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FULL RESEARCH PAPER

Validation of a QTL for resistance to ascochyta blight linked to resistance to fusarium wilt race 5 in chickpea (*Cicer arietinum* L.)

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Abstract Ascochyta blight caused by *Ascochyta rabiei* and fusarium wilt caused by *Fusarium oxysporum*. f. sp. *ciceris* are the two most serious diseases of chickpea (*Cicer arietinum*). Quantitative trait loci (QTL) or genes for ascochyta blight resistance and a cluster of resistance genes for several fusarium wilt races (*foc1*, *foc3*, *foc4* and *foc5*) located on LG2 of the chickpea map have been reported independently. In order to validate these results and study the linkage relationship between the loci that confer resistance to blight and wilt, an intraspecific chickpea recombinant inbred lines (RIL) population that segregates for resistance to both diseases was studied. A new LG2 was established using sequence tagged microsatellite sites (STMS) markers selected from other chickpea maps. Resistance to race 5 of *F. oxysporum* (*foc5*) was inherited as a single gene and mapped to LG2, flanked by the STMS markers TA110 (6.5 cM apart) and TA59 (8.9 cM apart). A QTL for resistance to ascochyta blight (QTL_{AR3}) was also detected on LG2 using evaluation data obtained separately in two cropping seasons. This genomic region, where QTL_{AR3} is located, was highly saturated with STMS markers. STMS TA194 appeared tightly linked to QTL_{AR3} and was flanked by the STMS markers TR58 and TS82 (6.5 cM apart). The genetic distance between *foc5* and QTL_{AR3} peak was around 24 cM including six markers within this interval. The markers linked to both loci could facilitate the pyramiding of resistance genes for both diseases through MAS.

Keywords *Cicer arietinum* · *Ascochyta rabiei* · *Fusarium oxysporum* · Molecular markers · Linkage analysis

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Introduction

Chickpea (*Cicer arietinum*) is an autogamous annual cool-season grain legume cultivated in arid and semi-arid areas across the six continents. It is valued for its high protein content and the absence of specific major anti-nutritional factors means that it is considered nutritional and healthy (Williams and Singh 1987; Gil et al. 1996). It is mostly used for human consumption and to a lesser extent for animal feed. Chickpea yield is

low and unstable with a global average below 0.8 t ha^{-1} (FAOSTAT 2005). Two fungal diseases, ascochyta blight (caused by *Ascochyta rabiei*; syn. *Phoma rabiei*) and fusarium wilt (caused by *Fusarium oxysporum* f. sp. *ciceris*), are important limiting factors for yield worldwide.

Ascochyta blight is the most destructive disease affecting chickpea in many farming regions of the world. *Ascochyta rabiei* can attack at any growth stage and affect all aerial parts of the plant, producing lesions with concentric rings of pycnidia and stem breakage due to girdling. Sources of resistance to ascochyta blight have been identified from *C. arietinum* and wild *Cicer* species (Singh and Reddy 1993; Collard et al. 2001; Chen et al. 2004). This resistance, available in cultivated chickpea, has been exploited in conventional breeding programmes, producing new resistant cultivars worldwide. Knowledge of the genetic bases of both virulence in *A. rabiei* and resistance in chickpea is essential in order to develop cultivars with more durable resistance. To date, the pathogen has been classified mainly into two broad pathotypes: pathotype I (less aggressive) and pathotype II (aggressive) (Chen et al. 2004); but further research is required to identify the genes that control aggressiveness. As for the host, early studies on the inheritance of blight resistance indicated that it could be conferred by one, two or three genes (Singh and Reddy 1983; Tewari and Pandey 1986; Dey and Singh 1993; Tekeoglu et al. 2000). Furthermore, evidence that resistance might be inherited as a quantitative trait has been reported (Muehlbauer and Kaiser, 1994). Resistance to blight is considered partial or incomplete in chickpea. Climatic conditions, inoculum density, pathotype variation and plant age all affect disease development. Thus, the use of RIL populations (homozygous lines) provides a more accurate evaluation of the disease than F_2 populations. Furthermore, RILs can be evaluated for reaction to different pathotypes and under differing environmental conditions. The use of RIL populations and molecular markers has contributed a great deal to the identification of quantitative trait loci (QTL) for resistance to ascochyta blight. Several QTL have been located on different maps developed by various authors and the STMS markers linked to these QTL have helped to assign them to linkage groups relating to the most extensive chickpea map (Winter et al. 2000). Two major QTL (QTL-1 and QTL-2) that confer resistance have been located on linkage group 4 (LG4) by different authors (Santra et al. 2000; Tekeoglu et al. 2002; Collard et al. 2003; Flandez-Galvez et al. 2003; Millán et al. 2003; Rakshit et al. 2003; Udupa and Baum 2003; Cho et al. 2004; Iruela et al. 2006). We suggest labelling them QTL_{AR1} and QTL_{AR2} (Iruela et al., 2006). These two QTL seem to confer resistance to pathotype II of *A. rabiei* according to the results of Udupa and Baum (2003) and Cho et al. (2004). QTL_{AR2} has been located in a genomic region with a high density of markers (Iruela et al. 2006) whereas QTL_{AR1} appeared in a loose genomic region. Other genes or QTL for resistance to blight have been reported on LG2 in a poorly saturated region (Udupa and Baum 2003; Cho et al. 2004; Cobos et al. 2006) and seem to be more associated with pathotype I of *A. rabiei* (Udupa and Baum 2003; Cho et al. 2004).

Fusarium wilt is another serious disease that affects chickpea, decreasing production in many countries. Eight pathogenic races (races 0, 1A, 1B/C, 2, 3, 4, 5 and 6) have been reported. In susceptible chickpea cultivars, races 1A, 2, 3, 4, 5 and 6 induce the wilting syndrome, whereas races 0 and 1B/C induce the yellowing syndrome. Races 0, 1A, 1B/C, 5 and 6 are found mainly in the Mediterranean region and California (see review by Jiménez-Gasco et al. 2004). In Spain, race 5 is the most virulent (Landa et al. 2004) and along with race 6 is the second most common after race 0 (Jimenez-Diaz et al. 1989). Breeding programmes have been developed using resistant desi cultivars but the pathogenic variability of the fungus is an added difficulty. Studies using inter and intraspecific populations of chickpea and random amplified polymorphic DNA (RAPD), inter simple sequence repeats (ISSR), sequence characterised amplified regions (SCAR) and sequence tagged microsatellite sites (STMS) markers indicated that resistance genes for fusarium wilt races 1, 3, 4 and 5 (*foc1*, *foc3*, *foc4* and *foc5*) are located on LG2, forming a cluster (Mayer et al. 1997; Ratnaparkhe et al. 1998a; Tullu et al. 1998; Winter et al. 2000; Sharma et al.

2004). Recently, one of the two genes that confers resistance to race 0 (Rubio et al. 2003) was mapped on LG5 (Cobos et al. 2005). However, the second gene for race 0 has been located on LG2 (unpublished data).

LG2 of the chickpea map is interesting because it contains resistance genes for fusarium wilt and QTL for ascochyta blight resistance, the two most important diseases worldwide. From the point of view of breeding, it is very important to know the linkage relationship (distance) between the QTL for resistance to blight and the resistance genes for fusarium wilt. This information could help to apply marker-assisted selection (MAS) for these two traits simultaneously, requiring a high number of tightly-linked markers flanking the QTL or genes. This study focused on a chickpea RIL population segregating for both diseases and mapped markers located on LG2, which enabled the linkage between the two diseases to be examined.

Materials and methods

Ascochyta blight and fusarium wilt resistance evaluations

A chickpea RIL population of 111 $F_{6:7}$ individuals derived from the intraspecific cross ILC3279 × WR315 was used. ILC3279 is a kabuli line from the former Soviet Union (maintained by the International Centre for Agricultural Research in the Dry Areas (ICARDA), Aleppo, Syria), which is resistant to ascochyta blight and susceptible to wilt. WR315 is a desi landrace from central India (maintained by the International Crop Research Institute for the Semi-Arid Tropics (ICRISAT); Patancheru, India), which is resistant to all races of fusarium wilt and susceptible to blight. The single seed descent method was employed for RIL population development.

Ascochyta blight resistance reaction of RILs and parents were scored in field trials in 2002 and 2003 (Iruela et al. 2006). The RIL population was also evaluated for wilt reaction under controlled conditions in a growth chamber. Colonised filter paper cultures of *F. oxysporum* f. sp. *ciceris* race 5 (kindly provided by Dr. Muehlbauer, Washington State University, Pullman, USA) were cultured in potato-dextrose broth (24 g l^{-1}) at 25°C with light for one week to produce liquid cultures of the pathogen. The liquid cultures were filtered through cheesecloth to remove mycelia. The spore suspension was then pelleted by centrifugation at low speed (3000 rpm) for 3 min. After the supernatant was discarded, the conidia were diluted with sterile water to obtain a concentration of 10^6 spores ml^{-1}. Parents and RILs seedlings at the three to four nodal stages were inoculated following the method described by Bhatti et al. (1990). The inoculated plants were grown in perlite in a growth room with a temperature regime of 25 and 22°C (12 h/12 h) under fluorescent light. The plants were watered daily and supplied with nutrient solution once a week after inoculation. Fusarium wilt incidence, scored as % of dead plants, was recorded 4 weeks after inoculation. RILs with 0–30% dead plants were considered resistant and RILs with 70–100% dead plants were considered susceptible.

Construction of molecular map and QTL analysis

The RIL population was genotyped for 10 STMS markers (GA16, TA37, TA53, TA59, TA103, TA110, TA194, TR19, TR58, TS82) and the SCAR marker CS27 selected from LG2 of both interspecific and intraspecific chickpea maps (Winter et al. 2000; Tekeoglu et al. 2002; Udupa and Baum 2003).

For DNA extraction, about 100 mg of young leaf tissue was excised, frozen immediately in liquid nitrogen and stored at –80° C. DNA was isolated using DNAZOL (Invitrogen). The STMS primer sequences and amplification conditions employed were described by Winter et al. (1999). The SCAR CS27, developed from the RAPD $CS27_{700}$ by Mayer et al. (1997), was analysed according to the protocol defined by these authors. Amplification products from STMS except TA37 were electrophoresed in 2.5% Metaphor agarose (Biowhitaker Molecular Application) gels. TA37 was analysed in 10% polyacrylamide gels and the SCAR CS27 in gels composed of a mixture of 1% SeaKem agarose

and 1% NuSieve agarose (Hispanlab SA). PCR fragments were stained with ethidium bromide.

Goodness of fit to the expected 1:1 segregation ratio of marker loci was tested using the χ^2 test. Linkage analysis was performed using JOINMAP 3.0 (Van Ooijen and Voorrips 2001) with a minimum LOD score of 3 and a maximum recombination fraction of 0.25. Kosambi's function was applied to estimate map distances in centiMorgans. MAPQTL 5 software (Van Ooijen, 2004) was employed to locate putative QTL for ascochyta blight resistance considering the disease evaluation data from the two cropping seasons. The interval mapping (IM) method with a mapping step size of 1 cM was applied, determining the significance thresholds for the LOD score through the permutation test (number of iterations = 1000, $P = 0.05$) (Churchill and Doerge 1994). The coefficient of determination (R^2) of the marker most closely linked to a QTL was used to estimate the percentage of the total phenotypic variation explained by the QTL.

Results

RIL population tested for reaction to wilt race 5 resulted in 50 resistant and 56 susceptible plants. This data fitted a 1:1 segregation ratio suggesting that a single gene controlled resistance to fusarium wilt race 5 (*foc5*) in this population. The resistant parental line (WR315) did not display symptoms of wilt and the susceptible parental line (ILC3279) had 100% dead plants.

The 10 STMS and the SCAR CS27, selected from previous chickpea maps because of their presence on LG2, revealed polymorphism between the parental lines and fitted the expected 1:1 ratio well when they were used to genotype the whole RIL population. As expected, all analysed markers and the locus *foc5* formed a single linkage group (LG2) covering a genetic distance of 62 cM and showing a maximum and minimum distance between markers of 14.1 and 1.3 cM, respectively (Fig. 1). The resistance gene *foc5* was flanked by the STMS markers TA110 (6.5 cM apart) and TA59 (8.9 cM apart). The SCAR CS27 was located 12.3 cM from this resistance gene. The utilisation of locus-specific STMS markers meant that the LG2 obtained could be aligned with other LG2 previously reported in different populations. The order of the STMS markers on LG2 was identical to that found by Udupa and Baum (2003) and Tekeoglu et al. (2002), employing RIL populations derived from intra and interspecific crosses, respectively. Though the order of the markers was the same as that found by Tekeoglu et al. (2002), genetic distances between the STMS TA194 and TA53 were considerably different. TA53 was 4.8 cM compared to 80.8 cM apart in the LG2 reported by Tekeoglu et al. (2002). Difference in the order of the markers was observed when compared to the interspecific *Cicer* map of Winter et al. (2000). However, marker TA194 was tightly linked to TR58 and TS82 markers in both studies.

The AUDPC data obtained from the evaluations for ascochyta blight in each cropping season (Iruela et al. 2006) were considered separately. This disease reaction data was tested for associations with single markers contained on LG2. Five of them (GA16, TS82, TA194, TR58 and TA53), covering a map distance of 21.3 cM, were found to be significantly associated ($P < 0.001$) with resistance in 2002; in 2003, on the other hand, only one marker (TA194) was found to be associated. Interval mapping located a QTL for blight resistance (suggested name QTL_{AR3}) on this LG2 in both years (Fig. 2). This QTL had a maximum LOD value of 5.9 in 2002 and 2.5 in 2003 and significance level of 1.8 in both years. QTL_{AR3} explained 22.6% and 11.3% of the total phenotypic variation of blight reaction using 2002 and 2003 evaluation data, respectively. In both years, the QTL peak coincided with the position of STMS TA194, which was flanked by the STMS TR58 and TS82 (6.5 cM apart). The distance between TA194 and *foc5* was around 24 cM.

Discussion

Microsatellite-based markers, such as STMS, have shown a significant degree of polymorphism in spite of the monotony of the chickpea genome, previously reported using isozymes, RFLP,

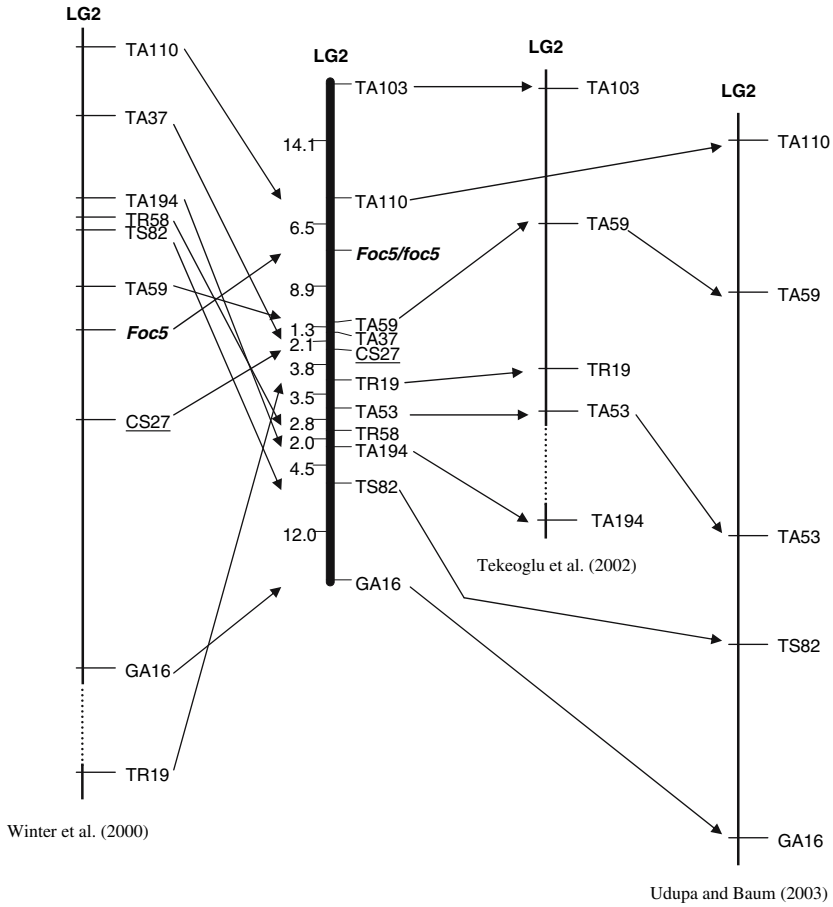

Fig. 1 Linkage group obtained in the chickpea RIL population ILC3279 × WR315 (black bar) and its alignment through common markers included in LG2 of Winter et al. (2000), Tekeoglu et al. (2002) and Udupa and Baum (2003). Map distances are in cM. Fusarium wilt race 5 resistant gene is in *bold*, the SCAR marker is *underlined* and the remainder of the markers are STMS

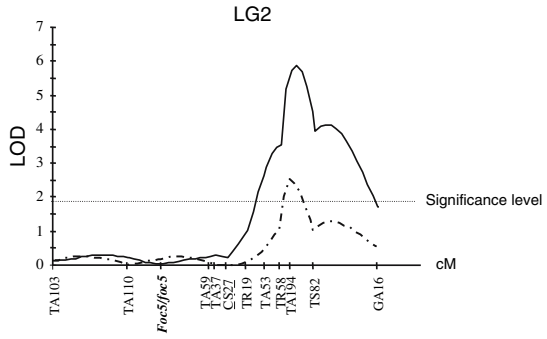

Fig. 2 QTL for ascochyta blight resistance obtained in the chickpea RIL population ILC3279 × WR315 evaluated in 2002 (—) and 2003 (–·–·). Fusarium wilt race 5 resistant gene is in *bold*, the SCAR marker is *underlined* and the remainder of the markers are STMS

RAPD, DAF and AFLP (see review by Winter et al. 2003). All STMS markers chosen from interspecific and intraspecific maps (Winter et al. 2000; Tekeoglu et al. 2002; Udupa and Baum 2003) were polymorphic, thus validating the potential of STMS in MAS. Differences in the order of markers compared with the linkage group of Winter et al. (2000) and a greater genetic distance between TA194 and TA53 in Tekeoglu et al. (2000) were observed. These may be due to the different origin of the RIL population used, which were derived from interspecific crosses in the case of Winter et al. (2000) and Tekeoglu et al. (2002), and intraspecific in this study. Furthermore, different software packages based on different procedures could affect the order or distance between markers. Population size is another factor to take into account; large populations would give a more accurate order of the markers. Consensus maps using different chickpea mapping populations that segregate for common markers across the populations, as reported in other crops (Doligez et al. 2006; Song

et al. 2004), could be one way of obtaining a more accurate chickpea map (Tekeoglu et al. 2002).

Resistance to race 5 of *F. oxysporum* was monogenic and was mapped to LG2, considering common STMS markers related to the reference chickpea genetic map of Winter et al. (2000). This result confirms previous reports of the monogenic nature of resistance to race 5 in WR315 (Sharma et al. 2005). Using another source of resistance, ICC4958, Tekeoglu et al. (2000) also demonstrated monogenic inheritance to race 5. This gene for resistance to race 5 present in ICC4958 was also located on LG2, linked to genes for resistance to races 1, 3 and 4 (Ratnaparkhe et al. 1998a, b; Tekeoglu et al. 2000; Winter et al. 2000). Genes for resistance to races 1, 3 and 4 present in WR315 have been also mapped to LG2 (indicative marker CS27) and could be considered to be the same as the one present in ICC4958 (Mayer et al. 1997; Tullu et al. 1998; Sharma et al. 2004). The gene conferring resistance to race 5 present in WR315 could be also considered the same as the one in ICC4958.

In addition to fusarium wilt resistance genes reported on LG2, genes or QTL associated with resistance to ascochyta blight were also found on this LG (Udupa and Baum 2003; Cho et al. 2004; Cobos et al. 2006). A major locus and a tightly linked QTL, which confer resistance to pathotype I and II respectively, were identified by Udupa and Baum (2003), who used the same resistant source employed in this study (ILC3279). Cho et al. (2004), using a different resistant parental line (FILP84-92C), also reported a major gene (Ar19) for resistance to pathotype I on LG2. In both studies, the genes or QTL were located in a poorly saturated genomic region and the closest marker was the STMS GA16 (around 20 cM apart). More recently, using an interspecific RIL population, another QTL for resistance to blight was located on LG2, flanked by a RAPD and a ISSR markers (14.1 cM apart) and the STMS TA103 was over 20 cM away from the peak of the QTL (Cobos et al. 2006). In this case, the resistance source was ILC72. Both markers flanked the QTL reported by Cobos et al. (2006) were monomorphic in the intraspecific population used in this study. There would need to be a higher density of markers around the genes in question in order to know whether these genes or QTL are or not the same as those present in different parental lines, and also in order to use MAS for resistance. This study detected a QTL far away from STMS TA103 (>30 cM) and around 20 cM from GA16. This QTL might be the same as that reported by Cobos et al. (2006) (indicative marker TA103), and possibly the same as those reported by Udupa and Baum (2003) and Cho et al. (2004) (indicative marker GA16). STMS TA194, which was tightly linked to the QTL, was not present in the maps reported by the aforementioned authors. However, STMS markers flanking the QTL such as TA53 and TS82 were present in the map defined by Udupa and Baum (2003), but located more than 25 cM away from the gene for pathotype I (ar1) or the QTL for pathotype II (ar2a) of ascochyta blight. The latter were closer to GA16, located midway between TS82 and ar1 or ar2a.

As mentioned previously, the order of markers in a linkage group can be affected by different factors. Furthermore, experimental error in the disease score might have contributed to a different order. Udupa and Baum (2003) phenotyped the RILs for pathotype I on the basis of a bimodal rather than continuous distribution, where the score of the RILs with intermediate reactions might contribute to the experimental error. Cho et al. (2004) reported a major gene (Ar19) for pathotype I on LG2+6, mapped between TR19 and GA16. They suggested that Ar19 appeared to provide most of the quantitative resistance to pathotype I and, to a lesser extent, resistance to pathotype II. In a previous study, using the same RIL population as in the current study as well as the same scoring data obtained during 2002 and 2003, two strong QTL (QTL_{AR1} and QTL_{AR2}) located on LG4 were reported in the second year only, suggesting that different pathotypes might be present in each of the evaluated years (Iruela et al. 2006). The QTL obtained on LG2 was more important in the first year and had only a slight presence in the second year. These results suggest that QTL_{AR3} on LG2 could be the same as the QTL or genes for resistance to pathotype I of *A. rabiei* proposed by the cited authors. More work needs to be done to saturate the genomic region of LG2 where these genes or QTL have

been detected in order to get a more accurate validation. TA194 could be a good reference marker for verification.

In conclusion, this study has confirmed that the loci responsible for the two most economically important diseases of chickpea appear as a cluster on LG2. Complex clusters of disease resistance genes are common in plant genomes . Examples of *R* genes that are present in clusters include *Rp1, Rpp5, Xa21, Pto, Dm3, I2, N*, *M* and the *Cf* genes (Takken et al. 2000). In *Arabidopsis*, 109 of the 149 NB-LRR genes reside in 40 clusters ranging in size from two to eight genes, while the remaining 40 genes exist as singletons (Meyers et al. 2003). These clusters can span large chromosome segments and confer resistance to different races of the same pathogen as well as to different pathogens. For example, a common bean map revealed numerous resistance gene clusters, including the co-location of genes for resistance to two fungal diseases, anthracnose and rust (Miklas et al. 2006). Resistance genes to powdery mildew *(Rmd*-c), Phytophthora stem and root rot *(Rps*2), and an ineffective nodulation gene *(Rj*2) have been mapped within a cluster on linkage group J in soybean (Polzin et al. 1994). From the point of view of chickpea breeding, the genetic distance (around 24 cM) between both loci (*foc5* and TA194) do not appear to pose a problem for pyramiding resistance to fusarium wilt race 5 and the QTL_{AR3} for ascochyta blight. The closely linked STMS markers to both loci could be used, via MAS, to achieve these objectives.

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