DEVELOPMENT OF MOLECULAR MARKERS LINKED TO YELLOW VEIN MOSAIC RESISTANCE IN OKRA

[*Abelmoschus esculentus* (L.) Moench]

By

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(2013-11-105)

THESIS

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DECLARATION

I hereby declare that the thesis entitled **"Development of molecular markers linked to yellow vein mosaic resistance in okra [*Abelmoschus esculentus* (L.) Moench]"** is a bonafide record of research work done by me during the course of research and that it has not been previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

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ABBREVIATIONS

%	Percentage
µg	Microgram
AFLP	Amplified Fragment Length Polymorphism
AP-PCR	Arbitrarily Primed Polymerase Chain Reaction
BF	Bio fertilizers
bp	Base pair
BSA	Bulk Segregant Analysis
cc	cubic centimetre
cm	Centimetre
CPBMB	Centre for Plant Biotechnology and Molecular Biology
CTAB	Cetyl Trimethyl Ammonium Bromide
DNA	Deoxyribonucleic Acid
DAF	DNA Amplification Fingerprinting
DNase	Deoxyribonuclease
dNTPs	Deoxyribo Nucleoside Triphosphate
EDTA	Ethylene Diamine Tetra Acetic acid
EMS	Ethyl methane sulphonate
EST	Expressed sequence tags
g	Gram
ha	Hectare
ISSR	Inter Simple Sequence Repeat
Kb	Kilo basepairs
L	Litre
M	Molar
mg	Milligram
ml	Millilitre
ME	Milling Energy
mM	Milli mole
NC	Neem Cake

ng	Nanogram
NAA	1-Naphthaleneacetic acid
NILs	Near Isogenic Lines
°C	Degree Celsius
OD	Optical Density
PCR	Polymerase Chain Reaction
pH	Hydrogen ion concentration
PIC	Polymorphic Information Content
pM	Pico molar
PVP	Poly vinyl pyrolidone
QTL	Quantitative Trait Loci
RAPD	Random Amplified Polymorphic DNA
RED	Recommended Dose Fertilizers
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
SCAR	Sequence Characterized Amplified Region
SCMV	Sugercane Mosaic Virus
SNP	Single Nucleotide Polymorphism
SSR	Simple Sequence Repeats
SSCP	Single Stranded Conformational Polymorphism
STS	Sequence Tagged Polymorphism
SVM	Soybean Mosaic Virus
TAE	Tris Acetate EDTA
TE	Tris EDTA
TYLCV	Tomato Yellow Leaf Curl Virus
TMV	Tomato Mosaic Virus
ToLCNDV	Tomato leaf curl New Delhi virus
TSWV	Tomato Spotted Wilt Virus
U	Unit

UV	Ultra violet
V	Volts
WBD	Witches Broom Disease
WSMV	Wheat streak mosaic virus
YVMV	Yellow vein mosaic virus
β	Beta
µl	Microlitre

Dedicated to
Lord Balaji,
my Amma
Appa
and Brother

Introduction

1. INTRODUCTION

Okra [*Abelmoschus esculentus* L. Moench], is a vegetable crop belonging to the family Malvaceae and originated in tropical Africa (Purseglove, 1987). It is an important crop which is widely cultivated in different parts of the world mostly for human consumption and also for industrial use as fibre (Alegbejo *et al.*, 2008).

India ranks first in the world with a production of 578400 tons (72% of the total world production) of okra from over 498000 ha (FAOSTAT, 2012). Andhra Pradesh is the leading okra producing state which has production of around 1184200 tons from an area of 78900 ha with a productivity of 15 tons/ha followed by West Bengal (862100 thousand tons from 74000 ha with 11.7 tons/ha productivity) (IIVR, 2013). Kerala produced 278 million tones of vegetables from 0.24 million ha in 2010-2011 (Agriinfo, 2011). It depends on the neighboring states for meeting a major share of its vegetable requirement. It is estimated that, about 60 per cent of the vegetable requirements of the state is met from outside sources and an amount of Rs.850 crores are spent yearly in this way (Gopalakrishnan, 1999).

Okra is highly susceptible to yellow vein mosaic virus, which causes a significant yield loss in okra. Infection of 100 per cent plants in a field is very usual and yield losses ranges from 50 per cent to 94 per cent depending on the stage of crop growth at which infection occurs (Sastry and Singh, 1974). Many agencies including private seed companies are working on various aspects of genetic improvement of okra against YVMV disease. White flies are the vectors for YVMV and hence, most of the work in this line is around the white flies' management. Newly emerging biotypes of whitefly, different virus strains and breakdown of resistance in okra varieties and hybrids were the major challenges (Sanwal *et al.*, 2014). Commercially available varieties and hybrids are highly susceptible to the YVMV disease (Deshmukh *et al.*, 2011). Conventional breeding in India has resulted in the development of resistant varieties such as Parbhani

Kranthi, Pusa Sawani, Arka Abhay, Arka Anamika, Varsha Uphar *etc.* The performance of these varieties is found to be highly region specific, often depending on the races of the virus prevailing. Thus it is necessarily to breed varieties highly resistant to YVMV.

Marker-assisted selection (MAS) provides opportunities for enhancing the response from selection because molecular markers can be applied at the seedling stage, with high precision and reductions in cost. MAS could be easily applied, but is often not necessary because the resistances are selected phenotypically. In quantitative disease resistances, MAS would be very useful, but the individual QTL often have small effects. Additionally, only a few monogenic resistances are durable and only a few QTL with high effects have been successfully transferred into elite breeding material (Farokhzadeh and Fakheri, 2014).

As of now, there is no marker system for the identification of YVMV resistance gene in okra. By considering the potential of molecular markers, an attempt was made to identify the RAPD (Random Amplified Polymorphic DNA) and ISSR (Inter Simple Sequence Repeats) markers linked with the YVMV resistance gene. RAPD is proven to be the best marker since they are simple, dominant, less technology intensive, cheap and does not require pre-sequencing for designing primers (Aladele *et al.,* 2008). As such, RAPD markers have been used widely in various genetic diversity studies of okra, including genetic mapping and identification of quantitative trait loci (QTLs) (Queiroz *et al.,* 2003; Brown *et al.,* 2005). ISSR targets the highly variable microsatellite regions of the nuclear genome providing a large number of polymorphic fragments (Gupta *et al.,* 1994). Therefore, this technique has been used extensively to evaluate genetic diversity both within and between plant populations in angiosperms and gymnosperms (Osborn *et al.*, 2005). The high level reproducibility of ISSR markers has already well established in many crops and hence these markers offer the potential for direct usage in MAS (Bornet and Branchard, 2001; Reddy *et al.*, 2002).

The present study was carried out with the objective to identify RAPD and ISSR markers linked with yellow vein mosaic virus resistance in okra [*Abelmoschus esculentus* L. Moench]. For this, Bulk Segregant Analysis (BSA) (Michelmore *et al.,* 1991) using the DNA from resistant variety Parbhani Kranthi, susceptible variety Salkeerthi and bulked DNA of resistant as well as susceptible F_2 plants from the cross Salkeerthi X Parbhani Kranthi were employed.

Review of Literature

2. REVIEW OF LITERATURE

The investigations on Development of molecular markers linked to yellow vein mosaic resistance in okra [*Abelmoschus esculentus* (L.) Moench] was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Kerala Agricultural University, Vellanikkara with the objective of identifying the molecular markers linked to YVMV resistance in okra.

A detailed review on the available literature in this line is presented below

2.1 Introduction

Okra [*Abelmoschus esculentus* (L.) Moench] Family: Malvaceae is an annual, herbaceous plant having erect growth habit and bisexual flowers. The cultivated okra has somatic chromosome number 2n = 130 and is considered to be an amphidiploid of *Abelmoschus tuberculatus* (2n = 58) and an unknown species with 2n=72 (Datta and Naug, 1968). Okra is one of the most important vegetable crops cultivated throughout the world. Less price and high consumer demand, makes farmers grow okra widely during rainy and summer seasons (Tiwari *et al.*, 2012). Okra was first found in former Abyssinia (present Ethiopia), and was later distributed to the Caribbean, South America, North America, Africa, India, and Eastern Mediterranea. Some of the important varieties of okra being grown in India are Pusa Makhmali, Pusa Sawani, IIHR 20-31, Pujab Padmini, Arka Anamika, Parbhani Kranti, Selection-2, Arka Abhey *etc.* For export purposes, mostly hybrid varieties are used (Indian Horticulture Database, 2011).

2.2 Botany and Taxonomy of Okra

Okra was previously included in the genus *Hibiscus*. Later, it was designated to *Abelmoschus*, which is distinguished from the genus *Hibiscus* by the characteristics of the calyx: spatulate, with five short teeth, connate to the corolla and caduceus after flowering (Terrell and Winters, 1974). Although about 50 species have been described, eight are most widely accepted (IBPGR, 1990).

There is significant variation in the chromosome numbers and ploidy levels in *Abelmoschus*. The lowest chromosome number known is $2n = 56$ for *A. Angulosus* (Ford, 1938) and the highest are close to 200 for *A. Caillei* (Siemonsma, 1982). Even within *A. esculentus*, chromosome numbers $2n = 72$, 108, 120, 132 and 144 are in regular series of polyploids with $n = 12$ (Datta and Naug, 1968).

The flowers are about 2 inches in diameter, with five white to yellow petals with a red or purple spot at the base of each petal. The flowers are almost actinomorphic. The perianth consisted of 5 sepals and 5 distinct petals. Calyx is completely fused as flower develops, splits longitudinally as flower opens and falls with corolla after anthesis. Pollen fertility is maximum in the period between one hour before and after opening of flower. It takes 2 to 6 hours for pollination and fertilization. Stigma is receptive at opening of flower and hence, bud pollination is not effective in okra (Padda, 1975).

Okra is cultivated for its fibrous fruits or pods containing round, white seeds and is mainly propagated by seeds. Okra plants are characterized by indeterminate growth. Flowering is continuous but highly dependent upon biotic and abiotic stresses. The okra pods are harvested when immature and high in mucilage, but before becoming highly fibrous. Generally the fibre production in the fruit starts from 6th day onwards of fruit formation and a sudden increase in fibre content from 9th day is observed (Nath, 1976).

2.3 Pollination

Venkitaramini (1952) observed that flower bud initiation, flowering, anthesis and stigma receptivity are influenced by genotype and climatic factors like temperature and humidity. From studies made on three okra varieties, flower buds are initiated at 22-26 days and the first flower opened 41-48 days after sowing. Once initiated, flowering continues for 40-60 days. Anthesis was

observed between 6 a.m. and 10 a.m. Anthers dehisce before flower opening, and hence self pollination may occur at anthesis. The dehiscence of anthers is transverse and complete dehiscence occurs in 5-10 minutes (Purewal and Randhawa, 1947). Stigma receptivity was also observed the day before flowering (50-70%) and the day after (1-15%). Flowers opened only once in the morning and closed after pollination on the same day and in the following morning, the corolla withered. Although insects are unnecessary for pollination and fertilization, the flowers are very attractive to bees and the plants are cross-pollinated. Cross pollination up to the extent of 4-19% (Shalaby, 1998) with maximum of 42.2% (Mitidieri and Vencovsky, 1998) has been reported.

2.4 Pests and diseases of okra

Although okra is considered a robust crop, under large-scale commercial production, yield losses are very high due to the incidence of a number of biotic and abiotic stresses. Okra is tolerant to most insect pests specifically during wet season because of the profuse growth of foliage, but diseases are common because of the wet–warm condition of the environment. However, during summer or second crop, whiteflies are the most damaging pest with occasional diseases. One of the major constraints identified in production is the increasing incidence of insect pests, diseases and nematodes, sometimes resulting in substantial yield losses.

Serious efforts to screen germplasm for viral resistance and utilization of resistance sources are pending. Several pests also cause serious damages on okra, such as the tomato fruit worm (TFW) (*Helicoverpa armigera*), the most destructive pest of okra. Powdery mildew and root rot are becoming serious problems. *Cercospora* leaf blight causes complete defoliation in seed crop with a yield loss of up to 43.6% (Pandey and Pandey, 2003). Jassids, mites and fruit borers infest okra plants during various growth stages of the crop and yield reduction to the extent of 54-66% has been reported due to de-saping by jassids if the crop remains unprotected (Satpathy *et al.*, 2004). In summer crop, mite causes

maximum yield loss of up to 25%. Shoot and fruit borer, infests okra both in the vegetative and fruiting stage of the crop and damage varies from 23-51% in various states of India (Satpathy and Rai, 1998).

2.4.1 Yellow vein mosaic virus disease

Viruses pose serious constraints to okra production. Okra is susceptible to at least 19 plant viruses (Brunt *et al.*, 1990; Swanson and Harrison 1993). These viruses severely affect okra production in terms of yield and fruit quality. Among the viruses yellow vein mosaic virus (YVMV) causes significant losses in the okra production. YVMV disease of okra was first reported in 1924 (Kulkarni, 1924) during the erstwhile Bombay Presidency in India. All cultivars and land races of okra succumb to YVMV, indicating the absence of resistance to this virus in *A. esculentus*. Several varieties exhibited tolerance/resistance to this virus at the time of release, but this tolerance or resistance has broken down with time (Kousalya, 2005).

2.4.2 Causal organisms

Okra yellow vein mosaic disease is caused by YVMV, which is a species of the genus Begomovirus, belongs to the family Geminiviridae (Fauquet and Stanley, 2005). Okra YVMV is believed to have originated in India (Usha, 2008). Viruses of the genus Begomovirus are transmitted by the ubiquitous whitefly *Bemisia tabaci* and are the most numerous and economically most destructive viruses among geminiviruses.

2.4.3 Disease symptoms

The viral nature of disease was first established by Uppal *et al.* (1940), who named it as yellow vein mosaic. Capoor and Verma (1950), described the symptomatology and host range. The characteristic symptoms of this disease are

homogenous interwoven network of yellow vein enclosing islands of green tissues within. Initially the leaves exhibit only yellow colored veins but in the later stages, the entire leaf turns completely yellow or at times fully cream colored. The chlorophyll content of the leaf is partially or completely destroyed. Infected plants remain stunted and produce very small leaves. Most of the affected plants develop thickening of veins on their lower sides. Hardly one or few fruits, often deformed, small, pale in color and tough in texture are produced on infected plants (Singh, 1990).

2.4.4 Assessment of losses

Sastry and Singh (1974) estimated that if the plants are infected within 20 days after germination the loss in yield was recorded up to 98 per cent. In plants that were infected at 35 and 50 days following germination, the loss was estimated to be 83 and 49 per cent respectively. Yellow vein mosaic causes severe damage in bendhi when the infection occurs in 30 days after sowing and is reported to occur all over the plants as well as in the lower hills of India (Arumugam and Muthukrishan, 1980). Sharma and Arora (1993) estimated the loss between 50-90 per cent because of yellow vein mosaic virus. Nath and Saikia (1993) also reported the relationship between crop age and yield losses caused by okra yellow vein mosaic virus; maximum 94.42 per cent and minimum yield losses 32.65 per cent were recorded for plants infected at 35 and 63 days after sowing. The disease infects at all the stages of crop growth and severely reduces growth and yield by 50 to 94 per cent (Aparna *et al.*, 2012).

2.4.5 Management of Yellow Vein Mosaic Virus

2.4.5.1 Screening for resistance to YVMV.

Naraini and Seth (1958) in their screening experiment for YVMV inferred that, *Hibiscus manihot* var. *pungens, H crinitus, H. vititolius*

and H. panduraetormis were immune. Sandhu *et al.* (1974) in their screening test found that accession E 31830, ' Asuntem Koko' from Ghana (*A. manihot* (L) Medicus ssp. *manihot)* was immune to YVMV. Raghupathy *et al.* (2000) screened 12 okra cultivars, including the highly susceptible Pusa sawani and MDU-1. The disease was absent in the highly resistant cultivars B0-1 and HRB-55. Resistant cultivars were KS-404, HRB 9-2, Hy.8, P-7, Parbhani Kranti, Sel-10 and Sel-4. BO-2 was susceptible and MDV-1 and Pusa Sawani recorded 90.83 and 91.53 per cent respectively.

Dhankhar (1996) developed YVMV resistant okra "Varsha Uphar" out of the cross between Lam Selection I x Parbhani Kranti. Fugro and Rajput (1999), using a partial diallel mating system involving nine genotypes, developed 36 F_1 hybrids, of which Sel-4 x Parbhani Kranti, Pusa Sawani x Punjab-7, Sel-4 x BO-1, Sel-4 x Punjab-7 and Sel-4 x Sel-10 were free from YVMV. Pathak *et al.* (1997) reported that the crosses Parbhani Kranti x HR-55 and Parbhani Kranti EC 16511 were found to be resistant to YVMV. Deo *et al.* (2000) found that Parbhani Kranti and its hybrid Parbhani Kranti x HRB-9-2 were highly resistant to YVMV. Rattan and Bindal (2000) in their programme to develop okra hybrids resistant to YVMV found that lines 407, 409, 417, 430 were completely resistant.

Prakash (1986) also studied the cross compatibility between *A. esculentus* and *A. manihot.* Wild species like *A. tetraphyllus, A. manihot* ssp. *tetraphyllus, A. ficulneus A. tnoschatus* and *Hibiscus huegetic* were resistant to yellow vein mosaic virus. Interspecific hybridization was done in the above resistant wild species with the locally adapted high yielding variety, Kiran. Normal fruits and seeds are produced in the cross combinations involving *A. tetraphyllus* and *A. manihot.* Among the cultivated varieties, Arka Anamika, Parbhani Kranti and Vijay F_1 hybrid were found promising in the southern region of Kerala.

Sheela (1994) attempted for combining the economic attributes of cultivars and yellow vein mosaic disease resistance of wild relatives. Varietal difference in compatibility of *A. esculentus* with the donor parents, *A. caillei* and *A. tetraphyllus* was noticed. Reciprocal crosses registered higher compatibility than the direct crosses. Natural crossing of *A. tetraphyllis* with *A. essculentus* and *A. caillei* was also observed. She further observed a higher proportion of low yielding YVMV resistant types similar to the wild types in F_2 and F_2 M_2 populations indicating strong genetic mechanisms preventing recombinations. However, more recombinants appeared in the F_2 M_2 generation indicating the breakage of undesirable linkage through irradiation.

Crop improvement by developing YVMV disease resistant varieties is more economical and environmentally safer than crop management using chemicals. Earlier efforts led to the development and release of cultivars like Pusa Sawani (Singh *et al.,* 1962) and MDU-1 with field resistance to YVMV. In a field trial with 22 genotypes exposed to whiteflies *(B. tabaci)* carrying okra yellow vein mosaic virus, cv. Arka Anamika remained free from disease and five other genotypes were highly resistant (Bora *et al.,* 1992). Nath and Saikia (1993) screened 14 okra varieties for resistance to okra yellow vein mosaic virus by artificial inoculation and natural infection. None of the entries were immune to the disease.

A comparative study of six resistant/tolerant *A. esculentus* varieties from various regions of India with Pusa Sawani during rabi 1987, kharif 1988 and kharif 1989 was conducted by Mathews *et al.* (1993) and they reported that the lowest incidence of Okra yellow vein mosaic virus was recorded in Sel 4 and Arka Anamika. Pusa Sawani and AROH- 1 were found to be the most susceptible. Interspecific hybridization followed by selection in the segregating generations is an effective method for obtaining YVMV resistant recombinants. Lowest level of virus infection was recorded for Punjab-7 and Parbhani Kranthi by Singh *et al.* (1993). Sharma *et al.* (1993) also confirmed the

resistance of Parbhani Kranthi to okra YVMV. Handa and Gupta (1993) inferred the tolerance nature of Parbhani Kranthi to YVMV of okra. Dhankhar (1996) reported that out of 20 parents and their 51 hybrids, only parent Parbhani Kranthi and 11 hybrids displayed high level of resistance to YVMV.

2.4.5.2 Genetics of resistance

The inheritance of this disease has been studied by number of research workers since 1962. There are contradictory reports with no definite conclusion for nature of resistance to yellow vein mosaic virus disease in okra, which may be due to complexity of the disease and more so on getting congenial environment for the establishment of its vector, the whitefly. Nevertheless, efforts are continuing to deter the spread of this deadly viral disease by involving wild relatives as a source of resistance at several institutions (Dhankar and Kumar, 2012).

Varma (1952) studied the relationship of YVMV and its vector white fly. Though a single insect was able to transmit the virus, the minimum number of flies required to produce 100 percent infection was about 10. The first visual symptom is the clearing of small veins, which usually starts at various points near the leaf margins in about 15 – 20 days after inoculation of plants. Affected plants flowers early and chemical control of the disease is difficult. Destruction of alternative hosts, control of white fly and other sucking insects and uprooting and burying of infected plants are some of the measures to reduce the vector population and also the diseased. Wild Okra species such as *A. pungens, A. crinitus, H. vitifolius, H. panduraeformis* are immune to this virus. During the last two decades several resistant varieties have been developed which are giving sustainable high yields in virus prone areas.

Two *Abelmoschus* species, viz., *A. manihot* (L.) Medik and *A. manihot* (L.) Medik ssp. *manihot*, resistant to Okra yellow vein mosaic (YVM) were crossed to *A. esculentus* cv. 'Pusa Sawani', a susceptible culture. The hybrids are

resistant and partially fertile. Segregation pattern for disease reaction in F_2, BC_1 and subsequent generations of the two crosses revealed that resistance to YVM is controlled by a single dominant gene in each species (Jambhale and Nekhar, 1981).

2.5 Molecular markers

In current scenario, the DNA markers have become the marker of choice for the study of crop genetic diversity has revolutionized the plant biotechnology. Techniques are being developed to more precisely, quickly and cheaply assess genetic variation. There is no single molecular approach for many of the problems facing gene bank managers, and many techniques complement each other. However, some techniques are clearly more appropriate than others for some specific applications like crop wise diversity and taxonomy studies (Kumar *et al.*, 2009).

With the use of molecular markers technique, it is now possible to hasten the transfer of desirable genes among varieties and to introgress novel genes from related wild species. Polygenic characters which were previously very difficult to analyse using traditional plant breeding methods, would now be easily tagged using molecular markers. It would also be possible to establish genetic relationships between sexually incompatible crop plants.

Techniques which are particularly promising in assisting selection for desirable characters involves the use of molecular markers such as random-amplified polymorphic DNAs (RAPDs), restriction fragment length polymorphisms (RFLPs), microsatellites and PCR-based DNA markers such as sequence characterized amplified regions (SCARs), sequence-tagged sites (STS) and inter-simple sequence repeat amplification (ISSR), amplified fragment length polymorphic DNAs (AFLPs) and amplicon length polymorphisms (ALPs) using F_2 and back-cross populations, near-isogenic lines, doubled haploids and recombinant inbred lines (Mohan *et al.*, 1996).

2.5.1 PCR- based molecular marker techniques

Applications of PCR based molecular markers include assessment of genetic variability, characterization of germplasm, identification and fingerprinting of genotypes, estimation of genetic distance, detection of monogenic and quantitative trait loci (QTL), marker assisted selection and in identification of resistant genes. The techniques used for cultivar identification are designed to detect the presence of specific DNA sequences or combination of sequences that uniquely identify the plant. In almost every major crop, molecular markers are associated with every character and genes of interests have been identified (Gurta *et al.*, 1999).

2.5.2 Polymerase Chain Reaction (PCR)

The Polymerase Chain Reaction (PCR) technique has been the basis of a growing range of new techniques for genome analysis, based on the selective amplification of genomic DNA fragments (Saiki *et al.*, 1988). Williams *et al.* (1990) reported the use of PCR with short oligonucleotide primers of arbitrary (random) sequence to generate markers, the basis of Random Amplified Polymorphic DNA (RAPD).

Welsch and McClelland (1990) also reported on Arbitrarily Primed Polymerase Chain Reaction (AP-PCR). DNA Amplification Fingerprinting (DAF) was also reported as another technique of PCR used in various experiments (Anolles *et al.*, 1991). The PCR reaction requires deoxynucleotides, DNA polymerase, primer, template and buffer containing magnesium (Taylor, 1991). Typical PCR amplification utilizes oligonucleotide primers which hybridize to complementary strands. The product of DNA synthesis of one primer serves as template for another primer. The PCR process requires repeated cycles of DNA denaturation, annealing and extension with DNA polymerase enzyme, leading to amplification of the target sequence. This results in an exponential increase in the

number of copies of the region amplified by the primer (Saiki *et al.*, 1988). The technique can be applied to detect polymorphism in various plants, animals, bacterial species and fungi.

The introduction of the PCR technique has revolutionized standard molecular techniques and has allowed for the proliferation of new tools for detecting DNA polymorphism (Hu and Quiros, 1991). The electrophoresis pattern of fragments generated by each primer for one isolate can be used as DNA fingerprints for assaying diversity (Tommerup *et al.*, 1998). Polymorphism between two individuals is generally scored as presence or absence (non-amplification) of a particular DNA fragment. The absence may result from deletion of a priming site or insertion rendering site too distant for successful amplification. Insertion can change the size of a DNA fragment without preventing its amplification (Williams *et al.*, 1990). The use of BSA in combination with PCR-based molecular markers has proven to be a very powerful technique for identifying markers tightly linked to or co segregating with, genes underlying monogenic traits [Agrama and Moussa (1996); Cho *et al.* (1996); Nakamura *et al.* (2001); Rostoks *et al.* (2002) and Shen *et al.* (2003)].

2.5.3 Molecular markers for identification of resistance genes

Identification of molecular markers linked to disease resistance genes facilitates marker-assisted selection (MAS) for achieving gene combinations in breeding programs (Sharp *et al.* 2001). Various molecular marker systems such as RFLP, RAPD, ISSR, AFLP and microsatellites (SSRs) have been widely used to tag resistance genes in all the crops.

2.5.3.1 Random Amplified Polymorphic DNAs (RAPD)

Martin *et al.* (1991) used RAPD marker system to identify the markers linked to the *pto* locus giving resistance to the bacteria *Pseudomonas syringae*

using the near isogenic lines in tomato. Similar marker system was used by Chalmers *et al.* (1992) to isolate markers linked to genetic factors contributing to the milling energy (ME) of barley grain. They applied RAPD marker system for bulked segregant analysis (BSA), on doubled haploids. Agrama and Scott (2006) used bulk segregant analysis to identify RAPD markers linked to TYLCV and TMV resistance. They revealed that the resistance against Tomato Yellow Leaf Curl Virus (TYLCV) and Tomato Mosaic Virus (ToMoV) were mainly examined by two QTL in two populations and one QTL in another. For all of the resistance QTL detected, the favourable allele was provided by the resistant parents. The presence of three different sources of TYLCV and ToMoV resistance; and the markers in tight linkage with them provide a means of systematically combining multiple resistance genes.

Naqvi *et al.* (1995) used the bulked segregant RAPD analysis for rapid identification of DNA markers linked to *Pi-10t* blast resistance gene in rice. They used Pooled DNA extracts from five homozygous blast-resistant (RR) and five susceptible (rr) BC_3 F_2 plants, derived from a CO39 × Tongil cross. Agwanda *et al.* (1997) identified the RAPD markers for resistance to coffee berry disease, *Colletotrìchuin kahawae*, in arabica coffee. Random amplified polymorphic DNA (RAPD) has proved useful in tagging resistance genes in a number of crops, including apples (Gianfranceschi *et al.*, 1994; Yang and Kruger, 1994), barley (Poulsen *et al.*, 1995), Brassica napus (Foisset *et al.*, 1995), rice (Nair *et al.*, 1995), sunflower (Mouzeyar *et al.*, 1995) and wheat (Talbert *et al.*, 1996). Sun *et al.* (1997) used 340 RAPD primers to identify the molecular markers linked to the *Yr15* stripe rust resistance gene of wheat. Six polymorphic bands among the susceptible and the resistant lines were identified. The genetic linkage of the polymorphic markers was tested on segregating F_2 population. Robert *et al.* (1999) identified molecular markers for the detection of the yellow rust resistance gene *Yr17* in wheat. They have used RAPD primers, for polymorphism, using a F_2 progeny of the cross between VPM1 (resistant) and Thesee (susceptible). They found that RAPD marker OP-Y15580 was closely linked to the *Yr17* gene.

Bulked segregant analysis (BSA) was used to identify four RAPD markers linked to Tomato Yellow Leaf Curl Virus resistance in 17.3cM region on chromosome 6 (Chague *et al.,* 1997). Also, BSA was applied in a recent study (Griffiths and Scott, 2000) to identify RAPD markers linked to Tomato Mosaic Virus resistance derived from *L. Chilense* accession LA1932. Sartorato *et al.* (2000) reported that the RAPD and SCAR markers linked resistance genes to angular leaf spot in common bean. They identified three RAPD markers in the F_2 population, OPN02890, OPAC142400 and OPE04650. These markers were mapped in coupling phase at 5.9, 6.6 and 11.8 cM from the resistant gene respectively. The OPN02890 fragment was transformed in to a SCAR marker. The polymorphism observed after amplification was identical to the one revealed with corresponding RAPD marker. Tullu *et al.* (2003) identified RAPD molecular markers linked to the anthracnose resistance gene in a recombinant inbred line (RIL) population developed from lentils, by a cross of eston lentil, the susceptible parent, and PI 320937 as resistant parent.

Hansen *et al.* (1997) carried out Bulked segregant analysis to identify RAPD markers in oilseed rape (*Brassica napus* L.) that were linked to a male fertility restorer gene for *Ogura* cytoplasmic male sterility. After screening 960 primers, 14 RAPD markers were mapped to a 25 cM region including the restorer locus, a mapping population of 242 F_2 individuals being employed. The map was used to select 11 markers that were investigated for polymorphisms between the restorer donor line and 46 recipient lines. A set of four RAPD markers, one in coupling phase with the restorer allele and three with the non-restorer allele, which were informative in all 46 combinations, were used in marker assisted selection of plants homozygous for the restorer allele. A total of 906 homozygous restored plants were found among the 4605 BC1F2 plants analysed. Phenotypic data of a subset of the classified plants was compared with the RAPD data and the expected number of recombinants was calculated from the map data. A close

correspondence between the expected and observed numbers of plants with a deviating phenotype was found.

Smiech *et al.* (2000) carried out RAPD analysis with the use of 271 primers. Out of 271 primers 28 generated stable polymorphism and so were tested for linkage to resistance gene against Tomato Spotted Wilt Virus (TSWV). Bulk segregant analysis (BSA) was applied to F_2 segregating progeny developed from resistant X susceptible parents. As a result, 5 primers enabling them to distinguish between resistant and susceptible forms were detected. Barua *et al.* (1993) found that RAPD markers are the most efficient and cost effective means of isolating molecular markers linked to genes located on introgressed DNA segments. They identified RAPD markers linked to a *Rhynchosporium secalis* resistance locus in barley through BSA on near-isogenic lines. Penner *et al.* (1993) observed ten polymorphic fragments between the bulks constructed for crown rust resistance but only one polymorphic DNA fragment was found to be tightly linked to the crown rust resistance gene. Similarly, Poulsen *et al.* (1995) identified a 2.7 kb RAPD marker, linked to leaf rust resistance gene in barley generated by the primer OPU-02. Results of earlier studies indicated that, the RAPD markers linked to trait of interest can easily be identified using bulked segregant analysis and may be used effectively breeding programmes.

Chen *et al.* (2004) carried out RAPD analysis between a near-isogenic line (NIL) Yr5/6 x Avocet S carrying the resistance gene *Yr5* against wheat stripe rust and its susceptible parent Avocet S, using the *Yr5* gene donor parent *Triticum spelta* album as control. Amplified product is separated on 4 per cent polyacrylamide gel electrophoresis. They suggested that using denaturing PAGE-silver staining could increase the level of DNA polymorphisms detected in wheat and also improve the repeatability of RAPD analysis. Kumar *et al.* (2006) screened 104 wheat recombinant inbred lines (RILs) from a cross between resistant parents (HD 29) and susceptible (WH 542) to karnal bunt (KB) disease

using random amplified polymorphic DNA (RAPD) markers. Marker OPM-20 showed apparent association with resistance to KB.

Poleg *et al.* (2000) performed Bulked Segregant Analysis to identify markers associated with genes conferring Zucchini Yellow Mosaic Virus resistance. One SSR marker was found to be tightly linked to the Zym-1 gene. A second SSR was found to be associated with another locus involved in ZYMV resistance. NILs were used to identify four RAPD markers associated with Cauliflower Mosaic Virus resistance. Araujo *et al.* (2002) used RAPD technique to identify molecular markers linked to blast resistance gene *Pi-ar* utilizing bulked segregant analysis in somaclone of rice cultivar araguaia, by using random primers for two parental DNAs from the resistant donor SC09 and 'Araguaia'. RAPD markers have been widely used as effective tools for the indirect selection of disease resistance genes once the linkages between markers and the resistance genes have been established (Haley *et al.*, 1993; Miklas *et al.*, 1993; Carvalho *et al.*, 1998; Castanheira *et al.*, 1999).

Malik *et al.* (2007) reported that PCR technique and bulked segregant analysis was used to identify DNA marker linked to leaf rust resistant gene in F_2 segregating population. The F_2 population derived from the cross involving leaf rust susceptible, SK-7 and resistant parent, PBG- 8881 with *Lr29* resistant gene was scored for disease resistance. The F_2 population segregated into resistant and susceptible plants in a ratio of 3:1 showed monogenic inheritance. The primer 60-5 amplified a polymorphic molecule of 1100 base pairs from the genomic DNA of resistant plant. This DNA molecule can be used as DNA marker to identify leaf rust resistant plants in a breeding programme for developing rust resistant wheat cultivars. The bulk segregant analysis technique was used to resistance gene (s) for CMV in 'Yamatouri' family. Two bulks were prepared, a resistant bulk consisting of DNA from 10 F_2 individuals (homozygous resistant plants) and a susceptible bulk consisting of DNA from 10 F_2 individuals (homozygous

susceptible plants). Another bulk DNAs from 10 BCs individuals (backcross with susceptible) were also included using RAPD markers (Daryono *et al.,* 2010).

Maiti *et al.* (2010) developed molecular markers linked to Mungbean Yellow Mosaic India Virus (MYMIV) resistance to facilitate genotyping of urdbean and mungbean germplasms for MYMIV-reaction. Resistance-linked molecular markers were successfully developed from consensus motifs of other resistance (R) gene or R gene homologue sequences. Applying linked marker assisted genotyping, plant breeders can carry out repeated genotyping throughout the growing season in absence of any disease incidence. Two MYMIV-resistance marker loci, YR4 and CYR1, were identified and of these two, CYR1 completely linked with MYMIV-resistant germplasms co-segregating with MYMIV-resistant F_2, F_3 progenies of urdbean. It was demonstrated that these two markers could be efficiently employed together in a multiplex-PCR reaction for genotyping both *V. mungo* and *V. Radiate* germplasms from field grown plants and also directly from the seed stock. This method of genotyping would save time and labour during the introgression of MYMIV-resistance through molecular breeding, as methods of phenotyping against begomoviruses are tedious, labour and time intensive.

2.5.3.2 Inter Simple Sequence Repeats (ISSR)

Inter-Simple Sequence Repeats (ISSRs) uses the microsatallite sequences directly for DNA amplifications using PCR. ISSR-PCR amplification use a single primer composed of a microsatallite sequence anchored at the 3′ or 5′ end by 2-4 arbitrary nucleotides (Fang and Roose, 1997). ISSR markers are universal, easy, repeatable useful to develop gene tagging and can be used for finding markers linked to the gene of interest. ISSR has been proposed as a new source of genetic markers which overcomes the technical limitations of RFLP and RAPD (Ratnaparkhe *et al.,* 1998).

The inter-simple sequence repeat (ISSR) PCR using primers based on dinucleotide, tetranucleotide or pentanucleotide repeats has now come into fashion among researchers (Zietkiewicz *et al.,* 1994). ISSRs have been used for cultivar identification in maize (Kantety *et al.,* 1995; Pejic *et al.,* 1998), potatoes (Prevost and Wilkinson 1999), trifoliate orange (Fang *et al.*, 1997), wheat (Nagaoka and Ogihara 1997), bean (Metais *et al.,* 2000) and *Diplotaxis* (Martin and Yelamo, 2000). The ISSR-directed approach in combination with bulked segregant analysis (BSA) showed wide application in plant and animal genome mapping. It can be extremely useful in: (1) identifying the markers at clusters of disease resistance gene, (2) filling large gaps in linkage maps, (3) developing sequence-tagged microsatallite sites and (4) providing marker enrichment at desired regions.

Fahmy (2006) used ISSRs-PCR technique to get molecular markers for blast resistance. Fifteen primers were used to obtain ISSRs markers for blast disease but only 12 succeeded in the DNA amplification. As a general conclusion, ISSRs are considered good molecular markers for blast disease study, especially when comparing parents, F1 and F2 individual plants (El-Sayed *et al.,* 2013). Marczewski *et al.* (2004) discovered that an ISSR marker UBC864600 was linked to the QTL *Plrv.4* for PLRV resistance. A pseudo-SCAR marker UBC864AC600 showed strong amplification in the resistant parent, but was absent in the susceptible parent, whereas another marker UBC864800 was linked to the susceptible allele.

Rai *et al.* (2013) identified PCR based markers linked to gene(s) confering resistance to Tomato leaf curl New Delhi virus (ToLCNDV). Their study covers the generation of a BC_1F_1 population derived from a reciprocal cross between a ToLCNDV tolerant accession of *Solanum habrochaites* LA1777 and susceptible cultivar 15SBSB (*Solanum lycopersicum* L.). Genetic analysis of 135 plants of BC_1F_1 population indicated that three dominant genes confer resistance to, ToLCNDV in the accession *S. habrochaites* LA1777. Using Bulk-Segregant-

Analysis, they identified an ISSR marker, which produced a 564 bp fragment in the tolerant wild accession and also in the tolerant bulk sample. The identified marker has been validated in a set of 18 diverse tomato accessions and can be used as a diagnostic marker to assist marker-assisted-breeding for ToLCNDV tolerance in tomato.

2.5.3.3 Restriction Fragment Length Polymorphism (RFLP)

A pooled sampling approach can rapidly identify restriction fragment length polymorphism (RFLP) or randomly amplified polymorphic DNA (RAPD) markers linked to disease resistance genes, as reported by Michelmore *et al.* (1991) for the *Dm5/8* locus in lettuce (*Lactuca sativa* L.) and McMullen *et al.* (1994) for the *wsm2* and *wsm3* loci in maize against MMV. Analysis of an F_2 population originating from the cross Pa405 and Oh28 revealed that three genes (Wsm1 on chromosome 6S near the RFLP marker umc85, Wsm2 on chromosome 3 near the RFLP marker umc102, and Wsm3 on chromosome 10 near the RFLP marker umc163) were involved in resistance to WSMV in maize (McMullen and Louie 1991; McMullen *et al.,* 1994). Borovkova *et al.* (1995) reported on the use of BSA analysis for the identification of several RAPD and RFLP markers linked to the stem rust resistance gene *rpg4* in barly. Recombinant inbred lines have been used for constructing molecular maps in maize (Burr and Burr 1991), *Arabidopsis* (Reiter *et al.,* 1992), and tomato (*Lycopersicon esculentum* Mill. (Paran *et al.,* 1995), as well as mapping disease resistance genes in rice (*Oriyza sativa* L.) (Wang *et al.,* 1994) and in maize (Kyetere *et al.,* 1995).

RFLP markers have served as tools in the identification of genes in many plants such as those for resistance to *Pseudomonas syringae*, tobacco mosaic virus and tomato spotted wilt virus (Martin *et al.,* 1991; Ohmori *et al.,* 1995; Stevens *et al.*, 1995). This method was developed by Michelmore *et al.* (1991) to map Dm genes of *Lactuca sativa* conferring resistance to *Bremia lactucae.* With this approach, DNA samples from susceptible or resistant plants from a segregating

population are bulked separately. The comparison of the bulks using RAPD or RFLP markers allows the identification of markers linked to the gene of interest. The target region is then tested against a random genetic background. Fang *et al.* (1998), evaluated resistance to citrus tristeza virus (CTV) in 554 progeny of 10 populations derived from *Poncirus trifoliata.* A dominant gene (*Ctv*) controlled CTV resistance in *P. trifoliata.* 21 dominant PCR-based DNA markers were identified as linked to *Ctv* by bulked segregant analysis. Resistance of European winter- barley germplasm rests mainly on the recessive *ym4* gene, which has been mapped on the long arm of chromosome 3H using RFLP and RAPD markers (Graner *et al.*, 1996; Weyen *et al.* 1996). However, this resistant gene is only effective against BaMMV and BaYMV-1 and has been overcome by a new strain, BaYMV-2 (Huth 1989). Evaluation of a large number of exotic accessions revealed various resistant lines displaying different specificity against individual members of the Barley Yellow Mosaic Virus complex (Goetz and Friedt, 1993; Ordon and Friedt, 1993).

Hanson *et al.* (2000) reported on mapping of a DNA fragment introgressed into cultivated tomato presumably from the wild species *L. hirsutum* Humb. and Bonpl. Which are found to be associated with TYLCV resistance. To locate introgressions of wild tomato alleles in TYLCV-resistant tomato line H24, its DNA was digested with six restriction enzymes and probed with 90 RFLP markers evenly spaced throughout the genome. Plants of a F_2 cross between H-24 and a susceptible tomato line were probed with randomly amplified polymorphic DNA (RFLP) markers linked to the targeted regions and F_3 families were developed by self pollination of F_2 plants that carried none, one, or both introgressions in either homozygous or heterozygous states. Results indicated that F_3 families homozygous for the introgression on chromosome 11 were resistant to TYLCV at both locations.

Brotman *et al.* (2005) studied in melon, that the Fom-1 gene confers monogenic resistance against the soil-borne fungus *Fusarium oxysporum* sp.

melonis, races 0 and 2, while the closely linked Prv gene specifies resistance against the papaya ring spot virus. Markers linked to these resistance (R) genes were identified using two recombinant inbred line populations, derived from crosses between *Cucumis melo* Vedrantais and *C. melo* PI 161375, and between *C. melo* Vedrantais and *C. melo* PI 414723, respectively. Using bulked segregant analysis, as well as systematic scoring of the mapping populations, they developed two amplified fragment length polymorphism markers, two random amplified polymorphic DNA markers and five restriction fragment length polymorphism (RFLP) markers linked to this locus. Four of the RFLP sequences bear homology to nucleotide-binding site–leucine-rich repeat R genes, indicating the presence of a significant R-gene cluster in this locus.

Polje *et al.* (2006) identified that chromosome regions carried potential QTLs for high grain yield in two synthetic maize populations - B73xMo17 and L1 xMo17 by bulk segregant analysis (BSA). Genome analysis of F_3 families was carried out with 58 RFLP markers. Allele frequency differences were detected at four RFLP loci in chromosomes 1, 2, 6 and 10 (B73xMo17). Only one region at chromosome 6 was identified in both populations, but with two different RFLP markers. Bulk segregant analysis was shown to be a quick and informative method for identification of chromosome regions which determine high yield expression in maize, i.e. for identification of RFLP markers closely linked to potential genes involved in expression of the trait.

2.5.3.4 Amplified Fragment Length Polymorphism (AFLP)

Bulked segregant analysis (BSA) has been proven to be very effective for identification of closely linked markers in target regions (Michelmore *et al.* 1991), especially combined with AFLPs (Ballvora *et al.* 1995 and Thomas *et al.* 1995). AFLP analysis is a genetic mapping technique that uses selective amplification of a subset of restriction enzyme-digested DNA fragments to generate a unique fingerprint for a particular genome. By using fluorescently based AFLP analysis,

Hartl *et al.* (1999) carried out the screening of a total 7654 DNA fragments for linkage to wheat powdery mildew resistance gene *Pm1c* in common wheat. Juergens *et al.* (2010) carried out three-years' field trials to get detailed information on the genetics of TuYV resistance derived from the re-synthesised *B. napus* line 'R54' and to develop closely linked markers. Bulked-segregant marker analysis for this resistance locus identified two closely linked SSR markers along with six closely linked and three co-segregating AFLP markers. Two AFLP markers were converted into co-dominant STS markers, facilitating efficient marker-based selection for TuYV resistance.

In wheat, AFLP markers for rust resistance, *Sr2* (Hayden *et al.*, 2004), *Sr39* (Gold *et al.*, 1999), *Lr19* (Prins *et al.*, 2001) and *Lr39* (Raupp *et al.*, 2001) were identified. Furthermore, there have been efforts to isolate and characterize leaf rust resistance genes by high resolution mapping for *Lr1* (Ling *et al.*, 2003) as well as map-based cloning for *Lr10* (Feuillet *et al.*, 2003) and *Lr21* (Huang *et al.*, 2003). Xinhua *et al.* (2009) reported that the use of an AFLP molecular marker linked to the TuMV resistance gene is an efficient method to improve pak-choi breeding. A dominant gene, *TuRBCH01*, has been mapped and used in resistant breeding. Amplified fragment length polymorphism (AFLP) technique and bulked segregant analysis (BSA) method were used to study the F_2 population. An AFLP marker (EccMctt3) linked to TuMV resistance gene with 7.8cM map distance was identified.

Cai *et al.* (2003) used a combination of the amplified fragment length polymorphism (AFLP) technique and bulked segregant analysis (BSA) in a large F_2 population in order to identify molecular markers linked to the *rhm* gene for resistance to Southern Corn Leaf Blight (SCLB). Ashutosh *et al.* (2007) identified the AFLP markers linked to the male fertility restorer gene of CMS *Brassica juncea* by using 64 primer combinations and converted it in to SCAR marker. They used BSA method to segregate the sterile and fertile lines. AFLP in conjunction with BSA has been successfully used to identify markers linked to

fertility restorer gene in petunia (Bentolila *et al.* 1998; Bentolila and Hanson, 2001), sorghum (Klein *et al.* 2001), radish (Imai *et al.* 2003; Koizuka *et al.* 2003; Murayama *et al.* 2003), rapeseed (Janeja *et al.* 2003), sunflower (Horn *et al.* 2003) and sugar beet (Touzet *et al.* 2004; Hagihara *et al.* 2005).

Shou *et al.* (2006) used AFLP analysis on two parents and their F_2 resistant and susceptible bulks for identification of markers linked to bacterial wilt resistance gene in tomato. They got a total of 4200 distinguishable amplified bands. 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. Brito *et al.* (2010) characterized the inheritance of coffee resistance gene(s) to race II of coffee leaf rust pathogen to identify and map AFLP molecular markers linked to the coffee leaf rust resistance trait. Using AFLP markers, Thakur *et al.* (2014) successfully identified markers linked with different alleles of the gene offering resistance to bacterial wilt disease in chilli. They used the BSA on F_2 population derived from a cross of NILs, susceptible Pusa Jwala and resistant Anugraha.

2.5.3.5 Simple Sequence Repeats (SSRs)

Microsatellites, or simple sequence repeats (SSRs), are stretches of DNA consisting of tandemly repeated short units of 1–6 base pairs in length. The uniqueness and the value of microsatellites arise from their multiallelic nature, codominant inheritance, relative abundance, extensive genome coverage and simple detection by PCR using unique primer, that flanks the microsatellite and hence the microsatellite locus (Powell *et al.*, 1996). Lokko *et al.* (2005) identified SSR and AFLP markers associated with resistance to CMD (Cassava Mosaic Virus) in a resistant landrace, using F_1 progenies derived from a cross between the CMD resistant landrace TME7 and the susceptible line TMS30555. Bulk segregant analysis (BSA) was performed using parents, resistant and susceptible DNA pools. An SSR marker, SSRY28-180, donated by the resistant parent was linked with resistance to CMD. Marker-trait association detected by regression

analysis showed that the marker, accounted for 57.41% of total phenotypic variation for resistance. The analysis further showed that another SSR marker, SSRY106-207 and an AFLP marker, E-ACC/M-CTC-225, accounted for 35.59% and 22.5% of the total phenotypic variation for resistance, respectively.

Bolek *et al.* (2005) developed molecular mapping of F_2 population derived from the interspecific cross of the highly tolerant *Gossypium barbadense* cv. Pima S-7 and the susceptible *G. hirsutum* cv. Acala 44 was phenotyped for disease incidence and severity.. The F_2 phenotypic distribution of these parameters (number of healthy leaves, node number, leaf weight, stem weight, and total shoot weight) suggested that resistance is polygenic inherited. Microsatellites were used to reveal polymorphism between resistant and susceptible parents. A total of 255 simple sequence repeat (SSR) primer pairs were screened over bulks constituted by 10 resistant and 10 susceptible progeny. Sixty markers were used to analyze quantitative trait loci (QTLs). Three loci (CM12, STS1, 3147-2) had large effect on resistance to Verticillium wilt. Two loci were located on LG-1 and one on LG-2 and both linkage groups are located on chromosome 11.

Lillemo *et al.* (2008) used SSR markers to identify molecular markers associated with the resistance to powdery mildew in wheat. Two major QTLs were identified, one on chromosome 7D and the other on chromosome 1B, corresponding to the adult plant rust resistance loci (*Lr34/Yr18* and *Lr46/Yr29)* respectively. Wu *et al.* (2014) identified and mapped powdery mildew resistance gene *VU-PM1* in the Chinese asparagus bean landrace ZN016 using SSR and SNP markers. The powdery mildew resistance gene was mapped to a locus on *LG9* flanked by SSR markers *CLM0305* and *CLM0260*, with a genetic distance of 2.0 cM and 5.0 cM. Gemenet *et al.* (2010) identified four candidate markers (p-umc2189, p-bnlg1179 and p-bnlg1014 and p-umc1542) using SSR molecular markers in F_2 population derived from a cross of drought susceptible and tolerant maize landraces using bulked segregant analysis (BSA).

Xu *et al.* (1999) studied RFLP, SSR and AFLP markers in maize for high resolution mapping of loci conferring resistance to sugarcane mosaic virus. Yu *et al.* (1993) used simple sequence repeats as genetic marker for the identification of the chromosomal location of *Rsv* gene which confer resistance to soybean mosaic virus (SVM). They found SSR marker SM176 which is a soybean heat shock protein gene closely linked to the *Rsv* gene with 0.5 cM distance. Using 50 SSR primers Mittal and Boora, (2005) tagged the gene for leaf blight resistance in sorghum; they used the resistant and susceptible bulked DNA samples for screening and found 38 primes giving polymorphism for leaf blight disease.

Kumbhar *et al.* (2013) developed F_2 population from a cross between rice (*Oryza sativa* L.) genotypes, 70 (highly susceptible to blast) and RDN 98-2-3-5-14 (resistant to blast), to study the inheritance of blast resistance and to identify the marker associated with resistance. The F_2 population segregated in 3:1 ratio for resistance: susceptible under hot spot conditions for blast suggesting monogenic control of resistance in this population. Bulk segregant analysis conducted using a total of 25 SSR markers identified two SSRs to be polymorphic between the parents and the corresponding bulks. One of these SSR markers (RM204) which has been reported to be mapped on the short arm of chromosome 6 and in close proximity of blast resistance gene/QTLs in other studies, showed expected segregation ratio (1:2:1) for single gene model in the F_2 population. This marker was found significantly associated with blast resistance on regression analysis.

2.5.4 Molecular markers in okra

2.5.4.1 AFLP markers in okra

Okra has received little attention with respect to its source of origin and genetic diversity, particularly at the molecular level. Phenotypic description (morphology, pod characteristics and seed germination) and AFLP (amplified fragment length polymorphism) analysis were performed on Greek and

international genotypes. Whereas morphological descriptors did not separate the accessions according to their geographical origin, AFLP analysis revealed a low level (12%) of polymorphism and distinct geographical groupings. Greek germplasm separated into three distinct groups with no overlap between them on the basis of molecular markers. A higher degree of genetic heterogeneity was found (UPGMA analysis) among the accessions of the Boyiatiou group than in the Pylaias group, whereas the occurrence of some common phylogenetic characteristics made separation on the basis of morphology alone difficult. The results from AFLP markers indicate that Greek germplasm constitutes a significant pool of variation with respect to morphological parameters, pod characteristics and seed germinability (Kyriakopouloua *et al.*, 2014).

Boonsirichai *et al.* (2006) studied gamma radiation-induced YVMD tolerant okra mutants and other commercial okra varieties at DNA level. They found that DNA extraction method that utilized sodium dodecyl sulphate (SDS) and potassium acetate to precipitate other biomolecules was a suitable method to use for DNA finger printing of okra. The MFLP finger printing technique was superior to the AFLP technique in finding polymorphisms among different okra varieties. Also polymorphisms between the YVMD-tolerant mutant lines and their parental variety could be detected, indicating that gamma radiation could induce some changes at DNA level in these plants.

2.5.4.2 RAPD markers in okra

There are few reports on molecular markers used in okra, and these have been limited to the use of random amplified polymorphic marker (RAPD) markers (Aladele *et al.*, 2008; Martinello *et al.*, 2001) and sequence related amplified polymorphism (SRAP) (Gulsen *et al.*, 2007) for cultivars and germplasm characterization. Simple sequence repeats (SSRs) developed for *Medicago truncatula* was found useful in okra cultivars (Sawadogo *et al.*, 2009). Haq *et al.* (2013) assessed thirty nine okra genotypes for genetic variability using Random

Amplified Polymorphic DNA (RAPD) markers. Twenty polymorphic RAPD primers amplified 111 DNA fragments, with an average of 5.5 fragments per primer. Among 39 okra genotypes, 107 fragments (96%) were found to be polymorphic. The UPGMA cluster analysis placed okra genotypes into seven main clusters. 'Sabzpari 2001' and 'Acc.No.019221' had shown maximum similarity (83%) while the minimum similarity (44.14%) was observed between the genotypes 'Punjab Selection' and 'Acc.No.019217'.

Kaur *et al.* (2013) used forty RAPD primers and eight quantitative traits for the assessment of genetic diversity and establishing phenotypic relationships in a set of 70 okra germplasm lines. Aladele *et al.* (2008) studied 93 accessions of okra which comprised of 50 West African genotypes (*Abelmoschus caillei*) and 43 Asian genotypes (*A. esculentus*) using RAPD markers. The molecular analysis showed that all the thirteen primers used revealed clear distinction between the two genotypes. Twenty-two genotypes of *Abelmoschus esculentus L.* Moench, including resistant varieties, hybrid varieties and susceptible varieties for YVMV infection, were collected and analyzed for genetic diversity using 20 random primers. The study showed the separation of individuals on the basis of their response towards YVMV infection with some exceptions. This study revealed that RAPD is effective, promising and informative for estimating the extent of genetic diversity as well as to determine the pattern of genetic relationships between different genotypes of *A. esculentus*. Nwangburuka *et al.* (2011) collected 29 okra accessions from different agro-ecological regions in Nigeria and analysed them using RAPD technique to access genetic variability. The amplification products of okra DNA assayed with OPM 18 and OPM 16 respectively revealed outstanding band patterns among the accessions signifying genetic differences amongst them.

2.5.4.3 SSR markers in Okra

Sawadogo *et al.* (2009) designed sixteen pairs of primers to amplify SSR regions of *Medicago truncatula,* same primers were used to amplify genomic

DNA samples of 20 different okra accessions collected from different regions Burkina Faso. These primers amplified a number of fragments that range from 1-16 with the size range between 396-506 bps. Each accession was scored for the presence or absence of the bands and phylogenetic analysis of this data clustered the 20 accessions into five different groups. Two okra accessions were distinctly different from other 18, based on molecular marker as well as morphological features of their fruits. One of the primers, MT-27 amplified a unique 440 bps PCR product of the 2 okra accessions. PCR product was sequenced and based on the sequence information, sequence specific primers were designed to PCR amplify the genomic DNA of all the okra accessions. Schafleitner (2013) mined okra transcriptome sequences for simple sequence repeat (SSR) markers. From 935 non-redundant SSR motifs identified in the unigene set, 199 were chosen for testing in a germplasm set, resulting in 161 polymorphic SSR markers. From this set, 19 markers were selected for a diversity analysis on 65 okra accessions comprising three different species, revealing 58 different genotypes and resulting in clustering of the accessions according to species and geographic origin.

2.5.4.4 ISSR markers in Okra

ISSRs are DNA fragments of about 100-3000 base pairs located between adjacent, oppositely oriented microsatellite regions. ISSRs are amplified by PCR using microsatellite core sequences as primers with a few selective nucleotides as anchors into the non-repeat adjacent regions (16-18 bp). About 10 fragments from multiple loci are generated simultaneously, separated by gel electrophoresis and scored by the presence or absence of fragments of particular size. ISSR primers are composed of microsatellite sequences either unanchored (Gupta *et al.*, 1994; Wu *et al.*, 1994) or anchored at the 5` or 3` end by two or four arbitrary nucleotides (Zietkiewicz *et al.*, 1994; Fang and Roose, 1997; Fang *et al.*, 1998). The sequence between the two binding sites in opposite orientation within suitable distance is amplified and loss or gains of binding sites are detected as band polymorphism (Yang and Meerow, 1996). The addition of a different base at the

5` or 3` end renders their binding sites more specific and reproducible (Barth *et al.*, 2002).

ISSR method has been used extensively to identify and determine relationships at the species and cultivar levels (Martins *et al.*, 2003). This method is widely applicable because it does not need sequence data for primer construction and is rapid, inexpensive and randomly distributed throughout the genome. The ISSR method has been reported to produce more complex marker patterns than the RAPD approach, which is advantageous when differentiating closely related cultivars (Parsons *et al.*, 1997; Chowdhury *et al.*, 2002). In addition, ISSR markers are more reproducible than RAPD markers (Goulao and Oliveira, 2001), because ISSR primers, designed to anneal to a microsatellite sequence, are longer than RAPD primers, allowing higher annealing temperatures to be used. Also, because of the multi locus fingerprinting profiles obtained, ISSR analysis can be applied in studies involving genetic identity, parentage, clone identification, strain identification and taxonomic studies of closely related species. In addition, ISSRs are considered useful in gene mapping studies (Godwin *et al.*, 1997). Genome analysis in woody perennials using ISSR has been shown possible in all crops (Charters *et al.*, 2000).

For acceleration of the breeding program for development of okra resistant genotypes using molecular approaches, help in detection of molecular markers related to resistance or susceptible genotypes and subsequent decrease of the period for development of new resistance cultivar in okra. Bulked Segregant Analysis (BSA) can be used to identify markers linked to a gene of interest. This process is highly efficient because it detects even a small percentage of polymorphisms in F_2 individual progeny tests. The use of BSA in combination with PCR-based molecular markers has proven to be a very powerful technique for identifying markers tightly linked to or cosegregating with, genes underlying monogenic traits [Agrama and Moussa 1996 ; Cho *et al.*, 1996 ; Nakamura *et al.*, 2001; Rostoks *et al.* 2002 and Shen *et al.*, 2003]. Inter-Simple Sequence Repeats

(ISSRs) are a new type of DNA markers, which involve the use of microsatallite sequences directly for DNA amplifications using PCR. ISSR-PCR amplification use a single primer composed of a microsatallite sequence anchored at the 3′ or 5′ end by 2-4 arbitrary nucleotides (Fang and Roose, 1997).

Yuan *et al.* (2014) employed Inter-simple sequence repeat (ISSR) markers to investigate the genetic diversity and differentiation of 24 okra genotypes. In this study, the PCR products were separated by electrophoresis on 8% nondenaturing polyacrylamide gel and visualized by silver staining. 22 ISSR primers produced 289 amplified DNA fragments, and 145 (50%) fragments were polymorphic. The 289 markers were used to construct the dendrogram based on the unweighted pair-group method with arithmetic average (UPGMA) cluster analysis. The dendrogram indicated that 24 okras were clustered into 4 geographically distinct groups. The average polymorphism information content (PIC) was 0.531929, which showed that the majority of primers were informative. The high values of allele frequency, genetic diversity, and heterozygosity showed that primer-sample combinations produced measurable fragments. The mean distances ranged from 0.045455 to 0.454545. The dendrogram indicated that the ISSR markers succeeded in distinguishing most of the 24 varieties in relation to their genetic backgrounds and geographical origins.

Younis *et al.* (2015) collected twenty nine okra accessions (*Abelmoschus esculentus L.*) from different locations, which were morphologically characterized. Significant differences were observed between some accessions for all quantitative characters studied while variations were detected and described for the qualitative characters. Forty two ISSR primers and five AFLP combinations were used to determine the level of polymorphism, molecular fingerprinting, and identification of unique markers and estimation of genetic distances for the 29 okra accessions. The ISSR primers amplified 508 fragments of which 415 were polymorphic. The number of positive and negative unique markers was 103 and was useful in identifying 24 genotypes out of the 29 accessions. Moreover, five

AFLP primer combinations yielded 449 amplicons, the total number of polymorphic amplicons was 439. Genetic similarity matrices estimated from ISSR and AFLP data, depicted that similarity coefficient ranged from 0.68 – 0.90 and 0.51-0.82 respectively.

2.6 Gene action

Only a few attempts have been made to study genetics of okra resistance/susceptibility to YVMV. There is no definite agreement for how to achieve inheritance of resistance to YVMV. Singh *et al.* (1962) found that two recessive alleles at two loci conferred resistance in inter varietal crosses of okra. Thakur (1976) implicated two complementary genes that govern resistance to YVMV under natural epiphytotic conditions. Dhillon (1978) agreed with these results and stated that additive component of variance was predominant compared to dominant gene effects. They also concluded that the genes governing YVMV resistance were influenced by environmental conditions and were temperature sensitive. There might be a possibility of polygenic control of YVMV in okra. Arumugam and Muthukrishnan (1980) and Jambhale and Nerkar (1981) revealed that resistance to YVMV was controlled by a single dominant gene. Sharma and Dhillon (1983) suggested that there are two complementary genes governing resistance to YVMV. Dhankar and Mishra (2005) confused the issue by reporting that inheritance of resistance to YVMV is under control of two complementary genes following Mendelian segregation.

Nerkar and Jambhale (1986) reported a single dominant gene for resistance to yellow vein mosaic virus in *A. manihot* and *A. manihot sp. manihot*, According to Irulappan (1991), additive genetic variance was important for virus incidence while dominance predominated for yield and individual fruit weight. Rajini and Manju (1999) also studied the gene action for okra and reported the presence of additive as well as dominant genes for the incidence of yellow vein mosaic.

Ali *et al.* (2000) also studied the inheritance of yellow vein mosaic virus and reported that the tolerance of YVMV in IPSA okra-I is quantitative with two possible major factors and dependent on gene dosage with incomplete dominant gene action. Inheritance of resistance to YVMV in okra was studied in crosses involving three resistances (Arka Anamka, Punjab Padmini, and Arka Abhay) and three susceptible parents (Pusa Sawani, Local and Pusa Makhamali). Inheritance patterns suggested that resistance to YVMV was controlled by two complementary dominant genes in susceptible X susceptible and susceptible X resistance crosses, where as in resistance X resistance crosses, two duplicate dominant genes were involved (Pullaiah *et al.,* 1998). Vashisht *et al.* (2001) studied the genetics of resistance to yellow vein mosaic virus, based on 9 generations derived from crosses involving resistance (Parbhani Kranthi) and susceptible (P-8, Punjab Padmini, Pusa Sawani and Pusa Makhamali). It was reported that additive gene effects were more significant than dominance gene effects.

Two *Abelmoschus* species, *A. manihot* (L.) Medik and *A. manihot* (L.) Medik ssp. *manihot,* resistant to YVMV were crossed to *A. esculentus* cv. Pusa Sawani, a susceptible variety. The hybrids were resistant and partially fertile. Segregation pattern for disease reaction in F_2, BC1 and subsequent generations of the two crosses revealed that resistance to YVMV is controlled by a single dominant gene in each species. Similarly in interspecific crosses between *A. manihot* and *A. tetraphyllus*, a single dominant gene controlled the resistance (Jambhale and Nerker 1981; Dutta 1984). The nature of inheritance for resistance to YVMV in okra in an interspecific cross of *A. esculentus* cv. Hisar Unnat and *A. manihot* (L.) Medikus ssp. manihot was studied and the resistance showed Mendelian segregation as per the condition governed by two complimentary dominant genes (Sharma and Sharma 1984 and Dhankhar and Mishra 2005). There is also a report that two recessive genes are responsible for the resistance in okra (Singh *et al.* 1962). *A. tuberculatus* (2n =58), *A. angulosus* (2n =138) and *A. manihot* (2n = 66) carry the virus without exhibiting symptoms. It was reported

that a variety of the cultivated species, IC-1542, also proved to be a 'symptomless' carrier (Nariani and Seth, 1958). Arora *et al.* (2008) conducted experiments with segregating generations of two YVMV resistant cultivars (Punjab-8 and Parbhani Kranti) and two susceptible cultivars (Pusa Sawani and Pusa Makhmali). The qualitative analysis for segregation of resistance and susceptible plants in F_2 and back cross generations indicated that the genes governing the resistance in different resistant parents were different and when these genes were brought together in the F_1 their effect was duplicated. In the crosses involving Resistant X Susceptible parents, the presence of single dominant gene controlling YVMV resistance was confirmed along with some minor genes.

Materials and Methods

3. MATERIALS AND METHODS

The study on "Development of molecular markers linked to yellow vein mosaic resistance in okra [*Abelmoschus esculentus* (L.) Moench]" was carried out at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Kerala Agricultural University during the period of 2013-2015. The materials used and methodologies adapted in the study are described below.

3.1 MATERIALS

3.1.1 Collection of seed material

Seeds of the two okra genotypes *viz.*, Parbhani Kranthi which is resistant to YVMV and Salkeerthi, which is susceptible to YVMV, were collected from MDKV (Marathwada Krishi Vidyapeeth) Parbhani, Maharastra and KAU (Kerala Agricultural University) respectively (Table 3.1). The F_2 progenies of the cross Salkeerthi X Parbhani Kranthi were used for screening against Yellow Vein Mosaic disease and for identifying the marker governing the resistance in okra, using RAPD and ISSR markers.

3.1.2 Source information on parental lines

The 'Parbhani Kranthi' has been developed by the MDKV by interspecific hybridization. It is derived from back cross of *Abelmoschus manihot* x *A. esculentus*. Two back crosses were made using Pusa Sawani (*A. esculentus*) as a recurrent parent followed by selection in the subsequent generations. It is a yellow vein mosaic virus (YVMV) resistant variety isolated in $BC2F_9$ generation. Plants flower in 45-60 days; fruits are dark green in colour, smooth and slender.

The popular *A. esculentus* variety 'Salkeerthi' developed by the Department of Olericulture, College of Horticulture, KAU, Vellanikkara, has a wide range of acceptability owing to its early bearing habit, excellent fruit quality, attractive light green pods and high yield, but it is highly susceptible to YVMV. Hence it cannot be grown during summer when the disease is more prevalent. An attempt is made to develop a mapping population to find the marker offering resistance to YVMV.

Table 3.1. Source information on parental lines

Sl. No	Information	Parent 1	Parent 2
1	Varietal name	Parbhani Kranthi	Salkeerhti
2	Acc. No.	AE-190	AE-202
3	Reported Chromosomal number	2n= 130	2n= 130
4	Crop duration	Annual type	Annual type
5	Pod colour	Dark green	Light green
6	Length of pod	9-10 cm	12-14 cm
7	Reaction to YVMV	Resistant	Susceptible
8	Number of plants selected for crossing	6 completely resistant plants	10 completely susceptible plants

3.1.3 Laboratory chemicals and glassware's

The chemicals used in this study were of AR grade procured from HIMEDIA, SRL, Merck India Ltd., and SISCO research Laboratories. The Taq DNA polymerase, Taq buffer and molecular weight marker (λ DNA */Hind III* + *Eco RI* double digest) were supplied by Banglore Genei. RNase A from Sigma,

USA was used. The plastic wares used for the study were purchased from Tarsons India Ltd. and Axygen, USA.

3.1.4 Equipment and machinery

The equipments available at the Centre for Plant Biotechnology and Molecular Biology, College of Horticulture were used for the study. Centrifugation was done in KUBOTA 6500 high speed cooling floor model centrifuge. Dai Ki-S1010 (Dai Ki Scientific Co.) was used for the incubation of the DNA at 4°C. The PCR was done in Applied Biosystem verti and Agilent SureCycler-8800, Thermocyclers and agarose gel electrophoresis was done in horizontal gel electrophoresis system (Bio-Rad). Gel DOC-It T^M Imaging system UVP (USA) was used for imaging the gel and gel pictures were analysed using Quantity One software (Bio-Rad). The list of laboratory equipments used for the study is provided in Annexure I.

3.2 METHODS

3.2.1 Development of mapping population

3.2.1.1 Field screening of parental lines for disease response

For identifying highly resistant and susceptible plants in the parental lines, field screening was carried out in CPBMB farm. Field screening of both the parents was done simultaneously in the same field in the summer months of February-June 2014. No artificial inoculation methods were followed since the heavy population of white flies observed in the summer months was sufficient to ensure the disease occurrence. There were no YVMV symptoms on both the lines till 35 days from the date of sowing and the symptoms started appearing from the

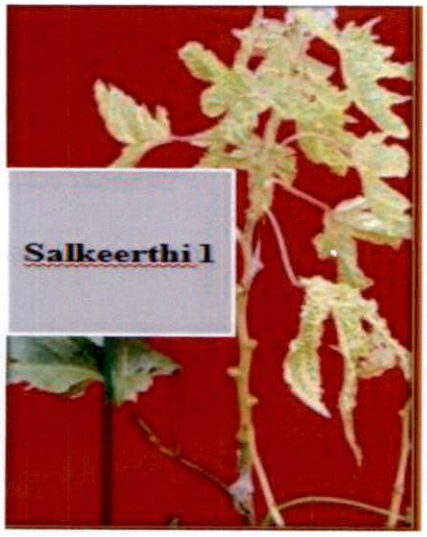
fifth week onwards. Initially, the vein clearing, followed by yellowing of leaves and gradual reduction in plant growth were observed in infected plants. Parbhani Kranthi plants which were supposed to be resistant also started showing symptoms. Those plants which were resistant till the end of the crop were only selected for the crossing programme. Leaf samples from the crossed plants were collected and high quality DNA was extracted and maintained. The parental genotypes, resultant F_1 and F_2 generations received timely management and care as per the package of practice recommendation of Kerala Agricultural University (2011).

3.2.1.2 Raising of F_1 populations

Hand emasculation was done between 4 and 6 p.m., one day before anthers dehisce. Anthers were removed carefully using a pair of forceps and the emasculated flowers were bagged. Next day morning, before 9 a.m., the butter paper bags were removed and pollination was done by the pollen of the bagged flower from the male parent. After pollination, the flowers were again bagged and properly tagged. The bags were removed three days after pollination. The seeds were harvested from the crossed plants and subsequently used for raising F_1. The raising of F_1 plants was carried out in the month of September- December 2014. 180 F_1 plants were raised in the CPBMB farm and morphological observations were recorded. All the F_1 plants were free from disease symptoms.

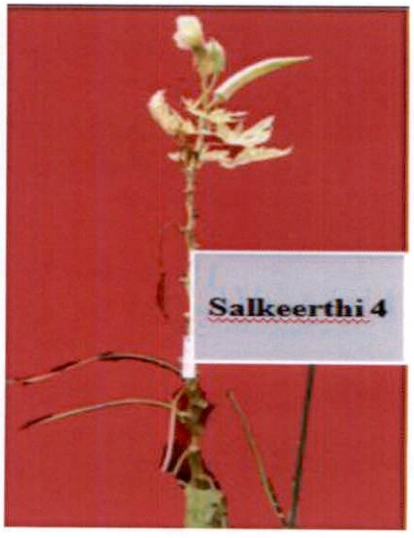
3.2.1.3 Raising of F_2 populations

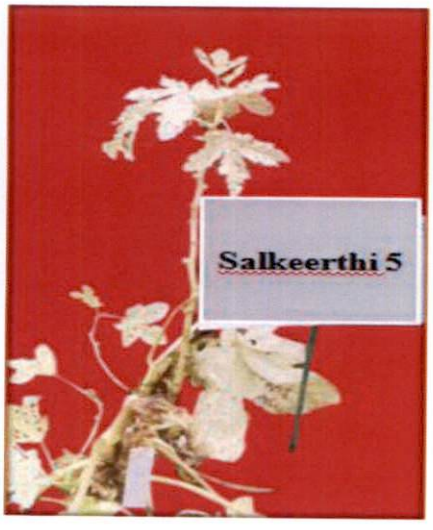
The F_1 plants were selfed to produce F_2 seeds. The F_2 populations were field screened during January-May 2015. 200 plants were screened to understand the disease response and the susceptible and resistant plants were identified. DNA from 7 susceptible and 7 resistant plants were isolated and bulked separately. This bulked DNA was used to perform Bulked Segregant Analysis (BSA). Identification of an ISSR or RAPD marker linked with the gene governing


Salkeerthi 1


Salkeerthi 2


Salkeerthi 3


Salkeerthi 4


Salkeerthi 5

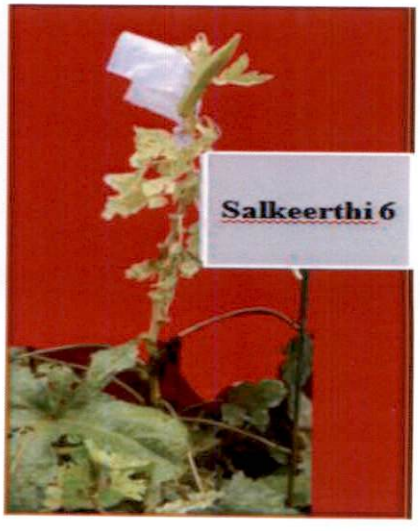

Salkeerthi 6

Plate 4.1: The highly susceptible Salkeerthi plants identified through the screening of parental lines. These lines were used as female parents in the crossing programme.

Plate 4.2: The highly resistant Parbhani Kranthi plants identified through the screening of parental lines. These lines were used as male parents in the crossing programme.

Plate 3.1: Field view of Salkeerthi plants screened to identify the susceptible ones.

Plate 3.2: Field view of Parbhani Kranthi plants screened to identify the resistant ones.

Plate 3.3: Field view of the F_2 plants screened to identify the YVMV resistant and susceptible ones.

resistance to the yellow vein mosaic virus disease in okra through bulked segregant analysis (to enable marker assisted selection) was done.

3.2.2 Morphological observations

Twenty plants were randomly selected from each parental species and F_1 hybrid for taking the observations. Eight quantitative and sixteen qualitative parameters were recorded and for the comparison of morphological characters, guidelines as per the International Plant Genetic Resources Institute (IPGRI, 2002) were followed. The characters observed were of the following:

1. **Plant growth habit:** Erect/Medium/Procumbent
2. **Stem:**

a) **Intensity of green colour:** Light/Medium/Dark

3. **Leaf**

a) **Size:** Small/medium/large

b) **Tip:** Pointed/blunt

c) **Vein colour:** Light green/green/dark green

4. **Flower**

a) **Petal colour:** Cream/Yellow/Purple

b) **Petal base colour:** Inside only/Both sides

c) **Diameter:** Small/Medium/Large

5. **Fruit**

a) **Immature fruit colour:** Yellowish green/Green /Dark green /Red

b) **Mature fruit colour:** Light green/Green/Light red/Red/Purple

c) **Surface between ridges:** Concave/Flat/Convex

d) **Shape of apex:** Narrow acute/Acute/Blunt

6. **Seed**

a) **Colour:** Green/Brown

b) **Hairiness:** Smooth/Hairy

c) **Seed shape:** Round/Depressed

7. **Plant height (cm)**

The height of the plant was measured from the base of the plant to tip at 90 days after sowing.

8. **Petiole length (cm)**

Length of petiole of seventh leaf of each plant was recorded at 60 days after sowing.

9. **Number of ridges per pod**

The number of ridges per pod from both the parents and their F_1 were recorded.

10. **Days to flower**

Number of days taken from the date of sowing to date of first flowering of the plants was recorded and expressed in mean values.

11. **Days to first harvest**

The number of days taken to harvest the first fruit in the observation plants in each genotype was taken and their average was taken to get the days to first harvest.

12. **Fruit length**

Three fruits were harvested from each observation plant at seven days after flowering and the fruit length was measured from basal cap to the tip of fruit.

13. Number of fruits per plant

The total number of fruits from each plant was harvested, counted and mean was worked out.

14. Yield per plant

The green fruit weight per plant of all pickings was recorded and the same was expressed in grams.

3.2.3 Scoring of okra populations for YVMV incidence

Observations on disease incidence and disease severity were recorded as per the approved methods. Disease severity was scored using 0-5 scale suggested by Deo *et al.*, 2000)

Grade	Per cent leaves infected
0	No symptoms
1	<25
2	25-50
3	51-75
4	76-90
5	>90

3.2.3.1 Disease assessment

Per cent disease incidence (PDI) was calculated using the formula given below:

$$\text{PDI} = \frac{\text{No. of plants infected}}{\text{Total no. of plants observed}} \times 100$$

Per cent disease severity (PDS) was calculated using the formula given below:

$$\textbf{PDS} = \frac{\text{Sum of all numerical ratings}}{\text{Total no. of plants observed}} \times \frac{100}{\text{maximum grade}}$$

Based on the per cent disease incidence and severity, coefficient of infection (CI.) was calculated as suggested by Dater and Mayee (1981)

$$(\text{CI}) = \frac{\text{Per cent disease incidence (PDI)} \times \text{Per cent disease severity}}{100}$$

Based on the Cl the genotypes were categorized into five categories as shown below:

CI	Category
0-4	Highly Resistant (HR)
4.1-9	Resistant (R)
9.1-19	Moderately Resistant (MR)
19.1-39	Moderately Susceptible (MS)
39.1-69	Susceptible (S)
69.1-100	Highly Susceptible (HS)

3.2.4 Molecular analysis

Molecular marker analysis was carried out on okra with two different marker system *viz.*, RAPD and ISSR.

3.2.4.1 Genomic DNA extraction

Fresh immature leaves were collected in the early morning hours, brought to laboratory in ice and used. The surface of the leaves was cleaned by washing with sterile water and wiped with 70 per cent ethanol and the leaves were kept at -80 ^{o}C till being used. CTAB method (Doyle and Doyle, 1987) was used for the extraction of genomic DNA.

3.2.4.1.1 Reagents used in DNA extraction

A. CTAB buffer (2X):

- 2 per cent CTAB (W/V)
- 100 mM Tris base (pH 8)
- 20 mM EDTA (pH 8)
- 1.4 M NaCl
- 1.0 per cent polyvinyl pyrrolidin (PVP)
- 0.2 per cent 2-β mercaptoethanol

B. chloroform: isoamyl alcohol (24:1 v/v)

C. Chilled isopropanol

D. Wash buffer

- 76 per cent ethyl alcohol
- 10 mM ammonium acetate

E. Ethanol 70 and 100 per cent

F. TE buffer:

- 10 mM Tris (pH 8)
- 1 mM EDTA (pH 8)

G. Sterile distilled water

Reagent A. and F. were autoclaved and stored at room temperature.

List of chemicals used in this study are given in Annexure II.

3.2.4.1.2 Procedure for total genomic DNA extraction

Preheated 5-7.5 ml of CTAB buffer (2X) was added in 50 ml Oakridge centrifuge tube to 60 °C in a water bath. 1.0 gm of leaf tissue was ground with a pinch of polyvinyl pyrrolidin (soluble) and 50µl of 2 per cent 2-β-mercaptoethanol in 60°C CTAB isolation buffer using a preheated mortar and pestle. The samples were incubated at 65°C for 30 (15-60) minutes with occasional gentle swirling. Equal volume of chloroform: isoamyl alcohol (24:1) mixture was added to the tube, mixed gently by inversion and centrifuged (Kubota 6500) at 12000 rpm for 20 minutes at room temperature. The contents got separated in to three distinct phases.

- Aqueous top layer - DNA with small quantity of RNA
- Middle layer - Protein and fine particles
- Lower layer - Chloroform, pigments and cell debris

The top aqueous layer was transferred to a sterile centrifuge tube, equal volume of chloroform: isoamylalcohol (24:1) was added and mixed by gentle inversions and subsequently centrifuged at 12,000 rpm for 20 minutes at room temperature. The aqueous phase was transferred into a clean centrifuge tube and 0.6 volume (3 ml) of chilled isopropanol was added and mixed by gentle inversions till the DNA got precipitated. These tubes were kept at -20°C for half an hour for complete precipitation. After the expiry of time, tubes were centrifuged at 12,000 rpm for 15 minutes at 4°C and the supernatant was gently poured off. The DNA pellet was washed with 20 ml of wash buffer (Ammonium acetate 5ml) with centrifugation at 1,000 rpm for 5 minutes. Then, the supernatant was carefully removed and again washed with 70 per cent ethanol and spun in tubes for 5 min at 10,000 rpm and the ethanol decanted. The pellet was air dried, dissolved in 50µl sterile distilled water and stored at -20 °C.

3.2.4.2 Assessing the quality of DNA by electrophoresis

The quality assessment of the isolated DNA was done by agarose gel electrophoresis (Sambrook *et al.,* 1989).

3.2.4.2.1 Reagents and Equipments

A. Agarose (Promega) 0.8 per cent (w/v)

B. 50X TAE buffer (pH 8.0)

- Tris base -242 g.

- Glacial acetic acid -57.1 ml.

- 0.5 mM EDTA -100 ml.

C. Tracking/loading dye (6X) – Bangalore Genei

- Bromophenol blue

- Glycerol

D. Ethidium bromide (SRL) (stock 10 mg/ml; working concentration 0.5 µg/ml)

E. Electrophoresis unit- Bio-Rad power PAC 1000, gel casting tray, comb

F. UV translluminator- (Herolab R)

G. Gel documentation and analysis system- BioRad Gel DOC-ItTM imaging system.

Composition of reagents is provided in Annexure III.

3.2.4.2.2 Procedure for agarose gel electrophoresis

The gel tray was prepared by sealing the ends with tape. Comb was placed in gel tray about 1 inch from one end of the tray and positioned vertically such that the teeth are about 1 to 2 mm above the surface of the tray. 0.8 per cent agarose was prepared in a glass beaker by dissolving 0.8 g agarose in 100 ml 1X TAE buffer (2 ml from 50X TAE buffer stock was made up to 100 ml with distilled water). The solution was microwaved for 60 seconds until the agarose got dissolved and solution become clear. Solution was allowed to cool for 45 ^{o}C under room conditions and at this point 5µl ethidium bromide from 10 mg/ml stock solution was added and mixed well. This warm gel solution was poured into the casting tray to a depth of 5 mm and the gel was allowed to solidify for about 30 minutes at room temperature. The comb and the tape used for sealing the gel tray were gently removed and the tray was placed in the electrophoresis chamber. The gel was covered with 1X TAE buffer till the wells are submerged. Samples for electrophoresis were prepared by adding 1 µl of 6X gel loading dye for every 5µl of DNA sample and mixing them well. 6µl of DNA and dye mixture was loaded per well. A suitable molecular weight marker (λ DNA *Eco*RI / *Hind* III double digest- Bangalore Genei) was also loaded in one lane. Electrophoresis was carried out at 70 V until the dye migrated two-third the length of the gel. The quality of the DNA was assessed based on the criteria that the intact DNA appeared as orange fluorescent bands. The degraded ones appeared as a smear because of the presence of a large number of DNA fragments which differed only in few bases. The presence of protein maybe observed as a thick white patch which will get restricted in the wells itself. The RNA contamination may be observed as thick band with size less than 100bp.

3.2.4.3 Gel documentation

The gel containing the electrophoresed DNA was viewed under UV transilluminator for the presence of DNA. The DNA was fluoresce under UV light

due to ethidium bromide dye. The image was documented on gel documentation system (BioRad Gel DOC-ItT^{M} imaging system). The gel profile was examined for intactness, clarity of DNA and contamination with RNA/Protein.

3.2.4.4 Purification of DNA by removing the RNA contamination

The DNA which had RNA as contaminant (as observed from the electrophoresis) was purified by RNase treatment and subsequent precipitation (Sambrook *et al.,* 1989).

3.2.4.4.1 Reagents used for RNase treatment

I. Chloroform: Isoamyl alcohol (24:1 v/v)

II. Chilled isopropanol (100 %)

III. 70 per cent ethanol

IV. 1 percent RNase

One per cent solution of RNase was prepared by dissolving RNase in TE buffer, at 100°C for 15 minutes to inactivate residual DNase. The solution was cooled to room temperature, dispensed into aliquots and stored at -20°C.

3.2.4.4.2 Procedure

For 50 µl DNA sample, 1 µl of 1 per cent RNase solution was added and incubated at 37°C in dry bath for 40 minutes. The total volume was made up to 250 µl with distilled water. Equal volume of chloroform: isoamyl alcohol (24:1) mixture was added and mixed gently. The contents were centrifuged at 12,000 rpm for 15 minutes at 4°C. The aqueous phase was transferred into a fresh micro centrifuge tube and equal volume of chloroform: isoamyl alcohol (24:1) was added and centrifuged at 12,000 rpm for 15 minutes at 4°C. The aqueous phase

was transferred into a clean centrifuge tube and 0.6 volume of chilled isopropanol was added and mixed by 2-3 gentle inversion till the DNA was precipitated. For complete precipitation the contents were kept at -20°C for half an hour. The contents were centrifuged thrice at 10,000 rpm for 15 minutes at 4°C. The pellet was air dried and dissolved in 50 µl sterile distilled water and stored at -20°C. The samples were loaded on 0.8 per cent agarose gel at constant voltage of 70V to test the quality and to find whether there is any shearing during RNAse treatment.

3.2.4.5 Assessing the quality and quantity of DNA using spectrophotometer (NanoDrop ND-1000)

The purity of DNA was further checked using NanoDrop spectrophometer.
Note: Nucleic acid shows absorption maxima at 260 nm whereas proteins show peak absorbance at 280 nm. Absorbance has been recorded at both wavelengths and the purity is indicated by the ratio OD260/OD280. A value between 1.8 and 2.0 indicated that the DNA is pure and free from proteins and RNA. When the ratio is <1.8 it means that the sample is RNA contaminated and when the ratio is >2.0 it means that sample is protein contaminated.

The quantity of DNA in the pure sample was calculated using the relation.
1OD at 260 nm = 50 µg DNA/ml
Therefore OD260 x 50 gives the quantity of DNA in ng/µl

3.2.4.5.1 Procedure followed for checking the quantity of DNA using NanoDrop spectrophotometer

The option nucleic acid was selected in the softwareND 1000 installed in the connected computer. With the sampling arm open, 1µl grade I water was pipetted onto the lower measurement pedestal. Sampling arm was closed and spectral measurement was initiated using the operating software. The sample column was automatically drawn between the upper and lower measurement pedestals and the spectral measurement was made. Then the reading was set to

zero with the sample blank. Subsequently, 1µl of sample was pipetted out onto the measurement pedestal and 'measure' option was selected. When the measurement has been completed, the sampling arm was opened and the sample was wiped from both the upper and lower pedestals using a soft laboratory tissue paper.

3.2.5 Molecular markers analysis

Two types of markers, RAPD (Random Amplified Polymorphic DNA) and ISSR (Inter Simple Sequences Repeats) were used in this study. Under each marker analysis system, bulked DNA of F_2 generations of okra was amplified along with the parental DNA simultaneously with the selected primer. The presence of characteristic marker in relation to the resistance to YVMV was examined for each primer.

3.2.5.1 DNA amplification conditions

The PCR conditions required for effective amplification in RAPD and ISSR analyses included appropriate proportions of the components of the reaction mixture. The reaction mixture included template DNA, assay buffer A or B, $MgCl_2$, Taq DNA polymerase, dNTPs and primers. The aliquot of this master mix was dispensed into 0.2 ml PCR tubes. The PCR was carried out in Verti Thermal Cycler (Applied Biosystems, USA) or Sure cycler 8800 (Agilent) thermal cyclers. The thermocycler was programmed for desired number of cycles and temperatures for denaturation, annealing and polymerisation.

3.2.5.2 RAPD assay

Good quality genomic DNA (40ng/µl) isolated from okra leaf samples were used in the RAPD analysis. An initial screening of RAPD primers was performed using the bulked DNA to select the RAPD primers with good resolving power.

PCR amplification was performed in a 20 µl reaction mixture and the composition of the reaction mixture consisted of,

a) Genomic DNA (40 ng)	-	1.0 µl
b) 10X Taq assay buffer A	-	2.5 µl
c) $MgCl_2$	-	1.5µl
c) dNTPs mix (10 mM each)	-	1.0 µl
d) Taq DNA polymerase (3U)	-	0.3 µl
e) Primer (10 pM)	-	1.5 µl
f) Autoclaved distilled water	-	12.2 µl
Total volume	-	20.0 µl

The PCR amplification was carried out with the following thermal profile

Total number of cycles	- 40
Initial denaturation	- 94°C for 4 minutes
Denaturation	- 94°C for 45 seconds
Primer annealing	- 37°C for 1 minute
Primer extension	- 72°C for 2 minutes
Final extension	- 72°C for 8 min

Incubation at - 4°C to hold the samples till electrophoresis.

3.2.5.2.1 Screening of RAPD primers

Eighty four primers (Sigma Technologies) were screened for RAPD analysis and listed in Table 3.2. Primers were selected from literatures based on previous studies on RAPD analysis (Nwangburuka et *al.*, 2011; Aladele *et al.*, 2008; Kaur *et al.*, 2013; Haq *et al.*, 2013), using resistant and susceptible DNA of okra. The amplified products were electrophoresed, along with 100 bp DNA ladder (Sigma, USA) on 1.5% agarose gel using 1X TAE buffer stained with

ethidium bromide. The profile was visualized under UV (312 nm) transilluminator and documented for further analyses. The documented RAPD profiles were carefully examined for polymorphism among amplicons.

Table 3.2. Details of RAPD primers used for initial screening

Sl. No.	RAPD primers used for screening okra	Nucleotide Sequence (5'-3')
1	OPA 01	5'CAGGCCCTTC3'
2	OPA 02	5'TGCCGAGCTG3'
3	OPA 03	5'AGTCAGCCAC3'
4	OPA 04	5'AATCGGGCTG3'
5	OPA 06	5'GGTCCCTGAC3'
6	OPA 08	5'GTGACGTAGG3'
7	OPA 09	5'GGGTAACGCC3'
8	OPA 10	5'GTGATCGCAG3'
9	OPA 12	5'TCGGCGATAG3'
10	OPA 16	5'AGCCAGCGAA3'
11	OPA 27	5'GAAACGGGTG3'
12	OPA 28	5'GTGACGTAGG3'
13	OPAH 1	5'TCCGCAACCA3'
14	OPAH 3	5'GGTTACTGCC3'
15	OPAH 5	5'TTGCAGGCAG3'
16	OPAH 6	5'GTAAGCCCCT3'
17	OPAH 9	5'AGAACCGAGG3'
18	OPB 08	5'GTCCACACGG3'
19	OPB 1	5'GTTTCGCTCC3'
20	OPB 10	5'CTGCTGGGAC3'
21	OPB 11	5'GTAGACCCGT3'
22	OPB 13	5'TTCCCCCGCT3'

23	OPB 14	5'TCCGCTCTGG3'
24	OPB 15	5'GGAGGGTGTT3'
25	OPB 16	5'TTTGCCCGGA3'
26	OPB 19	5'ACCCCCGAAG3'
27	OPB 02	5'TGATCCCTGG3'
28	OPB 03	5'CATCCCCCTG3'
29	OPB 06	5'TGCTCTGCCC3'
30	OPB 07	5'GGTGACGCAG3'
31	OPB 9	5'TGGGGGACTC3'
32	OPC 01	5'TTCGAGCCAG3'
33	OPC 02	5'GTGAGGCGTC3'
34	OPC 03	5' GGGGGTCTTT3'
35	OPC 04	5'GGTACGATGC3'
36	OPC 05	5'GATGACCGCC3'
37	OPC 06	5'GAACGGACTC3'
38	OPC 07	5'GTCCCGACGA3'
39	OPC 08	5'TGGACCGGTA3'
40	OPC 09	5'CTCACCGTCC 3'
41	OPC 11	5'AAAGCTGCGG3'
42	OPC 13	5'AAGCCTCGTC3'
43	OPC 14	5'TGCGTGCTTG3'
44	OPC 15	5'GACGGATCAG3'
45	OPC 17	5'TTCCCCCCAG3'
46	OPC 18	5'TGAGTGGGTG3'
47	OPC 19	5'GTTGCCAGCC3'
48	OPC 20	5'ACTTCGCCAC3'
49	OPD 07	5'TTGGCACGGG3'
50	OPD 08	5'GTGTGCCCCA3'
51	OPD 10	5'GGTCTACCAC3'
52	OPD 14	5'CTTCCCCAAG3'

53	OPD 15	5'CATCCGTGCT3'
54	OPD 18	5'GAGAGCCAAC3'
55	OPD 20	5'ACCCGGTAAC3'
56	OPE 05	5'CTGAGAATCC3'
57	OPE 07	5'AGATGCAGCC3'
58	OPF 09	5'CCAAGCTTCC3'
59	OPG 08	5'TCACGTCCAC3'
60	OPJ 17	5'ACGCCAGTTC3'
61	OPK 01	5'TGGCGACCTG3'
62	OPK O7	5'AGCGAGCAAG3'
63	OPL 08	5'AGCGAGCAAG3'
64	OPL 12	5'GGGCGGTACT3'
65	OPL 18	5'ACCACCCACC3'
66	OPM 16	5'GTAACCAGCC3'
67	OPM 18	5'CACCATCCGT3'
68	OPO 04	5'AAGTCCGCTC3'
69	OPP 13	5'GGAGTGCCTC3'
70	OPP 16	5'CCAAGCTGCC3'
71	OPP 17	5'TGACCCGCCT3'
72	OPP 19	5'GGGAAGGACA3'
73	OPU 03	5'CTATGCCGAC3'
74	OPU 07	5'CTACAGTGAG3'
75	OPU 13	5'GGCTGGTTCC3'
76	OPV16	5'ACACCCCACA3'
77	OPX 17	5'GACACGGACC3'
78	OPY 02	5'CATCGCCGCA3'
79	OPY 17	5'ACCCCCGAAG3'
80	RN 07	5'CAGCCCAGAG3'
81	RN 08	5'ACCTCAGCTC3'
82	RY 08	5'AGGCAGAGCA3'

83	S 12	5'CCTTGACGCA3'
84	S11	5'GTAGACCCGT3'

3.2.5.2.2 Bulk Segregant Analysis (BSA) of okra for YVMV resistance using RAPD primers

Bulk segregant analysis (Michelmore *et al.*, 1991) was carried out with DNA from resistant parent (Parbhani Kranthi), susceptible parent (Salkeerthi), F_2 resistant bulk (F_2R) and F_2 susceptible bulk (F_2S). Susceptible and resistant bulk consisted of DNAs from 7 plants.

Table: 3.3. List of selected RAPD primers for BSA

Sl. No	Primer	Sequence
1	OPA 02	5'TGCCGAGCTG3'
2	OPA 03	5'AGTCAGCCAC3'
3	OPA 04	5'AATCGGGCTG3'
4	OPA 06	5'GGTCCCTGAC3'
5	OPA 08	5'GTGACGTAGG3'
6	OPA 10	5'GTGATCGCAG3'
7	OPA 12	5'TCGGCGATAG3'
8	OPA 16	5'AGCCAGCGAA3'
9	OPA 28	5'GTGACGTAGG3'
10	OPB 03	5'CATCCCCCTG3'
11	OPB 06	5'TGCTCTGCCC3'
12	OPB 07	5'GGTGACGCAG3'
13	OPB 09	5'TGGGGGACTC3'
14	OPB 11	5'GTAGACCCGT3'
15	OPB 15	5'GGAGGGTGTT3'
16	OPB 16	5'TTTGCCCGGA3'
17	OPC 02	5'GTGAGGCGTC3'

18	OPC 06	5'GAACGGACTC3'
19	OPC 08	5'TGGACCGGTG3'
20	OPC 13	5'AAGCCTCGTC3'
21	OPC 15	5'GACGGATCAG3'
22	OPC 18	5'TGAGTGGGTG3'
23	OPC 19	5'GTTGCCAGCC3'
24	OPC 20	5'ACTTCGCCAC3'
25	OPD 07	5'TTGGCACGGG3'
26	OPD 08	5'GTGTGCCCCA3'
27	OPD 10	5'GGTCTACCAC3'
28	OPD 18	5'GAGAGCCAAC3'
29	OPF 09	5'CCAAGCTTCC3'
30	OPL 08	5'AGCGAGCAAG3'
31	OPL 12	5'GGGCGGTACT3'
32	OPL 18	5'ACCACCCACC3'
33	OPM 16	5'GTAACCAGCC3'
34	OPM 18	5'CACCATCCGT3'
35	OPO 04	5'AAGTCCGCTC3'
36	OPP 13	5'GGAGTGCCTC3'
37	OPP 16	5'CCAAGCTGCC3'
38	OPV16	5'ACACCCCACA3'
39	OPX 17	5'GACACGGACC3'

3.2.5.2.3 Co-segregation analysis using RAPD primers

RAPD primers which gave polymorphism in BSA was selected and used for Co-segregation analysis. It was carried out with individual DNA from resistant parent, susceptible parent, F_2 resistant and F_2 susceptible.

3.2.6 ISSR analysis

Good quality genomic DNA (30 ng/µl) isolated from okra leaf samples were used in the ISSR analysis. The ISSR primers were initially screened using the bulked DNA for selecting the primers with good resolving power.

PCR amplification was performed in a 20 µl reaction mixture and the composition of the reaction mixture consisted of,

a) Genomic DNA (30 ng)	- 2.0 µl
b) 10X Taq assay buffer B	- 2.5 µl
c) $MgCl_2$	- 1.5 µl
c) dNTPs mix (10 mM each)	- 1.0 µl
d) Taq DNA polymerase (3U)	- 0.4 µl
e) Primer (10 pM)	- 1.5 µl
f) Autoclaved distilled water	- 11.1 µl
Total volume	- 20.0 µl

The PCR amplification was carried out with the following thermal profile

Total number of cycles	= 40
Initial denaturation	– 94°C for 4 minutes
Denaturation	– 94°C for 45 seconds
Primer annealing	– 49°C to 65°C for 1 minute
Primer extension	– 72°C for 2 minutes
Final extension	– 72°C for 10 minutes

Incubation at – 4 °C to hold the sample

3.2.6.1 Screening of ISSR primers

Eighty two primers (Sigma Technologies) were initially screened in the ISSR analysis using bulked DNA of resistant and susceptible parents and listed in Table 3.3. Primers were selected from literatures based on previous studies in ISSR analysis in okra (Yuan *et al.*, 2014; Joshi *et al.*, 2007; Javan *et al.*, 2012).

Table 3.4. Details of ISSR markers used for initial screening

Sl. No.	Primers	Nucleotide sequence
1	HB 10	5'-GAGAGAGAGAGACC-3'
2	HB 12	5'- CACCACCACGC-3'
3	ISSR 10	5'- GTGTGTGTGTGTGTGTGTGTGTGTGTG-3'
4	ISSR 03	5'- CTCTCTCTCTCTCTCTTG-3'
5	ISSR 04	5'- GAGAGAGAGAGAGG-3'
6	ISSR 05	5'- CACACACACACACAC-3'
7	ISSR 06	5'- GTGTGTGTGTGTCC-3'
8	ISSR 08	5'- TGTGTGTGTGTGTGYCATGCACATTGTGT-3'
9	ISSR 09	5'- TGTGTGTGTGTGTGGCACATGCARTGTGT-3'
10	ISSR 22	5'- ATTATTATTATTATTCAT-3'
11	ISSR 23	5'-ACACACACACACACT-3'
12	ISSR 25	5'-ACACACACACACACG-3'
13	ISSR 26	5'- CTCCTCCTCGC-3'
14	ISSR 3	5'- TTATTATTATTATTACTT-3'
15	ISSR 5	5'- ATTATTGTTGTTGTTTTC-3'
16	ISSR 6	5'- TTATTATTATTATTATAA-3'
17	ISSR 7	5'- ATTATTGTTGTTGTTGTA-3'
18	ISSR 8	5'- ATTATTATTATTATTGTA-3'

19	ISSR 9	5'- TTATTATTATTATTATTACT-3'
20	ISSRP1	5'-CACGCACGCACGCACG-3'
21	Oligo ISSR 04	5'- ACACACACACACACACC- 3'
22	Oligo ISSR 05	5'- CTCTCTCTCTCTCTTG- 3'
23	Oligo ISSR 06	5'- GAGAGAGAGAGAGAGAC- 3'
24	Oligo ISSR 07	5'- CTCTCTCTCTCTCTTG- 3'
25	Oligo ISSR 08	5'- GAGAGAGAGAGAGAGAT- 3'
26	Oligo ISSR 15	5'- TCCTCCTCCTCCTCC- 3'
27	Oligo SPS 03	5'- GACAGACAGACAGACA- 3'
28	Oligo SPS 08	5'- GGAGGAGGAGGA- 3'
29	P1	5'- GACACGACACGACACGACAC-3'
30	P2	5'- AGCAGCAGCAGCGT-3'
31	P2	5'- GAAGTGGGGAAGTGGG-3'
32	P4	5'-GTGTGTGTGTGTGTGTC-3'
33	P5	5'-CTCTCTCTCTCTCTCTA-3'
34	P6	5'-TCTCTCTCTCTCTCC-3'
35	P9	5'-CTCTCTCTCTCTCTAC-3'
36	R 11	5'- GATCATCATCATCATCATCATCATCATC-3'
37	S07	5'- TCCGGATGCTG-
38	S1	5'- GTTGTTGTTGTTGTT-3'
39	S2	5'- CTGACTGACTGACTGA-3'
40	S3	5'- CCACCACCACCACCA-3'
41	S4	5'- GTCGTCGTCGTCGTC-3'
42	UBC 354	5'- AGAGAGAGAGAGAGAGY-3'
43	UBC 807	5'- AGAGAGAGAGAGAGAGT- 3'
44	UBC 808	5'-AGAGAGAGAGAGAGAGC-3'
45	UBC 809	5'- GAGAGAGAGAGAGAGAG- 3'
46	UBC 810	5'- GAGAGAGAGAGAGAGAT- 3'
47	UBC 811	5'- GAGAGAGAGAGAGAGAC- 3'

48	UBC 812	5'- GAGAGAGAGAGAGAGAA-3'
49	UBC 813	5'- CTCTCTCTCTCTCTCTT- 3'
50	UBC 814	5'- CTCTCTCTCTCTCTCTA- 3'
51	UBC 815	5'- CTCTCTCTCTCTCTCTG- 3'
52	UBC 816	5'- CACACACACACACACAT- 3'
53	UBC 817	5'- CACACACACACACACAA- 3'
54	UBC 818	5'- CACACACACACACACAG- 3'
55	UBC 820	5'- GTGTGTGTGTGTGTGTC- 3'
56	UBC 823	5'- TCTCTCTCTCTCTCTCC- 3'
57	UBC 825	5'- ACAGACACACACACACT- 3'
58	UBC 826	5'- ACACACACACACACACC- 3'
59	UBC 827	5' - ACACACACACACACACG- 3'
60	UBC 835	5'- AGAGAGAGAGAGAGAGYC- 3'
61	UBC 836	5'- AGAGAGAGAGAGAGAGYA- 3'
62	UBC 840	5'- GAGAGAGAGAGAGAGAYT- 3'
63	UBC 841	5'- GAGAGAGAGAGAGAGAY-3'
64	UBC 843	5'- CTCTCTCTCTCTCTCTRA- 3'
65	UBC 844	5'- CTCTCTCTCTCTCTCTRC- 3'
66	UBC 845	5'- CTCTCTCTCTCTCTCTRG- 3'
67	UBC 846	5'- CACACACACACACACART-3'
68	UBC 848	5'- CACACACACACACACARG-3'
69	UBC 854	5'- TCTCTCTCTCTCTCTCRG- 3'
70	UBC 855	5'- ACACACACACACACACYT-3'
71	UBC 856	5'- ACACACACACACACACYA- 3'
72	UBC 857	5' - ACACACACACACACACYG- 3'
73	UBC 865	5' - CCGCCGCCGCCGCCGCCG-3'
74	UBC 866	5'- CTCCTCCTCCTCCTCCTC- 3'
75	UBC 873	5'- GACAGACAGACAGACA-3'
76	UBC 880	5'- GGAGAGGAGAGGAGA-3'
77	UBC 890	5'- VHVGTGTGTGTGTGTGT- 3'

78	UBC 892	5'-TAGATCTGATATCTGAATTCCC-3'
79	UBC 895	5'- AGAGTTGGTAGCTCTTGATC-3'
80	UBC 899	5'-CATGGTGTTGGTCATTGTTCCA -3'
81	UBC 900	5'-ACTTCCCCACAGGTTAACACA-3'
82	UBC S2	5'- CTCTCTCTCGTGTGTGTG- 3'

3.2.6.2 Bulk Segregant Analysis (BSA) of okra for YVMV resistance

Bulk segregant analysis was carried out with DNA from resistant parent (Parbhani Kranthi), Susceptible parent (Salkeerthi), resistant bulk (F_2R) and susceptible bulk (F_2S). Susceptible and resistant bulk consisted of DNAs from 7 plants.

Table: 3.5. List of selected ISSR primers for BSA

Sl. No.	Primer	Sequence
1	ISSR 9	5'- TTATTATTATTATTATTACT-3'
2	ISSR 6	5'- TTATTATTATTATTATAA-3'
3	ISSR 22	5'- ATTATTATTATTATTCAT-3'
4	ISSR 23	5'-ACACACACACACACT-3'
5	ISSR 26	5'- CTCCTCCTCGC-3'
6	ISSR 8	5'- ATTATTATTATTATTGTA-3'
7	P6	5'-TCTCTCTCTCTCTCC-3'
8	P9	5'-CTCTCTCTCTCTCTAC-3'
9	UBC 808	5'-AGAGAGAGAGAGAGAGC-3'
10	UBC 811	5'- GAGAGAGAGAGAGAGAC- 3'
11	UBC 812	5'- GAGAGAGAGAGAGAGAA-3'
12	UBC 840	5'- GAGAGAGAGAGAGAGAYT- 3'
13	UBC 841	5'- GAGAGAGAGAGAGAGAY-3'
14	UBC 844	5'- CTCTCTCTCTCTCTCTRC- 3'
15	UBC 845	5'- CTCTCTCTCTCTCTCTRG- 3'

16	UBC 848	5'- CACACACACACACACARG-3'
17	UBC 854	5'- TCTCTCTCTCTCTCTCRG- 3'
18	UBC 857	5' - ACACACACACACACACYG- 3'
19	UBC 865	5' - CCGCCGCCGCCGCCGCCG-3'
20	UBC 866	5'- CTCCTCCTCCTCCTCCTC- 3'
21	UBC 873	5'- GACAGACAGACAGACA-3'
22	UBC 880	5'- GGAGAGGAGAGGAGA-3'
23	UBC 890	5'- VHVGTGTGTGTGTGTGT- 3'

3.2.6.3 Co-segregation analysis

Co-segregation analysis was carried out with individual DNA from resistant parent, susceptible parent, selected F_2 resistant and F_2 susceptible plants, using ISSR primers which showed polymorphism previously in bulk analysis as mentioned in the result chapter.

Results

4. RESULTS

The study, "Development of molecular markers linked to yellow vein mosaic resistance in okra [*Abelmoschus esculentus* (L.) Moench]" was conducted at the Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Kerala Agricultural University during the period of 2013-2015. The objective of the study was to identify an ISSR or RAPD marker linked with the gene governing resistance to the yellow vein mosaic virus disease in okra through bulked segregant analysis to enable marker assisted selection. The results of various aspects of the investigations are presented in this chapter.

During field screening of parental lines as mentioned in the materials and methods chapter, 6 highly resistant Parbhani Kranthi and 10 highly susceptible Salkeerthi plants were identified and used in the crossing programme.

4.1 Morphological observations on susceptible, resistant and F_1 accessions

Morphological parameters were recorded from the fully grown up plants at fruiting stage. Observations were taken on randomly selected twenty resistant Parbhani Kranthi and twenty susceptible Salkeerthi accessions and their F_1 progenies. The comparative performance of the qualitative and quantitative characters of parents and F_1 plants is presented in Table 4.1 and 4.2.

Table 4.1. Qualitative characters on parents and their F_1

Sl. No.	Characteristics	Parbhani Kranthi	Salkeerthi	F_1
1.	Plant growth habit	Erect	Erect	Erect
2.	Stem color	Green	Light Green	Green
3.	Leaf size	Medium	Large	Medium
4.	Leaf tip	Pointed	Pointed	Pointed
5.	Leaf vein color	Light green	Light green	Light green
6.	Flower: petal color	Yellow	Yellow	Yellow
7.	Flower: Petal base color (Purple)	Inside only	Inside only	Inside only
8.	Flower size	Medium	Medium	Medium
9.	Immature fruit color	Green	Light green	Green
10.	Mature fruit color	Green	Yellowish green	Green
11.	Fruit: Shape of apex	Narrow	Narrow	Narrow
12.	Fruit: Constriction of basal part	Absent	Absent	Absent
13	Fruit: Surface between ridges	Flat	Flat	Flat
14.	Seed: Color	Green	Green	Green
15.	Seed: Hairiness	Smooth	Smooth	Smooth
16.	Seed shape	Round	Round	Round

Table 4.2. Quantitative characters on parents and their F_1

Sl. No.	Characteristics	Mean		
		Parbhani Kranthi Mean	Salkeerthi Mean	F_1 Mean
1.	Plant height (cm)	79.2	83.0	80.3
2.	Days to flower	39.32	35.2	36.3
3.	Days to first harvest	46.5	41.3	42.8
4.	Number of fruits per plant	7	9	11
5.	Number of ridges per pod	5	5	5
6.	Yield per plant (gm)	193.0	231.2	210.9
7.	Petiole length (cm)	21.9	23.7	25.4
8.	Fruit length (cm)	8.2	12.5	11.9

1. **Plant height**

   Height of the plant was maximum in Salkeerthi (83.0 cm) while Parbhani Kranthi and F_1 hybrid recorded 79.2 cm and 80.3 cm respectively.

2. **Days to flower**

   Days to first flower initiation was minimum in Salkeerthi (35.2 days) while Parbhani Kranthi and F_1 hybrid were late in flowering (39.32 and 36.3 days) respectively.

3. **Days to first harvest**

   Salkeerthi took 41.3 days for the first fruit harvest where as Parbhani Kranthi and F_1 recorded 46.5 and 42.8 days respectively.

4. **Number of fruits per plant**

   F_1 produced more number of fruits (11) when compared to Parbhani Kranthi (7) and Salkeerthi (9).

5. **Number of ridges per pod**

   Number of ridges per pod was same (5) in Parbhani Kranthi, Salkeerthi and their F_1 progeny.

6. **Yield per plant**

   The estimated mean yield per plant was highest in Salkeerthi (231.2 gm/plant), followed by F_1 (210.9 gm/plant) and Parbhani Kranthi (193.0 gm/plant).

7. **Petiole length**

   Petiole length was recorded maximum in F_1 (25.4 cm) when compared to Salkeerthi (23.7 cm) and Parbhani Kranthi (21.9 cm).

8. **Fruit length**

   Fruit length was maximum in Salkeerthi (12.5 cm) while Parbhani Kranthi and F_1 hybrid recorded 8.2 cm and 11.9 cm, respectively.

4.3. Response of okra populations to YVMV

Sl. No.	Genotypes and their resultant F_1 and F_2 generations	PDI	PDS	CI	Disease reaction
1.	Parbahni Kranthi	40	16	6.4	Resistant
2.	Salkeerthi	100	99.67	99.67	Highly susceptible
3	F_1 generation	0	0	0	Highly resistant
4	F_2 generation	60	12	7.2	Resistant

4.2 Disease response of okra populations

The disease response of parental, F_1 and F_2 populations is presented in Table 4.3. Parbhani Kranthi was resistant with 40 PDI where as Salkeerthi was highly susceptible. F_1 population was highly resistant and F_2 population was resistant.

4.3 Isolation of quality DNA for molecular analysis

Initially the CTAB method of (Roger and Bendich, 1994) was used for the isolation of genomic DNA. The quality of DNA was not satisfactory with presence of high amounts of polyphenolic compounds and polysaccharides. Subsequently an improved semi automated rapid method in okra, as reported by Singh and Kumar (2012) was also tried but the results were not better than the CTAB method.

Subsequently, genomic DNA isolation through the modified CTAB method (Doyle and Doyle, 1987) was attempted. Treatment with β-

mercaptoethanol was given for removing the color of DNA due to polyphenols. The chloroform: isoamyl alcohol (24:1) treatment was given three times and the DNA was washed with ammonium acetate. RNase treatment and further precipitation gave sufficient quantity of good quality DNA from leaf sample. The agarose gel electrophoresis showed clear and discrete bands with no protein or RNA contamination (Fig. 4.1 and 4.2). Spectrophotometric analysis gave the acceptable ratio of UV absorbance (A260 /280) between 1.8 and 2.06 (Table 4.4).

4.4 Molecular marker analysis

The protocol for different marker assays, RAPD and ISSR were validated with bulked DNA of okra accessions. Different primers were screened with the bulked genomic DNA, using the validated protocols.

4.4.1 Random Amplified Polymorphic DNA (RAPD) analysis

Eighty four RAPD primers were used in the initial screening based on their ability to amplify the genomic DNA, with the thermal settings mentioned earlier under the material and methods section (3.2.5.2). Based on the type of bands and nature of amplification (Table 4.5), 39 RAPD primers were selected to perform bulk segregant analysis.

Table. 4.4. Quality and quantity of DNA isolated from the okra accessions, assessed by NanoDrop spectrophotometer PK (Parbhani Kranthi), S (Salkeerthi)

Sl. No	Plant used	UV absorbance at 260 nm (A260)	UV absorbance at 280 nm (A280)	UV absorbance at $A_{(260/280)}$	Quantity (ng/µl)
1	S 1	6.897	3.342	2.06	344.85
2	S 2	31.279	15.485	2.02	1563.95
3	S 3	4.303	2.254	1.90	215.15
4	S 4	10.872	5.760	1.88	543.6
5	S 5	22.268	11.413	1.95	1113.4
6	S 6	20.567	10.552	1.80	1059.24
7	S 7	5.422	3.037	1.79	271.10
8	S 8	14.063	7.401	1.87	1254.11
9	S 9	16.277	8.428	1.82	880.90
10	S 10	10.708	5.677	1.88	535.40
11	PK 1	2.078	1.122	1.85	103.89
12	PK 2	11.359	5.729	1.98	576.94
13	PK 3	9.567	5.045	1.85	478.37
14	PK 4	16.930	8.225	2.06	846.49
15	PK 5	9.358	5.045	1.85	476.92
16	PK 6	4.845	2.691	1.80	242.25
17	F_2 Resistant 1	3.621	1.948	1.86	181.05
17	F_2 Resistant 2	5.526	2.995	1.85	276.25
18	F_2 Resistant 3	2.473	1.293	1.91	123.65
20	F_2 Resistant 4	3.027	1.642	1.84	151.36
21	F_2 Resistant 5	5.525	2.863	1.93	276.17
22	F_2 Resistant 6	1.556	0.795	1.96	77.8
23	F_2 Resistant 7	6.093	3.395	1.80	304.67
24	F_2 Susceptible 1	3.288	1.827	1.80	164.39
25	F_2 Susceptible 2	1.638	0.861	1.90	81.92
26	F_2 Susceptible 3	2.069	1.110	1.80	104.66
27	F_2 Susceptible 4	5.495	2.814	1.95	274.74
28	F_2 Susceptible 5	5.523	2.863	1.93	276.17
29	F_2 Susceptible 6	3.198	1.539	1.90	348.51
30	F_2 Susceptible 7	4.783	2.495	1.92	239.17

Fig 4.1: The gel profile obtained by electophoresis of DNA samples of susceptible Salkeerthi plants isolated using modified Doyle and Doyle (1987) method, after RNase treatment

M: Marker Lambda DNA (*Eco* RI/ *Hind* III digest 1000 bp ladder), **B:** Blank, **S1 to S10:** Salkeerthi.

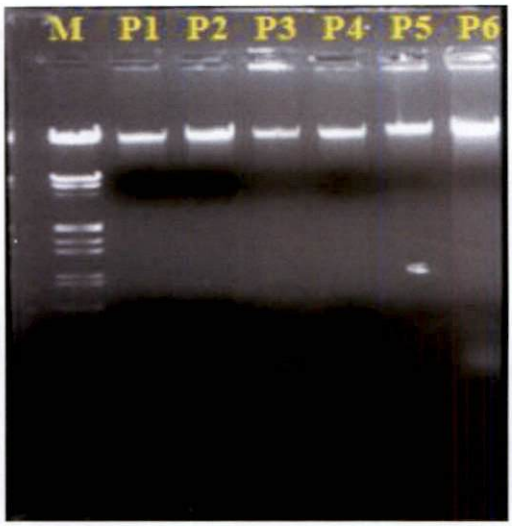


Fig 4.2: The gel profile obtained by electophoresis of DNA samples of resistant Parbhani Kranthi plants isolated using modified Doyle and Doyle (1987) method, after RNase treatment

M: Marker Lambda DNA (*Eco* RI/ *Hind* III digest 1000 bp ladder),

P1 to P6: Parbhani Kranthi

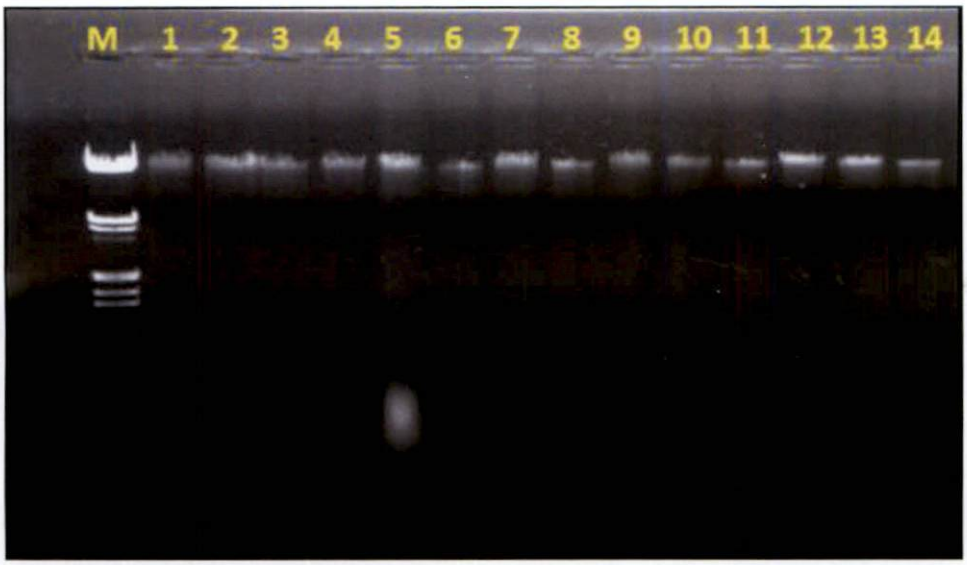


Fig 4.3: The gel profile obtained by electophoresis of DNA samples of resistant F_2 plants and susceptible F_2 plants isolated using modified Doyle and Doyle (1987) method, after RNase treatment

M: Marker Lambda DNA (*Eco* RI/ *Hind* III digest 1000 bp ladder), **1-7:** Susceptible F_2 plants and **8-14:** Resistant F_2 plants.

Table 4.5. Details of DNA amplification with the 84 RAPD primers in okra

Sl. No.	RAPD primers used for screening okra	Amplification pattern			
		No of bands	Types of bands		Remarks
			Distinct	Faint	
1	OPA 01	3	1	2	Not selected
2	OPA 02	11	2	9	Selected
3	OPA 03	6	3	3	Selected
4	OPA 04	5	3	2	Selected
5	OPA 06	8	3	5	Selected
6	OPA 08	8	5	3	Selected
7	OPA 09	2	0	0	Not selected
8	OPA 10	5	4	1	Selected
9	OPA 12	9	5	4	Selected
10	OPA 16	7	4	3	Selected
11	0PA 18	2	1	1	Not selected
12	OPA 27	0	0	0	Not selected
13	OPA 28	5	3	2	Selected
14	OPAH 1	0	0	0	Not selected
15	OPAH 3	0	0	0	Not selected
16	OPAH 5	0	0	0	Not selected
17	OPAH 6	0	0	0	Not selected
18	OPAH 9	0	0	0	Not selected
19	OPB 07	6	3	2	Selected
20	OPB 09	6	3	3	Selected
22	OPB 01	0	0	0	Not selected
23	OPB 10	0	0	0	Not selected
24	OPB 11	10	4	6	Selected
25	OPB 13	2	0	2	Not selected
26	OPB 14	1	1	0	Not selected

27	OPB 15	5	3	2	Selected
28	OPB 16	8	4	4	Selected
29	OPB 19	1	1	0	Not selected
30	OPB 02	3	1	2	Not selected
31	OPB 03	6	0	6	Selected
32	OPB 6	8	0	8	Selected
33	OPC 01	0	0	0	Not selected
34	OPC 02	5	4	1	Selected
35	OPC 04	0	0	0	Not selected
36	OPC 05	0	0	0	Not selected
37	OPC 06	9	4	5	Selected
38	OPC 07	3	1	2	Not selected
39	OPC 08	5	2	3	Selected
40	OPC 11	0	0	0	Not selected
41	OPC 12	3	2	1	Not selected
42	OPC 13	8	3	5	selected
43	OPC 14	0	0	0	Not selected
44	OPC 15	5	2	3	Selected
45	OPC 17	2	2	1	Not selected
46	OPC 18	8	0	8	Selected
47	OPC 19	8	6	2	Selected
48	OPC 20	7	2	5	Selected
49	OPD 07	8	6	2	Selected
50	OPD 08	6	3	3	Selected
51	OPD 10	9	5	4	Selected
52	OPD 14	3	0	3	Not selected
53	OPD 15	0	0	0	Not selected
54	OPD 18	6	4	2	Selected
55	OPD 20	0	0	0	Not selected
56	OPE 12	0	0	0	Not selected

57	OPE 16	2	0	2	Not selected
58	OPF 09	10	0	10	Selected
59	OPG 08	0	0	0	Not selected
60	OPJ 17	2	2	0	Not selected
61	OPK 01	0	0	0	Not selected
62	OPK O7	2	1	1	Not selected
63	OPL 08	7	1	6	Selected
64	OPL 12	8	2	6	Selected
65	OPL 18	11	5	6	Selected
66	OPM 16	5	2	3	Selected
67	OPM 18	9	4	5	Selected
68	OPO 04	6	3	3	Selected
69	OPP 13	10	7	3	Selected
70	OPP 16	11	7	4.	Selected
71	OPP 17	0	0	0	Not selected
72	OPP 19	3	0	3	Not selected
73	OPU 03	0	0	0	Not selected
74	OPU 07	0	0	0	Not selected
75	OPU 13	0	0	0	Not selected
76	OPV16	10	2	8	Selected
77	OPX 17	4	1	3	Selected
78	OPY 02	0	0	0	Not selected
79	OPY 17	1	0	0	Not selected
80	RN 07	0	0	0	Not selected
81	RN 08	0	0	0	Not selected
82	RY 08	0	0	0	Not selected
83	S 12	0	0	0	Not selected
84	S11	0	0	0	Not selected

Table 4.6. BSA on okra population using 39 RAPD primers

Sl. No.	Primer	Sequence	Banding pattern
1	OPA 02	5'TGCCGAGCTG3'	Monomorphic
2	OPA 03	5'AGTCAGCCAC3'	Monomorphic
3	OPA 04	5'AATCGGGCTG3'	Monomorphic
4	OPA 06	5'GGTCCCTGAC3'	Monomorphic
5	OPA 08	5'GTGACGTAGG3'	Monomorphic
6	OPA 10	5'GTGATCGCAG3'	Monomorphic
7	OPA 12	5'TCGGCGATAG3'	Monomorphic
8	OPA 16	5'AGCCAGCGAA3'	Monomorphic
9	OPA 28	5'GTGACGTAGG3'	Monomorphic
10	OPB 03	5'CATCCCCCTG3'	Monomorphic
11	OPB 06	5'TGCTCTGCCC3'	Monomorphic
12	OPB 07	5'GGTGACGCAG3'	Monomorphic
13	OPB 09	5'TGGGGGACTC3'	Monomorphic
14	OPB 11	5'GTAGACCCGT3'	**Polymorphic**
15	OPB 15	5'GGAGGGTGTT3'	Monomorphic
16	OPB 16	5'TTTGCCCGGA3'	Monomorphic
17	OPC 02	5'GTGAGGCGTC3'	Monomorphic
18	OPC 06	5'GAACGGACTC3'	Monomorphic
19	OPC 08	5'TGGACCGGTG3'	Monomorphic
20	OPC 13	5'AAGCCTCGTC3'	Monomorphic
21	OPC 15	5'GACGGATCAG3'	Monomorphic
22	OPC 18	5'TGAGTGGGTG3'	Monomorphic
23	OPC 19	5'GTTGCCAGCC3'	Monomorphic
24	OPC 20	5'ACTTCGCCAC3'	Monomorphic
25	OPD 07	5'TTGGCACGGG3'	Monomorphic
26	OPD 08	5'GTGTGCCCCA3'	Monomorphic
27	OPD 10	5'GGTCTACCAC3'	Monomorphic

28	OPD 18	5'GAGAGCCAAC3'	Monomorphic
29	OPF 09	5'CCAAGCTTCC3'	Monomorphic
30	OPL 08	5'AGCGAGCAAG3'	Monomorphic
31	OPL 12	5'GGGCGGTACT3'	Monomorphic
32	OPL 18	5'ACCACCCACC3'	**Polymorphic**
33	OPM 16	5'GTAACCAGCC3'	Monomorphic
34	OPM 18	5'CACCATCCGT3'	Monomorphic
35	OPO 04	5'AAGTCCGCTC3'	Monomorphic
36	OPP 13	5'GGAGTGCCTC3'	Monomorphic
37	OPP 16	5'CCAAGCTGCC3'	Monomorphic
38	OPV16	5'ACACCCCACA3'	Monomorphic
39	OPX 17	5'GACACGGACC3'	Monomorphic

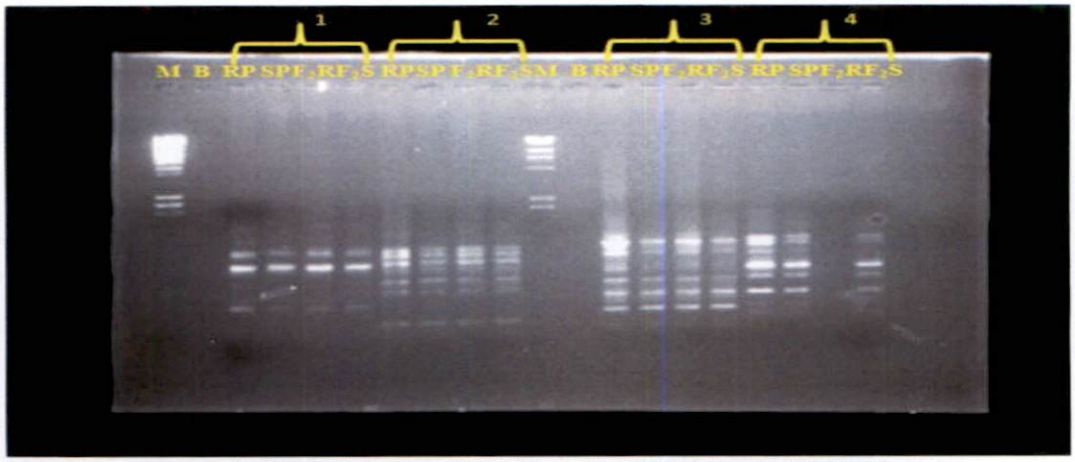
The analysis of result on individual primers is detailed here under.

4.3.1.1 OPC 15

RAPD assay using the primer OPC 15, followed by electrophoresis on 1.5 percent agarose gel generated a maximum of five amplicons in all lanes under study. The molecular weight of the band varied from 200 bp to 1 kb [Fig. 4.4(1)]. The amplicons generated were monomorphic with no association to YVMV resistance.

4.3.1.2 OPP 13

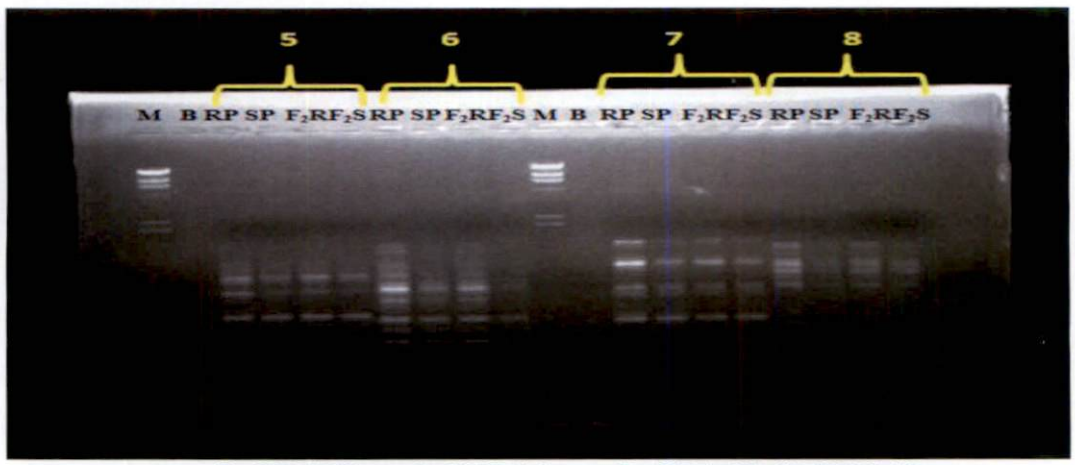
When both the parental and bulked DNA of F_2 were analysed using the RAPD primer OPP 13, ten amplicons were generated. The pattern of amplification is shown in Fig. 4.4(2). The molecular weight of the amplicons ranged from 250 to 1 kb. The amplicons generated were monomorphic with no association to YVMV resistance.



1. OPC 15 2. OPP 13 3. OPL 12 4. OPV 16

Fig. 4.4: Amplification pattern of both parental lines and bulked DNA of F_2 generated using RAPD primers OPC 15, OPP 13, OPL 12 and OPV 16

M: 1kb Molecular marker, **B:** Blank, **RP:** Resistant parent, **SP:** Susceptible parent, **F_2R:** F_2 resistant bulk and **F_2S:** F_2 susceptible bulk.



5: OPC 18 6: OPP 16 7: OPA 03 8: OPC 13.

Fig. 4.5: Amplification pattern of both parental lines and bulked DNA of F_2 generated using RAPD primers OPC 18, OPP 16, OPA 03 and OPC 13

M: 1kb Molecular marker, **B:** Blank, **RP:** Resistant parent, **SP:** Susceptible parent, **F_2R:** F_2 resistant bulk and **F_2S:** F_2 susceptible bulk.

4.3.1.3 OPL 12

On an average, eight amplicons were obtained with the RAPD primer OPL 12. The pattern of amplification is shown in Fig. 4.4(3). The molecular weight of the amplicons ranged from 100 bp to 1.5 kb. Even though the marker generated large numbers of amplicons, they were monomorphic for the character YVMV resistance.

4.3.1.4 OPV 16

Amplification with OPV 16 primer generated an average ten amplicons in both the parental lines, in F_2 resistant and susceptible bulk. The molecular weight of the band varied from 0.1 to 1.5 kb [Fig. 4.4(4)]. All amplicons generated from this primer were monomorphic.

4.3.1.5 OPC 18

Total of eight amplicons were generated on amplification of DNA using RAPD primer, OPC 18 and electrophoresed on 1.5 percent agarose gel. No polymorphism was observed between the parents and bulked DNA of resistant and susceptible F_2 lines [Fig. 4.5(5)].

4.3.1.6 OPP 16

RAPD assay using the primer OPP 16 followed by electrophoresis on 1.5 percent agarose gel has generated a maximum of eleven amplicons in both the parents and bulked DNA of resistant and susceptible F_2 lines. The molecular weight of the band varied from 0.1 to 1.5 kb [Fig. 4.5(6)]. The amplicons generated were monomorphic with no association to YVMV resistance.

4.3.1.7 OPA 03

When both the parents and bulked DNA of resistant and susceptible F_2 lines were analysed using the RAPD primer OPA 03, six amplicons were generated. The pattern of amplification is shown in Fig. 4.5(7). The molecular

weight of the amplicons ranged from 0.25 to 1 kb. The amplicons generated were monomorphic with no association to YVMV resistance.

4.3.1.8 OPC 13

On an average eight amplicons were obtained with the RAPD primer OPC 13. The pattern of amplification is shown in Fig. 4.5(8). The molecular weight of the amplicons ranged from 200 bp to 900 bp. Through this marker generated a large number of amplicons, they were monomorphic for the YVMV resistance.

4.3.1.9 OPC 06

Amplification with OPC 06 primer generated an average of nine amplicons in all the lanes. The molecular weight of the band varied from 0.2 to1.5 kb [Fig. 4.6 (9)]. All amplicons generated from this primer were monomorphic.

4.3.1.10 OPA 16

Total of seven amplicons were generated on amplification of DNA using RAPD primer OPA 16 which electrophoresed on 1.5 percent agarose gel. No polymorphism was observed between the parents and bulked DNA of resistant and susceptible F_2 lines [Fig. 4.6(10)].

4.3.1.11 OPA 04

RAPD assay using the primer OPA 04, followed by electrophoresis on 1.5 percent agarose gel generated a maximum of five amplicons in all lanes under study. The molecular weight of the band varied from 0.25 to 0.9 kb [Fig. 4.6(11)]. The amplicons generated were monomorphic with no association to YVMV resistance.

4.3.1.12 OPC 08

When the DNA of parents and bulked DNA of F_2 resistant and susceptible were analysed using the RAPD primer OPC 08, five amplicons were generated. The pattern of amplification is shown in Fig. 4.7(12). The molecular weight of the

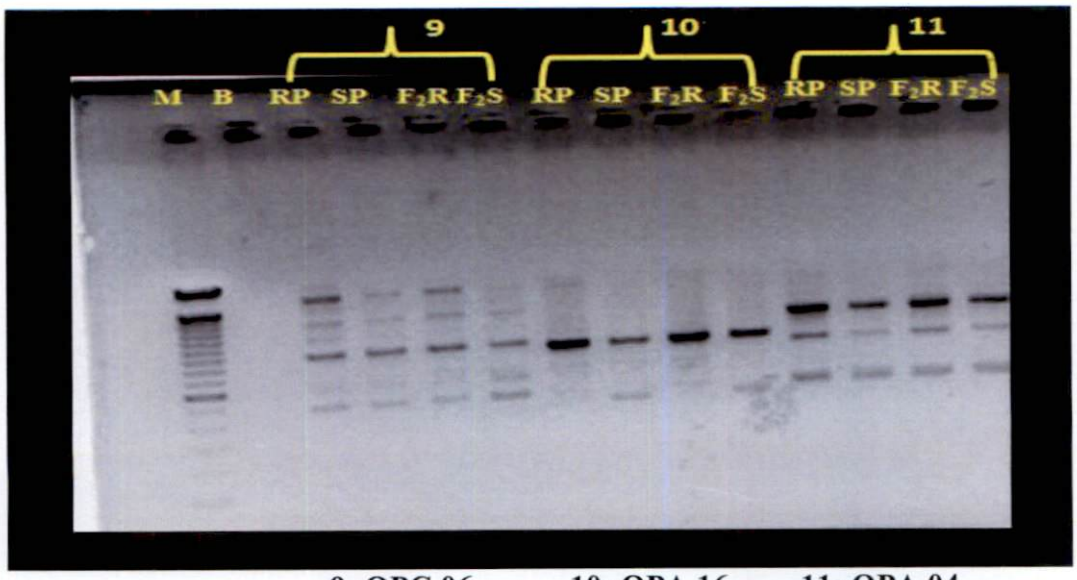


Fig. 4.6: Amplification pattern of both parental lines and bulked DNA of F_2 generated using RAPD primers OPC 06, OPA 16 and OPA 04

M: 1kb Molecular marker, **B:** Blank, **RP:** Resistant parent, **SP:** Susceptible parent, **F_2R:** F_2 resistant bulk and **F_2S:** F_2 susceptible bulk.

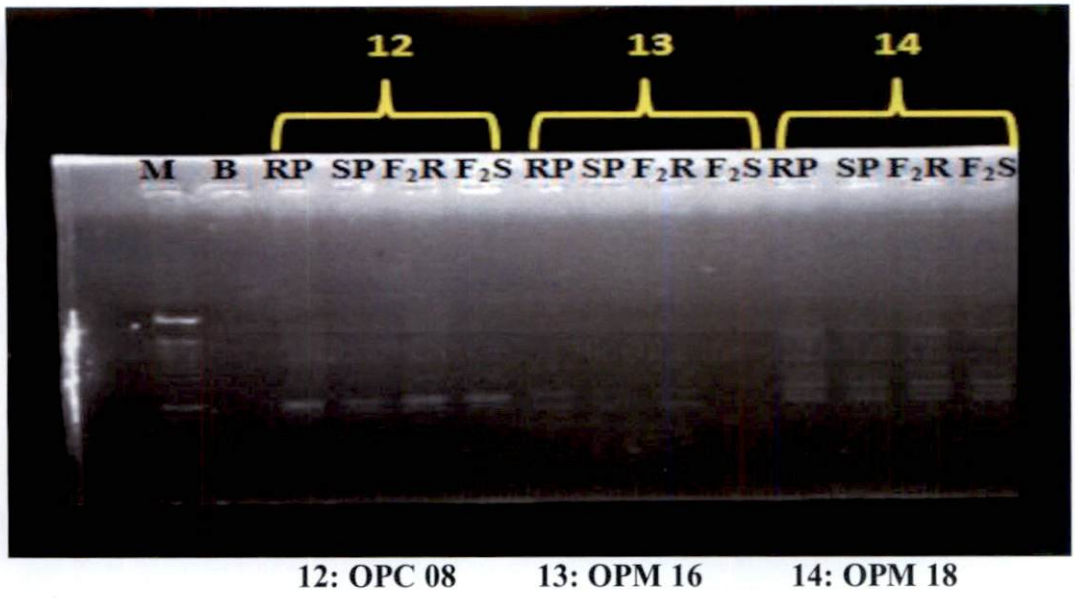


Fig. 4.7: Amplification pattern of both parental lines and bulked DNA of F_2 generated using RAPD primers OPC 08, OPM 16 and OPM 18

M: 1kb Molecular marker, **B:** Blank, **RP:** Resistant parent, **SP:** Susceptible parent, **F_2R:** F_2 resistant bulk and **F_2S:** F_2 susceptible bulk.

amplicons ranged from 0.2 to 1 kb. The amplicons generated were monomorphic with no association to YVMV resistance.

4.3.1.13 OPM 16

On an average five amplicons were obtained with the RAPD primer OPM 16. The pattern of amplification is shown in Fig. 4.7(13). The molecular weight of the amplicons ranged from 0.2 to 0.7 kb. The amplicons generated were monomorphic with no association to YVMV resistance.

4.3.1.14 OPM 18

Amplification with OPM 18 primer generated an average nine amplicons in each lane. The molecular weight of the band varied from 0.1 to 1.5 kb [Fig. 4.7(14)]. All amplicons generated from this primer were monomorphic.

4.3.1.15 OPC 20

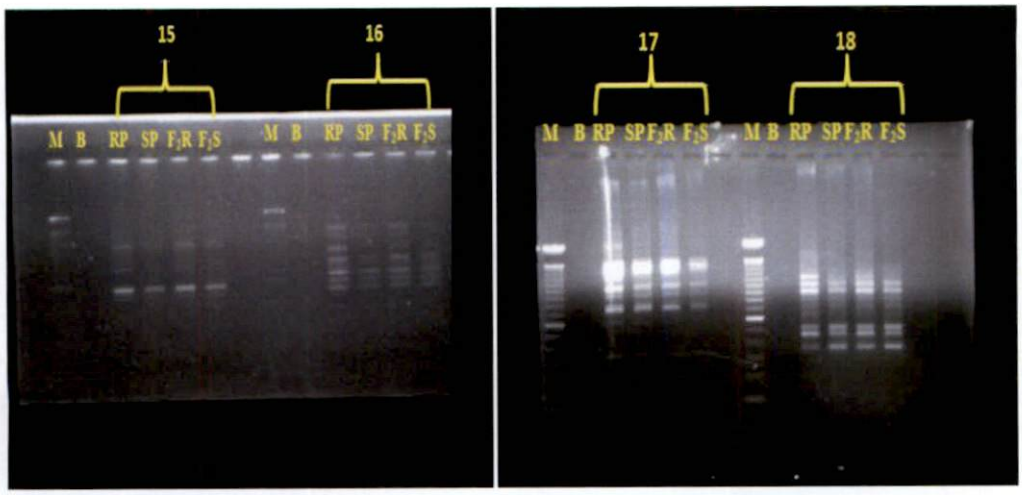
Total of seven amplicons were generated on amplification of DNA using RAPD primer OPC 20 which electrophoresed on 1.5 percent agarose gel. No polymorphism was observed between the parental lines and the bulked DNA of F_2 resistant and susceptible lanes [Fig. 4.8(15)]. All amplicons generated from this primer were monomorphic.

4.3.1.16 OPB 16

RAPD assay using the primer OPB 16 followed by electrophoresis on 1.5 percent agarose gel generated a maximum of eight amplicons in all the lanes under study. The molecular weight of the band varied from 100bp to 1.5 kb [Fig. 4.8(16)]. The amplicons generated were monomorphic with no association to YVMV resistance.

4.3.1.17 OPA 06

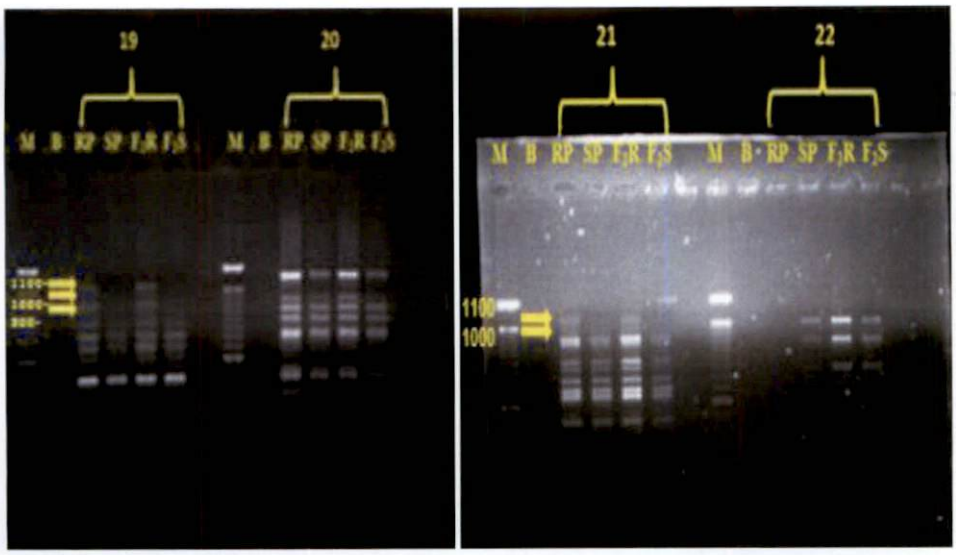
When the DNA of parents and bulked DNA of F_2 resistant and susceptible were analysed using the RAPD primer OPA 06, eight amplicons were generated.



15: OPC 20 **16: OPB 16** **17: OPA 06** **18: OPA 12.**

Fig. 4.8: Amplification pattern of both parental lines and bulked DNA of F_2 generated using RAPD primers OPC 20, OPB 16, OPA 06 and OPA 12

M: 1kb Molecular marker, **B:** Blank, **RP:** Resistant parent, **SP:** Susceptible parent, **F_2R:** F_2 resistant bulk and **F_2S:** F_2 susceptible bulk.



19: OPB 11 **20: OPC 19** **21: OPL 18** **22: OPB 07**

Fig. 4.9: Amplification pattern of both the parental lines and bulked DNA of F_2 generated using RAPD primers OPB 11, OPC 19, OPL 18 and OPB 07

M: 1kb Molecular marker **B:** Blank, **RP:** Resistant parent, **SP:** Susceptible parent, **F_2R:** F_2 resistant bulk and **F_2S:** F_2 susceptible bulk.

The pattern of amplification is shown in Fig. 4.8(17). The molecular weight of the amplicons ranged from 200 bp to 1.7 kb. The amplicons generated were monomorphic with no association to YVMV resistance.

4.3.1.18 OPA 12

On an average nine amplicons were obtained with the RAPD primer OPA 12. The pattern of amplification is shown in Fig. 4.8(18). The molecular weight of the amplicons ranged from 0.2 to 1.8 kb. Through this marker, large number of amplicons were generated, which were monomorphic for the character YVMV resistance.

4.3.1.19 OPB 11

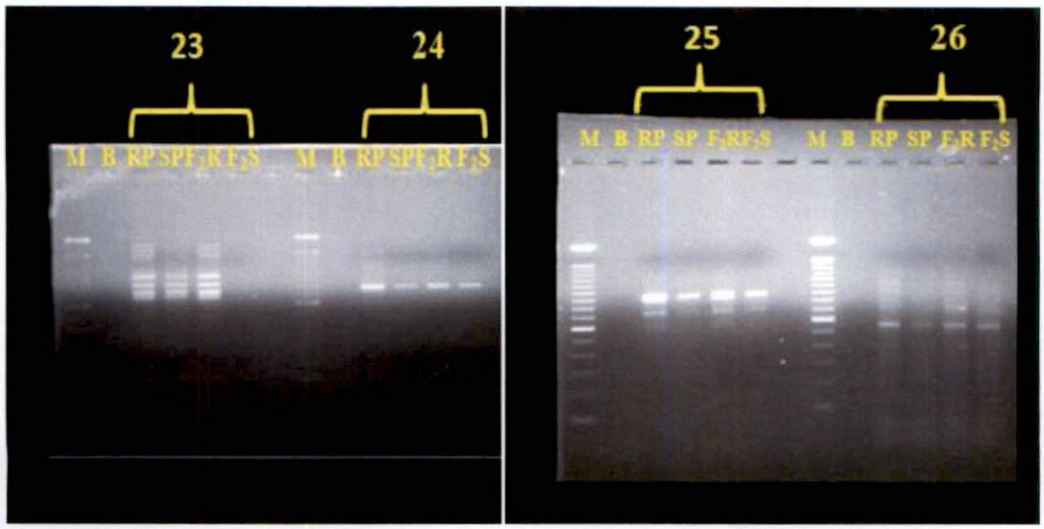
Using the primer OPB 11 an average of ten amplicons were obtained in each accession. The pattern of amplification is shown in Fig. 4.9(19). The molecular weight of the bands varied from 100 bp to 1.1 kb. Amplicons obtained with this primer at 800, 1000 and 1100 bp were distinctly polymorphic for resistance to YVMV. The polymorphic bands of 1.1, 0.9 and 0.8 kb were present in parent resistant and in F_2 resistant bulk but absent in parent susceptible and F_2 susceptible bulk. This primer was able to differentiate clearly between resistant and susceptible lines.

4.3.1.20 OPC 19

Amplification with OPC 09 primer, generated on an average eight amplicons in all lanes. The molecular weight of the band varied from 0.1 to 1.5 kb [Fig. 4.9(20)]. All amplicons generated from this primer were monomorphic.

4.3.1.21 OPL 18

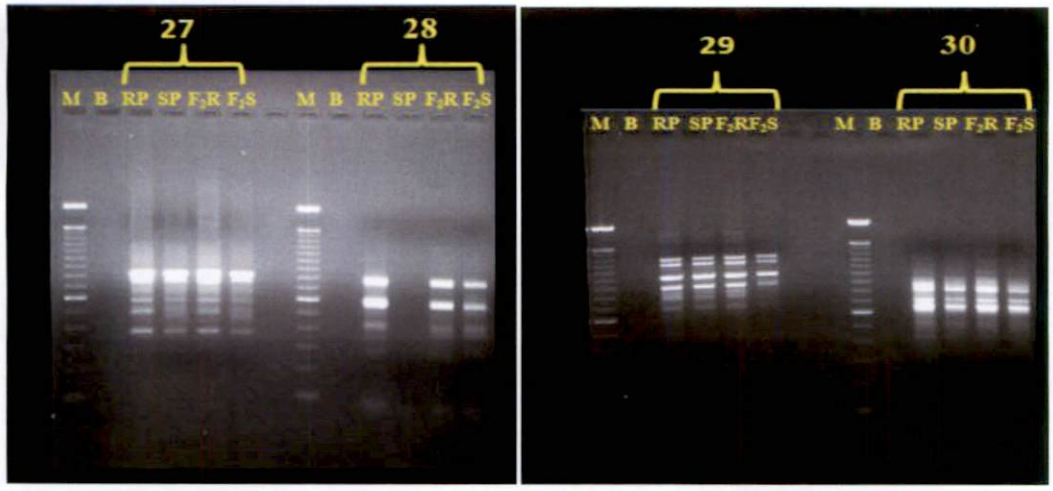
Using the primer OPL 18, an average of eleven amplicons was obtained in each accession. The pattern of amplification is shown in Fig. 4.9(21). The molecular weight of the bands varied from 100 bp to 1 kb. Amplicons obtained with this primer were distinctly polymorphic for resistance to YVMV. The



23: OP0 04 **24: OPX 17** **25: OPA 08** **26: OPA 10**

Fig. 4.10: Amplification pattern of both parental lines and bulked DNA of F_2 generated using RAPD primers OP0 04, OPX 17, OPA 08 and OPA 10

M: 1kb Molecular marker, **B:** Blank, **RP:** Resistant parent, **SP:** Susceptible parent, **F_2R:** F_2 resistant bulk and **F_2S:** F_2 susceptible bulk.



27: OPD 07 **28: OPC 02** **29: OPD 10** **30: OPF 09.**

Fig. 4.11: Amplification pattern of both parental lines and bulked DNA of F_2 generated using RAPD primers OPD 07, OPC 02, OPD 10 and OPF 09

M: 1kb Molecular marker, **B:** Blank, **RP:** Resistant parent, **SP:** Susceptible parent, **F_2R:** F_2 resistant bulk and **F_2S:** F_2 susceptible bulk.

polymorphic bands of 1 and 1.1 kb were present in resistant parent and in F_2 resistant bulk but absent in susceptible parent and F_2 susceptible bulk. This primer was able to differentiate clearly between resistant and susceptible lines.

4.3.1.22 OPB 07

Total of five amplicons were generated on amplification of DNA using RAPD primer OPB 07 which electrophoresed on 1.5 percent agarose gel. No polymorphism was observed between the parents, and F_2 resistant and F_2 susceptible lines [Fig. 4.9(22)].

4.3.1.23 OPO 04

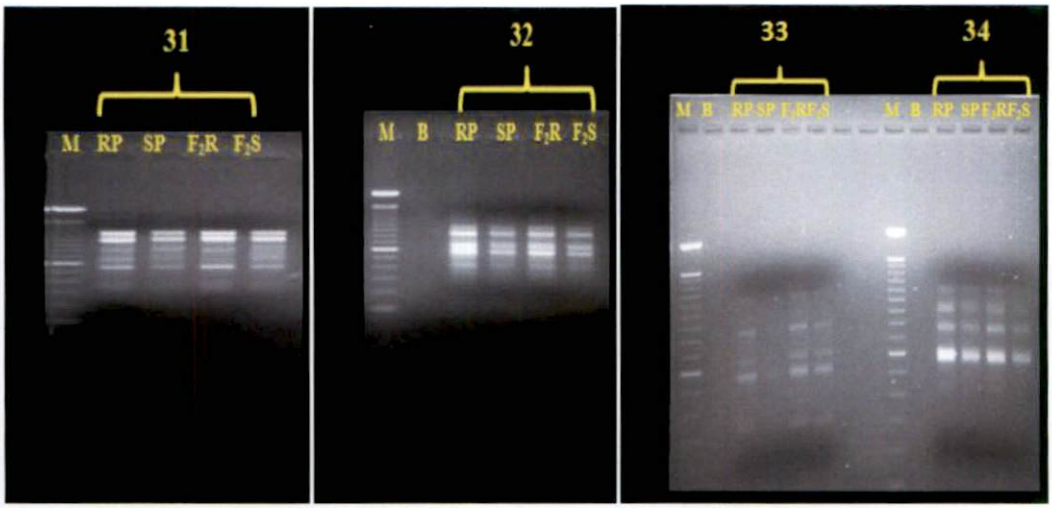
RAPD assay using the primer OPO 04 followed by electrophoresis on 1.5 percent agarose gel generated a maximum of six amplicons in both the parents and F_2 resistant lane but there is no amplification in F_2 susceptible lane under study. The molecular weight of the band varied from 0.2 to 0.9 kb [Fig. 4.10(23)]. The amplicons generated were monomorphic with no association to YVMV resistance.

4.3.1.24 OPX 17

When the DNA of parents and bulked DNA of resistant and susceptible F_2 were analysed using the RAPD primer OPX 17, four amplicons were generated. The pattern of amplification is shown in Fig. 4.10(24). The molecular weight of the amplicons ranged from 0.1 to 1 kb. The amplicons generated were monomorphic with no association to YVMV resistance.

4.3.1.25 OPA 08

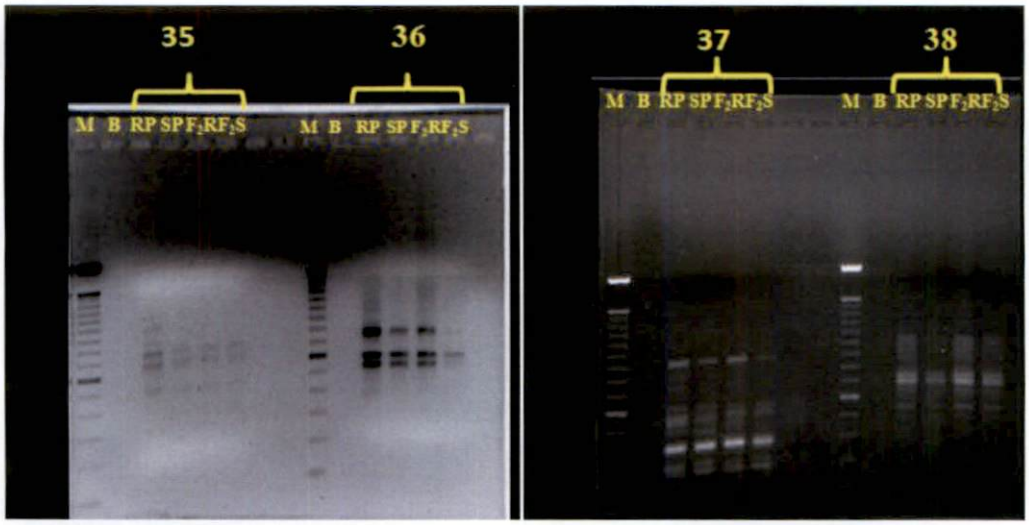
On an average five amplicons were obtained with the RAPD primer OPA 08. The pattern of amplification is shown in Fig. 4.10(25). The molecular weight of amplicons ranged from 0.1 to 0.9 kb. The amplicons generated were monomorphic with no association to YVMV resistance.



31: OPD 18 **32: OPB 03** **33: OPD 08** **34: OPB 06**

Fig. 4.12: Amplification pattern of both parental lines and bulked DNA of F_2 generated using RAPD primers OPD 18, OPB 03, OPD 08 and OPB 06

M: 1kb Molecular marker, **B:** Blank, **RP:** Resistant parent, **SP:** Susceptible parent, **F_2R:** F_2 resistant bulk and **F_2S:** F_2 susceptible bulk.



35: OPB 15 **36: OPB 09** **37: OPA 02** **38: OPA 28**

Fig. 4.13: Amplification pattern of both parental lines and bulked DNA of F_2 generated using RAPD primers OPB 15, OPB 09, OPA 02 and OPA 28

M: 1kb Molecular marker, **B:** Blank, **RP:** Resistant parent, **SP:** Susceptible parent, **F_2R:** F_2 resistant bulk and **F_2S:** F_2 susceptible bulk.

4.3.1.26 OPA 10

Amplification with OPA 10 primer generated on an average, five amplicons in each lane. The molecular weight of the band varied from 0.1 to 1.2 kb [Fig. 4.10(26)]. All amplicons generated from this primer were monomorphic.

4.3.1.27 OPD 07

Total of eight amplicons were generated on amplification of DNA using RAPD primer OPD 07 and electrophoresed on 1.5 percent agarose gel. No polymorphism was observed between the parents, resistant and susceptible bulked DNA of F_2 [Fig. 4.11(27)]. All amplicons generated from this primer were monomorphic.

4.3.1.28 OPC 02

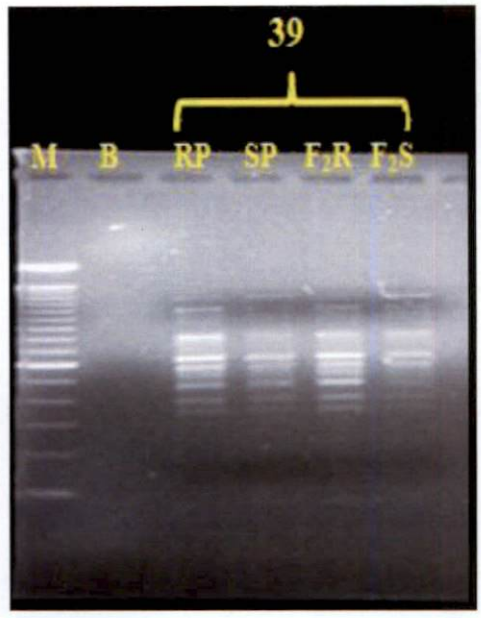
RAPD assay using the primer OPC 02 followed by electrophoresis on 1.5 percent agarose gel generated a maximum of five amplicons in the resistant parent, F_2 resistant and F_2 susceptible but there is no amplification in susceptible parent . The molecular weight of the band varied from 0.1 to 1 kb [Fig. 4.11(28)]. The amplicons generated were monomorphic with no association to YVMV resistance.

4.3.1.29 OPD 10

When the DNA of parents and bulked DNA of resistant and susceptible F_2 were analysed using the RAPD primer OPD 10, nine amplicons were generated in all lanes. The patteren of amplification is shown in Fig. 4.11(29). The molecular weight of the amplicons ranged from 0.25 to 1 kb. The amplicons generated were monomorphic with no association to YVMV resistance.

4.3.1.30 OPF 09

On an average, six amplicons were obtained with the RAPD primer OPF 09. The pattern of amplification is shown in Fig. 4.11(30). The molecular weight



39: OPF 09

Fig. 4.14: Amplification patterns both the parental lines and bulked DNA of F_2 generated using RAPD primer OPF 09

M: 1kb Molecular marker, **B:** Blank, **RP:** Resistant parent, **SP:** Susceptible parent, **F_2R:** F_2 resistant bulk and **F_2S:** F_2 susceptible bulk.

of the amplicons ranged from 0.3 to 0.5 kb. The amplicons were generated in all lanes monomorphic for the character YVMV resistance.

4.3.1.31 OPD 18

Amplification with OPD 18 primer, generated on an average six amplicons in all lanes. The molecular weight of the band varied from 0.1 to 0.9 kb [Fig. 4.12(31)]. All amplicons generated from this primer were monomorphic.

4.3.1.32 OPB 03

Total of seven amplicons were generated on amplification of DNA using RAPD primer OPB 03 which electrophoresed on 1.5 percent agarose gel. No polymorphism was observed between the parents, resistant and susceptible bulked DNA of F_2 [Fig. 4.12(32)]. All amplicons generated from this primer were monomorphic.

4.3.1.33 OPD 08

RAPD assay using the primer OPD 08 followed by electrophoresis on 1.5 percent agarose gel generated a maximum of five amplicons in all the lanes. The molecular weight of the band varied from 0.1 to 0.9 kb [Fig. 4.12(33)]. The amplicons generated were monomorphic with no association to YVMV resistance.

4.3.1.34 OPB 06

When the DNA of parents and bulked DNA of resistant and susceptible F_2 were analysed using the RAPD primer OPB 06, six amplicons were generated. The pattern of amplification is shown in Fig. 4.12(34). The molecular weight of the amplicons ranged from 0.2 to 1 kb. The amplicons generated were monomorphic with no association to YVMV resistance.

4.3.1.35 OPB 15

On an average, five amplicons were obtained with the RAPD primer OPB 15. The pattern of amplification is shown in Fig. 4.13(35). The molecular weight

of the amplicons ranged from 0.1 to 0.7 kb. The amplicons generated were monomorphic for the character YVMV resistance.

4.3.1.36 OPB 09

Amplification with OPB 09 primer generated on an average, six amplicons in all the lanes. The molecular weight of the band varied from 0.1 to 0.9 kb [Fig. 4.13(36)]. All amplicons generated from this primer were monomorphic.

4.3.1.37 OPA 02

Total of eleven amplicons were generated on amplification of DNA using RAPD primer OPA 02 which electrophoresed on 1.5 percent agarose gel. No polymorphism was observed between the parents, bulked resistant and susceptible F_2 [Fig. 4.13(37)]. All amplicons generated from this primer were monomorphic.

4.3.1.38 OPA 28

RAPD assay using the primer OPA 28 followed by electrophoresis on 1.5 percent agarose gel generated a maximum of ten amplicons in the resistant parent, F_2 resistant and F_2 susceptible but there was no amplification in susceptible parent . The molecular weight of the band varied from 100bp to 700bp [Fig. 4.13(38)]. The amplicons generated were monomorphic with no association to YVMV resistance.

4.3.1.39 OPF 09

RAPD assay using the primer OPF 09 followed by electrophoresis on 1.5 percent agarose gel generated a maximum of nine amplicons in all the lanes. The molecular weight of the band varied from 0.3 to 1.2 kb [Fig. 4.14(39)]. The amplicons generated were monomorphic with no association to YVMV resistance.

4.3.1.40. Cosegregation analysis with RAPD primers OPB 11 and OPL 18

Cosegregation analysis with the two RAPD primers OPB 11 and OPL 18 which were found linked with disease resistance of YVMV was performed and

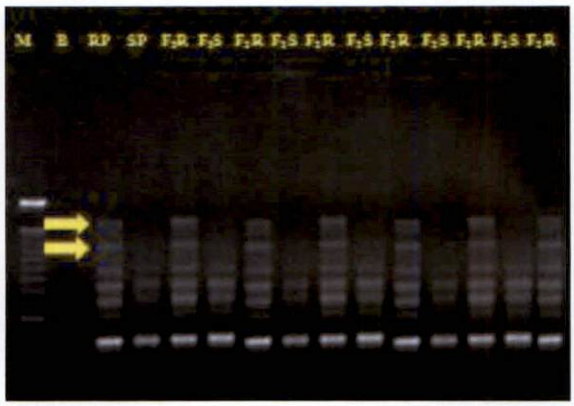


Fig. 4.15: Co-segregation of RAPD primer OPB 11 with YVMV resistance in F_2 individuals of the cross Salkeerthi X Parbhani Kranthi

M: 1kb Molecular marker, **B:** Blank, **RP:** Resistant parent, **SP:** Susceptible parent, **F_2R:** F_2 resistant and **F_2S:** F_2 susceptible.

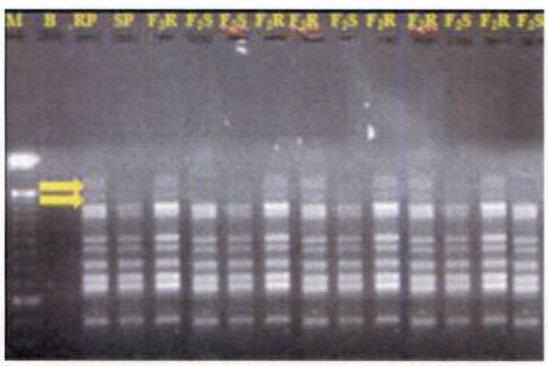


Fig. 4.16: Co-segregation of RAPD primer OPL 18 with YVMV resistance in F_2 individuals of the cross Salkeerthi X Parbhani Kranthi

M: 1kb Molecular marker, **B:** Blank, **RP:** Resistant parent, **SP:** Susceptible parent, **F_2R:** F_2 resistant and **F_2S:** F_2 susceptible.

results are presented in Fig. 4.15. and Fig. 4.16. The markers were found segregating with the gene for resistance.

4.3.2 Inter Simple Sequence Repeat (ISSR) analysis

Eighty two ISSR primers were used in the initial screening based on their ability to amplify the genomic DNA, with the thermal settings mentioned under the Materials and Methods section (3.2.5.3). Based on the types of bands and nature of amplification (Table 4.7), twenty three ISSR primers were selected to perform bulk segregant analysis. Details of the BSA using the selected primers are presented in (Table 4.8).

Table 4.7. Details of amplification with the 82 ISSR primers in okra

Sl. Num.	ISSR primers used for screening okra	Amplification pattern			
		No. of bands	Type of bands		Remarks
			Distinct	Faint	
1	HB 10	0	0	0	Not selected
2	HB 12	0	0	0	Not selected
3	ISSR 10	0	0	0	Not selected
4	ISSR 03	1	1	1	Not selected
5	ISSR 04	3	2	1	Not selected
6	ISSR 05	0	0	0	Not selected
7	ISSR 06	3	0	0	Not selected
8	ISSR 08	0	0	0	Not selected
9	ISSR 09	0	0	0	Not selected
10	ISSR 23	8	3	5	Selected
11	ISSR 22	8	2	6	Selected
12	ISSR 25	0	0	0	Not selected
13	ISSR 26	10	6	4	Selected

14	ISSR 3	1	1	0	Not selected
15	ISSR 5	3	3	0	Not selected
16	ISSR 6	8	3	5	Selected
17	ISSR 7	1	0	1	Not selected
18	ISSR 8	4	1	3	Not selected
19	ISSR 9	9	4	5	Selected
20	ISSRP1	0	0	0	Not selected
21	Oligo ISSR 04	0	0	0	Not selected
22	Oligo ISSR 05	0	0	0	Not selected
23	Oligo ISSR 06	0	0	0	Not selected
24	Oligo ISSR 07	0	0	0	Not selected
25	Oligo ISSR 08	0	0	0	Not selected
26	Oligo ISSR 15	0	0	0	Not selected
27	Oligo SPS 03	0	0	0	Not selected
28	Oligo SPS 08	0	0	0	Not selected
29	P1	0	0	0	Not selected
30	P2	0	0	0	Not selected
31	P3	3	1	2	Not selected
32	P4	2	0	2	Not selected
33	P5	2	0	2	Not selected
34	P6	11	5	6	Selected
35	P9	8	6	2	Selected
36	R 11	0	0	0	Not selected
37	S07	0	0	0	Not selected
38	S1	0	0	0	Not selected
39	S2	0	0	0	Not selected
40	S3	0	0	0	Not selected
41	S4	0	0	0	Not selected
42	UBC 354	0	0	0	Not selected
43	UBC 807	0	0	0	Not selected

44	UBC 808	6	2	4	Selected
45	UBC 809	0	0	0	Not selected
46	UBC 810	3	0	3	Not selected
47	UBC 811	8	3	5	Selected
48	UBC 812	8	4	4	Selected
49	UBC 813	1	1	1	Not selected
50	UBC 814	0	0	0	Not selected
51	UBC 815	0	0	0	Not selected
52	UBC 816	0	0	0	Not selected
53	UBC 817	0	0	0	Not selected
54	UBC 818	1	1	0	Not selected
55	UBC 820	1	1	0	Not selected
56	UBC 823	3	3	0	Not selected
57	UBC 825	0	0	0	Not selected
58	UBC 826	0	0	0	Not selected
59	UBC 827	1	0	1	Not selected
60	UBC 835	5	3	2	Selected
61	UBC 836	0	0	0	Not selected
62	UBC 840	9	3	6	Selected
63	UBC 841	9	4	5	Selected
64	UBC 843	0	0	0	Not selected
65	UBC 844	9	5	4	Selected
66	UBC 845	4	2	2	Selected
67	UBC 846	1	0	1	Not selected
68	UBC 848	8	3	5	Selected
69	UBC 854	8	4	4	Selected
70	UBC 855	1	1	0	Not selected
71	UBC 856	3	1	2	Not selected
72	UBC 857	9	4	5	Selected
73	UBC 865	9	4	5	Selected

74	UBC 866	6	3	3	Selected
75	UBC 873	7	2	5	Selected
76	UBC 880	5	2	3	Selected
77	UBC 890	5	4	1	Selected
78	UBC 892	0	0	0	Not selected
79	UBC 895	0	0	0	Not selected
80	UBC 899	0	0	0	Not selected
81	UBC 900	1	0	1	Not selected
82	UBC S2	0	0	0	Not selected

Table 4.8. Details on the BSA on okra populations segregating for YVMV resistance, using ISSR primers

Sl. No.	Primer	Sequence	Annealing temperature	Banding pattern
1	ISSR 9	5'- TTATTATTATTATTATTACT-3'	62.2 ^{o}C	Monomorphic
2	ISSR 6	5'- TTATTATTATTATTATAA-3'	51.9 ^{o}C	Monomorphic
3	ISSR 22	5'- ATTATTATTATTATTCAT-3'	51.2 ^{o}C	**Polymorphic**
4	ISSR 23	5'-ACACACACACACACT-3'	53.0 ^{o}C	Monomorphic
5	ISSR 26	5'- CTCCTCCTCGC-3'	51.5 ^{o}C	Monomorphic
6	ISSR 8	5'- ATTATTATTATTATTGTA-3'	49.9 ^{o}C	**Polymorphic**
7	P6	5'-TCTCTCTCTCTCTCC-3'	54.7 ^{o}C	Monomorphic
8	P9	5'-CTCTCTCTCTCTCTAC-3'	57.1 ^{o}C	Monomorphic
9	UBC 808	5'-AGAGAGAGAGAGAGAGC-3'	49.8 ^{o}C	Monomorphic
10	UBC 811	5'- GAGAGAGAGAGAGAGAC- 3'	54 ^{o}C	Monomorphic
11	UBC 812	5'- GAGAGAGAGAGAGAGAA-3'	56.2 ^{o}C	Monomorphic
12	UBC 840	5'- GAGAGAGAGAGAGAGAYT- 3'	50.8 ^{o}C	Monomorphic
13	UBC 841	5'- GAGAGAGAGAGAGAGAY-3'	51 ^{o}C	Monomorphic
14	UBC 844	5'- CTCTCTCTCTCTCTCTRC- 3'	53.5 ^{o}C	Monomorphic
15	UBC 845	5'- CTCTCTCTCTCTCTCTRG- 3'	59.7 ^{o}C	Monomorphic

16	UBC 848	5'- CACACACACACACACARG-3'	56.7 °C	Monomorphic
17	UBC 854	5'- TCTCTCTCTCTCTCTCRG- 3'	54.6 °C	Monomorphic
18	UBC 857	5' - ACACACACACACACACYG- 3'	62.1 °C	Monomorphic
19	UBC 865	5' - CCGCCGCCGCCGCCGCCG-3'	61.5 °C	Monomorphic
20	UBC 866	5'- CTCCTCCTCCTCCTCCTC- 3'	55.5 °C	Monomorphic
21	UBC 873	5'- GACAGACAGACAGACA-3'	59 °C	**Polymorphic**
22	UBC 880	5'- GGAGAGGAGAGGAGA-3'	52 °C	Monomorphic
23	UBC 890	5'- VHVGTGTGTGTGTGTGT- 3'	58.1 °C	Monomorphic

4.3.2.1 ISSR 9

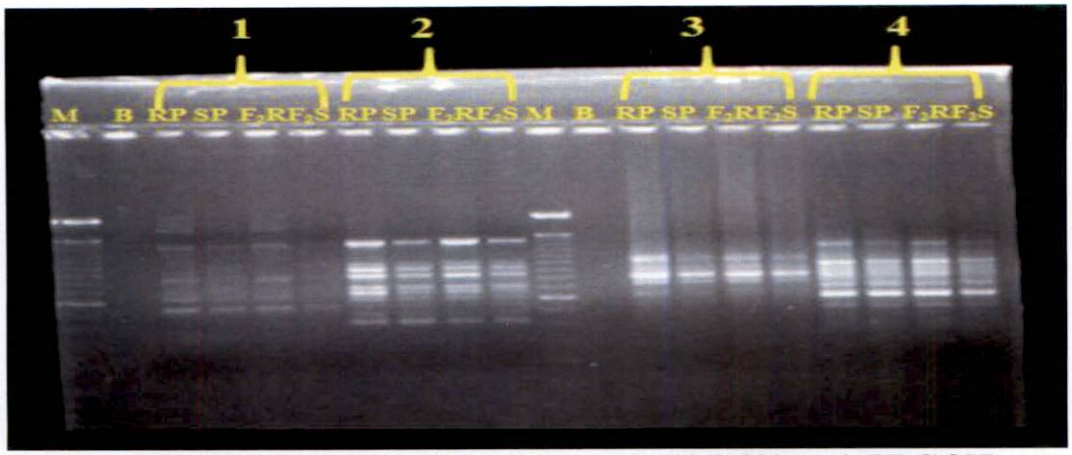
Total of nine amplicons were generated on amplification of DNA using ISSR 9 primer which electrophoresed on 1.5 percent agarose gel. No polymorphism was observed between the parental lines resistant and susceptible F_2 bulk [Fig. 4.17(1)]. All amplicons generated from this primer were monomorphic.

4.3.2.2 ISSR 6

ISSR assay using the primer ISSR 6 followed by electrophoresis on 1.5 percent agarose gel generated a maximum of eight amplicons in each lane. The molecular weight of the band varied from 0.1 to 1.3 kb [Fig. 4.17(2)]. The amplicons generated were monomorphic with no association to YVMV resistance.

4.3.2.3 UBC 808

When the DNA of parents, bulked DNA of resistant and susceptible F_2 were analysed using the ISSR primer UBC 808, six amplicons were generated. The pattern of amplification is shown in Fig. 4.17(3). The molecular weight of the amplicons ranged from 0.25 to 1.1 kb. The amplicons generated were monomorphic with no association to YVMV resistance.



1: ISSR 9 **2: ISSR 6** **3: UBC 808** **4: UBC 857**

Fig. 4.17: Amplification pattern of both parental lines and bulked DNA of resistant and susceptible F_2 generated using ISSR primers ISSR 9, ISSR 6, UBC 808 and UBC 857

M: 1kb Molecular marker, **B:** Blank, **RP:** Resistant parent, **SP:** Susceptible parent, **F_2R:** F_2 resistant bulk and **F_2S:** F_2 susceptible bulk.

5: UBC 841 **6: UBC 811** **7: UBC 844** **8: UBC 840**

Fig. 4.18: Amplification pattern of both the parental lines and bulked DNA of resistant and susceptible F_2 generated using ISSR primers UBC 841, UBC 811, UBC 844 and UBC 840

M: 1kb Molecular marker, **B:** Blank, **RP:** Resistant parent, **SP:** Susceptible parent, **F_2R:** F_2 resistant bulk and **F_2S:** F_2 susceptible bulk.

4.3.2.4 UBC 857

On an average nine amplicons were obtained with the ISSR primer UBC 857. The pattern of amplification is shown in Fig. 4.17(4). The molecular weight of the amplicons ranged from 0.2 to 1.2 kb. Through this marker large number of amplicons were generated, and they were monomorphic for the character YVMV resistance.

4.3.2.5 UBC 841

Amplification with UBC 841 primer, generated on an average nine amplicons in each lane. The molecular weight of the band varied from 0.25 to 1kb [Fig. 4.18(5)]. All amplicons generated from this primer was monomorphic.

4.3.2.6 UBC 811

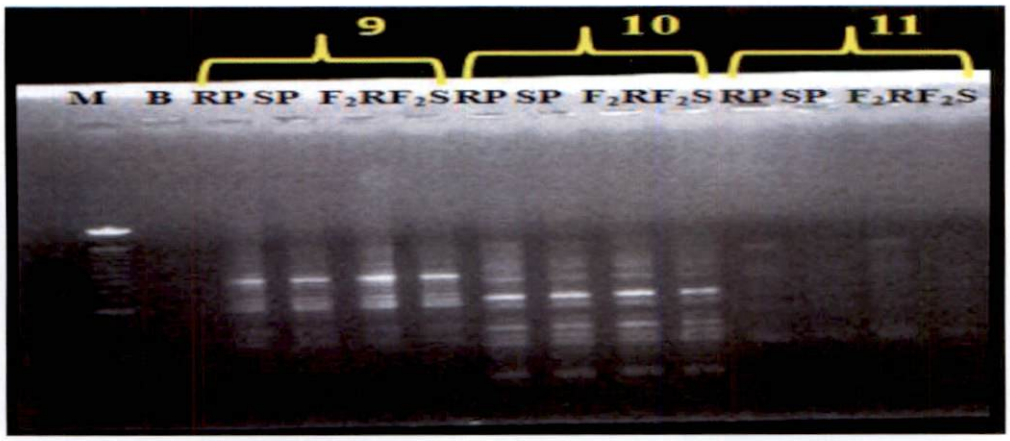
Total of eight amplicons were generated on amplification of DNA using ISSR primer UBC 811 which electrophoresed on 1.5 percent agarose gel. No polymorphism was observed between the parental lines, bulked DNA of resistant and susceptible lanes [Fig. 4.18(6)]. All amplicons generated from this primer were monomorphic.

4.3.2.7 UBC 844

ISSR assay using the primer UBC 844 followed by electrophoresis on 1.5 percent agarose gel generated nine amplicons in each lane. The molecular weight of the band varied from 0.1 to 1 kb [Fig. 4.18(7)]. The amplicons generated were monomorphic with no association to YVMV resistance.

4.3.2.8 UBC 840

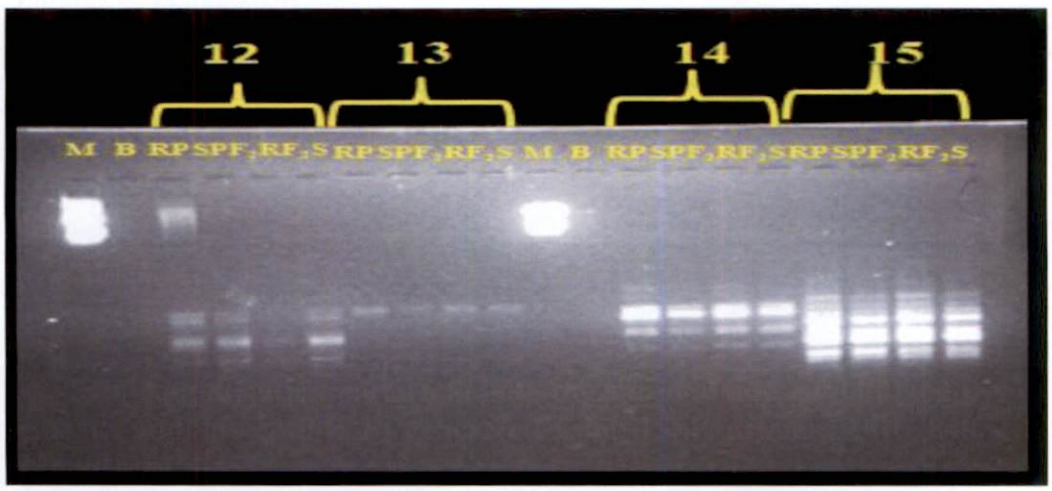
When the DNA of parents and bulked DNA of F_2 were analysed using the ISSR primer UBC 840, a maximum of seven amplicons were generated across each lane. The pattern of amplification is shown in Fig. 4.18(8). The molecular weight of the amplicons ranged from 0.25 to 1.5 kb. The amplicons generated were monomorphic with no association to YVMV resistance.



9: UBC 848 **10: UBC 812** **11: UBC 845**

Fig. 4.19: Amplification pattern of both the parental lines and bulked DNA of resistant and susceptible F_2 generated using ISSR primers UBC 848, UBC 812 and UBC 845

M: 1kb Molecular marker, **B:** Blank, **RP:** Resistant parent, **SP:** Susceptible parent, **F_2R:** F_2 resistant bulk and **F_2S:** F_2 susceptible bulk.



12: UBC 890 13: UBC 880 14: UBC 866 15: UBC 865

Fig. 4.20: Amplification pattern of both the parental lines and bulked DNA of resistant and susceptible F_2 generated using ISSR primers UBC 890, UBC 880, UBC 866 and UBC 865

M: 1kb Molecular marker, **B:** Blank, **RP:** Resistant parent, **SP:** Susceptible parent, **F_2R:** F_2 resistant bulk and **F_2S:** F_2 susceptible bulk.

4.3.2.9 UBC 848

On an average eight amplicons were obtained with the ISSR primer UBC 848. The patteren of amplification is shown in Fig. 4.19(9). The molecular weight of the amplicons ranged from 0.1 to 0.9 kb. Through this marker large numbers of amplicons were generated, and they were monomorphic for the character YVMV resistance.

4.3.2.10 UBC 812

Amplification with UBC 812 primer generated on an average eight amplicons in each lane. The molecular weight of the band varied from 0.25 to 1.5 kb [Fig. 4.19(10)]. All amplicons generated from this primer were monomorphic.

4.3.2.11 UBC 845

Total of six amplicons were generated on amplification of DNA using ISSR primer UBC 845 which electrophoresed on 1.5 percent agarose gel. No polymorphism was observed between the parents and bulked DNA [Fig. 4.19(11)]. All amplicons generated from this primer were monomorphic.

4.3.2.12 UBC 890

ISSR assay using the primer UBC 890 followed by electrophoresis on 1.5 percent agarose gel has generated five amplicons in each lane. The molecular weight of the band varied from 0.4 to 0.9 kb [Fig. 4.20(12)]. The amplicons generated were monomorphic with no association to YVMV resistance.

4.3.2.13 UBC 880

When the DNA of both the parents, bulked DNA of resistant and susceptible F_2 were analysed using the ISSR primer UBC 880, a maximum of 5 amplicons were generated across each lane. The pattern of amplification is shown in Fig. 4.20(13). The molecular weight of the amplicons ranged from 0.25 to 0.5 kb. The amplicons generated were monomorphic with no association with YVMV resistance.

4.3.2.14 UBC 866

On an average, six amplicons were obtained with the ISSR primer UBC 866. The pattern of amplification is shown in Fig. 4.20(14). The molecular weight of the amplicons ranged from 0.2 to 0.9 kb. The amplicons generated were monomorphic with no association with YVMV resistance.

4.3.2.15 UBC 865

Amplification with UBC 865 primer has generated on an average, nine amplicons in each lane. The molecular weight of the band varied from 0.1 to 1 kb [Fig. 4.20(15)]. All amplicons generated from this primer were monomorphic.

4.3.2.16 ISSR 23

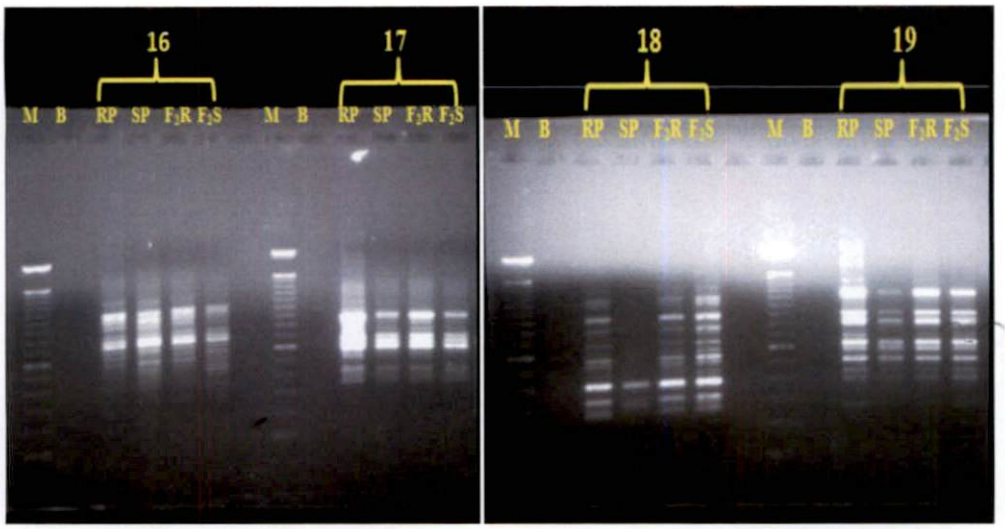
Total of eight amplicons were generated on amplification of DNA using ISSR 23 primer, which electrophoresed on 1.5 percent agarose gel. No polymorphism was observed between the parents and bulked DNA of resistsant and susceptible F_2 [Fig. 4.21(16)]. All amplicons generated from this primer were monomorphic.

4.3.2.17 ISSR 26

ISSR assay using the primer ISSR 26 followed by electrophoresis on 1.5 percent agarose gel generated, a maximum of ten amplicons in each lane. The molecular weight of the band varied from 0.3 to 1.5 kb [Fig. 4.21(17)]. The amplicons generated were monomorphic with no association to YVMV resistance.

4.3.2.18 P6

When the DNA of both the parental lines, bulked DNA of resistant and susceptible F_2 were analysed using the ISSR primer P6, a maximum of eleven amplicons were generated in all lanes except in susceptible parent which produced only two amplicons. The patteren of amplification is shown in Fig. 4.21(18). The molecular weight of the amplicons ranged from 0.2 to 1.5 kb. The amplicons generated were monomorphic with no association to YVMV resistance.



16: ISSR 23 **17: ISSR 26** **18: P6** **19: P9**

Fig. 4.21: Amplification pattern of both the parental lines and bulked DNA of resistant and susceptible F_2 generated using ISSR primers ISSR 23, ISSR 26, P6 and P9

M: 1kb Molecular marker, **B:** Blank, **RP:** Resistant parent, **SP:** Susceptible parent, **F_2R:** F_2 resistant bulk and **F_2S:** F_2 susceptible bulk.

Fig. 4.22: Amplification pattern of both the parental lines and bulked DNA of F_2 generated using ISSR primer UBC 854

M: 1kb Molecular marker, **B:** Blank, **RP:** Resistant parent, **SP:** Susceptible parent, **F_2R:** F_2 resistant bulk, **F_2S:** F_2 susceptible bulk and **20:** UBC 854.

4.3.2.19 P9

On an average ten amplicons were obtained with the ISSR primer P9. The pattern of amplification is shown in Fig. 4.21(19). The molecular weight of the amplicons ranged from 250 bp to 2500 bp. Through this marker, large no of amplicons were generated, which were monomorphic for the character YVMV resistance.

4.3.2.20 UBC 854

Amplification with UBC 854 primer generated on an average eight amplicons in each lane. The molecular weight of the band varied from 0.25 to 1.5 kb [Fig. 4.22(20)]. All amplicons generated from this primer were monomorphic.

4.3.2. 21 ISSR 8

Average of eleven amplicons was obtained in each lane, on DNA amplification with the primer ISSR 8. The pattern of amplification is shown in Fig. 4.23. The molecular weight of the bands varied from 350 bp to 1100bp. Amplicon obtained at 0.5 kb was distinctly polymorphic for resistance to YVMV. The polymorphic band of 0.5 kb was present in parent resistant and in F_2 resistant bulk but was absent in susceptible parent and F_2 susceptible bulk.

4.3.2.22 UBC 873

UBC 873 has yielded an average of twelve amplicons in each lane. The pattern of amplification is shown in Fig. 4.24. The molecular weight of the bands varied from 350 bp to 2600bp. Amplicon obtained at 900 bp was distinctly polymorphic for resistance to YVMV. This marker was present in resistant parent and in F_2 resistant bulk but absent in susceptible parent and F_2 susceptible bulk.

4.3.2.23 ISSR 22

DNA amplification with the primer ISSR 22 yielded six amplicons in susceptible parent and F_2 susceptible bulk, and five amplicons in resistant parent and F_2 resistant bulk. The pattern of amplification is shown in Fig 4.25. The

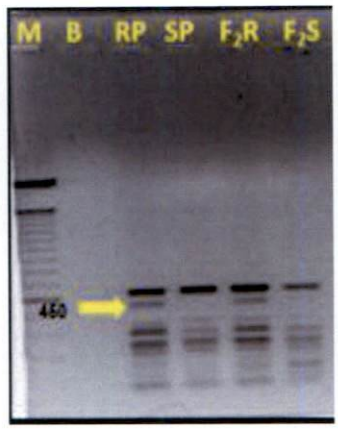


Fig. 4.23: Amplification pattern of both the parental lines and bulked DNA of F_2 generated using ISSR 8 primer

M: 1kb Molecular marker, **B:** Blank, **RP:** Resistant parent, **SP:** Susceptible parent, **F_2R:** F_2 resistant bulk and **F_2S:** F_2 susceptible bulk.

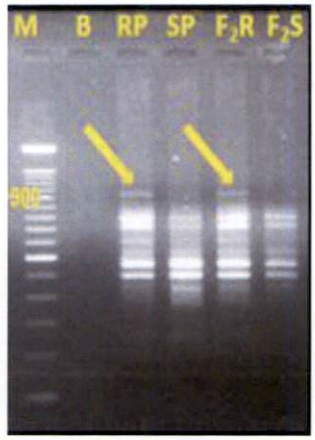


Fig. 4.24: Amplification pattern of both the parental lines and bulked DNA of F_2 generated using UBC 873

M: 3 kb Molecular marker, **B:** Blank, **RP:** Resistant parent, **SP:** Susceptible parent, **F_2R:** F_2 resistant bulk and **F_2S:** F_2 susceptible bulk.

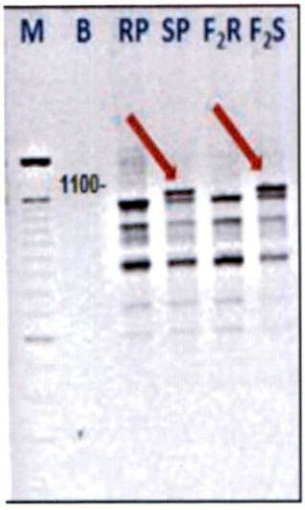


Fig. 4.25: Amplification pattern of both the parental lines and bulked DNA of F_2 generated using primer ISSR 22

M: 1kb Molecular marker, **B:** Blank, **RP:** Resistant parent, **SP:** Susceptible parent, **F_2R:** F_2 resistant bulk and **F_2S:** F_2 susceptible bulk.

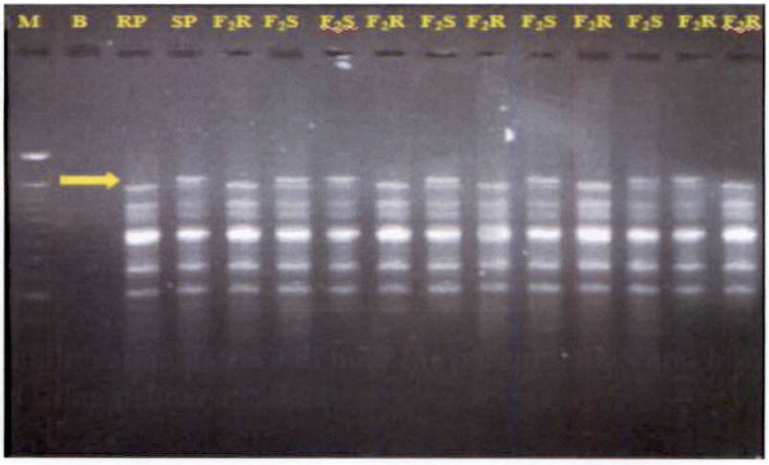


Fig 4.26: Co-segregation of ISSR 22 marker with YVMV resistance in F_2 individuals of the cross Salkeerthi X Parbhani Kranthi

M: 1kb Molecular marker, **B:** Blank, **RP:** Resistant parent, **SP:** Susceptible parent, **F_2R:** F_2 resistant and **F_2S:** F_2 susceptible.

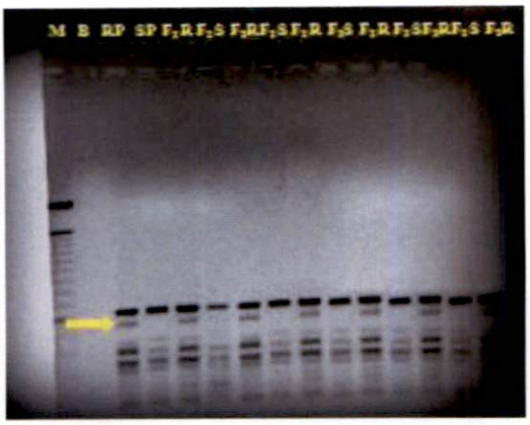


Fig 4.27: Co-segregation of ISSR 8 marker with YVMV resistance in F_2 individuals of the cross Salkeerthi X Parbhani Kranthi

M: 1kb Molecular marker, **B:** Blank, **RP:** Resistant parent, **SP:** Susceptible parent, **F_2R:** F_2 resistant and **F_2S:** F_2 susceptible.

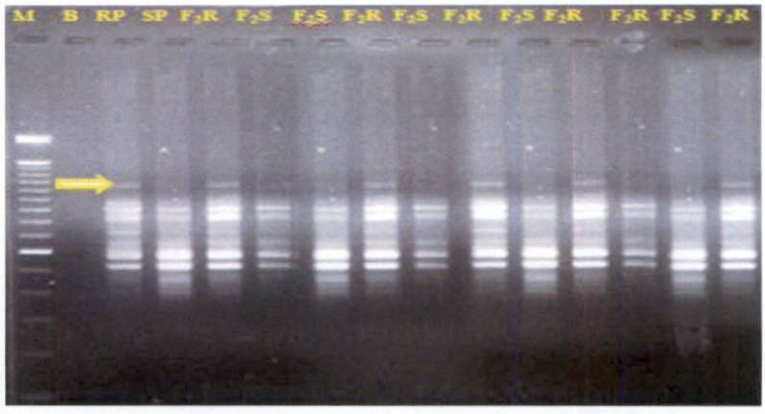


Fig 4.28: Co-segregation of ISSR primer UBC 873 with YVMV resistance in F_2 individuals of the cross Salkeerthi X Parbhani Kranthi

M: 1kb Molecular marker, **B:** Blank, **RP:** Resistant parent, **SP:** Susceptible parent, **F_2R:** F_2 resistant and **F_2S:** F_2 susceptible.

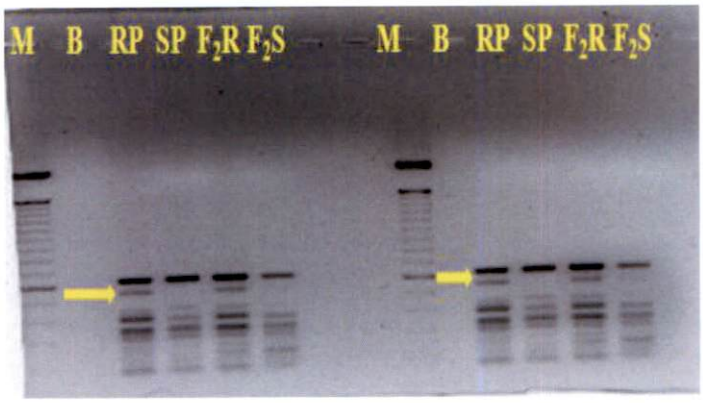


Fig 4.29: Reproducibility of primer ISSR with YVMV resistance in F_2 individuals of the cross Salkeerthi X Parbhani Kranthi

M: 1kb Molecular marker, **B:** Blank, **RP:** Resistant parent, **SP:** Susceptible parent, **F_2R:** F_2 resistant and **F_2S:** F_2 susceptible.

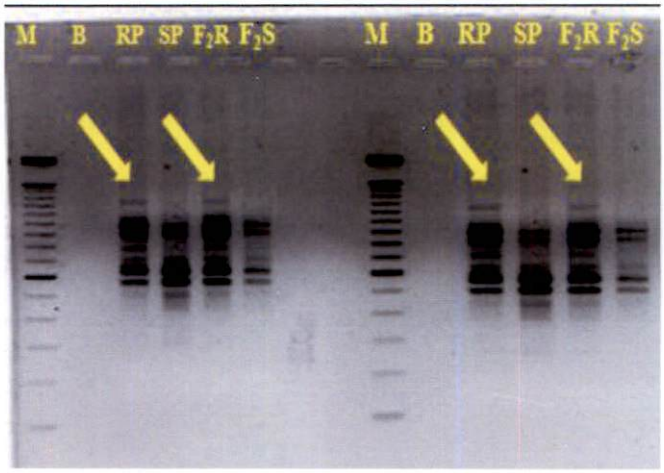


Fig 4.30: Reproducibility of primer ISSR UBC 873 with YVMV resistance in F_2 individuals of the cross Salkeerthi X Parbhani Kranthi

M: 1kb Molecular marker, **B:** Blank, **RP:** Resistant parent, **SP:** Susceptible parent, **F_2R:** F_2 resistant and **F_2S:** F_2 susceptible.

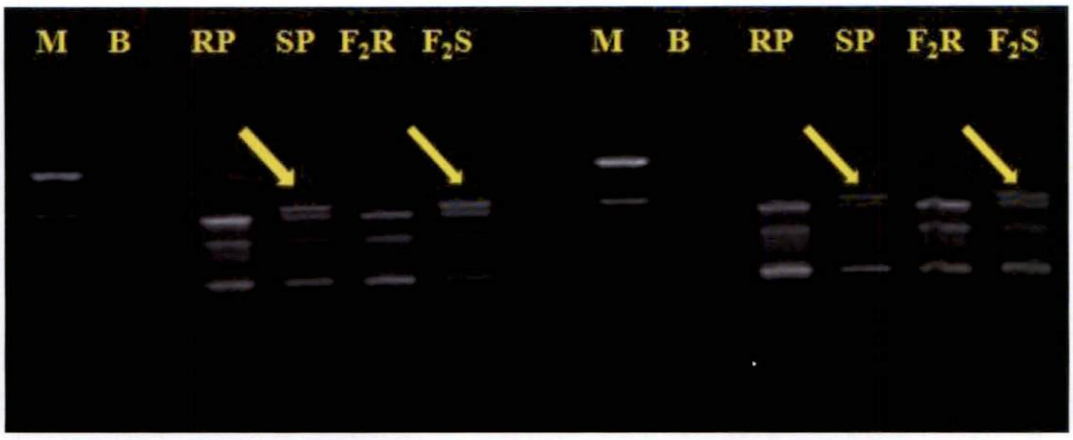


Fig 4.31: Reproducibility of primer ISSR 22 with YVMV resistance in F_2 individuals of the cross Salkeerthi X Parbhani Kranthi

M: 1kb Molecular marker, **B:** Blank, **RP:** Resistant parent, **SP:** Susceptible parent, **F_2R:** F_2 resistant and **F_2S:** F_2 susceptible.

molecular weight of the bands varied from 1000 bp to 2000bp. Amplicon at 1100 bp was distinctly polymorphic for the resistance to YVMV. This marker was present in susceptible parent and in susceptible F_2 bulk but absent in resistant parent and resistant F_2 bulk.

4.3.2.24 Cosegregation analysis with ISSR primers ISSR 8, ISSR 22 and UBC 873

Cosegregation analysis with the three ISSR primers ISSR 8, ISSR 22 and UBC 873 which were found linked with disease resistance of YVMV was performed and results are presented in Fig. 4.26, Fig. 4.27 and Fig 4. 28. The markers were found segregating with the gene for resistance.

4.3.3 Reproducibility of ISSR primers

Reproducibility was confirmed 2 times in each ISSR primer using same PCR programmes and conditions but in different PCR machines. The identified markers were reproducible for all primers (ISSR 8, UBC 873 and ISSR 22), as shown in Fig. 4.29, Fig. 4.30 and Fig. 4.31.

Discussion

5. DISCUSSION

Okra (*Abelmoschus esculentus* (L.) Moench) (Family: Malvaceae), also known as lady's finger, bhindi and gumbo is an important vegetable crop of the tropics and subtropics. The major problem in okra cultivation is the lack of location specific varieties/hybrids tolerant/resistant to biotic (diseases and pests) as well as abiotic stresses. In summer, when the environmental conditions are conducive for crop growth it is severely attacked by sucking pests and viral diseases. Among the viral diseases, yellow vein mosaic virus (YVMV) is one of the most destructive diseases transmitted by vector white fly (*Bemisia tabaci*). The characteristic symptoms are vein clearing, stunting, reduction in the number of leaves and vein chlorosis of leaves. The reported yield reduction due to this disease infection is in the range of 50 to 94 per cent depending on the stage of the crop growth at which infection occurs (Aparna *et al.*, 2012).

Conventional breeding involving germplasm collection, selection and heterosis breeding has resulted in the release of a number of varieties. Sharma *et al.* (1993) concluded that a variety "Parbhani Kranthi" was resistant to YVMV disease in okra and Kousalya *et al.* (2005) reported that the variety "Salkeerthi" has a wide range of acceptability owing to its early bearing habit, excellent fruit quality, attractive light green pods and high yield, but it is highly susceptible to YVMV. Hence it cannot be grown during summer when the disease is more prevalent. Also the transgenic approach shows potential for the genetic improvement for the crop using wide set of transgenes currently available which may confer viral resistance (Tripathi *et al.*, 2008).

The YVMV disease cannot be controlled adequately by chemical means. Frequent pickings, high operational cost and residues of pesticides entering food chain are the limiting factors for the chemical control of this disease (Prashanth *et al.*, 2008). Crop improvement by developing YVMV disease resistant varieties is more economical and environmentally safer than crop management using

chemicals. Several wild species of cultivated okra showed high degree of resistance to YVMV. However, resistant varieties developed by various research organizations by breeding methods such as interspecific hybridization and heterosis, have started showing sign of susceptibility probably due to the arrival of new virus strains. Hence it is imperative to find diverse sources of resistance to YVMV and evolve YVMV resistant varieties by marker assisted selection.

Genetic knowledge on the inheritance of resistance is important in breeding for resistance. Study on the nature of inheritance showed that YVMV resistance is monogenic and homozygous dominant (Arora *et al.,* 2008). The mode of resistance is still uncertain and the resistance is less influenced by environmental conditions such as temperature, pH and soil nature (Scott and Kasai, 2004).

Molecular markers like RAPD, RFLP, SSR, ISSR, AFLP and CAPS are commonly used to characterize genetic diversity within or between populations or groups of individuals because they typically detect high levels of polymorphism (Kumari, 2013). RAPD (Parani *et al.*, 1997) is a simple technique which detects polymorphism and has been used for molecular characterisation in several crop plants such as *Oryza sativa* L. (Chakravarthi and Naravaneni, 2006), *Piper nigrum* L. (Keshavachandran *et al.*, 2005) and *Manihot esculenta* crantz. (Santha, *et al.*, 2005), ISSR is a highly sensitive, reproducible and dominant marker system which has been successfully applied in genetic and evolutionary studies of many species, including fingermillet (Salimath *et al.,* 1995), wheat (Nagaoka & Ogihara, 1997), rice (Joshi *et al.,* 2000) and *Vigna* (Ajibade *et al.,* 2000).

Hence the present study "Development of molecular markers linked to yellow vein mosaic virus resistance to okra (*Abelmoschus esculentus* (L.) Moench)" was conducted. The methodology involved was BSA (Bulk Segregant Analysis). The study programme involved the following aspects.

1. Development of mapping population

2. Identification of molecular markers for yellow vein mosaic virus resistance gene form okra genotypes using RAPD and ISSR assay.

The results obtained on various aspects are discussed here.

5. 1 Development of mapping population

5.1.1 Identification of genotypes

In this study, there were two genotypes such as Parbhani Kranthi and Salkeerthi which are resistant and susceptible to YVMV respectively was used. Controlled crosses were made by selecting most susceptible plants of Salkeerthi and most resistant plants of Parbhani Kranthi as the pollen parent to generate the F_1 seeds.

Whenever a study is designed with the objective to screen any specific gene of interest, the best strategy is to choose near Isogenic Lines which differ only for the specific character. When no characterization of the gene of interest is done and sequence of the synthetic gene is not working in the concerned population, the alternate is to select the accessions differing widely in character expression and to go for Bulk Segregant analysis (BSA) (Michelmore *et al.*, 1991). Ultimately, the best strategy is to practice BSA with NILs and to analyse the polymorphism using powerful and dominant markers such as RAPD and ISSR.

5.1.2 Field screening of parental generations

In this study to identify highly resistant and susceptible plants in the parental lines, field screening of both the parents were done simultaneously in the same field in the summer months. No artificial inoculation methods were followed since the heavy population of white flies observed in summer months was sufficient to ensure the disease occurrence. There were no YVMV symptoms on

both the lines till 35 days from the date of sowing and the symptoms started appearing from the fifth week onwards. Vein clearing followed by yellowing of leaves and gradual reduction in plant growth were observed in infected plants. Parbhani Kranthi plants which were supposed to be resistant also started showing symptoms. Hence the plants which were resistant till the end of the crop were only selected for the crossing programme.

Earlier efforts led to the development and release of cultivars like Pusa Sawani (Singh *et al.,* 1962) and MDU-1 with field resistance to YVMV. In a field trial with 22 genotypes exposed to whiteflies *(B. tabaci)* carrying okra yellow vein mosaic virus, cv. Arka Anamika remained free from disease and five other genotypes were highly resistant (Bora *et al.,* 1992). Nath and Saikia (1993) screened 14 okra varieties for resistance to okra yellow vein mosaic virus by artificial inoculation and natural infection. None of the entries were immune to the disease.

5.1.3 Screening of F_1 generations

The raising of F_1 plants was carried out in the month of September-December 2014. 180 F_1 plants were raised and the morphological observations were recorded. All the F_1 plants were free from disease symptoms. The F_1 plants were selfed to produce F_2 seeds.

Arora *et al.* (2008) has reported that resistance to YVMV is offered by homozygous resistant condition that is responsible for resistant character. So among all F_1 plants that were crossed, all plants were found to be resistant to YVMV. This indicates the gene governing resistance in Parbhani Kranthi is dominant in action.

Mazumder *et al.* (1996) conducted experiments on the incidence of YVMV and its vector *Bemisia tabaci* in the cultivars Pusa Sawani, Parbhani Kranthi and M-31. Lower disease incidence of white fly populations were

recorded in crop sown between February 25 and March 20. He gave the statement that even ten whiteflies are sufficient to transfer virus to plants and hence produce the disease symptoms. Positive significant association was observed between disease incidence and white fly populations, high temperature, relative humidity (RH) of evening, rainfall and rain days.

Dhankhar (1996) developed YVMV resistant okra "Varsha Uphar" out of the cross between Lam Selection I x Parbhani Kranti. Fugro and Rajput (1999) used a partial diallel mating system involving nine genotypes, developed 36 F_1 hybrids, of which Sel-4 x Parbhani Kranti, Pusa Sawani x Punjab-7, Sel-4 x BO-1, Sel-4 x Punjab-7 and Sel-4 x Sel-10 were free from YVMV. Pathak *et al.* (1997) reported that the crosses Parbhani Kranti x HR-55 and Parbhani Kranti EC 16511 were found to be resistant to YVMV. Deo *et al.* (2000) found that Parbhani Kranti and its hybrid Parbhani Kranti x HRB-9-2 were highly resistant to YVMV. Rattan and Bindal (2000) in their programme to develop okra hybrids resistant to YVMV found that lines 407, 409, 417, 430 were completely resistant.

5.1.4 Screening of F_2 generations

Segregating population of 200 plants were raised in open field and screening was done naturally for confirmation of disease resistance. F_2 plants are reported to offer certain advantages over other mapping populations (DH lines, NIL's *etc.*,) because segregation is fixed (Benjamin and Burr, 1999). However plants from such segregating populations have to be grouped according to accurate phenotypic screening of the trait.

Hansen *et al.* (1997) carried out Bulked segregant analysis to identify RAPD markers in oilseed rape (*Brassica napus* L.) that were linked to a male fertility restorer gene for *Ogura* cytoplasmic male sterility. After screening 960 primers, 14 RAPD markers were mapped to a 25 cM region including the restorer

locus. A mapping population of 242 F_2 individuals were employed. Phenotypic data of a subset of the classified plants was compared with the RAPD data and the expected number of recombinants was calculated from the map data. A close correspondence between the expected and observed numbers of plants with a deviating phenotype was found.

Mehram and Dhapke (1981) reported that the hybrid between *A. esculentus* and *Abelmoschus tetraphyllus* was spreading in habit, dwarf in stature and highly male sterile. Interspecific crosses between two cultivars of *A. Esculentus*, a cultivar of YVMV resistant *A. manihot* and one of the reciprocals were successful. On comparison of parental F_1, F_2, BC_1 and BC_2 genotypes, heterosis over the better parent was observed for number of fruits per plant, plant height and number of branches (Sharma and Dhillon, 1983).

Bolek *et al.* (2005) developed molecular mapping of F_2 population derived from the interspecific cross of the highly tolerant *Gossypium barbadense* cv. Pima S-7 and the susceptible *G. hirsutum* cv. Acala 44 which phenotyped for disease incidence and severity. The F_2 phenotypic distribution of these parameters (number of healthy leaves, node number, leaf weight, stem weight, and total shoot weight) suggested that resistance is polygenic inherited. Microsatellites were used to reveal polymorphism between resistant and susceptible parents. A total of 255 simple sequence repeat (SSR) primer pairs were screened over bulks constituted by 10 resistant and 10 susceptible progeny. Sixty markers were used to analyze quantitative trait loci (QTLs). Three loci (CM12, STS1, 3147-2) had large effect on resistance to Verticillium wilt. Two loci were located on LG-1 and one on LG-2 and both linkage groups are located on chromosome 11.

Gemenet *et al.* (2010) identified four candidate markers (p-umc2189, p-bnlg1179 and p-bnlg1014 and p-umc1542) using SSR molecular markers in F_2 population derived from a cross of drought susceptible and tolerant maize landraces using bulked segregant analysis (BSA).

Kumbhar *et al.* (2013) developed F_2 population from a cross between rice (*Oryza sativa* L.) genotypes, 70 (highly susceptible to blast) and RDN 98-2-3-5-14 (resistant to blast), to study the inheritance of blast resistance and to identify the marker associated with resistance. The F_2 population segregated in 3:1 ratio for resistance: susceptible under hot spot conditions for blast suggesting monogenic control of resistance in this population. Bulk segregant analysis conducted using a total of 25 SSR markers identified two SSRs to be polymorphic between the parents and the corresponding bulks. One of these SSR markers RM204 which has been reported to be mapped on the short arm of chromosome 6 and in close proximity of blast resistance gene/QTLs in other studies showed expected segregation ratio (1:2:1) for single gene model in the F_2 population. This marker was found significantly associated with blast resistance on regression analysis.

5.2 Morphological characterization

The morphological characters including both qualitative and quantitative were observed as per the guidelines recommended by International Plant Genetic and Resources Institute (IPGRI, 2002). Evaluation of quantitative characters on F_1 in comparison with parental lines showed significant variation for the traits like plant height, petiole length, days to flowering, days to first harvest, number of fruits per plant and yield per plant.

5.3 Genomic DNA used for molecular characterization

Several published DNA extraction methods have been tried and found unsuitable for okra, due to the high levels of charged polysaccharides and polyphenolic compounds within the tissues. Other published protocols developed specifically for okra or plants with high amounts of polyphenolic compounds are complex and require extensive centrifugation steps, often with the use of

Ammonium acetate as a washing buffer (Kochko and Hamon, 1990; Meena *et al.*, 2014). After trying several modified protocols, the present DNA extraction method was developed for okra for further marker analysis.

The main obstacle preventing the extraction of DNA from green leaves of all members of the genus *Abelmoschus* is that, even after grinding in liquid nitrogen, the resulting suspension is very sticky and it is almost impossible to obtain DNA by a mini-preparation. This consistency is principally due to large amounts of polysaccharides produced during photosynthesis (Kochko and Hamon, 1990). For this reason it's necessary to purify total DNA using two to three leaf stage seedlings.

Modified Doyle and Doyle (1987) method was found to yield good quality DNA. In the standard protocol C: I treatment was given once but in modified method treatment was given three times to remove the excess mucilage. The DNA pellet was progressively washed with the wash buffer (76 per cent ethanol and 10mM ammonium acetate) to remove the stickiness. Treatment with the wash buffer progressively removes the brown color and stickiness of the DNA. In the CTAB method DNA isolated was unable to dissolve in the water due to its stickiness but the DNA from modified Doyle and Doyle method obtained was easily dissolved in distilled water.

The protocols tried mostly produced DNA with brown color. The brown coloration in the DNA pellets was mostly due to polyphenol and mucilage contamination (Couch and Fritz, 1990). Plants can vary considerably in the amount and number of secondary metabolites they produce and it is unlikely that any one technique for DNA extraction can be developed (Loomis, 1974). This developed DNA extraction protocol can be used to isolate nuclear DNA from a variety of other plant species especially high in polyphenols, tannins and mucilage.

5.3.1 Purification and quantification of DNA

Zimmermann *et al.* (1998) reported the methods for purifying nucleic acids from cell extracts through techniques such as extraction/precipitation, chromatography, centrifugation and affinity separation. In the present study, the quality of DNA was tested by subjecting it to agarose gel electrophoresis as well as spectophotometric method. DNA was visualized on 0.8 per cent agarose gel under UV light by ethidium bromide staining. A DNA sample is reported as of high quality if it had a band of high molecular weight with little smearing and a low amount of RNA (Wettasingf and Peffley, 1998). The DNA extracted showed less amount of RNA.

In the spectrophotometric method, the ratio of optical density at 260 and 280 nm was worked out to test the quality. The absorbance ratio was calculated as OD at 260/280, for the various samples. Those samples with ratio between 1.8 and 2.0 were considered to be of high quality. All the samples under the study recorded a ratio between 1.8 and 2.06 (Thakur *et al.*, 2014).

5.3.2 Bulk Segregant Analysis with RAPD and ISSR primers

Molecular characterization of the susceptible and resistant okra genotypes and their F_2 susceptible and resistant population was carried out with the objective of identifying marker for YVMV resistance in okra. Powerful, dominant (RAPD) and highly reproducible (ISSR) markers were used for the characterization.

BSA was carried out with the DNA from resistant parent (Parbhani Kranthi), susceptible parent (Salkeerthi), resistant bulk (7 most resistant plants from F_2) and susceptible bulk (7 most susceptible plants from F_2). The segregating population of F_2 plants originated from the resistant F_1 plants by selfing. The specific RAPD and ISSR primers which are reportedly suitable for the plants

belonging to Malvaceae family and supplied by SIGMA, Pvt, Ltd., Thrissur was used for BSA.

BSA involves comparing two pooled DNA samples of individuals from a segregating population originating from a single cross and the advantages of BSA over other genetical techniques for gene "tagging", is well demonstrated (Michelmore *et al.,* 1991). Within each bulk, the individuals are identical for the trait or gene of interest but are arbitrary for all other genes. BSA removes the need for screening the entire mapping population with every primer. The minimum size of the bulk is determined by the allelic frequency with linked loci and generally it is recommended that the number of plants from F_2 population used for DNA bulking should not be less than 5 or should not exceed 10 (Michelmore *et al.,* 1991).

5.4 Molecular marker analysis

As of now, there is no marker system for the identification of YVMV resistance gene in Okra. By considering the potential of molecular markers, an attempt to identify RAPD (Random Amplified Polymorphic DNA) and ISSR (Inter Simple Sequence Repeats) markers linked with the YVMV resistance gene has been made. RAPD proved to be the best markers since they are simple, less technology intensive, cheap and does not require pre-sequencing for designing primers (Williams *et al.,* 1990; Aladele, 2008; Azmat and Khan, 2010). As such, RAPD markers have been used widely in various genetic diversity studies of okra, including genetic mapping and identification of quantitative trait loci (QTLs) (Queiroz *et al.,* 2003; Brown *et al.,* 2005). ISSR targets the highly variable microsatellite regions of the nuclear genome providing a large number of polymorphic fragments (Gupta *et al.,* 1994). Therefore, this technique has been used extensively to evaluate genetic diversity both within and between plant populations in angiosperms and gymnosperms (Osborn *et al.,* 2005). The high level reproducibility of ISSR markers is already well established in many crops

and hence these markers offer the potential for direct usage in MAS (Bornet and Branchard, 2001; Reddy *et al.*, 2002).

5.4.1 RAPD analysis

The RAPD technique was developed by Williams *et al.*, (1990) and the technique relies on the differential enzymatic amplification of small DNA fragments using PCR with arbitrary decamer primers. In RAPD markers, polymorphism results from the changes in the sequence of the primer binding site. Usually RAPD markers are dominant in nature (Waugh and Powell, 1992) because polymorphisms are detected as the presence or absence of bands. In the present study the use of high quality DNA helped in getting reproducible bands using standardized conditions for the thermal cycler.

However, the advantages of RAPD include simplicity, rapidity, requirement for only a small quantity of DNA and ability to generate polymorphism (Cheng *et al.*, 1997). The RAPD amplification can be classified in to two types: constant (monomorphic) and variable (polymorphic) between the genotypes. These differences can be used to examine and establish systemic relationships (Handrys *et al.*, 1992).

In the present study 84 RAPD primers have been screened and 39 primers were selected based on the number of bands and nature of amplification. Out of 39 primers selected only 2 makers such as OPB 11 and OPL 18 are capable of yielding polymorphic bands which are linked with resistance to YVMV.

The RAPD primer OPB 11 was capable of yielding 3 polymorphic bands with the amplicons of 800, 1000 and 1100 bp in resistant parent and F_2 resistant lanes, but was completely absent in all parent susceptible and F_2 susceptible bulk. The assay has generated distinctly polymorphic banding patterns to differentiate the resistant okra lines from the susceptible lines.

Another RAPD primer OPL 18 which produced distinct polymorphic bands of 1 and 1.1 kb were present in resistant parent and in F_2 resistant bulk but absent in susceptible parent and F_2 susceptible. This primer was able to differentiate clearly between resistant and susceptible lines. Amplicons obtained with this primer were distinctly polymorphic for the resistance to YVMV. With these clear marker patterns, this RAPD was found to be highly successful in differentiating the YVMV resistant and susceptible lines.

Sartorato *et al.* (2000) reported that the RAPD and SCAR markers linked to resistance gene to angular leaf spot in common bean. They identified three RAPD markers in the F_2 population. The polymorphism observed after amplification was identical to the one revealed with corresponding RAPD marker.

5.4.2 ISSR analysis

The marker system called ISSR (Inter Simple Sequence Repeats) is a PCR based method that access variation in the numerous microsatellite regions dispersed throughout the genome. Zietkiewicz *et al.*, (1994) reported that primers based on microsatellites are utilized to amplify inter simple sequence repeat sequences in the DNA. When the primer successfully locates two microsatellite regions within an amplifiable distance away on the two strands of the template DNA, the PCR reaction generates a band of a particular molecular weight for that locus representing the intervening stretch of DNA between the microsatellites. The method uses a single oligonucleotide primer composed of 4 to 10 tri or di nucleotide repeats ending with 3'- or 5'- anchor sequence.

The ISSR marker requires small amounts of DNA and does not require information on DNA sequence. ISSR primers are designed from SSR motifs and can be undertaken for any plant species containing a sufficient number and distribution of SSR motifs in the genome (Gupta *et al.*, 1996; Buhulikar *et al.*,

2004). ISSR targets the highly variable microsatellite regions of the nuclear genome providing a large number of polymorphic fragments (Gupta *et al.,* 1994). Therefore this technique has been used extensively to evaluate genetic diversity both within and between plant populations in angiosperms and gymnosperms (Osborn *et al.*, 2005). The high level reproducibility of ISSR markers is already well established in many crops and hence these markers offer the potential for direct usage in MAS (Bornet and Branchard, 2001; Reddy *et al.*, 2002).

The inter-simple sequence repeat (ISSR) markers have been widely used in studies on genetic diversity and variability of wild populations. They are better than others since they do not require prior information of the DNA sequence, have low development costs, and have high transferability to other plant species, and generate a greater number of polymorphic fragments (Barth *et al.*, 2002; Brandão *et al.*, 2011).

In this study, ISSR 8 yielded distinct polymorphic band of size 500 bp. This band was present in resistant parent and resistant F_2 lane but absent in susceptible parent and F_2 susceptible. This ISSR primer is tightly linked with the YVMV disease resistant gene. Since it is missing in all the susceptible lines and present only in resistant lines, it is clear that the single dominant gene is contributing for resistance. Many diseases are reported to be dominant in nature (Parlevliet *et al.*, 1985; Caranta *et al.*, 1997; Niks *et al.*, 2000; Lindhout, 2002). Its presence itself leads to high level of resistance and others will be contributing towards its expression. Another primer UBC 873 produces the distinct polymorphic amplicon of 900 bp size in resistant clones alone. Apart from the 950 bp all other amplicons are monomorphic. This marker appears to be a very important and since it is present in both the resistant parent and F_2 resistant lane, it could be inferred that this marker is associated with YVMV resistance in okra. More over it could be observed that this gene has some kind of segregating action. This kind of segregating gene action was previously reported by Tan and Yeung

(1988). This gene could be targeted for developing resistant line in marker assisted selection.

ISSR 22 also showed the polymorphism at 1100 bp especially in parent susceptible and F_2 susceptible lanes but was absent in parent resistant and F_2 resistant. The banding pattern generated by the primer ISSR 22 shows that this marker is associated with a gene which has low level contribution to resistance, which confirmed through Co-segregation analysis. Obviously, the presences of this marker in all the susceptible lines show that this gene is an indispensible part of the gene groups which contribute towards susceptibility to YVMV in okra. .

5.5 Possible applications of RAPD and ISSR primers in Marker Assisted Selection (MAS)

Breeders want to incorporate agronomically interesting resistance traits in their breeding material. Marker assisted selection is a technique that can facilitate this process by the selection of genes that control traits of interest such as disease resistance. PCR based markers for *Sw*-5 gene of tomato spotted wilt virus resistant have been used in tomato MAS (Shi *et al.*, 2011). In the context of MAS, ISSR 8, ISSR 22 and UBC 873, OPB 11 and OPL 18 markers can be effectively utilized for tracing favourable allele (s) (dominant or recessive) across generations and identify the most suitable individuals among the segregating progeny based on allelic composition across a part or the entire genome.

5.6 Future line of work

The detection of reproducible molecular markers for identifying resistant gene bearing plants in the breeding population was a challenge till date. The present study had revealed 5 markers in RAPD and 3 markers in ISSR which could be directly used in marker assisted selection. The future line of work includes

1. Validation of identified ISSR markers using individuals F_2 and F_3
2. Development of SCAR markers from RAPD markers
3. Identifying the gene(s) governing resistance to YVMV
4. Marker phenotyping of 200 F_2 plants.

Summary

SUMMARY

The study entitled "Development of molecular markers linked to yellow vein mosaic resistance in okra [*Abelmoschus esculentus* (L.) Moench]" was carried out at the Center for Plant Biotechnology and Molecular Biology, College of Horticulture, Vellanikkara during the period 2013-2015. The objective of the study was to identify the RAPD and ISSR markers linked to the gene offering resistance to yellow vein mosaic virus disease in okra. Species of okra viz., Parbhani Kranthi and Salkeerthi varieties former being resistant and latter susceptible to YVMV disease were collected from MKV, Parbhani, Maharastra and Kerala Agricultural University, respectively. The F_2 mapping population was developed using resistant and susceptible plants of the varieties and used for identification of markers through BSA.

The salient findings of the study are as follows:

1. Field screening of parents such as Salkeerthi (susceptible) and Parbhani Kranthi (resistant) was done during Feb-June 2014. Truly resistant six Parbhani Kranthi plants and truly susceptible ten Salkeerthi plants were identified through field screening and crossing was done between selected plants of Salkeerthi X Parbhani Kranthi. Twenty four morphological observations including eight quantitative and sixteen qualitative characters were recorded on both the accessions and their F_1 hybrid.

2. 180 F_1 plants were raised during Sept-Dec 2014. F_1 populations were morphologically uniform and no plant expressed YVMV symptoms. F_1 plants were selfed to obtain the F_2 seeds.

3. Evaluation of qualitative and quantitative characters on F_1 in comparison with parental lines showed significant variation for the traits like immature fruit color, mature fruit color, stem color, plant height, petiole length, days to flowering, days to first harvest, number of fruits per plant and yield per plant.

4. 200 F_2 plants were field screened during Jan-May 2015. Seven highly resistant and seven highly susceptible plants were identified and DNA was isolated from each one separately and the DNA of resistant F_2 plants and susceptible F_2 were bulked separately.

5. Co efficient of infection on Parbhani Kranthi, Salkeerthi, F_1 and F_2 population was found resistant, susceptible, highly resistant and resistant respectively.

6. The protocol suggested by Doyle and Doyle (1987) with slight modification (chloroform: isoamyl alcohol treatment was given two times to remove the protein contamination and washed with 76 per cent ethanol plus 10mM ammonium acetate) was found ideal for isolation of genomic DNA from the okra species. The RNA contamination was completely removed through RNase treatment.

7. The quality and quantity of DNA was analysed using NanoDropR ND-1000 spectrophotometer. The absorbance ratio ranged from 1.80-2.06, which indicated good quality DNA and the recovery, was high with 1563 ng/µl of DNA.

8. Protocol for RAPD and ISSR assay in okra were standardised with the various quantities of DNA, PCR mixtures and conditions for DNA amplification.

9. Two molecular marker systems namely, RAPD and ISSR were employed for identification of markers linked YVMV resistance. A total of 84 RAPD primers and 82 ISSR primer pairs were initially screened for their ability to amplify the DNA fragments. Out of these, 39 RAPD primers and 23 ISSR primer pairs were selected based on the number of bands and nature of amplification for performing bulk segregant analysis (BSA).

10. Out of thirty nine RAPD primers selected, two primers OPB11 and OPL 18 produced distinct polymorphism in relation to YVMV resistance. OPB 11 yielded markers at 800, 1000 and 1100 bp size where as OPL 18 yielded distinct markers at 1000 and 1100 bp.

11. Out of twenty three ISSR primers screened two primers ISSR 8 and UBC 873 yielded distinct polymorphism in relation to YVMV resistance and ISSR 22 primer gave a distinct marker in relation to YVMV susceptibility.

12. Co-segregation analysis was performed using individual DNA of resistant parent, susceptible parent, resistant F_2 and susceptible F_2 individuals using RAPD primers OPB 11 and OPL 18. OPB 11 primer produced three distinct markers in all resistant F_2 individuals.

13. Co-segregation analysis was performed using individual DNA of resistant parent, susceptible parent, resistant F_2 and susceptible F_2 individuals using ISSR primers ISSR 8, UBC 873 and ISSR 22. ISSR 8 primer produced distinct marker of 500 bp in all resistant F_2 individuals.

14. OPB 11, OPL 18, ISSR 8, UBC 873 produced distinct polymorphic bands in individual DNA bulk with respect to YVMV resistance and ISSR 22 produced distinct polymorphic bands in individual bulk with respect to YVMV susceptibility.

15. The RAPD and ISSR markers identified through this study could be employed in marker assisted selection aimed to develop YVMV resistance and high yielding okra varieties.

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Annexures

ANNEXURE I

List of laboratory equipments used for the study

Refrigerated centrifuge : Kubota 6500,

Japan

Horizontal electrophoresis system : BioRad, USA

Thermal cycler : Veriti Thermal Cycler
(Applied Biosystem, USA)

Gel documentation system : BioRad, USA

Nanodrop® ND-1000 spectrophotometer :

Nanodrop®Technologies Inc. USA

ANNEXURE II

Reagents required for DNA isolation

Reagents:

1. 2x CTAB extraction buffer (100 ml)

CTAB (Cetyl trimethyl ammonium bromide)	:	2g
Tris base	:	1.21 g
EDTA	:	0.745 g
NaCl	:	8.18 g
PVP	:	1.0 g

Adjusted the pH to 8 and made up final volume up to 100 ml.

2. Chloroform- Isoamyl alcohol (24:1 v/v)

To chloroform (24 parts), isoamyl alcohol (1 part) was added and mixed properly.

3. Chilled isopropanol

Isopropanol was stored in refrigerator at 0°C and was used for the study.

4. Wash buffer

Ethyl alcohol 76 ml and distilled water 24 ml.
Ammonium acetate 0.077 g
Mix the 0.077 gm ammonium acetate in 100 ml 76% ethyl alcohol and mix well.

5. Ethanol (70 %)

To the 70 parts of absolute ethanol (100 %), 30 parts of sterile distilled water was added to make 70 per cent ethanol.

6. TE buffer (pH 8, 100 ml)

Tris HCl (10 mM) : 0.1576 g
EDTA (1 mM) : 0.0372 g (The solution was prepared, autoclaved and stored at room temperature).

ANNEXURE III

Composition of Buffers and Dyes used for Gel electrophoresis

1. TAE Buffer 50X

Tris base	:	242 g
Glacial acetic acid	:	57.1 ml
0.5M EDTA (pH 8.0)	:	100 ml

2. Loading Dye (6X)

0.25% bromophenol blue

0.25% xylene cyanol

30% glycerol in water

3. Ethidium bromide

The dye was prepared as a stock solution of 10 mg/ml in water and was stored at room temperature in a dark bottle.

DEVELOPMENT OF MOLECULAR MARKERS LINKED TO YELLOW VEIN MOSAIC RESISTANCE IN OKRA

[*Abelmoschus esculentus* (L.) Moench]

By

VINUTHA J. S.
(2013-11-105)

ABSTRACT OF THE THESIS
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ABSTRACT

Yellow vein mosaic is the most serious disease leading to 50-94 per cent yield loss in okra. None of the chemical measures are successful to save an infected plant and breeding the resistant varieties is the most accepted strategy in the management of this disease. Molecular breeding through marker assisted selection is more advantageous over the conventional breeding since it will help to assure the presence of the gene in the breeding lines and avoid the selection of pseudo-resistant line. As of now, there are no markers for the identification of YVMV resistance gene in okra. Hence the study was undertaken with the objective to identify ISSR and RAPD markers linked with the gene governing resistance to the yellow vein mosaic virus disease in okra through bulked segregant analysis to enable marker assisted selection.

In the present investigation, two okra genotypes namely Parbhani Kranthi (YVMV resistant) and Salkeerthi (YVMV susceptible, early bearing, excellent fruit quality) were used. Field screening of both the parental lines was done simultaneously in the same field during February-May 2014. No artificial inoculation methods were followed since the heavy population of white flies observed in the summer months was sufficient to ensure the disease occurrence and crossing was done between the selected plants (Salkeerthi X Parbhani Kranthi). The seeds were harvested from the crossed plants and subsequently used to raise F_1 population.

The raising of F_1 plants was carried out in the month of September-December 2014 and the morphological characteristics and disease response of 180 F_1 plants were recorded. All the F_1 plants were free from disease symptoms. The F_1 plants were selfed to produce F_2 seeds. The F_2 population with 200 plants was field screened during January-April 2015. Seven highly susceptible and 7 highly resistant plants were identified, DNA isolated from each, resistant and susceptible DNAs bulked separately and used for Bulked Segregant Analysis (BSA). For the

extraction of good quality DNA, the CTAB method (Doyle and Doyle, 1987) may be modified by avoiding the liquid nitrogen while grinding the plant tissue and additionally washing the DNA pellet with wash buffer to remove the mucilage content.

Evaluation of quantitative characters on F_1 in comparison with parental lines showed variation for the traits such as plant height, petiole length, days to flowering, days to first harvest, number of fruits per plant and yield per plant.

Two molecular marker systems namely, RAPD and ISSR were employed for identification of markers linked with YVMV resistance. A total of 84 RAPD primers and 82 ISSR primers were initially screened for their ability to amplify the DNA fragments. Out of these, 39 RAPD primers and 24 ISSR primers were selected based on the number of bands and nature of amplification. In BSA, two RAPD primers OPB11 and OPL 18 yielded markers linked with resistance to YVMV. OPB11 produced distinct polymorphic bands of 800, 1000 and 1100 bp sizes whereas, OPL 18 produced polymorphic bands of 1000 and 1100bp. Two ISSR primers ISSR 8 and UBC 873 yielded distinct polymorphic bands in relation to YVMV resistance. The ISSR 8 and UBC 873 yielded the markers at 500 and 900 bp, respectively. Another primer ISSR 22 gave a distinct marker at 1100 bp size, linked to susceptibility to YVMV. Co-segregation analysis was performed using individual DNA of resistant parent, susceptible parent, resistant F_2 and susceptible F_2 using RAPD primers OPB 11, OPL 18 and ISSR primers namely ISSR 8, ISSR 22 and UBC 873. OPB 11 produced three distinct markers in all resistant F_2 individuals. ISSR 8 produced distinct marker of 500 bp in all resistant F_2 individuals. ISSR markers were found to be reproducible and they are recommended for use in marker assisted selection for resistance to YVMV in okra.