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Fine Mapping of *HWC2*, a Complementary Hybrid Weakness Gene, and Haplotype Analysis Around the Locus in Rice

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Abstract Hybrid weakness is a reproductive barrier. In rice, the hybrid weakness caused by two complementary genes—*HWC1* and *HWC2*—has been surveyed extensively. However, their gene products and the molecular mechanism that causes hybrid weakness have remained unknown. We first performed fine mapping of *HWC2*, narrowing down the area of interest to 19 kb. We thereby identified five candidate genes. Second, we performed haplotype analysis around the *HWC2* locus of 33 cultivars. With 15 DNA markers examined, all the 13 *Hwc2-1* carriers share the same haplotype for consecutive 14 DNA markers. As for *hwc2-2* carriers, five out of 20 have the haplotypes relatively similar to those of *Hwc2-1* carriers. However, the other haplotypes differ remarkably from them. These results are useful to identify the *HWC2* gene and to study rice varietal differentiation.

Keywords Reproductive barrier · Linkage analysis · Haplotype analysis · Hybrid weakness

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Introduction

Hybrid weakness is a reproductive barrier. It can be defined as weak growth occurring in hybrids derived from crosses between two normal strains. According to its degree or symptom, it is also called hybrid lethality, hybrid abnormality, or hybrid necrosis. This phenomenon has been observed in many plant species including *Arabidopsis thaliana* (Bomblies et al. 2007) and *Phaseolus vulgaris* (Shii et al. 1980).

In Asian cultivated rice (*Oryza sativa*), two hybrid weakness phenomena from different cross combinations have been reported (for a review, see Sato 1997). One reportedly exists in some Indian rice crosses (Oka 1957), the other in the cross between a Peruvian cultivar Jamaica and Japanese lowland cultivars such as Norin 8 (Amemiya and Akemine 1963). Among the two hybrid weakness phenomena, the latter phenomenon has been studied more extensively. Genetically, this hybrid weakness is caused by

two complementary genes: *Hwc1*from Jamaica and *Hwc2* from Japanese lowland cultivars. According to the new gene nomenclature system for rice (McCouch 2008), we hereafter change our description of the gene symbols, as shown in Table 1. The distribution of the two genes was surveyed by Sato and Hayashi (1983) and Sato and Morishima (1987). Among the cultivars they surveyed, most of temperate Japonica cultivars carry *Hwc2-1*, although few tropical Japonica and Indica cultivars carry this gene. None of the 30 strains of wild relatives (*Oryza rufipogon* and *Oryza nivara*) carries *Hwc2-1*. As for *HWC1*, Jamaica is reportedly the only carrier of *Hwc1-1* gene. From these results, Sato and Morishima (1988) inferred that the *Hwc2-1* gene arose at an early stage of differentiation of temperate Japonica cultivars.

The molecular mechanism of the hybrid weakness has remained unknown. To understand the mechanism, we should clarify the causal genes and their gene products. We selected a map-based cloning strategy to identify the two causal genes: *HWC1* and *HWC2*. We performed linkage analysis of *HWC1* (Ichitani et al. 2007) and thereby located this locus on the long arm of chromosome 1, narrowing down the area of interest to 60 kb. We also performed linkage analysis of *HWC2* (Ichitani et al. 2001) and tagged this locus between the two restriction fragment length polymorphism (RFLP) markers on the long arm of chromosome 4. However, we have not located this gene on a physical map. In the present study, we performed high-resolution mapping of *HWC2*, narrowing down the area of interest to 19 kb. We also performed haplotype analysis around the *HWC2* locus and identified linkage disequilibrium around this locus.

Results

Fine mapping using the F_2 population from the cross between Nipponbare and Kasalath

First, we selected the F_2 population from the cross between the two cultivars, Nipponbare and Kasalath, as a mapping population (Table 2) for two reasons: first, our preliminary experiments indicated that Nipponbare carries *Hwc2-1*, whereas Kasalath carries neither *Hwc1-1* nor *Hwc2-1*. The second reason is that high-density rice linkage maps have already been constructed using the same cross combination (Kurata et al. 1994; Harushima et al. 1998). This DNA marker information is also useful for *HWC2* mapping. Our previous study (Ichitani et al. 2001) showed that *HWC2* is encompassed by the two RFLP markers, *XNpb264* and *XNpb197*. In the present study, a sequence-tagged site (STS) marker C11112 and a cleaved amplified polymorphic sequence (CAPS) marker C1016 (Table 3, Fig. 1a, b) were used for selecting recombinants around the *HWC2* locus in 2003 because the two markers encompass the two RFLP markers and show polymorphism between the two cultivars. In all, 209 recombinants between the two DNA markers were selected from 1,190 F_2 plants. Among them, 165 recombinants were crossed to Jamaica to determine each genotype for the *HWC2* locus. This means that the number of F_2 plants in 2003 was equivalent to 939 (=1,190×165/209). Then, these recombinants were analyzed for the genotypes of the DNA markers encompassed by C11112 and C1016 to localize the *HWC2* locus. Results of this analysis showed that the *HWC2* locus was located between 93*14.1 and RM5473 and that the target region could be narrowed down to 298 kb.

In 2004, 37 recombinants between two DNA markers, 93*14.1 and S13714, were selected from 2,100 F_2 plants from the same cross combination. Among them, 33 recombinants were crossed to Jamaica to determine each genotype for the *HWC2* locus. This means that the number of F_2 plants in 2004 was equivalent to 1,873 (=2,100×33/37). Nine recombinants were found in the three-way cross population consisting of 890 plants. Consequently, the *HWC2* locus was located between dG264H and RM5473; the target region was narrowed down to 150 kb. As for the *HWC1* locus, the area of interest was narrowed to 60 kb using 883 F_2 plants (Ichitani et al. 2007). Therefore, we inferred that crossover events were suppressed around the *HWC2* locus in this cross combination. In Fig. 1b, recombination events per kilobase pair of 50 recombinants between

Table 1 Gene Symbols Frequently Used in This Study According to the New Gene Nomenclature System for Rice (McCouch 2008)

	Old		New	
	Gene full name	Gene symbol	Gene full name	Gene symbol
Locus/gene	*Hybrid weakness c1*	*Hwc1*	*Hybrid WEAKNESS C1*	*HWC1*
Dominant allele	*Hybrid weakness c1*	*Hwc1*	*Hybrid weakness c1-1*	*Hwc1-1*
Recessive allele	*hybrid weakness c1*	*hwc1*	*hybrid weakness c1-2*	*hwc1-2*
Locus/gene	*Hybrid weakness c2*	*Hwc2*	*Hybrid WEAKNESS C2*	*HWC2*
Dominant allele	*Hybrid weakness c2*	*Hwc2*	*Hybrid weakness c2-1*	*Hwc2-1*
Recessive allele	*hybrid weakness c2*	*hwc2*	*hybrid weakness c2-2*	*hwc2-2*

Table 2 The Cross Combinations for *HWC2* Fine Mapping

Cross combination						
Parent carrying *Hwc2-1*	Parent carrying *hwc2-2*	Year	No. of plants	DNA marker pairs for recombinant seedlings	Marker distance (kb)	No. of recombinants
Nipponbare	Kasalath	2003	1,190	C11112/C1016	3,027	209
Nipponbare	Kasalath	2004	2,100	93*14.1/S13714	314	37
Nipponbare	Kasalath	2004	890[a]	93*14.1/S13714	314	9
Akage	Asominori	2005	159	d54950/RM3843	240	0
Kinmaze	Asominori	2005	149	174*4D/RM3843	252	1
Nipponbare	Katakutara	2005	173	C174*12/RM3843	174	2
Akihikari	Katakutara	2005	317	d54950/RM3843	240	7
Nekken 2	Kasalath	2005	233	174*4D/S13714	260	13
Nekken 2	Silewah	2005	70	174*4D/RM3843	252	4
Nekken 2	Kasalath	2006	634	RM3687/S13714	209	5
Kinmaze	IR24	2006	370	174*4D/RM5473	245	13
Kinmaze	Asominori	2006	58	174*4D/RM5473	245	0
Akihikari	Katakutara	2006	1,475	174*4D/RM5473	245	29
Koshihikari	Te-Tep	2006	931	RM5503/RM3836	1,448	146
Akihikari	Nanjing 11	2006	399	RM3687/S13714	209	5
J-147	Asominori	2006	284	174*4D/RM5473	245	1
Akage	Asominori	2006	262	d54950/RM5473	245	0
Taichung 65	Ashkata	2006	364	RM5503/RM3836	1,448	54
Akihikari	Bhutmuri	2006	208	RM3687/RM5473	193	4
Akihikari	Kele	2006	313	RM3687/RM5473	193	2
Hayakogane	Nanjing 11	2006	459	RM3687/S13714	209	4
Taichung 65	Milyang 23	2006	237	RM5503/RM3836	1,448	29

[a] Three-way cross combination (Nipponbare×Kasalath)×Jamaica. The other cross combinations were F_2 generation

93*14.1 and S13714 were calculated for each pair of neighboring DNA markers. We observed the tendency that recombination events per kilobase pair were smaller for the DNA marker pairs that were closer to the *HWC2* locus, supporting the inference presented above.

Fine mapping using the F_2 population from other cross combinations

We examined 1,101 F_2 plants from other six cross combinations in 2005 to avoid the recombination suppression problem (Table 2). One useful recombinant was obtained from the cross between Kinmaze and Asominori. The recombination event occurred between C111*4 and C111*8, localizing the *HWC2* locus between C111*4 and RM5473. In 2006, we examined 5,994 F_2 plants from 13 cross combinations (Table 2). We confirmed the result in 2005 and obtained one useful recombinant from the cross between Kinmaze and IR24. The recombination event occurred between KGC4M28 and KGC4M29, localizing the *HWC2* locus between KGC4M28 and RM5473. From 2003 to 2006, RM5473 has remained the nearest DNA markers from the telomere of the long arm. These results showed that the position of *HWC2* was confined to about the 19-kb region between the two DNA markers, KGC4M28 and RM5473, both of which were located on the Nipponbare bacterial artificial chromosome (BAC) clone OSJNBa0053K19 (Table 2, Fig. 1c)

Candidate gene analysis

In all, five open reading frames (ORFs) were found between KGC4M28 and RM5473 in Nipponbare genome (Feng et al. 2002; Fig. 1c, Table 4). In an Indica cultivar Guangluai 4 genome, of which 2.3 Mb of three contiguous segments of chromosome 4 was sequenced (Feng et al. 2002), corresponding ORFs were found in the same order (data not shown). According to the Rice Full-Length cDNA Consortium (2003), all ORFs but ORF4 are expressed.

In fact, ORF1 has some similarity with a blight resistance gene *Xa1* (Yoshimura et al. 1998) from IRBB1, a near-isogenic line for bacterial blight genes (Ogawa et al. 1991), and has NBS-LRR motif, which is detected in most plant pest resistance genes. A reported full-length cDNA

Table 3 Primer Sequences Designed and Used for Fine Mapping of the *HWC2* Locus

Marker	Kind of DNA marker (restriction enzyme)	Primer sequence(5′–3′)		Position in Nipponbare chromosome 4 (AP008210)		Source
				From	To	
C11112	STS	F	CCAGCAACAGGGGATGAAGC	30325704	30325548	RGP[a]
		R	CAGGCATAAAACGGAGTGGC			
RM5503	SSR	F	GGGAAGAAGATAGGAGATGG	30397622	30397822	McCouch et al. 2002
		R	CTCTGGGTACACTTCACGAG			
RM1250	SSR	F	GAAACCACGACTAGGCATCG	31296033	31296199	McCouch et al. 2002
		R	CTTCCACAAGGTCTCGCTTC			
0093No3	CAPS (*Rsa*I)	F	TGTCCCATATCCTCCTTCAC	31335526	31335717	This study
		R	ACATGGCACACTAGGCTCAC			
93*14.1	dCAPS (*Hin*dIII)	F	CTTTGTCTCTCTTTTTCTTCCACTAAG	31412533	31412724	This study
		R	AGTGCAAAAGTATTTCCGTG			
174*4D	STS	F	CCGCATGCGCCAAGAAATCC	31465900	31466061	This study
		R	TCACGGCGCCACCACACGAC			
d54950	STS	F	CCATCATGCTGAACAAGCTCATTGGAT	31477258	31477426	This study
		R	TACTCCATCGTGTCCGTGTC			
RM3687	SSR	F	CTCCTGAGAAGTGGGGACTG	31517541	31517704	McCouch et al. 2002
		R	AGTCCTCCATGCATGTGACC			
KGC4M1	STS	F	CCGATCAGGTGACCAAACTT	31533990	31534079	This study
		R	GATGCATTGACCATGGAAGA			
KGC4M2	STS	F	AACGGATCTGGATAGGACCA	31536677	31536812	This study
		R	GGAGTTGTCTTCTTCCACTGC			
C174*12	CAPS (*Rsa*I)	F	CCTTTGCTGGTGTCAAGTCA	31543923	31544160	This study
		R	ACTGGTGCTTCCTGGTCCTA			
KGC4M3	STS	F	TGTTGAAATAAGACGGCTTGG	31559019	31559107	This study
		R	CGTTAGCTTCGCTTTCAGTG			
dG264H	dCAPS (*Hin*dIII)	F	ACCAGGAGATGACAACACAG	31560681	31560890	This study
		R	ATCCGCTCCAAATCAGCAAAGCT			
C174*15	CAPS (*Mbo*II)	F	CAGTGGGCTTGTGGGTAAGT	31572635	31572793	This study
		R	GCACAGCACAGTCAAAGTGG			
111*1	dCAPS (*Hin*dIII)	F	AGAGATGTTAGCAATTTCAATG	31588152	331588361	This study
		R	CTGTTCCTATCATTTGTGCAACCAAG			
KGC4M5	STS	F	AATCCCATCGCCCTTGTT	31611674	31611742	This study
		R	CTGCTGCTGCGGAGACAC			
C111*4	CAPS (*Mbo*II)	F	TTGGGAGGAAAATAGCTAAGGA	31627739	31627938	This study
		R	TTGCTGTAAGTGGCTGGGTA			
KGC4M10	STS	F	AATTGATTACTGGATGGCTTGATAA	31630713	31631087	This study
		R	GGAAGTAGACGAAATCGATGGTAT			
KGC4M20	STS	F	ACGAGGCGATGTGTCATGT	31644658	31644749	This study
		R	GCCAGTCCAAACGAACACTTAT			
C111*8	CAPS (*Mbo*II)	F	GTCAGCAGACAACCAGGTGA	31668298	31668529	This study
		R	CAGCATTATGGTTGAGCATG			
KGC4M21	STS	F	CGGAAGGTCAAAATTAATCAGAG	31678330	31696684	This study
		R	ACAGCAATCATCCTTTTCAGTAGAT			
KGC4M8	STS	F	TGCCTTTTGCTTACCACTGA	31690516	31690609	This study
		R	AGATCCGGCGGAAGAGAA			
KGC4M28	CAPS (*Hin*dIII)	F	GTATGCAAGATGTATCCATTTTTGTGTC	31690926	31691142	This study
		R	CATTTTCTTCTTCAGTGATATCAAAGTGC			
KGC4M29	CAPS (*Rsa*I)	F	CAATCACTTGAGGAACTTTACATCCA	31695274	31695409	This study
		R	AAGAATGGAGCTGCAGAGACACTAAAT			
KGC4M23	CAPS (*Hpa*I)	F	GTGGTTGGGATCGGAATTG	31699144	31699571	This study
		R	TCACTACCGTTATATGTTCACGAAT			
KGC4M30	dCAPS (*Pst*I)	F	CAATATAAATGTTGTTTTGTAAGCATTCTA	31701502	31701604	This study
		R	TCTTTACTAAATATATCGTTTTTCTTCTGC			

Table 3 (continued)

Marker	Kind of DNA marker (restriction enzyme)	Primer sequence(5′–3′)		Position in Nipponbare chromosome 4 (AP008210)		Source
				From	To	
IBA44	STS	F	CTGGACGATATCCACGAACC	31704500	31704669	This study
		R	TGGGACCAGGTAGGACTTTG			
KGC4M31	dCAPS (*Hpa*I)	F	GCCCTTAGTGTCATAGAGAGCATAA	31705085	31705215	This study
		R	TGTTTGATCATGACCTTGAGAGTT			
KGC4M27	STS	F	TGTGTGATACAATAACACCCAATG	31709144	31709269	This study
		R	ACCTAGTTAAATTCCAACGTCCAA			
RM5473	SSR	F	ACACGGAGATAAGACACGAG	31710458	31710562	McCouch et al. 2002
		R	CGAGATTAACGTCGTCCTC			
KGC4M18	STS	F	TGCTTGTGAAAAAGAGGGAAT	31716481	31716558	This study
		R	GCTGTGAACAGACCATAGTATTGAA			
RM3843	SSR	F	ACCCTACTCCCAACAGTCCC	31717543	31717696	McCouch et al. 2002
		R	GGGGTCGTACGCTCATGTC			
S13174	STS	F	CGGTTGCAGTGATCGAATTG	31726177	31726399	RGP
		R	CCAAATTCCCTGCCAACGAC			
KGC4M52	STS	F	GTTGTTGCGTATTCTTTGGATTC	31810027	31810150	This study
		R	CGCAATAATAATACAGGATAAACATAAAA			
RM3836	SSR	F	ACTGTGGAGTACAGGTCGGC	31845478	31845603	McCouch et al. 2002
		R	GAAACGGAAACGAAACCCTC			
C1016	CAPS (*Hae*III)	F	CACGCTCTTTCTATGTTTCC	33352175	33352823	RGP, modified in this study
		R	ATTACAACCACCCCTCCTC			

[a] http://rgp.dna.affrc.go.jp/E/publicdata/caps/index.html

AK105096 covers only 20% of ORF1; its sequence coincides completely with the 3′ region of ORF1. The number of amino acids in ORF1 is similar to that in XA1. Therefore, longer full-length cDNA corresponding to ORF1 might exist in Nipponbare genome. In addition, ORF2 exhibits similarity with the human *RING3* gene (Thorpe et al. 1996); ORF2 and RING3 share bromodomain. Although ORF3 has no similarity with known genes, this gene is expressed. ORF4 has only 12 residues and has no similarity with a known protein. Also, ORF5 encodes a protein similar to LSD1, which represses transcription by demethylating histone H3 at K4, whose methylation is linked to active transcription (Shi et al. 2004).

Haplotype analysis

Banding patterns of 15 DNA markers around *HWC2* locus of 33 cultivars were arranged in Fig. 2. The result of χ^2 test for independence indicated that significant deviation from the expected ratio ($P<0.01$) was observed between the genotype for the *HWC2* locus and the banding patterns of all DNA markers except KGC4M1 ($P=0.55$), IBA44 ($P=0.41$), and S13714 ($P=0.09$). As for IBA44 and S13714, the banding patterns not represented by Nipponbare were very few, leading to nonsignificant results. The two markers were designed based on the insertion/deletion between Nipponbare and Kasalath. As a whole, linkage disequilibrium was observed around the *HWC2* locus, supporting the result of the fine mapping (Fig. 1c). The banding patterns of KGC4M29 and KGC4M31 coincided with the genotypes for the *HWC2* locus. All cultivars produced a single band when using the KGC4M31 primer pair. The polymerase chain reaction (PCR) products were digested with the restriction enzyme *Hpa*I in all the *hwc2-2* carriers, although they were not digested with the enzyme in any *Hwc2-1* carriers. As for KGC4M29, one PCR product was produced and was not digested with *Rsa*I in *Hwc2-1* carriers. In contrast, the PCR products were all digested in *hwc2-2* carriers except for a few cultivars: before the digestion with *Rsa*I, Te-Tep showed one band with the same migration distance as the other cultivars. After the digestion, the band proved to be composed of two PCR products: one was digested with *Rsa*I, the other not. The four cultivars, Nanjing 11, Guangluai 4, Culture 340, and Silewah, produced no bands with the KGC4M29 primer pair.

For all the DNA markers except KGC4M1, the banding pattern carried by Nipponbare was mostly shared by *Hwc2-1* carriers. Among them, Koshihikari, Akihikari, and Taichung 65 are generally classified as temperate Japonica. The *Hwc2-1* carriers share the same haplotype ranging from KGC4M5 to KGC4M52. On the other hand, the other

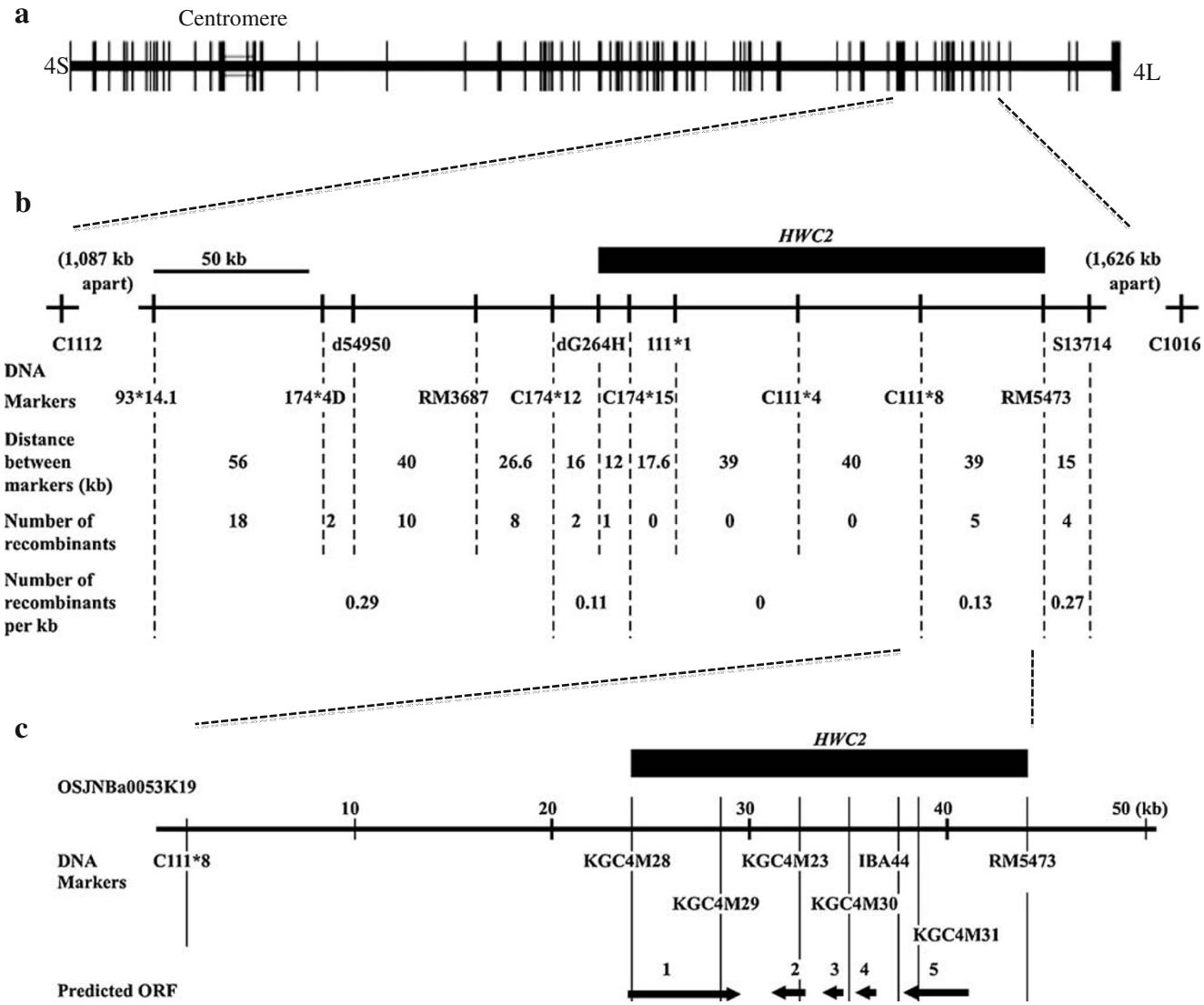

Fig. 1 Location of *HWC2* on rice chromosome 4. **a** Approximate location of the C1112 and C1016, initially used DNA markers for recombinants around the *HWC2* locus from the cross between Nipponbare and Kasalath, on the high-density linkage map of chromosome 4 modified from Harushima et al. (1998). **b** Fine mapping of *HWC2* using the cross between Nipponbare and Kasalath. A total of 50 recombinants between two DNA markers, 93*14.1 and S13714, were crossed to Jamaica and were analyzed for the order of *HWC2* and the internal DNA markers. **c** The 19-kb region containing *HWC2*, narrowed down by using all the cross combinations in Table 2, on the rice BAC clone OSJNBa0053K19 and positions of ORFs predicted using RiceGAAS (Sakata et al. 2002).

banding patterns and null bands were not shared by *Hwc2-1* carriers but by *hwc2-2* carriers. Among them, IR24, Milyang 23, Guangluai 4, and Kasalath are generally classified as Indica whereas Jamaica and Silewah are generally classified as tropical Japonica, and Norin 11, Iburiwase, and Asominori are generally classified as temperate Japonica. With 15 DNA markers scattered on the 276 kb of Nipponbare genome covering *HWC2* gene, the cultivars examined were classified into 13 haplotypes. The relationship between haplotypes is portrayed in Fig. 2. The cultivars were largely divided into two groups: one composed of *Hwc2-1* carriers and *hwc2-2* carriers whose haplotypes were relatively similar to those of *Hwc2-1* carriers and the other composed of *hwc2-2* carriers whose haplotypes differ remarkably from those of *Hwc2-1* carriers.

Discussion

Our previous study showed that the *HWC2* locus was located within the 3.9-cM region between two RFLP markers: *XNpb264* and *XNpb197*. The present study localized this gene on 19-kb region of the chromosome 4 physical map, where five candidate genes were annotated. We first selected the F_2 population from the cross between Nipponbare and Kasalath because of high DNA polymor-

Table 4 Candidate Genes in the *HWC2* Region with the aid of RiceGAAS (Sakata et al. 2002)

ORF	Protein	Rice ESTs	EST source	Corresponding predicted genes in	
				RAP-DB[a]	MSU rice genome annotation[b]
1	Putative XA1	AK105096[c] AB002266[d]	Nipponbare IRBB1	Os04g0622600	LOC_Os04g53160
2	putative RING3	AK067276[c]	Nipponbare	Os04g0623100	LOC_Os04g53170
3	Unknown protein	AK105231[c]	Nipponbare	Os04g0623200	LOC_Os04g53180
4	Hypothetical protein			None	None
5	Putative LSD1	AK064902[c]	Nipponbare	Os04g0623300	LOC_Os04g53190

[a] The Rice Annotation Project (2008)

[b] Ouyang et al. (2007)

[c] The Rice Full-Length cDNA Consortium (2003)

[d] Yoshimura et al. (1998)

phism and accumulation of DNA marker information. However, the candidate region was not narrowed down to less than 150 kb even though approximately 6,500 gametes were analyzed. We further examined 14,190 gametes from 18 kinds of other cross combinations and finally narrowed down the area of interest to 19 kb. In the cross between Nipponbare and Kasalath, chromosomal rearrangement such as inversion and insertion near the *HWC2* locus probably suppressed crossover events thereby.

Bomblies et al. (2007) showed that hybrid necrosis in *A. thaliana* was caused by complementary effect of two genes and that one causal gene encodes NB-LRR protein. This fact suggests that ORF1 can be a good candidate of *HWC2*. ORF2 was annotated to contain bromodomain, which is widely distributed among the different enzymes that acetylate, methylate, or remodel chromatin; it also has the ability to bind acetylated histone tails in vivo (De la Cruz et al. 2005). Therefore, it has been thought that chromatin-remodeling enzymes can utilize the bromodomain to find and/or act on their targets. Shi et al. (2004) showed that LSD1 specifically demethylates histone H3 lysine 4, which is linked to active transcription. ORF5, a homolog of LSD1, might catalyze the same reaction. Unusual expression of ORF2 or ORF5, working together with *HWC1*, might affect expression of other genes, engendering hybrid weakness.

Result of the haplotype analysis was consistent with the result of fine mapping. Among the DNA markers examined, the genotypes of KGC4M29 and KGC4M31 completely coincided with those for *HWC2*. The recognition site in KGC4M29 was located in ORF1, whereas that of KGC4M31 was in ORF5 (Fig. 1c). The results of the haplotype analysis suggest that ORF1 and ORF5 are the good candidates of *HWC2* gene, as inferred from their predicted functions.

The haplotype analysis also offered interesting results from the perspective of rice varietal differentiation. The *Hwc2-1* carriers share the same haplotype ranging from KGC4M5 to KGC4M52. The *hwc2-2* carriers have different haplotypes, most of which are distinct from those of the *Hwc2-1* carriers. Many haplotypes in *hwc2-2* carriers and only two haplotypes in *Hwc2-2* carriers led us to the following inferences: (1) the *hwc2-2* alleles have been differentiated at sequence level, some similar to *Hwc2-1*, others not. (2) The "gain-of-function" mutation from *hwc2-2*, probably of haplotype 3, to *Hwc2-1* occurred. (3) *Hwc2-1* gene diffused in temperate Japonica, dragging adjacent genes with it. To identify the origin and diffusion process of *Hwc2-1* and adjacent genes, we are undertaking larger haplotype analysis around *HWC2*. The DNA markers developed in this study will be very useful for that purpose. After cloning of *HWC2*, we also plan to perform the sequence analysis of *HWC2* to clarify the relation between the haplotypes and *HWC2* alleles or sequence variants.

Results of our previous study (Ichitani et al. 2001) indicated that *HWC2* and *Xa1* loci are closely linked to each other. A Japanese cultivar, Kogyoku, shows an example of conserved linkage block covering *hwc2-2* and *Xa1* surviving the selection in rice breeding history in Japan, where *Hwc2-1* carrier temperate Japonica cultivars have been prevalent (Sato and Hayashi 1983). Amemiya and Akemine (1963) reported that Kogyoku, which was originally written in Chinese characters and was called Kidama in Amemiya and Akemine (1963), does not carry *Hwc2-1*. Kogyoku carries a bacterial blight gene *Xa1*, which was introduced into a susceptible cultivar IR24 background by backcrossing to develop an isogenic line IRBB1 (Ogawa et al. 1991). The cDNA sequence of *Xa1* (AB002266; Yoshimura et al. 1998) of IRBB1 differed greatly from the corresponding sequence of Nipponbare, a typical temperate Japonica cultivar in Japan. The haplotype of the 15 DNA markers of Kogyoku is also very different from that of Nipponbare (Fig. 2). Nevertheless, we compared the electrophoresis patterns of the two cultivars of the 36 STS markers scattering on all the chromosomes with the result that the two cultivars share the same patterns except for one marker on chromosome 10

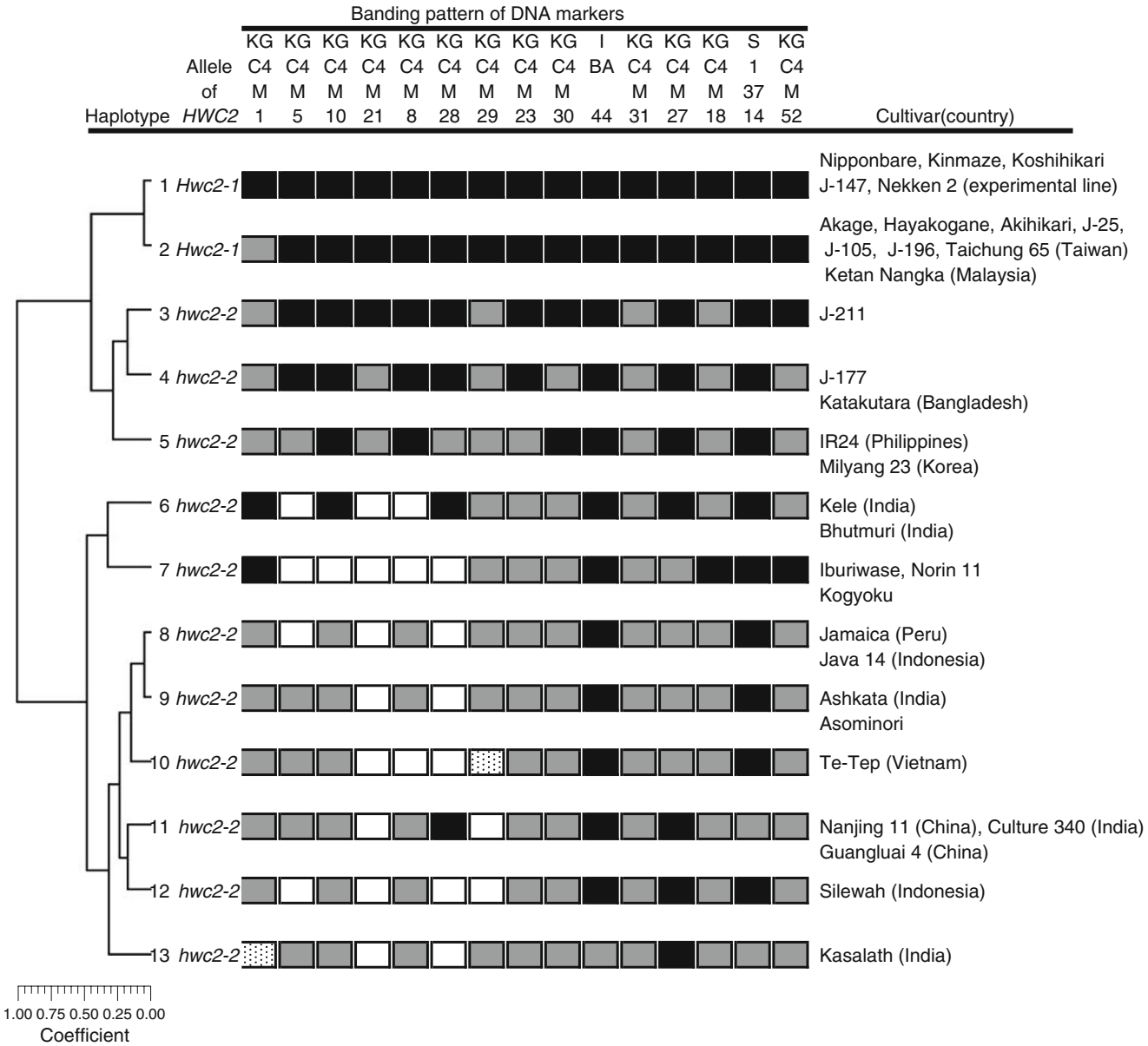

Fig. 2 Haplotype diversity around the *HWC2* locus. Banding patterns of 15 DNA markers around *HWC2* locus from KGC4M1 to KGC4M52 were analyzed for 33 cultivars whose genotypes of the *HWC2* locus had been identified, including parental cultivars of the fine mapping of the *HWC2* gene (Table 2). When the countries from which cultivars are originated are not specified, they are from Japan. The same banding patterns as Nipponbare are indicated by *solid rectangles*; the other patterns are indicated by *shaded* and *dotted rectangles*. *Open rectangles* indicate that no bands appeared. To the *left* is the genetic relationship among the 13 haplotypes by UPGMA cluster analysis. Each fragment size of the 15 DNA markers was treated as a unique characteristic and scored as present (1) or absent (0). The data matrix was used to calculate genetic similarities using the Jaccard (1908) coefficient.

(Ichitani et al., unpublished results). These facts imply that the chromosomal segment encompassing *hwc2-2* and *Xa1* gene pair of Kogyoku originated from Indica or tropical Japonica was introduced to temperate Japonica background by introgression. A similar story might be applied to *hwc2-2* carrier Japanese cultivars Norin 11, Iburiwase, and Asominori, and the opposite case might be an *Hwc2-1* carrier Malaysian cultivar Ketan Nangka, which is generally classified into tropical Japonica (Fig. 2).

Recently, two blast resistance gene have been mapped proximal to the *HWC2* locus: (1) a strong quantitative trait locus of field rice blast resistance named *Pikahei-1*(t) from upland rice cultivars Kahei cosegregated with RM5473 and mapped within 300 kb (Xu et al. 2008). (2) A blast field resistance gene *Pi39*(*t*) from Chubu 111 cosegregated with RM5473 and RM3843 (Terashima et al. 2008). A blight resistance gene *Xa2* (He et al. 2006) from a Vietnamese cultivar Te-Tep and a gall midge resistance gene *Gm7*

(Sardesai et al. 2002) from an Indica cultivar RP2333 are also located proximal to the *HWC2* locus. These resistance genes might have been introgressed into the local cultivars grown in the region where the above pests have been prevalent and have contributed to the adaptability of these cultivars. The alleles of the *HWC2* locus must have been dragged along by these resistance genes. Discussion of the distribution of *HWC2* gene should include the effect of linked genes on regional adaptability. The haplotype analysis showed that the banding pattern of KGC4M1 was independent of the genotype for the *HWC2* locus, although they are linked closely to each other genetically. This independence suggests that a small patch of chromosomal region around the *HWC2* locus might have been under selective pressure. Sato and Morishima (1987) reported a latitudinal cline of *HWC2* gene: *Hwc2-1* is more frequent in the more northern regions. The conserved haplotype around *HWC2* locus that is observed in *Hwc2-1* carriers might reflect the existence of linkage block adjusting to the environment of high latitudes.

We are performing closer linkage analyses of both *HWC1* and *HWC2* genes to clone them. Identification of these genes will contribute to understanding of the molecular mechanism causing hybrid weakness and rice varietal differentiation.

Materials and methods

Mapping strategy

The F_2 or three-way cross populations in which the *HWC2* gene segregated (Table 2) were grown in nurseries. At the seedling stage, recombinants between two DNA markers encompassing the *HWC2* locus were selected and transplanted individually in plastic pots. The detection of the genotypes of the recombinants for the *HWC2* locus followed the procedure described by Ichitani et al. (2001). They were crossed with *Hwc1-1* carrier Jamaica as a pollen donor. After harvest, hybrid seeds were dried at 50°C for 5 days to break dormancy. They were sown on Petri dishes containing 5-mm-deep tap water and left in the dark at 28°C for 5 days. The genotypes of each recombinant were estimated to be *Hwc2-1*/*Hwc2-1* if all hybrid plants showed inhibition of root elongation (usually less than 1 cm), *hwc2-2*/*hwc2-2* if they showed normal root elongation (usually more than 5 cm), and *Hwc2-1*/*hwc2-2* if hybrid plants segregated. A few intermediate phenotypes were transplanted in pots and grown for 1 month to check if hybrid weakness symptom as reported in our previous paper (Saito et al. 2007) was observed or not. The genotypes of internal DNA markers between the two initial encompassing markers of *HWC2* (Table 3) were tested to narrow down the area of interest.

DNA marker analysis

The DNA was extracted according to Dellaporta et al. (1983) with some modifications. The PCR conditions for STS, simple sequence repeat (SSR), and CAPS markers used in this study (Table 3) were: 95°C for 10 min, 40 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 1 min, except that the annealing temperature of KGC4M29 was 60°C instead of 55°C. Those for derived CAPS (dCAPS) markers were: 95°C for 10 min, ten cycles of 94°C for 30 s, 55°C for 2 min, and 72°C for 30 s, followed by 25 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a final extension of 72°C for 1 min, except that the annealing temperature of KGC4M30 was 50°C instead of 55°C. The PCR mixture (5 μl) contained 10-ng template DNA, 200 mM of each dNTP, 0.2 μM of primers, 0.25 units of Taq polymerase (AmpliTaq Gold; Applied BioSystems), and 1× buffers containing $MgCl_2$. As for KGC4M23, which contains GC-rich sequences, we used Takara LA Taq with GC buffer I (Takara Bio Inc.) instead of AmpliTaq. The PCR condition for KGC4M23 was 40 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s, followed by a final extension of 72°C for 1 min. 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 irradiation. The PCR products of CAPS and dCAPS markers were digested by respective restriction enzymes (Table 3) before electrophoresis.

Development of new DNA markers

Because the target region has been narrowed, no published DNA markers were present there. Therefore, we developed new PCR-based DNA markers. We collected DNA sequences covering the target region of three cultivars, Nipponbare, 93-11, and Guangluai 4. The Nipponbare chromosome 4 was sequenced completely (Feng et al. 2002). 93-11 is a parental cultivar of a superhybrid rice grown in China. It belongs to Indica: its draft sequence was produced by whole-genome shotgun sequencing (Yu et al. 2002). Guangluai 4 belongs to Indica. Its 2.3 Mb of three contiguous segments of chromosome 4 was sequenced (Feng et al. 2002). We also selected a Kasalath BAC clone K0211H09 covering the *HWC2* region by BLAST searching for BAC end sequences (http://rgp.dna.affrc.go.jp/blast/runblast.html, Katagiri et al. 2004). Shotgun sequencing was performed for the clone. We have already constructed a BAC library of Jamaica, which consists of 19,282 clones with average insert size of 104 kb, to clone *HWC1* gene from Jamaica. From the BAC library, we selected three Jamaica BAC clones covering the *HWC2* region by PCR screening using the most tightly

linked DNA markers of *HWC2* at that point using three-dimensional BAC pools as template DNA. Shotgun sequencing was performed also for these clones. The sequences of Kasalath and Jamaica consisted of several contigs and were therefore incomplete, but they provided sufficient information for the development of new DNA markers. The complete sequences of the two cultivars covering the *HWC2* region will be published elsewhere in the near future. We made alignments of the sequences of the five cultivars using DNAsis Pro (Hitachi Software Engineering Co. Ltd.) and detected substitutions and insertion/deletion (indels). We tried to produce STS markers by designing primers encompassing indels using Primer 3 (Rozen and Skaletsky 2000) if the indel size was longer than three bases. Regarding substitutions and indels shorter than four bases, we searched for restriction enzyme recognition sites along the polymorphic sequences with DNAsis Pro. We tried to produce CAPS markers by designing primers encompassing restriction enzyme recognition sites using Primer 3 if restriction enzyme recognition sites were present. Otherwise, we tried to make dCAPS markers by designing dCAPS primers including necessary mismatches using dCAPS finder 2.0 (Neff et al. 2002) and reverse primers using Primer 3.

Gene annotation

ORFs in the Nipponbare genomic DNA sequences containing the *HWC2* gene and the corresponding Guangluai 4 genomic sequences were predicted using a rice genome automated annotation system (RiceGAAS; Sakata et al. 2002 or http://RiceGAAS.dna.affrc.go.jp).

Haplotype analysis

We examined the genotypes of 15 DNA markers in the target region (Fig. 2) of 33 cultivars whose genotypes of the *HWC2* locus had been identified, including parental cultivars of the fine mapping of *HWC2* gene (Table 2). Actually, J-25, J-105, J-147, and J-177 were provided by Dr. Y. I. Sato, Research Institute for Humanity and Nature, Japan. The National BioResource Project, Japan, provided J-196 and J-211. Kogyoku and Guangluai 4 were provided by the Genebank of the National Institute of Agrobiological Sciences, Japan.

Cluster analysis of haplotypes

Genetic relations among the 13 haplotypes of *HWC2* region (Fig. 2) were evaluated using the 15 DNA markers. Each fragment size was treated as a unique characteristic and scored as present (1) or absent (0). The data matrix was used to calculate genetic similarities using the Jaccard (1908) coefficient. A phenogram of 13 haplotypes was constructed using unweighted pair-group method with an arithmetic mean (UPGMA) using software (NTSYS-pc ver. 2.2; Exeter Software).

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