Note

Quantitative trait loci analysis of grain appearance in *Oryza sativa* L. ‘Emi-no-kizuna’

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A quantitative trait locus (QTL) analysis was conducted on grain appearance in Emi-no-kizuna, a rice cultivar that has superior and stable appearance of the brown rice grain and high tolerance to high temperature stress, by using F₃ lines derived from Emi-no-kizuna and Tomohonami. The investigation was performed 2013 and 2015. In summer 2013 the air temperature was higher and the larger differences in grain appearance were observed. In the QTL analysis, a highly contributing locus, *qGA8*, was detected at the end of the short arm of chromosome 8. Because trends of reduced the occurrence of white immature kernel and increased the percentage of perfect grain were observed in Emi-no-kizuna genotype in both years, *qGA8* is likely to be an important QTL that is dominant in the superior grain appearance of Emi-no-kizuna. Also, *qGA8* was linked to a QTL associated with days to heading. Another QTL, *qGA7*, associated with grain appearance was detected on chromosome 7 in 2013. Because no negative correlation was found between the genotype of *qGA8* and thousand grain weight, it should be possible to breed cultivars that possess Emi-no-kizuna genotype *qGA8* without a decrease in thousand grain weight.

Key Words: Emi-no-kizuna, grain appearance (GA), high temperature tolerance, *Oryza sativa* L., quantitative trait locus (QTL), white immature kernel (WIK).

Introduction

Recently, in Japan, air temperatures have been rising in summer and causing problems with the grain appearance of paddy rice. Paddy rice tends to accumulate insufficient starch when it is exposed to high temperatures during the grain filling period, resulting in increase of white immature kernel (WIK) and lowering of grain appearance (GA) (Nagato and Ebata 1960, Tashiro and Wardlaw 1991). Low GA decreases sales prices, farmers' incomes and milling quality. Therefore, it is very important to breed a paddy rice cultivar that is highly tolerant to high temperature stress and produces few WIK, even when exposed to high temperatures during the grain filling period. The degree of occurrence of WIK upon exposure to high temperatures during grain filling is diverse and differs among cultivars (Iida *et al.* 2002, Nagato and Ebata 1965) and this trait is inherited (Tabata *et al.*

2005). Therefore, it is possible to breed a paddy cultivar with high tolerance to high temperature stress. In fact, cultivars have already been bred that have stable GA even under high temperatures; examples are ‘Tentakaku’ (Yamaguchi *et al.* 2006), ‘Akisakari’ (Tomita *et al.* 2009), ‘Nikomaru’ (Sakai *et al.* 2010) and ‘Natsuhonoka’ (Wakamatsu *et al.* 2016). However, considering that warming is predicted to continue and the problem of high WIK levels is anticipated to worsen (Morita 2008), we need to continue breeding cultivars that have further tolerance to high temperature stress.

‘Emi-no-kizuna’ has a stable and superior GA and high tolerance to high temperature stress. Even in 2010, when the summer was the hottest on record in Japan, almost no deterioration was observed in GA (Nagaoka *et al.* 2016). Thus, Emi-no-kizuna can be a useful genetic resource for breeding rice cultivars with high temperature tolerance. Quantitative trait locus (QTL) information would be very useful for efficiently adding the high temperature tolerance trait of Emi-no-kizuna to new cultivars, but the relevant QTLs have not yet been revealed. There have been QTL analyses of GA under exposure to high temperatures during grain filling (Kobayashi *et al.* 2013, Tabata *et al.* 2007, Wada *et al.*

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2015), but it would be important to organize the appropriate genetic background for high GA under natural conditions before evaluating high temperature tolerance. In this study, we conducted a QTL analysis of GA by using hybrid progenies of Emi-no-kizuna under natural conditions.

Materials and Methods

Plant materials

Plant materials were cultivated in paddy fields at the Central Region Agricultural Research Center, NARO (Joetsu City, Niigata Prefecture: 37°6'59"N, 138°16'14"E).

In 2012, an F₂ population derived from Emi-no-kizuna and 'Tomohonami' was planted. Tomohonami is a cultivar that possesses the rice blast resistance gene *pi21* discovered from upland rice and its genetic background is similar to that of 'Koshihikari'; it is prone to producing WIK and has poor resistance to high temperature stress (Saka *et al.* 2010). The seeds were sown on 17 April, and the plantlets were transplanted on 11 May. After the plants had matured, 180 individual plants were randomly sampled.

In 2013 and 2015, we grew 180 lines (F₃) from the 180 individual plants and used them for QTL analysis. The rice was sown on 17 April in 2013 and 21 April in 2015, and was transplanted on 17 May in 2013 and 15 May in 2015, respectively. The day on which panicles had emerged on about 50% of the plants belonging to a line was considered to be the heading date of the line. The number of days from the day of transplantation to the heading date was considered the days to heading (DH). After the plants had matured, 10 plants were sampled per line.

In both years, 5 g/m² each of N, P₂O₅, and K₂O was applied as a basal dressing. No additional fertilizer was applied.

Air temperature measurement

Air temperature was measured at 10 min intervals by using a thermometer, "Ondotori Jr." (model RTR-52A; T&D Corporation, Nagano, Japan) at a height of about 80 cm from the ground surface at the level of the rice panicles. The occurrence of WIK increases drastically when the average temperature during 20 days after heading is over 26 to 27°C (Terashima *et al.* 2001, Wakamatsu *et al.* 2007). Therefore, we used the mean air temperature 20 days after heading (hereinafter referred to as the "ripening temperature") as an index of air temperature.

Evaluation of GA and thousand grain weight

GA and thousand grain weight (GW) were evaluated by using a grain quality inspector (model RGQ120A; Satake Corporation, Hiroshima, Japan). After maturing, all brown rice grains were collected. The percentages of perfect grains (PG), milky white grains (MW), basal white grains (BW), and white back grains (WB) were determined and used as indexes of GA. Although the grain quality inspector is not able to recognize the difference between white back and white belly, we regarded injury to the side face of grains as

white back because white belly grains were not detected by visual observation in both parents and all lines. For the F₃ lines and parents, the mean of 10 plants was used as the value for each line. Note that here we sometimes refer to MW, BW and WB collectively as WIK.

QTL analysis

DNA was extracted from each F₂ individual plant in accordance with the method of Monna *et al.* (2002). Genotype was investigated by using 243 single nucleotide polymorphism (SNP) markers (Ebana *et al.* 2010, Nagasaki *et al.* 2010) positioned throughout the genome. Linkage maps were prepared by using MAPMAKER/EXP 3.0 (Lander *et al.* 1987). For the mapping function, Kosambi's function was used (Kosambi 1944). QTL analysis was conducted by using the composite interval mapping method and Windows QTL Cartographer 2.5 (Wang *et al.* 2006). A threshold value corresponding to a significance level of 5% was determined by 1000 permutations. When the obtained LOD score exceeded the threshold value, it was judged that a QTL was detected. In the analysis, the percentages of grains that were arcsine transformed were used to standardize dispersion.

Results

Air temperature transitions

Changes in the mean air temperature and mean duration of sunshine (hereinafter referred to as the "duration of sunshine") during the first 20 days in each year are shown in Fig. 1, together with the distribution of heading dates. Ripening temperature was higher in 2013 and duration of sunshine was longer in 2013. In 2015, ripening temperature was higher during the early period of heading. On the other

Fig. 1. Changes in mean air temperature and mean duration of sunshine during the first 20 days. Lines connecting circles denote air temperature; and solid and dotted lines show duration of sunshine. Double arrows show the heading date distribution in each year. Numbers in parenthesis are the numbers of days to heading. The values for duration of sunshine were cited from records at Takada Meteorological Station, Japan Meteorological Agency (37°6'N, 138°15'E, <http://www.jma.go.jp/jma>).

hand, in 2013, there was not large difference in ripening temperature, though it was slightly higher in the middle period of heading.

Distribution of traits in the F_3 lines and correlations among traits

The frequency distributions of each trait in the F_3 lines are shown in Fig. 2. The frequencies of occurrence of each percentage of PG and WIK, DH and GW showed continuous distributions in both years. The percentage of PG was greater in 2015. In 2015, the percentage of MW was similar to, or slightly larger than, that in 2013. The percentage of BW and WB were higher in 2013. The percentage of GW in 2015 was smaller than in 2013 in the F_3 lines and also in the parents. There were significant correlations between DH and percentages of PG and WIK in both years (Table 1). In 2013, no significant correlation was observed between GW and percentages of PG and WIK.

QTL analysis

The results of the QTL analysis are shown in Fig. 3 and Table 2. In 2013, a QTL associated with percentage of PG was detected on chromosome 7; a QTL associated with percentage of BW and DH were detected on chromosome 8. In 2015, a QTL associated with percentage of PG and DH was detected on chromosome 8. Of these loci, the QTL associated with percentage of BW (detected in 2013) and the QTL associated with percentage of PG (detected in 2015) were detected in the same region at the end of the short arm of chromosome 8; they had high percentages of phenotypic variation explained by each QTL (PVE) of 51.8% and 70.9%, respectively. The QTL associated with GA on chromosome 7 and the QTL detected on chromosome 8 for the same trait were named *qGA* (*Grain Appearance*) 7 and *qGA8*, respectively (Fig. 3). No QTL was found that was associated with percentage of MW or WB or with GW in both years.

Fig. 2. Score distributions of percentages of PG and WIK, DH and GW in an F_3 lines derived from Emi-no-kizuna and Tomohonami. Arrows show scores for the parents. E and T denote Emi-no-kizuna and Tomohonami, respectively. Y axes denote number of F_3 lines. PG, perfect grains; MW, milky white grains; BW, basal white grains; WB, white back grains; DH, days to heading; GW, thousand grain weight.

Table 1. Correlation coefficients among measures of percentages of PG and WIK, DH and GW

		2013						2015					
		PG	MW	BW	WB	DH	GW	PG	MW	BW	WB	DH	GW
2013	PG	1.00											
	MW	-0.92 ***	1.00										
	BW	-0.75 ***	0.59 ***	1.00									
	WB	-0.78 ***	0.66 ***	0.48 ***	1.00								
	DH	0.72 ***	-0.69 ***	-0.59 ***	-0.46 ***	1.00							
	GW	0.08 ns	-0.09 ns	-0.02 ns	0.06 ns	0.21 **	1.00						
2015	PG	0.55 ***	-0.53 ***	-0.45 ***	-0.43 ***	0.01 ns	0.54 ***	1.00					
	MW	-0.49 ***	0.46 ***	0.34 ***	0.37 ***	-0.02 ns	-0.49 ***	-0.91 ***	1.00				
	BW	-0.65 ***	0.63 ***	0.60 ***	0.56 ***	-0.01 ns	-0.57 ***	-0.70 ***	0.49 ***	1.00			
	WB	-0.31 ***	0.31 ***	0.27 ***	0.21 **	0.04 ns	-0.33 ***	-0.51 ***	0.34 ***	0.35 ***	1.00		
	DH	0.67 ***	-0.64 ***	-0.59 ***	-0.49 ***	0.17 *	0.86 ***	0.72 ***	-0.61 ***	-0.68 ***	-0.44 ***	1.00	
	GW	0.15 *	-0.12 ns	-0.17 *	-0.06 ns	0.10 ns	0.27 ***	0.22 **	-0.17 *	-0.18 *	-0.17 *	0.32 ***	1.00

***, ** and *: Significant at levels of 0.1%, 1% and 5%, respectively. ns: Not significant at a level of 5%.

PG, perfect grains; MW, milky white grains; BW, basal white grains; WB, white back grains; DH, days to heading; GW, thousand grain weight.

Fig. 3. QTL regions associated with GA and DH. Major SNP markers are shown on the right of the chromosomes. Bars on the left side of the chromosomes indicate the intervals above the LOD threshold values. Triangles denote the positions of LOD peaks. Black and open triangles indicate the peaks of QTLs where values increase in the Emi-no-kizuna and Tomohonami genotypes, respectively. Hatched arrows indicate where QTLs are likely to exist. Chromosomes on which no QTLs were detected are not shown.

PG, perfect grains; BW, basal white grains; DH, days to heading.

Relationship between the *qGA8* genotype and each trait

The relationships between the *qGA8* genotype and percentages of PG and WIK, as well as number of DH and GW, are shown in **Fig. 4**. In the F_3 lines, the Emi-no-kizuna genotype showed trends of high percentage of PG and low occurrence of WIK in both years. The Emi-no-kizuna genotype also showed a trend toward increased number of DH; heading date tended to be delayed by a few days. In 2015, the Tomohonami genotype of *qGA8* showed a trend toward small GW.

Discussion

In both years, the percentages of PG and WIK, as well as DH and GW in the F_3 lines between Emi-no-kizuna and Tomohonami showed continuous distributions (**Fig. 2**). This suggested that several genes were involved in GA, DH and GW in Emi-no-kizuna. Ripening temperatures in almost all F_3 lines were above 26°C in 2013 (**Fig. 1**); this was therefore the year when GA was more prone to deterioration. In fact, the lower percentage of PG, higher occurrence of WIK, and larger difference were observed in 2013 compared to in 2015 (**Fig. 2**).

Significant correlations were observed among the percentages of PG and WIK in both years, regardless of trait (**Table 1**). From these results, we deduced that the genetic

Table 2. QTLs detected by composite interval mapping analysis and corresponding to GA and DH

Year	Trait	Chr.	Nearest marker	Peak position (cM)	LOD	Additive effect ^a	PVE ^b	LOD threshold ^c
2013	PG	7	aa07005154	98.8	4.36	4.05	8.9	3.28
	BW	8	aa08000727	9.9	3.88	-4.78	51.8	3.57
	DH	8	aa08000792	35.7	7.28	1.98	35.4	5.22
2015	PG	8	aa08000002	0.0	6.77	7.41	70.9	5.19
	DH	8	aa08000792	35.7	6.79	2.12	33.3	4.52

^a Additive effect was positive in the Emi-no-kizuna genotype.

^b Percentage of phenotypic variation explained by each QTL.

^c LOD threshold was the value that was significant at the 5% level in 1000 permutations.

PG, perfect grains; BW, basal white grains; DH, days to heading.

factors that increases the percentage of PG, or reduces occurrence of WIK, were common. On the other hand, in both years, a significant positive correlation was observed between DH and percentage of PG; and significant negative correlations were observed between DH and percentages of WIK (**Table 1**). These results suggested that GA in Emi-no-kizuna are genetically associated with heading characteristics.

QTL analyses were conducted on the F_3 lines for the percentages of PG and WIK, DH and GW. In 2013, a QTL associated with percentage of PG and a QTL associated with percentage of BW were detected on chromosomes 7 and 8, respectively. In 2015, a QTL associated with percentage of PG was detected on chromosome 8 (**Fig. 3**, **Table 2**). In both years, PVEs of the QTLs detected at the end of the short arm of chromosome 8 were very high (**Table 2**). In this region, GA was classified for each genotype. Regardless of whether a QTL was detected or not, trends of increased percentage of PG and reduced occurrence of WIK were observed in the Emi-no-kizuna genotype (**Fig. 4**). From these results, the QTL (*qGA8*) is likely to suppress the occurrence of WIK in Emi-no-kizuna in a versatile manner and is thus likely to be a dominant and important QTL in increasing percentage of PG. Tabata *et al.* (2007) reported a QTL at the end of the short arm of chromosome 8 that was associated with the occurrence of WB, but it is difficult to simply compare their results with ours because they used materials, markers, and evaluation methods that differed from ours. It will be important precisely to evaluate this locus under higher temperatures by preparing near isogenic lines (NILs) for *qGA8* via backcrossing. We did not detect a QTL associated with percentage of PG in 2013 and percentage of BW in 2015, respectively, in the *qGA8* region. This was likely because the occurrence pattern of WIK and the distribution pattern of percentages of PG and BW differed between 2013 and 2015 (**Fig. 2**). Particularly, because occurrence of BW is enhanced under high temperature condition (Morita 2008), the distribution pattern of percentage of BW might be made clearly in 2013. On the other hand, a QTL associated with DH was detected on chromosome 8 in

Fig. 4. Relationships between the genotype of *qGA8* in F_3 lines and percentages of PG and WIK, DH and GW. The genotype of the SNP marker aa08000031 was used as the index. In bars: E, Emi-no-kizuna genotype; H, heterogeneous; T, Tomohonami genotype. Arrows show values for the parents.

PG, perfect grains; MW, milky white grains; BW, basal white grains; WB, white back grains; DH, days to heading; GW, thousand grain weight.

both years (Fig. 3, Table 2). The locus was therefore likely to exist near the SNP marker locus aa08000792. In the vicinity of this region, Wang *et al.* (2002) have reported a QTL associated with heading, and Lin *et al.* (2003) have reported the presence of *Hd5*. We are unable to discuss in any detail the disagreement or agreement of our results with these past reports, but it is likely that Emi-no-kizuna also has a gene associated with heading characteristics in this region. This QTL is linked to *qGA8*, and thus lines that had the Emi-no-kizuna genotype *qGA8* allele showed a trend toward late heading (Fig. 3). In order to evaluate net effect

of *qGA8*, we need to have taken measures to actively break the linkage by using DNA markers in breeding NILs. In 2013, a QTL (*qGA7*) associated with percentage of PG was detected on chromosome 7 (Fig. 3, Table 2). This QTL had a smaller PVE than *qGA8*, and no QTL was detected in this region in 2015. On chromosome 7, *Apq1*, which suppresses reduction in GA under high temperature stress, has been found in the *Indica* cultivar ‘Habataki’ and reported, but its location is slightly different from that of *qGA7* (Murata *et al.* 2014). Also, another QTL associated with the occurrence of white belly has been reported (Tan *et al.* 2000), but little

information is available. It will be a future task to breed NILs for *qGA7* and investigate the effects of the locus alone and the additive effects with *qGA8*.

In 2015, significant correlations were observed between GW and the percentages of PG and WIK (Table 1), and a trend toward reduced GW was observed in Tomohonami genotype *qGA8* (Fig. 4). The insufficiency of photosynthetic assimilation products by not only high temperature but also lack of sunshine during the grain filling period results in both an increase in the occurrence of WIK and a decrease in grain weight (Morita 2008). Because sunshine was relatively lacking in summer 2015 (Fig. 1), photosynthetic assimilation products likely became insufficient, resulting in insufficient grain filling and a slight reduction in GW. The above findings suggest that it is possible to breed cultivars that possess Emi-no-kizuna genotype *qGA8* without a decrease in thousand grain weight.

Acknowledgments

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