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ORIGINAL ARTICLE

Detection of QTLs for cold tolerance of rice cultivar 'Kuchum' and effect of QTL pyramiding

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Abstract

Key message **A QTL for cold tolerance at the booting stage of rice cultivar 'Kuchum' was detected and delimited into a 1.36 Mb region, and a cold-tolerant line was developed by QTL pyramiding.**

Abstract Low temperature in summer causes pollen sterility in rice, resulting in a serious loss of yield. The second most widely grown rice cultivar in Japan, 'Hitomebore', has been developed as a cultivar highly tolerant to low temperature at the booting stage. However, even 'Hitomebore' exhibits sterility at a temperature lower than 18.5 °C. Further improvement of cold tolerance of rice is required. In the present study, QTLs for cold tolerance in a Bhutanese rice variety, 'Kuchum', were analyzed using backcrossed progenies and a major QTL, named *qCT-4*, was detected on chromosome 4. Evaluating cold tolerance of seven types of near isogenic lines having 'Kuchum' alleles around *qCT-4* with a 'Hitomebore' genetic background, *qCT-4* was delimited to a region of ca. 1.36 Mb between DNA markers 9_1 and 10_13. Homozygous 'Kuchum' alleles at *qCT-4* showed an effect of increasing seed fertility by ca. 10 % under cold-water treatment. Near isogenic lines of 'Hitomebore' having 'Silewah' alleles of *Ctb1* and *Ctb2* and a 'Hokkai PL9' allele of *qCTB8* did not exhibit higher cold tolerance than that of 'Hitomebore'. On the other hand, a *qLTB3* allele derived from a Chinese cultivar 'Lijiangxintuanheigu' increased cold tolerance of 'Hitomebore', and pyramiding of the *qCT-4* allele and the *qLTB3* allele further increased seed fertility under cold-water treatment. Since NILs of 'Hitomebore' with the 'Kuchum' allele of *qCT-4* were highly similar to 'Hitomebore' in other agronomic traits, the *qCT-4* allele is considered to be useful for developing a cold-tolerant cultivar.

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Introduction

Low temperature at the booting stage of rice causes pollen sterility and results in yield loss, severe damage of which was experienced in 1993 in Japan. The developmental stage of rice most sensitive to low temperature has been revealed to be the early microspore stage just after the tetrad stage (Satake and Hayase 1970). Abnormality of sugar metabolism in anthers has been reported to be responsible for inhibition of pollen development and a decrease of fertile pollen grains, which result in inability of anther dehiscence (Nishiyama 1984).

Rice cultivar 'Hitomebore' has been developed as a cultivar highly tolerant to low temperature at the booting

stage (Sasaki et al. 1994) and is the second most widely grown rice cultivar following 'Koshihikari' in Japan. The low-temperature damage in 1993 in the Tohoku region of Japan caused replacement of the major cultivar 'Sasanishiki', which is relatively sensitive to low temperature, by 'Hitomebore'. However, even 'Hitomebore' exhibited sterility in some areas in 1993 (Sasaki et al. 1994). Further improvement of cold tolerance of rice is thus required. In previous rice breeding efforts for further improvement of cold tolerance using genetic resources as parents, inferior traits such as long culm, late maturity, low yield accompanying cold tolerance have made it difficult to develop a commercial cultivar having high yield and good quality. Development of DNA markers linked closely with cold tolerance genes is considered to be effective for breaking linkage drag to remove undesirable alleles derived from cold-tolerant parents.

Many quantitative trait loci (QTLs) for cold tolerance at the booting stage have been reported, i.e., *Ctb1*, *Ctb2* (Saito et al. 1995, 2001, 2010), *qCT-1*, *7*, *11* (Takeuchi et al. 2001), *qCTB1*, *2a*, *2b*, *3*, *5*, *6*, *7*, *9*, *12* (Andaya and Mackill 2003), *qRCT3*, *6a*, *6b, 7* (Dai et al. 2004), *qCTB1-1, 1-2, 8* (Kuroki et al. 2007, 2011), *qCTB-1-1*, *4-1*, *5-2*, *10-2*, *11-1* (Xu et al. 2008), *qPSST-3*, *7*, *9* (Suh et al. 2010), *qLTSPKST10.1* (Ye et al. 2010), *qCTB3-Silewah* (Mori et al. 2011), and *qLTB3* (Shirasawa et al. 2012). However, it is difficult to compare the effects of these reported QTLs, since the methods for evaluation of cold tolerance and tested cultivars are different between these studies. Mori et al. (2011) have detected a QTL for cold tolerance of 'Silewah' different from that of 'Silewah' detected by Saito et al. (1995), suggesting that effects of QTLs depend on environmental conditions for cold tolerance evaluation. Temperature, day length, and parental lines used for production of segregating populations for genetic studies may have an influence on the effect of QTLs. However, a study comparing the effects of several different cold tolerance QTLs in rice has not been reported.

There are few examples of introduction of the cold tolerance QTLs into major cultivars in rice breeding (Saito et al. 2001; Zhou et al. 2012). This is probably due to small effect of each QTL. To develop a highly cold-tolerant line, QTL pyramiding may be required. Successful QTL pyramiding in rice has been reported for improvement of grain size (Wang et al. 2012a) and yield potential (Wang et al. 2012b). Kuroki et al. (2011) have reported pyramiding effects of QTLs for cold tolerance at booting stage, i.e., *qCTB8.1*, *qCTB1.1*, and *qCTB1.2*, but seed fertilities of all pyramiding lines were not significantly higher than those of the lines having a single QTL. Comparing seed fertilities of different genotypes of three QTLs, Shinada et al. (2014) suggested pyramiding effect of QTLs for cold tolerance at fertilization stage in rice.

In our previous investigations, pollen dispersal from flowers of 'Kuchum', a cultivar in Bhutan, was observed under low temperature conditions, suggesting that this cultivar has high cold tolerance. Cold tolerance of 'Kuchum' cannot be examined by the cold-deep-water irrigation method (Matsunaga 2005), which is commonly used for cold tolerance evaluation of breeding lines by rice breeders, because it has a long culm length, ca. 150 cm, and a late flowering trait. Although high cold-tolerant lines, e.g., 'Tohoku 196', have been developed from progenies of 'Kuchum', development of an elite line usable as a commercial cultivar from progeny of 'Kuchum' has been unsuccessful because of unfavorable traits of 'Kuchum', i.e., long culm, late flowering, red-colored grains, and low yield. In the present study, QTLs for cold tolerance in 'Kuchum' were analyzed, and a major QTL was detected on chromosome 4. A chromosomal region containing this QTL, named *qCT-4* (QTL for Cold Tolerance at booting stage on chromosome 4), was delimited by developing near isogenic lines (NILs) of 'Hitomebore' with a chromosomal segment from 'Kuchum'. Furthermore, the effect of combining a 'Kuchum' allele at *qCT-4* with a cold tolerance *qLTB3* allele of 'Lijiangxintuanheigu' identified in our previous study (Shirasawa et al. 2012) was investigated. Effects of other QTLs, i.e., *Ctb1* and *Ctb2* derived from 'Silewah' and *qCTB8* from 'Hokkai PL9', under a genetic background of 'Hitomebore' were also examined.

Materials and methods

Plant materials

An F_1 hybrid between 'Kuchum', a gene source of cold tolerance, and 'Chiyohonami', which has relatively high cold tolerance, was backcrossed once with 'Chiyohonami', and selfing was repeated. A BC_1F_6 plant named 'BI6-12' was crossed with 'Chiyohonami' for production of an F_2 population of 96 plants named CKC1 (BC_2F_2 between 'Kuchum' and 'Chiyohonami'), which were used for QTL analysis of cold tolerance. Two populations of BC_6F_4 plants named EBS6 with 78 plants and EBS8 with 187 plants obtained by six backcrossings of 'Hitomebore' with a hybrid between 'Kuchum' and 'Hitomebore' were also used for QTL analysis. Pedigrees of these plant materials are shown in Supplementary Fig. 1. A breeding line having cold tolerance genotype in *qCT-4*, named 'Tou 1380', and one having cold tolerance genotype in *qLTB3*, named 'Ouu 415', were also used for QTL pyramiding. 'Ouu 415' has been developed by repeated backcrossing of a hybrid between 'Hitomebore' and 'Lijiangxintuanheigu' to 'Hitomebore'.

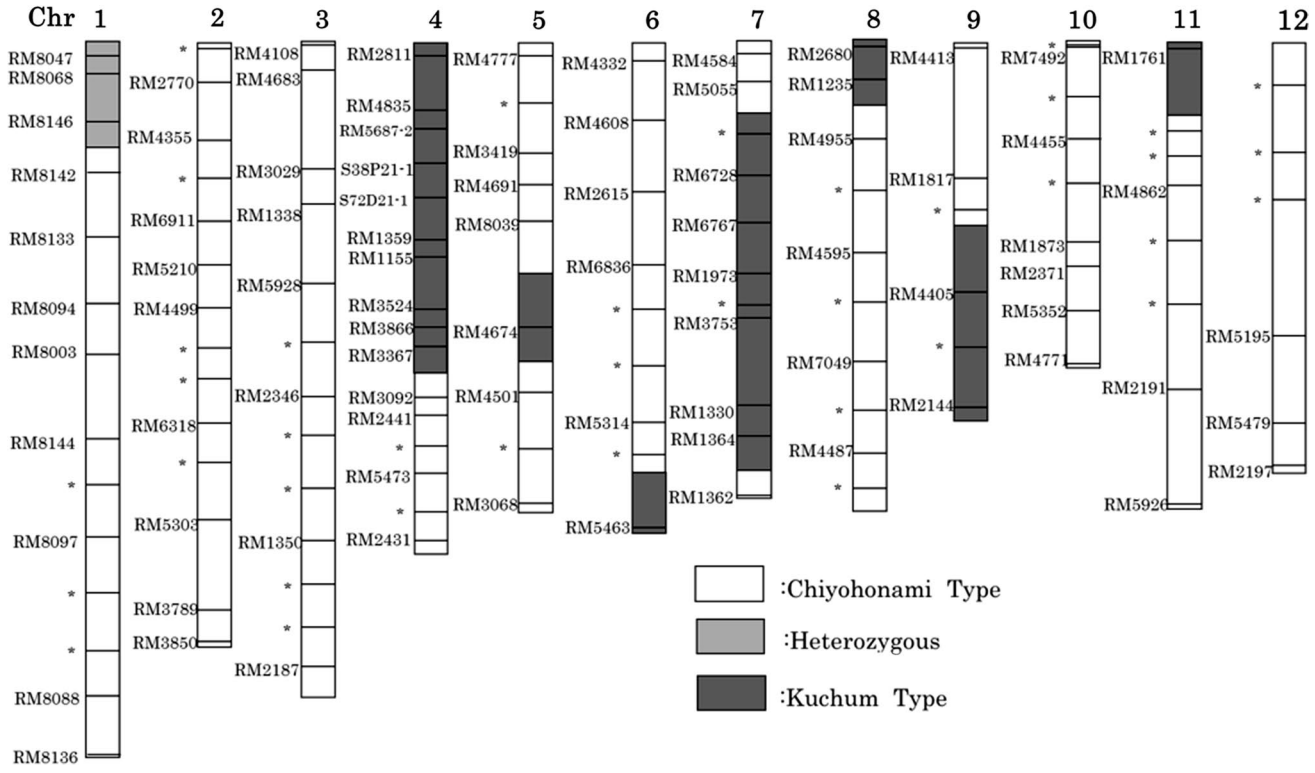

Fig. 1 Graphical genotyping of 'BI6-12' having chromosomal segments of 'Kuchum' with a 'Chiyohonami' genetic background. Stars without marker names indicate SSR markers showing no polymorphism between 'Kuchum' and 'Chiyohonami'

Evaluation of cold tolerance of plants

Populations for QTL analysis and developed NILs were grown in a paddy field equipped for cold tolerance evaluation (Miyagi Prefectural Furukawa Agricultural Experiment Station, Osaki, Miyagi, Japan) and were treated by the cold-deep-water irrigation method (Matsunaga 2005). In this paper, "cold tolerance" means only cold tolerance at booting stage. Seeds were sown in the middle of April and seedlings were planted with 41.6 plants/m^2 (24 × 10 cm) in the field with basal fertilizer of 40 kg N/ha for cold tolerance evaluation in the middle of May. Cold-water treatment was carried out from the beginning of June to the beginning of September. The water temperature was controlled at 18.5 °C (at 19.0 °C only for CKC1) and the water depth was 15 or 25 cm depending on plant heights. Seed fertility, which is the percentage of the number of fertile seeds in the number of florets, of 15 panicles from three plants was investigated. Three blocks were set for each line every year for 6 years. Dunnett's test or Tukey–Kramer's test was performed for multiple comparison, and the difference between two lines was analyzed by the Student's *t* test.

Genotyping of plants and QTL analyses

According to Thomson and Henry (1995), genomic DNA was extracted from fresh leaves by TPS solution (100 mM Tris–HCl pH 8.0, 10 mM ethylenediaminetetraacetic acid (EDTA), 1 M KCl), precipitated with isopropanol, washed with cold ethanol, and dissolved in 1/10 TE solution (1 mM Tris–HCl pH 8.0, 0.1 mM EDTA). Using the extracted genomic DNA as a template, DNA fragments were amplified by polymerase chain reaction (PCR) under the thermal cycling condition of 7 min denaturation at 94 °C, followed by 35 cycles of 1 min denaturation at 94 °C, 1 min annealing at 55 °C, and 2 min extension at 72 °C, and a final 10 min extension at 72 °C. PCR products were separated by electrophoresis on 3 % (w/v) agarose (Sigma, Type I-A) gel and detected by staining with ethidium bromide.

DNA polymorphism between 'Kuchum' and 'Chiyohonami' was analyzed using 125 SSR markers, and graphical genotyping of 'BI6-12' and QTL analysis using CKC1 were carried out with 87 markers, which showed polymorphism between 'Kuchum' and 'Chiyohonami'. Confirmation and delimitation of a detected QTL were performed using EBS6 and EBS8 with three markers, i.e.,

RM5687-2, A20I0241-4, and S38P21-1, and seven markers, i.e., RM2811, RM3658, RM7472, S72D08-2, S96E05-1, RM5687-2, and A20I0241-4 (Supplementary Table 1), respectively. A linkage map was constructed using Mapmaker/Exp Ver3.0 (Lincoln et al. 1992), and QTL was analyzed with the composite interval mapping method using Windows QTL Cartographer ver2.5 (Wang et al. 2006). The LOD threshold for QTL significance was determined by a permutation test (1000 replications) with a significance level $P = 0.05$.

Development of NILs of 'Hitomebore' with QTL regions of 'Kuchum', 'Silewah', and 'Hokkai PL9'

From the BC_6F_4 population between 'Kuchum' and 'Hitomebore', 20 lines of seven types named C1 to C7 having different lengths of a chromosome segment around a QTL, i.e., *qCT-4*, of 'Kuchum' were developed. Simultaneously, 14 lines of four types that are segregants having alleles of 'Hitomebore' type of *qCT-4* derived from the same parental plants as those of C1, C4, C5, and C7, which were named C1(-), C4(-), C5(-), and C7(-), respectively, were also developed for evaluation of the effect of *qCT-4* on cold tolerance. Three lines of BC_6F_4, BC_6F_6, and BC_6F_7 were used for evaluation of other traits important for rice production. Among them, 'Tou 1380' and 'Tou 1381' belonging to group C1 have 'Kuchum' genotype in the region between RM2811 and S38P21-1 and 'Tou 1489' has 'Kuchum' genotype in the region between 9_1 and S38P21-1. 'Tou 1380' was used as a parent for QTL pyramiding.

An F_1 hybrid between 'Silewah', a cold-tolerant cultivar in Indonesia, and 'Hitomebore' was backcrossed four times with 'Hitomebore', and 16 NILs of six types named S1 to S6 having 'Silewah' alleles at *Ctb1, Ctb2*, or both were selected (Supplementary Fig. 2). Three lines of S3 having 'Hitomebore' alleles in the *Ctb1–Ctb2* region derived from the same parental plants, which was named S3(-), were also developed. Eight NILs of two types named H1 and H2 having a 'Hokkai PL9' allele at *qCTB8* with a 'Hitomebore' background were developed by four backcrossings of 'Hitomebore' to an F_1 hybrid with 'Hokkai PL9'. Three lines named H2(-) that have lost the 'Hokkai PL9' allele at *qCTB8* by segregation were also selected.

QTL pyramiding

For combining the cold tolerance *qCT-4* allele and the cold tolerance *qLTB3* allele, 'Tou 1380' and 'Ouu 415' were crossed and F_2 plants were obtained. Using DNA markers Indel_2, RM5687-2, and S38P21-1 for *qCT-4* and RM6970, RM7000, and RM7389 for *qLTB3*, 18 F_2 plants of four genotypes homozygous for 'Kuchum', 'Lijiangxintuanheigu', and 'Hitomebore' alleles at *qCT-4* and *qLTB3* were selected. There were six lines of group L1 with the 'Hitomebore' alleles at both *qCT-4* and *qLTB3* and four lines of group L2 with the 'Kuchum' allele at *qCT-4* and the 'Hitomebore' allele at *qLTB3*. There were three lines of group L3 with the 'Hitomebore' allele at *qCT-4* and the 'Lijiangxintuanheigu' allele at *qLTB3*, and five lines of group L4 with the 'Kuchum' allele at *qCT-4* and the 'Lijiangxintuanheigu' allele at *qLTB3*.

Investigation of agronomical traits

In a rice paddy field at the Miyagi Prefectural Furukawa Agricultural Experiment Station (Osaki, Miyagi, Japan), heading date, maturity date, culm length, panicle length, number of panicles per plant, grain yield, grain weight of 1000 seeds, grain quality, and frequency of lodging plants of the developed lines were investigated. The grain quality was rated from 1 (excellent) to 5 (poor) based on percentage of normal semi-transparent grains, and the frequency of lodging plants was rated from 0 (no lodging) to 4 (severe lodging) according to angle from upright. Resistance to leaf blast was evaluated by the upland nursery test with natural infection boosted by spreading infected leaves. Resistance was rated twice from 0 (no symptom) to 10 (dying of the whole plant) according to Asaga (1981) at the end of June and the beginning of August.

Results

QTL analysis of cold tolerance of 'Kuchum'

DNA polymorphism between 'BI6-12' and 'Chiyohonami' was detected in 17 SSR markers among the 87 markers showing polymosphism between 'Kuchum' and 'Chiyohonami', and graphical genotyping indicated that 'BI6-12' has segments of chromosomes 1, 4, 5, 6, 7, 8, 9, and 11 from 'Kuchum' (Fig. 1). Frequency distribution of seed fertility of cold-water treated plants in CKC1, which is F_2 between 'BI6-12' and 'Chiyohonami', is shown in Supplementary Fig. 3. QTL analysis of seed fertility of CKC1 detected significant LOD scores at loci of RM8146 on chromosome 1 and S72D21-1 on chromosome 4. In the QTL of S72D21-1 on chromosome 4, 'Kuchum' alleles showed a beneficial effect of increasing seed fertility under cold treatment, i.e., cold tolerance. The additive effect, dominance effect, and explained variance of this QTL were 37.0, 9.87, and 35.2 %, respectively (Table 1). Although LOD score and explained variance of the QTL near RM8146 on chromosome 1 were larger than those of the QTL near S72D21-1 on chromosome 4, the QTL on chromosome 4 was further investigated because the QTL on chromosome 1 had negative additive effect.

Table 1 QTL analysis of cold tolerance of'Kuchum'

Population name[a]	Chr	QTL region	Maker nearest to a LOD peak	Maximum of LOD	Additive effect[b]	Dominance effect	Variance explained (%)
CKC1	1	RM8068–RM8146	RM8146	10.71	−16.0	−31.7	47.0
	4	RM4835–S72D21-1	S72D21-1	6.33	37.0	9.87	35.2
EBS6	4	RM5687-2–S38P21-1	RM5687-2	5.67	6.51	3.72	28.1
EBS8	4	RM2811–RM3658	RM3658	6.69	5.42	0.37	15.1
	4	RM7472–A20I0241-4	S96E05-1	3.08	4.99	2.63	8.0

Seed fertilities (%) of plants grown in a field with deep water controlled at 18.5 °C (19.0 °C for CKC1) were used as a parameter of cold tolerance

[a] CKC1 is an F_2 population obtained by a cross between 'Chiyohonami' and 'BI6-12', which is a backcrossed inbred line between 'Kuchum' as a donor with 'Chiyohonami' as a recurrent parent. EBS6 and EBS8 are BC_6F_4 populations obtained by backcrossings of 'Kuchum' as a donor with 'Hitomebore' as a recurrent parent

[b] Additive effect indicates effect of an allele of 'Kuchum'

The QTL on chromosome 4 was again analyzed using two BC_6F_4 populations, EBS6 and EBS8 (Supplementary Fig. 1), developed by backcrossing of 'Kuchum' with a recurrent parent 'Hitomebore' and marker-assisted selection using S72D21-1. EBS6 and EBS8 were derived from two BC_6F_1 plants, and independently developed by selfing, possibly having different lengths of a QTL region and genetic background. Frequency distribution of seed fertility in these backcross populations are shown in Supplementary Fig. 3. Analysis using EBS6 detected a significant LOD peak at RM5687-2 on chromosome 4, which has an additive effect of 6.51, a dominance effect of 3.72, and an explained variance of 28.1 % (Table 1). In EBS8, significant LOD peaks were detected at loci of RM3658 and S96E05-1 on chromosome 4 having additive effects of 5.42 and 4.99 and explained variances of 15.1 and 8.0 %, respectively. No QTLs for culm length and heading date were detected in EBS6 and EBS8, indicating that the detected QTL for cold tolerance is not due to high plant height, which sometimes causes inadequate cold-water treatment of developing flowers, nor to difference of the time most sensitive to low temperature.

In these QTL analyses using backcross progenies with recurrent parents 'Chiyohonami' and 'Hitomebore', a significant QTL having a high additive effect was detected in a region of ca. 14.3 Mb from RM3658 to S72D21-1. This QTL was named *qCT-4* (QTL for Cold Tolerance at booting stage on chromosome 4). Replacement of homozygous 'Hitomebore' alleles at this QTL with homozygous 'Kuchum' alleles is expected to contribute to an increase in seed fertility of ca. 10 % under cold treatment.

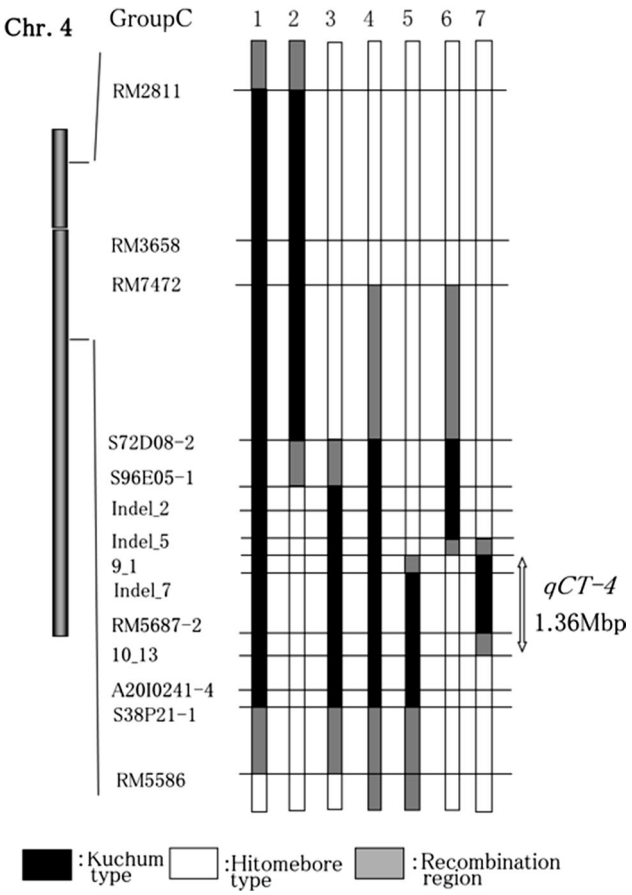

Fig. 2 Graphical genotyping of NILs developed for delimitation of the QTL region of *qCT-4*

Table 2 Cold tolerance evaluation of NILs for delimitation of the QTL region of *qCT-4*

Group or cultivar	Region of substitution	Geno-type[a]	Number of lines[b]	Seed fertility (%)					
				2008	2009	2011	2012	2013	2014
C1	RM2811–S38P21-1	+	3 (7)	53.3[c]	63.4[c]	76.2[c]	60.8[c]	76.6[c]	
C1(−)		−	5	29.9[a]	50.4[a]				
C2	RM2811–S72D08-2	+	2		45.7[b]	60.2[b]	43.4[b]	63.7[b]	
C3	S96E05-1–S38P21-1	+	5 (3)		55.3[a]	74.8[c]	61.5[c]	71.2[c]	
C4	S72D08-2–S38P21-1	+	2			78.1[c]	63.1[c]	72.9[c]	55.9[a]
C4(−)		−	3			67.6[a]	54.5[a]	65.9[c]	43.9[b]
C5	Indel7–S38P21-1	+	3					72.5[c]	52.8[a]
C5(−)		−	3					64.2[b]	45.8[b]
C6	S72D08-2–Indel_5	+	2			68.1 [a]	56.2[a]	61.2[b]	
C7	9_1–RM5687-2	+	3					70.0[c]	47.6[a]
C7(−)		−	3					66.7[c]	44.4[b]
Hitomebore		−		38.9[a]	52.4[a]	68.0[a]	53.7[a]	65.4[a]	53.4[a]

[a] *Plus* indicates a line having 'Kuchum' genotypes of DNA markers in the substituted region and *minus* indicates a line having 'Hitomebore' alleles at the corresponding region generated by segregation

[b] Number of *lines* in parentheses shows the number of lines in 2008 and 2009

[c] Data are average values. Repetition of each line was one in 2008 and three from 2009 to 2014. Repetition of 'Hitomebore' was two in 2008, 15 in 2009 and 2011, 18 in 2013, and 9 in 2012 and 2014

[d] *Different letters* represent significant difference (5 %) in comparison with 'Hitomebore' by Dunnet's test

[e] Generations of NILs are as follows; Group C1, 7 lines of BC_6F_3–BC_6F_4 in 2008 and 2009, 2 lines of BC_6F_6–BC_6F_8 in 2011 to 2013 and one line of BC_6F_8–BC_6F_{10} in 2011 to 2013; Group C1(−), BC_6F_3–BC_6F_4; Group C2, BC_6F_4, BC_6F_6–BC_6F_8; Group C3, 3 lines of BC_6F_4 in 2009, 3 lines of BC_6F_6–BC_6F_8 and 2 lines of BC_6F_8–BC_6F_{10} in 2011 to 2013; Group C4 and C4(−), BC_6F_8–BC_6F_9, BC_6F_{11}; Group C5 and C5(−), BC_6F_9–BC_6F_{10}; GroupC6, BC_6F_8–BC_6F_{10}; Group C7, BC_6F_{10}–BC_6F_{11}

Cold tolerance of NILs having a 'Kuchum' allele at *qCT-4* with a 'Hitomebore' genetic background

Twenty NILs having a QTL region of *qCT-4* from 'Kuchum' with a 'Hitomebore' genetic background were classified into seven groups according to the positions of recombination break points (Fig. 2; Table 2). Group C1 lines having 'Kuchum' alleles in a region from RM2811 to S38P21-1, which was developed first, showed significantly higher seed fertilities under cold stress than those of 'Hitomebore' and lines of C1(-) having 'Hitomebore' alleles in this QTL region, which was obtained by segregation in a population of group C1, in repeated cold tolerance evaluations for 5 years, indicating that *qCT-4* has a significant effect on cold tolerance. Groups C2 lines having 'Kuchum' alleles in a region from RM2811 to S72D08-2 showed lower seed fertilities under the cold stress than those of 'Hitomebore' in the repeated cold tolerance evaluations for 4 years. On the other hand, lines of groups C3 and C4 having 'Kuchum' alleles in regions from S96E05-1 to S38P21-1 and from S72D08-2 to S38P21-1, respectively, showed significantly higher seed fertilities than those of 'Hitomebore' in three of the four tested years. These results suggest that the cold tolerance gene in *qCT-4* is located in an 8.8 Mb region between S72D08-2 and RM5586.

Using progenies of NILs in groups from C1 to C4, recombinants within the 8.8 Mb region between S72D08-2 and RM5586 were further selected, and eight lines of three groups, i.e., group C5 to group C7 having shorter genomic regions derived from 'Kuchum' were developed (Fig. 2). Lines of groups C5 and C7 grown under the cold stress showed significantly higher seed fertilities than that of 'Hitomebore' in 2013. Although the lines of C5 and C7 did not show significantly higher seed fertilities than that of 'Hitomebore' in 2014, C5(-) lines and C7(-) lines having 'Hitomebore' alleles in the QTL region showed significantly lower seed fertilities than that of 'Hitomebore', suggesting that only the seed fertility of 'Hitomebore' was abnormally high in 2014. On the other hand, seed fertilities of lines of group C6 were comparable to those of 'Hitomebore' in the evaluations for 2 years of the three tested years. From these results, *qCT-4* can be inferred to be in a region of ca. 1.36 Mb between DNA markers 9_1 and 10_13. Supporting data for significant effect of this region on cold tolerance were obtained using other NILs, C8 to C10 (Supplementary Fig. 4, Supplementary Table 2), although number of plants used for cold tolerance evaluation of these lines were small.

Table 3 Cold tolerance of NILs having *Ctb1*, *Ctb2* with the 'Hitomebore' background

Group or cultivar	QTL	Substituted region (kb)	Geno-type[a]	Generation	Number of lines	Seed fertility (%)			
						2008	2009	2010	2011
S1	*Ctb1*	541	+	BC_4F_5	2	19.1[a]			
S2	*Ctb2*	1327	+	BC_4F_5	1	27.1[a]			
S3	*Ctb1*, *Ctb2*	2438	+	BC_4F_7–BC_4F_9	3		49.5[b]	47.6[b]	56.7[b]
S3(−)		2438	−	BC_4F_7	3		48.8[b]		
S4	*Ctb1*, *Ctb2*	3377	+	BC_4F_5	4	26.7[a]			
S5	*Ctb1*, *Ctb2*	3708	+	BC_4F_4	2	25.7[a]			
S6	*Ctb1*, *Ctb2*	5445	+	BC_4F_5	4	32.7[a]			
Hitomebore						33.1[a]	57.1[a]	64.5[a]	69.2[a]

[a] *Plus* indicates a line having 'Silewah'-type alleles in *Ctb1* and *Ctb2* loci. *Minus* indicates a 'Hitomebore'-type segregant

[b] Data show average values. There were three repetitions (one in S5 group) in each line or cultivar. There were six repetitions of 'Hitomebore' (nine in 2008)

[c] *Different letters* represent significant difference (5 %) in comparison with 'Hitomebore' by Dunnet's test in 2008 and 2009 and by Student's *t* test in 2010 and 2011

Table 4 Cold tolerance of NILs having *qCTB8* with the 'Hitomebore' background

Group or cultivar	QTL	Substituted region (kb)	Geno-type[a]	Generation	Number of lines	Seed fertility (%)		
						2008	2009	2011
H1	*qCTB8*	3229	+	BC_4F_3	5	35.4[a]		
H2	*qCTB8*	1325	+	BC_4F_5–BC_4F_6	3		48.4[a]	65.7[a]
H2(-)		1325	−	BC_4F_5	3		45.6[a]	
Hitomebore						36.9[a]	55.8[a]	69.2[a]

[a] *Plus* indicates a line having 'Hokkai PL9'-type alleles in a *qCTB8* locus. *Minus* indicates a 'Hitomebore' type segregant

[b] Data show average values. There were three repetitions in each line or cultivar. There were six repetitions of 'Hitomebore'

[c] The same letters represent no significant difference (5 %) in comparison with 'Hitomebore' by Student's *t* test in 2008 and 2011 and by Dunnet's test in 2009

Cold tolerance of NILs having *Ctb1, Ctb2,* or *qCTB8* with a 'Hitomebore' background

Ctb1 is linked with *Ctb2* on chromosome 4, and NILs having each or both cold tolerance alleles at *Ctb1* and *Ctb2* derived from 'Silewah' with a 'Hitomebore' genetic background were developed. Lines of group S1 having only the 'Silewah' *Ctb1* allele and group S2 having only the 'Silewah' *Ctb2* allele showed slightly lower seed fertilities under cold stress than that of 'Hitomebore' in 2008, but the differences were not significant (Table 3). NILs having 'Silewah' alleles in chromosomal regions of different lengths covering both *Ctb1* and *Ctb2*, i.e., groups S3 to S6, were obtained (Supplementary Fig. 2, Supplementary Table 3). Lines of S3 showed significantly lower seed fertilities than those of 'Hitomebore' in the evaluations for 3 years, and lines of groups S4, S5, and S6 showed slightly lower seed fertilities in 2008. Eight NILs of two groups, H1 and H2, having different lengths of a chromosomal region containing *qCTB8* from 'Hokkai PL9' with a 'Hitomebore' genetic background were also developed (Supplementary Fig. 2, Supplementary Table 3). The lines of group H1 having 'Hokkai PL9' alleles in a 3.2 Mb region did not show higher seed fertility than that of 'Hitomebore' in 2008, and neither did the lines of group H2 having 'Hokkai PL9' alleles in a region of 1.3 Mb in 2009 and 2011 (Table 4). Furthermore, the lines of S3 and those of H2 showed seed fertilities comparable to those of S3(−) lines and H2(−) lines, respectively, having 'Hitomebore' alleles in the QTL region. These results indicate that *Ctb1, Ctb2,* and *qCTB8* are ineffective for enhancing the cold tolerance of 'Hitomebore'.

Effect of QTL pyramiding

The NIL having the 'Kuchum' *qCT-4* allele was crossed with the line having the 'Lijiangxintuanheigu' *qLTB3* allele for pyramiding of QTLs and cold tolerance of 18 lines at F_3 and F_4 generations was evaluated in 2012 and 2013 (Table 5). In both years, lines of group L4 having the cold

Table 5 Cold tolerance of NILs having tolerance alleles of *qCT-4* and *qLTB3*

Group	QTL[a]		No. of lines	Seed fertility (%)	
	qCT-4	*qLTB3*		2012	2013
L1	−	−	6	55.2[a]	67.0[a]
L2	+	−	4	66.2[b]	78.4[b]
L3	−	+	3	72.7[b,c]	81.5[b,c]
L4	+	+	5	75.5[c]	82.5[c]
Hitomebore	−	−	1	51.5	72.6
Tou 1380	+	−	1	66.3	76.9
Ouu 415	−	+	1	65.3	80.5

[a] *Plus* indicates 'Kuchum' genotype in the *qCT-4* locus or 'Lijiangxintuanheigu' genotype in the *qLTB3* locus. *Minus* indicates 'Hitomebore' type

[b] Data show average values. There were three repetitions in each line or cultivar

[c] *Different letters* represent significant difference (5 %) by Tukey–Kramer's test

tolerance alleles at both *qCT-4* and *qLTB3* showed significantly higher seed fertility than lines of group L1 having 'Hitomebore' alleles in these QTL regions under the cold-stress condition. Seed fertilities of the L4 lines were significantly higher than those of lines of group L2 having the 'Kuchum' allele at *qCT-4*. Although the L4 lines showed higher seed fertilities than those of lines of group L3 having the 'Lijiangxintuanheigu' allele at *qLTB3,* the differences were not significant in either 2012 or 2013.

Agronomic traits of NILs having the 'Kuchum' *qCT-4* allele with the 'Hitomebore' genetic background

Agronomic traits of cold-tolerant lines having the 'Kuchum' *qCT-4* allele, which were named 'Tou 1380', 'Tou 1381', and 'Tou 1489', were investigated in the field under normal conditions. Days to heading of 'Tou 1380' and 'Tou 1381' were one to 2 days shorter than that of 'Hitomebore', and all the three NILs had slightly shorter culm lengths than 'Hitomebore' (Supplementary Table 4). The other traits, i.e., panicle length, grain yield, grain weight, lodging degree, grain quality, and eating quality, were comparable between the NILs and 'Hitomebore'. 'Tou 1489' was especially similar to 'Hitomebore'.

Discussion

Evaluation of cold tolerance of the many NILs having chromosomal segments from 'Kuchum' delimited the QTL for cold tolerance of 'Kuchum', i.e., *qCT-4*, into a region of ca. 1.36 Mb between 9_1 and 10_13, which are located at 14.9 and 16.3 Mb, respectively, on the short arm of chromosome 4. As other QTLs on chromosome 4, *Ctb1, Ctb2* (Saito et al. 1995), *qCTB4-1*, and *qCTB4-2* (Xu et al. 2008) have been reported. Comparison with the 'Nipponbare' genome sequence (IRGSP build5: http://rgp.dna.affrc.go.jp/E/IRGSP/Build5/) indicates that *Ctb1* and *Ctb2* are present between the sites of RM17341 at 28.8 Mb and RM17645 at 35.1 Mb from the end of the short arm, *qCTB4-1* being between RM518 at 2.0 Mb and RM6700 at 2.8 Mb, and *qCTB4-2* being between RM7200 at 4.0 Mb and RM8213 at 4.4 Mb, suggesting that *qCT-4* is different from the four previously reported QTLs and is a novel QTL for cold tolerance at the booting stage.

The additive effect of *qCT-4* was from 4.99 to 6.51 in the analysis using BC_6F_4. Corresponding to this, the NILs having the 'Kuchum' allele at *qCT-4* as homozygotes showed an increase in seed fertility of ca. 10 % under cold stress. QTLs for cold tolerance at the booting stage so far reported have shown relatively small additive effects, generally less than 10 % of seed fertility (Takeuchi et al. 2001; Xu et al. 2008; Kuroki et al. 2011). The small additive effects of these QTLs and *qCT-4* suggest the importance of pyramiding of several QTLs for improvement of cold tolerance. *qLTB3* has been identified as a QTL for cold tolerance at the booting stage in the terminal region of the long arm of chromosome 3 in Chinese cultivar 'Lijiangxintuanheigu' by Shirasawa et al. (2012). The lines having the cold tolerance alleles at both *qCT-4* and *qLTB3* showed significantly higher seed fertilities than the lines having the *qCT-4* allele, but the difference of seed fertilities between the lines having both QTL alleles and the lines having only the *qLTB3* allele was not significant. Although the cause of this non-significant difference is unknown, it may be necessary to evaluate cold tolerance of these lines under a more severe condition lower than 18.5 °C to exhibit enhanced cold tolerance by QTL pyramiding.

Ctb1 and *Ctb2* have been identified on chromosome 4 using a BC_1F_1 population between 'Norin-PL8' having high cold tolerance derived from a cross between 'Silewah' and 'Kirara 397', which has relatively high cold tolerance. Replacement of the linked DNA marker XNpb267 from 'Kirara 397' type to 'Silewah' type has increased seed fertility by 8.4 % (Saito et al. 1995, 2001). *qCTB8* has been identified on the short arm of chromosome 8 by QTL analysis using F_2 progeny of a hybrid between 'Hokkai PL9' having high cold tolerance and 'Hokkai 287' (Kuroki et al. 2007). Cold-tolerant lines, e.g., 'Hokkai IL1' having cold tolerance alleles at *Ctb1* and *Ctb2*, 'Hokkai IL2' having cold tolerance alleles at *qCTB8*, and 'Hokkai IL3' having such alleles at both *Ctb1* and *qCTB8*, have been developed in Hokkaido, Japan. However, seed fertilities of NILs having *Ctb1, Ctb2*, or *qCTB8* with 'Hitomebore' background were not higher than that of 'Hitomebore' under the cold-stress condition. The effect of these QTLs may be dependent on genetic

backgrounds of the developed NILs or environmental factors of cold tolerance evaluation. 'Hitomebore' has high cold tolerance, which is derived from 'Koshihikari'. Three QTLs for cold tolerance at the booting stage in 'Koshihikari', i.e., *qCT-1, qCT-7*, and *qCT-11*, have been detected on chromosomes 1, 7, and 11 (Takeuchi et al. 2001). 'Hitomebore' has been reported to have 'Koshihikari' alleles at all these QTLs (Shirasawa et al. 2007). It may be inferred that *Ctb1, Ctb2*, and *qCTB8* do not exhibit a pyramiding effect with *qCT-1, qCT-7*, and *qCT-11*.

The QTL for cold tolerance of 'Kuchum' was delimited to a region of ca. 1.36 Mb between 9_1 and 10_13. In the published 'Nipponbare' genome sequence between these DNA markers, 57 genes have been annotated. Among them, 32 genes have been reported to be expressed in inflorescence and anthers (Sato et al. 2011). Since the functions of these genes are unknown and a genomic sequence of 'Kuchum' in this 1.36 Mb region has not been determined, it is difficult to speculate as to which candidate gene is responsible for cold tolerance. Further narrowing of the QTL region is required for identification of the cold tolerance gene in 'Kuchum'.

In the breeding to introduce QTLs from various genetic resources into a leading cultivar, care should be taken to avoid accompanying undesirable traits. Heading date of 'Norin-PL8' has been reported to be slightly late (Abe et al. 1989). In the NILs having the 'Kuchum' allele at *qCT-4* with the 'Hitomebore' genetic background developed in the present study, such a delay of flowering was not observed. Grain yield and quality were not influenced by incorporation of the cold tolerance *qCT-4* allele into 'Hitomebore'. In the delimited QTL region of ca. 1.36 Mb, genes for evidently undesirable traits were not found, and DNA markers were developed in this short region. These markers will be useful for developing a cold-tolerant cultivar using 'Kuchum' as a gene source of cold tolerance, and the NILs having the 1.36 Mb region from 'Kuchum' with the 'Hitomebore' genetic background developed in the present study are considered to be useful lines for developing a highly cold-tolerant leading cultivar. One of the NILs having the shortest genomic region of 'Kuchum', i.e., group C7, will be released as a candidate of a new cultivar or a new breeding material.

Author contribution statement TE designed the research, developed the NILs of 'Hitomebore' having qCT-4, and performed evaluation of cold tolerance and BC developed 'BI6-12' and performed QTL analysis. KW and KS controlled the deep water irrigation system for evaluation of cold tolerance. TY and TU developed NILs of 'Hitomebore' having Ctb1, Ctb2 and qCTB8. TA, AS, TM, and TY developed DNA markers near qCT-4 and performed DNA marker-assisted selection of NILs. TN wrote the manuscript. All authors read and approved the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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