primers and 2,340 SRAP combined random primers, a primary map surrounding *Ms-cd1* was constructed. Eight markers closely linked to the target gene were identified, among which the closest one on each side to *Ms-cd1* was 0.53 and 5.04 cM, respectively. Markers linked closely to the *Ms-cd1* gene will enrich resources of molecular marker of *Ms-cd1* locus; also serve to lay the foundations for molecular-assisted selection in breeding program, as well as fine mapping and map-based cloning of *Ms-cd1* gene.

Key words: Sequence-related amplified polymorphism, DNA markers, linkage map, Chinese kale.

INTRODUCTION

Male sterility is defined as the failure of plants to produce functional anthers, pollen, or male gametes while the female reproduction is normal (Kaul, 1988). Based on its inheritance or origin, male sterility may be divided into nuclear male sterility (NMS), also called genic male sterility (GMS) and cytoplasmic male sterility (CMS). CMS systems are still convenient for the production of hybrid seeds because of their mode of maintenance and restoration of fertility (Budar and Pelletier, 2001). But genic male sterility (GMS) systems are regarded as a promising alternative to CMS, due to several advantages, such as the stable and complete male sterility; no negative cytoplasmic effect on yield as CMS might do (Yi et al., 2006). The percentage of male-sterile plants in an ideal male-sterile line should be 100% and the combining ability of its major economic traits should also be high. In order to obtain such a high quality male-sterile line, it is crucial to search the male sterility source and reveal the underlying genetic mechanism. A dominant male-sterility gene *Ms-cd1* was identified as a spontaneous mutation in a spring cabbage line 79-399-3 (Fang et al., 1997), which has been well utilized in commercial cabbage hybrid seed production. The male sterility controlled by *Ms-cd1* showed abnormal callous degeneration and failure of microspore separation during anther development and

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Abbreviations: GMS, Genic male sterility; **DGMS,** dominant male sterility; **NMS,** nuclear male sterility; **CMS** cytoplasmic male sterility; **SRAP,** sequence-related amplified polymorphism; **CTAB,** cetyltrimethylammonium bromide; **BSA,** bulked segregant analysis; **MAS,** marker-assisted selection; **SNP,** single nucleotide polymorphism; ***Ms-cd1,*** male sterility dominant gene. **PCR,** polymerase chain reactions; **RAPD,** random amplified polymorphic DNA; **ORFs,** open reading frames; **SSR,** simple sequence repeat; **SCAR,** sequence characterized amplified region; **ERPAR,** extended random primer amplified region; **AFLP,** amplified fragment length polymorphism.

temperature sensitivity (Fang et al., 1997), enabling creation of a homozygous (*Ms-cd1/Ms-cd1*) genotype by selfing heterozygous plants under permissive temperatures. However, identification of the homozygous male-sterile *Ms-cd1/Ms-cd1* plants by testcrossing with male-fertile testers is a time-consuming, laborious process because of the biennial nature of cabbage; since male-sterile plants can only be selected when the crop is flowering. This requires vernalization by either over winter or cold treatment during the warm season.

Light-microscopic studies indicated that meiotic division stopped at the tetrad stage in sterile plants of 79-399 (Fang et al., 1997). Nevertheless, nothing is available about the molecular mechanism of the *Ms-cd1* gene in stopping meiotic division. Isolation of the *Ms-cd1* gene is therefore regarded as an important work, not only for developing specific PCR markers for MAS, but for understanding the mode of action of these genes. In previous studies, molecular markers linked to this gene were developed, including a RAPD marker with a linkage distance of 7.8 cM (Wang et al., 1998), a SCAR marker converted by the previous RAPD marker (Wang et al., 2000a) and an ERPAR marker (Wang et al., 2000b). Some differentially expressed genes during bud development were detected by cDNA-AFLP, which suggested that the dominant *MS-cd1* gene may disrupt proper separation of pollen from treds and leading to the suppressed expression of a number of genes (Lou et al., 2007). Microarray analysis identified some differentially expressed genes between fertile and sterile individuals belong to anther developmental pathway and especially those expressed in tapetum cells (Kang et al., 2008). Wang et al. (2005) indirectly mapped *MS-cd1* onto linkage group O9, corresponding to chromosome 3 of *Brassica oleracea*. This region corresponds to the top of chromosome 5 in *Arabidopsis thaliana* through the comparative genomics analysis. Because traditional breeding is a rather long procedure, cheap, fast and reliable PCR-based markers were to be made available for the *Ms-cd1* locus, this would greatly improve the efficiency of breeding programs using the *Ms-cd1* allele. However, the genetic distance between these markers and *MS-cd1*was not close enough for practical utilization in marker assisted selection. Hence, it was needed to construct a genetic linkage map of *Ms-cd1* gene and seek suitable flanking marker.

Sequence-related amplified polymorphism (SRAP) was developed by Li and Quiros (2001) for the purpose of amplifying open reading frames (ORFs). The SRAP marker system is a simple and efficient method that can be adapted for a variety of purposes in different species, including map construction, gene tagging, genomic and cDNA fingerprinting and map-based cloning (Sun et al., 2007; Li et al., 2003; Gao et al., 2007). The SRAP system is more reproducible than that of RAPD and is less complicated than AFLP (Li and Quiros, 2001).

The purpose of the present study was to construct a genetic linkage map of *Ms-cd1* gene in Chinese kale through combining SRAP molecular technologies with BSA method and to gain more markers linked closely to the *Ms-cd1* locus. Achievement of suitable flanking markers will found the solid base on the molecular-assisted selection in the breeding program and map-based cloning of *Ms-cd1* in the future research.

MATERIALS AND METHODS

Plant material

Three individuals of Chinese kale came from a BC_4 population, which was derived from a cross between male-sterile broccoli and a Chinese kale line as the recurrent parent.

DNA extraction

Genomic DNA was extracted from freeze-dried leaf tissue using a modified cetyltrimethylammonium bromide (CTAB) method (Haymes 1996). DNA concentration and purity was measured by a Beckman spectrophotometer (NanoDrop ND-1000) at an absorption wavelength ratio of 260 nm versus 280 nm.

Primers

In this experiment, several molecular markers closely linked to the *Ms-cd1* gene in *B. oleracea* were screened out from 800 SRAP primers (Li and Quiros, 2001; Wang et al., 2005; Budak et al., 2004) and 3,200 compound primers of SRAP primers, AFLP primers (Vos et al., 1995) and SSR primers derived from *Brassica rapa* (Suwabe et al., 2002; Choi et al., 2007).

SRAP procedure

SRAP PCR amplification was the same as that of Li and Quiros (2001). DNA amplification fragments were separated on a denaturing 8% acrylamide gel and visualized using silver staining (Ricci et al., 1999).

Data analysis

The polymorphic bands were scored independently as being either present or absent in each genotype. Presence or absence of each fragment was recoded as “1” or “0”, where “1” indicated the presence of a specific allele and “0” indicated its absence and a letter “u” denoted it was missing. Only strong, reproducible and clearly distinguished bands were used in the analysis. The segregation of each marker was analyzed and linkage analysis of the BC_4 population was carried out using the program JoinMap® version 3.0 (http://www.kyazma.nl/). Marker order was determined with a log odds (LOD) score threshold of 3.0 and map distances were estimated by the Kosambi function (Kosambi, 1944).

RESULTS

SRAP analysis

In addition to the primer combination of the standard SRAP method, five different primer combinations were used in the method, for example SRAP-SSR, SRAP- AFLP

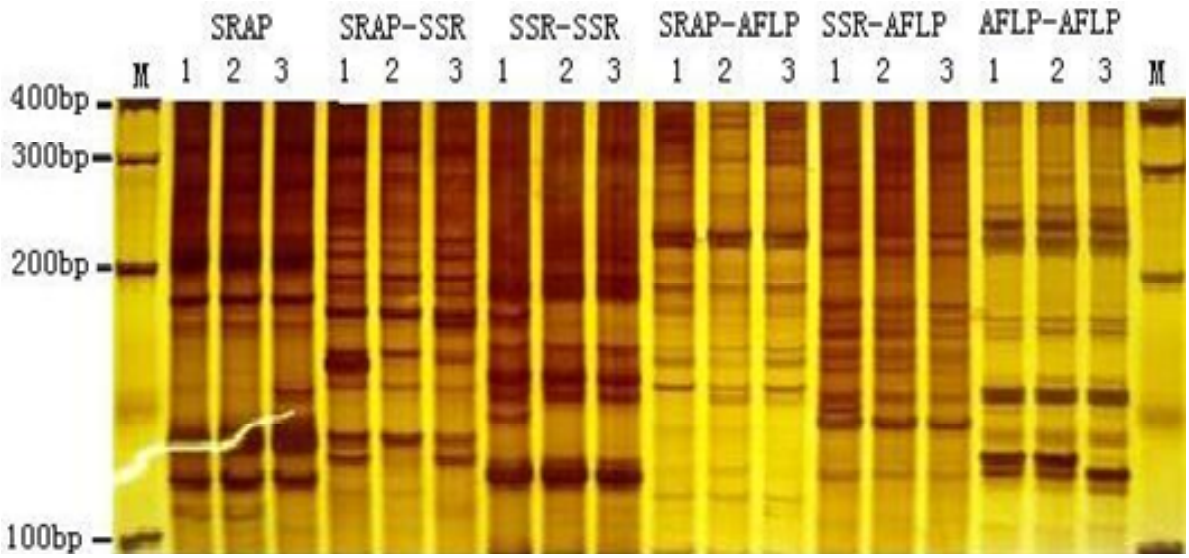

Figure 1. Examples of amplification patterns by various primer combinations denoted at the top of the photograph. The three samples (1 to 3) were taken from the BC_4 population of Chinese kale. M: 100 bp ladder as a DNA size standard.

Table 1. Description of six styles of combinations of primers in screening the markers linked to the *Ms-cd1*.

Primer combinations	Primer sequences (5'-3')	Primer character
SRAP- SRAP	BMe6F: TGAGTCCAAACCGGACA	SRAP
	BEm9R: GACTGCGTACGAATTCAG	SRAP
SRAP-SSR	CuMe7F: TGAGTCCTTTCCGGTCC	SRAP
	ENA14F: CTTACGGTGGAAATGCTG	SSR
SSR-SSR	Ni2E12F: TTATCTGCTTGTCTTGGGGC	SSR
	FITO07.1R: TAAAATTTAATCGCAGCGCG	SSR
SRAP-AFLP	BMe12F: TGAGTCCAAACCGGAGA	SRAP
	M31: GATGAGTCCTGAGTAAAAA	AFLP
SSR-AFLP	BC64F: TTCCGTCCCTTCCCTAAACA	SSR
	M35: GATGAGTCCTGAGTAAACA	AFLP
AFLP-AFLP	E67: GACTGCGTACCAATTCGCA	AFLP
	M40: GATGAGTCCTGAGTAAAGC	AFLP

SSR-SSR, SSR-AFLP and AFLP-AFLP (Figure 1). This resulted in multiple unspecified priming sites on each DNA strand. The results showed that six styles of SRAP combined random primers can be used to detect polymorphic bands (Table 1). For the three selected samples from the Chinese kale BC_4 population, including two fertile plants and one sterile plant, good DNA amplifications were obtained by using the five primer combinations method. For the 224 primer combinations that were screened, 38.4% of the primers produced polymorphic bands. A total of 1659 bands were detected, among which 116 (6.99%) were polymorphic within the group of primer combinations (Table 2). These bands ranged in size from 100 to 400 bp and were shared by the three individuals.

Genetic mapping of *Ms-cd1*

In our experiment, 226 individuals from a Chinese kale BC_4 segregating populations were screened SRAP primers. A total of 800 standard SRAP primer combinations, 300 SSR primer combinations, 1425 SRAP-AFLP primer combinations and 615 SRAP-SSR primer combinations were screened and 8 markers closely linked to the *MS-cd1* gene were identified based on the bulked segregant analysis (BSA) method (Michelmore et al., 1991) (Table 3). The polymorphisms had consistent amplifications and exhibited clear banding patterns. Using the 8 identified markers, a linkage map was constructed (Figure 2). The two flanking markers, ENA14F-CoEm7R and BMe11f-CoEm8R, were located on either side of the *Ms-cd1* gene

Table 2. Statistics of the polymorphic bands detected by the SRAP technology with compound primers from the BC_4 population of Chinese kale.

Parameter	SRAP	SRAP-SSR	SSR-SSR	SRAP-AFLP	SSR-AFLP	AFLP-AFLP	Total
Total number of bands	304	284	267	348	254	202	1659
Number of polymorphic bands	24	15	23	25	21	8	116
Polymorphic ratio (%)	7.89	5.28	8.61	7.18	8.27	3.96	6.99

Table 3. Description of primers linked to the *MS-cd1* gene in the linkage group of 8 markers in Chinese kale.

Name	Primer sequence (5'-3')	Primer type	Length	Marker type
BEm12-RM52	BEm12R: GACTGCGTACGAATTCTC	SRAP	160	SRAP-AFLP
	M52: GATGAGTCCTGAGTAACCC	AFLP		
Fme2-CuEm18R	Fme2: TGAGTCCAAACCGGAGC	SRAP	150	SRAP
	CuEm18R: GACTGCGTACGAATTCCT	SRAP		
Fme4-M31	Fme4: TGAGTCCAAACCGGACC	SRAP	190	SRAP-AFLP
	M31: GATGAGTCCTGAGTAAAAA	AFLP		
Fme4-OD3R	Fme4: TGAGTCCAAACCGGACC	SRAP	230	SRAP
	OD3R: CCAAAACCTAAAACCAGGA	SRAP		
CoEm17R-E37	CoEm17R: GACTGCGTACGAATTCCA	SRAP	210	SRAP-AFLP
	E37: GACTGCGTACCAATTCACG	AFLP		
ENA14F-CoEm7R	ENA14F: CTTACGGTGGAAATGCTG	SSR	141	SSR-SRAP
	CoEm7R: GACTGCGTACGAATTATG	SRAP		
Bme11f-CoEm8R	Bme11f: TGAGTCCAAACCGGAAC	SRAP	121	SRAP
	CoEm8R: GACTGCGTACGAATTAGC	SRAP		
CuMe6F-E37	CuMe6F: TGAGTCCTTTCCGGTAA	SRAP	130	SRAP-AFLP
	E37: GACTGCGTACCAATTCACG	AFLP		

with a distance of 0.53 and 5.04 cM. Segregation of polymorphic bands generated by the ENA14F-CoEm7R marker was observed (Figure 3). Although these markers are dominant, they could be reasonably efficient for use in marker-assisted selection (MAS) when they are associated in the repulsion phase with the genes for selection (He et al., 2008).

DISCUSSION

In the present study, SRAP technology was used in combination with BSA to develop markers linked to the *Ms-cd1* gene. We screened 800 SRAP primers, 2,340 SRAP combined random primers and identified eight SRAP markers linked to the *Ms-cd1* gene. Finally, a primary map of *Ms-cd1* gene in Chinese kale was constructed. In the previous study by Wang et al. (2000a, 2000b) and Lou et al. (2007), RAPD, SCAR and AFLP markers were identified as being linked to the *Ms-cd1* gene in a small population and located only on one side. In the present study, we detected closer flanking markers on both sides of *Ms-cd1* gene in *B. oleracea*. The markers tightly linked with the *Ms-cd1* identified in this study will be useful in marker-assisted selection of male-sterile plants and will facilitate the isolation of the *Ms-cd1* gene by map-based cloning.

SRAP technique has been widely used in genetic diversity analysis and construction of linkage map in *Brassica napus* (Riaz et al., 2001; Li and Quiros, 2001; Sun et al., 2007; Gao et al., 2007). In this study, SRAP primers were randomly combined with AFLP primers or SSR primers which were revealed successfully in polymorphism screening. The results indicated an extra primer resource for SRAP technology in addition to standard SRAP method. Furthermore, this new concept can be extended to the primers used in single nucleotide polymorphism (SNP), RAPD and as normal PCR primers.

In summary, a primary map of *Ms-cd1* gene in Chinese kale was constructed through screening SRAP primers and SRAP combined random primers. Based on the information of linkage map, suitable flanking markers will be chosen and used to screen the recombinant plant in a larger BC_4 population in future research. In addition, markers linked closely to the *Ms-cd1* gene will also serve to lay the foundations for future fine mapping and map-based cloning of *Ms-cd1* gene.

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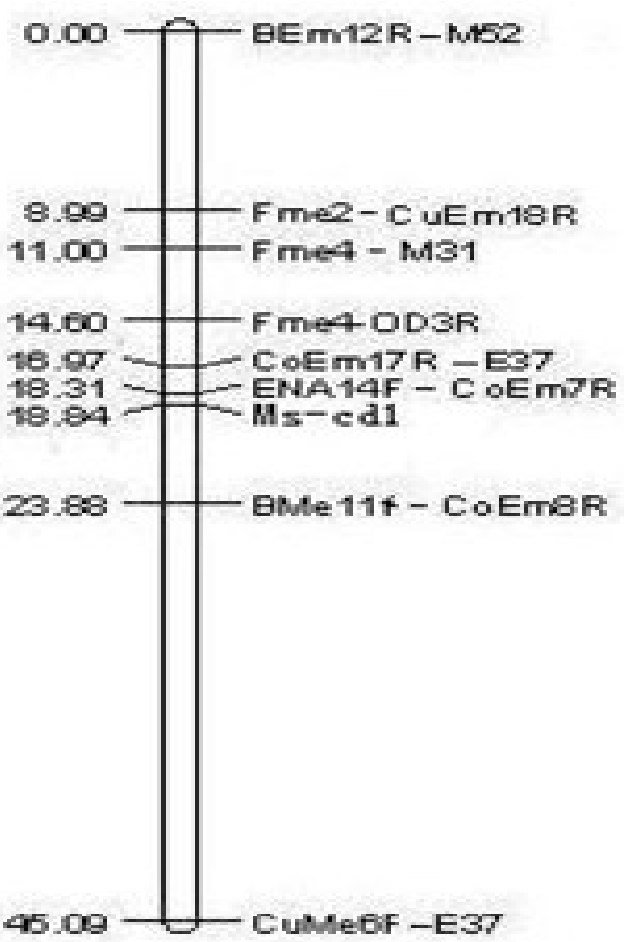

Figure 2. Genetic linkage map of the *Ms-cd1* locus constructed for a Chinese kale BC_4 population. Genetic distances (cM) are listed to the left of the map and marker loci to the right of the map.

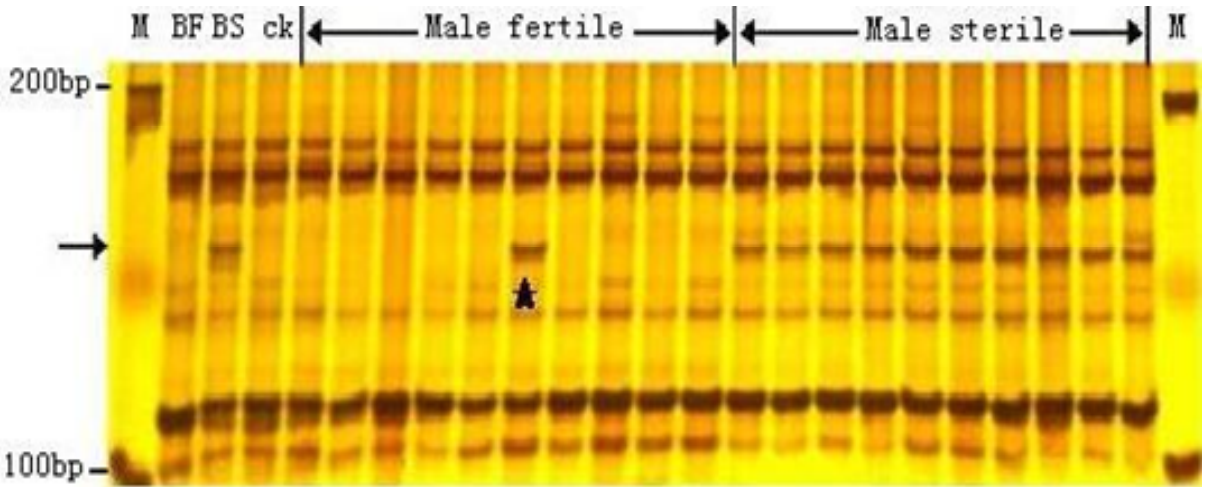

Figure 3. The amplification results of SRAP marker ENA14F-CoEm7R for individual plants in a BC_4 segregating population of Chinese kale. M: 100 bp DNA ladder; BF: male fertile bulk; BS: male sterile bulk; and ck: fertile parent; pentacle shows recombinant plant. Arrow head indicates the position of the marker.

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