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## Genetic studies for breeding of rice cultivars with superior grain appearance and lodging resistance from the rice cultivar ‘Emi-no-kizuna’

Ichiro Nagaoka<sup>a</sup>, Hideki Sasahara<sup>b</sup>, Kei Matsushita<sup>c</sup>, Hideo Maeda<sup>c</sup>, Shuichi Fukuoka<sup>c</sup> and Utako Yamanouchi<sup>c</sup>

<sup>a</sup>Central Region Agricultural Research Center, NARO, Joetsu, Japan; <sup>b</sup>Western Region Agricultural Research Center, NARO, Fukuyama, Japan; <sup>c</sup>Institute of Crop Science, NARO, Tsukuba, Japan

### ABSTRACT

We conducted a quantitative trait locus (QTL) analysis of grain appearance (GA) and agronomic traits of rice, using 128 recombinant inbred lines derived from ‘Emi-no-kizuna’ and ‘Tomohonami’. We detected two promising QTLs associated with GA: *qGA4* on chromosome 4 and *qGA8* on chromosome 8. *qGA4* contributed highly to the greater percentage of perfect grains of the Emi-no-kizuna genotype. In the same region, we detected other QTLs associated with panicle number and spikelet number per panicle. In near-isogenic lines (NILs) in which Emi-no-kizuna alleles were introgressed in the genomic region of only the *semi-dwarf 1* (*sd1*) locus (NIL\_1) and both the *sd1* locus and *qGA8* (NIL\_2), respectively, the percentage of perfect grains was significantly higher and the percentages of milky white, basal white, and white back grains were significantly lower than in Tomohonami; and the percentages of milky white and white back grains of NIL\_2 were significantly lower than those of NIL\_1. These results suggest that introgression in the *sd1* region could improve GA, and that the addition of *qGA8* could further improve GA. The culm lengths of the NILs were significantly shorter than that of Tomohonami, indicating improved lodging resistance. Grain weight of NIL\_2 was significantly smaller than that of NIL\_1, suggesting that the effect of *qGA8* could be pleiotropic, or the gene that underlies *qGA8* could be linked with genes associated with grain weight.

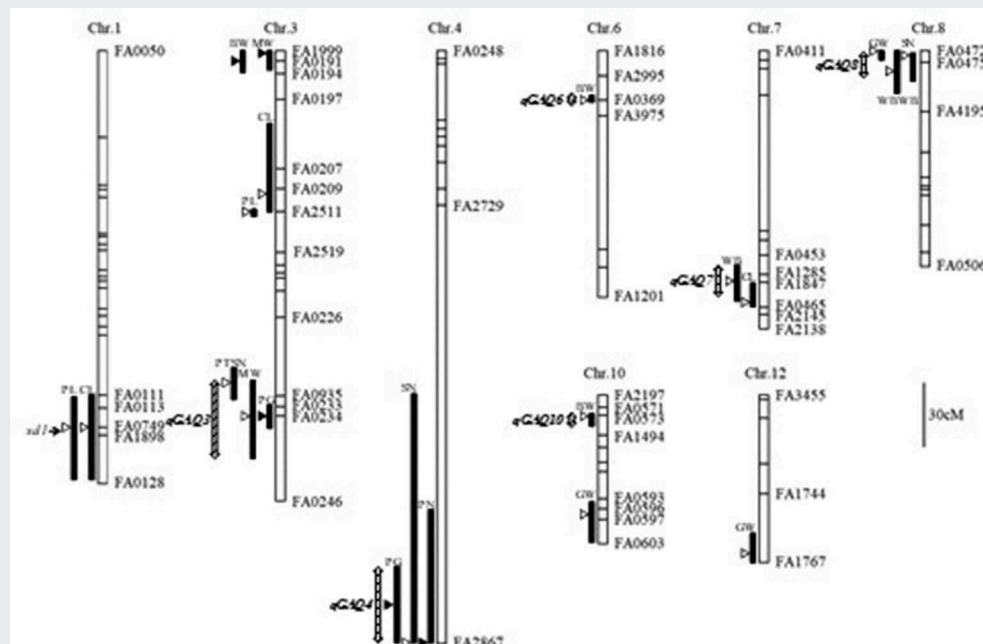
**Abbreviations:** ANOVA: analysis of variance; AT20: mean air temperature in the 20 days after heading; BW: basal white grain; CL: culm length; DAH: days after heading; GA: grain appearance; GW: 1000-grain weight; LOD: logarithm of odds; MW: milky white grain; NIL: near-isogenic line; PG: perfect grain; PL: panicle length; PN: panicle number; PTSN: putative total spikelet number; PVE: percentage of phenotypic variation explained; QTL: quantitative trait locus; RIL: recombinant inbred line; SN: spikelet number per panicle; SNP: single nucleotide polymorphism; WB: white back grain

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Emi-no-kizuna; grain appearance; high temperature tolerance; quantitative trait locus; rice; *sd1*



## Introduction

Summer air temperatures in Japan are rising, spoiling the grain appearance (GA) of paddy rice. A typical problem is increased chalkiness due to insufficient starch accumulation (Morita, 2008). Lowered GA decreases the market value of the rice and thus the farmers' incomes, and milling quality. As warming is predicted to continue and cause even more serious problems (Morita, 2008), it is necessary to breed cultivars that produce less chalkiness, particularly under high temperatures during the grain filling period.

The occurrence of chalkiness differs widely among cultivars (Iida, Yokota, Kiriha & Suga, 2002; Nagato & Ebata, 1965) and is heritable (Tabata, Iida & Ohsawa, 2005). It is thus likely that we can breed cultivars with improved GA. Quantitative trait loci (QTLs) associated with GA have been reported (Kobayashi et al., 2013; Tabata et al., 2007; Wada et al., 2015), and we identified QTLs for GA in the paddy rice cultivar Emi-no-kizuna, which has stable and superior GA (Nagaoka et al., 2017). These QTLs should prove useful for the improvement of GA, but the concrete effects of QTLs identified from Emi-no-kizuna have not yet been evaluated.

Relative to the popular cultivar Koshihikari, Emi-no-kizuna has a shorter culm length, lower spikelet number per panicle, lower 1000-grain weight (GW), and a higher panicle number (Sasahara et al., 2018). As GA is negatively correlated with the number of unhulled rice grains (Asano, Kanno & Endo, 2011) and GW (Takita, 1985), these traits should be investigated, because the superior GA of Emi-no-kizuna might be the result of the lower spikelet number or GW, i.e. yield.

On the other hand, the area of paddy fields under cultivation per farmer has been steadily increasing in Japan (Yagi, 2016). The resultant need to save costs has thus increased the demand for cultivars with not only superior GA but also lodging resistance. Hence, the *semi-dwarf 1* (*sd1*) locus (Sasaki et al., 2002), which is very effective for improving lodging resistance, should be increased in breeding. However, as introgressing the genomic region of the *sd1* locus might reduce GA (Terao & Hirose, 2015) the relationship between GA and *sd1* of Emi-no-kizuna should be investigated.

From this perspective, for effective breeding of rice cultivars with superior GA from Emi-no-kizuna without reduction of yield, we investigated the effects of QTLs for GA identified from Emi-no-kizuna and the relationships between GA and several agronomic traits via QTL analysis and evaluation of near-isogenic lines (NILs) by using hybrid and introgressed progeny of Emi-no-kizuna.

## Materials and methods

### Plant materials and examination of individual plants

We grew 180 recombinant inbred lines (RILs; F<sub>7</sub>) derived from Emi-no-kizuna and Tomohonami by the single-seed

descent method and the two parents in 2016 in a paddy field at the Central Region Agricultural Research Center (Joetsu, Niigata, Japan: 37°12'29"N, 138°21'50"E). Tomohonami is derived from upland rice. It has the rice blast resistance gene *pi21* and a genetic background similar to that of Koshihikari; it is prone to chalkiness and has poor resistance to heat stress (Saka et al., 2010). Seeds were sown on 26 April 2016, and 50 seedlings of each line were transplanted on 24 May in two ridges at intervals of 18 cm × 18 cm. The lines were spaced 36 cm apart. As a basal fertilizer, 5 g/m<sup>2</sup> each of N, P<sub>2</sub>O<sub>5</sub>-equivalent, and K<sub>2</sub>O-equivalent (Nyouso-kasei toku No. 45; JA Higashinohon Kumiai Shiryō Co., Gunma, Japan) was applied. No additional fertilizer was used.

To ensure uniform grain-filling conditions, from among the 180 lines we used only the 128 lines in which panicles emerged within 3 days of the parents' heading date. At 15 days after heading (DAH) we measured the culm length (CL), panicle length (PL), and panicle number (PN) in eight average plants per line or per parent as described by Sunohara and Horisue (1995). At 40 DAH we harvested the panicles of the same eight plants per line or per parent. We counted the spikelet number per panicle (SN) of the panicles used to measure the PL. All grains of each plant were hulled and examined. The heading date of each line was determined as the date when 50% of the panicles had headed. 'Days to heading' was determined as the number of days from the date of transplanting to the heading date. We defined PN × SN as the putative total spikelet number (PTSN) of a plant.

### Air temperature measurement

The air temperature at the level of the panicles, about 80 cm above the ground surface, was recorded every 10 min on a data logger (model RTR-52A; T&D, Nagano, Japan).

### Evaluation of GA and grain traits

We evaluated the GA and GW by using a grain quality inspector (model RGQI20A; Satake Co., Hiroshima, Japan). GW was based on 14.0% moisture content. All grains were examined and classified into perfect grains (PGs), milky white grains (MWs), basal white grains (BWGs), or white back grains (WBGs); the percentages of each were used as indexes of GA. Although the grain quality inspector cannot distinguish between white back and white belly, we regarded any blemish on a side face of a grain as white back, because we did not detect any white belly grains by

visual inspection in any of the lines or parents. We refer to MWs, BWs, and WBs collectively as chalkiness.

### QTL analysis

DNA was extracted from each  $F_6$  plant as described by Monna et al. (2002). We investigated the genotype by using 175 single nucleotide polymorphism (SNP) markers (Kim et al., 2016) spread throughout the genome. Linkage maps were prepared in MAPMAKER/EXP v. 3.0 software (Lander et al., 1987) using Kosambi's mapping function (Kosambi, 1944). QTL analysis was conducted by using composite interval mapping in QTL Cartographer v. 2.5 software (Wang, Basten & Zeng, 2006). We determined a threshold value corresponding to a significance level of 5% by using 1000 permutations. When the logarithm of odds (LOD) score exceeded the threshold value, we concluded that a QTL was detected. In the analysis, the percentages of grains were arcsine-transformed to standardize dispersion.

### Breeding and evaluation of NILs

Tomohonami and its progeny were backcrossed three times to Emi-no-kizuna. During backcrossing, we selected mother plants in which the genotypes of SNP markers at promising QTLs were heterozygous. Individuals were selected from the  $BC_3F_2$  generation in 2016, and the  $BC_3F_4$  lines were grown in 2018. We selected NIL\_1 and NIL\_2, in which Emi-no-kizuna alleles were introgressed only in the genomic regions of the *sd1* locus and QTLs associated with GA. We compared several agronomic traits of these NILs with those of Tomohonami and Emi-no-kizuna. Seeds were sown on 18 April and seedlings were transplanted on 16 May. Plants were grown and evaluated as for the RILs. Following a significant analysis of variance (ANOVA)

result at  $P < 0.05$ , the significance of differences was tested by Tukey's test.

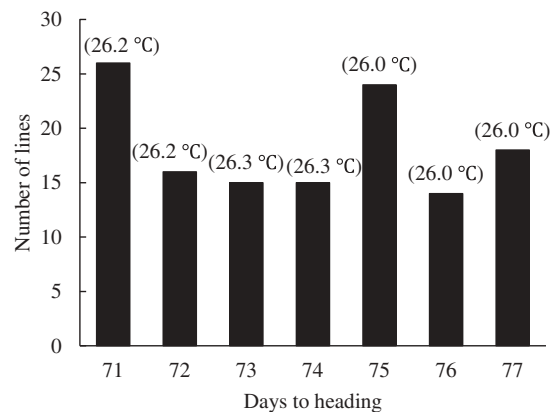
## Results

### Distribution of heading dates and air temperature

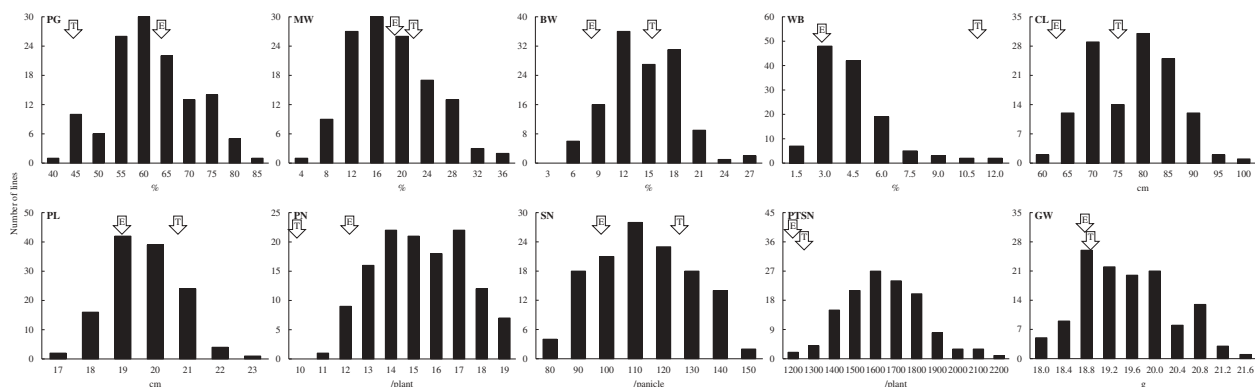
The number of days to heading of the 128 RILs ranged from 71 to 77, and that of both Emi-no-kizuna and Tomohonami was 74 days (Figure 1). The mean air temperature during the 20 DAH (AT20) ranged from 26.0 to 26.3°C, with no clear differences.

### Distributions of traits in the RILs, and correlations among traits

All traits showed continuous distributions (Figure 2). Emi-no-kizuna had a higher percentage of PGs, lower percentages of MWs, BWs, and WBs, shorter CLs and PLs, a lower SN, and a larger PN than Tomohonami (Figure 2). The



**Figure 1.** Distribution of days to heading in an  $F_7$  hybrid population between Emi-no-kizuna and Tomohonami. Values within parentheses denote the mean air temperature in the 20 days after heading. Days to heading of both parents were 74.



**Figure 2.** Frequency distributions of each trait in an  $F_7$  hybrid population between Emi-no-kizuna and Tomohonami.

Arrows, values of the parents: E, Emi-no-kizuna; T, Tomohonami;

PG, perfect grain; MW, milky white grain; BW, basal white grain; WB, white back grain; CL, culm length; PL, panicle length; PN, panicle number; SN, spikelet number per panicle; PTS, putative total spikelet number; GW, 1000-grain weight.

percentages of PGs, MWs, BWs, and WBs showed mostly significant correlations with PN, SN, and GW (Table 1).

### QTL analysis

In the analysis of traits related to GA, two QTLs associated with the percentage of PGs were detected, one on chromosome (Chr.) 3 and one on Chr. 4 (Figure 3, Table 2). Two QTLs associated with the percentage of MWs were detected, both on Chr. 3. Three QTLs associated with the percentage of BWs were detected, one on each of Chrs. 3, 6, and 10. Two QTLs associated with the percentage of WBs were detected, one on Chr. 7 and one on Chr. 8. The QTLs associated with the percentage of WBs on Chrs. 7 and 8 corresponded with *qGA7* and *qGA8*, respectively (Nagaoka et al., 2017).

Of the loci associated with GA, the QTL associated with the percentage of PGs on Chr. 4 explained 34.5% of phenotypic variation (percentage of phenotypic variation explained: PVE). At the end of the short arm of Chr. 3, we detected QTLs associated with the percentages of MWs and BWs at almost the same location. On the long arm of Chr. 3, QTLs associated with the percentages of PGs and MWs and PTSN were detected at almost at the same location. On the long arm of Chr. 4, QTLs associated with the percentage of PGs, PN, and SN were detected at almost the same location. On the short arm of Chr. 8, QTLs associated with the percentage of WBs, SN, and GW were detected at almost the same location. We designated these newly detected QTLs associated with GA as *qGA3*, *qGA4*, *qGA6*, and *qGA10*, respectively (Figure 3).

The QTLs associated with CL and PL were detected at almost the same location on Chr. 1 with high PVEs (56.4% and 25.4%, respectively, Table 2).

### Evaluation of NILs

In the NILs, Emi-no-kizuna alleles were introgressed in the genomic region of the *sd1* locus in NIL\_1 and of the *sd1*

locus and of *qGA8* in NIL\_2, and Tomohonami alleles were found in all other regions (Figure 4).

The percentage of PGs of NIL\_1 was significantly higher and those of MWs, BWs, and WBs were significantly lower than those of Tomohonami (Table 3). The percentages of MWs and WBs of NIL\_2 were significantly lower than those of NIL\_1. The GW of NIL\_2 was significantly lower than those of Tomohonami and NIL\_1. The CLs of the NILs were significantly shorter than that of Tomohonami.

### Discussion

We tested RILs derived from Emi-no-kizuna and Tomohonami. The parents headed on the same day, and all selected RILs headed within 3 days of that date. The AT20 was 26.0 to 26.3°C and was similar among heading dates (Figure 1). When AT20 exceeds around 26–27°C, the occurrence of chalkiness increases strongly (Terashima et al., 2001; Wakamatsu, Sasaki, Uezono & Tanaka, 2007). It is therefore likely that the grain filling conditions in this study were nearly uniform among the parents and RILs, and that the environmental conditions were conducive to a high incidence of chalkiness.

All traits investigated had continuous distributions (Figure 2), suggesting that several genes were associated with each trait. The percentages of PGs, MWs, BWs, and WBs showed mostly significant correlations with PN, SN, and GW (Table 1), suggesting that the traits were genetically correlated with the GA of Emi-no-kizuna.

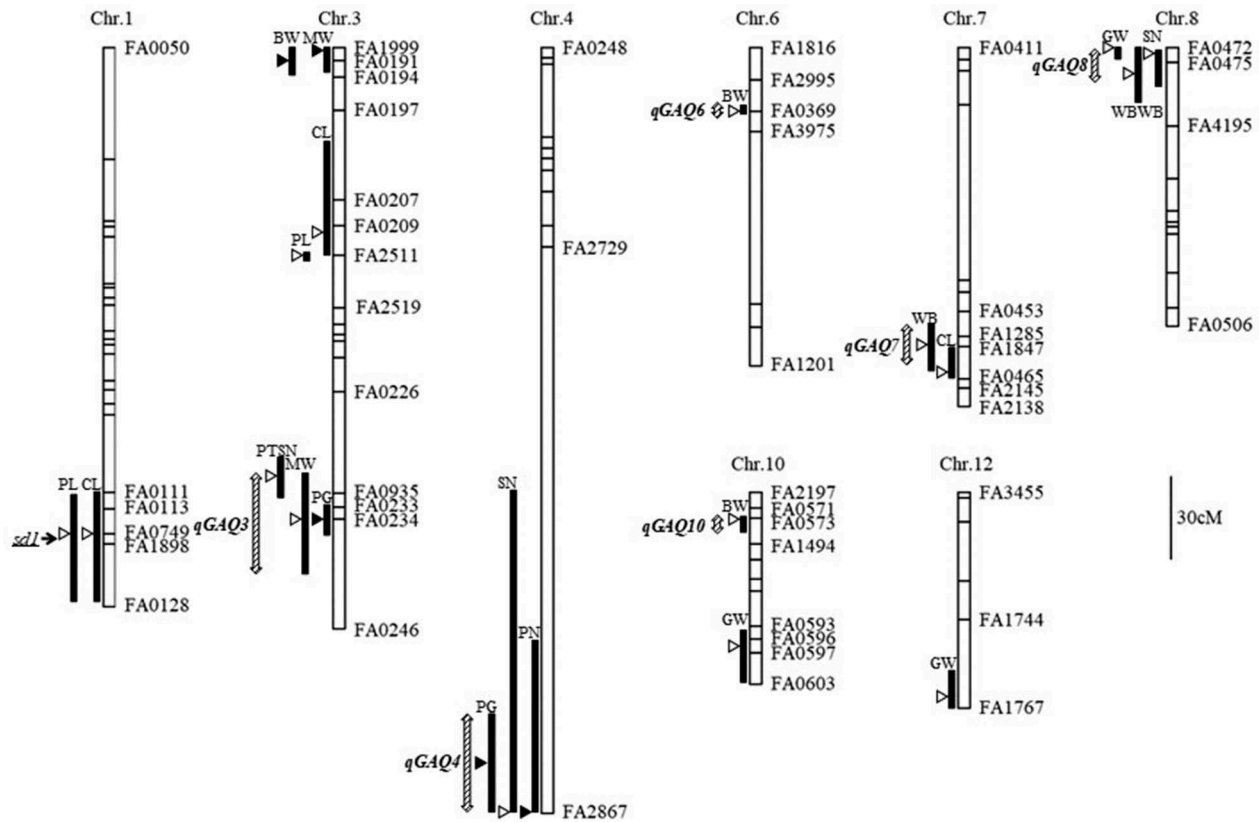
Of the detected QTLs associated with GA, the Emi-no-kizuna allele of *qGA4*, on the long arm of Chr. 4, increased the percentage of PGs, with a high PVE of 34.5%. In the same region, QTLs associated with PN and SN were detected (Figure 3, Table 2). It is thus possible that the effect of *qGA4* is due to the combination of PN and SN alleles. Although increases in PN and decreases in SN in the Emi-no-kizuna genotype could have been caused by a single factor because PN and SN are in a trade-off relationship (Kuroda, Abe, Ishibashi, Hirano & Murata, 1999), it is also possible that decreases in SN reduced the sink capacity per stem and resulted in

Table 1. Coefficients of correlation between traits.

	PG	MW	BW	WB	CL	PL	PN	SN	PTSN
MW	-0.87***								
BW	-0.62***	0.30***							
WB	-0.57***	0.47***	0.15ns						
CL	-0.12ns	0.06ns	0.19*	0.16ns					
PL	-0.25**	0.25**	0.13ns	0.22*	0.55***				
PN	0.26**	-0.14ns	-0.25**	-0.29**	-0.09ns	-0.23**			
SN	-0.35***	0.26**	0.24**	0.35***	0.24**	0.44***	-0.68***		
PTSN	-0.16ns	0.18*	0.03ns	0.12ns	0.21*	0.31***	0.23**	0.55***	
GW	-0.21*	0.26**	0.12ns	0.15ns	-0.19*	0.01ns	-0.07ns	-0.14ns	-0.28**

\*\*\*, \*\*, and \*: Significant at levels of 0.1%, 1%, and 5%, respectively. ns: Not significant at the 5% level according to Pearson product-moment correlation analysis.

PG, perfect grain; MW, milky white grain; BW, basal white grain; WB, white back grain; CL, culm length; PL, panicle length; PN, panicle number; SN, spikelet number per panicle; PTSN, putative total spikelet number; GW, 1000-grain weight.



**Figure 3.** Locations of quantitative trait loci (QTLs) associated with each trait.

Major markers are shown on the right of each chromosome. Bars on the left of each chromosome denote intervals equal to, or higher than, the logarithm of odds (LOD) value threshold. Triangles: location of LOD peak;  $\blacktriangleright$  Emi-no-kizuna increased;  $\square$  Tomohonami increased. Shaded arrows: Loci where QTLs associated with GA were estimated to exist. The positions of the *sd1* locus are shown on the left side. QTLs were not detected on chromosomes 2, 5, 9 and 11. Locations of *qGA7* and *qGA8* are from Nagaoka et al. (2017).

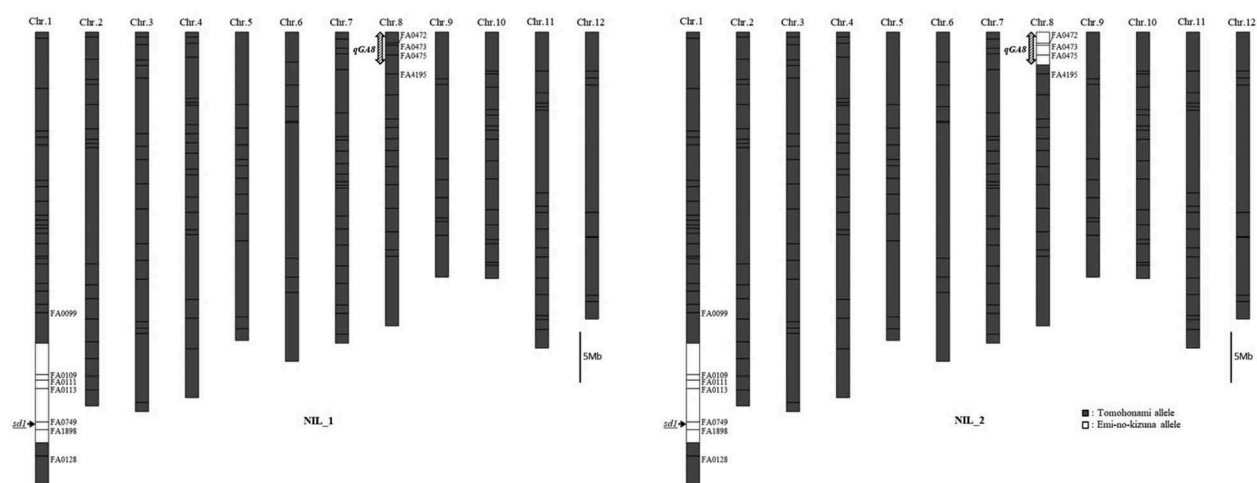
PG, perfect grain; MW, milky white grain; BW, basal white grain; WB, white back grain; CL, culm length; PL, panicle length; PN, panicle number; SN, spikelet number per panicle; PTSN, putative total spikelet number; GW, 1000-grain weight.

**Table 2.** Quantitative trait loci associated with traits detected by composite interval mapping analysis.

Trait	Chr.	Nearest marker	Peak position (cM)	LOD	Additive effect	PVE (%)	LOD threshold value
CL	1	FA0749	162.8	29.35	-6.31	56.4	3.35
PL	1	FA0749	163.1	10.17	-0.54	25.4	2.88
MW	3	FA1999	1.0	6.78	2.25	18.1	2.88
BW	3	FA0191	4.4	4.72	1.26	11.9	2.97
CL	3	FA0209	60.9	6.43	-2.41	8.3	3.35
PL	3	FA2511	69.7	2.97	-0.28	6.5	2.88
PTSN	3	FA0935	143.5	3.11	-71.54	14.1	2.90
PG	3	FA0234	158.1	2.96	1.59	8.2	2.77
MW	3	FA0234	158.1	4.27	-1.57	9.9	2.88
PG	4	FA2867	239.9	3.58	3.24	34.5	2.77
PN	4	FA2867	256.9	18.96	1.27	40.3	3.44
SN	4	FA2867	256.9	26.85	-12.37	52.3	3.57
BW	6	FA0369	21.3	3.08	-1.10	8.9	2.97
WB	7	FA1847	98.8	4.54	-0.91	10.8	2.87
CL	7	FA0465	109.3	5.30	-2.77	6.9	3.35
GW	8	FA0472	0.0	3.86	-0.25	10.2	3.08
SN	8	FA0472	2.9	4.08	-3.96	5.4	3.57
WB	8	FA0475	6.9	5.85	-1.07	16.0	2.87
BW	10	FA0573	8.7	3.19	-1.09	9.2	2.97
GW	10	FA0596	51.3	4.35	-0.27	11.2	3.08
GW	12	FA1767	69.8	3.59	-0.28	12.1	3.08

Additive effect refers to the Emi-no-kizuna allele.

PG, perfect grain; MW, milky white grain; BW, basal white grain; WB, white back grain; CL, culm length; LOD, the logarithm of odds; PL, panicle length; PN, panicle number; SN, spikelet number per panicle; PTSN, putative total spikelet number; PVE, percentage of phenotypic variation explained by each quantitative trait locus; GW, 1000-grain weight.



**Figure 4.** Graphical genotypes of near-isogenic lines NIL\_1 and NIL\_2.

Important markers are shown on the right side of each chromosome (from IRGSP v. 1.0). The positions of *sd1* and *qGA8* are shown on the left side. Closed and opened regions: Tomohonami and Emi-no-kizuna alleles, respectively. Shaded arrows: Region thought to contain the indicated QTL.

**Table 3.** Several agronomic traits of near-isogenic lines (NILs).

	Heading date (month/day)	AT20 (°C)	PG (%)	MW (%)	BW (%)	WB (%)	CL (cm)	PL (cm)	PN (/plant)	SN (/panicle)	PTSN (/plant)	GW (g)	Putative yield (g/plant)
NIL_1	8–3	26.2	74.9c	9.4b	8.3b	3.8b	72.8b	18.6a	16.2a	122.6a	1981a	19.6a	38.7a
NIL_2	8–3	26.2	80.7b	4.5c	8.0b	2.4c	72.8b	19.4a	17.0a	128.2a	2175a	18.6b	40.4a
Tomohonami	8–2	26.1	54.9d	13.1a	19.0a	8.7a	77.8a	19.4a	15.0a	114.4a	1728a	20.1a	34.9a
Emi-no-kizuna	8–3	26.2	93.8a	2.1d	1.2c	0.7d	71.4b	17.8a	17.2a	90.4b	1551a	20.1a	31.2a
ANOVA			***	***	***	***	**	ns	ns	***	ns	***	ns

\*\*\* and \*\*: Significant at the levels of 0.1 and 1%, respectively. ns: Not significant at the 5% level. Values followed by the same letters do not differ significantly at the 5% level according to the Tukey test.

Putative yield is defined as  $PTSN \times GW/1000$ .

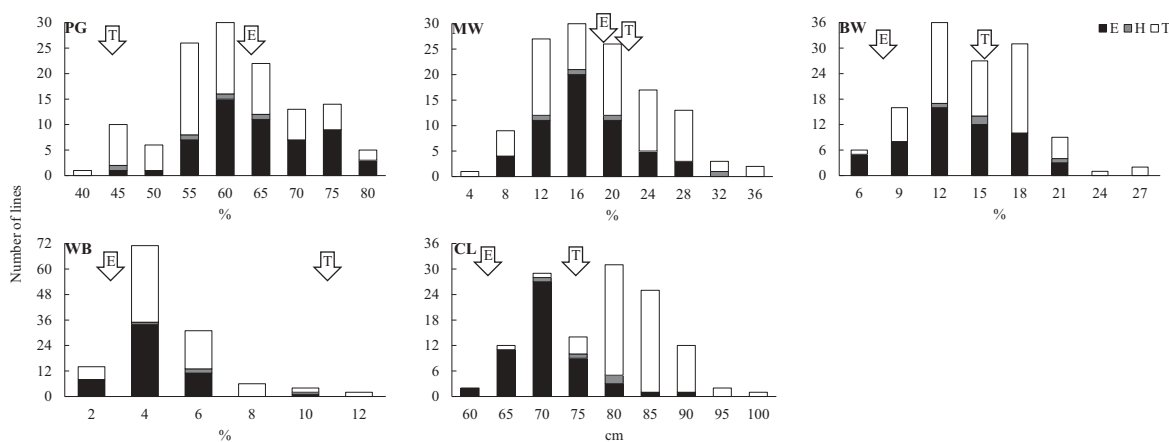
ANOVA, analysis of variance; AT20, the mean air temperature in the 20 days after heading; PG, perfect grain; MW, milky white grain; BW, basal white grain; WB, white back grain; CL, culm length; PL, panicle length; PN, panicle number; SN, spikelet number per panicle; PTSN, putative total spikelet number; GW, 1000-grain weight.

improved grain filling. This explanation agrees with the findings of Shiotsu, Liu, Bian, Toyota and Kusutani (2008), who noted that the grain filling performance was improved in plants with a large PN and a small SN. Of course, Emi-no-kizuna might have a gene associated with GA in the region that might be linked with genes associated with PN and SN, because a QTL associated with the percentage of WBs has been reported in this region (Kobayashi, Genliang, Shenghai & Tomita, 2007). The evaluation of these traits with NILs will offer an effective approach to reveal the details. In addition, several genes have been reported in this region, including *d17*, which controls tillering (Umehara et al., 2008), and *SPIKE* (Fujita et al., 2013), *GPS* (Takai et al., 2013), and *LSCHL4* (Zhang et al., 2014), which are involved in the number of spikelets, and should be taken into consideration in future research.

On the short arm of Chr. 3, we detected QTLs associated with increased percentages of MWs and BWs in the presence of the Emi-no-kizuna genotype (Figure 3, Table 2). Although we do not have enough information,

GA of Emi-no-kizuna might be improved by introgressing the Tomohonami allele in this region.

We expect that *qGA8*, detected in our previous study (Nagaoka et al., 2017), will be useful for the improvement of GA. On the other hand, we detected a QTL associated with CL on Chr. 1 with a high PVE (56.4%) (Figure 3, Table 2), which we suspect resulted from an effect of the *sd1* locus. The Emi-no-kizuna allele should be introgressed in this region, because the lodging resistance of Tomohonami is not strong (Saka et al., 2010). However, as it is unclear whether the *sd1* locus of Emi-no-kizuna affects GA or not we bred NILs from Tomohonami by introgressing Emi-no-kizuna alleles in the genomic regions of the *sd1* locus and *qGA8* (Figure 4). The percentage of PGs of NIL\_1 was significantly higher and the percentages of MWs, BWs, and WBs were significantly lower than those of Tomohonami (Table 3). Although no QTL associated with GA was detected near the *sd1* locus (Figure 3), the percentages of PGs and chalkiness showed associations with the genotype of the *sd1* locus in F<sub>7</sub> lines (Figure 5). These results suggest that there is some genetic factor which improves GA



**Figure 5.** Relationships between the genotype of the *sd1* locus in  $F_7$  lines and grain appearance and culm length. The genotype of marker FA0749 was used as the index of the genotype of *sd1* locus. In bars: E, Emi-no-kizuna genotype; H, heterogeneous; T, Tomohonami genotype. In arrows: E, Emi-no-kizuna parent values; T, Tomohonami parent values; PG, perfect grain; MW, milky white grain; BW, basal white grain; WB, white back grain; CL, culm length.

around the *sd1* locus of Emi-no-kizuna. We suggest two possibilities. One is genetic linkage between *sd1* locus and genes associated with GA, because QTLs associated with GA have been reported near *sd1* locus (Dong et al., 2018; Zhu et al., 2018). The other is a pleiotropic effect of the *sd1* gene. Moreover, the percentages of MWs and WBs of NIL\_2 were significantly lower than those of NIL\_1 (Table 3). These results suggest that GA could be further improved by the introgression of Emi-no-kizuna alleles in the *qGA8* region, and that *qGA8* reduces the incidence of not only WB but also MW. In addition, the CLs of the NILs were significantly shorter than that of Tomohonami (Table 3), indicating an improvement of lodging resistance. It would thus be possible to improve both GA and lodging resistance, especially by introgressing Emi-no-kizuna alleles in the region of the *sd1* locus.

On the other hand, the GW of NIL\_2 was significantly lower than that of NIL\_1 and Tomohonami (Table 3). As an explanation for the detection of QTLs associated with GW in the same region as *qGA8* (Figure 3, Table 2), the effect of *qGA8* may be pleiotropic. Although the reduction of GW might be slight, because there was no significant difference in PTSN (and therefore in putative yield) between NIL\_1 and NIL\_2 (Table 3), further analysis of the relationship between *qGA8* and yield performance is needed. As we cannot rule out a genetic linkage between genes associated with GA and GW, fine mapping in this region is desirable. Moreover, the concrete and additive effects of QTLs associated with GA, including heat tolerance, should be multilaterally investigated via the evaluation of NILs.

## Conclusion

Introgression of Emi-no-kizuna alleles in the region of the *sd1* locus could improve GA. In addition, introgression of

Emi-no-kizuna alleles in the region of *qGA8* could further improve GA while reducing GW.

## Disclosure statement

No potential conflict of interest was reported by the authors.

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