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The novel methods of development of the maintainer and cytoplasmic male sterile lines with different cytoplasms based on chromosome single-segment substitution lines in rice

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Abstract: The present study was carried out with the objective of developing new parental lines of hybrid rice based on chromosome single-segment substitution lines (SSSLs), on which the restorer and maintainer genes *Rf3* (*rf3*) and *Rf4* (*rf4*) were harbored in rice. To develop the new 3-line hybrid, the SSSL W23-19-06-06-11 with the genotype *Rf3Rf3/Rf4Rf4* [a strong restorer line for wild abortive (WA), dwarf-wild abortive (DA), and Yegong (Y) cytoplasmic male sterile (CMS) lines] was crossed 4 times as the male parent with 4 CMS lines: 2 typical WA-type CMS lines of Bobai A and Zhenshan 97A, 1 typical Y-type CMS line of Y-Huanong A, and 1 typical DA-type CMS line of Xieqingzao A. In the BC_3F_2 populations, the male sterile plants (MSPs) with WA-, Y-, and DA-type CMS sources and nuclear background of W23-19-06-06-11 and their maintainer plants (MPs) were developed by backcrossing and marker-assisted selection. All the MSPs showed 100% pollen and spikelet sterility and the panicle length ranged from 20 to 22.6 cm. The extent of incomplete panicle exertion in the entries ranged from −40.7 to −48.5. The pollen grain viability assay with I_2KI of the MSPs revealed 100% pollen sterility, consisting of both typical and spherical abortive pollen grains. The MPs showed normal pollen (spikelet) fertility. A genome-wide DNA marker survey revealed that the numbers of the substituted chromosome segments carried by the MSPs and MPs were 0.8 and 0.6, while the lengths of the substituted chromosome segments corresponding to the *Rf3* (or *Rf4*) locus were, on average, 26.2 cM and 17.0 cM (or 15.3 cM and 15.1 cM).

Key words: Three-line hybrid rice, chromosome single segment substitution line, development of restorer line, maintainer line, molecular marker-assisted selection

1. Introduction

Cytoplasmic male sterility (CMS), a maternally inherited trait failing to produce functional pollen, is a useful tool for the production of hybrid seeds in crops. CMS can be restored by fertility restorer (*Rf*) genes associated with nuclear genes encoding pentatricopeptide repeat proteins (Hanson and Bentolila, 2004). Wild-abortive (WA), Yegong (Y), and dwarf-wild abortive (DA) lines, discovered in China, belong to the *indica* sporophytic CMS systems, and they all possess typical aborted pollens (Yuan and Virmani, 1988; Cai, 2002; Xie et al., 2002).

Since the development of the first CMS line in China, many hybrids have been released in China, India, Vietnam, the Philippines, Indonesia, and Bangladesh. Rice hybrids are cultivated in more than 50% of the rice area in China. Of cultivated hybrids, 95% in China and in the tropics have wild WA-type cytoplasm derived from *Oryza sativa* (Yuan, 1993). This cytoplasmic uniformity of hybrid varieties may lead to the genetic vulnerability of hybrids in the long run, as was the case of southern corn blight on US maize hybrids carrying T-type cytoplasm (Hooker, 1974). There is thus an urgent need for cytoplasmic diversification of the male sterility source for hybrid rice breeding. In our previous work, we constructed a library of 1123 single-segment substitution lines (SSSLs) in rice using Hua-jing-xian 74 (HJX74), an elite *indica* variety from South China, as a recipient, and 24 accessions, including 14 *indica* and 10 *japonica* collected worldwide, as donors (Zhang et al., 2004). Since each SSSL contains only one donor homozygous chromosome segment with a high level of uniformity of the genetic background, the SSSLs were widely used to detect quantitative trait loci (QTLs) for traits of agronomic importance (Zhang et al., 2012), to assess allelic variation (Teng et al., 2012), and to clone the gene by map-based cloning (Wang et al., 2012) in rice. The SSSL W23-19-06-06-11 from the SSSL library, carrying the genotype *Rf3Rf3/Rf4Rf4*, is a strong restorer line for WA-, Y-, and DA-CMS (Cai et al., 2013). Of these, *Rf3* from

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recipient HJX74 was introgressed on chromosome 1 from recipient HJX74, and *Rf4* was introgressed on chromosome 10 from donor Lemont (a *japonica* variety from the United States). In this paper, we report the development of a set of new male sterile lines (MSPs) with WA-, Y-, and DA-CMS cytoplasms, the nuclear background of W23-19-06-06-11 and their corresponding maintainer lines (MPs), and the molecular characterization of the MSPs and the MPs.

2. Materials and methods

2.1. Plant materials

Two typical WA-type CMS lines of Bobai A (BbA) and Zhenshan 97A (ZsA), a typical Y-type CMS line of Y-Huanong A (HnA), and a typical DA-type CMS line of Xieqingzao A (XqA) were used as female parents (A-lines), with the SSSL W23-19-06-06-11 as the recurrent parent. The parental lines and their derived progenies were grown in the experimental station of South China Agricultural University in 2007 and 2012, respectively.

2.2. SSR marker analysis

To detect the *Rf3* and *Rf4* genes for fertility restoration, the SSR markers RM1, RM220, RM304, RM5373, RM258, and RM6100, which showed polymorphism between the SSSLW23-19-06-06-11 and A-lines and appeared closely linked to the 2 genes, were selected on the rice microsatellite maps (McCouch et al., 2002). PSM348 and PSM354 linked to the 2 genes were developed by Cai et al. (2013). Miniscale DNA extraction was carried out according to the procedure described by Zheng et al. (1995). PCR was conducted according to Panaud et al. (1996). The PCR products were separated through electrophoresis on 6% polyacrylamide gel. Bands were visualized by a silver staining process.

2.3. Population construction for development and molecular analysis of MSPs and MPs

The backcross process is illustrated in the Figure. The SSSLW23-19-06-06-11 carrying the *Rf3Rf3/Rf4Rf4* genotype was crossed as the male parent with BbA, ZsA, HnA, and XqA, respectively, and then backcrossed with the F_1. In the 4 BC_1F_{1s} populations, 20 plants for each cross were genotyped using 8 polymorphic SSR markers closely linked to the 2 *Rf* loci on chromosomes 1 (*Rf3*) and 10 (*Rf4*), and 5 plants carrying the genotype *Rf3rf3/Rf4rf4* in each cross were selected for further backcrossing to W23-19-06-06-11, producing a population of 4 $BC_2F_{1s.}$ From BC_2F_{1s}, 40 plants for each cross were genotyped using SSR markers, and 20 plants carrying the genotype *Rf3rf3/Rf4rf4* in each cross were selected for further backcrossing to W23-19-06-06-11, producing BC_3F_{1s}. Moreover, the 20 selected plants (*Rf3rf3/Rf4rf4*) of the BC_2F_{1s} populations were crossed as the female parent with HJX74 to develop BC_3F_{1m}. In BC_3F_{1s} and BC_3F_{1m}, 20 plants for each cross were genotyped using SSR markers, and 10 plants carrying the genotype *Rf3rf3/Rf4rf4* in each cross were selected for self-pollination to produce BC_3F_{2s} and BC_3F_{2m}, respectively. From the 4 BC_1F_1 to the 4 BC_3F_2 progenies, 205, 108, 42, and 30 polymorphic SSR markers on average were selected genome-wide and were used to detect the *Rf3* and *Rf4* genotypes and genetic background. In each generation, the plants whose chromosome segments produced maximal coverage of the recurrent parent W23-19-06-06-11 genome were selected for further backcrossing.

2.4. Estimation of number and length of substituted chromosome segments

In the 4 BC_3F_{2s} and 4 BC_3F_{2m} populations, the contents of donor genomes, including donor segment number (DSN)

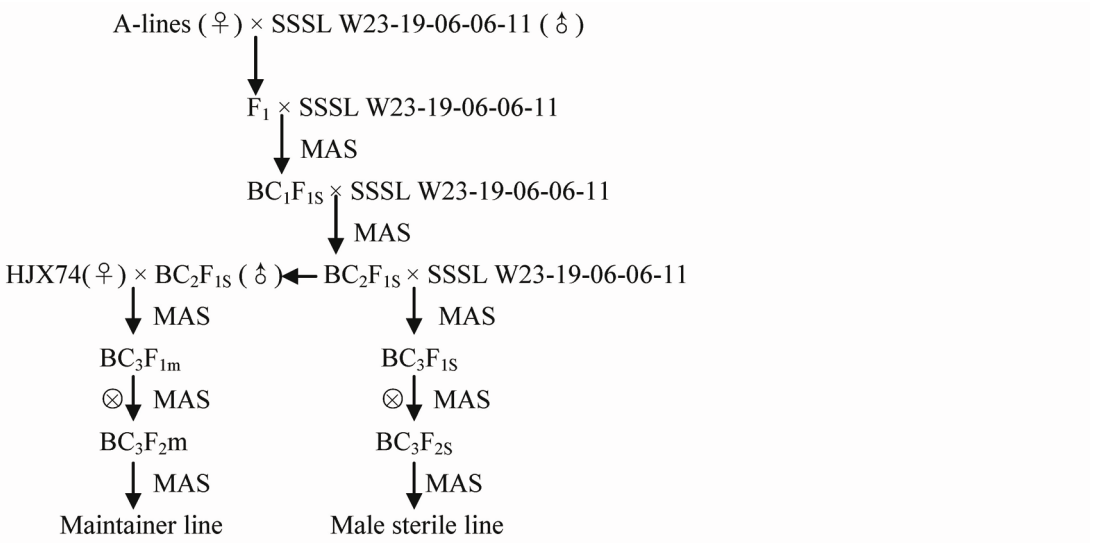

Figure. Schematic diagram of backcross generations. *MAS: Marker-assisted selection.

and donor segment length (DSL) with the *Rf3* (or *Rf4*) locus, carried by the MSPs and their MPs, were directly analyzed based on graphical genotypes as described by Hospital (2002). A chromosome segment flanked by one marker of donor type (DD) and one marker of recipient type (DR) was considered a 50% donor and 50% recipient genome. The length of DD plus the length of 2 half DRs was considered to be the estimated length of a DSL.

2.5. Fertility scoring for all BC_3F_2 plants

Pollen fertility and seed-setting rate were used as the main criteria for the evaluation of fertile and sterile plants. Mature anthers were harvested, and the pollen was stained with a 1% I_2KI solution. Pollen fertility and natural seed-setting rate in the case of bagged panicles, percentage of enclosed panicles (unexerted), panicle length (in cm), and exertion (in percentage) were measured as described by Zhu (1979). All the plants selected in the BC_3F_{2s} (BC_3F_{2m}) segregation populations had to be tested for each of the crosses.

3. Results

3.1. Polymorphic SSR markers

Out of 815 SSR primers surveyed, 205 (25.2%) and 197 (24.2%) showed polymorphism among W23-19-06-06-11 and BbA and ZsA, 219 (26.9%) were polymorphic between W23-19-06-06-11 and HnA, and 199 (24.4%) were polymorphic between W23-19-06-06-11 and XqA. The average size of the intervals between polymorphic markers in the 4 crosses ranged from 6.9 cM to 7.6 cM, with a mean of 7.3 cM (Table 1). In the process of developing BC_3F_2 populations through MAS, the polymorphism SSR markers were used for genotyping and background selection.

3.2. Development and characterizations of the *indica* MSPs

The 63, 79, 59, and 84 *indica* MSPs (namely WH74A1, WH74A2, YH74A, and DH74A) with the genotype of S (*rf3rf3/rf4rf4*), possessing WA-, Y-, and DA-type CMS and genetic background of W23-19-06-06-11, were selected via MAS in the 4 BC_3F_{2s} populations. All the entries showed 100% pollen and spikelet sterility and the panicle length ranged from 20 to 22.6 cm. The extent of incomplete panicle exertion in the entries ranged from –40.7 to –48.5. The pollen grain viability assay of the MSPs with I_2KI revealed 100% pollen sterility, consisting of both typical and spherical abortive pollen grains. The MSPs exhibited stronger stems and tillers; longer, thicker, and erect top leaves; and incomplete panicle exertion, which had CMS morphological characters similar to their corresponding CMS lines and differences from each other.

3.3. Development and characterizations of the *indica* MPs

The 47, 55, 61, and 53 *indica* MPs (namely WH74M1, WH74M2, YH74M, and DH74M) with the genotype of

Table 1. Polymorphic SSR markers between SSSL W23-19-06-06-11 and A-lines.

Description of markers	A-lines				Average
	BbA	ZsA	XqA	HnA	
Total number of markers tested	815	815	815	815	815
No. of polymorphic markers	205	197	219	199	205
Ratio of polymorphism of markers (%)	25.2	24.2	26.9	24.4	25.2
Average size of the interval between polymorphic markers (cM)	7.3	7.6	7.5	6.9	7.3
Distribution of polymorphic markers on 12 chromosomes					
Chr. 1	29	28	28	28	28
Chr. 2	18	18	18	24	19
Chr. 3	21	21	20	23	21
Chr. 4	16	13	12	13	14
Chr. 5	14	12	14	12	13
Chr. 6	13	13	14	16	14
Chr. 7	13	14	15	12	14
Chr. 8	15	12	13	13	13
Chr. 9	15	16	17	14	16
Chr. 10	21	20	13	18	18
Chr. 11	13	14	15	13	14
Chr. 12	17	16	20	13	17

N (*rf3rf3/rf4rf4*) and nuclear background of W23-19-06-06-11 (corresponding to WH74A1, WH74A2, YH74A, and DH74A, respectively) were selected via MAS in the 4 BC_3F_{2m} populations. All the entries showed that the pollen (spikelet) fertility, panicle length, and percentage of unexerted panicles of the MPs were 78.7%, 94.4%, 21.0 cm, and +2.4%, respectively.

3.4. DSNs and DSLs in the MSPs

Using a total of 197–219 polymorphism SSR markers between W23-19-06-06-11 and A-lines, the 285 MSPs with the genotype of S (*rf3rf3/rf4rf4*) were identified, and then a genome-wide scan was carried out to determine the homozygous or heterozygous status of the plants (Table 2). The DSNs carried by the MSPs ranged from 0.3 to 1.5, with a mean of 0.8. The DSLs corresponding to the locus ranged from 19.0 to 42.8 cM with a mean of 26.2 cM for *Rf3*, and from 11.2 to 20.0 cM with a mean of 15.3 cM for *Rf4*.

3.5. DSNs and DSLs in the MPs

Using the SSR markers, the 216 MPs with the genotype of N (*rf3rf3/rf4rf4*) were selected, and then a genome-wide scan was carried out to determine the homozygous or heterozygous status at the locus in the plants (Table 3). The DSNs carried by the MPs ranged from 0.3 to 1.5, with a mean of 0.6; the DSLs, corresponding to the locus, ranged from 10.8 to 23.2 cM with a mean of 17.0 cM for *Rf3*, and from 9.3 to 20.4 cM with a mean of 15.1 cM for *Rf4*.

4. Discussion

In hybrid rice seed production, the 3-line system is widely used. Two major fertility restorer genes, *Rf3* and *Rf4*,

Table 2. Substituted chromosome segments in the MSPs in the S (*rf3rf3/rf4rf4*) genotype.

Cross	No. of plants	Number of genetic background segments	Position[1] P1	P2	P3	P4	Marker[2]	Length (cM)
Substituted chromosome segments corresponding to *Rf3* locus								
BbA/W23-19-06-06-11	63	0.9	18.2	23.3	30.5	49.0	RM86--RM220-RM1-RM283-PSM572-PSM348-PSM354-RM259--RM581	19.0
ZsA/W23-19-06-06-11	79	0.6	23.3	24.7	38.8	49.0	RM220--RM1-RM283-PSM572-PSM348-PSM354-RM259--RM581	19.9
HnA/W23-19-06-06-11	59	0.3	18.2	23.3	38.8	49.0	RM86--RM220-RM1-RM283-PSM572-PSM348-PSM354-RM259--RM581	19.0
XqA/W23-19-06-06-11	84	1.5	18.2	23.3	58.1	69.0	RM86--RM220-RM-PSM572-PSM348-PSM354-RM259-RM23--PSM433	42.8
Average	71	0.8	19.5	23.7	41.6	54.0		26.2
Substituted chromosome segments corresponding to *Rf4* locus								
BbA/W23-19-06-06-11	63	0.9	48.8	49.0	61.4	70.0	RM258--PSM25599-PSM25510-RM304-RM5373-RM6100-PSM168-RM271-PSM169--RM147	16.8
ZsA/W23-19-06-06-11	79	0.6	45.7	48.8	59.4	61.4	PSM455--RM258-PSM25599-PSM25510-RM304-RM5373-RM6100-PSM168-RM271--PSM169	13.2
HnA/W23-19-06-06-11	59	0.3	45.7	48.8	57.5	59.4	PSM455--RM258-PSM25599-PSM25510-RM304-RM5373-RM6100-PSM168--RM271	11.2
XqA/W23-19-06-06-11	84	1.5	32.0	48.8	59.4	61.4	PSM166--RM258-PSM25599-PSM25510-RM304-RM5373-RM6100-PSM168-RM271--PSM169	20.0
Average	71	0.8	43.1	48.9	59.4	63.1		15.3

[1]P indicates the position of the substituted segment on chromosome 1 or 10. P1 and P4 indicate the position of the maximum length of the substituted segment; P2 and P3 indicate the positions of the minimum length of the substituted segment. The single hyphen in the middle of markers indicates chromosome substitution segments. The end markers of the triadic hyphens are side markers of substitution segments, which indicate that segment recombination might appear. [2]RM code indicates the markers described by McCouch et al. (2002), and PSM code indicates the markers designed by the State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresources, South China Agricultural University, China. The same notes apply in Table 3.

Table 3. Substituted chromosome segments in the MPs in the N (*rf3rf3/rf4rf4*) genotype.

Cross	No. of plants	Number of genetic background segments	Position[1] P1	P2	P3	P4	Marker[2]	Length (cM)
Substituted chromosome segments corresponding to *Rf3* locus								
HJX74/(BbA/3/W23-19-06-06-11)	47	0.5	18.2	23.3	38.8	49.0	RM86--RM220-RM1-RM283-PSM348-PSM354-RM259--RM581	23.2
HJX74/(ZsA/3/W23-19-06-06-11)	55	0.3	18.2	23.3	30.5	39.0	RM86--RM220-RM1-RM283- PSM572-PSM348-PSM354--RM259	14.0
HJX74/(HnA/3/W23-19-06-06-11)	61	0.3	23.3	24.7	30.5	39.0	RM220--RM1-RM283- PSM572-PSM348-PSM354--RM259	10.8
HJX74/(XqA/3/W23-19-06-06-11)	53	1.5	23.3	24.7	38.8	49.0	RM220--RM1-RM283-PSM348-PSM354-RM259--RM581	19.9
Average	54	0.6	20.8	24.0	34.7	44.0		17.0
Substituted chromosome segments corresponding to *Rf4* locus								
HJX74/(BbA/3/W23-19-06-06-11)	47	0.5	48.8	49	57.5	58.9	RM258--RM25599-RM304-RM5373-PSM168--PSM344	9.3
HJX74/(ZsA/3/W23-19-06-06-11)	55	0.3	32.0	45.5	58.9	59.4	PSM166--RM5620-RM304-RM5373-PSM344-RM3773--RM271	20.4
HJX74/(HnA/3/W23-19-06-06-11)	61	0.3	49.0	49.3	58.9	59.4	RM25599--RM25510-RM304-RM5373-PSM344-RM3773--RM271	10.0
HJX74/(XqA/3/W23-19-06-06-11)	53	1.5	32.0	45.5	58.9	59.4	PSM166--RM5620-RM304-RM5373-PSM344-RM3773--RM271	20.4
Average	54	0.6	40.5	47.3	58.6	59.4		15.1

are required for the production of viable pollen in WA-type CMS and DA-type CMS, and these genes have been mapped to chromosomes 1 and 10, respectively (Xie et al., 2002; Balaji Suresh et al., 2012). Markers linked to the gene can be used to select plants possessing the desired trait, and markers distributed throughout the genome can be used to select plants that are genetically similar to the recurrent parent (Hospital et al., 1992). SSSLs were found to be a powerful tool to identify, map, and clone QTLs for complex traits in rice (Liu et al., 2010; Wang et al., 2012; Zhang et al., 2012). In this research, newly developed MSPs carrying WA-, Y-, and DA-type CMS sources and sharing a uniform genetic background of W23-19-06-06-11, and their MPs, were selected by SSR markers closely linked to the *Rf3* and *Rf4* genes and traditional backcrossing based on SSSLs. This method presents new approaches to transferring *Rf* genes into adapted cultivars through a backcrossing program and MAS in an active hybrid rice breeding program.

The length and content of donor chromosome segments after a series of backcrosses is very important for the plant breeder who is interested in the development of stocks with desired genetic traits. Stam and Zeven (1981) reported that the size of a donor chromosomal segment was influenced by chromosome length, as well as by the number of backcross generations. During backcrossing, all donor chromosome segments would be heterozygous and any recombination in these segments would result in a reduction of the donor segment length. In our research, the numbers of substituted chromosome segments on average, carried by the MSPs and MPs, were 0.84 and 0.65. The lengths of the substituted chromosome segments with the *Rf3* (or *Rf4*) locus, carried by the MSPs and MPs, were 26.2 cM and 17.0 cM (or 15.3 cM and 15.1 cM), respectively. These results were similar to the assumption of Naveira and Barbadilla's (1992) formula to predict the average length of the donor segments of the backcross progeny in rice.

The success of hybrid seed production in rice largely depends on the genetic purity of the CMS and maintainer lines. Since the newly developed MSPs with WA-, Y-, and DA-type CMS sources and their MPs possessed a high

level of uniformity of the genetic background of W23-19-06-06-11 and small substituted chromosome segments, they could not only be useful in the near future to address the issue of genetic vulnerability of a single source of sterility-inducing cytoplasm, but also in the fine-mapping and cloning of *Rf3* and *Rf4* in rice.

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