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ORIGINAL PAPER

A quantitative trait locus for cold tolerance at the booting stage on rice chromosome 8

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Abstract A quantitative trait locus (QTL) for cold tolerance at the booting stage of a cold-tolerant rice breeding line, Hokkai-PL9, was analyzed. A total of 487 simple sequence repeat (SSR) markers distributed throughout the genome were used to survey for polymorphism between Hokkai-PL9 and a cold-sensitive breeding line, Hokkai287, and 54 markers were polymorphic. Single marker analysis revealed that markers on chromosome 8 are associated with cold tolerance. By interval mapping using an F_2 population between Hokkai-PL9 and Hokkai287, a QTL for cold tolerance was detected on the short arm of chromosome 8. The QTL explains 26.6% of the phenotypic variance, and its additive effect is 11.4%. Substitution mapping suggested that the QTL is located in a 193-kb interval between SSR markers RM5647 and PLA61. We tentatively designated the QTL as *qCTB8* (*q*uantitative trait locus for *c*old *t*olerance at the *b*ooting stage on chromosome *8*).

Introduction

Rice is a cold-sensitive plant that has its origin in tropical or sub-tropical areas. Spikelet fertility of rice decreases when rice plants are exposed to a low temperature, especially at the booting stage, due to the failure of microspore development under low temperature conditions (Satake and Hayase 1970). This sterile type of cold injury is a very serious problem both at high latitudes (e.g., Hokkaido, which is the northernmost island of Japan) and in uplands at low latitudes (e.g., Yunnan Province in China) (Dai et al. 2004) because it inevitably leads to yield reduction. For example, in 1993, rice production in Hokkaido was only 40% of the average crop.

To overcome the problem of damage caused by low temperature, rice breeders have been making efforts to develop more cold-tolerant cultivars. Two tropical *japonica*s, Silewah and Padi Labou Alumbis, were found to be cold-tolerant (International Rice Research Institute 1978). Their cold tolerance was introduced into Japanese breeding lines by backcross breeding, and cold-tolerant lines, Norin-PL8 and Norin-PL11, were developed in the late 1980s (Abe et al. 1989). Although Norin-PL8 and Norin-PL11 are more cold-tolerant than conventional cold-tolerant cultivars, they have unfavorable traits, including long duration to heading, long culm, and worse eating quality. Despite continuous efforts to improve their agronomical traits, no commercial cultivar has been established by utilizing cold tolerance of the two lines.

Genetic analysis has shown that cold tolerance is a complex trait and that many genes participate in it. Futsuhara and Toriyama (1966) revealed that cold tolerance of the temperate *japonica* cultivar Somewake is controlled by four or more loci and linked to morphological marker genes, d_2 on chromosome 1, *bc* on chromosome 3, *Pr* on chromosome 4, and *gh* and *nl* on chromosome 5. Nishimura (1995) showed by diallel analysis that two loci are involved in cold tolerance of the temperate *japonica* cultivar Hayayuki. However, no gene for cold tolerance had

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been mapped by these studies. Recently, many DNA markers have been developed, and quantitative trait locus (QTL) analysis using them has enabled identification of the chromosomal regions involved in cold tolerance. For example, Takeuchi et al. (2001) identified three QTLs for cold tolerance of the temperate *japonica* cultivar Koshihikari on chromosomes 1, 7, and 11. A study on chromosomal location of QTLs for cold tolerance at the booting stage in Norin-PL8 indicated that at least two regions on chromosomes 3 and 4 are responsible for cold tolerance (Saito et al. 1995). Moreover, it was revealed that the QTL for cold tolerance on chromosome 4 of Norin-PL8 consists of two closely linked genes, *Ctb1* and *Ctb2*, and *Ctb1* was delimited to 7 open reading frames (ORFs) in the 56-kb region (Saito et al. 2004). As a result of these QTL studies, many molecular markers for cold tolerance have been developed. The markers can be used for development of cold-tolerant cultivars through marker-assisted selection (MAS). However, Saito et al. (2001) observed that cold tolerance of near-isogenic lines (NILs) harboring both *Ctb1* and *Ctb2* is lower than that of Norin-PL8, indicating that cold tolerance of Norin-PL8 cannot be fully explained by *Ctb1* and *Ctb2*. Therefore, pyramiding of the QTLs for cold tolerance is necessary for achieving a high level of cold tolerance. Identification of a novel QTL for cold tolerance will contribute to effective pyramiding of QTLs for cold tolerance through increasing the number of target QTLs.

Here we report detection of the QTL for cold tolerance at the booting stage of chromosome 8. In the cold-tolerant breeding line Hokkai-PL9, which is descended from Norin-PL11, a QTL for cold tolerance was identified on chromosome 8, where no QTL has ever been reported, suggesting that a novel gene is involved in the cold tolerance of Hokkai-PL9. We developed new simple sequence repeat (SSR) markers on chromosome 8 and used them for interval mapping and substitution mapping. The region responsible for the QTL was narrowed down to a 193-kb interval.

Materials and methods

Plant materials

F_2, F_3, and F_7 progenies derived from a cross between Hokkai-PL9 and the cold-sensitive breeding line Hokkai287 were used for analysis of cold tolerance. A cold tolerant breeding line, Hokkai-PL9, is descended from Norin-PL11, which is developed by backcross breeding using two cold-tolerants, Padi Labou Alumbis (a Malaysian tropical *japonica*) and Hayayuki (a temperate *japonica* cultivar in Hokkaido) as donors of cold tolerance. Hokkai-PL9 has more improved agronomical traits than those of Norin-PL11.

Evaluation of cold tolerance

Cold tolerance was evaluated by the cool water irrigation method (Futsuhara and Toriyama 1964) in a paddy field (National Agricultural Research Center for Hokkaido Region, Sapporo, Japan). The field was irrigated with cool water controlled at 19.4°C from the primordial stage to the completion of heading (from 24 June to 25 August in 2004 and from 28 June to 8 September in 2005). The depth of water was about 20 cm. In 2004, the F_2 population for interval mapping and the F_7 lines for detection of the association were seeded on 16 April and transplanted on 1 June in the field. In 2005, the F_3 and F_7 populations for substitution mapping were seeded on 15 April and transplanted on 27 May in the field. Planting pattern of the F_2, F_3, and F_7 populations was one plant per plot, while that of the F_7 lines was five plants per plot with two replications. After ripening of the seeds, cold tolerance was evaluated on the basis of mean seed fertility of five panicles per individual or line.

DNA extraction and PCR amplification

DNA was extracted from leaves following the method described by Monna et al. (2002) with minor modifications. Simple sequence repeat (SSR) markers were amplified in 10 μl of mixture containing 10 mM Tris-Cl (pH 8.3), 1.5 mM $MgCl_2$, 0.001% gelatin, 0.1 mM of each deoxyribonucleotide, 0.2 μM of each primer, and 0.02 unit/μl AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA). The thermal cycles used were as follows: 1 cycle at 94°C for 4 min, followed by 45 cycles at 94°C for 1 min, 55 or 60°C for 1 min and 72°C for 2 min, and finally 1 cycle at 72°C for 7 min. PCR products were separated by electrophoreses in 4% (w/v) MetaPhor agarose (Cambrex, East Rutherford, NJ).

New SSR markers on the short arm of chromosome 8 were developed (Table 1). Identification of SSR and primer designing were performed using the online Simple Sequence Repeat Identification Tool (SSRIT; Temnykh et al. 2001; http://www.gramine.org/db/searches/ssrtool) and the online primer design tool Primer3 (Rozen and Skaletsky 2000; http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi), respectively, based on the complete sequence of chromosome 8 that were released from the International Rice Genome Sequencing Project (IRGSP, http://www.rgp.dna.affrc.go.jp/cgi-bin/statusdb/status.pl). Primers were then tested to detect for polymorphism between Hokkai-PL9 and Hokkai287. All of these primers were designed to have an annealing temperature of 55°C.

QTL mapping for cold tolerance

SSR markers covering 12 chromosomes of rice (Temnykh et al. 2001) were surveyed for polymorphism between

Table 1 New SSR markers on the telomeric half of the short arm of chromosome 8

Marker	Forward primer (5′–3′)	Reverse primer (3′–5′)	Motif	BAC/PAC clone (GenBank Accession No.)	
RM38-2[a]	GCGCCATTGATGACTAATTG	ATGGAAGAGGCAAGCAGAAG	(tc)18	OJ1613_G04	(AP003896)
PLA53	GATCCTTGCTGCATGTTCG	CCATGATGAAAAACCACAAAAA	(gca)8	P0473D02	(AP005542)
PLA41	ACACCCTACCAAACGAGCTG	GCCGCCATAGTATTCCTTCC	(ct)23	P0025F03	(AP004381)
PLA27	CGTAGCTATCTATAGCCACGAGAG	AATCCGATCAGGCCTTCCCTTTCG	(ag)18	P0665F09	(AP005506)
PLA46	GGCCTTGTGCTTGTTTAGGA	GGCTTGAGAGCGTTTGTAGG	(tcta)9	P0571B09	(AP005526)
PLA61	AAGTGGTGGCGAGACTGC	ATGAGAACCCCGTCACTGTC	(gcg)7	OJ1349_D05	(AP005467)
PLA62	TCTCCTCGACGATTTATGAACA	ACCAAGAGCCACGTCGTAAG	(caga)8	OJ1349_D05	(AP005467)
PLA19	TTCGATATGCAAGTGATGATGATG	TACTCTCCTCCAAGAAAACAAGCA	(gt)15	P0672D01	(AP004635)
MR902B	AAAAGCATATAGAGGCACCGGGTA	CCGTCAGGTTTTCATCTGATGAAC	(ag)16	P0455A11	(AP004692)

[a] RM38-2 has the same target as that of RM38, but sequences of primers are revised for more efficient amplification of the PCR product

Hokkai-PL9 and Hokkai287. The 59 F_7 lines were genotyped using the polymorphic SSR markers, and also their cold tolerance was evaluated. For each polymorphic marker, mean seed fertility of F_7 lines with homozygous Hokkai-PL9 allele (HkPL9 group) was compared to that of F_7 lines with homozygous Hokkai287 allele (Hk287 group). The experimentwise threshold value was estimated by 1,000 permutations following the method described in Churchill and Doerge (1994).

Detection of a QTL for cold tolerance was carried out using the F_2 population with 288 individuals. Calculation of genetic distances and interval mapping were performed using the computer program MAPL97 (Ukai et al. 1991, 1995).

For substitution mapping (Paterson et al. 1990) of the QTL, a single F_6 plant between Hokkai-PL9 and Hokkai287 that is heterozygous for the QTL region was selected. Its selfing F_7 population (P1-1) was genotyped. Furthermore, five F_2 recombinants within the QTL region were selected, and their selfing F_3 populations (P2-1, 2, 3, 4, and 5) were genotyped. In each population, individuals were divided into the three genotypic groups (HkPL9 group, Hk287 group and Heterozygous group). The mean seed fertilities in the HkPL9 and Hk287 groups were compared by the Wilcoxon rank sum test using the statistical program R ver 2.2.1 (R Development Core Team 2005).

Results

Single marker analysis for cold tolerance

A total of 487 SSR markers (Temnykh et al. 2001) distributed throughout the genome were used to survey the polymorphism between Hokkai-PL9 and Hokkai287, and 54 markers were polymorphic (Fig. 1). The percentage of polymorphism ranged from 2.4 (chromosome 5) to 31.8% (chromosome 11), and totally was 11.1% over 12 chromosomes. The polymorphism frequency was much lower than that of a *japonica* × *indica* cross determined by using SSR markers (Andaya and Mackill 2003) and was as low as that of a *japonica* × *japonica* cross using restriction fragment length polymorphisms (RFLP) and random amplified polymorphic DNA (RAPD) markers (Takeuchi et al. 2001).

The association between cold tolerance and the polymorphic SSR markers was tested using the 59 F_7 lines between Hokkai-PL9 and Hokkai287. A continuous distribution was observed in the F_7 lines, indicating quantitative inheritance of cold tolerance (Fig. 2). Cold tolerance of the F_7 lines with Hokkai-PL9 allele (HkPL9 group) was compared with that of the F_7 lines with Hokkai287 allele (Hk287 group) for the 54 polymorphic markers. Highest *Z*-value was observed in RM38 on chromosome 8, though its *Z*-value was not higher than the experimentwise threshold at 5% level ($Z = 3.19$). To verify the association between cold tolerance and chromosome 8, we found ten more polymorphic markers on the short arm of chromosome 8 from 48 SSR markers developed by McCouch et al. (2002) and used them for the single marker analysis. Statistically significant differences between genotypic groups were found in four markers (Table 2). It is noteworthy that the markers on chromosome 8 are significantly associated with cold tolerance, since no QTL for cold tolerance at the booting stage has ever been reported on chromosome 8.

Interval mapping for cold tolerance

For interval mapping of the QTL for cold tolerance on chromosome 8, we developed eight more polymorphic markers based on the IRGSP genome sequence (Table 1). A total of 288 plants of the F_2 population were genotyped using the 19 SSR markers and evaluated for cold tolerance (Fig. 3). A linkage map with 19 markers covering the telomeric half of the short arm of chromosome 8 (corresponding to 0–4.7 Mb in the IRGSP genome sequence) was

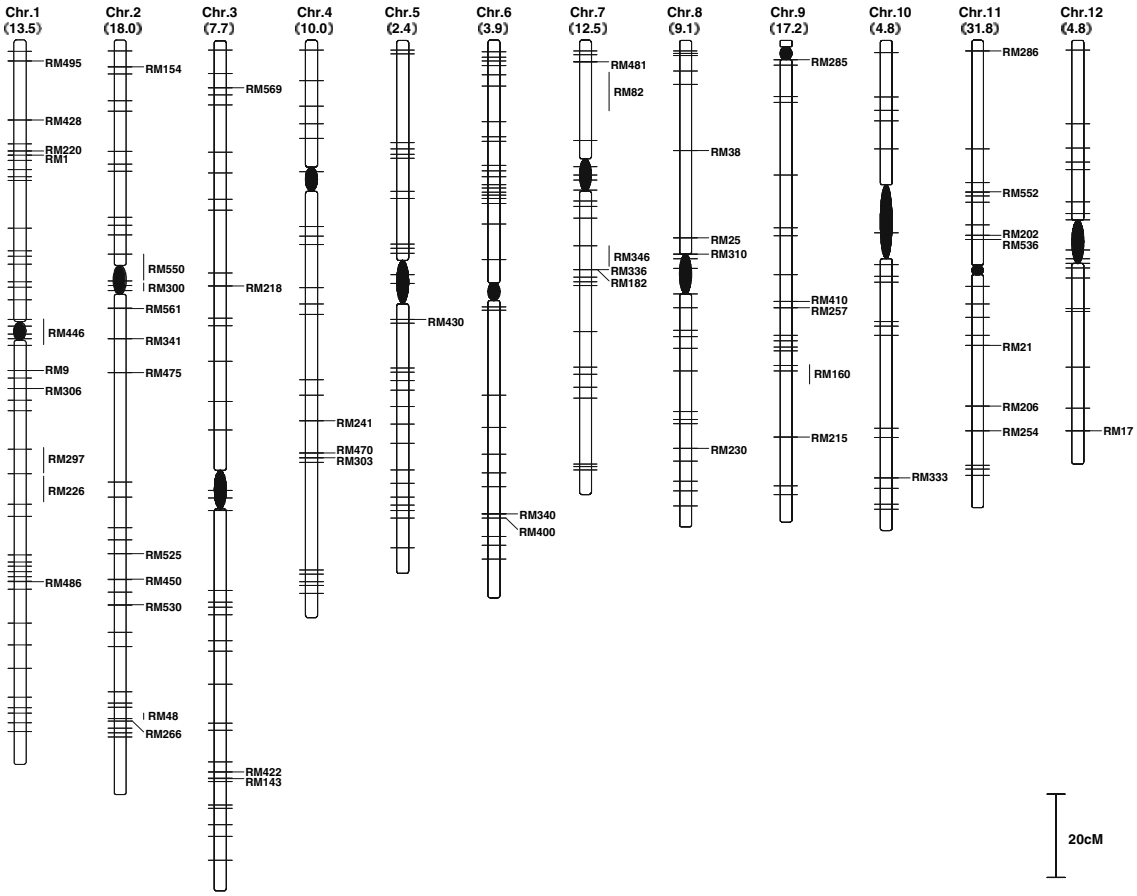

Fig. 1 Chromosomal positions of the polymorphic markers between Hokkai-PL9 and Hokkai287. The polymorphic markers are indicated by marker names, while *vertical lines* represent the probable intervals of the markers with low LOD score in map construction (Temnykh et al. 2001). The *horizontal lines* represent the positions of SSR markers used in the survey for polymorphic markers. Approximate positions of centromeres are shown by *solid ellipses*. The percentage of polymorphic markers on each chromosome is indicated in *parenthesis*

constructed. The log-likelihood (LOD) plot for cold tolerance reached the maximum score of 10.6 in the proximity of RM6670 (Fig. 4). Phenotypic variance explained (PVE) and additive effect (AE) of the QTL were 26.6 and 11.4%, respectively. The QTL with a comparatively large effect was tentatively designated *qCTB8* (*q*uantitative trait locus for *c*old *t*olerance at the *b*ooting stage on chromosome *8*).

Mean seed fertility of F_2 plants between Hokkai-PL9 and Hokkai287 under normal temperature condition was 88.9%, and the variance of seed fertility under normal temperature was significantly smaller than that under low temperature (37.7 vs. 245.9, $P < 0.001$ by F test). The result suggests that the seed fertility variation under low temperature condition is largely caused by the genetic variation of cold tolerance in the population and therefore *qCTB8* must be associated with cold tolerance rather than sterility.

Substitution mapping of *qCTB8*

An F_7 population derived from an F_6 plant that is heterozygous between PLA46 and RM5428 (Fig. 5) was segregated in cold tolerance, and cold tolerance of the HkPL9 group was significantly higher than that of the Hk287 group (Fig. 6). The F_6 plant had other heterozygous segments on chromosome 2 (including RM550, RM300, RM561, RM341, and RM475) and chromosome 6 (including RM340 and RM400), but the association between these seven markers and cold tolerance in the F_7 population was not significant (data not shown). Therefore, segregation of cold tolerance in the F_7 population was considered to be largely accounted for by the target loci on chromosome 8. The phenotypic difference between genotypic groups was 18%, coinciding with AE of *qCTB8* estimated by interval mapping (11.4%). This result indicates that *qCTB8* is located between PLA46 and RM5428.

To narrow down the *qCTB8* region, we developed five F_3 populations (P2-1, 2, 3, 4, and 5) from F_2 recombinants in the interval from RM38-2 to RM3572, and levels of cold tolerance were compared between genotypic groups in each population (Fig. 5). Significant differences in cold tolerance between genotypic groups were found in P2-1 and P2-2 (21.0% in P2-1 and 14.3% in P2-2), whereas the difference

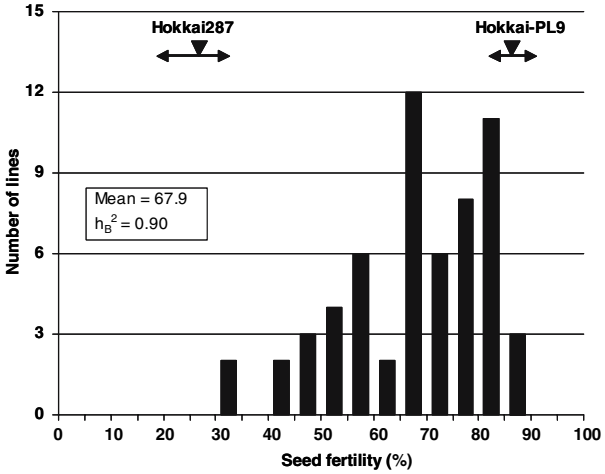

Fig. 2 Frequency distribution of cold tolerance in F_7 lines. Cold tolerance was evaluated as seed fertility after cool-water treatment. The ranges and means of the parents are represented by *arrows* and *solid triangles*, respectively

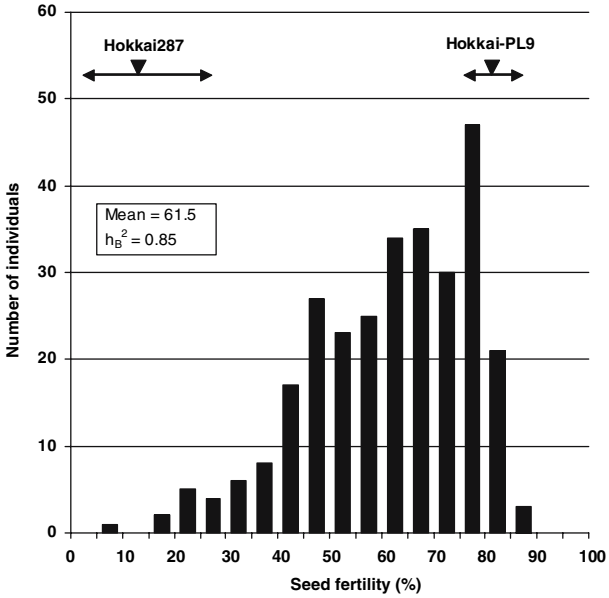

Fig. 3 Frequency distribution of cold tolerance in F_2 population. Cold tolerance was evaluated as seed fertility after cool-water treatment. The ranges and means of the parents are represented by *arrows* and *solid triangles*, respectively

Table 2 Association between cold tolerance and SSR markers on chromosome 8

Marker	Position[a] (Mb)	Mean seed fertility (Number of lines)		*Z*-value[b]
		HkPL9 group	Hk287 group	
RM5911	0.1	72.9 (30)	63.3 (27)	2.5092
RM6356	1.6	72.2 (30)	64.1 (27)	2.1416
RM38	2.1	72.9 (31)	62.2 (27)	2.8917
RM5647	2.9	74.2 (28)	61.4 (29)	3.5437[c]
RM6670	3.0	75.1 (27)	61.0 (30)	3.8677[c]
RM3819	3.0	75.1 (27)	61.0 (30)	3.8677[c]
RM5434	3.1	75.1 (27)	61.0 (30)	3.8677[c]
RM5428	3.2	74.1 (27)	61.9 (30)	3.1645
RM3572	3.9	71.3 (24)	65.6 (35)	1.3579
RM6999	4.0	71.3 (24)	65.6 (35)	1.3579
RM5556	4.6	69.6 (26)	66.6 (33)	0.6107

[a] Chromosomal positions of the markers are indicated based on the IRGSP genome sequence

[b] The significance of the difference between mean seed fertility of genotypic groups was tested by the Wilcoxon rank sum test

[c] Significant at 5% level by the permutation test (Churchill and Doerge 1994)

in P2-5 was not significant, suggesting that *qCTB8* is located distal from PLA61. Differences in cold tolerance between genotypic groups were also found in P2-3 and P2-4 (10.5% in P2-3 and 16.2% in P2-4), suggesting that *qCTB8* was located proximal from RM5647.

According to these results, it is likely that *qCTB8* is located on the 1.7 cM interval between RM5647 and PLA61 (Fig. 5). The region corresponds to 193 kb on the IRGSP genome sequence and is covered by the P1-derived artificial chromosome (PAC) clone P0443G08 (GenBank accession No. AP004461) and bacterial artificial chromosome (BAC) clone OJ1349_D05 (GenBank accession No. AP005467).

Discussion

We mapped *qCTB8* to a 1.7 cM interval by substitution mapping. The 1.7 cM *qCTB8* candidate region corresponds to 193 kb on the IRGSP genome sequence, and approximately 30 ORFs are predicted in it. One of the ORFs encoded monodehydroascorbate reductase (MDAR), which was reported to be up-regulated in the rice anther by cold treatment at the young microspore stage, which is the most sensitive stage to cold weather (Imin et al. 2006). The ORF for MDAR was located in the vicinity of RM6670 between RM5647 and RM6670, coinciding with the observation of the LOD peak in the interval mapping. Therefore, MDAR is thought to be one of the promising candidate genes for *qCTB8*.

We employed hundreds of SSR markers developed by Temnykh et al. (2001) to survey polymorphism between Hokkai-PL9 and Hokkai287. However, they are not sufficient for interval mapping of the QTL because the percentage of polymorphism was low in the materials we used. To facilitate the mapping of *qCTB8*, new SSR markers were developed in the *qCTB8* region using the publicly available rice (cv. Nipponbare) genome sequence. Some of the thousands of SSR markers that have been developed for rice (McCouch et al. 2002) were also used in the interval

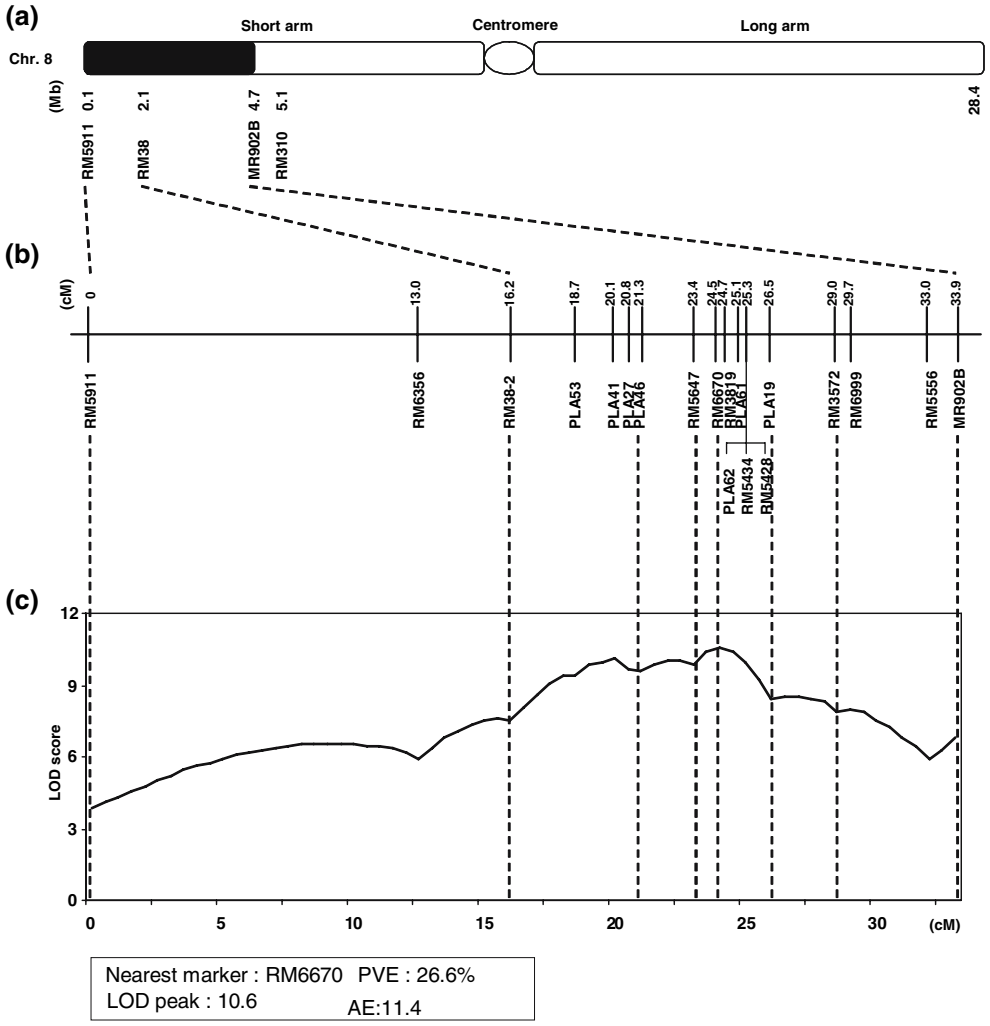

Fig. 4 Interval mapping for cold tolerance. **a** Chromosomal positions of the markers based on the IRGSP genome sequence. **b** Genetic linkage map of the markers. **c** LOD plot for cold tolerance covering the telomeric half of the short arm of chromosome 8

mapping. Mapping of *qCTB8* would not be successful without the newly developed markers, because *qCTB8* was mapped in the region without the previously reported SSR markers. The rice genome sequence provides us with a large number of markers (e.g., ca. 19000 SSR markers by IRGSP 2005) that enable high-resolution mapping in a low-polymorphic population (e.g., the population derived from the *japonica* × *japonica* cross in this study) through increasing the chance for detection of polymorphism.

F_1 plants between Norin-PL11, which is the donor of *qCTB8*, and Hoshinoyume, which is a cold-tolerant commercial cultivar in Hokkaido, were shown to be more cold-tolerant than the parents (unpublished). That study suggests that *qCTB8* is useful for improving cold tolerance of Hoshinoyume. Therefore, it is likely that *qCTB8* can contribute to cold tolerance breeding in Hokkaido. However, no commercial cultivar has been bred from progenies of Norin-PL11. Saito et al. (2001) reported that a gene for cold tolerance, *Ctb2*, of Norin-PL8 could be linked with late heading, which is unfavorable in the region with short summer such as Hokkaido. One possible reason why Norin-PL11 could not been utilized as a parent of a commercial cultivar is that cold tolerance of Norin-PL11 is linked with unfavorable traits as well as in the case of Norin-PL8. In conventional cold tolerance breeding, population size is relatively small because the field for cold tolerance evaluation is limited. Large-scale and precise screening for cold tolerance by MAS might be effective for removing linkage between cold tolerance and unfavorable traits. The cold-sensitive material in this study, Hokkai287, is a somaclonal variant of Kirara397, which is a leading cultivar in Hokkaido. Since Hokkai287 has good eating quality, Hokkai287 can be a commercial cultivar if its cold tolerance is improved. The *qCTB8* flanking SSR markers developed here can easily distinguish the Hokkai-PL9 genotype from the Hokkai287 and Hoshinoyume genotypes by PCR and

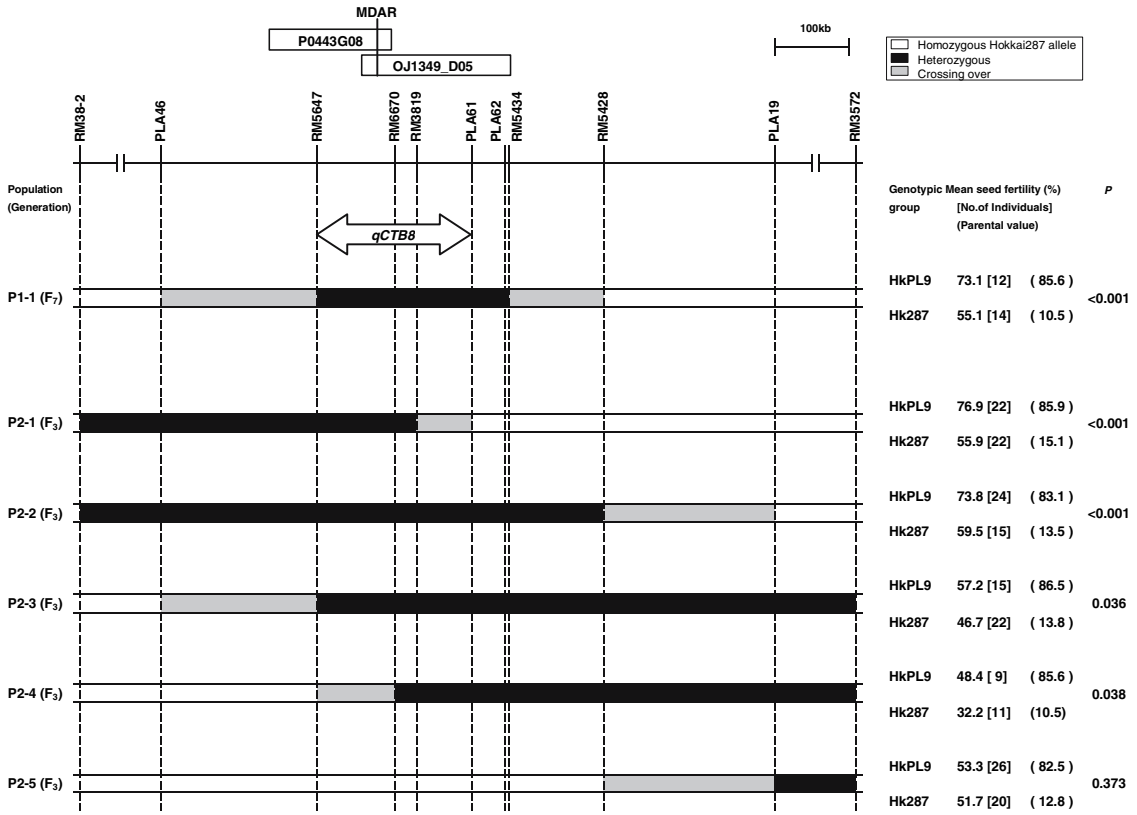

Fig. 5 Substitution mapping of *qCTB8*. Graphical genotypes of the F_3 and F_7 substitution lines are illustrated. Positions of markers are based on the IRGSP genome sequence. *Solid* and *open boxes* represent heterozygous allele and homozygous Hokkai287 allele, respectively, while the regions of potential recombination are shown as *shaded boxes*. Mean seed fertility of the HkPL9 group was compared with that of the Hk287 group by the Wilcoxon rank sum test

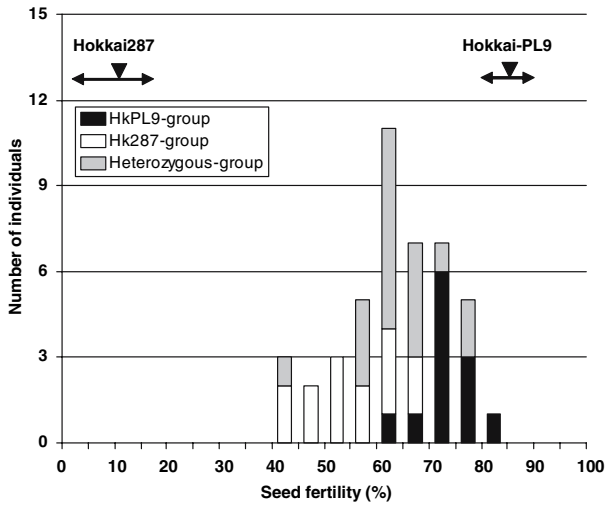

Fig. 6 Frequency distribution of cold tolerance in the P1-1 population. Frequency distribution of cold tolerance is shown classified by genotypic groups. Cold tolerance was evaluated as seed fertility after cool-water treatment. The ranges and means of the parents are represented by *arrows* and *solid triangles*, respectively

agarose gel electrophoresis. The markers are readily applicable for breeding of improved cold-tolerant cultivars by introduction of *qCTB8* into Hokkai287 and Hoshinoyume.

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