Breeding Science 65: 216–225 (2015)
doi:10.1270/jsbbs.65.216

Research Paper

Detection of QTLs for white-back and basal-white grains caused by high temperature during ripening period in *japonica* rice

Takuya Wada*[1)], Katsunori Miyahara[1)], Jun-ya Sonoda[2)], Tadashi Tsukaguchi[3)], Masayuki Miyazaki[1,7)], Masao Tsubone[1,7)], Tsuyu Ando[4,8)], Kaworu Ebana[5)], Toshio Yamamoto[5)], Norio Iwasawa[6,9)], Takayuki Umemoto[6,10)], Motohiko Kondo[6)] and Masahiro Yano[5,11)]

[1)] *Fukuoka Agriculture and Forestry Research Center*, 587 Yoshiki, Chikushino, Fukuoka 818-8549, Japan
[2)] *Kagoshima Prefectural Institute for Agricultural Development*, 2200 Ohno Kinpou-chou, Minami-Satsuma, Kagoshima 899-3401, Japan
[3)] *Faculty of Bioresources and Environment Science, Ishikawa Prefectural University*, 1-308 Suematsu, Nonoichi, Ishikawa 921-8836, Japan
[4)] *Institute of the Society for Techno-Innovation of Agriculture, Forestry and Fisheries*, 446-1 Ippaizuka, Kamiyokoba, Tsukuba, Ibaraki 305-0854, Japan
[5)] *National Institute of Agrobiological Science*, 2-1-1 Kannondai, Tsukuba, Ibaraki 305-8602, Japan
[6)] *NARO Institute of Crop Science*, 2-1-18 Kannondai, Tsukuba, Ibaraki 305-8518, Japan
[7)] *Present address: Fukuoka Prefectural Office*, 7-7 Higashi-koen, Hakata-ku, Fukuoka, Fukuoka 812-8577, Japan
[8)] *Present address: National Institute of Agrobiological Science*, 2-1-1 Kannondai, Tsukuba, Ibaraki 305-8602, Japan
[9)] *Present address: Ibaraki Agricultural Research Center, Agricultural Research Institute*, 3402 Kamikunii, Mito, Ibaraki 311-4203, Japan
[10)] *Present address: NARO Hokkaido Agricultural Research Center*, NARO, 1 Hitsujigaoka, Toyohira, Sapporo, Hokkaido 062-8555, Japan
[11)] *Present address: NARO Institute of Crop Science*, 2-1-18 Kannondai, Tsukuba, Ibaraki 305-8518, Japan

There is increasing evidence that global warming affects the development of rice. High temperatures during ripening increase the ratio of undesirable chalky grains followed by deteriorating grain appearance quality. In order to detect quantitative trait loci (QTLs) controlling the occurrence of white-back and basal-white chalky grains of brown rice, QTL analysis was performed using recombinant inbred lines derived from a cross between two strains, 'Tsukushiroman' (sensitive to heat stress) and 'Chikushi 52' (tolerant of heat stress). The F_7 and F_8 lines were exposed to heat stress during the ripening period in two locations, Fukuoka and Kagoshima, in Japan. QTLs for white-back grains and basal-white grains were detected on chromosomes 1, 3, and 8, and those for basal-white grains were detected on chromosomes 2, 3, and 12. QTLs on chromosome 8 for white-back grains were shared in the plants grown in both locations. Near-isogenic lines (NILs), which harbored a segment from 'Chikushi 52' on chromosome 8 with the genetic background of 'Tsukushiroman', showed relatively lower ratios of white-back grains than 'Tsukushiroman'. Therefore, insertion of the 'Chikushi 52' genomic region of the QTL on chromosome 8 can improve the quality of rice when it is grown under heat stress conditions.

Key Words: rice, QTL, tolerance to high temperature, white-back grain, basal-white grain.

Introduction

Rice that is exposed to high temperature during ripening develops "chalky," inferior grains. Grain quality is an important factor that determines the value of the product, so the decline in quality leads to a loss of income for rice farmers. There are several types of chalky grains, including white-back (WB), basal-white (BW), white-belly, and milky-white grains, all caused by heat stress conditions during ripening.

Nagato (1952) studied the environmental factors that caused the occurrence of white-core, white-belly, and milky-white grains, and reported that chalkiness was affected by various factors, including the quantity of solar radiation, nutritional condition, and the location of kernels on the panicle. Furthermore, Nagato and Ebata (1965) and Tashiro and Ebata (1975) revealed that high temperature during ripening increased the ratio of WB, BW, and milky-white grains of rice. They also observed variations in the ratio of WB grains among rice cultivars. Tsubone *et al.* (2008) and

Communicated by K. Okuno
Received October 24, 2014. Accepted March 9, 2015.
*Corresponding author (e-mail: tawada@farc.pref.fukuoka.jp)

Wakamatsu *et al.* (2004, 2005) revealed that the ratios of WB and BW grains increased when the average temperature during ripening exceeds 27°C; in addition, they noted that there was great variation in the ratio of chalky grains among *japonica* rice cultivars under high-temperature conditions. These observations suggest that the occurrence of chalky grains of rice is controlled partly by genetic factors and partly by environmental factors.

Several studies have been performed to detect chromosomal regions controlling the occurrence of grain chalkiness. Tabata *et al.* (2007) reported that quantitative trait loci (QTLs) on chromosomes (chr.) 1, 2, and 8 were involved in the occurrence of WB grains. They used recombinant inbred (RI) lines derived from a cross between two strains, 'Chiyonisihiki' (sensitive to heat stress) and 'Koshijiwase' (tolerant of heat stress). Kobayashi *et al.* (2007) also detected QTLs for WB grains on chr. 3, 4, and 6, using F_2 and F_3 populations derived from a cross between 'Hanaechizen' (tolerant of heat stress) and 'Niigatawase' (sensitive to heat stress). Using RILs derived from the same cross, they also detected the same QTLs as in the case of F_2 and F_3 populations, and identified another QTL for WB on chr. 9, followed by the successful validation of the effects of the QTLs on chr. 6 and 9 using near-isogenic lines (NILs) (Kobayashi *et al.* 2013). Ebitani *et al.* (2008) detected QTLs for WB grains on chr. 2, 5, 6, 8, and 10 and QTLs for milky-white grains on chr. 1 and 7 by using chromosome segment substitution lines derived from a cross between the *japonica* cultivar 'Koshihikari' and the *indica* cultivar 'Kasalath'.

We previously developed a promising line named 'Chikushi 52 (CH)', a *japonica* cultivar that is tolerant of high temperature during ripening. This line is derived from a cross between 'Hitomebore' (Sasaki *et al.* 1994) and 'Yumetsukushi' (Imabayashi *et al.* 1995). These progenitors are not genetically closely related to 'Koshijiwase' or 'Hanaechizen', which were analyzed by Tabata *et al.* (2007) and Kobayashi *et al.* (2007), respectively. Therefore, we wished to explore whether 'Chikushi 52' harbored a new genetic factor controlling grain quality. To date, no genetic study has been reported on the grain quality of CH.

The objective of this study was to identify the QTLs controlling the occurrence of WB and BW grains in brown rice using RI lines (F_7 and F_8) derived from a cross between CH and 'Tsukushiroman' (TR), another *japonica* cultivar, under different levels of heat stress treatment, in two locations (Fukuoka and Kagoshima). We detected these QTLs and confirmed their effects on the appearance of WB grains by examining advanced backcrossed progeny, the BC_4F_3 and BC_4F_4 NILs.

Materials and Methods

Plant materials

TR is sensitive to heat stress and tends to show many chalky grains when it ripens under these conditions. On the other hand, CH is tolerant of heat stress and shows relatively lower chalky grains than TR, even when it ripens under high temperature. We conducted a genetic analysis of 88 RILs developed from a cross between TR and CH by means of single seed descent. QTLs were detected in the F_7 lines and F_8 lines in 2009 and 2010, respectively.

To validate allelic differences among detected QTLs, we developed NILs, such as BC_4F_3 and BC_4F_4. F_1 plants produced by a cross between TR and CH were backcrossed with TR. We conducted foreground selection on BC_3F_1 plants using simple sequence repeat (SSR) markers and cleaved amplified polymorphic sequences (CAPS) markers linked with the QTLs that we had detected (**Supplemental Table 1**). In addition to conducting foreground selection, after backcrossing, we also conducted background selection for the polymorphic DNA markers distributed on all chromosomes to confirm the recovery of the genome of the recurrent parent, TR. Finally, we developed NILs (BC_4F_3 and BC_4F_4), which harbored a CH segment with the genetic background of TR.

Heat stress treatments

In order to detect stable QTLs for improvement of grain quality, four different heat stress treatments were applied in this study: hot water irrigation (Kasaneyama *et al.* 1999, Tsubone *et al.* 2008) (**Fig. 1**A), early transplantation in the field (**Fig. 1**B), cultivation in a greenhouse during midsummer (**Fig. 1**C), and cultivation in a Temperature Gradient Chamber (TGC, **Fig. 1**D) (Horie *et al.* 1995). Irrigation with hot water (approximately 35°C) was known to increase the air temperature around each rice panicle 0.5 or 1 degrees higher than that of the ambient condition. Early transplantation in the field was done in the Kagoshima Prefectural Institute for Agricultural Development, Japan (Lat: 31°28′N, Lon: 130°20′E). Stable high temperatures exceeding 27°C were maintained during the ripening stage in this treatment. Plants cultivated in a greenhouse at the Kagoshima Prefectural Institute experienced even higher temperatures than those in the open field. Finally, in the TGC of Ishikawa Prefectural University (Lat: 36°30′N, Lon: 136°36′E, **Fig. 1**D), specific air temperature gradients were generated in order to evaluate the heat stress tolerance shown by rice plants under different temperatures. Hot water irrigation and early transplantation were used to detect QTLs influencing the ratios of WB and BW. All four heat stress treatments were employed to validate QTLs for WB grains using NILs.

Evaluation of grain quality of RILs field experiments

RI lines (F_7, F_8) were sown on April 23rd and April 23rd and transplanted on May 15th and May 12th in 2009 and 2010, respectively, at Fukuoka Agricultural Research Center (Lat: 33°31′N, Lon: 130°30′E) in Japan. Four plants of each RILs were raised in the sandy paddy field of the hot water irrigation facility (Tsubone *et al.* 2008), using a randomized block design with two replications. The spacing was 30 cm between rows and 15 cm between plants in each row. The amount of applied basal nitrogen fertilizer was 5 g/m^2; no

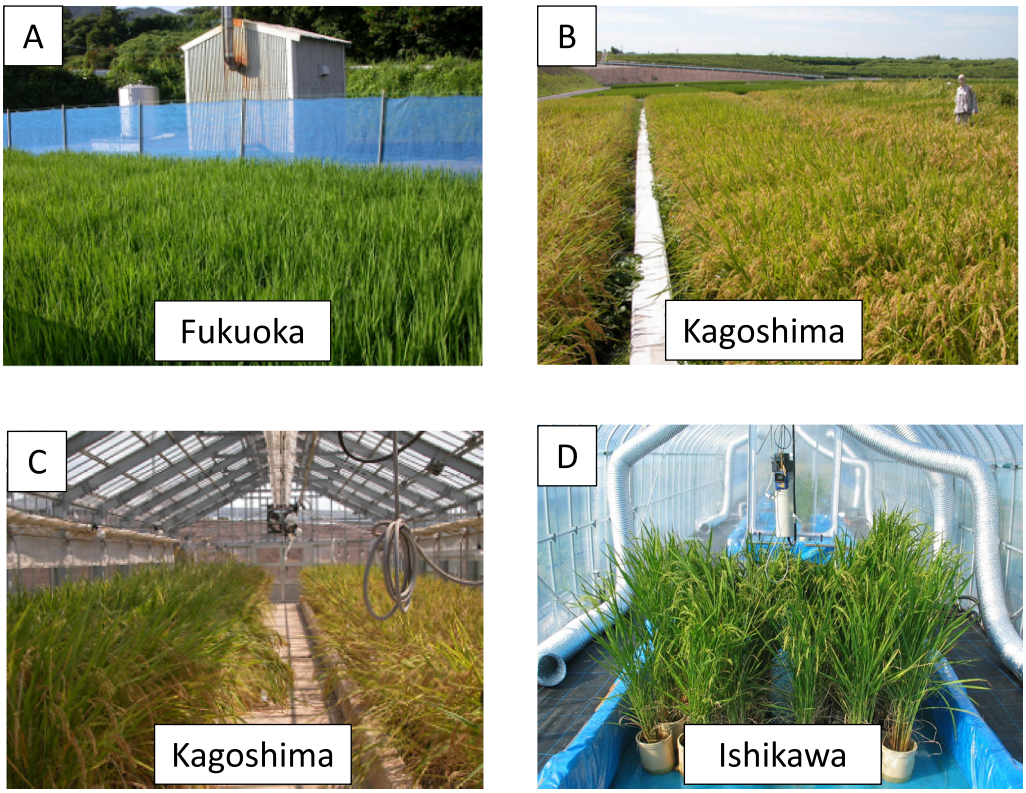

Fig. 1. High temperature treatment through (A) hot water irrigation, (B) early transplantation, (C) greenhouse cultivation, and (D) Temperature Gradient Chamber (TGC).

top dressing nitrogen was applied. Hot water irrigation was started after the first RIL headed (reached the stage at which half of the panicles were visible) and continued until 20 days after the last RI line headed. Air temperature during ripening was measured with Data Logger TR-52 (T&D Corporation, Matsumoto, Nagano, Japan). Two plants in each row of RIL plants were harvested at maturity and examined for the occurrence of chalky grains.

In order to evaluate the effect of early transplantation in the field, seeds of RILs were sown on April 28th and April 27th and transplanted on May 19th and May 20th in 2009 and 2010, respectively, in the paddy field at Kagoshima Prefectural Institute for Agricultural Development. Ten plants in each RIL were planted, using a randomized block design with two replications. Air temperature during ripening was measured with meteorological observatory equipment E-834 with hygrothermograph E-734 and pyrheliometers H0621 (Yokogawa, Denshikiki Co., Ltd., Shibuya, Tokyo, Japan). The spacing was 30 cm between rows and 15 cm between plants in each row. The amount of nitrogen applied was 2.5 g/m^2. One plant in each row of RIL was harvested at maturity and examined for the occurrence of chalky grains.

In both experiments, the number of days to heading (DTH) was counted, starting from the date of transplantation.

Validation of detected QTLs in WB grains

To test the effects of heat stress in the hot water irrigation facility in Fukuoka, seeds of NILs were sown on May 23rd and April 30th and transplanted to 1/5000 a Wagner pots that contained 5 g/m^2 nitrogen in each pot, on June 10th and May 21st in 2012 and 2013, respectively. One "plant" consisted of three seedlings, and two plants of each line were transplanted to each Wagner pot for each experiment. After the flowering of most spikelets had occurred, the plants were moved to the hot water irrigation facility and exposed to heat stress. Air temperature was measured with the same equipment as in the evaluation of RILs. At maturity, all the plants were harvested in order to measure the occurrence of chalky grains.

To evaluate the effects of heat treatment in the paddy field and greenhouse at Kagoshima, seeds of NILs were sown on April 22nd and transplanted on May 17th in the open paddy field and in soil in the greenhouse of Kagoshima Prefectural Institute for Agricultural Development in 2013. The amount of nitrogen applied was 4 kg/10 a. Ten plants in each line were raised, using a randomized block design with two replications. The spacing was 30 cm between rows and 15 cm between plants in each row. Air temperature and amount of solar irradiation were measured with the same equipment as in the evaluation of RILs. One plant in each row of NIL was harvested at maturity and the grain quality was measured.

To examine the effects of heat treatment in the TGC, seeds of NILs were sown on April 25th and transplanted on

May 22nd to 1/5000 a Wagner pots, each of which contained 25 g/m^2 nitrogen in 2013. One "plant" consisted of three seedlings, and eight plants of each line were used. Before heading occurred, topdressing fertilizer containing 15 g/m^2 nitrogen was applied to each pot. After heading of all lines had occurred, all the pots were moved into the TGC at Ishikawa Prefectural University and exposed to heat stress. Air temperature during ripening was measured with Data Logger RTR-53 (T&D Corporation, Matsumoto, Nagano, Japan). All the plants of NILs were harvested at maturity.

Evaluation of grain quality

In each experiment, grains of each RILs and NILs were dried and husked. The samples were sieved with 1.6-mm sifter. The ratio of chalky grains was determined from examination of 100 grains from each plant. The classification of immature grains was based on the criteria of Nagato and Ebata (1965). Grains with a white portion on the back side that exceeded half the total grain in length were considered WB grains. Grains with a white portion on the basal side were considered BW grains.

DNA isolation and genotyping

Genomic DNA was extracted from fully developed leaves of a single plant of each RILs (F_7) and NILs (BC_4F_3) by the cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson 1980). We screened more than 2,000 SSR markers from those reported by IRGSP (2005) and McCouch *et al.* (2002) to obtain polymorphic markers between the parental lines. PCR amplification of SSR markers used 40 ng DNA, 0.4 μM of each primer, 100 μM of each dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.5 units of Go Taq DNA polymerase (Promega Corporation, Fitchburg, Wisconsin, USA) in 20 μL of reaction mixture. The amplification profile 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 5 min at 72°C. PCR was performed in a T1 thermocycler (Biometra, Goettingen, Germany). Amplified products were electrophoresed in 3.0% agarose gel or 12.0% polyacrylamide non-denaturing gel. Agarose gels were stained with ethidium bromide, and polyacrylamide gels were stained with Gel Red (Biotium, Hayward, California, USA).

Single nucleotide polymorphism (SNP) markers showing polymorphism between the parental lines were selected from the genome-wide information about SNPs from Nagasaki *et al.* (2010). The genomic DNA of both parental lines was genotyped using these SNPs under Illumina GoldenGate BeadArray platform (Illumina, Inc.). Polymorphic SNPs were converted to CAPS or derived cleaved amplified polymorphic sequences (dCAPS) markers (**Supplemental Table 1**). Amplified products of these CAPS and dCAPS markers were digested with the corresponding restriction enzymes (**Supplemental Table 1**) and electrophoresed in 3.0% agarose gel.

QTL analysis

Linkage analysis was performed with MAPMAKER/EXP 3.0 (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 times permutations (Churchill and Doerge 1994), as implemented by the QTL Cartographer. The regions that showed significance in at least one year were nominated as QTLs in this experiment.

Results

Variation in grain quality in RILs

Average temperatures during the ripening period of RILs and their parental lines under hot water irrigation in 2009 and 2010 ranged from 26.7 to 28.0°C and from 28.9 to 29.3°C, respectively. Ambient temperature during that experiment in 2009 and 2010 was from 26.2°C to 27.2°C and from 28.3°C to 28.5°C, respectively. Plants that received early transplantation in Kagoshima in 2009 and 2010 experienced temperatures that ranged from 27.2 to 28.6°C and from 28.1 to 28.6°C, respectively. Although the average temperatures experienced by several lines were lower than 27°C in 2009 due to early flowering, most of the RI lines and their parental lines were exposed to high temperatures exceeding 27°C.

CH showed earlier heading than TR in all the experiments and the DTH of RILs ranged from 58 to 82 days, showing transgressive segregation beyond DTH of both parents (**Fig. 2**A). However, the grain weight (GW) of both parents was almost the same or showed only slight differences in all the experiments, whereas the GW of RILs had a wider distribution than did that of the parental lines (**Fig. 2**B). The ratio of WB grains of CH was lower than that of TR in the hot water irrigation group in 2009 and 2010, as well as lower than that of the early transplantation group of 2009 (**Fig. 2**C). Although the ratio of WB of RILs in 2009 was generally distributed between the values of both parents in the hot water irrigation group, transgressive segregation to higher ratios in 2009 was observed. In 2010 most of the RILs showed lower ratios of WB than both parents. But it showed a wider distribution in the early transplantation group (**Fig. 2**C). The frequency distribution of the ratio of WB was mostly continuous in both experiments (**Fig. 2**C). The ratio of BW grains followed a similar trend to that of the WB grains. These facts showed that not only environmental conditions but also multiple genetic factors contributed to the occurrence of WB and BW of RILs (**Fig. 2**D).

Correlation coefficients of the ratio of WB, BW, GW and DTH of RILs

Correlation analysis was carried out among all traits in

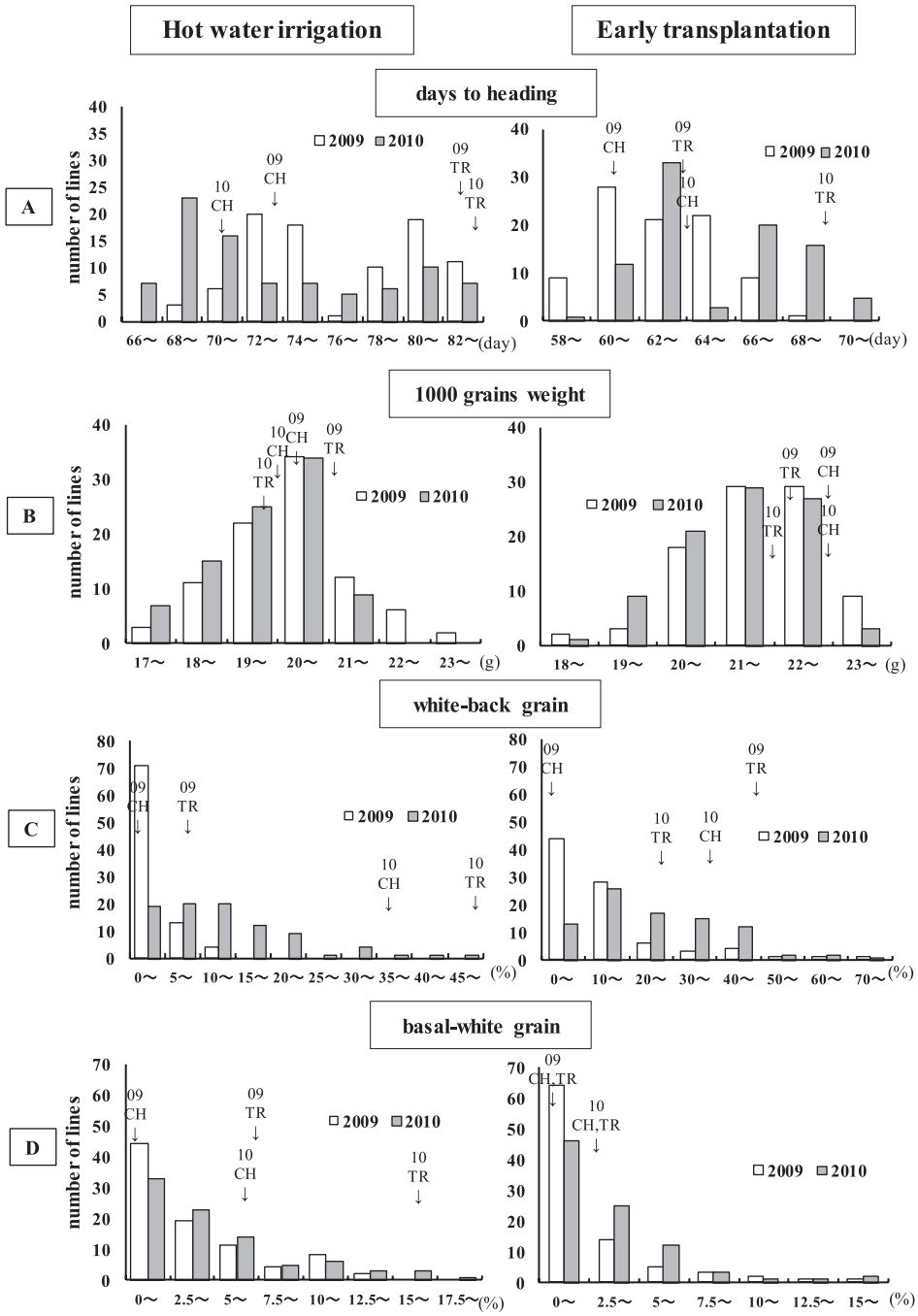

Fig. 2. Frequency distributions of (A) days to heading, (B) 1000-grain weight, (C) ratio of white-back grains, and (D) ratio of basal-white grains of recombinant inbred lines in the experiments using hot water irrigation (left) and early transplantation (right). CH: 'Chikushi 52', TR: 'Tsukushiroman'.

two experiments (**Table 1**). Although correlation coefficients of DTH were highly significant among two years in two experiments (r = 0.931** and 0.894**), those of WB and BW ratios were not significant except for the ratio of BW in the early transplantation group (r = 0.388**). The correlation coefficients of WB and BW with DTH were significant only in the early transplantation group of 2009 (r = 0.636** and 0.348**) and were not significant in the hot water irrigation group. GW was adversely correlated with the ratio of WB only in the early transplantation group of 2009 (r = –0.213*), but it was correlated with the ratio of BW in hot water irrigation of 2009 and 2010 (r = –0.340**, –0.409**) and with the early transplantation group of 2009 (r = –0.526**).

QTLs for the occurrence of WB and BW grains

Although we searched more than 2,000 SSR markers, only 70 markers were polymorphic between the parental lines. In order to cover the gap regions, where marker density was low, we screened 6 SNPs from the collection of SNPs

Table 1. Correlation coefficient of ratio of white-back grains, ratio of basal-white grains, 1000-grain weight, and days to heading of recombinant inbred lines

(A) Hot water irrigation in Fukuoka

Year	Trait name	2009				2010			
		WB[a]	BW[a]	GW[b]	DTH[b]	WB[a]	BW[a]	GW[b]	DTH[b]
2009	White-back grains ratio	1							
	Basal-white grains ratio	0.512 **	1						
	1000-grains weight	–0.034	–0.340 **	1					
	Days to heading	0.173	–0.112	0.336 **	1				
2010	White-back grains ratio	0.115	0.069	–0.145	0.125	1			
	Basal-white grains ratio	0.208 *	0.041	–0.056	–0.102	0.379 **	1		
	1000-grains weight	–0.178	–0.049	–0.014	0.134	0.068	–0.409 **	1	
	Days to heading	0.186	–0.115	0.425 **	0.931 **	0.119	–0.079	0.081	1

(B) Transplanting in early season in Kagoshima

Year	Trait name	2009				2010			
		WB[a]	BW[a]	GW[b]	DTH[b]	WB[a]	BW[a]	GW[b]	DTH[b]
2009	White-back grains ratio	1							
	Basal-white grains ratio	0.232 *	1						
	1000-grains weight	–0.213 *	–0.526 **	1					
	Days to heading	0.636 **	0.348 **	–0.313 **	1				
2010	White-back grains ratio	–0.050	0.037	0.036	–0.001	1			
	Basal-white grains ratio	0.109	0.388 **	–0.065	0.049	0.455 **	1		
	1000-grains weight	–0.100	–0.297 **	0.710 **	0.130	0.073	–0.016	1	
	Days to heading	0.613 **	0.358 **	–0.299	0.894 **	0.062	0.092	0.205	1

[a] WB and BW mean white-back grains ratio and basal-white-grains ratio, respectively.
[b] GW and DTH mean one-thousand grains weight and days to heading, respectively.
** and *: significant at 1% and 5% levels, respectively.

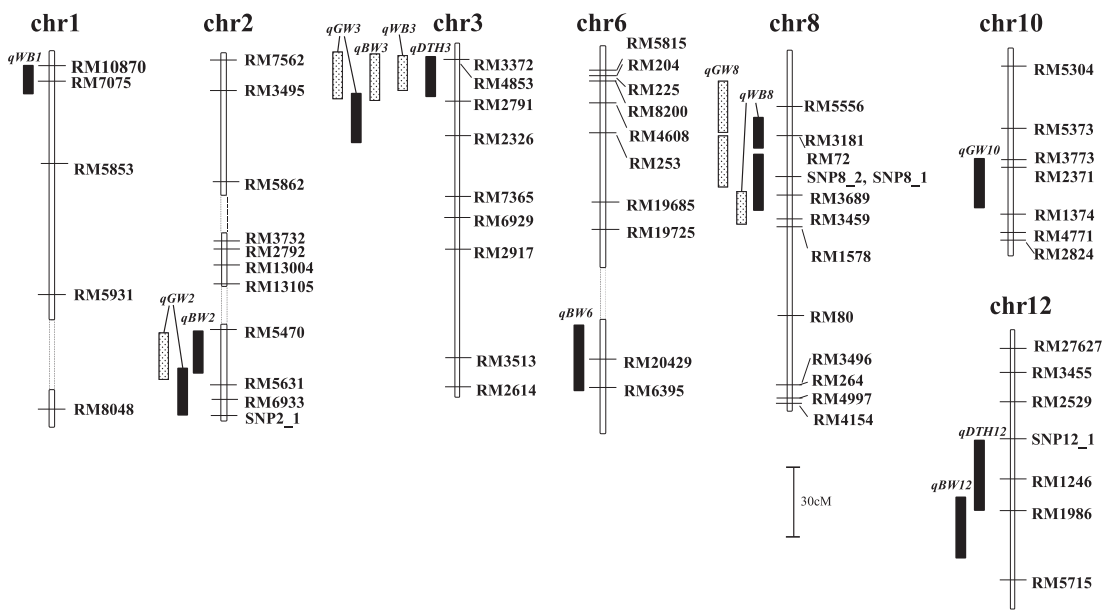

Fig. 3. Putative quantitative trait loci (QTLs) for white-back grains, basal-white grains, grain weight, and days to heading. WB: white-back, BW: basal-white, GW: grain weight, DTH: days to heading. Solid bars indicate QTLs detected in Fukuoka (in hot water irrigation group). Dotted bars indicate QTLs detected in Kagoshima (in early transplantation group). The length of bars indicates the significant regions beyond threshold values, based on the 2000 times permutation test. As QTLs for DTH were commonly detected in almost the same regions in both experiments, only QTLs detected in Fukuoka were indicated.

reported by Nagasaki *et al.* (2010). In total, we used 76 markers to construct a linkage map of 12 chromosomes (**Supplemental Fig. 1**). On the basis of this linkage map, we performed QTL analysis of WB, BW, GW, and DTH (**Fig. 3**, **Table 2**). QTLs associated with WB were detected on chr. 1, 3, and 8. The QTLs on chr. 8 (*qWB8*) were commonly mapped in the interval between RM5556 and RM1578. LOD scores of the *qWB8* detected in plants that

Table 2. Putative quantitative trait loci (QTLs) for white-back, basal-white, 1000-grain weight, and days to heading of rice grains detected by composite interval mapping

<Hot water irrigation in Fukuoka>								<Transplantation in early season in Kagoshima>							
Trait	QTL[a]	Chr.	Nearest marker[b]	Year	LOD	AE[c,d]	PVE[c]	Trait	QTL[a]	Chr.	Nearest marker[b]	Year	LOD	AE[c,d]	PVE[c]
White-back	*qWB1*	1	RM7075	2010	4.02	–3.82	15.6	White-back	*qWB3*	3	RM4853	2009	8.90	–8.39	30.5
	qWB8	8	SNP8_1	2009	4.34	–1.44	18.5		*qWB8*	8	RM3689	2009	2.21	–4.45	7.4
		8	RM72	2010	3.30	–4.40	12.9								
Basal-white	*qBW2*	2	RM5470	2009	2.93	–1.17	11.0	Basal-white	*qBW3*	3	RM4853	2009	4.59	–1.41	17.9
	qBW6	6	RM20429	2010	2.78	1.40	10.7								
	qBW12	12	RM1986	2010	3.35	1.60	12.9								
1000 grain weight	*qGW2*	2	RM6933	2010	2.29	–0.39	11.9	1000 grain weight	*qGW2*	2	RM5470	2009	4.22	0.39	13.4
											RM5470	2010	2.78	0.34	9.5
	qGW3	3	RM2791	2009	2.86	–0.32	13.6		*qGW3*	3	RM4853	2009	6.78	0.48	20.5
	qGW10	10	RM2371	2009	2.79	–0.33	14.3		*qGW8*	8	RM5556	2009	3.19	0.37	11.9
										8	RM5556	2010	6.92	0.61	31.5
										8	RM72	2010	6.44	0.58	27.5
Days to heading	*qDTH3*	3	RM4853	2009	43.50	–3.98	87.5	Days to heading	*qDTH3*	3	RM4853	2009	25.66	–2.19	71.8
		3	RM4853	2010	29.35	–4.74	75.3			3	RM4853	2010	38.12	–3.03	83.8
	qDTH12	12	SNP12_1	2009	3.96	–0.68	2.7		*qDTH12*	12	SNP12_1	2010	4.29	–0.65	3.9

[a] QTLs were detected based on the basis of 2000 times permutations.
[b] Nearest markers denote the relevant markers that were closest to the peak of QTLs.
[c] AE: additive effect, PVE: Percentage of total phenotypic variance explained in each QTL
[d] Minus values of additive effect indicate that 'Chikushi 52' alleles decrease the trait values.
[e] The significant threshold of LOD value ($P < 0.05$) of WB, BW, GW, and DTH in 2009 and those in 2010 of Fukuoka was 1.85, 2.00, 2.40, 1.81, 2.36, 2.03, 2.42, 2.41, respectively. That for WB, BW, GW, and DTH in 2009 and those in 2010 of Kagoshima was 2.39, 2.04, 2.14, 2.01, 2.4, 2.35, 2.37, and 2.19, respectively.

experienced hot water irrigation were 4.34 and 3.30 in 2009 and 2010, respectively. The percentage of phenotypic variation explained (PVE) by *qWB8* was 18.5% and 12.9% in 2009 and 2010, respectively. LOD score of the *qWB8* detected in plants that experienced early transplantation was 2.21 in 2009. The PVE of *qWB8* was 7.4% in 2009. The CH alleles lowered the ratio of WB throughout all the QTLs and contributed to the improvement of grain quality. QTLs associated with BW were identified on chr. 2, 3, 6, and 12. All the QTLs were detected during a single year, and no common QTLs were detected between both experiments. Although the CH alleles containing the *qBW2* and *qBW3* decreased the ratio of BW, the TR allele containing *qBW6* and *qBW12* also decreased it. QTLs for GW were detected on chr. 2, 3, 8, and 10. The QTLs on chromosome 8 (*qGW8*) were mapped in the interval between RM5556 and RM1578 in multiple experiments in Kagoshima. Although CH allele of *qGW2*, *qGW3*, and *qGW10*, which were detected with hot water irrigation in Fukuoka, had a negative effect on GW, the CH alleles of *qGW2*, *qGW3*, and *qGW8*, which were detected with early transplanting in Kagoshima, had an effect of increasing GW. QTLs for DTH were commonly detected on chr. 3 and 12; in particular, the QTL on chr. 3 was responsible for most of the difference in DTH between RILs. CH alleles of *qDTH3* and *qDTH12* shortened the DTH.

Validation of QTLs for the occurrence of WB grains

In order to validate the effect of QTLs on chr. 8, we developed NILs, named NIL01 and NIL02. Both lines contained a segment of CH between RM3181 and RM3689 on chr. 8 with the genetic background of TR. NIL02 harbored longer upstream region than NIL01 to RM5556. In addition to common small segments on the long arm of chr. 12, NIL01 harbored small segment on the short arm of chr. 7 and the long arm of chr. 12, and NIL02 harbored small segment on the long arm of chr. 1, 4, 6, and 12 (**Fig. 4**). The tolerance of those lines to heat stress was evaluated in the hot water irrigation facility (Fukuoka), in early transplantation (Kagoshima), under heat stress in a greenhouse (Kagoshima), and in a TGC (Ishikawa) (**Tables 3**, **4**).

In the hot water irrigation facility, NIL01 and NIL02 showed a significantly lower ratio of WB (38.0% and 47.5% for NIL01 in 2009 and 2010, and 41.0% and 33.0% for NIL02 in 2009 and 2010) than did TR (60.5% and 62% in 2009 and 2010) (**Table 3**). Both lines also showed heavier 1000-grain weight than did TR. Similar results were obtained in plants from the early transplantation, heat stress in greenhouse, and TGC groups; in these groups, NIL01 and NIL02 showed a lower WB than did TR (**Table 4**). These results clearly validated the effects of allelic differences at putative QTLs on chr.8 (*qWB8*) between two parental lines. In a case of GW, NIL01 showed similar or slightly heavier GW than TR, but NIL02 showed clearly higher GW than TR (**Table 4**).

Discussion

Tsubone *et al.* (2008) and Wakamatsu *et al.* (2004, 2005) indicated that the ratio of WB and BW grains increases if average air temperature during ripening exceeds 27°C.

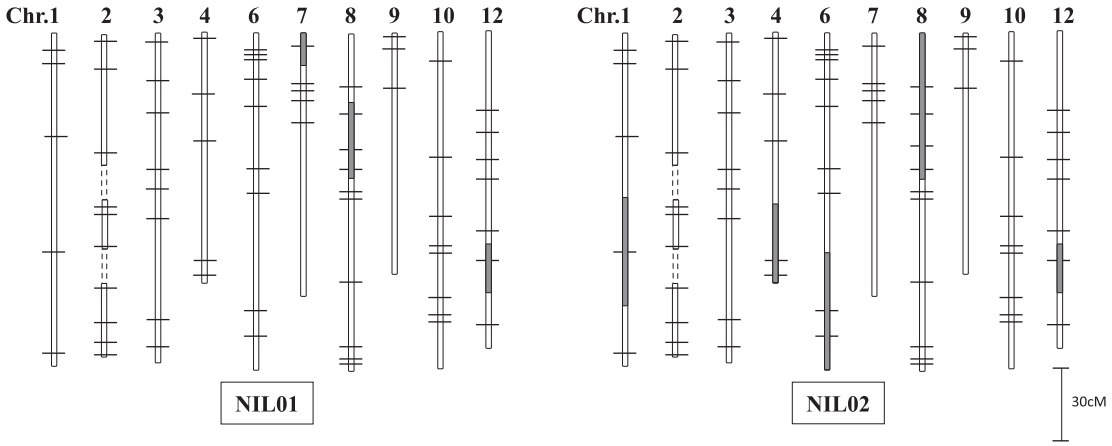

Fig. 4. Graphical genotype of near-isogenic lines (NILs) used for the validation of quantitative trait loci (QTLs) for white-back grains. We arbitrarily determined the recombination point to be the midpoint between markers of different genotypes: white, homozygous for 'Tsukushiroman' segment; gray, homozygous for 'Chikushi 52' segment.

Table 3. Ratio of white-back grains of NILs and the genotypes of SSR markers linked to the QTLs for ratio of white-back grains, 1000-grain weight, and days to heading

NIL No.	Genotype for SSR markers linked to the QTLs[a]					2012				2013				
	Chr. 3	Chr. 8 WB, GW				DTH (days)	Temp.[b] (°C)	GW (g)	WB (%)	DTH (days)	Temp.[b] (°C)	GW (g)	WB (%)	χ^2 test
	RM 3372	RM 5556	RM 3181	RM 3689	RM 223									
NIL01	TR	TR	CH	CH	TR	67	27.5	20.0	38.0	78	29.0	21.5	47.5	**[c]
NIL02	TR	CH	CH	CH	TR	67	27.5	21.2	41.0	77	29.1	21.5	33.0	**
Tsukushiroman	TR	TR	TR	TR	TR	67	27.5	19.8	60.5	78	29.0	20.0	62.0	–
Chikushi 52	CH	CH	CH	CH	CH	59	27.8	20.2	15.0	66	28.5	20.4	49.0	**

[a] This experiment was conducted in the hot water irrigatin facility in Fukuoka. CH: Chikushi 52 allele, TR: Tsukushiroman allele, WB: white-back grains, GW: 1000-grain weight, DTH: days to heading.
[b] Temp. means average temperature during the 20 days after heading.
[c] The symbols, ** of Cochran-Mantel-Haenszel Chi-Squared test showed that the ratio of white-back grains of each NIL was significant at 1% level, compared to that of 'Tsukushiroman'.

Table 4. Ratio of white-back grains of near-isogenic lines (NILs) and their recurrent parent 'Tsukushiroman' under different high-temperature treatments

NIL No.	Kagoshima 1[a]				Kagoshima 2[a]				Ishikawa 1[a]				Ishikawa 2[a]			
	DTH[c] (days)	Temp.[b] (°C)	GW[c] (g)	WB[c] (%)	DTH[c] (days)	Temp.[b] (°C)	GW[c] (g)	WB[c] (%)	DTH[c] (days)	Temp.[b] (°C)	GW[c] (g)	WB[c] (%)	DTH[c] (days)	Temp.[b] (°C)	GW[c] (g)	WB[c] (%)
NIL01	66	28.6	21.0	69.3**	65	29.4	20.1	28.0**	86	26.0	23.8	6.3**	86	28.5	23.3	35.0**
NIL02	66	28.6	21.2	61.3**	65	29.4	20.5	27.5**	86	26.0	24.2	8.5**	86	28.5	24.4	40.5**
Tsukushiroman	66	28.6	20.6	84.7	66	29.4	20.2	50.0	86	26.0	22.9	23.8	86	28.5	23.0	76.3

[a] Kagoshima 1: early transplantation in open paddy field; Kagoshima 2: high-temperature treatment in greenhouse; Ishikawa 1: heat treatment in area 1 of Temperature Gradient Chamber (TGC); Ishikawa 2: heat treatment in area 2 of TGC.
[b] Temp. means average temperature during the 20 days after heading.
[c] DTH: days to heading, GW: 1000-grains weight, WB: white-back grains.
[d] The symbols, ** of Chi-Squared test showed that the ratio of white-back grains of each NIL was significant at 1% level, compared to that of 'Tsukushiroman'.

Improving tolerance to heat stress is one of the major objectives in the present rice breeding program in Japan. In this study, we identified several QTLs that affected the quality of grain grown under heat stress. Among them, QTLs for WB on chr. 8 (*qWB8*) were detected in different heat treatments, including hot water irrigation in Fukuoka and early transplantation in Kagoshima (**Table 2**, **Fig. 3**). Those QTLs appeared to be relatively more stable and exhibited larger effects than other QTLs. Furthermore, their effects could be validated by using NILs (**Tables 3**, **4**). It was also clear that chromosome segments of these QTLs derived from CH decreased the ratio of WB and improved the plants' tolerance to heat stress. In previous studies, several QTLs for grain quality under heat stress were identified. Kobayashi *et*

al. (2007) detected QTLs for WB on chr. 3, 4, and 6. In addition, Tabata *et al.* (2007) detected QTLs for WB on chr. 1, 2, and 8. On the basis of a comparison of chromosomal location, the locations of QTLs we detected in this study did not coincide with the QTLs which were identified by Kobayashi *et al.* (2007). But *qWK8*, which was detected in the study of Tabata *et al.* (2007) might be the same as *qWB8* in this study, because both of them were detected in the short arm of chr. 8. Although the nearest markers of both QTLs were different, the source cultivar of *qWK8*, 'Koshijiwase' and that of *qWB8*, 'Chikushi 52' harbor the common ancestors, Norin 1 and Norin 22. Further study is required to clarify the location and function of *qWK8* and *qWB8*.

Wan *et al.* (2005) reported that QTLs for the occurrence of chalky grains were located in the interval G1149-R727 (RFLP markers) on chr. 8 using chromosome segment substitution lines (CSSLs) derived from the cross between 'IR24' (*indica*) and 'Asominori' (*japonica*). According to the Rice TOGO Browser (Nagamura *et al.* 2011), the physical locations of G1149 and R727, which were the nearest markers in the study of Wan *et al.* (2005), were very close to those of RM3181 and RM3689, both of which were the nearest markers of *qWB8* detected in this study. The comparison of chromosomal location indicated that it is very difficult to conclude how this QTL is related to others in this study. Further fine mapping or cloning of gene will be necessary in order to answer this question.

The ratio of chalky WB and BW grains of CH was lower than that of TR, except in the early transplantation group in Kagoshima in 2010. Although the temperature during 20 days after heading of CH and TR were similar (28.1, 28.4°C, respectively) in Kagoshima in 2010, solar irradiation of CH and TR during 5 days after heading was different; 99.6 MJ/m^2 of CH and 65.82 MJ/m^2 of TR. Wakamatsu *et al.* (2009) reported shading treatment mitigated the occurrence of WB. Accompanied with the difference of solar irradiation, the maximum temperature during 5 days after heading of CH was about 2 degrees higher than that of TR. Those differences of climate condition might lead to the difference of WB between CH and TR.

The temperature during rice ripening in 2009 was lower than that in 2010. These differences in environmental conditions might be responsible for the lower correlation of WB and BW between 2009 and 2010. Clearly, not only genetic regions but also environmental conditions affected the difference in the occurrence of WB and BW. In our QTL analyses over two years, only the QTL region of chr. 8 was shared in both years. These results might also be caused by differences in environmental conditions.

As mentioned, there were QTLs for GW (*qGW8*) in the vicinity of the QTLs for WB on chr. 8. The CH alleles on chr. 8 increased GW and decreased the ratio of WB. Wakamatsu *et al.* (2007) reported that classification of grains of 'Hinohikari' that matured under heat stress showed that WB grains had lighter GW than did fully matured grains. In addition, Tabata *et al.* (2007) mentioned that the ratio of WB grains was negatively correlated with GW among RI lines derived from a cross between 'Chiyonishiki' and 'Koshijiwase'. Both studies suggest that the appearance of WB grains tends to be associated with a decline in GW. A higher ratio of WB could cause lighter GW due to insufficient grain filling. Wan *et al.* (2005) and our experiments detected QTLs for chalky grains (WB) and those for GW in a narrow region. There are two possible explanations for the apparent association. First, both QTLs might be different and might be located in close proximity to one another. Second, the same QTL may have a pleiotropic effect on WB and GW. Furthermore, there was a slight difference of GW between NIL01 (20.0 g) and NIL02 (21.2 g) in the 2012 experiment of the study in Fukuoka, and similar tendency was observed through the experiments in early transplantation, greenhouse and TGC (**Table 4**). This suggested that there might be another region controlling GW around RM5556.

Based on the study conducted by Wan *et al.* (2005) using CSSLs derived from the cross between 'IR24' and 'Asominori', Liu *et al.* (2010) indicated that CSSL50-1, which carried an 'IR24' segment on chr. 8 with the genetic background of 'Asominori', exhibited a higher ratio of chalky grains than did the recurrent parent 'Asominori'. Liu *et al.* (2010) also pointed out that CSSL50-1 showed relatively faster grain filling rate during the early ripening stage than did 'Asominori' and suggested that the enzyme activities related to starch synthesis changed in CSSL50-1 and led to the occurrence of chalky grains during the later stage. Yamakawa *et al.* (2008) reported that there were many starch/carbohydrate-metabolizing enzyme genes on chr. 8, such as *SSIIIa*, *Amy3D*, *Amy3E*, *AGPS2*, and *ISA1*. Out of them, *AGPS2* (ADP-Glucose pyrophosphorylase2) was located between RM3181 and RM3689, which covered inner region of *qWB8*. Since other enzyme genes were not predicted to be located in the vicinity of the QTLs detected in this study, it was also suggested that these enzyme genes might be also involved in the allelic differences in these QTLs. Further study using more advanced backcross progeny, from which the small remaining segments is especially removed, will be required to prove the relationship between those enzyme genes and QTLs for WB and GW detected in this study.

Compared with the QTLs for WB, QTLs for BW were unstable and were detected in only one experiment over the course of the two years. We tried to validate the effect of *qBW2* with NILs but could not confirm its effect (data not shown). Kobayashi *et al.* (2007) also detected QTLs for BW on chr. 6 in only a single year, although they found QTLs for WB on chr. 6 in two years. These facts suggested that the ratio of BW tends to be affected mainly by environmental conditions.

Recently, a great deal of genetic information on rice grain quality has been reported (Ebitani *et al.* 2008, Kobayashi *et al.* 2007, 2013, Liu *et al.* 2010, Tabata *et al.* 2007, Wan *et al.* 2005). In this study, we identified a new QTL for WB on chr. 8, and confirmed that the CH allele

improved grain quality. The new information that has been reported in this study is certainly useful for improving grain quality under heat stress conditions. However, it should be noted that the allele effects of these QTLs could be affected by the genetic background of the cultivar and on the particular environmental conditions of the growing season. To improve our understanding of the genetic control of grain quality and its interaction with environmental conditions, we need further studies that will undertake cloning of QTLs and clarify the molecular function of the genes that contain them.

Acknowledgment

This work was supported by a grant from the Ministry of Agriculture, Forestry and Fisheries of Japan ('Genomics for agricultural innovation', QTL3004, and 'Development of mitigation and adaptation techniques to global warming in the sectors of agriculture, forestry, and fisheries', 1203).

Literature Cited

Churchill, G.A. and R.W. Doerge (1994) Empirical threshold values for quantitative trait mapping. Genetics 138: 963–971.

Ebitani, T., Y. Yamamoto, M. Yano and M. Funane (2008) Identification of quantitative trait loci for grain appearance using chromosome segment substitution lines in rice. Breed. Res. 10: 91–99.

Horie, T., H. Nakagawa, J. Nakano, K. Hamotani and H.Y. Kim (1995) Temperature gradient chambers for research on global environment change. III. A system designed for rice in Kyoto, Japan. Plant Cell Environ. 18: 1064–1069.

Imabayashi, S., Y. Hamachi, K. Furuno, H. Nishiyama, Y. Matsue, M. Yoshino and T. Yoshida (1995) A new rice cultivar 'Yumetsukushi'. Bull. Fukuoka Agric. Res. Cent. 14: 1–10.

International Rice Genome Sequencing Project (IRGSP) (2005) The map-based sequence of the rice genome. Nature 436: 793–800.

Kasaneyama, H., K. Itoh, S. Abe, K. Kobayashi, K. Hirao, T. Matui and T. Hoshi (1999) Improvement of grain and eating quality for rice breeding in Niigata prefecture XVI. Test of ripening ability at hot water irrigation in rice plant. Hokuriku Crop Sci. 34: 21–23.

Kobayashi, A., B. Genliang, Y. Shenghai and K. Tomita (2007) Detection of quantitative trait loci for white-back and basal-white kernels under high temperature stress in *japonica* rice varieties. Breed. Sci. 57: 107–116.

Kobayashi, A., J. Sonoda, K. Sugimoto, M. Kondo, N. Iwasawa, T. Hayashi, K. Tomita, M. Yano and T. Shimizu (2013) Detection and verification of QTLs associated with heat-induced quality decline of rice (*Oryza sativa* L.) using recombinant inbred lines and near-isogenic lines. Breed. Sci. 63: 339–346.

Kosambi, D.D. (1943) The estimation of map distances from recombination values. Ann. Eugen. 12: 172–175.

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

Liu, X., T. Guo, X. Wan, H. Wang, M. Zhu, A. Li, N. Su, Y. Shen, B. Mao, H. Zhai *et al.* (2010) Transcriptome analysis of grain-filling caryopses reveals involvement of multiple regulatory pathways in chalky grain formation in rice. BMC Genomics 11: 730.

McCouch, S.R., L. Teytelman, Y. Xu, K.B. Lobos, K. Clare, M. Walton, B. Fu, R. Maghirang, Z. Li, Y. Xing *et al.* (2002) Development and mapping of 2240 new SSR markers for rice (*Oryza sativa* L.). DNA Res. 9: 199–207.

Murray, M.G. and W.F. Thompson (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. 8: 4321–4326.

Nagamura, Y., B.A. Antonio, Y. Sato, A. Miyao, N. Namiki, J.-I. Yonemaru, H. Minami, K. Kamatsuki, K. Shimura, Y. Shimizu *et al.* (2011) Rice TOGO browser: a platform to retrieve integrated information on rice functional and applied genomics. Plant Cell Physiol. 52: 230–237.

Nagasaki, H., K. Ebana, T. Shibaya, J.-I. Yonemaru and M. Yano (2010) Core single-nucleotide polymorphisms—a tool for genetic analysis of the Japanese rice population. Breed. Sci. 60: 648–655.

Nagato, K. (1952) On the occurrence of white core, milk-white grain, and white belly in rice plant. Jpn. J. Crop Sci. 21: 26–27.

Nagato, K. and M. Ebata (1965) Effects of high temperature during ripening period on the development and the quality of rice kernels. Jpn. J. Crop Sci. 34: 59–66.

Sasaki, T., S. Abe, K. Matsunaga, E. Okamoto, K. Nagano, K. Tanno, Y. Chiba, A. Kano and K. Uematsu (1994) A new rice cultivar 'Hitomebore'. Bull. Miyagi Furukawa Agric. Exp. Sta. 2: 1–17.

Tabata, M., H. Hirabayashi, Y. Takeuchi, I. Ando, Y. Iida and R. Ohsawa (2007) Mapping of quantitative trait loci for the occurrence of white-back kernels associated with high temperatures during the ripening period of rice (*Oryza sativa* L.). Breed. Sci. 57: 47–52.

Tashiro, T. and M. Ebata (1975) Studies on white belly rice kernel III. Effect of ripening conditions on occurrence of white belly kernel. Jpn. J. Crop Sci. 44: 86–92.

Tsubone, M., T. Ogata and T. Wada (2008) Effective selection method of rice varieties with resistance to high-temperature during ripening period by warm water treatment. Rep. Kyushu Br. Crop Sci. Soc. Jpn. 74: 21–23.

Wakamatsu, K., T. Tanogashira, M. Takemure and K. Mori (2004) Effects of high air temperature at ripening period on the grain quality of rice in Kagoshima Prefecture. Rep. Kyushu Br. Crop Sci. Soc. Jpn. 70: 10–12.

Wakamatsu, K., T. Tanogashira, Y. Komaki and T. Higashi (2005) Effects of high air temperature in the ripening period on the grain quality of rice in warm regions and its varietal differences. Rep. Kyushu Br. Crop Sci. Soc. Jpn. 71: 6–9.

Wakamatsu, K., O. Sasaki, I. Uezono and A. Tanaka (2007) Effects of high air temperature during the ripening period on the grain quality of rice in warm regions of Japan. Jpn. J. Crop Sci. 76: 71–78.

Wakamatsu, K., O. Sasaki and A. Tanaka (2009) Effects of the amount of insolation and humidity during the ripening period on the grain quality of brown rice in warm regions of Japan. Jpn. J. Crop Sci. 78: 476–482.

Wan, X.Y., J.M. Wan, J.F. Weng, L. Jiang, J.C. Bi, C.M. Wang and H.Q. Zhai (2005) Stability of QTLs for rice grain dimension and endosperm chalkiness characteristics across eight environments. Theor. Appl. Genet. 110: 1334–1346.

Wang, S., C.J. Basten and Z.B. Zeng (2012) Windows QTL Cartographer 2.5. Raleigh, NC: Department of Statistics, North Carolina State University. (http://statgen.ncsu.edu/qtlcart/WQTLCart.htm)

Yamakawa, H., T. Ebitani and T. Terao (2008) Comparison between locations of QTLs for grain chalkiness and genes responsive to high temperature during grain filling on the rice chromosome map. Breed. Sci. 58: 337–343.