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ORIGINAL PAPER

Fine mapping a QTL *qCTB7* for cold tolerance at the booting stage on rice chromosome 7 using a near-isogenic line

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Abstract Low temperature at the booting stage is a serious abiotic stress in rice, and cold tolerance is a complex trait controlled by many quantitative trait loci (QTL). A QTL for cold tolerance at the booting stage in cold-tolerant near-isogenic rice line ZL1929-4 was analyzed. A total of 647 simple sequence repeat (SSR) markers distributed across 12 chromosomes were used to survey for polymorphisms between ZL1929-4 and the cold-sensitive japonica cultivar Towada, and nine were polymorphic. Single marker analysis revealed that markers on chromosome 7 were associated with cold tolerance. By interval mapping using an F_2 population from ZL1929-4 × Towada, a QTL for cold tolerance was detected on the long arm of chromosome 7. The QTL explained 9 and 21% of the phenotypic variances in the F_2 and F_3 generations, respectively. Recombinant plants were screened for two flanking markers, RM182 and RM1132, in an F_2 population with 2,810 plants. Two-step substitution mapping suggested that the QTL was located in a 92-kb interval between markers RI02905 and RM21862. This interval was present in BAC clone AP003804. We designated the QTL as *qCTB7* (quantitative trait locus for cold tolerance at the booting stage on chromosome 7), and identified 12 putative candidate genes.

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Introduction

Rice (*Oryza sativa* L.) is one of the three most important food crops in the world. Cold stress is a common problem of rice cultivation, and is a crucial factor affecting global food production. About 30.7 million ha of rice is grown in China and extend over a wide area ranging from 53°27′N to 18°90′N. Almost the entire area can be affected by cold injury resulting from low temperatures, and annual losses are 3–5 million tonnes of rice grain (Li and Guo 1993). Rice is a cold-sensitive plant that has its origin in tropical or sub-tropical areas. Spikelet fertility of rice decreases because the rapidly growing booting and reproductive tissues are very sensitive to low temperatures, especially at the stages ranging from pre-meiotic mother cells to microspores and pollen (Nishiyama 1982; Dai et al. 2002). Low temperatures at the booting stage cause degeneration of young microspores, and hypertrophy and dissolution of tapetal cells, interrupting or decreasing the supply of nutrients from the anther walls to the pollens (Hayase et al. 1969; Nishiyama 1976; Satake 1989). Consequently, it is imperative to screen for cold tolerance at this growth stage and to understand the genetic and molecular basis of cold tolerance.

Genetic analysis has shown that cold tolerance is a very complex trait involving many genes. Futsuhara and Toriyama (1966) showed that cold tolerance in the temperate

japonica cultivar, Somewake, was controlled by four or more loci and linked to morphological marker genes, d2 (dwarf) on chromosome 1, bc (brittle culm) on chromosome 3, Pr (purple hull) on chromosome 4, and gh (glabrous hull) and nl (neck leaf) on chromosome 5. Nishiyama (1995) showed that two loci were involved in cold tolerance of the temperate japonica cv. Hayayuki. Our understanding of the genetic basis of complex quantitative traits has been greatly enhanced by the recent development of molecular markers. This has enabled the identification and mapping on all rice chromosomes of many QTLs associated with cold tolerance at the booting stage over the last decade. For example, Li et al. (1997) identified two QTLs on chromosome 1 and one QTL on chromosome 12, using a BC_1F_1 population. Takeuchi et al. (2001) identified three QTLs on chromosomes 1, 7, and 11 using a doubled-haploid (DH) population from a cross between tolerant-temperate and sensitive-temperate japonica genotypes. Saito et al. (2001) identified closely linked QTLs *Ctb1* and *Ctb2* on chromosome 4; these were related to cold tolerance and anther length. *Ctb1* was subsequently fine-mapped and putative candidate genes were identified (Saito et al. 2004). Liu et al. (2003) identified three QTLs on chromosomes 1, 6, and 7 in cold-tolerant wild rice introgression lines. Andaya and Mackill (2003) identified nine QTLs on chromosomes 1, 2, 3, 5, 6, 7, 9, and 12 using a set of recombinant inbred lines derived from a cross between temperate japonica cv. M-202 and tropical indica cv. IR50. Dai et al. (2004) mapped nine QTLs using an F_2 population consisting of 250 individuals and four were validated using the F_3 population. Kuroki et al. (2007) detected a QTL on the short arm of chromosome 8 and mapped it to a 193-kb interval. Although QTLs for cold tolerance at the booting stage have been mapped on all 12 chromosomes, only one was narrowed down to a 100 kb region (Saito et al. 2004) and none has been cloned.

Near-isogenic lines are excellent materials for fine mapping and map-based cloning of the individual genetic components of complex quantitative traits. Some QTLs in rice were cloned by map-based cloning using NIL materials, such as *Gn1a* for grain number, *qGY2*-1 for grain yield, *qUVR*-10 for ultraviolet-B (UVB) resistance, and *qSH1* for seed shattering (Ashikari et al. 2005; He et al. 2006; Ueda et al. 2005; Konishi et al. 2006, respectively).

Kunmingxiaobaigu (KMXBG), cultivated in Kunming, Yunnan Province, for more than 300 years (Cheng 1993), is one of the most low temperature-tolerant landraces at all growth stages, whereas Towada is one of the least tolerant varieties identified during collaborative studies between Japan and China (Horisue et al. 1988). We developed a set of cold-tolerant NILs by backcrossing KMXBG as donor to Towada, and selecting cold-tolerant individuals in each generation of backcrossing. In our previous study, one of the cold-tolerant NILs was selected as a parent to construct a segregating population, and eight QTLs were mapped on chromosomes 1, 4, 5, 10, and 11 (Xu et al. 2008). Dai et al. (2004) mapped QTL *qRCT7* with major effect (20.6%) on the long arm of chromosome 7 using F_2 and F_3 populations generated from KMXBG × Towada. This QTL was not detected in our previous study because the segment containing *qRCT7* was not present in the particular cold-tolerant NIL parent that was used (Xu et al. 2008).

In the present work, we studied another cold-tolerant NIL, ZL1929-4, in which we detected and mapped a QTL for cold tolerance with major effect at the booting stage on the long arm of chromosome 7. By fine mapping and cloning of cold-tolerance genes, we are trying to establish functional roles for genes involved in cold tolerance and ultimately to use those genes in breeding modern cold-tolerant rice varieties.

Materials and methods

Plant materials

ZL1929-4 (hereafter, ZL1929) is a BC_6F_4 cold-tolerant NIL developed by backcrossing KMXBG as donor to the cold-sensitive Japanese commercial *japonica* cv. Towada, and selecting cold-tolerant individuals in each generation of backcrossing. Two F_2 populations were used in fine-mapping. One F_2 population consisting of 204 plants, derived from an F_1 plant of ZL1929 × Towada, was grown at the experimental farm, Yunnan Academy of Agricultural Sciences, Kunming (altitude 1,916 m), in the summer of 2007. The second and larger F_2 population comprising 2,606 plants derived from the same cross was grown at the China Agricultural University Experiment Station at Sanya (18°N, 109°E), Hainan, in the winter of 2007. All F_3 families derived from the smaller population, and F_3 families derived from all recombinants identified in the larger F_2 population from Sanya, along with the parents, were grown at Kunming, in the summer of 2008.

Evaluation of cold tolerance

Cold tolerance of ZL1929 and Towada

Plants of ZL1929 and Towada were individually harvested after treatments at the booting stage in three different environments, viz. Kunming (low temperature), Beijing (low temperature), and Beijing (normal temperature), respectively. Air temperature data were obtained from public records and water temperatures were measured daily by us. Starting from the differentiation of young panicles to the milky mature stage, cold treatment in Kunming was

applied by naturally low atmospheric temperatures of 18–21°C, and cold water at about 19.5°C provided to a depth of 30 cm (Xu et al. 2008). ZL1929 and Towada were grown at the China Agricultural University Experiment Station in Beijing, where the daily mean atmospheric temperatures were 25–31°C during the booting stage. The cold treatment in Beijing was as follows: eight rice seedlings each of ZL1929 and Towada were transplanted in plastic pots. Extra tillers were removed from each plant in the pot, leaving four tillers of each plant to avoid overcrowding and to promote better growth. Five healthy plants per pot showing uniform development stage were selected and one tiller each from the five plants was tagged. At the microsporogenesis stage the plants were moved to a controlled environment incubator maintained at 15 ± 0.5°C for 7 days. Microsporogenesis was estimated by the distance between the auricles of the flag and penultimate leaves. An interval of −4 (flag leaf auricle below the penultimate leaf auricle) to +2 cm (flag leaf auricle above the penultimate leaf auricle) was the indicative of the correct stage (Satake and Hayase 1970). As much as 13 cold tolerance-related traits (Zeng et al. 2006), including plant height, panicle length, inter-node length below the panicle, panicle neck length, flag leaf length, flag leaf width, penultimate leaf length, penultimate leaf width, first elongating inter-node length, full grains per panicle per spike, blighted grains per panicle, total grains per panicle and mean spikelet fertility, were evaluated. For each trait, the mean phenotypic values of eight plants were compared between ZL1929 and Towada. Spikelets of KMXBG, ZL1929, and Towada were collected one day before anthesis in Kunming. Pollen grains taken from anthers were stained with 1% KI–I_2 solution and examined by light microscopy (Olympus IX71). Pollens with a round shape and dark blue color were considered to be fertile; otherwise they were recorded as sterile.

Cold tolerance of populations

Cold tolerances of the F_2 population and F_3 families were evaluated at Kunming in the summers of 2007 and 2008, respectively. Thirty-day-old seedlings were transplanted in normally irrigated plots with 20 plants in a single row with 12.5 and 25-cm spacing between plants and rows, respectively. In 2007, 204 random F_2 plants and parental control were planted in each plot. In 2008, each of the 204 F_3 families and parental controls was represented by a row of 15–20 plants. The recombinant F_3 families selected from the larger F_2 population were planted in plots of 30–40 plants. All plants flowered within 7 days. Cold treatments of all F_2 plants, F_3 families, and parents were applied as described above. The Kunming environment provided sufficiently low temperatures for discrimination of cold-tolerant and cold-sensitive parental genotypes. Cold tolerances of F_2 plants were evaluated by the spikelet fertilities of the main panicles at the seed ripening stage, and F_3 families were evaluated as mean spikelet fertilities of the main panicles from 10 to 15 plants in each line. Most of the recombinant F_2 plants were heterozygous, but homozygous recombinant individuals were selected within the respective F_3 families. Cold tolerances of recombinants were evaluated as mean spikelet fertilities of the main panicles from 8 to 10 selected homozygous plants in each family.

DNA extraction and molecular marker analysis

DNA was extracted from leaves following the CTAB method described by Rogers and Bendich (1988) with minor modifications. A total of 647 SSR markers evenly distributed over all 12 chromosomes (mean marker interval 2.4 cM, entire genome 1,526.8 cM) (International Rice Genome Sequencing Project, 2005) were used to examine polymorphisms among KMXBG, the NIL (ZL1929), and Towada parents (Temnykh et al. 2000; McCouch et al. 1988, 2002). When PCR products had the same band size for KMXBG and ZL1929, but were different from Towada, it was assumed that the SSR marker was potentially linked to a cold-tolerance locus. These markers were validated in a one-way ANOVA. Additional SSR markers located near the polymorphic SSR markers were chosen from the Gramene database (http://www.gramene.org) and used to detect further polymorphisms between the parents. All SSR markers showing polymorphisms were used to genotype the entire 204 random F_2 population. Recombinant plants in the large F_2 population were identified by using the two markers RM182 and RM1132 flanking the putative QTL. Homozygous recombinants were detected among individuals in each recombinant F_3 family using the same markers. Molecular markers within the flanked region were screened to detect polymorphisms that would permit further genotyping of the recombinant lines. The markers included SSR (International Rice Genome Sequencing Project 2005) and one intron length polymorphism marker (Wang et al. 2005). The PCR and electrophoretic methods were described by Xu et al. (2008).

Data analysis

Linkage map construction was performed using Mapmaker/Exp 3.0 (Lincoln et al. 1992), and the Kosambi function was used to convert recombination values to genetic distances. QTL analysis was carried out by interval analysis with Map Manager QTXb20 (Manly et al. 2001). A LOD score of 3.0 was used as threshold to declare the presence of a putative QTL. The percentage variation explained (general contribution) by each QTL, and the additive and dominance effects were estimated.

The mean values of cold-related trait differences between ZL1929 and Towada were compared by *T* tests using the statistical program SPSS for Windows, version 11.0 (SPSS Inc. 2002). In each recombinant family, the mean phenotypic value for spikelet fertilities of the main panicles for homozygous recombinant individuals were compared with those of the ZL1929 and Towada controls using the SAS statistical software package (SAS Institute 2000). Recombinant lines were grouped based on the genotypes of the homozygous recombinants they contained and the mean phenotypic value of spikelet fertilities of the main panicles. In fine mapping of the position of *qCTB7*, a recurrent substitution mapping strategy as described by Paterson et al. (1990) was used.

Results

Characterization of the ZL1929 NIL

Phenotypic evaluations and comparisons of cold tolerance-related traits for ZL1929 and Towada using data collected in Kunming and Beijing are shown in Table 1. Under normal temperature conditions in Beijing, there were no significant differences in all investigated traits between ZL1929 and Towada. Under the two cold treatment regimes, there were no significant differences except for four grain-related traits, viz. full grains per panicle, blighted grains per panicle, total grains per panicle, and mean spikelet fertility (Table 1; Fig. 1). Pollen fertility for Towada (25%) was much lower than that for ZL1929 (80%) and KMXBG (nearly 100%) in Kunming (Fig. 1). These results indicated that ZL1929 was very similar to Towada except for the traits associated with spikelet fertility of main panicle. The effects of other cold-related traits on spikelet fertility were eliminated in the genetic background of near-isogenic line ZL1929. Thus, spikelet fertility was an appropriate index to evaluate cold tolerance at the booting stage in the ZL1929 × Towada segregating population.

One-way analysis of variance (ANOVA) and interval mapping for cold tolerance

A total of 183 (28.3%) out of the 647 markers were polymorphic between KMXBG and Towada. The percentage of polymorphism ranged from 23.2 (chromosome 5) to 32.3% (chromosome 11). The polymorphism frequency was much lower than that of a *japonica* × *indica* cross studied by Andaya and Mackill (2003). Nine SSR markers, viz. RM81A on chromosome 1, RM1221 on chromosome 3, RM3608, RM7237, and RM6432 on chromosome 7, RM331 on chromosome 8, RM409 and RM215 on chromosome 9, and RM552 on chromosome 11, showing clear polymorphisms between ZL1929 and Towada, were used to screen the 204 random F_2 plants from ZL1929 × Towada.

One-way ANOVA of the relationship between the SSR markers and spikelet fertility showed that RM3608, RM7237, and RM6432 on long arm of chromosome 7 were significantly associated with spikelet fertility ($P < 0.005$), whereas the other six markers were not significantly

Table 1 Comparisons of cold tolerance-related traits between ZL1929 and Towada under different treatment conditions in Kunming and Beijing at the booting stage

Trait	Kunming (LT)		Beijing (LT)		Beijing (NT)	
	Towada	ZL1929	Towada	ZL1929	Towada	ZL1929
Plant height (cm)	70.3	72.6	92.5	89.2	104	102.2
Panicle length (cm)	16.5	16.9	16.7	16.2	18.4	21.3
Inter-node length below the panicle(cm)	27.2	29.0	30.9	27.6	35.3	36.1
Panicle neck length (cm)	4.8	5.7	2.3	2.5	8.2	7.9
Flag leaf length (cm)	23.6	26.6	29.2	30.3	27.9	33.2
Flag leaf width (cm)	1.47	1.38	1.40	1.50	1.40	1.62
Penultimate leaf length (cm)	29.7	33.6	48.7	48.2	39.2	47.7
Penultimate leaf width (cm)	1.30	1.24	1.25	1.33	1.24	1.52
First elongating inter-node length (cm)	2.57	2.88	2.75	3.03	4.13	4.58
Full grains per panicle per spike	30.7	60.2*	16.0	53.0*	126.8	135.8
Blighted grains per panicle	74.7	35.8*	53.5	40.7*	8.2	14.8
Total grains per panicle	105.3	96.0	69.5	93.7*	135	150.6
Mean spikelet fertility (%)	30.4	60.7*	22.5	57.4*	93.9	90.2

LT low temperature, *NT* normal temperature

* Trait means of ZL1929 and Towada in the same treatment are significantly different ($P < 0.01$)

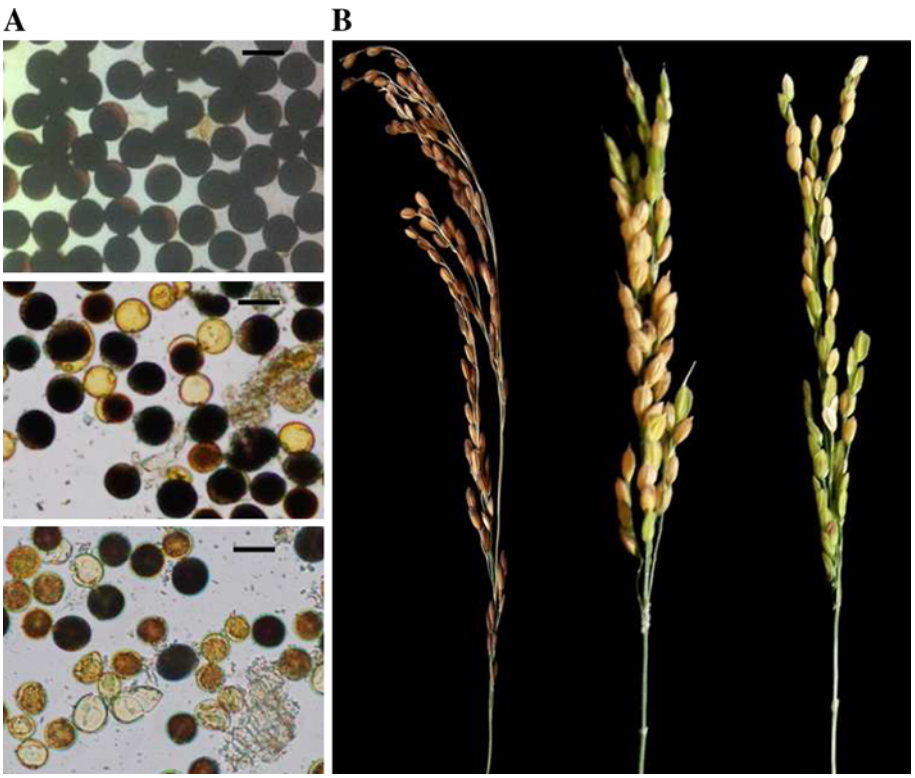

Fig. 1 Fertilities of KMXBG, ZL1929, and Towada under cold treatment condition at the booting stage in Kunming. **a** Pollen fertilities of KMXBG (*upper panel*), ZL1929 (*middle panel*), and Towada (*lower panel*). *Scale bars* 50 μm. **b** Spikelet fertilities of KMXBG (*left panel*), ZL1929 (*middle panel*), and Towada (*right panel*)

associated with it. Interactions among the nine polymorphic markers showed no significant association with cold tolerance in the F_2 population. The results thus indicated that the three chromosome 7 markers were linked to spikelet fertility of the main panicle under cold stress. Five additional polymorphic SSR markers near the linked markers were used to genotype the 204 F_2 individuals (Fig. 2a). QTL analysis of mean spikelet fertility of the main panicles revealed a significant peak between markers RM182 and RM1132 with a LRS score of 35.8 (LOD = 7.74), and explaining 9% of the phenotypic variance. KMXBG-derived allele contributed an increasing effect on mean spikelet fertility of the main panicles. We designated the locus *qCTB7*. QTL analysis of the F_3 family data further confirmed the unique QTL peak between markers RM182 and RM1132 with a LRS score of 51.5 (LOD = 11.2), the QTL accounting for 21% of the phenotypic variance (Table 2). The above results demonstrated that *qCTB7* was responsible for mean spikelet fertility of the main panicles under cold treatment in ZL1929. It appeared that *qCTB7* was a stable locus and amenable to fine mapping and eventual cloning.

Fine mapping of *qCTB7*

To further refine the position of *qCTB7*, the larger F_2 population was subjected to molecular analysis. Among the 2,810 F_2 plants (plus the previous 204 plants), a total of 63 recombinants between markers RM182 and RM1132 were detected (Fig. 2b). These recombinants were genotyped with three SSR markers, RM21862, RM21868, and RM21870, and one intron length polymorphism marker identified within the interval RM182–RM1132 (Fig. 2c, d). In each of the 63 recombinant families, homozygous recombinant individuals identified with the appropriate markers were evaluated for mean spikelet fertilities of the main panicles and the mean phenotypic value of each trait for each recombinant F_3 family was compared to those of ZL1929 and Towada. As much as 12 genotypic groups were identified (Fig. 2c). Group A1 contained 17 recombinants between RM182 and RM6432; all were significantly different ($P < 0.001$) from Towada in spikelet fertility, but were not different from ZL1929. The reciprocal A2 group of 12 recombinant families for the same region differed significantly from ZL1929, but not from Towada. Thus, the A group confined *qCTB7* to a region downstream of RM182. Using the same procedure, the B and C groups restricted *qCTB7* to a region downstream of RM6432 and RM5623, respectively, and the F groups placed the QTL upstream of RM1132. The most important groups, D1 and D2, identical in genotype between markers RM7237 and RM5508, but with allelic orientations for spikelet fertilities reversed relative to the parents ZL1929 and Towada; group D1 was significantly different from Towada, whereas group D2 was not different. The relationships for ZL1929 were the opposite. Thus, *qCTB7* was located in the region between markers RM7237 and RM5508. This conclusion was further confirmed by the

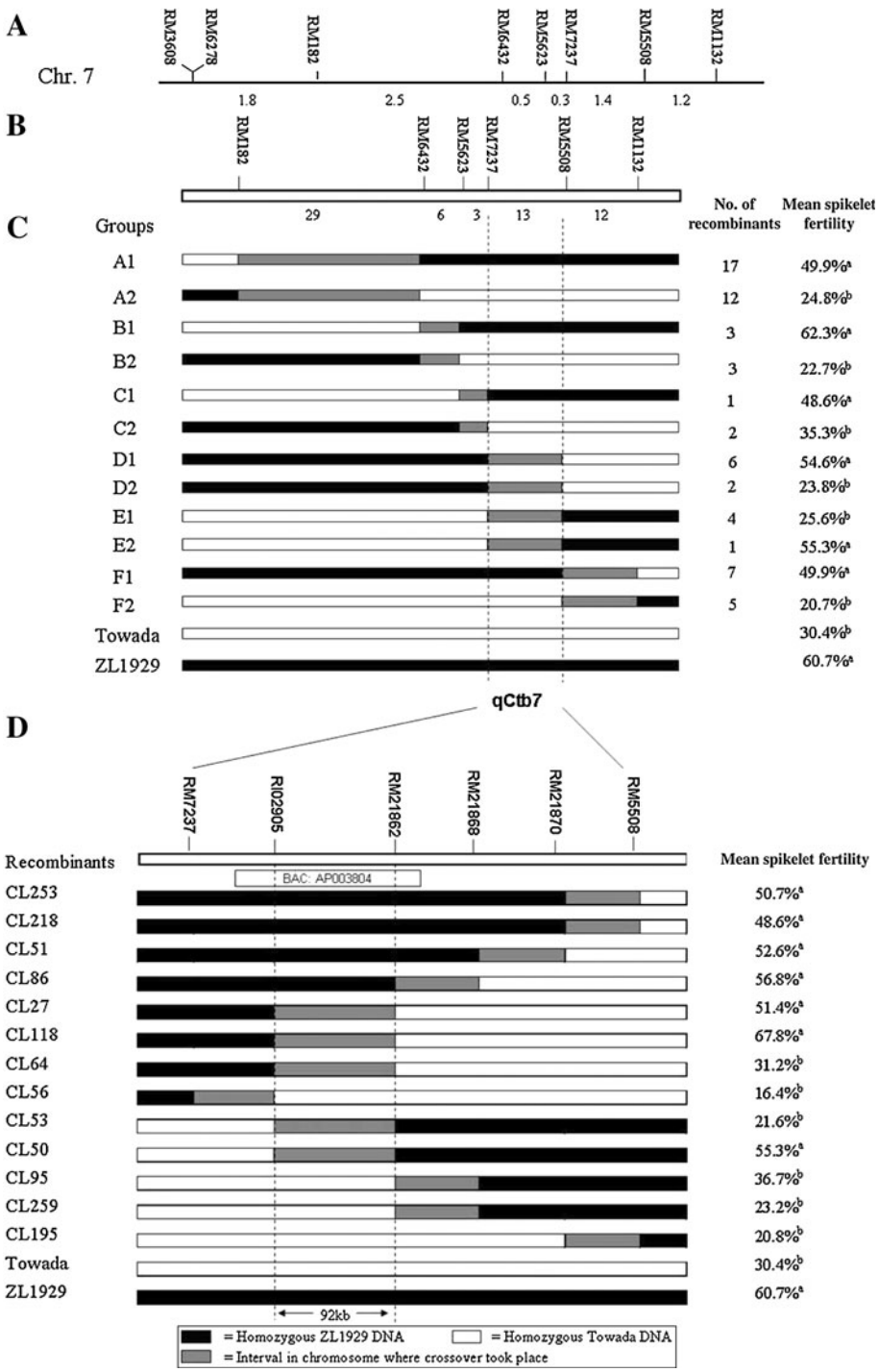

Fig. 2 Fine mapping of *qCTB7* by a two-step substitution strategy **a** The genetic linkage map (in cM) of *qCTB7* region on chromosome 7 based on 204 F_2 plants. *Numbers* below the *line* indicate genetic distance between adjacent markers. **b** High-resolution linkage map of the *qCTB7* region produced with 2,810 F_2 plants. The number of recombinants between adjacent markers is indicated under the linkage map. **c** Progeny testing of homozygous recombinants delimited the *qCTB7* locus to the region between markers RM7237 and RM5508. The 63 recombinants were grouped into 12 groups based on genotypes. The numbers of recombinants in each group and phenotypic difference of each group from the controls ZL1929 and Towada for mean spikelet fertility are shown on the right. **d** Fine mapping of *qCTB7*. The 13 recombinants between markers RM7237 and RM5508 are listed on the *left*. Phenotypic differences of each recombinant family from the controls ZL1929 and Towada for mean spikelet fertility are listed on the *right*. An "*a*" following the phenotypic value indicates that the mean phenotypic value of recombinant was not significantly different from that of ZL1929 at $P < 0.001$; a "*b*" indicates that the mean phenotypic value of recombinant was not significantly different from that of Towada

groups E, where results similar to those described for groups D were obtained in regard to E1 and E2.

For a more precise determination of the QTL location, four further markers were developed to subdivide the interval RM7237–RM5508; this permitted 13 recombinants to be further genotyped as above (Fig. 2d). The recombinant CL56 placed *qCTB7* in a region downstream of RM7237, recombinants CL253, CL218, and CL195 placed *qCTB7* in a region upstream of RM5508, recombinant CL51 placed it in a region upstream of RM21870, and CL86, CL95, and CL259 placed it upstream of RM21868. The most informative recombinants were CL27, CL118, CL64, CL53, and CL50 with identical genotypes between markers RI02905 and RM21862. Recombinants CL64 and CL53 were significantly different from ZL1929 in spikelet fertility, and not different from Towada, whereas recombinants CL27, CL118, and CL50 showed the reverse relationship. Thus, *qCTB7* must reside in the interval

Table 2 QTL analyses of spikelet fertility of the main panicle in the F_2 and F_3 generations of ZL1929 × Towada

Population	Interval	LRS	LOD[a]	Phenotypic variance[b] (%)	Add[c] (%)	Dom[d] (%)
F_2	RM182–RM1132	35.8	7.74	9	6	4
F_3	RM182–RM1132	51.5	11.2	21	12	3

[a] Likelihood ratio statistic (LRS) value was divided by 4.6 to obtain the equivalent logarithm of the odds (LOD) score (Manly et al. 2001)

[b] Phenotypic variance explained by the QTL

[c] Additive effect associated with KMXBG

[d] Dominance effect associated with KMXBG

Table 3 Candidate genes in the *qCTB7* region

ORF	Putative protein function	Full-length cDNA	Number of exons	Gene size (bp)	ESTs source tissues	EST number
Os07g0575800	Hydrolase	AK060302	7	1,146	Leaf, panicle, stem	14
Os07g0575900	DUF946 family protein	AK105640	1	1,653	Leaf, panicle, callus, stem, root, seed	56
Os07g0576000	Prenyltransferase	AK102382	11	981	Leaf, panicle, stem, flower, callus	52
Os07g0576100	OsGH3.10, GH3 homolog	–	3	1,437	–	0
Os07g0576500	OsGH3.9, GH3 homolog	AK106839	1	1,326	Panicle	1
Os07g0576600	Hypothetical protein	–	3	732	Panicle	2
Os07g0577300	Glycoside hydrolase	AK242837	5	1,497	Callus, flower, seed, panicle	31
Os07g0577400	Ubiquitin-conjugating enzyme E2	AK111080	4	609	Leaf	2
Os07g0577500	Conserved hypothetical protein	AK100831	1	1,062	Leaf, panicle, stem, flower, callus	16
Os07g0577600	Lhca2 protein	AK119176	5	792	Stem, leaf, panicle, root, flower, callus, seed	1,051
Os07g0577700	Succinyl-CoA ligase	AK243282	7	996	Panicle, leaf, callus, stem, root, flower	90
Os07g0577900	Target SNARE coiled-coil region domain containing protein	AK062833	4	1,101	Root, flower, stem	8

Data from http://www.ncbi.nlm.nih.gov/sites/entrez

RI02905–RM21862. This 92-kb region is spanned by BAC clone AP003804 (Fig. 2d).

Candidate genes in the 92-kb target region

Based on the available rice genome sequence and annotation databases (NCBI: http://www.ncbi.nlm.nih.gov/mapview/maps.cgi?taxid=4530&chr=7; TIGR: http://rice.plantbiology.msu.edu/), we found the accurate physical locations of RI02905 and RM21862 on chromosome 7. There were 12 putative genes in the 92-kb target region of the *Japonica* rice genome (cultivar: Nipponbare). This region is entirely covered by the BAC clone AP003804 (Table 3). Full-length cDNAs or ESTs corresponding to all, except Os07g0576100, were available. Ten genes (other than Os07g0576100, Os07g0577400) showed hits to ESTs expressed in reproductive tissue.

Discussion

Effect of cold stress at the booting stage

The reproductive stage includes the two most cold-sensitive stages, the booting stage (Microsporogenesis) and the flowering stage (anthesis) (Dai et al. 2002). The critical temperature for cold stress at booting (17–20°C) is higher than at flowering (15–17°C) (Li and Guo 1993). In the present study, the natural field conditions during the reproductive stage (19.5°C) in Kunming were within the critical cold temperature range for booting stage injury. Pollen fertility observations revealed that cold injury had occurred before anthesis. In addition, cold treatments in Beijing were conducted only at booting, and give the similar spikelet fertilities to Kunming (Table 1). These results indicated that spikelet sterility in Kunming was mainly caused by cold stress at the booting stage. Zeng et al. (2006) reported that plant height, panicle length, and 10 other traits were associated with cold tolerance and spikelet fertility during cold stress in a Yunnan rice core collection. Similar results were obtained by Suh et al. (2009). In the present study, we used near-isogenic lines and only the spikelet fertility traits of cold-tolerant ZL1929 were significantly different from the cold-sensitive Towada (Table 1). This indicated that the cold-tolerance QTL/gene in ZL1929 had a direct effect on spikelet fertility. Moreover, the total grains per panicle for Towada (69.5) were much lower than that for ZL1929 (93.7) under the cold treatment condition in Beijing (cold temperature), and very

different from those recorded at Kunming under cold temperatures and Beijing under normal temperatures. This indicated that critical low temperatures with short days at the booting stage not only affect spikelet fertile, but also spikelet development.

qCTB7 is a stable cold-tolerance locus

In earlier studies, Takeuchi et al. (2001) detected the QTL *qCT-7* between S1563 and W146 on chromosome 7 using a doubled-haploid (DH) population from a cross between tolerant-temperate and sensitive-temperate japonica varieties. Dai et al. (2004) detected the QTL *qRCT7* between RM182 and R1789 on chromosome 7 using an F_2 population consisting of 250 individuals of a cross between KMXBG and Towada. In this study, we mapped QTL *qCTB7* for cold tolerance on the long arm of chromosome 7, explaining 9 and 21% of the phenotypic variance in the F_2 and F_3 generations, respectively, and fine mapped its location to a 92-kb interval between markers RI02905 and RM21862. The genetic and physical locations of these markers (http://www.gramene.org/markers/index.html) indicate that *qCTB7*, *qRCT7*, and *qCT-7* may be the same locus. The three QTLs were detected in different genetic backgrounds and environments, but the phenotypic variances explained at 21, 20.6, and 22.1%, respectively, were quite similar. Cold tolerance at the booting stage in a Yunnan rice core collection was significantly associated ($P < 0.005$) with molecular marker RM7237 (unpublished data) indicating that the effect of the locus is stable in different genetic backgrounds and is likely a major effect QTL. It is also possible that the cold-tolerance allele at this locus may be conserved in rice evolution and act as a physiological switch in response to cold stress. In our former studies (Xu et al. 2008), in order to escape the hybrid sterility that is common in inter-subspecies rice crosses, but to ensure a distant geographical relationship, we developed cold-tolerant NILs using *Japonica* cultivars from Yunnan and Japan. The polymorphism frequency between Towada and KMXBG was much lower than that of the *japonica* × *indica* cross analyzed by Andaya and Mackill (2003). Although there were five recombinant lines between RI02905 and RM21862, we could not find polymorphic molecular markers in the 92-kb interval.

Analysis of possible candidate genes

No major cold-tolerance gene effective at the booting stage had been reported previously (Saito et al. 2004; Xu et al. 2008). We searched for candidate genes for *qCTB7* using the available sequence annotation database (http://www.ncbi.nlm.nih.gov/; http://rice.plantbiology.meu.edu/). Of 12 genes in the target region of the cultivated rice Nipponbare genome (Table 3), five could be related to response to cold or other stress responses, and were therefore the more likely candidates. These included two auxin response genes, Os07g0576100 and Os07g0576500, two hydrolase genes Os07g0575800 and Os07g0577300, and one ubiquitin-conjugating enzyme E2 gene Os07g0577400.

The phytohormone auxin plays a central role in almost every aspect of plant growth and development and several auxin-responsive genes have been implicated in both biotic (e.g. pathogen infection (Ding et al. 2008)) and abiotic stress responses (e.g. desiccation, low temperature, and salinity (Hannah et al. 2005; Jain and Khurana 2009; Song et al. 2009)). Primary auxin response genes, which are categorized in three major classes, viz. auxin/indole-3-acetic acid (Aux/IAA), GH3, and small auxin-up RNA (SAUR) (Guilfoyle 1999), induce very rapid transcript accumulations of a large number of genes. We found two GH3 homologs, Os07g0576100 and Os07g0576500, that are putative indole-3-acetic acid-amido synthetases named OsGH3.10 and OsGH3.9, respectively (Jain et al. 2006), in the *qCTB7* region. Members of the GH3 gene family encode enzymes that adenylate indole 3-acetic acid (IAA) to form amino acid conjugates, thereby preventing the accumulation of excessive free auxin, and are involved in auxin homeostasis (Staswick et al. 2005). In addition, GH3 enzymes catalyze amido conjugation to salicylic acid and jasmonic acid (Staswick et al. 2002). Twelve members of the GH3 gene family were identified in rice using sequences of full-length cDNA clones available from KOME and analysis of the whole genome sequence of rice. Tos17 insertion mutants of rice GH3 genes, OsGH3.5 and OsGH3.7, showed low fertility or sterile phenotypes (Jain et al. 2006). ESTs of Os07g0576500 (OsGH3.9) were reported to express in panicles and no EST was recorded for Os07g0576100 (OsGH3.10) (Table 3). Os07g0576100 (OsGH3.10) and Os07g0576500 (OsGH3.9) are considered a sister pair and to represent a local duplication event. It is therefore possible that Os07g0576500 (OsGH3.9) plays an important role in cold tolerance at the booting stage.

Os07g0575800 and Os07g0577300 are putative and expressed glycosyl hydrolase (glucan endo-1,3-beta-glucosidase) genes; β-glucosidases (E.C. 3.2.1.21) are ubiquitous. Glucosylation (reversible by the appropriate glucosidase) can affect various characteristics of the glucosylated moiety (the aglycone), including reactivity, solubility, and transport (Li et al. 2001). Many roles for glucosidases in plants have been postulated (reviewed in Esen 1993); some are capable of affecting cell wall properties (Gerardi et al. 2001; Li et al. 2001), which could be a crucial function in protecting cells from the physical deformations associated with freezing. In stress responses, β-glucosidases commonly release active molecules from inert precursors. The various released molecules include a

variety of antimicrobials (Cicek and Esen 1998; Sue et al. 2000), phytohormones (Brzobohaty et al. 1993), and at least one antioxidant (Chong et al. 2002). Stress-related roles were also suggested for several β-glucosidases of unknown function on the basis of their stress-responsive expressions (Chen et al. 2002; Thorlby et al. 2004; Spano et al. 2005).

Os07g0577400 is considered to be ubiquitin-conjugating enzyme E2, and may take part in cellular responses to stress. Ubiquitination also plays a crucial role in responses to cold (Ishitani et al. 1998; Dong et al. 2006). Recently, Zhou et al. (2010) reported that overexpression of a soybean ubiquitin-conjugating enzyme gene *GmUBC2* enhanced drought and salt tolerance through modulating abiotic stress-responsive gene expression in *Arabidopsis*.

Potential exploitation in rice cold-tolerance improvement

Cold tolerance in rice is a major distinguishing factor in classifying the two major subspecies of *Oryza sativa*, *japonica*, and *indica* (Glaszmann et al. 1990). The *indicas* are more sensitive to cold stress than the *japonicas*. *Japonicas* could be used for cold-tolerance improvement and to diversify *indica* germplasm. Fine mapping of *qCTB7* on chromosome 7 thus provides useful information for cold-tolerance breeding permitting large-scale and precise screening for cold-tolerant genotypes by marker-assisted selection. In further studies, we will validate these candidate genes by sequence analysis of parental lines, including the coding and promoter regions, and carry out gene expression analyses using reproductive tissues of cold-treated parental plants at the booting stage. The most promising candidate genes will be utilized in genetic transformation and functional analyses.

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