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Identification of DNA markers linked to H_2 locus of fusarium wilt resistance in chickpea (*Cicer arietinum* L.)

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Abstract

Resistance to chickpea wilt caused by *Fusarium oxysporum* f.sp. *ciceris* race 1 is governed by two to three genes. The DNA marker linked to H_1 locus is already available. In order to identify DNA marker linked to H_2 locus of wilt resistance, the recombinant inbred lines derived from the cross K 850 (late wilting) x WR-315 (resistant), segregating for only H_2 locus were utilized. The recombinant inbred lines showed 1:1 segregation for late wilting and resistance. Seventy-nine random oligonucleotide primers of 10 to 11 base pairs were used to study the polymorphism in parents. The primer A07C amplifies an extra band of 417 bp in susceptible parent and co-segregate in susceptible bulk. The DNA marker $A07C_{417}$ showed monogenic segregation ratio of 1:1 in the recombinant inbred lines. The linkage analysis indicated that the $A07C_{417}$ marker is linked to H_2 locus and susceptibility and were separated by 21.7 centi Morgan (cM). The RILs of another cross JG-62 x WR-315 segregate for both H_1 and H_2 loci; consequently the DNA markers linked to H_1 and H_2 also showed independent segregation in the RILs of a cross JG-62 x WR-315. The $A07C_{417}$ marker was also found linked to H_2 locus of wilt susceptibility in different genotypes tested. The DNA marker $A07C_{417}$ showed linkage with H_2 locus across genetic backgrounds. The identification of DNA markers linked to both H_1 and H_2 of wilt resistance will facilitate marker-assisted selection and pyramiding of resistance genes to susceptible varieties.

Key words: Chickpea, wilt resistance, *Fusarium oxysporum*, DNA marker, recombinant inbred lines

Introduction

Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *ciceris* is one of the most widespread diseases of chickpea (*Cicer arietinum* L.). The disease is prevalent in all the chickpea growing areas of world [1, 2]. In the Indian sub-continent and the Mediterranean regions, the disease is very severe causing annual yield losses of 10-15 percent and more than 80 percent under favourable conditions [3]. The pathogen persists in soil as well as in seeds [4]. In India, the inoculum is spread to all chickpea growing regions of the country and race 1, of the eight races reported, is the most widespread [5, 6]. The chemical control of the disease is difficult because of its soil borne nature. Therefore, cultivation of resistant varieties is most important to reduce the yield loss. Stable resistant sources are available in the cultivated germplasm of chickpea [7]. However, development and maintenance of uniform wilt sick plots for testing breeding lines to develop resistant varieties is very difficult. Furthermore, the severity of disease is affected by inoculum concentration, virulence and environmental conditions [8]. The development of reliable alternative screening technique is very important for a sound wilt resistance breeding programme in chickpea. The identification of reliable DNA markers closely linked to resistant genes increases the efficiency of selection of resistant genotypes at very early stage of growth in the absence of wilt sick plots.

The genetics of wilt resistance against race 1 indicated three independent loci designated as H_1, H_2 and H_3 govern resistance to wilt [7]. However, our own and a few other studies indicated two major independent loci, H_1 and H_2 determine resistance to race 1 in chickpea [9-11]. The dominant alleles at both H_1 and H_2 loci result in early wilting and recessive at any one (h_1h_1 H_2_or

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$H_1\_h_2h_2$) produce late wilting and recessive alleles at both the loci (h_1h_1 h_2h_2) result in resistance. The primer pair CS-27F/CS-27R, termed as allele-specific associated primer (ASAP), that amplifies a fragment of 700bp linked to the allele for susceptibility at H_1 locus was developed [12]. We validated the marker in different chickpea genotypes and found it to be highly reliable in identifying the chickpea genotypes with susceptible allele at H_1 locus [13, 14]. However, alone this marker is not sufficient to identify resistant and susceptible genotypes /segregants as there are two independent loci governing resistance. There is a need to identify DNA markers linked to H_2 locus to screen a large number of breeding lines for complete resistance and to pyramid resistant genes into superior but susceptible varieties. Two RAPD markers linked to H_2 locus were reported [15]. However, the identified RAPD markers failed to show linkage across genotypes. Therefore, we continued our efforts to identify new DNA markers linked to H_2 locus and the results are presented here under.

Materials and methods

Plant material

The recombinant inbred lines (RILs) derived from two crosses K 850 x WR-315 (Population 1) and JG-62 x WR-315 (Population 2) were used for this study. The mapping population (Population 1) was obtained by crossing the late wilting genotype, K 850 ($h_1h_1H_2H_2$) with resistant WR-315 (h_1h_1 h_2h_2). The parents segregate only for H_2 locus. The 164 RILs (F_8 generation) of this cross were phenotyped for their wilt reaction by growing in the wilt sick plot considered as the standard for testing of chickpea wilt maintained at International Crop Research Institute for Semi Arid Tropics (ICRISAT) Patancheru, Hyderabad [16].

For population-2, the early wilting susceptible JG-62 (H_1H_1 H_2H_2) was crossed to resistant WR-315 (h_1h_1 h_2h_2). The parents differ at both the loci and the RILs (F_6 generation) showed digenic inheritance for wilt resistance [9]. Seventy-five RILs were used to study the segregation of markers identified in the mapping population. The DNA markers were also tested in four late wilting varieties A-l, BG-256, ICCV-2, ICCV 4958 and one early wilting cultivar, Karikadle for validation along with resistant cultivar WR-315.

DNA extraction

The genomic DNA was extracted from vegetative buds and young leaves of individual plants of both the RIL populations and the parental genotypes by following CTAB extraction method with little modification [17]. The DNA samples were diluted to a working concentration of 20-25 ng/µl and stored for further PCR amplification.

PCR amplification

Seventy-two random oligonucleotide primers (Operon Techn. Inc. Alamedas, USA) of 10 nucleotide length and seven primres with 11-nucleotide length (Sigma Aldrich Ltd) were used in the present study. Initial screening of the markers was done by bulked segregant analysis in the mapping population (population 1) [18]. The DNA of parents (K 850 and WR-315) was amplified individually using 79 primers to identify the primers producing polymorphic bands in parents. PCR amplification was carried out using Master Thermal Cycler 5331-Eppendorf version 2.30, 31-09, Germany, The initial denaturation was at 95°C for 5 minutes followed by 40 cycles of 1 minute at 94°C, 1 min at 36°C, 2 min at 72°C and final 8 min extension at 72°C.

Electrophoresis

The PCR products amplified with RAPD primers were resolved on 1.4 % agarose gels; stained with ethidium bromide and visualized under UV light (Uvitec, Cambridge. England). The primers, which showed polymorphism in the parents, were selected for further analysis. The DNA isolated from five homozygous resistant and five homozygous susceptible RILs were bulked separately to form resistant and susceptible bulk. The DNA of resistant and susceptible bulks was amplified individually using the primers which showed polymorphism in parents. The markers that showed polymorphism in the bulks and appeared to be associated with resistance or susceptibility were used subsequently to screen the entire mapping population - RILs- individually.

Segregation of DNA markers linked to H_1 and H_2 in population 2

Only one marker $A07C_{417}$ amplified by primer A07C (5'GAAACGGGTGCA3') was found linked to H_2 locus in the mapping population. The segregation of $A07C_{417}$ linked to H_2 locus was studied along with already identified CS27 marker [12] linked to H_1 in the RILs of Population 2. The DNA amplification for the primer A07C was carried out as mentioned earlier [15]. The PCR programme for amplification of CS27 marker linked to H_1 was carried out as reported earlier [12].

The DNA from late wilting varieties A 1, BG 256, ICCV 2, ICCV 4958 and the early wilting cultivar Karikadle was also tested for the presence of DNA markers linked to H_1 and H_2 loci.

Statistical analysis

The 164 recombinant inbred lines of mapping population showed 1:1 segregation for susceptibility (late wilting) and resistance. The data generated by DNA markers were recorded in a binary fashion. As they are dominant markers, scoring was based on presence or absence of a band. The linkage between the marker and H_2 locus was established by testing wilt reaction and marker for 1:1:1:1 segregation ratio in the mapping population. The linkage between markers and resistance gene was calculated using the MAPMAKER programme [19]. The map was constructed using a LOD score of three.

Joint segregation of markers linked to H_1 and H_2 loci in population 2

The parental genotypes JG-62 and WR-315 showed polymorphism for both the DNA markers - $CS27_{700}$ linked to H_1 and $A07C_{417}$ linked to H_2. The goodness of fit for 1:1 segregation ratio was tested for each marker separately. The joint segregation of both the markers for 1:1:1:1 ratio was tested for digenic inheritance.

Results and discussion

DNA markers-based selection in chickpea is mainly limited by the availability of polymorphic markers. Molecular markers such as isozyme, RFLP and RAPD were reported to have less polymorphism in chickpea [20]. However RAPD markers were used successfully to map chickpea wilt resistance genes [21]. In the present study, seventy-nine primers were used to identify DNA marker that shows polymorphism between K 850 and WR-315. Of the four primers polymorphic between parents only one marker ($A07C_{417}$) showed polymorphism in parental lines and resistant and susceptible bulks. The application of RAPD primers for marker analysis has been reported earlier in chickpea [12, 22].

The primer A07C produced a unique band of 417bp in susceptible parent and susceptible bulk (Fig. 1). Thus, it is apparently linked to the locus for late wilting (H_2 locus). The marker was highly reproducible. In chickpea, BSA of F_3 plants of an intra specific cross (C-104 x WR-315) was used for identification of RAPD markers associated with resistance (H_1 locus) to race 1 of Fusarium wilt [12] and BSA of RILs to identify RAPD markers linked to H_2 locus [15]. The primer A07C was used for amplification of 164 RILs; the polymorphic band was present in 94 lines and absent in 70 (Fig. 2). The chi-square analysis of goodness of fit for 1:1 ratio was non-significant (χ^2 value = 22). The results indicate that

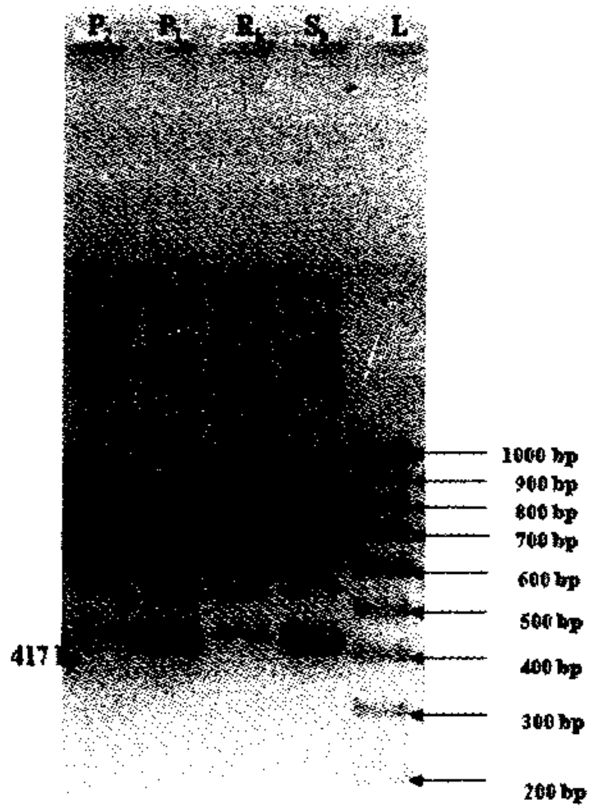

Fig. 1. Parental polymorphism and co-segregation of $A07C_{417}$. P_2 = WR 315; P_1 = JG 62; R_b = Resistant bulk; S_b = Susceptible bulk; L = Ladder

the $A07C_{417}$ segregated as alleles at a single locus. The RILs also showed monogenic 1:1 segregation for wilt reaction i.e. late wilting and resistance.

Considering the wilt reaction and molecular marker together, the RILs were classified into four categories (presence of DNA marker and resistance; presence of DNA marker and susceptible; absence of DNA marker and resistance and absence of DNA marker and susceptible) to examine for the independent assortment. The chi-square analysis for goodness of fit for 1:1:1:1 segregation of $A07C_{417}$ and wilt reaction indicated a significant deviation from 1:1:1:1 ratio (Table 1), suggesting that the marker $A07C_{417}$ is linked to H_2 locus of wilt resistance. More than expected number of susceptible lines were positive for the DNA marker conversely, less than expected number of resistant lines had the DNA marker. The results indicate there is a linkage between the susceptibility and the marker ($A07C_{417}$). The distance between the marker and H_2 locus was determined using MAPMAKER programme. The marker ($A07C_{417}$) and the H_2 locus were at 21.7 cM apart. In chickpea, the RAPD marker linked to H_1 locus of wilt susceptibility was identified initially which was later converted to SCAR markers [12].

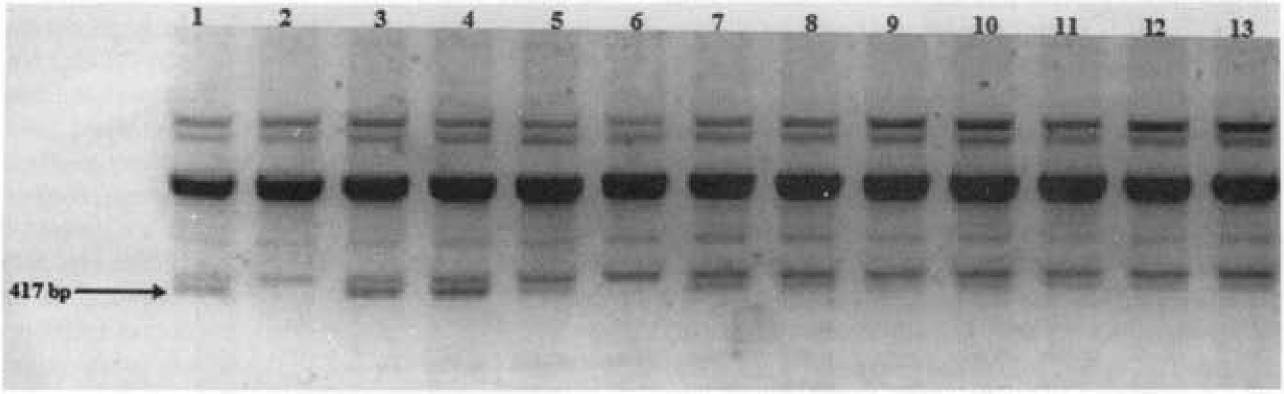

Fig. 2. The recombinant inbred lines of JG 62 x WR 315 showing segregation for $A07C_{417}$ DNA marker

Table 1. Joint segregation of marker $A07C_{417}$ and resistance in recombinant inbred lines of K850 x WR-315

Items	Total RILs	MS	mS	MR	mR
Observed (O)	164	65	15	29	55
Expected (E)	164	41	41	41	41
O-E		24	-26	-12	14

χ^2 for 1:1:1:1 ratio = 38.83; M = Presence of marker; m = absence of marker; R = Resistant; S = Susceptible

With this in chickpea, the DNA markers linked to both H_1 and H_2 loci of Fusarium wilt resistance are available. Both the markers are linked to susceptibility alleles. The markers enable to identify susceptible lines (early and late wilting) at very early stage of growth. Such of the lines/segregants showing presence of both or any one of the markers can be eliminated in the early generation. Conversely, segregants without these DNA markers are the ones that carry resistant genes at the two major loci. Therefore, these markers will greatly increase the speed and efficiency of conventional breeding for wilt resistance by reducing the need for subjective disease screening. Both the markers are linked to susceptibility; susceptibility is dominant over resistance at both the loci. Therefore, these markers identify both homozygous and heterozygous susceptible plants for elimination retaining only homozygous resistant plants.

However, it may be emphasized here that the DNA marker, $A07C_{417}$ has to be validated in different genetic backgrounds and mapping populations for its application in wilt resistance breeding and gene pyramiding. RAPD markers linked to H_2 locus were identified earlier [15]. But they failed to show linkage in different crosses and genetic background. However, $A07C_{417}$ identified in this study showed monogenic segregation of 1:1 in the recombinant inbred lines of another cross JG-62 X WR-315 (Table 2). The segregation of $A07C_{417}$ was independent of the segregation of the marker $CS27_{700}$ linked to H_1 in the cross (Table 3). It is reported that the two genes H_1 and H_2 segregate independently [10, 11], consequently the linked RAPD markers also segregate independently.

Table 2. Segregation for markers CS 27_{700} and $A07C_{417}$ in RILs of JG-62 x WR-315

Marker	+ve	-ve	Total	χ^2-test(1:1)
$CS27_{700}$	43	32	75	NS
$A07C_{417}$	37	38	75	NS

NS = Non significant; +ve: presence of markers, -ve: absence of markers.

Table 3. Joint segregation of markers $CS27_{700}$ and $A07C_{417}$ in the RILs of JG-62 x WR 315

No. of RILs	+ +	+ -	- +	- -	χ^2 -test (1:1:1:1)
Observed	22	21	15	17	NS

NS = Non significant; ++ = Presence of both marker, +- = Presence of $CS27_{700}$ absence of $A07C_{417}$; - + = Absence of $CS27_{700}$ presence of $A07C_{417}$; - - = Both absent.

Five diverse genotypes - A-1 (national check, widely adopted, late wilting), BG 256 (high yielding suitable for north India, late wilting), ICCV 4958 (drought tolerant, late wilting), ICCV-2 (Kabuli type, late wilting) and Karikadle (local cultivar, resistant to pod borer, early wilting) were selected for testing the DNA markers. The CS27 marker linked to H_1 locus was absent in late wilting genotypes (A_1 ICCV 2, ICCV 4958 and BG 256) and the DNA marker $A07C_{417}$ linked to H_2 locus was present in all the 4 genotypes (Fig. 3). Both the DNA marker CS27 and $A07C_{417}$ were present in early wilting Karikadle. The results support that the DNA markers $A07C_{417}$ and CS27 can be successfully used for identification of genotypes with resistance at both H_1 and H_2 loci. The RAPD marker identified in this study was found to be more reliable than the markers identified earlier [15]. It was validated in different genetic backgrounds. After the pioneering work of Williams *et al.* (1990), the RAPD technique has become one of the essential tools in molecular breeding [23]. Numerous RAPD markers linked to biotic and abiotic stress tolerance, genes of agronomic importance have been reported in different crops. Therefore $A07C_{417}$ along with $CS27_{700}$ can be used for introgression of the resistance genes from WR-315 and other resistant lines to susceptible varieties.

However, RAPD markers have certain limitation and the reproducibility of the RAPD technique is questionable. We believe that conversion of a simple RAPD fragment into a more reliable marker such as SCAR and CAPS are more beneficial to breeding programmes using MAS. The RAPD marker linked to H_1 locus was later converted to reliable SCAR marker [12]. Therefore, DNA fragment ($A07C_{417}$) linked to H_2 locus was excised from the gels, purified and cloned into a plasmid vector, and determined their nucleotide sequence. The sequence is as given below.

Attempts are being made to design specific primers to convert RAPD marker to more reliable and co dominant markers.

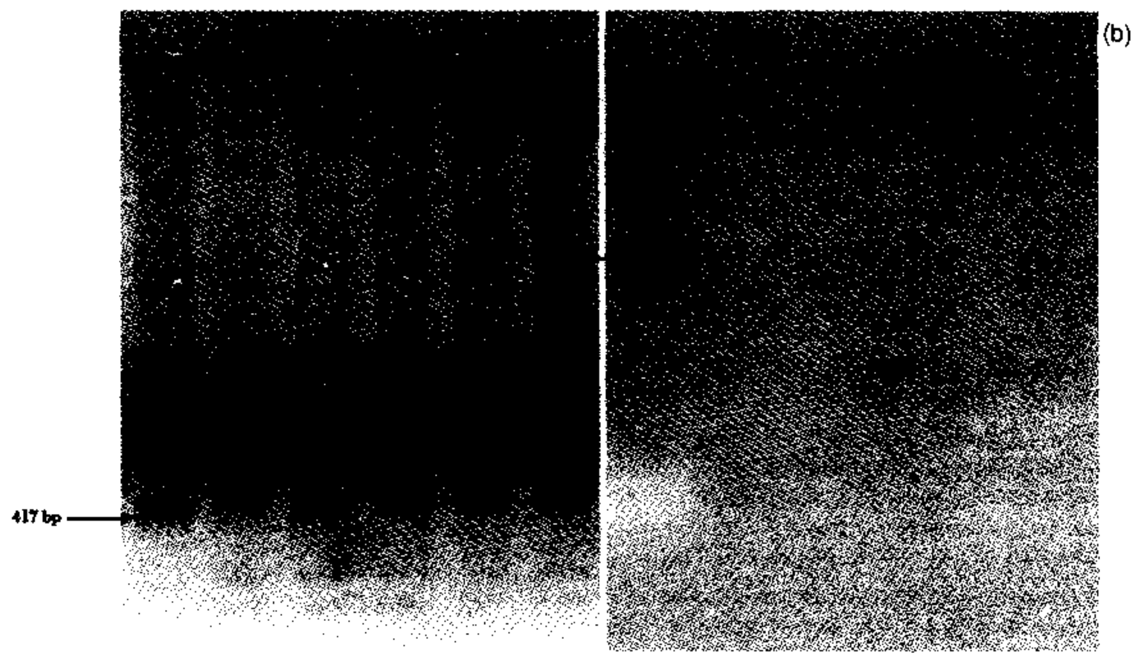

Fig. 3. Validation of DNA marker (a) $A07C_{417}$ and (b) $CS27_{700}$ across six different genotypes. 1 = Karikadle; 2 = WR 315; 3 = A_1; 4 = BG 256; 5 = ICCV2; 6 = ICCV 4958

GAAACGGGTGCAGTGTGTAAGTTTGGAAACTGTTTTGGAGTTTACATCTCTCATATAAATAGGGAATCCAA
GAAGCGTGTTACTAATGCATGTTTTCCTTTTTTTGCCAAATATAATTACAGCAAGGGCACCAACTATGGAC
AACCAGGAGGTTGATAACACATCCCAGACTATCAAACGGAGATTACCTAGGGAAATAAAGCTAAAACTTGC
TAAAGTTGCTAGATTAGCGGTACTGGCTAACTCTCCATTCCTGATATTGTGCTTGTAGAAATACATACAAT
TTCATATATTTTATTTTGATGTATATAAACATTGTCATATTTACTTGTGTTTTTTTGTTGCAGCAGGCAAG
CCAAGGGAAAGTATCAACGGAGTTGATTAATCGTCTTATGAGTAGTCTTGGGCACCCGTTTC

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