Mapping QTL conferring speckled snow mold resistance in winter wheat (Triticum aestivum L.)

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Speckled snow mold caused by Typhula ishikariensis is one of the most devastating diseases of winter wheat in Hokkaido, Japan and parts of the Pacific Northwest region of USA. Münstertaler is a winter wheat landrace from Switzerland that has very high resistance to snow mold and superior freezing tolerance. Quantitative trait loci (QTL) for resistance to speckled snow mold were identified in a doubled haploid population derived from a cross between Münstertaler and susceptible variety Ibis, both under field conditions and controlled environment tests. Composite interval mapping analysis revealed a major QTL on chromosome 5D from Münstertaler, and on chromosome 6B from Ibis. Flanking microsatellite marker cfd 29 for the QTL on chromosome 5D was about 5 cM distant from vernalization requirement gene Vrn-D1, suggesting that the QTL on chromosome 5D is located on a cold-stress-related gene cluster along with Vrn-D1 and freezing tolerance gene Fr-D1. The QTL on chromosome 6B from Ibis was located on the centromere region flanking QTn.mst-6B, which is reported to increase plant tiller number.

Key Words: winter wheat, snow mold resistance, quantitative trait loci, doubled haploid lines.

Introduction

Speckled snow mold, caused by the fungus Typhula ishikariensis S. Imai, is a serious disease of winter wheat in Hokkaido, Japan, as well as parts of Idaho and Washington States in the Pacific Northwest region of the USA (Amano 1987, Bruehl et al. 1966, Bruehl 1982). Snow mold develops on plants covered by snow for more than 100 days (Tomiyama 1955). Disease-resistant cultivars offer the most effective control, but identification and transfer of snow mold resistance genes to new cultivars has been difficult because development of snow mold is greatly influenced by environmental conditions and requires several years of field testing. Thus, resistance to speckled snow mold caused by T. ishikariensis is an important objective in wheat breeding programs for Hokkaido, Japan and the Pacific Northwest region of USA in where speckled snow mold is most major snow mold of winter wheat under prolonged snow cover.

Varietal differences in resistance to snow mold were reported by Tomiyama (1955). In the early 1960s, over 8,200 accessions of wheat from the USDA world wheat collection were screened for snow mold resistance in field tests. Only 10 lines, originating in Russia, Turkey, and Afghanistan, were considered promising sources of resistance (Bruehl et al. 1966, Bruehl 1982). Bruehl et al. (1966) examined resistance to T. ishikariensis in both controlled environment and field tests and observed hybrid vigor or multiple gene dominance in the F2, and multiple gene action in the F3 and F4. Based on a half-diallel cross with controlled environment testing for resistance, Amano (1982) reported that resistance to T. ishikariensis was attributable to two or three putative genes, and was characterized by overdominance, i.e., the most resistant varieties bore an excess of recessive resistance genes. Based on the analysis of a half-diallel cross and resistance-testing under field conditions, Iriki and Kuwabara (1993) reported the alleles for snow mold resistance were dominant. However, efforts to breed for snow mold resistance have met with comparatively limited success, due to complex inheritance of resistance genes, the confounding effects of cold-hardening before snow cover on susceptibility, and the dependence on snow fall conditions for efficient phenotyping.

In common wheat, major loci controlling freezing tolerance have been identified on the long arm of group 5 chromosomes (Galiba et al. 1995). Loci controlling flowering
response to vernalization (Vrn) that determine spring/winter habit and vegetative frost tolerance (Fr) have been identified at corresponding positions across Triticeae species (Galiba et al. 1995, Iwaki et al. 2002, Snape et al. 1998, Sutka 2001, Tóth et al. 2003, Yan et al. 2003). Freezing tolerance corresponds with a cluster of genes encoding C-repeat binding factors (CBF), and copy number, haplotype variation at the Vrn-A1 and central Fr-A2 loci are associated with frost tolerance in hexaploid wheat (Zhu et al. 2014).

During winter snow cover, a decrease in preformed defense reserves occurs that coincides with depletion of fructan in the wheat plants. Snow mold resistant winter wheat varieties reportedly accumulate higher fructan content earlier in crowns and metabolize carbohydrates more slowly under snow cover compared to susceptible varieties (Kiyomoto and Bruehl 1977, Yoshida et al. 1998). Considering those models for snow mold resistance, it is likely that snow mold resistance is a complex of stress-induced resistance that is systemic and effective against a broad spectrum of snow mold pathogens.

The need to maintain agronomic traits and the complex inheritance of snow mold resistance makes breeding for snow mold resistance in winter wheat difficult. The knowledge and availability of DNA markers closely linked to snow mold resistance and selection would afford a powerful tool in identifying resistant lines. Münstertaler is highly resistant to both freezing and snow mold damage (Gaudet and Kozub 1991, Iriki et al. 2001, Kleijer 1988, Kuwabara et al. 1996, Nishio et al. 2008, Torada et al. 2006). The current study was conducted to better understand the genetics of snow mold resistance by mapping QTL in a doubled-haploid population derived from the highly snow mold resistant variety Münstertaler and susceptible variety Ibis.

### Controlled environment (CE) tests for snow mold resistance

Resistance to speckled snow mold of the DH lines and parents was determined under controlled environment according to the methods of Nishio et al. (2008). Seeds were imbibed in water for 4 days at 4°C and four germinated seedlings of a single line were transplanted into a pot (12.7 cm diam) containing 1200 g (air-dry equivalent) of Thatuna silt loam soil mix (TSL, Thatuna silt loam), vermiculite, and washed river sand (90:5:5, w/w). Three pots were prepared for each DH and parental line (i.e., 3 replicates per line) for a total of 12 plants per line or parent. After transplanting, 2 g of slow-release fertilizer (Osmocote Pro 20-4-8 with IBDU & Minors, The Scotts Company, Marysville, OH) was applied to each pot, and plants were pre-hardened for 14 days at 20°C. Soil was saturated during pre-hardening, and then allowed to dry to the desired water potential before cold-hardening began. Based on a previously developed soil moisture release curve, 2 day interval weight-basis watering was used to maintain the desired soil matric potential over the cold-hardening period (Nishio et al. 2008). Plants were cold-hardened at 4°C ± 0.5°C for 3 wk with –0.1 MPa soil matric potential (dry soil) in a Convirón GR48 growth room (Convirón Environments Limited, Winnipeg, Canada). Light intensity was adjusted to 350 μmol·m⁻²·s⁻¹ with a 12 hr photoperiod for pre-hardening and 330 μmol·m⁻²·s⁻¹ with an 8 hr photoperiod for cold-hardening.

Isolate TB12 of T. ishikariensis biotype B (Nishio et al. 2008) was cultured on potato dextrose agar (PDA) at 10°C for 2 wk in petri dishes. A small piece of hyphae was transferred to sterile oat kernels and bran (1:1) and incubated for 30 days at 10°C. All batches of inoculated media were mixed for uniformity in a container prior to inoculation of test plants. After cold-hardening, the pathogen-colonized oat and bran medium (10 g) was spread on the soil surface of each pot. Plants were then covered with 15 cm² of wet cotton (~25 g of dry weight) and plastic film and incubated in the dark at 10°C (Kawakami and Abe 2003, Nakajima and Abe 1990, Nishio et al. 2008). Plants were incubated with the snow mold pathogen for 30 days. The cotton and plastic film were then removed, and pots were moved to a greenhouse at 20°C, where they were exposed to a 12 hr photoperiod. Regrowth of each plant was evaluated in the greenhouse. Three days after removal of the cotton, total number of tillers were counted and then cut 5 cm above the soil line. In CE tests, tiller survival rate was evaluated by determining the percentage of tillers that re-grew 1 wk after cutting. Each experiment was arranged in a completely randomized design with three replicates; each replicate included four individual plants. Each CE experiment was arranged in a randomized complete block design with three replicates, where blocks were separated by a space in the growth chamber.

### Materials and Methods

#### Plant materials

A population of 99 F₁-derived doubled-haploid (DH) lines was developed from a cross between Münstertaler and Ibis (Nishio et al. 2010). Münstertaler originates from Switzerland, is highly resistant to snow mold, and awned (Gaudet and Kozub 1991, Iriki et al. 2001, Kleijer 1988, Kuwabara et al. 1996, Nishio et al. 2008, Torada et al. 2006). Ibis is a snow mold susceptible, awnless winter wheat cultivar from Germany (Amano 1987, Iriki and Kuwabara 1992, 1993, Iriki et al. 2001, Nishio et al. 2008). Crosses were made between single plants of each parent in a greenhouse in 2003–04. In 2004–05, doubled-haploid lines were developed from greenhouse-grown F₁ plants using the maize pollination procedure (Inagaki and Tahir 1990). Both cultivars possess winter type alleles for Vrn-A1, B1, and D1 genes, and there was no spring type in the DH lines. Seed from individual DH plants was multiplied in the greenhouse for inoculation tests.
Field experiments for snow mold resistance

The DH lines and parents were tested for snow mold resistance in field plots at the National Agricultural Research Center for Hokkaido Region, Sapporo (43.0°N, 141.3°E) and Memuro (42.9°N, 143.0°E). The DH and parental lines were sown in early October of 2007 and 2009 at Sapporo and 2009 and 2011 at Memuro. Each experimental unit contained 30 plants and consisted of a single 2.0 m long row, which was spaced 0.75 m laterally from the adjacent row; there were three replications at Sapporo in 2007, and no replications at Sapporo in 2009 or at Memuro in 2009 and 2011. Individual plots represented the experimental units (plots). In the Sapporo 2007 field test, inoculated plots were arranged as a randomized block with three replications, and data represent the mean of the replications. Nitrogen was applied at a rate of 40 kg ha⁻¹ at seeding. Isolate PR75D of *T. ishikariensis* biotype A (Iriki and Kuwabara 1992, Kawakami and Abe 2003, Matsumoto et al. 1982) was cultured in the same manner as described for controlled environment experiments. In late October, each experimental unit was inoculated with 10 g of PR75D bearing inoculum. In field tests, plant survival rate was evaluated by determining the percentage of individual plants in each plot that re-grew 2 wk after snow melt. Plots were covered by snow for 124 days (28 Nov to 30 Mar) in 2007–2008, and 115 days (15 Dec to 8 Apr) in 2009–2010 at Sapporo, 120 days (4 Dec to 2 Apr) in 2009–2010, and 131 days (3 Dec to 11 Apr) in 2011–2012 at Memuro.

DNA extraction

Two grams of leaf tissue were ground in a mortar with liquid nitrogen, 10 ml of extraction buffer (1.5% cetyltrimethylammonium bromide (CTAB), 75 mM Tris, pH 8.0, 15 mM Na₂EDTA, pH 8.0, 1.05 M NaCl) was then added and the resultant slurry incubated at 65°C for 30 min (Murray and Thompson 1980). DNA was purified in 24:1 chloroform/isoamyl alcohol, precipitated from the aqueous phase by the addition of isopropanol and then washed in 70% ethanol. DNA samples were dissolved in distilled water and DNA concentration was determined by measuring optical density at 260 nm.

Molecular markers

Münstertaler and Ibis were screened at the USDA Regional Genotyping Lab (Pullman, WA, USA) to assess the polymorphism existing between them. Nine hundred microsatellite (SSR) markers, based on the map of Gupta et al. (2002), Guyomarc’h et al. (2002), Pestsova et al. (2000), Röder et al. (1998), Somers et al. (2004), Song et al. (2005), Sourdille et al. (2001) and Torada et al. (2006), were screened for polymorphism between parents and then polymorphic markers were tested on the DH population. PCR reactions were conducted in a 15 μl volume using a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA). The amplification program began with 1 cycle of primary denaturation at 94°C for 5 min, followed by 42 cycles of 94°C for 1 min denaturing, 51°C or 61°C for annealing and 72°C for extension. One additional cycle was performed at 72°C for 5 min for final elongation of the PCR product. Each 15 μl PCR reaction contained 5–10 ng of template DNA, 1 × PCR Mix, 1.7 mM MgCl₂, 200 nM of each primer, 200 μM of each dNTPs and 0.2 U Taq polymerase (Promega, Madison, WI, USA). PCR products were separated on a Sub-Cell® Model 96/192 (Bio-Rad, Hercules, CA, USA) electrophoresis apparatus with 4% agarose gel. The gels were run in 1 × TBE at a constant 100 V for 2 hrs and were visualized after staining with ethidium bromide for 30 min.

QTL analysis

MAPMAKER/Exp v3.0b (Lander et al. 1987) was used to construct the wheat microsatellite map. Logarithm of odds (LOD) threshold of 3 and 50 cM for the ordering were set for grouping. QTL analysis was performed using Windows QTL Cartographer 2.5 (Basten et al. 1996) using the composite interval mapping function.

Results

Snow mold resistance

For both CE and field tests, DH lines showed a continuous distribution in tiller or plant survival (Fig. 1). The two parental lines were situated at the extremes of the distribution in the field test, whereas survival of Münstertaler was less than some progeny lines in CE tests. The correlation coefficients of plant survival among the five snow mold tests are shown in Table 1. All plant survival after five snow mold inoculation environments were significantly correlated with each other. The correlation coefficients among different field environments (year of places) were greater than between CE and field tests (Table 1).

Marker analysis

Three hundred forty-eight markers were polymorphic
between the parents, of which 330 markers were located onto 26 linkage groups, covering a total genetic distance of 3,326 cM. Chromosome identity for linkage group was determined based on anchor microsatellite markers (Röder et al. 1998) and the wheat consensus map (Somers et al. 2004), and partial maps were obtained for all 21 wheat chromosomes. Average marker interval across the 26 linkage groups was 10.1 cM and ranged from 5.7 (1A) to 17.5 cM (7D), with an average of 12.7 markers per linkage group.

**Quantitative trait mapping**

QTL analysis revealed that regions on chromosomes 1B, 4A, 4B, 5D, and 6B were associated with speckled snow mold resistance in CE and field tests; of these, the regions on chromosomes 5D and 6B were significantly associated with speckled snow mold resistance in multiple environments (Table 2). The QTL on chromosome 5D derived from Münstertaler was detected in both CE tests and three field tests (Fig. 2), whereas the QTL on chromosome 6B from Ibis was detected in two field tests. The Münstertaler allele on chromosome 5D explained 9.49% of phenotypic variation for CE tests, and 12.01%, 14.12%, and 20.56% for the 2007 (Sapporo), 2009 (Memuro) and 2011 (Memuro) field tests, respectively.

**Discussion**

We found a major snow mold resistance QTL on the long arms of chromosome group 5. 

### Table 1. Correlation coefficients between plant survival after snow mold in controlled environment (CE) and four field tests

| Environment | 2006 CE | 2007 Sapporo | 2009 Sapporo | 2009 Memuro |
|-------------|---------|--------------|--------------|-------------|
| 2007 Sapporo| 0.345** | –            | –            | –           |
| 2009 Sapporo| 0.223** | 0.580**      | –            | –           |
| 2009 Memuro | 0.270** | 0.360**      | 0.325**      | –           |
| 2011 Memuro | 0.229*  | 0.644**      | 0.530**      | 0.416**     |

* P < 0.05, ** P < 0.01.

### Table 2. Quantitative trait loci (QTL) analysis estimates for plant survival in controlled environment (CE) and four field tests after snow mold for 99 winter wheat doubled haploid lines from the cross Münstertaler × Ibis. QTL are described by chromosome location, flanking markers, logarithm of the odds (LOD), coefficients of determination (R²), and additive effect of the significant QTL regions detected by the composite interval mapping

| Year | Environment | Chromosome | Locus | LOD | R² (%) | Additive Effect | Threshold LOD scores | Resistant source |
|------|-------------|------------|-------|-----|--------|-----------------|----------------------|-----------------|
| 2006 | CE          | 4A         | barc 59.2–gwm 637 | 2.66 | 9.22 | 4.30 | 2.38 | Ibis
|      |             | 5D         | gwm 215–cdf 29   | 2.87 | 9.49 | 4.30 |
| 2007 | Sapporo     | 5D         | gwm 215–cdf 29   | 4.43 | 12.01 | 4.40 | 3.10 | Münstertaler
|      |             | 6B         | ac 22–barc 136   | 7.92 | 23.18 | 8.49 |
|      |             | 6B         | barc 79–barc 178 | 4.24 | 11.35 | 5.91 |
| 2009 | Sapporo     | 4B         | hbg 211–gwm 125  | 4.75 | 18.08 | 11.29 | 3.25 | Münstertaler
|      |             | 6B         | gwm 104–ac 22    | 4.22 | 14.03 | 10.20 |
| 2009 | Memuro      | 1B         | barc 240–gwm 419 | 4.29 | 14.39 | 4.59 | 2.40 | Münstertaler
|      |             | 5D         | gwm 215–cdf 29   | 4.35 | 14.12 | 4.44 |
| 2011 | Memuro      | 5D         | gwm 583–barc 286 | 3.39 | 10.54 | 5.80 | 3.06 | Münstertaler
|      |             | 5D         | gwm 215–cdf 29   | 6.66 | 20.56 | 8.04 |

**Haplotype analysis of major QTL for snow mold resistance**

Haplotype of cfd 29 for the major snow mold resistance QTL on chromosome 5D for Münstertaler showed polymorphisms between current elite cultivar in Hokkaido (Yumechikara, Kitanokaori, Kitahonami, and Hokkai 264) (Fig. 3).
Therefore, the QTL on chromosome 5D flanking the cold-wheat consensus microsatellite map (Somers et al. 2017) also reported QTL for snow mold resistance in infection, plants with large tiller numbers are likely to survive by escaping infection. Thus, the QTL on chromosome 6B could be related an allele for increasing tiller number. In CE tests, a comparatively shorter cold-hardening period than field tests may have resulted in insufficient tiller increase, which resulted in the smaller differences in survival rate between parental lines compared to field test. The divergent effects of QTL on chromosome 6B between Sapporo and Memuro could be attributed to relatively longer snow cover period during the field test at Memuro (120 and 131 days for 2009 and 2011, respectively) compared to Sapporo (124 and 115 days for 2007 and 2009, respectively) that differentiate snow mold development environments. The QTL on chromosome 5D from Münstertaler was not detected only in 2009 Sapporo with shortest snow cover period. The major QTL for fructan content in the grain of winter wheat have been reported on chromosome 6D and 7A (Huynh et al. 2008) and were not associated with QTL for snow mold resistance in this study. Further study of fructan content in cold-acclimated crowns is needed to elucidate the interaction between carbohydrate accumulation and snow mold resistance.

In this study and that of Torada et al. (2005), no DH line had the same level of resistance as Münstertaler in the field test (Fig. 1). We suspect the possible reason for reduced survival of progeny lines compared to the resistant parent was the absence of those minor QTL. No cold acclimation or plant growth related QTL have been reported in that region, and further analysis between the snow mold resistance components needs to be investigated. Detection of a QTL for susceptibility in Münstertaler may explain decreased resistance in the DH lines. However, the number of markers in the minor QTL are still small and further analysis is necessary to elucidate the location and interactions between them.

These results will provide an insight for molecular marker-assisted selection (MAS) in snow mold resistance of winter wheat. Münstertaler has pretty good resistance to snow mold, but it has very long culms that are prone to lodging and late maturity, neither of which is desirable. Further research is necessary to validate the effectiveness of these QTL in other genetic backgrounds and potential epistasis effects on the negative traits to study the effect of the polymorphism of the flanking markers in other wheat varieties. For practical breeding programs, negative linkage of the QTL to inferior agronomic traits also needs to be validated.

**Author Contribution Statement**

Z.N., N.I. and T.D.M. designed the study, and N.I developed the plant materials. Z.N., M.I. and T.T. performed the snow mold inoculation experiments. Z.N. and T.D.M. analyzed the data and wrote the paper.

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