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Genetic basis of hybrid breakdown in a Japonica/Indica cross of rice, *Oryza sativa* L.

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Abstract Reproductive barriers often arise in hybrid progeny between two varietal groups of Asian cultivated rice (*Oryza sativa* L.), Japonica and Indica. Hybrid breakdown showing poor growth habit, and complete sterility was found in the backcrossed progeny derived from a cross between a Japonica variety, Asominori, and an Indica variety, IR24. We employed RFLP analysis in the segregating population to study the genetic basis underlying hybrid breakdown. It was found that the hybrid breakdown is caused by a set of two nuclear genes, which were symbolized as *hwe1* and *hwe2*. The parental varieties, Asominori and IR24, carry *hwe1*$^+$*hwe1*$^+$*hwe2hwe2* and *hwe1hwe1hwe2*$^+$*hwe2*$^+$ genotypes, respectively, whereas the progenies that showed a weakness performance carry the double recessive genotype (*hwe1hwe1hwe2hwe2*). Abnormality was not observed in the progenies that carry the other genotypes, indicating that a single dominant allele at either locus is necessary for normal growth. Based on linkage analysis with RFLP markers, the *hwe1* locus was located between RFLP markers *R1869* and *S1437* on chromosome 12 and the *hwe2* locus was located between *R3192* and *C1211* on chromosome 1. The genetic basis was reconfirmed using near-isogenic lines carrying the genes with reciprocal genetic backgrounds. The present study provides clear evidence, viewed by previous workers, that hybrid breakdown is attributed to complementary genes from both parents.

Keywords Reproductive barrier · Rice (*Oryza sativa* L.) · Hybrid breakdown · RFLP (restriction fragment length polymorphisms)

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Introduction

In F_1 hybrids or their progeny between two distantly related taxa, sterile or lethal individuals are frequently observed in animals and plants. These phenomena, which are referred as reproductive barriers, promote species development and maintenance in nature. However, the reproductive barriers generally become the major obstacles in cross breeding, because they prevent gene exchange between varieties belonging to two different groups.

Asian cultivated rice (*Oryza sativa* L.) has numerous varieties that was classified into two distinct varietal groups, Japonica type and Indica type. In F_1 hybrids between these two varietal groups, various reproductive barriers, such as hybrid sterility (Oka 1974; Ikehashi and Araki 1986), hybrid weakness (Oka et al. 1957; Sato and Morishima 1987) and certation (Nakagahra 1972), have been observed. There are several genetic factors that affect F_1 abnormalities. Recently, allelic interaction at a single locus (Kitamura 1962; Ikehashi and Araki 1986; Nakagahra 1972) and inter-locus (non-allelic) interaction at two independent loci (Oka 1974; Sato and Morishima 1987) are two major hypotheses as a model of genic disharmony. Many F_1 sterility genes have been identified, and some of them have already been localized on the rice genetic map (Yanagihara et al. 1995).

Hybrid breakdown is a difficult problem to solve because of its complex mode of inheritance. Stebbins (1950) proposed that hybrid breakdown is due to disharmonious interaction between a combination of the genes of parental species. This hypothesis was held in the hybrid breakdown of rice (Oka 1957; Oka and Doida 1962; Sato and Morishima 1988; Fukuoka et al. 1998a). However, the map locations of the genes for hybrid breakdown have not been determined, except the one reported by Fukuoka et al. (1998a). The map of hybrid breakdown genes gives us valuable information for gene cloning, for understanding the mechanism at the molecular level, and for overcoming the problem in cross breeding.

In our previous report (Kubo et al. 1999), we developed a series of chromosome segment substitution lines (CSSLs) between a Japonica variety and an Indica variety of rice, which aims to precisely map the genes controlling various quantitative traits. In the process of development of the CSSLs, hybrid breakdown was found in the BC_3F_2 populations. In this paper, we describe the genetic basis of the hybrid breakdown and linkage maps of the causal genes.

Materials and methods

Plant materials

A Japonica rice variety, Asominori, and an Indica variety, IR24, were used as the parents in the original crossing. The F_1 hybrids exhibited a vigorous plant type and about 60% seed-setting in the paddy field condition. The recombinant inbred (RI) lines derived from a cross between Asominori and IR24 (Tsunematsu et al. 1996), were crossed and successively backcrossed with Asominori. Two hundred and sixty eight BC_3F_1 plants were genotyped using 116 RFLP markers distributed evenly on the rice genome in order to select the candidate plants for a series of chromosome segment substitution lines (CSSLs) (Kubo et al. 1999). The RFLP genotypes of BC_3F_1 in the previous study were used as reference data in this study. The BC_3F_2 and BC_3F_3 populations were employed for genic analysis. BC_1F_3 populations developed from the cross of Asominori/IR24//Asominori were also used. Another series of the CSSL carrying an Asominori segment with a IR24 genetic background (BC_2F_4 generation) (Aida et al. 1997) was used to confirm the genetic basis of hybrid weakness.

DNA extraction and RFLP analysis

DNA was extracted from frozen leaf samples using the CTAB method (Murray and Thompson 1980). The isolated DNA (2.0 μg) was digested with restriction enzymes (*Bgl*II, *Dra*I, *Eco*RV, *Hin*dIII and *Kpn*I), separated by 0.8% agarose-gel electrophoresis and blotted onto Hybond N^+ membranes (Amersham) by capillary transfer on a 0.4 N NaOH solution. The 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. (1998a), were used. DNA labeling, hybridization and signal detection was conducted using the ECL detection system (Amersham).

Data analysis

Recombination values were estimated with the maximum-likelihood equation (Allard 1956). Obtained values were converted into map distances (cM) using the Kosambi function (Kosambi 1944).

Results

Characteristics of hybrid breakdown

In the process of production of a series of CSSLs, segregations of weak plants were observed in some BC_3F_2 populations. The weak plants were characterized by a small number of tillers, a short culm and panicle, a pale green leaf and absence of seed setting under natural field condition. More leaves were formed when the weak plants were grown in the glasshouse than in the field, but the matured seeds were never obtained. The pollen was completely sterile. Moreover, the number of pollen per anther from the weak plants was notably less than that of normal plants. Reciprocal crosses between the weak plant and Asominori did not give any matured cross seed. These results suggested that the weak plant had no reproducibility. Because the parents (BC_3F_1 plants) of the segregating populations showed normal growth habitats, this phenomenon was proposed to be a specifically hybrid breakdown. This hybrid breakdown was found in both F_2 populations derived from the reciprocal crosses between Asominori and IR24, suggesting that it is not due to a cytoplasmic effect.

Mapping of the hybrid breakdown gene, *hwe1*

The segregation of the weak plants in BC_3F_2 and BC_3F_3 is shown on Table 1. The frequencies of weak plants were markedly lower (0.7–13.3%) than the expected Mendelian proportion (25.0%). To find the causes for hybrid breakdown, RFLP analysis was conducted in the segregating populations. RFLP genotypes at 116 loci in the BC_3F_1 showed that 15 of 16 BC_3F_1 plants producing the weak segregants possessed a heterozygous substitut-

Table 1 Segregation for hybrid breakdown in the BC_3F_2 and BC_3F_3 populations

Population		No. of plants			Percentage of weak plants	χ^2 (3:1)	χ^2 (15:1)
		Normal	Weak	Total			
BC_3F_2	8	52	8	60	13.3	4.36*	5.14*
	55	34	4	38	10.5	4.25*	1.19^{ns}
	117	36	1	37	2.7	9.81**	0.79^{ns}
	122	52	5	57	8.8	8.01**	0.62^{ns}
	211	58	2	60	3.3	15.02***	0.87^{ns}
	262	54	6	60	10.0	7.20**	1.44^{ns}
BC_3F_3	117-1	138	13	151	8.6	21.64***	1.43^{ns}
	117-3	149	1	150	0.7	47.37***	7.98**
	211-10	112	4	116	3.4	28.74***	1.55^{ns}
	262-12	142	8	150	5.3	30.94***	0.22^{ns}

*, ** and *** Represents the significance levels of $P < 0.05$, 0.01 and 0.001

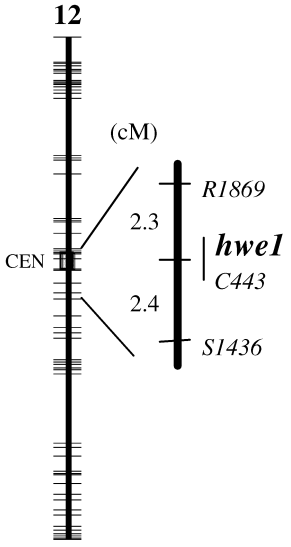

Fig. 1 Linkage map showing the location of the *hwe1* locus for hybrid breakdown. Left: RFLP framework map of chromosome 12 quoted from Harushima et al. (1998a); right: *hwe1* map constructed from the BC_3F_3 population (n = 150) in this study

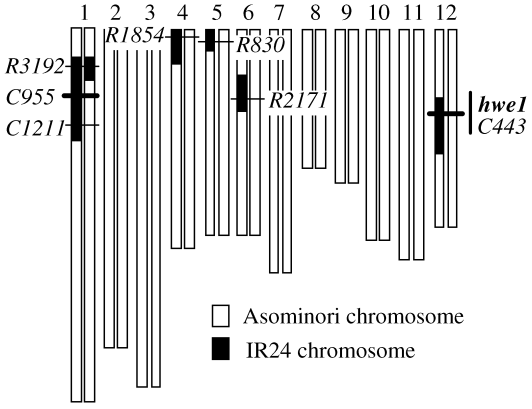

Fig. 2 Graphical genotype of plant, BC_3F_2 117-3, carrying heterozygous alleles at *C443* on chromosome 12 as well as at *C955* on chromosome 1. The positions of RFLP markers used for segregation analysis in the selfed progeny are shown

ed segment at the region between RFLP markers *R1869* and *C751* on chromosome 12. It was expected that a single recessive gene on this substituted segment should be responsible for the hybrid breakdown, although the weak plants appeared at lower frequency in the segregating populations. RFLP analysis in a BC_3F_3 population (BC_3F_3 262-12, n = 150) showed that all the weak plants carried IR24 homozygous alleles for RFLP marker *C443* on chromosome 12, whereas the normal plants carried heterozygous or Asominori homozygous alleles. It was clear that a recessive gene from IR24 caused hybrid breakdown and the gene tightly linked to *C443* on chromosome 12. Even though four complementary gene sets, '*Hwa*', '*hwb*', '*Hwc*' and '*hwd*', have been already reported (Oka 1957; Amemiya and Akemine 1963; Fukuoka et al. 1998a), there was no reported gene for hybrid weakness on chromosome 12. Therefore, this novel gene was designated as *hwe1* (hybrid weakness-e-1). Linkage analysis revealed that the *hwe1* locus was located between *R1869* and *S1436* with a distance of 2.3 cM and 2.4 cM, respectively, on chromosome 12 (Fig. 1). The donor parent IR24 carries the recessive *hwe1* allele, whereas the recurrent parent, Asominori, carries the dominant *hwe1*$^+$ allele. The recessive homozygous alleles, *hwe1hwe1*, caused hybrid breakdown.

Significant segregation distortion was observed at RFLP markers around the *hwe1* locus. The mapping population (BC_3F_3 262-12) segregated into 77 Asominori homozygous plants, 65 heterozygous plants and 8 IR24 homozygous plants at RFLP marker *C443*, which showed the most significant distortion (χ^2 test for 1:2:1, $P < 0.0001$). This segregation distortion around the *hwe1* locus brought about the reduced frequency of the weak plants.

Identification of the complementary factor, *hwe2*

The BC_3F_2 117 and BC_3F_3 117-3 distinctly exhibited a low frequency of weak plants (2.7 and 0.7%) (Table 1). The plant, BC_3F_2 117-3, carried a heterozygous segment on each of chromosomes 1, 4, 5 and 6 as well as the *hwe1* region of chromosome 12 (Fig. 2). In the progeny of BC_3F_2 117-3, there were 36 plants carrying IR24 homozygous alleles at *C443*, which could cause hybrid weakness (Table 2). Among them, actually, only a single plant showed weakness. The remaining 35 plants were normal. RFLP analysis revealed that this weak plant carried the IR24 homozygous alleles at *C443* and Asominori homozygous alleles at *C955* on chromosome 1. The other substituted segments on chromosomes 4, 5 and 6 showed no relationship with the segregation of the weak plants. This result suggested that the hybrid breakdown was due to the interaction between the gene linked to *C955* on the Asominori genome and the *hwe1* gene from IR24. The second gene on the Asominori genome was designated as *hwe2* (hybrid weakness-e-2). For further analysis, the segregation of weakness was examined in the selfed progenies of the plants carrying IR24 homozygous alleles for *C443* and heterozygous alleles for *C955* (i.e. the genotype *hwe1hwe1hwe2*$^+$*hwe2*), which was selected from BC_3F_3 and BC_1F_2 populations. The BC_1F_2 was developed by crossing Asominori/IR24//Asominori. The segregation ratios of the weak plants were markedly skewed from a theoretical 3:1 ratio in all the populations (Table 3). RFLP analysis using 123 individuals from BC_1F_3 1-8 (n = 220) showed that the weak plants were homozygous for Asominori alleles at *C955* and the normal plants had heterozygous or homozygous alleles for IR24. It was concluded that *hwe2* interacting with *hwe1* was located on the short arm of chromosome 1. The *hwe2* locus was mapped between *R3192* and *C1211* with a distance of 18.0 and 11.2 cM, respectively. There was no recombinant found between the *hwe2* locus and *C955* (Fig. 3).

Table 2 Segregation of hybrid breakdown and RFLP markers *C443* and *C955* in BC_3F_3 117-3 (See Fig. 2). The numbers in parenthesis are the number of segregants

Marker	Genotype[a]	*C955* (Chr. 1)			
		AA	AI	II	Total
C443 (Chr.12) *hwe1*	AA	Normal (5)	Normal (19)	Normal (10)	34
	AI	Normal (18)	Normal (40)	Normal (22)	80
	II	Weak (1)	Normal (19)	Normal (16)	36
	Total	24	78	48	150

[a] AA: Homozygous for Asominori; AI: Heterozygous; II: Homozygous for IR24

Table 3 Segration for hybrid breakdown in the selfed progenies (BC_3F_4, BC_1F_3) of the plants carrying *hwe1* homozygous and *hwe2* heterozygous alleles

Population		No. of plants			Percentage of weak plants	χ^2 (3:1)
		Normal	Weak	Total		
BC_3F_4	117-3-20	282	18	300	6.0	57.76***
	117-3-46	311	19	330	5.8	65.17***
BC_1F_3	1-8	202	18	220	8.2	33.19***
	1-15	3195	11	206	5.3	42.47***

*** Represents the significance level of $P < 0.001$

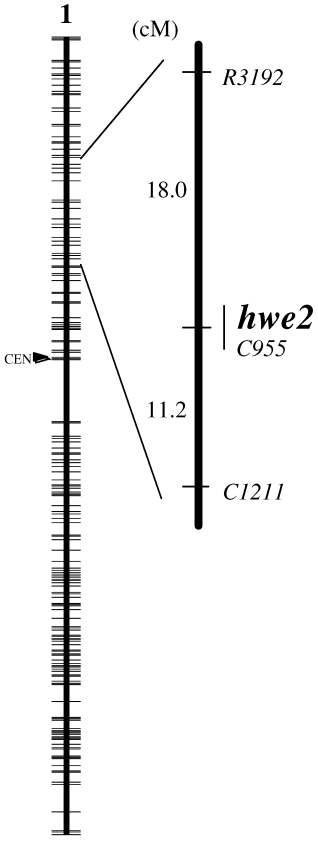

Fig. 3 Linkage map showing the location of the *hwe2* locus for hybrid breakdown. Left: RFLP framework map of chromosome 1 quoted from Harushima et al. (1998a); right: *hwe2* map constructed from the BC_1F_3 population (Asominori/IR24//Asominori) (n = 123) in this study

The RFLP marker *C955* linked to the *hwe2* locus showed the most remarkable segregation distortion (χ^2 test for 1:2:1, P = 0.0001). There were 13 Asominori homozygous plants showing the weakness, with 64 heterozygous plants and 46 IR24 homozygous plants. This implies the reduction of the Asominori allele around the *hwe2* locus.

Although the segregation distortions were observed at both genes, it was clearly demonstrated that the double recessive genotype *hwe1hwe1hwe2hwe2* caused hybrid breakdown. The other eight genotypes obtained through the combination of two alleles at *hwe1* and *hwe2* showed a normal plant type. The genotype of the parental varieties, Asominori and IR24, could be expressed as *hwe1*$^+$*hwe1*$^+$*hwe2hwe2* and *hwe1hwe1hwe2*$^+$*hwe2*$^+$, respectively.

Further evidence for the genetic basis

A reciprocal set of the chromosome segment substitution series between Asominori and IR24 has been developed using backcrossing and marker-assisted selection (MAS) in our previous studies (Aida et al. 1997; Kubo et al. 1999). The hybrid breakdown, which was found in the backcrossed progeny with the Asominori genetic background, could be explained by the set of two genes, *hwe1* and *hwe2*. The hybrid breakdown was observed also in the backcross progeny (BC_2F_4) with an IR24 genetic background. The parental plants of the weak segregants had heterozygous segments at the region including the *hwe2* locus on chromosome 1. It was confirmed that the hybrid breakdown observed in the population with an IR24 genetic background was due to *hwe2* by linkage analysis using RFLP marker *C955*. This conclusion was consistent with the finding that the two genes, *hwe1* and *hwe2* cause hybrid breakdown.

Discussion

Hybrid breakdown was found in the progeny derived from the cross between the Japonica and Indica varieties. Owing to complete sterility, the weak plants were unable to produce seeds for the next generation. The hybrid breakdown was caused by a set of two genes, *hwe1* derived from IR24 and *hwe2* derived from Asominori. Additional evidence for the genetic basis of duplicate recessive genes was obtained through the introgression analysis of *hwe2* in IR24. It is generally accepted that hybrid sterility should be due to complex epistatic interaction at multiple loci (Li et al. 1997a; Wang et al. 1998). On the other hand, a simple genetic basis has been assumed in many cases of hybrid weakness, such as two complementary genes in wheat (Caldwell and Compton 1943), in barley (Wiebe 1934) and in rice (Oka 1957; Fukuoka et al. 1998a; Sato and Morishima 1988). Our results based on DNA markers provides certain evidence for the hypothesis that hybrid breakdown is due to the complementary genes (Stebbins 1950; Oka 1957).

Geographic distribution of F_1-weakness genes (Sato and Morishima 1987), F_2-chlorosis genes (Sato and Morishima 1988) and hybrid-breakdown genes (Fukuoka et al. 1998b) have been surveyed in rice varieties. Investigating the allelic distribution in *O. sativa* and its wild relatives would allow a better understanding of the process of rice speciation, and serve as a useful criterion for the selection of a parental variety in a crossing program. In the previous study, it was concluded that those hybrid weakness genes would not affect rice varietal differentiation because of the specificity of the gene distribution. Despite interest in evolutionary genetics and usefulness in crossbreeding, it would be difficult to investigate *hwe1* and *hwe2* genes because of the following reasons. First, weak plants appear unstable at low frequency in a segregating population, which results in misjudgment of whether the tested variety is a gene carrier or not. Second, we cannot use the double recessive homozygous (weak) plant for crossing because of its reproductive defect.

Interestingly, distorted segregations were consistently observed at both *hwe1* and *hwe2* loci in all the populations used in this study. RFLP markers linked with *hwe1* and *hwe2* also revealed distorted segregation. For instance, at *C443* tightly linked with the *hwe1* locus, the mapping population segregated into 77 Asominori homozygous plants, 65 heterozygous plants and 8 IR24 homozygous plants. This reduced frequency of heterozygous plants implies that the distortion was due to the preferential transmission of gametes with the Asominori allele in self-pollination of the heterozygous plants, rather than lethality of weak plants at the seedling stage. The *hwe2* locus is located near the gametophyte gene *ga9* on chromosome 1 (Maekawa and Kita 1985), but there is no gene affecting gametic selection around the *hwe1* locus on chromosome12. It should be noted that the normal segregation at *C443* (linked to *hwe1*) and the lightened distortion at *C955* (linking to *hwe2*) was observed in the selfed progeny of double heterozygous plants (Table 2). We inferred that the distortion was possibly due to non-allelic interaction between *hwe 1* and *hwe2* as a pleiotropic effect. However, there is no other data supporting this inference. Non-allelic interactions causing distorted segregation have been detected based on RFLP loci in F_2 populations between the Japonica variety, and the Nipponbare and Indica variety, Kasalath (Harushima et al. 1998b). If our inference is correct, then *hwe* genes bring about a drastic elimination of the Japonica-Indica recombinants in the sporophytic phase, as well as the gametophytic phase, in hybrid progeny.

The rice blast resistance gene (Nakamura et al. 1997) and the fertility restoring gene for cytoplasmic male sterility (Zhang et al. 1997) are located near the *hwe1* and *hwe2* loci, respectively. Recently, genes causing hybrid sterility and hybrid breakdown have been mapped by the method of QTL analysis (Wu et al. 1995; Li et al. 1997a). The QTLs for reproductive barriers were mapped at the same region with QTLs for grain yield (Li et al. 1997b). The tendency of linkage between the deleterious genes and the useful genes brings out the serious problem, termed linkage drag, in rice breeding. Marker-assisted selection based on the linkage map enables us to effectively produce the introgressive hybrids free from deleterious genes in the early generation.

Finally, it is also interesting to characterize the hybrid breakdown genes at the molecular level. In mammals, e.g. in mouse; the cloning of hybrid sterility genes has been attempted and several candidate genes have been obtained (Trachtulec et al. 1997). Very little is known about the hybrid breakdown except for the fact that they are complementary genes. The genetic map information in the present study will be valuable for cloning of the duplicate genes and resloving the entire mechanism.

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