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ORIGINAL PAPER

Identification and linkage mapping of complementary recessive genes causing hybrid breakdown in an intraspecific rice cross

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Abstract One outcome of hybrid breakdown is poor growth, which we observed as a reduction in the number of panicles per plant and in culm length in an F_2 population derived from a cross between the genetically divergent rice (*Oryza sativa* L.) cultivars 'Sasanishiki' (*japonica*) and 'Habataki' (*indica*). Quantitative trait locus (QTL) analysis of the two traits and two-way ANOVA of the detected QTLs suggested that the poor growth was due mainly to an epistatic interaction between genes at QTLs located on chromosomes 2 and 11. The poor growth was likely to result when a plant was homozygous for the 'Habataki' allele at the QTL on chromosome 2 and homozygous for the 'Sasanishiki' allele at the QTL on chromosome 11. The results suggest that the poor growth found in the F_2 population was due to hybrid breakdown of a set of complementary genes. To test this hypothesis and determine the precise chromosomal location of the genes causing the hybrid breakdown, we performed genetic analyses using a chromosome segment substitution line, in which a part of chromosome 2 from 'Habataki' was substituted into the genetic background of 'Sasanishiki'. The segregation patterns of poor growth in plants suggested that both of the genes underlying the hybrid breakdown were recessive. The gene on chromosome 2, designated *hybrid breakdown 2* (*hbd2*), was mapped between simple sequence repeat markers RM3515 and RM3730. The gene on chromosome 11, *hbd3*, was mapped between RM5824 and RM1341.

Keywords *Oryza sativa* · Poor growth · Epistatic interaction · Linkage mapping · QTL mapping

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Introduction

Hybrid breakdown is defined as inviability or poor growth and sterility found in F_2 or later or backcross progeny from a cross between genetically divergent parents (Dobzhansky 1970), and it serves as a reproductive isolating barrier. This phenomenon is often observed in intraspecific crosses between *Oryza sativa* ssp. *japonica* and ssp. *indica*, which are the primary rice subspecies cultivated in Asia (Oka 1988; Fukuoka et al. 1998, 2005; Kubo and Yoshimura 2002, 2005; Matsubara et al. 2007). A set of complementary genes, which produce an effect significantly distinct from the others as a result of epistatic interaction between alleles, is always involved in intrinsic isolating barriers, such as the hybrid breakdown (Oka 1957; Sato et al. 1984; Fukuoka et al. 1998, 2005; Kubo and Yoshimura 2002, 2005) and F_1 inviability (Amemiya and Akemine 1963; Ichitani et al. 2001; Matsubara et al. 2003). The contribution of such complementary genes to reproductive isolation has been discussed in relation to divergence among populations (Dobzhansky 1970; Lynch and Force 2000; Coyne and Orr 2004).

Following the development of DNA markers in rice, QTL analyses have been performed to detect those genes

underlying hybrid breakdown in segregating populations such as F_2 or recombinant inbred lines (Wu et al. 1995; Li et al. 1997). Based on QTL analysis alone, however, it is difficult to elucidate the precise genetic basis for such hybrid breakdown, owing to the segregation of genetic factors other than the responsible genes. To address this problem, near-isogenic lines or chromosome segment substitution lines (CSSLs), in which each line has a particular chromosomal segment from the donor parent substituting for the corresponding segment in the host parent, can be used for detailed analysis. Recently, CSSLs have been developed in rice (Kubo et al. 2002; Ebitani et al. 2005; Rice Genome Resource Center URL: http://www.rgrc.dna.affrc.go.jp/index.html.en). The use of such genetic materials should facilitate our understanding of the effects on phenotype, the mode of inheritance, and the chromosomal location of each gene underlying a particular case of hybrid breakdown.

During a breeding program to improve seed production through hybridization of the rice cultivars 'Sasanishiki' (*japonica*) and 'Habataki' (*indica*), both of which have desirable traits for seed production (Kobayashi et al. 1990; Nagata et al. 2002; Ashikari et al. 2005), poor-growth segregants were observed in the F_2 population. In this study, we performed genetic analyses to better understand the poor growth habit in 'Sasanishiki' × 'Habataki' progeny. We first conducted QTL analysis to determine roughly how many genes and which chromosomal regions were involved in the hybrid breakdown. We then performed more detailed analyses, including characterization of the poor growth in plants and linkage mapping, using a CSSL in which a single chromosome segment from 'Habataki' was substituted for the corresponding segment in the genetic background of 'Sasanishiki'. We report the mode of inheritance and the chromosomal location of the complementary genes underlying the hybrid breakdown found in this intraspecific cross.

Materials and methods

Plant materials

All plant materials were cultivated and observed in an experimental paddy field at the National Institute of Agrobiological Sciences, Tsukuba, Japan. Seeds were sown in mid-April, and 1-month-old seedlings were then transplanted into the paddy field. The rice cultivars 'Sasanishiki' and 'Habataki', their progeny, and the CSSL SL-407 were used in this study.

'Sasanishiki' is a *japonica* cultivar that produces a large number of panicles per plant. 'Habataki' is an *indica* cultivar that is characterized by a semi-dwarf habit and a larger number of seeds per panicle than 'Sasanishiki' (Kobayashi et al. 1990; Nagata et al. 2002; Ashikari et al. 2005). The F_2 population ($n = 355$) used for QTL analysis was obtained by self-pollination of F_1 plants from a cross between 'Habataki' and 'Sasanishiki'. SL-407 was a CSSL in which a part of chromosome 2 of 'Habataki' was substituted into the genetic background of 'Sasanishiki' through successive backcrossing and marker-assisted selection (based on data from Rice Genome Resource Center). For a further genetic analysis, SL-407 was crossed with both 'Sasanishiki' and 'Habataki', and the resultant F_1 progeny were self-pollinated to obtain F_2 populations. These F_2 populations were used for a linkage mapping of each target QTLs as a single Mendelian factor.

Phenotypic evaluation

We chose the traits panicle number per plant (PN) and culm length (CL) in the primary F_2 plants ('Habataki' × 'Sasanishiki') as phenotypic characters diagnostic of poor growth. We categorized two phenotypic classes, poor-growth and normal types, which differed mainly in PN, based on visual observation in the secondary F_2 populations.

DNA marker analysis

Genomic DNA was extracted from 2-month-old seedlings by the cethyltrimethylammonium bromide method according to the techniques described by Murray and Thompson (1980) with slight modification. The entire genome of F_2 plants ('Habataki' × 'Sasanishiki') was genotyped based on 146 DNA markers, which consisted of 143 simple sequence repeat (SSR; McCouch et al. 2002) and three indel (insertion/deletion) markers. To add makers in the large intervals between markers, we developed three new indel markers using a polymorphism database published by Shen et al. (2004). Primer sequences of the three indel markers were as follows: 5′-GTGCTGCTTGTGCTGATGAT-3′ and 5′-GCTGCTGCTCATCTCGATCT-3′ for Ba35I24a, 5′-TGCATCGTCTAGCTACAAAACA-3′ and 5′-TAAGGGTCCCATAGCAAACG-3′ for Bb38P21a, and 5′-GGGGCAAAGAAGAGGAATTT-3′ and 5′-TTCCTCCTCTCCGATCCTCT-3′ for Bb77A02.

For PCR amplification, we used a 5-μl reaction volume containing 1 μl template DNA (20 ng μl^{-1}), 0.5 μl 10× PCR buffer, 25 mM $MgCl_2$, 2 mM each dNTP, 0.05 μl *Taq* DNA polymerase (5 U μl^{-1}), 0.1 μl of a 20 pM solution of each primer, and 2.55 μl H_2O. Amplification was performed for 35 cycles (1 min at 94°C, 1 min at 55°C, and 1 min at 72°C), followed by 7 min at 72°C. Amplified products were electrophoresed in 3% agarose gel to detect polymorphisms.

Map construction and QTL analysis

A linkage map of 146 SSR and indel markers was made using 355 F_2 plants from the cross ‘Habataki’ × ‘Sasanishiki’. Linkage order and genetic distances of marker loci were calculated using MAPMAKER/Exp 3.0 (Lander et al. 1987). The total length of the map was 1587.8 cM, with an average interval of 10.9 cM between adjacent markers (Fig. S1).

QTL analyses were performed using composite interval mapping (CIM; Zeng 1993, 1994) as implemented by the program Zmapqtl (model 6) of the software package QTL Cartographer version 2.5 (http://www.statgen.ncsu.edu/qtl-cart/WQTLCart.htm; see also Basten et al. 2005). CIM was run with a 10-cM window and five background cofactors. Tests were performed at 2-cM intervals, and cofactors were selected via forward–backward stepwise regression using the program SRmapqtl. Genome-wide threshold values ($\alpha = 0.05$) for declaring the presence of QTLs were estimated from 1000 permutations (Churchill and Doerge 1994; Doerge and Churchill 1996). One-LOD confidence intervals for the positions of QTLs were defined based on the CIM results. To evaluate whether epistatic interactions between detected QTLs were involved in the hybrid breakdown, two-way ANOVAs were performed. Significance levels were corrected by the Bonferroni method for multiple testing on the basis of the number of interaction tests (Sokal and Rohlf 1995).

Bulked segregant analysis

To detect the genetic factors controlling poor growth, we used a bulked segregant analysis in the F_2 population (SL-407 × ‘Habataki’) according to the method described by Lynch and Walsh (1998). We identified the poor-growth segregants based on visual observation. The total DNA of 22 F_2 plants showing poor growth was mixed to make one bulk sample. To map a causal gene, the bulk samples of DNA were screened using DNA markers.

Results

Identification of QTLs for poor growth in the F_2 population

In the primary F_2 population derived from ‘Habataki’ × ‘Sasanishiki’, we observed extremely poor growth in some plants, which we considered to be due to hybrid breakdown. However, the two diagnostic traits PN and CL showed normal distributions and continuous variation in the F_2 plants (Fig. 1a, b). Thus, it was difficult to reliably classify poor growth in some F_2 segregants. Therefore, we used QTL analysis for the two traits to diagnose the poor growth based on 146 DNA markers. Three QTLs for PN were detected on chromosomes 2, 3, and 11, and 6 for CL were detected on chromosomes 1, 2, 5, 8, 9, and 11. Of the nine detected QTLs, two on chromosomes 2 and 11 were shared and the range of the one-LOD support confidence interval, suggesting that they were identical loci and the two loci may be involved in the poor growth habit observed in the F_2 population (Table 1; Fig. S1).

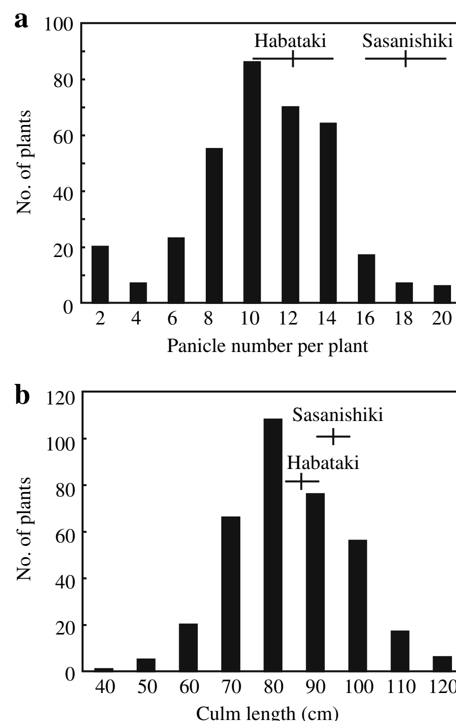

Fig. 1 Frequency distribution of panicle number per plant (**a**) and culm length (**b**) in the F_2 population from a cross between ‘Habataki’ and ‘Sasanishiki’. *Vertical* and *horizontal bars* represent the mean value and ±1 SD of the parents, respectively

To detect epistatic interactions among the all QTLs identified, two-way ANOVA was conducted on the genotype classes at the pair of QTLs. The results clearly demonstrated a significant epistatic interaction only between QTLs on chromosomes 2 and 11 for both traits ($P < 0.001$ for PN; $0.001 < P < 0.01$ for CL). To examine the effect of the allele combination of the QTLs, we compared phenotypic values for all genotype classes for the QTLs on chromosomes 2 and 11 (Fig. 2a, b). It was obvious that marked reductions of PL and CL were observed when an F_2 plant was homozygous for the ‘Habataki’ allele at the QTL on chromosome 2 and homozygous for the ‘Sasanishiki’ allele at the QTL on chromosome 11 (allele combination of *H/H S/S* in the middle of Fig. 2a,b). This observation supports the existence of epistasis between the QTLs resulting in the poor growth habit. According to the definition of Dobzhansky (1970), the poor-growth phenotype and the genetic feature observed in the F_2 progeny from this cross between

Table 1 QTLs for panicle number per plant and culm length detected in an F_2 population of 'Habataki' × 'Sasanishiki'

Trait	Chromosome	Position of QTL (cM)	Nearest marker	LOD score	*a*	*d*	R^2
Panicle number	2	104.7	RM3515	16.2	2.0	1.3	18.3
	3	120.2	RM6736	3.7	1.0	−0.8	3.8
	11	62.3	RM5824	11.9	−1.8	1.3	12.7
Culm length	1	164.6	RM1068	27.1	9.9	2.9	25.1
	2	114.8	RM1367	4.4	3.0	2.6	3.6
	5	140.2	RM7452	8.4	−5.7	8.4	7.9
	8	41.0	RM1148	4.2	3.5	3.7	4.5
	9	60.9	RM6235	6.6	4.6	8.1	5.8
	11	68.2	RM1355	4.7	−2.5	4.6	4.2

a is the additive effect; the sign of the additive effect corresponds to the direction of the effect of the 'Sasanishiki' allele. *d* is the dominance effect. R^2 is the proportion of variance explained by the QTL. The significance threshold for an experiment-wide error rate of $\alpha = 0.05$ was <3.5 for panicle number per plant and <3.7 for culm length

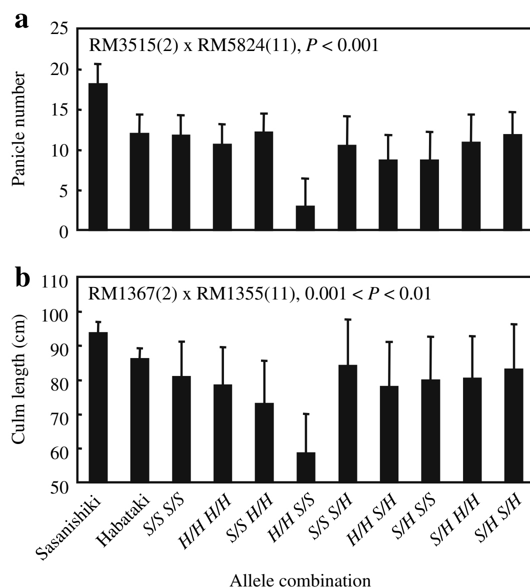

Fig. 2 Panicle number per plant (**a**) and culm length (**b**) of nine genotype classes of the QTLs on chromosomes 2 and 11 in the F_2 population. *S/S S/S* indicates the genotype of the nearest marker loci on chromosomes 2 and 11, respectively. *S* and *H* indicate the alleles from 'Sasanishiki' and 'Habataki' at the nearest marker locus, respectively. *S/S* and *H/H* indicate homozygous alleles and *S/H* indicates heterozygous alleles at the marker locus. *Error bars* represent 1 SD

genetically divergent cultivars could be classified as hybrid breakdown.

A CSSL showing the hybrid breakdown

The results of QTL analysis and two-way ANOVA suggested that the hybrid breakdown was due mainly to an epistatic interaction or a complementary effect of the two QTLs on chromosomes 2 and 11, although we could not completely rule out an additive contribution of the other detected QTLs. Therefore, we focused on the two QTLs with the complementary effect as the main factors causing the hybrid breakdown. To examine more precisely the genetic basis and the chromosomal location of the genes underlying the hybrid breakdown, we performed genetic analyses using a CSSL. In CSSL SL-407, a part of chromosome 2 from 'Habataki' was substituted into the genetic background of 'Sasanishiki'. The genotype of SL-407 was surveyed based on 166 DNA markers (163 SSRs and three indel markers) covering the whole genome (data from the Rice Genome Resource Center). The length of the chromosomal segment from 'Habataki' introduced into 'Sasanishiki' was beyond the two-LOD confidence interval of QTLs detected for PN (98.3–110.7 cM) and CL (100.7–130.2 cM; Fig. 3). Therefore, SL-407 was expected to show a poor-growth phenotype. Indeed, SL-407 showed a reduced panicle number and a shorter culm length (Fig. 4a). In addition, SL-407 exhibited a decreased growth rate during the seedling stage, with chlorosis in the young leaf. During the ripening stage, the phenotype of SL-407 was characterized by delayed heading and a reduced panicle length, followed by lower seed production than those of the parents (Fig. 4b).

Inheritance mode and linkage mapping of a causal gene on chromosome 2

To examine the mode of inheritance for the gene responsible for the hybrid breakdown, we scored the growth habit of 150 F_2 plants derived from a cross between SL-407 and 'Sasanishiki'. A total of 118 plants were scored as normal, and 32 showed poor growth. The segregation ratio of normal to poor-growth phenotypes fit the Mendelian ratio of 3:1 ($\chi^2 = 1.08$, ns), suggesting that the poor growth was

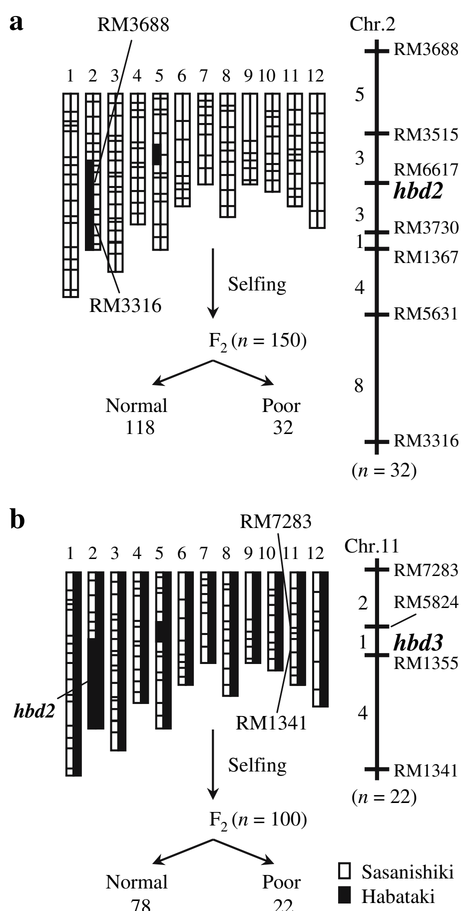

Fig. 3 The chromosomal locations of the genes *hbd2* (**a**) and *hbd3* (**b**) underlying the hybrid breakdown. Genotypes of F_1 plants of SL-407 × 'Sasanishiki' (**a**) and F_1 plants of SL-407 × 'Habataki' (**b**). Markers are indicated to the right of each chromosome, and the number of recombinants is shown on the *left*

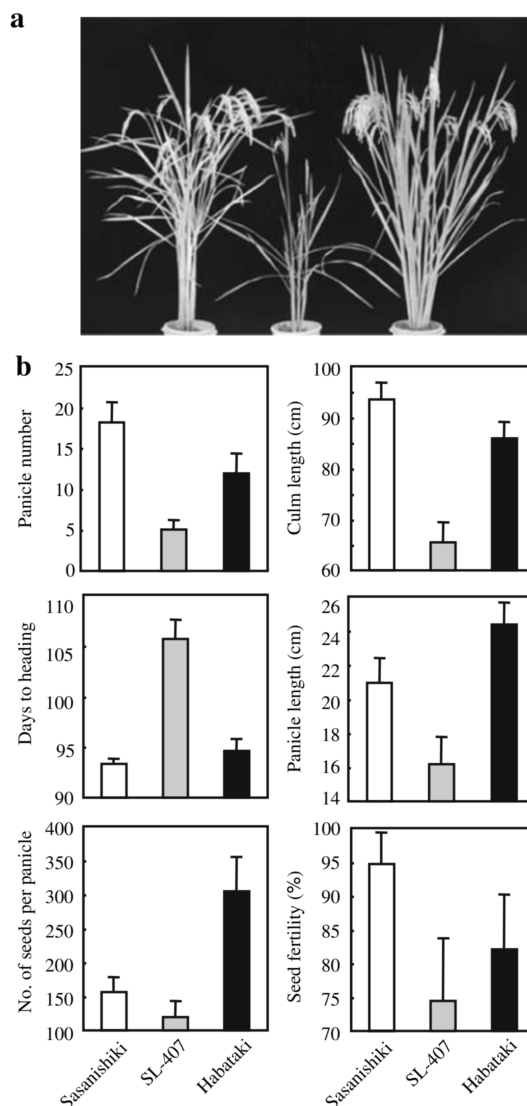

Fig. 4 Poor growth habit observed in advanced backcross progeny of 'Sasanishiki' × 'Habataki'. **a** Plant stature of 'Sasanishiki' (*left*), SL-407 (*middle*), and 'Habataki' (*right*). **b** Comparison of morphological characteristics between SL-407 (*gray bars*) and the parents (*open bars*, 'Sasanishiki'; *black bars*, 'Habataki'). *Error bars* indicate 1 SD

controlled by a single recessive gene (Fig. 3a). The recessive gene was tentatively designated as *hbd2* (*hybrid breakdown 2*).

To determine the precise chromosomal location of the gene for the poor growth, we selected 32 plants that were homozygous recessive at *hbd2*. Based on a linkage analysis of these plants using SSR markers, we were able to map the *hbd2* in the interval between RM3515 and RM3730 on the long arm of chromosome 2, and no recombinant was observed between *hbd2* and RM6617 (Fig. 3a).

Linkage mapping of the gene interacting with *hbd2*

The results of QTL analysis and two-way ANOVA suggested that a pair of alleles from 'Sasanishiki' on chromosome 11 was involved in the hybrid breakdown. To test this hypothesis, we produced an F_2 population from a cross between SL-407 and 'Habataki'. If the gene on chromosome 11 were involved in the hybrid breakdown, then the normal phenotype and the poor-growth phenotype should segregate at the ratio of 3:1, because all F_2 plants were homozygous for the 'Habataki' *hbd2* allele. In this analysis, the two classes of growth were distinguished on the basis of PN, the feature controlled by genes from the SL-407 parent. In the F_2 population of SL-407 × 'Habataki', 78 plants were scored as normal and 22 as showing poor growth. The segregation ratio of normal to poor-growth phenotypes fit the expected ratio of 3:1 ($\chi^2 = 0.48$, ns), indicating that the poor growth was controlled by another single recessive gene (Fig. 3b). The recessive gene was tentatively designated as *hbd3* (*hybrid breakdown 3*).

To map the *hbd3* gene, a bulked segregant analysis was carried out. DNA was extracted from each plant showing poor growth and then mixed into one bulk sample. The results obtained in the QTL analysis and ANOVA (Table 1; Fig. 2) suggested that the *hbd3* locus was on chromosome 11, so the bulk DNA sample was screened using 11 DNA makers on that chromosome. The analysis clearly indicated that the causal gene was located around RM1355 on chromosome 11, because the bulk DNA sample was homozygous for the 'Sasanishiki' allele at the RM1355 locus. To determine the precise chromosomal location of *hbd3*, for each plant that made up the bulk sample, we determined the genotype of four DNA markers that were expected to be linked with *hbd3*. The resulting map showed that *hbd3* was in the interval between RM7283 and RM1341 on the long arm of chromosome 11, and no recombinant was observed between *hbd3* and RM1355 (Fig. 3b).

Discussion

Breeders use hybridization to combine desirable phenotypic characters from two or more cultivars or species into a single cultivar. Sometimes genetic recombination results in the production of new and desirable characters not found in either parent. When hybridization occurs between genetically divergent parents, however, the disruption of coadapted gene combinations in a parent can manifest as a reproductive isolation barrier (Dobzhansky 1936; Muller 1942; Orr 1996). The view that such isolating barriers are caused by a set of complementary genes, known as a Dobzhansky–Muller incompatibility, has been frequently observed in both plants and animals (Coyne and Orr 2004; Moyle and Graham 2005).

Hybrid breakdown, which is caused by the disruption of a set of complementary genes, can be exhibited as poor growth in F_2 or backcrossed progeny. Numerous studies have reported hybrid breakdown in hybridization between distantly related rice cultivars (Oka 1957; Fukuoka et al. 1998, 2005; Kubo and Yoshimura 2002; Matsubara et al. 2007). In this study, we identified another case of hybrid breakdown that was exhibited as poor growth and was controlled by a set of complementary genes. In this case, the genotype of 'Sasanishiki' was *Hbd2/Hbd2 hbd3/hbd3*, whereas that of 'Habataki' was *hbd2/hbd2 Hbd3/Hbd3*. No plants with poor growth were observed in the F_1 population (*Hbd2/hbd2 Hbd3/hbd3*) owing to the recessive inheritance mode of these genes (data not shown). Therefore, the expected segregation ratio of the normal to poor-growth phenotype in the F_2 population would be 15:1. In this regard, the genetic basis of this hybrid breakdown was different from that of the three cases reported previously (Oka 1957; Fukuoka et al. 1998, 2005). In those cases, the complementary effect was not affected by a dominant allele at one locus, so the segregation ratio of the normal to poor-growth phenotype was 11:5 in the F_2 of the intraspecific crosses. Regardless of these differences, however, all of these cases are consistent with a Dobzhansky–Muller incompatibility caused by a set of complementary genes.

In the present study, one and five QTLs with no sign of epistasis were detected for PN and CL, respectively. Although their contribution to the hybrid breakdown might be subtle, it would have been difficult to determine the boundary between the normal and poor-growth phenotypes in the primary F_2 segregating population if we had used only a single trait in scoring. After QTL analyses for PN, however, we recognized that this trait was a good indicator for distinguishing between the poor-growth and normal phenotypes, because only one additional QTL (on chromosome 3) with relatively small effect was involved in the regulation of PN (Table 1). Thus, we were able to distinguish the poor-growth phenotype in the secondary F_2 populations derived from a cross between SL-407 × 'Habataki'.

Panicle number is an important component of yield in rice, and QTLs for PN have been detected in many studies (Lin et al. 1996; Zhuang et al. 1997; Xiao et al. 1998; Liao et al. 2001; Luo et al. 2001; Brondani et al. 2002; Hittalmani et al. 2002; Obara et al. 2004; Xu et al. 2004; Tian et al. 2006; Yoon et al. 2006; You et al. 2006). Among these, a QTLs PN have been mapped in approximately the same chromosomal regions as *hbd2* (on chromosome 2; Obara et al. 2004) and *hbd3* (on chromosome 11; Liao et al. 2001). Based on comparison of the map positions, it was difficult to elucidate the allelic relationship between *hbd2* and *hbd3* and these QTLs. Thus, cloning studies will be necessary to clarify these relationships.

We also observed slightly lower mean PN values in genotype classes *HH/SH* and *SH/SS* than in class *SS/HH*, although the differences were not statistically significant (Fig. 2). As noted above, PN is a good criterion for hybrid breakdown, so this result may suggest a dose-dependent effect of the *hbd2* and *hbd3* alleles. Because we performed multiple comparisons only for PN among the possible genotype classes in the primary F_2 population, however, it is not possible to draw firm conclusions about the dose dependence of these loci. Thus, it will be necessary to develop near-isogenic lines and lines combining each allele in these loci in order to more reliably investigate the potential dose effects.

Several sets of complementary recessive genes underlying hybrid breakdown (manifested as poor growth) have been mapped to particular chromosomal regions using DNA markers in *japonica* × *indica* cultivars (chromosomes 7 and 10, Fukuoka et al. 1998; chromosomes 1 and 12, Kubo and Yoshimura 2002; chromosomes 6 and 11, Fukuoka et al. 2005; chromosome 2, Matsubara et al. 2007).

Although the 'Sasanishiki' (*japonica*) cultivar was used as a common parent in three distinct intraspecific crosses, different genomic regions were involved in hybrid breakdown depending on the lineage of *indica* cultivars used as the other parent (Fukuoka et al. 1998, 2005; this study). These findings suggest that many genomic regions may cause hybrid breakdown in crosses between *japonica* and *indica* cultivars.

Hybrid breakdown caused by a complementary effect of different chromosomal regions would be a serious problem for breeding via hybridization. If a favorable allele were linked closely to a deleterious allele, the deleterious allele would then segregate along with the favorable allele (Lynch and Walsh 1998). If a deleterious allele, such *hbd2* in 'Habataki', were linked closely to an allele favorable in rice breeding, the favorable allele could not be transferred to the progeny or may be eliminated due to breeders selecting out those plants with the deleterious phenotype (linkage drag). Therefore, to avoid such problems in future breeding programs, it is important to recognize those cultivars that carry such deleterious alleles and to determine the precise chromosomal location of the genes.

Recently, several genes at QTLs have been cloned using a map-based strategy (Yano et al. 2000; Ashikari et al. 2005; Ren et al. 2005; Konishi et al. 2006). In this study, the chromosomal locations of *hbd2* and *hbd3* were coarsely determined, which will allow us to perform molecular cloning of *hbd2* and *hbd3* using this map-based strategy. Elucidation of the structure and function of those genes involved in hybrid breakdown will lead us to a better understanding of the molecular nature of hybrid breakdown in rice.

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