Research Article

Chromosomal Location of HCA1 and HCA2, Hybrid Chlorosis Genes in Rice

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Many postzygotic reproductive barrier forms have been reported in plants [1]: hybrid weakness, hybrid necrosis, and hybrid chlorosis. In this study, linkage analysis of the genes causing hybrid chlorosis in F2 generation in rice, HCA1 and HCA2, was performed. HCA1 and HCA2 are located respectively on the distal regions of the short arms of chromosomes 12 and 11. These regions are known to be highly conserved as a duplicated chromosomal segment. The molecular mechanism causing F2 chlorosis deduced from the location of the two genes was discussed. The possibility of the introgression of the chromosomal segments encompassing HCA1 and/or HCA2 was also discussed from the viewpoint of Indica-Japonica differentiation.

1. Introduction

Many postzygotic reproductive barrier forms have been reported in plants [1]: hybrid weakness, hybrid necrosis, and hybrid chlorosis. The latter has been observed often in the F1 generation from crosses among wheat (Triticum aestivum L.) and its relatives [2–6]. This phenomenon resulted from the complementary action of a pair of dominant genes. Research for distribution of these genes contributed greatly to the study of the origin of wheat.

Hybrid chlorosis in F2 generation has been reported only in rice (Oryza sativa L.) [7] and interspecific crosses among Melilotus species [8]. Sato et al. [7] incidentally found a case of hybrid chlorosis in the F2 population from a cross between two Japanese native cultivars: J-147 and J-321. Its first symptom was discoloration of the second or third leaf (Figure 1). The yellowish part expanded gradually. Then the whole plant died within 20 days [9], yielding no seed. The phenomenon was caused by a set of mutually independent duplicated recessive genes, named hca-1 and hca-2 by Sato and Morishima [9]. According to the new gene nomenclature system for rice [10], we changed our description of the gene symbols, as shown in Table 1.

Rice is classified into two types: Indica-type and Japonica-type. Sato and Morishima [9] examined the distribution of HCA1 and HCA2. The experimentally obtained results can be summarized as follows. (1) The hca2-1 gene is widely distributed in native Japonica-type cultivars, whereas many Indica-type cultivars carry its dominant allele, Hca2-2. (2) J-147 carries hca1-1. This gene is probably rare because the occurrence of F2 chlorosis has not been reported in crosses between Taichung 65, which carries hca2-1, and many cultivars except for J-147. The mode of inheritance differs between wheat hybrid chlorosis and that of rice, but the distribution of causal genes is related to varietal differentiation in both cases.

We are interested in genes conferring the postzygotic reproductive barrier in rice, and we have mapped these genes in the rice genome with the aid of DNA markers [11–14]. We produced hybrids from crosses between J-147 and several cultivars to verify the results of Sato and Morishima [9]. From them, we incidentally found chlorotic plants in the F2 population from the cross between J-147 and a Philippine Indica-type cultivar IR24. We have never seen chlorotic plants in F2 population from the cross between IR24 and rice cultivars except J-147. Moreover, no reports in
Table 1: Gene symbols frequently used in this study according to the new gene nomenclature system for rice [10].

| Gene symbol     | Sato and Morishima (1988) [9] | This study | Gene full name                  | Cultivars harboring chlorosis-causing gene                  |
|-----------------|-------------------------------|------------|---------------------------------|------------------------------------------------------------|
| Locus/gene      | hca-1                         | HCA1       | HYBRID CHLOROSIS A1             | J-147                                                      |
| Recessive allele| hca-1                         | hca1-1     | hybrid chlorosis a1-1            |                                                            |
| Dominant allele | hca-1<sup>+</sup>             | Hca1-2     | Hybrid chlorosis a1-2            |                                                            |
| Locus/gene      | hca-2                         | HCA2       | HYBRID CHLOROSIS A2             |                                                            |
| Recessive allele| hca-2                         | hca2-1     | hybrid chlorosis a2-1            | Akihikari, Asominori IR24, Milyang 23, Many Japonica-type cultivars [9] |
| Dominant allele | hca-2<sup>+</sup>             | Hca2-2     | Hybrid chlorosis a2-2            |                                                            |

Figure 1: Hybrid chlorosis caused by hca1-1 and hca2-1. Seedlings in an F<sub>4</sub> line from the cross between J-147 and IR24 are shown 10 days after sowing date. A chlorotic plant is located at the center. The neighboring green plants are normal.

2. Materials and Methods

2.1. Plant Materials. Five rice cultivars were used for this study: J-147, Akihikari, Asominori, Milyang 23, and IR24. Dr. Sato of the Research Institute for Humanity and Nature provided J-147, Dr. Atsushi Yoshimura of Kyushu University provided Asominori and IR24 for this study. Dr. Yoshimichi Fukuta of Japan International Research Center for Agricultural Sciences provided Akihikari and Milyang 23. Akihikari and Milyang 23 are the parents of a set of recombinant inbred (RI) lines developed by Fukuta et al. [15]. Asominori and IR24 are the parents of another set of RI lines developed by Tsunematsu et al. [16]. No report in the relevant literature describes the appearance of chlorotic plants in progeny from a cross between Asominori and IR24, or from a cross between Akihikari and Milyang 23, although hybrid breakdown phenomena were reported for the cross between Asominori and IR24 [17, 18], and in the cross between Akihikari and Milyang 23 [19]. J-147, Akihikari, and Asominori are generally categorized as Japonica-type, whereas Milyang 23 and IR24 are generally categorized as Indica-type. J-147 was crossed with four cultivars: Akihikari, Asominori, Milyang 23, and IR24. F<sub>2</sub> populations from the above cross combinations were grown in nurseries. The plant spacing was 3 x 3 cm.

Chlorotic plants were segregated in the F<sub>2</sub> population from the cross between J-147 and IR24 (see Section 3). The normal plants were transplanted to a paddy field in the experimental farm of Kagoshima University to harvest self-pollinated seeds. Approximately 80 plants in each of 16 F<sub>3</sub> lines were grown in the nursery for the segregation of chlorotic plants. Among them, normal plants in the F<sub>3</sub> lines in which chlorotic plants segregated were transplanted in the same way as the F<sub>2</sub> generation. Approximately 80 plants in each F<sub>4</sub> line were also grown in the nursery for segregation of the chlorotic plants.

2.2. Linkage Analysis of HCA1 and HCA2. F<sub>4</sub> lines in which only HCA1 or HCA2 gene was expected to segregate were subjected to linkage analysis using DNA markers. Preliminary analysis using a small number of plants detected the approximate locations of HCA1 and HCA2. Then, we selected the F<sub>4</sub> lines segregating chlorotic plants in which one locus is fixed for a recessive chlorosis-causing allele, whereas the other locus is heterozygous and the heterozygous chromosomal region encompasses the locus that is extended most. These lines were used for construction of linkage map of HCA1 or HCA2. Linkage analysis was conducted using a computer program (MapDisto ver. 1.7; Lorieux [20]). Map distances were estimated using the Kosambi function [21]. After the linkage analysis, the F<sub>2</sub> populations from the cross between J-147 and IR24 were grown again.
- J147 (Japonica-type) x IR24 (Indica-type) 
  \[ \text{hca1-1/hca1-1 Hca2-2/Hca2-2} \] 

- F1: Hca1-2/hca1-1 Hca2-2/hca2-1 
  - Self-pollinated seeds from normal plants were harvested to produce the F3 generation. 
  - Self-pollinated seeds from normal plants in F3 lines in which chlorotic plants were segregated were harvested to produce F4 generation.

- F2: 
  - The distal regions of the short arm of chromosomes 11 and 12 are highly conserved as a duplicated chromosomal segment [24, 25, 26].

- F3: 
  - Chlorotic plants were all homozygous of J147 allele at the RM27421 locus, HCA1 was mapped on the distal end of the short arm of chromosome 12.
  - TF4 27–10: Chlorotic plants were all homozygous of IR24 allele at the E30794 locus, HCA2 was mapped on the distal end of the short arm of chromosome 11.

- F4: 
  - TF4 29–30: Construction of linkage map around HCA1 locus (Figure 3): cosegregation of HCA1 and RM27404 was detected.
  - TF4 23–19: Construction of linkage map around HCA2 locus (Figure 3): cosegregation of HCA2 and RM25969 was detected.

Figure 2: Flow chart showing the breeding of the plant materials for mapping HCA1 and HCA2 and for verifying that hca1-1 and hca2-1 were sufficient to cause hybrid chlorosis in the F2 population from the cross between J147 and IR24. Arrows with a solid line indicate the flow of generation of plant materials. Arrows with a dotted line indicate the flow of information on linkage between chlorosis genes and DNA markers.

2.3. DNA Marker Analysis. The DNA of plant materials except for the F2 population was extracted using the process explained by Dellaporta et al. [22] with some modifications. The DNA of the F2 population was extracted according to the experimental protocols of the Rice Genome Project (RGP) (http://rgp.dna.affrc.go.jp/E/rgp/protocols/index.html, written in Japanese) with some modifications [14]. The PCR conditions for indel and SSR markers used for this study 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 with subsequent final extension of 72°C for 1 min. The PCR mixture (5 μL) contained 1 μL of template DNA, 200 mM of each dNTP, 0.2 μM of primers, 0.25 units of Taq polymerase (AmpliTaq Gold; Applied BioSystems), and 1 × buffer containing MgCl2. The PCR products were analyzed using electrophoresis in 10% (29:1) polyacrylamide gel with subsequent ethidium bromide staining. Then they were viewed under ultraviolet light irradiation. Most PCR-based DNA markers used for this study have already been published. Some primer pairs did not perform well. Therefore, we redesigned them. Details of PCR-based DNA marker design were reported in our previous papers [13, 14].

3. Results
The F2 populations of the four cultivars with J-147 all produced both normal plants and chlorotic plants (Table 2).
The ratios of normal plants and chlorotic plants were fitted to 15:1, the expected ratio deduced from the segregation of two independent recessive genes. These results indicated that J-147 carries hca1-1 gene and that Akihikari, Asominori, Milyang 23, and IR24 carry the hca2-1 gene.

Indica-type cultivars and Japonica-type cultivars have frequently shown DNA polymorphism between them. This is true for Asominori and IR24, and for Akihikari and Milyang 23. Using the DNA polymorphism between the two pair of cultivars, DNA marker-based linkage maps were constructed [15, 16]. Results of our recent study indicated much polymorphism between Indica-type cultivars and J-147, and little polymorphism between Japonica-type cultivars and J-147 [14]. Therefore, the progeny from the cross between Indica-type cultivars and J-147 were more suitable for mapping genes. We selected the progeny from the cross between IR24 and J-147 as a mapping population for HCA1 and HCA2 because the linkage map constructed from the cross between Asominori and IR24 covered almost the whole genome, whereas that constructed from Akihikari and Milyang 23 had some large gaps, suggesting that some chromosomal regions exist with no DNA polymorphism.

Seeds of normal plants in the F2 population were harvested to produce the F3 generation. The segregation of chlorotic plants was examined for 16 F3 lines from each F2 plant. Only normal plants appeared in 10 lines. Chlorotic plants segregated in six lines. In the six lines, the ratios of normal plants: chlorotic plants were all fitted to 15:1. In the F3 generation, the expected ratio of lines fixed for normal plants, those showing 3 normal: 1 chlorotic segregation, and others showing a 15 normal: 1 chlorotic segregation, was 7:4:4. However, no lines showing 3 normal: 1 chlorotic segregation appeared. Then, six F3 lines in which segregated chlorotic plants were transplanted to a paddy field, and the seeds of normal plants were harvested to produce F4 generation.

The segregation of chlorotic plants was examined for 211 F4 lines from each F3 plant. The line named TF4 27-10 was a progeny from an F3 line TF3 27. In TF4 27-10, 70 normal plants and 10 chlorotic plants segregated, showing the maximum chlorotic plant ratio among the 10 TF3 27-derived F4 lines tested in our first experiment in the F4 generation and significantly deviated from 15:1 ratio. Another line named TF4 23-5 was the progeny of an F3 line TF3 23. In TF4 23-5, 80 normal plants and 7 chlorotic plants segregated, fitted to a 15:1 ratio. We performed a preliminary linkage analysis using a bulked DNA composed of 20 normal plants from TF4 27-10 and another bulked DNA composed of 20 normal plants from TF4 23-5, and 39 DNA marker scattered on all the 12 chromosomes. Because of successive self-pollination, the ratio of heterozygous chromosomal region reduced to approximately 0.25 in the F4 generation. HCA1 and/or HCA2 are expected to be located on heterozygous chromosomal region of the lines in which chlorotic plants segregated. Among the DNA markers, four showed heterozygosity in both lines. Then DNA from six chlorotic plants in both F4 lines was analyzed individually. Possible linkage was observed between chlorosis and KGS1739 [14], a DNA marker located on the short arm of chromosome 12: no homozygotes of IR24 allele were detected. This result suggests that HCA1 was located on the chromosomal region. Then we analyzed 7 chlorotic plant and 54 normal plants in TF4 27-10 line individually. Results showed that the cosegregation of HCA1 and the more distal marker RM27421 [23] was detected: all 7 chlorotic plants in this line were homozygous for J-147 allele at the RM27421 locus, whereas all 54 normal plants were heterozygous or homozygous for the IR24 allele at the locus.

Based on RFLP-based linkage analysis using the F2 population from the cross between Nipponbare and Kasalath, Nagamura et al. [24] reported that the distal regions of the short arms of rice chromosomes 11 and 12 are highly conserved as a duplicated chromosomal segment. Antonio et al. [25] confirmed the high degree of conservation of duplicated segments in these regions using four other mapping populations. Moreover, Wu et al. [26] generated physical maps covering most of the duplicated regions. RM27421 was within the duplicated region on chromosome 12 (Figure 3). These experimentally obtained results and the fact that the hybrid chlorosis was controlled by a couple of recessive duplicate genes led us to the idea that HCA2 might be located on the distal region of the short arm of chromosome 11. Our preliminary analysis using an F4 line named TF4 29-30 showed the cosegregation of HCA2 and E30794, an STS marker designed by RGP (http://rgp.dna.affrc.go.jp/E/publicdata/caps/index.html), located on the distal region of the short arm of chromosome 11: all 20 chlorotic plants in this line were homozygous for IR24 allele at the E30794 locus, whereas all 68 normal plants were heterozygous or homozygous for J-147 allele at the locus.

To construct linkage maps around the HCA1 and HCA2 loci, we selected F4 lines in which one locus is fixed for a recessive chlorosis-causing allele and the other locus is on the heterozygous region extending farthest. Using these lines (HCA1: TF4 33-21, HCA2: TF4 23-19, see Figure 2),

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**Table 2: Segregation for normal and chlorotic plants in the four F2 populations.**

| Cross combination         | Normal | Chlorotic | Total | $\chi^2$ (15:1) | $P$   |
|---------------------------|--------|-----------|-------|-----------------|-------|
| J-147 × Milyang 23       | 56     | 1         | 57    | 1.966           | 0.161 |
| J-147 × Akihikari        | 59     | 1         | 60    | 2.151           | 0.142 |
| J-147 × IR24             | 62     | 5         | 67    | 0.168           | 0.682 |
| J-147 × Asominori        | 60     | 2         | 62    | 0.968           | 0.325 |
we conducted linkage analysis of each gene using the DNA markers shown in Table 3. The DNA marker sources were the International Rice Genome Sequencing Project [23], RGP, McCouch et al. [28], Chen et al. [29], Monna et al. [30], and Ichitani et al. [14]. The linkage analysis of $HCA_1$ gene and six DNA markers using 13 chlorotic plants and 102 normal plants in TF$_4$ 33-21 showed that $HCA_1$ was located at the distal end of the short arm of chromosome 12 and that it cosegregated with RM27404 (Figure 3): all 13 chlorotic plants in this line were homozygous for J-147 allele at the RM27404 locus, whereas 102 normal plants were heterozygous or homozygous for IR24 allele at the locus. The ratio of 102 : 13 did not fit to the expected ratio 3 : 1. Cosegregation of $HCA_1$ and RM27404 and distorted segregation of the linked DNA markers (Table 4) showed that the distorted segregation of $HCA_1$ resulted from gametophytic reproductive barrier gene(s), often found in the cross between Indica-type and Japonica-type (see Section 4).

The linkage analysis of $HCA_2$ gene and seven DNA markers using 23 chlorotic plants and 96 normal plants in TF$_4$ 23-19 showed that $HCA_2$ was located at the distal end of the short arm of chromosome 11 and that it cosegregated with RM25969 (Figure 3): all 23 chlorotic plants in this line were homozygous for IR24 allele at the RM25969 locus, whereas 96 normal plants were heterozygous or homozygous for J-147 allele at the locus. The ratio of 23 chlorotic plants: 96 normal plants fitted the expected ratio 1 : 3. The segregation of the tightly linked DNA markers of $HCA_2$ also fitted the expected ratio 1 : 2 : 1 (Table 4). RM202 and RM5731, showing distorted segregation, were inherited independently of $HCA_2$. Harushima et al. [27] constructed an often-cited restriction fragment length polymorphism (RFLP) marker-based high-density linkage map for rice, in which some RFLP markers have been sequenced. Based on the Nipponbare genome sequence, the relation between our linkage map and that by Harushima et al. [27] is shown in Figure 3.

Using F$_4$ generation, we located $HCA_1$ and $HCA_2$ genes on the rice genome. However, the ratio of normal plants to chlorotic plants was often distorted from the expected one; that is, the chlorotic plants were often significantly fewer than
Table 3: Primer sequences designed and used for mapping HCA1 and HCA2 loci.

| Marker name | Kind of DNA marker | Primer sequences (5′–3′) | Location on IRGSP pseudomolecules Build05 | Source |
|-------------|--------------------|--------------------------|------------------------------------------|--------|
|             |                    |                          | Chromosome | Position From | Position To | |
| RM25969     | SSR                | F TAAATTTGGTTGTCTACGCATGG | 11         | 364229       | 364418     | [23] |
|             |                    | R CTGCTCCAGATTAGGAGCCAG  |            |               |            | redesigned in this study |
| E30794      | Indel              | F TCTGCCTATGTATTTGTGCTTAAT | 11         | 679545       | 679744     | RGP |
|             |                    | R AAGTAACACAACGAAGGAGCAAC |            |               |            | redesigned in this study |
| S1284       | Indel              | F ACATTCAACTGATCACAGCC   | 11         | 2186184      | 2186392    | RGP |
|             |                    | R AGCTCTGCACTAGGATGATG   |            |               |            | |
| RM5599      | SSR                | F AATTTTGTGCTGTTGTGAA    | 11         | 3810354      | 3810494    | [28] |
|             |                    | R CTCAATATATCCATCCAC     |            |               |            | |
| S21074      | Indel              | F TGCTATAGGGTGTTGATGC    | 11         | 5588871      | 5589049    | RGP |
|             |                    | R TTTCAAGCTGAGAGAGCAT    |            |               |            | |
| RM202       | SSR                | F CCAGCAAGCATGTATAATGA   | 11         | 9050541      | 9050718    | [29] |
|             |                    | R CAGATTGAGAGATTGCCTCC   |            |               |            | |
| RM5731      | SSR                | F CTTCACACCTAAGCTTCCCTC  | 11         | 10005222     | 10005423   | [28] |
|             |                    | R CGATGCACCTAGCGCATC     |            |               |            | |
| RM27404     | SSR                | F GCAGCGATTGAGGTGAGAA    | 12         | 204766       | 204863     | [23] |
|             |                    | R GACCGTGCCATTTGTCCAG    |            |               |            | redesigned in this study |
| RM27421     | SSR                | F TCAACTCCATCTACTTCTACC  | 12         | 468463       | 468538     | [23] |
|             |                    | R GCTGCTGGTACTCTTAGAG    |            |               |            | |
| KGS1739     | Indel              | F AGAGACGCAGGAGCTGTCCTA  | 12         | 1999528      | 1999818    | [14, 30] |
|             |                    | R CATGACCCCTCTATGGCAATTAT|            |               |            | |
| RM6296      | SSR                | F CCCACGCCTTCCTGTCCCT    | 12         | 3200580      | 3200734    | [28] |
|             |                    | R TCCTGCTGCAGGGTGTAG     |            |               |            | |
| RM27695     | SSR                | F CTATAAGAGTCCGGAGGGGTATTGT | 12      | 4828865      | 4829000    | [23] |
|             |                    | R GGGAGAGGATGTGAATGAG    |            |               |            | redesigned in this study |
| RZ869       | Indel              | F TTGTGATTTGTGCTGATG     | 12         | 7739507      | 7739753    | RGP |
|             |                    | R TATCAATCCATCCCACTC     |            |               |            | |

expected. Therefore, we again produced the F2 generation from the cross between J-147 and IR24 to confirm that hca1-1 and hca2-1 are sufficient to cause chlorosis in F2 generation with the aid of tightly linked DNA markers.

A total of 503 F2 plants were classified into 481 normal plants and 22 chlorotic plants. The ratio 481:22 looked slightly skewed towards normal plants but fitted the expected ratio 15:1 ($\chi^2 = 3.033$, $P = 0.08$). Genotypes at the RM25969 and RM27404 loci were analyzed for all F2 plants (Table 5). No homozygotes of J-147 allele at the RM27404 locus and IR24 allele at the RM25969 locus were present in normal plants. In contrast, all chlorotic plants were homozygotes of J-147 allele at RM27404 locus and IR24 allele at the RM25969 locus. This result indicated that hca1-1 and hca2-1 were sufficient to cause chlorosis in F2 population from the cross between J-147 and IR24. Segregation of
Table 4: Segregation of DNA markers linked with HCA1 and HCA2 in the mapping population of these genes from the cross between J-147 and IR24.

| Marker | Genotype \(^a\) | I | H | J | n | \(\chi^2\) (1:2:1) | P |
|--------|----------------|---|---|---|---|-------------------|---|
| HCA1 (chr. 12) | | | | | | | |
| RM27404 | 13 | 59 | 43 | 115 | 15.730 | <0.001 |
| RM27421 | 12 | 59 | 44 | 115 | 17.887 | <0.001 |
| KGS1739 | 11 | 61 | 43 | 115 | 18.235 | <0.001 |
| RM6296 | 15 | 62 | 38 | 115 | 9.904 | 0.007 |
| RM27695 | 19 | 59 | 37 | 115 | 5.713 | 0.057 |
| RZ869 | 21 | 61 | 33 | 115 | 2.930 | 0.231 |

HCA2 (chr. 11)

| Marker | Genotype \(^a\) | I | H | J | n | \(\chi^2\) (1:2:1) | P |
|--------|----------------|---|---|---|---|-------------------|---|
| RM25969 | 31 | 65 | 23 | 119 | 2.092 | 0.231 |
| E30794 | 28 | 68 | 23 | 119 | 2.849 | 0.241 |
| S1284 | 27 | 68 | 24 | 119 | 2.580 | 0.275 |
| RM5599 | 33 | 61 | 25 | 119 | 1.151 | 0.562 |
| S21074 | 36 | 63 | 20 | 119 | 4.714 | 0.095 |
| RM202 | 42 | 58 | 19 | 119 | 8.966 | 0.011 |
| RM5731 | 43 | 55 | 21 | 119 | 8.815 | 0.012 |

\(^a\) I, H, and J respectively denote homozygote for IR24 allele, and heterozygote and homozygote for J-147 allele.

Table 5: Segregation of DNA markers linked with HCA1 and HCA2 in the F2 population (n = 503) derived from the cross between J-147 and IR24. Numbers of chlorotic plants are shown in parentheses.

| Genotypes for RM27404\(^a\) on chr. 12 | I | H | J | Total | \(\chi^2\) (1:2:1) | P |
|----------------------------------------|---|---|---|-------|-------------------|---|
| Genotypes for RM25969 on chr. 11 | I | 38 (0) | 60 (0) | 22 (22) | 120 | 2.917 | 0.233 |
| H | 94 (0) | 139 (0) | 37 (0) | 270 | 2.917 | 0.233 |
| J | 37 (0) | 66 (0) | 10 (0) | 113 | 2.917 | 0.233 |
| Total | 169 | 265 | 69 | | | |

\(^a\) I, H, and J respectively denote homozygote for IR24 allele and heterozygote, and homozygote for J-147 allele.

RM25969 fitted the expected ratio 1:2:1, although that of RM27404 did not fit the expected ratio: homozygotes of IR24 allele were more numerous than expected, and homozygotes of J-147 allele were much fewer than expected. This result is consistent with that of F4 generation. The distorted segregation of chlorotic plants in F3 generation was thought to result from abnormal segregation at the chromosomal region around the HCA1 locus.

4. Discussion

In this study, HCA1 and HCA2 were located on the respective distal regions of the short arms of chromosomes 12 and 11. These regions are known to be highly conserved as a duplicated chromosomal segment [24–26]. According to the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/index.shtml) [31], 30 genes with known functions are located in common in the areas of interest of chromosomes 11 and 12. Therefore, HCA1 and HCA2 are thought to be mutually homoeologous genes, and the loss-of-function mutations of these genes are thought to be the cause of hybrid chlorosis. Based on the symptom, the causal genes of hybrid chlorosis should be involved in chlorophyll synthesis or chloroplast metabolism.

Recently, two papers reported the reciprocal disruption of duplicate genes causing reproductive barrier in interspecific and intraspecific rice crosses. Yamagata et al. [32] reported that the reciprocal loss of duplicated gene encoding mitochondrial ribosomal protein L27 causes hybrid pollen sterility in F1 hybrids of O. sativa and O. glumaepatula. Mizuta et al. [33] reported disruption of duplicated genes DOPPELGANGER1 (DPL1) and DOPPELGANGER2 (DPL2) causing pollen sterility: independent disruption of DPL1 and DPL2 occurred, respectively, in Indica-type and Japonica-type. DPLs encode highly conserved, plant-specific small proteins. In Arabidopsis thaliana, recessive embryo lethality is caused by a disruption of duplicated histidinol-phosphate amino-transferase genes encoding for a protein that catalyzes an important pathway leading to histidine incorporated into proteins [34]. The hybrid chlorosis described in this study might offer a new example of a reproductive barrier caused by disruption of duplicate genes.

The segregation of HCA2 and its tightly linked DNA marker RM27404 was significantly distorted from the expected ratio: the frequencies of Japonica-type alleles were
smaller than expected. The segregation distortion of the distal end of the short arm of chromosome 12 was reported in the populations derived from the crosses between Indica-type cultivars and Japonica-type cultivars: IR24 and Asominori [16], and Milyang 23 and Akihikari [35]. The peak of distortion was at a RFLP marker XNpb193 (= G193), and the frequencies of Japonica-type alleles were smaller than expected in both crosses. Therefore, the segregation distortion observed in this study might result from the same genetic factor(s).

Haplotype analysis around reproductive barrier genes might shed new light on varietal differentiation. Kuboyama et al. [13] performed haplotype analysis around the HWC2 locus. Carriers of the weakness-inducing allele Hwc2-1, most of which are categorized as temperate Japonica-type, share the same haplotype in the 200 kb region between the two DNA markers, KGC4M5 and KGC4M52. The carriers of hwc2-2 have different haplotypes, most of which are distinct from those of Hwc2-1 carriers. These results suggested that Hwc2-1 diffused in temperate Japonica-types, dragging adjacent genes with it. The hca2-1 gene is mainly carried by Japonica-type cultivars. Therefore, the haplotype analysis of HCA2 might engender new findings related to Japonica-Indica differentiation. We are undertaking haplotype analysis of HCA2 using a core collection of world rice [36] and a minicollection of Japanese rice landrace [37].

Before the experiment, we expected that Akihikari and Asominori, both generally classified as Japonica-type, carry hca2-1 gene whereas Milyang 23 and IR24, both generally classified as Indica-type, carry the wild type Hca2-2. Based on that expectation, we produced an experimental design in which J-147 was crossed with a set of RI lines so that linkage between HCA2 and DNA markers was detectable by combining the genotype of each RI line of DNA markers on the whole genome, which had been analyzed by the breeders of the RI lines, and the segregation of chlorotic plants in the F2 population between J-147 and each RI line. The experimental design using RI lines for mapping a reproductive-barrier-related gene was successful in a hybrid line. The experimental design using RI lines for mapping a reproductive-barrier-related gene was successful in a hybrid line. The experimental design using RI lines for mapping a reproductive-barrier-related gene was successful in a hybrid line. But, contrary to our expectation, all four cultivars carry hca2-1 gene. Milyang 23 is a descendant of IR24. Therefore, hca2-1 gene of Milyang 23 might derive from IR24. Zhao et al. [38] used a 1,536 SNP panel genotyped across 395 rice diverse accessions to study genomic-wide pattern of polymorphism, to characterize population structure, and to infer the introgression history, revealing that most accessions exhibit some degree of admixture, with many individuals within a population sharing the same introgressed segment because of artificial selection. Therefore, a high probability exists that the HCA2 locus and its surrounding chromosomal region of IR24 were introgressed from a Japonica-type cultivar. However, another possibility exists: loss-of-function mutation occurred at the HCA2 locus independently in Indica-type cultivars. The origin of Hca2-2 allele of J-147 is also interesting. Ichitani et al. [14] reported that J-147 shares the same banding patterns of 38 out of 39 PCR-based DNA markers scattered on the whole genome with the three cultivars generally classified as temperate Japonica-type. This result indicates that J-147 can be categorized as temperate Japonica-type. A DNA marker S1284 (Table 3, Figure 3), located at 2.2 Mbp from distal end of short arm of chromosome 11 (IRGSP pseudomolecules Build05), clearly discriminates Japonica-type and Indica-type, and J-147 carries the Japonica-type allele [14]. Hca2-2 allele of J-147 might also be the result of the introgression of small chromosomal segment of the distal end of the short arm of chromosome 11.

To identify the causal genes, we are undertaking closer linkage analysis and linkage disequilibrium analysis of both HCA1 and HCA2 genes. Identification of causal genes will contribute to the study of rice varietal differentiation, gene duplication, and chlorophyll synthesis or chloroplast metabolism.

**Abbreviations**

- RGP: Rice genome project
- PCR: Polymerase chain reaction
- SSR: Simple sequence repeat marker
- RI: Recombinant inbred
- Indel: Insertion/deletion
- RFLP: Restriction fragment length polymorphism

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