BIOLOGIA PLANTARUM 57 (2): 274-280, 2013 DOI: 10.1007/s10535-012-0294-9

Allelic differentiations and effects of the *Rf3* and *Rf4* genes on fertility restoration in rice with wild abortive cytoplasmic male sterility

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Abstract

To reveal the allelic differentiations at the two genes for fertility restoration (*Rf*) on chromosomes 1 (*Rf3*) and 10 (*Rf4*), 15 chromosome single segment substitution lines (SSSLs) with the *Rf3* locus and 18 SSSLs with the *Rf4* locus were crossed with Bobai A (BbA), a cytoplasmic male sterility line with wild abortive type of cytoplasm (WA-CMS), respectively. Based on the pollen and seed fertility of the F_1 hybrids, the *Rf3* and *Rf4* genes were each classified into four alleles, namely *Rf3*-1, *Rf3*-2, *Rf3*-3, and *Rf3*-4 for *Rf3*, and *Rf4*-1, *Rf4*-2, *Rf4*-3, and *Rf4*-4 for *Rf4*. Out of the 33 SSSLs, an SSSL W23-19-06-06-11 carrying the genotype *Rf3*-4*Rf3*-4/*Rf4*-4*Rf4*-4 possessed the strongest restoring ability for BbA. To determine the genetic effects of *Rf3* and *Rf4* for WA-CMS, one BC_3F_2 population possessing the genetic background of W23-19-06-06-11 was generated from the cross between W23-19-06-06-11 and BbA by backcrossing and marker-assisted selection. In the BC_3F_2 population, the plants carrying the *Rf3Rf3/Rf4Rf4*, *Rf3Rf3/rf4rf4*, and *rf3rf3/Rf4Rf4* genotypes were selected and their phenotyping for pollen and spikelet fertility were evaluated. The result showed that under the genetic background of SSSL W23-19-06-06-11, the effect of *Rf4* appeared to be slightly larger than that of *Rf3* and their effects were additive for WA-CMS system. These studies will lead to the transfer of *Rf* genes into adapted cultivars through marker-assisted selection in active hybrid rice breeding programs.

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

Introduction

Cytoplasmic male sterility (CMS), a maternally inherited condition in which a plant carrying mitochondrial defects is unable to produce functional pollen, has been observed in numerous species (Bentolila *et al.* 2002) and, in many cases, male fertility can be restored by fertility restorer (*Rf*) genes associated with nuclear genes encoding pentatricopeptide repeat (PPR) proteins (Chase and Babay-Laughnan 2004, Hanson and Bentolila 2004). In rice, wild abortive (WA) discovered in China, belongs to the sporophytic cytoplasmic male sterility (CMS) system and it possesses typical aborted pollens (Yuan 1977, Lin and Yuan 1980). The inheritance of fertility restoration in the WA-CMS system has been extensively investigated and the chromosomal locations of the two *Rf* genes (*Rf3* and *Rf4*) have also been determined. Zhang *et al.* (1997) mapped the *Rf3* gene using restriction fragment length polymorphism (RFLP) on chromosome 1. 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 of *Rf3*. Tan *et al.* (1998) mapped two quantitative trait loci (QTLs), one on the middle of the long arm and one on the short arm of chromosome 10. Jing *et al.* (2001) mapped *Rf* locus (*Rf4*) governing fertility restoration on the long arm of chromosome 10. Zhang *et al.* (2002) mapped the *Rf4* gene at 0.9 cM from an RFLP marker (Y3-8) on chromosome 10. Sheeba *et al.* (2009) also mapped the *Rf4* gene at 1.2 cM from an SSR marker (RM6100) on chromosome 10.

Indica rice exhibits genetic diversity for *Rf* genes and *Rf* alleles for CMS in the AA genome of the genus *Oryza* may be a gene family that originated from a common ancestor evolutionarily and clustered in a small region in

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Received 20 April 2012, *accepted* 18 September 2012.

Abbreviations: CMS - cytoplasmic male sterility; MAS - marker-assisted selection; PCR - polymerase chain reaction; PPR - pentatricopeptide repeat; QTL - quantitative trait loci; *Rf* - fertility restorer gene; RFLP - restriction fragment length polymorphism; SSSLs - single segment substitution lines; WA - wild abortive.

Acknowledgements: This work was supported by National Natural Science Foundation of China (30830074) and Department of Education of Anhui Province Natural Science Foundation of China (No. KJ2010B155).

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the nuclear genome (Li *et al.* 2007). Ikehashi and Araki (1986) proposed an allelic interaction model for explaining the mechanism of hybrid sterility. According to the model, there are three alleles at the wide compatibility locus (S_5). The *S5*-i, *S5*-j, and *S5*-n alleles are present in *indica*, *japonica*, and wide compatibility varieties, respectively. Zhang and Lu (1989) identified three loci (*Sa*, *Sb*, and *Sc*) for F_1 pollen sterility from diallel crosses between Taichung 65, a *japonica* variety, and five near-isogenic lines which were derived from five *indica* donors by successive backcrosses (Oka 1974). Three additional sterility loci, namely *Sd*, *Se*, and *Sf*, were found by test crosses between the near-isogenic lines of Taichung 65 and a number of other varieties (Zhang *et al.* 1994). Long *et al.* (2008) found a locus for *indica-japonica* hybrid male sterility, *Sa*, comprising two adjacent genes, *SaM* and *SaF*, and proposed a two-gene/three-component interaction model for this hybrid male sterility system. Chen *et al.* (2008) proposed triallelic genetic model at the *S5* locus, namely *indica* (*S5*-i), *japonica* (*S5*-j), and compatibility gene (*S5*-n).

We have constructed a library of 1,123 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, Xi *et al.* 2006). 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 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.* 2008, 2009, 2010), and to clone the gene by map-based cloning (Wang *et al.* 2012) in rice. In this study, we selected 33 of the SSSLs, which carried the *Rf3* or *Rf4* genes on substituted segments, to detect the allelic differentiation and genetic effect of the genes for fertility restoration in rice.

Materials and methods

Plants: The SSSLs in the library were developed by using Hua-Jing-Xian 74 (HJX74), an elite genotype from *indica* variety as a recipient, and 24 accessions including 14 *indica* and 10 *japonica* accessions collected worldwide as donors (Zhang *et al.* 2004, Xi *et al.* 2006). Development of the SSSLs through backcrossing and SSR marker-assisted selection (MAS) was described by Xi *et al.* (2006). For this study, 33 SSSLs from 13 donors were selected (Table 1), each containing only one in the HJX74 genetic background. Fifteen of the SSSLs substituted segment with the *Rf3* or *Rf4* loci from a donors carried the *Rf3* locus on chromosome 1, and eighteen of the SSSLs carried the *Rf4* locus on chromosome 10. One typical wild-abortive CMS line of Bobai A (BbA) was used as female parent crossed with all the SSSLs and HJX74. These plant materials and their progenies were all planted in the experimental field in South China Agricultural University Campus.

Table 1. Recipient HJX74 and donors used to develop SSSLs. Variety according to He *et al.* (2003).

Genotype	Code	Origin	Variety	SSSLs
HJX74	W0	China	*indica*	
Amol 3 (Sona)	W02	Iran	*indica*	3
BG367	W04	Bangladesh	*indica*	2
Suyunuo	W07	China	*indica*	1
IR64	W08	IRRI	*indica*	1
Nanyangzhan	W10	China	*japonica*	1
Basmati370	W11	Pakistan	*indica*	4
Lianjian33	W14	China	*indica*	2
IRAT261	W18	Nigeria	*japonica*	5
Chenglongshuijing	W20	China	*indica*	2
Khazar	W22	Iran	*japonica*	3
Lemont	W23	USA	*trop. japonica*	5
Star bonnet 99	W24	USA	*trop. japonica*	2
IAPAR9	W27	Brazil	*trop. japonica*	2

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.

Allelic differentiation test for the *Rf* genes: Thirty-three SSSLs and HJX74 were crossed as the male parent with BbA. Allelic differentiations of the *Rf3* and *Rf4* loci were studied by evaluating pollen and spikelet fertility in F_1 progenies of the BbA/SSSLs and BbA/HJX74 crosses. Twenty spikelets 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 panicle length and percentage of wrapping panicle neck were recorded from 20 plants for each of the materials.

Genetic effect analysis of the *Rf* genes: An SSSL carrying the *Rf3*-4*Rf3*-4/*Rf4*-4*Rf4*-4 genotype, W23-19-06-06-11, was identified to be a strong restorer line in this study. The SSSL was crossed as the male parent with BbA and then backcrossed with the F_1. In the BC_1F_1, the plants with the *Rf3*-4*rf3*/*Rf4*-4*rf4* genotype, were selected by MAS. The selected plants were backcrossed to the SSSL for two more generations. In BC_3F_2 population, the plants carrying the genotypes *Rf3*-4*Rf3*-4/*Rf4*-4*Rf4*-4, *Rf3*-4*Rf3*-4/*rf4rf4* or *rf3rf3*/*Rf4*-4*Rf4*-4, were selected by MAS, and used to analyze the genetic effect of the *Rf* genes.

SSR marker analysis: A total of 205 polymorphic SSR markers were selected genome wide 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 Dellaporta *et al.* (1983). The PCR was conducted according to Panaud *et al.* (1996) with minor modifications. The PCR products were separated through electrophoresis on 6 % (m/v) polyacrylamide gel. Bands were visualized by a silver staining.

Results

Allelic differentiations of the *Rf3* and *Rf4* genes: Multiple comparison analysis for pollen fertility and spikelet fertility in the F_1 plants, derived from testcrosses of HJX74 and 33 SSSLs with BbA, indicated that four levels of restoration for the CMS were showed both at the *Rf3* and in the *Rf4* genes (Table 2). In the *Rf3* gene, the SSSLs derived from the W22 and W07 donors had 10.9 and 16.2 % (40.6 and 46.7 %) of pollen (spikelet) fertility respectively, with an average of 13.6 % (43.7 %). The restoration in the SSSLs was classified into the first level in the *Rf3* gene and the allele was named *Rf3*-1. The SSSLs from the W11 and W23 donors had 34.9 % (59.3 %) of pollen (spikelet) fertility on average at the second level of restoration and with *Rf3*-2 allele in the *Rf3* gene. The pollen (spikelet) fertility in the SSSLs from W10 and W02 was 47.9 % (69.7 %) ranging the third level of restoration which was controlled by the *Rf3*-3 allele. The restoration of the SSSLs from W18, W04, W27 and W14 were at the same level as HJX74 (W0), which carried the *Rf3*-4 allele. In the *Rf4* gene, the SSSLs from the W14 donor with the *Rf4*-1 allele showed 46.5 % (77.3 %) pollen (spikelet) fertility at the first level of restoration. The SSSLs from W02, W08, W18, and W22 displayed the same restoring ability as HJX74 with 64.8 % (85.4 %) of pollen (spikelet) fertility on average which were classified into the second level of restoration controlled by the *Rf4*-2 allele. The SSSLs from W04, W24, and W27 were at the third level of restoration with the *Rf4*-3 allele, exhibiting 70.9 % (93.2 %) of pollen (spikelet) fertility on average. At the top (fourth) level of restoration, the SSSLs from W11, W23, and W20, which carried the *Rf4*-4 allele, had 78.0 % (92.8 %) of pollen (spikelet) fertility on average.

Under the genetic background of HJX74 with the *Rf4*-2 allele, the genetic effects of the *Rf3* alleles were classified into four levels, namely *Rf3*-1, *Rf3*-2, *Rf3*-3, and *Rf3*-4 with the fertility restoration from weak to strong. In similar manner, the genetic effects of the *Rf4* alleles were classified into four levels, *Rf4*-1, *Rf4*-2, *Rf4*-3, and *Rf4*-4 under the genetic background of HJX74 with the *Rf3*-4 allele. The pollen grain viability assay with I_2-KI revealed that the percentage of fertile pollen was increasing according to the order of genetic effects of the *Rf3* and *Rf4* alleles from weak to strong (Fig. 1*A-H*).

Genotypes of some donors at the *Rf3* and *Rf4* loci: Based on the allelic differentiation of the *Rf3* and *Rf4* loci in the 33 SSSLs, we could validate the genotypes of some donors at the two *Rf* loci (Table 3). Out of 9 parents

Table 2. Allelic differentiations of the *Rf3* and *Rf4* loci based on the pollen (spikelet) fertility in F_1 plants from the crosses of BbA/SSSLs. Means ± SE.

Rf3 locus alleles	Donors to SSSLs	Fertility [%] pollen	spikelet	*Rf4* locus alleles	Donors to SSSLs	Fertility [%] pollen	spikelet
Rf3-1	W22	10.9 ± 1.1a	40.6 ± 1.2a	*Rf4*-1	W14	46.5 ± 2.2a	77.3 ± 0.9a
	W07	16.2 ± 1.6b	46.7 ± 1.6b	*Rf4*-2	W02	62.4 ± 1.0b	84.9 ± 0.7bc
	average	13.6 ± 1.4	43.7 ± 1.4		W08	65.3 ± 1.8b	85.5 ± 2.1bc
Rf3-2	W11	31.7 ± 0.4c	58.2 ± 0.6c		W0(CK)	65.3 ± 1.2b	83.7 ± 0.8b
	W23	38.1 ± 1.6d	60.3 ± 1.3c		W18	65.4 ± 1.7b	86.2 ± 1.0c
	average	34.9 ± 1.0	59.3 ± 1.0		W22	65.8 ± 1.5b	86.9 ± 2.6bc
Rf3-3	W10	45.4 ± 0.9e	64.3 ± 1.2d		average	64.8 ± 1.4	85.4 ± 1.4
	W02	50.4 ± 1.3f	75.0 ± 0.9e	*Rf4*-3	W04	69.9 ± 1.4c	91.4 ± 1.7d
	average	47.9 ± 1.1	69.7 ± 1.1		W27	70.7 ± 1.7c	95.5 ± 1.4e
Rf3-4	W18	60.7 ± 1.5g	79.4 ± 0.5f		W24	72.0 ± 0.9c	92.6 ± 0.7de
	W0 (CK)	65.3 ± 1.2h	83.7 ± 0.8g		average	70.9 ± 1.3	93.2 ± 1.2
	W04	66.3 ± 0.8h	84.0 ± 2.7g		W11	77.2 ± 1.6e	93.8 ± 0.9de
	W27	66.7 ± 1.0h	89.6 ± 1.4h		W23	78.2 ± 1.4e	91.2 ± 1.6d
	W14	68.5 ± 1.2h	89.6 ± 1.4h	*Rf4*-4	W20	78.6 ± 1.5e	93.3 ± 0.7de
	average	65.5 ± 1.1	84.8 ± 1.2)		average	78.0 ±1.5	92.8 ±1.1

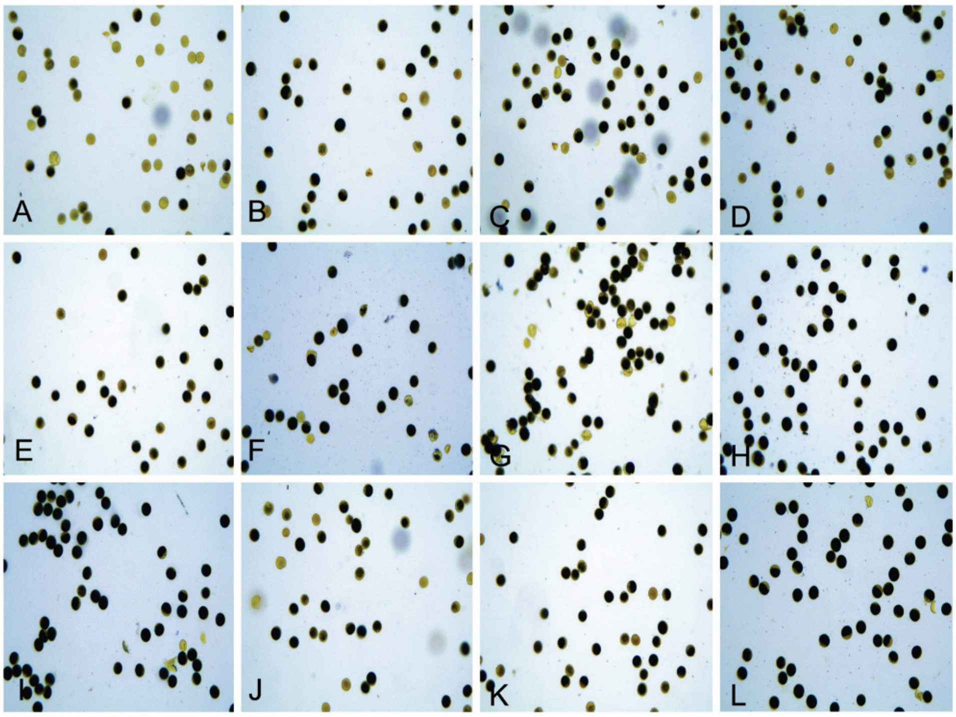

Fig. 1. I_2-KI stainability of pollen grains of the F_1 plants (BbA/SSSLs) and BC_3F_2 individuals generated from the cross between the SSSL W23-19-06-06-11 and BbA. *A* - BbA/SSSLs (*Rf3*-1*Rf3*-1/*Rf4*-2*Rf4*-2); *B* - BbA/SSSLs (*Rf3*-2*Rf3*-2/*Rf4*-2*Rf4*-2); *C* - BbA/SSSLs (*Rf3*-3*Rf3*-3/*Rf4*-2*Rf4*-2); *D* - BbA/SSSLs (*Rf3*-4*Rf3*-4/*Rf4*-2*Rf4*-2); *E* - BbA/SSSLs (*Rf3*-4*Rf3*-4/*Rf4*-1*Rf4*-1); *F* - BbA/SSSLs (*Rf3*-4*Rf3*-4/*Rf4*-2*Rf4*-2); *G* - BbA/SSSLs (*Rf3*-4*Rf3*-4/*Rf4*-3*Rf4*-3); *H* - BbA/SSSLs (*Rf3*-4*Rf3*-4/*Rf4*-4*Rf4*-4); *I* - BC_3F_2 individuals with the genotype *Rf3Rf3*/*Rf4Rf4*; *J* - BC_3F_2 individuals with the genotype *Rf3Rf3*/*rf4rf4*; *K* - BC_3F_2 individuals with the genotype *rf3rf3*/*Rf4Rf4*; *L* - HJX74 (CK) with the genotype *Rf3*-4*Rf3*-4/*Rf4*-2*Rf4*-2.

including donors and HJX74, the BG367, IAPAR9, carrying the *Rf3*-4*Rf3*-4/*Rf4*-3*Rf4*-3 genotype, possessed the strongest restoring ability for BbA, and Khazar, carrying the *Rf3*-1*Rf3*-1/*Rf4*-2*Rf4*-2 genotype, possessed the weakest restoring ability. HJX74 carrying the genotype of *Rf3*-4*Rf3*-4/*Rf4*-2*Rf4*-2 showed the middle restoring ability. The materials with the genotypes of *Rf3*-4*Rf3*-4/*Rf4*-4*Rf4*-4 and *Rf3*-1*Rf3*-1/*Rf4*-1*Rf4*-1 were not found. It indicated that it was naturally difficult to find the germplasms possessing the extreme restoring ability for the CMS in rice.

Table 3. Genotypes at the *Rf3* and *Rf4* loci in the recipient and some donors of the SSSLs.

Donor	Code	Alleles of *Rf3* locus	Alleles of *Rf4* locus
Khazar	W22	*Rf3*-1	*Rf4*-2
Basmati 370	W11	*Rf3*-2	*Rf4*-4
Lemont	W23	*Rf3*-2	*Rf4*-4
Amol 3 (Sona)	W02	*Rf3*-3	*Rf4*-2
Lianjian33	W14	*Rf3*-4	*Rf4*-1
HJX74		*Rf3*-4	*Rf4*-2
IRAT 261	W18	*Rf3*-4	*Rf4*-2
BG367	W04	*Rf3*-4	*Rf4*-3
IAPAR 9	W27	*Rf3*-4	*Rf4*-3

Genetic effects of two *Rf* loci: An SSSL, W23-19-06-06-11, with the *Rf3*-4*Rf3*-4/*Rf4*-4*Rf4*-4 genotype was selected to analyze the genetic effects of the *Rf3*-4 and *Rf4*-4 genes. The plants, carrying the genotypes of *Rf3*-4*Rf3*-4/*Rf4*-4*Rf4*-4, *Rf3*-4*Rf3*-4/*rf4rf4*, and *rf3rf3*/*Rf4*-4*Rf4*-4, were selected by MAS in the BC_3F_2 population generated from the cross between W23-19-06-06-11 and BbA. Pollen and spikelet fertility, panicle length, and wrapping panicle neck in the BC_3F_2 plants were evaluated (Table 4, Fig. 1*I*-*L*). The results showed that the pollen (spikelet) fertility, panicle length, and wrapping panicle neck ratio were 82.8 % (94.8 %), 0.22 m and -21.3 % in the plants with the *Rf3*-4*Rf3*-4/*Rf4*-4*Rf4*-4 genotype, 47.1 % (91.9 %), 0.23 m, and -30.3 % in the plants with the *Rf3*-4*Rf3*-4/*rf4rf4* genotype, and 58.1 % (92.2 %), 0.22 m, and -26.2 % in the plants with the genotype of *rf3rf3*/*Rf4*-4*Rf4*-4, respectively. So, the genetic effects on pollen (spikelet) fertility and wrapping panicle neck ratio would be *Rf3*-4*Rf3*-4/*Rf4*-4*Rf4*-4 > *rf3rf3*/*Rf4*-4*Rf4*-4 > *Rf3*-4*Rf3*-4/*rf4rf4*, and the effect of *Rf4* appeared to be slightly larger than that of *Rf3* in the CMS-BbA.

Furthermore, the variations of pollen (spikelet) fertility and wrapping panicle neck ratio were not correlated with panicle length in the BC_3F_2 plants (Table 4).

Table 4. Pollen and spikelet fertility, panicle length, and wrapping panicle neck of BC$_3$F$_2$ individuals generated from the cross between the SSSL W23-19-06-06-11 and BbA. Means ± SE.

Genotype	Pollen fertility [%]	Spikelet fertility [%]	Panicle length [m]	Wrapping neck [%]	Number of plants
Rf3Rf3/Rf4Rf4	82.8 ± 0.6	94.8 ± 0.5	0.22 ± 0.1	-21.3 ± 0.7	53
Rf3Rf3/rf4rf4	47.1 ± 0.4	91.9 ± 0.8	0.23 ± 0.1	-30.3 ± 0.8	55
rf3rf3/Rf4Rf4	58.1 ± 0.5	92.2 ± 0.6	0.22 ± 0.2	-26.2 ± 0.9	57
Average	62.7 ± 0.5	93.0 ± 0.6	0.23 ± 0.1	-29.9 ± 0.8	55

Discussion

Demand for QTL analysis of complex traits is increasing because of its potential to facilitate the effective manipulation of traits of importance in crop breeding (Yano and Sasaki 1997, Peleman and van der Voort 2003). SSSLs were found to be a powerful tool to identify, to map, and to clone QTLs for complex traits in rice (Liu *et al*. 2008, 2009, 2010, Wang *et al*. 2012, Zhang *et al*. 2012). Teng *et al*. (2012) classified the *Wx* gene into five alleles, *wx*, *Wx*-t, *Wx*-g1, *Wx*-g2, and *Wx*-g3, by the use of SSSLs in the HJX74 genetic background. In this study, the *Rf3* and *Rf4* genes were each classified into four alleles, namely *Rf3*-1, *Rf3*-2, *Rf3*-3, and *Rf3*-4, and *Rf4*-1, *Rf4*-2, *Rf4*-3, and *Rf4*-4, which had restoring ability for CMS-BbA from weak to strong under the HJX74 genetic background. Those results indicated the SSSLs were useful materials to assess allelic variation in the genes for complex traits.

In rice, two dominant restorer genes, *Rf3* and *Rf4*, are suggested to be responsible for the fertility restoration of WA-CMS (Zhang *et al*. 1997, Tan *et al*. 1998, Zhang *et al*. 2002). In this research, the effect of *Rf4* appeared to be slightly larger than that of *Rf3* and a certain level of additive effect is present at *Rf3* and *Rf4* loci under the HJX74 genetic background. Similar results had 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* (Yao *et al*. 1997, Zhuang *et al*. 2001, Sattari *et al*. 2008). Furthermore, it can be concluded that the variations of pollen (spikelet) fertility and wrapping panicle neck ratio were not correlated with panicle length. These results were the same as those obtained by Zhu (1979) and Zhang *et al*. (1993).

This study provided the information on genetic effects of *Rf3* and *Rf4* in some materials and classified four alleles each at the *Rf3* and *Rf4* loci using SSSLs. An SSSL, W23-19-06-06-11, with the strongest restoring ability for the CMS, was identified from the 33 SSSLs tested. By the use of the SSSL, the strongest *Rf4*-4 allele would be transferred into adapted cultivars through MAS in active hybrid rice breeding programs.

Japonica type of varieties was considered not carrying functional *Rf* genes (Zhu 2000). In the present study, some donors of the Japonica type, such as IRAT261 (W18) with the genotype *Rf3*-4*Rf3*-4/*Rf4*-2*Rf4*-**2**, Lemont (W23) with the genotype *Rf3*-2*Rf3*-2/*Rf4*-4*Rf4*-4, and IAPAR9 (W27) with the genotype *Rf3*-4*Rf3*-4/ *Rf4*-3 *Rf4*-3, contributed with functional alleles at the *Rf3* and/or *Rf4* loci. The pedigrees of some of the donors show that they are not typical Japonica ones, such as IRAT261 which was a line selected from Columbia/M312A, where Columbia and M312A are of Indica and Japonica types, respectively (Zhu 2000). These newly identified restorer genes at the *Rf3* and *Rf4* loci in the Japonica lines might be useful for the exploitation and utilization of heterosis in Japonica rice in the future.

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