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Tagging and mapping a second resistance gene for *Fusarium* wilt race 0 in chickpea

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Abstract A second gene conferring resistance to the chickpea wilt pathogen, *Fusarium oxysporum* f. sp *ciceris* race 0, has been mapped to linkage group 2 (LG2) of the chickpea genetic map. Resistance to race 0 is controlled by two genes which segregate independently; one present in accession JG62 ($Foc0_1/foc0_1$) and mapping to LG5 and the second present in accession CA2139 ($Foc0_2/foc0_2$) but remaining unmapped. Both genes separately confer complete resistance to race 0 of the wilt pathogen. Using a Recombinant Inbred Line (RIL) population that segregated for both genes (CA2139 × JG62) and the genotypic information provided by two markers flanking $Foc0_1/foc0_1$ ten resistant lines containing the resistant allele $Foc0_2/foc0_2$ were selected. Genotypic analysis using these ten resistant lines paired with ten susceptible RILs, selected in the same population, revealed that sequence tagged microsatellite sites (STMS) markers sited on LG2 were strongly associated with $Foc0_2/foc0_2$. Linkage analysis, using data from two mapping populations (CA2139/JG62 and CA2156/JG62), located $Foc0_2/foc0_2$ in a region where genes for resistance to wilt races 1, 2, 3, 4 and 5 have previously been reported and which is highly saturated with tightly-linked STMS markers that could be used in marker-assisted selection (MAS).

Keywords *Cicer arietinum* · Genetic maps · Marker-assisted selection · STMS

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Introduction

Chickpea (*Cicer arietinum*) is a major source of high-protein food and animal feed and is the world's third most important pulse crop after beans (*Phaseolus vulgaris*) and peas (*Pisum sativum*) (FAOSTAT 2007). Wilt caused by *Fusarium oxysporum* f. sp. *ciceris* (FOC) is one of the most devastating diseases of chickpea and is widespread in almost all chickpea growing countries, causing losses which may be total (see review by Sharma and Muehlbauer 2007). The pathogen penetrates through the roots until it reaches the xylem where it reduces or blocks completely

water transport to aerial parts. The infected plants finally wilt and die. To date, eight races of the pathogen have been reported (0, 1A, 1B/C, 2, 3, 4, 5 and 6), which are differentiated by the reactions they elicit in a differential set of cultivars (Haware and Nene 1982; Jiménez-Diaz et al. 1993; Halila and Strange 1996).

Knowledge of the inheritance of race-specific wilt resistance is a basic requirement for the identification and integration of resistance genes in linkage maps and for their use in marker-assisted selection (MAS) in order to develop new high yielding cultivars that are resistant to the pathogen. Up to three genes control resistance to the different races of FOC. Resistance to race 0 (FOC-0) is governed by two genes, each of them conferring complete resistance separately. In contrast, resistance to race 1 is controlled by three genes that segregate independently (h_1, h_2 and H_3) each one separately delaying wilting and any combination of two conferring complete resistance. Three genes have been described for resistance to race 2, two for race 4 and one for races 3 and 5 (see review Sharma and Muehlbauer 2007).

MAS based on the use of DNA markers linked closely to wilt resistance genes is an efficient strategy for pyramiding different resistance genes. Markers sited in linkage group 2 (LG2) of chickpea close to resistance genes for Fusarium wilt races 1, 2, 3, 4 and 5, which form a cluster, have been reported (see review Sharma and Muehlbauer 2007). In other cultivated species, disease resistance genes have also been reported to form clusters (Parniske et al. 1997; Song et al. 1997). However, in previous studies we found resistance to race 0 to be digenic (Rubio et al. 2003) and one of the two genes (*Foc0$_1$*/*foc0$_1$*), closely flanked by two markers (OPJ20$_{600}$ and TR59), mapped in a different linkage group, LG3 (Cobos et al. 2005), which corresponds to LG5 of the map devised by Winter et al. (2000). The second gene (*Foc0$_2$*/*foc0$_2$*) was not mapped, but we have considered the possibility that it could be located in LG2 because this linkage group, which was not present in the map of Cobos et al. (2005), contains clusters of other genes conferring resistance to wilt. Thus, if *Foc0$_2$*/*foc0$_2$* were mapped it might be possible to combine both genes by MAS to give durable resistance to FOC-0.

The present study aims to test the hypothesis that *Foc0$_2$*/*foc0$_2$* is located in LG2 by attempting to tag this second gene for resistance to *Fusarium* wilt race 0 using an RIL population segregating for the gene and *Foc0$_1$*/*foc0$_1$* (Rubio et al. 2003), the map developed by Cobos et al. (2005) and DNA markers (STMS: Sequence Tagged Microsatellite Sites) located on LG2 (Winter et al. 2000).

Materials and methods

Plant material

Two $F_{6:7}$ recombinant inbred lines (RIL) populations derived from intraspecific crosses CA2156 × JG62 (susceptible × resistant) and CA2139 × JG62 (both resistant) that segregate respectively for one (*Foc0$_1$*/*foc0$_1$*) and two genes (*Foc0$_1$*/*foc0$_1$* and *Foc0$_2$*/*foc0$_2$*) controlling FOC-0 resistance (Rubio et al. 2003) were used in this study. In order to identify STMS markers that tagged *Foc0$_2$*/*foc0$_2$*, 10 resistant and 10 susceptible lines were chosen from the population CA2139 × JG62, based on the data of Rubio et al. (2003), obtained after field evaluation for 2 years. This allowed STMS markers associated with resistance to be obtained quickly. Two markers flanking the gene *Foc0$_1$*/*foc0$_1$* (OPJ20$_{600}$ and TR59) (Cobos et al. 2005) were used to eliminate plants with this gene and allow selection of plants with only the second, unmapped resistance gene (*Foc0$_2$*/*foc0$_2$*). STMS markers were chosen from LG2 of different chickpea genetic maps (Winter et al. 2000; Tekeoglu et al. 2000; Abbo et al. 2005). In a second step, only STMS markers associated with disease reaction were integrated into the chickpea genetic map developed by Cobos et al. (2005). One hundred and sixty $F_{6:7}$ recombinant inbred lines (RILs) derived from both populations (CA2156 × JG62 and CA2139 × JG62) were genotyped for STMS markers allowing us to obtain a more accurate positioning of the markers.

PCR analysis

For DNA extraction, about 100 mg of young leaf tissue was harvested and immediately frozen in liquid nitrogen. DNA was isolated using the Plant DNAZOL® Reagent (invitrogen™). The PCR procedures, the primer names and sequences for STMS markers have been described by Winter et al. (1999).

Amplification conditions were as described by Winter et al. (1999): 20 µl reactions containing: 20–

40 ng DNA, buffer (Tris–HCl (pH 8.0) 10 mM, KCl 50 mM, EDTA 1 mM, Triton X-100 0.1%; glycerol 50% (*v*/*v*)), 1.5 mM $MgCl_2$, 250 μM of each dNTP, 2 μM of primer and 0.5 unit of Taq DNA polymerase (Biotools). Thirty-five cycles of PCR were performed in a DNA Thermal Cycler (Perkin Elmer Cetus 9700). The DNA was first denatured for 2 min at 94°C, annealing was at 55°C for 50 s, and elongation at 60°C (TAA repeats) or 72°C (GA and GAA repeats) for 50 s followed by a final extension at 60°C for 5 min.

PCR products of STMS were electrophoresed on 3% Metaphor agarose gel (Biowhitaker Molecular Application) in 1× TBE buffer. In all STMS that did not display well-differentiated polymorphism, their amplification products were separated on the ABI Prism Genetic Analysers A310 (Applied Biosystems) at the Central Service for Research Support (SCAI) of Cordoba University. PCR fragments labelled with fluorescence dye phosphoramidites were loaded, separated on the capillaries and detected by the system's laser. Fluorescence emissions were analysed by GeneScan software version 2.1, using internal-lane size standards (400HD ROX) and the system's local southern method for automatic size calling of peak positions. The results were interpreted with the Genotyper version 3.7 NT.

Statistical analysis

Segregation of marker loci was tested for the expected Mendelian ratio of 1:1 using χ^2 tests. Linkage analysis was performed using JOINMAP 3.0 (Van Ooijen and Voorrips 2001). A LOD score threshold of 3 and a maximum recombination fraction of 0.25 were employed as general linkage criteria to establish linkage groups. The Kosambi mapping function was used to determine cM distances between markers (Kosambi 1994).

Phenotypes of FOC-0 resistance in RIL population CA2139 × JG62 fitted the expected 3:1 (resistant to susceptible) segregation ratio for two independent genes (Rubio et al. 2003). In this case, resistance was treated as a quantitative trait (percentage of dead plants) because the allelic state for both resistance genes of the resistant lines could not be reliably determined. QTL analysis was performed using MAPQTL 5 software (Van Ooijen 2004). Interval Mapping (IM) (Lander and Botstein 1989; Van Ooijen 1992) with a mapping step size of 1 cM was applied to identify putative resistance QTL in each linkage group, considering the available data set from the 2 years' evaluation separately. The multiple-QTL models (MQM) mapping method (Jansen 1993; Jansen and Stam 1994), using the closest markers to detected QTL as cofactors, was applied. The significance of a QTL presence was determined empirically employing the permutation test of Churchill and Doerge (1994) with 1,000 replications. The coefficient of determination (R^2) for the marker most tightly linked to a QTL was used to estimate the proportion of the total phenotypic variation explained by the QTL.

Results

Analyses of both resistant and susceptible lines that segregated for the gene $Foc0_2/foc0_2$, showed a strong association with alleles of twelve STMS, chosen because they were sited on LG2 and were polymorphic between CA2139 and JG62. These results suggest that the second gene for disease reaction to wilt race 0 ($Foc0_2/foc0_2$) is linked to markers associated with LG2, confirming our hypothesis.

In order to map the $Foc0_2/foc0_2$ gene, complete RIL populations derived from two crosses (CA2156 × JG62 and CA2139 × JG62) were genotyped for the 12 STMS markers cited above. The segregation ratios of these markers fitted to the Mendelian ratio of 1:1 in both populations analysed. As expected, all of them were linked, forming a linkage group (LG2) and were integrated into the map of Cobos et al. (2005). The 12 markers on LG2 tested in this study covered a genetic distance of 38.44 cM, with an average distance of 3.49 cM between markers. The longest distance between markers was 12.38 cM and the shortest was 0.4 cM (Fig. 1). A cluster of six markers (TA200, TS47, TA59, TA27, TA96 and TA37) was found in a genomic region where Sharma and Muehlbauer (2007) reported five resistance genes to wilt (*foc-1, foc-2, foc-3, foc-4* and *foc-5*) using an intraspecific RIL population (Fig. 1).

A significant association between resistance reactions from both years and all markers in LG2 was detected using Kruskal Wallis analysis ($P<0.001$). Single interval mapping analysis, using the map of Cobos et al. (2005) plus the newly mapped LG2, permitted the identification of two QTL for resistance

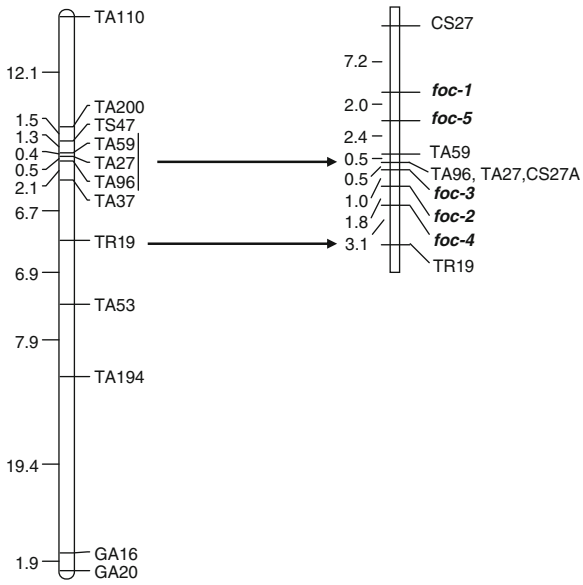

Fig. 1 Consensus linkage group 2 of the chickpea genome obtained from two RIL populations derived from crosses CA2156 × JG62 and CA2139 × JG62 (cross-hatched bar). Alignment of markers and wilt resistance genes on LG2, reported by Sharma and Muehlbauer (2007), are shown on the right (*open bar*). Map distances are in cM

to FOC-0, one in LG2 and other in LG5. The QTL in LG5 has previously been reported by Cobos et al. (2005) and TR59 was the closest marker. The QTL on LG2, closely flanked by two markers (TS47 and TA59), was located in a genomic region with a high density of STMS markers which are also linked to other wilt race-specific resistance genes (Sharma and Muehlbauer 2007). The marker TA59 was the closest to the new QTL and explained between 21.8% (1999 data) and 26.2% (2001 data) of the total phenotypic variation of resistance with maximum LOD-scores of 4.37 and 5.41, respectively. MQM analysis, employing the markers TA59 (LG2) and TR59 (LG5) as cofactors, detected two strong QTL in both years, with maximum LOD score values for LG2 and LG5 of 5.0 and 9.15 in the first year, and 6.14 and 8.52 in the second one, respectively (Fig. 2).

Discussion

Resistance to race 0 of FOC in chickpea is controlled by two genes that segregate independently; one of these genes was found to be present in the accession JG62 ($Foc0_1/foc0_1$) and the other in CA2139 ($Foc0_2/foc0_2$) (Rubio et al. 2003). $Foc0_1/foc0_1$ was located in LG5 of the chickpea map, closely flanked by RAPD and STMS markers ($OPJ20_{600}$ and TR59 respectively) (Cobos et al. 2005). The genotypic information provided by both markers ($OPJ20_{600}$ and TR59) has been useful in this study for the elimination of lines containing $Foc0_1/foc0_1$ and the selection of lines in which only the second resistance gene, ($Foc0_2/foc0_2$) derived from CA2139, was present. It was then possible to find associations between this gene and STMS markers sited on LG2.

In general, the order of STMS markers on LG2 in this study was similar to those of chickpea maps

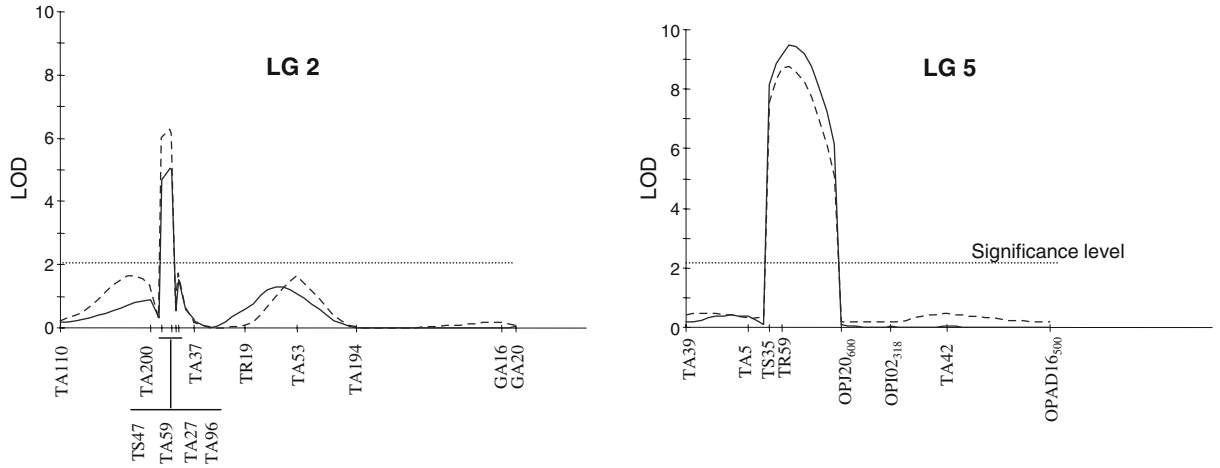

Fig. 2 QTL for Fusarium wilt race 0 resistance located on LGs 2 and 5 of the chickpea genome obtained in the RIL population CA2139 × JG62 evaluated during the years 1999 (*continuous line*) and 2001 (*dotted line*)

reported by other authors (Winter et al. 2000; Tekeoglu et al. 2000; Udupa and Baum 2003; Sharma et al. 2004; Cho et al. 2004; Abbo et al. 2005, Iruela et al. 2007) despite a few small discrepancies for some markers sometimes included in a narrow window. Different populations (inter- or intra-specific), numbers of markers studied, software employed and /or population size also may explain these few discrepancies. Good alignment of our sequence was obtained with that of LG2 reported by Sharma and Muehlbauer (2007), who mapped a cluster of five resistance genes to wilt (*foc-1, foc-2, foc-3, foc-4* and *foc-5*) in this linkage group using an intraspecific RIL population.

The QTL in LG5 located between the markers $OPJ20_{600}$ and TR59 has already been detected by Cobos et al. (2005). They found that this locus was coincident with the position of $Foc0_1/foc0_1$ in the RIL population CA2156 × JG62. The new QTL in LG2 found in the RIL population CA2139 × JG62 reported in this paper could be considered a second gene, $Foc02/foc0_2$ as proposed by Rubio et al. (2003). It should now be mapped more accurately in a population segregating only for this gene. Our results confirm our hypothesis about the possible location of the locus $Foc0_2/foc0_2$ in LG2 of chickpea where other genes for resistance to races 1, 2, 3, 4, and 5 of the pathogen have been reported (Ratnaparkhe et al. 1998a, b; Tullu et al. 1998; Tekeoglu et al. 2000; Winter et al. 2000; Sharma et al. 2004, 2005; Sharma and Muehlbauer 2007). Furthermore, QTL or genes for resistance to Ascochyta blight of chickpea and several potential resistance and pathogenesis-related genes have also been detected in LG2 (Hüettel et al. 2002; Pfaff and Kahl 2003; Udupa and Baum 2003; Cho et al. 2004; Cobos et al. 2006; Iruela et al. 2007). Therefore, this LG may be considered a hot spot for pathogen defence in chickpea (Millan et al. 2006) in line with the clusters of disease resistance genes reported in other cultivated species (Parniske et al. 1997; Song et al. 1997).

In conclusion, the locus $Foc0_2/foc0_2$ was located in a narrow genomic window (<5 cM) that is highly saturated with tightly interlinked STMS markers. These markers could be used in MAS and together with the markers linked to $Foc0_1/foc0_1$ in LG5 (Cobos et al. 2005) would allow us to pyramid both genes for resistance to race 0 in a single individual in order to provide durable resistance against race 0 of FOC. In some cases, markers closely linked to a gene or QTL are not polymorphic in other segregating populations. For resistance to race 0 of FOC the high number of markers available would be likely to allow us to solve this problem. The linkage relation of $Foc0_2/foc0_2$ with loci for resistance to race 1, 2, 3, 4 and 5 in the same LG should be studied.

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