Theor Appl Genet (2005) 110: 205–211
DOI 10.1007/s00122-004-1798-2

ORIGINAL PAPER

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Genetic dissection of embryo sac fertility, pollen fertility, and their contributions to spikelet fertility of intersubspecific hybrids in rice

Received: 7 May 2004 / Accepted: 12 August 2004 / Published online: 1 December 2004
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Abstract The partial sterility of hybrids has been a major barrier for utilization of the strong heterosis expressed in hybrids between *Oryza sativa* ssp. *indica* and *O. sativa* ssp. *japonica*. Wide-compatibility varieties, comprising a special class of germplasm, are able to produce fertile hybrids when crossed to both *indica* and *japonica* varieties. However, all the work on wide compatibility and majority of studies on *indica/japonica* hybrid sterility reported so far were based only on spikelet fertility; thus, it is not known to what extent male and female gamete abortions influence hybrid sterility. In this study, we investigated pollen fertility, embryo sac fertility, and spikelet fertility in an F_1 population of 202 true hybrid plants derived from a three-way cross (‘02428’/‘Nanjing 11’//‘Balilla’). A partial regression analysis showed that the pollen and embryo sac fertility contributed almost equally to spikelet fertility. QTL analysis based on a linkage map of 191 polymorphic marker loci identified two QTLs for pollen fertility, one QTL for embryo sac fertility, and three QTLs for spikelet fertility. The *S5* locus, previously identified as a locus for wide compatibility by spikelet fertility analysis, is a major locus for embryo sac fertility, and a QTL on chromosome 5 had a major effect on pollen fertility. These two loci coincided with the two major QTLs for spikelet fertility. The study also detected a QTL on chromosome 8, showing a large effect on spikelet fertility but no effect on either pollen or embryo sac fertility. Very little interaction among the QTLs was detected. The implications of the findings in rice breeding programs are discussed.

Communicated by C. Möllers

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Introduction

The Asian cultivated rice (*Oryza sativa* L.) can be classified into two main subspecies, *indica* and *japonica*. Although hybrids developed by crossing varieties within the same subspecies have achieved great success and contributed considerably to rice yield increase in recent years, the utilization of strong heterosis in the F_1s between the two subspecies has been difficult. One of the main difficulties is the partial sterility that frequently occurs in the F_1s of such hybrids (Kato et al. 1928).

The genetic basis of the intersubspecific hybrid sterility has been extensively investigated in the last several decades. Based on the analysis of near isogenic lines, Oka (1974) proposed a “duplicated lethal” model that involved *s* alleles at two genetically duplicated loci to explain the genetic basis of hybrid sterility. The finding of wide-compatibility varieties, comprising a special class of rice germplasm able to produce fertile hybrids when crossed to both *indica* and *japonica* varieties (Ikehashi and Araki 1984, 1986), brought hope for overcoming the reproductive barrier between the two subspecies and has attracted considerable research interests in the rice community. Recent studies, making use of molecular marker technology and high-density molecular marker linkage maps, not only confirmed the presence of such a locus for wide compatibility as reported by Ikehashi and Araki (1986), but also determined the precise location of the locus in the rice genome (Liu et al. 1992, 1997; Zheng 1992; Yanagihura et al. 1995). Moreover, a series of additional loci were also identified as causing hybrid sterility in *indica/japonica* crosses (Ikehashi and Wan 1996; Wang et al. 1998).

However, it should be mentioned that all the work on wide compatibility and majority of studies on *indica/japonica* hybrid sterility reported so far have been based only on spikelet fertility, which is directly a function of male gamete fertility, female gamete fertility, and affinity between the uniting male and female gametes. Cytological investigation of the intersubspecific sterility

revealed both male gamete abortions (Wang et al. 1991, 1992; He et al. 1994; Teng et al. 1996; Zhu et al. 1996) and female gamete abortions (Yokoo 1984; Li 1988; Ling et al. 1991; Li and Ouyang 1992; Liu et al. 1993, 1997; Zhu et al. 1998), as well as reduced dehiscence of the anthers (Maeka et al. 1991; Liu et al. 1993). However, it is not known how these cytological abnormalities are genetically controlled or to what extent they influence hybrid sterility.

In the study reported in this paper, we assayed pollen fertility, embryo sac fertility, and spikelet fertility of an F_1 population derived from a three-way cross that was previously used for mapping and identification of the wide-compatibility gene as well as QTLs for hybrid sterility (Liu et al. 1997). The analyses determined unambiguously the genetic bases of the pollen fertility and embryo sac fertility in relation to spikelet fertility.

Materials and methods

Experimental plant population

A three-way cross population, '02428'/'Nanjing 11'// 'Balilla', was constructed for this study. '02428' is a *japonica* variety with high wide compatibility (Liu et al. 1992, 1996), 'Nanjing 11' is an *indica* variety developed by the Agriculture Academy of Jiangsu Province, China, and 'Balilla' is a *japonica* variety from Italy. 'Nanjing 11' and 'Balilla' are regarded as typical *indica* and *japonica* varieties, respectively, that have been widely used as testers for *indica–japonica* compatibility analyses in rice breeding programs in China (Gu et al. 1993).

In conducting this study, a cross was made between '02428' and 'Nanjing 11' in the summer rice-growing season of 2001 in Wuhan, China, and the F_1 was crossed with 'Balilla' in the winter season of 2001/2002 in Hainan Island, China. The resulting F_1 plants were planted in the 2002 rice-growing season in the experimental farm of Huazhong Agriculture University, Wuhan. A population of 202 F_1 plants from the three-way cross was obtained for the study. At tillering stage, each plant was divided into three parts by peeling off the tillers and replanting, and each part was allowed to grow to maturity. One part of each plant was used for DNA extraction, the second part for embryo sac and pollen collection, and the third part for examining spikelet fertility. Spikelet fertility was scored as the seed-setting rate of three panicles randomly collected from each plant, and embryo sac and pollen fertility were examined using the methods described below.

Embryo sac and pollen-fertility evaluation

The whole-stain clearing method (Yang 1986) was used for evaluating embryo sac fertility. About 100–150 mature spikelets from various parts of different panicles of a plant were randomly collected and immediately immersed in a fixative [methanol:acetic acid (3:1)]. The sample was then placed in a vacuum for 30 min and incubated for 24 h at room temperature, after which the tissue was stored in 70% methanol at 4°C. Before staining, the samples were transferred to 70% ethanol, with the lemma and palea removed to expose the ovary to ethanol. The tissue was then processed through an ethanol series (50, 30, and 15%) and finally transferred into distilled water. The whole mature ovary was stained in Ehrlich's haematoxylin (Wang 1992) for 20–40 min and washed with distilled water for 24 h, followed by washing with tap water three to four times until the color of tissue turned from purple to blue. The tissue was dehydrated by passing it through an ethanol series and cleared by incubation in 100% methyl salicylate three times—more than 1 h for the first and the second incubation, and at least 24 h for the third incubation. Fertility of the embryo sacs was examined under a microscope. About 100 embryo sacs were examined for each plant.

About five to eight mature flowers from various parts of different panicles of a plant were collected for pollen-fertility investigation. Pollen from different spikelets were mixed, stained with I_2–KI solution, and observed under a microscope. A total of 300–800 pollens per plant were analyzed.

DNA marker assay

Total cellular DNA was extracted using essentially the protocol of Murray and Thompson (1980). A total of 191 nuclear microsatellite (SSR) and cleaved amplified polymorphic sequence (CAPS) (Konieczny and Ausubel 1993) markers were used for map construction. The primer pairs of the RM series and MRG series were designed according to Temnykh et al. (2000, 2001) and McCouch et al. (2002), respectively. Another two primer pairs, H3878-1, H4698-1, were derived from the published sequence (http://ncbi.nlm.nih.gov). Two CAPS markers, C11 and RG213, were converted from the RFLP markers on the basis of the sequence information.

Polymerase chain reaction (PCR) was performed in a 20-μl reaction volume containing 30–50 ng of the template DNA, 10 mM Tris-HCl, 50 mM KCl, 0.1% Triton X-100, 1.8 mM $MgCl_2$, 0.1 mM dNTP, 0.2 μM primer pairs, and 1 U *Taq* DNA polymerase. DNA amplification protocol included an initial 3 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, 1.5 min at 72°C, and a final extension for 5 min at 72°C in a thermocycler (MJ Research, USA). PCR products were separated on 6% polyacrylamide denaturing gels, and the amplified DNA fragments were silver-stained as described by Bassam et al. (1991).

Data processing and statistical analysis

As described previously by Liu et al. (1997), only the markers polymorphic between '02428' and 'Nanjing 11'

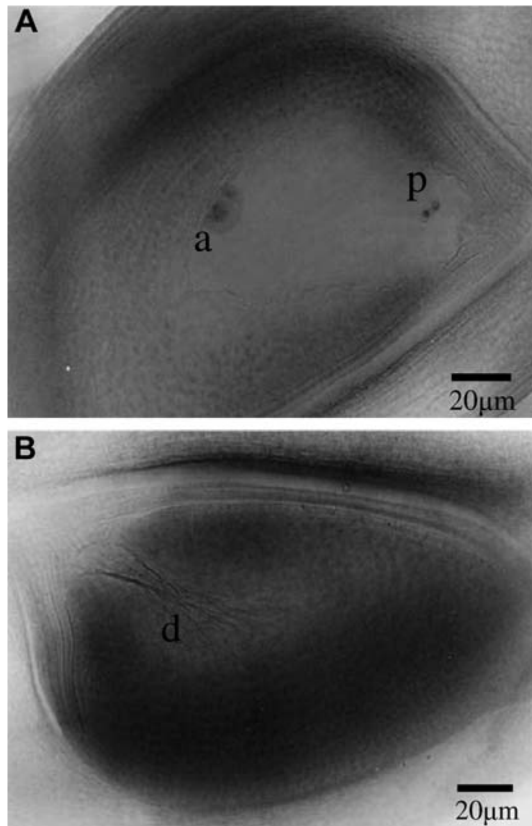

Fig. 1 (**A**) Fertile embryo sac with visible antipodal cell (*a*) and polar nucleus (*p*). (**B**) Sterile embryo sac with degenerated cells (*d*)

were informative for such analysis. Thus, data for markers polymorphic between '02428' and 'Nanjing 11' were scored and analyzed using the method for backcross populations in map construction and QTL analysis.

An SSR marker linkage map was constructed using MAPMAKER/EXP, version 3.0, at a LOD score of 3.0 (Lincoln et al. 1992). QTLs governing pollen fertility, embryo sac fertility, and spikelet fertility were resolved by composed interval mapping, using QTL Cartographer, version 1.15 (Basten et al. 2001). Analysis of variance (ANOVA) using marker genotypes as the classes was conducted using the statistical package STATISTICA (StatSoft 1995).

Results

Segregation of embryo sac fertility, pollen fertility, and spikelet fertility

A normal mature rice embryo sac has antipodal cells, two polar nuclei, two synergid nuclei, and an egg nucleus. Although the synergids and egg nucleus are sometimes not clearly visible by the whole-stain clearing method, normal embryo sacs can easily be distinguished from abortive embryo sacs (Fig. 1). Ovaries with normal embryo sac structure (visible antipodal cells and polar nucleus) were regarded as fertile, whereas those showing degenerated cells were classified as sterile. Embryo sac fertility in the three-way cross population varied from a low of 20% to a high of 100%, and showed an apparent bimodal distribution (Fig. 2).

Pollen grains that were round and darkly stained were regarded as fertile, and all others were classified as sterile. Pollen fertility also varied widely in the population from 0% to almost 100%, with apparently continuous distribution (Fig. 2), as did spikelet fertility.

Simple correlations were calculated pairwise to assess the relationship of embryo sac fertility, pollen fertility, and spikelet fertility. It was shown that spikelet fertility is highly significant and about equally correlated with embryo sac fertility (0.620) and pollen fertility (0.624). There is also a weak correlation between embryo sac fertility and pollen fertility (0.311, significant at $P \leq 0.01$ level).

A multiple regression analysis was also conducted using spikelet fertility as the dependent variable and the other two traits as independent variables (Table 1). The results again showed that spikelet fertility is highly and about equally dependent on embryo sac fertility and pollen fertility, as indicated by both partial correlation coefficients and the determination coefficients.

The linkage map

Based on 202 true hybrid plants, a linkage map consisting of 191 loci was constructed using MAPMAKER/EXP at LOD 3.0 (map not shown). The total length of this map was 1,761.4 cM, with an average interval of 9.2 cM between adjacent markers. The 191 loci were

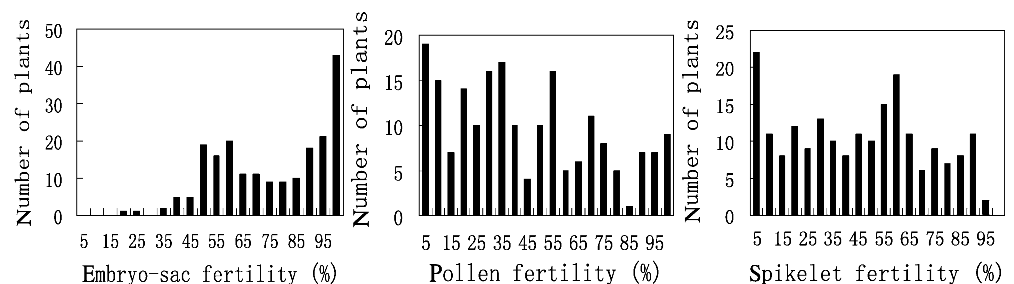

Fig. 2 Distributions of embryo sac fertility, pollen fertility and spikelet fertility of 202 F_1 plants of three-way cross '02428'/'Nanjing 11'//'Balilla'

Table 1 Multiple linear regression analysis of spikelet fertility as dependent variable and pollen fertility and embryo sac fertility as independent variables

Variable	Partial regression coefficient	SE	Partial correlation coefficient	Determination coefficient
Intercept	−21.663	4.476		
Pollen fertility	0.444	0.045	0.579	0.335
Embryo sac fertility	0.619	0.062	0.585	0.342

placed into 12 linkage groups at LOD 3.0, and these markers had a good coverage of all 12 chromosomes according to the published map (Temnykh et al. 2000, 2001). The linear order of makers in this map accorded well with that of Temnykh et al. (2000, 2001).

QTLs for embryo sac fertility, pollen fertility, and spikelet fertility

The genomic locations of the QTLs resolved using QTL Cartographer (Basten et al. 2001) for the three traits with LOD threshold 2.5 are illustrated in Fig 3.

Two QTLs were detected for pollen fertility. The QTL detected on chromosome 5, *pf5*, demonstrated a large effect on pollen fertility, and the effect of the other QTL on chromosome 12, *pf12*, was also substantial (Table 2). QTL *pf5* coincided with the *f5* locus for spikelet fertility identified previously by Wang et al. (1998) in a different three-way cross population (‘Balilla’/Dular//‘Nanjing 11’). In addition, *pf12* corresponded well with a QTL for spikelet fertility detected by Liu et al. (1997).

Only one QTL (*ef6*) with major effect was resolved for embryo sac fertility (Table 2). The genomic location of this QTL corresponded well with the *S5* locus for wide compatibility identified previously by spikelet fertility (Liu et al. 1992, 1997).

Three QTLs were identified as showing significant effects on spikelet fertility (Table 2). The one on chromosome 5, *spf5*, appeared to have much larger effect than the other two on chromosomes 6 and 8, *spf6* and *spf8*, respectively. The locations of *spf5* and *spf6* corresponded well with the QTLs for pollen fertility and embryo sac fertility, respectively (Fig 4). In addition, *spf8* had a similar location with *f8* for spikelet fertility identified previously by Wang et al. (1998).

Possible interactions between QTLs for each trait

As described above, two and three QTLs were detected for pollen fertility and spikelet fertility, respectively. To assess possible effects of interactions between loci on these two traits, ANOVAs were performed using the genotypes of the most closely linked markers as groups. The results of two-way ANOVA for pollen fertility indicated that the two loci did not have significant interaction effect on the trait (data not shown). A three-way ANOVA of spikelet fertility also indicated that

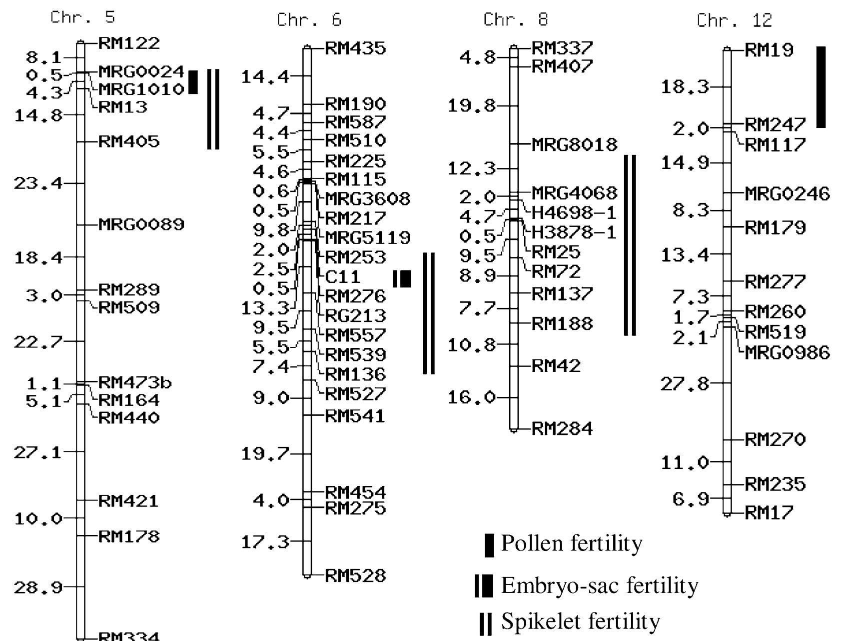

Fig. 3 The chromosome locations of QTLs for embryo sac fertility, pollen fertility, and spikelet fertility detected by QTL Cartographer, version 1.15

Table 2 QTLs controlling pollen fertility, embryo sac fertility and spikelet fertility in the '02428'/'Nanjing 11'// 'Balilla' population detected at LOD 2.5, with composite interval mapping, using QTL Cartographer, version 1.15

Trait	Interval	QTL	LOD	Effect	Variance (%)
Pollen fertility	MRG1010–RM13	*pf5*	10.10	26.48	21.69
	RM19–RM247	*pf12*	5.52	20.78	12.50
Embryo sac fertility	C11–RM276	*Ef6*	30.87	29.83	52.20
Spikelet fertility	RM13–RM405	*spf5*	11.96	26.75	25.22
	RG213–RM557	*spf6*	5.14	17.64	11.01
	H4698-1–H3878-1	*spf8*	4.01	15.66	8.69

there is very little interaction between paired loci or among the three loci, except one interaction of small effect ($F=4.81$, $P=0.03$) between RG213 (*spf5*) and H3878-1 (*spf8*). These results indicated that, essentially, these loci acted independently in determining pollen fertility and spikelet fertility.

Effects and modes of gene actions at the QTLs

There were two genotypes at each of the loci in this three-way cross: a genotype composed of a 'Nanjing 11' allele (*i*) and 'Balilla' allele (*j*) and a genotype composed of a '02428' allele (*n*) and a 'Balilla' allele (*j*). The mode of gene action was the same at all the six QTLs identified for the three traits (Tables 2, 3, 4, 5, 6). The *n*/*j* genotype was significantly more fertile than the *i*/*j* genotype at all loci, indicating that *indica*/*japonica* heterozygote reduced both embryo sac fertility and pollen fertility, which consequently caused reduced spikelet fertility. Conversely, the *n*/*j* genotype is favorable for embryo sac fertility, pollen fertility, and spikelet fertility.

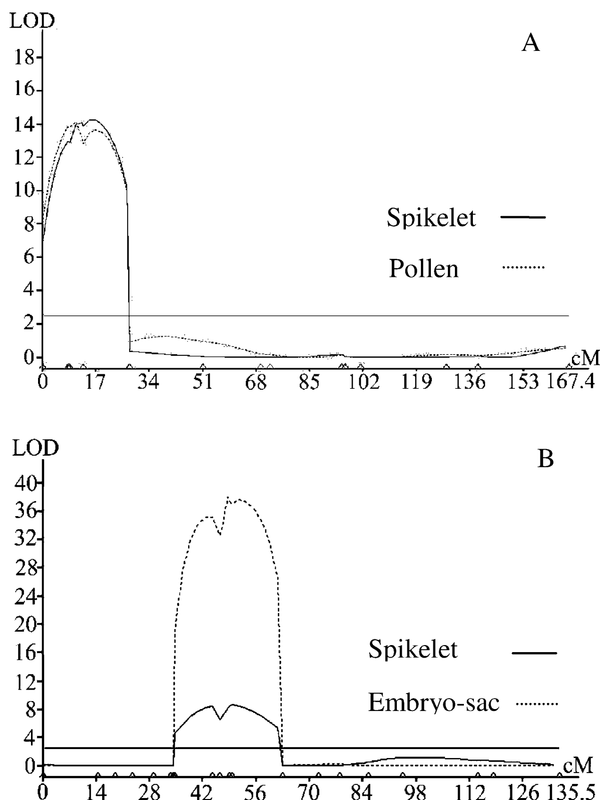

Fig. 4 (**A**) LOD curves of QTLs for pollen fertility and spikelet fertility on chromosome 5. (**B**) LOD curves of QTLs for embryo sac fertility and spikelet fertility on chromosome 6

Discussion

Wide compatibility that overcomes hybrid sterility in intersubspecific crosses in rice has been traditionally analyzed using spikelet fertility as the criterion (Ikehashi and Araki 1986; Gu et al. 1993), by which a number of loci or QTLs for wide compatibility as well as intersubspecific sterility has been identified (Ikehashi and

Table 3 Spikelet fertility for each of the three-locus combinations, based on the genotypes of the most closely linked marker loci

RM13 (*spf5*)	RG213 (*spf6*)	H3878-1 (*spf8*)	Spikelet fertility (%)
1[a]	1	1	16.20
1	1	0[b]	37.30
1	0	1	30.50
1	0	0	41.24
0	1	1	33.77
0	1	0	50.86
0	0	1	60.59
0	0	0	73.55

[a]Genotype 1 of each locus was composed of an allele from 'Nanjing 11' and an allele from 'Balilla'
[b]Genotype 0 of each locus was composed of an allele from '02428' and an allele from 'Balilla'

Table 4 Pollen fertility for each of the two-locus combinations, based on the genotypes of the most closely linked marker loci

RM13 (*pf5*)	RM19 (*pf12*)	Pollen fertility (%)
1[a]	1	22.90
1	0[b]	45.89
0	1	49.63
0	0	62.99

[a, b]Genotype designations are the same as in Table 3

Table 5 Embryo sac fertility for each genotype of the *ef6* locus

RM276 (*ef6*)	Embryo sac fertility (%)
1[a]	57.69
0[b]	87.36

[a, b]Genotype designations are the same as in Table 3

Table 6 Pollen fertility, embryo sac fertility and spikelet fertility for each combination of the two major QTLs, based on the genotypes of the most closely linked marker loci

RM13 (*pf5*, *spf5*)	RM276 (*ef6*, *spf6*)	Pollen fertility (%)	Embryo sac fertility (%)	Spikelet fertility (%)
1[a]	1	29.05	55.76	25.42
0[b]	1	54.58	59.74	42.93
1	0	30.36	80.65	35.75
0	0	52.29	94.15	67.13

[a, b]Genotype designations are the same as in Table 3

Wan 1996; Liu et al 1997; Zhang et al. 1997; Wang et al. 1998). In general, spikelet fertility is equally critically dependent on both male and female gamete fertility. It has been reported that both male and female gamete abortions occur in *indica*/*japonica* hybrids that greatly reduce the fertility of the hybrids (Yokoo 1984; Li 1988; Ling 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. 1998).

In this study, we quantified the importance of male and female gamete abortions in determining *indica*/*japonica* hybrid sterility, and found that male and female gamete abortions contributed almost equally to the intersubspecific hybrid sterility. Genetic dissection identified two major loci (QTLs) governing spikelet fertility; one of them (*spf5*) corresponded to a major locus specifying pollen fertility (*pf5*) and the other (*spf6*) corresponded to a major locus conditioning embryo sac fertility (*ef6*). Our results clearly showed that *ef6* is the same as the previously identified *S5* locus for wide compatibility (Ikehashi and Araki 1986; Liu et al. 1992, 1997; Yanagihara et al. 1995), whereas *pf5* specifying pollen fertility coincided with the *f5* locus for *indica*/*japonica* hybrid sterility previously reported by Wang et al. (1998). Zhuang et al. (2002) also mapped a locus for pollen fertility on chromosome 5 located in the same vicinity as the one identified in this study.

In addition to the two major QTLs for pollen and embryo sac fertility, *spf8* also has substantial effect on spikelet fertility, which is apparently independent of pollen fertility and embryo sac fertility. Recent results of a cytological study by Liu et al. (2004) showed that, in addition to pollen and embryo sac fertility, reduced affinity (or compatibility) between the uniting gametes is also an important cause for sterility in an *indica*/*japonica* hybrid. Whether *spf8* is a QTL for affinity (compatibility) between the uniting gametes remains to be investigated in future studies.

Previous studies repeatedly showed that '02428' is a wide compatibility variety with the "neutral" allele at the *S5* locus as a wide-compatibility gene (Liu et al. 1992, 1996, 1997). The data obtained in this study showed that the '02428' allele at the *pf5* locus also has a positive effect on fertility in heterozygote (*n*/*j*) with the 'Balilla' allele, as compared with the heterozygote of the 'Nanjing 11' allele with the 'Balilla' allele (*i*/*j*). Moreover, *pf5* seems to have a more prominent effect on spikelet fertility than does the *ef6* locus, as evidenced by the relative effects of *spf5* and *spf6*. However, because '02428' has many *japonica* characteristics and is regarded essentially as a *japonica* variety, it remains to be determined whether the '02428' allele at the *pf5* locus is a wide-compatibility gene for pollen fertility. The same issue also applies to the remaining QTLs (e.g., *pf12* and *spf8*).

It should be noted that the population used in this study was derived from the same three-way cross as used for the study by Liu et al. (1997). However, there were several discrepancies observed in this study as compared with the results of Liu et al. (1997). The first difference is that the *spf5* locus governing spikelet fertility via pollen fertility (*pf5*) observed in this study was not detected in the previous study, which is accounted for by a gap in the linkage map of Liu et al. (1997) that occurred at exactly the same genomic location as *spf5* (or *pf5*). The second difference occurred in the shape of the distribution curve of the spikelet fertility in the '02428'/'Nanjing 11'//'Balilla' F_1 population. This is largely due to the fluctuations of the environmental conditions in the two years when the experiments were conducted in the field. Another difference is the failure of this study in detecting a minor QTL for spikelet fertility on chromosome 2 resolved by Liu et al. (1997), also likely to be the results of environmental fluctuations.

The present findings might have significant implications in rice breeding programs. Utilization of the wide-compatibility gene (*S5n*) for development of intersubspecific hybrids has been a practice in many rice breeding programs. However, it has been frequently found that the *S5n* gene alone is not sufficient for producing *indica*/*japonica* hybrids with normal fertility. We now understand that the wide-compatibility gene at *S5* only overcomes the sterility caused by embryo sac abortion, and that a gene from the *pf5* (*spf5*) locus for overcoming pollen sterility is also essential for normal fertility hybrids. Moreover, a gene governing the affinity between the uniting gametes may also help, should there exist such a locus. This can easily be achieved with marker-assisted selection using the markers identified in the present study.

Acknowledgements This research was supported in part by a grant from the National Special Key Project on Functional Genomics and Biochips of China, and a grant from the National Natural Science Foundation of China.

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