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Development of CAPS Marker Linked to H_2 Locus of *Fusarium* Wilt Resistance in Chickpea

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ABSTRACT

Background: *Fusarium* wilt is one of the widely distributed biotic stress of chickpea limiting its productivity worldwide. The major problem limiting the resistance breeding is screening of germplasm and breeding lines for disease resistance. To address such problems, identification of molecular marker closely linked to resistance locus is an effective strategy.

Methods: The RAPD marker $A07C_{417}$ closely linked H_2 locus of *Fusarium* wilt resistance locus has been converted into SCAR and the loss of initial polymorphism was recaptured by alignment of consensus sequences from SCAR amplified locus for the identification of common motifs. Further, the linkage association of this marker with *Fusarium* wilt resistance locus has been reaffirmed using *Fusarium* wilt response of RILs phenotyped for wilt reaction using wilt sick pots and also wilt sick plots.

Result: Identification of single nucleotide polymorphism at the consensus SCAR locus between susceptible and resistant cultivar, enabled development of CAPS marker through suitable restriction enzyme *Aci* I making it an effective codominant marker system for resistance deployment via marker assisted selection.

Key words: CAPS, Chickpea, *Fusarium*, Linkage, SNP.

INTRODUCTION

Fusarium wilt caused by the *Fusarium oxysporum* f. sp. *ciceri* (FOC) is one of the major yield constraining elements in chickpea. The production of chickpea is seriously abridged by *Fusarium* wilt in all chickpea growing areas of the world and it has been reported from at least 33 countries, causing an annual yield loss of upto 100 *per cent* under favourable conditions (Cortes and Hav, 2000; Chattopadhya and Mohapatra, 2015). *Fusarium oxysporum* f. sp. *ciceri* (Foc) is a soil born pathogen and it also survives in the hilum region of seed as chlamydospore-like structures (Haware *et al.,* 1978). Till date, two pathotypes (yellowing and wilting) and eight distinct physiological races of pathogen (0, 1A, 1B, 2, 3, 4, 5 and 6) have been reported, of which six races cause wilting symptoms, whereas races 0 and 1B/C cause yellowing symptoms (Kelly *et al.,* 1994; Del Mar Jimenez-Gasco and Jimenez-Diaz, 2003; Jimenez-Fernandez *et al.,* 2013).

The genetic analysis of wilt resistance has indicated that the resistance to *Fusarium oxysporum* f. sp. *ciceri* race 0 (FOC 0) is governed by means of one or two independent genes (Tekeoglu *et al.,* 2000 and Rubio *et al.,* 2003); resistance to FOC 2 is conferred by a single recessive gene (Sharma and Muehlbauer, 2007); resistance to FOC 3 by monogenic (Sharma *et al.,* 2004) and resistance to race 4 (FOC 4) is monogenic recessive (Tullu *et al.,* 1998 and Sharma *et al.* 2005) as well as digenic recessive (Tullu *et al.,* 1999), but resistance to race 5 (FOC 5) is governed by single gene (Tekeoglu *et al.,* 2000 and Sharma *et al.,* 2005). The present study, is concentrated on race 1A, which is overwhelming in peninsular India in a pestilence manner, where resistance to FOC 1 is conditioned by two to three independent loci designated as H_1, H_2 and H_3 (Upadhyaya *et al.,* 1983; Singh *et al.* 1987 and Brinda and Ravikumar, 2005). Dominant alleles at both loci (H_1H_1 H_2H_2) results in early wilting reaction, homozygous recessive allele at either one of the loci (H_1_ h_2h_2 or h_1h_1 H_2_) produces late wilting reaction and the homozygous recessive allele at both the loci ($h_1h_1h_2h_2$) confer resistance to *Fusarium* wilt (Brinda and Ravikumar, 2005).

It is very difficult to manage this particular disease due to soil borne nature of pathogen (Chaithra *et al.,* 2019), however, the major problem encountered for the development of high yielding wilt resistant genotypes is the divergence of the pathogen and also the development and maintenance of uniform wilt sick plots. Further the severity of disease is also affected by inoculum concentration, virulence and environment conditions (Jimenez-Gasco and

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Perez-Artes, 2001). To overcome all these drawbacks, deployment of host plant resistance and development of resistant cultivar through marker assisted selection (MAS) is one of the best means to combat this problem. Therefore, identification of reliable DNA markers closely linked to resistant loci increase the efficiency of selection of resistant genotypes and also for the introgression of resistance loci against *Fusarium* wilt.

Several molecular markers linked to *Fusarium* wilt resistance have been reported in chickpea. However, there are only a few attempts to specifically identify molecular marker linked to H_1 or H_2 loci independently ($CS27_{700}$; Mayer *et al.,* 1997 and $A07C_{417;}$ Soregaon *et al.,* 2007). RAPD (Random Amplified Polymorphic DNA) markers are generally dominant, sensitive and non-stringent with poor reproducibility (Williams *et al.,* 1990; Jones *et al.,* 1997 and Bardakci, 2001). In the last few decades, there are growing number of cases, in which RAPD markers have been used to develop either allele-specific or locus-specific primers. SCAR (Sequence Characterized Amplified Regions)/CAPS (Cleaved Amplified Polymorphic Sequence) marker were developed by cloning and sequencing of amplified products of an RAPD marker offers more advanatges over RAPD marker as they detect only single locus, less sensitive to changing reaction condition and they can be potentially converted into co-dominant markers (Paran and Michelmore, 1993; Zheng *et al.,* 1999; Zhang and John, 2001 and Guiterrez *et al.,* 2006).

In this contest, in the present study an attempt was made to convert RAPD marker $A07C_{417}$ closely linked H_2 locus of *Fusarium* wilt resistance in chickpea (Soregaon *et al.,* 2007) into SCAR marker. However, loss of initial polymorphism among the parental genotypes in the SCAR amplified amplicons has been recovered by development of co-dominant CAPS marker and we also report our effort in ascertaining the linkage of this CAPS marker to *Fusarium* wilt resistance using recombinant inbred lines (RILs) screened over the seasons for wilt reaction.

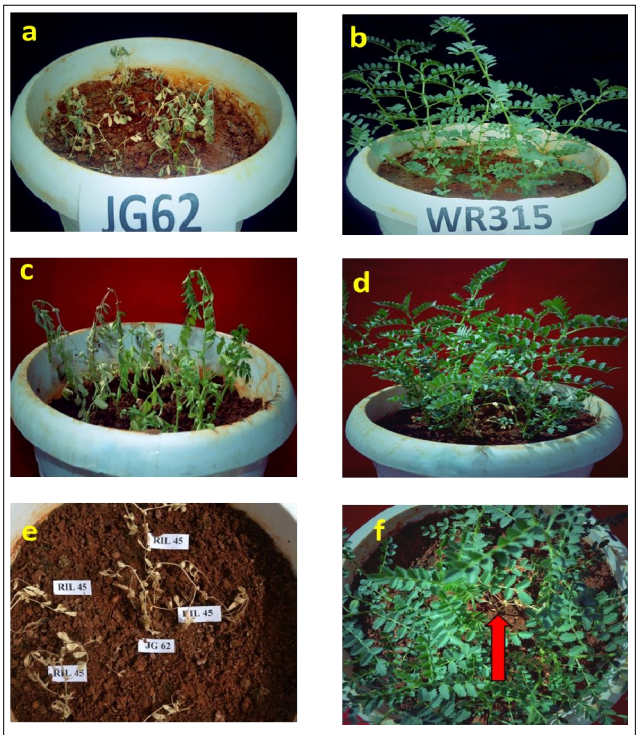
MATERIALS AND METHODS

Plant material and DNA extraction

The experiment was conducted in *rabi* session of 2016-17 at Department of Plant Biotechnology, University of Agricultural Sciences (UAS), GKVK, Bangalore, India. The experimental material consists of two parental genotypes, JG62, an early wilting genotype ($H_1H_1H_2H_2$), highly susceptible to *Foc* race 1 and WR315 genotype ($h_1h_1h_2h_2$) resistant to race 1A, race 2, race 3, race 4 and race 5 of *Fusarium* wilt (Upadhyaya *et al.,* 1983; Mayer *et al.* 1997 and Sharma *et al.* 2005) and also 117 F_{12} RILs derived from the cross JG62 X WR315. Total genomic DNA was extracted from vegetative buds and young leaves of two parental lines and individual RILs population by Cetyl Trimetyl Ammonium Bromide (CTAB) method as described in Doyle and Doyle (1987) with little modifications. The quality of DNA was assessed on 0.8 per cent agarose gel prepared in 1X TBE buffer followed by visualization and documentation using Alpha digidoc system (Alpha Innotech Corporation, USA) and quantification was done by nanodrop (Eppendorf Bio spectrometer).

Phenotyping: Evaluation of RILs for *Fusarium* wilt resistance

The RILs and parental lines were phenotyped for wilt reaction in green house using wilt sick pots developed at Department of Plant Biotechnology, UAS, GKVK, Bangalore. The wilt sick pots (30 cm diameter) were developed using the single spore culture of *Fusarium oxysporum* race 1A by following the standard procedure (Brinda and Ravikumar, 2005). The presence of pathogen in the pots was confirmed by growing the susceptible cultivar JG-62 plants (5-6 plants per pot). The disease screening using wilt sick pots was unambiguous and reliable as the early wilting susceptible genotype JG62 showed complete wilting within 30 days in wilt sick pots (Fig 1). The six seeds of each RILs were sown in wilt sick pot along with JG-62 as susceptible check at the centre in each wilt sick pots. The RILs were classified as susceptible if 60 per cent of the plants recorded wilting symptoms and death on 60th day and remaining as resistant (Haware and Nene, 1982) (Fig 1). Similarly, the data on the wilt reaction of RILs in the early generation over two years (*Rabi* 2007 and *Rabi* 2008) in wilt sick plots maintained at ICRISAT, Hyderabad was also used for the present study.



Where, a: Susceptible parent (JG-62), b: Resistant parent (WR-315), c: Susceptible RIL, d: Resistant RIL, e: Suceptible RIL at 60th day with JG-62 at center, f: Resistant RIL with JG-62 at center.

Fig 1: Phenotyping of RILs for *Fusarium* wilt reaction in wilt sick pots.

Design and amplification of SCAR marker

The five different SCAR primers were developed on the basis of available sequence information of an RAPD marker $A07C_{417}$ associated with H_2 locus of wilt resistance (Soregaon *et al.,* 2007) for the specific amplification of the target locus using Primer3 software. The primer sequence ranged from 15 bp in A07CSCAR-2 to 25 bp in A07CSCAR-3 and A07CSCAR-5 (Table 1). The primers were synthesised from Sigma-Aldrich Corporation (United nation).

CAPS marker development and analysis

SCAR amplified products produced monomorphic amplicons and did not differentiate the parental lines even after the optimization of PCR parameters. To recover the loss of initial polymorphism, SCAR amplified products from both the parental lines were sequenced and the consensus sequence obtained were analysed by means of sequence homology using BioEdit software to identify common motifs and appropriate restriction enzyme was selected for the development of CAPS marker. Restriction endonuclease digestion assay of parental lines and RILs were carried out using restriction enzyme *Aci* I in a 15 µl of SCAR amplified product in a reaction volume of 25 µl with 3U of restriction enzyme *Aci* I and 1X appropriate buffer based on supplier manual recommendation (New England Biolabs, Inc.) and restriction digested products were resolved on 3% agarose gel in 1X TBE buffer stained with 0.05 µg/ml ethidium bromide.

Statistical analysis

The segregation of CAPS marker among RIL population was tested for goodness-of-fit test (χ^2) for the segregation ratio of 1:1 and Goodness-of-fit test of CAPS marker was also performed together with 60th day wilting percentage for digenic inheritance ratio of 1:1:1:1 to determine the linkage. The percentage of plants infected on the 60th days in each RIL was used to compare the two classes for marker and the difference was compared using t-test.

RESULTS AND DISCUSSION

Development of SCAR/CAPS marker

The amplification of DNA of parental lines JG-62 (susceptible) and WR-315 (resistant) using five different SCAR primer sets produced monomorphic bands. The SCAR primer A07CSCAR-3 producing single, specific predicted size in both susceptible (JG62) and resistant parent (WR315) was selected and amplified products from both parental lines were sequenced. Comparison of consensus sequence information of SCAR amplified products from both the parental lines JG-62 and WR-315 enabled the identification of single nucleotide polymorphism (SNP) with a restriction site mutation for restriction enzyme *Aci* I (C▼CGC/GGC▲G) specific to resistant parent WR-315 at 219 bp (Fig 2), which clearly distinguished the susceptible and resistant parents. The restriction enzyme digestion of monomorphic SCAR product from both the parents JG-62 and WR-315 produced an expected electrophoretic banding pattern of two bands of size 212 bp and 159 bp in resistant parent WR-315 and single band of 371 bp in susceptible parent JG-62 (Fig 3).

The essential requirement of any MAS programme involves the identification of polymorphic markers that are closely linked to trait of interest and their validation. RAPD markers are the first generation marker system extensively used for germplasm classification in addition to their usefullness in gene tagging and mapping studies owing to their major attributes such as use of universal primers, short period with cost effectiveness, requirement small quantity of DNA for analysis and also non requirement of cloned DNA probes and hybridization filters (Ranade *et al.,* 2001). Although, RAPD markers provide an efficient assay for polymorphism as the differences in amplicon primarily results from the differences in their priming site or amplifying DNA segment from one parent but not in the other and used successfully to map chickpea wilt resistance loci (Soregaon *et al.,* 2007 and Babayeva *et al.,* 2018), the technique is dominant and sensitive to modification in PCR reaction condition, eventually resulting in poor reproducibility. These limitations has led to the successful conversion of RAPD marker linked to different traits into allele-specific or locus-specific primers (SCAR/CAPS) and it has been reported in melon, tomato, faba bean, black gram and mung been by Zheng *et al.* (1999); Zhang and John (2001); Gutierrz *et al.* (2006); Prashanti *et al.* (2011); Dhole and Reddy (2013) respectively.

Table 1: Sequence of SCAR primers derived from polymorphic DNA fragment of an RAPD marker $A07C_{417}$.

Primer	Sequence (5′-3′)	Annealing tem. (°C)
A07CSCAR-1	(F)-GGGAATGCAAGAAGCGT	54
	(R)-ACGGGTGCCCCAAGACT	
A07CSCAR-2	(F)-GAAACGGGTGCAGTG	55
	(R)-GAAACGGGTGCCCAA	
A07CSCAR-3	(F)-CGGGTGCAGTGTGTAAGTTTGGAA	65
	(R)-ACGGGTGCCCAAGACTACTCATAAG	
A07CSCAR-4	(F)-AATTACAGCAAGGGCACCAAC	62
	(R)-GTTGATACTTTCCCTTGGCTTGC	
A07CSCAR-5	(F)-CTGGCAAGGAAGACACCGCTAACA	65
	(R)-CCCTACCGACATACCAGTGAACAAA	

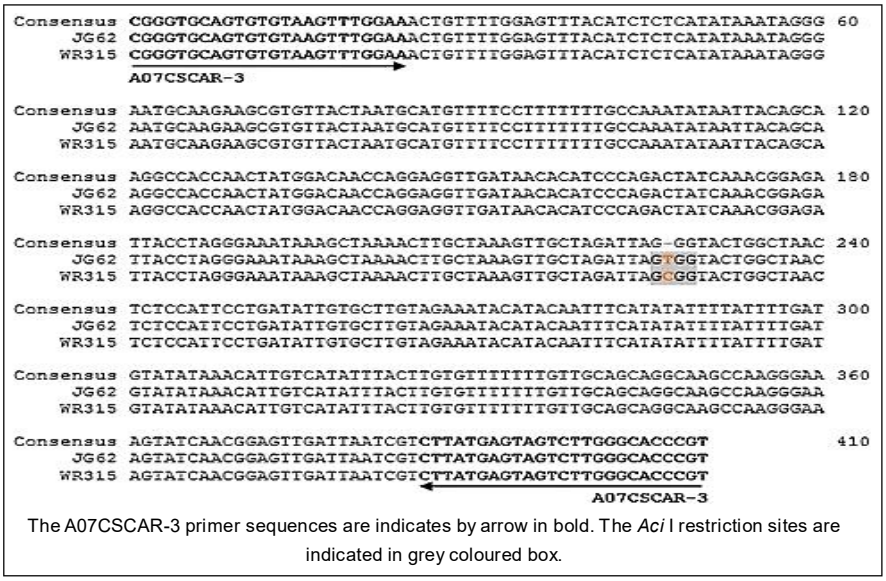


Fig 2: Alignment of A07CSCAR-3 amplified nucleotide sequences from susceptible parent JG62 and resistant parent WR315.

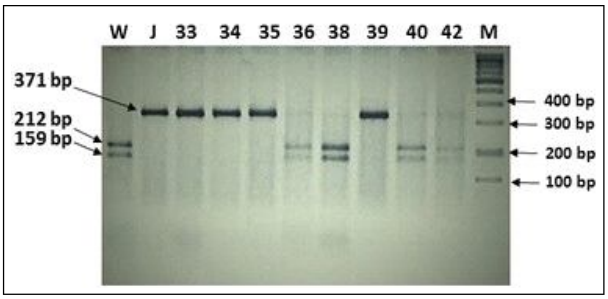


Fig 3: Electrophoretic banding pattern of CAPS marker.

Segregation and linkage analysis of CAPS marker

The χ^2 test for monogenic inheritance of CAPS marker and wilt reaction displayed an expected 1:1 segregation ratio in RILs population. Among 117 RILs, 61 RILs produced CAPS banding pattern similar to WR-315. The digenic analysis of wilt reaction and marker was carried out to determine the linkage between markers and wilt resistance. The joint segregation of CAPS marker together with 60th day wilt reaction in all the three seasons indicated significant deviation from 1:1:1:1 ratio in the RILs suggesting linkage between CAPS marker and wilt reaction (Table 2). Further in an effort to map the CAPS marker on to the sequence based physical map of chickpea by *in silico* approach using BLAST search tool of NCBI showed significant similarity with LOC101491241 on chromosome Ca4 (E-value = 0.0) of the chickpea genome with a putative function of wound responsive family protein determined using chickpea transcriptome database.

The first DNA markers linked to the *Fusarium* wilt race 1A at H_1 locus was identified by Mayer *et al.* (1997) using allele specific associated primer (ASAP) $CS27_{700}$. Similarly, RAPD marker $A07C_{417}$ linked to H_2 locus have been identified by Soregaon *et al.* (2007). Both of these allele specific markers were developed in different genetic backgrounds segregating independently for each locus. Independent segregation of markers linked to H_1 ($CS27_{700}$) and H_2 ($A07C_{417}$) locus were also confirmed using RILs of cross JG-62 x WR-315 revealing reliability of both these DNA markers (Brinda and Ravikumar, 2005; Soregaon and Ravikumar, 2010). Along with these linked markers, many SSR markers linked to wilt resistance have been identified by several researchers (Radhika *et al.,* 2007; Gowda *et al.,* 2009; Patil *et al.,* 2014 and Jingadae *et al.,* 2015). However, many of these markers need to be validated in different genetic background for their association with wilt resistance and it is also necessary to develop high density of polymorphic markers in these region, which will enable fine mapping of resistance locus (M Perez-de-Castro *et al.,* 2012). Hence, identifying markers tightly linked to resistance loci/QTL will provide an effective solution for resistance deployment *via* MAS particulary in case of complex inheritance pattern of disease resistance.

In the present investigation, SNP has been identified in the monomorphic SCAR locus of RAPD primer $A07C_{417}$, which is able to differentiate between susceptible and resistant genotypes enabling the development of reproducible codominant CAPS marker. The mapping population (RILs) used were developed by hybridization of highly susceptible (JG62) and resistant parent (WR315) segregating independently for both H_1 and H_2 locus potentially generating a full range of variability for wilt resistance. Further, to overcome the problems associated with disease screening of segregating population in wilt sick plots, development of standardized disease screening using uniform wilt sick pots in green house enabled efficient screening of segregating population. Disease screening of RILs for wilt reaction in wilt sick pots and wilt sick plots enabled detection of linkage association of CAPS marker with H_2 locus of *Fusarium* wilt resistance. Thus, identification

Table 2: Joint segregation of CAPS marker and wilt reaction in RILs of chickpea.

Wilt sick		Observed (O)	Expected (E)	O-E	$(O-E)^2$	$(O-E)^2/E$	χ^2
Plot	JS	40.00	29.25	10.75	115.56	3.95	15.00*
(*Rabi*-2007)	JR	16.00	29.25	-13.25	175.56	6.00	
	WS	22.00	29.25	-7.25	52.56	1.79	
	WR	39.00	29.25	9.75	95.06	3.25	
Wilt sick	JS	47.00	29.25	17.75	315.06	10.77	45.83*
Plot	JR	9.00	29.25	-20.25	410.06	14.01	
(*Rabi*-2008)	WS	48.00	29.25	18.75	351.56	12.01	
	WR	13.00	29.25	-16.25	264.06	9.02	
Wilt sick	JS	45.00	29.25	15.7	248.06	8.48	32.43*
pot	JR	11.00	29.25	-18.25	333.06	11.38	
	WS	44.00	29.25	14.75	217.56	7.43	
	WR	17.00	29.25	-12.25	150.06	5.13	

J = JG-62; S = Susceptible; W = WR315; R = Resistance; *Significant at 5%.

of SNP and the development of codominant CAPS marker in this study may find its usefulness in precise genetic mapping and marker assisted trait introgression of resistance locus into elite cultivar.

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