Theor Appl Genet (2008) 116:1117–1127
DOI 10.1007/s00122-008-0740-4

ORIGINAL PAPER

Fine mapping and candidate gene analysis of *hwh1* and *hwh2*, a set of complementary genes controlling hybrid breakdown in rice

Wenzhu Jiang · Sang-Ho Chu · Rihua Piao · Joong-Hyoun Chin · Yong-Mei Jin · Joohyun Lee · Yongli Qiao · Longzhi Han · Zongze Piao · Hee-Jong Koh

Received: 29 December 2007 / Accepted: 25 February 2008 / Published online: 12 March 2008

Abstract Hybrid breakdown (HB), a phenomenon of reduced viability or fertility accompanied with retarded growth in hybrid progenies, often arises in the offspring of intersubspecific hybrids between *indica* and *japonica* in rice. We detected HB plants in F_8 recombinant inbred lines derived from the cross between an *indica* variety, Milyang 23, and a *japonica* variety, Tong 88-7. HB plants showed retarded growth, with fewer tillers and spikelets. Genetic analysis revealed that HB was controlled by the complementary action of two recessive genes, *hwh1* and *hwh2*, originating from each of both parents, which were fine-mapped on the short arm of chromosome 2 and on the near centromere region of the long arm of chromosome 11, respectively. A comparison of the sequences of candidate genes among both parents and HB plants revealed that *hwh1* encoded a putative glucose-methanol-choline oxidoreductase with one amino acid change compared to *Hwh1* and that *hwh2* probably encoded a putative hexose transporter with a six amino acid insertion compared to *Hwh2*. Investigation of the distribution of these alleles among 54 *japonica* and *indica* cultivars using candidate gene-based markers suggested that the two loci might be involved in developing reproductive barriers between two subspecies.

Keywords Rice · Hybrid breakdown · Fine mapping · Complementary gene interaction

Communicated by Q. Zhang.

W. Jiang · S.-H. Chu · R. Piao · J. Lee · Y. Qiao · H.-J. Koh (✉)
Department of Plant Science and Research Institute of Agriculture and Life Sciences, Seoul National University,
Seoul 151-921, South Korea
e-mail: heejkoh@snu.ac.kr

J.-H. Chin
Plant Breeding, Genetics and Biotechnology,
International Rice Research Institute, Los Baños, Laguna,
DAPO BOX 7777, Manila, Philippines

Y.-M. Jin
School of Agricultural Biotechnology and Center for Agricultural Biomaterials, Seoul National University, Seoul 151-921,
South Korea

L. Han
Crop Science Research Institute,
Chinese Academy of Agricultural Sciences,
100081 Beijing, China

Z. Piao
Crop Research Institute,
Shanghai Academy of Agricultural Sciences,
201106 Shanghai, China

Introduction

Hybrid breakdown (HB) is a reproductive barrier observed only in the F_2 or later generations of interspecific or intersubspecific crosses (Stebbins 1950). In rice, HB causes reduced tiller numbers, retarded growth with short culms and panicles, chlorosis or necrosis of leaves, poor seed setting, and retarded root growth. This phenomenon can be regarded as the opposite of hybrid vigor. HB is believed to be controlled by the interaction of genes from both the parents. Since the first report of this phenomenon in rice (Oka 1957), genetic analyses have indicated that HB is mostly controlled by the complementary interaction of unlinked loci *Hwa1* and *Hwa2* (Oka 1957), *hwd1* (chr. 10) and *hwd2* (chr. 7) (Fukuoka et al. 1998), *hwe1* (chr. 12) and *hwe2* (chr. 1) (Kubo and Yoshimura 2002), *hwg1* (chr. 6) and *hwg2* (chr. 11) (Fukuoka et al. 2005), and *hbd2* (chr. 2) and *hbd3* (chr. 11) (Matsubara et al. 2007; Yamamoto et al.

2007). Epistatic interactions have also been suggested as a model to explain this phenomenon (Kubo and Yoshimura 2005; Li et al. 1997). HB is one of the postzygotic reproductive barriers that occur after mating or fertilization and lead to hybrid inviability or sterility (Coyne 1992). Such postzygotic reproductive barriers have important roles in speciation, along with prezygotic reproductive barriers that prevent mating or fertilization (Futuyma 1986). The mechanisms of postzygotic reproductive barriers have been studied through hybrid inviability/sterility phenomena in many organisms, such as *Drosophila* (Muller and Pontecorve 1940), mice (Oka et al. 2004), and plants (Oka 1957; Christie and MacNair 1984; Bomblies et al. 2007). The study of postzygotic reproductive barriers is important not only for the breeding programs that seek to develop new varieties with useful genes from other interspecies or intersubspecies, but also for providing evolutionary clues in species speciation due in part to restricted gene flow across diverged populations and accelerated genetic differentiation accomplished by postzygotic reproductive barriers (Bomblies and Weigel 2007).

The complementary gene model for the occurrence of hybrid inviability (e.g., hybrid necrosis, hybrid weakness, and HB), known as the Bateson–Dobzhansky–Muller (BDM) model, was first suggested by Bateson (1909), followed by Dobzhansky (1936) and Muller (Muller 1942; Muller and Pontecorve 1940). This model could explain the hybrid incompatibilities phenomenon with negative interactions between different regions of the nuclear genome as well as interactions between one or more regions of the nuclear genome and some component of the cytoplasm such as the chloroplast or mitochondrial genome (Burke and Arnold 2001). However, this model does not explain why the neutral (or beneficial) mutations within a given lineage would produce strongly disadvantageous viabilities when combined in a hybrid background (Burke and Arnold 2001). Lynch and Force (2000) proposed another model for the mechanism of hybrid inviability with the complete loss of functional genes of a duplicated pair, in which recombinant hybrid progeny have both defective genes and would be nonviable, whereas their parents could be normal with one copy each of the normal and defective gene. Only one set of BDM genes causing hybrid lethality has been completely cloned in *Drosophila* (Brideau et al. 2006). The result demonstrated that *lethal hybrid rescue* (*Lhr*) from functionally diverged *Drosophila simulans* suppressed *Drosophila melanogaster hybrid male rescue* (*Hmr*) duplication, causing lethality in F_1 hybrid males. In *Arabidopsis*, autoimmune response has been suggested as an example of the BDM model, where a continuous autoimmune response is activated to cause leaf necrosis by gene interaction between the *NB-LRR* disease resistance gene from one parent and an unknown gene from the other parent (Bomblies et al. 2007).

We have identified HB plants in a recombinant inbred population derived from a cross between an *indica* variety, Milyang 23, and a *japonica* variety, Tong 88-7. In the present study, we conducted genetic analysis and map-based cloning to sequence two candidate genes for hybrid breakdown, and developed gene-specific DNA markers for the candidate genes using sequence differences between normal and HB plants.

Materials and methods

Plant materials

The two HB lines, RIL-132 and RIL-166, were previously detected from recombinant inbred lines (RILs) composed of a set of 166 F_8 lines derived by single-seed decent (SSD) method from a cross between an *indica* variety, Milyang 23 (a Korean *tongil-type* rice, derived from an *indica* × *japonica* cross and similar to *indica* in its genetic make-up), and a temperate *japonica* variety, Tong 88-7 (originating from the Jilin Province of China). For the genetic analysis and fine mapping, we used an F_2 population from the cross between Milyang 23 and Tong 88-7, backcrossed F_2 populations (BC_1F_2 and BC_3F_2) developed by backcrossing RIL-166 to both parents, and the BC_3F_3 populations.

Phenotypic analysis

Agronomic traits including days to heading (DTH), culm length (CL), panicle length (PL), panicle number (PN), spikelet number (SN), and spikelet fertility (SF) were evaluated in the parental varieties, RIL-132, and RIL-166. To categorize the backcrossed F_2 plants (which generally did not show clear distinct agronomic traits), we used the PN as the key character for distinguishing HB lines from normal lines. All backcrossed F_2 individuals were progressed to produce backcrossed F_3 lines to confirm their phenotype based on the segregation pattern in the F_3 lines.

DNA analysis

DNA was extracted from the leaves of each individual from mapping populations and their parents according to the methods of Causse et al. (1994). PCR was performed in a reaction volume of 20 µl containing 40 ng of template DNA, 0.2 µM of each primer, 200 µM of each dNTP, 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, and 0.5 U of *Taq* DNA polymerase. Amplification was carried out in a PTC100 96U Thermocycler (MJ Research, USA) using the following conditions: 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, and a final extension of 5 min at 72°C. PCR products were separated in 3.0% agarose gels.

BSA and linkage analysis

Twelve normal plants and 12 HB plants were selected from two primary BC_1F_2 populations, respectively, and DNA was extracted from individual plants to perform bulked segregant analysis (BSA) (Michelmore et al. 1991). Equal amounts of DNA from each of 12 individual samples were combined into a single bulked sample for genotyping. After BSA, the two hybrid breakdown genes were determined, and fine mapping was conducted with neighboring STS and SSR markers. The STS markers were developed by designing primers based on the differences in DNA sequences between *indica* and *japonica* rice (Chin et al. 2007), which are available at http://www.ncbi.nlm.nih.gov/ (for *indica*) and at http://www.rgp.dna.affrc.go.jp/ (for *japonica*). Primer sequences and the amplified length of developed DNA markers used in this study are listed in Table 1. Linkage analysis was conducted using MAPMAKER version 3.0 software (Lander et al. 1987). Map distances were estimated by the Kosambi function (Kosambi 1944).

Sequencing of candidate genes

The full-length genomic DNA sequence of candidate genes was divided into several segments to design specific PCR primers. These primers were used to amplify genomic DNA from Milyang 23, Tong 88-7, and RIL-166. PCR products were purified using a PCR purification kit (Bioneer, Korea) for TA cloning. Purified PCR product was introduced into the pGEM-T Easy Vector (Promega, USA), and transformed into *E.coli* strain DH5α. The recombinant plasmid was sequenced with an ABI Prism 3730 XL DNA Analyzer (PE Applied Biosystems, USA). Sequence alignment was performed with the BLAST network service (National Center for Biotechnology Information, NCBI) and ClustalW (European Bioinformatics Institute, EBI).

dCAPS marker analysis

The primers that began two base mismatches in the 5′ direction from the SNP of interest were designed using the dCAPS Finder 2.0 program (http://helix.wustl.edu/dcaps/dcaps.html). The forward primer was 24–30 nucleotides in length, with the reverse primer located up to 150–200 bp away in the 3′ end. The PCR products were digested with appropriate restriction enzymes and analyzed on horizontal 3% agarose gels.

Distribution of *hwh* alleles in *indica* and *japonica* varieties

To investigate the distribution of *hwh* alleles at two HB loci, genotyping was performed in 54 varieties, including 21 *indica* and 33 *japonica* varieties, using each *hwh* gene-specific DNA marker developed in this study. The name and origin of 54 varieties were presented in Table 5.

Results

Morphological characterization

The two HB lines, RIL-132 and RIL-166, could be easily distinguished from normal plants and their parents by their

Table 1 PCR-based molecular markers used in fine mapping of hybrid breakdown genes

Marker	Chr.	Marker type	Size (bp)	Forward primer (5′–3′)	Reverse primer (5′–3′)
S0298A	2	STS	198	TTCCCAACTTTTTCTTCAAA	GCCTAGTATTAGGATGGGACA
S0298B	2	STS	202	GGGAAGCACATCTGAATCTA	TCTCCTTTTCTCCCTCTTCT
RM13608	2	SSR	95	CAGCACGCAACGCAAATCAGC	CAGTCACCTCAACACGCACACG
S0299N	2	dCAPS/ *Bts*I	189	TGGCCGTGGATTGGGAACATTCAGCAG	AGCTGCCCATTATCATCTGAA
S0299J	2	STS	191	TAGTAGCTCACTGCTCAACG	ACGGTGTTTGCTTGCTTG
S0299A	2	STS	176	CCAAAACTCTGAAGAAATGG	ACCACCCTTGCATTAGTTTA
S0299G	2	STS	248	GAACGAACAGACAATGATTAGTT	CTCCGATTAATATGAGAATTT
S0299B	2	STS	187	TGCCAACCCAGATATGTAAT	GGTAGTGTGTGTGTGAGGTG
S1158A	11	STS	192	CGTATATAGCTGCGTGAACA	AAAATCGAGCAAGCTATGAA
S1159A	11	STS	158	AGGTTCAGGCCTTCTTATTC	TCCGTTAGGGATATGAGCTA
S1159B	11	STS	202	GATCGGGAACATTCATAAAA	CTTGCATCTACGGTTTTGAC
S1159C	11	STS	189	TGGTTCTTGAGCGAATAAAT	GGTTTCCACCAAGAACATAG
RM26694	11	SSR	109	GCGGCGCAAATCTTCGATCC	TCAGATCGCTCCCACCATACTCG
S1159E	11	STS	161	GGCAATTCTCTCATCTCTTG	TTGAGCTAAGCCTTCTGAAT
RM26697	11	SSR	253	CAGCGACTAACCGAACCCACTCC	ACCAGAGCGGGCTGTTGAGC
S1164A	11	STS	180	AACTGGCAAGCATAGAGAAA	TGTCCACAGAATTGTAGCAG

unusually weak appearance, including inferior rooting ability and leaf discoloration in the tip of leaf blades at the tillering stage (Fig. 1a). The HB lines had at most five panicles, a short culm (47.8 cm, RIL-132; 38.6 cm, RIL-166), short panicles (19.1 cm, RIL-132; 18.9 cm, RIL-166), and fewer SN (113, RIL-132; 111, RIL-166), whereas their parents had 9–12 panicles, 70–76 cm CL, 21–27 cm PL, and 139–201 SN (Table 2). DTH and SF were not different between the HB lines and parents. F_1 plants from crosses between the two HB lines, RIL-132 and RIL-166, exhibited the same phenotype as the parents, indicating that the two HB lines were homozygous and identical in HB genotype (Fig. 1b).

Fig. 1 Phenotypes of HB lines and their parents. **a** Tillering stage. **b** Mature grain stage

Genetic segregation of HB

Genetic analysis of 200 F_2 plants from the cross between Milyang 23 and Tong 88-7 revealed 188 normal plants and 12 HB plants, which fits the expected ratio of 15:1, indicating that a pair of complementary recessive genes controlled HB (Table 3). The F_1 plants were normal with sterile panicles, as were most of F_1 hybrids between *indica* and *japonica*. Based on the genetic analysis and unique nature of HB, wherein the breakdown only occurs in the F_2 or later generations of interspecific or intersubspecific crosses (Stebbins 1950), it is reasonable to assume that each of two recessive genes originated from each parent, respectively. To confirm this assumption, genetic analyses were conducted on the four backcrossed F_2 populations of BC_1F_2 and BC_3F_2 created by backcrossing RIL-166 to each of Tong 88-7 and Milyang 23. The segregation ratios fit the expected ratio of 3:1 for normal plants to HB plants, confirming that HB was controlled by one pair of complementary recessive genes originating from each parent, Tong 88-7 and Milyang 23, respectively (Table 3; Fig. 2). The two complementary recessive genes were putatively named *hwh1* (*hybrid weakness h1*) from Milyang 23, and *hwh2* (*hybrid weakness h2*) from Tong 88-7, following the nomenclature in previous reports of "*Hwa*", "*hwb*", "*Hwc*", "*hwd*", "*hwe*", "*hwf*", and "*hwg*".

Fine mapping of the HB gene, *hwh1*

To map the *hwh1* gene from Milyang 23, BSA was initially conducted on 144 BC_1F_2 plants derived from the cross between RIL-166 and Tong 88-7 to identify markers linked to the *hwh1* gene. Among 63 markers, including 29 SSR

Table 2 Agronomic traits for parents, hybrid breakdown lines, and their F_1 crosses

Pedigree	DTH	CL ± SE	PL ± SE	PN ± SE	SN ± SE	SF ± SE
Milyang 23	117	70.4 ± 1.2	27.3 ± 0.3	12 ± 0.3	201 ± 2	87.6 ± 1.3
Tong 88–7	91	76.7 ± 1.4	21.0 ± 0.4	9 ± 0.4	139 ± 2	87.0 ± 0.7
Milyang 23/Tong 88–7 (F_1)	103	81.4 ± 0.9	28.7 ± 0.5	12 ± 0.2	211 ± 3	52.3 ± 1.6
RIL-132 (RIL, F_8)	103	47.8 ± 1.1	19.1 ± 0.3	5 ± 0.2	113 ± 1	74.5 ± 1.4
RIL-166 (RIL, F_8)	99	38.6 ± 1.2	18.9 ± 0.2	5 ± 0.1	111 ± 2	91.5 ± 1.6
RIL-166/Milyang 23 (BC_1F_1)	110	76.5 ± 1.3	26.7 ± .04	11 ± 0.3	191 ± 8	45.3 ± 1.5
RIL-166/Tong 88-7 (BC_1F_1)	100	71.2 ± 1.5	23.1 ± 0.7	13 ± 0.2	191 ± 8	87.7 ± 1.5
RIL-132/RIL-166 (F_1)	105	43.4 ± 1.1	20.0 ± 0.4	6 ± 0.1	116 ± 3	87.7 ± 1.0

DTH days to heading, *CL* culm length, *PL* panicle length, *PN* panicle number, *SN* spikelet number, *SF* spikelet fertility, *SE* standard error

Table 3 Genetic analysis of hybrid breakdown in rice

Pedigree	Popul.	No. of plants			Percentage of HB plants	X^2 (3:1)	X^2 (15:1)
		Normal	HB plant	Total			
Milyang 23/Tong 88-7	F_2	188	12	200	6.0	38.5**	0.02NS
RIL-166/Tong 88-7	BC_1F_2	113	31	144	21.5	0.93NS	44.44**
RIL-166/3*Tong 88-7	BC_3F_2	98	33	131	25.2	0.00NS	80.21**
RIL-166/Milyang 23	BC_1F_2	121	29	150	19.3	2.56NS	43.82**
RIL-166/3*Milyang 23	BC_3F_2	173	49	222	22.1	1.02NS	94.85**

* $P < 0.05$, ** $P < 0.01$, *NS*: not significant at the 0.05 level

and 34 STS markers that were distributed over the 12 chromosomes, S0288 and S0298A on chromosome 2 demonstrated a significant polymorphism between the bulked DNA samples of normal and HB plants in the BC_1F_2 population. Consequently, additional SSR and STS markers on chromosome 2 were further used in the BSA. Linkage analysis among *hwh1* and the selected 12 markers in 144 BC_1F_2 plants revealed that the HB gene *hwh1* was located between markers S0298A and S0299B at a distance of 1.0 and 0.8 cM, respectively (Fig. 3b).

To further refine the position of the *hwh1* gene, an additional large mapping population containing 978 BC_1F_3 plants was derived from two BC_1F_2 plants. The flanking markers S0298A and S0299B were used to survey all 144 BC_1F_2, 133 BC_3F_2, and 978 BC_1F_3 plants, which resulted in detection of four recombinants in BC_1F_2 (9820-21, 9820-23, 9820-30, and 9820-96) and five recombinants in BC_1F_3 (9820-21-3, 9820-21-3-5, 9820-21-3-6, 9820-21-3-83, and 9820-96-37). In addition, seven novel DNA markers, including four STS, one SSR, and two dCAPS between S0298A and S0299B, were designed based on the rice genomic database (http://www.ncbi.nlm.nih.gov/ for *indica* and at http://www.rgp.dna.affrc.go.jp/ for *japonica*). A total of eight markers (six new and two flanking) were used to genotype the nine recombinants. In the individuals 9820-21, 9820-21-3, 9820-21-5 and 9820-21-6, the crossover occurred somewhere between S0299N and S0299J, while in 9820-21-83 and 9820-96-37, the crossover occurred somewhere between S0299N and S0299J (Fig. 3c). Consequently, the location of the *hwh1* gene was identified between the marker loci S0299N and S0299J, originating from the BAC clone, BAC OJ1234_B11, and the physical distance between these two markers was 11.8 kb. Based on available sequence annotation databases (http://www.tigr.org, http://www.gramene.org), there was only one predicted gene, a putative glucose-methanol-choline (GMC) oxidoreductase family protein (LOC_Os02g40840) (Fig. 3d). The LOC_Os02g40840 gene was sequenced from Milyang 23, Tong 88-7, and RIL-166. Three nucleotide polymorphisms were detected in the transcript region; two did not affect the translated amino acid, but the 5204th nucleotide T in Milyang 23 and RIL-166 was changed to G in Tong 88-7, changing the amino acid from Tyrosine (Tyr; 763th amino acid) in Milyang 23 and RIL-166 into Asparagine (Asp) in Tong 88-7 (Fig. 4a). To verify the SNP in the nucleotide sequence of the *hwh1* gene, we designed a dCAPS marker (S0299M) and screened the BC_1F_2 population derived from RIL-166/Milyang 23 as parents. We observed that Tong 88-7 had a shorter fragment, while Milyang 23 and RIL-166 exhibited a longer fragment. In the BC_1F_2 population, the S0299M genotypes co-segregated exactly with phenotypes (Fig. 4b).

Fine mapping of the HB gene, *hwh2*

The same strategy was applied for fine mapping of *hwh2*, which originated from Tong 88-7. BSA was conducted on 150 BC_1F_2 plants derived from the cross between RIL-166 and Milyang 23. Among the 63 markers used in BSA for *hwh2*, S1159C on chromosome 11 was detected as a polymorphic marker between the bulked DNA samples of normal and HB plants from the BC_1F_2 population. Additional STS markers flanking S1159C were surveyed for the BC_1F_2 plants, resulting in the detection of 11 additional STS markers linked to the *hwh2* gene. Through linkage analysis with the 12 markers in the BC_1F_2 population, the *hwh2* gene was mapped between markers S1158A and S1159B (S1159C mapped at the same locus as S1159B) at a distance of 0.7 and 0 cM, respectively (Fig. 5b). All of the HB plants carried Tong 88-7 homozygous alleles for S1159B on chromosome 11, whereas the normal plants carried heterozygous or Milyang 23 homozygous alleles.

To further refine the position of the *hwh2* gene, 147 BC_3F_2 plants (population entry 96288), 222 BC_3F_2 (entry 96292), 1062 BC_3F_3 plants derived from one individual of BC_3F_2 (entry 96292), and 544 BC_3F_3 population developed from one individual of BC_3F_2 (entry 96292) were screened. Five additional markers near S1158A and S1159C were identified, and then three recombinants from the BC_1F_2 population, six recombinants from the BC_3F_2, and four recombinants from the BC_3F_3 were detected by surveying with markers between S1158A and S1164A. Genotyping 13 recombinants with the 8 markers mapped the *hwh2* gene between S1159A and S1159C, with a physical distance of 117.2 kb (Fig. 5c). According to the Institute for Genome Research Rice Genome Annotation database (http://

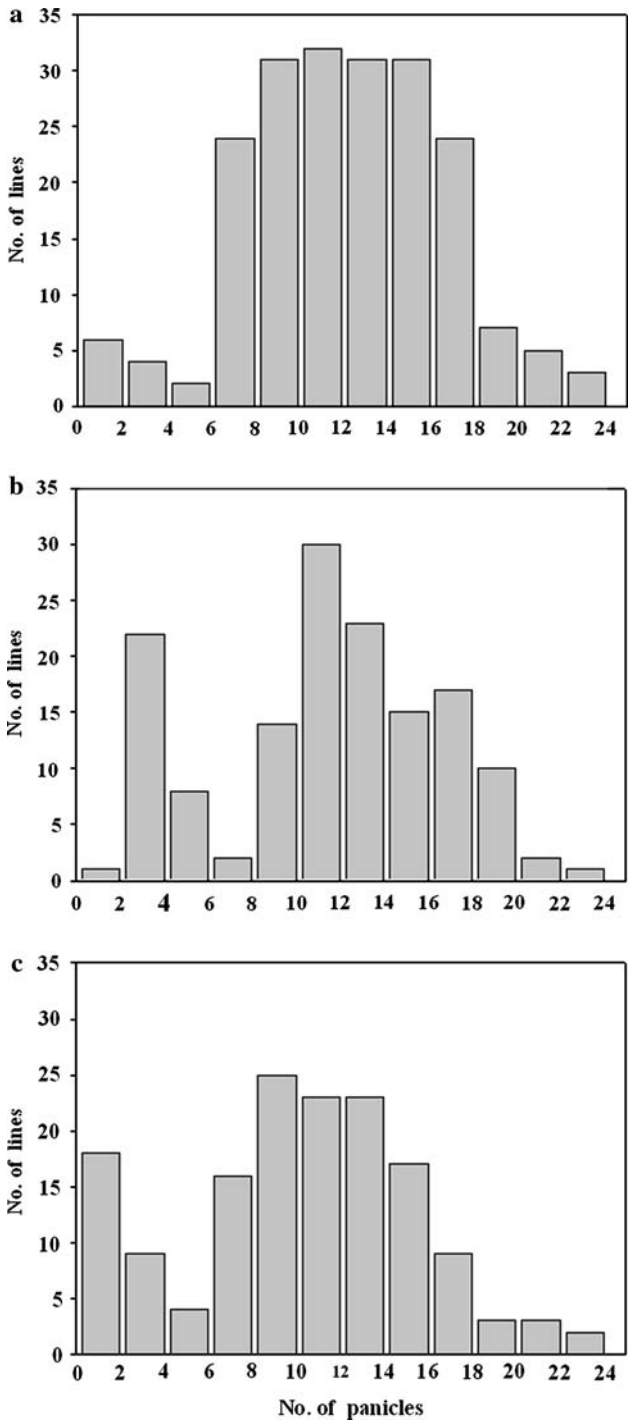

Fig. 2 The segregation of tiller number observed in the F_2 population of Milyang 23/Tong 88-7 (**a**), and BC_1F_2 populations RIL-166/ Tong 88-7 (**b**) and RIL-166/Milyang 23 (**c**)

www.tigr.org/tdb/e2k1/osa1/), this region contains 12 genes: 3 encoding hypothetical proteins, and 6 encoding retrotransposon protein, hexose transporter, putative AT hook motif-containing protein, and expressed proteins. BLAST analysis with the amino acid sequence of the hypothetical proteins revealed no homologue proteins reported in any other organisms. In addition, there was no cDNA sequence of these proteins reported and the protein sequences were relatively short. Thus, these proteins were excluded in analyzing the candidate genes for *hwh2*. Three genes, an expressed protein (LOC_Os11g28600), a putative hexose transporter (LOC_Os11g28610), and a putative AT hook motif-containing protein (LOC_Os11g28650), were chosen and sequenced for Milyang 23, Tong 88-7, and RIL-166. No polymorphisms were detected in the AT hook motif-containing protein and expressed protein, but one insertion/deletion polymorphism (InDel) region 18 nucleotides long was detected in the hexose transporter (Fig. 6a). The 18 nucleotides from 1402nd to 1419th were inserted in the Milyang 23 with a leading insert of 6 amino acids from 469th to 474th, compared to Tong 88-7 and RIL-166. An STS marker (S1159H) for the sequenced gene, a putative hexose transporter, was designed to verify the InDel in the nucleotide sequence of the *hwh2* gene. Genotype analysis for S1159H among the BC_1F_2 population revealed that the S1159H genotypes exhibited co-segregation with phenotypes, with a longer fragment for Milyang 23 and a shorter fragment for and Tong 88-7 and RIL-166 (Fig. 6b).

Distribution of *hwh* genes in rice varieties

To investigate *hwh* gene distribution among *indica* and *japonica* cultivars, 21 *indica* and 33 *japonica* rice varieties were genotyped using the dCAPS marker S0299M for *hwh1* and the STS marker S1159H for *hwh2* (Table 4). In the *indica* group, seven varieties possessed the *hwh1/Hwh2* genotype, while other varieties had the *Hwh1/Hwh2* genotype. In the *japonica* group, 27 of 33 varieties had the *Hwh1/hwh2* genotype, five varieties that represented the tropical *japonica* group had the *Hwh1/Hwh2* genotype (Table 5), and one variety had the *hwh1/Hwh2* genotype. We also tested these two markers in other HB plants detected in F_3 lines derived from Dasanbyeo (Korean *indica* variety)/TR22183 (Chinese *japonica* variety) and the reciprocal TR22183/Dasanbyeo crosses (Fig. 7a). The HB plants from the F_3 lines were found to have an *hwh1/hwh2* genotype, whereas one of the normal plants had *Hwh1/hwh2* genotype from Dasanbyeo/TR22183 crosses and the other normal plant had *hwh1/Hwh2* genotype from TR22183/Dasanbyeo crosses (Fig. 7b).

Discussion

Recently, Matsubara et al. (2007) and Yamamoto et al. (2007) independently reported the same two rice HB genes, *hbd2* on chromosome 2 and *hbd3* on chromosome 11, even though they used different *japonica* parents and a common *indica* parent [Sasanishiki (*japonica*) and Habataki (*indica*)

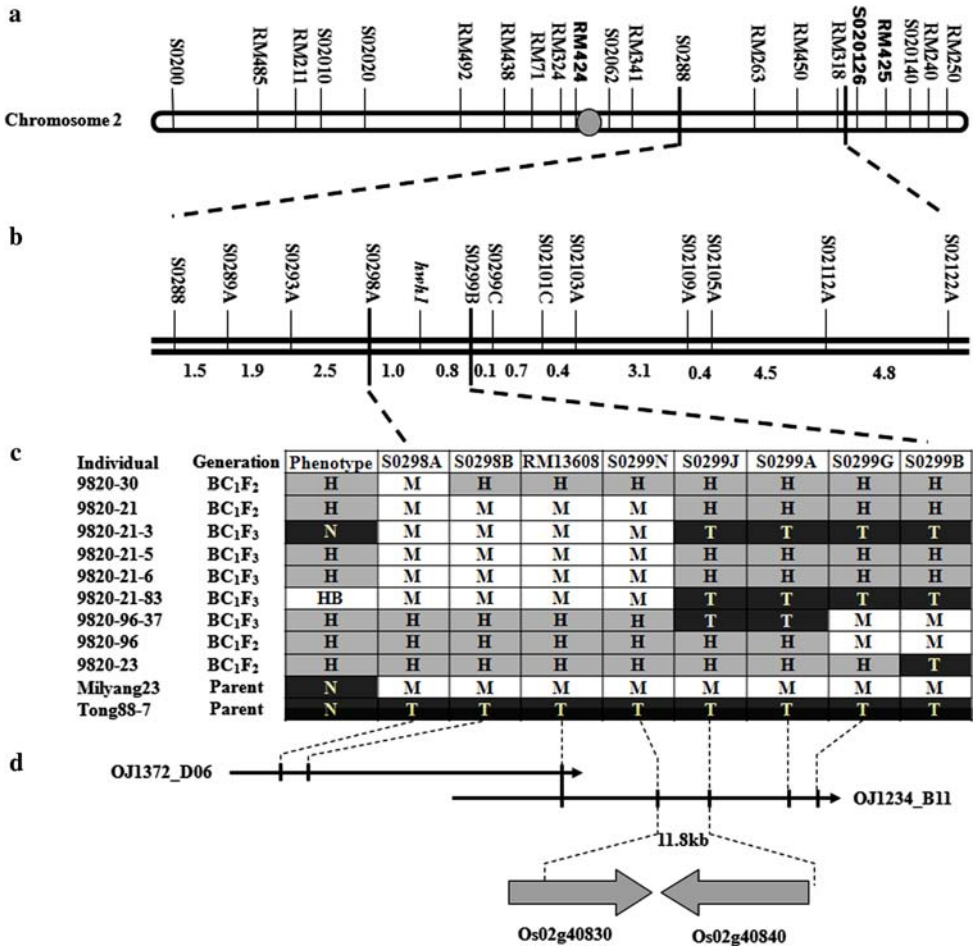

Fig. 3 Genetic and physical maps of the *hwh1* gene. **a** Linkage map of chromosome 2 constructed using RILs derived from the cross between Milyang 23 and Tong 88-7. **b** High resolution genetic map of the *hwh1* locus in the BC_1F_2 population. **c** Genotypes of key recombinants at the loci of markers and *hwh1*. **d** Physical map of the *hwh1* region in the *japonica* Nipponbare from GenBank accessions. Phenotypes, *N* normal plant, *HB* HB plant, *H* normal plant but segregating in offspring. Genotypes, *M* Milyang 23 type, *T* Tong 88-7 type, *H* heterozygote

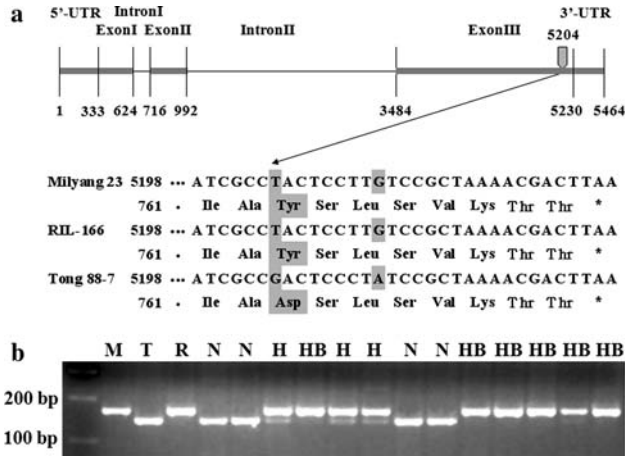

Fig. 4 DNA sequence alignment and DNA marker analysis. **a** The structure of candidate gene LOC_Os02g40840 and single nucleotide polymorphism of Milyang 23, Tong 88-7, and RIL-166. **b** Co-segregation analysis in RIL-166/Tong 88-7 BC_1F_2 population using dCAPS marker (S0299M, one-base mismatched forward primer generated a *Sal*I specific site, 5′-cgtattgcctcgcaaatggcatcgtc-3′; reverse primer, 5′-tggtcctcgtaacaatcatatacgct-3′). *M* Milyang 23, *T* Tong 88-7, *R* RIL-166, Phenotypes: *N* normal plant, *H* segregating, *HB* HB plant

for the Matsubara group and Koshihikari (*japonica*) and Habataki (*indica*) for the Yamamoto group]. Interestingly, the mapped locations of these two genes are similar to the two *hwh* genes identified in this study, *hbd2* to *hwh1* and *hbd3* to *hwh2*. We conducted genotype analysis using specific DNA markers, S0299M for the *hwh1* and S1159H for the *hwh2*, as described in Table 4, on the cultivars Sasanishiki, Koshihikari, and Habataki. We found that the genotype of Sasanishiki and Koshihikari was *Hwh1/hwh2* and the genotype of Habataki was *hwh1/Hwh2*. Accordingly, it is highly possible that the HB genes reported by Matsubara et al. (2007) and Yamamoto et al. (2007) are the same as the HB genes identified in this study. Further evaluation is needed to determine whether these genes are identical or not. If the genes are identical, it appears that HB may be caused by a few sets of genes distributed in *indica* and *japonica* varieties. The genotype analysis among 54 rice cultivars revealed that *hwh* alleles were widely distributed in rice cultivars; 33.3% of *indica* and 3.0% of *japonica* varieties had a genotype *hwh1/Hwh2*, whereas 0% of *indica* and 81.8% *japonica* varieties had a genotype *Hwh1/hwh2*

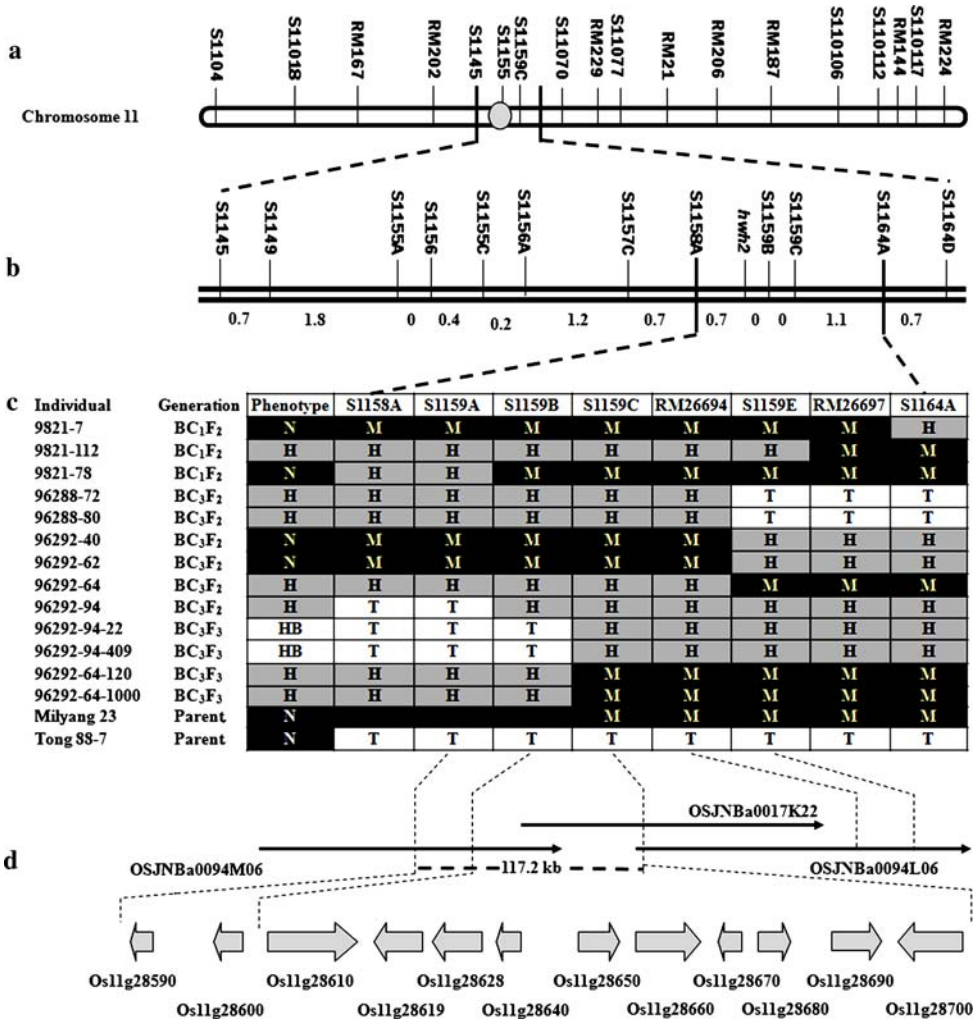

Fig. 5 Genetic and physical maps of the *hwh2* gene. **a** Linkage map of chromosome 11 constructed using RILs derived from the cross between Milyang 23 and Tong 88-7. **b** High resolution genetic map of the *hwh2* locus in the BC_1F_2 population. **c** Genotypes of key recombinants at the loci of markers and *hwh2*. **d** Physical map of the *hwh2* region in the *japonica* Nipponbare from GenBank accessions. Phenotypes: *N* normal plant, *HB* HB plant, *H* normal plant but segregating in offspring. Genotypes: *M* Milyang 23 type, *T* Tong 88-7 type, *H* heterozygote

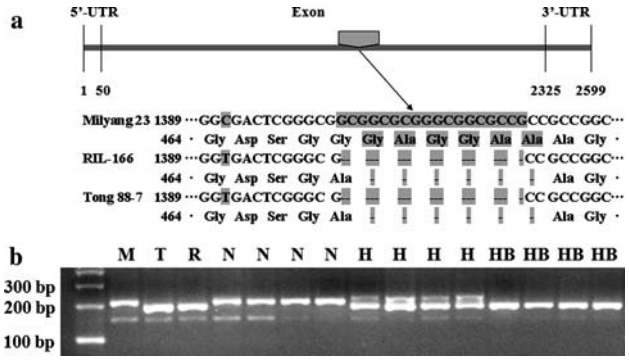

Fig. 6 DNA sequence alignment and DNA marker analysis. **a** The structure of candidate gene LOC_Os11g28610 and InDel of Milyang 23, Tong 88-7, and RIL-166. **b** Co-segregation analysis in the RIL-166/Tong 88-7 BC_1F_2 population using STS marker (S1159H, 5′-tggaagtggacggagaacgt-3′; reverse primer, 5′-atcaggacgtccttggtgtaca-3′). *M* Milyang 23, *T* Tong 88-7, *R* RIL-166, Phenotypes: *N* normal plant, *H* segregating, *HB* HB plant

(Table 4), indicating that HB plants would be easily produced in the progenies of *indica* and *japonica* varieties. It was also notable that all of the five tropical *japonica* varieties had *Hwh1/Hwh2* genotype just like *indica* varieties. The developed DNA markers for *hwh1* and *hwh2* can be used to avoid the creation of HB plants, especially in marker-assisted selection breeding programs. With the DNA markers, we observed that the HB plants from reciprocal crosses between *indica* variety (Dasanbyeo) and *japonica* variety (TR22183) have the *hwh1/hwh2* genotype (Fig. 7). The occurrence of HB plants might decrease the breeding efficiency and cause loss of favorable alleles in rice breeding programs when the favorable alleles are closely linked to

Table 4 Gene distribution of *hwh1* and *hwh2* in *indica* and *japonica* rice

	Genotypic group				Total
	Hwh1/Hwh2	*hwh1/Hwh2*	*Hwh1/hwh2*	*hwh1/hwh2*	
Indica	14	7	0	0	21
Japonica	5	1	27	0	33

Refer to Table 5 for the varietal names belonging to each genotypic group

Table 5 Rice varieties grouped by the genotypes at the *hwh* loci

Indica variety			*Japonica* variety		
Genotypic group[a]	Variety	Origin	Genotypic group[a]	Variety	Origin
Hwh1/Hwh2	China1039	China	*Hwh1/Hwh2*	Shennong 27	China
	Diantun 502	China		Azucena	Philippines
	ARC 10239	India		CP-SLO	Philippines
	Chinsurah Boro II	India		L201	USA
	IR 20	IRRI		Malagkit Sinaguing	Philippines
	IR 36	IRRI			
	IR 64	IRRI	*hwh1/Hwh2*	Hapcheon 1	Korea
	IR 72	IRRI			
	IRSR-3	IRRI			
	Tadukan	Philippines	*Hwh1/hwh2*	Changbai 9	China
	Tetep	Philippines		Tong 88-7	China
	Cheongcheongbyeo	Korea		Yan 304	China
	Gogowierie	Surinam		Chujing 27	China
	Zenith	USA		Dianjingyou 1	China
				Dianxi 1	China
hwh1/Hwh2	Bonni	India		Hexi 41	China
	Sakha local	India		Kunmingxiaobaigu	China
	Habataki	Japan		Yinguang	China
	Dasanbyeo	Korea		Yunjing 17	China
	Milyang 23	Korea		Yunjing 19	China
	Nampungbyeo	Korea		TR22183	China
	Satbyeolbyeo	Korea		Fujihikari	Japan
				Hitomebore	Japan
				Koshihikari	Japan
				Nipponbare	Japan
				Sasanishiki	Japan
				Towada	Japan
				Dongjinbyeo	Korea
				Gopumbyeo	Korea
				Hwacheongchalbyeo	Korea
				Ilpumbyeo	Korea
				Nakdongbyeo	Korea
				Nampyeongbyeo	Korea
				Samgwangbyeo	Korea
				Sindongjinbyeo	Korea
				Ungwangbyeo	Korea

[a] Refer to Table 4 for genotypic group

HB alleles (Okuno 1999; Lynch and Walsh 1998). Thus, our finding of candidate genes of *hwh* and the developed DNA markers will help minimize the occurrence of such undesirable situations.

The GMC oxidoreductase, which is likely encoded by the *Hwh1* gene, is an enzyme involved in the oxidation–reduction reaction, and its enzyme super-family includes choline oxidase, choline dehydrogenase, methanol oxidase, and cholesterol oxidase (Cavener 1992). Structural analysis indicated that the putative GMC oxidoreductase protein (LOC_Os02g40840) is composed of GMC oxidoreductase N-terminal FAD-binding domain (IPR000172, 310th-41st amino acid), long-chain fatty acid alcohol dehydrogenase (IPR012400, 3rd-769th amino acid) and fumarate reductase/succinate dehydrogenase flavoprotein N-terminal domain (IPR003953, amino acid 264th-299th amino acid) (InterProScan, http://www.ebi.ac.uk/InterProScan/). Among the GMC gene cluster, glucose dehydrogenase (GLD) and glucose oxidase (GOX) have been characterized as involved in development and immunity in *Drosophila* (Iida

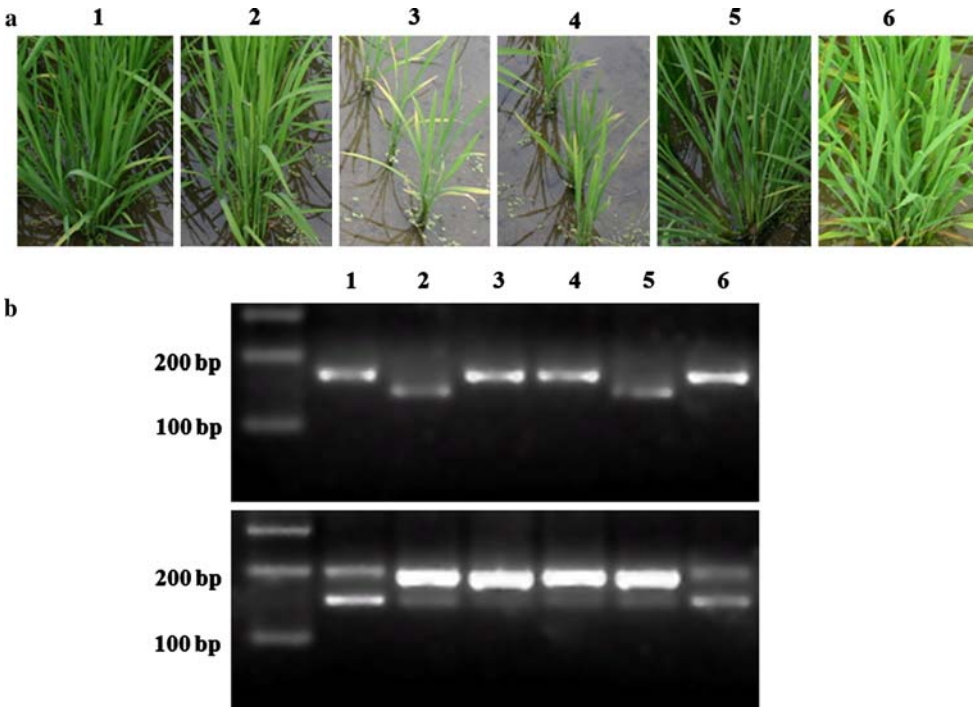

Fig. 7 Phenotypic and genotypic analysis of *hwh* genes in F_3 lines derived from reciprocal crosses between Dasanbyeo (*indica*) and TR22183 (*japonica*). **a** Phenotypes of parents and lines. **b** Genotypes of parents and lines, *above*: dCAPS marker S0299M, *bottom*: STS marker S1159H. *1* Dasanbyeo, *2* TR22183, *3* HB plant from Dasanbyeo/TR22183, *4* HB plant from TR22183/Dasanbyeo, *5* normal plant from Dasanbyeo/TR22183, *6* normal plant from TR22183/Dasanbyeo

et al. 2007; Yang and Cox-Foster 2005). However, the specific roles of other GMC oxidoreductases have not been reported in plants.

The candidate protein for *Hwh2*, the putative hexose transporter protein (LOC_Os11g28610), contains three domains: a sugar transporter superfamily, including sugar transporter 1 (IPR005829, 63rd–68th amino acid), and 2 (IPR005829, 105th–130th amino acid), a general substrate transporter (IPR005828, 7th–742nd amino acid), and a major facilitator superfamily domain (IPR007114, 7th–731st amino acid) (InterProScan, http://www.ebi.ac.uk/InterProScan/). These integral membrane proteins are responsible for the binding and transport of various carbohydrates, organic alcohols, acids, ions and other substrates in a wide range of plants. Sugar transport proteins play a crucial role in the cell-to-cell and long-distance distribution of sugars throughout the plant (Williams et al. 2000). A wide range of sugars including tetroses, pentoses, hexoses, sugar acids, and sugar alcohols (except disaccharides) are transported through sugar transport proteins. Plants encode around 100 putative monosaccharide transporters within the major facilitator superfamily, yet only a few have been studied (Reinders et al. 2005). To date, monosaccharide transporters comprising seven subfamilies and at least 53 genes have been predicted through phylogenetic analysis of the *Arabidopsis* genome (http://www.biologie.uni-erlangen.de/mpp/TPer/index_TP.shtml), and less than dozen of these genes have been reported (Johnson et al. 2006).

Since the known functions of both the genes are limited, and there is no report or evidence of direct interaction, it is challenging to make any model or hypothesis to explain the mechanism of the HB phenomenon caused by the interaction of both proteins. Both genes do relate to carbohydrate metabolism. The interaction may cause unbalanced or unfavorable conditions in cells through the combination of *hwh2*, changing the substrate transport rate, and *hwh1*, altering the substrate catalyzing efficiency. A comparison analysis of these protein activities among HB plants and their parents would be a good starting point to further explore the mechanisms of HB, and we plan to apply other general molecular biology tools such as gene transformation or gene knockouts in further studies.

This study reports the genetic analysis and cloning of genes *hwh1* and *hwh2*, which may help improving our understanding of the molecular mechanisms controlling HB. The developed DNA markers for *hwh1* and *hwh2* can be used to predict the possible occurrence of HB plants in breeding programs utilizing inter-subspecific hybridization in rice.

Acknowledgments This research was supported by a grant (code#CG3111) from the Crop Functional Genomics Center of the 21st Century Frontier Research Program funded by the Ministry of Science and Technology, Republic of Korea.

References

Bateson W (1909) Heredity and variation in modern lights. In: Seward AC (ed) Darwin and modern science, Cambridge University Press, Cambridge, pp 85–101

Bomblies K, Lempe J, Epple P, Warthmann N, Lanz C, Dangl JL, Weigel D (2007) Autoimmune response as a mechanism for a

Dobzhansky-Muller-Type incompatibility syndrome in plants. PLoS Biol 5(9):1962–1972

Bomblies K, Weigel D (2007) Hybrid necrosis: autoimmunity as a potential gene-flow barrier in plant species. Nat Rev Genet 8:382–393

Brideau NJ, Flores HA, Wang J, Maheshwari S, Wang X, Barbash DA (2006) Two Dobzhansky-Muller genes interact to cause hybrid lethality in Drosophila. Science 314(5803):1292–1295

Burke J, Arnold ML (2001) Genetics and the fitness of hybrids. Annu Rev Genet 35:31–52

Causse MA, Fulto TM, Cho YG, Ahn SN, Chuncongse J, Wu K, Xiao J, Yu Z, Ronald PC, Harrington SE, Second G, McCouch SR, Tanksley SD (1994) Saturated molecular map of the rice genome based on an interspecific backcross population. Genetics 138:1251–1274

Cavener DR (1992) GMC oxidoreductases. A newly defined family of homologous proteins with diverse catalytic activities. J Mol Biol 223:811–814

Chin JH, Kim JH, Jiang W, Chu SH, Woo MO, Han L, Brar D, Koh HJ (2007) Identification of subspecies-specific STS markers and their association with segregation distortion in rice (*Oryza sativa* L.). J Crop Sci Biotech 10(3):175–184

Christie P, MacNair MR (1984) Complementary lethal factors in two North American populations of the yellow monkey flower. J Hered 75:510–511

Coyne JA (1992) Genetics and speciation. Nature 355:511–515

Dobzhansky TH (1936) Studies on hybrid sterility. II. Localization of sterility factors in *Drosophila pseudoobscura* hybrids. Genetics 21:113–135

Fukuoka S, Namai H, Okuno k (1998) RFLP mapping of the genes controlling hybrid breakdown in rice (*Oryza sativa L.*). Theor Appl Genet 97:446–499

Fukuoka S, Newingham MCV, Ihitiaq M, Nagamine T, Kawase M, Okuno K (2005) Identification and mapping of two new loci for hybrid breakdown in cultivated rice. Rice Genet Newsl 22:29–31

Futuyma DJ (1986) Evolutionary biology. 2nd edn. Sinauer, Sunderland

Iida K, Cox-Foster DL, Yang X, Ko WY, Cavener DR (2007) Expansion and evolution of insect GMC oxidoreductases. BMC Evol Biol 7:75–85

Johnson DA, Hill JP, Thomas MA (2006) The monosaccharide transporter gene family in land plants is ancient and shows differential subfamily expression and expansion across lineages. BMC Evol Biol 6:64

Kosambi DD (1944) The estimation of map distances from recombination values. Ann Eugen 12:172–175

Kubo T, Yoshimura A (2002) Genetic basis of hybrid breakdown in a *japonica/indica* cross of rice, *Oryza sativa* L. Theor Appl Genet 105:906–911

Kubo T, Yoshimura A (2005) Epistasis underlying female sterility detected in hybrid breakdown in a *japonica-indica* cross of rice (*Oryza sativa* L.). Theor Appl Genet 110:346–355

Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newburg L (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174–181

Li Z, Pinson SRM, Paterson AH, Park WD, Stancel JW (1997) Genetics of hybrid sterility and hybrid breakdown in an intersubspecific rice (*Oryza sativa* L.) population. Genetics 145:1139–1148

Lynch M, Force AG (2000) The origin of interspecific genomic incompatibility via gene duplication. Am Nat 156:590–605

Lynch M, Walsh B (1998) Genetics and analysis of quantitative traits. Sinauer, Sunderland, pp 379–429

Matsubara K, Ando T, Mizubayashi T, Tto S, Yano M (2007) Identiication and linkage mapping of complementary recessive genes causing hybrid breakdown in an intraspecific rice cross. Theor Appl Genet 115:179–186

Michelmore RW, Paran I, Kesseli KV (1991) Identification of markers linked to disease resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. Proc Natl Acad Sci USA 88:9828–9832

Muller HJ (1942) Isolating mechanisms, evolution and temperature. Biol Symp 6:71–125

Muller HJ, Pontecorve G (1940) Recombinants between *Drosophila* species the F_1 hybrids of which are sterile. Nature 146:199–200

Oka A, Mita A, Sakurai-Yamatani N, Yamamoto H, Takagi N, Takano-Shimizu T, Toshimori K, Moriwaki K, Shiroishi T (2004) Hybrid breakdown caused by substitution of the X chromosome between two mouse subspecies. Genetics 166(2):913–924

Oka HI (1957) Phylogenetic differentiation of cultivated rice. XV. Complementary lethal genes in rice. Jpn J Genet 32:83–87

Okuno K (1999) Geographical distribution of genes causing hybrid breakdown in varietal crosses of Asian cultivated rice. Genetic Res Crop Evol 46:13–17

Reinders A, Panshyshyn JA, Ward JM (2005) Analysis of transport activity of Arabidopsis sugar alcohol permease homolog *AtPLT5*. J Biol Chem 280:1594–1602

Stebbins GL Jr (1950) Isolation and the origin of species. In: Stebbins GL Jr (ed) Variation and evolution in plants. Columbia University Press, New York, pp 189–250

Williams LE, Lemoine R, Sauer N (2000) Sugar transporters in higher plants—a diversity of roles and complex regulation. Trends Plant Sci 5(7):283–290

Yamamoto E, Takashi T, Morinaka Y, Lin S, Kitano H, Matsuoka M, Ashikari M (2007) Interaction of two recessive genes, *hbd2* and *hbd3*, induces hybrid breakdown in rice. Theor Appl Genet 115:187–194

Yang X, Cox-Foster DL (2005) Impact of an ectoparasite on the immunity and pathology of an invertebrate: evidence for host immunosuppression and viral amplification. Proc Natl Acad Sci USA 102(21):7470–7475