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Research Article

Interspecific detection of polymorphism using sequence tagged microsatellites (STMS) in chickpea

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Abstract

Chickpea is an important grain legume of the semiarid tropics and warm temperate zones, and form one of the major components of human diet. Genetic mapping in chickpea was initially hampered due to limited availability of genomic resources and early reliance on dominant markers. The development of STMS and other co-dominant markers has greatly improved our understanding of the chickpea genome. In the present study a set of 129 $F_{6:7}$ recombinant inbred lines (RILs) obtained from an interspecific cross of *Cicer arietinum* ((ICC4958, resistant) × *Cicer reticulatum* (ICC489777, susceptible) was used to analyze genetic diversity pattern using twenty five polymorphic STMS markers. Since the accession ICC4958 is resistant to Fusarium oxysporum, and parental line ICC489777 is susceptible, the segregation of underlying resistance loci could also be followed along with the evaluation of genetic diversity and molecular mapping in chickpea using these polymorphic markers.

Key words : RIL, recombinant inbred lines; STMS, sequence tagged microsatellite sites; Co-dominant; *Cicer arietinum*

Introduction

Chickpea (*Cicer arietinum* L.), is the third most important cool season food legume in the world after dry beans and peas (FAOSTAT, 2004). It has been cultivated mainly in the Indian subcontinent, West Asia, and North Africa, but recently large acreages have been introduced in the Americas and Australia. Chickpea is a diploid with $2n = 2x = 16$ (Arumuganathan and Earle, 1991) having a genome size of approximay931Mbp (www.rbgkew.org.uk/cval). It is a highly self-pollinated crop with an out crossing rate of less than 1%. It serves as an important source of protein in human diet and plays an important role in the enrichment of soil fertility.The genetic advance for yield in chickpea is low because of limited genetic variation present in the germplasm and therefore is classified a recalcitrant crop (Van Rhenen et al., 1993).

Lack of desired genetic variation in available germplasm of cultivated species necessitates the exploitation of related annual species for genetic improvement. Interspecific or wide hybridization has been identified as a potential means of increasing genetic variation and introduction of resistance genes in cultivated species from wild species.

Among the biotic stresses that affect chickpea, fusarium wilt caused by *F. oxysporum* Schlechtend.:Fr. f. sp. *ciceris* has been reported in many countries as a major yield-limiting factor (Nene and Reddy, 1987; Haware, 1990). The pathogen is either soilborne or seed transmitted (Kraft et al., 1994) and can survive in the soil in the absence of the host for at least 6 yr (Stevenson et al., 1995). The most practical and economical method for controlling the disease is through the use of resistant cultivars.

Polymorphic molecular markers are the prerequisite for mapping disease resistance genes as well understanding molecular breeding activites. Molecular markers have been used to establish linkage maps for many crop species (O'Brien, 1993) and they have been utilized to determine gene number for particular traits and for gene tagging (Paterson et al., 1991; Lee, 1995). However, the availability of reasonable number of polymorphic markers in chickpea is

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very low (Muehlbauer and Singh, 1987; Gaur and Slinkard, 1990; Kazan et al., 1993; Simon and Muehlbauer, 1997).One of the main reasons for this may be attributed to the low level of genetic diversity present in the cultivated gene pools of these species, atleast with the detection tools that are currently available (Varshney et al., 2007).

Many important disease resistance genes have been mapped and tagged in various crops (Staub et al., 1996; Mohan et al., 1997). RAPD markers (Williams et al., 1990; Welsh and McClelland, 1990) are simple and fast and have been employed widely for mapping genomes (Torres et al., 1993; Hunt and Page, 1995) and for tagging resistance genes (Staub et al., 1996; Mohan et al., 1997). Isozyme analysis has revealed insufficient polymorphism to be useful in finding tags for fusarium wilt and ascochyta blight resistance genes (Kusmenoglu et al., 1992). DNA marker systems such as RAPD, ISSR, sequence tagged microsatellite sites (STMS), and amplified fragment length polymorphism (AFLP) overcome the problem of minimal polymorphism and allow more detailed analysis of the genome.Genes conferring resistance to fusarium wilt in chickpea have been tagged with RAPD and ISSR markers (Ratnaparkhe et al., 1998;Cobos et al.,2005).

Use of molecular markers to analyze genetic diversity and mode of inheritance of race specific resistant genes along with their location in the chickpea genome through construction of high density genetic map will be useful in identification of genes/QTLs associated wilt resistance as well as for understanding extensive molecular breeding in chickpea. Thus, the objective of the present study was to analyze polymorphic STMS markers in 129, resistant and susceptible individuals of an inter-specific mapping population (*C.arietinum* ICC4958,resistant × C.reticulatum PI489777 ,susceptible) to fusarium wilt.

Materials and methods

A set of 129 $F_{6:7}$ recombinant inbred lines (RILs), obtained from cross between cultivated chickpea line *Cicer arietinum* ICC4958 × *Cicer reticulatum* ICC489777.The seeds of RILs and parents were procured from Dr, F.J. Muehlbauer (USDA-ARS ,Washington state university, Pullman, USA).and grown in the experimental field of NIPGR, Delhi, India during the crop season 2006-07 for genomic DNA isolation and collection of seeds. Leaves from 10- to 12-day-old seedlings were harvested from each inbred lines and DNA extracted using the CTAB method (Khan et al.,2004). From the primers developed in the laboratory of NIPGR (Sethy et al., 2006, unpublished), 25 polymorphic STMS primer pairs (Table -1) were selected to represent the whole nuclear genome. The Tm value of the primers was between 41^0C and 49^0C.Amplification reactions were carried out in Gene Amp PCR system 2700, 96-well DNA thermal cycler (Applied Biosystems Singapore).Each 25µl reaction mix comprised 13.4 µl sterile-distilled water, 0.1 µl (.1U) of Taq polymerase (Titanium) ,2.5 µl of 10× PCR with $MgCl_2$ (supplied with enzyme with a camposition of 100mM Tris-HCl ,15 mM $MgCl_2$ and 500 mM KCl,pH 8.3), 1.0µl of dNTP (10 mM equimolar solution of each dATP, dCTP, dGTP, and dTPP), 1µl of each forward and reverse primers (10µM solution) and 5µl of template DNA (10 ng/µl). Touchdown PCR cycles were used for amplification .The PCR conditions are as follows: PCR profile consisting of 18 cycles of 94^0C for 1 minute denaturing, and 72^0C for 1 minute extension.Annealing temperatures (30s) were progressively decreased by 0.5 degree every cycle from 64^0C to 55^0C .The PCR reaction continued for 30 additional cycles at 94^0C for 1 minute 55^0C for 1 minute and 72^0C for 1 min.The reaction ended with a 10-min extension at 72^0C.Amplification products from ×California,USA,Sequi-GenGT) and visualised by silver staining (Promega Silver Sequencing System,Wisconsin, USA).

Results and discussion

The primer pairs used in this study were designed against microsatellite motifs of variant size (total repeat length), type (simple, imperfect, campound and interrupted) and composition (AT rich and GC rich). So, the annealing temperature (Tm) varied significantly. In order to amplify the correct microsatellite loci stringent amplification conditions were employed.Using touchdown amplification profile we were able to amplify the NCPGR series of microsatellite markers (Table-1).

Polymorphism in the form of length variant bands as well as multiple bands were observed between the parental lines.The 129 resistant and susceptible RILs were amplified along with the mapping parents i.e. *C.arietinum* (ICC4958, resistant) × *C.reticulatum* (PI489777 , susceptible).

The polymorphic bands were scored in a spreadsheet format, with ‘A’ representing the first parental band,’B’ representing the second parental band and ‘H’ representing the heterozygote individuals.The 25 polymorphic markers analyzed in this study will be incorporated into the inter-specific genetic linkage map to tag genes conferring resistance to fusarium wilt in chickpea (*C.arietinum* ICC4958 × *C.reticulatum* PI489777) using STMS markers.

The linkage map in chickpea based on molecular markers is less well developed when compared to maps of other crops. Difficulties of mapping the *Cicer* genome are due to the minimal amount of polymorphism available. The first genetic linkage map of *Cicer* genome consisted of four linkage groups based on isozyme markers (Gaur and Slinkard, 1990). Later, a map of 10 linkage groups was reported using three separate F_2 populations and included 28 isozyme, 44 RAPD, 9 RFLP, and 6 other markers (Simon and Muehlbauer, 1997). A linkage map of *Cicer* based on a RIL population and using STMS was reported (Winter et al 1999**;** Halila et al., 2008). Use of recombinant inbred lines instead of an F_2 population is advantageous for mapping fusarium blight resistance genes because nearly homozygous lines are scored rather than individual heterozygous plants. There was little segregation within RILs and this simplified scoring disease reactions. Seed sterility was not a problem in the RILs although the lines were developed from an interspecific cross. Interspecific crosses (*C. arietinum* x *C. reticulatum*) were also used for mapping isozyme and DNA markers in chickpea (Gaur and Slinkard, 1990; Kazan et al., 1993; Simon and Muehlbauer, 1997).

In conclusion, through this study we have substantially increased the number of markers that reveal polymorphism in cultivated chickpea.Sufficient markers are now available for a skeleton map of cultivated chickpea ,provided that a sufficiently wide crosses is used.

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Table 1. List of polymorphic microsatellite markers

S.No.	Polymorphic Markers	Microsatellite motifs
1	NCPGR 5	(CT)19
2	NCPGR 6	(GA)12(GA)3(GA)4(GA)10(GA)6
3	NCPGR 17	(GA)12(GA)3(GA)4(GA)9
4	NCPGR 50	(CT)13(CA)11
5	NCPGR 90	(GA)20TA(GA)5
6	NCPGR 107	(CA)15(CA)>20
7	NCPGR 117	(CT)2TC(CT)21
8	NCPGR 136	(CT)16(CT)18
9	NCPGR 141	(CT)25
10	NCPGR 182	(CT)24TT(CT)2
11	NCPGR 200	(GA)8AA(GA)31AA(GA)9
12	NCPGR	(CA)4(CA)10(TA)4
13	NCPGR	(TA)2(CA)13
14	NCPGR	(CT)10
15	NCPGR	(CA)23(TA)2
16	NCPGR	(CT)14(CA)13
17	NCPGR	(GA)8(CA)4
18	NCPGR	(CT)12TT
19	NCPGR	(GA)19
20	NCPGR	(CA)12
21	NCPGR	(GA)12(GA)3(GA)4(GA)10
22	NCPGR	(GA)20TA
23	NCPGR	(GA)8AA(GA)13
24	NCPGR	(GA)12(GA)2
25	NCPG2	(GA)4(GA)9

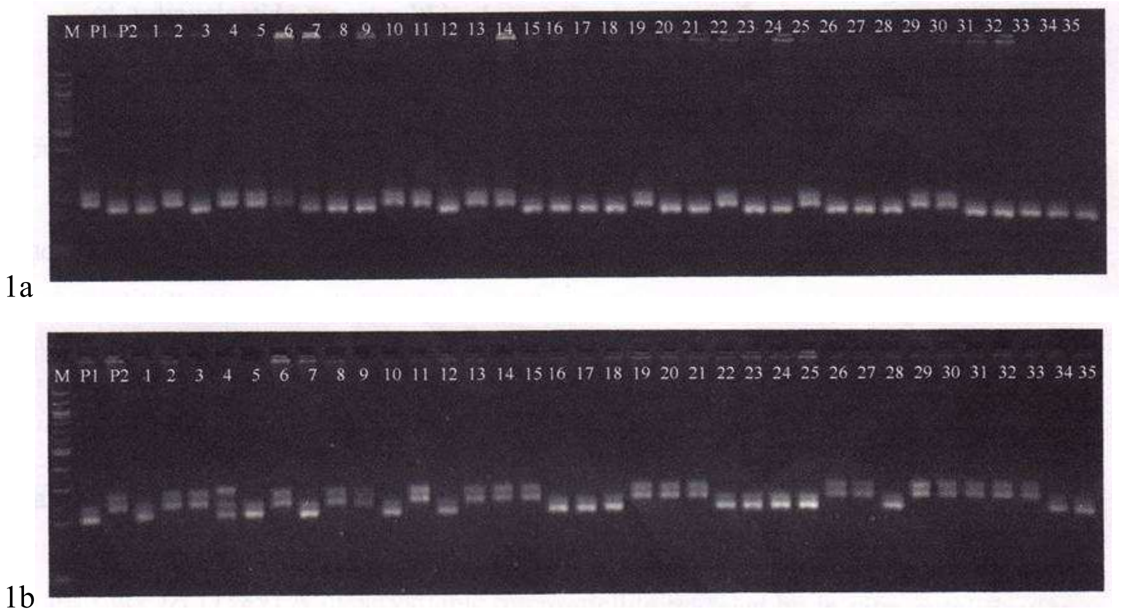

Fig.-1 Genetic variation revealed by primer pairs a NCPGR 37, b NCPGR 57