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Genetic effects of F_1 pollen sterility genes *S-b*, *S-d* and *S-e* in rice (*Oryza sativa* L.)

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Abstract: An experimental population commonly used in genetic analyses of gene or quantitative trait loci (QTLs) in rice is chromosome segment substitution lines (CSSLs). In the present study, with the typical *indica* variety Guangluai 4 as a donor and *japonica* variety Taichung 65 as a recipient, seven CSSLs carrying F_1 pollen sterility genes *S-b*, *S-d*, *S-e*, *S-b*/*S-d*, *S-b*/*S-e*, *S-d*/*S-e*, and *S-b*/*S-d*/*S-e* were obtained by specific selection for the target genes, non-specific selection for the genome of the recurrent parents in four backcross populations (BC_1F_2, BC_2F_2, BC_3F_2 and BC_3F_3). We evaluated the genetic effect of the F_1 pollen sterility genes using 35 F_1 hybrid individuals in crosses derived from CSSLs and Taichung 65. Pollen fertility of F_1 hybrid plants was observed and the results indicated that the single genes *S-b*, *S-d* and *S-e* can cause 67.7%, 14.6% and 53.2% of pollen sterility, respectively. Multiple genes *S-b*/*S-d*, *S-b*/*S-e*, *S-d*/*S-e*, and *S-b*/*S-d*/*S-e* can cause 76.6%, 85%, 68.7%, and 93% of pollen sterility, respectively.

Keywords: chromosome segment substitution lines; donor; marker-assisted selection; recipient

Rice (*Oryza sativa* L.) is one of the most important crops feeding about half of the world's population. At present, with a reduction of the global cultivated rice land area, breeding more productive rice varieties becomes an urgent task for rice breeders (Li *et al*. 2006; Ali *et al*. 2010). F_1 hybrids between *indica* and *japonica* subspecies usually demonstrate very strong hybrid vigour or heterosis, which has attracted considerable research interest with the hope of using such heterosis in hybrid rice production (Qiu *et al*. 2005). However, varying degrees of hybrid sterility are commonly seen in crosses between *indica* and *japonica* subspecies, and the partial or complete sterility of the hybrids is a serious constraint for utilizing heterosis in hybrid rice breeding (Oka 1957; Liu *et al*. 1997; Long *et al*. 2008).

Apart from female gamete abortion, male gamete abortion (F_1 pollen sterility) is considered to play a key role in *indica-japonica* hybrid sterility (Oka 1974; Jing *et al*. 2007). Zhang and Lu (1989, 1993) put forward the "Specific Compatibility Hypothesis". According to the hypothesis, F_1 pollen sterility was the main form of hybrid sterility and there were at least six genes, namely *S-a*, *S-b*, *S-c*, *S-d*, *S-e* and *S-f*, controlling the pollen sterility of F_1 hybrids. The degree of pollen sterility caused by the allelic interaction at the loci varied with the particular gene and the gene number: the higher the level of heterozygosity at the six genes, the higher pollen and spikelet sterility is observed. Up to now, five loci, *S-a*, *S-b*, *S-c*, *S-d* and *S-e*, have been located in the genetic map of rice (Zhang & Zhang 2001; Li *et al*. 2002; Zhuang *et al*. 2002; Yang *et al*. 2004; Li *et al*. 2006; Zhu *et al*. 2008). Among them, three loci, *S-b*, *S-c* and *S-d*, have already been located in the physical map, and one locus, *S-a*, has been cloned by positional cloning strategy (Long *et al*. 2008).

Chromosome segment substitution lines (CSSLs) are important genetic stocks for investigating the function and regulation of single genes and useful for gene cloning. In rice, several CSSLs have been produced (Zhu *et al*. 2009; Ramos *et al*. 2016). The

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goals of the present study were (1) construction of CSSLs containing F_1 pollen sterility genes *S-b*, *S-d* and *S-e*, (2) validation of these genes using the CSSLs, and (3) estimation of the gene effect on the hybrid sterility trait.

MATERIAL AND METHODS

Plant materials and growth conditions. Guangluai 4 used as a donor in this study is a typical *indica* variety in South China's Guangdong province. Taichung 65 is a typical *japonica* variety, which was used as a recipient. First, the cross between Guangluai 4 and Taichung 65 was carried out, then a series of backcrosses with Taichung 65 as the recurrent parent was performed. A total of 240 BC_3F_3 individual plants as well as the parents and 35 F_1 individuals (CSSLs/Taichuang 65) were planted in the experimental farm of Nanyanghu, Jining city, Shandong province of China.

DNA preparation and PCR protocol. DNA was extracted from fresh young leaves using the CTAB method (Murray & Thompson 1980). The PCR was conducted with minor modifications by Panaud *et al*. (1996). The protocol for PCR was as follows: the template DNA was subjected to denaturation at 94°C for 5 min, followed by 35 cycles (denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, and primer extension at 72°C for 1 min), and a final extension at 72°C for 5 min. The PCR products were separated through electrophoresis on a 6% non-denaturing polyacrylamide gel. Bands were revealed using a silver staining procedure.

Genetic background detection and molecular marker linked with *S-b*, *S-d*, and *S-e*. For genetic background detection, 180 SSR markers selected from dense rice microsatellite maps (McCouch *et al*. 2002) were used to survey the polymorphism between Guangluai 4 and Taichung 65. Polymorphic markers were used for the genetic background detection of CSSLs. For selection of CSSLs carrying F_1 pollen sterility genes, six markers were used for *S-b*, *S-d*, and *S-e* (Table 1). Bulked segregant analysis (BSA) was employed to evaluate the uniformity of the genetic background in the CSSLs.

Pollen fertility analysis. The identification of pollen fertility was assessed as described by Zhang and Lu (1989). Ten florets per panicle were collected from the upper one-third portion of the panicle and fixed in a FAA solution of 6% (v/v) formaldehyde, 89% (v/v) alcohol, and 5% (v/v) acetic acid. The pollen phenotypes of rice were observed in a 1% I2-KI (potassium iodide) solution under a microscope. Pollen was classified as fertile (round and full dark), empty abortive (irregular and yellow) and staining abortive (round and partial dark) based on their shape and stainability. Pollen fertility of hybrids was observed in F_1 hybrid plants and different types of individuals from F_2 populations.

RESULTS

Construction of CSSLs. The procedure for the development of CSSLs is summarized in Figure 1. The F_1 plants derived from Guangluai 4/Taichung 65 were selfed to produce an F_2 generation. In the F_2 population, individuals similar to Taichung 65 in plant height and heading date were selected to backcross with Taichung 65 to produce BC_1F_2 population con-

Table 1. The primers linked with rice F_1 pollen sterility genes *S-b*, *S-d* and *S-e*

F_1 pollen sterility locus	Chromosome	Linked markers	Sequence of marker (5'-3')
S-b	5	PSM60	F: CCCATCCAGGTCACCACCACAAT R: AATCCGAATCGCATCAGAAGCAG
		PSM202	F: CGGAATCAATGGAAGGTTT R: CCCTTGACTTCCCATTCT
S-d	1	PSM43	F: CGTAGTGGTCCATCGGAGGC R: TGAGCTGAGCTGCGGCAAG
		RM84	F: TAAGGGTCCATCCACAAGATG R: TTGCAAATGCAGCTAGAGTAC
S-e	12	PSM597	F: GCCTGATGCTACTCGCCAC R: CAGCCATTGTTAATTTCTACGATG
		PSM459	F: CTGCATTGCGCTTGGTGT R: GATCCTGAAGTGGTTGTGGC

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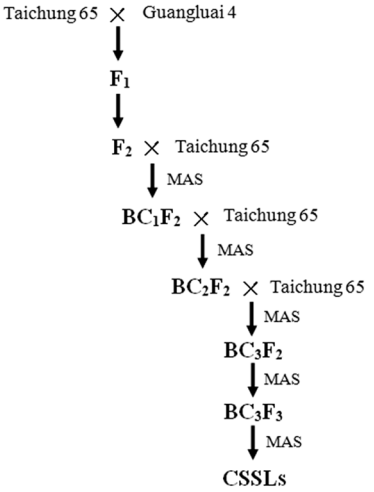

Figure 1. The development of seven chromosomal segment substitution lines with Guangluai 4 as donor and Taichung 65 as recipient; marker-assisted selection (MAS) was used for the selection of F_1 pollen sterility genes *S-b*, *S-d*, and *S-e*

taining 240 individuals. For each backcross only individuals carrying the genes *S-b*, *S-d* and *S-e* derived from Guangluai 4 were used. With marker-assisted selection (MAS), 48 BC_1F_2 plants were backcrossed with Taichung 65 to produce 305 BC_2F_2 plants. In the same way, 245 BC_3F_2 individuals derived from 20 BC_2F_2 plants were obtained. MAS with a whole-genome survey of 245 BC_3F_2 enabled the identification of 13 plants homozygous for Taichung 65 in the majority of genomic regions except for one or two heterozygous substituted segments from the donor with sterility genes. These plants were selfed to produce 13 BC_3F_3 lines (650 individuals). Finally, a total of seven CSSLs carrying one or multiple F_1 pollen sterility genes (*S-b*, *S-d*, *S-e*, *S-b*/*S-d*, *S-b*/*S-e*, *S-d*/*S-e*, and *S-b/S-d/S-e*) were obtained.

Detection of residual segments and uniformity of genetic background in the CSSLs. One hundred and twenty-nine SSR markers displayed polymorphism between parents and the rate of polymorphism was 71.67%. To evaluate the uniformity of the genetic background in the seven CSSLs, a round of examination for the genetic background was carried out and Bulked Segregant Analysis (BSA) was employed with polymorphic markers. Six BSAs (245 BC_3F_2 individuals) were tested using 129 polymorphic SSR markers selected in non-target chromosomal regions. From 774 PCR products, residual segments or heterozygous segments in non-target chromosomal regions were detected in 48 PCR products. The percentage of PCR products of residual segments was 6.2% in the BC_3F_2 population (Table 2). All residual segments in the population were treated as non-target segments for further selection in 245 BC_3F_2 individuals. The second round of detection for donor residual segments was done in the BC_3F_3 generation. From 1600 PCR products in 10 candidate CSSLs, 20 PCR products were found to reveal donor alleles. The percentage of PCR products of residual segments was 1.3%. The lines with residual segments were eliminated. These results implied that the homozygosity of Taichung 65 alleles in non-target chromosomal regions or the uniformity of the Taichung 65 genetic background in the seven CSSLs was greater than 98.8%.

Table 2. Residual segments in the BC_3F_2 population of rice

Chromosome	Marker	Genotype of six BSAs
	RM109	heterozygote
2	RM112	heterozygote
	RM154	heterozygote
	RM85	heterozygote
3	RM218	heterozygote
	RM442	heterozygote
4	RM131	heterozygote
7	RM298	heterozygote

BSA – bulked segregant analysis

Genetic effects of F_1 pollen sterility genes *S-b*, *S-d* and *S-e*. The results indicated that the single genes *S-b*, *S-d* and *S-e* can cause 67.7%, 14.6% and 53.2% of pollen sterility (Figure 2), respectively. While two genes *S-b/S-d*, *S-b/S-e* and *S-d/S-e* can cause 76.6%, 85% and 68.7% of pollen sterility (Figure 3),

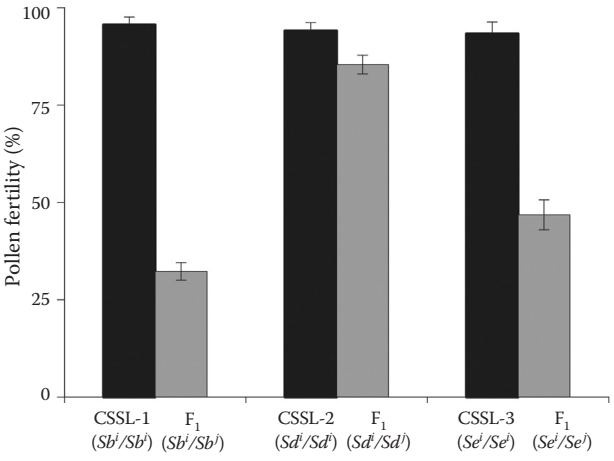

Figure 2. Genetic effects of single rice F_1 pollen sterility genes *S-b*, *S-d*, and *S-e* evaluated in three F_1 hybrids derived from CSSL-1/Taichung 65, CSSL-2/Taichung 65 and CSSL-3/Taichung 65

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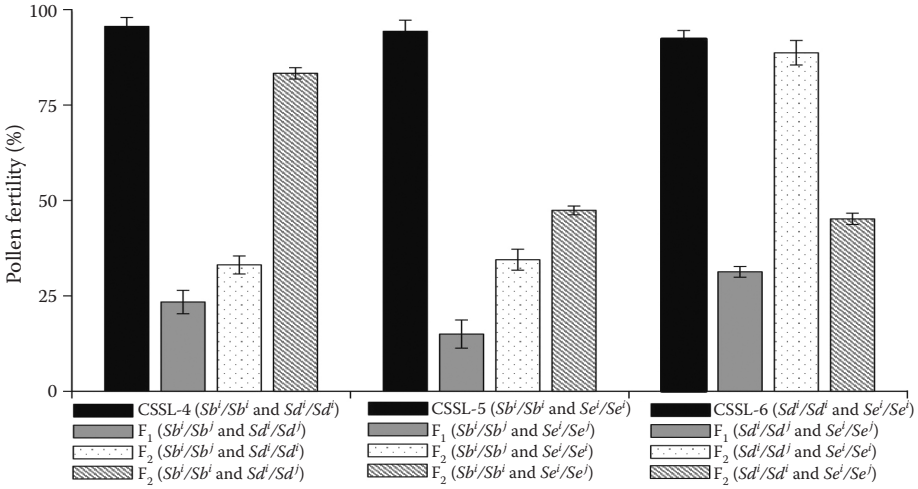

Figure 3. Genetic effects of double rice F_1 pollen sterility genes *S-b*/*S-d*, *S-b*/*S-e*, and *S-d*/*S-e* evaluated in three F_1 hybrids and six different types of F_2 individuals derived from CSSL-4/Taichuang 65, CSSL-5/Taichung 65 and CSSL-6/Taichung 65

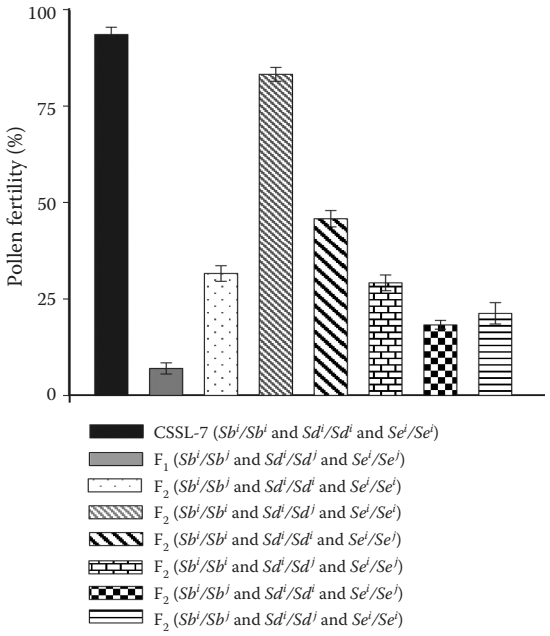

Figure 4. Genetic effects of multiple rice F_1 pollen sterility genes *S-b*/*S-d*/*S-e* evaluated in F_1 hybrid plants and six different types of F_2 individuals derived from CSSL-7/Taichung 65

respectively, and multiple genes *S-b*/ *S-d*/*S-e* can cause 93% of pollen sterility (Figure 4).

DISCUSSION

The hybrid sterility between *indica* and *japonica* subspecies of Asian cultivated rice is a complex trait. The genetic basis of F_1 partial sterility has been intensively studied. An allelic interaction model may explain the mechanism of hybrid sterility (Ikehashi & Araki 1986, 1988). According to the model, there were three alleles at the *S5* gene, $S5^i$, $S5^j$ and $S5^n$, representing *indica*, *japonica* and a widely compatible variety, respectively (Chen *et al*. 2008). The heterozygote of *indica*/*japonica* ($S5^i/S5^j$) produced semi-sterile panicles, resulting from the partial abortion of female gametes carrying $S5^j$, whereas the heterozygote consisting of the $S5^n$ allele with either of the other two alleles, e.g. $S5^n/S5^i$ or $S5^n/S5^j$, would be fully fertile. So far, a series of female sterility genes including major QTLs was identified and mapped (Wan *et al*. 1993, 1996; Wan & Ikehashi 1995; Wang *et al*. 1998; Liu *et al*. 2001; Song *et al*. 2005).

It seemed that two approaches may be able to overcome F_1 sterility between *indica-japonica* subspecies varieties. The one was to screen widely compatible varieties. A number of these varieties produced fertile F_1 hybrids when crossed to both *indica* and *japonica* varieties and thus they were called widely compatible varieties. Ikehashi and Araki (1984) recognized the significance of this phenomenon and then proposed a genetic basis for F_1 sterility and wide compatibility. The finding of the neutral allele $S5^n$ excited rice breeders to screen widely compatible varieties from a large amount of *indica* and *japonica* subspecies through extensive test-crosses and some were gathered. The widely compatible varieties were currently the main tools for breaking the sterility barriers between *indica* and *japonica* subspecies and have made an important contribution to rice breeding. Recently, however, some *indica-japonica* hybrids

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which had the neutral allele $S5^n$ have been tested for their yield potential. Such hybrids showed strong heterosis, but their seed set was unstable in adverse conditions. In fact, besides $S5^n$, there are neutral alleles of other genes. It seemed that more neutral alleles should be pyramided to make varieties widely compatible. The other was breeding and utilization of *indica*-compatible *japonica* lines by introducing the S^i allele into *japonica* varieties through continuous backcrosses. *Indica*-compatible *japonica* lines carried S^i/S^i at the hybrid sterility loci. When crossed with *indica* varieties, the F_1 not only had a strong vigour, but also was fully fertile. Such a kind of breeding program is being undertaken and some excellent *indica*-compatible *japonica* lines have already been cultivated (Ding *et al*. 2002; Guo *et al*. 2016). This approach needs to pyramid F_1 pollen sterility genes which carry S^i/S^i to the same *japonica* variety. Hence, searching for F_1 pollen sterility genes and evaluating their genetic effect are very important for developing *indica*-compatible *japonica* lines. The development of the CSSLs of F_1 pollen sterility genes not only enriched and perfected the 'Specific Compatibility Hypothesis' but also provided the strong basis for breeding *indica*-compatible *japonica* lines with molecular marker-assisted selection.

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