Research Paper

Colocalization of QTLs for hull-cracked rice and grain size in elite rice varieties in Japan

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The control of insects that consume cereal grains is important for the production and storage of grains. Hull-cracked rice, which has splits in the hull, becomes more susceptible to insects both in the paddy field and during storage. The development of varieties with a low frequency of hull-cracked rice is the most economical and effective strategy to avoid insect damage and the environmental risks from agricultural chemical entering rice grains. In this study, we identified that QTLs for the frequency of hull-cracked rice and for grain width are located on the same chromosome using recombinant inbred lines derived from a cross between the elite rice varieties in Hokkaido, Japan, which are from the same pedigree and are genetically closely related. These QTLs were detected close to different molecular markers, which were separated by 1,101,675 bp, on chromosome 5 in the reference Nipponbare genome. In addition, low coefficient values of the phenotype were found between hull-cracked rice and grain size. These results suggested that the ratio of hull-cracked rice is independent of grain size. Using these QTLs, new varieties with low hull-cracked rice could be developed regardless of grain size.

Key Words: elite variety, grain size, hull-cracked rice, myINDEL, QTLs, rice.

Introduction

Rice grains are susceptible to insect damage. During the ripening stage in the paddy field, rice grains are injured by rice leaf bug *Trigonotylus caelestialium*, decreasing the grain quality at market in Japan (Ishimoto 2007, Ito 2004). World-wide, stored rice is susceptible to the lesser grain borer *Rhyzoperha dominica* (Chanbang *et al.* 2008a, 2008b, Kavallieratos *et al.* 2012). Rice grains are enclosed in a hull formed by two leaves, palea and lemma. Hull-cracked rice, which has splits in the hull, becomes more susceptible to grain borer attack. Nymphal development depends on feeding on grains with cracked hulls. The appearance and frequency of hull-cracked rice may reflect in the density of *T. caelestialium* in the field (Ishimoto 2007, Ito 2004), while *R. dominica* selects grains with hull-cracked rice for reproduction (Kavallieratos *et al.* 2012).

Damage from the insects can cause significant reductions in yields. Agricultural chemical control of insects has been

used in rice cultivation, but being able to omit chemical use is important for food safety in the production of grain cereals, the cost of chemicals, and environmental concerns. Development of varieties with lower frequencies of hull-cracked rice is the most economical and effective strategy to avoid insect damage and environmental risks from chemical use in rice. However, rice breeding programs for hull-cracked rice are difficult because of inadequate knowledge of the genetic basis of this trait.

Imbalance between hull size (sink) and grain size (source) may generate hull-cracked rice. Increased grain size is major objectives in rice breeding programs, and spikelet hulls could restrict grain growth, thus determining grain size (Li and Li 2016). Many QTLs for grain size have been identified for diverse grain size traits (reviewed by Huang *et al.* 2013), such as the difference between subspecies levels, *indica-japonica*. Today, the number of QTLs for grain size is increasing (Zhao *et al.* 2017, Zhen *et al.* 2017). Therefore, it is unclear which genes play a major role in the control grain size in the local rice varieties.

Current elite rice varieties in Japan show high grain quality and good eating quality for the market in Japan. Based on the grain shape, rice is divided into three classes, long, medium, and short, for which (grain length)/(grain width)

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values are more than 3.0, 2.0–3.0, and smaller than 2.0, respectively. Preferences for grain shapes in rice depend on the cultures in local regions (Calingacion *et al.* 2014). Short, round grains are favored in Japan, Korea, and northern China. Rice breeding programs have established many varieties with high yield and good eating quality in Hokkaido, Japan (Fujino *et al.* 2017). Elite rice varieties tend to have many tillers with short panicles. Without a change in grain size, rice production, as measured using 1,000-grain weight, has improved during rice breeding programs.

QTL mapping is an effective way to dissect the genetic basis of quantitative traits, including most of agronomic traits. QTLs for various agronomic traits have been identified during the past two decades (<http://www.gramene.org>). Many types of molecular marker sets, restriction fragment length polymorphisms (RFLPs), simple sequence repeats (SSRs), insertion/deletions (InDels), and single nucleotide polymorphisms (SNPs) have been listed. However, the efficiency of the application of these markers in studies, in which polymorphisms have been detected within materials, are dependent on the genetic relationships between varieties. The set-up of molecular markers that are polymorphic between parental lines is a key step for performing QTL mapping, but this is time consuming and labor-intensive when the parents are genetically closely related. In the pedigree of Japanese modern rice varieties, a small number of varieties and superior landraces have been frequently used as parental varieties (Yamamoto *et al.* 2010). This efficiency directly facilitates QTL analysis as genetic studies. Recently, several kinds of SNP chips have been used (Shanmugavadivel *et al.* 2017, Singh *et al.* 2017, Wang *et al.* 2017). In addition, genotype-by-sequencing (GBS) by next-generation sequencing (NGS) has been applied in rice (Chen *et al.* 2016, De Leon *et al.* 2016, Furuta *et al.* 2017, Zaid *et al.* 2017), because NGS has enabled the discovery of numerous SNPs. However, there is the cost of laboratory research on QTL analysis using primary mapping populations. Furthermore, there are too many datasets for breeders to handle alone.

Here, we reported QTLs for hull-cracked rice in rice. The frequency of hull-cracked rice has been increasing in elite rice varieties in Hokkaido, Japan, during rice breeding programs (Fujino *et al.* 2017). Using recombinant inbred lines (RILs) between the elite varieties in Hokkaido, a single QTL with a large effect on hull-cracked rice has been identified on chromosome 5. In addition, QTL analyses for grain size, length, width, and thickness were carried out. Another QTL on chromosome 5 increased grain width and thickness. Finally, we propose an analysis flow for NGS data for customizing DNA marker development for laboratory research, which was named myINDEL.

Materials and Methods

Plant material

Two *japonica* elite rice varieties, *Hoshinoyume* (HS) and

Kitakurin (KK), were used in this study as the parental varieties. Both were bred in rice breeding programs in Hokkaido, Japan, for the same current market class in Japan. HS was registered in 1996 and it shows a high frequency of hull-cracked rice. KK is a progeny of HS and was registered in 2014, but it has a low frequency in hull-cracked rice (**Supplemental Fig. 1**). To identify QTLs controlling hull-cracked rice and grain size, we developed mapping populations of RILs derived from the cross between HS and KK.

Phenotype evaluation

Four agronomic traits were evaluated in accordance with Fujino *et al.* (2017). In brief, the frequency of hull-cracked rice, grain length, grain width, and grain thickness were evaluated. We evaluated the values of varieties and RILs over two years, 2013 and 2014, because agro-economical traits are influenced by environmental conditions such as temperature during growth periods.

Plant material (**Supplemental Fig. 1**), re-sequencing, DNA analysis (**Supplemental Table 1**), and QTL analysis are described in **Supplemental Text 1**.

Results

Variations in the four traits of the ratio of hull-cracked rice and grain size

There was a large difference in the frequency of hull-cracked rice between the varieties (**Fig. 1**, **Table 1**). HS showed high levels of hull-cracked rice, 62.0% in 2013 and 71.5% in 2014, whereas KK showed low levels of hull-cracked rice, 2.2% in 2013 and 4.8% in 2014. In contrast, the differences in grain size were small and the grain shapes

Fig. 1. Panicles with a high ratio of hull-cracked rice in Hoshinoyume (HS, upper) and with a low ratio in Kitakurin (KK, lower). Scale indicates 1 cm.

belonged to the same market class, 1.89 of HS and 1.77 of KK in (grain length)/(grain width) ratio (**Table 1**).

To identify QTLs controlling hull-cracked rice and grain size, we developed mapping populations of RILs derived from the cross between HS and KK. There was wide variation with a continuous distribution in the frequency of hull-cracked rice, ranging from 2.2% to 71.5% (**Fig. 2, Supplemental Table 2**). Conversely the variations in grain size, grain length, grain width, and grain thickness were all small. High coefficients of phenotype between years, 0.75–0.81, were detected in all four traits, suggesting that these traits were stable for environmental conditions. There was a low coefficient between hull-cracked rice and grain size (**Supplemental Table 3**). Among the traits for grain size, low coefficients were observed between grain length and grain width and between grain length and grain thickness, whereas high positive coefficients were detected between grain width and grain thickness, 0.66 in 2013 and 0.76 in 2014 (**Supplemental Table 3**). Therefore, (grain length)/(grain width) of grain shape in the RILs showed that they were in the same market class, with narrow ranges of 1.68–1.98 in 2013 and 1.70–2.00 in 2014.

Table 1. Parental phenotypes

Trait	Year	Kitakurin (KK)	Hoshinoyume (HS)
Hull-cracked rice (%)	2013	2.2	62
	2014	4.8	71.5
Grain length (mm)	2013	5.18 ± 0.23	5.28 ± 0.25
	2014	5.21 ± 0.28	5.25 ± 0.28
Grain width (mm)	2013	2.93 ± 0.19	2.79 ± 0.14
	2014	2.97 ± 0.17	2.84 ± 0.13
Grain thickness (mm)	2013	1.98 ± 0.11	1.94 ± 0.14
	2014	1.99 ± 0.15	1.98 ± 0.08
Grain length/Grain width	2013	1.77	1.89
	2014	1.75	1.85

Fig. 2. Frequency distributions of hull-cracked rice and grain size in recombinant inbred lines ($n = 96$) derived from the cross between Hoshinoyume (HS) and Kitakurin (KK). A, the ratio of hull-cracked rice, B, grain length, C, grain width, D, grain thickness evaluated in 2013.

Polymorphisms between the parental genomes

To develop molecular markers for the parents, the genome sequences of HS and KK were determined using NGS methods. A total of 152.4 Mb of reads in HS and 150.7 Mb of reads in KK were obtained (**Supplemental Table 4**). Trimmed sequences were then mapped to the Nipponbare sequence (IRGSP 1.0). Finally, we had sequences, which covered more than 90% of the rice genome in >10 depth (**Supplemental Table 4**). SNPs were distributed over the genome (**Supplemental Table 5**). The distributions of SNPs varied along chromosomes, ranging from 7,750 on chromosome 5 to 38,807 on chromosome 11 in HS and from 8,457 on chromosome 5 to 37,214 on chromosome 11 in KK.

A total of 360,038 SNPs between HS and KK were distributed over the genome, ranging from 11,914 on chromosome 5 to 56,076 on chromosome 11 (**Supplemental Table 6**). Although the average SNP density per 1 Mb was 965.0 over the genome, the distributions of SNPs varied along the chromosomes, ranging from 397.1 on chromosome 5 to 1933.7 on chromosome 11 (**Fig. 3, Supplemental Table 7**). The SNP density per 1 Mb along the chromosomes was compared. The highest density of SNPs was 6,854, located in the 16–17 Mb region on chromosome 2,

Fig. 3. SNPs distribution between Hoshinoyume (HS) and Kitakurin (KK) over the genome. Horizontal bars indicate the INDEL markers used for QTL analysis. Numbers (1–12) indicate rice chromosome numbers. The frame map is based on the Nipponbare genome (IRGSP 1.0).

whereas the lowest density was 39 observed in the 40–41 Mb region on chromosome 1 (**Supplemental Table 7**). Although HS and KK, a progeny of HS, are genetically closely related, SNPs on the chromosomal regions confirmed their identity at the sequence level. The chromosomal regions with high and low SNP identity might be from different origins and shared origins, respectively. The longest chromosomal regions with SNPs more than the chromosomal averages were two 6-Mb long sections in the 3–8 Mb region on chromosome 5 and the 18–24 Mb region on chromosome 6, whereas the longest region with less than the chromosomal average was the 14 Mb length in the 7–23 Mb region on chromosome 12 (**Supplemental Table 7**).

Next, polymorphic InDels, except for SSRs, between HS and KK over the genome were targeted for primer design for PCR as molecular markers. Among the 36,736 InDels in HS and 36,315 in KK (**Supplemental Table 8**), we focused on InDels of 20–2,000 bp in size over the genome, which are easier to handle in experiments in the laboratory. Sixty-five InDel markers showed clear polymorphisms between the parents, and these markers were used for QTL analysis (**Supplemental Table 1**).

QTLs with large effects on the frequency of hull-cracked rice and grain size

A single QTL with a large effect on the frequency of hull-cracked rice was detected near INDEL01046 on chromosome 5, *qHC5*, which accounted for 20.0% in 2013 and 21.9% in 2014 of total phenotypic variation. The KK allele decreased the ratio of hull-cracked rice (**Table 2**).

A total of six QTLs for grain size were detected between the two years (**Table 2**). A QTL, *qGW5*, with large effects, 23.5–31.6% of total phenotypic variation, for grain width and grain thickness was detected at INDEL01608 on chromosome 5 in both years. The KK alleles increased grain width and thickness. A single QTL, *qGL11*, for grain length was detected at INDEL01036 on chromosome 11 in 2014 only. This QTL accounted for 21.4% of total phenotypic

variation. The KK allele decreased grain length.

Both QTLs for hull-cracked rice and grain width, *qHC5* and *qGW5*, were detected at the 26.0–28.0 Mb region on chromosome 5 (**Table 2**, **Supplemental Fig. 2**).

Discussion

The control of insects in cereal grains is important for the production and storage of cereal grains. Hull-cracked rice, which has splits in the hull, becomes more susceptible to insects both in the rice field and during storage (Chanbang *et al.* 2008a, 2008b, Ishimoto 2007, Ito 2004, Kavallieratos *et al.* 2012). The development of varieties with low rates of hull-cracked rice is the most economical and effective strategy to avoid insect damage and reduce the environmental risks from chemicals applied to eliminate these insects on rice. In this study, we identified a QTL for hull-cracked rice near INDEL01046, *qHC5*, and a QTL for grain width near INDEL01608, *qGW5*, located on chromosome 5, and these QTLs were detected close to different molecular markers, which were separated by 1,101,675 bp in the reference Nipponbare genome. There were 10 plants with recombinations between these markers (**Supplemental Fig. 2**, **Supplemental Table 9**). In addition, there were low coefficient values of the phenotype between hull-cracked rice and grain size (**Supplemental Table 3**). These results suggested that hull-cracked rice is independent of grain size, but these QTLs located in a 1.1 Mb region on chromosome 5. The KK allele showed the opposite functions on the traits, a decrease in the frequency of hull-cracked rice at *qHC5* and enlargement of grain width at *qGW5*.

QTLs for grain width and thickness, *qGW5* and *qGT5*, were detected on the same chromosomal region near INDEL01608 on chromosome 5. There is a possibility that *qGW5* for grain width had pleiotropic effects on grain thickness. QTLs with pleiotropic effects on grain shape have been found frequently in rice (Bai *et al.* 2010, Fan *et al.* 2006, Li *et al.* 2011, Mao *et al.* 2010, Song *et al.* 2007, Zhen

Table 2. QTLs for hull-cracked rice and grain size

Trait	Year	QTL	NMM	Chromosome	Position	LOD	PVE (%)	AE	Threshold
Hull-cracked rice (%)	2013	<i>qHC5</i>	INDEL01046	5	26,862,898	7.35	20.0	−7.30	4.54
	2014	<i>qHC5</i>	INDEL01046	5	26,862,898	7.47	21.9	−8.00	5.14
Grain length (mm)	2013	—	—	—	—	—	—	—	3.45
	2014	<i>qGL11</i>	INDEL01086	11	22,018,392	6.32	21.4	−0.050	3.24
		<i>qGL4</i>	INDEL01036	4	13,370,937	3.27	10.0	0.034	
Grain width (mm)	2013	<i>qGW5</i>	INDEL01608	5	27,964,573	8.29	23.5	0.034	3.35
		<i>qGW2</i>	INDEL01022	2	28,432,250	5.22	14.2	0.029	
		<i>qGW4</i>	INDEL01033	4	695,303	3.87	2.3	−0.012	
		<i>qGW11</i>	INDEL01082	11	9,156,370	3.88	4.9	−0.018	
	2014	<i>qGW5</i>	INDEL01608	5	27,964,573	12.13	31.6	0.040	4.44
		<i>qGW2</i>	INDEL01022	2	28,432,250	5.13	11.3	0.024	
		—	—	—	—	—	—	—	
Grain thickness (mm)	2013	<i>qGT5</i>	INDEL01608	5	27,964,573	6.95	28.1	0.021	3.40
	2014	<i>qGT5</i>	INDEL01608	5	27,964,573	9.11	26.2	0.021	3.42
		<i>qGT2</i>	INDEL01022	2	28,432,250	4.49	16.7	0.018	

NMM; nearest molecular marker, PVE; phenotypic variation explained, AE; additive effect

Position is based on the Nipponbare genome (IRGSP 1.0). Threshold was calculated using 1,000 permutation tests.

et al. 2017). Additionally, advanced backcrossed progenies derived from a single combination between *japonica* *Koshihikari* and *indica* *IR64* confirmed the complexity of the control of grain size, with 65 QTLs identified (Nagata *et al.* 2015). Conventional breeding to develop improved varieties with low levels of hull-cracked rice has been conducted. The QTLs, *qHC5* and *qGW5*, found in this study could have great potential value in rice breeding programs. Using these QTLs, new varieties with lower levels of hull-cracked rice could be developed instead of changes in grain size.

In this study, we performed QTL analysis using elite rice varieties in a local region that is genetically closely related. SSR markers among several kinds of DNA markers are useful for genetic studies in rice because they frequently show multiple alleles (McCouch *et al.* 2002). Considerable efforts in screening for polymorphic SSR markers between such varieties are critical for QTL analysis. To simplify such efforts, we can directly compare the genome sequences obtained from NGS. Without abundant SNPs, small numbers of polymorphic InDels could be sufficient for QTL analysis using primary mapping population. The significant impact of InDel markers is the easier manipulation by PCR methods in the laboratory.

We proposed this analysis flow as myINDEL, which customizes DNA marker development using NGS sequences in varieties that are genetically closely related (Fig. 4). At first, InDel regions over the genome were predicted by programs (for example Pindel (Ye *et al.* 2009) and Breakdancer (Chen *et al.* 2009)). Second, InDels were chosen based on the demands, the chromosomal position, polymorphism, and InDel

size. Third, primers were designed. Finally, polymorphism of InDel was validated by the experiment. myINDEL is breeder-friendly for the handling of huge data sets from NGS and could facilitate the identification of QTLs for sophisticated traits in elite varieties used in plant breeding programs. The dataset of SNPs in NGS sequences is too large to use for QTL analysis with the primary mapping populations, but is a way to detect polymorphism between varieties that are genetically closely related. The validated InDel markers will be used for not only QTL analysis but also in further studies including marker-assisted selection, fine mapping, and map-based cloning.

Elite traits with favorable values have been improved using germplasm within the similar genetic pool, or sometimes using exotic germplasm, in plant breeding programs (Fujino *et al.* 2015, 2017, Shinada *et al.* 2014). Associations between QTLs for elite traits and chromosomal genotypes may be from genetically different origins or by spontaneous mutations generated during rice breeding programs within identical chromosomal regions, such as loss-of-function *hd5* controlling heading date in rice (Fujino *et al.* 2013) and low temperature germinability *qLTG3-1* (Fujino and Iwata 2011, Fujino and Sekiguchi 2011). Improved understanding of the genetic basis of elite traits could improve the strategies to develop further elite varieties.

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Fig. 4. myINDEL, an analysis flow for customizing the development of DNA markers between varieties that are genetically closely related.

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