QTL pyramiding for improving of cold tolerance at fertilization stage in rice

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Vigorous cold tolerance at the fertilization stage (CTF) is a very important characteristic for stable rice production in cold temperature conditions. Because CTF is a quantitatively inherited trait, pyramiding quantitative trait loci (QTLs) using marker-assisted selection (MAS) is effective for improving CTF levels in rice breeding programs. We previously identified three QTLs controlling CTF, qCTF7, qCTF8 and qCTF12, using backcrossed inbred lines derived from a cross between rice cultivar Eikei88223 (vigorous CTF) and Suisei (very weak CTF). However, pyramiding of these QTLs for the application of MAS in practical rice breeding programs have not yet been elucidated. In this study, we examined the effect of pyramiding QTLs for improvement of CTF level using eight possible genotype classes from the 152 F3 population derived from a cross between Eikei88223 and Suisei. Increasing of CTF levels in combinations between qCTF7 and qCTF12 and between qCTF8 and qCTF12 were detected. Furthermore, we compared the haplotype pattern around the QTLs for CTF among the rice cultivars from Hokkaido. These results are useful for improvement of new cultivars with high CTF levels using MAS and identification of genetic resources with the novel QTL(s) for CTF.

Key Words: Oryza sativa L., cold tolerance, fertilization stage, rice breeding programs, QTL pyramiding.

Introduction

Many important agronomic traits including yield, disease resistance and stress tolerance are typically governed by a number of genes known as quantitative trait loci (QTLs). To date, most QTL mapping studies have focused on QTL discovery and estimations of the contribution to phenotypic variation by each QTL within a population. Pyramiding QTLs using marker-assisted selection (MAS) is effective for achieving the desirable phenotypic level of a quantitative trait in plant breeding programs (Ashikari and Matsuoka 2006, Nagai et al. 2012). However, the outcomes of pyramiding QTLs are not always as expected, for example because of epistatic interactions between QTLs (Bovill et al. 2010, Miedaner et al. 2006). Furthermore, QTL expression often varies according to the genetic background (Bovill et al. 2010, Iwata and Fujino 2010). Therefore, the arrangement of many QTLs and an evaluation of the interactions between QTLs are needed to improve complex traits using MAS in plant breeding programs.

Cold temperature is one of the most common environmental stresses that affects plant growth and development and places a major limit on plant productivity. Tolerance to cold temperature is an important objective in plant breeding programs (Kelly et al. 2010). A number of QTL analyses showed that tolerance to cold temperature is a quantitative trait controlled by many genes and affected by natural environmental conditions (Dai et al. 2004, Jompuk et al. 2005, Knoll et al. 2008, Shinada et al. 2013, Suh et al. 2010). Pyramiding QTLs to improve cold tolerance has been regarded as the most valuable use of molecular markers linked to QTLs. However, few studies have examined the outcomes of pyramiding QTLs for cold tolerance.

Rice is one of the most important staple foods in the world, eaten by 50% of the world’s population and originates in tropical regions. Cold temperature is one of the major constraints in rice production in high latitude or high altitude areas. Cold temperature causes retardation during rice growth, from seed germination to the grain filling stage. Rice plants are sensitive to cold temperature, especially in the reproductive phase, which can lead to a decrease in rice yields (Dai et al. 2004, Kuroki et al. 2007, Mori et al. 2011, Saito et al. 1995, Shinada et al. 2013).

The fertilization stage, which ranges from pollen maturation to complete fertilization, was shown to be one of the most sensitive to cold temperature in rice (Satake and Koike 1983, Shinada et al. 2013). Vigorous cold tolerance at the...
fertilization stage (CTF) is a very important characteristic for stable rice production in cold temperature conditions in higher latitude or higher altitude areas. Variations in CTF levels have been reported among rice cultivars from Hokkaido (Shinada et al. 2013, Tanno et al. 2000). Pollen maturation, pollen germination and pollen tube elongation were shown to be involved in the control of CTF levels (Shinada et al. 2013). We previously identified three QTLs controlling CTF, \( q_{\text{CTF7}} \), \( q_{\text{CTF8}} \) and \( q_{\text{CTF12}} \), using backcrossed inbred lines derived from a cross between rice cultivar Eikei88223 (vigorous CTF) and Suisei (very weak CTF) (Shinada et al. 2013). However, pyramiding effects of these QTLs for the application of MAS in practical rice breeding programs have not yet been elucidated.

The specific objective of this study was to evaluate the effect of pyramiding of QTLs controlling CTF levels using an F\(_3\) population derived from a cross between Eikei88223 and Suisei. Furthermore, we analyzed haplotype patterns around the QTLs for CTF among the rice cultivars from Hokkaido. We discussed which QTL combinations contribute effective improvement of CTF in rice breeding programs.

Materials and Methods

**Plant materials**

The three QTLs controlling CTF were identified previously using backcrossed inbred lines derived from a cross between Eikei88223 and Suisei (Shinada et al. 2013). To elucidate genetic interactions between these QTLs, the F\(_2\) population composed of 1754 plants derived from this cross was developed. This F\(_2\) population was classified into eight genotype classes of QTLs, \( q_{\text{CTF7}} \), \( q_{\text{CTF8}} \) and \( q_{\text{CTF12}} \) using simple sequence repeat (SSR) markers. For the evaluation of CTF, plants with poor growth and different heading day in each panicle were omitted from the analysis. A total 152 plants were finally selected and selfed to produce F\(_3\) generations used for evaluation of CTF. Equalizing the genetic background is needed to evaluate the effect of pyramiding of QTLs derived from an exotic germplasm. However, much time will be dedicated to the development and combination of near isogenic lines for the evaluation of each QTL and their combination. Both parental cultivars, Eikei88223 and Suisei, are the rice cultivars from Hokkaido, which is one of the northern limits of rice cultivation in the world. These two cultivars were in a genetically close relationship. Previously, a total of 949 SSR markers distributed throughout the genome were surveyed to detect polymorphisms between these cultivars for the QTL mapping. Only 87 markers (9.2\%) exhibited polymorphisms (Shinada et al. 2013). Because of similarities in genetic background, in this study, we developed experimental materials selected directly from the F\(_2\) population.

A total of 17 rice cultivars from Hokkaido were used to elucidate the relationships between haplotype patterns around these QTLs and CTF levels. These cultivars were breeding lines established between 1988 and 2006. The CTF level of these cultivars was referenced from Shinada et al. (2013).

**Evaluation of CTF**

The evaluation of CTF was carried out in accordance with Shinada et al. (2013). One germinated seed was sown into a 500 ml plastic pot and grown in a greenhouse. To avoid damage by cold temperature at the booting stage, 30-day-old plants were grown in a growth chamber at 26/20°C day/night under a natural sunlight intensity and day length until the start of the cold temperature treatment. When at least two panicles in a plant headed (i.e. the appearance of the panicle from the sheath of the flag leaf), the pot was moved to a growth chamber at 17.5/17.5°C day/night with 50\% shading of natural sunlight intensity for the cold temperature treatment; two to four panicles were examined in each plant to evaluate CTF. After 15 days’ cold temperature treatment, plants were moved to and grown in a growth chamber at 26/20°C until the seed maturing stage. The percentage of seed fertility was used as a parameter of cold tolerance. As a control, the seed fertility of two or three plants in each genotype class kept in a growth chamber without cold temperature treatment was examined.

For the comparison of means of seed fertility among eight genotype classes, Steel-Dwass test was conducted using JMP (SAS Institute, Cary, NC, USA).

**DNA analysis**

Total DNA was extracted from the leaves of each plant using a modified CTAB method (Murray and Thompson 1980). Genotyping with SSR markers was performed as described by Fujino et al. (2004). The genotype of each QTL in the F\(_2\) population was determined using RM5711 for

**Table 1. Summary of SSR markers within three QTL regions**

| chromosome | CTF QTL | Marker   | Marker position\(^a\) | No. of alleles |
|------------|---------|----------|-----------------------|---------------|
| 7          | \( q_{\text{CTF7}} \) | RM20875  | 1,274,622             | 2             |
|            |         | RM3196   | 2,044,280             | 2             |
|            |         | RM5752   | 2,567,873             | 3             |
|            |         | RM20973  | 2,769,995             | 2             |
|            |         | RM5711*  | 3,142,204             | 6             |
|            |         | RM21030  | 3,501,880             | 4             |
|            |         | RM21052  | 3,770,907             | 3             |
|            |         | RM21076  | 4,028,130             | 2             |
|            |         | RM21078  | 4,028,526             | 2             |
| 8          | \( q_{\text{CTF8}} \) | RM22674* | 8,497,626             | 2             |
|            |         | RM22679  | 8,580,100             | 3             |
|            |         | RM22684  | 8,806,201             | 3             |
|            |         | RM22780  | 10,798,150            | 2             |
| 12         | \( q_{\text{CTF12}} \) | RM3331   | 23,494,445            | 2             |
|            |         | RM28580  | 24,324,781            | 5             |
|            |         | RM28669* | 25,556,822            | 3             |
|            |         | RM28678  | 25,736,191            | 3             |
|            |         | RM1227   | 27,338,885            | 3             |
|            |         | RM28825  | 27,466,213            | 2             |

\(^a\) Position of marker based on location of the start of SSR repeat in IRGSP build 1.0 in RAP-DB (http://rapdb.dna.affrc.go.jp).

* The nearest markers to the LOD peak for each QTL.
qCTF7, RM22684 for qCTF8 and RM28661 for qCTF12. A total of 19 SSR markers (Gramene, http://www.gramene.org) around three QTLs were examined in 17 cultivars to evaluate the haplotype pattern of the QTLs (Table 1). These markers showed polymorphism between Eikei88223 and Suisei. Nine markers spanning 2.8 Mb for qCTF7, four markers spanning 2.3 Mb for qCTF8, and six markers spanning 4.1 Mb for qCTF12 were used. Fragment size was determined using polyacrylamide gel (33 cm wide, 42 cm length) electrophoresis for sequence analysis (Fujino et al. 2004).

Results

Evaluation of CTF in each genotype class

To elucidate genetic interactions between QTLs for CTF, an F2 population composed of 1754 plants derived from the cross between Eikei88223 and Suisei was classified into eight-genotype classes (class 0, class 7, class 8, class 12, class 7 + 8, class 8 + 12, class 7 + 12 and class 7 + 8 + 12) based on the genotype of the three QTLs (Fig. 1). A total 152 plants in eight classes, with 14–29 plants in each class, were selected and selfed, 152 F3 plants were used for evaluation of CTF. The mean of seed fertility in each class was then compared after cold temperature treatment.

The seed fertilities of plants without cold temperature treatment were over 90% in every class. On the other hand, the seed fertility in the eight genotype classes with cold temperature treatment showed a wide range of 18.2–63.1%, which was within the range of the parental cultivars. An increase in seed fertility after cold temperature treatment was observed, with an increase in the number of QTLs (Fig. 1).

![Graph showing seed fertility](image)

Fig. 1. Seed fertility after cold temperature treatment of plants with homozygous alleles for all eight QTL regions in the F1 population derived from a cross between Eikei88223 and Suisei. Data represent the mean value ± SD. n represents the number of individuals. Mean values with the same letters are not significantly different (P > 0.05, Steel-Dwass test)

Increasing of CTF level by pyramiding QTLS

To evaluate pyramiding effect of each QTL, we compared the means of seed fertility between genotype classes in all combinations (Fig. 1). Although, the mean of seed fertility of class 7, class 8 and class 12 was 32.9%, 32.5% and 38.9%, respectively, which were higher than that of class 0, no significant differences were detected among these four groups owing to the relatively large standard deviation (SD). The means of seed fertility of class 7 + 12 (53.3%) and class 8 + 12 (54.5%) were significantly higher than that of class 0. On the other hand, class 7 + 8 (35.3%) was not significantly different from class 0. A significant difference was detected between class 7 + 8 + 12 (63.1%) and class 0 (18.2%).

Haplotype patterns of chromosomal regions around the three QTLs

To elucidate the role of the three QTLs in the variation of CTF levels among the other cultivars from Hokkaido, haplotype patterns around these QTLs were compared in 17 cultivars. The number of alleles at each locus varied from two to six (Table 1). At least nine, six and 11 haplotype patterns were detected for qCTF7, qCTF8 and qCTF12 regions, respectively among the 17 cultivars. These were more complex than the haplotype patterns on the short arm of chromosome 3 among the cultivars from Hokkaido (Fujino and Iwata 2011). Haplotype patterns of all three QTL regions in Eikei88223 were different from those in Suisei (Fig. 2). There were no cultivars that had the same haplotype patterns as Eikei88223 in all three QTL regions. Furthermore, the same haplotype patterns at qCTF7 and qCTF12 as Suisei were only detected in Ginpu, which is the parental line of Suisei (Fig. 3).

Association of haplotype patterns around QTLs and CTF levels

We compared haplotype patterns around QTLs with CTF levels. The same haplotype pattern to Eikei88223 near each QTL regions is shown in yellow in Fig. 2. Six cultivars had same haplotype pattern as Eikei88223 within the qCTF7 region. CTF levels of these cultivars varied from 32.8% to 78.4%. Only Ginpu had the same haplotype pattern as Eikei88223 within the qCTF8 region. This cultivar showed low CTF levels. Four cultivars had same haplotype pattern as Eikei88223 in the qCTF12 region, showing higher CTF levels, 64.8–92.6%. Only two cultivars, Kitaake and Kirara397, had the same haplotype patterns as Eikei88223 in both qCTF7 and qCTF12 (Fig. 2). In contrast, Hokkai No. 302, Kitaibuki and Hokuikumochi No. 87 showed higher CTF levels, 79.0–92.6%. However these three cultivars had different haplotype patterns from Eikei88223 in all three QTL regions.
Discussion

The MAS scheme of QTL pyramiding has enabled the quick and accurate selection of lines with a target trait (Ashikari and Matsuoka 2006, Nagai et al. 2012). However, quantitative traits are generally result from: (1) a combination of multiple QTLs; (2) their interaction with other loci; and (3) their interaction with environments that affect their expression (Wade et al. 2001). In order to identify the efficient combinations of QTLs for the improvement of quantitative traits in plant breeding programs, it is necessary to arrange a number of QTLs and evaluate interactions between them. In this study, we evaluated pyramiding effect of three QTLs for CTF. Furthermore, we compared the haplotype pattern around the QTLs for CTF among rice cultivars from Hokkaido. These results are useful for improvement of new cultivars with high CTF levels using MAS and identification of genetic resources with the novel QTL(s) for CTF.

An increase in seed fertility after cold temperature treatment was observed, with an increase in the number of QTLs. This result clearly supported the previous results of QTL analysis (Shinada et al. 2013). Only one QTL for CTF was not sufficiently effective to improve CTF level. However, pyramiding of $qCTF7$ and $qCTF12$ (class 7 + 12) or $qCTF8$ and $qCTF12$ (class 8 + 12) showed significantly higher seed fertilities than that of plants with no QTL (class 0) and Suisei. These two combinations of QTLs for CTF will be useful for improving CTF levels in rice breeding programs.

Previously, a wide phenotypic variation for CTF was
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Shinada, H., N. Iwata, T. Sato and K. Fujino (2013) Genetical and morphological characterization of cold tolerance at the fertilization demonstrated among rice cultivars from Hokkaido (Shinada et al. 2013, Tanno et al. 2000). Characterizing haplotype patterns of the three QTL regions in these cultivars can help to determine the allelic distribution in different CTF sources and aid in the choice of vigorous CTF parents for future QTL mapping studies and breeding programs. In this study, two cultivars from Hokkaido possessed a same haplotype pattern as that of Eikei88223 within the qCTF7 and qCTF12 regions. However, there were no cultivars that had the same haplotype patterns as Eikei88223 in all three QTL regions. The cultivars with the same haplotype patterns as Eikei88223 may be rare among this local gene pool. On the other hand, Hokkai No. 302, Kitaibuki, and Hokuikumochi No. 87 with vigorous CTF possessed different haplotype patterns from that of Eikei88223 within all three QTL regions. These three cultivars may possess other QTLs for CTF. Understanding the genetic bases of CTF involved in these three cultivars will be useful for improving CTF in rice breeding program.

The results in this study may be also useful to understand the genetic interactions between each QTL. Although, due to the relatively large standard deviation (SD), no significant differences were detected among five classes (class 7 + 12, 8 + 12, 7, 8 and 12), the mean of seed fertility of class 7 + 12 (53.3%) and class 8 + 12 (54.5%) was higher than those of class 7 (32.9%), class 8 (32.5%) and class 12 (38.9%). Furthermore, although probability in the two-way ANOVA for qCTF7 and qCTF8 was not significant (P = 0.27), due to the relatively large SD, the mean of seed fertility of class 7 + 8 (35.3%) was almost equal to those of class 7 (32.9%) and class 8 (32.5%) and class 12 (38.9%) (Fig. 1). These results might suggest epistatic interaction in the combination between qCTF7 and qCTF8 and additive effects in the combination between qCTF7 and qCTF12 and between qCTF8 and qCTF12. In order to verify this hypothesis, additional analysis using the methods that can perform evaluation of CTF more precisely would be necessary.

A larger SD of the percentage of seed fertility in selected plants for eight genotype classes was observed than that in the parental cultivars (Fig. 1). These results suggested that some factors might disturb the precise evaluations of CTF. In this study, the genotypes of each QTL were determined using a single SSR marker. These markers may show weak linkage to the target QTL. Furthermore, variations in CTF levels involved pollen maturation, pollen germination and pollen tube elongation under cold temperature (Shinada et al. 2013). Seed fertility on CTF may result from the complex interactions of these factors. There may also be a limitation in evaluating genetic effects of QTLs or interactions between QTLs on the phenotype of CTF using only seed fertility as an index. Evaluating the dissected phenotype of CTF may help in identifying the genuine gene effect of each QTL.
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