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Quantitative Trait Loci for Cold Tolerance of Rice Recombinant Inbred Lines in Low Temperature Environments

Wenzhu Jiang[1,6], Yong-Mei Jin[2,6], Joohyun Lee[2], Kang-le Lee[2], Rihua Piao[2], Longzhi Han[3], Jin-Chul Shin[4], Rong-De Jin[5], Tiehua Cao[5], Hong-Yu Pan[1], Xinglin Du[1,*], and Hee-Jong Koh[2,*]

Low temperature is one of the major environmental stresses in rice cultivation in high-altitude and high-latitude regions. In this study, we cultivated a set of recombinant inbred lines (RIL) derived from Dasanbyeo (*indica*) / TR22183 (*japonica*) crosses in Yanji (high-latitude area), Kunming (high-altitude area), Chuncheon (cold water irrigation) and Suwon (normal) to evaluate the main effects of quantitative trait loci (QTL) and epistatic QTL (E-QTL) with regard to their interactions with environments for cold-related traits. Six QTLs for spikelet fertility (SF) were identified in three cold treatment locations. Among them, four QTLs on chromosomes 2, 7, 8, and 10 were validated by several near isogenic lines (NILs) under cold treatment in Chuncheon. A total of 57 QTLs and 76 E-QTLs for nine cold-related traits were identified as distributing on all 12 chromosomes; among them, 19 QTLs and E-QTLs showed significant interactions of QTLs and environments (QEIs). The total phenotypic variation explained by each trait ranged from 13.2 to 29.1% in QTLs, 10.6 to 29.0% in E-QTLs, 2.2 to 8.8% in QEIs and 1.0% to 7.7% in E-QTL × environment interactions (E-QEIs). These results demonstrate that epistatic effects and QEIs are important properties of QTL parameters for cold tolerance at the reproductive stage. In order to develop cold tolerant varieties adaptable to wide-ranges of cold stress, a strategy facilitating marker-assisted selection (MAS) is being adopted to accumulate QTLs identified from different environments.

INTRODUCTION

Low temperature, one of the limiting factors in world rice production, causes yield losses of about 10% per year (Wu and Grag, 2003). In rice, cold injury can be caused by exposure to cool weather or cold irrigation water at any developmental stage. During the reproductive stage, rice is highly susceptible; cold stress during this time causes many types of phenotypic damage, such as delayed heading (which extends the flowering period), incomplete panicle exertion and degeneration of spikelet sterility (Jiang et al., 2010; Ye et al., 2009). The critical nightly temperature capable of inducing cold damage ranges from 13 to 15°C, and varies depending on cultivars and the duration of the low temperature (Farrell et al., 2006). In a previous study, Satake (1976) concluded that the thresholds of air temperature capable of inducing cold damage during the reproductive stage were 20°C for cold-sensitive varieties and 15°C for cold-tolerant varieties. Naturally low temperature areas, such as Kunming (China), a high-altitude area in a sub-tropical region, and Yanji (China), a high-latitude area, are ideal places for screening cold tolerance (Dai et al., 2004; Jiang et al., 2010; Xu et al., 2008). To minimize the field uncertainty of low temperature patterns, cold tolerance screening must be performed in various screening facilities, including natural conditions and artificially controlled environments (Jiang et al., 2010).

Molecular marker-based QTL analyses have greatly improved over time, thus it has become possible to analyze complex traits. Many studies using QTL mapping for cold tolerance during the reproductive stage have been conducted using various rice populations. The QTLs for cold tolerance of Norin-PL8 were identified in the introgressions on chromosomes 3 and 4, and the fine mapping of the QTLs on chromosome 4 revealed that the QTLs were comprised of two closely linked genes, *Ctb1* and *Ctb2*; furthermore, the *Ctb1* gene, encoding an F-box protein, was isolated by map-based cloning (Saito et al., 1995; 2001; 2004; 2010). Li et al. (1997) identified two QTLs on chromosomes 1 and 12 using BC_1F_1 and F_2 populations. Takeuchi et al. (2001) identified three QTLs on chromosomes 1, 7 and 11 using a double haploid (DH) population. Liu et al. (2003) mapped three QTLs on chromosomes 1, 6 and 7 in a cold tolerant, wild rice introgression line. Andaya and Mackill (2003) identified nine QTLs on chromosomes 1, 2, 3, 5, 6, 7, 9 and 12 using a RIL population. Dai et al. (2004) mapped nine QTLs on

[1]College of Plant Science, Jilin University, Changchun, 130062, China, [2]Department of Plant Science, Research Institute of Agriculture and Life Sciences, and Plant Genomics and Breeding Institute, Seoul National University, Seoul 151-921, Korea, [3]Key Laboratory of Crop Germplasm Resources and Biotechnology, Ministry of Agriculture, Institute of Crop Science, Chinese Academy of Agricultural Sciences, Beijing 100081, China, [4]National Institute of Crop Science, Rural Development Administration, Suwon 441-857, Korea, [5]Jilin Academy of Agricultural Sciences, Changchun 130124, China, [6]These authors contributed equally to this work.
*Correspondence: heejkoh@snu.ac.kr (HJK); duxinglin2004@163.com (XD).

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chromosomes 1, 3, 4, 6, 7, 10 and 12 for cold tolerance during the reproductive stage using a F_2 and F_3 population derived from Towada (cold sensitive parent) and Kunmingxiaobaigu (cold tolerant parent); however, Xu et al. (2008) identified nine QTLs on chromosomes 1, 4, 5, 10 and 11 using BC_5F_3 NILs derived from the same parents. Additionally, several groups have recently mapped many QTLs for cold tolerance during the booting stage, such as QTLs on chromosomes 1 and 10, using a RIL population (Kuroki et al., 2009), QTLs on chromosomes 3, 7 and 9 using RIL population (Suh et al., 2010), QTLs on chromosome 10 using F_2, BC_1F_1 and BC_2F_1 populations and QTLs on chromosomes 3, 4 and 1 using a RIL population (Mori et al., 2011). Although QTLs for cold tolerance during the reproductive stage have been mapped on all 12 chromosomes, only two QTLs (*qCTB-8* and *qCTB-7*) were narrowed down to a 200 kb region (Kuroki et al., 2007; Zhou et al., 2010). Furthermore, only one cold tolerance gene (*Ctb1*) has been cloned (Saito et al., 2010).

Cold tolerance during the reproductive stage, one of the most genetically complex traits in rice, is intrinsically quantitative and exhibits substantial environmental variation (Jiang et al., 2010; Mori et al., 2011). Therefore, it is influenced by multiple QTLs, which may have different effects in any given environment. The QTL identification must be based on phenotypic data from multiple environments that are representative of a range of target environments, and the QTL × environment interaction (QEI) patterns must be investigated before the usefulness of a QTL can be determined. Although QEI has been the focus of many studies in rice (Fan et al., 2005; Kovi et al., 2011; Li et al., 2001; 2005; Talukder et al., 2005; Zhuang et al., 2002), many of these studies have treated this phenomenon as a nuisance, focusing primarily on QTL main effects. Thus, potentially valuable patterns of QEI may have been neglected, due to the absence of appropriate statistical methods, which allow for meaningful exploration and useful descriptions of QEI.

In order to identify QTLs for cold tolerance during the reproductive stage, in the present research, we quantified additive and epistatic QTLs with regard to their interactions with environments using an RIL population grown in four different cold stress locations.

MATERIALS AND METHODS

Plant materials

An RIL population (F_9) consisting of 152 lines was produced from a cross between the rice varieties Dasanbyeo and TR22183. Dasanbyeo, a genetically divergent *tongil*-type variety developed in Korea, is sensitive to cold temperatures (Park et al., 2004). TR22183, a temperate *japonica* variety developed in northern China, is highly tolerant to low temperatures.

For phenotypic evaluation of cold tolerance, the RIL population was planted in four different low temperature locations (Suwon, Chuncheon, Kunming, and Yanji). A molecular map, consisting of 216 markers, was developed with the DNA extracted from F_9 lines as previously described by Cho et al. (2007) and Jiang et al. (2011). To confirm the QTL effects of cold tolerance, four lines harboring one or more target QTL regions were selected from the RIL population for developing NILs. These lines were backcrossed with their parent, Dasanbyeo, twice to produce a set of BC_2F_1 generations. A total of 139 markers for NILs were used background and foreground selection. After marker selection, several BC_2F_1 plants containing *japonica* segments harboring target QTLs (alleles from TR22183), and a small portion of other segments originating from *japonica* varieties, were selected and selfed twice to produce BC_2F_3 generations. Finally, cold tolerance was evaluated using the BC_2F_3 line of each introgression type and their parents in a cold water irrigation nursery in Chuncheon in 2005 (BC_2F_3) and 2006 (BC_2F_4).

Field trials and trait evaluation

The field trials and meteorological environments in this experiment were described in our previous paper (Jiang et al., 2010). The RIL populations and parents were cultivated in the year 2003 in four diverse locations, i.e., the Experimental Farm of Seoul National University (Korea, 127°36′E, 37°51′N, 74 m a.s.l.), the Chuncheon substation of the National Institute of Crop Science, RDA (Korea, 127°2′E, 37°16′N, 36 m a.s.l.), Yunnan Academy of Agricultural Sciences (Kunming, China, the high-altitude area, 102°41′E, 25°1′N, 1916 m a.s.l.) and Yanbian Academy of Agricultural Sciences (Yanji, China, the high-latitude area, 129°24′E, 42°46′N, 242 m a.s.l.). Among these four locations, Suwon was designated as the control location and Chuncheon, Kunming and Yanji as cold treatment locations. From the tillering to the ripening stage, the daily average temperature was generally higher than 20°C in Suwon and Chuncheon; however, it was frequently below 20°C in Yanji and Kunming. In Chuncheon, cold water at 17°C was irrigated for a 5 cm depth from 20 days after transplanting through the ripening stage; therefore, chilling damage was only induced by cold water irrigation in this case. In Kunming and Yanji, the cold treatment was naturally conducted in the field, due to low atmospheric temperatures. The water levels were maintained at a 10 cm and 5 cm depth in Kunming and Yanji, respectively. The diurnal temperature range was narrower in Kunming than in Yanji. The rainfall in the four experimental locations was mainly concentrated between May and September. The amount of rainfall was highest in Chuncheon and was lowest in Yanji. Yanji had the longest sun duration; the other three locations had similar sun durations. The average wind speed was similar in the three cold treatment locations. All experiments were designed as complete blocks with 8-12 plants per replication and three replications per location. Weeds, diseases and insects were strictly controlled throughout the entire rice growth period in all experimental locations.

A total of nine traits related to cold tolerance were evaluated in the RIL population across four locations. These traits included days to heading (DTH), culm length (CL), panicle length (PL), panicle exsertion (PE), panicle number (PN), spikelet number (SN), spikelet fertility (SF), grain weight (GW) and phenotypic acceptability (PA).

Data analysis

An analysis of variance (ANOVA) for each RIL population receiving cold treatments was performed using the SAS PROC MIXED procedure (SAS Institute, 1999). Variance components for estimating broad sense heritability (h^2) were estimated by a model, wherein all factors were considered randomly using the restricted maximum likelihood (REML) option of the SAS PROC VARCOMP procedure (SAS Institute 1999). The h^2 of cold tolerance over locations was estimated as:

$h^2 = \delta_\alpha^2 / (\delta_\alpha^2 + \delta_{\alpha\beta}^2 / j + \delta_\varepsilon^2 / jk)$, where δ_α^2, $\delta_{\alpha\beta}^2$ and δ_ε^2 represent the genotype, genotype × environment interaction and residual variance components, respectively; *j* represents the number of location and *k* represents the replicates (Hill et al., 1998).

QTL analysis

QTL analysis for target traits was performed based on a genetic linkage map. In the analysis, we performed composite interval mapping using a mixed linear approach and the computer soft-

Table 1. Means and phenotypic variations in the RIL population and parents for days to heading (DTH), culm length (CL), panicle length (PL), panicle exsertion (PE), panicle number (PN), fertile spikelet number (FSN), sterile spikelet number (SSN), spikelet number (SN), spikelet fertility (SF), grain weight (GW) and phenotypic acceptability (PA) in 2003 and 2004, and their broad-sense heritabilities (h^2)

Trait	Location	Parents			RIL population				h^2
		Dasanbyeo	TR22183	Diff[a]	Mean	Range	Skewness	Kurtosis	
DTH	Suwon	116d[b]	99c	*	107d	93-129	0.79	-0.70	68.1
	Chuncheon	126c	106b	*	120b	101-157	1.59	4.48	
	Yanji	133b	106b	*	116c	100-139	0.23	-0.50	
	Kunming	141a	126a	*	131a	120-158	1.01	0.77	
CL	Suwon	78.0a	84.7a	*	73.0a	48.3-126.7	1.44	2.98	70.6
	Chuncheon	54.1b	66.5b	*	48.4d	25.7-96.8	1.33	2.89	
	Yanji	49.3b	61.3b	*	59.3c	24.7-118.3	1.25	2.49	
	Kunming	40.0c	52.3c	*	47.2d	18.9-84.7	0.85	2.12	
PL	Suwon	24.4a	25.7a	NS	22.3b	15.2-29.8	0.18	0.26	82.0
	Chuncheon	20.7b	22.8b	*	19.9c	12.3-28.3	-0.09	1.16	
	Yanji	24.7a	26.4a	*	22.3b	15.3-28.4	-0.08	0.29	
	Kunming	20.2b	22.3b	*	19.9c	10.6-26.6	-0.01	0.67	
PE	Suwon	2.8a	5.5a	*	2.5c	-5.2-16.2	0.50	0.62	64.1
	Chuncheon	-1.1b	3.0b	*	-3.6d	-19.0-6.7	0.33	0.51	
	Yanji	-2.0b	2.4c	*	10.9b	-2.0-26.2	0.33	2.38	
	Kunming	-4.3c	0.6c	*	-2.6d	-13.9-10.5	0.01	1.23	
PN	Suwon	11b	9b	NS	12b	4-21	0.18	0.78	46.0
	Chuncheon	8b	7b	NS	7d	1-14	0.59	0.75	
	Yanji	22a	16a	*	21a	11-34	1.34	3.14	
	Kunming	12b	18a	*	13b	2-38	1.09	2.09	
SN	Suwon	235a	246b	NS	180c	72-343	0.65	1.08	40.7
	Chuncheon	158b	233b	*	164c	54-274	0.09	0.06	
	Yanji	176b	288a	*	195b	68-327	0.41	0.26	
	Kunming	118c	201c	*	149d	27-239	0.10	0.13	
SF	Suwon	83.0a	85.0a	NS	81.8a	63.0-93.4	-1.52	3.16	53.3
	Chuncheon	40.8b	70.2b	*	41.3d	0-81.3	0.85	-0.70	
	Yanji	23.8c	86.8a	*	59.4c	0-90.5	0.98	0.59	
	Kunming	24.7c	67.7c	*	22.3d	0-72.6	0.72	-0.60	
GW	Suwon	25.8a	25.3a	NS	25.0a	17.6-31.4	0.08	-0.30	58.6
	Chuncheon	24.3b	22.0c	*	21.6c	10.7-33.7	-0.01	0.85	
	Yanji	17.3c	24.1b	*	21.4c	9.7-30.3	-0.09	0.05	
	Kunming	22.0a	24.2b	*	23.9b	16.7-29.7	-0.29	-0.40	
PA	Chuncheon	7a	3a	*	5.8a	3-9	-0.25	-0.37	61.3
	Yanji	9a	3a	*	6.0a	3-9	-0.17	-1.30	
	Kunming	9a	3a	*	6.1a	3-9	-0.05	-0.90	

[a]* and NS signify a difference between Dasanbyeo and TR22183 at the 5% significance level and at a non-significant level with an LSD test, respectively.
[b]Means followed by the same letter within a given trait are not significantly different at the 5% level among locations, as verified by an LSD test

ware QTLMapper 1.0 (Wang et al., 1999). The value of the likelihood ratio (LR) corresponding to $P \leq 0.005$ (equivalent to LOD = 2.79 for $df = 3$) was used for estimation of main effect QTL (QTL). To determine the empirical significance threshold for declaring a QTL, 10,000 permutations were performed to calculate the thresholds of the LOD score using the software Qgene 3.06 for Macintosh (Kim et al., 2004; Nelson, 1997). The threshold of the LOD score ($P = 0.05$) by the permutation test ranged from 2.96 to 3.02 for various traits. For epistatic QTLs (E-QTLs) and QTL × environment interactions (QEIs), the LR value corresponding to $P \leq 0.001$ was used as the threshold for claiming the presence of putative epistatic QTLs and QEIs. The genetic effects of the QTL components were further tested using the Bayesian re-sampling procedure. The effect of the QTL was the accumulated effect expressed in the same way across different locations, while the interaction effect was deviated because of the specific environment. In a specific environment, the total effects of a QTL should include the main effects plus QEI effects of the environment. The proportion of total phenotypic variance explained (PVE) was estimated by

Table 2. Correlation coefficients between cold-related traits in the RIL population across locations

	DTH[a]	CL	PL	PE	PN	SN	SF	GW
CL	0.231**							
PL	0.278**	0.603**						
PE	0.053	0.737**	0.317**					
PN	-0.494**	0.116	0.046	0.079				
SN	0.338**	0.538**	0.558**	0.408**	-0.107			
SF	-0.109	0.198*	-0.038	0.275**	0.055	-0.042		
GW	-0.102	0.094	0.216**	0.001	0.073	-0.126	0.264**	
PA	0.154	-0.269**	-0.066	-0.299**	-0.198*	-0.114	-0.398**	-0.287**

[a]Refer to Table 1 for abbreviations

**Significant at $P < 0.01$, * Significant at $P < 0.05$

Table 3. Mean square value for agronomic traits of the RIL population across locations

Source	DF[a]	DTH[b]	CL	PL	PE	PN	SN	SF	GW	PA
Location (L)	3	56242.5**	67650.8**	1643.9**	5323.8**	18614.3**	232921.0**	375309.3**	1270.4**	4.9**
Replication	2	35.5	26.6	10.9	0.9	9.9	951.1	171.0	39.3**	1.3
Genotype (G)	154	545.4**	1254.9**	58.1**	123.2**	56.4**	16463.8**	2150.0**	59.1**	21.3**
G×L	462	43.9**	135.5**	12.9**	22.6**	29.7**	3393.3**	852.6**	41.5**	9.6**
Error	1240	24.4	13.6	4.9	4.1	7.0	755.0	75.1	7.6	0.8

[a]Degree of freedom

[b]Refer to Table 1 for abbreviations

** Significant at $P < 0.01$, * Significant at $P < 0.05$

the sum of phenotypic effects (R^2) of each QTL or epistatic QTL or QEIs for each location. The loci in which QTLs have been identified have been labeled according to their chromosome and their position on the chromosome. Hence, the three regions containing QTLs on chromosome 8 were labeled 8.1, 8.2, and 8.3, with 8.1 being the uppermost.

RESULTS

Phenotypic variation

The variability parameters for nine cold tolerance-related traits of the RIL population and parents are listed in Table 1. Large differences between the two parents were observed in all nine cold tolerance evaluating parameters. TR22183 contributed towards characteristics of strong cold tolerance and its related-traits (tall stature, panicle exertion length, large spike and more grains, high fertility and good PA). The values of these traits suggested that TR22183 was more tolerant to low temperatures than Dasanbyeo in the three cold treatment locations. The values of CL, PL, PE, SN, SF and PA were largely reduced in the RIL population and parents in the cold treatment locations, indicating that the treatments were successful for evaluating substantial differences in cold-related traits. Results of the skewness and peak of distribution curves of the nine traits are presented in Table 1. The distribution curves for most traits were found to be platykurtic, with a kurtosis value of less than 3. The RIL population was similar to Dasanbyeo in PL, GW and PA, and was similar to TR22183 in DTH, CL, PE, PN, SN and SF. The continuous distribution of parental classes indicated that major, as well as minor, genes contributed to the cold tolerance traits.

The correlation traits for cold tolerance of the RIL population at the booting stage (across all locations) are listed in Table 2. The SF was positively correlated with CL, PE and GW, and negatively correlated with the PA. The PA was negatively correlated with CL, PE, PN, SF and GW. Unexpectedly, DTH had no significant correlation with SF or PA, indicating that a late heading date did not affect the SF across locations. The ANOVA revealed that all 9 traits exhibited significant differences in location, genotype, and location × genotype (Table 3). Broad sense heritability (h^2) of the cold-related traits estimated across locations was relatively large in the RILs ranging from 40.7% for SN to 82% for PL, although the experimental locations were extremely diverse (Table 1).

QTL for each trait

Details of the QTLs identified for each trait are provided in Supplementary Table 1, and a summary of the QTL positions on the molecular map is given in Fig. 1. A total of 36 loci revealed QTLs overall. Of these 36 loci, 10 revealed QTLs for DTH, 4 in both Suwon (control) and Chuncheon, 3 in Yanji and 2 in Kunming. Only 2 of these loci (3.1 and 8.2) contained QTLs in more than one cold treatment location. A total of 6 loci revealed QTLs for CL, 4 in Chuncheon, 3 in both Suwon and Kunming and 1 in Yanji. Of these, 2 loci (2.1 and 4.3) were identified in 3 cold treatment locations, while a single locus (7.3) was identified in 2 cold treatment locations. A total of 7 loci revealed QTLs for PL, 2 in both Suwon and Kunming and 3 in both Chuncheon and Yanji. Three of these loci (4.1, 5.1, and 5.3) were identified in 2 locations. A total of 9 loci revealed QTLs for PE, 2 in Suwon, and 4 in Chuncheon, Yanji, and Kunming. One QTL (4.3) was identified in 3 cold treatment locations, while 3 QTLs (1.1, 7.2, and 7.3) were identified in 2 locations. A total of 3 loci revealed QTLs for PN, 2 in Suwon and 1 in both Chuncheon and Yanji. Of these, 1 QTL (3.4) was identified in 2 cold treatment locations. A total of 3 loci revealed QTLs for SN, 1 in both Suwon

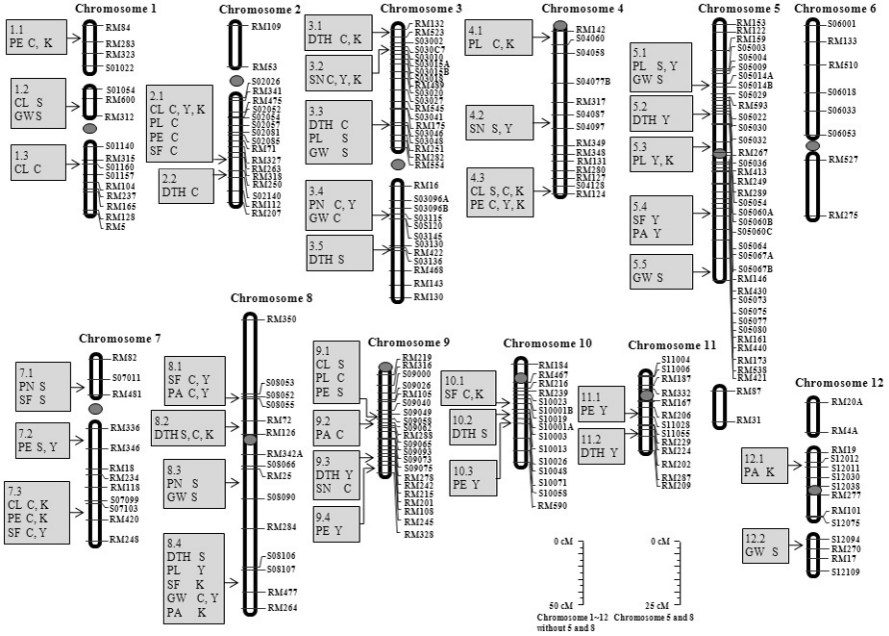

Fig. 1. Linkage map of Dasanbyeo × TR22183 mapping population showing the location of QTLs for the cold-related traits for each of the four treatments (S, Suwon; C, Chuncheon; Y, Yanji; K, Kunming).

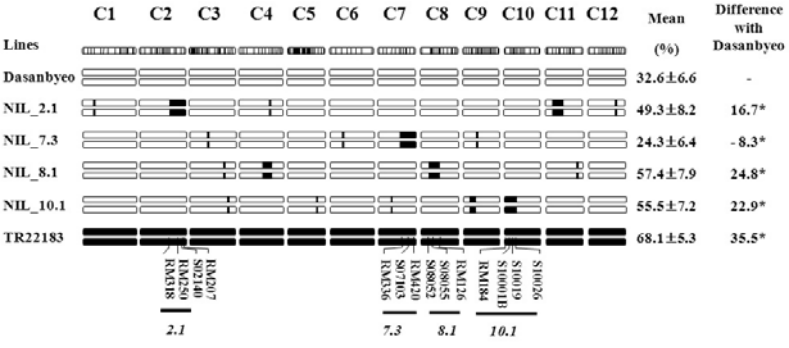

Fig. 2. Genomic composition of NILs derived from backcrossing Dasanbyeo to RILs. Black bar: TR22183 homozygote segment; white bar: Dasanbyeo homozygote segment. * SF of NIL was significantly different from Dasanbyeo at $P < 0.001$ based on the *t*-test. ns, not significant. DTH, days to heading; CL, culm length; PL, panicle length; PE, panicle exsertion; PN, panicle number; SN, spikelet number; SF, spikelet fertility; GW, grain weight; PA, phenotypic acceptability.

and Kunming, and 2 in Chuncheon and Yanji. One QTL (3.2) was identified in 3 cold treatment locations, while 1 QTL (4.3) was identified in 2 locations. A total of 7 loci revealed QTLs for SF, 4 in Chuncheon, 3 in Yanji, 2 in Kunming and 1 in Suwon; of these, 3 (7.3, 8.1, and 10.1) were identified in 2 locations. A total of 8 loci revealed QTLs for GW, 6 in Suwon, 2 in Chuncheon and 1 in Yanji; of these, only 1 QTL (8.4) was identified in 2 locations. A total of 5 loci revealed QTLs for PA, 4 loci were indentified in 1 location; however, 1 loci (QTL 8.1) was identified in 2 locations.

By sorting the loci by location, most QTLs were identified in Chuncheon (26 QTLs) followed by Suwon (21 QTLs) and Yanji (20 QTLs), and the least number of QTLs was in Kunming (17 QTLs).

Pleiotropic effects of identified QTLs

Among the 36 regions revealing QTLs, a total of 13 QTLs were identified for more than one trait in the same location, showing evidence of pleiotropy (Fig. 1). Nine loci displayed pleiotropy only in one location, while 4 loci (4.3, 7.3, 8.1, and 8.4) displayed pleiotropy in two locations. The TR22183 allele increased CL and PE at locus 4.3, but decreased CL and PE at locus 7.3 in both Chuncheon and Kunming. At locus 8.1, the TR22183 allele increased SF, but decreased PA in both Chuncheon and Yanji. At locus 8.4, the TR22183 allele decreased SF and GW, but increased PA and PL in both Kunming and Yanji. In the 6 loci showing pleiotropy, SF was affected by the locus as well as some agronomic traits and PA, suggesting that SF has a relationship with some cold-related traits through cold treatment.

QTL × environment interaction (QEI)

For analysis of various genetic components in the mixed model, we conducted a Bayesian test for parameter estimation and a significance test of QTL genetic main effects and QEI effects by cutting one genotype each round, using the mixed model approach. Of the 36 regions revealing QTLs, a total of 19 loci showed significant QEIs. On average, 2 QEIs were identified in each trait, with 1 for CL, PN, and PA, 2 for DTH, SN and GW, 3 for PL and PE and 4 for SF (Table 4 and Supplementary Table 2). Sixteen loci showed significant QEIs in one trait. For 2 loci, 5.1 (PL and GW) and 8.4 (DTH and GW), 2 traits showed significant QEIs, while 7.3 had significant QEIs in three traits (CL, PE and SF). The total phenotypic variance explained by each trait ranged from 2.1% (CL) to 8.8% (SN) in QEIs.

Table 4. Additive and epistatic QTL effects with environment interaction across four locations

Traits	QTL i	Marker interval i	QTL j	Marker interval j	LOD	Suwon	Chuncheon	Yanji	Kunming	R^2	Interaction[a]
DTH[b]	3.1	RM132-RM523	12	RM270-RM17	10.7		1.47*		-1.34*	1.5	AEi
	5	S05060A-S05060B	8.4	S08107-RM477	8.45	1.25*				1.1	AEj
						1.88*	-1.77*			2.4	AAE
CL	2.1	RM318-RM250	9	RM245-RM328	9.32	-2.16*			2.29*	1.7	AAE
	5	RM249-RM289	5	S05064-S05067A	14.03	2.14*			-2.13*	1.9	AAE
	7	RM118-S07099	7.3	S07103-RM420	5.67		2.55*		-1.59*	2.1	AEj
PL	1	RM323-S01022	5.3	RM289-S05054	10.22			-0.45*	0.47*	2.1	AEj
	1	RM165-RM128	9.1	S09049-S09058	5.71			0.47*	-0.44*	1.7	AAE
	2.1	RM318-RM250	5	S05036-RM413	8.36	0.49*				1.4	AAE
	3	RM175-S03046	5	S05009-S05014A	6.52		0.38*		-0.29*	1.3	AAE
	4.1	RM142-S04060	4	S04077B-RM317	8.97		-0.36*		0.32*	1.2	AEi
	5.1	S05014B-S05029	12	S12030-S12038	8.84	-0.30*		0.31*		1.5	AEi
PE	4	RM348-RM131	10	RM467-RM216	5.67		-0.93*			1.9	AAE
	4.3	S04128-RM124	12	RM277-RM101	13.02		-0.59*			1.4	AEi
	7	S07011-RM481	7	RM234-RM118	5.62	-0.70*				1.3	AAE
	7.3	S07103-RM420	8	RM350-S08053	9.41		0.43*		-0.47*	0.9	AEi
	11.1	S11028-S11055	11	RM202-RM287	10.3			-0.81*		1.7	AEi
PN	1	S01157-RM104	7.1	S07011-RM481	6.08			-0.95*		1.8	AAE
	3	RM545-S03041	8.3	RM25-S08090	6.24		-0.97*	1.59*		5.8	AAE
	3.4	S03115-S03120	3	S03136-RM468	8.02	0.69*		-0.89*		2.9	AEi
SN	3.2	S030C7-S03010	3	RM489-S03020	20.34		8.94*		-12.55*	3.4	AEi
	4.2	S04087-S04097	4	RM348-RM131	6.98	-8.33*		7.19*		5.4	AEi
	4	RM348-RM131	10	S10019-S10001A	6.13		7.55*			1.8	AAE
	5	RM146-RM430	11	RM187-RM332	6.24		6.54*			1.0	AAE
	9	S09093-S09073	9	RM278-RM242	4.78	7.66*			-4.95*	1.5	AAE
SF	2.1	RM318-RM250	6	S06033-S06053	4.31				4.78*	1.7	AAE
	3	S03010-S03015A	8	RM284-S08106	5.59	3.86*			-3.70*	1.3	AAE
	3	RM468-RM143	4	RM142-S04060	6.03		4.03*			1.3	AAE
	5	S05067A-S05067B	7.1	S07011-RM481	10.88	4.24*				1.1	AEj
	7.3	S07103-RM420	8	S08066-RM25	12.26		-4.81*	5.43*		1.8	AEi
	8.1	S08052-S08055	8	S08066-RM25	8.99		3.38*	-4.73*		1.7	AEi
	10.1	S10001B-S10019	10	S10013-S10026	6.84		-6.08*		6.33*	2.2	AEi
GW	5.1	S05104A-S05014B	6	RM133-RM510	4.54	0.62*				1.1	AEi
	8.4	S08107-RM477	9	RM316-S09000	7.68	-0.64*				1.1	AEi
	9	S09075A-S09075B	12	RM277-RM101	5.48	-0.66*				1.0	AAE
PA	3	RM132-RM523	5	RM593-S05022B	7.05				0.40*	2.1	AAE
	4	S04077B-RM317	9.2	RM288-S09065	5.96		-0.39*		0.50*	5.3	AAE
	5.4	S05080-RM161	5	RM538-RM421	4.25			0.30*		2.7	AEi

[a]Type of QTL × environment interactions. *AEi* and *AEj* interaction between i^{th} and j^{th} marker interval with the environment, respectively; AAE interaction between epistatic QTL with the environment

[b]Refer to Table 1 for abbreviations.

Contribution of QTL parameters

In the four cold treatment locations, a total of 57 QTLs and 76 pairs of E-QTLs were identified in 9 traits. Among them, 19 QTLs and E-QTLs clearly showed significant QEIs. The total phenotypic variation explained by each trait ranged from 13.2% (PA) to 29.1% (DTH) in QTLs, 10.6% (DTH) to 29.0% (PL) in E-QTLs, 2.2% (GW) to 8.8% (PA) in QEIs and 1.0% (GW) to 7.7% (PA) in E-QEIs. The total of PVE by each trait ranged from 37.9% (PE) to 53.7% (PL) (Supplementary Table 2).

QTL validation for cold tolerance in NILs

Four NILs and their parents were evaluated for cold tolerance in a cold water irrigation nursery in Chuncheon (Fig. 2). NIL_2.1, NIL_8.1 and NIL_10.1 contained the TTR22183 allele at the QTL 2.1, 8.1 and 10.1 on chromosomes 2, 8 and 10, respectively. The SF of NIL_2.1, NIL_8.1, and NIL_10.1 was 16.7%,

24.8% and 22.9%, respectively, which was significantly higher than that of the recurrent parent Dasanbyeo; this suggested that the enhancement of cold tolerance in these NILs was caused by the effect of the TR22183 alleles at QTLs 2.1, 8.1 and 10.1. NIL_7.3 contained TR22183 segments in the QTL 7.3 region on chromosome 7. As expected, the average of SF in NIL_7.3 was 24.3%, which was significantly lower than that of Dasanbyeo; this indicated that the effect of cold tolerance was from the Dasanbyeo allele. These results coincided with the direction of the additive effects of 7.3 in QTL analysis. These results also demonstrated that the QTLs reliably affected the cold tolerance of certain genotypes harboring different genetic backgrounds.

DISCUSSION

Cold stress is one of the major environmental concerns in rice cultivation, especially in areas of high altitude and latitude (Dai et al., 2004; Jiang et al., 2010). SF was decreased when rice plants were exposed to low temperatures during the reproductive stage, including the booting and flowering stages (Satake, 1976). SF has been extensively used for evaluating cold tolerance during the reproductive stage (Dai et al., 2004; Jiang et al., 2010; Kuroki et al., 2007; Suh et al., 2009; Takeuchi et al., 2001; Zhou et al., 2010). Among the 9 cold-related traits evaluated, SF was severely damaged in Dasanbyeo and the RIL population, and even in a cold-tolerant variety, TR22183, in the three cold treatment locations (Table 1). Importantly, this indicated that SF could be conveniently used as a criterion for the routine selection of cold tolerance. The PA score showed highly significant differences between the parents and the RIL population in the cold treatment locations (Table 1). PA has been discussed as a complementary index of SF for cold tolerance in multi-locational screenings in previous studies (Javier and Toledo, 2001; Jiang et al., 2010; Lee, 2001). Additionally, heading was delayed, and the values of CL, PL, PE, SN and GW were reduced in the RIL population and in their parents in the cold treatment locations (Table 1). These results indicated that multi-locational cold tolerance screening could successfully evaluate substantial differences in cold-related traits, and thus were suitable for the QTL study.

In the three cold treatment locations, 6 loci revealing QTLs were identified as associating with SF (Table 1, Supplementary Table 1). Among them, 3 loci (2.1, 5.4 and 8.4) were only identified in 1 location, while the other 3 (7.3, 8.1 and 10.1) were identified in 2 locations. All of these QTLs displayed pleiotropy in 2 or more traits, with the exception of 10.1. Among them, 3 QTLs, 5.4, 8.1 and 8.4, displayed pleiotropic effects in SF and PA in 1 or 2 cold treatment locations. During the intermediate stages of cloning and confirming these QTLs, we developed NILs corresponding to QTLs, including 2.1, 7.3, 8.1 and 10.1 using molecular marker-assisted selection through foreground and background selection (Fig. 2). After developing the NILs under cold water irrigation treatment in Chuncheon, we verified that each genomic region on the target QTLs controlled cold tolerance. QTL 8.1, identified in both Chuncheon and Yanji, had the largest variation, which could explain 20.4% of the variation of SF caused by naturally low temperatures in Yanji. In addition, this QTL had a strong additive effect (11.7), and increased the SF by 11% in genotypes carrying the allele from TR22183. This QTL was located near the centromeric region on chromosome 8 (52-55 cM in IRGSP database), which differs from *qCTB8* which has been mapped to a 1.7 cM interval between RM5647 (12.1 cM in IRGSP database) and PLA61 (12.9 cM in IRGSP database) by substitution mapping (Kuroki et al., 2007). QTL 2.1 (on the long arm of chromosome 2), which was first reported here, was identified only in Chuncheon; however, it demonstrated pleiotropy in 4 traits including SF, CL, PL and PE. The TR22183 allele at this locus increased CL, PL, PE and SF in Chuncheon. QTL 7.3 (on the long arm of chromosome 7) was observed to lead to small additive effects in SF in both Chuncheon and Yanji. At QTL 7.3, the TR22183 allele decreased SF, CL and PE in Chuncheon, indicating that genes of cold tolerance are derived not only from the cold tolerant variety, but also from the cold sensitive variety. In a similar interval, a QTL for cold tolerance at the booting stage was also identified in previous studies (Dai et al., 2004; Takeuchi et al., 2001; Zhou et al., 2010). Ye et al. (2010) identified that QTL qLTSPKST10.1 was located between S10010.9 and S10014.4 on chromosome 10 in Lijiangheigu from Yunnan in Southern China. We identified QTL 10.1, which was located between S10001B and S10019 on chromosome 10. The interval of qLTSPKST10.1 (10.9-14.4 cM) was overlapped with that of QTL 10.1, indicating that these 2 QTLs may be controlled by the same cold tolerant gene. Xu et al. (2008) also identified the same QTL (qCTB-10-2) in another rice variety, Kunmingxiaobaigu, from Yunnan Southern China. Although QTL 5.4 and 8.4 displayed pleiotropy in SF and PA, respectively, whether they were affected by cold tolerance remains unclear. Therefore, these QTL effects must be confirmed by further use of the NIL strategy.

In the last two decades, advances in QTL mapping technology have provided a powerful tool for the genetic dissection of complex traits, such as yield and cold tolerance. To date, for cold tolerance, approximately 40 QTLs located on all chromosomes have been reported in various cross combinations (Andaya and Mackill, 2003; Dai et al., 2004; Kuroki et al., 2007, 2009; Li et al., 1997; Liu et al., 2003; Mori et al., 2011; Oh et al., 2004; Saito et al., 1995; Suh et al., 2010; Takeuchi et al., 2001; Xu et al., 2008; Ye et al., 2010; Zhou et al., 2010). In these studies, only main effect QTL for cold tolerance was analyzed; however, an attempt to understand the contributions of the other important genetic components, including epistasis and GEI was not made. The importance and magnitude of epistatic QTL and QEI effects in rice have been discussed for complex traits including yield, agronomic traits, quality, drought tolerance and nitrogen efficiency (Fan et al., 2005; Kovi et al., 2011; Li et al., 2005; Talukder et al., 2005; Zhuang et al., 2002). In this study, the QTLs, E-QTLs, QEIs and E-QTLs for 9 traits were indentified using the basis of the best linear unbiased prediction (BLUP) of the mixed linear model approach (Supplementary Table 2). Interestingly, the total phenotypic variations explained by E-QTLs for some traits such as CL, PL, PE, SN, GW and PA were much higher than those explained by the corresponding additive QTLs. Relatively large phenotypic variations explained by QEIs and E-QEIs were found in SN, SF, PN and PA compared to additive and epistatic effects without environment effects; this suggested that these traits were very sensitive to the environment, leading to phenotypic plasticity. Phenotypic plasticity of complex traits arises in nature from interactions between QTLs and environments at the molecular level (Yang et al., 2007). When QEIs are present, a genotype displays good growing performance only in specific environments, whereas the lack of QEIs allows a genotype to adapt to various environments (Kovi et al., 2011). For SF, 3 out of 6 QTLs (7.3, 8.1 and 10.1) in cold treatment locations displayed significant evidence of QEIs (Table 4). This indicated that the effects of these loci were enhanced more greatly under cold stress, providing evidence for why SF was affected more significantly by low temperatures than normal temperatures. In contrast, QTLs 2.1, 5.4 and 8.4 were not involved in QEIs (Table 4), suggesting that

these QTLs affected SF more or less on phenotypic plasticity, irrespective of environment. Both of these two types of loci could enhance cold tolerance in a specific or broad cold environment, thus it is important to pay attention to pyramiding these two types of genes through marker-assisted selection (MAS) in cold-tolerant rice breeding.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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