MOLECULAR CHARACTERIZATION OF CHILLI (*Capsicum annuum* L.) GENOTYPES FOR TAGGING BACTERIAL WILT RESISTANCE GENE

By

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THESIS

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DECLARATION

I hereby declare that the thesis entitled **"Molecular characterization of chilli (*Capsicum annuum* L.) genotypes for tagging bacterial wilt resistance gene"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

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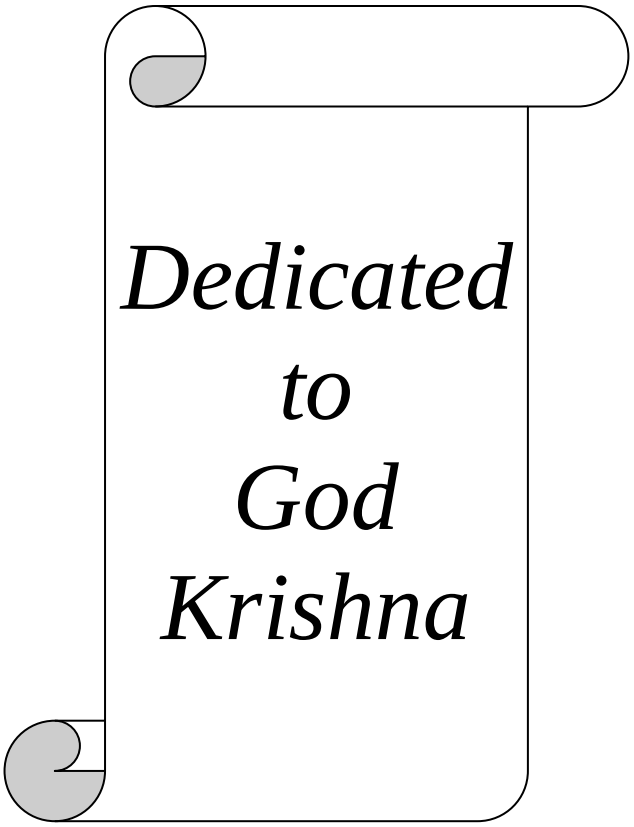
Dedicated
to
God
Krishna

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ABBREVIATIONS

A	Adenine
AASTATS	Amino acid statistics
AFLP	Amplified Fragment Length Polymorphism
bp	Base pairs
β	Beta
BLAST	Basic Local Alignment Search Tool
BPH	Black plant hopper
BSA	Bulk Segregant Analysis
C	Cytosine
CAPs	Cleaved amplified polymorphic sequences
cm	Centimeter
cM	Centi Morgan
CPBMB	Centre for Plant Biotechnology and Molecular Biology
cfu	Colony forming unit
°C	Degree Celsius
DNA	Deoxyribo Nucleic Acid
dNTPs	Deoxyribo Nucleoside Triphosphate
EDTA	Ethylene Diamine Tetra Acetic acid
EPS	Extra Cellular Polysaccharide
FLA	Fluorescent Image analyzing system
FYM	Farm yard manure
G	Guanine
GC	Guanine- Cytosine
γ	Gamma
g	Gram
HR	Hypersentitive response
HPR	Host plant resistance
hr	Hour (s)
ha	Hectares
IAA	Indole Acetic Acid
IARI	Indian Agricultural Research Institute
ICAR	Indian Council of Agricultural Research
IIHR	Indian Institute of Horticulture Research
IPTG	Isopropylthio-β-D-galactoside
ISSR	Inter-simple sequence repeats
KAU	Kerala Agricultural University
kb	Kilo base pairs
L	Litre
LB	Luria Broth
LBA	Luria Bretani Agar
LRR	Leucine rich repeat
Mb	Mega base pairs
M	Mole
MAPK	Mitogen activated protein kinase
MAS	Marker Assited selection
MDH	Maternal doubled haploid

MR	Moderately resistant
min	Minute(s)
ml	Millilitre
mM	Millimole
mg	Milligram
µg	Microgram
µl	Microlitre
µM	Micromole
N	Amino
NBS	Nuclotide Binding site
ng	Nanogram
nm	Nanometer
NASTATS	Nucleic Acid Statistics
NCBI	National Centre for Biotechnology Information
NIL	Near isogenic line
NTSyS	Numerical Taxonomy System of Multivariate Statistical Program
OD	Optical Density
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
pH	Hydrogen ion concentration
PR	Pathogenesis Related
PVY	Potato Y potyvirus
PCD	Programmed cell death
%	Percentage
QTL	Quantitative Trait Loci
R	Resistance
RAPD	Random Amplified Polymorphic DNA
RGCs	Resistant gene candidates
RFLP	Restriction Fragment Length Polymorphism
RGA	Resistance Gene Analogues
RILs	Recombinant inbreed lines
ROIs	Reactive oxygen intermediates
RNA	Ribo Nucleic Acid
RNAse	Ribonuclease
RP	Resistant parent
RB	Resistant bulk
rpm	Rotations per minute
S	Susceptible
SAR	Systemic acquired resistance
STS	Sequence tagged sites
SSLP	Simple sequence length polymorphism
SCAR	Sequnced Characterized Amplified Region
SDS	Sodium Dodecyl Sulphate
SP	Susceptible parent
SB	Susceptible bulk
SSR	Simple sequence repeats
sec	Second (s)

SAHN	Sequential Agglomerative Hierarchical Nested Clustering
T	Thymine
TAE	Tris Acetate EDTA
TE	Tris EDTA
TEMED	N, N, N, N- Tetramethylene ethylene diamine
TSMV	Tomato spotted wilt virus
TZC	Triphenyl Tetrazolium Chloride
U	Unit
UPGMA	Unweighted Pair Group Method of Arithmetic Averages
USA	United State of America
UV	Ultra Violet
V	Volts
v/v	Volume by volume
w/v	Weight by volume
W	Watt
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
YAC	Yeast Artificial chromosome

Introduction

1. INTRODUCTION

Bacterial wilt caused by *Ralstonia solanacearum* (Smith) Yabuuchi *et al* is a serious problem in crops of economic importance grown in tropics and subtropics of the world. In susceptible genotypes, the inoculum multiplies in xylem vessels and sudden wilting of plants occurs due to blockage of water conduction. Warm, humid, tropical condition with sunshine promotes bacterial wilt disease. Yield loss in susceptible varieties varies from 30 per cent under mild infection to 100 per cent under the severe infection.

Erwin.F.Smith observed the occurrence of wilt disease caused by *Pseudomonas solanacearum* E.F.Smith in solanaceous plants for the first time (Smith, 1896). The first report of the occurrence of bacterial wilt in chilli due to *Ralstonia solanacearum* (Smith) Yabuuchi *et al* was recorded from USA (Rolfs, 1898). Now it is prevalent in different parts of India and world. In India the incidence of bacterial wilt of chilli was reported by Shekhawat *et al*., (1978) and they observed that the causal organism of this disease is endemic in India throughout the West coast, Central and Deccan Plateau of Karnataka, Western Maharashtra and Madhya Pradesh, Eastern plains of Assam, West Bengal, Orissa and Chotta Nagpur Plateau on potato, tomato, brinjal and chilli. The disease has also been reported from Madhya Pradesh (I.C.A.R, 1969) and Karnataka (Khan *et al*., 1979). The incidence of bacterial wilt was also reported from Java (Schwarz, 1926), Philippines (Reinking, 1919), Ceylon (Park, 1932) and Florida (Rolfs, 1898).

Important crops like chilli, potato, tomato, brinjal, groundnut, ginger etc. are hosts of this pathogen. Chilli is raised in an area of 1776 thousand hectares in the World, with a production of 7182 thousand tones. India ranks first in area (8.31 lakh ha) and production (8.46 lakh ha) in the world. Though chilli is grown through out India, Andhra Pradesh leads in area and production, which accounts for 46 per cent of the chilli production in India. The important chilli producing states are Andhra Pradesh, Maharashtra, Tamil Nadu, Karnataka, West Bengal, Bihar and Assam. These states together share 96% of total area under cultivation. During 2002-2004,

India exported 81.5 thousand tones of chilli valued at Rs. 35,511.25 Lakhs. The cultivation of chilli in Kerala is limited to 638 ha with an annual production of 670 tones. The disease incidence is greatly influenced by environmental conditions and resistance/susceptibility reaction of the host.

The conventional control measures by chemicals and sanitation measures are not very effective in controlling the disease. Worldwide control strategies for disease, consist of breeding wilt resistant cultivars and success stories have been reported in tomato, chilli and eggplant. The breeding strategies, pedigree and backcross methods usually adopted to move disease resistance genes are constrained by the influence of environment on the disease expression and absence of inoculum at appropriate time during the growing season. It is also time consuming and labour intensive.

In the process of breeding for durable disease resistance by employing multiple genes, breeders frequently encounter the problem of epistatic interaction among the resistance genes confounding selection through conventional means. These problems in conventional resistance breeding can be over come by identifying molecular markers linked to disease resistance genes (gene tagging) and using these markers for marker assisted selection (MAS). This eliminates the need for target trait expression for selection.

Recombinant DNA technology offers tools for cloning the tagged resistance gene and for evolving durable resistant genotypes, cutting across species boundaries. It simplifies screening for traits that are highly complex provided a closely linked marker to the phenotype is identified.

In chilli, resistant varieties to bacterial wilt have been developed by conventional means and are recommended for cultivation in Kerala. These are Ujwala, Manjari (Gopalakrishnan and Peter, 1991) and Anugraha (Markose, 2003). According to Markose (2003), the bacterial wilt resistance gene imparting resistance in Ujwala and Anugraha is monogenic and incompletely dominant. Anugraha is a hybrid derivative of the cross Ujwala x Pusa Jwala.

The present study involves molecular characterization of resistant and susceptible genotypes using PCR based RAPD and SCAR markers for tagging bacterial wilt resistant gene. Resistant variety Ujwala, susceptible variety Pusa Jwala, its hybrid derivative with bacterial wilt resistance, Anugraha and segregating F_2 population of cross Ujwala x Pusa Jwala were used as source of DNA for molecular marker analysis.

The main objectives of the study were the following:

1. Molecular characterization of susceptible and resistant genotypes through RAPD analysis for detecting polymorphism between them.
2. Cloning and sequencing of polymorphic amplicon linked to bacterial wilt resistance.
3. Conversion of RAPD markers into SCAR marker by designing longer primers.
4. Confirming the efficiency of SCAR marker in distinguishing susceptible and resistant genotypes.

Review of Literature

2. REVIEW OF LITERATURE

The research programme "Molecular characterization of chilli (*Capsicum annuum* L.) genotypes for tagging bacterial wilt resistance gene" was selected for developing a molecular marker linked to bacterial wilt resistance in chilli. The relevant literature on various aspects of the investigation is reviewed in this chapter.

2.1. Bacterial wilt disease of chilli

Bacterial wilt in chilli caused by *Ralstonia solanacearum* (Smith) Yabuuchi *et al* is an important problem limiting its cultivation in humid tropics and subtropics of the world. The loss due to the same in chilli is estimated to be 30-100 per cent. This can be reduced by growing resistant cultivars. Over one hundred years have elapsed since Erwin.F.Smith published the first description of *Pseudomonas solanacearum* E.F.Smith that causes a wilt disease of solanaceous plants (Smith, 1896).

The first report of the occurrence of bacterial wilt of chilli due to *Ralstonia solanacearum* (Smith) Yabuuchi *et al* was recorded from USA (Rolfs, 1898). It is prevalent in different parts of India and world. In India the incidence of bacterial wilt of chilli was reported by Shekhawat *et al*., (1978) and they observed that the causal organism of this disease is endemic in India throughout the West coast, Central and Deccan plateau of Karnataka, Western Maharashtra and Madhya Pradesh, Eastern plains of Assam, West Bengal, Orissa and Chotta Nagpur Plateau on potato, tomato, brinjal and chilli and also reported from Madhya Pradesh (I.C.A.R, 1969) and Karnataka (Khan *et al*., 1979). The incidence of bacterial wilt was also reported from Java (Schwarz, 1926), Philippines (Reinking, 1919), Ceylon (Park, 1932) and in Florida (Rolfs, 1898).

Yabuuchi *et al.* (1992) transferred several species of the rRNA homology group II *Pseudomonas*, including *Pseudomonas solanacearum* to the genus *Burkholderia*. Sequencing information of 16S rRNA genes and polyphasic taxonomy led to the proposal of genus *Ralstonia* and the pathogen was renamed as *Ralstonia solanacearum* (Yabuuchi *et al.*, 1995).

2.2. Symptomatology of the disease

The Pathogen enters through the root system and it is believed that a wound is necessary for the entry (Kelman, 1953; Walker, 1952). Walker (1952) reported that the pathogen first enters into the intercellular spaces of cortex, from there it moves to pith and xylem vessels and wilting of plants is due to vascular plugging. The roots and lower part of the stem appearing normal from outside, show a browning of vascular bundles and a water socked appearance in the root (Walker, 1952). Dwarfing and stunting of the plants may also occur (Kelman, 1953; Young, 1946).

Chupp and Sherf, (1960) reported entry of the bacterium through natural opening in the plant. Visible symptom occurs within 2 to 8 days of entry of the pathogen into the host plant. Generally the first expression of the disease is yellowing and wilting of the lower leaves of plants. Eventually dark brown to black areas develop due to decay of root system and the whole plant dies off. A very characteristic distinct indication of bacterial wilt is the appearance of bacterial ooze from the injured vascular regions (Ashrafuzzaman and Islam, 1975).

2.3. Methods of Artificial Inoculation

Several artificial inoculation methods are effective in distinguishing reaction of genotypes with different resistance levels to bacterial wilt.

Winstead and Kelman (1952) evaluated the relative effectiveness of various procedures to inoculate susceptible and resistant tomato plants. Preliminary tests with naturally infested soils or diseased plant debris showed the superiority of pure cultures for inoculation under greenhouse conditions. They aimed at developing a procedure that would ensure uniform and rapid development of bacterial wilt. Pathogenecity test of bacterial cultures by inoculating tomato plants by stem puncture method lead to rapid wilting and at the end of 14 days, all inoculated plants were severely wilted.

Wang (1971) reported an artificial inoculation of tomato by leaf clipping method. Lin *et al.* (1974) inoculated tomato plants by clipping off leaf tips with scissors dipped in a suspension of bacterial culture and obtained wilting to the same extend as found in plants grown in naturally infested field. Sheela (1982) reported that dipping of seedlings in fresh bacterial ooze just before transplanting followed by inoculation of seedlings in the leaf axils resulted in 100 per cent infection in susceptible brinjal seedlings.

Kishun and Chand (1988) conducted an experiment with seven inoculation methods by using 25 days old susceptible tomato cv. Pusa Ruby in nutrient solution can be successfully used for evaluation of virulence of *Pseudomonas solanacearum* and the best method was leaf axil inoculation. Butranu *et al*. (1994) several inoculation techniques tested, petiole-clipping method was found to be the most effective for resistance screening.

2.4. Mechanism of wilting

The pathogen is first detected in the xylem vessels which later progresses into the intercellular spaces of cortex and pith causing lysigenous cavities. Break down of plant tissues due to bacterial wilt is attributed to the cellulase and poly-galacturonase enzymes produced by the bacterium (Hussain and Kelman, 1957).

Severe wilting is caused by vascular plugging even when inoculated with weakly virulent strains. All the virulent strains produced an extracellular slime, a wilt inducing material (Hussain and Kelman, 1958). They suggested that the virulent strains after entry into the host multiply rapidly in xylem and form slime in abundance which causes a marked increase in the viscosity of vascular stream and they interfere with water movement resulting in wilting. Bacterial colonization of the stem results in browning of the xylem, foliar epinasty and lethal generalized wilting (Buddenhagen and Kelman, 1964).

2.5. Source of Resistance

Empig *et al.* (1962) studied 11 varieties and two strains of pepper for bacterial wilt resistance and found that 'Pasites', 'All Big' and 'World Wonder' gave the lowest disease index. Mihov (1969) performed cross between *Capsicum* species and observed that peppers produced from crosses between *C. annuum* and *C. fasciculatum* were resistant and of good quality. Rahim and Samraj (1974) screened nine chilli varieties for resistance against *P. solanacearum* and found the variety 'Khandari' as highly resistant and 'Pungent Pride', 'Cherry Red', 'Vattal', 'Dark Purple' and 'Long Red' as moderately resistant.

Yazawa *et al.* (1980) compared the hybrids of three *C. annuum* cultivars with *C. chinense* '3341' from Bolivia and found that the vigorous Murasaki x 3341 was resistant to *P. solanacearum*. Goth *et al.* (1983) reported that 'KAU cluster' (Manjari) was resistant to four race 1 isolates and one race 3 isolate of *P. solanacearum*.

Peter *et al.* (1984) evaluated four Indian hot chilli cultivars' Pant C-1', 'KAU cluster', 'White khandari' and 'Chuna' along with six US cultivars for reaction to nine isolates of *P. solanacearum* (race 1 and race 3) and found that Pant C-1 was the most resistant one. Thomas (1985) also observed resistance to bacterial wilt in 'KAU cluster'.

At Kerala Agricultural University, Girijadevi and Peter (1987) made crosses of two hot pepper lines viz., 'Manjari' and 'Pant C-1' with five sweet pepper lines *viz*., Hungarian Wax, Sweet Red Cherry Pickling, Early Clwonder, Cubenelle and Yolowonder. All the F_1s were susceptible or moderately susceptible indicating the recessive nature of inheritance of resistance to bacterial wilt.

Gopalakrishnan and Thomas (1989) after conducting studies on resistance to bacterial wilt and inheritance of clusterness in chilli concluded that the accession, CA 33 which was further improved to the status of a variety "Manjari" was resistant to bacterial wilt disease. Gopalakrishnan and Peter (1991) studied 146 accessions of *Capsicum* species for resistance to bacterial wilt in a wilt sick soil after artificial inoculation and found that CA 219 (Ujwala) and CA 33 (Manjari) were highly resistant with good dry chilli yields. Jyothi (1992) also revealed resistance in chilli variety Manjari against bacterial wilt.

Markose (2003) developed a hybrid derivative by advanced selection from the cross Ujwala x Pusa Jwala, named Anugraha, which is resistant to bacterial wilt and having characteristics of Pusa Jwala. She crossed the susceptible variety Pusa Jwala to resistant variety Ujwala followed by two back crosses on susceptible variety and advancing to the F_1 generation to BC_2F_7. She revealed that the inheritance of resistance to bacterial wilt was monogenic and incompletely dominant.

2.6. Biochemical basis of resistance

The resistant varieties possess certain defense mechanisms such as physical and biochemical barriers to restrict the entry and growth of the pathogen in host cells. Muller (1959) and Cruickshank (1963) stated that a host might have two kinds of defence factors viz., Prohibitins and phytoalexins. Prohibitins are passive chemical barriers while phytoalexins are active biochemical barriers against infection. The

principAL antimicrobial substances biosynthesized by phanerogame are alkaloids, glycosides, sulphur compounds, unsaturated lactones, fatty acids, phenols, quinines and their derivatives and essential oils.

The chemical compounds which inhibit the pathogen are classified as pre-infectional and post-infectional inhibitors (Russel, 1978). Pre-infectional inhibitors in plant are mainly catechol, procatechuic acids, phenols, terpenes, flavanoids and tomatine (Roddick, 1974).

2.6.1. Defence mechanisms of the host

Phenolics

Phenolics in high concentrations are toxic to plant cells themselves (Tepper and Anderson, 1984) and the synthesis of phenolics is activated after infection and it rapidly suppresses the pathogen development (Vidhyasekharan, 1990).

Mahadevan (1966) reported orthodihydroxy phenolics substance which is highly toxic and plays a major role in disease resistance. They are easily oxidized by polyphenol oxidase and peroxidase to highly reactive quinines which are effective inhibitors of sulphydryl enzymes thereby preventing the metabolic activities of host and parasitic cells (Mahadevan, 1970).

Bajaj (1988) reported that chlorogenic acid and caffeic acid were the most important phenolics compounds involved in disease resistance mechanisms. Rajan (1985) reported that after artificial inoculation, total phenols were higher in roots and shoots of susceptible variety, whereas OD phenol content was increased and remained at a higher level in resistant variety.

Sitaramaiah *et al*. (1985) and Geetha (1989) were unable to correlate the total phenol content to resistance/susceptibility to bacterial wilt in brinjal. Kuc (1964) and Rajan (1985) observed a negative correlation between resistance and total phenol content in tomato and they suggested that the lower levels of phenolics in the roots of the resistant line might be due to the increased rate of oxidation of phenolics.

Gopinath and Madalageri (1986) and Sadhankumar (1995) indicated a high significant correlation of phenol with resistance and suggested a possible role of phenols in the mechanism of wilt resistance in brinjal and tomato respectively. Markose (1996) reported that polyphenol content was significantly higher in resistant variety 'Ujwala' in all the plant parts at various growth stages than the susceptible variety 'Pusa Jwala' before and after inoculation. On artificial inoculation, there was increased content in both varieties but resistant variety Ujwala had significantly higher content than susceptible variety.

Proteins

Enhanced protein synthesis appears to be a universal phenomenon in host pathogen interactions. *De novo* synthesis of new proteins was also reported (Dewit and Bakkar, 1980; Tani and Yamamoto, 1979). When the protein synthesis is inhibited by introducing inhibitors such as blasticidin S, puromycin and cycloheximide, resistance of the varieties breaks down. This clearly suggests that the protein synthesis is an important factor in disease resistance. The synthesized proteins may not be inhibitory to the pathogens. They mostly activate the synthesis of defence chemicals. The preformed existing proteins may not be involved in the disease resistance process (Gabriel and Ellingboe, 1982; Dewit and Bakkar, 1980).

Leach *et al*. (1983) detected entirely new protein which was different from pathogenesis related proteins (PR-proteins). It appeared when lipo-polysaccharide of *Pseudomonas solanacearum* was infiltrated into the leaves of tobacco cultivar. Chander

(1994) indicated that the resistant chilli line contained less total nitrogen and true protein. Markose (1996) reported higher protein content in roots of resistant variety Ujwala as compared to susceptible variety Pusa Jwala.

Host enzyme

Some of the host enzymes play an important role in disease resistance peroxidase and polyphenol oxidases are the key enzymes which are responsible for synthesis of quinones from phenolics. Quinones are highly bactericidal and fungitoxic (Rama and Dunleavy, 1975). Hence sometimes the increased activity of these enzymes might be responsible for disease resistance. Maine and Kelman (1961) observed that Polyphenol oxidase activity was much greater in infected than in healthy stem tissues and suggested that its activity may be involved directly or indirectly in resistance of host plants to pathogenic micro organisms including *Pseudomonas*. Retig (1974) conducted studies on the role of polyphenol oxidase in *Fusarium* wilt resistance in tomato and observed a very high increased activity in both roots and stems of the resistant plant after inoculation. No increase in activity was found in susceptible plants.

Duan *et al*. (1994) studied correlation between bacterial wilt resistance and polyphenol oxidase activity in groundnut and reported that no significant difference was observed between resistant and susceptible genotypes before inoculation. Shan and Tan (1994) studied the role of esterase activity in bacterial wilt disease resistance in groundnut and observed increased activity only in the susceptible cultivars. Resistant genotype, ‘Ujwala’ showed significantly higher peroxidase and polyphenol oxidase activity than the susceptible variety ‘Pusa Jwala’ before and after inoculation in all the plant parts (Markose, 1996).

2.7. Anatomical basis of bacterial wilt resistance

Rapid deposition of compounds such as lignin (Friend *et al.*, 1973) and callose (Beckman *et al.*, 1982) on cell wall or near the point of penetration are certain mechanisms to prevent the entry of pathogen. Lignification leads to thickening of secondary cell wall and it acts as a physical barrier for the development of pathogen. The highly developed cells prevented colonization of the pathogen in host cells. Lignification occurred at the site of penetration of the pathogen and this barrier was resistant to cellulose and mascerating enzymes of the pathogen (Ride and Pearce, 1979).

Baldacci (1977) opined that EPS (Extra Cellular Polysaccharide) responsible for vascular plugging, a chemically unidentified fraction which alters the membrane permeability is produced by the pathogen. The bacterium also produced IAA which can initiate tylose formation and increase cell wall plasticity.

Grimault *et al.* (1994) conducted histological studies in bacterial wilt resistant and susceptible tomato cultivars by light and electron microscopy, to investigate nature of barriers involved in the limitation of bacterial spread in resistant cultivars. They reported that tyloses occluded the colonized vessels and the contiguous one, limiting the bacterial spread. Other reactions involved in resistance or susceptibility were the occurrence of gums, break down of cell wall or modifications of primary cell wall. They concluded that the limitation of bacterial spread associated with resistance of tomato varieties to bacterial wilt was mainly attributed to an induced, non specific physical barrier.

Grimault and Prior (1994) were carried out experiments on tomato to investigate the mechanism of resistance to *P. solanacearum*. Colonization frequencies and bacterial densities observed in plants grafted on resistant or susceptible root stock showed that resistance was correlated to the limitation of bacterial spread in the lower part of the stem.

Markose (1996) conducted anatomical studies in bacterial wilt resistant and susceptible chilli cultivars and she reported the resistant variety have higher degree of secondary xylem development coupled with thick and compact arrangement of piliferous layers and cortical cells acted as a physical barrier preventing the entry of the pathogen.

2.8. Molecular basis of R-gene mediated resistance

Plants have developed elaborate and sophisticated defense system against microbial invaders. The defense mechanisms evolved by plants ranges from passive-mechanical or preformed chemical barriers to active responses that provide host-or cultivar-specific resistance (Jackson and Taylor, 1996). Hierarchy of events that constitute host defense begins with the recognistion of pathogen arivulence (*Avr*) gene encoded elicitors (Crute and Pink, 1996; Nimchuk *et al.*, 2001) by the host R-gene encoded receptors (Chen *et al.*, 1998). Flor (1971) proposed the "gene-for gene model".

Through his classic work, it became evident that the outcome of many host parasite interactions is governed by matching gene pairs viz., resistance (R) and avirulence (*Avr*) genes, respectively (Crute, 1985). Intense efforts with numerous sets of R-Avr proteins emphasized these two direct interactions (Jia *et al.*, 2000; Tang *et al.*, 1996).

Dangl and Jones (2001) reported the inconsistency of 'gene model' to support all cases of host pathogen interactions led to the formulation of 'guard hypothesis'. According to this hypothesis, a given R protein acts as molecular antennae that recognize interactions between pathogen avirulence factors and their targets in host cell. This is accomplished not by the direct binding of R-protein to the elicitor but by detecting physiological changes in the cell following pathogen attack. These changes may take in many forms, they could involve the binding of an effector protein to its

target, the detection of covalent modification or proteolytic cleavage both inside and outside the cell (Schneider, 2002) all constituting the essence of "guard hypothesis".

Elicitor activation of plant R genes either directly or indirectly initiates protein kinase cascades that eventually result in the transcriptional activation of batteries of plant defense genes, such as pathogenesis related (PR) genes. The defense genes impair pathogen ingress through several mechanisms including proteolysis accumulation of reactive oxygen intermediates (ROIs) and nitrogen species and activation of MAPK (mitogen activated protein kinase) cascades (Cohn *et al.*, 2001; Martin, 1999; Yang *et al.*, 1997; Jackson and Taylor, 1996). This also leads to the collapse of challenged plant cells by a localized programmed cell death (PCD), termed the 'hypersensitive response' (Stakman, 1915).

Ross (1961) reported that hypersensitive response' (HR) results in establishment of systemic acquired resistance (SAR) that immunizes the entire plant against further infections. Ward *et al.* (1991) studied accompanying activation of PR genes and cellular decompartmentalization finally leading to strengthened plant resistance.

Fluhr (2001) observed that in R genes a number of protein motifs, in a variety of combinations that are characteristic of receptors in yeast, *Drosophila* and Vertebrates (Whitham *et al.*, 1994, Parker *et al.*, 1997, Nurnberger *et al.*, 2004). Hulbert *et al.* (2001) studied that R genes are typically expressed at low levels in healthy, unchallenged plant in readiness for the detection of attack.

In *Arabidopsis*, lettuce and soybean (Witsenboer *et al.*, 1995) at particular genomic regions, R genes conferring resistance to viral, bacterial and fungal pathogens are loosely clustered approximately 1-2 cm apart. The organization of resistance gene in clusters therefore provides building blocks for rapidly evolving genes with new recognition capabilities or act as reservoirs of unique specificities (Meyers *et al.*, 1998a and b).

2.8.1. Classification of R-genes

Presence of conserved domains has permitted the grouping of R genes into five classes (Hammond-Kosak and Jones, 1997).

1. NBS-LRR class:

R-genes belonging to this class encodes cytoplasmic receptor like proteins with either on N-terminal TIR (homology to *Drosophilla* developmental Toll protein and mammalian Interleukin-1 Receptors [IL-1R] or a CC/LZ (Coiled-Coil/Lenuine Zipper) domain followed by a nucleotide binding site (NBS) and a C-terminal LRR domain.

Alber (1992) reported that R gene with N-terminal LZ/CC domains are *Arabidopsis RPS2*, *RPM1* and tomato *I2* genes. O'Nell and Greene (1998) identified TIR region in mammals, insects and plants that spans about 0.5 Kb in R genes in an ancient signaling domain induced by biotic and abiotic stress.

Martin *et al*. (2003) isolated most of the R genes so far, belonging to NBS-LRR class. R gene belonging to this class confer resistance to a number of organisms including viral, bacterial, oomycetes and fungal pathogens and even to nematodes and aphids.

2. LRR-TM class:

This class encodes for a putative transmembrane receptor with a large extra-cytoplasmic LRR domain. Jones *et al*. (1994) reported genes *Cf-2*, *Cf-4*, *Cf*-5 and *Cf-9* from tomato that confers resistance to different fungal races of *Cladosporium fulvum*.

3. LRR-TM kinase class:

Proteins encoded by this class contains an amino terminal extracellular leucine rich repeat motif joined by a transmembrane domain to a cytoplasmic C-terminal/serine/threanin protein kinase domain as seen in rice *Xa21* and *Xa26* (Song *et*

al., 1995). *Arabidopsis FLS2* (Gomez and Boller, 2000) and Wheat *LRK10* gene (Feuillet *et al*., 1997).

4. Kinase class:

This class contain only a serine-threonine kinase domain genes like barley *Rpg1* (Horvath *et al*., 2003) and tomato *Pto* (Martin *et al*., 1993) come in this groups.

5. Other R-gene:

Genes that belong to other R gene classes do not fit the mold of a receptor or signaling component involved in pathogen recognition. Examples include barley *Mlo* (Buschges *et al*., 1997), maize *HM1* and *RTM1* (Chisholm *et al.,* 2000) from *Arabidopsis*. The *HM1* gene of maize is also unlike other known R-genes in that it codes for an enzyme, HC toxin reductase that detoxifies the toxin made by race 1 *Cochlibolus carbonum* (Johal and Briggs, 1992).

2.9. Molecular characterization

The detection of naturally occurring DNA sequence polymorphisms between individuals and using that as molecular markers for crop improvement represent one of the recent developments in molecular biology. The pace of DNA-based mapping and DNA-based markers selection has been accelerated in the past few years by the advent of the PCR (Saiki *et al*., 1988; Mullis and Faloona, 1987; Mullis *et al*., 1986). This facilitated the development of marker-based gene tags, map-based cloning of agronomically important genes, variability studies, phylogenetic analysis and marker-assisted selection of desirable genotypes.

Target genes in a segregating population can be identified with the assistance of DNA markers to accelerate traditional breeding programme called as Marker Assisted

Selection (MAS). Protein and isozyme markers have been used in many crops. The major limitation of using these markers is insufficient is polymorphism among closely related cultivars. Because proteins are the products of gene expression, they may vary in different tissues, developmental stages and environments (Beckman and Soller, 1990). On the other hand, DNA markers give a much higher degree of polymorphism and stability.

The molecular markers are especially advantageous for agronomic traits and are used to tag traits as resistance to pathogens, insects, and nematodes, tolerance to abiotic stresses, quality parameters and quantitative traits. Molecular marker studied using near isogenic lines (Martin *et al.*, 1991), recombinants inbreed lines (Mohan *et al.*, 1994) or bulked segregant analysis (Michelmore *et al.*, 1991) has accelerated the mapping of many genes in different plant species. PCR based molecular markers (e.g. RAPD, SCAR, CAPs, STS and SSLP) are preferred over hybridization based markers like RFLP. Among the PCR based markers, RAPD and Microsatellite have been exploited in genome mapping, DNA fingerprinting, study of genetic diversity and gene tagging (Gupta and Varshney, 2000; Xiao *et al.*, 1996).

Genetic Markers of heritable entities that are associated with economically important traits can be used by plant breeders as selection tools (Darvasi and Soller, 1994). Restriction fragment length polymorphism (RFLP) analysis is a powerful tool for developing precise high-density molecular genetic maps because it reveals reliable and stable polymorphism. Markers based upon DNA probes have introduced a new dimension to the development of genetic maps and the mapping of agronomically and physiologically important characters. (Wyman and White, 1980).

Wongse *et al.* (1994) reported the tagging of a powdery mildew resistance gene in tomato using RFLP markers. Microsatellites have proved to be potentially useful markers for genome mapping and DNA fingerprinting (Yu *et al.*, 1999). Simple sequence repeat polymorphism has been demonstrated to be a powerful tool in gene tagging

and QTL analysis (Blair and McCouch, 1997; Xiao *et al*., 1996), pedigree analysis and marker-assisted breeding (Ayres *et al*., 1997).

2.9.1. Molecular markers in mapping disease resistance genes in crops

Molecular markers have opened up new opportunities for studies in host plant resistance (HPR). They hold good promise to trace genes conferring resistance to bacterial pathogens. Molecular genetic maps are now available for solanaceous crops and other field crops. If the genes for bacterial pathogen can be tagged by tight linkage with molecular markers, selection efficiency can be increased and the time and money in moving these genes from one varietals background to another can be greatly reduced. Hence the molecular marker technique has made it feasible to map the major genes, polygene and candidate genes associated with resistance to bacterial wilt.

Many of the identified major genes for bacterial wilt resistance have been mapped to specific chromosomal locations of genome using the molecular marker technique. The strategy followed to tag major genes includes parental and progeny survey, followed by construction of near isogenic line (NIL). Since construction of NIL is, time consuming and tedious, major genes can be mapped by another alternative strategy, bulk Segregant analysis (Michelmore *et al*., 1991).

Jena *et al.* (1992) reported BPH resistance gene has been introduced into cultivated rice lines from a distantly related species of *Oryza* and the gene has been mapped with a DNA marker by RAPD and bulked segregant analysis method. Zhu *et al*. (1993) mapped blast resistance gene, *Pikh* in rice with RAPD marker using double haploid lines, and the linked RAPD marker was later mapped torice chromosome 8.

Stevens *et al.* (1995) mapped *SW-5* which confers resistance to tomato spotted wilt virus (TSMV) in tomato and acts as a dominant gene between two RFLP markers

(CT 27 and CT 220) near telomeric area of chromosome 9, and also identified one RAPD marker that is localized within 0.5 cM of *SW-5*. Chague *et al.* (1996) identified six RAPD marker linked to the *SW-5* gene using bulked Segregant analysis. One of the coupling markers close to the gene was used to develop a SCAR marker.

2.9.2. Random Amplified Polymorphic DNA (RAPD)

The RAPD technique is based on the use of single arbitrary oligonucleotides as primers for PCR (Williams et al., 1990). These primers are synthetically produced random DNA sequences, approximately 10 nucleotides in length, with 50-60 per cent GC content. RAPD markers have been proposed as an alternative to RFLP (Williams *et al.*, 1990) and require less DNA, do not require Southern blotting and radioactive labeling, and are relatively quick to assay. It is inherited in a Mendelian fashion and can be generated without any prior knowledge of the target DNA sequence (Welsh *et al.*, 1991). It is inherited as dominant markers, where the presence of a particular band is dominant, and its absence is recessive (Tingley and Tufo, 1993).

Co-dominant RAPD markers are comparatively rare. They have been identified when manifested as two bands of different gel nobilities that exhibit complete repulsion-phase linkage in a segregating population (Schulz *et al.*, 1994). RAPD analysis has found applications in population studies (Welsh *et al.*, 1991), biosystematics (Stiles *et al.*, 1993), gene tagging (Naqvi and Chattoo, 1995) and fingerprinting (Mackill, 1996; Virk *et al.*, 1995).

Michelmore (1995) provided new opportunities for accessing the great diversity of disease resistance genes in various crop plants by RAPD marker. Zhang *et al.* (1997) identified six RAPD markers associated with the nuclear fertility-restoring gene *Rf-3*. Three of these OPK 5_{800}, OPV 10_{1100} and OPW 1_{350} were mapped on chromosome 1.

Yi *et al.* (1998) reported that the root-knot nematode resistance gene *Rk* which confers resistance to races 1 and 3 of the root-knot nematode (*Meleoidogyne incognita*) has been mapped in tobacco with RAPD markers and they used two variety NC528, KY14 and three Rk-resistant DNA bulks, and three Rk-susceptible bulks generated from F_1-derived maternal doubled haploid (MDH) individuals were screened with the primers for bulked Segregant analysis.

2.9.3. Sequenced Characterized Amplified Region (SCAR)

A SCAR is a genomic DNA fragment at a single genetically cloned locus that is identified by PCR amplification using a pair of specific oligonucleotide primers (Williams *et al.*, 1991). Mispriming error amounted in replication studies due to frequently observed problems with reproducibility over all RAPD profiles and specific bands, to overcome this problem, Paran and Michelmore (1993) converted RAPD fragments to simple and robust PCR markers, termed sequenced characterized amplified region (SCAR). It similar to the RAPD method but uses longer primers, which are generally composed of between 18 and 24 bases. By increasing the specificity of the primers, the results become more reproducible and more specific (Hernandez *et al.*, 1999).

Naqvi *et al.* (1995) reported the identification of two RAPD markers (OPF 6_{2700} and OPH 18_{2400}) linked to '*Pi-10*' blast resistance gene in rice. These markers were converted into SCARs and based on differences in the length of SCARs alternate alleles were considered for the indirect selection of *Pi-10*.

Kasai *et al.*, (2000) developed SCARs based on nucleotide differences within resistance gene like fragments isolated from a potato plant carrying the *Ryadg* gene, which confers extreme resistance to potato Y potyvirus (PVY). It originates from *Solanum tuberosum* subsp. *andigena*, and a susceptible potato plant. The SCAR marker RYSC3 was generated only in genotypes carrying *Ryadg* gene.

Sugita *et al.* (2004) reported RAPD markers linked to the L^3 locus by applying the BSA method to two doubled haploid (DH) population in *Capsicum*. The co-dominant RAPD markers E 18_{272} and E 18_{286} were converted into SCAR markers by molecular cloning and nucleotide sequencing and they mapped at a distance of 4.0 cM from the L^3 locus.

Potato virus Y (PVY) is the only potyvirus infecting *Capsicum annuum* L., a monogenic dominant gene *Pvr4* confers resistance to PVY pathogen. Andres *et al.* (2004) conducted BSA to search for RAPD markers linked to the *Pvr4* gene using segregating progenies obtained by crossing a homozygous resistant 'Serrano Criollo de Morelos-334' with a homozygous susceptible 'Yolo Wonder' cultivar. Eight hundred decamer primers were screened to identify one RAPD marker UBC 19_{1432} linked in repulsion phase to *Pvr4*. This marker was converted into a dominant SCAR marker SCUBC 19_{1423}.

Quirin *et al.* (2005) rreported SCAR primers for the detection of *phyto.5.2* a major QTL for resistance to *Phytophthora capsici* Leon. in pepper. They screened genotypes of *C. annuum* and *C. chinese* with a series of RAPD primers. One primer, OpDO4, amplified a single band only in those *C. annuum* and *C. chinense* genotypes showing the highest level of resistance and amplified product was cloned, sequenced and converted to a SCAR marker. These primers were observed to define locus on *Capsicum* chromosome 5, which was tightly linked to *phyto.5.2*.

Techawongstein *et al.* (2006) studied the inheritance of pungency in *Capsicum* by SCAR marker and they crossed the highly pungent variety "Yodson" (YS), local commercial variety of Thailand and the low pungent variety. "YuYi" (YY) from China. They conducted bulked Segregant analysis and reported capsaicinoid content, broad sense heritability (h_b^2) and DNA finger print with UBC 20 SCAR primer. They compared results among F_2 populations and their parents. The SCAR primer for UBC

20 could amplify a common fragment of 1700 bp in both low and high pungency chilli plants of all generation.

Julio *et al*. (2006) reported that SCAR markers linked to three disease resistances viz., black root rot, blue-mold and potato virus Y (PVY) were reported in a *Nocotiana tabaccum* L. accession by AFLP assay. Tomato spotted wilt virus (TSMV) is a serious disease in tobacco (*Nicotiana tabaccum* L.). The breeding line "Polalta" contains a single dominant gene conferring resistance to TSMV that was introgressed from *N. alata* Link and Otto. The DNA bulks from susceptible and resistant doubled haploid lines derived from a cross between susceptible cultivar "K326' and "Polalta" were analysed to identify resistance gene by AFLP technology and bulked segregant analysis. Four AFLP fragment were successfully converted to SCAR marker (Moon and Nicholson, 2007).

Kim *et al*. (2008) developed molecular marker linked to the L4 locus conferring resistance to tobamovirus pathotype in pepper plants, they performed AFLP with 512 primer combinations for susceptible 'S pool' and Resistant 'R pool'. A total of 19 primer pairs produced scorable bands in the R pool. Further screening with these primers pairs was done on DNA bulks from T102, a $BC_{10}F_2$ derived from T10 by backcrossing. Each bulk was made by pooling of DNA of five homozygous individuals from a T10 population, which was a near-isogenic BC_4F_2 generation for the L4 locus. AFLP markers were finally selected and designated L4-a, L4-b and L4-c, whereas no recombination for L4-b was seen in 20 individuals of each DNA bulk. L4-b was successfully converted into a simple 340 bp SCAR marker designated L4SC340, which mapped 1.8 cM from the L4 locus in T102 and 0.9 cM in another $BC_{10}F_2$ population.

2.10. TAGGING OF DISEASE RESISTANT GENES WITH MOLECULAR MARKERS

2.10.1. Tagging major resistant genes with RAPD markers

The process of locating genes of interest via linkage of markers is referred as "gene tagging" (Chawla, 2002). RAPD markers are suitable for tagging major gene controlling disease resistance, pest resistance etc. RAPD markers are suitable for tagging major genes controlling disease resistance, pest resistance etc. In rice, gene conferring resistance to blast was tagged using RAPD markers (Zhu *et al.*, 1993). Wang *et al.* 1995 tagged major genes for thermo sensitive genetic male sterility in rice by RAPD markers.

Martin *et al.* (1991) surveyed polymorphism for bacterial wilt against *Pseudomonas* in the near isogenic line (NILs) of tomato through RAPD and identified three markers. These markers were identified to be linked to '*Pto*' gene conferring resistance to the bacterial wilt.

Mohan *et al.* (1994) and Nair *et al.* (1995) used BSA and tagged RAPD markers OPF 8 and OPF 10 to the rice *Gm2* gene that confers resistance to biotype 1 of gall midge in rice. The *Gm4t* gene for gall midge resistance was also tagged by RAPD markers E 20_{570} and E 20_{583}, which were then converted into sequenced tagged sites.

Bulk Segregant analysis on F_2 population of Nipponbare x Kasalath cross was used to determine RAPD markers in a specific interval in the middle of the chromosome 6 of rice for tagging the photoperiod sensitivity gene. Fourteen markers tightly linked to the photoperiod sensitivity gene were identified. They were converted into STS by cloning and sequencing the polymorphic fragments (Monna *et al.*, 1995).

Naqvi and Chattoo (1995) reported the identification of two RAPD markers OPF 6_{2700} and OPH 18_{2400} linked to *Pi-10* blast resistance gene in rice. These markers were converted into SCARs and based on the polymorphisms appearing as differences

in the length of the SCARs alternate were considered for the indirect selection of *Pi-1* RAPD analysis was conducted with the combined use of near isogenic lines and BSA to tag the bacterial blight resistance genes *Xa*-21, *Xa*-3, *Xa*-4 and *xa*-5 in rice (Yoshimura *et al.*, 1995; Zhang *et al.*, 1994). From the survey of 260 decamer primer OPAC 5 was detected to amplify specifically a 0.9 Kb fragments from the DNA of bacterial blight susceptible plants. The distance between the RAPD marker OPAC 5_{900} and *Xa*-13 was estimated to be 5.3 cM (Zhang *et al.*, 1996).

Procunier *et al.* (1997) tagged the R-gene T_{10} with SCAR marker linked to loose smut resistance gene which caused by *Ustilago Segatium tritici* in wheat. Chunwongse *et al.* (1997) developed high-resolution genetic map in tomato and tagged *Lv* resistance locus, which confers powdery mildew resistance by RAPD marker. Tai *et al.* (1999) studied expression of the *Bs2* pepper gene, which confers resistance to bacterial spot disease in tomato.

Shen *et al.*, 1998 surveyed two F_2 populations and one BC_1 population have been used to confirm the linkage of the markers for fertility restorer gene *Rf*-3 gene and they tagged with RAPD markers OPB 18_{1000} at a distance of 5.3 cM in chromosome 1.

Jeon *et al.* (1999) used BSA and tagged RAPD marker OPD 7 to the rice *bph 1* gene conferring resistance to biotype 1 of BPH. OPD 7 yielded a 700 bp fragment, which was present in resistant parent, and resistant F_2 plants but absent in susceptible parent and susceptible F_2 plants. Chromosomal regions surrounding the *Bph-1* were examined with additional RFLP and microsatellite markers on chromosome 12 to define the location of the RAPD marker and *Bph-1*.

Sandhu *et al.* (2002) carried out RAPD analysis of herbicide-resistant Brazilian rice lines produced via mutagenesis. Among eighty random primers tested, 10 were selected for a detailed study of RAPD markers that could tag herbicide resistance genes. Resistant and susceptible lines produced variation in the RAPD patterns and certain

bands were found only in certain lines. Twenty lines resistant to herbicide were selected as a result of this study.

Caranta *et al.* (1997) reported the tagging of a powdery mildew *Leveillula taurica* (Lev.) Arnaud resistance gene (*Lv*) in tomato using RAPD and RFLP markers. DNA from a resistant and a susceptible cultivar were screened with 300 random primers and four primers yielded fragments that were unique to the resistant line and also linked to the resistance gene in F_2 population. One of these amplified fragments with OP248, with a molecular weight of 0.7 Kb, was subsequently mapped to chromosome 12.

2.10.2. QTLs tagged with RAPD markers

Extending the molecular technique from mapping the major genes to the QTL, resulted in the identification of both major and minor genes conferring resistance to pathogens and insect pest. Genetic dissection of several quantitative traits in to single Mendelian factors in tomato and also many QTLs have been classified using DNA markers in various crop plants such as in maize (Edwards *et al.*, 1992) and tomato (Chague *et al.*, 1997; Bernacchi, 1998).

Nishi *et al.* (2003) found QTL linked to bacterial wilt resistance in tobacco. The susceptible variety 'Michinoku l' and the resistant variety 'W6' were screened for AFLP polymorphisms with 3072 primer combinations. They identified 117 polymorphic markers and these markers were analyzed in 125 doubled haploid lines for analyzing the association between the markers and bacterial wilt resistance and they drawn a linkage map consisting of 10 linkage group. One QTL for bacterial wilt resistance was identified on a 32 cM linkage group consisting of 15 markers.

2.10.3. Tagging of gene with AFLP and RFLP markers

In case of white backed plant hopper (WBPH), NILs having individual resistance genes *viz*., *Wbph1, Wbph2, Wbph3,* and *Wbph4* have been developed with 'IR 36' background. RFLP analysis has been carried out to identify the chromosomal segments introgressed from the resistant donor into the respective NILs. Segregating population for each gene has been produced by making cross between susceptible IR 36 and NILs for each gene. Using this NILs, he mapped *Wbph 1* gene from chromosome 7 with an RFLP marker RG146. (McCouch, 1990).

Wongse *et al*. (1994) reported the tagging of a powdery mildew resistance gene in tomato using RFLP markers. Hittalmani *et al*. (1995) developed a PCR-based marker using RFLP marker RG64, which is tightly linked to blast resistant gene *Pi-2* and generated three PCR products known as sequence tagged sites (STS). The amplified products were cleaved with restriction enzymes *HaeII* and produced specific amplicon.

Singh (2002) reported molecular tagging of rice blast resistance gene *Pikh* using PCR based markers and AFLP. The causal pathogen is *Magnaporthe grisea*.

2.11. Bulk segment analysis (BSA)

Bulk segment analysis is a rapid procedure for identifying markers in specific regions of the genome. This method involves using two pooled DNA samples of individuals from a segregating population originating from a single cross. Within each pool, or bulk, the individuals are identical for the trait or gene of interest but are arbitrary for all other gene (Michelmore *et al*., 1991). BSA removes the need for screening the entire mapping population with every primer. Poulson *et al*. (1995) suggested that when bulks are constructed from enough individuals, the BSA is sufficiently robust to cope with the low level of phenotypic misclassification.

Wang *et al.* (1995) used RAPD markers and BSA to identify molecular markers linked to *Pi-ta* gene in rice which resides on chromosome 12 and flanking between SP4B9 and SP9F3 RAPD markers. Three RAPD markers OPK 17_{1400}, OPA 7_{550} and OPB 10_{450} were reported to be co-segregated with resistance phenotype of neck blast in Gumei 2. Resistance gene was located between OPK 17_{1400} and OPA 7_{550} having genetic distance of 2.4 cM to OPK 17_{1400} and 7.5 cM to OPA 7_{550}.

Nirmal Jyothi *et al.* (2001) used RAPD makers and BSA to identify molecular markers linked to (*Magnaporthe grisea* Cav.) disease resistance in rice. RAPD analysis and BSA were followed to identify four phenotype specific markers for yellow stem borer resistance in rice. The markers CI_{320} and K_{695} were linked with resistance phenotype whereas markers AH 5_{660} and C 4_{1300} were linked with susceptibe phenotype (Selvi *et al.*, 2002).

Milla *et al.* (2005) identified RAPD and SCAR markers linked to the blue mold resistant tobacco cultivars which is caused by fungal pathogen *Peronospora tabacina*. BSA was used to screen for polymorphisms between DNA bulk from susceptible and resistance cultivar using 1216 RAPD primers. Fifteen RAPD markers were identified as being linked to the major resistance locus to blue mold and two RAPD markers flanking the most likely QTL position were converted to SCAR markers.

Minamiyama *et al.* (2005) studied the character of pungency in pepper (*Capsicum annuum* L.) which is controlled by a single recessive gene (c) and developed a molecular marker linked to the c-locus using two segregating F_2 population (TM2 and TF2) derived from crosses between pungent and non-pungent peppers in *C. annuum*. Using the RAPD technique in combination with a bulked Segregant analysis, two RAPD markers, OPD 20_{800} and OPY 9_{800} were obtained.

Moon and Nicholson (2007) studied the resistance source in tobacco for tomato spotted wilt virus (TSMV) in the breeding line 'Polalta', which contains a TSMV

resistance gene introgressed from the wild relative *Nicotiana alata*. He applied AFLP technology and BSA to identify markers linked to TSMV resistance. Primer combinations of 128 numbers were used to screen one resistant and two susceptible bulks and found that 48 potential markers linked to the TSMV resistance and from them four AFLP fragments were converted to SCAR markers.

2.12. Cloning of disease resistant gene

Johal and Brigg (1992) cloned disease resistance genes *HM1* which confers resistance to *Cochlibobolus carbonum* from corn by transposon tagging. One marker that co-segragated with *Pto* was used to isolate a single YAC clone and which provide resistance to different strains of *Pseudomonas syringae* has been isolated by map based cloning from tomato. *Pto* and *RPS2,* which provides resistance to different strains of *Pseudomonas syringae*, have been isolated by map-based cloning from tomato and *Arabidopsis* respectively (Bent *et al*., 1994; Martin *et al*., 1993).

Martin *et al*. (1991) analyzed a pair of tomato near isogenic lines that differ for a region on chromosome 5 that contains a gene *Pto* conferring resistance to *Pseudomonas syringe* pv. tomato. One hundred forty four random primers were screened on these lines, and seven amplified products were identified that were present in one but not the other line; they further investigated; three were confirmed by segregation analysis to be tightly linked to the *Pto* gene.

The first plant R gene to be cloned was the maize *Hm1* gene by transposon tagging (Johal and Briggs, 1992). Martin *et al*. (1993) first clone the tomato resistance gene, *Pto*.

Transposon tagging using activator (Ac) or dissociation (Ds) elements from corn enabled cloning of the *N* gene, which confers resistance to tobacco mosaic virus (Whitham *et al*., 1994). Similarly, isolation of the tomato *cf-9* gene for resistance to

Cladosporium fulvum by transposon tagging and transgenic plants expressing the bacterial avirulence factor Avr 9 (Jones *et al.*, 1994).

Recently a candidate sequence for *Prf* has been isolated by map-based cloning that share sequence similarity to *RPS2*, *N* and *L6*. This *Prf* gene was initially identified by mutagenesis and this tomato mutants altered in bacterial disease resistance provide evidence for a new locus i.e., *Pto* locus controlling pathogen recognition (Salmeron *et al.*, 1994).

As a first step in cloning, Miyamoto *et al.* (1996) finely mapped the blast resistance *Pi-6* near the terminal region of chromosome two using RFLP and RAPD markers. This gene co-segregated with RAPD (b-1) and RFLP (Rz123) markers.

Seah *et al.* (1998) studied close genetic linkage of RGCs (resistant gene candidates) with R gene loci in many plant species and demonstrates the potential of PCR strategy as a more feasible alternative to transposon tagging and map based cloning, these two most commonly used techniques in R gene isolation.

Dilbirligi *et al.* (2004) reported that around 46 resistance genes have been clones from numerous plant species by the techniques of either transposon tagging or map based cloning and some of them are enlisted in Table 1.

Species	R gene (Pathogen)	Approach to isolate gene	Functional domain	Reference
Maize	*Hm1*(*Cochliobolus carbonum*)	Map-based	HC toxin reductase	Johal and Briggs,1992
Tobacco	*N* (Tobacco mosaic virus)	Transposon tagging	NBS-LRR	Whitham *et al.*1994
Flax	*L6* (*Melamspora lini*)	Transposon tagging	NBS-LRR	Lawrence *et al.*1995
Arabidopsis	*RPM1*(*Pseudomonas syringae* pv. *maculicola*)	Map-based	NBS-LRR	Grant *et al.* 1995
Arabidopsis	*RPS 2* (*P. syringae* pv. *Phaseolica*)	Map-based	NBS-LRR	Mindrions *et al.* 1994
Arabidopsis	*RPS 4* (*P. syringae* pv. *Phaseolica*	Map-based	NBS-LRR	Grassmann *et al.* 1999
Arabidopsis	*RPP 5* (*Pernospora parasitica*)	Map-based	NBS-LRR	Parker *et al.* 1997
Tomato	*Cf-2 (Cladosporium fulvum)*	Map-based	LRR	Dixon *et al.* 1996
Tomato	*Cf-4 (C. fulvum)*	Map-based	LRR	Thomas *et al.* 1997
Tomato	*Cf-9 (C. fulvum)*	Transposon tagging	LRR	Jones *et al.* 1994
Tomato	*Cf-5 (C. fulvum)*	Map-based	LRR	Dixon *et al.* 1998
Tomato	*Pto* (*P. syringae* pv. tomato)	Map-based	Ser/Thr kinase	Martin *et al.* 1993
Tomato	*Prf* (*P. syringae* pv. tomato)	Map-based	NBS-LRR	Salmeron *et al.* 1996
Rice	*Xa 21* (*Xanthomonas oryzae* pv. *oryzae*)	Map-based	NBS-LRR-kinase	Song *et al.* 1995

Table 1. Disease resistance (R) genes isolated from different plant species

Materials and Methods

3. MATERIALS AND METHODS

The study on "Molecular characterization of chilli (*Capsicum annuum* L.) genotypes for tagging bacterial wilt resistance gene" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period 2006-2008. The materials used and methodologies adopted are discussed in this chapter.

3.1. MATERIALS

3.1.1. Plant Materials

Seeds of the three chilli genotypes *viz*., Ujwala, Pusa Jwala and Anugraha with varying levels of resistance were collected from different institutions (Table 2 and Plate 1) and F_2 progenies of the cross Ujwala x Pusa Jwala were selected for phenotypic screening against bacterial wilt and for genotyping by RAPD and SCAR markers (Plate 2). The seedlings were raised in pots containing sterilized soil medium and were subjected to artificial inoculation to confirm the resistance/susceptibility to bacterial wilt caused by *Ralstonia solanacearum*. Anugraha is the hybrid derivative of cross Ujwala x Pusa Jwala with resistance to bacterial wilt from Ujwala.

Table 2. Chilli genotypes and their sources

Genotypes	Source
Ujwala	Kerala Agricultural University, Thrissur
Pusa Jwala	IARI, New Delhi
Anugraha	Kerala Agricultural University, Thrissur

a. Resistant variety (Ujwala)

b. Susceptible variety (Pusa Jwala)

c. Anugraha

Plate 1. Genotypes used for study

3.1.2. Laboratory chemicals and glassware

The chemicals used in the present study were of good quality (AR grade) procured from Merck India Ltd., SRL, HIMEDIA and SISCO Research Laboratories. The Taq DNA polymerase, dNTPs, Taq buffer and molecular weight marker (λDNA /*Hind*III +*Eco*RI double digest) were supplied by Bangalore Genei. The random primers were obtained from Operon Technologies, USA. The SCAR primers were synthesized from Integrated DNA Technologies, USA. The plastic wares used for the study were purchased from Tarsons India Ltd. and Axygen, USA.

3.1.3 Equipment and machinery

The equipment available at the Centre for Plant Biotechnology and Molecular Biology and the Bioinformatics Centre, College of Horticulture was used for the present study. Centrifugation was done in KUBOTA centrifuge. Dai Ki-S1010 (Dai Ki Scientific Co.) was used for the incubation of culture. The PCR was done in Eppendorf Master Cycler (Eppendorf, USA) and agarose gel electrophoresis done in horizontal gel electrophoresis system (BIO-RAD). Gel DOC-It ™ Imaging system UVP (USA) was used for imaging the gel. The list of laboratory equipments used for the study is provided in Appendix I.

3.2. METHODS

3.2.1. Development of segregating population

3.2.1.1. Hybridization and development of F_1 plants

The hybridization experiment was done in pots. The bacterial wilt resistant variety Ujwala and susceptible variety Pusa Jwala were grown in pots containing

sterilized soils. The crop was raised as per package of practices of Kerala Agricultural University (KAU, 2002). The bacterial wilt resistant variety Ujwala was crossed with susceptible variety Pusa Jwala for development of F_1 hybrids (Plate 2c), where in Ujwala was the ovule parent and Pusa Jwala was the pollen parent. Female flower for crossing was emasculated at 5-6 pm, one day prior to anthesis. Artificial pollination was done early morning at 7 am on the day of anthesis. F_1 plants were produced by sowing seeds from ripe hybrid fruits.

3.2.1.2. Development of F_2 progeny

The F_2 plants raised from a single F_1 hybrid were used as mapping population. The F_1 plants were selfed to obtain F_2 generation seeds and F_2 generation seeds were sown in pots for developments of F_2 progeny (Plate 2d).

3.2.2. PHENOTYPING OF GENOTYPES FOR BACTERIAL WILT RESISTANCE

3.2.2.1. Standardization of artificial inoculation technique

A pot culture study was carried out using the susceptible variety Pusa Jwala, to standardize inoculation technique in chilli. The seeds were sown in sterilized pots containing 1:1:1 mixture of sand, soil and FYM. Sterilization of the medium was carried out with 40 per cent formaldehyde solution. The fresh bacteria ooze from wilted plants was used for inoculation. The bacterial ooze was collected in 100 ml of sterile distilled water and the optical density (OD) of the suspension was adjusted to 0.3 at 600 nm containing 10^8 cfu/ml. Screening was done during the month of October in open condition. Thirty days after sowing, when the seedlings are at 5-6 leaf stage they were transplanted to small polythene bags, and inoculated with fresh bacterial ooze. In each treatment 10 plants were screened and 5 plants were maintained as control.

The following four methods of inoculation were tested for identifying the best method. Root dipping was done before transplanting and the rest were given after transplanting.

i) Root dipping:

Seedlings were lifted carefully and roots washed with sterile distilled water to remove the soil particles. Then roots were dipped in bacterial suspension for 30 minutes and seedlings transplanted in polythene bags containing sterilized soil.

ii) Stem-puncturing:

A drop of bacterial suspension was placed in the axils of second and third expanded leaves below the stem apex and then a needle was forced into the stem through the drop. A piece of cotton dipped in bacterial suspension was then placed in the punctured axils to ensure sufficient inoculum. The treatment was given immediately after transplanting.

iii) Soil drenching:

The soil in the pot was drenched by pouring 30 ml of inoculation around the base of seedling.

iv) Soil drenching with wounding:

The lateral roots of each transplanted seedling were cut along one side of the plant by inserting the blade of a knife, 1.5 cm away from the collar region to a depth of about 4 cm. Then the soil was drenched by pouring 30 ml of the inoculum around the base of the seedlings.

Plants were observed for 15 days for the incidence of bacterial wilt. The wilted plants were subjected to ooze test to confirm the association of pathogen with the wilt (Plate 3).

The severity of wilt incidence was scored according to the resistance/susceptibility based on the scoring system by Mew and Ho (1976) and details are given below:-

Disease Rating	**Per cent survival**
R (Resistant)	80 or above
MR (Moderately resistant)	60 – 80
MS (Moderately susceptible)	40 – 60
S (susceptible)	less than 40

Based on the observations made on disease reaction, the per cent wilt incidence was calculated using the following formula:

$$\text{Per cent of wilt incidence} = \frac{\text{No. of plants affected by wilt}}{\text{Total no. of plants under observation}} \times 100$$

3.2.2.2. Phenotyping of genotypes for bacterial wilt resistance

The F_2 progenies of the cross Ujwala x Pusa Jwala were tested along with susceptible and resistant check Pusa Jwala and Ujwala respectively for their resistance to bacterial wilt infection. F_1 plants of cross Ujwala x Pusa Jwala and Anugraha, its hybrid derivative resistance to bacterial wilt with resistance to bacterial wilt were also included for study. Artificial inoculation was given by soil drenching method big wounding of 30 days old transplanted seedling at 5-6 leaf stage. In each group 20 plants were screened. The severity of wilt incidence was scored according to the resistance/susceptibility based on the scoring system proposed by Mew and Ho (1976).

3.2.3. MOLECULAR CHARACTERISATION OF CHILLI GENOTYPES BY RAPD MARKER

Molecular characterization of selected chilli genotypes and F_2 progenies for tagging bacterial wilt resistance gene was done with RAPD (Plate 4).

3.2.3.1. Standardization of genomic DNA isolation

Isolation of good quality genomic DNA is one of the most important prerequisites for doing RAPD and SCAR analysis. The procedures reported by Doyle and Doyle (1987) and Rogers and Bendich (1994) for the extraction of nucleic acids were compared for the isolation of genomic DNA from chilli with slight modifications. Tender leaves from the selected plants were collected early in the morning and used for the genomic DNA isolation.

3.2.3.1.1. Procedure reported by Doyle and Doyle (1987)

Reagents

1. Extraction buffer (4X)
2. Lysis buffer
3. TE buffer
4. Isopropanol
5. Chloroform: isoamyl alcohol (24:1, v/v)
6. 5 % sarcosin
7. Ethanol 100 and 70%

Details of composition of reagents are provided in the Appendix II.

Procedure

Leaf sample (1 g) was ground to a fine powder in excess of liquid nitrogen using a mortar and pestle. A pinch of sodium metabisulphite and 50 µl of β- mercaptoethanol

were added. Later 3 ml extraction buffer was also added. The homogenate was transferred to a 50 ml oakridge tube containing 4 ml prewarmed lysis buffer and 1 ml sarcosin. The contents were mixed well and maintained at 65^0C for 15 minutes. Equal volume of chloroform: isoamyl alcohol (24:1) mixture was added to the tube, mixed gently by inversion and centrifuged at 10000 rpm for 15 minutes at 4^0C. The contents got separated into three distinct phases. The upper aqueous phase containing DNA was pipetted out into a fresh 50 ml oakridge tube. Then 0.6 volume of ice cold chilled isopropanol was added and the contents were mixed gently. The sample was then incubated at -20^0C for 30 minutes to 10 hrs to precipitate the DNA completely. The DNA was then pelleted by centrifuging at 10000 rpm for 15 minutes at 4^0C. The isopropanol was poured out and the pellet was washed with 70 percent alcohol. The pellet was air dried to remove alcohol and finally dissolved in 250 µl TE buffer.

3.2.3.1.2. Procedure reported by Rogers and Bendich (1994)

Reagents

1. 2 X CTAB extraction buffer
2. 10% CTAB solution
3. TE Buffer
4. Isopropanol
5. Chloroform: isopropanol (24:1, v/v)
6. Ethanol 70% and 100%

The details of preparation of reagents are provided in Appendix II.

Procedure

Leaf sample (1 g) was weighed accurately and ground using liquid nitrogen. The ground tissue was transferred to a 50 ml oakridge tube containing 5 ml prewarmed 2X CTAB extraction buffer and 60 µl β-mercaptoethanol. The contents were mixed and incubated at 65^0C for 15-20 minutes. Then equal volume of chloroform: isoamyl

alcohol mixture was added, mixed gently by inversion and centrifuged at 10000 rpm for 10 minutes at 4^0C. The supernatant was transferred to a fresh tube and 1/10 volume of 10% CTAB solution was added. Equal volume of chloroform: isoamyl alcohol was added again, mixed gently and centrifuged at 10000 rpm for 15 minutes at 4^0C. The aqueous phase was transferred to another fresh tube and 1/6 volume of isopropanol was added and kept at -20^0C for 30 minutes for complete precipitation of DNA. The DNA was pelleted by centrifuging at 10000 rpm for 15 minutes at 4^0C. The supernatant was discarded and the pellet was washed with 70 percent ethanol. The pellet was air dried and dissolved in 250 µl TE buffer.

3.2.3.2. Purification of DNA

The DNA isolated contained RNA as contaminant and was purified by phenol precipitation and RNAse treatment (Sambrook *et al.*, 1989).

Reagents

1. Phenol: chloroform mixture (1:1, v/v)
2. Chilled isopropanol
3. 70 per cent ethanol
4. TE buffer
5. Chloroform : isoamyl alcohol (24:1, v/v)
6. One per cent RNAse

The RNAse A from Sigma, USA was used to prepare RNAse. One per cent solution was prepared by dissolving RNAse A in TE buffer at 100^0C for 15 minutes. The solution was cooled to room temperature, dispensed into aliquots and stored at -20^0C.

The procedure followed for DNA purification is as follows:

RNAse solution (2 µl) was added to 100 µl DNA sample and incubated at 37^0C in dry bath (Genei, Thermocon) for 1 hour. The volume was made up to 250 µl with distilled water and equal volume of phenol: chloroform mixture was added. This was then centrifuged at 12,000 xg for 10 minutes at 4^0C. The aqueous phase was collected in a fresh micro centrifuge tube and added equal volume of chloroform: isoamyl alcohol (24:1). Again it was centrifuged at 12,000 xg for 10 minutes at 4^0C. The above two steps were repeated and finally precipitated DNA from the aqueous phase with 0.6 volume of chilled isopropanol. The mixture was then incubated at -20^0C for 30 minutes and centrifuged at 10,000 rpm for 15 minutes at 4^0C. The pellet of DNA obtained was washed with 70 per cent ethanol. The pellets were air dried and dissolved in 250 µl TE buffer.

3.2.3.3. Assessing the quality of DNA by electrophoresis

The quality of isolated DNA was evaluated through agarose gel electrophoresis (Sambrook *et al.*, 1989).

Reagents

1. Agarose - 0.7 per cent (for genomic DNA)
   - 1.2 per cent (for PCR samples)
2. 50X TAE buffer (pH 8.0)
3. Tracking dye (6X)
4. Ethidium bromide (stock 10 mg/ml; working concentration; 0.5 µg/ml)
5. UV transilluminator (Herolab ^{R})
6. Electrophoresis unit, power pack, gel casting tray, comb
7. Gel documentation and analysis system Gel DOC-It ^{TM} Imaging system UVP (USA)

(Composition of reagents is provided in Appendix III).

The procedure followed for agarose gel electrophoresis is as follows:

1X TAE buffer was prepared from the 50 X TAE stock solutions. Agarose (1%) was weighed and dissolved in TAE buffer by boiling. Then ethidium bromide was added at a concentration of 0.5 μg ml^{-1} and mixed well. The open end of gel casting tray was sealed with a cellotape and kept on a horizontal surface. The comb was placed desirably and the dissolved agarose was poured on to the tray. The gel was allowed to set for 30 minutes after which the comb was removed carefully. The tray was kept in the electrophoresis unit with well side directed towards the cathode. 1X TAE buffer was added to the tank. Then DNA sample (5 μl) along with the tracking dye (1 μl) was loaded into the wells using a micropipette carefully. λDNA / *Eco*RI+*Hind*III double digest was used as a molecular marker. After closing the tank, the anode and cathode ends were connected to the power pack and the gel was run at a constant voltage (100V) and current (50 A). The power was turned off when the tracking dye reached $2/3^{rd}$ length of the gel.

Then the gel was taken from the electrophoresis unit and viewed under UV light in a transilluminator. The DNA fluoresces under UV light due to ethidium bromide dye. The quality of DNA was judged by clarity of DNA band. The image was documented and saved in gel documentation system (Gel DOC-It ^{TM} Imaging system UVP, USA).

3.2.3.4. Assessing the quality and quantity of DNA by NanoDrop method

The purity of DNA was further analysed by using NanoDrop® ND-1000 spectrophotometer. A 1 μl sample was used for assessing the purity of DNA. The absorbance of nucleic acid samples were measured at a wavelength of 260 nm and 280 nm. The purity of DNA was assessed by the ratio OD_{260}/OD_{280}. A ratio of 1.8 to 2.0 indicated pure DNA. The quantity of DNA in the pure sample was calculated using the formula $OD_{260} = 1$ is equivalent to 50 μg double stranded DNA.

1 OD at 260 nm = 50 µg/ml DNA

Therefore OD_{260} x 50 gives the quantity of DNA in µg/ml.

3.2.3.5. RAPD (Random Amplified Polymorphic DNA) analysis

The good quality genomic DNA isolated from chilli leaf by Rogers and Bendich (1994) method were subjected to RAPD as per the procedure reported by Williams *et al.* (1990). Random decamer primers supplied by 'Operon Technologies' USA with good resolving power were used for amplification of DNA. Decamer primers for RAPD assay were selected after an initial screening study of primers.

3.2.3.5.1. Screening of random primers for RAPD analysis

Forty seven decamer primers in the series OPE, OPAH, OPN, OPP, OPS and OPY were screened with genomic DNA of resistant variety Ujwala and susceptible variety Pusa Jwala as the templates. Details of primers used for screening is given in (Table 3a and Table 3b). Genomic DNA at the concentration of 25 ng was subjected to amplification using selected random primers.

The amplification reaction was carried out in an Eppendorf Master Cycler (Eppendorf, USA). A master mix without the template DNA was prepared using the reaction mixture for the required number of reactions. From this, master mix, 18 µl was pipetted into each PCR tube. Template DNA (2 µl) was added. PCR amplification was performed in a 20 µl reaction mixture as constituted below:

Composition of the reaction mixture for PCR (20.0 µl)

Genomic DNA (25 ng)	1.0 µl
10X Taq assay buffer	2.0 µl
dNTP mix (10 mM each)	1.0 µl
$MgCl_2$ (25 mM)	1.0 µl
Decamer primer (10 pM)	1.5 µl
Taq DNA polymerase (1U)	0.3 µl
Autoclaved distilled water	12.2 µl
Total volume	= 20.0 µl

The PCR tubes were kept in the thermal cycler and were run in the following programme:

94^0C for 5 minutes	-Initial denaturation	
94^0C for 1 minute	-Denaturation	40 cycles
37^0C for 1 minute	-Primer annealing	
72^0C for 2 minutes	-Primer extension	
72^0C for 2 minutes	-Final extension	
4^0C for infinity	to hold the sample	

The amplified products were run on 1.2 per cent agarose gel using 1X TAE buffer stained with ethidium bromide along with marker (λDNA / *Hind* III + *Eco* RI double digest). The profile was visualized under UV (312 nm) transilluminator and documented using gel documentation system Gel DOC-It ™ Imaging system UVP (USA). The documented RAPD profiles were carefully examined for polymorphism. Number of bands produced by each primer was counted and tabulated. Those primers, which gave good amplification with more than seven bands, were selected for further studies.

Table 3 (a). List of Operon decamer primer series OPAH, OPP and OPN used for screening chilli genotypes

Sl. No	Primer	Nucleotide Sequence (5'–3')
1	OPAH 4	CTC CCC AGA C
2	OPAH 5	TTG CAG GCA G
3	OPAH 9	AGA ACC GAG G
4	OPAH 13	TGA GTC CGC A
5	OPAH 16	CAA GGT GGG T
6	OPAH 17	CAG TGG GGA G
7	OPAH 18	GGG CTA GTC A
8	OPAH 19	GGC AGT TCT C
9	OPAH 20	GGA AGG TGA G
10	OPP 1	GTA GCA CTC C
11	OPP 3	CTG ATA CGC C
12	OPP 4	GTG TCT CAG G
13	OPP 5	CCC CGG TAA C
14	OPP 6	GTG GGC TGA C
15	OPP 7	GTC CAT GCC A
16	OPN 1	CTC ACG TTG G
17	OPN 3	GGT ACT CCC C
18	OPN 5	ACT GAA CGC C
19	OPN 6	GAG ACG CAC A
20	OPN 7	CAG CCC AGA G
21	OPN 8	ACC TCA GCT C

Table 3 (b). List of Operon decamer primer series OPS, OPY and OPE used for screening chilli genotypes

Sl. No	Primer	Nucleotide Sequence (5'–3')
1	OPS 1	GTT TCG CTC C
2	OPS 2	TGA TCC CTG G
3	OPS 3	CAT CCC CCT G
4	OPS 5	TCG GCC CTT C
5	OPS 6	TGC TCT GCC C
6	OPS 9	TGG GGG ACT C
7	OPS 10	CTG CTG GGA C
8	OPS 11	GTA GAC CCG T
9	OPS 12	CCT TGA CGC A
10	OPS 18	CCA CAG CAG T
11	OPS 19	ACC CCC GAA G
12	OPS 20	GGA CCC TTA C
13	OPY 1	GTG GCA TCT C
14	OPY 3	ACA GCC TGC T
15	OPY 5	GGC TGC GAC A
16	OPY 7	AGA GCC GTC A
17	OPY 9	AGC AGC GCA C
18	OPY 11	AGA CGA TGG G
19	OPE 3	CCA GAT GCA C
20	OPE 5	TCA GGG AGG T
21	OPE 6	AAG ACC CCT C
22	OPE 8	TCA CCA CGG T
23	OPE 9	CTT CAC CCG A
24	OPE 10	CACCAGGTG A
25	OPE 13	CCC GAT TCG G
26	OPE 14	TGC GGC TGA G

3.2.3.5.2. Bulk Segregant analysis of chilli genotypes

Bulked Segregant Analysis was carried out with resistant parent (Ujwala), susceptible parent (Pusa Jwala), resistant bulk (resistant F_2 s), susceptible bulk (susceptible F_2 s) and Anugraha. Resistant bulk and susceptible bulk consisted of 5 each resistant and susceptible F_2 plants that originated from a single F_1 by selfing. The resistant and susceptible F_2 progenies were identified by Phenotyping of F_2 plants after artificial inoculation with bacterial ooze. Twenty two selected primers after primer screening belonging to OPE, OPAH, OPN, OPP, OPS and OPY series were used for bulked segregant analysis. The details of primers used are given in Table 9.

PCR was carried out as per the procedure followed for screening of primers mentioned in section 3.2.3.5.1. The amplified products of five groups of genomic DNA for each primer were run on 1.2 per cent agarose gel using 1X TAE buffer stained with ethidium bromide along with λDNA / *Hind*III +*Eco*RI double digest marker. The documented RAPD profile for 22 selected decamer primers were carefully examined for polymorphism among susceptible parent, resistant parent, resistant bulk, susceptible bulk and near isogenic line of Pusa Jwala. The number of monomorphic and polymorphic bands produced by each primer was recorded and per cent of polymorphism for each primer was calculated as given below:

$$\text{Per cent polymorphism} = \frac{\text{No. of polymorphic bands}}{\text{Total no. of bands}} \times 100$$

The primers which showed polymorphism in resistant and susceptible genotypes were selected. The polymorphic band which was present in resistant genotypes and absent in susceptible genotypes was eluted for developing SCAR maker.

3.2.3.5.3. Co-segregation analysis

Individuals of resistant bulk, susceptible bulk, resistant parent, susceptible parent, and Anugraha were again screened with RAPD primer OPS 1 which gave phenotype specific bands in resistant parents and resistant bulks using the same PCR ingredients and temperature profile. PCR Products were run in 1.2 per cent agarose gel to see the co-segregation of particular bands with respective phenotypes (Plate 12).

3.2.3.5.4. Analysis of amplification profiles

Amplification profiles of resistant parent, resistant bulk, susceptible parent, susceptible bulk and Anugraha were compared with each other and bands of DNA fragments scored manually as (1) or (0) depending upon the presence or absence of particular band respectively. The data was analysed using Numerical Taxonomy System of Multivariate Statistical Programme (NTSYS) software package (Rohlf, 1990). The SIMQUAL programme was used to calculate Jaccard's coefficient, a common estimator of genetic identity. Clustering was done using Sequential Agglomerative Hierarchial Nested Clustering (SAHN) routine and a dendrogram was constructed using Unweighted Pair Group Method of Arithmetic Averages (UPGMA) by Sneath and Sokal (1973) using NTSYS package.

3.2.3.6. MOLECULAR CLONING

3.2.3.6.1. Detection of trait specific markers

The primer OPS 1 gave 1.24 kb polymorphic band in resistant parent and resistant bulk to bacterial wilt resistance. Five RAPD reactions with template DNA from the resistant genotype Ujwala were set up as mentioned in section 3.2.3.5.1. One

negative control reaction was also set with the genomic DNA of susceptible genotype Pusa Jwala. After amplification, the products were resolved on 1.2 per cent agarose gel.

3.2.3.6.2. Gel elution of PCR amplified fragments

The specific DNA band amplified by OPS 1 primer in resistant parent, Ujwala was eluted from 1.2 per cent agarose gel using AxyPrep DNA Gel Extraction Kit (Axygen, Biosciences). Elution was carried out as per the manufacturer's guide lines:

1. DNA fragment of interest was excised from the gel using a sterile, sharp scalpel avoiding much exposure to UV on transilluminator.
2. Gel slice was weighed in a colorless 1.5 ml micro centrifuge tube.
3. 3X gel volume of gel solubilization buffer (w/v) were added.
4. The gel was resuspended in gel solubilization buffer by vortexing. It was heated at 75^0C until the gel was completely dissolved. Intermittent vortexing was given every 2-3 minutes to accelerate gel solubilization.
5. 0.5X gel solubilization buffer volume of binding buffer was added and mixed properly.
6. Once the gel slice was completely dissolved, DNA fragments less than 400 bp was supplemented by adding 1X gel volume of isopropanol and mixed briefly by inversion.
7. Solubilized gel slice was transferred into the spin column that was assembled in the 2 ml collection tube and centrifuged at 12,000 xg for 1 minute.
8. The filtrate was discarded and 500 µl of wash buffer was added to the spin column and centrifuged at 12,000 xg for 30 seconds.
9. The filtrate was discarded and 700 µl of desalting buffer was added.
10. Centrifugation was carried out at 12,000 xg for 30 seconds.
11. As a second wash, 700 µl of desalting buffer was added.

12. Centrifugation was carried out at 12,000 xg for 30 seconds to ensure the complete removal of salt.
13. The filtrate was discarded. The spin column was again placed on collection tube.
14. Column was again centrifuged for 1 minute at 12,000 xg to remove any residual buffer.
15. Spin column was transferred to a fresh 1.5 ml centrifuge tube. The eluent was prewarmed at 65 ^{0}C to improve the elution efficiency.
16. To elute the DNA, 25 µl of eluent was added to the centre of the spin column. It was allowed to stand for 1 minute at room temperature. Then it was centrifuged at 12,000 xg for 1 minute.
17. Eluted DNA fragments were checked on 0.7 per cent (w/v) agarose gel and stored at – 20 ^{0}C.

3.2.3.7. TRANSFORMATION OF *E. coli*

3.2.3.7.1. Preparation of competent cells

Competent cells of *Escherichia coli* DH 5α strain for plasmid transformation were prepared following the protocol of Mandel and Higa (1970).

The media used were LB medium and LBA medium. (Details of media prepared are given in the Appendix IV).

The steps followed for competent cell preparation were as follows:

Day 1:

1. Eighteen hrs old *Escherichia coli* DH 5α strain, single colony was inoculated to 3 ml LB medium in sterile condition and incubated overnight at 37^{0}C on a shaker set at 160 rpm.

Day 2:

1. Three ml overnight grown culture was aseptically transferred to 50 ml LB broth and inoculated for 4 hrs at 37^0C on a shaker set at 160 rpm until OD_{600} reached 0.4-0.5. The growth of culture was monitored at every 30 minutes.

2. The cells were aseptically transferred to a sterile disposable ice cold 50 ml polypropylene tube.
3. The culture was cooled to 0^0C on ice for 10 minutes.
4. The cell suspension was centrifuged at 5000 rpm for 10 minutes at 4^0C.
5. The supernatant obtained was carefully discarded and the pellet obtained was gently resuspended in 10 ml ice cold filter sterilized 0.1 M $CaCl_2$.
6. The tubes were kept on ice for 10 minutes and the cell suspension was centrifuged at 5000 rpm for 10 minutes at 4^0C.
7. The supernatant was decanted and the pellet was resuspended in 2 ml of ice cold filter sterilized 0.1 M $CaCl_2$.

Day 3:

1. Chilled glycerol (400 µl) was added to the cell suspension and mixed well using a sterile micro tip.

2. The competent cells prepared were stored at -70^0C as aliquots of 100 µl in chilled 1.5 ml micro centrifuge tubes.

3.2.3.7.2. Screening of competent cells

Transformation of competent cells with a plasmid having ampicillin resistance (pUC 18) was carried out to check the competence and purity of competent cells. The procedure followed for screening of plasmid is as follows:

1. The competent cells stored at -70^{0}C were thawed over ice for 10 minutes.
2. Plasmid DNA (10 µl) was added to 100 µl competent cells. Negative control was placed simultaneously without adding plasmid.
3. The cells were kept in ice for 40 minutes. Heat shock was given at 42^{0}C for 2 minutes in a dry bath and plunged in ice for 5 minutes.
4. LB medium (250 µl) was added to the cells and incubated at 37^{0}C for 1 hour on a shaker set at 120 rpm.
5. The transformed cells (100 µl) were plated on LBA ampicillin medium and incubated overnight at 37^{0}C in a shaker (100 rpm). The recombinant clones alone can grow on ampicillin plate.

3.2.3.8. CLONING OF ELUTED DNA

The eluted product was cloned in pGEM-T vector (Fig.1) using pGEM-T Easy Vector System supplied by Promega, USA.

3.2.3.8.1. Ligation with pGEM-T vector

The pGEM-T Easy vector and control insert was centrifuged briefly to collect contents at the bottom of the tubes. The pGEM-T Easy vector contained ampicillin resistance and Lac Z region as marker. Ligation reaction was set up in 0.5 ml microfuge tubes as follows:

(Procedure followed as per the manufacturer's protocol).

1. Reaction mixture was prepared as described below:

2X rapid ligation buffer	- 5.0 µl
pGEM-T Easy Vector (50 ng)	- 1.0 µl
PCR product	-1.0 µl
T4 DNA ligase (3 units/ µl)	-1.0 µl
Deionised water	- 2.0 µl
	= 10 µl

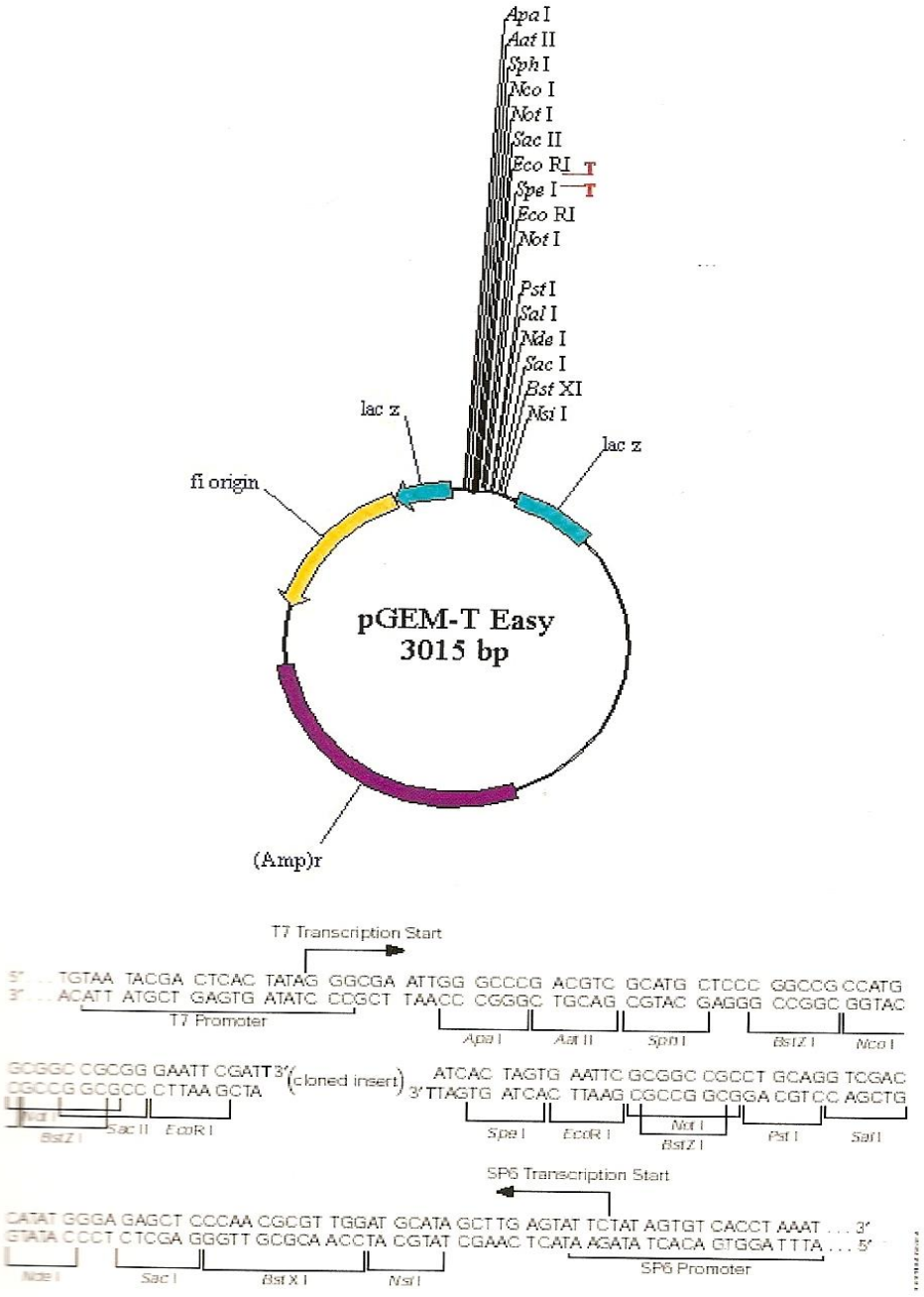


Fig. 1. pGEM-T Easy Vector (Promega) used for cloning of RAPD product.

The *lac*Z region, promoter and multiple cloning sites are shown in the figure. The top strands of the sequence shown correspond to the RNA synthesized by T7 RNA polymerase and bottom strands correspond to the RNA synthesized by SP6 RNA polymerase.

2. The reaction mixture was incubated for one hour at room temperature. Then it was kept at 4^0C overnight. Next day it was taken for transformation in competent cells of *E. coli.*

3.2.3.8.2. Transformation of competent cells with ligated DNA

Materials

1. LB media
2. IPTG-0.5 M dissolved in water
3. Ampicillin-10%
4. X-gal – 10 mg/ml in DMSO

The procedure followed for transformation of competent cells with ligated DNA and blue white screening is as follows:

1. The vial containing competent cells was thawed on ice.
2. The recombinant plasmid was added to the vial containing competent cells, contents mixed gently and kept on ice for 40 minutes.
3. The tube was rapidly taken from ice, heat shock was given at 42^0C exactly for 90 seconds without shaking and placed back on ice for 5 minutes.
4. Under sterile conditions, 250 µl of LB broth was added and the tube was inverted twice to mix the cells and LB broth.
5. The tube was incubated at 37 ^{0}C for one hour with shaking.
6. Aliquots containing 250 µl of the transformed cells were plated and LB/ ampicillin (50 mg/l) plates layered with IPTG (6 µl) and X-gal (60 µl). (Stock: Ampicillin-5 mg/ ml in water, IPTG-200 mg/ ml in water, X-gal–20 mg /ml in DMFO) and incubated overnight at 37 ^{0}C.
7. The recombinant clones were selected based on blue-white screening.

3.2.3.8.3. Isolation of recombinant plasmid DNA

Plasmid DNA was isolated from single white colonies using alkaline mini-prep procedure as given by Birnboim and Doly (1979).

Reagents

Solution I (Resuspension buffer)

Solution II (Lysis buffer)

Solution III (Neutralization buffer)

(Chemical compositions of the solutions are given in Annexure IV)

3.2.3.8.3.1. Procedure for plasmid DNA isolation

1. Cells were harvested from overnight grown recombinant *E. coli* culture containing plasmid DNA by centrifugation at 12,000 rpm for 1 minute at 4^0C.
2. 100 µl of ice cold solution I was added to the bacterial pellet and resuspended.
3. To the above, 200 µl of freshly prepared lysis buffer was added, mixed gently by inverting the tube for five times.
4. 150 µl of ice cold solution III was added to the tubes, mixed well and kept on ice for 5 minutes.
5. The contents were centrifuged at 12,000 rpm for 5 minutes at 4^0C and the pellet was discarded.
6. To the supernatant, 0.6 volume of ice cold isopropanol was added and kept at room temperature for 5 minutes.
7. The contents were centrifuged at 12,000 rpm for 5 minutes at 4^0C and the supernatant was discarded.
8. The pellet was rinsed with 1 ml of 70 per cent (v/v) ethanol and mixed gently.
9. The tube was centrifuged at 12,000 rpm for 10 minutes at 4^0C.
10. The supernatant was discarded and the pellet air dried for 10 minutes.
11. Pellet was finally dissolved in 30 µl TE buffer.
12. Plasmids isolated were checked by electrophoresis and documented.

3.2.3.9. CONFIRMATION OF PRESENCE OF INSERT

3.2.3.9.1. Confirmation of recombinant plasmid DNA using RAPD reaction

PCR was carried out as described in section 3.2.3.5.1, with DNA from plasmid of white colony, blue colony and resistant parent with OPS 1 primer. The template DNA was diluted at 1:10 ratio. The PCR products were analysed on 1.2 per cent agarose gel as described in section 3.2.3.3. The gel was examined for the presence of DNA band of size 1.24 kb in plasmid DNA from white colony.

3.2.3.10. MAINTENCE OF CLONES

3.2.3.10.1. Preparation of pure culture of recombinant bacteria

In laminar air flow, single white colony from the transformed plate was taken by using flame sterilized bacterial loop. This was streaked on LBA media containing the antibiotic ampicillin. Ampicillin resistance was used as marker. The plate was incubated overnight at 37°C for multiplication of the bacteria. Then it was stored at 4°C for further use.

3.2.3.10.2. Preparation of stabs

Pure culture of recombinant *E. coli* was raised in culture tubes. For this the LBA medium containing antibiotic ampicillin was melted and poured into storage vial, aseptically and allowed to solidify. Single colony of recombinant bacterial colonies was carefully lifted with a sterile bacterial loop. The loop loaded with bacteria was plunged into the solid medium and the cultures were incubated overnight at 37°C. The stabs showing good growth of bacteria were further stored in refrigerator at 4°C till further use.

3.2.3.10.3. Glycerol culture

In a laminar flow, recombinant colony from the transformed plate was taken using flame-sterilized loop. This was plunged into LB medium containing the antibiotic, ampicillin (50 mg/l). It was incubated at 37^0C in a shaker at 120 rpm overnight. The next day aliquots (800 µl) of cell culture were added to 100 per cent glycerol (200 µl) aseptically and stored at -20^0C.

3.2.3.11. SEQUENCING OF DNA CLONES

The stabs of recombinant clones were sent to Bangalore Genei (www.bangaloregenei.com) for sequencing. Sequencing was done with SP6 primer to obtain 5'-3' sequence information of the insert from the reverse region, using automated sequencer (ABI – 31100 Genetic Analyzer, USA). To obtained full length sequence information, primer walking of DNA clone was also carried out.

3.2.3.12. THEORETICAL ANALYSIS OF SEQUENCE

The sequence information obtained from the Bangalore Genei was named as '*Chilli seq 1*' (Fig. 3.) and further analyzed for its characterization.

3.2.3.12.1. Vector screening

The sequence obtained was subjected to vector screening to remove vector regions from the clones. Vector screening was done by using VecScreen tool available in NCBI (Fig. 4.). The sequences obtained after automated sequencing were subjected to vector screening using the NCBI to remove vector regions from the clones and the remaining sequence named as *'Chilli seq 2'*(Fig. 5.).

3.2.3.12.2. Nucleic acid sequence analysis

The nucleotide sequence of *Chilli seq 2* was compared with published sequence in public database using BLAST tool offered by NCBI. Homology search was carried out using Basic Local Alignment Search Tool (BLAST).

The following BLAST programes were used:

1. Nucleotide- nucleotide BLAST (blastn)

2. Protein- protein BLAST (blastp)

The BLAST programme blastn and blastp was obtained from (http:// www.ncbi.nm.nih.gov/blast/; (Altschul et al., 1997). To find the open reading frame of the insert nucleotide sequence, the programme ORF finder of NCBI was used (www.ncbi.nlm.gov/gorf/gorf). Nucleotide composition of the given sequence was determined by nucleotide statistics (NASTATS) tool offered by Biology Workbench (http://seqtool.sdsc.edu/). Restriction sites available in the DNA fragment for the restriction enzymes were detected by restriction site analysis (TACG) offered by Biology Workbench.

3.2.3.12.3. Amino acid analysis

Physical and chemical properties of the given protein from the deduced amino acid were determined by amino acid statistics (AASTATS) tool offered by Biology Workbench. The secondary structure prediction of the amino acid sequences was done by using SOPMA tool. Hydrophobicity analysis of the sequences was done by Kyte and Doolittle hydropathy plots (Kyte and Doolittle, 1982).

3.2.4. MOLECULAR CHARACTERIZATION OF CHILLI GENOTYPES BY SCAR MARKER

3.2.4.1. SCAR primer designing:

For designing longer primers of 24 bp and 23bp length the sequence of cloned RAPD fragment OPS 1_{1240} was used (Fig. 12.).

The following criteria were considered during SCAR primer designing:

1. Each primer contained the original 10 bases of RAPD primer plus the next 14 internal bases from the end.

2. The forward and reverse primers were selected from end sequences in such a way that:

    a) The end sequences selected should have GC content 40-50 per cent.
    b) Melting temperature (Tm = 4 GC + 2 AT) ranged between 57^0C to 65^0C.
    c) The distance between the primers ranged from 200 to 400 base pairs.
    d) It is preferable to have GC content at 3' end.
    e) There should not be any complementarity between forward and reverse primers.
    f) Repeats of single base should not appear within the primer sequence.
    g) The distance between forward and reverse primer should be greater than 200 bp.
    h) Each primer should be 24 to 27 bp long.
    i) Primer sequences had no palindromic sequences or repetitive sequences.
    j) For designing primers, the sequence of the forward primer was taken as such and for the reverse primer, the reverse complementary sequence was taken.

Based on above information, nucleotide sequence of forward and reverse primers was designed. The forward and reverse primers were selected from both two ends of RAPD fragment regions.

3.2.4.3. PCR analysis with SCAR primer

A pair of 24 and 23 base pairs oligonucleotide primers synthesized based on the end sequences of RAPD clone OPS 1_{1240} were used as SCAR primers for amplifying DNA from of resistant parent (Ujwala), susceptible parent (Pusa Jwala), resistant bulk, susceptible bulk and resistant progenies (Plate 18). PCR was carried out with SCAR specific primers in Eppendorf Master Cycler (Eppendorf, USA).

Composition of the reaction mixture for PCR analysis was the following:-

a) Genomic DNA (25 ng) - 1.0 µl
b) 10X Taq assay buffer - 2.5 µl
c) dNTP mix (10 mM) - 1.0 µl
d) Forward primer (8 pM) -1.0 µl
e) Reverse primer (8 pM) -1.0 µl
f) Taq DNA polymerase (0.3 U) -2.0 µl
g) Autoclaved distilled water -16.5 µl
= 25.0 µl

The total volume for one PCR reaction was 25.0 µl. The reaction was set in a 200 µl microfuge tube chilled over ice flakes. A momentary spin was given for the reaction and was set in thermal cycler for polymerase chain amplification under suitable programme. A positive control was set up using standard reference for amplification of SCAR primer in resistant parent and resistant bulk. A negative control was also kept without template DNA.

The following thermal cyclers were used to amplify the template DNA with SCAR primer:

Step 1: 94 ^{0}C for 2 min - initial denaturation
Step 2: 94 ^{0}C for 45 sec - denaturation
Step 3: 65 ^{0}C for 1 min – annealing
Step 4: 72 ^{0}C for 2 min - extension
Step 5: 72 ^{0}C for 10 min - final extension
Step 6: 4 ^{0}C for infinity to hold the sample

30 cycles

The PCR product was run on 1.5% agarose and documented and interpreted.

Results

4. RESULTS

The study on "Molecular characterization of chilli (*Capsicum annuum* L.) genotypes for tagging bacterial wilt resistance gene" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period 2006-2008. The results of different experiments are described in this chapter.

4.1. Development of segregating generation

Crosses were made using the resistant variety Ujwala as the ovule parent and susceptible variety Pusa Jwala as the pollen parent and F_1 plants were generated (Plate 2c). Fifty F_2 plants (Plate 2d) were raised from a single F_1 plant by selfing and they were used as segregating population for tagging the bacterial wilt resistance gene.

4.2. PHENOTYPING OF GENOTYPES FOR BACTERIAL WILT RESISTANCE

4.2.1. Standardization of artificial inoculation technique

A pot culture study was carried out to standardize artificial inoculation techniques in chilli for bacterial wilt incidence using the susceptible variety Pusa Jwala. Artificial inoculation was done 30 days after sowing, with fresh bacterial ooze, when the seedlings were at 5-6 leaf stage. At this stage they were transplanted to small polythene bags. Artificial inoculation was done by 4 different methods *viz*., root dipping, stem-puncturing, soil drenching and soil drenching with wounding, and plants were scored for bacterial wilt incidence. Plants were maintained in open condition (Plate 3). The susceptible genotype showed complete wilting within 5 days of inoculation (Plate 3b).

Among the 4 methods evaluated, maximum wilt incidence was observed in soil drenching with wounding method (90%), followed by soil drenching method (80%) and then root dipping method (80%). The lowest wilt incidence (70%) was

a. Resistant parent (Ujwala)

X

b. Susceptible parent (Pusa Jwala)

c. F_1 Hybrid of Ujwala x Pusa Jwala

d. F_2 Progeny of Ujwala x Pusa Jwala

Plate 2. Development of F_1 and F_2 Progeny

observed in stem-puncturing method (Table 4). The wilted plants were subjected to ooze test and the presence of causal organism, *Ralstonia solanacearum* was confirmed (Plate 3c). The artificial inoculation method, soil drenching with wounding was selected for phenotyping of genotypes for bacterial wilt resistance.

4.2.2. Phenotyping of genotypes for bacterial wilt resistance

Thirty F_2 progeny along with the resistant parent Ujwala, susceptible parent Pusa Jwala, F_1 of cross Ujwala x Pusa Jwala and Anugraha were screened by artificial inoculation technique. Artificial inoculation was given by soil drenching with wounding method. The wilt symptoms were observed 5 days after inoculation under open condition. The symptom started as leaf drooping followed by complete wilting, and death of the plant occurred within 10 days (Plate 3b). The genotypes were classified into four different categories according to Mew and Ho (1976) system.

Among the five groups evaluated, the lowest wilt incidence was observed in Ujwala (10%) followed by the genotype Anugraha (20%), both of which were rated as resistant (R). Seventy per cent wilt incidence was found in F_2 progeny and 80 per cent wilt incidence was found in F_1 plants, both of which were rated as susceptible (S). Maximum wilt incidence (85%) was found in the genotype Pusa Jwala and it was categorized as susceptible (S). The wilted plants showed positive response to bacterial ooze test. The reaction of genotypes to bacterial wilt incidence is given in (Table 5).

4.3. MOLECULAR CHARACTERISATION OF CHILLI GENOTYPES WITH RAPD MARKER

Molecular characterization of selected chilli genotypes and segregating F_2 population for tagging bacterial wilt resistance gene was done with RAPD marker (Plate 4).

a. Phenotyping of genotypes for bacterial wilt incidence

b. Complete wilting of chilli plant

c. Bacterial ooze test

Plate 3. Symptom of bacterial wilt and its confirmation

Table 4. Standardization of artificial inoculation techniques in Pusa Jwala chilli genotype by four methods

Sl. No.	Methods	No. of plants artificially inoculated	Wilting percentage
1.	Stem-puncturing	10	70
2.	Root dipping	10	80
3.	Soil drenching	10	80
4.	Soil drenching with wounding	10	90

Table 5. Phenotyping of genotypes for bacterial wilt incidence

Sl. No.	Genotype/generation	No. of plants artificially inoculated	Per cent survival (%)	Disease reaction
1.	Ujwala	20	90	R
2.	Pusa Jwala	20	15	S
3.	Anugraha	20	80	R
4.	F_1 Hybrid	20	20	S
5.	F_2 Plants	30	30	S

Disease Rating	Per cent survival
R (Resistant)	80 or above
MR (Moderately resistant)	60 – 80
MS (Moderately susceptible)	40 – 60
S (susceptible)	less than 40

a. Resistant variety (Ujwala)

b. Susceptible variety (Pusa Jwala)

c. F_2 progeny

d. Anugraha

Plate 4. Genotypes and generation used for molecular characterization of chilli genotypes

4.3.1. Standardization of genomic DNA isolation

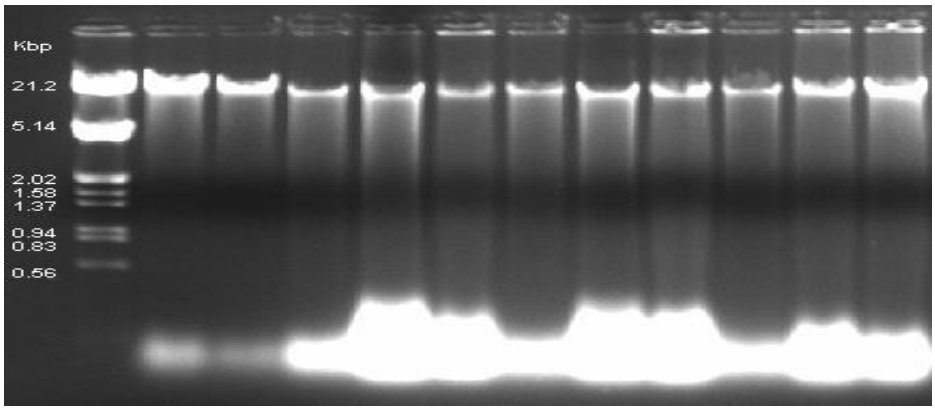
The protocols suggested by Doyle and Doyle (1987) and Rogers and Bendich (1994) were compared for the extraction of genomic DNA from chilli. The quality of DNA isolated using the aforesaid protocols was assessed using agarose gel electrophoresis. Good quality genomic DNA was obtained using Rogers and Bendich (1994) protocol (Plate 5a). The DNA isolated by this method appeared as clear and distinct band in the agarose gel. The DNA samples isolated using Doyle and Doyle (1987) method showed less discrete bands with more RNA contamination Plate 5b). So the method Rogers and Bendich (1994) was selected for isolation of genomic DNA. The DNA isolated by Rogers and Bendich (1994) methods was purified by RNAse treatment (Plate 5c).

4.3.2. Isolation and quantification of genomic DNA in chilli genotypes

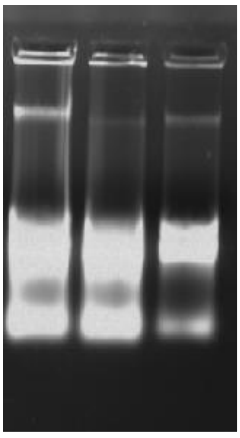
The quality and quantity of DNA thus isolated was analyzed using both electrophoretic and spectrophotometric method (NanoDrop®ND-1000 spectrophotometer). In all cases intact clear narrow band indicated non-degraded DNA was obtained. The ratio of absorbance ranged between 1.8-1.9 which indicated that quality of DNA was good and presented as (Table 6). The DNA, thus isolated, after appropriate dilutions were used as templates for RAPD and SCAR analysis.

4.3.3. RAPD (Random Amplified Polymorphic DNA) analysis

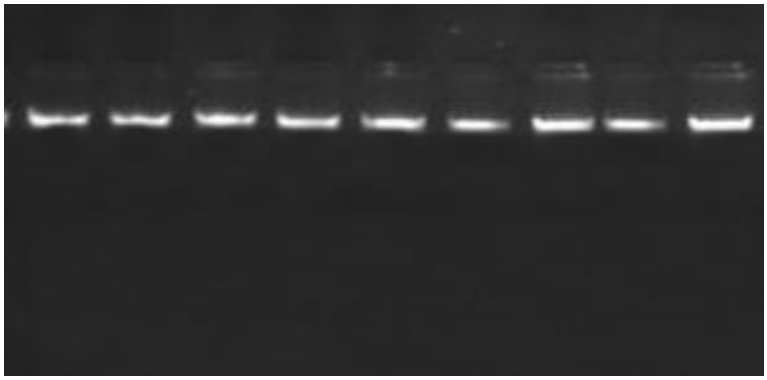
After isolation of good quality genomic DNA, the chilli genotypes were subjected to RAPD analysis. The different experiments carried out under this include screening of random primers, bulked segregant analysis and co segregation of chilli genotypes using selected primers and finally the analysis of results using NTSYS pc. (ver 2.1).



a. DNA isolated by Rogers and Bendich method



b. DNA isolated by Doyle and Doyle method



c. DNA isolated by Rogers and Bendich method after RNAse treatment

Plate 5. DNA isolated by different methods and purification

Table 6. Quality and quantity of DNA isolated from chilli genotypes as determined by NanoDrop method

Genotype/generation	Absorbance			Quantity (µg/g)	Quality
	A_{260}	A_{280}	$A_{260/280}$		
Ujwala	0.328	0.180	1.84	246.00	Good
Pusa Jwala	0.292	0.163	1.82	219.00	Good
Anugraha	0.312	0.169	1.90	234.00	Good
Hybrid (F_1)	0.287	0.157	1.80	215.25	Good
Resistant F_2 (R1)	0.296	0.164	1.81	222.00	Good
Resistant F_2 (R2)	0.340	0.187	1.84	255.00	Good
Resistant F_2 (R3)	0.309	0.171	1.81	231.7	Good
Resistant F_2 (R4)	0.313	0.172	1.82	234.8	Good
Resistant F_2 (R5)	0.318	0.174	1.83	238.00	Good
Susceptible F_2 (S1)	0.313	0.173	1.85	234.75	Good
Susceptible F_2 (S2)	0.301	0.164	1.81	225.75	Good
Susceptible F_2 (S3)	0.326	0.176	1.83	244.50	Good
Susceptible F_2 (S4)	0.310	0.168	1.84	232.50	Good
Susceptible F_2 (S5)	0.318	0.175	1.82	238.5	Good

4.3.3.1. Screening of primers for RAPD analysis

The amplification pattern produced in resistant genotype Ujwala and susceptible genotype Pusa Jwala by forty seven primers belonging to six Operon series viz., OPE, OPAH, OPN, OPP, OPS and OPY (Table 3a and 3b.) are presented in Table 7 and Table 8. The selected 22 random primers were OPE 3, OPE 5, OPE 6, OPAH 5, OPAH 19, OPN 3, OPN 7, OPP 3, OPP 4, OPP 5, OPP 7, OPS 1, OPS 2, OPS 3, OPS 5, OPS 9, OPS 10, OPS 18, OPY 1, OPY 3, OPY 5 and OPY 7.

a) Screening of OPE series primers

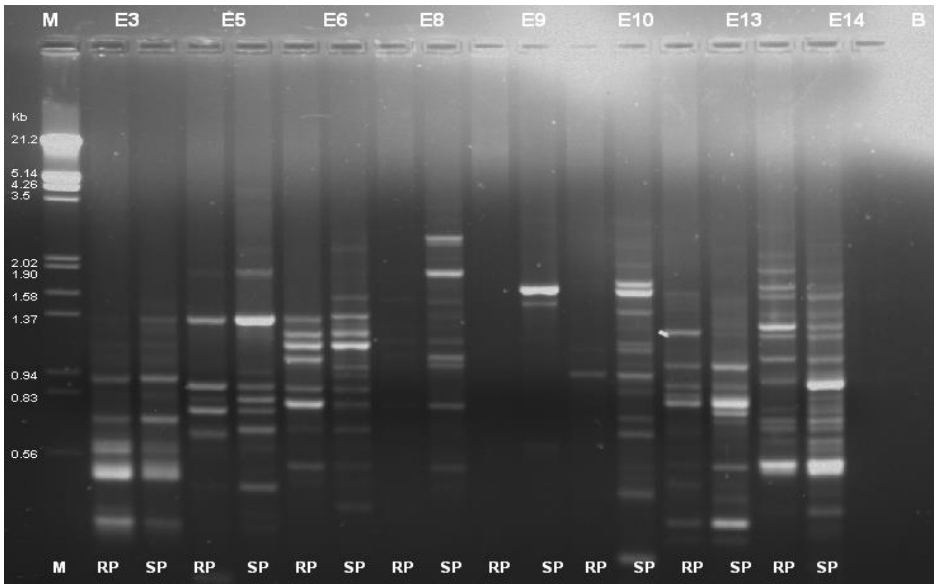
Eight primers in this series were screened. The amplification pattern obtained for different primers in OPE series are presented in the Table 7 and Plate 6a. The number of bands ranged between zero and sixteen and the molecular weight of bands varied from 3.37 kb to 0.13 kb. All primers of this series gave good amplification with more number of discrete bands. The primers OPE 3, 5 and 6 were selected for further analysis. Poor amplification was obtained for the primer OPE 9 and no polymorphism in OPE 13.

b) Screening of OPAH series primers

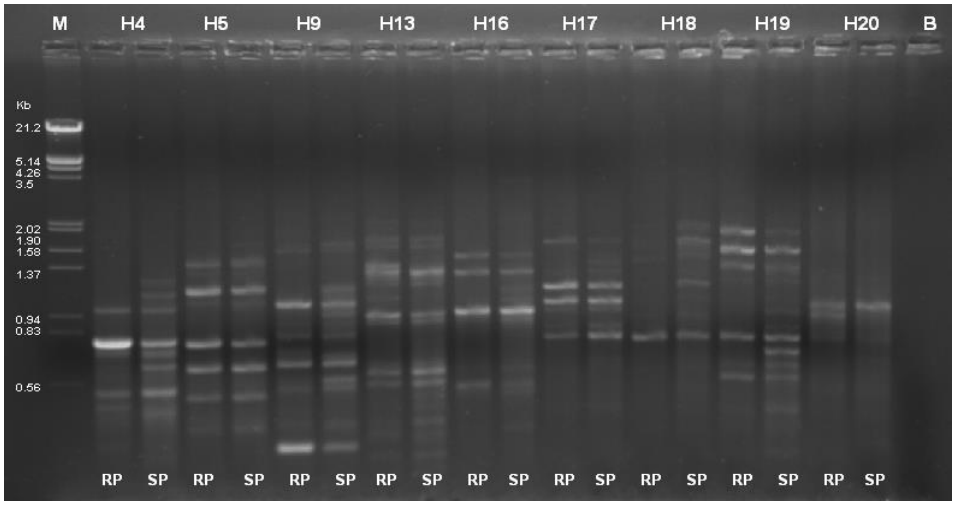
Results of screening of the nine primers in OPAH series are given in the Table 7 and Plate 6b. The number of amplicons obtained using the primers in this series ranged between 1 and 11 and the molecular weight of bands varied from 2.84 kb to 0.164 kb. All the primers gave good amplification pattern. For further study, OPAH 5 and OPAH 19 were selected due to distinct banding pattern with good quality amplification and reproducibility obtained in these primers.

c) **Screening of OPN series primers**

Six primers under this series were screened. The amplification pattern obtained for different primers in OPE series are given in the (Table 7 and Plate 7a). The number of amplicons obtained using the primers in this series ranged between 3



(a) Amplification with OPE series primers



(b) Amplification with OPAH series primers

M: Marker Lambda DNA / *Eco*RI / *Hind* III
RP: Resistant parent, SP: Susceptible parent, B: Blank
OPE 3, OPE 5, OPE 6, OPE 8, OPE 9, OPE 10, OPE 13, OPE 14
OPAH 4, OPAH 5, OPAH 9, OPAH 13, OPAH 16, OPAH 17
OPAH 18, OPAH 19, OPAH 20

Plate 6. Screening of resistant parent Ujwala and susceptible parent Pusa Jwala with OPE and OPAH series primers

and 15 and the molecular weight of bands varied from 3.1 kb to 0.23 kb. All primers of this series gave good amplification with more number of discrete bands and OPN 3 and OPN 7 were selected for further analysis.

d) Screening of OPP series primers

Six primers in this series were screened. The amplification pattern obtained for different primers in OPP series are given in the (Table 7 and Plate 7b). The number of bands ranged between zero and fifteen and the molecular weight of bands varied from 3.25 kb to 0.24 kb. OPP 3, 4, 5 and OPP 7 gave good amplification with more number of discrete bands and were selected for further analysis.

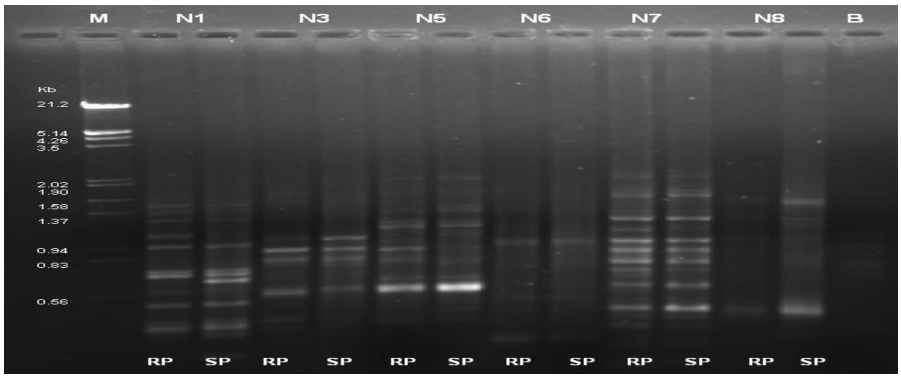
e) Screening of OPY series primers

Results of screening with six primers in OPY series are given in the Table 8 and Plate 7c. The number of amplicons obtained using the primers in this series ranged between 4 and 10 and the molecular weight of bands varied from 1.53 kb to 0.25 kb. All the primers of the OPY series namely OPY 1, 3, 5, 7, 9 and OPS 11 were selected due to distinct banding pattern with good quality amplification and reproducibility, obtained in these primers.

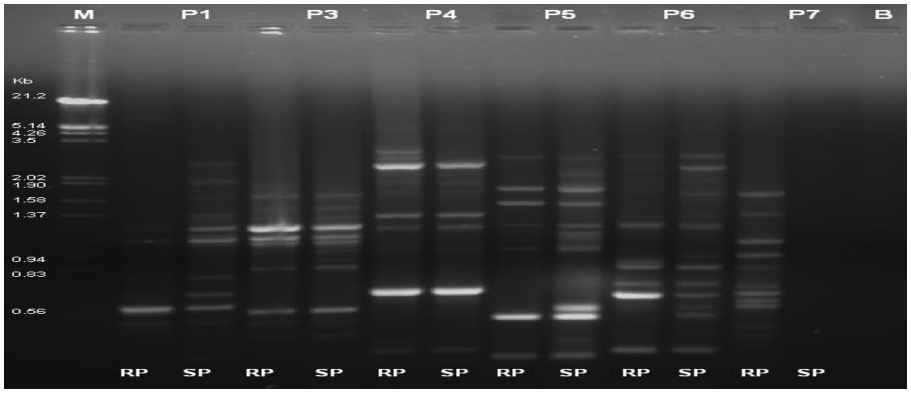
f) Screening of OPS series primers

Results of screening of the twelve primers in OPS series are presented in the Table 8 and Plate 8. Number of bands produced by the primers varied from 4 to 8, and the molecular weight of bands varied from 2.7 kb to 0.173 kb. All the primers gave good amplification pattern. For further study, OPS 1, 2, 3, 5, 9, 10, 11, 18 and OPS 20 were selected due to distinct banding pattern with good quality amplification and reproducibility obtained in these primers.

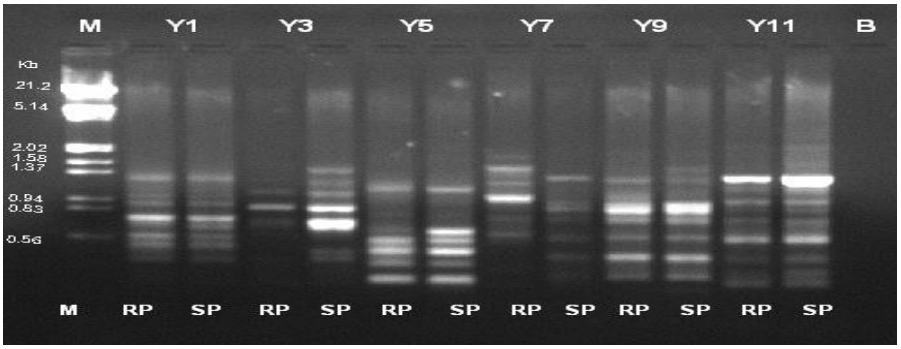
Out of 47 primers tested, 22 primers were selected for bulk Segregant analysis. They are OPE 3, OPE 5, OPE 6, OPAH 5, OPAH 19, OPN 3, OPN 7, OPP 3, OPP 4, OPP 5, OPP 7, OPS 1, OPS 2, OPS 3, OPS 5, OPS 9, OPS 10, OPS 18, OPY 1, OPY 3, OPY 5 and OPY 7.



(a) Amplification with OPN series primers



(b) Amplification with OPP series primers



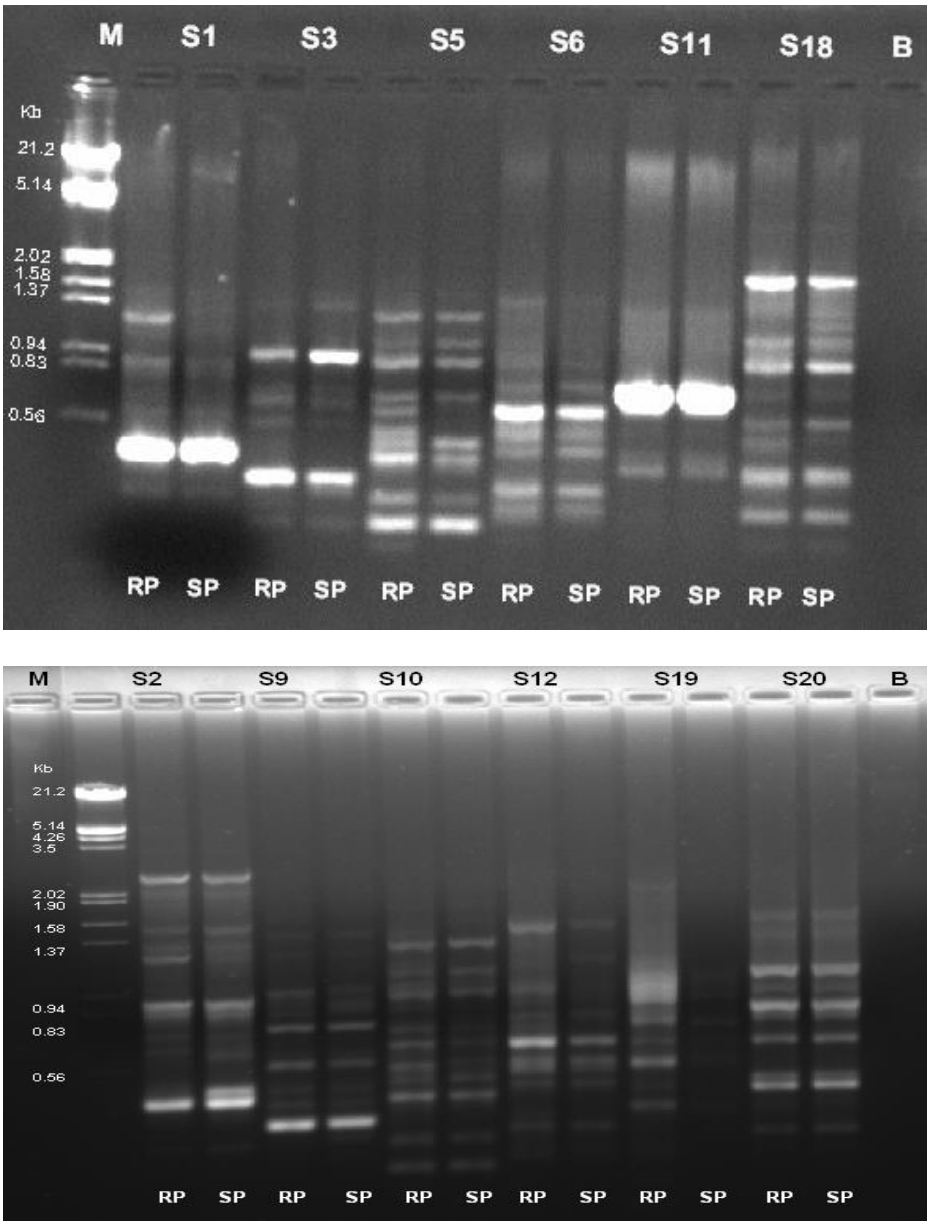
(c) Amplification with primers in OPY series

M: Marker Lambda DNA / *Eco*RI / *Hind* III

RP: Resistant Parent, SP: Susceptible Parent, B: Blank

OPN 1, OPN 3, OPN 5, OPN 6, OPN 7, OPN 8, OPP 1, OPP 3, OPP 4, OPP 5, OPP 6, OPP 7, OPY 1, OPY 3, OPY 5, OPY 7, OPY 9, OPY 11

Plate 7. Screening of resistant parent Ujwala and Susceptible parent Pusa Jwala with OPN, OPP and OPY series primers



M: Marker Lambda DNA / *Eco*RI / *Hind* III
RP: Resistant parent, SP: Susceptible parent, B: Blank
OPS 1, OPS 2, OPS 3, OPS 5, OPS 6, OPS 9, OPS 10, OPS 11, OPS 12, OPS 18, OPS 19, OPS 20 primers

Plate 8. Screening of resistant parent Ujwala and susceptible parent Pusa Jwala with OPS series primers

Table 7. Amplification patterns of chilli genotypes with OPE, OPAH, OPN and OPP series primers

Sl. No	Primer	No. of bands (RP)	No. of bands (SP)	Amplification pattern
1	OPE 3	9	10	Good
2	OPE 5	7	10	Good
3	OPE 6	9	13	Good
4	OPE 8	5	10	Good
5	OPE 9	0	3	Poor
6	OPE 10	4	15	Good
7	OPE 13	11	11	Good
8	OPE 14	15	16	Good
9	OPAH 4	5	8	Good
10	OPAH 5	6	7	Good
11	OPAH 9	7	11	Good
12	OPAH 13	11	9	Good
13	OPAH 16	4	7	Good
14	OPAH 17	5	8	Good
15	OPAH 18	1	5	Average
16	OPAH 19	7	10	Good
17	OPAH 20	3	1	Average
18	OPN 1	11	13	Good
19	OPN 3	7	8	Good
20	OPN 5	12	13	Good
21	OPN 6	5	5	Good
22	OPN 7	15	14	Good
23	OPN 8	3	4	Average
24	OPP 1	4	15	Good
25	OPP 3	11	13	Good
26	OPP 4	11	9	Good
27	OPP 5	7	12	Good
28	OPP 6	11	13	Good
29	OPP 7	11	0	Good

RP: Resistant parent, SP: Susceptible parent

Table 8. Amplification patterns of chilli genotypes with OPS and OPY series primers

Sl. No	Primer code	No. of bands (RP)	No. of bands (SP)	Qualities of Amplification pattern
1	OPS 1	8	7	Good
2	OPS 2	10	11	Good
3	OPS 3	8	9	Good
4	OPS 5	11	10	Good
5	OPS 6	6	10	Good
6	OPS 9	10	10	Good
7	OPS 10	12	11	Good
8	OPS 11	4	4	Average
9	OPS 12	10	8	Good
10	OPS 18	18	10	Good
11	OPS 19	7	5	Good
12	OPS 20	11	10	Good
13	OPY 1	7	8	Good
14	OPY 3	4	7	Good
15	OPY 5	7	8	Good
16	OPY 7	7	6	Good
17	OPY 9	7	8	Good
18	OPY 11	9	10	Good

RP: Resistant parent, SP: Susceptible parent

4.3.3.2. Bulk Segregant analysis (BSA) using selected RAPD primers

Out of the twenty two selected primer screened for detection of polymorphism among resistant parent, resistant bulk, susceptible parent, and susceptible bulk and Anugraha. Only OPS1 distinguished the individuals genetically with respect to bacterial wilt resistance. The particular polymorphic band present in resistant genotypes was eluted for cloning. The numbers of bands produced in each genotype by the 22 selected primers are presented in next sheet.

OPE 3

Five amplicons were observed on the agarose gel for the DNA amplified with the primer OPE 3 (Plate 9a). Only one band was polymorphic and the rest were monomorphic among the genotypes. This primer showed 20 per cent polymorphism. The molecular weight of the products ranged between 0.83 kb and 0.28 kb. Almost all bands were present in all resistant and susceptible genotypes. This primer was unable to differentiate between the resistant and susceptible genotypes.

OPE 5

Ten amplicons were observed on the agarose gel for the DNA amplified with the primer OPE 5 (Plate 9b). Among them five bands were polymorphic and the rest were monomorphic among the genotypes. This primer showed 50 per cent polymorphism. The molecular weight of the products ranged between 5.53 kb and 0.33 kb. One band of sizes 0.57 kb was absent in susceptible bulk. Almost all other bands were present in all resistant and susceptible genotypes. The amplicons obtained with this primer were almost monomorphic for the genotypes selected. This primer was unable to differentiate between the resistant and susceptible genotypes.

OPE 6

Amplification with this primer generated eight amplicons of which two were polymorphic. The molecular weight of the amplicon ranged between 3.44 kb and

0.55 kb (Plate 9c). Twenty five per cent polymorphism was obtained with this primer. Unique bands shared by resistant/susceptible genotypes were not obtained. The maximum number of nine bands was observed for the resistant bulk, susceptible parent and Anugraha. This primer was unable to differentiate between the resistant and susceptible genotypes.

OPAH 5

Six amplicons were observed on the agarose gel for the DNA amplified with the primer OPY 7 (Plate 9d). Two bands were monomorphic and the rest were polymorphic among the genotypes. This primer showed 33 per cent polymorphism. The molecular weight of the products ranged between 1.38 kb and 0.16 kb. Almost all other bands were present in all resistant and susceptible genotypes. This primer was unable to differentiate between the resistant and susceptible genotypes.

OPAH 19

Amplification with this primer generated seven amplicons of which two were polymorphic. The molecular weight of the amplicons ranged between 2.09 kb and 0.38 kb (Plate 9e). Twenty nine per cent polymorphism was obtained with this primer. The maximum number of nine bands was observed for the resistant bulk, susceptible parent, susceptible bulk and Anugraha. Unique bands shared by resistant/susceptible genotypes were not obtained.

OPN 3

A total of five amplicons were obtained after DNA amplification with the primer OPN 3. The pattern of amplification is shown in (Plate 9f). The amplicons obtained with this primer were almost monomorphic for the genotypes selected. The molecular weight of the bands varied from 1.65 kb to 0.22 kb. No polymorphism detected by this primer. Unique bands shared by resistant/susceptible genotypes were not obtained.

OPN 7

Amplification with this primer generated twelve amplicons of which four were polymorphic and exhibited about 33 percent polymorphism. The molecular weight of the amplicons ranged between 2.31 kb and 0.09 kb (Plate 9g). Unique bands shared by resistant/susceptible genotypes were not obtained.

OPP 3

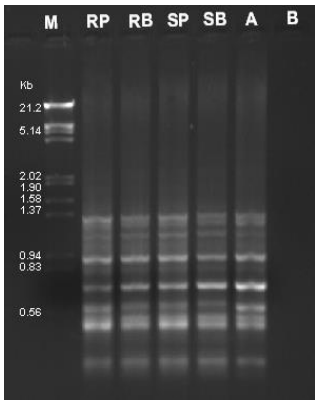
Eight amplicons were observed on the agarose gel for the DNA amplified with the primer OPP 3 (Plate 9h). Among them two band was polymorphic and the rest were monomorphic among the genotypes. This primer showed 25 per cent polymorphism. The molecular weight of the products ranged between 4.05 kb and 0.29 kb. Two bands of sizes 0.43 kb and 0.29 kb were absent in Pusa Jwala (susceptible). Almost all other bands were present in all resistant and susceptible genotypes. The amplicons obtained with this primer were almost monomorphic for the genotypes selected. This primer was unable to differentiate between the resistant and susceptible genotypes.

OPP 4

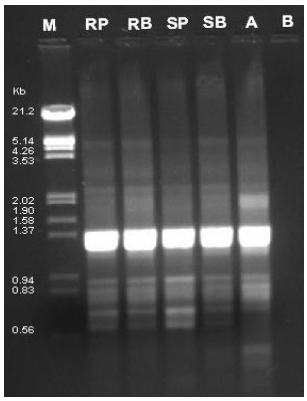
A total of six amplicons were obtained after DNA amplification with the primer OPP 4. The pattern of amplification is shown in (Plate 9i). Two bands were polymorphic and the rest were monomorphic among the genotypes. This primer showed 33 per cent polymorphism. The molecular weight of the products ranged between 3.16 kb and 0.21 kb. Almost all other bands were present in all resistant and susceptible genotypes. This primer was unable to differentiate between the resistant and susceptible genotypes.

OPP 5

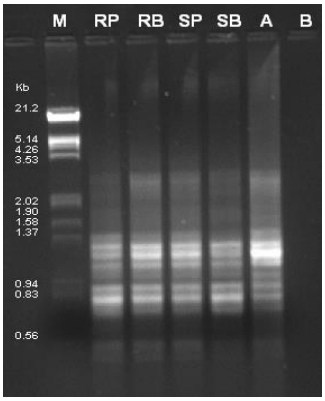
Six amplicons were observed on the agarose gel for the DNA amplified with the primer OPP 3 (Plate 10a). Three bands were polymorphic and the rest were monomorphic among the genotypes. This primer showed 50 per cent polymorphism.



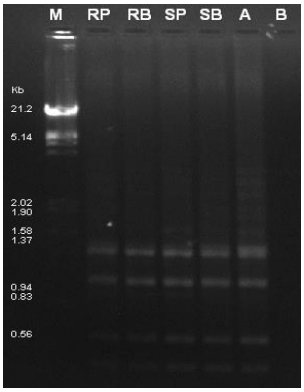
(a) OPE 3



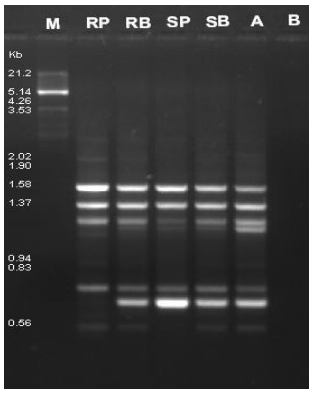
(b) OPE 5



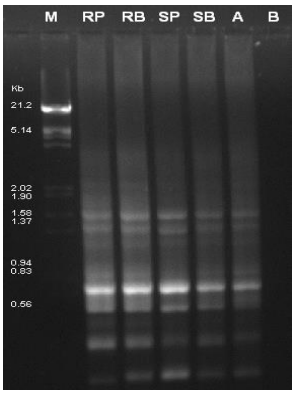
(c) OPE 6



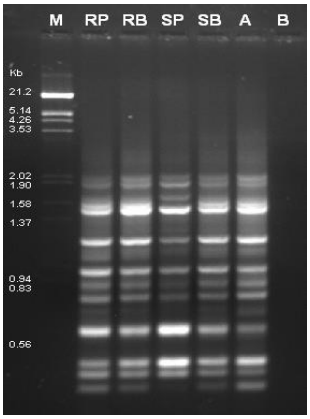
(d) OPAH 5



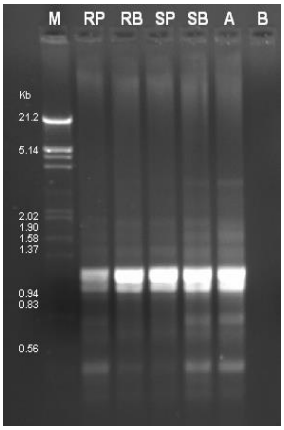
(e) OPAH 19



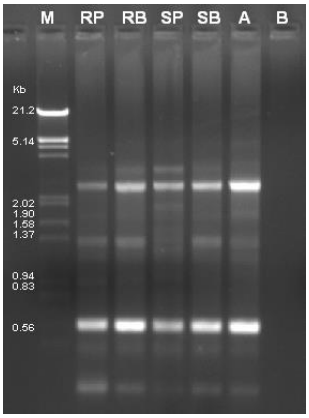
(f) OPN 3



(g) OPN 7



(h) OPP 3



(i) OPP 4

M: λDNA / *Hind*III+*Eco*RI, RP: Resistant Parent, RB: Resistant Bulk
SP: Susceptible Parent, SB: Susceptible Bulk, A: Anugraha, B: Blank

Plate 9. Bulk segregant analysis with selected primers from OPE, OPAH, OPN and OPP series

The molecular weight of the products ranged between 3.2 kb and 0.12 kb. This primer was able to differentiate between the resistant and susceptible genotypes.

OPP 7

Twelve amplicons were observed on the agarose gel for the DNA amplified with the primer OPP 7 (Plate 10b). Six bands were polymorphic and the rest were monomorphic among the genotypes. Poor amplification obtained in susceptible parent. This primer showed 50 per cent polymorphism. The molecular weight of the products ranged between 3.39 kb and 0.29 kb. This primer was unable to differentiate between the resistant and susceptible genotypes.

OPS 1

Nine amplified products were produced out of which 6 bands were polymorphic. The PCR analysis with this primer showed maximum polymorphism (66.67%). The number of bands ranged from 3 and 9. The RAPD profiles generated by OPS 1 are shown in the (Plate 10c). One distinct band of molecular weight 1.24 kb was seen in the resistant parent and resistant bulk. It was assessed that the band was considered unique to resistance to bacterial wilt. The size of the amplicons varied from 1.58 kb to 0.2 kb.

OPS 2

RAPD profile with the primer OPS 2 displayed 13 amplification products and two of them were polymorphic. The size of the amplicons varied from 2.68 kb to 0.13 kb. No amplication was obtained in susceptible bulk (Plate 10d). This primer exhibited 15 per cent polymorphism. The band with 0.31 kb size was absent in susceptible genotype Pusa Jwala while it was present in Anugraha. Unique bands shared by resistant/susceptible genotypes were not obtained.

OPS 3

Ten amplicons were observed on the agarose gel for the DNA amplified with the primer OPS 3 (Plate 10e). Five bands were polymorphic and the rest were

monomorphic among the genotypes. The molecular weight of the products ranged between 1.14 kb and 0.06 kb. Almost all bands were present in all resistant and susceptible genotypes. This primer was unable to differentiate between the resistant and susceptible genotypes.

OPS 5

Using OPS 5, twelve amplicons were obtained and three of them were polymorphic (Plate 10f). The molecular weight of these markers ranged between 1.86 kb and 0.09 kb. The band of size 0.31 kb was absent in susceptible parent and susceptible bulk genotypes and it was present in resistant genotypes (resistant parent, resistant bulk and Anugraha). This primer exhibited polymorphism of 25 per cent among the different genotypes. Unique bands shared by resistant/susceptible genotypes were not obtained.

OPS 9

OPS 9 primer exhibited 20 percent polymorphism. Five amplified products were produced out of which 1 band was polymorphic. The number of bands ranged from 4 and 5. The molecular weight of these markers ranged between 2 kb and 0.12 kb. The RAPD profiles generated by OPS 9 are shown in the (Plate 10g). This primer was unable to differentiate between the resistant and susceptible genotypes.

OPS 10

Eight amplicons were observed on the agarose gel for the DNA amplified with the primer OPS 10 (Plate 10h). Seven bands were monomorphic and the only one was polymorphic among the genotypes. This primer showed 12.5 per cent polymorphism. The molecular weight of the products ranged between 1.02 kb and 0.14 kb. One bands of sizes 1.02 kb was absent in susceptible parent. Almost all other bands were present in all resistant and susceptible genotypes. This primer was unable to differentiate between the resistant and susceptible genotypes.

OPS 18

Using OPS 18, thirteen amplicons were obtained and seven of them were polymorphic (Plate 10i). The molecular weight of these markers ranged between 2.25 kb and 0.09 kb. This primer exhibited polymorphism of 54 per cent among the different genotypes. Unique bands shared by resistant/susceptible genotypes were not obtained.

OPY 1

Ten amplicons were obtained with OPY 1 primer on BSA assay and five of them were polymorphic (Plate 11a). This primer showed 50 per cent polymorphism. The size of the bands ranged from 1.27 kb to 0.29 kb. Five bands of sizes 1.27 kb, 0.88 kb, 0.73 kb, 0.56 kb and 0.49 kb were common to all genotypes. This was unique bands that could differentiate the resistant and susceptible genotypes.

OPY 3

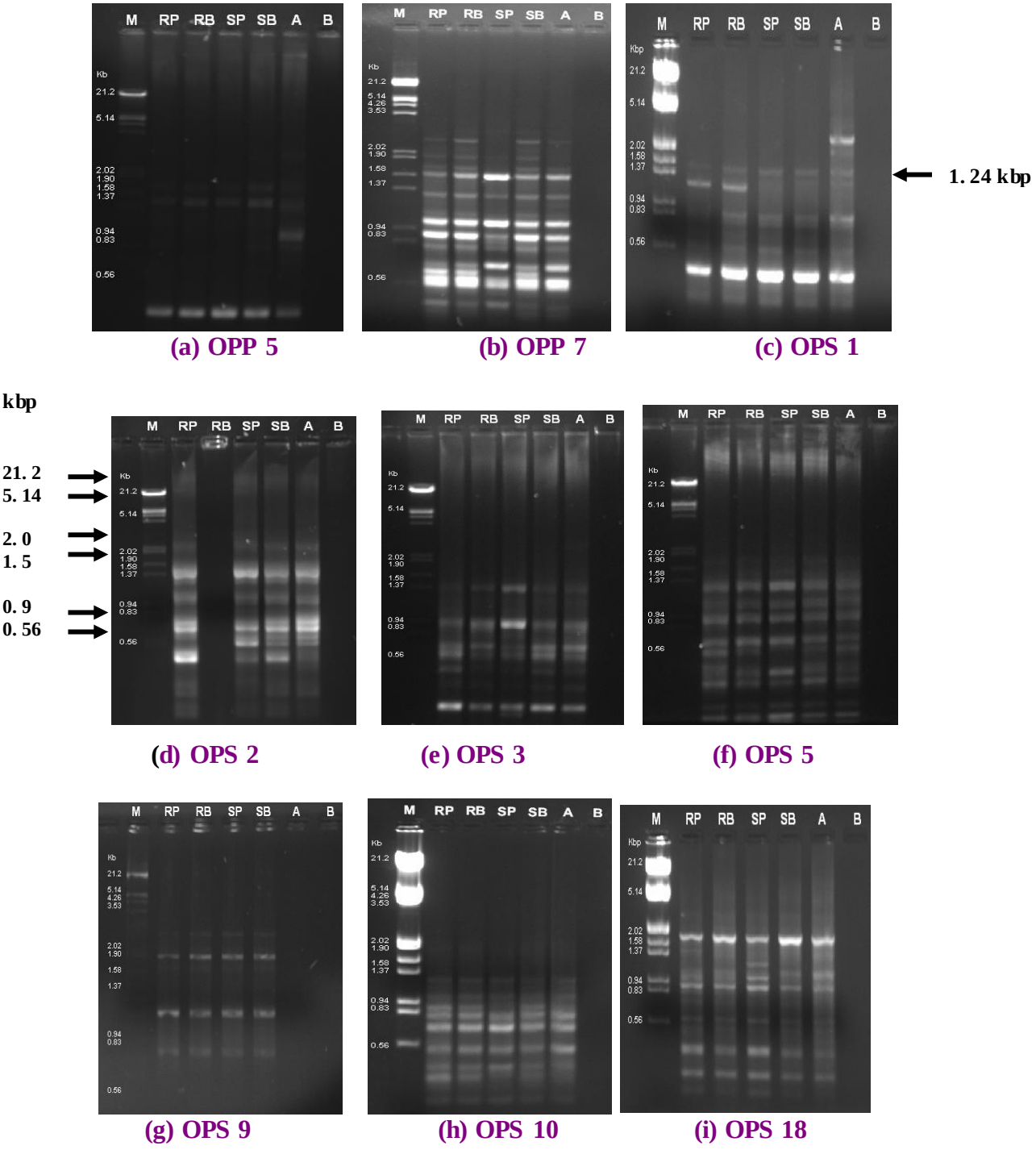
Amplification with this primer generated ten amplicons of which two were polymorphic. The molecular weight of the amplicons ranged between 1.41 kb and 0.27 kb (Plate 11b). Twenty per cent polymorphism was obtained with this primer. Unique bands shared by resistant/susceptible genotypes were not obtained.

OPY 5

Ten amplicons were observed on the agarose gel for the DNA amplified with the primer OPY 5 (Plate 11c). Six bands were monomorphic and the rest were polymorphic among the genotypes. This primer showed 40 per cent polymorphism. The molecular weight of the products ranged between 2.74 kb and 0.17 kb. This primer was unable to differentiate between the resistant and susceptible genotypes.

OPY 7

Using OPY 7, seven amplicons were obtained and four of them were polymorphic (Plate 11d). The molecular weight of these markers ranged between



M: λDNA / *Hind*III+*Eco*RI, RP: Resistant Parent, RB: Resistant Bulk
SP: Susceptible Parent, SB: Susceptible Bulk, A: Anugraha, B: Blank
(Arrow indicates polymorphic band used for cloning of DNA)

Plate 10. Bulk segregant analysis with selected primers from OPP and OPS series

1.71 kb and 0.33 kb. The band of size 0.33 kb was present in susceptible parent and susceptible bulk genotypes and it was absent in resistant genotypes (resistant parent, resistant bulk and Anugraha). This primer exhibited polymorphism of 57 per cent among the different genotypes. Unique bands shared by resistant/susceptible genotypes were not obtained.

Table 9 shows the number of amplification products, number of polymorphic bands and percentage polymorphism calculated for each of the 22 selected primers.

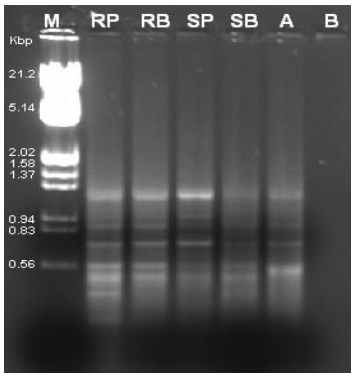
4.3.3.3. Co-segregation analysis

Co-segregation analysis was carried out with resistant parent, resistant bulk, susceptible parent, and susceptible bulk, Anugraha and individuals of resistant and susceptible bulk with selected primer OPS 1. The result is presented in Plate 12. One band of size 1.24 kb was present in resistant parent, in resistant bulk and 5 resistant F_2's but it was absent in susceptible parent, susceptible bulk and 5 susceptible F_2's.

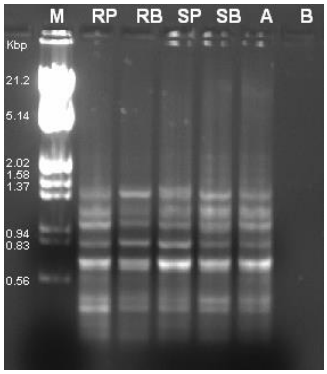
4.3.3.4. Analysis of amplification profiles

The RAPD data generated through bulk segregant analysis were analyzed for similarity by using the NTSYSpc Version 2.0. Genetic similarity values were estimated using Jaccard's similarity coefficient and Unweighed Pair Group Method using Arithmetic averages (UPGMA) for cluster analysis. Dendrogram was constructed using the SAHN programme of NTSYS. The genetic similarity matrix pertaining to the pooled data obtained from the 22 primers is given in Table 10.

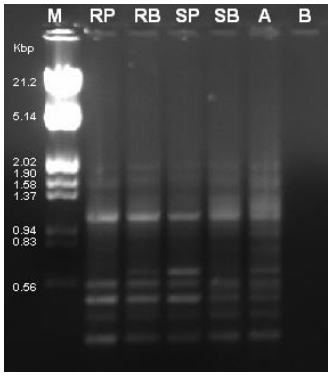
The highest (93 per cent) genetic similarity was noticed between resistant parent (RP) and resistant bulk (RB). The lowest (77 per cent) similarity was noticed between resistant parent and susceptible parent (SP). Anugraha showed 91 per cent similarity with the susceptible bulk.



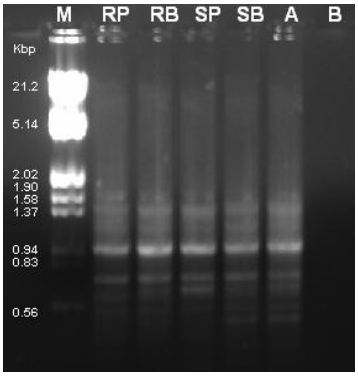
(a) OPY 1



(b) OPY 3



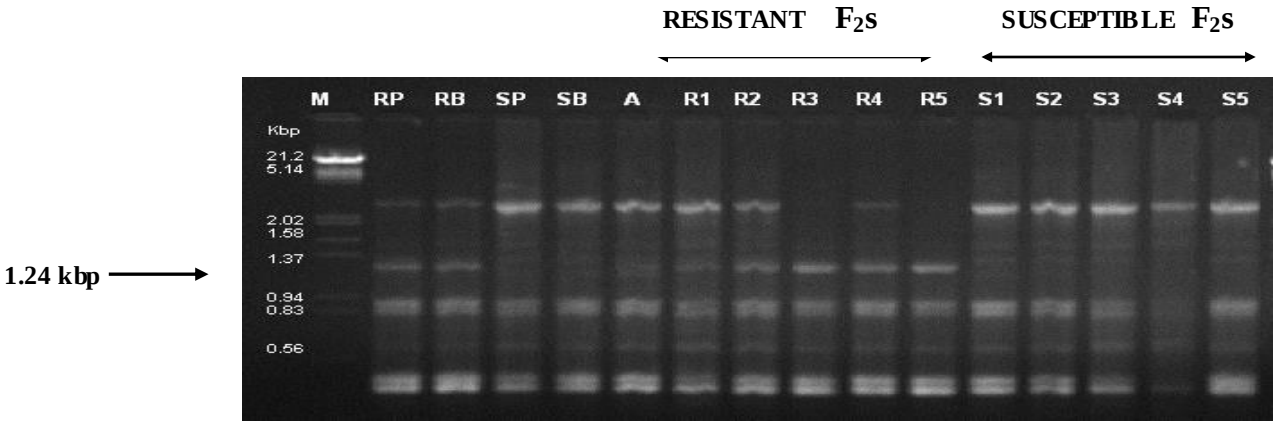
(c) OPY 5



(d) OPY 7

M: λDNA / *Hind*III+*Eco*RI
RP: Resistant Parent
RB: Resistant Bulk
SP: Susceptible Parent
SB: Susceptible Bulk
A: Anugraha
B: Blank

Plate 11. Bulk segregant analysis with selected primers from OPY series



RP: Resistant Parent, RB: Resistant Bulk, SP: Susceptible Parent, SB: Susceptible Bulk, A: Anugraha, R1, R2, R3, R4, and R5: Resistant F_2s; S1, S2, S3, S4, S5 – Susceptible F_2s, B: Blank (Arrow indicates polymorphic band)

Plate 12. Co-segregation analysis in chilli genotypes with OPS 1 primer

Table 9. Amplification products, polymorphic bands and percentage polymorphism of selected RAPD primers in bulk segregant analysis

Sl. No.	Primer	No. of bands	No. of Polymorphic bands	Percentage polymorphism (%)
1	OPE 3	5	1	20
2	OPE 5	10	5	50
3	OPE 6	8	2	25
4	OPAH 5	6	2	33
5	OPAH 19	7	2	29
6	OPN 3	5	0	0
7	OPN 7	12	4	33
8	OPP 3	8	2	25
9	OPP 4	6	2	33
10	OPP 5	6	3	50
11	OPP 7	12	6	50
12	OPS 1	9	6	66.67
13	OPS 2	13	2	15
14	OPS 3	10	5	50
15	OPS 5	12	3	25
16	OPS 9	5	1	20
17	OPS 10	8	1	12.5
18	OPS 18	13	7	54
19	OPY 1	10	5	50
20	OPY 3	10	2	20
21	OPY 5	10	4	40
22	OPY 7	7	4	57

The phenetic representation of similarity coefficients among 5 genetic groups of chilli is presented in (Fig. 2). In the dendrogram all the 5 genotypes were divided into one distinct major cluster, at 93 per cent similarity and the genotype susceptible parent joined them separately. The main cluster was again divided into two sub clusters viz., sub-Cluster (1A) and sub-cluster (1B). The sub-Cluster 1A had two genotypes, resistant parent and resistant bulk grouped together. Susceptible bulk and Anugraha formed the second group in the second major sub-cluster (1B).

Based on estimated Genetic Similarity Matrix using Jaccard's coefficient, the closely related genotypes were Ujwala (resistant parent) and resistant bulk (resistant), with a genetic similarity of 0.93 followed by Anugraha and susceptible bulk with a similarity coefficient of 0.91. The genotype susceptible parent, 'Pusa Jwala' was widely separated from other genotypes in the main clusters at a genetic similarity value of 0.8. The minimum similarity value of 0.769 was obtained between resistant parent Ujwala and susceptible parent Pusa Jwala.

The dendrogram revealed high degree of relatedness between the resistant parent and resistant bulk and between susceptible bulk and Anugraha genotypes. The resistant parent and susceptible parent showed maximum dissimarity.

4.4. MOLECULAR CLONING

4.4.1. Detection and elution of specific amplicons

A distinct band of size ~1.24 kb was observed in resistant parent Ujwala, resistant bulk and five resistant F_2 when the genomic DNA was amplified using OPS 1 primer. In order to elute the DNA from the specific band, four RAPD reactions with the same primer and common template DNA (Ujwala) were set. The RAPD products were resolved on 1.2 per cent agarose gel (Plate 14) and the band of interest was cut from each lane, pooled and eluted. The specific band of size ~1.24 kb was absent in negative control indicating that it is unique to the resistant genotype (Plate 12).

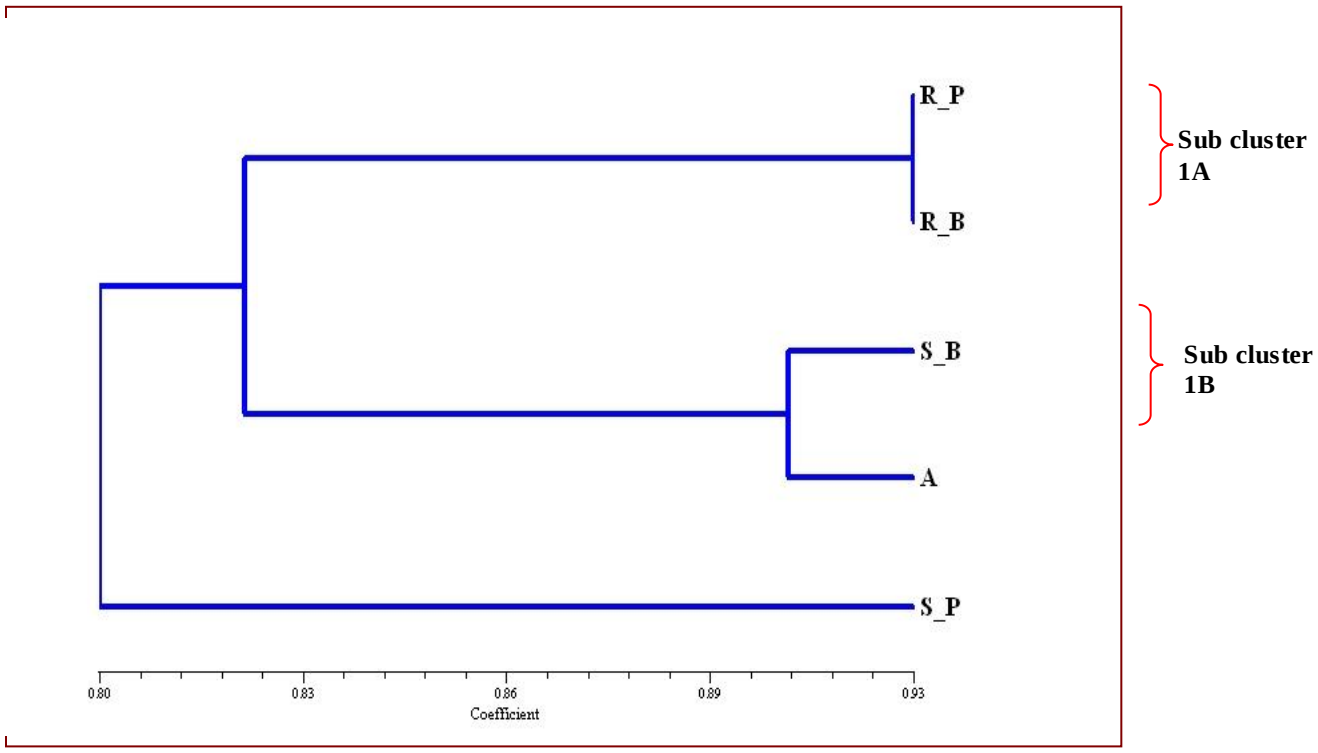


Fig. 2. Dendrogram derived from bulk segregant analysis with 5 chilli groups

Rows\Cols	R_P	R_B	S_P	S_B	A
R_P	1.0000000				
R_B	0.9270833	1.0000000			
S_P	0.7696078	0.8125000	1.0000000		
S_B	0.8186275	0.8333333	0.8137255	1.0000000	
A	0.8039216	0.8229167	0.7892157	0.9068627	1.0000000

Table 10. Genetic similarity matrix obtained using RAPD data

RP: Resistant parent, RB: Resistant bulk, SP: Susceptible parent
SB: Susceptible bulk, A: Anugraha

The eluted product (3 μl) was checked on 0.8 per cent agarose gel and band of the same molecular weight was observed. The bands obtained after elution were of good quality (Plate 15). The eluted product when subjected to agarose gel electrophoresis showed good recovery. This eluted product was used for cloning and sequencing study.

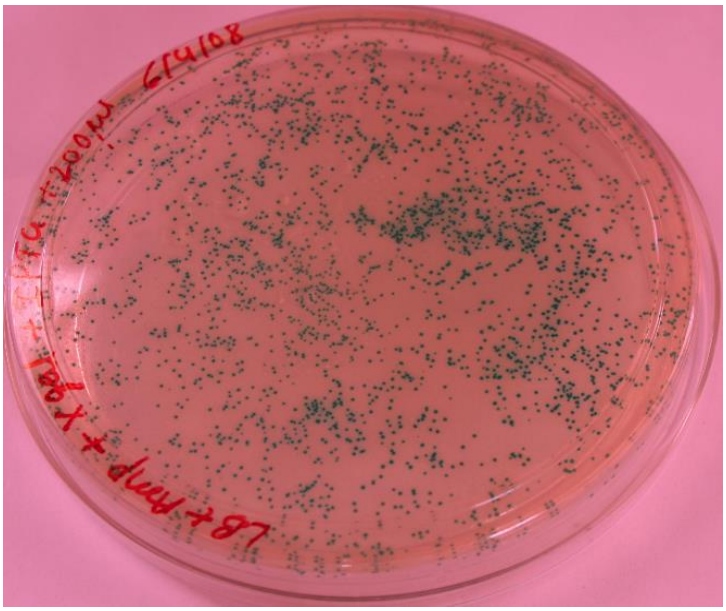
4.4.2. TRANSFORMATION AND CLONING

4.4.2.1. Preparation and screening of competent cells

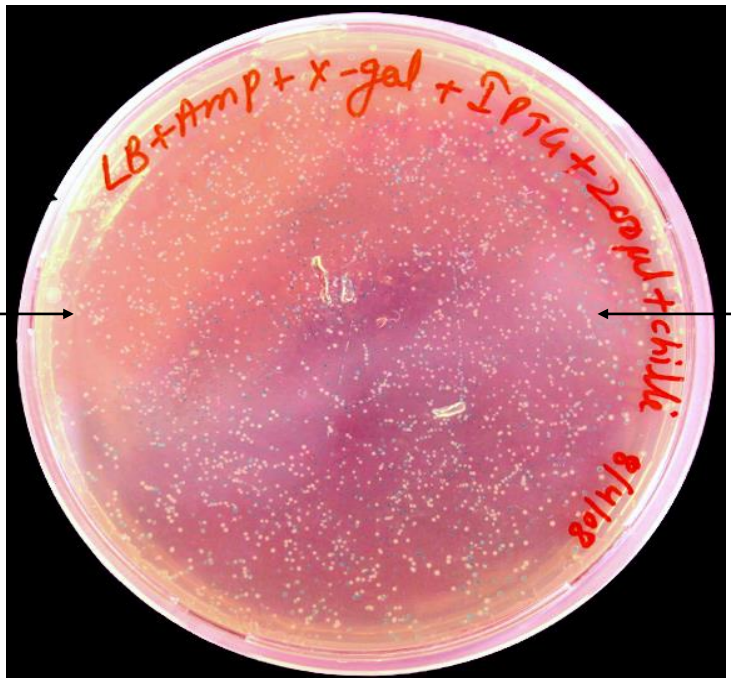
Competent cells were prepared from *E. coli* JM 109 strain as per the procedure described in Section 3.10.1. Large number of colonies was obtained when the competent cells were checked for competence by transformation using the pUC 18 plasmid containing ampicillin resistance marker. The colonies showed luxuriant growth, with no other contamination in LBA ampicillin plates (Plate 13a). Thus the competent cells prepared were found to be efficient for transformation and further cloning purposes.

4.4.2.2. Transformation of *E. coli* with ligated DNA

The eluted DNA was ligated into pGEM-T vector. After confirmation of competence, the ligated DNA was transferred into competent *E. coli* JM109 cells using the heat shock method at 42^0C. When the transformed *E. coli* cells were grown in LBA ampicillin plates overlaid with X-gal and IPTG, a combination of blue and white colonies were obtained after overnight incubation confirming successful transformation (Plate 13b). No growth was observed on negative plate.



a. Competent cell colonies growing in ampicillin media



White colony

Blue colony

b. Blue and white colonies in the transformation plate

Plate 13. Screening of competent cells for transformation with RAPD product

4.4.3. CONFIRMATION OF RECOMBINATION

4.4.3.1. Screening of the transformed colonies

The LB ampicillin agar plates containing the transformed colonies were screened for recombinant plasmid. Four white colonies were picked from each plate and were grown in LB ampicillin media separately. One blue colony was also inoculated in order to set the negative control. Plasmid DNA was isolated as described in section 3.2.1.1 and subjected to electrophoresis on 0.7 per cent (w/v) agarose gel along with molecular weight marker (λDNA /*Hind* III+ *Eco*RI). The plasmid DNA isolated from white colony had higher molecular weight when compared with plasmid DNA isolated from blue colonies (Plate 16). This confirmed the presence of DNA insert in plasmid of white colonies.

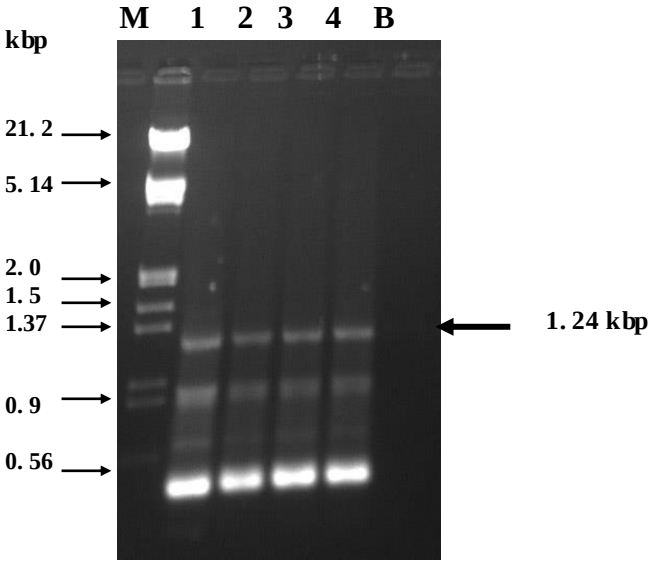
4.4.3.2. Detection of the insert by RAPD amplification

The plasmid from white colony was checked for the presence of insert by PCR confirmation. The plasmid DNA from blue as well as white colony was amplified using selected random primer OPS 1. A positive control RAPD reaction was set up using the resistant parent, Ujwala as template. The PCR products were checked on 1.2 per cent (w/v) agarose gel along with molecular weight marker (λDNA /*Hind* III+*Eco*RI).

Single amplified band of expected size, 1.24 kb which is exactly similar to the genomic DNA amplification product, was obtained in plasmid isolated from white colony, confirming the presence of insert in the plasmid. No amplification was detected in plasmid isolated from blue colony (Plate 17). The insert used for transformation was also loaded along with the amplified plasmid PCR products, to confirm the presence of insert in the plasmid.

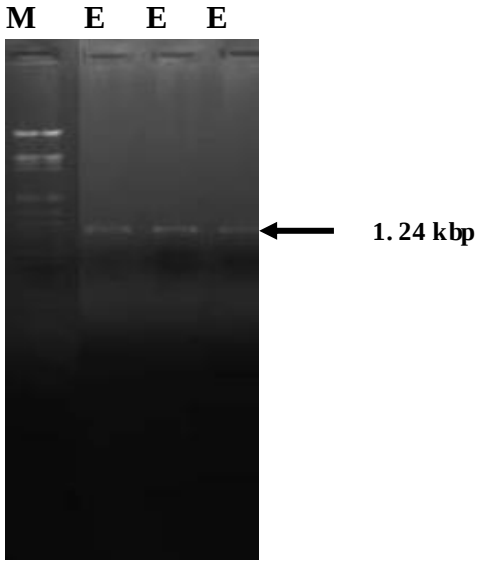
4.4.4. Sequence analysis of the cloned fragment

Sequence information obtained (referred as *Chilli seq 1*) for the fragment with OPS 1 primer had a size of 1506 bp (Fig. 3).



M: MW marker, Lane 1 to 4-Resistant genotype, B: Blank

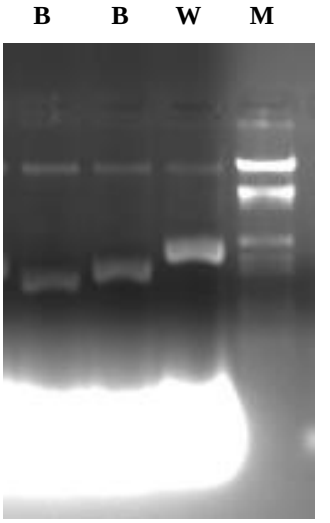
Plate 14. RAPD amplification of resistant chilli genotype, Ujwala with OPS 1 Primer



E: Eluted amplicon

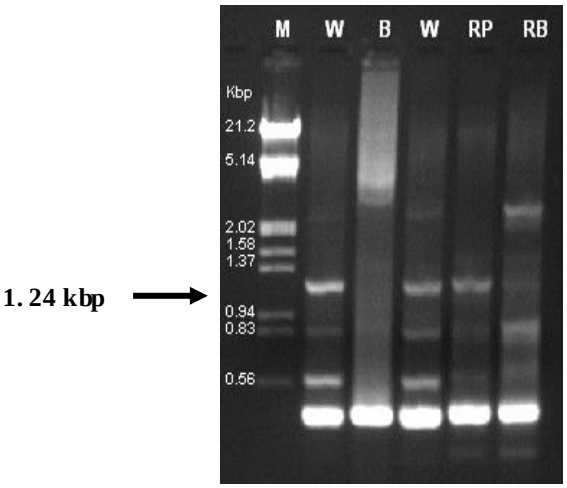
(Arrow indicates 1.24 kb amplicon eluted)

Plate 15. Eluted band obtained from resistant genotype



M: MW marker, B: Plasmid DNA from blue colonies, W: Plasmid DNA from White colonies

Plate 16. Plasmid DNA isolated from recombinant and non recombinant colonies



M: MW marker, B: Plasmid DNA from blue colonies, W: Plasmid DNA from White colonies, RP: Resistant plant genomic DNA

Plate 17. Confirmation of recombination with RAPD reaction

(Arrow indicates 1.24 kb, polymorphic amplicon)

Reverse SCAR primer

The sequences obtained after automated sequencing were subjected to vector screening using the VecScreen tool in NCBI to remove vector regions from the clones (Fig. 4). The region starting from 1 to 69 bases and 510 to 1506 bases showed similarity with the pGEM-T Easy Vector sequence, and it was deleted from the original sequence. The sequence of 440 bases named as *Chilli seq 2* obtained after vector screening is presented in (Fig. 5).

4.4.4.1. Nucleotide analysis

4.4.4.1.1. Homology search

Homology of the sequence obtained from the cloned product of chilli (*Chilli seq 2*) with the other reported sequence was analyzed using BLAST search tool. It has shown 79 per cent identity to *Capsicum annuum* clone pC.a.D Ty1-copia retrotransposon nonfunctional polyprotein gene, 100 per cent identity to ethylene responsive element binding protein C2 mRNA, 90 per cent identity to lipid transfer protein III gene in *Capsicum annuum*, 94 per cent identity to *Capsicum annuum* clone A1-4 PR-protein gene and 94 per cent identity with beta-galactosidase (BG1) (Fig. 6). The sequences were translated into amino acid sequence. The amino acid sequence of *Chilli seq 2* was obtained using Biology Workbench (Fig.7). The BLASTp search of this translated amino acid sequences producing significant alignments are given in (Fig. 8).

4.4.4.1.2. Detection of Open Reading Frame (ORF)

Open reading frame identified in the sequence *Chilli seq 2* using NCBI tool 'ORF Finder' contained no ORF.

4.4.4.1.3. Nucleic acid statistics

Nucleic acid statistics of the *Chilli seq 2* obtained in the Biology Workbench is presented in Table 11. The fragment had low GC content (38.9%) and high AT content (61.1%).

AATCAGGCTATGCATCCACGCGTTGGGAGCTCCCATATGGTCAACCTGCAGGCGGCCGCACTAG
TGATTGTTTCGCTCCATCATTTAGTTGAGGCACTTCAGTGGCACTCATTGGAGTTTTTGCTGAATT
ATAGTTCTTCATGTTTTCGTCAACGAGGATTTCATTGATGTAGTTGGACTGTGATAAAGTGATTCC
TTTGGCAGTTCGGAGCACCTCCACTCCAAGAAAGAAGTTTAATTCCCAAGATCCTTAATGGAAA
ACGCCCACAAGACGAGCAATAATCTGATTAACCTCTGAGTTGTGATTCCCTGTAATAATGATATC
GTCTATATAAACCAGTGCGTACAAAATATTGTTTGATACTTTGTGAATAAAGAGTGAGGCATCAG
ATTCTGACTTGACAAATCCTAGATAAAGAAAAAATGTCTTAAGTTCATTGTACCACGCCCGAGG
GGCTTGCTTGAGGCAATATATTACTTTATTTAGACGACATATGTGGGAGCGAAACAATCCCGCGG
CCATGGCGGCCGGGAGCATGCGACGTCGGGCCCAATTCGCCCTATAGTGAGTCGTATTACAATT
CACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTT
GCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCC
AACAGTTGCGCAGCCTGAATGGCGAATGGACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGT
GTGGTGGTTACGCCCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTT
CTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGGGGCTCCCTTT
AGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAAACTTGATTAGGGTGATGGTTCAC
GTAGTGGGCCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAAT
AGTGGACTCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGTCTATTCTTTTGATTTATAA
GGGATTTTGCCGATTTCGGCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTAACGCGAA
TTTTAACAAAATATTAACGCTTACAATTTCCTGATGCGGTATTTTCTCCTTACGCATCTGTGCGGT
ATTTCACACCGCATCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTT
CTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATT
GAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTGCGGCATTTT
GCCTTCCTGTTTTTGCTCACCCAAAACGCTGGTGAAAGTAAAAATGCTGAAATCTTGGGTGCCGA
GTGGGTTCCTC

Fig. 3. Nucleotide sequence of *Chilli seq 1* amplicon

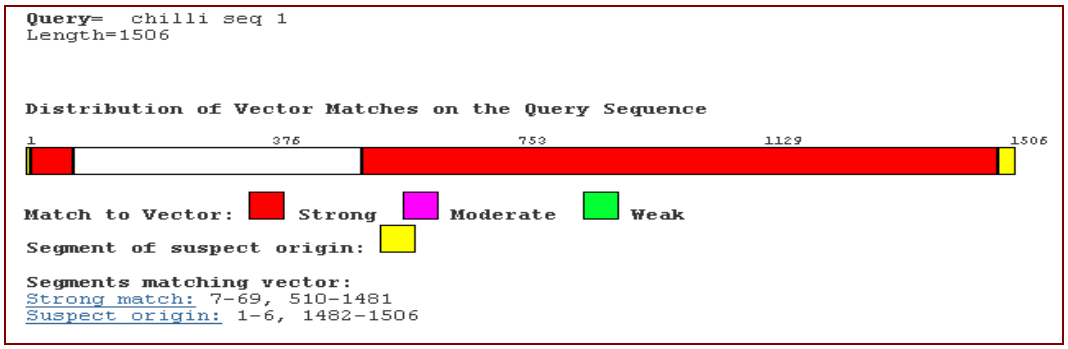
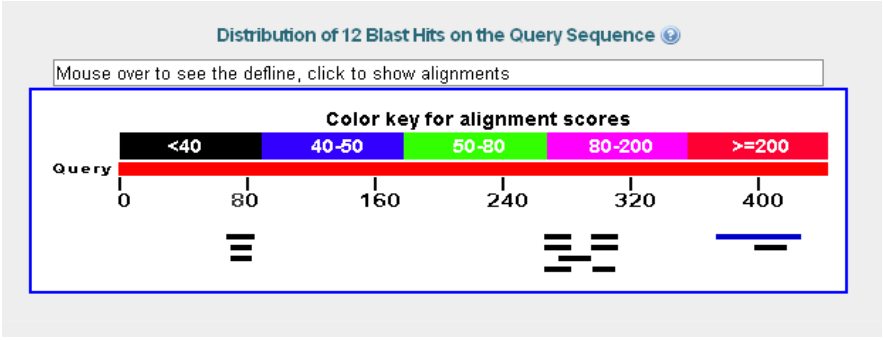


Fig. 4. VecScreen output of *Chilli Seq 1*

GTTTCGCTCCATCATTTAGTTGAGGCACTTCAGTGGCACTCATTGGAGTTTTTGCTGAATTATAGTTC
TTCATGTTTTCGTCAACGAGGATTTCATTGATGTAGTTGGACTGTGATAAAGTGATTCCTTTGGCAG
TTCGGAGCACCTCCACTCCAAGAAAGAAGTTTAATTCCCAAGATCCTTAATGGAAAACGCCCACAA
GACGAGCAATAATCTGATTAACCTCTGAGTTGTGATTCCCTGTAATAATGATATCGTCTATATAAAC
CAGTGCGTACAAAATATTGTTTGATACTTTGTGAATAAAGAGTGAGGCATCAGATTCTGACTTGACA
AATCCTAGATAAAGAAAAAATGTCTTAAGTTCATTGTACCACGCCCGAGGGGCTTGCTTGAGGCAA
TATATTACTTTATTTAGACGACATATGTGGGAGCGAAAC

Fig. 5. *Chilli seq 2* - nucleotide sequence after deleting vector sequence



Legend for links to other resources: U UniGene E GEO G Gene S Structure M Map Viewer

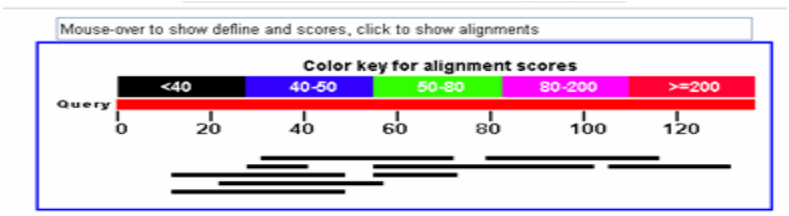
Sequences producing significant alignments:
(Click headers to sort columns)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
DQ644591.1	Capsicum annuum clone pC.a.D Ty1-copia retrotransposon nonfunc	46.4	46.4	12%	4e-06	79%	
AY789636.1	Capsicum annuum ethylene responsive element binding protein C2	31.9	31.9	3%	0.099	100%	
AY496107.1	Capsicum annuum ripening regulated protein DDTFR10/A mRNA, pa	31.9	31.9	3%	0.099	100%	
AJ276631.1	Capsicum annuum partial kn gene for Knolle protein, promoter regi	28.3	28.3	4%	1.2	90%	
AY876012.1	Capsicum annuum lipid transfer protein III gene, complete cds	28.3	28.3	4%	1.2	90%	
EF064262.1	Capsicum annuum clone A1-4 PR-protein gene, partial cds	26.5	26.5	3%	4.2	94%	
AJ005588.1	Capsicum annuum gene encoding 5-epi-aristolochene synthase, ex	26.5	26.5	3%	4.2	100%	
AB206919.1	Capsicum annuum catf1 gene for acyl-transferase, partial cds	26.5	26.5	3%	4.2	94%	
AY819029.1	Capsicum annuum cultivar Thai Hot acyltransferase (Pun1) gene, cc	26.5	26.5	3%	4.2	94%	
AY819028.1	Capsicum annuum cultivar Hot 1493 acyltransferase (Pun1) gene, c	26.5	26.5	3%	4.2	94%	
AY029226.1	Capsicum annuum beta-galactosidase (BG1) mRNA, complete cds	26.5	26.5	3%	4.2	100%	
AB080296.2	Capsicum annuum PBG1 mRNA for beta-galactosidase, complete cd	26.5	26.5	3%	4.2	100%	

Fig. 6. *Chilli seq 2* producing significant alignments in nucleotide BLAST analysis

```
  1   X  V  S  L  H  H  L  V  E  A  L  Q  W  H  S  L  E  F  L  L
  1   CNNGTTTCGCTCCATCATTTAGTTGAGGCACTTCAGTGGCACTCATTGGAGTTTTTGCTG
 21   N  Y  S  S  S  C  F  R  Q  R  G  F  H  -  C  S  W  T  V  I
 61   AATTATAGTTCTTCATGTTTTCGTCAACGAGGATTTCATTGATGTAGTTGGACTGTGATA

 41   K  -  F  L  W  Q  F  G  A  P  P  L  Q  E  R  S  L  I  P  K
121   AAGTGATTCCTTTGGCAGTTCGGAGCACCTCCACTCCAAGAAAGAAGTTTAATTCCCAAG

 61   I  L  N  G  K  R  P  Q  D  E  Q  -  S  D  -  P  L  S  C  D
181   ATCCTTAATGGAAAACGCCCACAAGACGAGCAATAATCTGATTAACCTCTGAGTTGTGAT

 81   S  L  -  -  -  Y  R  L  Y  K  P  V  R  T  K  Y  C  L  I  L
241   TCCCTGTAATAATGATATCGTCTATATAAACCAGTGCGTACAAAATATTGTTTGATACTT

101   C  E  -  R  V  R  H  Q  I  L  T  -  Q  I  L  D  K  E  K  M
301   TGTGAATAAAGAGTGAGGCATCAGATTCTGACTTGACAAATCCTAGATAAAGAAAAAATG

121   S  -  V  H  C  T  T  P  E  G  L  A  -  G  N  I  L  L  Y  L
361   TCTTAAGTTCATTGTACCACGCCCGAGGGGCTTGCTTGAGGCAATATATTACTTTATTTA

141   D  D  I  C  G  S  E  T
421   GACGACATATGTGGGAGCGAAAC
```

Fig. 7. Deduced amino acid sequence of *Chilli Seq* 2 obtained using Molecular Tool Kit



Distance tree of results NEW

Sequences producing significant alignments:		Score (Bits)	E Value
gb|ABQ44212.1|	galactinol synthase [Capsicum annuum]	23.1	0.90
gb|ACC62519.1|	Bax inhibitor [Capsicum annuum]	21.9	2.2
gb|AAM97542.1|	catalase 3 [Capsicum annuum]	20.8	4.8
emb|CAA59170.1|	dimethylallyltransferase [Capsicum annuum]	20.8	5.0
gb|ACB05669.1|	chloroplast chlorophyll a/b binding protein [C...	20.4	5.6
gb|ABM97654.1|	PR protein [Capsicum annuum]	20.0	8.3
gb|ABG75660.1|	NADH dehydrogenase subunit [Capsicum annuum va...	19.6	9.9

Fig. 8. Protein BLAST of deduced amino acid sequence of *Chilli Seq* 2

4.4.4.2. Restriction analysis

Major restriction sites deduced for the fragment *Chilli seq 2* are provided in Table 12. It contains frequent cutter *Eco*RV and the rare cutter *Mbo*I with a single restriction site in the sequence. The restriction map is represented in (Fig. 9).

4.4.4.3. Amino acid sequencnalysis

Deduced amino acid sequence of protein analyzed and proportion of each amino acid calculated using AASTATS of Biology Workbench are presented in Table 13. The major amino acids deduced from the cloned fragment *Chilli seq 2* were Leucine, Serine, Isoleucine, Glutamine, Arginine and Glutamate.

4.4.4.4. Secondary structure prediction

The hydropathy plot analysis revealed that there is no transmembrane region in the cloned sequence, *Chilli seq 2* (Fig. 10). The secondary structure of proteins predicted by SOPMA programme of ExSpasy tool is presented in Fig. 11. The amino acid deduced from *Chilli seq 2* had 51.09 per cent alpha helix, 8.03 per cent extended strands, 7.30 per cent beta turns and 33.58 per cent random coils. It also indicated that the protein is made up of 67 hydrophilic and 70 hydrophobic amino acids.

4.5. MOLECULAR CHARACTERIZATION OF CHILLI GENOTYPES BY SCAR MARKER

4.5.1. SCAR Primer designing

Based on the parameters described in Section 3.2.4.1, a pair of SCAR primers was designed. For designing longer primers of 24 bp and 23 bp length the sequence of RAPD fragments OPS 1(*Chilli seq 2*) were used (Fig. 12).

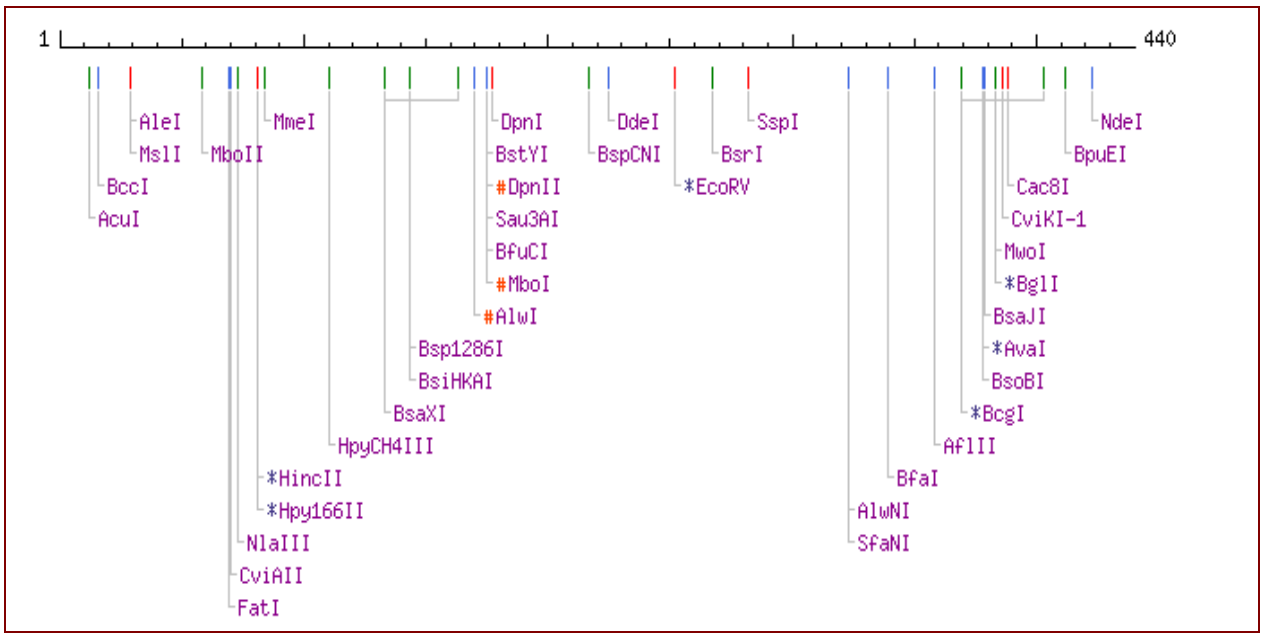


Fig. 9. Restriction analysis of *Chilli Seq* 2 using Biology Workbench

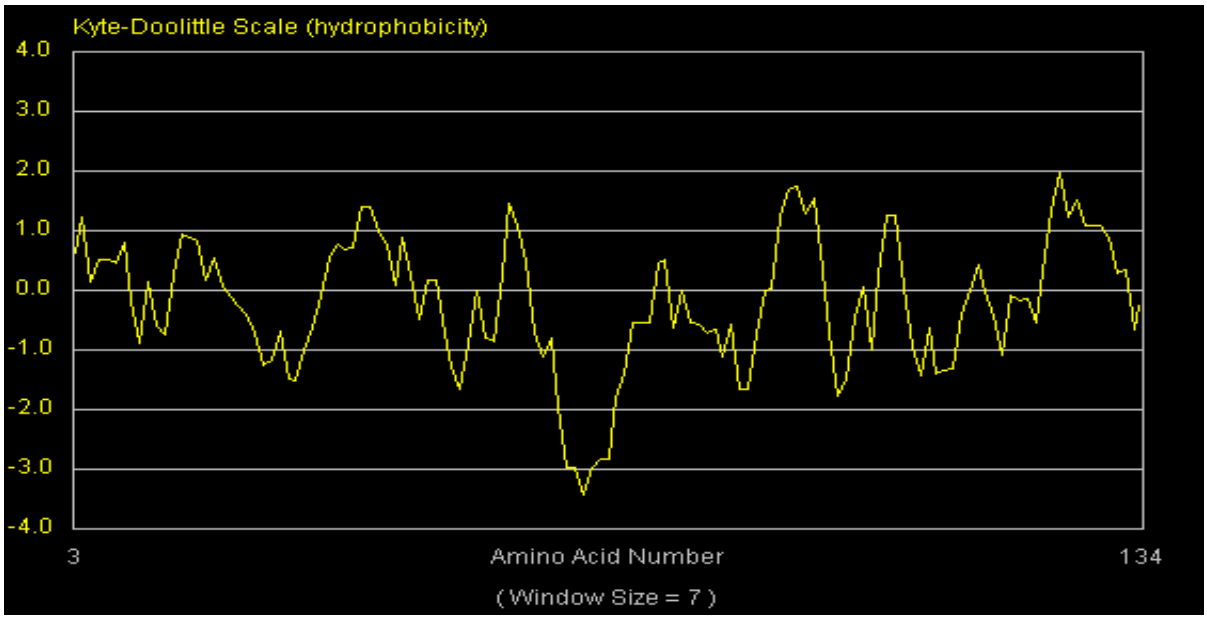
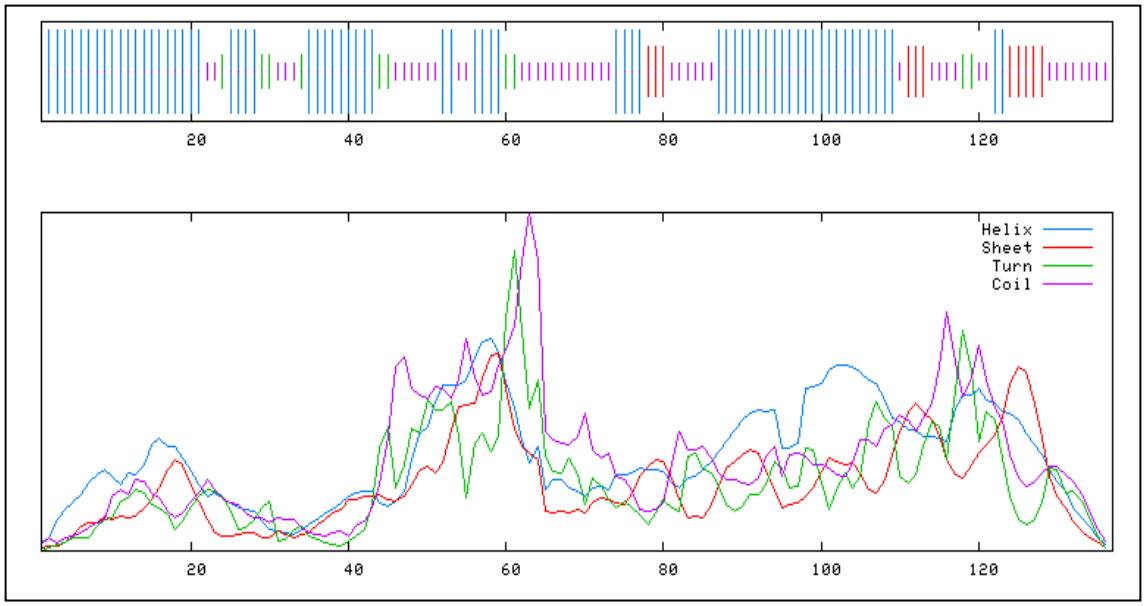


Fig. 10. Doolittle hydropathy plot for deduced protein of *Chilli Seq* 2




```
        10        20        30        40        50        60        70
         |         |         |         |         |         |         |
XVSLHHLVEALQWHSLEFLLNYSSSCFRQRGFHCSWTVIKFLWQFGAPPLQERSLIPKILNGKRPQDEQS
hhhhhhhhhhhhhhhhhhhhhcchhhhttcccthhhhhhhhhttccccchhcchhhhttccccccc
DPLSCDSLYRLYKPVRTKYCLILCERVRHQILTQILDKEKMSVHCTTPEGLAGNILLYLDDICGSET
ccccchhheeeccccccchhhhhhhhhhhhhhhhhhhhhhhhceeeccccttcchheeeeeccccccc

Sequence length :   137
```

```
SOPMA :
    Alpha helix      (Hh) :     70 is   51.09%
    3 10  helix      (Gg) :      0 is    0.00%
    Pi helix         (Ii) :      0 is    0.00%
    Beta bridge      (Bb) :      0 is    0.00%
    Extended strand  (Ee) :     11 is    8.03%
    Beta turn        (Tt) :     10 is    7.30%
    Bend region      (Ss) :      0 is    0.00%
    Random coil      (Cc) :     46 is   33.58%
    Ambigous states  (?)  :      0 is    0.00%
    Other states          :      0 is    0.00%
```

Fig. 11. The secondary structure of protein predicted by SOPMA tool

Table 11. Nucleotide statistics of *Chilli Seq 2* obtained using NASTATS

Sl.No.	Nucleotide	Total	Percentage
1.	A	131	29.8%
2.	T	138	31.4%
3.	C	80	18.2%
4.	G	91	20.7%
5.	A + T	269	61.1%
6.	C + G	171	38.9%

Table 12. Restriction sites of *Chilli Seq 2* with common restriction enzymes

Sl.No.	Name of restriction enzyme	Recognition sequence	No. of cuts	Position of restriction(bp)
1.	*AflII*	C'TTAA_G	1	360
2.	*BstKTI*	G_AT'C	1	180
3.	*BfaI*	GC C'TA_G	1	341
4.	*DpnI*	GA'TC	1	179
5.	*EcoRV*	GAT'ATC	1	254
6.	*FatI*	CATG	1	71
7.	*MboI*	GATC	1	177
8.	*MseI*	T'TA_A	4	167
9.	*NdeI*	CA'TA_TG	1	425
10.	*RsaI*	GT'AC	2	277
11.	*SspI*	AAT'ATT	1	284

Table 13. Deduced amino acid composition of *Chilli Seq 2* using AASTATS Biology Workbench

Amino acid group		Amino acid	Number	Mol %
Non polar		Gly	6	4.38
		Ala	3	2.19
		Trp	3	2.19
		Val	6	4.38
		Leu	21	15.33
		Ile	8	5.84
		Met	1	0.73
		Pro	7	5.11
		Phe	5	3.65
Polar	Unchancharged	Ser	12	8.76
		Cys	7	5.11
		Asn	3	2.19
		Thr	6	4.38
		Tyr	5	3.65
		Gln	8	5.84
	Basic	Lys	7	5.11
		Arg	8	5.84
		His	6	4.38
	Acidic	Asp	6	4.38
		Glu	8	5.84

Accordingly two primers forward and reverse primers were designed for OPS 1_{1240} with melting temperature of 68^0C and 70^0C and GC content of 41.6 and 52.17 per cent respectively. Both primer sequences had no palindromic or repetitive sequences. The forward primer has starting bases of the *Chilli seq* 2 included all the 10 bases of the random primer whereas the reverse primer was terminus including 10 bases of the random primer. The forward and reverse primers designed for $OPS1_{1240}$ were 24 and 23 bp long.

Primers were synthesized from Integrated DNA Technologies, USA. Details of primers viz., annealing temperature, melting temperature and GC% used for the amplification of resistant parent, resistant bulk and 5 F_2's are given in Table 14. Primers sequences and length of primers are provided in Table 15.

4.5.2. PCR amplification using SCAR primers

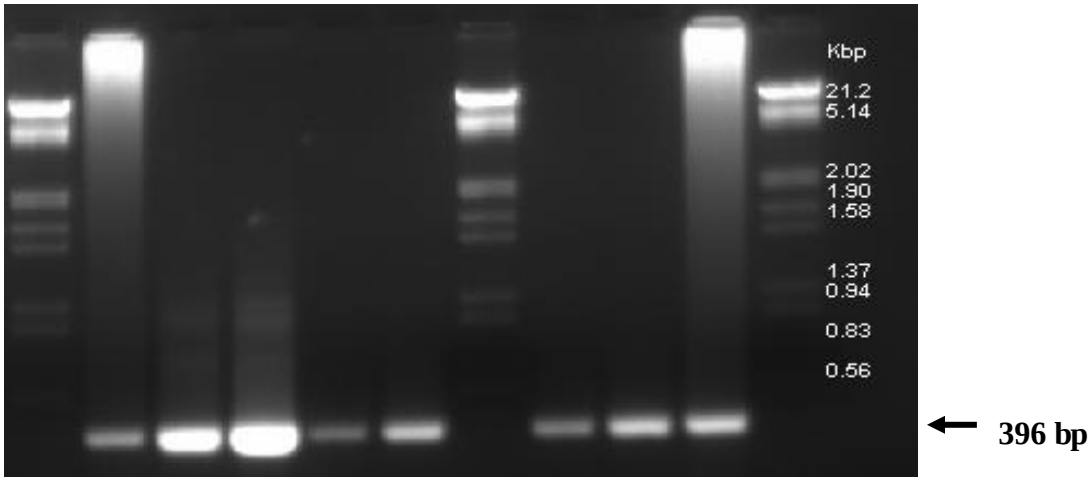
A pair of 24 mer oligonucleotide primers synthesized based on the end sequences of the RAPD clone OPS 1_{1240} were used for amplification of DNA of the resistant parent (Ujwala), susceptible parent (Pusa Jwala), resistant bulk, susceptible bulk and individuals of resistant bulk. The product was subjected to electrophoresis along with molecular weight marker (λDNA /*Hind* III+ *Eco*RI). The gel document revealed an amplicon of 396 bp in all the genotypes tested (Plate 18). So there was no polymorphism between the resistant and susceptible genotypes. The amplicon produced by SCAR primers were of length 396 bp while the amplicon produced by OPS1 primer was 1.24 kbp. It was also seen that the SCAR marker of OPS 1_{1240} was smaller than the RAPD marker OPS 1_{1240}.

Forward SCAR primer

GTTTCGCTCCATCATTTAGTTGAGGCACTTCAGTGGCACTCATTG
GAGTTTTTGCTGAATTATAGTTCTTCATGTTTTCGTCAACGAGGA
TTTCATTGATGTAGTTGGACTGTGATAAAGTGATTCCTTTGGCAG
TTCGGAGCACCTCCACTCCAAGAAAGAAGTTTAATTCCCAAGAT
CCTTAATGGAAAACGCCCACAAGACGAGCAATAATCTGATTAAC
CTCTGAGTTGTGATTCCCTGTAATAATGATATCGTCTATATAAAC
CAGTGCGTACAAAATATTGTTTGATACTTTGTGAATAAAGAGTGA
GGCATCAGATTCTGACTTGACAAATCCTAGATAAAGAAAAAATG
TCTTAAGTTCATTGTACCACGCCCGAGGGGCTTGCTTGAGGCAAT
ATATTACTTTATTTAGACGACATATGTGGGAGCGAAAC

Reverse SCAR primer

Fig. 12. SCAR primers designed from end sequences of *Chilli seq 2*



RP: Resistant parent, RB: Resistant bulk, SP: Susceptible parent,
SB: Susceptible bulk, A: Anugraha, R1 and R2: Resistant F_2s, S1 Susceptible F_2

Plate 18. Amplification of chilli genotypes with SCAR primers

Table 14. Details of annealing temperatures, melting temperatures and GC content of SCAR primers

Sl. No	Primer	Annealing Temperature	Tm (^{0}C)	GC%
1	forward primer	63^{0}C	68	41.6
2	reverse primer	65^{0}C	70	52.17

Table 15. Details of SCAR primers used in SCAR analysis

Sl.No	Primer	Primer sequence	Length (bp)
1	Forward Primer	5’ GTTTCGCTCCATCATTTAGTTGAG 3’	24
2	Reverse Primer	5’ GTTTCGCTCCCACATATGTCGTC 3’	23

Discussion

5. DISCUSSION

Chilli (*Capsicum annuum* L.) is an important vegetable cum spice crop grown in India. Bacterial wilt incited by *Ralstonia solanacearum* is one of the wide spread and severe diseases in chilli. Bacterial wilt by *Ralstonia solanacearum* causes damage to many crops of economic importance in the tropical and subtropical regions of the world. To prevent the yield losses due to this menace, cultivation of resistant varieties and chemical protection are the major strategies followed currently. Use of chemicals is not generally desired due to serious environmental hazards. Breeding for resistant varieties is an effective approach to eliminate the use of chemicals and minimize crop loss. Although conventional breeding methods have given excellent results, they are time consuming and expensive. Moreover, the pathogen populations adapt to resistant cultivars at a faster rate. This necessitates the exploration of more efficient selection and breeding strategies than those currently existing.

Molecular marker offers a great potential in breeding for disease and pest resistance. Genetic engineering and biotechnology hold great potential for plant breeding as they promise to expedite the time taken to produce crop varieties with desirable characters. With the use of molecular techniques, it would now be possible to hasten the transfer of desirable genes among varieties and to introgress novel genes from related wild species. Techniques which are particularly promising in assisting selection for desirable characters involve the use of molecular markers such as RAPD, RFLP, SSR, AFLP, SCAR, STS and ISSR using F_2 and backcross populations, and also NILs, RILs and doubled haploids.

Progress has been made in mapping and tagging many agriculturally important genes with molecular markers, which forms the foundation for marker-assisted selection. Molecular markers have several advantages over the traditional phenotypic markers that were previously available to plant breeders. Using recombinant DNA

technology, desirable cloned genes could be transferred to the varieties of different crops within a shorter time. Use of molecular markers gives accurate information about the susceptibility/resistance of the genotypes to a particular disease at an early stage of plant growth compared to field screening with artificial inoculation. It also eliminates environmental influence in resistance/susceptibility reaction of genotypes in field screening.

Availability of molecular markers linked to resistance genes will help in identifying plants carrying these genes in various generations without subjecting them to pathogen infection. No doubt, when reliable markers are identified and gene is tagged with them they would prove to be a very powerful tool especially in screening and selection for quantitative and qualititative characters there by reducing the burden of plant breeders to a great extent.

Through conventional breeding, bacterial wilt resistant varieties such as Ujwala, Manjari (Gopalakrishnan and Peter, 1991), Anugraha (Markose, 2003) have been developed at KAU. The present study aimed to develop a molecular marker linked to the bacterial wilt resistance trait found in resistant variety Ujwala. The methodology followed is bulk segregant analysis and co-segregation analysis. The technical programme involved the following aspects:

1. Development of segregating F_2 generation for the trait under consideration
2. Phenotyping of genotypes for bacterial wilt resistance
3. Molecular characterization of chilli genotypes with RAPD marker for getting polymorphism between resistant and susceptible genotypes
4. Conversion of RAPD marker into SCAR marker for tagging bacterial wilt resistance gene.

The results obtained on various aspects are discussed here under.

5.1. Development of segregating F_2 generation for trait under consideration

Crosses were made using the resistant variety Ujwala as the ovule parent and susceptible variety Pusa Jwala as the pollen parent, and F_1 plants were generated (Plate 2c). Fifty F_2 plants (Plate 2d) were raised from a single F_1 plant by selfing and they were used as segregating population for tagging the bacterial wilt resistance gene. Segregating population for the character under consideration is necessary to know the gene expression under homozygous dominant, homozygous recessive and heterozygous condition. It will also reveal monogenic or polygenic nature of the character. F_2 plants offer certain advantages over other mapping populations (DH lines, NILs etc.) because segregation is fixed (Benjamin and Burr, 1991).

The usual method to locate and compare loci regulating quantitative traits (QTLs) requires a segregating population of plants with each one genotyped with molecular markers. However, plants from such segregating population can also be grouped according to phenotypic expression of trait and tested for differences in allele frequency between the population bulks.

5.2. Phenotyping of genotypes for bacterial wilt resistance

5.2.1. Standardization of artificial inoculation technique

The genotype used was the susceptible variety Pusa Jwala. Artificial inoculation was given to 30 days old seedlings at 5-6 leaf stage with fresh bacterial ooze. Four different methods of inoculation were compared *viz*., root dipping, stem-puncturing, soil drenching and soil drenching with wounding, and scoring was done according to Mew and Ho (1976) system.

Among the 4 methods evaluated, maximum wilt incidence was observed in soil drenching with wounding method (90%). Winstead and Kelman (1952), and Husain and Kelman (1958) have also reported about the suitability of this method for artificial

inoculation in tomato. So this method was selected for phenotyping of genotypes for bacterial wilt resistance. The stem inoculation technique helped in rapid development of disease symptoms, as there was direct introduction of the pathogen into the vascular tissues, which enabled faster movement and multiplication of the pathogen. James (2001) also made similar observations and reported stem puncturing in tomato to induce 100 per cent wilt incidence one week after inoculation.

The root dipping method allowed for a more passive introduction of the bacteria into the plant. This is in accordance with observations made by Kelman and Sequeira (1965) that mechanical damage to roots in the field results in rapid increase in the incidence and severity of bacterial wilt. Severing of the secondary roots provided many opportunities for bacterial infection. As natural infection mainly occurs through wounded roots, soil drenching with root severing technique may mimic the field condition. In addition, soil drenching has an advantage that the plant growth is not distorted, as observed to occur sometimes with the stem puncture stabbing method.

Being younger (30 day old), the seedlings succumbed to wilt more rapidly. The reason for this could be that the seedlings have thinner cortical cells compared to older plants that make the entry of pathogen easier. Phenotypic screening indicated a continuous variation for the trait with a larger proportion of lines falling towards susceptibility. Reports by Winstead and Kelman (1952), and Celine (1981) also highlighted that wilting was more in juvenile stage as compared to one observed in adult stage.

5.2.2. Phenotyping of genotypes for bacterial wilt resistance

Resistant parent Ujwala, susceptible parent Pusa Jwala, F_1 and F_2 of cross Ujwala x Pusa Jwala and Anugraha, were inoculated artificially by soil drenching with wounding method and scoring was done by Mew and Ho (1976) system. Observations revealed that Ujwala was resistant with 90 per cent survival, Pusa Jwala was susceptible with 15 per

cent survival and Anugraha was resistant (R) with 80 per cent survival. F_1 and F_2 plants were susceptible with 20 and 30 per cent survival. According to (Gopalakrishnan and Peter, 1991) Ujwala was resistant to bacterial wilt and it was observed in present study. According to Markose (1996 and 2003), percent of survival in Ujwala, Pusa Jwala, F_1, F_2 of cross Ujwala x Pusa Jwala and Anugraha were 94, 17, 22, 33 and 92 per cent respectively.

Phenotyping of genotypes with artificial inoculation classified the varieties, and F_1 and F_2 progenies into various groups according to the degree of susceptibility/resistance reaction. It also threw light on the genetic basis of bacterial wilt resistance.

The data generated in this experiment was used for genetic analysis of bacterial wilt resistant gene as shown below:

Ujwala (Resistant) x Pusa Jwala (Susceptible)

(rr) ↓ (RR)

F_1 (Rr) - 80% wilting (Susceptible)

↓

F_2 (Rr, rR, RR, rr) - 70% wilting (Susceptible-RR, Rr, rR)

- 30% survival (rr)

Based on the above it can be concluded that bacterial wilt resistant character in Ujwala is homozygous recessive in nature. Since F_1 showed 80 per cent wilting, the character can be under the control of polygene.

Reports from Asian Vegetable Research and Development Centre, Taiwan (AVRDC, 1975) also revealed that multiple recessive genes acting additively controlled resistance to bacterial wilt in tomato.

The finding of Rajan (1985) revwaled that the bacterial wilt resistance mechanism in tomato variety 'Sakthi' is monogenic and incompletely dominant supports the present study. Singh (1961) reported that multiple recessive genes control the resistance to bacterial wilt whereas Akiba et al. (1972) observed that it being governed by a pair of dominant genes.

At Kerala Agricultural University, Girijadevi and Peter (1987) made crosses of two hot pepper lines viz., 'Manjari' and 'Pant C-1' with five sweet peppers. All the F_1s were susceptible or moderately susceptible indicating the recessive nature of inheritance of resistance to bacterial wilt.

Varghese (1991) reported monogenic recessive gene action in brinjal for wilt resistance after evaluating six generations of the cross 'Surya' x 'Pant Rituraj'. Momma and Sakata (1993) reported that the bacterial wilt resistance was partially recessive as there was incomplete dominance towards susceptibility.

Markose (1996) studied the inheritance of bacterial wilt resistance using resistant variety Ujwala and susceptible variety Pusa Jwala by developing six generations of P_1, P_2, F_1, F_2, B_1 and B_2. Performance of the six generations showed that the resistance in Ujwala is monogenically inherited and is incompletely dominant. A survival of 23.3 per cent plants in F_1 indicated that the gene action for wilt resistance makes it rather easy to manipulate this trait in breeding programme.

For tagging bacterial wilt resistance gene, contrasting genotypes for the character of concern are needed. The F_2 population used in the study was developed from the parents showing maximum and minimum bacterial wilt percent which is the most effective and excellent method for developing linked markers. The DNA of thirty F_2 plants tested was isolated prior to the artificial inoculation.

The R or MR reaction exhibited by the varieties might be due to their biochemical make up which will restrict wilt development.

5.3. Molecular characterization of chilli genotypes

Molecular characterization of selected chilli genotypes and F_2 population for tagging bacterial wilt resistance gene was done with RAPD and SCAR marker.

5.3.1. Genomic DNA used for molecular characterization

Isolation of good quality genomic DNA is one of the most important prerequisites for doing RAPD and SCAR analysis. The procedures reported by Doyle and Doyle (1987) and Rogers and Bendich (1994) for the extraction of nucleic acids were compared for the isolation of genomic DNA from chilli with slight modifications. The method suggested by Rogers and Bendich (1994) was the best for the isolation of the genomic DNA from chilli since distinct bands without shearing were obtained. Recovery of DNA was also high.

Tender leaves were chosen for DNA extraction in all the cases. Babu (2000) reported that the quality and quantity of DNA isolated was best when tender leaves were used as compared to mature and half mature leaf samples. Tender leaves contain actively dividing cells with lesser concentration of extra nuclear materials like protein, oil, carbohydrates, fats, and other metabolites that interfere with the isolation of nucleic acids. Fu *et al.* (2003) also reported that the use of young leaves was best for DNA isolation for RAPD assay.

The DNA isolated using the Rogers and Bendich (1994) protocol was appeared as clear and distinct band with less RNA contamination in the agarose gel compared with that of Doyle and Doyle (1987) method (Plate 5a and 5b). Micheli *et al.* (1994) reported that RNA in the genomic DNA preparation often influences the reproducibility of RAPD patterns. So, an attempt was made to remove the contaminants by treatment with Ribonuclease A. Intact DNA bands were obtained after the treatment (Plate 5c).

The quality and quantity of DNA thus isolated was analyzed using both electrophoretic and spectrophotometric methods (NanoDrop® ND-1000 spectrophotometer). In all cases intact clear narrow band indicated non-degraded DNA. The absorbance of nucleic acid samples were measured at a wavelength of 260 nm and 280 nm. The purity of DNA was assessed by the ratio OD_{260}/OD_{280}. The ratio of absorbance ranged between 1.80-1.90 which indicated that quality of DNA was good (Table 6). The DNA, thus isolated, after an appropriate dilution was used as template for RAPD and SCAR analysis.

5.3.2. RAPD (Random Amplified Polymorphic DNA) analysis

The RAPD technique was developed by Williams *et al*. (1991). This technique relies on the differential enzymatic amplification of small DNA fragments using PCR with arbitrary oligonucleotide primers. In RAPD markers, polymorphism results from changes in either the sequence of the primer binding site (which prevents stable association with the primer) or from mutations like insertion, deletion, inversion etc. (which alter the size or prevent the successful amplification of a target DNA). Usually RAPD markers are dominant in nature (Waugh *et al*., 1992) because polymorphisms are detected as the presence or absence of bands. This procedure is rapid, requires only small amounts of DNA, which need not be of high quality, and involves no radioactivity.

Williams *et al.* (1990) reported that even a single base change in the primer sequence could cause a complete change in the set of amplified DNA segments. They also found that the GC content in the 10-mer primer influenced the amplification and a GC content of 40 per cent or more in the primer sequence was needed to generate detectable levels of amplified products. Welsh and McClelland (1990) observed that primers of similar length but different sequence gave a different set of pattern since the template-primer interactions were different. Fakuoka *et al.* (1992) reported that in rice,

increased GC content of the primers in the range of 40-60 per cent tended to increase the number of amplification products.

The utility of RAPD characterization can further be increased by sequencing the termini of specific RAPD markers and designing longer primers for more specific amplification, a technique called Sequence Characterized Amplified Regions (SCAR).

5.3.2.1. Screening of primers for RAPD analysis

Random decamer primer kits obtained from Operon Technologies, USA were used for the present assay. Operon primers are popular among researchers working on RAPD analysis mainly because of ease of availability and better results. The resistant Ujwala and susceptible Pusa Jwala parent were initially screened with six kits of Operon primer series viz., OPE, OPAH, OPN, OPP, OPS and OPY the nucleotide sequence of which are presented in Table 3a and Table 3b.

Twenty two primers which gave more than seven bands were used for molecular characterization of selected genotypes through bulk segregant analysis. These were OPE 3, OPE 5, OPE 6, OPAH 5, OPAH 19, OPN 3, OPN 7, OPP 3, OPP 4, OPP 5, OPP 7, OPS 1, OPS 2, OPS 3, OPS 5, OPS 9, OPS 10, OPS 18, OPY 1, OPY 3, OPY 5 and OPY 7. Screening of those primers with maximum amplification of DNA of resistant and susceptible genotypes minimized the labour and input required for the total RAPD analysis. Sharma *et al.* (2004) and Uma *et al.* (2004) have used random primers from different Operon primer series in their RAPD studies.

5.3.2.2. Bulk Segregant analysis (BSA)

Bulked Segregant Analysis was carried out with resistant parent (Ujwala), susceptible parent (Pusa Jwala), resistant bulk (5 resistant F_2 plants), susceptible bulk (5 susceptible F_2 plants) and Anugraha. Resistant bulk and susceptible bulk consisted of 5

each resistant and susceptible F_2 plants originated from a single F_1 by selfing. The resistant and susceptible F_2 were identified by phenotyping of F_2 plants after artificial inoculation with bacterial ooze.

Twenty two primers, selected after primer screening, belonging to OPE, OPAH, OPN, OPP, OPS and OPY series were used for bulked segregant analysis. The number of bands produced in each genotype by the 22 primers selected was presented in Table 9. Out of the twenty two selected primers screened for polymorphism, OPS 1 produced a polymorphic band of size 1.24 kb in resistant genotypes. It was absent in Anugraha. An OPS 5 produced a band of size 0.31 kb but was faint. The pictographs developed by the 22 selected primers are shown in Fig. 13, Fig. 14, Fig. 15, Fig. 16, Fig. 17 and Fig.18.

Bulked segregant analysis was employed to identify RAPD markers linked to a gene with major effects for bacterial wilt resistance. BSA involves comprising two pooled DNA samples of individuals from a segregating population originating from a single cross (Michelmore *et al*., 1991). Within each pool or bulk, the individuals are identical for the trait or gene of interest but are arbitrary for all other genes. BSA removes the need for screening the entire mapping population with every primer. The minimum size of the bulk is determined by the frequency with which linked loci might be detected as polymorphic between the bulked samples. BSA has been used to screen alternatively for RAPD and RFLP (Monna *et al*, 1995; Chague *et al* 1996). The information content in an individual RAPD maker is very low. It is only when many of these anonymous markers are used to define a genome that they begin to have utility (Williams *et al.*, 1990).

5.3.2.3. Co-segregation analysis

Co-segregation analysis was carried out with resistant parent, resistant bulk, susceptible parent, and susceptible bulk, Anugraha and individuals of resistant and

Primer	Band (kb)	Genotypes				
		1	2	3	4	5
OPY 1	1.27	■	■	■	■	■
OPY 1	1.08	■	■			
OPY 1	0.99	■	■	■		
OPY 1	0.88	■	■	■	■	■
OPY 1	0.73	■	■	■	■	■
OPY 1	0.63			■		
OPY 1	0.56	■	■	■	■	■
OPY 1	0.49	■	■	■	■	■
OPY 1	0.40	■	■			
OPY 1	0.29	■	■			
Primer	**Band (kb)**	**1**	**2**	**3**	**4**	**5**
OPY 3	1.45	■	■	■	■	■
OPY 3	1.22	■	■		■	■
OPY 3	1.03	■	■	■	■	■
OPY 3	0.83	■	■	■	■	■
OPY 3	0.66	■	■	■	■	■
OPY 3	0.62	■	■	■	■	■
OPY 3	0.43	■	■	■	■	■
OPY 3	0.38	■	■	■	■	■
OPY 3	0.31	■	■		■	■
OPY 3	0.27	■	■	■	■	■
Primer	**Band (kb)**	**1**	**2**	**3**	**4**	**5**
OPY 5	2.74		■	■	■	■
OPY 5	1.94	■	■	■	■	■
OPY 5	1.49				■	■
OPY 5	1.22	■	■	■	■	■
OPY 5	0.91				■	■
OPY 5	0.49		■	■		■
OPY 5	0.39	■	■	■	■	■
OPY 5	0.30	■	■	■	■	■
OPY 5	0.24	■	■	■	■	■
OPY 5	0.17	■	■	■	■	■

■ Presence of band □ Absence of band

1. Resistant Parent **2.** Resistant Bulk **3.** Susceptible Parent **4.** Susceptible Bulk **5.** Anugraha

Fig. 13. Pictograph indicating the banding pattern developed with the primers OPY 1, OPY 3 and OPY 5

Primer	Band (kb)	Genotypes				
		1	2	3	4	5
OPY 7	1.71	■	■	■	■	■
OPY 7	1.3	■	■		■	■
OPY 7	0.99	■	■	■	■	■
OPY 7	0.63	■	■	■	■	■
OPY 7	0.49	■	■	■		■
OPY 7	0.39	■	■		■	■
OPY 7	0.33				■	■
Primer	**Band (kb)**	**1**	**2**	**3**	**4**	**5**
OPS 1	1.58					■
OPS 1	1.48					■
OPS 1	1.26					■
OPS 1	1.24	■	■			
OPS 1	1.24			■	■	■
OPS 1	0.76		■	■	■	■
OPS 1	0.38		■	■	■	■
OPS 1	0.23	■	■	■	■	■
OPS 1	0.2	■	■	■	■	■
Primer	**Band (kb)**	**1**	**2**	**3**	**4**	**5**
OPS 2	2.68	■		■	■	■
OPS 2	1.5	■		■	■	■
OPS 2	1.3	■		■	■	■
OPS 2	0.97	■		■	■	■
OPS 2	0.83	■		■	■	■
OPS 2	0.55	■		■	■	■
OPS 2	0.48	■		■	■	■
OPS 2	0.42	■		■	■	■
OPS 2	0.37	■		■	■	■
OPS 2	0.31	■			■	■
OPS 2	0.26	■		■	■	
OPS 2	0.16	■		■	■	■
OPS 2	0.13	■		■	■	■

■ Presence of band □ Absence of band

1. Resistant Parent **2.** Resistant Bulk **3.** Susceptible Parent **4.** Susceptible Bulk **5.** Anugraha

Fig. 14. Pictograph indicating the banding pattern developed with the primers OPY 7, OPS 1 and OPS 2

Primer	Band kb)	Genotypes				
		1	2	3	4	5
OPS 3	1.14	■	■	■	■	■
OPS 3	0.50	■	■	■	■	■
OPS 3	0.45		■			
OPS 3	0.39			■		
OPS 3	0.30	■	■	■	■	■
OPS 3	0.23	■	■	■	■	■
OPS 3	0.17	■	■		■	■
OPS 3	0.11				■	■
OPS 3	0.09	■	■	■	■	■
OPS 3	0.06			■		
Primer	**Band kb)**	**1**	**2**	**3**	**4**	**5**
OPS 5	1.86				■	
OPS 5	1.25	■	■	■	■	■
OPS 5	1.10	■	■	■	■	■
OPS 5	0.85	■	■	■	■	■
OPS 5	0.76	■	■	■	■	■
OPS 5	0.58	■	■	■	■	■
OPS 5	0.38	■	■	■	■	■
OPS 5	0.31	■	■			■
OPS 5	0.20	■	■	■	■	■
OPS 5	0.16	■	■	■	■	■
OPS 5	0.09	■	■	■	■	
OPS 5	0.07	■	■	■	■	■
Primer	**Band(kb)**	**1**	**2**	**3**	**4**	**5**
OPS 9	2.00	■	■	■	■	■
OPS 9	1.50		■	■		
OPS 9	1.20	■	■	■	■	■
OPS 9	0.30	■	■	■	■	■
OPS 9	0.12	■	■	■	■	■
Primer	**Bandkb)**	**1**	**2**	**3**	**4**	**5**
OPS 10	1.02	■	■		■	■
OPS 10	0.82	■	■	■	■	■
OPS 10	0.69	■	■	■	■	■
OPS 10	0.57	■	■	■	■	■
OPS 10	0.39	■	■	■	■	■
OPS 10	0.27	■	■	■	■	■
OPS 10	0.23	■	■	■	■	■
OPS 10	0.14	■	■	■	■	■

■ Presence of band □ Absence of band

1. Resistant Parent **2.** Resistant Bulk **3.** Susceptible Parent **4.** Susceptible Bulk **5.** Anugraha

Fig. 15. Pictograph indicating the banding pattern developed with the primers OPS 3, OPS 5, OPS 9 and OPS 10

Primer	Band (kb)	Genotypes				
		1	2	3	4	5
OPS 18	1.75	■				
OPS 18	1.54			■		■
OPS 18	1.25			■		
OPS 18	1.05	■	■	■	■	■
OPS 18	0.99	■	■	■		
OPS 18	0.80	■	■	■	■	■
OPS 18	0.58	■	■			
OPS 18	0.40	■	■	■	■	■
OPS 18	0.32				■	
OPS 18	0.19	■	■	■	■	■
OPS 18	0.12	■	■	■	■	■
OPS 18	0.09	■	■	■		
Primer	**Band (kb)**	**1**	**2**	**3**	**4**	**5**
OPAH 5	1.38			■		■
OPAH 5	1.09	■			■	■
OPAH 5	0.97	■	■	■	■	■
OPAH 5	0.63	■	■	■	■	■
OPAH 5	0.26	■	■	■	■	■
OPAH 5	0.16	■	■	■	■	■
Primer	**Band (kb)**	**1**	**2**	**3**	**4**	**5**
OPAH 19	2.09	■	■	■	■	■
OPAH 19	1.69	■	■	■	■	■
OPAH 19	1.36	■	■	■	■	■
OPAH 19	1.25		■	■	■	■
OPAH 19	0.62	■	■	■	■	■
OPAH 19	0.52		■	■	■	■
OPAH 19	0.38	■	■	■	■	■
Primer	**Band (kb)**	**1**	**2**	**3**	**4**	**5**
OPE 3	0.83	■	■	■	■	■
OPE 3	0.57	■		■	■	■
OPE 3	0.45	■	■	■	■	■
OPE 3	0.36	■	■	■	■	■
OPE 3	0.28	■	■	■	■	■

■ Presence of band □ Absence of band

1. Resistant Parent **2.** Resistant Bulk **3.** Susceptible Parent **4.** Susceptible Bulk **5.** Anugraha

Fig. 16. Pictograph indicating the banding pattern developed with the primers OPS 18, OPAH 5, OPAH 19 and OPE 3

Primer	Band (kb)	Genotypes 1	2	3	4	5
OPE 5	3.82	■	■	■	■	■
OPE 5	1.38	■	■	■	■	■
OPE 5	2.47					■
OPE 5	1.38	■	■	■	■	■
OPE 5	0.86	■	■	■	■	■
OPE 5	0.65	■	■	■	■	■
OPE 5	0.54	■	■	■		
OPE 5	0.43	■	■	■		
OPE 5	0.33					■
Primer	**Band (kb)**	**1**	**2**	**3**	**4**	**5**
OPE 6	3.44		■	■	■	■
OPE 6	1.59	■	■	■		■
OPE 6	1.37	■	■	■	■	■
OPE 6	1.18	■	■	■	■	■
OPE 6	1.06	■	■	■	■	■
OPE 6	0.75	■	■	■	■	■
OPE 6	0.69	■	■	■	■	■
OPE 6	0.55	■	■	■	■	■
Primer	**Band (kb)**	**1**	**2**	**3**	**4**	**5**
OPN 3	1.65	■	■	■	■	■
OPN 3	0.72	■	■	■	■	■
OPN 3	0.56	■	■	■	■	■
OPN 3	0.36	■	■	■	■	■
OPN 3	0.22	■	■	■	■	■
Primer	**Band (kb)**	**1**	**2**	**3**	**4**	**5**
OPN 7	2.31	■	■	■	■	■
OPN 7	1.99	■	■	■	■	■
OPN 7	1.61			■		
OPN 7	1.43	■	■			■
OPN 7	1.27	■	■	■	■	■
OPN 7	0.81	■	■	■	■	■
OPN 7	0.51	■	■	■	■	■
OPN 7	0.42	■	■		■	■
OPN 7	3.40	■	■	■	■	■
OPN 7	0.25	■	■	■	■	■
OPN 7	0.19	■	■	■	■	■
OPN 7	0.09	■	■		■	■

■ Presence of band □ Absence of band

1. Resistant Parent **2.** Resistant Bulk **3.** Susceptible Parent **4.** Susceptible Bulk **5.** Anugraha

Fig. 17. Pictograph indicating the banding pattern developed with the primers OPE 5, OPE 6, OPN 3 and OPN 7

Primer	Band (kb)	Genotypes				
		1	2	3	4	5
OPP 3	2.53	■	■	■	■	■
OPP 3	1.87	■	■	■	■	■
OPP 3	1.57	■	■	■	■	■
OPP 3	1.13	■	■	■	■	■
OPP 3	0.92	■	■	■	■	■
OPP 3	0.43	■	■		■	■
OPP 3	0.29	■	■		■	■
Primer	**Band (kb)**	**1**	**2**	**3**	**4**	**5**
OPP 4	3.16	■	■	■	■	■
OPP 4	2.43			■		
OPP 4	1.53	■	■		■	■
OPP 4	0.50	■	■	■	■	■
OPP 4	0.37	■	■	■	■	■
OPP 4	0.21	■	■	■	■	■
Primer	**Band (kb)**	**1**	**2**	**3**	**4**	**5**
OPP 5	3.20					■
OPP 5	1.59	■	■	■	■	■
OPP 5	1.17	■	■	■	■	■
OPP 5	0.61			■	■	■
OPP 5	0.46				■	■
OPP 5	0.12	■	■	■	■	■
Primer	**Band (kb)**	**1**	**2**	**3**	**4**	**5**
OPP 7	3.39	■	■		■	■
OPP 7	2.63	■	■		■	
OPP 7	2.25	■	■		■	■
OPP 7	1.93	■	■	■	■	■
OPP 7	1.40	■	■	■	■	■
OPP 7	0.96	■	■		■	■
OPP 7	0.78	■	■	■	■	■
OPP 7	0.65	■	■		■	■
OPP 7	0.50	■	■	■	■	■
OPP 7	0.43	■	■	■	■	■
OPP 7	0.39	■	■		■	■
OPP 7	0.27	■	■	■	■	■

■ Presence of band □ Absence of band

1. Resistant Parent **2.** Resistant Bulk **3.** Susceptible Parent **4.** Susceptible Bulk **5.** Anugraha

Fig. 18. Pictograph indicating the banding pattern developed with the primers OPP 3, OPP 4, OPP 5 and OPP 7

susceptible bulk with selected primer OPS 1. The result is presented in Plate 12. One band of size 1.24 kb was present in resistant parent, resistant bulk and 5 resistant F_2's but it was absent in susceptible parent, susceptible bulk and 5 susceptible F_2's.

The close analysis of segregation pattern of these markers with resistant phenotype helps in selection of resistant lines within large breeding population. Jena *et al.* (2002) used RAPD analysis to tag a gene for resistance to BPH in rice. Out of 19 co-dominant markers, one primer OPA 16 was linked with BPH resistant gene (0.52 cM). Further, the associated markers can be converted to SCAR markers, which are co dominant. Linkage analysis should be done with all the F_2 progeny to confirm its close linkage with the bacterial wilt resistance gene.

Bulk segregant analysis and co-segregation analysis reduces the error associated with molecular markers in distinguishing polymorphism among resistant and susceptible individuals. Co-segregation analysis crosschecked the information produced by bulk segregant analysis.

5.3.2.4. Analysis of amplification profiles

The scored data of all the 22 primers was used for similarity based analysis using NTYSYS pc. (ver.2.1). The SIMQUAL programme was used to calculate Jaccard's coefficient, a common estimator of genetic identity (Table 10). Based on estimated Genetic Similarity Matrix using Jaccard's coefficient, the highest (93 per cent) genetic similarity was noticed between a resistant parent (RP) and resistant bulk (RB). The lowest (77 per cent) similarity was noticed between resistance parent and susceptible parent (SP). Anugraha showed 91 per cent similarity with susceptible bulk.

The dendrogram revealed high degree of relatedness between the resistant parent and resistant bulk and between susceptible bulk and Anugraha genotypes. The resistant parent and susceptible parent showed maximum dissimarity.

The genetic similarity values are generally high when RAPD markers are used to study polymorphism among accessions within a species. In case of variation studies among individuals of different species, the similarity values tend to decrease indicating a higher difference at DNA level.

5.4. TRANSFORMATION AND CLONING OF DNA

Transformation may be described as the uptake of any DNA molecule by any type of cell, regardless of whether the uptake results in a detectable change in the cell, or whether the cell involved is that of bacterial, fungal, animal or plant. Uptake and stable retention of a plasmid is usually detected by looking for expression of the genes carried by the plasmid. In order to undergo transformation, the cell must become competent. Bacteria, such as *E. coli* may be artificially induced to become competent. *E. coli* cells are normally sensitive to the growth inhibitory effects of the antibiotics ampicillin and tetracycline. The success of transformation is measured in terms of transformation efficiency.

5.4.1. Detection and elution of specific amplicon

A distinct band of size ~1.24 kb was observed in resistant parent Ujwala, resistant bulk and five resistant F_2 when the genomic DNA was amplified using OPS 1 primer. In order to elute the DNA from the specific band, four RAPD reactions with the same primer and common template DNA (Ujwala) were set. The specific band of size ~1.24 kb was absent in negative control Pusa Jwala indicating that it is unique to the resistant genotype.

5.4.2. Preparation and screening of competent cells

Aseptic condition was maintained through out the preparation of competent cells, since contamination in competent cells can produce white colonies after blue

white screening. *E. coli* cells that were soaked in ice cold salt solution were more efficient at DNA uptake than unsoaked cells. Possibly $CaCl_2$ causes the DNA precipitates onto the outside of the cells, or perhaps the salt is responsible for some kind of change in the cell wall that improves DNA binding. The actual movement of DNA into competent cells is stimulated by briefly raising the temperature to 42^0C.

Competence of *E. coli* JM 109 cells was confirmed by transforming them with plasmid (pUC18) containing selectable marker ampicillin resistance. A selectable marker is simply a gene that provides a transformed cell with a new characteristic, one that is not possessed by a non-transformant. High frequency of transformation was obtained with the competent cells prepared (Plate 13). Only those competent cells of E. *coli* that have taken up a pUC18 plasmid are able to form colonies on an agar medium that contains ampicillin, do not produce colonies on the selective medium. Plating onto selection medium enables transformants to be distinguished from non-transformants.

5.4.3. Ligation of eluted DNA

The specific amplicon eluted from the resistant genotype Ujwala (amplified with OPS 1 primer) was of ~1.24 kb size that could be conveniently ligated with pGEM-T (Promega) Easy Vector System I. insertion of eluted DNA fragment into the plasmid destroys the integrity of the genes present on the molecules. The vector was custom made by cutting with *Eco*RV and adding 3'-terminal thymidine to both ends. These single 3'-T overhangs at the insertion site improve the efficiency of ligation of a PCR product into the plasmid by preventing recircularization of the vector and provide a compatible overhang for the product generated by *Taq* polymerase. This thermostable enzyme often adds a single deoxyadenosine, in a template independent fashion, to the 3' ends of the amplified fragments (Clark, 1998). The high copy number pGEM-T Easy Vector contain T7 and SP6 RNA polymerase promoter flanking a multiple cloning site within the *lac*Z region. This facilitates blue-white screening of recombinants by insertional inactivation of β-galactosidase. This vector also possesses multiple

restriction sites within the *lac*Z gene. It was found that incubation of ligation reaction at room temperature for one hour followed by overnight incubation at 4^0C yields maximum number of transformants.

5.4.4. Transformation of *E. coli* with recombinant plasmid

Ligated product containing the specific amplicon was used to transform the *E. coli* cells, which could be later picked up from the media containing X-gal, and IPTG based on blue-white screening. The *E. coli* cells were given a shock treatment by abruptly increasing the temperature from zero to 42^0C for a short period of 90 seconds. The treated bacteria are more likely to take up foreign DNA. Temperature is an important factor to obtain the highest transformation efficiency.

The *E. coli* cells after transformation when plated on LBA plate overlaid with X-gal/IPTG; produced blue and white colonies after overnight incubation. The pGEM-T easy vector carries a gene called *lacZ*. Which code for part of the enzyme β-galactosidase. Cloning with pUC18 involves insertional inactivation of the *lacZ* gene, with recombinants identified because of their inability to synthesize β- galactosidase.

Screening for β-galactosidase presence or absence is in fact quite easy. Rather than assay for lactose being split to glucose and galactose, a slightly different reaction catalalysed by the enzyme is tested. This involved a lactose analogue called X-gal which is broken by β-galactosidase to a product that is colored deep blue. If X-gal is added to the agar, along with ampicillin, the non-recombinant colonies, the cells of which synthesize β-galactosidase, will be coloured blue, whereas recombinant colonies with a disrupted *lacZ* gene are unable to poduce β- galactosidase and hence will be white. This system is called Lac selection.

During transformation, the host encoded and vector encoded protein regions of β-galactosidase undergoes α-complementation to form enzymatically active proteins. The *E. coli* cells that are not transformed with the recombinant plasmid can further

utilize the chromogenic substrate X-gal and appear as blue colonies (Ullman *et al.*, 1967). The *E. coli* cells carrying recombinant plasmids which are not capable of α-complementation and developed into white colonies.

Competence of *E. coli* JM 109 cells was confirmed by transforming the cells with a pUC18 plasmid containing an ampicillin resistance marker. There was luxuriant growth of the competent cells in LBA- ampicillin plates overlaid with X- gal and IPTG, indicating high transformation efficiency. *E. coli* cells alone could not grow in the media containing ampicillin, since they lacked the resistance encoding sequence. But all the competent cells harbouring the plasmid could grow in that media.

5.4.4. CONFIRMATION OF RECOMBINATION

5.4.4.1. Detection of the insert by RAPD amplification

The plasmid from white colony was checked for the presence of insert by PCR confirmation. The plasmid DNA from blue as well as white colony was amplified using selected random primer OPS 1. A positive control RAPD reaction was set up using the DNA of resistant parent, Ujwala as template. Single amplified band of 1.24 kb, which is exactly similar to the genomic DNA amplification product, was obtained in plasmid DNA isolated from white colony, confirming the presence of insert in the plasmid. No amplification was detected in plasmid DNA isolated from blue colony (Plate 17). The insert used for transformation was also loaded along with the amplified plasmid PCR products, to confirm the presence of insert in the plasmid.

5.4.5 Sequencing of the cloned fragment

The cloned fragment when sequenced by automated sequencing with SP6 primer provided the 5'-3' sequence data in the upstream direction since it is a reverse primer. By this the precise order of nucleotides in a piece of DNA can be determined.

5.5. Sequence analysis of the cloned fragment

Sequence information obtained (referred as *Chilli seq 1*) for the fragment with OPS 1 primer had a size of 1506 bp (Fig. 3). The sequences obtained after automated sequencing were subjected to vector screening using the VecScreen tool in NCBI to remove vector regions from the clones (Fig. 4). The region starting from 1 to 69 bases and 510 to 1506 bases showed similarity with the pGEM-T Easy Vector sequence, and it was deleted from the original sequence. The sequence of 440 bases named as *Chilli seq 2* obtained after vector screening is presented in Fig. 5.

Homology of the sequence obtained from the cloned product of chilli (*Chilli seq 2*) with the other reported sequence was analyzed using blastn search tool. It has shown 79 per cent identity to *Capsicum annuum* clone pC.a.D Ty1-copia retrotransposon nonfunctional polyprotein gene, 100 per cent identity to ethylene responsive element binding protein C2 mRNA, 90 per cent identity to lipid transfer protein III gene in *Capsicum annuum*, 94 per cent identity to *Capsicum annuum* clone A1-4 PR-protein gene and 94 per cent identity with beta-galactosidase (BG1) (Fig. 6). The sequences were translated into amino acid sequence. The amino acid sequence of *Chilli seq 2* was obtained using Biology Workbench (Fig. 7). The BLASTp search of this translated amino acid sequences producing significant alignments are given in Fig. 8. Open reading frame identified in the sequence *Chilli seq 2* using NCBI tool 'ORF Finder' contained no ORF.

Nucleic acid statistics of the *Chilli seq 2* obtained in the Biology Workbench is presented in Table 11. The fragment had low GC content (38.9%) and high AT content (61.1%).

Major restriction sites deduced for the fragment *Chilli seq 2* are provided in Table 12. It contains frequent cutter *Eco*RV and the rare cutter *Mbo*I and had single restriction site in the sequence. The restriction map is represented in Fig. 9. Deduced amino acid sequence of protein analyzed and proportion of each amino acid calculated

using AASTATS of Biology Workbench are presented in Table 13. The major amino acids deduced from the cloned fragment *Chilli seq 2* were Leucine, Serine, Isoleucine, Glutamine, Arginine and Glutamate.

The hydropathy plot analysis revealed that there is no transmembrane region in the cloned sequence, *Chilli seq 2* (Fig. 10). The secondary structure of proteins predicted by SOPMA programme of ExSpasy tool is presented in Fig. 11. The amino acid deduced from *Chilli seq 2* had 51.09 per cent alpha helix, 8.03 per cent extended strands, 7.30 per cent beta turns and 33.58 per cent random coils. It also indicated that the protein is made up of 67 hydrophilic and 70 hydrophobic amino acids.

5.6. MOLECULAR CHARACTERIZATION OF CHILLI GENOTYPES BY SCAR MARKER

5.6.1. SCAR Primer designing

A pair of SCAR primers was designed. For designing longer primers of 24 bp and 23 bp length the sequence of RAPD fragments OPS 1(*Chilli seq 2*) were used (Fig. 12). A pair of 24 mer oligonucleotide primers designed which linked to *Tm-1* locus to have sequences identical to 5' endmost 24 bases of the cloned RAPD DNA which confers tomato mosaic virus resistance in tomato (Ohmori *et al.,* 1996). Eight RAPD markers were converted into SCAR markers for onion linkage maps in *Allium* species. Each SCAR marker contained the original 10 bases of the RAPD primer plus the next 14 internal bases from the end (Masuzaki *et al.*, 2007)

Primers were synthesized from Integrated DNA Technologies, USA. Details of primers viz., annealing temperature, melting temperature and GC% used for the amplification of resistant parent, resistant bulk and 5 F_2's are given in Table 14. Primers sequences and length of primers are provided in Table 15.

5.6.2. PCR amplification using SCAR primers

A pair of 24 mer oligonucleotide primers synthesized based on the end sequences of the RAPD clone OPS 1_{1240} were used for amplification of the resistant parent (Ujwala), susceptible parent (Pusa Jwala), resistant bulk, susceptible bulk and individual of resistant bulk. The gel document revealed an amplicon of 396 bp in all the genotype tested (Plate 18). Polymorphism could not be detected between the resistant and susceptible genotypes. It was also seen that the SCAR marker of OPS 1_{1240} was smaller than the RAPD marker OPS 1_{1240}.

SCAR primers derived from the sequences of AFLP fragments AR257 and AS168 failed to generate phenotype-specific amplification and instead a single monomorphic band was produced in both parents in rice (Jain *et al.*, 2004).

5.7. Future line of work

1. Design SCAR primers with full length sequence data and test its efficiency in distinguishing resistant and susceptible genotypes
2. Isolate mRNA from resistant genotypes after artificial inoculation with pathogen, followed by cDNA synthesis and gene prediction.
3. Molecular characterization of susceptible and resistant genotypes with more number of primers and clone the polymorphic band present in resistant genotypes.

Summary

6. SUMMARY

The study on "Molecular characterization of chilli (*Capsicum annuum* L.) genotypes for tagging bacterial wilt resistance gene" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period 2006-2008. The main objective of the study was to develop a molecular marker for bacterial wilt resistance in chilli with RAPD assay and SCAR marker. The genotypes used for study were resistant varieties Ujwala, Anugraha, and susceptible variety Pusa Jwala, F_1 and F_2 progenies of cross Ujwala x Pusa Jwala.

The salient findings of the study are summarized below:

1. F_1 plant was raised by crossing resistant variety Ujwala as the ovule parent and susceptible variety Pusa Jwala as the pollen parent. F_1 plant of cross Ujwala x Pusa Jwala was selfed and segregating F_2 progenies for bacterial wilt incidence were raised.

2. A pot culture study was carried out to standardize artificial inoculation techniques in chilli for bacterial wilt incidence using the susceptible variety Pusa Jwala. Seedlings at 5-6 leaf stage, 30 days after sowing were inoculated with fresh bacterial ooze. Four different methods *viz*., root dipping, stem-puncturing, soil drenching and soil drenching with wounding were compared and plants were scored for bacterial wilt incidence as per the Mew and Ho (1976) system. Soil drenching with wounding method recorded 90 per cent wilting within 15 days. So this method was used for phenotyping of genotypes for bacterial wilt incidence.

3. The genotypes were phenotyped after artificial inoculation and classified into two different categories according to Mew and Ho (1976) system. Ujwala was ranked as resistant with 90 per cent survival, Pusa Jwala was susceptible with 15

per cent survival, and Anugraha was resistant with 80 per cent survival. In F_1 and F_2 of cross Ujwala x Pusa Jwala, the survival percentage was 20 and 30, respectively.

4. The protocols suggested by Doyle and Doyle (1987) and Rogers and Bendich (1994) were compared for the isolation of genomic DNA. Tender leaves from the selected plants were used for the genomic DNA isolation. The protocol suggested by Rogers and Bendich (1994) was found to be the best for the isolation of genomic DNA from chilli. The quantity and quality of DNA was analyzed by both electrophoresis and spectrophotometric method (NanoDrop® spectrophotometer). The absorbance ratio ranged from 1.80-1.90 which indicated good quality of DNA.

5. The RNA contamination was completely removed through RNAse treatment, which resulted in DNA with no impurities and suitable for RAPD and SCAR analysis.

6. Primer screening was done to select primers with good amplification. Forty seven random decamer primers belonging to six Operon series were screened. Twenty two primers which gave more than seven bands were used in RAPD analysis.

7. Bulk segregant analysis was done with genotypes Ujwala, Pusa Jwala, Anugraha and resistant and susceptible bulk. Of the 22 primers used in bulked segregant analysis, only one primer, OPS 1 produced a DNA fragment, specific to resistant phenotypes. The unique band of size 1.24 kb was present in resistant genotype Ujwala and resistant bulk. It was absent in susceptible parent and susceptible bulk.

8. Co-segregation analysis of individuals of resistant bulk and susceptible bulk with OPS 1_{1240} primer revealed its presence in resistant genotypes. Co-

segregation analysis was also done with Anugraha. a light band was present corresponding to the unique amplicons by OPS 1_{1240}.

9. The scored data based on RAPD banding pattern was used to construct a dendrogram using the NTSYS pc. (ver 2.1) software. The dendrogram, revealed that the closely related genotypes were Ujwala (resistant parent) and resistant bulk (resistant), with a genetic similarity coefficient of 0.93, followed by Anugraha and susceptible bulk with a similarity coefficient of 0.90. The susceptible parent, 'Pusa Jwala' was widely separated from other genotypes in the main clusters at a genetic similarity value of 0.8. The minimum similarity value of 0.769 was obtained between resistant parent Ujwala and susceptible parent Pusa Jwala.

10. The polymorphic gene fragment 1.24 kb obtained with the RAPD assay with OPS 1_{1240} primer using template DNA from the resistant genotype Ujwala was eluted and cloned into pGEM-T vector and the product was used to transform competent cells of *E. coli* JM 109 cells. A combination of blue and white colonies was obtained after overnight incubation confirming successful transformation.

11. Presence of insert was checked by RAPD amplification with OPS 1_{1240} primer. An amplicon of 1.24 kb was obtained in plasmid of white colony where as it was absent in plasmid of blue colony.

12. The cloned fragment was sequenced in automated sequencer. Sequencing as well as primer walking with SP6 universal primer gave the sequence data for 1506 bases and was named as *Chilli seq 1*. Vector screening was perfomed to remove the vector sequence from *Chilli seq 1* using the VecScreen tool in NCBI. The insert sequence of 440 bases was obtained. This sequence was named as *Chilli seq 2*.

13. *Chilli seq 2* was subjected to nucleotide Blast search. It revealed significant levels of homology with *Capsicum annuum* lipid transfer protein III gene, clone A1-4 PR-protein gene and ethylene responsive element binding protein C2 of chilli. The sequence was deposited in the public domain.

14. Open reading frame was searched in *Chilli seq 2* using NCBI tool" ORF Finder" and no ORF was found.

15. The nucleic acid composition of the *Chilli seq 2* was analysed using NASTATS tools available in Biology Workbench. The fragment had low GC content (38.9%) and high AT content (61.1%).

16. Restriction analysis of *Chilli seq 2* revealed distribution patterns of cleavage sites of different restriction enzymes and it contained frequent cutter *Eco*RV and rare cutter *Mbo*I.

17. The secondary structure predicted for *Chilli seq 2* contained 51.09 per cent alpha helix, 8.03 per cent extended strands, 7.30 per cent beta turns and 33.58 per cent random coils and it showed that alpha helices are the major structural components.

18. The aminoacid and nucleic acid composition of the *Chilli seq 2* were analysed using AASTAT tools available in Biology Workbench. The major amino acids deduced from the sequence were Leucine, Serine, Isoleucine, Arginine and Glutamate.

19. Kite-Doolittle hydropathy plot showed that *Chilli seq 2* contained more number of hydrophobic amino acids.

20. One pair of gene specific SCAR primer was designed based on the end sequences of *Chilli seq 2*. Forward and reverse primers had a melting temperature of 68^0C and 70^0C respectively and sequence length of 24 to 23 bp.

21. The amplification cycle was altered to give specific amplification pattern with SACR primers. The annealing temp was raised from 37^0C (for RAPD) to 65^0C and number of cycle reduced to 30. Amplicon size of the fragment obtained was approximately 396 bp in all the genotypes tested. Polymorphism could not be detected between the resistant and susceptible genotypes. This may be due to the absence of full length sequence information of cloned fragment.

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Appendices

APPENDIX-I

List of Laboratory Equipments Used For the Study

Spectrophotometer	Spectronic Genesys-5, Spectronic Instrument, USA
Refrigerated centrifuge	Kubota, Japan
Horizontal electrophoresis system	Biorad
Thermal cycler	Mastercycler personal, Eppendorf
Gel documentation system	1. Gel DOC-It ™ Imaging system UVP (USA) 2. Phosphor Imager FLA-5100 Fuji

APPENDIX-II

Composition of Reagents Used for DNA Isolation

1. Doyle and Doyle method

4X Extraction Buffer

Sorbitol – 2.5 g

Tris HCl – 4.8 g

EDTA – 0.74 g

Dissolved in about 80 ml of distilled water, adjusted the pH to 7.5 and made up to 100 ml with distilled water.

Lysis buffer

Tris HCl (1 M, pH 8) – 20 ml (15.76 g per 100 ml)

EDTA (0.2 ml) – 20 ml (9.305 g per 100 ml)

NaCl (5 M) – 40 ml (29.22 g per 100 ml)

Distilled water – 20 ml

CTAB – 2 g (Dissolved in 20ml distilled water and then added to the remaining components).

5% Sarcosin

Sarcosin – 5 g

Distilled water – 100 ml

TE Buffer

10 mM Tris (pH 8)

1 mM EDTA (pH 8)

2. Rogers and Bendich (CTAB) method

2X CTAB Extraction Buffer

CTAB (2%, v/v)

100 mM Tris buffer (pH 8)

20 mM EDTA (pH 8)

1.4 M NaCl

10% CTAB Solution

10% CTAB (w/v)

0.7 M NaCl

TE Buffer

10 mM Tris (pH 8)

10 mM EDTA (pH 8)

APPENDIX-III

Composition of Buffers and Dyes used for gel electrophoresis

1. **TAE Buffer 50X (for 1 litre)**

   242 g Tris base

   57.1 ml glacial acetic acid

   100 ml 0.5 M EDTA (pH 8.0)

2. **TBE Buffer 10X (for 1 litre)**

   54 g Tris base

   27.5 g Boric acid

   20 ml 0.5 M EDTA (pH 8.0)

3. **Loading Dye (6X)**

   0.25% bromophenol blue

   0.25% xylene cyanol

   30% glycerol in water

4. **Formamide Dye**

   Formamide – 10 ml

   Xylene cyanol – 10 mg

   Bromophenol blue – 10 mg

   0.5 M EDTA (pH 8.0) – 200 µl

APPENDIX – IV

Composition of reagents used for cloning and transformation studies

I. Reagent used for competent cell preparation and cloning

1. Solution A

Ice- cold 100 mM $CaCl_2$

2. Luria Bertani (LB) broth

Tryptone	- 10 g
Yeast Extract	- 5 g
NaCl	- 5 g
pH adjusted to 7 ± 0.2	
Distilled water	- 1 L

3. Luria Bertani Agar medium

Tryptone	- 10 g
Yeast Extract	- 5 g
NaCl	- 5 g
Agar	- 20 g
pH adjusted to 7 ± 0.2	
Distilled water	- 1 L

II. Reagents used for plasmid isolation

a) Solution I (Resuspension buffer)

50 mM glucose

25 mM Tris

10 mM EDTA

b) Solution II (Lysis buffer)

2 N NaOH

1 per cent SDS

c) Solution III (Neutralization buffer)

5M Potassium acetate (60 ml)

Glacial acetic acid (11.5 ml)

Distilled water (28.5 ml)

Abstract

MOLECULAR CHARACTERIZATION OF CHILLI (*Capsicum annuum* L.) GENOTYPES FOR TAGGING BACTERIAL WILT RESISTANT GENE

By

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ABSTRACT OF THETHESIS

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ABSTRACT

Investigation on "Molecular characterization of chilli (*Capsicum annuum* L.) genotypes for tagging bacterial wilt resistance gene" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period 2006-2008.

Bacterial wilt caused by *Ralstonia solanacearum* (Smith) Yabuuchi *et al*. is the most important problem of chilli cultivation in warm humid tropics. The loss due to this varies from 30-100 per cent. Use of chemicals and field sanitation are not sufficient for controlling the disease. World wide approach is to use resistant varieties. KAU has developed and released bacterial wilt resistant varieties *viz*., Ujwala, and Anugraha for cultivation.

The present investigation was taken up to develop a molecular marker for bacterial wilt resistance gene through molecular characterization of resistant and susceptible genotypes. The genotypes used for the study were Ujwala, Pusa Jwala, Anugraha and F_2 population of the cross between Ujwala and Pusa Jwala. The genotypes were phenotyped for bacterial wilt incidence through artificial inoculation with *Ralstonia solanacearum*. The varieties Ujwala and Anugraha were resistant. Pusa Jwala, F_1 and F_2 progenies were susceptible.

The molecular marker techniques used were RAPD and SCAR. Genomic DNA was isolated by Rogers and Bendich (1994) method. Forty seven decamer primers belonging to six Operon primer series *viz*., OPE, OPAH, OPN, OPP, OPS and OPY were used for primer screening. Twenty two primers which gave more than seven bands were used for molecular characterization of selected genotypes through bulk segregant analysis. OPS 1 primer amplified a DNA fragment of 1.24 kb in resistant parent and resistant bulk. Co-segregation analysis was also done with OPS 1 primer with individuals of susceptible and resistant bulk.

The polymorphic band produced by OPS 1 primer in resistant parent and resistant bulk was eluted and cloned in pGEM-T vector, and was transformed into *E. coli* JM 109 cells. Recombination of the insert was confirmed through RAPD reaction with OPS 1 primer. The cloned fragment was sequenced to obtain the nucleotide sequence information and was named as *Chilli seq 1*.

The sequence obtained after vector sequence deletion was named as *Chilli seq 2* and it contained only 440 bp in place of 1240 bp. It was subjected to nucleotide blast search. It revealed significant levels of homology with *Capsicum annuum* ethylene responsive element binding protein C2, lipid transfer protein III gene, clone A1-4 PR-protein gene and ripening regulated protein DDTFR10/A gene of chilli deposited in the public domain. The sequence was also subjected to various sequence analysis using bioinformatics tools, which include ORF finder, SOPMA, NEB cutter, Hydropathy plot, NASTATS and AASTATS tools of Biology Workbench.

Based on the end sequences of the cloned RAPD fragment, SCAR primers were designed. The efficiency of SCAR primer to distinguish resistant and susceptible genotypes was tested but no polymorphism was detected. The SCAR primer OPS 1_{1240} amplified a fragment of 396 bp in all the genotypes tested. Full sequence data of the cloned RAPD fragment was not available for SCAR primer designing. More efforts needed to get full-length sequence data.