

RESEARCH ARTICLE

Development of molecular map and identification of QTLs linked to *Fusarium* wilt resistance in chickpea

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Abstract

A number of genetic maps for *Fusarium* wilt resistance in chickpea have been reported in earlier studies, however QTLs identified for *Fusarium* wilt resistance were unstable. Hence, the present study aims to map novel molecular markers and to identify QTLs for *Fusarium* wilt resistance in chickpea. An intraspecific linkage map of chickpea (*Cicer arietinum* L.) was constructed using F₁₀–F₁₁ recombinant inbred lines (RILs) derived from a cross between K850 and WR315 segregating for H₂ locus. A set of 31 polymorphic simple sequence repeat (SSR) markers obtained by screening 300 SSRs and were used for genotyping. The linkage map had four linkage groups and coverage of 690 cM with a marker density of 5.72 cM. The RILs were screened for their wilt reaction across two seasons in wilt sick plot at International Crop Research Institute for Semi-Arid Tropics (ICRISAT), Hyderabad, India. Five major quantitative trait loci (QTLs) were detected in both seasons for late wilting (60 days after sowing). A stable QTL (GSSR 18-TC14801) for wilt resistance was identified in both the seasons, and the QTL explained a variance of 69.80 and 60.80% in 2007 and 2008 rabi respectively.

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Introduction

Chickpea (*Cicer arietinum*, $2n = 2x = 16$) is a major cool season legume crop of India with a genome size of ~738 Mbp (Jain *et al.* 2013; Varshney *et al.* 2013). Though about 65% of the world acreage and 68% production are accounted in India, the production is still not adequate to meet the domestic demand due to its low productivity (850 kg/ha). The major causes for low productivity of chickpea are low yield potential and susceptibility of improved present day cultivars to various biotic and abiotic stresses (Gowda *et al.* 2011). *Fusarium* wilt, caused by *Fusarium oxysporum ciceri* (FOC), can cause an annual yield losses of up to 10–15%, under epidemics can be devastating and may cause 100% yield loss under favourable conditions. Among eight races reported till now (Jimenez-Diaz *et al.* 1993), race 1A, is more prevalent in peninsular India in epidemic form. Genetics of resistance to race 1A (FOC1) suggested that two to three major independent loci H₁, H₂ and H₃ (Upadhyaya *et al.* 1983a, b; Singh *et al.* 1987; Brinda and Ravikumar 2005) govern wilt resistance in chickpea. Dominant alleles at both H₁ and H₂ loci cause early wilting and dominance at any one locus (either H₁ or H₂) lead to late wilting,

while homozygous recessive at H₁ and H₂ loci confer complete resistance (Brinda and Ravikumar 2005). Even though high level of resistance to FOC race 1A is available in the cultivated species, the progress in the development of high-yielding wilt-resistant varieties is very slow. Since, the selection for wilt resistance in segregating population and maintenance of wilt sick plot is difficult task, hence marker-assisted selection (MAS) will be an efficient strategy to accelerate pyramiding of different resistance genes for *Fusarium* wilt resistance in chickpea. In chickpea, a large number of molecular markers, molecular maps and QTLs linked to *Fusarium* wilt resistance are available (Millan *et al.* 2010; Nayak *et al.* 2010; Gujaria *et al.* 2011; Thudi *et al.* 2011; Choudhary *et al.* 2012; Hiremath *et al.* 2012; Barman *et al.* 2014; Patil *et al.* 2014). However, these reported QTLs are derived from mapping studies with limited population size and the identified QTLs were not validated across environments and in different genetic background. Hence, it is important to explore novel markers to develop linkage maps and to identify stable QTLs linked to *Fusarium* wilt resistance for reliable selection of resistant genotypes from segregating populations.

In this context, the study was conducted to develop a linkage map using genic and genomic simple sequence repeat (SSR) markers to tag H₂ locus and to identify QTLs linked to

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wilt resistance for race 1A, which is prevalent in peninsular India.

Materials and methods

Plant material and DNA extraction

The experimental material consist of a set of 141 intraspecific F₁₀–F₁₁ recombinant inbred lines (RILs) segregating for (H₂ locus) wilt resistance derived by single seed descent method from intraspecific cross between K850 (susceptible late wilter, h₁h₁H₂H₂) and WR315 (resistant, h₁h₁h₂h₂) (Upadhyaya *et al.* 1983a). Good quality genomic DNA was extracted from vegetative buds and young leaves of RILs along with their parental genotypes by following cetyl trimethyl ammonium bromide (CTAB) method of extraction with slight modifications (Doyle and Doyle 1987). DNA quantification was done by nanodrop (Eppendorf Biospec-trometer) and stored in –20°C freezer for genotyping.

Phenotyping of RILs for *Fusarium* wilt reaction

The RILs were phenotyped for wilt reaction, based on the evaluation made over two seasons rabi 2007 (F₇) and 2008 rabi (F₈) in wilt sick plot at International Crop Research Institute for Arid and Semi-arid Tropics (ICRISAT), Patancheru, Hyderabad, India (Sharma *et al.* 2005; Ravikumar *et al.* 2007). The wilt incidence was measured on the basis of number of wilted plants to the total number of plants in each row and expressed as per cent wilt incidence. The average of wilt incidence was calculated based on values from two replications. The percentage wilting was recorded on 30 days after sowing (DAS) and 60 DAS (Haware and Nene 1982). The death due to wilting was confirmed by observing the discolouration of the xylem by uprooting the plants.

SSR marker analysis

A set of 300 novel microsatellite markers (table 1 in electronic supplementary material at <http://www.ias.ac.in/jgenet/>) developed at National Institute of Plant Genome

Research (NIPGR), New Delhi, along with earlier reported SSR markers were used to survey parental polymorphism (Jhanwar *et al.* 2012; Kujur *et al.* 2013). The genic SSRs were developed from transcriptome sequence of various tissues of chickpea variety ICC4958, using next generation sequencing platforms (Garg *et al.* 2011). The genomic markers are based on the draft genome sequence of Desi chickpea (Jain *et al.* 2013).

The parental lines K850 and WR315 were tested for polymorphism with SSR markers in 15 µL reactions using thermal cycler (Mastercycler gradient, Eppendorf, Hamburg, Germany). The PCR products from each tube along with 5 µL of loading dye were separated electrophoretically using 3.0% agarose gels containing 0.05 µg/mL ethidium bromide. The amplification products were examined under UV light and photographed using Alpha digidoc 1000 system (Alpha Innotech Corporation, USA) gel documentation system (figures 1 and 2). The polymorphic markers were used to genotype all the RILs and the data generated was recorded was subsequently used for mapping studies.

Linkage and QTL analysis

The segregation of markers was tested for goodness of fit to the expected ratio of 1:1 using chi-square (χ^2) test ($P < 0.05$). Linkage mapping was performed using G-Mendel 2.0 programme of iMAS 1.0.1 (integrated marker assisted selection) developed by ICRISAT, Hyderabad. A minimum LOD of 2.5 and maximum recombination fraction of 0.4 were set as threshold value for linkage group determination. The marker ordering is validated by Monte Carlo and bootstrap methods. Haldane mapping function was used to convert recombination fraction into map distances in centimorgans (cM).

QTL analysis was performed using QTL Cartographer ver. 2.5 (Basten *et al.* 1994). The composite interval mapping was used to identify putative *Fusarium* wilt resistance QTLs in every linkage group considering the wilt data of 2007 and 2008. A walk speed of 2 cM and a minimum LOD score of 3.0 were used for declaring the presence of a

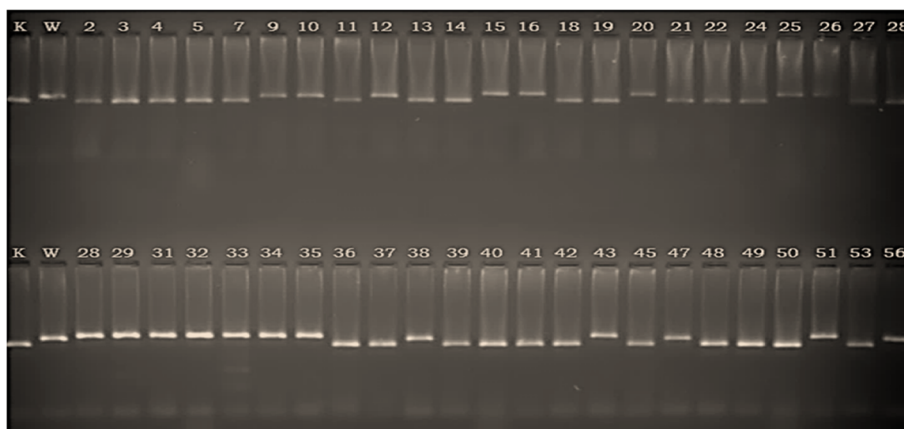


Figure 1. Segregation pattern of genic marker (EST-SSR 3) across chickpea RILs and parents K850 (K) and WR315 (W).

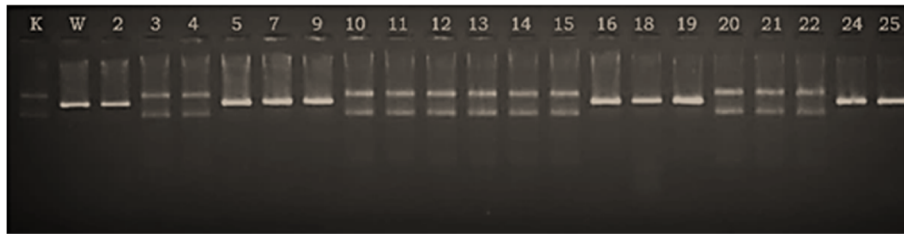


Figure 2. Segregation pattern of genomic marker (GSSR 50) across chickpea RILs and parents K850 (K) and WR315 (W).

putative QTL. The presence of putative QTL in an interval was tested by using a critical value for LOD threshold of 3.0 as determined by WQTL Cartographer using the Bonferroni chi-square approximation (Zeng 1994).

Results

Phenotyping of RILs for *Fusarium* wilt reaction

In the present investigation, 141 RILs derived from the cross between WR315 (resistant parent) and K850 (late wilter) were phenotyped for wilt incidence. At 60 DAS during 2007 and 2008 seasons, the distribution of RILs showed continuous distribution with respect to per cent of wilt incidence in both seasons (figure 3).

Linkage map construction

Among 300 SSRs screened for parental polymorphism, a total of 280 (93.3%) SSRs produced scorable amplicons, of which 31 (10.3%) primers were found to be consistently polymorphic between the parental genotypes. The 31 polymorphic primers consist of 23 genomic SSRs and eight genic SSRs. Among the 23 polymorphic genomic SSRs, 15 were novel and remaining eight were mapped in earlier studies using different mapping populations, whereas all the eight genic SSRs used in the present study were novel and were not mapped in any of the earlier studies.

All the markers analysed except four showed expected 1:1 ratio (data not shown). In linkage analysis 23 marker were mapped to four linkage groups covering a total genetic distance of 690 cM with an average marker density of 30 cM. The length of the linkage group ranged from 12.8 (LG 2) to

588.60 cM (LG 1). The number of markers mapped per linkage group varied from 2 (LG 2) to 14 (LG 1). The highest marker density was observed in LG 2 with an average marker density of 6.4 cM and the least marker density of 42.04 cM was observed in LG 1 (table 1).

QTLs for *Fusarium* wilt resistance

The current linkage map was used to detect QTLs using the data obtained on wilt reaction of RILs for two years. A total of five major QTLs (LOD \geq 3.0) for late wilting (60 DAS) of which, two QTLs (GSSR 18-TC14801 and GSSR 11-EST SSR 3) in 2007 rabi and three QTLs (TR 24-EST SSR 21, EST SSR 21-EST SSR 65 and GSSR 18-TC14801) were identified in 2008 rabi (table 2). The QTLs GSSR 18-TC14801 and GSSR 11-EST SSR 3 explained phenotypic variance of 69.80 and 68.80% with an additive gene effects of -25.0 and 24.4, respectively. Similarly, three QTLs such as TR 24-EST SSR 21, EST SSR 21-EST SSR 65 and GSSR 18-TC14801, explained phenotypic variance of 56.80, 56.20 and 60.80% with an additive gene effects of 21.98, 21.94 and -22.33, respectively (figure 4).

Discussion

Resistance to *F. oxysporium* race 1 is reported to be controlled by 2–3 genes (Upadhyaya *et al.* 1983a, b; Singh *et al.* 1987; Brinda and Ravikumar 2005). The presence of two major genes H₁ and H₂ in dominant homozygous conditions cause early wilting and any one in dominant condition causes late wilting (Brinda and Ravikumar 2005). Mayer *et al.* (1997) first reported linkage of an allele-specific marker (CS27A) to H₁ locus of FOC 1. However, the mapping population segregate for only H₂ locus and late wilting and the

Table 1. Features of linkage map developed using SSR markers and intraspecific mapping population derived from a cross between K850 and WR315.

Linkage group	Size in (cM)	No. of mapped markers	Average marker density
LG I	588.6	14	42.04
LG II	12.8	2	6.40
LG III	38.3	3	12.76
LG IV	50.3	4	12.57

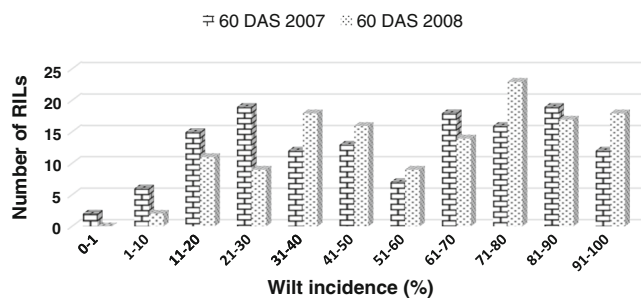


Figure 3. Frequency distribution of RILs for *Fusarium* wilt incidence at 60 DAS during 2007 and 2008 rabi seasons.

Table 2. List of QTLs identified for *Fusarium* wilt resistance in chickpea using RILs segregating for H₂ locus.

Seasons	Linkage group	Flanking marker	QTL position (cM)	Max. LOD	R ² (%)	Genetic effect		Donor parent
						Additive	Dominant	
Rabi 2007	1	GSSR 18-TC14801	340.71	8.90	69.80	-25.00	0.0	Female
	1	GSSR 11-EST SSR 3	559.81	3.05	68.80	24.43	0.0	Male
Rabi 2008	1	TR 24-EST SSR 21	32.01	4.43	56.80	21.98	0.0	Male
	1	EST SSR 21-EST SSR 65	95.51	3.13	56.20	21.94	0.0	Male
	1	GSSR 18-TC14801	316.71	4.43	60.80	-22.33	0.0	Female

QTLs identified in this study targets the locus other than H₁ locus.

The continuous distribution of RILs with respect to per cent of wilt incidence in both the seasons indicate involvement of many minor genes along with major genes governing the trait. At 60 DAS, majority of RILs (>60%) showed more than 50% wilt incidence which confirms genotypic constitution of the parents involved such as WR315 (h₁h₁h₂h₂) and K850 (h₁h₁H₂H₂). The disease reaction of RILs showed continuous variation suggesting polygenic inheritance of wilt reaction along with major genes (Upadhyaya *et al.* 1983a, b).

Linkage map construction

The per cent polymorphism observed in the present study (10.3%) is in accordance with majority of earlier studies namely, Tullu *et al.* (1998) (14%), Radhika *et al.* (2007) (9.5 and 11.57%), Gowda *et al.* (2009) (13.45%), Nayak *et al.* (2010) (16.7%) and Sabbavarapu *et al.* (2013) (20.8%). However, when compared with reports by Ratnaparkhe *et al.*

(1998) (38%) and Patil *et al.* (2014) (21.30%), the per cent polymorphism obtained in the present study was rather low (10.3%). The possible reason could be the use of intraspecific mapping population and more number (84.3%) of genic markers (which are developed from conserved regions of the genome) employed in the present study, unlike interspecific mapping population and ISSR markers used by Ratnaparkhe *et al.* (1998).

Segregation distortion observed in the mapping population (12.9%) was relatively less compared to that reported by Tullu *et al.* (1999), Winter *et al.* (2000), Radhika *et al.* (2007) and Flandez-Galvez *et al.* (2003) in chickpea. The observed segregation distortion is in favour of the maternal alleles (K850) which was in agreement with Flandez-Galvez *et al.* (2003). Segregation distortion affects the estimation of map distances and the order of markers and, hence, affect linkage map construction and QTL analysis. Though, Winter *et al.* (2000) observed different amounts of segregation distortion for different classes of markers, segregation distortion was less related to the class of affected markers than to the genomic region where they resided.

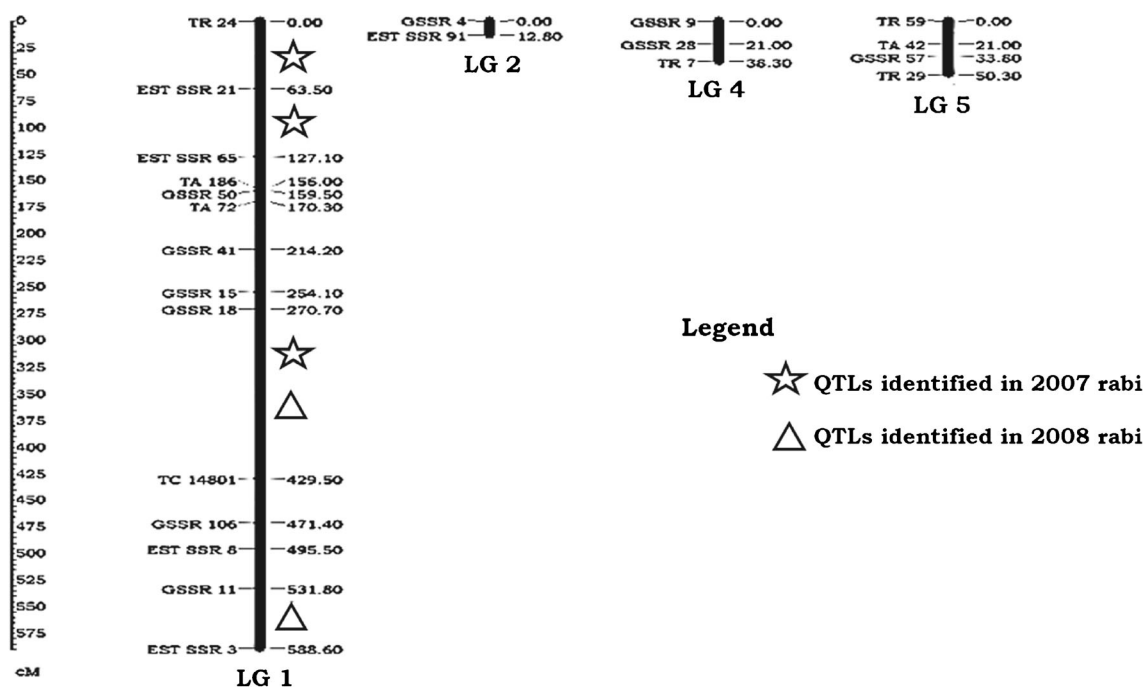


Figure 4. Genetic linkage map depicting marker position and QTLs associated with *Fusarium* wilt resistance in chickpea.

Linkage group 1 (LG 1) of present study composed of 14 markers and spanning a length of 588.60 cM may correspond to LG II of map of Radhika *et al.* (2007) as it contains STMS loci TR24, TA186 and TA72. However, it may also correspond to LG IV of Millan *et al.* (2010) and Sabbavarapu *et al.* (2013) due to the presence of a STMS marker TA72. The LG 4 composed of four markers and comprising a length of 50.30 cM may equated to LG I of map of Radhika *et al.* (2007) since both the maps shared three STMS markers (TR59, TA42 and TR29) in common. This group may also correspond to LG V of Sabbavarapu *et al.* (2013) and Millan *et al.* (2010) due to the presence of STMS markers TR59 and TR29 respectively. However, this group may also be equated with LG IV of Patil *et al.* (2014) due to the presence of a STMS marker TR29. This clearly indicated the lack of sufficient number of markers for joining the corresponding linkage groups and the other reason could be attributed to the use of novel markers in the present study for the development of linkage map. Among 31 SSR markers, 23 SSRs (eight genic and 15 genomic) were informatively mapped on four linkage groups, of which, 16 were novel markers and were mapped for the first time in chickpea. Hence, it is an addition to already existing integrated map by Nayak *et al.* (2010).

QTLs for *Fusarium* wilt resistance

All the five QTLs reported in the study were for late wilting (60 DAS) and no QTLs were identified for early wilting (30 DAS) since the experimental material used in the study were derived from a cross between a late wilter (K850) and a resistant (WR315) parent and hence, the RILs segregate for H₂ locus alone. The QTLs identified in the study were major with R^2 more than 50% and LOD > 4. It is interesting to note that a QTL identified in 2007 rabi and another QTL identified in 2008 rabi were apparently in the same locus, having the same flanking markers GGSR 18 and TC 14801 with slightly different positions. However, difference observed between phenotypic variance and LOD can be accounted for differences in environmental conditions between two trial seasons. The presence of common flanking markers indicates the same genomic regions. In addition, negative additive effect for the both QTLs indicated the favourable alleles were donated by female parent. Hence, it can be considered as relatively stable and apparently common QTL. The similar report of presence of apparently common QTL for *Ascochyta* blight resistance in chickpea was reported by Stephens *et al.* (2014). However, as the distance between flanking markers spanning the identified stable QTL (GGSR 18 and TC 14801) is more, therefore, further efforts are required to fine map the QTL region in order to identify more closely-linked flanking markers for successful introgression of the QTL using markers-assisted selection (MAS) and map-based cloning of identified *Fusarium* resistant QTL.

The remaining three QTLs namely (GSSR 11-EST SSR 3) in 2007 rabi and (TR 24-EST SSR 21 and EST SSR

21-EST SSR 65) in 2008 rabi were also major QTLs with high additive effect indicated the contributing alleles were from male parent WR315. The QTLs found during 2008 rabi (TR 24-EST SSR 21 and EST SSR 21-EST SSR 65) were very close to each other while the second QTL (GSSR 11-EST SSR 3) of 2007 was at the other end of linkage group 1.

The major QTL (GSSR 18-TC14801) present in LG 1 for *Fusarium* wilt resistance which was contradictory with majority of earlier reports (Ratnaparkhe *et al.* 1998; Tekeoglu *et al.* 2000; Winter *et al.* 2000; Millan *et al.* 2006; Sharma and Muehlbauer 2007; Cobos *et al.* 2009; Gowda *et al.* 2009; Millan *et al.* 2010; Barman *et al.* 2014; Patil *et al.* 2014; Varshney *et al.* 2014) where the QTLs for FOC 1 were mapped on LG 2 and few studies reported the QTL on other linkage group, Cobos *et al.* (2005) reported a QTL on LG 3 and Sabbavarapu *et al.* (2013) reported two novel QTLs for *Fusarium* wilt race 1 on LG 6 in a different mapping population derived from same resistant parent (WR315).

All the QTLs reported in the present study for wilt resistance against race 1 were located on LG 1 and explained a higher phenotypic variation ranging from 53.34 to 71.41% and had a higher LOD score of 8.90%. The QTLs detected were not reported so far in any of the earlier studies, hence, the QTLs may be considered as novel. In earlier studies, locating the additional QTLs on other linkage groups (LG 2, LG 3 and LG 6) accounted for a lesser proportion of genetic variation compared to the current study. This variation could be due to various factors, such as use of different methodology, different genetic background, different phenotypic techniques (sick plot versus sick pot), different types of markers and interspecific versus intraspecific crosses.

The identification of closely-linked markers or stable QTLs for the *Fusarium* wilt resistance genes, will facilitate introgression of resistant genes from *Fusarium* wilt resistant cultivars carrying individual genes into commercially competitive chickpea varieties, as witnessed in the recent study by Varshney *et al.* (2014). In earlier studies, efforts were also made to map QTLs/genes and markers flanking QTLs for different *Fusarium* wilt races. For instance, markers flanking *Foc0* locus (OPJ20₆₀₀ and TR59), *Foc1* locus (TA110 and H₃A₁₂), *Foc2* locus (H₃A₁₂ and TA96), *Foc3* locus (TA96 and TA194), *Foc4* locus (TA96 and CS27) and *Foc5* locus (TA59 and TA96), determining resistance to race 0 (Cobos *et al.* 2005), race 1 (Gowda *et al.* 2009), race 2 (Gowda *et al.* 2009), race 3 (Sharma *et al.* 2004; Gowda *et al.* 2009), race 4 (Winter *et al.* 2000; Sharma *et al.* 2004) and race 5 (Cobos *et al.* 2009) respectively, were reported.

Conclusion

A set of 31 novel molecular markers were mapped in the present study and stable QTL (GSSR 18-TC14801) across seasons for *Fusarium* wilt resistance was identified. Since the distance between the flanking markers is high, validation of

the identified stable QTL and further efforts to fine map the region to identify more closely-linked markers is needed so that the identified stable QTL can be effectively made use in chickpea breeding programme.

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