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Inheritance and linkage of a gene for resistance to race 4 of fusarium wilt and RAPD markers in chickpea

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Summary

Several races of *Fusarium oxysporum* Schlechtend.:Fr f. sp. *ciceris* (Padwick) Matuo and K. Sato cause economic losses from wilting disease of chickpea (*Cicer arietinum* L.). While the genetics of resistance to race 1 have been reported, little is known of the genetics of resistance to race 4. We undertook a study to determine the inheritance of resistance and identified random amplified polymorphic DNA markers (RAPDs) linked to the gene for resistance. For the investigation, we used 100 F_5 derived F_7 recombinant inbred lines (RILs) that had been developed from the cross of breeding lines C-104 x WR-315. Results indicated that resistance is controlled by a single recessive gene. The RAPD markers previously shown to amplify fragments linked to race 1 resistance also amplified fragments associated with race 4 resistance. The RAPD loci, CS-27_{700}, UBC-170_{550} and the gene for resistance to race 4 segregated in 1:1 ratios expected for single genes. Both RAPD markers were located 9 map units from the race 4 resistance locus and were on the same side of the resistance gene. Our results indicated that the genes for resistance to race 1 and 4 are 5 map units apart. The need to determine the genomic locations of race specific resistance genes and the possibility that these genes are clustered to the same genomic region should be investigated.

Introduction

Fusarium wilt caused by *Fusarium oxysporum* Schlechtend.: Fr. f. sp. *ciceris* (Padwick) Matuo & K. Sato is an economically important disease of chickpea growing areas worldwide including production areas in the United States (Buddenhagen & Workneh, 1988; Trapero-Casas & Jiménez-Diaz, 1985).

The use of resistant cultivars is the most economical, environmentally safe and effective means of controlling the disease. However, physiological races of the wilt pathogen which are capable of overcoming resistance have been identified (Kaiser et al., 1994). Extensive screening at the International Crops Research Institute for Semi-Arid Tropics (ICRISAT) (Haware & Nene, 1982) and in Spain (Jiménez-Diaz et al., 1989) has indicated that there are seven distinct races of *Fusarium oxysporum* f. Sp. *ciceris*. Races 1, 2, 3 and 4 were reported from India (Haware & Nene, 1982), while races 0, 5, and 6 were reported from Spain (Jiménez- Diaz et al., 1989). Breeding programs at national and international centers have developed and released resistant chickpea cultivars; however, these cultivars have not maintained resistance across locations (Infantino et al., 1996) due to the prevalence of location-specific races of the wilt pathogen.

To date, the genetic constitutions and inheritance pattern of genes conferring resistance to only race 1 have been reported. Using F_1s, F_2 plants and F_3 families of different crosses, three independently function-

ing resistance loci designated as H_1, H_2, and H_3; and breeding lines carrying each of these genes have been identified (Upadhyaya et al., 1983a; 1983b; Singh et al., 1987a). Singh et al., (1987a) suggested the genetic constitution for C-104, a line susceptible to race 1 (late wilting), to be $H_1H_1h_2h_2h_3h_3$, the homozygous recessive gene at the second locus confers late wilting. WR-315 ($h_1h_1h_2h_2h_3h_3$), a resistant line, differs from C-104 only at the first locus. These were the two parental lines used in the present study. No information is available on the inheritance pattern of the gene(s) conferring resistance to race 4, one of the most virulent of the wilt races (El-Hadi, 1993).

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 can be difficult, particularly when large numbers of breeding lines are involved. Identification of molecular markers for indirect selection of the gene(s) for resistance would facilitate breeding. Two Random Amplified Polymorphic DNA (RAPD) markers, $UBC\text{-}170_{550}$ and $CS\text{-}27_{700}$, were shown to be closely linked to the genes for resistance to fusarium wilt race 1 (Mayer et al., 1997). No other molecular markers have been shown to be associated with fusarium wilt resistance in chickpea.

The present study describes the inheritance of resistance to race 4 of fusarium wilt and the identification of closely linked molecular markers. The relationship of the identified markers to the genes for resistance to races 1 and 4 is also discussed.

Materials and methods

Plant materials

A set of 100 recombinant inbred lines (RILs) segregating for fusarium wilt resistance were derived by single-seed descent from a cross of germplasm lines, WR-315 and C-104, which were reported to be resistant and susceptible, respectively, to races 1, 2 and 4 (Haware & Nene, 1982). The cross was made at ICRISAT and F_3 families were provided to the USDA-ARS Grain Legume Genetics and Physiology Research Unit at Pullman, Washington, USA. Each of the 100 F_3 families was advanced by single seed descent to the F_5. The lines were then increased and 100 F_5derived F_7families were used to study the inheritance of resistance to race 4. The same RILs were used in our previous study of resistance to race 1 (Mayer et al., 1997) except that they were advanced an additional generation.

Culture preparation

Inoculum was prepared from soil-tube cultures of *F. oxysporum* f. sp. *ciceris* race 4. The culture was single spored onto 2% water agar and increased on fresh Potato-Dextrose Agar onto which a sterile filter paper was placed. Three petri plates were inoculated with race 4 and incubated at 22 ± 1 °C for 5 days under continuous white fluorescent light to facilitate conidial development. When the filter paper was completely colonized by the fungus, colonies representative of wild type were aseptically removed and placed in petri plates for drying for 5 days in the transfer hood. The dried filter papers were cut into small pieces and aseptically transferred to petri plates which were then sealed with parafilm and stored at 4 °C. Pieces of filter paper were used to produce liquid cultures of the pathogen following procedures described by Bhatti (1990). Conidia were strained through four layers of cheese cloth to remove mycelial mats; conidia in the filtrate were pelleted by centrifugation at 3000 rpm for one minute; the supernatant was discarded; the conidia were then washed twice with sterile water; counted with a hemacytometer and adjusted to 1×10^6 spores ml^{-1}. Working cultures were obtained from stocks every 4–6 months to avoid loss of virulence.

Host inoculation

Ten to twelve seeds of each of the 100 F_5 derived F_7 RILs and the two parents were surface disinfected in 5% chlorox (NaOCl) for 5 min, rinsed in water, then air-dried, germinated in a germinator at 22 °C and sown in flats containing coarse perlite in the greenhouse at 21–26 °C. The entire experiment was repeated. At the third to fourth node stage of growth, test plants were carefully removed from the perlite, the roots submerged into the inoculum solution and about one-fourth to one-fifth were excised as described in peas (Haglund & Kraft, 1979) and chickpeas (Bhatti, 1990). After about a 5 min exposure, the plants were replanted into the same perlite. Scoring of the RILs as either resistant when 0–10% wilted, susceptible when 90–100% wilted or segregating when plants segregate into 3 susceptible and 1 resistant was made over the next 60 days and compared to non-inoculated controls, which had been grown, pruned and inoculated in the same manner but with sterile distilled water. Also,

included in these experiments were the known fusarium wilt differential lines, (Haware & Nene, 1982; Jiménez- Diaz et al., 1989), 5 germplasm lines and 4 chickpea cultivars (Table 4). The greenhouse data was further corroborated by isolating the pathogen from the sixth node of plants which had been scored as susceptible. For the isolation, stem sections from the sixth node were surface disinfected with 5% chlorox for 2 min, rinsed in water and incubated on fresh PDA. If the 'wild type' of the fusarium wilt pathogen grew out from the nodal tissue, the plant was considered as infected.

PCR analysis

We followed the RAPD analysis described by Mayer et al. (1997) to screen primers on the DNA samples of the parental lines. Of the 370 RAPD primers tested previously (Mayer et al., 1997) and an additional 50 primers used to screen the parental lines that were used to develop the RILs, we used only those that revealed polymorphisms to determine linkage of the marker loci with the gene for resistance to fusarium wilt race 4.

Tests of Goodness of fit to Mendelian ratios were conducted using the Linkage 1 computer program (Suiter et al., 1983). Linkage relationships between the genes for resistance to races 1 and 4, and between the gene for race 4 and the markers were estimated by a 2-point analysis using the method of Burr et al. (1988).

Results and discussion

A total of ninety-nine F_5 derived F_7 RILs were tested for resistance to race 4 of fusarium wilt. Forty-eight and 46 of the lines were resistant and susceptible, respectively, and five of the lines segregated for wilt reaction. The RILs that segregated for wilt reaction yielded a total of 37 susceptible and 14 resistant plants indicating that the gene for resistance was recessive. Single seeds from F_7 seed stock of the segregating RILs were grown in order to develop lines homozygous for wilt reaction. The resulting F_8 progenies were then screened against race 4 of fusarium wilt. The data on the reaction to race 4 of this latter group of lines was then combined with the original data: from the combined data, 51 RILs were susceptible and 48 were resistant (Table 1). The data closely fit a 1:1 segregation ratio which suggested that a single gene conferred resistance to race 4 in the cross of C-104 with WR-315 (Table 1).

Table 1 Single locus goodness of fit to a 1:1 ratio for *Fusarium oxysporum* f. sp. *ciceris* race 4 resistance and genetic markers used in linkage analysis in the cross of C-104 and WR-315

Locus	+	−	χ^2	Probability
CS-27_{770}	54	46	0.64	0.42
UBC-170_{550}	52	48	0.16	0.69
Race 4	51(S)	48(R)	0.04	0.76

+ = presence of an amplified product.
− = absence of an amplified product.

Of the 420 random oligonucleotide primers used to survey the susceptible and resistant parental lines, two of the primers designated as CS-27 (5′-AGTGGTCGCG) and UBC-170 (5′-ATCTCTCCTG) amplified DNA fragments associated with susceptibility and resistance to race 4 of fusarium wilt, respectively. The additional 50 primers we tested on the parental lines did not reveal any polymorphism that cosegregated with the gene for resistance. CS-27 and UBC-170 were previously reported (Mayer et al., 1997) to amplify genomic regions associated with susceptibility and resistance, respectively, to race 1 of fusarium wilt. Using this RAPD data and the genotypic data for race 4, the locus for resistance to race 4 was determined to be located 9 map units from the loci corresponding to CS-$27_{700}$and UBC-170_{500} (Table 2). We found that the two loci that confer resistance to races 1 and 4 were 5 map units apart with a standard error of 0.032 (Table 3). According to Mayer et al., (1997) the loci corresponding to CS-27_{700} and UBC-170_{500} were separated by 6 map units and each were 7 map units from the gene conferring resistance to fusarium wilt race 1. The higher recombination frequency between the markers seems to be unexpected given their distances from the resistance genes. One probable reason as suggested by Mayer et al., (1997) could be the continuing segregation within the recombinant inbred lines. Different linkage data could be obtained when the marker data of genotypes in the F_5 and wilt data of $F_{5:7}$ and $F_{5:8}$ lines are used in an analysis. An analysis of distances between the markers and resistance genes, and other RAPD markers indicated that both of the RAPD markers were positioned on the same side relative to the genes for resistance to races 1 and 4 (data not shown). Chickpea differential lines, cultivars and germplasm lines were surveyed with the two markers associated with resistance to race 4 (Table 4). In several lines, resistance was associated with the presence of CS-27_{700} while in other lines susceptibility was asso-

Table 2. Joint segregation of F_5 derived F_7 recombinant inbred lines from the cross of C-104 and WR-315 for resistance to *Fusarium oxysporum* f. sp. *ciceris* race 4 and two RAPD markers, contingency χ^2, and the estimated recombination (r) between the resistance locus and marker loci

Locus	Reaction to race 4				χ^2	r	Probability
	Susceptible		Resistant				
	+	−	+	−			
$CS\text{-}27_{700}$	45	6	9	39	48.2	0.09	<0.010
$UBC\text{-}170_{550}$	9	42	42	6	48.3	0.09	<0.010

+ = presence of an amplified product.
− = absence of an amplified product.
S = Susceptible to fusarium wilt.
R = Resistant to fusarium wilt.

Table 3. Joint segregation of F_5 derived F_7 recombinant inbred lines from a cross of C-104 and WR-315 for resistance to *Fusarium oxysporum* f. sp. *ciceris* races 1 and 4, contingency χ^2, and the estimated recombination (r) between the resistance loci

Locus	Reaction to race 1				χ^2	r	Probability
	Susceptible		Resistant				
	S	R	S	R			
Race 4	44	4	5	42	62.4	0.05	<0.010

S = Susceptible to fusarium wilt.
R = Resistant to fusarium wilt.

ciated with the presence of $UBC\text{-}170_{550}$. The presence of a fragment amplified by $CS\text{-}27_{700}$ in resistant chickpea lines (e.g., 12-07/10054) indicated that the marker is present in both coupling or repulsion phases with the resistance gene in chickpea germplasm. Haley et al., (1993) also explained a similar situation where a RAPD marker linked to resistance in coupling was also present in a few susceptible cultivars and in the susceptible backcross progenies. We have not observed lines exhibiting the absence of $CS\text{-}27_{700}$ linked to susceptibility and absence of $UBC\text{-}170_{550}$ linked to resistance. This could probably be explained by the fact that we surveyed relatively few lines among the array of chickpea germplasm and those lines did not represent the available range of germplasm lines and cultivars.

The number of polymorphisms associated with resistance to races 1 and 4 of fusarium wilt were lower than the number of polymorphisms reported with regard to resistance in other plant systems. In common bean (*Phaseolus vulgaris* L.), using 306 10-mer primers, Haley et al., (1993) found 14 polymorphic markers that were associated with resistance to *Uromyces appendiculatus* (Pers.:Pers.) Unger var. appendiculatus. In muskmelon (*Cucumis melo* L.), two markers, one apparently linked to resistance and the other linked to susceptibility to *Fusarium oxysporum* f. sp. *melonis* race 1, were reported from an evaluation of 320 10-mer primers (Wechter et al., 1995). Of the 420 primers tested on the parental lines, 349 amplified DNA fragments. Only 14% of these showed polymorphism. Moreover, results from isozyme analysis have revealed insufficient polymorphism to be useful for tagging wilt resistance genes in the C-104 and WR-315 population (unpublished data). The lack of additional linked markers based on isozymes and RAPDs might suggest minimal divergence between the parental lines. C-104 (ICC-4928) is a cross between Punjab 7 (desi type) and Rabat (kabuli type) varieties and was released in 1960 in Punjab, India. WR 315 (ICC 8933) is a wilt resistant collection from the Kanpur area of India. The lines should have revealed more polymorphisms than observed considering the two types of chickpea (desi and Kabuli) used in the crossing to develop the RTLs. The lack of detectable genetic polymorphisms in cultivated germplasm is not uncommon, particularly for chickpea (Oram et al., 1987 and Kazan and Muehlbauer, 1991), soybean (*Glycine* sp.) (Keim et al., 1990) and common bean (Gepts, 1991). This low level of polymorphism might suggest the need to exploit the potential of wild species for identifying other markers linked to these resistance genes. Additonal markers would be of value for introgression of resistance genes from wild species to otherwise acceptable backgrounds and for the expansion of genetic map of *Cicer*.

Table 4. Survey of the reactions of chickpea differential lines, cultivars and germplasm lines to *Fusarium oxysporum* f. sp. *ciceris* race 4 and the associated markers

Line/ cultivar	Wilt reaction race 4	Markers	
		CS-27_{700}	UBC-170_{550}
JG-62	S	+	–
C-104	S	+	–
L-550	S	+	–
BG-212	R		+
WR-315	R	–	+
ICCV-4	R	–	+
P-2445	S	+	+
K-850	R	–	+
Chafa	I/S[a]/S[b]	+	+
WR-350	R	–	+
12-071/10054	R	+	+
CPS-1	R	–	+
JG-74	R	–	+
Annigeri	R/S[a]/S[b]	–	+
DZ-10-4	S	+	+
ICC-14093	S	+	–
Shanho	S	+	+
CADU-54	S		
+PV-60	S	+	na
CA188178	R	+	+
UC-5	S	+	–
Suratato-77	R	–	+
Sonora-80	R	–	+
ICCV-5	R	+	+

R=Resistant, S=Susceptible, I=Intermediate, na=not available.
[a] =El-Hadi, 1993.
[b] =Haware and Nene, 1982.

CS-$27_{700}$and UBC-170_{500}DNA fragments that were identified as linked to the gene for resistance to race 1 of fusarium wilt by Mayer et al., (1997) were also linked to the locus that controls resistance to race 4 indicating a possible cluster of resistance genes. Identification of molecular markers on opposite sides of these genes would be especially useful for introgression into otherwise susceptible germplasm. The genomic positions of the genes for resistance to the wilt races needs to be determined and the possiblity that other wilt resistance genes may also be present in the cluster should be investigated.

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