

Development of molecular markers for crown rot resistance in wheat: Mapping of QTLs for seedling resistance in a 2-49 x Janz population.

B.C.Y. Collard^{1, 3}, R.A. Grams¹, W.D. Bovill¹, C.D. Percy¹, R. Jolley¹, A. Lehmensiek¹, G. Wildermuth² and M.W. Sutherland^{1, 4}.

¹Centre for Rural and Environmental Biotechnology, University of Southern Queensland, Toowoomba QLD 4350, Australia.

²Leslie Research Centre, Queensland Department of Primary Industries & Fisheries, Toowoomba QLD 4350, Australia.

³Present address: Plant Breeding, Genetics and Biotechnology Division, International Rice Research Institute, Philippines.

⁴Corresponding author, email: marksuth@usq.edu.au

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Abstract

Crown rot, caused by *Fusarium pseudograminearum*, is an important disease of wheat in Australia and elsewhere. In order to identify molecular markers associated with partial seedling resistance to this disease, bulked segregant analysis and QTL mapping approaches were undertaken using a population of 145 doubled haploid lines constructed from '2-49' (partially resistant) x 'Janz' (susceptible) parents. Phenotypic data indicated that the trait is quantitatively inherited. The largest QTLs were located on chromosomes 1D and 1A, and explained 21% and 9% of the phenotypic variance, respectively. Using the best markers associated with five QTLs identified by

composite interval mapping (CIM), the combined effect of the QTLs explained 40.6% of the phenotypic variance. All resistance alleles were inherited from '2-49' with the exception of a QTL on 2B, which was inherited from 'Janz'. A minor QTL on 4B was loosely-linked (19.8 cM) to the *Rht1* locus in repulsion. None of the QTLs identified in this study were located in the same region as resistance QTLs identified in other populations segregating for Fusarium head blight, caused by *Fusarium graminearum*.

Additional keywords: *Triticum aestivum* – Fusarium head blight - plant height - bulked segregant analysis - QTL mapping.

Crown rot, caused by *Fusarium pseudograminearum*, is an important disease of wheat in many parts of the Australian wheat belt and regions of North America where it causes significant losses in yield (Klein et al., 1991; Burgess et al., 2001; Backhouse et al., 2004; Smiley et al., 2004). The disease has also been reported in South Africa, Italy, Egypt and Syria, and the fungus is known to occur in Morocco and Argentina (Burgess et al., 2001). Fungal hyphae originating from crop residues initiate infection of wheat plants through the crown region and colonise the lower internodes. Estimates for economic losses caused by crown rot in Australia are of the order of AUS \$56 M per year (Brennan and Murray, 1998). Although management practices can reduce the severity of the disease, the use of resistant cultivars would be the most effective way to control the disease (Swan et al., 2000). However, to date, only sources of partial resistance have been identified (Wildermuth and McNamara, 1994). In particular the line 2-49 has been identified in field trials as an important source of crown rot resistance. This incomplete resistance can also be detected in seedling pot trials (Wildermuth and McNamara, 1994; Wildermuth et al., 2001; Wallwork et al., 2004).

Breeding for resistance to crown rot has been hampered by a lack of knowledge concerning the genetic basis of resistance. Furthermore, phenotypic evaluation for crown rot resistance is very difficult. Phenotypic evaluation is most commonly conducted in field trials, in which plants are grown to harvest maturity in inoculated rows, collected from the field and rated in the laboratory. For some sources of resistance, strong correlations between a seedling screening method and field resistance have been reported ($R^2 = 0.60$; Wildermuth and McNamara, 1994). However, both methods are time-consuming, laborious and relatively expensive, thus impeding breeding efforts. Therefore, the identification of genetic markers that are

tightly-linked to disease resistance loci could be valuable tools for the development of resistant cultivars, using marker-assisted selection (MAS).

The primary objective of this study was to investigate the genetics of resistance and identify loci associated with crown rot. The breeding line '2-49' is a hexaploid white spring wheat which has been identified as carrying high levels of partial resistance in Australian environments. This resistance is currently being introgressed into elite materials in several breeding programmes. Initially, bulked segregant analysis (BSA) (Michelmore et al., 1991) was conducted in a '2-49' (partially resistant) x 'Janz' (susceptible) doubled haploid (DH) population. Subsequent mapping of this population has been conducted in order to identify additional loci that were not detected by BSA.

Materials and Methods

Plant material

A population consisting of 153 DH lines of wheat, *Triticum aestivum* L., was produced using the maize pollination method (Kammholz et al., 1996) from a '2-49' (partially resistant) x 'Janz' (susceptible) F1. Seed was supplied by the Leslie Research Centre, Queensland Dept of Primary Industries and Fisheries, Toowoomba.

Phenotypic evaluation of crown rot resistance

A seedling trial was performed in the glasshouses at the Leslie Research Centre, Toowoomba according to the method described by Wildermuth and McNamara (1994). Briefly, 13 seeds for each DH line were sown per pot. . Seedling infection in 10 plants/pot was rated 21 days after planting using a five-class scale where: 0 = no infection; 1 = 0-25%; 2 = 25-50%; 3 = 50-75% and 4 = 75-100%. Three leaf

sheaths were rated for each individual plant to produce a disease score out of 12. Overall mean scores for each DH line, which were expressed as percentage infection based on the highly susceptible cultivar ‘Puseas’, were used for QTL analysis. Each line was replicated three times in a randomised row-column design. Each line was tested at least twice in independent experiments. One factor ANOVA was performed to determine the genetic variation between lines of the DH population using SPSS (Version 11, SPSS Inc. Chicago, IL, USA)

DNA extractions and molecular marker analysis

DNA was extracted from seedling plant material using a phenol: chloroform method or using a DNA Easy kit (Qiagen, Cat. No. 69106). Microsatellite (SSR) primers were synthesised by Invitrogen (Australia) using published primer sequences (Röder et al., 1998a; Röder et al., 1998b; Pestova et al., 2000; Gupta et al., 2002; Song et al., 2002). Additional SSR primer sequences were obtained from the Grain Genes website (<http://wheat.pw.usda.gov>) using ‘quick queries’: Xwmc SSRs, developed by the Wheat Microsatellite Consortium; Xbarc SSRs, developed by P. Cregan and Q. Song; and Xcfd and Xcfa SSRs, developed by P. Sourdille (Guyomarc’h et al. 2002; Sourdille et al. 2001). Eight sequence-tagged site (STS) primers associated with Fusarium head blight (FHB) resistance on chromosome 3BS (STS3B-49, STS3B-52, STS-55, STS3B-58, STS3B-66, STS3B-80, STS3B-138 and STS3B-142) were selected from Liu and Anderson (2003). PCR was performed according to the protocol described in Lehmensiek et al. (2004). PCR amplification products were visualised by polyacrylamide gel electrophoresis and silver staining using a modified published method (Sourdille et al., 1998).

Bulked segregant analysis

Based on the phenotypic data for the 2-49/Janz population, equal amounts of DNA from 18 partially resistant lines and from 18 highly susceptible lines were pooled to form resistant and susceptible 'bulks'. A total of 168 SSRs, evenly-spaced along chromosomes, were screened across the bulks. Polymorphic SSRs indicating a possible association with resistance were used to genotype the individuals making up the bulks. If there was a possible association with resistance, the marker was used to genotype the entire population.

Map construction

A linkage map was constructed using MapManager QTX (version b20) using the Kosambi mapping function (Manly et al., 2001). Linkage groups were formed by using the 'make linkage groups' command using a linkage evaluation criterion of $P = 0.001$. Known chromosome positions of SSR markers from consensus wheat maps were used to assign chromosome numbers. Double crossovers occurring between three markers spaced <10 cM apart were assumed to be genotyping errors and were scored as missing data.

QTL analysis

Simple interval mapping (SIM) and composite interval mapping (CIM) were performed by Map Manager QTX in order to identify QTLs. Significance levels for highly-significant (HS), significant (S) and suggestive (Sg) QTLs, recommended for reporting QTLs by Lander and Kruglyak (1995), were determined with 10,000 permutations using 1 cM steps. CIM was performed by including into the background unlinked markers with the highest likelihood ratio statistic (LRS) values exceeding the

significance threshold. LRS scores were converted into LOD value by a direct transformation where $LRS = 4.6 \times LOD$ (Liu, 1998). Chromosome maps were made using MapChart Version 2.1 (Voorrips, 2002). Multiple regression (simultaneous model) using single markers with the highest LOD values from CIM was performed using SPSS (Version 11, SPSS Inc. Chicago, IL, USA), in order to estimate the combined effect of QTLs.

Segregation of *Rht1* morphological marker

Segregation of the *Rht1* locus was determined using a gibberellic acid (GA) sensitivity test (Richards, 1992). Briefly, pots (50 mm x 75 mm) were placed into aluminium flats and filled with the potting medium (50% vermiculite: 50% perlite). Five seeds of each line were then planted (1 seed/pot). The GA solution (4 ppm) was regularly added to the bottom of the flats to ensure that the pots were standing in 1-2 cm of solution throughout the experiment. After 3 weeks, 5 seedlings per line were rated by measuring the distance between the seed and the ligule of the second leaf, and a mean extension score was calculated for each line. A Latinised row/column design was used in order to conduct spatial analysis to eliminate any variation arising from local environmental effects on the elongation response. Correlation analysis (using Pearson's coefficient; 2-tailed test) was performed to determine if there was a relationship between the *Rht1* gene and seedling resistance (SPSS Version 11, SPSS Inc. Chicago, IL, USA).

Results

Phenotypic distribution

A total of 145 DH lines were phenotyped. The phenotypic distribution of disease scores for seedling resistance was continuous suggesting that resistance was quantitatively inherited (Figure 1). Analysis of variance indicated genetic variation

between DH lines ($P < 0.001$). Mean parental disease scores were 39.3% for 2-49 and 68.1% for Janz relative to the highly susceptible Puseas. The mean disease score of the population was 50.8%. The range of disease scores was between 18.0 and 91.2%, indicating transgressive segregation.

Linkage map

Over 400 SSRs were screened for polymorphism between 2-49 and Janz of which 46% were polymorphic. One hundred and forty two SSRs that gave clear reproducible bands were used to genotype the mapping population. From these 142 SSRs, 165 alleles were scored. Two out of the 8 STS primer pairs (STS3B-52 and STS3B-138) associated with FHB produced clear and reproducible polymorphisms and were also used for mapping. A total of 27 linkage groups were constructed, covering a total map distance of 1879.3 cM. The average marker spacing was 15.2 cM. Twenty six out of the 27 linkage groups could be assigned to known chromosomes or chromosome segments based on consensus maps. Due to a lack of markers within the proximal regions of chromosomes 2A, 3A, 3B, 6D and 7A, two linkage groups were detected for each of these chromosomes. One small linkage group could not be assigned to any chromosome due to linkage between SSR markers putatively from different chromosomes. Ten markers remained unlinked.

Bulked segregant analysis and QTL analysis

Polymorphisms between partially resistant and susceptible bulks were detected with markers located on chromosomes 1D, 2A and 4B, and these markers were subsequently used to genotype the mapping population. Mapping was extended to cover the entire genome, in order to identify additional QTLs controlling resistance.

SIM detected QTLs on chromosomes 1A, 1DL, 2AS, 2BS and 7BS. There were two peaks (with equal LOD scores) detected using SIM on chromosome 1A. When CIM was used, QTLs were also detected on 1AL, 1DL, 2BS and 7BS, in addition to another QTL that was detected on chromosome 4BL. Using CIM, a more clearly defined QTL peak was detected on 1AL that was just below the highly significant threshold while the QTL on chromosome 2AS dropped below the suggestive significance level. The results for SIM and CIM are reported in Figure 2 and Table 1. Multiple regression, using the best 5 markers (on 1D, 1A, 2B, 4B and 7B) identified by CIM, explained 40.6% of the phenotypic variance. The putative resistance QTLs were all inherited from 2-49 except for the QTL on chromosome 2B, which was inherited from Janz (Table 2). No epistatic interactions were detected between QTLs or unlinked markers using the interactions function in Map Manager QTX.

Segregation of *Rht1* morphological markers

The distribution of the mean seedling extension lengths indicated two peaks between 5-8 cm and 11-15 cm (Figure 3). Lines that were 9 or 10 cm in length were examined closely and classified into tall or dwarf classes based on measurements for all replicates. The occurrence of intermediate lines was attributed to environmental effects and uneven germination. Three lines were excluded from the data set, since they could not be confidently grouped with either size class. The ratio of 62: 77 (tall: dwarf) was consistent with a 1:1 segregation ratio, which would be expected for the segregation of a single gene in a doubled haploid population (Chi square test $P = 0.20$). A small but significant correlation was detected between seedling extension and seedling resistance ($r = -0.17$; $P = 0.036$), indicating that in general taller plants were more resistant.

Discussion

Detection of QTLs

Since the QTL on 1DL is confined by tightly-linked flanking markers (2.1 cM) and explains a relatively large proportion of the phenotypic variance, this QTL would be an ideal candidate for marker-assisted selection. The detection of an important resistance locus in the D genome is also consistent with the observation that durum wheat cultivars are generally susceptible to crown rot. Interestingly, this QTL is in the same region as a major gene for powdery mildew (*Pm24*) which appears to be tightly linked to the SSR marker Xgwm337 (Huang et al., 2000), the only polymorphic marker common to both studies. Further investigations are required to determine the proximity of these resistance loci.

The most likely position for the QTL on 1AL is between the markers Xwmc120 and Xwmc312, which are 18.4 cM apart. All publicly available SSR markers in this region were screened in order to more precisely determine the position of the QTL. However, other SSR markers did not map to this specific region or were not polymorphic. We are currently investigating alternative markers types in this region. Targeted mapping of amplified fragment length polymorphism (AFLP) markers using bulked segregant analysis could be an efficient strategy to map this specific chromosomal region (Campbell et al., 2001). The large confidence interval and width of the QTL peak on 1A indicates that the QTL position was not well resolved. One explanation for the inability to resolve this QTL could be the presence of multiple linked QTLs (Liu, 1998).

The identification of a QTL from Janz on chromosome 2BS was consistent with the transgressive segregation of the disease phenotype observed within the population. This suggests that the effectiveness of recognised resistance QTLs in

backcrossing programs will vary between recurrent backgrounds since current phenotyping methods are not sensitive enough to consistently detect single genes of minor effect that may be present in susceptible materials. The inheritance of disease resistance alleles from susceptible wheat parents has been reported by a number of recent studies (Waldron et al., 1999; Navabi et al., 2004; Steiner et al., 2004).

The development of interval mapping methods for detecting QTLs have greatly expedited the discovery of QTLs for important traits in many crop species (Doerge, 2002). Simple interval mapping (SIM) and composite interval mapping (CIM) are two widely-used methods. Although the two methods often produce similar results, differences in QTL identification have been reported (Borem et al., 1999; Falak et al., 1999; Cai and Morishima, 2000). CIM is considered to be more statistically powerful than SIM because the method incorporates additional markers into the statistical model in addition to the adjacent pair of linked markers used in SIM (Liu, 1998; Zeng, 1993; Zeng, 1994). Therefore, the QTL detected on 2AS, which was only identified using SIM, requires further confirmation. The validation of all of these QTLs in other 2-49-related populations is currently in progress.

Bulked segregant analysis (BSA) is a gene tagging technique, first proposed by Michelmore et al. (1991). Despite the large size of the wheat genome, low levels of polymorphism and existence of numerous repeated sequences, this approach has been successfully used to identify markers associated with disease resistance QTLs, for example Chantret et al. (2000) and Czembor et al. (2003). The identification by BSA of a major QTL on 1DL is consistent with the claims that BSA will generally only detect QTLs with large effects (Liu et al., 2001; Bourdoncle and Ohm, 2003) (Czembor et al., 2003). The detection of this QTL by SIM and CIM is consistent with the expectation that interval mapping methods will confirm QTLs identified using

BSA (Tang et al., 2000). The failure of BSA to detect the QTL on 1A may be explained by the relatively small number of markers tested within this region during BSA. The detection of suggestive QTLs on 2AS and 4BL indicates that BSA conducted at a sufficient screening density can also detect some QTLs with small effects (i.e. $R^2 < 10\%$).

Position of crown rot QTL from previous study

To date, there is only one other report of markers associated with crown rot resistance (Wallwork et al., 2004). In this study the cultivar Kukri expressed a partial adult plant resistance, significantly less effective than that expressed by 2-49 in the same trials. Nevertheless, using a Kukri x Janz doubled-haploid population these authors have identified a QTL for resistance on chromosome 4B (Wallwork et al., 2004). This QTL is located in the same region near Xgwm251 (between the SSR marker Xgwm251 and AFLP marker ACG-CAC) as the minor QTL detected in this study. Our preliminary screening indicates that 2-49 and Kukri do not share common marker alleles in this region of 4B (other than Xgwm251), consistent with their unrelated pedigrees. Kukri does not show partial resistance to crown rot in seedling tests. Further work is required to determine whether these are distinct QTLs or manifestations of a single QTL of minor effect which shows varied timing of expression in different backgrounds.

Linkage between *Rht1* and QTLs for crown rot resistance

The *Rht1* gene was mapped because field observations by one of us indicated that plant height and crown rot resistance may be correlated. The demonstration of a correlation between *Rht1* and resistance and the identification of a minor QTL for

resistance on 4B (significant in CIM but not SIM), approximately 20cM from *Rht1* has confirmed this observation. This linkage was also observed by Wallwork et al. (2004) using a SIM approach. In ‘2-49’, the resistance QTL is linked in repulsion to the *Rht1* allele conferring the preferred semi-dwarf habit. However, given the distance between the two loci, this linkage should be able to be broken from ‘2-49’ during backcrossing into *Rht1* backgrounds, producing semi-dwarf resistance recombinant genotypes that retain the desirable 4B resistance allele. Nine such recombinant lines were detected in the ‘2-49’ x ‘Janz’ population.

Correlations with Fusarium head blight QTLs

Fusarium head blight (FHB), caused by *Fusarium graminearum*, is a devastating disease of wheat world-wide (Miedaner, 1997). We are currently investigating the possibility of a linkage between resistance to *F. graminearum* and resistance to *F. pseudograminearum*. Major QTLs in ‘Sumai-3’ and ‘Frontana’ and their derivatives conditioning resistance to FHB have been identified on 3B (Waldron et al., 1999; Anderson et al., 2001; Bourdoncle and Ohm, 2003) and 3A (Steiner et al., 2004) respectively. Additional QTLs have been identified on chromosomes 6B (Anderson et al., 2001), 5A (Buerstmayr et al., 2003; Steiner et al., 2004) and 2A and 2B (Waldron et al., 1999; Zhou et al., 2002). STS primers (Liu and Anderson, 2003) were used for mapping 3BS because these markers were specifically developed for FHB resistance and known SSR primers for markers in this region failed to produce clear and reproducible polymorphisms in our laboratory. However, in the 2-49 x Janz population, none of the identified QTLs for crown rot resistance coincided with the regions containing head blight resistance QTLs identified in the above studies. Although QTLs associated with crown rot resistance were also identified on

chromosomes 2A and 2B, they were not located in the same regions as the QTLs for FHB.

This is the first report investigating the genetic basis for crown rot resistance in hexaploid wheat seedlings, based on the construction of a framework map. Seedling resistance to crown rot in the '2-49' x 'Janz' population appears to be controlled by two major QTLs on chromosomes 1D and 1A and at least three other minor QTLs. We are currently conducting seedling trials of independent but related populations and field trials, in order to determine if the QTLs controlling seedling resistance in '2-49' are associated with adult plant resistance. We are also genotyping a set of Northern Region (Australian) wheat genotypes, to test the utility of the SSR markers for marker-assisted selection.

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Table 1: QTLs for seedling resistance to *F. pseudograminearum* detected using simple interval mapping (SIM) and composite interval mapping (CIM).

Chr.	Source of resistance allele	SIM				CIM			
		Best flanking markers	Max LOD	R ² (%)	Sig. level ¹	Best flanking markers	Max LOD	R ² (%)	Sig. level
1AL	2-49	Xwmc24-Xbarc148 & Xwmc120-Xwmc312	3.2	10	S	Xwmc120-Xwmc312	4.0	9	S
1DL	2-49	Xcfd19-Xwmc216	7.5	21	HS	Xcfd19-Xwmc216	8.5	21	HS
2AS	2-49	Xgwm339-Xgwm425	1.8	6	Sg	Xgwm558-Xgwm294	1.0	2	NS
2BS	Janz	Xgwm388-Xbarc349.1	1.7	5	Sg	Xgwm388-Xbarc349.1	1.9	4	Sg
4BL	2-49	Xgwm251-Xwmc349	0.9	3	NS	Xgwm165-Xgwm251	1.9	4	Sg
7BS	2-49	Xgwm400-Xwmc476	2.3	8	Sg	Xgwm400-Xwmc476	2.5	6	Sg

¹ Sig. level – significance level determined by 10,000 permutation tests.

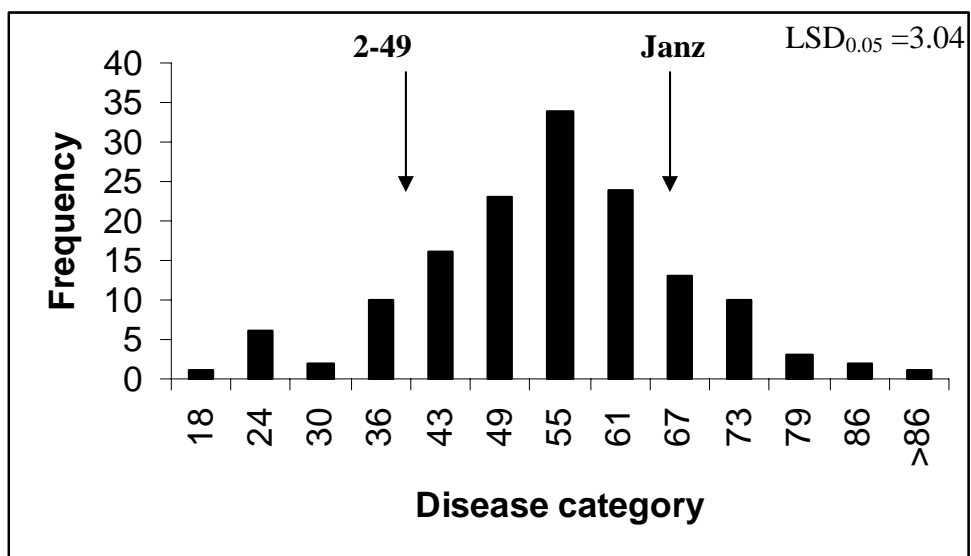
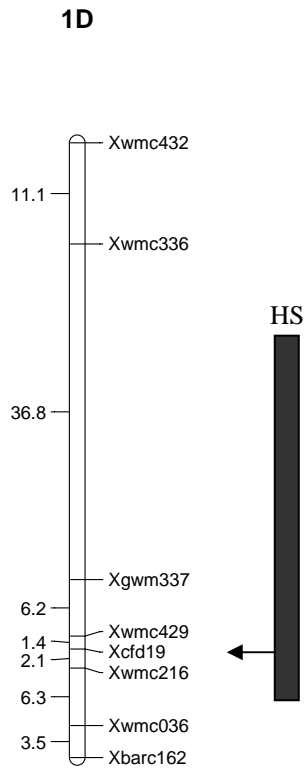
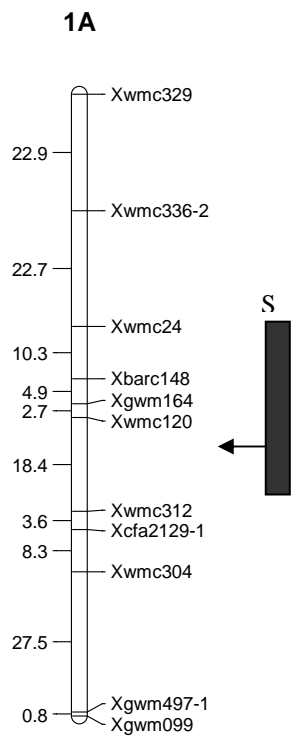


Figure 1. Histogram of disease score categories for seedling resistance for 145 DH lines. Mean disease scores for the parental lines are indicated by arrows.

(a)



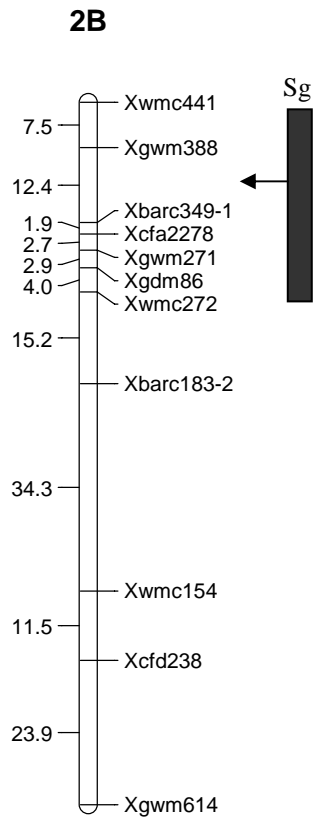
(b)



(c)



(d)



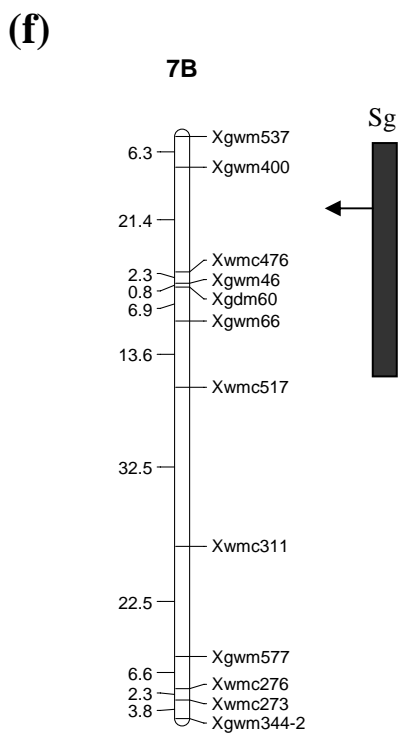
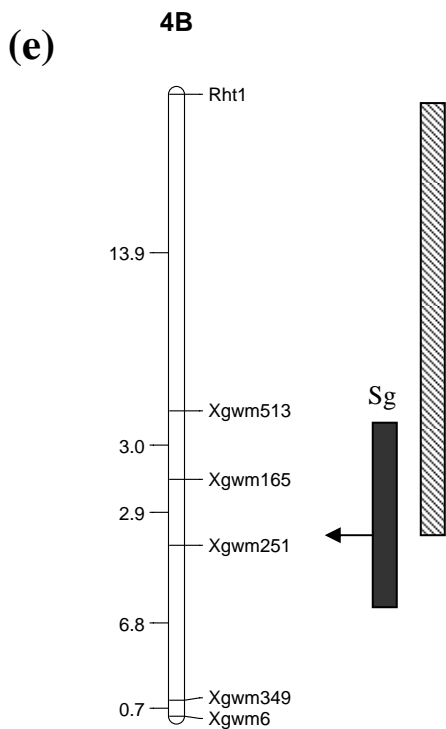


Figure 2. Chromosome positions of QTLs for seedling resistance on: 1D (a); 1A (b); 2A (c); 2B (d); 4B (e); and 7B (f). Chromosome regions exceeding highly significant (HS), significant (S) and suggestive (Sg) thresholds are indicated by filled vertical bars. Arrows indicate the most likely position of QTLs, as determined by maximum LOD peaks. The hatched bar on chromosome 4B indicates the corresponding region for the major QTL for crown rot resistance detected by Wallwork et al. (2004).

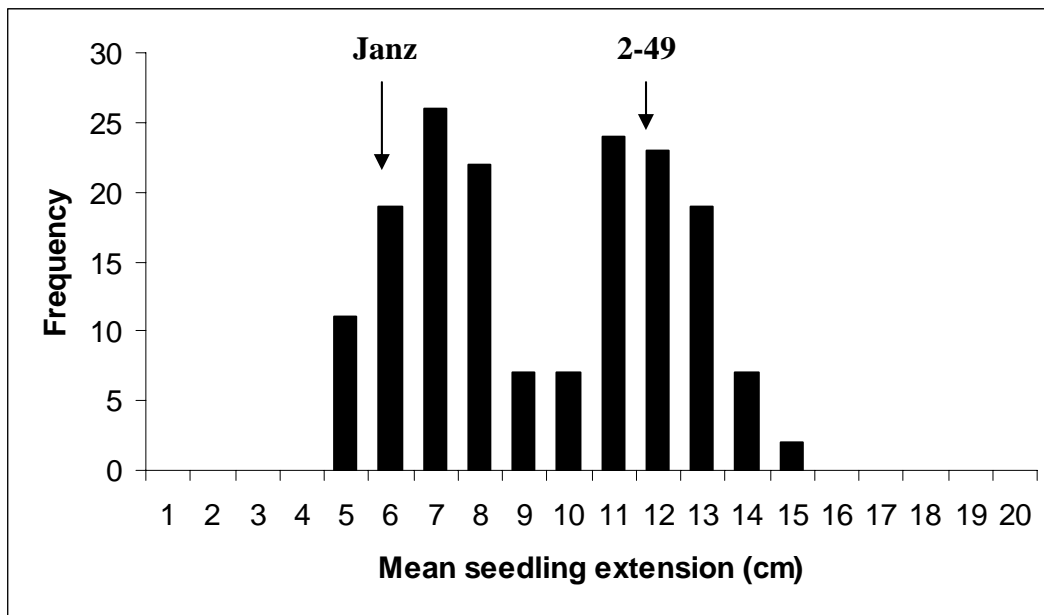


Figure 3. Distribution of seedling extension distance (cm) from seed to ligule of second leaf for the '2-49' x 'Janz' population. Mean lengths for the parental lines are indicated by arrows.

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