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ORIGINAL PAPER

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Molecular mapping of *Fusarium oxysporum* f. sp. *ciceris* race 3 resistance gene in chickpea

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Abstract Sequence-tagged microsatellite site (STMS) and sequence-tagged site (STS) markers linked closely to *Fusarium oxysporum* f. sp. *ciceris* race 3 resistance gene in chickpea were identified, and linkage between three wilt resistance genes was elucidated. The resistance to race 3 in chickpea germplasm accession WR-315 was inherited as a single gene, designated *foc-3,* in 100 F_7 recombinant inbred lines derived from the cross of WR-315 (resistant) × C-104 (susceptible). The *foc-3* gene was mapped 0.6 cM from STMS markers TA96 and TA27 and STS marker CS27A. Another STMS marker, TA194, at 14.3 cM, flanked the gene on the other side. Linkage between *foc-3* and two other chickpea wilt resistance genes, *foc-1* (syn. h_1) and *foc-4*, was established. *foc-3* was mapped 9.8 cM from *foc-1* and 8.7 cM from *foc-4*, whereas *foc-1* and *foc-4* are closely linked at 1.1 cM. The identification of closely linked markers to resistance genes will facilitate marker-assisted selection for introgression of the race 3 resistance gene to susceptible chickpea lines.

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Introduction

Wilt caused by a Deuteromycetes fungus *Fusarium oxysporum* Schlechtend.: Fr. f. sp. *ciceris* (Padwick) Matuo & K. Sato, is one of the most destructive diseases of chickpea (*Cicer arietinum* L.), the third most important pulse crop in the world after bean (*Phaseolus vulgaris* L.) and peas (*Pisum sativum* L.) (FAO 1998). The disease is prevalent in almost all chickpea growing areas of the world, including the Indian subcontinent, Iran, Peru, Syria, Ethiopia, Mexico, Spain, Tunisia, Turkey, and the United States (Westerlund et al. 1974; Nene et al. 1989; Halila and Strange 1996) and is capable of causing 100% yield loss. The pathogen persists in soil as well as on seed (Haware et al. 1978, 1986). Eight races of the pathogen have been reported, of which six (1A, 2, 3, 4, 5, and 6) cause wilting syndrome and are economically more important when compared to races 0 and 1B/C that cause yellowing syndrome (Haware and Nene 1982; Jimenez-Diaz et al. 1993; Kelly et al. 1994). It is difficult to manage the disease either through crop rotation or application of chemicals because of its soil-borne nature. Cultivation of varieties possessing resistance to specific races of the pathogen prevalent in a region or locality is the most economical disease management strategy (Jalali and Chand 1992). Evaluation of a large number of host cultivars and breeding lines for resistance to specific races of the pathogen under field as well green house conditions is laborious, time consuming, costly, and is affected by inoculum concentration and environmental conditions (Jimenez-Gasco et al. 2001; Landa-Blanca et al. 2001).

Marker-assisted selection (MAS) based on the use of DNA markers linked closely to wilt resistance genes can be used to screen large numbers of chickpea breeding lines for the presence of these genes and to pyramid them in agronomically superior varieties. The paucity of genetic polymorphism among different chickpea lines, however, is considered a main handicap in the development of closely linked molecular markers for different chickpea wilt resistance genes (Mayer et al. 1997). Molecular markers such as isozymes, restriction fragment

length polymorphism, and random amplified polymorphic DNA (RAPD) were reported to have minimal polymorphism in chickpea (Udupa et al. 1999). Nevertheless, RAPD markers were successfully used to map chickpea wilt resistance genes *foc-1* (syn. h_1) and *foc-4* that govern resistance to races 1 and 4, respectively (Mayer et al. 1997; Tullu et al. 1999). Of late, inter-simple sequence repeat (ISSR) and sequence-tagged microsatellite sites (STMS) markers have been developed for chickpea and used to map chickpea wilt resistance genes *foc-4* and *foc-5* (race 5 resistance gene) (Ratnaparkhe et al. 1998; Tekeoglu et al. 2000; Winter et al. 2000).

Initial evidence shows that chickpea wilt resistance genes may be clustered in the genome. Tekeoglu et al. (2000) studied the linkage between two genes, *foc-4* and *foc-5*, using a mapping population derived from an interspecific cross between *C. reticulatum* and *C. arietinum*, and found them on the same linkage group and separated by 11.2 cM. The presence of these two genes in the same linkage group was further confirmed by Winter et al. (2000), and the possibility of clustering of *foc-4, foc-5 and foc-1* was suggested.

Genes governing resistance to races 0, 2, 3, and 6 of *F. oxysporum* f. sp. *ciceris* have not been mapped. Moreover, the genetics of resistance in chickpea to race 3 and race 6 has not been explored. In the present paper, we report the genetic mapping of the race 3 resistance gene (designated as *foc-3*) and linkage between *foc-1*, *foc-3*, and *foc-4* resistance genes using RAPD, STS, ISSR, and STMS markers. The study aims at the exploitation of marker-assisted selection for introgression of wilt resistance genes into elite chickpea varieties and, ultimately, to isolate the genes through positional cloning.

Materials and methods

Plant material and fungal strains

One hundred F_7-derived recombinant inbred lines (RILs) from a cross between two *C. arietinum* lines, WR-315 and C-104, were used to map the fusarium wilt resistance genes. Phenotype of these lines and another line, P2245, to races 1, 3, and 4 of the *F. oxysporum* f. sp. *ciceris* was evaluated as per the procedure described later. WR-315 is resistant, whereas C-104 and P2245 are susceptible to these races. Reaction of WR-315 and C-104 to these races was similar to that obtained by Tullu (1996). Seed of wilt differentials and other chickpea lines was obtained from Regional Plant Introduction Station, Washington State University, Pullman, Wash., USA. Isolates of race 3 were obtained from the International Crops Research Institute for Semi-Arid Tropics (ICRISAT), Patancheru, India and were stored at −20°C on filter paper discs inoculated with the fungus. For this study, the race 3 isolate of the pathogen was revived from filter paper discs on V8 juice agar (150 ml V8 juice, 3 g calcium carbonate, and 16 g agar per liter of the medium) plates and multiplied on liquid V8 juice medium. A single-spore culture of the revived isolate was used to inoculate plants of susceptible chickpea line P-2245 (Tullu 1996). The pathogen was re-isolated from fourth internode stem sections of infected plants of P-2245 as described by Tullu et al. (1998). Single-spore cultures of the isolate established on V8 juice agar medium were used to inoculate a set of 20 chickpea differential lines (Tullu 1996) to determine the race of the isolate. One single-spore isolate was used to inoculate the plants of the mapping population.

Inoculation procedure and disease scoring

Inoculum was prepared by growing the race 3 isolate on liquid V8 medium. Cultures were grown at 25°C for 20 days on a shaker at 100 rpm under continuous light provided by white fluorescent tubes. The cultures were passed through four layers of cheesecloth under aseptic conditions and the number of microconidia per milliliter of the spore suspension was calculated using a hemocytometer (improved Neubauer). Final concentration of the microconidia in the inoculum was adjusted to 1×10^6 spores per milliliter by adding sterile water to the spore suspension. Seed of RILs, parents and chickpea lines was pre-germinated and grown in 20×10×2.25-in trays filled with perlite. At the 4–5 leaf stage, plants were uprooted from perlite, the lowermost fifth portion of roots of individual plants was cut, and the roots were dipped in the inoculum for 5 min. The inoculated plants were transplanted into 20×14×4.25-in trays containing a mixture of perlite and potting soil in the ratio of 1:1 (v/v). Uninoculated plants of the parents served as control for this experiment, and control plants were of three types: (1) plants not uprooted, (2) plants uprooted and replanted, and (3) plants uprooted. The lowermost fifth portion of the roots was cut, dipped for 5 min in sterile distilled water, and replanted. Nutrient solution of 10% N, 10% P_2O_5, 10% K_2O, 0.025% Mg, 0.0034% B, 0.0018% Cu (chelated), 0.025% Fe (chelated), 0.0125% Mn (chelated), 0.00045% Mo, and 0.00125% Zn was applied to inoculated as well as control trays before transplanting plants into them and once during the second week after inoculation. Nutrient solution was applied twice a week thereafter. The inoculated and control plants were incubated at a temperature regime of 26°C/22°C for 12/12 h and 16 h illumination provided by fluorescent bulbs.

The experiment was conducted in a completely randomized design and was replicated three times with ten plants in each replication. The number of wilted and live plants in each line were noted at weekly intervals 3 weeks after inoculation, and final disease data were recorded 8 weeks after inoculation. Reaction of RILs was based on wilt incidence where 0–10% = resistance, 11–89% = segregating, and 90–100% = susceptible. Chickpea genotypes with 11–89% wilt incidence were scored as intermediate in reaction as these were not the segregating populations. Data for the reaction of the RILs to races 1 and 4 was taken from Tullu (1996) who used same population of RILs.

DNA extraction, PCR amplification, and electrophoresis

Genomic DNA from the RILs and chickpea lines was isolated from vegetative buds and young leaves as per Doyle and Doyle (1990). Twenty ISSR primers, 15–23 nucleotides in length (University of British Columbia, Vancouver, British Columbia, Canada), 75 STMS primers (Winter et al. 1999), four 10-mer primers of UBC series (University of British Columbia, Vancouver, British Columbia, Canada), two 10-mer primers of CS series (designed in our laboratory), and an STS primer were used in the present study. STS primer CS27A was developed from RAPD primer CS27 of Mayer et al. (1997) and was shown to be linked to an allele of the gene for resistance to fusarium wilt race 1. Initial screening of the markers was done by slight modification of the bulked segregant analysis (Michelmore et al. 1991). DNA isolated from homozygous resistant or susceptible RILs was not combined for bulked segregant analysis. Instead, DNA of five homozygous resistant and five homozygous susceptible RILs and two parents was amplified individually using different primers. The markers that appeared to be associated with resistance or susceptibility were used subsequently to screen the entire mapping population.

PCR amplification for RAPD and STS primers was carried out according to Mayer et al. (1997). The PCR program for RAPD comprised 40 cycles of 94°C for 20 s, 36°C for 1 min, and 72°C for

2 min. PCR with ISSR and STMS primers was performed as described previously (Ratnaparkhe et al. 1998; Winter et al. 1999).

PCR products amplified with ISSR, RAPD, and STS primers were resolved on 1.4% agarose gels, whereas STMS markers were resolved on 6% polyacrylamide gels. Agarose gels were stained with ethidium bromide and visualized under UV light. Polyacrylamide gels were silver stained and visualized in white light.

Statistical analysis

Disease reactions of RILs to different races of the pathogen and marker data were analyzed by chi-square to determine goodness-of-fit to an expected segregation ratio of 1 resistant:1 susceptible. Data generated by different markers were recorded in a binary fashion. ISSR, STS, and RAPD are dominant markers, and scoring was based on presence or absence of a band. Linkage between markers and resistance genes was established using the MAPMAKER program (Lincoln et al. 1992). The map was constructed using a LOD score of 4.0 and the Kosambi function of the MAPMAKER program (Kosambi 1944).

Results

Genetics of wilt resistance in chickpea

Of the 100 RILs tested for reaction to race 3 of *F. oxysporum* f. sp. *ciceris*, 44 were resistant, 51 were susceptible, and 5 lines segregated for resistance. Susceptible RILs took an average of 3–5 weeks for complete wilting. Our data showed that plants of the susceptible parent (C-104) wilted within 24 days after inoculation, whereas the resistant parent (WR-315) did not develop wilt symptoms. Chi-square analysis of disease reaction data of RILs indicated a good fit to the 1:1 (susceptible:resistance) segregation ratio expected for a single gene conferring resistance to race 3 in WR-315 (Table 1). The gene was designated as *foc-3*, according to the nomenclature proposed for fusarium wilt resistance genes in chickpea by Tekeoglu et al. (2000). RILs also segregated in the ratio of 1:1 for resistance and susceptibility to race 1 as well as race 4, indicating that resistant and susceptible parents differ only with respect to a single gene for resistance to these races.

Bulked segregant analysis and identification of markers linked to *foc-3*

Polymorphism detected with RAPD and ISSR primers was low compared to STMS primers. Based on the data obtained after amplification of DNA of five resistant and five susceptible RILs, we could select 16 primers that appeared to be linked to the resistance gene. However, a total of 53 (50 STMS, two 10-mer and one STS) primers were used to amplify genomic DNA of the entire population of 100 RILs to identify markers linked to the resistance genes. STMS primer TA96 amplified two bands of 280 bp and 260 bp, respectively, in resistant and susceptible RILs, whereas STS primer CS27A amplified a band of 565 bp only in susceptible RILs. The size of bands amplified with another STMS primer, TA27 in resistant RILs (241 bp), and susceptible RILs (235 bp) differ only by 6 bp. DNA fingerprints of 38 RILs and parents obtained with TA96 and that of 18 RILs obtained with CS27A are presented in Figs. 1 and 2.

Analysis of amplification data generated by 53 primers revealed that TA96 was linked to *foc-3* at a distance of 0.6 cM, whereas TA27 and CS27A co-segregated with TA96 (Fig. 3). These markers segregated in the ratio of 1:1 in the RIL population for resistance and susceptibility with only one recombination in case of TA96 and TA27 and three for CS27A (Table 2). TA96, TA27, and CS27A flanked the resistance gene on one side, whereas TA194 was mapped on the other side of the gene at a distance of 14.3 cM.

Table 1 Reaction of F_7-derived recombinant inbred lines to three races of *Fusarium oxysporum* f. sp. *ciceris*

Race	Resistant	Susceptible	χ^2 (1:1)	Probability
1	48	46	0.04	0.85
3	44	51	0.52	0.54
4	48	51	0.09	0.77

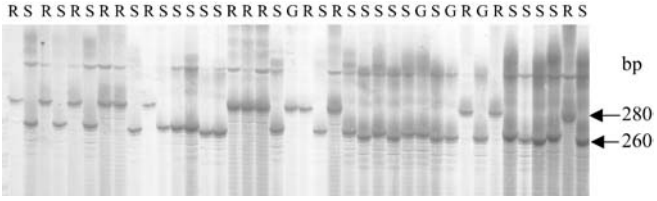

Fig. 1 Segregation of sequence-tagged microsatellite site (STMS) marker TA96 in 38 recombinant inbred lines of chickpea parents WR-315 and C-104. *Second to the last lane* and the *last lane* represent WR-315 (resistant) and C-104 (susceptible). Loci corresponding to resistant and susceptible parents and recombinant inbred lines (RILs) are shown with *arrows*. Disease reaction of RILs and parents to *Fusarium oxysporum* f. sp. *ciceris* race 3 is given at the *top* (*R* resistant, *S* susceptible, *G* segregating)

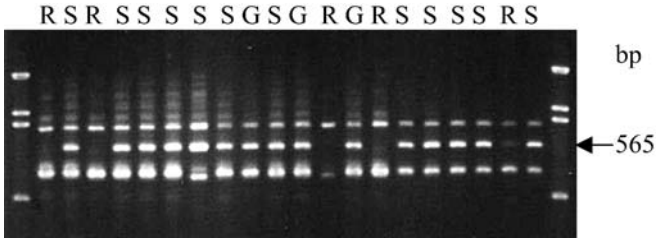

Fig. 2 DNA fingerprints of 18 RILs and parents WR-315 and C-104 obtained with sequence-tagged site (STS) primer CS27A. *First* and *last lanes* are DNA size standards. *Second to the last* and *last lanes* are WR-315 and C-104, respectively. The marker band, indicated by the -ase reaction of RILs and parents to *Fusarium oxysporum* f. sp. *ciceris* race 3, is given at the *top* (*R* resistant, *S* susceptible, *G* segregating)

Table 2 Joint segregation of markers TA27, TA96, and CS27A with resistance and susceptibility in homozygous recombinant inbred lines

Marker	A		B		χ^2	Probability	Correlation
	Resistant	Susceptible	Resistant	Susceptible			
TA27	46	1	0	50	93.1	<0.0001	0.98
TA96	44	1	0	52	93.0	<0.0001	0.98
CS27A[a]	44	1	2	56	91.2	<0.0001	0.94

[a] *A* denotes the absence and *B* the presence of the marker band in STS primer CS27A

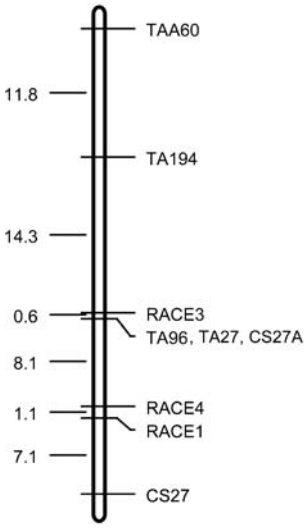

Fig. 3 Linkage map of the chickpea genomic region surrounding wilt resistance genes *foc-1*, *foc-3*, and *foc-4.* Markers starting with *TA* are STMS, *CS27* is a RAPD marker and *CS27A* is a STS marker. *Numbers on the left* are distances in centiMorgans. (See text for more detail)

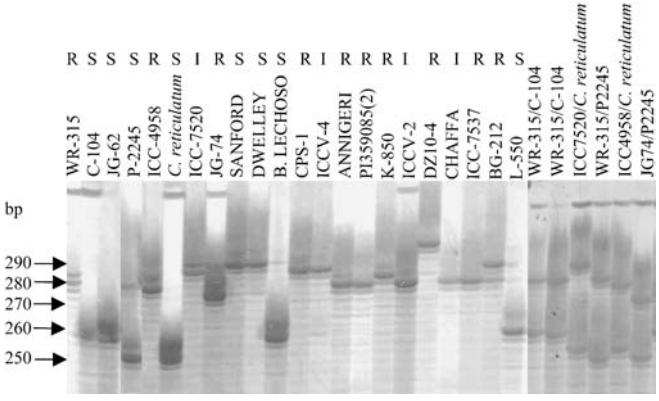

Fig. 4 Amplification of genomic DNA of different *Cicer* lines and F_1s with STMS primer TA96. Names of the lines and hybrids are at the *top*. The reaction of *Cicer* lines to *Fusarium oxysporum* f. sp. *ciceris* race 3 is given on the top (*R* resistance, *S* susceptible, *I* intermediate)

foc-3, *foc-1*, and *foc-4* are linked

Analysis of disease resistance as well as marker data showed that *foc-3*, *foc-1*, and *foc-4* were linked (Fig. 3). *foc-4* and *foc-1* were mapped 0.6 cM apart, whereas *foc-3* was 8.7 cM from *foc-4* and 9.8 cM from *foc-1*. *foc-4* was 8.1 cM and *foc-1* 9.2 cM from TA27, TA96, and CS27A. *foc-4* and *foc-1* were linked to CS27 at a distance of 8.2 cM and 7.1 cM, respectively. CS27 was 16.9 cM from *foc-3* and 16.3 cM from TA96, TA27, and CS27A. The markers TA96, TA27, CS27, and CS27A have already been reported to be situated on linkage group II of the *Cicer* linkage map (Winter et al. 2000) indicating that *foc-3*, *foc-1*, and *foc-4* are also located on linkage group 2.

Validation of TA96 and TA27 in different genetic backgrounds

Twenty-one *C. arietinum* lines and one *C. reticulatum* (PI489777) line collected from different regions of the world were evaluated for reaction to race 3, and genomic DNA of the lines was amplified with TA96 and TA27. Ten lines were resistant to race 3, four intermediate in reaction, and eight susceptible (Fig. 4). In addition, *C. reticulatum* and P-2245 were also tested for reaction to races 1, 2, 4, and 5 and found susceptible to all the races under test. The size of the bands amplified by TA96 in different genetic backgrounds varied from 250 bp (P-2245, *C. reticulatum,* susceptible) to 306 bp (DZ 10-4, resistant), whereas it varied from 201 bp (*C. reticulatum*, susceptible) to 273 bp (DZ 10-4, resistant) for TA27. In addition to a 250 bp band, P-2245 also amplified another band of 278 bp corresponding to loci of resistant line ICC-4958. Out of the eight susceptible lines, six amplified bands with a size between 250–260 bp with TA96 compared to 274–286 bp bands amplified in resistant and intermediate lines. Only exceptions were susceptible lines, Sanford and Dwelley, that amplified loci (286 bp) corresponding to resistant lines with TA96. To verify the utility of TA96 in the identification of *Cicer* hybrids, we tested two interspecific (*C. arietinum* × *C. reticulatum*) and four intraspecific (*C. arietinum* × *C. arietinum*) hybrids using TA96 (Fig. 4). Since, one of the parents of the F_1s was resistant to race 3 and the other one susceptible except for ICC-7520 (intermediate) × *C. reticulatum* (susceptible)-derived F_1, two bands, one corresponding to resistant/intermediate parent and another to susceptible parent, were amplified by each of the hybrids (Fig. 4). Like F_1s, the size of bands amplified by TA96 and TA27 in F_7 RILs was either similar to resistant parent or susceptible parent. Four RILs segregated for TA96 loci compared to that of three in the case of TA27.

One RIL amplified a band of intermediate size (238 bp) compared to those of 241 bp and 235 bp amplified in parents with TA27. Overall data suggest that TA96 and TA27 can be used for introgression of the gene for resistance to race 3 from WR-315 and other resistant lines to improved germplasm.

Discussion

The resistance to races 1 and 4 of chickpea wilt pathogen has been reported to be governed by three and two loci respectively (Kumar and Haware 1982; Singh et al. 1987; Tullu et al 1999). However, monogenic inheritance for race 1 and 4 resistance in WR-315 obtained in the present study confirms the results of Mayer et al. (1997) for race 1 and Tullu et al. (1998) for race 4. WR-315 and C-104, the parents of the mapping population, belong to the same species and, as expected for chickpea, polymorphism detected with ISSR primers was low. We observed high polymorphism with STMS markers that might be attributed to the highly mutable nature of the chickpea microsatellite loci (Udupa and Baum 2001) and conservation of microsatellite flanking sequences in WR-315 and C-104. Variation in size of marker bands observed in different chickpea lines generated by TA27 and TA96 might be due to either variable number of tandem repeats or non-variable number of tandem repeat mutations in microsatellites amplified by these primers, as has been observed by Choumane et al. (2000) for chickpea.

TA27, TA96, and CS27A were mapped 0.6 cM apart from *foc-3* and can be used in MAS for *foc-3* either independently or in combination. Markers TA96, CS27A, and CS27 and the *foc-4* and *foc-5* loci have been mapped earlier on linkage group 2 of the chickpea genetic linkage map (Winter et al. 2000). The results presented here demonstrate that *foc-1 and foc-3* can also be assigned to linkage group 2 based on the location of these markers. Additionally, we confirmed the position of *foc-4* on linkage group 2 and in close proximity to *foc-1*. Among these genes, *foc-1* and *foc-4* are closer to each other as compared to *foc-3*. The results obtained by us and those obtained previously (Tullu et al. 1998; Tekeoglu et al. 2000; Winter et al. 2000) indicate two clusters of fusarium wilt resistance genes on linkage group 2: one that contains the *foc-1* and *foc-4* and the other that comprises the *foc-3* and *foc-5* resistance genes.

Since the markers TA96, TA27, and CS27 mapped in the present study have also been used in some recent genetic maps of the *Cicer* genome (Winter et al. 1999, 2000; Cho et al. 2002; Tekeoglu et al. 2002; Udupa and Baum 2003), the position of genes for resistance and other traits observed in different populations can be assigned to a particular region of the chickpea genome. The results presented here and those by Udupa and Baum (2003) contribute to the emerging picture of a hot spot for resistance against two diseases (fusarium wilt and ascochyta blight) on linkage group 2 of the map of Winter et al. (2000). The region containing the *foc-1/foc-4* cluster also harbors QTL for resistance against pathotypes I and II of *Ascochyta rabiei*. Apart from resistance genes, other genes involved in pathogen defense are also located in the same region of linkage group 2. The region containing *foc* gene clusters also harbors sequences with high homology to pathogenesis-related genes such as a Thaumatin-like protein (PrP 5) gene or the gene coding for *N*-hydoxycinnamoyl-benzoyltransferase that catalyses one of the first steps in the production of phytoalexins (Benko-Iseppon et al. 2003).

In other host pathogen systems, resistance genes have been reported to be clustered in chromosomal regions spanning from a few cM to 20 cM covering several megabases (Holub 1997; Hulbert et al. 1997). It has been observed that *R* genes lie physically close to each other in species with higher gene density, whereas in species with lower gene density, the resistance genes are usually separated by a few hundred kilo bases or more (Hulbert et al. 2001). Thus, clustering of fusarium and blight resistance genes in chickpea is not unusual. Also, the situation in chickpea where different types of genes involved in plant defense, e.g., resistance genes and pathogenesis-related genes, are closely linked has been described for many plants (review in Hulbert et al. 2001). There are eight different races of fusarium causing wilt in chickpea, and resistance to some of them is governed by more than one gene. Allelic tests will have to be performed and genetic stocks must be developed for all of them as a basis for complete understanding of the complex genetics of resistance, which is a prerequisite for pyramiding of resistance genes to obtain durable resistant varieties in future. However, tagging of fusarium resistance genes with tightly linked co-dominant and dominant DNA markers as demonstrated here will contribute in pyramiding wilt resistance genes in chickpea.

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