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Detection of QTLs for Cold Tolerance at the Booting Stage in Near-isogenic Lines Derived from Rice Landrace Lijiangxintuanheigu

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Abstract

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Chilling damage significantly reduces grain yield in rice, while the utilisation of major quantitative trait loci (QTLs) can potentially improve rice yielding. Mapping of QTLs for 5 cold tolerance-related traits at the booting stage was conducted with SSR markers, including composite interval mapping (ICIM). 105 near-isogenic lines, derived from a backcross between Lijiangxintuanheigu (LTH, cold-tolerant landrace) and Towada (cold-sensitive cultivar) were analysed. Phenotype values were investigated under five cold-stress environments and analysed by the best linear unbiased prediction (BLUP). Twenty-one QTLs were identified on chromosomes 1, 2, 3, 4, 6, 7, 10 and 11, and the amount of variation (R^2) explained by each QTL ranged from 7.71 to 29.66%, with five co-located QTL regions. Eight novel major loci (*qSF-2*, *qSF-6a*, *qSF-7*, *qGW-6*, *qDGWP-4*, *qDSWPP-4*, *qDWPP-1* and *qDWPP-4b*) were detected in several environments and by using BLUP. Their alleles were derived from the cultivar LTH with R^2 variance from 12.24 to 29.66%. These favourable QTLs would help to elucidate the genetic mechanism of cold tolerance and provide strategies for breeding of high-yielding rice.

Keywords: *Oryza sativa*; quantitative trait loci; near-isogenic lines; cold tolerance at the booting stage

Rice (*Oryza sativa* L.) is one of the most important staple crops, and is responsible for feeding nearly a half of the world's population. However, about 15 million hectares of rice fields suffer from the risk of weather extremes in 24 countries (Cruz *et al*. 2013). Even though a low temperature affects the rice plants in any stage of growth, which at the booting stage significantly reduces the potential yield, with up to 10% losses per year (Tazib *et al*. 2015), which frequently occurs in Korea (Endo *et al*. 2016), Japan (Shimono *et al*. 2016), Australia and China (Zhu *et al*. 2015). This problem is particularly severe in high-altitude *japonica* rice regions in the Yunnan plateau of China (Yang *et al*. 2013). With global climate change, it is urgent to develop cold-tolerant rice cultivars for improved production in these regions.

Cold tolerance at the booting stage (CTB) in rice is a quantitatively inherited trait controlled by multiple genes (Shimono *et al*. 2016). According to the Gramene QTL database (http://www.Gramene.org/), more than 60 quantitative trait loci (QTLs) have been identified on rice chromosomes based on various cross combinations and environments. However, most of the analyses showed that the contribution to R^2 by each QTL was below 15%, and has large physical intervals, because of difficulties in the regulation of phenotype-specific transcription factors (Li *et al*. 2017). For instance, mapping

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of QTL was performed with the large effects that were related to spikelet fertility and anther length, including *Ste1* (32.1%), *Ste2* (19.4%), *qCT-1* (31.1%), *qCTB2a* (16.8%), *QTL2.1* (16.7%), *qLTB3* (24.4%), *Ctb-1* (24.4%), *qCT-7* (22.1%), *qRCT7* (20.6%), *QTL8.1* (24.8%), *qLSPKST10.1* (20.5%), *QTL10.1* (22.9%), and a part of QTL clusters distributed on chromosome (Jiang *et al*. 2011; Shirasawa *et al*. 2012). Recently, several QTLs were fine mapped, such as *qCTB8* (Kuroki *et al*. 2007), *qCTB-7* (Zhou *et al*. 2010), *qAL09-2/qAL10-2* and *qAL09-3/qAL10-3* (Tazib *et al*. 2015), *qCTR5* and *qCTR12* (Shimono *et al*. 2016), *qCT-3-2* (Zhu *et al*. 2015) and *qLTB3* (Ulziibat *et al*. 2016). Only *Ctb1*, *Ctb2* and *CTB4a* have been cloned (Saito *et al*. 2010; Zhang *et al*. 2017), and little is known about the underlying mechanisms for CTB. Therefore, further dissection of main-effect QTL will be required to facilitate the molecular breeding.

Yunnan province of China is one of the largest genetic and ecological centres of diversity for rice germplasm in the world. The wide range of Yunnan rice cropping regions with latitude (N: 21–29°) and elevation (76.4–2695 m a.s.l.) will not only provide an ideal place for the evaluation, breeding, and application of rice cold tolerance, but also generate many cold-tolerant landraces at CTB (Cui *et al*. 2017). Previous studies showed that Lijiangxintuanheigu (LTH) is one of the most cold-tolerant cultivars among 148 world cultivars (Shirasawa *et al*. 2012), and contains two pairs of additive, dominant and epistatic major genes with heritability up to 80.11% (Yang *et al*. 2013). However, the genetic factors of cold tolerance have not been characterised in a comprehensive way. Here, we report the stable QTL identification of LTH and provide novel alleles for improvement of CTB.

MATERIAL AND METHODS

Plant materials. A set of 105 NIL (BC_4F_8 and BC_4F_9) populations was developed by backcrossing of LTH (as donor) to Towada (as recipient). LTH is the most cold-tolerant *japonica* landrace at the booting stage of Yunnan province in China (Shirasawa *et al*. 2012), and Towada derived from Japan is a cold susceptible elite *japonica* cultivar with high yield and quality (Yang *et al*. 2013).

Evaluation of CTB. The field experiments were conducted in two consecutive years and three sites, i.e. in Baiyi (25.06°N, 102.41°E, altitude 2160 m), Xundian (25.22°N, 102.43°E, altitude 2325 m) and Yuxi (23.18°N, 101.18°E, altitude 1730 m) in the Yunnan province, China. All implementing methods are described below (Table 1). Each plot consisted of 20 plants that were planted in a single row with 15-/25-cm spacing between plants and rows according to a completely randomized block design with two replications. In Baiyi, the plants of near-isogenic line (NIL) and two parents were irrigated with cold water (C_W) (16–19°C) and at a depth of about 25 cm from tillering stage (20 days after transplanting) to grain maturity (Endo *et al*. 2016). Cold injury was applied under natural low air temperature (NLT_a) in Xundian and Yuxi. Air temperature during the whole growth stage was obtained from the Yunnan local meteorological observatory. The air and water temperatures from booting to milky stages were measured daily. The indices were measured at maturity, including spikelet fertility of the main panicle (SF), 1000-grain weight (GW), dry grain weight per panicle (DGWP), dry straw weight per panicle (DSWPP) and dry matter weight per panicle

Table 1. Details of field experiments and cold treatment of rice at different years and locations

Item	BC_4F_8 (2011)			BC_4F_9 (2012)	
	Baiyi	Xundian	Yuxi	Baiyi	Yuxi
Cold treatment method	C_W	NLT_a	NLT_a	C_W	NLT_a
Sowing date	March 21	March 21	March 21	March 23	March 23
Transplant date	May 12	May 13	May 13	May 15	May 14
Harvest date	Sep 23	Sep 20	Sep 16	Sep 25	Sep 17
Range of T_a whole growth stage (°C)	13.6–23.7	14.4–24.9	18.9–26.8	13.7–25.6	19.7–27.5
Range of T_a from booting to milky stages (°C)	15.4–20.3	15.9–20.4	17.2–21.7	16.1–20.6	17.5–21.9
Minimum T_w from booting to milky stages (°C)	16.6	17.3	18.5	16.8	18.1
Mean T_w from booting to milky stages (°C)	18.3 ± 0.14	19.1 ± 0.36	20.6 ± 0.41	18.6 ± 0.66	20.2 ± 0.37

C_W – continuous deep cold-water irrigation; NLT_a – natural low atmospheric temperature; T_a – atmospheric temperature; T_w – water temperature

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(DWPP). The mean of each phenotype was from ten middle plants per line.

Statistical analysis of phenotypic data. The phenotypic data were analysed by SPSS (Ver. 20.0, 2011). The analysis of joint variances on traits in five environments was conducted using SAS statistical software (Ver. 9.4, 2013), and comparisons of means between environments for each trait were implemented by Duncan's test ($P < 0.05$). The best linear unbiased prediction (BLUP) was estimated following the method of Liu *et al.* (2008).

Genotyping of plants. Genomic DNA was extracted from fresh leaves according to the CTAB method (Rogers & Bendich 1988). A total of 480 SSR markers distributed at regular intervals (around 3–5 cM) on 12 rice chromosomes was used to examine polymorphism between the parents. Each PCR reaction contained 1.5 µl of 10 × loading buffer (20 mM Tris-HCl pH 8.0, 50 mM KCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 50% glycerol), 2.0 µl of 20 ng DNA, 1.0 µl of 330 nM each forward and reverse primer, 0.5 µl of 250 µM each dNTP, 1.0 µl of 0.6 units Taq polymerase. The thermal cycling included: 1 min denaturation at 94°C, 35 cycles of 30 s denaturation at 94°C, 40 s annealing at 55°C, 45 s extension at 72°C, with a 5 min final extension at 72°C(PTC-200 Thermocycler, Germany). The PCR products were separated by electrophoresis in 6% acrylamide gels and stained with ethidium bromide.

Linkage map construction and QTL analysis. A genetic map comprising 180 microsatellite markers and covering 1820.6 cM of the genome with the average distance between the markers being 15.67 cM was constructed (Figure 1) using MAP functionality in the IciMapping V4.0 software (http://www.isbreeding.net/). According to the phenotypic value and BLUP of NIL, QTL analyses were carried out by an inclusive composite interval mapping (ICIM) method (Meng *et al.* 2015) with BIP functionality in the same software with PIN of 0.001, walking speed of 1 cM. A logarithm of the odds (LOD) threshold was used as 1000 permutations test. The R^2 explained by each QTL and additive effects were estimated. QTL nomenclature followed the method of McCouch (2008).

RESULTS

Phenotypic variation for cold tolerance. LTH contributed to the strongly cold tolerance-associated characters and its phenotypic values more greatly

Table 2. Phenotypic performance of spikelet fertility of the main panicle (SF), 1000-grain weight (GW), dry grain weight per panicle (DGWP), dry straw weight per panicle (DSWPP) and dry matter weight per panicle (DWPP) of rice parents and their NIL population at the booting stage under five cold stress environments

Traits	Year	Baiyi					Xundian					Yuxi				
		parental mean		NIL population			parental mean		NIL population			parental mean		NIL population		
		LTH	Towada	mean ± SD	skewness	kurtosis	LTH	Towada	Mean ± SD	skewness	kurtosis	LTH	Towada	Mean ± SD	skewness	kurtosis
SF (%)	2011	81.4	27.1	61.5 ± 15.8^b	1.229	−4.371	81.8	36.3	65.5 ± 20.4^b	2.614	−3.256	85.3	44.1	79.3 ± 6.4^a	2.525	3.077
	2012	82.9	24.1	64.4 ± 16.5^b	2.831	3.585						83.8	42.6	72.8 ± 6.3^a	1.864	4.265
GW (g)	2011	23.5	17.7	17.9 ± 5.7^b	−1.344	−0.956	18.8	18.2	11.1 ± 3.6^c	1.548	−0.250	23.9	22.8	25.1 ± 2.7^a	1.093	−3.114
	2012	20.5	16.4	19.0 ± 3.3^b	2.018	−0.518						22.5	21.9	23.3 ± 3.7^a	1.086	2.164
DGWP (g)	2011	24.2	12.7	15.7 ± 5.5^b	2.195	−3.679	17.8	16.2	15.4 ± 2.8^{bc}	1.589	0.200	28.81	19.9	20.0 ± 4.6^a	1.471	−0.504
	2012	14.9	10.1	12.6 ± 4.1^c	1.512	−2.188						27.75	18.4	22.0 ± 5.6^a	1.503	−0.042
DSWPP (g)	2011	39.2	26.1	41.2 ± 9.6^a	1.063	0.700	33.4	23.9	28.0 ± 7.8^b	1.254	0.731	23.61	13.3	19.1 ± 3.2^c	0.630	1.337
	2012	40.3	27.6	30.5 ± 8.2^b	1.607	0.653						25.67	18.5	23.2 ± 5.6^c	1.585	2.718
DWPP (g)	2011	63.4	38.9	61.7 ± 17.3^a	1.450	0.808	51.2	40.0	34.4 ± 8.1^c	1.316	0.383	52.42	33.2	39.1 ± 6.7^{bc}	0.410	−0.738
	2012	55.3	37.7	41.1 ± 10.0^b	1.432	−0.350						53.42	36.9	45.2 ± 10.2^b	1.173	1.352

SD – standard deviation; the values followed by a common letter in the same column are not significantly different at $P < 0.05$ (Duncan tested)

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than Towada (Table 2). All traits for NIL displayed continuous distribution with median values between the two parents, which showed the extent of greater phenotypic variability. The means comparison between circumstances shows that the environments had a significant effect on traits (Table 2). The skewness and kurtosis value of studied traits was almost beyond 1, with positively skewed slightly towards LTH, which indicates that it contained major genes (Table 2).

QTLs for SF and GW. Four significant QTLs for SF were detected on chromosomes 2, 6 and 7 (Table 3, Figure 1). The R^2 ranged from 15.39 to 29.66%, the *qSF-2*, *qSF-6a* and *qSF-7* were detected in two or three environments and BLUP, which had a positive effect on the allele from LTH. Two significant QTLs (*qGW-6* and *qGW-10*) for GW were located on chromosomes 6 and 10 (Table 3, Figure 1), which explained 12.24–24.53% of the total variation, and the *qGW-6* was detected in three environments and the BLUP value.

QTLs for DGWP, DSWPP and DWPP. DGWP was controlled by six QTLs located on chromosomes 1, 4, 10 and 11 (Table 3, Figure 1), and they explained 10.01–20.97% of the phenotypic variation. Among them, the *qDGWP-1a* and *qDGWP-4* were detected in different environments and BLUP. Five QTLs for DSWPP were detected on chromosomes 1, 3, 4 and 6, with R^2 varying from 7.71 to 21.65% (Table 3, Figure 1). The *qDSWPP-4* and *qDSWPP-6a* were detected in two environments and BLUP. Four QTLs for DWPP were located on chromosomes 1, 4 and 10 (Table 3, Figure 1), which accounted for 12.26–23.89% of the total variation, and the *qDWPP-1* and *qDWPP-4b* were detected in different environments and BLUP.

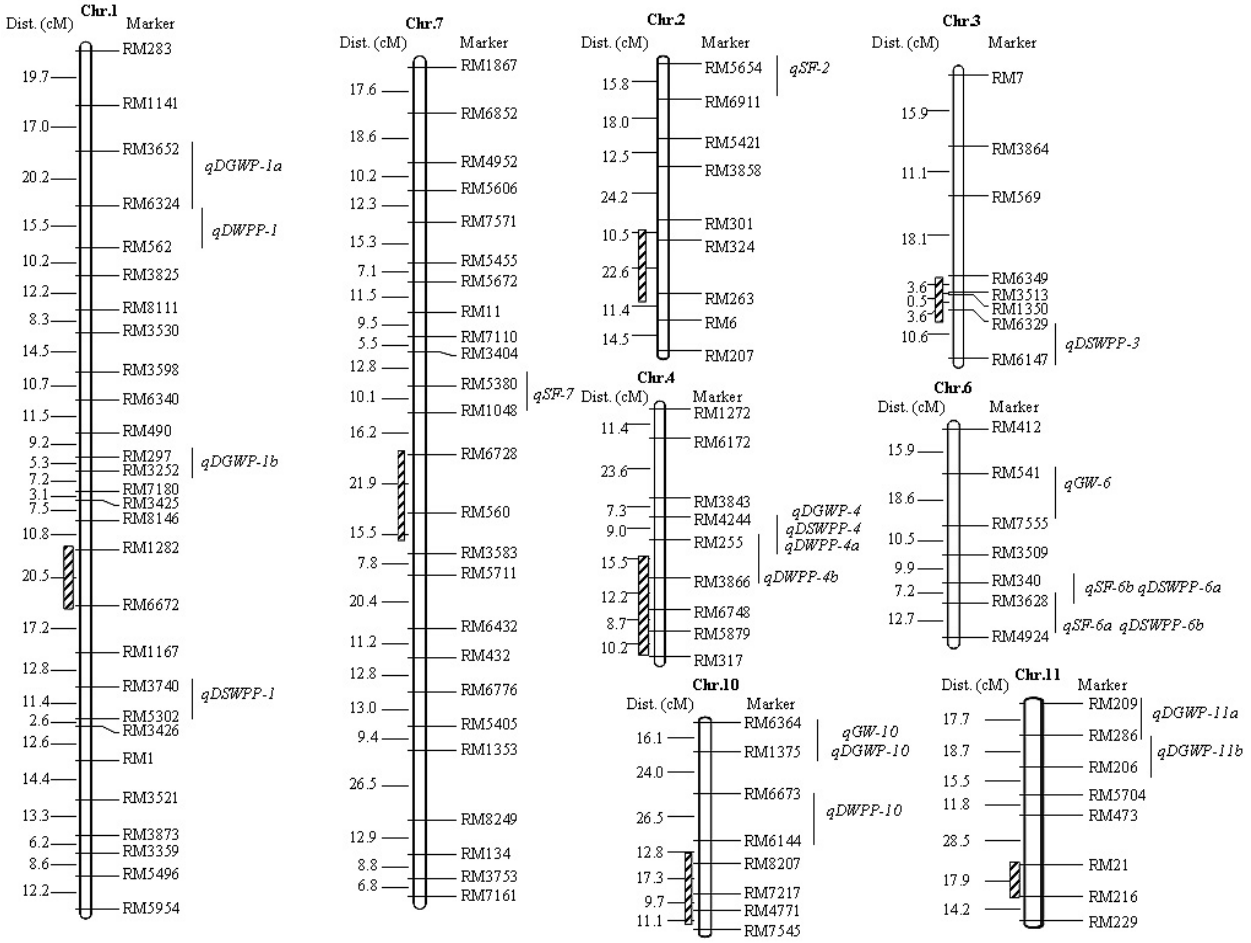

Figure 1. Chromosomal positions of QTLs for cold tolerance at the booting stage in rice; ▤ represents a dense region of QTLs in previous studies; the map distances expressed in centiMorgans (cM) were calculated using the Kosambi function, which are shown on the left; the markers and QTL names are shown to the right of the linkage group

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Table 3. Rice QTL for spikelet fertility of the main panicle (SF), 1000-grain weight (GW), dry grain weight per panicle (DGWP), dry straw weight per panicle (DSWPP) and dry matter weight per panicle (DWPP) identified in five cold stress environments

Trait	Year/location	Chr.	Locus	Marker interval	LOD	R^2/%	AE
SF	2011/Baiyi	2	*qSF-2*	**RM5654-RM6911**[a]	4.41	15.44	11.13
	2012/Yuxi				4.83	16.66	5.40
	2011/Baiyi	6	*qSF-6a*	**RM3628-RM4924**[a]	4.88	21.09	9.72
	2012/Baiyi				5.49	17.95	7.81
	2011/Xundian				6.23	16.82	5.34
	2012/Yuxi	6	*qSF-6b*	RM340-RM3628	5.94	15.39	7.67
	2011/Baiyi	7	*qSF-7*	**RM5380-RM1048**[a]	3.68	29.66	7.54
	2012/Baiyi				9.02	19.11	10.13
GW	2011/Baiyi	6	*qGW-6*	**RM541-RM7555**[a]	3.72	24.53	4.54
	2012/Baiyi				3.19	12.24	1.65
	2011/Xundian				3.62	17.51	2.74
	2011/Baiyi	10	*qGW-10*	RM6364-RM1375	5.03	22.14	–4.87
DGWP	2011/Baiyi	1	*qDGWP-1a*	**RM3652-RM6324**[a]	4.74	18.43	3.19
	2012/Baiyi				3.32	12.23	0.24
	2011/Xundian				5.42	18.52	7.19
	2011/Yuxi				4.46	20.97	0.33
	2011/Yuxi	1	*qDGWP-1b*	RM297-RM3252	3.62	17.95	4.41
	2012/Baiyi	4	*qDGWP-4*	**RM4244-RM255**[a]	5.13	20.82	3.04
	2011/Xundian				6.15	18.43	6.11
	2011/Baiyi	10	*qDGWP-10*	RM6364-RM1375	3.49	15.41	-4.19
	2012/Yuxi	11	*qDGWP-11a*	RM209-RM286	3.44	10.01	3.59
	2012/Yuxi	11	*qDGWP-11b*	**RM286-RM206**[a]	3.89	11.32	3.67
DSWPP	2011/Baiyi	1	*qDSWPP-1*	RM3740-RM5302	3.03	16.41	–3.91
	2011/Xundian	3	*qDSWPP-3*	RM6329-RM6147	3.05	7.71	3.97
	2011/Xundian	4	*qDSWPP-4*	**RM4244-RM255**[a]	3.51	15.82	2.66
	2011/Baiyi				3.42	21.65	2.45
	2011/Xundian	6	*qDSWPP-6a*	**RM340-RM3628**[a]	4.06	18.17	3.87
	2012/Yuxi				6.80	19.27	7.22
	2012/Yuxi	6	*qDSWPP-6b*	RM3628-RM4924	7.37	13.04	6.79
DWPP	2012/Yuxi	1	*qDWPP-1*	**RM6324-RM562**[a]	3.94	15.24	3.97
	2012/Baiyi				6.88	17.25	4.06
	2011/Xundian	4	*qDWPP-4a*	RM4244-RM255	4.93	16.10	3.74
	2011/Baiyi	4	*qDWPP-4b*	**RM255-RM3866**[a]	3.19	19.21	4.65
	2012/Baiyi				3.71	23.89	0.29
	2011/Yuxi				4.62	19.90	0.07
	2012/Yuxi	10	*qDWPP-10*	RM6673-RM6144	3.01	12.26	3.60

Chr – chromosome on which the QTL was located; [a]QTL were also detected in the marker interval using the best linear unbiased prediction value; LOD – additive logarithm of odds value; R^2 – proportion of the total variance explained by each QTL; AE – additive effect, the negative additive effect value indicates effects from Towada, and positive values indicate effects from Lijiangxintuanheigu; bold – negative additive effect

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DISCUSSION

In this study, twenty-one QTLs were identified with phenotypic values, and among them, 11 QTLs were mapped in multi-environments and the BLUP value. These results showed that the environment contributed to the varying power related QTL detection (Table 3, Figure 1). Most of the previously detected QTL was based on spikelet sterility and anther length (Shimono *et al.* 2016). It is difficult to compare the effects of these reported QTL for different methods and cultivars. According to the Gramene QTL database, some QTLs in previous studies are relatively little located either overlapped or adjacent to the regions identified in our study. For instance, the *qDGWP-1a* was located in a similar interval with a grain yield QTL (*qDTY1.2*) mapped by Sandhu *et al.* (2014). The *qDSWPP-6a* was mapped in a similar location with *qPBN6*, *qGYPP6-1*, the same as *qDGWP-11b* compared with *qPBN11*, *qSBN11* and *qPN11* by Yang *et al.* (2017). The eight major-effect QTLs might represent novel genes, whose alleles were contributed by LTH (Table 3).

In general, three methods have been described for evaluating cold tolerance at the booting stage in rice, including deep cold-water irrigation (16–19°C) (Endo *et al.* 2016), in a phytotron and in high-altitude environments with naturally low temperatures in Yunnan of China (Zhu *et al.* 2015; Zhang *et al.* 2017), who reported that critical temperatures are between 15°C and 17°C (cold-tolerant genotypes) or between 17°C and 20°C (cold-sensitive genotypes) (Saito *et al.* 2010). Moreover, LTH was successfully evaluated using the C_W method at 18.5°C or 18.8°C of water temperature (Shirasawa *et al.* 2012; Ulziibat *et al.* 2016). For these reasons, both C_W and NLT_a (Table 1) were chosen to study the population over several years. The yield of LTH (with high culm length) and Towada was 4650 and 9300 kg/ha under favourable temperature, respectively, especially Towada had a higher yield in comparison with ordinary rice varieties (7200 kg/ha). After being exposed to C_W and NLT_a, their yield was reduced up to 4350 and 3300 kg/ha, respectively. However, several high-yielding (> 9450 kg/ha) strains with similar plant type of Towada were developed through cold intensive selection. Herein, our results revealed that the *qSF-2*, *qSF-6a* and *qSF-7* were stable, and the R^2 ranged from 15.44 to 29.66%, with positive alleles from LTH (Table 3). However, none of these QTLs were coincident with those reported for LTH previously. For instance, *qLTB3* from LTH was identified as a 1.2-Mb region between RM7000 and RM3719 markers on chromosome 3 (Shirasawa *et al.* 2012); furthermore this QTL was delimited within a region of about 35 kb that contains six genes (Ulziibat *et al.* 2016), and *qLTB3* appeared a small effect to reinforce cold tolerance under C_W (Fukushima *et al.* 2017). Although transgenic rice plants with LTH allele (Os03g0806700) have been obtained, they have been unable to perform a reliable cold tolerance test in an isolated greenhouse (Ulziibat *et al.* 2016), and this might make it difficult to apply in breeding new cultivars. There are several possibilities to speculate the reason for different results: (*i*) LTH allele has a strong effect on cold tolerance, which would be a highly valuable genetic factor for rice breeding using the MAS strategy, (*ii*) some regions on chromosomes 3, 6, 10, and 11 have a limited number of polymorphic markers. However, novel QTLs suggest that LTH might be a multiple major QTL, and (*iii*) the effects of QTLs differ depending on the genetic background and environments of cold exposure (Endo *et al.* 2016), and which provided caution for the use of genetic resources and evaluation method in practical breeding of rice.

In this study, several QTLs were co-localized in the interval of RM4244-RM255 (DWPP, DSWPP and DGWP), RM340-RM3628 and RM3628-RM4924 (SF and DSWPP), RM6364-RM1375 (GW and DGWP) (Table 3, Figure 1). The close aggregation suggests that there is a strong genetic control of these traits despite the large effect from different environments. Other studies have also reported that there were significant positive correlations between grain weight per panicle and 1000-grain weight and spikelet fertility (Wainaina *et al.* 2015). Our previous investigation observed that chilling stress increased accumulation levels of soluble sugars, soluble protein, proline, malondialdehyde, peroxidase and catalase activities in the rice flag leaves and anthers (Yang *et al.* 2014). These results suggested that there may also be a limitation in evaluating QTL at CTB using only SF or anther characteristics as an index, especially because SF variation in cold tolerance depended on the number of differentiated microspores, pollen survival rate, proportion of viable pollen grains and fertilization efficiency (Shimono *et al.* 2016; Fukushima *et al.* 2017), physiological characters and high-yield traits should also be considered. Extraordinarily, the major QTLs (*qDGWP-1a* and *qSF-6a*) of this study not only have a high additive effect but also possess

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consistent expression in multi-environments, and which had at least one, or more positive alleles of QTLs (Table 3). Therefore, further characterization of two QTLs would provide beneficial information for breeding and cloning genes at CTB.

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