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Microsatellite DNA Markers Indicate Quantitative Trait Loci Controlling Resistance to Pea Root Rot Caused by *Fusarium avenaceum* (Corda ex Fries) Sacc

[1,2]W.J. Li, [1]J. Feng, [1]K.F. Chang, [3]R.L. Conner, [1]S.F. Hwang, [4]S.E. Strelkov,
[5]B.D. Gossen and [6]D.L. McLaren
[1]Crop Diversification Centre North, Alberta Agriculture and Rural Development,
Edmonton, AB, T5Y 6H3, Canada
[2]Biotechnology and Germplasm Resources Institute, Yunnan Academy of Agricultural Sciences,
Kunming, Yunnan, 650223, P.R. China
[3]Agriculture and Agri-Food Canada (AAFC), Morden, MB, R6M 1Y5, Canada
[4]Department of Agricultural, Food and Nutritional Science, University of Alberta,
Edmonton, AB, T6G 2P5, Canada
[5]AAFC, Saskatoon, SK, S7N 0X2, Canada
[6]AAFC, Brandan, MB, R7A 5Y3, Canada

Abstract: To identify Quantitative Trait Loci (QTL) controlling root rot resistance of field pea (*Pisum sativum* L.), 213 Simple Sequence Repeats (SSR) were screened against a population of Recombinant Inbred Lines (RIL) derived from crosses between a moderately resistant cultivar Carman and a susceptible cultivar Reward. Phenotypic data were obtained following inoculation of pea plants with *Fusarium avenaceum* (Corda ex Fries) Sacc. in field experiments conducted during 2009 and 2010. Linkage analysis based on a single factor ANOVA indicated that four markers were associated with root rot resistance. QTL analysis based on these four markers identified a QTL on Chromosome VII that explained 21.7% of the variance in resistance. The microsatellite markers that are closely linked to this QTL may be useful for stacking QTLs from Carman and other resistance sources to develop cultivars with superior fusarium root rot resistance.

Key words: Simple sequence repeats, quantitative trait loci, *Fusarium avenaceum*, root rot, pea

INTRODUCTION

Fusarium root rot is a major disease of field pea worldwide (Kraft and Pfleger, 2001). This disease is caused by various *Fusarium* species and *F. solani* is the most extensively studied (Tu, 1992) but other species, including *F. avenaceum* and *F. oxysporum*, can also be important components of this disease complex (Xue, 2003). Recently, *F. avenaceum* has been isolated more frequently than any of the other fungal species in field surveys of field pea conducted in North Dakota (Chittem *et al*., 2010) and Alberta (Feng *et al*., 2010).

Deployment of genetically resistant cultivars has the potential to be an effective and efficient approach to root rot control. Selection and breeding for resistance requires the screening of candidate sources. However, traditional methods for resistance screening take a long time, require space for the production of many plants of each parent and progeny line and involve the uprooting of each plant for evaluation. The availability of DNA markers linked to the genes controlling resistance permits more efficient selection strategies based on marker genotyping. Among the markers frequently used in plant breeding, microsatellite or SSR have advantages such as co-dominant inheritance, multi-allelic nature, relative abundance and extensive coverage of the genome (Agarwal *et al*., 2008). They consist of short tandemly repeated nucleotide motifs flanked by conserved sequences which allow the detection of polymorphisms by standard Polymerase Chain Reaction (PCR) techniques. Development of microsatellite markers is time consuming and expensive. However, once developed, they are easily maintained and shared among laboratories.

Numerous microsatellite markers covering each of the seven chromosomes of field pea have been developed from different genotypes (Burstin *et al*., 2001; Loridon *et al*., 2005). These markers can be used to map various pea genes of interest, including those that confer disease resistance. Therefore, in the current study, the microsatellite markers developed by Loridon *et al.* (2005)

Corresponding Author: Sheau-Fang Hwang, Crop Diversification Centre North, Alberta Agriculture and Rural Development, Edmonton, AB, T5Y 6H3, Canada

were selected and used to identify QTLs linked to the resistance against root rot caused by *F. avenaceum* in a pea RIL population derived from crosses between the resistant cultivar Carman and the susceptible cultivar Reward. The objectives of this study were to identify QTLs for *F. avenaceum* resistance in Carman and to estimate their position and genetic effects.

MATERIALS AND METHODS

Population development: Crosses between the root rot-resistant field pea cultivar Carman and the susceptible cultivar reward were made in a greenhouse. A recombinant inbred population ($F_{2:10}$) consisting of 161 lines was developed from individual F_2 seeds by the single-seed-descent method (Johnson and Bernard, 1962).

Root rot assessment: A single-spore isolate of *F. avenaceum* was cultured on sterilized wheat grain in autoclaved plastic bags for 3 weeks to ensure complete colonization of the grain. The grains were dried in a fume hood and used as inoculum. The RIL population was divided into two subpopulations which were tested for *F. avenaceum* resistance in 2009 (76 RILs) and 2010 (85 RILs) in field experiments conducted at the Morden Research Station, MB, Canada. For each subpopulation, 15 seeds from each RIL were mixed with 10 g of inoculum and planted into a single row, 1 m in length. Rows of the parents Carman and Reward were included as check treatments in each group of 15 to 20 RILs. The trials were laid out in a randomized complete block design with three replications. At the end of the growing season, all of the plants in each plot were rated for root rot severity on a 0-9 scale (Table 1). The root rot data were analyzed using the GLM procedure in SAS v. 9.1.3 (SAS Institute, Cary, NC). Significant differences between treatments were assessed using the Least Significant Difference (LSD) multiple comparisons test.

DNA extraction: Seedlings were generated from each RIL or parental cultivar in a greenhouse set for a 24°C day/18°C night temperature regime and a 16 h photoperiod. Genomic DNA was extracted from one leaf of a 14 day old seedling using the DNeasy plant mini kit (Qiagen Canada, Mississauga, ON) following the manufacturer's instructions. The concentration and quality of extracted DNA were confirmed with a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA) and adjusted to 20 ng μL^{-1} with deionized water.

Table 1: An assessment scale for root rot severity (0-9) in field pea

Scale	Description of seedling	Description of lower stem and roots
0	Healthy	No lesions
1-2	Slight	Slight necrosis or few small lesions, total infected area <5% (1) or 5-10% (2)
3-4	Mild	Scattered necrosis or small lesions, total infected area 10-25% (3) or 25-50% (4)
5-6	Moderate	Extensive necrosis or large lesions, total infected area 50-75% (5) or >75% (6)
7-8	Severe	Extensive necrosis, or girdling lesions on lower stem and roots, roots developed (7), or little or no roots (8)
9	Dead plants	

Microsatellite markers: Two hundred and thirteen primer pairs corresponding to microsatellite DNA that were well-distributed over the seven pea chromosomes were selected for the study. The primer sequences had been previously described by Loridon *et al.* (2005) and the primers were synthesized by Integrated DNA Technologies (Coralville, IA).

Primer screening and RIL genotyping: PCR was conducted using DNA from 'Carman' and 'Reward' to screen all the primer pairs for polymorphisms. The resulting polymorphic primer pairs were used to genotype 32 of the most resistant and 32 of the most susceptible RILs from the two subpopulations (total 64 RILs). From these RILs, an amplified band corresponding to a band in 'Carman' (resistant parent) or 'Reward' (susceptible parent) was taken to indicate that the marker locus from that parent was present in the RIL. Primer pairs that produced a marker associated with resistance among these 64 lines were used to genotype each of the 161 RILs. For all PCRs performed, each 20 µL of reaction consisted of 40 ng of genomic DNA, 0.2 µM of each primer and 10 µL of 2× PCR master mix (Promega, Madison, WI). Amplification was carried out in a My Cycler thermal cycler (Bio-Rad Canada, Mississauga, ON). The annealing temperature was chosen according to the primers used and the extension time was 1 min. The PCR products were electrophoresed on 3% MetaPhor Agarose (Lonza, Allendale, NJ). DNA bands were visualized using GelGreen (Omega Bio-Tek, Norcross, GA) and photographed with a UV transilluminator (Bio-Rad Canada, Mississauga, ON).

Data analysis: Segregation of each polymorphic marker in the RIL population was analyzed for goodness of fit to an expected ratio of 1:1 based on a chi-square test at $p>0.05$. A single factor ANOVA which produces a one-way analysis of variance for a quantitative dependent variable (resistance) by a single factor (marker), was performed for each marker to establish its association with resistance in the populations. A significant association between a marker and resistance was declared at $p<0.05$. QTL

analysis was performed using Mapmaker v. 3.0b (Lander *et al*., 1987). The RIL (ri self) genetic model, minimum LOD (logarithm of likelihood ratio) score of 3 and Kosambi genetic distances estimation (maximum recombination of 30%) were used for map elaboration. The Mapmaker files were analyzed for QTL detection using QTL Cartographer v. 2.5 (http:// statgen. ncsu. edu/ qtlcart /WQTLCart.htm). Interval Mapping (IM) (Ooijen, 1992) was used with a mapping step size of 1 cM. LOD thresholds for QTL significance were empirically determined with a permutation method consisting of 1000 replications (Churchill and Doerge, 1994) and LOD thresholds were established at a significance level of 5%. The R^2 value from the resulting IM genetic model was accepted as the percentage of phenotypic variation explained by the identified QTL.

RESULTS

Root rot assessment: The frequency distribution of severity among the RILs indicates the presence of quantitative variation for resistance to *F. avenaceum* in the population (Fig. 1). The parental cv. Carman was moderately resistant to root rot, with a mean rating of 4.1 (range 4.0-4.3) in 2009 and 4.1 (4.0-4.3) in 2010 and cv. Reward was susceptible, with a mean rating of 4.9 (4.7-5.6) in 2009 and 5.8 (5.6-6.1) in 2010. The RILs showed considerable variation in severity, with an overall mean rating of 4.5 (3.3-6.1) in 2009 and 5.2 (3.5-6.8) in 2010. Lines more resistant than 'Carman' or more susceptible than 'Reward' were identified by Fisher's LSD test from both subpopulations which indicated that transgressive segregation for resistance occurred in the crosses.

Primer screening, RIL genotyping and QTL mapping: Among the 213 primer pairs, 55 produced polymorphic bands from DNA samples of the two parental cultivars. Most of these primer pairs amplified single clear bands of different length in 'Carman' and 'Reward'. When used to genotype the 64 selected RILs, four primer pairs produced polymorphisms that showed association with disease severity (data not shown). These four markers were then evaluated using the entire RIL population (Fig. 2). Since the disease severity data were taken in alternative years, genotype data obtained from each of these four markers was sorted into two subpopulations (2009 and 2010) and analyzed separately.

The Chi square test indicated that the four polymorphic markers were present at a ratio of 1:1 in at least one subpopulation, suggesting that these primers were specific to a single locus (Table 2). However, when the chi square test was conducted based on the entire population, only marker AA160 showed a 1:1 ratio. The other three markers showed a segregation distortion with deviation being biased towards either 'Carman' (AA416) or 'Reward' (AB60 and AD53) alleles.

Markers AB60 in 2009 and AA416 and AD53 in 2010 had probability values of $p<0.05$ in one-way ANOVAs of severity which indicates a linkage with a putative QTL. According to Loridon *et al*. (2005), marker AA160 was located in the region between AA416 and AD53. Thus,

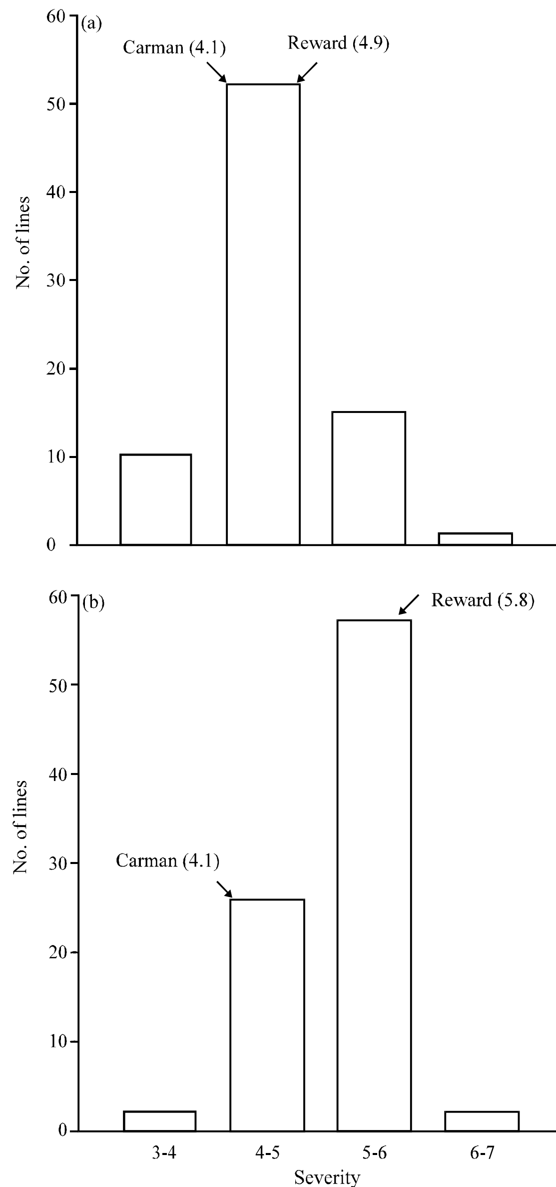

Fig. 1(a-b): Reactions of pea recombinant inbred lines to *Fusarium* root rot, measured using a 0-9 rating scale (a) Subpopulation 2009 with 76 lines and (b) Subpopulation 2010 with 85 lines

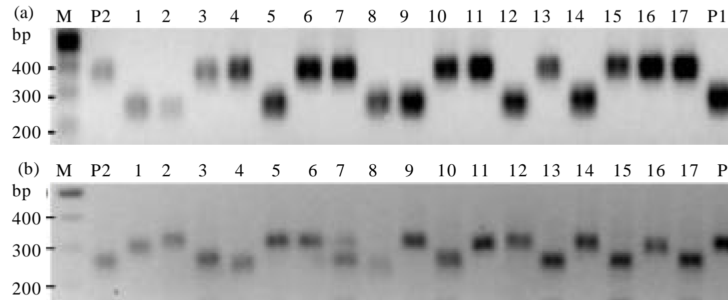

Fig. 2(a-b): Polymorphisms detected using the microsatellite markers (a) AB60 and (b) AD53 across recombinant inbred lines, P1: Resistant parent, cv. Carman, P2: Susceptible parent, cv. Reward, 1-17: Selected recombinant inbred lines. M: DNA marker

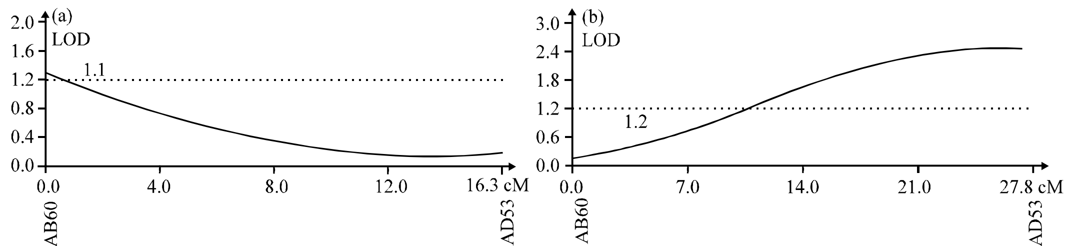

Fig. 3(a-b): Interval QTL mapping analysis of the *Fusarium* root rot resistance located on LGVII of the pea genome with subpopulations of pea recombinant inbred lines (a) Subpopulation 2009 and (b) Subpopulation 2010, dotted lines represent the threshold LOD scores estimated using a permutation test

Table 2: Microsatellite markers producing polymorphic bands between cultivars Carman and Reward and in the recombinant inbred lines

Marker[1]	Band (bp) from Carman	Band (bp) from Reward	Subpopulation	p-value of chi square (1:1)[2]		p-value of F test[3]
AA416	200-300	200-300[4]	2009	0.37		
			2010	0.04	0.03	0.30
						0.04
AA160	300-400	200-300	2009	1.00		
			2010	0.24	0.39	0.08
						0.52
AD53	300-400	200-300	2009	0.17		
			2010	0.02	0.01	0.55
						0.0006
AB60	200-300	300-400	2009	0.04		
			2010	0.16	0.01	0.02
						0.26

[1]Marker designations according to Loridon *et al.* (2005), [2]Chi-square test for goodness of fit to an expected allelic 1:1 ratio for individual years and the entire population, [3]F test in the single factor ANOVA to examine the association of markers with resistance, [4]The size of the band was smaller than that from 'Carman'

each of these four markers was included in linkage analysis. Data from both subpopulations indicated that AB60 and AD53 were linked. These two markers defined a 16.3 cM segment in the 2009 subpopulation and 27.8 cM in the 2010 subpopulation (Fig. 3). Based on the interval analysis, a QTL was located in a region around AA160 and AD53 which explained 6.7% of the variation in root rot reaction among the 76 RILs in 2009 and 21.7% of the variation in root rot reaction among the 85 RILs in 2010. These two markers were mapped on the distal end of chromosome VII (Loridon *et al*., 2005), so the QTL must be located on the distal end of VII.

DISCUSSION

The continuous distribution of root rot reaction values among the RILs observed from the two subpopulations indicated that the resistance in this population was quantitative. This may reflect the contributed functions of several QTLs and the effect of

the environment. In the current study, alternative QTLs were not associated with resistance or susceptibility to fusarium root rot, possibly due to the relatively small size of the population assessed and the low density of markers. On the other hand, the difference in mean root rot severity between the two subpopulations indicates that the effect of environment on the expression of root rot resistance was complex.

Transgressive segregation was observed among the RILs in each of the two subpopulations. This phenomenon is not uncommon in breeding populations, for example, QTLs for *Aphanomyces* root rot resistance have been shown to be derived from the susceptible parents (Pilet-Nayel *et al*., 2002). According to Jinks and Pooni (1976), superior progeny genotypes can occur when both parents are phenotypically similar but differ in their genetic background. This was likely the case in our population. In the current study, segregation distortion was observed on both of the two QTL markers, with the deviation being biased towards the ‘Reward’ alleles. Segregation distortion may arise from genetic, physiological and environmental causes and the relative contribution of each of these factors may differ in specific populations (Xu *et al*., 1997). In RIL populations such as the one used in the current study, it is difficult to distinguish genetic from environmental causes of distorted allele frequencies because the G×E interaction becomes more pronounced during the progress of selfing.

Several QTLs in field pea that confer partial resistance to root rot caused by *F. oxysporum* (Dirlewanger *et al*., 1994; Coyne *et al*., 2000), *A. euteiches* (Pilet-Nayel *et al*., 2002, 2005; Hamon *et al*., 2011) and *F. solani* (Weeden and Porter, 2007; Feng *et al*., 2011) have been identified. The two QTLs for *F. solani* resistance identified by Weeden and Porter (2007) and Feng *et al*. (2011), as well as eight of the QTLs for *A. euteiches* resistance identified by Hamon *et al*. (2011), were located on chromosome VII. In the current study, a major QTL was also mapped on chromosome VII. The population used in the current study is derived from the same parents as the population used by Feng *et al*. (2011) and the same markers were identified to flank the QTL. Furthermore, one of the nearest markers (AA416) linked to one of the eight QTLs identified by Hamon *et al*. (2011) was also linked to *F. avenaceum* resistance in the current study. Thus, we conclude that the same QTL controls resistance to both *F. avenaceum* and *F. solani* and that this QTL may also contribute to the resistance to *A. euteiches*.

Almost 80% of the phenotypic variation was not explained by the detected QTL which may indicate the presence of other resistance genes or QTLs. As a result, we are screening more microsatellite markers. Additional polymorphic markers will be used to genotype a larger RIL population. Once a marker is shown to be associated with resistance to root rot, more markers in its vicinity will be screened.

The current study provides valuable information for future pea breeding activities, in particular with respect to two aspects of breeding for resistance to root rot in field pea. Firstly, the identified QTL appears to control resistance to more than one *Fusarium* species (*F. solani* and *F. avenaceum*) and so merits integration into new cultivars. Secondly, the two identified markers (AA160 and AD53) can be used in marker-assisted selection (MAS) to develop root rot resistant pea cultivars. The advantages of using MAS include ease of observation and scoring, freedom from genotype-environment interactions and independence of plant growth stage, season, location and agronomic practices. The markers identified in the current study can be used to identify the specific QTL for resistance but could also be useful in subsequent assessments in the search for novel sources of root rot resistance and for the pyramiding of resistance genes.

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