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QTLs conferring cold tolerance at the booting stage of rice using recombinant inbred lines from a *japonica* × *indica* cross

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Abstract Low temperature stress is common for rice grown in temperate regions and at high elevations in the tropics. The most senstive stage to this stress is booting, about 11 days before heading. Japonica cultivars are known to be more tolerant than indicas. We constructed a genetic map using 191 recombinant inbred lines derived from a cross between a temperate *japonica*, M-202, and a tropical *indica*, IR50, in order to locate quantitative trait loci (QTLs) conferring cold tolerance. The map with a total length of 1,276.8 cM and an average density of one marker every 7.1 cM was developed from 181 loci produced by 175 microsatellite markers. Cold tolerance was measured as the degree of spikelet sterility of treated plants at a 12 °C temperature for 5 days in the growth chamber. QTLs on chromosomes 1, 2, 3, 5, 6, 7, 9 and 12 were identified to confer cold tolerance at the booting stage. The QTL contribution to the phenotypic variation ranged from 11 to 17%. The two QTLs with the highest contribution to variation, designated *qCTB2a* and *qCTB3*, were derived from the tolerant parent, M-202, each explaining approximately 17% of the phenotypic variance. Two of the eight QTLs for cold tolerance were contributed by IR50.

Keywords Low temperature · Spikelet sterility · Rice · QTL · Genetic map

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Introduction

Molecular markers have facilitated the identification of chromosomal regions associated with many complex traits in rice (McCouch and Doerge 1995). A rice genetic linkage map comprised of simple sequence repeats (SSRs) or microsatellite markers was constructed recently with a map density of one SSR marker per 6 centimorgans (cM) (Temnykh et al. 2000). Having a high frequency of occurrence genome-wise, a high mutation rate and therefore a high level of polymorphism, these microsatellite markers are becoming increasingly important as markers of choice in genetic studies. It was estimated that around 5,700–10,000 of these uniformly distributed and highly diverse markers are present in the rice genome (McCouch et al. 1997). The rice genome sequence published by Goff et al. (2002) indicated the presence of one potential SSR marker every 8,000 bp for a total of 48,351.

Genetic markers are useful in overcoming limitations in breeding for difficult traits like cold tolerance. The ability of rice to tolerate low temperatures is a major distinguishing factor in classifying the two major subspecies of *Oryza sativa, japonica* and *indica* (Glaszmann et al. 1990). The *japonicas* are more cold tolerant than the *indicas* and predominate in temperate and subtropical environments. The *indica* germplasm has many desirable alleles for improving agronomic characters, stress resistance or grain quality. *Indicas* could be used for varietal improvement and to diversify *japonica* germplasm, because reliance on a limited gene pool has resulted in the narrowing of the genetic base (Dilday 1990). When used as donors for important traits, cold tolerance must be selected to maintain the high level needed for production in temperate and cool rice-growing areas.

Low temperatures dramatically reduce grain yield if the stress coincides with the sensitive period during the reproductive stage of rice by inducing spikelet sterility due to failure of microspore development (Satake 1989) or a decrease in the number of pollen grains per anther (Murai et al. 1991). It was shown using mutants from

Taichung 65 that pollen development was inhibited and the embryo sac was malformed in some mutants that resulted in lower seed fertility when treated with cool water at 19 °C (Nagasawa et al. 1994). Exposure at 15 °C/10 °C day/night temperature reduces spikelet number and fertility up to 90% for susceptible genotypes (Jacobs and Pearson 1999), and can also affect the overall grain and eating quality (Nishimura 1993). The degree of cold damage depends on the length of the low temperature period and the maximum and minimum temperature (Hosoi 1990).

In spite of the significance of booting-stage cold tolerance in rice cultivation, however, the genetic mechanism of low temperature resistance at this stage is not well known. Nishimura and Hamamura (1993), subjecting plants to 15 °C for 10 days, observed that cold tolerance seemed to be controlled by dominant alleles. It was estimated that four or more genes controlled cold tolerance of a *japonica* cultivar Somewake and that low temperature resistance was dominant over susceptibility (Nagasawa et al. 1994). Studies have been conducted to locate chromosomal regions responsible for cold tolerance at the vegetative stage, but there were only a few instances of quantitative trait loci (QTLs) being identified at the booting stage. Saito et al. (1995), using the cool irrigation water method in field screening, identified segments from chromosomes 3 and 4 in Norin-PL8 controlling booting-stage tolerance. These segments appeared to be the introgressed regions from a tolerant Indonesian tropical *japonica* cultivar, Silewah. Li et al. (1997) identified two QTLs on chromosome 1 and one on chromosome 12 that, in indica-japonica hybrids, were associated with low-temperature induced sterility in the heterozygous state. Recently, Takeuchi et al. (2001) identified QTLs on chromosomes 1, 7 and 11 based on a doubled-haploid (DH) population from a cross between two temperate *japonica* cultivars.

The appropriate screening procedure in evaluating low-temperature sensitivity at the booting stage in rice is an important issue in measuring the trait. The procedure of screening rice in the field using cool irrigation water has been considered a reliable method of screening (Nagasawa et al. 1994; Nishimura 1995), although air temperature may not be necessarily controllable under field conditions. In addition, the differential maturity of test lines or the duration and growth stage by which stress is imposed add to the complexity in data interpretation. Thus, it is imperative to study the genetics of booting-stage cold tolerance in the growth chamber or greenhouse to minimize the problems imposed by differences in maturity of the test materials.

The major objective of this study was to locate genetic loci or QTLs associated with rice cold tolerance at the booting stage measured in a controlled environment facility. We used recombinant inbred lines (RILs) developed from a cross between the widely grown California *japonica* M-202 and the tropical *indica* IR50. The attributes of the genetic map, comprised mainly of microsatellite markers, were also described.

Materials and methods

Mapping population

A temperate *japonica* California rice cultivar, M-202, and an *indica* cultivar IR50, developed at the International Rice Research Institute (IRRI), were crossed to generate the mapping population. Widely grown in California for its overall tolerance to low temperature, M-202 is a photoperiod insensitive, early maturing, semidwarf, and has fully exserted panicles, good seedling vigor, and superior tolerance to low temperature-induced spikelet sterility. IR50, being adapted to tropical environments, is cold sensitive at both seedling and booting stages.

A single F_1 seed from the M-202/IR50 cross was advanced to the F_2 generation to develop the RILs. Since the F_1 of this intersubspecific cross exhibited partial sterility, the F_1 plant was grown in the greenhouse and the panicles covered with glassine bags to prevent cross-pollination. In the F_2, only the plants that exhibited normal spikelet fertility were advanced via the single-seed descent method. The panicles of selected plants were bagged during the advancement to prevent outcrossing. A total of 191 RILs were extracted in the F_5 generation and the resulting F_6 seeds were used in the experiments.

Marker analysis

DNA was extracted from young rice leaves as described by Mackill (1995). A total of 300 commercially available rice microsatellite primer pairs (Research Genetics, Huntsville, Ala., USA) were initially surveyed for polymorphism with DNA from the parents and the F_1 using 96-well gels performed in the ABI Prism 377 DNA sequencer (Applied Biosystems, USA).

The polymerase chain reaction (PCR) procedures were as described by Ni et al. (2002), with some modifications. Briefly, a 15-µl reaction mixture containing 1.5 µl of PCR buffer (20 mM Tris pH 8.0, 50 mM KCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 50% glycerol), 50 ng of DNA, 330 nM of each forward and reverse primer, 250 µM of each dNTPs, 330 nM of rhodamine dye-labeled fluorescent dUTP and 0.6 units of *Taq* polymerase. The samples were prepared in a 96-well amplification plate for amplification using the GeneAmp PCR System 9700 thermocycler (Applied Biosystems). The PCR conditions were as follows: a 2-min hold at 95 °C; 25 cycles of 95 °C for 15 s, 55 °C for 1 min, 72 °C for 30 s; and a final extension step at 72 °C for 5 min. Protocols for preparing gels, sample preparation, sample loading, running the electrophoresis using the ABI Prism 377 DNA sequencer followed the manufacturer's recommendations (Applied Biosystems). Fragment analysis was performed using the GeneScan software (Applied Biosystems).

Microsatellite markers that detected polymorphism between the two parents were noted for band size and intensity and were used to assay the RIL population. Preparations for PCR and electrophoresis were made to allow multiplexing up to four microsatellite primer pairs in a single PCR and eight markers during the ABI Prism 377-run using two fluorescent labels.

Booting-stage cold tolerance screening

On 27 May 2000, 20 seeds of each RIL were seeded in germination trays (TLC Polyform Inc, Minn., USA) containing Sunshine Mix #1 growing medium (SunGro, Wash., USA). The trays were placed in a Conviron PGV36 (Controlled Environments Ltd., Winnipeg) walk-in growth chamber set at 25 °C/20 °C 12-h day/12-h night temperature regimes. Ten plants from each of the 191 RILs and the two parents were transplanted in a 4-L cylindrical plastic pot containing 2 kg of pulverized dry soil and 10 g of commercial grade fertilizer (15-15-15, $N–P_2O_5–K_2O$) on 10 June. Seedlings were arranged in a circular pattern in the pot. Small pots containing two seedlings of each RIL were prepared to monitor fertility of greenhouse-grown rice plants. Every week, extra tillers were removed from each plant

in a pot, leaving a maximum of two tillers per plant to avoid overcrowding and to promote better growth.

Approximately 2 weeks before booting, or when the distance between the auricles of the flag leaf and the penultimate leaf of the tiller was within 2 cm, tillers were tagged and pots were taken to the growth chamber. The growth chamber condition was set as follows: 12 °C constant temperature, 12-h light period, 800 µmol m^{-2} s^{-1} irradiance and 72% relative humidity. After 5 days, treated pots were taken back to the greenhouse until maturity. At maturity, tagged tillers were harvested, processed and measured for the following data: total number of spikelets per panicle (TGP) and the number of fully filled (FFS), partially filled (PFS) and undeveloped spikelets (US) per panicle. Spikelet fertility was expressed as SFPa, SFPb and USP, where SFPa = FFS/TGP × 100; SFPb = (FFS+PFS)/TGP × 100, and; USP = US/TGP × 100. Only the tillers that headed 13–16 days after the start of treatment were included in data analysis.

Linkage and QTL analysis

The linkage map was constructed using MAPMAKER 2.0 (Lander et al. 1987), a version of the linkage program that runs on a Macintosh computer. The GROUP command was set at a LOD = 5.0 to identify initial linkage groupings, and markers that did not belong to the initial group even at LOD = 1.0 were not included in the analysis. A total of 175 microsatellite markers producing 181 loci were used in constructing the linkage map. The genetic map had a total length of 1,276.8 cM with an average distance using a Kosambi function (Kosambi 1944) of 7.1 cM between two markers.

The PLABQTL program (Utz and Melchinger 1996) that performs composite interval mapping (CIM) (Jansen and Stam 1994; Zeng 1994) via the multiple regression approach was used to identify QTLs for spikelet fertility measures. Critical LOD threshold values in declaring the presence of a QTL through CIM were calculated using the chi-square approximation method (Zeng 1994). Using this method, at experimentwise errors of $P = 0.05$ and 0.01, the critical thresholds were equivalent to LOD = 3.52 and 4.22, respectively.

Results and discussion

Linkage map construction

The number of surveyed markers per chromosome ranged from 15 to 41, while the percentage polymorphism ranged from 27 to 88% (Table 1). The average polymorphism was 67% for the *indica* × *japonica* cross.

The 181 microsatellite loci provided an average density of one marker for every 7.1-cM genetic distance, spanning the 1,277-cM total length of all 12 rice chromosomes. Clustering and distribution of microsatellite loci coincided with the observation of Temnykh (2000), though genetic distances here were shorter than the 1,822 cM derived from the IR64/Azucena DH population or the 1,814 cM from the Milyang23/Gihobyeo RIL population (Cho et al. 1998). The lengths of the rice chromosomes ranged from 39 to 170 cM. As expected, chromosome 8 was the shortest due to the limited number of polymorphic markers identified on that chromosome.

Marker segregation

All 181 microsatellite loci included in constructing the genetic map were tested for segregation distortion. Distortion was detected using the χ^2-test for goodness of fit to the expected allelic frequency of 1:1 *indica* allele: *japonica* allele. The actual allele frequency for the RIL population was calculated at 0.52 and 0.48 for the IR50 and M-202 alleles, respectively. Of the 181 mapped loci, 12% were skewed in favor of the IR50 allele while 5% were skewed towards the M-202 allele (Fig. 1). The allelic frequency for IR50 ranged from 0.19 to 0.72, with all the markers on chromosomes 5, 6, 9,10 and 11 having a normal allelic distribution and segregation pattern (Fig. 1). Portions of chromosomes 1, 3 and 8 showed marker segregation distortions skewed towards the japonica allele.

Table 1 Number of microsatellite markers surveyed and used for map construction per chromosome, the degree of polymorphism, and chromosome length in cM for the rice linkage map

Chromosome no.	No. of surveyed SSRs	% Polymorphism	Chromosome length (cM)
1	41	61	164.9
2	40	75	139.4
3	28	61	170.5
4	19	84	116.2
5	24	88	120.0
6	27	67	107.5
7	24	58	97.0
8	30	27	39.1
9	20	65	72.5
10	16	75	78.6
11	16	81	91.5
12	15	67	79.6
Total	300	67.4	1,276.8

Segregation distortion patterns in rice were compared on several molecular maps derived from different types of crosses and populations (Xu et al. 1997). While distorted segregation and allele distribution was also observed in other RIL or DH populations (Huang et al. 1997; Cho et al. 1998), and commonly observed in *indica-japonica* crosses, the direction of skewness appeared to vary in different populations and chromosomal regions. Some of the reasons for the observed segregation distortion in linkage mapping involving diverse parents may be caused by a partial lethal-factor (Cheng et al. 1998) or the presence of gametophytic (ga) or sterility genes (S) (Xu et al. 1997). It was also observed that the cause of distortion may be due to a large proportion of non-functional pollen in *indica*/*japonica* crosses (Lin et al. 1992). In the present study, only fertile plants were advanced from the F_2 generation, so this may be the cause for much of the distorted segregation.

Overall, the microsatellite marker framework map developed based on a RIL population from a tropical *indica* IR50 and a temperate *japonica* M-202 should be useful in examining important traits in both tropical and temperate environments. The genetic linkage map was comparable in terms of total length, marker distribution and detected regions having marker segregation distortion to other maps and populations used in QTL detection.

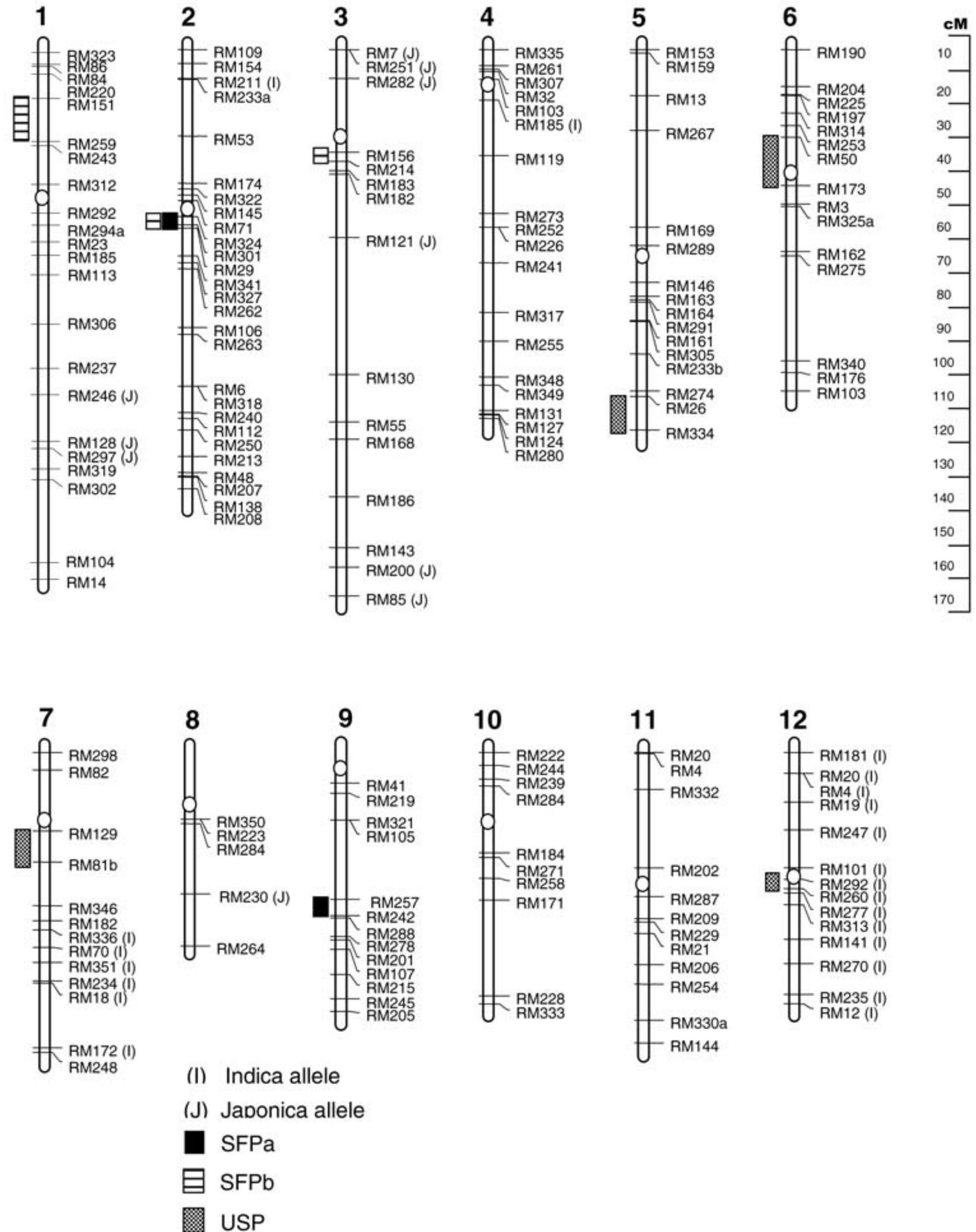

Fig. 1 Microsatellite linkage map developed using 191 RILs derived from an *indica* × *japonica* cross and the chromosomal regions showing markers with distorted segregation, and chromosomal location of putative QTLs detected for SFPa, SFPb and USP. The *circles* indicate the approximate position of the centromeres on each chromosome drawn to scale, and whose orientation was from the short to the long arm according to the map by Temnykh et al. (2000). The map distances expressed in centimorgans (cM) were calculated using the Kosambi function. Skewed markers and the direction of skewness were noted. The critical LOD threshold values calculated using the chi-square approximation method (Zeng 1994) at experimentwise errors of $P = 0.05$ and 0.01 were equivalent to LOD = 3.52 and 4.22, respectively

Phenotypic variation and QTL detection

Preliminary evaluation of cold tolerance at the booting stage of the parental lines M-202 and IR50 at 12 °C showed that low-temperature damage, as measured by a reduction in spikelet fertility (SFPa), became evident for the susceptible parent starting at 4 days of low-temperature treatment (data not shown). Though M-202 also suffered some damage, the rate of decline was not as abrupt as in IR50 in the first 5 days. SFPa dropped from 70 to 60% for M-202 and from 30 to 20% for IR50 at 4 to 5 days of treatment. In contrast, SFPb for IR50 was low at 6 days of treatment while M-202 maintained a value of about 70% even up to 7 days. Though the screening for booting-stage cold tolerance may be performed for 4 to 6 days based on the parental data, the duration of treatment was set at 5 days to evaluate the RILs.

Some RILs suffered severe necrosis, yellowing and even death upon returning the materials to the greenhouse under normal temperature, in addition to the failure of panicles to emerge and panicle malformation in susceptible lines. Necrosis was identified to be under the influence of a major QTL on chromosome 12 (data not shown). By eliminating affected entries, only 150 RILs were included in the data analysis.

The average spikelet fertility for M-202 was 65 and 75% for SFPa and SFPb, respectively, compared to 3% for IR50 in both measurements (Fig. 2). RIL values ranged from 0 to 80% with a mean of 24% for SFPa and 4–88% with a mean of 42% for SFPb. USP reached 62% for IR50 compared to 8% for M-202 while the RILs ranged from 0 to 75% and averaged 15%. All the traits had a continuous distribution, indicating quantitative inheritance of those traits. The distribution of SFPa and

Table 2 Chromosome location, estimated position, flanking microsatellite markers, coefficient of determination (R^2) and additive effects of the QTLs for percentage spikelet fertility and undeveloped spikelet percentage. Panicles were treated approximately 14 days before emergence in the growth chamber for 5 days at a 12 °C constant temperature. SFPa, only the fully-filled grains were used in calculating spikelet fertility; SFPb, both fully and partially filled grains were used in calculating spikelet fertility

QTL	Chrom. no.	Marker interval	Interval	LOD distance (cM)	R^2	Additive	DPE[b] effect
% Spikelet fertility							
SFPa					(17.5)[a]		
qCTB2a	2	RM324–RM301	2.6	6.0**	16.8	5.83	M
qCTB9	9	RM257–RM242	5.1	3.7*	10.7	4.68	M
SFPb					(27.7)		
qCTB1	1	RM151–RM259	14.5	5.1**	14.6	5.81	M
qCTB2b	2	RM324–RM301	2.6	3.6*	10.5	4.02	M
qCTB3	3	RM156–RM214	3.1	5.9**	16.5	5.76	M
% Undeveloped spikelet (USP)					(15.9)		
qCTB5	5	RM26–RM334	10.4	4.3**	12.8	–10.63	M
qCTB6	6	RM50–RM173	14.9	4.3**	12.6	7.42	I
qCTB7	7	RM129–RM81b	10.1	4.0*	11.7	5.13	I
qCTB12	12	RM292–RM260	3.1	3.9*	11.6	–9.11	M

*,** – indicates experimentwise $P = 0.05$ and 0.01 equivalent to critical LOD score of 3.52 and 4.22, respectively

[a] Number in parenthesis denotes the total contribution of the detected QTL to the phenotypic variation

[b] DPE, direction of phenotypic effect; M and I indicate M-202 and IR50, respectively

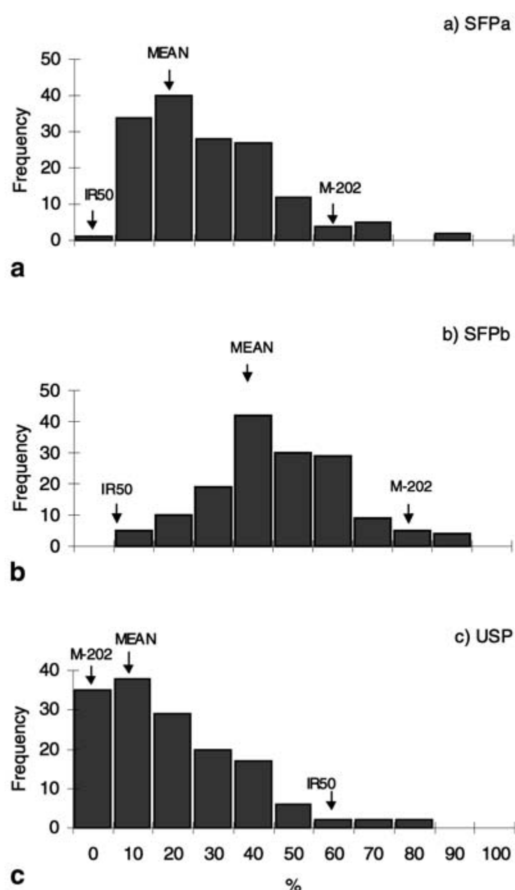

Fig. 2 Frequency distribution of percentage spikelet fertility (*SFPa, SFPb*) and undeveloped spikelet percentage (*USP*). SFPa was computed using only the fully filled spikelets, while SFPb used both partially and fully filled spikelets in computing spikelet fertility. The RILs, M-202 and IR50 means were indicated by *arrows*

USP were skewed towards the susceptible parent IR50 and tolerant parent M-202, respectively (Fig. 2).

SFPa was controlled by two QTLs located on chromosomes 2 and 9 (Table 2, Fig. 1) designated as *qCTB2a* and *qCTB9*, respectively. The QTL *qCTB2a* flanked by markers RM324–RM301 accounted for 17% of the phenotypic variance at LOD = 6.0 while *qCTB9* accounted for 11%. The direction of phenotypic effect for the two QTLs was towards the *japonica* parent. Both loci combined accounted for 18% of the phenotypic variation. Using SFPb as the measure of spikelet fertility, the QTL on chromosome 2 identified in SFPa was also detected, designated as *qCTB2b*. It accounted for only 11% of the phenotypic variance with a LOD value of 3.6. Two additional QTLs flanked by RM151–RM259, *qCTB1*, on chromosome 1 and by RM156–RM214, *qCTB3*, on chromosome 3 accounted for 15% (LOD = 5.1) and 17% (LOD = 5.9) of the phenotypic variance, respectively. The QTLs associated with undeveloped spikelets were located on chromosomes 5, 6, 7 and 12. The QTLs *qCTB5* and *qCTB12* located on chromosomes 5 and 12, respectively, accounted for 13 and 12% of the phenotypic variance and were associated with a reduced number of under-developed grains from the tolerant parent M-202. The direction of the phenotypic effect for the other two QTLs, designated *qCTB6* on chromosome 6 and *qCTB7* on chromosome 7, was towards IR50 and accounted for 13 and 12% of the phenotypic variance, respectively. We observed that undeveloped spikelets appeared to be an early sign of injury due to exposure of young panicles to low temperature at the booting stage. The M-202 alleles on chromosomes 5 and 12 appeared to contribute to tolerance of rice to this early sign of cold injury. Two additional QTLs were also detected on chromosomes 6 and 7, with alleles derived from IR50 con-

tributing to the reduction of undeveloped spikelets due to cold stress, even though the variety was considered susceptible.

To our knowledge, these are the first QTLs to be identified controlling cold tolerance at the booting stage using RILs treated in the growth chamber. Previous QTLs mapped using the cool irrigation water method identified regions on chromosomes 3 and 4 that were responsible for cold tolerance at the booting stage (Saito et al. 1995). The QTL on chromosome 3 detected in this study, however, was not at the same position as that detected in that study. Recently, QTLs on chromosomes 1, 7 and 11 were determined to control cool-temperature tolerance at the booting stage in a cross between two *japonica* rice cultivars (Takeuchi et al. 2001). Likewise, the QTLs detected in this study appear to be different from those identified in that population.

Eight regions on eight rice chromosomes were associated with the ability to tolerate low temperature at the booting stage. The method we employed, using the growth chamber to impose cold stress at a specific reproductive stage for all the RILs, minimized the complications imposed by environmental factors in field screening such as the cool water irrigation method as observed by other authors (Saito et al. 1995; Takeuchi et al. 2001). It was recognized that measuring cold tolerance at the booting stage may be complicated by differential heading time and unpredictable field conditions in screening procedures involving the cold irrigation water (Saito et al. 1995), even though it has been considered a reliable method of screening (Nagasawa et al. 1994; Nishimura 1995).

Our effort to further partition spikelet fertility into SFPa, SFPb and USP resulted in the detection of a greater number of QTLs that may influence booting-stage cold tolerance. The approach probably detected different QTLs for tolerance during fertilization vs tolerance during grain filling that is possibly mediated through maintenance of photosynthesis and/or assimilate transport into developing grains. Clearly, the production of fully filled spikelets involved having normal pollen and embryosac, successful fertilization, and normal seed setting and grain-filling. More sets of genes were probably associated with cold tolerance at the reproductive stage if tolerance components were identified and partitioned.

We have succeeded in identifying the genetic loci associated with cold tolerance in a cross between the two main subspecies that represent tolerant (*japonica*) and susceptible (*indica*) rice germplasm. Our screening method using the growth chamber, instead of the cool irrigation water method, allowed us to target specifically the phase of the reproductive stage most sensitive to cold stress and to minimize the effect of differential maturity and stress duration. The microsatellite markers identified in this study that were closely associated with QTLs controlling the expression of tolerance to low temperature-induced spikelet sterility may be useful in facilitating the selection and development of tolerant rice lines for temperate environments. The microsatellite map we generated, and the RIL population we developed from a tropical *indica* IR50 and a temperate *japonica* M-202, should be useful in examining important traits in both tropical and temperate environments. With the RILs, the QTLs identified under controlled environment conditions can be tested in the field, because seeds can easily be produced for use in replicated experiments.

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