DEVELOPMENT OF TRAIT RELATED MARKERS FOR BACTERIAL WILT RESISTANCE IN TOMATO (*Solanum lycopersicum* L.)

By

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THESIS

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DECLARATION

I hereby declare that the thesis entitled **"Development of trait related markers for bacterial wilt resistance in tomato (*Solanum lycopersicum* L.)"** 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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CERTIFICATE

Certified that the thesis entitled **"Development of trait related markers for bacterial wilt resistance in tomato (*Solanum lycopersicum* L.)"** is a record of research work done independently by Ms. Sameera Karumannil (2005-11-137) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, associateship or fellowship to her.

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Sameera Karumannil


Dedicated to
My Loving
Family

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ABBREVIATIONS

A	Adenine
AASTATS	Amino acid statistics
AFLP	Amplified Fragment Length Polymorphism
ACRI	Agriculture College and Research Institute
bp	Base pairs
β	Beta
BLAST	Basic Local Alignment Search Tool
C	Cytosine
cm	Centimeter
cM	Centi Morgan
CPBMB	Centre for Plant Biotechnology and Molecular Biology
cfu	Colony forming unit
°C	Degree Celsius
DAT	Days after transplanting
DNA	Deoxyribo Nucleic Acid
dNTPs	Deoxyribo Nucleoside Triphosphate
EDTA	Ethylene Diamine Tetra Acetic acid
FLA	Fluorescent Image Analyzing system
G	Guanine
γ	Gamma
g	Gram
HPKV	Himachal Pradesh Krishi Viswa Vidyalaya
hr	Hour (s)
IARI	Indian Agricultural Research Institute
IIHR	Indian Institute of Horticulture Research
IPTG	Isopropylthio-β-D-galactoside
KAU	Kerala Agricultural University
kb	Kilo base pairs
L	Litre
LB	Luria Broth
LBA	Luria Bretani Agar
Mb	Mega base pairs
M	Mole
min	Minute(s)
ml	Millilitre
mM	Millimole
μg	Microgram
μl	Microlitre
μM	Micromole
ng	Nanogram
nm	Nanometer
NASTATS	Nucleic Acid Statistics
NCBI	National Centre for Biotechnology Information

NTSyS	Numerical Taxonomy System of Multivariate Statistical Program
OD	Optical Density
ORF	Open Reading Frame
OUAT	Orissa University of Agriculture and Technology
PCR	Polymerase Chain Reaction
pH	Hydrogen ion concentration
%	Percentage
QTL	Quantitative Trait Loci
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RGA	Resistance Gene Analogues
RNA	Ribo Nucleic Acid
RNase	Ribonuclease
rpm	Rotations per minute
SDS	Sodium Dodecyl Sulphate
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
TZC	Triphenyl Tetrazolium Chloride
U	Unit
UPGMA	Unweighted Pair Group Method of Arithmetic Averages
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

Tomato (*Solanum lycopersicum* L.) is considered as one of the most important widely grown and consumed vegetable crops in the world. India is the sixth largest producer of tomato in the world with an area of 0.50 million hectares under cultivation and with a productivity of 17.4MT/ha (Chamber *et al*., 2006). Tomato is used as a fresh vegetable and also can be processed and canned. Nutritionally tomato is a significant dietary source of vitamin A and C. Furthermore, recent studies have shown the importance of lycopene, a major component of red tomatoes, which has antioxidant properties that can help to resist cancer and heart diseases.

One of the major constraints of tomato cultivation in many tropical and subtropical countries is the incidence of bacterial wilt caused by *Ralstonia solanacearum* (Smith) Yabuuchi *et al*. This soil-borne pathogen has different races and wide host range of over 200 plant species (Buddenhagen *et al*., 1962). Tomato bacterial wilt is mostly caused by Race 1 strains that are highly variable in their genotype and aggressiveness on tomato. The disease is widespread in most of the tomato growing states of India causing yield losses up to 90 per cent. Kerala, being characterized by warm, humid tropical climate and acidic soil, is a hot spot for bacterial wilt disease.

The bacterium invades the vascular tissues from wounded roots or natural openings, which occurs subsequent to the emergence of secondary roots (Kelman and Sequeira, 1965). Bacterial colonization of stem results in browning of the xylem, foliar epinasty and lethal generalized wilt. As of other vascular plant diseases, chemicals are not effective and sanitation measures are difficult to apply.

Although bacterial wilt is a disease of major economic importance, little is known about the molecular mechanisms governing resistance. In tomato, resistance to *Ralstonia solanacearum* is polygenic and several loci governing

resistance have been reported. Breeding for resistance remains as the best strategy even if this property may fluctuate from breeding areas to cropping areas due to extreme variability and adaptation of the pathogen. It is doubtful that any single line of tomato exists that carries resistance to all strains of *Ralstonia solanacearum*, since tomato breeding programmes are usually specific for geographic locations and hundreds of different strains of the pathogen are present worldwide.

So, if the disease is to be overcome genetically, a more coordinated worldwide approach is required. The International SOL Genome Project including more than 30 countries all over the world was initiated for developing a web based Solanaceae bioinformatics framework that will link sequences, phenotypes and habitats to promote scientific discovery. They have selected tomato as the reference species for genome sequencing and India is making a major contribution by sequencing the fifth chromosome.

DNA marker technology has been used in commercial plant breeding programmes since the early 1990s. The markers linked to disease resistance can be used for marker assisted selection (MAS) and this will speed up the breeding of new, superior cultivars as it allows rapid identification of plants containing genes of interest. In addition markers linked to resistance genes may also be useful for cloning and sequencing the genes. This thesis research entitled "Development of trait related markers for bacterial wilt resistance in tomato (*Solanum lycopersicum* L.)" was undertaken as part of International SOL Genome Project with the following objectives.

1. To characterize selected tomato genotypes for reaction to bacterial wilt.
2. To detect and characterize trait related markers with special reference to bacterial wilt.

Review of Literature

2. REVIEW OF LITERATURE

The review of literature on the crop, causal organism of bacterial wilt, its symptomatology, artificial inoculation, source of resistance, molecular characterization of tomato genotypes, and resistant genes and QTL is briefly dealt in this chapter.

2.1. The Crop

Tomato (*Solanum lycopersicum* L.) is one of the most important vegetable crops grown in India. Since 1800, tomatoes are being used as food all over the world. Miller in 1978 gave the name *Lycopersicon esculentum* to cultivated tomato. More recently based on much molecular and morphological information, a new taxonomic classification of tomato and readoption of *Solanum lycopersicum* for the cultivated tomato have been suggested (Majid, 2007). It belongs to the family Solanaceae, and the genus *Lycopersicon* includes the cultivated tomato and eight wild species, all of which originated from the western Andes of South America. The wild species of tomato are *L. pimpinellifolium, L. cheesmani, L. chilense, L. hirsutum, L. peruvianum, L. parvifolium, L. chinense* and *L. pennellii.* The cherry tomato *L. esculentum* var. *cerasiforme* is considered as the most likely ancestor of cultivated tomatoes. Almost all disease resistance genes in cultivated tomato originated from wild tomato species (Thamburaj and Singh, 2001). Bacterial wilt resistance originated from wild tomato, particularly *L. esculentum* var. *cerasiforme* and *L. pimpinellifolium* and most tomato resistance sources tend to be small-fruited.

As a crop plant, tomato is one of the best-characterized plant systems. It has a relatively small genome of 950 Mb per haploid nucleus, (Arumuganathan and Earle, 1991) and features such as diploidy, self-pollination, and a relatively short generation time make it amenable to genetic analysis.

The wild species bear a wealth of genetic variability. Less than 10 per cent of the total genetic diversity in the *Lycopersicon* gene pool is found in *L. esculentum* (Miller and Tanksley, 1990). The cultivated tomato, *Lycopersicon esculentum*, is genetically depleted compared to its wild relatives due to domestication and modern plant breeding (Rick and Chetelat, 1997).

2.2. The Pathogen

Bacterial wilt caused by the soil-borne bacterium *Ralstonia solanacearum* (Smith) Yabuuchi *et al*. is a devastating plant disease in the tropical and subtropical humid regions of the world. The pathogen has an extremely wide host range. The first incidence of the disease was reported from Italy (Walker, 1952).

Over one hundred years 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). Hedayathullah and Saha (1941) first reported the incidence of bacterial wilt disease in tomato from India.

Pseudomonas solanacearum is a complex pathogen, differing in host range and pathogenecity. Geographical variation occurs in the organism. Buddenhagen *et al*. (1962) classified *Pseudomonas solanacearum* isolates from a wide range of hosts in Central and South America, based on host range, pathogenecity and colony appearance on TZC medium, into 3 races viz. race 1, race 2 and race3.

1. Race 1 (Solanaceous strain) – It has wide host range, distributed throughout the lowlands of tropics and subtropics. They attack tomato, tobacco and many solanaceous crops and other weeds.
2. Race 2 (Musaceous strain) – This is restricted to *Musa* spp. and a few perennial hosts. Initially limited to American tropics and spreading to Asia.

3. Race 3 (Potato strain) – Restricted to potato and few alternate hosts in tropics and subtropics.

Based on the ability to oxidize various carbon sources and other bacteriological reactions, Hayward (1964) classified *Pseudomonas solanacearum* into biotypes or biochemical types namely biotype I, biotype II, biotype III and biotype IV.

1. Biotype I – doesn't oxidize disaccharides and sugar alcohols.
2. Biotype II – oxidize only disaccharides.
3. Biotype III – oxidizes both disaccharides and sugar alcohols.
4. Biotype IV – oxidizes only hexahydric alcohols.

Later, two new races were proposed, one from ornamental ginger as race 4 (Aragaki and Quinon, 1965) and the other from mulberry as race 5 (He *et al*., 1983).

In a study of thirty tomato isolates of *P. solanacearum* from Assam and Orissa, it was concluded that all isolates belonged to race 1 (Addy *et al*., 1980). Granada and Sequeira (1983) documented the survival of *P. solanacearum* in the rhizosphere and reported that the bacterium invades the roots of presumed non-hosts such as bean and maize.

Cook and Sequeira (1988) used RFLP technique to study the relationship between biovars I to IV of Hayward and races 1, 2 and 3 of Buddenhagen *et al*. They divided *P. solanacearum* into two groups viz. Group I (includes strains of race 1, biovars III and IV) and Group II (includes strains of race 1 biovar 1 and races 2 and 3). The gel pattern suggested that race 3 is a homogeneous group and fell into three distinct groups representing strains from different geographical origin. In contrast, race 1 strains exhibited highly variable RFLP gel patterns suggesting that race 1 is highly heterogeneous. Another recent classification of *P. solanacearum*, based on RFLP and other genetic fingerprinting studies (Hayward,

2000), is into Division I (biovars 3, 4 and 5 originating in Asia) and Division II (biovars 1, 2 A and 2T, originating in South America).

Kumar *et al*. (1993) differentiated twelve isolates of *P. solanacaerum* from solanaceous hosts into biovars following Hayward's classification. All the isolates from tomato, potato, aubergine and bell pepper were identified as biovar III or a sub type in biovar III. 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).

Thwaites *et al*. (1997) studied the genetic variation among strains of *R. solanacearum* using polymerase chain reaction with random primers. A transposon induced mutant *R. solanacearum* has lost pathogenecity on its natural host, banana; but is still retaining the ability to wilt tomato.

Paul (1998) identified bacterial wilt infecting tomato and chilli isolates as race 1 biovar III. Studies conducted on the isolates of *R. solanacearum* from tomato, brinjal and chilli suggested that the pathogen belongs to race 1, biovar III and biovar IV (Mathew *et al*., 2000).

Variability studies conducted on the isolates of *R. solanacearum* of tomato, brinjal and chilli from different locations of Kerala showed the existence of pathogen belonging to race 1, race 3 and biovar III, III A and V (James, 2001 and Mathew, 2001).

2.3. Symptomatology

Walker (1952) reported that the first expression of the disease is wilting of lower leaves of the plants followed by wilting of the entire plant. Dwarfing or

stunting may also occur. The bacterium enters through the root system and it was believed that a wound is necessary for the entry (Walker, 1952; Kelman, 1953; Chupp and Sherf, 1960). The entry is reported to also occur through natural opening of the plant (Hildebrandt, 1950). Chupp and Sherf (1960) reported that the bacteria could enter at the points of origin of secondary roots. The roots and lower parts of the stem show browning of vascular bundles and water soaked areas appear in the roots. The pathogen is reported to enter into the uninjured roots also (Libman *et al*., 1964). The pathogen first enters into the intercellular spaces of the cortex from where it moves to pith and xylem vessels leading to vascular plugging and wilting of the plants (Walker, 1952).

In advanced stages, dark brown to black areas develop due to decay of root system and the whole plant dies off. A very characteristic indication of bacterial wilt is the appearance of bacterial ooze from the injured vascular regions (Ashrafuzzaman and Islam, 1975). It is reported that break down of plant tissues by the pathogen is due to the cellulase and polygalaturonase enzymes produced by the pathogen. Continued tissue decay and plugging finally result in death of the plant. Visible symptoms of the disease occur within two to eight days after the entry of the pathogen into the host plant (Kelman, 1953; Chupp and Sherf, 1960).

According to Kelman (1954), the virulence might be explained, at least in part by the quantitative difference in EPS (extra cellular polysaccharides). The bacterium can produce IAA, which helps in initiation of tylose formation and increases cell wall plasticity. Ethylene production is also associated with the disease development. Sequeira (1993) reported that there is no cytological evidence for how the bacterium reaches the vascular system. It is assumed that the pathogen has to digest its way through the primary wall of the weakened cortical cells as well as of the tracheary elements, where it is exposed between the spiral thickenings. Allen (1997) reported that *R. solanacearum* passes much of its life cycle living in harmony or in an uneasy truce with its plant hosts.

2.4. Artificial inoculation

Several inoculation techniques have been tried by many workers for testing the pathogenecity and for evaluating resistance to *Ralstonia solanacearum*.

The first attempt on standardization of the inoculation technique to test the pathogenecity of different strains of *R. solanacearum* on respective hosts was done by Winstead and Kelman (1952). They aimed a procedure that would ensure uniform and rapid development of bacterial wilt. For this, they tried (1) stem puncturing at the third node below apex, (2) cutting the lateral roots along one side and pouring bacterial suspension over soil, (3) pouring bacterial suspension over soil without injury to roots, and (4) dipping roots in bacterial suspension. Symptoms of wilt became first evident in those plants inoculated by stem puncture at third node below the apex. At the end of 15 days all plants were dead. Inoculation by pouring bacterial suspension over soil without injury to roots was not an effective procedure.

Kelman (1954) tested the pathogenecity of bacterial cultures by inoculating tomato plants by stem puncture method. All cultures produced rapid wilting and at the end of 14 days, all inoculated plants were severely wilted.

Using stem puncture by means of a hypodermic syringe on eight weeks old tomato plants, Husain and Kelman (1958) noticed intermediate to advanced symptoms of wilting after 10 days of inoculation. Wang (1971) used leaf clipping for artificial inoculation in tomato. Root inoculation also had been tested on different hosts and found effective (Khan *et al.,* 1979*;* He *et al*., 1983; Swanepoel and Young, 1988 and Paul, 1988).

Chellemi *et al*. (1994) inoculated six strains of *P. solanacearum* onto 19 tomato genotypes using stem puncture technique and the seedlings were transplanted to naturally infested soil. Disease incidence ranged from 30 per cent

to 94 per cent. Hawaii 7997, Hawaii 7998 and CRA 66 had the lowest incidence of wilt disease. Kelaniyangoda *et al*. (1999) screened several tomato genotypes in three batches for bacterial wilt tolerance in naturally infested soil and artificially inoculated soil. Each batch was screened for two consecutive seasons and approximately 22 genotypes were identified as bacterial wilt tolerant.

2.5. Source of resistance

Schaub and Baver (1994) reported that, in the field trials conducted at North Carolina (USA), cultivars Louisiana Pink and T-414 from Puerto Rico showed good resistance to bacterial wilt. A further source of resistance was reported in *Lycopersicon pimpinellifolium* (PI 127805A), which had partial dominance at seedling stage, and the resistance was controlled by recessive genes (Abeygunawardena and Siriwardena, 1963). The expression of resistance in a variety is a function of the age of the plant and changes in temperature (Acosta *et al*., 1964).

In an experiment conducted by Henderson and Jerkins (1972) to evaluate resistance in several genotypes, they found the genotypes such as Venus, Saturn and Beltsville-3814 to be resistant to bacterial wilt. Similarly from the work carried out by Ahuja and Waite (1974), they observed more than 90 per cent survival of the seedlings in genotypes BWN-514, BWN-16, BWN-17, and BWN-7755 against the attack from pathogen *P. solanacearum*.

A diallel test involving six cultivars Walter, CRA 66, H 7741, Venus, VC-4 and Llanos de Coke was performed by Graham and Yap (1976). They observed that high level of wilt resistance was attained in a breeding procedure of repeated selfing and selection followed by intercrossing of resistant selections. In Florida, Sonoda and Augustine (1978) found Hawaii 7997, CRA 66 and PI 126408 to be the best resistant sources for bacterial wilt.

Ramachandran *et al.* (1980) evaluated 36 tomato lines for their resistance to bacterial wilt in Kerala. They observed resistance in La-Bonita and CL-132 d-0-1-19GS cultivars. Celine (1981) collected 78 lines to isolate resistant ones and the field screening indicated the tolerance of LE-79, a line from AVRDC, Taiwan. Tikoo *et al*. (1983) reported the presence of two independent genes for wilt resistance. The resistance was reported to be governed by multiple recessive genes in CRA 66 Sel A from Hawaii and by single dominant gene in 663-12-3 from Taiwan. Sreelathakumari (1983) reported a complimentary and hypostatic type of digenic recessive gene system for wilt resistance.

Goth *et al*. (1983) found that the wilt resistance in cultivar Venus and the line CL-32d-0-1-19GS from Taiwan was broken down when *Meloidogyne incognita* larvae were added at the rate of 100/10 cm pot at the time of inoculation with bacteria. He also suggested that nematode should be considered as a factor in the development of bacterial wilt resistant lines.

Narayanankutty (1985) reported that the susceptible check Pusa Ruby showed 100 per cent susceptibility in an experiment to develop new sources of resistance to bacterial wilt. He observed resistance in F2 generation of Saturn X LE 79. Rigorous screening under natural as well as artificial conditions and subsequent selection in the Department of Olericulture, College of Horticulture, Vellanikkara resulted in the development of two bacterial wilt resistant varieties namely Sakthi (LE-79) and Mukthi (LE-79-5). Rajan and Peter (1986) reported a monogenic incompletely dominant gene action in the resistant line LE-79. Hanudin (1987) reported resistance in cultivars Intan, Ratna, CI 32-6-125-d-0, AV 22 and AV 15. Nirmaladevi (1987) observed that resistance to bacterial wilt in the genotype CRA 66 Sel A was under polygenic control.

Peter *et al*. (1992) reported resistance in the lines LE-214, LE-217, LE-79, LE-70 LFG, LE-79 DG and LE-79 SPF. Evaluation for bacterial wilt resistance by Sadhankumar (1995) revealed consistent resistance of Sakthi and Mukthi. He

obtained four additional resistance sources (LE-214, CAV-5, LE-415 and LE-382-1) and also reported that genes responsible for resistance in these lines were recessive. Chellemi *et al*. (1994) evaluated 30 tomato genotypes for resistance to *R. solanacearum* and observed that the disease incidence ranged from zero in Hawaii 7997, GA 219 and GA 1565 to 83 per cent in Solarset.

Rani (2000) reported that the F1 hybrids LE 415 X Mukthi, LE 415 X Sakthi, LE 415 X BWR-1 and Sakthi X Mukthi are resistant to bacterial wilt. Kurian and Peter (2001) evaluated F1 hybrids of bacterial wilt tolerant/resistant genotypes Sakthi, LE 214 and LE 206 with HW 208F, St 64, Ohio 8125, TH 318 and Fresh market, and they found that these hybrids were completely susceptible to bacterial wilt. Sadashiva *et al*. (2002) reported that the hybrids TLBR-3 X IIHR 2202, TLBR-3 X IIHR 2200, TLBR-4 X IIHR 2200, TLBR-3 X IIHR 2199 and TLBR-6 X IIHR 2202 are having combined resistance to bacterial wilt and tomato leaf curl virus. A high yielding tomato variety, Anagha (LE-415), with built-in resistance to bacterial-wilt has been developed by the scientists at the Department of Olericulture, College of Horticulture of the Kerala Agricultural University (KAU), Vellanikkara (Anon., 2003).

2.6. Molecular characterization

Many of the complications of a phenotype-based assay can be mitigated through direct identification of genotype with a DNA based diagnostic assay. For this reason, DNA based genetic markers are being integrated into several plant breeding systems and are expected to play an important role in the future of plant breeding programme (Tingey and del Tufo, 1993).

DNA marker technology has been used in commercial plant breeding programmes since the early 1990s and has proved helpful for the rapid and efficient transfer of useful traits into agronomically desirable varieties and hybrids (Tanksley *et al*., 1989). Several Polymerase Chain Reaction (PCR) based and

hybridization based DNA marker techniques can be used for the characterization of genetic variability in pathogens and molecular tagging of disease resistance genes. DNA markers linked to specific resistance gene can be used in marker-assisted selection (MAS) for resistance breeding, gene pyramiding and map-based cloning of the resistance gene (Sharma, 2003).

2.6.1. Random Amplified Polymorphic DNA (RAPD)

Williams *et al*. (1990) developed a technique that employs random primers in a Polymerase Chain Reaction (PCR) to rapidly generate polymorphic markers that can be used to create genetic linkage maps. They also reported that RAPD is a dominant marker inherited in Mendalian fashion. Klein-Lankhorst *et al*. (1991) developed a new DNA polymorphism assay based on the amplification by the PCR of random DNA segments, using single primers of arbitrary nucleotide sequence. Combining two primers in a single PCR further increased the original RAPD assay. They could identify three chromosome 6 specific RAPD markers by comparing the fingerprints of *L. esculentum, L. pennellii* and *L. esculentum* chromosome 6 substitution line LA 1641. One of the RAPD markers was found to be tightly linked to the nematode resistance gene, *Mi*.

Martin *et al*. (1991) identified the presence of three markers that were generated by random primers to be polymorphic in the Near Isogenic lines (NILs) of tomato. These markers were reported to be linked to *Pto* gene conferring resistance to the bacteria, *Pseudomonas*. It has been shown that RAPD markers can vary according to experimental conditions like $MgCl_2$ concentration (Wolf *et al*., 1993), Taq polymerase quality and quantity (Schiewater and Ender, 1993) and type of thermal cycler (Wolf *et al*., 1993).

Chunwongse *et al*. (1994) succeeded in tagging of a powdery mildew resistant gene (*Lv*) in tomato using RAPD and RFLP markers. DNA from a resistant and a susceptible cultivar was screened with 300 random primers that

were used to amplify DNA of resistant and susceptible plants. Four RAPD markers linked to *Sw-5* gene, which confers resistance to spotted wilt virus in tomato, were identified by Chague *et al*. (1996). They have reported that two of these markers, R_2 and S_1, are tightly linked to this gene.

Yui *et al*. (1999) obtained four RAPD markers, which are useful for preliminary selection of bacterial wilt resistance, introduced from a bacterial wilt resistant parent Hawaii 7998. F_2 population derived from the cross between susceptible parent Tomato Parental Line 5 (TPL 5) and Hawaii 7998 was screened for these markers. Two markers, namely RA 12-13450 and RA 12-291600, were found to be linked to a resistance gene with large effect.

RAPD analysis was made to identify polymorphic markers between resistant bulk and susceptible bulk DNA of *Solanum phureja* with 300 random primers. The primer OGP09 produced a 960 bp reproducible band only in resistant clones, linking to the wilt resistance in the population (Gang *et al.*, 2002).

Archak *et al*. (2002) reported low levels of polymorphism using RAPDs in tomato and difficulties in molecular characterization of cultivars in other diploid, autogamous solanaceae species. The homogeneity between accessions of *Solanum torvum* regarding high bacterial wilt tolerance was confirmed by the genetic homogeneity (zero per cent polymorphism) revealed using 168 RAPD primers. *S. torvum* has been identified as a potential source of bacterial wilt disease for cultivated Solanacea species (Clain *et al*., 2004).

2.6.2. Amplified Fragment Length Polymorphism (AFLP)

Vos *et al*. (1995) described a noval technique for DNA fingerprinting, named AFLP. The AFLP technique is based on detection of genomic restriction fragment by PCR reaction, and can be used for DNAs of any origin and

complexity. They illustrated how this method can be best used in fingerprinting of genomic DNA of a number of organisms and plants including tomato. For tomato, DNA primer combinations with a total of six selective nucleotides were used, three selective bases for both *Eco*RI and *Mse*I primer. They also reported that the AFLP procedure is insensitive to the template DNA concentration. Saliba *et al*. (2000) reported that the use of AFLP markers to discriminate tomato lines was more efficient than RAPD markers, but did not reveal much polymorphism.

PCR based markers such as amplified fragment length polymorphism (AFLP), resistance gene analogues (RGAs) and simple sequence repeats (SSR) were evaluated and used to construct a molecular linkage map of tomato using F_6 recombinant inbred lines of the cross Hawaii 7996 X Wva 700. High-resolution detection was achieved with the non-radioactive silver staining detection method (Balatero *et al*., 2002).

Menezes *et al*. (2003) identified the presence of 21 AFLP primers revealing DNA bands unique to genotypes resistant to tomato spotted wilt virus and five primers revealing DNA bands associated with susceptibility. He has surveyed a total 170 AFLP primers for screening the tomato genotypes.

One hundred and seventeen markers were identified by the amplified fragment length polymorphism (AFLP) method between W6, a variety with resistance and Michinoku 1, a commercial wilt-susceptible variety, using 3,072 primer combinations (Nishi *et al*., 2003). These markers were analyzed in 125 doubled haploid lines, derived from F_1hybrids between W6 and Michinoku 1, and a linkage map consisting of ten linkage groups was drawn. The resistance phenotype of each of these lines was investigated on the basis of the average of disease severity obtained from field trials over two growing cycles. Quantitative trait loci (QTL) analysis was performed on the marker phenotypes and the resistance phenotype of each line. One QTL for the bacterial wilt resistance of W6 and DNA markers associated with this QTL were identified on a linkage group

consisting of 15 markers, 32 cM in length. This QTL explained more than 30% of the variance in resistance among these lines.

Gang *et al*. (2005) analyzed the feasibility and efficiency of the identification of the molecular markers linked to bacterial wilt resistance in potato (*Solanum phureja*) using BSA (Bulked Segregant Analysis). Genomic position of molecular markers in the linkage map was identified using AFLP, and primer combinations ATG/CTC and AAC/CAC were considered as markers associated with the resistance which where located on chromosome 1 and 12.

Chuan *et al*. (2006) made a cross between bacterial wilt resistant tomato variety "T51A" and susceptible variety 'T9230' for mapping bacterial wilt resistance gene(s). Through inoculation test of its F1 and F2 progeny, it was proved that the resistance of 'T51A' to bacterial wilt was controlled by one heterozygous gene and cytoplasm. With 64 *Eco*RI/*Mse*I primer combinations, AFLP analysis was performed on two parents and their F2 resistant and susceptible bulks. A total of about 4200 distinguishable bands were amplified, of which two were stable. Genetic linkage analysis of the two polymorphic DNA fragments with the resistance gene(s) was tested in the F2 segregating population derived from the cross between 'T51A' and 'T9230'. The DNA fragment AAG/CAT was found closely linked to one of the bacterial wilt resistant genes, with a genetic distance of 6.7 cM that was tentatively named RRS-342. The cloned fragment AAG/CAT was sequenced and then successfully converted to a SCAR marker, which can be used more conveniently in marker-assisted selection for tomato resistance to bacterial wilt gene.

A recessive gene *Ol-2* confers complete resistance to tomato powdery mildew, a recently identified disease that occurs in open field and protected environment to cause serious damage to tomato crops. Riccardia *et al*. (2007) reported that eight new amplified fragment length polymorphism (AFLP) markers

are tightly linked to the *Ol-2* gene for resistance, adding useful mapping information to the chromosome 4 region where this gene is located.

2.7. RESISTANCE GENES (*R* genes) and QTL (Quantitative Trait Loci)

Plants have developed a wide array of defense responses to control pathogen invasion. Among those, the presence or absence of complementary pairs of resistance (R) genes in the host and avirulence genes (*avr*) in the invading microorganisms determine the outcome of many plant–pathogen interactions. In the elicitor–receptor model proposed to account for this gene-for-gene theory (Flor, 1971), *avr* genes encode elicitors that serve as ligands for receptors encoded by *R* genes, which trigger a complex defense response. Most of these genes contain a leucine-rich repeat (LRR) domain, which function in mediating protein-protein interactions. Several cloned *R* genes belong to the nucleotide binding site (NBS)-LRR class (Staskawicz, 2001).

Many of these *R* gene products share structural motifs, which indicate that resistance to diverse pathogen may operate through similar pathways. The use of molecular markers and interval mapping is a powerful approach that permits the identification and genetic mapping of loci controlling a trait of interest. Several quantitative trait loci (QTL) have been shown to play a role in resistance to bacterial wilt in different studies using different populations and at different geographic locations (Danesh *et al.*, 1994).

Martin *et al*. (1992) reported that resistance to *Pseudomonas syringae* pv *tomato* (*Pst*) is conferred by a single dominant locus, *Pto*. Multistep positional cloning strategy was used to isolate the *Pto* locus. They identified RFLP and RAPD markers that are tightly linked to this locus in near isogenic lines of tomato. Tomato YAC library was screened with TG 538 marker and 5 YACs ranging from 400-600 kbp that carry sequences derived from the *Pto* region were identified.

Later, Martin *et al*. (1993) constructed a high-resolution linkage map containing 18 molecular markers and spanning 20 centi Morgans of tomato chromosome 5 for the region containing *Pto.* One marker identified, TG 538, co-segregated with *Pto* and therefore provides a starting point for chromosome walking. This gene code for Serine / Threonine rich protein kinase conferring resistance to *Psuedomonas syringae* pv *tomato*.

Thouquet *et al*. (1996) developed a molecular map of F_2 population of Hawaii 7996 X Wva 700 using RFLP. Nine independent resistance tests were conducted and identified that the most important QTL was located on chromosome 6, and another QTL on chromosome 4 and a weaker putative QTL at other map positions.

In tomato, the *Cf-4* and *Cf-9* resistance genes map to the same location but confer resistance to *Cladosporium fulvum* through recognition of different avirulence determinants (AVR4 and AVR9). Thomas *et al*. (1997) reported the cloning and characterization of *Cf-4* gene, which encodes a membrane-anchored extra cellular glycoprotein. DNA sequence comparison suggests that Cf-4 and Cf-9 are derived from a common progenitor sequence. Both *Cf-4* and *Cf-9* are located within a 36 kbp region comprising five tandemly duplicated homologous genes.

Deberdta *et al*. (1999) showed that the presence of the *Mi* gene was associated with a marked decrease in bacterial wilt resistance. They suggested that at least one gene governing part of the bacterial wilt resistance is closely linked or allelic to the *Mi* gene in the tomato lines Caraibo, CRA66 and Cranita. Lee *et al*. (2002) investigated expression of lactoferrin (LF) gene, a cationic iron-binding glycoprotein in transgenic tomato plants. Resistance of these transgenic plants was determined by inoculation of *Ralstonia solanacearum* isolates and it is reported that 44 to 55 per cent of plants survived until maturity.

Deslandes *et al*. (2003) did the identification and mapping of RRS1, a Single Recessive Locus in *Arabidopsis thaliana* (Nd-1 ecotype) that confers resistance to *R. solanacearum. RRS1-R* is the first characterized *R* gene conferring resistance to *R. solanacearum*, and its characterization, the elucidation of its mode of action, as well as its intergeneric transfer in various crops are important steps in facilitating the elaboration of new approaches to disease control.

Chi *et al*. (2004) introduced the Arabidopsis *NPR 1* (nonexpressor of PR genes) gene into a tomato cultivar, which possesses heat tolerance and resistance to tomato mosaic virus. Disease screens against eight important tropical diseases revealed that, in addition to innate ToMV resistance, the tested transgenic lines conferred significant level of enhanced resistance to bacterial wilt and *Fusarium* wilt.

2.8. MOLECULAR CLONING

The identification of resistant germplasm in wild species of agronomically important crops and the subsequent introgression into commercial cultivars has been the major focus of many plant breeders. Most plant breeders employed dominant and semi-dominant genes in breeding programmes because of ease and efficacy in which they could be introduced into agronomically acceptable cultivars. The fact that most characterized resistance genes were inherited as single genetic loci allowed plant biologists to employ genetic strategies to clone *R* genes. The development of transposon tagging and map-based cloning strategies in the 1980s seemed like the best method of choice to clone *R* genes from plants. In tomato several *R* genes have been so far cloned and sequenced (Staskawicz, 2001).

The term gene cloning refers to the isolation and amplification of an individual gene sequence by insertion of that sequence into a vector molecule (e.g. plasmid, phage, cosmid) where it can be replicated. The precise order of the

nucleotide sequence of the insert can be determined by DNA sequencing and this is important to know the function of a gene (Chawla, 2002).

2.8.1. Transformation in *E. coli*

Cohen *et al*. (1972) showed that Calcium chloride treated *E.coli* cells are effective recipients for plasmid DNA. During transformation a restriction deficient strain of *E.coli* is used as a transformable host. It is found that *E.coli* cells and plasmid DNA effectively interact in an environment of calcium ions and low temperature (0 – 5°C). A subsequent heat shock (3°C – 45°C) is important. The calcium chloride influences the cell wall permeability, which plays an important role in binding of DNA to the cell surface. The actual uptake of DNA is stimulated by the brief heat shock (Old and Primrose, 1994).

Sambrook *et al*. (1989) reported restriction analysis of plasmid DNA as a method of finding the desired recombinants within a small number of randomly chosen transformed cells. Many of the cloning vectors carry a short segment of *E.coli* DNA that contains the regulatory sequences and the coding information for the first 146 amino acids of the β-galactosidase gene (*lac Z*). This coding region contains a polycloning site that does not disrupt the reading frame but results in the harmless interpolation of a small number of amino acids into amino terminal fragment of β-galactosidase. The carboxy terminal portion of β-galactosidase is coded by the host cells of this type of vectors. The host encoded and vector encoded proteins together constitute the active protein (Ullmann *et al*., 1967).

The recognition of lac^+ bacteria that result from α complementation is easy because they form blue colonies in presence of the chromogenic substrate X-gal (Horwitz *et al*., 1964). The insertion of a fragment of foreign DNA into the polycloning site of the plasmid results in the production of an amino terminal fragment that is not capable of complementation. White colonies are formed by the bacteria carrying recombinant plasmid while the non-recombinants produce

blue colonies. Screening of these colonies can be done visually to recognize bacteria that carry putative recombinant plasmids.

2.8.2. Cloning and sequencing of plant disease resistance genes

Several resistance genes have been cloned in recent years and have been shown to occur in gene clusters. A major breakthrough was the cloning and characterization of the maize *Hm1*disease resistance gene. The *Hm1* gene control resistance to certain isolates of *Cochliobolus carbonum* and was cloned by transposon tagging employing maize transposon, *Mu*. This gene code for NADPH-dependent reductase that inactivates the toxin produced by the invading fungus (Johal and Briggs, 1992). Another resistance gene, *Pto*, was isolated from tomato in the next year. The gene encodes a Ser / Thr kinase activity and is unrelated to the *Hm1* gene (Martin *et al*., 1993).

The pace of cloning *R* genes increased in 1994 with the cloning and characterization of *R* genes for resistance to several classes of pathogens, including viral, bacterial and fungal pathogens. Staskawicz *et al*. (1995) reported the coning of *RPS2* gene in Arabidopsis, *N* gene in tobacco, *Cf-9* gene in tomato and *L6* gene of flax. Subsequent analyses of the predicted polypeptides revealed that all four proteins leucine rich repeat motifs, suggesting that plants may share common mechanisms for disease resistance to diverse pathogens. The sequencing of these genes further validated the idea that disease resistance gene share common protein motifs. Song *et al*. (1997) succeeded in cloning *Xa*21 gene in rice encoding a receptor-like protein kinase. The sequence analysis of this gene family indicated that evolution followed recombination, duplication and transposition events and not every member of an evolving gene family is functional in conferring resistance.

Two members of a multigene family, designated as complex *I2C* were isolated from the *I2 Fusarium oxysporum lycopersici* race 2 resistance loci in

tomato (Ori *et al*., 1997). The genes showed similarity to the group of isolated plant resistance genes that encode cytoplasmic proteins containing a nucleotide binding site motif and leucine-rich repeats (LRRs). The members of the *I2C* family were mapped to five genomic positions.

Thus it could be summarized that several workers have attempted to unravel the disease reaction and resistance mechanisms in tomato for bacterial wilt incidence. DNA marker based detection is one among the advanced technologies being exploited for the purpose.

Materials and Methods

3. MATERIALS AND METHODS

The study on development of trait related markers for bacterial wilt resistance in tomato (*Solanum lycopersicum* L.) was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during the period 2005 to 2007. Description of the materials used and methodology adopted in the study has been furnished in this chapter.

3.1. MATERIALS

3.1.1. Plant material

Seeds of the 15 tomato genotypes with varying levels of resistance were collected from different institutions viz., IARI, New Delhi; IIHR, Bangalore; Himachal Pradesh Krishi Viswa Vidyalaya (HPKV), Palampur; Orissa University of Agriculture and Technology (OUAT), Bhubaneswar; Agriculture College and Research Institute (ACRI), Periyakulam and Kerala Agricultural University (KAU), Thrissur (Table 1). The seedlings were raised in earthen pots containing sterilized soil medium and were subjected to artificial inoculation to confirm the resistance/susceptibility to bacterial wilt caused by *Ralstonia solanacearum*.

Table 1. Tomato genotypes evaluated and their sources

Genotype	Source
Sakthi, Mukthi, Anagha, LE-474, LE-66, LE-1-2, LE-20	KAU, Thrissur
Hawaii 7998, BL-333-3-1	HPKV, Palampur
BT-1, BT-218, BTH-102-1-2-2	OUAT, Bhubaneswar
Pusa Ruby	IARI, New Delhi
Arka Vikas	IIHR, Bangalore
PKM-1	ACRI, Periyakulam

3.2.2. Chemical, glassware and plastic ware

The chemicals used for the study were of good quality (AR grade) from various agencies including MERCK, SRL and HIMEDIA. Molecular Biology Grade enzymes and buffers were supplied by Bangalore Genei Ltd. All the plastic ware used was obtained from Axygen and Tarson India Ltd. The primers were obtained from Operon Technolgies, USA. γ^{32} P dATP was obtained from Board of Radiation and Isotope Technology (BRIT), Mumbai.

3.2.3. Equipment items and machinery

The equipment items available at the Centre for Plant Biotechnology and Molecular Biology and Bioinformatics Centre, College of Horticulture were used for the present study. The PCR was done in thermal cycler of model Mastercycler personal (Eppendorf) and horizontal gel electrophoresis system (BIO-RAD) was used for agarose gel electrophoresis. Alpha Imager 1200 was used for imaging and documenting the agarose gel. AFLP was performed in Sequencing Cell apparatus (BIO-RAD) and the gel was analyzed using Fluorescent Image Analyzing System FLA-5100 (FUJI PHOTO FILM Co., Ltd.). The list of laboratory equipments used for the study is provided in Appendix I.

3.2. METHODS

3.2.1. CONFIRMATION OF GENOTYPIC RESPONSE TO BACTERIAL WILT INCIDENCE

Raising the crop

The 15 tomato genotypes were grown in greenhouse (50% shade) and open condition during October 2006 to January 2007. 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. This solution

was applied at the rate of 1.5 L per pot and covered with polythene sheets. After one week, the polythene sheets were removed and the pots were kept open for another 7 days to remove the residual formalin content. Twenty eight days after sowing, the seedlings were transplanted to individual pots filled with the same sterilized medium. Both in greenhouse and open condition, a minimum of twenty plants per genotype were maintained in two replications and the management practices as per the package of practices recommendations of Kerala Agricultural University (KAU, 2002) were followed.

Artificial inoculation

Artificial inoculation was performed 10 days after transplanting. The fresh bacterial ooze from wilted plants in the tomato field in the campus was used as the inoculum. 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 estimated at 10^8cfu/ml. Both sets of seedlings were subjected to artificial inoculation by means of a combination of the following methods of inoculation.

i) Stem-puncture inoculation technique

A drop of bacterial suspension was placed in the axils of sec and third expanded leaves below the stem apex and then a needle was forced into the stem through the drop. A thin piece of cotton dipped in bacterial suspension was then placed in the punctured axils to ensure sufficient inoculum.

ii) Soil drenching with wounding

The lateral roots of each 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 30ml of the inoculum around the base of the seedlings.

C) Disease scoring

The inoculated plants were observed daily up to 14 days, for the development of disease symptoms. Symptoms start as leaf drooping, followed by complete wilting of the plant within a few days. The recently wilted plants usually remain green (Plate 1a). The disease infection was confirmed through ooze test. The stem of the wilted plant was sliced and the base was placed in a test tube containing clear water and allowed to stand for few min (Plate 1b).

Based on the observations made on disease reaction, the percent wilt incidence was calculated using the formula,

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

The rating and classification of resistance / susceptibility was done based on the scoring system proposed by Mew and Ho (1976) as shown in Table 2.

Table 2. Score chart for bacterial wilt disease

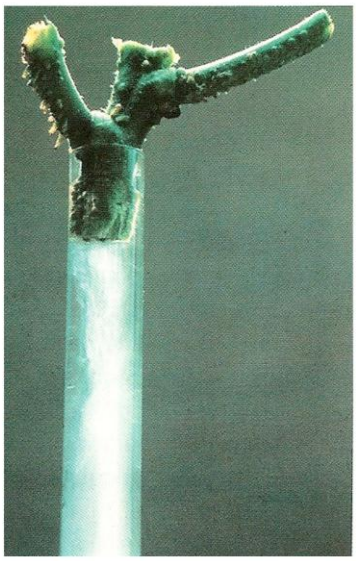
Disease Reaction	Per cent wilt incidence
R (Resistant)	Less than 20
MR (Moderately resistant)	20 to 40
MS (Moderately susceptible)	40 to 60
S (susceptible)	More than 60

3.2.2. MOLECULAR CHARACTERIZATION

3.2.2.1. Standardization of genomic DNA isolation

Isolation of good quality genomic DNA from tomato is a pre-requisite for RAPD and AFLP analysis. The procedures reported by Doyle and Doyle (1987) and Rogers and Bendich (1994) for the isolation of DNA were tried for genomic

a. Complete wilting of the tomato plant



b. Bacterial ooze test

Plate 1. Symptoms of bacterial wilt and its confirmation

DNA isolation in tomato. The tender leaves from selected 15 genotypes collected early in the morning were used for DNA isolation.

3.2.2.1.a) DNA isolation by Doyle and Doyle method

The original protocol along with modifications like the use of β-mercaptoethanol and sodium metabisuphite was followed so as to obtain good quality DNA.

Reagents used

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

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

Procedure

- Tender leaf tissue (0.5 g) was ground in excess liquid nitrogen and the following reagents were added.

  1X Extraction buffer -3 ml
  Sodium metabisuphite -10 mg
  β-mercaptoethanol -50 µl

- The homogenate was transferred individually into 50 ml Oakridge centrifuge tubes containing pre-warmed lysis buffer (4 ml) and added 1 ml of 5 per cent sarcosine.
- The mixture was maintained at 65^0C for 10 to 15 min in water bath with gentle mixing.

- Equal volume of Chloroform: Isoamyl alcohol mixture was added and mixed again.
- The homogenate was centrifuged at 10,000 rpm for 15 min at 4°C.
- The upper aqueous phase was saved and transferred to a fresh centrifuge tube after checking the volume.
- To this, 0.6 volume of chilled isopropanol was added and mixed gently, and then incubated at –20°C for 30 min for complete precipitation of DNA.
- The DNA was pelleted by centrifugation at 10,000 rpm for 15 min at 4°C.
- The supernatant was discarded and the pellet was washed with 70 per cent alcohol, followed by absolute alcohol.
- The pellet was air dried for 30 min, resuspended in 250 µl TE buffer and stored at –20°C for further use.

3.2.2.1.b) DNA isolation method by Rogers and Bendich protocol

The original protocol along with modifications like the addition of β-mercaptoethanol and changing the quantity of extraction buffer was followed.

Reagents used

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

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

Procedure

- Tender leaf tissue (1 g) was ground in excess liquid nitrogen and 4 ml of 2X extraction buffer and 100 µl β-mercaptoethanol were added.

- The ground tissue was transferred into a 50 ml Oakridge tube containing 3 ml prewarmed extraction buffer. The contents were mixed well and incubated at 65°C for 15 min.
- Equal volume of Chloroform: Isoamyl alcohol mixture was added, mixed gently by inversion and centrifuged at 10,000 rpm for 10 min at 4°C.
- The upper aqueous phase containing DNA was transferred to a fresh 50 ml oakridge tube, and $1/10^{th}$ volume of 10 per cent CTAB solution was added and mixed gently by inversion.
- Equal volume of Chloroform: Isoamyl alcohol mixture was added, mixed gently to form an emulsion and centrifuged at 10,000 rpm for 10 min at 4°C.
- The upper aqueous phase was collected in a fresh Oakridge tube and 0.6 volume of chilled isopropanol was added, and mixed gently to precipitate the DNA. It was incubated at –20°C for 30 min.
- The contents were centrifuged at 10,000 rpm for 5 min at 4°C to pellet the DNA.
- The supernatant was discarded and the pellet was washed with 70 per cent ethanol followed by absolute alcohol.
- The pellet was air dried for 30 min, resuspended in 250 µl TE buffer and stored at –20°C until further use.

3.2.2.2. PURIFICATION OF DNA

The DNA isolated was purified from RNA contamination by phenol precipitation and RNase treatment (Sambrook *et al*., 1989).

Reagents used

1. Phenol: Chloroform mixture (1:1, v/v)
2. Chilled isopropanol
3. Ethanol (70%)

4. TE buffer
5. Chloroform:Isoamyl alcohol (24:1; v/v)
6. RNase

The RNase A from Sigma Aldrich, USA was used to prepare RNase. One per cent solution was prepared by dissolving RNase A in TE buffer at 100°C for 15 min. The solution was cooled to room temperature, dispensed into aliquots and stored at –20°C.

The procedure followed for DNA purification is as follows:

- RNAse solution (2 µl) was added to 100 µl DNA sample and incubated at 37°C in dry bath (Genei, Thermocon) for 1 hr.
- The volume was made up to 250 µl with distilled water and equal volume of Phenol: Chloroform mixture was added.
- Centrifuged at 12,000 xg for 10 min at 4°C.
- Collected the aqueous phase in a fresh microfuge tube and added equal volume of Chloroform: Isoamyl alcohol (24:1).
- Centrifuged at 12,000 xg for 10 min at 4°C.
- The above two steps were repeated and finally precipitated DNA from the aqueous phase with 0.6 volume of chilled isopropanol.
- Incubated the mixture at –20°C for 30 min and centrifuged at 10,000 rpm for 15 min at 4°C.
- The pellet of DNA was washed with 70 per cent ethanol.
- Air dried the DNA pellets and resuspended in 250 µl TE buffer and stored at –20°C until further use.
- Two micro litre of this purified DNA was run on agarose gel to check for complete removal of RNA.

3.2.2.3. ESTIMATION OF QUALITY OF DNA

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

Materials for agarose gel electrophoresis (AGE)

1) Agarose (Genei, Low EEO) - 0.7 per cent (for genomic DNA)
- 1.2 per cent (for PCR samples)

2) 50X TAE buffer (pH 8.0)

3) 6X Loading/ Tracking dye

4) Ethidium bromide solution (stock 10 mg/ml; working concentration: 0.5μg/ml)

5) UV transilluminator (Herolab ^{R})

6) Electrophoresis unit, power pack, gel casting tray, comb

7) Gel documentation and analysis system (Alpha imager TM 1200)

Composition of reagents is provided in Appendix III.

Procedure for AGE

1. The 50X stock solution of TAE buffer was diluted to 1X concentration.
2. The required quantity of agarose (1%) was weighed and dissolved completely in 1X TAE buffer by boiling.
3. The solution was cooled to lukewarm temperature (55°C) and ethidium bromide was added at a concentration of 0.5 μg /ml as an intercalating dye of DNA, which will help in its visualization in UV rays, and mixed well.
4. The open ends of the gel-casting tray were sealed using cello tape and placed on a horizontal leveled platform. The comb was placed properly and the solution of agarose was poured into the gel-casting tray.
5. The gel was allowed to set for 30 to 45 min at room temperature. After solidification, the comb was removed carefully and the tape was pulled off the gel-casting tray.
6. The gel was placed in an electrophoresis unit (BioRad) containing 1X TAE buffer with the wells directed towards the cathode. Required quantity of 1X TAE buffer was added so as to submerge the gel to a depth of 1cm.

7. A piece of parafilm was pressed on a solid surface and 1 µl 6X loading dye was dispensed in small quantity on the tape. A quantity of 3 to 5 µl of DNA was added to each slot and mixed well by pipetting in and out for 2 to3 times.
8. The samples were carefully loaded in the wells using a micropipette. The λ DNA /*Eco*RI + *Hind*III double digest (Bangalore Genei) was loaded in one of the wells as the molecular weight marker.
9. The cathode and anode of the electrophoresis unit were connected to the power supply. The power supply was turned on and the gel was run at constant voltage (100 volts) till the tracking dye reached 2/3rd length of the gel.
10. Then the current was disconnected and the gel was removed from the electrophoresis unit.
11. The gel was placed in a gel documentation and analysis system (Alpha Imager TM 1200), and bands were visualized under UV light in a transilluminator and the gel image was documented. Thus the quality of DNA extracted was ensured.

3.2.2.4. QUANTIFICATION OF DNA

The genomic DNA isolated and detected as good through agarose gel electrophoresis were further analyzed for its quantity using UV spectrophotometer (Spectronic Genesys 5). The sample DNA (3 µl) was diluted to 500 µl with sterile water. The absorbance was measured at 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 50µg 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.

3.2.2.5. RAPD (Random Amplified Polymorphic DNA) ASSAY

After isolation of good quality of genomic DNA from all the selected genotypes, RAPD analysis was performed for characterizing them. Random Amplified Polymorphic DNA (RAPD) is a PCR based molecular marker that uses arbitrarily selected decamer primers to amplify a set of DNA fragments that are located at random positions in the genome. Since the primers are 10 nucleotides long, they can be annealed at a number of locations in the genome giving a number of amplification products. These products are separated on 1.2 per cent agarose gel. Different banding patterns can be observed depending on the genetic make up of individuals.

An RAPD reaction mixture contains different constituents like template DNA, random primer, enzyme, dNTPs, $MgCl_2$ and assay buffer which are subjected to repeated cycles of denaturation, primer annealing and extension in a thermal cycler.

3.2.2.5.1. Protocol for RAPD

The basic RAPD procedure suggested by Williams *et al*. (1990) was suitably modified to suit tomato genome amplification. The reaction was carried out in a thermal cycler (Mastercycler personal, Eppendorf) using the following programme with heated lid condition.

Initial denaturation	- 94°C for 5 min	
Denaturation	- 94°C for 1 min	} 40 cycles
Primer annealing	- 37°C for 1 min	
Primer extension	- 72°C for 2 min	
Final extension	- 72°C for 2 min	

4°C for infinity to hold the sample.

RAPD was performed in a total reaction volume of 20 µl and each reaction had the following components.

a) Genomic DNA (25 ng)	- 2.0 µl
b) 10X Taq assay buffer	- 2.0 µl
c) d NTP mix (10 mM each)	- 1.0 µl
d) $MgCl_2$ (25 mM)	- 1.0 µl
d) Decamer primer (10 pM)	- 1.5 µl
e) Taq DNA polymerase (1U)	- 0.3 µl
f) Autoclaved distilled water	- 12.2 µl
Total volume	: 20.0 µl

A master mix was prepared for the required number of reactions adding all the components of reaction mix except the primer. Aiquots of the master mix (18 µl) was pipetted out into each of the 0.2 ml PCR tubes placed on ice followed by addition of 2 µl DNA into each tube separately. A brief spinning was given for the reaction, the tubes were loaded in the thermal cycler and the RAPD programme was run.

The amplified products were separated on 1.2 per cent agarose gel using 1X TAE buffer stained with ethidium bromide. The profile was visualized under UV (312 nm) transilluminator and documented using gel documentation system (Alpha Imager TM 1200).

3.2.2.5.2. Screening of random primers for RAPD

Primer screening was carried out to identify the best primers for RAPD analysis. A total of 40 deacamer primers under two different Operon series, viz. OPS (OPS 1 to OPS 20) and OPY (OPY 1 to OPY 20) were screened for amplification of the genomic DNA extracted from the genotype Sakthi, using the

thermal cycler mentioned under section 3.2.2.5.1. Table 3 and 4 shows the nucleotide sequence of each of the 40 primers screened.

After initial screening, the primers showing good amplification with five or more reproducible bands were selected and utilized for further characterization of 15 genotypes. From the banding pattern observed, the amplicons were rated as good (showing clear reproducible bands), average (faint bands, but reproducible) and poor (faint but not reproducible), and only those primers that displayed more than 50 per cent 'good' bands with higher number of amplicons were selected for further study.

The total number of bands along with the number of polymorphic bands obtained in all 15 genotypes with each of 14 primers selected was recorded. Percent polymorphism was calculated using the following formula.

$$\text{Per cent polymorphism} = \frac{\text{Total number of polymorphic bands X 100}}{\text{Total number of bands}}$$

3.2.2.5.3. Analysis of RAPD amplification profiles

Amplification profiles of 15 genotypes were compared with each other and the bands of DNA fragment scored manually as (1) or (0) depending on 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 Version 2.0 package.

Table 3. Nucleotide sequences of OPS primer screened for RAPD assay

Sl. No.	Primer	Nucleotide Sequence (5' – 3')
1	OPS 1	GTTTCGCTCC
2	OPS 2	TGATCCCTGG
3	OPS 3	CATCCCCCTG
4	OPS 4	GGACTGGAGT
5	OPS 5	TCGGCCCTTC
6	OPS 6	TGC TCT GCCC
7	OPS 7	GGTGACGCAG
8	OPS 8	GTCCACACGG
9	OPS 9	TGGGGGACTC
10	OPS 10	CTGCTGGGAC
11	OPS 11	GTAGACCCGT
12	OPS 12	CCTTGACGCA
13	OPS 13	TTCCCCCGCT
14	OPS 14	TCCGCTCTGG
15	OPS 15	GGAGGGTGTT
16	OPS 16	TTTGCCCGGA
17	OPS 17	AGGGAACGAG
18	OPS 18	CCACAGCAGT
19	OPS 19	ACCCCCGAAG
20	OPS 20	GGACCCTTAC

Table 4. Nucleotide sequences of OPY primer screened for RAPD assay

Sl. No	Primer	Nucleotide Sequence (5' – 3')
1	OPY 1	GTGGCATCTC
2	OPY 2	CATCGCCGCA
3	OPY 3	ACAGCCTGCT
4	OPY 4	GGCTGCAATG
5	OPY 5	GGCTGCGACA
6	OPY 6	AAGGCTCACC
7	OPY 7	AGAGCCGTCA
8	OPY 8	AGGCAGAGCA
9	OPY 9	AGCAGCGCAC
10	OPY 10	CAAACGTGGG
11	OPY 11	AGACGATGGG
12	OPY 12	AAGCTTGCGA
13	OPY 13	GGGTCTCGGT
14	OPY 14	GGTCGATCTG
15	OPY 15	AGTCGCCCTT
16	OPY 16	GGGCCAATGT
17	OPY 17	GACGTGGTGA
18	OPY 18	GTGGAGTCAG
19	OPY 19	TGAGGGTCCC
20	OPY 20	AGCCGTGGAA

Resolving power (Prevost and Wilkinson, 1999) was used to identify the primers that would distinguish the accessions most efficiently. Resolving power (Rp) of a primer was calculated as the sum of 'band informativeness' of all the bands produced by the primer. Band informativeness (Ib) is = 1-(2 x |0.5-p|) where 'p' is the proportion of accessions containing the band. Resolving power of the primer is represented as: Rp= ∑Ib. Finally the data obtained was pooled together to generate a combined dendrogram.

3.2.2.6. AFLP (Amplified Fragment Length Polymorphism) ASSAY

AFLP is a highly sensitive and reproducible method for detecting polymorphism throughout the genome. It is based on PCR amplification of genomic restriction fragments generated by specific restriction enzymes and oligonucleotide adapters of a few nucleotide bases (Vos *et al*., 1995). The technique of AFLP involves mainly 3 steps.

1. Restriction endonuclease digestion of DNA and ligation of adapters.
2. Amplification of restriction fragments.
3. Gel analysis of amplified fragments.

Different combinations of *Eco*RI and *Mse*I primer pairs are used for different AFLP reactions. This method allows specific co-amplification of 50-100 restricted fragments in each reaction and detection on denaturing polycrylamide gels. The resultant banding pattern can be documented and analysed for polymorphisms either manually or using analytical software.

3.2.2.6.1. Protocol for AFLP assay

The AFLP analysis was carried out using AFLP^{R} Analysis system I kit (Invitrogen Corporation, USA) according to manufacturer's instructions.

Composition of different reagents used in AFLP analysis is given in Appendix IV. The procedure followed involved the following steps:

A) Restriction digestion of genomic DNA

The following components were added to a 1.5 ml microfuge tube and the digestion reaction was set up.

Components	**Quantity per sample**
5X reaction buffer	5 µl
Sample DNA (250 ng)	2 µl
*Eco*RI / *Mse*I	2 µl
Distilled water	16 µl
Total volume	: 25 µl

The contents were mixed gently and a brief centrifugation was given. The mixture was incubated for 2hrs at 37°C. The mixture was again incubated at 70°C for 15 min to inactivate the restriction endonuclease. Then the tubes were placed on ice and a brief centrifugation was given.

B) Ligation of adapters

Adapter ligation solution (24 µl) and T_4 DNA ligase (1 µl) were added to the digested DNA. The contents were mixed gently and centrifuged briefly. These tubes were incubated at 20 ± 2°C for 2 hrs. The ligation mixture was diluted 10 times by adding 90 µl TE buffer to 10 µl of the reaction mixture and mixed well. The unused portion of the reaction mixture was stored at –20°C.

C) Preamplification reaction

PCR amplification was carried out in two consecutive steps. In the first reaction, called preamplification, the ligated product was amplified with an *Mse*I primer containing one selective nucleotide (N+1) and an *Eco*RI primer containing no selective nucleotide (N+0). The reaction was set up as given below. The following components were added to a 0.2ml thin walled microfuge tube.

Components	**Volume**
Diluted DNA (1:10 ligated product)	5 µl
Pre-amp primer mix	40 µl
10X PCR buffer + Mg	5 µl
Taq DNA polymerase (5 U/µl)	1 µl
Total volume	: 51 µl

The contents in the tubes were mixed gently and briefly centrifuged to collect the reaction. The tubes were placed in a thermal cycler and PCR was performed for 20 cycles at 94°C for 30 sec, 56°C for 60 sec and 72°C for 60 sec.

Following amplification, 1:50 dilution of the PCR product was done as follows: 3 µl of the amplified product was transferred to a fresh 0.2 ml microfuge tube containing 147µl of TE buffer. Both the diluted as well as undiluted reaction products were stored at –20°C.

D) Primer labeling

The selective N+2 *Eco*RI primer was end labeled by phosphorylating the 5'end with γ^{32}P dATP using T_4 kinase. ^{32}P labeled primers were preferred as they gave a better resolution of the PCR products on the gels and the reaction products were less prone to degradation due to auto radiolysis. The labeling reaction was

set up as given below. The following components were added to a 0.2 ml microfuge tube.

Components	**Volume (for 10 samples)**
*Eco*RI primer	- 1.8 µl
5X kinase buffer	- 1.0 µl
γ^{32}P dATP	- 2.0 µl
T_4 kinase	- 0.2 µl
Total volume	: 5 µl

The contents were mixed gently, centrifuged briefly and then incubated at 37°C for one hr. Following incubation, the enzyme was heat inactivated at 70°C for 10 min. The tube was centrifuged briefly to collect the contents.

Simultaneously, the 30-330 bp AFLP ladder (Invitrogen) was also labeled using γ^{32}P dATP. The labeling reaction was set up as given below.

30-330 bp AFLP ladder	- 2 µl
5X Exchange reaction buffer	- 1 µl
γ^{32}P ATP	- 1 µl
T_4 polynucleotide kinase	- 1 µl

The components were mixed thoroughly, collected by brief centrifugation and incubated for 10 min at 37°C. Then the reaction was inactivated at 65°C for 15 min. An equal volume (5µl) of TE buffer was added to the reaction mixture followed by 20 µl of denaturing solution. The solution was incubated at 70°C for 5 min and stored at –20°C.

E) Selective amplification

The 1:50 dilution of the amplified product was used as template for the sec amplification reaction called selective amplification using *Mse*I and *Eco*RI primer containing 3 selective nucleotides (N+3). The sequence of the *Eco*RI and *Mse*I primers used are listed below.

1. E + ACG (5’ AGACTGCGTACCAATTCACG 3’)
2. E + AGC (5’ AGACTGCGTACCAATTCAGC 3’)
3. E + AGG (5’ AGACTGCGTACCAATTCAGG 3’)
4. E + AAG (5’ AGACTGCGTACCAATTCAAG 3’)
5. M + CAC (5’ GATGAGTCCTGAGTAACAC 3’)
6. M + CAA (5’ GATGAGTCCTGAGTAACAA 3’)
7. M + CAT (5’ GATGAGTCCTGAGTAACAT 3’)
8. M + CTT (5’ GATGAGTCCTGAGTAACTT 3’)

The reaction for selective amplification was set up as given below.

A) For each primer pair, the following components were added to a 0.2 ml microfuge tube and it was marked as Mix 1.

Components	**Volume (for 10 samples)**
Labelled *Eco*RI primer	5 µl
MseI primer	45 µl
Total volume	: 50 µl

B) The following components were added to another 0.2 ml microfuge tube that was marked as Mix II.

Components	**Volume (for 10 samples)**
Distilled water	79 µl
10X PCR buffer	20 µl
Taq DNA polymerase	1 µl
Total volume	: 100 µl

C) The final selective amplification reaction was assembled by adding 5 µl of pre-amplified, diluted template DNA, 5 µl of Mix I and 10 µl of Mix II to a 0.2 ml microfuge tube. The contents were mixed gently and a brief spin was given.

The tubes (20 µl reaction volume) were placed in a thermal cycler for selective amplification with the following conditions: one cycle at 94°C for 1 min, 65°C for 1 min and 72°C for 1 hr 30 min. Next the annealing temperature was lowered in each cycle by 0.7°C during 12 cycles. This was followed by 23 cycles at 94°C for 30 sec, 56°C for 30 sec, and 72°C for 1 min. After completion of amplification, the reaction was held at 4°C. The products were then stored at –20°C for further use.

3.2.2.6.2. PolyAcrylamide Gel Electrophoresis (PAGE):

The amplified products of AFLP reaction were separated on denaturing polyacrylamide gel. Polyacrylamide gel was prepared by co-polymerization of acrylamide and a cross linker, N' N'-methylene bisacrylamide. The ratio of acrylamide to bisacrylamide (19:1 is commonly used) and total concentration of acrylamide in gel determine the pore size of the matrix and its sieving effect on the DNA molecule as they pass through it. To keep the DNA denatured, the sequencing gel was prepared with a high concentration of urea (7-8 M). Formamide dye was added to the gel to increase its denaturing capacity. DNA sequencing gels are typically very thin (0.2-0.4 mm). Electrophoresis was done at high voltage (1200-1300 V) and the power was adjusted to maintain the gel temperature at 55 to 60°C. Electrophoresis took about 2 hrs time.

Procedure for PAGE:

1) The electrophoresis plates, spacers and sharks tooth comb of the BIORAD gel sequencer apparatus were thoroughly washed with distilled water. Then washed with ethanol and dried with tissues.
2) On the thermostatic plate 50 µl repeller silane was evenly spread using a tissue paper, which aids in easy transfer of gel on to a filter paper.
3) Then the spacers were placed on either side of the thermostatic plate and the other glass plate was placed over it.
4) This gel-casting unit was then fitted by tightening the clamps on both sides of the plates and provided a support. Then it was kept horizontally on a leveled surface such that the sec plate faced downwards.
5) Four per cent denaturing acrylamide-bis acrylamide solution was prepared and stored in amber coloured bottles for further use. The gel mix was prepared in a beaker adding the following components.

Acrylamide solution	- 11.5 ml
TBE buffer	- 15 ml
Urea	- 31.5 g
Ammonium per sulphate (APS)	- 0.1 g in 1 ml distilled water
TEMED	- 50 µl

6) The prepared gel mix was immediately injected into the gel-casting unit through the bottom side using a syringe. Injection was done slowly to avoid trapping of air bubbles.
7) The sharks toothcomb was inserted immediately at the top of the gel-casting unit, in between the two plates, with the even edge facing the gel.
8) The gel was allowed to set for 30 to 45 min at room temperature. After polymerization, the gel-casting unit was placed vertically in the sequencing cell apparatus (BIO-RAD).
9) The electrophoresis tank and the reservoir in the thermostatic plate were filled with 0.5X TBE buffer.

10) The assembly was then connected to a power pack and subjected to pre-running at 45 W for 30 min to warm the gel to 50°C.
11) After pre-running, the comb was taken out and the top surface of the gel was flushed out using a syringe filled with buffer immediately before loading.
12) Then the comb was re-inserted into the gel surface with even side up to create wells.
13) Formamide dye, equal to the total reaction volume (20 µl) was added to the sample and denatured at 95°C for 5 min in a thermal cycler. After this, the tubes were placed on ice immediately to prevent renaturation.
14) Then the samples and denatured molecular weight ladder was loaded (8 µl) in the wells and the gel was run at 40 W constant power and 50°C temperature for 1.5 to 2 hrs until the xylene cyanol was two-third down the length of the gel.

3.2.7.2. Gel drying

After electrophoresis, the plates were removed from the apparatus; the buffer was poured off and allowed to cool to room temperature. Using a flat spatula the top glass plate was removed slowly without disturbing the gel. Then a Whatman filter paper cut to the size of the gel was placed over the gel and pressed gently. The filter paper was lifted slowly so that the gel was sticking to it. The gel was then covered with a cling film and dried under vaccum in a gel drier (BIORAD) at 80°C for about 2 hrs.

3.2.7.3. Gel exposure and Scanning

After dying, the gel wrapped with cling film was exposed to an Imaging Plate (IP) and kept in a BAS cassette (FUJIFILM) for about 20 min. The IP plate accumulates and stores radiation energy while it is exposed. This plate

has an image-recording layer consisting of polyester base material densely coated with accelerated phosphorescent fluorescent material of fine crystals. The recording surface of the IP was scanned with a laser beam inside the Fluorescent Image Analyzing System FLA-5100 (FUJI PHOTO FILM Co., Ltd.) and the digital image was recorded in the analyzer unit. The image was then analyzed using the Multigage software (FUJIFILM).

3.2.7.4. Data analysis of AFLP gel profile

Each AFLP band was treated as a unit character and was scored manually as independent binary codes (1 for presence and 0 for absence). Only distinct and well-resolved fragments were scored. The resulting data were used to construct an UPGMA (Unweighted Pair Group Method with Arithmetic means) dendrogram using the software package NTSYS (Rohlf, 1990).

3.2.2.8. MOLECULAR CLONING

3.2.2.8.1. Detection of trait specific markers

The primer OPS 1 gave polymorphism between the genotypes with respect to bacterial wilt resistance. The RAPD reaction was repeated thrice using the same primer to confirm the reproducibility. Since the intensity of the specific band was less, ten RAPD reactions with template DNA from the resistant genotype Mukthi were set up as mentioned in section 3.2.2.5.1. One negative control reaction was also set with the genomic DNA of susceptible genotype Arka Vikas. After amplification, the products were resolved on 1.2 per cent agarose gel. The band of interest was eluted, pooled and reamplified with the same primer.

3.2.2.8.2. Gel elution of specific amplicon

Products obtained in different RAPD reactions were loaded separately on 1.2 per cent (w/v) agarose gel and desired band in each case was eluted using AxyPrep DNA Gel Extraction Kit (Axygen, Biosciences). Procedure as per the manufacturer's guidelines was followed.

- DNA fragment of interest was excised from the gel using a sterile, sharp scalpel avoiding much exposure to UV on a transilluminator.
- Gel slice was weighed in a colourless 1.5 ml microfuge tube.
- Added 3X gel volume of gel solubilization buffer (w/v).
- The gel was resuspended in gel solubilization buffer by vortexing. Heated the gel containing gel solubilization buffer at 75°C until the gel was completely dissolved. Intermittent vortexing was given every 2 to 3 min to accelerate gel solubilization.
- Added 0.5X gel solubilization volume of binding buffer and mixed properly.
- A spin column was placed in a 2 ml collection tube. The 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 min.
- The filtrate was discarded. Added 500 µl of wash buffer to the spin column and centrifuged at 12,000 xg for 30 sec.
- Discarded the filtrate and 700 µl of desalting buffer was added and centrifuged at 12, 000 xg for 30 sec.
- A sec wash, by adding 700 µl of desalting buffer, followed by centrifugation at 12,000 xg for 30 sec was done to ensure the complete removal of salt. The filtrate was discarded and spin column was again placed in collection tube.
- Column was again centrifuged for 1 min at 12,000 xg to remove any residual buffer.

- Spin column was transferred to a fresh 1.5 ml centrifuge tube. The eluent was prewarmed at 65°C to improve the elution efficiency. To elute the DNA, 25 µl of eluent was added to the centre of the spin column. It was allowed to stand for 1 min at room temperature. Then centrifuged at 12,000 xg for 1 min.
- Eluted DNA fragments were checked on 0.7 per cent (w/v) agarose gel and stored at – 20°C for further cloning works.

3.2.2.9. TRANSFORMATION

3.2.2.9.1. Preparation of competent cells

Competent cells for plasmid transformation were prepared following the protocol of Mandel and Higa (1970).

Medium prepared: LB medium and LBA medium. (Details of media prepared are given in the Appendix V)

The steps followed for competent cell preparation were as follows:

Day 1:

- Inoculated 18 hr old *Escherichia coli* JM 109 strain, single colony to 3 ml LB medium in sterile condition and incubated overnight at 37°C on a shaker set at 160 rpm.

Day 2:

- Aseptically transferred 3 ml overnight grown culture to 50 ml sterile LB broth and inoculated for 4 hrs at 37°C on a shaker set at 160 rpm until OD_{600} reached 0.4 to 0.5. The growth of culture was monitored at every 30 min.
- The cells were aseptically transferred to a sterile, disposable ice-cold 50 ml polypropylene tube.
- The culture was cooled to 0°C on ice for 20 min.
- The cell suspension was centrifuged at 3500 rpm for 10 min at 4°C.

- The supernatant obtained was carefully discarded and the pellet obtained was gently resuspended in 10 ml ice-cold, filter sterilized 0.1M $CaCl_2$.
- The tubes were kept on ice for 20 min and the cell suspension was centrifuged at 5000 rpm for 10 min at 4°C.
- The supernatant was decanted and resuspended the pellet in 2 ml of ice- cold filter sterilized 0.1 M $CaCl_2$. The tubes were kept on ice for 18 hrs.

Day 3:

- Chilled glycerol (4 ml) was added to the cell suspension and mixed well using a sterile micro tip.
- The competent cells prepared were stored at -70°C as aliquots of 100 µl in chilled 1.5 ml microfuge tubes covered with aluminium foil until further use.

3.2.2.9.2. 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,

- The competent cells stored at –70°C were thawed over ice for 10 min.
- Plasmid DNA (10 µl) was added to 100 µl competent cells. Negative control was placed simultaneously without adding plasmid.
- The cells were kept in ice for 40 min. Heat shock was given at 42°C for 90 sec in a water bath and plunged in ice for 5 min.
- LB medium (250 µl) was added to the cells and incubated at 37°C for 1 hr on a shaker set at 120 rpm.

- The transformed cells (100 µl) were plated on LBA/ampicillin and incubated overnight at 37°C in a shaker (100 rpm). The recombinant clones alone can grow on ampicillin plate.

3.2.2.10. 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.

3.2.2.10.1. Ligation

The pGEM-T Easy vector and control insert was centrifuged briefly to collect contents at the bottom of the tubes. Ligation reaction was set up in 0.5ml microfuge tubes as follows,

Ingredients	**Standard reaction**	**Positive control**	**Background control**
Rapid ligation buffer (2X)	5 µl	5 µl	5 µl
pGEM-T Easy Vector (50 ng)	1 µl	1 µl	1 µl
Eluted DNA	1 µl	--	--
Control insert DNA	--	2 µl	--
T_4 DNA ligase (3 Weiss units/ µl)	1 µl	1 µl	1 µl
Deionized water	2 µl	1 µl	3 µl
Final volume	10 µl	10 µl	10 µl

The reactions were mixed and incubated for 1hr at room temperature and then incubated at 4°C overnight. It was taken on the next day for transformation in competent cells of *E. coli.*

3.2.2.10.2. Transformation of ligated product and recombinant selection

Reagents prepared:

1. Ampicillin → 5 mg/ml in water
2. IPTG → 200 mg/ml in water

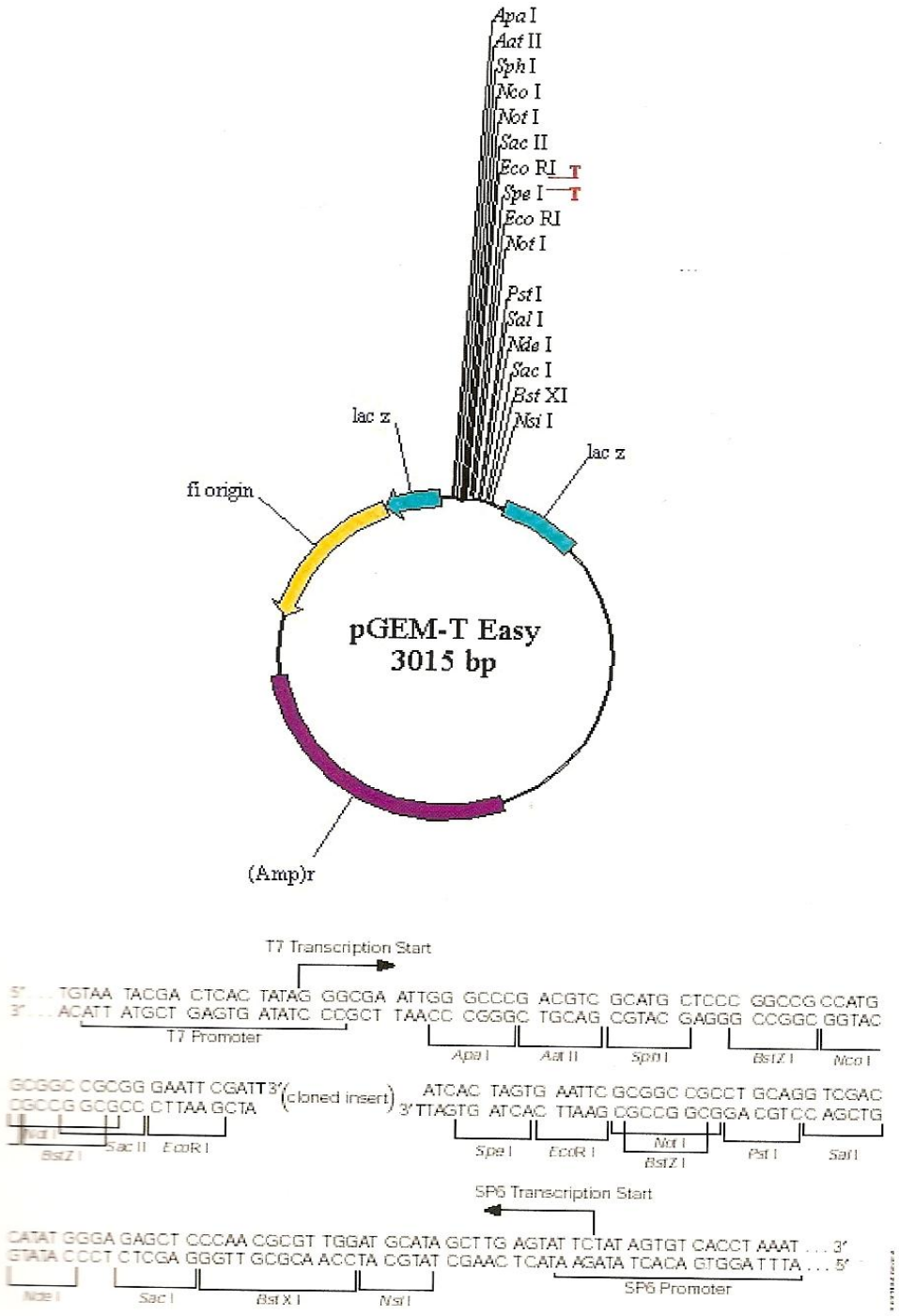


Fig. 1. pGEM-T Easy Vector (Promega) used for cloning RAPD product. The *lac*Z region, promoter and multiple cloning sites are shown in the figure. The top strands of the sequence shown corresponds to the RNA synthesized by T7 RNA polymerase and bottom strands corresponds to the RNA synthesized by SP6 RNA polymerase.

3.X-Gal → 10 mg/ml in DMSO

The procedure followed for DNA transformation and blue white screening is as follows:

The ligated PCR product was added to 100 µl of competent cells and kept on ice for 40 min. Heat shock was given at 42°C for 2 min in a water bath and plunged in ice for 5 min. LB medium (250 µl) was added to the cells and incubated at 37°C for 1 hr on a shaker set at 120 rpm. The aliquots of cells (100 µl) were plated on LBA/Ampicillin (5 mg/ml)/X-Gal (20 mg/ml) plates and incubated overnight at 37°C.

3.2.2.10.3. Isolation of recombinant plasmid DNA

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

Reagents prepared:

Resuspension buffer (Solution I)

Lysis buffer (Solution II)

Neutralization buffer (Solution III)

LB medium

Ampicillin (10%)

(Details of the reagents prepared are given in Appendix V)

Procedure:

- Cells were harvested from overnight grown recombinant *E. coli* culture from white colonies containing plasmid DNA by centrifugation at 10,000 rpm for 5 min.
- The supernatant was discarded and the bacterial pellet was suspended in ice-cold resuspension buffer (100 µl).
- The freshly prepared lysis buffer (200 µl) was added to the pellet and mixed gently by inverting the tubes for 5 min.

- Ice-cold neutralization buffer (150 µl) was added, vortexed gently and kept on ice for 5 min.
- Centrifuged at 12,000 xg for 10 min at 4°C and the supernatant was transferred into a fresh Eppendorf tube.
- The DNA was precipitated with two volume of ethanol at room temperature and vortexed.
- Incubated at room temperature for 2 min and centrifuged at 12,000 xg for 5 min at 4°C.
- The supernatant was removed and pellet was rinsed with 70 per cent ethanol.
- The pellet was air dried and resuspended in 30 µl autoclaved double distilled water.
- Five microlitre of the plasmid suspension was checked on agarose gel and the image was documented.

3.2.2.11. CONFIRMATION OF DNA CLONES

3.2.2.11.1. Confirmation of recombinant plasmid DNA using RAPD

The recombinant plasmid DNA isolated by alkali lysis method was amplified by PCR (Eppendorf Mastercycler Personnel) in 50 µl reaction mix using OPS 1 primer. The thermal settings followed were:

i) Initial denaturation at 94°C for 5 min
ii) Denaturation at 94°C for 1 min
iii) Primer annealing at 37°C for 1 min
iv) Primer extension at 72°C for 2 min } 40 cycles
v) Final extension at 72°C for 2 min
vi) Cooling at 4°C to hold the sample

Each reaction mixture for PCR had the following reagents:

a. Plasmid DNA

b. Taq assay buffer
c. dNTP mix
d. Random primer
e. Taq polymerase

The total volume was made up to 20 µl with autoclaved distilled water. All the reaction mixtures were set in 0.2 ml microfuge tubes chilled over ice flakes. A momentary spinning was given for the reaction and set in thermal cycler for polymerase chain amplification under suitable program with heated lid condition. The RAPD product was checked on 1.2 per cent agarose gel and documented.

3.2.2.11.2. Restriction digestion of recombinant plasmid DNA

Purity of the recombinant plasmid DNA was checked on 0.7 per cent agarose gel before proceeding with the restriction digestion. The reaction mix was prepared as given below in a sterile 0.2 ml microfuge tube.

Contents	**Quantity**
Plasmid DNA	10 µl
Restriction enzyme, *Eco*RI	1 µl
Restriction buffer	2 µl
Autoclaved distilled water	7 µl
Total volume	20 µl

The reaction mix was incubated at 37°C for 1 hr. The restriction digestion was arrested by adding 1 µl 0.5 M EDTA and then keeping on ice for 15 min. The restriction digestion was confirmed by running the digest in 0.7 per cent agarose gel and documented.

3.2.2.12. MAINTENANCE OF CLONES

3.2.2.12.1. Preparation of pure culture of recombinant bacteria

Materials prepared

LBA medium and Ampicillin 5 per cent

In a laminar flow, single white colony from the transformed plate was taken by using flame-sterilized bacterial loop. This was streaked on LBA plate containing ampicillin (50mg/l). The plate was incubated overnight at 37°C and further stored at 4°C.

3.2.12.2. Preparation of stabs

Materials prepared

LBA medium and ampicillin (5%)

The LBA medium containing antibiotic ampicillin (50 mg/l) was melted and poured into storage vial, aseptically and allowed to solidify. Single colony of recombinant bacterial colony was carefully lifted with a sterile bacterial loop. The loop loaded with bacteria was plunged into the solid medium and incubated at 37°C overnight in the culture tube. The stabs showing good growth of bacteria were further stored in refrigerator at 4°C.

3.2.12.3. Glycerol culture

Materials prepared

LB liquid and ampicillin (5%)

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°C in a shaker at 120 rpm overnight. On the next day aliquots (800 μl) of cell culture was added to 100 per cent glycerol (200 μl) aseptically and stored at –20°C.

3.2.9. SEQUENCING OF DNA CLONES

The stab of the recombinant clone was sent to DNA sequencing facility, Genei, Bangalore (www.bangaloregenei.com) for sequencing. Details of the vector cloned and size of the insert were provided. 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, that uses fluorescent labeled dye terminators and fluorescent labeled primers. The Sanger's method of sequencing was reported to be adopted by the firm.

3.2.10. THEORETICAL ANALYSIS OF SEQUENCE

The sequence information obtained from the firm was named as '*Tomato seq1*' and further analyzed for its characterization.

3.2.10.1. Vector screening

The sequence of pGEM-T Easy Vector was downloaded from the site (www.promega.org). The vector sequence was aligned with the sequence obtained using bl2seq tool offered by NCBI (www.ncbi.nlm.nih.gov) to remove the vector region from the clone. The aligned vector sequence was deleted and the remaining sequence named as '*Tomato seq 2*'.

3.2.10.2. Homology search

The nucleotide sequence '*Tomato seq 2*' was compared with the sequence available in the database using BLAST tool offered by NCBI. Nucleotide-nucleotide blast (blast n) was carried out for homology search. The BLAST programme 'blastn' (Altschul *et al*., 1997) provided by NCBI (www.ncbi.nlm.nih.gov/Blast/Blast.cgi) was utilized for the purpose. The nucleotide sequence of the insert was pasted in the BLAST web page and the

programme was run at default settings. The best sequence alignment of the search results were noted and saved.

3.2.10.3. Detection of Open Reading Frame (ORF)

The programme 'ORF finder' (www.ncbi.nlm.nih.gov/gorf/gorf.html) of NCBI was used to find the open reading frame of the insert nucleotide sequence. The nucleotide sequence was copied and pasted in the displayed box and clicked on 'OrfFind'. The displayed web page showed ORF sequence in all reading frames. Open reading frames available in the entire region were noted and saved. The displayed amino acid sequence of the reading frames were pasted in a notepad and BLASTp search was performed. The results obtained were saved for further interpretation.

3.2.10.4. Detection of nucleotide statistics

Nucleotide composition of the sequence obtained was determined by nucleotide statistics tool provided by Biology Workbench (http://seqtool.sdsc.edu/). Initially the nucleotide sequence of the insert was saved in the workbench. The site was entered and the nucleotide tool NASTATS was selected to obtain the details of nitrogen bases in the DNA fragment.

3.2.10.5. Restriction analysis

Restriction sites available in the DNA fragment for the restriction enzymes were detected by restriction analysis tool offered by NEB cutter (http;//tools.neb.com/NEBcutter2). Important sites available for manipulation were selected and interpreted.

3.2.10.6. Amino acid analysis

Physical and chemical properties of the given protein from the deduced amino acid sequence were determined using amino acid statistics tool (AASTATS) offered by Biology Workbench. The site was entered and selected for protein tools. Deduced amino acid sequence of the DNA sequence was saved in this Biology Workbench. The sequence was selected and appropriate tools were chosen as per instructions in the site.

Other analyses carried out were Kyte and Doolittle (Kyte and Doolittle, 1987) hydropathy plot using Hydrophobicity plot tool of Molecular tool kit (www.vivo.colostate.edu/molkit/index.html) and secondary structure prediction using SOPMA programme of ExPASY tool (www.expasy.org/tools).

Results

4. RESULTS

The results of the study conducted on Development of trait related markers for bacterial wilt resistance in tomato at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara are presented in this chapter under different subheads.

4.1. Evaluation of genotypes for bacterial wilt resistance

Seedlings belonging to the 15 tomato genotypes transplanted and raised in earthen pots containing sterile mixture of farmyard manure, sand and soil in the ratio 1:1:1 recorded good establishment rate. The establishment and growth performance of seedlings was better in the greenhouse than in open culture (Plate 2 and Plate 3). The survival rate of seedlings 10 days after transplanting is given in Table 5. The individual seedlings when artificially inoculated with bacterial ooze through stem puncture and soil drenching with wounding; the wilt symptoms were observed 3 days after inoculation under open condition. The symptom started as leaf drooping followed by complete wilting and death of the plant. The wilted plants showed positive response to bacterial ooze test. When sliced stem of the wilted plant was placed in a test tube containing clear water and allowed to stand for few minutes, streaming of bacteria was observed confirming the presence of bacteria.

Based on the observations made on wilt incidence, the genotypes were classified into four different categories following Mew and Ho (1976) system. The percent of wilt incidence and reaction of each genotype to bacterial wilt disease are presented in Table 6. The reaction of genotypes to bacterial wilt incidence is given in Plate 4a, 4b and 4c. The plants started wilting from third day onwards and continued upto six days after inoculation.

a. Tomato seedlings 10 days after transplanting

b. Tomato genotypes at flowering stage

Plate 2. Growth performance of tomato genotypes in greenhouse

Table 5. Survival rate of seedlings 10 days after transplanting

Sl. No.	Genotype	No. of plants transplanted		Per cent survival	
		Green house	Open condition	Green house	Open condition
1.	Anagha	28	22	100	100
2.	Arka Vikas	27	20	100	96
3.	BL-333-3-1	26	20	100	98
4.	BT-1	28	20	100	96
5.	BT-218	24	21	100	98
6.	BTH-102-1-2-2	24	20	100	94
7.	Hawaii 7998	28	23	100	98
8.	LE-1-2	26	20	100	98
9.	LE-20	28	20	100	96
10.	LE-66	28	22	100	96
11.	LE-474	26	20	100	100
12.	Mukthi	28	23	100	100
13.	PKM-1	27	20	100	98
14.	Pusa Ruby	24	20	100	98
15.	Sakthi	28	20	100	100

Plate 3. Tomato seedlings (10 DAT) maintained in open condition

Plate 4a. Reaction of tomato genotypes to artificial inoculation with *R. solanacearum*

Plate 4b. Reaction of tomato genotypes to artificial inoculation with *R. solanacearum*

Plate 4c. Reaction of tomato genotypes to artificial inoculation with *R. solanacearum*

Among the fifteen genotypes evaluated, the lowest wilt incidence was observed in Anagha (10%) followed by Sakthi (15%), Mukthi (15%), Hawaii 7998 (15%) and LE-66 (18%) and all these genotypes were categorized as resistant (R). The genotypes LE-474, LE-20 and LE-1-2 were observed to be moderately resistant (MR) with 20 to 40 per cent bacterial wilt incidence. The moderately susceptible (MS) category included the genotypes BT-1, BT-218 and BTH-102-1-2-2 and the wilt incidence ranged between 40 and 60 per cent. Pusa Ruby was found to be the most susceptible (S) genotype recording 90 per cent wilt incidence. Arka Vikas (80%), PKM-1 (75%) and BL-333-3-1 (70%) were the other genotypes grouped as susceptible since the wilt incidence was more than 60 per cent. The seedlings maintained in the greenhouse failed to take disease infection and were healthy and robust in appearance even at flowering stage (Plate 2b).

4.2. MOLECULAR CHARACTERIZATION

Genomic DNA of each of the 15 genotypes was isolated, purified and subjected to RAPD and AFLP assay for molecular characterization.

4.2.1. Isolation and quantification of genomic DNA

The protocols suggested by Doyle and Doyle (1987) and Rogers and Bendich (1994) were used with modifications for the isolation of genomic DNA from tender tomato leaves. The quality of DNA isolated by both the methods was tested using agarose gel electrophoresis. Better quality DNA with distinct bands and lesser RNA contamination was obtained in all the genotypes with the Rogers and Bendich method upon electrophoresis on 0.8 per cent agarose gel (Plate 5a). The DNA samples isolated by Doyle and Doyle method showed less discrete bands with more RNA contamination (Plate 5b).

Table 6. Genotypic response of tomato to bacterial wilt disease

Sl. No.	Genotype	Per cent wilt incidence	Disease reaction
1.	Anagha	10	R
2.	Arka Vikas	80	S
3.	BL-333-3-1	70	S
4.	BT-1	58	MS
5.	BT-218	50	MS
6.	BTH-102-1-2-2	45	MS
7.	Hawaii 7998	15	R
8.	LE-1-2	35	MR
9.	LE-20	25	MR
10.	LE-66	18	R
11.	LE-474	26	MR
12.	Mukthi	15	R
13.	PKM-1	75	S
14.	Pusa Ruby	90	S
15.	Sakthi	15	R

R – Resistant MR – Moderately Resistant

S – Susceptible MS – Moderately Susceptible

RNase treatment was given for all the DNA preparations in order to remove RNA (Sambrook *et al*., 1989). The electrophoretic profile after RNase treatment revealed uniform discrete bands from all the samples indicating good quality DNA free from RNA and other contaminants (Plate 5c).

The quality of DNA isolated by Rogers and Bendich method from the 15 tomato genotypes was checked using spectrophotometry. The ratio of absorbance at 260nm to that at 280nm ranged from 1.78 to 1.88 indicating relatively pure DNA in the samples. The ratio A_{260}/A_{280} was highest for the genotype Mukthi and least for the genotype Arka Vikas.

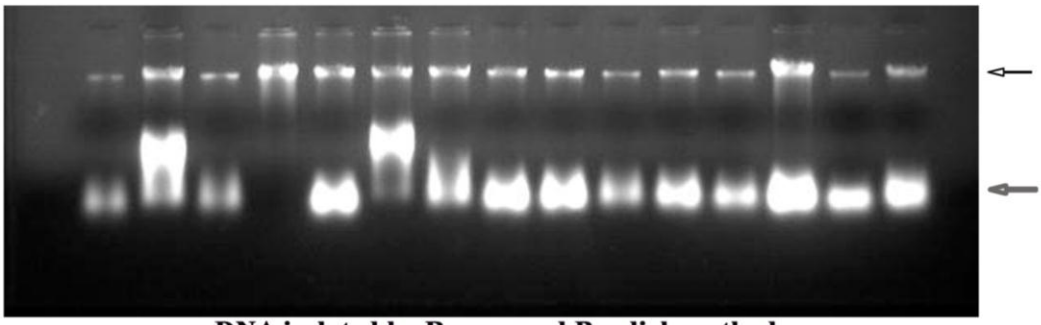
Quantity of DNA isolated from each genotype using the Rogers and Bendich protocol was calculated. The quantity of DNA ranged between 219 and 255 µg/g of tissue. Maximum quantity of DNA was isolated in the genotype BT-1 and minimum quantity of DNA was isolated from the genotype LE-1-2. The quality and quantity of DNA isolated from each genotype are given in Table 7.

The DNA isolated by Rogers and Bendich method was further used for RAPD and AFLP analysis.

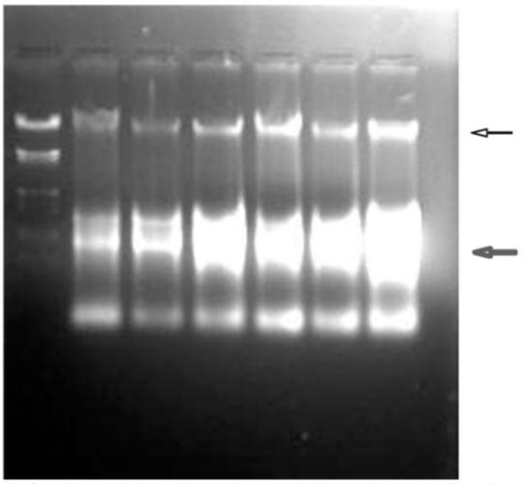
4.2.2. Random Amplified Polymorphic DNA (RAPD) assay

RAPD assay was carried out with the good quality DNA isolated from the 15 tomato genotypes using the random decamer primers. The list of genotypes used for RAPD analysis is

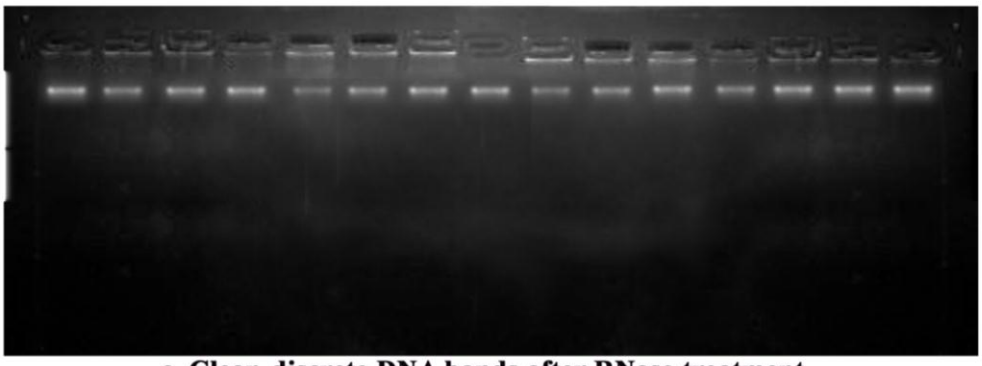
Category	**Genotype**
Resistant	Anagha, Sakthi, Mukthi, LE-66, Hawaii 7998
Moderately resistant	LE-1-2, LE-20, LE-474
Moderately susceptible	BT-1, BT-218, BTH-102-1-2-2
Susceptible	Pusa Ruby, Arka Vikas, BL-333-3-1, PKM-1



a. DNA isolated by Rogers and Bendich method



b. DNA isolated by Doyle and Doyle method



c. Clear, discrete DNA bands after RNase treatment

⇦ DNA bands ⇦ RNA contamination

Plate 5. Standardization of genomic DNA isolation in tomato

Table 7. Quality and quantity of DNA isolated from tomato genotypes by Rogers and Bendich method

Genotype	Absorbance at 260nm	Absorbance at 280nm	Absorbance 260/280	Quantity (µg/g)
1. Anagha	0.301	0.164	1.81	225.75
2. Arka Vikas	0.296	0.164	1.78	222.00
3. BL-333-3-1	0.326	0.176	1.83	244.50
4. BT-1	0.340	0.187	1.84	255.00
5. BT-218	0.310	0.168	1.84	232.50
6. BTH-102-1-2-2	0.334	0.183	1.83	250.50
7. Hawaii 7998	0.328	0.180	1.84	246.00
8. LE-1-2	0.287	0.157	1.80	215.25
9. LE-20	0.311	0.170	1.81	233.25
10. LE-66	0.310	0.168	1.86	232.00
11. LE-474	0.312	0.179	1.85	234.00
12. Mukthi	0.313	0.173	1.88	234.75
13. PKM-1	0.314	0.168	1.82	235.50
14. Pusa Ruby	0.292	0.163	1.82	219.00
15. Sakthi	0.318	0.174	1.83	238.00

4.2.2.1. Screening of primers

Forty random primers belonging to two different Operon primer series were screened using the DNA isolated from the genotype Sakthi to select primers showing good amplification for use in genotype screening. Among the 40 decamer primers tried, 35 primers, which gave good amplification were selected and used for further characterization.

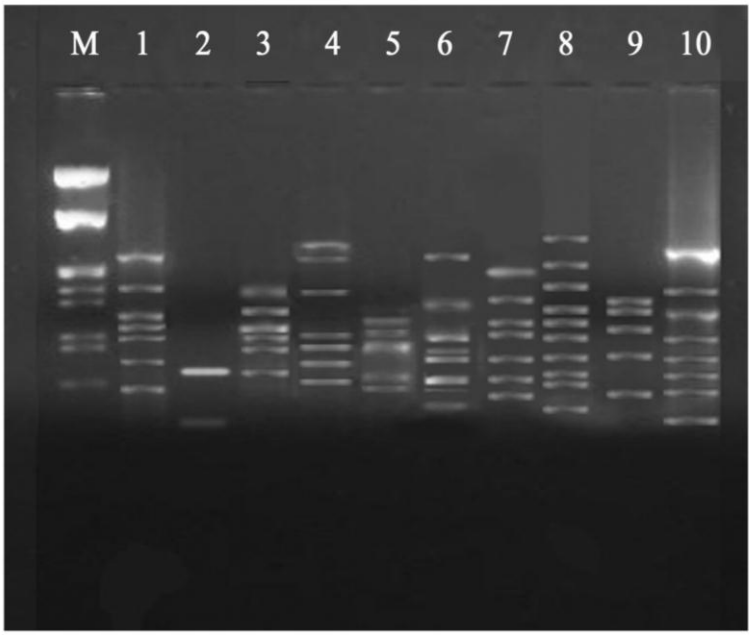
4.2.2.1. a) Screening with OPS series

Twenty primers belonging to the OPS series were screened using DNA from the genotype Sakthi. The number of amplicons obtained using the primers in this series ranged between 2 and 10 and the molecular weight of bands varied from 3.1 kb to 0.3 kb. The amplification pattern obtained for the primers of this series are shown in Plate 6, and the number of amplification products generated by each primer of the series is given in Table 8.

The primer, OPS 2 produced only two amplicons and the amplification was poor and hence not utilized for further characterization. All other primers from this series gave good amplification with five or more bands and were used for further screening of 15 genotypes.

4.2.2.1. b) Screening with OPY series

The amplification pattern obtained for the primers of this series is presented in Plate 7 and Table 9. The number of bands obtained using the primers in this series ranged from 4 to 8 and the product size ranged between 3 kb and 0.25 kb. The primers OPY 4, OPY 5, OPY 12 and OPY 16 gave less than five bands and hence were not utilized for further genotypic screening. The remaining sixteen primers of this series produced good amplification with five or more bands and therefore selected for characterizing the 15 genotypes.



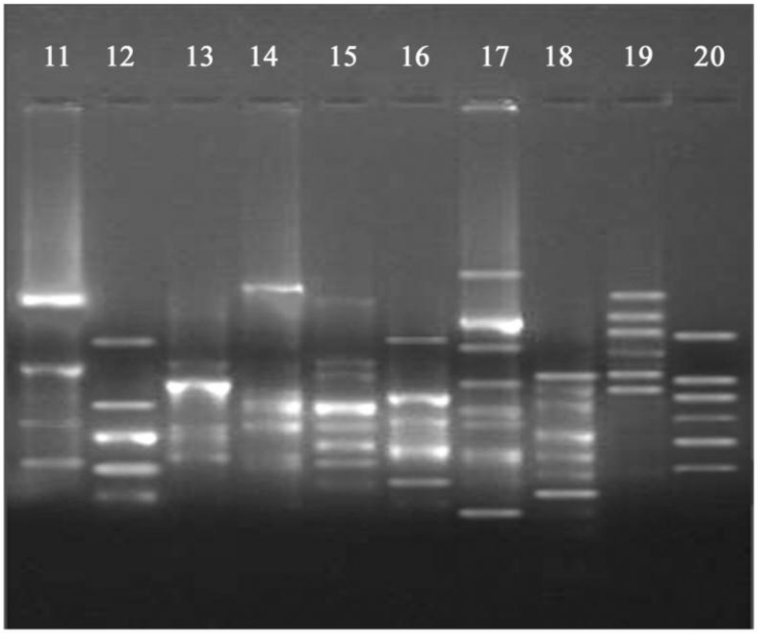


Plate 6. Screening of OPS series (1-20) primers for RAPD assay

Table 8. Number of bands and amplification pattern of OPS primers

Sl. No	Primer code	No. of bands	Amplification pattern
1	OPS 1	7	Good
2	OPS 2	2	Poor
3	OPS 3	6	Good
4	OPS 4	8	Good
5	OPS 5	5	Good
6	OPS 6	9	Good
7	OPS 7	7	Good
8	OPS 8	10	Good
9	OPS 9	5	Good
10	OPS 10	8	Good
11	OPS 11	4	Good
12	OPS 12	5	Good
13	OPS 13	5	Good
14	OPS 14	5	Good
15	OPS 15	8	Good
16	OPS 16	5	Good
17	OPS 17	9	Good
18	OPS 18	7	Good
19	OPS 19	6	Good
20	OPS 20	6	Good

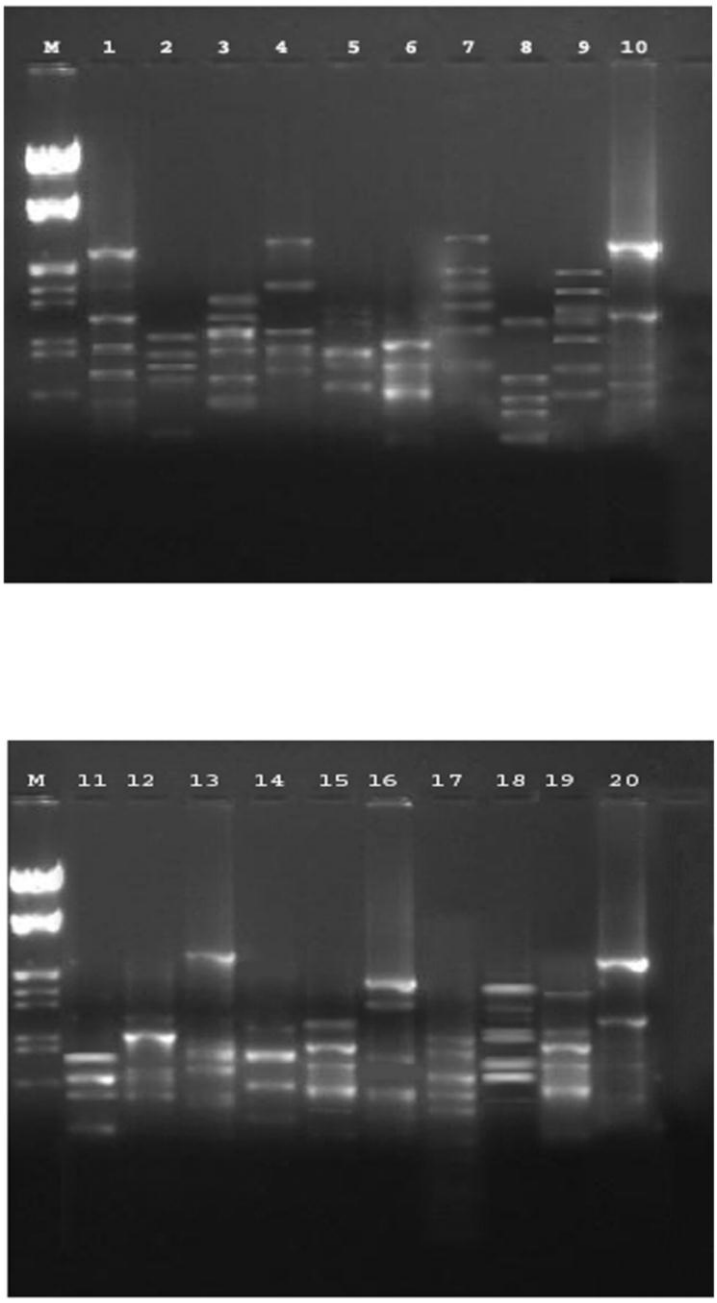


Plate 7. Screening of OPY series (1-20) primers for RAPD assay

Table 9. Number of bands and amplification pattern of OPY primers

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

4.2.2.2. RAPD profiles of tomato genotypes

The fifteen tomato genotypes differing in reaction to bacterial wilt disease were screened using the selected random primers belonging to the Operon primer series OPS and OPY.

Based on the results obtained from primer screening, the genotypes were characterized with the selected 35 primers. The number of amplicons and nature of amplicons produced in each genotype by the 35 primers selected are presented in Table 10 (A) and (B). Out of the 35 primers used for amplification of 15 genotypes, ten primers of OPS series and four primers of OPY series displayed good amplification with clear, reproducible bands with number of amplicons ranging from 5 to 13.

4.2.2.2. a) RAPD profile with selected primers from OPS primer kit

Based on the number of amplicons and nature of amplicons, ten primers were selected from this series for characterizing the tomato genotypes. The details of the amplification pattern generated by each primer are described below.

OPS 1

Seven amplicons were obtained after DNA amplification with the primer OPS 1. Among the seven bands produced, two clear, intense bands of sizes 2.5 kb and 1.2 kb were found to be monomorphic among the fifteen genotypes. The molecular weight of the bands obtained ranged between 2.5 kb and 0.5 kb. One unique band of size ~1.5 kb was shared by the resistant genotypes Sakthi, Mukthi and LE-66. The moderately resistant genotypes LE-20 and LE-1-2 and moderately susceptible genotype BTH-102-1-2-2 also showed the presence of this specific band and it was absent in all the four susceptible genotypes. The resistant genotypes Anagha and Mukthi shared two more bands of sizes ~1 kb and 0.5 kb. A polymorphic band of size 0.79 kb was observed in three resistant genotypes and

Table 10.A) Number and nature of amplicons observed in the 15 genotypes with the selected OPS primers

Primer	1		2		3		4		5		6		7		8		9		10		11		12		13		14		15	
	a	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**A**	**b**
OPS 1	3	A	3	A	2	A	2	A	2	A	5	G	3	G	3	G	7	G	5	G	6	G	7	G	3	G	6	G	7	G
OPS 3	4	A	3	G	1	A	6	G	6	G	6	G	6	A	3	A	3	G	6	G	6	G	6	G	6	G	5	G	4	G
OPS 4	11	A	10	A	5	P	7	P	11	A	11	A	10	A	5	P	6	P	9	A	10	G	8	P	10	A	10	A	9	G
OPS 5	5	G	5	G	5	G	5	G	5	G	5	G	-	P	5	A	5	G	5	G	5	G	5	G	5	G	5	A	5	G
OPS 6	8	G	5	A	3	A	7	G	7	G	9	G	9	G	5	A	8	G	8	G	8	G	8	G	8	G	9	G	9	G
OPS 7	5	A	6	A	2	P	6	G	5	G	6	G	5	G	5	G	6	G	6	G	7	G	7	G	7	G	7	G	7	G
OPS 8	10	G	10	G	4	A	5	A	8	G	9	G	9	G	9	G	10	G	10	G	11	G	11	G	11	G	11	G	11	G
OPS 9	2	A	6	A	-	P	4	A	4	A	6	A	6	A	-	P	-	P	6	A	5	G	5	G	-	P	6	G	6	A
OPS 10	8	G	8	G	4	A	8	G	7	G	8	G	7	G	5	G	5	G	8	G	8	G	8	G	7	G	6	A	7	G
OPS 11	6	A	6	A	4	A	3	G	2	G	3	G	3	A	3	G	5	A	4	G	3	G	3	G	5	G	3	G	6	G
OPS 12	5	A	5	A	4	A	2	A	3	A	6	A	4	A	4	A	5	A	4	A	5	A	5	A	6	P	5	A	6	P
OPS 13	5	G	5	G	3	A	4	A	3	A	5	G	3	G	-	P	-	P	3	P	5	G	5	A	3	A	4	P	5	A
OPS 14	5	G	5	A	5	A	5	A	5	A	4	A	5	P	5	A	5	A	5	A	5	A	5	A	5	A	4	A	4	P
OPS 15	7	A	8	G	5	A	5	A	5	G	8	G	7	G	5	A	8	G	8	G	8	G	8	G	8	G	8	P	8	G
OPS 16	4	G	4	A	1	G	3	A	4	G	3	G	4	G	1	A	5	G	5	G	4	A	4	G	4	P	5	G	4	G
OPS 17	9	G	7	A	7	A	7	A	7	A	9	A	8	A	7	A	9	A	8	A	9	A	9	A	8	A	9	G	9	A
OPS 18	7	G	7	G	6	A	5	A	7	G	7	G	7	G	4	G	7	G	7	G	7	G	7	G	7	G	7	A	6	G
OPS 19	8	G	7	A	3	A	6	A	7	A	2	A	6	A	-	P	6	A	6	G	6	A	6	A	6	G	6	A	6	A
OPS 20	5	A	5	A	4	P	5	A	5	A	4	A	6	A	3	P	6	A	6	A	6	A	6	A	6	A	6	A	8	A

T1. PKM 1, T2. Sakthi, T3. BL 333-3-1, T4. Arka Vikas, T5. Hawaii 7998, T6. LE 66, T7. LE 474, T8. BT-1, T9. LE 1-2, T10. Anagha, T11. Mukthi, T12. BTH 102-1-2-2, T13. Pusa Ruby, T14. BT 218, T15. LE 20.

a – Number of amplicons
b – Nature of amplicons

P – Poor
A – Average
G - Good

Table 10.B) Number and nature of amplicons observed in the 15 genotypes with the selected OPY primers

Primer	T 1		T 2		T 3		T 4		T 5		T 6		T 7		T 8		T 9		T 10		T 11		T 12		T 13		T 14		T 15	
	a	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**A**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**B**	**a**	**b**
OPY 1	4	G	6	G	5	G	-	P	-	P	5	A	-	P	-	P	5	G	5	G	5	A	5	G	6	G	-	P	6	G
OPY 2	5	A	4	A	4	A	5	G	3	P	-	P	4	A	3	G	5	P	4	P	-	P	2	A	6	A	5	A	4	A
OPY 3	-	P	-	P	-	P	-	P	-	P	-	P	-	P	-	P	-	P	-	P	-	P	-	P	-	P	-	P	4	A
OPY 6	-	P	-	P	-	P	-	P	-	P	-	P	-	P	-	P	-	P	-	P	-	P	-	P	2	G	1	A	2	G
OPY 7	7	G	7	G	5	G	7	G	7	G	7	G	7	G	4	G	4	A	7	G	4	G	7	G	7	G	6	G	6	G
OPY 8	-	P	-	P	-	P	-	P	-	P	-	P	-	P	-	P	-	P	11	G	11	G	-	P	10	G	11	G	11	G
OPY 9	7	G	1	P	2	A	6	G	6	G	5	G	4	G	-	P	4	G	5	G	2	A	3	G	6	G	3	G	5	G
OPY 10	3	A	7	A	3	A	3	A	3	P	4	P	4	A	-	P	3	A	8	G	1	P	3	A	2	A	3	A	3	A
OPY 11	2	A	2	A	2	A	3	A	3	A	3	G	3		-	P	1	G	3	G	3	G	2	G	3	G	2	A	2	A
OPY 13	7	A	7	A	6	A	5	A	5	A	5	A	5	A	5	A	5	A	5	A	4	A	5	A	4	P	4	P	4	P
OPY 14	-	P	-	P	-	P	-	P	-	P	-	P	7	A	-	P	-	P	7	A	-	P	-	P	7	A	7	A	7	P
OPY 15	8	G	8	G	8	G	9	G	9	G	10	G	2	A	1	P	9	G	10	G	6	G	9	G	9	G	8	G	8	G
OPY 17	2	A	2	A	2	A	2	A	2	A	2	A	2	A	2	A	2	A	2	A	2	A	2	A	2	A	2	A	2	A
OPY 18	12	A	12	A	12	A	12	A	12	A	12	A	12	A	12	A	12	A	12	A	12	A	12	A	12	A	12	A	12	A
OPY 19	-	P	-	P	-	P	-	P	-	P	-	P	-	P	-	P	-	P	-	P	-	P	-	P	5	G	-	P	5	G
OPY 20	-	P	-	P	-	P	-	P	6	G	-	P	-	P	-	P	-	P	-	P	-	P	-	P	11	G	11	G	6	G

T1. PKM 1, T2. Sakthi, T3. BL 333-3-1, T4. Arka Vikas, T5. Hawaii 7998, T6. LE 66, T7. LE 474, T8. BT-1, T9. LE 1-2, T10. Anagha, T11. Mukthi, T12. BTH 102-1-2-2, T13. Pusa Ruby, T14. BT 218, T15. LE 20.

a – Number of amplicons
b – Nature of amplicons

P – Poor
A – Average
G - Good

one susceptible genotype (Pusa Ruby). This primer exhibited 71 per cent polymorphism and resolving power of 4.28. The RAPD profile generated by this primer is shown in Plate 8a.

OPS 3

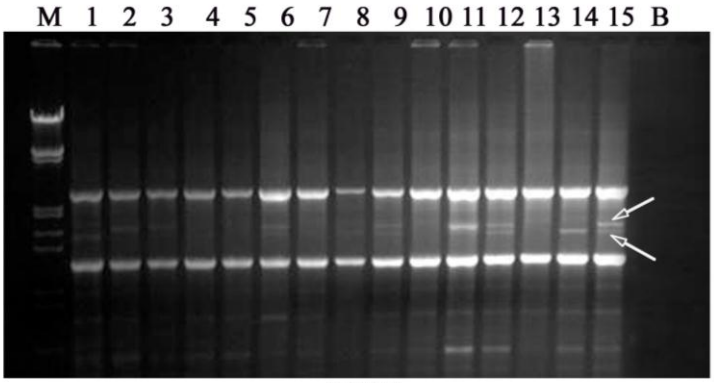
RAPD assay with this primer displayed six scorable amplicons and five of them were polymorphic (Plate 8b). The size of the amplicons ranged between 1.3 kb and 0.6 kb and the polymorphism observed was 83 per cent. The monomorphic band produced was of size 1.1 kb and was very intense. Poor amplification was obtained in case of genotype BL-333-3-1 with only one amplicon of size 1.1 kb. All the six amplicons were present in the resistant genotypes Anagha, Mukthi, LE-66 and Hawaii 7998, and moderately resistant genotype LE-474. An amplicon of size 1.3 kb was present in all the resistant genotypes and absent in the susceptible genotype BL-333-3-1 and moderately susceptible genotype BT-218. Two amplicons of sizes 1 kb and 0.74 kb were shared by all the resistant genotypes and two susceptible genotypes, Arka Vikas and Pusa Ruby. Amplicon of size 0.6 kb was absent in susceptibe genotype BL-333-3-1 and moderately susceptible genotype BT-1. The resolving power obtained with this primer was 1.86.

OPS 5

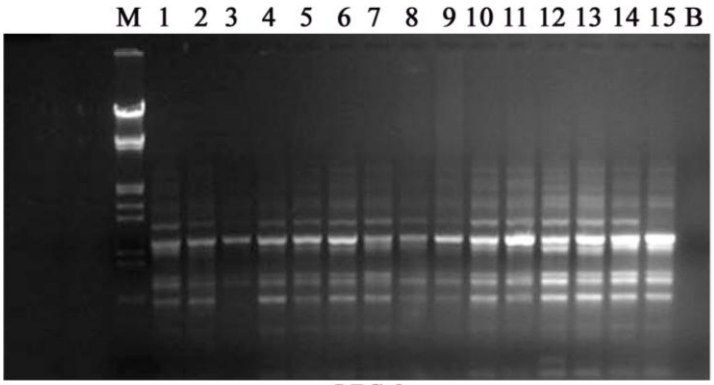
The RAPD profile generated by this primer displayed five unique fragments of different sizes. All the five bands were monomorphic among the genotypes (Plate 8c) and this primer exhibited zero per cent polymorphism. The molecular size of the fragments ranged between 1.3 kb and 0.4 kb. The resolving power calculated for this primer was zero.

OPS 6

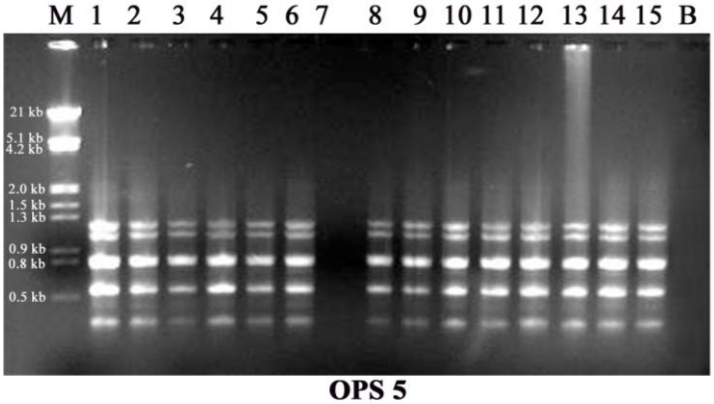
The primer OPS 6 gave ten markers when the amplification products were viewed on agarose gel electrophoresis after RAPD reaction. There were three clear and distinct monomorphic bands of sizes 1.3 kb, 0.8 kb and 0.6 kb. The



OPS 1



OPS 3



OPS 5

M- Molecular weight marker, 1- PKM 1, 2- Sakthi, 3- BL 333-3-1, 4- A.Vikas, 5-Hawaii 7998, 6- LE 66, 7- LE 474, 8- BT-1, 9- LE 1-2, 10- Anagha, 11-Mukthi, 12- BTH 102-1-2-2, 13- Pusa Ruby, 14- BT 218, 15- LE 20, B-Blank
(Arrow indicates polymorphic bands)

Plate 8. RAPD profile of tomato genotypes with primers OPS 1, 3 and 5

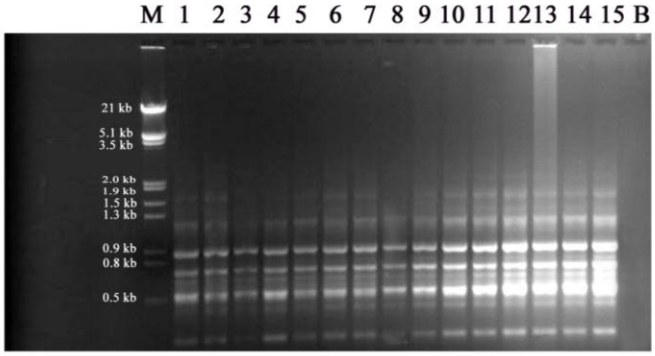
umber of polymorphic bands observed was seven and the per cent polymorphism was 70. The resolving power of this primer was 2.66. A marker of molecular weight 1.8 kb was absent in the susceptible genotypes Arka Vikas and BL-333-3-1 and moderately susceptible genotype BT-1. This band was absent in Hawaii 7998 also, which is a resistant genotype. Another unique marker of size 0.67 kb was present in the resistant genotypes Hawaii 7998 and LE-66 and moderately resistant genotype LE-474 and absent in all susceptible genotypes. The RAPD profile obtained with this primer is shown in Plate 9a.

OPS 7

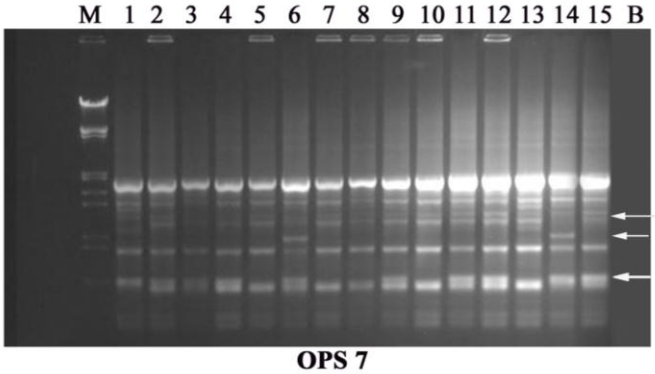
This primer produced nine amplicons and seven of them were polymorphic (Plate 9b). The polymorphism displayed was 77 per cent and the resolving power obtained was 3.98. Two amplicons of sizes 0.8 kb and 1.8 kb was common to all genotypes. The resistant genotypes Anagha and Mukthi and moderately resistant genotype LE-20 shared a common band of size 1.2 kb and it was also present in the susceptible genotype Pusa Ruby. A band of molecular weight 0.9 kb was present in the resistant genotype LE-66 and moderately resistant genotype LE-20. The susceptible genotypes, PKM-1 and BL-333-3-1 did not amplify the 0.4 kb fragment. Unique bands shared by resistant/susceptible genotypes were not obtained.

OPS 8

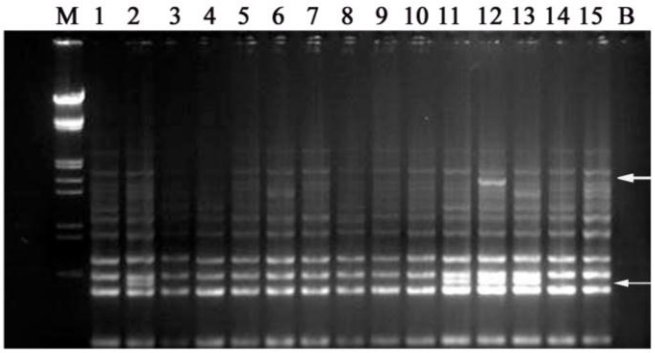
The primer OPS 8 gave maximum number of amplicons among the 14 selected primers (Plate 9c). A total of 13 bands were obtained and nine of them were polymorphic. The molecular weight of the amplicons obtained using this primer ranged from 2.3 kb to 0.3 kb. The band of size 2.3 kb was present in all the resistant genotypes and absent in all susceptible genotypes, except PKM-1. The 1.3 kb marker was amplified in susceptible genotype Pusa Ruby alone. The resistant genotypes Sakthi and Mukthi shared a unique band of size 0.5 kb and this was also present in the susceptible genotype Pusa Ruby and moderately



OPS 6



OPS 7



OPS 8

M- Molecular weight marker, 1- PKM 1, 2- Sakthi, 3- BL 333-3-1, 4- A.Vikas, 5-Hawaii 7998, 6- LE 66, 7- LE 474, 8- BT-1, 9- LE 1-2, 10- Anagha, 11-Mukthi, 12- BTH 102-1-2-2, 13- Pusa Ruby, 14- BT 218, 15- LE 20, B-Blank
(Arrow indicates polymorphic bands)

Plate 9. RAPD profile of tomato genotypes with primers OPS 6, 7 and 8

susceptible genotype BTH-102-1-2-2. This primer disclosed a polymorphism of 69 per cent and the highest resolving power (4.54).

OPS 10

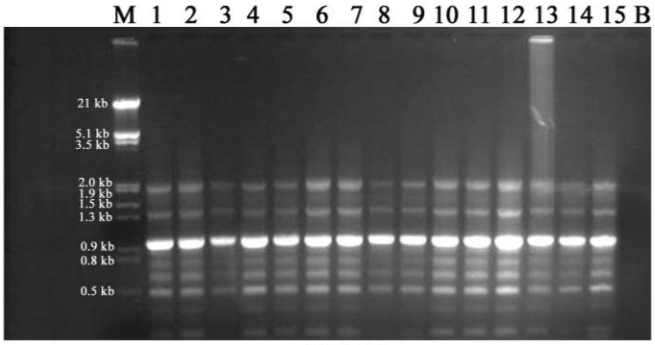
RAPD assay with this primer produced eight amplification products out of which four were polymorphic. The amplification pattern is shown in Plate 10a. The size of the amplicons ranged between 2.1 kb and 0.3 kb. All the amplicons were present in resistant genotypes Sakthi, Mukthi, Anagha and LE-66. The percentage polymorphism obtained using the primer OPS 10 was 50 per cent. This primer was unable to distinguish between bacterial wilt resistant and susceptible genotypes. The resolving power obtained for this primer was 2.58.

OPS 11

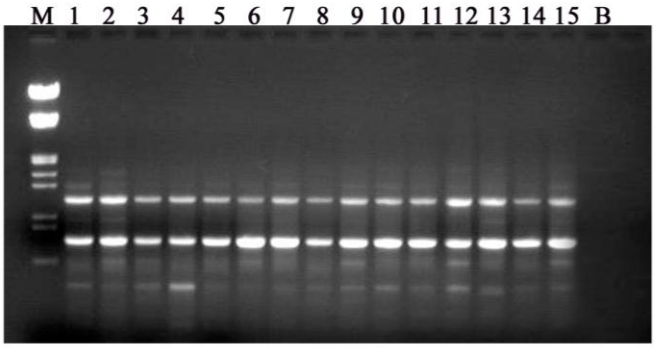
RAPD profile with the primer OPS 11 displayed 6 amplification products and three of them were polymorphic. The size of the amplicons varied from 1.7 kb to 0.4 kb. Three bands (1.7 kb, 0.7 kb and 0.4 kb) were present in all the resistant and susceptible genotypes (Plate 10b). This primer exhibited 50 per cent polymorphism. The band with I kb size was absent in resistant genotypes Anagha, Mukthi, Hawaii and LE-66 while it was present in two susceptible genotypes, PKM-1 and Pusa Ruby. The resolving power observed for this primer was 2.44.

OPS 15

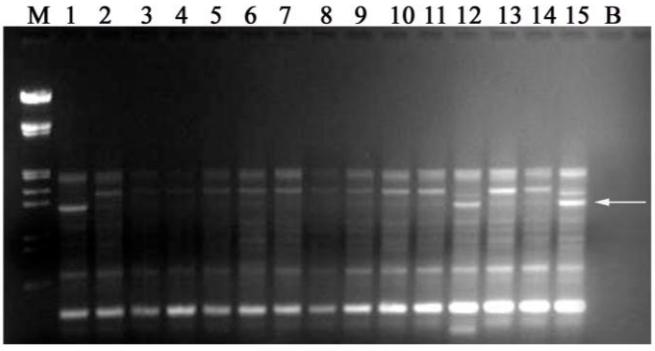
The primer OPS 15 generated ten amplicons on RAPD analysis of the tomato genotypes (Plate 10c). The bands ranged in molecular weight from 2.1 kb to 0.5 kb. Six bands were found to be polymorphic among the genotypes. The Three bands of sizes 1.2 kb, 1.1 kb and 1 kb were present in four resistant genotypes. One of the susceptible genotypes also (Pusa Ruby) shared these bands. Amplicons of sizes 1.2 kb and 1.1 kb were absent in susceptibe genotypes Arka Vikas and BL-333-3-1 and moderately susceptible genotype BT-1. The per cent polymorphism exhibited by this primer was 60 and the resolving power was 2.38.



OPS 10



OPS 11



OPS 15

M- Molecular weight marker, 1- PKM 1, 2- Sakthi, 3- BL 333-3-1, 4- A.Vikas, 5-Hawaii 7998, 6- LE 66, 7- LE 474, 8- BT-1, 9- LE 1-2, 10- Anagha, 11-Mukthi, 12- BTH 102-1-2-2, 13- Pusa Ruby, 14- BT 218, 15- LE 20, B-Blank
(Arrow indicates polymorphic bands)

Plate 10. RAPD profile of tomato genotypes with primers OPS 10, 11 and 15

OPS 18

A total of seven amplicons were obtained after DNA amplification with the primer OPS 18. The pattern of amplification is shown in Plate 11a. The amplicons obtained with this primer were almost monomorphic for the genotypes selected. The molecular weight of the bands varied from 1.6 kb to 0.4 kb. There were three polymorphic bands and the observed polymorphism was 43 per cent. Four bands of sizes 1.6 kb, 1 kb, 0.6 kb and 0.29 kb were shared by the resistant and susceptible genotypes. This primer did not amplify the 1.5 kb and 1.4 kb fragments in the susceptible genotype Arka Vikas and moderately susceptible genotype BT-1. However, these two fragments were observed in all other susceptible and resistant genotypes. Three monomorphic bands could be clearly identified in the amplification pattern generated. The resolving power disclosed by this primer was 1.02.

4.2.2.2. b) RAPD profile with selected primers from OPY primer kit

Sixteen primers from this series were used for RAPD analysis, but only four primers that produced clear, reproducible bands were chosen for further experiments. The descriptions of the RAPD profile produced by the four selected primers are given below.

OPY 1

Amplification with this primer generated six amplicons of which three were polymorphic. The molecular weight of the amplicons ranged between 1.4 kb and 0.5 kb (Plate 11b). There was no amplification in genotypes Arka Vikas and Hawaii 7998. Fifty per cent polymorphism was obtained with this primer. Unique bands shared by resistant/susceptible genotypes were not obtained. This primer showed the lowest resolving power value of 2.52.

OPY 7

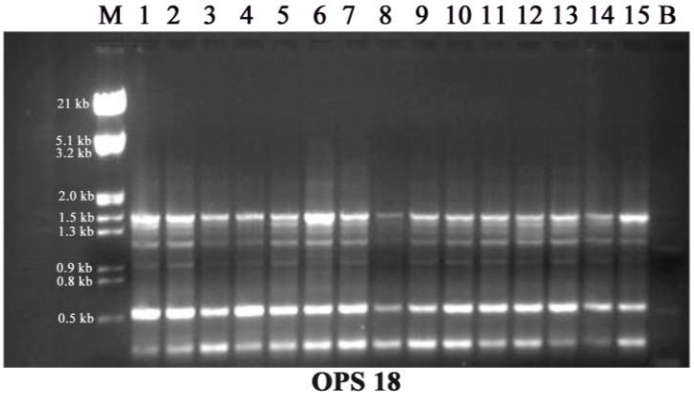
Seven amplicons were observed on the agarose gel for the DNA amplified with the primer OPY 7 (Plate 11c). Only one band was monomorphic and the rest were polymorphic among the genotypes. This primer showed 86 per cent polymorphism. The resolving power observed was 1.66. The molecular weight of the products ranged between 1.8 kb and 0.5 kb. Two bands of sizes 1.8 kb and 1 kb were absent in Mukthi (resistant) and BL-333-3-1 (susceptible). Almost all other bands were present in all resistant and susceptible genotypes. This primer was unable to differentiate between the resistant and susceptible genotypes.

OPY 9

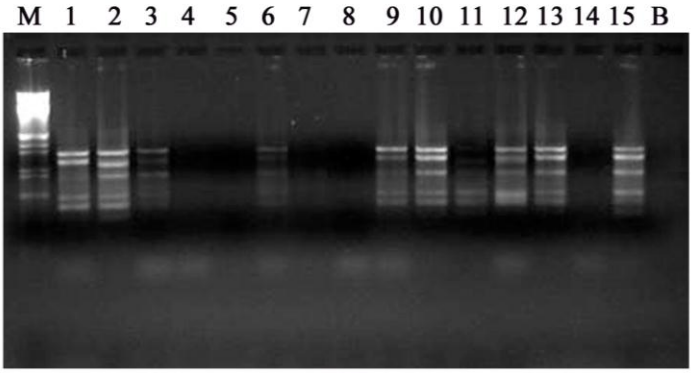
Using OPY 9, ten markers were obtained and nine of them were polymorphic (Plate 12a). The molecular weight of these markers ranged between 2 kb and 0.28 kb. The 0.4 kb marker was present in all resistant and susceptible genotypes. Poor amplification was observed in genotypes Sakthi, BL-333-3-1 and Mukthi. The band of size 0.6 kb was present in two susceptible genotypes (PKM-1 and Arka Vikas) and it was absent in four resistant genotypes (Sakthi, Mukthi, Anagha and LE-66). This primer exhibited polymorphism of 90 per cent among the different genotypes and the resolving power obtained was 4.54.

OPY 15

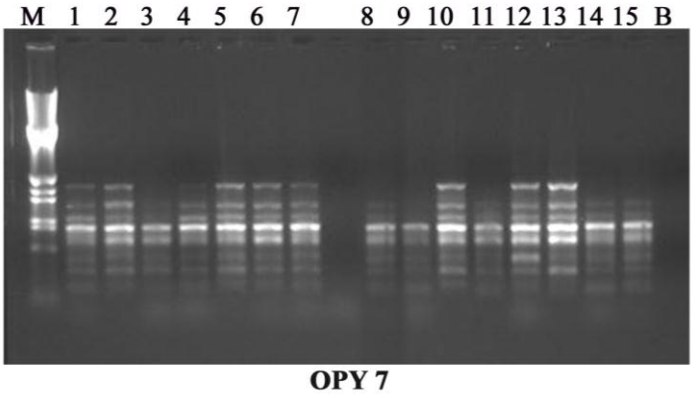
Six markers were obtained with OPY 15 primer on RAPD assay and three of them were polymorphic (Plate 12b). This primer showed 50 per cent polymorphism. The size of the bands ranged from 3.3 kb to 0.5 kb. Four bands of sizes 1.3 kb, 1.1 kb, 0.6 kb and 0.5 kb were common to all resistant and susceptible genotypes. There were no unique bands that could differentiate the resistant and susceptible genotypes. The resolving power for this primer was 4.26. Overall, 110 amplification products were generated by the fourteen decamer primers of which 70 (63.6%) were polymorphic. The number of amplified products for various primers varied from 5 to 13 with an average of 8 bands per primer, while the number of polymorphic bands ranged from 0 to 9 with



OPS 18



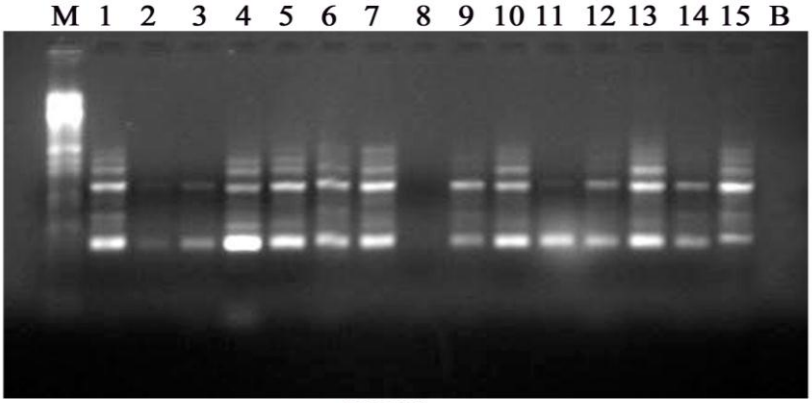
OPY 1



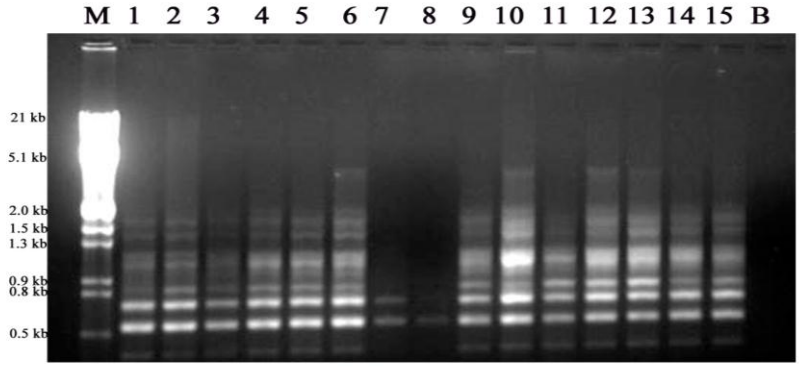
OPY 7

M- Molecular weight marker, 1- PKM 1, 2- Sakthi, 3- BL 333-3-1, 4- A.Vikas, 5-Hawaii 7998, 6- LE 66, 7- LE 474, 8- BT-1, 9- LE 1-2, 10- Anagha, 11-Mukthi, 12- BTH 102-1-2-2, 13- Pusa Ruby, 14- BT 218, 15- LE 20, B-Blank

Plate 11. RAPD profile of tomato genotypes with primers OPS 18, OPY 1 and OPY 7



OPY 9



OPY 15

Molecular weight marker, 1- PKM 1, 2- Sakthi, 3- BL 333-3-1, 4- A.Vikas, awaii 7998, 6- LE 66, 7- LE 474, 8- BT-1, 9- LE 1-2, 10- Anagha, 11-Mukthi, BTH 102-1-2-2, 13- Pusa Ruby, 14- BT 218, 15- LE 20, B-Blank

Plate 12. RAPD profile of tomato genotypes with OPY 9 and 15

an average of 5 per primer. Resolving power of the 14 random decamer primers selected for the study was calculated using the formula $Rp = \Sigma Ib$, where 'Ib' is the 'band informativeness' calculated as $Ib = 1 – (2 \times |0.5-p|)$, where 'p' is the proportion of genotypes containing the band. The resolving power of the selected primers ranged from a value of 6.96 for the primer OPY 1 to a value of 18.4 for the primer OPS 8. Table 11 shows the number of amplification products, number of polymorphic bands and resolving power calculated for each of the 14 selected primers.

Amplification pattern of tomato genotypes using 14 selected random primers with the number of polymorphic bands obtained in each genotype is given in Table 12.

4.2.2.3. Data analysis of RAPD profiles

Data 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) were employed for cluster analysis. Dendrogram was constructed using the SAHN programme of NTSYS. The genetic similarity matrix pertaining to the pooled data obtained from the 14 primers is given in Table 13.

The similarity coefficient among the genotypes ranged from 0.60 and 0.89. The UPGMA based dendrogram suggested that the 15 genotypes are grouped into two main clusters, I and II (Fig. 2). The genetic distance between two main clusters was 0.12. The first main cluster was subdivided into two sub clusters with 13 genotypes and there were two genotypes representing the second main cluster.

Based on estimated Genetic Similarity Matrix using Jaccard's coefficient, the closely related genotypes were Arka Vikas (susceptible) and Hawaii 7998 (resistant), with a genetic similarity of 0.88 followed by 0.87 between Anagha and

Table 11. Amplification pattern and the level of polymorphism observed with the selected RAPD primers in the 15 genotypes of tomato

Sl. No.	Primer code	No:of bands	No:of polymorphic bands	Per cent polymorphism	Resolving power
1	OPS 1	7	5	71	4.28
2	OPS 3	6	5	83	1.86
3	OPS 5	5	0	00	0.00
4	OPS 6	10	7	70	2.66
5	OPS 7	9	7	77	3.98
6	OPS 8	13	9	69	4.54
7	OPS 10	8	4	50	2.58
8	OPS 11	6	3	50	2.44
9	OPS 15	10	6	60	2.38
10	OPS 18	7	3	43	1.02
11	OPY 1	6	3	50	1.66
12	OPY 7	7	6	86	4.26
13	OPY 9	10	9	90	2.52
14	OPY 15	6	3	50	4.54
	Total	110	70		

Table 12. Amplification pattern of 15 tomato genotypes with the selected 14 primers

Primer		T1	T2	T3	T4	T5	T6	T7	T8	T9	T 10	T 11	T 12	T 13	T 14	T 15
OPS 1	**a**	3	3	2	2	2	5	3	3	7	5	6	7	3	6	7
	b	1	1	0	0	0	3	1	1	5	3	4	5	2	4	5
OPS 3	**a**	4	3	1	6	6	6	6	3	3	6	6	6	6	5	4
	b	3	2	0	5	5	5	5	2	2	5	5	5	5	4	3
OPS 5	**a**	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	b	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
OPS 7	**a**	5	6	2	6	5	6	5	5	6	6	7	7	7	7	7
	b	3	4	0	4	3	4	3	3	4	4	5	5	5	5	5
OPS 8	**a**	10	10	4	5	8	9	9	9	10	10	11	11	11	11	11
	b	6	6	0	1	4	5	5	5	6	6	7	7	7	7	7
OPS 10	**a**	8	8	4	8	7	8	7	5	5	8	8	8	7	6	7
	b	4	4	0	4	3	4	3	1	1	4	4	4	3	2	3
OPS 11	**a**	6	6	4	3	2	3	3	3	5	4	3	5	5	3	6
	b	4	4	2	1	0	1	1	1	3	2	1	3	3	1	4
OPS 15	**a**	7	8	5	5	5	8	7	5	8	8	8	9	8	8	8
	b	3	4	1	1	1	4	3	1	4	4	4	4	4	4	4
OPS 18	**a**	7	7	6	5	7	7	7	4	7	7	7	7	7	7	6
	b	4	3	2	1	3	3	3	0	3	3	3	3	3	3	2
OPY 1	**a**	4	6	5	-	-	5	-	-	5	5	5	5	6	-	6
	b	1	3	2	-	-	2	-	-	2	2	2	2	3	-	3
OPY 7	**a**	7	7	5	7	7	7	7	4	4	7	4	7	7	6	6
	b	3	3	1	3	3	3	3	0	0	3	0	3	3	5	5
OPY 9	**a**	7	1	2	6	6	5	4	-	4	5	2	3	6	3	6
	b	6	0	1	5	5	4	3	-	3	4	1	2	5	2	5
OPY 15	**a**	8	8	8	9	9	10	2	1	9	10	6	9	9	8	8
	b	7	7	7	8	8	9	1	0	8	9	5	8	8	7	7

a – Total number of bands

b – Number of polymorphic bands

T1- PKM 1, T2- Sakthi, T3- BL 333-3-1 T4- A.Vikas T5- Hawaii 7998

T6- LE 66, T7- LE 474, T8- BT-1, T9- LE 1-2, T 10- Anagha,

T11- Mukthi, T 12- BTH 102, T 13- P.Ruby , T 14- BT 218, T 15- LE 20

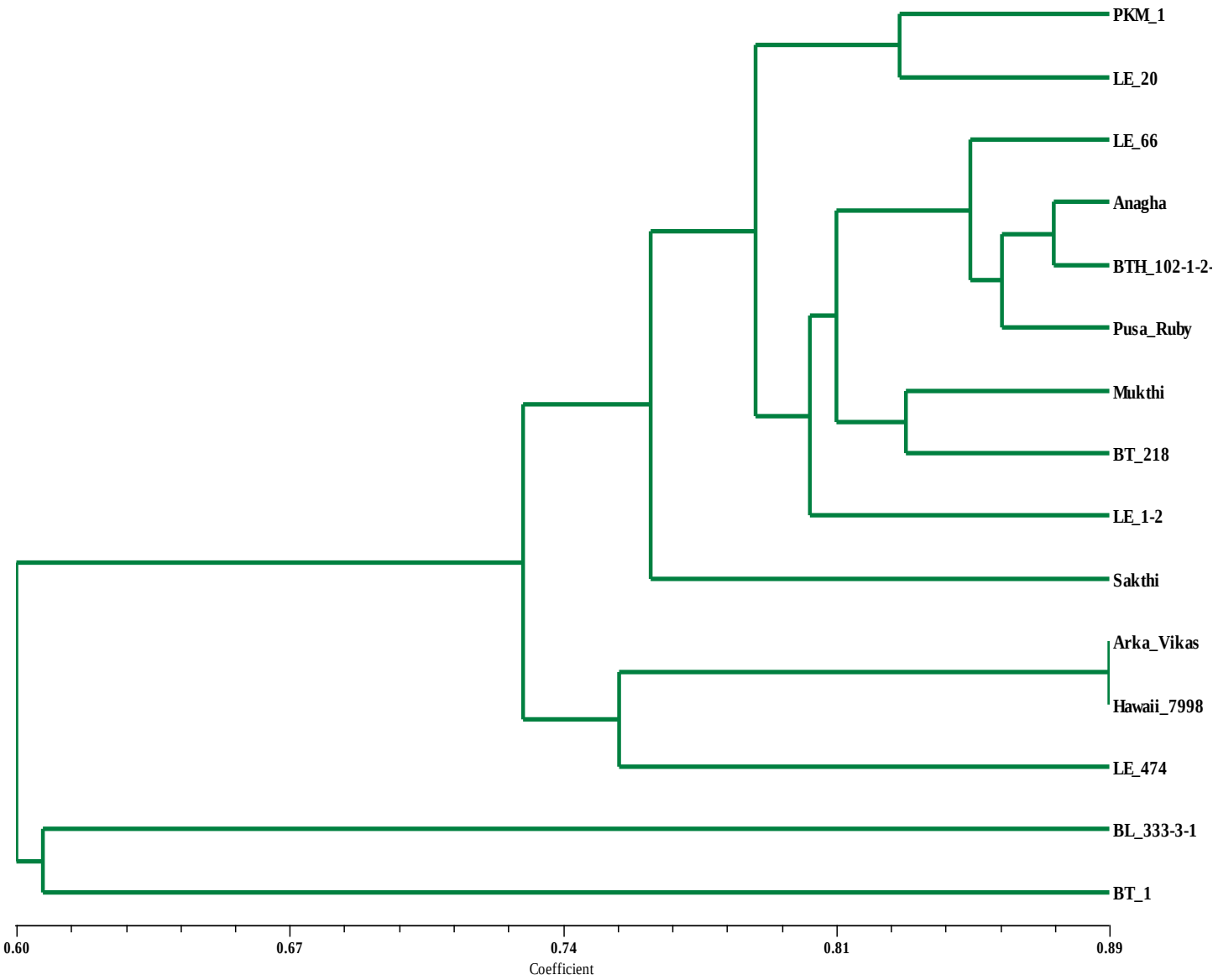


Fig. 2. Dendrogram derived from RAPD analysis of 15 tomato genotypes

Table 13. Genetic similarity matrix obtained using RAPD data

Rows\Cols	PKM_1	Sakthi	BL_333-3-	Arka_Vika	Hawaii_79	LE_66	LE_474	BT_1	LE_1-2	Anagha	Mukthi	BTH_102-1	Pusa_Ruby	BT_218	LE_20
PKM_1	1.0000000														
Sakthi	0.7916667	1.0000000													
BL_333-3-	0.5934066	0.6547619	1.0000000												
Arka_Vika	0.7472527	0.6966292	0.6447368	1.0000000											
Hawaii_79	0.7608696	0.7111111	0.6410256	0.8875000	1.0000000										
LE_66	0.8300000	0.7700000	0.5957447	0.7717391	0.8043478	1.0000000									
LE_474	0.7241379	0.6904762	0.5064935	0.7160494	0.7974684	0.7500000	1.0000000								
BT_1	0.5853659	0.5609756	0.6031746	0.6438356	0.6621622	0.5632184	0.6617647	1.0000000							
LE_1-2	0.7676768	0.7422680	0.6321839	0.6666667	0.6989247	0.8181818	0.6976744	0.6329114	1.0000000						
Anagha	0.8118812	0.7700000	0.5789474	0.7912088	0.8241758	0.8613861	0.8117647	0.5813953	0.8181818	1.0000000					
Mukthi	0.7156863	0.7789474	0.5604396	0.7032967	0.7173913	0.7821782	0.7590361	0.6172840	0.8105263	0.8367347	1.0000000				
BTH_102-1	0.7884615	0.7821782	0.5612245	0.7113402	0.7244898	0.8365385	0.7444444	0.5494505	0.8118812	0.8725490	0.8300000	1.0000000			
Pusa_Ruby	0.8217822	0.7979798	0.5894737	0.7717391	0.7849462	0.8529412	0.7500000	0.5882353	0.7745098	0.8712871	0.8100000	0.8461538	1.0000000		
BT_218	0.7789474	0.7311828	0.5882353	0.6808511	0.7311828	0.7835052	0.7325581	0.6219512	0.8131868	0.8404255	0.8333333	0.8144330	0.8210526	1.0000000	
LE_20	0.8316832	0.7211538	0.5510204	0.6400000	0.6700000	0.7924528	0.6666667	0.5280899	0.7843137	0.7924528	0.7500000	0.8207547	0.8190476	0.8315789	1.0000000

BTH-102-1-2-2. The genotype Sakthi was widely separated from other genotypes in the first sub clusters at a genetic similarity value of 0.75. The minimum similarity value of 0.50 was obtained between LE-474 and BL-333-3-1.

The resistant genotype Sakthi was separated from other resistant genotypes Mukthi, Anagha and LE-66 by a similarity value of 0.77. The susceptible genotype Pusa Ruby showed 82 per cent similarity with PKM-1, 74 per cent with Arka Vikas and 59 per cent with BL-333-3-1.

The dendrogram revealed high degree of relatedness among the 15 tomato genotypes selected for the study and no specific demarcation between resistant and susceptible genotypes.

4.2.3. Amplified Fragment Length Polymorphism (AFLP) assay

AFLP analysis was carried out with the DNA samples isolated from 10 selected tomato genotypes, which included four resistant genotypes (Anagha, Sakthi, Mukthi and LE-66), one moderately resistant genotype (LE-474), four susceptible genotypes (Pusa Ruby, Arka Vikas, BL-333-3-1 and PKM-1) and one moderately susceptible genotype (BT-1). The DNA samples were pre-amplified and the diluted pre-amplified DNA was used for selective amplification with radiolabled *Eco*RI and *Mse*I primer pairs. Five *Eco*RI and *Mse*I selective primer combinations were tested for their ability to detect the polymorphism between the 10 selected genotypes.

4.2.3.1. Assay with E+ACG/M+CAC combination

The genomic DNA of ten selected tomato genotypes gave 41 scorable AFLP markers when this primer combination was used for screening. This primer pair produced 29 polymorphic bands and the percent polymorphism observed was 70.73. The amplification pattern generated by this primer pair is given in Plate 13.

Maximum number of bands was obtained with the moderately resistant genotype LE-474. The resistant genotypes Anagha, LE-474 and LE-66 showed the amplification of ~150bp fragent and this was abent in susceptible genotypes Pusa Ruby and Arka Vikas. Another fragment of size ~90 bp was absent in all resistant genotypes and present in three susceptible genotypes (Pusa Ruby, BT-1 and PKM-1).

4.2.3.2. Assay with E+AGC/M+CAA combination

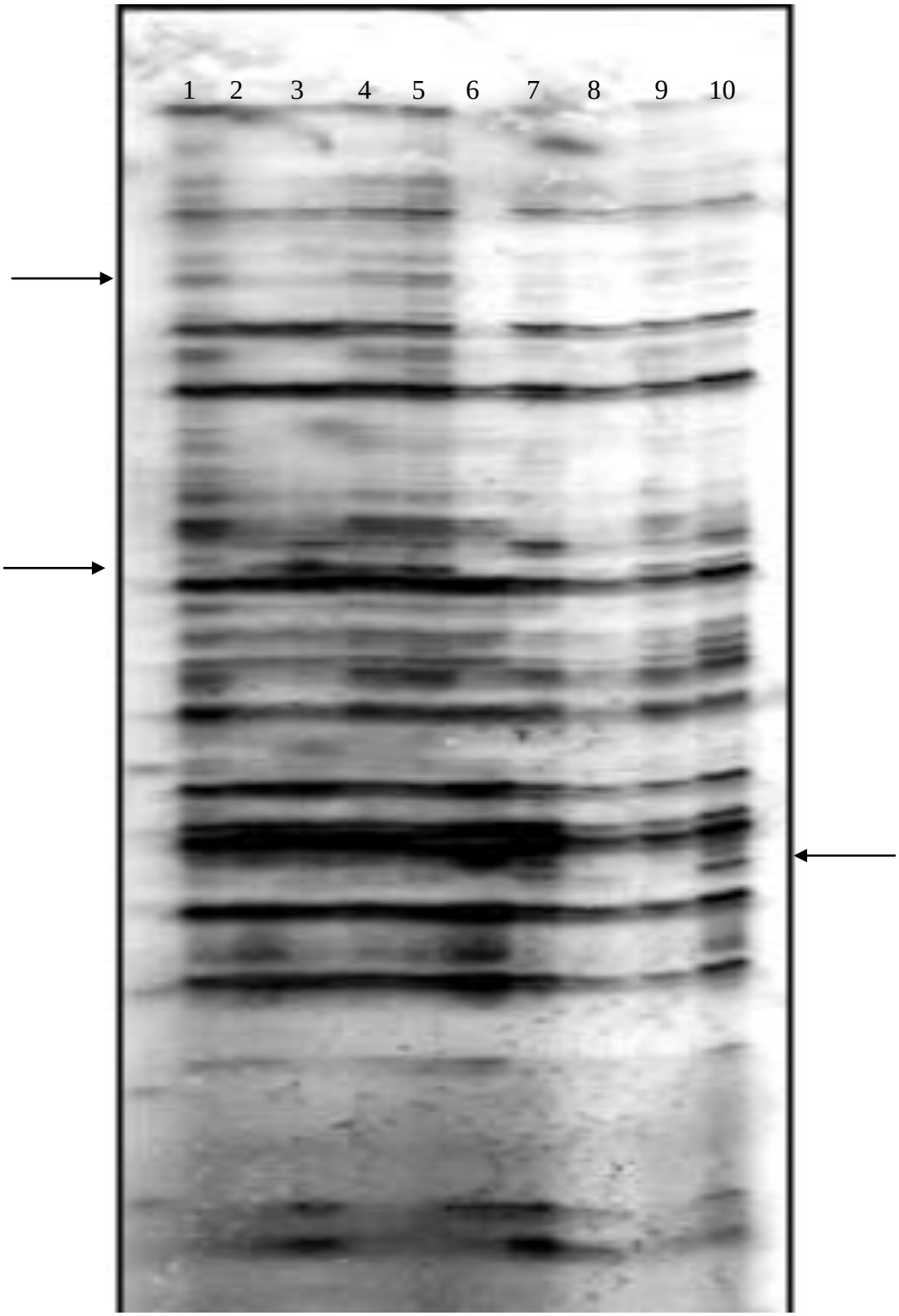
The AFLP profile obtained with this primer pair showed the maximum number of amplicons with a total of 64 fragments (Plate 14) out of which 23 were polymorphic to all the genotypes. The percent polymorphism obtained with this primer pair was 36. Maximum number of amplicons was obtained in the genotype PKM-1. One trait specific marker of ~60 bp was observed in susceptible genotypes Pusa Ruby, Arka Vikas, BL-333-3-1 and PKM-1 and was absent in the four resistant genotypes. However, such a specific polymorphism was not observed in the moderately susceptible genotype BT-1 and the resistant genotype Anagha.

4.2.3.3. Assay with E+AGG/M+CAA combination

The amplification pattern produced by this primer combination is represented in Plate 15. This primer pair yielded a total of 49 scorable markers. Twenty markers were polymorphic recording 40.81 per cent polymorphism. All the 49 markers were present in the genotype LE-66. One amplicon of size ~290 bp was present in all the susceptible genotypes and one resistant genotype (Anagha).

4.2.3.4. Assay with E+AAG/M+CAT combination

This primer combination produced 25 amplification products of which nineteen were polymorphic. The Plate 16 shows the AFLP pattern produced by

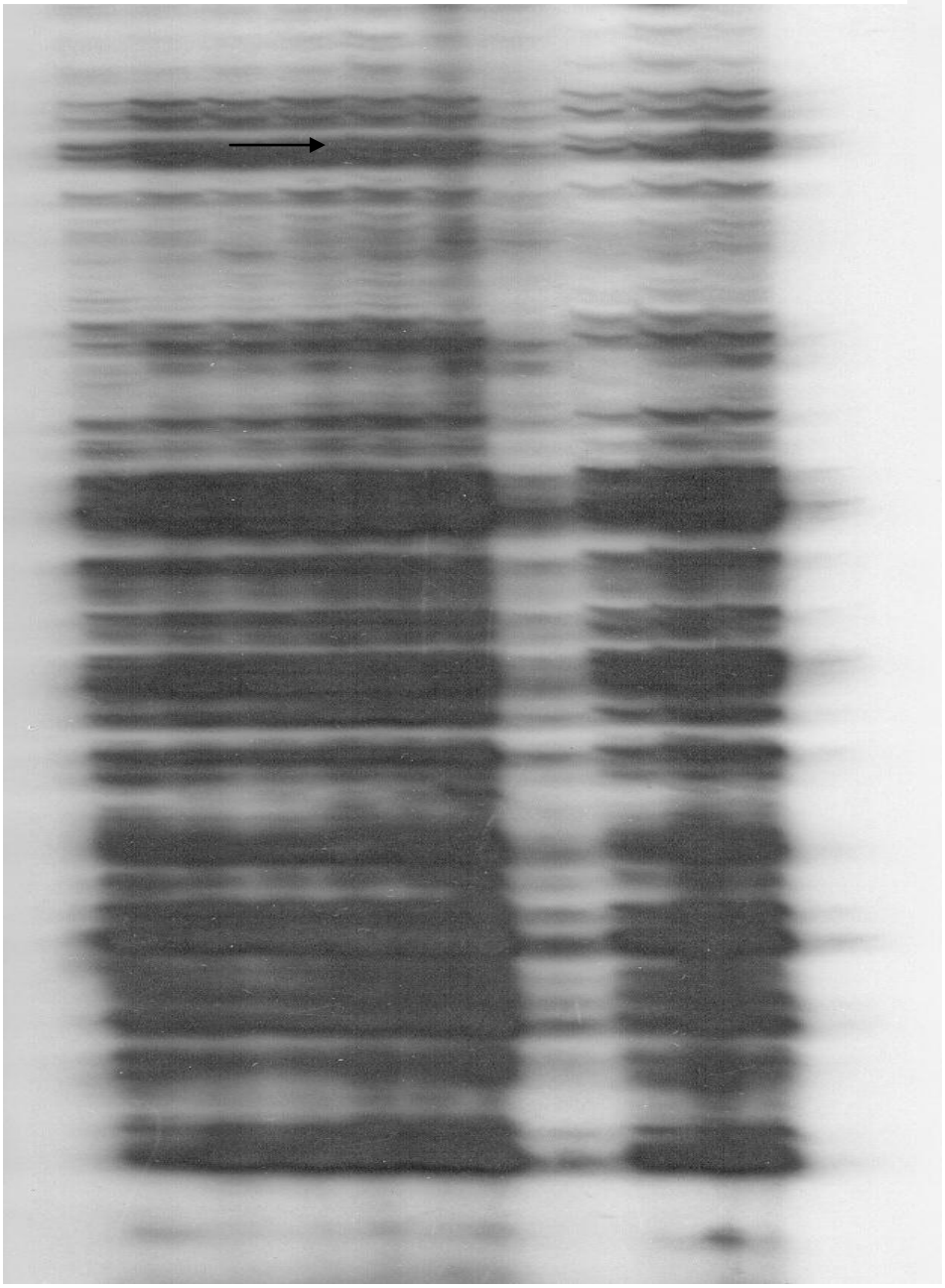


1. Anagha, 2. Sakthi, 3. Mukthi, 4. LE-474, 5. LE-66, 6. Pusa Ruby
7. BT-1, 8. Arka Vikas, 9. BL-333-3-1, 10. PKM-1
(Arrow indicates polymorphic bands)

Plate 13. AFLP profile with E+ACG/M+CAC primer pair

1 2 3 4 5 6 7 8 9 10

Plate 14. AFLP profile with E+AGC/M+CAA primer pair



1. Pusa Ruby, 2. BT-1, 3. Arka Vikas 4. BL-333-3-1, 5. PKM-1, 6. Anagha, 7. Sakthi, 8. Mukthi, 9. LE-474, 10. LE-66
(Arrow indicates polymorphic bands)

Plate 15. AFLP profile with E+AGG/M+CAA primer pair

this primer pair. The maximum number of amplicons was observed in genotype LE-66 and the percent polymorphism exhibited by this combination was 76. The amplification produced by this primer was comparatively less.

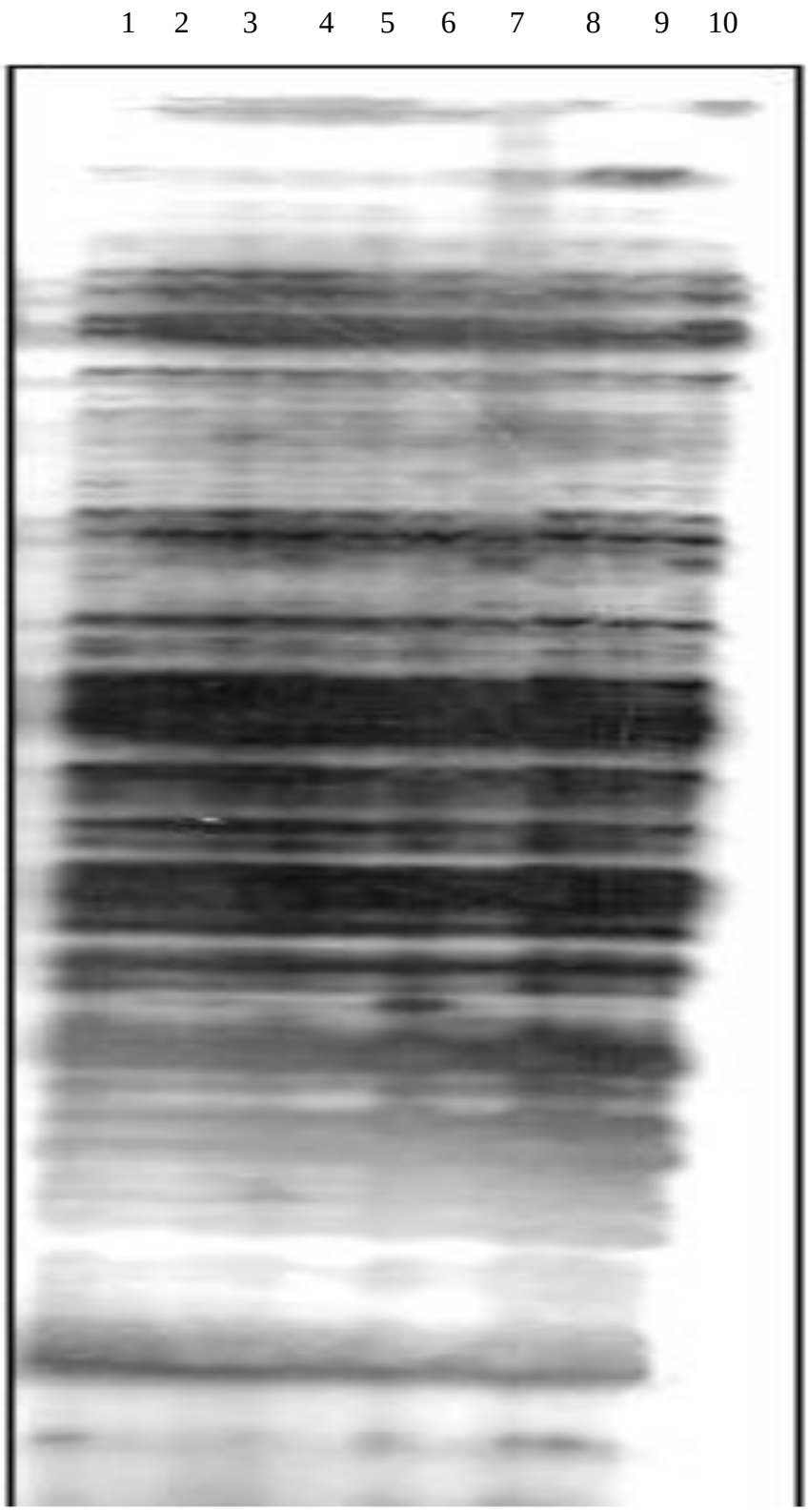
4.2.3.5. Assay with E+AGC/M+CTT combination

This primer pair generated 30 amplified products and the genomic DNA from Arka Vikas showed the maximum amplification (Plate 17). There were 24 polymorphic bands and the percent polymorphism (80 %) observed was relatively higher compared to other primer pairs used in the study. The susceptible genotype Arka Vikas showed poor amplification.

A total of 209 reproducible amplification fragments were generated across all the genotypes using the five primer pair combinations in AFLP assay. The number of AFLP bands detected per primer combination varied from 25 to 64 with an average of 41.8 bands per primer pair. Of the 209 bands visualized, 115 (55%) were polymorphic with an average of 23 polymorphic fragments amplified per primer combination. The fragment sizes determined by comparing the amplicons with the standard DNA ladder ranged from ~320 bp to ~30 bp. Table 14 summarizes the data obtained from AFLP studies.

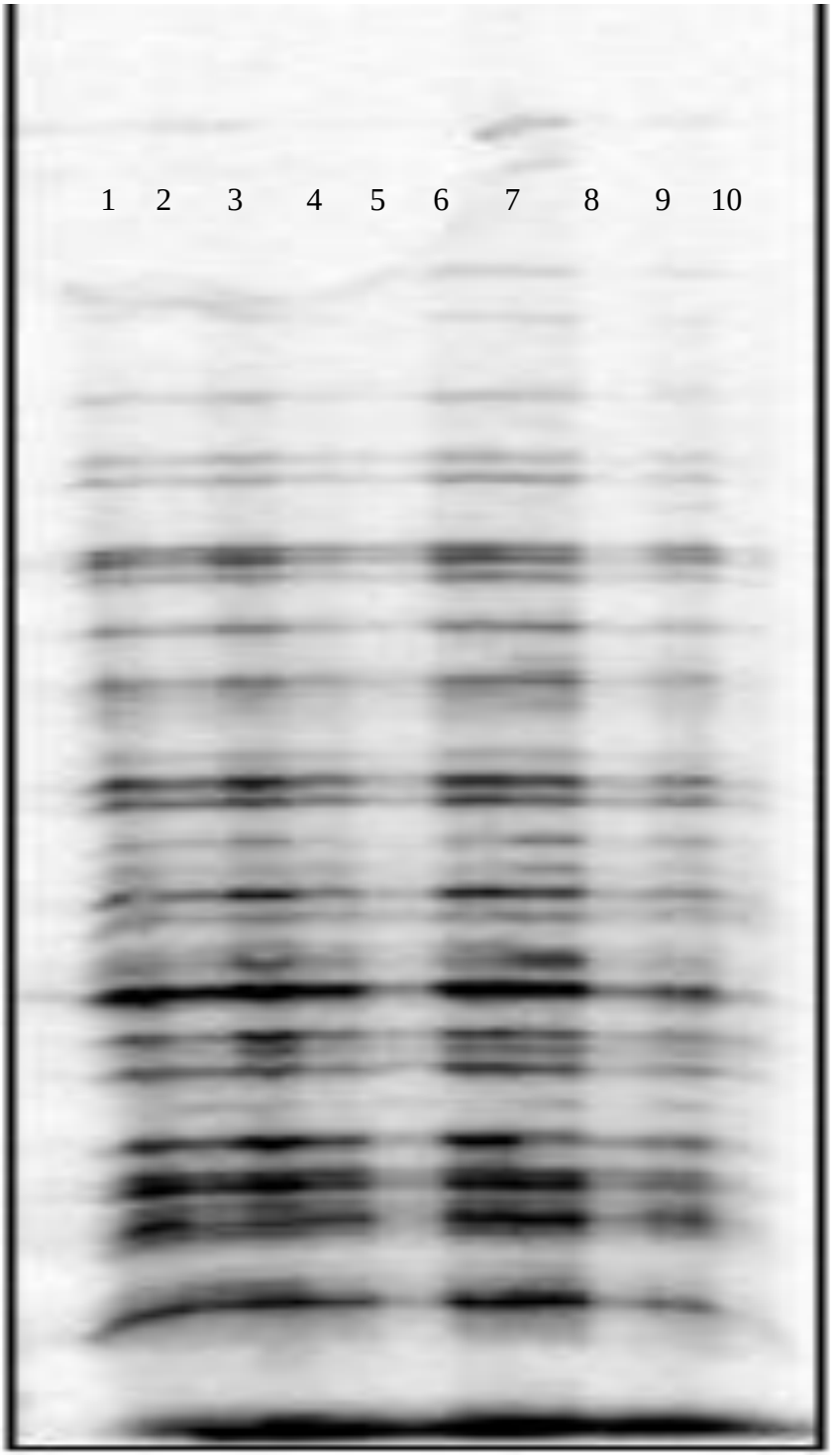
Table 14. Amplification pattern with the five primer pairs in AFLP

Primer pair (*Eco*R1/*MSe* 1)	**Total No. of bands**	**No. of polymorphic bands**	**Per cent polymorphism**
E+ACG/M+CAC	41	29	70.73
E+AGC/M+CAA	64	23	36.00
E+AGG/M+CAA	49	20	40.81
E+AAG/M+CAT	25	19	76.00
E+AGC/M+CTT	30	24	80.00
Total	209	115	



1. Anagha, 2. Sakthi, 3. Mukthi, 4. LE-474, 5. LE-66, 6. Pusa Ruby
7. BT-1, 8. Arka Vikas, 9. BL-333-3-1, 10. PKM-1

Plate 16. AFLP profile with E+AAG/M+CAT primer pair



1. Anagha, 2. Sakthi, 3. Mukthi, 4. LE-474, 5. LE-66, 6. Pusa Ruby
7. BT-1, 8. Arka Vikas, 9. BL-333-3-1, 10. PKM-1

Plate 17. AFLP profile with E+AGC/M+CTT primer pair

4.2.4. Data analysis in AFLP Assay:

The data was analyzed using the Numerical Taxonomy System of Multivariate Statistical Program (NTSyS) software package NTSYSpc Version 2.0. The banding patterns were scored as zero and one based on the absence and presence of bands.

The UPGMA dendrogram (Fig. 3) based on Jaccard's coefficient of genetic distance suggested the existence of two major clusters. The genetic distance between two main clusters was 0.03. The similarity coefficient among the genotypes ranged from 0.74 to 0.87. The first major cluster was divided into two subclusters. The susceptible genotype BL-333-3-1 and resistant genotype LE-66 formed the second subcluster. The phenetic representation of similarity coefficients among the 10 tomato genotypes is presented in Table 15.

The highest genetic similarity was noticed between the resistant genotype Anagha and susceptible genotype PKM-1 (87%) and the least between the resistant genotype LE-66 and susceptible genotype Pusa Ruby (65%). The subcluster A was constituted by eight genotypes which includes three resistant and susceptible genotypes each. The resistant genotypes Sakthi and Mukthi joined together at a similarity coefficient of 0.84. The susceptible genotypes Pusa Ruby and Arka Vikas were clustered together at 85 per cent similarity. The resistant genotype LE-66 and the susceptible genotype BL-333-3-1 were clustered together forming the second sub cluster (B).

4.3 MOLECULAR CLONNING

4.3.1. Detection and elucation of specific amplicon

A distinct band of size 1.5kb was observed in resistant genotypes Sakthi, Mukthi, LE-66 and moderately resistant genotypes LE-20, LE-1-2 when the genomic DNA was amplified using ops 1 primer. In order to elute the DNA from

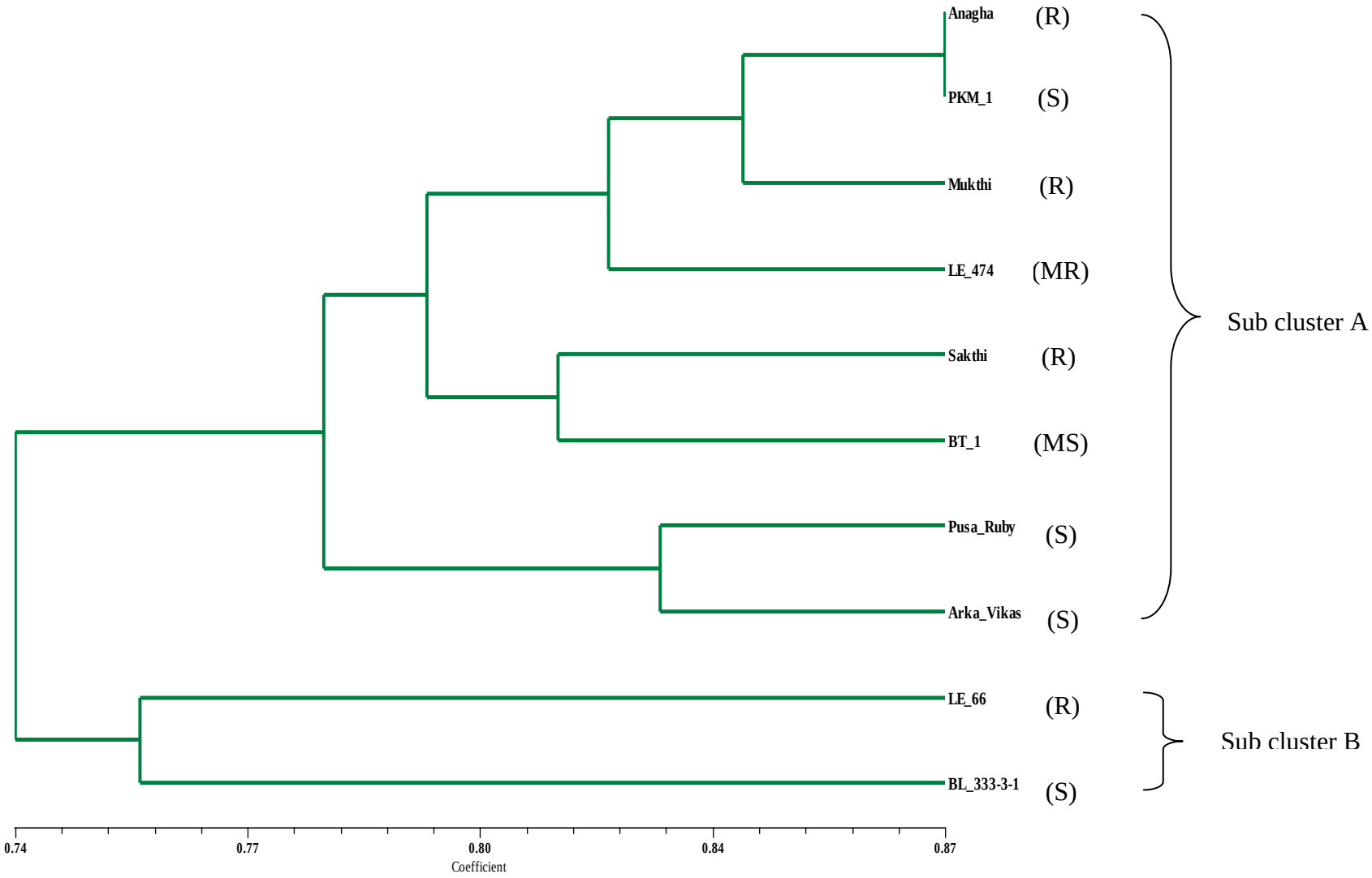


Fig. 3. Dendrogram obtained from AFLP data of 10 tomato genotypes

Table 15. Similarity matrix of the 10 tomato genotypes using AFLP data

Rows\Cols	Anagha	Sakthi	Mukthi	LE_474	LE_66	Pusa_Ruby	BT_1	Arka_Vika	BL_333-3-	PKM_1
Anagha	1.0000000									
Sakthi	0.8144330	1.0000000								
Mukthi	0.8410256	0.8423913	1.0000000							
LE_474	0.8163265	0.7230769	0.8148148	1.0000000						
LE_66	0.7658537	0.7360406	0.7024390	0.7300000	1.0000000					
Pusa_Ruby	0.8144330	0.7945946	0.8128342	0.7230769	0.6521739	1.0000000				
BT_1	0.7980296	0.8157895	0.7777778	0.7450000	0.8020305	0.7512690	1.0000000			
Arka_Vika	0.7661692	0.7819149	0.8000000	0.7295918	0.6586538	0.8306011	0.7755102	1.0000000		
BL_333-3-	0.7886598	0.7210526	0.7395833	0.7606383	0.7552083	0.7393617	0.7085427	0.7010309	1.0000000	
PKM_1	0.8719212	0.8090452	0.8442211	0.8383838	0.7874396	0.8181818	0.8650000	0.8150000	0.8020305	1.0000000

the specific band, ten RAPD reactions with the same primer and common template DNA (Mukthi) were set along with one negative control having template DNA from the susceptible genotype Arka Vikas. The RAPD products were resolved on 1.2 per cent agarose gel (Plate 18a) and the band of interest was cut from each lane, pooled and eluted. The specific band of size ~1.5kb was absent in negative control indicating that it is unique to the resistant genotype.

The eluted product (3µl) was checked on 0.8 per cent agarose gel and band of the same molecular weight was observed. The eluted product when again reamplified (Plate 18b) using the same primer, an amplicon with higher intensity and good quality was obtained after elution (Plate 18c). This eluted product was used for cloning and sequencing study.

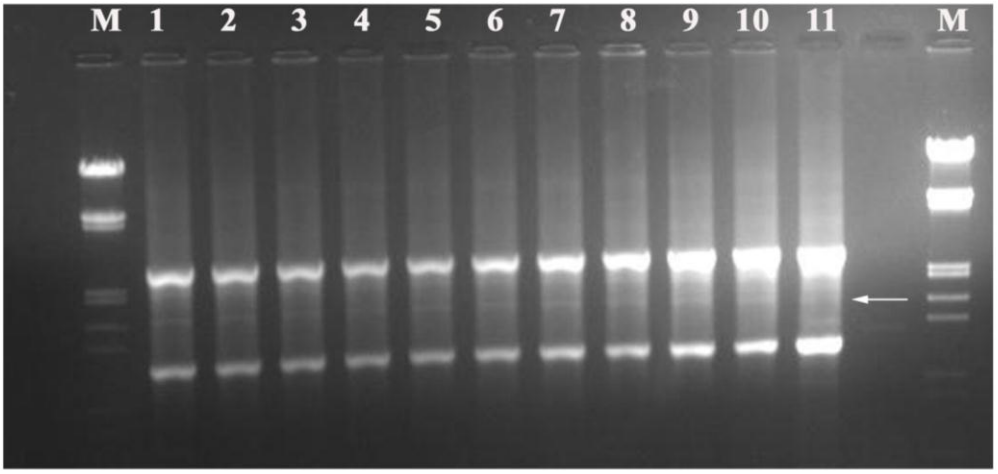
4.3.2. TRANSFORMATION AND CLONING

4.3.2.1. Preparation and screening of competent cells

Competent cells prepared from *E. coli* JM 109 strain as per procedure described on section 3.2.2.9.1 were highly competent and showed high degree of transformation efficiency when transformed with a plasmid (pUC 18) containing ampicillin resistance marker (Plate 19a). The survival rate was high and thus the competent cells prepared was found to be efficient for further cloning works.

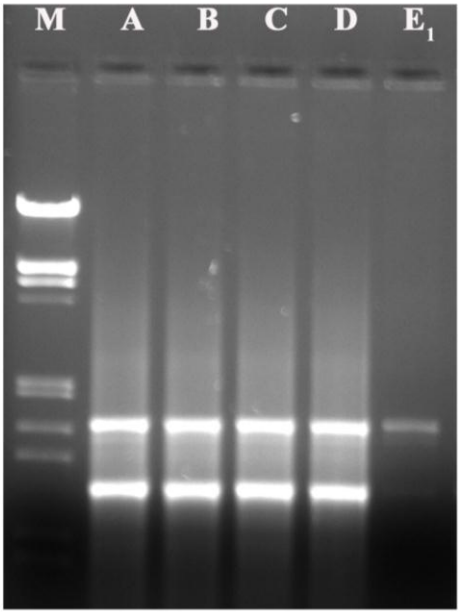
4.3.2.2. Transformation of DNA

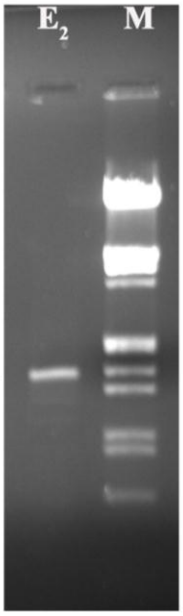
The eluted DNA was inserted in pGEM-T Easy Vector and the ligated product was transferred to the *E. coli* JM 109 cells using the heat shock method at 42°C. Both blue and white colonies were observed when the transformed *E.coli* cells were cultured in ampicillin media overlaid with X-Gal and IPTG confirming a successful transformation (Plate 19b). No growth was observed on negative plate.



M - MW marker, Lane 1 to 10 - Resistant genotypes, Lane 11 - Negative control

a. RAPD amplification of resistant tomato genotypes with OPS 1 primer





Lane 1 to 4 - Reamplified product
E_1 - Pooled, eluted amplicon (3µl)

E_2 - Reamplified, eluted amplicon

(Arrow indicates specific amplicon eluted)

b. Reamplification of eluted DNA with OPS1 primer

c. Reamplified, eluted DNA on

Plate 18. Amplification and elution of DNA from resistant tomato genotypes

4.3.4. CONFIRMATION OF RECOMBINATION

4.3.4.1. Checking the presence of recombinant plasmid

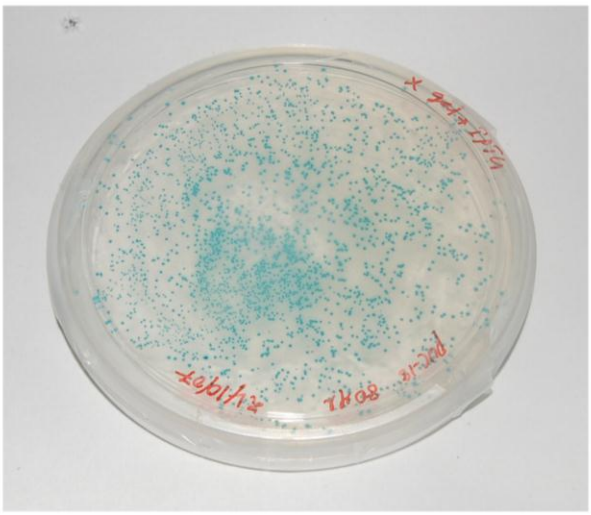
The agar/ampicillin plates containing the transformed colonies were screened for recombinant plasmid. The blue colonies and white colonies picked up from the transformation plates were multiplied separately in LB broth and plasmids isolated were electrophoresed. The plasmid isolated from the white colonies had a higher molecular weight when compared to the plasmid isolated from blue colonies as it lacked the insert (Plate 20a). This confirms the presence of the insert in the plasmid.

4.3.4.2. Detection of the insert by RAPD amplification

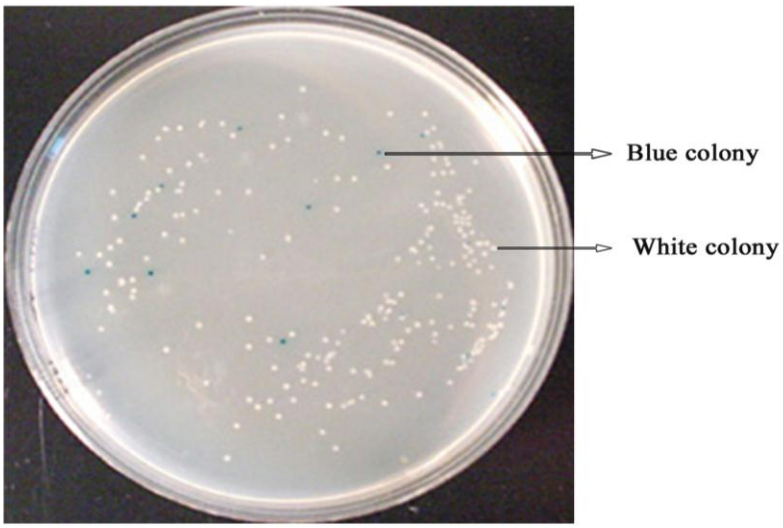
The plasmid was checked for the presence of insert by PCR amplification. The plasmid DNA was used as template and the insert was amplified using the selected random primer OPS 1.A positive control RAPD reaction was set up using the plant genomic DNA as template. Single amplified band of required size, 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 20b).

4.3.4.3 Checking the presence of insert in vector by restriction digestion:

Presence of insert in the plasmid was further confirmed by restriction digestion with *Eco*R1. The digested product when checked on 0.7 per cent agarose gel showed two bands (Plate 20c), one small band of size exactly similar to the amplicon of insert and another band of size equal to that of plasmid. The high molecular weight band corresponded to pGEMT vector and the low molecular weight band confirmed the presence of insert in the plasmid.



a. Competent cell colonies growing in ampicillin media



b. Blue and white colonies in the transformation plate

Plate 19. Screening of competent cells and transformation with RAPD product

4.3.4. SEQUENCE ANALYSIS OF THE CLONED FRAGMENT

Sequence information obtained (referred as *Tomato seq 1*) for the fragment with OPS 1 primer had a size of 681 bp. The details are presented below.

>*Tomato seq 1*

GAAACCATACGCATCCCACGCGTTGGGAGCTCTCCCACTATGGTCGACCTCGC
AGGCGGCCGCACTCACGTGATTATTCTTTTGATTTATAAGGGATTTTGCCGAT
TTCGGCCTATTGGTTACAAAATGAGCTGATTTAACAAAAATTTAACGCGAATT
TTAACAAAATATTAACGCTTACAATTTCCTGATGCGGTATTTTCTCCTTACGC
ATCTGTGCGGTATTTCACACCGCATCAGGTGGCACTTTTCGGGGAAATGTGCG
CGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCAT
GAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATG
AGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTT
CCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATC
AGTTGGGTGCACGAGTGGGTTACATCCAAACTGGATCTCCACAGCCGGTAAG
ATCCTTGAGAGTTTTCCCCCCGAAAACGTTTTCCATGATGACCCTTTTAAGGT
TCTGCTATGTGGCGCCGTTATTATCCCTTATTGACCCGGACAAAACACTTCGG
TGGCGGAAAGGATTCGCAAATGATTGGTTCACCCACCCCCCACCCC//

The electropherogram developed for this sequence is shown in Fig. 4. The coloured peaks indicate the bases. When this sequence was aligned with the pGEM-T Easy Vector sequence, the region starting from 75th base to 617th base showed similarity and was deleted from the original sequence. The sequence named as *Tomato seq 2* obtained after sequence editing (vector screening) is presented below.

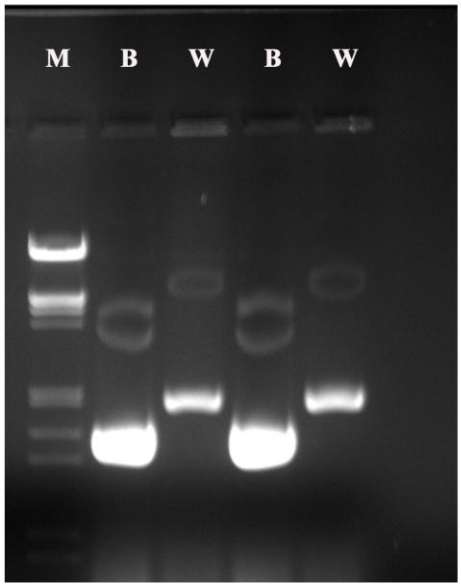
>*Tomato seq 2*

GAAACCATACGCATCCCACGCGTTGGGAGCTCTCCCACTATGGTCGACCTCGC
AGGCGGCCGCACTCACGTGATCCGGACAAAACACTTCGGTGGCGGAAAGGAT
TCGCAAATGATTGGTTCACCCACCCCCCACCCC//

4.3.4.1. Nucleotide analysis

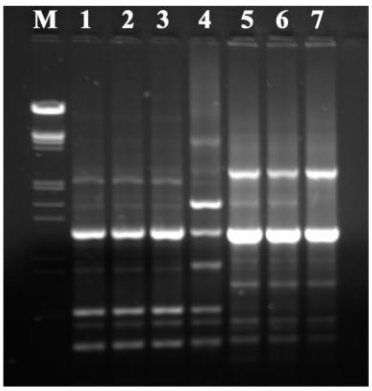
4.3.4.1.1. Homology search

Homology of the sequence obtained from the cloned product of tomato (*Tomato seq 2*) with the other reported sequence was analyzed. The sequence showed significant homology to tomato (*Solanum lycopersicum*) genomic DNA



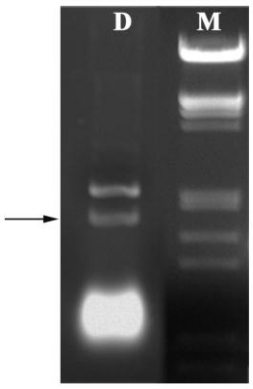
M - MW marker, B - Plasmid DNA from blue colonies,
W - Plasmid DNA from white colonies

a. Plasmid isolated from blue and white colonies



M - Molecular weight marker
Lane 1 to 3 - Plasmid DNA from white colonies
Lane 4 - Plasmid DNA from blue colonies
Lane 5 to 7 - Plant genomic DNA

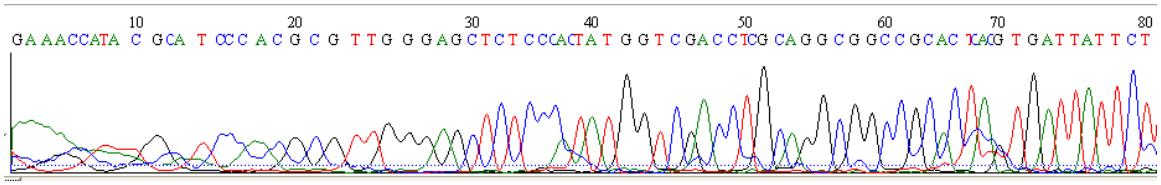
b. Confirmation of recombination with RAPD reaction

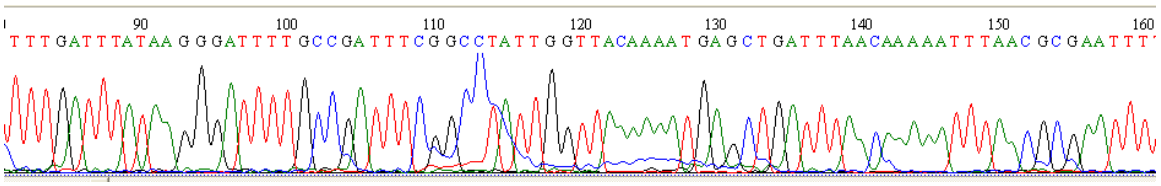


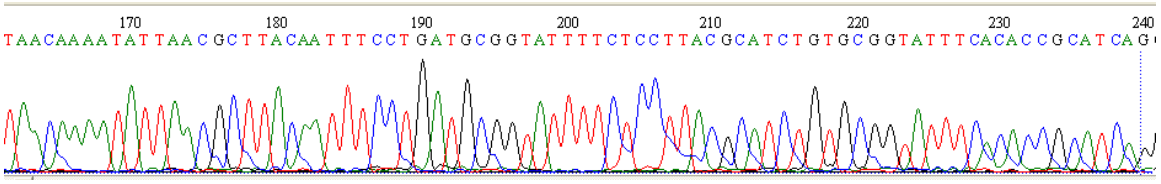
D - *Eco*R1 digested recombinant plasmid
M - Molecular weight marker
Arrow indicates insert DNA

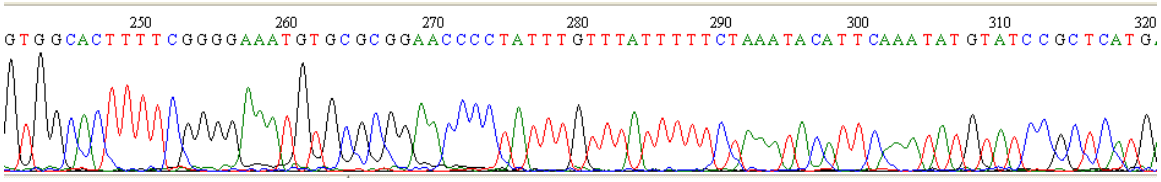
c. Restriction digestion of recombinant plasmid with *Eco*R1

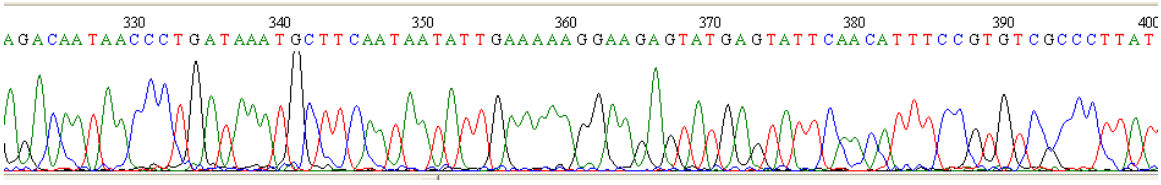
Plate 20. Plasmid DNA isolation and confirmation of recombination in plasmid DNA


10 20 30 40 50 60 70 80
GAAACCATA C GCA T CCC ACG C G TTG G GAGCTCTCCCACTAT GGTCGACCTCG CAGGCGGCCGCAC TACG T GATTATTCT


90 100 110 120 130 140 150 160
TTTGATTTATAAGGGATTTTGCCGATTTCGGCCTATTGGTTACAAAATGAGCTGATTTAACAAAAATTTAACGCGAATTT


170 180 190 200 210 220 230 240
TAACAAAATATTAACGCTTACAATTTCCTGATGCGGTATTTTCTCCTTACGCATCTGTGCGGTATTTCACACCGCATCAG


250 260 270 280 290 300 310 320
GTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATG


330 340 350 360 370 380 390 400
AGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTAT

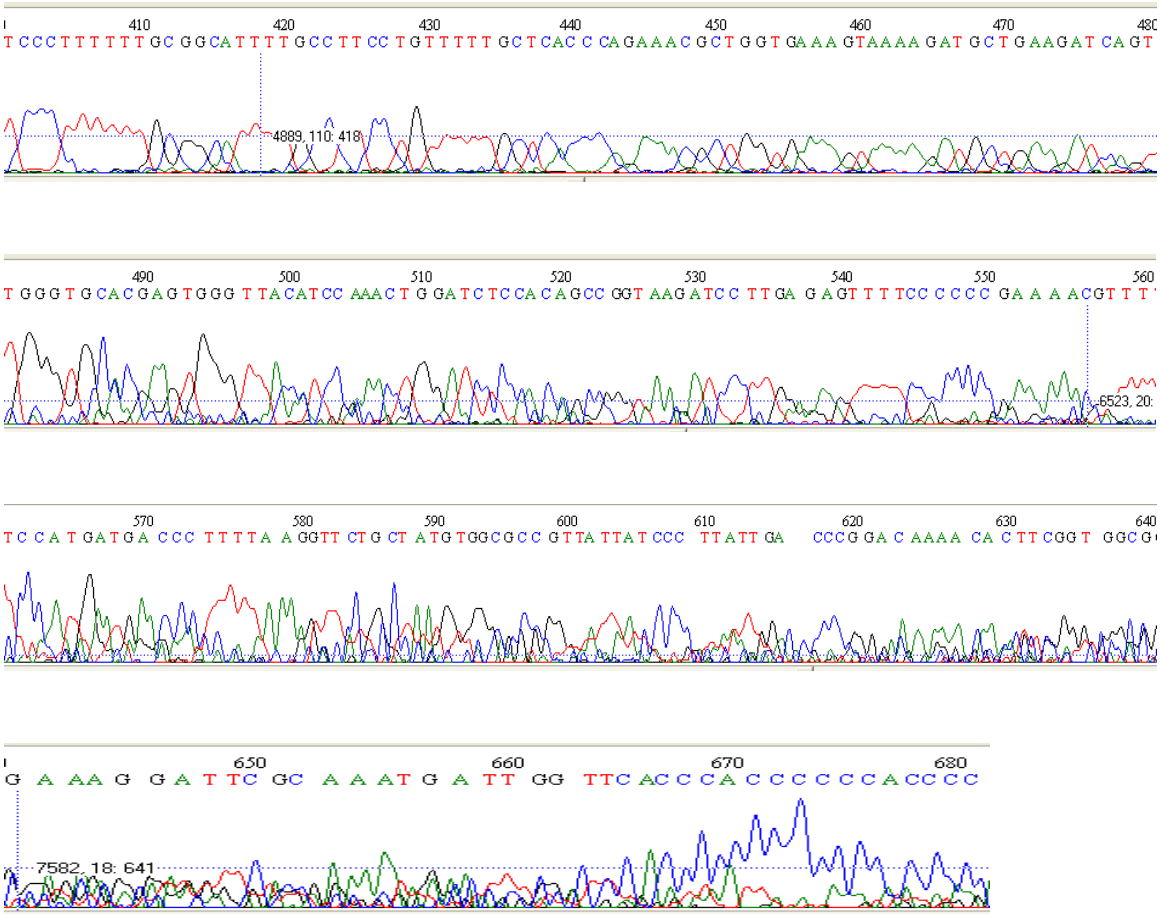


Fig. 4. Electropherogram developed for the cloned fragment

sequence and sequence of *RNase LER* gene for T-2 type RNase deposited in the public domain database using blastn search tool (Fig. 5). It has shown 91 per cent identity to *Solanum lycopersicum* chromosome 11 clone, 100 per cent identity to *Solanum lycopersicum* chromosome 8 clone, 84 per cent identity to *Solanum lycopersicum* chromosome 9 clone and 95 per cent identity with *Solanum lycopersicum RNase LER* gene for T-2 type RNase. The amino acid sequence for this DNA fragment was obtained using Biology Workbench (Fig. 6).

4.3.4.1.2. Detection of Open Reading Frame (ORF)

Open reading frame identified in the sequence *Tomato seq 2* using NCBI tool 'ORF Finder' contained two ORFs. The largest ORF (named as ORF_1) was present on +3 reading frame with 136 bases coding for 45 amino acids in length (Fig. 7). When this significant open reading frame of *Tomato seq 2* was subjected to BLASTp search, it detected homology with AREB like protein of *Lycopersicon esculentum* (Fig. 8). The second ORF (named as ORF_2) was encoded in –1 reading frame and it was of length 111 bases coding for 36 amino acid residues (Fig. 9). The BLASTp search of ORF_2 showed homology with *GRAS4* gene of *Solanum lycopersicum* (Fig. 10).

4.3.4.1.3. Nucleic acid statistics

Nucleic acid statistics of the *Tomato seq 2* obtained in the Biology Workbench is presented in Table 16. The fragment had high GC content (60.1%) and low AT content (39.9%).

4.3.4.2. Restriction analysis

Major restriction sites deduced for the fragment *Tomato seq 2* are provided in Table 17. Both the frequent cutter *Alu*I and the rare cutter *Not*I had a single

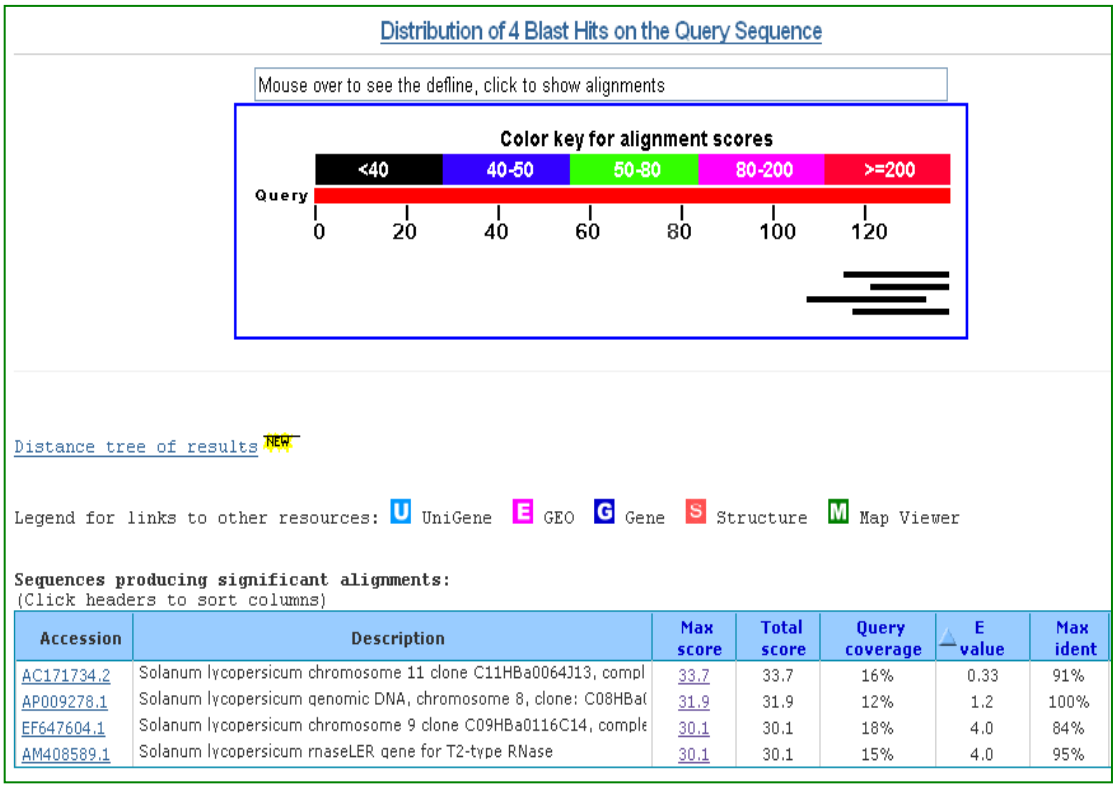


Fig. 5. Results of homology search of *Tomato seq 2* obtained using Blast tool

```
  1   E  T  I  R  I  P  R  V  G  S  S  P  T  M  V  D  L  A  G  G
  1   GAAACCATACGCATCCCACGCGTTGGGAGCTCTCCCACTATGGTCGACCTCGCAGGCGGC

 21   R  T  H  V  I  R  T  K  H  F  G  G  G  K  D  S  Q  M  I  G
 61   CGCACTCACGTGATCCGGACAAAACACTTCGGTGGCGGAAAGGATTCGCAAATGATTGGT

 41   S  P  T  P  H  P
121   TCACCCACCCCCCACCCC
```

Fig. 6. Deduced amino acid sequence of *Tomato seq 2* obtained using Molecular Tool Kit

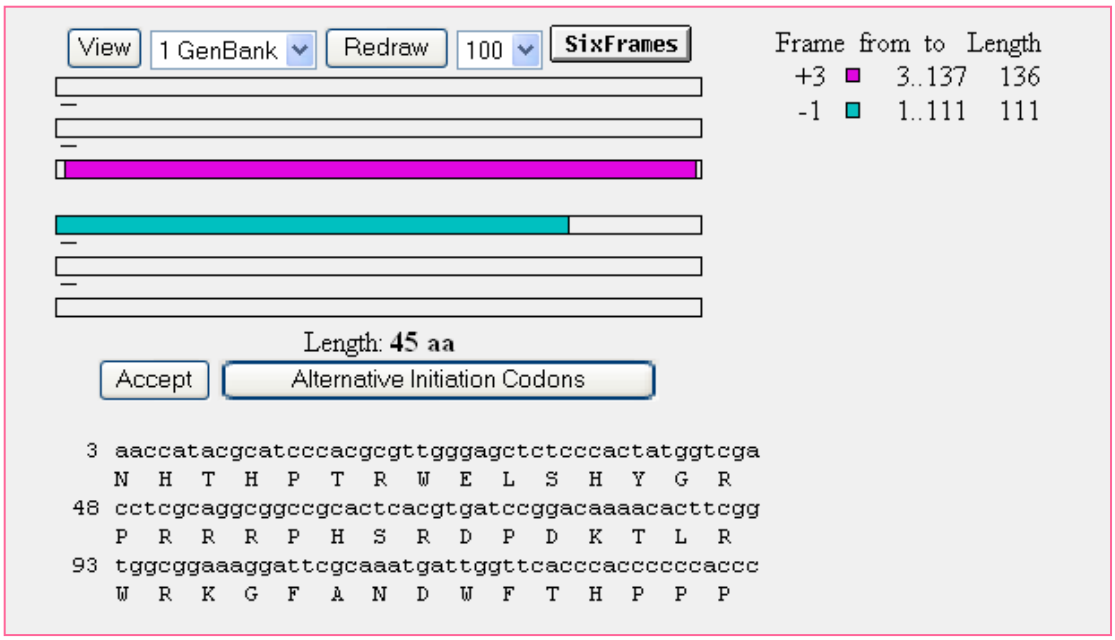


Fig. 7. Open reading frame of *Tomato seq 2* with its largest ORF (ORF_1) and amino acid sequence

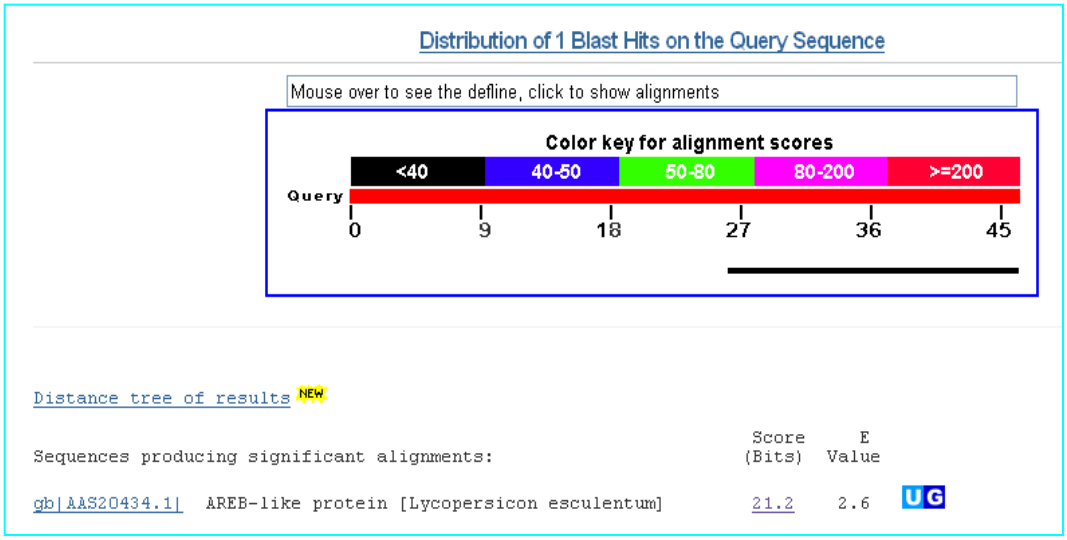


Fig. 8. Results of homology search of ORF_1 obtained using pBLAST tool

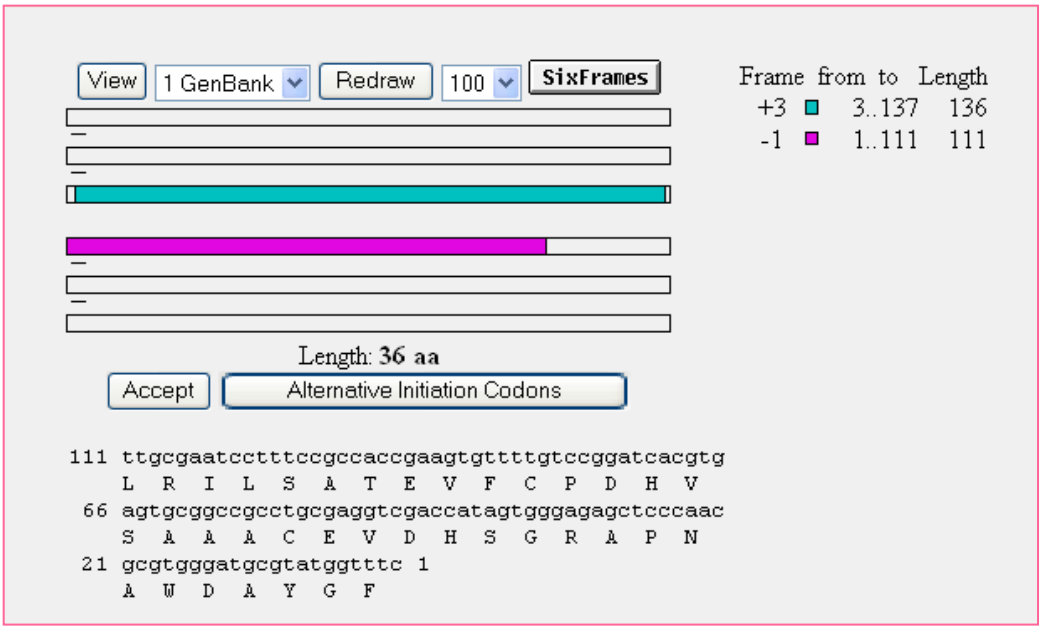


Fig. 9. Open reading frame of *Tomato seq 2* with its second ORF (ORF_2) and amino acid sequence

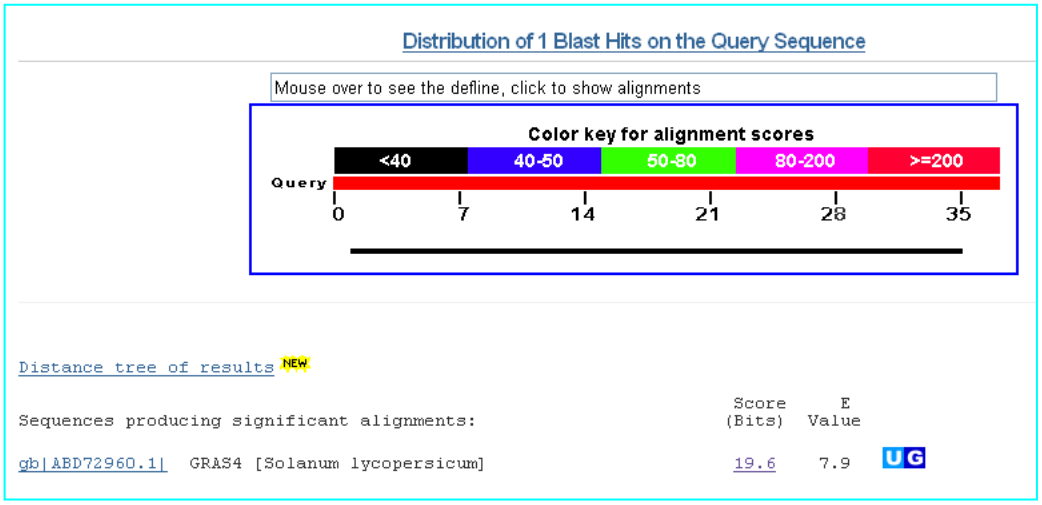


Fig. 10. Results of homology search of ORF_2 obtained using pBLAST tool

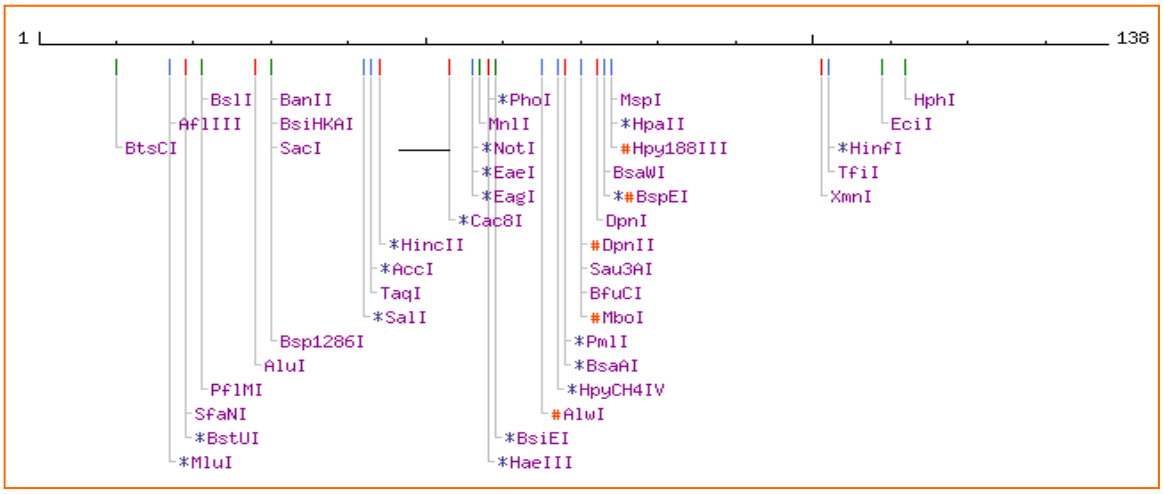


Fig. 11. Restriction analysis of *Tomato seq 2* using Biology Workbench

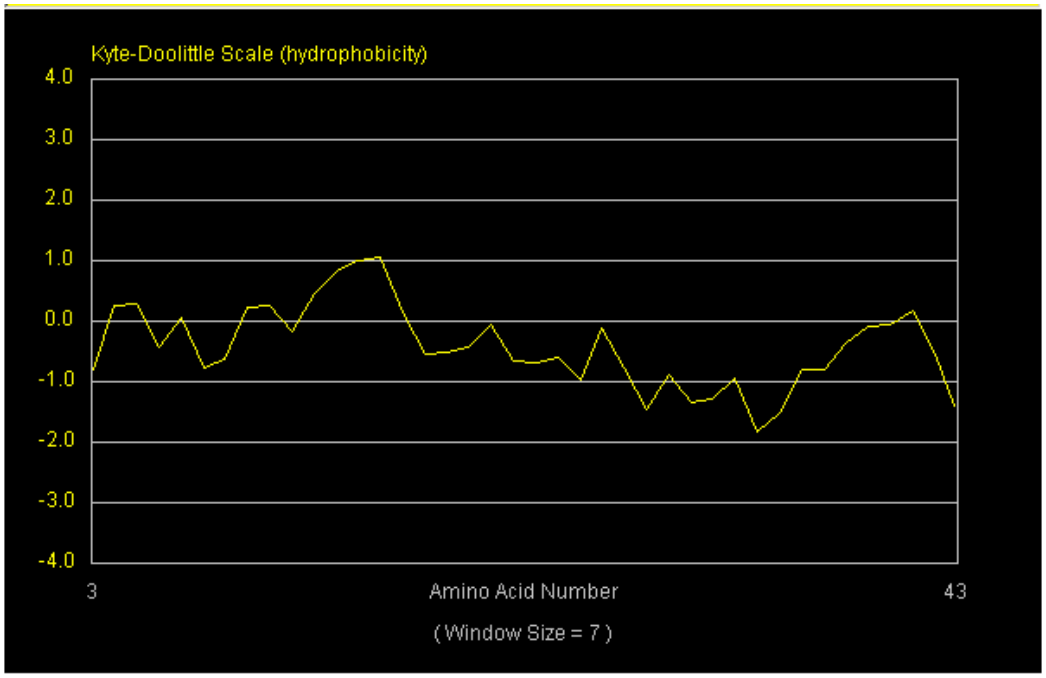
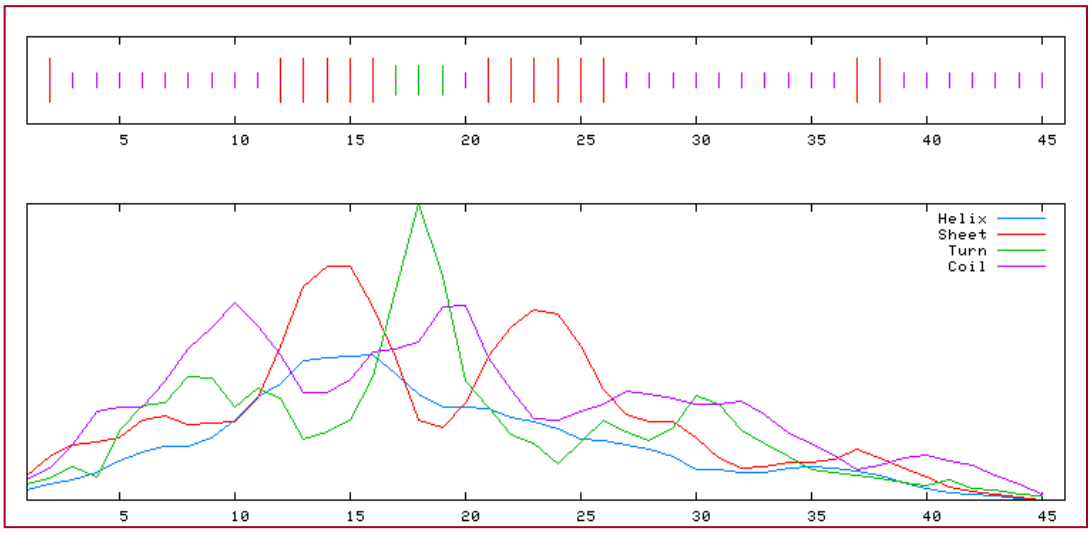


Fig. 12. Doolittle hydropathy plot for deduced protein of *Tomato seq 2*




```
         10         20         30         40
          |          |          |          |
ETIRIPRVGSSPTMVDLAGGRTHVIRTKHFGGGKDSQMIGSPTPHP
eeeccccccccceeeeettteeeeeeccccccccccceeccccccc

Sequence length :    46
```

```
SOPMA :
    Alpha helix       (Hh) :      0 is    0.00%
    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) :     16 is   34.78%
    Beta turn         (Tt) :      3 is    6.52%
    Bend region       (Ss) :      0 is    0.00%
    Random coil       (Cc) :     27 is   58.70%
    Ambigous states   (?)  :      0 is    0.00%
    Other states           :      0 is    0.00%
```

Fig. 13. The secondary structure of protein predicted by SOPMA tool

restriction site in the sequence. It lacked restriction sites for *EcoR*I, *Hind*III, *Xma*I and *Bam*HI. The restriction map is represented in Fig. 11.

4.3.4.3. Amino acid sequence analysis

Deduced amino acid sequence of protein analyzed and proportion of each amino acid calculated using AASTATS of Biology Workbench are presented in Table 18. The major amino acids deduced from the cloned fragment *Tomato seq 2* were Glycine, Proline and Threonine.

4.3.4.3. Secondary structure prediction

The hydropathy plot analysis revealed that there is no transmembrane region in the cloned sequence, *Tomato seq 2* (Fig. 12). The secondary structure of proteins predicted by SOPMA programme of ExSpasy tool is presented in Fig. 13. The amino acid deduced from *Tomato seq 2* had 34.78 per cent extended strands, 6.52 per cent beta turns and 58.7 per cent random coils. It also indicated that the protein is made up of 22 hydrophilic and 24 hydrophobic amino acids.

Table 16. Nucleotide statistics of *Tomato seq 2* obtained using NASTATS

Sl.No.	Nucleotide	Total	Percentage
1.	A	32	23.2%
2.	T	23	16.7%
3.	C	50	36.2%
4.	G	33	23.9%
5.	A + T	55	39.9%
6.	C + G	83	60.1%

Table 17. Restriction sites of *Tomato seq 2* with common restriction enzymes

Sl.No.	Name of restriction enzyme	Recognition sequence	No. of cuts	Position of restriction	Position of restriction fragments (bp)
1.	*Alu*I	AG'CT	1	30	30, 108
2.	*Dpn*I	GA'TC	1	74	64, 74
3.	*Eco*RI	G’AATT_C	0	0	0
4.	*Hae*III	GG'CC	1	60	60, 78
5.	*Not*I	GC'GGCC_GC	1	58	58, 80
6.	*Mbo*I	'GATC_	1	72	66, 72
7.	*Sac*I	G_AGCT'C	1	32	32, 106
8.	*Sal*I	G'TCGA_C	1	44	44, 94
9.	*Taq*I	T'CG_A	1	45	45, 93

Table 18. Deduced Amino acid composition of *Tomato seq 2*

Amino acid group		Amino acid	Number	Mol %
Non polar		**Gly**	7	15.22
		Ala	1	2.17
		Val	3	6.52
		Leu	1	2.17
		Ile	4	8.70
		Met	2	4.35
		Pro	5	10.87
		Phe	1	2.17
Polar	Unchancharged	*Ser*	4	8.70
		Thr	5	10.87
		Gln	1	2.17
	Basic	*Lys*	2	4.35
		Arg	4	8.70
	Acidic	*His*	3	6.52
		Asp	2	4.35
		Glu	1	2.17

Discussion

5. DISCUSSION

Tomato, *Solanum lycopersicum* L., is the most economically important vegetable crop in the world second to potato. Tomato cultivation in the tropics is hampered by bacterial wilt, a devastating soil-borne disease caused by *Ralstonia solanacearum* (Smith) Yabuuchi *et al*. The disease is wide spread in India, causing yield losses to the extent of 90 per cent and is the main limiting factor for tomato cultivation in Kerala. The warm, humid tropical climate and acidic soil prevailing in Kerala favours the incidence of bacterial wilt.

In the absence of effective chemical control measures, breeding for resistance is likely to remain as the most adopted strategy for disease management. Asian Vegetable Research and Development Centre, Taiwan, National Agricultural Research Systems and private seed companies have worked on the development of bacterial wilt resistant genotypes. However, the resistance is unstable because of the wide genetic diversity of the pathogen. Moreover, it is complex being controlled quantitatively and is strongly influenced by environmental conditions such as temperature, pH and moisture content of the soil. Interference of environmental factors also hinder effective selection in segregating population as there may be symptomatic plants in resistant entries and symptomless plants in susceptible entries. This necessitates the integration of molecular marker technologies into conventional breeding programmes.

At this context, the study entitled “Development of trait related markers for bacterial wilt resistance in tomato (*Solanum lycopersicum* L.)” was undertaken to characterize selected tomato genotypes, and to detect and characterize trait related markers with special reference to bacterial wilt.

The result of screening different tomato genotypes to bacterial wilt, their molecular characterization, cloning and sequencing of trait related markers are discussed in this chapter.

5.1. Screening of genotypes to bacterial wilt:

Fifteen tomato genotypes were evaluated for their reaction to bacterial wilt during October 2005 to September 2007. Both the nursery and the seedlings were raised in earthen pots containing sterile soil medium. The medium was sterilized with formaldehyde solution to avoid the inhabiting inoculum of *Ralstonia solanacearum* in the soil, which may otherwise interfere with artificial screening.

Thirty eight day old seedlings were used for artificial inoculation through stem puncture and soil drenching with wounding, which were earlier reported by Winstead and Kelman (1952) and Husain and Kelman (1958). Uniform inoculum density of *Ralstonia solanacearum* was maintained in all the treatments. The genotypes started wilting within three days indicating the effectiveness of the methods, in evaluating the reaction of genotypes to bacterial wilt incidence. 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. Moreover, sufficient inoculum was ensured by keeping a piece of cotton dipped in bacterial suspension in the punctured leaf axils. One disadvantage of this method was the extra care necessary to the seedlings to avoid physical damage due to the needle inserted into the stem for inoculation.

The root severing 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 ample opportunity for bacterial infection. As natural infection mainly occur through wounded roots, soil drenching with root severing technique may mimic the field condition. In addition, soil drenching had an advantage that

the plant growth was not distorted, as observed to occur sometimes with the stem puncture stabbing method.

Being younger (38 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. 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. It was suggested that resistance in younger plants could not be compared to that found in older plants (Mew and Ho, 1976).

5.2. Reaction of genotypes to bacterial wilt incidence:

The fifteen genotypes of tomato screened using the stem inoculation method and soil drenching with wounding showed different degrees of resistance and susceptibility. It was interesting to observe the disease reaction in the selected genotypes under greenhouse condition. The plants were inoculated 10 days after transplanting by which time those grown in the greenhouse had put forth better vegetative growth (Plate 2). None of the genotypes expressed disease symptoms within seven days after inoculation. Many scientists have reported that variation in environmental factors influence bacterial wilt incidence and disease reaction in tomato (Arnold and Brown, 1968; Krausz and Thurston, 1975; Scott *et al*., 1992; Hanson *et al*., 1996). The robust nature of the crop observed in the greenhouse condition might have helped the plants to defend the inoculum. Hence the growth conditions and stage of plant growth are to be standardized for artificial inoculation with *Ralstonia solanacearum*.

The seedlings grown in open condition responded to artificial inoculation. Based on the observations made, the genotype Anagha with 10 per cent wilt incidence was found to be more resistant to the disease. The genotypes Sakthi, Mukthi and Hawaii7998 recorded 15 per cent wilt incidence and in the genotype

LE 66 it was 18 per cent. According to the classification suggested by Mew and Ho (1976), these five genotypes were grouped under the resistant category since the incidence was below 20 per cent. The resistance in Hawaii7998 can be traced to PI 127805A, a *L. pimpinellifolium* line (Gibert *et al*., 1973). Many researchers (Ramachandran *et al*., 1980; Celine, 1981; Rajan and Peter, 1986; Peter *et al*., 1992; Sadhankumar, 1995; Bose, 1999) have reported the resistance of the genotypes Sakthi and Mukthi to bacterial wilt earlier. One of the reasons could be that both these genotypes evolved from a common line, CL 32d-0-1-19GS which showed resistance to bacterial wilt as per the reports of Ramachandran *et al*. (1980). The resistance of the genotypes to bacterial wilt could also be due to the introgression of resistance genes from wild species like *Lycopersicon pimpinellifolium* or from *L. esculentum* var. *cerasiforme*, the progenitor of cultivated tomato (Prior *et al*., 1994). Another reason might be the higher amount of total phenol and OD phenol content as observed by Bose (1999).

The resistant genotype Sakthi, which was reported as "not affected by wilt" by Celine (1981) recorded 10 per cent disease incidence in the present study. This may be attributed to the fact that the inoculum density and temperature can alter the expression of resistance. Krausz and Thurston (1975) illustrated this in their earlier reports.

The genotypes LE-20, LE-474 and LE-1-2 with wilt incidence of 25, 26 and 35 per cent respectively were classified as moderately resistant. These are known sources of resistance released from elsewhere. The congenial conditions in Kerala might be the reason for the change in disease reaction as reported in several other genotypes (Goth *et al.*, 1983).

The genotypes BTH-102-1-2-2, BT-218 and BT-1 were moderately susceptible to bacterial wilt with an incidence of 45, 50 and 58 percent respectively. The genotype BT-1 showed moderate susceptibility in an experiment conducted by Bose (1999).

The remaining four genotypes included in the study were highly susceptible. Pusa Ruby recorded 90 per cent wilt incidence followed by Arka Vikas (80%). This is in accordance with earlier reports of Sadhankumar (1995) and Paul (1998) in Pusa Ruby. The genotypes PKM-1 and BL 333-3-1 also showed 75 and 70 per cent of wilt incidence.

5.3. MOLECULAR CHARACTERIZATION

Molecular characterization of the tomato genotypes selected for the study was carried out with the objective of developing trait specific markers for bacterial wilt resistance. RAPD (Random Amplified Polymorphic DNA) assay and AFLP (Amplified Fragment Length Polymorphism) assay were used for characterization.

5.3.1. ISOLATION OF GENOMIC DNA

Isolation of genomic DNA of superior quality and quantity is a pre-requisite for any molecular biology based study. DNA was isolated from fresh, tender leaves collected early in the morning so as to minimize the interference of polyphenols. 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. Zhang and Mc Stewart (2000) reported that DNA extraction from plants is preferentially performed from young tissues due to the lower content of polyphenols, polysaccharides and other secondary metabolites, which co-precipitate with DNA in the extraction procedure, inhibit DNA digestion and RAPD assay, presumably by irreversible interactions with DNA. The use of tender leaves for DNA isolation in tomato has been reported by Martin *et al.* (1991), Archak *et al.* (2002) and Langella *et al.* (2004).

Protocols reported by two groups of scientists, Doyle and Doyle (1987) and Rogers and Bendich (1994), were tested for suitability of DNA isolation in tomato. The DNA isolated by the latter method gave discrete bands with minimum RNA contamination (Plate 5a). The spectrophotometric readings showed that the quantity of DNA isolated by this method was good enough to carry out RAPD and AFLP assay. The DNA yield obtained ranged from 219 to 255 μg/g of tissue. The extracted DNA was of high quality as it showed an absorbance ratio ranging between 1.78 and 1.88 at 260/280.

Liquid nitrogen was used for homogenization of the leaf tissue, and this could be one of the reasons for obtaining intact DNA. Liquid nitrogen freezes the tissues and helps to prevent degradation of nucleic acid. Similar observations have been made by Lodhi *et al.* (1994) in grapevine cultivars, Sharma *et al.* (2002) in sorghum, chickpea, wheat and soybean, and Padmalatha and Prasad (2006) in medicinal and aromatic plants.

The presence of certain substances can hamper DNA isolation procedures and reactions such as DNA amplification, restriction and cloning. Like many other plant species, tomato tissues contain high levels of tannin and polyphenolic compounds that were problematic in good quality DNA isolation. During tissue homogenization phenolics become oxidized, covalently bind to DNA giving it a brown colour. The irreversible binding produces a gelatinous material, which is hard to separate from organelles and the DNA become unsuitable for amplification and digestion analysis. This problem was overcome by the addition of β-mercapto ethanol and Poly Vinyl Pyrrolidone (PVP) along with the extraction buffer. β-mercapto ethanol is a reducing agent, which protect DNA from peroxidase and polyphenol oxidase. β-mercapto ethanol disrupts the protein disulfide bonds and is thereby capable of initiating protein degradation. PVP complexes with secondary plant products especially polyphenols and tannins by binding them with hydrogen bonds and can be separated from DNA by centrifugation. Nesbit *et al.* (1995) and Padmalatha and Prasad (2006) have

reported the use of β-mercapto ethanol and PVP for overcoming phenolic contamination.

The cationic detergent, CTAB (cetyltrimethylammonium bromide), present in the extraction buffer helps in lysis of cell membrane and release of nucleic acids. On the other hand, it acts as selective precipitant of nucleic acids in the solution. CTAB forms a complex with polysaccharides and prevents co-precipitation of polysaccharides with nucleic acids. By these actions, CTAB must have helped in the recovery of relatively pure DNA in the present study. The DNA is soluble in presence of CTAB at high salt concentration (1.4M NaCl). In addition, NaCl present in the extraction buffer would also have helped in removal of polysaccharides. Sharma *et al.* (2002) reported that nucleic acids form tight complexes with polysaccharides creating a gelatinous pellet that contains embedded DNA, and polysaccharides co-precipitate with DNA after alcohol addition during DNA isolation leading to viscous solutions. Certain polysaccharides are also known to inhibit RAPD reactions. They distort the results in many analytical applications and lead to many wrong interpretations (Padmalatha and Prasad, 2006). Therefore, their removal is of great importance in DNA isolation.

EDTA present in the extraction buffer chelate the Mg^{2+} ion which is an essential co-factor for the enzyme to act and prevents the indigenous endonucleases which act on nucleic acids. EDTA is also a major component of TE (Tris EDTA) buffer in which the DNA is dissolved finally. It plays the same role of that of a chelating agent here also.

After the tissue homogenization and addition of extraction buffer, the samples were incubated at 65°C for 15 minutes. This is because high temperature can accelerate the release of DNA from the nucleus and inactivate DNases, and other enzymes that can destroy DNA.

The DNA isolated by the Rogers and Bendich (1994) protocol was free from chlorophyll and other pigments. This could be due to the fact that this protocol involves two treatments with chloroform: isoamyl alcohol (24:1). These treatments ensure the removal of chlorophyll and other colouring substances such as pigments, dyes, etc.

The DNA isolated was found to be contaminated with some amount of RNA (Plate 5a). Large amounts of RNA in the sample can chelate Mg^{2+} ions and reduce the yield of polymerase chain reaction (PCR). The contaminating RNA that precipitates along with DNA causes interference with DNA amplification involving random primes and improper priming of DNA templates during thermal cycle amplification. The presence of RNA in the genomic DNA preparation often influences the reproducibility of RAPD patterns (Micheli *et al.*, 1994). In order to overcome the problem of RNA contamination, the extracted DNA samples were treated with RNase A. This treatment resulted in DNA with no impurities, very much suitable for RAPD analysis (Plate 5c). The importance of RNase treatment in order to yield RNA-free pure DNA was also reported by Lodhi *et al.* (1994), Archak *et al.* (2002), Archak *et al.* (2003) and Padmalatha and Prasad (2006).

5.3.2. RAPD ASSAY

Random Amplified Polymorphic DNA (RAPD) is a powerful technique for determining inter and intra-specific DNA variation. Wiliams *et al*. (1990) for the first time demonstrated that single primers of arbitrary sequence can be used to amplify genomic DNA segments and the polymorphism can be detected between the amplified products of different individuals. It is a dominant marker system that is inherited in a Mendelian fashion.

In an RAPD reaction, a single species of primer binds to the genomic DNA at two different sites on opposite strands of the DNA template. A discrete DNA product is formed if these sites are within an amplifiable distance of each

other. Each primer will direct the amplification of several discrete loci in the genome thus making the assay an efficient method to screen for nucleotide sequence polymorphisms between individuals. This marker system has been used in many research applications involving the detection of DNA, sequence polymorphism, isolation of markers linked to various traits, varietal identification and parentage analysis.

5.3.2.1. Primer screening

Random decamer primers obtained from Operon Technologies, USA were used for the present study. Operon primer kits are easily available and yield good results. For these reasons, they are popular with researchers working on RAPD analysis. The use of Operon primers has been reported by Archak *et al.* (2002) and Rajput *et al.* (2006) in tomato and Clain *et al.* (2004) in *Solanum torvum.*

A total of 40 random decamer primers belonging to the OPS and OPY series were screened initially and the primers for further analysis were selected based on the number and nature of amplicons. Thirty five primers that showed good and average amplification were selected and employed for RAPD profiling of the 15 tomato genotypes. The number of amplification products produced by both sets of primers together during primer screening ranged from two to ten. This difference in the number of amplification products could be due to the fact that even a single base change in the primer sequence could lead to a complete change in the set of DNA fragments amplified as reported by Williams *et al.* (1990). They have also reported that GC content in the decamer primer influenced the amplification and a GC content of 40 percent or more in the primer sequence was needed to generate detectable levels of amplified products.

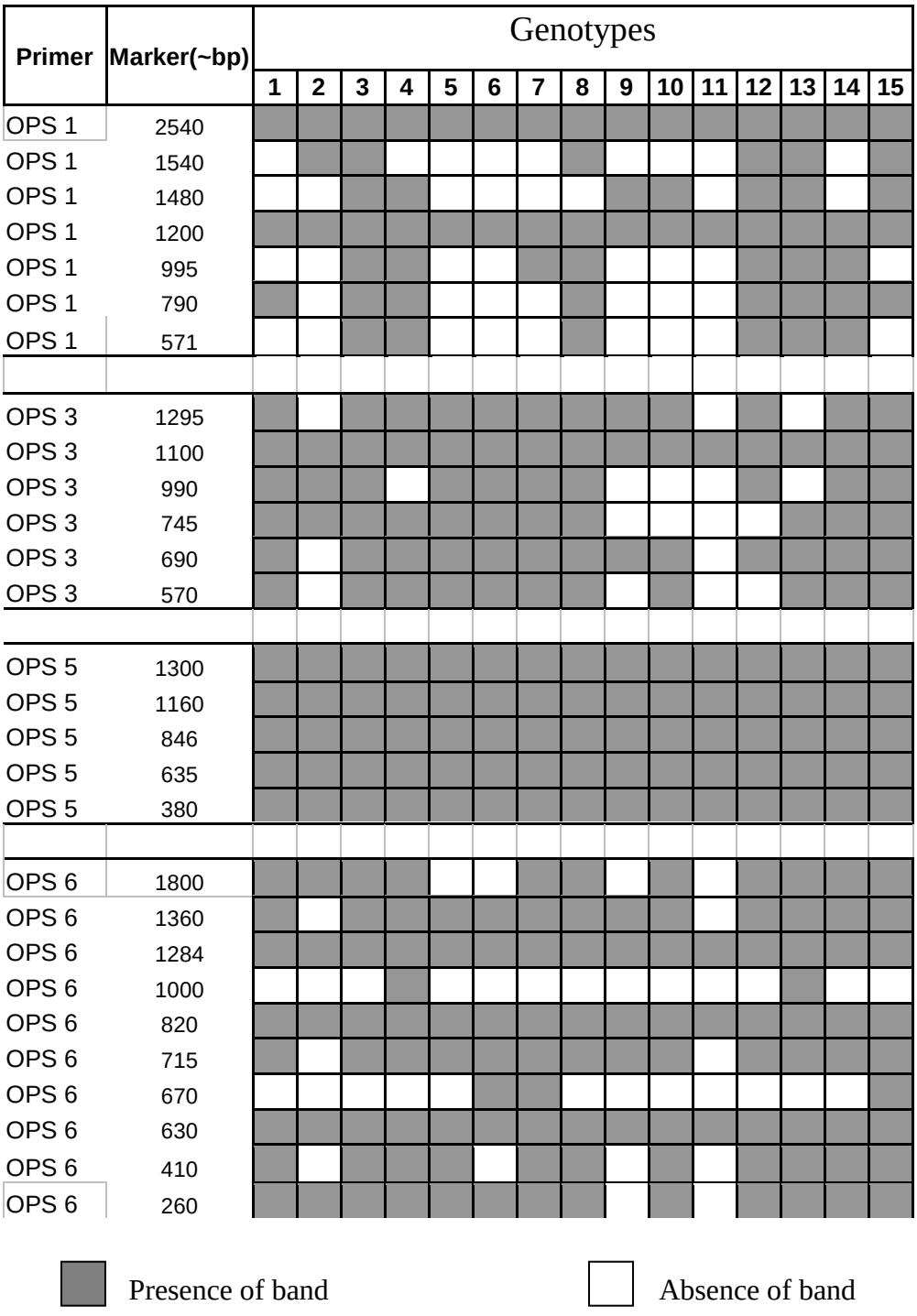
5.3.2.2. RAPD profiling of tomato genotypes

The decamer primers that yielded clear and reproducible banding pattern were selected for amplifying the genomic DNA isolated from the fifteen tomato genotypes. For each primer, fifteen reactions plus a negative control was set up. The negative control comprised of all the ingredients in the RAPD reaction mixture excluding the template DNA. The negative control was maintained in order to ensure that amplification was due to the tomato genomic DNA alone.

Out of the 35 primers selected after primer screening, 14 primers namely OPS 1, OPS 3, OPS 5, OPS 6, OPS 7, OPS 8, OPS 10, OPS 11, OPS 15, OPS 18, OPY 7, OPY 9, OPY 15 and OPY 18 could display good amplification with total number of bands ranging from five to thirteen. The size of amplicons ranged from 3.2 kb to 0.3 kb. The per cent reproducibility of RAPD was found to be 40 and this is in accordance with the reports of Rajput *et* al. (2006) that RAPDs are difficult to reproduce. Numerous studies have reported the variation of RAPD markers according to experimental conditions including the type of thermal cycler used.

A total of 110 RAPDs were obtained with the 14 selected primers. The range of polymorphic markers per primer was zero (OPS 5) to thirteen (OPS 8 and OPY 9) and the percentage polymorphism ranged from zero (OPS 5) to 90 (OPY 9). The primer OPS 20 exhibited the maximum polymorphism with nine polymorphic bands out of 10 amplicons. The pictographs developed by the 14 selected primers are shown in Fig. 14, Fig. 15, Fig. 16 and Fig.17.

Though the percentage polymorphism was as high as 90 per cent, the polymorphism was not significant in relation to disease reaction to bacterial wilt. Only one primer, OPS 1, produced one unique band of size ~1.5kb in the resistant genotypes Sakthi, Mukthi and LE-66 and moderately resistant genotypes LE-20 and LE1-2, and moderately susceptible genotype BTH 102-1-2-2. This particular



1. Anagha **2.** Sakthi **3.** Mukthi **4.** Hawaii 7998 **5.** LE-66 **6.** LE 20 **7.** LE-474 **8.** LE-1-2 **9.** BTH-102-1-2-2 **10.** BT-218 **11.** BT-1 **12.** BL-333-3-1 **13.** PKM-1 **14.** Arka Vikas **15.** Pusa Ruby

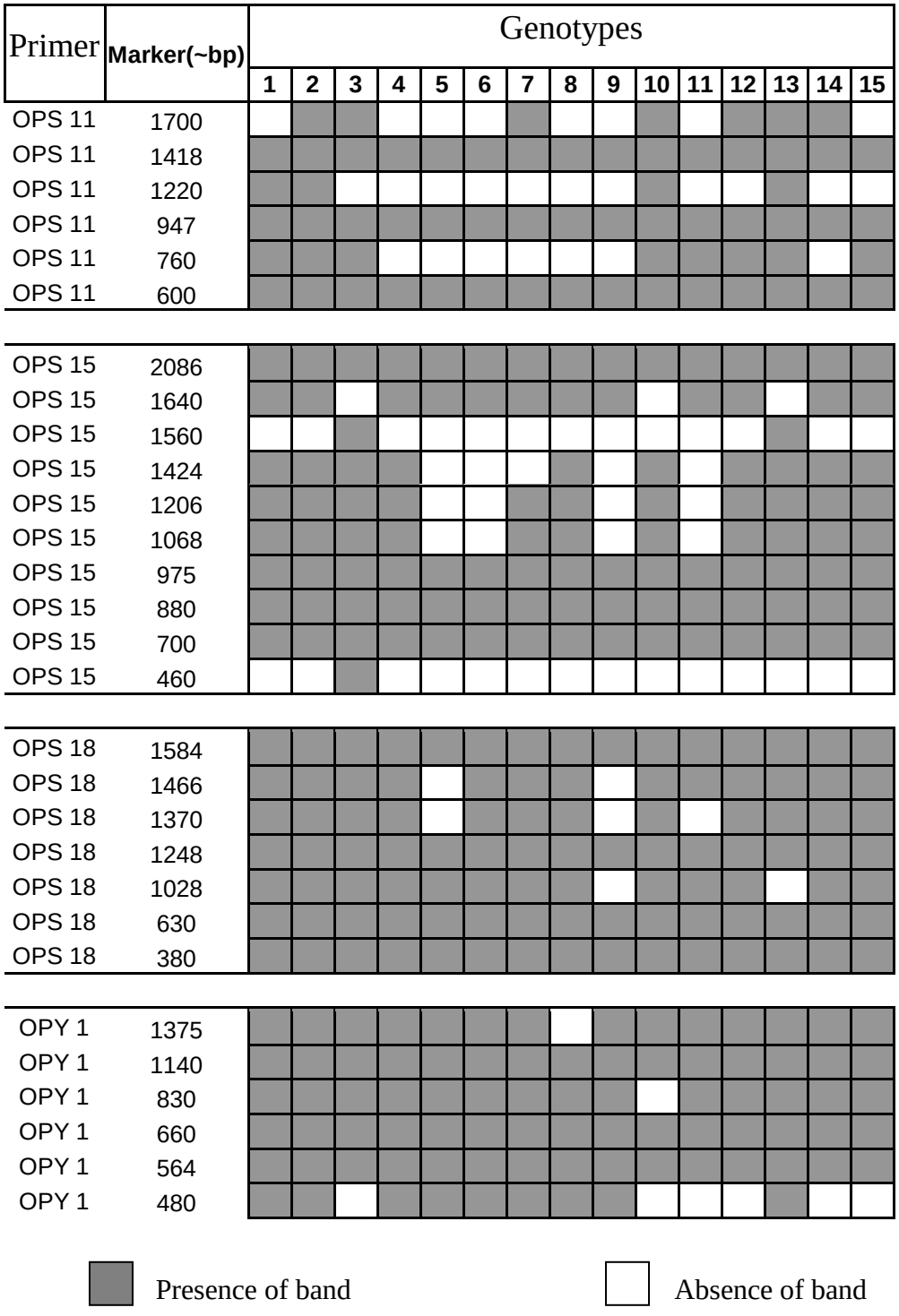
Fig. 14. Pictograph indicating the banding pattern developed with the

Primer	Marker(~bp)	Genotypes														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
OPS 7	1820	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
OPS 7	1400	■	■	■	■	■	■	■	■	■	■	□	■	■	■	■
OPS 7	1320	□	□	□	■	□	□	□	□	□	■	□	□	■	□	□
OPS 7	1230	■	□	■	■	□	□	□	■	□	□	□	□	■	■	□
OPS 7	1113	■	■	■	■	■	■	■	■	■	□	□	■	□	■	□
OPS 7	930	□	□	□	□	□	□	□	□	□	□	□	□	■	□	■
OPS 7	820	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
OPS 7	587	■	■	■	□	■	□	□	■	□	■	□	■	■	□	■
OPS 7	415	■	■	■	■	■	■	■	■	■	□	□	■	□	■	■
OPS 8	2475	□	■	■	■	□	■	■	■	□	■	□	■	■	■	■
OPS 8	1800	■	■	■	■	□	■	■	■	■	■	□	■	■	■	■
OPS 8	1534	■	■	■	■	□	□	■	■	■	■	□	■	■	■	□
OPS 8	1418	■	■	□	■	□	□	□	■	□	■	□	■	■	■	■
OPS 8	1330	■	□	■	■	□	□	□	□	□	□	□	□	■	□	□
OPS 8	1140	■	□	■	■	□	■	■	■	■	■	□	■	■	■	■
OPS 8	1040	□	□	□	□	□	□	□	□	■	□	□	□	□	□	□
OPS 8	864	■	■	■	■	■	■	■	■	■	■	□	■	■	■	■
OPS 8	675	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
OPS 8	570	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
OPS 8	500	■	■	■	□	□	□	□	■	□	□	□	□	□	□	□
OPS 8	450	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
OPS 8	285	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
OPS 10	2110	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
OPS 10	1441	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
OPS 10	1067	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
OPS 10	820	■	□	■	■	■	■	□	■	□	■	■	■	■	■	■
OPS 10	696	■	□	■	■	■	■	■	■	■	■	■	■	■	■	■
OPS 10	554	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
OPS 10	420	□	■	■	□	■	□	□	■	□	■	□	□	□	■	■
OPS 10	335	■	■	■	□	■	■	■	■	□	■	□	□	■	■	■

■ Presence of band □ Absence of Band

1. Anagha **2.** Sakthi **3.** Mukthi **4.** Hawaii 7998 **5.** LE-66 **6.** LE 20 **7.** LE-474 **8.** LE-1-2 **9.** BTH-102-1-2-2 **10.** BT-218 **11.** BT-1 **12.** BL-333-3-1 **13.** PKM-1 **14.** Arka Vikas **15.** Pusa Ruby

Fig. 15. Pictograph indicating the banding pattern developed with the primers OPS 7, OPS 8 and OPS 10



1. Anagha **2.** Sakthi **3.** Mukthi **4.** Hawaii 7998 **5.** LE-66 **6.** LE 20 **7.** LE-474 **8.** LE-1-2 **9.** BTH-102-1-2-2 **10.** BT-218 **11.** BT-1 **12.** BL-333-3-1 **13.** PKM-1 **14.** Arka Vikas **15.** Pusa Ruby

Fig. 16. Pictograph indicating the banding pattern developed with the primers OPS 11, OPS 15 and OPS 18 AND OPY 1

Primer	Marker(~bp)	Genotypes														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
OPY 7	1770	■	■	■	□	■	■	■	□	□	■	□	□	□	■	■
OPY 7	1270	■	■	■	■	■	■	■	□	□	■	■	□	■	■	■
OPY 7	1025	■	■	■	■	■	■	■	□	□	■	□	□	■	■	■
OPY 7	850	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
OPY 7	675	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
OPY 7	540	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
OPY 7	480	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
OPY 9	1956	■	□	□	□	■	■	■	□	■	■	□	□	■	■	■
OPY 9	1584	■	□	□	□	■	■	□	□	■	■	□	□	■	■	□
OPY 9	1400	■	□	■	□	■	■	■	□	■	■	□	■	■	■	■
OPY 9	1093	■	□	■	■	■	■	■	■	■	■	■	■	■	■	■
OPY 9	658	■	□	□	■	□	□	□	□	■	■	□	■	■	□	■
OPY 9	578	□	□	□	□	■	■	□	□	■	■	□	□	□	□	□
OPY 9	430	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
OPY 15	3280	■	□	■	□	□	□	□	□	□	□	□	□	□	■	■
OPY 15	1760	■	■	■	■	■	■	□	□	□	■	■	■	■	■	■
OPY 15	1524	■	■	■	■	■	■	□	□	□	■	■	■	■	■	■
OPY 15	1250	■	■	■	■	■	■	□	■	□	■	■	■	■	■	■
OPY 15	1133	■	■	■	■	■	■	□	■	□	■	■	■	■	■	■
OPY 15	1043	□	□	□	□	■	■	□	□	□	□	□	■	□	■	■
OPY 15	880	■	■	■	■	■	■	□	■	□	■	■	■	■	■	■
OPY 15	756	■	■	■	■	■	■	■	■	□	■	■	■	■	■	■
OPY 15	620	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
OPY 15	480	■	■	■	■	■	■	□	■	□	■	■	■	■	■	■

■ Presence of band □ Absence of band

1. Anagha **2.** Sakthi **3.** Mukthi **4.** Hawaii 7998 **5.** LE-66 **6.** LE 20 **7.** LE-474 **8.** LE-1-2 **9.** BTH-102-1-2-2 **10.** BT-218 **11.** BT-1 **12.** BL-333-3-1 **13.** PKM-1 **14.** Arka Vikas **15.** Pusa Ruby

Fig. 17. Pictograph indicating the banding pattern developed with the primers OPY 7, OPY 9 and OPY15

band was absent in all the four susceptible genotypes. Hence, these bands obtained could be the markers contributing resistance to bacterial wilt disease. Though LE-20 and LE-1-2 were designated as moderately resistant and BTH 102-1-2-2 as moderately susceptible ones in the present study; these are included in the resistant genotypes at the respective centres from where these genotypes were released. Since RAPD polymorphisms are the result of either a nucleotide base change that alters the primer binding site, or an insertion or deletion within the amplified region, polymorphisms are usually noted by the presence or absence of an amplification product from a single locus (Williams *et al*., 1990).

Resolving power (Rp) was calculated for each of the 14 primers selected in the study. Resolving power provides a modest indication of the ability of the primers to distinguish between cultivars (Prevost and Wilkinson, 1999). The resolving power of the primers in the present study ranged from zero (OPS 5) to 4.54 (OPS 8 and OPY 9). This indicates that the primer OPS 8 and OPY 9 were most efficient in amplifying the genomic DNA. Nair (2005) has reported the use of resolving power to determine the value of primers in RAPD reaction.

5.3.2.3. RAPD Dendrogram analysis

Amplicons in the RAPD profiles of the 14 selected primers were scored as discrete variables using one to indicate the presence and zero to indicate the absence of amplicon. Pair wise similarities between the genotypes evaluated based on Jaccard's coefficient ranged between 5.71 and 9.14. As a whole, high levels of pair-wise similarity were obtained among the genotypes studied. Overall high levels of pair wise similarity and low value of mean marker diversity have been reported suggesting limited genetic variation in tomato cultivars grown in India. Similar reports also exist in tomato accessions from other regions of the world including both primary and secondary centres of diversity. Existence of very low genetic diversity within cultivated tomatoes could be attributed to self-pollination, artificial selection and founder effect (Archak *et al*., 2002).

It is important to note that the dendrogram constructed from the RAPD data using the fourteen selected primers revealed high similarity (50-88%) among most of the 15 genotypes selected for the study (Fig. 2). No distinct grouping was observed for the susceptible and resistant genotypes studied in relation to bacterial wilt.

The resistant genotype Hawaii7998 and the susceptible genotype Arka Vikas showed the maximum similarity of 88 per cent. This is mainly due to the unique banding pattern observed in these two genotypes with most of the OPS primers used in the study. All the resistant genotypes shared more than 70 per cent similarity between them. The minimum similarity (50%) was observed between the moderately resistant genotype LE-474 and moderately susceptible genotype BL-333-3-1. It was noted that the genotype BT-1 showed less than 65 per cent similarity with all other genotypes used in the study.

A clear demarcation between the genotypes with respect to resistance/susceptibility to bacterial wilt was not obtained with the primers screened. This indicates the need for more number of random primers and more sensitive assay systems to be exploited for discerning the genetic basis of disease reaction in tomato genotypes. Chandrashekhara *et al.* (2003) have also reported the high level of similarity (60-84 %) in tomato by the use of RAPD analysis. The lack of polymorphism in RAPD analysis between *Solanum torvum* accessions and difficulties for molecular characterization of cultivars in other diploid, autogamous Solanaceae species has been reported by Clain *et al.* (2004).

5.3.3. AFLP ASSAY

Amplified Fragment Length Polymorphism (AFLP) technology is a new technique for fingerprinting genomic DNA. DNA polymorphisms identified using AFLP are typically inherited in Mendelian fashion and can be extensively used for determining the identity of a specific DNA sample or to assess the relatedness

between samples or as the source of genetic markers to generate linkage maps or to identify molecular markers linked to phenotypic traits and genetic loci (Vos *et al*., 1995). This technique is able to detect small sequence variations and produce markers that are reliable and reproducible.

5.3.3.1. AFLP characterization of tomato genotypes

In the present study, AFLP analysis was carried out using five random combinations of *Eco*RI and *MseI* primer pairs. To prepare an AFLP template, the genomic DNA isolated from each of the five resistant and five susceptible genotypes were digested with two restriction endonucleases simultaneously, one having 6-bp recognition site (*Eco*RI) and other a 4-bp recognition site (*Mse*I). When used together, these enzymes generate small DNA fragments that will amplify well and are in the optimal size range (<1 kb) for separation on denaturing polyacrylamide gels (Savelkoul *et al.*, 1999). Due to primer design and amplification strategy, the *Eco*RI–*Mse*I fragments are preferentially amplified rather than *Eco*RI–*Eco*RI and *Mse*I–*Mse*I fragments. The AFLP primers are generally 17 to 21 nucleotides in length and anneal perfectly to their target sequences.

The five primer combinations evaluated in the study detected a total of 209 amplified fragments across all the genotypes among which 115 were polymorphic. The polymorphism observed was 55 per cent and thus AFLP assay was more effective in detecting genetic diversity among the genotypes. This effectiveness of the AFLP technique compared to other molecular techniques like RAPD, isozymes and RFLP may be due to a more efficient detection of single nucleotide changes at sites for restriction and selective amplification that can be easily resolved and detected in a denaturing poly acrylamide gel under appropriate conditions (He and Prakash, 1997).

Maximum number of DNA fragments (64) was amplified by E+AGC/M+CAA combination. The most important factor determining the number of restriction fragments is the number of selective nucleotides in the selective primers. In the present study, all the selective primers contained three selective nucleotides. The highest polymorphism of 80 per cent was observed with the primer combination E+AGC/M+CTT. Distinct polymorphism among the genotypes was observed with all the primer combinations, but it was not relevant with respect to disease reaction.

However, specific marker for resistance/susceptibility to bacterial wilt could be detected in E+ACG/M+CAC, E+AGG/M+CAA and E+AGC/M+CAA primer combinations. The resistant genotypes showed the absence of an amplicon of size ~90 bp with E+ACG/M+CAC primer combination while it was present in susceptible genotypes Pusa Ruby, BT-1 and PKM-1. The primer pair E+AGG/M+CAA provided a unique amplicon of size ~290 bp in all the susceptible genotypes.

5.3.3.2. Analysis of AFLP dendrogram

The pair-wise similarity matrix obtained on the basis of pooled data of AFLP profiles generated from the 10 genotypes using the selected five primer combinations revealed similarity values ranging between 0.65 and 0.87 (Table 15). Higher similarity values in AFLP assay indicate the high genetic uniformity among the genotypes studied. As in the case of RAPD assay, the AFLP data was also not good enough to demarcate resistant and susceptible genotypes. This was quite expected since the polymorphism scored in different primer combinations did not specify resistant or susceptible genotypes. However, the susceptible genotypes Arka vikas and Pusa Ruby were found to have 86 per cent similarity and and formed a unique cluster. Doganlar *et al*. (2002) have reported that AFLP markers might not be more polymorphic than RAPDs in *Solanum torvum* because of the strong homologies between the genomes of Solanaceae species. He also

reported that AFLP assay should improve resolution power by providing more scorable markers. In the present study, the least similarity was observed between the resistant genotype LE-66 and susceptible genotype Pusa Ruby.

The dendrogram obtained from the AFLP data clearly revealed that all the 10 genotypes selected for AFLP analysis were related to each other (Fig. 3). The resistant genotypes Sakthi and Mukthi, having similar pedigree, were found to have 84 per cent similarity. Another resistant genotype Anagha showed high similarity with Sakthi and Mukthi (81% and 84% respectively). The susceptible genotypes Pusa Ruby and Arka Vikas were joined together showing 83 per cent similarity. The moderately resistant genotype LE-474 stood as a distinct genotype and it showed above 81 per cent similarity with resistant genotypes Anagha and Mukthi. The susceptible genotype BL-333-3-1 and resistant genotype LE-66 formed a separate cluster. There was 86 per cent similarity between BT-1 and PKM-1, which showed susceptible reaction to bacterial wilt in pot culture screening. The least similarity value (65%) was shown by the genotypes LE-66 (resistant) and Pusa Ruby (susceptible).

5.3.4. TRANSFORMATION AND CLONING OF DNA

Transformation may be described as the stable, heritable uptake of exogenous DNA into a host cell. In order to undergo transformation, the cell must be competent and bacteria such as *E. coli* may be artificially induced to become competent. The success of transformation is measured in terms of transformation efficiency.

5.3.4.1. Preparation and screening of competent cells

Strict aseptic condition was maintained through out the preparation of competent cells, since contamination in competent cells can produce white colonies after blue-white screening. . The cells were made permeable to plasmid

DNA by chilling the cells in presence of divalent cations such as $CaCl_2$. The $CaCl_2$ affected affected the cell walls and might be responsible for binding of DNA to the cell surface (Old and Primrose, 1994). Competence of *E. coli* JM 109 cells was confirmed by transforming them with an uncut plasmid (pUC 18) containing ampicillin resistance marker. High frequency of transformation was obtained with the competent cells prepared (Plate 19a). The competent cells harbouring the plasmid alone could grow in ampicillin containing medium. If any other ampicillin resistant bacteria thrive in the transformation plate, they will give white colonies that could be misinterpreted as transformed cells.

5.3.4.2. Ligation of eluted DNA

The specific amplicon eluted from the resistant genotype (amplified with OPS 1 primer) was of ~1.5 kb size that could be conveniently ligated with pGEM-T (Promega) Easy Vector System I. 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 providing 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 or selection 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°C yields maximum number of transformants.

5.3.4.3. Transformation of DNA

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°C for a short period of 90 seconds. The treated bacteria are more likely to take up foreign DNA. The 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 contained polycloning sites inside the β-galactosidase encoding gene (*lac*Z). Insertion of a foreign sequence will disrupt the reading frame of β-galactosidase encoding gene. 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 plasmid were not capable of α-complementation and developed into white colonies.

5.3.5. CONFIRMATION OF RECOMBINATION

Plasmids isolated from white and blue colonies gave bands with different molecular weights. Plasmid DNA from the blue colony gave a band corresponding to 3 kb, which is the actual size of the plasmid. The plasmid from white colonies had higher molecular weight than the pGEM-T vector and the size was ~4.5 kb indicating the presence of insert (Plate 20a).

RAPD reaction with OPS 1 primer amplified fragments of expected size both in blue and white colonies (Plate 20b). This confirmed the presence of insert in the vector. Usually the PCR confirmation for recombination is done with specific primers. The presence of complimentary sequences for the RAPD primer can be the reason for positive result in the non-recombinants. Hence the recombination was further confirmed by restriction analysis.

Restriction analysis using *Eco*RI enzyme having recognition sequence on either side of the polycloning site could cut the insert from the vector. Two separate bands corresponding to the vector and the insert were obtained (Plate 20c) confirming the presence of insert in the vector.

5.3.6. SEQUENCING AND SEQUENCE ANALYSIS

5.3.6.1. 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. The sequence obtained after cloning, was subjected to vector screening to delete the sequences of vector, if any present. Vector screening of the sequence showed significant similarity with the pGEM-T Easy Vector which was used for cloning. Hence, the region showing similarity to the vector was deleted and only the insert sequence was retrieved. The actual insert sequence data obtained was of 138 bases.

5.3.6.2. Analysis of sequence data

Homology search through BLAST is a heuristic method to find the highest scoring locally optimal alignments between a query sequence and a database sequence (Altschul *et al*., 1997). The BLAST analysis can determine the sequence homology to predict the identity and function of the query sequence. It was interesting to note that the sequence (*Tomato seq 2*) obtained from the bacterial

wilt resistant tomato with the Operon primer OPS 1 displayed high homology to the genomic DNA sequence of tomato (*Lycoperison esculetum/Solanum lycopesicum*) in chromosome 11, 8 and 9 and the sequence for *RNse LE* for T-2 type RNase (Fig. 5). Thoquet *et al*. (1996) reported one QTL on chromosome 11 imparting field resistance to *Ralstonia solanacearum* in F_2 clones of Hawaii 7996 X Wva 700 cross. He also reported three more QTL, one on chromosome 6 and two on chromosome 4. Two putative new QTL were found on chromosomes 3 and 8. In addition, a weak putative QTL was detected on chromosome 10.

The *Tomato seq 2* showed significant identity with *RNase LER* gene in tomato. Tomato *RNase LE* is induced by phosphate deficiency and wounding and may play a role in macromolecular recycling as well as wound healing. Abel and Cock (1998) purified and characterized *RNase LE*, which are synthesized in remarkably high amounts in cultivated tomato cells. They reported that *RNase LE* is specific for single stranded RNA substrates and extra cellular space is the location for *RNase LE* function.

LeBrasseur *et al*. (2002) made an ivestigation to whether the RNases are involved in the wound response in *Arabidopsis thaliana*. They noticed that as in other plant systems, several activities are induced with various timings in damaged leaves, stems and seedlings in *Arabidopsis*, including at least three bifunctional nucleases, capable of degrading both RNA and DNA, as well as RNS1, a member of the ubiquitous RNase T_2 family of RNases. RNS1 is induced systemically after wounding, and preliminary results point to a role in defense against several different pathogens. In addition, RNS1 induction is independent of all known signals controlling wounding responses in Arabidopsis. The strong induction of *RNS1* is particularly interesting because it occurs both locally and systemically following wounding. The systemic induction of this RNase indicates that members of this family may be involved in defense mechanisms in addition to their previously hypothesized functions in nutrient recycling and remobilization. The presence of polymorphic band related to RNase of T-2 family

in the resistant genotypes in the present study is also indicative of its involvement in the defense.

Open reading frame is the part of protein coding gene and the longest ORF of the DNA sequence contains the protein-coding region. It starts with an initiation codon and ends with a termination codon (Old and Primrose, 1994). When the ORFs of the cloned sequence were examined, *Tomato seq 2* encoded the largest ORF (ORF_1) in +3 reading frame with a length of 136 bases and code for peptide of 46 amino acids. The second ORF (ORF_2) was encoded in –1 reading frame having a length of 111 bases coding for 36 amino acid residues. When the two ORFs displayed for *Tomato seq 2* were subjected to BLASTp tool, ORF_1 revealed high homology with AREB like protein (*Lycopersicon esculentum*) and ORF_2 with GRAS 4 protein (*Solanum lycopersicum*). These are also stress-related proteins reported in tomato.

When the *Tomato seq 2* was subjected to nucleotide statistics tool, it indicated high G + C content (Table 17) suggestive of eukaryotic DNA. High G + C content also indicate stability of the DNA sequence.

The deduced amino acid composition showed presence of both polar and non-polar amino acids. The hydrophobicity plot analysis delineated the hydrophobic character of the protein (Fig. 12). Regions with values below zero are hydrophilic in character and thus likely to be exposed on the surface of a folded protein.

In the present study, fifteen genotypes obtained from various centres were screened for resistance/susceptibility to bacterial wilt disease. These genotypes were characterized at molecular level using RAPD and AFLP assay to understand the genetic basis behind the resistance/susceptibility. The specific amplicon, which was found unique to the resistant genotypes, was eluted and cloned in pGEM-T Easy Vector and sequenced. The Blast search revealed high homology

with genomic DNA sequence and ribonuclease gene of tomato in the public database. Biochemical characterization and sequencing of *RNase LE* gene have already revealed its role in wound healing and defense mechanism. Efforts have to be made to obtain more sequence data through primer walking, and to isolate the full-length gene for further exploitation. Confirmation with more genotypes and development of SCAR marker based on the sequence data would be the next option for developing trait related marker for bacterial wilt resistance in tomato.

Summary

6. SUMMARY

An investigation on "Development of trait related markers for bacterial wilt resistance in tomato (*Solanum lycopersicum* L.)" was conducted at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara, Thrissur from October 2005 to September 2007. The main objectives of the study were characterization of selected tomato genotypes and detection and characterization of trait related markers with special reference to bacterial wilt. Fifteen tomato genotypes used for the study were obtained from different institutions in India.

The salient findings of the study are as follows:

1) The genotypes raised in pots containing sterile soil maintained in greenhouse (50% shade) and open conditions were subjected to artificial inoculation by both stem puncturing and soil drenching with wounding.

2) The genotypes raised in open condition showed symptoms of bacterial wilt within 3 days. Bacterial ooze test was performed to confirm the infection by *Ralstonia solanacearum*. However plants maintained and inoculated under greenhouse condition failed to take infection.

3) Based on percentage of wilt incidence, the genotypes Anagha, Sakthi, Mukthi, Hawaii 7998 and LE-66 were grouped into resistant category. The genotypes LE-474, LE-20 and LE-1-2 were found to be moderately resistant. The genotypes BTH-102-1-2-2, BT-218 and BT-1 were moderately susceptible. The genotypes Pusa Ruby, Arka Vikas, PKM-1 and BL-333-3-1 were found to be susceptible to bacterial wilt.

4) The genotypes were further characterized at molecular level using RAPD and AFLP markers. Genomic DNA was isolated from the fresh tender tomato leaves collected early in the morning.

5) The protocols suggested by Doyle and Doyle (1987) and Rogers and Bendich (1994) were tried for DNA isolation. The latter method was found to give discrete DNA bands with lesser RNA contamination. The quality and quantity of DNA isolated was estimated by spectrophotometric analysis and the values obtained indicated good yield and purity of DNA.

6) DNA preparations were treated with RNase and this resulted in DNA with no impurities and very suitable for RAPD and AFLP analysis.

7) Forty random decamer primers from two Operon series (OPS and OPY) were screened for RAPD assay. Out of these, 35 primers with good amplification were selected.

8) RAPD assay of fifteen tomato genotypes was performed with the selected 35 primers. Out of these, 14 primers yielded clear and reproducible bands in genotype characterization. The selected 14 primers generated a total of 110 RAPD amplified fragments. The percentage of polymorphism ranged between 43 and 90 per cent.

9) Resolving power of the random primers was calculated as per Prevost and Wilkinson (1999). Highest resolving power was obtained for the primer OPS 8 and OPY 9(4.54) and lowest resolving power was obtained for the primer OPS 5 (zero).

10) The RAPD amplification patterns were scored and the pooled data was analysed using NTSYSpc (version 2.0) software to determine the genetic similarity among the genotypes. A dendrogram was constructed based on

Jaccard's coefficient and UPGMA cluster analysis. The similarity value ranged from 0.50 to 0.88 per cent indicating relatedness among the genotypes. No clear demarcation was observed between the resistant and susceptible genotypes with respect to bacterial wilt disease.

11) RAPD assay could not identify the specific marker for resistance/susceptibility to bacterial wilt disease except with OPS 1 primer, which gave a unique band of size ~1.5 kb in resistant genotypes Sakthi, Mukthi and LE-66.

12) AFLP assay was carried out using the genomic DNA isolated from ten selected genotypes (5 each from resistant and susceptible group). Five different primer pairs (*Eco*RI and *Mse*I) were used for AFLP analysis.

13) AFLP assay could detect a total of 219 amplicons, out of which 115 were polymorphic. The average polymorphism per primer was found to be 23. Polymorphic banding pattern with respect to bacterial wilt was observed in the assay with E+ACG/M+CAC and E+AGC/M+CAA primer pairs.

14) The AFLP profile generated was scored and data was analysed using NTSYS software. Maximum similarity was observed between the resistant genotype Anagha and susceptible genotype PKM-1 (87%).

15) A dendrogram was constructed based on the pooled data of AFLP. Distinct grouping of resistant and susceptible genotypes were not obtained as in the case of RAPD dendrogram.

16) For elution of specific amplicon, RAPD assay with OPS 1 primer using template DNA from the resistant genotype Mukthi was carried out. The eluted DNA was pooled and reamplified to get an intense band.

17) The amplicon eluted was effectively ligated with pGEM-T Easy Vector and the product was used to transform the competent cells prepared. Transformant was screened through blue-white screening.

18) Plasmid was isolated from blue and white colonies and the electrophoretic profile confirmed the presence of insert in it. Further confirmation tests were done using RAPD amplification with the same primer (OPS 1) and restriction analysis.

19) Sequencing of cloned fragment with SP6 primer gave the sequence data for 681 bases and was named as *Tomato seq 1*. Vector editing was perfomed to remove the vector sequence from *Tomato seq 1* and the sequence of the insert alone (138 bases) was obtained. This sequence was named as *Tomato seq 2*.

20) The BLAST homology search revealed significant homology with genomic DNA of tomato chromosome 11, 9 and 8 and the sequence for *RNase LE* gene.

21) The cloned fragment had largest ORF of size 136 bases that coded 46 amino acids. The fragment was found to have high GC content (60.1%) and low AT content (39.9%).

22) Restriction analysis revealed that the fragment has restriction sites for frequent cutter *Alu*I and rare cutter *Not*I.

23) The major amino acids deduced from the sequence were Glycine, Proline and Threonine. Kite-Doolittle hydropathy plot showed that *Tomato seq 2* contained more number of hydrophobic amino acids.

24) The secondary structure predicted for *Tomato seq 2* contained extended strands, beta turns and random coils.

25) The sequence information from *Tomato seq 2* showed that they are counterparts of tomato chromosome 11, 8 and 9 and the sequence for *RNase LE* gene of tomato. This ribonuclease is induced by wounding and has role in defense mechanism.

References

REFERENCES

(Anonymous). 2003. March 27. Bacterial-wilt resistant tomato. *The Hindu.* 12p.

Abel, S. and Cock, M. 2001. Secretory acid ribonucleases from tomato, *Lycopersicon esculentum* Mill. *Methods in Enzymology*. 341: 351-368.

Abeygunawardena, D.V.W. and Siriwardena, A.A.P. 1963. Studies on resistance in tomato to bacterial wilt. *Trop. Agric.* 119: 55-66.

Acosta, J.C., Gilbert, J.C. and Quino, V.L. 1964. Heritability of bacterial wilt resistance in tomato. *Proc. Am. Soc. Hort. Sci*. 84: 455-462.

Addy, S.K., Das, G.C., Thakuria, D. and Rath, P.K. 1980. Studies on wilt of tomato. *J. Res. Assam agric. Univ.* 1:62-67.

Ahuja, M. and Waite, B.M. 1974. Field resistance of tomato to bacterial wilt during the rainy season in Elsalvado. *Proc. Am. Soc. Hort. Sci.* 94: 120-121.

Allen, C. 1997. Directions for future research on bacterial pathogenicity. *Reports of the second international bacterial wilt symposium,* Gosier, Guadeloupe, France, 22-27 June. INRA, Paris. pp.155-156.

Altschul, S.F., Thomas, L.M., Schaffer, A.A., Zhang, J., Zhang, S., Miller, W. and Lipman, D.J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25: 3389-3402.

Aragaki, M. and Quino, V.L. 1965. Bacterial wilt of ornamental gingers (Hedychium spp.) caused by *Pseudomonas solanacearum*. *Plant Disease Rep.* 49: 38-39.

Archak, J.L., Karihaloo, A. and Jain, A. 2002. RAPD markers reveal narrowing genetic base of Indian tomato cultivars. *Curr. Sci.* 82: 1139-1143.

Archak, J.L., Gaikwad, A.B., Gautam, D., Rao, E.V.V.B., Swamy, K.R. M. and Karihaloo, J.L. 2003. DNA fingerprinting of Indian cashew (*Anacardium occidentale* L.) varieties using RAPD and ISSR techniques. *Euphytica.* 230: 397-404.

Arnold, M.H. and Brown, S.J. 1968. Variation in the host-parasite relationship of a crop disease. *J. agric. Sci.* 71: 19-36.

Arumuganathan, K. and Earle, E.D. 1991. Nuclear DNA content of some important plant species. *Plant Mol. Biol. Rep*. 9: 208-218.

Ashrafuzzaman, H. and Islam, T. 1975. Bacterial wilt in tomato – a review. *Bangladesh Hort.* 3: 37-44.

Babu, T.P.H. 2000. RAPD analysis to assess the genetic stability in tissue culture derived black pepper (*Piper nigrum* L.) plants. MSc(Ag) thesis, Kerala Agricultural University, Thrissur, 83p.

Balatero, C.H., Hautea, D.M., Hanson, P.M. and Narciso, J.O. 2002. Development of molecular markers for marker-assisted breeding for bacterial wilt resistance in tomato. *Philipp. agric. Scientist.* 85(2): 170-181.

Birnboim, H.C. and Doly, J. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* 7: 1513-1523.

Bose, S.C. 1999. Screening and biochemical characterization of tomato genotypes for resistance to bacterial wilt. MSc(Hort) thesis, Kerala Agricultural University, Thrissur, 64p.

Buddenhagen, I.W., Sequeira, L. and Kelman, A. 1962. Designation of races of *Pseudomonas solanacearum*. *Phytopathology*. 52: 726.

Celine, V.A. 1981. Genetic cataloguing of tomato germplasm towards isolation of line(s) resistant to bacterial wilt. MSc(Hort) thesis, Kerala Agricultural University, Thrissur, 69p.

Chague, V., Mercier, J.C., Guenard, M., de Courcel, A. and Vedel, F. 1996. Identification and mapping on chromosome 9 of RAPD markers linked to *Sw-5* in tomato by bulked segregant analysis. *Theor. Appl. Genet.* 92: 1045-1051.

Chamber, J.N., Mistry, N.C. and Singh, B. 2006. Indian Horticulture Database-2005. National Horticulture Board, Gurgaon, 361p.

Chandrashekhara, A.C., Kulkarni, R.S. and Muniyappa, V. 2003. Genetic distance of four species of tomato as revealed by RAPD marker. *Indian J. Genet.* 63(3): 275-276.

Chawla, H.S. 2002. *Introduction to Plant Biotechnology*. Oxford and IBH Publishing Co. Pvt. Ltd., New Delhi, 538p.

Chellemi, D.O., Dankers, H.A. and Olson, S.M. 1994. Evaluating bacterial wilt-resistant tomato genotypes using a regional approach. *J. Am. Soc. Hort. Sci.* 119 (2): 325-329.

Chi, L.W., Fang, L.C., Wei, W.J., Lung, C.M., Mei, L.Y., Sun, Y.N., black, L., Green, S.K., Fen, W.J. and Ping, C.C. 2004. Transgenic tomato plants

expressing the Arabidopsis *NPR1* gene display enhanced resistance to a spectrum of fungal and bacterial diseases. *Transgenic Res.* 13 (6): 567-581.

Chuan, Y. 2006. Identification of AFLP markers linked to bacterial wilt resistance gene in tomato. *Theor. Appl. Genet*. 28(2): 195-199.

Chunwongse, J., Bunn, T.B., Crossman, C., Jian, J. and Tanksley, S.D. 1994. Chromosomal localization and molecular marker tagging of the powdery mildew resistance gene (*Lv*) in tomato. *Theo. Appl. Genet.* 89: 76-79.

Chupp, C. and Sherf, A.F. 1960. *Vegetable Diseases and their Control.* The Ronald Press Co., New York, 695p.

Clain, C., Da Silva, D., Fock, I., Vaniet, S., Carmeille, A., Gousset, C., Sihachakr, D., Luisetti, J., Kodja, H. and Besse, P. 2004. RAPD genetic homogeneity and high levels of bacterial wilt tolerance in *Solanum torvum* Sw. (Solanaceae) accessions from Reunion Island. *Plant Sci.* 166: 1533-1540.

Clark, J.M. 1998. Novel non-templated nucleotide addition reactions catalyzed by prokaryotic and eukaryotic DNA polymerases. *Nucleic Acids Res.* 16: 9677-9686.

Cohen, J.D., Chang, A.C.Y. and Hsu, L. 1972. Non chromosomal antibiotic resistance in bacteria: genetic transformation of *E.coli* by R factor DNA. *Proc. Nat. Acad. Sci. USA*. 69: 2110-2114.

Cook, D.R. and Sequeira, L. 1988. The Use of Restriction Fragment Length Polymorphism (RFLP) Analysis in Taxonomy and Diagnosis. *Bacterial Wilt Newsl.* 4: 4.

Danesh, D., Aarons, S., McGill, G.E. and Young, N.D. 1994. Genetic dissection of oligogenic resistance to bacterial wilt in tomato. *Mol. Plant Microbe Interact*. 7: 464-471.

Deberdta, P., Queneherveb, P., Darrassea, A. And priora, P. 1999. Increased susceptibility to bacterial wilt in tomatoes by nematode galling and the role of the *Mi* gene in resistance to nematodes and bacterial wilt. *Plant Pathol.* 48: 408-414.

Deslandes, L., Olivier, J., Theulieres, F., Hirsch, J., Feng, D.X., Bittner-Eddy, P., Beynon, J. and Marco, Y. 2002. resistance to Ralstonia solanacearum in Arabidopsis thaliana is conferred by the recessive RRS1-R gene, a member of a novel family of resistance genes. Proc. Natl. Acad. Sci. *99: 2404-2409.*

Doganlar, S., Frary, A., Daunay, R.N., Lester, S.D. and Tanksley, S.D. 2002. A comparative genetic linkage map of egg plant (*Solanum melongena*) and its implication for genome evolution in the Solanaceae. *Genet.* 161: 1697-1711.

Doyle, J.J. and Doyle, J.L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissues. *Phytochem. Bull.* 19: 11-15.

*Flor, H.H. 1971. Current status of the gene-for-gene concept. *Annu. Rev. Phytopathology*. 9: 275-296.

Gang, G., Ping, J.L., Yu, Q.D., Yong, L., Qin, Y.G. Yann, D.J. 2005. Identification of common AFLP markers linked with resistance to bacterial wilt in potato. *Acta Botanica Boreali Occidentalia Sinica*. 25 (2): 269-274.

Gang, G., Yu, Q.D., Yong, L., Ping, J.L. and Xiang, F.L. 2002. A RAPD marker linked to the potato resistance to bacterial wilt. *Acta Phytopathologica Sinica.* 32(3): 267-271.

Gilbert, J.C., Tanaka, J.S., and Takeda, K.Y. 1973. 'Kewalo' tomato. *HortSci.* 9: 481-482.

Goth, R.W., Peter, K.V. and Webb, R.E. 1983. Effect of root-knot nematode in bacterial wilt of tomato. *Phytopathology.* 73: 966.

Graham, K.M. and Yap, T.C. 1976. Studies on bacterial wilt 1: Inheritance of resistance to *Pseudomonas solanacearum* in tomato. *Malaysian agric. Res*. 5: 1-8.

Granada, G.A. and Sequeira, L. 1983. Survival of Pseudomonas solanacearum *in soil, rhizosphere and plant roots.* Canadian J. Microbiol. *29: 433-440.*

Hanson, P.M., Licardo, O., Hanudin, Wang, J.F. and Chen, J.T. 1998. Diallel analysis of bacterial wilt resistance in tomato derived from different sources. *Plant Disease*. 82: 74-78.

Hanudin. 1987. Evaluation of tomato cultivars to *P. solanacearum* (E.F. Smith) injection. *Hort. Abstr*. 59: 9122.

Hayward, A.C. 1964. Characteristics of *Pseudomonas solanacearum*. *J. Appl. Bact.* 27: 265-277.

Hayward, A.C. 2000. *Ralstonia solanacearum*. *Encyclopedia of Microbiol.* 4: 32-42.

He, G. and Prakash, C.S. 1997. Identification of polymorphic DNA markers in cultivated peanut (*Arachis hypogaea* L.). *Euphytica.* 97: 143-149.

He, H.Y., sequeira, L. and Kelman, A. 1983. Characteristics of strains of *Pseudomonas solanacearum* from China. *Plant Disease.* 67: 1357-1361.

Hedyathullah, S. and Saha, J.C. 1941. Bacterial wilt disease of tomato. *Sci. Cult.* 7: 226-227.

Henderson, W.R. and Jerkins, S.F. 1972. Venus and Saturn: two new tomato varieties combining desirable horticultural features with Southern bacterial wilt resistance. *Bull. Agric. Exp. Stn. North Caroline State Univ.* 444: 13-17.

Hildebrandt, A.C. 1950. Some important galls and wilts of plants and the inciting bacteria. *Biol. Rev.* 14: 259-272.

Horwitz, J.P., Chua, J., Curby, R.J., Tomson, A.J. and Fisher, B.E. 1964. Substrates for cytochemical demonstration of enzyme activity. *J. med. Chem*. 7: 574-576.

Husain, A. and Kelman, A. 1958. Relation of slime production to mechanisms of wilting and pathogenicity of *Pseudomonas solanacearum*. *Phytopathology*. 48: 155-165.

James, D. 2001. Molecular characterization of *Ralstonia solanacearum* (Smith) yabuuchi *et al.* causing bacterial wilt in solanaceous vegetables. MSc(Ag) thesis, Kerala Agricultural University, Thrissur, 104p.

Johal, G. and Briggs, S. 1992. Reductase activity encoded by the *HM1* disease resistance gene in maize. *Sci*. 258: 985-987.

KAU. 2002. *Package of Practices Recommendations Crops 2002.* Directorate of Extension, Kerala Agricultural University, Thrissur, 278p.

Kelaniyangoda, D.B., Heart, L.G. and hemachandra, B. 1999. Sources of bacterial wilt (*Ralstonia solanacearum* E.F. Smith) disease resistance in tomato. *Tropical Agriculturist*. 152: 19-29.

*Kelman, A. 1953. The bacterial wilt caused by *Pseudomonas solanacearum*. A literature review and bibliography. *North Carolina agric. Exp. Stn. tech. Bull*. 99: 194p.

*Kelman, A. 1954. The relationship of pathogenicity in *P. solanacearum* to colony appearance on a tetrazolium medium. *Phytopathology*. 44: 693-695.

Kelman, A. and Sequeira, L. 1965. Root-to-root spread of *Pseudomonas solanacearum. Phytopathology*. 55: 305-309.

Khan, A.N.A., Shetty, K.S. and Patil, R.B. 1979. Occurrence of bacterial wilt of chilli in Karnataka and its relationship to the wits of other solanaceous crops. *Phytopathology*.32: 507-511.

Klein-Lankhorst, R.M., Vermunt, A., Weide, R., Liharska, T. and Zabel, P. 1991. Isolation of molecular markers for tomato (*Lycopersicon esculentum*) using random amplified polymorphic DNA (RAPD). *Theor. Appl. Genet.* 83: 108-114.

*Krausz, J.P. and Thurston, H.D. 1975. Breakdown of resistance to *Pseudomonas solanacearum* in tomato. *Phytopathology*. 65: 1272-1274.

Kumar, V., Singh, B.M. and Sugha, S.K. 1993. Variation in isolates of *Pseudomonas solanacearum* from Himachal Pradesh. *Indian J. Mycol. Plant Pathol.* 23: 232-236.

Kurian, A. and Peter, K.V. 2001. Heterosis for quality traits in tomato. *J. Tropic. Agric.* 39: 13-16.

Kyte, J. and Doolittle, R.F. 1982. A simple method for displaying hydropathic character of a protein. *J. Mol. Biol*. 157: 105-132.

Langella, R., Ercolano, M.R., Monti, L.M., Frusciante, L. and Barone, A. 2004. Molecular marker assisted transfer of resistance to TSWV in tomato elite lines. *J. hort. Sci. Biotech.* 79(5): 806-810.

LeBrasseur, N.D., MacIntosh, G.C., Amador, M.A.P., Saitoh, M. and Green, P.M. 2002. Local and systemic wound-induction of RNase and nuclease activities in *Arabidopsis*: RNS1 as a marker for a JA-independent systemic signaling pathway. *The Plant J*. 29 (4): 393-403.

Lee, J.J., Coyne, D.P., Clemente, T.E. and Mitra, A. 2002. Partial resistance to bacterial wilt in transgenic tomato plants expressing antibacterial lactoferrin gene. *J. Am. Soc. hort. Sci.* 127 (2): 158-164.

*Libman, G., Leach, J.G. and Adams, R.E. 1964. Role of certain plant-parasitic nematodes in infection of tomatoes by *Pseudomonas solanacearum*. *Phytopathology*. 54: 151-156.

Lodhi, M.A., Ye, G.N., Weeden, N.F. and Reisch, B.I. 1994. A simple and efficient method for DNA extraction from grapevine cultivars, *Vitis* species and Ampelopsis. *Plant Mol. Biol. Rep.* 12(1): 6-13.

Majid, R.F. 2007. Genome mapping and molecular breeding in tomato. *int. J. Plant Genomics.* 2007: 1-52.

Mandel, M. and Higa, A. 1970. Calcium dependent bacteriophage DNA infection. *J. Mol. Biol*. 53: 159-162.

Martin, G. B., Brommonschenkel, S. H., Chunwongse, J., Frary, A., Ganal, M. W., Spivey, R., Wu, T., Earle, E. D. and Tanksley, S. D. 1993. Map based cloning of a protein kinase gene conferring disease resistance in tomato. *Sci.* 262: 1432-1436.

Martin, G., Vincente, C., Ganal, M., Miller, L., Tanksley, S.D., de Vincente, C., Nester, E.W. and Verma, D.P.S. 1992. Towards positional cloning of the pto bacterial resistance locus from tomato. *Advances in molecular genetics of plant-microbe interactions.* July 1992. International Symposium of Plant-Microbe Interactions, Seattle, Washington, USA. 14: 451-455.

Martin, G.B., Williams, J.G. K. and Tanksley, S.D. 1991. Rapid identification of markers linked to *Pseudomonas* resistance gene in tomato by using random primers and near isogenic lines. *Proc. Natl. Acad. Sci.*, USA. 88: 2336-2340.

Mathew, S. K., Girija, D., Devi, S.N., Sadhankumar, P.G. and Rajan, S. 2000. Variability in isolates of *Ralstonia solanacearum* affecting solanaceous vegetables in Kerala. *Veg. Sci.* 27: 189-191.

Mathew, S.K. 2001. Biocontrol of *Ralstonia solanacearum* E.F. Smith causing bacterial wilt in solanaceous vegetable crops, *ICAR Project Annu. Rep*., Kerala Agricultural University, Thrissur, 28p.

Menezes, C.B., Maluf, W.R., Faria, M.V., Nascimento, I.R. and Stevens, M.R. 2003. AFLP markers linked to spotted wilt virus resistant genes in tomato [abstract]. In: *Abstract, Plant and Animal Genomes XI Conference;* 11-15, January, 2003, California. 505p. Abstract No. 5.1.5.

Mew, T.W. and Ho, W.C. 1976. Varietal resistance to bacterial wilt in tomato. *Plant Disease Reporter*. 60: 264-268.

Micheli, M.R., Bova, R., Pascale, E. and Ambrosio, E.O. 1994. Reproducible DNA fingerprinting with the random amplified polymorphic DNA (RAPD) method. *Nucleic Acids Res.* 22: 1921-1922.

Miller, J.C. and Tanksley, S.D. 1990. RFLP analysis of phylogenetic relationships and genetic variation in the genus *Lycopersicon*. *Theor. Appl. Genet.* 80: 437-448.

Nair, S. 2005. Variability in chakkarakolli (*Gymnema sylvestre* R. BR.) using morphological, biochemical and molecular markers. M. Sc. (Ag.) thesis, Kerala Agricultural University, Thrissur, 125p.

Narayanankutty, C. 1985. Evaluation of a set of non-segregating and segregating populations of tomato for field resistance to bacterial wilt. MSc(Hort) thesis, Kerala Agricultural University, Thrissur, 70p.

Nesbit, K.A., Potts, B.M., Vaillancourt, R.E., Wests, A.K. and Reid, J.B. 1995. Partitioning and distribution of RAPD variation in a forest tree species, *Eucalyptus globules* (Myrtaceae). *J. Heredity*. 74: 628-637.

Nirmaladevi, S. 1987. Studies on genetic resistance to bacterial wilt (*Pseudomonas solanacearum* E.F. Smith) and root-knot nematode (*Meloidogyne incognita* Kofoid and White, 1919; Chitwood, 1949) in tomato (*Lycopersicon esculentum* Mill.). Ph.D. thesis, University of Agricultural Sciences, Bangalore, 122p.

Nishi, T., Tajima, T., Noguchi, S. Ajsaka, H. and Negishi, H. 2003. Identification of DNA markers of tobacco linked to bacterial wilt resistance. *Theor. Appl. Genet.* 106(4):765-70.

Old, R.W. and Primrose, S.B. 1994. *Priciples of Gene manipulation: An Introduction to Genetic engineering*. Oxford Blackwell Scientific Publications, London, 474p.

Ori, N., Eshed, Y., Paran, I., Presting, G., Aviv, D. Tanksley, s., Zamir, D. and Fluhr, R. 1997. The *I2C* family from the wilt disease resistance locus *I2* belongs to the nucleotide binding, leucine-rich repeat superfamily of plant resistance genes. *Plant Cell*. 9: 521-532.

Padmalatha, K. and Prasad, M.N.V. 2006. Optimization of DNA isolation and PCR protocol for RAPD analysis of selected medicinal and aromatic plants of conservation concern from peninsular India. *African J. Biotech.* 5(3): 230-234.

Paul, T.S. 1998. Biochemical and biological bases of resistance in solanaceous vegetables against bacterial wilt incited by *Ralstonia solanacearum* (Smith) Yabuuchi *et al*. Ph.D. thesis, Kerala Agricultural University, Thrissur, 278p.

Peter, K.V., Gopalakrishnan, T.R., Rajan, S. and Sadhankumar, P.G. 1992. *Breeding for resistance to bacterial wilt in tomato, egg plant and pepper*. (eds. Hartman, G.L. and Hayward, A.C.). Proceedings of an international conference held at Kaohsiung, Taiwan, 381p.

Prevost, A. and Wilkinson, M.J. 1999. A new system for comparing PCR primers applied to IISR fingerprinting of potato cultivars. *Theor. appl. Genet.* 98: 107-112.

Prior, P., Grimault, V. and Schmit, J. 1994. Resistance to bacterial wilt (*Pseudomonas solanacearum)* in tomato: Present status and prospects. In: (eds. Hayward, A.C. and Hartman, G.L.) Bacterial wilt: The

disease and its causative agent *Pseudomonas solanacearum*. CAB Interanational, Wallingford, England. pp.209-223.

Rajan, S. and Peter, K.V. 1986. Incomplete dominance of bacterial wilt resistance. *Tomato Gen. Coop. Rep.* 36: 24.

Rajput, S.G., Wable, K.J., Sharma, K.M., Kubde, P.D. and Mulay, S.A. 2006. Reproducibility testing of RAPD and SSR markers in tomato. *African J. Biotech.* 5(2): 108-112.

Ramachandran, C., Gopalakrishnan, P.K. and Peter, K.V. 1980. Evolving high yielding tomato varieties with resistance to bacterial wilt. Trichur, Kerala, *Annu. Rep. Kerala agric. Univ.* 181p.

Rani, P.R. 2000. Heterosis in bacterial wilt resistant tomato. MSc(Hort) thesis, Kerala Agricultural University, Thrissur, 62p.

Riccardia, L., Lottib, C., Stefano, P., Yuling, B., Pim, L. and Giovannia, C. 2007. Further isolation of AFLP and LMS markers for the mapping of the Ol-2 locus related to powdery mildew (*Oidium neolycopersici*) resistance in tomato (*Solanum lycopersicum* L.). *Plant Sci.* 172(4): 746-755.

Rick, C.M. and Chetelat, R.T. 1997. Past, present, and future prospects for using wild germplasm resources for tomato improvement. Proceedings of the 1st International Conference on the Processing Tomato. *Am. Soc. hort. Sci*. pp.145-149.

Rogers, S.O. and Bendich, A.J. 1994. Extraction of total cellular DNA from plants, algae and fungi. *Plant Mol. Biol. Manual.* 91: 1-18.

Rohlf, F.J. 1990. NTSYS-PC. Numerical taxonomy and multivariate analysis system. Exeter Software. *Euphytica.* 68: 69-76.

Sadashiva, A.T., Reddy, M.K., Singh, T.H., Balaram, M.V., Prasad, B.C.N., Prasanna, K.M., Satyanarayana, H.V. and Naveen, L.R. 2002. Breeding tomato (*Lycopersicon esculentum* Mill.) for combined resistance to bacterial wilt and tomato leaf curl virus. International Conference on Vegetables, 11-14 Nov., Bangalore. *Abstract.* 47p.

Sadhankumar, P.G. 1995. Incorporation of resistance to fruit cracking in a bacterial wilt resistant genetic background in tomato. Ph.D. thesis, Kerala Agricultural University, Thrissur, 146p.

Saliba-Columbani, V., Causse, M., Gervais, L. and Philouze, J. 2000. Efficiency of RFLP, RAPD and AFLP markers for the construction of an intraspecific map of the tomato genome. *Genome.* 43: 29-40.

Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual.* Second ed. Cold Spring Harbor Laboratory Press, New York, USA, 1322p.

Savelkoul, P.H.M., Aarts, H.J.M., de Haas, J., Dijkshoorn, L., Duim, B., Otsen, M., Rademaker, J.L.W., Schouls, L. and Lenstra, J.A. 1999. Amplified fragment length polymorphism analysis: the state of an art. *J. Clin. Microbiol.* 37(10): 3038-3091.

Schaub, I.O. and Baver, L.D. 1944. Research and farming (In) 67th *Rep. North Carolina Agric. Exp. Stn*. 4: 27-31.

Schiewater, B. and Ender, A. 1993. Different thermostable DNA polymerases may amplify different RAPD products. *Nuc. Acids Res.* 21: 4647-4648.

Scott, J.W., Somodi, G.C. and Jones, J.B. 1992.Comparison of inoculation techniques for screening tomato genotypes for bacterial wilt resistance.

(eds. Hartman, G.L. and Hayward, A.C.). Proceedings of an international conference held at Kaohsiung, Taiwan, 381p.

Sequeira, L. 1993. Bacterial wilt: Past, present and future. *ACIAR proceedings.* 45: 12-21.

Sharma, A.D., Gill, P.K. and Singh, P. 2002. DNA isolation from dry and fresh samples of polysaccharide-rich plants. *Plant Mol. Biol. Rep.* 20: 415a-415f.

Sharma, T.R. 2003. Molecular diagnosis and application of DNA markers in the management of fungal and bacterial plant diseases. *Indian J. Biotech.* 2: 99-109.

*Smith E.F. 1896. A bacterial disease of the tomato, egg plant and Irish potato (*Bacillus solanacearum* nov. sp.). *U.S. Dept. Agric. Div. Veg. Phys. and Pathol. Bull.* 12: 1-28.

Sneath, P.H.A. and Sokal, R.R. 1973. *Principles of Numerical Taxonomy.* W. H. Freeman and Co., San Fransisco, 359p.

Song, W.Y., Wang, G.L., Chen, L.L., Kim, H.S., Pi, L.Y., Holsten, T., Gardner, J., Wang, B., Zhai, W.X., Zhu, L.H., Fauquet, C. and Ronald, P. 1995. A receptor kinase-like protein encoded by the rice disease resistance gene *Xa*21. *Sci*. 270: 1804-1806.

Sonoda, R.M. and Augustine, J. 1978. Reaction of bacterial wilt-resistant tomato lines to *Pseudomonas solanacearum* in Florida. *Plant Disease Rep.* 62: 464-466.

Sreelathakumari, I. 1983. Incorporation of two main sources of bacterial wilt F1 generation of tomato (Lycopersicon esculentum (L.) Karst.). MSc(Hort) thesis, Kerala Agricultural University, Thrissur, 75p.

Staskawicz, B.J. 2001. Genetics of plant-pathogen interactions specifying plant disease resistance. *Plant Physiol.* 125: 73-76.

Staskawicz, B.J., Ausubel, F.M., Baker, B.J., Ellis, J.G. and Jones, J.D.G. 1995. Molecular genetics of plant disease resistance. *Sci.* 268: 661-667.

Swanepoel, A.E. and Young, B.W. 1988. Characteristics of South African strains of *Pseudomonas solanacearum*. *Plant Disease.* 72: 403-405.

Tanksley, S.D., Young, N.D., Paterson, A.H. and Boneirbale, M.W. 1989. RFLP mapping in plant breeding: a new tool for an old science. Biotech. *7: 257-264.*

Thamburaj, S. and Singh, N. 2001. *Textbook of Vegetables, Tuber Crops and Spice*, Directorate of information and publications of agriculture, IARI, New Delhi, 469p.

Thomas, C.M., Jones, D.A., Parniske, M., Harrison, K., Balint-Kurti, P.J., Hatzixanthis, K. and Jones, J.D.G. 1997. Characterization of the tomato Cf-4 *gene for resistance to* Cladosporium fulvum *identifies sequences that determine recognitional specificity in Cf-4 and Cf-9.* The Plant Cell. *9(12): 2209-2224.*

Thouquet, P., Olivier, J., Sperisen, C., Rogowsky, P., Laterrot, H. and Grimsley, N. 1996. Quantitative trait loci determining resistance to bacterial wilt in tomato cultivar Hawaii 7996. *Mol. Plant Microbe Interactions.* 9 (9): 826-836.

Thwaites, R., Eden-Green, S., Mansfield, J. and Seal, S. 1997. Studies on the molecular basis for pathogenecity and host specificity in strains of *Ralstonia solanacearum* pathogenic to banana. *Bacterial Wilt Disease*. (eds. Prior, P.H., Allen, C. and Elphinstone, J.). Springer Publications, New York. pp.197-199.

Tikoo, S.K., Anand, N. and Kishun, R. 1983. Presence of two independent genetic systems for resistance to bacterial wilt (*Pseudomonas solanacearum)* in tomato. *Proceedings of the 15th International Genetics Congress*. December, 1983. pp.12-23.

Tingey, S.V. and del Tufo, J.P. 1993. Genetic analysis with random amplified polymorphic DNA markers. *Plant Physiol.* 101: 349-352.

Ullmann, A., Jacob, F. and Monod, J. 1967. Characterization by *in vitro* complementation of a peptide corresponding to an operator-proximal segment of the β-galactosidase structural gene of *Eschersia coli*. *J. Mol. Biol*. 24: 339-345.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijter, A., Pot, J., Peleman, J., Kuiper, M. and Zabeau, M. 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res.* 23(21): 4407-4414.

*Walker, J.C. 1952. Disease of Vegetable Crops.1st ed. McGraw Hill Book Co., New York, 529p.

Wang, J.F. 1971. Basic protocols for conducting research on tomato bacterial wilt caused by *Pseudomonas solanacearum*. AVRDC. pp.23.

Wang, Y.F., Wang, M., Wang, D.Y. and Wang, L. 1998. Studies on heterosis in some processing tomatoes (*Lycopersicon esculentum* Mill.). *Acta Agricultura Shanga*. 14: 329-349.

Williams, J.G.K., Kubelisk, A.R., Livak, K.J., Rafalski, J.A. and Tingey, S.K. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* 18: 6531-6535.

*Winstead, N.N. and Kelman, A. 1952. Inoculation techniques for evaluating resistance to *Pseudomonas solanacearum*. *Phytopathology*. 42: 628-634.

Wolf, K., Schoen, E.D. and Peters-Van Rijin, J. 1993. Optimizing the generation of random amplified polymorphic DNAs in chrysanthemum. *Theor. Appl. Genet.* 86: 1033-1037.

*Yabuuchi, E., Kosako, Y., Yano, I., Hotta, H. and Nishiuchi, Y. 1995. Transfer of two *Burkholderia* and an *Alcaligenes* species to *Ralstonia gen. nov.* Proposal of *Ralstonia pickettii (*Ralston, Palleroni and Doudoroff, 1973). Comb. nov., *Ralstonia solanacearum* (Smith, 1896) Comb. Nov. and *Ralstonia eutropha* (Davis, 1969) Comb. nov. *Microbiol. Immunol.* 39: 897-904.

*Yabuuchi, E., Kosako, Y., Yano, I., Hotta, H., Hashimoto, Y., Ezaki, T. and Arakawa, M. 1992. Proposal of *Burkholderia* gen nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Pallermi and Holmes, 1981) Comb. nov. *Microbiol. Immunol.* 39: 1251-1255.

Yui, M., Monma, S., Hirai, M. Nishimura, S., Ukai, Y. and Enomoto, S. 1999. Random Amplified Polymorphic DNA (RAPD) markers for the

selection of tomatoes resistant to bacterial wilt. *Bull. Natl. Res. Inst. Veg., Ornamental Plants and Tea.* 14: 189-198.

Zhang, J. and McStewart, J.D. 2000. Economical and rapid method for extracting cotton genomic DNA. *J. Cotton Sci.* 4: 193-201.

(* Originals not seen)

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
Vertical electrophoresis system cell	Biorad Sequi-Gen® GT sequencing
Thermal cycler	Mastercycler personal, Eppendorf
Gel dryer	Biorad, Model 583
Gel documentation system	1. Alpha Imager 2. Phosphor Imager FLA-5100 Fuji

APPENDIX-II

Composition of Reagents Used for DNA Isolation

1. Doyle and Doyle method

4X Extraction Buffer

Sorbitol – 2.5g

Tris HCl – 4.8g

EDTA – 0.74g

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

Lysis buffer

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

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

NaCl (5M) – 40ml (29.22g per 100ml)

Distilled water – 20ml

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

5% Sarcosin

Sarcosin – 5g

Distilled water – 100ml

TE Buffer

10mM Tris (pH 8)

1mM EDTA (pH 8)

2. **Rogers and Bendich (CTAB) method**

2X CTAB Extraction Buffer

CTAB (2%, v/v)

100mM Tris buffer (pH 8)

20mM EDTA (pH 8)

1.4M NaCl

10% CTAB Solution

10% CTAB (w/v)

0.7M NaCl

TE Buffer

10mM Tris (pH 8)

10mM EDTA (pH 8)

APPENDIX-III

Composition of Buffers and Dyes used for gel electrophoresis

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

   242g Tris base

   57.1ml glacial acetic acid

   100ml 0.5M EDTA (pH 8.0)

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

   54g Tris base

   27.5g Boric acid

   20ml 0.5M EDTA (pH 8.0)

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

   0.25% bromophenol blue

   0.25% xylene cyanol

   30% glycerol in water

4. **Formamide Dye**

   Formamide – 10ml

   Xylene cyanol – 10mg

   Bromophenol blue – 10mg

   0.5M EDTA (pH 8.0) - 200µl

APPENDIX – IV

Composition of Reagents used for AFLP Reaction

1. **5X Reaction Buffer**

   50 mM Tris-HCl (pH 7.5)

   50 mM Mg-acetate

   250 mM K-acetate

2. ***Eco* RI/*Mse* I**

   1.25 units/µl each in 10 mM Tris-HCl (pH 7.5)

   50 mM NaCl

   0.1 mM EDTA

   1 mM DTT

   0.1 mg/ml BSA

   50% glycerol (v/v)

   0.1% Triton® X-100

3. **Adapter/ligation Solution**

   Eco RI/*Mse* I adapters

   0.4 mM ATP

   10 mM Tris-HCl (pH 7.5)

   10 mM Mg-acetate

   50 mM K-acetate

4. **T4 DNA Ligase**

   1 unit/µl in 10 mM Tris-HCl (pH 7.5)

   1 mM DTT

   50 mM KCl

   50% glycerol (v/v)

5. **TE Buffer**

   10 mM Tris-HCl (pH 8.0)

   0.1 mM EDTA

6. **5X Kinase Buffer**

   350 mM Tris-HCl (pH 7.6)

   50 mM $MgCl_2$

   500 mM KCl

   5 mM 2-mercaptoethanol

7. **T4 Kinase**

   10 units/µl in 50 mM Tris-HCl (pH 7.6)

   25 mM KCl

   1 mM 2-mercaptoethanol

   0.1 µM ATP

   50% glycerol (v/v)

8. **10X PCR Buffer plus Mg**

   200 mM Tris-HCl (pH 8.4)

   15 mM $MgCl_2$

   500 mM KCl

9. ***Eco*RI primers**

   27.8 ng/µl

10. ***Mse*I primers**

    6.7 ng/µl

    dNTPs

APPENDIX – V

Composition of reagents used for cloning and transformation studies

1. Luria Bertani (LB) broth

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

2. Luria Bertani Agar medium

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

2. Plasmid isolation solutions

a) Solution I (Resuspension buffer)

50 mM glucose

25 mM Tris

10 mM EDTA

b) Solution II (Lysis buffer)

2N 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)

DEVELOPMENT OF TRAIT RELATED MARKERS FOR BACTERIAL WILT RESISTANCE IN TOMATO (*Solanum lycopersicum* L.)

By

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(2005-11-137)

THESIS

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ABSTRACT

Tomato is one of the commercially important vegetable crops grown throughout the world, both for the fresh market and processed food industries. It is the second most important vegetable in India. Bacterial wilt caused by *Ralstonia solanacearum* (Smith) Yabuuchi *et al*. is a major constraint for tomato production in many tropical and sub-tropical regions. The area under tomato cultivation in Kerala is limited due to the incidence of this disease. The warm, humid tropical climate and acidic soil favours the bacterial wilt incidence in Kerala.

The crop loss estimated by bacterial wilt attack is to the extent of 90 per cent. Chemical control measures are not effective as the pathogen is soil-borne. The mechanism of bacterial wilt resistance is cryptic and mainly dependent on environment and strain of the pathogen. Molecular level approaches can provide the key to unravel this complex genetic disease and can help a long way in breeding resistant genotypes.

The study entitled "Development of trait related markers for bacterial wilt resistance in tomato (*Solanum lycopersicum* L.)" was undertaken at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara during the period 2005-2007 to characterize selected tomato genotypes and to detect markers linked to bacterial wilt resistance/susceptibility. Fifteen tomato genotypes obtained from various centers were raised under open condition and greenhouse condition in earthen pots containing sterile soil medium during the peak period of infection.

The seedlings were artificially inoculated to confirm the genotypic response to bacterial wilt. The greenhouse grown seedlings defended the infection. Out of the 15 genotypes evaluated, five (Anagha, Sakthi, Mukthi, Hawaii 7998 and LE-66) were resistant, three (LE-20, LE-474 and LE-1-2) were moderately resistant, three were moderately susceptible (BT-1, BT-218 and BTH-

102-1-2-2) and four (Pusa Ruby, Arka Vikas, BL-333-3-1 and PKM-1) were susceptible.

Further, the genotypes were subjected to molecular characterization using RAPD and AFLP assay. Genomic DNA was isolated by two protocols and the protocol suggested by Rogers and Bendich (1994) was found to be the ideal one for DNA isolation from tomato leaves.

Forty random decamer primers were screened for RAPD profiling and 35 of these were selected for genotype screening. Out of this, only 14 primers that displayed clear, reproducible bands were selected for further analysis. Almost all the primers yielded monomorphic banding pattern, except OPS 1, which yielded a specific fragment unique to resistant genotypes. The dendrogram obtained from the pooled data indicated high genetic similarity among the genotypes studied.

A more sensitive assay, AFLP was performed with five combinations of *Eco*RI and *Mse*I based primer pairs. This technique could display the genetic diversity among the genotypes in a better way compared to RAPD marker analysis. Two markers linked to bacterial wilt susceptibility were obtained using the primer combinations E+ACG/M+CAC and E+AGC/M+CAA.

The RAPD specific amplicon obtained in resistant genotypes, with the primer OPS 1 was eluted and cloned in pGEM-T Easy Vector and was transformed into *E. coli* JM 109 cells. The recombination efficiency was assessed through blue-white screening. Recombination of the insert was confirmed through RAPD reaction and restriction analysis. The cloned fragment was sequenced to obtain the nucleotide sequence information.

The sequence obtained after vector screening was named as *Tomato seq 2* was subjected to Blast search. It revealed significant levels of homology with genomic DNA of tomato in chromosome 11, 9 and 8, and the sequence for T-2

Type *RNase LER* gene of tomato deposited in the public domain. The sequence was also subjected to various sequence analyses using bioinformatics tools, which include ORF finder, SOPMA, NEB cutter, Hydropathy plot, NASTATS and AASTATS tools of Biology Workbench.

Scientists have already reported QTL conferring resistance to bacterial wilt on tomato chromosome 11 and 8. Moreover, the role of RNase LE in wound healing and defense mechanism is well established. So the future line of works should be focused on development of SCAR marker based on sequence data for its use as a trait related marker. Efforts are also to be made to isolate and characterize the full-length gene.