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ORIGINAL PAPER

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Identification and confirmation of three neutral alleles conferring wide compatibility in inter-subspecific hybrids of rice (*Oryza sativa* L.) using near-isogenic lines

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Abstract Wide-compatibility varieties (WCVs) are a special class of rice germplasm that is able to produce fertile hybrids when crossed to both *indica* and *japonica* subspecies. Previous studies determined 'Dular' and 02428 as two WCVs and identified a number of QTLs as having large effects on fertility of inter-subspecific hybrids. In this study, we developed five near-isogenic lines (NILs) for three of the QTLs, *f5*, *f6* and *S5*, by backcrossing and marker-assisted selection, using 'Dular' and 02428 as the donors and 'Zhenshan 97' as the recipient. Three of the NILs each carried one introgressed allele, and two NILs each carried two introgressed alleles in combinations. The NILs were testcrossed to an *indica* tester 'Nanjing 11' and a *japonica* tester 'Balilla'. The results showed that the *f5* allele from 'Dular' (*f5*-Du) is a neutral allele conferring wide compatibility, with a large effect on both pollen and spikelet fertility, and the *f6* allele from 'Dular' (*f6*-Du) is a neutral allele for spikelet fertility with smaller effect. The *S5* allele from 02428 (*S5*-08) was confirmed to be a neutral allele for spikelet fertility. It is likely that *f6* and *S5* are the same locus as deduced by their genomic locations and effects. The results also showed that even in combination, two neutral alleles of different loci were not able to produce normal fertility hybrids in typical *indica–japonica* crosses. The implications of the findings in rice breeding programs are discussed.

Introduction

The strong hybrid vigor in the F_1s between *indica* and *japonica* subspecies of Asian cultivated rice (*Oryza sativa* L.) has attracted a large amount of research interest, with the hope for developing hybrid rice by making use of such heterosis (Yang et al. 1962; Chu et al. 1964; Yuan 1987). However, hybrid sterility frequently occurs in such inter-subspecific crosses; the fertility of *indica–japonica* hybrids varies widely from fully fertile to almost completely sterile, with the majority of such hybrids showing significantly reduced fertility (Kato et al. 1928; Oka 1988; Liu et al. 1996; Zhang et al. 1997).

Wide-compatibility varieties (WCVs) are a special class of rice germplasm that is able to produce fertile hybrids when crossed to both *indica* and *japonica* varieties (Ikehashi and Araki 1984). The discovery of WCVs brought hope for breaking the fertility barrier between *indica* and *japonica* subspecies and provided a possibility for exploiting the very strong heterosis demonstrated in crosses between the two subspecies. Consequently, there has been considerable interest in understanding the mechanisms underlying wide compatibility and hybrid sterility. Ikehashi and Araki (1986), among others, proposed an allelic interaction model for explaining the mechanism of hybrid sterility and wide compatibility. According to this model, there are three alleles at the *S5* locus: a neutral allele (S_5^n), an *indica* allele (S_5^i), and a *japonica* allele (S_5^j). A zygote formed of the S_5^n allele with either of the other two alleles, e.g., S_5^n S_5^i or S_5^n S_5^j, would be fully fertile, whereas a zygote of S_5^i S_5^j would be partly sterile. Ikehashi and Araki (1986) also located the *S5* locus on chromosome 6. Utilization of the wide-compatibility gene (S_5^n) for development of inter-subspecific hybrids has been a practice in many rice breeding programs (Araki et al. 1988; Ikehashi 1991).

However, it was soon realized that the mechanisms underlying hybrid sterility and wide compatibility are rather complex, which cannot be explained by the *S5* locus alone. In addition to *S5*, a number loci causing female gamete abortion by allelic interaction (Yanagihara et al. 1992; Wan et al. 1993, 1996) and QTLs for defective female gametophyte development were also identified in an inter-subspecific cross (Liu et al. 2001).

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Male gamete abortion was also considered to play a key role in *indica–japonica* hybrid sterility. Zhang and Lu (1989, 1993) and Zhang et al. (1994) identified six loci for F_1 pollen sterility and proposed that hybrid sterility in *indica–japonica* hybrids was mainly caused by allelic interactions at the F_1 pollen sterility loci. Moreover, there were also gametophyte genes that were reported as causing male gamete abortion or selective fertilization and distorted segregation in inter-subspecific hybrids (Lin et al. 1992, 1993; Kinoshita 1995; Lu et al. 2000).

Two WCVs, 'Dular' and 02428, have been widely used in *indica–japonica* hybrid rice breeding programs in China and other parts of the world. Liu et al. (1997), using a three-way cross (02428/'Nanjing 11'//'Balilla') population and a molecular marker linkage map, identified a major locus for wide compatibility that corresponded well to the *S5* locus reported previously (Ikehashi and Araki 1986; Liu et al. 1992; Zheng et al. 1992; Yanagihara et al. 1995) and two minor loci located on chromosomes 2 and 12, respectively. Wang et al. (1998), also using a three-way cross ('Balilla'/'Dular'// 'Nanjing 11') population, resolved five QTLs as conferring significant effects on hybrid fertility, with the one on chromosome 5 (*f5*) showing the largest effect, followed by *f6*, also seeming to correspond to the *S5* region.

However, several issues remain to be resolved. The most important issue concerns whether the alleles from WCVs identified above are neutral alleles conferring wide compatibility or *indica–japonica* alleles for hybrid sterility. Compatibility tests demonstrated that WCVs vary greatly in both compatible spectra and levels of compatibility (Pan et al. 1990; Liu et al. 1996; Zhang et al. 1997), indicating that the WCVs differ in their alleles for wide compatibility or hybrid sterility. Previous results (Liu et al. 1992; Liu et al. 1997) established beyond doubt that the *S5* allele from 02428 is a neutral allele. However, based on the results of the three-way cross by Wang et al. (1998), it could only be inferred that the identified QTL alleles had high compatibility with the *indica* tester, despite the observation that 'Dular' has both a wide spectrum and high level of compatibility (Ikehashi and Araki 1988; Gu et al. 1993; Liu et al. 1996). Thus, it is necessary to test the 'Dular' alleles for their compatibility with *japonica* testers.

Second, it can be inferred that *f6* and *S5* are located in similar chromosomal regions based on molecular marker linkage maps (Liu et al. 1997; Wang et al. 1998). However further assessment is needed to ascertain whether they are indeed the same locus. Moreover, the allele from 'Dular' at this locus seemed to have a much smaller effect on hybrid fertility than the one from 02428, based on the data from the two populations (Liu et al. 1997; Wang et al. 1998), which again needs to be assessed under a common genetic background.

The study reported in this paper was undertaken to determine whether the *f5* and *f6* alleles from 'Dular' are neutral alleles conferring wide compatibility and to characterize the three loci (*f5*, *f6*, and *S5*) with respect to their effects on wide compatibility, using a set of near-isogenic lines (NILs) developed in this work.

Materials and methods

Plant materials

According to the results of previous studies, 'Dular', an *indica* WCV from India conferring both a wide spectrum and high level of wide compatibility when crossed to a range of *indica* and *japonica* varieties (Liu et al. 1996; Zhang et al. 1997), exhibited large effects on *indica–japonica* hybrid fertility at the *f5* locus on chromosome 5 and the *f6* locus on chromosome 6 (Wang et al. 1998). These two alleles were referred to as *f5*-Du and *f6*-Du, respectively. 02428, a *japonica* WCV widely used in inter-subspecific hybrid rice breeding programs in China, carried an allele for wide compatibility at the *S5* locus (Liu et al. 1992; Liu et al. 1997) that was referred to as *S5*-08. 'Zhenshan 97', a typical *indica* cultivar and the parent for a number of widely cultivated elite hybrids, did not have wide compatibility and accordingly, the 'Zhenshan 97' alleles at these loci were referred to as *f5*-ZS, *f6*-ZS, and *S5*-ZS, respectively.

In this study, 'Zhenshan 97' was used as the recurrent parent, 'Dular' as the donor parent for the *f5*-Du and *f6*-Du alleles and 02428 as the donor parent for the *S5*-08 allele. In addition, 'Balilla', a typical temperate *japonica* variety introduced from Italy, was used as tester for compatibility to *japonica* and 'Nanjing 11', a typical *indica* variety developed by Jiangsu Academy of Agricultural Sciences, China, was used as tester for compatibility to *indica*. Both 'Balilla' and 'Nanjing 11' have been designated as testers for screening WCVs in Chinese rice breeding programs (Gu et al. 1991).

Development of NILs

The *f5*-Du, *f6*-Du, and *S5*-08 alleles were introgressed from the respective parental lines into 'Zhenshan 97' by successive backcrossing, combined with selection using molecular markers. In this scheme, the progeny of each backcross was selected for the presence of the target allele, using two or more flanking markers tightly linked to the target locus. Five simple sequence repeat (SSR),—RM122, MRG0200 (forward: 5′-CTTGCC-TAACCCGTCTTGAC-3′; reverse: 5′-TCGATGTGT-TGTCTTGTCCC-3′), MRG0259 (forward: 5′-TGGTC-TTTCAAGAATGGGACA-3′; reverse: 5′-TGGACTA-GCTTCCCTTGAGC-3′), RM413, and RM267—and two restriction fragment length polymorphic (RFLP) markers—R830 and R3166, flanking the *f5* locus (Wang et al. 1998; Temnykh et al. 2000, 2001; McCouch et al. 2002)—were used for selecting the presence of the *f5*-Du allele. Four SSR markers, RM225, RM314, RM253, and RM276 (Liu et al. 1997; Wang et al. 1998; Temnykh

et al. 2000, 2001), were used for selecting the presence of both *f6*-Du and *S5*-08 alleles. Positive individuals were then used for the next backcrossing.

In the BC_3F_1, RFLP and SSR markers (Table 1) were used to "clean up" the introgressed chromosomal segments from WCVs for other hybrid sterility loci reported in previous studies (Liu et al. 1997; Wang et al. 1998; Song et al. 2005).

In the BC_5F_1 or BC_6F_1, the selected individuals were assayed using molecular markers for recovering the genetic background of 'Zhenshan 97'. A total of 117 SSR markers, polymorphic between 'Dular' and 'Zhenshan 97' and distributed evenly on the 12 chromosomes, were used in the 'Zhenshan 97' × 'Dular' cross. Similarly, a total of 118 SSR markers were used in the 'Zhenshan 97' × 02428 cross. Individuals with the highest proportion of the recurrent parent genotypes were kept as NILs of 'Zhenshan 97'. Further, the neutral alleles of different loci were combined by inter-crossing the NILs.

Compatibility tests and field planting

The resulting NILs with heterozygous genotypes at the target loci were testcrossed to 'Balilla' for compatibility to *japonica*. The NILs homozygous for the introgressed alleles and 'Zhenshan 97' (as a control) were testcrossed to 'Nanjing 11' for compatibility to *indica*.

The progenies of compatibility tests and all the other *indica–japonica* hybrids were planted in the summer rice growing season at the experimental farm of Huazhong Agricultural University, Wuhan, China. The planting time was 19 May 2003 and 16 May 2004. Thus, the planting placed the temperature sensitive stage for fertility in late July and early August, at which time the average daily temperature was 30.6°C in 2003 and 29.7°C in 2004, favorable for the fertility of the inter-subspecific hybrids (Li et al. 1996; Lu et al. 2002).

Seedlings of 25 days were transplanted, and the planting density was 16.5 cm between plants in a row, 26.4 cm between rows, with 12 plants per row. Field management followed essentially the normal agricultural practices. Irrigation of the field was maintained to avoid drought stress.

Trait measurements

For pollen fertility, one or two panicles per plant were sampled at the time of heading and fixed in 70% (v/v) ethanol. Six florets per panicle were taken from the upper, middle, and lower portions of the panicle. One anther per floret was collected, and the six anthers from the same panicle were mixed and spread on a microscope slide. Pollens were stained with an I_2–KI solution containing 0.1% (w/v) iodine and 1% (w/v) iodine potassium. More than 500 pollen grains from each individual were observed with a microscope for estimating percentage of fertile stainable pollen. Spikelet fertility of each plant was scored as seed setting rate on the basis of four to five panicles.

Molecular marker assay

The experiment procedures for RFLP assay, including DNA isolation, digestion, electrophoresis, and Southern blot hybridization, were essentially as described previously (Liu et al. 1997). The SSR primers of the RM series were designed according to Temnykh et al. (2000, 2001) and those of the MRG series were according to the rice genome sequences of Monsanto Company that were made available by McCouch et al. (2002). SSR analysis was carried out essentially according to the procedures described by Wu and Tanksley (1993).

Data processing and statistical analysis

Local linkage maps for the *f5*, *f6*, and *S5* genomic regions were constructed using MAPMAKER/EXP 3.0 (Lincoln et al. 1992a) with a LOD threshold of 3.0, which was also used for searching loci governing pollen fertility and spikelet fertility, using MAPMAKER/QTL 1.0 at a LOD threshold of 3.0 (Lincoln et al. 1992b).

Results

The NILs

Five individuals were selected. The selected individuals were heterozygous at the target genomic regions and

Table 1 Markers used to "clean up" the introgressed chromosomal segments from wide-compatibility varieties (WCVs) at loci for hybrid sterility other than *f5*, *f6*, and *S5* resolved in previous studies

Mapping population	QTL	Chromosome location	Interval	Polymorphic markers used in this study
'Balilla'/'Dualr'//'Nanjing 11' (Wang et al. 1998)	*f1*	1	R3192–RG532	RG532, RM272, RM84
	f3	3	C603–G144	G144, RM347, RM282, RM251
	f8	8	RG333–C1121A	RG333, RM310, RM44
02428/'Nanjing 11'//'Balilla' (Liu et al. 1997)	QTLs	2	RG151–C560–RG324	RM183, RM526, RM599, RM318
		12	R1534–RZ816–G1112A–RG98	RM491, RM101, RM155, RM270, RM235
02428/'Nanjing 11'//'Balilla' (Song et al. 2005)	*pf5*, *spf5*	5	RM1024–RM13	RM1024, MRG0259, RM413
	pf12	12	RM19–RM247	RM19, RM247
	spf8	8	RM5068–RM25–RM137	RM38, RM25, RM483

homozygous for the 'Zhenshan 97' alleles in the rest of the genome with exceptions only in a few regions. In particular, these individuals were homozygous for the 'Zhenshan 97' alleles for markers representing all the regions known to harbor loci (or QTLs) for *indica–japonica* hybrid sterility (Table 1), as reported in previous studies (Liu et al. 1997; Wang et al. 1998; Song et al. 2005). According to the alleles and combinations they carried, these individuals were referred to as NILs designated ZS(*f5*-Du/*f5*-ZS), ZS(*f6*-Du/*f6*-ZS), ZS(*S5*-08/*S5*-ZS), ZS(*f5*-Du/*f5*-ZS, *f6*-Du/*f6*-ZS), and ZS(*f5*-Du/*f5*-ZS, *S5*-08/*S5*-ZS), respectively. The recovery rates of the recurrent parent genome were 98.75, 98.75, 97.92, 96.67, and 96.67% for these NILs, as determined by molecular marker assays.

Compatibility tests to the *indica* variety

Ten hybrid plants per genotype identified using tightly linked molecular markers from the testcrossed populations of the NILs homozygous for the introgressed alleles to 'Nanjing 11' were selected for trait measurements for compatibility to *indica*. The pollen and spikelet fertility data (Table 2) showed that all the crosses produced normal fertile hybrids, when compared to the control cross, 'Zhenshan 97' × 'Nanjing 11'.

Forty-eight plants per NIL were genotyped from the self-pollinated progenies of the NILs heterozygous for the target loci (Table 3), from which the fertility data were taken. All the three genotypes of the self-pollinated progenies from each of the NILs also showed normal fertility. In addition, no distorted segregation of the three genotypes was observed (Table 3). Thus, all three alleles that were transferred to 'Zhenshan 97' from the WCVs had complete compatibility with the *indica* alleles.

Compatibility tests to the *japonica* variety

Fertility distributions in the segregating populations

Molecular marker assay of the plants in the field planting of 2003 using tightly linked markers identified a total of 131 hybrid plants from the cross ZS(*f5*-Du/*f5*-ZS, *f6*-Du/*f6*-ZS) × 'Balilla', 130 hybrid plants from ZS(*f5*-Du/*f5*-ZS, *S5*-08/*S5*-ZS) × 'Balilla', 65 hybrid plants from ZS(*f5*-Du/*f5*-ZS)×'Balilla', 59 hybrid plants from ZS(*f6*-Du/*f6*-ZS) × 'Balilla', and 55 hybrid plants from ZS(*S5*-08/*S5*-ZS) × 'Balilla'. The two two-locus testcross populations were planted again in 2004. After assaying of the plants using molecular markers, 12 plants for each of the four genotypes in the two segregating populations were evaluated for pollen and spikelet fertility.

In the progenies from ZS(*f5*-Du/*f5*-ZS, *f6*-Du/*f6*-ZS) × 'Balilla' and ZS(*f5*-Du/*f5*-ZS, *S5*-08/*S5*-ZS) × 'Balilla', the distributions of pollen fertility segregation were clearly bimodal, with an apparent valleys at approximately 50–65% of fertile pollen (Fig. 1a, c), indicating a simple genetic control in each case most likely by a single locus. However, the distributions of spikelet fertility seemed to be more complex, with three apparent peaks in both populations (Fig. 1b, d).

In the segregating population of ZS(*f5*-Du/*f5*-ZS) × 'Balilla', distributions of both pollen and spikelet fertility appeared to be bimodal (Fig. 1e, f), again indicating

Table 2 Pollen and spikelet fertility of the progenies in various testcross populations with 'Nanjing 11' as the tester

Cross	Pollen fertility (average % ± SD)	Spikelet fertility (average % ± SD)
ZS(*f5*-Du/*f5*-Du, *f6*-Du/*f6*-Du) × 'Nanjing 11'	90.88 ± 3.31	80.12 ± 3.02
ZS(*f5*-Du/*f5*-Du, *S5*-08/*S5*-08) × 'Nanjing 11'	89.50 ± 4.04	80.16 ± 2.36
ZS(*f5*-Du/*f5*-Du) × 'Nanjing 11'	87.88 ± 4.45	79.11 ± 4.01
ZS(*f6*-Du/*f6*-Du) × 'Nanjing 11'	89.38 ± 5.53	74.97 ± 4.64
ZS(*S5*-08/*S5*-08) × 'Nanjing 11'	90.25 ± 4.13	74.24 ± 7.67
'Zhenshan 97' × 'Nanjing 11'	87.25 ± 4.17	73.98 ± 9.75

Table 3 Pollen and spikelet fertility of the three different genotypes in the self-pollinated progenies of near-isogenic lines with a single heterozygous target allele

Population	Genotype	Number of individuals	Pollen fertility (average % ± SD)	Spikelet fertility (average % ± SD)	χ^2 (1:2:1)
ZS(*f5*-Du/*f5*-ZS)	*f5*-Du/*f5*-Du	13	96.05 ± 2.49	88.45 ± 5.79	0.0521 ($P > 0.95$)
	f5-ZS/*f5*-ZS	12	96.33 ± 2.97	84.87 ± 4.42	
	f5-Du/*f5*-ZS	23	96.83 ± 2.48	84.11 ± 5.06	
ZS(*f6*-Du/*f6*-ZS)	*f6*-Du/*f6*-Du	12	96.56 ± 2.69	84.00 ± 3.18	0.3021 ($P > 0.75$)
	f6-ZS/*f6*-ZS	10	96.69 ± 2.70	85.41 ± 2.73	
	f6-Du/*f6*-ZS	26	95.81 ± 3.00	84.64 ± 2.47	
ZS(*S5*-08/*S5*-ZS)	*S5*-08/*S5*-08	11	96.74 ± 2.75	86.93 ± 3.98	0.0521 ($P > 0.95$)
	S5-ZS/*S5*-ZS	13	95.63 ± 2.20	84.49 ± 4.30	
	S5-08/*S5*-ZS	24	96.25 ± 2.36	85.47 ± 3.76	

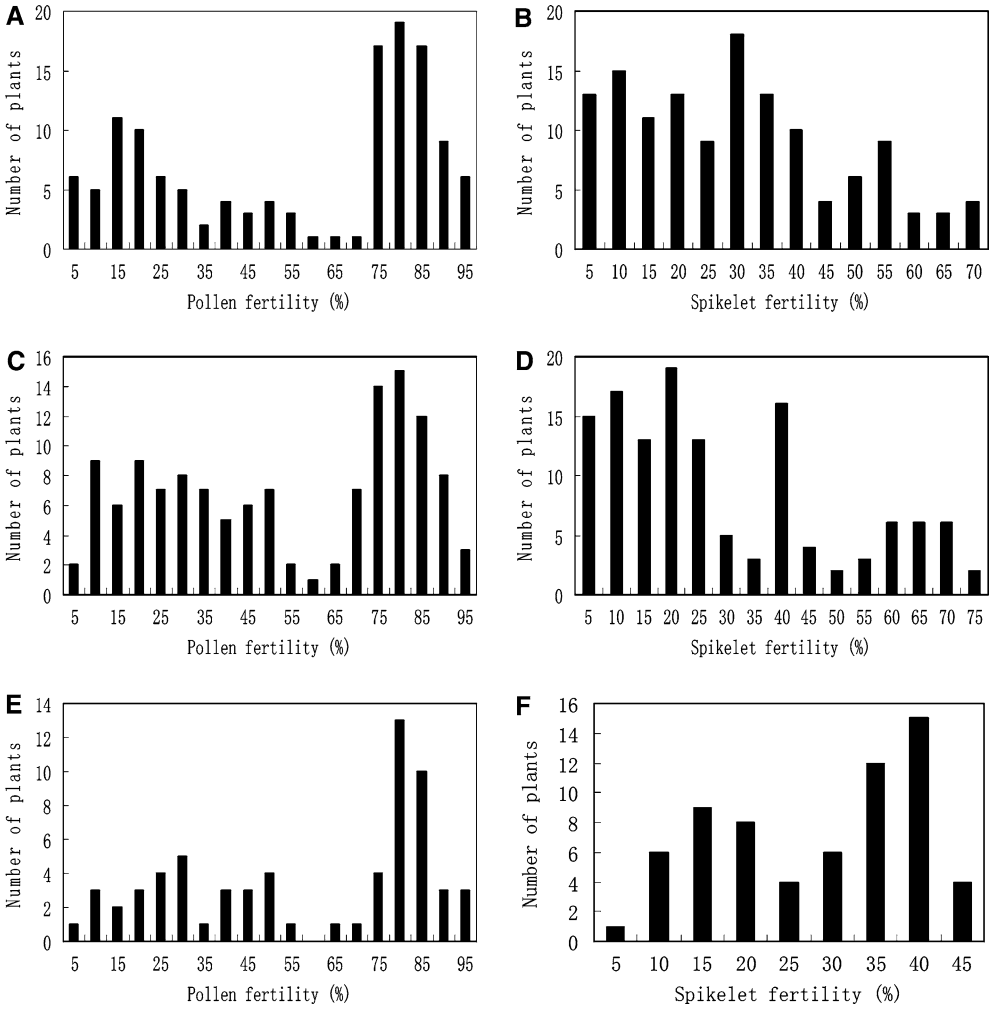

Fig. 1 Distributions of pollen and spikelet fertility in the testcross populations: **a**, **b** ZS(*f5*-Du/*f5*-ZS, *f6*-Du/*f6*-ZS) × 'Balilla'; **c**, **d** ZS(*f5*-Du/*f5*-ZS, *S5*-08/*S5*-ZS) × 'Balilla'; **e**, **f** ZS(*f5*-Du/*f5*-ZS) × 'Balilla'

a likely single-locus control. However, no significant segregation was observed in the progenies from the crosses of ZS(*f6*-Du/*f6*-ZS) × 'Balilla' and ZS(*S5*-08/*S5*-ZS) × 'Balilla', with the majority of pollen fertility in the range of 4–45% and spikelet fertility between 1% and 17% (data not shown).

The effects of f5, f6, and S5

The results of compatibility tests of the NILs are presented in Tables 4 and 5. Comparison between the two genotypes for each of the three loci (Table 4) showed that the two genotypes at the *f5* locus had the largest differences in both pollen and spikelet fertility; the genotype having the *f5*-Du allele produced much higher fertility than the other genotype, whereas differences between the two genotypes at the other two loci were not significant.

Similar to the results of single-locus analysis, testcrosses of two-locus genotypes (Table 5) also showed that *f5* was the only locus causing statistically significant difference in pollen fertility. All the two-locus genotypes involving the *f5*-Du allele had much higher pollen fertility than those having the *f5*-ZS allele.

Testcrosses of the two-locus genotypes also detected the effects of the other two loci (*f6* and *S5*) on spikelet fertility, in addition to the large effects of the *f5* locus. It is clear from Table 5 that the effects of these two loci were dependent both on the genotypes of the *f5* locus and on the environmental conditions. In 2003, the effects of *f6* and *S5* on spikelet fertility were significant only in the presence of the *f5*-Du allele, while in 2004, both *f6* and *S5* had significant effects on spikelet fertility in both the presence and absence of the *f5*-Du allele. However, the amounts of effects of the two loci were much larger in the presence of the *f5*-Du allele than otherwise.

Comparison of the data provided in Table 5 also indicated that the magnitudes of differences in spikelet fertility caused by allelic substitutions at *f6* and *S5* were similar within each year, suggesting that the magnitudes of gene effects at the two loci were similar.

To evaluate further the relative effect of each locus on pollen and spikelet fertility, the data obtained in 2003 were assessed using MAPMAKER/QTL 1.0. The analysis resolved one major QTL with very large effect on both pollen and spikelet fertility in all three segregating

Table 4 Pollen and spikelet fertility of the various genotypes (identified using tightly linked molecular markers) in the testcross populations with 'Balilla' as the tester

Population	Marker genotype	Pollen fertility (average % ± SD)	Spikelet fertility (average % ± SD)
ZS(*f5*-Du/*f5*-ZS) × 'Balilla'	*f5*-Du/B	79.79 ± 5.86**	35.13 ± 3.95**
	f5-ZS/B	27.50 ± 14.15	14.72 ± 5.71
ZS(*f6*-Du/*f6*-ZS) × 'Balilla'	*f6*-Du/B	15.92 ± 12.20	6.59 ± 6.04
	f6-ZS/B	20.90 ± 16.38	6.01 ± 6.08
ZS(*S5*-08/*S5*-ZS) × 'Balilla'	*S5*-08/B	29.02 ± 13.30	10.42 ± 6.51
	S5-ZS/B	27.69 ± 14.66	10.35 ± 6.69

**Significant difference from the genotype with the counterpart 'Zhenshan 97' allele at the 0.01 probability level

Table 5 Pollen and spikelet fertility of the various two-locus genotypes (identified using tightly linked molecular markers) in the testcross populations with 'Balilla' as the tester

Year	Population	Genotype	Pollen fertility (average % ± SD)	Spikelet fertility (average % ± SD)
2003	ZS(*f5*-Du/*f5*-ZS, *f6*-Du/*f6*-ZS) × 'Balilla'	*f5*-Du/B, *f6*-Du/B	78.44 ± 6.09 A[a]	51.04 ± 10.63 A[a]
		f5-Du/B, *f6*-ZS/B	80.73 ± 7.36 A	30.03 ± 5.99 B
		f5-ZS/B, *f6*-Du/B	22.49 ± 15.18 B	12.07 ± 8.51 C
		f5-ZS/B, *f6*-ZS/B	24.96 ± 16.51 B	11.90 ± 7.40 C
2003	ZS(*f5*-Du/*f5*-ZS, *S5*-08/*S5*-ZS) × 'Balilla'	*f5*-Du/B, *S5*-08/B	78.29 ± 7.50 A	54.30 ± 11.95 A
		f5-Du/B, *S5*-ZS/B	76.48 ± 6.60 A	29.16 ± 7.69 B
		f5-ZS/B, *S5*-08/B	28.01 ± 16.03 B	13.00 ± 6.39 C
		f5-ZS/B, S *5*-ZS/B	25.72 ± 13.39 B	10.58 ± 6.57 C
2004	ZS(*f5*-Du/*f5*-ZS, *f6*-Du/*f6*-ZS) × 'Balilla'	*f5*-Du/B, *f6*-Du/B	81.37 ± 8.52 A	63.83 ± 3.77 A
		f5-Du/B, *f6*-ZS/B	75.84 ± 10.22 A	34.82 ± 3.43 B
		f5-ZS/B, *f6*-Du/B	16.54 ± 10.13 B	19.03 ± 4.66 C
		f5-ZS/B, *f6*-ZS/B	17.03 ± 11.82 B	12.71 ± 4.61 D
2004	ZS(*f5*-Du/*f5*-ZS, *S5*-08/*S5*-ZS) × 'Balilla'	*f5*-Du/B, *S5*-08/B	74.50 ± 9.34 A	60.03 ± 6.93 A
		f5-Du/B, *S5*-ZS/B	72.64 ± 9.55 A	35.51 ± 4.19 B
		f5-ZS/B, *S5*-08/B	20.51 ± 11.01 B	21.19 ± 5.36 C
		f5-ZS/B, *S5*-ZS/B	23.64 ± 12.13 B	15.10 ± s3.21 D

[a]Ranked using Duncan's LSR test at the 0.01 probability level. The comparisons are valid only among the four genotypes in the same population

Table 6 Relative effects on pollen and spikelet fertility of the target loci evaluated using MAPMAKER/QTL 1.0

Population	Locus	Flanking markers	Trait	LOD	Var. (%)	Effect[a]
ZS(*f5*-Du/*f5*-ZS, *f6*-Du/*f6*-ZS) × 'Balilla'	*f5*	MRG0200-MRG0259	Spikelet fertility	27.5	61.9	28.6
			Pollen fertility	53.0	84.5	55.5
	f6	RM253-RM276	Spikelet fertility	5.7	18.0	15.5
ZS(*f5*-Du/*f5*-ZS, *S5*-08/*S5*-ZS) × 'Balilla'	*f5*	MRG0200-MRG0259	Spikelet fertility	25.0	61.6	31.2
			Pollen fertility	47.2	82.9	50.9
	S5	RM253-RM276	Spikelet fertility	7.8	24.1	19.5
ZS(*f5*-Du/*f5*-ZS) × 'Balilla'	*f5*	MRG0200-MRG0259	Spikelet fertility	24.2	82.0	20.4
			Pollen fertility	28.1	86.4	52.3

[a]Effect of the genotype having the WCV allele on fertility (percentage)

populations located in the interval between MRG0200 and MRG0259 on chromosome 5 corresponding to the *f5* locus (Table 6; Fig. 2). The analysis also detected significant effects of *f6* and *S5* on spikelet fertility in the ZS(*f5*-Du/*f5*-ZS, *f6*-Du/*f6*-ZS) × 'Balilla' and ZS(*f5*-Du/*f5*-ZS, *S5*-08/*S5*-ZS) × 'Balilla' populations, respectively (Table 6; Fig. 2). In both cases, the alleles from WCVs contributed to the increase of spikelet fertility. Table 6 also revealed that the effect of *S5* seemed to be larger than *f6*, using *f5* as the reference. The locations of the *f5*, *f6*, and *S5* loci on the molecular linkage maps are shown in Fig. 2.

Discussion

Cytological investigation of the inter-subspecific sterility revealed that both male and female gamete abortions and reduced affinity between the uniting gametes, as well as anther indehiscence and non-synchronization of male and female gamete development in the same spikelet, all contribute to hybrid sterility (Yokoo 1984; Li 1988; Ling et al. 1991; Maeka et al. 1991; Wang et al. 1991, 1992; Li and Ouyang 1992; Liu et al. 1993, 1997, 2004; He et al. 1994; Teng et al. 1996; Zhu et al. 1996). Recently, Song

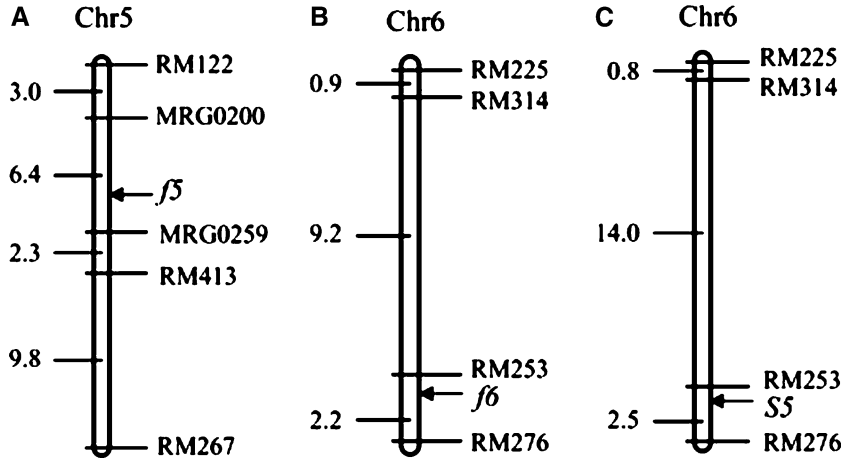

Fig. 2 The locations of *f5*, *f6*, and *S5* loci in the molecular marker linkage maps. The *arrow* on each chromosomal region indicates the position of the LOD peak. **a** Map location of *f5* locus, determined using the populations of ZS(*f5*-Du/*f5*-ZS, *f6*-Du/*f6*-ZS) × 'Balilla', ZS(*f5*-Du/*f5*-ZS, *S5*-08/*S5*-ZS) × 'Balilla', and ZS(*f5*-Du/*f5*-ZS) × 'Balilla'. **b** Map location of *f6* locus, determined using the population of ZS(*f5*-Du/*f5*-ZS, *f6*-Du/*f6*-ZS) × 'Balilla'. **c** Map location of *S5* locus determined using the population of (*f5*-Du/*f5*-ZS, *S5*-08/*S5*-ZS) × 'Balilla'

et al. (2005) identified a major QTL for male gamete abortion and a major QTL for female gamete abortion in a segregating population. Their results determined that the *S5* locus on chromosome 6, previously identified as a locus for wide compatibility by spikelet fertility analysis, was a major locus for embryo sac fertility, and a QTL on chromosome 5 had a major effect on pollen fertility. Both of the loci had large effects on spikelet fertility.

The most important finding of the present study is the determination of *f5* as a locus for *indica–japonica* hybrid sterility and identification of *f5*-Du from 'Dular' as a neutral allele that is able to increase the fertility of the hybrids when crossed to both *indica* and *japonica* varieties. In particular, the results clearly demonstrated that the *f5*-Du allele exerted a large effect on hybrid fertility by specifically increasing pollen fertility, thus proving to be a neutral allele for pollen fertility. In the *indica* genetic background provided by 'Zhenshan 97', this allele can produce normal fertile hybrids when testcrossed to the *indica* tester. When testcrossed to the *japonica* tester, this allele could increase the pollen fertility (I_2–KI stainability) by more than 50% and the spikelet fertility by over 20%, although the fertility of the testcross progenies with the *japonica* tester was still far from normal, presumably because of allelic differences in numerous other loci. Interestingly, the *f5* locus appeared to be located in the same genomic region as the *pf5* (*spf5*) locus identified by Song et al. (2005). In addition, Zhuang et al. (2002) also mapped a locus for pollen fertility on chromosome 5 located in the same vicinity. Thus, quite likely, the *f5* locus identified using 'Dular' and *pf5* (*spf5*) using 02428 are the same locus.

Another important finding is the confirmation of *f6* as a locus for *indica–japonica* hybrid sterility, at which the *f6*-Du allele from 'Dular' is a neutral allele conferring wide compatibility. The *f6* and *S5* loci appeared to have high similarities, as demonstrated by the *f6*-Du and *S5*-08 alleles: both alleles had effects only on spikelet fertility, not on pollen fertility; the magnitudes of effects caused by substitutions of the two alleles were also similar, both of which were much smaller than the *f5*-Du allele; the two loci are located in the same genomic interval on chromosome 6. Thus, it is reasonable to assume that *f6* is also a locus for embryo sac fertility and hence, *f6* and *S5* are most likely to be the same locus.

It should be also noted that previous studies showed that cool temperature could significantly reduce the pollen fertility and spikelet fertility of *indica–japonica* hybrids, even in the presence of wide compatibility genes (Li et al. 1996, 1997; Lu et al. 2002). To avoid the influence of temperature on hybrid sterility in the present study, the planting dates for all the experimental materials were arranged such that the entire reproductive development stage was under high-temperature conditions. We also planted the F_1s of 'Dular' × 'Balilla' and 02428 × 'Nanjing 11' in the same field in 2003 and 2004 as controls, both of which showed normal pollen fertility and spikelet fertility in both years, indicating that the observed fertility segregations in all the populations were not affected by temperature.

A notable feature observed from the results of this study and the study of Song et al. (2005) is the large influence of the male fertility on spikelet fertility. Theoretically, a few normal pollen grains would be enough for a flower to set seed. Thus the large effect of the *f5* locus on pollen fertility strongly indicates the severe scarcity of fertile pollen in the *indica–japonica* hybrids, suggesting that the actual fertility (or germinability) of the pollen produced by the *indica–japonica* hybrids is much lower than observed with I_2–KI staining. Lin et al. (1992) found that in *indica–japonica* hybrids, the germinability of the pollen was less than 10% in the hybrids, and a large portion of the pollen was not functional, although 45–55% of the pollen grains appeared to be morphologically normal. In addition, anther indehiscence may also be a cause for spikelet sterility, as was found in the F_1 hybrid between rice varieties 'Silewah' and 'Hayakogane' (Maeka et al. 1991). In any case, shortage in the

supply of fertile pollen may diminish the effects of other components of spikelet fertility such as embryo sac fertility. This may especially be the case in a cross between a typical *indica* variety like 'Zhenshan 97' and a typical *japonica* variety like 'Balilla', in which the hybrid may be heterozygous at the majority, if not all, of the loci for *indica–japonica* hybrid sterility and adding one neutral allele for embryo-sac fertility to the hybrid may not increase fertility much because of the unavailability of fertile pollen. This may provide an explanation for the large dependence of the effects of *S5* and *f6* on *f5*, such that the effects of *S5* and *f6* were significant only in the presence of the *f5*-Du allele.

The results also have significant implications for *indica–japonica* hybrid rice breeding programs. Most of the current breeding programs only use the *S5* locus to manipulate the fertility of the hybrids, due largely to the limited information from previous studies. The results of Song et al. (2005) and this study clearly indicate that alleles at *S5* locus alone is far from sufficient to overcome hybrid sterility of *indica–japonica* crosses, because the *f5* locus plays a more important role in determining spikelet fertility by exerting a major effect on pollen fertility. Moreover, the results further showed that, even in combination, the alleles of the two loci *f5* and *S5* (*f6*) are still not sufficient to produce normal fertility hybrids in a typical *indica–japonica* cross like 'Zhenshan 97'/'Balilla'. Hence, to achieve normal spikelet fertility, more neutral alleles conferring wide compatibility should be introduced. In this connection, it should be noted that it has been repeatedly reported that 'Dular' has both a wide-compatible spectrum and a high level of compatibility, as it produces highly fertile hybrids when crossed to a wide range of *indica* and *japonica* varieties (Pan et al. 1990; Liu et al. 1996; Zhang et al. 1997). So far, 'Dular' has been identified to carry neutral alleles at a number of loci, including *f5*, *f6* (*S5*), *S7*, *S8*, *S9*, *ga11*, and *ga14* (Wan et al. 1996; Lu et al. 2000) and confirmed to be a very useful WCV. However, this variety is agronomically undesirable because of tall and thin culm and poor yielding. Thus, it may be a better strategy to transfer these identified neutral alleles into elite *indica* or *japonica* varieties by marker-assisted selection and the resulting introgression lines can then be selectively used in inter-subspecific hybrid rice breeding programs.

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References

Araki H, Toya K, Ikehashi H (1988) Role of wide-compatibility genes in hybrid rice breeding. In: Hybrid rice. IRRI, Manila, pp 79–83

Chu LH, Chou YC, Tau C (1964) Breeding studies on hybridization between *O sativa* L subsp *hsien* and O *sativa* L subsp *keng* in cultivated rice (in Chinese with English abstract). Acta Agron Sin 3:69–84

Gu MH, Pan XB, Chen ZX, Yang WC (1991) A study on compatibility of standard wide compatibility testers of rice in China (in Chinese with English abstract). Sci Agric Sin 24:27–32

Gu M, You A, Pan X (1993) Analysis on allelic relationship of wide compatibility genes among several WCVs (*Oryza sativa* L.). Sci Agric Sin 26:13–21

He GH, Zheng JK, Yin GD, Yang ZL (1994) Gamete fertility of F1 between Indica and Japonica (in Chinese with English abstract). Chin J Rice Sci 8:177–180

Ikehashi H (1991) Genetics of hybrid sterility in wide hybridization in rice. In: Bajaj YPS (ed) Biotechnology in agriculture and forestry, vol 14. Rice. Springer, Berlin Heidelberg New York, pp 113–127

Ikehashi H, Araki H (1984) Variety screening of compatibility types revealed in F1 fertility of distant cross in rice. Jpn J Breed 34:304–313

Ikehashi H, Araki H (1986) Genetics of F1 sterility in remote crosses of rice. In: IRRI (ed) Rice genetics. IRRI, Manila, pp 119–130

Ikehashi H, Araki H (1988) Multiple alleles controlling F1 sterility in remote crosses of rice (*Oryza sativa*). Jpn J Breed 38:283–291

Kato S, Kosaka H, Hara S (1928) On the affinity of rice varieties as shown by fertility of hybrid plants. Bull Sci Fac Agric Kyushu Univ 3:132–147

Kinoshita T (1995) Report of the committee on gene symbolization. Rice Genet Newsl 12:94–125

Li XQ (1988) Improving the fertility of *indica–japonica* hybrids by using the wide-compatibility gene (in Chinese). Hybrid Rice 31–33

Li BJ, Ouyang XZ (1992) Cytological study on the abortion of spikelets of F1 from *indica* and *japonica*. In: Yuan LP (ed) Current status of two line hybrid rice research (in Chinese with English abstract). Agricultural, Beijing, pp 286–289

Li HB, Zhang Q, Liu AM, Zou JS, Chen ZM (1996) A genetic analysis of low-temperature-sensitive sterility in *indica–japonica* hybrids. Plant Breed 115:305–309

Li HB, Wang J, Liu AM, Liu KD, Zhang Q, Zou JS (1997) Genetic basis of low-temperature-sensitive sterility in *indica–japonica* hybrids of rice as determined by RFLP analysis. Theor Appl Genet 95:1092–1097

Lin SY, Ikehashi H (1993) A gamete abortion locus detected by segregation distortion of isozyme locus *EST-9* in wide crosses (*Oryza sativa* L.). Euphytica 67:35–40

Lin SY, Ikehashi H, Yanagihara S, Kawashima A (1992) Segregation distortion via male gamete in hybrids between Indica and Japonica or wide-compatibility varieties of rice (*Oryza sativa* L.). Theor Appl Genet 84:812–818

Lincoln S, Daly M, Lander E (1992a) Constructing genetic maps with MAPMAKER/EXP 3.0. Whitehead Institute Technical Report, 2nd edn. Whitehead Institute, Cambridge

Lincoln S, Daly M, Lander E (1992b) Mapping genes controlling quantitative traits with MAPMAKER/QTL 1.1. Whitehead Institute Technical report, 2nd edn. Whitehead Institute, Cambridge

Ling DH, Ma ZR, Chen MF, Liang CY, He BS (1991) Types of male sterile mutants in somaclones from somatic cell culture of *indica* rice (in Chinese with English abstract). Acta Genet Sin 18:132–139

Liu A, Zhang Q, Li H (1992) Location of a gene for wide-compatibility in the RFLP linkage map. Rice Genet Newsl 9:134–136

Liu YS, Zhou KD, Yin GD, Luo WZ (1993) Preliminary cytological observations on female sterility of hybrids between *indica* and *japonica* rice (in Chinese with English abstract). Acta Biol Exp Sin 26:95–99

Liu KD, Zhou ZQ, Xu CG, Zhang Q, Saghai Maroof MA (1996) An analysis of hybrid sterility in rice using a diallel cross of 21 parents involving *indica*, *japonica* and wide compatibility varieties. Euphytica 90:275–280

Liu YS, Sun JS, Zhou KD (1997) Cytological basis causing spikelet sterility of intersubspecific hybrid in *Oryza sativa* (in Chinese with English abstract). Acta Biol Exp Sin 30:335–341

Liu KD, Wang J, Li HB, Xu CG, Liu AM, Li XH, Zhang Q (1997) A genome-wide analysis of wide compatibility in rice and the precise location of the *S5* locus in the molecular map. Theor Appl Genet 95:809–814

Liu YS, Zhu LH, Sun JS, Chen Y (2001) Mapping QTLs for defective female gametophyte development in an inter-subspecific cross in *Oryza sativa* L. Theor Appl Genet 102:1243–1251

Liu HY, Xu CG, Zhang Q (2004) Male and female gamete abortions, and reduced affinity between the uniting gametes as the causes for sterility in an *indica*/*japonica* hybrid in rice. Sex Plant Reprod 17:55–62

Lu CG, Takabatake K, Ikehashi H (2000) Identification of segregation-distortion-neutral alleles to improve pollen fertility of *indica–japonica* hybrids in rice (*Oryza sativa* L.). Euphytica 113:101–107

Lu CG, Wang CL, Zong SY, Zhao L, Zou JS (2002) Effects of temperature on fertility and seed set in intersubspecific hybrid rice (in Chinese with English abstract). Acta Agron Sin 28:499–504

Maeka M, Inuka T, Shinbashi N (1991) Spikelet sterility in F1 hybrids between rice varieties Silewah and Hayakogane. Jpn J Breed 41:359–363

McCouch SR, Teytelman L, Xu Y, Lobos KB, Clare K, Walton M, Fu B, Maghirang R, Li Z, Xing Y, Zhang Q, Kono I, Yano M, Fjellstrom R, DeClerck G, Schneider D, Cartinhour S, Ware D, Stein L (2002) Development and mapping of 2,240 new SSR markers for rice (*Oryza sativa* L.). DNA Res 9:199–207

Oka HI (1988) Origin of cultivated rice. Scientific Societies, Tokyo, pp 181–209

Pan XB, Gu MH, Chen ZX, Hu XY (1990) A comparative study on major wide compatibility varieties of rice. In: Yuan LP (ed) Current status of two line hybrid rice research. Agricultural, Beijing, pp 236–245

Song X, Qiu SQ, Xu CG, Li XH, Zhang Q (2005) Genetic dissection of embryo sac fertility, pollen fertility, and their contributions to spikelet fertility of intersubspecific hybrids in rice. Theor Appl Genet 110:205–211

Tanksley SD (1993) Mapping polygenes. Annu Rev Genet 27:205–233

Temnykh S, Park WD, Ayres N, Cartihour S, Hauck N, Lipovich L, Cho YG, Ishii T, McCouch SR (2000) Mapping and genome organization of microsatellite sequences in rice (*Oryza sativa* L.). Theor Appl Genet 100:697–712

Temnykh S, Declerck G, Luashova A, Lipovich L, Cartinhour S, McCouch S (2001) Computational and experimental analysis of microsatellites in rice (*Oryza sativa* L.): frequency, length variation, transposon associations, and genetic marker potential. Genome Res 11:1441–1452

Teng JL, Xue QZ, Wang YX (1996) Ultrastructural observations of pollen and anther wall developments between subspecies in rice (*Oryza sativa* L.) (in Chinese with English abstract). J Zhejiang Agric Univ 22:467–473

Wan J, Yanagihara S, Kato H, Ikehashi H (1993) Multiple alleles at a new locus causing hybrid sterility between a Korean *indica* variety and a Javanica variety in rice (*Oryza sativa* L.). Jpn J Breed 43:507–516

Wan J, Yamaguchi Y, Kato H, Ikehashi H (1996) Two new loci for hybrid sterility in cultivated rice (*Oryza sativa* L.). Theor Appl Genet 92:183–190

Wang YX, Yan JQ, Xue QZ, Shen SQ (1991) Cytological studies on partial male sterility of F1 hybrids between subspecies in rice (in Chinese with English abstract). Acta Agric Univ Zhejiangensis 17:417–422

Wang CL, Zhang ZL, Tang SZ, Shi JD (1992) Exploitation of heterosis between Indica and Japonica by three-line method. I. Differentiation between *indica–japonica* sterility and cytoplasmic male sterility (in Chinese with English abstract). Jiangsu J Agric Sci 8:1–7

Wang J, Liu KD, Xu CC, Li XH, Zhang Q (1998) The high level of wide-compatibility of 'Dular' has a complex genetic basis Theor Appl Genet 97:407–412

Wu KS, Tanksley SD (1993) Abundance, polymorphism and genetic mapping of microsatellites in rice. Mol Gen Genet 241:225–235

Yanagihara S, Kato H, Ikehashi H (1992) A new locus for multiple alleles causing hybrid sterility between an AUS variety and Javanica varieties in rice (*Oryza sativa* L.). Jpn J Breed 42:793–801

Yanagihara S, McCouch SR, Ishikawa K, Ogi Y, Maruyama K, Ikehashi H (1995) Molecular analysis of the inheritance of the *S*-it 5 locus, conferring wide compatibility in *indica*/*japonica* hybrids of rice (*O. sativa* L.). Theor Appl Genet 90:182–188

Yang SR, Shen XY, Gu VYL, Cao DJ (1962) The report of rice breeding by *indica–japonica* crosses (in Chinese). Acta Agron Sin 1:97–102

Yokoo M (1984) Female sterility in an *indica–japonica* cross of rice. Jpn J Breed 34:219–227

Yuan LP (1987) Strategies of hybrid rice breeding (in Chinese). Hybrid Rice 1–3

Zhang GQ, Lu YG (1989) Genetic studies on the hybrid sterility in cultivated rice (*Oryza sativa* L.) I Diallel analysis of the hybrid sterility among isogenic F1 sterility lines (in Chinese with English abstract). Chin J Rice Sci 3:97–101

Zhang GQ, Lu YG (1993) Genetic studies on the hybrid sterility in cultivated rice (*Oryza sativa* L.) II A genic model for F1 pollen sterility (in Chinese with English abstract). Acta Genet Sin 20:222–228

Zhang GQ, Lu YG, Zhang H, Yang JC, Liu GF (1994) Genetic studies on the hybrid sterility in cultivated rice (*Oryza sativa* L.) IV Genotypes for F1 pollen sterility (in Chinese with English abstract). Acta Genet Sin 21:34–41

Zhang Q, Liu KD, Yang GP, Saghai Maroof MA, Xu CG, Zhou ZQ (1997) Molecular marker diversity and hybrid sterility in *indica–japonica* rice crosses. Theor Appl Genet 95:112–118

Zheng K, Shen P, Qian H, Wang J (1992) Tagging genes for wide compatibility in rice via linkage to RFLP markers. Chin J Rice Sci 6:145–150

Zhu XH, Cao XZ, Zhu QS (1996) Cytological studies on spikelet sterility of *indica–japonica* hybrids in rice (in Chinese with English abstract). Chin J Rice Sci 10:71–78

Zhuang CX, Mei MT, Zhang GQ, Lu YG (2002) Chromosome mapping of the *S-b* locus for F1 pollen sterility in cultivated rice (*Oryza sativa* L.) with RAPD markers (in Chinese with English abstract). Acta Genet Sin 29:700–705