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Association mapping of genomic loci linked with Fusarium wilt resistance (*Foc*2) in chickpea

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Abstract

Improving plant resistance against Fusarium wilt (FW) is key to sustaining chickpea production worldwide. Given this, the current study tested a set of 75 FW-responsive chickpea breeding lines including checks in a wilt-sick plot for two consecutive years (2016 and 2017). Genetic diversity analysis using 75 simple sequence repeats (SSRs) revealed a total of 267 alleles with an average of 3.56 alleles per marker. The entire set was divided into two major classes based on clustering method and factorial analysis. Similarly, STRUCTURE analysis placed the 75 genotypes into three distinct sub-groups ($K = 3$). Marker-trait association (MTA) analysis using the generalized linear model approach revealed nine and eight significant MTAs for FW resistance in the years 2016 and 2017, respectively. Three significant MTAs were obtained for FW resistance following the mixed linear model approach for both years. The SSR markers CESSR433, NCPGR21 and ICCM0284 could be potentially employed for targeted and accelerated improvement of FW resistance in chickpea. To the best of our knowledge, this is the first report on association mapping of the genomic loci controlling FW (*Foc2*) resistance in chickpea.

Keywords: association mapping, *Cicer arietinum*, Fusarium wilt, SSR

Introduction

Chickpea is one of the most important grain legumes grown globally (FAO, 2017). Globally, 14.78 Mt chickpea is harvested annually from 14.56 Mha area (FAO, 2017). It plays a crucial role in providing protein-based nutrition security to the increasing global human population – especially in the developing countries (Graham and Vance, 2003; Jukanti *et al.*, 2012; Bohra *et al.*, 2014). Being a leguminous crop, chickpea improves soil nitrogen content by incorporating atmospheric nitrogen in association with root inhabiting active rhizobia (Graham and Vance, 2003).

Chickpea yield is seriously challenged by a range of biotic and abiotic stresses. Among the various biotic stresses, vascular wilt and Ascochyta blight caused by *Fusarium oxysporum* f. sp. *ciceris* and *Ascochyta rabiei*, respectively remain the most devastating diseases, causing up to 90% yield losses (Sabbavarapu *et al.*, 2013; Jha *et al.*, 2020). Importantly, Fusarium wilt (FW) resistance is an essential prerequisite for variety identification and release system in major chickpea growing countries such as India. To date, a total of eight FW races (race 0, 1A, 1B/C, 2, 3, 4, 5 and 6) have been recognized in chickpea (Jiménez-Gasco and Jiménez-Diaz, 2003). Initial studies based on Mendelian genetics in chickpea have elucidated the genetic makeup of plant resistance against FW races 1A, 2, 3, 4 and 5 (Sharma *et al.*, 2005). However, genetic resistance of chickpea for FW races 1B, 1C and 6 remains to be studied.

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Trait-mapping studies in chickpea have provided a set of DNA markers linked to genomic regions controlling resistance to multiple FW races. Examples include DNA markers OPJ20600 and TR59 for *Foc0* race, TA110 and H3A12 for *Foc1* race, H3A12 and TA96 for *Foc2* race (Cobos *et al.*, 2005), TA196 and TR19 for *Foc4* (Mannur *et al.*, 2019) and TA59 for *Foc5* race (Castro *et al.*, 2010; Caballo *et al.*, 2019). Similarly, DNA markers for selection against FW race 1 (*Foc1*) and race 2 (*Foc2*) were reported (Gowda *et al.*, 2009). Other examples of FW mapping in chickpea include against races *Foc3* (Sharma *et al.*, 2004; Gowda *et al.*, 2009), *Foc4* (Winter *et al.*, 2000; Sharma *et al.*, 2004, 2005; Mannur *et al.*, 2019) and *Foc5* (Cobos *et al.*, 2009; Caballo *et al.*, 2019). However, these studies involved the analysis of mapping populations based on two FW-responsive parents with contrasting disease reactions (Varshney *et al.*, 2014; Mannur *et al.*, 2019).

In recent years, association genetics has been implemented in chickpea for the identification of significant gene–trait associations for a variety of important traits (Thudi *et al.*, 2014; Upadhyaya *et al.*, 2016a, b; Jha *et al.*, 2018). In this context, the current study identified significant marker-trait associations (MTAs) for FW (*Foc2*) using simple sequence repeat (SSR)-based association mapping of 75 chickpea genotypes. The significant MTAs will pave the way for rapid and targeted breeding of chickpea cultivars with enhanced resistance to *Foc2* race.

Materials and methods

A panel of FW-responsive 75 chickpea genotypes along with WR 315 (FW-resistant check) and JG 62 (FW-susceptible check) (see online Supplementary Table S1) was screened for two consecutive years 2016 and 2017 in FW (*Foc2*)-sick nursery at Indian Institute of Pulses Research (IIPR), Kanpur, India. The genotypes were sown in the first week of November in both years (2016 and 2017) in augmented block design arranged in five blocks, with each block containing 15 genotypes along with the checks. The genotypes were sown in 60 cm × 10 cm spacing and the row length was kept as 3 m. The susceptible check (JG 62) was planted after every two rows. Wilt incidence was recorded at three different stages i.e. pre-flowering, post flowering and post podding (from November to February). The disease incidence was calculated according the following method suggested by Sharma *et al.* (2016):

$$\%\ \text{disease incidence} = \frac{\text{Number of FW-infected plants} \times 100}{\text{Total number of plants}}$$

Based on the % disease incidence, genotypes displaying less than 10.0% incidence were classified as resistant, whereas genotypes having incidence between 10.1 and 20.0% were classified as moderately tolerant. Genotypes with 20.1–50.0% incidence were considered as moderately susceptible and genotypes showing >50.0% incidence were considered as susceptible.

DNA extraction and SSR analysis

Genomic DNA was isolated from 3 weeks old chickpea seedlings following the CTAB method (Saghai-Maroof *et al.*, 1984). A total of 125 SSRs were screened on the given set of genotypes, of which 75 SSRs yielded polymorphic fragments. The SSRs used here are reported previously by different research groups (Winter *et al.*, 1999, 2000; Sethy *et al.*, 2003, 2006; Nayak *et al.*, 2010; Gaur *et al.*, 2011; Gujaria *et al.*, 2011; Choudhary *et al.*, 2012) and the selected SSRs span entire eight linkage groups (LGs) of chickpea (online Supplementary Table S2).

Polymerase chain reaction (PCR) analysis

The PCR protocol was followed as suggested by Jha *et al.* (2018) and Bohra *et al.* (2020). A reaction mixture of 10 µl volume containing 5.9 µl of sterilized distilled water, 1.00 µl template DNA (25 ng), 0.5 µl of forward and 0.5 µl of reverse primers (5 µM), 1.00 µl 10× PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 1.00 µl dNTP mix (0.2 mM each of dATP, dGTP, dCTP and dTTP) and 0.1 µl *Taq* polymerase (5 U/µl) (Thermo Fisher Scientific Mumbai, India, Pvt. Ltd.) was prepared. This reaction mixture was polymerized by using G-40402 thermo cycler (G-STORM, Somerset, UK) using the touchdown PCR profile for amplification with initial denaturation at 94°C for 5 min followed by 10 cycles of touchdown 61–51°C, 30 s at 94°C, annealing for 30 s at 61°C (the annealing temperature for each cycle being reduced by 1°C per cycle) and extension for 30 s at 72°C. This was followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 51°C for 30 s, elongation at 72°C for 45 s and 10 min of final extension at 72°C. Amplified fragments were resolved on 3% agarose gel using 0.5× TBE running buffer and images were analysed with Quantity one software (Bio-Rad, CA 94547, USA).

Genetic diversity and population structure analysis

Genetic diversity parameters such as number of alleles per locus (N_a), gene diversity (H_e) and polymorphism information content (PIC) were computed with PowerMarker v. 3.25 (Liu and Muse, 2005). With 1000 bootstrap value, neighbourhood joining tree and factorial analysis was performed with DARwin v. 6.0.13 (Perrier and Jacquemoud-Collet, 2006). To determine population structure (Q) and the subpopulation (K) in the given set of genotypes,

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model-based analysis was conducted with STRUCTURE v. 2.3.4 (Pritchard *et al*., 2000). By applying admixture model, five independent runs were conducted with 200,000 Markov Chain Monte Carlo iterations for each K value ranging from 1 to 10 with a burn-in length of 200. In parallel, the best K value was obtained according to the ΔK method of Evanno *et al*. (2005) by processing the STRUCTURE results by STRUCTURE HARVESTER (Earl and von Holdt, 2012) (http://taylor0.biology.ucla.edu).

Association mapping for the identification of significant MTAs and putative candidate genes

The wilt disease scores and the genotypic data were analysed to discover significant MTAs using generalized linear model (GLM) and mixed linear model (MLM) models in TASSEL v. 3.0 (Bradbury *et al*., 2007; Zhang *et al*., 2010). The MTAs were detected at the thresholds of $P = 0.05$ and at $P = 0.01$. Furthermore, to find the possible candidate genes corresponding to the reported MTAs and the putative proteins encoded by these, we performed BLASTn search for the associated SSRs against the reference genome sequence of CDC frontier (Varshney *et al*., 2013). In parallel, the proteins were predicted for the corresponding sequences using InterPro (https://www.ebi.ac.uk/interpro/).

Results

Genetic diversity for FW resistance

Analysis of the FW response of the 75 chickpea genotypes during years 2016 and 2017 suggested a broad range of genetic variability. Figure S1 (see online Supplementary material) depicts the frequency distribution of disease incidence in the tested chickpea genotypes for the two consecutive years. Based on the average of disease scoring data of the two years, a total of 30 resistant (R), 34 moderately resistant (MR) and 11 susceptible (S) genotypes were obtained (online Supplementary Table S1).

SSR-based molecular diversity analysis

Assaying 75 chickpea genotypes with 75 SSRs revealed a total of 267 alleles with an average of 3.56 alleles per marker (online Supplementary Table S2). The number of alleles ranged from two to eight, while the PIC values varied between 0.17 and 0.77. Similarly, gene diversity ranged from 0.19 to 0.8 with an average value of 0.59.

As shown in Fig. 1, entire 75 genotypes were clustered into two groups according to unweighted neighbour joining. Cluster I contained 42 genotypes, while cluster II had 33 genotypes. Similarly, factorial analysis placed all the genotypes into two coordinates (online Supplementary Fig. S3).

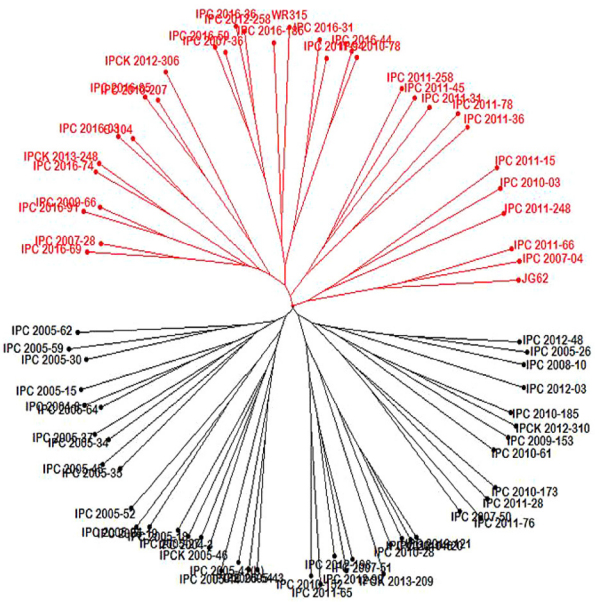

Fig. 1. Neighbour-joining trees of the 75 chickpea genotypes for FW resistance phenotyping.

Structure analysis

Population structure of the 75 chickpea genotypes was investigated with Bayesian approach using the STRUCTURE program. The ln $P(D)$ as well as Evanno's ΔK values identified three genetically distinct populations (i.e. $K = 3$) (Fig. 2 and online Supplementary Fig. S4).

Identification of MTAs for FW resistance

We used both GLM and MLM approaches for the detection of significant MTAs for FW (*Foc2*) resistance. Following GLM analysis, seven markers (NCPGR40, NCPGR180, NCPGR249, NCPGR231, NCPGR149, CESSR433 and ICCM0284) explained 7.9–45.5% of the phenotypic variance (PV) in the year 2016 (Table 1). Although MLM analysis showed significant association of three SSR markers CESSR433, ICCM0284 and NCPGR231, with PVs found in the range of 21.7–35.6% in the same year (Table 1). Figure 3 depicts the Q–Q plot for FW resistance in 2016 and 2017 based on GLM analysis. In year 2017, GLM revealed significant association with R^2 (11.7–37.5%) for seven SSR markers (NCPGR40, NCPGR180, NCPGR249, NCPGR231, NCPGR149, CESSR433 and ICCM 0284) (Table 2). However, association of the three markers CESSR433, ICCM0284 and NCPGR231 was evident through MLM analysis, with PVs extending up to 33.4% in the year

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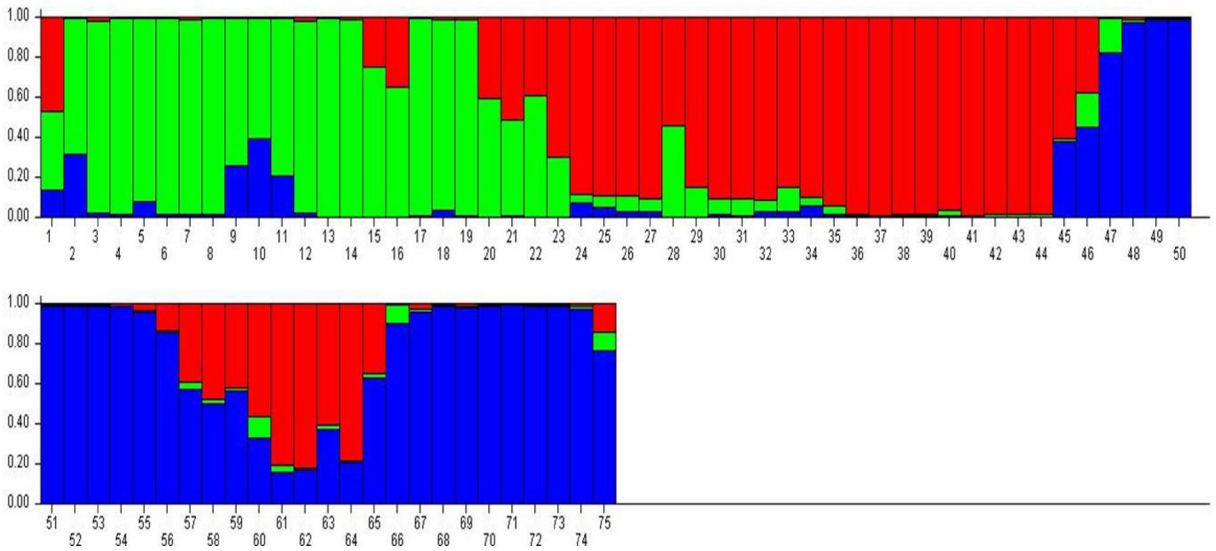

Fig. 2. Population structure analysis of 75 chickpea genotypes.

Table 1. Significant MTA for FW resistance in chickpea obtained in the year 2016 by Q GLM and MLM approaches of association mapping

GLM mean in year 2016	LG group	*P* value	R^2 (%)
NCPGR40	CaLG02	0.012*	11.4
NCPGR180	CaLG03	0.012*	11.3
NCPGR249	CaLG01	0.014*	7.9
NCPGR231	CaLG04	0.016*	19.9
NCPGR149	–	0.016*	19.8
CESSR433	CaLG01	0.004**	45.4
ICCM0284	CaLG06	0.001**	39.7
Significant MTA for FW resistance in chickpea obtained in 2016 in the year by Q MLM approach of association mapping			
MLM mean in year 2016	LG group	*P* value	R^2 (%)
CESSR433	CaLG01	0.009	31.5
NCPGR231	CaLG04	0.022	21.7
ICCM0284	CaLG06	0.0004	35.6

*Significant at 5% level.
**Significant at 1% level.

2017 (Table 2). The *Q*–*Q* plots for years 2016 and 2017 based on MLM are shown in Fig. 3.

Candidate gene identification

The candidate genomic regions showing significant association with FW resistance were BLASTed for gene prediction against the CDC frontier genome sequence (Varshney *et al.*, 2013). As a result, seven candidate genes with putative function (see Table 3) were predicted for significant MTAs for FW (*Foc2*) tolerance.

Discussion

Marker attributes such as genome abundance, reproducibility and cost-efficiency make SSR the preferred marker system for marker-assisted breeding programmes (Semagn *et al.*, 2006; Kalia *et al.*, 2011). In the current study, 267 alleles with an average of 3.56 alleles per marker were obtained following analysis of 75 FW-responsive chickpea genotypes with 75 SSRs. The level of heterozygosity ranged from 0.3 (NCPGR171) to 0.80 (NCPGR255) with a mean of 0.59. These results were in close agreement with earlier findings in chickpea (Upadhyaya *et al.*, 2008; Ghaffari *et al.*, 2014; Hajibarat *et al.*, 2015; Jha *et al.*, 2018).

Identification of MTAs for traits of breeding relevance has received greater attention for accelerating crop improvement. Some noteworthy examples of MTAs identification in chickpea include mapping of drought (Thudi *et al.*, 2014), heat stress (Jha *et al.*, 2018), seed weight, seed protein content (Upadhyaya *et al.*, 2016a) and grain zinc content (Upadhyaya *et al.*, 2016b).

The genetic underpinnings of FW resistance were elucidated earlier in chickpea (Winter *et al.*, 2000; Sharma *et al.*, 2004, 2005; Sabbavarapu *et al.*, 2013; Caballo *et al.*, 2019; Mannur *et al.*, 2019), and the MTAs in these studies were identified using biparental quantitative trait locus (QTL) mapping. However, the potential of association genetics had not previously been explored to identify genomic

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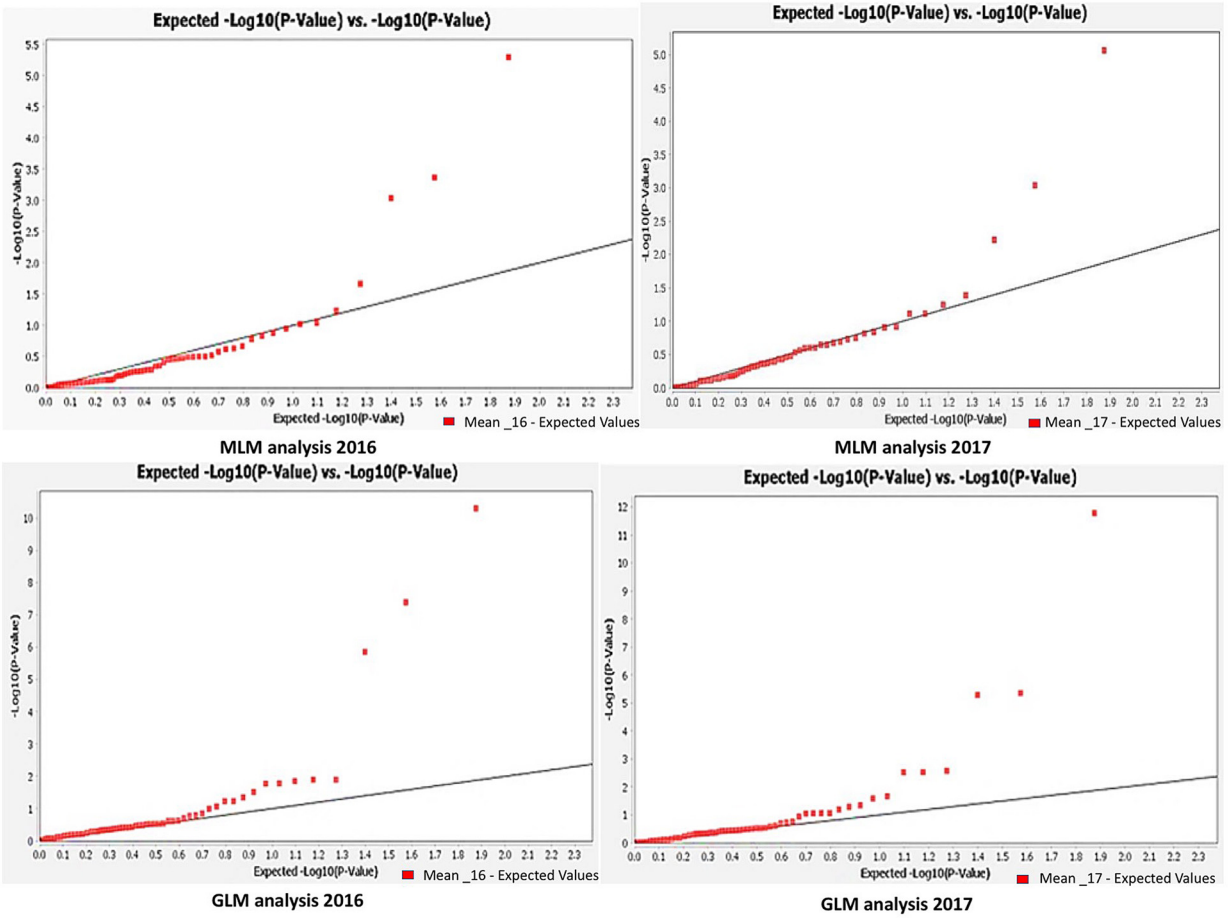

Fig. 3. *Q–Q* plots of AM for FW resistance in chickpea evaluated in 2016 and 2017 using MLM and GLM analyses.

regions underlying FW resistance. In the current study, significant association of the marker ICCM0284 with FW was detected on LG06 by both GLM and MLM approaches in both years (2016 and 2017). Earlier, Sabbavarapu *et al.* (2013) recorded two major QTLs on LG06 that explained up to 18% PV for FW resistance (race 1). In the current study, another significant MTA (NCPGR40) for wilt resistance was identified on LG02 by GLM analysis in two years. A similar QTL analysis reported QTLs for early and late wilt on LG02 (Patil *et al.*, 2014). Notably, chickpea LG02 harbours resistance gene(s)/QTLs for FW races 1 (*Foc1*), 3 (*Foc3*), 4 (*Foc4*) and 5 (*Foc*5) (Ratnaparkhe *et al.*, 1998; Tullu *et al.*, 1998; Winter *et al.*, 2000; Sharma *et al.*, 2004; Varshney *et al.*, 2014; Caballo *et al.*, 2019). The GLM and MLM analyses also detected the marker CESSR433 on LG01, showing linkage with wilt resistance in both years 2016 and 2017. Occurrence of QTLs for FW resistance in LG01 was reported earlier in chickpea (Jingade and Ravikumar, 2015). It is interesting to note that three SSR markers NCPGR231, CESSR433 and ICCM0284 displayed significant MTAs for FW tolerance consistently for two years in both GLM and MLM analyses. Thus, these SSR markers could be used as proxies for selecting desirable level of resistance against FW in chickpea.

Candidate genes underlying the marker intervals with their putative functions are presented in Table 3. The major genes underlying candidate genomic regions could be predicted as *Ca_23618*, *Ca_14730*, *Ca_13845*, *Ca_24529*, *Ca_03351*, *Ca_17303* and *Ca_20129*. The probable function of *Ca_23618* candidate gene is to code for a RING type zinc finger protein. Similarly, the candidate gene *Ca_14730* encodes a putative uncharacterized protein, while the possible protein coded by *Ca_20129* is a MYB-like protein. Among these, the role of the zinc finger protein domain in the plant disease response has been reported in barley (Shirasu *et al.*, 1999), rice (Xu and He, 2007) and *Arabidopsis* (Shi *et al.*, 2014). Similarly, MYB protein participating in disease resistance has been demonstrated in *Arabidopsis* (Mengiste *et al.*, 2003), potato (Tai *et al.*, 2013) and wheat (Al-Attala *et al.*, 2014; Shan *et al.*,

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Table 2. Significant MTAs for FW resistance in chickpea obtained in the year 2017 by Q GLM and MLM approaches of association mapping

Significant MTA for FW resistance obtained in the year 2017 by Q GLM approach of association mapping			
GLM mean in year 2017	LG group	*P* value	R^2 (%)
NCPGR249	CaLG07	0.002**	11.7
NCPGR180	CaLG03	0.002**	14.9
NCPGR40	CaLG02	0.002**	14.9
NCPGR231	CaLG04	0.021*	19.1
NCPGR149	–	0.02*	18.5
ICCM0284	CaLG06	0.0004**	37.5
CESSR433	CaLG01	0.0005**	36.7
Significant MTA for FW resistance in chickpea obtained in year 2017 by Q MLM approach of association mapping			
MLM mean in year 2017	LG group	*P* value	R^2 (%)
CESSR433	CaLG01	0.006**	24.2
NCPGR231	CaLG04	0.040*	19
ICCM0284	CaLG06	0.009**	33.4

*Significant at 5% level.
**Significant at 1% level.

2016). Previously, a set of candidate genes controlling FW resistance had been identified in chickpea that included *Ca_14301* encoding NB-ARC domain disease resistance protein, controlling tolerance against race *Foc4* (Mannur *et al.*, 2019), and *LOC101511605* encoding CBL-interacting serine/threonine-protein kinase 8-like for *Foc5* (Caballo *et al.*, 2019). Functional validation of these candidate genes and cloning of the causative gene could greatly assist precise incorporation of FW resistance into chickpea cultivars.

Conclusion

We investigated the disease response of 75 chickpea genotypes for FW using the association mapping approach. To the best of our knowledge, this is the first MTA analysis for FW (*Foc2*) in chickpea that analyses an FW-responsive chickpea collection with genome-wide SSRs. Based on the results of both GLM and MLM, three SSR markers NCPGR231, CESSR433 and ICCM 0284 consistently displayed significant associations with FW (*Foc2*) resistance across both years. However, fine mapping will be needed to precisely delineate the causative gene(s) underlying the candidate genomic regions for future research and breeding for FW resistance in chickpea.

Supplementary material

The supplementary material for this article can be found at https://doi.org/10.1017/S1479262121000228.

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Author contributions

UCJ conceived the idea and wrote the MS with AB, SP and NPS. SKC provided the material; ML and PRS conducted the phenotyping; RJ and VT performed the genotyping and analysis. AB edited the manuscript. All authors have read and approved the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

Table 3. Candidate genes underlying the marker intervals with their putative functions (based on InterPro)

Marker	Chromosome	Position	Effect	Strand	Gene	Function
CESSR433	CaLG01	18,130,935	Intron	+	*Ca_14730*	D7T6D5_VITVI putative uncharacterized protein OS = *Vitis vinifera* GN = VIT_05s0020g01270 PE = 4 SV = 1
NCPGR231	scaffold38	224,459	URR	+	*Ca_23618*	IPR001841; Zinc finger, RING-type
ICCM0284	CaLG04	22,202,321	DRR	–	*Ca_20129*	IPR017877; MYB-like
NCPGR40	CaLG06	45,528,875	DRR	–	*Ca_13845*	IPR001752; Kinesin, motor domain
NCPGR180	scaffold528	188,266	URR	–	*Ca_24529*	NMCP_ARATH putative nuclear matrix constituent protein 1-like protein OS = *Arabidopsis thaliana* GN = At5g65770 PE = 2 SV = 1
NCPGR249	CaLG07	1,208,942	CDS	+	*Ca_03351*	IPR001789; signal transduction response regulator, receiver domain
NCPGR149	CaLG07	9,513,960	DRR	+	*Ca_17303*	IPR006702; uncharacterized protein family UPF0497, trans-membrane plant

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