Theor Appl Genet (2007) 114:1407–1415
DOI 10.1007/s00122-007-0526-0

ORIGINAL PAPER

Fine mapping and allelic dosage effect of *Hwc1*, a complementary hybrid weakness gene in rice

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Received: 12 July 2006 / Accepted: 16 February 2007 / Published online: 21 March 2007

Abstract Hybrid weakness is a reproductive barrier that is found in many plant species. In rice, the hybrid weakness caused by two complementary genes, *Hwc1* and *Hwc2*, has been surveyed intensively. However, their gene products and the molecular mechanism that causes hybrid weakness have remained unknown. We performed linkage analyses of *Hwc1*, narrowed down the area of interest to 60 kb, and identified eight candidate genes. In the F_2 population, in which both *Hwc1* and *Hwc2* genes were segregated, plants were separable into four classes according to their respective phenotypes: severe type, semi-severe type, F_1 type, and normal type. Severe type plants show such severe symptoms that they could produce only tiny shoot-like structures; they were unable to generate roots. Genetic analyses using closely linked DNA markers of the two genes showed that the symptoms of the F_2 plants were explainable by the genotypes of *Hwc1* and *Hwc2*. Weakness was observed in plants that have both *Hwc1* and *Hwc2*. In *Hwc1* homozygote, the symptoms worsened and severe type or semi-severe type plants appeared. Consequently, *Hwc1* should have a gene dosage effect and be a semi-dominant gene. The dosage effect of *Hwc2* was recognizable, but it was not so severe as that in *Hwc1*. These results are useful to elucidate the mechanism that causes the hybrid weakness phenomenon and the role of each causal gene in hybrid weakness.

Communicated by Y. Xue.

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Introduction

Many reproductive barrier forms have been reported in many plant species, e.g., hybrid inviability, hybrid sterility, and hybrid breakdown. They are obstacles to overcome for plant breeders acquiring a new gene combination via hybridization. They have also been of great interest for scientists studying evolution and speciation (Stebbins 1958; Orr 1996). Among such reproductive barriers, weak growth occurring in hybrids derived from crosses between two normal strains is called hybrid weakness.

In Asian cultivated rice (*Oryza sativa* L.), two hybrid combinations causing hybrid weakness have been reported (for a review, Sato 1997). Oka (1957) first reported hybrid weakness, caused by a set of dominant complementary genes, in some Indian rice crosses. Amemiya and Akemine (1963) found another hybrid weakness in the cross between a Peruvian rice cultivar (Jamaica) and a Japanese cultivar (Norin 8). Chu and Oka (1972) found an interspecific hybrid weakness in African-cultivated rice *O. glaberrima*

and its wild relative *O. barthii* (formerly named *O. breviligulata*). These phenomena are conditioned by different pairs of complementary dominant genes.

Of the three hybrid-weakness phenomena described above, the weakness found in the cross between Jamaica and Norin 8 is caused by two complementary genes: *Hwc1* derived from Jamaica and *Hwc2* from Norin 8. Distribution of the gene-carrier was surveyed (Sato and Hayashi 1983; Sato and Morishima 1987). The *Hwc2* gene is prevalent among temperate Japonica, but not among tropical Japonica or Indica. Thirty strains of wild relatives (*O. rufipogon* and *O. nivara*) carried no *Hwc2*. From these results, Sato and Morishima (1988) inferred that the *Hwc2* gene arose at an early stage of differentiation of temperate Japonica types. On the other hand, 91 cultivated rice cultivars and 30 wild relatives examined were found not to carry *Hwc1* (Sato and Morishima 1987), indicating that *Hwc1* is a very rare gene and that Jamaica has been the only carrier of this gene up to now. These studies indicate that these two genes are very important for rice phylogeny, especially for differentiation of Japonica into temperate and tropical groups.

Anatomical and biochemical experiments to elucidate hybrid weakness were performed intensively by Amemiya and Akemine (1963). Their results can be summarized as follows. (1) Growth is normal in embryonic development and germination. (2) Root growth inhibition occurs earlier and more severely than that of shoots. (3) Cell enlargement, cell wall lignification, and cytoplasm elimination occur in hybrid roots, indicating the decrepitude of the meristematic region. (4) Recovery of root growth of the hybrid has never been observed in the embryo culture on media including $NaNO_2$, amino acids, vitamins, growth hormones, plant extracts, or carbohydrates. Our previous study (Saitou et al., submitted) indicated that this hybrid weakness event is temperature-dependent: high temperature (34°C) induces hybrids' recovery from weakness. Results also showed that root growth inhibition is observable as early as 5 days after sowing under 28°C conditions (Ichitani et al. 2001). Although many experimental results are available, molecular and biochemical mechanisms controlling hybrid weakness remain unknown.

To understand the mechanism causing the hybrid weakness, it is crucial that the two causal genes be isolated and the structure of the genes and the gene products be elucidated. Knowledge of that mechanism might also suggest useful tools for studying rice varietal differentiation if the gene structure that engenders the difference between the causal genes and their normal alleles were clarified. In our previous study (Ichitani et al. 2001), *Hwc2* gene was tagged by DNA markers in rice chromosome 4. In the present study, we report chromosomal location of *Hwc1* gene. We also report the dosage effect of the two genes on the weakness symptom.

Materials and methods

Linkage analysis

Four rice cultivars were used in linkage analyses: Jamaica, Kasalath, Taichung 65, and Nipponbare. Of them, Jamaica is the only carrier of *Hwc1* gene. Taichung 65 and Nipponbare carry *Hwc2* gene. Kasalath carries neither *Hwc1* nor *Hwc2*.

First, for rough chromosomal location of *Hwc1*, 43 F_2 plants derived from the cross between Jamaica and Kasalath were crossed with *Hwc2*-carrier Taichung 65 or Nipponbare as female. After harvest, hybrid seeds were dried at 50°C for five days to break dormancy. Subsequently, they were sown on Petri dishes containing tapwater 5 mm deep and left at 28°C for 5 days. Their roots were examined to estimate the genotype of the F_2 plants at the *Hwc1* locus, as described in Ichitani et al. (2001): The genotype of each F_2 plant was estimated to be *Hwc1Hwc1* if all hybrid plants showed inhibition of root elongation (less than 1 cm), *hwc1hwc1* if they showed normal root elongation (usually more than 5 cm) and *Hwc1hwc1* if hybrid plants segregated. To confirm the genotypes, hybrid plants were grown for 3 weeks, and root elongation was observed again. The DNA marker genotypes of some hybrid plants were analyzed to verify successful pollination. The F_2 plants were also analyzed for genotypes for 19 STS markers developed by the Rice Genome Project in Japan (RGP, http://www.rgp.dna.affrc.go.jp/publicdata/caps/index.html) and 30 simple sequence repeats (SSR) markers developed by the International Rice Microsatellite Initiative (IRMI) (McCouch et al. 2002). Independence between DNA markers and *Hwc1* gene of F_2 plants was tested using Chi-square analysis. Because a significant linkage relationship was detected between markers on the chromosome 1 and *Hwc1*, linkage order and recombination values among them were estimated using Mapmaker/Exp. 3.0 (Lander et al. 1987). Obtained recombination values were converted into genetic map distances (in centi-Morgans) using the Kosambi function (Kosambi 1944).

After rough mapping, fine mapping using a three-way cross was performed. The F_1 plants derived from the cross between Jamaica and Kasalath were crossed with Taichung 65. The progenies were divided into weak phenotype carrying both *Hwc1* and *Hwc2* genes, and normal phenotype carrying only *Hwc2* gene. The criterion dividing progenies into normal and weak phenotypes was root elongation, as described above. After DNA extraction, the progenies were analyzed for linkage between *Hwc1* and DNA markers. Finally, recombinants between the two DNA markers encompassing the *Hwc1* locus were selected from 833 F_2 plants from the cross between Jamaica and Kasalath. They were crossed with Taichung 65 for their *Hwc1* genotypes.

They were also analyzed for genotypes of neighboring SSRs and STS markers, many of which were constructed in this study with the aid of DNA sequence information released by RGP (Table 1). Primers were designed using 'Primer 3' (http://www.frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Searching for SSRs was done using SSRIT (Temnykh et al. 2001, http://www.gramene.org).

The DNA was extracted according to Dellaporta et al. (1983) with some modifications. The PCR conditions were: 95°C for 10 min, 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, followed by a final extension of 72°C for 5 min. The PCR mixture (5 µl) contained 10 ng template DNA, 200 µM of each d NTP, 0.2 µM of primers, 0.25 units *Taq* DNA polymerase (AmpliTaq Gold; Applied Biosystems), and 1× buffer containing $MgCl_2$. The PCR products were analyzed using electrophoresis in 10% (29:1) polyacrylamide gel or 3% agarose gel, followed by ethidium bromide staining and viewing under ultraviolet light radiation.

Gene annotation

Open reading frames (ORFs) were predicted using a rice-genome automated annotation system, RiceGAAS (http://www.RiceGAAS.dna.affrc.go.jp). Software integrated in RiceGAAS includes coding region prediction programs (GENSCAN, RiceHMM, FGENESH, MZEF), a splice site prediction program (SplicePredictor), homology search analysis programs (Blast, HMMER, ProfileScan, MOTIF), a tRNA gene prediction program (tRNAscan-SE), repetitive DNA analysis programs (RepeatMasker, Printrepeats), a signal scan search program (Signal Scan), a protein localization site prediction program (PSORT), and a program of classification and secondary structure prediction of membrane proteins (SOSUI).

Dosage effect of the *Hwc1* and *Hwc2* genes on weakness symptoms

The F_1 hybrids of Nipponbare and Jamaica were germinated and cultured at 34°C for 4 weeks. Then they were transplanted into pots and cultured outside. More than 150 seeds were obtained from the F_1 hybrids. These seeds were sown on 0.5× MS medium at 24°C under continuous illumination (ca. 20 µmol m^{-2} s^{-1}). The genotypes for *Hwc1* and *Hwc2* loci were estimated through tightly linked DNA markers, RMIBA6 for *Hwc1* (an SSR marker located between dIBA3 and RMKG11; see results) and C111*4 for *Hwc2* [a CAPS marker located between *XNpb264* and *Ph* (Ichitani et al. 2001), and cosegregating with *Hwc2* in more than 2,500 F_2 plants from the cross between Nipponbare and Kasalath (Kuboyama et al. unpublished data)].

Results

Chromosomal location of *Hwc1*

According to our primary linkage analysis of *Hwc1* gene using 43 F_2 plants, *Hwc1* was located on the long arm of rice chromosome 1; it was linked with an STS marker S5756 and an SSR marker RM2318 (McCouch et al. 2002) (Fig. 1).

Table 1 Primer sequences designed and used for fine mapping of the *Hwc1* locus

Marker	Primer sequence (5′–3′)	Product size in Nipponbare (bp)
RMKG1	F: GATGTCTCTTGCGTGCATTG	145
	R: GCCAAACCATTTTACTGACGA	
RMKG2	F: TTGACTTTTAAATCAGCAAGAACA	121
	R: TCGGACCATATGAGAAAGCA	
RMKG3	F: GCACCACTGGACCGTTCTAC	150
	R: CTTTTGCATGAACTGCATCG	
RMKG8	F: GGGTGAAGGTGATGACGTG	152
	R: TTGGAACGAAACGAATCTCC	
RMKG11	F: GCAGGGTTATGTCGCATTAAA	236
	R: CAAGCAAAAGCAGAGCATCA	
RMGK14	F: GCTGCGTCGGAGTAGGATA	181
	R: GCAGAATCGCACTCACCAT	
RMIBA6	F: AGTCCGCGACTTCCTATTCC	150
	R: TCCTAATTTTGATATTCTGCCAAG	
IBA2	F: CATTTTACATGAGCCCACCTT	281
	R: TAAGGGCGAAGGTAGAACGA	

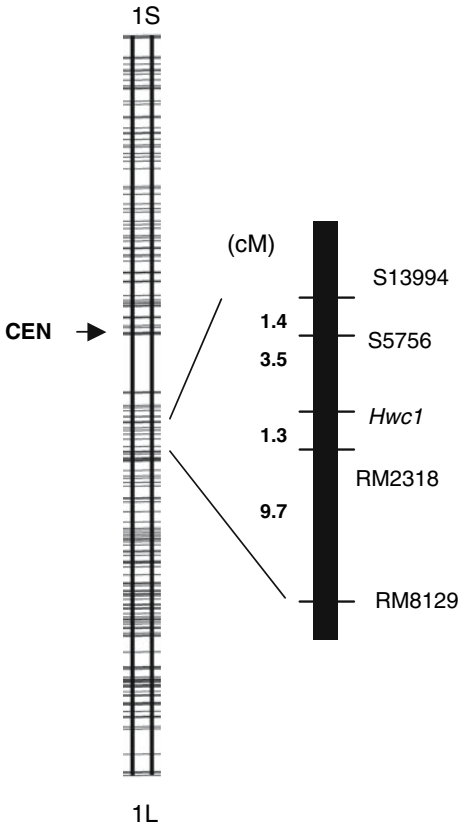

Fig. 1 Linkage map showing the location of the *Hwc1* locus. *Left* RFLP framework map of chromosome 1 quoted from Harushima et al. (1998). *Right Hwc1* map constructed from the F_2 population ($n = 43$) in this study

Fine mapping of *Hwc1*

The progeny from the three-way cross [Taichung 65 × (Jamaica × Kasalath)] were separated into 401 normal plants and 298 plants showing hybrid weakness. The observed ratio 401:298 did not fit to the expected ratio 1:1($\chi^2 = 15.177$, $P < 0.01$), probably because of zygotic reproductive barrier linked with the *Hwc1* locus (Harushima et al. 2001; see Discussion). Among them, all normal plants and 130 plants showing weakness were subjected to linkage analyses. The rest of the plants showing hybrid weakness died before DNA extraction. Results of the analyses indicated that *Hwc1* cosegregated with an SSR marker RM5 (Panaud et al. 1996). We produced three SSR markers, RMKG1–RMKG3, and one STS marker, IBA2, based on rice chromosome 1 DNA sequence (http://www.rgp.dna.affrc.go.jp/cgi-bin/statusdb/stattable.pl?chr=1&lab=RGP) (Table 1). Then we narrowed down the position of *Hwc1* gene between RMKG3 and IBA2. The former is located at the position of 10900 on the PAC clone P0704D04; the latter is located at the position of 132570 on the same PAC clone.

Finally, recombinants between RMKG3 and IBA2 were selected at the seedling stage from 833 F_2 plants from the cross between Jamaica and Kasalath. They were crossed to T65 to perform progeny tests for *Hwc1* genotypes. Three SSR markers, RMKG8, RMKG11, and RMKG14, and one dCAPS marker, dIBA3, at the *Hwc1* candidate region between RMKG3 and IBA2, were designed and subjected to linkage analysis (Table 1). One SSR marker, MRG2704 (acc. no.: AY020379), was also examined. Results of fine mapping are summarized in Fig. 2. The position of the *Hwc1* gene was confined to about the 60 kb region on the PAC clone P0704D04 between the two SSR markers, RMKG14 at the position of 54240 and RM5 at the position of 114960, and the gene cosegregated with MRG2704, dIBA3 and RMKG11.

Candidate gene analysis

Between RMKG14 and IBA2, 10 ORFs were found (Fig. 2; Table 2); eight of the ORFs have EST clones in DNA sequence databases. These show that they are transcribed. The first, ORF1, has DnaJ domain in its N-terminal; DnaJ is a member of the Hsp40 family of molecular chaperones, which is also called the J-protein family, the members of which regulate the activity of Hsp70s (Walsh et al. 2004). Proteins ORF3 and ORF4 have the SUMO motif (Hilgarth et al. 2004) in their amino acid sequences. Two others,

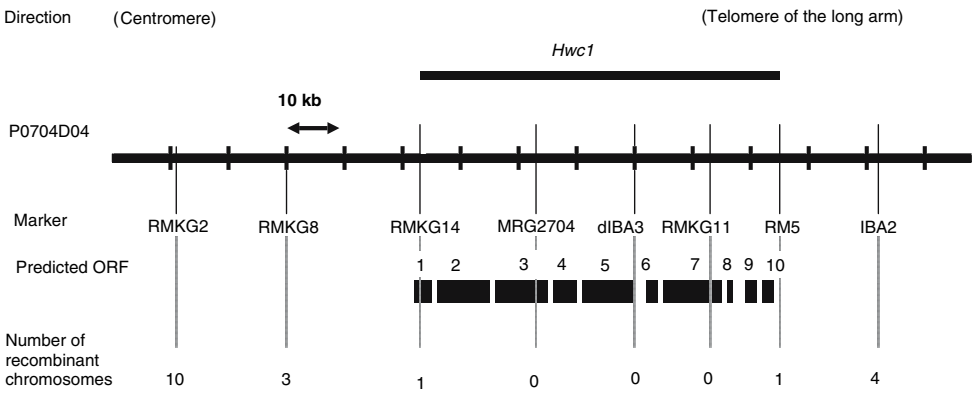

Fig. 2 Fine mapping of *Hwc1* on the rice PAC clone P0704D04 using 833 plants from the F_2 population from the cross Jamaica × Kasalath, and positions of ORFs predicted using RiceGAAS on the 60 kb region containing *Hwc1*

ORF 5 and ORF7, have similarity with *STYLOSA*, a flower development regulator in *Antirrhinum* (Navarro et al. 2004), which is expressed in all shoot meristems and later becomes confined to the adaxial domain and (pro)-vascular tissues. Neither ORF2 nor ORF6 shows any similarity with a known protein, but they have homology with rice ESTs. Both ORF 8 and ORF9 code a hypothetical protein and have no similarity with a known protein or with rice ESTs. The pentatricopeptide repeat PPR motif was found in ORF 10.

Dosage effect of the *Hwc1* and *Hwc2* genes on weakness symptom

Among F_2 populations between Nipponbare and Jamaica, normal and F_1-like plants (hereafter called normal type and F_1 type) were observed. In addition, two other classes of abnormal plants were encountered that had not been previously observed. One class of plants (severe type) had very tiny shoots and did not grow roots (Fig. 3). Germination of severe type was recognizable because of its embryo swelling one day after soaking in water. However, they did not grow further. The other class of plants (semi-severe type) had shorter shoots than F_1 plants and roots were rarely observed at 2 weeks after the sowing date (Fig. 3). The distribution of the length between the tip of second leaf and the base of the stem (hereafter called second leaf length) of the F_2 population is presented along with the four-group classification in Fig. 4. Although the distribution of plant height was rather continuous from semi-severe type to normal type, it was easy to distinguish the normal type from other types by their root growth. On the other hand, it is difficult to classify some F_2 plants as the semi-severe type and the F_1-like type. Therefore, second leaf length was tentatively adopted as the criterion to distinguish them. Plants with a second leaf length shorter than 10 cm were tentatively defined as semi-severe type. The remaining plants were defined as F_1 type plants.

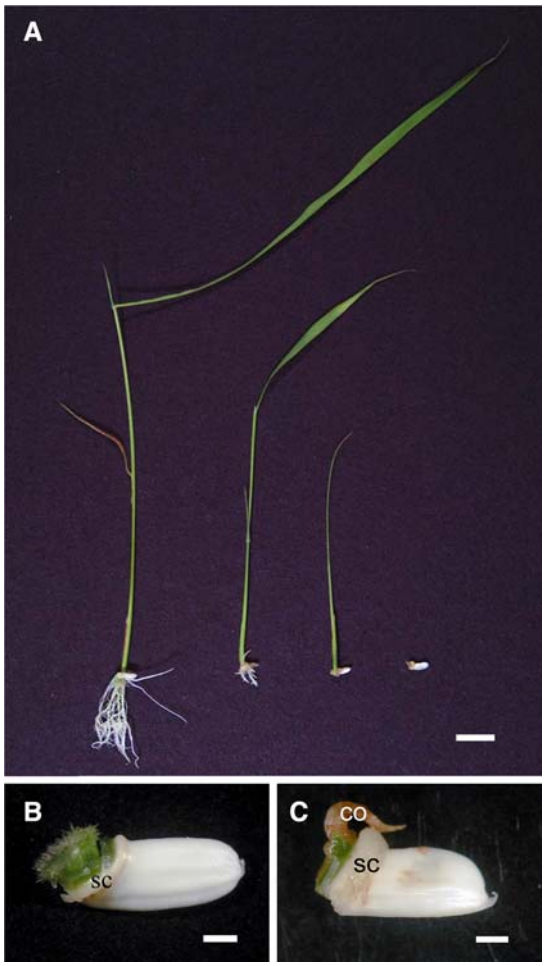

Fig. 3 Four plant types observed in F_2 population from the cross between Nipponbare and Jamaica. Seeds were sown on MS medium; fourteen days later, seedlings were classified into the following four types. **a** From left to right, normal type, F_1 type, semi-severe type and severe type. **b** and **c** Magnified pictures of severe-type plants. *sc* Scutellum, *co* coleoptile. A *bar of* **a** shows 1 cm and *bars of* **b** and **c** show 1 mm

To examine the relationship between the genotype of *Hwc1* and *Hwc2* and the weakness symptoms, F_2 progenies were analyzed using RMIBA6 and C111*4 (Table 3); RMIBA6 is

Table 2 Candidate genes in the *Hwc1* region

ORF	Protein	Rice ESTs	EST source	Tissue[a]
1	Heat-shock protein-like	AK065697	Nipponbare	Shoot
2	Unknown protein	AK105136	Nipponbare	Etiolated shoot
3	Putative amino acid transporters	AK105656	Nipponbare	Etiolated shoot
4	Unknown protein similar to amino acid or GABA permease	AK109289	Nipponbare	Shoot and root of germinating seeds
5	Putative STYLOSA protein	AK105049	Nipponbare	Green shoot
6	Unknown protein	AK101152	Nipponbare	Callus
7	Putative STYLOSA protein	AK105049	Nipponbare	Green shoot
8	Hypothetical protein	None		
9	Hypothetical protein	None		
10	Putative pentatricopeptide repeat (PPR)-containing protein	AK062372	Nipponbare	Etiolated shoot

[a] Information was obtained from The Rice Full-Length cDNA Consortium (2003)

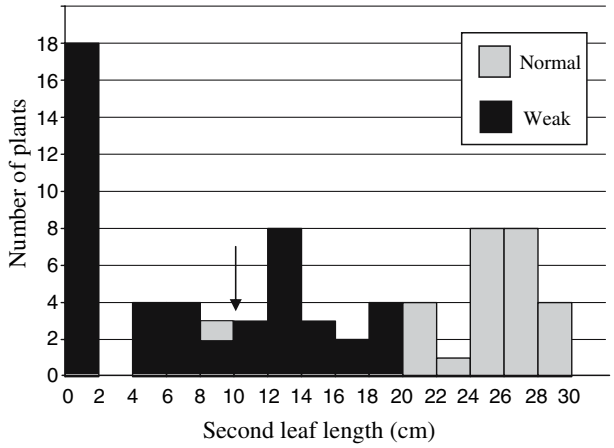

Fig. 4 The segregation of shoot growth observed in F_2 population of Nipponbare and Jamaica. Second leaf length of 74 F_2 plants was measured 14 days after sowing and used as an index of shoot growth. Normal type plants were distinguished from weak plants by their root growth and were subsequently painted *pale gray*. Plants showing symptoms of hybrid weakness are painted *dark gray*. An *arrow* shows the leaf length (10 cm) that divides semi-severe type and F_1 type

tightly linked with the *Hwc1* locus, so that the Jamaica allele of RMIBA6 indicates *Hwc1* and the Nipponbare allele of RMIBA6 indicates *hwc1*. On the other hand, C111*4 is tightly linked with *Hwc2*, so that the Nipponbare allele of C111*4 indicates *Hwc2*, and the Jamaica allele of C111*4 indicates *hwc2*. Segregation of the DNA marker linked with *Hwc1* was JJ:JN:NN = 57:43:21, not fitted to the expected ratio 1:2:1 ($\chi^2 = 28.817$, $P < 0.01$), skewed toward the Jamaica allele (Table 3). On the other hand, segregation of the DNA markers linked with *Hwc2* was JJ:JN:NN = 27:62:32, fitted to the expected ratio ($\chi^2 = 0.4876$, $0.90 > P > 0.75$). Normal plants were Nipponbare homozygotes for RMIBA6 or Jamaica homozygotes for C111*4, suggesting that their genotypes were either *hwc1/hwc1* -/- or -/- *hwc2/hwc2*. This result supports the genetic model proposed by Amemiya and Akemine (1963) that this hybrid weakness was attributable to a set of complementary dominant genes, *Hwc1 and Hwc2*.

Among the plants showing weakness, predicted homozygotes of *Hwc1* gene were severe or semi-severe phenotype, whereas the plants with the predicted heterozygotes of this gene were semi-severe or F_1 type. Thus, severe type plants were only observed in the predicted homozygotes of *Hwc1*. These results suggest that the *Hwc1* gene must be homozygous to express severe phenotype. They also indicate that *Hwc1* has a gene dosage effect on the degree of weakness, and that it is semi-dominant, although its penetrance is not complete.

The effect of *Hwc2* gene on the degree of weakness might be obscure. However, among the predicted homozygotes for *Hwc1* gene, the predicted homozygotes of *Hwc2* gene all showed severe phenotype, whereas predicted heterozygotes of *Hwc2* showed both severe and semi-severe types. Moreover, among the predicted heterozygotes of *Hwc1* gene, the ratio of semi-severe phenotype was much higher in predicted homozygotes of *Hwc2* gene (3 of 8) than in heterozygotes (2 of 22). These results indicate that *Hwc2* gene also has a dosage effect on *Hwc1* gene expressivity and that it is semi-dominant. In conclusion, the degree of weakness is determined mainly by the dosage effect of *Hwc1* gene. It is only slightly affected by the dosage effect of *Hwc2* gene.

Discussion

Many phenomena exist in relation to reproductive barriers that occur in F_1 or later generations in rice, such as hybrid inviability, hybrid breakdown, hybrid sterility, and cross-

Table 3 Relationship between weakness symptoms and predicted genotypes for the *Hwc1* and *Hwc2* loci with the aid of tightly linked DNA markers

Markers[a]		Predicted genotypes	Number of plants				
RMIBA6	C111*4		Severe	Semi-severe	F_1	Normal	Total
J/J	N/N	*Hwc1/Hwc1 Hwc2/Hwc2*	18				18
J/J	J/N	*Hwc1/Hwc1 Hwc2/hwc2*	16	13			29
J/N	N/N	*Hwc1/hwc1 Hwc2/Hwc2*		3	5		8
J/N	J/N	*Hwc1/hwc1 Hwc2/hwc2*		2	20		22
J/J	J/J	*Hwc1/Hwc1 hwc2/hwc2*				10	10
J/N	J/J	*Hwc1/hwc1 hwc2/hwc2*				13	13
N/N	N/N	*hwc1/hwc1 Hwc2/Hwc2*				6	6
N/N	J/N	*hwc1/hwc1 Hwc2/hwc2*				11	11
N/N	J/J	*hwc1/hwc1 hwc2/hwc2*				4	4
Total			34	18	25	44	121

[a] An SSR marker RMIBA6 was linked tightly with *Hwc1*. A CAPS marker C111*4 was linked tightly with *Hwc2*. For details, see text. 'J' shows Jamaica allele. 'N' shows Nipponbare allele

incompatibility. To date, three hybrid breakdown events have been analyzed genetically; their causal gene sets were mapped in rice (Fukuoka et al. 1998; Kubo and Yoshimura 2002, 2005). For other reproductive barriers such as hybrid sterility (Ikehashi and Araki 1986; Qiu et al. 2005) and cross-incompatibility (Matsubara et al. 2003), causal genes have already been mapped on a DNA-marker-based high-density linkage map. However, for genes controlling hybrid weakness in the F_1 generation, only the *Hwc2* gene was mapped on an RFLP-based linkage map (Ichitani et al. 2001). In this study, we fine-mapped *Hwc1*, narrowed down the area of interest to 60 kb, and identified eight putative candidate genes. We also fine-mapped *Hwc2* and narrowed down the area of interest to 150 kb on rice chromosome 4 (Kuboyama et al., unpublished data). The study of these reproductive barrier genes will advance phylogeny and evolutionary genetics of rice and other plant species.

Correspondence between *Hwc1* and other genes on rice chromosome 1 can be summarized as follows. An integrated map of RFLP markers and marker genes was constructed by Yoshimura et al. (1997). This integrated map is also available at http://www.shigen.nig.ac.jp/rice/oryzabase/genes/mapAction.do?categoryNo=0&chromosomeNo=1. In that map, six marker genes, *fs2* (fine stripe 2), *d18* (dwarf 18), *d2* (dwarf 2), *rl2* (rolled leaf 2), *spl6* (spotted leaf 6) and *eg* (extra glume), were located in this order among RFLP markers. A marker gene *rl2* is linked to an RFLP marker *XNpb368* with a genetic distance of about 10 centi-Morgans. *XNpb368* is closely linked with Y2820R and R2635. Because these two markers encompass *Hwc1*, *Hwc1* should be located near *rl2*, and between *rl2* and *spl6*. In addition, F_1 hybrids between Nipponbare and Jamaica have rolled leaves (Amemiya and Akemine 1963; Saitou et al. 2004). Therefore, it is interesting to examine the linkage relationship between *Hwc1* and *rl2*. A zygotic reproductive barrier causing segregation distortion was detected near the RFLP marker S13849 on chromosome 1 in the F_2 population from the cross between Nipponbare and Kasalath (Harushima et al. 2001); S13849 was linked with Y2820 and R2653. The segregation distortion around the *Hwc1* locus in this study might be attributable to the zygotic reproductive barrier described above.

Genetic analyses have revealed the gene dosage effect of *Hwc1* and *Hwc2* on hybrid weakness. The gene dosage effect was also reported in hybrid abnormalities of *Phaseolus vulgaris* (Shii et al. 1980) and in hybrid necrosis of *Gossypium* (Rooney and Stelly 1990). The *Phaseolus* case was also explained by the effects of two complementary dominant genes and the four classes of plants that are observed among the F_2 population. The number of plants within each class conformed to the 7:4:4:1 ratio. This result indicates that the severity of the inviability becomes stronger in proportion to the number of the dominant genes (Shii et al. 1980). Therefore, we infer that the two loci causing the *Phaseolus* hybrid inviability are both semi-dominant and contribute equally to this phenomenon. The plants belonging to the most severe class were homozygous dominant at both loci and have the expected frequency of 1/16. In the hybrid weakness between Nipponbare and Jamaica, because of the segregation distortion, we were unable to infer gene action considering only phenotype segregation. However, with the help of DNA markers, the gene dosage effect was also observed, and the causal genes did not contribute equally (Table 3): the gene dosage of *Hwc1* contributes more strongly to the severity of hybrid symptoms. Abnormal features are also different between the rice hybrid weakness and the *Phaseolus* case. The hybrid abnormalities reported by Shii et al. (1980) are chlorosis of leaves, necrosis, and retarded growth. In contrast, maintenance of root apical meristem (RAM) is the most common and pronounced problem of the hybrid weakness that occurs between Nipponbare and Jamaica (Amemiya and Akemine 1963; Saitou et al., unpublished data). We infer, therefore, that the genetic bases of both phenomena should differ. The *Gossypium* case was explained by intralocus and interlocus interaction, shown respectively by the dominant Le_2^{dav} gene from *G. davidsonii* at Le_2 locus and Le_1 and/or Le_2, dominant alleles from *G. hirsutum* at the Le_1 and Le_2 loci (Lee 1981). These two loci are thought to be on homoeologous chromosomal segments to each other on *G. hirsutum* allotetraploid genome (Samora et al. 1994). Increased gene dosage of Le_1 and Le_2 with Le_2^{dav} was reported to hasten necrosis (Rooney and Stelly 1990). No intralocus interaction is observed at *Hwc1* or *Hwc2*; their chromosomal regions were not homoeologous. Therefore, the *Gossypium* case is also genetically different from that of the present study. For the other hybrid weakness phenomena in which two dominant complementary genes are involved in rice (Oka 1957; Chu and Oka 1972), the symptoms were both characterized by inhibition of root elongation. However, no experiments were performed for allelic dosage effects using F_2 or later generations. Taking advantage of accumulation of rice genome sequences, further genetic analyses of these genes might increase our knowledge about hybrid weakness considerably.

Observation of hybrid development in the cross between Nipponbare and Jamaica shows that the hybrids have difficulty in the maintenance of RAM (Amemiya and Akemine 1963; Saitou et al., submitted). However, the growth of the shoot apical meristem (SAM) is also arrested in severe type plants of F_2, which indicates that *Hwc1 and Hwc2* are both involved in the SAM maintenance in parental lines, as in the RAM maintenance. The difference in susceptibility to the hybrid weakness between SAM and RAM is an interesting question to be solved in the future. As for genes related to the RAM maintenance, no reports have described genes

behaving in a semi-dominant manner as *Hwc1* does. As for genes related to the SAM maintenance, a mutation allele of an *Arabidopsis* gene *CLV1* was reported to behave in a semi-dominant manner (Clark et al. 1993). The mutant allele *clv1*, with strongest effect, disrupts the apical meristem structure by enlarging it and causing massive overproliferation. In addition, *CLV1* is known as a component of CLV receptor complex; the CLV signaling pathway is part of a negative feedback loop that maintains an appropriately sized stem cell reservoir in the SAM (Sharma et al. 2003). Probably, *Hwc1* is also involved in regulation of meristem maintenance. The strength of signal might be dependent on the gene dosage of *Hwc1*.

Gene annotation suggested that ten tentative ORFs are located on the target chromosomal region. One of them, ORF4, which is expressed on shoots and roots of germinating seeds (Table 2), can be a good candidate of *Hwc1*. Because homozygotes of both *Hwc1* and *Hwc2* genes show very short shoots as well as short roots, mutation in ORF1, ORF2, ORF3, and ORF10, all of which are expressed in shoots, might turn *hwc1* into *Hwc1*. Other candidate genes, ORF5 and ORF7 are similar to the *LEUNIG*, *Arabidopsis* homolog of *STYLOSA* (Navarro et al. 2004; Conner and Liu 2000). *LEUNIG* is expressed in roots and shoots in 10-day-old seedlings of *Arabidopsis*. Therefore, both ORF5 and ORF7 can be candidate genes. However, none of their homologs has been reported to act in a temperature-dependent manner. For candidate gene analysis, *Hwc2* should be also annotated. Much attention should be devoted to determining whether ORFs of two kinds of proteins that interact with each other are located separately in *Hwc1* and *Hwc2* candidate regions.

Now, we are undertaking closer linkage analyses of both *Hwc1* and *Hwc2* genes to clone them. Identification of theses genes will clarify the molecular mechanisms causing hybrid weakness and will provide a key to understanding rice varietal differentiation. In addition, gene identification will lead to better understanding of the molecular mechanisms controlling root and shoot formation.

Acknowledgments We gratefully acknowledge Drs. Izumi Kono at STAFF and Masahiro Yano at NIAS for valuable technical advice. This work was funded by the Ministry of Education, Culture, Sports, Science and Technology, and by the Ministry of Agriculture, Forestry and Fisheries of Japan (Green Technology Project GD-2005), Japan.

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