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ORIGINAL PAPER

Fine mapping of the *qCTS12* locus, a major QTL for seedling cold tolerance in rice

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Abstract The temperate *japonica* rice cultivar M202 is the predominant variety grown in California due to its tolerance to low temperature stress, good grain quality and high yield. Earlier analysis of a recombinant inbred line mapping population derived from a cross between M202 and IR50, an *indica* cultivar that is highly sensitive to cold stress, resulted in the identification of a number of QTL conferring tolerance to cold-induced wilting and necrosis. A major QTL, *qCTS12*, located on the short arm of chromosome 12, contributes over 40% of the phenotypic variance. To identify the gene(s) underlying *qCTS12*, we have undertaken the fine mapping of this locus. Saturating the short arm of chromosome 12 with microsatellite markers revealed that *qCTS12* is closest to RM7003. Using RM5746 and RM3103, which are immediately outside of RM7003, we screened 1,954 F_5-F_{10} lines to find recombinants in the *qCTS12* region. Additional microsatellite markers were identified from publicly available genomic sequence and used to fine map *qCTS12* to a region of approximately 87 kb located on the BAC clone OSJNBb0071I17. This region contains ten open reading frames (ORFs) consisting of five hypothetical and expressed proteins of unknown function, a transposon protein, a putative NBS-LRR disease resistance protein, two zeta class glutathione S-transferases (*OsGSTZ1* and *OsGSTZ2*), and a DAHP synthetase. Further fine mapping with markers developed from the ORFs delimited the QTL to a region of about 55 kb. The most likely candidates for the gene(s) underlying *qCTS12* are *OsGSTZ1* and *OsGSTZ2*.

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Introduction

One important strategy for increasing crop productivity is to minimize losses due to biotic and abiotic stresses by developing more stress-tolerant varieties (Boyer 1982; Khush 1999). In temperate and high elevation areas, low temperature stress remains one of the most important limitations on production. Like other plants originating from tropical or subtropical climates, cultivated rice (*Oryza sativa* L.) is sensitive to temperatures below 15–20°C (Yoshida et al. 1996; Nakagahra et al. 1997). Under low temperature conditions, some common injuries include poor germination, seedling stunting, yellowing or withering, reduced tillering, delayed heading, and sterility (Kaneda and Beachell 1974; Mackill and Lei 1997). Given the wide-ranging effects of low temperature on rice and the serious impact on productivity, tolerance to this stress is of considerable importance to temperate rice growing regions of the world as well as to high altitude regions in South and Southeast Asia (Sthapit and Witcombe 1998).

Efforts to develop rice cultivars with improved cold tolerance (CT) typically focus on seedling and reproductive developmental stages, which have the greatest

impact on yield. In the case of rice seedlings, low temperatures can retard growth, and lead to poor stand establishment as well as delayed and non-uniform crop maturation. Seedling cold tolerance is of particular importance to direct seeding culture systems as planting typically coincides with the lowest temperatures of the season. Genetic analysis of seedling cold tolerance has resulted in the identification of a number of single genes and QTL that appear to be involved in different responses to cold stress. Kwak et al. (1984) identified a single dominant gene controlling leaf yellowing *Cts1(t)* while Nagamine (1991) reported a major gene for leaf withering, *Cts2(t)*. Although these studies suggest that major genes are involved in seedling cold tolerance, studies involving QTL analysis support the idea that cold tolerance is a complex trait involving multiple genes. Misawa et al. (2000) found that as many as 13 QTL, located on chromosomes 1, 3, 9 and 11, are associated with low temperature response when seedlings at the 2-leaf stage were subjected to 3 days at 4°C. More recently, Zhang et al. (2005) reported the identification of four putative QTL for seedling cold tolerance located on chromosomes 1, 3, 7 and 11. One QTL on chromosome 11, designated as *qSCT-11*, explained 30% of the phenotypic variance.

Of the two major subspecies of *O. sativa*, *indica* and *japonica*, the majority of rice grown in temperate regions are *japonicas* which possess better cold tolerance than *indicas* (Glaszmann et al. 1990; Mackill and Lei 1997). To determine the basis for the difference in low temperature tolerance between *japonica* and *indica*, Andaya and Mackill (2003) developed a recombinant inbred line mapping population using the cold tolerant California temperate *japonica* cultivar M202 and the cold sensitive *indica* cultivar IR50. Analysis of this population for seedling cold tolerance led to the identification of a number of M202 derived QTL that confer tolerance to cold-induced wilting and necrosis observed in the cold-sensitive *indica*, IR50. Of these loci, a major QTL affecting cold-induced wilting and necrosis, designated as *qCTS12* and which accounted for more than 40% of the phenotypic variation, was mapped to the short arm of chromosome 12.

Many studies have reported the identification of rice genes differentially expressed under low temperature stress conditions (Watanabe and Hirai 2002; Reyes et al. 2003; Wang et al. 2003; Morsy et al. 2005) and the improved cold tolerance in transgenic rice due to the expression of several genes (Saijo et al. 2000; Sato et al. 2001; Takesawa et al. 2002; Garg et al. 2002; Xiong and Yang 2003). However, although a number of QTL affecting seedling cold tolerance have been identified, the genes underlying these loci have yet to be isolated and characterized. The current challenge is to identify these genes, determine their mode of action, and devise ways of exploiting them to improve rice productivity under low temperature stress.

Here we report the fine mapping of the *qCTS12* locus to a 55 kb region containing eight candidate genes. Two of these genes are members of the zeta class of glutathione S-transferases (*OsGSTZ1* and *OsGSTZ2*). Over-expression of *OsGSTZ1* in transgenic rice has previously been demonstrated to enhance germination and seedling growth at low temperature (Takesawa et al. 2002). Taken together, these results suggest that *OsGSTZ1*, and possibly *OsGSTZ2*, are responsible for the tolerance to cold-induced wilting and necrosis conferred by *qCTS12*.

Materials and methods

Plant materials and DNA extraction

The recombinant inbred lines (RILs) used in this study were developed from a cross between M202 and IR50 (Andaya and Mackill 2003). A total of 1,954 RILs composed of 496 F_{10}, 518 F_6 and 940 F_5 individuals, each derived from independent F_2 plants and advanced via single seed descent, were screened with the microsatellite markers RM5746 and RM3103 which flank *qCTS12*. Recombinants between these markers were subjected to phenotyping for tolerance to cold-induced wilting and genotyping with additional molecular markers.

A high-throughput method was used to rapidly extract DNA from rice seedlings for PCR amplification of microsatellite markers. Approximately 1 cm^2 leaf tissue was placed into each well of a 96-deep well plate (PGC Scientific, Minneapolis, Minn.). Four 3 mm glass beads (Fisher Scientific, Pittsburgh, Penn.) and 400 µl $T_{0.1}E$ buffer (10 mM Tris–HCl, 0.1 mM EDTA, pH 8.0) were added to each well. Plates were capped, inserted in Minibeadbeater-96™ (Biospec Products, Bartlesville, Okla.) and agitated for 3 min. Samples were incubated at 65°C for 10 min, and 120 µl of 5 M potassium acetate was added. Samples were mixed by brief vortexing, and incubated on ice for 15 min. Plates were centrifuged briefly to pellet debris and 180 µl of each supernatant was transferred to new plates containing 120 µl isopropanol. The samples were vortexed gently and chilled in a −20°C freezer for 30 min to precipitate DNA. The samples were spun for 15 min before removing supernatants and air-drying pellets. Pellets were dissolved in 50 µl $T_{0.1}E$ buffer and 2 µl of the DNA was used per PCR reaction.

Seedling cold tolerance phenotyping

Seedling cold tolerance was assessed as described by Andaya and Mackill (2003) with some modifications as follows. For better seedling growth, six seeds from each of the 61 recombinants and the parental lines, M202 and IR50, were sown in 98-cell plug flats (Morton's Horticultural Products, McMinnville, Tenn.) containing fine field soil from the Rice Facility at Davis, CA. The flats were placed in a greenhouse until the seedlings reached the 3-leaf stage. They were then transferred to a Conviron PGV36 walk-in growth chamber (Controlled Environments Ltd., Winnipeg, Canada) with the following settings: 9°C constant temperature, 12 h light period provided by sixteen 400 W metal halide and twelve 100 W mercury vapor lamps, an average of 300 μmol m^{-2} s^{-1} irradiance and 70% RH. Inside the growth chamber, the flats were placed in tanks with 6 cm water depth to fully submerge the flats. Water temperature was maintained at an average of 20°C using a 300 W submersible aquarium water heater and a recirculation pump. Using this modification, necrosis appeared first as a sign of cold injury followed by wilting. Phenotyping was performed using a Randomized Complete Block Design with three replications. Plant reaction to cold stress as exhibited by necrosis and/or wilting was scored at 8, 10, 12, and 14 d of treatment using the scale of 1 (tolerant) to 9 (susceptible) as described in the Standard Evaluation Systems in rice (IRRI 1988). CT scores for the recombinants and parental lines were derived from the average of the scores from the four rating periods.

Two additional phenotyping experiments were conducted using either the original seeds of the 61 recombinants (F_5, F_6 and F_{10} seeds) or the progeny seeds derived from these lines (F_6, F_7 and F_{11}), and coded as E2 and E3, respectively. The experimental design was the same as the first phenotyping experiment (E1) except for the following changes: the use of Premier Promix BX (Premier Horticulture, Inc., Quakertown, Penn.) commercial soil instead of field soil, relative humidity of 85%, and scoring at 10, 12, 14 and 16 d of treatment. The average CT scores for each of the three experiments and the overall mean scores were calculated for QTL analysis.

Marker development and genotyping

Primers for microsatellite markers on the short arm of chromosome 12 (McCouch et al. 2002; http://www.gramene.org/) were obtained and used to survey the parents, M202 and IR50, for polymorphism. Subsequent analysis using the informative markers revealed that three markers, RM5746, RM7003 and RM3103, were closest to *qCTS12*. DNA sequences for 24 contigs covering the region between RM5746 and RM3103 were accessed from the Gramene database (http://www.gramene.org) and analyzed using the SSR Identification Tool (Temnykh et al. 2001; http://www.gramene.org/db/searches/ssrtool) and Primer3 (Rozen and Skaletsky 2000; http://www.frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Initially, primer pairs were designed to amplify 3 to 4 microsatellites per contig (designated CT). Polymorphic markers were identified and used for fine mapping (Table 1). Additional microsatellite markers were developed as needed.

Microsatellite marker analyses were as previously described (Andaya and Mackill 2003) with minor modifications. PCR reactions consisted of 1X PCR buffer (10 mM Tris–HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% Gelatin), 0.5 M Betaine, 240 nM of each primer, 20 μM of each dNTP, 375 nM Fluorescein-12 or Tetramethylrhodamine-6 labeled dUTPs (Molecular Probes, Eugene, Ore.), 10–50 ng template DNA and 0.6 units *Taq* polymerase in a final volume of 8 μl. Amplifications were performed using MJ Research PTC-200 thermal cyclers (Waltham, Mass.) and the following PCR profile: 95°C for 1 min, 35 cycles of 95°C for 15 s, 55°C for 1 min, 72°C for 30 s, with a final extension of 72°C for 5 min. PCR products were ran on an ABI 377 DNA sequencer with ROX-500 size standard using manufacturer recommended protocols and fragment analysis was made using GENESCAN ver. 3.1.2 (Applied Biosystems, Foster City, Calif.)

The two closest microsatellite markers, CT37-1 and CT38-1, which flank the *qCTS12* region, are located on a single BAC clone, OSJNBb0071I17 (Genebank Accession, AL954853). Publicly available annotation of this region (http://www.tigr.org) indicates the presence of 10 open-reading frames (ORFs) (Table 2). Using Web Primer (http://www.seq.yeastgenome.org/cgi-bin/web-primer/), primer pairs were designed to amplify regions from each ORF (Table 2). Genomic DNA for amplifying ORF-based markers were extracted using the procedure of Tai and Tanksley (1990). PCR amplification was performed as previously described (Andaya and Tai 2005). PCR reactions were performed in a 20 μl volume consisting of 200 ng DNA, 300 nM each primer, 1X PCR buffer (10 mM Tris–HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% Gelatin), 2 μM Fluorescein-12 or Tetramethylrhodamine-6 labeled dUTPs, 100 μM dNTPs, 1.5% DMSO, and 2 units Taq Polymerase. Amplifications were performed using the following profile: 94°C for 5 min, 30 cycles of 94°C for 1 min, 55°C for 2 min and 72°C for 3 min, with a final extension of 72°C for 7 min. PCR products were

Table 1 Polymorphic microsatellite markers designed from the sequences of the 24 BAC clones spanning the RM5746-RM3103 interval

Marker	Motif	GeneBank Clone Acc	Forward primer	Reverse primer	Size in M202, IR50
CT9	(cgc)7	AL713901	tcctctcatccacccaaatc	tcgaaccaacccaaacaaac	210, 240
CT10	(ga)12	AL713901	ttgtgtgtgttggggttttc	gtgaggaccaaacgagcatt	153, 147
CT11	(ta)15	AL713901	aacgtgttgcccatgctatc	cttcccttgataaggacattca	151, 290
CT18	(at)12	AL731879	tctagctgcacgtgcgtaat	gggaggggaggtgagtttag	183, 175
CT23	(ca)7	BX664711	tttatactccatccgtttgataatg	ttcctccgcgttttataaggt	151, –
CT26	(ta)21	BX649218	tcctatgtgcacgtccttca	gtcccttcaccaaactccaa	225, 175
CT27	(tat)25	BX649218	acccagcgtgcgtatgtact	gaaggaagagttaatctaggaagagg	148, 130
CT28	(ta)16	BX649218	gccaatttgaaccagctgtc	tcttgcaaatgttgtgtgctt	270, 327
CT31	(ta)16	BX000560	tcttgcaaatgttgtgtgctt	gccaatttgaaccagctgtc	270, 327
CT33	(taa)25	BX000560	ttaatctaggaagagggtggtg	acccagcgtgcgtatgtact	137, 120
CT36	(at)11	AL954853	agtcaaaaagagcggggaat	tccgcattaatccaatacga	209, 187
CT37	(at)13	AL954853	gctaagcgttttctaaacgaca	cgttaccaaccgggactaaa	148, –
CT37-1	(tgc)8	AL954853	gcaggctctcattctgacg	cgctagggaacgacagagaa	225, –
CT38-1	(ta)6	AL954853	aaattttatatacacgcatgcaaac	cccgccctctagtatgatctc	337, –
CT38-2	(ct)6	AL954853	gttgctcgaaggaatggaaa	ataccaaggcctcgatctca	186, 184
CT38-3	(ggc)5	AL713952	tctccgatcatgtcgattca	cggctgcaaatccttctcta	235, 223
CT38-4	(cgc)6	AL713952	ctccagcaagaggaaagacg	gtagaggccgtgccagtagt	223, 226
CT38-5	(gag)5	AL713952	ggaaggcggctaaggattta	ctttcgctggtatcgtcgag	239, 239
CT39	(tct)8	AL713952	tcttgccttcttggattcgt	tccgatccatctcaacaaca	–, 198
CT39-1	(ggc)5	AL713952	cccagctgacgaggaatcta	gctgctccagtgagtccaat	–, 225
CT52	(at)19	BX664712	cgccggcaaatgaattatac	ctgtttgctgcatggttga	295, 251
CT53	(at)11	BX664712	aagatagcttgctgctttcaa	aacataaaatttgtgggaacaaga	191, 197
CT57	(ta)11	AL831805	caaccctttagattcaaatcttatctt	agtcgcgcttttgatctgtt	245, 289
CT69	(atct)12	AL713939	cggtctgcattacaggatga	gaagtgtcacatccgtccaa	216, –
CT70	(aat)22	AL713939	gactagaaagaacgggacctg	agtagctgggggtttccaat	190, 213
CT73	(aat)13	AL713935	cgactaaattgaacttttggtgaa	gtgcacccgtgtttctcttt	227, 200
CT75	(ct)9	AL731740	atgtcggcctcacagttttt	ggtcgagagagcgagtgaga	223, –
CT85	(ct)21	AL928782	cggaggagaggagagagaca	ttttcagaagaaaacaaacaagg	190, 210
CT86	(ct)21	BX842241	ggggaagaagagagactaggg	tctgtccccagaattcagagat	163, 183
CT87	(ag)9	BX842241	aacccaagaacgaatgatgc	tggacgtacgaatgatcgaa	214, 212
CT88	(tc)8	BX842241	gagctcttcgaccgcatc	aacagctttgaatctttgattgc	170, 166
CT90	(aag)9	AL954160	cagcagccaaacaaaaacaa	gctagctgctcggaggttc	202, 220
CT92	(ag)10	AL954160	cagattgatttgtttgagggcta	aaaggatttcttttgtatttcattg	135, 165
CT95	(tc)10	AL831806	aaaggatttcttttgtatttcattg	cagattgatttgtttgagggcta	135, 165
CT97	(ttc)9	AL831806	ctagctgctcggaggttcg	aaaaagaagcaaacaacatcg	167, 184
CT99	(ac)12	AL732648	ttctgttaagctggagaagaaaca	ttgcaagaaactcagcatgg	177, 179
CT103	(ta)53	AL731756	gtcgctctcgctccataatc	catgcatgcgatgtcgtaat	–, 135

run on an ABI 377 DNA sequencer with ROX-1000 size standard and fragment sizes analyzed using GENESCAN ver. 3.1.2 (Applied Biosystems, Foster City, Calif.)

Mapping and QTL analysis

The additional polymorphic microsatellite markers (RM) on the short arm of chromosome 12 were genotyped and added to the previously reported linkage map (Andaya and Mackill 2003) using a Macintosh version MAPMAKER 2.0 (Lander et al. 1987). The initial map position of *qCTS12* was determined using PLABQTL (Utz and Melchinger 1996). Interval mapping was performed to fine map *qCTS12* by analyzing the phenotypic and CT marker data from the recombinants with MAPMAKER/QTL (Lander and Botstein 1989). Phenotypic and genotypic data management, analyses of variance, and correlation analyses were performed using MS EXCEL (Microsoft Corp., Redmond, Wash.)

Results

Identification of recombinants for fine mapping

Since the initial identification of *qCTS12*, analysis of the rice genome sequence has led to the identification and characterization of thousands of additional microsatellite markers (McCouch et al. 2002; http://www.gramene.org). Given this resource, we were able to identify a set of 14 polymorphic markers for refining the *qCTS12* locus map position. In the previous report,

Table 2 Open-reading frames between the microsatellite markers CT37-1 and CT38-1 in BAC clone OSJNBb0071I17 (GeneBank Accession, AL954853) and ORF-based markers designed to fine map *qCTS12*

ORF product[a]	Transcript length[a] (bp)	No. Exon[a]	Marker	Forward primer	Reverse primer	Size in M202, IR50
Hypothetical protein (HP1)	390	2	CT690	acgtcttccaggaacagcttg	atttgtcaatggacaccgca	–
Expressed protein (EP1)	4,025	13	CT700A	tcttggtaactggactgctca	gggggaaacagcaagtaacaa	1,000
			CT700B	agagagttggcaaggcctgta	tttttcttgccaggacctca	850
			CT700C	gatgaaggtgctgggcatgt	gtttttgccccttccaatct	900
			CT700D	agccctcttttctcctcgat	ctggtgaagagaagtcctcgt	–
			CT700E	tgaggaactcagatggtttca	tggagaaatacctgaattccc	1,050
			CT700F	tcctgtcgatatgagaccaca	tgcgatgatatatccctcaca	1,050
NBS-LRR (NBS)	4,719	4	CT710A	tgaagaacaagcttggggaa	cctggattcacaattgcttcc	–
			CT710B	caacaacccatgacccaaaa	ggagcaaacaacttttccca	1,200
			CT710C	agcagaagcagcacatggta	cgcttcaaagcaagcaagaa	950
			CT710D	ggaaagaatgggagcagaat	tgtcgatgtagcgaaggtgtt	1,000
			CT710E	ctttatcgtttgcggcatgta	gcagtttggggcattgtttt	1,000
			CT710F[b]	tcaaatccctacacatacgca	ccgcatccgtacgttcatt	1,000, 950
Glutathione S-transferase (GST)	1,276	10	CT720A[b]	cgaacggataggtccatgaat	gccgagacacacgtgtaactt	1,000,1,150
			CT720B	tgaagacaaatatccccagca	aaacacatccgcctgtacaca	1,100
			CT720C	gaagcaccccaatttcagaa	tcctcctcctcagctagcaa	1,100
Maleylacetoacetate isomerase (MAAI)	1,059	9	CT730A	tctgaaaaaggaccatcttgc	tccaagtttgtggctcatca	750
			CT730B	gacattggaattgcagtatgc	tctcatcggggcttagctta	900
			CT730C	tacagttccatcttggcttgc	ttcatttgatgacaaattccg	1,000
Expressed protein (EP2)	3,328	19	CT740A	tgtgttgacccgttcttgatg	tcagcaatcaggtctgaactg	1,000
			CT740B	tcgcagccattttgggataa	tttgcggtacctccccact	950
			CT740C[b]	ttgtttcatgaattcaggggc	taggtggagatttgtgcttgc	900, –
Hypothetical protein (HP2)	363	2	CT750	tctgtatgttatagccggcca	tgaagtggagttttggagca	–
Hypothetical protein (HP3)	519	2	CT760	tgtgatcatgcatgtttcgg	gcggtgatgatttatatgcaa	800
Transposon protein (TP)	687	4	–	–	–	–
DAHP synthetase I family (DAHP)	1,470	10	CT780A	atggagggaagtggacaaagt	ccaacagatttaaatgcgcc	1,000
			CT780B[b]	tcctgctattttccctccaca	tgcagcagaatttggtctca	760, 780

[a] Information from Gramene and TIGR (http://www.gramene.org; http://www.tigr.org)

[b] Polymorphic markers

the locus was closely associated with RM101. With eight of the markers reducing the gap between RM247 and RM101 (14 cM apart) on the short arm of chromosome 12 and subsequent linkage and QTL analyses, the locus position was found to be most closely associated with RM7003 (Fig. 2a).

The markers RM5746 and RM3103, which flank RM7003, were used in screening for recombinants around *qCTS12*. Based on the original recombinant inbred line mapping population of 191 individuals (Andaya and Mackill 2003), the RM5746-RM7003 and RM7003-RM3103 intervals are 1.5 cM and 2.7 cM apart, respectively. A total of 61 recombinants were identified by screening 1,954 RILs derived from independent F_2 plants (F_{10}=496, F_6=518, and F_5=940); 27/61 recombinants between RM5746-RM7003 and 34/61 recombinants between RM7003-RM3103.

Cold tolerance phenotyping

The 61 recombinants identified using microsatellite markers were subjected to cold tolerance phenotyping with the parental types M202 and IR50 serving as tolerant and susceptible controls. The analysis of variance for each phenotyping experiment and the combined analysis are presented in Table 3. The coefficients of variation (%) were 12.36, 10.11 and 11.19 for E1, E2 and E3 respectively. In all analyses, differences among genotypes were highly significant. Combined analysis also revealed significant genotype by experiment interaction. Broadsense heritability, computed using variance components estimates from combined analysis of three experiments for cold tolerance, was very high (h^2_B=0.98).

The assay used for cold tolerance screening in the growth chamber is highly reproducible. The CT scores for the controls, M202 and IR50, were consistent in all experiments. Likewise, tolerance ratings between the original recombinant lines and their progenies also showed similar scores as expected since the lines are essentially fixed.

The frequency distribution of cold tolerance scores is presented in Fig. 1. The overall mean cold tolerance ratings for M202 and IR50 were 1.31 (±0.08 SD) and 8.44 (±0.21 SD), respectively. Susceptibility of IR50 was evident as the cultivar approached a rating near 9

Table 3 Individual and combined analyses of phenotyping experiments to measure cold tolerance at the seedling stage using 61 recombinants and the two parents

Source of variation	Degrees of freedom	Mean square		
		E1	E2	E3
Genotype (G)	62	18.851 **	14.362 **	15.060 **
Replication	2	0.359 NS	3.351 **	0.082 NS
Error	124	0.354	0.205	0.284
CV (%)		12.36	10.11	11.19
		Combined		
Experiment (E)	2	5.9884 **		
Reps within E	6	1.2641 **		
Genotype	62	45.0344 **		
G × E	124	1.6279 **		
Pooled error	372	0.2810		
V_G=16.80				
V_E=0.28				
V_{GE}=0.45				
h^2_B=0.98				

NS not significant, V_G genetic variance, V_E environmental variance, V_{GE} genetic × environment variance, h^2_B broadsense heritability

**Significant at 1% level

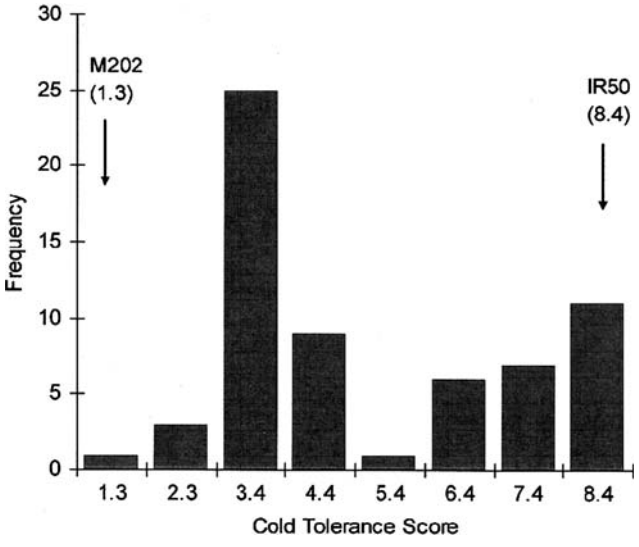

Fig. 1 Frequency distribution of cold tolerance scores based on the overall average of four scoring periods from the three phenotyping experiments. The mean scores of M202 and IR50 are indicated by *arrows*

at 8 to 10 d of cold treatment, whereas M202 remained at a score of below two even after 14 to 16 d. The majority of the recombinants were classified either as tolerant to moderately tolerant (CT score of 3–4; 59%) or susceptible (CT score of 8–9; 39%) to cold stress. A distinct bimodal frequency distribution pattern of CT scores is evident, indicative of a major gene controlling CT.

High resolution mapping and candidate gene identification

To identify and develop additional molecular markers between RM5746 and RM3103, sequences of the 24 BAC clones in the region between the two markers were obtained from the Gramene database (http://www.gramene.org). The RM5746-RM7003 interval was approximately 1.68 Mb containing 16 BAC/PAC clones while the interval RM7003-RM3103 was 688 kb spanned by eight clones. A minimum of three SSR markers (designated CT*N*) per BAC clone were designed to saturate the region. Initially, we identified 96 microsatellite markers and surveyed them for polymorphism. Using the genotypic information for the 30 CT*N* polymorphic SSR markers and the average cold tolerance score of the 61 recombinants in the first phenotyping experiment, a correlation analysis was performed as a preliminary analysis to identify the marker closest to *qCTS12*. The correlation coefficient (r) is highest with the marker CT37 with r=0.78. In contrast, the r value for the RM markers RM5746, RM7003 and RM3103 were 0.39, 0.67, and -0.37, respectively. These results suggest that the position of *qCTS12* has to be close to CT37. A second set of 20 SSR markers (designated CT*N*-*n*) was developed from which seven useful markers were identified. In total, 116 SSR markers in the region were examined, 37 of which were found to be polymorphic (Table 1) and were used for fine mapping in conjunction with the cold tolerance ratings derived from the combined analysis of three separate phenotyping experiments.

The closest microsatellite markers flanking *qCTS12* are CT37-1 and CT38-1, which are located 87 kb apart on a single BAC clone, OSJNBb0071I17 (Genebank Accession, AL954853). Six recombinants (RILs MIa 977, MIa 2993, MIa 333, MI 646, MI 81-4, and MIa 2097) within the region flanked by CT37-1 and CT38-1 were identified. All except MIa 2097 were tolerant to cold-induced wilting and necrosis (Fig. 2d). The high resolution genetic and physical maps of the *qCTS12* locus are shown in Fig. 2b and c. Based on the current gene annotation for BAC clone OSJNBb0071I17 (http://www.tigr.org), there are ten putative open-reading frames in the 87 kb target region (Fig. 2c, Table 2). The ORFs encode three hypothetical (HP1, HP2, HP3) and two expressed proteins (EP1, EP2) of unknown function, a transposon protein (TP), a putative NBS-LRR disease resistance protein (NBS), two glutathione S-transferases (GST, MAAI), and a DAHP synthetase (DAHP).

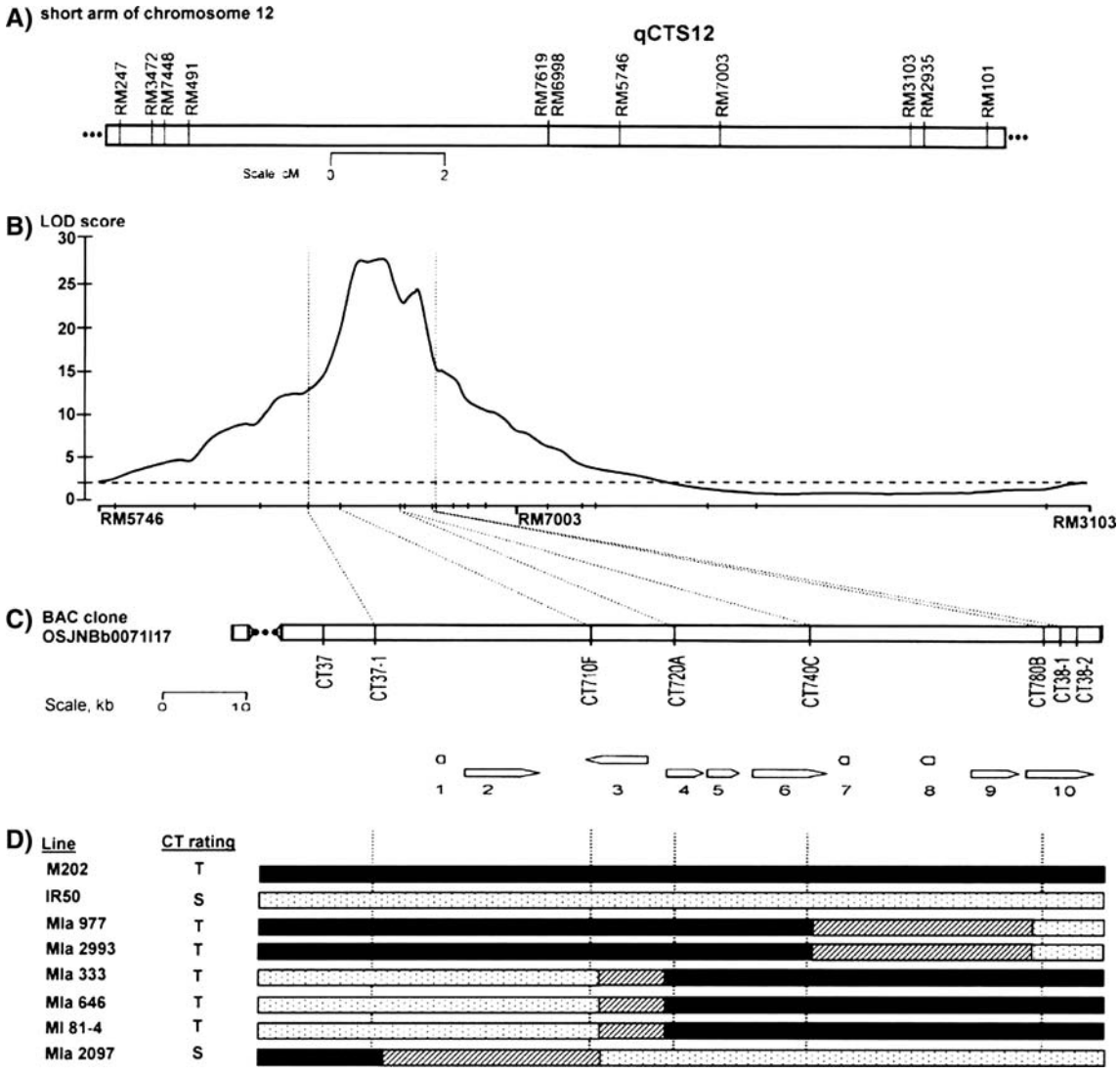

Fig. 2 **a** Position of *qCTS12* locus on the short arm of chromosome 12 after additional microsatellite markers in the RM247-RM101 interval were mapped. **b** Interval mapping of *qCTS12* on the short arm of chromosome 12 using the 61 recombinants. **c** Candidate genes within the region flanked by the microsatellite markers CT37-1 and CT38-1. Legend: 1=hypothetical protein (HP1), 2=expressed protein (EP1), 3=NBS-LRR disease resistance protein (NBS), 4=Glutathione S-transferase (GST), 5=Maleylacetoacetate isomerase (MAAI), 6=expressed protein (EP2), 7–8=hypothetical protein (HP2, HP3), 9=transposon protein (TP), and 10=DAHP synthetase (DAHP). **d** Genotypes and phenotypes of the 6 recombinants between CT37-1 and CT38-1. *Solid bars*=homozygous M202 allele; *dotted bars*=homozygous IR50 allele; *hatched bars*=site of recombination

Sequences of these putative ORFs were taken from the Gramene database (http://www.gramene.org) to design PCR-based markers. From a total of 26 ORF-based primer pairs, only four (CT710F, CT720A, CT740C and CT780B) detected polymorphisms (Table 2). The locus is further delimited to a 55 kb region flanked by CT710F and CT780B, which contains all the ORFs except HP1 and EP1. Interval analysis indicates that the QTL peak with LOD score=27.2 is located within the interval defined by CT710F and CT740C.

Discussion

In an earlier study by Andaya and Mackill (2003), major QTL were identified for seedling cold tolerance expressed as cold-induced wilting tolerance (CIWT) and cold-induced necrosis tolerance (CINT), and designated *qCTS12a* and *qCTS12b*, respectively. Though the injuries manifested as wilting and necrosis appeared to be distinct, both traits were strongly correlated. Since *qCTS12a* and *qCTS12b* were mapped to the same position in chromosome 12, there is a distinct

possibility that the two QTL are the same. Thus, the new designation *qCTS12* conferring tolerance to wilting and necrosis is used here.

Using over 1,900 RILs derived from a cross between M202 and IR50, we have defined an interval of about 55 kb flanked by the ORF-based markers CT710F and CT780B on the short arm of chromosome 12 that contains the *qCTS12* locus. The eight putative ORFs in the delimited region encode three proteins (two hypothetical and one expressed) of unknown function, a NBS-LRR disease resistance protein, two glutathione S-transferases, a transposon protein, and a DAHP synthetase.

The two glutathione S-transferases (GSTs) at the *qCTS12* locus are *OsGSTZ1* (annotated as a glutathione S-transferase, GST) and *OsGSTZ2* (annotated as a maleylacetoacetate isomerase, MAAI), which are both members of the zeta class of GSTs. GSTs are enzymes that perform a wide array of functional roles using glutathione as a cosubstrate or coenzyme (Marrs 1996; Dixon et al. 2002) and are encoded by large gene families (Marrs 1996; Dixon et al. 2002; Soranzo et al. 2004). These enzymes are thought to contribute to tolerance against various stresses such as cold, salt and drought through detoxification of xenobiotic compounds and reactive oxygen species (Marrs 1996; Takesawa et al. 2002).

In plants, GSTs are divided into five classes based on gene sequence identity: phi, tau, theta, zeta and lambda (Dixon et al. 2002). The rice genome contains three zeta class GSTs, two of these, *OsGSTZ1* and *OsGSTZ2*, are tandemly arranged on chromosome 12 (Soranzo et al. 2004; Tsuchiya and Nakamura 2004). Members of this class have been shown to have maleylacetoacetate isomerase activity which catalyzes the conversion of maleylacetoacetate to fumarylacetoacetate, the penultimate step in the tyrosine/phenylalanine catabolic pathway (Thom et al. 2001). Importantly, over-expression of *OsGSTZ1* in transgenic rice has been shown to result in enhanced germination and growth of seedlings at low temperature (Takesawa et al. 2002). This finding, taken together with our genetic mapping analysis, strongly suggests that we have identified the *qCTS12* gene(s) responsible for tolerance to cold-induced wilting and necrosis in rice seedlings.

Given the role of GSTs as detoxifying enzymes and their possible involvement in various stress responses, *OsGSTZ1* and *OsGSTZ2* appear to be the most likely candidates for *qCTS12*. Furthermore, the finding of Takesawa et al. (2002) that over-expression of *OsGSTZ1* results in enhanced germination and growth of rice seedlings at low temperature strongly suggests that *OsGSTZ1* is *qCTS12*, but does not rule out the possible involvement of *OsGSTZ2* or the six other candidate genes. Confirmation of the identity of the gene or genes underlying the tolerance to cold-induced wilting and necrosis conferred by *qCTS12* awaits functional complementation studies.

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