MARKER ASSISTED SELECTION FOR INTROGRESSION OF BACTERIAL BLIGHT (BB) RESISTANCE GENES IN RICE (*Oryza sativa* L.)

By

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CERTIFICATE - I

This is to certify that this dissertation entitled, **“Marker Assisted Selection for Introgression of Bacterial Blight (BB) Resistance Genes in Rice (*Oryza sativa* L.)**” submitted for the degree of **Doctor of Philosophy** in the subject of Biotechnology and Molecular Biology to the Chaudhary Charan Singh Haryana Agricultural University, Hisar, is a bonafide research work carried out by **Rekha Malik (Admn. No. 2008BS9D),** under my supervision and that no part of this dissertation has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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This is to certify that this thesis entitled, **“Marker Assisted Selection for Introgression of Bacterial Blight (BB) Resistance Genes in Rice (*Oryza sativa* L.).”** submitted by **Rekha Malik** to the Chaudhary Charan Singh Haryana Agricultural University, Hisar, in partial fulfillment of the requirements for the degree of **Doctor of Philosophy**, in the subject of Biotechnology and Molecular Biology, has been approved by the Student's Advisory Committee after an oral examination on the same in collaboration with an External Examiner.

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CONTENTS

LIST OF TABLES

LIST OF PLATES

Plate No.	Description
1	Electrophoretic pattern of purified high molecular weight genomic DNA free from protein and RNA extracted from the parental genotypes of rice.
2	Electrophoretic pattern of PCR amplified fragments of parental rice genotypes using STS marker pTA248 specific for *Xa21*gene.
3.	Electrophoretic pattern of PCR amplified fragments of parental rice genotypes using STS marker RG136 (Digested with R.E *Hinf* I) specific for *xa13* gene.
4.	Electrophoretic pattern of PCR amplified fragments of parental rice genotypes using STS marker RG556 (Digested with R.E *Dra* I) specific for *xa5* gene.
5	Electrophoretic pattern of purified high molecular weight genomic DNA free from protein and RNA extracted from F_1 plants of cross CSR-30 x Pusa Basmati-1460.
6	Foreground selection at F_1 generation (CSR-30 x Pusa Basmati-1460) by screening of plants using *Xa21* linked marker pTA248.M- 100 bp ladder, D (Donor parent) - IRBB-60, R (Recurrent parent) – CSR-30. 1-28:F_1 plants of the cross CSR-30 x Pusa Basmati-1460. Arrow indicates plants having *Xa21* gene.
7	Foreground selection at F_1 generation (CSR-30 x Pusa Basmati-1460) by screening of plants using *xa13* linked marker RG136 (Digested with R.E *Hinf* I).M- 100 bp ladder, D (Donor parent) - IRBB-60, R (Recurrent parent) – CSR-30. 1-28:F_1 plants of the cross CSR-30 x Pusa Basmati-1460. Arrow indicates plants having *xa13* gene.
8	Electrophoretic pattern of purified high molecular weight genomic DNA free from protein and RNA extracted from four F_1 plants of cross CSR-30 x Pusa Basmati-1460.
9	Foreground selection at F_1 generation (CSR-30 x Pusa Basmati-1460) by screening of plants using *Xa21* linked marker pTA248.M- 100 bp ladder, D (Donor parent) - IRBB-60, R (Recurrent parent) – CSR-30. 1-4:F_1 plants of the cross CSR-30 x Pusa Basmati-1460. Arrow indicates plants having *Xa21* gene.
10	Foreground selection at F_1 generation (CSR-30 x Pusa Basmati-1460) by screening of plants using *xa13* linked marker RG136 (Digested with R.E *Hinf* I).M- 100 bp ladder, D (Donor parent) - IRBB-60, R (Recurrent parent) – CSR-30. 1-4:F_1 plants of the cross CSR-30 x Pusa Basmati-1460. Arrow indicates plants having *xa13* gene.
11	Foreground selection at F_1 generation by screening of plants using *Xa21*, *xa13*&*xa5* linked marker pTA248, RG136 (digested with R.E *Hinf* I) & R556 (digested with R.E *Dra* I). M- 100 bp ladder. 1-6: F_1 plants of the cross CSR-30 x IRBB-60. Arrow indicates plants having *Xa21*, *xa13* and *xa5* BB resistance genes.
12	Electrophoretic pattern of purified high molecular weight genomic DNA free from protein and RNA extracted from 230 BC_1F_2 plants.
13	Foreground selection of 230 BC_1F_2 plants using *Xa21* linked marker pTA248.M- 100 bp ladder, D (Donor parent) - IRBB-60, R (Recurrent parent) – CSR-30. 1-230-BC_1F_2 plants. Arrow indicates plants having *Xa21* gene.
14	Foreground selection of BC_1F_2 plants 'positive' for *Xa21* using *xa5* linked marker RG556 (digested with R.E *Dra* I).M- 100bp ladder, D- (Donor parent) IRBB-60, R- (Recurrent parent) CSR-30. Arrow indicates plants having *xa5* gene.
15	Foreground selection of BC_1F_2 plants positive' for both *Xa21* and *xa5* using *xa13* linked marker RG136 (digested with R.E *Hinf* I).M- 100bp ladder D (Donor parent) - IRBB-60, R (Recurrent parent) - CSR-30. Arrow indicates plants having *xa13* gene.
16	Electrophoretic pattern of parental genotypes of rice with primers (RM 1125, 1134, 1136, 1146, 1150, 1152, 1164, 1178, 1182, 1186, 1189, 1208) showing polymorphism among them.M: Marker (100bp ladder). P1- IRBB-60, P2- CSR-30, P3- Pusa Basmati-1460, P4- HBC-19. Circles showing polymorphic bands.\

LIST OF FIGURES

ABBREVIATIONS

AFLP	Amplified fragment length polymorphism
bp	Base pair
BB	Bacterial blight
CAPS	Cleaved amplified polymorphic sequences
CTAB	Cetyl trimethyl ammonium bromide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
EDTA	Ethylene diamine tetra acetic acid
MAS	Marker-assisted selection
NTSYS-PC	Numerical taxonomy and multivariate analysis system programme
PCR	Polymerase chain reaction
QTL	Quantitative trait loci
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
Rpm	Revolution per minute
RNA	Ribonucleic acid
RNase	Ribonuclease
RPG	Recurrent parent genome
SSR	Simple sequence repeat
STS	Sequence tagged site
Taq	*Thermus aquaticus*
TBE	Tris Boric EDTA
TE	Tris EDTA
Tris	2 amino-2 (hydroxymethyl)-1, 3-propanediol
UPGMA	Unweighted pair group methods with arithmetic average
w/v	Weight by volume
Xoo	*Xanthomonas oryzae* pathovar *oryzae*

CHAPTER-I

INTRODUCTION

Rice (*Oryza sativa* L. $2n=24$) is an important cereal food crop that serves as a major carbohydrate source for nearly half of the world's population. It is a nutritious cereal crop that provides 20 per cent of the calories and 15 per cent of protein consumed by world's population (Bouman, 2003). Besides, being the chief source of carbohydrate and protein in Asia, it also provides minerals and fiber. Asia accounts for 90 per cent and 92 per cent of world's rice area and production respectively. In India, rice occupies an area of about 44 million hectares with production of 100.0 million tonnes (FAO, 2012) and thus, India is the largest rice growing country accounting for about one-third of the world acreage. The importance of rice is represented both by large productivity and by the superior quality of its proteins. It is a rich source of sulphur containing amino acids cysteine and methionine. Also, it is an excellent source of manganese, magnesium, thiamin, niacin and Vitamin B_6. Rice straw and bran are important animal feed in many countries. It is probably the most important grain with regard to human nutrition and calorie intake providing more than one fifth of the calories consumed worldwide by the human species (FAO, 2011).

Rice, a member of the family Poaceae, is widely grown in tropical and subtropical region. It is a self-pollinated monocot plant species that has the smallest genome among monocots with genome size of 389 Mb (IRGSP, 2005). The genus consists of 23 wild and weedy species and two cultivated species, *viz.*, the *O. sativa* and *O. glaberrima*. *Oryza sativa* is mainly domesticated in Asia while *Oryza glaberrima* in western tropical Africa. Among the various varieties of rice grown world-wide, fine grain aromatic (Basmati) rice constitute a small but special group of rice, which are considered best in quality.

Basmati rice is characterised by extra-long, super fine slender grains, pleasant and exquisite aroma, soft texture and extra elongation with least breadth wise swelling in cooking. Various basmati varieties are traditionally grown in the northern and north western parts of Indian subcontinent. The unique combination of grain, cooking and eating characteristics had evolved the aromatic Basmati as a prized commercial commodity (Khush and Dela Cruz, 2002). Basmati rice ensures higher returns to farmers as it is priced three times higher than non- Basmati rice in the international as well as in the Indian domestic markets. Globally, annual Basmati export market alone is valued at US$ 1.0 billion and is reported to be on the rise (Archak *et al*., 2011). Among the Basmati rice varieties exported from India, the traditional Basmati cultivars have superiority for their unmatched grain, cooking and eating quality over the recently developed evolved Basmati cultivars (Shobha Rani *et al*., 2006). The traditional Basmati rice of Indian sub-continent clinches higher price compared to relatively inferior Basmati rice varieties and long-grain *indica* rice types (Bhasin, 2000).

Bacterial blight (BB) caused by the rod-shaped bacterium, *Xanthomonas oryzae* pv. *Oryzae* (*Xoo*) is one of the most devastating diseases in rice (*Oryza sativa)* and serves as the major constraints for rice production and a major threat to sustainable Basmati rice production. The disease was first characterized in Fukuoka, Japan in 1884 (Ou, 1985). This disease, which occurs as vascular wilt at the early stages of crop growth (nursery to tillering) and as leaf blight at later stages (panicle initiation to flowering), severely affects production of rice cultivated in Asia. In the northern plains of India, including the State of Punjab, Haryana and Uttar Pradesh, the disease is a serious problem, as rice is grown under irrigated and high fertilizer input conditions that are conducive to disease development. It causes leaf wilting, affects photosynthesis, reduces 1000 grain weight and can cause yield loss typically ranging from 20 % to 50% (Sonti 1998; Hoan and Nghia, 2003), but in severe cases, it can cause as high as 80% yield reduction (Singh *et al*., 2005; Noh *et al*., 2007). However, it depends on rice growth stages, geographic locations or seasonal conditions (Noh *et al*., 2007).

Control measures for BB include cultural practices, chemical control, biological control, disease forecasting and most importantly, host genetic resistance. Since the chemical control for BB is not effective, the utilization of resistant varieties carrying resistance genes have been considered to be the most effective way to control the disease (Nino-Lui *et al.,* 2006). Most breeders are interested in utilizing BB resistant varieties and this goal is certainly achievable providing the availability of an easy strategy to identify resistance genes. In the case of BB resistance, so far, 38 BB resistance genes [27 dominant (*Xa1*, *Xa2*, *Xa3*, *Xa4*, *Xa7*, *Xa10*, *Xa11*, *X12*, *Xa14*, *Xa16*, *Xa17*, *Xa18*, *Xa21, Xa22* (t), *Xa23, Xa25 (t), Xa26, Xa27, Xa29, Xa30, Xa30 (t), Xa31 (t), Xa32 (t), Xa34, Xa35 (t), Xa36 (t), Xa38*) and 11 recessive (*xa5*, *xa5(t)*, *xa8*, *xa13*, *xa15*, *xa19, xa20, xa24, xa28 (t), xa31* and *xa32)*] have been identified in cultivated rice and the wild relatives (Natrajkumar *et al.,* 2012 and Chun *et al.,* 2012). The majority of BB resistance genes were identified in rice *O. sativa* sp. indica and wild rice *O. longistaminata*, *O. rufiprogon*, *O. minuta* and *O. officinalis*, while some of them were identified in *O. sativa* sp. japonica (Khush *et al*., 1989; Lee *et al*., 2003). Till date, out of the 38 BB genes, six are physically mapped (*Xa2, Xa4, Xa7, Xa30, Xa33* and *Xa38)* and six are cloned (*Xa1, xa5, xa13, Xa21, Xa26, Xa3 and Xa27*) (Liu *et al.,* 2007; Cheema *et al*., 2008; Bhasin *et al*., 2012; Natrajkumar *et al*., 2012). All these resistance genes follow a Mendelian pattern of major gene inheritance and express resistance to a diverse group of *Xoo* pathogens (Gu *et al*., 2005; Cheema *et al.,* 2008; Korinsak *et al*., 2009). A number of these resistance genes (*xa13, xa5, Xa4*, *Xa7, Xa21, Xa22* and *Xa23*) have been tagged by closely linked molecular markers (Chen *et al.,* 1997; Sonti1998; Rao *et al*., 2002; Porter *et al.,* 2003).

Most of these genes follow the classic gene-for-gene concept for the race-specific interaction between rice and *Xoo* (Flor, 1971). Some resistance genes are effective only in adult plants, while others are effective at all stages of growth. Some genes confer resistance to a broad spectrum of *Xoo* races, whereas others do so against only one or a few races. This observation could be influenced by particular geographical locations (Nino-Lui *et al.*, 2006). Several rice resistance genes are expressed at the highest level only in the adult stage (Century *et al.*, 1999; Panter *et al.,* 2002). *Xa21*-mediated resistance gene was shown to be expressed since the seedling stage and *xa13* gene showed broad resistance only in adult plants (Sidhu and Khush, 1978). However, *xa5* and *Xa4* gene could confer resistance at all growth stages and exhibit a broad spectrum of resistance to *Xoo* isolates (Adhikari *et al.,* 1995; Garris *et al.,* 2003; Arif *et al.,* 2008).

Resistant rice cultivars mainly based on a single resistance gene were developed but the large-scale and long-term cultivation of those varieties may enable the pathogen to overcome BB resistance. Historically, cultivation of rice varieties carrying single resistance gene for a long period has resulted in a significant shift in pathogen-race frequency and consequent breakdown of resistance (Mew *et al*., 1992). However, this can be delayed by pyramiding multiple resistance genes into rice cultivars. The evolution of new races has made the plant breeders to change their strategy from oligogenic resistance to gene pyramiding to provide durable resistance. Gene pyramiding provided a broad-spectrum of resistant cultivar which is economical and effective method for BB management (Babujee and Gnanamanickam, 2000). This technique is difficult using conventional breeding methods due to the dominance and epistasis effects of genes governing disease resistance. Moreover, genes with similar reactions to 2 or more races are difficult to identify and transfer through conventional approaches (Joseph *et al*., 2004; Sundaram *et al*., 2009 ; Rajpurohit *et al*., 2010). So, conventional breeding methods to improve rice cultivars for BB resistance have not found much success (Shin *et al.,* 2011).

Marker assisted selection (MAS) offers unique advantages to overcome some of these limitations. Marker assisted selection involves selection of plants carrying genomic regions that are involved in the expression of traits of interest through molecular markers. Marker-assisted backcross breeding (MABB), which involves two steps - (1) MAS for the gene of interest, known as foreground selection and (2) MAS for recovery of the recurrent parent genome, known as background selection (Hospital *et al*., 1992) is the most effective way of transferring specific gene(s) to agronomically superior variety/parental lines. So, the first step towards rice improvement via marker-based selection and map based cloning of the resistance genes is the identification of molecular markers that are tightly linked to the genes of interest and the availability of these molecular markers closely linked with each of the resistance genes makes the identification of plants with two and three genes possible. For carrying out foreground selection for bacterial blight resistance in rice several sequence tagged site (STS) markers RG556, RG136, pTA248, M5, Npb181 are available for *xa5*, *xa13, Xa21, Xa7* and *Xa4* respectively (Huang *et al.,* 1997; Singh *et al.,* 2001; Sundaram *et al.,* 2008; Shanti *et al.,* 2010; Xu *et al.,* 2012). The pTA248 marker is 0.2 cM from *Xa21* (Ronald *et al.,* 1992), RG136 marker is 3.8 cM from *xa13* (Zhang *et al.,* 1996), RG556 marker is 1.7

cM from *xa5* (Yoshimura *et al*., 1995), marker M5 is 0.2cM from *Xa7* (Porter *et al.,* 2003) and marker Npb181 is 1.7 cM from *Xa4* (Sun *et al.,* 2003). These markers have been employed to identify germplasm containing these genes (Blair and McCouch, 1997) and to develop rice cultivars with single and multiple resistance genes which are now widely cultivated in many countries (Singh *et al*., 2001; Sundaram *et al*., 2008; Perumalsamy *et al.,* 2010; Rajpurohit *et al.,* 2010; Fu *et al.,* 2012 and ; Suh *et al.,* 2013).

Background selection for recovery of recurrent parent genome is usually carried out using a set of simple sequence repeat (SSR) markers as these microsatellite markers are highly sequence specific, co-dominant, multiallelic, highly polymorphic, uniformly dispersed and efficiently analyzed by PCR (McCouch *et al*., 2002). Another effective PCR-based marker is Cleaved Amplified Polymorphic sequence (CAPS) (Konieczny and Ausubel, 1993; Dubcovsky, 2004; Li *et al.,* 2005). They are co-dominant genetic markers, but CAPS requires restriction endonuclease digestion to detect polymorphisms (SNP) in the region of interest. Numerous researchers have been interested in *Xa21, xa5* and *xa13* genes and marker assisted selection for pyramiding these important genes along with rapid background recovery of the recurrent parent genome, while maintaining the exquisite quality characteristics of rice, has been carried out which could be an effective approach for rice improvement (Singh *et al*., 2001; Xu and Crouch, 2008; Kim *et al.,* 2009; Shanti *et al.,* 2010; Ye, 2010; Suh *et al*., 2011; Huang *et al.,* 2012).

Basmati rice variety CSR-30 is very widely grown in Haryana and is extremely popular amongst rice farmers and consumers because of its aroma, high yield, medium slender grains and excellent cooking and eating qualities. Despite its popularity, CSR-30 is highly susceptible to bacterial blight (BB). The BB resistant rice variety is an important controlling method against this disease. The present investigation has, therefore, been planned to introgress effective BB resistance genes *Xa21, xa13* and *xa5* from BB-resistant rice varieties Pusa Basmati-1460 and IRBB-60 into BB-susceptible Basmati variety CSR-30 through marker-assisted selection (MAS). Pusa Basmati-1460 (IET 18990) has been developed by pyramiding BB resistance genes (*xa13* & *Xa21*) in the background of Pusa Basmati-1 through marker assisted backcross breeding by Indian Agricultural Research Institute under ICAR. IRBB-60, a near isogenic line in the background of IR24, carrying the four resistance genes *xa5, xa13, Xa4* and *Xa21* has been developed by IRRI, Phillipines.

Objectives:

1. To confirm the presence of resistance genes *xa13* and *Xa21* in the genotype Pusa Basmati-1460.
2. To pyramid these genes into Basmati varieties using Marker Assisted Selection.

CHAPTER-II

REVIEW OF LITERATURE

Rice (*Oryza sativa* L.), morsel for billions in the world, is a marvel of the grass family Graminae. It is a model cereal for genomic research because of its small genome and diploid nature (Bernier *et al*., 2008). In Asia, rice provides 35-80% of total calorie uptake (IRRI, 1997). Among the rice growing countries of the world, India has the largest rice acreage and ranks second in production. In India, rice occupies about 23.3 per cent of gross cropped area, it contributes 43 per cent of total food grain production and 46 per cent of total cereal production (FAO, 2012). Rice is one of the most diverse crops being grown as far north as Manchuria (China) and far south as Uruguay and New South Wales in Australia. Over 150,000 germplasm accessions of rice are being maintained at International Rice Research Institute (IRRI, The Philippines) and national research institutions in various countries, which consists of multitude of varieties adapted to a wide range of environment with enormous variability for traits such as plant type, yield, resistance to diseases, tolerance to abiotic stresses, taste and other quality parameters, having small and enriched genetic map (IRGSP, 2005).

2.1 Basmati rice

Various scented or aromatic rice varieties have been grown in Indian sub-continent for centuries. Of the various aromatic rice types, Basmati rice is considered the best. The word Basmati has been derived from two Sanskrit roots ‘Vas’ meaning aroma and ‘Mayap’ meaning ingrained from the beginning. While combining two ‘Mayap’ changed to ‘Mati’ making Vasamati and popularised as Basmati (Shobha Rani *et al.,* 2001). In India, cultivation of Basmati rice is concentrated in northern belt consisting of the districts of Karnal, Ambala and Kurukshetra in Haryana, Gurdaspur, Amritsar and Pathankot in Punjab, Saharanpur, Muzzafarnagar and Pilibhit in Uttar Pardesh and Dehradun and Nainital in Uttaranchal. Basmati rice is characterised by long slender grains, soft texture, intermediate amylose content, low gelatinization temperature, medium gel consistency, high elongation ratio and strong aroma (Singh *et al*., 2001). Basmati rice varieties are highly susceptible to bacterial blight (BB), thus making them a high-risk crop for farmers.

2.2. Bacterial blight disease

Bacterial blight (BB) is one of the most destructive bacterial diseases of rice in irrigated and rainfed lowland ecosystem and widespread throughout Asia. The disease has been reported to occur in Australia, the United States, and several rice growing countries of Latin America and Africa as well (Mew 1987). Farmers in the Fukuoka area of Japan have first seen BB disease during 1884–1910. The study of the disease started since then. Bacterial blight can cause damage at vegetative and reproductive stages of rice plants. High fertilizer input condition can induce the disease development (IRRI, 2004; 2010). Irrigation water is considered to contribute the spread of BB disease, as it carries the bacterial ooze that drop in the rice field water. The pathogen can survive around 15 days in the field water (Nino-Liu, 2006).

2.3 Causal organism and symptoms of BB disease

Bacterial blight is caused by rod-shaped bacterium with round ends 1-2×0.8-1 μm, monotrichous flagellum 6-8 μm, gram-negative, non-spore-forming and aerobic bacteria (Ishiyama, 1992). Colonies of *Xoo* are circular, convex, whitish yellow to straw yellow with smooth surface, entire margin and opaque against transmitted light (Figure 2.1). The bacterium may occur in two forms: i) the dry form, *Xoo* is found in the vascular vessel and xylem parenchyma of dried plants. If they are moistened by rain in winter, these dry form bacteria gradually die. ii) the growth form, bacterial cells are found in stubble and in the root system of perennial wild plants, especially *Leesia* spp. The pathogens survive in an inactive stage. The dry form can be activated and turn into the growth form after receiving moisture under favourable conditions.

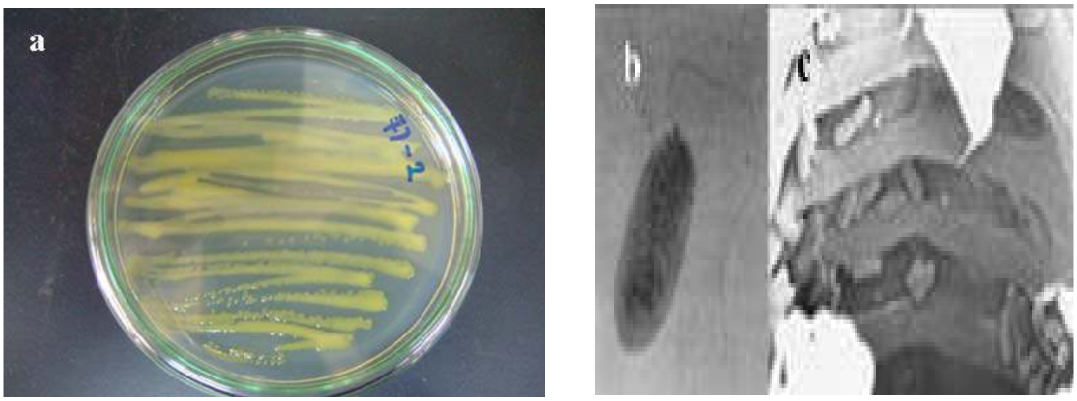

Figure 2.1: Characteristics of *Xanthomonas oryza* pv.*Oryzae* (a) colonies (b) rod shape of pathogen (c) *Xoo* in rice xylem vessel with scanning electron micrograph
Source: www.ceniap.gov.ve and www.apsnet.org (Khush *et al*., 1989)

The Bacterial blight is a vascular disease that spreads through the xylem vessels. Lesions usually begin at the margin, a few centimetre (cm) from the tip as water-soaked stripes. It can occur at all stages of the rice plants. At the seedling stage, the symptom first appeared as tiny water-soaked spots at the margin of the rice leaf blade. Then, it will enlarge and the rice plants turn yellow and wither. The symptom of the disease at the seedling stage is known as kresek (Ou, 1985) (Figure 2.2). The lesions may start at anywhere on the leaf blade at the site of an injury. The affected leaves will turn yellow, roll up and wilt rapidly. At the tillering and reproductive stages the symptom is known as leaf blight, a systemic infection that produces tannish-grey to white lesions along the vein. If plant produces panicles, the sterility percentage and number of immature grains will increase. Grains from diseased plants easily break during milling. Milky or opaque dewdrops of bacterial exudates on the surface of young lesions can be observed in the early morning. They dry up to form small, yellowish, spherical beads. They are shaken off by the wind and drop into the field water.

Figure 2.2: The BB disease symptom (a) leaf blight symptom occur on adult plant (b) kresek symptom occur on the seedling plant, (c) and (d) characteristics of BB symptom

2.4 The infection of *Xoo* in rice plants

Primarily, *Xoo* enters its hosts through hydathodes and wounds. Bacterial cells on the leaf surface may become suspended in guttation fluid as it exudes at night and then enter the plant by swimming, or passively as the fluid is withdrawn into the leaf in the morning. Bacteria multiply in the intercellular spaces of the underlying epithelial cells. It then enters and spreads into the plant through the xylem. Within a few days, bacterial cells and EPS (extracellular polysaccharide) fill the xylem vessels and ooze out from hydathodes, forming beads or strands of exudate on the leaf surface, a characteristic sign of the disease and a source of secondary inoculum (Nino-Liu *et al*., 2006). As the bacterium develops in the plant it can also spread into the mesophyll (Nino-Liu *et al*., 2005). The bacteria multiply outside the hydathodes of susceptible rice cultivars and gain entrance through them within 24 hours after spray inoculation. In contrary, they are trapped and embedded in a thin layer of exudates secrete from the water pores of resistant cultivars (Huang and Cleene, 1989). The hydathode apertures in susceptible rice plants are much larger due to the reduced growth of the outer edges, allowing *Xoo* to pass through freely. In the resistance varieties, bacterial cells are irregular in shape and enveloped by abundant fibrillar material (FM) and apparently dead.

2.5 Identification and molecular mapping of bacterial blight resistance genes

The identification and characterization of major genes for resistance and related factors have been a great deal to the success in plant breeding programs. To date, 38 BB resistance genes have been identified from cultivated rice and wild rice (Zhang, 2005; Chun *et al.,* 2012) as shown in table 2.1. Twenty seven BB genes are dominant (*Xa1*, *Xa2*, *Xa3*, *Xa4*, *Xa7*, *Xa10*, *Xa11*, *X12*, *Xa14*, *Xa16*, *Xa17*, *Xa18*, *Xa21, Xa22* (t), *Xa23, Xa25 (t), Xa26, Xa27, Xa29, Xa30, Xa30 (t), Xa31 (t), Xa32 (t), Xa34, Xa35 (t), Xa36 (t), Xa38*) and eleven are recessive genes (*xa5*, *-xa5(t)*, *xa8*, *xa13*, *xa15*, *xa19*, *xa20*, *xa24*, *xa28*, *xa31* and *xa32,* Nino-Lui *et al.*, 2006; Singh *et al.,* 2007; Natrajkumar *et al.,* 2012). Three resistance genes, *xa15*, *xa19* and *xa20* were induced by mutagenesis (Lee *et al.,* 2003). Six resistance genes, *Xa21*, *Xa1*, *xa5*, *xa13*, *Xa26* and *Xa27* have already been cloned and six resistance genes have been physically mapped (*Xa2, Xa4, Xa7, Xa30, Xa33 and Xa38)* (Chu *et al.*, 2006; Bhasin *et al.*, 2012).

Recessive resistance gene *xa5* was first reported by Murty and Khush, 1972. It was naturally found only within the *Aus* subpopulation of rice (Garris *et al.,* 2003). This recessive R gene was mapped to the telomeric region on short arm of chromosome 5 (Blair and McCouch, 1997). RFLP markers RG556, RG207, RZ390 and SSR markers RM122 and RM390 were closely linked to *xa5* gene (Yoshimura *et al.* 1995; Blair and Mc Couch, 1997). The *xa5* gene is particularly strong and has broad resistance to all BB isolates in Korea (Jeung *et al.,* 2006). Iyer-Pascuzzi and McCouch (2007) presented a set of CAPS markers for easy, quick and direct identification of cultivars carrying *xa5*-mediated resistance and provided evidence that these markers are 100% predictive of the presence of the *xa5* allele.

Gene *-xa5 (t)* was identified from rice cultivar Ajaya (IET8585). This variety is highly resistant to all pathotypes in India. Two SSR markers RM39 and RM31 on long arm of chromosome 5 were found to be tightly linked to the resistance gene (Rao, 2003). In the same way, gene *xa31 (t)* was identified in wild rice *O. Glaberrima* using SSR analysis. This recessive gene located on chromosome 5 had two flanking markers RM548 and RM593 with the distance of 1.7 and 1.1 cM, respectively (Singh *et al.,* 2007).

Gene *Xa7* is a dominant resistance gene directed against *Xoo* located on chromosome 6 and was originally identified in rice cultivar DV85. The resistance gene conveys resistance at flowering stage (Sidhu *et al.,* 1978). It was the most resistant to BB in Indonesia (Kadir *et al.,* 2004) and expressed low level of susceptibility to Korean BB races (Jena *et al.*, 2007). Chen *et al.* (2008) reported the high resolution mapping and the genetic prediction of resistance gene *Xa7*. Gene *Xa7* was mapped to the 0.21 cM interval between the STMS and SSR markers GDSSR02 and RM20593, respectively.

Table 2.1: Genes conferring resistance to different races of bacterial blight pathogen

Gene	Chromosome	Cultivar/Variety	Isolate/race	Reference
Xa1	4		Japanese race I and II	Sakaguchi, 1976
Xa2	4	Tetep	Japanese race I and II	Sakaguchi, 1976
Xa3	11	WaseAikokuChukogu-45 IR20, IR22	Japanese race and III	Ezuka *et al*., 1975
Xa-4	11	IR1529-680-3 TKM6	Philippine race I	Petpisit *et al*., 1977 Sidhu *et al*., 1978
xa5	5	IR1545-248, BJ- 1, IR291-7, DV85	Japanese races	McCouch, 1997 ; Petpisit *et al*., 1977; Singh *et al*., 1983
xa5(t)	5	Ajaya	Indian races	Rao, 2003
Xa6	not determined	Malagetsunsong IR994-102, IR1698-24, Zenith	Philippine race I	Sidhu *et al.,* 1978
Xa7	6	DV85, DV87	Philippine race I	Sidhu *et al*., 1978
xa8	7	PI231129	Philippine isolates	Sidhu *et al*., 1978
xa9	11	Sateng	Philippine isolates	Singh *et al*., 1983
Xa10	11	Cas209	Japanese isolates	Yoshimura *et al*., 1983
Xa11	not determined	IR8, RP9-3	Japanese isolates	Ogawa and Yamamoto, 1986
Xa12	4	Java 14	Japanese and Indonesian isolates	Kogyoku and Ogawa *et al*., 1978
xa13	8	BJ1	Philippine isolates	Zhang *et al*., 1996
Xa14	4	TN1	Japanese isolates	Taura *et al*., 1989
xa15	not determined	M41	Japanese isolates	Noda, 1989
Xa16	not determined	Tetep and IR24	Japanese isolates	Noda, 1989
Xa17	not determined	Asominori	Japanese isolates	Ogawa, 1989
Xa18	not determined	Toyonishiki	Burmese isolates	Ogawa and Yamamoto, 1986
xa19	not determined	XM5	Japanese isolates	Taura *et al*., 1991
xa20	not determined	XM6	Japanese isolates	Taura *et al*., 1992
Xa21	11	*O. longistaminata*	Philippine and Japanese isolates	Khush *et al*., 1990
Xa22	11	Zhachanglong	Chinese isolates	Lin *et al*., 1996
Xa23 *Xa23*	11	*O. nivara* *O. rufipogon*	Indian isolates PXO99	Kumar, 1999 Wang *et al*., 2006
xa24,	2	DV85, DV86 Aus29	Philippine race 6	Mir and Khush, 1990, Krush and Angeles, 1999 Lee *et al.,* 2001 Gao*et al*., 2002
Xa25	12	Minghui63	Philippine race 9	Chen *et al*., 2002
Xa26	11	Nep Bha Bong Minghui63	Philippine race 1,2,3 and 5	Lee *et al*., 2003 Sun *et al*., 2004
Xa-27	6	*O. minuta* , Arai Raj	Philippine race 2	Amante-Bordeos*et al*., 1992 Lee *et al.,* 2003
Xa28	not determined	Lota Sail	Philippine race 2	Lee *et al*., 2003
Xa29	1	*O. officinalis*	_	Tan *et al*., 2004
Xa30 *Xa30(t)*	11 4	CB30 *O. nivara*	Phillipine race 6	Jin *et al*., 2007 Singh *et al*., 2007
Xa31 *xa31*	4 5	Zhachanglong *O. glaberrimar*	PXO61 and OS105	Wang *et al*., 2009 Singh *et al*., 2007
xa32	6	6 *O. barthii*		Singh *et al*., 2007

Gene *xa32(t)* was identified in wild rice *O. barthii*. It was mapped with SSR markers using bulk segregation analysis (BSA). Bulk segregation analysis indicated presence of *O. Barthii* on the terminal region of chromosome 6 at a distance of 9.3 cM proximal to RM588 (Singh *et al.,* 2007).

Many BB resistance genes, i.e. *Xa3, Xa4, Xa6*, *Xa10, Xa21, Xa22, Xa23*, *Xa26*, *Xa30* and *xa9* have been reported to locate on chromosome 11. Most of them are multigene family and tightly linked together. Dominant resistance gene *Xa21* was introgressed from a wild species *O. Longistaminata* into *O. sativa* background (Khush *et al.*, 1989). It confers resistance to a broad range of *Xoo* strains. This resistance gene was first tagged with RAPD marker. Later, the RFLP marker RG103 had found to be tightly linked to this gene at a distance of 1.2 cM. Then, a PCR-based STS marker pTA248 was developed and used in MAS (Ronald *et al.,* 1992). Gene *Xa21* was the first BB resistance gene to be successfully cloned. It was isolated using map-based cloning and found to be a member of a complex locus located on long arm of chromosome 11. A functional marker PB7/8, derived from *Xa21* gene has been found to be very effective for selecting BB resistance *Xa21* gene in rice (Chunwongse *et al.,* 1993).

The *xa13* gene is fully recessive, conferring resistance only in the homozygous status (Khush and Angeles, 1999). This gene specifically confers resistance to the Philippine *Xoo* race 6, the most virulent race that is not overcomed by most of the reported R genes. The *xa13* gene was first discovered in the rice variety BJ1 and mapped on the long arm of rice chromosome 8 (Ogawa *et al.*, 1986; Zhang *et al.*, 1996; Sanchez *et al.*, 1999). It interacts strongly with other R genes such as *xa5, Xa4 and Xa21* (Li *et al.*, 2001).

Gene *Xa4/Xa26* locus was mapped to a region of about 1.68 cM. This locus co-segregated with marker R1506 had two flanking markers, RM224 and Y6855RA. These two flanking markers were 0.21 cM and 1.47 cM, respectively from marker R1506 (Yang *et al.,* 2003). Gene *Xa26* encodes a leucine-rich repeat (LRR) receptor kinase-like protein. The gene belongs to a multigene family consisting of four members (Sun *et al.,* 2004). Gene *Xa4* had the same copy numbers of *Xa26* family members from the rice line Minghui 63 (Xiang *et al.,* 2006). Another resistance gene from the rice line IR1188 was also identified and mapped in the same region on the chromosome 11. (Jantaboon *et al.,* 2004)

The resistance gene *Xa10* was identified from rice cultivar Cas 209 (Mew *et al.,* 1982; Yoshimura *et al.,* 1983). The *Xa10* locus was initially mapped between the RAPD marker $OO7_{2000}$ and RFLP marker CDO365 on the long arm of chromosome11 (Yoshimura *et al.,* 1995). *Xa21* and *Xa23* were located at the middle region of chromosome 11. *Xa21* co-segregated with RG103 (Ronald *et al.,* 1992) while *Xa23* was mapped to a region between markers RG1091 and G1465 (Zhang *et al.,* 1998). Therefore, *Xa10* locus is flanked by *Xa21* and *Xa23*. Fine mapping of *Xa10* revealed that the resistance gene was flanked between markers M491 and M419 on Nipponbare genome which consisted of six candidate genes (Gu *et al.,* 2008).

2.6 DNA markers and marker-assisted selection (MAS)

DNA markers or molecular markers are typically derived from a small region of DNA that show sequence polymorphism. Molecular markers increase the efficiency of backcrossing by allowing selection of genotype with the maximum percentage of recurrent parent genome (Hospital, 2005). Commonly four types of markers i.e RAPD, RFLP, AFLP and SSR are used and implemented in rice genomics. However, RAPD and RFLP markers have limitations. RFLP using southern analysis is laborious, time-consuming, costly and involves the use of radiochemical, while SSR marker is simple, accurate, efficient, cost-effective, represent single-loci and can detect high levels of polymorphism (Babu *et al.,* 2004).

Simple sequence repeats (SSRs) are tandem arrays of two to six nucleotide base pair repeats that occur ubiquitously throughout the genome (Tautz, 1989). The unique sequences flanking the SSR (also known as microsatellite) are generally conserved within a species. The flanking sequences are utilized to design forward and reverse primers to amplify the corresponding SSR loci, which are also referred to as sequence tagged microsatellite sites (STMS) markers (Beckmann and Soller, 1990). It constitutes excellent genetic markers with locus identity and can be multiplexed to achieve higher throughput (Mitchell *et al,* 1997). Cleaved Amplified Polymorphic Sequence (CAPS) are new generation codominant molecular markers based on the detection of single nucleotide polymorphisms and can be used for MAS but require restriction endonuclease digestion to detect SNP in an interesting region (Dubcovsky, 2004).

One of the major applications of molecular markers to rice breeding is using marker assisted selection. It would improve the efficiency of plant breeding through precise transfer of genomic regions of interest and accelerating the recovery of the recurrent parent genome. It is based on the principle that if a gene is tightly linked to an easily identifiable genetic marker, it may be more efficient to select in a breeding programme for the marker than for the trait itself. Therefore, MAS strategy is a way to capitalize on available markers and to incorporate valuable traits into elite lines that are suitable for cultivar release (Dubcovsky, 2004; Collard and Mackill, 2008).

Conventional breeding is primarily based on phenotypic selection of superior individuals among segregating progenies resulting from hybridization. Moreover, transfer of recessive genes through conventional breeding requires additional selfing generations after every backcross, a procedure that is prohibitively slow for most commercial breeding purpose. Thus, MAS has been used to transfer favourable alleles of genes/QTLs for biotic and abiotic stress resistance/tolerance into the desired rice genetic background (Babu *et al.,* 2004;

Toojinda *et al.,* 2005). Gandhi (2007) crossed a deep rooted upland japonica rice variety from the Philippines with a high yielding indica variety using MAS. The new variety MAS 946-1 consumes up to 60% less water than traditional varieties and became the first drought tolerant aerobic rice variety released in India.

Neeraja *et al*. (2007) developed submergence-tolerant Swarna-Sub1 from submergence-susceptible Swarna by carrying out MAS. Swarna-Sub1 shows a twofold or higher yield advantage over Swarna after submergence for 10 days or more during the vegetative stage (Septiningsih *et al*., 2009).

Wen and Gao (2011) introgressed the broad-spectrum blast resistant gene *Pi-9(t)* from the donor parent P2 into hybrid restorer Luhui17 by using molecular marker-assisted selection. Sixty eight lines carrying blast resistant gene *Pi-9(t)* were obtained, and four lines, WR1023, WR1043, WR1056 and WR1062, were identified to be homozygous backcross lines with resistant gene *Pi-9(t)* by molecular phenotyping identification

2.7 Molecular breeding for BB resistance

Historically, long-term cultivation of rice varieties carrying single resistance gene has resulted in a significant shift in pathogen-race frequency and consequent breakdown of resistance (Mew *et al*., 1992). One tangible solution to resistance breakdown is pyramiding of multiple resistance genes in the background of modern high yielding varieties. The probability of simultaneous pathogen mutations for virulence to defeat two or more effective genes is much lower than with a single gene (Mundt, 1990). Gene pyramiding is difficult to achieve using conventional breeding alone because of linkage with some undesirable traits that is very difficult to break even after repeated backcrossings (Tanksley *et al.,* 1989). When two or more genes are introgressed, phenotypic evaluation is unable to distinguish the effect of individual gene precisely since each gene confers resistance to and combats multiple races of the pathogen. Moreover, in the presence of a dominant and a recessive allele, the effect of the recessive gene is masked. The success of BB resistance gene pyramiding using MAS has been reported from several rice breeding programs.

Sanchez *et al*. (2000) successfully transferred three bacterial blight resistance genes (*xa5*, *xa13*, and *Xa21)*, to improve the resistance of the three NPT lines, IR65598-112 and the two sister lines IR65600-42 and IR65600-96 to *Xoo*, via a marker-aided backcrossing procedure. Fifty-nine BC_3F_2 near iso-genic lines (NILs) in the three NPT backgrounds containing one to three BB resistance genes in various combinations were developed through MAS for the resistance genes and phenotypic selection for the NPT. The BC_3F_3 NILs having more than one BB resistance gene showed a wider resistance spectrum and manifested increased levels of resistance to the *Xoo* races, as compared with those having a single BB resistance gene.

Singh *et al.* (2001) pyramided three BB resistance genes *xa5, xa13* and *Xa21* in PR106 cultivar using MAS and during testing with 17 *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) isolates under artificial inoculation and field conditions in Punjab and found that the combination of genes provided wider spectrum of resistance to the pathogen populations prevalent in the region. They found that *Xa21* was the most effective followed by *xa5*, whereas *xa13* gene was the least effective against *Xoo*.

Joseph *et al.* (2004) reported pyramiding of two BB resistance genes, *xa13* and *Xa21* along with grain and cooking quality characteristics and desirable agronomic features of the leading Basmati rice variety, PB-1, by a combination of phenotypic and molecular marker aided selection. Background analysis using 252 polymorphic AFLP markers detected 80.4 to 86.7% recurrent parent alleles in BC_1F_3 selections. Similarly, BB resistance genes *Xa21, xa5* and *Xa4* from IRBB60 were introgressed into a hybrid rice line, Shunhui 527 using MAS by Perez *et al.* (2008). The *Xa4+xa5+Xa21* pyramided lines expressed high levels of resistance to all the *Xoo* races used in the study.

Sundaram *et al.* (2008) were able to pyramid three BB resistance genes *xa5*, *xa13* and *Xa21* using marker assisted backcross breeding in an elite rice variety Samba Mahsuri from a donor line SS1113 along with its nearly 97% background recovery by BC_4F_1 through foreground and background selection during each backcross generation.

Basavraj *et al*. (2010) carried out the introgression of BB resistance genes *xa13* and *Xa21* into Pusa6B and PRR78 (the maintainer parent and the restorer parent of the hybrid Pusa RH10) using a marker assisted backcross breeding program. They were able to recover the recurrent parent genome ranging from 85.14 to 97.30% and 87.04 to 92.81% in the 10 best BC2F5 families of Pusa6B and PRR78, respectively.

Bharani *et al*. (2010) pyramided BB resistance genes (*Xa21*, *xa13* and *xa5*) through molecular marker assisted selection into high yielding susceptible rice cultivars ADT43 and ADT47. The genotypes with different resistance gene combinations were challenged with two *Xoo* isolates under field conditions. Plants with both dominant and recessive gene in homozygous condition either *Xa21/xa13 or Xa21/xa5* combinations were found to be resistant.

Kottapalli *et al.* (2010) introgressed three bacterial blight resistance genes *xa5, xa13* and *Xa21* into a fine grain rice variety, Samba Mahsuri using sequence tagged site (STS) markers linked to these genes. They adopted four different pyramiding schemes to minimize loss of recessive resistance genes in advanced backcross generations. They further demonstrated that there was no yield penalty due to pyramiding of multiple genes into the elite indica rice variety.

Rajpurohit *et al*. (2010) carried out the pyramiding of bacterial leaf blight resistance genes *Xa21* and *xa13* along with the semidwarfing gene sd-1 in the traditional Indian basmati rice cultivar Type 3 Basmati and marker assisted background profiling of selected BC_2F_3

progenies using rice SSR and ISSR markers. They found that the pyramided lines were BB resistant and had excellent cooking quality and strong aroma.

Shanti *et al*. (2010**)** reported the pyramiding of four BB resistance genes *Xa4, xa5*, *xa13* and *Xa21* through marker-assisted selection (MAS) in the BB susceptible high yielding rice cv. Mahsuri and parental lines of hybrid rice, maintainers IR58025B and Pusa 6B and the restorer lines KMR3 and PRR78 simultaneously. Only foreground selection was done using markers. Conventional breeding strategy was adopted for background selection. The pyramids showed very high level of disease resistance to 10 highly virulent isolates of *Xanthomonas oryzae* pv. *oryzae.* Grain quality parameters of the pyramids were found to be on par with that of the original genotype.

Huang *et al.* (2012) introgressed four bacterial leaf blight (BLB) resistance genes *Xa7, Xa21, Xa22* and *Xa23* into an elite hybrid rice restorer line Huahui 1035 by marker-assisted selection. Ten promising BLB resistant lines were identified in Huahui 1035 restorer background and their respective F1 hybrids with a cytoplasmic male sterile line i.e. Jinke 1A. Restorer lines with *Xa23* introgression i.e. HBQ809 and HBQ810 were found to be resistant to all eleven Chinese representative *Xoo* races, showing broad spectrum resistance to BB. Interestingly, the F_1 hybrids with *Xa23, Xa7* or *Xa7/Xa21* were found to be resistant to two severe epidemic *Xoo* races of China.

Pandey *et al.* (2012) aimed at targeted introgression of two major effective BB resistance genes *Xa21* and *xa13* from a high-yielding, medium slender grain-type semi-dwarf, BB-resistant rice variety, Improved Samba Mahsuri into the genetic background of two elite, low-yielding BB-susceptible traditional Basmati varieties, Taraori Basmati and Basmati 386, through the process of MAS for a single generation (BC_1) coupled with phenotype-based selection for short plant stature and high yield.

Salgotra *et al.* (2012) carried out the introgression of bacterial leaf blight resistance and aroma genes using functional marker-assisted selection in rice variety IRS 5441-2 (*Oryza sativa* L.) from BB resistant variety IRBB59 having three BB resistance genes *xa5*, *xa13* and *Xa21.* The BC_1F_3 recombinants with enhanced resistance to BLB, basmati quality and desirable agronomic traits derived in this study from the cross IRS 5441-2 9 IRBB59, were found to be equally effective against the most virulent BLB isolate, as the donor line IRBB59.

Suh *et al.* (2013) developed three elite advanced backcross breeding lines (ABL) of a BB-susceptible elite japonica rice cultivar, Mangeumbyeo, with three resistance genes (*Xa4* + *xa5* + *Xa21*) by foreground and phenotypic selection from an indica donor (IRBB57), using a marker-assisted backcrossing (MAB) breeding strategy. The background genome recovery of the ABL expressed more than 92.1% using genome-wide SSR marker analysis. Also these three ABL lines were found to be highly resistant to the 18 isolates of *Xoo* prevalent in Korea, with agronomic and grain quality traits similar to those of the recurrent parent.

CHAPTER-III

MATERIALS AND METHODS

I. MATERIALS

3.1 Plant Materials

Pusa Basmati-1460 (having *xa13* and *Xa21* BB resistance genes) and IRBB-60 (having *xa5, xa13, Xa4* and *Xa21* BB resistance genes), both BB resistant varieties, were used as donor varieties and CSR-30 which is BB susceptible but agronomically superior basmati variety was used as the recipient variety for the present study. The combination of two and three bacterial blight resistance genes *xa5, xa13* and *Xa21* were used for gene pyramiding (Table 3.1). Seeds of all the above varieties were obtained from Regional Rice Research Station, Kaul. All the seeds were raised in pots in the net house of Department of Molecular Biology and Biotechnology, college of Basic Sciences and Humanities and Rice Research Station, Kaul (Kaithal), CCS Haryana Agricultural University, Hisar.

Table 3.1: Bacterial blight resistance genes used for gene pyramiding

Gene	Isoline	Donor	Chromosome	Reference
xa-5	IRBB5	DZ192	5	Yoshimura *et al*. (1995)
xa-13	IRBB13	Long grain	8	Zhang *et al*. (1996
Xa-21	IRBB21	*O. longistaminata*	11	Ronald *et al*. (1992)

Experiment No.1: To confirm the presence of resistance genes in Pusa Basmati-1460 and IRBB-60

3.2 Molecular Markers

Three Sequence Tagged Site (STS) markers specific for the BB resistance genes pTA248 for *Xa21*, RG136 for *xa-13* and RG556 for *xa-5* were used to confirm the presence of these resistance genes in Pusa Basmati-1460 and IRBB-60 (Table 3.2). The pTA248 marker is 0.2 cM from *Xa21* (Ronald *et al.,* 1992), the RG136 marker is 3.8 cM from *xa13* (Zhang *et al.,* 1996) and the RG556 marker is 1.7 cM from *xa5* (Yoshimura *et al*., 1995). These primers were commercially synthesized from Eurofins Genomics India Pvt. Ltd.

Table 3.2: Sequence tagged site markers used for marker-assisted selection of resistance genes to *Xanthomonas oryzae* pv. *oryzae*.

STS marker and distance	Primer Sequence (5'-3')	Enzyme	R Genes for BB
pTA248 (0.2cM)	FAGACGCGGAAGGGTGGTTCCCGGA3' RAGACCGGTAATCGAAAGATGAAAA3'	–	*Xa21*
RG136 (3.8cM)	F TCCCAGAAAGCTACTACAGC R GCAGACTCCAGTTTGACTTC	*Hinf* I	*xa13*
RG556 (1.7cM)	F TAGCTGCTGCCGTGCTGTGC3' R AATATTTCAGTGTGCATCTC3'	*Dra* I	*xa5*

3.3 Chemicals/ Reagents

For Polymerase Chain Reaction, *Taq* DNA polymerase, magnesium chloride and dNTPs were obtained from New England Biolabs (NEB). All other chemicals used in the present investigation were of analytical grade and were purchased from Sigma Chemicals and NEB.

II. Methods

3.4 DNA isolation

3.4.1 Reagents

CTAB extraction buffer

Tris (pH 8.0)	0.2M
EDTA* (disodium, pH 8.0)	0.02M
Sodium chloride	1.4M
CTAB**	1.5%
β-Mercaptoethanol (Added prior to use)	2.0%

Wash I solution

Ethanol	76%
Sodium acetate	0.2M

Wash II solution

Ethanol	76%
Ammonium acetate	10mM

TE buffer

Tris (pH 8.0)	10mM
EDTA (pH 8.0)	1mM

*Ethylene diamine tetra acetic acid

** Cetyl trimethyl ammonium bromide

Genomic DNA was isolated from the young leaves of parental rice plants, Pusa Basmati-1460 and IRBB-60, both BB resistant varieties and CSR-30, BB susceptible variety, using CTAB extraction method of Murray and Thompson (1980) modified by Saghai-Maroof *et al.* (1984) and Xu *et al.* (1994), which was further modified in our laboratory.

- Five gram of the leaf tissue was hand homogenized to fine powder in liquid nitrogen using sterilized mortar and pestle. Finely ground leaf tissue was mixed with 10 ml of CTAB extraction buffer pre-warmed at 65°C in sterilized 50 ml polypropylene tubes. The samples were thoroughly mixed by inverting the tubes several times and were incubated in water bath at 65°C for 1.5 hours.

Contents of the tubes were mixed gently at an interval of 15 minutes, by inverting the tubes several times.

- After incubation, the samples were cooled to room temperature and 10 ml of chloroform: octanol (24: 1) solution was added. The samples were mixed by gently inverting the tubes several times and were centrifuged for 10 minutes at 8000 rpm.
- The upper aqueous phase was transferred in a clean sterilized centrifuge tube and again extracted with 10 ml of chloroform: octanol (24:1) solution.
- The extracted DNA was precipitated with equal volume of ice-cold isopropanol. The precipitated DNA was spooled out by using sterilized glass hooks and kept at room temperature for few minutes.
- The DNA samples were washed with Wash I solution for 20 minutes followed by washing with Wash II solution for 2 minutes.
- The DNA samples were then air dried at room temperature and subsequently dissolved in appropriate volume of TE buffer.

3.4.2 RNase treatment

The DNA was treated with the RNA degrading enzyme, RNase A (50µg/ml) and incubated at 37°C for 3-4 hours to remove the RNA contamination from the samples.

3.4.3 Purification of DNA

DNA samples, which did not form a clear solution and showed turbidity, were purified by treating with phenol. The samples were mixed with equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1) thoroughly until an emulsion formed. Samples were centrifuged at 8000 rpm for 10 minutes at room temperature (25°C). Aqueous phase was transferred to fresh sterilized eppendorf tube and again extracted with chloroform: isoamyl alcohol (24: 1 v/v). DNA from aqueous phase was transferred to a new sterilized eppendorf tube and precipitated by adding one-tenth volume of 3M sodium acetate (pH 5.2) and two and a half volumes of ice-cold ethanol. The contents of the tube were incubated at -70°C for 1 hour. Genomic DNA was then pelleted down by cenrifugation at 8000 rpm for 10 minutes at 4°C. Supernatant was carefully removed and pellet was washed with 70 per cent ethanol. Sample tubes were kept open at 37°C until last trace of ethanol had evaporated. The DNA pellet was dissolved in appropriate volume of TE buffer and stored at -20°C until further use.

3.4.4 Quantification of DNA

Quantity of genomic DNA for each genotype was estimated using an UV-visible spectrophotometer. DNA concentration was estimated by measuring absorbance (A) at 260 nm. Using the relationship of 1.0 O.D. at 260 nm equivalent to 50μg DNA per ml, the concentration of DNA was estimated from the following formula:

Concentration of DNA (μg/ml) = A_{260} x 50 x dilution factor.

3.4.5 Quality of DNA

Quality of DNA was checked both by UV-visible spectrophotometer and on 0.8 per cent (w/v) agarose gel electrophoresis. After running DNA on gel, it was stained with ethidium bromide and visualized under U.V light to check the intactness and shearing of DNA and RNA contamination. Single intact band of high molecular weight showed that DNA was pure. Using spectrophotometer, the absorbance at 260 nm and 280 nm of DNA was recorded and then ratio of A_{260}/A_{280} was calculated. The ratio of A_{260}/A_{280} = 1.8±0.05 was taken as pure DNA. If the $A_{260}/A_{280} > 1.8$, there is a RNA contamination and if this value < 1.8 then there is a protein contamination. Samples contaminated with RNA were given RNase A treatment to remove RNA and protein contaminated samples were treated with proteinase K and phenol: chloroform to remove proteins. The purified DNA was used for PCR amplification.

3.5 Polymerase Chain Reaction (PCR)

DNA extracted from the parental genotypes was used for PCR amplification using the specific STS primers. PCR reaction was carried out in 20 μl reaction mixture containing 50ng genomic DNA, 2 units of Taq DNA polymerase, 1X PCR Buffer (10mM Tris HCL, 1.5mM $MgCl_2$), 100μM each of dNTPs and 10 μM of primer.

The reactions were carried out in PTC- 100 programmable thermal cycler from (MJ Research and Biometra Personal). Following PCR conditions were used:

1.	Initial denaturation	94°C for 5 min.	30 cycles
2.	Denaturation	94°C for 30 sec.	
3.	Annealing	55°C for 1 min.	
4.	Extension	72°C for 1 min.	
5.	Final extension	72°C for 10 min.	

Amplified products were stored at -20°C till further use.

3.6 Agarose Gel Electrophoresis

Stock solutions

10X TBE Buffer

Tris	108g
Boric acid	55g
0.5M EDTA (pH-8.0)	40g
Final volume (Distilled H_2O)	1000m

6X loading dye

Sucrose	4.0g
Bromophenol blue	0.025g
Xylene cyanol	0.025g
Volume (Distilled H_2O	10ml

(Loading dye solution was stored at 4°C)

- After genomic DNA isolation the genomic DNA of all the genotypes was resolved by submerged horizontal gel electrophoresis in 0.8 per cent (w/v) agarose gel.
- The amplified product of pTA248 was electrophoretically resolved on a 1.5 % agarose gel containing 0.5 µg/ml of ethidium bromide in 1.0X TBE buffer and visualized under UV light.
- A 100 bp DNA ladder was used to estimate allele sizes in base pairs (bp). Polymorphisms in the DNA profiles were scored visually by comparing the alleles amplified by various primers in the parental genotypes and their size was calculated in reference to standard DNA ladder.
- For the amplified products of RG136 and RG556, 5 µl of PCR product was used for gel electrophoresis to check DNA amplification. The remaining PCR product was used for restriction digestion.
- The reaction mixture used for digestion of PCR product with the respective restriction enzyme consisted of 0.6 µl (10 U/µl) of restriction enzyme (*Hinf* 1 for RG136 amplicons and *Dra* 1 for RG556 amplicons), 2.5 µl of 10 X restriction buffer, 6.9 µl of sterile distilled water and 15 µl PCR product to a final volume of 25 µl of the reaction (Perumalsamy *et al.,* 2010). The reaction mixture was incubated for 4 h at 37°C and the products of restriction digestion were separated by gel electrophoresis (1.5% agarose) and visualized under UV light.

Procedure:

- Gel casting plate was washed with distilled water and dried. Plate was wiped with ethanol, air-dried and ends were sealed with tape.

- The agarose solution was prepared in 1X TBE buffer by boiling in microwave oven. The solution was cooled to 50-55°C and ethidium bromide was added in the gel at a concentration of 0.5µl/ml from a stock of 10mg/ml.
- Agarose gel was then poured into the gel casting plate with an appropriate comb inserted and allowed to solidify for 30 minutes. After setting of gel, sealing tapes were removed from both ends.
- Gel plate was then placed in electrophoresis chamber and submerged using 1X TBE buffer. The comb was removed gently using both hands.
- DNA samples were prepared by adding 1µl/ml of 6X loading dye solution. The samples were loaded in wells using appropriate micropipette.
- Cover was placed on electrophoretic unit and proper connections were made using power supply from M/S Atto, Japan.
- Electrophoresis was carried out at constant voltage (3V/cm) until dye had moved to three-fourth length in the gel.
- DNA segments resolved in gel were viewed under UV light and documented (UV wavelength of 350 nm) using gel documentation system (Pharmacia Biotech).

Experiment No. 2: Pyramiding of resistance genes in the basmati variety CSR-30

Crosses were made between CSR-30 x Pusa Basmati-1460 and CSR-30 x IRBB-60. The recurrent parent CSR-30 was taken as female and the donor parents Pusa Basmati-1460 and IRBB-60 as male parent for the crosses and the F_1s were obtained.

3.7 Marker Assisted Foreground Selection

In F_1 generation, sequence tagged site markers pTA248, RG136 and RG556 linked to *Xa21, xa13* and *xa5* genes respectively, were used to select plants with BB resistance alleles (Foreground selection). The F_1 plants having all the three BB resistance genes were back crossed with the recurrent parent CSR-30 (as male parent) and the BC_1F_1 seeds from these crosses were harvested. The BC_1F_1 plants were selfed to produce BC_1F_2 seeds. Among the BC_1F_2 plants, molecular markers linked to *Xa21, xa13* and *xa5* were again used to select plants with BB resistance alleles of the donor line. The BC_1F_2 plants positive for all the BB resistance genes were again backcrossed with CSR-30 to produce BC_2F_2 seeds.

3.8 Marker Assisted Background selection

To assess the relative contribution of the two parental genomes to the segregants and to identify positive genotypes having BB resistance genes with greater genetic similarity to the recurrent parent, a total of 300 microsatellite markers spanning the entire rice genome uniformly spread over 12 rice chromosomes based on the rice linkage maps of Akagi *et al.* (1996) and Temnykh *et al.* (2000) were used (Table 3.3). The original source, repeat motifs, primer sequences and chromosomal positions of these markers were selected from rice genome database. For background selection, genomic DNA was isolated from 35-day old foreground selected plants using CTAB method as described earlier.

Table 3.3: **List of the SSR markers used to study polymorphism between the parental genotypes.**

Sr. No.	Primer pair name	Primer pair sequence (5’-3’)
1	RM462	CCGCGAATCCATTCAGACTGC TCTAGGAGGAGATGGCGGAGTAGC
2	RM21	ACA GTA TTC CGT AGG CAC GG GCT CCA TGA GGG TGG TAG AG
3	RM114	ACA GTA TTC CGT AGG CAC GG GCT CCA TGA GGG TGG TAG AG
4	RM122	GAG TCG ATG TAA TGT CAT CAG TGC GAA GGA GGT ATC GCT TTG TTG GAC
5	RM164	TCT TGC CCG TCA CTG CAG ATA TCC GCA GCC CTA ATG CTA CAA TTC TTC
6	RM190	CTT TGT CTA TCT CAA GAC AC TTG CAG ATG TTC TTC CTG ATG
7	RM101	GTGAATGGTCAAGTGACTTAGGTGGC ACACAACATGTTCCCTCCCATGC
8	RM-230	GCC AGA CCG TGG ATG TTC CAC CGC AGT CAC TTT TCA AG
9	RM102	AAC TTT CCC ACC ACC ACC GCG G AGC AGC AGC AAG CCA GCA AGC G
10	RM106	CGT CTT CAT CAT CGT CGC CCC G GGC CCA TCC CGT CGT GGA TCT C
11	RG207	ATT GTT ACG TTT GGT GGG GG GCC ATG GCG ACT GTC AGT CG
12	RM 130	TGT TGC TGC CCT CAC GCG AAG GGT CGC GTG CTT GGT TTG GTT C
13	RM 206	CCC ATG CGT TTA ACT ATT CT CGT TCC ATC GAT CCG TAT GG
14	RM 224	ATC GAT CGA TCT TCA CGA GG GCT ATA AAA GGC ATT CGG G
15	RM 216	GCA TGG CCG ATG GTA AAG TGT ATA AAA CCA CAC GGC CA
16	RM 229	CAC TCA CAC GAA CGA CTG AC CGC AGG TTC TTG TGA AAT GT

17	RM 235	AGA AGC TAG GGC TAA CGA AC TCA CCT GGT CAG CCT CTT TC
18	RM 257	CAG TTC CGA GCA AGA GTA CTC GGA TCG GAC GTG GCA TAT G
19	RM 265	CGA GTT CGT CCA AGT GAG C CAT CCA CCA TTC CAC CAA TC
20	RM 272	AAT TGG TAG AGA GGG GAG AG ACA TGC CAT TAG AGT CAG GC
21	RM 240	CCT TAA TGG GTA GTG TGC AC TGT AAC CAT TCC TTC CAT CC
22	RM 138	AGC GCA ACA ACC AAT CCA TCC G AAG AAG CTG CCT TTG ACG CTA TGG
23	RM 208	TCT GCA AGC CTT GTC TGA TG TAA GTC GAT CAT TGT GTG GAC C
24	RM 159	GGG GCA CTG GCA AGG GTG AAG G CTT GTG CTT CTC TCT CTC TCT CTC TCT
25	RM 254	AGC CCC GAA TAA ATC CAC CT CTG GAG GAG CAT TTG GTA GC
26	RM 270	GGC CGT TGG TTC TAA AAT C TGC GCA GTA TCA TCG GCG AG
27	RM 220	GGA AGG TAA CTG TTT CCA AC GAA ATG CTT CCC ACA TGT CT
28	RM 236	GCG CTG GTG GAA AAT GAG GGC ATC CCT CTT TGA TTC CTC
29	RM 248	TCC TTG TGA AAT CTG GTC CC GTA GCC TAG CAT GGT GCA TG
30	RM 421	AGC TCA GGT GAA ACA TCC AC ATC CAG AAT CCA TTG ACC CC
31	RM 148	ATA CAA CAT TAG GGA TGA GGC TGG TCC TTA AAG GTG GTG CAA TGC GAG
32	RM 226	AGC TAA GGT CTG GGA GAA ACC AAG TAG GAT GGG GCA CAA GCT C
33	RM 184	ATC CCA TTC GCC AAA ACC GGC C TGA CAC TTG GAG AGC GGT GTG G
34	RM 204	GTG ACT GAC TTG GTC ATA GGG GCT AGC CAT GCT CTC GTA CC
35	RM 338	CAC AGG AGC AGG AGA AGA GC GGC AAA CCG ATC ACT CAG TC
36	RM 114	CAG GGA CGA ATC GTC GCC GGA G TTG GCC CCC TTG AGG TTG TCG G

37	RM23	CAT TGG AGT GGA GGC TGG GTC AGG CTT CTG CCA TTC TC
38	RM24	GAA GTG TGA TCA CTG TAA CC TAC AGT GGA CGG CGA AGT CG
39	RM25	GGA AAG AAT GAT CTT TTC ATG G CTA CCA TCA AAA CCA ATG TTC
40	RM26	GAG TCG ACG AGC GGC AGA CTG CGA GCG ACG GTA ACA
41	RM27	TTT TCC TTC TCA CCC ACT TCA TCT TTG ACA AGA GGA AAG AGG C
42	RM29	CAG GGA CCC ACC TGT CAT AC AAC GTT GGT CAT ATC GGT GG
43	RM30	GGT TAG GCA TCG TCA CGG TCA CCT CAC CAC ACG ACA CG
44	RM31	GAT CAC GAT CCA CTG GAG CT AAG TCC ATT ACT CTC CTC CC
45	RM34	GAA ATG GCA ATG TGT GCG GCC GGA GAA CCC TAG CTC
46	.RM35	TGG TTA ATC GAT CGG TCG CC CGA CGG CAG ATA TAC ACG G
47	RM36	CAA CTA TGC ACC ATT GTC GC GTA CTC CAC AAG ACC GTA CC
48	RM38	ACG AGC TCT CGA TCA GCC TA TCG GTC TCC ATG TCC CAC
49	RM39	GCC TCT CTC GTC TCC TTC CT AAT TCA AAC TGC GGT GGC
50	RM41	AAG TCT AGT TTG CCT CCC AAT TTC TAC GTC GTC GGG C
51	RM42	ATC CTA CCG CTG ACC ATG AG TTT GGT CTA CGT GGC GTA CA
52	RM44	ACG GGC AAT CCG AAC AAC C TCG GGA AAA CCT ACC CTA CC
53	RM47	ACT CCA CTC CAC TCC CCA C GTC AGC AGG TCG GAC GTC
54	RM48	TGT CCC ACT GCT TTC AAG C CGA GAA TGA GGG ACA AAT AAC C
55	RM49	TTC GGA AGT TGG TTA CTG ATC A TTG GAG CGG ATT CGG AGG
56	RM50	ACT GTA CCG GTC GAA GAC G AAA TTC CAC GTC AGC CTC C

57	RM51	TCT CGA TTC AAT GTC CTC GG CTA CGT CAT CAT CGT CTT CCC
58	RM53	ACG TCT CGA CGC ATC AAT GG CAC AAG AAC TTC CTC GGT AC
59	RM55	CCG TCG CCG TAG TAG AGA AG TCC CGG TTA TTT TAA GGC G
60	RM60	AGT CCC ATG TTC CAC TTC CG ATG GCT ACT GCC TGT ACT AC
61	RM70	GTG GAC TTC ATT TCA ACT CG GAT GTA TAA GAT AGT CCC
62	RM80	TTG AAG GCG CTG AAG GAG CAT CAA CCT CGT CTT CAC CG
63	RM81	GAG TGC TTG TGC AAG ATC CA CTT CTT CAC TCA TGC AGT TC
64	RM431	TCC TGC GAA CTG AAG AGT TG AGA GCA AAA CCC TGG TTC AC
65	RM82	TGC TTC TTG TCA ATT CGC C CGA CTC GTG GAG GTA CGG
66	RM83	ACT CGA TGA CAA GTT GAG G CAC CTA GAC ACG ATC GAG
67	RM84	TAA GGG TCC ATC CAC AAG ATG TTG CAA ATG CAG CTA GAG TAC
68	RM201	CTC GTT TAT TAC CTA CAG TAC C CTA CCT CCT TTC TAG ACC GAT A
69	RM202	CAG ATT GGA GAT GAA GTC CTC C CCA GCA AGC ATG TCA ATG TA
70	RM204	GTG ACT GAC TTG GTC ATA GGG GCT AGC CAT GCT CTC GTA CC
71	RM205	CTG GTT CTG TAT GGG AGC AG CTG GCC CTT CAC GTT TCA GTG
72	RM206	CCC ATG CGT TTA ACT ATT CT CGT TCC ATC GAT CCG TAT GG
73	RM207	CCA TTC GTG AGA AGA TCT GA CAC CTC ATC CTC GTA ACG CC
74	RM208	TCT GCA AGC CTT GTC TGA TG TAA GTC GAT CAT TGT GTG GAC C
75	RM209	ATA TGA GTT GCT GTC GTG CG CAA CTT GCA TCC TCC CCT CC
76	RM210	TCA CAT TCG GTG GCA TTG CGA GGA TGG TTG TTC ACT TG

77	RM211	CCG ATC TCA TCA ACC AAC TG CTT CAC GAG GAT CTC AAA GG
78	RM212	CCA CTT TCA GCT ACT ACC AG CAC CCA TTT GTC TCT CAT TAT G
79	RM213	ATC TGT TTG CAG GGG ACA AG AGG TCT AGA CGA TGT CGT GA
80	RM214	CTG ATG ATA GAA ACC TCT TCT C AAG AAC AGC TGA CTT CAC AA
81	RM215	CAA AAT GGA GCA GCA AGA GC TGA GCA CCT CCT TCT CTG TAG
82	RM216	GCA TGG CCG ATG GTA AAG TGT ATA AAA CCA CAC GGC CA
83	RM217	ATC GCA GCA ATG CCT CGT GGG TGT GAA CAA AGA CAC
84	RM218	TGG TCA AAC CAA GGT CCT TC GAC ATA CAT TCT ACC CCC GG
85	RM219	CGT CGG ATG ATG TAA AGC CT CAT ATC GGC ATT CGC CTG
86	RM220	GGA AGG TAA CTG TTT CCA AC GAA ATG CTT CCC ACA TGT CT
87	RM221	ACA TGT CAG CAT GCC ACA TC TGC AAG AAT CTG ACC CGG
88	RM222	CTT AAA TGG GCC ACA TGC G CAA AGC TTC CGG CCA AAA G
89	RM223	GAG TGA GCT TGG GCT GAA AC GAG GCA AGT CTT GGC ACT G
90	RM224	ATC GAT CGA TCT TCA CGA GG TGC TAT AAA AGG CAT TCG GG
91	RM225	TGC CCA TAT GGT CTG GAT G GAA AGT GGA TCA GGA AGG C
92	RM227	ACC TTT CGT CAT AAA GAC GAG GAT TGG AGA GAA AAG AAG CC
93	RM228	CTG GCC ATT AGT CCT TGG GCT TGC GGC TCT GCT TAC
94	RM229	CAC TCA CAC GAA CGA CTG AC CGC AGG TTC TTG TGA AAT GT
95	RM230	GCC AGA CCG TGG ATG TTC CAC CGC AGT CAC TTT TCA AG
96	RM231	CCA GAT TAT TTC CTG AGG TC CAC TTG CAT AGT TCT GCA TTG

97	RM232	CCG GTA TCC TTC GAT ATT GC CCG ACT TTT CCT CCT GAC G
98	RM233	CCA AAT GAA CCT ACA TGT TG GCA TTG CAG ACA GCT ATT GA
99	RM154	ACC CTC TCC GCC TCG CCT CCT C CTC CTC CTC CTG CGA CCG CTC C
100	RM234	ACA GTA TCC AAG GCC CTG G CAC GTG AGA CAA AGA CGG AG
101	RM235	AGA AGC TAG GGC TAA CGA AC TCA CCT GGT CAG CCT CTT TC
102	RM236	GCG CTG GTG GAA AAT GAG GGC ATC CCT CTT TGA TTC CTC
103	RM19	CAA AAA CAG AGC AGA TGA C CTC AAG ATG GAC GCC AAG A
104	RM238	GAT GGA AAG CAC GTG CAC TA ACA GGC AAT CCG TAG ACT CG
105	RM452	CTG ATC GAG AGC GTT AAG GG GGG ATC AAA CCA CGT TTC TG
106	RM239	TAC AAA ATG CTG GGT ACC CC ACA TAT GGG ACC CAC CTG TC
107	RM240	CCT TAA TGG GTA GTG TGC AC TGT AAC CAT TCC TTC CAT CC
108	RM241	GAG CCA AAT AAG ATC GCT GA TGC AAG CAG CAG ATT TAG TG
109	RM242	GGC CAA CGT GTG TAT GTC TC TAT ATG CCA AGA CGG ATG GG
110	RM243	GAT CTG CAG ACT GCA GTT GC AGC TGC AAC GAT GTT GTC C
111	RM244	CCG ACT GTT CGT CCT TAT CA CTG CTC TCG GGT GAA CGT
112	RM245	ATG CCG CCA GTG AAT AGC CTG AGA ATC CAA TTA TCT GGG G
113	RM246	GAG CTC CAT CAG CCA TTC AG CTG AGT GCT GCT GCG ACT
114	RM247	TAG TGC CGA TCG ATG TAA CG CAT ATG GTT TTG ACA AAG CG
115	RM248	TCC TTG TGA AAT CTG GTC CC GTA GCC TAG CAT GGT GCA TG
116	RM249	GGC GTA AAG GTT TTG CAT GT ATG ATG CCA TGA AGG TCA GC

117	RM250	GGT TCA AAC CAA GCT GAT CA GAT GAA GGC CTT CCA CGC AG
118	RM251	GAA TGG CAA TGG CGC TAG ATG CGG TTC AAG ATT CGA TC
119	RM252	TTC GCT GAC GTG ATA GGT TG ATG ACT TGA TCC CGA GAA CG
120	RM253	TCC TTC AAG AGT GCA AAA CC GCA TTG TCA TGT CGA AGC C
121	RM254	AGC CCC GAA TAA ATC CAC CT CTG GAG GAG CAT TTG GTA GC
122	RM255	TGT TGC GTG TGG AGA TGT G CGA AAC CGC TCA GTT CAA C
123	RM256	GAC AGG GAG TGA TTG AAG GC GTT GAT TTC GCC AAG GGC
124	RM257	CAG TTC CGA GCA AGA GTA CTC GGA TCG GAC GTG GCA TAT G
125	RM258	TGC TGT ATG TAG CTC GCA CC TGG CCT TTA AAG CTG TCG C
126	RM484	TCT CCC TCC TCA CCA TTG TC TGC TGC CCT CTC TCT CTC TC
127	RM260	ACT CCA CTA TGA CCC AGA G GAA CAA TCC CTT CTA CGA TCG
128	RM261	CTA CTT CTC CCC TTG TGT CG TGT ACC ATC GCC AAA TCT CC
129	RM262	CAT TCC GTC TCG GCT CAA CT CAG AGC AAG GTG GCT TGC
130	RM263	CCC AGG CTA GCT CAT GAA CC GCT ACG TTT GAG CTA CCA CG
131	RM1002	GAA CCA GAC AAG CAA AAC GG AGC ATG GGG ATT TAG GAA CC
132	RM1003	GAT TCT TCC TCC CCT TCG TG TTC CTG TCA GAA CAG GGA GC
133	RM1004	ACG ACC CCT CCT GGT TCT G CTC GTG GTT CTG GTC ACA AC
134	RM1013	GCT GCA ATG TCT TTC ACT GC GGC TTT GGG GGA AAT AGA AG
135	RM1015	TGT ATG ACT TTT TAG CAT TG CCA CAT TCA TTT AGA TGT TA
136	RM1018	ATC TTG TCC CAC TGC ACC AC TGT GAC TGC TTT TCT GTC GC

137	RM1019	GTT TGA ACA GTA GGA CTT GT AGA ACA TCT CAC ACT TCT CT
138	RM1026	GCC TCT GGC AGA ATA GCA TC TAT CAC TTT GCT GCC TAG GC
139	RM1036	CTC ATT TGT CGA TTG CCG TC ATG GGA GGA GTG ATC AAA CG
140	RM1038	CGG ATT TCA GAA TCC ATG GC TCG ATG CCT CGA TGT ATG TG
141	RM1080	AGA GCC CTC GTA AGC CAA AG GGT CGT GAA TCT CCT CCA AG
142	RM1083	CCT TGA TTG CAG CAT CCG TTG AGC CTT TTA CGA GAC GG
143	RM1085	GGG GAA AAA GGA ACA CCT TC ACA GGA CAG ACG ACA ATT GG
144	RM1093	AGG TTG ATG AAC CCG ATG AG CTA GCT GCA GAA CGG AGG AG
145	RM1099	CTC GGC GAA TCA GAG AAG AC ATC CTA ACG TGC CTA TCC CC
146	RM1100	GAA AGA GCG AAG GCG GTG TCT CTG TCT CTC TCG CTC TCG
147	RM1112	TCA GGA CAC ATG GCC CTT AC CAG CTC CTG ACA GAG CAC AC
148	RM1124	AAG CTA TCC CCC TTT TTG GC AGG GAT CGG TAG ACC CAA TC
149	RM1125	GGG GCC AGA GTT TTC TTC AG GTA CGC GCA GAA AAT GAG AG
150	RM1134	ACA CCC AAC TTT TCT CAC GC AGC TAG GGT TTC GAT CTC CC
151	RM1136	ATG TCA TCC AGA GTC GCC TC AGG ACG TAT TCA CAC ACG AC
152	RM1146	ACC CCG ATG ATC GAT TGT AC CCC TAT TCC CGT GTA AAT CG
153	RM1150	ACA GTG GCC ACA GTG TGT TG GGA TTC GGG AGG TTG ACG
154	RM1152	GCC TTT GTC CTT CAG TAG GC AGA GCG CCT GGG TAT AAT TG
155	RM1164	CGT TTC TCC GAG AAA AGT CG

		CAA GGT GGT CGT TGA GGC
156	RM1178	CAG TGG GCG AGC ATA GGA G ATC CTT TTC TCC CTC TCT CG
157	RM1182	GGC CCA GAT TCG ATG TAA TG AAA GCT TCT TCC GCT TCC TC
158	RM1186	AAT AAT CTG AGC CAG CTG CG CTG CGG GTA GGC AGC TAT AC
159	RM1189	AAC TGC CCA TTT GTC GTC GAC TCC GGA CTA GAC CAA TC
160	RM1208	GCA GGG GAC ACG AGA TTT C TGT GCT CTG CTT GGA GAG TG
161	RM1221	GAGTAGAGAGAGATGGCGGC AGG ATT AGC AGC GTT AAG CG
162	RM1227	ATG GTA GAG ACG AGA GAT GG GGA CCA CTC CAA CAA TTT TA
163	RM1233	TTC GTT TTC CTT GGT TAG TG ATT GGC TCC TGA AGA AGG
164	RM1235	GAA AAC TAA AAA GCA GAG GA AAG CTA TCC ATT TTG GAT TA
165	RM1236	AGA AAA GTT AAT TCC AAA GG CAA GGA ATT CTA GAG GAG TG
166	RM1270	TAC TAG TTC ACT ACC ACG CAG C GCA TTT CCC GCA ATG TAG AG
167	RM1272	TCT ATG GAT CTG CAT GCT GG CTG CCC TGT CCT TTT AAT CG
168	RM1297	TGC CTT ACA ACT CAA CGA CG TGC ACT CCC AGT TCA GTA CG
169	RM1313	TGT GTC TGA AAA CCA AGG GG CGT CCA AGC TGT TCG TTC TC
170	RM1321	CTT GCA TGA CTA CAC GAG TCG TAT CCT GAG CGA GAT CAG GG
171	RM1324	TGT TGA TCC CCT TGA TAG GG AGC AAG ATC AGC TAG CTG CC
172	RM1335	GCA TGC ATG AAT ATG ATG G AGA TCG AAC AAG AAG AGT GG
173	RM1337	GCT GAG GAG TAT CCT TTC TC

		ACC ATA GGA AGA TCA TCA CA
174	RM1340	TTC CAA ACT AGT GGG AAC GC CCT CAA CGC CAT GAA CCT C
175	RM1341	AAC CTG GAG GTG CTG GTC TC TTT CTC CCC CCC AAC CAC
176	RM1345	ACC ACC ACG CCA TTA GAG AC TGA GCA TCC CGT GCT GTC
177	RM1367	GCA TCG TTC ATG TAC ACT GG CTG CTA CGC TGC TAC TCC TAG
178	RM1374	TAG ATA TGT TGG GCC GGA AG AGA TCG ATG CCG TTT CAG AC
179	RM1388	CTG CTC AGT GCT CAA TGG AG TAG CTT GGA CTA GGG GCA TG
180	RM1812	CAG CTA GTG AGC TCC TAG TG GCT AAC CCA CCA ACT TAT TC
181	RM1843	GTC ACA ACT ACT TTT ACA CG CTA CTA ACA GAA GGC TAT GG
182	RM1817	TAG TAT TCT TTC CTT ACA GA ATT GAA AAC TTA ACA AAT AG
183	RM1761	ACG CTT AAA GAA CAT TTG AT GCG ATT AAC TTT TAA CCA TT
184	RM1789	GGA AAT GTA CAG ATG TGT GG CAA TCT CGC AAT TTT TCA TA
185	RM1841	ATT GGA ATA CCT AAA AGC TA GGA TGC CTT ATA TGA AAT AT
186	RM1925	AAT TCA TTC AAG CCT TGA TA ATT AGT TTC ACC AAA GCA AC
187	RM2006	GTG TGA CTC ATC CTA ATA CA AGT ACA ACG AAT CTG GAT AT
188	RM2144	ACA TTA TGA AAC GGA GGA AG GAA ATG ATG CAT CAG CAT TA
189	RM2187	GTC ATT TGA AGT AAA TCC GT GGT CTA CTT GCG AAA TAA GT
190	RM2318	CTT TTG CTC ATC CAT TCG CCT CTT CAT GCG ATA AAC AT
191	RM2422	AAC ATG GGA AAC ACT AAT AA AAG ATT TGA ACC ACA GTA GA

192	RM2468	TCC CCT GCC TCT AAT TAA TC AAG TCA AAG TGT CAA GAC CAA A
193	RM2486	CGT CTT CTC TGC AAC ATT AC CGA ACG CGT TTA GAC TAA TA
194	RM2529	CAT TAA AAT CAG TGG GAC TG AGG CAT TTC CTG ATA TGA TC
195	RM3134	GCA GGC ACA AAA GCA AAG AG AGG TGA AGG TGC ATT GTG TG
196	RM3137	GTT AGG AAT TCC ATG CTG CG TGC CCG TGC TCG ATA AGG
197	RM3138	TTG ACA AGA GAT CAA GGC GG GTG AAT GTT GAG CTG CAT GG
198	RM3143	AGC CTG GAT AAG ATG GTT CG CGA GAA GAC CCA GTT TCT GC
199	RM3152	ACA GGT TTG CAG ATT ACA TA CCC ATC TTT AAT ACC TTC AA
200	RM3153	CAC AAA GTT TCA AAT ATA GC GAT CTC ATG ATA GTC ACT CA
201	RM3165	GTT CCG GTC GGG ACT AGT TC GTG GAT GGG AGC AGG TGT AC
202	RM3248	AGA AGG TTG CTT TCT TGG CC CTT GCA AGG TCT GTT GCA TC
203	RM3295	TCG TGT CAT GCG ATC GAC GCT TCG ACT CGA CCA AGA TC
204	RM3331	CCT CCT CCA TGA GCT AAT GC AGG AGG AGC GGA TTT CTC TC
205	RM3394	CCC TTA CGT GCA GTA CAT T ATG CAG GCT ACT TAC TAG CG
206	RM3473	ATA TTG GAA GGA GCA ATC AC CGT AAT GTT GGT GAA GCA G
207	RM3590	GTC TTG CTG CAC CCT CTT TC CAC CAC TGC ACA CAA TCC AC
208	RM3564	CAT AAA CCG CTC GGC ATT G CTG ACT CAC AAG ACA ACA GGG
209	RM3598	CGA CTT CTC CTC CAT TTT CG CAA ATT CAC GCA GTG ACC AC

210	RM3648	TAC CCT TTC TTC CCC AAA CC ACC TCC TCC TCC ACT TCT CC
211	RM3691	GCT GAT GGT CAA AGA TCA GG ATG TGT CTG CTG GCA CAG AG
212	RM3717	AGC TCT ACC TTT GCT GTC GG AAC TCC CTA GAC CCA CCT GC
213	RM3744	CAG GTA AGT TTT CAT TTT CA GAG CAG GAG TAA CAG TTG TA
214	RM3761	CCT CAA CAA TAG CAC CAC CC CTG CAA GTC TGC AAG CAC AG
215	RM3796	ATT AGC CTT TAA TTC CAC TG ATA CAA ACA AAC AGC TTG TG
216	RM3720	GGA GGA GAG CTG GGA GAG C GTC GAT GGG CCG AAT CTC
217	RM3827	GGA CGG ATT GTA GGT AGG AC CCT TTC TTC AAT CTG CAT TC
218	RM3828	AAG CAT TAT TGA CCA CCA AC TCT GAT GTC CTT ATG TCA TGG
219	RM3969	AGG CTA AGT TCA TAT CCA AC ACC CTA TAA AGC ATA AAG GA
220	RM4069	ACA ATA ATC TTC AAA GAT GC AAT CAT TGT GAA GTT CAA TC
221	RM4128	AGT AAC TCG ATC AAA CTA AC AGA GTC CAT ATA GAA TTT CA
222	RM4244	GAT TAA TTT TCA CAT GAA TA GAA TTT GTA AAC TTA ATG AA
223	RM4266	AAT AAA TTG ATT AGC TTG AA GTT ATC CCA ATT ATG TGA TA
224	RM4348	GGT CAA GCT TAG TTA CTC CT ACA TTA ATC GTC TGC AAA TA
225	RM4355	GGG ATG AGA GTA GAA GGC A TAT ATG GCA AGC CTA GCG
226	RM4477	AGT AAA CAT GTC TTC GGG AT CAG TGC ATA TTC CAC TGG TA
227	RM4584	CCT ATT TAA TAT AGC ACC AG CCA TTT AAA CAT AGA AAA AC
228	RM4888	CTG GTG CTC AAT CAT CAT AT

		CAA GAT TCT TTT CCA AAC AA
229	RM5084	CAT GCT AAC TTG AGA CGA TC ATG AAA TTC ACT GTG GGA GT
230	RM5134	GAT TGG AGC TTG TTT TCT C CAC AAA TCA AAT ACA TCA CAG
231	RM5607	GAG TAG GAG GAG CCG AGG AG ACG TGG CCA ACT GGC TAT AC
232	RM5609	CGC CAG TGT CGA ATA TGA TG TCT TGG TGC AGT AGG TGC AC
233	RM5620	TCG ACT TGA AGC ATC ACA CC TCT GAA ATG TCA AGT GGG CC
234	RM5633	GTG TAG CTG CTA GGC CGA AC TTC CTT TCG CTA CGT TGG AC
235	RM5661	GTT GCT GGG CTT GAT CTT TG CTG TCA TGG CCC CTC ATT AC
236	RM5693	CTC TTT GTG TCT ACA AAA AC TGG TTA TTT TAA TAA GAT GC
237	RM5704	AAC GAA TGA TTA AAC ATC TA AAG CAG AGT CAA CAT ATT TA
238	RM5711	GTC CAT GCA TCC ATC TCT AG ACG GAA GGA ATA CGT CTG TA
239	RM5735	AGG CTT GTC CAA TAC GAT CG TTC TGT TGC TGT AGT TGC CG
240	RM5745	ATG CCA AGT GGA CGA TGT AC ACA TGT GGG TAG TGG GAT GG
241	RM5757	CCT GAG ACC ATA TGC TGC TG GAG GGA GCA TCA TTA GCT GG
242	RM5780	GCT GCT GCA TCT TCT ACT GC ACG CAC ATG CCT AAG CCT AG
243	RM5795	AAT CAG CAG GTG AGG ACG AG GAA CTG ATG GGA AAG CCA AC
244	RM5801	TTC GGT TAT CGA TGA GGA GG CAT CAT TGC GCC ATG TAC TC
245	RM5807	CTG CTG TTG CGT TGG AGT AC TGC TCC GCC ATG CCT AAC
246	RM5814	GCT CAT CAC TCC ATG AAT CG ATC AAG GCT CGC TAC TGC TC
247	RM5816	CTG CAT GCA TGA CTG CAT C TGA CTC TGA TCG ATC CAT GG

248	RM5819	CAC TCC AGA ACC CAG TAG TAG C ACC CAA CAA CTG GTA GAC GG
249	RM5841	CCT CTC TCT CTC TCT CCC CC TGT TAT TGG CAC GTG GTG TG
250	RM5847	TGA GAT GAG AGA TAG ACT CC AAC AGA TGA AGG CTA TTT TA
251	RM5851	GCT GTC GGG GAT GTA ATA CG GCT TTG CGG CTG GTT AAT TG
252	RM5862	TTA GTA CCT CAT CAT AGC TG CTC TAA TCT TCT CTC ATT ATC A
253	RM5864	ATT AGT ACC GTG TGG TCC GC GAC CGA ATT GGT GAT CGA TC
254	RM5879	ACC AGA GAT CGA TTG GTA GC GGC TGC CTA TCG AGG CTA AC
255	RM5887	ACC GAC GAC TCG TAC AAA GG CTC ACT TTG GGC TTT TGA CC
256	RM5894	ATC TCC CTA AGG CAC AAC CC CGT CAT GCG ATG TTC TCT TG
257	RM5900	TTC TAC GTT TGA CCG TCA TCT AGG AGC GTT TGT AGG AG
258	RM5911	CCC TCT TTT TAA GTC TGG GG GGT GCC TCC TTT CAA AGT TG
259	RM5914	TCT CCA CAT TTG ACT CAC GG AAC GGT GTG ATA AGG GAT GC
260	RM5925	GTA TAT TCC ATC GAG ATT AG ATG AAC TGG TAG GAT AAG TA
261	RM5939	CAC TCC ACT GCT GTT GCA CT CAC CTC GGT GAG GTT TCT TC
262	RM5944	GAG CCG CAT CAA CCA GTT AC CAG TAC AGC GCG CAC TAC AC
263	RM5950	CAG ACC GGC ATG ATG GTC GAG ATC CGG GTT CTT GGC
264	RM5954	CTC GTC CTC AAG TGC CTC TC TCG ACT CCT ACT CCG ACT CC
265	RM5955	ACA ACC TCC TGC AGT TCC AG TGG CTC TGC AAA GTT GTA CG
266	RM5963	CGA AAA GTG GGA AGC AAA TG GCG TAC CCC TAG TGG CTG TA
267	RM5970	CCC ATC TGG TTC ACC TTC AC AGG AGC AGC CTT TTG TCT TC

268	RM5982	ACC CGC CCT GCA AAT CAC CGT CGT GTT GTG ATG TGA TG
269	RM5989	CAA ACT CAT CAC CCA TTC GC AAG TAG GTG CCC TTT GCT CC
270	RM5990	TAG CCT CCC TCC TCT TCC TC AGA TGG AGG TGG AGG TGG AG
271	RM5996	CGA TTC GCT TCG TTT CCT AC AAA CCA ACA GCG ACA CGC
272	RM6011	TTT CAC CAT CCT TCT CTG CC CTG GGT ACT GCG TAG AGA TCG
273	RM6051	AGG CTG ATC CAA GAT CCA TG CCC GGA GGC TGA TTC TTG
274	RM6057	TCA TGT TGT TGC TCC TCC TG AGG GAG AGA GAC AGC AGC AG
275	RM6075	CCA ACG ACT TCC AGA AGG TC CGA AGA GGG CTA GAA TCA CG
276	RM6080	CAG AGG AAG CAA GGA GAT CG CCA TCG GGA GAG AAA GAG AG
277	RM6083	CGT AAA AGG TCC TCG TCG TC AGT AGC CTG CTC TCC ATT GG
278	RM6091	GCT GTC CTG TCC TTG AAT CC TGG TAG GCT GGT GAC ATG C
279	RM6120	TCA AGA ACG AGA AAG CCA CC CCG TGT AGA CGA CGA CGA C
280	RM6137	ACG GCG ACT TTG ACT CTC C GAC TCC CAC TCG TCA ACC AC
281	RM6156	GCT CCA AAA CCC AGA CTC TG TCG AGC AAG CGA GGG AAG
282	RM6174	TCG AGG TGG AGA AGC AGC TAG TCT TCG TGT CAC GCA GC
283	RM6187	GGA CGG TGT CTA CCA TGT C CAT TGC TGG AGA AAT CAC TAC
284	RM8012	CTGCATGGTTTAGGGTTTAT TACATACACCTGGGTGTACG
285	RM8014	ATA CTG AGA AGA AAA ACC AA ATA ACA GAC AAA TGA AAA CC
286	RM8029	CTT CAT TTC AGA ATG TAA GA ACC CAC ATT TAT TTA GAT GT
287	RM8045	TCG CGG TTA ATG TCA TCT GAC TGA CCC TAA AAC CAT ACA C

288	RM8061	GTC TAA TTT TCC TCC CTC CT TGT GTT GGC TTT GTT ATT G
289	RM8116	TCA CCT CCC AGA GAA GAG AAC G TTT CGG AAT ACG CGC CTA TCT T
290	RM8138	TGA TTA CAC CAT TAT ATT TGT T GAA AAG CTA AAA GTT AGT GG
291	RM8210	GTC CCA CAT GTC AGG ATG ATC TGC TAC TTG TGG AGG AG
292	RM8214	CCT AGC TTT CAG GAG CAA G CCC ACA ATG AGA AAC AGT TG
293	RM8243	CTC GTG CAA CCA TTA TAT TC ACC TTA GCT GTC CTG AAT TG
294	RM7000	CCC TTC TTT TCA ACT GAA TA TTG TAA CAA TGA ACT CGT TC
295	RM7003	GGC AGA CAT ACA GCT TAT AGG C TGC AAA TGA ACC CCT CTA GC
296	RM7006	CTC GTT TAT CCT CCC AGT GC CAC TTG TAT CCA GAA GCA GG
297	RM7018	CAT CGT TGA CCG CTG CTC AAT AAA CAG CAC GTG CTC CC
298	RM7081	CCG CAC TAC ACT GCA CTC C AAC TTG CTC ATG GAG TTG GG
299	RM7300	TCCGTATCCTAGTCGCGATC CGCCGTCATGACTCATACTC
300	RM7402	TAGTGGAACAAAGTTCCGCC ACCAGCATATGCGAACACAG

3.8.1 Simple Sequence Repeat markers Analysis

Three hundred SSR primer pairs were used to differentiate the parental genotypes Pusa Basmati-1460, CSR-30 and IRBB-60. Polymorphisms in the alleles amplified by different SSR markers of the parental genotypes were scored visually and their size was calculated using 100 bp DNA ladder. The SSR markers which amplified the unique alleles only in the recurrent parent CSR-30 were further used for the background selection to find out the recovery of recurrent parent genome in foreground selected plants positive for all the three BB resistance genes.

PCR conditions for SSR marker analysis

1.	Initial denaturation	94°C for 5 min.	
2.	Denaturation	94°C for 1 min.	35 cycles
3.	Annealing	55°C for 1 min.	
4.	Extension	72°C for 2 min.	
5.	Final extension	72°C for 10 min.	

The amplified products were resolved in 2.6% agarose gel.

3.8.2 Allele Scoring

DNA amplicons obtained with the polymorphic SSR markers analysis were scored visually for the presence (1) and absence (0) of parental alleles for all the three-gene pyramided BC_1F_2 genotypes and the parental genotypes. DNA banding pattern for each primer was scored by visual observations, where only clear and unambiguous bands were scored. The size (in nucleotide base pairs) of the amplified bands was determined based on its migration relative to molecular size marker (100bp DNA ladder from Bangalore Genie, India). Also the data was scored for IRBB-60 alleles as A and CSR-30 alleles as B for each primer–genotype combination.

3.8.3 Data Analysis

The DNA banding patterns obtained from SSR analysis for each primer were scored by visual observation. This 0/1 matrix was used to calculate the genetic similarity to estimate all pair-wise differences in the amplification products for all three-gene pyramided genotypes. The genetic similarity between these lines was evaluated by calculating the Jaccard similarity coefficient. Similarity coefficients were used for cluster analysis using sub program of NTSYS-PC (Rohlf, 2000). The level of similarity relationships among the three gene pyramided genotypes was determined. The dendrogram was constructed by unweighted pair group method with arithmetic averages (UPGMA) sub programme of NTSYS-PC. Principal component analysis (PCA) was done using the 'CPCA' sub-programme of NTSYS-PC software. The assessment of the genomic contribution of the parents in the three gene pyramided genotypes based on SSR marker data was carried out using the software programme Graphical Geno Types (GGT) Version 2.0 (Van Berloo 1999).

3.9 Artificial screening of BB resistance in the three-gene pyramided BC_1F_2 lines

3.9.1 Isolation of bacteria (*Xanthomonas oryzae* pv. *oryzae*)

- Infected rice leaves showing bacterial blight symptoms were collected from the BB infected fields of RRS Kaul.
- These leaves were surface-sterilized with 2% sodium hypochlorite for 1 min and washed twice with sterile distilled water.

- The leaves were then cut into 0.5 cm pieces and placed in 10 ml of sterile distilled water.
- The cells were allowed to ooze from leaves into sterile water and streaked for single-colony isolation on PSA plates.
- The *Xoo* isolate was multiplied and maintained on peptone sucrose agar (PSA) at 28°C. These isolates were preserved in glycerol at −70°C.

Composition of Peptone Sucrose Agar (PSA) media

Sucrose	5.0g
Sodium glutamate	1.0g
Ferrous sulphate	0.25g
Yeast extract	2.5g
Peptone	10.0g
Agar	15.0g
pH	6.0

3.9.2 Artificial Inoculation

Plants selected on the basis of molecular marker analysis from the BC_1F_2 generation carrying resistance genes (*xa5*, *xa13* and *Xa21*) individually and in combinations, along with the control, were inoculated with the predominant *Xoo* isolate prevalent in Haryana State using a bacterial suspension of 10^9cells/ml (Kauffman *et al.* 1973). The plants were clip inoculated at maximum tillering stage. The leaf blades were inoculated by clipping with scissors at 3 cm below the leaf tips. On an average five leaves per plant were inoculated and the disease incidence (DI) using 0-5 scale (Table 3.4) was measured 14 days after inoculation.

Table 3.4: Disease rating using 0-5 scale

Infection (%)	Score	Host response
0	0	Highly resistant (HR)
1 -10	1	Resistant (R)
10 -30	2	Moderately resistant (MR)
30 -50	3	Moderately susceptible (MS)
50 -75	4	Susceptible (S)
75 -100	5	Highly susceptible (HS)

3.10.1 Data collection on various physio-morphological traits of parental and pyramided BC_1F_2 genotypes

For every plant, observations were recorded on the following characters. The data was subsequently analyzed to determine the variability.

(i) Plant height (cm)

The plant height of fully mature plant was recorded from ground to the flag leaf.

(ii) Panicle length (cm)

The panicle length of fully mature plant was recorded.

(iii) Effective number of tillers per plant

Effective number of tillers per plant was recorded.

(iv) 1000 grain weight (grams)

1000 grain weight per plant of all the genotypes was weighed.

(v) Grain yield per plant (grams)

The total grain yield per plant of all the genotypes was weighed.

(vi) Length/Breadth ratio of seeds

The length/breadth ratio of five seeds from each plant was recorded using digital Vernier Caliper.

3.10.2 Statistical Methods

Mean: The mean value of each length-breadth ratio was worked out by dividing the totals by corresponding number of observation:

$$x = \frac{\Sigma X_i}{N}$$

Where,

X_i - any observation in i^{th} treatment

N - Total number of observations

CHAPTER-IV

EXPERIMENTAL RESULTS

Progress has been made in mapping and tagging many agriculturally important genes with molecular markers which forms the foundation of marker-assisted selection (MAS) in crop plants. Recent developments in DNA marker technology together with the concept of marker-assisted selection have been effectively utilized in crop improvement and germplasm characterization. While, there are several applications of DNA markers in breeding, the most promising for cultivar development is marker assisted selection (MAS). Marker assisted selection refers to the use of DNA markers that are tightly-linked to target loci as a substitute for or to assist phenotypic screening. Once molecular markers closely linked to desirable traits are identified, MAS can be performed in early segregating population and at early stages of plant development. Marker assisted selection can be used to pyramid resistance genes in order to enhance the durability of resistance. It greatly increases the efficiency and effectiveness for breeding compared to conventional breeding because genotypic screening is not affected by environment, as compared to phenotypic screening and selection may be carried out at seedling stage.

Bacterial leaf blight caused by the pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is one of the most widely distributed and devastating rice disease worldwide. The most effective approach to combat bacterial blight is the use of resistant varieties in combination with agricultural management practices. Conventional backcrosses usually succeed in transferring only one gene at a time. The large-scale and long-term cultivation of the conventionally-bred resistant varieties resulted in evolution of newer races of the pathogen leading to breakdown of resistance in rice varieties. Therefore, one way to delay such a breakdown is to pyramid multiple resistance genes into a variety through MAS. In this study, MAS was carried out to introgress three BB resistance genes *Xa21, xa13* and *xa5* into the basmati variety CSR-30.

Experiment No. 1: Confirmation of the presence of BB resistance genes in Pusa Basmati-1460 and IRBB-60

4.1 Isolation of Genomic DNA

The rice genotypes, Pusa Basmati-1460, IRBB-60 and CSR-30, were grown in the net house, Department of Molecular Biology and Biotechnology, CCSHAU, Hisar. Genomic DNA was isolated from the young leaves of 3-4 week old seedlings of the parental genotypes as per modified CTAB (cetyl trimethyl ammonium bromide) extraction method of Murray and Thompson (1980), modified by Saghai-Maroof *et al*. (1984) and Xu *et al*. (1994). The quantity and quality of extracted genomic DNA was checked both by agarose gel electrophoresis and UV spectrophotometer. Standardization of DNA isolation was performed with the following variations.

- **CTAB-** 1.5 % and 2 %
- **Incubation time-** 1hr, 1.5hr and 2hr. at 65ºC

Less amount of DNA was obtained at incubation time 2hr with 1.5% CTAB concentration in extraction buffer and at incubation time 1hr and 1.5 hr with 2% CTAB concentration in extraction buffer. Smeared DNA banding pattern was observed when DNA isolation was carried out using 1.5 % CTAB concentration in extraction buffer at incubation time 1hr with and with 2 % CTAB concentration in extraction buffer at incubation time 2hr. Good quality and high amount of DNA was obtained at the incubation time 1.5 hr at 65ºC using 1.5 % CTAB concentration in extraction buffer.

4.2 Qualitative and Quantitative Estimation of DNA

To determine the amount of genomic DNA, the quantity of DNA was estimated by taking UV absorbance at 260 nm of the genomic DNA samples and blank samples. To work out the amount of protein and RNA in the genomic DNA, the observation was also recorded at 280 nm using the same UV spectrophotometer. The ratio of absorbance at 260 nm and 280 nm equals to 1.8±0.05 showed that the DNA was free from contaminants like RNA and proteins etc. The ratio of absorbance at 260 nm and 280 nm of the parental genotypes Pusa Basmati-1460, IRBB-60 and CSR-30 calculated was 1.78, 1.75 and 1.72 respectively (Table 4.1). Genotype CSR-30 was treated with Phenol: chloroform to remove protein contamination. The quantity of DNA of the parental genotypes Pusa Basmati-1460, IRBB-60 and CSR-30 was 2203 µg/ml, 1462 µg/ml and 2473 µg/ml respectively. This showed that CTAB extraction protocol yielded high amount of genomic DNA (Table 4.1). The quality of genomic DNA of all the parental genotypes was also checked on agarose gel for its base pair size and RNA contamination. Resolution of genomic DNA on 0.8 per cent (w/v) submerged agarose gel showed a discrete single band of high molecular weight DNA (Plate 1). This showed that the genomic DNA of the parental genotypes was free from any mechanical or enzymatic degradation and was intact and of good quality.

Table: 4.1 Quantity and quality of Genomic DNA isolated from parental genotypes of rice following CTAB method

S. No.	Genotype	Quantity of DNA(µg/ml)	O.D of DNA at A260	O.D of DNA at A280	Ratio of A260/A280
1	Pusa Basmati-1460	2203	1.206	0.678	1.78
2	IRBB-60	1462	0.925	0.529	1.75
3	CSR-30	2473	1.345	0.782	1.72

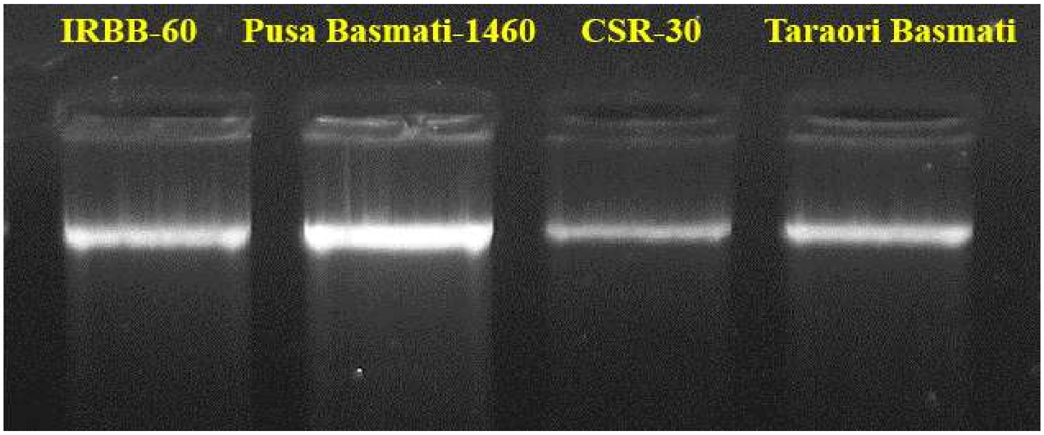

Plate 1: Electrophoretic pattern of purified high molecular weight genomic DNA free from protein and RNA extracted from the parental genotypes of rice.

4.3 STS markers analysis

Genomic DNA extracted from the parental genotypes IRBB-60, Pusa Basmati-1460 and CSR-30 was used for PCR amplification using the specific STS markers for each BB resistance genes (Table3.2).

4.3.1 Optimization of PCR amplification conditions

To optimize conditions for PCR amplifcation, PCR reactions were made with different concentrations of template DNA, primers, $MgCl_2$, dNTPs mix and Taq DNA polymerase. For this, varying concentrations of template DNA (100 ng, 150 ng and 200 ng), primer each Forward/Reverse (0.5 µM, 1.0 µM and 2.0 µM), $MgCl_2$ (0.8 mM, 1.0 mM and 2.0 mM) and Taq DNA polymerase (1.0 unit, 2.0 units and 3.0 units) in a reaction volume of 10 µl.

Amplified products with inconsistent band were obtained when the concentration of template DNA was 50 ng and that of $MgCl_2$ was 2 mM. With higher concentration of template DNA (200 ng) and $MgCl_2$ (2 mM)), smeared DNA banding pattern was produced. High annealing temperature of 65ºC gave no amplification or few bands in some genotypes. While at lower annealing temperature of 45ºC, some false bands were observed. However, the annealing temperature of 55ºC was found to be optimum for generating clear and reproducible bands. No significant difference was observed in banding pattern when the concentration of Taq DNA polymerase taken was either 1.0 unit or 2.0 units. Therefore, in subsequent PCR reactions, the concentration of Taq DNA polymerase was kept at 1.0 unit per reaction. Reproducible and clear banding patterns were obtained in a reaction mixture of 10 µl containing 100 ng template DNA, 100 µM of dNTPs mix, 0.5 µM of each primer

(Forward/Reverse), 1.0 mM of $MgCl_2$ and 1.0 units of Taq DNA polymerase. The PCR amplified products were resolved on 1.5 % agarose gel and visualized under U.V. light after staining with ethidium bromide.

4.3.2 Confirmation of BB resistance genes in the parental genotypes

The amplified product of marker pTA248 linked to BB resistance gene *Xa21* was resolved on 1.5 % agarose gel and visualised under U.V. light after staining with ethidium bromide. A specific band of 1000 bp was present only in the donor parental genotypes Pusa Basmati-1460 and IRBB-60 and absent in the variety CSR-30. So, the BB resistance gene *Xa21* was confirmed in donor parents Pusa Basmati-1460 and IRBB-60 (Plate 2).

For the gene *xa13*, PCR amplified product of marker RG136 was used for restriction digestion by restriction enzyme *Hinf* 1. The products of R.E digestion were separated on 1.5% agarose gel and visualised under U.V light after staining with ethidium bromide. Two bands of size 400 bp and 506 bp were present in the R.E digested PCR product of donor parental genotypes Pusa Basmati-1460 and IRBB-60 but absent in CSR-30. This confirmed the presence of BB resistance gene *xa13* in Pusa Basmati-1460 and IRBB-60 (Plate 3).

For the gene *xa5*, amplified product of marker RG556 was used for restriction digestion by restriction enzyme *Dra*1. The products of R.E digestion were separated on 1.5% agarose gel and visualised under U.V after staining with ethidium bromide. A band of size 450 bp was present in the R.E digested PCR product of donor parental genotype IRBB-60 and was absent in the variety CSR-30, thus confirming the presence of resistance gene *xa5* in donor parent IRBB-60 (Plate 4).

This, thus confirmed the presence of two BB resistance genes *Xa21* and *xa13* in donor parent Pusa Basmati-1460 and three BB resistance genes *Xa21*, *xa13* and *xa-5* in IRBB-60.

Experiment No. 2: Pyramiding of resistance genes in the basmati variety CSR-30

Crosses were made between CSR-30 x Pusa Basmati-1460 and CSR-30 x IRBB-60. The F_1 seeds of the above crosses were harvested.

4.4 Crosses	**No. of F_1 seeds**
CSR-30 x Pusa Basmati-1460	50
CSR-30 x IRBB-60	6

4.4.1 Cross CSR-30 x Pusa Basmati-1460

The fifty F_1 seeds of the cross CSR-30 x Pusa Basmati-1460 were grown in the net house out of which forty three seeds were germinated. Mini-scale DNA isolation was carried out from 35-day old seedlings from these forty three F_1 plants following CTAB method of Murray and Thompson (1980) to carry out the foreground selection. The quality of genomic

DNA of all the forty three F_1 plants was checked on agarose gel (Plate 5) and also the quantity of genomic DNA was calculated (Table 4.2).

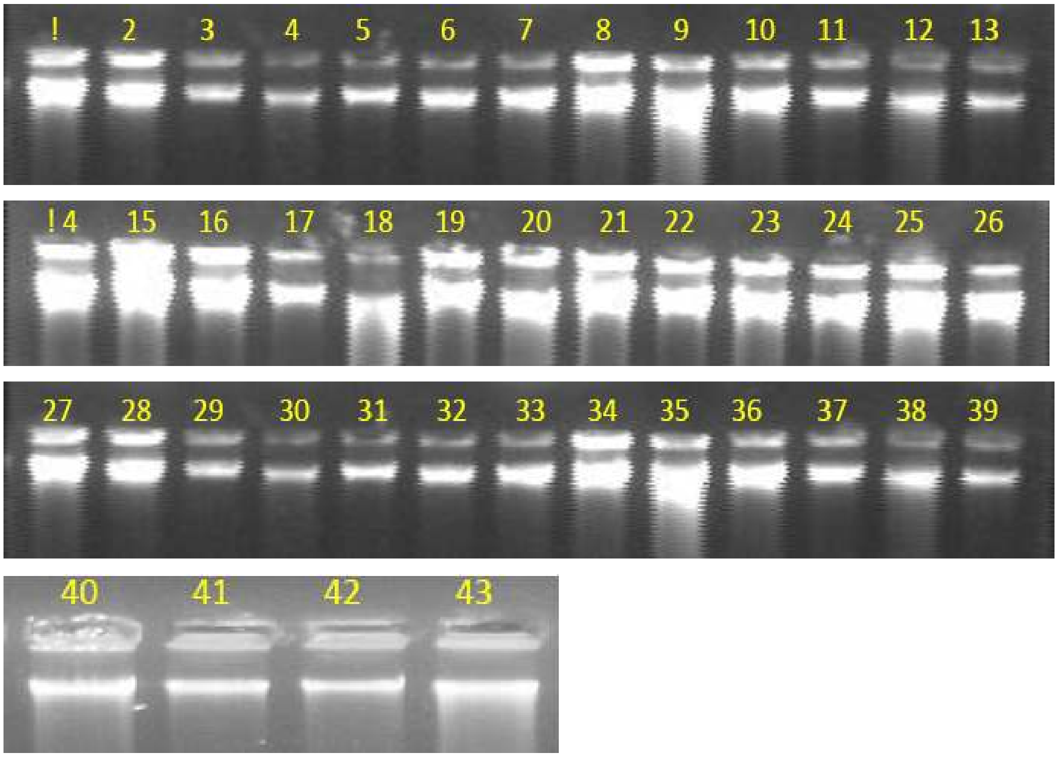

Plate5: Electrophoretic pattern of purified high molecular weight genomic DNA free from protein and RNA extracted from F_1 plants of cross CSR-30 x Pusa Basmati-1460.

Table 4.2: Quantity and quality of genomic DNA isolated from forty three F_1 plants of cross CSR-30 x Pusa Basmati-1460 following CTAB method

Genotypes	Quantity of DNA (µg/ml)	Ratio of A_{260}/A_{280}	Genotypes	Quantity of DNA (µg /ml)	Ratio of A_{260}/A_{280}
G-1	2090	1.85	G-23	2170	1.77
G-2	1990	1.81	G-24	2580	1.79
G-3	2080	1.76	G-25	2300	1.82
G-4	2010	1.78	G-26	2020	1.82
G-5	2090	1.80	G-27	2240	1.80
G-6	2150	1.74	G-28	2210	1.82
G-7	2220	1.75	G-29	2190	1.84
G-8	1910	1.73	G-30	2470	1.75
G-9	1890	1.72	G-31	2365	1.82
G-10	2160	1.83	G-32	2420	1.81
G-11	2240	1.73	G-33	2390	1.82
G-12	2460	1.75	G-34	2350	1.92
G-13	2340	1.82	G-35	2440	1.83
G-14	2260	1.75	G-36	2450	1.82
G-15	2250	1.76	G-37	2314	1.84
G-16	2320	1.76	G-38	2465	1.76
G-17	2150	1.73	G-39	2360	1.74
G-18	2200	1.74	G-40	2520	1.83
G-19	2110	1.84	G-41	2240	1.73
G-20	2160	1.79	G-42	2460	1.75
G-21	2330	1.75	G-43	2340	1.85
G-22	2166	1.78			

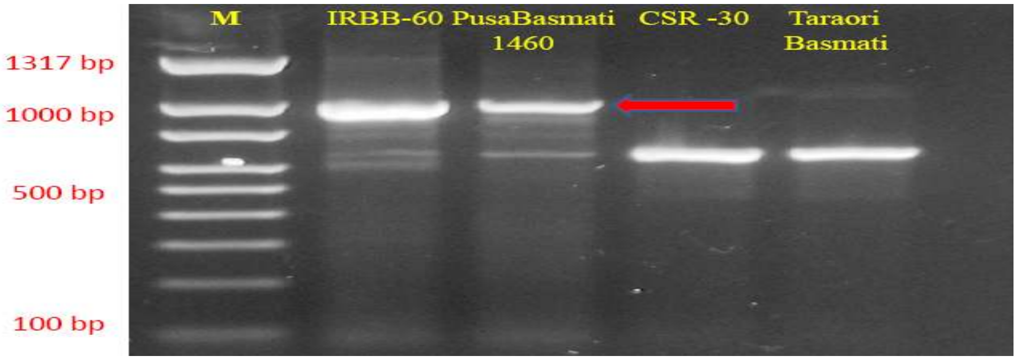

Plate2: Electrophoretic pattern of PCR amplified fragments of parental rice genotypes using STS marker pTA248 specific for *Xa21* gene. M- 100bp ladder
Arrow indicates 1000 bp band specific for *Xa21* gene

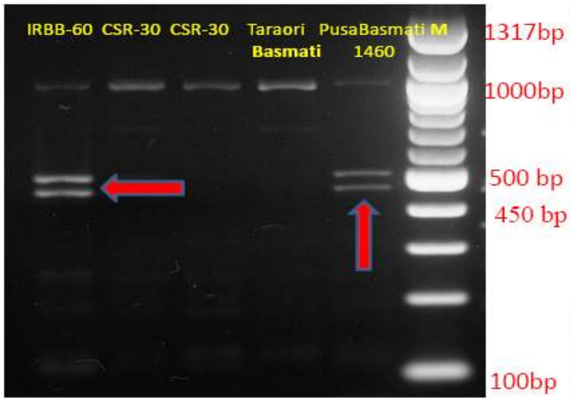

Plate 3

Electrophoretic pattern of PCR amplified fragments of parental rice genotypes using STS marker RG136 (Digested with R.E *Hinf* I) specific for *xa13* gene M- 100bp ladder
Arrow indicates 400 bp and 506 bp band specific for *xa13* gene

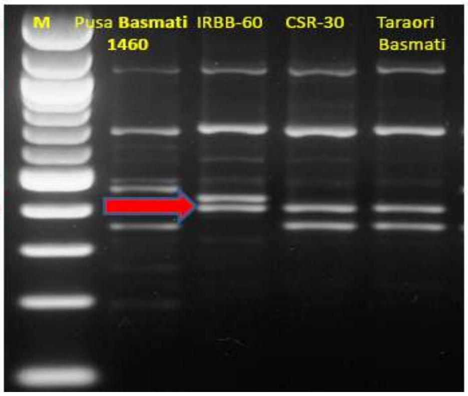

Plate 4

Electrophoretic pattern of PCR amplified fragments of parental rice genotypes using STS marker RG556 (Digested with R.E *Dra* I)
specific for *xa5* gene. M- 100bp ladder
Arrow indicates 450 bp band specific for *xa5* gene

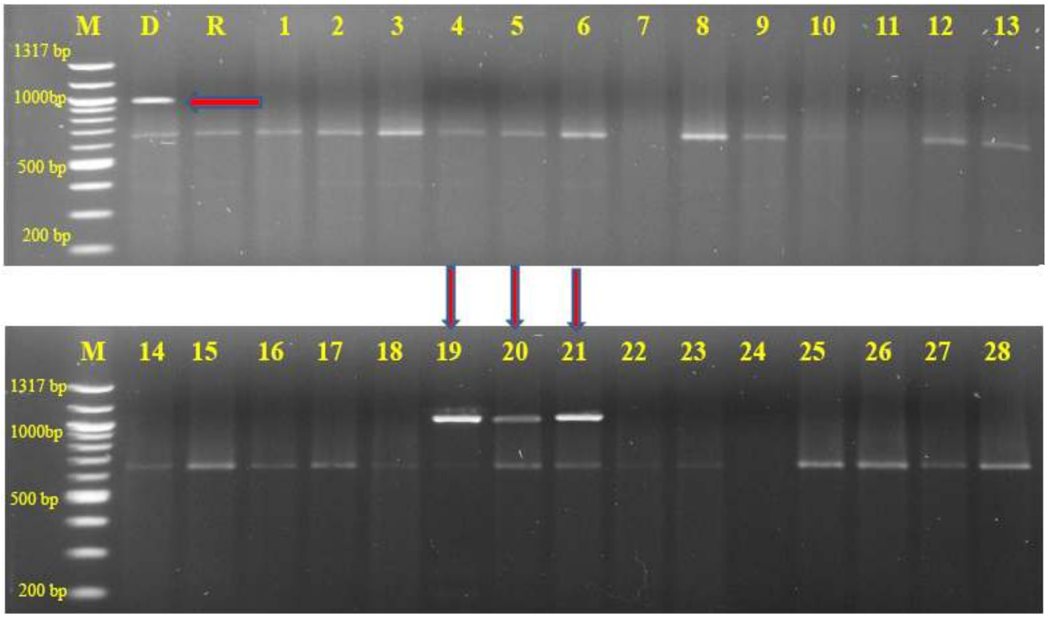

Plate 6: Foreground selection at F_1 generation by screening of plants using *Xa21* linked marker pTA248. M- 100 bp ladder. **D** (Donor parent) **- IRBB-60** **R** (Recurrent parent) **- CSR-30, 1-28:** F_1 plants of the cross CSR-30 x Pusa Basmati-1460
Arrow indicates 'positive' plant having *Xa21* gene (1000bp band)

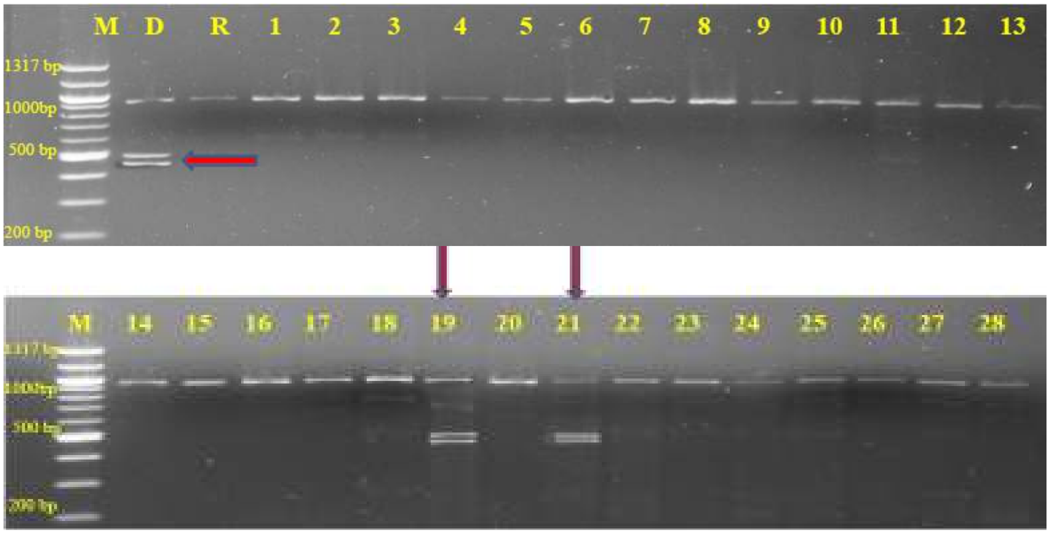

Plate 7: Foreground selection at F_1 generation by screening of plants using *xa13* linked marker RG136 (digested with R.E *Hinf* I).
M- 100 bp ladder. **D** (Donor parent) **- IRBB-60** **R** (Recurrent parent)**-CSR-30, 1-28:** F_1 plants of the cross CSR-30 x Pusa Basmati-1460
Arrow indicates 'positive' plant having *xa13* gene (400 and 506 bp band)

The presence of the resistance genes *xa13* and *Xa21* were checked in all the F_1 plants using the specific STS markers RG136 and pTA248, respectively. Out of forty three F_1 plants, two plants were found to have both the BB resistance genes *xa13* and *Xa21* (Plate 6 & Plate 7).

The two plants having both the resistance genes *xa13* and *Xa21,* failed to survive in the field. So, fresh crosses of CSR-30 x Pusa Basmati-1460 were made in the next growing season and the F_1 seeds of the cross were harvested. This time seed setting was very low.

Cross	**No. of F_1 seeds**
CSR-30 x Pusa Basmati-1460	4

The F_1 seeds of the cross, CSR-30 x Pusa Basmati-1460 were grown in the net house and genomic DNA was isolated from the 35-day old seedlings of these four F_1 plants following CTAB method. The quality and quantity of genomic DNA of the four F_1 plants is shown in plate 8 and table 4.3.

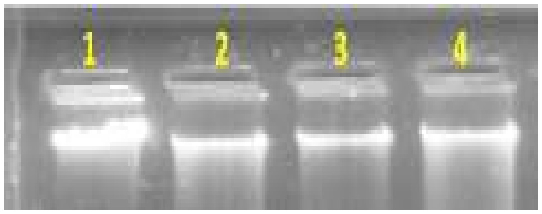

Plate 8: **Electrophoretic pattern of high molecular weight genomic DNA free from protein and RNA from four F_1 plants of cross CSR-30 x Pusa Basmati-1460**

S. No.	Genotype	Quantity of DNA (µg/ml)
1.	G-1	2520
2.	G-2	2200
3.	G-3	1605
4.	G-4	2510

Table 4.3: Quantity and quality of genomic DNA isolated from four F_1 plants of cross CSR-30 x Pusa Basmati-1460

The foreground selection was carried out in these F_1 plants using the specific STS markers pTA248 and RG136 to detect the presence of resistance genes *Xa21* and *xa13,* respectively. A total of two out of four F_1 plants were found to have both the BB resistance genes *Xa21* and *xa13* (Plate 9 and plate10).

Sowing of the F_1 plants having both BB resistance genes (*Xa21* and *xa13*) and the recurrent parent CSR-30 was done on 10th june 2012. Early sowing of recurrent parent CSR-30 was also done on 18th May 2012. The positive F_1 plants flowers early in 125 days and the recurrent parent CSR-30 flowers in 155 days. So backcrosses of F_1 plants were not made with recurrent parent CSR-30 due to non-synchronization of flowering time between the CSR-30 and positive F_1 plants and hence, selfed F_2 seeds were harvested.

4.4.2 Cross CSR-30 x IRBB-60

The six F_1 seeds of the cross CSR-30 x IRBB-60 were grown in the net house. Mini-scale DNA isolation was carried out from 35-day old seedlings from these six F_1 plants following CTAB method of Murray and Thompson (1980). The foreground selection was carried out to detect the presence of the resistance genes *xa13, xa-5* and *Xa21* in these F_1

plants using the specific STS markers RG136, RG556 and pTA248, respectively. A total of three F_1 plants were found to have all the three resistance genes *Xa21*, *xa13* and *xa-5* (Plate 11).

Backcrosses of the F_1 plants from the cross of IRBB-60 x CSR-30, having all the three resistance genes were made with the respective recurrent parent i.e. CSR-30. The BC_1F_1 seeds of this cross were harvested.

Cross	**No. of BC_1F_1 seeds**
F_1 x CSR-30	57

The fifty seven BC_1F_1 seeds were grown in the net house during the next growing season out of which forty seeds were germinated. These forty BC_1F_1 plants were selfed to produce BC_1F_2 seeds. A total of two hundred and fifty BC_1F_2 seeds were grown in the net house in the next growing season out of which two hundred and thirty seeds were germinated. Mini scale DNA isolation was carried out from 4-5 week old leaves of all the 230 BC_1F_2 plants following CTAB method. The genomic DNA of 230 BC_1F_2 plants were checked by electrophoresis on 0.8% agarose gels. Also the quantity and quality of the DNA was checked by U.V spectrophotometer by taking absorbance at 260 nm and also at 280 nm. The quantity of DNA ranged from 1122 μg/ml (G-89) to 2530 μg/ml (G-214). Genotypes that showed contamination of RNA were given RNase A treatment to remove RNA contamination and the genotypes which showed protein contamination were treated with phenol: chloroform to remove protein contamination. The DNA of each of the 230 BC_1F_2 genotypes gave a single intact band of high molecular weight when resolved on 0.8 per cent (w/v) agarose gel (Plate 12). This showed that CTAB extraction protocol yielded high amount of DNA (Table 4.4).

Table 4.4: Quantity and quality of genomic DNA isolated from 230 BC_1F_2 plants following CTAB method

BC_1F_2 plants	**Quantity of DNA (μg/ml)**	**Ratio of A_{260}/A_{280}**	**BC_1F_2 plants**	**Quantity of DNA(μg /ml)**	**Ratio of A_{260}/A_{280}**
G-1	2434	1.76	G-115	2120	1.85
G-2	1820	1.84	G-116	2220	1.86
G-3	1846	1.82	G-117	2345	1.85
G-4	1550	1.78	G-118	2465	1.81
G-5	1564	1.85	G-119	2344	1.83
G-6	1862	1.79	G-120	2123	1.82
G-7	1750	1.84	G-121	3214	1.79
G-8	1863	1.82	G-122	1182	1.82
G-9	1750	1.78	G-123	2213	1.84
G-10	1692	1.75	G-124	1365	1.80
G-11	2474	1.85	G-125	2475	1.80
G-12	2132	1.84	G-126	2341	1.79
G-13	2345	1.79	G-127	2215	1.79
G-14	2022	1.77	G-128	1324	1.85

G-15	1208	1.80	G-129	1563	1.84
G-16	2546	1.80	G-130	1238	1.82
G-17	1730	1.83	G-131	1786	1.80
G-18	1444	1.76	G-132	1754	1.79
G-19	1286	1.78	G-133	2234	1.75
G-20	1680	1.81	G-134	2432	1.87
G-21	1910	1.73	G-135	1234	1.82
G-22	2090	1.85	G-136	1457	1.75
G-23	1990	1.91	G-137	2344	1.75
G-24	2080	1.76	G-139	1234	1.70
G-25	2010	1.78	G-140	1129	1.79
G-26	2090	1.81	G-141	1324	1.78
G-27	2150	1.74	G-142	2345	1.70
G-28	2220	1.75	G-143	1540	1.82
G-29	1910	1.73	G-144	2140	1.75
G-30	1890	1.72	G-145	2180	1.76
G-31	2160	1.8	G-146	2170	1.77
G-32	2240	1.73	G-147	1480	1.83
G-33	2460	1.75	G-148	2490	1.83
G-34	2340	1.75	G-149	2350	1.84
G-35	1660	1.75	G-150	2300	1.82
G-36	3250	1.76	G-151	2100	1.76
G-37	2720	1.76	G-152	2420	1.75
G-38	2150	1.73	G-153	1800	1.83
G-39	2200	1.74	G-154	2180	1.84
G-40	2110	1.79	G-155	2120	1.83
G-41	1154	1.75	G-156	2200	1.78
G-42	2332	1.85	G-157	2471	1.76
G-43	2586	1.84	G-158	1900	1.77
G-44	2192	1.79	G-159	1200	1.84
G-45	1628	1.84	G-160	1630	1.84
G-46	2060	1.82	G-161	2040	1.76
G-47	2276	1.78	G-162	1770	1.84
G-48	1192	1.85	G-163	2180	1.84
G-49	1626	1.79	G-164	1300	1.82
G-50	2134	1.84	G-165	2080	1.77
G-51	1254	1.82	G-166	1780	1.76
G-52	1235	1.76	G-167	1890	1.82
G-53	1347	1.84	G-168	2090	1.85
G-54	2150	1.82	G-169	1990	1.91
G-55	1612	1.79	G-170	1080	1.76
G-56	2150	1.77	G-171	2010	1.78
G-57	2245	1.80	G-172	2090	1.81
G-58	2214	1.73	G-173	2150	1.74
G-59	1734	1.76	G-174	2220	1.75
G-60	1820	1.84	G-175	1910	1.73
G-61	1846	1.82	G-176	1890	1.72
G-62	2213	1.75	G-177	2160	1.80
G-63	2214	1.73	G-178	2240	1.73
G-64	2207	1.78	G-179	2460	1.75
G-65	1234	1.77	G-180	2340	1.85
G-66	1345	1.80	G-181	2660	1.75
G-67	2345	1.85	G-182	1250	1.76

G-68	2465	1.89	G-183	1720	1.76
G-69	2344	1.83	G-184	2150	1.73
G-70	1123	1.82	G-185	2200	1.74
G-71	1214	1.79	G-186	2110	1.83
G-72	1182	1.82	G-187	2160	1.82
G-73	2213	1.84	G-188	1830	1.75
G-74	1365	1.80	G-189	1860	1.84
G-75	2475	1.80	G-191	2170	1.82
G-76	2341	1.79	G-192	1680	1.82
G-77	2215	1.79	G-193	1700	1.82
G-78	1324	1.85	G-194	2090	1.85
G-79	1563	1.88	G-195	1990	1.91
G-80	1238	1.82	G-196	2080	1.76
G-81	1786	1.80	G-197	2010	1.78
G-82	1754	1.79	G-198	2090	1.81
G-83	2234	1.75	G-199	2150	1.74
G-84	2432	1.87	G-200	2220	1.75
G-85	1234	1.82	G-201	1910	1.73
G-86	1457	1.75	G-202	1890	1.85
G-87	2344	1.75	G-203	2160	1.77
G-88	1234	1.70	G-204	2240	1.76
G-89	1122	1.79	G-205	2460	1.78
G-90	1324	1.78	G-206	2340	1.81
G-91	2345	1.70	G-207	1660	1.74
G-92	1700	1.77	G-208	2250	1.75
G-93	1570	1.76	G-209	2720	1.73
G-94	1250	1.84	G-210	2150	1.73
G-95	1960	1.85	G-211	2200	1.74
G-96	1910	1.84	G-212	2110	1.76
G-97	2350	1.84	G-213	2160	1.82
G-98	1800	1.88	G-214	2530	1.75
G-99	2380	1.76	G-215	1860	1.84
G-100	1900	1.82	G-216	2170	1.82
G-101	2300	1.77	G-217	2680	1.82
G-102	1000	1.84	G-218	2020	1.88
G-103	2270	1.82	G-219	2240	1.84
G-104	1900	1.85	G-220	2210	1.75
G-105	1580	1.75	G-221	2190	1.82
G-106	1590	1.78	G-222	1870	1.81
G-107	1630	1.84	G-223	1960	1.82
G-108	1190	1.83	G-224	2420	1.74
G-109	1800	1.86	G-225	2590	1.83
G-110	2140	1.76	G-226	2350	1.82
G-111	1700	1.75	G-227	1840	1.80
G-112	1840	1.83	G-228	1950	1.83
G-113	2400	1.77	G-229	2810	1.82
G-114	2080	1.82	G-230	2220	1.86

4.5 Marker Assisted Foreground Selection

The 230 BC$_1$F$_2$ plants were subjected to foreground selection to check for presence of *Xa21* resistance gene using STS marker pTA248 (Plate 13). Seventy one plants amplified

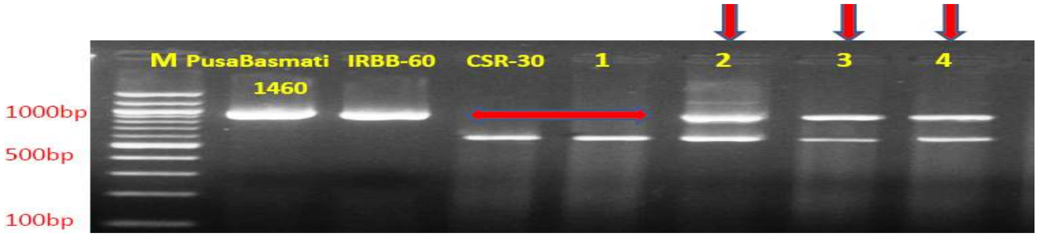

Plate 9: Foreground selection at F_1 generation (CSR-30 x Pusa Basmati-1460) by screening of plants using *xa21* linked marker pTA248

M- 100 bp ladder **IRBB-60 - (**Donor parent**)** **CSR-30** - (Recurrent parent)

1- 4 - F_1 plants of the cross CSR-30 x Pusa Basmati-1460

Arrow indicates 'positive' plant having *Xa21* gene (1000 bp band).

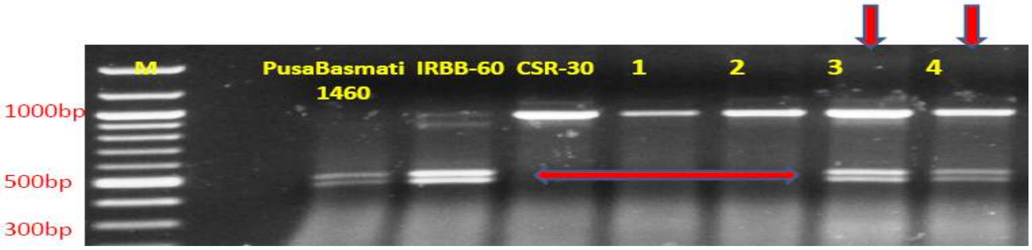

Plate 10: Foreground selection at F_1 generation by screening of plants using *xa13* linked marker RG136 (Digested with R.E *Hinf* I).

M- 100 bp ladder **IRBB-60 -** (Donor parent) **CSR-30 -** (Recurrent parent)

1- 4 - F_1 plants of the cross CSR-30 x Pusa Basmati-1460

Arrow indicates 'positive' plant having *xa5* gene (400 bp and 506 bp band).

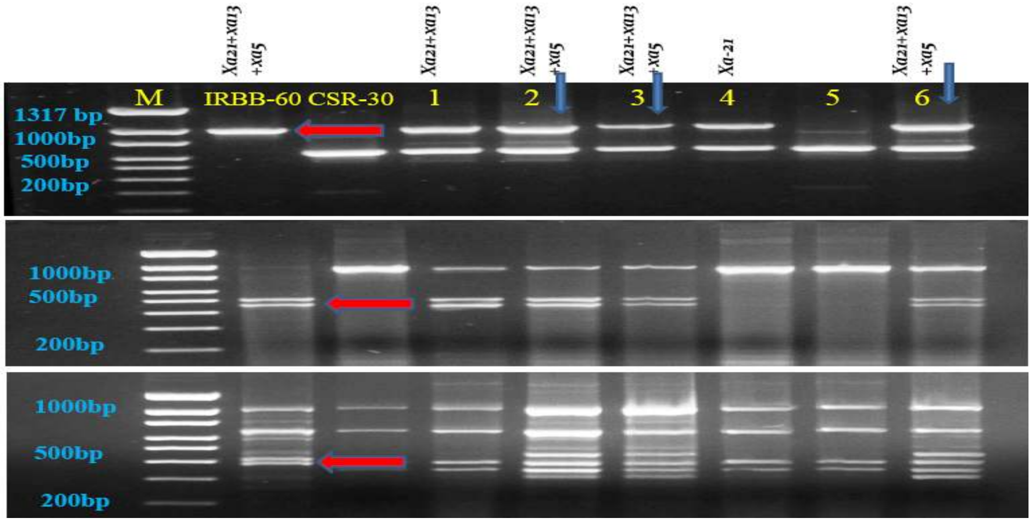

Plate 11: **Foreground selection at F_1 generation by screening of plants using *Xa21*, *xa13* & *xa5* linked marker pTA248, RG136 (digested with R.E *Hinf* I) & R556 (digested with R.E *Dra* I).**

M- 100 bp ladder. **1-6**: F_1 plants of the cross CSR-30 x IRBB-60.

Arrow indicates plants having *Xa21*, *xa13* and *xa5* BB resistance genes.

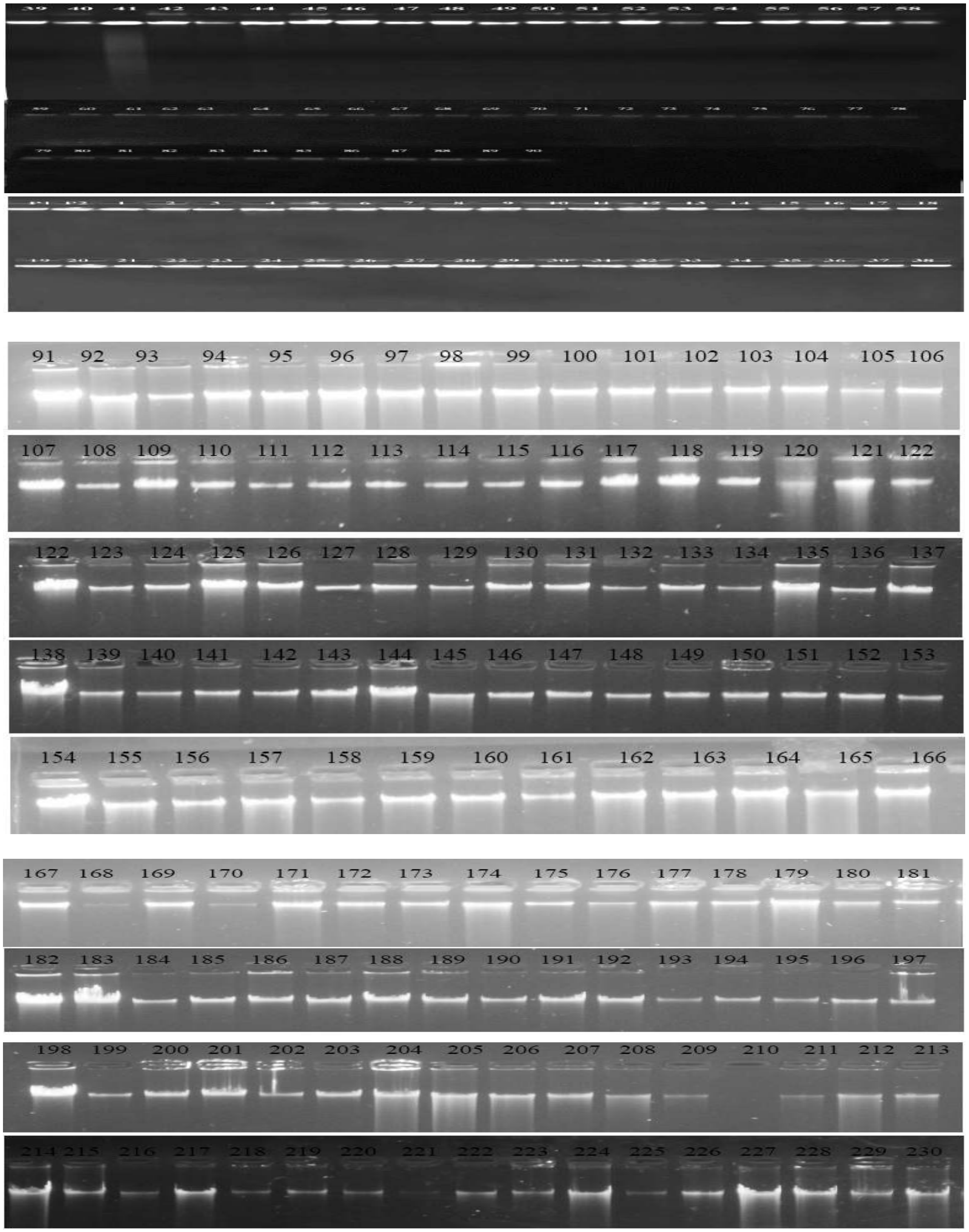

Plate 12: Electrophoretic pattern of purified high molecular weight genomic DNA free from protein and RNA extracted of 230 BC_1F_2 plants.

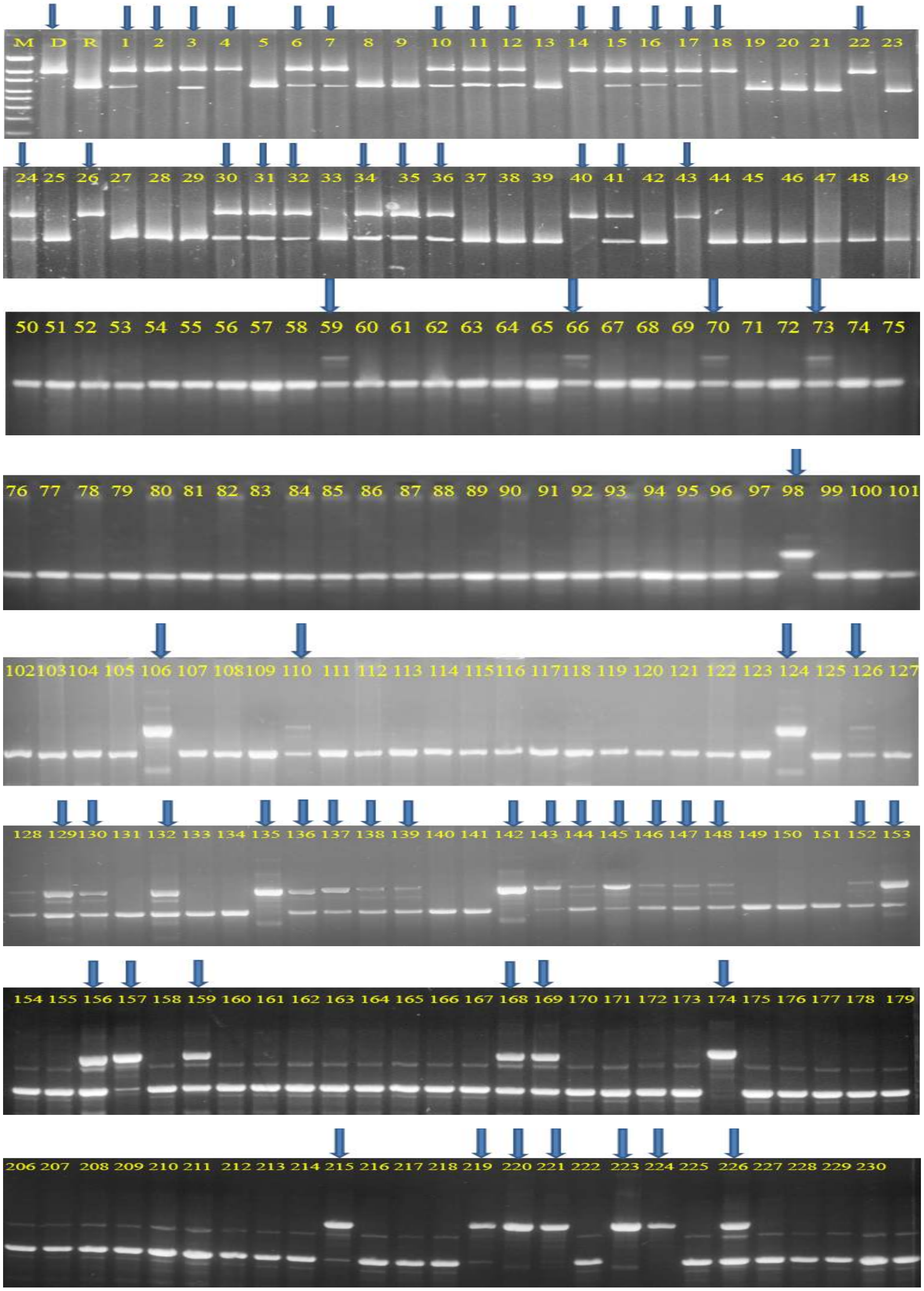

Plate 13: Foreground selection of 230 BC_1F_2 plants using *Xa21* linked marker pTA248.

M- 100bp ladder **D-** (Donor parent) **IRBB-60** **R-** (Recurrent parent) **CSR-30**

1-230 - BC_1F_2 plants **Arrow indicates plants having *Xa21* gene**

1000 bp band specific to *Xa21* gene. These plants that were positive for *Xa21* gene were then screened for presence of *xa5* resistance gene using STS marker RG556 (digested with *Dra* I) as shown in Plate 14. A band of size 450 bp specific to *xa5* gene was present in twenty four plants. Finally, these double positive twenty four plants having both *Xa21* and *xa5* resistance genes were screened for the presence of *xa13* resistance gene using STS marker RG136 (digested with *Hinf* I). Ten plants produced bands of size 400 bp and 506 bp specific to *xa13* gene (Plate 15). Thus, a total of 10/230 BC_1F_2 plants were found to have the three resistance genes *Xa21, xa13* and *xa5*.

Ten BC_1F_2 plants (G-1, G-3, G-6, G-7, G-10, G-11, G-17, G-22, G-169, G-187), were found to be positive for all the three resistance genes *Xa21, xa5* and *xa13.* Twenty six plants with various two gene combinations were obtained out of which a total of seventeen plants have resistance genes *Xa21* and *xa5,* four plants have resistance genes *Xa21* and *xa13* and five plants have resistance genes *xa13* and *xa5*. Fifty nine plants with single resistance gene (forty four plants with *Xa21*gene, nine plants with *xa5* gene and six plants with *xa13* gene) were obtained (Table 4.5).

Table 4.5: Number of BC_1F_2 plants with multiple resistance gene combinations

S.No	**Gene combinations**	**No. of BC_1F_2 plants**	**BC_1F_2 plants**
1	*Xa21/Xa21* *xa13/xa13* *xa5/xa5*	10	G-1, G-3, G-6, G-7, G-10, G-11, G-17, G-22, G-169, G-187
2	*Xa21/Xa21* *xa13/xa13*	4	G-12, G-36 , G-146, G-164
3	*Xa21/Xa21* *xa5/xa5*	17	G-2, G-4, G-66, G-70, G-73, G-128, G-132, G-135, G-138, G-139, G-153, G-159, G-168, G-194, G-198, G-215, G-220
4	*xa13/xa13* *xa5/xa5*	5	G-25, G-33, G-150, G-160, G-212
5	*Xa21/Xa21*	44	G-14, G-15, G-16, G-18, G-24, G-26, G-30, G-31, G-32, G-34, G-35, G-40, G-41, G-43, G-59, G-98, G-106, G-110, G-124, G-126, G-129, G-130, G-136, G-137, G-142, G-143, G-144, G-145, G-147, G-148, G-152, G-156, G-189, G-190, G-191, G-193, G-197, G-200, G-201, G-219, G-221, G-223, G-224, G-226
6	*xa13/xa13*	6	G-8, G-9, G-151, C-166, G-179, G-182
7	*xa5/xa5*	9	G-5, G-13, G-20, G-68, G-75, G-120, G-163, G-208, G-210

4.6 Marker Assisted Background Selection

4.6.1 Background Selection

Genomic DNA was isolated from the young leaves of 3-4 week old seedlings of the parental rice genotypes Pusa Basmati-1460, IRBB-60 and CSR-30 as per modified CTAB extraction method. A total of three hundred SSR primers distributed throughout the rice genome covering the 12 rice chromosomes were used for PCR amplification (Table 4.6).

PCR amplification conditions already stanadaradized for STS marker analysis were used for SSR markers. Reproducible and clear banding patterns were obtained in a reaction mixture of 10 µl containing 100 ng template DNA, 100 µM of dNTPs mix, 0.5 µM of each primer (Forward/Reverse), 1.0 mM of $MgCl_2$ and 1.0 units of Taq DNA polymerase with annealing temperature of 58°C for 1 minutes. These conditions were maintained throughout the amplification reaction. The PCR amplified products were resolved on 2.6 % agarose gel and visualized under U.V. light after staining with ethidium bromide.

Three hundred SSR markers were used to identify the polymorphism between parental genotypes Pusa Basmati-1460 and CSR-30 and between IRBB-60 and CSR-30. The list of primers used in this study, with their sequences is given in table 3.3. Out of 300 primers used, 286 primers produced amplifcation. Amplification for all the markers has been recorded. The PCR amplification of some of the markers showing polymorphc alleles between the parental genotypes Pusa Basmati-1460 and CSR-30 and between IRBB-60 and CSR-30 is shown in plates 16, 17, 18 & 19.

A total of 72 SSR markers produced polymorphism between the parents Pusa Basmati-1460 and CSR-30 whereas in parents IRBB-60 and CSR-30, a total of 104 SSR primers produced polymorphic alleles (Table 4.6 and Table 4.7). Polymorphic SSR markers amplifying unique alleles situated throughout the genome of recurrent parent CSR-30 were recorded. One hundred and four polymorphic markers situated on different chromosomes produced one hundred and twenty four unique alleles of CSR-30.

Table 4.6: Simple sequence repeat markers that are polymorphic between Pusa Basmati-1460 and CSR-30 (The number of polymorphic markers/chromosome No. are indicated in parentheses)

Chromosome locus	Total markers analysed	Primers that produce polymorphism	Percentage polymorphism (%)
1	28	RM 243, 1233, 1321, 2318, 3598, 5990, 8045	25
2	38	RM 29, 236, 240, 1038, 3248, 4355, 5780, 6137	21
3	29	RM 231, 1164, 1221, 3134, 3564, 5801, 5864, 8210	28
4	21	RM 252, 1018, 1112, 3473, 5879, 6156	29
5	20	RM 1843, 3796, 4244, 5816	20
6	22	RM 253, 1150, 1843, 3138, 3827, 8116	27
7	21	RM 214, 248,1085, 1093, 3394, 5711, 6011	33
8	25	RM 25, 138, 1019, 1235, 3761	20
9	25	RM 242, 270, 1099, 4348	16
10	22	RM 258, 1236, 1374	14
11	25	RM 254, 1233, 1812, 1761, 5704, 5735	24
12	24	RM 19, 240, 1080, 1134, 1136, 1146, 1227, 5864	33
Total	300	72	24

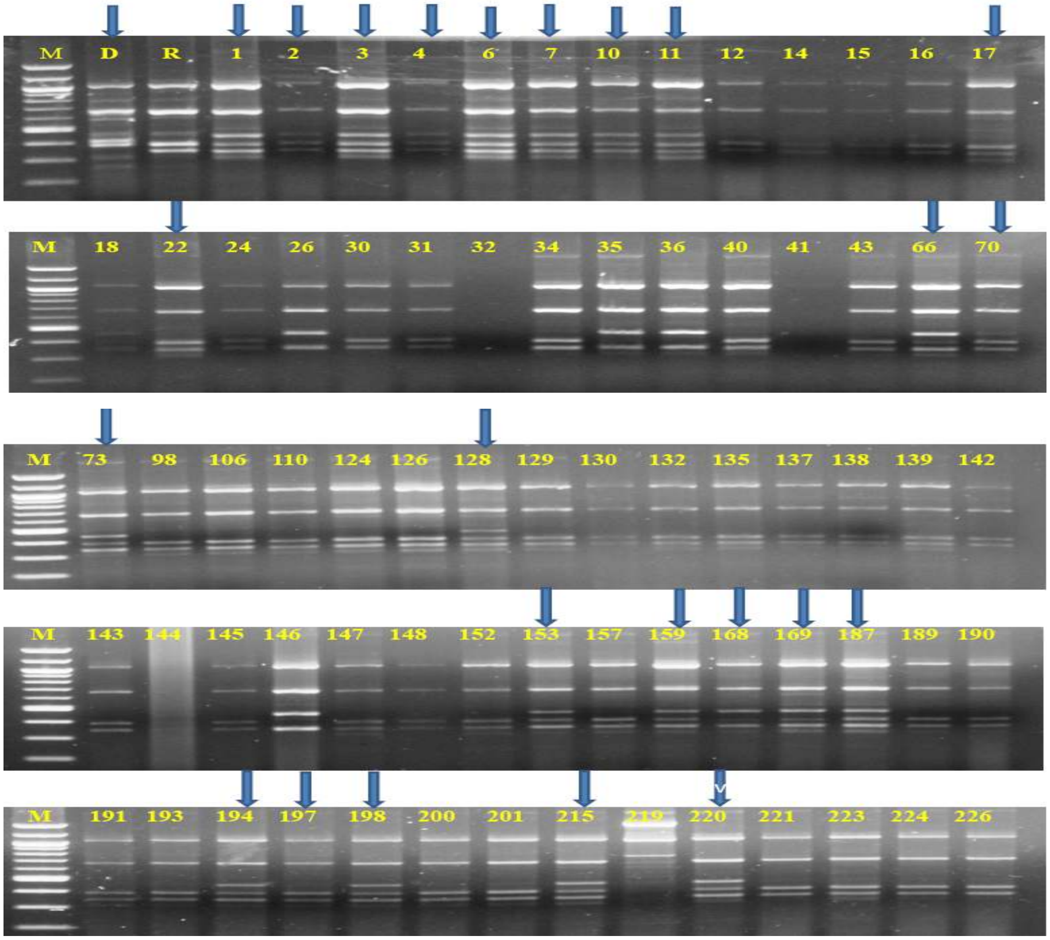

Plate 14: Foreground selection of BC_1F_2 plants 'positive' for *Xa21* using *xa5* linked marker RG556 (digested with R.E *Dra* I)

M- 100bp ladder **D-** (Donor parent) **IRBB-60 R-** (Recurrent parent) **CSR-30**

Arrow indicates plants having *xa5* gene

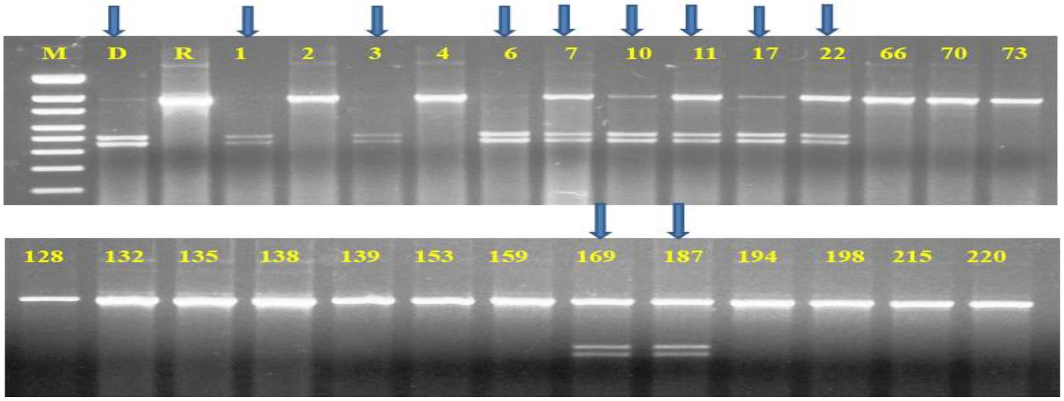

Plate 15: Foreground selection of BC_1F_2 plants positive' for both *Xa21* and *xa5* using *xa13* linked marker RG136 (digested with R.E *Hinf* I).

M- 100bp ladder **D** (Donor parent) - **IRBB-60** **R** (Recurrent parent) - **CSR-30**

Arrow indicates plants having *xa13* gene

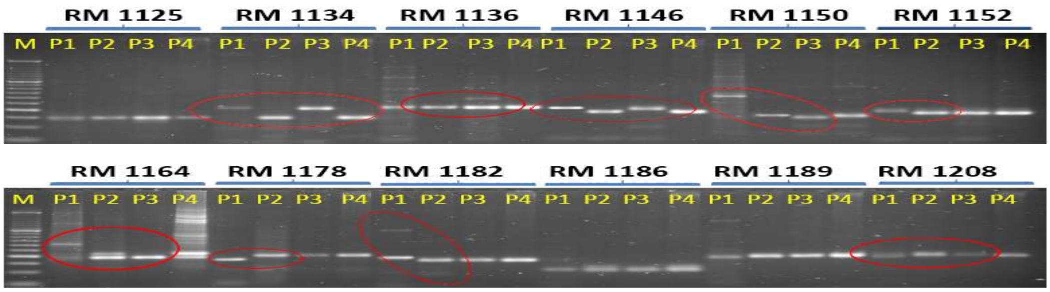

Plate 16

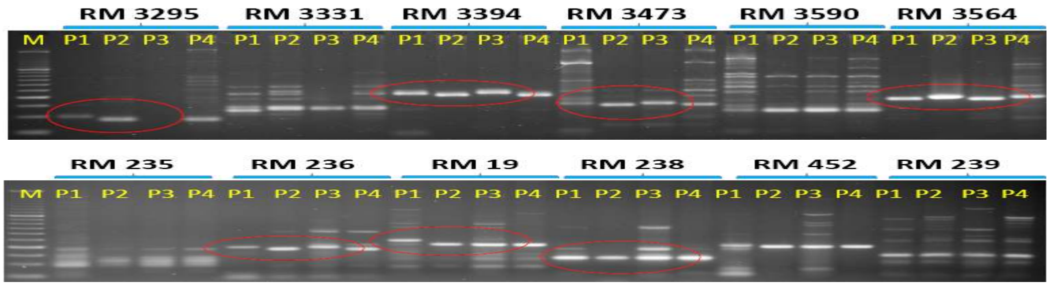

Plate 17

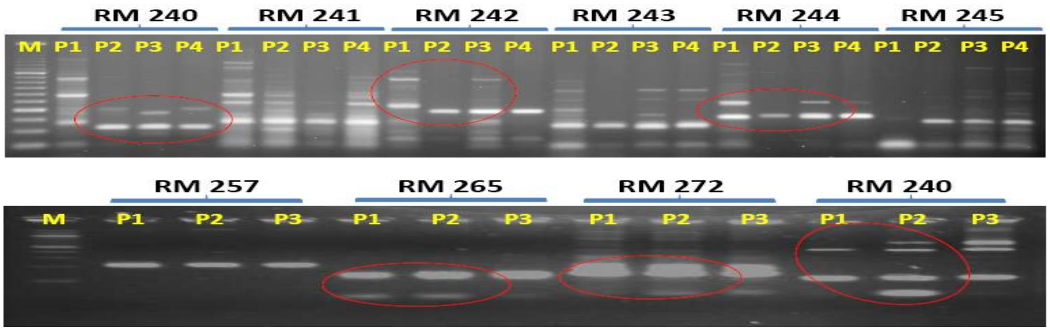

Plate 18

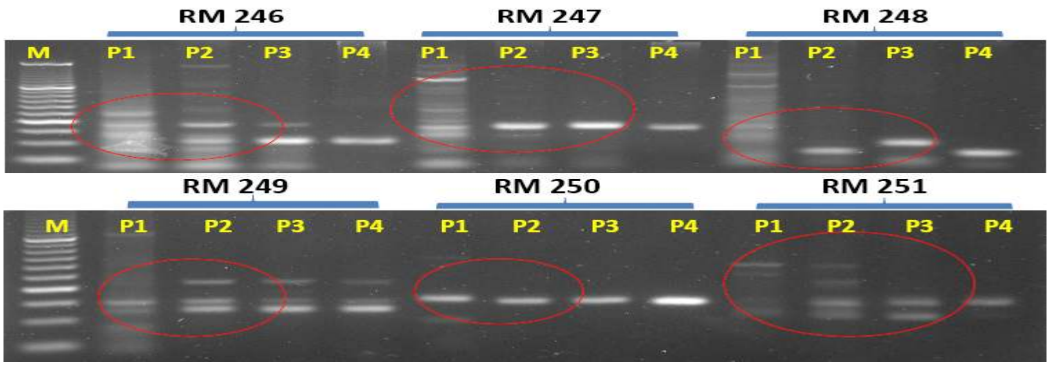

Plate 19

Plate 16, 17, 18 & 19: **Electrophoretic pattern of parental genotypes of rice with different primers showing polymorphism among them.**

M: Marker (100bp ladder)

P1- IRBB-60, **P2-** CSR-30, **P3-** Pusa Basmati-1460, **P4-** Taraori Basmati

Circles showing polymorphic bands.

Table 4.7: Simple sequence repeat markers that are polymorphic between parents IRBB-60 and CSR-30. (The number of polymorphic markers/ chromosome No. is indicated in parentheses).

Chromosome locus	Total markers analysed	Primers that produce polymorphism	Percentage polymorphism (%)
1	28	RM 220, 212, 238, 243, 1003, 1152, 1321, 3143, 3598, 5735, 6083, 8045)	55
2	38	RM 240, 208, 236, 29, 236, 452, 250, 1038, 1178, 1313, 1367, 3828, 4355, 5607, 5780, 5862, 6137)	47
3	29	RM 1164, 1221, 1324, 3564, 5801, 6080, 8210)	31
4	21	RM 252, 261, 1018, 1112, 1388, 3473, 6156)	38
5	20	RM 249, 1182, 3295, 3796, 3969, 4244)	30
6	22	RM 253, 1150, 1340, 3827, 8116)	23
7	21	RM 214, 1085, 1093, 1335, 2006, 3394, 5711, 5847, 8012)	43
8	25	RM 25, 138, 1019, 1235, 1345, 1789, 3153, 3761)	32
9	25	RM 205, 242, 245, 1099, 2144, 3744, 5661, 6174,	32
10	22	RM 244, 258, 1236, 1374, 5620)	23
11	25	RM 254, 254, 260, 1208, 1233, 1761, 3137, 5795)	32
12	24	RM 19, 247, 1080, 1134, 1146, 1337, 3331, 5851)	33
Total	300	104	35

4.6.2 Introgression of unique alleles in three gene pyramided BC_1F_2 genotypes

The SSR markers which produced polymorphism among the parental genotypes IRBB-60 and CSR-30 were used to screen the BC_1F_2 genotypes positive for three BB resistance genes (Plates 20, 21, 22, 23, 24 & 25).

The PCR amplified fragments were scored visually for the presence (1) and absence (0) of parental alleles for all the three gene pyramided BC_1F_2 genotypes and the parental genotypes IRBB-60 and CSR-30. Markers RM 29, RM 208, RM 220, RM 1150, RM 1152, RM 1182, RM 2006 and RM 2187 amplified only the recurrent parent allelic band in the ten three gene pyramided BC_1F_2 genotypes. Markers RM 212, RM 3598 and RM214 amplified recurrent parent allelic band in six genotypes and donor parent allele in four genotypes. Marker RM 206 amplified recurrent parent allelic band in seven genotypes and donor parent allele in two genotypes. Marker RM 8210 amplified recurrent parent allelic band in five genotypes and donor parent allele in five genotypes. Markers RM 6174 and RM 3744 amplified recurrent parent allelic band in four genotypes, donor parent allele in one genotype and heterozygous alleles in four genotypes. Markers RM 6083 and RM 3560 amplified recurrent parent allelic band in five genotypes, donor parent allele in two genotypes and heterozygous alleles in two genotypes. Marker RM 3473 amplified recurrent parent allelic band in six genotypes, donor parent allele in one genotype and heterozygous alleles in three genotypes. Marker RM 138 amplified recurrent parent allelic band in five genotypes, donor parent allele in two genotypes and heterozygous alleles in three genotypes. Marker RM 5780 amplified recurrent parent allelic band in five genotypes, donor parent allele in one genotype and heterozygous alleles in four genotypes. Marker RM 1235 amplified recurrent parent allelic band in three genotypes, donor parent allele in three genotypes and heterozygous alleles in four genotypes.

4.7 Genetic Relationship and Cluster Analysis of three- gene pyramided BC_1F_2 plants

The SSR data obtained was used to construct similarity matrices of the three gene pyramided BC_1F_2 rice genotypes positive for *Xa21, xa5* and *xa13* BB resistance genes, using 'Simqual' sub-programme of software NTSYS-PC. The allelic diversity data was used for 'Cluster Tree Analysis' sub-programme of the same software that revealed the genetic linkage and proximity among all these genotypes investigated.

4.7.1 Similarity Matrices

Similarity matrix of the three-gene pyramided BC_1F_2 rice genotypes revealed the genetic relationship among them (Table 4.8). The similarity indices between different genotypes ranged from 0.15 to 0.93 across all the genotypes. The maximum genetic similarity coefficent (0.93) between two three-gene pyramided BC_1F_2 rice genotypes was detected between G-3 with G-7 and G-3 with G-169 progenies

The genotypes G-3 and G-169 were the most closest to the recurrent parent CSR-30 with genetic similarity coefficient (0.88), followed by G-7 with CSR-30 with genetic similarity coefficient (0.85). The genetic similarity of the remaining seven genotypes (G-1, G-6, G-7, G-10, G-11, G-17 and G-22) with CSR-30 varied from 64% to 85%.The minimum genetic similarity coefficent (0.11) detected was between G-3 with donor parent IRBB-60 followed by G-7 with IRBB-60 and G-169 with IRBB-60 having genetic similarity coefficent (0.15) as analysed with the SSR marker data.

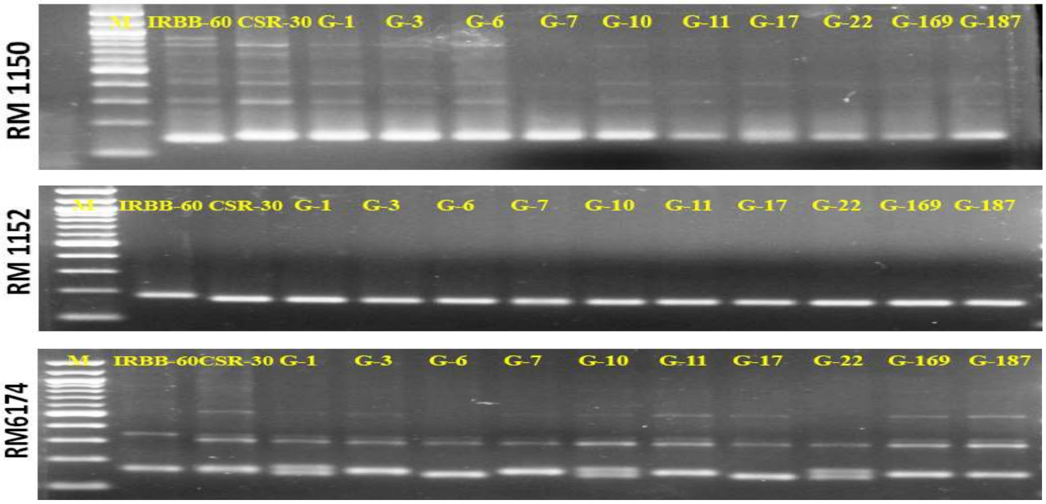

Plate 20

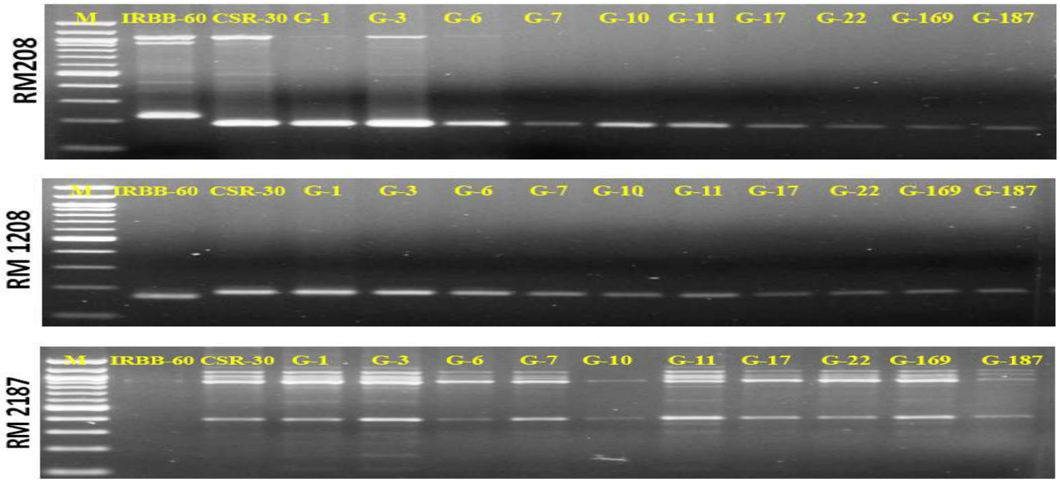

Plate 21

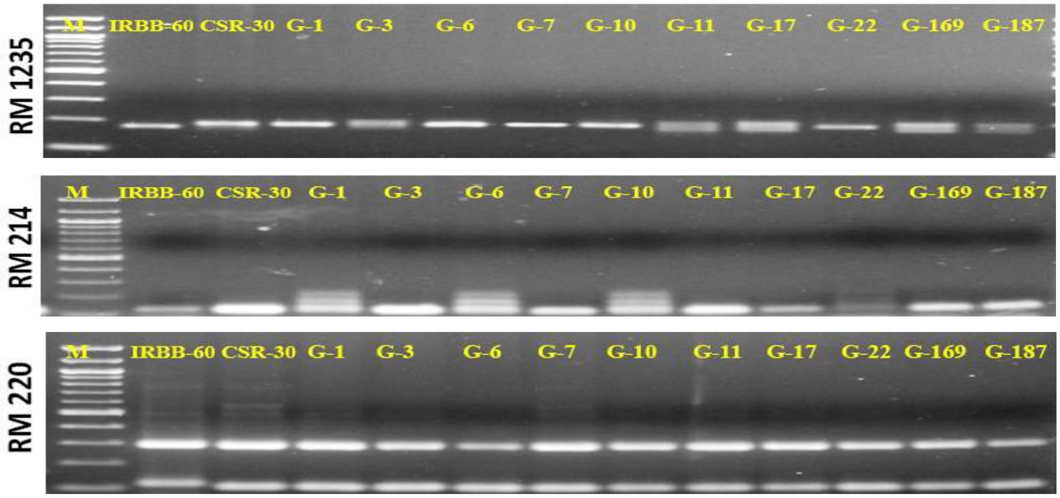

Plate 22

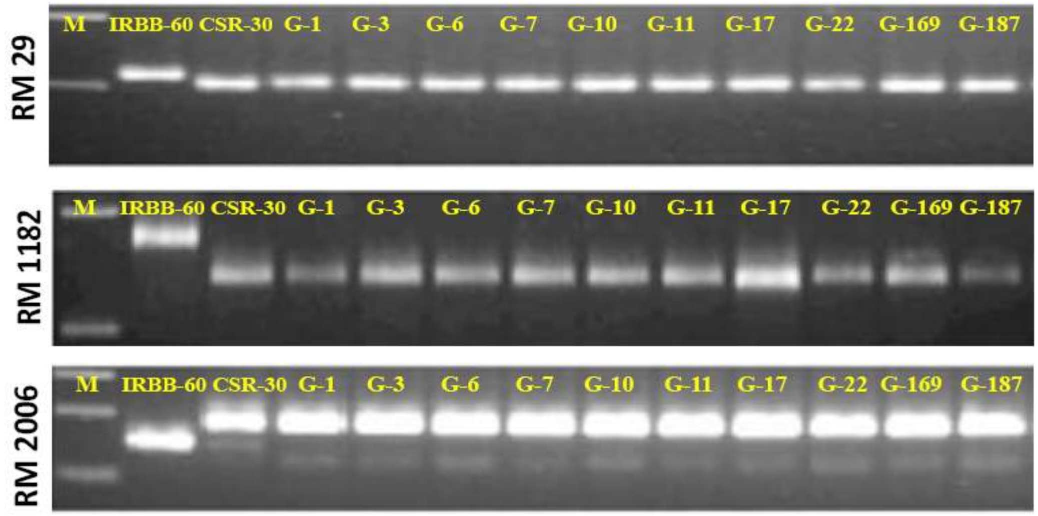

Plate 23

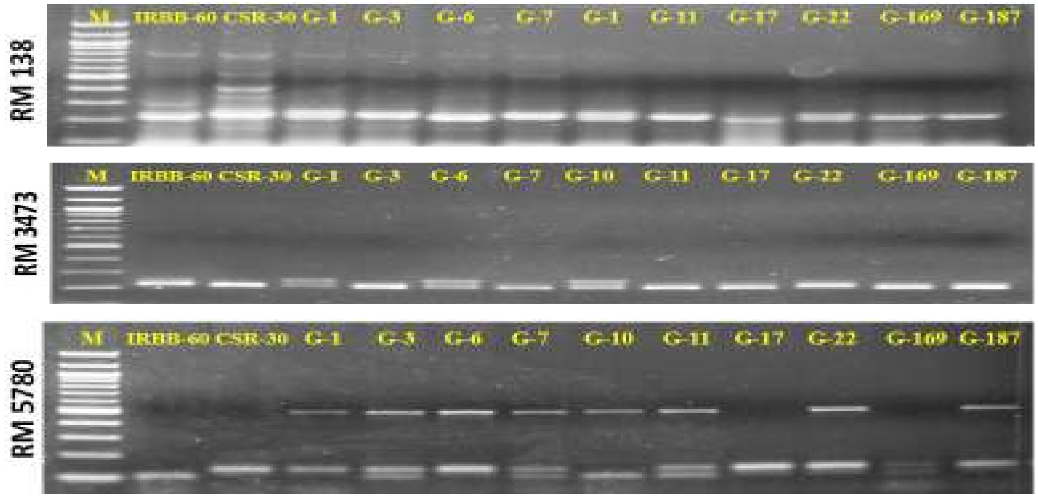

Plate 24

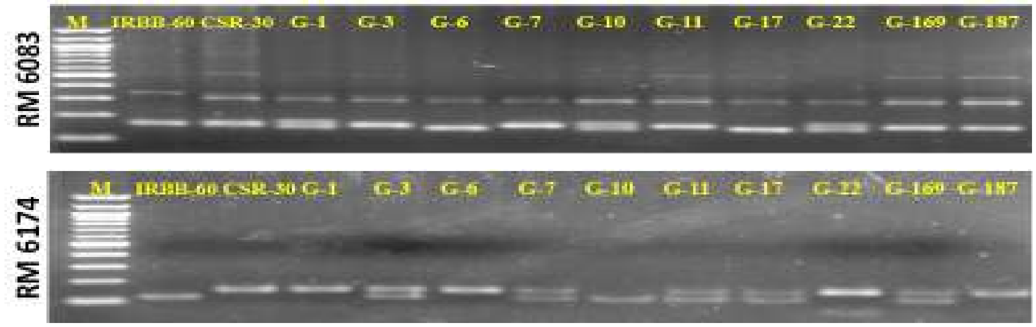

Plate 25

Plate 20, 21, 22, 23, 24 & 25: Background selection of the three-gene pyramided BC_1F_2 genotypes using parental polymorphic SSR markers.

IRBB-60 (Donor parent), **CSR-30** (Recurrent parent)

G represents PCR products obtained from ten BC_1F_2 three-gene pyramided genotypes

Table 4.8: Average similarity coefficient value calculated on the basis of similarity matrices of ten three-gene pyramided BC$_1$F$_2$ genotypes

	IRBB-60	CSR-30	G-1	G-3	G -6	G -7	G-10	G -11	G -17	G -22	G -169	G-187
IRBB-60	1.0000000											
CSR-30	0.0370370	1.0000000										
G-1	0.3148148	0.7222222	1.0000000									
G-3	0.1111111	0.8888889	0.6851852	1.0000000								
G -6	0.3888889	0.6481481	0.8888889	0.6851852	1.0000000							
G -7	0.1481481	0.8518519	0.7222222	0.9259259	0.7222222	1.0000000						
G-10	0.2777778	0.7592593	0.7407407	0.7592593	0.7407407	0.7222222	1.0000000					
G -11	0.2407407	0.7962963	0.6666667	0.7037037	0.8333333	0.8703704	0.7777778	1.0000000				
G -17	0.3333333	0.6666667	0.7592593	0.7037037	0.8333333	0.7777778	0.6481481	0.7962963	1.0000000			
G -22	0.1851852	0.8148148	0.7962963	0.7777778	0.7222222	0.7777778	0.7962963	0.7222222	0.6666667	1.0000000		
G -169	0.1481481	0.8888889	0.6481481	0.9259259	0.6481481	0.8888889	0.7592593	0.8703704	0.7407407	0.8148148	1.0000000	
G-187	0.1851852	0.8518519	0.6851852	0.8148148	0.6851852	0.7777778	0.7592593	0.8333333	0.7407407	0.7037037	0.8518519	1.0000000

4.7.2 Cluster Analysis

The genetic similarities among different genotypes obtained from the SSR marker system were analyzed to find out the clustering of progenies among each other and the parents. The cluster analysis was based on similarity matrices obtained with the unweighted pair group method using arithmetic average (UPGMA) clustering algorithm. The UPGMA cluster tree was divided into two groups (Figure 4.1). All the BC_1F_2 rice genotypes positive for *Xa21, xa5* and *xa13* BB resistance genes and the recipient parent CSR-30 fell in one group with two major sub-groups. Sub-group I consisted of recipient parent CSR-30 and three genotypes G-3, G-7 and G-169 with maximum similarity among them. Sub-group II consisted of the remaining seven genotypes G1, G6, G10, G11, G17, G22 and G187. As expected the donor parent IRBB-60 remained as a separate group. Among the three gene pyramided BC_1F_2 genotypes, G-3, G-7 and G-169 closely clustered along with the recipient parent CSR-30. Genotypes G-3 and G-7 showed 88% genetic similarity with CSR-30 and G-169 showed 85% genetic similarity with the same. The genotypes, which are lying nearer to each other in dendrogram are more similar to one another than those lying apart. The resemblance coefficient between the genotypes is the value at which their branches join.

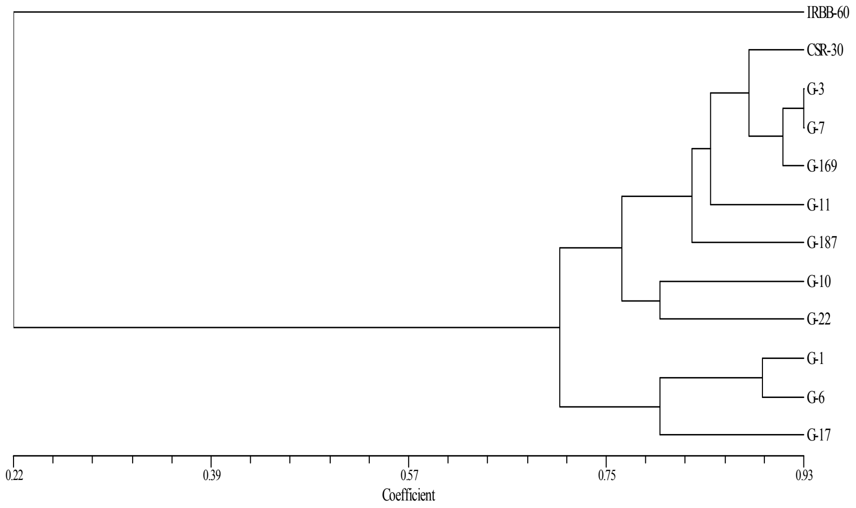

Figure 4.1: Dendrogram showing genetic similarity among the BC_1F_2 three-gene pyramided genotypes and parents as revealed by UPGMA cluster analysis.

4.7.3 Two Dimensional and Three Dimensional Principle Component Analysis

Similar clustering of the three gene pyramided BC_1F_2 genotypes and parental genotypes was also evident from the two dimensional principle component analysis (PCA) (Figure 4.2). The three dimensional PCA also revealed the similar clustering of the ten three gene pyramided BC_1F_2 genotypes and parental genotypes (Figure 4.3) as represented in the dendrogram. All the three gene pyramided BC_1F_2 genotypes and the recurrent parent CSR-30 fell in one group and donor parent IRBB-60 was out grouped. The three genotypes G-3, G-7 and G-169 were the most similar to each other as well as to the recipient parent CSR-30.

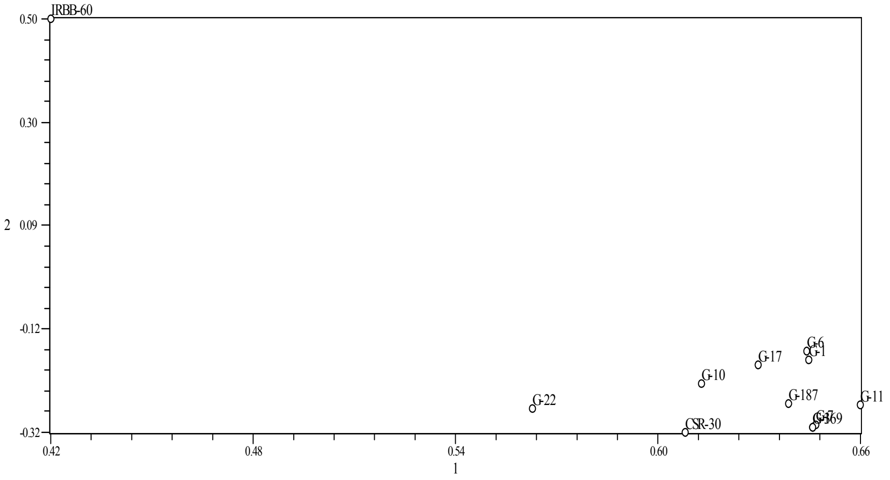

Figure 4.2: Two Dimensional PCA (Principle Component Analysis) Scaling of the ten BC_1F_2 three-gene pyramided genotypes and parents.

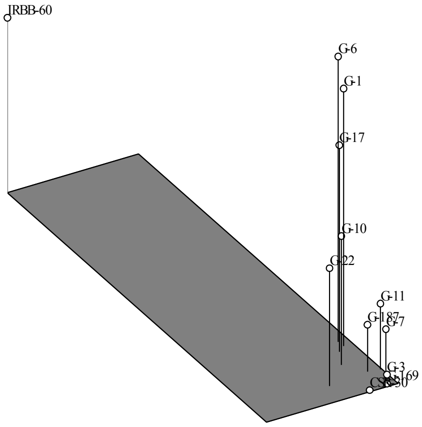

Figure 4.3: Three Dimensional PCA (Principle Component Analysis) Scaling of the ten BC_1F_2 three-gene pyramided genotypes and parents.

4.8: Assessment of recurrent parent genomic contribution

SSR marker data was also scored for IRBB-60 allele as A and CSR-30 allele as B for each primer-genotype combination. The assessment of the genomic contribution of the parents in the ten three gene pyramided BC_1F_2 genotypes based on SSR marker data was carried out using the software programme Graphical Geno Types (GGT) version 2.0. Markers RM 29, RM 208, RM 220, RM 1150, RM 1152, RM 1182, RM 2006 and RM 2187 amplified only the recurrent parent allelic band in the ten three gene pyramided BC_1F_2 genotypes. Markers RM 212, RM 3598 and RM 214 amplified recurrent parent allelic band in six genotypes and donor parent allele in four genotypes. Marker RM 206 amplified recurrent parent allelic band in seven genotypes and donor parent allele in two genotypes. Marker RM 8210 amplified recurrent parent allelic band in five genotypes and donor parent allele in five genotypes. Markers RM 6174 and RM 3744 amplified recurrent parent allelic band in four genotypes, donor parent allele in one genotype and heterozygous alleles in four genotypes. Markers RM 6083 and RM 3560 amplified recurrent parent allelic band in two genotypes, donor parent allele in five genotypes and heterozygous alleles in two genotypes. Marker statistics showed that markers RM 220, RM 212, RM 3598 and RM 1152 located on chromosome 1 recovered recurrent parent genome (RPG %) of 91.7%, 58.3%, 58.3%, and 91.7 % respectively. Markers RM208, RM29 and RM 5780 located on chromosome 2 showed RPG recovery of 91.7%, 50.7%, and 91.7 % respectively. Markers RM 2187, RM 8120 and RM 3574 located on chromosome 3 showed RPG recovery of 91.7%, 50.7%, and 25.0 % respectively. Markers RM 3473 located on chromosome 4 and RM 1182 located on chromosome 5 showed RPG recovery of 58.75 and 91.7% respectively. Markers RM 8116 and RM 1150 located on chromosome 6 showed RPG recovery of 58.3%, and 91.7 % respectively. Markers RM 214, RM 2006 and RM 6011 located on chromosome 7 showed RPG recovery of 58.3 %, 91.7 % and 33.3 % respectively. Markers RM 138 and RM 3761 located on chromosome 8 recovered RPG of 50 % and 25 % respectively. Markers RM 242, RM 2144, RM 6174 and RM 3744 located on chromosome 9 showed RPG recovery of 66.7 %, 16.7 %, 41.7% and 41.7 % respectively. Markers RM 244 and RM 1146 located on chromosome 10 showed RPG recovery of 91.7 % and 88.3 % respectively. Markers RM 1208 and RM 206 located on chromosome 11 showed a recovery of 91.7 % and 75.0 % RPG. The percentage recovery of recurrent parent genome in the three gene pyramided BC_1F_2 rice genotypes by the polymorphic SSR primers located on 12 chromosomes by marker statistical analysis is listed in the table 4.9. The recurrent parent genomic contribution in the three gene pyramided BC_1F_2 rice genotypes is well demonstrated further in the figures 4.4 and 4.5.

Table 4.9: Marker statistics showing the percentage recovery of recurrent parent genome in the three gene pyramided BC_1F_2 genotypes by the polymorphic SSR markers located on 12 chromosomes of rice.

Locus	Group	Position	A alleles	B alleles	H alleles	A (%)	B (%)	H (%)
RM 220	1	6.3	1	11	0	8.3	91.7	0.0
RM 212	1	7.6	5	7	0	41.7	58.3	0.0
RM 3598	1	38.8	5	7	0	41.7	58.3	0.0
RM 1152	1	124.8	1	11	0	8.3	91.7	0.0
RM 6083	1	155.2	6	3	3	50.0	25.0	25.0
RM 208	2	5.5	1	11	0	8.3	91.7	0.0
RM 5780	2	26.9	2	6	4	16.7	50.0	33.3
RM 29	2	52.4	1	11	0	8.3	91.7	0.0
RM 2187	3	113.0	1	11	0	8.3	91.7	0.0
RM 8210	3	144.5	6	6	0	50.0	50.0	0.0
RM 3564	3	151.5	6	3	3	50.0	25.0	25.0
RM 3473	4	128.5	2	7	3	16.7	58.3	25.0
RM 1182	5	3.0	1	11	0	8.3	91.7	0.0
RM 8116	6	2.3	4	7	1	33.3	58.3	8.3
RM 1150	6	121.7	1	11	0	8.3	91.7	0.0
RM 214	7	1.6	5	7	0	41.7	58.3	0.0
RM 2006	7	31.0	1	11	0	8.3	91.7	0.0
RM 6011	7	73.2	1	4	7	8.3	33.3	58.3
RM 138	8	12.0	3	6	3	25.0	50.0	25.0
RM 3761	8	112.6	3	3	6	25.0	25.0	50.0
RM 242	9	1.0	4	8	0	33.3	66.7	0.0
RM 2144	9	91.5	1	2	9	8.3	16.7	75.0
RM 6174	9	91.8	2	5	5	16.7	41.7	41.7
RM 3744	9	93.5	2	5	5	16.7	41.7	41.7
RM 244	10	3.2	1	11	0	8.3	91.7	0.0
RM 1146	10	57.5	1	7	4	8.3	58.3	33.3
RM 1208	11	10.3	1	11	0	8.3	91.7	0.0
RM 206	11	13.5	3	9	0	25.0	75.0	0.0
RM 19	12	8.9	1	11	0	8.3	91.7	0.0

A- Donor parent (IRBB-60)
B- Recurrent parent (CSR-30)
H- Heterozygous

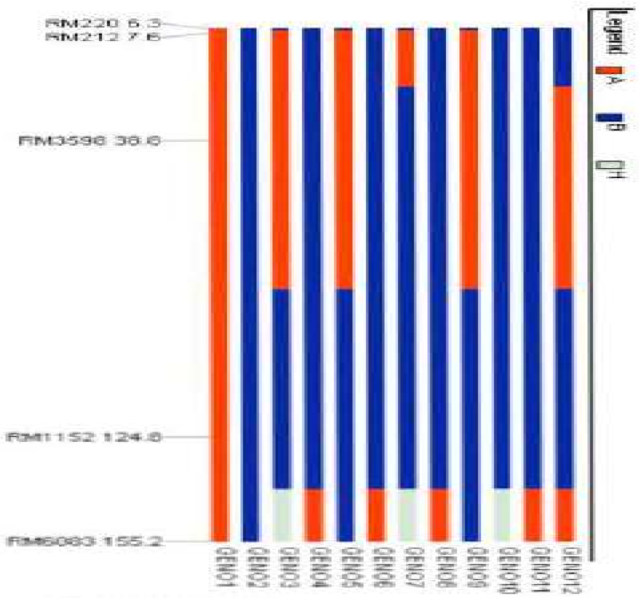

Figure 4.4: Demonstration of donor parent and recurrent parent genome in the ten three gene pyramided BC_1F_2 genotypes.

A- Donor parent (IRBB-60)

B- Recurrent parent (CSR-30)

H- Heterozygous

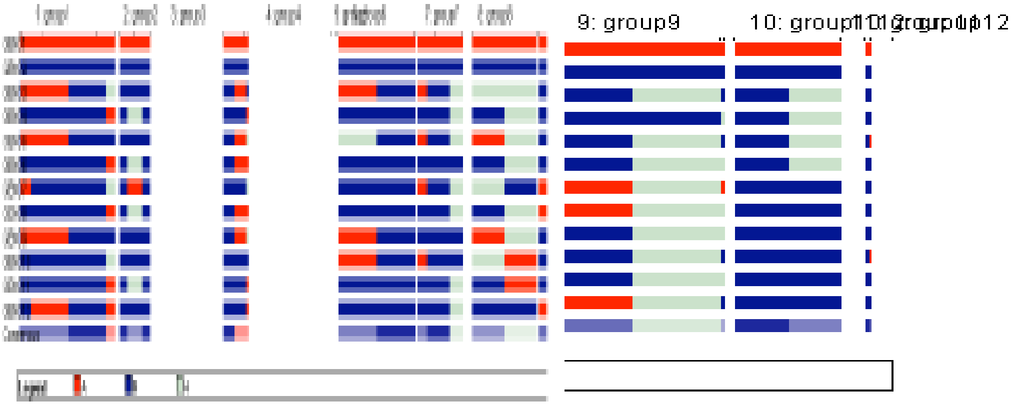

Figure 4.5: Analysis of recurrent parent genome introgression in the ten three gene pyramided BC_1F_2 genotypes by the polymorphic SSR primers located on 12 chromosomes of rice.

A- Donor parent (IRBB-60)

B- Recurrent parent (CSR-30)

H- Heterozygous

The percentage recovery of recurrent parent genome in the three-gene pyramided BC_1F_2 genotypes ranged from 44.2% to 78.9% as revealed by the global statistics using the GGT software. Genotypes G-1, G-3, G-6, G-7, G-10, G-11, G-17, G-22, G-169 and G-187 showed a percentage recovery of 44.1, 78.9, 45.6, 72.5, 64.9, 66.7, 54.9, 64.9, 76.2, 72.0 % respectively (Table 4.10).

Table 4.10: Global statistics showing the percentage of donor parent allele, recurrent parent allele and heterozygous alleles in the three gene pyramided BC_1F_2 genotypes.

Group	Genotypes	A (%)	B (%)	H (%)
1	IRBB-60	100.0	0.0	0.0
2	CSR-30	0.0	100.0	0.0
3	G-1	24.9	44.1	31.0
4	G-3	2.8	78.9	18.3
5	G-6	23.8	45.6	30.6
6	G-7	5.6	72.5	21.9
7	G-10	15.0	64.9	20.0
8	G-11	12.3	66.7	21.0
9	G-17	30.2	54.9	15.0
10	G-22	16.4	64.9	18.7
11	G-169	13.6	76.2	10.2
12	G-187	9.9	72.0	18.1

A- Donor parent (IRBB-60)
B- Recurrent parent (CSR-30)
H- Heterozygous

Similar results were obtained using both the softwares GGT and NTSYS. The BC_1F_2 genotypes (having three BB resistance genes *Xa21, xa5* and *xa13*) G-3, G-7 and G-169 had the maximum recurrent parent genome contribution and were most close to the recurrent parent CSR-30.

4.11. Evaluation of BB resistance characteristics of BC_1F_2 rice genotypes (having one to three BB resistance genes)

The pyramided lines were evaluated for their resistance to bacterial blight under glass house conditions using the *Xanthomonas oryzae* strain isolated from the BB infected fields of RRS, Kaul, CCSHAU, Hisar. Isolation of bacteria was done by adapting streak plate method as explained in material and methods. Well separated colonies of the isolate were picked up and streaked on Peptone sucrose agar (PSA) media and incubated at 28°C for 72 hours. The pure colonies obtained were again streaked on PSA slants and kept for incubation at 28°C for 72 hours. The cultures so obtained were stored in the refrigerator at 5°C, which served as a stock culture for further studies. The pyramided lines along with the control, were inoculated using a bacterial suspension of 10^{9}cells/ml. The leaf blades were inoculated by clipping with scissors at 3 cm below the leaf tips. On an average five leaves per plant were inoculated and the disease incidence using 0-5 scale was measured 14 days after inoculation.

As compared to the recurrent parent CSR-30, the pyramided lines with three gene and two gene combinations exhibited very high level of resistance to bacterial blight disease (Table 4.11). In addition, the lines containing either *Xa21* alone or *xa5* alone also exhibited moderate BB resistance. However, pyramided lines with *xa-13* gene alone were found to be susceptible to BB disease. The results indicated that the genes in combinations were more effective against the pathogen than a single gene.

Table 4.11: Disease reaction of BC_1F_2 rice genotypes containing 1 to 3 BB resistance genes to *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) (Five point rating scale for scoring/ screening of bacterial blight disease).

S.No.	Parents and pyramided BC_1F_2 genotypes	*Xa21*	*xa13*	*xa5*	Disease rating	Reaction category
1	IRBB-60	+	+	+	0	HR
2	CSR-30	-	-	-	5	HS
3	G-1*	+	+	+	1	R
4	G-2	+	-	+	1	R
5	G-3*	+	+	+	0	HR
6	G-4	+	-	+	2	MR
7	G-5	-	-	+	2	MR
8	G-6*	+	+	+	0	HR
9	G-7*	+	+	+	0	HR
10	G-8	-	+	-	3	MS
11	G-9	-	+	-	4	S
12	G-10*	+	+	+	1	R
13	G-11*	+	+	+	1	R
14	G-12	+	+	-	1	R
15	G-13	-	-	+	2	MR
16	G-14	+	-	-	2	MR
17	G-15	+	-	-	1	R
18	G-16	+	-	-	2	MR
19	G-17*	+	+	+	0	HR
20	G-18	+	-	-	1	R
21	G-20	-	-	+	2	MR
22	G-22*	+	+	+	1	R
23	G-24	+	-	-	2	MR
24	G-25	-	+	+	3	MS
25	G-26	+	-	-	3	MS
26	G-30	+	-	-	3	MS
27	G-31	+	-	-	2	MR

28	G-32	+	-	-	2	MR
29	G-33	+	-	-	2	MR
30	G-34	+	-	-	2	MR
31	G-35	+	-	-	3	MS
32	G-36	+	+	-	2	MR
33	G-40	+	-	-	2	MR
34	G-41	+	-	-	2	MR
35	G-43	+	-	-	2	MR
36	G-59	+	-	-	3	MS
37	G-66	+	-	+	1	R
38	G-68	-	-	+	3	MS
39	G-70	+	-	+	1	R
40	G-73	+	-	+	1	R
41	G-75	-	-	+	2	MR
42	G-98	+	-	-	2	MR
43	G-106	+	-	-	2	MR
44	G-110	+	-	-	2	MR
45	G-120	-	-	+	3	MS
46	G-124	+	-	-	2	MR
47	G-126	+	-	-	1	R
48	G-128	+	-	+	1	R
49	G-129	+	-	-	2	MR
50	G-130	+	-	-	2	MR
51	G-132	+	-	+	1	R
52	G-135	+	-	+	1	R
53	G-136	+	-	-	2	MR
54	G-137	+	-	-	2	MR
55	G-138	+	-	+	2	MR
56	G-139	+	-	+	1	R
57	G-142	+	-	-	1	R
58	G-143	+	-	-	2	MR
59	G-144	+	-	-	1	R
60	G-145	+	-	-	1	R
61	G-146	+	+	-	1	R
62	G-147	+	-	-	2	MR
63	G-148	+	-	-	2	MR
64	G-150	-	+	+	1	R
65	G-151	-	+	-	4	S
66	G-152	+	-	-	2	MR
67	G-153	+	-	+	1	R

68	G-156	+	-	-	2	MR
69	G-159	+	-+	-	1	R
70	G-160	-	+	+	2	MR
71	G-163	-	-	+	2	MR
72	G-164	+	+	-	1	R
73	G-168	+	-	+	1	R
74	G-166	-	+	-	3	MS
75	G-169*	+	+	+	1	R
76	G-179	-	+	-	4	S
77	G-182	-	+	-	4	S
78	G-187*	+	+	+	0	HR
79	G-189	+	-	-	2	MR
80	G-190	+	-	-	1	R
81	G-191	+	-	-	2	MR
82	G-193	+	-	-	3	MS
83	G-194	+	-	+	1	R
84	G-197	+	-	-	3	MS
85	G-198	+	-	+	1	R
86	G-200	+	-	-	2	MR
87	G-201	+	-	-	2	MR
88	G-208	-	-	+	3	MS
89	G-210	-	-	+	2	MR
90	G-212	-	+	+	2	MR
91	G-215	+	-	+	1	R
92	G-219	+	-	-	3	MS
93	G-220	+	-	+	2	MR
94	G-221	+	-	-	2	MR
95	G-223	+	-	-	2	MR
96	G-224	+	-	-	2	MR
97	G-226	+	-	-	2	MR

* indicates three gene pyramided genotypes

4.12 Yield and agro-morphological traits of three gene pyramided BC_1F_2 plants

Data was recorded for the yield and several agro- morphological traits in the pyramided BC_1F_2 plants and the parental genotypes (Table 4.12). Significant variation was observed among the pyramided lines and parental rice genotypes for plant height, effective number of tillers per plant, seeds per panicle, grain length/breadth ratio and yield per plant. Range for various agronomic traits in the pyramided BC_1F_2 plants is shown in the table 4.13

4.12.1 Plant height (cm)

Plant height of the parental genotypes CSR-30 and IRBB-60 was recorded 126 cm and 110 cm respectively. Plant height of the three gene pyramided BC_1F_2 plants varied between 120-129 cm. Genotype G-6 had a maximum height of 129 cm and genotype G-17 had a minimum height of 120 cm. All the genotypes (G-1(124 cm), G-3 (123 cm), G-7 (126 cm), G-10 (123 cm), G-11 (121 cm), G-17 (120 cm), G-22 (122 cm), G-169 (124 cm), G-187 (122 cm) except G-6 (129 cm) had plant height intermediate between the two parents

4.12.2 Panicle length (cm)

Panicle length of the parental genotypes CSR-30 and IRBB-60 was recorded 25.20±0.58 cm and 20.0±0.70 cm respectively. Panicle length of the pyramided BC_1F_2 plants varied between 20.08-26.80 cm. Genotype G-3 had a maximum panicle length of 26.20 cm±1.15 cm and genotype G-22 had a minimum panicle length of 20.08±1.63 cm. Genotypes G-6, G-7, G-10, G-11, G-17, G-169 and G-187 had panicle length of 24.60 cm, 21.94 cm, 23.50 cm, 25.80 cm, 21.40 cm, 23.00 cm and 22.80 cm respectively.

4.12.3 Effective number of tillers/ plant

Effective numbers of tillers in the parental genotypes CSR-30 and IRBB-60 was recorded 14 and 11 respectively. Effective numbers of tillers in the pyramided BC_1F_2 plants varied between 11 and 15. Genotypes G-7 and G-169 had maximum 15 effective number of tillers whereas minimum 11 effective number of tillers/ plant were observed in genotype G-11. The effective number of tillers in the genotypes (G-1(13), G-3 (14), G-6 (10), G-10 (12), G-17 (13), G-22 (12), G-187 (12) were intermediate between the two parents.

4.12.4 Seeds per panicle

Number of seeds per panicle in the parental genotypes CSR-30 and IRBB-60 was recorded 175 and 166 respectively. Number of seeds per panicle in the pyramided BC_1F_2 plants varied between166-179. Genotype G-3 had maximum 179 number of seeds per panicle and genotype G-22 had a minimum 166 number of seeds per panicle. Genotypes G-6, G-7, G-10, G-11, G-17, G-169 and G-186 had 169, 168, 172, 168, 176, 170, 171 and 174 number of seeds per panicle respectively.

4.12.5 Length/Breadth ratio of grain

Length/breadth ratio of grain in the parental genotypes CSR-30 and IRBB-60 was recorded to be 5.74±0.03 cm and 3.99±0.17 cm respectively. Length/breadth ratio of grain among pyramided BC_1F_2 plants varied between 5.33- 6.54 cm. Three genotypes (IC-3 (5.78 cm), IC-169-(5.76 cm) and IC-187 (6.54 cm) had a higher length/breadth ratio than the recipient parent CSR-30 (5.74 cm).

4.12.6 Grain Yield/ plant (g)

Grain yield per plant in the parental genotypes CSR-30 and IRBB-60 was recorded 33.20 g and 28.30 g respectively. Grain yield per plant in the pyramided BC_1F_2 plants varied between 30.33-34.25 g. A maximum grain yield of 34.25 g was recorded in genotype G-3 and minimum grain yield of 30.33 g was recorded in genotype G-10. All the genotypes (G-3, G-6, G-7, G-17, G-22, G-169 and G-187) except three genotypes (G-1, G-10 and G-11) recorded higher yields than CSR-30 as shown in the table. The genotypes G-3 (34.25 g/plant), G-7 (34.15 g/plant) and G-169 (33.80 g/plant) showed marginally higher yield than the recurrent parent CSR-30 (33.20 g/ plant).

Table 4.12: Agronomic traits and % recurrent parent genome recovery in the three gene pyramided BC_1F_2 genoypes grown in the field.

S. No.	BC_1F_2 plants	Plant height (cm)	Effective number of tillers	Panicle length (cm)	Seeds per panicle	length/ breadth ratio	1000 grain weight (g)	Yield/ plant(g)	RPG%
1	CSR-30	126	14	25.20±0.58	175	5.74±0.17	24.66	33.20	100
2	G-1	124	13	23.46±0.09	169	5.30±0.03	23.16	31.88	44.1
3	G-3	123	14	26.20±1.15	179	5.78±0.11	24.33	34.25	78.9
4	G-6	129	10	24.60±0.53	168	5.39±0.07	22.08	33.63	45.6
5	G-7	126	15	21.94±1.07	172	5.55±0.36	24.22	34.15	72.5
6	G-10	123	12	23.50±0.97	168	5.33±0.15	23.56	30.33	64.9
7	G-11	121	11	25.80±1.06	176	5.68±0.12	23.04	31.36	66.7
8	G-17	120	13	21.40±0.74	170	5.88±0.16	24.98	33.30	54.9
9	G-22	122	12	20.08±1.63	166	5.34±1.01	23.89	32.89	64.9
10	G-169	124	15	24.96.±0.70	171	5.76±0.79	21.33	33.80	76.2
11	G-187	122	12	22.80±0.96	174	6.54±0.15	22.36	33.22	72.0

Table 4.13: Range for various agronomic traits in three gene pyramided BC_1F_2 genotypes grown in the field

Trait	IRBB-60	CSR-30	BC_1F_2 plants
			Range
Plant height (cm)	110	126	121-129
Effective number of tillers/plant	11	14	10-15
Panicle length (cm)	20.0±0.70	25.20±0.58	20.08-26.20
Seeds per panicle	166	175	166-179
Length/Breadth ratio	3.99±0.17	5.74±0.03	5.33-6.54
1000 grain weight (g)	21.45	24.66	21.33-24.33
Total grain yield/plant (g)	28.30	33.20	30.33-34.25

The highest yielding genotype G-3 showed maximum recurrent parent genome recovery of 78.90 %. These results clearly indicated that there was no yield penalty due to pyramiding of three bacterial blight resistance genes into CSR-30. Genotype G-3 showed the best agronomic features with maximum recovery of recurrent parent genome RPG (78.9%) followed by G-169 with RPG 76.2% followed by line G-7 with RPG 72.5%.

Based on the agronomic evaluation, BB reaction and percentage recovery of recurrent parent genome three genotypes G-3, G-7 and G-169 were selected for further backcrossing with the recurrent parent CSR-30. These three gene pyramided BC_1F_2 genotypes were backcrossed with the recurrent parent CSR-30 and the BC_2F_2 seeds (151 seeds) were harvested. Also, the selfed BC_1F_3 seeds were harvested. These identified genotypes can be further improved through phenotypic selection as well as molecular markers for the development of potential BB resistance donors.

CHAPTER-V

DISCUSSION

Recently uses of DNA-based genetic markers have been extensively utilized as genetic markers for assessment of genetic diversity, cultivar identification and gene tagging. Introducing novel genes for agronomic traits such as yield, quality traits, disease resistance and abiotic stress tolerance facilitates the improvement of any crop species. These new genes are most successfully gleaned from the primary Molecular markers have been looked upon as tools for a large number of applications ranging from localization of a gene to improvement of plant varieties by marker-assisted selection. The history of the development of molecular markers gives evidence that they have been improved over the last two decades to provide easy, fast and automated assistance to scientists and breeders. Mapping and tagging of agriculturally important genes have been greatly facilitated by an array of molecular markers in crop plants. Marker-assisted selection (MAS) is gaining considerable importance as it would improve the efficiency of plant breeding through precise transfer of genomic regions of interest (foreground selection) and accelerating the recovery of the recurrent parent genome (background selection). With the development and availability of an array of molecular markers and dense molecular genetic maps in crop plants, MAS has become possible for traits both governed by major genes as well as quantitative trait loci (QTLs).

High level of susceptibility of basmati rice to bacterial blight, caused by the bacterium *Xanthomonas oryzae* pv. *oryzae*, is a serious constraint to basmati rice production which results in major yield loss. In addition, the grain and cooking quality of the basmati rice is severely affected by this disease (Rajpurohit *et al.,* 2010). Hence, in the present study, an attempt was made to develop high yielding BB resistant gene pyramided genotypes through MAS approach. Large scale and long term cultivation of varieties carrying single gene for resistance has resulted in evolution of new races of the pathogen leading to breakdown of resistance in these varieties. One of the ways to delay such a breakdown of BB resistance is to pyramid multiple resistance genes into rice cultivars. Combining genes/QTLs for BB resistance in a single genotype is difficult task and cannot be easily achieved using conventional breeding. Yoshimura *et al.* (1995) reported that action of one major gene may mask the action of another in conventional phenotypic selection. Accumulation of major genes for BB resistance in an elite genotype by conventional breeding is laborious and time-consuming when one or more genes are effective against all known isolates of the pathogen. Nowadays, advancement in molecular biology makes it possible and convenient to improve the BB resistance by transformation and marker assisted selection. However, transgenic technique is not only time-consuming and costly but there are some technical limitations also such as gene silence and unstable inheritance (Deng *et al.*, 2006). In view of these, present investigation was carried out to introgress the bacterial blight resistance genes *Xa21*, *xa13* and *xa5* into BB susceptible variety CSR-30 using marker assisted selection.

5.1 Marker assisted gene pyramiding

Marker assisted selection have distinctive priority in pyramiding of multigenes. The advantages of MAS include precision, speediness and independency for genotyping (Deng *et al.* 2006). Marker assisted selection is preferred over conventional phenotypic selection because it is simpler compared to phenotypic screening and selection may be carried out at seedling stage itself. Thus, MAS can be a great advantage in crops with a long generation time. The plants not having genes of interest can be discarded at early stage so as to reduce the time and labour. Molecular markers linked to particular gene could identify plants having that gene and hence, we can know the number of lines that need to be advanced in the further generation. The closely linked DNA markers can be used to accelerate the fixation of favourable alleles and hence increasing the efficiency of plant breeding with the maximum percentage of recurrent parent genome (Babu *et al.* 2004). Marker assisted pyramiding has been successfully applied in rice crop breeding programs to evolve high level of resistance using multiple resistance genes (Huang *et al*. 1997; Samis *et al*. 2002; Narayanan *et al*. 2004; Jena and Mackill, 2008; Bharathkumar *et al*. 2008; Kottapali *et al*. 2010; Huang *et al*. 2012).

5.2 Genomic DNA isolation

Genomic DNA was extracted from the parental genotypes and individual F_1's and back-crossed generation used in our study using CTAB method given by Murray and Thompson (1980) and modified by Saghai-Maroof *et al*. (1984) and Xu *et al*. (1994). DNA isolation was performed with the different concentrations of CTAB (1.5 % and 2 %) and different incubation time (1 hr, 1.5 hr and 2 hr) at 65°C. The optimum yield of good quality DNA was obtained when DNA was extracted with 1.5% CTAB at incubation temperature of 65°C for ninety minutes. The quantity of DNA extracted from the parental genotypes and F_1's and back-crossed generation ranged from 1120 to 2580 µg/ml. The quality and quantity of genomic DNA extracted was in tandem with the quality and quantity of DNA reported by various workers. Cheng *et al*. (2003) also reported that good quality and high amount of DNA was obtained using 1.5% of CTAB at incubation temperature of 65°C for seventy five minutes. Chunwongse *et al*. (1993), Williams and Ronald (1994) and Keng *et al*. (1998) also used CTAB method for DNA extraction using 1.5% CTAB with incubation at 65°C for ninety minutes.

Pirttila *et al.* (2001) followed the CTAB method for DNA extraction but used PVP with a concentration of 50 mg/0.5 g of leaf tissue containing high polysaccharide and polyphenolic components. Ribeiro and Lovato (2007) followed CTAB method (2% CTAB) for DNA isolation and used 2% PVP in the extraction buffer for minimizing polyphenolic compounds contamination.

However, Kermekchiev *et al.* (2009) carried out DNA extraction using nuclear extraction buffer (15% (w/v) sucrose, 50mM Tris-HCl (pH 8.0), 50 mM Na_2 –EDTA and 250 mM NaCl), lysis buffer (10 mM Tris-HCl (pH 8.0) and 1 mM Na_2-EDTA.3) and 10% (w/v) solution of Sodium Dodecyl Sulphate (SDS). Roychowdhury *et al.* (2012) also used nuclear extraction buffer containing 10% SDS at incubation temperature of 70 °C for fifteen minutes for DNA extraction. The quality and quantity of the isolated genomic DNA was comparable with the standardized CTAB protocol used in our study.

The template DNA to be used for SSR amplification should be of good quality and free of cellular contaminants. The DNA extraction protocol to a large extent affect the quality and quantity of DNA isolated, hence, in the present study emphasis was laid on standardization of DNA extraction method for high quality and good quantity of genomic DNA. Though SSR is a fast and simple technique for studying polymorphism, yet it is sensitive to template DNA, annealing temperature and DNA polymerase enzyme. But no protocol has universal application for all crop plants.

5.3 PCR amplification conditions

The PCR conditions need to be well defined to obtain reproducible patterns of DNA amplification. The effect of various concentrations of genomic DNA (100 ng, 150 ng and 200 ng), primer each Forward/Reverse (0.5 μM, 1.0 μM and 2.0 μM), $MgCl_2$ (0.8 mM, 1.0 mM and 2.0 mM) and Taq DNA polymerase (1.0 unit, 2.0 units and 3.0 units) in a reaction volume of 10 μl were investigated on amplified products. Analysis of the amplified products on agarose gel electrophoresis revealed that the DNA amplification was influenced by all these factors i.e. concentration of template DNA, $MgCl_2$, Taq. DNA polymerase and the annealing temperature. At low concentrations of DNA template, a few numbers of bands were observed, whereas, the high concentration of DNA template resulted in some non-reproducible bands. By changing the concentration of template DNA, both the yield and profile of PCR amplified products varied. High annealing temperature of 65ºC gave products with faint bands or no amplification. While at lower annealing temperature of 45ºC, some false bands were observed. However, the annealing temperature of 58 °C was found to be optimum for generating clear and reproducible bands. Reproducible and clear banding patterns were obtained in a reaction mixture of 10 μl containing 100 ng template DNA, 100 μM of dNTPs mix, 0.5 μM of each primer (Forward/Reverse), 1.0 mM of $MgCl_2$ and 1.0 unit of Taq DNA polymerase with annealing temperature of 58 °C for 1 minutes. Several other workers optimized concentration of various reagents and conditions for polymerase chain reactions in rice. Ramalingam *et al.* (2001) got the best banding pattern in the PCR reaction mixture of 20 µl containing 25–50 ng template DNA, 5 pmoles of each primer, 1X PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2 and 0.01% gelatin) and 1 unit of Taq polymerase. One minute primer annealing at 55 °C was the optimum for PCR amplification. Similarly, Basavraj *et al*. (2010) performed PCR reactions using a reaction volume of 25 µl and obtained the most consistent and reproducible results using 50 ng template DNA, 5 pmol of each primer, 0.05 mM dNTPs, 1X PCR buffer,1.8 mM $MgCl_2$ and 0.5 U of Taq DNA.

However, Sundaram *et al*. (2008) observed good amplification pattern by carrying out PCR using 5 ng DNA as template for amplification, 5 pmoles of each primer, 0.05 mM dNTPs, 1X PCR buffer 100 µM dUTP (Flourescein) and 1 U Taq DNA polymerase in a total volume of 12.5 µl. Primer annealing at 55^0C for 30 seconds was found to be optimum for their PCR amplification reactions. In a similar study, Kottapali *et al.* (2010) performed PCR using 50–100 ng template DNA, 50 ng of each primer, 0.05 mM dNTPs, PCR buffer (10 mM Tris/HCl. pH 8.4, 50 mM KCl, 1.8 mM MgCl2 and 0.01 mg gelatin/ml) and 0.6 U Taq DNA polymerase in a reaction mixture volume of 20 µl. Primer annealing was carried out at 55ºC for 30 seconds to obtain good amplification pattern. Huang *et al*. (2012) also obtained clear DNA banding pattern in PCR reaction mixture containing 50 ng template DNA, 50 ng of each primer, 0.05 mM dNTPs, 1X PCR buffer (10 mM TRIS, pH 8.4, 50 mM KCl, 1.8 mM $MgCl_2$ and 0.01 mg/ml gelatin) and 1 unit taq DNA polymerase in a volume of 20 µl.

5.4 Marker assisted foreground selection

It is the first level of selection that involves the use of markers called 'foreground markers' that are linked to the gene for a particular trait and are used to screen the target trait. These markers are useful for traits that have laborious phenotypic screening procedures or governed by recessive alleles. The already cloned and characterized bacterial blight resistance genes *Xa21, xa13* and *xa5* were used in our research for the introgression of these genes into bacterial blight susceptible basmati variety CSR-30. *Xa21* is a dominant resistance gene that encodes a receptor kinase containing NBS-LRR domains (Song *et al*. 1995), while *xa5* is a recessive resistance gene and encodes a variant form of transcription factor cIIa (Iyer and McCouch, 2004). The *xa13* resistance gene is also recessive in nature and has been shown to be a mutation in the promoter region of a gene that is a homolog of the nodulin MtN3 (Chu *et al*. 2006). In rice lines containing the dominant (susceptibility) allele of the gene, the expression of the nodulin homolog is up regulated upon infection with *Xoo*. It appears that the increased expression of this gene is necessary for *Xoo* to grow on rice. This up regulation does not occur in rice lines containing the resistance (recessive) *xa13* allele (Yang *et a*l. 2006). The apparently different modes of action of the three resistance genes used in our work might contribute to make the resistance in the two and three-gene pyramid lines quite durable.

Crosses were made between CSR-30 x Pusa Basmati-1460 (having two BB resistance genes) and CSR-30 x IRBB-60 (having three BB resistance genes). A total of 50 and 6 F_1

seeds of the crosses CSR-30 x Pusa Basmati-1460 and CSR-30 x IRBB-60 were harvested respectively. These F_1 plants were checked for the presence of BB resistance genes *Xa21, xa13* and *xa5* using a set of three STS markers pTA248, RG136 and RG556 linked to BB resistance genes *Xa21, xa13* and *xa15* respectively, for carrying out foreground selection. Marker pTA248 amplified a resistant allele of 1000 bp specific to *Xa21*gene, RG136 amplified resistant alleles of size 400 and 506 bp specific to *xa13* gene on digestion with restriction enzyme *Hinf* I. Marker RG556 amplified a resistant allele of 450 bp specific to *xa5* gene on digestion with restriction enzyme *Dra* I. These three STS markers pTA248, RG136 and RG556 showed highly effective results for the identification of resistant alleles of *Xa21, xa13* and *xa5* genes in our study. A total of two plants of the cross CSR-30 x Pusa Basmati-1460 were found to have BB resistance genes *Xa21* and *xa13* and three F_1 plants of the cross CSR-30 x IRBB-60 were found to be positive for the three resistance genes *xa13*, *xa5* and *Xa21*. The two F_1 plants of the cross CSR-30 x Pusa Basmati-1460 having BB resistance genes *xa13* and *Xa21* were selfed to produce F_2 seeds.

The F_1 plants of the cross CSR-30 x IRBB-60 having three BB resistance genes *xa13*, *xa5* and *Xa21* were crossed with the recurrent parent CSR-30 to get 57 BC_1F_1 seeds which were selfed to produce BC_1F_2 seeds. A total of 10/230 BC_1F_2 plants (G-1, G-3, G-6, G-7, G-10, G-11, G-17, G-22, G-169, G-187), were found to be positive for all the three resistance genes *Xa21, xa5* and *xa13.* Twenty six plants with various two gene combinations were obtained out of which a total of seventeen plants have resistance genes *Xa21* and *xa5,* four plants have resistance genes *Xa21* and *xa13* and five plants have resistance genes *xa13* and *xa5.* Fifty nine plants with single resistance genes (forty four plants with *Xa21*gene, nine plants with *xa5* gene and six plants with *xa13* gene) were obtained. In a similar study, Sundaram *et al*. (2008) made crosses between SS1113 (having BB resistance genes *Xa21, xa13* and *xa5*) and Samba Mahsuri and carried out foreground selection using the same set of STS markers pTA248, RG136 and RG556 which we used in our study. The positive F_1 plants were backcrossed with Samba Mahsuri. A total of 11/145 BC_1F_1 plants were found to have all the three resistance genes. The resulting BC_1F_1 lines were checked for presence of the marker linked to *Xa21* and *xa13* resistance allele in a heterozygous condition and the process was continued upto BC_4F_1 stage. Similarly, Kottapali *et al.* (2010) used the same three STS markers i.e. pTA248 linked to *Xa21* gene, RG136 linked to *xa13* gene and RG556 linked to *xa5* gene for carrying out the foreground selection. A total of 200 F_1 plants were found positive for three resistance genes *xa5, xa13* and *Xa21,* which were then selfed and simultaneously backcrossed with Samba Mahsuri to generate BC_2F_3 and BC_3F_1 seeds. Pandey *et al.* 2012, also carried out foreground selection of F_1 plants of the crosses between Taraori Basmati x Improved Samba Mahsuri (having *Xa21* and *xa13* BB genes) and Basmati 386 x Improved Samba Mahsuri using STS marker pTA248 linked to *Xa21* and *xa13*-prom linked to *xa13* gene and backcrossed them using Basmati varieties as a female parent. A total of 30/400 and 25/361 BC_1F_2 plants were found to be positive for the resistance genes *Xa21* and *xa13* in the crosses RP4693 and RP4694, respectively.

However, Suh *et al.* (2013) used different set of STS markers MP1 + MP2, T10Dw (digested with R.E *Rsa*I) and U1/I1 linked with resistance genes *Xa4, xa5* and *Xa2*1, respectively for the similar type of study and obtained F_1 plants (Mangeumbyeo (BB susceptible) x IRBB57 (BB resistant) having the three BB resistance genes (*Xa4, xa5* and *Xa21*). These F_1 plants were backcrossed with the recurrent parent and 28/288 BC_1F_1 progenies having three BB resistance genes were obtained on the basis of molecular marker analysis and phenotypic selection which were further backcrossed with the recurrent parent to obtain 32/ 536 BC_2F_1 and 42/645 BC_3F_1 plants. Shanti *et al.* (2010) carried out foreground selection of F_1 plants of the crosses Mahsuri/IRBB60 (donor for the four BB resistant genes), KMR3/IRBB60, PRR78/IRBB60, IR58025B/IRBB60, Pusa 6B/IRBB60 using three STS markers Nbp181, pTA248, RG136 tightly linked to *Xa4, Xa21* and *xa13* gene respectively and a SSR marker RM122 tightly linked to *xa5* gene. The BC_1 F_1 plants having all the four BB genes were backcrossed with the recurrent parent and this was continued up to the BC_3F_1 generation.

5.5 Marker assisted background selection

Marker-assisted background analysis of the segregants/recombinants is useful in determining the relative contribution of the progenitor parents. Molecular marker analysis with SSR markers gives a quick evaluation of the genetic background of the recombinants. In the present study, we used three hundred SSR markers for background selection of the three gene pyramided plants to derive maximum genetic background of recurrent parent CSR-30.

5.5.1 Background selection and recovery ratio of recurrent parent genome (RPG)

Three hundred SSR markers were used to identify the polymorphism between parental genotypes Pusa Basmati-1460 and CSR-30 and between IRBB-60 and CSR-30. A total of 72 and 104 SSR primers produced polymorphism between the genotypes Pusa Basmati-1460 and CSR-30 and genotypes IRBB-60 and CSR-30, respectively. The primers producing polymorphic alleles between IRBB-60 and CSR-30 were used to genotype BC_1F_2 plants having three resistance genes *Xa21, xa13* and *xa5*. The similarity indices between different genotypes ranged from 0.15 to 0.93 across all the genotypes. The genotypes G-3 and G-169 were the most closest to the recurrent parent CSR-30 with genetic similarity coefficient 0.88, followed by G-7 with CSR-30 with genetic similarity coefficient 0.85. The minimum genetic similarity coefficient 0.11 detected between three-gene pyramided BC_1F_2 rice genotypes was between G-3 with donor parent IRBB-60 followed by G-7 with IRBB-60 and G-169 with IRBB-60 having genetic similarity coefficent (0.15) as analysed with the SSR marker data. The background recovery among the three-gene pyramided BC_1F_2 rice genotypes varied from 44.20 % (G-1) to 78.90% (G-3) as revealed by the analysis of the SSR marker data using software Graphical Geno Types (GGT) version 2.0. Genotype G-3 showed the maximum recovery of recurrent parent genome (RPG) of 78.9% followed by G-169 with RPG 76.2% followed by line G-7 with RPG 72.5%. Theoretically with single backcrosse average background recovery should be 75%. In our study the background recovery varied from 44.20 % to 78.90%. Reduced background recovery in some lines is largely due to linkage drag of the donor genotype on the carrier chromosomes around three target genes *Xa21*, *xa13* and *xa5*. In a similar study, Sundaram *et al*. (2008) introgressed three BB resistance genes in the background of Sambha Mahsuri through marker-assisted selection. For background selection they used 139 microsatellite markers out of which 50 markers were found to be polymorphic between the parents SS113 and Sambha Mahsuri. These polymorphic markers were used to genotype the eleven plants having three BB resistance genes and identify the plant having maximum recurrent parent genome contribution. They recovered 96% of the recurrent parent genome at BC_4F_1 stage. Rajpurohit *et al*. (2010) also tested 209 rice SSR markers for background selection out of which a set of 95 markers showed polymorphism between the parents Type 3 Basmati and PR106-P2. Sixteen BC_2F_3 progenies with nearly Type 3 Basmati seeds were finally selected for background profiling using 95 SSR and 12 ISSR markers. On the basis of SSR markers, these lines showed background recovery from 81.57% (41-3-40) to 92.10% (29-1-35). Pyramid line 29-1-35 recovered maximum recurrent parent genome (92.0%) followed by line 31-4-2 with RPG (91.05%). Similarly, Basavraj *et al*. (2010) carried out marker assisted background selection in the 10 best BC_2F_5 families of Pusa6B and PRR78 using 74 STMS markers polymorphic between Pusa6B and Pusa146 and 54 STMS markers polymorphic between PRR78 and Pusa1460. They recovered the recurrent parent genome ranging from 85.14 to 97.30% and 87.04 to 92.81% in the 10 selected BC_2F_5 families of Pusa6B and PRR78, respectively. Pandey *et al*. (2012) in a similar study subjected the backcross-derived lines similar to their recurrent parents to background selection using 61 and 58 polymorphic SSR markers spanning all the 12 chromosomes for the breeding lines in the genetic background of Taraori Basmati and Basmati 386, respectively. Genome recovery ranged from 73.8 % (RP4693-47-4-4, RP4693-47-3-4) to 83.6 % (RP4693-101-3-3) and 75.9 % (RP4694-157-1-2, RP4694-157-3-2) to 82.7 % (RP4694-157-2-1) in the genetic background of Taraori Basmati and Basmati 386, respectively.

However, Shanti *et al.* (2010) adopted conventional breeding strategy instead of using background markers for carrying out background selection of foreground selected pyramided plants. Chen *et al.* (2001), on the other hand, used 10 pairs of AFLP primers in contrary to SSR markers as used in our study, to select for the genetic background of the recurrent parent while incorporating *Xa21* in ‘6078’, an elite restorer line. They developed an improved version of '6078 (*Xa21*)' with about 98.8% of the recurrent parent genome after three generations of backcrossing. Joseph *et al.* (2004) also used a total of 25 AFLP primer combinations to detect polymorphism between Pusa Basmati-1 and IRBB55 out of which eight primer pairs were found to be highly polymorphic between the parents. Genotyping of 21 BC_1F_3 plants revealed that percentage of recurrent parent genome varied from 80.4% in Pusa 32-2-6 to 86.72% in Pusa 75-4-14.

In the present study, we demonstrated the efficiency of the single backcross method with single backcrossing using marker-assisted foreground and background selections along with phenotypic selection for various agronomic traits in rice. Similar strategy was followed by Joseph *et al*. 2004; Gopalakrishnan *et al*. 2008. Gopalakrishnan *et al*. (2008) carried out marker-assisted selection for the two resistance genes in BC_1F_1, BC_1F_2 and BC_1F_3 generations. On background analysis using 252 polymorphic amplified fragment length polymorphism (AFLP) markers they detected 80.4 to 86.7% recurrent parent alleles in BC_1F_3 selections. However, in the studies by Singh *et al*. 2001; Narayanan *et al*. 2004; Perez *et al*. 2008, three backcrosses were attempted and selection of lines in the backcross generation was entirely based on foreground selection of target traits.

Background selection for large numbers of plants with markers at each backcross generation would be an expensive proposition. Therefore, we suggest, based on the results obtained in the present study, that when resources are limited, phenotypic selection combined with marker-assisted foreground and assessment of background genome recovery through marker analysis can accelerate backcross breeding programs and make them cost-effective. Different strategies have been followed for MAS (i) each target gene is transferred separately and then the plants carrying different genes in the same background are crossed to pyramid the genes and (ii) all the genes are pyramided through simultaneous foreground and background selection. We followed the second approach in this study and were able to pyramid one dominant (*Xa21*) and two recessive genes (*xa13* and *xa5*) simultaneously into the BB susceptible basmati variety CSR-30. The first strategy could have taken more time for pyramiding the same set of genes. Along with the resistant gene from the donor parent, more than 70% of recurrent parent genome was transformed in the population. It was possible to pyramid multiple genes and to recover maximum genetic background of CSR-30 in the shortest time by transferring the genes simultaneously using the second strategy. We select the target genes through foreground selection and then carried out background selection to find out the recovery of maximum recurrent parent genome in the pyramided lines. However, instead of going for more number of backcrosses with the recurrent parent, it is suggested that after one or two backcrosses, after confirming that more than 70% of alleles of the recurrent parent has been transferred, genotypes should be evaluated for yield and its attributes along with the quality traits. It is expected that some of the favourable genes from the donor parent, in addition to the resistance genes, may be exploited for better gene combination.

5.6 Evaluation of BB resistance characteristics of the BC_1F_2 pyramided plants

The pyramided lines were evaluated for their resistance to bacterial blight under glass house conditions using the *Xanthomonas oryzae* strain isolated from the BB infected fields of RRS, Kaul, CCSHAU, Hisar. The three gene pyramided BC_1F_2 plants derived in this study from the cross CSR-30 x IRBB-60, were found to be equally effective against the virulent *Xoo* strain as the donor line IRBB60. Also the pyramided lines having either *Xa21* or *xa5* resistance genes alone were found to be resistant or moderately resistant to the BB disease. However, pyramided lines with *xa13* gene alone were found to be susceptible to BB disease. The pyramided lines (two gene or three gene combinations) had a higher level of resistance and broader spectrum of resistance than parental lines or lines with a single gene. This might be due to interaction and/or complementation between the resistance genes. Li *et al.* 2006 reported that a high level of durable resistance to *Xoo* can be achieved by the cumulative effects of multiple QTLs, including the residual effects of defeated major resistance genes. They revealed a complex genetic network of epistatic effects between resistance genes and QTL's for resistance in rice. They reported that resistance to specific *Xoo* strains is governed by both major resistance genes with a qualitative effect that condition complete resistance and polygenes with a quantitative effect (QTL) that condition partial resistance. The interactions between alleles at the rice resistance loci and alleles at the corresponding avirulent loci in *Xoo* lead to complete resistance whereas interactions between rice QTL for resistance and corresponding aggressiveness loci in *Xoo* lead to partial resistance.

In a similar study, Singh *et al.* (2001) reported that the genes in combinations were more effective against the pathogen than a single gene, by inoculating the selected BC_2F_3 PR106 lines homozygous for each of the individual genes and with different combinations with the 17 isolates of *Xoo* prevalent in Punjab. Resistance gene *Xa21* was effective against 16 of the isolates from Punjab used in their study. Resistance gene *xa13* was effective against 6 isolates (PXo7, PXo8, PXo11, PXo14, PXo15 and PXo16), whereas a line with gene *xa5* was resistant to 14 isolates. They found that *Xa21* was the most effective gene, followed by *xa5* and that gene *xa13* was the least effective. Rajpurohit *et al.* (2010) also presented the similar results by recording disease reaction in forty BC_2F_3 progenies of Type 3 basmati containing individual *xa13* and *Xa21* genes or combination of both under artificial inoculation conditions using mixture of seven *Xoo* isolates. Their results showed that the progenies having both the resistance genes *Xa21* and *xa13* were highly resistant to BB disease than the progenies having individual resistance genes. However, progenies having *xa13* gene alone were found to be more effective than the progenies having *Xa21* gene. But in our study, the BC_1F_2 plants having *xa13* gene were less effective than the plants having *Xa21* or *xa5* gene.

Similarly, Pandey *et al.,* 2012 reported that the backcross-derived improved Basmati lines of traditional Basmati varieties Taraori Basmati and Basmati 386 developed through MAS at BC_1F_5 possessing a single resistance gene (i.e. either *Xa21* or *xa13*) displayed moderate resistance to BB, while lines possessing both *Xa21* and *xa13* showed significantly higher levels of resistance as the donor parent Improved Samba Mahsuri, PR106.

Huang *et al*., 2012, in an another study, introgressed four bacterial blight (BB) resistance genes, *Xa7, Xa21, Xa22* and *Xa23*, into an elite hybrid rice restorer line Huahui 1035, using marker-assisted selection and found that restorer lines (HBQ809 and HBQ810) with *Xa23* gene were resistant to all eleven Chinese representative *Xoo* races, showing broad spectrum resistance to BB. However, the restorer lines possessing *Xa7* or *Xa7*+*Xa21* were resistant to ten out of eleven *Xoo* races and the lines possessing *Xa22* were found to be resistant to six or five out of inoculated eleven *Xoo* races.

5.7 Evaluation of yield and agro-morphological traits of BC_1F_2 pyramided plants

In the present study, the foreground selection for target loci and background selection in favour of the recurrent parent genome was followed by phenotypic selection for agronomic traits of recurrent parent. Before advancing the generation, agronomic traits of the selected plants was analysed and only those plants which derived maximum genetic background of CSR-30 with the desirable features such as BB resistance, grain number per panicle, yield per plant etc. were advanced to the next generation. This approach greatly hastened the recovery of the recurrent parent genotype and phenotype as the number of plants finally subjected to background selection was far smaller than the original population. Similar studies were conducted by Basavaraj *et al*. (2010) and Rajpurohit *et a*l. (2010). They also studied the agro-morphological traits of the selected plants before advancing to the next generation. However, in earlier studies by Chen *et al*. (2001), Singh *et al*. (2001) and Deng *et al*. (2006), the identification of resistant plants was based only on marker assisted background analysis data and hence large numbers of plants were genotyped.

In the present study, significant variation was observed among the pyramided lines and parental rice genotypes for plant height, effective number of tillers per plant, seeds per panicle and yield per plant. Most of the three gene pyramided BC_1F_2 genotypes were on par or superior to the recurrent parent CSR-30 with respect to the agronomic traits. In the pyramided BC_1F_2 genotypes, plant height ranged from 120 cm-129 cm, whereas the plant height of recurrent parent CSR-30 was 126 cm. All the three gene pyramided BC_1F_2 genotypes except G-6 had plant height intermediate between the two parents. Panicle length of CSR-30 was 25.20 cm and that of the pyramided BC_1F_2 plants varied between 20.08-26.80 cm. Genotype G-3 had a maximum panicle length of 26.20cm±1.15 cm and genotype G-22 had a minimum panicle length of 20.08±1.63 cm. Effective numbers of tillers in CSR-30 were 14 and in the pyramided BC_1F_2 genotypes the effective number of tillers ranged from 11 to 15. Genotypes

G-7 and G-169 had maximum 15 effective numbers of tillers. Number of seeds per panicle in CSR-30 were 175 and varied from 166-179 in the pyramided BC_1F_2 plants. Genotype G-3 had maximum 179 seeds per panicle followed by G-11 having 176 seeds per panicle Grain yield per plant in the pyramided BC_1F_2 plants genotypes varied between 30.33-34.25 g whereas in CSR-30 the grain yield per plant was recorded 33.20 g. The genotypes G-3 (34.25g/plant), G-7 (34.15g/plant), and G-169 (33.80g/plant) showed marginally higher yield than the recurrent parent CSR-30 (33.20g/ plant). Genotype G-3 showed the best agronomic features with maximum recovery of recurrent parent genome RPG (78.9%) followed by G-169 with RPG (76.2%) followed by line G-7 with RPG (72.5%).

In a similar study, Sundaram *et al.,* 2008 evaluated yield and agro-morphological traits of four of the three-gene pyramid lines at BC_4F_6 generation along with the donor and recipient .The recipient parent, Samba Mahsuri recorded an overall mean grain yield of 4,739 kg/ha, while the donor parent recorded 4,486 kg/ha. Three of the pyramided lines showed grain yields on par with Samba Mahsuri. However, the test entries did not show any significant variation as compared to Samba Mahsuri in terms of flowering duration, panicles/m2, plant stature as well as other characters that are considered under distinctness, uniformity and stability (DUS) tests. Shanti *et al*. (2010) also studied the agronomic and quality traits of the BC_2F_4 improved lines of Pusa6B and found that most of these lines were on a par with Pusa6B with respect to yield and yield components. Significant variation was observed for various agro morphological traits among the pyramided lines. Three families Pusa1605-05-38-3-1 (52.44 q/ha) and Pusa1605-05-38-3-2 (51.89 q/ha) showed marginally higher yield than the original Pusa6B (48.30 q/ha). The highest yielding family Pusa1605-05-38-3-1 showed maximum RPG recovery (97.30%). Similarly, Basavraj *et al.,* 2010 also observed significant variation for the agronomic traits among the ten BC_2F_3 improved lines of Pusa6B and found that most of the families were on par with Pusa6B with respect to yield and yield components. The families Pusa1605-05-38-3-1 (52.44 q/ha) and Pusa1605-05-38-3-2 (51.89 q/ha) showed marginally higher yield than the original Pusa6B (48.30 q/ha). The highest yielding family Pusa1605-05-38-3-1 showed maximum RPG recovery (97.30%). Pandey *et al.* (2012) also evaluated agronomic and Basmati-type grain quality of 96 and 16 pyramid lines in the BC_1F_4 generation from the crosses RP4693 and RP4694, respectively. They also observed significant variation among the pyramided lines for most of the agronomic traits.

Traditional basmati varieties are highly susceptible to bacterial blight disease for which a number of effective genes have been tagged and cloned (Rajpurohit *et al.* 2010). More resistance genes need to be identified and pyramid together into the elite cultivars to ensure the durability of BB resistance. Most of major genes have been overcomed by new or unrecognized pathogen races. Fortunately, this can be prevented by combining with other resistance genes. Pyramiding of two or more resistance genes should lead to more durable resistance in rice. In this study, pyramiding genes conferring broad spectrum resistance through MAS were successfully conducted. The pyramided lines obtained in our study can be used as genetic resources for BB resistance in breeding programs that will be paving way for an environmental-friendly means to achieve a better disease management. Moreover, the success will facilitate future efforts to transfer combinations of BB resistance genes into other preferred rice cultivars.

CHAPTER-VI

SUMMARY AND CONCLUSION

Rice (*Oryza sativa* L.) is the principal food crop of half of the world's population and the present world population of 7.1 billion is likely to reach 7.7 billion by 2020 (world population clock, Bureau census). Thus, rice production must be increased by 50 per cent to meet the growing demand of ever-increasing population. Bacterial blight (BB), caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), has been one of the most serious diseases of rice, affecting production in irrigated and rain-fed lowland ecosystems throughout Asia, northern Australia, mainland Africa, the southern part of the United States and Latin America. The present investigation was, therefore, carried out to introgress three BB resistance genes *Xa21, xa13* and *xa5* from BB resistant donor varieties IRBB-60 and Pusa Basmati-1460 into the BB susceptible basmati variety CSR-30 through marker assisted selection. The plant material for the current investigation was obtained from Regional Rice Research Station, Kaul, CCSHAU, Hisar. Crosses were made between CSR-30 x IRBB-60 and CSR-30 x Pusa Basmati-1460. Foreground selection was carried out in F_1 generation and backcross generation used in our study to detect the presence of BB resistance genes *Xa21, xa13* and *xa5* using STS markers pTA248, RG136 and RG556, respectively. For background selection, a set of 300 SSR markers were used to find out the polymorphism between the parental genotypes IRBB-60 and CSR-30 and Pusa Basmati-60 and CSR-30. The primers amplifying polymorphic alleles between the parents IRBB-60 and CSR-30 were used to find out the recovery of recurrent parent genome in the three gene pyramided genotypes. The results obtained and conclusions drawn from this study are summarized as follows:

I Confirmation of the presence of BB resistance genes in Pusa Basmati-1460 and IRBB-60

1. Total genomic DNA was extracted from the young leaves of 3-4 week old seedlings of the parental genotypes using modified CTAB method. DNA concentration was estimated by using UV spectrophotometer and its quality was checked by taking absorbance at 260 and 280nm, which was very near to 1.8 for all the genotypes.
2. PCR amplification was carried out using the specific STS markers pTA248, RG136 and RG556 linked to BB resistance genes *Xa21, xa13* and *xa5,* respectively. The amplified product of the STS markers were resolved on 1.5 % agarose gel and visualised under U.V. light after staining with ethidium bromide.
3. A specific band of 1000 bp was present only in the donor parents Pusa Basmati-1460 and IRBB-60 and absent in the variety CSR-30, when amplified with STS marker

pTA248 linked to *Xa21* gene. So, the BB resistance gene *Xa21* was confirmed in donor parents Pusa Basmati-1460 and IRBB-60.

4. For the gene *xa13* and *xa5*, PCR amplified product of marker RG136 and RG556 were used for restriction digestion by restriction enzyme *Hinf* 1 and *Dra*1, respectively. Two bands of size 400 bp and 506 bp were present in the R.E digested PCR product of marker RG136 in the donor parental genotypes Pusa Basmati-1460 and IRBB-60 and a band of size 450 bp was present in R.E digested PCR product of marker RG556 in donor parental genotype IRBB-60 and were absent in CSR-30. This confirmed the presence of BB resistance gene *xa13* in Pusa Basmati-1460 and IRBB-60 and the presence of resistance gene *xa5* in donor parent IRBB-60.

II Pyramiding of resistance genes in the basmati variety CSR-30

Crosses were made between CSR-30 x Pusa Basmati-1460 and CSR-30 x IRBB-60. A total of 50 and 6 F_1 seeds of the crosses CSR-30 x Pusa Basmati-1460 and CSR-30 x IRBB-60 were harvested, respectively.

Cross CSR-30 x Pusa Basmati-1460

1. The fifty F_1 seeds of the cross CSR-30 x Pusa Basmati-1460 were grown in the net house and the presence of the resistance genes *xa13* and *Xa21* were checked in all the F_1 plants using the specific STS markers RG136 and pTA248, respectively. Two plants were found to have both the resistance genes *xa13* and *Xa21.*
2. The two plants having both the resistance genes *xa13* and *Xa21,* failed to survive in the field. So, fresh crosses of CSR-30 x Pusa Basmati-1460 were made in the next growing season and the F_1 seeds (4 seeds) of the cross were harvested.
3. The four F_1 seeds of the cross CSR-30 x Pusa Basmati-1460 were grown in the net house and foreground selection was carried out in these F_1 plants . A total of two out of four F_1 plants were found to have both the BB resistance genes *Xa21* and *xa13.* These F_1 plants were grown in the net house and selfed to get F_2 seeds.

Cross CSR-30 x IRBB-60

1. The six F_1 seeds of the cross CSR-30 x IRBB-60 were grown in the net house and the foreground selection was carried out to detect the presence of the resistance genes *xa13, xa-5* and *Xa21* in these F_1 plants using the specific STS markers RG136, RG556 and pTA248, respectively. A total of three F_1 plants were found to have all the three resistance genes *Xa21*, *xa13* and *xa-5.*
2. Backcrosses of the F_1 plants from the cross of IRBB-60 x CSR-30, having all the three resistance genes were made with the respective recurrent parent i.e. CSR-30. The BC_1F_1 seeds (fifty seven seeds) of this cross were harvested.

3. The fifty seven BC_1F_1 seeds were grown in the net house during the next growing season out of which forty seeds were germinated. These forty BC_1F_1 plants were selfed to produce BC_1F_2 seeds.
4. A total of two hundred and fifty BC_1F_2 seeds were grown in the net house in the next growing season out of which two hundred and thirty plants were germinated and mini scale DNA isolation was carried out from 4-5 week old leaves of all the 230 BC_1F_2 plants following CTAB method.
5. The 230 BC_1F_2 plants were subjected to foreground selection to check for presence of *Xa21* resistance gene using STS marker pTA248. Seventy one plants amplified 1000bp band specific to *Xa21* gene.
6. These seventy one plants that were positive for *Xa21* gene were then screened for presence of *xa5* resistance gene using STS marker RG556 (digested with *Dra* I). A band of size 450bp specific to *xa5* gene was present in twenty four plants.
7. Finally, these double positive twenty four plants having both *Xa21* and *xa5* resistance genes were screened for the presence of *xa13* resistance gene using STS marker RG136 (digested with *Hinf* I). Ten plants produced bands of size 400 bp and 506 bp specific to *xa13* gene.
8. Three hundred SSR markers were used to identify the polymorphism between parental genotypes Pusa Basmati-1460 and CSR-30 and between IRBB-60 and CSR-30.
9. A total of 72 SSR markers produced polymorphism between the parents Pusa Basmati-1460 and CSR-30 whereas a total of 104 SSR primers produced polymorphic alleles in parents IRBB-60 and CSR-30.
10. The primers polymorphic between the parents IRBB-60 & CSR-30 were used to genotype the ten three gene pyramided genotypes to find out the recovery of recurrent parent genome contribution in these plants.
11. The PCR amplified fragments were scored visually for the presence (1) and absence (0) of parental alleles for the ten three gene pyramided BC_1F_2 genotypes and the parental genotypes IRBB-60 and CSR-30.
12. The SSR marker data obtained was used to construct similarity matrices of the three gene pyramided BC_1F_2 rice genotypes using 'Simqual' sub-programme of software NTSYS-PC. The similarity indices between different genotypes ranged from 0.15 to 0.93 across all the genotypes. The genotypes G-3 and G-169 were the most closest to the recurrent parent CSR-30 with genetic similarity coefficient (0.88), followed by G-7 with CSR-30 with genetic similarity coefficient (0.85). The genetic similarity of the remaining seven genotypes

(G-1, G-6, G-7, G-10, G-11, G-17 and G-22) with CSR-30 varied from 64% to 85%.

13. Cluster tree analysis revealed that the BC_1F_2 rice genotypes positive for *Xa21, xa5* and *xa13* BB resistance genes and the recipient parent CSR-30 fell in one group with two major sub-groups. Sub-group I consisted of recipient parent CSR-30 and three genotypes G-3, G-7 and G-169 with maximum similarity among them. Sub-group II consisted of the remaining seven genotypes G1, G6, G10, G11, G17, G22 and G187. As expected the donor parent IRBB-60 remained as a separate group.

14. The percentage recovery of recurrent parent genome in the three-gene pyramided BC_1F_2 genotypes ranged from 44.2% to 78.9% as revealed by the global statistics using the GGT software.

15. The pyramided lines were evaluated for their resistance to bacterial blight under glass house conditions using the *Xanthomonas oryzae* strain isolated from the BB infected fields of RRS, Kaul, CCSHAU, Hisar. As compared to the recurrent parent CSR-30, the pyramided lines exhibited very high level of resistance to bacterial blight disease.

16. Data was recorded for the yield and several agro- morphological traits in the pyramided BC_1F_2 plants and the parental genotypes. It was found that the three-gene pyramided BC_1F_2 plants had CSR-30 like characteristics and their agronomic traits were either superior or on par with CSR-30.

17. The genotypes G-3 (34.25 g/plant), G-7 (34.15 g/plant) and G-169 (33.80 g/plant) showed marginally higher yield than the recurrent parent CSR-30 (33.20 g/ plant). Genotype G-3 showed the best agronomic features with maximum recovery of recurrent parent genome RPG (78.9 %) followed by G-169 with RPG (76.2 %) followed by line G-7 with RPG (72.5 %).

Based on the agronomic evaluation, BB reaction and percentage recovery of recurrent parent genome three genotypes G-3, G-7 and G-169 were selected for further backcrossing with the recurrent parent CSR-30. These three gene pyramided BC_1F_2 plants were backcrossed with the recurrent parent CSR-30 and the BC_2F_2 seeds and BC_1F_3 seeds were harvested. These identified genotypes can be further improved through phenotypic selection as well as molecular markers for the development of potential BB resistance donors. The BC_2F_2 seeds will be grown in the field. Foreground and background selection will be carried out and also the agro- morphological traits of the three gene pyramided BC_3F_2 plants along with the quality traits will be evaluated further, by another Ph.D student. Advanced basmati breeding lines, which will be derived through MAS and phenotypic selection, will, therefore, be of practical value in providing durable bacterial blight resistance in the basmati growing region and are expected to have a high impact on the yield stability and sustainability of basmati rice production.

BIBLIOGRAPHY

Adhikari, T.B., Vera Cruz, C.M., Zhang, Q., Nelson, R.J., Skinner, D.Z., Mew, T.W. and Leach, J.E. 1995. Genetic diversity of *Xanthomonas oryzae* pv. *Oryzae* in Asia. *Applied Microbiology and Biotechnology* **61**(3): 966-971.

Akagi, H., Yokozeki, Y., Inagaki, A. and Fujimura, T. 1996. Microsatellite DNA markers for rice chromosome. *Theoretical and Applied Genetics* **94**:61–67.

Archak, Sunil, Lakshminarayana Reddy, Nagaraju, V. and Javaregowda, 2011. High throughput multiplex microsatellite marker assay for detection and quantification of adulteration in Basmati rice (*Oryza sativa*) *Electrophoresis* **28**(14): 2396-2405.

Arif, M., Jaffar, M., Babar, M. and Sheikh, A.M. 2008. Identification of bacterial blight resistance genes *Xa4* in Pakistani rice germplasm using PCR. *African Journal of Biotechnology* **7**: 541-545.

Babu, R., Nair, S.K., Prasanna, B.M. and Gupta, H.S. 2004. Integrating marker assisted selection in crop breeding – Prospects and challenges. *Current Science* **7**(5): 607-619.

Babujee, L. and Gnanamanickam, S.S. 2000. Molecular tools for characterization of rice blast pathogen, *Magnaporthe grisea*, population and molecular marker-assisted breeding for disease resistance. *Current Science* **78**:248-257.

Basavaraj, S.H., Singh, V.K., Singh, A., Singh, A., Singh, A., Yadav, S., Ellur, R.K., Singh, D., Gopalakrishnan, S., Nagarajan, M., Mohapatra,T., Prabhu, K.V. and Singh A.K. 2010. Marker-assisted improvement of bacterial blight resistance in parental lines of Pusa RH10, a superfine grain aromatic rice hybrid. *Molecular Breeding* **26**: 293–305.

Beckmann, J.S. and Soller, M. 1990. Towards a unified approach to genetic mapping of eukaryotes based on sequence-tagged microsatellite sites. *Biotechnology* **8**: 930-932.

Bernier, J., Kumar, A., Venuprasad, R., Spaner, D., Verulkar, S., Mandal, N.P., Sinha, P.K., Peeraju, P., Dongre, P.R., Mahto, R.N. and Atlin, G. 2008. Characterization of the effect of a QTL for drought resistance in rice, qtl12.1, over a range of environments in the Philippines and eastern India. *Euphytica* **166 (2)**: 207-217.

Bharani, M., Nagarajan, P., Rabindaran,R., Saraswathi, R., Balasubramanian, P. and Ramalingam, J. 2010. Bacterial leaf blight resistance genes (*Xa21, xa13* and *xa5*) pyramiding through molecular marker assisted selection into rice cultivars. *Phytopathology and Plant protection* **43**: 1032-1043.

Bharathkumar, S., Paulraj,S.D., Brindha, P.V., Kavitha, S. and Gnanamanickam, S.S. 2008. Improvement of bacterial blight resistance in rice cultivars Jyothi and IR50 via marker-assisted backcross breeding. *Journal of Crop Improvement* **21**:101–116.

Bhasin, V.K. 2000. India and the emerging global rice trade. In *aromatic rices*. Eds: R.K Singh. U.S.Singh and G.S. khush. New Delhi: Oxford and IBH Publishing Company Pvt Limited.

Bhasin, H., Bhatia, D., Raghuvanshi, S., Lore, J.S., Sahi, G.K., Kaur, B., Vikal, Y. and Singh, K. 2012. New PCR-based sequence-tagged site marker for bacterial blight resistance gene *Xa38* of rice. *Molecular Breeding* **30**:607–611.

Blair, M.W. and McCouch, S.R. 1997. Microsatellite and sequence tagged site markers diagnostic for the rice bacterial leaf blight resistance gene *xa-5*. *Theoretical and Applied Genetics* **252**: 597-607.

Bouman, B.A.M. 2003. Water efficient management strategies in rice production. *International Rice Research Notes*, IRRI, Los Banos, Philippines. **16(2)**: 17-22.

Century, K.S., Lagman, R.A., Adkisson, M., Morlan, J., Tobias, R., Schwartz, K.A., Smith, J., Love, Ronald, P.C. and Whalen, M.C. 1999. Developmental control of *Xa21*-mediated resistance in rice. *Plant Journal* **20**: 231-236.

Cheema, K., Grewal, N., Vikal, Y., Sharma, R., Lore, J.S., Das, A., Bhatia, D.,Mahajan, R., Gupta, V., Bharaj, T.S. and Singh, K. 2008. A novel bacterial blight resistance gene from *Orzo nivara* mapped to 38 kb region on chromosome 4 L and to *Orzo sativa* L. *Genetics Research* **90**:397–407.

Chen, X., Temnykh, S., Xu, Y. and Cho, Y.G. 1997. Development of a microsatellite framework map providing genome-wide coverage in rice (*Oryza sativa* L.). *Theoretical and Applied Genetics* **95**: 553-567.

Chen, S., Xu, C.G., Lin, X.H and Zhang, Q. 2001. Improving bacterial blight resistance of '6087', an elite restorer line of hybrid rice, by molecular marker assisted selection. *Plant Breeding* **120**: 133-137.

Chen, S., Huang, Z., Zeng, L., Yang, J., Lui, Q.and Zhu, X. 2008. High-resolution mapping and gene prediction of *Xanthomonas oryza* epv. *Oryzae* resistance gene *Xa7*. *Molecular Breeding* **22**: 433-441.

Cheng, Y.J., Guo, W.W., Yi, H.L., Pang, X.M. and Deng, X. 2003.An efficient protocol for genomic DNA extraction from rice plant. *Plant Molecular Biology Reporter* **21**(2): 177-178.

Chu, Z., Fu, B.H., Yang, C., Xu, Z., Li, A., Sanchez, Y.J., Park, L., Bennetzen, Q., Zhang and Wang, S. 2006. Targeting *xa13*, a recessive gene for bacterial blight resistance in rice. *Theoretical and Applied Genetics* **112**: 455-461.

Chun, X., Hongqi, C. and Xudong, Z. 2012. Identification, Mapping, Isolation of the Genes resisting to Bacterial Blight and Breeding application in Rice. *Molecular Plant Breeding* **3**(12): 121-131**.**

Chunwongse, J., Martin, G.B. and Tanksley, S.D. 1993. Pre-germination genotypic screening using PCR amplification of half-seeds. *Theoretical and Applied Genetics* **86**(6): 694-698.

Collard, B.C.Y. and Mackill, D.J. 2008. Marker-assisted selection: an approach for precision plant breeding in the twenty-first century. *Philosophical Transactions of the Royal Society* **363**: 557 – 572.

Deng, Q., S. Wang, A., Zhang, H., Zhang and Li, P. 2006. Breeding rice restorer lines with high resistance to bacterial blight by using molecular marker-assisted selection. *Rice Science* **13**(1): 22-28.

Dubcovsky, J. 2004. Marker-assisted selection in public breeding programs: the wheat experience. *Crop Science* **44**: 1895-1896.

FAO, 2011and 2012.www.fao.org/rice/

Flor, H.H. 1971. Current status of the gene-for-gene concept.*Annual Review of Phytopathology* **9**: 275-276.

Fu, C., Wu, T., Liu, W., Wang,L., Li, J., Zhu, X., Huang, H. and Zhu, M. 2012.Genetic improvement of resistance to blast and bacterial blight of the elite maintainer line Rongfeng B in hybrid rice (*Oryza sativa* L.) by using marker-assisted selection. *African Journal of Biotechnology* **11**(67): 13104-13124.

Gandhi, D. 2007. UAS scientist develops first drought tolerant rice. Marker-assisted selection: an approach for precision plant breeding in the twenty-first century. *Philosophical Transactions of the Royal Society* **363**: 557 – 572.

Garris, A.J., McCouch, S.R. and Kresovich, S. 2003. Population structure and its effect on haplotype diversity and linkage disequilibrium surrounding the *xa5* locus of rice (*Orzo sativa* L.). *Genetics* **165**: 759-769.

Gopalakrishnan, S., Sharma, R.K., AnandRajkumar, K., Joseph, M., Singh, V.P., Singh, A.K., Bhat, K.V., Singh, N.K. and Mohapatra, T. 2008. Integrating marker assisted background analysis with foreground selection for identification of superior bacterial blight resistant recombinants in Basmati rice. *Plant Breeding* **127**:131–139.

Gu, K., Yang, B., Tian, B., Wu, L., Wang, D., Sreekala, C., Yang, F., Chu, Z., Wang, G.L., White, F.F. and Yin, Z. 2005. *R* gene expression induced by a type-III effect or triggers disease resistance in rice. *Nature* **435**: 1122–1125.

Gu, K., Sangha, J.S., Li, Y. and Yin, Z. 2008. High-resolution genetic mapping of bacterial blight resistance gene *Xa10*. *Theoretical and Applied Genetics* **116**(2): 155-163.

Hoan, N.T. and Nghia, N.H. 2003. Hybrid rice development and use in Vietnam. In: Virmani SS, Mao CX, Hardy B (eds) Hybrid rice for food security, poverty alleviation, and environmental protection. Fourth international symposium on hybrid rice, International Rice Research Institute, Vietnam, 357–371.

Hospital, F., Chavalet, C. and Mubsant, P. 1992. Using markers in gene introgression breeding programmes. *Genetics* **132**:1119–1210.

Hospital, F. 2005.Selection in backcross programs. *Philsophical Transactions of the Royal Society* **360**: 1503-1511.

Huang, N., Angeles, E.R., Domingo, J., Magpantay, G., Singh, S., Zhang, Q., Kumaravadivel, N., Bennett, J. and Khush, G.S. 1997.Pyramiding of bacterial resistance genes in rice: marker aided selection using RFLP and PCR. *Theoretical and Applied Genetics* **95**:313–320.

Huang, J.S. and Cleene, M.D. 1989. How rice plants are infected by *Xanthomonas campestris* pv. *oryzae*, pp. 31-42. *In Proceedings of the International Workshop on Bacterial Blight of Rice*. International Rice Research Institute, Manila, Philippines.

Huang, B., Xu, J.Y., Hou, M.S., Ali, J. and Mou, T.M. 2012. Introgression of bacterial blight resistance genes *Xa7, Xa21, Xa22* and *Xa23* into hybrid rice restorer lines by molecular marker-assisted selection. *Euphytica* **187**:449–459.

International Rice Research Institute (IRRI). 1973. Annual Report for 1972**.** International Rice Research Institute, Los Banos, Philippines.

IRGSP, (2005).The map-based sequence of the rice genome. *Nature* **436**: 793 – 800.

IRRI, International Rice Research Institute 1997.*Rice almanac*, 2nd ed. P.181.IRRI, Los Banos, Philippines.

IRRI, International Rice Research Institute, 2004. Rice Fact Sheet, Bacterial Leaf Streak.

IRRI. International Rice Research Institute, 2010. Rice Fact Sheet, Bacterial Blight.

Ishiyama, S. 1992. Studies of bacterial leaf blight of rice. Report Imp. Agric. Stn.Konosu. **45**: 233-261.

Iyer, A.S. and McCouch, S.R. 2004. The rice bacterial blight resistance gene *xa5* encodes a novel form of disease resistance. *Molecular Plant Microbial Interaction* **17**:1348–1354.

Iyer-Pascuzzi, A.S. and McCouch, S.R. 2007. Functional markers for *xa5*-mediated resistance in rice (*Orzo sativa* L). *Molecular Breeding* **19**: 191-296.

Jantaboon, J., Siangliw, M., Wongsaprom, C., Toojinda, T. and Vanavichit, A. 2004. Discovery of new bacterial blight resistance genes in backcross introgressed lines of KDML105, pp. 118. In Proceedings of the 1st International Conference on Rice for the Future. Kasetsart University, Bangkok, Thailand.

Jena, K.K., Jeung, J.U., Noh, T.H., Heu, S., Suh, J.P., Kim, K.Y., Shin, M.S., Reveche, M.Y., Pamplona, A., Vera-Cruz, C.M., Kim, Y.G. and Brar, D.S. 2007. Molecular breeding for bacterial blight resistance in japonica rice, pp.77-82. In The 2nd International Conference on Bacterial Blight of Rice, Nanjing, China.

Jena, K.K. and Mackill, D.J. 2008. Molecular markers and their use in marker-assisted selection in rice. *Crop Science* **48**:1266–1276.

Jeung, J.U., Heu, S.G., Shin, M.S., Cruz, C.M.V. and Jena, K.K. 2006. Dynamics of *Xanthomonas oryzae* pv. *Oryzae* populations in Korea and their relationship to known bacterial blight resistance genes. *The American Phytopathological Society* **96**(8): 867-875.

Joseph, M., Gopalakrishnan, S., Sharma, R.K., Singh, V.P., Singh, A.K., Singh, N.K. and Mohapatra, T. 2004. Combining bacterial blight resistance and basmati quality characteristics by phenotypic and molecular marker assisted selection in rice. *Molecular Breeding* **13**:377–387.

Kadir, T.S., Widiarta, I.N., Daradjat, A.A., Suryadi, I.L. and Hidayat, Y. 2004. Current status of bacterial blight of rice in Indonesia, pp.33. In The 1st International Conference on Bacterial Blight of Rice. Tsukuba Science City, Ibaraki,Japan.

Kauffman, H.E., Reddy, A.P.K., Hsien, S.P.Y. and Merca, S.D. 1973. An improved technique for evaluating resistance of rice varieties to *Xanthomonas oryzae*. *Plant Disease Response* **57**: 537–541.

Keng, H.W., Cho, Y.G., Yoon, U.H., Eun, M.Y. 1998. A rapid DNA extraction method for RFLP and PCR analysis from a single dry seed. *Plant Molecular Biology Reporter* **16**(1): 1-9.

Kermekchiev, M.B., Lyubka, I.K., Erika, E.V. and Barnes, W.M. 2009. Mutants of Taq DNA polymerase resistant to PCR inhibitors allow DNA amplification from whole blood and crude soil samples. *Nucleic Acids Research* **37**(5): 1-14.

Khush, G.S., Mackill, D.J. and Sidhu, G.S. 1989. Breeding rice for resistance to bacterial blight.In: Bacterial blight of rice, Proceedings of the International Workshop on Bacterial blight of Rice. International Rice Research Institute, Manila,Philippines. 177-207.

Khush, G.S. and Angeles, E.R. 1999. A new gene for resistance to race 6 of bacterial blight in rice, *Oryzasativa* L. *Rice Genetics Newsletter* **16**:92–93.

Khush, G.S. and Dela Cruz, N. 2002. Developing Basmati rices with high yield potential.International symposium on rice quality- Nottingham, U.K, November 24-27.

Kim, K.Y., Shin, M.S., Kim, W.J., Mo, Y.J., Nam, J.K., Noh, T.H., Kim, B.K. and Ko, J.K. 2009. Effective combination of resistance genes against rice bacterial blight pathogen. *Korean Journal of Breeding Science* **41**(3):244–251.

Konieczny, A. and Ausubel, F.M. 1993. A procedure for mapping *Arabidopsis* mutations using co-dominant ecotype-specific PCR-based markers. *Plant Journal* **4**: 403-410.

Korinsak, S., Sriprakhon, S., Sirithanya, P., Jairin, J., Korinsak, S., Vanavichit, A. and Toojinda, T. 2009. Identification of microsatellite markers (SSR) linked to a new bacterial blight resistance gene *xa33 (t)* in rice cultivar 'Ba70. *International Journal of Science and Technology* **3**:235–247

Kottapalli, K.R., Narasu, M.L. and Jena, K.K. 2010. Effective strategy for pyramiding three bacterial blight resistance genes into fine grain rice cultivar, Samba Mahsuri, using sequence tagged site markers. *Biotechnology* **32**:989–996.

Lee, K.S., Rasabandith, S., Angeles, E.R. and Khush, G.S. 2003. Inheritance of resistance to bacterial blight in 21 cultivars of rice.*Phytopathology* **93**(2): 147-152.

Li, Z.K., Sanchez, A., Angeles, E., Singh, S., Domingo, J., Huang, N. and Khush, G.S. 2001. Are the dominant and recessive plant disease resistance genes similar? A case study of rice R genes and *Xanthomonas oryzae* pv. *Oryzae* races. *Genetics* **159**:757–765.

Li, Z.K., Fu, B.Y., Gao, Y.M., Xu, J.L., Ali, J., Lafitte, H.R., Jiang, Y.Z., Rey, J., Vijayakumar, C., Maghirang, R., Zheng, T.Q. and Zhu, L.H. 2005. Genome-wide introgression lines and their use in genetic and molecular dissection of complex phenotypes in rice (*Orzo sativa* L.). *Plant Molecular Biology* **59**: 33-52.

Li, Z.K., M. Arif, D.B. Zhong, B.Y. Fu, J.L. Xu, J. Domingo-Rey, J. Ali, C.H.M.. Vijayakumar, S.B. Yu and Khush, G.S. 2006. Complex genetic networks underlying the defensive system of rice (*Orzo sativa* L.) to *Xanthomonas oryzae* pv. *oryzae*. *Proceedings of National Academy of Sciences* **103**(21): 7994-7999.

Liu, H., Yang, B., Hu, B. and Liu, F. 2007. Virulence Analysis and Race Classification of *Xanthomonas oryzae* pv. *Oryzae* in China. *Phytopathology* **155**(3): 129-139.

McCouch, S.R., Teytelman, L., Xu, Y.B., Lobos, K.B., Clare, K., Walton, M., Fu, B.Y., Maghirang, R., Li, Z.K., Xing, Y.Z., Zhang, Q.F., Kono, I., Yano, M., Fjellstrom, R., DeClerck, G., Schneider, D., Cartinhour, S., Ware, D. and Stein, L. 2002. Development and mapping of 2240 new SSR markers for rice (*Orzo sativa* L). *DNA Research* **9**:199–207.

Mew, T.W., Vera-Cruz, C.M. and Reyes, R.C. 1982. Interaction of *Xanthomonascampestris*pv. *Oryzae* and a resistant rice cultivar. *Phytopathology* **72**: 786-789.

Mew, T.W. 1987.Current status and future prospects of research on bacterial blight of rice. *Annual Review of Phytopathology* **25**: 359-382.

Mew, T.W., Cruz, C.M. and Medalla, E.S. 1992. Changes in the race frequency of *Xanthomonas oryzae* pv. *Oryzae* in response to the planting of rice. *Phytopathology* **72**: 786-789.

Mitchell, S.E., Kresovich, S., Jester, C.A. and Szewc-McFadden, A.K. 1997. Application of multiplex PCR and fluorescence-based, semi-automated allele sizing technology for genotyping plant genetic resources. *Crop Science* **37**: 617-624.

Mundt, C.C. 1990. Probability of mutation of multiple virulence and durability of resistance gene pyramids. *Phytopathology* **80** (3): 221–223.

Murray, M.G. and Thompson, W.F. 1980. Rapid isolation of high molecular weight plant. *Nucleic Acids Research* **8**: 4321-4325.

Murty, V.V.S. and Khush, G.S. 1972. Studies on the inheritance of resistance to bacterial blight in rice varieties, pp. 301-305.In Rice Breeding Research Institute, Los Banos, Philippines.

Narayanan, N.N., Baisakh, N., Vera Cruz, C.M., Gnanamanickam, S.S., Datta K and Datta, S.K. 2004. Molecular breeding: marker-assisted selection combined with biolistictransformation for blast and bacterial blight resistance in Indica rice (cv.CO39). *Molecular Breeding* **14**: 61–71.

Natrajkumar, P., Sujatha, K., Laha, G.S, Srinivasarao, K., Mishra, B., Viraktamath, B.C., Hari, Y., Reddy, C.S., Balachandran, S.M., Ram, T., Sheshumadhav, M., Shobharani, N., Neeraja, C.N., Ashokreddy, G., Shaik, H. and Sundaram, R.M. 2012. Identification and fine-mapping of *Xa33*, a novel gene for resistance to *Xanthomonas oryzae* pv.*oryzae*. *Phytopathology* **102**:222–228.

Neeraja, C.N., Maghirang-Rodriguez, R., Pamplona, A., Heuer, S., Collard, B.C.Y., Septiningsih, E.M., Vergara, G., Sanchez, D., Xu, K., Ismail, A.M. and MacKill, D.J. 2007. A marker-assisted backcross approach for developing submergence-tolerant rice cultivars. *Theoretical and Applied Genetics* **115**: 767– 776.

Nino-Liu, D.O. Ronald, P.C. and Bogdanove, A.J. 2005. A Simple method of mass inoculation of rice effective for both pathovars of *Xanthomonas oryzae,* and the construction of comparable sets of host cDNA libraries spanning early stages of bacterial leaf blight and bacterial leaf streak. *Journal of Phytopathology* **153**: 500-504.

Nino-Lui, D.O., Ronald, P.C. and Bogdanove, A.J. 2006. Pathogen profile of *Xanthomonas oryzae* pathovars: model pathogens of a model crop. *Molecular Plant Patho*logy **7**(5):303-324.

Noh, T.H., Lee, D.K., Kang, M.H., Shin, M.S. and Na, S.Y. 2003. Identification of new race of *Xanthomonas oryzae* pv. *Oryzae (Xoo)* in Korea. *Phytopathology* **93** (suppl):S66.

Noh, T.H., Lee, D.K., Park, J.C., Shim, H.K., Choi, M.Y., Kang, M.H. and Kim, J.D. 2007. Effect of bacterial leaf blight occurrence on rice yield and grain quality in different rice growth stage. *Plant Disease* **13**: 20-23.

Ogawa, T., Yamamoto, T., Khush, G.S. and Mew, T.W. 1986.The relationship between *Xa-3* and *Xa-6* for resistance to rice bacterial blight. *Rice Genetics Newsletter* **3**: 83-84.

Ou, S.H. 1985. Rice disease 2nd edition Commonwealth Mycology Institute, England.

Pandey, M.K., Shobha Rani, N., Sundaram, R. M., Laha, G. S., Madhav, M. S., Rao, S.K., Sudharshan, I., Varaprasad, G.S., SubbaRao, Sivaranjani, A. K. P.B.C. and Viraktamath. 2012. Improvement of two traditional Basmati rice varieties for bacterial blight resistance and plant stature through morphological and marker-assisted selection. *Molecular Breeding* DOI 10.1007/s11032-012-9779-7.

Panter, S.N., Hammond-Kosack, K.E., Harrison, K., Jones, J.D. and Jones, D.A. 2002. Developmental control of promoter activity is not responsible for mature onset of *Cf-9B*-mediated resistance to leaf mold in tomato. *Molecular Plant Microbes Interactions* **15**: 1099-1107.

Perez, L.M., Redoña, E.D., Mendioro, M.S., Vera Cruz, C.M. and Leung, H. 2008. Introgression of *Xa4*, *Xa7* and *Xa21* for resistance to bacterial blight in thermosensitive genetic male sterile rice (*Oryza sativa* L.) for the developmentof two-line hybrids. *Euphytica* **164**(3): 627-636.

Perumalsamy, S., Bharani, M., Sudha, M., Nagarajan, P., Arul, L., Sarawathi, R.,Balasubramanian, P. and Ramalingam, J. 2010. Functional marker-assisted selection for bacterial leaf blight resistance genes in rice (*Oryza sativa* L.). *Plant Breeding* **129**:400–406.

Pirttila, A.M., Hirsikorpi, M., Kamarainen, T., Jaakola, L. and Hohtola, A. 2001. DNA isolation methods for medicinal and aromatic plants. *Plant Molecular Biology* **19**(3): 273-278.

Porter, B.W., Chittoor, J.M., Yano, M., Sasaki, T. and White, F.F. 2003. Development and mapping of markers linked to the rice bacterial blight resistance gene *Xa7*. *Crop Science* **43**: 1484-1492.

Rajpurohit, D., Kumar, R., Kumar, M., Paul, P., Awasthi, A.A., Basha, P.O., Puri, A., Jhang, T., Singh, K. and Dhaliwal, H.S. 2010. Pyramiding of two bacterial blight resistance and a semi dwarfing gene in Type 3 Basmati using marker-assisted selection. *Euphytica* **178**:111–126.

RamalingamJ, Basharat HS, Zhang G (2001). Polymorphism of DNA markers linked to bacterial blight resistance genes in useful rice germplasm. IRRN. **26**: 23-24.

Rao KK, Lakshminarasu, M. and Jena, K.K. 2002. DNA markers and marker-assisted breeding for durable resistance to bacterial blight of rice. *Biotechnology Advances* **20**:33–47.

Rao, K.K. 2003. Molecular tagging of a new gene bacterial blight resistance gene in rice using RAPD and SSR markers. *Plant Breeding* **8**(1): 16-17.

Ribeiro, R.A. and Lovato, M.B. 2007. Comparative analysis of different DNA extraction protocols in fresh and herbarium specimens of the genus *oryza sativa*. *Genetics and Molecular Research* **6**(1): 173-187.

Rohlf, F.J. 2000. NTSYS-PC, numerical taxonomy and multivariate analysis system.Version 2.02, *Applied Biostatistical Inc, New York.*

Ronald, P.C., Albano, B., Tabien, R., Abenes, L., Wu, K.S., McCouch, S.R. and Tanksley, S.D. 1992. Genetic and physical analysis of the rice bacterial blight disease resistance locus, *Xa21.Molecular Genome and Genetics* **236**(1): 113-120.

Roychowdhary, R., Karmakar, J. and Deng, N. 2012. PCR compatible genomic DNA isolation from different tissues of rice (*Oryza sativa*). *Eurasian Journal of Biosciences* **6**: 85-90.

Saghai-Maroof, M.A., Soliman, K.M., Jorgensen R.A. and Allard, R.W. 1984. Ribosomal DNA spacer-length polymorphism in barley: Mendelian inheritance, Chromosomal-location and population dynamics. *Proceedings of National Academy of Sciences USA* **81**: 8014-8018.

Salgotra, R.K., Gupta, B. Reginald, B., Millwood, J., MuthukumarBalasubramaniam and Stewart, C.N. 2012. Introgression of bacterial leaf blight resistance and aroma genes using functional marker-assisted selection in rice (*Oryza sativa* L.) *Euphytica* **187**:313–323.

Samis, K., Bowley, S. and McKersie, B. 2002. Pyramiding Mn superoxide dismutase transgenes to improve persistence and biomass production in alfalfa. *Journal of Experimental Botany* **53**:1343–1350.

Sanchez, A.C., Ilag, L.L., Yang, D., Brar, D.S., Ausubel, F., Khush, G.S., Yano, M., Sasaki, T., Li, Z. and Huang, N. 1999. Genetic and physical mapping of *xa-13* a recessive bacterial blight resistance gene in rice. *Theoretical and Applied Genetics* **98**: 1022–1028.

Sanchez, A.C., Brar, D.S., Huang, N., Li, Z. and Khush, G.S. 2000. Sequence tagged site marker-assisted selection for three bacterial blight resistance genes in rice. *Crop Science* **40**: 792-797.

Septiningsih, E.M., Pamplona, A.M., Sanchez, D.L., Neeraja, C.N., Vergara, G.V., Heuer, S., Ismail, A.M. & Mackill, D.J. 2009. Development of submergence-tolerant rice cultivars: the Sub1 locus and beyond. *Annals of Botany* **103**: 151 – 160.

Shanti, M.L., Shenoy, V.V., Devi, G.L., Kumar, V.M., Premalatha, P., Kumar, G.N., Shashidhar, H.E., Zehr, U.B. and Freeman, W.H. 2010. Marker-assisted breeding for resistance to bacterial leaf blight in popular cultivars and parental lines of hybrid rice. *Journal of Plant Pathology* **92**(2):495–501.

Shin, M.S., Kim, K.Y., Park, H.S. and Ko, J.K. 2011. Breeding for resistance to bacterial blight in rice. *Korean Journal of Breeding* **43**:251–261

Shobha Rani, N., Mishra, B., Prasad, G.S.V., PrasadaRao, U., Subbaiah, S.V., Muralidharan, K. and Pasalu, I.C. 2001. Basmati rice- Heritage of India, DRR Technical Bulletin 2001-02, DRR, Hyderabad, pp:28.

Shobha Rani, N., Pandey, M.K., Prasad, G.S.V. and Sudharshan, I. 2006. Historical significance, grain quality features and precision breeding for improvement of export quality basmati varieties in India. *Indian Journal of Crop Sciences* **1**:29–41

Sidhu, G.S., Khush, G.S. and Mew, T.W. 1978. Genetic analysis of bacterial blight resistance in seventy four cultivars of rice, *Orzo sativa* L. from Indinesia. *Theoretical and Applied Genetics* **53**: 105-111.

Sidhu, G.S., Khush, G.S. and Mew, T.W. 1978. Genetic analysis of bacterial blight resistance in seventy four cultivars of rice, *Orzo sativa* L. from Indonesia. *Theoretical and Applied Genetics* **53**: 105-11.

Singh, S., Sindhu, J.S., Huang, N., Vikal, Y., Li, Z., Brar, D.S., Dhaliwal, H.S. and Khush, G.S. 2001. Pyramiding three bacterial blight resistance genes (*xa5, xa13* and *Xa21*) using marker-assisted selection into indica rice cultivar PR106. *Theoretical and Applied Genetics* **102**:1011–1015.

Singh, A.K., Mohapatra, T., Prabhu, K.V., Singh, V.P., Zaman, F.U., Mishra, G.P., Nandakumar, N., Minu, J., Gopalakrishnan, S., Garg, A., Tyagi, N.K., Prakash, P., Sharma, R.K., Shah, U.S. and Singh, S.K. 2005. Application of molecular markers in rice breeding: progress at IARI. *Euphytica* **187**:449–459. Paper presented at the advances in marker assisted selection workshop, International Rice Research Institute, Manila, Philippines, 21–24 February 2005.

Singh, K., Vikal, Y., Mahajan, R., Cheema, K.K., Bhatia, D., Sharma, R., Lore, J.S. and Bharaj, T.S. 2007. Three novel bacterial blight resistance genes identified, mapped and transfer to cultivated rice *O. sativa* L., pp. 82-84. In The 2nd International Conference on Bacterial Blight of Rice, Nanjing, China.

Song, W.Y., Wang, G.L., Chen, L.L., Kim, H.S., Pi, L.Y., Holsten, T., Gardner, J., Wang, B., Zhai, W.X., Zhu, L.H., Fauquet, C. and Ronald, P.C.1995. A receptor kinase-like protein encoded by the rice disease resistance gene, *Xa21. Science* **270**:1804–1806.

Sonti, R.V. 1998. Bacterial leaf blight of rice: new insights from molecular genetics. *Current Science* **74**:206–212.

Suh, J.P., Yang, S.J., Jeung, J.U., Pamplona, A., Kim, J.J., Lee, J.H., Hong, H.C., Yang, C.I., Kim, Y.G. and Jena, K.K. 2011. Development of elite breeding lines conferring Bph18 gene-derived resistance to brown plant hopper (BPH) by marker-assisted selection and genome-wide background analysis in japonica rice (*Oryzasativa* L.). *Field Crops Research* **120**:215–222.

Suh, J.P., Jeung, P., Ung., Noh, T. and Shin, M.S. 2013. Development of breeding lines with three pyramided resistance genes that confer broad-spectrum bacterial blight resistance and their molecular analysis in rice. *Rice* **6**:5.

Sun, Q., Wu, W., Qian, W., Hu, J., Fang, R. and He, C. 2003. High-qualitymutant libraries of *Xanthomonas oryzae* pv. *oryzae* and *X. campestris* pv. *campestris* generated by an efficient transposon mutagenesis system. *FEMS Microbiology Letters* **226**:145–150.

Sun, X., Cao, Y., Yang, Z., Xu, C., Li, X., Wang, S. and Zhang, Q. 2004. *Xa26*, a gene resistance to *Xanthomonas oryzae* pv. *Oryzae* in rice, encodes an LRR receptor kinase-like protein. *Plant Journal* **37**: 517-527.

Sundarum, R.M., Vishnupriya, M.R., Biradar, S.K., Laha, G.S., Reddy, G.A., Rani, N.S., Sharma, N.P. and Sonti, R.V. 2008. Marker-assisted introgressionof bacterial blight resistance in Samba Mashuri, an elite indica rice variety. *Euphytica* **160**(3): 411-422.

Sundaram, R.M., Vishnupriya, M.R., Laha, G.S., Rani, N.S,, Rao, P.S., Balachandran, S.M., Reddy, G.A., Sharma, N.P. and Sonti, R.V. 2009. Introduction of bacterial blight resistance into Triguna, a high yielding, mid-early duration rice variety. *Biotechnol*ogy **4**:400–407.

Tanksley, S.D. 1989. RFLP mapping in plant breeding: new tools. *Biotechnology***7:** 257–263.

Tautz, D. 1989. Hypervariability of simple sequences as a general source for polymorphic DNA markers. *Nucleic Acids Research* **17**: 6463-71.

Temnykh, S., Park, W.D., Ayres, N., Cartinhour, S., Hauck, N., Lipovich, L., Cho, Y.G., Ishii, T.and McCouch, S.R. 2000. Mapping and genome organization of microsatellite sequences in rice (*Oryza sativa* L.). *Theoretical and Applied Genetics* **100**:697–712.

Toojinda, T., Tragoonrung, S., Vanavichit, A., Siangliw, J.L., Jantaboon, J., Siangliw, M. and Fukai, S. 2005. Molecular breeding for rainfed lowland rice in the Mekong region. *Plant Production Science* **8**(3): 330-333.

Van Berloo, R. 1999 GGT: software for display of graphical genotypes. *Journal of Heredity* **90**:328–329.

Wen, S. and Gao, B. 2011. Introgressing Blast Resistant Gene *Pi-9(t)* into Elite rice Restorer Luhui 17 by Marker assisted selection. *Rice Genomics and Genetics* **2** (4): 708-714.

Williams, C. and Ronald, P.C. 1994. Rapid, homogenization-free isolation of rice DNA for PCR. *Nucleic Acids Research* **22**(10): 1917-1918.

World population clock, 2013.www.census.gov/popclock/

Xiang, Y., Cao, Y., Xu, C. and Li, X. 2006. *Xa3*, Conferring resistance for rice bacterial blight and encoding a receptor kinase-like protein, is the same as*Xa26*. *Theoretical and Applied Genetics* **113**(7): 1347-1355.

Xu, G. W., Magill, C. W., Schertz, K. F. and Hart, G. E. 1994. An RFLP linkage map of Sorghum bicolor (L.)Moench. *Theoretical and Applied Genetics* **89**: 139-145.

Xu, Y. and Crouch, J.H. 2008. Marker-assisted selection in plant breeding: from publication to practice. *Crop Sci*ence **48**: 391–407.

Xu, J., Jiang, J., Dong, J., Ali, J.and Mou, T. 2012. Introgression of bacterial blight (BB) resistance genes *Xa7* and *Xa21* into popular restorer line and their hybrids by molecular marker-assisted backcross (MABC) selection scheme. *African Journal of Biotechnology* **11**(33): 8225-8233.

Yang, Z., Sun, X., Wang, S. and Zhang, Q. 2003. Genetic and physical mapping of a new gene for bacterial blight resistance in rice. *Theoretical and Applied Genetics* **106**:1567-1472.

Yang, B., Sugio, A. and White, F.F. 2006. Os8N3 is a host disease susceptibility gene for bacterial blight of rice. *Proceedings of National Academy of Sciences* **103**:10503–10508.

Ye, G. 2010. Marker-assisted gene pyramiding for cultivar development. *Plant Breeding Review* **33**:219–256.

Yoshimura, A., Mew, T.W., Khush, G.S. and Moura, T. 1983. Inheritance of resistance to bacterial blight in rice cultivar Cas 209. *Phytopathology* **73**: 1409-1412.

Yoshimura, S.A., Yishimura, N., Iwata, McCouch, S.R., Abenes, M.L., Baraoidian, M.R., Mew, T.W and Nelson, R.J. 1995. Tagging and combining bacterial blight resistance genes in rice using RAPD and RFLP markers. *Molecular Breeding* **1**:375–387.

Zhang, G., Angeles, E.R., Abenes, M.L.P., Khush, G.S. and Huang, N. 1996. RAPD and RFLP mapping of the bacterial blight resistance gene *xa13* in rice. *Theoretical and Applied Genetics* **93**: 65-70.

Zhang, Q., Lin, S.C., Zhao, B.Y., Wang, C.L., Yang, W.C.,. Zhou, Y.L., Li, D.Y., Chen, C.B and Zhu, L.H. 1998. Identification and Tagging a new gene for resistance to bacterial blight (*Xanthomonas oryzae* pv. *oryzae*) from *O.rufipogon*. *Rice Genetics Newsletter* **15**: 138-141.

Zhang, Q. 2005. Highlights in identification and application of resistance genes to bacterial blight. *Chinese Journal of Rice Sciences* **19**:453–459**.**

ABSTRACT

Title of Thesis	:	Marker Assisted Selection for Introgression of Bacterial Blight (BB) resistance genes in rice (*Oryza sativa* L.)
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Admission No.	:	2008BS9D
Title of Degree	:	**Doctor of Philosophy**
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Key words: Bacterial blight, *Xanthomonas oryzae*, Simple sequence repeats, Marker assisted selection

The present investigation was undertaken to introgress the major BB resistance genes (*Xa21, xa13* and *xa5*) into BB susceptible rice variety CSR-30, from BB resistant donor varieties Pusa Basmati -1460 (having two BB resistance genes *Xa21* and *xa13*) and IRBB-60 (having three BB resistance genes *Xa21, xa13* and *xa5*) through marker assisted selection.

Crosses were made between CSR-30 x Pusa Basmati -1460 and CSR-30 x IRBB-60. Foreground selection was carried out in F_1 plants of both the crosses using specific STS markers pTA248, RG136 and RG556 linked to *Xa21, xa13* and *xa5,* genes respectively. In cross CSR-30 x Pusa Basmati -1460, four F_1 seeds were harvested. Foreground selection was carried out in which two plants were found to have both the BB resistance genes *Xa21*and *xa13.* These F_1 plants were grown in the net house and selfed to get F_2 seeds. In cross CSR-30 x IRBB-60, 6 F_1 seeds were harvested. Foreground selection was carried out and three plants were found to have three resistance genes *Xa21, xa13* and *xa-5*. Backcrosses of these positive F_1 plants were made with CSR-30 and the BC_1F_1 seeds of this cross harvested. These BC_1F_1 seeds were selfed to produce BC_1F_2 seeds. In foreground selection, 10/250 BC_1F_2 plants were found to have all the three resistance genes *Xa21, xa13* and *xa-5*. For background selection, 300 SSR primers were used to identify the polymorphism between parental genotypes Pusa Basmati-1460 and CSR-30 and between IRBB-60 and CSR-30, out of which 72 and 104 SSR markers produced polymorphic alleles between the parents Pusa Basmati-1460 and CSR-30 and parents IRBB-60 and CSR-30, respectively. Cluster tree analysis revealed that the three gene pyramided BC_1F_2 rice genotypes and the recipient parent CSR-30 fell in one group with two major sub-groups and the donor parent IRBB-60 remained as a separate group. The percentage recovery of recurrent parent genome in the three-gene pyramided BC_1F_2 genotypes ranged from 44.2% to 78.9% using GGT software. The pyramided lines exhibited very high level of resistance to bacterial blight disease when artificially inoculated with *Xanthomonas oryzae* strain isolated from the BB infected fields of RRS, Kaul, CCSHAU, Hisar. The agro-morphological traits of the three-gene pyramided BC_1F_2 genotypes were found to be either superior or on par with the recurrent parent CSR-30. Genotype G-3 showed the best agronomic features with maximum recovery of recurrent parent genome (RPG) 78.9% followed by G-169 with RPG 76.2% followed by line G-7 with RPG 72.5%. The plants having maximum recurrent parent genome were backcrossed with the recurrent parent CSR-30 and BC_2F_2 seeds were harvested. It is suggested that positive F_2 seeds of cross CSR-30 x Pusa Basmati -1460 should be backcrossed with CSR-30 for further work. However, in cross CSR-30 x IRBB-60, BC_2F_2 genotypes should be further evaluated for agronomic traits. This work demonstrates the successful application of MAS for targeted introgression of multiple resistance genes into premium quality rice variety CSR-30.

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