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ORIGINAL PAPER

Markers to a common bunt resistance gene derived from 'Blizzard' wheat (*Triticum aestivum* L.) and mapped to chromosome arm 1BS

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Abstract Common bunt, caused by *Tilletia caries* (DC.) Tul. & C. Tul. and *T. laevis* J.G Kuhn, is an economically important disease of wheat (*Triticum aestivum* L.) worldwide. The resistance in the winter wheat cultivar 'Blizzard' is effective against known races of common bunt in western Canada. The incorporation of resistance from Blizzard into field-ready cultivars may be accelerated through the use of molecular markers. Using the maize pollen method, a doubled haploid population of 147 lines was developed from the F_1 of the second backcross of Blizzard (resistant) by breeding line '8405-JC3C' (susceptible). Doubled haploid lines were inoculated at seeding with race T19 or T19 and L16 and disease reaction was examined under controlled conditions in 1999 and natural conditions in 2002, and 2003. Resistant:susceptible-doubled haploid lines segregated in a 1:1 ratio for bunt reaction, indicating single major gene segregation. Microsatellite primers polymorphic on the parents were screened on the population. Initial qualitative segregation analysis indicated that the wheat microsatellite markers *Xgwm374*, *Xbarc128* and *Xgwm264*, located on wheat chromosome 1BS, were significantly linked to the resistance locus. Qualitative results were confirmed with quantitative trait locus analysis. The genetic distance, calculated with JoinMap®, between the bunt resistance locus and overlapping markers *Xgwm374, Xgwm264* and *Xbarc128* was 3.9 cM. The three markers were validated on doubled haploid populations BW337/P9502&DAF1BB and Blizzard/P9514-AR17A3E evaluated for common bunt reaction in the growth chamber in 2007. These markers will be useful in selecting for the common bunt resistance from Blizzard and assist in identifying the resistance among potential new sources of resistance.

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Introduction

Common bunt caused by *Tilletia caries* (DC) Tul & C. Tul and *T. laevis* J.G Kuhn attacks both spring and winter wheat (*Triticum aestivum* L.) worldwide and may occur wherever these crops are grown (Hoffmann and Metzger 1976). Common bunt was first found in Canada in 1928 (Conners 1929), and has historically been a major economic disease of hexaploid wheat ($2n = 6 \times = 42$) and tetraploid durum wheat (*Triticum durum* Desf., $2n = 4\times = 28$) and was rated as a major cause of crop loss before 1950 (Cherewick 1953). Although appreciable yield losses now occur infrequently in western Canada, financial losses resulting from reduction of grade still amount to large sums annually (Bailey et al. 2003). This occurs because the commercial value of grain contaminated with even low levels of bunt (0.05% by weight) is reduced. Heavily bunted wheat is not accepted by grain traders and is even difficult to dispose of as feed (Bailey et al. 2003). Control of common bunt by chemical treatment of seed is possible, but this increases

costs, environmental pollution and hazard to human health (Williams 1990). Resistant cultivars are an effective, economical and environmentally sound tactic to protect wheat from common bunt while minimizing pesticide usage (Knox et al. 1998b; DePauw et al. 1999).

Fifteen common bunt resistance genes (*Bt1–15*) have been designated in wheat (Goates 1996). In a study to identify races prevalent in western Canada, genes *Bt1 to Bt10* were evaluated in a differential set (Gaudet and Puchalski 1989a). The *Bt5*, *Bt8* and *Bt10* genes were effective against all isolates while seven genes (*Bt1–4*, *6*, *7*, *9*) were defeated by the combined virulence from L-type and T-type races. The *Bt10* gene is widely used in wheat breeding programs because this resistance gene is effective against all known races of common bunt on the western Canadian prairies (Gaudet and Puchalski 1989a). A DNA marker closely linked to the *Bt10* gene was developed for the purpose of marker-assisted selection (Demeke et al. 1996; Laroche et al. 2000). This gene was mapped on to chromosome 6D (Menzies et al. 2006). However, with much of the elite germplasm in western Canada possessing *Bt10* (DePauw and Hunt 2001), there is a possibility that a new race of common bunt will evolve to overcome the *Bt10* resistance from increased selection pressure (Laroche et al. 2000; Goates 2002). To anticipate such an event, it is critical to identify and incorporate other sources of common bunt resistance into wheat germplasm. Efforts are ongoing to identify new sources of common bunt resistance, and quantitative trait loci (QTL) for resistance from the Canadian cultivar 'AC Domain' on chromosomes 1B and 7A were reported (Fofana et al. 2008). Microsatellite markers were recently reported from wild-grass species *Aegilops cylindrical* on chromosome 1B (Galaev et al. 2006). However, resistance from adapted wheat lines is easier to incorporate while also maintaining the end use quality for quicker selection progress.

The resistance in Blizzard winter wheat is effective against all North American races of dwarf bunt (Goates 2002), and European (Blazkova and Bartos 2002) and current United States races of common bunt (Sunderman et al. 1991). The application of the Blizzard resistance to wheat breeding would complement existing resistance genes. However, molecular markers linked to common bunt resistance in Blizzard and the chromosomal location of the gene are not known which prevents application of marker-assisted selection to wheat germplasm enhancement and variety development from this source of bunt resistance. The objective of this study was to locate the Blizzard resistance to a chromosome with mapped microsatellite markers, and to find a molecular marker or markers linked to the Blizzard source of resistance that can be effectively used in marker-assisted breeding of bread wheat to generate common bunt-resistant cultivars.

Materials and methods

Genetic materials for marker discovery

For marker discovery, a doubled haploid (DH) population was developed from the backcross F_1 of 8405-JC3C/Blizzard//2*8405-JC3C (abbreviated 8405/Blizzard) using the maize pollen method (Knox et al. 2000). The susceptible recurrent parent 8405-JC3C, derived from the cross Neepawa/Columbus//Pacific, is a hard red spring wheat cultivar with high yield and high protein. Blizzard, a winter wheat with common bunt resistance, is derived from a cross of Utah216C-12-10/Cheyenne/5/PI476212(SM4)/4/Burt/3/Rio/Rex//Nebred where 'PI 476212' contributed bunt resistance (Sunderman et al. 1991). The F_1 plants of the first cross were backcrossed twice with the susceptible recurrent parent 8405-JC3C to develop lines with spring habit possessing bunt resistance from Blizzard. The F_1 seeds at each backcross were inoculated with common bunt race T19 and the susceptible lines were discarded. The *T. caries* race T19 was used because it was avirulent on Blizzard. The non-infected BC_2F_1 plants were used to develop the doubled haploid population. The DH lines of 8405/Blizzard were spring growth habit.

Genetic materials for marker validation

Two additional doubled haploid populations were used for the validation of the molecular markers. The Blizzard/P9514-AR17A3E (abbreviated Blizzard/P9514) population consisted of 70 lines. The parent 'P9514-AR17A3E' was a resistant line (not the same resistance as the gene described in this report) selected from the cross HY393/Blizzard//2*HY393. The BW337/P9502&DAF1BB (abbreviated BW337/P9502) population consisted of 76 lines. The parent 'BW337' derives from a University of Saskatchewan line, 'CDC Teal', and a North Dakota line, 'ND2710' and was susceptible to common bunt. The parent 'P9502&DAF1BB' was a bunt-resistant line selected from the cross 8405-JC3C/Blizzard//2*8405-JC3C.

Phenotypic evaluations

Owing to the growth chamber space limitations, 78 of the 147 doubled haploid 8405/Blizzard lines were randomly selected for common bunt testing in a growth room experiment in 1999. Population size was based on the hypothesis that segregation of resistant and susceptible lines follows a 1:1 single major gene ratio and a similar 1:1 segregation applied to molecular variants of each marker. A minimum of 63 lines is required to determine linkage of at least 30% in a doubled haploid population at a type I error rate of 5% (Hanson 1959).

The 1999 growth room experiment of parent 8405-JC3C, check cultivars and 8405/Blizzard DH lines, were randomized in five replications of ten plants each for the purpose of sampling variation within the chamber. The entries were inoculated at seeding with the single *T. caries* race T19. Spores of common bunt were inoculated onto the seed by shaking the seed and spores in a Petri plate for at least 15 s to assure good infestation of the seed for maximum disease infection. Ten inoculated seeds were uniformly planted per row with eight rows in each 400 cm^2 flat containing soil on the bottom and peat on top. Flats were placed in growth chambers initially supplied with 12 h of light (200 μmol m^{-2} s^{-1} of photosynthetic photon flux density) at 15°C and 12 h dark at 12°C; at 3.5 weeks, the temperature for the light period was changed to 18°C; at 6 weeks, the light period was extended to 16 h; and at 11 weeks, the night temperature was raised to 15°C. Near maturity, individual plants were evaluated for bunt. The percentage incidence of bunt within each plot row was determined by totaling the number of diseased plants and dividing by the total number of plants ratable within the plot and multiplying by 100.

Doubled haploid progeny of the Blizzard/P9514 population were vernalized because Blizzard is a winter wheat. Seventy randomly selected Blizzard/P9514 DH lines were inoculated and planted in 2007 in a two replicate test as described above for the 8405/Blizzard population. On day 1, the test was seeded at 15°C with a 16/8 h light/dark cycle and light intensity as described above for the 8405/Blizzard population. On day 19, the temperature was reduced to 10°C. On day 21, the temperature was reduced to 5°C. On day 22, the temperature was further reduced to 2°C during the day and 3°C for the night. On day 40, the temperature was raised to 3°C night and 6°C day at 75% light intensity for 14 h in a 24 h cycle. On day 69, the temperature was further raised to 15°C with full light for 16 h. On day 113, the temperature was raised to 18°C and on day 118, the temperature was adjusted to 21°C during 16 h of light and 18°C for 8 h dark until the test was evaluated for common bunt reaction on day 146.

The marker validation population BW337/P9502 was also inoculated and grown in 2007 in a growth chamber test as described above for 8405/Blizzard population. On day 1, the test was seeded at 15°C with a 12/12 h light/dark cycle and light intensity as described above for the 8405/Blizzard population. On day 23, the temperature was raised to 18°C with lights on and 12°C with lights off. On day 43, the temperature was increased to 18°C during the day and the light cycle was changed to 16 h light and 8 h dark. On day 71, the temperature at night was raised to 15°C. The test was evaluated for common bunt reaction between days 100 and 107.

All 147 of the 8405/Blizzard doubled haploid lines were evaluated in field trials in 2 years. The field trials were grown in un-replicated, randomized single row plots under dry land conditions in 2002 and 2003 near Swift Current, SK. Inoculated 8405-JC3C parent and other controls listed in Table 1 occurred in blocked intervals in 2002, and randomly throughout the bunt nursery in 2003. The seeds were inoculated at seeding time with a 1:1 ratio mixture of common bunt races T19 (*T. caries*) and L16 (*T. Laevis*). These two races represent the virulence of races found on the Canadian Prairies. The inoculum of common bunt was blended by thoroughly shaking spores with the seed in a plastic seed tray. Each line was planted in a 2 m long row with uniform spacing of 45 cm between rows. The seeding rate was approximately 100 kernels per row. The planting dates were April 26 in 2002 and April 28 in 2003. Near maturity, bunt incidence was rated as the percentage of bunted spikes over the total spikes within a plot. A plant was considered bunted when a spike contained at least one bunt ball.

Control cultivars for the various tests of common bunt are listed in Table 1. The parents of the crosses used to develop the genetic populations were used as controls where possible. In the initial experiments evaluating the 8405/Blizzard population, the line 'BW553' possessing *Bt10* resistance was used as a resistant control in the growth chamber study because Blizzard did not have a spring habit. Originally, we thought the resistant line P9514-AR17A3E (HY 393/Blizzard//2*HY 393) possessed the major gene for bunt resistance from Blizzard, hence we used P9514-AR17A3E in field trials as a resistant control in place of Blizzard because P9514-AR17A3E had a spring growth habit. We later learned that P9514-AR17A3E did not possess the major bunt resistance gene for which we developed molecular markers. 'Neepawa' was used as a control with an intermediate bunt reaction and 'Biggar' was used as a susceptible bunt reaction control. Subsequently in 2005, to evaluate Blizzard common bunt reaction, Blizzard, 8405-JC3C, P9514-AR17A3E and Biggar were evaluated together for reaction to race T19 in a vernalized five replicate growth chamber test. The conditions of the test were as follows: the temperature was initially 15°C and decreased to 2°C during a 12 h light period and 4°C during 12 h dark period until day 55 when the temperature was elevated to 18°C for 12 h with lights on and 12°C for 12 h of dark. On day 76, the temperature was set at 18°C with 16 h of light and 12°C with 8 h of dark and on day 107, the temperature was adjusted to 18°C with 16 h of light and 15°C with 8 h dark until the lines were rated on day 149. Controls for the validation experiments involving BW337/P9502 and Blizzard/P9514 additionally included the susceptible line 'HY393'.

Genetic evaluations

Six of the most resistant and six of the most susceptible lines of the 8405/Blizzard population were evaluated with

Table 1 Mean common bunt incidence (%), measure of variation (standard error of the mean or standard deviation) and number of observations (*n*) of lines in the 8405-JC3C/Blizzard//2*8405-JC3C population grown in four environments, the Blizzard/P9514-AR17A3E and BW337/P9502&DAF1BB populations each grown in one growth chamber test

	8405-JC3C/Blizzard//2 × 8405-JC3C												BW337/P9502&DAF1BB			Blizzard/P9514-AR17A3E		
	Growth chamber 1999			Growth chamber 2005			Field 2002			Field 2003			Growth chamber 2007			Growth chamber 2007		
	n	Mean	SE of mean[a]	*n*	Mean	SE of mean[a]	*n*	Mean	SE of mean	*n*	Mean	SE of mean	*n*	Mean	SE of mean	*n*	Mean	SE of mean
Population	387	40.2	1.69	–[c]	–	–	147	8.8	10.25[b]	147	4.0	6.40[b]	145[d]	41.15	3.20	140	7.4	1.25
Parent																		
8405-JC3C	5	85.3	5.20	5	95.1	3.10	4	33.8	8.26[a]	10	19.8	1.46[a]	4	81.25	8.07	–	–	–
Blizzard	–	–	–	20	0	0	–	–	–	–	–	–	–	–	–	4	0.0	0.0
P9514-AR17A3E	–	–	–	5	44.7	7.20	3	0	0[a]	10	0	0[a]	–	–	–	4	32.5	4.79
BW337	–	–	–	–	–	–	–	–	–	–	–	–	8	92.94	3.53	–	–	–
P9502&DAF1BB	–	–	–	–	–	–	–	–	–	–	–	–	8	0.0	0.0	–	–	–
Checks																		
Biggar	7	96.2	2.48	5	100	0	4	36.3	5.54[a]	5	38.5	1.5[a]	1[d]	62.5	–	4	97.2	2.78
Neepawa	5	98	2	–	–	–	4	17.5	3.23[a]	5	8.5	2.69[a]	4	56.75	12.43	4	59.4	11.42
BW 553	5	0	0	–	–	–	–	–	–	–	–	–	4	0.0	0.0	–	–	–
HY393	–	–	–	–	–	–	–	–	–	–	–	–	2[d]	84.45	4.45	4	85.4	2.57

[a] Standard error of the mean

[b] Standard deviation

[c] No data

[d] Lines with three or fewer rateable plants of the ten seeds seeded were not included

markers in bulked segregant analysis (Michelmore et al. 1991). The DNA for bulked segregant analysis was isolated from seedlings of lines of the 8405/Blizzard population. The DNA of lines within a bulk was pooled and the two sets of bulked lines, plus 8405-JC3C and Blizzard were screened with microsatellite primers. Once a polymorphism between resistant and susceptible bulks was identified, individual lines of the bulks were evaluated with the putative marker. The DNA of the 147 individual doubled haploid lines was then tested with *Xgwm374*, *Xbarc128* and *Xgwm264*. DNA was extracted from seedlings of individual lines of the validation populations BW337/P9502 and Blizzard/P9514.

The DNA for PCR was isolated using a modified cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle 1987). When the plants reached the three or four leaf stage, 10 cm leaf segments from primary leaves were harvested for genomic DNA isolation. The concentration of DNA was quantified spectrophotometrically using a GeneQuant instrument to allow equalization of concentrations for PCR.

A total of 230 microsatellite primer pairs were tested for polymorphism between resistant and susceptible bulks and parents of the 8405/Blizzard population. Additional *barc* primers were evaluated in the region nearby *gwm* primers initially found linked to the resistance. Primer sequence, chromosome location and annealing temperature were obtained from Röder et al. (1998), Somers et al. (2004) and the GrainGenes database at http://wheat.pw.usda.gov.

The PCR reactions were performed in 0.2-ml strip tubes containing 25 µl of a reaction mixture consisting of 50 mM KCl, 10 mM Tris–HCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 µM microsatellite primers, 0.07 U μl^{-1} of *Taq* DNA polymerase and 2 ng μl^{-1} of genomic DNA. The DNA amplification was performed in an MJ Research, Inc., PTC-100 thermocycler at 94°C for 3 min, followed by 44 cycles of 94°C for 1 min, annealing (temperatures were dependent on the individual microsatellite primers) for 1 min, and 72°C for 1 min, followed by a final extension at 72°C for 10 min before cooling to 4°C. The annealing temperature of *Xgwm374* and *Xgwm264* was 60°C and *Xbarc*128 was 52°C. Amplification products were resolved by electrophoresis in mixed 2% metaphor and 1% agarose LE gels at 4 V cm^{-1} in TBE (0.045 M TRIS, 0.045 M Borate and 0.001 M EDTA) buffer and stained with ethidium bromide (0.5 µg ml^{-1}). The DNA banding patterns were visualized with UV light and recorded by a Kodak EDAS-290 digital camera imaging system.

Statistical and genetic analysis

An analysis of variance using SAS Institute Inc. (1999) was performed on common bunt incidence from the 8405/Blizzard growth chamber study. The model was a randomized complete block design of five replicates for the 8405/Blizzard population with lines considered fixed and replicates random, to determine if lines varied significantly from each other. The SAS GLM procedure generated least square means for bunt incidence from the growth chamber test. Simple means were generated from the two replicates for each of the BW337/P9502 and Blizzard/P9514 populations. The means were used for Chi-square goodness-of-fit and QTL analyses. Experimental units with fewer than four observations were not included in analyses. Means and standard errors were calculated on individual observations of the parents, checks and all lines of the population for each of the environments. The standard errors were used to calculate confidence limits, where applicable, with appropriate *t* value and degrees of freedom.

Frequency distributions of bunt incidence in the doubled haploid population, for each of the three experiments (one growth chamber and two field), were plotted to allow visual determination of susceptible and resistant groups for qualitative analysis. A class interval for percent bunt incidence was chosen that best displayed the bimodal distribution of lines into resistant and susceptible groups in the growth chamber tests. Owing to the difficulty to discriminate the resistant and susceptible groups from the graphs of the field test results, the resistant control and susceptible parent phenotypes were considered in applying classes to the data. A 95% confidence interval of the susceptible parent 8405-JC3C was used to generate the susceptible class interval for the 2002 field results. For 2003, due to the overall low bunt rating, only lines with a zero incidence of bunt were classed as resistant and all other lines were classed as susceptible. The one-gene segregation ratio was tested by Chi-square goodness-of-fit analysis with the correction for continuity because of one degree of freedom.

Putative markers were evaluated on all lines. Good infection development and subsequent characterization of lines in the growth room for bunt resistance allowed qualitative analysis of the data, and two-point linkage mapping between markers and the common bunt resistance gene. Quantitative assessment of growth room results was also done to strengthen the analyses. Emphasis was placed on the quantitative analysis of continuously distributed field results; however, the susceptible parent was used to derive a class interval of the susceptible lines for qualitative analysis of field results.

Qualitative association between the putative marker genotype and the common bunt phenotype was carried out by classifying the 78 doubled haploid lines of the 8405/Blizzard population from the growth chamber and the 147 doubled haploid lines of the 2002 and 2003 field tests into parental and non-parental groups. Recombination frequencies were calculated between microsatellite markers and the

common bunt resistance gene using the software JoinMap® (Van Ooijen 2006) with 1999 growth chamber data. Quantitative analysis of association between markers and bunt resistance was performed using Student's *t* test by comparing the mean bunt incidence of lines with the marker variant of the resistant parent and of the susceptible parent. The program MQTL (Tinker and Mather 1995) was also used to test the significance between the markers and resistance gene. Molecular markers were considered significantly associated with the resistance reaction if $P < 0.001$ in at least one environment or if $P < 0.01$ in more than one environment (Lander and Kruglyak 1995).

The SAS Proc Mixed procedure was used to estimate variances to obtain the percent variation explained by the markers (Knapp 2001; Knapp and Bridges 1990).

The markers found through analysis of the 8405/Blizzard population were validated on the BW337/P9502 population using both QTL and qualitative analysis and the Blizzard/P9514 population using QTL analysis.

Results

Marker discovery: segregation of bunt resistance

Analysis of variance performed on the growth chamber data of the 8405/Blizzard population indicated that the doubled haploid lines were significantly different in level of bunt resistance. A least significant difference (LSD) indicated that the more resistant lines (from 0 up to 40% disease incidence) were significantly different ($P < 0.05$) from both the more susceptible lines and susceptible parent. For example, a line designated P9502&DAM1AK with a mean bunt incidence of 36% was significantly different from the susceptible parent 8405-JC3C with a mean bunt incidence of 85%. Lines more susceptible to bunt (greater than 40% incidence) were significantly different from both the most resistant lines and the resistant check cultivar. For example, a line designated P9502&DAC1AE with a mean bunt incidence of 45% was significantly different from the resistant check BW553 with a mean bunt incidence of 0%.

The mean bunt infection levels of doubled haploid lines, susceptible controls and the parent 8405-JC3C grown in the growth chamber were much higher than for the means of field grown material (Table 1). Under controlled conditions, the 8405/Blizzard population was distributed bimodally, and a minimum occurred at about 40% incidence (Fig. 1). The spread in the distribution allowed qualitative analysis with a count of 41 lines with infection levels between 0 and 36% in the resistant peak, and a count of 37 lines with infection levels of 44% and greater in the susceptible peak. The counts of the lines within the susceptible and resistant classes indicated that segregation fit an expected ratio of one resistant to one susceptible in a Chi-square goodness-of-fit test ($\chi^2 = 0.115$, $P \geq 0.73$). The follow-up vernalized test of parents and checks showed Blizzard was completely resistant (0% incidence) to race T19, while the susceptible parent 8405-JC3C was 95.1% (SD 6.8) bunted and the susceptible control Biggar was 100% bunted (Table 1).

The distribution of common bunt scores of the 8405/Blizzard population grown in the field were compressed within the low end of the percentage scale for both 2002 (highest bunt incidence of 40% and test mean of 8.8%) and 2003 (highest bunt incidence of 35% and test mean of 4.0%) (Tables 1, 2). No clear separation of resistant and

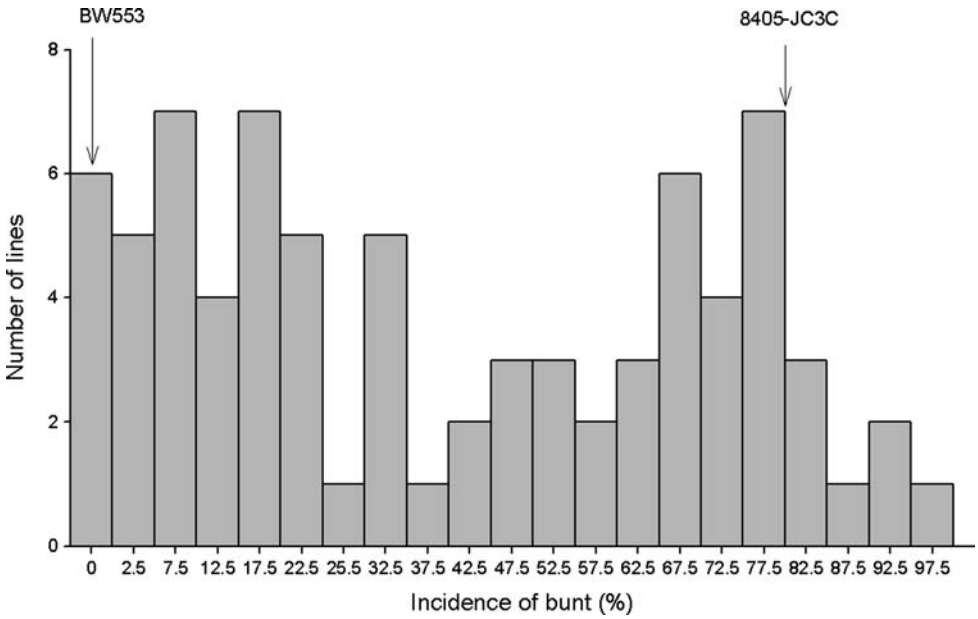

Fig. 1 Common bunt percent incidence of 78 doubled haploid lines from the 8405/Blizzard population grown in the growth chamber in 1999

Table 2 Distribution of common bunt incidence for class intervals 0% and 5% increments thereafter of field ratings on the 8405/Blizzard population grown in 2002 and 2003

	Number of progeny in 5% infection classes																				
	0	1–5	6–10	11–15	16–20	21–25	26–30	31–35	36–40	41–45	46–50	51–55	56–60	61–65	66–70	71–75	76–80	81–85	86–90	91–95	96–100
Field 2002	54	27	28	11	8	9	4	3	3	0	0	0	0	0	0	0	0	0	0	0	
Field 2003	74	39	19	6	7	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	

susceptible classes was possible based on the appearance of the distribution. Given that the data did not present a natural division between resistant and susceptible lines, we classified the lines from the field test of 2002 using the 95% confidence limit of 33.8 ± 26.3 for the susceptible parent 8405-JC3C to group susceptible lines with the remaining lines considered as resistant. The 81 resistant to 66 susceptible lines fit a 1:1 segregation ($\chi^2 = 1.333$, $P \geq 0.25$). With even lower infection levels in 2003, the zero phenotype was used to distinguish the resistant class and the remaining lines were considered susceptible. The segregation of 74 resistant to 73 susceptible fit a 1:1 ratio ($\chi^2 = 0.0$, $P = 1$). With such low bunt infection in the field nurseries, many lines considered susceptible in the growth chamber test escaped infection in the field, whereas several lines rated resistant or moderately resistant in the growth chamber test were rated susceptible in field tests with very low levels of infection.

Marker discovery: microsatellite markers, linkage analysis and genetic mapping

From 230 microsatellite primer pairs evaluated, 78 primer pairs generated polymorphisms between the parents Blizzard and 8405-JC3C. The *Xgwm374*, *Xbarc128* and *Xgwm264* amplified polymorphic DNA fragments that were associated with the resistance gene. The *Xgwm374* primers produced a 180 bp fragment in the susceptible parent 8405-JC3C and bulked susceptible lines, but in the resistant source Blizzard and bulked resistant lines the 180 bp fragment was absent. The *Xbarc128* primers produced a 250 bp fragment in 8405-JC3C and bulked susceptible lines, whereas, this fragment was absent in the resistant source and bulked resistant lines. As for *Xgwm264*, 190 and 175 bp fragments were present in Blizzard and bulked resistant lines, whereas a 180 bp fragment was present in 8405-JC3C and bulked susceptible lines.

When considering the 78 lines that were evaluated in the growth chamber for bunt, all three markers produced the same segregation pattern. The number of lines with the resistant parental type possessing the Blizzard marker molecular variant with bunt resistance was 38. The number of lines with the susceptible parental type possessing the 8405-JC3C marker molecular variant with bunt susceptibility was 37. Three non-parental types with the 8405-JC3C molecular variant and bunt resistance were observed. The segregation of 38 lines displaying the Blizzard marker molecular variant and 40 lines with the 8405-JC3C molecular variant fit an expected 1:1 segregation for the DNA polymorphism ($\chi^2 = 0.013$, $P = 0.91$). The co-segregation of marker and bunt resistance was significantly different ($\chi^2 = 22.15$, $P < 0.01$) from a 1:1:1:1 random segregation ratio indicating the markers were linked to the bunt resistance found in the 8405/Blizzard population.

Chi-square analysis of bunt results of the 8405/Blizzard population grown in the field with the markers did not show an association between the bunt resistance and the markers. In other words, there was no difference from a 1:1:1:1 random segregation ratio (2002: $\chi^2 = 5.49$, $P = 0.14$; 2003: $\chi^2 = 3.33$, $P = 0.34$). However, when we applied Student's *t* test to means of bunt reactions for all lines pooled by marker molecular variant, a significant association at the 0.001 level of significance was demonstrated between each molecular variant for bunt resistance for the growth chamber (78 lines) and 2002 (147 lines) environments. The mean of the group with the resistant parent molecular variant of *Xgwm374* was 13.0% common bunt incidence in the 1999 growth chamber test, compared with the 66.1% for the group with the susceptible parent molecular variant. In the 2002 field test, the means were 5.9 and 11.5% bunt incidence and in 2003 were 2.73 and 5.22% for resistant and susceptible parental molecular variant classes respectively. Given the co-segregation of markers, the means were similar for *Xbarc128* and *Xgwm264*. The *t* test was significant at the 0.05 level for the 2003 environment (147 lines). The demonstration of the association of markers *Xgwm374*, *Xgwm264* and *Xbarc128* with bunt using the *t* test led us to also apply simple interval mapping (SIM) using MQTL (Table 3). These results were consistent with our preliminary *t* test results. We also applied SIM to field results using the same 78 lines grown in the growth chamber. Although the markers were significantly associated with bunt resistance at the 0.05 level of significance when 147 lines were used (Table 3), the test statistic was much lower at 1.9 with a non-significant probability of 0.211 when 78 lines were considered.

Joinmap 3.0 results indicated that the recombination distance between the bunt resistance and overlapping markers

Table 3 Simple interval mapping (SIM) test statistic and probability for microsatellite markers associated with common bunt within population 8405/Blizzard population

Marker	1999 78 lines		2002 147 lines		2003 147 lines	
	Test statistic	*P*	Test statistic	*P*	Test statistic	*P*
Xgwm374	114.8	<0.001	11.4	<0.001	5.7	0.033
Xgwm264	114.8	<0.001	11.4	<0.001	5.7	0.033
Xbarc128	114.8	<0.001	10.9	<0.001	5.2	0.043

was 3.9 cM based on the analysis of the doubled haploid population grown in the growth chamber.

The estimation of variation in bunt incidence associated with marker *Xgwm374* was 81% in the 8405/Blizzard population tested in the growth chamber in 1999. The estimate for co-segregating markers *Xgwm264* and *Xbarc128,* was similar to *Xgwm374*.

Marker validation

In the test involving the BW337/P9502 population, the semi-dwarf control Biggar was crowded out and only one row could be rated. Despite this the common bunt incidence of the susceptible control Biggar and moderately susceptible controls HY393 and 8405-JC3C in the growth chamber tests for marker validation populations BW337/P9502 and Blizzard/P9514 indicated that conditions were optimal for common bunt development (Table 1). The BW337/P9502 population parent BW337 was highly susceptible and the P9502&DAF1BB parent was highly resistant. In the test of the Blizzard/P9514 population, Blizzard was completely resistant and P9514-AR17A3E was moderately resistant in the 2007 growth chamber study and completely resistant in the two field trials (Table 1). Segregation within the BW337/P9502 population showed a skewed resistant class and a skewed susceptible class (Fig. 2). There were 21 lines with the same complete resistance phenotype as P9502&DAF1BB, 17 lines moderately resistant (≤35% bunt incidence) and the remaining 38 were moderately susceptible to susceptible. This segregation fit a 1:1:2 (with two classes pooled) two gene ratio ($\chi^2 = 0.42$, $P \geq 0.810$). The Blizzard/P9514 population segregation was skewed to resistant with three moderately susceptible lines.

The Chi-square test for segregation of markers with bunt resistance clearly showed an association between each marker and Blizzard resistance in the BW337/P9502 population. The resistance-*Xbarc128* segregation was significantly different from a 1:1:1:1 random segregation ($\chi^2 = 28.42$, $P < 0.0001$) as was resistance-*Xgwm264* ($\chi^2 = 47.83$, $P < 0.0001$) and resistance-*Xgwm374* ($\chi^2 = 52.10$, $P < 0.0001$). Simple interval mapping supported the Chi-square analysis with each marker being associated with the bunt resistance evaluated quantitatively (Table 4). Because of the more complex segregation of major and minor genes in the Blizzard/P9514 population, only quantitative analysis was applied to bunt resistance at the marked locus. Once again, the *Xgwm264* and *Xgwm374* marked a QTL for common bunt resistance (Table 4). However, *Xbarc128* did not indicate a significant QTL at a probability level of 5%. The

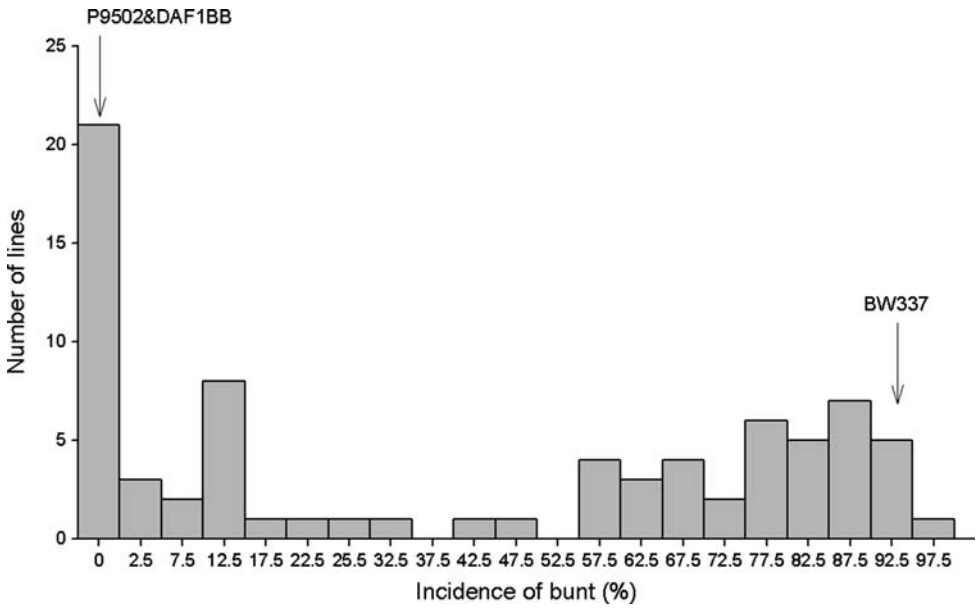

Fig. 2 Common bunt percent incidence of doubled haploid lines from the BW337/P9502 population grown in the growth chamber in 2007

Table 4 Simple interval mapping (SIM) test statistic and probability for microsatellite markers associated with common bunt within populations BW337/P9502 and Blizzard/P9514

Marker	Blizzard/P9514		BW337/P9502	
	Test statistic	*P*	Test statistic	*P*
Xgwm374	15.5	<0.001	107.1	<0.001
Xgwm264	15.5	<0.001	93.1	<0.001
Xbarc128	4.0	0.0793	26.3	<0.001

results of QTL analysis within the Blizzard/P9514 population are consistent with the map distances generated by JoinMap on the BW337/P9502 in which *Xgwm374* was placed 8.3 cM from the bunt resistance factor, *Xgwm264* was 9.8 cM away and *Xbarc128* was furthest away at 30.8 cM.

Discussion

It is obvious from the results that environment and segregation, and the interaction of the two affected the expression of bunt resistance in the 8405/Blizzard population. A higher infection in the lines when grown in the growth chamber compared with the field demonstrated the influence of environment. The difference in bunt incidence among environments that we observed agreed with similar studies (Gaudet and Puchalski 1989a, b; He and Hughes 2003; Knox et al. 1998a; Laroche et al. 2000). Difficulty in precisely classifying lines as resistant and susceptible to bunt, particularly with field results when the expression differential is low, is frequent in genetic studies of common bunt (He and Hughes 2003; Laroche et al. 2000; Knox et al. 1998a).

Optimally in the genetic analysis of common bunt resistance, environmental conditions should favor a high level of disease in the susceptible parent and a low level of disease in the resistant parent. Such conditions were achieved in the 1999 growth chamber test of 8405/Blizzard with the high infection level of the susceptible parent 8405-JC3C. In this test, Blizzard, the resistant parent, was not used as a control because it has a winter growth habit. However, resistance was expressed in the growth chamber test as indicated by zero incidence in the BW553 control possessing *Bt10* and the mean of the population was intermediate to the resistant control and susceptible parent. Furthermore, the range of zero to over 90% incidence in the distribution of bunt reactions, as seen in Fig. 1, indicated conditions were favorable to differentiate resistant and susceptible lines. In the separate vernalized test of the parents and Biggar, the resistance within Blizzard was confirmed as completely resistant to race T19 while 8405-JC3C and Biggar were very susceptible (Table 1).

The lower bunt incidence of 8405-JC3C in the 2002 (range 0–40% infection) and 2003 (range 0–35% infection) field trials with the 8405/Blizzard population indicated that those trials displayed suboptimal infection for good phenotypic characterization of the lines. The narrower distribution of the segregating lines indicated a lower infection level compared with the growth chamber test. The relatively low overall infection in these two environments was reinforced by the intermediate level of infection of the highly susceptible check Biggar and the moderate level of infection of the intermediate resistant cultivar Neepawa (Table 1). Although the 2002 and 2003 results were within the typical range for field environments, they did not allow for precise separation of resistant phenotypes from susceptible phenotypes for qualitative assessment of inheritance and marker linkage. Given the optimal level of infection of the population grown in the growth chamber, more weight was placed on results from the growth chamber than on the data from field trials.

Blizzard was consistently completely resistant to bunt across replications in the 2005 growth chamber test, yet the penetrance of the progeny of the 8405/Blizzard population ranged from complete resistance to moderately resistant in the 1999 growth chamber test. This indicated the breakup of major and modifier genes from Blizzard. Although details of the inheritance and level of expression of the Blizzard bunt resistance are not published, for other sources of resistance the level of penetrance can vary (Gaudet and Puchalski 1989b; Hoffmann and Metzger 1976). Genes such as *Bt8* are characterized simply as major genes (Waud and Metzger 1970), but vary in penetrance depending on the environment (Gaudet and Puchalski 1989b). The gene *Bt10* which is highly expressive and penetrant under conditions favorable to common bunt development while in a certain genetic background (Gaudet and Puchalski 1995) will segregate as a less penetrant phenotype in genetic backgrounds where minor modifier gene influences have been removed and the environment is favorable to disease (Laroche et al. 2000). Knox et al. (1998a) reported that 'SC8021-V2' expressed zero incidence of bunt, but the bunt reaction of progeny from a cross of SC8021-V2 demonstrated segregation of a major gene with incomplete resistance unless combined with a background of a modifier gene or genes. The pattern of segregation of the Blizzard resistance in our growth chamber test was similar to that produced by SC8021-V2.

Under controlled conditions of 1999 with the environment favoring common bunt development, a more precise assessment of lines was achieved because of the wide differential in disease expression. The resulting bimodal distribution seen in Fig. 1 indicated segregation of a major resistance gene. But, as with the SC8021-V2 (Knox et al. 1998a), given the range in incidence of bunt in the resistant

portion of lines (0–36% incidence) of the 8405/Blizzard population (Fig. 1), we expect Blizzard resistance ranged from incomplete to complete depending on variation in background, minor or modifier genes. Because Blizzard shows complete resistance, but the progeny show complete and moderate resistance, at least one additional gene to the major gene must be considered. With a two gene model where AABB is the completely resistant parental type and A is the major gene locus and B is the minor gene locus and aabb is the susceptible parental type, the progeny will segregate in a doubled haploid population 1:1:1:1. The AABB progeny will be completely resistant like Blizzard, the AAbb will be moderately resistant when A is without the additive modifier B for complete resistance, aaBB will be moderately susceptible because BB on its own expresses weak resistance and aabb is completely susceptible. This model would also explain the segregation in the BW337/P9502 population.

Although the 1:1 segregation of the bimodal distribution demonstrated a major resistance gene in Blizzard which could be assessed as a qualitative trait under optimal conditions for bunt expression, under suboptimal conditions the resistance appeared as a continuously distributed quantitative trait. The qualitative inheritance analysis of 1999 growth chamber results did not preclude the application of quantitative analysis which we undertook to confirm the relationship of resistance with the markers.

The high level of polymorphism (34%) between parents made microsatellite markers well suited to mark the Blizzard resistance gene in the 8405/Blizzard population. Among 78 polymorphic markers, we identified a linked marker to common bunt resistance using data from the 1999 growth chamber trial. We followed up the initial marker discovery with the discovery of other linked markers mapped to the region. Classical genetic analysis of *Xgwm374*, *Xbarc128* and *Xgwm264* showed close linkage with the resistance. The dominant DNA bands of markers *Xgwm374* and *Xbarc128* at 180 and 250 base pairs, respectively, are less desirable than codominant markers particularly with the presence of the dominant amplified fragment being in repulsion with the resistance. Fortunately, the *Xgwm264* produced codominant bands between resistant and susceptible parents and progenies at 190 and 175 base pairs for resistant and 180 base pairs for susceptible lines. The two dominant markers linked to Blizzard bunt resistance run counter to the general experience that microsatellites are codominantly inherited (Rafalski and Tingey 1993; Röder et al. 1998). A possible explanation for the dominance of the microsatellite markers associated with the Blizzard resistance locus is that the priming site is altered or missing in the resistant genotype, and is thus characterized by the absence of a PCR DNA fragment (Gill et al. 1991; Liu et al. 2001).

The significantly different means, as determined by the *t* test, for bunt incidence of the lines with the marker molecular variant of the susceptible parent compared with the lines with the marker molecular variant of the resistant parent for each of the three markers supported the findings of class data evaluated with the Chi-square test. The difference of 53.1% incidence in bunt between the class characterized by the resistant molecular variant (13.0%) and the class characterized by the susceptible molecular variant (66.1%) in the 1999 growth chamber study supports the presence of a major gene. This difference is particularly impressive given the environmental conditions favored a high level of bunt infection as demonstrated by the level of infection in the susceptible parental check and breadth of the distribution. The overall lower incidence of common bunt in the two field environments of the 8405/Blizzard population was not favorable to qualitative analysis of linkage between the markers and bunt resistance compared with the growth chamber test. However, quantitative analysis was still possible. Large populations helped to compensate for environments when gene expression was suboptimal. Further supporting evidence of association of the markers with bunt resistance was the linkage indicated by JoinMap and the significant QTLs generated by MQTL. The association between *Xgwm374*, *Xgwm264* and *Xbarc128* appeared to be very strong in the 8405/Blizzard population.

Assessment of common bunt incidence variation associated with microsatellite markers at two loci in a study by Fofana et al. (2008) was 32%. The conservative assessment of variation in common bunt expression associated with the locus marked by *Xgwm374*, *Xgwm264* and *Xbarc128* was markedly higher at 81%. As with the 1:1 segregation by qualitative analysis and the large differential in means associated with molecular variants, the variation associated with the Blizzard marker locus indicates a major allele for bunt resistance in Blizzard.

To confirm the linkage of the markers *Xgwm374*, *Xgwm264* and *Xbarc128* with bunt resistance in Blizzard, we evaluated the markers on two populations, Blizzard/P9514 and BW337/P9502, in which the Blizzard resistance was segregating. The BW337/P9502 population was made specifically for validation, whereas the Blizzard/P9514 population was made to see if the line P9514-AR17A3E possessed the major gene for bunt resistance from Blizzard. The extreme expression in common bunt between parental lines BW337 and P9502&DAF1BB was supported by the clear differential in the segregation of the progeny of the BW337/P9502 population. The segregation within BW337/P9502 indicated two genes for resistance came from P9502&DAF1BB which derived resistance from Blizzard. The pattern of segregation shown in Fig. 2 indicated a major gene that provided moderate resistance when alone

and a second minor gene that modified the major gene to provide complete resistance to common bunt race T19.

The line P9514-AR17A3E, we hypothesize derives minor genes for resistance from Blizzard and from the moderately susceptible parent HY393. The segregation of three moderately susceptible progeny in the Blizzard/P9514 population (Fig. 3) indicated that P9514-AR17A3E does not possess the major gene for bunt resistance from Blizzard. This allowed the major gene from Blizzard to segregate and be identified with QTL analysis (Table 4). The results from the marker validation populations not only confirmed linkage between the markers and bunt resistance, but further supported evidence from the 8405/Blizzard population that Blizzard possesses more than one gene for resistance to common bunt with differing levels of penetrance.

Somers et al. (2004) mapped *Xgwm374* and *Xbarc128* to the short arm of chromosome 1B and also to 2B, and they mapped *Xgwm264* to the short arm of 1B and to 3B. Therefore, it is likely that the Blizzard bunt resistance is located on the 1B short arm because 1B is common between the two sets of markers. The wheat ABD consensus map 'Ta-SSR-2004-1B' in the GrainGenes database of the USDA also reports these three markers on chromosome 1B.

Other bunt resistance genes have been located to various chromosomes including 1B. The *Bt10* gene is located to 6D by marker association (Menzies et al. 2006), *Bt1* is located to 2B (Sears et al. 1960; McIntosh et al. 1998), *Bt7* to 2D (McIntosh et al. 1998), *Bt4* and *Bt6* to 1B (Schmidt et al. 1969), and *Bt5* to 1B (McIntosh et al. 1998). The *Bt8* gene was not on chromosome 5A, 1B or 2D (Waud and Metzger 1970). The evidence that the Blizzard resistance was mapped to 1B indicates the resistance is controlled by a different gene from *Bt1*, *Bt7*, *Bt8* and *Bt10*, but further study is required to determine if it is different from *Bt4*, *Bt5*, *Bt6* or other named genes with unknown chromosome location. The resistance is unlikely to be genes *Bt4* or *Bt6* because race L16, which we used in the field evaluation, is virulent on these two genes (Hoffmann and Metzger 1976). Fofana et al. (2008) report a bunt resistance QTL locus on chromosome 1B that derives from the cultivar AC Domain. Two markers associated with this QTL, *Xgwm374* and *Xgwm264* are the same markers we found linked to Blizzard bunt resistance indicating the two genes may be the same. However, the factor we report on, unlike that of Fofana et al. (2008), was expressed strongly enough to be characterized as a Mendelian factor. Therefore, if the gene is the same the two sources may represent different alleles.

If there were any concern about how evaluating the Blizzard derived populations in an artificial environment would relate to field conditions, these concerns should be alleviated by the field evidence support of the results from the controlled environment study. In fact the controlled environment helped improve precision of marker detection. The three markers showed polymorphism in three populations, but further work is needed to determine how broadly applicable these markers are across potential parental genotypes. The difficulty of identifying the Blizzard phenotype under field conditions and the time and cost of space-limited environmentally controlled tests demonstrates the importance of molecular markers as a tool to accurately and effectively assess the phenotype for bunt resistance.

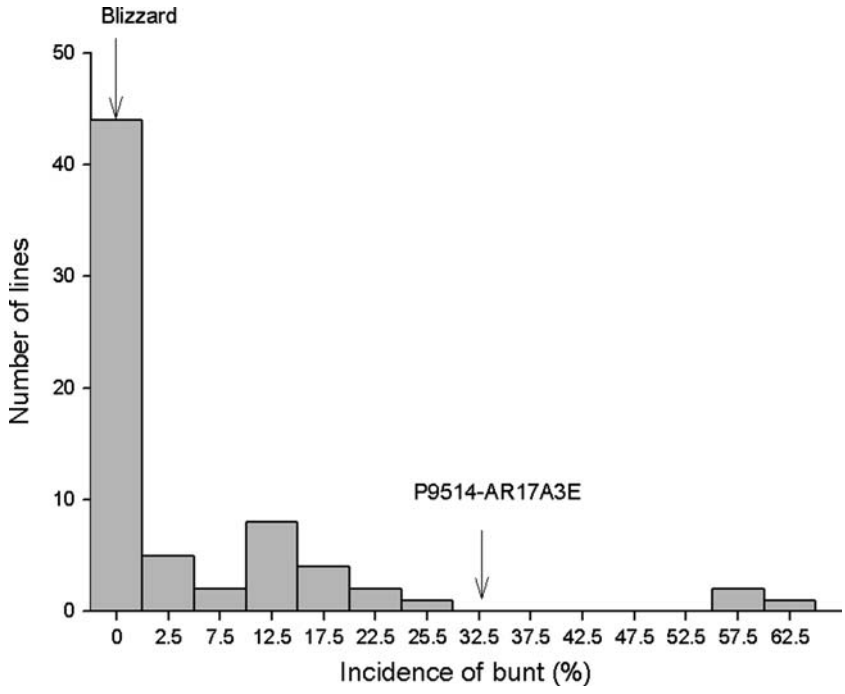

Fig. 3 Common bunt percent incidence of doubled haploid lines from the Blizzard/P9514 population grown in the growth chamber in 2007

Three closely linked markers to the same gene offer an advantage to breeders in selecting markers appropriate to their specific populations because any one marker may not be polymorphic in any given population. If *Xgwm374*, *Xgwm264* and *Xbarc128* are monomorphic in a particular population, breeders can check other markers in the same region for polymorphism (Somers et al. 2004). The distance between the Blizzard resistance and the three markers is close enough for breeders to make substantial genetic gain using the markers. These markers will provide a useful tool for fast and accurate selection of bunt-resistant progenies in early generations. The markers also provide a potentially powerful tool to pyramid the Blizzard resistance gene with other common bunt genes, particularly the *Bt10* gene for which a marker also exists (Demeke et al. 1996; Laroche et al. 2000). Pyramiding bunt resistance genes into wheat cultivars should increase the durability of resistance to common bunt.

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