

Molecular mapping of QTLs for resistance to early and late Fusarium wilt in chickpea

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Abstract

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A molecular map of chickpea was constructed using F₉:F₁₀ recombinant inbred lines from an intraspecific cross between Fusarium wilt susceptible (JG 62) and resistant (WR 315) genotypes. A total of 23 markers with LOD scores of > 3.0 were mapped on the recombinant inbred lines (RILs). Twenty sequence tagged microsatellites (STMS) and three amplified fragment length polymorphisms (AFLPs) covered 300.2 cM in five linkage groups at an average inter-marker distance of 13 cM. Early and late wilting due to Fusarium infection was recorded in RILs at 30 and 60 DAS, respectively. There was a significant variation among RILs for wilt resistance for both early and late wilting. QTLs associated with early (30 days after sowing (DAS)) and late (60 DAS) wilting are located on LG II. The flanking markers for these QTLs were the same as those of previous reports. Five STMS markers located on LG II of reference map (interspecific) were mapped on LG II of the present map (intraspecific) with minor changes in the order of markers indicating the conservation of these genomic regions across the *Cicer* species.

Keywords: chickpea; Fusarium wilt; QTL; STMS

Chickpea (*Cicer arietinum* L.) is the third most important food legume crop worldwide. Globally, it is cultivated in more than 57 countries and ranks the second in acreage after dry bean. However, it stands third in production after dry bean and pea with the productivity of about 913 kg/ha (FAO 2012). Nearly 66% of the world acreage and 67% of the global chickpea production is from India alone. However, productivity of chickpea in India is low (872 kg/ha). One of the main constraints to realize higher yield in the Indian subcontinent is the vascular disease Fusarium wilt caused by *Fusarium oxysporum* f.sp. *ciceris* (Padwick) Matauo & K. Sato. Annual yield loss from this disease, on average has been estimated to range from 10 to 15% (JALALI & CHAND 1992), but it is capable of causing 100% loss under favourable conditions. Among the eight races of the pathogen reported, race 1 is widespread in India. Since it is a soil-borne pathogen, its management by agronomic and plant protection measures is

difficult. Cultivation of varieties possessing resistance to specific races of the pathogen prevalent in a region is the most economical management strategy for this disease (ARVAYO-ORTIZ *et al.* 2012).

The development of wilt resistant varieties through traditional plant breeding methods requires about 6–7 generations with laborious disease screening and selection. Hence, methods which could accelerate the development of resistant variety are desirable. One such technology, the marker-assisted selection (MAS) could become an efficient strategy to accelerate wilt resistance breeding in chickpea. Chickpea has $2n = 2x = 16$ chromosomes with a relatively small genome size of 738.09 Mb (VARSHNEY *et al.* 2013). MAYER *et al.* (1997) was the first to report allele and locus specific molecular markers linked to Fusarium wilt in chickpea. Later several studies on inter- and intraspecific recombinant inbred line (RIL) populations revealed that the resistance genes (*foc1* and

foc3, *foc4* and *foc5*) against Fusarium wilt races 1, 3, 4 and 5 are organized in two adjacent resistance gene clusters on Linkage Group II (LG II). These two gene clusters are flanked by sequence-tagged microsatellite (STMS) markers GA16 and TA96 (*foc1–foc4* cluster) and TA96 and TA27 (*foc3–foc5* cluster), respectively (WINTER *et al.* 2000; SHARMA & MUEHLBAUER 2007; COBOS *et al.* 2009; GOWDA *et al.* 2009; HALILA *et al.* 2009). HALILA *et al.* (2009) located *foc 0₂/foc 0₂* in a region where genes for resistance to wilt races 1, 2, 3, 4 and 5 have previously been reported using data from two mapping populations (CA2139/JG62 and CA2156/JG62). In recent studies (GURJAR *et al.* 2012; SOREGAON & RAVIKUMAR 2012), few other minor genes were implicated in chickpea defence against Fusarium wilt.

GOWDA *et al.* (2009) identified new SSR markers H₃A₁₂ and TA110 flanking the *foc1* locus at 3.9 and 2.1 cM, respectively, while *foc 2* was tagged with SSR markers TA96 and H₃A₁₂ at a distance of 0.2 and 2.7 cM, respectively. Similarly, H₁B06y and TA194 markers flanked the *foc 3* locus at 0.2 and 0.7 cM, respectively. Thus the majority of the studies targeted to map genes specific to particular race/s. Resistance to race 1 (the most prevalent race in India) is governed by duplicate recessive genes (BAYRAKTAR & DOLAR 2012). It causes wilting either at seedling stage (early wilting) or after flowering stage (late wilting) depending upon the allelic constitution at the two duplicate loci. Dominant allele at both the loci (*H₁H₁H₂H₂*) gives early wilting; the recessive allele in homozygous form at either of these two loci will give late wilting (*H₁H₁h₂h₂/h₁h₁H₂H₂*) and recessive alleles at both the loci (*h₁h₁h₂h₂*) confer complete resistance. Cultivars such as JG 62, which carry neither of the recessive alleles, show early wilting, while WR 315 carrying recessive alleles at both the loci (*h₁h₁h₂h₂*) is completely resistant to race 1. However, RILs derived from these two genotypes showed a lot of variation in the timing of wilting indicating the influence of QTLs with a minor effect on major resistance genes (BRINDA & RAVIKUMAR 2005). Hence, in the present study F₉:F₁₀ RIL population of JG 62 × WR 315 was used to map the QTLs associated with early and late wilt resistance.

MATERIAL AND METHODS

DNA extraction, PCR amplification and electrophoresis. The mapping population was derived from the JG62 × WR315 cross. Ninety-four RILs out of total one hundred and twenty-five F₉:F₁₀ RILs

were randomly chosen to construct a molecular map and locate the QTLs associated with Fusarium wilt resistance. JG62 is an early wilting genotype highly susceptible to Foc races 1 to 5 (SHARMA & MUEHLBAUER 2007), while WR315 is resistant to them. During the year 2010 plant materials were propagated in the glasshouse at UAS (Dharwad) and genomic DNA was isolated from young leaves as per the procedure of DOYLE and DOYLE (1990). One hundred and ten chickpea STMS markers and 48 amplified fragment length polymorphism (AFLP) markers were used to screen parents and to identify polymorphic markers. Polymorphic markers were used for genotyping the RIL population.

STMS marker. One hundred and ten chickpea sequence tagged microsatellites (STMS) markers (LICHTENZVEIG *et al.* 2005; WINTER *et al.* 2000) were used to screen parents and to identify polymorphic markers. Each primer pair was screened for the ability to amplify a clear unilocus amplicon in both the parental DNAs of chickpea. A 20 µl PCR mix was used which consisted of 50–100 ng of genomic DNA template, 1× PCR reaction buffer, 2.5 mM MgCl₂, 0.2 mM of each dNTP, 10 pmol of each primer and 1 unit of Taq DNA polymerase. The amplification reactions were carried out in a PTC200 thermocycler (MJ Research, Inc., Waltham, USA). The thermocycling conditions (with minor variations) were: 2 min initial denaturation step followed by 35 cycles of 94°C for 30 s each, marker-specific annealing temperature for 30 s and 72°C for 45 s, then a final extension step of 5 min at 72°C. The parental polymorphism was detected by separating the PCR product directly on 2% agarose gel and visualizing under UV-light after staining with Sybre Safe (Life Technologies, Grand Island, USA). Different methods were used to genotype RILs (F₁₀ population) depending on the type of polymorphism identified. Amplicons with distinct length (> 10 bp) polymorphisms were separated directly on 2% agarose gel. The amplicons showing a small difference in the amplicon size (< 10 bp) were amplified by Multiplexed-Ready PCR using fluorescently labelled primers. The PCR products were resolved on an AB3730 capillary sequencer (AB Applied Biosystems, Carlsbad, USA), using GeneScan™-500 LIZ® size standard (AB). Genotyping was done using the AB GeneMapper programme.

AFLP marker. amplified fragment length polymorphism (AFLP) markers were generated as described by Vos *et al.* (1995), using one selective nucleotide for pre-amplification (*EcoRI*-C and *MseI*-A) and two selective nucleotides for selective amplifica-

tion. The AFLP products were resolved with AB3730 DNA sequencer as described for STMS marker above.

Phenotyping. Phenotyping of RIL population along with the parents against race 1 of *Fusarium* was carried out on a wilt sick plot at ICRISAT, Patancheru, Hyderabad. The RILs were evaluated using a randomized complete block design. Each RIL was sown in single rows of two-meter length with two replications during the post-rainy season 2009–2010. Since the resistance and susceptibility of the parents to *Fusarium* wilt were already established, the parents themselves were repeated after every twenty rows in each replication. The *Fusarium* wilt scores were recorded at 30 and 60 days after sowing (DAS). The wilt incidence was measured on the basis of the number of wilted plants to the total number of plants in each row and expressed as percent wilt incidence. The average of wilt incidence was calculated based on values from both the replications.

Map construction and QTL mapping. Twenty-eight STMS and five AFLP markers were found polymorphic. These polymorphic primers were used to screen the 94 $F_9:F_{10}$ RILs. Chi-square analysis ($P < 0.05$) was applied to test the segregation of the markers against the expected Mendelian segregation ratio of 1:1 for RILs. The linkage analysis was performed using MAPMANAGER programme (MANLY *et al.* 2001). A minimum LOD score of 3.0 was set as the threshold value for linkage group determination. Recombination fractions were converted into map distances in centimorgans (cM) using the Kosambi mapping function. These inter-marker distances were used to construct the linkage map by MAPCHART version 2.2 (VOORRIPS 2002).

Mean phenotypic data along with genotypic data and linkage map were used to identify the association of QTLs using MAPMANAGER QTX (MANLY

et al. 2001). The replication means of 94 RILs for wilt reaction were used for QTL mapping. QTL analysis was performed using simple interval mapping (HALEY & KNOTT 1992).

RESULT AND DISCUSSION

In the present study, one hundred and twenty-five RILs of chickpea derived from the cross between JG 62 (wilt susceptible both 30 DAS and 60 DAS) and WR 315 (early and late wilt resistant) were phenotyped for wilt reaction. The number of plants showing wilt symptoms in each line was recorded in percentage on 30th day for early wilting and 60th day for late wilting. The percent wilting at both the stages of crop growth is presented in Table 1 and Figure 1. WR 315 showed less than 10% wilt incidence (1.43% and 9.91% at 30 and 60 DAS, respectively) whereas JG 62 had more than 85% wilting (86.67% and 98.34% at 30 and 60 DAS, respectively); reconfirming their wilt resistance and susceptibility, respectively. The RILs showed bimodal distribution with respect to percent wilt incidence, indicating two genes govern the trait. At an early stage (30 DAS) less than 50% wilt incidence was noted in the majority of the RILs, whereas in the second stage (60 DAS) more than 50% of them showed wilt incidence (data not shown). This was expected due to the genotypic constitution of the parents (JG 62 ($H_1H_1H_2H_2$); WR 315 ($h_1h_1h_2h_2$)) involved. The genotyping of parental cultivars (JG 62 and WR

Table 1. Percent wilt incidence in the parents at 30 and 60 days after sowing (DAS)

	30	60
WR 315	1.43 ± 0.82	9.91 ± 0.10
JG 62	86.67 ± 5.77	98.34 ± 0.96

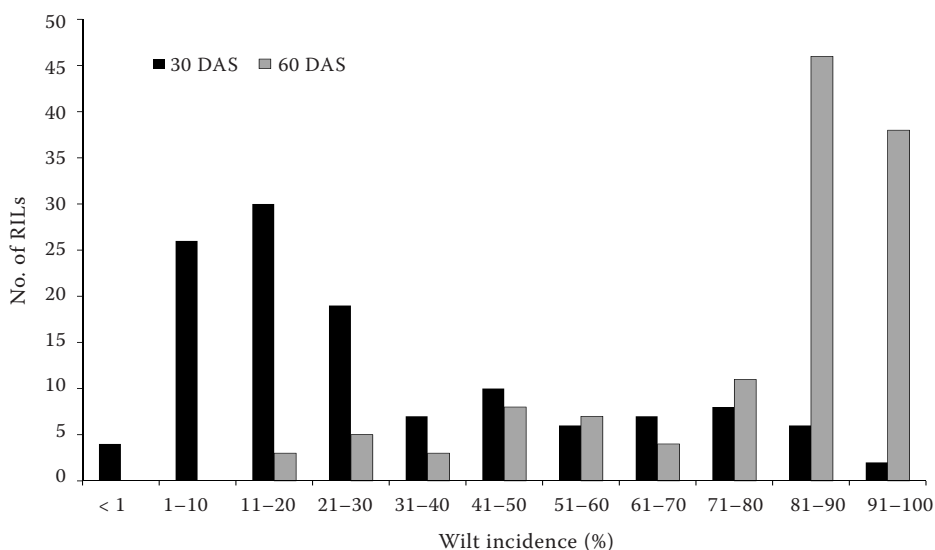


Figure 1. Frequency distribution of disease scores for *Fusarium* wilt under field conditions at 30 and 60 DAS. DAS – days after sowing; RILs – recombinant inbred lines

315) was done with the help of 110 STMS markers and 48 AFLP markers. Out of these, 98 markers produced scorable bands, while only 28 STMS and 5 AFLP markers were polymorphic. These polymorphic markers were used for generating the marker genotyping data on the population. The segregation ratio of individual markers in the RILs was tested using χ^2 test. Six out of thirty-three markers showed segregation distortion of which four were AFLP markers. Twenty STMS and three AFLP markers were mapped into five linkage groups (LG) that spanned a total length of 300.2 cM with an average marker density of 13 cM while eight STMS markers and two AFLP markers were unlinked. The length of LG ranged from 1.6 cM to 129.2 cM. The number of markers mapped per linkage group varied from two to eight. The smallest linkage group (LG I) is made up of two STMS markers, whereas the largest (LG II) comprised eight markers. LG V comprised only AFLP markers (Table 2 and Figure 2).

The percent polymorphism obtained in the present study (21.30%) was very low. This might be due to the involvement of two desi cultivars differing for wilt reaction in the development of the RIL population. In such situation, a low level of polymorphism in the population is expected, except for markers associated with resistance genes. Such a very low level of polymorphism was also reported in previous studies of RADHIKA *et al.* (2007) (9.5% and 11.57%), GOWDA *et al.* (2009) (13.45%) and NAYAK *et al.* (2010) (16.7%).

Interval mapping conducted with MAPMANAGER QTX software detected regions associated with wilt resistance. Two QTLs, one associated with early wilt resistance and the other with late wilt resistance, could be identified on linkage group 2 (LG II). The QTL associated with early wilt resistance explained

36% of phenotypic variability with the maximum LOD score of 9.19. QTL for late wilt resistance explained 16% of phenotypic variability with the LOD score of 3.53 (Table 3). For both the QTLs (identified in RILs) the nearest locus was TA59, which is also amplified in resistant parent WR 315. Hence the favourable alleles for both the QTLs were contributed from WR 315.

Linkage group	Size (in cM)	No. of mapped markers	Average marker density
LG I	1.6	2	0.8
LG II	129.2	8	16.15
LG III	86.2	6	14.36
LG IV	48.5	4	12.12
LG V	34.7	3	11.56

Major QTL for both the stages of wilt resistance is located on linkage group II (LG II). Marker interval for the QTL associated with wilt resistance at 30 DAS was TA27-TA59 and for 60 DAS it was TA27-TA110. The nearest marker associated with the highest LOD score for both the QTLs was TA 59. STMS marker TA 27 is the common flanking marker for both the QTLs. TA27 was one of the flanking markers for *loc3-loc5* cluster, while TA110 was one of the flanking markers for *loc1* (GOWDA *et al.* 2009). The QTL for early wilt resistance (at 30 DAS) covered a distance of 5.4 cM and explained 36% of phenotypic variation, whereas the interval length for QTL associated with

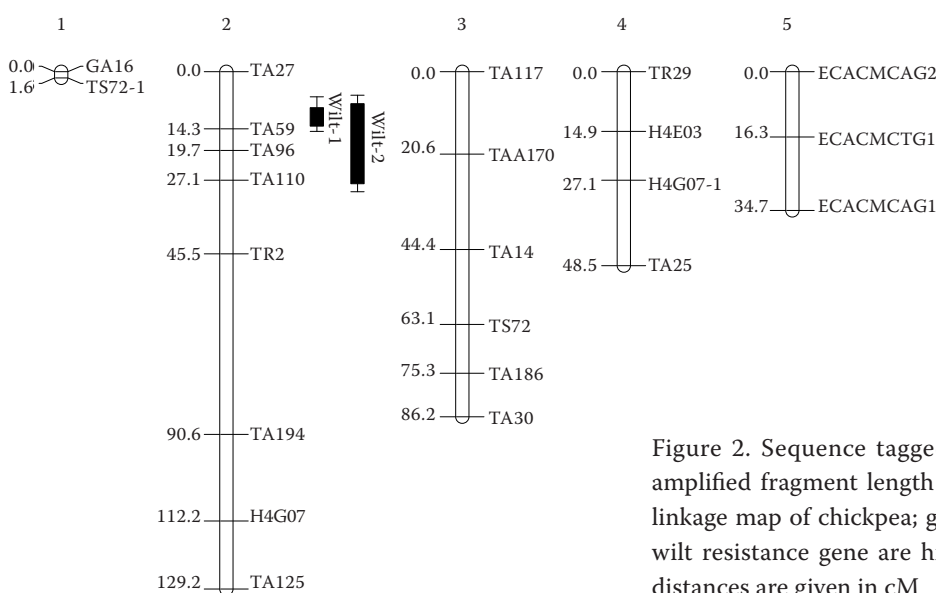


Figure 2. Sequence tagged microsatellite (STMS) and amplified fragment length polymorphism (AFLP) based linkage map of chickpea; genomic locations of QTLs for wilt resistance gene are highlighted to the left; marker distances are given in cM

Table 3. Quantitative trait loci (QTL) detected for Fusarium wilt resistance at 30 and 60 days after sowing (DAS) in chickpea

QTL	Linkage group	Flanking markers	Interval length (cM)	Nearest locus to the maximum LOD peak	Maximum LOD score	R ² (%)	Additive effect
Wilt 1 (at 30 DAS)	LG II	TA27-TA59	9.0	TA 59	9.19	36	-15.98
Wilt 2 (at 60 DAS)	LG II	TA27-TA110	22.2	TA59	3.53	16	-9.26

LOD – logarithm of the odds

late wilting is 22.2 cM and explained 16% of phenotypic variation. The Fusarium wilt scores recorded at 60 DAS also included the wilt scores of 30 DAS, hence there is an overlap in QTL mapping with a longer interval length for QTL associated with the combined scores. In earlier studies, resistance genes (*foc-0*, *foc-1*, *foc-2*, *foc-3*, *foc-4* and *foc-5*) against the races (0, 1, 2, 3, 4 and 5; *foc-0*, *foc-1*, *foc-2*, *foc-3*, *foc-4* and *foc-5*) were found in clusters on linkage group II (LG II) of chickpea reference map (WINTER *et al.* 2000; SHARMA & MUEHLBAUER 2007; GOWDA *et al.* 2009) and considered LG II as a hotspot of Fusarium wilt resistance genes (SHARMA & MUEHLBAUER 2007). QTLs detected in the present study are also located on LG II, confirming the earlier reports. In the present study, LG II is the largest linkage group comprising a third of the total markers in the genetic map, since the parents differed in wilt reaction and QTLs for wilt are located on LG II. As a result the markers linked to wilt resistance genes showed a high polymorphism and are located on LG II, resulting in the largest linkage group in the study.

WINTER *et al.* (2000) mapped QTLs for race 1 resistance along with single gene analysis for races 4 and 5 of Fusarium wilt. All of them mapped to the same linkage group (LG II) indicating a clustering of several Fusarium-wilt resistance genes around this locus. Comparison of genetic map of our study with reference map of WINTER *et al.* (2000) revealed similarity between LG II of present map with reference map (Figure 3). In the present map QTL for race 1 resistance is located close to the STMS marker TA59 as was also reported by WINTER *et al.* (2000). The marker similarity between interspecific map (WINTER *et al.* 2000) and intraspecific map of the present study and association of the same markers with QTL in other intraspecific crosses (HALILA *et al.* 2009; GOWDA *et al.* 2009) indicates that this genomic region is conserved across the species. However, the map distances differed, possibly due to the low number of markers used; while the marker order differed, possibly due to the intraspecific nature of the mapping population used in the present study.

The linkage analysis of Fusarium wilt resistance in chickpea by several researchers (WINTER *et al.*

2000; SHARMA & MUEHLBAUER 2007; GOWDA *et al.* 2009) using different mapping populations located wilt resistance genes against races, 0, 1, 2, 3, 4 on linkage group II (LG II) of the chickpea map and in a region which is highly saturated with tightly linked STMS markers that could be used for fine mapping. The presence of resistance genes/QTLs to several races of chickpea in a certain chromosomal segment (LG II) suggested the possibility of development of chickpea varieties with durable and broad spectrum of resistance to wilt. However, a higher density of markers in the area of these genes is still necessary to detect polymorphisms for marker-assisted selection (MAS) in different genetic backgrounds.

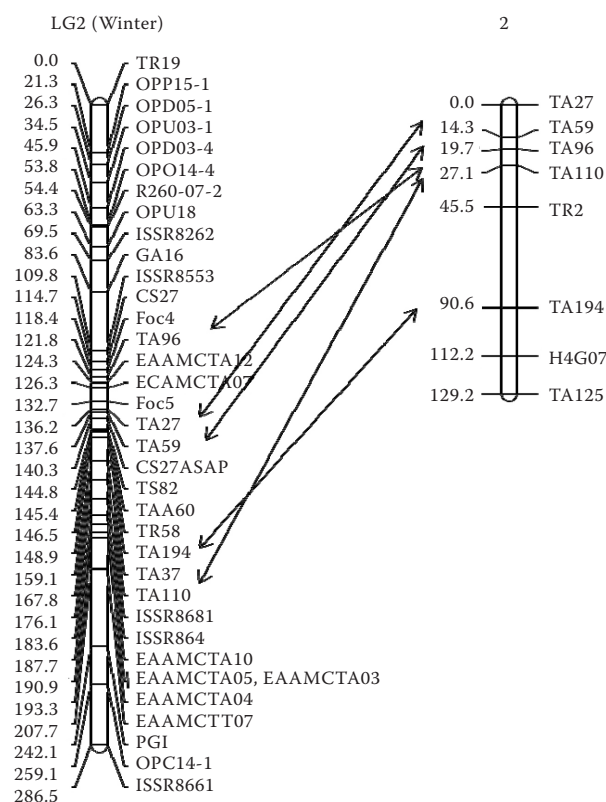


Figure 3. Comparison of sequence tagged microsatellites (STMS) and amplified fragment length polymorphism (AFLP) based chickpea linkage group 2 with LG II of WINTER *et al.* (2000) reference map

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