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Full Length Research Paper

A genetic male sterile line developed by molecular marker-assisted selection in Chinese cabbage (*Brassica rapa* ssp. *pekinensis*)

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A multiple allele inherited genetic male sterile line of Chinese cabbage 06s x 110 was used as the source of male sterility, and methods of crossing, backcrossing and selfing were applied to breed the male sterility to male fertile line of Chinese cabbage Y02. The SCAR marker syau-scr04 which linked to the male sterility gene *Ms*, was applied in the selection of *Ms* gene. The new male sterile line GMS_4 with similar botanical traits to Y02 with 100% male sterility and 100% male sterile plants, was bred successfully. The accuracy of marker syau-scr04 in determining the plant genotype was 100%. The results indicate that the marker could be applied in the marker-assisted selection of the genetic male sterile line in *Brassica* crops in *B.campestris* (AA, n = 10).

Key words: Chinese cabbage, marker-assisted selection (MAS), genetic male sterile line, breeding.

INTRODUCTION

Chinese cabbage (*Brassica rapa* sp. *pekinensis*) is a typically allogamous plant with bisexual flowers, which exhibits obvious heterosis. The utilization of male sterile line is an ideal way to product hybrids. Feng et al. (1995, 1996) discovered the multiple allele inherited male sterile material in Chinese cabbage, which inherited stably, 100% male sterility with no negative cytoplasmic effects, due to these advantages; it has become the focus of many breeders. According to the "genetic hypothesis of genic multiple allele male sterile gene in Chinese cabbage", the model considers that a single locus with three alleles, "*Ms"* for male sterility, "*ms*" for fertility and "Ms^f" for fertility restoration. In this theory, the dominant-recessive relationship of the three alleles is Ms^f>*Ms*>*ms*. In order that the male sterile gene could be used widely; a new breeding program was designed by Feng et al. (2007), which could transfer the main horticultural characteristics through continuous back crossing and the plant genotype was identified by test cross. The male sterile gene and horticultural characteristics could be transferred simultaneously. The method has been creating a series of excellent male sterile line in a variety of *Brassica* crops (Feng et al., 2007; Xin et al., 2009).

As a special inheritance mechanism, the process of directional transfer of the genetic male sterile line; two groups (Ms^fMs^f; Ms^fMs) and (Ms^fMs^f; Ms^fms), which exhibit the same fertile phenotype but different genotypes, would come out. The genotypes could not be distinguished in the parallel generation only by the fertility in flowers. In order to choose the desired genotypes (Ms^fMs and Ms^fms), test cross is required in each generation which is bound to cost more time and labor. Marker-assisted selection (MAS), which is stable, accurate and not affected by the development of plants or the environmental factors could accelerate the breeding process. Molecular markers of male sterility in Chinese cabbage have already been reported. Ying et al. (2003) identified sequence-tagged site (STS) markers which linked to the recessive male sterile gene. Shen et al. (2004) got a random amplified polymorphism DNA (RAPD) marker linked to the restoring gene. Zhang et al. (2008) identified a sequence characterized amplified region

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Abbreviations: SCAR, Sequence-characterized amplified region, MAS, marker-assisted selection.

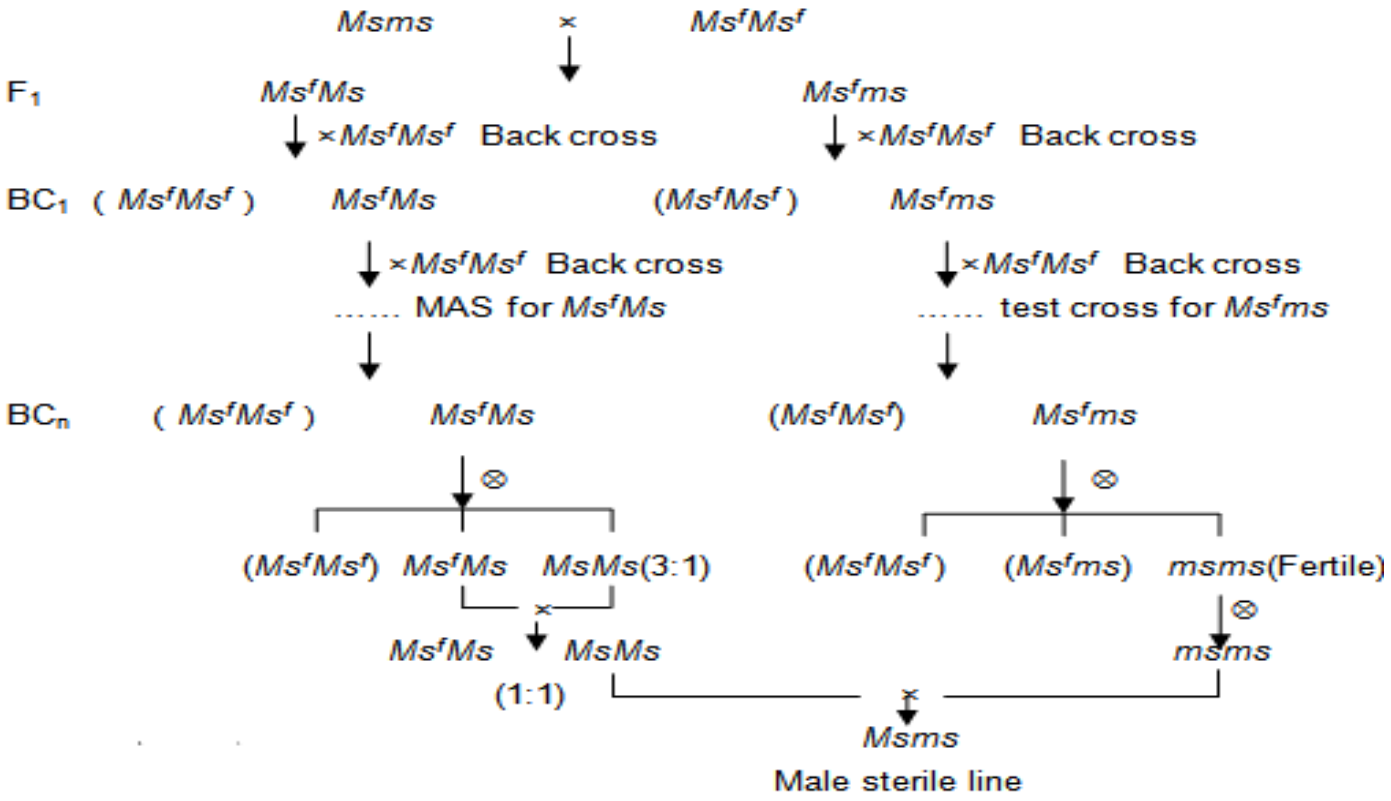

Figure 1. Genetic model for directional transfer of the multiple allele inherited male sterile line in Chinese cabbage based on MAS.

(SCAR) marker tightly linked to the dominant male sterile gene. Yuan et al. (2009) located the genic male sterile gene in the chromosome 4. Up to date, the studies have been concentrated on the development of markers and location of the male sterile gene, but there was no report on genetic male sterile line developed by using molecular marker-assisted selection. In the research, Chinese cabbage "Gong Gu Hua Rui" Y02 was used as the target line and the multiple allele inherited genetic male sterile line of Chinese cabbage 06sx110 was used as the source of male sterility, and the SCAR marker syau-scr04 which developed by Feng et al. (2009) was applied for selecting the male sterile gene *Ms*. The new male sterile line was bred successfully.

MATERIALS AND METHODS

Plant materials

Male sterile source material: male sterile line in Chinese cabbage 06s x 110 (*Msms*); the male sterile plants of "AB" line in Chinese cabbage 06sx090 (*MsMs*).

Target line: Chinese cabbage "Gong Gu Hua Rui" Y02 (Ms^fMs^f).

Candidate marker used for selecting the target gene

The SCAR marker syau-scr04 developed by Feng et al. (2009) in previous study was used for selecting the target gene *Ms*.

The primers: 5'-AGGATATATCTTGGCTCACGAG-3' and 3'-CATCAATAGTGGCGTATGTCTG-5', at a distance of 2.5 cM to *Ms* gene, the annealing temperature is 58℃.

DNA extraction and PCR analysis

Genomic DNA was isolated from fresh leaves of the tested plants with the improved CTAB (Williamson et al., 1994). The isolated genomic DNA was detected by 0.8% agarose gel and UV2000 ultraviolet spectrophotometer. PCR amplification was carried out in a volume of 10μL, containing sterile distilled water 6.6μL, 10×PCR buffer including Mg^{2+} 1μL, 2.5mM dNTPs 0.8μmol, 2.5U/μL *Taq polymerase* 0.1μL, 0.5μmol/L primers 1μL and 0.5μL template genomic DNA. The PCR was carried out in a BIO-RAD iCycler thermocyler with the profile as follows: 94℃ for 5 min, followed by 30 cycles at 94℃ for 1min, annealing temperature 58℃ for 1min, 72℃ for 2 min and ended by extension at 72℃ for 5 min.

Genetic model for directional transfer of the multiple allele inherited male sterile line by using MAS

In the "genetic hypothesis of genic multiple-allele male sterile gene in Chinese cabbage", the target line with the genotype Ms^fMs^f should use the male sterile line as the source of the multiple alleles. According to the genetic model for directional transfer of the multiple allele inherited male sterile line in Chinese cabbage (Figure 1), the desirable genotypes and botanical characters were obtained by continuous back crossing. Two groups with different genotypes but shared an identical fertile phenotype (Ms^fMs^f; Ms^fMs) and

Table 1. Results of the genotype check for transfer male sterile line in BC1.

Code	Combination	Fertile plants: sterile plants	Theoretical ratio(X20.05, 1 = 3.841)
BC1	06sx110×((06sx110×Y02)-3×Y02)-6	32:26	1:1(0.431)

(*Ms^{f}Ms^f*; *Ms^{f}ms*) could appear in the breeding process and be selected respectively. The plants with the genotype *Ms^{f}Ms* were selected by using MAS and the plants with the genotype *Ms^{f}ms* could be identified by test cross according to the segregated ratio for fertility/sterility in the corresponding population. After 4 generations back crossing, the backcross progenies of plants identified as *Ms^{f}Ms* and *Ms^{f}ms* could be selected, in which the "AB" line (*Ms^{f}Ms*, *MsMs*) and the "temporary maintainer" (*msms*) were developed by selfing and sib cross. Afterwards, the male sterile plant (*MsMs*) of the "AB" line crossed with its corresponding "temporary maintainer" (*msms*), the male sterile line with 100% male sterility and male sterile plants was bred. Formula for the sample size "n": n≥lg (0.01)/lg (1-p), p: probability of occurrence for target individuals.

RESULTS

Genotyping of "Y02"

The genotype could be distinguished by test cross with the Chinese cabbage male sterile line (*Msms*). 96 F_1 plants, which were totally fertile and were obtained from the cross between 06s x 110 as the female parent and the target line Y02. It showed that the genotype of Y02 is *Ms^{f}Ms^f*.

Verification of marker syau-scr04 between the 2 parents

Marker syau-scr04 was used to identify polymorphisms between the male sterile plants 06s x 090 (*MsMs*) of the "AB" line and the male fertile plants Y02 (*Ms^{f}Ms^f*) of the target line. The results indicated that syau-scr04 was polymorphic and co-dominant.

Directional transfer of the multiple allele inherited male sterile line

Results of the directional breeding

Following the model in Figure 1, the F_1 population which shared the same fertile phenotype included 2 genotypes, *Ms^{f}Ms* and *Ms^{f}ms*. The 2 genotypes could be identified by selfing progenies segregated ratio:

Ms^{f}Ms⊗→*Ms^{f}Ms^f* , 2*Ms^{f}Ms* , *MsMs* 3:1 (fertile:sterile)

Ms^{f}ms⊗→*Ms^{f}Ms^f* , 2*Ms^{f}ms* , *msms* all fertile.

The F_1 plants with the genotypes *Ms^{f}Ms* and *Ms^{f}ms* were selected and backcrossed with the target line Y02 (*Ms^{f}Ms^f*) respectively. In each BC generations, plant with the genotype *Ms^{f}Ms* was selected by MAS, and *Ms^{f}ms* was selected by test cross, while those of the plants with the genotype *Ms^{f}Ms^f* were eliminated. After 4 generations continuous backcrossing with the target line Y02, the genes *Ms* and *ms* were kept.

Selection results for male sterile gene *Ms* using MAS

For the model in Figure1, the group including 2 genotypes (*Ms^{f}Ms^f* and *Ms^{f}Ms*) appeared in each BC generations. The genotypes of plants in BC_1 were identified by test cross (Table 1). The accuracy of marker syau-scr04 was verified in BC_2. 50 plants were selected randomly in BC_2, and marker syau-scr04 was used to select plants with gene *Ms*, simultaneously, the accuracy of MAS was checked by 50 plants crossed with male sterile line 06s x 110. For the results, 22 plants showed *Ms*-specific bands, and it was consistent with the results of test cross (Table 2). It showed that the accuracy was 100% and the marker syau-scr04 could be applied to assist selection of plants with genotype *Ms^{f}Ms*. 13 plants (at least 7) with similar botanical characters to the target line Y02 were selected randomly in BC_3, using marker syau-scr04 for selective amplification. The results showed that No. 2, 4, 5, 6, 8, 9, 11 plants showed *Ms*-specific bands. 13 plants (at least 7) were selected randomly in BC_4 and marked with syau-scr04, the results revealed that No. 3, 4, 5, 8, 9, 12 plants showed *Ms*-specific bands, of which No. 8 plant was selected and selfed. The selfing progenies resulted in the fertile plants and sterile plants segregated in a ratio of 3:1. The inheritance pattern: *Ms^{f}Ms*⊗→*Ms^{f}Ms^f*, *Ms^{f}Ms*, *MsMs*.

Selection results for male fertile gene *ms* using test cross

In this research, we employed conventional test cross for the plants with genotype *Ms^{f}ms*. According to the model in Figure 1, the group including 2 genotypes (*Ms^{f}Ms^f* and *Ms^{f}ms*) appeared in each BC generations. For the plants with genotype *Ms^{f}ms* in each generation, at least 7 plants with the similar botanical characters to the target line Y02 were selected randomly and crossed with the male sterile line 06sx110 to test the ideal genotype (Table 3). No. 1 plant with genotype *Ms^{f}ms* was selected in BC_4 and selfed The selfing progenies identified all fertile in phenotype.. The inheritance pattern: *Ms^{f}ms*⊗→*Ms^f Ms^f, Ms^{f}ms, msms.*

Table 2. Results of the genotype tested with syau-scr04 and testcross in BC_2.

Code	Screening results (yau-scr040)	Fertile plants: Sterile plants	Theoretical ratio ($X^2_{0.05,1} = 3.841$)	Genotype
1	-	52:0	all fertile	Ms^fMs^f
2	=	28:26	1:1(0.018)	Ms^fMs
3	=	21:24	1:1(0.089)	Ms^fMs
4	-	47:0	all fertile	Ms^fMs^f
5	=	27:25	1:1(0.019)	Ms^fMs
6	-	48:0	all fertile	Ms^fMs^f
7	-	50:0	all fertile	Ms^fMs^f
8	-	45:0	all fertile	Ms^fMs^f
9	=	26:21	1:1(0.340)	Ms^fMs
10	-	39:0	all fertile	Ms^fMs^f
11	-	41:0	all fertile	Ms^fMs^f
12	=	25:23	1:1(0.571)	Ms^fMs
13	=	26:30	1:1(0.161)	Ms^fMs
14	=	25:19	1:1(0.568)	Ms^fMs
15	-	45:0	all fertile	Ms^fMs^f
16	-	51:0	all fertile	Ms^fMs^f
17	=	24:16	1:1(0.148)	Ms^fMs
18	-	47:0	all fertile	Ms^fMs^f
19	=	22:20	1:1(0.023)	Ms^fMs
20	-	42:0	all fertile	Ms^fMs^f
21	-	48:0	all fertile	Ms^fMs^f
22	-	50:0	all fertile	Ms^fMs^f
23	=	23:20	1:1(0.093)	Ms^fMs
24	=	28:26	1:1(0.018)	Ms^fMs
25	-	37:0	all fertile	Ms^fMs^f
26	=	25:23	1:1(0.571)	Ms^fMs
27	-	55:0	all fertile	Ms^fMs^f
28	=	25:26	1:1(0.000)	Ms^fMs
29	-	54:0	all fertile	Ms^fMs^f
30	-	41:0	all fertile	Ms^fMs^f
31	=	28:25	1:1(0.075)	Ms^fMs
32	-	46:0	all fertile	Ms^fMs^f
33	-	44:0	all fertile	Ms^fMs^f
34	=	20:27	1:1(0.765)	Ms^fMs
35	=	16:25	1:1(1.561)	Ms^fMs
36	=	23:14	1:1(1.729)	Ms^fMs
37	=	21:24	1:1(0.089)	Ms^fMs
38	-	38:0	all fertile	Ms^fMs^f
39	-	36:0	all fertile	Ms^fMs^f
40	-	46:0	all fertile	Ms^fMs^f
41	=	36:27	1:1(1.016)	Ms^fMs
42	-	52:0	all fertile	Ms^fMs^f
43	-	43:0	all fertile	Ms^fMs^f
44	-	45:0	all fertile	Ms^fMs^f
45	=	24:17	1:1(0.878)	Ms^fMs
46	-	51:0	all fertile	Ms^fMs^f
47	=	22:24	1:1(0.042)	Ms^fMs
48	-	47:0	all fertile	Ms^fMs^f
49	=	29:20	1:1(1.306)	Ms^fMs
50	-	43:0	all fertile	Ms^fMs^f

-: Without the Ms-specific band; =: With the Ms-specific band.

Table 3. Results of the genotype check for transfer male sterile line of temporary maintainer line in back cross generations.

	Code Combination	Fertile plants : sterile plants	Theoretical ratio($X^2_{0.05,1}$ = 3.841)
BC_1	06sx110×((06sx110×Y02)-1×Y02)-1	35:10	3:1(0.067)
BC_2	06sx110×(((06sx110×Y02)-1×Y02)-1×Y02)-4	29:8	3:1(0.081)
BC_3	06sx110×((((06sx110×Y02)-1×Y02)-1×Y02)-4×Y02)-4	38:13	3:1(0.006)
BC_4	06sx110×(((((06sx110×Y02)-1×Y02)-1×Y02)-4×Y02)-4×Y02)-1	30:12	3:1(0.127)

Table 4. Observation on botanical traits of the new male sterile line and the target line.

Traits	GMS_2	GMS_4	Y02
Plant height (cm)	16.250	18.458	19.642
Angular divergence (cm)	45.953	39.142	38.433
Leaf length (cm)	15.320	21.833	21.333
Leaf width (cm)	12.413	14.200	14.017
Petiole length (cm)	7.350	9.250	9.192
Petiole width (cm)	1.595	2.017	2.000
Plant mass (kg)	0.095	0.159	0.161

Date in the table is the average of 60 plants.

Breeding of the new male sterile line

No. 8 plant in BC_4 was selected by using MAS, namely, its genotype was Ms^fMs, the selfing progenies of which segregated for 3:1. In the selfing group, sib crossing was made between 6 fertile plants (Ms^fMs^f or Ms^fMs) and the male sterile plants ($MsMs$) respectively. The phenotype of sib crossing progenies segregated for 1:1, which showed that the new "AB" line was obtained. The inheritance pattern: $MsMs\times Ms^fMs^f\rightarrow Ms^fMs$ (all fertile); $MsMs\times Ms^fMs\rightarrow Ms^fMs$, $MsMs$ 1:1 (fertile: sterile).

No.1 plant was selected through test cross in BC_4 and its genotype was Ms^fms, the selfing progenies of which were all fertile. 16 plants were selected randomly from the selfing progenies and crossed with the male sterile plants ($MsMs$). In case the progenies were all sterile, the new temporary maintainer line was obtained. The inheritance pattern: $MsMs\times Ms^fMs^f\rightarrow Ms^fMs$ (all fertile); $MsMs\times Ms^fms\rightarrow Ms^fMs$, $Msms$ 1:1(fertile: sterile); $MsMs\times msms\rightarrow Msms$ (all sterile).

The new male sterile line GMS_4 could be bred based on the male sterile plants of the new "AB" line and the new "temporary maintainer", the inheritance pattern: $MsMs\times msms\rightarrow Msms$ (all sterile). 200 plants were investigated, which identified that the sterility frequency and sterility degree all reached 100%.

Observation on botanical traits of the new male sterile line and the target line

The optimal number of the backcross generation was determined according to the similarity between Y02, the male sterile line GMS_2 in BC_2 and the new male sterile line GMS_4 (Table 4). It could be found that the male sterile line bred in BC_4 generation was almost approaching to the recurrent parent Y02 in botanical traits, which ultimately reached the objective of directional transfer.

DISCUSSION

Thus far, MAS has been widely used in crops, corn (Gao et al., 2008) and rice (Liang et al., 2004, Sang et al., 2006) included. In the present research, we employed MAS for breeding of the male sterile line, which could accelerate the breeding process.

According to the genetic model for directional transfer of the multiple allele inherited male sterile line (Li et al., 2006, 2009), for the target line with genotypes Ms^fMs^f, it is appropriate to use the male sterile line ($Msms$) as the source of the multiple alleles. There are two parts of continuous backcrossing (Liu et al., 2010), one is "$Ms^fMs\times Ms^fMs^f$", and the other is "$Ms^fms\times Ms^fMs^f$", of which the progenies are all fertile. The progenies in BC generations are determined by test cross with plants of known genotype. If we could utilize the markers which linked to gene *Ms* and *ms* to identify the suited plants, it could accelerate the breeding process. In the study, the SCAR marker syau-scr04, developed by Feng et al. (2009), was applied to assist the selection of plants contained gene *Ms*. Nevertheless, the markers linked to *ms* have not been exploited yet. Consequently, conventional test cross was employed to identify plants with gene *ms*. The development of markers tightly linked to gene *ms* is a critical program in the present time.

Brassica crops are widely planted all over the world, it is the best way to utilize MAS for the genotype identification in the process of the male sterile line breeding. Presently, limited markers which tightly linked to the multiple allele inherited male sterile gene have been developed, and it is rarely that the markers drew on the male sterile line breeding in *Brassica* crops. The utility of the markers in MAS is not only determined by the diatance between the marker and the target gene but by the difference in the genetic background of the 2 parents. Henceforth, it is significant to develop more markers with shorter distance and higher stability.

The GMS_4, a new male sterile line with 100% male sterility and 100% male sterile plants, was bred by using the SCAR marker syau-scr04 for the selection of the object gene *Ms* and test cross for the target gene *ms*.

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