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ORIGINAL PAPER

Fine mapping of *S32(t)*, a new gene causing hybrid embryo sac sterility in a Chinese landrace rice (*Oryza sativa* L.)

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Abstract Ketan Nangka, the donor of wide compatibility genes, showed sterility when crossed to Tuanguzao, a landrace rice from Yunnan province, China. Genetic and cytological analyses revealed that the semi-sterility was primarily caused by partial abortion of the embryo sac. Genome-wide analysis of the linkage map constructed from the backcross population of Tuanguzao/Ketan Nangka//Ketan Nangka identified two independent loci responsible for the hybrid sterility located on chromosomes 2 and 5, which explained 18.6 and 20.1% of phenotypic variance, respectively. The gene on chromosome 5 mapped to the previously reported sterility gene *S31(t)*, while the gene on chromosome 2, a new hybrid sterility gene, was tentatively designated as *S32(t)*. The BC_1F_2 was developed for further confirmation and fine mapping of *S32(t)*. The gene *S32(t)* was precisely mapped to the same region as that detected in the BC_1F_1 but its position was narrowed down to an interval of about 1.9 cM between markers RM236 and RM12475. By assaying the recombinant events in the BC_1F_2, *S32(t)* was further narrowed down to a 64 kb region on the same PAC clone. Sequence analysis of this fragment revealed seven predicted open reading frames, four of which encoded known proteins and three encoded putative proteins. Further analyses showed that wide-compatibility variety Dular had neutral alleles at loci *S31(t)* and *S32(t)* that can overcome the sterilities caused by these two genes. These results are useful for map-based cloning of *S32(t)* and for marker-assisted transferring of the neutral allele in hybrid rice breeding.

Keywords *Oryza sativa* · *Indica–japonica* hybrids · Hybrid sterility · Fine mapping

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Introduction

Semi-sterility has been a major barrier for the utilization of strong heterosis between *indica* and *japonica*, which are two subspecies of Asian cultivated rice (*Oryza sativa* L.). Cytological investigations have revealed that many factors contribute to sterility, including male gamete abortions, female gamete abortions, affinity between the uniting male and female gametes, and reduced dehiscence of anthers (Liu et al. 2004). In addition, environmental conditions, especially low temperature, can also greatly reduce the fertility in inter-subspecific hybrids (Li et al. 1997).

Ikehashi and Araki (1986) first proved that the sterility in F_1 of inter-subspecific hybrids was caused by an allelic interaction at the *S5* locus on chromosome 6,

where *indica* and *japonica* varieties have alleles $S5^i$ and $S5^j$, respectively, but some *javanica* varieties have a neutral allele $S5^n$. The $S5^i/S5^j$ genotype produces semi-sterile panicles due to partial abortion of female gametes carrying $S5^j$. Such an abortion does not occur if the $S5^n$ allele is present in such genotypes as $S5^n/S5^i$ and $S5^n/S5^j$. The parent homozygous for $S5^n$ is called a wide-compatibility variety (WCV). As more rice varieties were screened for WCV, a series of other loci causing hybrid sterility, such as *S7*, *S8*, *S9*, *S15*, *S16, S17*, *S29*, *S30* and *S31* were identified and mapped on rice chromosomes (Wan et al. 1993, 1996; Wan and Ikehashi 1995; Zhu et al. 2005a, b; Zhao et al. 2006).

To date, more than 30 loci controlling pollen fertility or spikelet fertility in hybrids between *indica* and *japonica* have been identified, but some were just detected as QTLs with minor effects and have not been confirmed. To conduct marker-assisted selection (MAS) in hybrid breeding or map-based cloning of these genes, it is necessary to deal with each QTL as a single Mendelian factor and to localize each QTL precisely on the linkage map (fine mapping) using appropriate populations. Populations derived from near-isogenic lines (NILs) or special genotypes on the basis of the primary mapping are most suitable for confirming and fine mapping of target QTLs.

Tuanguzao, a landrace from Yunnan province, China, is a valuable source of multiple resistances to major diseases (e.g., rice stripe virus) and stresses (e.g., cold). Ketan Nangka is a *javanica* variety carrying wide compatibility genes (WCGs) at loci *S5* and *S15* (Wan and Ikehashi 1996). However, the typical semi-sterility shown in the F_1 hybrids between Ketan Nangka and Tuanguzao is a genetic barrier for the introduction of desirable genes in Tuanguzao. Detection and fine mapping of the locus (loci) controlling hybrid sterility and identification of the corresponding neutral alleles are key to pyramid WCGs by MAS and further to utilize desirable genes in Tuanguzao.

The main objectives of this study are to: (1) determine the cytological basis for semi-sterility in the F_1 hybrid between Tuanguzao and Ketan Nangka and identify the neutral alleles that can overcome the sterility; (2) map the genes controlling hybrid semi-sterility and conduct fine mapping of the newly identified sterility gene, *S32(t)*.

Materials and methods

Parental materials and mapping populations

Three parents were used in this study. Ketan Nangka is a *javanica* variety from Indonesia carrying neutral alleles at the loci *S5* and *S15* (Wan and Ikehashi 1996). Tuanguzao belongs to a landrace of Yunnan province in China. Dular, a landrace variety from India, confers both a wide spectrum and high level of wide-compatibility when crossed to a series of *indica* and *japonica* varieties (Zhang et al. 1997).

A backcross population (Tuanguzao/Ketan Nangka//Ketan Nangka) consisting of 238 individuals was used for the genome-wide detection of the loci controlling hybrid semi-sterility between Tuanguzao and Ketan Nangka. Based on the initial mapping results of the *S32(t)* locus, BC_1F_1 plants heterozygous at locus *S32(t)* [$S32^{ti}(t)/S32^{kn}(t)$] but homozygous at locus *S31(t)* [$S31^{kn}(t)/S31^{kn}(t)$] with lower than 50% spikelet fertility were selected to produce a BC_1F_2 population for further confirmation and fine mapping of *S32(t)*. F_1 hybrids of Tuanguzao/Dular and Ketan Nangka/Dular were made to identify the WCGs at loci *S31(t)* and *S32(t)*.

The three parents and their F_1s plus the 238 individuals in the BC_1F_1 population were planted in the rice growing seasons of 2003 at the experimental farm of Nanjing Agricultural University, Jiangsu Province, China. The parents and 1,050 individuals of the BC_1F_2 population were planted in 2005.

Fertility examination

Ten individual plants from each Tuanguzao, Ketan Nangka and their F_1 hybrids were examined for pollen fertility. Six florets from three panicles in each plant were collected 1–2 days before flowering. One anther per floret from the same plant was mixed and stained with 1% iodine potassium iodide (I_2–KI) solution, and four views were observed per plant under microscope.

Embryo sac fertility was evaluated by the ferrovanadium–haematoxylin method. One hundred to one hundred and fifty mature florets from ten plants of Tuanguzao, Ketan Nangka and their F_1 hybrids were collected 1–2 days before flowering and immediately fixed in FAA solution (containing an 18:1:1 mixture of 70% ethanol, formalin and acetic acid). The standard protocol was used to obtain thin histological sections, which were mounted on slides to be examined and photographed with a photomicroscope (Olympus BH-2).

The affinity between pollen and stigma was examined by observing the behavior of the pollen grains on the stigma after pollination. Twenty florets were collected 30 min after flowering to examine the adherence of pollen on stigma, pollen germination, and elongation of the pollen tube by confocal laser scanning microscopy (Leica SP2). The in vitro pollen germination and fertilization status of embryo sacs were tested

according to the methods of Daniela (2003) and Liu (2004), respectively.

The spikelet fertility of individual plants of the parents, F_1 hybrids, BC_1F_1 and BC_1F_2 population was determined by counting fertile and sterile spikelets on the upper half of three main panicles after maturity.

Molecular marker screening, development and assay

The simple sequence repeat (SSR) markers used in the initial mapping were obtained from Gramene (http://www.gramene.org/microsat/) based on the SSR linkage map constructed by McCouch (2002). The polymorphisms between Tuanguzao and Ketan Nangka were screened using 711 SSR and 15 expressed sequence tag (EST) markers covering the whole rice genome. The procedure for DNA amplification by PCR was as follows: 94°C for 5 min, followed by 35 cycles of 30 s at 94°C, 30 s at 55°C, 1 min at 72°C with a final extension of 7 min at 72°C. Amplified DNA products were analyzed by gel electrophoresis on 8% native polyacrylamide gels in 0.5× TBE buffer and visualized by silver staining. Markers that showed evident polymorphism and were evenly distributed on the chromosomes were used to construct the linkage map.

Based on the distribution of the polymorphic SSR markers and the initial mapping of *S32(t)*, more SSR and insertion/deletion (InDel) markers were designed for confirmation and fine mapping of *S32(t)*. The SSR loci were detected from the corresponding sequence in the region of the *japonica* rice Nipponbare according to the SSR identification tool (SSRIT; http://www.gramene.org/microsat), and SSR markers were designed using the Primer5.0 software. The BLAST (basic local alignment search tool) search in NCBI database was conducted to find InDel sequence divergence between the *indica* rice 93–11 and the *japonica* rice Nipponbare around the locus *S32 (t)* (http://www.ncbi.nlm.nih.gov /BLAST/). InDel markers were designed around the sequence divergence including 10–100 bp InDel using the Primer5.0 software.

Linkage map construction and QTL mapping

An SSR marker linkage map was constructed using MAPMAKER/EXP, version 3.0 (Lander et al. 1987). Composite interval mapping was applied for mapping genes controlling hybrid sterility through QTL Cartographer, version 2.0. The experiment-wise LOD (log of the odds ratio) threshold significance level was determined from 1,000 permutation tests (Churchill and Doerge 1994), as implemented by the QTL Cartographer. The LOD value for the level of significance for QTLs in this study was 2.89 ($P = 0.05$). Analysis of variance (ANOVA) using marker genotypes as the classes was conducted using the general linear model (GLM) procedure of SAS (SAS Institute 1989).

Results

The mechanism of the hybrid sterility between Tuanguzao and Ketan Nangka

The pollen and spikelets of the two parents, Tuanguzao and Ketan Nangka, were normal, whereas typical spikelet semi-sterility was observed in their F_1 hybrids. To elucidate the mechanism of the sterility in F_1 hybrids, pollen fertility, in vitro pollen germination, embryo sac fertility, fertilized ovaries, and spikelet fertility were examined and compared to control parent strains (Table 1). The results revealed that both pollen fertility and in vitro pollen germination rate of the F_1 hybrids were similar to those of the parents. In addition, the spikelet fertility of F_1 hybrids was not restored to normal levels after hand pollination with pollen from each parent (Table 1). Furthermore, no distinction was observed between F_1 hybrids and the two parents when examining the number of pollen grains adhered to stigmas, pollen germination, and pollen tube elongation using confocal laser scanning microscopy (data not shown).

Analysis of histological sections of mature embryo sacs revealed that the frequency of abnormal embryo sacs in F_1 hybrids was significantly higher than in parental controls (Table 1). There were three different types of abnormalities observed in the mature embryo sacs of the F_1 hybrids. In the first type, embryo sac differentiation was incomplete and embryo sacs failed to develop into functional embryos with eight cells. In the second type, embryo sacs did not undergo differentiation and darkly stained degenerated embryo sacs and nucellus cells were visible in the ovule. In the third type, embryo sacs were empty and lacked visible cells (Fig. 1). The fertilization rate of ovaries measured 2 days after flowering was subsequently lower in F_1 hybrids than in parents. Taken together, these results suggested that the partial abortive embryo sac was the main reason for the hybrid sterility in the F_1 hybrid between Tuanguzao and Ketan Nangka.

Further examination showed that spikelet fertility of the F_1 plants of Tuanguzao/Ketan Nangka was not distinct from that of its reciprocal F_1 (Table 2). Considering the similarity of bagged seed-setting rate, open seed-setting rate and fertilized ovary rate

Table 1 The fertility-related traits of two parents Tuanguzao and Ketan Nangka and their F_1 hybrid

	Tuanguzao	Ketan Nangka	F_1
Pollen fertility (%)	95.4 ± 7.8	92.7 ± 3.6	90.2 ± 8.6
In vitro pollen germination rate (%)	89.4 ± 9.2	78.2 ± 5.3	84.3 ± 6.9
Normal embryo sac (%)	85.3 ± 7.7	88.9 ± 9.5	50.4 ± 13.7 A[a]
Fertilized ovaries (%)	89.9 ± 2.3	90.5 ± 1.3	52.2 ± 8.7 A
Bagged seed-setting rate (%)	86.4 ± 2.6	89.2 ± 5.4	43.3 ± 12.1 A
Open seed-setting rate (%)	87.2 ± 1.8	86.1 ± 4.1	49.4 ± 5.5 A
Supplementary pollination with Ketan Nangka (%)			51.3 ± 7.7 A
Supplementary pollination with Tuanguzao (%)			49.9 ± 10.3 A

[a] Shows no significant difference among six traits at significance level of 1% based on *t*-test

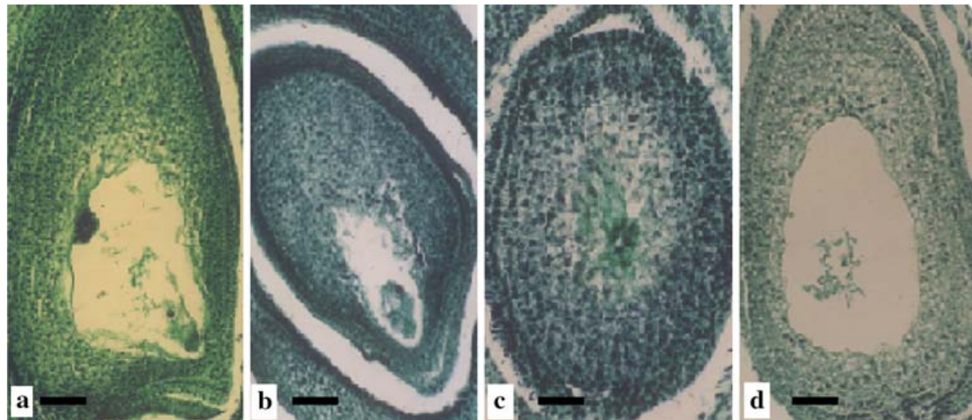

Fig. 1 Normal embryo sacs of parents (**a**) and various abnormal embryo sacs of Tuanguzao/Ketan Nangka (**b–d**). **a** Normal embryo sacs of parents with visible antipodal cells and polar nucleus. **b** Embryo sac differentiation was incomplete and embryo sacs failed to develop into functional embryos with eight cells. **c** Embryo sacs did not undergo differentiation and darkly stained degenerated embryo sacs and nucellus cells were visible in the ovule. **d** Embryo sacs were empty and lacked visible cells. *Bar* 30 μm

Table 2 The spikelet fertility of three parents and their F_1 hybrids

Parents and crosses	Mean ± SD (%)
Tuanguzao	87.2 ± 1.8
Ketan Nangka	86.1 ± 4.1
Dular	91.7 ± 1.1
Tuanguzao/Ketan Nangka	49.4 ± 5.5 A[a]
Ketan Nangka/Tuanguzao	46.2 ± 7.4 A
Tuanguzao/Dular	80.9 ± 12.1
Ketan Nangka/Dular	83.5 ± 8.7

[a] Shows no significant difference among six items at significance level of 1% (*t*-test)

(Table 1) we concluded that cytoplasmic gene(s) and environment factors had no noticeable impact on the fertility of F_1 hybrids. No statistically significant differences were observed in normal embryo sac frequency, fertilized ovary rates and seed setting rates, revealing that the seed setting rate correlated well with the normal embryo sacs rate (Table 1). Therefore, in this study, we used the seed setting rate as a convenient indicator for estimating normal embryo sac rate.

The distribution of spikelet fertility in BC_1F_1 and the linkage map

Spikelet fertility in the BC_1F_1 of Tuanguzao/Ketan Nangka//Ketan Nangka ranged widely from 3 to 97% without a clear grouping tendency (Fig. 2). The segregation pattern of highly fertile and partly sterile individuals deviated significantly from the expected 1:1 ratio. There were more individuals in the high-fertility group than in the low-fertility group, implying that a substantial portion of female gametes with Tuanguzao alleles was aborted in the F_1 hybrids.

Out of 711 SSR and 15 EST markers used to detect polymorphisms between Tuanguzao and Ketan Nangka, 339 SSR markers (47.8%) and one EST marker (0.07%) showed polymorphisms. The frequency of polymorphism varied among the chromosomes, ranging from 36.8 to 66.7%.

Based on the 238 individuals in the BC_1F_1 population, a linkage map consisting of 124 markers was constructed using MAPMAKER/EXP (Fig. 3). The total length of this map was 1,746.5 cM, with an average interval of 14.1 cM between adjacent markers. The 124

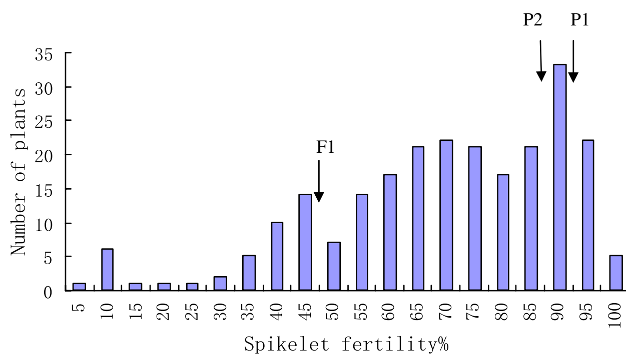

Fig. 2 Distribution of spikelet fertility of 238 plants in BC_1F_1 population of Tuanguzao/Ketan Nangka//Ketan Nangka. P_1, P_2 and F_1 represent Tuanguzao, Ketan Nangka and Tuanguzao/Ketan Nangka, respectively

markers had an even distribution on 12 chromosomes and the order of these markers on each chromosome was consistent with those described by Temnykh (2000), McCouch (2002) and Wu (2002). Therefore, the map was suitable for QTL mapping.

Genes for hybrid sterility in BC_1F_1 and mode of gene actions

A genome-wide analysis was carried out to search for genes having significant effects on hybrid sterility using QTL Cartographer (version 2.0), at a threshold LOD score of 2.89 (Table 3). As shown in Fig. 3, we have identified two loci. The first, with a LOD score of 15.1 on chromosome 5, was tightly linked to RM413 and explained 20.1% of the phenotypic variance. This QTL may correspond to *S31(t)* according to its chromosomal location (Zhao et al. 2006). The second locus was located on chromosome 2 with a LOD score of 14.5 and a close linkage to RM236, and explained 18.6% of the phenotypic variance. This QTL was different from all previously reported hybrid sterility loci, and was designated as *S32(t)* following the hybrid sterility nomenclature.

A two-way ANOVA, based on the genotypes of the markers closely linked to *S31(t)* and *S32(t)*, respectively, was carried out to characterize the mode of gene action in this two-locus system (Table 4). The results revealed no significant interaction between the two loci suggesting that the two loci acted independently. Thus, fertility reduction is likely a result of allelic interaction within each of the two loci, as in the case of a number of hybrid sterility loci identified in previous studies (Ikehashi and Araki 1986; Wan et al. 1996).

There were two kinds of genotypes at each locus in BC_1F_1 of Tuanguzao/Ketan Nangka//Ketan Nangka. One was homozygous (S^{kn} S^{kn}) with two alleles from Ketan Nangka, and the other was heterozygous (S^{ti} S^{kn}), carrying the allele S^{ti} of Tuanguzao and the allele S^{kn} of Ketan Nangka. For each locus, the fertility of homozygotes was significantly higher than that of heterozygotes (Table 5). For different allelic combinations, plants homozygous at both loci exhibited the highest fertility, followed by plants homozygous at only one locus, plants heterozygous at both loci exhibiting the lowest fertility. Very significant differences existed among the three genetic combinations (Table 6). These results confirmed that the hybrid sterility was due to allelic interaction at each locus and that genetic effects of the two loci were additive.

Table 3 QTLs controlling hybrid sterility detected in the BC_1F_1 of Tuanguzao/Ketan Nangka//Ketan Nangka population

QTLs	Chromosome	NML[a]	LOD score	Effect	Variance (%)
S32(t)	2	RM236	14.5	21.5	18.6
S31(t)	5	RM413	15.1	21.5	20.1

[a] Nearest marker locus of putative QTLs

Detection of wide compatibility genes at loci *S31(t)* and *S32(t)*

F_1 hybrids of Tuanguzao/Ketan Nangka showed typical spikelet sterility while those of Ketan Nangka/Dular and Tuanguzao/Dular were normal (Table 2). Based on the model of allelic interaction (Ikehashi and Araki 1986), for three given A, B and N varieties, if the A/B hybrid but not the A/N or B/N hybrid shows gamete abortion at a locus *SX* by allelic interaction between SX^a and SX^b, the variety N may possess a neutral allele SX^n. Based on the observed normal fertility in F_1 hybrids of Ketan Nangka/Dular and Tuanguzao/Dular, we believe that Dular should carry the neutral alleles $S31^n(t)$ and $S32^n(t)$ at loci *S31(t)* and *S32(t)*, respectively. The WCGs can overcome the sterility caused by these two loci.

Confirmation and fine mapping of *S32(t)*

For confirming and fine mapping of *S32(t)*, it was important to clean out the effect of *S31(t)* and generate a fertility separation population in which the sterility was controlled only by *S32(t)*. In this study, BC_1F_1 (Tuanguzao/Ketan Nangka//Ketan Nangka) plants heterozygous at the *S32(t)* locus [$S32^{ti}(t)/S32^{kn}(t)$] and homozygous at the *S31(t)* locus [$S31^{kn}(t)/S31^{kn}(t)$] with spikelet fertility lower than 50% were selected to produce the BC_1F_2 mapping population. Theoretically, this BC_1F_2 population should be desirable for confirming

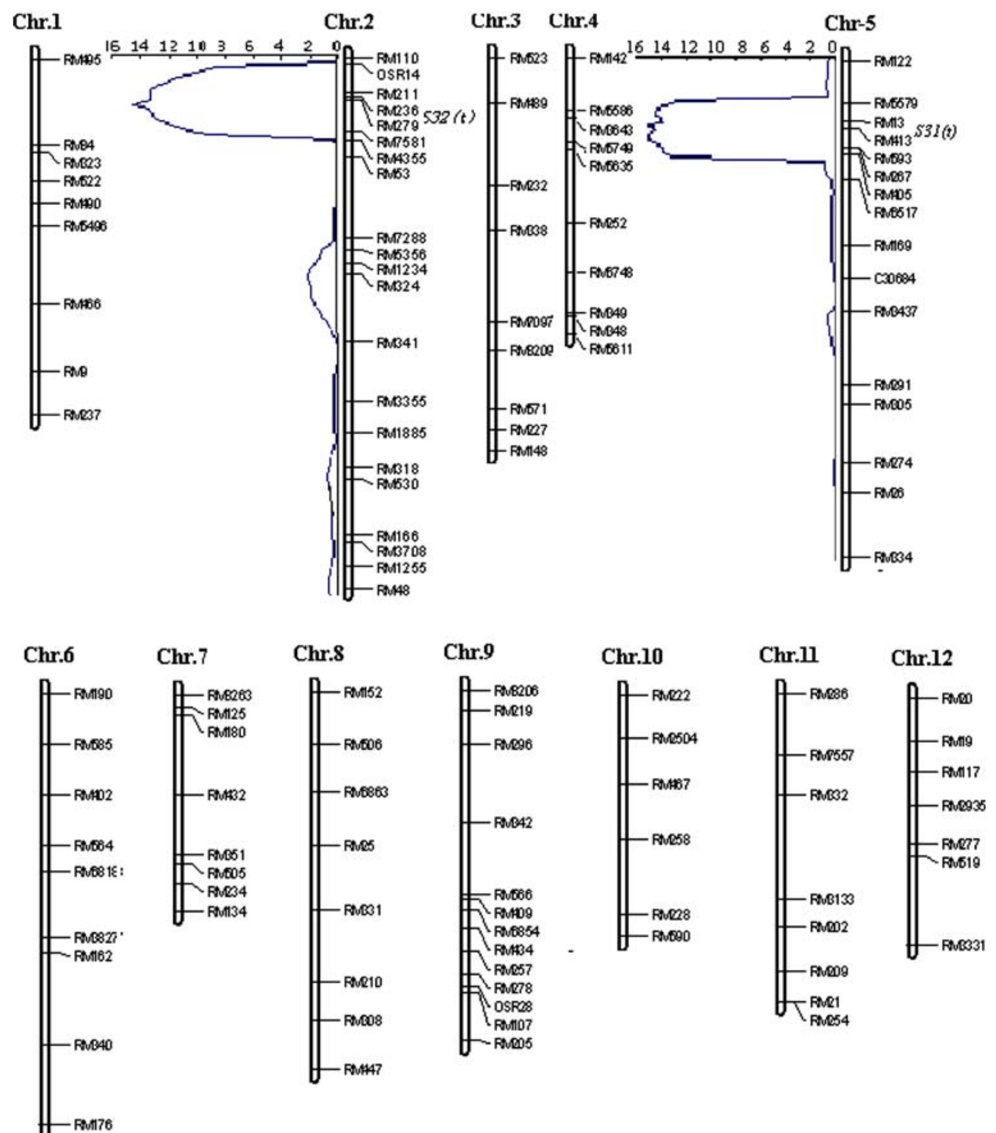

Fig. 3 Chromosome locations of two loci controlling hybrid fertility. The molecular linkage map constructed by SSR and EST markers based on BC_1F_1 population of Tuanguzao/Ketan Nangka//Ketan Nangka

Table 4 A two-way ANOVA using one marker locus from each of the genomic regions showing effects on hybrid fertility

Source of variation	Degree of freedom	Mean square	*F*-value	Probability
RM236	1	1.9025	68.89	0.0001
RM413	1	1.6211	58.70	0.0001
RM236 × RM413	1	0.0124	0.45	0.5029
Error	218	0.0276		

and fine mapping of the *S32(t)* locus since the fertility in this population is solely controlled by *S32(t)*.

Fertility separation in each BC_1F_2 line derived from the corresponding BC_1F_1 plant indicated the validity of selection. Spikelet fertility of 1,050 plants from the BC_1F_2 population showed a bimodal distribution with an apparent valley at about 65% (Fig. 4). The individuals with spikelet fertility rates higher than 65% were classified as fertile, while those with spikelet fertility lower than 65% were considered partially sterile. The numbers of fertile and partially sterile individuals were consistent with the expected 1:1 segregation ratio. These results indicated that the spikelet fertility of the BC_1F_2 population was mainly controlled by *S32(t)*.

Using 1,050 plants from BC_1F_2, a linkage map covering the *S32(t)* region was constructed using eight markers surrounding the *S32(t)* locus on the basis of our initial mapping. *S32(t)* was detected in the same location as that derived from the BC_1F_1 analysis, which was located on the interval between SSR markers RM236 and RM279 (Fig. 5).

In the BC_1F_2 population, plants homozygous for the Tuanguzao genotype [$S32^{ti}(t)/S32^{ti}(t)$] showed similar spikelet fertility to those homozygous for the Ketan Nangka genotype [$S32^{kn}(t)/S32^{kn}(t)$]. However, both genotypes had significantly higher spikelet fertility than the $S32^{ti}(t)/S32^{kn}(t)$ heterozygous plants (Table 5).

Table 5 Spikelet fertility for each of genotype of the most closely linked marker locus in BC_1F_1 and BC_1F_2 population

Mapping population	Marker	Genotype	Number of plants with spikelet fertility							Total	Mean fertility (%)
			30	40	50	60	70	80	90		
BC_1F_1	*S32(t)* (RM236)	Ketan Nangka	1	3	6	8	20	26	58	122	75.7 A[a]
		F_1	10	11	13	25	21	13	17	110	57.6 B
	S31(t) (RM413)	Ketan Nangka	1	2	2	6	22	21	54	108	76.6 A
		F_1	10	12	18	25	16	20	18	119	57.9 B
BC_1F_2	*S32(t)* (RM236)	Tuanguzao	2	0	2	8	9	13	21	68	70.2 A
		Ketan Nangka	1	3	2	9	24	12	31	127	74.7 A
		F_1	32	15	22	60	19	19	26	193	45.6 B

[a] A, B show significant difference between among genotypes at the significance level of 1% (*t*-test)

Table 6 Spikelet fertility for each of the two-locus combinations in BC_1F_1 population, based on the genotypes of the most closely linked marker loci

S32(t) (RM236)	*S31(t)* (RM413)	Number	Spikelet fertility (%)
Ketan Nangka	Ketan Nangka	60	83.2 A[a]
Ketan Nangka	F_1	55	67.5 B
F_1	Ketan Nangka	46	67.9 B
F_1	F_1	61	49.1 C

[a] A, B and C show significant difference between genotypes at 1% (*t*-test)

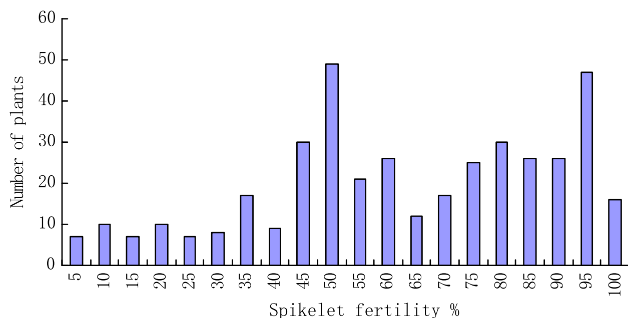

Fig. 4 Distribution of spikelet fertility of 1,050 plants in BC_1F_2 population of Tuanguzao/Ketan Nangka//Ketan Nangka. P_1, P_2 and F_1 represent Tuanguzao, Ketan Nangka and Tuanguzao/Ketan Nangka, respectively

Interestingly, the number of plants with the $S32^{kn}(t)/S32^{kn}(t)$ genotype was significantly higher than that with the $S32^{ti}(t)/S32^{ti}(t)$ genotype. The lower number of $S32^{ti}(t)/S32^{ti}(t)$ plants clearly indicated that gametes carrying the Tuanguzao alleles in the hybrid were partially abortive (Table 5).

More SSR markers between RM236 and RM279 were developed to narrow down the genomic region containing the *S32(t)* locus. Three hundred and twenty-five plants with spikelet fertility higher than 85% or lower than 40% were selected for fine mapping of the *S32(t)* locus. The location of *S32(t)* was further narrowed to a smaller interval of 1.9 cM between SSR markers RM236 and RM12475 (Fig. 5).

Identification of a 64 kb genomic fragment containing the *S32(t)* locus

Based on the sequence of the *japonica* rice Nipponbare provided by the Gramene database (http://www.gramene.org/), the distance between the two tightly linked SSR markers RM236 and RM12475 was about 600 kb, including seven overlapping PAC clones (AP005294, AP005721, AP005776, AP005649, AP005288, AP005191 and AP005412) (Fig. 6). More SSR and InDel markers were developed for each PAC clone to analyze recombinant events of 325 plants with extreme phenotypes. The mapping analysis identified three *S32(t)*-co-segregating markers including L2173, L2163 and L2226, four and two recombinant events between RM236 and *S32(t)* and between L2165 and *S32(t)*, respectively. The recombinant frequency increased at the markers flanking RM236 and L2165. These data indicated that the *S32(t)* locus was located on a 64 kb genomic fragment bordered by markers RM236 and L2165 on a single PAC clone (AP005294).

Discussion

The wide compatibility gene $S5^n$ has been incorporated into breeding lines to utilize the strong heterosis of *indica–japonica* hybrids in hybrid rice breeding (Araki et al. 1988; Ikehashi 1991; Yuan 1992). However, with the broadening of parents used in rice breeding, some hybrids still show semi-sterility despite the presence of the $S5^n$ allele in one of the parents. In order to take full advantage of the strong heterosis of inter-subspecific hybrids, it is necessary to accumulate the WCGs found in useful genetic backgrounds to overcome various sterilities. The genetic study of hybrid sterility led to the finding of a neutral allele, or wide compatibility allele. In this study, the wide compatibility variety Dular was found to have neutral alleles at loci *S31(t)* and *S32(t)* that can overcome the sterility in F_1 hybrids between

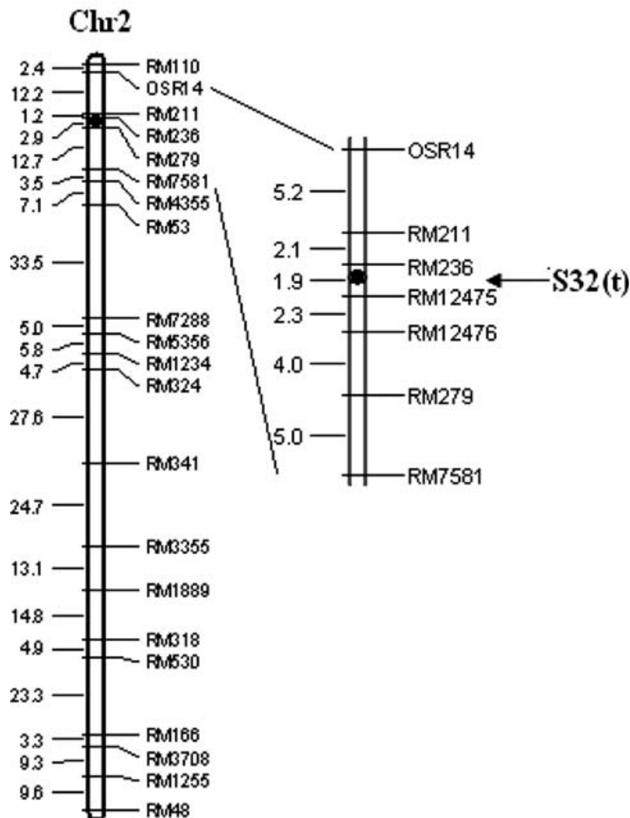

Fig. 5 Chromosome location of *S32(t)*. *A S32(t)* location detected in BC_1F_1 population. *B S32(t)* location detected in BC_1F_2 population

Tuanguzao and Ketan Nangka. The mapping of sterile loci and the detection of WCGs were useful for the introduction of the desirable traits in Tuanguzao by MAS in inter-subspecific hybrid rice breeding.

In this study, two independent loci responsible for hybrid sterility in the F_1 between Tuanguzao and Ketan Nangka were detected on chromosomes 2 and 5, respectively. Up to now, two sterility loci, *S29(t)* and *S22(t)*, have been reported on chromosome 2. *S29 (t)* was found to confer hybrid sterility between Bai Mi Fen and Ketan Nangka and is located on the long arm of chromosome 2 (Zhu et al. 2005a). It appears to be different from the sterility locus on chromosome 2 identified in this study based on its chromosomal location. *S22(t)* was previously detected in the backcross progeny of *O. sativa* and *O. glumaepatula* and was located on the short arm of chromosome 2, tightly linked with RFLP marker *S910* (Sobrizal et al. 2000). *S22(t)* was a pollen sterility locus that produced 50% pollen fertility and normal spikelet fertility under the heterozygous condition. In this study, we showed that genes at the locus on chromosome 2 control F_1 embryo sac fertility, and produced normal pollen and partial abortion of embryo sacs under the heterozygous condition. We therefore concluded that the newly identified locus is different from the two previously reported sterility loci on chromosome 2. Following the hybrid sterility nomenclature, this new locus was tentatively designated as *S32(t)*. The genetic location of the second sterility locus identified in this study on chromosome 5 is the same as that of the previously reported sterile gene *S31(t)*.

The progenies derived from the individuals with special genotypes are suitable for confirming and fine mapping of target gene because the effect of the non-target genes were excluded and the target gene acted as a single Mendelian factor in this population. The *S32(t)* locus was successfully confirmed and fine mapped to a 64 kb region using the BC_1F_2 population derived from BC_1F_1 plants with target genotype, confirming the validity and efficiency of this method. Fine mapping of *S32(t)* was useful for map-based cloning and incorporating WCGs by MAS.

Gene prediction analysis suggests there are seven putative open reading frames (ORF) on the 64-kb DNA fragment covering the *S32(t)* locus. One of these ORFs encodes a protein similar to the DNA-binding domain of squamosa promoter binding protein that was known to be important for flower

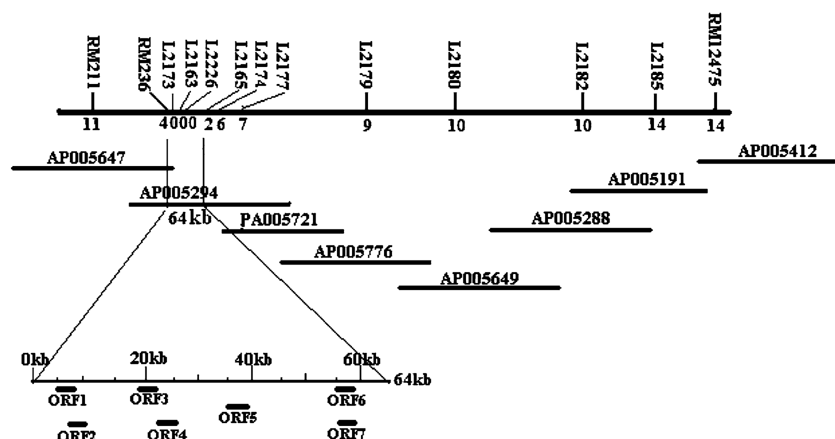

Fig. 6 High-resolution genetic and physical maps of the *S32(t)* locus and genes prediction. *RM* SSR markers, *L* In-Del markers. The *number* denotes the recombinant plants corresponding to marker

development in *Arabidopsis* (Silvia et al. 2004; Beth et al. 2005). Since little is known about the characteristics of the genes for the hybrid semi-sterility caused by interactions between the *indica* and *japonica* alleles, the bioinformatics analysis of the 64 kb genomic sequence did not provide enough useful information to identify a most likely candidate gene for the *S32(t)* locus. Additional experiments are needed to clone *S32(t)* and reveal its biochemical function, such as further fine mapping with a larger mapping population, developing functional markers based on sequence information, and sequencing and expression analysis of each putative gene in the two parent strains. Once a candidate gene is identified, it is essential to conduct transformation experiment to confirm its genetic function.

The hybrid sterility between *indica* and *japonica* in current Chinese cultivars was mostly due to allelic interaction at *S5* (Wan et al. 1993), whereas the hybrid sterility between some landraces of Yunnan province, China, such as Dabai, Luanjiaolong and Xiaobaigu, and Ketan Nangka was caused by *S16* on chromosome 1 (Wan and Ikehashi 1995). In this study, a genome-wide analysis revealed no allelic interaction at *S5* or *S16* in the hybrid between Yunnan landrace Tuanguzao and Ketan Nangka. Instead, two other loci, *S31(t)* and *S32(t)* were identified. Another hybrid sterility locus *S29(t)* was detected in the hybrid between Ketan Nangka and Baimifen, another landrace from Yunnan province (Zhu et al. 2005a). Ikehashi (2000) suggested that gamete-abortion-causing alleles can not be retained in cross-pollinated populations while neutral alleles may be retained. He also indicted that the gamete-abortion-causing allele can only be retained in the course of isolation and self-pollination. Only after such isolated groups were hybridized, the existence of gamete-abortion-causing alleles is revealed. Special existence of gamete-abortion -causing alleles at *S16*, *S29(t)* and *S32(t)* in landraces from Yunnan province reflects their unique origin, diffentiation, relatively isolated habitats and the resultant diverse compatibility. The Yunnan Province in China has been recognized as a center of rice genetic diversity, not only in morphological traits but also in compatibility. In a word, both morphological traits and hybrid sterility genes of these landraces have a wide and complex differentiation. As a result, the hybrid sterile loci exhibit the characteristics of wide separation. Additional new loci will be identified through expansion and analysis of the fertility of hybrids between landraces and other distant groups. This will allow for the transfer of available genes in Yunnan landraces and for the collection of more WCGs.

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