Theor Appl Genet (2005) 110: 346–355
DOI 10.1007/s00122-004-1846-y

ORIGINAL PAPER

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Epistasis underlying female sterility detected in hybrid breakdown in a Japonica–Indica cross of rice (*Oryza sativa* L.)

Received: 26 May 2004 / Accepted: 13 October 2004 / Published online: 10 November 2004

Abstract Epistasis is considered to be a primary genetic basis of hybrid breakdown. We found novel epistatic genes causing hybrid breakdown in an intraspecific cross of cultivated rice (*Oryza sativa* L.). F_2 progeny derived from a cross between a Japonica variety, Asominori, and an Indica variety, IR24, showed segregation of high sterility for seeds, even though the reciprocal F_1 hybrids showed about 60% seed fertility. Backcross populations (BC_3F_2, BC_3F_3), obtained from repeated backcrossing with Asominori, showed the segregation of causal genes in a simple Mendelian fashion. Using these populations, we identified that this sterility was hybrid breakdown caused by interaction among three nuclear genes distributed on the both parental genomes. These new genes, designated as *hsa1*, *hsa2*, and *hsa3*, were found to be involved in female gamete development by histological examination. The Indica parent IR24 has a sterile allele, *hsa1-IR*, which was located at near RFLP marker *G148* on chromosome 12, whereas the Japonica parent Asominori has two sterile alleles, *hsa2-As* on chromosome 8 (close to *G104*) and *hsa3-As* on chromosome 9 (close to *RM285*). Female gametes carrying the *hsa1-IR*, *hsa2-As*, and *hsa3-As* alleles aborted in *hsa1-IR* homozygous plant, leading to seed sterility and selective elimination of the specific allelic combination. This study provides direct evidence that hybrid breakdown is attributed to epistatic interaction of genes from both parents and suggests that complicated mechanisms has been developed for hybrid breakdown during the evolution of rice.

Communicated by Q. Zhang

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Introduction

Hybridization of rice cultivars frequently shows various forms of reproductive barrier, such as hybrid sterility, hybrid breakdown, inviability, certation, and chlorosis. Hybrid sterility occurs in both interspecific and intraspecific crosses of rice. Cultivated rice, *Oryza sativa* L., is grown throughout Asia and other continents and possesses great variation in morphological and physiological characteristics. Since Kato (1930) classified cultivated rice into two major groups, Japonica and Indica, based on intervarietal hybrid sterility, the hybrid sterility in intraspecific crosses has been widely studied.

In rice, most intraspecific hybrid sterility is caused by disharmonious interactions between nuclear genes derived from their respective parents. In some cases, however, sterility is caused by cytoplasmic-nuclear interactions (Shinjyo 1975) or chromosomal aberrations (Henderson et al. 1959). There are two major genetic models for F_1 hybrid sterility: allelic interaction at a single locus (Kitamura 1962; Ikehashi and Araki 1986; Wan et al. 1996) and epistatic interaction between loci (Oka 1974; Tomita 1996). To date, approximately 30 loci responsible for F_1 sterility have been reported in both interspecific and intraspecific crosses of cultivated rice and its wild relatives, and the genetic mechanisms have been well characterized, whereas only a few studies have focused on hybrid breakdown (F_2 sterility, Kitamura 1962; Oka and Doida 1962; Oka 1978; Yokoo 1984). Stebbins (1950) proposed that hybrid breakdown is controlled by complementary genes, and this hypothesis has been supported by the findings of many rice geneticists.

Now that high-density rice linkage maps (Causse et al. 1994; Harushima et al. 1998) are available, we can survey the whole genome of the hybrid progeny. Using the high-density linkage maps, a considerable number of quantitative genetics studies have been conducted, allowing the number and putative locations of genes responsible for hybrid sterility and hybrid breakdown in

Japonica–Indica crosses to be determined (Wu et al. 1995; Li et al. 1997; Wang et al. 1998). These studies indicated that hybrid sterility as well as hybrid breakdown is caused by multiple genes interacting with one another. In *Drosophila*, several studies have reported that complex genic interaction was responsible for hybrid sterility (Davis et al. 1994; Orr and Irving 2001). Although it is apparent that gene interaction plays a role in hybrid sterility and hybrid breakdown, its molecular basis has not been fully understood.

We have previously developed a reciprocal series of chromosome segment substitution lines [(CSSLs) Kubo et al. 2002], derived from a cross between the Japonica variety Asominori and the Indica rice variety IR24. The reciprocal CSSLs produced by backcrossing and marker-assisted selection (MAS) had either a single or a few donor segments contained within an otherwise uniform genetic background from the recurrent parent. This provides an effective approach to clarifying the complex genetics of reproductive barriers.

A better understanding of genetic mechanism for hybrid breakdown will aid in figuring out how genes work in evolution and increasing the availability of distantly related genetic resources in rice breeding. The objective in the present study was to characterize the genetic mechanism of hybrid breakdown found in a cross between Japonica and Indica rice varieties. To dissect genes responsible for hybrid breakdown, we analyzed the CSSL lines and their derivative backcross populations. We showed direct evidence that hybrid breakdown was caused by epistatic interaction at three loci and identified their detailed positions on rice chromosomes. This study has also characterized their respective gene effects and revealed that these genes were responsible for female gamete abortion.

Materials and methods

Plant materials

The Japonica rice variety Asominori and the Indica variety IR24, both of which are fully fertile, were used as the parental varieties. Reciprocal F_1 hybrids were about 60% fertile when grown in paddy field conditions at Fukuoka, Japan. The F_2 generation exhibited a wide variation in sterility, ranging from complete sterility to full fertility, regardless of cytoplasm. The experimental materials used in this study were derivative backcross populations obtained in the process of production of a CSSL series (Kubo et al. 2002). The CSSL series has been produced by repeated backcrossing with Asominori and MAS. This carried one or a few IR24 substitution segments against an Asominori genetic background with an Asominori cytoplasm. For MAS, a total of 268 BC_3F_1 plants obtained from backcrossing with Asominori have been genotyped, using 116 RFLP markers scattered over the rice genome. The 268 populations derived from self-pollinated BC_3F_1 plants were examined for hybrid sterility. BC_3F_3 and additional backcrossed BC_4F_2 populations were used for DNA marker analysis. The RFLP genotypes of BC_3F_1 plants were used to infer the positions of substituted segments. The BC_3F_3 populations, with the exception of population 133-6, were cultivated in 1999. BC_3F_3 population 133-6 and the BC_4F_2 populations were cultivated in 2001. In all experiments, plants uniformly headed by early September.

Measurement of spikelet fertility

Three panicles with fully ripened grains were collected from each individual plant and evaluated for spikelet fertility. Filled and unfilled spikelets were counted. Spikelet fertility is equal to the number of filled grains divided by the total number of filled and unfilled grains. In this study, panicles with less than 40, 40–70, 70–85, and more than 85% fertile spikelets were classified as highly sterile, semi-sterile, partially sterile and fertile, respectively.

DNA extraction and DNA marker analyses

DNA was extracted from frozen leaf samples, using the CTAB method (Murray and Thompson 1980). To perform RFLP analysis, 2 μg isolated DNA was digested with restriction enzymes, separated by 0.8% agarose gel electrophoresis, and blotted onto Hybond-N^+ membranes (Amersham) by capillary transfer, mediated by 0.4 M NaOH. Blotted membranes were rinsed in 2×SSC, dried, and baked at 120°C for 20 min. DNA clones previously mapped by Tsunematsu et al. (1996) and Harushima et al. (1998) were used. DNA labeling, hybridization, and signal detection were conducted using the ECL detection system (Amersham). SSR marker *RM285*, located on chromosome 9 (Temnykh et al. 2000), was also used for gene mapping.

Data analyses

Recombination values were estimated using the maximum likelihood equation (Allard 1956). Obtained values were converted into genetic map distances (in centi-Morgans), using the Kosambi function (Kosambi 1944).

Histological experiments

The pollen and embryo sac of sterile plants were examined using a light microscope. Pre-flowering panicles from highly sterile, semi-sterile plants, and Asominori were collected and fixed in formalin acetic acid alcoholic solution. Fixed samples were stored at 6°C and used for microscopic examination. Pollen grains were stained by 1% acetocarmine solution and compared

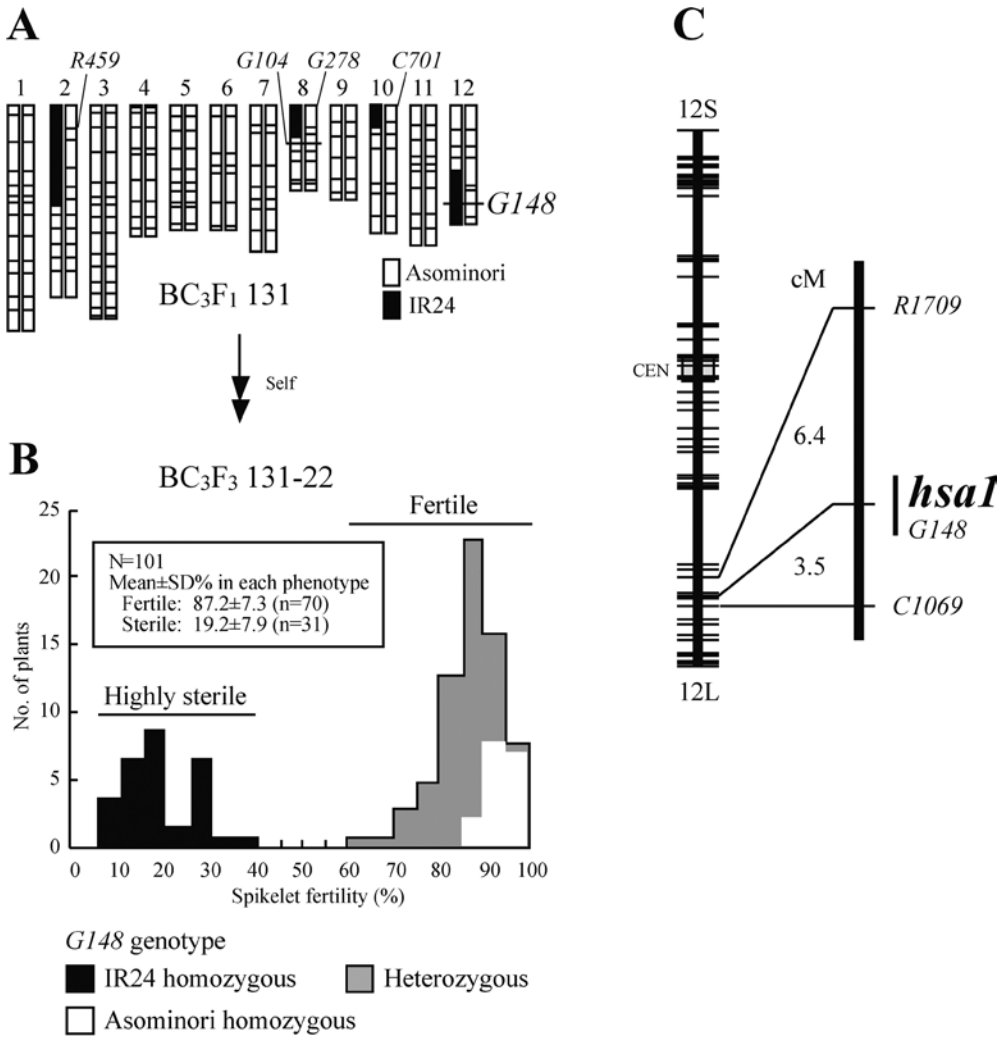

Fig. 1 **a** Graphical genotype of the BC_3F_1-131 plant. The *horizontal lines* on the graphical genotype indicate the locations of the 116 RFLP loci used for whole genome survey in BC_3F_1. RFLP markers used for segregation analysis are shown. The position of RFLP locus *G104* is also shown to indicate that the *hsa1*-interacting locus, *hsa2* (see Fig. 4), was homozygous for Asominori alleles in the BC_3F_1-131 plant. **b** Frequency distribution of spikelet fertility in BC_3F_3-131-22, classified by RFLP genotype at *G148* on chromosome 12. **c** Linkage map showing the location of *hsa1* for hybrid breakdown. *Left* RFLP framework map of chromosome 12, constructed by Harushima et al. (1998). *Right* *hsa1* linkage map constructed from BC_3F_3-131-22

with the stained normal pollen of Asominori. Embryo sacs were embedded in paraplast and sectioned, then stained by 1% Safranin O and 0.5% FastGreen FCF solutions.

Results

Identification of a spikelet sterility gene derived from IR24

Initially, we investigated segregation of spikelet sterility in BC_3F_2 populations developed for production of CSSL series. The genotypes of 268 BC_3F_1 plants have been observed using 116 RFLP markers scattered over the rice genome (Kubo et al. 2002). The positions of the 116 RFLP markers were shown on the graphical genotype (Fig. 1a). Self-pollinated progenies (mainly $n = 20$–50 in each population) of 268 BC_3F_1 plants were examined for spikelet sterility. Thirty-three of 268 BC_3F_2 populations showed segregation of high spikelet sterility (about 10–30% fertility). These highly sterile segregants had erect panicles with empty spikelets and were easily distinguishable from the fertile and semi-sterile segregants in the field. Segregations in BC_3F_3 were also investigated because of the small populations in the BC_3F_2. We found two major types of segregation patterns: monogenic segregation (3:1 for fertile:highly sterile) and digenic segregation (15:1 for fertile:highly sterile) (Table 1). High sterility was fixed in the self-pollinated progenies of the highly sterile plants (Table 1). The inheritance pattern suggested that this spikelet sterility may be characterized as hybrid breakdown and controlled by more than two nuclear genes.

The chromosome location of a causal gene for high sterility was inferred by comparing the retained substitution segments in BC_3F_1. Thirty-one of 33 BC_3F_1 plants commonly carried a substituted segment at the long arm of chromosome 12, suggesting that a causal gene should exist in this region. BC_3F_3 population 131-22 ($n = 101$), a 3:1 segregating population, was analyzed using the RFLP markers located on the substituted segments. The progenitor of BC_3F_3-131-22 (BC_3F_1-131) carried heterozygous segments on chromosomes 2, 8, 10,

Table 1 Segregations for spikelet sterility in BC_3F_2 and BC_3F_3 populations

Population	No. of plants			χ^2 value	
	Fertile	Highly sterile	Total	3:1	15:1
BC_3F_2					
131	42	16	58	0.21(NS)	
133	25	1	26	6.20*	0.25(NS)
136[a]	48	1	49	13.78**	1.42(NS)
BC_3F_3					
131-22 (F)[b]	70	31	101	1.75(NS)	
131-45 (F)	84	23	107	0.70(NS)	
133-6[a] (F)	104	13	117	12.04**	4.72*
136-47[a] (F)	89	8	97	14.52**	0.66(NS)
131-1 (HS)	0	20	20		
131-8 (HS)	0	20	20		
131-19 (HS)	0	20	20		

NS Not significant
Significance levels: $^{*}P < 0.05$, $^{**}P < 0.001$
[a]Semi-sterility and partial sterility also segregated in these populations; these were classified into "fertile" type
[b]The phenotype of BC_3F_3 parent is enclosed in *parentheses*: *F* fertile, *HS* highly sterile

and 12 (Fig. 1a). BC_3F_3-131-22 showed a clear bimodal segregation consisting of 70 fertile plants (63.7–99.2% fertility) and 31 sterile plants (6.1–39.3% fertility) (Fig. 1b). RFLP analysis showed that all highly sterile plants were homozygous for the IR24 allele at *G148* (chromosome 12), whereas fertile plants were heterozygous or homozygous for the Asominori allele. The segregation of sterility had no relation to the other retained segments of chromosomes 2 (RFLP marker *R459* was observed), 8 (*G278*), and 10 (*C701*). These results indicated a tight linkage between the sterility gene and *G148*. Because no gene for hybrid breakdown located around *G148* on chromosome 12 has been reported, the new gene was designated as hybrid sterility-a-1 (*hsa1*). The gene *hsa1* was linked to *R1709* and *C1069* with map distances of 6.4 cM and 3.5 cM, respectively (Fig. 1c). Hereafter, we call the sterile allele from IR24 *hsa1-IR* and the normal allele from Asominori *hsa1-As*.

There was a variation in fertility ranging from 63.7% to 99.2% in the fertile class (Fig. 1b). Mean spikelet fertility of *G148* heterozygous plants (85.1%, $n = 53$) was significantly lower than that of Asominori homozygous plants (93.3%; $n = 17$; *t*-test, $P < 0.0001$). This slight sterility was commonly observed in most segregating populations, suggesting that it might be due to the heterozygous segment around the *hsa1* locus.

hsa1-interacting genes

Because there was no common retained segment except for the *hsa1* region in all the BC_3F_1 plants, it could be presumed that the sterility factor derived from IR24 was only *hsa1-IR*, and the other(s) was (were) derived from Asominori. Two populations, BC_3F_3-136-47 and BC_3F_3-133-6 (Table 1), were used to identify the genes interacting with *hsa1-IR*. The BC_3F_3-136-47 population showed a 15:1 segregation ratio, whereas BC_3F_3-133-6 showed neither 15:1 nor 3:1 segregations (χ^2 for 15:1 = 4.72, $P = 0.03$; χ^2 for 3:1 = 12.04, $P < 0.001$). The BC_3F_1 progenitors of these two populations carried eight independent substitution segments on different chromosomes (Figs. 2a, 3a). RFLP analysis showed that seven of eight highly sterile plants were IR24 homozygotes at *G148* as well as Asominori homozygotes at *G104* in the BC_3F_3-136-47 population (Fig. 2b). Almost all the semi-sterile plants carried IR24 homozygous alleles for *G148* and heterozygous alleles for *G104*, whereas the fertile plants carried either IR24 homozygous alleles for both *G148* and *G104* or other genotypes. This implies that the Asominori allele at a gene linked to *G104* interacted with *hsa1-IR*. In the same manner as *G104*, the tight relationship between *hsa1-IR* and the Asominori allele for *C152* (chromosome 9) was observed in the BC_3F_3-133-6 population (Fig. 3b). No other retained segments correlated with high sterility in the two

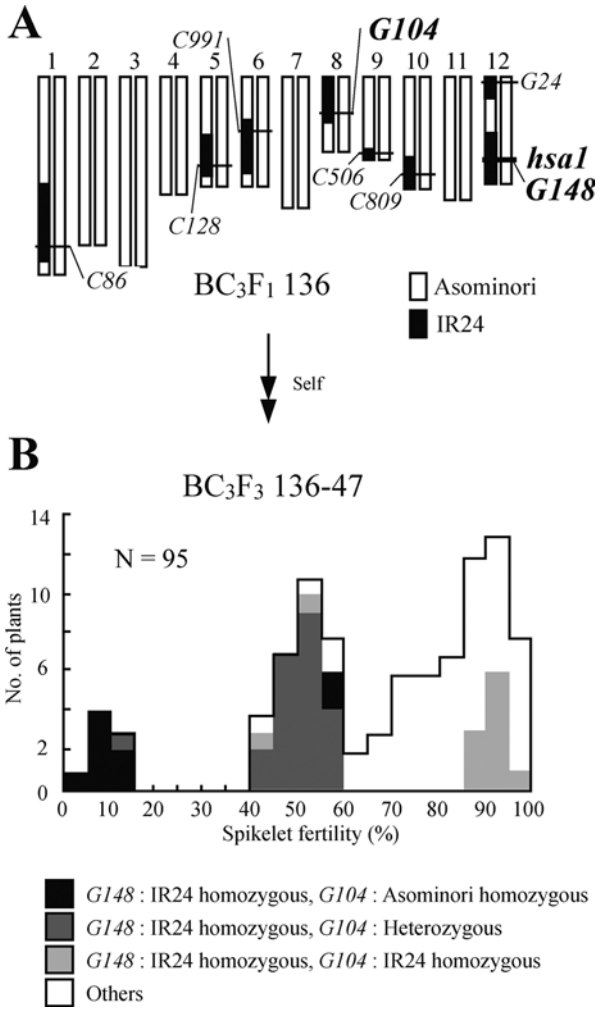

Fig. 2 **a** Graphical genotype of a plant, BC_3F_1-136. The positions of RFLP markers used for segregation analysis are shown. **b** Frequency distribution of spikelet fertility in BC_3F_3-136-47, classified by RFLP genotypes at *G148* and *G104* loci

populations was observed. These results suggested that two interacting loci of *hsa1* (designated as *hsa2* and *hsa3*) were located on chromosome 8 and on chromosome 9, respectively, and that Asominori alleles at these loci interact with *hsa1-IR*. In addition, it was thought that the semi-sterility could be due to heterozygous alleles for *hsa2* and *hsa3* under the *hsa1-IR* homozygous state. The precise positions of the genes were examined using near-isogenic plants for these genes.

Mapping of *hsa2*

A plant, BC_3F_2-15-7, which was a semi-sterile segregant observed in one of the 33 segregating populations, was selected as a near-isogenic plant for the genetic analysis of *hsa2*. The BC_3F_2-15-7 plant carried homozygous alleles for *G148* and heterozygous alleles for *G104* (genotype *hsa1-IR*/*hsa1-IR* *hsa2-As*/*hsa2-IR*) with an otherwise uniform Asominori genetic background. The self-pollinated progeny, BC_3F_3-15-7, clearly segregated into three fertility classes: fertile, semi-sterile, and highly sterile (Fig. 4a). There were 54 fertile, 40 semi-sterile, and five highly sterile plants. At RFLP marker *G104* on chromosome 8, 51 out of 54 fertile plants were homozygous for IR24 alleles, all 39 semi-sterile plants were heterozygous, and four of the five highly sterile plants were homozygous for Asominori alleles. Although the segregation ratio was biased toward IR24 alleles (χ^2 for 1:2:1 = 46.55, $P < 0.001$), the result clearly showed that the interaction between the Asominori allele at *hsa2* on chromosome 8 and the *hsa1-IR* allele caused spikelet sterility. Linkage analysis showed that *hsa*2 was located between RFLP markers *G104* and *C347* with distances of 2.1 cM and 2.6 cM, respectively (Fig. 4b). Apparently, Asominori carried the sterile allele at *hsa2* locus (*hsa2-As*), IR24 carried the normal allele (*hsa2-IR*), and the *hsa1-IR*/*hsa1-IR* *hsa2-As*/*hsa2-As* genotype showed high sterility. In addition, the heterozygote for *hsa2* showed semi-sterility. Self-pollinated progenies (BC_3F_4) of the highly sterile and fertile segregants were fixed with their respective parental phenotypes, whereas those of the semi-sterile plants segregated once again (data not shown).

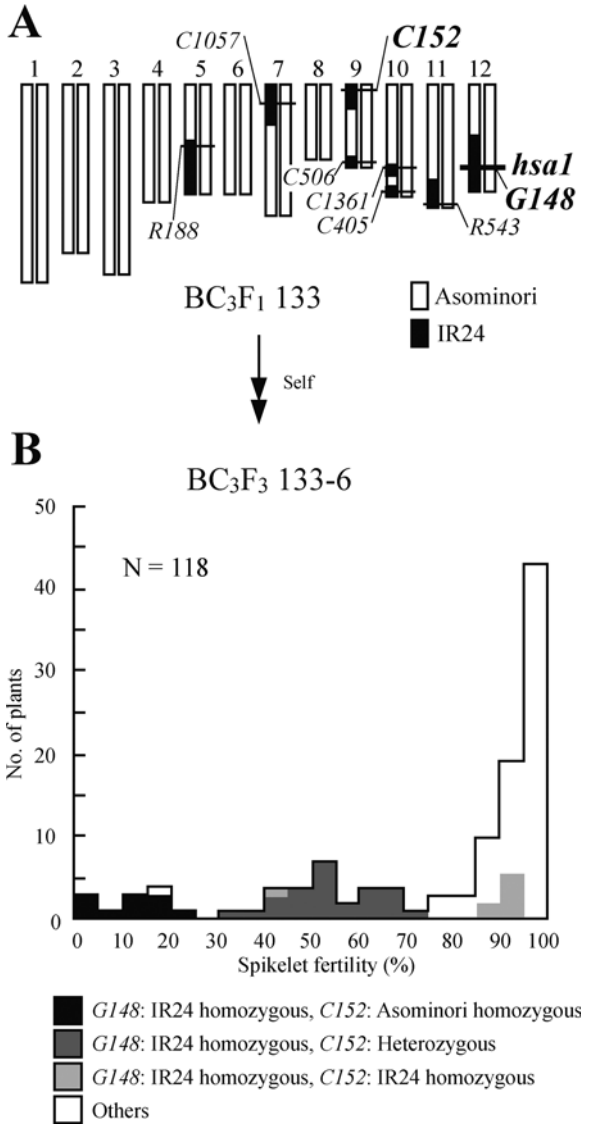

Fig. 3 **a** Graphical genotype of a plant, BC_3F_1-133. The positions of RFLP markers used for segregation analysis are shown. **b** Frequency distribution of spikelet fertility in BC_3F_3-133-6, classified by RFLP genotypes at *G148* and *C152* loci

Mapping of *hsa3*

A BC_4F_2-133-7-4 plant carrying the *hsa1-IR*/*hsa1-IR* *hsa3-As*/*hsa3-IR* genotype was selected using the RFLP markers *G148* and *C152*. The BC_4F_2-133-7 population was a sister line of BC_3F_2-133-6 (both were derived from a single plant, BC_3F_1-133, see Fig. 3a). The self-pollinated progeny of the BC_4F_2-133-7-4 plant segregated into three classes: fertile, semi-sterile, and highly sterile. There were 66 fertile, 55 semi-sterile, and six highly sterile plants (Fig. 5a). Except for a few recombinants, the fertile, semi-sterile, and highly sterile plants were IR24 homozygotes, heterozygotes and Asominori homozygotes for *C152*, respectively. This result confirmed the presence of *hsa3* on chromosome 9 and further indicated that the Asominori allele at *hsa3* locus (*hsa3-As*) interacted with *hsa1-IR*. The *hsa3* locus was located between *RM285* and *R1164* on the linkage map of chromosome 9 (Fig. 5b). Similar to *hsa2*, self-pollinated progenies of the highly sterile and fertile segregants were fixed with their respective parental phenotypes, whereas those of the semi-sterile plants segregated once again (data not shown).

Histological characteristics

The histological manifestation of spikelet sterility was determined by microscopic examination and crossing

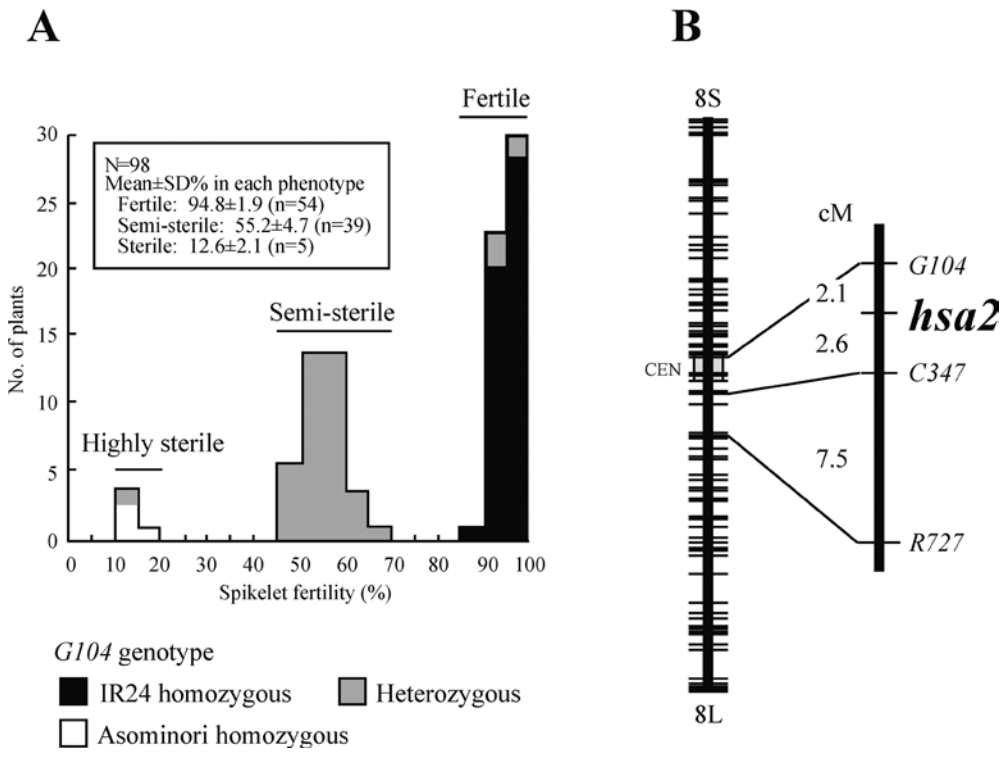

Fig. 4 Mapping of *hsa2* responsible for hybrid breakdown. **a** Frequency distribution of spikelet fertility in the selfed progenies of BC_3F_2-15-7, classified by RFLP genotype at *G104* on chromosome 8. **b** Linkage map showing the location of *hsa2* locus. *Left* RFLP framework map of chromosome 8 constructed by Harushima et al. (1998). *Right* *hsa2* linkage map constructed in this study

experiments. Fixed lines with high sterility (genotype *hsa1-IR*/*hsa1-IR* *hsa2-As*/*hsa2-As* *hsa3-As*/*hsa3-As*) derived from the self-pollination of highly sterile segregants in BC_3F_2-131 and other segregating populations were utilized for microscopic observation and crossing experiments. To characterize the semi-sterility, semi-sterile plants (genotype *hsa1-IR*/*hsa1-IR* *hsa2-As*/*hsa2-IR* *hsa3-As*/*hsa3-As*), which were obtained from the cross between fixed lines with high sterility and fertile lines (genotype *hsa1-IR*/*hsa1-IR* *hsa2-IR*/*hsa2-IR* *hsa3-As*/*hsa3-As*) derived from BC_3F_3-15-7, were also used for microscopic observation. The highly sterile and semi-sterile plants had normal anthers in appearance. No abnormalities were found in the pollen grains of highly

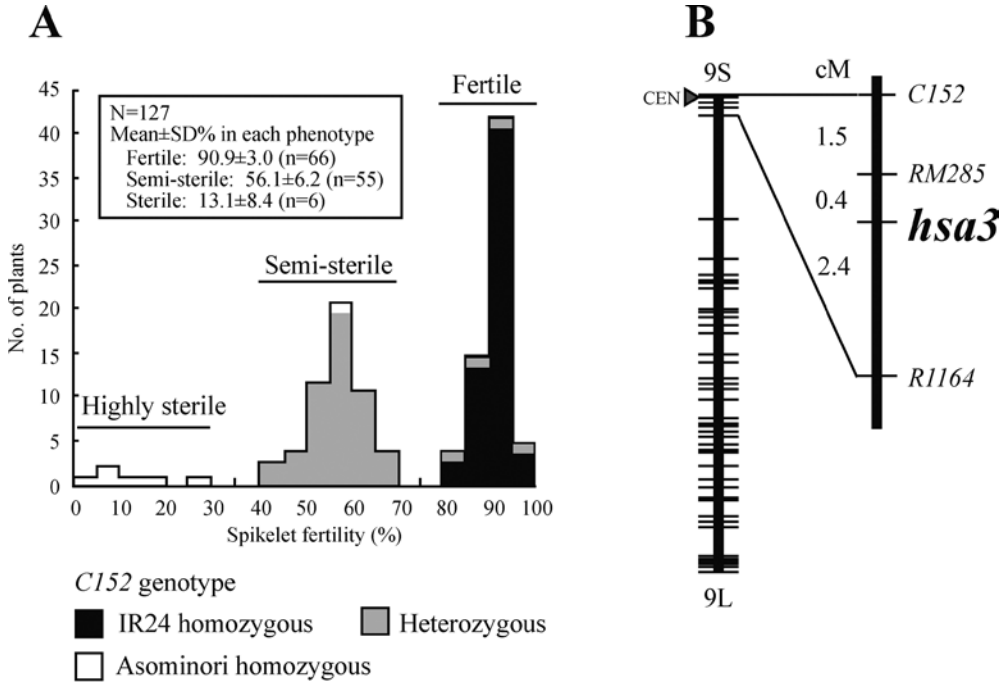

Fig. 5 Mapping of *hsa3* responsible for hybrid breakdown. **a** Frequency distribution of spikelet fertility in the selfed progenies of BC_4F_2-133-7-4, classified by RFLP genotype at *C152* on chromosome 9. **b** Linkage map showing the location of *hsa3* locus. *Left* RFLP framework map of chromosome 9 constructed by Harushima et al. (1998). *Right* *hsa3* linkage map constructed in this study

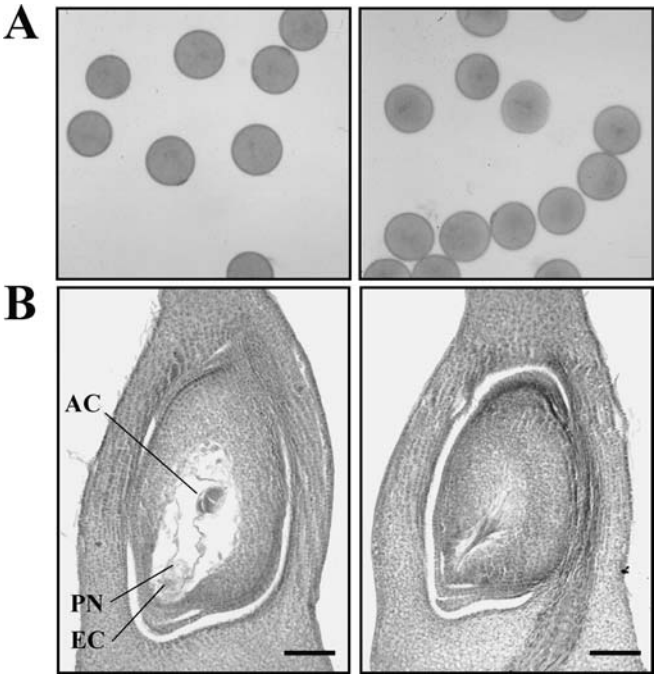

Fig. 6 Photomicrograph of the male and female gametes of Asominori (*left*) and a highly sterile plant (*right*). **a** Pollen grains stained by acetocarmin. **b** Embryo sacs stained by Safranin O and Fast Green FCF solutions. *Scale bars* = 0.1 mm. *AC* antipodal cell, *PN* polar nuclei, *EC* egg cell. Each gamete at mature stage was extracted from pre-flowering spikelets and used for microscopic observation

sterile and semi-sterile plants compared with the normal pollen grains of Asominori (Fig. 6a). Three nuclei were normally formed in the pollen grains from the highly sterile plants. However, the embryo sacs from highly sterile plants were aborted. Reproductive cells were formed and vacuolation took place in normal embryo sacs of Asominori, whereas the highly sterile plants did not show the normal formation of these reproductive cells (Fig. 6b). Furthermore, it was found that only a few embryo sacs developed normally within the panicles of highly sterile plants. On the other hand, approximately half of the embryo sacs within the panicles of semi-sterile plants developed normally, indicating that spikelet sterility is due to abortion of the female gamete.

To examine whether the gametes are functional or not, tests of reciprocal crosses were employed on highly sterile plants and both parents (Table 2). Crossed seeds were obtained at a similar rate with a positive control when the highly sterile plants were used to pollinate Asominori plants, whereas few matured seeds were obtained when the highly sterile plants were pollinated with Asominori pollen. Similar results were obtained in another test using IR24 plants. These observations showed that highly sterile plants produced functional pollen but not functional embryo sacs. This result supported the results of histological examination that the abortion of the female gamete caused high sterility.

Discussion

Genetic analyses using the near-isogenic populations showed that epistatic interaction between the triplet loci (*hsa1*, *hsa2*, and *hsa3*) was responsible for hybrid female sterility. The epistatic interaction degenerates embryo sac formation but not pollen resulting in spikelet sterility. The phenotypes of 27 genotypic combinations provided by three genes are summarized in Table 3. Triple homozygotes for *hsa1-IR*, *hsa2-As*, and *hsa3-As* exhib-

Table 2 Seed-settings in reciprocal crosses between HS plants and their parental varieties

Cross combination Female/male	Self-fertility of female plant (%)	No. of spikelets		Seed setting (%)
		Matured	Crossed	
HS 29-3/Asominori	5.3	1	109	0.9
HS 29-5/Asominori	7.1	0	41	0.0
HS 29-11/Asominori	7.5	3	84	3.6
HS 35-3/Asominori	2.8	2	165	1.2
HS 35-6/Asominori	1.9	2	103	1.9
HS 42-18/Asominori	17.3	22	200	11.0
HS 42-19/Asominori	19.4	9	82	11.0
Asominori/Asominori [a]	87.8	41	71	57.7
Asominori/HS 29-3	–	37	96	38.5
Asominori/HS 29-5	–	53	93	57.0
Asominori/HS 29-11	–	17	55	30.9
Asominori/HS 35-3	–	46	125	36.8
Asominori/HS 35-6	–	43	149	28.9
Asominori/HS 42-18	–	16	52	30.8
Asominori/HS 42-19	–	61	133	45.9
HS 46-2/IR24	9.9	1	46	2.2
HS 48-4/IR24	3.5	1	120	0.8
IR24/IR24 [a]	74.2	7	17	41.2
IR24/HS 46-2	–	8	19	42.1
IR24/HS 48-4	–	56	89	62.9

[a]Positive control

ited high sterility. The gene *hsa1-IR* was considered to be a sporophytic gene, because it segregated in a 3:1 ratio. Consequently, spikelet sterility was observed only in *hsa1-IR* homozygous classes. On the other hand, *hsa2-As* and *hsa3-As* seem to act gametophytically. It was inferred that the semi-sterility and distorted segregation in the mapping populations would result from abortion of the female gamete carrying *hsa2-As* and *hsa3-As* allele. We concluded that female gametes carrying the *hsa1-IR, hsa2-As*, and *hsa3-As* alleles abort under *hsa1-IR* homozygous plants. Although the phenotypes of 12 genotypic classes (underlined in Table 3) could not be observed in this study, we predict that they would be fertile based on their genotypic basis excepting a genotypic class (*hsa1 IR*/*hsa1-IR hsa2-As*/*hsa2-IR hsa3-As*/*hsa3-IR*). Stebbins (1950) earlier proposed that hybrid sterility can be classified into "F_1 sterility" and "F_2 sterility (hybrid breakdown)". In rice, it is known that F_1 sterility is caused by a gametophytic or sporogametophytic gene, whereas F_2 sterility is caused by a sporophytic gene. However, the present results showed that F_2 sterility is controlled by a complex epistatic interaction between sporophytic (*hsa1-IR*) and gametophytic genes (*hsa2-As* and *hsa3-As*). It has never been reported that epistatic interaction between three genes causes hybrid breakdown. A more detailed understanding of the molecular mechanism may offer the opportunity to clarify a part of developmental process of reproduction barrier.

The phenotypic effect of these genes was to reduce spikelet fertility to approximately 10–20%. The degree of spikelet fertility was directly influenced by the proportion of aborted female gametes. Therefore, 10–20% of female gametes with the *hsa1-IR hsa2-As hsa3-As* genotype were viable and functional escaping abortion, indicating an incomplete penetrance of these genes. The mean proportion of semi-sterility due to *hsa2* or *hsa3* heterozygous alleles was approximately 55% (Figs. 4, 5), assuming that 50% were derived from the female gametes with the normal allele, whereas 5% with the sterile allele escaped.

The backcross populations showed clear segregation for spikelet sterility, resulting in the resolution of three major epistatic genes. In the populations segregating for *hsa1*, a minor fertility variation ranging from 61% to 100% was observed. Slight sterility (about 61–85%) was closely related to the heterozygous segment at the region around the *hsa1* locus. It is most likely that *hsa1* or an unidentified sterility locus linked to *hsa1* causes slight sterility in the heterozygous condition. For instance, the F_1 sterility gene *S15* (Wan et al. 1996) and a QTL (Liu et al. 2001) for female gamete abortion have been mapped at that region on the RFLP linkage map in different Japonica–Indica crosses. Because the phenotype of *hsa1* heterozygotes was undetermined, it is shown as fertile or partially sterile in Table 3.

The reciprocal CSSLs series (Kubo et al. 2002) facilitated studying epistatic interactions underlying hybrid breakdown in the previous study (Kubo and Yoshimura 2002) and in this study. The reciprocal CSSLs consisted of an IR24 chromosome substituted series with an Asominori genetic background (called AIS) and an Asominori chromosome substituted series with an IR24 genetic background (called IAS). The AIS populations revealed that the introgression of *hsa1-IR* from IR24 caused high sterility against the Asominori genetic background in this study. The next question is whether the concurrent introgression of *hsa2-As* and *hsa3-As* into IR24 would cause high sterility, which asks how many genes are involved in the hybrid breakdown. If such introgression resulted in high sterility, the genetic basis could be explained by these three genes. Otherwise, we would have to consider the possibility that additional genes, hidden in the Asominori genome, play a role. Our recent experiments using IAS populations showed that highly sterile segregants appeared at the frequency of 3.3% in a population segregating for *hsa2* and *hsa3* with IR24 genetic background (preliminary data). This frequency of the high sterility enhances the possibility that the complete gene set could consist of three genes. This segregating population needs to be genotyped and examined in detail to determine the precise number of the related genes.

Many hybrid sterility and hybrid breakdown genes are supposed to evolve as varieties adapted to environmental variation in cultivated rice. However, few cases of epistatic interaction have been reported because, it is difficult to find epistatic interaction in segregating pop-

Table 3 Summary of spikelet fertility of the 27 genotypes provided by the three sterility loci. The *underlined* phenotypes are expectations based on the genetic basis, because they were not evaluated in this study

Genotype	*hsa1-As*/*hsa1-As*	*hsa1-As*/*hsa1-IR*	*hsa1-IR*/*hsa1-IR*
hsa2-IR/*hsa2-IR*			
hsa3-IR/*hsa3-IR*	Fertile	Fertile	Fertile
hsa3-As/*hsa3-IR*	Fertile	Fertile	Fertile
hsa3-As/*hsa3-As*	Fertile	Fertile	Fertile
hsa2-As/*hsa2-IR*			
hsa3-IR/*hsa3-IR*	Fertile	Fertile	Fertile
hsa3-As/*hsa3-IR*	Fertile	Fertile	Partial sterile
hsa3-As/*hsa3-As*	Fertile	Fertile	Semi-sterile
hsa2-As/*hsa2-As*			
hsa3-IR/*hsa3-IR*	Fertile	Fertile	Fertile
hsa3-As/*hsa3-IR*	Fertile	Fertile	Semi-sterile
hsa3-As/*hsa3-As*	Fertile	F or PS[a]	Highly sterile

[a] *F or PS* Not determined whether fertile or partial sterile in this study

ulations such as F_2 and BC_1F_1, even though QTL analysis have overcome this problem (Wu et al. 1995; Liu et al. 1997; Wang et al. 1998). The previous studies have revealed complementary genes for F_2 sterility, which were examined by classical genetic approach (Kitamura 1962; Oka 1978; Yokoo1984), but the detailed chromosome positions and molecular mechanisms remain unknown. In the present study, we first evaluated F_2 sterility genes as a single Mendelian factor in plants and animals. Thus, the series of chromosome substitution lines is a useful tool in the study of complex gene interactions as well as quantitative genetic studies for other various traits. The DNA markers identified in this study will be useful to increase the efficiency of selection in cross breeding as well as map-based cloning analysis.

Wu et al. (1995) widely investigated the QTLs relating to spikelet sterility in F_2 population in rice. Some QTLs were detected using a Japonica–Indica hybrid. One QTL was located near *hsa1* on chromosome 12, even though the other interacting QTLs were not located near *hsa2* and *hsa3* loci, but instead were on chromosomes 1 and 7. Recently, another study reported QTLs for the defective development of the female gametophyte located in the same region of *hsa1* on chromosome 12 (Liu et al. 2001). Moreover, as stated above, the gene proposed for F_1 female sterility, *S15* (Wan et al. 1996), is located near *hsa1*. We speculate that a gene complex responsible for female gamete development should exist in this region on chromosome 12. In addition, several allelic differentiations at the gene complex and other interacting loci on the different chromosomes would bring about various degrees of female sterility in the different cross combinations. Investigating the distribution and differentiation of alleles at the sterility loci is very important to understand the evolution of rice species. In animals such as *Drosophila* and *Mus*, several hybrid sterility genes have been cloned, and the molecular mechanisms have been studied (Trachtulec et al. 1997; Ting et al. 1998; Barbash et al. 2003). Cloning of the gene set, *hsa1*, *hsa2*, and *hsa3* might lead to a better understanding of the molecular mechanism controlling female gamete development as well as the evolutionary dynamics of reproductive isolation genes in rice.

Acknowledgements This study was partly supported by the Bio-oriented Technology Research Advancement Institution (BRAIN), Japan.

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