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Marker assisted characterization of chickpea genotypes for wilt resistance

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Marker assisted characterization of six chickpea genotypes differing for *Fusarium* wilt reaction was carried out using seven molecular markers reported by earlier workers linked to disease resistant/susceptibility. In the present study, four different markers (namely, CS-27, UBC-170, CS-27A and UBC-825) linked to susceptibility and three microsatellite based markers (TA-59, TA-96 and TR-19) linked to resistance allele were validated. It was observed that two Random Amplified Polymorphic DNA (RAPD) markers, CS-27 and UBC-170 and one sequenced characterized amplified region (SCAR) CS-27A$_{700}$ gave amplification of 700, 550 and 700 bp, respectively in susceptible genotype only as reported by earlier worker. The inter simple sequence repeat (ISSR) marker UBC-825 produced amplification of 1200 bp in susceptible genotypes (JG-62 and GG 4) and intermediate genotype (Chaffa). Three sequence tagged microsatellites site (STMS) primers (TA-59, TA-96 and TR-19) gave specific allele in wilt resistant genotypes. The PCR amplification of TA-59 primer generated two alleles, out of which the allele of 258 bp was present only in resistance genotypes. The alleles of 265 bp amplified by primer TA-96 was present only in resistance genotypes and absent in other genotypes. The marker TR-19 amplified allele of 227 bp in resistant genotypes. Further, the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) analysis of seed storage protein showed a difference in protein profile among studied genotypes but none of polypeptide fragment was specific to wilt resistance or susceptibility. In present study, the reported markers linked to susceptibility and resistance proved their effectiveness and further can be exploited for maker assisted selection (MAS) of wilt resistance breeding in chickpea.

Key words: Chickpea, *Fusarium* wilt, molecular markers, resistance, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE).

INTRODUCTION

Chickpea (*Cicer arietinum* L.; 2n = 2x = 16; genome size of 750 Mbp) is an important legume crop in most of the developing countries in the world and ranks third among food legumes in production (FAO, 2012). Chickpea is most important pulse crop of India and its adjoining countries account for 90% of the total world production (Gupta et al., 2011). However, the average annual world chickpea yield (0.78 tons/ha) is considered comparatively lower than its potential yield (Sudupak et al., 2002). One major reason for the low productivity of cultivated chickpea is its narrow genetic base and its sexual incompatibility with other wild species of *Cicer* in natural inter specific

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crosses (Abbo et al., 2003). Furthermore, various biotic (*Fusarium* wilt, *Aschochyta* blight, nematodes and pests) and abiotic stresses (drought and cold) severely reduce the yield (Croser et al., 2003). Vascular wilt caused by the deuteromycetes fungal pathogen *Fusarium oxysporum* f. sp. *Ciceri* is one of the major constraints in realization of the full yield potential (4 tons/ha) of chickpea. This disease causes huge yield losses (10 to 90%) annually (Singh and Reddy, 1991). Therefore, many chickpea breeding programmes are focused on improving the genetic potential, both to increase yield and to provide protection against biotic and abiotic stresses (Rao et al., 2007).

The use of wilt resistant chickpea genotypes, when they are available, is the most effective and eco friendly method of managing the disease (Sabbavarapu et al., 2013). Identifying *Fusarium* wilt race specific resistance genes and transferring them to adapted backgrounds is a major challenge for plant breeders. Direct assays (screening) for these genes may be difficult, particularly when large number of breeding lines is involved. Isozymes and total protein banding pattern have direct/indirect application in plant breeding programmes and host–pathogen interactions. These have been used for characterisation of particular plant genotypes, cultivars, inbred lines, screening variability in plant populations and mapping of chromosomes (Moore and Collins, 1983). Marker assisted selection (MAS) using DNA markers tightly linked to wilt resistance genes can be used to screen a large number of germplasm lines for the presence of these genes without actually subjecting them to the pathogen and to pyramid them into agronomically superior varieties. MAS are an accurate, easy as compared to conventional method, less time consuming and independent to environmental conditions. The genetic studies showed that the resistance to race 4 was monogenic recessive in some lines (Tullu et al., 1998; Sharma and Muehlbauer, 2005); whereas it was digenic recessive in Surutato-77 (Tullu et al., 1999).

Several studies are under taken to decipher the molecular marker closely linked to *foc*-4 resistance. Various markers namely, RAPD, SCAR, ISSR, STMS closely linked to *foc*-4 were reported (Sharma and Muehlbauer, 2007). Further validation of these markers in other genotypes is necessary to prove their efficiency to characterize chickpea genotypes. Thus, the present study was under taken with an objective to validate reported markers with selected chickpea genotypes showing different reaction to wilt disease.

MATERIALS AND METHODS

Plant material

Six chickpea genotypes differing in wilt disease reaction (Rathod and Vakharia, 2011) that is, WR-315 and ICCV-2 (resistant), GG-1 (tolerant), GG-4 (susceptible), JG-62 (highly susceptible) and Chaffa (moderately susceptible) were procured from Castor and Pulses Research station, NAU, Navsari, for molecular characterization against *fusarium* wilt. All genotypes were sown in plastic pots and leaf samples were taken after 20 days of sowing.

Seed storage protein extraction and SDS PAGE

The seed storage protein was extracted according to procedure of Hameed et al. (2009). The procedure of Laemmli (1970) was used for the electrophoresis analysis of protein on vertical SDS PAGE (10%). The standard staining and de-staining procedures were used for visualization of clear protein fragments.

DNA isolation

Total genomic DNA was isolated from the young leaves following the CTAB method described by Rogers and Bendich (1988) with minor modifications. The quality and quantity were estimated by measuring O.D. at 260/280 and 260 nm, respectively in a Nanodrop spectrophotometer. Intactness of genomic DNA was checked on 0.8% agarose gel.

PCR amplification and electrophoresis

PCR amplification was performed following the procedure given by Sethy et al. (2006) with minor modifications. Seven molecular markers reported by earlier worker linked to wilt resistance were synthesized from MWG Biotech, Germany (Table 1). PCR amplifications were performed in 25 µl volumes using a thermal cycler (Bio-Rad, USA). The reaction mixtures contained 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 60 ng genomic DNA, 25 pmol of each primer, 1.5 mM $MgCl_2$, 2.5 mM of each dNTP and 1.5 units of Taq DNA polymerase (Biogene, USA). The temperature profile consisted of an initial denaturation step of DNA at 94°C for 3 min, followed by 40 cycles: 94°C for 1 min, 55 to 59°C for 1 min, and 72°C for 1 min. Annealing temperatures were optimized individually for each primer. After the final cycle, samples were incubated at 72°C for 10 min to ensure complete extension followed by hold at 4°C. PCR amplified products were mixed with 4 µl of 6x loading dye (0.25% bromophenol blue, 0.25% xylene cynol and 40% sucrose, w/v), electrophoresed on 1.5 or 3.0% agarose gel, stained with ethidium bromide and visualized under UV light.

Data analysis

The amplified products were visualized under UV light and photographed by Bio- Rad gel documentation system. The presence or absence of specific band linked to resistance/susceptible allele reported by earlier worker was recorded. For SDS PAGE analysis, the binary data was prepared using the presence and absence of polypeptide fragment as 1 and 0, respectively. The data was analyzed using numerical taxonomy and multivarient analysis (NTSYS-pc) system version 2.02i by Exeter software (Rohlf, 2004). Jaccard's similarity coefficient was calculated using SIMQUALK programme. A dendrogram was produced using mean of the Unweighted pair group method with arithmetic averages (UPGMA) analysis.

RESULTS AND DISCUSSION

Large number of molecular markers linked to different wilt resistance genes were identified and mapped (Sharma and Muehlbauer, 2007). The available molecular marker information may be used for MAS. In present study, two RAPD, one each for SCAR and ISSR, and three STMS

Table 1. Information regarding molecular markers employed for marker assisted characterisation of six diverse chickpea genotypes with their linkage distance from resistant (R) gene in centi Morgan (cM) and expected fragment size in base pair (bp). The molecular marker reported by earlier worker linked to disease resistance/susceptibility was used.

Source	Primer	Primer sequence	Distance from R gene (cM)	Expected fragment size (bp)
Tullu et al. (1999)	CS-27	AGT GGT CGC G	15.2	700
	UBC-170	ATC TCT CCT G	9.0	550
Mayer et al. (1997)	CS-27A	F – AGC TGG TCG CGG GTC AGA GGA AGA R – AGT GGT CGC GAT GGG GCC ATG GTG	3.3	700
Ratnaparkhe et al. (1998)	UBC-825	ACA CAC ACA CAC ACT	5.0	1200
Winter et al. (2000)	TA-59	F - ATC TAA AGA GAA ATC AAA ATT GTC GAA R - GCA AAT GTG AAG CAT GTA TAG ATA AAG	3.8	258
	TA-96	F –TGT TTT GGA GAA GAG TGA TTC R- TGT GCA TGC AAA TTC TTA CT	3.3	275
	TR-19	F - TCA GTA TCA CGT GTA ATT CGT R- CAT GAA CAT CAA GTT CTC CA	3.1	227

markers previously reported to linked with disease resistance gene were tested for their ability to differenttiate the wilt resistance and susceptible genotypes. It was observed that two RAPD markers CS-27 and UBC-170 reported by earlier worker linked to disease resistance gave amplification of 700 and 550bp, respectively in susceptible genotype only (Figure 1A and B). The SCAR marker CS-27A gave amplified product of 700 bp in susceptible genotypes only, the amplification was absent in other genotypes (Table 2 and Figure 1C). Further, the ISSR marker UBC-825 gave amplification of 1200 bp in susceptible and moderately susceptible genotypes. Three STMS primer (TA-59, TA-96 and TR-19) were utilized in present study to characterize chickpea genotype for *foc*-4 resistance. The PCR amplification of TA-59 primer generated two alleles, out of which the allele of 258 bp was observed only in resistant genotypes (Figure 1E). The alleles of 265 bp amplified by primer TA-96 was present only in resistant genotypes; whereas the same was absent in other genotypes (Table 2 and Figure 1F). The marker TR- 19 amplified allele of 227 bp in resistant genotypes. The seed storage proteins of chickpea genotypes separated on 10% SDS PAGE resolved a total number of 21 bands (Figure 2). In present study, differences have been observed among studied genotypes based on protein profiling but none of polypeptide fragment was specific to wilt resistance or susceptibility. However, dendrogram based on Nei's similarity coefficient could distinguish some sort of grouping among resistant and susceptible genotypes. Moderately resistant genotype Chaffa was laid on separate cluster while resistant genotype WR-315 was laid on sub-cluster of cluster-I. Moreover, another resistant and tolerant genotype that is, ICCV-2 and GG-1 was laid on same subcluster of cluster-II, the susceptible genotypes GG-4 and JG-62 were present in one cluster (Figure 3). These results may be further confirmed using large number of diverse chickpea accessions.

The seed storage protein profiling by SDS-PAGE had been exploited for inter and intra species diversity analysis in *cicer* (Asghar et al., 2003; Hameed et al., 2009). But it had not been exploited until today for characterization of disease resistance in cicer. Total seed storage protein profiling by SDS-PAGE revealed the presence of two unique protein of ~97 and ~100 kDa in pearl millet genotypes resistant to downey mildew (Mahatma et al., 2011). The molecular markers linked to either susceptibility or resistance have been effectively utilized for MAS. In present study, we have employed four different susceptibility linked markers (namely CS-27, UBC-170, CS-27A and UBC-825) which amplified specific fragment of reported size in susceptible genotypes only. Tullu et al. (1999) reported that the RAPD ($CS\text{-}27_{700}$) marker locus is linked to one of the resistance genes inferred from the F_2 phenotypic data. They found that the marker linked to the *fusarium* wilt resistant genes consistently amplified a distinct DNA fragment (700 bp) in the susceptible F_2 plants. Similar size of fragment was observed in susceptible genotypes (JG 62 and GG 4) in present study. The RAPD marker $CS\text{-}27_{700}$ and $UBC\text{-}170_{550}$ were also reported to link with the susceptibility (Tullu et al., 1998). The study on inheritance and linkage of a gene for resistance

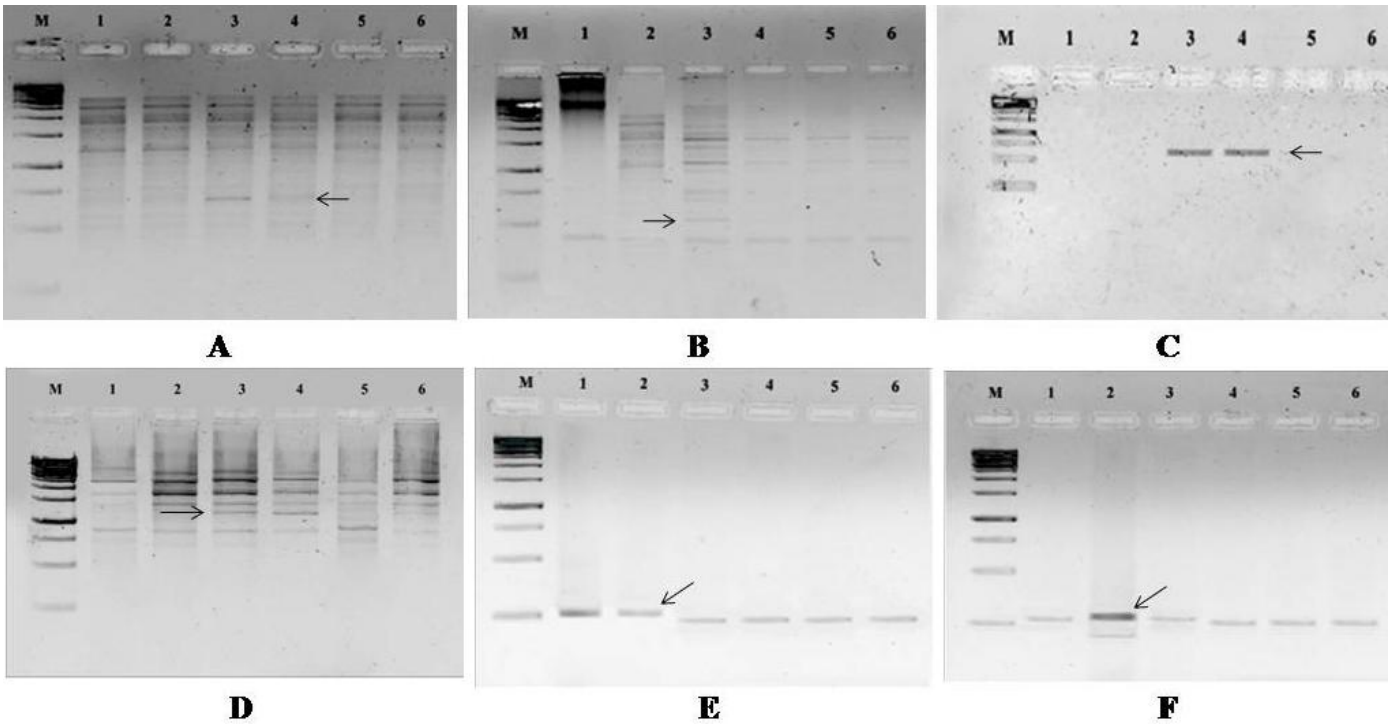

Figure 1. A subset of alleles revealed by primer pairs (A) CS-27, (B) UBC-170, (C) CS-27A, (D) UBC-825, (E) TA-59 and (F) TA-96 among six diverge genotypes of *Cicer arietinum* L. PCR products were separated on agarose gel along with DNA ladder and photographed by gel documentation system (Bio- Rad, USA). Lanes 1 to 6: M = 250 bp DNA ladder, 1 = WR-315, 2 = ICCV-2, 3 = JG-62, 4 = GG-4, 5 = GG-1 and 6 = Chaffa. The fragment of interest (described in Table 2) has been marked with arrow.

Table 2. Reaction of six diverse chickpea genotypes with seven molecular markers linked to disease resistance. The presence of specifically disease resistance/susceptibility linked DNA fragment was analysed using fragment size analysis tool available in Bio- Rad, USA by comparing it with standard DNA ladder.

Genotype	Wilt reaction	Markers linked to the resistance gene (bp)						
		RAPD		SCAR	ISSR	STMS		
		CS-27	UBC-170	CS-27A	UBC-825	TA-59	TA-96	TR-19
WR-315	Resistant	-	-	-	-	258	265	227
ICCV-2	Resistant	-	-	-	-	258	265	227
JG-62	Highly susceptible	700	550	700	1200	-	-	-
GG-4	Susceptible	700	550	700	1200	-	-	-
GG-1	Tolerant	-	-	-	-	-	-	-
Chaffa	Moderately susceptible	-	-	-	1200	-	-	-

to race 4 of *fusarium* wilt and RAPD markers in chickpea shown that these two RAPD markers were located 9 map unit from the race 4 resistance locus and were on the same side of resistance gene. The linkage of the CS 27_{700} marker with wilt susceptibility was established through study on fifteen genotypes with diverse background (Soregaon and Ravikumar, 2010). Ratnaparkhe et al. (1998) identified new ISSR primer (UBC-825_{1200}) by changing the anchored region of the ISSR primers previously reported to linked with disease resistance gene.

The repeat $(AC)_8T$ amplified a marker, UBC-825_{1200}, which was located 5.0 centi Morgan (cM) from the gene for resistance to *fusarium* wilt race 4 and was closer than other markers. The microsatellite based UBC-825 was also able to identify the intermediate reacting genotype. So, considering the ability to give the amplification in intermediate genotypes also, this marker should always be used with other reported markers in order to avoid miss leading conclusions. Three different STMS markers (TA-59, TA-96 and TR-19) linked to resistance allele by 3.8, 3.3 and 3.1 cM distance respectively were shown expected amplification in resistant genotypes only. Winter et al. (1999) characterized and mapped 120 STMS on the chickpea genome map. The primer TA-59, TA-96 and TR-19 were mapped on same linkage group on which gene for disease resistance was present (Winter et al., 2000).

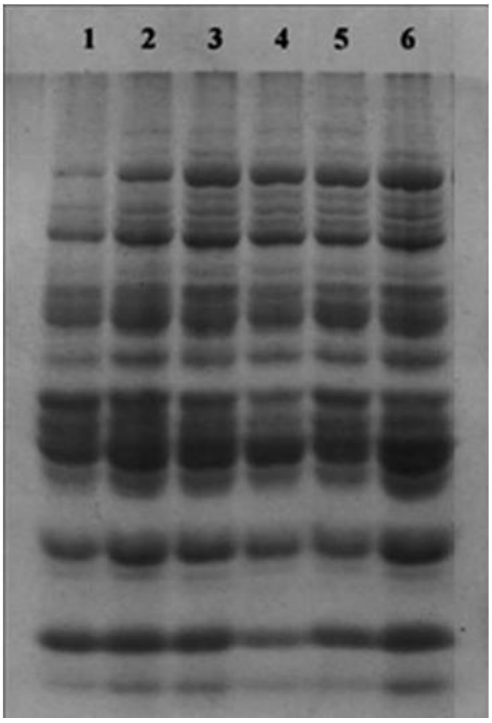

Figure 2. Seed storage protein profile generated by electrophoresis on 10% SDS PAGE. The PAGE resolved total 21 polypeptide fragments, but the fragment specific to susceptibility or resistance was absent. Lanes 1 to 6: 1 = WR-315, 2 = ICCV-2, 3 = JG-62, 4 = GG-4, 5 = GG-1 and 6 = Chaffa.

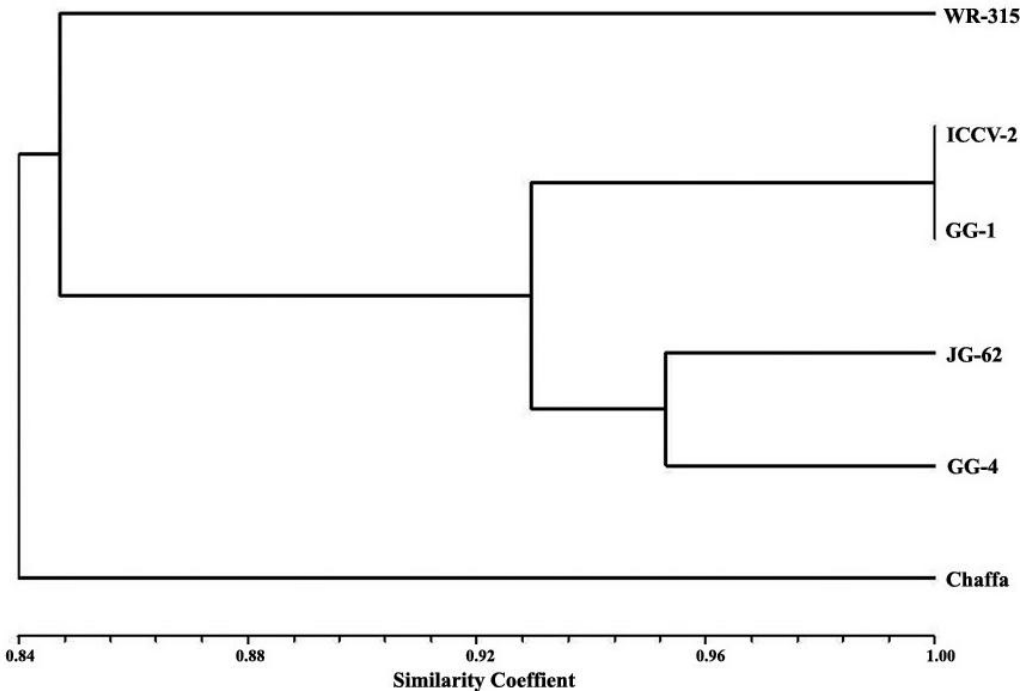

Figure 3. The dendrogram generated by NTSYS-pc (version 2.02i) based on UPGMA using Jaccard's coefficient of seed storage protein data. The dendrogram distinguished some sort of grouping among resistant and susceptible genotypes.

The amplification size of TA-59, TA-96 and TR-19 as characterized in *cicer* sp. were 278, 275 and 227 bp, respectively. Our results are closely consistent with reported results. These three STMS primers showed specific amplification pattern in resistant genotype, which can be effectively utilized for large scale screening in disease resistance breeding as well as for marker assisted breeding programme.

In the present study, seed storage protein profiling and seven different molecular markers (CS-27, UBC-170, CS-27A, UBC-825, TA-59, TA-96 and TR-19) linked to disease resistance were analyzed on six diverse chickpea genotypes. Though, seed protein fragment analysis offered clustering of genotypes, fragment specific to resistance or susceptibility was not reported. The molecular markers validated in this study could be effectively utilized for marker assisted selection in disease resistance breeding of chickpea.

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