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Identification of Quantitative Trait Loci for Spikelet Fertility at the Booting Stage in Rice (*Oryza sativa* L.) under Different Low-Temperature Conditions

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Abstract: Cold stress in rice is a critical factor limiting growth and yield in temperate regions. In this study, we identified quantitative trait loci (QTL) conferring cold tolerance during the booting stage using a recombinant inbred line population derived from a cross between a cold-susceptible Tongil-type cultivar Milyang23 and a cold-tolerant *japonica* cultivar Giho. A phenotypic evaluation was performed in a cold-water-irrigated field (17 °C) and a temperature-controlled (17 °C/17 °C air and water) greenhouse at the booting stage. Four QTL, including two on chromosome 1 and one each on chromosomes 6 and 9, were identified in the cold-water-irrigated field, with an R^2 range of 6.3%–10.6%. Three QTL, one on each of chromosomes 2, 6 and 9, were identified under the temperature-controlled greenhouse condition, with an R^2 range of 5.7%–15.1%. Among these, two QTL pairs on chromosomes 6 (*qSFF6* and *qSFG6*) and 9 (*qSFF9* and *qSFG9*) were detected in the cold treatments of both field and greenhouse screenings. Our results provide a reliable dual-screening strategy for rice cold tolerance at the booting stage.

Keywords: rice; recombinant inbred line; cold tolerance; booting stage; QTL

1. Introduction

Rice (*Oryza sativa* L.) is one of the most important staple crops worldwide and feeds almost half of the world's population. Rice is a crop that adapts well to various environmental conditions, as it is grown in many different locations under various climates. Rice is cultivated from 53° N in Northern China to 35° S in Australia and can be cultivated at elevations above 2000 m in the mountains of Nepal and India [1]. However, various environmental factors, such as low or high temperatures, drought, flooding and salinity can limit the growth and production of rice. Among these factors, low temperature is a major limiting factor in rice production because rice originates from tropical or subtropical regions, making it particularly sensitive to low temperature compared to other crops. Cold stress is one of the major limitations to rice growth and yield and leads to significant economic losses and food shortages in rice-cultivating countries around the world [2].

In Korea, tremendous reductions in yield were experienced in 1980 and 1993 due to low temperatures during the reproductive stage, and grain yields were decreased by 78% and 20%, respectively [3]. In Australia, losses in rice yield due to cold damage amount to $23.2 million per year on average [4], and annual yield loss of 3–5 million tons has been reported in China [5]. The Tohoku region, one of Japan's major rice-producing areas, suffered a 44% yield reduction due to cold damage in 1993. Therefore, development of rice cultivars with strong tolerance to cold stress is becoming an increasingly important objective for rice breeding in this region.

When rice plants are exposed to cold stress, various symptoms appear, such as poor germination, slow seedling growth, delayed vegetative growth, discoloration and poor grain fertility and filling, depending on the developmental stage [1]. These symptoms inhibit the normal growth of rice and ultimately lead to yield losses. Especially, cold stress at the reproductive stage causes serious spikelet sterility and reduces grain yields to a great extent. The reproductive stage can be divided into the booting stage and the fertilization stage. The booting stage occurs 10–12 days prior to heading when premeiotic mother cells develop to microspores and pollen matures [6]. Low temperatures can cause pollen abortion during microsporogenesis when pollen grains are being formed, which leads to spikelet sterility [7]. Therefore, the percentage of fertile spikelets is a key indicator of cold tolerance in rice during the reproductive stage [8,9]. The fertilization stage begins immediately after pollen maturation, and the complete fertilization stage consists of anther dehiscence, pollen germination, pollen tube elongation and fertilization [10].

Cold tolerance at the booting stage (CTB) is often evaluated based on spikelet fertility in the field using cold water irrigation at 18–19 °C. Using this method, large varietal differences and extensive quantitative trait loci (QTL) have been identified. For example, *Ctb*1 and *Ctb*2 derived from a *japonica* variety Norin-PL8 were identified on chromosome 4 using near-isogenic lines (NILs) [11]. *Ctb*1 was subsequently fine-mapped [12] and cloned as a gene encoding an F-box protein that constitutes part of the E3 ubiquitin ligase complex [13]. Five QTL for CTB were identified on chromosomes 1 (*qCTB1*), 2 (*qCTB2a* and *qCTB2b*), 3 (*qCTB3*) and 9 (*qCTB9*) using a recombinant inbred line (RIL) population derived from a *japonica* (M-202) and *indica* (IR50) cross [14]. A QTL for CTB, *qCTB*8, was identified on chromosome 8 using F_2, F_3 and F_7 populations derived from a cross between two temperate *japonica* cultivars Hokkai-PL9 and Hokkai287 [15]. Using NILs of a *japonica* landrace Kunmingxiaobaigu in the background of a *japonica* variety Towada, eight QTL for CTB were identified on chromosomes 1 (*qCTB1-1*), 4 (*qCTB4-1* and *qCTB4-2*), 5 (*qCTB5-1* and *qCTB5-2*), 10 (*qCTB10-1* and *qCTB10-2*) and 11 (*qCTB11-1*) [5], of which *qCTB4-1* was subsequently cloned as *CTB4a* encoding a leucine-rich repeat receptor-like protein kinase [16]. Three QTL for CTB were identified on chromosomes 3 (*qPSST-3*), 7 (*qPSST-7*) and 9 (*qPSST-9*) using a RIL population derived from a cross between a *japonica* variety Geumobyeo and a *javanica*-derived breeding line IR66160-121-4-4-2 [17].

Despite a number of QTL identified for CTB, only two have been cloned [13,16] and the gene mechanisms underlying most QTL are still largely unknown, partly due to complex quantitative nature of the trait and the lack of reliable phenotyping system [18]. In this study, we identified two reliable QTL for rice CTB by examining a RIL population derived from a cross between Milyang23 (Tongil-type) and Giho (*japonica*) using a dual phenotyping system with a cold-water-irrigated field condition and a temperature-controlled greenhouse condition. By evaluating major agronomic traits of the RIL population, we also report a QTL for CTB that is not affected by other traits such as days to heading, culm and panicle length.

2. Materials and Methods

2.1. Plant Material and Mapping Population

From a cross between Milyang23, a cold-susceptible Korean Tongil-type (*japonica–indica* cross) cultivar, and Giho, a cold-tolerant Korean *japonica* cultivar, a RIL population consisting of 162 lines (MGRIL) was developed. MGRIL was crossed in 1988 and was grown to the F_6 generation using the single seed descent (SSD) method, after which it was grown using the pedigree method. MGRIL is currently beyond the F_{20} generation [19,20]. Seeds of the 162 RILs and two parental lines were obtained from the genebank of the National Institute of Agricultural Sciences (NIAS), Rural Development Administration (RDA), Suwon, Republic of Korea. These 162 RILs and their two parental lines were planted in an experimental field for phenotypic observation at the National Institute of Crop Sciences (NICS), RDA, Suwon, Korea. Based on the phenotypic observation in the field, 139 of the 162 RILs were selected, and 23 lines were excluded because of seed degeneration or late or no heading.

These 139 RILs were then used to construct a linkage map to identify QTL controlling cold tolerance at the booting stage.

2.2. Genotyping Using SSR and SNP Markers

The total genomic DNA of 139 RILs and their parental lines was extracted from the leaves of 18- to 21-day-old seedlings using the CTAB method with minor modifications [21]. For simple sequence repeat (SSR) marker analysis, a total of 671 SSRs distributed at regular intervals (approximately 3–5 cM) on rice chromosomes were tested in a polymorphism survey between the two parents. Among the 671 SSRs, 424 polymorphic markers between the two parents were used for genotype analysis of the RILs. PCR analyses were carried out in a 20 µL reaction mixture containing 10 µM of each primer, 50 mM KCl, 1.5 mM MgCl2, 25 ng of template DNA and 0.02 U/µL of Taq polymerase (SolGent Co. Ltd., Daejeon, Korea). The PCR conditions consisted of one cycle of 8 min at 95 °C, followed by 35 cycles of 1 min at 94 °C, 30 s at 55 °C, 1 min at 72 °C and a final cycle of 10 min at 72 °C. For single nucleotide polymorphism (SNP) analysis, Illumina BeadXpress 384-plex SNP plates with the oligo pool assay (OPA) customized for *indica–japonica* were used (Illumina OPA ID: GS0011862-OPA; Rice OPA 3.1) [22]. PCR amplification and hybridization were performed using the protocol specified by the Golden Gate genotyping assay for Vera Code manual (Illumina, San Diego, CA, USA). The Vera Code 384-plex plate was scanned using the Illumina BeadXpress Reader (Genotyping Services Lab, IRRI), and raw intensity values were exported from Genome Studio software V1.1.0 (Illumina). Genotype calling was performed using Alchemy software [23].

2.3. Phenotyping of Cold Tolerance

Cold tolerance was evaluated using two different methods: (1) screening in a cold-water-irrigated field, and (2) screening in a temperature-controlled greenhouse facility. For the field screening, 139 RILs and the two parents were planted in a cold tolerance screening field at Chuncheon substation, NICS, RDA, Republic of Korea, in 2016 (Figure 1). Cold tolerance was evaluated according to the procedure of [17], with some modifications. Thirty-day-old seedlings were transplanted to a control plot and a cold-water-irrigated plot, with 40 plants in a single row and 30- and 15-cm spacing between rows and plants, respectively. In the cold-water-irrigated plot, cold water at 17 °C was irrigated during the entire period of rice growth from tillering (20 days after transplanting) to the grain maturity stage. The depth of the cold water was approximately 5 cm, and the temperature of the water ranged from 17 °C at the inlet to approximately 24 °C at the outlet in the 8 m-long screening plot (Figure 1). The temperature range of 19–20 °C inhibited the normal development of most agronomic characteristics of the RIL. Thus, we measured the temperature gradient within row using an electronic thermometer (SK-L200TII, SATO, Japan) to mark the 19–20 °C zone and evaluated plants grown in this region. In the control plot, approximately 24–26 °C water was supplied for normal growth. Spikelet fertility, days to heading (DH), culm length (CL), panicle length (PL) and panicle exsertion (PEX) were investigated in both cold-water-irrigated and control plots. Average data collected from five plants were used for QTL analysis.

In the temperature-controlled greenhouse facility, evaluation of cold tolerance at the booting stage was carried out according to the method provided by [24]. Sixteen germinated seeds of the 139 RILs and their parents were sown in a Wagner pot (175 mm width and 200 mm height) and grown under natural conditions. To ensure uniform growth, tillers, except for the main culm, were removed 30 days after seeding, and only twelve plants with the main stems were maintained per pot. Cold treatment began at the early booting stage (10–12 days before heading) when the auricle of the flag leaf was slightly below the auricle of the penultimate leaf according to the auricle distance method [6]. A total of 5–7 plants with uniform growth were tagged from each pot, and cold treatment was conducted under constant 17 °C air and 17 °C water with a depth of 15 cm. After 10 days of cold treatment, the pots were moved outside under natural conditions and grown until the mature stage. The percentage of seed

set was used as a parameter to measure cold tolerance at the booting stage. As a control, the spikelet fertility of plants under normal conditions without cold treatment was examined.

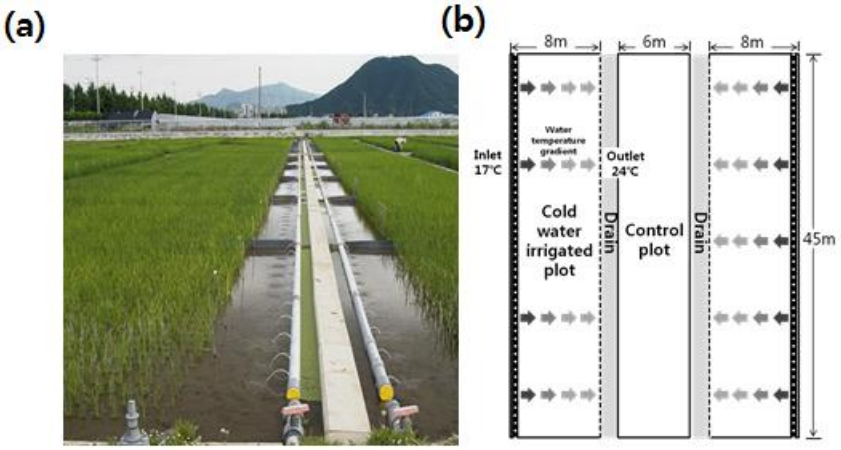

Figure 1. Views of the paddy field of Chuncheon substation used for cold tolerance screening (**a**) and diagram of the paddy field containing a cold-water-irrigated plotw and a control plot (**b**). In the cold-water-irrigated plot, 17 °C cold water was supplied through pipelines to a field at a 5 cm water depth during the entire rice growth period, from 20 days after transplanting to grain maturity. Depending on the water flow, a water temperature gradient was formed in the 8 m-long cold-water-irrigated plot, from 17 °C at the inlet to approximately 24 °C at the outlet. The normal plot with natural water temperature was used as a control.

2.4. Linkage Map Construction and QTL Analysis

A linkage map was constructed using MAPMAKER/EXP 3.0 with the Kosambi mapping function [25]. Out of 678 markers (424 SSRs and 254 SNPs) polymorphic between Milyang23 and Giho, 327 (168 SSRs and 159 SNPs) with reliable genotyping results were selected for the map construction after excluding overlapping markers with the same genetic position among the 139 RILs.

QTL were detected using composite interval mapping (CIM) with IciMapping Version 3.1, which consists of 1000 permutations [26]. The cofactors in CIM were automatically selected using forward–backward stepwise regression with F-in = 0.01, F-out = 0.01 and a window size of 10 cM [27]. The threshold LOD score ranged from 3.70 to 3.92 at an alpha 0.05 significance level across all traits.

3. Results

3.1. Phenotypic Variation in Parental Cultivars and RILs

In the field condition, the spikelet fertility of both Milyang23 (90.3% ± 1.73%) and Giho (90.0% ± 1.73%) was high under the control treatment without significant difference. However, under cold water treatment, the spikelet fertility of Milyang23 (51.5% ± 2.08%) was significantly lower than that of Giho (76.0% ± 5.13%). The spikelet fertility of the 139 RILs under cold water treatment ranged from 0% to 86.3%, with a mean of 30.6%. The distribution of RILs (skewness = 0.567, kurtosis = −0.481) showed that the population was slightly skewed toward lower spikelet fertility under cold water treatment (Table 1).

The spikelet fertility of Milyang23 (87.5% ± 4.03%) and Giho (89.9% ± 3.66%) under the greenhouse condition showed no significant difference in the control treatment. However, under cold water and cold air treatment, the spikelet fertility of Milyang23 (12.4% ± 2.33%) was significantly lower than that of Giho (55.7% ± 4.18%). The spikelet fertility of the 139 RILs under cold water treatment ranged from 0% to 82.7%, with a mean of 13.2%. Similar to the field evaluation, the distribution of RILs (skewness = 1.841, kurtosis = 3.405) showed that the population was slightly skewed toward lower spikelet fertility under cold water treatment (Table 1).

Major agronomic traits related to cold tolerance, including DH, CL, PL and PEX, were evaluated for the 139 RILs and the two parents in the field (Table 2). In the control plot, DH and PL of Miyang23 and Giho were not significantly different, but CL and PEX of Giho were slightly longer than those of Miyang23. However, in the cold-water-irrigated plot, cold-susceptible Milyang23 showed much poorer growth than Giho. In particular, the delay in DH, and the reduction in CL and PEX of Milyang 23 were greater than those of Giho. The DH of Milyang23 in the cold plot was delayed by 19 days (11 days in Giho) compared with the control plot. The CL and PEX of Milyang23 in the cold plot were decreased by 28 cm and 7 cm (11 cm and 4 cm in Giho), respectively, compared to the control plot.

Correlation analysis in the control revealed positive but weak correlations between spikelet fertility in the field (SFF) and CL (r = 0.149), PL (r = 0.120) and PEX (r = 0.206; Table 3). Interestingly, SFF in the cold treatment showed stronger positive correlations with CL (r = 0.430), PL (r = 0.195) and PEX (r = 0.518), indicating that plants with longer culm, longer panicle and better panicle exsertion might escape the cold water more easily than shorter plants and exhibit higher fertility. A weak but positive correlation between SFF and DH (r = 0.122) was observed in the cold treatment, which was absent in the control. Spikelet fertility in the greenhouse (SFG) was positively correlated with SFF (r = 0.363) in the control, but this correlation was weaker (r = 0.116) in the cold treatment. Additionally, correlation patterns between SFG and the agronomic traits were very different from those between SFF and the agronomic traits (Table 3), implying that different physiological and/or genetic mechanisms are involved in determining cold tolerance under field and greenhouse environments.

3.2. Linkage Map Construction

The percentage of polymorphism in the SSR and SNP markers per chromosome ranged from 38.9% to 97.2% and 46.4% to 86.7%, respectively (Table 4). The average polymorphism of the SSR and SNP markers between Milyang23 and Giho was 65.5% and 65.8%, respectively. In particular, on chromosome 5, the polymorphism of the SNP and SSR markers was 38.9% and 46.4%, respectively, showing the lowest polymorphism. From the 678 polymorphic markers (424 SSRs and 254 SNPs), we selected 327 (168 SSRs and 159 SNPs) for a linkage map construction after excluding markers with overlapping genetic position or unclear genotype data (Table 4). The genetic linkage map covered a total of 1564.0 cM, with an average interval size of 4.9 cM between markers (Figure S1).

3.3. Identification of QTL for Spikelet Fertility under Field and Greenhouse Conditions

QTL analysis using CIM identified four significant QTL for spikelet fertility in the field (SFF) under cold treatment on chromosomes 1 (*qSFF1-1* and *qSFF1-2*), 6 (*qSFF6*) and 9 (*qSFF9*), which explained 6.3%–10.6% of the observed phenotypic variance (Table 5, Figure 2). While *qSFF6* was identified as a spikelet fertility QTL also in the control plot, the others were detected only in the cold plot (Table 5, Table S1). Milyang23 contributed the allele for high spikelet fertility at *qSFF1-1*, whereas Giho contributed the allele for high spikelet fertility at *qSFF1-2*, *qSFF6* and *qSFF9*. Among the four QTL, *qSFF6* located between the markers id6004563 and id6005608 on chromosome 6 exhibited the highest additive effect: 25.8% increase in spikelet fertility was expected when the homozygous Milyang23 alleles were replaced by the homozygous Giho alleles (Table 5).

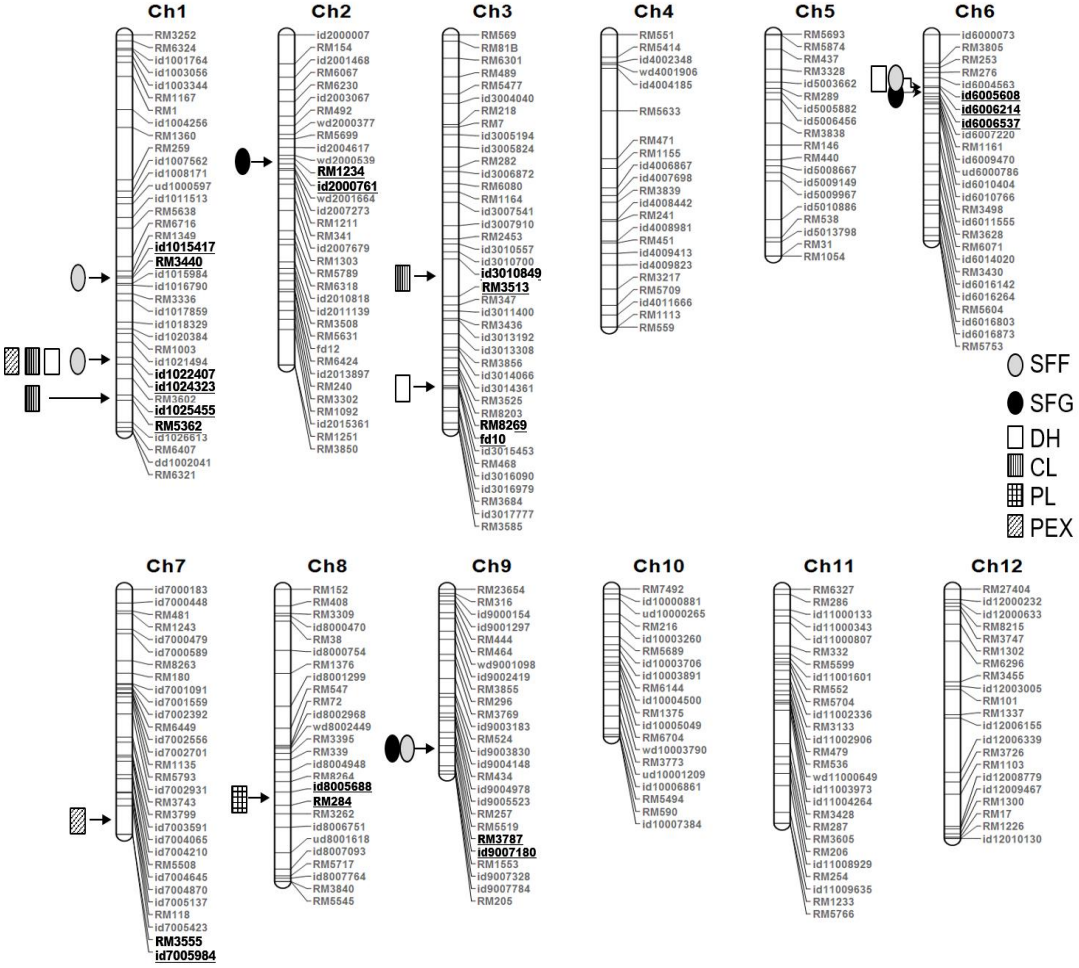

Figure 2. The linkage map and chromosomal locations of quantitative trait loci (QTL) for spikelet fertility and other agronomic traits detected in the recombinant inbred line (RIL) population. QTL detected through composite interval mapping analysis (IciMapping v3.2) are represented as boxes to the left of the chromosomes. Trait abbreviations are as follows. SFF: spikelet fertility in the cold-water-irrigated field; SFG: spikelet fertility in the temperature-controlled greenhouse; DH: days to heading; CL: culm length; PL: panicle length; PEX: panicle exsertion.

A total of three QTL were identified for spikelet fertility in the greenhouse (SFG) treated with cold water and cold air at the booting stage: *qSFG2*, *qSFG6* and *qSFG9* on chromosomes 2, 6 and 9, respectively, which explained 5.7–15.1% of the phenotypic variance (Table 5). All three SFG QTL were detected only in the cold treatment (Table 5, Table S1). The QTL positions of *qSFG6* and *qSFG9* overlapped with *qSFF6* and *qSFF9*, respectively, suggesting that these QTL would provide stable cold tolerance at the booting stage in both field and greenhouse conditions (Table 5). For all three SFG QTL, Giho contributed the allele for high spikelet fertility. Similar to the field analysis, *qSFG6* located between the markers id6006537 and id6007220 on chromosome 6 exhibited the highest additive effect: 17.0% increase in spikelet fertility was expected when the homozygous Milyang23 alleles were replaced by the homozygous Giho alleles (Table 5).

When the effects of each QTL on spikelet fertility was evaluated in the RIL population, plants carrying the allele for cold tolerance (Giho allele) at *qSFF6*/*qSFG6* and *qSFF9*/*qSFG9* exhibited significantly higher spikelet fertility than those carrying the allele for cold susceptibility (Milyang23 allele) in both cold-water-irrigated field and temperature-controlled greenhouse conditions (Figure 3), indicating that *qSFF6*/*qSFG6* and *qSFF9*/*qSFG9* confer reliable cold tolerance in both field and greenhouse environments.

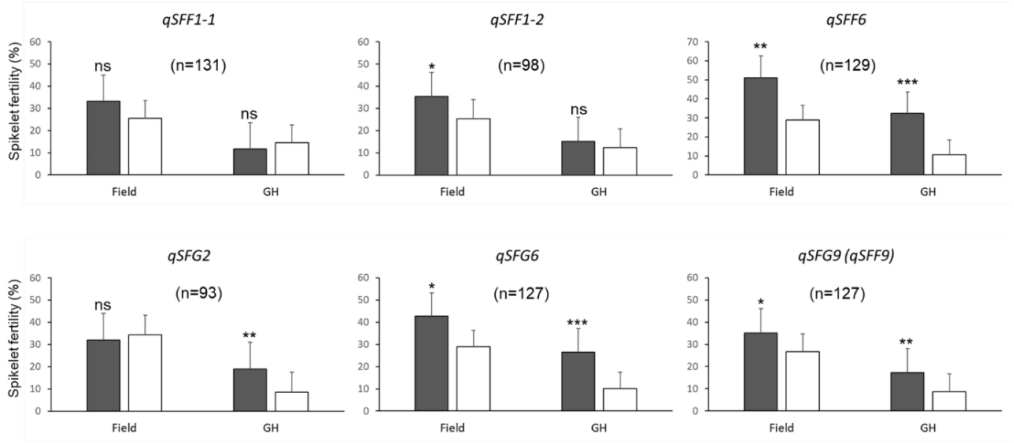

Figure 3. Effects of the cold tolerance QTL on spikelet fertility in cold-water-irrigated field and temperature-controlled greenhouse conditions. Gray bars and white bars indicate average spikelet fertility of RILs carrying alleles for cold tolerance and cold susceptibility at each locus, respectively. The alleles for cold tolerance were contributed by Giho at all QTL except for *qSFF1-1*, at which Milyang23 provided the allele for cold tolerance (see Table 5). *, **, ***: significantly different at $p < 0.05$, $p < 0.01$ and $p < 0.001$ based on the *t*-test. ns: not significant. Error bars indicate standard deviations.

3.4. QTL Identification for Agronomic Traits Related to Cold Tolerance

A total of 17 QTL for DH, CL, PL and PEX were identified in the cold-water-irrigated field (Table 6, Figure 2).

For DH, three QTL were detected on chromosomes 1 (*qDH1*), 3 (*qDH3*) and 6 (*qDH6*), which explained 9.2–24.8% of the phenotypic variance (Table 6). *qDH3* and *qDH3* were detected as the heading date QTL also in the control plot, while *qDH1* was detected only in the cold plot (Table 6, Table S1). The allele for late heading was contributed by Milyang23 at *qDH1* and *qDH3*, and by Giho at *qDH6*.

For CL, Four QTL were identified on chromosomes 1 (*qCL1-1* and *qCL1-2*), 3 (*qCL3*) and 10 (*qCL10)*, which explained 4.8–37.5% of the phenotypic variance. *qCL1-1* and *qCL3* were detected as QTL for culm length also in the control plot, while the others were detected only in the cold plot (Table 6, Table S1). The allele for long culm was contributed by Giho at *qCL1-1*, *qCL1-2*, *qCL10* and by Milyang23 at *qCL3*.

Seven QTL for PL was identified on chromosomes 1 (*qPL1-1*, *qPL1-2*), 3 (*qPL3-1*, *qPL3-2*), 8 (*qPL8-1*, *qPL8-2*) and 10 (*qPL10*) explaining 3.3–10.9% of the phenotypic variance. *qPL1-2* was the only QTL for panicle length detected in both control and cold treatments, while the others were detected only in the cold treatment (Table 6, Table S1). The allele for long panicle was contributed by Milyang23 at *qPL1-1*, *qPL3-1* and *qPL3-2*, and by Giho at *qPL1-2*, *qPL8-1*, *qPL8-2* and *qPL10*.

Three QTL for PEX were identified on chromosomes 1 (*qPEX1*), 7 (*qPEX7*) and 8 (*qPEX8*), which explained 7.1–25.1% of the phenotypic variance, respectively. *qPEX1* was detected as QTL for panicle exsertion also in the control, while the others were detected only in the cold treatment (Table 6, Table S1). The allele for long panicle exsertion was contributed by Giho for *qPEX1* and *qPEX7*, and by Milyang23 for *qPEX7*.

It should be noted that the QTL for DH (*qDH1*), CL (*qCL1-1*), PL (*qPL1-2*) and PEX (*qPEX1*) were identified at the similar position on chromosome 1 between markers id1022407 and id1025455, which also overlapped with the cold tolerance QTL *qSFF1-2* identified in the cold-water-irrigated field condition (Tables 4 and 5 and Figure 2). The allele for cold tolerance (Giho allele) at this locus was associated with early heading, long culm and long panicle exsertion. Additionally, *qDH6* detected between the markers id6004563 and id6005608 on chromosome 6 was at the position similar to *qSFF6* and *qSFG6* (Tables 4 and 5 and Figure 2). Unlike *qSFF1-2*, the allele for cold tolerance (Giho allele) at *qSFF6*/*qSFG6* was associated with late heading

Table 1. Variation in spikelet fertility in the RIL population and its parents under field and greenhouse conditions.

Evaluation Method [a]	Treatment	Parent (Mean ± SD)			RIL [c]						
		Milyang23	Giho [b]		Mean	Var	CV	Min	Max	Skewness	Kurtosis
Field	Control	90.3 ± 1.73	90.0 ± 1.73	ns	73.1	155.1	0.17	29.4	95.3	−0.919	0.982
	Cold	51.5 ± 2.08	76.0 ± 5.13	*	30.6	481.9	0.72	0.0	86.3	0.567	−0.481
Greenhouse	Control	87.5 ± 4.03	89.9 ± 3.66	ns	73.6	180.5	0.18	8.4	97.7	−1.457	4.198
	Cold	12.4 ± 2.33	55.7 ± 4.18	**	13.2	264.2	1.24	0.0	82.7	1.841	3.405

[a] In the field evaluation method, the control experiment was performed at a water temperature range of approximately 24–26 °C for normal growth. In the cold-water-irrigated plot, irrigation with cold water at 17 °C was performed during the entire rice growth period, from tillering (20 days after transplanting) to the grain maturity stage. In the greenhouse, cold treatment at the booting stage consisted of simultaneous treatment with cold air and cold water at a constant 17 °C. The water depth was 15 cm. After 10 days of cold treatment, the pots were moved outside to natural conditions, and the plants grew to the mature stage. [b] Differences between the two parents at * $p < 0.05$ and ** $p < 0.01$ based on the *t*-test. ns: not significant. [c] Var: variance; CV: coefficient of variation; Min: minimum; Max: maximum.

Table 2. Variation in agronomic traits in the RIL population and its parents in the control plot and the cold-water-irrigated plot.

Trait [a]	Treatment	Parent (Mean ± SD)			RIL [c]						
		Milyang23	Giho [b]		Mean	Var	CV	Min	Max	Skewness	Kurtosis
DH	Control	101.7 ± 1.15	100.7 ± 1.15	ns	98.7	30.2	0.06	84.0	130.0	2.124	9.583
	Cold	120.3 ± 2.52	111.3 ± 0.58	*	109.3	43.4	0.06	96.0	141.0	1.500	4.687
CL	Control	65.3 ± 1.53	77.0 ± 2.00	*	74.3	186.5	0.18	40.0	103.2	−0.085	−0.493
	Cold	37.0 ± 7.00	66.0 ± 1.73	*	50.9	168.1	0.26	24.6	81.6	0.080	−0.753
PL	Control	20.3 ± 1.15	22.5 ± 1.29	ns	20.7	4.8	0.11	15.4	25.4	0.100	−0.321
	Cold	18.3 ± 1.55	17.2 ± 0.35	ns	17.8	4.7	0.12	12.8	23.4	−0.073	−0.263
PEX	Control	1.4 ± 0.51	8.3 ± 0.42	**	5.1	11.2	0.66	−4.4	13.2	−0.036	0.105
	Cold	−5.5 ± 1.00	3.9 ± 0.32	**	-1.6	20.3	−2.67	−13.8	7.8	0.081	−0.420

[a] DH: days to heading (d); CL: culm length (cm); PL: panicle length (cm); PEX: panicle exsertion (cm). [b] Differences between the two parents at * $p < 0.05$ and ** $p < 0.01$ based on the *t*-test. ns: not significant.

Table 3. Correlation coefficients among major agronomic traits and spikelet fertility of the RIL population in the control and cold treatment.

Treatment	Trait [a]	DH	CL	PL	PEX	SFF	SFG
Control	DH	1	ns	ns	−0.177 **	ns	−0.213 **
	CL		1	0.211 **	0.455 **	0.149 **	ns
	PL			1	0.143 **	0.120 *	ns
	PEX				1	0.206 **	ns
	SFF					1	0.363 **
	SFG						1
Cold	DH	1	−0.259 **	−0.111 *	−0.241 **	0.122 *	ns
	CL		1	0.409 **	0.615 **	0.430 **	ns
	PL			1	0.399 **	0.195 **	ns
	PEX					0.518 **	0.124 *
	SFF					1	0.116 *
	SFG						1

[a] DH: days to heading; CL: culm length; PL: panicle length; PEX: panicle exsertion; SFF: spikelet fertility in the field; SFG: spikelet fertility in the greenhouse. DH, CL, PL and PEX were evaluated only in the field condition. Significant correlations are indicated with asterisks at * $p < 0.05$ and ** $p < 0.01$. ns: not significant.

Table 4. Summary of molecular markers used for genotyping and linkage map construction.

Chr.	No. of Tested Markers		No. of Polymorphic Markers [a]		% Polymorphism		No. of Markers Used for Map Construction [b]			Chromosome Length (cM)	Average Distance [c] (cM)
	SSR	SNP	SSR	SNP	SSR	SNP	SSR	SNP	Sum		
1	83	44	63	34	75.9	77.3	16	20	36	190.0	5.3
2	80	37	32	32	40.0	86.5	19	15	34	159.0	4.7
3	68	41	43	23	63.2	56.1	21	19	40	182.5	4.6
4	50	35	28	20	56.0	57.1	12	10	22	138.4	6.3
5	72	28	28	13	38.9	46.4	11	8	19	137.3	7.2
6	36	35	27	20	75.0	57.1	10	16	26	110.3	4.2
7	44	30	37	26	84.1	86.7	12	18	30	120.9	4.0
8	54	29	41	21	75.9	72.4	15	11	26	137.8	5.3
9	40	23	36	18	90.0	78.3	14	12	26	85.4	3.3
10	38	24	26	14	68.4	58.3	9	11	20	75.1	3.8
11	53	30	34	18	64.2	60.0	16	11	27	108.3	4.0
12	53	28	29	15	54.7	53.6	13	8	21	119.0	5.7
Sum(Mean)	671	384	424	254	(65.5)	(65.8)	168	159	327	1564.0	(4.9)

[a] Polymorphism was tested between Milyang23 and Giho, the parents of the RIL population used for linkage map construction and QTL mapping. [b] Markers with unclear genotyping results and overlapping genetic positions were excluded. [c] Average distance between the two adjacent markers used for map construction.

Table 5. QTL for spikelet fertility under cold treatment in the field and greenhouse conditions determined by composite interval mapping.

Evaluation Method	Trait [a]	QTLs	Chr.	Marker Interval			Position (Mb) [b]			Position (cM)	LOD [c]	R^2 [d]	Additive Effect [e]
Field	SFF	*qSFF1-1*	1	id1015417	~	RM3440	26.3	~	27.2	120	3.5	6.6	6.7
		qSFF1-2	1	id1024323	~	RM3602	38.3	~	39.0	160	5.6	10.6	−8.5
		qSFF6	6	id6004563	~	id6005608	7.1	~	8.7	39	3.4	6.5	−12.9
		qSFF9	9	RM3787	~	id9007180	20.0	~	20.8	75	3.3	6.3	−6.5
Greenhouse	SFG	*qSFG2*	2	RM1234	~	ud2000761	11.3	~	14.2	67	2.5	5.7	−3.7
		qSFG6	6	id6006537	~	id6007220	10.6	~	11.4	43	5.2	15.1	−8.5
		qSFG9	9	id9007180	~	RM1553	20.8	~	21.0	76	2.6	6.7	−4.0

[a] Trait abbreviations are as follows. SFF: spikelet fertility in the field; SFG: spikelet fertility in the greenhouse. [b] Os-Nipponbare-Reference-IRGSP 1.0 [28]. [c] LOD experiment-wise $p = 0.05$ was equivalent to the critical LOD score threshold of 2.5. [d] Percent phenotypic variance explained by the QTL. [e] Negative values indicate additive effects from Giho.

Table 6. QTL for other agronomic traits under cold-water-irrigated field determined by composite interval mapping.

Trait [a]	QTLs	Chr.	Marker Interval			Position (Mb) [b]			Position (cM)	LOD [c]	R^2 [d]	Additive Effect [e]
DH	*qDH1*	1	id1022407	~	id1024323	35.5	~	38.3	159	9.0	17.3	2.8
	qDH3	3	RM8269	~	fd10	31.4	~	31.5	159	5.0	9.2	2.1
	qDH6	6	id6004563	~	id6005608	7.1	~	8.7	39	11.2	24.8	−6.7
CL	*qCL1-1*	1	id1022407	~	id1024323	35.5	~	38.3	159	17.4	37.5	−6.8
	qCL1-2	1	id1025455	~	RM5362	40.0	~	41.1	174	5.8	9.9	−3.5
	qCL3	3	id3010849	~	RM3513	24.4	~	25.1	116	6.7	13.6	4.2
	qCL10	10	id10004500	~	RM1375	16.1	~	16.7	42	2.9	4.8	−2.5
PL	*qPL1-1*	1	id1016790	~	RM3336	28.6	~	28.6	124	5.6	6.5	0.7
	qPL1-2	1	RM3602	~	id1025455	39.0	~	40.0	173	8.5	10.8	−0.9
	qPL3-1	3	id3005194	~	id3005824	10.1	~	11.1	50	2.9	3.3	0.5
	qPL3-2	3	id3013192	~	id3013308	28.3	~	28.5	134	8.9	10.9	0.9
	qPL8-1	8	wd8002449	~	RM3395	10.3	~	13.7	77	5.2	6.0	−0.7
	qPL8-2	8	id8007764	~	RM3840	27.8	~	27.9	137	6.7	8.1	−0.8
	qPL10	10	id10003260	~	RM5689	12.1	~	13.6	23	6.0	7.3	−0.7
PEX	*qPEX1*	1	id1024323	~	RM3602	38.3	~	39.0	160	11.5	25.1	−2.3
	qPEX7	7	id7005423	~	RM3555	27.6	~	27.9	120	4.9	9.8	1.4
	qPEX8	8	RM8264	~	id8005688	19.8	~	20.8	94	3.3	7.1	−1.2

[a] Trait abbreviations are as follows. DH: days to heading (d); CL: culm length (cm); PL: panicle length (cm); PEX: panicle exsertion (cm). [b] Os-Nipponbare-Reference-IRGSP 1.0 [28]. [c] LOD experiment-wise $p = 0.05$ was equivalent to the critical LOD score threshold of 2.5. [d] Percent phenotypic variance explained by the QTL. [e] Negative values indicate additive effects from Giho.

4. Discussion

4.1. Potential Use of the Cold Tolerance QTL in Inter-Subspecies Rice Breeding

Cold stress is one of the major obstacles to stable rice production in the temperate rice-cultivating regions of the world. Between the two major rice subspecies, *japonica* varieties generally exhibit much stronger cold tolerance than *indica* varieties at all growth stages [29,30]. This often poses problems in *japonica–indica* cross breeding in the temperate region. In Korea, the *japonica–indica* inter-subspecies cross has been used over 40 years to develop nearly 60 high-yielding rice varieties categorized as the Tongil-type, which originates from the Korean "Green Revolution" in the 1970s [31]. Although significant yield enhancement has been achieved by the Tongil-type breeding programs in Korea, improving milled rice yield from around 5 t/ha in the 1970s to over 8 t/ha, cold susceptibility of the Tongil-type cultivars remains as the major limitation in achieving yield stability across years and environments [32].

Using a RIL population derived from a cross between the Tongil-type variety Milyang23 and the *japonica* variety Giho, we identified booting stage cold tolerance QTL of which the allele for cold tolerance was contributed by Giho at six of the seven QTL (Table 5). The favorable *japonica* alleles at these QTL provide genetic sources potentially useful for enhancing cold tolerance of the Tongil-type varieties. The two QTL pairs identified on chromosomes 6 (*qSFF6*/*qSFG6* and) and 9 (*qSFF9*/*qSFG9*) are especially promising as these were detected in both field and greenhouse conditions (Figure 3). To facilitate the use of the beneficial *japonica* alleles for cold tolerance in Tongil-type breeding programs, we are currently investigating the allelic composition of these QTL in 300 Korean rice varieties (263 *japonica* and 37 Tongil-type) released during 1979–2017 [33] to design cross combinations for efficiently pyramiding the desirable alleles and validating their effects under different genetic backgrounds.

4.2. Reliable Cold Tolerance QTL at the Booting Stage Identified by the Dual Screening System

In this study, we identified QTL associated with spikelet fertility under low temperature conditions using two different screening methods, namely, a cold-water-irrigated field and a temperature-controlled greenhouse. A total of seven QTL were identified–*qSFF1-1*, *qSFF1-2*, *qSFF6* and *qSFF9* in the field condition and *qSFG2*, *qSFG6* and *qSFG9* in the greenhouse condition (Table 5, Figure 2). Notably, two QTL pairs were detected in similar chromosomal regions in both screening conditions–*qSFF6* and *qSFG6* at 39–43 cM position on chromosome 6, and *qSFF9* and *qSFG9* at 75–76 cM position on chromosome 9. *qSFF6*/*qSFG6* and *qSFF9*/*qSFG9* detected in our study represent reliable cold tolerance QTL at the booting stage, as the alleles for cold tolerance contributed by Giho at these loci significantly increased spikelet fertility under cold treatments in both field and greenhouse conditions (Figure 3).

Many breeding programs rely on cold-water-irrigated field method for screening cold-tolerant rice genotypes, as it allows large amounts of material to be screened and because changes in agronomic traits associated with cold tolerance, such as DH, CL and PL, can be evaluated simultaneously [5,17]. However, since air temperature cannot be controlled under field conditions, characteristics such as early or late heading may allow plants to escape from cold water/air and reduce spikelet infertility [6,9,34]. *qSFF1-2* detected in our study in cold-water-irrigated field represents such case. In the marker intervals containing id1022407 or id1025455 as a flanking marker on chromosome 1 (35.5–40.0 Mb; IRGSP-1.0), *qSFF1-2*, *qDH1*, *qCL1-1*, *qPL1-2* and *qPEX1* were simultaneously identified (Tables 4 and 5, Figure 2). At this locus, the allele for cold tolerance contributed by Giho was associated with earlier heading, longer culm and more panicle exsertion, indicating that cold tolerance conferred by *qSFF1-2* is likely due to the rapid escape of young panicles from cold water. This is supported by the fact that *qSFF1-2* was not detected in the temperature-controlled greenhouse screening precisely targeting the booting stage (cold water and cold air treatment for 10 days during the booting stage). Similar to *qSFF1-2*, *qSFF6* on chromosome 6 was also co-located with a heading date QTL, *qDH6* (Tables 4 and 5, Figure 2). However, unlike *qSFF1-2*, the allele for cold tolerance contributed by Giho at *qSFF6* was associated with later heading. While it is possible that late heading might have contributed to high spikelet fertility by

allowing the plants to avoid cold air, the cold tolerance conferred by this locus may not be merely from cold escape due to late heading as the similar region was identified as a cold tolerance QTL, *qSFG6*, also in the temperature-controlled greenhouse screening.

Unlike *qSFF1-2* and *qSFF6*/*qSFG6*, the flanking markers of *qSFF1-1*, *qSFG2* and *qSFF9*/*qSFG9* did not overlap with QTL controlling other agronomic traits. While *qSFF1-1* and *qSFG2* were detected in only one of the two screening methods, the locus represented by *qSFF9*/*qSFG9* was identified in both cold-water-irrigated field and temperature-controlled greenhouse conditions, suggesting that *qSFF9*/*qSFG9* is a reliable cold tolerance QTL for the booting stage that is not affected by other traits such as heading time or culm length.

4.3. Comparison with Previously Identified Cold Tolerance QTL at the Booting Stage

qSFF6 and *qSFG6* explained 6.5% and 15.1% of the phenotypic variance in spikelet fertility, with the additive effects of 12.9% and 8.5% contributed by Giho in the cold-water-irrigated field and the temperature-controlled greenhouse conditions, respectively. *qSFF6* was located on chromosome 6 between id6004563 and id6005608 (7.1–8.7 Mb; IRGSP-1.0), and *qSFG6* was located between id6006537 and id6007220 (10.6–11.4 Mb; IRGSP-1.0). *qCTB6* previously identified near RM50 (6.4 Mb; IRGSP-1.0; [14]) and *qRCT6b* flanked by R1954–RM253 (4.9–5.4 Mb; IRGSP-1.0; [35]) on chromosome 6 are located relatively close to *qSFF6*/*qSFG6*, with similar additive effects on spikelet fertility (7.4% and 10.2% contributed by IR50 and Kunmingxiaobaigu, respectively). Although the chromosomal locations suggest that *qSFF6*/*qSFG6* may represent a different cold tolerance gene from *qRCT6b*, we were not able to precisely compare the locations of *qSFF6*/*qSFG6* and *qCTB6* as one of the flanking markers for *qCTB6* (RM173) was located on chromosome 5, not 6, of the rice pseudomolecule reference Os-Nipponbare-Reference-IRGSP-1.0 [28].

Within the *qSFF6*/*qSFG6* region, *qDH6* associated with DH under low temperature was identified by the same marker interval as *qSFF6* (Tables 4 and 5). The Giho allele for cold tolerance at *qSFF6* was associated with late heading. Previously, a heading date QTL *dth6* was identified on chromosome 6 flanked by markers RM539–RM527 (8.2–9.9 Mb; IRGSP-1.0; [36]), which overlaps with the *qSFF6*/*qDH6* interval (7.1–8.7 Mb; IRGSP-1.0). Fine-mapping and cloning of *qSFF6*/*qSFG6* and *dth6* are required to determine if a single gene underlying this locus has pleiotropic effects on cold tolerance and heading time, or there are two adjacent genes controlling each trait independently.

The other reliable QTL pair, *qSFF9*/*qSFG9*, was detected on chromosome 9 between RM3787–RM1553 (20.0–21.0 Mb; IRGSP-1.0) and explained 6.3% and 6.7% of the phenotypic variation in spikelet fertility under the cold-water-irrigated field and temperature-controlled greenhouse conditions, respectively (Table 5). The additive effects of *qSFF9* (6.5%) and *qSFG9* (4.0%) contributed by Giho were approximately half those of *qSFF6* (12.9%) and *qSFG6* (8.5%). Three cold tolerance QTL previously identified on chromosome 9 are located close to *qSFF9*/*qSFG9*–*qPSST-9* flanked by RM24427–RM24545 (16.4–18.2 Mb; IRGSP-1.0; [17]), *qCTB9* flanked by RM257–RM242 (17.7–18.8 Mb; IRGSP-1.0; [14]) and *cisc(t)* also flanked by RM257–RM242 (17.7–18.8 Mb; IRGSP-1.0; [37]). Both *qPSST-9* and *qCTB9* were identified as QTL affecting spikelet fertility under cold treatments and exhibited additive effects of 6.2% and 4.7% contributed by the tolerant parents IR66160-121-4-4-2 and M-202, respectively, which were similar to those of *qSFF9*/*qSFG9* [14,17]. While *cist(t)* was subsequently fine-mapped in a region with a single candidate gene encoding a pentatricopeptide repeat protein [38], it controls cold tolerance at the seedling stage and its effects on spikelet fertility under a cold temperature is unknown [37,38]. Further work is required to determine if *qSFF9*/*qSFG9*, *qPSST-9* and *qCTB9*/*cisc(t)* represent alleles of a same gene or different genes located in a similar chromosomal position.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/10/9/1225/s1, Figure S1: Linkage map from the Milyang23 × Giho RIL population using 168 SSR and 159 SNP markers, Table S1: QTL for spikelet fertility and other agronomic traits under control condition.

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References

1. Yoshida, S. Growth and development of the rice plant. In *Fundamentals of Rice Crop Science*; International Rice Research Institute: Los Baños, Philippines, 1981; pp. 1–61.
2. Xie, G.; Kato, H.; Imai, R. Biochemical identification of the *OsMKK6-OsMPK3* signalling pathway for chilling stress tolerance in rice. *Biochem. J.* **2012**, *443*, 95–102.
3. Lee, M. Low temperature tolerance in rice: The Korean experience. In Proceedings of the Conference: Increased Lowland Rice Production in the Mekong Region, Vientiane, Laos, 30 October–2 November 2000.
4. Farrell, T.; Fox, K.M.; Williams, R.L.; Fukai, S.; Reinke, R.F.; Lewin, L.G. Temperature constraints to rice production in Australia and Laos: A shared problem. In Proceedings of the Conference: Increased Lowland Rice Production in the Mekong Region, Vientiane, Laos, 30 October–2 November 2000.
5. Xu, L.M.; Zhou, L.; Zeng, Y.W.; Wang, F.; Zhang, H.; Shen, S.; Li, Z. Identification and mapping of quantitative trait loci for cold tolerance at the booting stage in a *japonica* rice near-isogenic line. *Plant. Sci.* **2008**, *174*, 340–347.
6. Satake, T.; Hayase, H. Male sterility caused by cooling treatment at the young microspore stage in rice plants: V. Estimations of pollen developmental stage and the most sensitive stage to coolness. *Jpn. J. Crop. Sci.* **1970**, *39*, 468–473.
7. Mackill, D.J.; Coffman, W.R.; Garrity, D.P. *Rainfed Lowland Rice Improvement*; International Rice Research Institute: Los Baños, Philippines, 1996.
8. Saito, K.; Miura, K.; Nagano, K.; Hayano-Saito, Y.; Saito, A.; Araki, H.; Kato, A. Chromosomal location of quantitative trait loci for cool tolerance at booting stage in rice variety 'Norin-PL8'. *Breed. Sci.* **1995**, *45*, 337–340.
9. Takeuchi, Y.; Hayasaka, H.; Chiba, B.; Tanaka, I.; Shimano, T.; Yamagishi, M.; Nagano, K.; Sasaki, T.; Yano, M. Mapping quantitative trait loci controlling cool-temperature tolerance at booting stage in temperate *japonica* rice. *Breed. Sci.* **2001**, *51*, 191–197.
10. Shinada, H.; Iwata, N.; Sato, T.; Fujino, K. Genetical and morphological characterization of cold tolerance at fertilization stage in rice. *Breed. Sci.* **2013**, *63*, 197–204.
11. Saito, K.; Miura, K.; Nagano, K.; Hayano-Saito, Y.; Araki, H.; Kato, A. Identification of two closely linked quantitative trait loci for cold tolerance on chromosome 4 of rice and their association with anther length. *Theor. Appl. Genet.* **2001**, *103*, 862–868.
12. Saito, K.; Hayano-Saito, Y.; Maruyama-Funatsuki, W.; Sato, Y.; Kato, A. Physical mapping and putative candidate gene identification of a quantitative trait locus *Ctb1* for cold tolerance at the booting stage of rice. *Theor. Appl. Genet.* **2004**, *109*, 515–522.
13. Saito, K.; Hayano-Saito, Y.; Kuroki, M.; Sato, Y. Map-based cloning of the rice cold tolerance gene *Ctb*1. *Plant. Sci.* **2010**, *179*, 97–102.
14. Andaya, V.C.; Mackill, D.J. QTLs conferring cold tolerance at the booting stage of rice using recombinant inbred lines from a *japonica* × *indica* cross. *Theor. Appl Genet.* **2003**, *106*, 1084–1090.
15. Kuroki, M.; Saito, K.; Matsuba, S.; Yokogami, N.; Shimizu, H.; Ando, I.; Sato, Y. A quantitative trait locus for cold tolerance at the booting stage on rice chromosome 8. *Theor. Appl Genet.* **2007**, *115*, 593–600.
16. Zhang, Z.; Li, J.; Pan, Y.; Li, J.; Zhou, L.; Shi, H.; Zeng, Y.; Guo, H.; Yang, S.; Zheng, W.; et al. Natural variation in *CTB4a* enhances rice adaptation to cold habitats. *Nat. Commun.* **2017**, *8*, 14788.

17. Suh, J.P.; Jeung, J.U.; Lee, J.I.; Choi, Y.H.; Yea, J.D.; Virk, P.S.; Mackill, D.J.; Jena, K.K. Identification and analysis of QTLs controlling cold tolerance at the reproductive stage and validation of effective QTLs in cold-tolerant genotypes of rice (*Oryza sativa* L.). *Theor. Appl. Genet.* **2010**, *120*, 985–995.
18. Yang, L.M.; Liu, H.L.; Zhao, H.W.; Wang, J.G.; Sun, J.; Zheng, H.L.; Lei, L.; Zou, D.T. Mapping quantitative trait loci and meta-analysis for cold tolerance in rice at booting stage. *Euphytica* **2019**, *215*, 89.
19. Cho, Y.G.; Kang, M.R.; Kim, Y.W.; Eun, M.Y.; Chung, T.Y.; Lee, Y.T. Development of RFLP framework map of rice (*Oryza sativa* L.) using recombinant inbreed population derived from Milyang23/Gihobyeo cross. *Korean J. Breed.* **1998**, *30*, 289–297.
20. Ji, H.; Kim, H.; Lee, G.; Yoon, U.; Kim, T.; Seol, Y.; Yun, D.; Koh, H.; Eun, M. Molecular genetic and physical map using PCR-based DNA markers with the recombinant inbred population derived from Milyang23/Gihobyeo cross. *Korean J. Breed.* **2012**, *44*, 273–281.
21. Murray, M.G.; Thompson, W.F. Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res.* **1980**, *8*, 4321–4325.
22. Thomson, M.J.; Zhao, K.; Wright, M.; McNally, K.L.; Rey, J.; Tung, C.; Reynolds, A.; Scheffler, B.; Eizenga, G.; McClung, A.; et al. High-throughput single nucleotide polymorphism genotyping for breeding applications in rice using the BeadXpress platform. *Mol. Breed.* **2012**, *29*, 875–886.
23. Wright, M.H.; Tung, C.W.; Zhao, K.; Reynolds, A.; McCouch, S.R.; Bustamante, C.D. Alchemy: A reliable method for automated SNP genotype calling for small batch sizes and highly homozygous populations. *Bioinformatics* **2010**, *26*, 2952–2960.
24. Tanno, H.; Kinoshita, M.; Kiuchi, H.; Hirayama, Y.; Kikuchi, H. Estimation of cool-weather tolerance at the flowering stage and its relationship with that of the booting stage in Hokkaido rice varieties. *Jpn. J. Crop. Sci.* **2000**, *69*, 493–499.
25. Lincoln, S.E.; Daly, M.J.; Lander, E.S. *Constructing Genetic Linkage Maps With MAPMAKER/EXP Version 3.0 A Tutorial and Reference Manual*; A Whitehead Institute for Biomedical Research Technical Report; Whitehead Institute for Biomedical Research: Cambridge, MA, USA, 1993.
26. Wang, J.; Li, H.; Zhang, L.; Li, C.; Meng, L. *Users' Manual of QTL IciMapping v3.1*; Institute of Crop Science, CAAS: Beijing, China; Crop Research Informatics Lab: Mexico City, Mexico, 2011.
27. Septiningsih, E.M.; Sanchez, D.L.; Singh, N.; Sendon, P.M.; Pamplona, A.M.; Heuer, S.; Mackill, D.J. Identifying novel QTLs for submergence tolerance in rice cultivars IR72 and Madabaru. *Theor. Appl. Genet.* **2012**, *124*, 867–874.
28. Kawahara, Y.; Bastide, M.; Hamilton, J.P.; Kanamori, H.; McCombie, W.R.; Ouyang, S.; Schwartz, D.C.; Tanaka, T.; Wu, J.; Zhou, S.; et al. Improvement of the Oryza sativa Nipponbare reference genome using next generation sequence and optical map data. *Rice* **2013**, *6*, 4.
29. Mackill, D.J.; Lei, X. Genetic variation for traits related to temperate adaptation of rice cultivars. *Crop. Sci.* **1997**, *37*, 1340–1346.
30. Pan, Y.; Zhang, H.; Zhang, D.; Li, J.; Xiong, H.; Yu, J.; Li, J.; Rashid, M.A.R.; Li, G.; Ma, X.; et al. Genetic analysis of cold tolerance at the germination and booting stages in rice by association mapping. *PLoS ONE* **2015**, *10*, e0120590.
31. Kim, B.; Kim, D.G.; Lee, G.; Seo, J.; Choi, I.Y.; Choi, B.S.; Yang, T.J.; Kim, K.S.; Lee, J.; Chin, J.H.; et al. Defining the genome structure of 'Tongil' rice, an important cultivar in the Korean "Green Revolution". *Rice* **2014**, *7*, 22.
32. Won, Y.J.; Ahn, E.K.; Lee, J.H.; Suh, J.P.; Lee, J.S.; Chang, J.K.; Choi, Y.H.; Hwang, T.S.; Lim, Y.T.; Ha, W.G.; et al. Regional yield variation of some Korean high-yielding rice varieties. *Korean J. Int. Agric.* **2014**, *26*, 414–418.
33. Lee, C.M.; Park, H.S.; Baek, M.K.; Suh, J.P.; Kim, C.S.; Lee, K.M.; Park, S.G.; Cho, Y.C. Characterization of grain-related traits of 300 Korean rice varieties. In Proceedings of the Conference: 2019 Korean Society of Breeding Science (KSBS) & Society for the Advancement of Breeding Research in Asia and Oceania (SABRAO) international conference on plant breeding for sustainable development, Gwangju, Korea, 2–5 July 2019.
34. Satake, T. Male sterility caused by cooling treatment at the young microspore stage in rice plants. XXIX. The mechanism of enhancement in cool tolerance by raising water temperature before the critical stage. *Jpn. J. Crop. Sci.* **1989**, *58*, 240–245.

35. Dai, L.; Lin, X.; Ye, C.; Ise, K.; Saito, K.; Kato, A.; Xu, F.; Yu, T.; Zhang, D. Identification of quantitative trait loci controlling cold tolerance at the reproductive stage in Yunnan landrace of rice, Kunmingxiaobaigu. *Breed. Sci.* **2004**, *54*, 253–258.
36. Oh, C.S.; Choi, Y.H.; Lee, S.J.; Yoon, D.; Moon, H.; Ahn, S. Mapping of quantitative trait loci for cold tolerance in weedy rice. *Breed. Sci.* **2004**, *54*, 373–380.
37. Lan, T.; Liang, K.J.; Chen, Z.W.; Duan, Y.L.; Wang, J.L.; Ye, N.; Wu, W.R. Genetic analysis and gene mapping of cold-indued seedling chlorosis in rice. *Hereditas* **2007**, *29*, 1121–1125.
38. Lan, T.; Wang, B.; Ling, Q.; Xu, C.; Tong, Z.; Liang, K.; Duan, Y.; Jin, J.; Wu, W. Fine mapping of *cisc(t)*, a gene for cold-induced seedling chlorosis and identification of its candidate in rice. *Chin. Sci. Bull.* **2010**, *55*, 3149–3153.