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QTL detection and candidate gene identification of *qCTB1* for cold tolerance in the Yunnan plateau landrace rice

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Abstract

Cold stress is one of the main abiotic stresses that affects rice growth and production worldwide. Dissection of the genetic basis is important for genetic improvement of cold tolerance in rice. In this study, a new source of cold-tolerant accession from the Yunnan plateau, Lijiangxiaoheigu, was used as the donor parent and crossed with a cold-sensitive cultivar, Deyou17, to develop recombinant inbred lines (RILs) for quantitative trait locus (QTL) analysis for cold tolerance at the early seedling and booting stages in rice. In total, three QTLs for cold tolerance at the early seedling stage on chromosomes 2 and 7, and four QTLs at the booting stage on chromosomes 1, 3, 5, and 7, were identified. Haplotype and linear regression analyses showed that QTL pyramiding based on the additive effect of these favorable loci has good potential for cold tolerance breeding. Effect assessment in the RIL and BC_3F_3 populations demonstrated that *qCTB1* had a stable effect on cold tolerance at the booting stage in the genetic segregation populations. Under different cold stress conditions, *qCTB1* was fine-mapped to a 341-kb interval between markers M3 and M4. Through the combination of parental sequence comparison, candidate gene-based association analysis, and tissue and cold-induced expression analyses, eight important candidate genes for *qCTB1* were identified. This study will provide genetic resources for molecular breeding and gene cloning to improve cold tolerance in rice.

Keywords Rice · Cold tolerance · QTL analysis · QTL pyramiding · Candidate gene

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Introduction

Rice (*Oryza sativa* L.) is one of the most widely adapted staple crops worldwide, providing about 21% of human nutrient intake and energy requirements. Rice is sensitive to cold stress since it is a thermophilic crop that has evolved in tropical and subtropical regions (Kovach et al. 2007). There are about 15 million hectares of fields under threat of cold stress in 24 rice-producing countries, including the major areas of Korea, Japan, and China (Sun et al. 2019). Estimates of 3–5 million tons of rice are lost annually due to cold stress (Zhu et al. 2015). Therefore, genetic improvement of cold tolerance has become one of the important targets of modern rice breeding for stress tolerance, so as to maintain rice production in rice-growing areas prone to cold stress.

Cold stress influences the growth and development of rice throughout different stages. Cold stress at the early seedling stage occurs when rice is exposed to temperature below 10℃ from germination to the emergence of primary leaves, which disrupts seed germination, delays seedling emergence, and significantly increases seedling mortality, resulting in substantial yield losses (Peterson et al. 1978). Cold stress at the booting stage occurs when rice is exposed to temperature below 20℃ during the period of microspore differentiation and pollen maturation. This disruption affects microtubules, interfering with mitosis and preventing the formation of normal pollen grains, which directly causes spikelet sterility and leads to serious yield losses (Satake and Hayase 1970). Survival rate and seed-setting rate are commonly used as indicators to assess cold tolerance at the early seedling and booting stages, respectively (Sun et al. 2019; Peterson et al. 1978). Due to the multiple affecting factors such as the heading date and external environment, it is often necessary to utilize several evaluation methods, including natural low-temperature in a high-altitude area, cold stress in deep water or in a phytotron, to accurately evaluate cold tolerance at the booting stage.

The dissection of the genetic structure of cold tolerance is the foundation for the improvement of cold tolerance through molecular breeding. Cold tolerance in rice is a complex quantitative trait with polygenic inheritance. To date, several QTLs for cold tolerance at the early seedling stage have been identified in different genomic regions in rice (Zhang et al. 2005; Ranawake et al. 2014; Yang et al. 2021), but only one QTL (*OsLTPL159*) has been cloned (Zhao et al. 2019). Meanwhile, several QTLs for cold tolerance at the booting stage have been detected through linkage analysis or genome-wide association studies using bi-parental mapping populations derived from *japonica/japonica* crosses or *japonica/indica* crosses, as well as diverse rice germplasms (Andaya and Mackill 2003; Xu et al. 2008; Xiao et al. 2018; Guo et al. 2020; Zhang et al. 2021). However, because of the difficulty and complexity of cold tolerance evaluation at the booting stage, only five QTLs (*qLTB3*, *qCTB7*, *qCTB10-2*, *qCTB8*, and *qCT-4*) have been further fine-mapped via the substitution mapping approach (Kuroki et al. 2007; Zhou et al. 2010; Shirasawa et al. 2012; Endo et al. 2016; Li et al. 2018), and only two QTLs (*Ctb1* and *CTB4a*) have been cloned as well as functionally characterized (Saito et al. 2010; Zhang et al. 2017). Therefore, further efforts are needed to

discover and fine-tune QTLs for cold tolerance at the early seedling and booting stages to provide new genetic resources for molecular breeding of cold tolerance in rice.

Yunnan province, located in the southwest plateau with high altitude and a diverse climate in the rice-growing area, is the largest center of rice genetic diversity in China and has bred unique and elite cold-tolerant germplasms. These plateau germplasms contain abundant favorable natural variations for cold tolerance that are valuable genetic resources for gene mining and variety improvement of cold tolerance in rice. In this study, we developed a recombinant inbred line population using the Yunnan plateau *japonica* landrace Lijiangxiaoheigu (LJXHG) and the cold-sensitive *indica* variety Deyou 17 (DY17). QTL mapping for cold tolerance at the early seedling and booting stages was performed using the RIL population. Haplotype and linear regression analyses were performed to clarify the breeding potential of QTL pyramiding among the identified QTLs. The effect of a stable locus, *qCTB1,* on cold tolerance at the booting stage was assessed in different segregation populations, and *qCTB1* was further fine-mapped under two different cold treatment conditions. Through the combination of fine mapping, parental sequence comparison, association analysis, and expression pattern analysis, candidate genes for *qCTB1* were identified. This study will lay the foundation for further cloning of cold tolerance genes and also provide new genetic resources for the genetic improvement of cold tolerance in rice.

Results

Phenotypic variation of cold tolerance in parents and the RIL population

Two parents and 130 RILs were evaluated for cold tolerance at the early seedling and booting stages to identify QTLs for cold tolerance in rice (Fig. S1 A-D). LJXHG and DY17 grew normally with no significant difference in survival rate (SR) under normal conditions, but LJXHG showed a higher SR than DY17 under different low temperature conditions (10 °C, 8 °C, and 6 °C) in the low-temperature incubator (Fig. 1A, B). In the evaluation of cold tolerance at the booting stage in 2014 (E1) and 2015 (E2), there was no significant difference in seed-setting rates under normal conditions (SS) between LJXHG and DY17, but LJXHG showed higher seed-setting rates under cold stress conditions (SSC) and relative seed-setting rates (RSS) than DY17 (Table 1; Fig. 1C, D). These results indicated that LJXHG was more cold-tolerant than DY17 at both the early seedling and booting stages.

There were great variations in SR, SSC, and RSS in the RIL population. The range of SR for RILs was 2–100%, with a mean of 36%. The ranges of SSC for RILs at E1 and E2 were 1–74% and 19–74%, with means of 41% and 51%, respectively; the ranges of RSS for RILs at E1 and E2 were 1–90% and 24–85%, with means of 53% and 61%, respectively (Table 1). The estimation of Pearson's correlation coefficients of SS, SSC, and RSS between E1 and E2 showed that these traits were significantly positively correlated between E1 and E2 respectively (Fig. S2A, B, C). SR, SSC, and RSS showed continuous distributions,
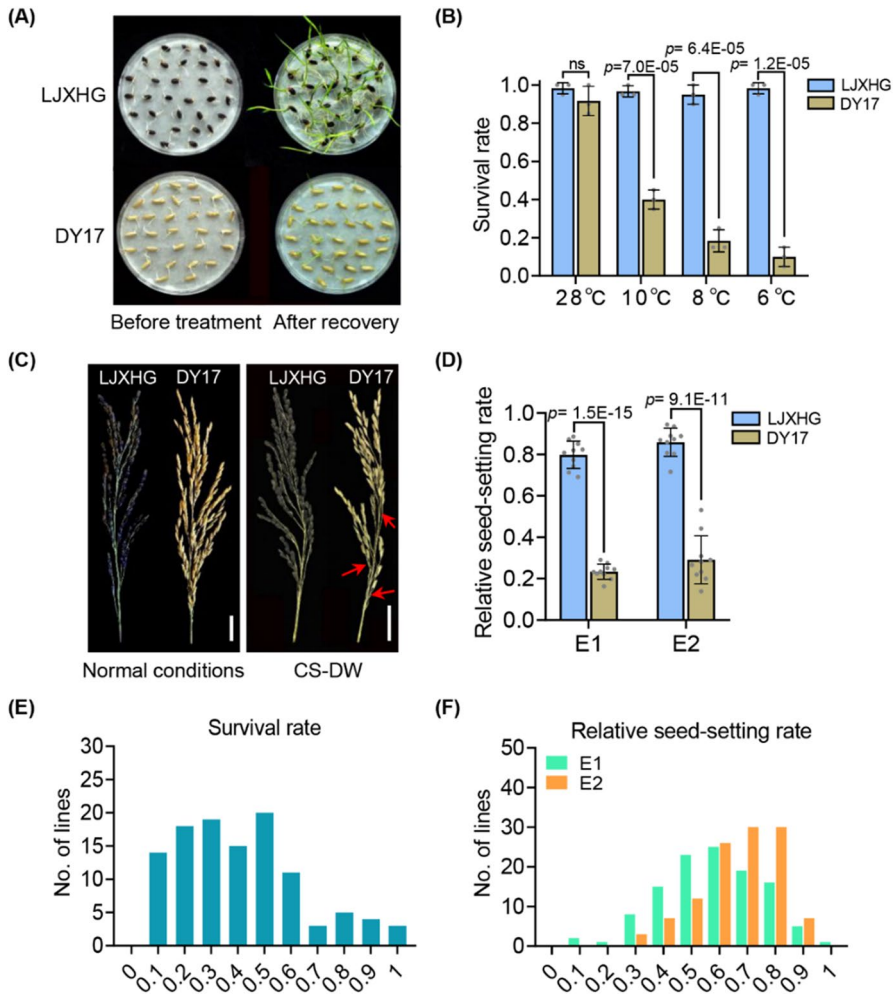
Fig. 1 Cold tolerance of parents and RIL population at the early seedling and booting stages. (**A**) Early seedlings of parents before cold treatment and after recovery for one week. (**B**) Comparison of the survival rate between parents under normal and different temperature conditions. (**C**) Panicles of parents under normal and cold stress in deep water conditions (CS-DW), bar, 2.5 cm. The red arrows indicate degraded or sterile spikelets. (**D**) Comparison of the relative seed-setting rate between parents in 2014 (E1) and 2015 (E2). (**E–F**) Frequency distributions of the survival rate (**E**) and relative seed-setting rate (**F**) in the RIL population

indicating that these traits were inherited quantitatively (Fig. 1E, F; Fig. S2D, E). SR and RSS (E2) displayed more or less deviations from normality, whereas SSC (E1, E2) and RSS (E1) exhibited normal distributions based on the Shapiro–Wilk test (Table 1).

Table 1 Statistics of cold tolerance among parents and the RIL population

Stage	Trait	Trail	Parents (Mean±SD)		RIL population					
			LJXHG	DY17	Minimum	Maximum	Mean	Standard Deviation	Median	Shapiro–Wilk (Significance)
Early seedling	SR		0.98±0.03	0.09±0.02	0.02	1.00	0.36	0.23	0.35	4.83E-04
Booting	SS	E1	0.93±0.04	0.88±0.04	0.65	0.99	0.77	0.09	0.78	1.65E-04
		E2	0.91±0.03	0.89±0.03	0.65	0.96	0.83	0.09	0.86	3.20E-06
	SSC	E1	0.75±0.06	0.21±0.08	0.01	0.74	0.41	0.15	0.42	0.42
		E2	0.80±0.08	0.28±0.14	0.19	0.74	0.51	0.13	0.52	0.08
	RSS	E1	0.80±0.06	0.23±0.09	0.01	0.90	0.53	0.18	0.54	0.32
		E2	0.88±0.09	0.32±0.16	0.24	0.85	0.61	0.14	0.63	0.01
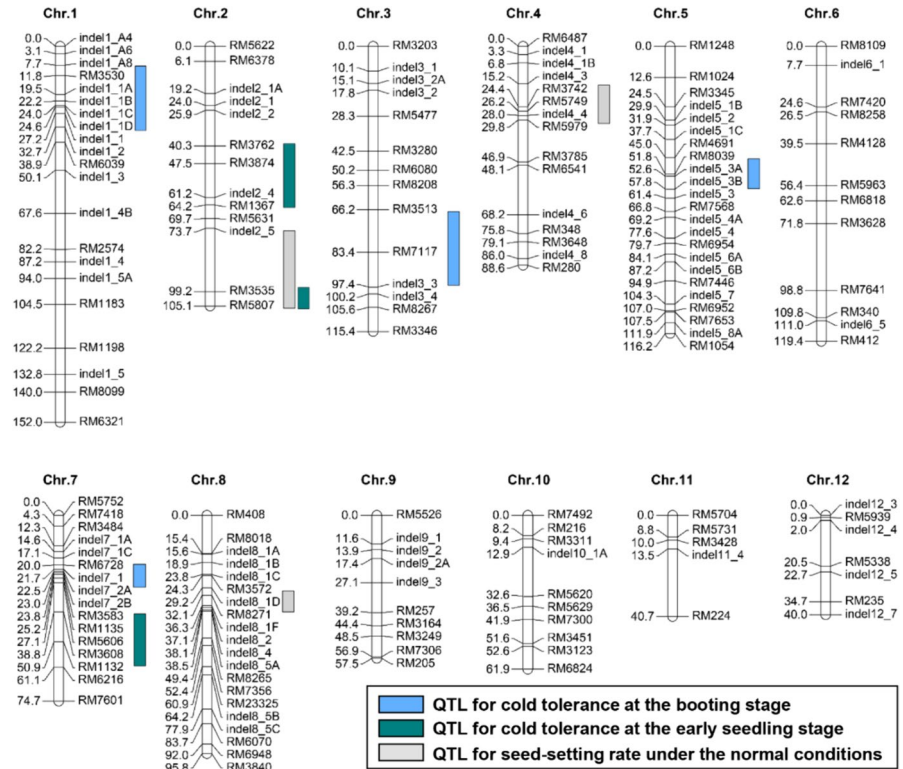
Fig. 2 The linkage map and chromosomal locations of putative QTLs for cold tolerance at the early seedling and booting stages

Linkage map construction and QTL detection

A genetic linkage map was constructed using 166 polymorphic markers distributed on the 12 rice chromosomes (Fig. 2; Table S1). The genetic linkage map covered a total length of 1,067.3 cM with a maximum interval size of 27.2 cM, a minimum interval size of 0.3 cM, and an average interval size of 7.3 cM between adjacent markers (Table S2).

A total of three QTLs for cold tolerance at the early seedling stage were detected on chromosomes 2 and 7, with phenotypic variations explained ranging from 3.02% to 5.03%. The LJXHG-derived alleles in all three QTLs increased SR at low temperatures (Table 2).

In total, four QTLs influencing SSC were detected on chromosomes 1, 3, 5, and 7. Three of them (*qSSC1*, *qSSC5*, and *qSSC7*) were identified in E1 and explained 8.60%, 4.60%, and 4.68% of the phenotypic variation, respectively. Two of them (*qSSC1* and *qSSC3*) were identified in E2, which explained 3.88% and 3.92% of the phenotypic variation, respectively. Except for *qSSC3*, the positive additive effects of the other three QTLs were all from LJXHG (Table 2).

Table 2 QTLs detected for cold tolerance at the early seedling and booting stages
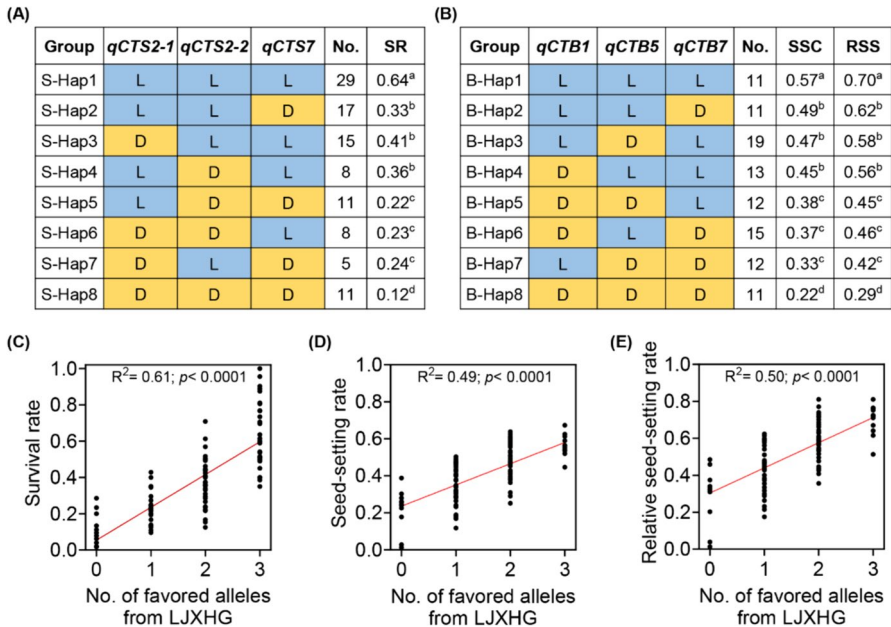
Stage	Trait	QTL	Trail	Chr	Marker interval	LOD	Add[a]	PVE(%)
Early seedling	SR	qCTS2-1		2	RM3762-RM1367	3.07	0.08	3.02
		qCTS2-2		2	RM3535-RM5807	4.92	0.12	5.03
		qCTS7		7	RM3608-RM6216	3.51	0.08	3.13
Booting	SS	qSS2	E1	2	indel2_5-RM5807	5.16	-0.04	1.52
		qSS4	E2	4	indel4_3-RM5979	2.62	0.03	1.84
		qSS8	E2	8	indel8_1D-indel8_4	3.92	-0.03	2.12
	SSC	qSSC1	E1	1	indel1_A8-indel1_2	4.44	0.06	8.60
			E2	1	indel1_A8-indel1_2	4.85	0.05	3.88
		qSSC3	E2	3	RM3513-indel3_3	5.15	-0.05	3.92
		qSSC5	E1	5	RM4691-indel5_3B	2.89	0.04	4.60
		qSSC7	E1	7	RM6728-RM1135	3.33	0.05	4.68
	RSS	qCTB1	E1	1	indel1_A8-indel1_2	4.10	0.07	6.92
			E2	1	indel1_A8-indel1_2	5.07	0.06	5.80
		qCTB3	E2	3	RM3513-indel3_3	4.66	-0.06	4.76
		qCTB5	E1	5	RM4691-indel5_3B	5.47	0.07	8.04
		qCTB7	E1	7	RM6728-RM1135	4.77	0.07	7.28

[a] The positive values of the additive effect indicate an effect from LJXHG, and the negative values indicate effect from DY17

In order to eliminate the effects of sterility caused by the incompatibility of the *indica* and *japonica* crossings when detecting QTLs for cold tolerance at the booting stage, only the RIL lines with SS above 65% under normal conditions were used. By using the RSS as the index, four QTLs for cold tolerance at the booting stage were detected on chromosomes 1, 3, 5, and 7, which were co-localized with the QTLs for SSC. Among them, three QTLs (*qCTB1*, *qCTB5*, and *qCTB7*) were identified in E1 that explained 6.92%, 8.04%, and 7.28% of the phenotypic variation, respectively. And two QTLs (*qCTB1* and *qCTB3*) were identified in E2 and explained 5.80% and 4.76% of the phenotypic variation, respectively. Except for *qCTB3*, the positive additive effects of the other three QTLs were all from LJXHG (Table 2).

QTL combinations for cold tolerance at the early seedling and booting stages

To explore the effects of different QTL combinations on cold tolerance at the early seedling and booting stages in rice, haplotype and linear regression analyses were performed for the identified QTLs whose positive additive effects were from LJXHG. Haplotype analysis for three QTLs (*qCTS2-1*, *qCTS2-2*, and *qCTS7*) conferring cold tolerance at the early seedling stage showed that the haplotypes containing more favorable loci exhibited stronger cold tolerance, and the haplotypes containing the same number of favorable loci exhibited similar cold tolerance (Fig. 3A). The same result was also observed in the haplotype analysis for three QTLs (*qCTB1*, *qCTB5,* and *qCTB7*) conferring cold tolerance at the booting stage (Fig. 3B). Linear

(A)

Group	qCTS2-1	qCTS2-2	qCTS7	No.	SR
S-Hap1	L	L	L	29	0.64[a]
S-Hap2	L	L	D	17	0.33[b]
S-Hap3	D	L	L	15	0.41[b]
S-Hap4	L	D	L	8	0.36[b]
S-Hap5	L	D	D	11	0.22[c]
S-Hap6	D	D	L	8	0.23[c]
S-Hap7	D	L	D	5	0.24[c]
S-Hap8	D	D	D	11	0.12[d]

(B)

Group	qCTB1	qCTB5	qCTB7	No.	SSC	RSS
B-Hap1	L	L	L	11	0.57[a]	0.70[a]
B-Hap2	L	L	D	11	0.49[b]	0.62[b]
B-Hap3	L	D	L	19	0.47[b]	0.58[b]
B-Hap4	D	L	L	13	0.45[b]	0.56[b]
B-Hap5	D	D	L	12	0.38[c]	0.45[c]
B-Hap6	D	L	D	15	0.37[c]	0.46[c]
B-Hap7	L	D	D	12	0.33[c]	0.42[c]
B-Hap8	D	D	D	11	0.22[d]	0.29[d]

(C), **(D)**, **(E)**

Fig. 3 Pyramiding of favorable QTLs from LJXHG for cold tolerance at the early seedling and booting stages. (**A-B**) Haplotype analyses of QTLs for cold tolerance at the early seedling (**A**) and booting (**B**) stages. (**C-E**) Allelic effects of favored QTLs from LJXHG for survival rate (**C**), seed-setting rate under cold stress conditions (**D**), and relative seed-setting rate (**E**) based on the linear regression. In A and B, the letters "L" and "D" represent the alleles that derive from LJXHG and DY17, respectively. In A and B, different letters indicate significant differences ($p < 0.05$, one-way ANOVA, Duncan test)

regression analysis showed that cold tolerance at the early seedling and booting stages increased gradually along with an increase in favorable loci from LJXHG, and the cold tolerance is positively correlated with the number of QTLs for cold resistance (Fig. 3C, D, E). These results indicate that QTL pyramiding based on the additive effects of favorable loci may be an effective way to improve cold tolerance in rice at both the early seedling and booting stages.

Effect assessment and fine mapping of *qCTB1*

Among the QTLs identified for cold tolerance at the booting stage, *qCTB1* could be detected in two consecutive years and explained a relatively high proportion of the phenotypic variation (Table 2). Moreover, *qCTB1* coincided with the previously reported QTLs for cold tolerance at the booting stage, indicating that *qCTB1* is a reliable and stable locus for cold tolerance. To further fine map *qCTB1*, the backcross inbred line populations were constructed through marker-assisted selection (Fig. S3).

To assess the application potential of *qCTB1*, we analyzed its effects in the RIL and BC_3F_3 populations. There was no significant difference in cold tolerance
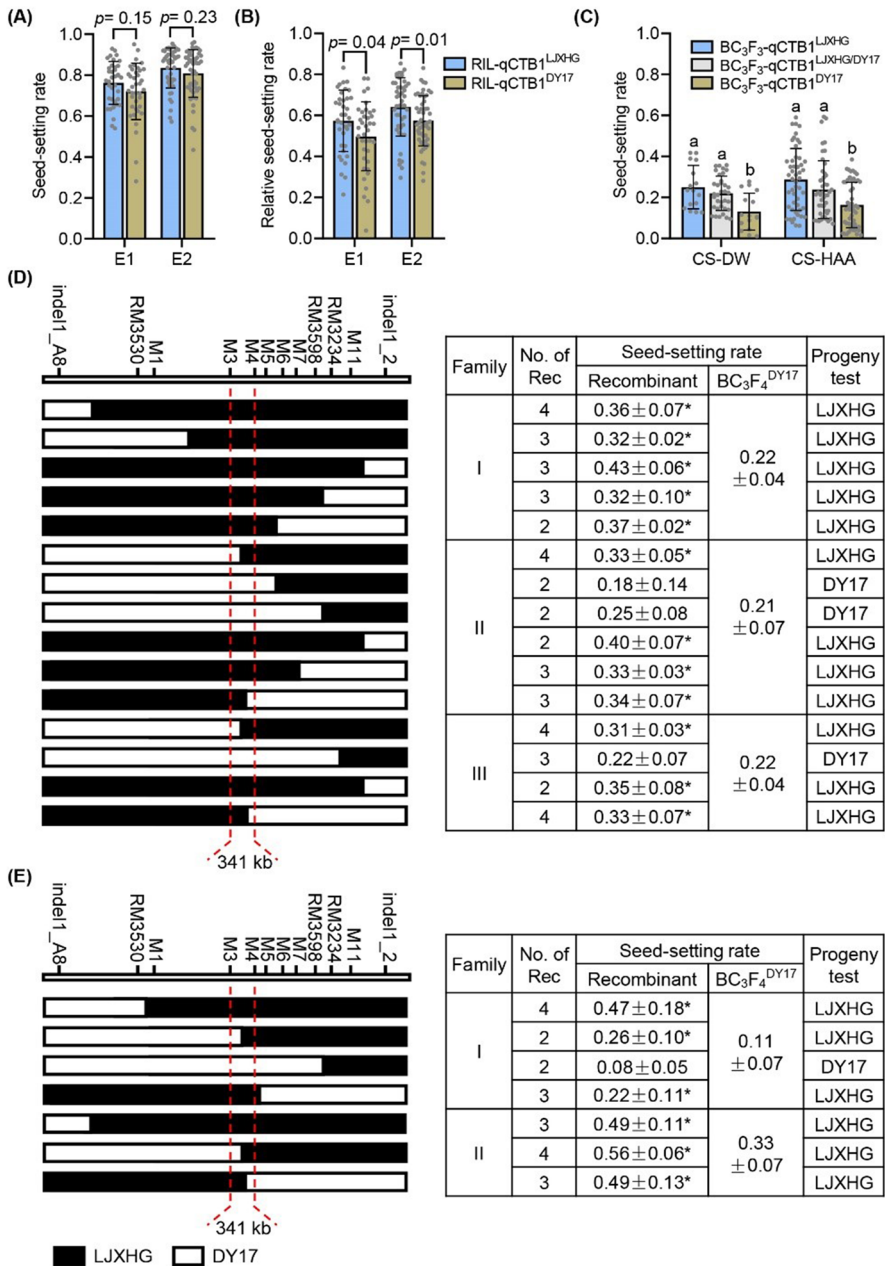
Fig. 4 Effect assessment and fine mapping of *qCTB1*. (**A-B**) Comparison of the normal seed-setting rate (**A**) and relative seed-setting rate (**B**) between the RIL lines with different genotypes of *qCTB1*. *p* values were calculated by Student's *t* tests. (**C**) Comparison of the seed-setting rate among BC$_3$F$_3$ individuals with different genotypes of *qCTB1* under CS-DW and CS-HAA conditions. Different letters indicate significant differences (*p* < 0.05, one-way ANOVA, Duncan test). (**D-E**) Fine mapping of *qCTB1* under cold stress conditions in deep water (**D**) and in a high-altitude area (**E**). The asterisks indicate significant differences in Student's *t* tests

between the RIL lines containing different genotypes of *qCTB1* under normal conditions (Fig. 4A), while the RIL lines containing the *qCTB1^{LJXHG}* allele showed stronger cold tolerance than those containing the *qCTB1^{DY17}* allele under CS-DW conditions (Fig. 4B). In addition, the BC₃F₃ individuals containing the *qCTB1^{LJXHG}* or *qCTB1^{LJXHG/DY17}* allele showed stronger cold tolerance than those containing the *qCTB1^{DY17}* allele under both the CS-DW and CS-HAA conditions (Fig. 4C).

Under cold stress conditions in deep water (CS-DW) (Fig. S1C, D), forty-four recombinants between markers indel1_A8 and indel1_2 were screened in a BC₃F₃ population of 1,083 individuals classified into three families. Ten polymorphic markers were identified between parents within the interval of indel1_A8 and indel1_2 and were used to detect the recombination sites of the recombinants. Homozygous BC₃F₄ progenies of the recombinants identified with the appropriate markers were evaluated for cold tolerance under CS-DW conditions. Based on the progeny test for homozygous recombinants within the family, *qCTB1* was located in the region between markers M1 and M6 in family I and between markers M3 and M4 in both families II and III (Fig. 4D).

Meanwhile, under cold stress conditions in a high-altitude area (CS-HAA, 1,974 m) (Fig. S1E), twenty-one recombinants between markers indel1_A8 and indel1_2 were screened in another BC₃F₃ population of 835 individuals classified into two families. Homozygous BC₃F₄ progenies of the recombinants identified with the appropriate markers were evaluated for cold tolerance under CS-HAA conditions. Based on the progeny test for homozygous recombinants within the family, *qCTB1* was located in the region between markers M3 and M5 in family I and between M3 and M4 in family II (Fig. 4E).

According to the progeny tests under the cold stress conditions in CS-DW and CS-HAA, *qCTB1* was finally located in the 341-kb interval between markers M3 and M4.

Candidate gene identification of *qCTB1*

To investigate the candidate genes of *qCTB1*, the sequence differences of the predicted genes within the fine-mapping interval were compared between LJXHG and DY17. Among the 52 genes acquired within the 341-kb interval, 29 genes contained polymorphic SNPs in the promoter regions or polymorphic non-synonymous SNPs in the coding regions between the parents. To further verify the associations between these 29 genes and cold tolerance at the booting stage, polymorphic SNPs in the promoters and polymorphic non-synonymous SNPs in the coding regions of these genes were used for candidate gene-based association analysis. By using 153 rice accessions and 480 polymorphic SNPs, a total of 65 significant SNPs were identified (Fig. 5A), which were located on 19 genes consisting of 17 genes encoding functional proteins and 2 genes encoding hypothetical proteins.

To further identify possible candidate genes, tissue and cold-induced expression patterns of the above 17 genes were analyzed. Tissue expression analysis based on the public rice expression databases showed that 6 genes were not expressed in the anther, while the other 11 genes were expressed in the anther at different levels
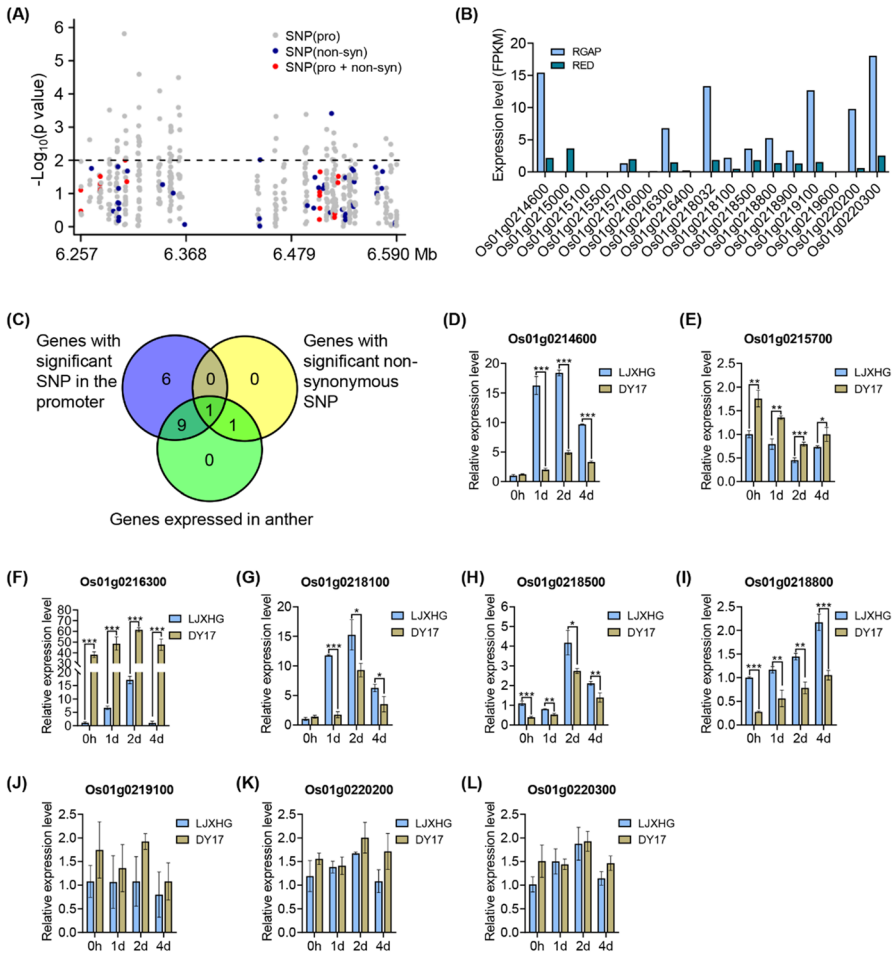
Fig. 5 Candidate gene analysis of *qCTB1*. (**A**) Candidate gene-based association analysis using SNPs in the promoter and non-synonymous SNPs in the coding region of genes within the fine-mapping interval of *qCTB1*. (**B**) Expression level of *qCTB1* candidate genes in the anthers based on the data from the RGAP and RED websites. (**C**) Comparison of the candidate genes containing significant SNPs in the promoter or significant non-synonymous SNPs with the candidate genes expressed in anther. (**D-L**) Cold-induced expression of candidate genes in parents at the booting stage

(Fig. 5B). Among these 11 genes, two (*Os01g0218032* and *Os01g0218900*) contained significant and polymorphic non-synonymous SNPs in the coding regions, and the remaining 9 genes contained significant and polymorphic SNPs only in the promoter regions (Fig. 5C). Cold-induced expression analysis at the booting stage showed that, except for *Os01g0219100*, *Os01g0220200*, and *Os01g0220300*, the other 6 genes containing significant SNPs in the promoters were differentially responsive to cold at the booting stage between the parents (Fig. 5D-L).

To sum up, a total of 8 important candidate genes were identified for *qCTB1*, including 2 genes (*Os01g0218032* and *Os01g0218900*)

Table 3 Details of the eight potential candidate genes for *qCTB1*

Gene ID	Annotation	Number of polymorphic SNPs between parents (pro/non-syn)[a]	Number of significant SNPs (pro/non-syn)	Cold induced expression in parents
Os01g0218032	DNA demethylase, DNG702	10/3	0/1	
Os01g0218900	PHD-type domain containing protein, OsPHD3	48/4	4/1	
Os01g0214600	GDSL-like lipase/acylhydrolase, OsGELP2	5/1	1/0	Differential
Os01g0215700	GDSL-like lipase/acylhydrolase, OsGELP6	39/0	10/0	Differential
Os01g0216300	GDSL-like lipase/acylhydrolase, OsGELP8	36/1	9/0	Differential
Os01g0218100	OsbHLH037	21/0	3/0	Differential
Os01g0218500	FLOWERING LOCUS T Like homolog, OsFTL1	23/1	5/0	Differential
Os01g0218800	SET-domain group protein 721, OsSDG721	22/3	3/0	Differential

[a] pro/non-syn, the SNPs in the promoter region, or the non-synonymous SNPs in the coding region

with significant non-synonymous SNPs in the coding regions and 6 genes (*Os01g0214600*, *Os01g0215700*, *Os01g0216300*, *Os01g0218100*, *Os01g0218500*, and *Os01g0218800*) with significant SNPs in the promoters that responded differently to cold between the parents (Table 3).

Discussion

Cold stress is one of the major abiotic environmental stresses affecting rice growth and development worldwide. Although physiological responses to cold stress have been studied, the genetic mechanism of cold tolerance in rice has not been fully understood. Dissection of the genetic structure of cold tolerance will provide a foundation for elucidating the genetic mechanism of cold tolerance and provide genetic resources for the molecular breeding of cold tolerance in rice.

There are various ways to evaluate the cold tolerance at the booting stage, including natural low temperatures, short-term deep cold-water irrigation, long-term deep cold-water irrigation, and artificial chamber environments. Natural low-temperature and long-term deep cold-water irrigation are simple and effort-saving methods that have been commonly used to evaluate cold tolerance at the booting stage (Sun et al. 2019; Xu et al. 2008). While short-term deep cold-water irrigation and an artificial chamber could better control the influence of factors such as heading date and external environment and are especially suitable for the cold tolerance evaluation of genetic populations with segregation at heading date (Xiao et al. 2018; Ye et al. 2010). To accurately assess cold tolerance at the booting stage, different cold treatment methods were flexibly utilized in this study. For example, for the RIL lines that differed in the heading date, the short-term deep cold-water irrigation method was used to reduce the possible impact of heading date differences. For the backcross inbred lines with no obvious segregation at the heading stage, two cold treatment methods, including short-term deep cold-water irrigation and natural low temperatures, were simultaneously utilized to ensure the accuracy of the cold tolerance of the key recombinants at the booting stage.

There are several traits associated with cold tolerance at the booting stage in rice, including seed-setting rate, number of filled grains per panicle, number of unfilled grains per panicle, and anther length (Zeng et al. 2009). Among them, seed-setting rate was widely used as the criterion for evaluating cold tolerance at the booting stage in rice. However, it is difficult to distinguish between the reduced seed-setting rate caused by gametophyte abortion and that caused by low temperature in the genetic population of *indica*/*japonica* crosses (Suh et al. 2010). In this study, only the RIL lines with a seed-setting rate greater than 65% under normal conditions were used to identify QTLs for cold tolerance at the booting stage. In addition, the relative seed-setting rate was used as the criterion in the present study. Through the QTL analysis, four QTLs for cold tolerance at the booting stage were identified, which were co-localized with the QTLs for SSC but were completely different from the QTLs for SS (Table 2). This indicated that the approach we took could effectively reduce the influence of incompatibility between *indica* and *japonica* crosses and thereby ensure the accuracy of QTL identification.

Cold tolerance is a complex quantitative trait with polygenic inheritance. By using linkage analysis and association analysis, the identification of QTLs for cold tolerance at the early seedling and booting stages has been previously reported. In this study, Lijiangxiaoheigu, an elite cold-tolerant landrace, was used to develop the recombinant inbred lines. Totally, three QTLs for cold tolerance at the early seedling stage and four QTLs for cold tolerance at the booting stage were identified (Table 2). Among the QTLs for cold tolerance at the early seedling stage, *qCTS2-2* was identified on chromosome 2, and a previous study also detected a locus for rice seed vigor at a similar interval using the Daguandao/IR28 RIL population (Wang et al. 2010). *qCTS7* was identified on chromosome 7, which is consistent with the study of Han et al. (2006), which identified QTL for low-temperature vigor of germination in a similar position using a Jileng 1/Milyang 23 $F_{2:3}$ population (Han et al. 2006). Among the QTLs for cold tolerance at the booting stage, *qCTB1* was detected on chromosome 1, and similar genomic regions were previously reported to harbor QTLs for cold tolerance at the booting stage using the M-202/IR50 RIL population and Kunmingxiaobaigu/Towada F_2 population (Andaya and Mackill 2003; Dai et al. 2004). Likewise, *qCTB7* was detected on chromosome 7, and a previous study also reported the co-localized QTL for cold tolerance at the booting stage on chromosome 7 in the IR66160-121–4-4–2/Geumobyeo RIL population (Suh et al. 2010). The discovery of these reported QTLs further demonstrated the reliability of the QTLs identified in this study. In addition, novel QTLs such as *qCTS2-1*, *qCTB3,* and *qCTB5*, were also discovered in this study, which would provide new genetic resources for molecular breeding of cold tolerance in rice. *qCTS2-1*, similar to *qCTS2-2* and *qCTS7*, showed significant effects in improving cold tolerance at the early seedling stage (Fig. 3A). *qCTB5*, one of the identified QTLs for cold tolerance at the booting stage, contributed the highest phenotypic variance for seed-setting rate, highlighting its crucial role in the genetic control of cold tolerance at the booting stage (Table 2).

Fine mapping of quantitative trait loci mainly depends on three key elements: crossover density, marker density, and recombinant phenotypes (Yang et al. 2012). As quantitative traits are severely affected by genetic backgrounds, environmental conditions, and gene-by-environment interactions, the ability to obtain an accurate recombinant phenotype is the ultimate determining factor for successful QTL fine mapping (Poland et al. 2009). In the fine mapping of *qCTB1*, two cold treatment methods, including CS-HAA and CS-DW, were simultaneously utilized for fine mapping *qCTB1* to accurately evaluate the cold tolerance of the recombinants. Moreover, progeny test was performed within the recombinant-derived progeny families, which could minimize the influence of genetic background and environmental differences to accurately assess the true genetic effects of recombinants. Through the substitution mapping, *qCTB1* was finally fine-mapped to a 341-kb interval (Fig. 4), which laid a good foundation for further cloning of *qCTB1*.

By using fine mapping, parental sequence comparison, candidate gene-based association analysis, and expression pattern analysis, eight important candidate genes were identified for *qCTB1* (Table 3). Notably, we also identified sequence variations in the promoter or coding regions of the eight candidate genes between M-202 and IR50, and between Kunmingxiaobaigu and Towada (Table S3). Among

them, two genes (*Os01g0218032* and *Os01g0218900*) possess significant non-synonymous SNPs in the coding regions. *Os01g0218032* encodes DNA demethylase DNG702, which is involved in DNA methylation reprogramming and plays an important role in zygote initiation and embryo development. Zhou et al. reported that the *dng702* mutant failed to produce fertile embryos, while the *dng701* and *dng704* mutants resulted in delayed or aborted embryo development (Zhou et al. 2021). *Os01g0218900* encodes the PHD-finger domain-containing protein OsPHD3. Plant homeodomain (PHD) is a class of transcriptional regulators whose primary function is to recognize various histone modifications and bind to DNA, playing an important role in plant growth and development. Yang et al. reported that the PHD-finger domain-containing protein *qCTB7* influenced cold tolerance at the booting stage by altering the morphology and cytoarchitecture of anthers and pollen (Yang et al. 2023). In addition, there were 6 genes with significant SNPs in the promoters that responded differently to cold between the parents. *Os01g0214600*, *Os01g0215700*, and *Os01g0216300* encode the GDSL-like lipase OsGELP2, OsGELP6, and OsGELP8, respectively. GDSL esterase/lipase protein (GELP) is a kind of hydrolytic enzyme that plays an important role in pollen development. Zhao et al. reported that *OsGELP34* determines rice male fertility by mediating lipid homeostasis in the anther (Zhao et al. 2020). Zhang et al. reported that *OsGELP34*, *OsGELP110*, and *OsGELP115* are key factors regulating the development of the outer wall of pollen in rice (Zhang et al. 2020). *Os01g0218100* encodes a bHLH family transcription factor, OsbHLH037. The bHLH transcription factor plays an important role in the regulation of cold tolerance and pollen development in plants (Chinnusamy et al. 2003; Niu et al. 2013; Fu et al. 2014). *Os01g0218500* encodes the FLOWERING LOCUS T (FT)-Like homolog OsFTL1, which regulates flowering and reproductive development in rice (Giaume et al. 2023). *Os01g0218800* encodes the TRITHORAX-like protein OsSDG721, which regulates plant height, pollen grain development, and salt tolerance in rice (Liu et al. 2021). Based on the biological function described above, it could be judged that these eight genes may be involved in the regulation of pollen fertility under cold conditions and are possible candidates for *qCTB1*. These findings provide an important basis and direction for subsequent gene function analysis of *qCTB1*. Further transgenic functional validation, such as gene knockout (CRISPR/Cas9) and overexpression, as well as molecular and genetic interaction analyses, are needed to verify the cold tolerance function of these candidate genes and to explore their potential interactions.

Materials and methods

Plant materials

Lijiangxiaoheigu, a cold-tolerant *japonica* landrace from the Yunnan plateau, was used as the donor parent for cold tolerance. An RIL population (F_9 and F_{10}) consisting of 130 lines was developed by single seed descent from an F_2 population of a cross between the cold-sensitive *indica* cultivar Deyou17 and Lijiangxiaoheigu

and was used to identify QTLs for cold tolerance at the early seedling and booting stages.

To further fine-map *qCTB1* that confers cold tolerance at the booting stage, back-cross inbred lines were constructed with the RIL lines containing homozygous *qCTB1^{LJXHG}* as the donor parent and Deyou17 as the recurrent parent through continuous backcross and marker-assisted selection (Fig. S3).

Cold tolerance evaluation and statistical analysis

The cold tolerances at the early seedling stage of parents and the RIL population were evaluated in the winter of 2016. The experiments were repeated twice, with three replications each time. For each line, at least 60 manually selected filled grains were treated at 42 ℃ for 3 days and then soaked in distilled water at 28 ℃ for 2 days to allow the seeds to germinate. Twenty germinated seeds with 5 mm long coleoptiles were then placed on filter paper in a 9 cm diameter petri dish covered with 10 ml of distilled water, submerging the seeds just below the water surface (Fig. S1A). The dishes were then placed in a growth chamber (14-h/10-h light/dark cycle and 85% relative humidity) (Fig. S1B). We initially subjected the parents to different temperature conditions (6 °C, 8 °C, 10 °C, and 28 °C) for 10 days. Given that parental phenotypic differences were most significant at 6 °C, we subsequently chose 6 °C as the appropriate temperature for evaluating the cold tolerance of the RIL population at the early seedling stage for 10 days. Following the treatments, the growth chamber was adjusted to 28 ℃ for one week to allow the seedlings to resume normal growth. Cold tolerance at the early seedling stage was evaluated as seedling survival rate (survival seedlings/total seedlings cold-treated).

Two cold treatment methods, including cold stress in deep water (CS-DW) and in a high-altitude area (CS-HAA, altitude 1,974 m), were adopted in this study to evaluate the cold tolerance at the booting stage. By using the CS-DW method, the F_9 RIL population was evaluated for cold tolerance in 2014 (E1), and the F_{10} RIL population underwent evaluation for cold tolerance in 2015 (E2). The BC_3F_3 and BC_3F_4 populations were evaluated for cold tolerance by using both CS-DW and CS-HAA methods in 2018 and 2019. For CS-DW, the plants were sown in late April at the Shang Zhuang Experimental Station of the China Agricultural University in Beijing (40°08'N, 116°10'E). Thirty-day-old seedlings were transplanted to the field in 12.5 cm × 25 cm plots. Five individuals from each line with main panicles at booting were labelled, transferred to deep cold water (16–18℃) for one week, and then transplanted back to the field (Fig. S1C, D). After harvest, seed-setting rates under normal conditions (SS) and cold stress conditions (SSC), as well as the relative seed-setting rates (RSS, the ratio of SSC to SS), were measured to evaluate the cold tolerance. For CS-HAA, the plants were sown in early April in the field of the Yunnan Academy of Agricultural Sciences in Songming county, Yunnan province (25°05'N, 102°72'E). About thirty-day-old seedlings were transplanted in the field in 15 cm × 25 cm plots. The heading date was investigated. Songming county is situated at an altitude of 1,974 m, and the minimum temperature from June to September is below the critical temperature for cold stress at the booting stage, which

makes it a natural low-temperature environment suitable for identifying cold tolerance at the booting stage (Fig. S1E). Cold tolerance was evaluated on the basis of the mean seed-setting rates of the main panicles.

Basic statistical analysis, the Shapiro–Wilk test, linear regression analysis, and multiple comparisons were performed by SPSS software version 21.0 (SPSS, Chicago, USA). The relevant graphics were drawn using GraphPad Prism 8 software (GraphPad Software, San Diego, California, USA).

DNA extraction and molecular marker analysis

Genomic DNA was extracted from the fresh leaves by a modified CTAB method (Rogers and Bendich 1988). A total of 1,600 SSR markers distributed on all 12 chromosomes were used in a polymorphism survey between the parents. SSR markers that showed obvious polymorphism between the parents and were evenly distributed throughout the rice genome were used for genotype analysis of the RIL population. We also designed additional indel markers on chromosome regions based on the genome sequences of the parents to locate QTLs for cold tolerance (Table S1). PCR amplification was performed using *Taq* DNA polymerase (TransGen Biotech, China) with the following conditions: one cycle at 95℃ for 5 min, followed by 35 cycles of denaturing at 95℃ for 30 s, annealing at 55℃ for 30 s, and extension at 72℃ for 40 s, with a final extension at 72℃ for 10 min (Eastwin, ETC-811 Thermocycler, China). PCR products were detected using 8% (w/v) denaturing polyacrylamide gel electrophoresis followed by silver staining.

Linkage map construction and QTL mapping

A total of 166 polymorphic markers, including 97 SSR markers and 69 indel markers (Table S2), were used to construct the genetic linkage map using the JoinMap4 software by maximum likelihood mapping (Ooijen et al. 2006). The primers of SSR markers were developed using the reported methods (McCouch et al. 2002; Temnykh et al. 2000), and the primers of indel markers were designed according to the genome sequences of the parents. The Kosambi function was used to convert recombination frequencies to map distances in centiMorgans (cM), and the other parameters were default values. The genetic linkage map was drawn by the Mapchart software (Voorrips 2002).

To ensure the accuracy of cold tolerance at the early seedling stage, the mean value of seedling survival rates obtained from two experiments was used as the phenotypic indicator to identify QTLs for cold tolerance at the early seedling stage. To exclude the influence of the incompatibility of the *indica* and *japonica* crossing, RIL lines with SS less than 65% were eliminated in E1 and E2, and finally, 115 RIL lines were used to identify QTLs for cold tolerance at the booting stage. The composite interval mapping (CIM) method was used to determine the QTL by the Windows QTL Cartographer software version 2.5 (Wang et al. 2012). The parameters in QTL Cartographer were set as follows: model 6 (standard model), forward and backward regression, a window size of 10 cM, cross type of Ri1, and a walk speed of 1 cM.

The threshold for the logarithm of odds (LOD) to declare the presence of putative QTLs was determined by a permutation test with 1,000 repetitions at a significance level of $p < 0.05$. The confidence intervals were calculated by a two-LOD drop from the estimated QTL positions. The QTL naming convention was as described by McCouch et al. (1997).

Fine mapping of *qCTB1*

In 2018, BC_3F_2 individuals heterozygous for *qCTB1* were selected. Three BC_3F_3 family populations were planted in Beijing, and two populations were planted in Kunming. These populations were assessed for cold tolerance under the CS-DW and CS-HAA conditions, respectively. The flanking markers of *qCTB1* were used to identify the recombinants. In 2019, the progenies of the recombinants were planted in Beijing and Kunming, respectively. The recombinant BC_3F_4 progeny families were planted in plots of 40 plants. Polymorphic markers were developed between indel1_A8 and indel1_2 and were used to genotype the progeny population (Table S1). In each recombinant-progeny family, the seed-setting rates of the homozygous recombinants were compared with those of the BC_3F_4-qCTB1^{DY17} controls.

To fine-tune the position of *qCTB1*, the substitution mapping strategy as described by Paterson et al. (1990) was used.

Candidate gene analysis

LJXHG and DY17 were sequenced on the HiSeq2000 platform with high sequencing depth. The adapters of the raw reads were trimmed, and reads containing more than 50% low-quality bases (quality value ≤ 5) were removed. The SNPs were identified by aligning the cleaned reads to the reference genome of Nipponbare.

To identify candidate genes for *qCTB1*, association analysis on genes within the fine-mapped interval of *qCTB1* were carried out using TASSEL 5.0 software (Bradbury et al. 2007). This analysis involved 153 rice accessions and 480 polymorphic SNPs located in the 2,000-bp promoters, as well as non-synonymous SNPs in the coding regions. The sequencing data and cold tolerance phenotype at the booting stage for the 153 rice accessions were obtained from our previously published data (Table S4) (Guo et al. 2020). 462,514 SNPs distributed on 12 chromosomes were used to perform principal component analysis through GCTA software (Yang et al. 2011).

Data for tissue expression in the anther were collected from the Rice Genome Annotation Project (http://rice.uga.edu/) and the Rice Expression Database (http://expression.ic4r.org/). For the cold-induced expression analysis, LJXHG and DY17 were transferred to a phytotron (16–17℃) at the booting stage, and young panicles were sampled at different time points. Total RNA was extracted using the RNApure Total RNA Kit (Aidlab, China), and cDNA was generated using the TRUEscript RT Kit (Aidlab, China). qRT-PCR was conducted using the QuantStudio (TM) 6 Flex System (Applied Bio-systems) with the following thermal cycling parameters: initial

denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing/extension at 60 °C for 1 min, and a final extension at 72 °C for 5 min. *OsActin1* (*Os03g0234200*) was used as the internal reference.

Author contributions Z.L., Jinjie Li, and P.Y. conceived and designed the experiments. H.G. performed the experiments and wrote the manuscript. Yongmei Guo and Y.Z. provided the experimental materials and conducted the field experiment. Z.L., Jinjie Li, and N.U.K. revised the manuscript. A.Z., Yunsong Gu, Jin Li, X.S., Z.Z., H.Z., Y.P., H.L., and Z.W. provided support and experimental guidance for this study. All authors read and approved the final manuscript.

Data availability The data or material used in the current study are available from the corresponding author upon request.

Declarations

Ethics approval We declare that these experiments comply with the ethical standards in China.

Conflict of interests The authors declare no conflicts of interest.

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