African Journal of Biotechnology Vol. 9(35), pp. 5623-5628, 30 August, 2010
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 ©2010 Academic Journals

Full Length Research Paper

Verification of the utility of molecular markers linked to the multiple-allele male-sterile gene *Ms* in the breeding of male-sterile lines of Chinese cabbage (*Brassica rapa*)

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Accepted 29 April, 2010

To verify the molecular markers linked to the genic multiple-allele male-sterile gene *Ms*, an F_1 plant, which was generated by crossing the inbred line a20 and the male-sterile plant of the genic multiple-allele male-sterile AB line, was backcrossed with an a20 plant to develop BC_4 and BC_5 populations. Sequence-characterized amplified region (SCAR) marker *syau_scr01* and simple sequence repeat (SSR) marker *syau_m13*, which were linked to *Ms*, exhibited polymorphism between the 2 parents. The accuracies of these 2 markers in determining the plant genotype was 85 and 91.7%, respectively. The accuracy reached 100% when the 2 markers were used in combination. These results indicate that these 2 markers can be applied in the marker-assisted selection of the genic multiple-allele male-sterile line of Chinese cabbage.

Key words: Chinese cabbage, genic multiple-allele male sterility, marker-assisted selection, simple sequence repeat, sequence-characterized amplified region.

INTRODUCTION

Chinese cabbage (*Brassica rapa* L.) is a typical example of an allogamous plant with bisexual flowers, which exhibits obvious heterosis. The pattern of hybrid seed production is very important for the efficient utilization of heterosis. A male-sterile line is a reliable and economical choice for the production of hybrid seeds. Feng et al. (1995, 1996) discovered the multiple-allele inherited male-sterile gene in Chinese cabbage, which exhibited stable and complete inheritance, 100% male sterility, and no negative cytoplasmic effects, etc, and owing to these characteristics, this gene has attracted attention of many breeders. The model considers that a single locus is occupied by 3 alleles: "*Ms*" allele for male sterility, "*ms*" for fertility, and "Ms^f" for fertility restoration. The dominant- recessive relationship among these alleles was Ms^f>*Ms*>*ms.*

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Abbreviations: SCAR, Sequence-characterized amplified region; **SSR,** simple sequence repeat; **MAS,** marker-assisted selection; **BSA,** bulk segregant analysis.

The genotypes of sterile and fertile male plants of an AB line were *MsMs* and Ms^fMs, respectively, while that of the temporary maintainer line was *msms*. The male-sterile line with 100% sterile male plants was obtained by crossing the male-sterile plant (*MsMs*) of the AB line with a plant of the temporary maintainer line (*msms*).

In order to broaden the application scope of such male-sterile lines, a breeding program was designed to enable the simultaneous transfer of main horticultural characteristics and the male sterility gene (Li et al., 2006). The male sterility gene, which was discovered in the cylindrical ecotype of Chinese cabbage, has been transferred in various ecotypes, thereby creating some excellent male-sterile lines (Wang et al., 2005; Li and Feng, 2006).

If the target locus has a special inheritance mechanism, it requires more time, labor, and space to select plants with desired genotypes; moreover, it is necessary to include testcrosses in each generation for the breeding of such plants (Wang et al., 2005; Yue and Feng, 2005; Li et al., 2009). Molecular markers linked to the *Ms* allele can be effectively applied for the rapid selection of the desired plants. In the previous studies conducted at our laboratory, we have identified 2 sequence-characterized amplified

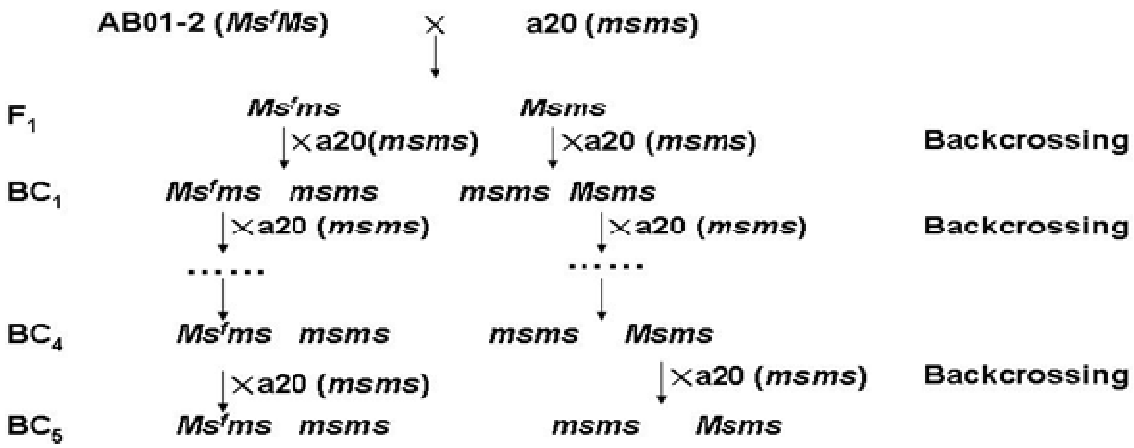

Figure 1. Genetic model for the breeding of a multiple-allele male-sterile line in Chinese cabbage.

region (SCAR) markers and 4 simple sequence repeat (SSR) markers linked to the *Ms* gene (Feng et al., 2009). With an aim to improve the application of marker-assisted selection (MAS) in the breeding of male-sterile lines, we investigated the efficacy of the aforementioned markers in the genotype identification of plants in this study.

MATERIALS AND METHODS

Plant materials

AB01-2, a fertile plant produced from a previously bred male-sterile AB line with a 1:1 segregation ratio of the sterile plants (AB01-1, *MsMs*) and fertile plants (AB01-2, *Ms^{f}Ms*) was used as the female parent and a20; an inbred line which was used as the male parent of the test population.

Development of the test population

According to the genetic hypothesis of the genic multiple-allele male sterility in Chinese cabbage proposed by Feng et al. (1996), a breeding program was designed for a new male-sterile line. An F_1 plant, generated by crossing AB01-2 and a20, was backcrossed with an a20 plant for 4 and 5 generations to obtain plants of BC_4 and BC_5 generations, respectively. The genotype of the sterile male plant in these 2 populations was *Msms*, while that of the fertile plant was *msms* (Figure 1).

Candidate markers used for the verification

The test population was verified using the following candidate markers: 2 SCAR markers, namely, *syau_scr01* and *syau_scr04*, and 4 SSR markers, namely, *syau_m13*, *cnu_m273*, *cnu_m030*, and *cnu_m295*, which were developed in a previous study of Feng et al. (2009) (Table 1).

DNA extraction and polymerase chain reaction analysis

Genomic DNA was isolated from fresh leaves of the parents and the BC_4 and BC_5 plants according to the procedure described by Guillemaut and Marechal-Drouard (1992). The polymerase chain reaction (PCR) system and the reaction profile described by Feng et al. (2009) were employed in this experiment.

RESULTS

Development of BC_4 population and phenotyping of the individuals

According to the model shown in Figure 1, among the 60 BC_4 plants, 33 were fertile and 27 were sterile males, which accorded with the theoretical segregation ratio of 1: 1 ($X^2_{0.05} = 0.417 < 3.841$).

Verification of candidate markers between the 2 parents

We used 6 primers to identify polymorphisms between the 2 parents, namely, AB01-2 and a20 (Table 1). The results indicated that *syau_scr01* and *syau_m13* were polymorphic, *syau_scr01* was co-dominant, and *syau_m13* was dominant (Figure 2).

Verification of the 2 markers in the BC_4 generation

All the BC_4 individuals were screened with *syau_scr01* and *syau_m13*. Screening with *syau_scr01* revealed that among the 60 BC_4 plants, 28 plants showed *Ms*-specific bands, of which 23 were sterile and 5 were fertile. Among the 32 plants from which the *Ms*-specific band could not be amplified, 28 were fertile and 4 were sterile. The accuracy of this estimation was 85% (Tables 2 and 3). Screening with *syau_m13* revealed that among all the 30 plants with the *Ms*-specific bands, 26 were sterile and 4 were fertile. Among the plants without the polymorphic band, 29 were fertile and only 1 was sterile. The accuracy of this estimation was 91.7% (Tables 2 and 3). When the

Table 1. Markers linked to the genic male sterility gene *Ms.*

Names of primers	Sequence (5'-3')	Type of markers	Genetic distance (cM)	Annealing temperature (℃)
syau_scr01	GCAAATTTGTCAAACTTCACC and TCCACCACATTACTTCCCAA	SCAR (dominant)	0.8	58
syau_scr04	AGGATATATCTTGGCTCACGAG and CATCAATAGTGGCGTATGTCTG	SCAR (co-dominant)	2.5	58
syau_m13	TGTTCCTGACTGGAAACTAGTGT and GTCAAAATGAGTCGTAAAGAAAGC	SSR (co-dominant)	3.3	59
cnu_m273	ATAAGGGCATCGCCTCAACA and TGCACGCATCCACATAAACA	SSR (co-dominant)	6.2	58
cun_m030	GAAACAAATTATTTAAAAATCAGACCA and TGGAACAATCCGTAAAACTATGC	SSR (co-dominant)	7.8	55
cnu_m295	GCTGCCTAATAGGGTGCTTG and AGAGCGCATTCAAGTCTGGT	SSR (co-dominant)	8.6	59

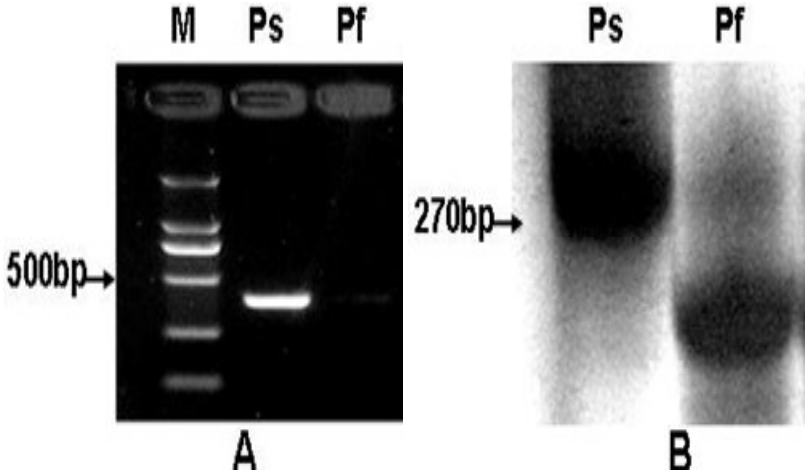

Figure 2. Amplification results of **(A)** *syau_scr01* and **(B)** *syau_m13* between 2 parents. M: DL2000 DNA ladder; Ps: AB01-2 (*Ms^{f}Ms*); Pf: a20 (*msms*).

2 markers were used in combination, the accuracy increased to 100% (Table 3).

Verification of the 2 markers in the BC_5 generation

The BC_5 population was developed by backcrossing a sterile male plant (*Msms*) of the BC_4 generation with an a20 (*msms*) plant. The accuracy of screening of this population with *syau_scr01* and *syau_m13* was 85 and 91.7%, respectively. The accuracy increased up to 100% when these markers were used in combination (Table 4).

DISCUSSION

In a breeding program, plants with the target genotype are generally selected through test-crossing. However, this method cannot be applied if the characteristics of the plants are sensitive to the environment (Luo et al., 2003; Jin et al., 2007). MAS offers good stability and efficiency. Up to now, MAS has been widely used in crops, including corn (Gao et al., 2008) and rice (Liang et al., 2004; Sang et al., 2006). The breeding of multiple-allele male-sterile lines of Chinese cabbage currently includes the transfer of botanical characteristics through crossing and back-crossing and the determination of genotypes through test-crossing with plants of known genotype. In the present study, we employed MAS for the breeding of the male-sterile line, thereby accelerating the breeding process.

Most molecular markers linked to the male sterility gene(s) in Chinese cabbage were obtained through the BSA (bulk segregant analysis) method (Ying et al., 2003;

Table 2. Testing results of *syau_scr01* and *syau_m13* in the BC4 population.

Code	Screening result			Phenotyping results	Code	Screening result			Phenotyping results
	syau_scr01	***syau_m13***	**combination**			***syau_scr01***	***syau_m13***	**combination**	
1	-	-	F	F	31	-	-	F	F
2	-	-	F	F	32	-	-	F	F
3	-	-	F	F	33	-	-	F	F
4	-	-	F	F	34	+	+	S	S
5	-	-	F	F	35	+	+	S	S
6	-	+	○	F	36	+	+	S	S
7	-	+	○	F	37	-	+	○	S
8	-	-	F	F	38	+	+	S	S
9	-	-	F	F	39	-	+	○	S
10	+	-	○	F	40	+	+	S	S
11	-	-	F	F	41	-	+	○	S
12	-	-	F	F	42	+	+	S	S
13	-	-	F	F	43	+	+	S	S
14	-	+	○	F	44	+	-	○	S
15	-	-	F	F	45	+	+	S	S
16	+	-	○	F	46	+	+	S	S
17	-	-	F	F	47	+	+	S	S
18	+	-	○	F	48	+	+	S	S
19	-	-	F	F	49	+	+	S	S
20	-	-	F	F	50	+	+	S	S
21	-	-	F	F	51	+	+	S	S
22	+	-	○	F	52	+	+	S	S
23	-	-	F	F	53	+	+	S	S
24	-	-	F	F	54	-	+	○	S
25	-	+	○	F	55	+	+	S	S
26	-	-	F	F	56	+	+	S	S
27	-	-	F	F	57	+	+	S	S
28	-	-	F	F	58	+	+	S	S
29	+	-	○	F	59	+	+	S	S
30	-	-	F	F	60	+	+	S	S

+: With the Ms-specific band; -: without the Ms-specific band; S: male sterile plant; F: fertile plant; ○: plants with inconsistent results.

Table 3. Accuracy of syau_scr01 and syau_m13 in the MAS in BC4 population.

Screening result with the markers			Phenotyping result		Accuracy (%)
Markers	**Genotypes**	**Number of plants**	**Sterile plants (*Msms*)**	**Fertile plants (*msms*)**	
syau_scr01	*Msms*	28	23	5	85%
	msms	32	4	28	
syau_m13	*Msms*	30	26	4	
	msms	30	1	29	91.7%
Combination	*Msms*	22	22	0	100%

Table 4. Accuracy of *syau_scr01* and *syau_m13* in the MAS in BC_5 population.

Screening result with the markers			Phenotyping result		Accuracy (%)
Markers	**Genotypes**	**Number of plants**	**Sterile plants (*Msms*)**	**Fertile plants (*msms*)**	
syau_scr01	*Msms*	25	21	4	85.5%
	msms	30	4	26	
syau_m13	*Msms*	28	1	27	92.7%
	msms	20	20	0	
Combination	*Msms*	23	0	23	100%

Shen et al., 2004, Song et al., 2006; Yi et al., 2006; Huang et al., 2007, Zhang et al., 2008; Yuan, et al., 2009; Feng et al., 2009). However, the utility of these markers in MAS is quite different. The utility of the markers in MAS is determined not only by the distance between the marker and the target genes but also by the difference in the genetic background of the 2 parents (Wang et al., 1998). In the 6 markers developed previously in our laboratory, only 2 were polymorphic between the 2 parents of the population used in the present study. Therefore, before successful cloning of the target gene, it is necessary to develop an effective MAS system including a set of markers, but not only 1 or 2 markers. The 2 potential markers that were identified in the present population were characterized by low cost, easy performance and high stability.

The most important concern in MAS is the accuracy of the selection of the target gene, which is determined by the distance between the markers and the gene. Before the development of tightly linked markers, 2 markers flanking the gene were used in combination to improve the selection procedure (Liu et al., 2006; Huang et al., 2006). The accuracy of the 2 markers used in this study, namely, *syau_scr01* and *syau_m13*, was 85 and 91.7%, respectively. The accuracy increased to 100% when these 2 markers were used in combination.

According to the genetic hypothesis of the genic multiple-allele male sterility in Chinese cabbage proposed by Feng et al. (1996), a single locus was occupied by 3 alleles: the "*Ms*" allele for male sterility, "*ms*" for fertility, and "Ms^f" for fertility restoration. The dominant-recessive relationship among these alleles was $Ms^f > Ms > ms$. The results of this study suggest that *syau_scr01* and *syau_m13* markers could be applied for the identification of plants containing the *Ms* gene. The selective efficiency of these markers can be improved if the markers linked to Ms^f are found.

Conclusion

SCAR marker *syau_scr01* and SSR marker *syau_m13*, which are linked to the genic multiple-allele male-sterile gene *Ms* in Chinese cabbage, were found to be applicable in the MAS of the male-sterile line breeding. If these 2 markers, which flank the gene, were used in combination, it was possible to achieve selective accuracy up to 100%.

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