Detection and validation of QTLs for milky-white grains caused by high temperature during the ripening period in *Japonica* rice

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The occurrence of chalky rice (*Oryza sativa* L.) grains caused by high temperature is a serious problem in rice production. Of the several kinds of chalky grains, milky-white grains are not well analyzed. The milky-white rice grain phenomenon is caused by genetic factors as well as environmental and nutritional conditions. To analyze the genetic control system for rice grain quality, we raised recombinant inbred lines from progeny produced from ‘Tsukushiroman’ (high temperature sensitive) and ‘Chikushi 52’ (high temperature tolerant) cultivars. Quantitative trait locus (QTL) analysis revealed that the QTL on chromosome 4, linked to the simple sequence repeat marker RM16424, contributed substantially to the occurrence of milky-white grains, as it was detected over two experimental years. To validate the effect of the QTL, we developed near isogenic lines that have the ‘Chikushi 52’ segment on the short arm of chromosome 4 in the ‘Tsukushiroman’ genetic background, and that had a lower milky-white grain ratio than that of ‘Tsukushiroman’ when exposed to high temperatures during the ripening period. These results suggest that the ‘Chikushi 52’ allele on chromosome 4 suppresses the occurrence of milky-white grains and improves rice grain quality under heat stress during the grain ripening period.

**Key Words:** rice, QTL, high temperature tolerance, milky-white rice grains.

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assimilate supply to the grain. On the other hand, the percentage of the core type milky-white grains increases with a rise in mean daily temperature after heading (Tsukaguchi et al. 2012). These complexities have made it difficult to accurately analyze the genetic system for the appearance of milky-white grains. In this study, we focused on the milky-white grains caused by high temperature and tried to detect QTLs for the occurrence of these grains in high-temperature conditions during the ripening period by using recombinant inbred lines (RILs) (Wada et al. 2015). Furthermore, we validated the effect of the detected QTL for the occurrence of milky-white grains by using near isogenic lines (NILs) that had the chromosome segment of ‘Chikushi 52’ in the genetic background of ‘Tsukushiroman’. Both the genetic information and the materials developed in the course of this study contribute to the efficient breeding of new cultivars with fewer milky-white grains which will address problems of rice quality deterioration due to global warming.

**Materials and Methods**

**Plant materials**

We developed 88 RILs from a cross between ‘Tsukushiroman’ (Hamachi et al. 2003) and ‘Chikushi 52’ cultivars by the single seed descent method for this study. ‘Tsukushiroman’ is a cultivar that has good eating quality, but when it is exposed to high temperatures during the ripening period, it has a high chalky grain ratio. ‘Chikushi 52’ is derived from a cross between ‘Hitomebore’ (Sasaki et al. 1994) and ‘Yumetsukushi’ (Imabayashi et al. 1995) and is characterized by its resistance to high temperature during its ripening period and rare incidence of chalky grains even when it matures under heat stress conditions. The F7 and F8 lines were used for field experiments in 2009 and 2010, respectively, and the linkage map (Supplemental Fig. 1) constructed in our previous study (Wada et al. 2015) was also used for the QTL analysis for the occurrence of milky-white rice grains.

For the production of NILs, hybrids of ‘Tsukushiroman’ and ‘Chikushi 52’ were backcrossed with ‘Tsukushiroman’. After backcrossing four times and one self-pollination event, BC4F2 plants were selected for with assorted markers: 70 simple sequence repeat (SSR), four cleaved amplified polymorphic sequences (CAPS), and two derived cleaved amplified polymorphic sequences (dCAPS) (Supplemental Fig. 1, Supplemental Table 1). Finally, we obtained two NILs that harbored a ‘Chikushi 52’ segment around the regions of RM16423, RM16424, XspI 8, and RM5324 on chromosome (chr.) 4, which includes one of the QTLs (qMW4.1) detected in this study in the genetic background of ‘Tsukushiroman’. Both NILs had slightly different genotypes and were designated NIL03 and NIL04 to follow NIL01 and NIL02, which were produced and used in our previous study to validate the QTLs for white-back grains (Wada et al. 2015). We used both NIL03 and NIL04 to test whether the effect of QTLs for milky-white grains was detected in the analysis of RILs.

**Evaluation of grain quality in RILs and QTL detection**

We cultivated the RILs at the Fukuoka Agricultural Research Center (33°31′N, 130°30′E) in Japan. The RILs (F7, F8) were sown on May 25th and May 17th and transplanted on June 15th and June 9th into 1/5000a Wagner’s pots in 2009 and 2010, respectively. The pots contained 3 kg paddy soil with 5 g/m² nitrogen fertilizer. Two hills of these seedlings each were planted in each pot. Just after each plant had fully matured, the pots were moved into a greenhouse with an average controlled temperature around 30°C. After 10 days of heat treatment, they were relocated to an open field and grown until harvest. After harvesting, the grains were dried and husked, and fully matured grains were obtained by sieving (1.6 mm mesh). One hundred grains from one plant were used to evaluate the proportion of milky-white grains. A grain that was chalky throughout more than half of its area when observed laterally was defined as a milky-white grain.

Linkage analysis was performed with MAPMAKER/EXP3.0 (Lander et al. 1987). The frequency of recombination between two markers was converted to genetic distance using Kosambi’s map function (Kosambi 1943). Composite interval mapping was performed using Windows QTL Cartographer 2.5 (Wang et al. 2012) with forward and backward regression. The experiment-wise logarithm of the odds (LOD) threshold significance level was determined by computing 2000 permutations (Churchill and Doerge 1994), as implemented in QTL Cartographer.

**Validation of detected QTLs for milky-white grains**

For validating the QTL, we used NIL03 and NIL04 which harbored the ‘Chikushi 52’ segment on chr. 4 in the ‘Tsukushiroman’ genetic background. The examinations were carried out in 2013 at the Fukuoka Agricultural Research Center. Furthermore, we repeated the growing experiment at Ishikawa Prefectural University (36°30’N, 130°30’E) in 2013 and 2014, as well as at Kagoshima Prefectural Institute for Agricultural Development (Lat: 31°28′N, Lon: 130°20′E) in 2014. At Fukuoka, the NILs and parent lines were sown on April 30th and planted on May 21st in 2013. At Ishikawa, the seeds were sown in paper pots (R-5, Nippon Beet Sugar Manufacturing, Tokyo, Japan) on April 25th and April 23rd, and seedlings were transplanted into 1/5000a Wagner’s pots on May 22nd and May 23rd in 2013 and 2014, respectively. Plants were grown under outdoor conditions until the start of the temperature treatment in both years. At Kagoshima, the NILs were sown on April 25th and transplanted into greenhouse beds on May 15th in 2014. Rows were spaced 30 cm apart, with 15 cm between plants in each row. Heat treatments were also applied in each locality. In Fukuoka, the heat treatment was executed in the same way as for the RIL test. In Ishikawa, the NILs were transferred to partitioned temperature-gradient chambers (Nakagawa et al. 2009) at full heading and grown in them until maturity. The NILs grown in Kagoshima were planted in a greenhouse. The temperature
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during the heat treatment was controlled at approximately 30°C in all experiments. The harvested grains were weighed per hill and evaluated in the same way as for the RILs test. The significance of the results was evaluated in Statcel 3 (OMS Inc., Tokorozawa, Japan) using Dunnett’s multiple range test or a t-test following Ishikawa (2013).

**DNA isolation and genotyping**
Genomic DNA was extracted from mature leaves of a single plant from each of the RILs (F$_6$) and NILs (BC$_4$F$_2$) by the CTAB method (Murray and Thompson 1980). Polymorphic SSR markers, CAPS markers, and dCAPS markers between parents were selected from our previous study (Wada et al. 2015). PCR amplification of SSR, CAPS, and dCAPS markers used 40 ng DNA, 0.4 μM each primer, 100 μM dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl$_2$, and 0.5 units GoTaq DNA polymerase (Promega Corporation, Fitchburg, Wisconsin, USA) in 20 μl of reaction mixture. The amplification program was as follows: 5 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 50°C, and 2 min at 72°C; and a final extension of 5 min at 72°C. The PCR was performed in a T1 thermocycler (Biometra, Goettingen, Germany). Amplified products of SSR markers and final products of CAPS and dCAPS markers digested with corresponding restriction enzymes were electrophoresed in a 3.0% agarose gel or a 12.0% polyacrylamide non-denaturing gel. Agarose gels were stained with ethidium bromide and polyacrylamide gels with Gel Red (Biotium, Hayward, California, USA).

**Results**

**QTL detection for milky-white grains**
The average temperatures during the 10 days of treatment in the greenhouse were between 30.2°C and 30.4°C for each RIL in 2009, and between 30.4°C and 31.8°C in 2010. The milky-white grain ratio in ‘Tsukushiroman’ was 34.0 and 30.7% in 2009 and 2010, respectively; this ratio in ‘Chikushi 52’ was 5.0 and 1.0% in 2009 and 2010, respectively. Additionally, the ratios of milky-white rice grains in the 88 RILs were distributed between 1.7 and 52.8% in 2009 and between 0 and 70.5% in 2010 (Fig. 1). The milky-white grain ratios in ‘Tsukushiroman’ and ‘Chikushi 52’ were significantly different from each other in both years. The correlation of the ratios for milky-white grains among RILs between in 2009 and in 2010 was significant (r=0.362**) (Fig. 2). We performed QTL analysis for the ratio of milky-white grains and detected QTLs on chr. 2 (qMW2), 4 (qMW4.1, qMW4.2), and 9 (qMW9) in 2009. Of these QTLs, only qMW4.1 was mapped in 2010. Therefore, only qMW4.1 was detected in both years among the four QTLs (Fig. 3, Table 1).

**Validation of the QTL for milky-white rice grains**
To validate the effect of qMW4.1 in suppressing the occurrence of milky-white grains, we developed NIL03 and NIL04. Both lines contained a segment of the ‘Chikushi 52’ allele at RM16423, RM16424, Xspl_8 and RM 3524 on chr. 4 in the genetic background of ‘Tsukushiroman’. In addition, both lines partially had ‘Chikushi 52’ segments on chr. 2, 4, and 6 (Fig. 4). The segment on chr. 2 might have included qMW2.

In the validation test at Fukuoka in 2013, the average
temperatures during the heat treatment of NIL03, NIL04, and ‘Tsukushiroman’ the recurrent parent were 31.4°C, 31.7°C, and 32.4°C, respectively. The days to heading and milky-white grain ratios in the NILs were 81 d, 50.0% and 81 d, 69.0%, and the milky-white grain ratios were significantly lower than that of ‘Tsukushiroman’ (76d, 85.0%). In the Fukuoka validation test, the grain weight and ratios of white-back grains didn’t differ significantly among the experimental plants (Table 2). At Ishikawa in 2013, NIL03 showed a significantly lower ratio for milky-white grains (18.8%) than did ‘Tsukushiroman’ (33.8%). The ratios of white-back grains were not significantly different. However, the grain weight of NIL03 was significantly lower than that of ‘Tsukushiroman’. In 2014 at Ishikawa, the milky-white grain ratios in NIL03 (13.8%) and NIL04 (10.5%) were significantly lower than those in ‘Tsukushiroman’ (28.8%), while the white-back grain ratios and grain weight weren’t significantly different. The treatment temperatures were 29.9°C in 2013 and 30.0°C in 2014 (Table 2). Finally, at Kagoshima in 2014, both NILs showed significantly lower milky-white rice grain ratios (12.3% and 11.3%) than that of ‘Tsukushiroman’ (28.7%), while the white-back grain ratios and grain weight were not significantly different. The treatment temperature at this site was between 30.2 and 30.3°C (Table 2).

Discussion

To accommodate the increasing demand for rice breeding as global climate change affects agricultural growing conditions, we identified a QTL for improving the appearance of brown rice exposed to high temperatures during the ripening period. Many studies on rice grain chalkiness have been

Fig. 3. Putative quantitative trait loci (QTLs) for milky-white rice grains detected by composite interval mapping. QTLs were detected on the basis of 2,000 permutations. The numbers show LOD value and the numbers in parentheses indicate the test year. Up and down arrows indicate that the ‘Chikushi 52’ allele increased and decreased the milky-white grain ratio, respectively. Length of the arrows indicates the regions of the QTLs.

Table 1. Putative quantitative trait loci (QTLs) for milky-white rice grains detected by composite interval mapping

| QTLa     | Chr. | Nearest marker | Year | LODb | AEbc, PVEb | PVEb |
|----------|------|----------------|------|------|------------|------|
| qMW2     | 2    | RM5470         | 2009 | 2.90 | 3.81 10.3  |      |
| qMW4.1   | 4    | RM16424        | 2009 | 2.57 | -3.43 8.2  |      |
| qMW4.2   | 4    | RM6906         | 2009 | 3.06 | -3.77 10.1 |      |
| qMW9     | 9    | DdeI19         | 2009 | 2.76 | -3.72 9.0  |      |

a QTLs were detected on the basis of 2,000 permutations.  
b LOD: logarithm of the odds, AE: additive effect, PVE: Percentage of total phenotypic variance explained in each QTL.  
c Negative values of the additive effect indicate that ‘Chikushi 52’ alleles decrease milky-white grains.  
d The significant threshold of LOD values (P<0.05) in 2009 and 2010 were 2.16 and 1.61, respectively.

Fig. 4. Graphical representation of the near-isogenic line (NIL) genotypes used for the validation of quantitative trait loci (QTLs) for milky-white rice grains. We arbitrarily determined the recombination point to be the midpoint between markers for different genotypes: white, homozygous for the ‘Tsukushiroman’ segment; gray, homozygous for the ‘Chikushi 52’ segment; dashed line, heterozygous segment.
published, and those that did not separated grain chalkiness into specific types identified three QTLs for grain chalkiness on chr. 5, 8, and 10 using RILs derived from Japonica rice parents ‘Koshihikari’ (low percentage of grain chalkiness) and ‘C602’ (high percentage of grain chalkiness) (Liu et al. 2012). Additionally, Chen et al. (2016) also reported three QTLs on chr. 5, 7, and 8 using RILs generated from Indica and Japonica rice parents. Both results indicated that chr. 5 and 8 have genomic regions that control rice grain chalkiness. To analyze the common mechanisms for every type of grain chalkiness, it might be effective to consider all types of chalkiness as a single trait. Our previous study for white-back rice grains was approximately 28°C after heading, but the ratio of milky-white grains in NILs was lower than that in ‘Tsukushiroman’. This result clearly suggests that the effect of qMW2 was low or negligible compared to that of qMW4.1.

Wakamatsu et al. (2010) reported that the occurrence of white-back rice grains was accelerated when the temperature was over 28°C after heading, but the ratio of milky-white grains increased when the temperature was over 30°C. Their study suggested that the conditions that caused white-back and milky-white grain discoloration were different. We previously reported that a genomic region on chr. 8 is linked to RM3181 and RM3689 and affects the ratio of white-back grain emergence (Wada et al. 2015). However, in that study, no QTLs for chalky grains were detected on chr. 4, even though the same RILs were tested in both the previous and the present study. The difference between these two studies was the treatment temperatures during the rice ripening period. The high-temperature treatment in our previous study for white-back rice grains was approximately 27°C, while the heat treatment in this current study for milky-white grains was closer to 30°C. These differences revealed that the qMW4.1 allele detected in this study was effective only at temperatures of approximately 30°C. In addition, it also clearly indicates that the genetic regions that control white-back and milky-white grain coloration are not identical. These results support those of Wakamatsu et al. (2010), as well as providing evidence that the mechanisms that cause white-back and milky-white grains are different. Generally, rice grain chalkiness is caused by inadequate accumulation of starch. In chalky rice grains, compound starch granules are loosely packed with the many

### Table 2. Ratios of milky-white, white-back and grain weight in NILs and the recurrent parent cultivar ‘Tsukushiroman’

|          | Fukuoka | Ishikawa | Kagoshima | Ishikawa |
|----------|---------|----------|-----------|----------|
| **2013** |         |          |           |          |
| Milky-white (%) | 50.0** | 18.8* | 12.3* | 13.8* |
| (81 d/31.4°C) (83 d/29.9°C) | (64 d/30.2°C) (90 d/30.0°C) |
| NIL03    |         |          |           |          |
| NIL04    | 69.0** | –       | 11.3* | 10.5** |
| (81 d/31.7°C) | (65 d/30.3°C) (90 d/30.0°C) |
| Tsukushiroman | 85.0   | 33.8    | 28.7     | 28.8    |
| (76 d/32.5°C) (81 d/29.9°C) | (64 d/30.2°C) (90 d/30.0°C) |
| **2014** |         |          |           |          |
| White-back (%) | 5.5 | 20.5 | 50.3 | 17.0 |
| NIL03    | 0.5    |          | 51.7     | 14.5    |
| NIL04    | 6.0    | 6.4**   | 11.6     | 7.1     |
| Tsukushiroman | 2.0   | 12.8    | 18.0     | 10.3    |
| Grains weight (g/hill) | 5.6 | 6.4** | 11.1 | 7.5 |
| NIL03    | 6.0    |          | 11.6     | 7.1     |
| NIL04    | 4.4    | 11.0    | 10.6     | 7.2     |
| Tsukushiroman | 4.4   | 11.0    | 10.6     | 7.2     |

* The asterisks, **, * indicate that the ratio of milky-white grains, white-back grains and grain weight in each NIL were significant at the 1% and 5% levels, respectively, compared to that of ‘Tsukushiroman’ using Dunnett’s test or t-test.

* The numbers in the parentheses are days to heading (left) and temperatures during the heat treatment (right).
intergranular air spaces. This results in the irregular reflection of light, which appears as a chalky coloration to the human eye (Tashiro and Ebata 1975b, Zakaria et al. 2002). Therefore, genes that are related to starch metabolism in both synthesis and degradation may also control the occurrence of chalky-colored rice grains. One of the starch debranching enzymes, ‘PULLULANASE’, removes α-1, 6 linkages of rice amylopectin, and the Pullulanase gene is located on the short arm of chr. 4 (Nakamura et al. 1996). Furthermore, the levels of short amylopectin chains in PULLULANASE mutant lines were higher than those in wild-type (Fujita et al. 2009). Conversely, Kai and Iba (2014) suggested that the unusual accumulation of heat shock proteins could be involved in heat stress tolerance. One of the many heat shock protein genes, HSP82, is also located on the short arm of chr. 4 (Yamakawa et al. 2008). Yamakawa et al. (2007) also reported that the transcriptional level of HSP82 was up-regulated three-fold in high temperature conditions. Furthermore, ETHYLENE RECEPTOR 2, a protein that affects starch accumulation, is also localized on chr. 4 (Wuriyanghan et al. 2009). These genes could influence rice grain chalkiness. To understand how qMW4.1 works on milky-white grains, we need to trace its specific location in the region between RM16423 and RM3524. When we can finely map the QTL, we may learn whether the effect is triggered by one of the genes mentioned above or by a novel gene.

Miyazaki et al. (2013) conducted a DNA microarray analysis to detect genes responsible for heat stress tolerance in developing grains of ‘Koshihikari’. They found that 1,695 out of 32,000 rice genes were up-regulated more than two-fold under high temperature conditions. Combining the approaches from QTL analysis and expression analysis could be a useful way to identify which gene has the most important role, accelerating the study of which gene functions can improve grain quality.

In this article, we studied the genetic region controlling the occurrence of milky-white rice grains. The conditions that increase the core and ring type milky-white grains are different; the core type is induced by high temperature during grain filling period, and the ring type is induced by an assimilation shortage of carbohydrates in the grains (Wakamatsu et al. 2010). The competition for assimilation is caused by low sunlight conditions and characterized by the production of too many spikelets in the rice panicle. In our study, the treatment temperatures were over 29.9°C, with the exception of the validation test at Ishikawa in 2013, the grain weight did not differ significantly between NILs and ‘Tsukushiron’. These facts suggested that the milky-white grains we detected in this study were mainly core type milky-white grains caused by high temperature. However, in normal field conditions, core and ring type milky-white grains often emerge at the same time, and the mechanisms of their occurrence are different (Kondo et al. 2012). Therefore, we need to identify the experimental conditions that induce ring type milky-white grains to find solutions for this type of grain quality degradation.

In this study, we detected four QTLs for milky-white grains and successfully validated one of them, qMW4.1. This QTL is not identical to the QTL Ebitani et al. (2008) reported, which indicates that there are several key genetic factors related to milky-white grains. In white-back and basal-white grains, the QTLs detected using different genetic sources were not identical (Ebitani et al. 2008, Kobayashi et al. 2007, 2013, Tabata et al. 2007, Wada et al. 2015). This implies that the mechanisms of grain chalkiness occurrence are highly complex. Although it is not easy to understand the whole mechanism for grain chalkiness, narrowing the genetic regions of the detected QTLs and identifying the genes that play major roles in the production of chalky grains, as well as increasing the amount of genomic information on temperature-sensitive genes in rice, would provide a roadmap for solving the grain chalkiness problem inherent in global warming conditions.

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