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Effects of the number of pollen grains on cold tolerance at the booting stage in rice lines with QTLs for cold tolerance

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ABSTRACT

This study was conducted to clarify the relationship between the number of pollen grains per anther and spikelet fertility under low temperature conditions in the rice cultivars and lines including lines with quantitative trait loci (QTL) for cold tolerance, adapted to the Tohoku region of northern Japan. Cold-water treatment decreased anther length, the number of pollen grains per anther, and spikelet fertility in all cultivars and lines. The number of pollen grains was proportional to anther length in all cultivars and lines and under all temperatures. Spikelet fertility decreased with decreasing the number of pollen grains in cold-water treatments at 18.5 and 19.3 °C. 'Ouu 415', with the *qLTB3* QTL for cold tolerance, had 28% more pollen grains and 9% higher spikelet fertility than the recurrent parent, 'Hitomebore', in the 18.5 °C cold-water treatment, suggesting that *qLTB3* increased both parameters. Lines with the *qCTB8* QTL for cold tolerance had significantly more pollen grains in two of the three years and significantly higher spikelet fertility in all three years in the 19.3 °C cold-water treatment, suggesting that *qCTB8* reinforced cold tolerance. *Ctb1*, a proposed cold-tolerance QTL, had no effect on the number of pollen grains or spikelet fertility. In conclusion, the cultivars and lines with more pollen grains had a higher cold tolerance. Some of QTLs were inferred to increase the number of pollen grains and reinforce cold tolerance.

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Anther; cold tolerance; spikelet fertility; pollen grain; QTL; rice

CLASSIFICATION

Genetic Resources Evaluation

Rice yield is often decreased by spikelet sterility caused by low temperatures at the booting stage in northern Japan's Tohoku region. Breeding for cold tolerance using a deep-water irrigation system produced the cold-tolerant cultivar 'Hitomebore' in 1991 (Matsunaga, 2005; Sasaki, 2005). Replacement of the cold-sensitive cultivar 'Sasanishiki' by 'Hitomebore' reduced damage caused by cool summers in Miyagi Prefecture, in the Tohoku region. Nevertheless, an unusual cool summer in 2003 reduced the yield of 'Hitomebore', indicating the need for cultivars with better cold tolerance that are adapted to the Tohoku region.

One promising method of breeding cold-tolerant cultivars is the introduction of quantitative trait loci (QTLs) for cold tolerance into elite cultivars by taking advantage of DNA marker-assisted selection. Recently, many QTLs for cold tolerance have been detected, including *qCT-7* (Takeuchi et al., 2001), *Ctb1* and *Ctb2* (Saito et al., 2004), *qCTB8* (Kuroki et al., 2007), and *qLTB3* (Shirasawa et al., 2012). However, their role in cold tolerance is not fully understood, and their introduction into elite cultivars in the Tohoku region has not always reinforced cold tolerance. To develop new rice cultivars with high cold tolerance, it is necessary to elucidate the mechanism or process responsible for sterility under low temperatures.

The sterility appears to result mainly from damage to the microspores at low temperatures during the booting stage, preventing them from developing into mature pollen grains. Satake and Shibata (1992) divided the pollen developmental process and subsequent fertilization into four components and found that the first three components (i.e. the number of differentiated microspores, the percentage of developed pollen, and the percentage of shed pollen grains) could explain 82% of varietal differences in cold tolerance. Among the many processes and factors related to spikelet sterility under low temperatures, the number of pollen grains has been studied most because it is easy to evaluate and effective. Nishiyama (1982, 1983) discovered that lower spikelet fertility in the upper part of the panicle was caused by fewer pollen grains than in spikelets in the lower part of the panicle. Hayashi et al. (1997) revealed that high nitrogen application decreased the number of microspores and pollen grains, resulting in lower spikelet fertility under low temperatures. Nakamura et al. (2000) pointed out that cultivars with more pollen grains had higher fertility under low temperatures. All of these studies suggest that the number of pollen grains is an important indicator of cold tolerance. However, genetic diversity in the number of pollen grains produced under

low temperatures is not fully understood. Here, our goal was to clarify the relationship between the number of pollen grains and spikelet fertility under low temperatures in rice cultivars and lines including lines with QTLs for cold tolerance.

Materials and methods

Rice cultivars and lines adapted to Japan's Tohoku region

We used four elite cultivars from the Tohoku region as the control cultivars: 'Masshigura', 'Akitakomachi', 'Hitomebore', and 'Fukuhibiki'. We compared their performance with the following cold-tolerant cultivars and lines: 'Ukei 898' and 'Ukei 965', *indica* lines derived from crosses among cold-tolerant *indica* cultivars (Figure 1, Yamaguchi & Yokogami, 2003); 'Lijiangxintuanheigu' ('LTH'), a local cultivar from China's Yunnan Province; 'Ouu 415' ('LTH'/'Hitomebore' × 4), with QTL *qLTB3* for cold tolerance, derived from 'LTH'; and 'Ukei 855' ('Kunmingxiaobaigu' [a cold-tolerant local cultivar from Yunnan Province]/'Hitomebore' × 2).

Rice lines with QTLs *qCTB8* and *Ctb1* for cold tolerance

'Hokkai IL3', a donor parent that contains the *qCTB8* and *Ctb1* cold-tolerance QTLs, was backcrossed with 'Fukuhibiki' (Figure 2). Four types of lines (FK lines) were developed that either had or lacked the cold-tolerance QTLs: FK81 (*qCTB8* +, *Ctb1* +), FK80 (*qCTB8* +, *Ctb1* -), FK01 (*qCTB8* -, *Ctb1* +), and FK00 (*qCTB8* -, *Ctb1* -). Plants were selected by DNA marker-assisted selection, using SSR markers, RM6670 for *qCTB8* (Kuroki et al., 2007) and PNK7 for *Ctb1* (Saito et al., 2010). A total of 16 lines (4 types × 4 lines) was developed and used.

Cultivation methods

Plants were grown in a paddy field or a deep-water irrigation system (Matsunaga, 2005) at the NARO Tohoku

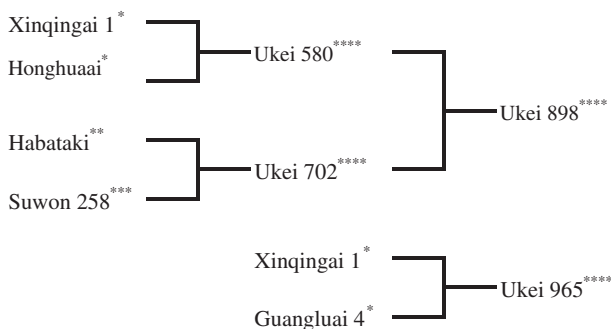


Figure 1. Lineage of *indica* lines with cold tolerance. **Indica* variety originated from China, ***Indica* variety developed in Japan, ****Indica* variety originated from Korea, *****Indica* line developed in Japan's NARO Tohoku agricultural research center.

Agricultural Research Center (Daisen city, Japan; 39°29'N, 140°29'E) in 2013 to 2015. The dates of seeding and transplanting were 18 April and 24 May in 2013, 16 April and 24 May in 2014, and 15 April and 28 May in 2015. The seedlings at a leaf age of 4.0–5.0 were transplanted by hand, one seedlings per hill, at a density of 30 hills m⁻² in the paddy field and 46 hills m⁻² in the deep-water irrigation system. Replication was one in the paddy field and two in the deep-water irrigation system. A compound fertilizer containing 16% N, 16% P₂O₅, and 16% K₂O was applied as basal dressing at a rate of 8 g m⁻² of N in the paddy field and a rate of 7 g m⁻² of N in the deep-water irrigation system. The water temperature in the deep-water irrigation system was set to 18.5 or 19.3 °C from 1 July to 31 August.

Evaluation of the number of pollen grains and spikelet fertility

Three or four panicles were harvested randomly just before flowering from each replication. Among them, more than 15 spikelets at the position third to fifth spikelets on the second and third primary rachis branches (Figure 3) were sampled and fixed in 50% ethanol, following the method of Satake (1989). After staining the spikelets with iodine–potassium iodide solution, we extracted a total of 12 anthers per treatment (3 anthers × 4 spikelets × 1 replication in the paddy field and 3 anthers × 2 spikelets × 2 replications in the deep-water irrigation system) and measured their length and the number of fully mature pollen grains under a microscope (Figure 4). After ripening, we sampled a total of 30 panicles per treatment (3 panicles × 5 hills × 2 replications) in the deep-water irrigation system and measured the fertility of spikelets at the positions shown in Figure 3.

Results

The number of pollen grains and spikelet fertility of the rice cultivars and lines adapted to Japan's Tohoku region

We observed clear varietal differences in anther length, the number of pollen grains, and spikelet fertility within the paddy field and cold-water treatments (Table 1). Spikelet fertility was observed visually to be >90% in all cultivars and lines in the paddy field, where the average temperature in the booting stage was more than 23.0 °C in every three years, which was clearly higher than 20.0 °C, the sensitive temperature for cold damage. Cold-water treatment delayed the heading date and decreased the culm length, anther length, number of pollen grains, and spikelet fertility in most cultivars and lines, especially at 18.5 °C. As the anther length increased, the number of pollen grains also increased in all cultivars and cultivation temperatures (Figure 5). As the number of pollen grains decreased, the

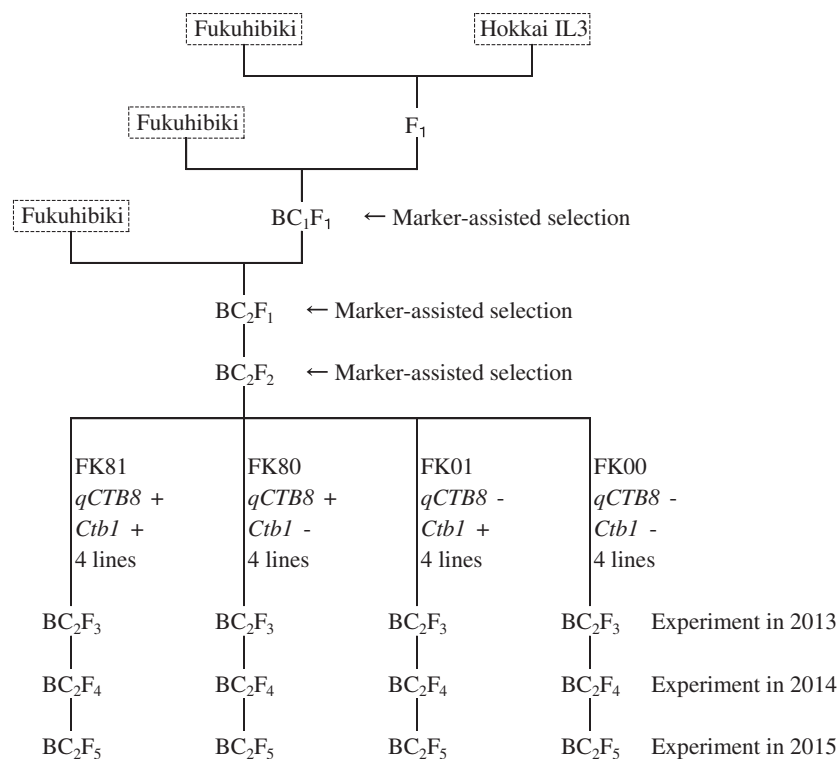


Figure 2. Lineage of the FK lines with the *qCTB8* and *Ctb1* cold-tolerance QTLs.

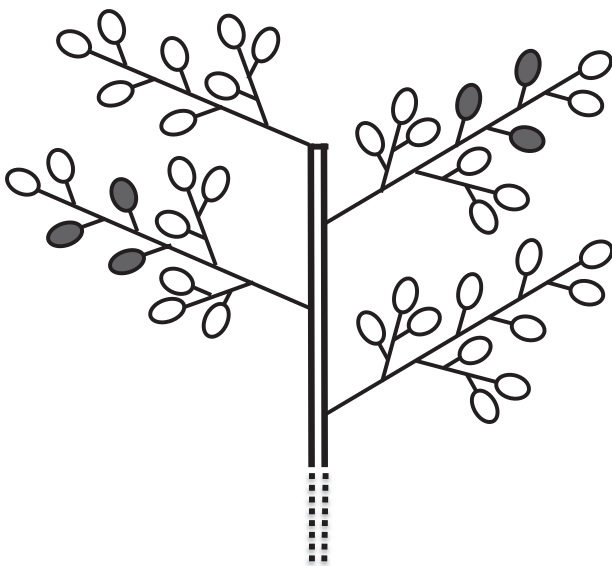


Figure 3. Positions of sampled spikelets within the panicle. The shaded spikelets were sampled just before flowering and their fertility was measured after ripening.

spikelet fertility also decreased in both cold-water treatments (Figure 6). The largest number of pollen grains was attained by 'LTH' under all temperature conditions (Table 1). However, the highest spikelet fertility was attained by 'Ukei 855' in both cold-water treatments. The number of pollen grains and spikelet fertility of 'Ouu 415' were not different from those of the recurrent parent, 'Hitomebore', in the 19.3 °C treatment and paddy field. However, in the 18.5 °C

treatment, 'Ouu 415' had 28% more pollen grains and 9% higher spikelet fertility than 'Hitomebore', although the differences were not significant. In addition, the culm length of 'Ouu 415' was slightly longer than that of 'Hitomebore' at all three temperatures although the differences were not significant.

Effects of the *qCTB8* and *Ctb1* cold tolerance QTLs on the number of pollen grains and spikelet fertility

In the paddy field, the FK lines with *qCTB8* had significantly longer anthers and significantly more pollen grains per anther than those without *qCTB8* (Table 2). In the 19.3 °C treatment, they also had significantly more pollen grains in two of the three years and significantly higher spikelet fertility in all three years. These lines also had a significantly longer culm under all temperature conditions, but had significantly delayed heading in the cold-water treatment. On the other hand, the FK lines with *Ctb1* did not have any significant differences in anther length, number of pollen grains, spikelet fertility, and heading date from those without *Ctb1* under the 19.3 °C treatment and the paddy field.

Relationships between the numbers of pollen grains in the paddy field and cold-water treatments

The number of pollen grains in the cold-water treatments was proportional to the number in the paddy field in all

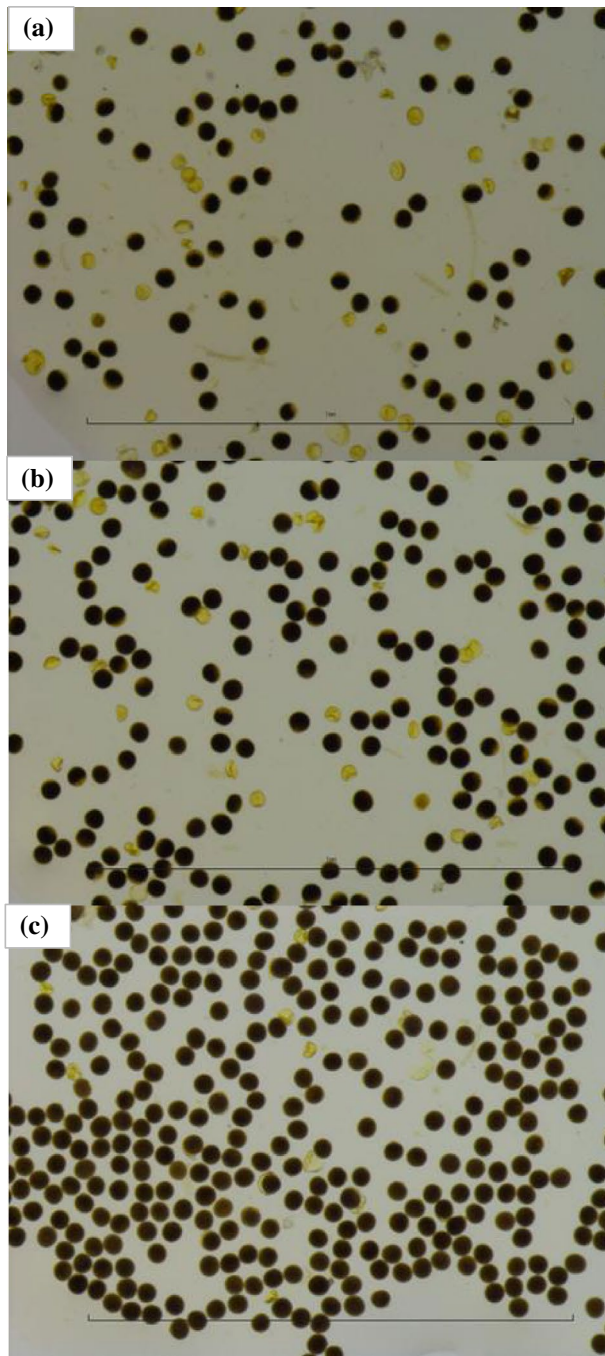


Figure 4. Pollen grains from 'Hitomebore' in the cold-water treatments and the paddy field. (a) 18.5°C, (b) 19.3°C, (c) normal temperature in paddy field. Bar = 1 mm.

treatments ($r > 0.9$, $p < 0.01$; Figure 7). The relationships in the FK lines were not clear owing to the small variation in the number of pollen grains.

Discussion

The number of pollen grains was related to spikelet fertility under low temperatures in the rice cultivars and lines we tested, including lines with QTLs for cold tolerance, in all

Table 1. Effects of the cold-water treatments on the number of pollen grains and spikelet fertility in rice cultivars and lines adapted to Japan's Tohoku region.

| Varieties and lines | Cold-water treatment at 18.5°C | | | | | Cold-water treatment at 19.3°C | | | | | Control in the paddy field | | | | |
|---------------------|--------------------------------|------------------|--------------------|--|--------------------------------------|--------------------------------|------------------|--------------------|--|---------------------------------------|----------------------------|-------------------|--------------------|--|--|
| | Heading date (month.day) | Culm length (cm) | Anther length (mm) | The number of pollen grains per anther | Percentage of spikelet fertility (%) | Heading date (month.day) | Culm length (cm) | Anther length (mm) | The number of pollen grains per anther | Percent-age of spikelet fertility (%) | Heading date (month.day) | Culm* length (cm) | Anther length (mm) | The number of pollen grains per anther | |
| Masshigura | 8.09 a | 64 a | 1.43 bc | 204 ab | 9 ab | 8.06 a | 68 ab | 1.58 abc | 318 ab | 47 b | 7.30 a | 75 | 2.06 ab | 1307 ab | |
| Akitakomachi | 8.13 ab | 69 ab | 1.14 a | 116 a | 7 ab | 8.09 ab | 74 abc | 1.31 a | 231 a | 40 ab | 8.02 a | 80 | 2.02 a | 1341 ab | |
| Fukuhibiki | 8.15 bc | 61 a | 1.23 ab | 100 a | 2 a | 8.11 abc | 66 ab | 1.38 ab | 250 ab | 17 a | 8.04 a | 74 | 1.98 a | 1157 a | |
| Hitomebore | 8.20 cd | 74 bc | 1.49 c | 398 abc | 65 c | 8.16 bc | 77 bcd | 1.64 bc | 612 abc | 89 d | 8.04 a | 84 | 2.16 ab | 1470 bc | |
| Ouu 415 | 8.22 d | 76 bc | 1.52 c | 508 bc | 71 cd | 8.16 bc | 82 cd | 1.63 bc | 651 abc | 89 d | 8.07 a | 87 | 2.24 b | 1461 bc | |
| LTH | 8.16 bc | 123 d | 1.78 d | 1071 d | 74 cd | 8.14 abc | 129 e | 1.95 d | 1201 d | 81 cd | 8.06 a | 131 | 2.51 c | 2007 d | |
| Ukei 855 | 8.23 d | 82 c | 1.60 cd | 650 c | 89 d | 8.17 c | 87 d | 1.74 cd | 870 cd | 94 d | 8.07 a | 88 | 2.29 bc | 1558 bc | |
| Ukei 898 | 8.19 cd | 60 a | 1.60 cd | 498 bc | 27 b | 8.16 abc | 66 ab | 1.66 bc | 685 bc | 55 bc | 8.07 a | 79 | 2.30 bc | 1674 c | |
| Ukei 965 | 8.19 cd | 58 a | 1.67 cd | 489 bc | 54 c | 8.16 bc | 63 a | 1.75 cd | 791 cd | 81 cd | 8.06 a | 78 | 2.48 c | 1655 c | |

Note: Average of three years (*average of 2014 and 2015). Values in a column labeled with different letters differ significantly ($p < 0.05$, the Bonferroni's correction).

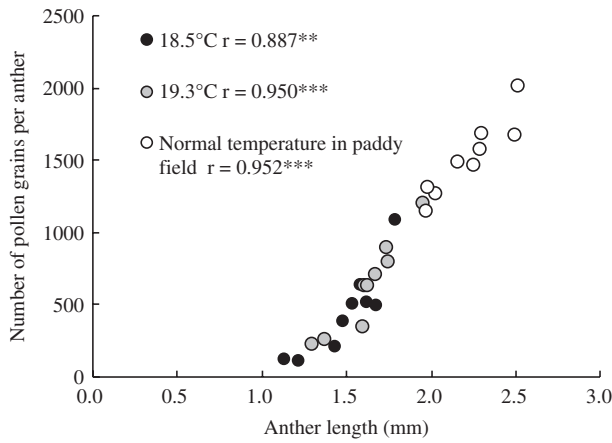


Figure 5. Relationships between anther length and number of pollen grains of rice cultivars and lines adapted to Japan's Tohoku region. Average of three years was indicated. Significance: ** $p < 0.01$; *** $p < 0.001$.

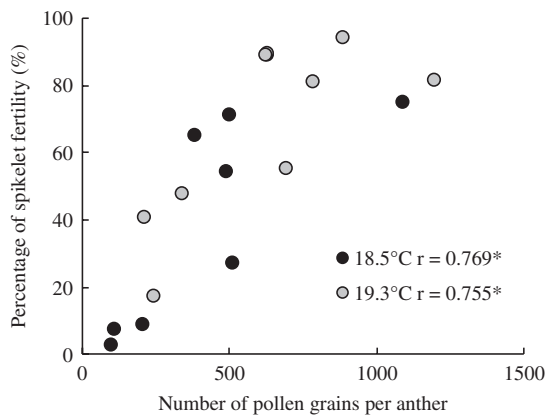


Figure 6. Relationships between number of pollen grains and spikelet fertility under cold-water treatments of rice cultivars and lines adapted to Japan's Tohoku region. Average of three years was indicated. Significance: * $p < 0.05$.

3 years. Nakamura et al. (2000) also suggested that the number of pollen grains was a primary, genetically determined factor, although they believed that other genetic factors were also involved.

'Ouu 415', with the cold-tolerance QTL *qLTB3*, had 28% more pollen grains and 9% higher spikelet fertility than the recurrent parent ('Hitomebore') in the cold-water treatment at 18.5°C (Table 1), suggesting that *qLTB3* increased both parameters. In addition, the FK lines with *qCTB8* produced significantly more pollen grains than those without it in two of the three years, and had higher spikelet fertility in all three years, suggesting that *qCTB8* reinforced cold tolerance by increasing the number of pollen grains (Table 2). In the experiment of 2015, however, *qCTB8* did not increase the number of pollen grains significantly, although increased the spikelet fertility significantly (Table 2). The spikelet fertility in 2015 was

Table 2. Effects of cold-water treatment on heading date, culm and anther lengths, number of pollen grains, and spikelet fertility in rice lines with or without the *qCTB8* and *Ctb1* QTLs for cold tolerance.

| Varieties and lines | QTLs for cold tolerance | | | | | | | | | | Control in the paddy field | | | | | | | | | |
|---------------------|--------------------------------|--------------|--------------------|------------------|--------------------|--|--------------------------------------|------|------|------|--------------------------------|------|------|--------------------|------------------|----------------------------|--|--|--|--|
| | Cold water treatment at 19.3°C | | | | | Cold water treatment at 18.5°C | | | | | Cold water treatment at 19.3°C | | | | | Control in the paddy field | | | | |
| | <i>qCTB8</i> | <i>Ctb1</i> | Heading date (m.d) | Culm length (cm) | Anther length (mm) | The number of pollen grains per anther | Percentage of spikelet fertility (%) | 2013 | 2014 | 2015 | 2013 | 2014 | 2015 | Heading date (m.d) | Culm length (cm) | Anther length (mm) | The number of pollen grains per anther | | | |
| FK81 | + | + | 8.15 | 70 | 1.50 | 297 | 34.5 | 19.3 | 62.3 | 8.05 | 78 | 2.16 | 1351 | | | | | | | |
| FK80 | + | - | 8.16 | 70 | 1.50 | 269 | 28.5 | 25.6 | 65.3 | 8.06 | 77 | 2.13 | 1354 | | | | | | | |
| FK01 | - | + | 8.11 | 68 | 1.34 | 180 | 2.3 | 4.3 | 32.6 | 8.05 | 75 | 2.04 | 1228 | | | | | | | |
| FK00 | - | - | 8.13 | 65 | 1.38 | 194 | 4.8 | 4.2 | 30.7 | 8.04 | 74 | 2.09 | 1272 | | | | | | | |
| ANOVA | | | ** | ** | ** | * | ** | ** | ** | NS | * | * | * | | | | | | | |
| | | <i>qCTB8</i> | NS | * | NS | NS | NS | NS | NS | NS | NS | NS | NS | | | | | | | |
| | | <i>Ctb1</i> | NS | * | NS | NS | NS | NS | NS | NS | NS | NS | NS | | | | | | | |
| | | Interaction | NS | * | NS | NS | NS | NS | NS | NS | NS | NS | NS | | | | | | | |

Note: Each value represents the average of 3 years. NS, not significant (ANOVA). * $p < 0.05$; ** $p < 0.01$.

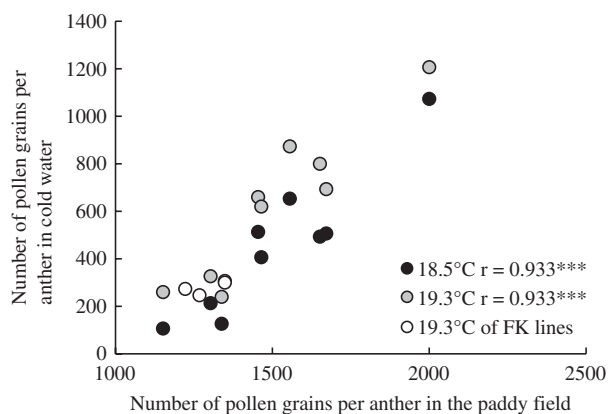


Figure 7. Relationships between number of pollen grains in the paddy field and numbers in cold-water treatments of rice cultivars and lines adapted to Japan's Tohoku region. Average of three years was indicated. Significance: *** $p < 0.001$.

higher than that in 2013 and 2014 probably due a little high water temperature. In the condition of high percentage of spikelets fertility, another factors differed from the number of pollen grains may affect spikelet fertility. On the other hand, FK lines with *Ctb1* did not show significant improvements in cold tolerance, even though Saito et al. (2004) reported that lines with *Ctb1* and *Ctb2* had longer anthers and higher spikelet fertility under low temperatures in Japan's Hokkaido region. These inconsistent results for *Ctb1* might be attributable to differences in climate between Tohoku and Hokkaido and to differences in the genetic background, including whether or not the lines also include *Ctb2*.

'Ouu 415', with *qLTB3*, and the FK lines with *qCTB8* had higher spikelet fertility and slightly longer culms than their recurrent parents (Tables 1 and 2), suggesting pleiotropic effects of the QTLs for cold tolerance. Sakata et al. (2014) reported that low temperature reduced the endogenous levels of bioactive gibberellins in developing anthers, resulting in fewer pollen grains and lower spikelet fertility. In contrast, Zhang et al. (2001) found that the number of pollen grains was decreased by the application of GA_3 and increased by the application of an inhibitor of gibberellin biosynthesis, although they did not measure the level of gibberellins in the developing anthers. Based on level of gibberellins in the developing anther and stem, some QTLs for cold tolerance might affect both spikelet fertility and culm length. FK lines with *qCTB8* revealed later heading date (Table 2), suggesting that *qCTB8* had a risk to bring delayed growth-type damage due to cool summer. Kuroki et al. (2011) reported that no QTLs for heading date were detected in the same chromosomal region of *qCTB8* in Japan's Hokkaido region. Therefore, interactions of *qCTB8* with climate condition of Tohoku region and/or genetic background were inferred to delay heading date.

Cultivars and lines with more pollen grains in the paddy field also had more pollen grains and higher spikelet fertility in the cold-water treatments (Figure 7), suggesting that varietal differences in cold tolerance can be evaluated from the anther length or the number of pollen grains under normal paddy field temperatures.

In conclusion, we found that cultivars and lines that produced many pollen grains had improved cold tolerance. Although two QTLs (*qLTB3* and *qCTB8*) appear to reinforce the cold tolerance, the effects of the cold tolerance QTLs were not large. This might make it difficult to use them to breed cold-tolerant cultivars. The next step in our research will be to detect new candidate QTLs for cold tolerance with larger effects and to elucidate how these QTLs or their genes act in the developing anther.

Disclosure statement

No potential conflict of interest is reported by the authors.

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