Identification and Molecular mapping of novel Bacterial leaf blight resistance genes in rice

Thesis submitted to the University of Hyderabad for the award of the degree of Doctor of Philosophy in Plant Sciences

K. Sujatha
(04LPPH08)

(Supervisor: Dr. B. Mishra)

Dept. of Plant Sciences
School of Life Sciences
University of Hyderabad, Hyderabad - 500 046
Andhra Pradesh, India

&

Directorate of Rice Research
Rajendranagar, Hyderabad – 500 030
Andhra Pradesh, India

September 2009

Sher - e - Kashmir
University of Agricultural Sciences & Technology of Jammu
Administrative Building, Chatha, Jammu(J&K) -180 009

Dr. B. Mishra
Vice Chancellor

CERTIFICATE

This is to certify that K. Sujatha carried out the research work embodied in the present thesis entitled "Identification and Molecular mapping of novel Bacterial Leaf Blight (BLB) resistance gene(s) in rice" and submitted for the degree of Doctor of Philosophy was accomplished for the full period prescribed under Ph.D. ordinances of the University, under my supervision at the Directorate of Rice Research, Rajendranagar, Hyderabad-500030 and in the Department of Plant Sciences, School of Life Sciences, University of Hyderabad, and I declare to the best of my knowledge that no part of this thesis was earlier submitted in part or in full, for the award of any research degree or diploma of any University.

(B. Mishra)
Supervisor

(Prof. P.B. Kirti)
Co-Supervisor

Head
Department of Plant Sciences

Dean 24/09/09
School of Life Sciences

Telephones : 91-191-2263714 (Office) 91-191-2471082 (Res)
Fax 91-191-2262073
E-mail: vc@skuast org, b9mishra@gmail com

DECLARATION

I hereby declare that the thesis entitled "Identification and Molecular mapping of novel Bacterial leaf blight resistance genes in rice" submitted to University of Hyderabad for the degree of Doctor of Philosophy in Plant Sciences is the result of original research work done by me at the Directorate of Rice Research (DRR), Hyderabad. I also further declare that no part of the thesis has been submitted for any other degree or diploma earlier.

K Sujatha

(K. Sujatha)
04LPPH08
Department of Plant Sciences
School of Life Sciences
University of Hyderabad
Hyderabad – 500046

Dedicated to

The holy feet of Lord Shri venkareshwara swamy

and

My dear parents

My brother

My husband

My little astronaut

Who have painstakingly helped me climb the ladders of my life

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Sujatha

(Sujatha)

List of Figures

List of Tables

List of important abbreviations

µ	:	Micro
^{0}C	:	Degree centigrade
cM	:	Centimorgan
cm	:	Centimeter
DNA	:	Deoxy ribo nucleic acid
dNTP	:	Deoxynucledotide 5' triphosphate
dATP	:	Deoxyadenosine 5' triphosphate
dCTP	:	Deoxycytidine 5' triphosphate
dGTP	:	Deoxyguanosine 5' triphosphate
dTTP	:	Deoxythymidine 5' triphosphate
kb	:	Kilo base pair
M	:	Molar
MAB	:	Marker-assisted breeding
MAS	:	Marker-assisted selection
Mb	:	Mega base pair
min	:	Minute
ml	:	Milli litre
mM	:	Milli molar
ng	:	Nanogram
PCR	:	Polymerase chain reaction
SSR	:	Simple sequence repeat
Xoo	:	*Xanthomonas oryzae* pv. *Oryzae*

Contents

Chapter 1

Introduction

Chapter I – Introduction

Rice (*Oryza sativa* L.) is the most important crop of India and is grown all over the country in diverse ecosystems. It is agronomically and nutritionally, most important food crop of the world serving as the staple food for nearly 2.4 billion people. It plays a pivotal role in Indian economy being staple food for two thirds of the population. With 44.62 million hectares planted to rice, India ranks first in area and second in production (96 million tons). 21% of the total calorie requirement in the Indian diet is being supplied by rice. It is a member of the Gramineae family, has a genome size of 0.45 $X10^9$ bp (Arumunagathan and Earle, 1991), which is one tenth the size of the human genome and is a model system for cereal genome analysis. Different genotypes of rice are adapted to a wide range of environmental conditions from tropical flooding to temperate dry land, making it an excellent example for real-life, adaptive responses.

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The production of rice in India has shown an increasing trend from 83 million tonnes to 93 million tonnes during 1997-98 to 2001-02 (http://www.fao.org/rice2004/en/pdf/nigam.pdf), and is presently around 96 million tonnes. But future projections indicate that the current levels of production may not be sufficient to feed the ever increasing population in our country. In order to meet the food demands for the future, concerted efforts are required to increase the rice productivity and minimize production losses due to pests and diseases. Even though the introduction of high yielding varieties (HYVs), development and adoption of modern rice production technologies have led to progressive growth in rice production, it also inadvertently increased the genetic

⊕ Rice, member of Gramineae family, has a genome size of 0.45 $X10^9$ bp (A.... 1991) which is one tenth the. . . . . genome analysis.

vulnerability of the crop to pests and diseases resulting in serious outbreak of pest and disease epidemics in several countries (Khush and Virmani, 1985). The productivity of rice is being affected by a number of biotic and abiotic stresses. Among the biotic stresses, bacterial leaf blight (BLB) is an important disease that results in significant yield reduction worldwide. The disease, in its severe form, is known to cause yield losses ranging from 74 to 81% (Srinivasan and Gnanamanickam 2005).

Bacterial leaf blight (BLB) is a vascular disease resulting in a systemic infection that produces tannish-grey to white lesions along the veins. Symptoms are observed at tillering stage, disease incidence increases with plant growth, peaking at the flowering stage (Gnanamanickam *et al.*, 1999). BLB is characterized by yellow lesions with wavy margins on leaf blades that may extend to the sheath. These lesions acquire a whitish straw color over a period of time. The severity and significance of damages caused by infection have necessitated the development of strategies to control and manage the disease, so as to reduce crop loss and to avert an epidemic. In the absence of effective chemical or other control agents against BLB pathogens, host resistance has gained enormous importance in controlling this disease.

Till now 31 resistance genes have been identified. Employing host resistance has been widely resorted for bringing control over the disease. Many major and minor genes have been identified and near isogenic lines (NILs) have been developed with a single major gene in a susceptible genomic background (IR24), to analyze virulence characteristics of the existing pathogen population. A gene-for-gene relationship was found in the interaction of the pathogen with every major resistance gene. In some areas, although a single gene confers resistance to the existing pathogen population, the large-

scale use of this gene results in the breakdown of resistance. In rice breeding programmes at International Rice Research Institute (IRRI) and in national rice improvement programmes in the Philippines, Indonesia and India, resistance genes (*Xa4, xa5,xa13, Xa7* and *Xa21)* are being transferred to commercially important rice varieties.

Resistance breeding is considered as the most economical and eco-friendly strategy for achieving disease resistance and yield stability. Introgression of the "R" gene(s) and gene pyramiding efforts through conventional breeding strategies based on plant phenotype selection are time consuming, resource intensive and sometimes inconclusive, necessitating the deployment of more efficient and accurate phenotyping methodologies. Molecular markers can support classical breeding in crop plants by saving time and labour in breeding programmes. Of the several DNA markers available, microsatellites or SSRs (simple sequence repeats) are considered to be ideal molecular genetic markers. Microsatellite sequences are abundantly present in eukaryotic genomes and have, therefore, been used for genome analysis of many crop plants including rice (Gupta *et al.*, 1994; Ramakrishna *et al.*, 1994; 1995). Presence of a high level of allelic diversity at these SSR loci makes them informative and valuable markers, with many applications in agriculture.

Widespread cultivation of varieties with single BLB resistance genes has led to predominance of *Xoo* races that can overcome the gene (Mew *et al.*, 1992). The deployment of rice cultivars that have multiple BLB resistance genes is expected to lead to more durable resistance. Through marker-assisted gene pyramiding two or more resistance genes like *Xa21*, *xa13, xa5* and *Xa4* have been incorporated in the genetic background of elite varieties (Huang *et al.*, 1997, Sanchez *et al.*, 2000, Singh *et al.*, 2001,

Joseph *et al.*, 2004, Sundaram *et al.*, 2008). Even though gene pyramid combinations like *Xa21* + *xa13* + *xa5* or *Xa21* + *xa5* or *Xa21* + *xa13* have been observed to possess high level of resistance against multiple isolates of *Xoo*, the durability of resistance in such gene pyramid lines has not been validated so far. In order to enhance the durability of resistance, it is desirable to identify and characterize new genes from wild relatives and durable resistant cultivars of rice so that they could be deployed along with *Xa21* or *xa13* or *xa5* in elite rice varieties. Towards this objective, the present research work entitled "Identification and Molecular mapping of novel Bacterial Leaf Blight resistance genes in rice" has been designed and carried out with the following objectives:

Objectives

1. To screen wild rice accessions and bacterial leaf blight resistant donor varieties in order to identify donors possessing novel resistance.
2. To investigate the inheritance of resistance in the identified genotypes and establish the novelty of the resistance genes
3. To tag and map the novel resistance gene(s) using molecular markers and to validate the tightly linked markers identified in the alternate populations
4. To identify the putative candidate genes possibly responsible for resistance in the donors through *in silico* analysis

Chapter 2

Review of Literature

Chapter II – Review of Literature

2.1 History of occurrence of the disease

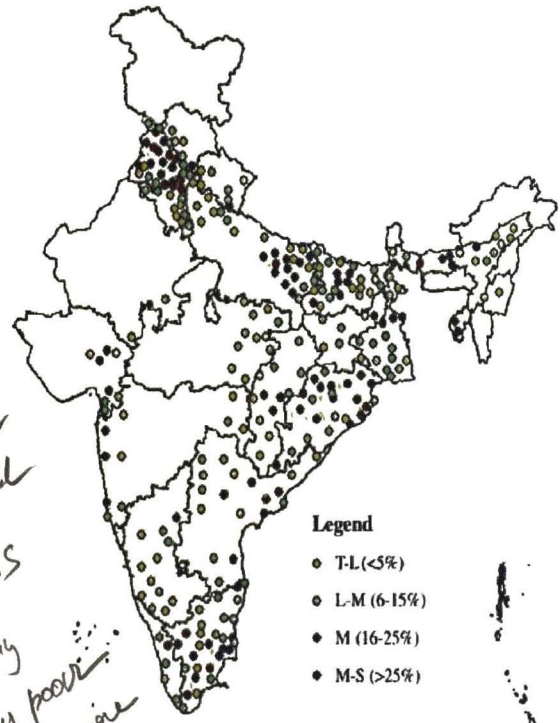
Bacterial blight of rice caused by *Xanthomonas oryzae* pv. *oryzae* is one of the most destructive diseases of rice in majority of the rice growing countries especially in Asia. Though the disease has been known in Japan since 1884, its bacterial nature was established only in 1922 (Ishiyama, 1922). Subsequently, the disease was reported from most of the rice growing countries. In India, the first authentic report of typical bacterial blight symptoms was made by Bhapkar and co-workers in 1960. The disease broke out in epidemic form in Shahabad district of Bihar during 1963 and since then it has spread fast to other rice growing regions of the country often causing considerable yield loss especially in high yielding varieties during rainy season. *Xanthomonas oryzae* pv. *oryzae* causing bacterial blight of rice has been listed as bioterrorism agent by the U.S. Department of Agriculture's Animal and Plant Health Inspection Service (APHIS) necessitating strict bio-safety measures to combat the spread of this disease.

In India, bacterial blight is considered as a serious production constraint especially in irrigated and rainfed lowland ecosystem. In Punjab and Haryana states of India, major epidemics occurred in 1979 and 1980; severe kresek was observed and total crop failure was reported (Mew, 1987).The disease was again reported in epidemic form during 1998 in Pallakad district of Kerala and since then it has become endemic in that region (Priyadarishini and Gnanamanickam, 1999).

2.2 Distribution of the disease

This disease is a major problem in kharif season (wet season) crop in rice growing regions of Punjab, Haryana, Uttaranchal, Bihar, West Bengal, Tripura, Assam, Tamil Nadu, eastern Uttar Pradesh and Andaman and Nicobar islands; coastal areas of Andhra Pradesh and Kerala and parts of Maharashtra, Chhattishgarh, Gujarat, Himachal Pradesh and Karnataka (Figure 1).

Figure 1: Map showing the distribution and severity of BLB in India



2.3 Nature and extent of damage

The extent of yield loss depends on the growth stages of the crop at which it is infected, the level of susceptibility of the cultivar, season, climatic condition and level of nitrogen fertilizer applied. This disease is a problem of rainy season (kharif). In general, late infection results in only slight reduction in yield, but the early infection leading to 'kresek' (wilt) causes far heavier losses and sometimes, the entire crop may be lost. Generally the stage between maximum tillering and booting is highly sensitive to disease infection, as it affects the yield significantly in terms of filled grain weight per hill and total yield. In a study, it was observed that when the disease occurred at the maximum tillering stage and onwards, the yield reduction approached 70% highly susceptible cultivars like TNI and , up to 20% in field tolerant cultivars like IR20 where as post-flowering occurrence of the disease resulted in 22% and 9%yield reduction in TN1 and IR20 respectively. In a study of extent of yield loss in 19 different cultivars. ~~the yield loss~~ varied from 6.12% in CR-44-35 to 74.2 % in the cultivar Bala. (Reddy and Kauffman 1973)

Depending on the stage of infection and severity of the disease under natural condition, the extent of yield loss has been reported to vary from 6-60% (Srivastava *et al.*, 1966). Some other workers have reported yield loss up to 50% depending on the variety, severity and stage of infection (Rao and Kauffman, 1971).

2.4 The Pathogen

Xanthomonas oryzae pv. *oryzae (Xoo)* is a gram –ve, non-spore forming and rod shaped bacterium which is motile with a single polar flagellum. Individual cells vary in length approximately from 1-2 µm and in width from 0-0.7µm. Colonies on solid culture medium are round, convex, mucoid and yellow in colour due to production of a non-

diffusible yellow pigment called xanthomonadin (a brominated, aryl polyene pigment), characteristics of the genus. The bacterium produces copious capsular extra-polysaccharide (EPS), which is important in the formation of droplets of bacterial exudates from the infected leaves, providing protection from desiccation and aiding in wind-and rain-borne dispersal.

2.4.1 Symptoms

This is a typical vascular disease and has two distinct phases of symptoms.

Figure2: Symptoms of Bacterial leaf blight

A. Kresek phase

B. Leaf blight phase

Kresek Phase

This is the most destructive phase of the disease in the tropics (Figure-2A) resulting from early systemic infection in the nursery or from seed infection. The leaves roll completely, droop, turn yellow or gray and ultimately the tillers wither away. In severe cases, the affected hills may be completely killed

Leaf blight Phase

Leaf blight phase is the most common phase (Figure-2B). The symptom starts as water soaked lesions on the tip of the leaves and increases in length downwards. Initially, the lesions are pale green in colour and later turn into yellow to straw colored stripes with wavy margins. As the disease advances, the lesion covers the entire leaf blade, turns white and later becomes grayish or blackish due to growth of various saprophytic fungi.

2.4.2 Virulence spectrum of the pathogen

Many studies have revealed the existence of variation in the virulence of the pathogen. Variation, in terms of the host-pathogen means a potential shift of the pathogen to match the resistance of a currently grown variety. X.*oryzae* pv. *oryzae* is highly variable and the dynamic nature of its pathogenicity is demonstrated by the continuous appearance of new pathogenic variants. Occurrence of highly virulent forms of X.*oryzae* pv.*oryzae* was one of the reasons of bacterial blight epidemic in North-Western India during 1979-1980.

Earlier work at All India Coordinated Rice Imrpovement Programme (AICRIP) classified the Indian isolates into eight groups. However, analysis of populations from north-western India revealed the presence of 11 virulence genotypes in X. *oryzae* pv. *oryzae* based on differentials (Gupta *et al.*, 1986). The number of races/pathotypes in a pathogen population depends on the differential systems used for recording the pathogenic reaction. In an analysis of 150 isolates of X. *oryzae* pv. *Oryzae* collected from 25 different locations, the isolates were broadly grouped into two pathotypes- Pathotype-I and Pathotype-II. Pathotype- I was avirulent on DV 85 but virulent on Cempocelak and Java-14 whereas pathotype-II was virulent on DV 85 but avirulent on Cempocelak and Java-14. Pathotype- I was further divided into two subgroups i.e. pathotype Ia and Ib based on their avirulence and virulence, respectively on the rice cultivar IR 20 (Reddy and Reddy, 1992). It was also reported that pathotype-I was present in Punjab, Eastern U.P., Tamil Nadu and Kerala, while pathotype- II was restricted to West Bengal.

2.4.3 Variability in the pathogen

The bacterial blight pathogen, *Xanthomonas oryzae* pv. *oryzae* is a highly variable and the dynamic nature of the pathogen is demonstrated by the continuous appearance of new pathogenic variants. Many workers have done classical pathotyping using a set of differentials (Mew, 1987). In addition to the classical studies, a number of molecular tools have been developed for studying the pathogen variability at the genetic level. Restriction Fragment Length Polymorphism (RFLP) has been used for analyzing the genetic diversity and phylogenetic relationships of *Xoo* in Asia (Leach *et al.*, 1992; Nelson *et al.*, 1994; Adhikari *et al.*, 1995; Ardales *et al.*, 1996). Using RFLPs, Ardales *et al.*, (1996) observed a high degree of genetic differentiation between the pathogen populations among sites, indicating substantial geographical differentiation between the pathogen populations in different ecosystem. Vera Cruz *et al.*, (1996) compared the efficiency of RFLP and rep-PCR in detecting the variation in the pathogen population and revealed that rep-primers detected the maximum polymorphism. George *et al.*, (1997) used two outwardly directed primers (p JEL1 and p JEL2) complementary to the sequences in IS1112, a repetitive element isolated from *Xoo* to fingerprint 71 bacterial isolates using polymerase chain reaction. Kosawang *et al.*, (2006) used amplified fragment length polymorphism fingerprinting to differentiate the genetic diversity among the isolates of *Xanthomonas oryzae* pv. *oryzae*.

2.4.4 Mode of entry and Infection

The bacterium gains entry into the host through wounds or natural openings called hydathodes that are densely distributed along the edge of the upper surface, predominantly near the leaf tips. Wounds on rice leaves caused by rain storms, strong

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2.4.4 Mode of entry and Infection

The bacterium gains entry into the host through wounds or natural openings called hydathodes that are densely distributed along the edge of the upper surface, predominantly near the leaf tips. Wounds on rice leaves caused by rain storms, strong

winds, insect damage and intercultural operations are common and are favourable avenues for entry. In the tropics, the leaf tips of rice seedlings are often cut before transplanting. In addition, roots are broken when the seedlings are pulled from the seedbed. The broken roots attract the bacterium. These are the important points of infection and are partly responsible for the kresek symptoms that are found in the tropics. The bacterium multiplies in the epitheme (loosely packed parenchymatous tissue) into which the vessels open. When sufficient bacterial multiplication has taken place in the epitheme, some of the bacteria invade the vascular system and some ooze out from the water pore.

2.5 Disease Scoring

Host plant resistance to bacterial blight pathogen can be measured using different parameters like disease incidence, disease severity, area under disease progress curve (AUDPC) and apparent infection rate (r). Among these, disease incidence (percentage of hills, plants or leaves diseased) and disease severity (lesion size or percentage of leaf area diseased) are most commonly adopted in research and evaluating breeding materials.

For assessing resistance based on the percentage of leaf area affected, the standard evaluation system (SES) developed at International Rice Research Institute (IRRI) is usually followed .

Scoring based on the absolute lesion length for characterizing host reaction as resistant (lesion length less or equal 4 cm), moderately resistant (lesion length 4.1-8 cm) and susceptible (lesion length> 8 cm) is also followed. Some workers categorize their cultures as resistant (lesion length less or equal 3 cm), moderately resistant (lesion length 3-6 cm), moderately susceptible (lesion length 6-9 cm) and susceptible (lesion length> 9 cm) (Chen *et al.*, 2000)

2.6 Strategies for management of bacterial blight

No single effective control measure is available for the control of the disease, and integrated measures are therefore suggested. These include the use of resistant cultivars, avoidance of flooding or deep water in the nursery, removal of primary sources of inoculum, spraying with chemicals in both nursery and field, avoidance of the use of nitrogenous fertilizers etc. Presently, the control of the disease is based on nutrition management and therefore host plant resistance is considered as the most effective mode to control the bacterial leaf blight disease.

2.6.1 Cultural control

Cultural practices useful for BB control vary depending on the location and disease incidence records. At the nursery stage, methods include seed disinfection proper nursery drainage, and removal of diseased plants, weeds and debris. Prior to transplanting, fields may be disinfected by burning rice straw left from the previous season. Weeds are removed from canals and ridges in order to reduce natural habitats for the pathogen and its dispersal through irrigation water. At the paddy field stage, judicious fertilization and proper plant spacing are the most recommended cultural methods of control (Mizukami and Wakimoto, 1969). Fertilization must avoid an excess of nitrogen as it stimulates rapid vegetative growth of the plant, which favours disease development. Application of fertilizers rich in potassium and phosphorus, as well as application of agrochemicals at the maximum tillering to booting stages or after a typhoon or a severe flood are common practices (Ho and Lim, 1979; Mizukami and Wakimoto, 1969).

2.6.2 Chemical control

Chemical control of BB in rice fields began in the 1950s with the preventative application of Bordeaux mixture (hydrated lime and copper sulfate) and the testing of several antibiotics, mercuric and copper compounds. Laboratory tests determined that streptomycin derivatives and mercuric compounds were most effective, but they were found to damage rice grains when sprayed at the heading stage in the field (Mizukami and Wakimoto, 1969). In the 1960s, different kinds of agrochemicals were developed from repeated field trials and made available on a large commercial scale, mostly in Japan. They were based on L-chloramphenicol, nickel-dimethyldithiocarbamate, dithianon and fentiazon. Most were unreliable, however, owing to variability in sensitivity among the pathogen population (Gnanamanickam *et al.*, 1999; Mizukami and Wakimoto, 1969; Ou, 1973). Although seed transmission of the disease is an uncertain source of primary inoculum, disinfection of rice seeds with mercuric compounds, antibiotic solutions or hot water is practised in several countries in tropical Asia. In temperate regions, chemical control of BB in nurseries and paddy fields includes the application of probenazole to the paddy water before and after transplanting the seedlings, in order to inhibit bacterial multiplication and prevent or retard the disease. Other chemicals such as tecloftalam, phenazine oxide and nickel dimethyldithiocarbamate are sprayed directly on plants (Goto, 1992; Mizukami and Wakimoto, 1969). However, chemical control of BB in the tropical monsoon climate of Asia is impractical, and no truly effective bactericide is commercially available for disease control (Lee *et al* ., 2003; Ou, 1973).

2.6.3 Biological control

It is an environmentally friendly and cost effective alternative to chemical control. Bacterial antagonists of Xoo have received particular attention as bio control candidates, largely because of their rapid growth, easy handling and effective colonization of the rhizosphere. In India, about 40 bacterial isolates antagonistic to Xoo were identified through plate and field assays. Among those antagonists native strains of the rice-associated rhizobacteria *Pseudomonas fluorescens* and *P. putida* strain V14i (also used in biocontrol of the rice sheath blight pathogen *Rhizoctonia solani*) significantly suppressed BB severity when sprayed on leaves (Sivamani *et al.*, 1987). Different species of Bacillus have been employed as seed treatment before sowing, root dips prior to transplanting and foliar sprays in the fields. In at least one study, BB was suppressed by almost 60%, and plant height and grain yield increased by two-fold. Although the mechanisms of BB suppression are not known, a recent investigation of bio control of the rice sheath blight disease has suggested that a rice systemic resistance response to the agents may be involved, as has been observed in other systems. Despite promising results such as these, biological agents have not seen widespread use in the control of BB. As chemical control is unavailable or impractical, breeding and deployment of resistant cultivars carrying major resistance (R) genes has been the most effective approach to controlling BLB.

2.6.4 Host plant resistance

A large number of studies conducted in several countries have identified the presence of 31 major genes (Laha *et al.*, 2009) conferring resistance to various races of the pathogen. These genes are designated as *Xa-1* to *Xa-31* (Table 1).

Table 1: List of genes conferring resistance to Bacterial leaf blight

Gene	Chromosome*	Nature of resistance†	Source/vaiet-ies analyzed	Features	References
Xa1	4	Dominant	Kogyoku	Cloned, NBS-LRR type; expression induced by wounding and bacterial inoculation; resistance to Japanese race I; ineffective against all Philippine races.	(Yoshimura et al., 1998)
Xa2	4	Dominant	Tetep	Linked to Xa1; possibly receptor-like kinase type; resistant to Japanese race II; susceptible to all Philippine races.	(Oryzabase, 2006)
Xa3	11	Dominant	Wase Aikoku 3, Java-14, Chugoku-45, Cempocelak	Other names: Xa4b, Xa6, Xa9, Xaw; resistance to Philippine races 1, 2, 4, 5 and all Japanese races at booting (adult) stage only; resistance to Philippine race 3 at all growth stages.	Ogawa and Yamamoto 1986; Oryzabase, 2006 Sun et al., 2004)
Xa4	11	Dominant	TKM 6, IR 20, IR 22	Linked to Xa26; resistance to Philippine races 1, 4, 5, 7, 8, and 10; most widely used before defeat in 1970s	(Wang et al., 2002)
xa5	5	recessive	Aus Boro lines (e.g. DZ192), DV 85, DV 86, DZ 78	Cloned; encodes TFIIA gamma subunit; resistance to Philippine races 1, 2, 3, 5, 7, 8, 9, and 10.	(Iyer and McCouch, 2004)
Xa7	6	Dominant	DV85, DV 86, DZ 78	Resistance to Philippine race 1 at booting stage and to Philippine races 2, 3, 5, 7, 8, and 10 at all growth stages.	Porter et al., 2003)
xa8	7	recessive	PI231129	Resistance to Philippine races 5 and 8.	(Sidhu et al., 1978; Singh et al., 2002)
Xa10	11	Dominant	Cas 209	Linked to Xa4; resistance to Philippine races 2, 5, and 7.	(Oryzabase, 2006; Yoshimura et al., 1983)
Xa11	Not determined	Dominant	IR8, IR944	Resistance to Japanese races IB, II, IIIA and V; ineffective against all Philippine races.	(Mew, 1987; Oryzabase, 2006)
Xa12	4	Dominant	Kogyoku, Tetep, Java-14	Also known as Xakg; resistance to Japanese race V.	(Mew, 1987; Oryzabase, 2006)
xa13	8	recessive	BJ1, Chinsurah Boro II	Cloned; homologous with the Medicago truncatulaMtN3gene; role in pollen development, resistance	(Chu et al., 2006)

Gene	Chromosome*	Nature of resistance†	Source/vaiet-ies analyzed	Features	References
				to Philippine race 6.	
Xa14	4	Dominant	TN1	Resistance to Philippine races 5 and 8.	(Oryzabase, 2006)
xa15	Not determined	recessive	M41, a Harebare mutant line	Created by thermal neutron irradiation of seed; wide spectrum of resistance to Japanese races.	(Gnanamanickam et al., 1999; Nakai et al., 1988)
Xa16	Not determined	Dominant	Tetep	Resistance to Japanese isolates H8581 and H8584.	(Oryzabase, 2006)
Xa17	Not determined	Dominant	Asominori	Resistance to Japanese isolate H8513.	(Oryzabase, 2006)
Xa18	Not determined	Dominant	IR24, Toyonishiki	Resistance to Burmese isolates BM8417 and BM8429; ineffective against all Philippine races.	Oryzabase, 2006)
xa19	Not determined	recessive	XM5	Created by mutagenesis (N-methyl-N-nitrosourea); resistance to Philippine races 1, 2, 3, 4, 5, and 6.	(Lee et al., 2003; Oryzabase, 2006)
xa20	Not determined	recessive	XM6	Created by mutagenesis (N-methyl-N-nitrosourea); resistance to Philippine races 1, 2, 3, 4, 5, and 6.	(Lee et al., 2003; Oryzabase, 2006)
Xa21	11	Dominant	*O longistaminata*	Cloned; receptor-like kinase type; developmentally regulated; resistance only at adult stage to Philippine races 1, 2, 3, 4, 5, 6, 7, 8 and 9.	(Song et al., 1995)
Xa22	11	Dominant	Zhachanglong	Linked to Xa26; broad spectrum resistance.	(Oryzabase, 2006; Sun et al., 2004)
Xa23	11	Dominant	*O. rufipogon*	Strong resistance at all growth stages to all Philippine races and most Japanese and Chinese races.	(Zhang et al., 1998, 2001)
Xa24	Not determined	recessive	DV86, DV85, Aus 295	Resistance to Philippine race 6.	(Khush and Angeles, 1999)
Xa25(a)	4	Dominant	HX-3, a somaclonal mutant of Minghui 63	Resistance to Philippine races 1, 3, and 4, and to several Chinese races.	(Gao et al , 2001)
Xa25(b)	12	Dominant	Minghui 63	Resistance to Philippine race 9.	(Chen et al., 2002)
Xa26	11	Dominant	Minghui 63	Cloned; receptor-like kinase; linked to Xa4and Xa3; broad-spectrum resistance to Philippine and Chinese races; affected	(Sun et al , 2004; Yang et al , 2003)

Gene	Chromosome*	Nature of resistance†	Source/vaiet-ies analyzed	Features	References
				quantitatively and qualitatively by genetic background of the host cultivar.	
Xa27	6	Semi Dominant	*O. minuta*	Cloned; no informative sequence similarities; resistance to Philippine races 2 and 5; involves induction by the pathogen; developmentally regulated or a dosage effect in the cv. CO39 genetic background.	(Gu et al., 2004, 2005; Lee et al., 2003)
xa28	Not determined	recessive	Lota Sail	Resistance to Philippine races 2 and 5.	(Lee et al., 2003)
Xa29(t)	1	Dominant	*O. officinalis*	*Xa29* is a tentative designation; not fully characterized.	(Tan et al., 2004)
Xa30(t)	4	Dominant	*O. nivara*	*Xa30* is a tentative designation	Cheema et al., 2008
Xa31(t)	4	Dominant	Zhachanglong	*Xa31*(t) is a tentative designation	Wang et al., 2008
Xa32(t)	Not determined	Dominant	*O. brachyantha*	*Xa32(t)* is a tentative designation	DRR,unpublished report
Xa33(t)	7	Dominant	*O. nivara*	*Xa33(t)* is a tentative designation	DRR,unpublished report
Xa34(t)	Not determined	recessive	*O. rufipogon*	*Xa34(t)* is a tentative designation	DRR,unpublished report

Courtesy: Nino-Liu et al., 2006 (Mol. Pl. Path., 7(5) :303

As chemical control is unavailable or impractical, breeding and deployment of resistant cultivars carrying major resistance (R) genes has been the most effective approach to controlling BLB. Till date, 31 BB resistance genes have been identified from diverse sources (Nino-Liu *et al.*, 2006; Cheema *et al.*, 2008; Wang *et al.*, 2008). Majority of these genes have been identified from cultivated rice *Oryza sativa;* but some are from related wild species like O. *longistaminata,* O. *rufipogon,* O. *minuta,* o. *officinalis* and O. *nivara.* Most of the 'R' genes are dominant in nature (e.g. *Xa21)* while some are recessive (e.g. *xa13).* Some of the 'R' genes are effective only in adult plants (e.g.*Xa21)* while others do not seem to be developmentally regulated. Some of the 'R' genes provide wide spectrum resistance against multiple isolates/strains (e.g. *xa13, Xa21, Xa23)* while others are narrow in their protective activity (e.g. *Xal).*So far, six BB resistant genes *viz., Xal, xa5, xa13, Xa21, Xa26 and Xa27,*have been cloned using different cloning strategies. Most of these 'R' genes to BB have been introgressed into the background of the susceptible indica cultivar IR 24 and are available as near isogenic lines (NILs).

These genes are available as near isogenic lines (IRBB lines). As of now, more than 30 bacterial leaf blight resistance genes have been identified (Laha *et al.,* 2009). Some of these genes (e.g. *Xa-4*) have been used widely in rice breeding through traditional breeding programme and these varieties are widely grown. A majority of the resistance genes are dominant in nature while a few like *xa5, xa8, xa9* and *xa13* are recessive in nature. *Xa21*, a dominant gene introgressed from *Oryza longistaminata* into *Oryza sativa* (IR24 background), located on chromosome 11 confers broad spectrum resistance to most isolates of *Xanthomonas oryzae* pv. *oryzae*, making it a valuable gene for resistance plant breeding. Another dominant gene *Xa23* introgressed from *O.*

rufipogon provides strong resistance to all the races of the pathogen in Philippines and Japan (Zhang *et al.*, 1998, 2001). Many of these genes are tagged by closely linked markers. These markers can be used for marker assisted selection (MAS) in rice breeding. Gene diversification is one of the approaches to sustain the resistance for a long period and this can be achieved by scouting genes, either from cultivated germplasm or wild species (Vikal *et al.*, 2004).

2.7 Wild Rice- A Reservoir of Resistance genes

Intense selection pressure for specific traits such as yield, due to domestication and modern breeding practices, has resulted in the narrowing of the genetic base of rice thus rendering it vulnerable to epidemics and disasters to several biotic and abiotic constraints. Although wild and unadapted germplasm is less desirable compared to high yielding varieties for its growth and yield potential, it is a unique reservoir of several useful traits. They are the unique sources of genes which confer resistance to several biotic and abiotic stresses such as *Xa21* and *Xa23* for bacterial leaf blight (Song *et al*, 1995; Zhang *et al.*, 1998), *Pi40* for blast (Jeung *et al.*, 2007), and *RTSV* for rice tungro virus (Sebastian *et al.*, 1996), *Gm1* and *Gm2* for gall midge (Himabindu *et al.*, 2007), *bph19* for brown plant hopper (Chen *et al.*, 2006), *Grh5* for green leafhopper (Fujita *et al.*, 2006), QTL for cold tolerance (Lou *et al.*, 2007), *qST1* and *qST3* for salt stress (Lee *et al.*, 2007) and *sub1A* for submergence tolerance (Neeraja *et al.*, 2007). Such agronomically important traits with resistance genes when incorporated into commercially important rice varieties will have an enormous impact on the productivity of rice. Using biotechnological tools several hybrids have been produced between *O. sativa* and many wild species (Khush *et al*, 1993).

On the basis of ease of gene transfer, wild species *O. nivara, O. rufipogon, O. glumaepatula, O. meridionalis, O. breviligulata, O. longistaminata* and the cultivated *O. sativa* and *O. glaberrima* constitute the primary gene pool. They share the AA genome and gene transfer can be accomplished through conventional hybridization and selection procedures. Species belonging to the *O. officinalis* complex constitute the secondary gene pool. Crosses between *O. sativa* and the species of this complex can be accomplished through embryo rescue technique. Since there is limited homology between the A genome of *O. sativa* and BB, CC, CCDD, EE, and FF genomes of wild species, only limited gene transfer is possible. Species belonging to *O. meyeriana, O. ridleyi*, and *O. schlechteri* complexes constitute the tertiary gene pool. Crosses between *O. sativa* and the species belonging to these complexes are extremely difficult to accomplish and gene transfer is rare if at all (Khush, 1997).

2.8 Mechanism of bacterial blight resistance

2.8.1 Biochemical basis

Studies on the multiplication of Xoo indicated that bacteriostasis occurs in resistant cultivars due to incompatible reactions. Bacteriostasis is correlated with early accumulation of bright yellow green fluorescent compound and host cell death in incompatible reactions (Reimers and Leach, 1991). It was also found that during incompatible reactions, lignin-like polymers formed rapidly whereas in compatible reactions these polymers were not deposited. Bacteriostasis and lignin-like polymer deposition were correlated with an increase in the activity of extracellular peroxidases during incompatible reactions (Reimers *et al.*, 1992). Peroxidases are formed in the last enzymatic step of lignin biosynthetic pathway, i.e., oxidation of cinnamyl alcohol into

free radical intermediates which are polymerized into lignin. Since Xoo primarily resides in the vascular tissues and does not directly penetrate the host cells, it is unlikely that lignin per se serves as a physical barrier to pathogen spread. Probably, the peroxidase activity, toxic phenolic compounds and free radicals that are formed during the lignin biosynthetic process, play an important role in the defense against Xoo in resistant cultivars.

2.8.2 Molecular basis

Classical genetic analysis of disease resistance has led to the identification of genes controlling pathogen recognition in both the plant and the pathogen (Flor, 1971). Race specific interaction in the bacterial blight disease of rice follows the gene for gene model according to which the incompatible interactions are the consequences of the positive function encoded by pathogen avirulence genes and corresponding host resistance genes (Kelemu and Leech, 1990). Gene for gene interactions have been described for numerous plant pathogen pairs (Song *et al.*, 1995). From a study of such interactions between several pathogens and their corresponding host plants, these workers concluded that a plant disease resistance is expressed by a single resistance (R) gene in the plant, that corresponds to a single avirulence (avr) gene in the pathogen. Plant disease resistance genes enable the host to recognize pathogens producing specific signal molecules. Production of these molecules is, in turn, controlled by avirulence (avr) genes in the pathogen. The recognition events specified by matching pairs of resistance and avr genes trigger the rapid induction of host defense response and ultimately result in phenotypic resistance (Kunnel, 1996). Disease susceptibility is the result of either the plant R gene or the pathogen avirulence gene being absent from the interacting organisms. Hopkins *et al.*,

(1992) have identified three independent clones from Xoo, each of which contains an avirulence gene (avr xa-5, avr Xa-7 and avr Xa-10) that controls bacterial elicitation in rice cultivars containing the *xa-5*, *Xa-7* and *Xa-10* resistance genes.

2.9 Inheritance of resistance

Considerable progress in the study of inheritance of resistance has been made at IRRI and in other tropical countries. Murty *et al.,* (1973) studied the resistance of Sigadis, TKM 6, BJ 1, Wase-Aikoku 3, Zenith PI215936 and B589-A4-18-1 and showed that at least three different genes were involved in the resistance of these cultivars and strains. Petpisit *et al.,* (1977) demonstrated that the resistance in IR20 and IR22 was governed by a dominant gene named *Xa4* where as the resistance in lines IR1415-284 and RP291-7 was controlled by a recessive gene named *xa5*. The two genes, designated *Xa4* and *xa5* respectively, assort independently. These two genes were also found in many other resistant rice cultivars (Sidhu *et al.,* 1978). Olufowote *et al.,* (1977) found *Xa4* in IR1330-3-2 and Pelita I/1, and *xa5* in Kele and Chinsurah Boro II.

Sidhu and Khush, (1978) identified a single gene that conferred resistance to five cultivars. The pattern of segregation indicated a monogenic recessive factor when plants are inoculated at the booting stage but it is monogenic dominant when the plants are inoculated during flowering. Sidhu and Khush, (1978) called this phenomenon "dominance reversal" and designated the gene as *Xa6*. Sidhu *et al.,* (1978) analyzed the genetics of resistance in 74 cultivars of which 18 were found to have *Xa4a*, 20 were found to have *Xa4b* and 32 were found to have *xa5*. They also found that resistance in DV85, DV86 and DZ78 is conditioned by two genes, *xa5* and a dominant gene at a later stage, designated *Xa7*. They further found that resistance in P1 231129 is conditioned by

a single recessive gene *xa8*. The genes *Xa7* and *xa8* segregated independently of *Xa4*, *xa5* and *Xa6*. Singh *et al.*, (1983) identified gene *Xa9* in cultivars Khao Lay Nhay and Sateng. Later studies showed that *Xa4b*, *Xa6* and *Xa9* are all allelic/similar to *Xa3*.

Ogawa *et al.*, (1987b) conducted genetic analysis of resistance of five cultivars belonging to BJ 1 group and found that they had two recessive genes. One of them was *xa5* and other was a new recessive resistance gene. The gene conveyed resistance to Philippine race 6 and was designated as *xa13*. Khush *et al.*, (1990) used *O. longistaminata* as a resistance source and found that the resistance in this accession is governed by a single dominant gene which they named as *Xa21*. Zhang *et al.*, (1998) studied the resistance of an inter-specific cross between a resistant wild species *Oryza rufipogon* and a susceptible rice cultivar, Jiagang 30, by inoculation test, inheritance studies, PCR analysis and gene tagging. They found that the gene for resistance derived from *O. rufipogon* to be a new gene located on chromosome 11 and designated it as *Xa23t*

2.10 Breeding for bacterial blight resistance

2.10.1. Conventional breeding

So far, 31 genes conferring resistance to different Xoo races have been identified and some of them have been incorporated into modern rice varieties. The exploitation of resistant gene *Xa-4* by conventional back cross-breeding resulted in the development of many resistant varieties that have played an important role in protecting rice from Xoo (Khush *et al.*, 1989). In India, TKM6 was used as a donor of bacterial blight resistance and several varieties (e.g. ABT 32, Govind, UR 20, IR 36, Karjat, Radha, Ramakrishna) were developed. The genotype Sigadis was used for developing varieties Ratnagiri and 68-1. BJI was used as a donor for BB genes and varieties PR 4141 and IET 8585 (Ajaya) were developed. However, large-scale and long-term cultivation of varieties carrying

only *Xa-4* has resulted in evolution of newer races of the pathogen leading to breakdown of resistance in rice varieties across several Asian countries. One way to delay such a breakdown of BB resistance is to pyramid/converge multiple resistance genes into rice cultivars. Yoshimura *et al.*, (1995a; 1995b) combined resistance genes in pairs *Xa-4/xa-5* and *xa-5/Xa-10* and showed that plants with two genes can have a higher level of resistance to Xoo than would be expected from the sum of the parental levels.

2.10.2. Marker assisted selection (MAS) and gene pyramiding

One of the most significant advances in the last decade for the development of improved crop varieties was the use of molecular markers to identify and track genes of interest (Paterson, 1996; Tanksley and McCouch, 1997). Breeders have traditionally developed improved varieties by selecting on the basis of phenotype. However, a plant's phenotype is determined by its genetic composition and the environment in which it is grown. Often, the effect of the environment masks genotypic effects. Molecular markers enabled breeders to exercise precise selection on genotypic differences (i.e., differences in DNA) rather than phenotypic differences, which has the potential to greatly increase selection efficiency and shorten the breeding cycle (Yencho *et al.,* 2000). The use of molecular markers linked to resistance genes accelerated gene pyramiding efforts and made the process more precise (Mohan *et al.,* 1997a). Marker-assisted selection (MAS) has been successfully deployed at International Rice Research Institute (IRRI) for pyramiding bacterial leaf blight resistance genes (Huang *et al.,* 1997), gall midge resistance in rice (Katiyar *et al.,* 2001a) and blast resistance genes (Hittalmani *et al.,* 2000).

Since RFLPs are very cumbersome and RAPDs are extremely sensitive to various reaction parameters, both these markers cannot directly be used for MAS. However, once these markers are converted into STS markers or SCARs as seen in the case of *Xa*-21 gene (Åbenes *et al*, 1993; Huang *et al*, 1997), they can be readily used for MAS. Specific amplicon polymorphisms (SAP) or cleaved amplicon polymorphisms (CAPs), generated between resistant and susceptible genotypes when the STS markers obtained by sequencing the RFLP clones were found to be monomorphic, have been used for MAS of *Pi*43 2(t) gene conferring resistance to blast and *xa*-5 and *xa*-13 genes against BLB (Hittalmani *et al.*, 1994; Blair and McCouch, 1997; Huang *et al.*, 1997). Hittalmani *et al.*, (1999; 2000) have used these SAP markers and RFLPs closely linked to the resistance genes in MAS to select agronomically superior lines into which the desired gene has been incorporated. In addition to SAP, STMS markers have also been found to be tightly linked to the *xa-5* gene (Blair and McCouch, 1997) and can be used for MAS. SCAR markers tightly linked to the gall midge resistance genes, *Gm*-2 and *Gm*-4(t) (Nair *et al.*, 1995a; 1996) and RFLPs cosegregating with the semidwarfing gene, *sd*-1 (Cho *et al.*, 1994) have facilitated their use in MAS. Nair *et al.*, (1995a) have also developed two allele-specific associated primers (ASAPs), one for each allele (resistant and susceptible), which could be used either individually used or multiplexed in a single PCR reaction to discriminate between resistant and susceptible plants for gall midge biotype 1.

Another scope for MAS is in gene introgression studies where Chen *et al.*, (2000) for example, have used it to introgress *Xa*-21 into Minghui 63, a restorer line widely used for hybrid rice production in China, which has become increasingly susceptible to BLB over a period of time. Using a PCR based MAS system comprising a marker that is a part of

Xa-21 and two markers located 0.8cM and 0.3cM on either side of *Xa*-21, an improved version of Minghui 63, which is exactly the same as the original except for a fragment of less than 3.8cM in length surrounding the *Xa*-21 locus, has been developed. Field examination has shown that the improved version of Minghui 63 shows significantly higher grain weight and spikelet fertility than the original genotype under heavily diseased conditions while they are identical when there is no disease stress. Rapid, nondestructive DNA isolation methods from leaf discs, leaf blades and half seeds have made it easier to implement MAS (Deragon and Landry, 1992; Wang *et al.*, 1993; Zheng *et al.*, 1995; Zhai *et al.*, 1996). Gene pyramiding refers to the combining of two or more major genes for resistance into a single plant genotype (Mundt, 1990). While the use of a single major gene limits the useful lifespan of resistant cultivars to a few years, gene pyramiding could delay resistance breakdown by conferring horizontal resistance effective against all prevalent pathotypes of the pathogen. Yoshimura *et al.*, (1995a) and Huang *et al.*, (1997) have combined two to four BLB resistance genes into a single line by MAS using RFLP, RAPD, SCAR and SAP markers. They have observed that pyramid lines show a wider spectrum and higher level of resistance than their single gene parental lines, through both ordinary gene action and quantitative complementation. Hittalmani *et al.*, (2000) have also observed a similar phenomenon when they carried out marker-assisted pyramiding of three major blast resistance genes. The gene-pyramided lines can be used to conduct quantitative analysis on the effect of interaction with other genes.

2.11 Improvement of rice varieties for Bacterial Blight (BB) through gene pyramiding :

Sundaram *et al.*, (2008; 2009) introgressed 3 major BB genes (*Xa 21*, *xa 13* and *xa 5*) into elite cultivars Samba Mahsuri (BPT 5204) and Triguna from a donor line SS1113

through marker aided selection. Huang *et al.*, (1997) pyramided four BB resistance genes (*Xa 4*, *xa 5*, *xa 13* and *Xa 21*) in to IR24. He developed PCR markers for two recessive genes (*xa 5* and *xa 13*) and used them for marker aided selection. Joseph *et al.*, (2004) introgressed *Xa21* and *xa13* into Pusa Basmati 1, the most popular high yielding basmati rice variety. Recombinants having enhanced resistance to BLB, basmati quality and desirable agronomic traits were obtained. Background analysis using 252 polymorphic AFLP markers detected 80.4 to 86.7 % recurrent parent alleles in the BC_1F_3 generations.

Singh *et al.*, (2001) pyramided 3 BLB resistant genes (*Xa21*, *xa13*, *xa5*) into PR106, which is widely grown in Punjab. The combination of genes provided a wider spectrum of resistance to the pathogen population prevalent in the region, *Xa21* was the most effective followed by *xa 5*.

Sanchez *et al.*, (2000) transferred 3 BLB resistance genes (*Xa21, xa13, xa5*) into 3 NPT lines (IR 65598-112, IR 65600-42 and IR 65600-96) with high yielding potential. Sequence tagged site (STS) markers for the two resistant genes were developed based on DNA sequences of their linked restriction fragment length polymorphism (RFLP) markers (RG 556 and RG 207 for *xa5* and RG 136 for *xa13*). Marker polymorphism for *xa5* was detected for digestion of RG 556 polymerase chain reaction (PCR) products with MaeII enzyme and digestion of RG 136 PCR product with Hinf I enzyme for *xa13*. The BC_3F_3 NILs having more than one BLB resistant gene showed a wider resistance spectrum and manifested increase levels of resistance of the Xoo races, as compared with those having a single resistant gene. Results showed that MAS reached an accuracy of 96% of identifying homozygous resistant plants for *xa5* and *xa13* respectively. These NPT NILs for BLB resistance provided valuable material for

breeding and genetic studies of single gene effects and interaction of several resistance genes.

Yoshimura *et al.*, (1995) used RFLP and RAPD markers to select homozygous rice lines for *Xa4*, *xa5* and *Xa10*. Lines carrying *Xa4* and *xa5* were more resistant to isolate of race 4 than were either of the parental lines (quantitative complementation). No such effects were seen for *Xa4+Xa10*. Thus, combination of resistance genes provides broader spectra of resistance through both ordinary gene action expected and quantitative complementation. A dominant BLB resistance gene, *Xa30t* located on Chr. 4 has been recently introgressed from wild rice accession *O. nivara* (Acc.81825) (Cheema *et al.*, 2008) in to PR106.

Chapter 3

Materials & methods

Chapter III – Materials and Methods

The experimental studies pertaining to "Identification and Molecular mapping of novel Bacterial leaf blight resistance genes in rice" was undertaken at the Directorate of Rice Research (DRR), Rajendranagar, Hyderabad. The objectives of the study were

1. To screen wild rice accessions and bacterial leaf blight resistant donor varieties in order to identify donors possessing novel resistance.
2. To investigate the inheritance of resistance in the identified genotypes and to establish the novelty of the resistance genes
3. To tag and map the novel resistance gene(s) using molecular markers and to validate the tightly linked markers in alternate mapping populations
4. To identify the putative candidate genes, which could be associated with resistance in the donors through *in silico* analysis

The materials and methods used for these studies are described below

3.1 Plant materials used and development of mapping population

3.1.1 Screening of Wild rice accessions

Seventy two wild rice introgression lines carrying introgressions from different species of *Oryza* namely *O. nivara* (8), *O. rufipogon* (32), *O. longistaminata* (7), *O. glaberrima* (4) *O. officinalis* (9) and *O. brachyantha* (12) and an elite variety Ajaya were screened for BLB resistance along with few susceptible cultivars TN1, BPT5204, Swarna and MTU1010 with seven hyper-virulent isolates of the bacterial pathogen collected from different hot spot locations of India. The details of wild rice accessions are given in Table 2.

Table 2: List of rice genotypes screened for BLB resistance

S. No.	Accession No.	Species
1	IR-31917-45-3-2	*O. nivara*
2	IR-7/033-62-15-B	*O. nivara*
3	IR-7/033-121-15-B	*O. nivara*
4	IR-7/033-4-1-127-B	*O. nivara*
5	IR-65482-17-511-5-7	*O. nivara*
6	IR-65483-118-25-31-7-1-5-B	*O. nivara*
7	IR-65483-141-2-4-4-2-5-B	*O. nivara*
8	IR-65483-147-2-7-4-B	*O. nivara*
9	IR-65600-81-5-3-2	*O. rufipogon*
10	IR-77981-19-7-B	*O. rufipogon*
11	IR-77981-34-34-B	*O. rufipogon*
12	IR-75083-19-22-21-1-B-B	*O. rufipogon*
13	IR-75862-139-2-5-22-B-B	*O. rufipogon*
14	IR-75862-143-5-38-22-B-B	*O. rufipogon*
15	IR-75862-221-2-1-3-B-B	*O. rufipogon*
16	IR-71033-4-1-123-B	*O. rufipogon*
17	IR-73885-1-4-3-2-1-6	*O. rufipogon*
18	IR-55423-01	*O. rufipogon*
19	IR-80310-12-B-1-3-B	*O. rufipogon*
20	IR-80314-4-B-1-3-B	*O. rufipogon*
21	IR-69502-6-SRN-3-V-BN-1-B	*O. rufipogon*
22	IR-80340-23-B-12-6-B	*O. rufipogon*
23	IR-77384-12-35-6-6-B	*O. rufipogon*
24	IR-77384-12-35-3-11-6-B	*O. rufipogon*
25	IR-77384-12-35-3-12-1-B	*O. rufipogon*
26	IR-73884-12-35-3-12-3-B	*O. rufipogon*
27	IR-71033-121-15	*O. rufipogon*
28	IR-73885-1-4-3-2-1-6	*O. rufipogon*
29	IR-55423-01	*O. rufipogon*
30	IR-80310-12-B-1-3-B	*O. rufipogon*
31	IR-80314-4-B-1-3-B	*O. rufipogon*
32	IR-69502-6-SRN-3-V-BN-1-B	*O. rufipogon*
33	IR-80340-23-B-12-6-B	*O. rufipogon*
34	IR-77384-12-35-6-6-B	*O. rufipogon*
35	IR-77384-12-35-3-11-6-B	*O. rufipogon*
36	IR-77384-12-35-3-12-1-B	*O. rufipogon*
37	IR-73884-12-35-3-12-3-B	*O. rufipogon*
38	IR-71033-121-15	*O. rufipogon*
39	IR-73885-1-4-3-2-1-6	*O. rufipogon*
40	IR-73678-6-9-B	*O. rufipogon*
41	IR-75870-5-8-5-B-2-B	*O. longistaminata*
42	IR-55423-01	*O. longistaminata*

43	IR-80310-12-B-1-3-B	*O. longistaminata*
44	IR-80314-4-B-1-3-B	*O. longistaminata*
45	IR-69502-6-SRN-3-UBN-1-B	*O. longistaminata*
46	IR-80340-23-B-12-6-B	*O. longistaminata*
47	IR-77384-12-35-3-6-6-B	*O. longistaminata*
48	IR-77384-12-35-3-11-6-B	*O. glaberrima*
49	IR-77384-12-35-3-12-1-B	*O. glaberrima*
50	IR-77384-12-35-3-12-15-B	*O. glaberrima*
51	IR-77384-2-B-1-4-5	*O. glaberrima*
52	IR-77384-2-B-4-2-1	*O. officinalis*
53	IR-77384-13-B-1-18-9	*O. officinalis*
54	IR-77384-17-3-2-11-2	*O. officinalis*
55	IR-77384-17-3-2-11-10	*O. officinalis*
56	IR-77384-17-3-2-13-4	*O. officinalis*
57	IR-77384-17-3-2-16-4	*O. officinalis*
58	IR-77384-17-3-18-1-6	*O. officinalis*
59	IR-77384-17-3-19-1-5	*O. officinalis*
60	IR-77384-17-3-19-3-4	*O. officinalis*
61	IR-77384-17-3-19-5-1	*O. brachyantha*
62	IR-77384-17-3-19-5-2	*O. brachyantha*
63	IR-77384-17-B-19-1-7	*O. brachyantha*
64	IR-77384-17-B-1-4-1	*O. brachyantha*
65	IR-77384-17-B-1-4-4	*O. brachyantha*
66	IR-65483-141-2-4-4-2-5-B	*O. brachyantha*
67	IR-77384-17-B-8-3-4	*O. brachyantha*
68	IR-77384-17-B-8-3-10	*O. brachyantha*
69	IR-77384-17-B-8-4-7	*O. brachyantha*
70	IR-65483-141-2-4-4-2-5-B	*O. brachyantha*
71	IR-77384-17-B-15-6-1	*O. brachyantha*
72	IR-77384-17-B-20-4-1	*O. brachyantha*
73	Ajaya (IET8585)	*O. sativa*
74	TN1	*O. sativa*
75	BPT 5204	*O. sativa*
76	Swarna	*O. sativa*
77	MTU1010	*O. sativa*

3.1.2 *Xanthomonas oryzae* p.v. *oryzae* (*Xoo*) *isolates*:

Seven different strains of bacterial leaf blight were isolated from infected leaf samples collected from various hot spot locations of India. The pathogenicity of these isolates was confirmed by inoculating them on susceptible plants (TN1) and they were further characterized

by inoculating them on a set of host differentials. These isolates are maintained at the Directorate of Rice Research, Hyderabad was used in the present study (Table 3).

Table 3: List of BLB isolates used for screening

S. No.	Name of Isolate	Origin
1	KAUL (DX-O84)	Kaul
2	FZB (DX-002)	Faizabad
3	PNT-8585 (DX-011)	Pantnagar
4	WR6 (DX-044)	Hyderabad
5	MTU (DX-049)	Maruteru
6	RPR4 (DX-066)	Raipur
7	CHN (DX-027)	Chinsurah

3.2 Screening for bacterial leaf blight resistance

3.2.1 Isolation and maintenance of bacterial cultures:

A small portion (7-10 cms) of leaves showing typical BLB symptoms was collected and surface sterilized by soaking it in 0.1% mercuric chloride or sodium hypochloride (%) for 30-45 second followed by 3-4 washing in sterile distilled water. It was then dried well with sterile blotting paper. These symptomatic leaves were cut into small portions (1-2 mm) by sterile scissors and put in the vial containing small amount of sterile distilled water for 20 min to let the bacterial cells streaming out from the diseased tissues through the cut ends. Using a sterile inoculation needle, a loopful of ooze streaked over the modified Hayward's medium (HM) or modified Wakimoto's medium (MWA). The plates were incubated at 28^0C for 96-120 hours (Suparyono *et al.,* 2004). Typical yellow, round, convex, mucoid and shiny colonies characteristic of *Xoo* were picked and transferred to agar slants and used for further studies. The bacteria were stored at 4^0C for short term storage and in 15% glycerol at -70^0C for long term preservation.

3.2.2 Preparation of Hayward's agar media:

Hayward's agar media was made using peptone (1% w/v), sucrose (2% w/v), disodium hydrogen orthophosphate (0.18% w/v), magnesium sulphate (0.03% w/v) and agar (2% w/v). All the ingredients except agar were dissolved in 600 ml of quartz distilled water, the pH of the medium was adjusted to 7.0 and added agar-agar, boiled to dissolve the agar and the volume was made to 1 liter with quartz distilled water. Aliquots of 250 ml medium were dispensed into 500 ml Erlenmeyer flasks. These flasks were plugged with cotton and autoclaved at 15 lbs pressure, 121^0C for 15 min.

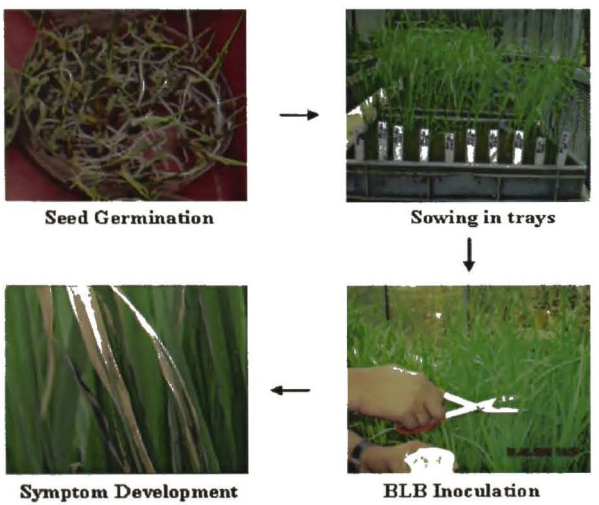
3.2.3 Mass multiplication of *Xanthomonas oryzae* pv. *oryzae*, plant inoculation and observation

Individual isolates of *Xoo* were mass multiplied on HM or MWA plates. The bacteria were streaked on HM or MWA plates and incubated at 28^0C for 96 hours. After the incubation period, the bacterial cells were harvested by adding sterile distilled water and the final concentration of approximately 10^8 cfu/ml was made (Preece, 1982). The plants at the maximum tillering stage were inoculated by leaf clip inoculation method which was developed at All Indian Coordinated Rice Improvement Project (AICRIP) (Kauffman *et al.*, 1973). In this method, top of fresh leaves of each plant are cut with a sterilized scissor dipped in freshly prepared bacterial solution. Observations were taken 14 days after inoculation by measuring the lesion length (in cm). The plants showing a lesion length of 4 cm or less were considered as resistant. (Chen *et al.*, 2000; Shanti *et al.*, 2001).

3.2.4 Screening of germplasm/mapping populations for bacterial leaf blight resistance

The genotype of each F_2 plant was inferred based on the phenotype shown by its progeny with respect to bacterial leaf blight resistance. A set of 20 F_3 seedlings from each F_2 line of the mapping populations along with 20 seedlings of the susceptible and resistant parents were inoculated by leaf clipping method (Kauffman *et al.*, 1973) (Figure 3) at 50-55 days after sowing (active tillering phase) with DX-066 of BLB *Xanthomonas oryzae* p.v. *oryzae* (*Xoo*). Plant reaction to the inoculation was recorded 14 days after inoculation. The lesion length in each plant was measured on five individual leaves and average lesion length was obtained. The F_3 plants were scored as either susceptible or resistant based on the lesion length obtained. An F_2 line was scored as homozygous susceptible if all its progenies exhibited susceptible reaction. On the other hand, if all the progenies of an F_2 line were resistant, it was scored as homozygous resistant. Those F_2 lines whose progenies exhibited segregation with respect to lesion length was scored as heterozygous resistant after statistically confirming goodness of fit for the 3:1 ratio through χ^2 tests (Gomez and Gomez, 1976).

Figure 3: Clip leaf inoculation method of screening



present in a separate slot with photos enlarged

3.3 Development of mapping populations:

One wild rice introgression line carrying introgression from *O. brachyantha* (IR-65483-141-2-4-4-2-5-B) and the variety Ajaya were found to be highly resistant to all the isolates in repeated tests. These genotypes i.e. IR-65483-141-2-4-4-2-5-B and Ajaya were used as donors and the bacterial leaf blight susceptible varieties- TN1 and Samba Mahsuri (BPT5204) were used as recipient parents for developing the mapping and alternate mapping populations. The susceptible variety TN1 (Taichung Native 1) was developed by crossing dwarf Chow-wu-gen with Tsai-Yuan-Chunj, while Samba Mahsuri, a fine-grained variety, was derived from the cross (GEB-24/TN1)/Mahsuri and released by Acharya N. G. Ranga Agricultural University (ANGRAU), Hyderabad.

Figure 4 A: Main Population for mapping

TN1 X IR-65483-141-2-4-4-2-5-B

↓

F_1

↓

F_2

↓

F_3

4 B. Alternate Population for validation

BPT5204 X IR-65483-141-2-4-4-2-5-B

↓

F_1

↓

F_2

↓

Figure 5 A: Main Population for mapping

TN1 X Ajaya

↓

F_1

↓

F_2

↓

F_3

5 B. Alternate Population for validation

BPT5204 X Ajaya

↓

F_1

↓

F_2

↓

F_3

The two donors IR-65483-141-2-4-4-2-5-B and Ajaya were crossed to the two recipients TN1 and BPT5204. The F_{1s} of all the four crosses were advanced to F_2 and F_3 generations. In the case of IR-65483-141-2-4-4-2-5-B, a mapping population consisting of 296 progeny tested F_2 individuals was developed by crossing IR-65483-141-2-4-4-2-5-B with TN1, which and was used for tagging and mapping studies (Figure 4A) while another mapping population consisting of 250 progeny tested F_2 individuals developed from the cross BPT5204/ IR-65483-141-2-4-4-2-5-B was used for validation of linked markers identified in the first population (Figure 4B)

In the case of Ajaya, a mapping population consisting of 400 progeny tested F_2 individuals was developed by crossing Ajaya with TN1, which and was used for tagging and mapping studies (Figure 5A), while another mapping population consisting of 196 progeny tested F_2 individuals developed from the cross BPT5204/Ajaya was used for validation of linked markers identified in the first population (Figure5B). A schematic diagram illustrating the development of mapping populations is given in Figure 4 and 5.

3.3.1 Development of F_2 mapping populations for allelism tests

Allelism tests were carried by crossing IR-65483-141-2-4-4-2-5-B with near isogenic lines (NILs) of IR24, possessing known single dominant BLB resistance genes, *viz.*, IRBB1 (possessing *Xa1*), IRBB4 (possessing *Xa4*), IRBB7 (possessing *Xa7*), IRBB21 (possessing *Xa21*) and IRBB26 (possessing *Xa26*). The F_2 populations were developed by selfing the F_1 plants and were then screened to study the segregation of resistance to BLB.

Ajaya was crossed with near isogenic line (NIL) of IR24, possessing BLB resistance gene *xa5* *viz.*, IRBB5. The F_2 population developed by selfing the F_1 plants were screened to study the segregation of resistance to BLB.

3.4 DNA isolation

3.4.1 Collection and preservation of leaf material

From each plant of the parental lines and F_2s growing in pots in the screen house, at 50-60 days after planting, leaves were collected in the morning and immediately stored at – 80^{0}C.

3.4.2 DNA isolation

DNA was isolated using a method modified from the protocol of Zheng *et al.*, (1995) which is detailed below

1. Two to three large leaf pieces (weighing ~2-3 g) were collected from the plants growing in pots in the screen house in a labeled plastic cover kept in freezer pack. After bringing the plastic cover to lab, they were immediately stored at -80^{0}C.
2. Leaf pieces were cut into small bits in a sterile porcelain mortar and liquid nitrogen was added till the leaf bits were completely immersed. The leaf bits were then ground into fine powder using a sterile porcelain pestle. While grinding, it was ensured that the leaf powder does not thaw by continuously adding liquid nitrogen.
3. Immediately after the grinding, 3-4 ml of extraction buffer (50 mM Tris HCl pH 8; 25mM EDTA, 300 mM NaCl and 1% SDS) was added to the mortar, which was then kept in a water bath maintained at 65^{0}C for about 5 min. Once the solution thaws, the contents from the mortar were transferred to 3-5 sterile, labeled 1.5 ml microcentrifuge tubes using a 1000 µl capacity micropipette using micropipette tips cut at their tip portion. The microcentrifuge tubes were incubated for 15 min at 65^{0}C.

4. After the incubation step, approximately equal volume of phenol: chloroform: isoamylalcohol (25:24:1) mixture (~400 µl) was added to each microcentrifuge tube. It was ensured that the pH of phenol used was ~8.0. The contents were mixed well by inversion for about 10 minutes and centrifuged at 10,000 rpm (~8000 g) for about 10 minutes at room temperature.

5. After centrifugation, the supernatant was aliquoted from the micro centrifuge tube into freshly labeled sterile 1.5 ml micro centrifuge tubes. Care was taken that the intermediate layer of insoluble proteins was not disturbed. If the supernatant was not clear, the phenol: chloroform: isoamylalcohol purification step was repeated once again. To the supernatant 5 µl of RNase (10 mg/ml) was added and incubated for 30 minutes at room temperature. One more treatment with an equal volume of chloroform (400 µl) was given and centrifuged at 10,000 rpm (~8000 g) for 10 minutes at room temperature.

6. To the clear aqueous supernatant, 1/8 volume of 3M sodium acetate (pH 5.2) and equal volume (500-600 µl) of chilled Isopropyl alcohol was added. The contents were mixed gently and centrifuged at 10,000 rpm (~8000 g) for 10 minutes at room temperature.

7. The supernatant was drained gently and about 200 µl of 70% ethanol was added to the pellet collected at the bottom of the micro centrifuge tube. The tube was tapped gently so that the pellet was disturbed. Centrifugation is done at 10,000 rpm (~8000 g) for 10 minute at room temperature. After this the supernatant was drained and the pellet was washed with 70% ethanol once again as described above.

8. Finally, the pellet was left for air-drying over night at room temperature with the tube cap open. After complete drying of pellet, depending on the size of the pellet, about 50-100 µl of sterile TE buffer (10mM Tris-HCl, pH 8.0 and 1mM EDTA) was added to the tube for dissolving the pellet. The DNA solution was then stored at 4^0C till further analysis.

3.4.3 Analysis of quality and quantity of DNA isolated

The quality and quantity of isolated DNA was checked through Ethidium bromide stained Agarose gel electrophoresis

A 0.8% Agarose gel was prepared as follows for electrophoresis of DNA samples

1. 0.3 g of agarose was weighed and added into a 250 ml conical flask.
2. One litre of 10X TBE was prepared as follows: 108 g of Tris base (Sigma, USA) was taken in 500 ml double distilled water, to which 55 g of Boric acid (Qualigens, India) was added, mixed well by stirring. This was followed by the addition of 9.8 g of Na_4EDTA and the volume was finally made up to 1000 ml using double distilled water. The solution after thorough mixing was filtered using 0.45 µm filter (Millipore, USA), autoclaved and stored at room temperature. This was used for preparation of 0.5X TBE, which was prepared by diluting 10X TBE twenty times with single distilled water.
3. 30 ml of 0.5X TBE was added to a 250 ml beaker and boiled gently in a microwave oven.
4. After the agarose was properly melted (when the solution was crystal clear with no floating particulate matter), the solution was taken out of the microwave oven and allowed to cool down.
5. Meanwhile, a gel-casting tray (CBS Scientific, USA) was washed thoroughly first with tap water and then with distilled water followed by rinsing with methanol.

6. The gel-casting tray was then placed in a sealing mechanism given by the supplier (CBS Scientific, USA) and a comb (containing 8 lanes) was arranged in its slot on the gel-casting tray.
7. When the boiled agarose had cooled down substantially (to about 45^0C), 2 µl of ethidium bromide solution (10mg/ml of distilled water) was added and mixed gently. Gloves were used while handling ethidium bromide.
8. The melted agarose was poured in the gel casting tray by carefully avoiding air bubbles and allowed to solidify (~20-30 min).
9. After solidification, the gel was removed from its casting tray and put into a gel tank, which was then filled with 0.5X TBE buffer till the buffer reaches 0.5 cm above the gel surface
10. Five microlitre of DNA solution was then mixed with ~1-2 µl of bromophenol blue dye (0.0025% bromophenol blue in 40% sucrose), loaded into the wells and the electrodes were connected to Power pack. (CBS Scientific, USA). DNA solutions of known concentration (50 ng/µl, 100 ng/µl, 150 ng/µl and 200 ng/µl) were also loaded along with the samples.
11. The samples were subjected to electrophoresis at 75 V for 20-30 minutes till the dye front reached $2/3^{rd}$ of the running length of the gel. The gel was then visualized under UV light in an Alpha Innotech gel documentation system (Alpha Innotech, USA) for checking the quality and quantity of DNA. Based on comparison with intensity of DNA solution of known concentration, the unknown samples were diluted to a final concentration of 50 ng/µl using 1X TE buffer.

3.5 Analysis using microsatellite molecular markers

Rice microsatellite markers were used in the present study for tagging and mapping the novel gene(s) identified in IR-65483-141-2-4-4-2-5-B and Ajaya. Initially, 282 SSR markers spread across the 12 chromosomes were tested to find out the polymorphic markers between resistant donors (IR-65483-141-2-4-4-2-5-B, Ajaya) and susceptible recipient (TN1). The details of these markers are given in Table 4.

Table 4 : List of microsatellite markers used to amplify the parents

S. No.	Chr. No.	Name of marker	Repeat Motif	F primer	R primer	Genomic position (MB)
1	1	RM495	(AGC)7	ATGATGATGGACGACGAC AACG	TGAATCCAAGGTGCAGAG ATGG	0 2
2	1	RM1282	(AG)17	TCGTGCAGGAGGTCTTCA TGG	TTGAGGATGGTAACGAAC CTTGC	0 54
3	1	RM1331	(AG)20	CAAACGGAGTGAGTACAT TAGC	TGATGTGTTCTGTACAGGT TCC	1 66
4	1	RM3740	(AG)16	CCCGTCACCAACTCACCA TAGG	CTAAGCCACCCTTACCAC CATCC	2 8
5	1	RM3652	(AG)14	TGAGGAGGTGGCGGAAGA GG	CTCTGATGCGTCCGAAGA GAGG	3.57
6	1	RM3917	(AT)31	TCTAGGGCTAGCTCTTCCA CTCAGG	CGAACGAACGAGGACGAA CG	3 8
7	1	RM6902	(TTA)19	CACAAACCGAAAGTTGCC CTTCC	TTCGGAGAAGACCGAGAC GTAGATGC	3.8
8	1	RM3426	(AG)18	CCGATGATGGAAGCACAG ATAGG	TTCTCATTCTCGAAGGCTT ACGG	4 05
9	1	RM3521	(CT)31	CCTTCGAGTGCATGTCCC	GAACACGAGGAGAATGAG CC	4 39
10	1	RM283	(AG)20	GGCATGAGAGTCTGTGAT GTTGG	TAGTACTGCTCCATCTGCC TTGG	4 88
11	1	RM3174	(AG)12	GCTTCAGGAGCTCTCCAA AGAGG	CTAGCTCCGTGTCAGCAA CACC	5
12	1	RM1118	(AG)12	TGCCACAGTGGAGAGAGA AAGG	CAGTGCTTGCTGTGTGTAG TATGC	5 56
13	1	RM151	(ATT)11	TGCCCTGGCGCAAATTTG ATCC	GCTAGAGGAGATCAGATG GTAGTGCATG	5.6
14	1	RM10353	(GC)10	GGACACTTTGAATGAAGG CAACC	TTGTTAGTTGGCGAAAGG AAGC	5 94
15	1	RM1201	(AG)14	GCTACGTACGAGCCCTAG TTACCG	TACCGCGCCACATATACA CAACC	7 15
16	1	RM5735	(AAC)14	TACACGAAGGCTTGTCCA ATACG	GCTTTCAATGCCTTCTCTC TTCTCC	7 4
17	1	RM243	(AG)18	CAGACTGCAGTTGCACGA TACTACG	GAAAGCTGCAACGATGTT GTCC	7 97
18	1	RM5644	(AAG)15	ATCCAAGCTCCTGAAATG CATCC	TGGTACATGCCCATATCA AGAAGC	8 27
19	1	RM577	(TA)9(CA)8	GCTTTCCCTCTAACCCCTC T	GGATGTACCGCTGACATG AA	8 4
20	1	RM579	(AG)25	TTCCGAGTGGTTATGCAA	TCGTGACCTGAGAAATTG	8 45

				ATGG	TGTCC	
21	1	RM3506	(AG)25	TTCCGAGTGGTTATGCAA ATGG	TCGTGACCTGAGAAATTG TGTCC	8 45
22	1	RM1151	(AG)13	GTAACAGCGACCGTTGGT TGG	CCCATCATCTGATGTCACA CTCC	8 57
23	1	RM583	(AAG)20	GTTGCGGTTTGTTCGTTCT TGC	TAGATCCCAGCAGACGGA TCAGC	9 1
24	1	RM580	(AAG)19	GAAATGGACTCGCTCCTA AACTGG	ACGAACTAGAGCATGGGC ACTCC	9 6
25	1	RM1061	(AC)49	ATGACTCTTGACAGTTGCC TTAGC	TGACTAGACTGACTGACA CGTAGGC	9.72
26	1	RM5644	(AAG)15	ATCCAAGCTCCTGAAATG CATCC	TGGTACATGCCCATATCA AGAAGC	9 82
27	1	RM572	(AG)14	CGCGGTTAATGTCATCTG ATTGG	CCATACTTCGAGATCCAA GACTGACC	9.86
28	1	RM3627	(AG)13	CTATGCCTCCGCCTCTATC TCC	CTCTCTGGACCTACCCGTC ATCC	10 3
29	1	RM1287	(AG)17	CCATTTGCAGTATGAACC ATGC	ATCATGCAATAGCCGGTA GAGG	10 83
30	1	RM493	(AAG)9	GTACGTAAACGCGGAAGG TGACG	CGACGTACGAGATGCCGA TCC	12 2
31	1	RM562	(AAG)13	GGAAAGGAAGAATCAGAC ACAGAGC	GTACCGTTCCTTTCGTCAC TTCC	14 6
32	1	RM594	(AG)15	TCGAGAGAGGGAGAGTGA GAACATGG	GCCTTCGCACATAAAGGA TGAACC	15 1
33	1	RM595	(AG)12	AGACTACAGGCTTGTTCA GATTGG	TTGAGCTGTGCATAGGTG AGG	15 19
34	1	RM466	(AG)17	CCTCACCATTGATCTCCAT CACC	ACTTGCCCTCCTCTTCTCA ACG	17 27
35	1	RM24	(AG)22	CTAAATTTCTGGCCGTAG GATCTTGG	GGGTAGTGGACGGCGAAT GC	19 3
36	1	RM7056	(AATT)6	GAGGATAGGCATCTCTCG AAGC	ACGATGAAACGTGTAGCA GTACG	23.11
37	1	RM5	(AG)15	CACACTCCCATGCTAACA ACTGG	CATCAAGAAGAGCAGTCC TGTGC	23 96
38	1	RM3475	(AG)22	ATGTTGTCGAGTCGTGGT AATGC	TATTCCTCGGTGTATGGGT CTCC	26 03
39	1	RM3143	(AC)17	AAAGCCTGGATAAGATGG TTCG	CTGTAGTTGCTGTTTGCCT GTCC	26 8
40	1	RM246	(CT)20	GAGCTCCATCAGCCATTC AG	CTGAGTGCTGCTGCGACT	27 6
41	1	RM1232	(AG)15	TCACTGGCTGTCTCTGTGG AGTGG	CGCTTTGCTTCCCTCCTCT GC	27 62
42	1	RM1244	(AG)11	GACAAGACAAACTGAGCA AACTCG	GGTGAACGTCCTGTCTGT AGTGC	28 68
43	1	RM1117	(AG)12	ACGGGAAAGATAAAGGTG ATCC	TGCAGCACACATTACAGT CACC	29 28
44	1	RM306	(GT)18(AT)8CT(GT)6	CAAGGTCAAGAATGCAAT GG	GCCACTTTAATCATTGCAT C	29 4
45	1	RM128	(AAG)9	TGATTTCTTGGAAGCGAA GAGTGAGG	CCTCCTTGTGCTCAGCCAT GC	30 73
46	1	RM3324	(AG)14	GGAGCTTGAGTTAAGACT GGTTGG	CTTCCCTCTCGTTCAAATC AATCC	31.71
47	1	RM297	(AG)36	ACAGGGCTATGCAGACAC AGTGC	AGCAAGCGAAGGGAAGTG ACC	32 09
48	1	RM1216	(AG)14	ACATCGCATGCAGACTGA TTAGC	CCAATGGAACAGTGACAC AAGC	32 1
49	1	RM212	(AG)14	AAGGTCAAGGAAACAGGG ACTGG	AGCCACGAATTCCACTTTC AGC	33
50	1	RM5501	(AG)27	GTTGGCGTACGTAGAGAG	CTTCATTGTCGCTGCCAGA	34.54

				GAGTACG	GC	
51	1	RM6547	(AGC)9	ATGACACACCACCACCAC CAACG	GGAAGCGCAGCATCAACA AAGG	34 68
52	1	RM315	(AT)4(GT) 10	GAGGTACTTCCTCCGTTTC AC	AGTCAGCTCACTGTGCAG TG	37 1
53	1	RM1198	(AG)14	TCACTTGGTCCTCCACAAA GAGC	CATCTTGTTTCTCTCTCCA TGTCTGC	37 59
54	1	RM431	(AG)16	GCTTGCTTGTATCTGCATT GGTAGG	GGGATGATCCACTCTCTGT TTGG	38 88
55	1	RM6827	(AAG)11	TGTGTAGCTGTCAGAAGA AACAGAGG	CACCCTGGAATTCTGCATT AACC	39 19
56	1	RM1387	(AG)44	GCTACCATGCACGAAACA GAGACG	ACCAAATGTGGCTGGCTG ATCG	40 2
57	1	RM1067	(AC)20	CTAGGTGGAGCAGAGACA ACAGC	TAACCGATGGAGAGAGAA TGTCTAGC	42 91
58	2	RM300	(AAC)21	GGGCTTAAGGACTTCTGC GAACC	AGCGATCCACATCATCAA ATCG	13 1
59	2	RM110	(AG)12	AAATTCGAAGCCATCCAC CAACG	GCCGACGAGGTCGAGTAG AAGG	1 32
60	2	RM7033	(AAAT)10	AGAATAACTCCAGCCCAC ACTGG	GCGGTGATTTCTGATGAC ATTCC	1.7
61	2	RM233A	(AG)11	TATTGCTAGAGCCTACCTT TCC	CCAACCAGTCTTACAATC AACC	2 06
62	2	RM5654	(AAG)29	ATTACGTGGGTCGAACCA GAGC	CAAGTTCGTTACAGCAGA GGAAAGC	3 42
63	2	RM7581	(AG)13	CTCTGTTGCAATGCCCAAT ACC	AAGGGAGTCCATGGAGAT GAGG	4 2
64	2	RM5862	(AAT)42	CCTCCTGAAGGGTAAAGG ATTGG	TCCACACATGATCGCTAC ATCG	6
65	2	RM174	(AAG)7	ATAAGCGACGCCAAGACA AGTCG	GGAAGCAAGAAGGAAAG AGAGATGG	7
66	2	RM3874	(AG)15	AAGCTGGGTGATCTTAGT TTGG	GAGAGTAGTGGCTATTTG CTTGC	23 8
67	2	RM5706	(AAT)21	GTGGCTTGATTTCTGCAAC TTCC	GCTGGTACCTACCAAGTA TCATTTCG	26 5
68	2	RM1385	(AG)39	GACAGGTAAGGTGTGGTG GTAAGG	AAACCTTTCTCAAACGCA CACG	26 66
69	2	RM526	(AATT)5	GCATCGGAATTTCATCAG AGAGC	AAGCAAGGCTCCTCAAAG AATCG	26 68
70	2	RM450	(AG)17	CAGTAGTACGCCGGATCA ACAGG	CCACTTGTTCCATCCACAT CTCC	28 65
71	2	RM6933	(AAT)34	AATGCCTAGCACTCATCCT TGC	AGGCACCCTACGATGAAA TAGTGG	29 33
72	2	RM14131	(ACG)7	CCAGCTGTTCGACTTCCTC ACC	GCGACGTAGATGACGGAC TTGG	34 29
73	2	RM213	(AG)11	ACAAGCAGATACTGACTG ATGC	CTTCTTTGCATCCAGACTT CC	34 6
74	2	RM14156	(AT)18	GATCGATCTTTAGTCCCGG TTGG	AACACAATTCACCGGTCC TTAGC	34 82
75	2	RM2848	(AT)20	TAGGGCTAGCTCTTCCACT CAGG	GGACGATCGTTAACGTGA AATGC	34 82
76	2	RM208	(AG)12	AGTACCACCACCATTCTCT GCAAGC	TCGATTGGCCATGAGTTCT CG	35 16
77	3	RM60	(AATT)6	CAAGTTCACCCGCCTTCTC G	TTTCCATCATTAGCAGGCA GTAGC	0 08
78	3	RM22	(AG)10	GTTGACAAACAGGCCCTG AAACC	GTATGGTCTGGAGACGAC GATCC	1 5
79	3	RM5474	(AG)21	GTGGGTTTGTGTTTGGAG AGACG	GTGTTGGTGAGCATAGCA GTTGG	3 7
80	3	RM545	(AG)30	CCTTCCCTGAAAGTATTCG	GAGAACGTCTTCATTGGA	4.9

				TTCTCC	TGTTCC	
81	3	RM14783	(AG)10	CTCCTCGAGCTTGACGATGG	GTACGCAACCCTACGAGAGACG	10 1
82	3	RM14845	(AT)13	CACATCAACGGTGTAGATTGTAGG	TAGTAGCGGTGATCTCATTGTGC	11 3
83	3	RM14965	(ACC)7	GAAGGCAACCAACCCGAAGG	CGAGGATCTGGGTGAGAATGG	13 4
84	3	RM15022	(AT)28	ACCAATCCGGGACTAAAGATCG	CACCGGTCCTTAGCTTCTGAGC	14.2
85	3	RM16090	(AT)30	TTCACTGTGAGAATTCGCTTGG	GACGACCGAAGTCCTAACTACAGC	28 3
86	3	RM520	(AG)10	ACGATAACGCCGACATCACTGG	GCTAAGCATCCACGGTTTCTCTCC	30 7
87	4	RM16303	(AT)40	CTCACTCTGTCCACAAATACAGG	GTTGGGTGGATAGACAGTGG	1.09
88	4	RM16396	(AT)19	CGATATATGGGAACAGCGAGTTGC	CTCTCGTACCACTGGCACTTCTTCC	3 42
89	4	RM16447	(AT)30	CGGGTCTGTCTTTCAGTTTGC	AGTGGCGTACTTGCTCTACTGC	4 76
90	4	RM16489	(AT)23	AACCAGCTAGCCCTTTGGAAGC	AGAGATGGATCAGCGCTTCTTGG	6 26
91	4	RM6156	(CCG)10	CGTCCGCACGCAAGAAGAAGG	CCGTACGTGTGGCTTCAGATTGG	7 8
92	4	RM16553	(AT)14	CATAGCCACTTATCGTTGTTACGC	TGTCCATCTATGACTGTCCACTACG	8 6
93	4	RM16569	(AT)31	ATAGACTTCACCCGGTACAACG	CTGGTATCGACATCGGCTAGG	9.72
94	4	RM16592	(ACAT)29	CTTAGCACGGACACTCATATTTGG	CACAATACGTTTGATGGCTTGC	11.36
95	4	RM16604	(AGAT)14	CGGTACATAATATACGGTCAGGATGC	TGGGTATTGCCTAACTGCTTTCC	11 52
96	4	RM16616	(AT)27	TAGGCACTGGGCTCAAGTAGTGG	TTCTCATCCTTCGCCATCTAACC	12.1
97	4	RM16652	(AT)90	TGACATTAGTTGTGGCAGATCC	CCTAGAATCTCATCTGTCTTCTGG	13 65
98	4	RM16694	(AT)37	GCGTGATAGATGGATCTGTTGG	CATCCGATAGTACTACCTCCATCC	14 8
99	4	RM1236	(AG)15	TCTCTCCGAAGGAGTGCTAGGG	CCAGCCTTAACACCATGTCTACG	15 3
100	4	RM16757	(AGAT)12	AACGGAAATGGCTGTTGAAGC	GGCATGAGGGCGTTTGTAGG	17 08
101	4	RM16790	(AGAT)5	TTTCTCTGCCTCATGCCACACACC	CAACTGGAGCATGGCTAGGTCACG	18 11
102	4	RM16801	(AAT)36	CGTTCAAGGAGCTTGTGTTGATCC	GGACCGATTTAAGTGAACGTTGATGG	18 34
103	4	RM6314	(AAG)11	CATGTCTGATATTGCGGTTCAGG	TCAAGCCCTGCCCAACTACG	18 6
104	4	RM6314	(AAG)11	CATGTCTGATATTGCGGTTCAGG	TCAAGCCCTGCCCAACTACG	18 62
105	4	RM185	(AGG)9	GGCTCTCCATCTCCATTGATCC	GAGTTGTTGGGAGGGAGAAAGG	18 7
106	4	RM417	(GA)7	CGGATCCAAGAAACAGCAG	TTCGGTATCCTCCACACCTC	19 4
107	4	RM16913	(AAT)51	GTGTACGTGTTGGCTCTCTGTACG	GATGTTGCTTGTGCTGCAACC	19.82
108	4	RM3643	(AG)14	GCTAAGCTAATCTGACCGGATCTACG	GATGGGCCGATTAACAAATTCC	20 1
109	4	RM17093	(AT)43	TATCGACGACGGCTGTTGAGAGG	GTTGGTTGGACCTTGGGTCTGG	22 99
110	4	RM3367	(AG)23	GCCTCGGCTGATGTAACC	GATATGTGCTGCTGTGTGC	24 26

				TTCC	TTGC	
111	4	RM17162	(AT)35	GATGTACCAGTCCAGTTA CAAAGACC	CCTTCAGAGTCTGCACAC AGG	24 44
112	4	RM17201	(AG)46	GATCGTTGCTGCTTTCAAT GAGG	AGTGTTTCACCTTGGACCC ATGC	25 19
113	4	RM1223	(AG)15	GGTGAGCAACTATCAAGA ATCACAGC	GCTGCTACCAGGTCAGAG TTGC	25 4
114	4	RM17263	(AAT)30	GGCTCCCAAGTGTAGTAT ACGG	TCTGTAATCGACTGATGTC TCG	26 28
115	4	RM3125	(AC)13	ACTAGCCACAGCCCATCT GTTCC	CCCGCTCAAATTCTTCACA ATCC	26 7
116	4	RM3125	(AC)13	ACTAGCCACAGCCCATCT GTTCC	CCCGCTCAAATTCTTCACA ATCC	26 73
117	4	RM241	(AG)28	GTTCATTTCGTGATCTCTG AGC	GCAGATTTACAGGTTTGCT AGG	26 8
118	4	RM17310	(AAT)28	CGATTTGAGCCGATACAT TAGGG	TGTTGGTGTCGGATTTCTC AACC	27 44
119	4	RM470	(AAG)14	CCCTCCCGTAGACCTTGTA CCC	CCACAGCTAACCAATCCT TCTCC	28 24
120	4	RM17353	(AT)13	GATGTGAATCATGGGAAC AAGAGC	GATGATGATGTGCTGCAT TTGG	28 5
121	4	RM303	(AT)13	ATCGATGTAGGTAGAGGG ACACC	CAGATCTAGTCGACATGG TTGG	28 7
122	4	RM17372	(AT)32	ATTATCCAAGGTCCAAAC CTACGC	GTGTGTCCCATCATCACA AACTAGC	28 73
123	4	RM17405	(AAT)36	GGTGTACGTATTAGCAGG TTTCG	CGAACTACCAACTCAAAT CACC	29 8
124	4	RM3217	(AG)23	GACCTAACCAATCGGACG AACG	CACTAGTGCTCTGTCTCTG CTTACCC	30 33
125	4	RM17473	(AT)34	TCTCTCCAGCTCCCTAAAC ATTCC	AGCGACACACTGTTCACC TTGC	31 05
126	4	RM5709	(AAT)24	TTTGGGATAAATGGGAGA GG	TCAGCTGGTTATTATGGA GAGG	32 09
127	4	RM349	(GA)16	TTGCCATTCGCGTGGAGG CG	GTCCATCATCCCTATGGTC G	32 4
128	4	RM17540	(AT)36	GTCATAGGGCACTCTAGT TTCG	CACAATGTGTTAGACCCTT CG	32 69
129	4	RM5950	(ACC)9	ATACGCTGGCTGCATGTT GACG	TACGACACGGCGGTGTTC TACC	33
130	4	RM17600	(AAT)114	CCTCGAAATGAATTGCAG TCGAACG	GTCTTGTGCCTTGTGCCGA TGG	33 81
131	4	RM17630	(AT)35	CGCTCAGTTCACGAAACC AACC	TCTCTGCACTCTGCATTAC ACACG	34 35
132	4	RM567	(AG)23	GCATACCGTAATGTTGGT GAAGC	AATAGCAACTGGGAGGAG GTAAGG	34 75
133	4	RM127	(AGG)8	CGAAGCTTTCGGTGGGAT AGC	ACCTTGAGCGAGTCCTTG AACG	34 75
134	4	RM17659	(AT)15	GGTATGTATGCTTCCTTCC CTTCC	ACCTGTGAGCATTCTTTGT TGG	34 88
135	5	RM1248	(AG)15	CTCTCAGGTGTGTTGTACA TTGTTCC	CTGCTCAAACAAGCAGCT AATGG	0 1
136	5	RM122	(GA)7A(G A)2A(GA) 11	GAGTCGATGTAATGTCAT CAGTGC	GAAGGAGGTATCGCTTTG TTGGAC	2 79
137	5	RM3334	(AG)15	GGGAAAGGAAGGAGCTCA ACG	AGAGGAGACTAATCACAG GGAATCG	0.6
138	5	RM5579	(AC)22	CAACAGAACCTGCCAACT CTTGC	TGCCTCATGGTAATAAGC CAAGC	1.8
139	5	RM2585	(AT)30	TCCGTCTTCTCTGAAGAAT CG	GTTGAGAGGTGTAGGTAT GAATCG	2 04

140	5	RM413	(AG)11	CCAATCTTGTCTTCCGGAT CTTGC	AGATAGCCATGGGCGATT CTTGG	2 1
141	5	RM548	(AG)12	GCTCTTCCAAACAACACC TTAGC	GACAAGAGAACATCGCTA GGAAGC	2 7
142	5	RM225	(AG)14	TATGTGGTTGGCTTGCCTA GTGG	TGCCCATATGGTCTGGAT GTGC	3 41
143	5	RM437	(AG)13	ATCCCTCCTCTGCTCAATG TTGG	TCAGGGAGGGTCCTAGCT ACTGG	3 8
144	5	RM2998	(AT)41	TCTAGATACACCGTCTCA ATGG	AAAGAGGTAGCTAGGTTG AAGG	7 1
145	5	RM289	G11(GA)1 6	TTCCATGGCACACAAGCC	CTGTGCACGAACTTCCAA AG	7 7
146	5	RM249	(AG)17	CAACTCCACTCCAGACTC AACTCC	GGTATGATGCCATGAAGG TCAGC	10 6
147	5	RM1237	(AG)15	CAGCACACATACTCTGGC TCTCC	CCGCGAGCTTTAGAAGAG AAGG	17 87
148	5	RM18613	(AG)10	AACGCCATTGAGCTCGTT CTTGC	CTACCACCGTGGCGAGTT CTGC	19 06
149	5	RM18614	(AG)12	TGGCGCAATATCTCTCTCA TTCC	TGCCACTTGTGTGTTGTTC TGC	19 07
150	5	RM163	(AG)15	CGCCTTTATGAGGAGGAG ATGG	AAACTCTTCGACACGCCTT GC	19 1
151	5	RM1090	(AG)12	AATATTAATGGCCGGAAC CGAAGG	GCAAACTTTGGGTGAGTG TTTGTGC	21 79
152	5	RM5592	(AT)23	CTCGCTCTTACAACTTTCA AGC	CACTTACCTCCACTTCTCA ACC	21 9
153	5	RM7446	(AAAT)22	CGTTGAGCCAAGAAGAAG AAAGG	TTTGAAGGCAGTTTCACTG ACG	24 8
154	5	RM480	(AC)30	TGGTACTCACCATGCAAG TAGAACG	ATGCTCAAGCATTCTGCA GTTGG	27 1
155	5	RM26	(GA)15	GAGTCGACGAGCGGCAGA	CTGCGAGCGACGGTAACA	27 3
156	6	RM19261	(AT)12	CGCATGCCAATTGATCCT ATCC	GCACGGTCTATTTCATCCA ACTCG	0 57
157	6	RM19271	(AGG)7	CCCTCGCATCACGATTAA CTAGC	ACGCCTCTGTACCGCAAG TACC	0.9
158	6	RM7399	(ATCG)6	CAGATATGATGTTCTTGCC CTTGC	GCTTGCCAGATCACCTAC CTACC	1 04
159	6	RM19289	(AT)24	TTTAACGTGGCAAATCCTC ACC	ATCTGCTCCAAAGAGCCA AACG	1 21
160	6	RM19290	(AG)27	TTTAACGTGGCAAATCCTC ACC	ATCTGCTCCAAAGAGCCA AACG	1 21
161	6	RM190	(AG)18	GCTACAAATAGCCACCCA CACC	CAACACAAGCAGAGAAGT GAAGC	1.76
162	6	RM400	(AAT)63	TTACACCAGGCTACCCAA ACTCG	TTGCTGAGTTCCCTCGTCT ATCC	2 8
163	6	RM204	(AG)42	CTAGCTAGCCATGCTCTCG TACC	CTGTGACTGACTTGGTCAT AGGG	3.1
164	6	RM585	(AG)45	CTAGCTAGCCATGCTCTCG TACC	CTGTGACTGACTTGGTCAT AGGG	3 16
165	6	RM225	(AG)14	TATGTGGTTGGCTTGCCTA GTGG	TGCCCATATGGTCTGGAT GTGC	3 4
166	6	RM444	(AT)12	TGCATCTTTCACCGTAGTC CTAGC	CTTGCTGGAGCTCGTAGA TGC	5.87
167	6	RM7088	(AGAT)6	CGCTGCAGAGAATGAATA ATCG	AATCCTGATCGATCTGAG TGATGC	6 6
168	6	RM5183	(AT)31	CGGGTGGTGTGAACCTAT TCC	CACGATTAACCATCGTGA GCTACC	8 29
169	6	RM257	(AG)30	CCGTGCAACTTAAATCCA AACAGG	GGAATCCTATATGAGCCA GTGATGG	17 66
170	6	RM553	(AG)10	TGTGTGGCCACTTTACTCA	GGAGAAGGTGGTTGCAGA	19 03

				ACC	AGC	
171	6	RM3	(AG)13	GTCACATTCCGTTTCCCAT CATTCC	CCTCACCTCACCACACGA CACG	26 86
172	6	RM30	(AG)13	GTCACATTCCGTTTCCCAT CATTCC	CCTCACCTCACCACACGA CACG	26 86
173	6	RM6926	(AAT)31	CGATCGGCCTATCTTTCTG TGC	GCTAGCAGTGGGATCATG TTTGG	28 2
174	6	RM494	(AAG)16	GGGATCGAGATAGACATA GACC	TCTGTACAGTGTCATTCCT TCC	30 5
175	7	RM3859	(AG)28	CTCATGCTTTCAGTCATTC AGTGC	TCCTGGATTCATGGTGTCT TTAGC	8 9
176	7	RM6872	(ACC)8	CACCACGATATCCACCTCT AGC	CCTAGGATGAACACTGAT GATGG	4.6
177	7	RM180	(AAT)10	CCTTCTCCTTCTTTCAGCT TCTGC	CAACTTGCTCTACTTGTGG TGAGG	5 7
178	7	RM6728	(AAT)16	TCACCCTTCTTGTCAAGCA AACC	GAGGATCAGGATAGAACA TGAGATGC	5 76
179	7	RM8007	(AT)13	CCGCATGTAATGTATGGT GTGC	TGAACACTGCCACTATCA GAGACG	7.74
180	7	RM500	(AAG)9	AGAAGTGCAGGTTGGCTC TGC	CACGAATCTCGGAGTGTC TAGGG	15 8
181	7	RM533	(AG)16	AAAGGCCGTACCTTTGCC TTCC	AGCTAGGGATCCATCCTC CAACC	17 4
182	7	RM21591	(AGAT)5	CCTGAATCTGCAGGACGA CAACG	TTTCTAGCCTTCACAGCGA ATGTGG	17 51
183	7	RM2752	(AT)35	GATCCGCTTGCACATGGA TAGG	TACACGTGTGAGCGAGCT TAGCC	22 49
184	7	RM21843	(AAAT)5	ATAAGTGCAGCCATGGTA TTCG	TGACTGAAAGTACTCACT CCGTTCC	22 73
185	7	RM21962	(AGG)7	CACCATCGAGGAGGTGAA GAAGC	GCTGAGGATGATCTGCGT GTCC	25 25
186	7	RM234	(AG)13	TTCAGCCAAGAACAGAAC AGTGG	CTTCTCTTCATCCTCCTCC TTGG	25 4
187	7	RM18	(GA)4AA (GA)(AG)1 6	TTCCCTCTCATGAGCTCCA T	GAGTGCCTGGCGCTGTAC	25 6
188	7	RM478	(AG)12	GGGTGGAGTGTAATAATA GCAAGC	AACACGTCCAAAGTCACA GAGC	25 8
189	7	RM473A	(AGAT)15	AAAGGATGTGGCGGTAGA ATGC	GACTCCAACCCTAAAGCC TCTCC	28 12
190	7	RM248	(AG)15	AGAGAGCAAGTTTGAAGC GAAGC	ACCAAGAGGGTAGCCTAG CATGG	29 28
191	8	RM1235	(AG)10	GAGAAACACAATCAGTGA CACC	CTGAAATTGCACTTCACTG G	1.2
192	8	RM3819	(AG)20	GAACAGAGGCGAGCGTTG TGG	AAGGCAACACCAAGAAAG CATCC	3
193	8	RM3374	(AG)16	ACCGAGCAGACAAAGAGT AGC	TGGTGATCTAGGTCAAGT TGG	4 7
194	8	RM10383	(AC)11	CTACACCTCATGTGGGTGT TTCC	CAGTCATCAGCAACATTT GTGTCC	6 47
195	8	RM10417	(AT)27	TGTACGCATACACACGCA ATACG	GGCCTATCCTCCCTATCGA ACG	6 99
196	8	RM3598	(AG)13	CACGGTGAGATAGAGACG AGATGG	CAATCACAAATTCACGCA GTGACC	7.34
197	8	RM3235	(AG)13	ATCTAATTCCAGTGGCGC AGAGG	TGGAGCTAAGTGAGAGCT AGTGATGG	7 51
198	8	RM1384	(AG)36	TGGTACGGGAGAACTGGT ACGC	AATCGAGCCAGCCTAGCA AGC	11 8
199	8	RM3409	(AG)17	GCATGGCTGATGCATTTCT TGC	GTACACGCGCGTTCGGTA GTGC	15 4

				ACC	AGC	
171	6	RM3	(AG)13	GTCACATTCCGTTTCCCAT CATTCC	CCTCACCTCACCACACGA CACG	26 86
172	6	RM30	(AG)13	GTCACATTCCGTTTCCCAT CATTCC	CCTCACCTCACCACACGA CACG	26 86
173	6	RM6926	(AAT)31	CGATCGGCCTATCTTTCTG TGC	GCTAGCAGTGGGATCATG TTTGG	28 2
174	6	RM494	(AAG)16	GGGATCGAGATAGACATA GACC	TCTGTACAGTGTCATTCCT TCC	30 5
175	7	RM3859	(AG)28	CTCATGCTTTCAGTCATTC AGTGC	TCCTGGATTCATGGTGTCT TTAGC	8.9
176	7	RM6872	(ACC)8	CACCACGATATCCACCTCT AGC	CCTAGGATGAACACTGAT GATGG	4 6
177	7	RM180	(AAT)10	CCTTCTCCTTCTTTCAGCT TCTGC	CAACTTGCTCTACTTGTGG TGAGG	5 7
178	7	RM6728	(AAT)16	TCACCCTTCTTGTCAAGCA AACC	GAGGATCAGGATAGAACA TGAGATGC	5 76
179	7	RM8007	(AT)13	CCGCATGTAATGTATGGT GTGC	TGAACACTGCCACTATCA GAGACG	7 74
180	7	RM500	(AAG)9	AGAAGTGCAGGTTGGCTC TGC	CACGAATCTCGGAGTGTC TAGGG	15 8
181	7	RM533	(AG)16	AAAGGCCGTACCTTTGCC TTCC	AGCTAGGGATCCATCCTC CAACC	17 4
182	7	RM21591	(AGAT)5	CCTGAATCTGCAGGACGA CAACG	TTTCTAGCCTTCACAGCGA ATGTGG	17 51
183	7	RM2752	(AT)35	GATCCGCTTGCACATGGA TAGG	TACACGTGTGAGCGAGCT TAGCC	22 49
184	7	RM21843	(AAAT)5	ATAAGTGCAGCCATGGTA TTCG	TGACTGAAAGTACTCACT CCGTTCC	22.73
185	7	RM21962	(AGG)7	CACCATCGAGGAGGTGAA GAAGC	GCTGAGGATGATCTGCGT GTCC	25 25
186	7	RM234	(AG)13	TTCAGCCAAGAACAGAAC AGTGG	CTTCTCTTCATCCTCCTCC TTGG	25 4
187	7	RM18	(GA)4AA (GA)(AG)1 6	TTCCCTCTCATGAGCTCCA T	GAGTGCCTGGCGCTGTAC	25 6
188	7	RM478	(AG)12	GGGTGGAGTGTAATAATA GCAAGC	AACACGTCCAAAGTCACA GAGC	25 8
189	7	RM473A	(AGAT)15	AAAGGATGTGGCGGTAGA ATGC	GACTCCAACCCTAAAGCC TCTCC	28 12
190	7	RM248	(AG)15	AGAGAGCAAGTTTGAAGC GAAGC	ACCAAGAGGGTAGCCTAG CATGG	29 28
191	8	RM1235	(AG)10	GAGAAACACAATCAGTGA CACC	CTGAAATTGCACTTCACTG G	1 2
192	8	RM3819	(AG)20	GAACAGAGGCGAGCGTTG TGG	AAGGCAACACCAAGAAAG CATCC	3
193	8	RM3374	(AG)16	ACCGAGCAGACAAAGAGT AGC	TGGTGATCTAGGTCAAGT TGG	4 7
194	8	RM10383	(AC)11	CTACACCTCATGTGGGTGT TTCC	CAGTCATCAGCAACATTT GTGTCC	6.47
195	8	RM10417	(AT)27	TGTACGCATACACACGCA ATACG	GGCCTATCCTCCCTATCGA ACG	6 99
196	8	RM3598	(AG)13	CACGGTGAGATAGAGACG AGATGG	CAATCACAAATTCACGCA GTGACC	7.34
197	8	RM3235	(AG)13	ATCTAATTCCAGTGGCGC AGAGG	TGGAGCTAAGTGAGAGCT AGTGATGG	7 51
198	8	RM1384	(AG)36	TGGTACGGGAGAACTGGT ACGC	AATCGAGCCAGCCTAGCA AGC	11 8
199	8	RM3409	(AG)17	GCATGGCTGATGCATTTCT TGC	GTACACGCGCGTTCGGTA GTGC	15 4

200	8	RM3459	(AG)20	GCTGGGCCTATATGGACT TTCG	AGGCAAGTCTTGGCACTG TAAGC	20 6
201	8	RM223	(AG)20	GCTGGGCCTATATGGACT TTCG	AGGCAAGTCTTGGCACTG TAAGC	20 64
202	8	RM1345	(AG)23	CGCACAAACCAAACACAC AACC	CTCGTGTCGTCTTCACCAA AGC	26
203	8	RM3496	(AG)25	GGTATACGGCCCTTCAAG TACACG	TGCAGAGAAAGAAGGGA AGATCG	27 8
204	9	RM8219	(AG)11	AGATAAGCAGGGATTGGA AAGG	GCAACAGGGAAGAATAGT ATGTCG	1 4
205	9	RM23819	(AT)38	CAATTCATAACGTCGGTC CTTCC	ATGGCTCTGTTTGCTGCAT GG	5.01
206	9	RM5899	(AAT)16	GCGTTGTTTAACCGTGGTC TTGC	AGCGTCTATTGACCGTGG TCTTGC	5 07
207	9	RM8206	(AG)16	AATCCACCTGGCCCTAAT CTTCC	CACTGCTGCTTCCTTCCTT CTGC	5 9
208	9	RM23865	(AG)10	TCATCCCATTCTCTTCCTC ACC	CATACGGCCATACAAATG AACC	6 2
209	9	RM23872	(AT)33	AACCTGCAGATACGACAA ATGG	CGAGTCCTGTGATATCTTC TCACC	6 3
210	9	RM24017	(AT)47	CCTGCTATTGTACCTGCTC TAATGC	CGTCAGATTACAGTGTCG CATCC	9 54
211	9	RM5777	(AAG)11	CGTCGCCATCTAGTTCTCT CACC	CGAGAGTGCATGTGGAGT GAGG	10 07
212	9	RM6854	(AGC)8	AGATGATCAATCCGTCTCT TGC	TGCTTTCTCTGTGATTCTC TGC	14 3
213	9	RM3700	(AG)15	CCTTTGCCGCCTTCTCTTG G	ACGAGTTCCCGGTTAACC TTACG	15 37
214	9	RM434	(AG)12	TCTCTAGTTGCCTCATCCC TCTAACC	GGCTCAACCTCTATATTTG CTGATCG	15 6
215	9	RM257	(AG)30	CCGTGCAACTTAAATCCA AACAGG	GGAATCCTATATGAGCCA GTGATGG	17 6
216	9	RM24540	(AT)33	ACTCTATTCCTCGATACCT TGG	GGAGCCCATACTCCATAT AGC	18 11
217	9	RM201	(AG)10	GTACTCTCGCCGTTCACAA CTCC	TTAGTGACCGGGATGACA CAGC	19 87
218	9	RM24653	(AT)29	TCTGCGAAATAAGTGGAA CG	GATGTCGCAAAGTTGAAA CC	19 9
219	9	RM215	(AG)16	GAGCAGCAAGAGCAGCAG AGG	CATGCTCGACTTCAGAAG CTTGG	20 8
220	9	RM24720	(AG)22	ATAAATGCCGGACAGGAA CACG	TCCGGAGAGAGCATAAGT CAACG	20 96
221	10	RM24881	(AT)40	TGAGGGATAACCTGACCA CACC	AAGGAGCACTGGTGGAGA AACC	0 31
222	10	RM474	(AT)13	TACACGAGGGAGTACTCG AATGG	CATGGAGGTATAGAAGAG CATTGG	1 79
223	10	RM216	(AG)12	GATGGTAAAGGAAGAACG TGTGC	CACTCATAGACGCATCAC ATAGCC	4 98
224	10	RM25110	(CCG)8	AGCGACGCACCAACCTCT CC	ATTCCACCAAACGCATTC AACG	5 75
225	10	RM311	(GT)3(GT AT)8(GT)5	TGGTAGTATAGGTACTAA ACAT	TCCTATACACATACAAAC ATAC	9 4
226	10	RM3283	(AG)13	AGCGGAATCAGATTGTGA CGAACTCC	CTCCCTGCCGATGAGCAA GTATCG	11 8
227	10	RM5147	(AT)36	CTAGGTATAGGGTATCGT CTTGG	AGGTCCTGACAATAAACT ACCG	14
228	10	RM1375	(AG)31	GCTTGTTGGCAGTTTGTAT TGTGG	GGTACACTAAGTGGGCAA ATCAGG	16 2
229	10	RM1873	(AT)18	CCAGACAAGCAACACCTA CTTCC	CAAACAGGCCCTAACTGA CAGG	17 37

230	10	RM258	(AG)11	CTCCCTGGCCTTTAAAGCT GTCG	GACGAACAGCAGCAGAAG AGAAGC	17.5
231	10	RM304	(AT)30	TCAAACCGGCACATATAA GACC	CGTTGTAGTGTCAGCAAG ATAGGG	18 21
232	10	RM309	(AC)14	CACGCACCTTTCTGGCTTT CAGC	AGCAACCTCCGACGGGAG AAGG	21 52
233	11	RM286	(AG)21	CTGGCCTCTAGCTACAAC CTTGC	AAACTCTCGCTGGATTCG ATAGG	0.3
234	11	RM6327	(AAG)18	GGAGCTGATACAACAATC AGACAGC	CGCTACGCTGCTCCAGATT AGG	0 3
235	11	RM3717	(AG)13	ATCCCTTTCCCTCAACCAA ATCC	AGCAAGCTCTACCTTTGCT GTCG	1 1
236	11	RM6690	(AAT)28	TTCCTCGGCTCTACATATA CTCC	CTTACCACTATGCAGGTA AGAGG	1.5
237	11	RM1812	(AT)16	CCTACCTCCAGTGAGAGC TAACC	ACGTGCATTTGTGTGGTTT AGG	2 3
238	11	RM332	(CTT)5-12-(CTT)14	GCGAAGGCGAAGGTGAAG	CATGAGTGATCTCACTCA CCC	2 8
239	11	RM6085	(AGG)9	GATGATGGCGACCACCAG TAGG	CCAGCAGCTAAACCCTTC TTGC	3
240	11	RM167	(AG)19	CTCCGAGTCCGACCACAA GG	TCCAGCCCTTCCTATCATA TTGC	4 05
241	11	RM555	(AG)11	TTGACATGCGAAATGGAG ATGG	TTGGATCAGCCAAAGGAG ACC	4 3
242	11	RM5704	(AAT)20	TTGATCACCTACACGTAC ACATGC	AAGGTCCTTTGAGCATTC AGTCG	5 4
243	11	RM4469	(AT)34	CCTATGCGTAGGTTCTCAT TGTGC	AAGGGAGGGACGTAGAAT AGATTTGG	6.1
244	11	RM6115	(CCG)8	CGCCATAGTCGATGACAT TGTACG	CAACGTGTCGCTTTAACA CAACC	8
245	11	RM536	(AG)16	TACCAGGATCATGTTTCTC TCC	ACTGTGAGATTGACTGAC AGTGG	8 89
246	11	RM202	(CT)30	CAGATTGGAGATGAAGTC CTCC	CCAGCAAGCATGTCAATG TA	8 9
247	11	RM5857	(AAT)31	TGGTATGTGGCAATTGGA TGG	AAGAGTCCTTGTGAGAGT CCTGTACC	11 75
248	11	RM26666	(AT)10	GCTAAATATGC1CCACCA CTGC	TTGGATAAGAGGGACACT CTGG	15 32
249	11	RM26703	(ACAT)6	TGCTGGTACATGTTGCTTT CAGG	GCCATATGGGCACATACC ATGC	16 38
250	11	RM287	(AG)15	GGCTACACCTACACGCGA GAACC	AGATGCATGGAATGCCTG TTTGG	16 61
251	11	RM229	(TC)11(CT)5 C3(CT)5	CACTCACACGAACGACTG AC	CGCAGGTTCTTGTGAAAT GT	18 3
252	11	RM26910	(CCG)7	GACCACCGCTCACCGTTG TAGG	GTTGCTTTGTGGAGCGCTG ACG	19 89
253	11	RM206	(AG)33	ATCGATCCGTATGGGTTCT AGC	GTCCATGTAGCCAATCTTA TGTGG	21 62
254	11	RM2191	(AT)22	TAGTTGTTCGGAGAACAT GG	ACACAAAGACACCTCGTA TGC	24 2
255	11	RM2191	(AT)22	TAGTTGTTCGGAGAACAT GG	ACACAAAGACACCTCGTA TGC	24 2
256	11	RM4069A	(AT)13	CCGAGACTTCAGAGTGCT CACC	CTATATTGCCGTGGCTCAT TAGTGG	25 8
257	11	RM144	(AAT)7	CATGTTGTGCTTGTCCTAC TGC	AGCTAGAGGAGATCAGAT GGTAGTGC	28 17
258	12	RM286	(AG)21	CTGGCCTCTAGCTACAAC CTTGC	AAACTCTCGCTGGATTCG ATAGG	0 3
259	12	RM6327	(AAG)18	GGAGCTGATACAACAATC AGACAGC	CGCTACGCTGCTCCAGATT AGG	0 3

260	12	RM3717	(AG)13	ATCCCTTTCCCTCAACCAA ATCC	AGCAAGCTCTACCTTTGCT GTCG	1 1
261	12	RM6690	(AAT)28	TTCCTCGGCTCTACATATA CTCC	CTTACCACTATGCAGGTA AGAGG	1 5
262	12	RM1812	(AT)16	CCTACCTCCAGTGAGAGC TAACC	ACGTGCATTTGTGTGGTTT AGG	2 3
263	12	RM332	(CTT)5-12-(CTT)14	GCGAAGGCGAAGGTGAAG	CATGAGTGATCTCACTCA CCC	2 8
264	12	RM6085	(AGG)9	GATGATGGCGACCACCAG TAGG	CCAGCAGCTAAACCCTTC TTGC	3
265	12	RM167	(AG)19	CTCCGAGTCCGACCACAA GG	TCCAGCCCTTCCTATCATA TTGC	4 05
266	12	RM555	(AG)11	TTGACATGCGAAATGGAG ATGG	TTGGATCAGCCAAAGGAG ACC	4 3
267	12	RM5704	(AAT)20	TTGATCACCTACACGTAC ACATGC	AAGGTCCTTTGAGCATTC AGTCG	5 4
268	12	RM4469	(AT)34	CCTATGCGTAGGTTCTCAT TGTGC	AAGGGAGGGACGTAGAAT AGATTTGG	6 1
269	12	RM6115	(CCG)8	CGCCATAGTCGATGACAT TGTACG	CAACGTGTCGCTTTAACA CAACC	8
270	12	RM536	(AG)16	TACCAGGATCATGTTTCTC TCC	ACTGTGAGATTGACTGAC AGTGG	8 89
271	12	RM202	(CT)30	CAGATTGGAGATGAAGTC CTCC	CCAGCAAGCATGTCAATG TA	8 9
272	12	RM5857	(AAT)31	TGGTATGTGGCAATTGGA TGG	AAGAGTCCTTGTGAGAGT CCTGTACC	11 75
273	12	RM26666	(AT)10	GCTAAATATGCTCCACCA CTGC	TTGGATAAGAGGGACACT CTGG	15 32
274	12	RM26703	(ACAT)6	TGCTGGTACATGTTGCTTT CAGG	GCCATATGGGCACATACC ATGC	16 38
275	12	RM287	(AG)15	GGCTACACCTACACGCGA GAACC	AGATGCATGGAATGCCTG TTTGG	16 61
276	12	RM229	(TC)11(CT)5 C3(CT)5	CACTCACACGAACGACTG AC	CGCAGGTTCTTGTGAAAT GT	18 3
277	12	RM26910	(CCG)7	GACCACCGCTCACCGTTG TAGG	GTTGCTTTGTGGAGCGCTG ACG	19 89
278	12	RM206	(AG)33	ATCGATCCGTATGGGTTCT AGC	GTCCATGTAGCCAATCTTA TGTGG	21 62
279	12	RM2191	(AT)22	TAGTTGTTCGGAGAACAT GG	ACACAAAGACACCTCGTA TGC	24 2
280	12	RM2191	(AT)22	TAGTTGTTCGGAGAACAT GG	ACACAAAGACACCTCGTA TGC	24 2
281	12	RM4069A	(AT)13	CCGAGACTTCAGAGTGCT CACC	CTATATTGCCGTGGCTCAT TAGTGG	25 8
282	12	RM144	(AAT)7	CATGTTGTGCTTGTCCTAC TGC	AGCTAGAGGAGATCAGAT GGTAGTGC	28 17

The identified polymorphic SSR markers were used for bulk segregant analysis. PCR was performed with using equal quantity (~100 ng) of genomic DNA collected from 10 samples of each of homozygous resistant and homozygous susceptible F_2 plants derived from the cross TN1/ IR-65483-141-2-4-4-2-5-B or TN1/Ajaya and bulked to constitute the resistant bulks (RB) and

susceptible bulks (SB) respectively along with resistant and susceptible parents (IR-65483-141-2-4-4-2-5-B/Ajaya and TN1 respectively).

3.5.1: PCR conditions used for analysis of SSRs

The SSR markers were used for assessing the polymorphism between the donor and recipient parents following PCR conditions described by Chen *et al* (1997b) with minor modifications. PCR was set up in a 10 µl reaction volume in a Perkin Elmer Thermal cycler (Perkin Elmer, USA). The ingredients of the PCR mixture included 10X PCR buffer of Bangalore Genei (10mM Tris, pH 8.4, 50 mM KCl, 1.8 mM $MgCl_2$ and 0.01mg/ml gelatin), 0.1 mM of dNTPs, 200 nM of primers, 1.0 Unit of Taq polymerase (Bangalore Genei, India) and 20 ng of genomic DNA. The following cycling conditions were adopted: Initial denaturation for 5 min at 94^0C, followed by 35 cycles of denaturation at 94 °C for 30-s, annealing at 55^0C for 30-s and extension at 72^0C, followed by a final extension at 72^0C for 7 min. The PCR products were resolved on 3.5% agarose gels (US Biochemicals, USA) in 1X TBE buffer in a mid size electrophoresis unit (CBS Scientific, USA) for 3 hours at 100V, stained with ethidium bromide (0.5 µg/ml) and photographed under UV light.

Polymorphic SSR markers were scored for co-segregation with the trait phenotype through bulked segregant analysis involving resistant and susceptible bulks prepared from 10 each of homozygous resistant and homozygous susceptible F_2 individuals derived from the cross IR-65483-141-2-4-4-2-5-B/TN1. Markers which displayed bulk specific amplification were subjected for analysis in the entire mapping population. This strategy was followed in order to quickly identify the tentative chromosomal location of the novel gene as well as SSR marker(s) co-segregating with the trait phenotype. Once the tentative chromosome location was known, all

the SSR markers on that particular chromosome were further analyzed in the entire F_2 population. The size of the amplified fragments was calculated as described earlier with 100 bp DNA ladder (MBI Fermentas, Lithuania) as size reference standard.

For fine mapping of novel genes in IR-65483-141-2-4-4-2-5-B and Ajaya a set of microsatellite markers which are located on Chr. 1 (Table 5) and Chr. 8 (Table 6) of rice, respectively were used. These markers were selected based on their relative positions in the SSR linkage map available at http://gramene.org and Class I SSR map of IRGSP 2005 (complete details and primer information available at http://www.gramene.org). Of the 37 microsatellite markers selected for fine mapping in IR-65483-141-2-4-4-2-5-B, 29 were from the RM series and eight markers *viz.*, RMCG1 to RMCG8 were specifically designed based on flanking sequence of repeat motifs identified in between the flanking markers identified in coarse mapping. Of the 45 microsatellite markers selected for fine mapping in Ajaya, 39 were from the RM series and six markers *viz.*, RMAFM1 to RMAFM6 were specifically designed based on flanking sequence of repeat motifs identified in between the flanking markers identified in coarse mapping.. The list of the SSR markers was given in the Table 5 and 6

Table 5: List of microsatellite markers on chromosome 1 used for fine mapping in IR-65483-141-2-4-4-2-5-B

S. No.	Name of marker	Repeat Motif	F primer	R primer	Physical Position (Mb)
1	RM10773	(AT)42	CCACCTATAGAAATAGGTCCATGC	TAGAACATGTCACCATCCAAGG	12 14
2	RM10839	(AG)16	ATGCCATGAATGTAACCGAGACG	AGAGAGCAATCCATGCATCTTCC	13.56
3	RM10855	(AT)47	CCAAACAAGATGTAGTGGGAACATCC	CAAGATGATCAGTGGGCTATTCTTGG	14 05
4	RM10874	(AT)18	GTACCTTCGAGAATTTAGGCAAGC	CAAGCTCCAAATGAGCTGAACC	14 42
5	RM10916	(AG)23	CATCTCAAAGTCTCAAACCCTAGCC	CTCGTGCTCCCTGTACCTACTCC	15 05
6	RM10920	(ACAT)13	GCGTTGCAGCGGAATTTGTAGG	CCCTGCTTCTCTCGTGCAGTCG	15.10
7	RM10922	(AG)15	TCGAGAGAGGGAGAGTGAGAACATGG	GCCTTCGCACATAAAGGATGAACC	15.14
8	RM10923	(AAT)8	TTCCGTGCCCAACTTTAAT	CCTAACACCATAGGAGTGCAA	15 16

			TGC	CAGC	
9	RM10926	(AG)10	CCCACTCTTGCAGCAGTTC TCG	GTCAGACTCGTCGCCACTTCA CC	15 37
10	RM10928	(CCGG)5	TCGCCCTTTCACCTCCCTA ACC	CTATCGGTGGCTCAAAGGGAT GG	15 44
11	RM10930	(AAT)19	AATTGGACTTTACGAGCC TGTGC	TCTCGTCCTCACCTCAAGAGTG G	15 53
12	RMCG1	(AGAT)37	ACGGTTTGGAAGTGTTCG TAGG	TGGTACTGCATAATCTCAGCA TCG	15 74
13	RM10940	(AT)16	GTGATTCAGCCTTAGCAG ATGG	ATCTTCTCCGAGGTATACTCAA GC	15 79
14	RM10941	(AGAT)5	GAGGGTTTGTAGGATGAC ATGTGG	TAATGGCAGAGGAGATTGCTT GG	15 82
15	RM10948	(AAT)19	CAACCGGGACTATTGTGG AGTTCG	CGAGCGAATGCGCAAGCTAGG	15 88
16	RMCG2	(AGAT)6	GCGCACGTACATACTACT GAAGG	TAGATTTGTACGCAGGACTTG G	15 91
17	RM10954	(AG)11	CTATTGGGCGTTGGTCTAG TGG	CAACGAAATCATCCCTAGCTT CC	16.07
18	RMCG3	(AT)13	AGAGGTTGTCGCAATAGC AGAGC	AACATGAGAACTCGTGGAAAG TGG	16.15
19	RM10963	(AT)26	TCCAGACTGTGACGAGCA GTACC	ATAACCAGGATCTACCCACAT ACCC	16 29
20	RMCG4	(AT)37	CCACACGATAGATTGGGT CAGC	ACAATGGTTGGATGCATGTTG G	16 30
21	RM10967	(AGAT)6	TGGCGACTTGTGAGTGTTT ATAGG	CAAATGCTGTTAGGTCGCTAC G	16 43
22	RMCG5	(AG)12	TACCCGAGGGTAGATCAG ATGG	CCTTTCACCGTGTTCGCTAGG	16 53
23	RM10974	(ACAT)7	ACATCGATCACACAAGAA GCCAAAGG	GGCATGCAACATGGACGCTAC C	16 70
24	RM10975	(AGC)10	CAGATGGTAGCGTCCATG TTGC	TTCCTACTCCACTCTACCTGGC TTCC	16 70
25	RMCG 6	(AT)12	TTATTCCGGGTATGTCCCT GTC	CAAGGACGTATTCGATGTGAG C	16.97
26	RMCG 7	(GT)14	GGTTGAGCTTTGTGCTAA GTGC	ACCTAGCCGTCTAGGAGTCCT C	16 97
27	RMCG8	(AT)50	CTACTAGAGTCACTGAGG CTGC	AGATGGTGCACTGCTTGATTG C	16 98
28	RM10979	(AT)21	TGTCCACCATCAATAGAT GACTCC	TTTCTTTCTCCCAGTTCGCTAC C	16 99
29	RM10980	(AT)25	TGTGAAGCACATCCAGTG ATCC	GGGATGAGTGACACTTGTTAA TGG	17.15
30	RM10986	(AGGC)6	TGGAGAGGAATGGGTGAG AAGC	CCTCTACAGTCCTCCACCAGA GG	17 27
31	RM10988	(AAT)22	GTCTTCGCAATTAAGCCAT TGGAACG	GGGATAGACAAGCGCACCTAA TCACC	17 27
32	RM10989	(AT)30	GGAAGAGTGCTCTAGTGG CAAGC	ACCAAACAAGCCCTGAGACAT CC	17 28
33	RM10990	(AG)14	ACTTTGACCTCCTTTAGCA TCC	GGCCAATGTATAATCGTCTTCC	17 30
34	RM10995	(AGC)10	TCTTCCAGGATCCGCCTTC TCC	GGAAGATCTCCCGCAGCTCAC C	17 57
35	RM10998	(AT)11	TAATCTGGTAAGGCGGTT GAGAATCG	CTGCCACCACGGAGATACTAA CATGC	17 69
36	RM11002	(AT)30	GCAAAGGACAACGACTCA TACAGAGG	TCCGGGTGAAATGCTACAACG	17 80
37	RM11033	(AT)35	TGTTTCAGAGTTCAGAGTC ACACG	AGGACAAGCCCACTTATTGAA CC	18 67

Table 6: List of microsatellite markers on chromosome 8 used for fine mapping in Ajaya

S. No.	Name of marker	Repeat Motif	F primer	R primer	Physical position
1	RM23168	(CCG)8	CTCCGACGTGCTCCTCTTC TCC	GAGACATTTCGTCGAACGAG TGG	21 01
2	RM23248	(AG)23	GACAACTCCATATCAACG CAAAGC	GAGGATGGTTGTTCACTTGT TTGG	22 47
3	RM23283	(AT)40	TGATCTTGGATAACTCGGT CCTACC	CACAGGGTGATTCATGTGTG TCC	23 12
4	RM23310	(AT)47	GATGAGGCCCAGATTTAC ATTTCC	CTAAGACTTCCTGACGATGT CAATGC	23 54
5	RM23327	(AT)37	AGACGAAGGTAGTGCATA ATAGGG	TTGGATTGTTACGTGCACAG G	23.65
6	RM23344	(AG)22	ATGATTGCATCTGCATCAC TGC	ATACCTGTTTCCAATGCGTA GCC	24 07
7	RM23349	(AT)33	TAGAGTCGTGCAAGGGTA TTGAATGG	GTTGTGATGGATTGTAGCGG TTGG	24 14
8	RM23362	(AAG)1 9	CTCATCTCCGCCCTTGATT CC	GCCCATCAACCTCGTCTTCA CC	24 47
9	RM23377	(AG)23	GTGGAAACCACCCAACCC AATCC	CTACTCGCGCGTGGAGTTGA AGG	24 75
10	RM23386	(AT)33	AGGTTGACCTGTGTGAGT AGCAAGG	ACATCGCCAACCATCTCAAG G	24 83
11	RM23399	(AT)24	GACTGTTCAGCACACACA ACC	GAGCTCCCATACATACACAT CTCC	24 96
12	RM23408	(AT)47	CCATCTCAACTCCTTCGTT TACTGC	TCGACTGTTTGCTTGGAATA GGC	25 08
13	RM23419	(AT)42	ACCTGAGCTCGATGGTTCT TTCC	GCTCCCACCAAGTATTCCTA TCG	25 22
14	RM23433	(AG)22	CTGCAACACCAGTAACTC TAGATCG	CCAAGAACAGCAAGAACAA CC	25 37
15	RM23440	(AT)23	CACGAGTGAGCTTAGTGG TTTGG	GCTGGATATGCTTGTGTTGA TTGG	25 56
16	RM23447	(AG)15	TACCGACCTGATTCGAAT GAACC	TACGGATGTCGAGGAGAAG TTCG	25 65
17	RM23460	(AG)34	GAAGAGAGCTGATTCAGG AGAGAGC	ATAGTTAGGCAGCAACAGC AACG	25 92
18	RM23468	(AG)24	GCGGTAACAAACCAACCA ACC	AAAGCAGGACACAGTCACA CAGG	26 14
19	RM23471	(AT)23	GATGCCGAACACACCCTT AGC	TCACCTGTACTAGCCACGTA GTCG	26 2
20	RM23478	(AGG)7	CGACGCAGGGTTTAGATA GAGTGC	GTTCTCGTTCCGATGGCTAG ACG	26 3
21	RM23484	(AT)12	CGCAACAAAGTGCAGAGA ATCACC	GCTTACAACCTCAACAAGCA TTCACC	26 37
22	RM23489	(AG)10	AGCAGGAGGAGGAGAAG GGAAGG	CGAGCCACTGCTTCTTCTGT GC	26 4
23	RM23496	(ACC)7	CCTTCCTCCACCTCGCTTG C	CTCCAGCTGCAACCAACCAA CC	26 5
24	RM23497	(AGC)7	ATGTACAATCCGAGTGCT CCTAGTGG	GGGCACCATCTCCATCTCTC C	26 51
25	AFM2	(CAG)7	TAGTGACGAGACGAGTCG TCGT	CCATCTCCTCGCCGTGCGAG CT	26 61
26	AFM3	(CGG)6	TCGGACTTCTCCACCGTGT ACG	AAAGACCCAATCGCACCAA ACC	26 62
27	AFM1	(GA)26	CTCAGATGGAACTGAAGG CCAC	CAACTGGCTAATATGGTGCT CC	26 62
28	RM447	(AAG)8	ACGGGCTTCTTCTCCTTCT	TCCCTTGTGCTGTCTCCTCTC	26 64

			CTCC	C	
29	AFM4	(TC)12	ACACGCACGAACGCATGG CCTG	AGAACATGACACGCATGCTT CC	26 64
30	RM23512	(AT)20	GGACACATGGTAGTTTAG ATGAGC	GGATTCTTATCCTCCTGTTTG G	26 73
31	RM23518	(AT)44	TTTCGCTTCTGAGTTGGCA GAGG	GACCTGAACAACGTGCCAA ACG	26 81
32	RM23518	(AT)44	TTTCGCTTCTGAGTTGGCA GAGG	GACCTGAACAACGTGCCAA ACG	26 82
33	AFM6	(AT)35	CGTGCGGTTTCTCTTTTTG TTATTA	TGCGCAAACCTAGCTAAAAC CC	26 83
34	AFM5	(AT)40	ACCATATTTTCCTCGATCG TCAG	CGCCGCTATCGACGACCTGA AC	26 91
35	RM23529	(AG)17	CTCGAGCTACATGAGCCA ATGC	GCCTCCTAACATGAAGACCA TATCC	26 97
36	RM23535	(AG)18	TGTGGAGTGTGGAGGCGA GAGC	AGGCACCCACCCACTGACTT CC	27.1
37	RM23545	(AT)37	CAACGCTATTGATGCCTCT CTGC	ATCCTCTGCCATCGTCTTCTT CG	27 22
38	RM23553	(AT)43	TCACTGTACGTGCACTATC CTAACC	GGAGACAAATGTATTGGGA GTAGG	27 25
39	RM23570	(AG)22	TAGACAATGGTCTGCAAC TCTGC	CAAGGAGATTGGATTGCTGT ACC	27 39
40	RM23574	(AAT)30	TGGGATGAACGAGCGTGT ATGG	ATGCTAATTTCCTGACTGAC CCAACC	27 44
41	RM23575	(AT)29	GAGAGAGGCGACAGGAA GACG	TTAGAGACCGTGGGAATGAT GG	27 44
42	RM23578	(AAT)48	AGCGATTCAGAACGAATC AACG	TGCCAAAGCTACACAAATCT GACC	27 47
43	RM23593	(AG)25	GGTATACGGCCCTTCAAG TACACG	TGCAGAGAAAGAAGGGAAG ATCG	27 83
44	RM23601	(AC)31	TGCGTGTAACTGTTTCGCT TGC	CCCTTGAGCTGATGCACATA CG	27 89
45	RM23649	(AT)35	GAGCGTCATGTCGTACTGT GG	AAGAGAGTGTTTGGGAACT AGGG	28 34

3.6 Linkage analysis and molecular mapping

Linkage analysis and map construction were performed using MAPMAKER/EXP, version 3 (Lander *et al.*, 1987). The genotype data for the polymorphic markers were used in the analysis, together with the genotypic data with respect to resistance/susceptibility for the individual F_2 plants, which in turn was deduced through progeny testing at F_3. Linkage groups were obtained using two-point analysis with a log-likelihood of odds (LOD) score of 4.0 and maximum recombination level of 0.3. This step was implemented by using the 'group' command. Linked markers within the linkage groups were ordered using multipoint analysis with 'compare', 'suggest subset' and 'try' commands. Best order of marker was then confirmed with the 'ripple'

command, using a minimum LOD of 4.0. Finally, the map distance was calculated using 'map' command. The map distances were converted into centiMorgans, using the Kosambi (1944) function. Linkage maps were prepared for the different mapping populations separately.

3.7 Physical localization of markers on the rice genome

The draft genome sequence of Japonica was downloaded from the Beijing Genomics Institute Japonica genome sequence database (available at http://www.rice.genomics.org.cn/rice/index2.jsp) and opened using the software Bioedit (available at http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The forward and reverse primer sequences were used as the reference points and the exact physical position of the primers was identified using the 'find' command of the software.

3.8 Identification of putative candidate genes

The exact physical position of the forward reverse primers of the markers closely linked to the novel gene was first identified as described earlier. Using the markers as reference points, the genomic sequence in between the flanking markers and/or in the vicinity of the closest marker was downloaded from Japonica rice genome and analyzed using the online software FGENESH (available at http://www.softberry.com). All genes with clear open reading frames (ORFs) were identified and putative function for these genes was annotated using BLAST-P utility (available at http://www.ncbi.nlm.nih.gov) to identify the putative function of each gene identified in the region.

3.9 Cloning

3.9.1 Gel elution:

The DNA fragments to be cloned and sequenced were eluded from agarose gel using Wizard® SV Gel and PCR Clean-Up System (Promega) by the following procedure.

1. A 1.5ml microcentrifuge tube was weighed and the weight was recorded.
2. The DNA fragment of interest was excised in a minimal volume of agarose using a clean scalpel or razor blade. The gel slice was transferred in to the weighed microcentrifuge tube and the weight was recorded. The weight of the empty tube was subtracted from the total weight to obtain the weight of the gel slice
3. Membrane Binding Solution was added at the rate of 10µl of solution per 10mg of agarose gel slice.
4. The mixture was vortexed and incubated at 50-65°C for 10 minutes or until the gel slice is completely dissolved.
5. The dissolved gel mixture was transfered to the SVMinicolumn assembly and incubated for 1 minute at room temperature.
6. The SV Minicolumn assembly was centrifuged at 14,000rpm for 1 minute and the liquid in the collection tube was discarded.
7. The column was washed by adding 700µl of Membrane Wash Solution, previously diluted with 95% ethanol
8. The SV Minicolumn assembly was centrifuged for 1 minute at 14,000rpm and placed back in the collection Tube.
9. The wash was repeated with 500µl of Membrane Wash Solution and the SV Minicolumn assembly was centrifuged for 5 minutes at 14,000rpm.
10. The SV Minicolumn assembly was removed from the centrifuge, the collection tube was emptied and the column was recentrifuged for 1 minute with the micro-centrifuge lid open (or off) to allow evaporation of any residual ethanol.
11. The SV Minicolumn was transfered to a clean 1.5ml microcentrifuge tube. 50µl of Nuclease-Free Water was applied directly to the center of the column without touching the membrane with the pipette tip.

12. The column was incubated at room temperature for 1 minute and centrifuged for 1 minute at 14,000rpm
13. The SV Minicolumn was discarded and the centrifuge tube containing the eluted DNA was stored at 4°C or .20°C.

3.9.2 Cloning:

The eluted fragments were cloned in to a vector using QIAGEN PCR Cloning Kit by the following procedure.

1. Appropriate number of tubes of QIAGEN EZ Competent Cells and SOC medium were thawed.
2. 1–2 µl of ligation-reaction mixture per tube of QIAGEN EZ Competent Cells was added, mixed gently, and incubated on ice for 5 min.
3. The tube(s) were heated in a 42°C water bath or heating block for 30 s without shaking and incubated on ice for 2 min.
4. 250 µl of SOC medium per tube was added and 100 µl of transformation mixture was used for plating onto LB agar plates containing ampicillin.
5. The plates were incubated at room temperature until the transformation mixture was absorbed into the agar. The plates were inverted and incubated at 37°C overnight (e.g., 15–18 h).
6. The positive colonies were identified by blue white screening and were confirmed by colony PCR.

3.9.3 Plasmid isolation

Plasmid was isolated using QIA prep spin miniprep kit by the following procedure

1. 5ml culture was aliquoted into fresh falcon tube from the overnight grown cultures. The tubes were spun at 3000 rpm for 10min at 4°C and supernatant was discarded gently

2. The pellet was re-suspended (bacterial cells) in 250µl P1 buffer and tapped gently .250µl of buffer P2 and 350µl of buffer N3 were added and mixed thoroughly by inverting the tube 4-6 times.

3. The tubes were centrifuged at 13000 rpm for 10 minutes in a table top microcentrifuge and the supernatant was collected and applied to QIA prep spin column by pipetting.

4. The tubes were centrifuged at high speed for one minute and the flow through was discarded.

5. 0.5ml PB buffer was added and the columns were centrifuged for one minute and the flow through was discarded

6. 0.75ml of PE buffer was added, centrifuged for one minute at high speed and the flow through was discarded.

7. The empty columns were spun for one minute more to remove residual wash buffer. The columns were placed in fresh micro centrifuge tubes and 53µl of TE buffer was added. The tubes were incubated at room temperature for one minute and centrifuged for one minute at high speed

Chapter 4

Results

Chapter IV – Results

Bacterial Leaf Blight (BLB) is a major bacterial disease of rice causing significant economic losses to farmers. Since chemical control of the disease is ineffective, resistance breeding is the best option to overcome this problem. In this regard, more than 30 genes have been identified and characterized. But due to the dynamic nature of the pathogen, there is an imminent need to identify novel genes which can combat the bacterium and protect the rice plant from the attack of the pathogen. The present study was thus conducted with the objectives to identify novel genes from promising donors identified from a set of germplasm consisting of wild rice introgression lines of different species of *oryza* and durable resistant cultivars, tag and map them with SSR markers. The results of the study are detailed below.

4.1 Screening of wild rice accessions

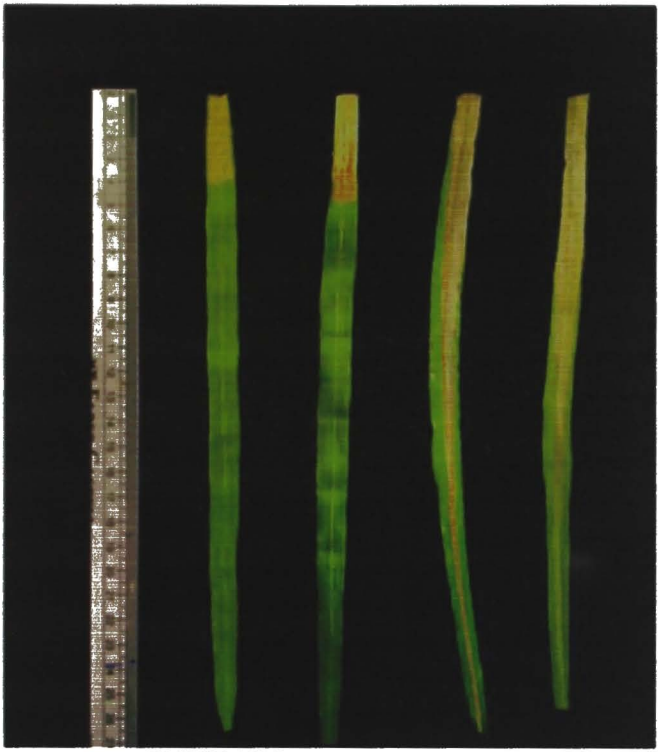
A total of 72 rice lines possessing introgressions from wild rice species belonging to *O. nivara* (8), *O. rufipogon* (32),*O. longistaminata* (7), *O. glaberrima* (4), *O. officinalis* (9) and *O. brachyantha* (12) obtained from International Rice Research Institute, Phillipines were screened for their resistance to bacterial leaf blight along with a few BLB susceptible cultivars with seven hyper virulent isolates of *Xanthomonas oryzae* pv. *oryzae* collected from different hotspot locations of India (Table 3). One introgression line of *O. brachyantha* (IR-65483-141-2-4-4-2-5-B) and the cultivar Ajaya were found to be consistently resistant (avg. lesion length less than 4 cm), while two introgression lines of *O. nivara* (IR-65483-118-25-31-7-1-5-B, IR-65483-141-2-4-4-2-5-B), six

introgression lines of *O. rufipogon* (IR-77981-19-7-B, IR-77981-34-34-B, IR-75862-139-2-5-22-B-B, IR-75862-139-2-5-22-B-B, IR-75862-143-5-38-22-B-B, IR-75862-221-2-1-3-B-B), two introgression lines of *O. longistaminata* (IR-80310-12-B-1-3-B, IR-80314-4-B-1-3-B) and one introgression line of *O. officinalis* (IR-77384-13-B-1-18-9) were moderately resistant (avg. lesion length 4-8 cm) when compared with the susceptible checks, i.e., TN1, Samba Mahsuri (BPT5204), Swarna and MTU1010 (avg. lesion length more than 15 cm). The details of resistant wild rice accessions are given in the Table 7.

Table7: List of resistant and moderately resistant wild rice introgression lines.

S. No.	Accession No.	Species	Average lesion length (cm) ± SD	Category
1	IR-65483-118-25-31-7-1-5-B	*O nivara*	6 5±1 2	Moderately resistant
2	IR-65483-141-2-4-4-2-5-B	*O nivara*	7.2±0.7	Moderately resistant
3	IR-77981-19-7-B	*O rufipogon*	5.5±0.8	Moderately resistant
4	IR-77981-34-34-B	*O rufipogon*	4 6±1 4	Moderately resistant
5	IR-75083-19-22-21-1-B-B	*O rufipogon*	5 2±0 5	Moderately resistant
6	IR-75862-139-2-5-22-B-B	*O rufipogon*	5±0 4	Moderately resistant
7	IR-75862-143-5-38-22-B-B	*O rufipogon*	7.6±0.9	Moderately resistant
8	IR-75862-221-2-1-3-B-B	*O rufipogon*	6.9±1.1	Moderately resistant
9	IR-80310-12-B-1-3-B	*O longistaminata*	4.5±1.6	Moderately resistant
10	IR-80314-4-B-1-3-B	*O longistaminata*	7 1±1 5	Moderately resistant
11	IR-77384-13-B-1-18-9	*O officinalis*	6±1 2	Moderately resistant
12	IR-65483-141-2-4-4-2-5-B	*O brachyantha*	3.02.±0.27	Resistant
13	Ajaya (IET8585)	*O sativa*	3 47±0.58	Resistant
14	TN1	*O sativa*	19 5±2.2	Susceptible
15	Samba Mahsuri	*O sativa*	16.9±2.4	Susceptible
16	Swarna	*O sativa*	18.1±2.42	Susceptible
17	MTU1010	*O. sativa*	19.5±1.6	Susceptible

Figure6: Comparison of average lesion lengths of donors and recepients



In order to study the inheritance of BLB resistance in the resistant lines identified and to tag and map the resistance genes, crosses were made between the two donors, IR-65483-141-2-4-4-2-5-B (an *O. brachyantha* introgression line) and Ajaya with two BLB susceptible varieties TN1 and BPT 5204. The F_1s of the four crosses were advanced by selfing for developing F_2 mapping populations for inheritance and mapping studies

Table 8: Resistance reaction of the donors as compared to the NILs carrying different known BLB resistance genes and their combination

S. No	Genotypes	WR-8	FZB	KPT7/99	CHN/Jhing	MTU-3	Kaul-05-2	RPR-4	Mean lesion Length (cm)
1	IR-65483-141-2-4-4-2-5-B	2 2 ± 0 23	3 ± 0 35	3 1 ± 0 15	3 7 ± 0 25	3 ± 0 16	3 2 ± 0 55	3±0 20	3 02 ± 0 27
2	Ajaya (IET8585)	3 ± 0 52	4 2 ± 0 23	4 2 ± 0 95	4 ± 0 88	3 8 ± 1 00	4 1 ± 0 25	3 8±0 23	3 47 ± 0 58
3	IRBB21 (*Xa21*)	3 5 ± 0 21	4 5 ± 0 56	4 ± 0 32	3 4 ± 0 55	4 2 ± 0 29	2 5 ± 0 65	4 2±0 82	3 75 ± 0 48
4	IRBB13 (*xa13*)	3 5 ± 0 43	2 5 ± 0 65	2 5 ± 0 36	3 6 ± 0 59	3 5 ± 0 93	3 9 ± 0 36	3±0 13	3 21 ± 0 49
5	IRBB5 (*xa5*)	5 5 ± 1 2	7 5 ± 1 3	7 ± 0 35	6 5 ± 0 9	7 33 ± 1 2	7 8 ± 0 70	8 0±1 25	7 08 ± 1 27
6	*xa5+xa13*	3 2 ± 0.96	2 ± 0 56	2.5 ± 0 98	3 3 ± 0 35	3 0 ± 0 85	2 6 ± 0 32	2 73±0 25	2 76 ± 0 61
7	*xa5+Xa21*	3 6 ± 0.95	4 5 ± 0 56	4 ± 0 63	2 8 ± 0 75	4 0 ± 0 34	2 5 ± 0 33	4 1±0 21	3 64 ± 0 53
8	*xa13+Xa21*	2 1 ± 0 56	3 ± 0 57	2 6 ± 0 20	2 5 ± 0 63	2 5 ± 0 92	3 2 ± 0 22	3 8±0 11	2 81 ± 0 45
9	*xa5+xa13 +Xa21*	2 ± 0.96	1 2 ± 0.45	2 7 ± 0 11	2.0 ± 0 23	2 4 ± 0 55	1 97 ± 0 95	2 1±0.13	2 ± 0 48
10	*Xa4+xa5 +xa13+Xa21*	1.53 ± 0 40	2 5 ± 0.4	1 5 ± 0.4	2 ± 0 3	1 8 ± 0 3	2 67 ± 0 14	2 00 ± 0 2	1 99 ± 0 36
11	TN1	21.1 ± 1.63	22.1 ± 2.0	20 6 ± 1 56	17 5 ± 2 6	15 6 ± 2 3	21 7 ± 2 3	18 1±3 2	19 5 ± 2 2
12	BPT5204	18.4 ± 3 20	16 ± 2 5	17 5 ± 1 95	16.6 ± 2 01	15 9 ± 2 0	17.7 ± 3 01	16 3±2 5	16 9 ± 2 4
13	Swarna	17.6 ± 2.4	18 2 ± 3.2	21 8 ± 2.10	18.4 ± 3 6	16 6 ± 2 5	17 3 ± 1 26	17 4±1 9	18 1 ± 2 42
14	MTU1010	17.4 ± 3.0	18 6 ± 0 9	21 6 ± 2 95	18.5 ± 2 3	20 6 ± 1	22 5 ± 0 48	17 6±0 62	19 5 ± 1 6

4.2 Bacterial blight resistance in the *O. brachyantha* introgression line IR-65483-141-2-4-4-2-5-B

4.2.1 Study of inheritance of BLB resistance in the *O.brachyantha* introgression line IR-65483-141-2-4-4-2-5-B

In order to study the inheritance of BLB resistance in the *O.brachyantha* introgression line IR-65483-141-2-4-4-2-5-B, it was crossed with BLB susceptible cultivars TN1 and Samba Mahsuri (BPT5204) as explained in materials and methods. The F_1s were screened using a hyper-virulent isolate DX-066 (Raipur) of BLB pathogen The F_1s of both the crosses were observed to be resistant to BLB with an average lesion length of

3.5 cms. The F_1s were selfed to generate F_2 mapping populations consisting of 296 individuals and 250 individuals, respectively from the cross with TN1/ IR-65483-141-2-4-4-2-5-B and BPT5204/ IR-65483-141-2-4-4-2-5-B respectively. In the F_2 generation of the cross TN1/IR-65483-141-2-4-4-2-5-B, the average lesion length ranged from 3.5 cm to 20 cm. Of the 296 F_2 plants, 225 were observed to be resistant, while 71 were susceptible which fitted well in a segregation ratio of 3R:1S (χ^2= 0.16, P=0.68) indicating the possible involvement of a single dominant gene in governing resistance in this source. Progeny testing of the 296 individuals revealed 70 plants to be homozygous resistant, 155 to be heterozygous resistant and 71 to be homozygous susceptible, which fitted well with the classical Mendelian ratio of 1:2:1 (χ^2= 0.66, P=0.71) (Table 9). The average lesion lengths in the F_2 population of the cross BPT 5204/ IR-65483-141-2-4-4-2-5-B ranged from 3 cm to 18.5 cm. Out of 250 plants screened, 195 were resistant and 55 were susceptible (3R:1S, (χ^2= 1.20,P=0.27)). F_2 progeny testing revealed 58 plants to be homozygous resistant, 137 to be heterozygous resistant and 55 to be homozygous susceptible [1:2:1, (χ^2= 2.37, P=0.30)] (Table 10). These results confirm the action of a single dominant resistance gene in IR-65483-141-2-4-4-2-5-B.

Table 9: Frequency of resistant and susceptible plants in the F_1, F_2, F_3 populations derived from the cross TN1/ IR-65483-141-2-4-4-2-5-B

Population	Number of plants			Total	χ^2 Value	*P* value
	Resistant (<5cm)	Segregating	Susceptible (>5cm)			
F_1	24	-	-	24	-	-
F_2	224	-	72	296	0.16 (3R:1S)	0.68
F_3	70	154	72	296	0.66 (1:2:1)	0.71

Table 10: Frequency of resistant and susceptible plants in the F_1, F_2, F_3 populations derived from the cross BPT 5204/ IR-65483-141-2-4-4-2-5-B

Population	Number of plants			Total	χ^2 Value	*P* value
	Resistant (<5cm)	Segregating	Susceptible (>5cm)			
F_1	15	-	-	15	-	-
F_2	195	-	55	250	1.20 (3R:1S)	0.27
F_3	58	137	55	250	2.37 (1:2:1)	0.30

4.2.2 Allelism test of IR-65483-141-2-4-4-2-5-B with near isogenic lines (NILs) of IR24 possessing known, dominant resistance genes

In order to assess whether the donor IR-65483-141-2-4-4-2-5-B possessed any of the known dominant BLB resistance genes, it was crossed with near isogenic lines of IR24 possessing known single dominant BLB resistance genes, viz., IRBB1 (*Xa1*), IRBB4 (*Xa4*), IRBB7 (*Xa7*), IRBB21 (*Xa21*) and IRBB26 (*Xa26*). The F_1s derived from these crosses were then selfed to generate F_2 plants, which were then screened for resistance to BLB. In this study, it was observed that the F_1s of all the five crosses were resistant and the F_2 plants segregated for BLB resistance in a typical ratio of 15R:1S (Table 11) indicating that the resistance gene in IR-65483-141-2-4-4-2-5-B is non-allelic to *Xa1*, *Xa4*, *Xa7*, *Xa21* and *Xa26* and the gene could be novel.

Table 11: Allelism tests for known dominant resistance genes.

S.No.	Cross	No of F_2 plants		χ^2 Value	*P* value
		Resistant	Susceptible		
1	IR-65483-141-2-4-4-2-5-B/ IRBB1 .	166	6	2.23 (15R:1S)	0.13
2	IR-65483-141-2-4-4-2-5-B/ IRBB4	140	5	1.94 (15R:1S)	0.16
3	IR-65483-141-2-4-4-2-5-B/ IRBB7	181	8	1.31 (15R:1S)	0.25
4	IR-65483-141-2-4-4-2-5-B/ IRBB21	203	18	1.35 (15R:1S)	0.24
5	IR-65483-141-2-4-4-2-5-B/ IRBB26	126	12	1.41 (15R:1S)	0.23

4.2.3 Parental Polymorphic survey and Bulked segregating Analysis (BSA):

Parental polymorphism survey was carried out using SSR markers spread across all the 12 chromosomes of rice. The polymorphic markers were then subjected to bulked segregant analysis (BSA) (Michelmore *et al.*, 1991) to identify the tentative location of the resistance gene. Out of 282 SSR markers tested, 261 (71.1%) were monomorphic and 106 (28.8%) were polymorphic between the parents IR-65483-141-2-4-4-2-5-B and TN1 (Table12). Polymorphism percentage ranged from as low as 9.4% for markers on chromosome 1 to 55.5% with respect to markers on chromosome 11. The details of polymorphic SSR markers on each chromosome are listed in Table 12.

Table 12 : List of parental polymorphic SSR markers

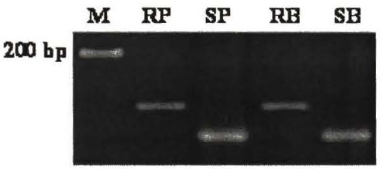
S. No.	Chromosome No.	Name of marker	Physical position (Mb)
1	1	RM3174	5
2	1	RM582	9.19
3	1	RM8094	11.23
4	1	RM595	15.19
5	1	RM5	23.96
6	1	RM3143	26.8
7	1	RM14	35
8	2	RM110	1.32
9	2	RM12690	6
10	2	RM13063	12.268
11	2	RM13263	18.187
12	2	RM3874	23.8
13	2	RM263	25.88
14	2	RM208	35.16
15	3	RM3467	5.97
16	3	RM14723	9.19
17	3	RM15288	18.94
18	3	RM15494	23.67
19	3	RM85	34
20	3	RM448	35
21	4	RM307	3.15
22	4	RM16396	3.42
23	4	RM16447	4.76
24	4	RM16569	9.72
25	4	RM16592	11.36
26	4	RM16309	11.59
27	4	RM16913	19.82
28	4	RM17093	22.99
29	4	RM3367	24.26
30	4	RM17162	24.44
31	4	RM470	28.24
32	4	RM17352	28.47
33	4	RM17405	29.8

34	4	RM5709	32.09
35	5	RM1248	0.06
36	5	RM122	0.27
37	5	RM413	2.15
38	5	RM289	7.78
39	5	RM13	8.58
40	5	RM249	10.67
41	5	RM164	19.11
42	5	RM39	24
43	5	RM274	26.82
44	5	RM480	27.15
45	5	RM26	27.32
46	6	RM190	1.76
47	6	RM19603	5.84
48	6	RM19819	10.32
49	6	RM20023	15.62
50	6	RM20168	19.48
51	6	RM3	26.86
52	6	RM30	27.25
53	7	RM295	0.41
54	7	RM3859	8.91
55	7	RM21591	17.51
56	7	RM70	19.38
57	7	RM2752	22.49
58	7	RM18	25.65
59	7	RM248	29.28
60	8	RM1384	11.84
61	8	RM42	19.96
62	8	RM223	20.64
63	8	RM3459	20.64
64	8	RM5493	26.14
65	8	RM6765	26.37
66	8	RM23460	26.54
67	9	RM23872	6.27
68	9	RM24022	9.68
69	9	RM434	15.6
70	9	RM24542	18.11

71	9	RM24654	19.9
72	9	RM215	20.88
73	9	RM24717	20.89
74	10	RM24864	0.13
75	10	RM474	1.79
76	10	RM216	4.98
77	10	RM1375	16.2
78	10	RM1873	17.37
79	10	RM1937	17.48
80	10	RM333	21.92
81	11	RM6288	2.15
82	11	RM332	2.82
83	11	RM6085	3.02
84	11	RM167	4.05
85	11	RM4469	6.18
86	11	RM6115	8
87	11	RM202	8.9
88	11	RM5777	10.07
89	11	RM5857	11.75
90	11	RM26671	15.5
91	11	RM26702	16.37
92	11	RM287	16.6
93	11	RM260	18
94	11	RM206	21.62
95	11	RM2191	24.27
96	11	RM224	26.75
97	12	RM1080	0.9
98	12	RM28067	14.74
99	12	RM28102	15.95
100	12	RM511	17.44
101	12	RM28217	18.57
102	12	RM28346	20.98
103	12	RM28363	21.21
104	12	RM3331	23.52
105	12	RM6693	23.83
106	12	RM28706	25.99

All the 106 polymorphic SSR markers were tested for bulked-segregant analysis (BSA) using the pooled DNA samples isolated from ten each of homozygous resistant and homozygous susceptible F_2 plants of the cross TN1/ IR-65483-141-2-4-4-2-5-B as described in the materials and methods. The polymorphic SSR markers on Chromosomes 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 did not show any bulk specific amplification pattern, but the SSR marker RM595 located on chromosome 1, showed bulk specific amplification, wherein the resistant parent and resistant bulk exhibited fragments of similar size (150bp) while the susceptible parent and susceptible bulk showed similar alleles (130bp) (Figure 7).

Figure 7: Bulk specific amplification of RM595



M-Molecular weight marker (100bp ladder), RP- Resistant parent, IR-65483-141-2-4-4-2-5-B SP- Susceptible parent TN1, RB-Resistant bulk, SB- Susceptible bulk

4.2.4 Molecular mapping of the novel BLB resistance gene in IR-65483-141-2-4-4-2-5-B

The SSR marker RM595, which displayed bulk specific amplification was analyzed for its segregation pattern in all the 296 individuals constituting the F_2 mapping population and the linkage distance was calculated. The marker RM595 amplified polymorphic fragments of size 150 bp in IR-65483-141-2-4-4-2-5-B and 130 bp in TN1. This marker

amplified the resistant parent specific allele in homozygous condition (i.e. RR) in 74 F_2 plants, and both resistant and susceptible parent specific fragments in heterozygous condition (Rr) in 152 F_2 plants, and the susceptible parent (i.e. TN1) specific fragment in homozygous condition (i.e. rr) in 70 F_2 plants in a typical Mendelian ratio of 1:2:1 with respect to RR:Rr:rr ($\chi^2 = 0.32$, $P = 0.85$). A total of 48 recombinants were observed with respect to this marker out of the 296 F_2 plants screened and the genetic distance of the marker from the resistance gene was observed to be 10.2 cM.

Since RM595 located on 15.19Mb on Chr. 1 exhibited linkage with bacterial leaf blight resistance gene, a set of 20 SSR markers in the vicinity of RM595 (in a genomic region spanning from 12Mb to 18Mb) were tested for parental polymorphism. Out of them, 7 markers, viz., RM10773, RM10839, RM10855, RM10874, RM10916, RM11002 and RM11033 were polymorphic between IR-65483-141-2-4-4-2-5-B and TN1. These markers were then tested for their segregation pattern in the F_2 mapping population. The marker-trait segregation pattern exhibited by these markers was given below.

RM10773:

The marker amplified polymorphic fragments of size 330 bp in IR-65483-141-2-4-4-2-5-B and 380 bp in TN1 and a total of 157 recombinants were observed out of 296 F_2 individuals screened. The marker was at genetic distance of 32.5 cM away from the gene.

RM10839:

RM10839 amplified polymorphic fragments of 305 bp in IR-65483-141-2-4-4-2-5-B and 340 bp in TN1. Co-segregation analysis with the marker revealed 131 recombinants out of 296 F_2 individuals tested , with the marker being located at a genetic distance of 26.4 cM.

RM10855:

The marker RM10855 amplified polymorphic fragments of size 175 bp in IR-65483-141-2-4-4-2-5-B and 185 bp in TN1. A total of 108 recombinants were observed out of the 296 F_2 plants screened, with this marker being 21.5 cM away from the gene.

RM10874:

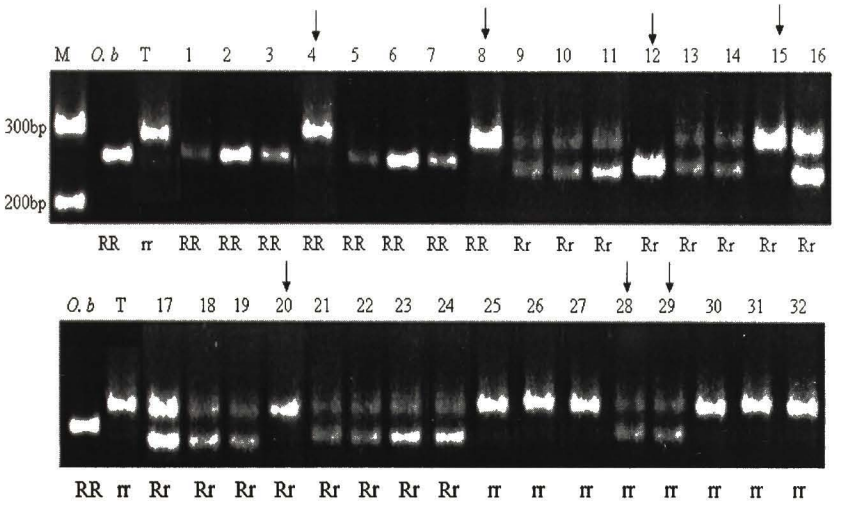
The marker RM10874 displayed amplification of a fragment of size 90 bp in IR-65483-141-2-4-4-2-5-B and 105 bp in TN1 and a total of 96 recombinants were observed out of 296 F_2 individuals screened. The marker was at genetic distance of 19.1 cM away from the gene.

RM10916:

The marker RM10916 displayed amplification of a fragment of size 270 bp in IR-65483-141-2-4-4-2-5-B and 290 bp in TN1 and a total of 72 recombinants were observed out of 296 F_2 individuals screened. The marker was at genetic distance of 14 cM away from the

gene. The amplification pattern of RM10916 in a set of 32 F_2 plants was given in Figure 8.

Figure 8 : Segregation pattern of RM10916 in the F_2 population of the cross TN1 x IR-65483-141-2-4-4-2-5-B



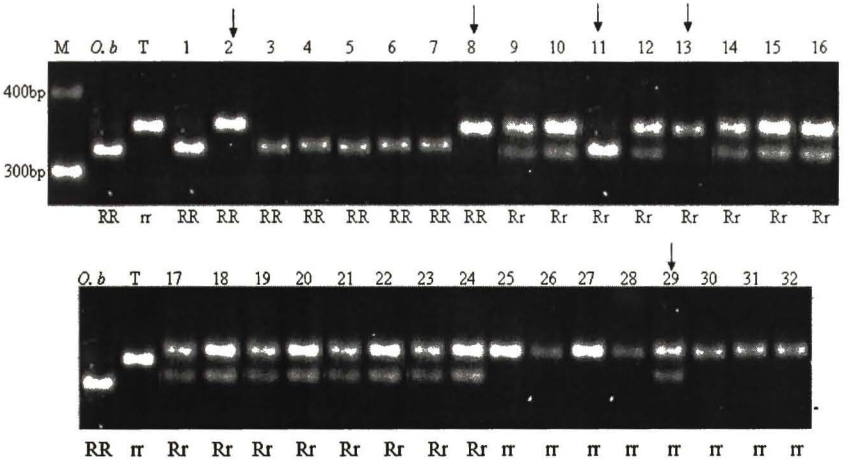
O.b-O.brachyantha, T-TN1, M-Molecular weight marker (100bp ladder). Numbers on top of the gel represent the F_2 plant while the genotype of the plant (as deduced from progeny testing at F_3) is represented at the bottom of the gel. RR and rr indicate homozygous resistant and homozygous susceptible F_2 respectively, while Rr represents heterozygous resistant plants. Arrows on top of the gel indicate recombinants

RM11002:

The marker RM11002 displayed amplification of a fragment of size 330 bp in IR-65483-141-2-4-4-2-5-B and 360 bp in TN1 and a total of 80 recombinants were observed out of 296 F_2 individuals screened. The marker was at genetic distance of 15.2 cM away from

the gene. The amplification pattern of RM11002 in a set of 32 F_2 plants was given in Figure 9.

Figure 9 : Segregation pattern of RM11002 in the F_2 population of the cross TN1 x IR-65483-141-2-4-4-2-5-B



O.b-O.brachyantha, T-TN1, M-Molecular weight marker (100bp ladder). Numbers on top of the gel represent the F_2 plant while the genotype of the plant (as deduced from progeny testing at F_3) is represented at the bottom of the gel. RR and rr indicate homozygous resistant and homozygous susceptible F_2 respectively, while Rr represents heterozygous resistant plants. Arrows on top of the gel indicate recombinants

RM11033:

The marker RM11033 displayed amplification of a fragment of size 90 bp in IR-65483-141-2-4-4-2-5-B and 105 bp in TN1 and a total of 88 recombinants were observed out of

296 F_2 individuals screened. The marker was at genetic distance of 17.9 cM away from the gene.

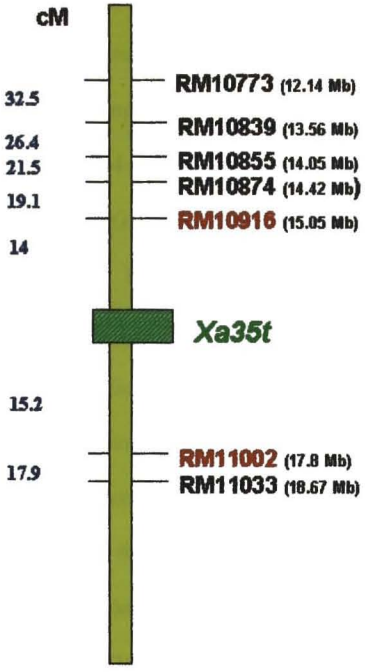
The segregation pattern of all the above said markers was tabulated in the Table 13.

Table 13 : Recombination pattern of the polymorphic SSR markers

S.No.	SSR marker	Physical position (Mb)	No of recombinants	Genetic distance (cM)
1	RM10773	12.145	157	32.5
2	RM10839	13.563	131	26.4
3	RM10855	14.051	108	21.5
4	RM10874	14.424	96	19.1
5	RM10916	15.052	72	14.0
6	RM11002	17.805	80	15.2
7	RM11033	18.673	88	17.9

Data analysis using MAPMAKER software as described in materials and methods mapped the markers RM10773, RM10839, RM10855, RM10874, and RM10916 on one side of the gene, while the markers RM11002 and RM11033 were mapped on the other side. Since no other BLB resistance gene has been mapped in the vicinity of 16 MB genomic region on Chr. 1 and since the resistance reaction of the gene was different from other known, dominant BLB resistance genes and based on allelism tests, the gene was confirmed to be novel and was tentatively named as *Xa35t*. Figure 10 depicts the linkage map of Chr. 1 showing the relative position of the newly identified gene *Xa35t* when analyzed with polymorphic SSR markers. Since none of these markers are close enough for routine use in marker-assisted breeding, fine mapping analysis was attempted using a set of SSR markers located in the vicinity of RM10916 and RM11002, which were the closest and flanking the gene.

Figure 10 : Coarse map of *Xa35t* on Chr.1



The SSR markers RM10916 and RM11002 were found to be flanking *Xa35t* at a distance of 14 and 15.2cM respectively.

4.2.5 Fine mapping of *Xa35t*:

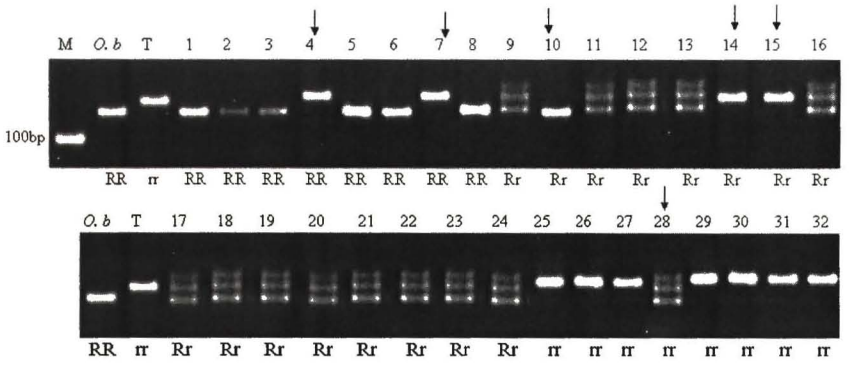
To further narrow down the genomic region where the novel BLB resistance gene from the introgression line IR-65483-141-2-4-4-2-5-B is located, a set of nine hyper-variable SSR markers located in the genomic region spanned by the SSR markers RM10916 and RM11002 were used for fine mapping analysis in the F_2 mapping population derived from the cross TN1/ IR-65483-141-2-4-4-2-5-B. RM10916 is located at 15.052Mb on the rice genome (Pseudo molecule 4 of *O. sativa* subsp. *Japonica,* cv. Nipponbare), while RM11002 is located at 17.805 Mb. In addition to the SSR markers available from Gramene database (http://gramene.org) in the genomic region defined by RM10916 and RM11002, the genomic region was also mined for new set of SSR markers. A set of of nine SSR markers from RM series and a set of eight new SSR markers (RMCG1 to RMCG8) were designed and synthesized based on the microsatellite repeats identified in the genomic between RM10916 and RM11002. Among these, four markers from RM series, viz., RM10920, RM10963, RM10974 and RM10975 and four newly designed SSR markers, viz., RMCG5, RMCG6, RMCG7 and RMCG8 were observed to be polymorphic between the parental lines (IR-65483-141-2-4-4-2-5-B and TN1). The details of marker-trait co-segregation pattern of the above said markers were given below.

RM10920:

The marker displayed amplification of IR-65483-141-2-4-4-2-5-B specific fragment in homozygous fashion in 74 F_2 plants, heterozygous amplification pattern in 150 F_2 plants and TN1 specific fragment in homozygous condition in 72 F_2 plants thus segregating in the ratio of 1:2:1 (χ^2 = 0.08, P = 0.96). Based on the amplification pattern the marker

could predict the trait phenotype in 70 of 70 homozygous resistant plants, 150 of 154 heterozygous resistant plants, and 72 out of 72 homozygous susceptible plants (Table 14). The marker was at a genetic distance of 9.4 cM away from the gene. The amplification pattern of RM10920 in a set of 32 F_2 plants was given in Figure 11.

Figure11 : Segregation pattern of RM10920 in the F_2 population of the cross TN1 x IR-65483-141-2-4-4-2-5-B



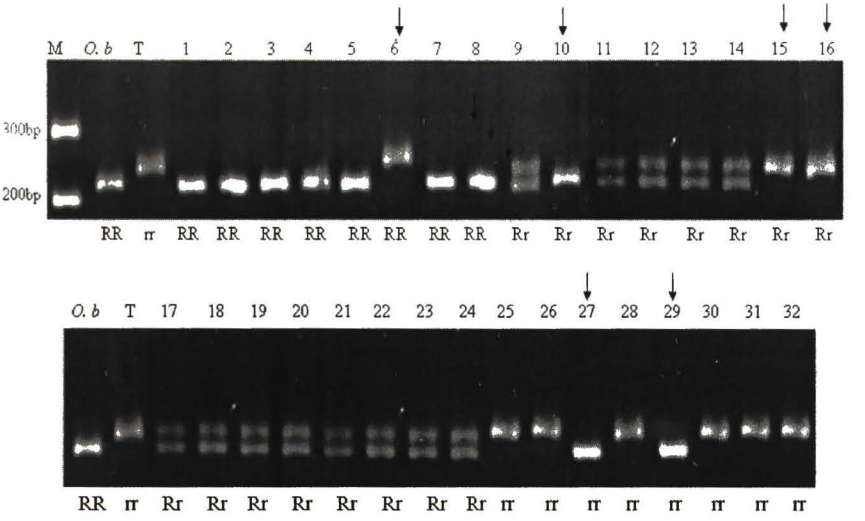
O. b-O. brachyantha, T-TN1, M-Molecular weight marker (100bp ladder). Numbers on top of the gel represent the F_2 plant while the genotype of the plant (as deduced from progeny testing at F_3) is represented at the bottom of the gel. RR and rr indicate homozygous resistant and homozygous susceptible F_2 respectively, while Rr represents heterozygous resistant plants. Arrows on top of the gel indicate recombinants

RM10963:

The marker displayed amplification of IR-65483-141-2-4-4-2-5-B specific fragment in homozygous fashion in 70 F_2 plants, heterozygous amplification pattern in 162 F_2 plants and TN1 specific fragment in homozygous condition in 64 F_2 plants thus segregating in the ratio of 1:2:1 (χ^2 = 2.89, P = 0.23). Based on the amplification pattern the marker could predict the trait phenotype in 70 of 70 homozygous resistant plants, 154 of 154 heterozygous resistant plants and 64 out of 72 homozygous susceptible plants (Table 14).

The marker was at a genetic distance of 7.5 cM away from the gene. The amplification pattern of RM10963 in a set of 32 F_2 plants was given in Figure 12.

Figure 12 : Segregation pattern of RM10963 in the F_2 population of the cross TN1 x IR-65483-141-2-4-4-2-5-B



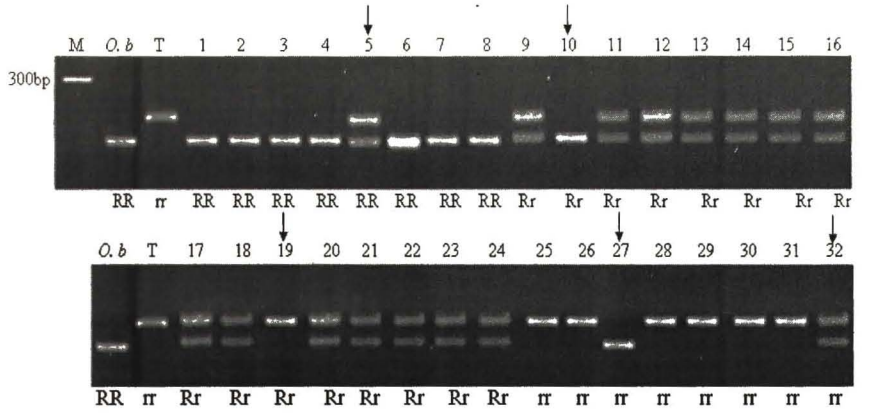
O. b-O. brachyantha, T-TN1, M-Molecular weight marker (100bp ladder). Numbers on top of the gel represent the F_2 plant while the genotype of the plant (as deduced from progeny testing at F_3) is represented at the bottom of the gel. RR and rr indicate homozygous resistant and homozygous susceptible F_2 respectively, while Rr represents heterozygous resistant plants. Arrows on top of the gel indicate recombinants

RMCG5:

The marker displayed amplification of IR-65483-141-2-4-4-2-5-B specific fragment in homozygous fashion in 76 F_2 plants, heterozygous amplification pattern in 155 F_2 plants and TN1 specific fragment in homozygous condition in 65 F_2 plants thus segregating in the ratio of 1:2:1 (χ^2 = 1.47, P = 0.47). Based on the amplification pattern the marker could predict the trait phenotype in 70 of 70 homozygous resistant plants, 154 of 154 heterozygous resistant plants, and 65 out of 74 homozygous susceptible plants (Table 14).

The marker was at a genetic distance of 6 cM away from the gene. The amplification pattern of RMCG5 in a set of 32 F_2 plants was given in Figure 15.

Figure 13 : Segregation pattern of RMCG5 in the F_2 population of the cross TN1 x IR-65483-141-2-4-4-2-5-B



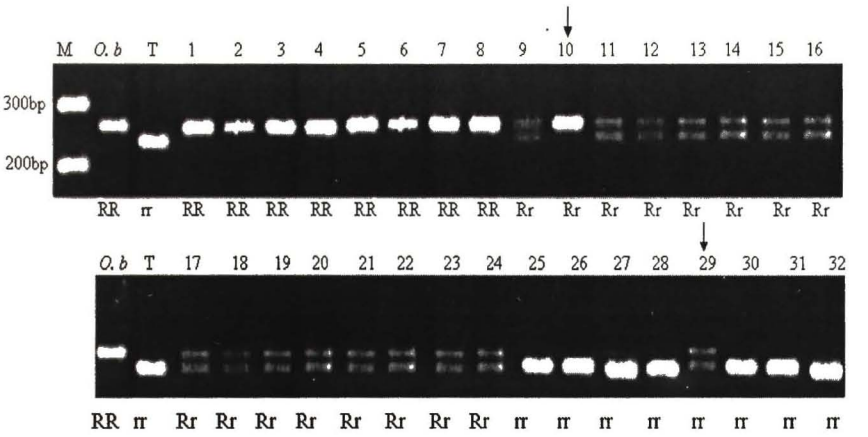
O.b-O.brachyantha, T-TN1, M-Molecular weight marker (100bp ladder). Numbers on top of the gel represent the F_2 plant while the genotype of the plant (as deduced from progeny testing at F_3) is represented at the bottom of the gel. RR and rr indicate homozygous resistant and homozygous susceptible F_2 respectively, while Rr represents heterozygous resistant plants. Arrows on top of the gel indicate recombinants

RM10974:

The marker displayed amplification of IR-65483-141-2-4-4-2-5-B specific fragment in homozygous fashion in 70 F_2 plants, heterozygous amplification pattern in 153 F_2 plants and TN1 specific fragment in homozygous condition in 73 F_2 plants thus segregating in the ratio of 1:2:1 (χ^2 = 0.39, P = 0.82). Based on the amplification pattern the marker could predict the trait phenotype in 70 of 70 homozygous resistant plants, 153 of 154 heterozygous resistant plants, and 72 out of 72 homozygous susceptible plants (Table 14).

The marker was at a genetic distance of 1.85 cM away from the gene. The amplification pattern of RM10974 in a set of 32 F_2 plants was given in Figure 14.

Figure14 : Segregation pattern of RM10974 in the F_2 population of the cross TN1 x IR-65483-141-2-4-4-2-5-B



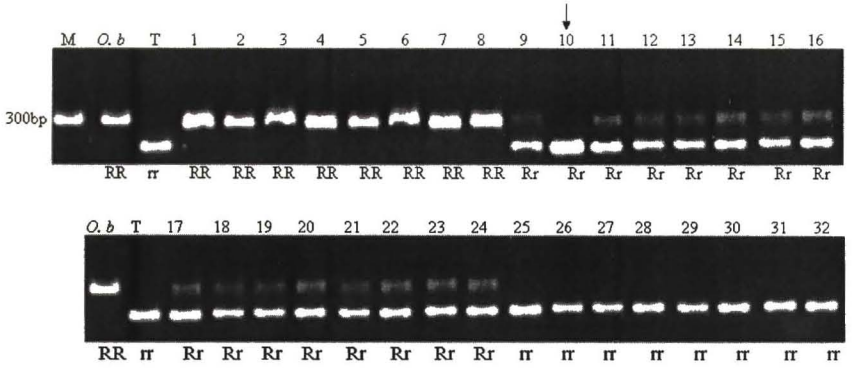
O. b-O. brachyantha, T-TN1, M-Molecular weight marker (100bp ladder). Numbers on top of the gel represent the F_2 plant while the genotype of the plant (as deduced from progeny testing at F_3) is represented at the bottom of the gel. RR and rr indicate homozygous resistant and homozygous susceptible F_2 respectively, while Rr represents heterozygous resistant plants. Arrows on top of the gel indicate recombinants

RM10975:

The marker displayed amplification of IR-65483-141-2-4-4-2-5-B specific fragment in homozygous fashion in 71 F_2 plants, heterozygous amplification pattern in 155 F_2 plants and TN1 specific fragment in homozygous condition in 70 F_2 plants thus segregating in a ratio of 1:2:1 (χ^2 = 0.66, P = 0.71). Based on the amplification pattern the marker could predict the trait phenotype in 70 of 70 homozygous resistant plants, 154 of 154

heterozygous resistant plants and 70 out of 72 homozygous susceptible plants (Table 14). The marker was at a genetic distance of 0.82 cM away from the gene. The amplification pattern of RM10975 in a set of 32 F_2 plants was given in Figure 15.

Figure 15 : Segregation pattern of RM10975 in the F_2 population of the cross TN1 x IR-65483-141-2-4-4-2-5-B



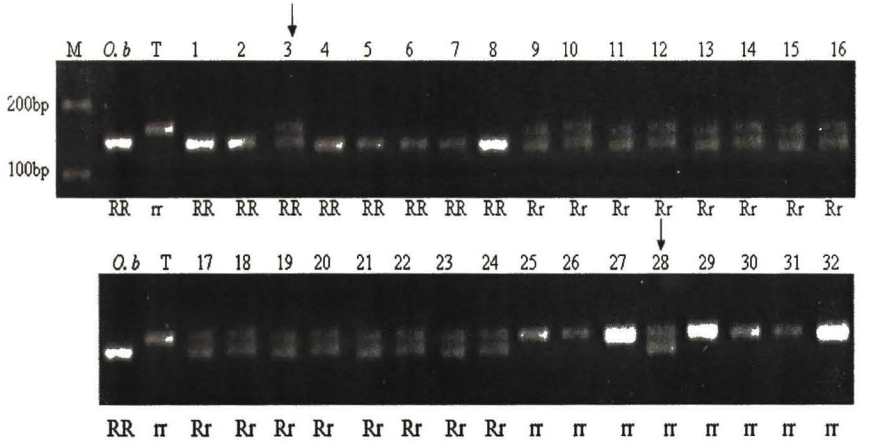
O. b-O.brachyantha, T-TN1, M-Molecular weight marker (100bp ladder). Numbers on top of the gel represent the F_2 plant while the genotype of the plant (as deduced from progeny testing at F_3) is represented at the bottom of the gel. RR and rr indicate homozygous resistant and homozygous susceptible F_2 respectively, while Rr represents heterozygous resistant plants. Arrows on top of the gel indicate recombinants

RMCG6:

The marker displayed amplification of IR-65483-141-2-4-4-2-5-B specific fragment in homozygous fashion in 71 F_2 plants, heterozygous amplification pattern in 154 F_2 plants and TN1 specific fragment in homozygous condition in 71 F_2 plants thus segregating in the ratio of 1:2:1 (χ^2 = 0.48, P = 0.78). Based on the amplification pattern the marker could predict the trait phenotype in 70 of 70 homozygous resistant plants, 154 of 154

heterozygous resistant plants and 71 out of 72 homozygous susceptible plants (Table 14). The marker was at a genetic distance of 1.8 cM away from the gene. The amplification pattern of RMCG6 in a set of 32 F_2 plants was given in Figure 16.

Figure 16 : Segregation pattern of RMCG6 in the F_2 population of the cross TN1 x IR-65483-141-2-4-4-2-5-B



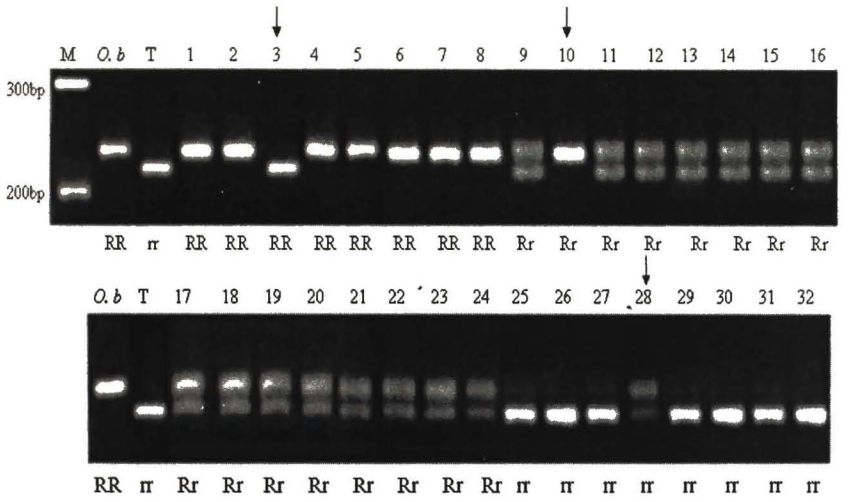
O. b-O. brachyantha, T-TN1, M-Molecular weight marker (100bp ladder). Numbers on top of the gel represent the F_2 plant while the genotype of the plant (as deduced from progeny testing at F_3) is represented at the bottom of the gel. RR and rr indicate homozygous resistant and homozygous susceptible F_2 respectively, while Rr represents heterozygous resistant plants. Arrows on top of the gel indicate recombinants

RMCG7:

The marker displayed amplification of IR-65483-141-2-4-4-2-5-B specific fragment in homozygous fashion in 63 F_2 plants, heterozygous amplification pattern in 155 F_2 plants and TN1 specific fragment in homozygous condition in 78 F_2 plants thus segregating in

the ratio of 1:2:1 (χ^2 = 2.17, P = 0.33). Based on the amplification pattern the marker could predict the trait phenotype in 63 of 70 homozygous resistant plants, 154 of 154 heterozygous resistant plants, and 72 out of 72 homozygous susceptible plants (Table 14). The marker was at a genetic distance of 5.6 cM away from the gene. The amplification pattern of RMCG7 in a set of 32 F_2 plants was given in Figure 17.

Figure 17 : Segregation pattern of RMCG7 in the F_2 population of the cross TN1 x IR-65483-141-2-4-4-2-5-B

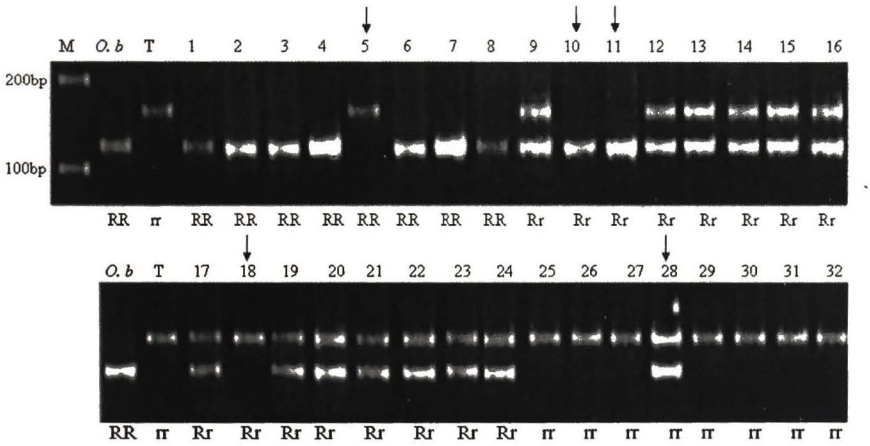


O. b-O. brachyantha, T-TN1, M-Molecular weight marker (100bp ladder). Numbers on top of the gel represent the F_2 plant while the genotype of the plant (as deduced from progeny testing at F_3) is represented at the bottom of the gel. RR and rr indicate homozygous resistant and homozygous susceptible F_2 respectively, while Rr represents heterozygous resistant plants. Arrows on top of the gel indicate recombinants

RMCG8:

The marker displayed amplification of IR-65483-141-2-4-4-2-5-B specific fragment in homozygous fashion in 66 F_2 plants, heterozygous amplification pattern in 160 F_2 plants and TN1 specific fragment in homozygous condition in 70 F_2 plants thus segregating in the ratio of 1:2:1 (χ^2 = 2.05, P = 0.35). Based on the amplification pattern the marker could predict the trait phenotype in 66 of 70 homozygous resistant plants, 148 of 148 heterozygous resistant plants and 70 out of 72 homozygous susceptible plants (Table 14). The marker was at a genetic distance of 8.4 cM away from the gene. The amplification pattern of RMCG8 in a set of 32 F_2 plants was given in Figure 18.

Figure 18 : Segregation pattern of RMCG8 in the F_2 population of the cross TN1 x IR-65483-141-2-4-4-2-5-B



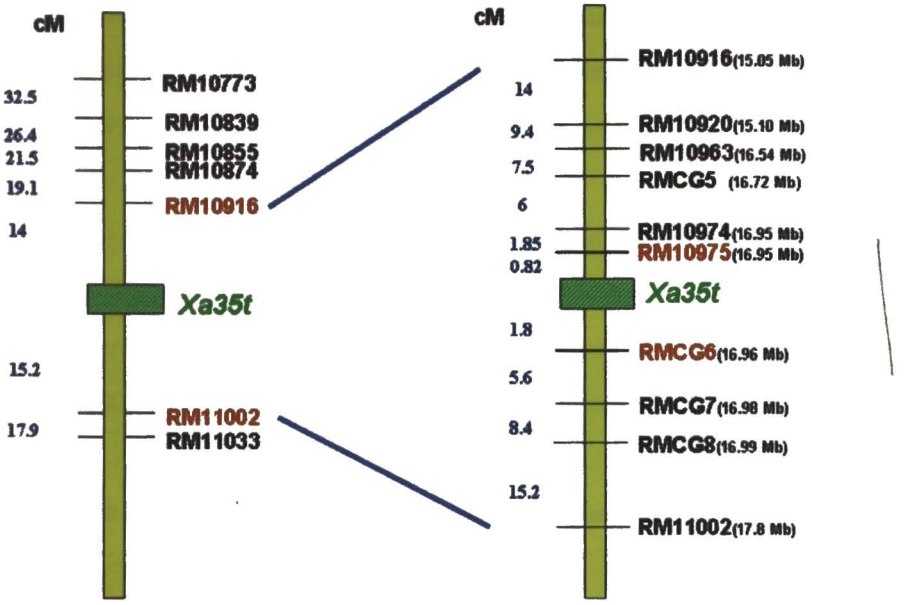
O. b-O. brachyantha, T-TN1, M-Molecular weight marker (100bp ladder). Numbers on top of the gel represent the F_2 plant while the genotype of the plant (as deduced from progeny testing at F_3) is represented at the bottom of the gel. RR and rr indicate homozygous resistant and homozygous susceptible F_2 respectively, while Rr represents heterozygous resistant plants. Arrows on top of the gel indicate recombinants

Table 14: Segregation pattern observed in the F_2 population derived from the cross TN1/ IR-65483-141-2-4-4-2-5-B

S. No.	Marker	Physical position (Mb)	Segregation pattern			Genetic distance (cM)	Segregation ratio
			No of plants showing IR-65483-141-2-4-4-2-5-B specific allele in homozygous condition (RR)	No of plants showing heterozygous amplification (Rr)	No of plants showingTN1 specific allele in homozygous condition (rr)		
1	RM10920	15 101	66	144	86	9 4	1:2:1(χ2=0 08,P=0.96)
2	RM10963	16 548	74	156	66	7 5	1;2:1(χ2=2 89,P=0.23)
3	RMCG5	16.722	80	149	67	6	1·2:1(χ2=1.47,P=0 47)
4	RM10974	16.952760	74	147	75	1.85	1:2:1(χ2=0.39,P=0.82)
5	RM10975	16.952922	75	149	72	0.82	1:2:1(χ2=0.66,P=0 71)
6	RMCG6	16.968	75	148	73	1 8	1·2:1(χ2=0.48,P=0 78)
7	RMCG7	16.982	67	149	80	5 6	1.2:1(χ2=2.17,P=0.33)
8	RMCG8	16.997	70	154	72	8 4	1:2:1(χ2=2.05,P=0 35)

When the data obtained from the SSR markers used for fine mapping of the novel gene was analyzed using Mapmaker software, the markers RM10920, RM10963, RMCG5 RM10974 and RM10975 were observed to be located on one side of the gene at a genetic distance of 9.4 cM, 7.5 cM, 6 cM 1.85 cM and 0.82cM respectively while RMCG6, RMCG7 and RMCG8 were located on the other side of the gene at a genetic distance of 1.8 cM, 5.6 cM, and 8.4 cM respectively (Figure 19). RM10975 and RMCG6 were observed to be very close to the gene and flanking it.

Figure 19 :Genetic linkage map of the genomic region in the vicinity of *Xa35t* on Chr.1



The SSR markers RM10975 and RMCG6 were found to be close to the gene and flanking the novel resistance gene *Xa35t* at a genetic distance of 0.82 and 1.8cM respectively

4.2.6 Validation of the linked SSR markers in alternate mapping population

The utility of the identified SSR markers to predict resistance to bacterial leaf blight was validated in a progeny-tested F_2 mapping population consisting of 250 plants derived from the cross BPT5204/ IR-65483-141-2-4-4-2-5-B. The closely linked markers RM10974, RM10975, RMCG6, and RMCG7 exhibited polymorphism between the parents IR-65483-141-2-4-4-2-5-B and BPT5204 and their co segregation with respect to resistance/ susceptibility was studied in the above mentioned mapping population.

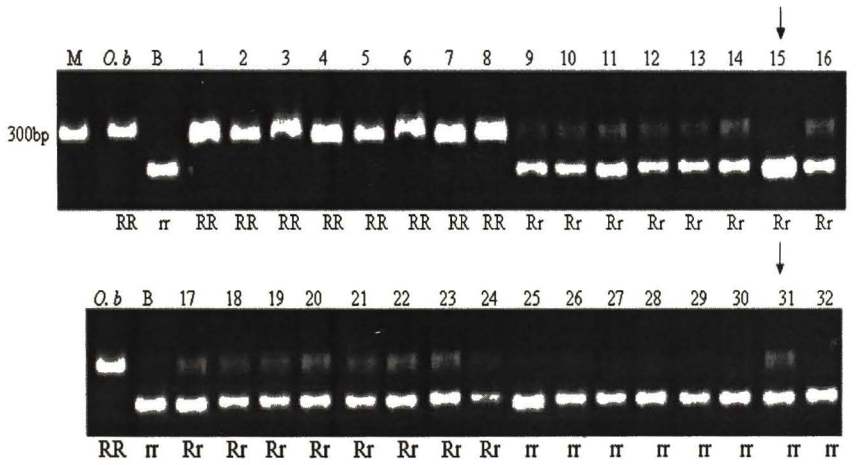
RM10974:

The marker could predict the trait phenotype in 58 of 58 homozygous resistant plants, all the 137 heterozygous resistant plants, and 54 out of 55 homozygous susceptible plants. The marker was at genetic distance of 2 cM away from the gene.

RM10975:

The marker could predict the trait phenotype in 58 of 58 homozygous resistant plants, all the 137 heterozygous resistant plants, and 54 out of 55 homozygous susceptible plants. The marker was at genetic distance of 0.8 cM away from the gene. The amplification pattern of RM10975 in a set of 32 F2 plants was given in Figure 20.

Figure 20 : Segregation pattern of RM10975 in the F_2 population of the cross BPT5204 x IR-65483-141-2-4-4-2-5-B

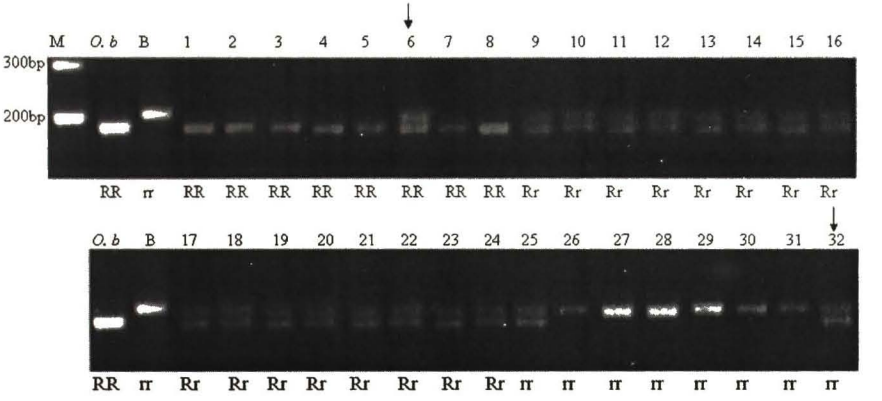


O. b-O. brachyantha, B-BPT5204, M-Molecular weight marker (100bp ladder). Numbers on top of the gel represent the F_2 plant while the genotype of the plant (as deduced from progeny testing at F_3) is represented at the bottom of the gel. RR and rr indicate homozygous resistant and homozygous susceptible F_2 respectively, while Rr represents heterozygous resistant plants. Arrows on top of the gel indicate recombinants

RMCG6

The marker could predict the trait phenotype in 57 of 58 homozygous resistant plants, 137 of 137 heterozygous resistant plants, and 55 out of 55 homozygous susceptible plants. The marker was at genetic distance of 2.2 cM away from the gene. The amplification pattern of RMCG6 in a set of 32 F_2 plants was given in Figure 21.

Figure 21 : Segregation pattern of RMCG6 in the F_2 population of the cross BPT5204 x IR-65483-141-2-4-4-2-5-B



O. b-O. brachyantha, B-BPT5204, M-Molecular weight marker (100bp ladder). Numbers on top of the gel represent the F_2 plant while the genotype of the plant (as deduced from progeny testing at F_3) is represented at the bottom of the gel. RR and rr indicate homozygous resistant and homozygous susceptible F_2 respectively, while Rr represents heterozygous resistant plants. Arrows on top of the gel indicate recombinants

RMCG7:

The marker could predict the trait phenotype in 58 of 58 homozygous resistant plants, 135 of 137 heterozygous resistant plants, and 54 out of 55 homozygous susceptible plants. The marker was at genetic distance of 4.8 cM away from the gene.

Table15: Comparison of the recombination distances in the two populations

Marker	Recombination distance in the cross TN1/IR-65483-141-2-4-4-2-5-B (cM)	Recombination distance In the cross BPT5204/ IR-65483-141-2-4-4-2-5-B (cM)
RM10974	1.85	2
RM10975	0.82	0.8
RMCG6	1.8	2.2
RMCG7	5.6	4.8

Linkage analysis of the marker segregation data in both the populations using Mapmaker software revealed similar marker order as well as recombination distances with the resistance gene in both the mapping populations, thus validating the markers.

4.2.7 Analysis of amplification pattern of the *Xa35t* linked SSR markers in a set of rice varieties

To examine whether the linked markers would be of use in introgression of the novel gene into elite, but BLB susceptible cultivars, DNA samples from a set of eight high priority bacterial leaf blight susceptible cultivars (TN1, Samba Mahsuri (BPT5204), Swarna, MTU1001, MTU1010, Tellahamsa, Krishnahamsa and IR64) along with the resistant wild rice accession IR-65483-141-2-4-4-2-5-B, were amplified by PCR using the closest flanking markers RM10975 and RMCG6 (Table 16).

Table 16: Amplification pattern of the novel gene linked SSR markers in a set of parental lines

S.No.	Name of the Genotype	Fragment Size (bp)	
		RM10975	RMCG6
1	IR-65483-141-2-4-4-2-5-B	300	190
2	TN1	260	220
3	Samba Mahsuri (BPT5204)	260	220
4	Swarna	260	220
5	MTU1001	260	220
6	MTU1010	260	220
7	Tellahamsa	260	220
8	Krishnahamsa	260	220
9	IR64	260	220
10	Basmati370	260	220

The marker RM10975 amplified a fragment of size 300 bp in the BLB resistant introgression line IR-65483-141-2-4-4-2-5-B while the remaining nine genotypes yielded fragments of size 210 bp. With respect to the marker RMCG6, the susceptible cultivars amplified 220 bp, while in case of IR-65483-141-2-4-4-2-5-B, a fragment of size 190 bp was observed to be amplified (Figure 22 and 23).

Figure 22 :Analysis of RM10975 in a set of high priority BLB susceptible cultivars

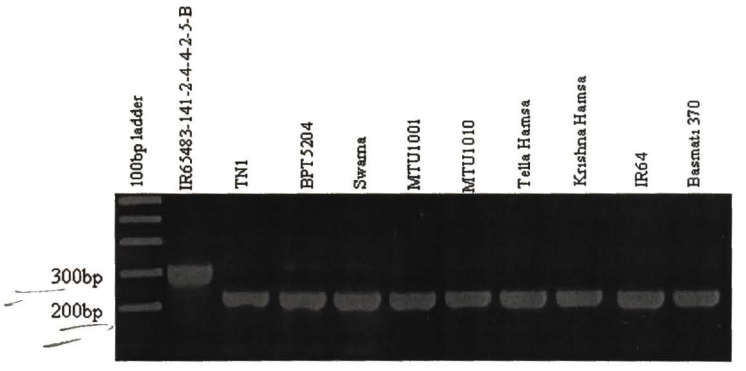
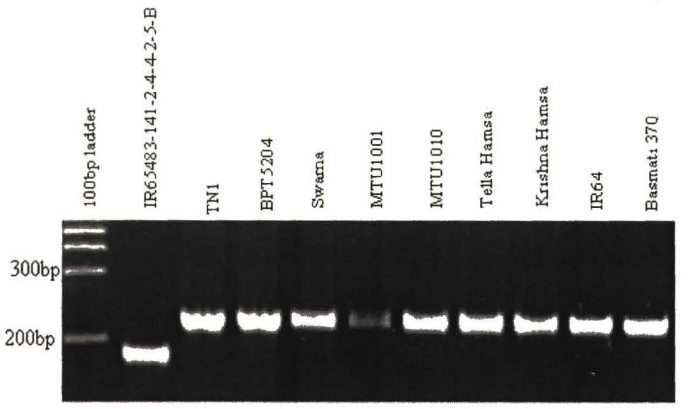


Figure 23 :Analysis of RMCG6 in a set of high priority BLB Susceptible cultivars



4.2.8 Analysis of genes present in the genomic region on Chromosome 1 flanked by SSR markers closely linked to *Xa35t*

In the fine structure genetic linkage map of *Xa35t*, the SSR marker RM10975 was located at a genetic distance of 0.82 cM on one side of the gene and another SSR marker RMCG6 was located on the other side of the gene at a genetic distance of 1.8 cM (Figure 19). The forward primer of RM10975 was observed to be physically located (start position) in the Japonica genome (http://rise.genomics.org.cn) at 16.952 Mb while the reverse primer of RMCG6 was physically located (end position) in the Japonica genome at 16.968 Mb. The intervening genomic region consisting of 16 kb of sequence was downloaded from the Japonica genome sequence database (http://rise.genomics.org.cn) and analyzed for putatively expressed genes as explained in materials and methods. A total of four genes were observed to be present in the genomic region flanked by RM10975 and RMCG6.

The average gene density was one gene for every ~ 4 kb of sequence. Of the four genes identified in the region, two genes belong to No apical meristem super family, one was a hypothetical protein and the other is PTZOO265 gene involved in multidrug resistance. The details of the list of the genes, their size in base pairs, amino acids along with their putative function(s) were given in the Table 17.

Table 17: List of genes present in the genomic region flanked by SSR markers RM10975 and RMCG6 which are closely linked to the novel gene

Gene No.	Gene start (bp)	Gene end (bp)	Gene size (bp)	No. of amino acids	Putative function of protein encoded by the gene and proteins showing homology to the gene
1	16952913	16954012	1002	333	No apical meristem super family
2	16958704	16960517	1149	382	No apical meristem super a family
3	16960783	16962569	950	316	Hypothetical protein
4	16963639	16965379	408	135	PTZ00265, Multidrug resistance

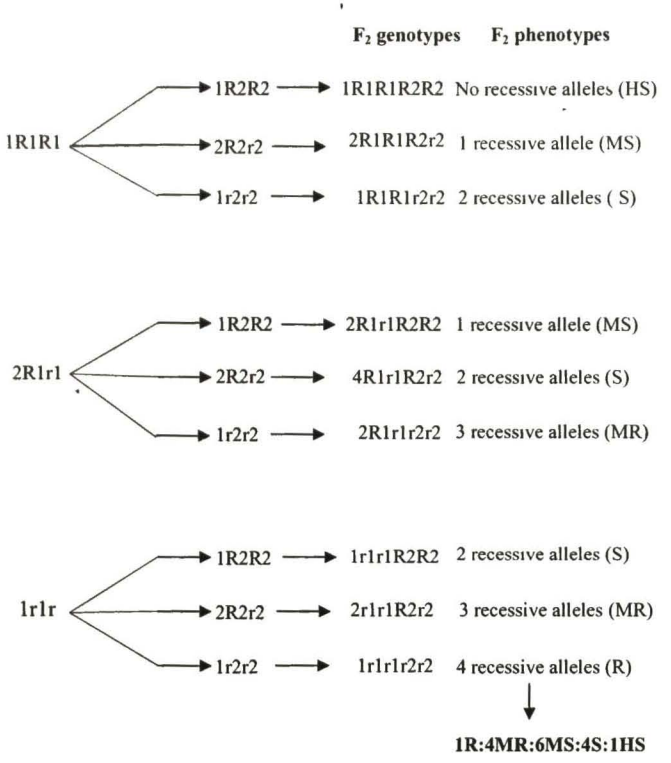
4.3 BLB resistance in Ajaya

4.3.1 Study of inheritance of BLB resistance in Ajaya

In order to study the inheritance of BLB resistance in Ajaya, it was crossed with two BLB susceptible cultivars TN1 and Samba Mahsuri (BPT5204). The F_1s were screened using the DX-066 (Raipur) isolate of BLB pathogen. The F_1s of both the crosses were observed to be intermediate to both the parents in terms of BLB resistance with an average lesion length of 12.5 cm and 11.2 cm in the cross with TN1 and BPT5204 respectively. The F_1s were selfed to generate F_2 mapping populations consisting of 400 individuals and 196 individuals in the crosses with TN1 and BPT5204 respectively.

In the F_2 generation of the cross TN1/Ajaya, the average lesion lengths ranged from 3.0 cm to 23.5 cm. Of the 400 F_2 plants screened for BLB resistance 25 plants were observed to be resistant (avg. lesion length < 4 cm), 105 were moderately resistant (avg. lesion length 4-8 cm), 132 were moderately susceptible (avg. lesion length 8-12 cm), 110 were susceptible (avg. lesion length 12-16 cm) and 28 were highly susceptible (avg. lesion length >16 cm). This fitted well in a segregation ratio of 1:4:6:4:1 (χ^2= 4.15, P=0.38) with respect to resistance, moderate resistance, moderate susceptibility, susceptibility and high susceptibility indicating the possible involvement of two incompletely dominant resistance genes with equal effects interacting additively (Figure 25).

Figure 25: Schematic illustration of segregation of two incompletely dominant genes governing resistance in Ajaya



The F_3 progeny of all the 25 resistant plants were observed to be uniformly resistant indicating that they were homozygous resistant with respect to both the resistance genes. The F_3 progeny of all the 28 highly susceptible plants were also uniformly susceptible indicating that they were homozygous susceptible with respect to both the resistance genes. The F_3 progeny of the moderately resistant, moderately susceptible and susceptible phenotypic classes segregated in various proportions. (Table 18).

Table 18: Frequency of the different phenotypic classes in the F_2 and segregation of F_3 lines of TN1/ Ajaya

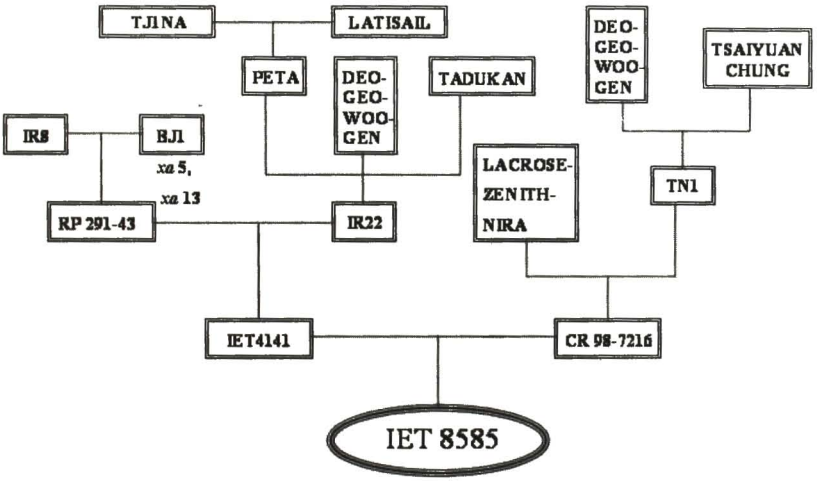
S. No.	Phenotypic class	Predicted genotype	Frequency	F_3 Segregation pattern				
				R	MR	MS	S	HS
1	Resistant (R)	r1r1r2r2	25	25	-	-	-	-
2	Moderately resistant (MR)	R1r1r2r2/ r1r1R2r2	105	segregated in 1R:2MR:1MS ratio				
3	Moderately Susceptible (MS)	R1R1r2r2/ r1r1R2R2	44	-	-	44	-	-
		R1r1R2r2	88	Segregated in1R:4MR:6MS:4S: 1HS ratio				
4	Susceptible (S)	R1r1R2R2/ R1R1R2r2	110	segregated in 1HS:2S:1MS ratio				
5	Highly Susceptible (HS)	R1R1R2R2	28	-	-	-	-	28

The average lesion lengths in the F_2 population of the cross BPT 5204/ Ajaya ranged from 3.2cm to 19.6cm. Out of 196 plants screened, 10 were resistant, 38 were moderately resistant, 89 were moderately susceptible, 43 were susceptible and 16 were highly susceptible. This fitted well in a segregation ratio of 1:4:6:4:1 (χ^2= 8.03, P=0.09) with respect to resistance, moderate resistance, moderate susceptibility, susceptibility and high susceptibility. The F_3 progeny of the ten resistant plants were uniformly resistant and the progenies of the 16 highly susceptible plants were uniformly susceptible indicating that they were homozygous resistant and homozygous susceptible respectively at both the gene loci. The F_3 progeny of the other phenotypic classes segregated in various proportions (Table 19). These results confirm the action of two incompletely dominant genes acting additively in governing resistance in Ajaya.

Table 19: Frequency of the different phenotypic classes in the F_2 and segregation of F_3 lines of BPT5204/ Ajaya

S.No.	Phenotypic class	Predicted genotype	Frequency	F_3 Segregation pattern				
				R	MR	MS	S	HS
1	Resistant (R)	r1r1r2r2	10	10	-	-	-	-
2	Moderately resistant (MR)	R1r1r2r2/ r1r1R2r2	38	segregated in 1R:2MR:1MS ratio				
3	Moderately Susceptible (MS)	R1R1r2r2/ r1r1R2R2	28	-	-	28	-	-
		R1r1R2r2	61	Segregated in1R:4MR:6MS:4S: 1HS ratio				
4	Susceptible (S)	R1r1R2R2/ R1R1R2r2	43	segregated in 1HS:2S:1MS ratio				
5	Highly Susceptible (HS)	R1R1R2R2	16	-	-	-	-	16

Figure 26: Ajaya pedigree



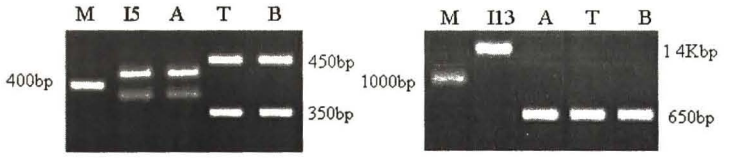
4.3.2 Screening of Ajaya with PCR based markers, which are linked to known BLB resistance genes

Since it was identified earlier that Ajaya possesses two incompletely dominant resistance genes, an attempt was made to look into its pedigree. It was observed that Ajaya has a complex pedigree and was originally derived from a cross between the lines IET4141 and CR98-7216. When the pedigree of these two lines was analyzed (Figure 26), it was observed that eight landraces, namely Tjina, Latisail, Peta, Tadukan, Deogeowoogen, Tsaiyuanchung, Lacrose-zenithnira and BJ1 and 4 cultivars namely TN1, IR8, IR22 and RP291-43 were part of their pedigree. The landrace BJ1 originating from Bangladesh is known to possess the bacterial leaf blight resistance genes *xa5* and *xa13*. Based on these observations, it was hypothesized that Ajaya may possess either or both *xa5* and *xa13*. In order to test this hypothesis, the genomic DNA of, Ajaya was analyzed with molecular markers RG556 and *xa13*-prom, which are very close (~ 0.1 cM) to *xa5* and *xa13* genes, respectively. In addition to Ajaya, the near isogenic lines carrying the resistance genes *xa5* (IRBB5) and *xa13* (IRBB13) along with two susceptible varieties TN1 and BPT5204 were also analyzed with the gene linked markers mentioned above. The details of screening are as follows:

The marker RG556 after PCR amplification and subsequent restriction digestion with the restriction enzyme DraI showed amplification of a 390 bp and a 410 bp fragment in IRBB5 (NIL with *xa5* gene) and Ajaya, while fragments of sizes 350 bp and 450 bp were observed to be amplified in the case of the BLB susceptible cultivars, TN1 and BPT5204, respectively (Figure 27A).

The maker *xa13*-prom amplified a fragment of size 1.4 kb in IRBB13 (NIL possessing *xa13*) and a 0.65 kb fragment in Ajaya and the susceptible cultivars TN1 and Samba Mahsuri (Figure 27B).

Figure 27 :Analysis of *xa5* (RG556) and *xa13* (xa13 prom) linked markers in Ajaya



A RG556 B xa13 prom

M- Molecular weight marker (1 kb ladder), I5-IRBB5, I13- IRBB13, A-Ajaya, T-TN1, B-BPT5204

4.3.3 Allelism test with near isogenic line (NIL) of IR24 possessing *xa5*

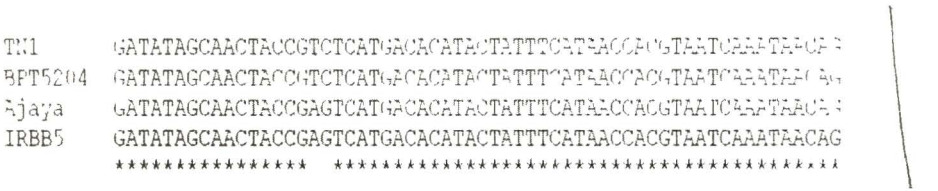
In order to confirm whether Ajaya possessed *xa5* gene as hypothesized from its pedigree, it was crossed with the NILs of IR24, IRBB5 which possessed *xa5*. It was observed that the F_1s derived from the cross IRBB5/Ajaya were resistant and all the 177 F_2 plants were also observed to be uniformly resistant with out any segregation indicating the possibility that one of the resistance genes in Ajaya could be *xa5*.

4.3.4 Sequencing the functional polymorphic region specific to the resistant allele of *xa5*:

In order to confirm the presence of *xa5* in Ajaya, the functional polymorphic region in the second exon of the *xa5* gene was PCR amplified in the genotypes Ajaya, IRBB5, TN1 and BPT5204, cloned and sequenced as explained in materials and methods. It was observed that Ajaya possessed the 2 bp polymophism (TC → AG substitution), which

was similar to that of IRBB5, while the other two susceptible cultivars did not exhibit the 2 bp substitution at the functional polymorphic region of *xa5* candidate gene (Figure 28) This confirmed that one of the resistance genes in Ajaya is indeed *xa5*.

Figure 28: Sequence alignment of the functional polymorphic region in second exon of *xa5*

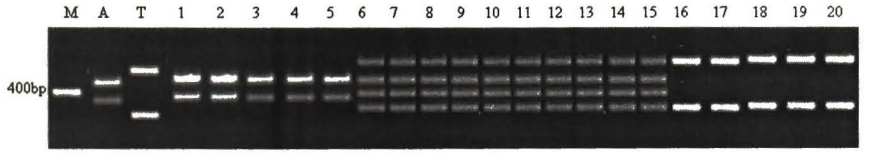


Since one of the resistance gene in Ajaya was found to be *xa5* and the other being a novel gene, before going for mapping the second gene the genotype of all the individuals of the F_2 mapping population with respect to the first R locus was studied. This data was required in order to predict the genotype of the individuals with respect to the second resistance gene locus as the interaction between the genes is additive. In this interaction the genotype of a plant cannot be known just based on the F_3 segregation data. For eg. In the cross Ajaya/TN1, there are 105 moderately resistant plants which segregated in the ratio of 1 resistant: 2 moderately resistant: 1 susceptible in the F_3 which indicates that they are heterozygous at one or the other locus. In order to study the genotype of these plants at the second locus, the data of which is required for mapping the novel gene the genotype of the plants at the *xa5* locus was required.

4.3.5 Analysis of segregation pattern of the *xa5* gene linked marker RG556 in the mapping population

The CAPS marker RG556 linked to *xa5* was analyzed in the F_2 mapping population derived from the cross TN1 /Ajaya to study the segregation pattern. The marker amplified two fragments of sizes 390 bp and 410 bp in Ajaya while fragments of sizes 350 bp and 450bp were amplified in case of TN1. This marker amplified the resistant parent specific allele in homozygous condition (i.e. rr) in 102 F_2 plants, and both resistant and susceptible parent specific fragments (Rr) in 204 F_2 plants, and the susceptible parent (i.e. TN1) specific fragment in homozygous condition (i.e. RR) in 94 F_2 plants in a typical Mendelian ratio of 1:2:1 with respect to rr:Rr:RR (χ^2 = 0.48, P = 0.78). The marker amplified the resistant parent specific allele in homozygous condition (i.e. rr) in all the 25 resistant plants, 50 of 105 moderately resistant plants and 20 of 25 moderately susceptible plants. It showed heterozygous specific amplification in 55 of 105 moderately resistant plants, 88 of 132 moderately susceptible plants and 60 of 110 susceptible plants. It showed homozygous susceptible specific amplification in 24 of 132 moderately susceptible plants, 50 of 110 susceptible plants and in all the 28 highly susceptible plants. Figure 29 depicts the amplification of RG556 in a random set of 20 F_2 plants.

Figure 29 :Analysis of RG556 in the F_2 population of the cross TN1 x Ajaya



A-Ajaya, T-TN1, M-Molecular weight marker (100bp ladder). 1-20 are random set of F_2plants

4.3.6 Prediction of Genotype of individual F_2 plants based on F_3 segregation.

The genotype of all the 400 F_2 individuals of the cross TN1/Ajaya was predicted based on their F_3 segregation pattern as under .

- As all the 25 resistant F_2 plants in the population were uniformly resistant and showed no segregation in the F_3 and their frequency being 1 out of 16, they were considered as homozygous at both the resistance loci and their genotype was assumed to be r1r1r2r2. .
- Moderately resistant plants segregating in the ratio of 1R:2MR:1S in F_3 were supposed to be heterozygous at one or the other of the 2 loci, carry 3 recessive alleles and their genotype was assumed to be R1r1r2r2/r1r1R2r2.
- Those moderately susceptible plants whose F_3 progeny lines are uniformly moderately susceptible with out any segregation were assumed to be homozygous at both the loci and supposed to carry 2 recessive alleles and so have the genotype R1R1r2r2/r1r1R2R2.
- Those moderately susceptible plants whose F_3 progeny lines segregated in1R:4MR:6MS:4S:1HS ratio were assumed to be heterozygous at both the genic loci and supposed to carry 2 recessive alleles and so have the genotype R1r1R2r2.
- Susceptible plants whose F_3 progeny lines segregated in1HS:2S:1MS ratios were assumed to carry one recessive allele and have the genotype R1r1R2R2/R1R1R2r2.
- Since the 28 highly susceptible plants were uniformly susceptible and showed no segregation in the F_3 and their frequency being 1 out of 16,

they were considered as homozygous at both the resistance loci and their genotype was assumed to be R1R1R2R2

Since one of the resistance genes in Ajaya was identified to be *xa5*, and the interaction between the two resistance genes being additive, the genotype of each of the 400 F_2 individuals with respect to the second resistance locus was predicted based on both the F_3 segregation data and marker genotype at *xa5* locus (RG556) as shown in Table 20.

Table 20: Prediction of genotype at R2 locus based on F_3 and genotype at R1/*xa5* locus

S. No.	Phenotypic class	Frequency	F_3 segregation	Predicted genotype based on F_3	Genotype at R1/*xa5* locus	Predicted genotype at R2 locus
1	Resistant (R)	25	All R	r1r1r2r2	r1r1	r2r2
2	Moderately resistant (MR)	50	1R:2MR:1MS	r1r1R2r2/ R1r1r2r2	r1r1	R2r2
		55	1R:2MR:1MS		R1r1	R2r2
3	Moderately Susceptible (MS)	20	All MS	r1r1R2R2/ R1R1r2r2/ R1r1R2r2	r1r1	R2R2
		24	All MS		R1R1	r2r2
		88	1R:4MR:6MS : 4S:1HS		R1r1	R2r2
4	Susceptible (S)	50	1HS:2S:1MS	R1R1R2r2/ R1r1R2R2	R1R1	R2r2
		60	1HS:2S:1MS		R1r1	R2R2
5	Highly Susceptible HS)	28	All HS	R1R1R2R2	R1R1	R2R2

4.3.7 Tagging and mapping of the second BLB resistance gene in Ajaya:

One of the genes governing BLB resistance in Ajaya was identified to be *xa5* based on linked marker analysis, allelic tests and sequencing analysis. An attempt was made to tag and map the second BLB resistance gene in Ajaya. A parental polymorphism survey was carried out using SSR markers spread across all the 12 chromosomes of rice. The polymorphic markers were then subjected to bulked segregant analysis (BSA) as explained in materials and methods to identify the tentative location of the resistance gene. Out of 282 SSR markers tested, 205 (72.6 %) were monomorphic and 77 (27.3 %)

were polymorphic between the parents Ajaya and TN1. Percentage polymorphism ranged from as low as 10.7 % for markers on chromosome 2 to 44.8 % with respect to markers on chromosome 11. The details of Polymorphic SSR markers on each chromosome were listed in the Table 21.

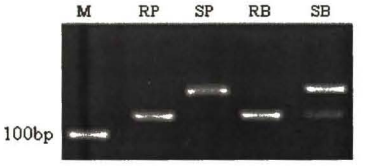
Table 21: List of parental polymorphic SSR markers

S. No.	Chromosome No.	Name of marker	Physical position
1	1	RM428	3.3
2	1	RM3174	5.09
3	1	RM583	8.32
4	1	RM577	8.37
5	1	RM3506	8.45
6	1	RM1151	8.57
7	1	RM582	9.19
8	1	RM8094	11.23
9	1	RM493	12.26
10	1	RM490	15.3
11	1	RM5638	20.93
12	1	RM1387	40.2
13	2	RM110	1.32
14	2	RM3874	23.8
15	2	RM263	25.88
16	3	RM6	29.5
17	3	RM6933	29.33
18	4	RM307	0.3
19	4	RM16396	3.42
20	4	RM16569	9.72
21	4	RM3367	24.26
22	4	RM17162	24.44
23	4	RM470	28.24
24	4	RM17405	29.8
25	4	RM5709	32.09
26	5	RM1248	0.1
27	5	RM122	2.79
28	5	RM413	2.1
29	5	RM289	7.7
30	5	RM13	8.4
31	5	RM249	10.6
32	5	RM164	19.11
33	5	RM39	23.4
34	5	RM274	26.82
35	5	RM480	27.1

36	5	RM26	27.3
37	7	RM5211	0.5
38	7	RM3859	8.9
39	7	RM70	19.2
40	7	RM2752	22.49
41	7	RM18	25.6
42	7	RM248	29.28
43	8	RM1384	11.8
44	8	RM42	19.96
45	8	RM223	20.64
46	8	RM5493	26
47	8	RM6765	26.37
48	8	RM23345	24.07
49	8	RM23476	26.26
50	9	RM110	1.32
51	9	RM263	25.88
52	9	RM3874	23.8
53	9	RM6	29
54	9	RM6933	29.33
55	10	RM24881	0.31
56	10	RM474	1.79
57	10	RM216	4.98
58	10	RM1375	16.2
59	10	RM1873	17.37
60	11	RM332	2.8
61	11	RM6085	3
62	11	RM167	4.05
63	11	RM555	4.3
64	11	RM4469	6.1
65	11	RM6115	8
66	11	RM5857	11.75
67	11	RM26666	15.32
68	11	RM26703	16.38
69	11	RM287	16.61
70	11	RM260	18.3
71	11	RM206	21.62
72	11	RM2191	24.2
73	12	RM1080	0.9
74	12	RM28064	14.7
75	12	RM28222	18.6
76	12	RM28346	20.9
77	12	RM1986	21.2

All the 77 polymorphic SSR markers were then tested for BSA using the pooled DNA samples isolated from ten each of homozygous resistant and homozygous susceptible F_2 plants derived from the cross TN1/Ajaya as described in the materials and methods. The polymorphic SSR markers on Chromosomes 1, 2, 3, 4, 6, 7, 9, 10, 11 and 12 did not show any bulk specific amplification pattern. Two SSR markers on Chr.5 namely RM122 (2.79Mb) and RM13 (8.4Mb) and one marker on Chr. 8, *viz.*, RM23476 (26.263 Mb) showed bulk specific amplification; where in the resistant parent and resistant bulk exhibited fragments of similar size while the susceptible parent and susceptible bulk showed similar alleles (Figure 30).

Figure 30 : Bulk specific amplification of RM23476



M-Molecular weight marker (100bp ladder), RP-Resistant parent Ajaya, SP-Susceptible parent TN1, RB-Resistant bulk, SB-Susceptible bulk

The marker RM23476was then tested with all the 400 individuals constituting the F_2 mapping population and the genetic distance was calculated (Since the markers RM122 and RM13 are known to be linked to *xa5*, one of the gene conferring resistance in Ajaya they were not genotyped in the population).

The marker RM23476 amplified the resistant parent specific allele in homozygous condition (i.e. RR) in 97 F_2 plants, and both resistant and susceptible parent specific

fragments in heterozygous condition (Rr) in 196 F_2 plants, and the susceptible parent (i.e. TN1) specific fragment in homozygous condition (i.e. rr) in 107 F_2 plants in a typical Mendelian ratio of 1:2:1 with respect to rr:Rr:RR (χ^2 = 0.66, P = 0.71). A total of 134 recombinants were observed in the 400 F2 plants screened and the genetic distance of the marker was observed to be 15.1 cM.

Since RM23476 located on 26.263 Mb on Chr. 8 exhibited linkage with the resistance gene in Ajaya, a set of 30 SSR markers in the vicinity of RM23476 were tested for parental polymorphism. Of these, nine markers, viz., RM23283, RM23345, RM23433, RM23471, RM23478, RM23535, RM23574, RM23601 and RM23649 were polymorphic between Ajaya and TN1. These markers were then tested for their segregation pattern in the F_2 mapping population. The marker-trait segregation pattern exhibited by these markers is given below.

RM23283:

The marker amplified polymorphic fragments of size 250 bp in Ajaya and 300 bp in TN1 and a total of 196 recombinants were observed in the F_2 population consisting of 400 individuals. The marker was observed to be at a genetic distance of 28.3 cM from the gene

RM23345:

The marker amplified polymorphic fragments of size 340 bp in Ajaya and 360 bp in TN1. A total of 185 recombinants were observed in the F_2 population consisting of 400 individuals. The marker showed a linkage distance of 26.5 cM from the gene.

RM23433:

The marker amplified polymorphic fragments of size 330 bp in Ajaya and 380 bp in TN1 and a total of 176 recombinants were observed in the F_2 population consisting of 400 individuals. The marker was at a genetic distance of 24.9 cM from the gene

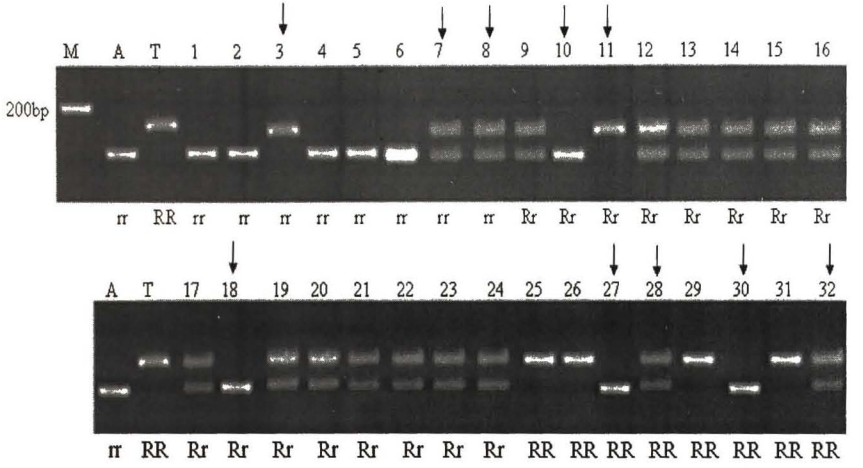
RM23471:

The marker RM23471 displayed amplification of a fragment of size 90 bp in Ajaya and 105 bp in TN1 and a total of 110 recombinants were observed in the F_2 population consisting of 400 individuals. The marker was at a genetic distance of 16.1 cM from the gene.

RM23478:

The marker RM234789 displayed amplification of a fragment of size 150 bp in Ajaya and 190 bp in TN1 and a total of 103 recombinants were observed in the F_2 population consisting of 400 individuals. The marker was at a genetic distance of 14.8 cM from the gene. The amplification pattern of RM23478 in a set of 32 F_2 plants was given in Figure 31.

Figure 31 : Segregation pattern of RM23478 in the F_2 population of the cross TN1 x Ajaya

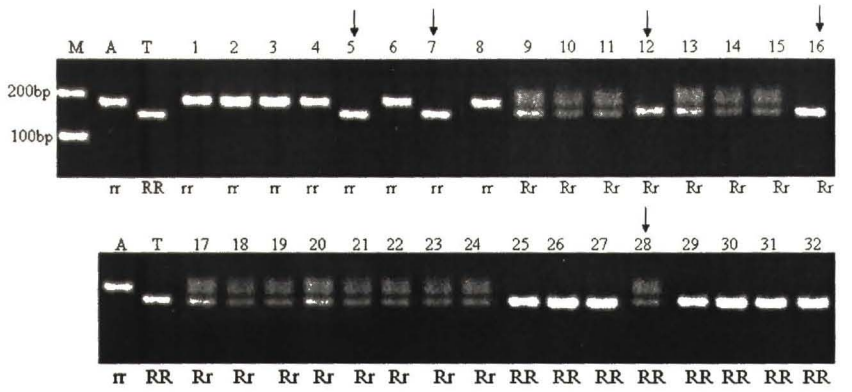


A-Ajaya, T-TN1, M-Molecular weight marker (100bp ladder). Numbers on top of the gel represent the F_2 plant while the genotype of the plant (as deduced from progeny testing at $F_3$and marker genotype of RG556) is represented at the bottom of the gel. rr and RR indicate homozygous resistant and homozygous susceptible F_2 respectively, while Rr represents heterozygous susceptible plants. Arrows on top of the gel indicate recombinants

RM23535:

The marker RM23535 displayed amplification of a fragment of size 170 bp in Ajaya and 190 bp in TN1 and a total of 107 recombinants were observed in the F_2 population consisting of 400 individuals. The marker was at genetic distance of 15.1 cM from the gene. The amplification pattern of RM23535 in a set of 32 F_2 plants was given in Figure 32.

Figure 32 : Segregation pattern of RM23535 in the F_2 population of the cross TN1 x Ajaya



A-Ajaya, T-TN1, M-Molecular weight marker (100bp ladder). Numbers on top of the gel represent the F_2 plant while the genotype of the plant (as deduced from progeny testing at $F_3$and marker genotype of RG556) is represented at the bottom of the gel. rr and RR indicate homozygous resistant and homozygous susceptible F_2 respectively, while Rr represents heterozygous susceptible plants. Arrows on top of the gel indicate recombinants

RM23574:

The marker RM23574 displayed amplification of a fragment of size 250 bp in Ajaya and 290 bp in TN1 and a total of 136 recombinants were observed in the F_2 population consisting of 400 individuals. The marker was at a genetic distance of 18.3 cM from the gene.

RM23601:

The marker RM23601 displayed amplification of a fragment of size 90 bp in Ajaya and 125 bp in TN1 and a total of 141 recombinants were observed in the F_2 population consisting of 400 individuals. The marker was at genetic distance of 20.4 cM from the gene.

RM23649:

The marker RM23601 displayed amplification of a fragment of size 120 bp in Ajaya and 105 bp in TN1 and a total of 170 recombinants were observed in the F_2 population consisting of 400 individuals. The marker was at a genetic distance of 24.6 cM from the gene. The segregation pattern of all the above said markers was tabulated in Table 22.

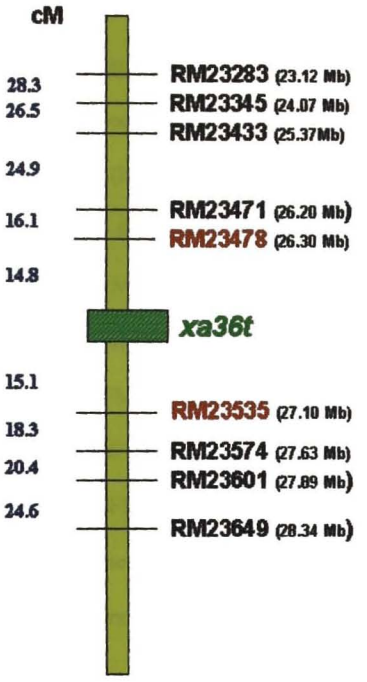
Table 22 : Recombination pattern of the polymorphic SSR markers

S.No.	SSR marker	Physical position (Mb)	No of recombinants	Genetic distance (cM)
1	RM23283	23.120	196	28.3
2	RM23345	24.075	185	26.5
3	RM23433	25.373	176	24.9
4	RM23471	26.203	110	16.1
5	RM23478	26.302	103	14.8
6	RM23535	27.102	107	15.1
7	RM23574	27.637	136	18.3
8	RM23601	27.898	138	20.4
9	RM23649	28.348	170	24.6

Data analysis using Mapmaker software mapped the markers RM23283, RM23345, RM23433, RM23460, RM23471 and RM23478 on one side of the gene, while RM23535, RM23574, RM23601 and RM23649 were mapped on the other side. Figure 33 depicts the linkage map of Chr. 8 showing the relative position of the second BLB resistance gene in Ajaya. Since no BLB resistance gene has been reported in the genomic region flanked by RM23478 and RM23535 other than *xa13* and since it was observed that the second BLB resistance gene in Ajaya is non-allelic to *xa13*, it was assumed that the gene could be novel and was tentatively named as *xa36t*. Since none of these markers are close enough

for routine use in marker-assisted breeding, fine mapping analysis was attempted using a set of SSR markers located in the vicinity of RM23478 and RM23535, which were the closest and flanking the gene.

Figure 33 : Coarse map of *xa36t* on Chr.8



The SSR markers RM23478 and RM23535 were found to be flanking *xa36t* at a distance of 14.8 and 15.1cM respectively.

4.3.8 Fine mapping of *xa36t*

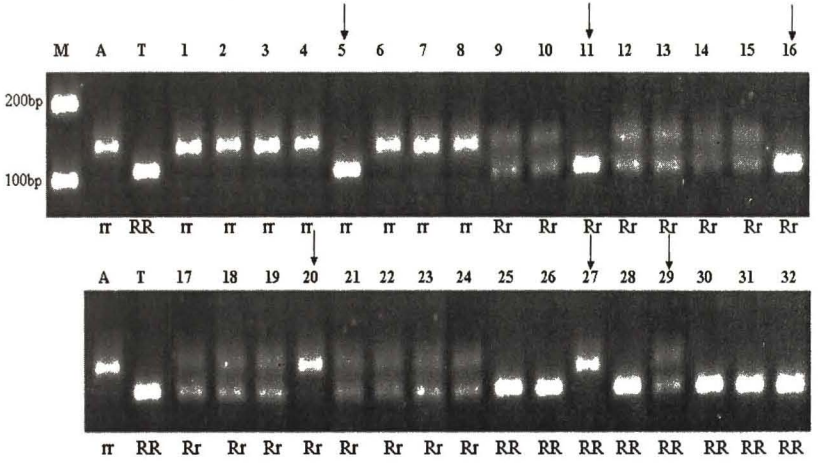
To further narrow down the genomic region where the novel BLB resistance gene, *xa36t* is located, a set of nine hyper-variable SSR markers located in the genomic region defined by the SSR markers RM23478 and RM23535 were used for fine mapping analysis in the F_2 mapping population derived from the cross TN1/Ajaya. RM23478 is located at 26.302 Kb on the rice genome (Pseudomolecule 4 of *O. sativa* subsp. *Japonica*, cv. Nipponbare), while RM23535 is located at 27.102 Kb. In addition to the SSR markers available from Gramene database (http://www.gramene.org) in the genomic region defined by RM23478 and RM23535, the genomic region was also mined for new set of SSR markers. Thus a total of nine RM series SSR markers (viz. RM23439, RM23452, RM23464, RM23469, RM23477, RM3484, RM23489, RM23496 and RM447) and six newly designed SSR markers (RMAFM1 to RMAFM6) were identified. Of these, three markers from RM series, viz., RM23489, RM23496 and RM447 and five newly designed SSR markers, viz., RMAFM1, RMAFM2, RMAFM4, RMAFM5 and RMAFM6 were polymorphic between the parental lines (Ajaya and TN1). The details of marker co-segregation pattern of the above markers were discussed below.

RM23489:

The marker displayed amplification of Ajaya specific fragment in homozygous fashion in 82 out of 104 homozygous resistant F_2 plants, heterozygous amplification pattern in 213 out of 188 heterozygous susceptible F_2 plants and TN1 specific fragment in homozygous condition in 105 out of 108 homozygous susceptible plants in a typical Mendelian ratio of 1:2:1 (χ^2 = 4.33, P = 0.11). Based on the amplification pattern of the marker in combination with RG556 it was possible to predict the trait phenotype in 21 of 25

resistant plants, 73 of 105 moderately resistant plants, 109 of 132 moderately susceptible plants, 85 of 110 susceptible plants and 20 out of 28 highly susceptible plants (Table 23) The marker was at a genetic distance of 13.6 cM away from the gene. The amplification pattern of RM23489 in a set of 32 F_2 plants was given in Figure 34.

Figure 34 : Segregation pattern of RM23489 in the F_2 population of the cross TN1 x Ajaya

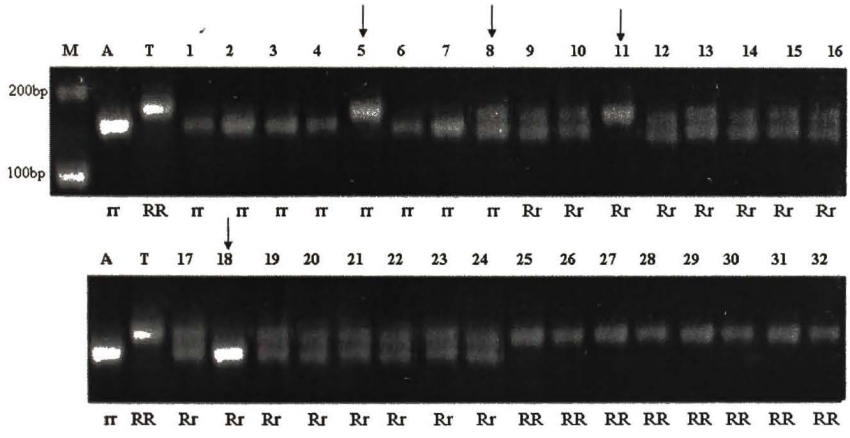


A-Ajaya, T-TN1, M-Molecular weight marker (100bp ladder). Number on top of the gel represents the F_2 plant number while the genotype of the plant (as deduced from progeny testing at $F_3$and marker genotype of RG556) is represented at the bottom of the gel. rr and RR indicate homozygous resistant and homozygous susceptible F_2 plants respectively, while Rr represents heterozygous susceptible plants. Arrows on top of the gel indicate recombinants

RM23496:

The marker displayed amplification of Ajaya specific fragment in homozygous fashion in 100 of 104 homozygous resistant F_2 plants, heterozygous amplification pattern in 192 of 188 heterozygous susceptible F_2 plants and TN1 specific fragment in homozygous condition in 108 of 108 homozygous susceptible F_2 plants in a typical Mendelian ratio of 1:2:1 (χ^2 = 0.96, P = 0.61). Based on the amplification pattern of the marker in combination with RG556 it was possible to predict the trait phenotype in 22 of 25 resistant plants, 82 of 105 moderately resistant plants, 96 of 132 moderately susceptible plants, 84 of 110 susceptible plants and 26 out of 28 highly susceptible plants (Table 23).The marker was at genetic distance of 12.2 cM away from the gene. The amplification pattern of RM23496 in a set of 32 F_2 plants was given in Figure 35.

Figure 35 : Segregation pattern of RM23496 in the F_2 population of the cross TN1 x Ajaya

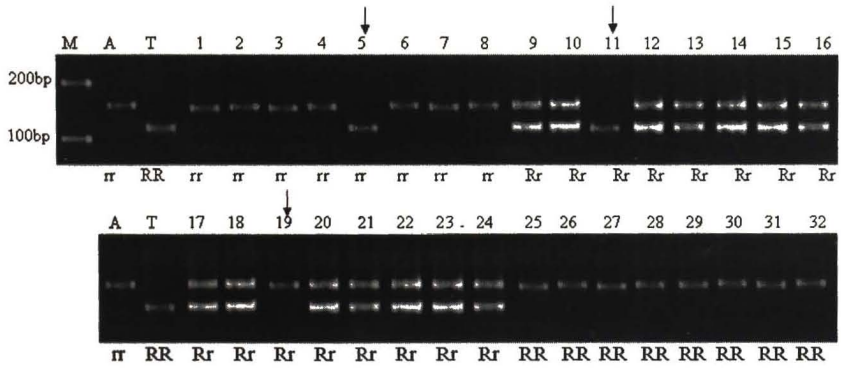


A-Ajaya, T-TN1, M-Molecular weight marker (100bp ladder). Number on top of the gel represents the F_2 plant number while the genotype of the plant (as deduced from progeny testing at $F_3$and marker genotype of RG556) is represented at the bottom of the gel. rr and RR indicate homozygous resistant and homozygous susceptible F_2 plants respectively, while Rr represents heterozygous susceptible plants. Arrows on top of the gel indicate recombinants

RMAFM2:

The marker displayed amplification of Ajaya specific fragment in homozygous fashion in 99 of 104 homozygous resistant F_2 plants, heterozygous amplification pattern in 192 of 188 heterozygous susceptible plants and TN1 specific fragment in homozygous condition in 109 of 108 homozygous susceptible plants in a typical Mendelian ratio of 1:2:1 (χ^2 = 1.14, P = 0.56). Based on the amplification pattern of the marker in combination with RG556 it was possible to predict the trait phenotype in 24 of 25 resistant plants, 97 of 105 moderately resistant plants, 102 of 132 moderately susceptible plants, 104 of 110 susceptible plants and 26 out of 28 highly susceptible plants (Table 23). The marker was at a genetic distance of 4.3 cM away from the gene. The amplification pattern of RMAFM2 in a set of 32 F_2 plants was given in Figure 36.

Figure 36 : Segregation pattern of RMAFM2 in the F_2 population of the cross TN1 x Ajaya

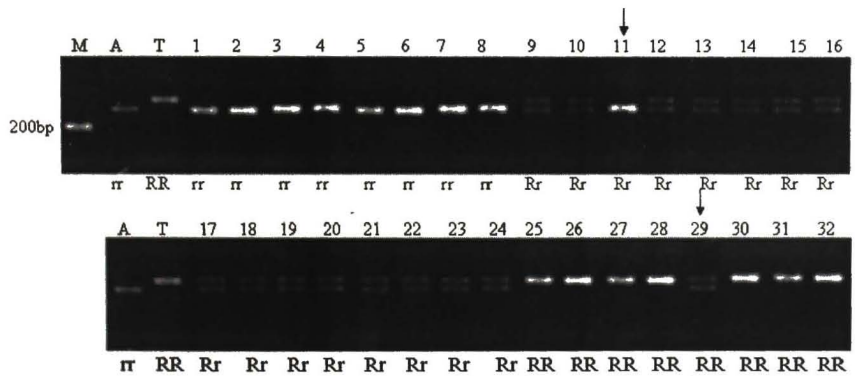


A-Ajaya, T-TN1, M-Molecular weight marker (100bp ladder). Numbers on top of the gel represent the F_2 plant while the genotype of the plant (as deduced from progeny testing at $F_3$and marker genotype of RG556) is represented at the bottom of the gel. rr and RR indicate homozygous resistant and homozygous susceptible F_2 respectively, while Rr represents heterozygous susceptible plants. Arrows on top of the gel indicate recombinants

RMAFM1:

The marker displayed amplification of Ajaya specific fragment in homozygous fashion in 105 of 104 homozygous resistant F_2 plants, heterozygous amplification pattern in 184 of 188 heterozygous susceptible plants and TN1 specific fragment in homozygous condition in 111 of 108 homozygous susceptible F_2 plants in a typical Mendelian ratio of 1:2:1 (χ^2 = 2.74, P = 0.25). Based on the amplification pattern of the marker in combination with RG556 it was possible to predict the trait phenotype in 24 of 25 resistant plants, 102 of 105 moderately resistant plants, 127 of 132 moderately susceptible plants, 106 of 110 susceptible plants and 28 out of 28 highly susceptible plants (Table 23). The marker was at a genetic distance of 1.9 cM away from the gene. The amplification pattern of RMAFM1 in a set of 32 F_2 plants was given in Figure 37.

Figure 37 : Segregation pattern of RMAFM1 in the F_2 population of the cross TN1 x Ajaya

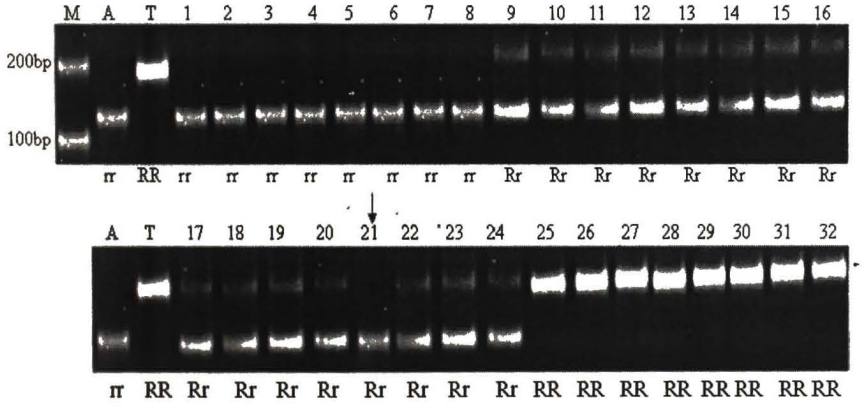


A-Ajaya, T-TN1, M-Molecular weight marker (100bp ladder). Numbers on top of the gel represent the F_2 plant while the genotype of the plant (as deduced from progeny testing at $F_3$and marker genotype of RG556) is represented at the bottom of the gel. rr and RR indicate homozygous resistant and homozygous susceptible F_2 respectively, while Rr represents heterozygous susceptible plants. Arrows on top of the gel indicate recombinants

RM447:

The marker displayed amplification of Ajaya specific fragment in homozygous fashion in 105 of 104 homozygous resistant F_2 plants, heterozygous amplification pattern in 187 of 188 heterozygous susceptible plants and TN1 specific fragment in homozygous condition in 108 of 108 homozygous susceptible plants in a typical Mendelian ratio of 1:2:1 (χ^2 = 1.73, P = 0.42). Based on the amplification pattern of the marker in combination with RG556 it was possible to predict the trait phenotype in 25 of 25 resistant plants, 103 of 105 moderately resistant plants, 131 of 132 moderately susceptible plants, 108 of 110 susceptible plants and 26 out of 28 highly susceptible plants (Table 23). The marker was at a genetic distance of 0.87 cM away from the gene. The amplification pattern of RM447 in a set of 32 F_2 plants was given in Figure 38.

Figure 38 : Segregation pattern of RM447 in the F_2 population of the cross TN1 x Ajaya

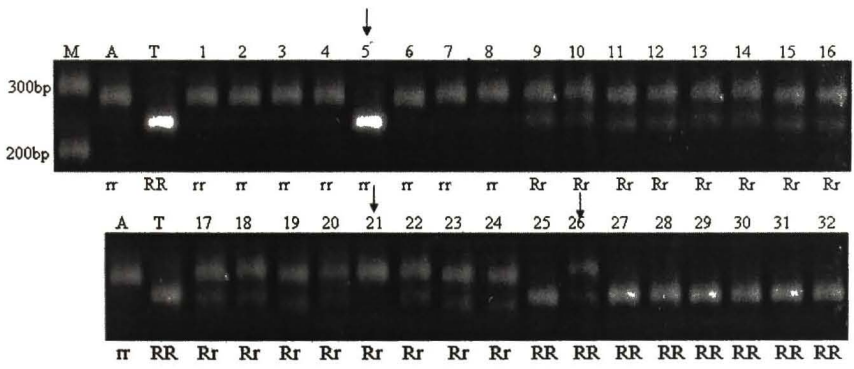


A-Ajaya, T-TN1, M-Molecular weight marker (100bp ladder). Numbers on top of the gel represent the F_2 plant while the genotype of the plant (as deduced from progeny testing at F_3 and marker genotype of RG556) is represented at the bottom of the gel. rr and RR indicate homozygous resistant and homozygous susceptible F_2 respectively, while Rr represents heterozygous susceptible plants. Arrows on top of the gel indicate recombinants

RMAFM4:

The marker displayed amplification of Ajaya specific fragment in homozygous fashion in 101 of 104 homozygous resistant F_2 plants, heterozygous amplification pattern in 191 of 188 heterozygous susceptible plants and TN1 specific fragment in homozygous condition in 108 of 108 homozygous susceptible plants in a typical Mendelian ratio of 1:2:1 (χ^2 = 1.05, P = 0.59). Based on the amplification pattern of the marker in combination with RG556 it was possible to predict the trait phenotype in 24 of 25 resistant plants, 97 of 105 moderately resistant plants, 123 of 132 moderately susceptible plants, 106 of 110 susceptible plants and 27 out of 28 highly susceptible plants (Table 23). The marker was at genetic distance of 3.1 cM away from the gene. The amplification pattern of RMAFM4 in a set of 32 F_2 plants was given in Figure 39.

Figure 39 : Segregation pattern of RMAFM4 in the F_2 population of the cross TN1 x Ajaya

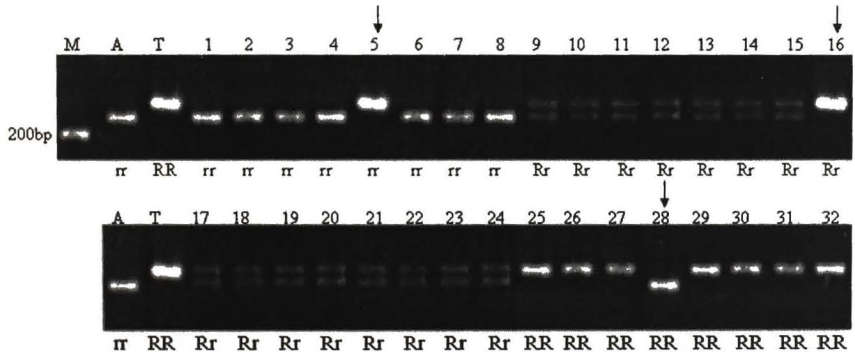


A-Ajaya, T-TN1, M-Molecular weight marker (100bp ladder). Numbers on top of the gel represent the F_2 plant while the genotype of the plant (as deduced from progeny testing at F_3 and marker genotype of RG556) is represented at the bottom of the gel. rr and RR indicate homozygous resistant and homozygous susceptible F_2 respectively, while Rr represents heterozygous susceptible plants. Arrows on top of the gel indicate recombinants

RMAFM5:

The marker displayed amplification of Ajaya specific fragment in homozygous fashion in 101 of 104 homozygous resistant F_2 plants, heterozygous amplification pattern in 184 of 188 heterozygous susceptible F_2 plants and TN1 specific fragment in homozygous condition in 115 of 108 homozygous susceptible plants in a typical Mendelian ratio of 1:2:1 (χ^2 = 3.54, P = 0.17). Based on the amplification pattern of the marker in combination with RG556 it was possible to predict the trait phenotype in 22 of 25 resistant plants, 93 of 105 moderately resistant plants, 118 of 132 moderately susceptible plants, 99 of 110 susceptible plants and 25 out of 28 highly susceptible plants (Table 23).The marker was at genetic distance of 6.2 cM away from the gene. The amplification pattern of RMAFM5 in a set of 32 F_2 plants was given in Figure 40.

Figure 40 : Segregation pattern of RMAFM5 in the F_2 population of the cross TN1 x Ajaya

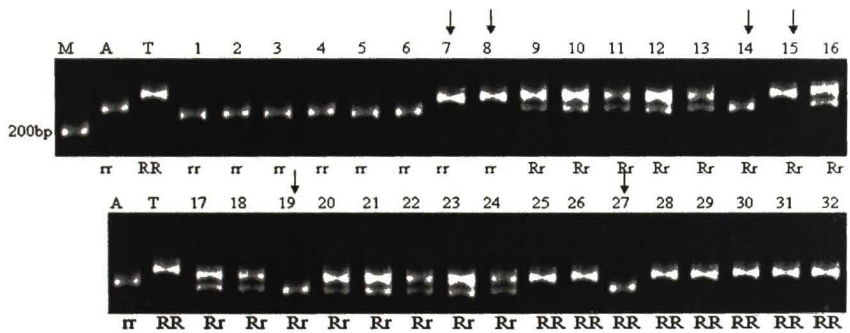


A-Ajaya, T-TN1, M-Molecular weight marker (100bp ladder). Numbers on top of the gel represent the F_2 plant while the genotype of the plant (as deduced from progeny testing at $F_3$and marker genotype of RG556) is represented at the bottom of the gel. rr and RR indicate homozygous resistant and homozygous susceptible F_2 respectively, while Rr represents heterozygous susceptible plants. Arrows on top of the gel indicate recombinants

RMAFM6:

The marker displayed amplification of Ajaya specific fragment in homozygous fashion in 88 of 104 homozygous resistant F_2 plants, heterozygous amplification pattern in 198 of 188 heterozygous susceptible plants and TN1 specific fragment in homozygous condition in 96 of 108 homozygous susceptible plants in a typical Mendelian ratio of 1:2:1 (χ^2 = 0.22, P = 0.89). Based on the amplification pattern of the marker in combination with RG556 it was possible to predict the trait phenotype in 21 of 25 resistant plants, 92 of 105 moderately resistant plants, 110 of 132 moderately susceptible plants, 97 of 110 susceptible plants and 20 out of 28 highly susceptible plants (Table 23). The marker was at genetic distance of 10.1 cM from the gene. The amplification pattern of RMAFM6 in a set of 32 F_2 plants was given in Figure 41.

Figure 41 : Segregation pattern of RMAFM6 in the F_2 population of the cross TN1 x Ajaya



A-Ajaya, T-TN1, M-Molecular weight marker (100bp ladder). Numbers on top of the gel represent the F_2 plant while the genotype of the plant (as deduced from progeny testing at $F_3$and marker genotype of RG556) is represented at the bottom of the gel. rr and RR indicate homozygous resistant and homozygous susceptible F_2 respectively, while Rr represents heterozygous susceptible plants. Arrows on top of the gel indicate recombinants

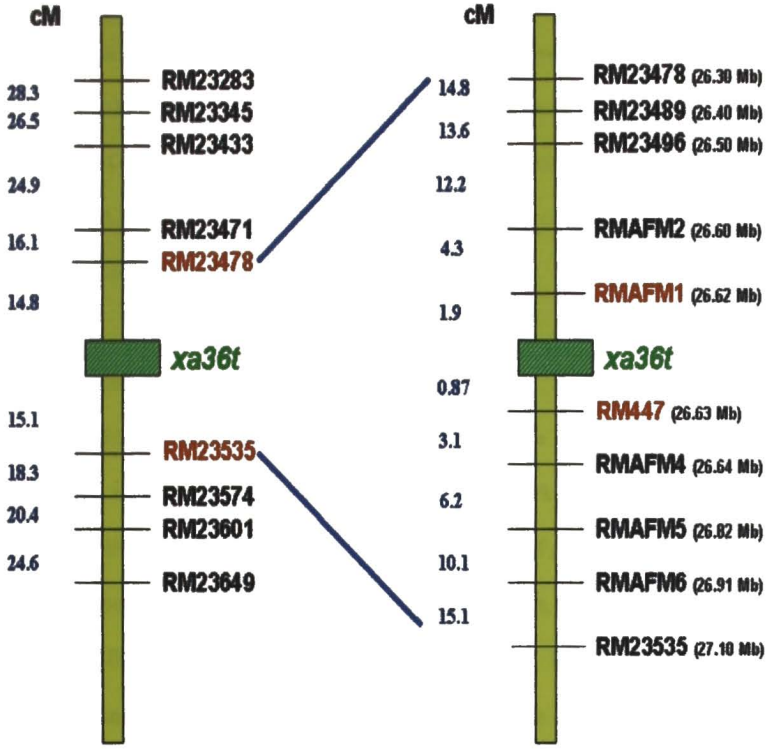
Table 23: Segregation pattern of polymorphic markers in the F_2 population derived from TN1/Ajaya

S. No.	SSR marker	Physical position (Mb)	No. of plants showing amplification of Ajaya allele in homozygous condition i.e., r2r2	No. of plants showing heterozygous amplification i.e., R2r2	No. of plants showing amplification of TN1 allele in homozygous condition i.e, R2R2	Segregation ratio	Genetic distance (cM)
1	RM23489	26 401	82	213	105	1 2 1(λ=4 33, P=0 11)	13 6
2	RM23496	26 500	100	192	108	1 2 1(λ=0 96, P-0 61)	12 2
3	RMAFM2	26 607	99	192	109	1 2 1(λ=1 14, P=0 56)	4 3
4	RMAFM1	26 624	105	184	111	1 2 1(λ=2 74, P=0 25)	1 9
5	RM447	26 637	105	187	108	1 2 1(λ=1 73, P=0 42)	0 87
6	RMAFM4	26 643	101	191	108	1 2 1(λ=1 05, P=0 59)	3 1
7	RMAFM5	26 825	101	184	115	1 2 1(λ=3 54, P=0 17)	6 2
8	RMAFM6	26 914	88	198	96	1 2 1(λ=0 22, P=0 89)	10 1

The markers RM23489, RM23496, RMAFM2 and RMAFM1 were observed to be located on one side of the gene, while RM447, RMAFM4, RMAFM5 and RMAFM6 were located on the other side. The closest flanking SSR markers RM447 and RMAFM1 were observed to be located at a genetic distance of 0.87 and 1.9 cM respectively from the gene.

When the data obtained from the SSR markers used for fine mapping of the novel gene was analyzed using Mapmaker software, the markers RM23489, RM23496, RMAFM2 and RMAFM1 were observed to be located on one side of the gene at a genetic distance of 13.6 cM, 12.2 cM, 4.3 cM and 1.9 cM respectively , while RM447, RMAFM4, RMAFM5 and RMAFM6 were located on the other side of the gene at a genetic distance of 0.87 cM, 3.1 cM, 6.2 cM and 10.1cM respectively (Figure 42). RM447 and RMAFM1 were very close to the gene and flanking it.

Figure 42 :Genetic linkage map of the genomic region in the vicinity of *xa36t* on Chr.8



The SSR markers RM447 and RMAFM1 were found to be close to the gene and flanking the novel resistance gene *xa36t* at a genetic distance of 0.87 and 1.9cM respectively

4.3.9 Validation of the linked SSR markers in alternate mapping population

The *xa5* linked CAPS marker RG556 and the SSR markers identified to be closely linked to the resistance gene *xa36t* as observed in the F_2 population of the cross TN1/Ajaya were validated in a progeny-tested F_2 population consisting of 196 plants derived from the cross BPT5204/Ajaya. The closest marker RM447 was monomorphic between Ajaya and BPT5204. RG556 and the other closer and flanking polymorphic markers RMAFM1, RMAFM2, RMAFM4, and RMAFM5 were studied for their co segregation with respect to resistance/ susceptibility in the alternate population.

4.3.9.1 Genotyping at *xa5* locus with RG556:

The marker RG556 linked to *xa5* was amplified in the whole F_2 population of the cross BPT5204/Ajaya to study the segregation pattern. This marker amplified the resistant parent specific allele in homozygous condition (i.e. rr) in 42 F_2 plants, and both resistant and susceptible parent specific fragments (Rr) in 106 F_2 plants, and the susceptible parent (i.e. TN1) specific fragment in homozygous condition (i.e. RR) in 48 F_2 plants in a typical Mendelian ratio of 1:2:1 with respect to rr:Rr:RR ($\chi^2 = 1.67$, P=0.43). The marker amplified the resistant parent specific allele in homozygous condition (i.e. rr) in all the 10 resistant plants and homozygous susceptible allele in all the 16 highly susceptible plants indicating that *xa5* conditions resistance in the alternate population derived from the cross BPT5204/Ajaya (Figure 43). The marker amplified the resistant parent specific allele in homozygous condition in 20 of 38 moderately resistant plants and 17 of 89 moderately susceptible plants. It showed heterozygous specific amplification in 18 of 38 moderately resistant plants, 51 of 89 moderately susceptible plants and 23 of 43 susceptible plants. It

showed homozygous susceptible specific amplification in 21 of 89 moderately susceptible plants and 20 of 43 susceptible plants. This data along with the F_3 phenotypic data was used to predict the corresponding genotype at the second resistance gene locus of each of the individual plants constituting the alternate population.

Figure 43 :Analysis of the CAPS marker RG556 in the F_2 population of the cross BPT5204 x Ajaya

A-Ajaya, B-BPT5204, M-Molecular weight marker (100bp ladder). 1-10 are resistant F_2 plants, 11-26 are highly susceptible F_2 plants

4.3.9.2 Segregation of markers linked to *xa36t* on Chr8

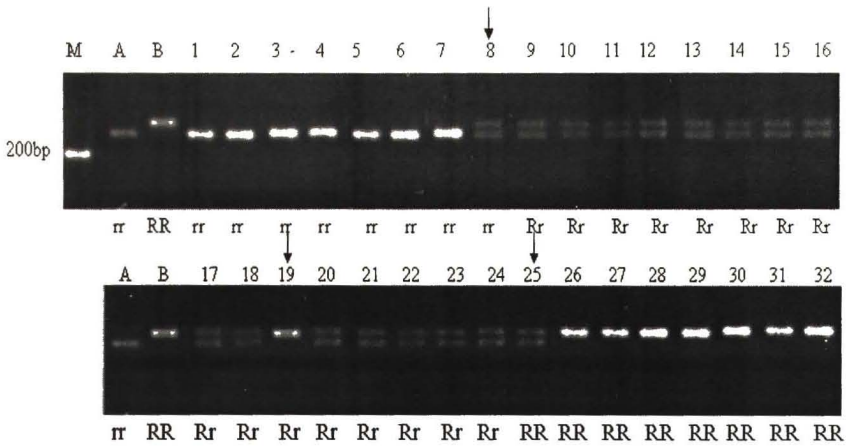
RMAFM2:

The marker in combination with RG556 could predict the trait phenotype in eight of the ten resistant plants, 35 of 38 moderately resistant plants, 81 of 89 moderately susceptible plants, 42 of 43 susceptible plants and 14 out of 16 highly susceptible plants. The marker was observed to be at a genetic distance of 5.4 cM away from the gene.

RMAFM1:

The marker in combination with RG556 could predict the trait phenotype in nine of the ten resistant plants, 36 of 38 moderately resistant plants, 86 of 89 moderately susceptible plants, 42 of 43 susceptible plants and 15 out of 16 highly susceptible plants. The marker showed a linkage distance of 2.1 cM from the gene. The amplification pattern of RMAFM1 in a set of 32 F2 plants was given in Figure 44.

Figure 44 : Segregation pattern of RMAFM1 in the F_2 population of the cross BPT5204 x Ajaya



A-Ajaya, B-BPT5204, M-Molecular weight marker (100bp ladder). Numbers on top of the gel represent the F_2 plant while the genotype of the plant (as deduced from progeny testing at $F_3$and marker genotype of RG556) is represented at the bottom of the gel. rr and RR indicate homozygous resistant and homozygous susceptible F_2 respectively, while Rr represents heterozygous susceptible plants. Arrows on top of the gel indicate recombinants

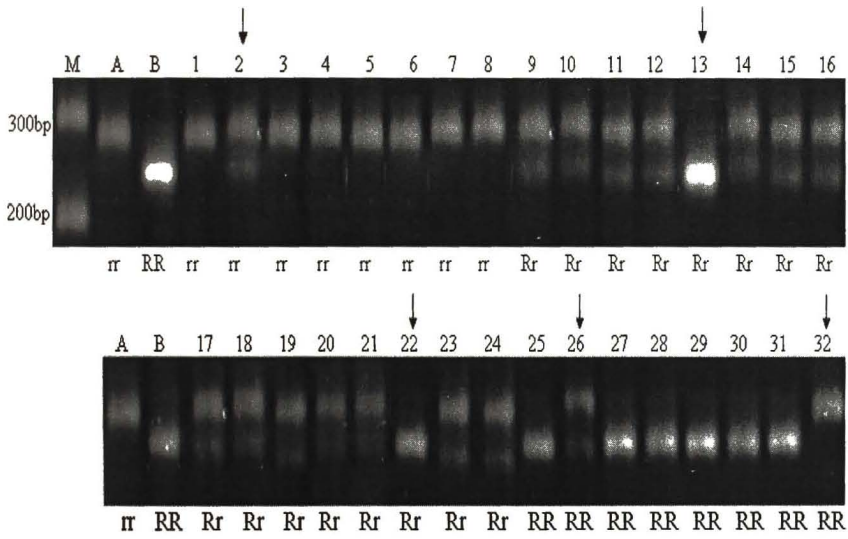
RMAFM5:

The marker in combination with RG556 could predict the trait phenotype in seven of the ten resistant plants, 34 of 38 moderately resistant plants, 81 of 89 moderately susceptible plants, 39 of 43 susceptible plants and 13 out of 16 highly susceptible plants. The marker was at genetic distance of 7.5 cM away from the gene.

RMAFM4:

The marker in combination with RG556 could predict the trait phenotype in eight of the ten resistant plants, 34 of 38 moderately resistant plants, 87 of 89 moderately susceptible plants, 40 of 43 susceptible plants and 15 out of 16 highly susceptible plants. The marker was at genetic distance of 4.2 cM from the gene. The amplification pattern of RMAFM4 in a set of 32 F_2 plants was given in Figure 45.

Figure 45 : Segregation pattern of RMAFM4 in the F_2 population of the cross BPT5204 x Ajaya



A-Ajaya, B-BPT5204, M-Molecular weight marker (100bp ladder). Numbers on top of the gel represent the F_2 plant while the genotype of the plant (as deduced from progeny testing at F_3 and marker genotype of RG556) is represented at the bottom of the gel. rr and RR indicate homozygous resistant and homozygous susceptible F_2 respectively, while Rr represents heterozygous susceptible plants. Arrows on top of the gel indicate recombinants

Table 24: Comparison of the recombination distances in the two populations

Marker	Recombination distance in TN1/Ajaya (cM)	Recombination distance in BPT5204/Ajaya (cM)
RMAFM2	4.3	5.4
RMAFM1	1.9	2.1
RMAFM4	3.1	4.2
RMAFM5	6.2	7.5

Linkage analysis of the marker segregation data in both the populations using Mapmaker software revealed similar marker order as well as recombination distances with the resistance gene (Table 24). Thus the above markers could effectively validate the gene in the alternate population and the gene could be precisely mapped in between the markers RMAFM1 and RMAFM4.

4.3.10 Analysis of amplification pattern of the novel gene linked SSR markers in a set of rice varieties

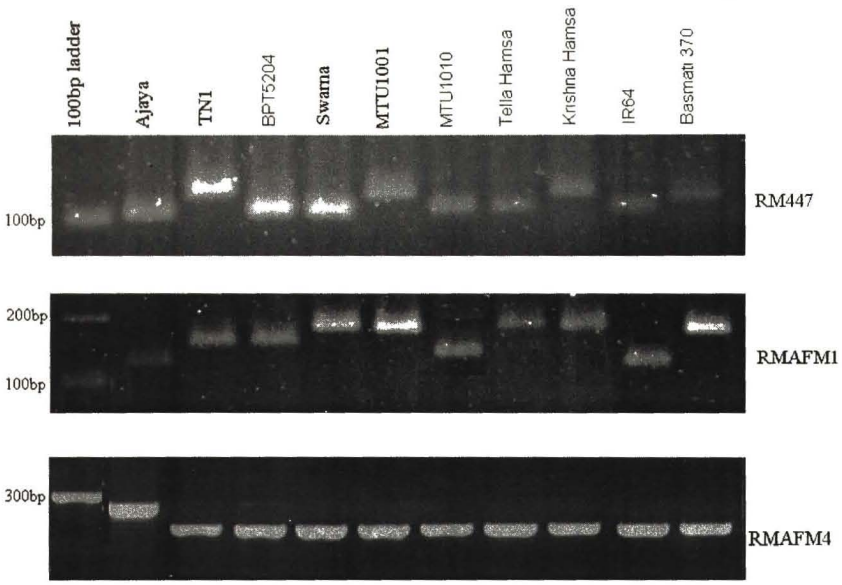
To examine whether the linked markers would be of use in introgression of the novel gene into other BLB susceptible cultivars, DNA samples from a set of nine high priority bacterial leaf blight susceptible cultivars (TN1, Samba Mahsuri (BPT5204), Swarna, MTU1001, MTU1010, Tellahamsa, Krishnahamsa, IR64 and Basmati370) along with the Ajaya, were amplified by PCR using the closest flanking markers RM447 and RMAFM1 and RMAFM4 (Table 25).

Table 25: Amplification pattern of the novel gene linked SSR markers in a set of parental lines

S. No.	Name of the Genotype	Fragment Size (bp)		
		RM447	RMAFM1	RMAFM4
1	Ajaya	110	160	290
2	TN1	130	170	270
3	Samba Mahsuri (BPT5204)	110	170	270
4	Swarna	110	180	270
5	MTU1001	130	180	270
6	MTU1010	110	160	270
7	Tellahamsa	110	180	270
8	Krishnahamsa	130	180	270
9	IR64	110	150	270
10	Basmati370	120	180	270

The marker RM447 amplified a fragment of size 110 bp in Ajaya while the nine susceptible genotypes yielded fragments (i.e. alleles) of size 110-130 bp. With respect to the marker RMAFM1, the susceptible cultivars amplified alleles in the size range 150-180 bp, while in case of Ajaya, a fragment of size 160 bp was observed to be amplified (Figure 46). The marker RMAFM4 amplified a fragment of size 300 bp in Ajaya and 290bp in all the nine susceptible genotypes. The flanking marker combination of RM447-RMAFM1 can be used for introgression of the gene from Ajaya in to susceptible cultivars TN1, MTU1001, Krishnahamsa and Basmati370. For introgression in to other susceptible cultivars Samba Mahsuri, Swarna, Tellahamsa and IR64 the marker combination of RMAFM1-RMAFM4 can be used. Since both the markers RM447 and RMAFM1 are monomorphic between Ajaya and MTU1010, the marker RMAFM4 can be used singly or in combination with the other relatively closer polymorphic markers for introgression of the gene from Ajaya.

Figure 46 :Analysis of flanking markers in a set of high priority BLB susceptible cultivars



4.3.11 Analysis of genes present in the genomic region on Chromosome 1 flanked by SSR markers closely linked to the novel Bacterial leaf blight resistance gene

Based on fine structure linkage map, the SSR marker RM447 was located at a genetic distance of 0.87 cM on one side of the gene and RMAFM1 was located on the other side of the gene at a genetic distance of 1.9 cM (Figure 42). The forward primer of RMAFM1was observed to be physically located (start position) in the Japonica genome (http://rise.genomics.org.cn) at 26.624bp while the reverse primer of RM447 was physically located (end position) at 26.637 bp. The intervening genomic region consisting of 13 kb of sequence was downloaded from the Japonica genome sequence database (http://rise.genomics.org.cn) and analyzed for presence of putative genes using the

software Fgenes H (http://www.softberry.com). The functionality of the genes was analyzed using the BLAST tool (Altschul *et al* 1990) available online at http://www.ncbi.nlm.gov.in. A total of three genes were observed to be present in the genomic region flanked by RMAFM1 and RM447. The average gene density was one gene for every ~ 4.33 kb of sequence. Of the three genes identified in the region, one is Nodulin gene; one was SUA7 a yeast gene coding for the transcription factor TFIIB and the other was a gene belonging to plant peroxidase super family. The details of the list of the genes along with their size in base pairs, amino acids along with their putative function(s) were given in Table 26.

Table 26: List of genes present in the genomic region flanked by SSR markers RMAFM1 and RM447

Gene No.	Gene start (bp)	Gene end (bp)	Gene size (bp)	No. of amino acids	Putative function of protein encoded by the gene and proteins showing homology to the gene
1	26625582	26630530	1665	554	Nodulin gene
2	26630866	26633866	1722	573	SUA7,Transcription factor TFIIB
3	26635013	26637736	843	280	Plant peroxidase super family

Chapter 5

Discussion

Chapter V – Discussion

Bacterial leaf blight (BLB) of rice caused by *Xanthomonas oryzae* pv. *Oryzae* is one of the most destructive diseases of rice, particularly in the *kharif* season crop in India Since chemical control is ineffective, development and deployment of BLB resistant cultivars is the only practical strategy for managing the disease. However, large scale and long term cultivation of cultivars possessing single genes (*Xa3* in Japonica rice and *Xa4* in Indica rice) have resulted in the appearance of virulent strains which can overcome resistance. The breakdown of resistance to bacterial blight in the rice variety Asakaze in Japan is a famous example of the instability of major genes (Ezuka and Sakaguchi, 1978). In 1972, an isolate strain, designated "Isabela," from the northern part of the Philippines was found to cause breakdown of *Xa4* conferred resistance (IRRI, 1973) and in 1975, IRRI cultivars carrying the *Xa4* gene became susceptible to bacterial blight not only in the Philippines (Mew *et al.*, 1992) but also in many parts of India, Indonesia, and China (Huang *et al.*, 1997).

Resistance conferred by the major gene *Xa21* has also been reported to be overcome in some parts of India and Indonesia (Sirisha *et al.*, 2004; Goel *et al.*, 1998). One strategy to delay such breakdown of resistance is to provide a broad-spectrum of resistance by combining multiple genes having complementary resistance spectra, in a single plant genotype through the strategy of gene pyramiding (Babujee and Gnanamanickam, 2000). Gene pyramiding would thus result in development of genotypes with more durable resistance through individual gene action and quantitative complementation (Ogawa *et al.*, 1987; Yoshimura *et al.*, 1995b). The present study was therefore designed to identify, broad spectrum BLB resistance

genes from wild rice introgression lines of rice and durable BLB resistant cultivars, to tag and map them with tightly linked SSR markers

A total of 72 wild rice introgression lines carrying introgressions from the wild species *O. nivara* (8), *O rufipogon* (32),*O. longistaminata* (7), *O. glaberrima* (4), *O. officinalis* (9) and *O.brachyantha* (12), obtained from the International Rice Research Institute, Phillipines were screened for their resistance to BLB with seven hyper-virulent isolates of *Xanthomonas oryzae* pv. *oryzae*. One line carrying introgression from *Oryza brachyantha* (FF genome) i.e. IR-65483-141-2-4-4-2-5-B in the background of the *O. sativa* indica cultivar IR56 and the variety Ajaya (IET 8585) showed resistance reaction consistently with all the isolates tested under both the glasshouse and field conditions. Further IR-65483-141-2-4-4-2-5-B was observed to be superior in terms of BLB resistance when compared with NILs of IR24 carrying the single BLB resistance genes *xa5*, *xa13*, *Xa21* and was on par with respect to resistance reaction with the NILs of IR24 carrying four BLB resistance genes *Xa4*, *xa5*, *xa13* and *Xa21*. Ajaya showed higher average lesion length when compared to the NILs of IR24 possessing four BLB resistance genes *Xa4*, *xa5*, *xa13* and *Xa21* but was on par with NILs of IR24 possessing the individual genes *xa13* and *Xa21* and was significantly superior to the NIL of IR24 possessing *xa5* (Table 8). Based on these observations, it was hypothesized that these genotypes may possess one or more novel BLB resistance genes.

The introgression line IR-65483-141-2-4-4-2-5-B was developed from the cross of IR56 with101231 (an accession of *O. brachyantha*) by embryo rescue technique (Brar and Khush, 1997). Genetic analysis in the F_2 population derived from the cross IR-65483-141-2-4-4-2-5-B/TN1 revealed the action of a single dominant resistance gene

governing resistance. Out of the 296 F_2 plants screened for resistance, 225 plants were resistant, while 71were susceptible which fitted well in a segregation ratio of 3R:1S (χ^2= 0.16, P=0.68). Progeny testing of the 296 individuals revealed that the F_2 plants segregated in a classical Mendelian ratio of 1:2:1 (χ^2= 0.66, P=0.71) with respect to homozygous resistant, heterozygous resistant and homozygous susceptible individuals. The F_2 population consisting of 250 individuals of the cross IR-65483-141-2-4-4-2-5-B /BPT5204 also showed segregation in 3R:1S ratio (195 resistant and 55 susceptible) (χ^2= 1.2, P=0.27). Progeny testing of the F_2 lines, as observed earlier segregated in a ratio of 1:2:1, (χ^2= 2.37, P=0.30) with respect to homozygous resistant, heterozygous resistant and homozygous susceptible individuals. These results confirm the action of a single dominant gene in conferring resistance in IR-65483-141-2-4-4-2-5-B

In order to identify whether the dominant BLB resistance gene identified in IR-65483-141-2-4-4-2-5-B is allelic to any of the known, dominant BLB resistance genes, allelism tests were carried out with NILs of IR24 carrying known single dominant resistance genes, viz. *Xa1, Xa4, Xa7, Xa21* and *Xa26*. Similar allelism tests were conducted by Ogawa *et al.*, 1978, Ogawa and Yamamoto, (1986), Sidhu *et al.*, (1978; 1979), while studying inheritance and novelty of BLB resistance genes. The F_2 plants derived from the crosses of IR-65483-141-2-4-4-2-5-B with IRBB1, IRBB4, IRBB7, IRBB21 and IRBB26 segregated in a phenotypic ratio of 15R:1S indicating that the resistant gene is non-allelic to any of the above said dominant BLB resistance genes. However, since DRR, Hyderabad did not possess IR24 NILs carrying other known dominant resistance genes, the novelty of the gene in IR-65483-141-2-4-4-2-5-B, could not be confirmed with certainty. It was hypothesized that if the gene is novel, it

should be mapped to a genomic location, which is hitherto unreported for any known dominant BLB resistance genes. Hence, a molecular mapping analysis was carried out using SSR markers in order to identify the chromosomal location of the putatively novel BLB resistance gene in IR-65483-141-2-4-4-2-5-B and to identify markers closely linked to the gene for possible use in marker-assisted selection.

A total of 282 SSR markers were used for the survey of parental polymorphism in the preliminary coarse mapping analysis with coverage of ~ 23 markers per chromosome. Out of the 282 SSR markers analyzed, 106 displayed polymorphism between the resistant and susceptible parents (i.e. IR-65483-141-2-4-4-2-5-B and TN1) covering all the 12 chromosomes of rice, (8.8 markers per chromosome), giving an approximate coverage of one marker every 4 cM. The parental polymorphic SSR markers were then analyzed through the strategy of bulked-segregant analysis (BSA) for identification of markers displaying co-segregation with trait phenotype. The strategy of BSA has been used earlier for tagging and mapping of many agronomically important genes in rice like white back plant hopper resistance genes (Tan *et al.*, 2004), *Xa30t* (Cheema *et al.*, 2008) and QTLs associated with heat tolerance (Zhang *et al.*, 2009). In the present study also, the strategy of BSA was successful in identifying the tentative localization of the new BLB resistance on Chr. 1 using the SSR marker RM595.

While tagging and mapping the putatively novel BLB resistance gene present in, IR-65483-141-2-4-4-2-5-B progeny testing at F_3 generation under a stringent phenotyping procedure as described in materials and methods ensured correct identification of homozygotes and heterozygotes in F_2 with no escapes. The choice of a early segregating population like F_2, which was used in the present study for tagging

and mapping BLB resistance gene(s) is considered to be useful, as it saves time and resources in tagging and mapping compared to development and utilization of advanced generation populations like recombinant inbred lines and near isogenic lines, which take a lot of time for development (Michelmore *et al.*, 1991; Haley *et al.*, 1994; Biradar *et al.*, 2004).

In bulked-segregant analysis, RM595 showed association with trait phenotype. Any marker that assorts independently of resistance gene will display amplification of fragments specific for both resistant and susceptible parents in both resistant bulk (RB) as well as susceptible bulk (SB), whereas a co-dominant marker closely linked to the target gene will show only susceptible parent specific fragment in the SB, and only the resistant parent specific fragment in the RB. In the present study, based on the bulked-segregant analysis, it was hypothesized that the BLB resistance gene may be close to the marker RM595, since the marker showed clear bulk specific amplification pattern. This marker when analyzed in the entire mapping population, showed a linkage distance of 10.2 cM with the gene.

In order to identify the tentative map location of the putatively novel BLB resistance gene on Chr. 1, 20 SSR markers selected from the vicinity of the genomic region where RM595 is located were tested for parental polymorphism. Of these, seven were polymorphic. When these polymorphic SSRs were tested for their marker-trait segregation pattern in the F_2 mapping population, two markers *viz.*, RM10916 (15.052Mb) and RM11002 (17.805Mb) were observed to be the closest and flanking the BLB resistance gene at a genetic distance of 14cM and 15.2cM respectively. Even though these markers were not close enough to be used for marker-assisted selection

of the gene, the fact that these two markers flank the resistance gene helped to narrow down the gene to a physical region of 2.75Mb on Chr. 1 which served as a starting point for fine-mapping analysis.

For carrying out fine-mapping analysis, a set of nine hyper-variable RM series SSR markers (http://www.gramene.org) and eight newly designed SSR markers (RMCG1 to RMCG8) located in the genomic region spanned by RM10916 and RM11002 were used. The gene was mapped at a distance of 0.82 cM from the marker RM10975 and 1.8 cM away from RMCG6 with both these markers flanking the gene. The markers RM10920, RM10963, RMCG5, RM10974 and RM10975 were observed to be located on one side of the gene at a genetic distance of 9.4 cM, 7.5 cM, 6 cM 1.85 cM and 0.82cM respectively while RMCG6, RMCG7 and RMCG8 were located on the other side of the gene at a genetic distance of 1.8 cM, 5.6 cM, and 8.4 cM respectively.. Tan et al., (2004) reported a dominant BLB resistance gene *Xa29t* from the wild species *O. officinalis* and mapped it on Chr. 1 in the marker interval of two RFLP markers C904 (13.6MB) and R596 (15.4MB). Hence the gene identified in the present study spanned by the flanking markers RM10975 (16.952MB) and RMCG6 (16.968) was considered as novel and has been designated as *Xa35t.*

Even though the two closest flanking markers RM10975 (0.82cM) and RMCG6 (1.8cM) showed a few recombinants, they both are as such individually close enough for effective use in marker-assisted selection. Further, the fact that the two SSR markers flank the novel BLB resistance helps in accurate prediction of the presence of the gene with less than 1% error. If the selection accuracy (calculated based on recombination frequencies observed in F_2 population) while using RM10975 and RMCG6 individually to predict BLB is 99.2% and 98.2%, respectively, then based on

the product rule of probability, the combined use of the markers RM10975 and RMCG6 enhances the selection efficiency to 99.03%. Biradar et al., (2004), in their efforts to tag and map a major, dominant rice gall midge resistance gene, *Gm1* have highlighted the utility of flanking markers in marker-assisted selection (MAS) of *Gm1*.

The utility of the SSR markers linked to the novel BLB resistance gene was validated in an alternative F_2 mapping population derived from the cross IR-65483-141-2-4-4-2-5-B /Samba Mahsuri. It was observed that the map location and linkage distances of the SSR markers were identical to those observed in the F_2 mapping population derived from the cross IR-65483-141-2-4-4-2-5-B /TN1, thus validating the linkage and utility of the flanking SSR markers. Marker validation studies help in testing the efficacy of flanking markers in predicting the presence of gene of interest in a different genetic background. Biradar *et al.*, (2004), Rathour *et al.*, (2008) and Sheeba *et al.*, (2005) validated the presence of *Gm1* (a rice gall midge resistance gene), *Pi-z*, (a blast resistance gene) and Rf, (a fertility restorer gene), respectively in alternate populations using SSR markers. Such validations are necessary for successful utilization of linked markers in marker-assisted breeding programs.

The present study relied on utilization of rice SSR markers for tagging and mapping of the new BLB resistance gene from the *Oryza brachyantha* introgression line (IR-65483-141-2-4-4-2-5-B). SSR markers have been widely used in tagging and mapping of the disease resistance genes in rice. A dominant gall midge resistance gene was tagged and mapped on Chr. 9 by using SSR markers (Biradar *et al.*, 2004). In addition to being helpful in tagging and coarse mapping, SSR markers are highly useful in fine

mapping also as demonstrated in the present study. Blair *et al.*, (2003) used SSR markers for fine mapping of the bacterial blight resistance gene *xa5* while Chu *et al.*, (2006a) used these markers for fine mapping *xa13* and recently Cheema *et al* , (2008) used these for fine mapping *Xa30(t).* In rice, so far more than 20,000 SSR markers have been identified and been mapped, both genetically and physically (IRGSP, 2005), giving an average saturation of one marker per 19.2 kb or one marker per ~0.1 cM. This coverage should be sufficient for fine mapping and map based cloning of agronomically important genes in rice. In fact, the genes *xa5* (Iyer and Mc Couch., 2004) and *xa13* (Chu *et al.*, 2006b) were cloned based on information derived from fine mapping analysis using SSR markers.

As on date, a total of 31 BLB resistance genes have been identified and most of these have been mapped on the rice genome using molecular markers (Laha *et al.,* 2009). In the present study, the new gene identified from *O. brachyantha* introgression line IR-65483-141-2-4-4-2-5-B has been mapped on Chr. 1 in the genomic region flanked by the two SSR markers, RM10975 (16.952Mb) and RMCG6 (16.968Mb).

In order to utilize the novel BLB resistance gene *Xa35t* effectively through marker-assisted breeding, it is imperative to study the amplification pattern of the flanking markers RM10975 and RMCG6 in a set of high priority bacterial leaf blight susceptible recipient lines into which the novel gene can be introgressed. With this objective, the amplfication pattern of the flanking markers RM10975 and RMCG6 was tested in a set of eight high priority, BLB susceptible rice varieties in order to assess the polymorphism of the two markers. Both RM10975 and RMCG6 displayed unique polymorphism between resistant donors and susceptible rice varieties and

hence can be considered highly useful either singly or in combination for marker-assisted introgression of *Xa35t* into high priority BLB susceptible rice cultivars.

In addition to tagging and fine mapping of *Xa35t*, in the present study, an attempt was made to analyze the genomic region flanked by the closest SSR markers RM10975 and RMCG6 for putatively expressed candidate genes through an *in-silico* approach. Such an *in-silico* approach was earlier used by Sharma *et al.*, (2005) for identification of the candidate gene involved in *Pi-k*^{h} mediated resistance to blast. The genomic region of Japonica rice genome flanked by the closely linked SSR markers RM10975 and RMCG6 (spanning 16kb region) was downloaded and analyzed for putative candidate genes. A total of four genes were identified in the region analyzed of which two genes belonged to a No apical meristem super family, one was a hypothetical protein and one gene PTZoo265, a multidrug resistance gene. The No apical meristem gene can be assumed to be most probable candidate responsible for resistance in IR-65483-141-2-4-4-2-5-B based on its physical position and the segregation pattern of the closely linked markers RM10975 and RMCG6. The No apical meristem gene belongs to the NAC domain super family, the members of which are known to code for transcription factors (Ren *et al.*, 2000; Xie *et al.*, 1999; Duval *et al.*, 2002). NAC proteins are involved in transcriptional control of a variety of plant developmental processes, including formation of the shoot apical meristem, floral organs and lateral shoots (Souer *et al.*, 1996; Aida *et al.*, 1997; Xie *et al.*, 2000) as well as in plant hormonal control and in defense responses against stress and viral infections (Xie *et al.*, 1999; Ren *et al.*, 2000; Collinge and Boller, 2001; Nakashima *et al.*, 2007). Several NAC domains containing proteins have been reported to have plant resistance function in species like Chillies, Potato, Turnip and Rice (Ren *et al.*, 2000;

Collinge and Boller, 2001; Oh *et al.*, 2005; Lin *et al.*, 2007b). A NAC domain containing protein has been identified as the putative candidate for BLB resistance mediated by BLB resistance gene *Xa7* (Chen *et al.*, 2008) and for another gene reported to be allelic to *Xa7* in a japonica restorer line Zhenhui 084 (Zhang *et al.*, 2009).

Till date five BLB resistance genes have been identified from five wild rice species of rice namely *Xa21* identified from an accession of *O. longistaminata* (Khush *et al.*, 1990), *Xa23* from *O.rufipogon* (Zhang *et al.*, 1998), *Xa27* from *O. minuta* (Gu *et al.*, 2004), *Xa29* from *O.0fficinalis* (Tan *et al.*, 2004) and recently another gene, *Xa30t*, identified from the wild rice *O. nivara* (Cheema *et al.*, 2008). Similar to these efforts, through the present study, a novel BLB resistance gene, *Xa35t* has been identified from an introgression line of *O. brachyantha* (IR-65483-141-2-4-4-2-5-B), tagged and fine mapped on Chr. 1 using SSR markers. A set of putatively expressed genes, which could be candidate(s) for *Xa35t* and a set of very closely linked flanking SSR markers have been identified paving the way for marker-assisted introgression of the gene into elite rice varieties.

Identification and mapping of novel BLB resistance gene(s) from Ajaya :

In addition to the introgression line of *O. brachyantha* (IR-65483-141-2-4-4-2-5-B), in which a novel BLB resistance gene was identified through the present study, the popular BLB resistant check variety, Ajaya, which was released for cultivation by the Directorate of Rice Research was also observed to be resistant to most of the BLB isolates tested in the present study. The cultivar Ajaya is a medium duration (130-135 days) variety released for cultivation under irrigated ecosystems. Since its release in

1992, by the central varietal release committee it has performed well in the coordinated trials conducted by AICRIP and has been used as a national and an international check for screening the germplasm. Hence an effort was made to study the inheritance of BLB resistance in Ajaya and to identify, tag and map the resistance gene(s) present in Ajaya.

In order to study the inheritance of BLB resistance in Ajaya it was crossed with two BLB susceptible cultivars TN1 and Samba Mahsuri (BPT5204). The F_1s of both the crosses were observed to be intermediate to both the parents with respect to average lesion length indicating the involvement of incompletely dominant gene(s) in governing resistance. In the F_2 generation of the cross TN1/Ajaya consisting of 400 F_2 plants screened for BLB resistance, 25 were resistant (avg. lesion length < 4 cm), 105 were moderately resistant (avg. lesion length 4-8 cm), 132 were moderately susceptible (avg. lesion length 8-12 cm), 110 were susceptible (avg. lesion length 12-16 cm) and 28 were highly susceptible (avg. lesion length >16 cm). This fitted well in a segregation ratio of 1:4:6:4:1 (χ^2= 4.15, P=0.68) with respect to resistance , moderate resistance, moderate susceptibility, susceptibility and high susceptibility indicating the possible action of two incompletely dominant resistance genes with equal effects interacting additively (Figure 25). This can be considered as a case of quantitative inheritance of resistance governed by two loci. The classical example explaining such an interaction was grain color in Wheat by Nilsson-Ehle. On crossing two wheat genotypes possessing dark red and white grain colour, the F_1 showed light red grain colour and the F_2 segregated in the ratio of 1dark red:4medium red:6light red:4pale red :1white (Nilsson-Ehle 1909). Such interactions were also reported in case of inheritance of tolerance to high concentration of soil Boron in pea (Bagheri *et*

al., 1996), inheritance of palmitic acid content in soya bean (Erickson *et al.*, 1988) and inheritance of ray floret length in *Senecio cambrensis* (Ruth Ingram and Noltie, 1984).

Two earlier studies on inheritance of BLB resistance in Ajaya reported the action of two independently segregating dominant genes (Saini *et al.*, 1996) and a single recessive gene (Kameswara Rao *et al.*, 2003). However in our study the additive interaction between two incompletely dominant resistance genes was observed to condition resistance in Ajaya which was also validated in an alternate population. Additive /dosage effects has been reported between the BLB resistance genes *Xa1* and *Xa3* in Java14 (Kaku, 1997) and between *Xa1* and *Xa4* in the cultivar IR20 (Kaku, 1999). The increased level of resistance conferred by more than one gene governing resistance to a single pathogen race has been described as quantitative complementation (Sanchez *et al.*, 2000). The increased level of resistance of pyramided lines expressed as reduced lesion length has been reported in several gene-pyramiding studies on BLB resistance (Yoshimura *et al.*, 1995b; Huang *et al.*, 1997; Sanchez *et al.*, 2000; Singh *et al.*, 2001; Joseph *et al.*, 2004; Sundaram *et al.*, 2008; 2009). This increased resistance has been hypothesized to be due to synergistic action and/or complementation between the resistant genes used in the pyramid lines.

According to the pedigree of Ajaya (Figure 25), BLB resistance is thought to be contributed by one its parental line BJ1, a landrace reported to possess two resistance genes *xa5* and *xa13* (Ogawa and Yamamoto, 1986). Since genetic analysis in the F_2 mapping population revealed the action of two genes in conferring resistance in Ajaya, its allelic status was tested with respect to the *xa5* linked marker RG556 (Blair

and Mc Couch, 1997) and a functional marker for *xa13*, *xa13*-prom , (chu *et al.*, 2006b) along with two susceptible varieties TN1 and BPT5204. PCR Analysis with RG556 showed resistant allele specific amplification in Ajaya similar to IRBB5 while TN1 and BPT5204 amplified the susceptible allele indicating that *xa5* could be one of the genes conferring resistance in Ajaya. Davierwala *et al.*, (2001) predicted the possible presence of *xa5* in Ajaya and several other rice varieties based on PCR analysis with RG556. Analysis with *xa13*-prom primer showed amplification of susceptibility specific allele in Ajaya similar to TN1 and BPT5204 indicating that the second resistance gene in Ajaya is not *xa13*. Based on these observations, it can be assumed that while Ajaya may possess *xa5* gene inherited from BJ1, it certainly does not possess *xa13*, since the functional marker for the gene, *xa13*-prom did not reveal amplification of the resistance linked allele in Ajaya.

In order to confirm the presence of *xa5* in Ajaya, allelism test was carried out with IR24 NIL carrying recessive resistance genes *xa5*. The F_2 population derived from the cross Ajaya/IRBB5 was uniformly resistant and did not segregate for susceptibility indicating that *xa5* is one of the genes conferring resistance in Ajaya. In case of the gene *xa5*, the functional polymorphism specific for resistance was due to a 2-bp pair substitution, i.e. TC (susceptible genotypes) to AG (resistant genotypes) in the exon-2 of the candidate gene for *xa5*, Transcription factor IIAγ, leading to a change in a single amino acid i.e. Valine (susceptible) to Glutamic acid (resistant) as reported by Iyer and Mc Couch *et al.*, (2004). Sequence analysis of the 260 bp fragment amplified in this functional polymorphic region of the second exon of *xa5* in Ajaya showed AG substitution while the susceptible genotypes TN1 and BPT5204 showed TC

substitution. Thus from the results of the present study, it is conclusively clear that one of BLB resistance genes in Ajaya is *xa5*.

In an earlier study Kameswara Rao *et al.*, (2003) reported presence of a gene, which is non-allelic to *xa5* to be responsible for resistance in Ajaya and mapped it on the long arm of Chr. 5 between the markers RM39 (14.5cM from the gene) and RM31 (17.7cM from the gene), while *xa5* was mapped on the short arm of Chr. 5 (Blair and McCouch, 1997). However in the present study, through all the three approaches followed, i.e. marker analysis with RG556, allelism tests with IRBB5 and sequence analysis of the functional polymorphic region of *xa5* proved that *xa5* is indeed one of the candidate genes in Ajaya. The better level of resistance shown by Ajaya (displaying avg. lesion length of 3.87 cm) when compared to IRBB5 (a NIL harboring *xa5*, which displayed avg. lesion length of 7.08 cm) is because of the additive effect of the second resistance gene as evident from the F_2 genetic analysis. The recessive nature of resistance conferred by *xa5* was reported by Murthy and Khush, (1972), Olufowote *et al.*, (1977) and Blair *et al.*, (2003). However in the present study, *xa5* exhibited incomplete dominance confirming the results as obtained by Li *et al.*, (2001), who also observed incomplete dominance of *xa5*.

Since one of the resistance genes in Ajaya was identified to be *xa5*, and the interaction between the two resistance genes was observed to be additive, the genotype of each of the 400 F_2 individuals with respect to the second resistance locus was predicted based on both the F_3 segregation data and marker genotype at *xa5* locus as under

- The 25 resistant plants in the F_2 population of the cross TN1/Ajaya were assumed to have the genotype r1r1r2r2 (as they were uniformly resistant and

showed no segregation in the F_3) and were predicted to be homozygous (r2r2) at the second resistance locus as they showed homozygous resistant (r1r1) amplification at *xa5* locus

- The 50 moderately resistant plants whose genotype was assumed to be r1r1R2r2/R1r1r2r2 (as their F_3 segregated in the ratio of 1R:2MR:1S) were supposed to be heterozygous at the second locus (R2r2), as they were homozygous resistant (r1r1) with respect to *xa5* locus
- The second set of 55 moderately resistant plants whose genotype was assumed to be R1r1r2r2 were supposed to be homozygous resistant (r2r2) at the second locus as they were heterozygous with respect to *xa5* (R1r1)
- The 20 moderately susceptible plants whose F_3 progeny lines were uniformly moderately susceptible with out any segregation in the F_3 were assumed to be homozygous at both the loci and supposed to have the genotype r1r1R2R2. Their genotype at the second resistance locus was predicted to be homozygous susceptible(R2R2) as they showed homozygous resistant (r1r1)amplification with respect to *xa5*
- The 24 moderately susceptible plants whose F_3 progeny lines were uniformly moderately susceptible with out any segregation in the F_3 were assumed to have the genotype R1R1r2r2 and their corresponding second resistance locus was predicted to be homozygous resistant (r2r2) as they were homozygous susceptible (R1R1) at *xa5* locus
- The 88 moderately susceptible plants whose F_3 progeny lines segregated in1R:4MR:6MS:4S:1HS ratio were assumed to be heterozygous at both the genic loci and supposed to have the genotype R1r1R2r2. Their second

resistance locus was predicted to be heterozygous (R2r2) as they showed heterozygous susceptible amplification (R1r1) with respect to *xa5*

- The 50 Susceptible plants whose F_3 progeny lines segregated in1HS:2S:1MS ratio were assumed have the genotype R1r1R2R2/R1R1R2r2 and were predicted to be heterozygous (R2r2) as they were homozygous susceptible (R1R1) at *xa5* locus
- The second set of 60 susceptible plants were assumed have the genotype R1r1R2R2/R1R1R2r2 and were predicted to be homozygous susceptible (R2R2) at the second resistance locus as they were heterozygous susceptible (R1r1) at *xa5* locus
- All the 28 highly susceptible plants whose progeny was uniformly susceptible and showed no segregation in the F_3 were assumed to be homozygous at both the resistance loci and their genotype was supposed to be R1R1R2R2. Their genotype with respect to the second resistance locus was predicted to be homozygous susceptible (R2R2) as they were homozygous susceptible (R1R1) with respect to *xa5*

The genotype of each of the individual F_2 plants of the population of the cross TN1/Ajaya with respect to the second resistance locus as predicted from the F_3 segregation and marker data at *xa5* locus (RG556) was used for mapping the second resistance in Ajaya

In order to study the precise location of the second resistance gene in Ajaya, a parental polymorphism survey was carried out with a set of 282 SSR markers covering the 12 chromosomes of rice. Out of 282 primers tested, only 77 were observed to be polymorphic. The marker RM23476 located at 26.26 Mb on Chr. 8 showed bulk specific amplification and when checked in the whole mapping

population, was observed to be at a linkage distance of 15.1 cM In order to identify the map location of the second resistance gene in Ajaya on Chr. 8, 30 SSR markers in the vicinity of RM23476 were tested for parental polymorphism, of which, 10 were polymorphic. When these polymorphic SSRs were then tested for their marker-trait segregation pattern in the F_2 mapping population, two markers *viz.*, RM23478 and RM23535 were observed to be the closest and flanking the novel gene at a genetic distance of 14.8 and 15.1 cM respectively. These two markers helped to narrow down the gene to a physical region of 800 Kb on Chr. 8, thus serving as a starting point for fine-mapping analysis. Fine-mapping analysis was carried out with a set of nine hyper-variable SSR markers and six newly designed SSR markers (RMAFM1 to RMAFM6) located in the genomic region spanned by RM23478 and RM23535. The gene was mapped in the marker interval of RM447 and RMAFM1 with both of them flanking it. This gene, which has been mapped approximately at a distance of 100 Kb from another gene on Chr.8 i.e. *xa13* can be considered as novel, since no other BLB resistance gene has been reported in this region and hence it was designated as *xa36(t)*. The markers RM23489, RM23496, RMAFM2 and RMAFM1 were observed to be located on one side of the gene at a genetic distance of 13.6 cM, 12.2 cM, 4.3 cM and 1.9 cM respectively, while RM447, RMAFM4, RMAFM5 and RMAFM6 were located on the other side of the gene at a genetic distance of 0.87 cM, 3.1 cM, 6.2 cM and 10.1cM respectively. The closest flanking markers RM447 and RMAFM1 span a physical distance of 13 kb which could serve as the starting point for *in-silico* identification of putative candidates for *xa36t* and possibly, map based cloning of the gene.

There were only four recombinants out of 400 F_2 plants analyzed with respect to the marker RM447 while five recombinants were observed with respect to the marker RMAFM1. Even though the two markers showed a few recombinants, their flanking nature helps them for effective use in marker-assisted selection. The selection efficiency of the marker RM447 was observed to be 99.2% as it is at a genetic distance of 0.8 cM from the gene while that for RMAFM1, it was 98.2% as it is 1.8cM from the gene. The combined use of the flanking markers RM447 and RMAFM1 enhances the selection efficiency to 99.03%, based on the product rule of probability (Biradar et al., 2004). In fact, in the present study, a total of 102 F_2 plants possessed Ajaya specific allele for both RM447 and RMAFM1 in homozygous condition and all these plants were observed to be homozygous resistant, indicating the utility of flanking markers in screening for resistance trait. Since RM447 was monomorphic between Ajaya and BPT5204 the other closely linked markers RMAFM2, RMAFM1, RMAFM4 and RMAFM5 were validated in the alternative F_2 mapping population derived from the cross Samba Mahsuri/Ajaya. It was observed that the map location and linkage distances of the SSR markers were identical to those observed in the F_2 mapping population derived from the cross TN1/Ajaya , thus validating the linkage and utility of the flanking SSR markers.

In the present study, the new gene identified from Ajaya and fine mapped on Chr. 8 at a distance of ~100 kb from *xa13* has been tentatively named as *xa36t*. The utility of the flanking markers identified, for marker assisted introgression of the *xa 36t* gene was evaluated by studying their amplification pattern in a set of high priority bacterial leaf blight susceptible recipient lines into which the novel gene can be introgressed. The flanking marker combination of RM447-RMAFM1 can be used for introgression

of the gene from Ajaya in to susceptible cultivars TN1, MTU1001, Krishnahamsa and Basmati370 as these markers were polymorphic between the Ajaya and the recipient parents. Since the marker RM447 was monomorphic between Ajaya and BPT5204, Swarna, MTU1010, Tellahamsa and IR64, the flanking marker combination of RMAFM1 and RMAFM4 is suggested for marker-assisted introgression of this gene to the above mentioned susceptible rice cultivars. Since both the markers RM447 and RMAFM1 were monomorphic between Ajaya and MTU1010, the marker RMAFM4 can be used singly or in combination with the other relatively closer polymorphic markers for introgression of *xa36t*.

The genomic region flanked by the closest SSR markers RM447 amd RMAFM1was analysed for putative candidate genes through an *in-silico* approach. The genomic region of Japonica rice genome flanked by the the markers RM447 amd RMAFM1 (~ 13Kb) was downloaded and analyzed for putative candidate genes. A total of three genes were identified in the region analyzed. Gene density within this region is about one gene every 4.3 kb, against the published predictions of one gene every 9.9 kb (International Rice Genome Sequencing Project 2005). One of the genes identified in this genomic region belonged to Nodulin super family. The candidate gene for *xa13* has been identified to be a nodulin gene, which are usually expressed in the root nodules of plants belonging to leguminosae family (chu *et al.*, 2006b). Nodulin related genes are found in several species like nematodes, insects, and animals, although the biochemical functions of their proteins are unknown. Yang *et al.*, (2006) reported about 17 nodulin related genes distributed all over the rice genome. It is hypothesized that the nodulin gene identified in the present study, which is mapped at a distance of about 100kb away from *xa13* could be one of the candidates for *xa36t*. The second

gene which was observed to be the most probable candidate for *xa36t* based on the segregation of the closely linked flanking markers was SUA7, a gene from *Sacharomyces cerevisiae* (yeast), which codes for the general transcription factor TFIIB which is involved in the transcription of genes in interaction with the enzyme RNA Polymerase II. A related factor of TFIIB called the BRF1 acts as a subunit and of the yeast transcription factor TFIIIB and plays a role in RNA Polymerase III initiation analogous to the role played by TFIIB for RNA polymerase II in its interaction with TATA Box Binding protein (Colbert and Hahn, 1992). The other gene identified in the genomic region of interest was a plant peroxidase gene. Peroxidases are involved in lignin biosynthesis whose accumulation was observed in the resistance mediated by BLB resistance genes *xa5, Xa7*, and *Xa10* (Reimers and Leach, 1991). Although the role for peroxidases in defense responses has not been clearly demonstrated, increases in peroxidase activity have been correlated with infection in many plant species. Peroxidases are involved in production of active oxygen species, which may play various roles in reduction of pathogen viability and spread (Mehdy *et al.*, 1996; Tenhaken *et al.*, 1995). In rice, induction of specific peroxidases has been correlated with resistance to BLB (Chittoor *et al.*, 1997; Hilaire *et al.*, 2001). Cloning and sequencing of the above said putative candidate genes for *xa36t* may help in precisely identifying and characterizing the candidate gene associated with resistance controlled by *xa36t.*

In general, the 'R' proteins are predicted to act as receptors to bind specifically to a pathogen produced ligand, which is produced directly or indirectly by the *avr* gene present in the pathogen. This direct interaction of the 'R' protein and *Avr* ligand results in activation of the plant defense response, which ultimately results in the expression

of resistance. Therefore, the function of the 'R' gene depends on the presence of a recognizable *Avr* ligand in the pathogen. The *Avr* ligands, which may be interacting with *Xa35t and Xa36t,* need to be identified through intensive host-pathogen interaction studies

Out of the five dominant BLB resistance genes identified from five wild rice species i.e. *Xa21* from *O.longistaminata*, *Xa 23* from *O rufipogon*, *Xa27t* from *O minuta, Xa 29t* from *O.officinalis* and *Xa30t* from *O.nivara*, *Xa21* is the most effective and the most deployed resistance gene in rice genotypes. The gene has been introgressed into many elite rice varieties like PR106 (Singh *et al.*, 2001), Pusa Basmati1 (Joseph *et al*, 2004), Samba Mahsuri (Sundaram *et al.*, 2008), Swarna and IR64 and many hybrid rice parental lines like Minghui 63 (Chen *et al.*, 2000), Zhenshan 97A (Zhang *et al.*, 2002). Recently, *Xa30t* identified from an *O. nivara* accession (Cheema *et al.*, 2008) was introgressed into the variety PR114. The novel resistance gene, *Xa35t* identified from an introgression line of *O. brachyantha* in the present study could complement the resistance mediated by *Xa21* in hybrid rice. The genes *Xa35t* and *xa36t* can be deployed either singly or in combination with other major BLB resistance genes *xa5*, *xa13* and *Xa21* to obtain broad spectrum resistance against BLB. The tightly linked flanking markers developed in the present study can be used to track the introgression of these genes in to elite backgrounds. The, putatively expressed genes, which could be candidates for *Xa35t* and *xa36t*, could help in Map based cloning and functional dissection of the novel genes identified.

Chapter 6

Summary & conclusions

VI – Summary and Conclusions

Bacterial leaf blight of rice caused by *Xanthomonas oryzae* pv *oryzae* is one of the most destructive diseases of rice in majority of the rice growing countries especially in Asia. Since chemical control of the disease is not economical, cultivation of rice varieties possessing one or more bacterial blight resistance genes has been recommended. So far 31 Bacterial leaf blight resistance genes have been identified. Extensive cultivation of varieties containing single resistance genes has resulted in frequent breakdown of resistance due to emergence of virulent strains of the disease across many locations in India. Therefore there is a need for continuous search for the new/novel sources of resistance and their introgression along with major BLB resistance genes in to elite varieties to achieve durable resistance. The present study was thus carried out with the objective of identification and mapping of novel bacterial leaf blight (BLB) resistance gene(s) in rice.

The results obtained from the present study are summarized as follows:

- 72 wild rice introgression lines carrying introgressions from the wild species *O. nivara*, *O. rufipogon, O. longistaminata*, *O. glaberrima*, *O. officinalis* and *O. brachyantha* obtained from International Rice Research Institute and the international BLB resistant check variety Ajaya were screened for bacterial leaf blight resistance with seven hyper virulent isolates collected from different parts of India. One *O. brachyantha* introgression line namely IR-65483-141-2-4-4-2-5-B, and the elite variety Ajaya were found to be resistant consistently with all the

isolates tested when compared with susceptible checks, i.e., TN1, Samba Mahsuri (BPT5204), Swarna and MTU1010.

- Genetic analysis in the F_2 population of both the crosses TN1/ IR-65483-141-2-4-4-2-5-B and BPT/ IR-65483-141-2-4-4-2-5-B revealed the action of a single dominant gene in governing resistance in IR-65483-141-2-4-4-2-5-B
- In order to assess whether the resistance gene in IR-65483-141-2-4-4-2-5-B was allelic to any of the known dominant BLB resistance genes, it was crossed with near isogenic lines of IR24 possessing known single dominant BLB resistance genes, *viz.*, IRBB1, IRBB4, IRBB7, IRBB21 and IRBB26. The F_1s derived from these crosses segregated for BLB resistance in a typical ratio of 15:1 (resistant: susceptible) indicating that the resistance gene in IR-65483-141-2-4-4-2-5-B was different from *Xa1, Xa4, Xa7, Xa21* and *Xa26* and could be novel.
- In order to tag and map the dominant BLB resistance gene in IR-65483-141-2-4-4-2-5-B bulked-segregant analysis was carried out at F_2 generation using 106 parental polymorphic SSR markers spread across the 12 rice chromosomes.
- The SSR marker RM595 located on chromosome 1, showed bulk specific amplification. This marker when tested with the individuals constituting the bulks and the remaining individuals of the F_2 mapping population for cosegregation, showed a linkage distance of 10.2cM from the gene.
- Since no other previous study has reported presence of any dominant BLB resistance gene on Chr. 1 and since the BLB resistance gene identified in IR-65483-141-2-4-4-2-5-B was non-allelic to the known dominant BLB resistance genes, it was assumed that the gene is novel and was tentatively named as *Xa35t.*

- Linkage analysis with, a set of 37 SSR markers in the vicinity of RM595 mapped *Xa35t* in the marker interval of RM10975 (0.82cM) and RMCG6(1.8cM). The markers RM10920, RM10963, RMCG5 RM10974 and RM10975 were observed to be located on one side of the gene at a genetic distance of 9.4 cM, 7.5 cM, 6 cM 1.85 cM and 0.82cM respectively while RMCG6, RMCG7 and RMCG8 were located on the other side of the gene at a genetic distance of 1.8 cM, 5.6 cM, and 8.4 cM respectively
- In order to validate the linkage distances of the identified markers, they were analyzed for their segregation pattern in a progeny tested alternate mapping population consisting of 250 F_2 individuals derived from the cross BPT5204/ IR-65483-141-2-4-4-2-5-B. The linkage distances of the closely linked markers were observed to be identical to those observed in the mapping population derived from the cross TN1/ IR-65483-141-2-4-4-2-5-B thus validating these markers.
- The amplification pattern of the closest markers RM10975 and RMCG6 was analyzed in a set of high priority BLB susceptible rice varieties along with IR-65483-141-2-4-4-2-5-B. Both RM10975 and RMCG6 displayed polymorphism between BLB resistant and susceptible genotypes indicating the possibility of using these markers either individually or together in marker-assisted transfer of *Xa35t* into elite rice varieties.
- The physical interval flanked by the closest markers RM10975and RMCG6 was analyzed *in silico* for putatively expressed candidate genes. A set of four genes were identified in this interval of which a gene encoding a No apical meristem protein appears to be the best candidate for *Xa35t*.

- A study on the inheritance of resistance in Ajaya using the F_2 populations of the crosses TN1/Ajaya and BPT/Ajaya revealed an additive interaction between two incompletely dominant genes with equal effects
- According to the pedigree of Ajaya its resistance was conferred by 2 genes, *xa5* and *xa13* contributed by one its parental line BJ1. The allelic status of Ajaya was therefore tested with respect to linked markers to *xa5* (RG556) and *xa13* genes (*xa 13* prom). PCR analysis with RG556 showed resistant specific amplification in Ajaya as in IRBB5 while analysis with xa13 promoter primer showed susceptible specific amplification as in TN1 and BPT5204. This indicated that one of the two resistance genes in Ajaya could be allelic to *xa5* and the other non allelic to *xa13*.
- In order to further assess whether Ajaya possessed *xa5*, allelism test was conducted with a near isogenic line of IR24 possessing the BLB resistance gene *xa5* (IRBB5). The F_2 progeny of the cross Ajaya /IRBB5 was uniformly resistant with out any segregation indicating that one of the resistance genes in Ajaya is *xa 5*. Sequence analysis of the functional polymorphic region in the second exon of *xa5* in Ajaya further proved the presence of *xa5*.
- The 400 F_2 individuals of the population of the cross TN1/Ajaya were genotyped with respect to RG556 and this data was used along with the F_3 segregation data to predict the genotype at the second resistance locus.
- Bulked-segregant analysis carried out using 77 parental polymorphic SSR markers spread across the 12 rice chromosomes revealed the linkage of the second

resistance gene in Ajaya with an SSR marker RM23476 located on Chromosome 8 (26. 26Mb) which showed a genetic distance of 15.1cM.

- Linkage analysis with, a set of 45 SSR markers in the vicinity of RM23476 mapped the gene in the marker interval of RMAFM1 and RM447. The markers RM23489, RM23496, RMAFM2 and RMAFM1 were observed to be located on one side of the gene at a genetic distance of 13.6 cM, 12.2 cM, 4.3 cM and 1.9 cM respectively , while RM447, RMAFM4, RMAFM5 and RMAFM6 were located on the other side of the gene at a genetic distance of 0.87 cM, 3.1 cM, 6.2 cM and 10.1cM respectively
- Since RM447 was monomorphic between the parents Ajaya and BPT5204. The other closer and flanking markers RMAFM1, RMAFM2, RMAFM4, and RMAFM5 were checked for their validity in the alternate population of the cross BPT5204/Ajaya. These markers showed similar marker order and identical linkage distances with the gene as in the main population of the cross TN1/Ajaya and the gene could be precisely mapped in between the markers RMAFM1 and RMAFM4.
- The utility of the flanking markers identified, for marker assisted introgression of the *xa 36t* gene was evaluated by studying their amplification pattern in a set of high priority bacterial leaf blight susceptible recipient lines into which the novel gene can be introgressed. The flanking marker combination of RM447-RMAFM1 can be used for introgression of the gene from Ajaya in to susceptible cultivars TN1, MTU1001, Krishnahamsa and Basmati370 as these markers were polymorphic between Ajaya and the recipient parents. Since the marker RM447

was monomorphic between Ajaya and BPT5204, Swarna, MTU1010, Tellahamsa and IR64, the flanking marker combination of RMAFM1 and RMAFM4 is suggested for marker-assisted introgression of this gene to the above mentioned susceptible rice cultivars. Since both the markers RM447 and RMAFM1 were monomorphic between Ajaya and MTU1010, the marker RMAFM4 can be used singly or in combination with the other relatively closer polymorphic markers for introgression of *xa36t.*

- The physical interval flanked by the closest markers RM447 and RMAFM1 was analyzed *in silico* for putatively expressed candidate genes. A set of three genes were identified in this interval of which a gene from yeast, SUA7 encoding the transcription factor TFIIB appears to be the best candidate for *xa36t.*

To conclude, from the present study, two novel BLB resistance genes *Xa35t,* a dominant gene from an introgression line of *O. brachyantha* i.e. IR-65483-141-2-4-4-2-5-B, tagged and fine mapped on Chr.1 and *xa36t,* an incompletely dominant gene from Ajaya, tagged and fine mapped on Chr.8 using SSR markers were identified. Further, putative candidate genes which could be possibly associated with *Xa35t* and *xa36t* mediated resistance have also been identified.

Chapter 7

List of References

Chapter VII – List of References

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