DOI: 10.1007/s10535-013-0385-2 BIOLOGIA PLANTARUM 58 (2): 241-246, 2014

Comparative analysis of fertility restoration genes for WA, Y, and DA cytoplasmic male sterility in rice

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Abstract

Rice chromosome single segment substitution line (SSSL) W23-19-06-06-11 with the genotype *Rf3Rf3/Rf4Rf4*, a strong restorer line for wild-abortive (WA) cytoplasmic male sterility (CMS), was recently identified from the SSSL library. To investigate the genetic mode of *Rf* genes and the genetic relationship among WA, yegong (Y), and dwarf-wild-abortive (DA) CMS systems, the plants derived from three BC_3F_2 populations involving W23-19-06-06-11 and the three CMS lines, that carried the *Rf3Rf3/Rf4Rf4*, *Rf3Rf3/rf4rf4*, and *rf3rf3/Rf4Rf4* genotypes and WA-, Y-, and DA-CMS cytoplasm, were selected and their pollen and spikelet fertility were evaluated. The results show that the genetic effect displayed a trend of Y-CMS > WA-CMS > DA-CMS in the genetic background of W23-19-06-06-11, the effect of *Rf4* appeared to be slightly larger than that of *Rf3*, and their effects were additive for the three CMS systems. Two pairs of dominant genes governed the fertility restoration in pollen and spikelet in the W23-19-06-06-11 which indicates that the genetic mode of the *Rf* genes was a qualitative character for the three CMS systems.

Additional key words: marker-assisted selection, *Oryza sativa*, pollen fertility, single segment substitution lines.

Introduction

Hybrid rice technology offers a potentially viable option for increasing rice yield potential beyond the level of inbred high-yielding cultivars by exploiting heterosis or hybrid vigour on a commercial scale. Cytoplasmic male sterility (CMS) combined with a fertility restoration system has been found to be the most efficient genetic tool in commercializing this technology in rice (Lin and Yuan 1980, Virmani and Wan 1988). CMS in plants is a maternally inherited and mediated by mitochondrial genomes and by the interaction of mitochondrial and nuclear genes (Chase 2007, Luo *et al*. 2013).

Wild abortive (WA), yegong (Y), and dwarf-wild-abortive type (DA) CMS belong to the sporophytic sterility system. They possess typical aborted pollens (Lin and Yuan 1980, Yuan and Virmani 1988, Cai 2002, Xie *et al*. 2002). Among these CMS, the inheritance of fertility restoration in the WA-CMS system has been extensively investigated. It was discovered that fertility restoration is controlled by two independent dominant nuclear genes with one stronger in action than the other (Young and Virmani 1984, Tan *et al*. 2008). Some studies have revealed that the two fertility restorer genes are additive in their inheritance giving rise to an F_2 segregation ratio of 15:1 (fertile:sterile) (Sattari *et al*. 2008). It was also reported that the restoration of fertility in IR36 is governed by two independent and dominant genes of which one appears to be stronger than the other (Raj and Virmani 1988). Bharaj *et al*. (1995) reported that the stronger gene is located on chromosome 7 and the weaker one on chromosome 10 in IR36. Yao *et al*. (1997) identified two *Rf* loci on chromosomes 1 (*Rf3*) and 10 (*Rf4*) and showed that the effect of *Rf4* is larger than that

Submitted 16 May 2013, *last revision* 1 July 2013, *accepted* 15 July 2013.
Abbreviations: BT - boro type II; CMS - cytoplasmic male sterility; DA - dwarf-wild-abortive; HL - Honglian; MAS - marker-assisted selection; PCR - polymerase chain reaction; PPR - pentatricopeptide repeat; QTL - quantitative trait loci; *Rf* - fertility restorer gene; RFLP - restriction fragment length polymorphism; SCSs - substituted chromosome segments; SSSLs - single segment substitution lines; WA - wild abortive; Y - Yegong.
Acknowledgements: This work was supported by the National Natural Science Foundation of China (30830074 and 2013GA710018) and the Department of Education of Anhui Province Natural Science Foundation of China (No. KJ2010B155).
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of *Rf3*. Currently, there are over 17 *Rf* alleles for different CMS systems being reported in rice, and *Rf2*, *Rf3*, and *Rf17* are mapped on chromosomes 2, 1, and 4, respectively (Zhang *et al*. 1997, Li *et al*. 2007, Fujii and Toriyama 2009). Others, including *Rf4* for the WA-CMS system, *Rf1* for BT-CMS, *Rf5* and *Rf6* for HL-CMS, and *qRf-10-2* for DA-CMS, lay on the long arm of chromosome 10 and are closely linked to form a gene cluster (Tan *et al*. 1998, Xie *et al*. 2002, Zhang *et al*. 2002, Liu *et al*. 2004, Wang *et al*. 2006, Ngangkham *et al*. 2010).

A library of 1 123 single-segment substitution lines (SSSLs) in rice has been constructed by the State Key Laboratory for Conservation and Utilization of Subtropical Agro-bioresources, the South China Agricultural University (Zhang *et al*. 2004, Xi *et al*. 2006). Since each SSSL contains only one donor homozygous chromosome segment with a high uniformity of the genetic back-ground of Hua-jing-xian 74 (HJX74), an elite cultivar belonging to the *indica* variety from South China as recipient, the SSSLs are widely used to detect QTLs for the traits of agronomic importance (Zhang *et al*. 2012), to assess allelic variation (Teng *et al*. 2012), to analyze the interaction of gene-by-environment (Liu *et al*. 2010), and to clone the gene by map-based cloning (Wang *et al*. 2012) in rice. The SSSL W23-19-06-06-11 carrying the genotype *Rf3Rf3/Rf4Rf4,* a strong restorer line for WA-CMS, was recently identified from the SSSL library (Cai *et al*. 2013). *Rf3* on chromosome 1 is introgressed from recipient HJX74, and *Rf4* on chromosome 10 from donor cv. Lemont (an *japonica* variety from America). In this study, we selected the SSSL W23-19-06-06-11 to detect the genetic relationship among WA-, Y-, and DA-CMS systems, and genetic effects and genetic mode of the *Rf3* and *Rf4* genes for fertility restoration in rice.

Materials and methods

Plants: One typical WA-CMS line of Zhenshan97A (ZsA), a typical Y-CMS line of Y-HuanongA (HnA), and a typical DA-CMS line of *Oryza sativa* L. cv. XieqingzaoA (XqA) were used as female parents with the SSSL W23-19-06-06-11 as male parent. These parents and their progenies were planted in the experimental field in the Agricultural University Campus, South China.

Primers: To detect the *Rf3* and *Rf4* genes for fertility restoration, some primers of SSR markers, such as RM1, RM220, RM304, RM5373, RM258, and RM6100 were selected on the rice microsatellite maps (McCouch *et al.* 2002, Mishra *et al*. 2003). The primers of markers, PSM348 (F: 5'-GATGAGGTTAGGTTGGTGCC-3', R: 5'-GTAGAATCAACTCGAGCGGC-3') and PSM354 (F: 5'-ACAAGCTAAGGTAGTGTCCATG-3', R: 5'-CAT TTTACCTCAGGCTCTTCA-3'), were developed in this study.

Population construction for genetic analysis of the *Rf* genes: The SSSL W23-19-06-06-11 carrying the *Rf3Rf3/Rf4Rf4* genotype was crossed as the male parent with ZsA, HnA, and XqA, respectively, and then backcrossed with the F_1. In the three BC_1F_1, the plants with the *Rf3rf3/Rf4rf4* genotype were selected by marker-assisted selection (MAS). The selected plants were backcrossed to the SSSL for two more generations. In the three BC_3F_2 populations, the plants carrying the genotypes *Rf3Rf3/Rf4Rf4*, *Rf3Rf3/rf4rf4*, or *rf3rf3/Rf4Rf4* were selected by MAS and used to analyze the genetic relationship among WA-, Y-, and DA-CMS systems, genetic effects, and mode of the *Rf3* and *Rf4* genes. Spikelets of the selected plants were collected at flowering. Anthers were taken from the spikelets to determine pollen fertility. Pollen fertility and seed-setting rate were used as the main criteria for the evaluation of fertile and sterile plants. The pollens were stained with 1 % (m/v) KI solution. The numbers of stainable pollens and un-stainable pollens in each individual were counted under an optical microscope. Morphological features like the panicle length and percentage of wrapping panicle neck were recorded from 20 plants for each variant.

SSR marker analysis: A total of 197 - 219 SSR markers showing polymorphism between SSSL W23-19-06-06-11 and the three CMS lines (A-lines), involving ZsA, HnA, and XqA, respectively, were selected and used to detect the *Rf3* and *Rf4* genotypes and genetic background. Mini-scale DNA extraction was carried out according to the procedure described by Zheng *et al*. (1995). The PCR products were separated by electrophoresis on a 6 % polyacrylamide gel. Bands were visualized by silver staining.

Estimation of number and length of substituted chromosome segments: In the three BC_3F_2 populations, the number of segments and the length of substituted chromosome segments (SCSs), carried by BC_3F_2 individuals with the genotype of *Rf3Rf3/Rf4Rf4*, were estimated based on graphical genotypes (Hospital 2002). A chromosome segment flanked by two markers of donor type (DD) is considered as the donor genome, a chromosome segment flanked by two markers of recipient type (RR) is considered as the recipient genome, and a chromosome segment flanked by one marker of donor type and one marker of recipient type (DR) is considered as the 50 % donor and 50 % recipient genome. In other words, the length of DD plus the length of two half DR was considered to be the estimated length of a SCS.

Results

Eight hundred and fifteen SSR markers, distributed across the 12 chromosomes selected on the rice microsatellite maps (McCouch *et al*. 2002) and designed using the *Primer Premier v5.0* software by screening the genomic sequence in the *Rf* region of the *japonica* cultivar Nipponbare (http://rgp.dna.affrc.go.jp/IRGSP/index.html) with *SSRIT* software (http://www.gramene.org/microsat/), were used to survey the polymorphism between each CMS line and SSSL W23-19-06-06-11. The numbers of polymorphic markers between W23-19-06-06-11 and A-lines, such as ZsA, HnA, and XqA were 197, 219, and 199, respectively, with a polymorphism ratio from 24.2 to 26.9 %. The polymorphic markers were used for MAS in the process of BC_3F_2 population development which consisted of selecting the *Rf3* and *Rf4* genotypes and genetic background. The average size of the intervals between polymorphic markers in the three crosses ranged from 6.9 to 7.6 cM (Table 1).

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

	ZsA	HnA	XqA	Average
Number. of markers tested	815	815	815	815
Number of polymorphic markers	197	219	199	205
Polymorphism of markers [%]	24.2	26.9	24.4	25.1
Interval between polymorphic markers [cM]	7.6	6.9	7.5	7.3

Table 2. Pollen (spikelet) fertility [%] in the BC_3F_2 individuals generated from the crosses between SSSL W23-19-06-06-11 and A-lines. Mean of three replicates ± SE. * - Number of plants with the respective genotype.

Cross	*Rf3Rf3/ Rf4Rf4*	*Rf3Rf3/ rf4rf4*	*rf3rf3/ Rf4Rf4*
ZsA/ W23-19-06-06-11	89.3±0.4 (94.6±0.6) *43	40.8±0.7 (53.2±2.0) *32	48.6±0.5 (67.7±1.6) *41
HnA/ W23-19-06-06-11	78.3±0.4 (90.2±0.4) *46	31.5±0.6 (45.2±1.4) *45	39.2±0.4 (56.6±1.1) *46
XqA/ W23-19-06-06-11	84.3±0.5 (92.4±0.6) *45	45.5±0.6 (67.3±1.8) *40	56.7±0.9 (79.8±1.7) *37

An SSSL W23-19-06-06-11, with the *Rf3Rf3/Rf4Rf4* genotype, was selected to analyze the genetic effects of the *Rf3* and *Rf4* genes, as well as the genetic relationship among WA-, Y-, and DA-CMS systems. The plants derived from the three BC_3F_2 populations involving W23-19-06-06-11 and WA-, Y-, and DA-CMS lines that carried the *Rf3Rf3/Rf4Rf4*, *Rf3Rf3/rf4rf4*, and *rf3rf3/Rf4Rf4* genotypes, and the three types of cytoplasm, were selected and their pollen and spikelet fertilities were evaluated (Table 2). The results show that the pollen and spikelet fertility were 84.0 and 92.4 % in the plants with the *Rf3Rf3/Rf4Rf4* genotype, 39.3 and 55.2 % in the plants with the *Rf3Rf3/rf4rf4* genotype, and 48.2 and 68.0 % in the plants with the genotype of *rf3rf3/Rf4Rf4*, respectively. So, the genetic effects on pollen and spikelet fertility would be *Rf3Rf3/Rf4Rf4* > *rf3rf3/Rf4Rf4* > *Rf3Rf3/rf4rf4*, and the effect of *Rf4* appeared to be slightly larger than that of *Rf3* in the three CMS systems. The pollen and spikelet fertility were 59.6 and 71.8 % in the plants of ZsA/W23-19-06-06-11, 49.7 and 64.0 % in the plants of HnA/W23-19-06-06-11, and 62.2 and 79.8 % in the plants of XqA/W23-19-06-06-11 which indicates that the increasing order of genetic effects of the three CMS would be Y-CMS > WA-CMS > DA-CMS in the genetic background of W23-19-06-06-11.

When 205 SSR markers on average were used to analyze the genetic background and average length of SCSs with the *Rf3* and *Rf4* loci of the three BC_3F_2 individuals, carrying the genotype *Rf3Rf3/Rf4Rf4* (Table 3), the results show that the numbers of SCSs carried by the BC_3F_2 plants of ZsA/W23-19-06-06-11 were 0.9, carried by the BC_3F_2 plants of HnA/W23-19-06-06-11 were 0.8, and carried by the BC_3F_2 plants of XqA/W23-19-06-06-11 were 1.1, with a mean of 0.9. The length of the SCSs carried by the BC_3F_2 individuals corresponding to the *Rf3* and *Rf4* loci were on average 18.0 and 11.5 cM, respectively.

To analyze the genetic mode of the *Rf* genes, the plants carrying the *Rf3Rf3/Rf4Rf4* genotype and a less number of genetic background segments were selected by MAS in the three BC_3F_2 populations, and their pollen and spikelet fertilities were evaluated (Table 4). The pollen (spikelet) fertility of the BC_3F_2 individuals from the cross of ZsA/W23-19-06-06-11 corresponding to 2, 5, 11, 19, and 3 plants were 78.6 (80.5), 83.7 (88.4), 89.2 (94.5), 94.8 (96.9), and 95.6 % (98.1 %), respectively, and the BC_3F_2 individuals from the cross of HnA/W23-19-06-06-11 corresponding to 18, 7, 6, 5, and 3 plants, had 67.7 (80.0), 70.5 (83.4), 78.8 (83.6), 82.7 (85.0), and 86.9 % (88.3 %) pollen (spikelet) fertility, respectively. The pollen (spikelet) fertility corresponding to 8, 14, 13, and 8 plants showed 77.6 (86.6), 83.2 (92.4), 88.3 (95.4), and 92.1 % (97.5 %), respectively, in the BC_3F_2 individuals of XqA/W23-19-06-06-11. It can be concluded from the distribution of pollen and spikelet fertility of the BC_3F_2 plants that two pairs of dominant genes governed the fertility restoration in pollen and spikelet in the W23-19-06-06-11. So, the genetic mode of the *Rf* genes was a qualitative character for the three CMS systems.

Table 3. Substituted chromosome segments corresponding to *Rf3* or *Rf4* locus in the BC_3F_2 plants (A-lines/W23-19-06-06-11) carrying the genotype *Rf3Rf3/Rf4Rf4*. P1 and P4 indicate the positions of maximum length of a substituted segment, P2 and P3 indicate the positions of minimum length of a substituted segment. The *single hyphen* in the middle of markers indicates chromosome substitution segments. The end markers of the *double hyphens* are side markers of a substitution segment which indicates that a segment recombination might appear. The RM code indicates the markers described in McCouch *et al*. (2002), and the PSM code indicates the markers designed by the State Key Laboratory for Conservation and Utilization of Subtropical Agro-bioresources, South China Agricultural University, China.

Locus	Cross	Number of plants	Number of segments	P1	P2	P3	P4	Marker	Length [cM]
Rf3	ZsA/W23-19-06-06-11	53	0.9	23.3	25.0	38.8	49.0	RM220--RM1-RM283-PSM572-PSM348-PSM354-RM259--RM581	19.9
	HnA/W23-19-06-06-11	46	0.8	18.2	23.3	38.8	49.0	RM86--RM220-RM1-RM283-PSM348-PSM354-RM259--RM581	23.3
	XqA/W23-19-06-06-11	45	1.1	23.3	24.7	30.5	39.0	RM220--RM1-RM283-PSM572-PSM348-PSM354--RM259	10.8
Rf4	ZsA/W23-19-06-06-11	53	0.9	45.7	48.8	59.0	61.0	PSM455--RM258-PSM25599-PSM25510-RM304-RM5373-RM6100-PSM168-RM271--PSM169	13.2
	HnA/W23-19-06-06-11	46	0.8	45.7	48.8	58.0	59.0	PSM455--RM258-PSM25599-PSM25510-RM304-RM5373-RM6100-PSM168--RM271	11.2
	XqA/W23-19-06-06-11	45	1.1	49.0	49.3	58.9	59.4	RM25599--RM25510-RM304-RM5373-RM6100-PSM344-RM3773--RM271	10.0

Table 4. Distribution of pollen and spikelet fertility of the BC_3F_2 plants (A-lines/W23-19-06-06-11) with *Rf3Rf3/Rf4Rf4*. Means of three replicates ± SE. * - Individual rows represented different lines of the same cross.

Cross	Number of plants*	Pollen fertility [%]	Range [%]	Spikelet fertility [%]	Range [%]
ZsA/W23-19-06-06-11	2	78.6 ± 0.3	77.7 - 80.0	80.5 ± 6.9	79.9 - 81.1
	5	83.7 ± 2.2	80.7 - 84.9	88.4 ± 3.7	87.8 - 90.0
	11	89.2 ± 1.9	87.7 - 89.6	94.5 ± 0.5	92.2 - 94.6
	19	94.8 ± 3.1	90.1 - 95.0	96.9 ± 2.2	94.7 - 97.4
	3	95.6 ± 0.7	95.0 - 95.7	98.1 ± 1.9	98.1 - 98.4
HnA/W23-19-06-06-11	18	67.7 ± 1.5	65.1 - 69.7	80.0 ± 4.3	75.2 - 81.7
	7	70.5 ± 2.5	70.0 - 71.7	83.4 ± 1.8	82.1 - 84.4
	6	78.8 ± 1.0	77.8 - 79.8	83.6 ± 1.9	83.5 - 84.8
	5	82.7 ± 2.4	80.6 - 83.6	85.0 ± 0.8	84.8 - 85.2
	3	86.9 ± 2.9	85.1 - 88.2	88.3 ± 1.6	85.3 - 89.7
XqA/W23-19-06-06-11	8	77.6 ± 3.6	77.0 - 79.1	86.6 ± 3.6	78.5 - 88.5
	14	83.2 ± 1.7	80.3 - 86.3	92.4 ± 1.0	88.6 - 94.6
	13	88.3 ± 1.7	85.6 - 89.4	95.4 ± 2.8	93.6 - 95.7
	8	92.1 ± 0.9	90.0 - 93.8	97.5 ± 0.9	96.3 - 98.2

Discussion

The numbers and working model of *Rf* genes are specific to each of the CMS types within a plant species (Gabay-Laughnan and Laughnan 1994). Moreover, differences in degree of fertility restoration in different cytoplasmic backgrounds had been reported by Pradhan and Jachuck (1999), who attributed such differences to the infuence of the female genotype and/or differential penetrance of restorer genes in different CMS lines. Luo *et al*. (2013) reported that the new mitochondrial gene, *WA352*, which originated recently in wild rice, confers CMS-WA and CMS-DA because the encoded protein interacts with the nuclear-encoded mitochondrial protein COX11. In this context, the genetic effect showed a trend of Y-CMS > WA-CMS > DA-CMS in the genetic background of W23-19-06-06-11, the effect of *Rf4* appeared to be slightly larger than that of *Rf3*, and their effects were

additive for the three CMS systems. Similar results (Yao *et al*. 1997, Sattari *et al*. 2008, Zhuang *et al*. 2001) reported that the two fertility restorer genes are additive in their inheritance, and the effect of *Rf4* appeared to be larger than that of *Rf3*. Moreover, we show that two pairs of dominant genes governed the fertility restoration in pollen and spikelet in the W23-19-06-06-11 (Table 4) which indicates that the genetic mode of the *Rf* genes was a qualitative character for the three CMS systems.

The length of donor chromosome segments after a series of backcrosses is very important for the plant breeder who interests in the development of stocks with desired genetic traits. Several authors have derived equations to predict this length (Naveira and Barbadilla 1992). Stam and Zeven (1981) show that the size of a donor chromosomal segment is influenced by chromosome length, as well as by the number of back-cross generations. During backcrossing, all donor chromosome segments would be heterozygous and any recombination in these segments would result in reduction of the donor segment length. In our research, the mean of the segments of inherited background of the BC_3F_2 individuals was 0.9, whereas the average lengths of the SCSs, corresponding to *Rf3* and *Rf4* loci, were 18.0 and 11.5 cM, respectively. These results were similar to the assumption of the Naveira and Barbadilla (1992) formula predicting the average length of the donor segments of the backcross progeny in rice.

This study has provided for the first time information on effects of *Rf3* and *Rf4* in Y-CMS and DA-CMS and genetic relationship among WA-, Y-, and DA-CMS systems in the nuclear genetic background of SSSL W23-19-06-06-11. It should lead to the new approaches to alloplasmic line breeding and the transfer of *Rf* genes into adapted cultivars through a backcrossing program in an active hybrid rice breeding program.

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