Genetic and epistatic effects for grain quality and yield of three grain-size QTLs identified in brewing rice (Oryza sativa L.).

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Title: The genetic and epistatic effects of three QTLs for rice (*Oryza sativa* L.) grain size and influence to traits related to yield and grain quality using QTL pyramiding lines.

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Abstract

Rice (*Oryza sativa* L.) in Japan is not only a food staple, but also an important material for the Japanese alcoholic beverage, *sake*. The grain used in *sake* brewing has different characters from the cooking rice grain, including a large grain size and high white-core expression rate (WCE). Because large sized grains often have a heavy grain weight and higher yield, this trait is also important for cooking rice. Chalky grains, such as white-core or white-belly grains, are not ideal as cooking rice. Here, we report that three grain size quantitative trait loci (QTLs; *qGL4-2*, *qGWh5*, *qGWh10*), derived from the brewing cultivar, Yamadanishiki, affect grain shape, chalky-grain rate, and yield, using near isogenic and pyramiding lines in the genetic background of the cooking cultivar, Koshihikari. First, these QTLs influenced multiple components of grain shape, where epistatic effects were detected between *qGL4-2* and *qGWh5,* for grain width and thickness; and between *qGL4-2* and *qGWh10,* for grain length. Therefore, these QTLs may coordinate to control grain shape. Second, lines harboring *qGWh5* or *qGWh10* at the Yamadanishiki allele exhibited increased WCE, whereas lines with *qGL4-2* and *qGWh10* exhibited decreased white-belly rate (WBR). Thus, grain shape is associated with the occurrence of chalky grain, where the chalky-type depends on the QTL. Finally, we used total panicle weight of plants as a simplified rice yield index, and a promising line pyramiding *qGL4-2* and *qGWh5* emerged. In conclusion, *qGL4-2* would be useful for the breeding of cooking rice, to decrease WBR; while *qGWh5* and *qGWh10* were definitely more beneficial for that of brewing rice, to increase grain weight and WCE.

Keywords:

grain shape, QTL, epistasis, grain quality, rice yield, pyramiding line

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Compliance with ethical standards

Conflict of interest: The authors declare that they have no conflict of interest.

Authors' contributions

SO and MY designed experiments, SO, KI and KH performed genotyping, SO conducted phenotyping and data analysis and SO and MY wrote the manuscript. All authors read and approved the final manuscript.

Introduction

Rice (*Oryza sativa* L.) is one of the most important crops in the world, with over 50% of the global population consuming it as their main daily food source. In Japan, rice is not only a food staple, but also a raw material for the Japanese alcoholic beverage, *sake*. *Sake* is brewed from steamed rice, *Aspergillus oryzae*, *Saccharomyces cerevisiae* and water. Special brewing rice cultivars have been created to brew *sake*, which have some different characteristics from cooking rice cultivars in Japan. These cultivars have a larger grain size and weight, and higher white-core expression rate (WCE), which are related to the *sake*-brewing process. For example, large grains are fitted to high-grade polishing (such as 50%) to remove the large amounts of protein and fat around the surface layer of the grain. This increases the WCE of the rice, allowing it to absorb water before steaming and thus increasing the quality of the fermentation process. This process generates high quality *koji,* a saccharifying starch of the steamed rice produced by *Aspergillus oryzae*, and is a very important process for brewing flavorful *sake* (Nagato and Ebata 1959, Aramaki et al. 1995, Yanagiuchi et al. 1997). Breeding of brewing rice varieties selects for these desirable traits. Examination and reporting of the genetic basis of these traits is therefore necessary to improve brewing rice cultivars.

On the other hand, many QTLs and genes associated with grain size and weight have been identified by mapping populations derived from cooking rice cultivars, creating increases in yield to satisfy the growing global demand for food (Huang et al. 2013). The genetic pathway controlling grain shape has been elucidated by many studies (Song et al. 2007, Weng et al. 2008, Shomura et al. 2008, Li et al. 2011, Wang et al. 2012, Ishimaru et al. 2013, Duan et al. 2014, Hu et al. 2015, Liu et al. 2015, Wang et al. 2015a, Wang et al. 2015b, Si et al. 2016, Hu et al. 2018, Zhao et al. 2018). The current state of knowledge holds that the pathways are comprised of the ubiquitin-proteasome and phytohormone pathways, as well as transcriptional regulatory factors, P450 family proteins, mitogen-activated protein kinase cascades, and many others (Li and Li 2016, Li et al. 2018). However, despite all of these efforts, a perfect understanding of the genetic control of rice grain shape has not yet been reached, and many existing QTLs have still not been isolated. The identification of QTLs for grain shape and grain weight in brewing rice cultivars is useful to improve both cooking and brewing rice cultivars.

Rice grain quality, such as grain chalkiness, is also a crucial factor determining both the rate of grain breakage after milling and the market value of the rice (Fitzgerald et al. 2009). Many QTLs associated with grain quality are distributed across all rice chromosomes (He et al. 1999, Tan et al. 2000, Liu et al. 2011, Zheng et al. 2012, Lu et al. 2013, Peng et al. 2014, Chen et al., 2016, Yun et al. 2016, Zhao et al. 2016, Qiu et al. 2017, Wang et al. 2017). These traits are also affected by environmental factors (Morita 2000, Wada et al. 2014). Many QTLs affecting grain shape have been found to also affect grain chalkiness (Qiu et al. 2017, Wang et al. 2017). Increases in the percentage of chalky grains strongly correlated with increases in grain width (GWh) and grain length (GL) (Gong et al. 2017, Wang et al. 2017). Among isolated genes relating to grain shape, genes such as *GW2*, *GW7*/*GL7*, *GW8*/*OsSPL16* and *GS9* have been found to influence chalkiness (Song et al. 2007, Wang et al. 2012, Wang et al. 2015a, Wang et al. 2015b, Zhao et al. 2018). This indicates the importance of elucidating the relationship between QTLs associated with grain shape and chalky grain.

In our previous study, we detected important QTLs for grain shape, grain weight and WCE using a primary mapping population derived from a cross between Koshihikari, an elite cooking cultivar in Japan; and Yamadanishiki, the most popular Japanese brewing rice cultivar (Okada et al. 2017). Of these QTLs, the genetic effects of *qGWh5* and *qGWh10* on chromosomes 5 and 10, respectively, have already been validated. It was also found that *qGWh5* and *qGWh10* affected both GWh and grain weight (Okada et al. 2018a, Okada et al. 2018b). Since *qGL4-2* on chromosome 4 has not been validated for grain size, confirmation of the genetic effect is essential (Okada et al. 2017). Moreover, *qGWh10* has also been identified as a QTL for WCE (Okada et al. 2017). Therefore, our objectives in this study were to clarify (1) the genetic and epistatic effects of the three QTLs, *qGL4-2*, *qGWh5,* and *qGWh10,* for grain shape; (2) the degree of contribution of the three QTLs to grain chalkiness; and (3) the extent of the QTLs' influence on rice yield indices. To achieve these objectives, we have developed near isogenic and pyramiding lines (NILs and PYLs) for the QTLs, which have one, two or three Yamadanishiki alleles on the genetic background of Koshihikari. These lines were usable for detailed analysis of epistatic effects, as well as of phenotyping traits under pyramiding lines of QTLs (Mackay 2014). The best combination can be proposed for rice yield, because it is known that increase of sink size such as grain size, often leads to trade-offs in other organs (Song et al. 2007, Fang et al. 2016, Guo et al. 2018). In this study, we verified whether these QTLs were effective for cooking rice as well as brewing rice cultivars, by measuring rice yield indices (excluding percentage of ripening grains) and total panicle weight per plant (TPW). Panicle weight is a simple first screening indicator of rice yield (Okada et al. 2018b).

Materials and methods

Development of near isogenic and pyramiding lines

Two rice plants were selected from the BC_4F_2 population described by Okada et al. (2018a). The population was consecutively backcrossed with Koshihikari four times for F_1, which was derived from a cross between Koshihikari and Yamadanishiki, and the resultant BC_4F_1 population was self-pollinated. Of the two plants, one was heterozygous at both *qGL4-2* and *qGWh5,* and Koshihikari homozygous at *qGWh10*; and the other was heterozygous at both *qGL4-2* and *qGWh5,* and Yamadanishiki homozygous at *qGWh10* (Supplemental Fig. 1). The plants were genotyped by 335 single nucleotide polymorphism markers between two cultivars using a BeadStation 500G system (Illumina, San Diego, CA, USA), using the same method and array as Okada et al. (2018a). These plants were self-pollinated, and we selected lines harboring one QTL (*qGL4-2*_NIL, *qGWh5*_NIL and *qGWh10*_NIL), two QTLs (*qGL4-2+qGWh5*_PYL, *qGL4-2+qGWh10*_PYL and *qGWh5+qGWh10*_PYL) and three QTLs (*qGL4-2+qGWh5+qGWh10*_PYL), using DNA markers enclosing each QTL (Supplemental Fig. 1, Supplemental Table). The description of these lines represented the number of Yamadanishiki homozygous combinations at each QTL allele. These lines were genotyped at regions where Yamadanishiki chromosomal segments still existed, based on the genotypes in the BC_4F_2 generation (Supplemental Fig. 1, Supplemental Table). During genotyping, we sampled bulked seedling leaves of each line cultivated in 2016 and 2017, and extracted this DNA; however, these seedlings were only used for leaf sampling. The corresponding graphical genotypes are shown in Supplemental Fig. 2.

Trait evaluation

For trait evaluation, the lines and parents were cultivated at an experimental field at Kobe University Food Resources Education and Research Center (Kasai City, Hyogo Prefecture Japan; 34.88°N, 134.86°E) in 2016 and 2017. First, we evaluated the flowering date of each line and parent, and days to heading (DTH) was calculated. Second, we counted panicle number (PN), using 10-20 plants, while excluding plants facing paths and showing abnormality. 45 days after the heading date, 10 plants were harvested and air-dried for three days. Three of these plants, which displayed an average PN for each line, were used to measure spikelet number per panicle (SN). The 10 plants were also used to measure GWh, GL, grain thickness (GT), milky-white grain rate (MWR), WCE, basal-white grain rate (BWR), white-belly grain rate (WBR), 1000-grain weight (TGW) and TPW after dehulling. TPW was measured by an electric balance to an accuracy of 0.01g, as a simple rice yield index. For evaluation of GWh, GL, GT, MWR, BWR and WBR, we selected more than 300 grains, excluding immature and cracked grains after dehulling, and measured these traits using the cooking rice grain mode of a RGQI20A rice grain analyzer (SATAKE Corporation, Higashi-Hiroshima City, Hiroshima Prefecture, Japan). These selected grains were weighed and converted to TGW. We further selected 100 grains to measure WCE, using visual inspection with a light box.

Statistical analysis

We used R (ver. 3.4.1, R Core Team 2017) for all statistical analyses. We performed Dunnett's test for all traits to compare Koshihikari with each line. In addition, three-way analyses of variance (ANOVA) were conducted for GWh, GL and GT to detect epistasis between QTLs, using Koshihikari and all lines.

Results

Grain shape

In the NILs and PYLs we developed, the three traits for grain shape were very different from the recurrent parent, Koshihikari. First, compared with Koshihikari, all lines had significantly larger values for GWh and GT (Table 1). Therefore, all QTLs at Yamadanishiki alleles had effects on GWh and GT. Moreover, *qGWh5+qGWh10*_PYL had the greatest GWh and GT of all lines, excluding GT in 2017, and the PYL was obviously larger in GWh and GT than NILs harboring *qGWh5* or *qGWh10*. GWh and GT of *qGL4-2+qGWh5*_PYL were smaller than those of *qGWh5*_NIL, although in the case of *qGL4-2+qGWh10*_PYL, GWh and GT increased in comparison with *qGL4-2*_NIL and *qGWh10*_NIL. A three-way ANOVA demonstrated an epistatic effect between *qGL4-2* and *qGWh5* for GWh and GT over two years (Table 2). The GWh and GT values of *qGL4-2+qGWh5*_PYL were smaller than a predicted value, assuming an additive effect of these QTLs (Fig. 1b and c). Furthermore, epistasis between *qGWh5* and *qGWh10* was detected for GT both years. However, since the trend of epistasis differed between 2016 and 2017

(Supplemental Fig. 3), we could not determine whether this interaction was true.

*qGL4-2*_NIL and *qGWh10*_NIL had larger GL values than Koshihikari (Table 1). Therefore, *qGL4-2* and *qGWh10* at Yamadanishiki alleles seemed to increase GL. However, the GL of *qGL4-2+qGWh10*_PYL was smaller than a value predicted from the effects of these QTLs (Fig. 1a), indicating the existence of an epistatic effect for GL between these QTLs (Table 2).

Grain quality

Chalky grain appearance was substantially different in the parents because Yamadanishiki was bred for high WCE (Okada et al. 2017) and thus had higher WCE than Koshihikari (Table 3). The MWR of Yamadanishiki also was higher than that of Koshihikari. Because MWR was measured by a rice grain analyzer using the cooking rice grain mode, large-sized white-core grains of Yamadanishiki were probably evaluated as milky-white grains. Conversely, the BWR and WBR of Yamadanishiki were lower than those of Koshihikari.

For the MWR, *qGWh5+qGWh10*_PYL and *qGL4-2+qGWh5+qGWh10*_PYL were higher than Koshihikari over two years (Table 3). The WCE of all lines was lower in 2016 than 2017, and only *qGWh10*_NIL and *qGL4-2+qGWh10*_PYL had a consistently higher WCE than Koshihikari (Table 3). On the other hand, for BWR, no significant difference was detected over two years; and excluding *qGWh5*_NIL and *qGWh5+qGWh10*_PYL, the WBR of the lines was lower than in Koshihikari (Table 3). In particular, the WBR of *qGL4-2+qGWh10*_PYL was much lower than that of *qGL4-2*_NIL or *qGWh10*_NIL (Table 3).

DTH and traits associated with rice yield

All lines showed a similar DTH to Koshihikari, and it was found that these lines showed early heading in Japan. However, the DTH values of the lines, excluding *qGWh5*_NIL, were shorter than in Koshihikari (Table 4).

We evaluated TGW, PN, SN and TPW as rice yield traits. For TGW, all lines showed significant differences between 2016 and 2017 (Table 4), and all QTLs influenced TGW, as well as grain shape. Of the three QTLs, *qGWh10* had the largest effect on TGW. Furthermore, the PYLs exhibiting *qGWh5* and *qGWh10* (*qGWh5+qGWh10*_PYL and *qGL4-2+qGWh5+qGWh10*_PYL) had the largest TGW, which were close to that of Yamadanishiki (Table 4). The TGWs of these PYLs were clearly heavier than those of *qGWh5*_NIL or *qGWh10*_NIL (Table 4).

None of the lines showed significant changes in PN, SN or TPW values over two years (Table 4). However, The averages of PN of *qGWh5+qGWh10*_PYL and *qGL4-2+qGWh5+qGWh10*_PYL were lower than in Koshihikari in 2017 (Table 4). In addition, the TPW of *qGL4-2+qGWh5*_PYL was significantly heavier than that of Koshihikari in 2017. In 2016, the TPW also tended to be large, although we did not detect any significant differences ($P = 0.056$; Table 4).

Discussion

In our previous study, *qGL4-2*, *qGWh5* and *qGWh10* were detected as the QTLs displaying the main effects of the population derived from crossing Koshihikari and Yamadanishiki (Okada et al. 2017). In this study, we also validated the genetic effects of *qGWh5* and *qGWh10* for GWh, as well as the grain weight of *qGWh5* and *qGWh10* (Okada et al. 2018a and 2018b). In addition, these QTLs have already been elucidated by various previous genetic studies (*qGL4-2*; Redona and Mackill 1998, Yoshida et al. 2002, Dang et al. 2015 and Zeng et al. 2016, *qGWh5*; Yoshida et al. 2002 and Nagata et al. 2015, *qGWh10*; Huang et al. 1997, Nelson et al. 2011, Wang et al. 2017 and Zhen et al. 2017). In the present study, the genetic effect of *qGL4-2* was newly validated for GL (Table 1). Moreover, it became clear that *qGL4-2* and *qGWh10* at Yamadanishiki alleles also had an effect on all three components of grain shape. The result of *qGWh10* supported the results of Zhen et al. (2017). On the other hand, it was found that *qGWh5* also influenced GT, besides the genetic effects for GWh and grain weight, which was clarified in our previous study (Table1; Okada et al. 2018a and 2018b). The results of the present study suggested that the main genetic effects for grain shape of *qGL4-2*, *qGWh5* and *qGWh10* were GL, GWh, and GWh, respectively (Table 1). Moreover, we also detected novel epistatic effects for grain shape, which were between *qGL4-2* and *qGWh5* for GWh and GT, and between *qGL4-2* and *qGWh10* for GL (Table 2). These results presumed that these combined QTLs functioned on the same trait regulatory pathway. We then proposed that the pathway regulating grain shape was associated with the QTLs (Supplemental fig. 5). In this pathway, *qGL4-2* may function in the same pathway as *qGWh5* to control GWh and GT, and *qGWh10* to control GL (Supplemental fig. 5). Thus, it is expected that *qGL4-2* functions upstream of *qGWh5* and *qGWh10*, and that *qGWh5* and *qGWh10* belong to different pathways. In future work, it will be important to further analyze these QTLs, as the developed lines exhibit some chromosomal substituted segments except in regions of the three QTLs (Supplemental Fig. 2). Thus, it is important to elucidate whether epistatic effects result from a direct interaction at the molecular level, such as an interaction between proteins, or from another indirect mechanism.

In the present study, it was clear that the three QTLs associated with grain shape also contributed to grain chalkiness, with the exception of BWR (Table 3). First, MWR and WCE can be considered to be linked, because Yamadanishiki exhibiting a high WCE also exhibited a high MWR (Table 3). *qGWh5+qGWh10*_PYL and *qGL4-2+qGWh5+qGWh10*_PYL had a higher MWR than Koshihikari, suggesting that this phenomenon results from increasing grain size and weight (Table 1, 3 and 4). In the case of cooking rice cultivars, milky-white grain color is often explained by exposure to high temperatures during the ripening stage (Tsukaguchi et al. 2012). However, in a brewing rice cultivar, exposure to high temperatures during the ripening stage increases the size of the white-core (Ishii et al. 2008), and it becomes very difficult to distinguish between milky-white and white-core grain colors. Yet white-core grains in brewing rice cultivar are often found in vigorous or superior rice grains (Nagato and Ebata 1959), and during the ripening stage, the WCE of Yamadanishiki matures better at normal than at high temperatures

(Okada et al. 2017, Okada and Yamasaki 2019). Therefore, the cultivation conditions of 2017 were considered to have been better for white-core expression, since in the present study the percentage of basal-white Koshihikari grains, often induced by high temperature, was lower in 2017 than 2016 (Nagato and Ebata 1965). WCEs in the 2017 lines were higher than those of Koshihikari for NILs harboring *qGWh5* or *qGWh10*; and WCEs of *qGWh5+qGWh10*_PYL were much higher than that of *qGWh5*_NIL or *qGWh10*_NIL (Table 3), and *qGWh5* and *qGWh10* are thus thought to contribute to WCE. The genetic effect of *qGWh10* was consistent with the result of the initial QTL analysis (Okada et al. 2017). Moreover, Okada et al. (2017) reported that grain weight, or GWh and WCE, had a positive correlation, suggesting that *qGWh5* and *qGWh10,* which mainly affect GWh and grain weight can partly explain the positive correlation. Next, the WBR of the lines tended to be lower than that of Koshihikari, which was different from MWR and WCE (Table 3). Because the NILs with *qGL4-2* or *qGWh10* had a low WBR, *qGL4-2* and *qGWh10* are thought to have a genetic effect on WBR. The QTLs also have a genetic effect on GL (Table 1), and in a previous study, GL was usually negatively correlated with percentage of chalky-grain (Wang et al. 2017). In addition, *GW8*/*OsSPL16*, *GL7*/*GW7* and *GS9* at alleles increasing the GL also exhibited decreased degrees of chalkiness (Wang et al. 2012, Wang et al. 2015a, Wang et al. 2015b, Zhao et al. 2018). Therefore, the low WBR of NILs harboring *qGL4-2* and *qGWh10* at Yamadanishiki alleles would result from increasing GLs. Interestingly, *qGWh10* had two effects on the occurrence of chalky-grain; that is, *qGWh10* at Yamadanishiki alleles increased WCE, but decreased WBR (Table 3). This phenomenon may indicate that there are different causes for different types of chalky-grain, but it is possible that two QTLs link tightly, affecting results from some substituted segments in the genetic background. Thus, it is suggested that these materials are useful for elucidating the relationship between grain shape and chalky-grain.

The lines harboring *qGL4-2* or *qGWh10* headed slightly earlier than Koshihikari (Table 4). Zhu et al. (2018) and Sun et al. (2018) reported minor-effect QTLs affecting heading date near *qGL4-2* and *qGWh10*, respectively. The two QTLs may thus have a minor effect on DTH, although they were not detected by our QTL mapping in a primary mapping population (Okada et al. 2017). Fine-mapping for these QTLs should still be conducted, as it is possible that two QTLs are tightly co-located in these regions.

Of the rice yield indices measured in this study, the TGW of all lines was heavier than in Koshihikari, and all QTLs associated with grain shape were also thought to contribute to grain weight (Table 4). A novel finding was that *qGL4-2* at Yamadanishiki alleles also increased grain weight. In particular, *qGWh5* and *qGWh10* were considered to be very important QTLs for grain weight in Yamadanishiki, since the PYL of *qGWh5* and *qGWh10* had the heaviest weight, where the TGW of Koshihikari increased by more than 3.54 g. In a previous study, it was reported that genes associated with grain size also affected the PN and SN (Song et al. 2007, Fang et al. 2016). In the present study, the PN and SN of all NILs were not consistently different from those of Koshihikari over two years (Table 4). However, PNs of *qGWh5+qGWh10*_PYL and *qGL4-2+qGWh5+qGWh10*_PYL were significantly lower than in

Koshihikari in 2017; whereas average values of SNs of NILs carrying *qGWh10* were less than in Koshihikari, but not to a significant degree (Table 4). This is because *qGWh5*+*qGWh10*_PYL and *qGL4-2*+*qGWh5*+*qGWh10*_PYL, which had heavy grain weight, did not have high TPWs (Table 4). Thus it may be hypothesized that a trade-off exists between TGW and PN, although this is still uncertain. On the other hand, *qGL4-2*+*qGWh5*_PYL had the highest TPW, suggesting that the combination of these QTLs is better for rice yield.

In conclusion, *qGL4-2*, *qGWh5* and *qGWh10* at Yamadanishiki alleles, which we analyzed in detail, had various genetic effects on the grain shape, chalky-grain and yield indices. In particular, *qGWh5* and *qGWh10* at Yamadanishiki alleles were beneficial for cultivating brewing rice, as they cause increased grain size, grain weight and WCE; while *qGL4-2* at Yamadanishiki alleles are useful in the cultivation of cooking rice, as WBR is decreased. In the present study, we conducted rice yield line screening using TPW. Our results suggested that *qGL4-2*+*qGWh5*_PYL is promising for increased rice yield, and that PYL should be measured per plot for rice yield. Our results should provide beneficial information for the cultivation of cooking as well as brewing rice.

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Table1 Grain size of near isogenic and pyramiding lines.

Line	Grain width (mm)		Grain length (mm)		Grain thickness (mm)	
	2016	2017	2016	2017	2016	2017
qGL4-2 _NIL	2.97 ± 0.02 **	3.02 ± 0.04 ***	5.28 ± 0.03 ***	5.36 ± 0.03 ***	2.05 ± 0.01 ***	2.08 ± 0.01 ***
qGWh5 _NIL	3.03 ± 0.02 ***	3.11 ± 0.02 ***	5.18 ± 0.04	5.24 ± 0.02	2.04 ± 0.02 ***	2.10 ± 0.01 ***
qGWh10 _NIL	3.05 ± 0.01 ***	3.05 ± 0.02 ***	5.25 ± 0.02 ***	5.34 ± 0.03 ***	2.07 ± 0.01 ***	2.10 ± 0.01 ***
qGL4-2+qGWh5 _PL	3.00 ± 0.02 ***	3.03 ± 0.03 ***	5.23 ± 0.03 **	5.29 ± 0.04 ***	2.03 ± 0.02 ***	2.07 ± 0.02 ***
qGL4-2+qGWh10 _PYL	3.09 ± 0.01 ***	3.11 ± 0.01 ***	5.26 ± 0.02 ***	5.37 ± 0.03 ***	2.11 ± 0.01 ***	2.12 ± 0.01 ***
qGWh5+qGWh10 _PYL	3.15 ± 0.02 ***	3.17 ± 0.01 ***	5.19 ± 0.01	5.32 ± 0.03 ***	2.18 ± 0.07 ***	2.13 ± 0.01 ***
qGL4-2+qGWh5+qGWh10 _PYI	3.14 ± 0.02 ***	3.14 ± 0.02 ***	5.23 ± 0.05 *	5.31 ± 0.02 ***	2.13 ± 0.01 ***	2.14 ± 0.01 ***
Koshihikari	2.94 ± 0.02	2.92 ± 0.01	5.19 ± 0.02	5.22 ± 0.04	1.96 ± 0.01	2.01 ± 0.01
Yamadanishiki	3.20 ± 0.01	3.24 ± 0.01	5.52 ± 0.02	5.57 ± 0.01	2.06 ± 0.01	2.04 ± 0.01

The trait values show "mean value" ± "standard deviation".

"*", "**" and "***" indicate significance at $P<0.05$, $P<0.01$ and $P<0.001$, respectively, using Dunnett's multiple comparison method to compare Koshihikari with each line.

Table 2 QTL-by-QTL interactions of three grain size QTLs.

	Grain width						Grain length						Grain thickness					
	2016			2017			2016			2017			2016			2017		
	F value	P value		F value	P value		F value	P value		F value	P value		F value	P value		F value	P value	
qGL4-2 ×*qGWh5*	44.5	<0.001	***	172.4	<0.001	***	0.2	0.665		20.6	<0.001	***	60.9	<0.001	***	140.6	<0.001	***
qGL4-2 ×*qGWh10*	2.3	0.135		0.3	0.5729		10.2	0.002	**	39.0	<0.001	***	12.8	<0.001	***	0.4	0.535	
qGWh5 ×*qGWh10*	1.6	0.212		5.4	0.0234	*	0.9	0.349		1.1	0.301		9.5	0.003	**	11.1	0.001	**
qGL4-2 ×*qGWh5*×*qGWh10*	0.8	0.381		24.9	<0.001	***	6.8	0.011	*	2.2	0.141		0.2	0.686		65.8	<0.001	***

"*", "**" and "***" indicate significance at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

Table 3 Grain quality of near isogenic and pyramiding lines.

Line	Milky-white grain rate (%)		White-core expression rate (%)		Basal-white grain rate (%)		White-belly grain rate (%)	
	2016	2017	2016	2017	2016	2017	2016	2017
qGL4-2 _NIL	8.4 ± 1.9 ***	3.7 ± 1.5	1.8 ± 1.2	6.5 ± 3.0	18.8 ± 3.5	18.8 ± 3.3 *	3.9 ± 1.2 ***	2.8 ± 0.8 *
qGWh5 _NIL	23.6 ± 6.5	7.8 ± 1.7 ***	2.9 ± 2.5	13 ± 5.8 **	17.2 ± 3.0	18.1 ± 2.6	9.3 ± 1.7	4.3 ± 1.1
qGWh10 _NIL	10.3 ± 2.6 ***	4.8 ± 1.3	5.8 ± 2.3 *	12 ± 4.4 *	17.4 ± 2.2	20.0 ± 4.3 **	3.4 ± 1.0 ***	1.4 ± 0.6 ***
qGL4-2+qGWh5 _PYL	16.6 ± 3.4	4.2 ± 1.2	1.6 ± 1.6	13 ± 3.7 **	17.5 ± 2.7	18.6 ± 3.8	7.3 ± 2.1 ***	2.6 ± 0.9 **
qGL4-2+qGWh10 _PYL	10.9 ± 3.2 ***	2.3 ± 0.9	5.8 ± 2.2 *	14 ± 5.1 **	16.6 ± 3.5 *	13.9 ± 2.9	3.1 ± 1.0 ***	0.9 ± 0.4 ***
qGWh5+qGWh10 _PYL	29.6 ± 4.3 **	10.1 ± 2.8 ***	3.7 ± 1.4	22 ± 7.9 ***	17.1 ± 2.7	25.1 ± 4.2 ***	6.2 ± 1.3 ***	3.8 ± 1.5
qGL4-2+qGWh5+qGWh10 _PYL	28.7 ± 5.1 **	9.4 ± 1.9 ***	3.9 ± 1.5	20 ± 5.2 ***	23.6 ± 5.0	25.1 ± 3.4 ***	6.1 ± 1.4 ***	2.6 ± 0.8 ***
Koshihikari	21.4 ± 6.9	3.3 ± 0.8	2.8 ± 1.7	6.4 ± 3.5	21.1 ± 4.0	14.9 ± 3.1	11.8 ± 3.2	4.1 ± 1.3
Yamadanishiki	39.3 ± 5.4	56 ± 4.2	69.4 ± 5.2	89 ± 4.7	13.0 ± 1.8	5.0 ± 1.4	4.0 ± 0.8	3.1 ± 1.4

The trait values show "mean value" ± "standard deviation".

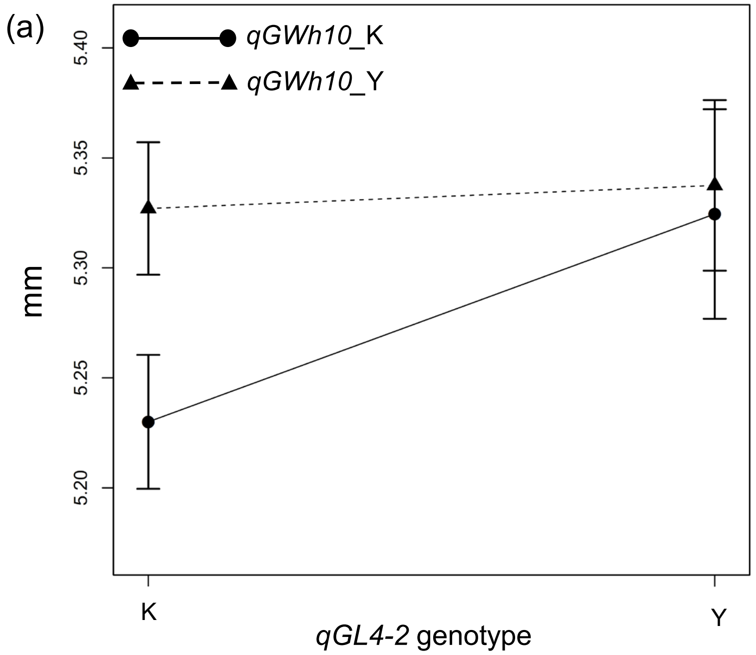
"*", "**" and "***" indicate significance at $P<0.05$, $P<0.01$ and $P<0.001$, respectively, compared between Koshihikari and each lines with Dunnett's multiple comparison method.

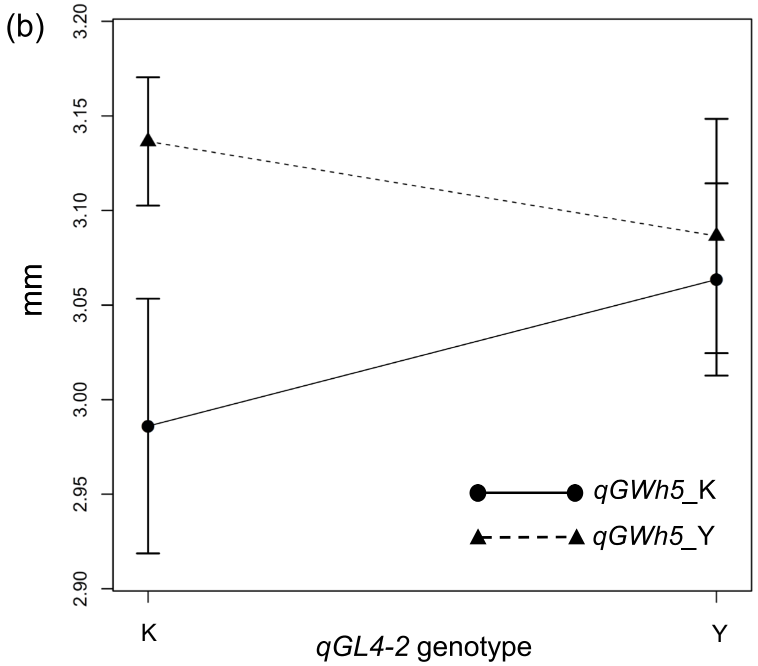
Table 4 Characteristic of heading date and rice yield of each near isogenic and pyramiding line.

Line	Days to heading		1000-grain weight (g)		Panicle number		Spikelet number per panicle		Total panicle weight (g)	
	2016	2017	2016	2017	2016	2017	2016	2017	2016	2017
qGL4-2 _NIL	100 ± 1 ***	101 ± 1 ***	#### ± 0.23 ***	#### ± 0.47 ***	14 ± 3	11 ± 2	126 ± 41	123 ± 28	51.18 ± 11.30	35.26 ± 3.99
qGWh5 _NIL	103 ± 1 ***	104 ± 2	#### ± 0.33 ***	#### ± 0.24 ***	13 ± 3	11 ± 1	138 ± 37	133 ± 32	47.09 ± 8.83	38.95 ± 5.17
qGWh10 _NIL	101 ± 1 ***	102 ± 1 ***	#### ± 0.31 ***	#### ± 0.42 ***	14 ± 2	10 ± 1	118 ± 31	121 ± 29	47.49 ± 6.64	32.66 ± 4.49
qGL4-2+qGWh5 _PYL	101 ± 2 ***	102 ± 2 **	#### ± 0.49 ***	#### ± 0.58 ***	14 ± 4	12 ± 3	137 ± 34	136 ± 33	55.12 ± 9.10	46.08 ± 5.76 ***
qGL4-2+qGWh10 _PYL	99 ± 1 ***	101 ± 1 ***	#### ± 0.19 ***	#### ± 0.25 ***	13 ± 3	10 ± 2	115 ± 28	118 ± 29	44.26 ± 8.63	32.84 ± 4.33
qGWh5+qGWh10 _PYL	100 ± 1 ***	101 ± 2 ***	#### ± 0.42 ***	#### ± 0.43 ***	13 ± 2	9 ± 2 *	123 ± 34	128 ± 29	51.15 ± 5.05	35.01 ± 4.20
qGL4-2+qGWh5+qGWh10 _PYI	99 ± 1 ***	101 ± 1 ***	#### ± 0.43 ***	#### ± 0.43 ***	13 ± 3	9 ± 1 **	132 ± 36	127 ± 28	50.61 ± 7.34	35.56 ± 2.84
Koshihikari	105 ± 1	105 ± 1	#### ± 0.38	#### ± 0.27	15 ± 3	11 ± 2	134 ± 34	131 ± 35	46.07 ± 9.93	34.51 ± 6.05
Yamadanishiki	119 ± 1	125 ± 1	#### ± 0.34	#### ± 0.31	14 ± 4	12 ± 2	no data	72 ± 17	33.3 ± 10.9	27.4 ± 3.26

The trait values show "mean value" ± "standard deviation".

"*", "**" and "***" indicate significance at $P<0.05$, $P<0.01$ and $P<0.001$, respectively, using Dunnett's multiple comparison method to compare Koshihikari with each line


(a)
qGWh10_K
qGWh10_Y
5.40
5.35
5.30
5.25
5.20
mm
K
Y
qGL4-2 genotype


(b)
3.20
3.15
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3.05
3.00
2.95
2.90
mm
qGWh5_K
qGWh5_Y
K
Y
qGL4-2 genotype

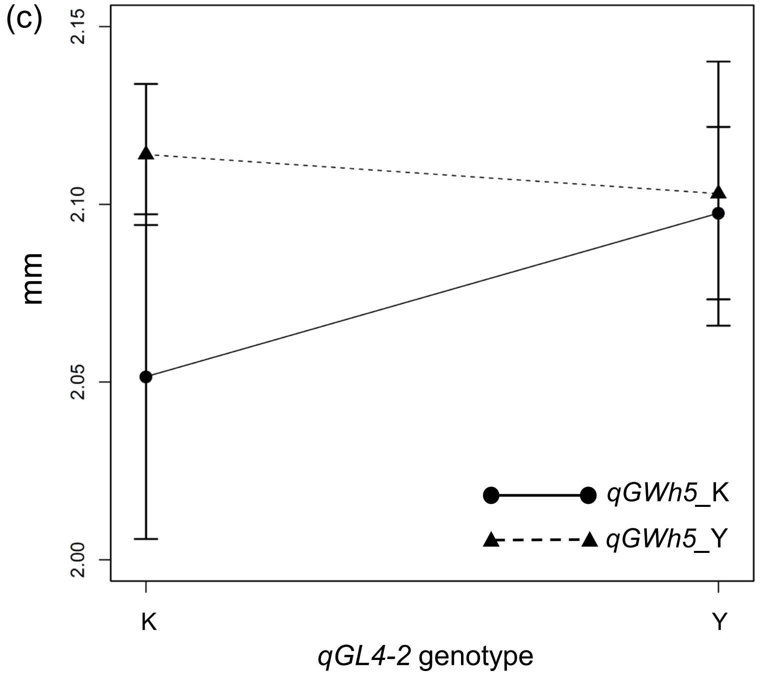
(c)
2.15
2.10
2.05
2.00
mm
qGWh5_K
qGWh5_Y
K
Y
qGL4-2 genotype

Figure caption

Fig. 1 Plots of the epistatic effect between QTLs for GL (a), GWh (b), and GT (c) in 2017. “K” and “Y” represent Koshihikari homozygous and Yamadanihsiki homozygous genotypes of QTLs, respectively.