Theor Appl Genet (1998) 96 : 348–353

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Inheritance of inter-simple-sequence-repeat polymorphisms and linkage with a fusarium wilt resistance gene in chickpea

Received: 10 August 1997 / Accepted: 6 October 1997

Abstract The inheritance of an inter-simple-sequence-repeat (ISSR) polymorphism was studied in a cross of cultivated chickpea (*Cicer arietinum* L.) and a closely related wild species (*C. reticulatum* Lad.) using primers that anneal to a simple repeat of various lengths, sequences and non-repetitive motifs. Dinucleotides were the majority of those tested, and provided all of the useful banding patterns. The ISSR loci showed virtually complete agreement with expected Mendelian ratios. Twenty two primers were used for analysis and yielded a total of 31 segregating loci. Primers based on $(GA)_n$ repeats were the most abundant while primers with a $(TG)_n$ repeat gave the largest number of polymorphic loci. Nucleotides at the 5′ and 3′ end of the primers played an important role in detecting polymorphism. All the markers showed dominance. We found an ISSR marker linked to the gene for resistance to fusarium wilt race 4. The marker concerned, UBC-855_{500}, was found to be linked in repulsion with the fusarium wilt resistance gene at a distance of 5.2 cM. It co-segregated with CS-27_{700}, a RAPD marker previously shown to be linked to the gene for resistance to fusarium wilt race 1, and was mapped to linkage group 6 of the *Cicer* genome. This indicated that genes for resistance to fusarium wilt races 1 and 4 are closely linked. The marker UBC-855_{500} is located 0.6 cM from CS-27_{700} and is present on the same side of the wilt resistance gene. To our knowledge this is the first report of the utility of an ISSR marker in gene tagging. These markers may provide valuable information for the development of sequence-tagged microsatellite sites (STMS) at a desired locus.

Communicated by P. M. A. Tigerstedt

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Key words Microsatellite · PCR · Disease-resistance · Mapping

Introduction

Chickpea (*Cicer arietinum* L.) is the third most important pulse crop in the world, and ranks first in the Indian subcontinent and the Mediterranean basin (FAO 1994). The annual growth rate of chickpea production has been very slow at 1.9%, and yields have risen at the rate of only 0.6% annually. The main difficulty in increasing yield is susceptibility to diseases such as fusarium wilt caused by *Fusarium oxysporum* Schlecht emd.: Fr. f. sp. *ciceris* (Padwick) Matuo & K. Sato and ascochyta blight, *Ascochyta rabiei* (Pass.) Lab. Fusarium wilt is reported from many countries of Asia, Africa, North and South America with yield-losses ranging from 10–90% (Srivastava et al. 1984; Jiménez-Diaz et al. 1993). The existence of pathogenic races of *F. oxysporum* is well established, with races 1, 2, 3, and 4 reported from India (Haware and Nene 1982) and races 0, 5 and 6 from Spain (Jiménez-Diaz et al. 1989). In many national and international chickpea breeding programs, improving resistance to fusarium wilt is a major objective. Current screening tests for resistance to fusarium wilt are time-consuming and inconsistent. Therefore, screening tests must be repeated to ensure accuracy. This necessitates the exploration of more efficient selection and breeding strategies than are currently employed. Two markers, CS-27_{700} and UBC-170_{550}, have been identified previously that amplified fragments linked to fusarium wilt race-1 resistance with 7% recombination (Tullu 1996; Mayer et al. 1997). Commonly used molecular markers have shown limited polymorphism in chickpea (Simon and Muehlbauer 1997). The existing chickpea map consists of 96 markers comprising isozyme, morphological markers, RFLP and RAPDs, and was made from genetical analyses of interspecific crosses between *C. arietinum* and

its wild relatives *C. reticulatum* and *C. echinospermum* (Gaur and Slinkard 1990; Kazan et al. 1993; Simon and Muehlbauer 1997). In recent years, a new class of molecular marker, the microsatellite marker, has been shown to be more useful and to be highly polymorphic. Microsatellites or simple-sequence repeats (SSRs) are short tandem repetitive DNA sequences with a repeat length of a few (1–5) base pairs (Litt and Luty 1989). The sequences are abundant, dispersed throughout the genome and are highly polymorphic in comparison with other molecular markers (Akkaya et al. 1992; Morgante and Olivieri 1993; Wang et al. 1994). Because of their short repeat length and limited interaction at individual loci, microsatellites can be readily studied via polymerase chain reaction (PCR) amplification (Moore et al. 1991; Lagercrantz et al. 1993). The abundance of microsatellites in the chickpea genome is shown by the hybridization-based multilocus fingerprinting technique using a variety of synthetic oligonucleotide probes of varying sequence and length (Weising et al. 1992; Sharma et al. 1995). Multilocus fingerprinting methods have been developed using oligonucleotides based on simple-sequence repeats as primers in PCR amplifications (Gupta et al. 1994; Zietkiewicz et al. 1994; Wu et al. 1994; Tsumura et al. 1996). This technique enables amplification of genomic DNA and provides information about many loci simultaneously. Here we have studied the inheritance of ISSR polymorphisms using 131 recombinant inbred lines (RILs) derived from the cross of *C. arietinum* (ICC-4958), a cultivated chickpea germplasm line with resistance of fusarium wilt, and *C. reticulatum* (PI 489777), the closest wild relative of the cultivated species. We also report a ISSR marker linked to the gene for resistance to fusarium wilt race 4.

Materials and methods

Plant material

The chickpea population used in this study was a set of 131 F_6-derived F_7 recombinant inbred lines (RILs) obtained from a cross of *C. arietinum* (ICC-4958) and *C. reticulatum* (PI 489777). The RILs were developed by the single-seed-descent procedure. Scoring for resistance to fusarium wilt in the two parents and 131 RILs was done in the glasshouse.

DNA extraction

DNA was isolated from vegetative buds and leaf tissues of the parents and RILs using the microprep method of Doyle and Doyle (1987). One gram of each leaf sample was submerged in liquid nitrogen and then ground to fine powder. The powder was quickly transferred to a tube containing 7.5 ml of ice-cold extraction buffer (0.35 M sorbitol, 0.1 M Tris, 5 mM EDTA, pH 7.5). The tube was briefly shaken and 7.5 ml of nuclei lysis buffer (2 M NaCl, 0.2 M Tris, 50 mM EDTA, 2% CTAB, pH 7.5) was then quickly added, followed by 3 ml of 5% sarkosyl solution. Sample sets were incubated in a 65°C water bath for 20 min. After incubation, the tubes were allowed to cool for a few min and 18 ml of chloroform/isoamyl alcohol (24 : 1) was added to each tube. The tubes were then centrifuged at 500 g for 15 min. The aqueous layer was removed and extracted again with a 15-ml chloroform mixture. Finally DNA was precipitated with chilled ethanol and was suspended in 1 ml of TE buffer.

Culture preparation and inoculation procedures

Inoculum was prepared from a single-spored fungal isolate grown on sterile filter paper placed on potato-dextrose-agar (PDA) as described in Tullu (1996). When the filter paper was completely colonized by the fungus, only colonies representative of wild-type were aseptically removed and placed in a fresh Petri dish to dry for 5 days in a laminar flow hood. The dried filter paper was cut into pieces aseptically using a pair of sterile scissors. Pieces of filter paper were then used to prepare the primary inoculum. The conidial concentration was adjusted to 1×10^6 spores ml^{-1} with a hemacytometer. Twelve to twenty seeds of each RIL were grown in the glasshouse (21–26°C) in single rows in plastic trays filled with sterile coarse perlite. When the seedlings reached the 3–4 nodal stage, they were carefully removed from the perlite, pruned while submerged in the spore suspension, and after about 5 min in the spore suspension were re-planted into the perlite. Plants were then scored as susceptible or resistant over the next 2 months.

Oligonucleotide primers

One-hundred primers of 15–23 nucleotides in length (UBC set #9) were obtained from the Biotechnology Laboratory, University of British Columbia, Vancouver, British Columbia, Canada. Twenty two polymorphic primers which gave clear and consistent banding patterns were used to study segregation.

PCR amplification and electrophoresis

PCR amplification was performed in 10 mM Tris-HCl pH 8.3, 50 mM KCl, 0.1% Triton × 100, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 0.24 μm of primer, 30 ng of genomic DNA per 25 μl of reaction vol and 1 unit of *Taq* polymerase. PCR amplifications were done using a Perkin Elmer Cetus 9600, with de-naturation at 94°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 2 min for 35 cycles with a 10-min final extension at 72°C. For (AT)-rich sequences, primer annealing was carried out at 36°C. PCR products were separated on 2% agarose gels, then stained with ethidium bromide and scored for the presence or absence of bands. Since ISSR markers are dominant, a locus was considered to be polymorphic if the band was present in one parent and not in the other.

Results

Selection of ISSR markers and their inheritance

In this study, 38 primers out of 100 were found to be polymorphic, and 26 were monomorphic; the other primers did not show amplification. From the primers that gave amplification products, we selected 22 that gave the clearest bands. A total of 190 bands were amplified of which 31 bands were scored; unstable and weak bands were not scored. Out of 31 bands, 29 segregated in a 1 : 1 (presence : absence) ratio. The number of bands for each primer which produced a

polymorphic banding pattern varied from 2 (UBC-843) to 16 (UBC-888) in the size range of 200 bp (UBC-885) to 3 kb (UBC-840). The polymorphism with simple-sequence repeats was primer-dependent. In our study the $(GA)_n$ primers were most abundant while primers with $(TG)_n$ repeats gave rise to the largest number of polymorphic loci followed by $(AC)_n$ repeats. The sequences of the 22 primers that were selected, together with the number of polymorphic bands amplified in the parental lines, are shown in Table 1. The marker was designated according to the primer number, followed by the molecular weight in base pairs. Figure 1 shows a representative picture of PCR-amplified DNA fragments using primer UBC-840.

Marker UBC-855_{500} linked to the fusarium wilt resistance gene

The 131 RILs were inoculated with race 4 of fusarium wilt. The scoring for the disease reaction of each RIL was either resistant (0–10% wilted plants) or susceptible (90–100% wilted plants). However, a few RILs had an intermediate type of reaction (11–89%) and were not used for the analysis. The two parents ICC-4958 and *C. reticulatum* (PI 489777) were resistant and susceptible, respectively, to race 4. We surveyed 131 RILs with ISSR primers and identified that UBC-855_{500} amplified a DNA region closely associated with the fusarium wilt race-4 resistance gene. The amplification pattern revealed that UBC-855_{500} was amplified in all the susceptible lines and was absent in the resistant lines. However, the marker was also amplified in five resistant lines. The amplification of the UBC-855_{500} band in resistant lines was most likely due to recombination between the gene and the marker. The genotypic data were then used to determine the degree of linkage of the marker and its relative position to the locus conferring resistance to race 4 of fusarium wilt. Linkage analysis was performed using the Mapmaker program (Lander et al. 1987) with a Lod score of 7.0. Goodness of fit to the expected 1 : 1 Mendelian ratio for

Table 1 Segregation of ISSR fragments in chickpea RILs developed from a cross of ICC-4958 and PI 489777. "a" indicates band presence, "b" indicates band absence, *R* purine, *Y* pyrimidine; *N* any nucleotide; *B* indicates C, G or T; *D* as A, G, or T; *H* as A, C, or T; and *V* as A, C, or G

Primer	Sequence	Fragment size (bp)	Observed ratio a : b	χ^2 (1 : 1)	*P*
UBC807	AGAGAGAGAGAGAGAGT	829	71 : 60	0.4618	0.25–0.50
UBC809	AGAGAGAGAGAGAGAGG	1400	65 : 66	0.0038	0.90–0.95
UBC810	GAGAGAGAGAGAGAGAT	1100	62 : 69	0.1870	0.50–0.75
UBC811	GAGAGAGAGAGAGAGAC	1600	65 : 66	0.0038	0.90–0.95
UBC823	TCTCTCTCTCTCTCTCC	940	70 : 61	0.3091	0.50–0.75
UBC823	TCTCTCTCTCTCTCTCC	1800	71 : 60	0.4618	0.25–0.50
UBC825	ACACACACACACACACT	900	67 : 64	0.0343	0.75–0.90
UBC825	ACACACACACACACACT	1100	63 : 68	0.0594	0.75–0.90
UBC830	TGTGTGTGTGTGTGTGG	1300	73 : 48	0.8587	0.25–0.50
UBC840	GAGAGAGAGAGAGAGAYT	1000	53 : 78	2.3580	0.10–0.25
UBC840	GAGAGAGAGAGAGAGAYT	1300	67 : 64	0.0343	0.75–0.90
UBC842	GAGAGAGAGAGAGAGAYG	1200	65 : 66	0.0038	0.95–0.97
UBC843	CTCTCTCTCTCTCTCTRA	1050	95 : 36	13.2862	< 0.005
UBC855	ACACACACACACACACYT	375	64 : 67	0.0343	0.75–0.90
UBC855	ACACACACACACACACYT	500	90 : 41	9.1641	< 0.005
UBC856	ACACACACACACACACYA	1800	63 : 68	0.0954	0.75–0.90
UBC858	TGTGTGTGTGTGTGTGRT	400	65 : 66	0.0038	0.95–0.97
UBC859	TGTGTGTGTGTGTGTGRC	1800	60 : 71	0.4618	0.25–0.50
UBC860	TGTGTGTGTGTGTGTGRA	725	60 : 71	0.4618	0.25–0.50
UBC864	ATGATGATGATGATGATG	425	83 : 48	4.6755	0.02–0.50
UBC866	CTCCTCCTCCTCCTCCTC	900	61 : 70	0.3091	0.50–0.75
UBC868	GAAGAAGAAGAAGAAGAA	700	77 : 54	2.0190	0.10–0.25
UBC880	GGAGAGGAGAGGAGA	1050	74 : 57	1.1030	0.25–0.50
UBC880	GGAGAGGAGAGGAGA	1400	72 : 59	0.6450	0.25–0.50
UBC880	GGAGAGGAGAGGAGA	1600	74 : 57	1.1030	0.25–0.50
UBC884	HBHAGAGAGAGAGAGAG	450	68 : 63	0.0954	0.75–0.90
UBC884	HBHAGAGAGAGAGAGAG	1150	60 : 71	0.4618	0.25–0.50
UBC884	HBHAGAGAGAGAGAGAG	1600	80 : 51	3.2099	0.05–0.10
UBC885	BHBGAGAGAGAGAGAGA	1100	78 : 53	2.3850	0.10–0.25
UBC885	BHBGAGAGAGAGAGAGA	1500	79 : 52	2.7824	0.05–0.10
UBC888	BDBCACACACACACACA	600	63 : 68	0.0954	0.75–0.90

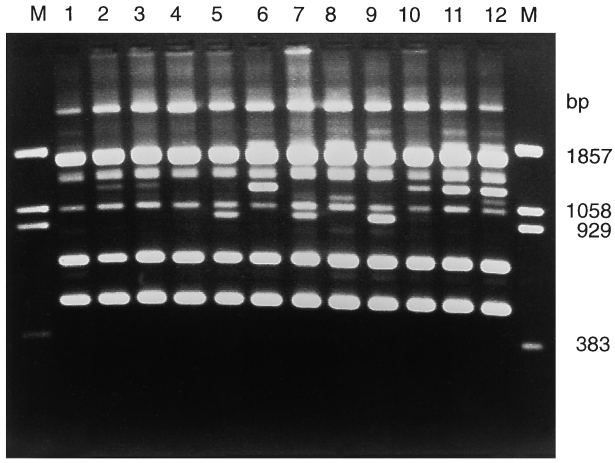

Fig. 1 PCR-amplified inter-simple-sequence-repeat patterns on 2% agarose gels. *M* represents the marker pBR 322 *Bst*N1 digest. *Lanes 1–12* represents chickpea RILs amplified with primer UBC 840

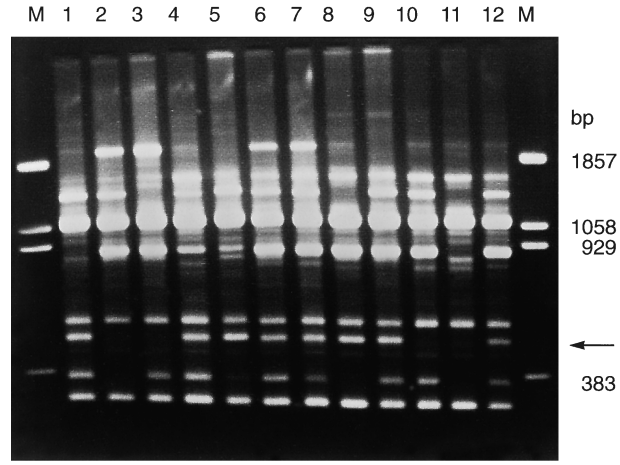

Fig. 2 PCR-amplified inter-simple-sequence-repeat patterns on 2% agarose gels. *M* represents the marker pBR 322 *Bst*N1 digest. *Lane 1–12* represents chickpea RILs amplified with primer UBC 855. The marker linked with fusarium wilt is indicated by an *arrow*

each segregating locus was determined by chi-square. Based on these data, we estimated that UBC-855_{500} is located 5.2 cM away from the gene for resistance to fusarium wilt race 4.

The genes for resistance to race 1 and race 4 are linked

Two markers, CS-27_{700} and UBC-170_{550}, amplify fragments linked to fusarium wilt race-1 resistance in RILs developed from a cross between C-104 and WR-315 (Tullu 1996; Mayer et al. 1997). Both the RAPD markers are located 7 cM from the resistance gene and are on the same side. In this study UBC-855_{500} co-segregated in coupling with CS-27_{700}. The CS-27_{700} locus was mapped to linkage group 6 of the *Cicer* genome (Simon and Muehlbauer 1997). UBC-855_{500} is 0.6 cM from CS-27_{700}. This indicated that the genes for resistance to race 1 and 4 are also closely linked. Such linked markers and a complete genetic map can be used to investigate if there are one or more additional genes segregating in the population which can account for the different classes of resistance as reported by Kumar and Haware (1982) and Upadhyaya et al. (1983). The electrophoretic pattern of the PCR-amplified DNA fragment using primer UBC-855 is shown in Fig. 2.

Discussion

The ISSR loci we studied showed virtually complete agreement with the expectations of Mendelian segregation, which is necessary for their use in genome mapping and population studies. The Mendelian inheritance of the ISSR markers indicated that most lines are relatively unbiased and show low heterozygosity. The lines would thus appear to constitute a suitable population for the mapping of major disease resistance genes. The choice of parents for the construction of the interspecific mapping populations was based on the fact that there is very little polymorphism within chickpea accessions. The availability of RILs as permanent mapping populations will greatly facilitate the mapping of other genes.

The degree of polymorphism detected in the present study is comparatively higher than previously reported using RFLP markers (Simon and Muehlbauer 1997). This may be due to the high resolving power of PCR-based markers for detecting polymorphism. The probability of finding a marker within a specified distance of a target gene depends on the genome size, the number of primers screened, and the sequence divergence in the region scanning the target gene. Since chickpea has little genetic diversity it was necessary to screen many ISSR primers to identify a marker linked to the gene for resistance to fusarium wilt race 4. In our study, 90% of PCR products were common between *C. arietinum* and *C. reticulatum*, indicating that microsatellites are highly conserved in both species. This is not surprising considering the large amount of structural and functional homology that exists between the two species. In this study, the markers showing skewed ratios were amplified from *C. reticulatum.* The possible reason for skewed ratios might be preferential chromosome elimination (Goshen et al. 1982) or selective elimination of particular zygotes (Zamir and Tadmor 1986).

Identifying fusarium wilt race-specific resistance genes and transferring them to adapted backgrounds are major challenges. To-date, only genes conferring resistance to race 1 have been reported. For race 1, three genes are reported to control resistance; complete resistance is conferred when any two of the loci are present, while partially recessive alleles in a

homozygous condition delay wilting (Singh et al. 1987). Screening techniques for evaluating fusarium wilt resistance have already been developed and standardized; however, they are costly in terms of time and effort. Several sources of fusarium wilt resistance have been reported, and it is possible that the resistance in these lines involves distinct genes. At present, no information is available on the chromosomal location of race-specific resistance genes in the *Cicer* genome. Our study indicated that genes for fusarium wilt race 1 and race 4 map to the same location and are present on linkage group 6. Further, the marker UBC-855_{500} can be used for selecting the fusarium wilt-resistant lines.

Advantages of UBC-855_{500} over RAPD markers

The results confirm the ability of primers, based on SSR motifs, to produce fragments that are useful as genetic markers. The ISSR markers possess a number of relative advantages over RFLP and RAPD markers for use in high-resolution mapping and marker-assisted indirect selection of traits in plant-breeding applications. The ISSR marker procedure is simple, quick, reliable and can process many samples per day compared to RFLP. The amplification conditions with ISSR markers are very stringent, which allows high reproducibility as compared to RAPD markers. The UBC-855_{500} marker can provide valuable information for the selection of fusarium wilt. This marker is closer to the fusarium wilt resistance gene than other RAPD markers and can be used to accurately select resistant lines. It can also provide information for the development of sequence-tagged microsatellite sites (STMS) linked to the fusarium wilt resistance. Finding STMS at a desired locus is very expensive and time consuming. The microsatellite-primed PCR can speed up the identification of such markers.

Conclusions

In summary, our results indicate that simple-sequence repeats can be used as highly informative markers across a wide range of chickpea mapping and breeding populations. The marker UBC-855_{500} can be of potential use in marker-assisted selection for fusarium wilt resistance. Marker-assisted selection (MAS) makes it possible to screen for disease resistance without the need to inoculate the segregating plant material. Pyramiding genes for resistance into a single genetic background would also be facilitated by marker-assisted selection.

Acknowledgements This work was supported by the McKnight Foundation through a collaborative project between the Institute of Biological Chemistry and the Department of Crop and Soils of Washington State University, Pullman, Washington, USA, and the Plant Molecular Biology Unit, National Chemical Laboratory, Pune, India. M. B. Ratnaparkhe and D. K. Santra wish to thank the McKnight foundation for providing the Fellowship that made this research possible. Helpful suggestions by Drs. C. A. Ryan, T. W. Okita, V. R. Franceschi, P. K. Ranjekar and V. S. Gupta are gratefully acknowledged. We also thank Drs. S. S. Jones and C. J. Coyne for a critical review of the manuscript, D. Tinnemore for technical assistance, and A. Kahriman for assistance with the computer analysis.

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