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Development of Novel Microsatellite Markers Using Genome Sequence Information in Chickpea (*Cicer arietinum* L.)

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Abstract

Fusarium wilt (FW) is a major disease of chickpea (*Cicer arietinum* L.) limiting the productivity in all the chickpea growing areas. Several DNA markers linked to FW resistance were reported earlier. However, these markers were identified based on limited size of mapping population and limited number of markers representing wide genomic region and not reliable for Marker Assisted Selection (MAS). In the present study, QTLs and/or markers linked to wilt resistance in genetic msap were *in-silico* physically mapped on chickpea genome sequence. Majority of FW linked markers mapped to chromosome (Ca) 2, 4 and 6 in sequence based physical map. The highest number of markers and QTLs were located on Ca2 indicating hotspot for wilt resistance. Scanning of genomic regions of FW resistance flanked by TA110-H3A12, TA200-TA37 and TA27-TA59 on Ca2, and CaM1402-CaM1101 on Ca6, covers a region of 14.41 Mb and 13.58 Mb, respectively covering 1027 genes and 1385 genic SSR repeats. Four hundred and nine genic SSR primer pairs were designed from identified genes and out of which 165 primers were synthesised and checked for amplification in chickpea. All newly designed primers except seven gave expected amplicon size and hence will be used for fine mapping the *Fusarium* wilt resistance loci.

Chickpea is the second most cool season legume crop after common bean (*Phaseolus vulgaris*) with a genome size of H"738Mb (Varshney *et al.,* 2013). Even though chickpea production has increased, the productivity (962 kg/ha) has remained low (Anonymous, 2016). The low productivity is due to several biotic and abiotic stresses that affect the crop at all growth stages.

Fusarium wilt caused by soil borne fungal pathogen *Fusarium oxysporum* f. sp. c*iceri* (FOC) is a serious pathogen of chickpea. Among eight race of FOC (0, 1A, 1B/C, 2, 3, 4, 5 and 6) (Jimenez-Diaz *et al.,* 1993), race 1 is predominant in peninsular India. On an average the annual yield loss from this disease is estimated to be 10 per cent which can go up to 90 per cent under favourable conditions (Singh and Reddy, 1991). Being soil borne, the management of FW is difficult. Cultivation of FW resistant varieties can be an effective management strategy to reduce the yield loss. However, development of resistant varieties for wilt through traditional breeding methods and maintenance of wilt sick plot is difficult and time consuming. Hence, molecular breeding could become an efficient strategy to speed up the development of wilt resistant genotypes.

Mayer *et al.,* (1997) was first to report molecular markers CS-27_{700} (allele specific) and UBC-170 (locus specific) linked to resistance genes for FW against race 1 (*Foc1*). Sharm *et al.,* (2004) mapped the resistance loci *Foc1*, *Foc3* and *Foc4* on linkage group (LG) 2. Gowda *et al.,* (2009) also mapped *Foc1*, *Foc2* and *Foc3* loci on LG2 and Barman *et al.,* (2014) mapped *Foc1* on LG2. Sabbavarapu *et al.,* (2013) identified the two new QTL for FW resistance for race 1 *i.e., FW-Q-APR-6-1* and *FW-Q-APR-6-2* on LG6 with phenotypic variation 10.4 and 18.8 per cent, respectively. Patil *et al.,* (2014) mapped two QTLs associated with early and late wilting on LG2, i.e., *Wilt 1* and *Wilt 2,* respectively.

The two wilt resistance loci located on LG2 and LG6 could be super loci function against many races. It is important to fine map these loci to determine the candidate genes for resistance and to develop reliable robust markers for MAS. To drive fine mapping of QTLs linked to wilt resistance, development of microsatellite, SNP and indel markers in the genomic region controlling wilt resistance is important. The development of markers in any genomic region has now become simpler and easy. High throughput NGS provides a rapid means of discovery of genes/markers

based on genome/transcriptome sequences. Recently chickpea genome has been sequenced and its genome sequence information is available in the database (Varshney *et al.,* 2013). The physical mapping of QTLs associated with FW resistance and development of polymorphic markers in QTL regions would facilitate fine mapping of QTLs and development of robust markers of MAS.

Based on the information available 29 markers closely linked to wilt resistance to race 1 and/or markers flanking QTLs for the FW resistance were selected for BLAST (Basic Local Alignment Search Tool) analysis against chickpea genome in the NCBI (National Center for Biotechnology Information) database. Out of 29 markers, CS-27 is an Allele Specific Associated Primer marker, A07C is a RAPD marker and the remaining 27 were SSR markers. The forward and reverse primer sequences of selected markers were used as query sequences for homology search in chickpea genome sequence in NCBI. The following parameters *viz*., (a) sequence producing significant alignment with lowest E-value (Expect value), (b) maximum identity, (c) no gaps, and (d) both primer pair sequences should fall on same chromosome on +/- strands opposite to one another with in 500bp were used for placing query sequence on the sequence based physical map of chickpea.

Four *Foc1* loci flanked by CS27-TA96 (Sharma et al., 2004), TA110-H3A12 (Gowda *et al.,* 2009), TA110-TA200 (Millan *et al.,* 2010), and TA200-TA37 (Barman *et al.,* 2014) on LG2; and four QTLs *FW-Q-APR-6-1* and *FW-Q-APR-6-2* (Sabbavarapu *et al.,* 2013) on LG6, *Wilt 1* and *Wilt 2* (Patil *et al.,* 2014) on LG2 flanked by CaM1402-CaM1101, CaM1125-TA22, TA27-TA59 and TA27-TA110, respectively were selected from genetic map for *in silico* physical map studies. In sequence based physical mapping, genes were identified from the genomic region flanked by these markers covering QTLs/resistant loci using NCBI map viewer (http://www.ncbi.nlm.nih.gov/projects/sviewer). All the genes identified were scanned for SSRs (mono, di, tri, tetra, penta and hexanucleotide repeats) using online Webstat software (http://purl.oclc.org/NET/websat/). Primer designing was tried using webstat software for all the genic SSR repeats identified (Martins *et al.,* 2009). A few genic SSR primers were selected for synthesis based on following two criteria, (a) SSR should repeat more than seven times and (b) avoid mono-nucleotide repeats These synthesised primers were used for amplification of genomic DNA of three chickpea genotypes.

Among 29 markers selected for *in silico* physical mapping, five markers (CS-27, TS47, TR2, H1A12, and NCPGR58) did not follow the criteria mentioned above; hence these markers were no longer considered for the study. Eight SSR markers were mapped on the unplaced genomic scaffold and in this region we identified genes and genic microsatellites for which primers were designed for future use. The remaining 16 markers were mapped to chromosomes. In chickpea till now four QTLs (Sabbavarapu *et al.,* 2013; Patil *et al.,* 2014) and four genomic regions (Sharma *et al.,* 2004; Gowda *et al.,* 2009; Millan *et al.,* 2010; Barman *et al.,* 2014) for *Fusarium* wilt resistance has been reported independently.

The physical mapping of four QTLs using flanking genetic markers resulted in the identification of only two chromosomal regions (Table I). Similarly out of four *Foc1* loci only two could be physically mapped using flanking markers.

Totally by physical mapping four chromosomal regions were identified for FW resistance. The chromosomal region one is a combined genomic region of two *Foc1* loci (flanked by TA110-TA200 and TA110-H3A12) and one QTL (flanked by TA27-TA110). This region one was mapped to *C. arietinum* chromosome 2 (Ca 2) covering 6.84 Mb genomic region. This genomic region covers 310 genes out of which 49 were uncharacterised in NCBI database (Table 1). Totally from all the genes in this region, 288 genic SSR repeats comprises of 203 mono, 57 di, 19 tri, seven tetra, one penta and one hexanucleotide repeats were observed. The second chromosomal region flanked by markers TA37and TA200 was mapped to Ca2 flanking 1.73 Mb genomic region which covers about 62 genes out of which 19 were uncharacterised in NCBI database (Table 1). Ninety genic SSR repeats were identified, out of which 57

TABLE I

In silico physical mapping of DNA markers linked to FW resistance in chickpea genome sequence.

Genetic map				*In silico* physical map					
QTLs\resistant loci	Flanking markers	LG	References	Chromo-some	Position of flanking marker (bp)	Distance covered (Mbp)	No. of genes	No. genic SSR markers	Primers designed
Foc 1 locus	TA110-H3A12	2	Gowda *et al.*, 2009	Ca2	9410378-16257461	6.84	310	288	74
Foc1 locus	TA200-TA37	2	Barman *et al 2014*	Ca2	15459860-17196573	1.73	62	90	55
Wilt-1 (QTL)	TA27-TA59	2	Patil *et al.*, 2014	Ca2	17196296-23029285	5.83	135	204	141
FW-Q-APR-6-1	CaM1402-CaM1101	6	Sabbavarapu *et al.*, 2013	Ca6	42884010-56466494	13.58	520	803	139

mono, 25 di, seven tri, one penta, two hexa and zero tetranucleotide repeat. Both the *Foc1* loci were present on LG2 in genetic map reported.

The third region flanked by TA27-TA59 covering 5.83 Mb of genomic region comprises 135 genes out of which 46 were uncharacterised in NCBI database (Table 1). Two hundred and four genic SSR repeats have been identified comprises 135 mono, 53 di, 14 tri, one tetra, one penta and zero hexanucleotide repeats.

The fourth region flanked by markers CaM1402-CaM1101 was physically mapped on Ca6. These markers cover 13.58 Mb genomic regions. This genomic region covers 520 genes out of which 40 were uncharacterised in NCBI data base (Table I). This genomic region contains about 803 genic SSR repeats out of which 522 were mono, 182 di, 64 tri, four tetra, 30 penta and one hexanucleotide repeat.

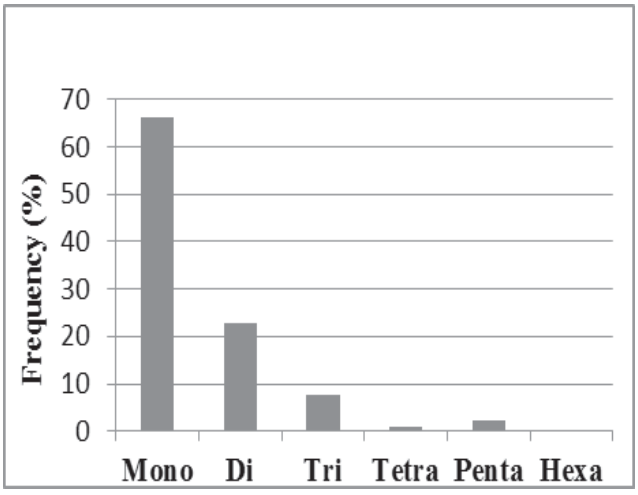

Fig. 1: Frequency of microsatellite repeats found in FW resistance regions of chickpea genome sequence.

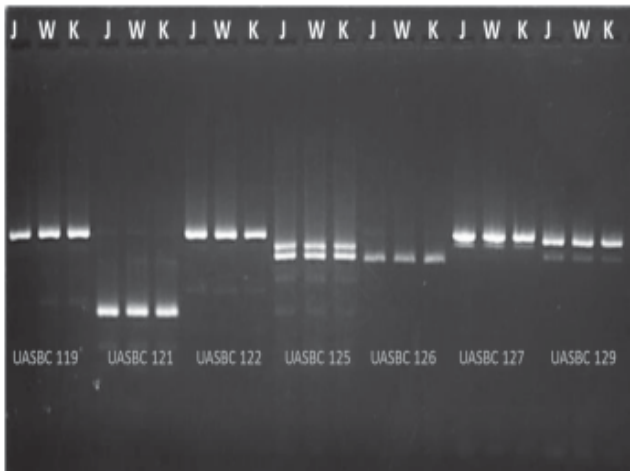

Fig. 2**:** Amplification of genomic DNA by newly synthesised genic SSR primers

The four physically mapped regions contain 1027 genes and 1385 SSR repeats. Among SSR repeats mononucleotide repeats were highest in frequency followed by di, tri and penta nucleotide repeats (Fig 1). Totally 409 primer pairs were designed for genic SSRs of which 270 were in Ca2 and 139 were in Ca6. From 409 SSR primers, 165 were selected for synthesis. These synthesised SSRs were tested for amplification of chickpea genomic DNA of three genotypes JG62, WR315 and K850. Out of 165 primer pairs synthesised 158 showed perfect amplification and produced the expected product size in all the three genotypes (Fig. 2). Hence, the new primers developed based on *in silico* physical mapping of FW resistance loci will be useful for fine mapping the genomic region linked to wilt resistance. The development of closely linked markers support marker assisted selection in chickpea breeding.

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