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Inheritance and Molecular Mapping of Wilt Resistance Gene(s) in Castor (*Ricinus communis* L.)

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ABSTRACT

The inheritance of resistance to castor wilt derived from 'Haritha' and '48-1' conferring resistance to race 1 of the pathogen *Fusarium oxysporum* F.sp. *ricini* has been determined. The F_2 individuals and backcross F_1 families of two crosses Haritha and '48-1' (resistant) × 'Kranthi' (susceptible) were screened against *Fusarium* wilt in combined tests which indicated dominant control by single locus of tightly linked gene(s). Bulked segregant analysis (BSA) was carried out on F_2 and BC_1F_1 individuals with 186 RAPD primers that resulted in the identification of two RAPD markers linked to resistance that distinguished the bulks. Of these, the RAPD markers OPH-12_{4973} and OPJ-15_{4268} were tightly linked to 'Haritha' and '48-1' at a distance of 5.0 cM and 7.0 cM to wilt resistance. These closely linked markers to the wilt resistance gene(s) provide a valuable basis for marker-assisted selection in castor breeding programs.

Keywords: bulk segregant analysis, back cross, locus, marker-assisted selection, RAPD
Abbreviations: RAPD, randomly amplified polymorphic DNA; **RFLP**, restricted fragment length polymorphism; **Fw**, *Fusarium* wilt; **cM**, centimorgan; **PCR**, polymerase chain reaction; **LOD**, logarithm of odds

INTRODUCTION

Castor (*Ricinus communis* L.) wilt incited by *Fusarium oxysporum* f.sp is a major disease in all castor-growing regions (Nanda and Prasad 1974). It is a seed- and soil-borne disease that often damages more than 50% of the plants in affected field and accounts for more than 90% loss of yield (Patel *et al*. 1991a). In certain endemic areas even the leading wilt resistant castor hybrid 'GCH-4' turned out to be susceptible to *Fusarium* wilt (Patel *et al*. 1991a). The resistant variety, 'DCS-9' showed wilt incidence of 60% in experiments indicating gradual breakdown of resistance against wilt (Anjani *et al*. 2004). A field experiment in Andhra Pradesh on screening castor varieties for wilt resistance indicated that cultivar '48-1' has less wilt incidence (5%) followed by cv. 'DCS-9' (Pushpavathi *et al*. 1998). The pot culture experiments revealed that the nematode *Rotylenchus reniformis* alone and in combination with wilt reduced plant growth of wilt susceptible castor hybrid, 'GAUCH-1' and wilt resistant hybrid, 'GCH-4' and they found that the interaction was synergistic when the two pathogens were inoculated together in both castor hybrids (Patel *et al*. 2000).

Several hypotheses have been proposed regarding the mode of action of genes conferring resistance to *F. oxysporum* in castor, with most studies indicating *F. oxysporum* in the inter line castor hybrids studied was controlled by at least 2 loci (Suf_1 and Suf_2), in some hybrids by the recessive allele *Suf1*, with modifiers being present in some cases (Sviridov 1986). Involvement of additive gene action is reported in some of the crosses studied and suggested that wilt resistance in castor probably is governed by polygenes. Inheritance of *Fusarium* wilt resistance was studied by evaluating F_2, BC_1 and BC_2 segregating generations of a cross involving 'VP-1' as a susceptible female and '48-1' as a resistant male. The results showed that the F_1 was susceptible and F_2 generation segregated in a 9: 7 ratio indicating the role of two recessive genes showing complementary epistasis in the inheritance of wilt resistance (Chandramohan 2002). Wilt resistance to *F. oxysporum ricini* was governed by two complementary genes in 48-1 (Rao *et al*. 2005).

Inheritance of resistance was studied and random amplified polymorphic DNA (RAPDs) markers linked to gene for resistance was identified in chickpea (*Pisum sativum*) (Tullu *et al*. 1998). The RAPD fragments associated with race 4 resistance segregated in a 1: 1 ratio expected for a single gene (Tullu *et al*. 1998). The F_2 population of chickpea consisting of 174 plants was analysed by RFLP and RAPD markers. A partial linkage map was constructed which included 3 morphological characters, 4 microsatellite loci and 2 RAPD loci markers linked to *F. oxysporum* wilt 6 cM from *Fusarium* wilt (Dirlewanger *et al*. 1994).

RAPD markers have been widely used to tag resistance genes in many crop species (Nedim *et al.* 2008; Suvendu *et al*. 2008; Nisar *et al*. 2011; Shabnum *et al.* 2011). To date there is no available genetic map and limited genomic information for castor (Chan *et al*. 2010) and there are no attempts at identification of molecular markers linked to wilt resistance in castor. The present study was undertaken to study the inheritance of wilt and identify DNA markers linked to the wilt locus of castor.

MATERIALS AND METHODS

Plant material and mapping populations

The castor cultivars 'Haritha' and '48-1' constitute resistance sources and these varieties were originally released from Acharya N.G Ranga agricultural university, India for resistance to *Fusarium* wilt. It was found to confer resistance to wilt (Ashok *et al*. 2006). 'Kranthi' is the susceptible variety for *Fusarium* wilt (Anjani 2004). Crosses were made between 'Haritha' and '48-1' with Kranthi and the genomic DNA was isolated from individual plants of F_2 and BC_1F_1.

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Original Research Paper

Screening for resistance to *Fusarium* wilt

1. Preparation of wilt sick plot

Wilt sick plot was developed (Nene *et al*. 1981) by incorporating debris of the diseased plants followed by repeated cultivation of susceptible varieties. The infected plants debris was obtained from the field and maintained on potato dextrose agar (PDA) medium. For mass multiplication of the pathogen, the sorghum seed was first steamed in 2% sucrose solution and then inoculated with fungal discs in sorghum seeds and incubated for 21 days at room temperature. The cultured inoculum was incorporated in to the wilt sick plot.

2. Root dip inoculation technique

The *Fusarium* spores were harvested into sterile distilled water (Desai and Dange 2003). After about 30 min of adding to sterile distilled water the conical flasks were vigorously shaken for 10 min. The suspension was filtered through double-layered muslin cloth. Spore counts were made using a heamocytometer (Bright-Line™, Sigma-Aldrich). The spore suspension was diluted to a concentration of 10^6 spores per ml. Surface sterilized seeds were grown in sterile sand and ten-day-old seedlings were uprooted and the roots were clipped before dipping them in spore suspension for 3 min. Then the seedlings were transplanted into pots with sterile soil and all the pots were kept at atmospheric temperature (26.6 to 35.9°C). Disease reaction of the F_2 and BC_1F_1 individual families was determined separately for each cross in race 1 using wilt sick plot as well as in root dip inoculation method. Observations on resistance/susceptibility were recorded in the field on individual F_2 and BC_1F_1 plants.

DNA isolation and quantification

The genomic DNA was extracted from the parents ('Kranthi', 'Haritha' and '48-1'), F_1, F_2 and BC_1F_1 plants according to the protocol Dellaporta *et al*. (1983). Freeze dried (-80°C) and lyophilized young leaves obtained from 30-35days old plants were ground to a fine powder and used for DNA extraction. The concentration of DNA was quantified using an Eppendorf biophotometer (Hamburg, Germany) and diluted to 25 ng/µl for PCR analysis. Phenotypic measurements for each trait were used to score individual F_2 plants and 10 most resistant and 10 most susceptible plants were chosen for BSA (Michelmore *et al*. 1991). After quantification equimolar amounts of DNA from the 10 F_2 individuals employed to constitute both the resistant and susceptible pools were prepared and bulked samples were screened over 186 Oligo-nucleotide primers. Markers present in both the wilt resistant F_2 bulk and 'Haritha' and '48-1' absent in the wilt susceptible F_2 bulk and 'Kranthi' were consider to segregate with *Fusarium* wilt. Markers that were strong candidates for association with *Fusarium* wilt disease response based on chi-square analysis were screened using the entire F_2 and BC_1F_1 individuals to linkage analysis

PCR analysis

Oligo-nucleotide primers from Operon Technologies Inc. (Alameda, CA, USA), 186 in total, were screened for polymorphism between the parental lines and also the resistant/susceptible bulks. The primers that showed polymorphism between the bulks were tested on 10 plants that constituted the resistant and susceptible bulks. The primers that generated reproducible polymorphism in individual plant assays were selected for genotyping the F_2 and BC_1F_1 individuals.

RAPD reactions were performed in the Perkin-Elmer 9600 thermal cycler (Barnstead Thermolyne Corp., Iowa, USA). The reaction was performed in a 20 µl reaction mix, containing 25 ng/ul of genomic DNA as template, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 2 mM of each dNTP, 5 ρmoles/µl of primer, and 0.3 U of *Taq* DNA polymerase (Banglore Genei Pvt. Ltd.). The PCR programme was set as follows: Initial denaturation at 94°C for 3 min followed by denaturation at 94°C for 1 min, annealing at 40°C for 2 min and extension at 72°C for 2 min and these steps were repeated for 45 cycles with a final extension at 72°C for 5 min and hold at 4°C. The RAPD products and the marker (λ-DNA digested with *Eco*RI and *Hind*III) were resolved using 1.8% agarose gels. Ethidium bromide stained gels were visualized and documented by gel documentation system (Syngene Biosystems, UK).

Statistical analysis

Segregation ratios were checked with Chi-square tests. Linkage analysis was performed using the computer-aided program MAPMAKER (Lander *et al*. 1987), which applies the maximum likelihood method for determination of recombination values and the Kosambi's mapping function (Kosambi 1944) for correcting the recombination values to cM distances. The logarithm of odds (LOD) score of 3.0 was used as a linkage threshold. MAPMAKER can carry out both two-point and multipoint linkage analysis. While the first is used to derive pair-wise linkage distances between any two segregating loci taken at a time, the multipoint linkage analysis derives the best possible locus order and also a map using the data on all segregating loci at one time.

RESULTS AND DISCUSSION

All the plants of the resistant parents ('Haritha' and '48-1') were resistant to *Fusarium* wilt with 8.88 and 9.44 disease incidence, respectively. The susceptible parent Kranthi showed complete susceptible reaction with wilt incidence of 81.33%. The segregation ratio of the F_2 generations of each cross to the *Fusarium* wilt fits a 3: 1 resistant: susceptible Mendelian segregation ratio (χ^2= 0.39, P= 0.53; χ^2= 1.12, P= 0.28) (**Table 1**). The BC_1F_1 families of the crosses 'Haritha' x 'Kranthi' and '48-1' x 'Kranthi' segregation ratio fit a 1: 1 resistant: susceptible Mendelian ratio (χ^2= 4.1, P= 0.042; χ^2= 1.38, P = 0.24) and suggested that *Fusarium* wilt resistance in 'Haritha' and '48-1' were controlled by a single dominant gene.

The DNA from the resistant 'Haritha' and '48-1' cultivars, susceptible analogue 'Kranthi' and their F_1 was subjected to RAPD screening. A total of 186 oligo-nucleotide primers were tested, of which 16 and 21 primers generated polymorphism between the parents in the two crosses, respectively. All these primers were utilized for Bulk segregant analysis. The identified polymorphic primers were used to

Table 1 segregation for resistance to *Fusarium* wilt in the F_2 and BC_1F_1 generations of the cross 'Haritha' and '48-1' x 'Kranthi'.

Generation	Observed frequency		Ratio tested	χ^2	*P*
	Resistance (RR)	Susceptible (rr)			
Haritha x Kranthi (field screening)					
F_2	184	53	3:1	0.75	0.3865
BC_1F_1	57	50	1:1	0.34	0.5598
RDIT (root dip inoculation)					
F_2	173	51	3:1	0.48	0.4884
BC_1F_1	54	46	1:1	0.50	0.4795
48-1 x Kranthi (field screening)					
F_2	182	55	3:1	0.32	0.5716
BC_1F_1	56	48	1:1	0.48	0.4884
RDIT (root dip inoculation)					
F_2	179	52	3:1	0.64	0.4237
BC_1F_1	56	49	1:1	0.34	0.5598

Table 2 Segregation of two markers linked to wilt in a F_2 and BC_1F_1 populations from ('Haritha' x 'Kranthi') and ('48-1' x 'Kranthi').

Generation	Observer frequency		Ratio tested	χ^2	*P*
	Resistance (RR)	Susceptible (rr)			
OPH-12$_{4973}$ (Haritha x Kranthi)					
F_2	82	23	3:1	0.39	0.5323
BC_1F_1	54	34	1:1	4.10	0.0429
OPJ-15$_{4268}$ (48-1 x Kranthi)					
F_2	77	19	3:1	1.12	0.2899
BC_1F_1	50	38	1:1	1.38	0.2401

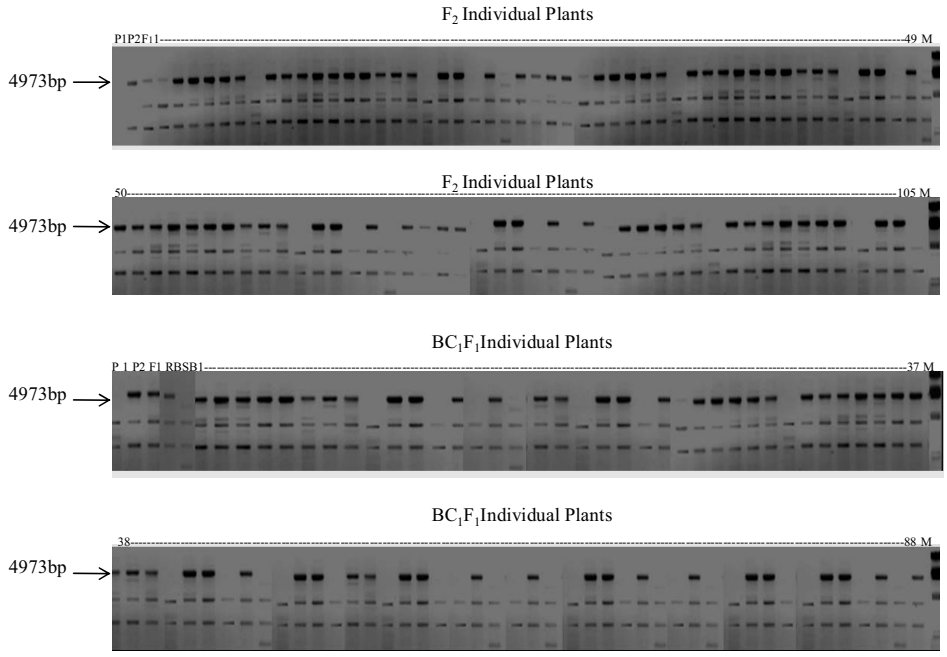

Fig. 1 Genotyping of the 105 F_2 and 88 BC_1F_1 individuals derived from the cross between Haritha x Kranthi along with parents (*P1* resistant parent Haritha, *P2* susceptible plant Kranthi) and F_1 with OPH-12_{4973}. Arrow indicates *Eco*RI and *Hin*dIII digested λ-DNA marker, RB (resistant bulk), SB (susceptible bulk).

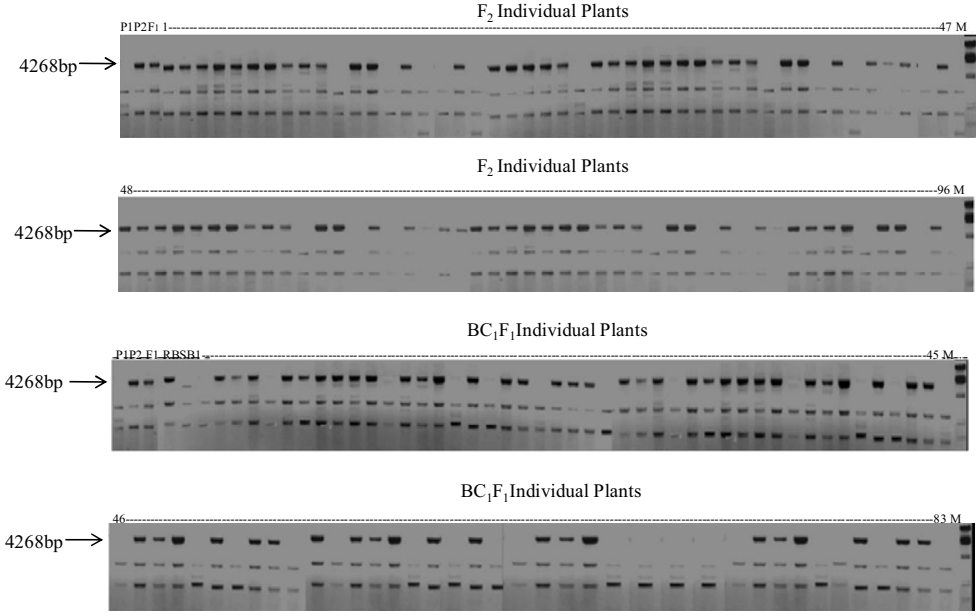

Fig. 2 Genotyping of the 96 F_2 and 88 BC_1F_1 individuals derived from the cross between 48-1 x Kranthi along with parents (*P1* resistant parent 48-1, *P2* susceptible plant Kranthi) and F_1 with OPJ-15_{4268}. Arrow indicates *Eco*RI and *Hin*dIII digested λ-DNA marker, RB (resistant bulk), SB (susceptible bulk).

screen the F_2 and BC_1F_1 mapping populations which resulted in the identification of two markers associated with the *Fusarium* wilt resistant phenotype. Of these, OPH-12_{4973} marker was closest to the resistant Haritha wilt locus and OPJ-15_{4268} marker was closest to the resistant wilt '48-1' locus (**Figs. 1, 2**).

The segregation ratio of the two linked markers was in agreement with Mendelian segregation ratios of 3: 1 and 1: 1 for dominant markers (**Table 2**). The 'Haritha' resistant gene was linked by OPH-12_{4973} with genetic distance of 5.0 cM while the resistant gene of '48-1' was linked by OPJ-15_{4268} with genetic distance of 7.0 cM (**Fig. 3**).

Resistance to *Fusarium* wilt controlled by dominant single locus has been successfully transferred to suitable agronomic background. However, evolution of physiological races of the pathogen that overcome widely used resistant genes is a continuous process necessitating the identification of new broad spectrum sources of resistance. To date, there is no available genetic map and limited genomic information for the castor crop (Chan *et al.* 2010) and there are no attempts to search for molecular markers linked to wilt in castor. This report is the first successful example of identification of molecular markers linked to wilt resistance in castor. The RAPD markers OPH-12_{4973} and OPJ-15_{4268} were tightly linked to 'Haritha' and '48-1' at a distance of 5.0 cM, 7.0 cM, respectively to wilt resistance. These closely linked markers to the wilt resistance gene(s) provide a valuable basis for marker-assisted selection in castor breeding programs.

Molecular markers linked to a gene of interest are the milestones and these tags are useful starters for identification of resistant genes. Once the molecular markers closely linked to desirable traits are detected, MAS can be performed in early segregating populations and at early stages

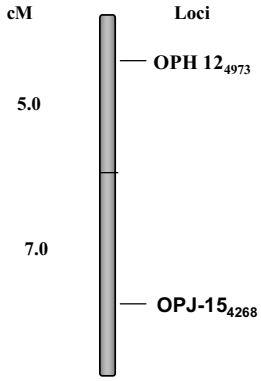

Fig. 3 Molecular mapping of wilt resistance genes.

of plant development (Zhang *et al*. 2003; Francia *et al*. 2005). Therefore, it is safe to say that molecular markers will gain more and more influence on plant breeding in future and will speed up breeding processes considerably. In view of the potential development of new strategies, the future for improvement of polygenic traits through DNA markers appears bright. Moreover, by adopting new and novel marker systems like EST-SSRs, SNPs, microarrays, indeed, it may be possible to select best varieties for breeding in castor.

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